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Development of a Targeted NGS Oncology Assay for Detection and Reporting Comprehensive Genomic Profiling

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INTRODUCTION

Next-generation sequencing (NGS) is a research tool that allows for the detection of cancer-associated somatic mutations, focal copy number aberrations, and gene fusions.[1] Current scientific and clinical research indicates these genomic variants may be associated with favorable or unfavorable responses to specific targeted therapeutic regimens.[2,3,4] The recent emergence of immunotherapeutic agents that enable the immune system to destroy cancer cells has driven research to identify additional biomarkers that may be associated with therapeutic response to these agents. Speculation that immune cells are recruited to the tumor by the presentation of mutant antigens[5] has led to the investigation of several biomarkers associated with increased somatic mutation rates. Comprehensive sequencing of the genes involved in DNA repair pathways may identify variants that lead to deficiencies in these pathways. These deficiencies may manifest themselves as microsatellite instability[6] (MSI), the extension of small repetitive sequence elements within the genome, or as an increase in overall tumor mutational burden (TMB)[7], an estimate of the total number of mutations observed in a tumor sample, expressed in mutations/Megabase of DNA.

We developed an NGS assay for FFPE tissue samples that can detect a variety of DNA variants. Variants detected by the assay have been associated with response to targeted therapies and immune checkpoint inhibitors.[2,3,6,7] This assay covers over 500 genes, including several target genes that have been associated with oncology clinical research, and requires low amounts of input FFPE material. This assay-to-report workflow that allows streamlined utilization for clinical research.

MATERIALS AND METHODS

Gene content was prioritized based on variant prevalence in solid tumors. To support robust TMB estimation, additional genomic regions were added in order to bring the coding sequence footprint to >1MB. To enable MSI status assessment, coverage of a diverse set of microsatellites throughout the genome was added. The MSI analysis solution makes use of in-sample standards incorporated as internal references.

The assay uses Ion AmpliSeq™ technology with automated templating on the Ion Chef™ System and sequencing on the Ion Torrent GeneStudio™ S5 sequencing platform. An automated variant calling and TMB reporting workflow is provided within Ion Reporter™ Software.

RESULTS

Over 500 genes with DNA based alterations and over 50 RNA fusion drivers are included in the assay. Of the genes measured for DNA alterations, 169 cover important cancer hotspots, 333 cover copy number variants (CNV), and 227 genes have full coding sequence (CDS) coverage for detection of truncating variants. The total genomic coverage of the assay is 1.50MB with 1.1MB of exonic sequence, to support high-confidence TMB estimation. A diverse set of microsatellite markers targeting MSI locations comprising of mono- and di-nucleotide repeats ranging from 7 to 34 bp are included for MSI status assessment. RNA content for the assay is more fully described in poster ST091 “Development of a comprehensive next-generation sequencing assay for gene-fusions detection in solid tumors”.

FFPE tumor samples from a variety of tissue types were sequenced using the assay. The assay displays high (>95%) uniformity and consistent read depth (>2200x) to support robust variant calling at low allele frequency. An excellent variant calling (SNV/INDEL) performance was observed, with sensitivity ranging from 98%-100% and PPV ranging from 88%-100%, depending upon the variant type. CNV detection sensitivity and specificity were 89%-99% and 100%, respectively. In-silico TMB assessment using publicly available whole-exome cancer sequencing data resulted in a correlation of $R^2 > 0.90$ (0-40 mut/mb) in pan-cancer and specific cancer types including lung, colorectal and melanoma. MSI test performance was assessed using 192 samples with known MSI status. An overall performance of 96% sensitivity and 99% specificity was observed.

Figure 1. Summary of the Assay Content

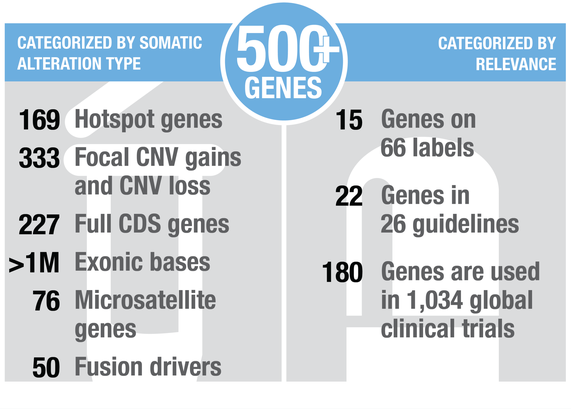
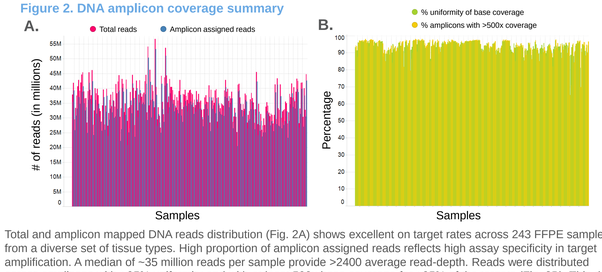


Table 1. Oncomine Comprehensive Assay Plus Cancer Gene Targets

CNV gain genes						Full CDS coverage								
	Hotspot genes													
CNV gain only	CNV gain and hotspot			Hotspot only		CNV loss								CDS only
AIRC81	ABL1	FGFR4	PIK3C2B	ACVR1	MYOD1	ABRAXAS1	CDKN1A	FANCA	MAP3K1	POLE	SOX9	CALR
CTNND2	ABL2	FLT3	PIK3CA	ATP1A1	NSD2	ACVR1B	CDKN1B	FANCC	MAP3K4	POT1	SPEN	CIITA
DDR1	AKT1	FLT4	PIK3CB	AURKB	NTSC2	ACVR2A	CDKN2A	FANCD2	MAPK8	PPM1D	STAG2	CYP2D6
EMSY	AKT2	FOXA1	PIK3R2	BCR	NTRK2	ADAMTS12	CDKN2B	FANCE	MEN1	PPP2R2A	STK11	ERCC5
FGF3	AKT3	GATA2	PIM1	BMP5	NUP93	ADAMTS2	CDKN2C	FANCF	MGA	PRDM1	SUFU	FAS
FGF4	ALK	GNAS	PLCG1	BTK	PAX5	AMER1	CHEK1	FANCG	MLH1	PRDM9	TAP1	ID3
FGF9	AR	H3F3A	PPP2R1A	CACNA1D	PIK3CD	APC	CHEK2	FANCI	MLH3	PRKAR1A	TAP2	KLHL13
FGF19	ARAF	H3F3B	PPP6C	CD79B	PIK3CG	ARHGAP35	CIC	FANCL	MRE11	PTCH1	TBX3	MTUS2
FGF23	AURKA	IDH2	PRKACA	CSF1R	PTPRD	ARID1A	CREBBP	FANCM	MSH2	PTEN	TCF7L2	PSMB8
FYN	AURKC	IKBKB	PTPN11	CTNNB1	RGS7	ARID1B	CSMD3	FAT1	MSH3	PTPRT	TET2	PSMB9
GLI3	AXL	IL7R	PXDNL	CUL3	RHOA	ARID2	CTCF	FBXW7	MSH6	RAD50	TGFBR2	PSMB10
IGF1R	BCL2	KDR	RAC1	CYSLTR2	RPL10	ARID5B	CTLA4	FUBP1	MTAP	RAD51	TNFAIP3	RNASEH2C
MCL1	BCL2L12	KIT	RAF1	DDR2	SIX1	ASXL1	CUL3	GATA3	MUTYH	RAD51B	TNFRSF14	RPL5
MDM2	BCL6	KLF5	RARA	DROSHA	SIX2	ASXL2	CUL4A	GNA13	NBN	RAD51C	TP53	RPL22
MYCL	BRAF	KRAS	RET	E2F1	SNCAIP	ATM	CUL4B	GPS2	NCOR1	RAD51D	TP63	RUNX1T1
RPS6KB1	CARD11	MAGOH	RHEB	EPAS1	SOS1	ATR	CYLD	HDAC2	NF1	RAD52	TPP2	SDHC
RPTOR	CBL	MAPK1	RICTOR	FGF7	SOX2	ATRX	CYP2C9	HDAC9	NF2	RAD54L	TSC1	SOCS1
YAP1	CCND1	MAP2K1	RIT1	FOXL2	SRSF2	AXIN1	DAXX	HLA-A	NOTCH1	RASA1	TSC2	STAT1
YES1	CCND2	MAX	ROS1	FOXO1	STAT5B	AXIN2	DDX3X	HLA-B	NOTCH2	RASA2	USP9X	STAT4
	CCND3	MDM4	SETBP1	GLI1	TAF1	B2M	DICER1	HNF1A	NOTCH3	RB1	VHL	TMEM132D
	CCNE1	MECOM	SF3B1	GNA11	TGFBR1	BAP1	DNMT3A	INPP4B	NOTCH4	RBM10	WT1	UGT1A1
	CDK4	MEF2B	SLCO1B3	GNAQ	TRRAP	BARD1	DOCK3	JAK1	PALB2	RECQL4	XRCC2	ZBTB20
	CDK6	MET	SMC1A	HIF1A	TSHR	BCOR	DPYD	JAK2	PARP1	RNASEH2A	XRCC3	
	CHD4	MITF	SMO	HIST1H1E	WAS	BLM	DSC1	JAK3	PARP2	RNASEH2B	ZFHX3	
	DDR2	MPL	SPOP	HIST1H2BD		BMPR2	DSC3	KDM5C	PARP3	RNF43	ZMYM3	
	EGFR	MTOR	SRC	HIST1H3B		BRCA1	ELF3	KDM6A	PARP4	RPA1	ZRSR2	
	EIF1AX	MYC	STAT3	HRAS		BRCA2	ENO1	KEAP1	PBRM1	RUNX1		
	ERBB2	MYCN	STAT6	IDH1		BRIP1	EP300	KMT2A	PDCD1	SDHA		
	ERBB3	MYD88	TERT	IL6ST		CASP8	EPCAM	KMT2B	PDCD1LG2	SDHB		
	ERBB4	NFE2L2	TOP1	IRF4		CBFB	EPHA2	KMT2C	PDIA3	SDHD		
	ESR1	NRAS	TSHR	IRS4		CD274	ERAP1	KMT2D	PGD	SETD2		
	EZH2	NTRK1	U2AF1	KCNJ5		CD276	ERAP2	LARP4B	PHF6	SLX4		
	FAM135B	NTRK3	USP8	KLF4		CDC73	ERCC2	LATS1	PIK3R1	SMAD2		
	FGFR1	PCBP1	XPO1	KNSTRN		CDH1	ERCC4	LATS2	PMS1	SMAD4		
	FGFR2	PDGFRA	ZNF217	MAP2K2		CDH10	ERRFI1	MAP2K4	PMS2	SMARCA4		
	FGFR3	PDGFRB	ZNF429	MED12		CDK12	ETV6	MAP2K7	POLD1	SMARCB1		

Figure 2. DNA amplicon coverage summary



Total and amplicon mapped DNA reads distribution (Fig. 2A) shows excellent on target rates across 243 FFPE samples from a diverse set of tissue types. High proportion of amplicon assigned reads reflects high assay specificity in target amplification. A median of ~35 million reads per sample provide >2400 average read-depth. Reads were distributed across amplicons with >95% uniformity and with at least 500x base coverage for >95% of the targets (Fig. 2B). This data indicates the assay will be capable of detecting variants with low allele frequencies (>5%)

Table 2. Representative variant calling performance using commercially available controls

SNV

		TP	FP	FN	%Sen.	%PPV
AOHC Seraseq	Hotspot	1243	0	17	98.7	100
		34	0	0		
AOHC Seraseq	De-novo	938	4	16	98.32	99.5
		na	na	na		

INDEL

		TP	FP	FN	%Sen.	%PPV
AOHC Seraseq	Hotspot	53	0	1	98.3	100
		4	0	0		
AOHC Seraseq	De-novo	9	2	0	100	88.2
		6	0	0		

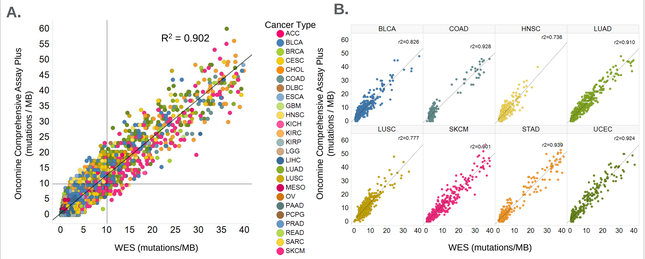
To assess small-variant calling performance we performed sequencing experiments using the AcroMetrix™ Oncology Hotspot Control® (AOHC) that contains hundreds of SNVs and tens of indels covered, and Seraseq™ Tri-Level Tumor Mutation DNA Mix v2 containing tens of SNV/INDELs by Oncomine Comprehensive Assay Plus. Nine replicates of AOHC and two replicates of Seraseq™ were sequenced. Performance was calculated as %sensitivity and %PPV (positive predictive value) in detection and measured independently for hotspot and de-novo variants. SNV hotspot and de-novo performance was assessed at variant allele frequency LOD of >= 5%, INDEL hotspots at LOD of 10% and de-novo INDEL at LOD of 20%.

Table 3. Representative copy-number variant calling performance

	TP	FN	TN	Sensitivity	Specificity
CNV Gain	33	1	12	97%	100%
CNV loss	18	2	12	90%	

Copy number detection performance was assessed against known copy-number status from internally characterized solid tumor FFPE samples (n=5) and commercially available ATCC™ (n=3) and Coriell Institute for Medical Research (n=14) cell lines. For the FFPE samples, known truth was determined using an orthogonal Oncomine NGS assay; for ATCC™ cell-lines, known truth was derived from the literature and publicly available genotyping expression arrays. For Coriell cell-lines, positive calls were inferred using the reported cytogenetic abnormalities and aneuploidy status. Gene-level copy-gain is called when the lower confidence interval (CI) of the copy number estimate is >4; copy-loss is called when the CI upper bound <2. For specificity calculations, wild-type copy-number estimates (autosomal copy number within (1.8, 2.2)) were considered as negative, while CN estimates outside that range were called positive.

Figure 3. In-Silico comparison of the assay TMB with Whole Exome Sequencing (WES)



Scatter plots showing the correlation between the targeted assay (y-axis) and WES (x-axis) mutation counts. WES data was downloaded from TCGA MC3[9]. In-silico analysis was performed to characterize TMB performance of the targeted sequencing assay. Rate of nonsynonymous somatic mutations was computed for WES TMB. Mutations were limited to the targeted assay for predicted TMB. WES TMB strongly correlated ($R^2 > 0.9$) with the assay TMB in pan-cancer analysis (Fig. 3A). Figure 3B displays similar distribution for selected cancer types. Strong correlation ($R^2 > 0.9$) was observed for colorectal adenocarcinoma (COAD), lung adenocarcinoma (LUAD), skin cutaneous melanoma (SKCM) and stomach adenocarcinoma (STAD).

Figure 4. Oncomine Comprehensive Assay Plus TMB Correlates with Whole Exome Sequencing (WES)

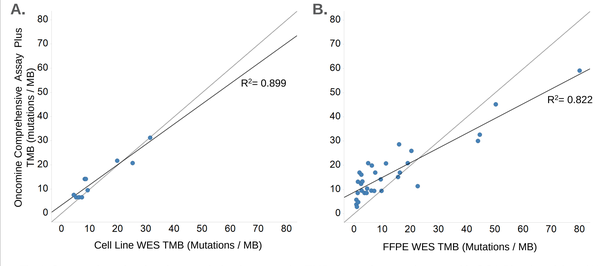


Fig 4. Scatter plots showing the correlation between the TMB scores for samples run with OCA Plus (y-axis) and Whole-Exome Sequencing (WES, x-axis). A. Correlation between TMB scores calculated after sequencing using OCA Plus versus provided WES TMB values for ten cancer cell lines from Friends of Cancer Research[10] (FOCR). B. Correlation between TMB scores calculated after sequencing using OCA Plus for 35 FFPE tumor samples, versus TMB calculated based on WES for the tumor samples and matched normals.

Figure 5. OCA Plus TMB Estimates for FFPE Samples Correlate with MSI Status

Oncomine Comprehensive Assay Plus TMB scores can separate microsatellite instable (MSI) samples from microsatellite stable (MSS) samples. For this study, 20 colorectal FFPE tumors previously typed for microsatellite instability (MSI) were sequenced using the Oncomine Comprehensive Assay Plus, which was used to generate TMB estimates. TMB scores were significantly different between the 12 MSI and 8 MSS samples (student t-test p-value = 1.13E-06).

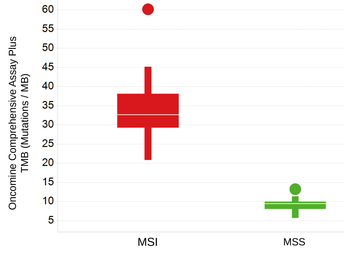


Table 4: Summary of the MSI test performance using reference tests

OCA Plus Results	Reference Test Results	
	Positive	Negative
Positive (MSI-High)	TP=23	FP=2
Negative	FN=1	TN=166
Sensitivity	**96%**	
Specificity	**99%**	

The Oncomine Comprehensive Assay Plus provides a test for assessing microsatellite status in tumor samples by utilizing variation in the semiconductor-based sequencing signals. The MSI algorithm measures the amount of change in the homopolymer length of MSI target markers present on the panel relative to the known control. An overall MSI score is calculated and MSI status is determined using the upper and lower bound thresholds of the MSI score. For testing, a set of 192 samples (FFPE and cell-lines) were selected. These samples came from multiple tissue types (including colorectal, gastric, and endometrial cancer) and had known MSI status. For HapMap cell-lines and known normal tissue samples, a known truth of MSS was assumed. Samples were sequenced using OCA Plus and MSI score and status were reported by the DNA software workflow. An excellent performance with overall sensitivity of 96% and specificity of 99% was observed.

Figure 6. Schematic flow-diagram of the complete workflow

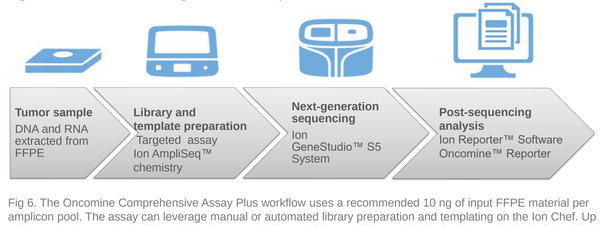


Fig 6. The Oncomine Comprehensive Assay Plus workflow uses a recommended 10 ng of input FFPE material per amplicon pool. The assay can leverage manual or automated library preparation and templating on the Ion Chef. Up to four samples can be multiplexed on the Ion 550 chip to achieve sufficient read depth. The analysis pipeline utilizes a custom variant calling optimized for solid-tumor samples, quantification of somatic mutations for TMB, and a novel algorithm that leverages the unique signal processing properties inherent in semi-conductor sequencing for MSI detection. The analysis solution is optimized as a tumor sample only workflow for genomic profiling of clinical research samples.

CONCLUSIONS

A targeted NGS assay was developed to support comprehensive genomic profiling and routine clinical research in oncology. The assay includes cancer relevant gene content to support accurate reporting multiple variant types. The design (optimized genomic footprint, CDS coverage and included MSI markers) and optimized informatics workflow support characterization of mutational signatures including SNV, INDEL and CNVs, and measures important immuno-oncology biomarkers such as TMB and MSI with a strong correlation with orthogonal methods.

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