Development and Evaluation of a Lung Cancer Fusion NGS Panel

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Abstract

Introduction: Targeted therapies are available for patients with non-small cell lung cancer (NSCLC) patients with gene arrangements of ALK, ROS1, RET and NTRK1, and MET exon-14 skipping alteration. Detection of these type of variants is needed for determining specific treatment. To most effectively use clinical samples, especially FFPE samples with poor quality and quantity, we developed a lung cancer fusion NGS panel with RNA input using a multiplex PCR-based method for Illumina sequencing platforms. In our single-well PCR-based NGS assay, the amplicons are designed to detect the gene rearrangement by 1) targeting specific breakpoints of known fusion transcripts and 2) assessing the expression balance or imbalance between 3' and 5' ends of the mRNA of the kinase genes, which does not require prior knowledge of fusion partners or breakpoints. The panel contains 92 specific fusion variant amplicons involving ALK, ROS1, RET, NTRK1, FGFR3, NRG1 and PBX1 as well as the MET exon 14 skipping alteration. Here, we evaluated the performance of the Lung Cancer Panel on samples from various sources including cell line samples, commercial FFPE reference samples and clinical FFPE samples.

Methods: A total of 40 samples with confirmed known fusions from various sources (cell line, manufactured references, clinical) were initially used for a fusion detection concordance study. The library was prepared using RNA ranging from 10 ng - 50 ng. Subsequently, a total of 27 clinical NSCLC samples were tested: 26 FFPE RNA samples and one fresh-frozen RNA sample. 20 - 25 ng RNA input was used for the library preparation for each clinical sample. The libraries were pooled and sequenced on the Illumina MiSeq or NextSeq instruments. The data was analyzed using Pillar Biosciences' PiVAT software (Pillar Variant Analysis Toolkit).

Results: For the 40 samples with known variants, all known fusions including ALK, ROS1, RET, NTRK1, and MET exon 14 alterations were detected without false positives. Out of the 27 clinical lung cancer samples, two samples didn't produce enough sequencing reads due to low library yields (not included in the 40). The remaining 25 samples were successfully processed: 10 with ALK fusion detected targeted breakpoints, one with a RET fusion called by expression imbalance, and one sample with MET exon 14 skipping.

Conclusions: The Pillar Biosciences lung cancer fusion NGS assay targets important fusion variants in lung cancer and can be applied on FFPE samples of varying quality.

Methods and Assay Design

Product Specifications	
Number of Genes	7
Variant Type	Fusion RNA Transcripts
Variant Number	92 previously reported fusions plus MET exon 14 skipping
Average Amplicon Size	141 bp
Input	≥ 25 ng recommended
Number of Pools	1
Sample Types	RNA from FFPE, tissue

Table 1 – Product specifications

Kinase (exon #s)	Partner Gene (exon #s)
ALK(19, 20)	CLTC (31) EML4 (2,3,6,13,14,15,17,18,20) HIP1(21,28,30) KIF5B(15,17,24) KLC1(9) MSN(11) STRN(3) TFG(4,5,6) TPM3(8) TPR(15)
FGFR3(17,18)	TACC3(4,8,9,10,11)
NRG1(4,6)	CD74(6,8) SLC3A2(2,5) VAMP2(4)
NTRK1 (10,12,13)	CD74(3,8) MPRIP(14,18,21) TFG(5) TPM3(8) TPR(6,21)
RET(8, 11, 12)	CCDC6(1) CUX1(10) KIF5B(15,16,22,23,24) NCOA4(7) TRIM33(11,14)
ROS1(32, 34, 35, 36)	CCDC6(5) CD74(6) CLTC(31) EZR(10) GOPC(4,8) LRIG3(16) MSN(9) SDC4(2,4) SLC34A2(4,13) TFG(4) TPM3(8)
PBX1 (3)	TCF3 (16)
MET(15)	KIF5B(24)
Exon Skipping	
MET exon 14	

Table 2 – Variants assayed in the lung cancer fusion NGS panel.

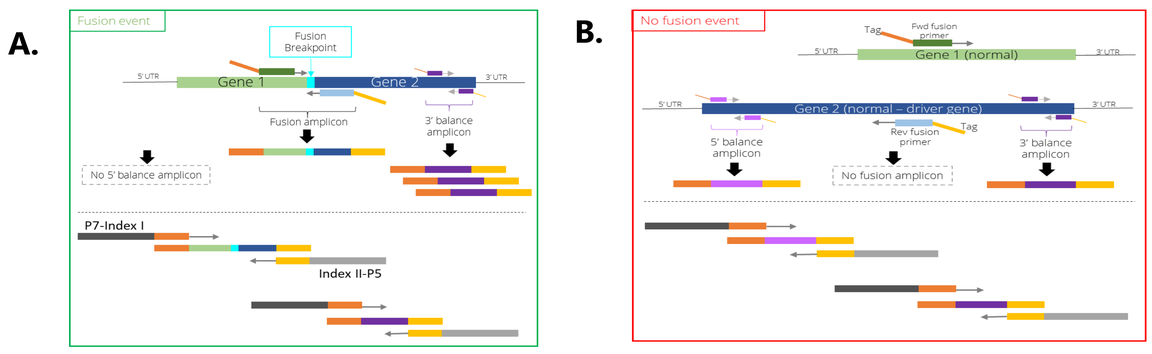

Figure 1 – A. In fusion transcripts, Gene Specific PCR (GSPCR) primers flanking fusion breakpoints amplify chimeric fusion transcripts. 3′ balance amplicon primers targeting the kinase genes are over expressed in the fusion transcripts; **B.** In normal transcripts, no fusion products are amplified and the 3′ and 5′ balance amplicons for the kinase genes are expressed at similar levels. The normalized ratio of coverage depths of 3′ and 5′ balance amplicons can be used as a predictor of gene fusions (Fig 2.).

Sample	Source	Sample #
LC2/ad	Sigma	4, 5
CR560513	Origene	6
A549	NCI	7
HCC78	DSMZ	12
KM12	NCI	13
H2228	ATCC	24
H3122	NCI	27

Sample	Source	Sample #
Control 1	SZB	10
Control 2	SZB	11
Control 3	SZB	25
Control 4	SZB	28
Control 5	SZB	31
Control 6	SZB	36
Control 7	SZB	40

Table 3 – Cell line and control RNA samples used in Fig 2. (SZB- Shanghai Zhengu Biotech)

Results and Conclusions

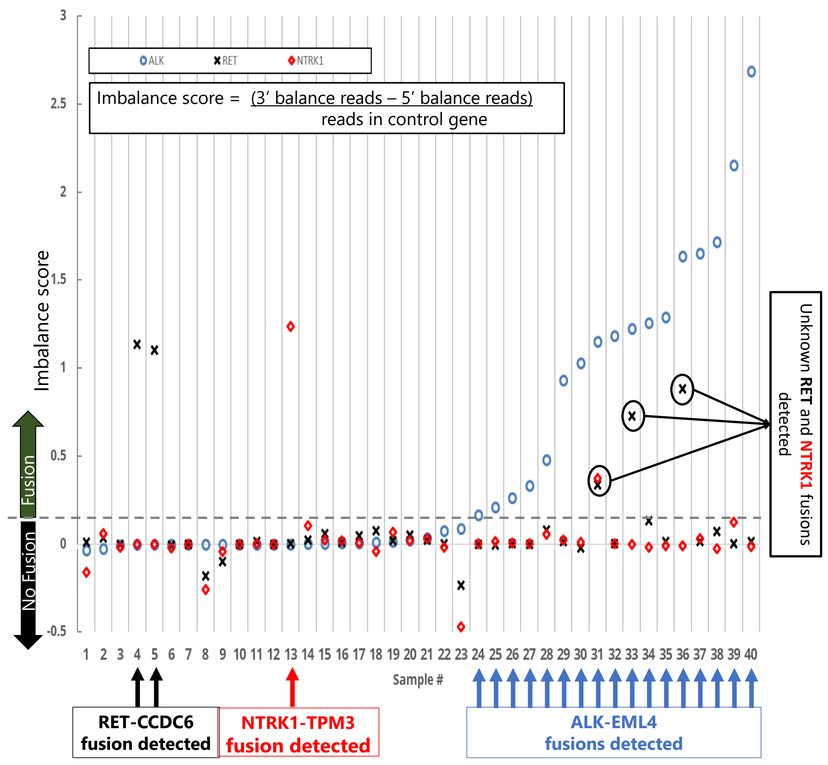

Figure 2 – 3′ and 5′ imbalance scores were calculated for each of the kinase genes assayed in the panel (see key, Table 2, Figure 1). Samples are of clinical origin and commercially available cell lines (Table 3). Results from ALK, RET, and NTRK1 are shown. An imbalance score of 0.15 was empirically defined as a threshold (dashed line); samples with imbalance scores above 0.15 were found to have a fusion with the given kinase while samples with imbalance score below 0.15 did not have a fusion with the given kinase. ALK(ex20) fusions with ELM4(ex6, ex13, ex20) were detected among samples 24-40. Samples 33 and 36 have known ALK(ex20)-EML4(ex13) fusions as well as an unknown RET fusion identified by high imbalance score. Sample 31 has a known ALK(ex20)-EML4(ex20) fusion transcript and also has two unknown fusions identified by high imbalance scores (RET and NTRK1). Samples 31 and 36 are manufactured reference RNA samples and thus multiple fusion events are not abnormal.

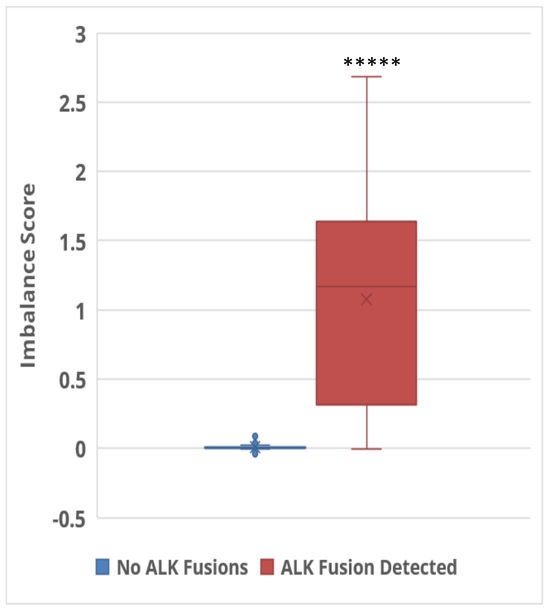

Figure 3 – ALK imbalance score in samples with detected ALK fusions (red) are significantly higher than samples without a detected ALK fusion (blue) (t-test; *****=$p<10^{-7}$).

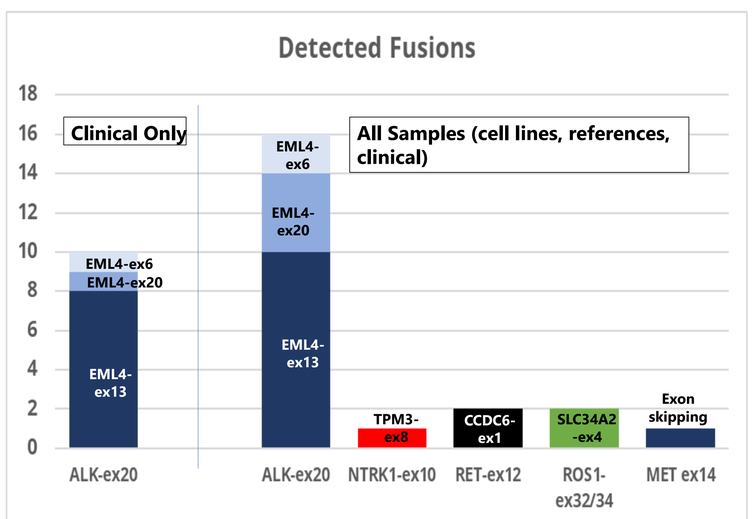

Figure 4 – Fusions detected in samples tested during panel evaluation. Ten ALK(ex20) fusions were detected in the clinical samples tested; eight ALK(ex20)-EML4(ex13) fusions (variant 1), one ALK(ex20)-EML4(ex20) fusion (variant 2), and one ALK(ex20)-EML4(ex6) fusion (variant 3) were detected. Across all samples tested, including samples derived from cell lines, 16 total ALK fusions, one NTRK1(ex10)-TPM2(ex8) fusion, two RET(ex12)-CCDC6(ex1) fusions, and two ROS1(ex32/34)-SLC34A2(ex4) fusions were detected. Additionally, MET exon 14 skipping was measured in one sample. A minimum of 50 fusion reads is required to make a positive call. The reads supporting the fusions in this dataset ranged from 462 to 230k with 76k to 131k on-target reads.

Panel Evaluation Summary:

- The Pillar Biosciences lung cancer fusion NGS panel is a robust assay for the detection of RNA fusion transcripts with as little as 10 ng of RNA input.
- The workflow is simple and allows for same day loading of the MiSeq instrument, even when starting from FFPE RNA.
- The assay was able to directly detect fusion transcripts from several different driver gene kinases, including ALK, NTRK1, RET and ROS1.
- Imbalance scores are a reliable predictor of fusion events, even in the absence of known fusions.