Single-vial Amplification Based NGS with Rapid Turn-Around-Time for Interrogation of Variants in Tumors

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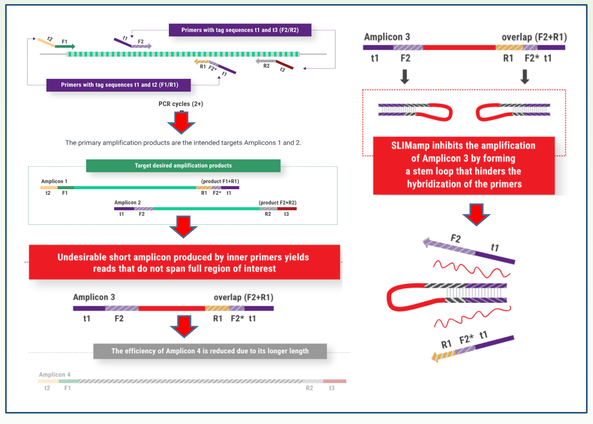
Introduction

Targeted Next Generation Sequencing (NGS) is the primary assay for interrogation of variants in tumors, in molecular laboratories. In samples with limited amount of tumor material, NGS libraries that use amplification methodologies have been successfully sequenced for identification of targetable variants. However, total DNA input, PCR artifacts, compromised DNA quality, turn-around-time and ease-of-use, are factors that hamper the universal adoption of NGS assays in routine diagnostics. We evaluated the ONCOReveal panel with single tube Stem-Loop Inhibition Mediated Amplification (SLIMamp) technology (Pillar Biosciences) for accuracy and sensitivity of detection of variants harbored in solid tumors.

Methods

Fifty-six previously tested FFPE samples harboring 26 different clinically relevant variants present in 9 genes were included in the evaluation. Variants present were previously detected using either, TruSeq Amplicon Cancer Panel (Illumina) (N=48) or Sanger sequencing (N=8). 6/56 samples were wildtype. Sensitivity studies ranging from 2.5 ng – 20 ng input DNA, were performed with 3 samples that harbored clinically relevant variants. NGS libraries, using the ONCOReveal Multi-Cancer Panel (Pillar Biosciences) were prepared with DNA input ranging from 2- 90 ng. For each run, up to 24 samples were normalized, pooled and run using the MiSeq reagent kit V2 (Illumina). Data analysis including sequence alignment, variant calling and annotation was performed using FASTQ files, with the Pillar Variant Analysis Toolkit (PiVAT). FASTQ files were also analyzed on NextGENE for comparison.



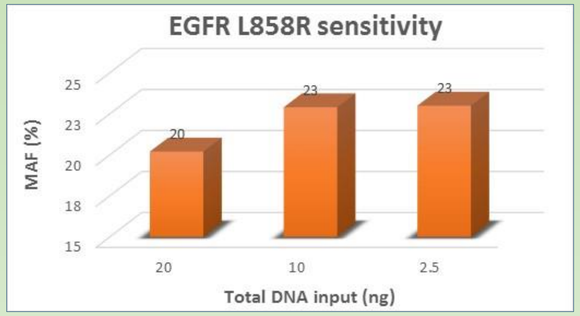
Schematic of SLIMamp technology used in the ONCOReveal assay: Increased specificity is obtained by inhibition of amplification of the stem-loop structure.

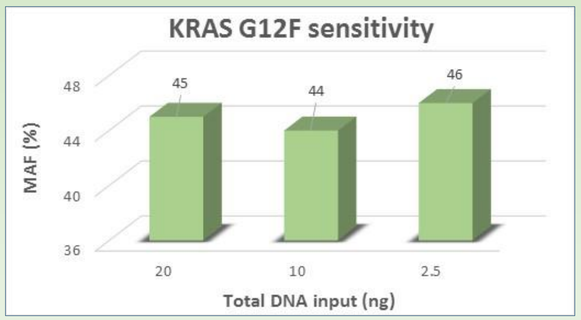
Accuracy

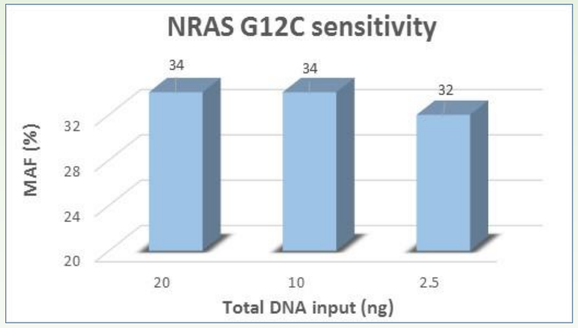
	VARIANTS(TruSeq)	MAF(%)	VARIANTS(ONCOReveal)	MAF(%)
1	BRAF D449E	18	BRAF D449E	22
2	BRAF V600E	5	BRAF V600E	6.3
3	BRAF V600E	3	BRAF V600E	4.3
4	BRAF V600E	1.6	BRAF V600E	1.5
5	BRAF V600E	10	BRAF V600E	12
6	EGFR E745_A750del12	28	EGFR E745_A750del12	30
7	EGFR E745_A750del12	21	EGFR E745_A750del12	17
8	EGFR E746_A750del15	53	EGFR E746_A750del15	37
9	EGFR E746_A750del15	85	EGFR E746_A750del15	82
10	EGFR E746_A750del15	35	EGFR E746_A750del15	36
11	EGFR E746_A750del15	Sanger	EGFR P747_S752del18	21
12	EGFR P747_S752del18	3.2	EGFR P747_S752del18	1.4
13	EGFR E746_S752delinsV	54	EGFR E746_S752delinsV	46
14	EGFR L858R	73	EGFR L858R	75
15	EGFR L858R	24	EGFR L858R	20
16	EGFR L858R	36	EGFR L858R	33
17	EGFR L858R	9	EGFR L858R	9.1
18	KIT A502_Y503dup	35	KIT A502_Y503dup	38
19	KIT W557_K558del	52	KIT W557_K558del	63
20	KRAS G12C	27	KRAS G12C	27
21	KRAS G12C	8	KRAS G12C	6
22	KRAS G12C	34	KRAS G12C	34
23	KRAS G12C	16	KRAS G12C	15
24	KRAS G12C	11	KRAS G12C	10
25	KRAS G12D	36	KRAS G12D	34
26	KRAS G12D	36	KRAS G12D	37
27	KRAS G12D	29	KRAS G12D	33
28	KRAS G12D	40	KRAS G12D	39
29	KRAS G12D	25	KRAS G12D	27
30	KRAS G13D	22	KRAS G13D	21
31	KRAS G13D	18	KRAS G13D	20
32	KRAS G12F	44	KRAS G12F	45
33	KRAS G12R	31	KRAS G12R	29
34	KRAS G12S	Sanger	KRAS G12S	44
35	KRAS G12V	Sanger	KRAS G12V	48
36	KRAS G12V	Sanger	KRAS G12V	16
37	KRAS G12V	12	KRAS G12V	14
38	KRAS V14I	8	KRAS V14I	7
39	MET c.3082 +2 T>A, p.?	70	MET c.3082 +2 T>A, p.?	71
40	NRAS Q61K	19	NRAS Q61K	40
41	NRAS Q61R	75	NRAS Q61K	78
42	PIK3CA E545K	36	PIK3CA E545K	33
43	PIK3CA H1047R	6	PIK3CA H1047R	5.4
44	STK11 D343N	30	STK11 D343N	33
45	STK11 K48*	6	WILDTYPE	
46	STK11 c.592_597 +5 delinsA	27	STK11 c.592_597 +5 delin	19
47	TP53 V173M	34	TP53 V173M	46
48	WILDTYPE	NGS	WILDTYPE	
49	WILDTYPE	NGS	WILDTYPE	
50	WILDTYPE	NGS	WILDTYPE	
51	WILDTYPE	NGS	FLT3 D835Y	18
52	WILDTYPE (BRAF)	Sanger	BRAF G469R	24.4
53	WILDTYPE (EGFR)	Sanger	BAD TOO MANY LOW% VARIANTS	
54	FAILURE	Sanger	FAILURE	
55	FAILURE	Sanger	FAILURE	
56	FAILURE	NGS	FAILURE	

Forty-nine of the fifty-three samples tested, showed identical results with similar MAF(%) detected in both ONCOReveal and TruSeq assays. The ONCORevel detected 2 additional variants, not interrogated in the Truseq panel.

Sensitivity



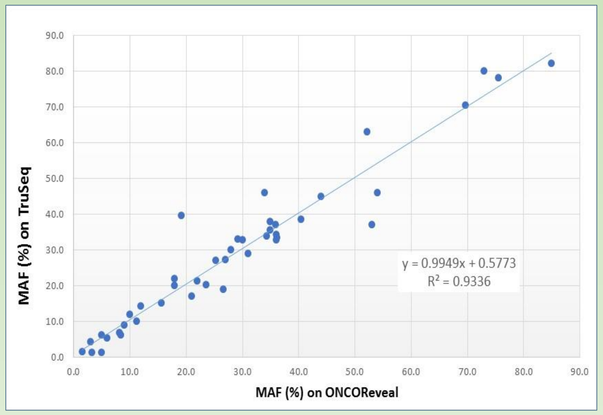




Three samples with clinically relevant variants (KRAS G12F, EGFR L858R & NRAS G12C) were tested at concentrations of 20, 10 and 2.5 ng total input DNA. The MAF (%) across the three different concentrations were compared.

The figures show comparable MAF(%) values obtained for all 3 variants at the three concentrations tested.

Mutant Allele Fraction (MAF%) comparison



The percentage of mutant alleles in the forty three samples tested (Mutant Allele Fraction [MAF]) in both assays shows excellent correlation.

Conclusions

- ❑ Interrogation of variants in solid tumors using the SLIMamp technology with the ONCOReveal panel can identify actionable alterations, including missense variants and indels in tumors where the input DNA is as low as 2.5ng.
- ❑ Assays that use the technology can reliably detect variants with 3% MAF in samples with low input DNA.
- ❑ Variant analysis using PiVAT software provides rapid annotation and interpretation of genomic alterations
- ❑ The simplicity of a single-vial library preparation coupled with a rapid turn-around-time of 3-4 days from sample to answer, allows for viable implementation of SLIMamp technology in molecular laboratories.

Results

- All but one of the 53 previously sequenced samples were successfully sequenced using SLIMamp technology. One sample that yielded a wildtype result by Sanger did not pass the NGS quality control metrics used for analysis. Three samples failed to yield sequences in both assays.
- Samples analyzed with Sanger had insufficient DNA for the TruSeq assay. These samples were successfully sequenced using the ONCOReveal assay.
- Targeted alterations in the samples, included missense variants (N=35), indels (N=11) and a splice variant.
- The mutant allele fraction (MAF) percentage in the samples, ranged from 3% to 80%. and showed excellent correlation (R^2=0.94).
- The “on target” percentage of the ONCOReveal assay was >99% and average coverage obtained across the samples was 3731X.
- Sensitivity studies demonstrated that missense variants with MAF of 3% or more were reliably detected at 2.5 ng input DNA.

Reference: Schenk, D., et al., 2017. PLoS One. 2017 Jul 12;12(7):e0181062. doi: 10.1371/journal.pone.0181062.