Robust Low Frequency Somatic Variant Detection in cfDNA Using a SLIMamp® Based liquid Biopsy Assay

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ABSTRACT

Introduction. Detection of variants in cell-free DNA (cfDNA), particularly those at exceptionally low variant allele frequency (VAF), is important for early-stage treatment decision, therapy monitoring, recurrence detection, and improving survival for cancer patients. However, adoption of this technology is limited by challenges such as insufficient cfDNA amount, distinguishing real signals from noise, lack of kitted Liquid Biopsy (LBx) panels for local NGS (Next Generation Sequencing) testing, and cost effectiveness. To address these challenges, we developed oncoReveal™ Core LBx, a cost-effective, unique molecular identifier (UMI) free, 446 amplicon research-use-only (RUO) panel and assessed its performance in calling low-frequency somatic variants. We verified the panel's efficacy on both clinical and contrived samples.

Methods. Standard reference control samples from Seraseq® were used at VAFs ranging from 0.1-5%, at 10ng and 30ng DNA input, for a total of 37 replicates. cfDNA was extracted from plasma samples (8 - 30ng) from 16 patients, 2 healthy donors, 2 standard reference controls and 1 wild-type control sample. NGS libraries were prepared using the oncoReveal™ Core LBx panel and sequenced on Illumina's NextSeq™ 550 platform, targeting an average of 30M paired-end reads per sample and variants were called using Pillar's PiVAT® (**Pi**llar Biosciences **V**ariant **A**nalysis **T**oolkit).

Results. We were able to detect low-frequency variants, including in samples with low DNA input (<10ng). We detected 88% and 96% of the expected variants in 0.25% VAF at DNA inputs of 10ng and 30ng, respectively. High-priority variants with known drug response or pathogenicity were detected down to 0.1% VAF. All variants were detected in higher VAF samples. In negative samples, we had >99.99% per-position specificity.

Conclusion. We demonstrate here a low-cost, kitted, amplicon-based (RUO) liquid biopsy panel that can detect clinically significant, high priority variants down to 0.1% VAF, without the use of UMIs. The panel can be run at scale on the mid-throughput Illumina NextSeq 550 NGS platform, enabling greater opportunity for laboratories to perform liquid biopsy-based tumor profiling within their own laboratories. The panel was able to amplify and detect variants even in samples with limited DNA input <10ng. Further, the panel's performance was not affected by the choice of extraction strategy used.

EXPERIMENT DESIGN

- Amplicons are designed to be as small as possible to facilitate detection of tumor ctDNA, which is predicted to average around 145bp in length based on the size of DNA fragment protected by the nucleosome
- Amplicons range from 54 to 129 bp in length, avg 79.2, med 71
- **Positive standard reference samples:**
  - Seraseq® ctDNA Complete™ Mutation Mix v1 (number of mutations = 13),
  - Seraseq® ctDNA Mutation Mix v2 (number of mutations = 24)
- **Targeted LoD**: 0.25% at 30ng, 0.5% at 10ng
- **Negative standard reference samples:** Anchor Molecular Reference, Seraseq® wildtype
- **Clinical samples:** Human cfDNA normal sample, cancer patient plasma samples (including but not limited to colorectal, breast, lung, and pancreatic carcinoma).

Table 1. Sample table listing different classes and types of samples tested as part of the study. Breakdown of positive standard reference samples, negative standard reference samples, and clinical samples is provided. Samples with expected mean allele frequency (in %) are delineated. For positive standard reference samples, allele frequency within the targeted limit of detection (LoD) range is shaded in light blue. Cancer patient samples from a variety of cancer types and tissue biopsy results were also screened with the panel. Cancer patient samples were run in biological duplicates.

Sample Class	Sample Type	Input (ng)	Expected Allele Frequency (%)								Total
			0	0.125	0.25	0.5	1	2.5	5	unknown	
Positive Standard Reference Samples (Seraseq®)	Complete™ Mutation Mix v1	10	0	1	2	4	2	1	1	0	11
		30	0	1	3	2	2	1	1	0	10
	Mutation Mix v2	10	0	1	3	4	1	0	0	0	9
		30	0	1	3	2	1	0	0	0	7
Negative Standard Reference Samples	Anchor Molecular	20-30	6	0	0	0	0	0	0	0	6
	Seraseq® Wildtype	30	1	0	0	0	0	0	0	0	1
Clinical Samples	Normal donor	10-30	12	0	0	0	0	0	0	0	12
	Cancer patients	8-30	0	0	0	0	0	0	0	16	16
Total			19	4	11	12	6	2	2	16	72

RESULTS

Pillar Biosciences oncoReveal Core LBx Overview

Table 2. Genes covered in oncoReveal Core LBx. Core LBx's assay design includes 104 genes with SNV/indel coverage. The full CDS of the TP53 gene is covered within the panel, with additional 10 genes having sufficient coverage to enable copy number amplification (CNA). Further, primers for 127 genes are also present in the Core LBx assay, with 25 genes for microsatellite instability (MSI), but they are not listed in this table.

AKT1	ALK	APC	AR	ARAF	ARID1A	ARID2	ASXL1	ATM	ATRX	AXIN2	AXL
B2M	BCOR	BRAF	CARD11	CCND1	CDH1	CDK4	CDK6	CDKN2A	CIC	CREBBP	CTCF
CTNNB1	EGFR +CNA	EP300	ERBB2 +CNA	ERBB3	ESR1	EZH2	FBXW7	FGFR1 +CNA	FGFR2 +CNA	FGFR3 +CNA	FLCN
FOXL2	GATA3	GLI1	GNA11	GNAQ	GNAS	HNF1A	HRAS	IDH1	IDH2	IKZF1	JAK1
KDM5A	KIT +CNA	KRAS	MAP2K1 +CNA	MAP2K2	MAPK1	MED12	MET +CNA	MLH1	MLL2	MRE11A	MSH6
MTOR	MYC	MYOD1	NCOR1	NF1	NFE2L2	NOTCH1	NRAS	NTRK1	NTRK3	PAK7	PDCD1
PDGFRA +CNA	PIK3CA +CNA	PIK3R1	POLE	PPP2R1A	PTCH1	PTEN	PTPN11	PTPRD	PTPRS	RAC1	RAF1
RB1	RET	RHEB	RHOA	RIT1	RNF43	ROS1	RUNX1	SF3B1	SMAD4	SMO	SOX9
SPOP	STAT5B	STK11	TCF7L2	TP53 Full CDS	TSC1	U2AF1	VHL				

Pillar Biosciences oncoReveal Core LBx Workflow

Figure 1. Overview of oncoReveal Core LBx's workflow. Analysis of cfDNA samples begins with isolation of cfDNA, followed by amplification of oncoReveal Core LBx target sequences. High-quality libraries are sequenced to high coverage, approximately 30M clusters, on an Illumina machine. The resulting reads are aligned to the human genome with PiVAT and the alignments are further refined to reduce errors. Finally, variants are called and then polished using a novel noise suppression algorithm.

Performance Summary on Standard Reference Control Samples

(A) Positive Percent Agreement (PPA)

Sample Type	Input (ng)	Variant Allele Frequency (%) – All Variants							Tier 1 Variants	
		0	0.125	0.25	0.5	1	2.5	5	0.125	0.25
Complete™ Mutation Mix v1	10	0	46%	96%	100%	100%	100%	100%	40%	100%
	30	0	62%	100%	100%	100%	100%	100%	20%	100%
Mutation Mix v2	10	0	75%	85%	100%	100%	0	0	75%	92%
	30	0	92%	94%	100%	100%	0	0	100%	100%
Combined	10	0	65%	88%	100%	100%	100%	100%	55%	94%
	30	0	81%	96%	100%	100%	100%	100%	55%	100%

(B) Negative Percent Agreement (NPA)

Background	N	ROI	Sites	Possible variants	FPs	Per-position NPA	Per-variant NPA
AM	6	11,401	68,406	342,030	2	99.997%	99.999%
Human normal	12	11,401	136,812	684,060	19	99.997%	99.997%
Seraseq WT	1	11,401	11,401	57,005	5	99.956%	99.991%

Table 3. Performance summary of oncoReveal LBx on standard reference samples. (A) Positive percent agreement (PPA) and (B) negative percent agreement (NPA) are shown for the standard reference control samples tested in this study. (A) The panel shows a PPA > 95% at the target LoD of 0.25% at 30ng and 0.5% at 10ng. **However, the higher tier variants are detected as low as 0.25% at 10ng and 0.125% at 30ng.** (B) The panel maintains a very high NPA, except Seraseq wildtype (WT) sample, in which a higher number of false positives (FPs) were observed. The sample's false positives were reproducible across 3 replicates as well as when tested on other Pillar panels, raising the possibility that they are likely artifacts of this contrived material.

RESULTS

Sequencing Summary

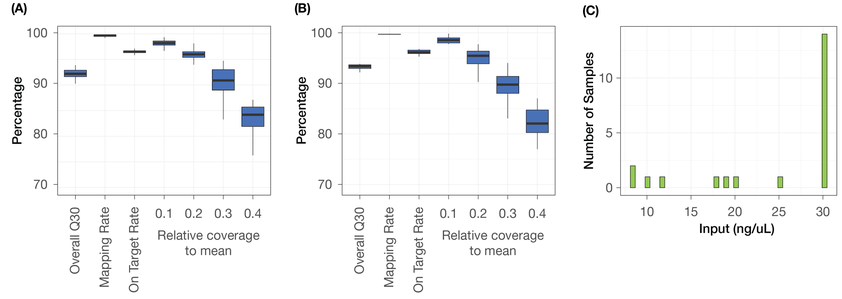

Figure 2. Sequencing summary across experiments. Sequencing statistics summary are shown for (A) standard reference and clinical normal samples, and (B) plasma samples from cancer patients. All the samples were sequenced to a mean depth of 43M. Percentage of reads with overall Q30, mapping rate and on target rate were observed to be >90%. Coverage uniformity, as measured by percentage of read with coverage depth > 20% of mean, was also observed to be >90% across all samples. (C) The DNA input from plasma of cancer patients had a wide range from 8.2 ng/uL to 30 ng/uL. Regardless of the DNA input, high quality sequencing data was generated from each sample.

Clinical Results

Table 4. Variants expected within the cancer patient's plasma samples and detected by Core LBx. Plasma sample from the 16 cancer patients were analyzed through Core LBx and resulting variants were compared to variants characterized using tissue NGS results. Variants highlighted in **black and bold** font type were covered by Core LBx. The assay was able to detect variants in most of the samples, with the lowest VAF of 0.2%.

Sample ID	Tissue NGS Result	Variants Detected by Core Assay (VAF)
S1	RB1 splice site 26S-1G>T, **TP53 G266E**, CREBP R1446L	TP53 G266E (17.13%)
S2	**KRAS G12D, TP53 R175H**	KRAS G12D (0.3%), TP53 R175H(0.2%)
S3	**APC R283*, KRAS G12V, FBXW7 R505C**, SMAD2 R182*, DNMT3A R688fs817#	APC R283* (11.62%), KRAS G12V (40.92%), FBXW7 R505C (26.19%)
S4	**BRAF V600E**, PMS2, Y255*, **TP53 R306*, APC T1556fs*3**, ASXL1, D8644fs*3	BRAF V600E (38.63%), TP53 R306* (56.46%)
S5	**KRAS G12V**, APC S1315, **TP53 P142del, TP53 P152fs*14**	
S6	ATM D2708N, **GNAS R201H, TP53 R175H, TP53 R248W**	GNAS R201H (0.49%), TP53 R175H (1.09%), TP53 R248W (2.74%)
S7	FBXW7 V341fs*1, **TP53 R273H**	TP53 R273H (0.97%)
S8	**TP53 F134fs*15**	
S9	**NFE2L2 V32G**	
S10	**PIK3CA V105-N107>D**, PIK3C2B R1349W	PIK3CA V105-N107>D (2.29%)
S11	**BRAF V600E TP53 C242fs*5**	
S12	**PIK3CA E545K**, CHECKR117G, CDH1 Q610*, DNMT3A Y735C	PIK3CA E545K (4.3%)
S13	**PIK3CA M1043I, TP53 I255S**, SMAD4 splice site 788-1G>A	
S14	**KRAS G12V TP53 V272L**	
S15	APC<1431fs*42, **FBXW7 R505C TP53 S127P**	FBXW7 R505C (7.89%)
S16	APC pF773fs, pN1531fs, **KRAS G13D, PIK3CA N345K**, RNF43 Y719fs, **TP53 Y234C**	KRAS G13D (10.24%), PIK3CA N345K (7.98%), TP53 Y234C (11.94%)

CONCLUSIONS

- The Pillar oncoReveal™ Core LBx (RUO) panel and secondary analysis achieve high sensitivity at low VAF in contrived samples
- The panel performs well with low DNA input, reaching 100% sensitivity at 0.5% VAF for 10ng DNA
- Characterizing patient plasma shows that the panel is usable with a very broad range of VAF
- Specificity is high across synthetic normal and human normal cfDNA samples
- The Pillar oncoReveal Core LBx panel now provides laboratories with a high-performing off the shelf RUO kit to perform liquid biopsy-based tumor profiling within their own laboratories on standard mid-throughput NGS systems.