Analytical validation of the Oncomine Pan-Cancer Cell-Free Assay

Introduction

Advances in next-generation sequencing (NGS) technologies have enabled highly sensitive detection of genetic variation in a background of wild-type alleles, allowing researchers to obtain information on solid tumors by detection of low-frequency variants present in circulating tumor DNA and RNA. Assessing tumor-derived somatic variants from blood plasma has the potential to provide minimally invasive tumor profiling, decreased testing cost relative to traditional tissue biopsy [1], and rapid turnaround time. There is increasing evidence [2-6] for the clinical utility of cancer-derived somatic mutation detection in the management of advanced solid tumors, with National Comprehensive Cancer Network (NCCN) non-small cell lung cancer (NSCLC) guidelines (version 2.2019) now addressing the use of plasma-based cell-free and circulating tumor nucleic acids testing in certain clinical circumstances. These cases include when an invasive tissue biopsy is unable to be obtained or if there is insufficient material for molecular characterization to be performed using the primary biopsy. Importantly, solid tumor biopsy specimens that are suitable for genomic profiling are unable to be obtained from an estimated ~20% of NSCLC patients with EGFR tyrosine kinase inhibitor (TKI) resistance [1], highlighting the need for alternative strategies to assess molecular drivers of resistance in advanced solid tumors.

There are a wide range of gene alterations that impact therapy across selection indications, and molecular testing for these targets is important for both selection of appropriate targeted therapies and avoidance of therapies that are unlikely to provide clinical benefit. Thus, management of advanced solid tumors is becoming increasingly reliant on molecular testing of multiple genes from a single sample.

The Ion Torrent™ Oncomine™ Pan-Cancer Cell-Free Assay is a massively parallel, high-throughput, next-generation sequencing assay, designed to simultaneously detect somatic DNA variants, copy number variants, and gene fusions in cell-free total nucleic acid (cfTNA) across 52 unique genes. The gene content was selected based on curation of available peer-reviewed literature, clinical trial information, and both NCCN and European Society for Medical Oncology (ESMO) guidelines—driving inclusion of genes for NSCLC: *EGFR* (including both *EGFR* TKI–sensitizing and acquired resistance mutations), *ALK* fusions, *ROS1* fusions, and *BRAF* mutations. In addition, the assay enables highly sensitive detection of mutations related to acquired resistance and mechanisms of action of targeted drugs for NSCLC, such as *EGFR* T790M mutations, kinase domain mutations in *ALK*, *MET* amplification, and *ERBB2* amplification.

Here we describe an analytical validation approach to assess the performance characteristics of the Oncomine Pan-Cancer Cell-Free Assay using both commercially available, pre-characterized cell-free reference materials as well as clinical plasma samples from three solid tumor indications procured from a commercial biorepository.

Materials and methods

Clinical research whole-blood specimens (n = 73) consisting of healthy (10), breast cancer (9), colorectal cancer (CRC) (32), and NSCLC (22) subjects (Figure 1) were collected in K_2EDTA tubes, and plasma was separated by centrifugation within 8 hours of collection. cfTNA was isolated from 4 mL of plasma using the Applied Biosystems™ MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit on the Thermo Scientific™ KingFisher™ Flex system. Extracted cfTNA was quantitated using the Invitrogen™ Qubit™ dsDNA HS Assay Kit. Pre-characterized cell-free reference materials were purchased from SeraCare (Milford, MA). Libraries were prepared using 20.8 ng cfTNA input following the Oncomine Pan-Cancer Cell-Free Assay user guide. Templating and sequencing were performed using the Ion 550™ Kit on the Ion Chef™ and Ion GeneStudio™ S5 systems. Alignment to human genome assembly (also known as hg19) and variant calling were performed using Torrent Suite™ and Ion Reporter™ software version 5.10, respectively.

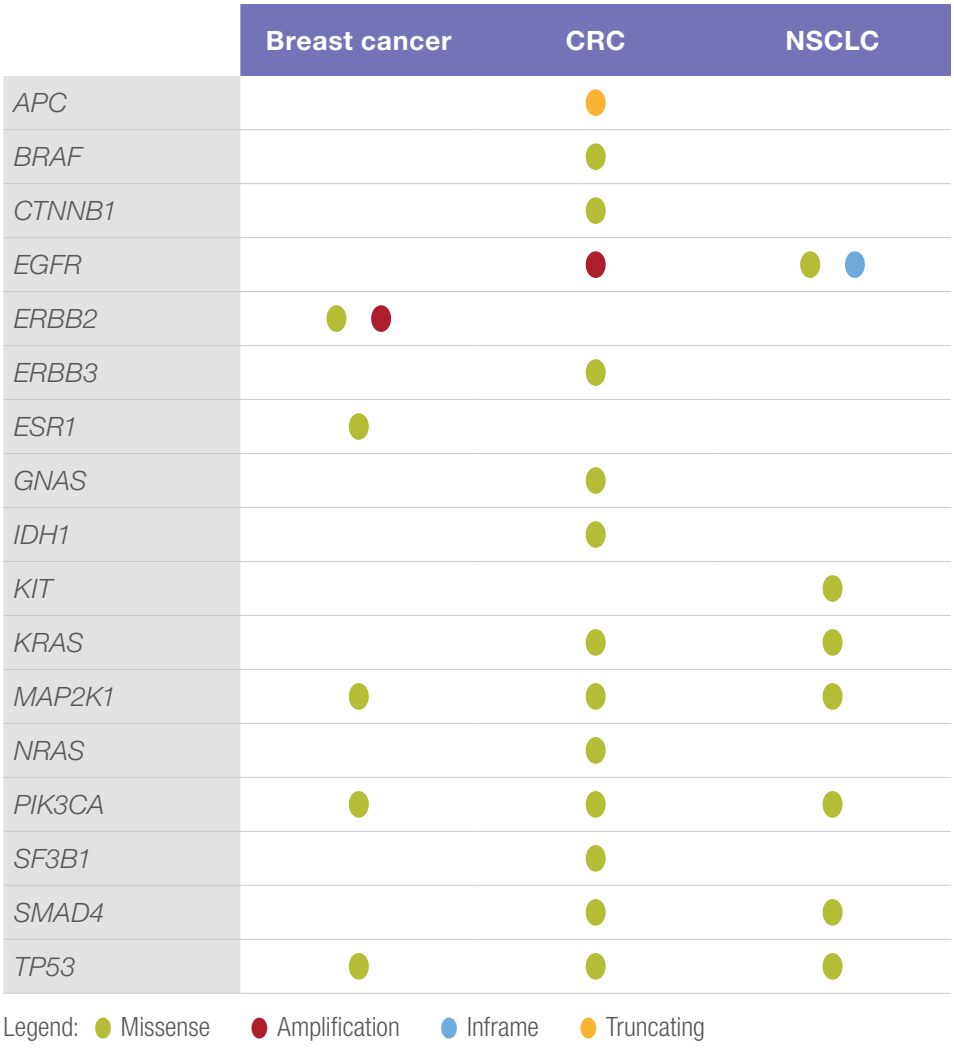

Figure 1. Mutational landscape across sample sets.

Analytical validation design

This validation was performed in a College of American Pathologists (CAP)–accredited and Clinical Laboratory Improvement Amendment (CLIA)–regulated laboratory. It was designed according to the joint guidelines of Americal Society of Clinical Oncology (ASCO) and CAP on circulating tumor analysis [1] and CAP-accredited Molecular Pathology Assay Validation requirements checklist items (MOL.30785, Molecular Pathology Checklist 08.22.2018). The Oncomine Pan-Cancer Cell-Free Assay detects four major classes of alterations: single-nucleotide variant (SNV), insertion or deletion (indel), copy number variation (CNV), and gene fusions. Analytical validation studies were performed for SNVs, indels, CNVs, and fusions independently, with analytical sensitivity (limit of detection (LOD)), specificity, accuracy, and precision evaluated for each variant class. Performance characteristics were established using commercially available, pre-characterized control materials followed by verification using clinical samples; the analytical sensitivity and precision were verified in clinical specimens for SNVs, indels, and CNVs, and analytical specificity was assessed using cfTNA isolated from healthy subjects.

Analytical sensitivity—(LOD)

The sensitivity of an assay is the percent of true positives at a given allele frequency that are detected by the assay. Here, the analytical sensitivity of the assay was determined using commercially available materials containing pre-characterized variants across allele frequencies (AFs) for SNVs, indels, CNVs, and fusions. Each sample was tested with the Oncomine Pan-Cancer Cell-Free Assay to evaluate variant detection status at decreasing alteration frequencies, with each level tested in six replicates by two operators. The LOD values for SNVs, indels, and fusions were determined by calculating the sensitivity of variant detection at each input level. The LOD for CNVs was calculated following the classical approach protocol for CNVs in Clinical and Laboratory Standards Institute (CLSI) document EP17-A. The LOD was verified in samples for SNV, indel, and CNV variant classes, but was not verified in samples for fusions due to limitations on sample availability.

Analytical sensitivity—SNVs and indels

The LOD was assessed in fragmented, pre-characterized control specimens and verified on orthogonally genotyped clinical research specimens (Table 1). Orthogonal genotyping was performed in external CLIA-regulated laboratories. The AFs evaluated were 5%, 2.5%, 1%, 0.5%, and 0.1%. The analytical sensitivity at the 0.5% AF is >99% (99.9–100% at 95% confidence interval) and the analytical sensitivity at 0.1% AF is 80% (75–85% at 95% confidence interval).

Analytical sensitivity—CNVs

The analytical sensitivity for CNVs was determined using cfTNA from both healthy subjects and pre-characterized commercially available controls, and was subsequently verified on clinical research specimens. To determine the LOD for CNV calls, the CLSI EP17-A classical approach was applied. Specimens from 10 healthy subjects were analyzed to assess variability in CNV calls in copy number–neutral specimens, and replicate testing (n = 6 replicates, 2 operators) of pre-characterized control specimens was carried out to characterize variability in CNV calls in low-positive samples (defined as a fold change of less than 2). Per CLSI EP17-A guidelines, the LOD is defined as the value where 95% of measurements in a true positive sample are detectable above subjects without CNV. The LOD was then verified in orthogonally confirmed clinical research samples. Table 2 outlines the calculated CNV LOD as well as the clinical research sample–verified analytical sensitivity for CNV detection. The LOD for CNV calling was set at the clinical research sample–verified fold-change value. The analytical sensitivity is >99.9% above the clinical research sample–validated 1.34-fold change (99.9–100% at 95% confidence interval).

Table 1. Analytical sensitivity—SNVs and indels.

Sample description	AF	True positive	False negative	Sensitivity
SeraCare Seraseq™ ctDNA Complete Mutation Mix (Cat. No. 0710-0528)	5%	78	0	100%
SeraCare Seraseq™ ctDNA Complete Mutation Mix (Cat. No. 0710-0529)	2.5%	78	0	100%
SeraCare Seraseq™ ctDNA Complete Mutation Mix (Cat. No. 0710-0530)	1%	78	0	100%
SeraCare Seraseq™ ctDNA Complete Mutation Mix (Cat. No. 0710-0531)	0.5%	78	0	100%
Fragmented Thermo Scientific™ AcroMetrix™ Oncology Hotspot material (custom order)	0.1%	404	100	80%
LOD verification in orthogonally confirmed clinical research tissue samples				
Sample type	Variant	Observed AF		
Plasma—stage IV colorectal cancer	*KRAS* p.G12D	0.09%		
Plasma—stage IV colorectal cancer	*NRAS* p.Q61K	0.13%		
Plasma—stage IV colorectal cancer	*KRAS* p.G12D	0.12%		

Table 2. Analytical sensitivity—CNVs.

Gene	Mean fold change in healthy subjects	Mean fold change in low-positive samples	CLSI EP17-A LOD
ERBB2	**1.06 (SD = 0.03)**	1.80 (SD = 0.03)	1.20
MET	**1.08 (SD = 0.03)**	1.88 (SD = 0.02)	1.18
Combined	**1.07 (SD = 0.03)**	1.84 (SD = 0.025)	1.19
LOD verification in orthogonally confirmed clinical research samples			
Sample type	Variant	Fold change	
Plasma—stage IV colorectal cancer	*EGFR* amplified	1.45	
Plasma—stage II breast cancer	*ERBB2* amplified	1.34	

Analytical sensitivity—fusions

The analytical sensitivity of fusion detection was determined by spike-in studies where pre-characterized RNA containing 13 unique fusions were pooled into cfTNA of healthy subjects at decreasing mean fusion fraction, resulting in 4 dilution points. Each dilution point was run in 6 replicates. The analytical sensitivity is >99.9% above 0.4% fusion frequency (corresponding to a mean of 34 fusion copies across the 13 fusions as determined by droplet digital PCR; >99.9–100% at 95% confidence interval). The relationship between input level and the number of unique molecular families detected is illustrated in Figure 2 and analytical sensitivity of fusion detection is summarized in Table 3. No clinical research samples were available to verify the analytical sensitivity due to limitations on availability of gene fusion–positive samples.

Analytical accuracy

The accuracy of an assay is a measure of overall assay performance that takes both positive and negative data into account. The accuracy of the Oncomine Pan-Cancer Cell-Free Assay was assessed using commercially available, pre-characterized control materials engineered to mimic circulating tumor nucleic acids, plasma specimens in clinical research with available genotype-matched solid tumor tissue, and samples from healthy subjects. Only those clinical research specimens with variants detected for which corresponding genotype-matched tumor tissue results were available were included in the accuracy study. A representative approach was taken where a subset of alterations detected by the assay comprising the main variant classes (SNV, indel, CNV, and gene fusion) were compared to results obtained via orthogonal methods (droplet digital PCR for detecting SNVs, indels, and CNVs, and qPCR for detecting fusions; performed in an external laboratory). Orthogonal confirmation assays with tissue were performed at external laboratories.

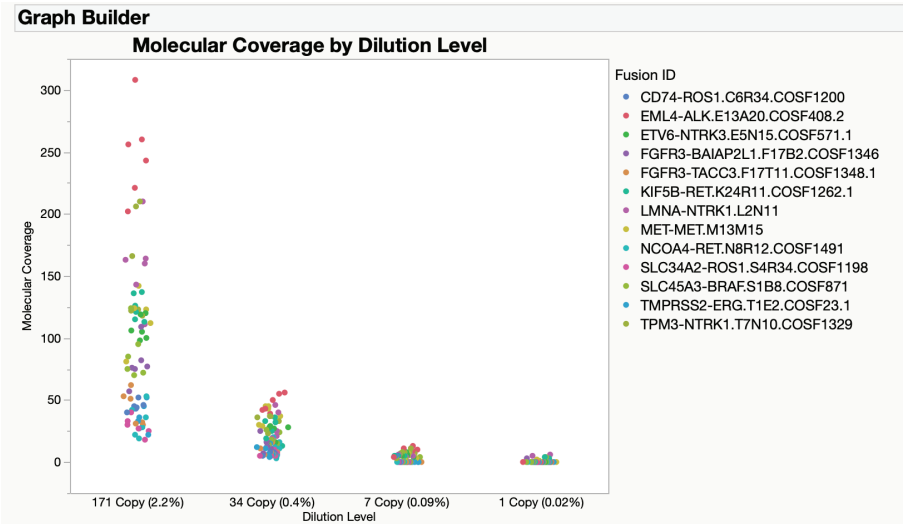

Figure 2. Unique molecular coverage across fusion fraction dilutions.

Table 3. Analytical sensitivity—fusions.

Dilution	Mean fusion copies (ddPCR)	True positive	False negative	Sensitivity
2.2% fusion fraction	171	78	0	100%
0.4% fusion fraction	34	78	0	100%
0.09% fusion fraction	7	43	35	55.1%
0.02% fusion fraction	1	6	78	7.8%

Analytical accuracy—SNVs and indels

The overall accuracy of the Oncomine Pan-Cancer Cell-Free Assay for SNVs and indels detected above the 0.1% AF is 99.7% (99.6–99.8% at 95% confidence interval). Technical replicates of pre-characterized control materials were incorporated into the accuracy characterization to further evaluate assay performance. Orthogonal genotyping data from clinical research specimens was obtained from external laboratories. All orthogonally confirmed SNV and indel variants (n = 17) in clinical research tissue samples were detected in subjects with stage IV colorectal cancer.

Analytical accuracy—CNVs and fusions

The overall accuracy of the Oncomine Pan-Cancer Cell-Free assay for CNVs detected above 1.34-fold change is 99.8% (99.3–100% at 95% confidence interval). The overall accuracy of the assay for fusions detected above 0.4% (an average of 34 fusion copies) is >99.9% (99.9–100% at 95% confidence interval). The technical replicates of pre-characterized control materials were incorporated into the accuracy characterization to further evaluate assay performance. Orthogonal CNV data from clinical research specimens was obtained from external laboratories. Accuracy studies for fusion detection did not include clinical research specimens due to limitations on sample availability.

Analytical specificity

The analytical specificity for each variant class was determined by testing 10 healthy-subject plasma samples (5 male, 5 female, age range 21–68 years old). The results for variants detected above the established LOD are summarized in Table 4.

Precision

The precision of the assay was determined by evaluating variability in variant detection both within a run (repeatability) and across runs, operators, and sequencing instruments (reproducibility). Precision was determined for each variant class using a combination of pre-characterized control specimens and 35 clinical research specimens with variants spanning the reportable range of the assay. Analysis was performed by evaluating pairwise concordance of variant detection within a run (single operator) and across runs (multiple operators and instruments). The precision of the assay was characterized with SNVs and indels above the 0.5% AF, CNVs above 1.34-fold amplification, and fusions above 0.4% fusion fraction.

Intra-run and inter-run pairwise concordance (repeatability and reproducibility, respectively) for pre-characterized controls are described in Table 5. A total of 16 pairwise comparisons of technical replicates for SNVs/indels and 8 pairwise comparisons for CNVs/fusions were performed to characterize repeatability. Twelve pairwise comparisons of technical replicates for SNVs/indels and six pairwise comparisons for CNVs/fusions were performed to characterize reproducibility.

Inter-run pairwise concordance (reproducibility) for 35 clinical research specimens is described in Table 6.

Table 4. Analytical specificity for different classes of variants.

Accession number	Median molecular coverage	FP* (SNVs/indels)	FP (CNVs)	FP (fusions)	Specificity (SNVs/indels)	Specificity (CNVs)	Specificity (fusions)
cfTNA-001	1,779	0	0	0	100.00%	100.00%	100.00%
cfTNA-002	5,042	0	0	0	100.00%	100.00%	100.00%
cfTNA-003	4,289	0	0	0	100.00%	100.00%	100.00%
cfTNA-004	1,874	2	0	0	99.79%	100.00%	100.00%
cfTNA-005	3,777	1	0	0	99.90%	100.00%	100.00%
cfTNA-006	2,528	0	0	0	100.00%	100.00%	100.00%
cfTNA-007	4,051	1	0	0	99.90%	100.00%	100.00%
cfTNA-008	1,813	0	0	0	100.00%	100.00%	100.00%
cfTNA-009	3,536	1	0	0	99.90%	100.00%	100.00%
cfTNA-010	2,116	2	0	0	99.79%	100.00%	100.00%
				Average (95% CI)	**99.9%** (99.88–99.98%)	**>99%** (>99–100%)	**>99%** (>99–100%)

* FP: false positives.

Table 5. Assay precision in pre-characterized controls.

Variant classification	SNV/indel	CNV	Fusion
True positive	208	16	104
False positive	2	0	0
False negative	0	0	0
Repeatability (within run precision)	**98%**	**>99%**	**>99%**
Variant classification	**SNV/indel**	**CNV**	**Fusion**
True positive	156	12	78
False positive	1	0	0
False negative	0	0	0
Repeatability (within run precision)	**99%**	**>99%**	**>99%**

Table 6. Assay precision in clinical research specimens.

Variant classification	SNV/indel	CNV	Fusion
Concordant cases	34	34	35
Discordant cases	1	1	0
Overall concordance (clinical samples)	**97%**	**97%**	**>99%**

Assay performance in clinical research specimens

To evaluate assay performance, we procured and screened 63 clinical research plasma specimens spanning three solid tumor indications: breast cancer (BC, n = 9), colorectal cancer (CRC, n = 32), and lung cancer (LC, n = 22). cfTNA was extracted from 4 mL plasma, resulting in an average nucleic acid yield of approximately 60 ng, well above the target input of 20.8 ng. Across the 63-sample cohort, 39 cases had at least one SNV and/or indel driver variant detected with a mean AF of 0.95% (range 0.07–11.3% AF). For the subset of circulating tumor variants detected that had genotype-matched solid tumor results from orthogonal methods (n = 17 SNVs, n = 2 CNVs), we observed 100% cross-tissue (plasma vs. solid tumor) concordance.

Evaluation of the mutational landscape in the CRC cell-free cohort (n = 32 cases; 26 stage IV, 4 stage III, 1 stage II, 1 stage I) provides insight into recurrently altered genes in cfTNA: we observed 11 oncogenic alterations (34%) in the *KRAS* codon 12/13, 11 deleterious *TP53* alterations (34%), 5 truncating *APC* mutations (16%), and 4 *PIK3CA* oncogenic alterations (13%). These four genes are also recurrently altered in large-scale solid tumor profiling projects such as The Cancer Genome Atlas (TCGA) CRC cohort. A similar analysis of our LC cohort (n = 22; 10 stage IV, 3 stage III, 3 stage II, 2 stage I, and 4 unknown stage) reveals 11 deleterious *TP53* alterations (34%), 3 oncogenic *EGFR* mutations (9% of cases, one case had dual *EGFR* alterations), and 2 deleterious *SMAD4* variants. *TP53* is commonly altered across tumor types in TCGA data, and *EGFR* alterations provide relevant insights in NSCLC per NCCN guidelines.

Taken together, the cfTNA yield obtained from clinical research samples and the associated cell-free mutational landscapes demonstrate the ability of the Oncomine Pan-Cancer Cell-Free Assay to generate meaningful data from plasma samples.

Summary and conclusions

We have presented an approach to analytically validate the Oncomine Pan-Cancer Cell-Free Assay in a CLIA-regulated, CAP-accredited laboratory. The validation is summarized in Table 7, demonstrating that the assay is a highly sensitive, specific, accurate, and precise method to simultaneously detect SNVs, indels, CNVs, and fusions in cfTNA.

Table 7. Validation summary of the Oncomine Pan-Cancer Cell-Free Assay.

Validation component	SNV/indel	CNV	Fusion
Analytical sensitivity	>99.9% at 0.5% AF 80% at 0.1% AF	>99% at >1.34- fold amplification	>99% at 0.4%
Analytical specificity	>99.9%	>99.9%	>99.9%
Accuracy	>99%	>99%	>99%
Repeatability	98%	>99%	>99%
Reproducibility	99% in reference materials 97% in clinical samples	99% in reference materials 97% in clinical samples	>99% in reference materials >99% in clinical samples
Positive predictive value	98.4% above 0.5% AF 93.7% above 0.1% AF	>99% above 1.34-fold amplification	>99% above 0.4%

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