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Development of a Comprehensive Next-Generation Sequencing Assay for Gene-Fusion Detection in Solid Tumors

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INTRODUCTION

Gene fusions caused by chromosomal rearrangements play an important role in oncogenesis, the progression of cancer and the selection of targeted therapies. Next-generation sequencing (NGS) using RNA as the input material enables sensitive, specific and precise detection of potentially clinically relevant gene fusions[1,2,3]. Gene fusion drivers have preferred partners that are prevalent and frequently reported as well as a diverse set of partner isoforms that are less frequently reported. The availability of sufficient input tumor sample material is a limiting factor for routine testing. Therefore, we have developed an NGS solution appropriate for small amounts of FFPE oncology tissue to detect solid tumor gene fusion biomarkers in clinical research.

MATERIALS AND METHODS

Fusion content was prioritized based on relevant, verified fusion isoforms reported in the literature and by collaborators, as well as the prevalence of solid tumor fusion driver genes in public databases (e.g., COSMIC). A comprehensive literature survey was undertaken to identify isoforms reported from clinical samples that supported unambiguous characterization of the fusion breakpoint.
For targeted detection, the assay was designed to detect specific isoforms for more than 50 fusion driver genes, as well as intragenic variation in key genes. Because novel combinations of partners may generate a novel fusion isoform, an algorithmic solution was used to detect fusions with any combination of driver and partner gene that exists within the panel design.
An imbalance algorithm was developed to assess partner-agnostic fusions in key fusion driver genes. (see Poster ST131: “A partner agnostic approach for gene fusion detection with targeted next-generation sequencing,” presented by Amir Marcovitz, for more details on novel fusion detection.)
The assay uses Ion AmpliSeq multiplex PCR chemistry with manual or automated library preparation, automated templating on the Ion Chef, and sequencing on the Ion Torrent GeneStudio™ S5 sequencing platform with the Ion 540 chip, using 20ng of input RNA per sample. The automated analysis workflow is supported by optimized algorithms for sample QC, secondary read analysis, fusion calling, and reporting in Torrent Suite and Ion Reporter software. Sample data presented utilized the Ion Chef for library preparation and templating and sequenced with an RNA-only sample workflow.
Streamlined access to reporting of variant relevance is enabled by Oncomine™ Reporter[4].

RESULTS

The Oncomine Comprehensive Assay Plus (OCA Plus) primer designs support targeted identification of over 1300 targeted isoforms from more than 50 key prevalent or actionable fusion driver genes, including ALK, RET, ROS1, NTRK1/2/3 and FGFR1/2/3. Intragenic fusion events are also assayed by targeted detection for MET, EGFR, BRAF and AR genes. A development study using commercially available RNA controls (SeraCare Seraseq™ Fusion Mix v3) confirmed detection of major clinically relevant isoforms. Testing of 11 fusion-containing cell lines confirmed detection of additional isoforms and driver genes and showed concordance to the on-market Oncomine Comprehensive Assay v3 (OCAv3), also sequenced on S5 platform. All the expected fusions were detected with 100% sensitivity and specificity.
The automated workflow required 20ng input of FFPE material (10ng per pool). Sample to report turnaround time was less than five days.
The assay also reported fusion events in relevant driver genes by using a novel statistically significant expression imbalance algorithm comparing 5’- and 3’- end gene expression for detecting novel partners of the oncogenic driver genes including NTRK1/2/3, ALK and RET. Non-targeted fusions with either novel fusion partners or novel breakpoints were also reported by assessing the significance of supporting mapped sequencing read information. (see Poster ST131 presented by Amir Marcovitz, for more details on novel fusion detection.)

Figure 1. Schematic flow-diagram of the complete workflow

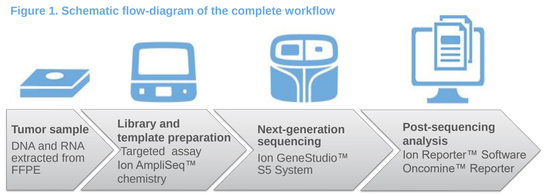
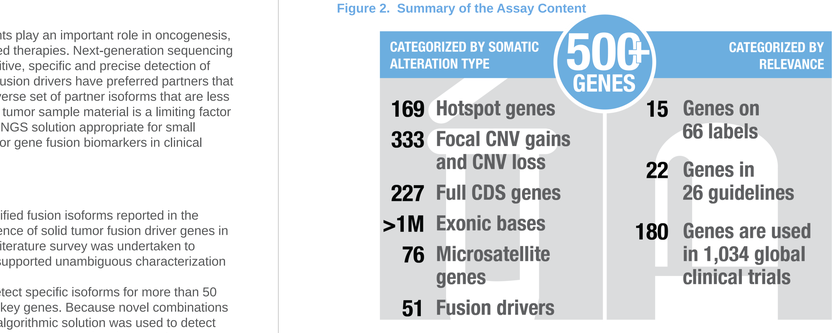


Figure 2. Summary of the Assay Content



Driver	Isoform Count
ALK	211
BRAF	188
FGFR2	116
NTRK1	102
RET*	89
ROS1	58
NTRK3	50
NTRK2	46
FGFR3	42
ESR1	37
FGFR1	37
RAF1	36
NRG1	35
MET	32
MYB	22
ERG	21
NUTM1	16
EGFR	14
ETV1	14
CDKN2A	12
ERBB2	12
TERT	10
MYBL1, YAP1, PPARG, RELA, RARA, TFE3, ETV4, RSPO3, CDKN2B-AS1, ERBB4, RSPO2, ETV5, MAP3K8, NOTCH2, PRKACA, PRKACB, STAT6, AKT2, AKT3, BRCA1, NOTCH1, NOTCH3, PIK3CA, PIK3CB, TFEB, AKT1, AR	< 10 each
Total	**1,314**

Gene	Intragenic Variants
AR	19
BRAF	5
EGFR	8
MET	5
NTRK1	1
RELA	1
Total	**41**

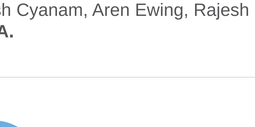


Figure 3. Fusion detection in FFPE control

Drivers Tested in Controls	
ALK	NTRK1
BRAF	NTRK3
EGFR	PPARG
ERG	RET
FGFR3	ROS1
MET	

Figure 3: Fusion detection in an in-house TriFusion control sample (mix of three cell lines) displayed as normalized reads per 1.5 million mapped reads. OCA Plus assays detected fusion targets only covered by the panel. OCA Plus results were also compared with Oncomine™ Comprehensive v3 (OCAv3) RNA panel. Relative distribution of reads between the two NGS assays is consistent across fusion targets. Control contains EML4-ALK.E6bA20 fusion, which is sometimes also detected as EML4-ALK-E6bA20 fusion.

Figure 4. Fusion detection in commercially available cell-line control

Digital PCR copies/rxn
238
459
208
319
368
332
246
310
360
331
648
382
254
242
206
415

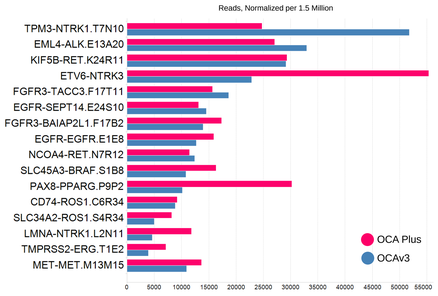


Figure 4: Fusion detection in SeraCare Seraseq™ Fusion RNA Mix v3 control sample displayed as normalized reads per 1.5 million mapped reads. Control sample contains 16 fusions and all were detected by OCA Plus assay. Relative distribution of normalized read counts from OCAv3 is consistent with the copies of the fusion molecules in the samples (showed in the table to the left). OCA Plus results were also compared with OCAv3 RNA panel. Relative distribution of reads between the two NGS assays is consistent across fusion targets.

Figure 5. Distribution of mapped reads across different assay types

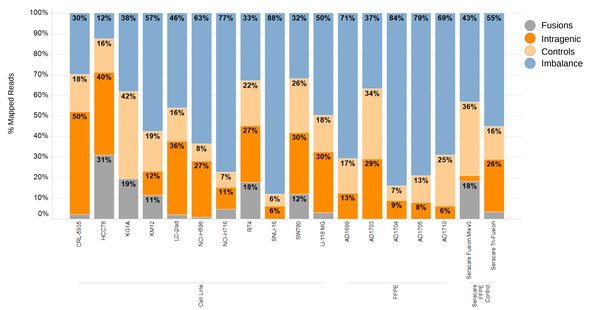


Figure 5: Histogram showing abundance of reads as % Mapped Reads across four major classes of RNA target assay types on OCA Plus assay. Fusion: Targeted fusions with defined breakpoint; Intragenic: intragenic fusion events and associated splice variants (e.g. exon-skipping), Controls: Gene expression control assays for normalization and QC, Imbalance: Assays supporting de-novo (i.e. partner agnostic) fusion detection in six driver genes. A mix of 11 cell-lines, five FFPEs and two commercially available controls were tested with two replicates of each test sample. % Mapped Reads across assay types vary primarily by the gene-fusion present in the sample. Imbalance assays are expected to take up a considerable proportion of mapped reads as they represent the expression of the underlying normal genes.

Figure 6. Fusion detection in fusion positive lung cancer cell-lines

A

	Fusion	Cell line
1	Total Lung RNA	Ambion
2	EML4-ALK.E6A20	CRL-5935
3	CCDC6-RET.C1R12	LC-2/ad
4	SLC34A2-ROS.S4R32 (Limited)	HCC78
5	GOPC-ROS1.G7R36	U-118 MG
6	FGFR3-TACC3.F17Intron17T4	RT4
7	FGFR3-BAIAP2L1.F17B2.COSF1346	SW780
8	FGFR1OP2-FGFR1	KG1A
9	CD44-FGFR2.C1F3	SNU-16
10	FGFR2-COL14A1	NCI-H716
11	TPM3-NTRK1	KM12

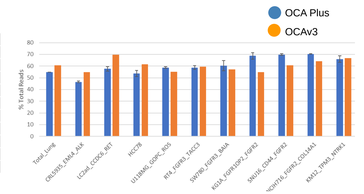


Figure 6A: Histogram showing mapped reads across test samples (x-axis). Positive signal is displayed on y-axis as percentage of the total reads from the samples. Eleven fusion positive lung cancer cell lines were tested along with a Total Lung RNA samples. Expected fusions in each test sample are listed in the table to the left. Detection sensitivity and mapped read fraction from OCA Plus assay was also compared with OCAv3. Relative distribution of reads is consistent between the two RNA assays across test samples

B

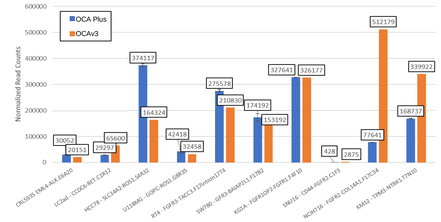


Figure 6B. Histogram show positive detection the expected fusions across test samples (x-axis). Positive signal is displayed on y-axis as percentage of the total reads from the samples (Figure 6A) and as reads normalized to total mapped reads (Figure 6B). Eleven fusion positive lung cancer cell lines were tested along with a Total Lung RNA samples. Expected fusions in each test sample are listed in the table to the left. Detection sensitivity from OCA Plus assay was also compared with OCAv3 Relative distribution of reads is consistent between the two RNA assays across test samples. Figure 6B also shows relative abundance of fusion reads from each driver genes.

CONCLUSIONS

A comprehensive NGS assay was developed to support clinical research in oncology for detecting relevant RNA structural alterations from solid tumor FFPEs. Minimal input material requirement and rapid sample to report time will have a high impact on clinical research.

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ACKNOWLEDGEMENTS

Patients and their families for donation of samples to scientific studies and for public access.

TRADEMARKS/LICENSING

Conflict of Interest: The authors are employees of Thermo Fisher.

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