Validation of a Comprehensive Next-generation Sequencing Liquid Biopsy Assay for Clinical Diagnostics and Clinical Trial Applications

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Introduction

Background: Next-generation sequencing of cell-free circulating solid tumor nucleic acid from blood samples, known as "liquid biopsy", provides a powerful non-invasive approach to detect solid tumor derived somatic variants in a massively parallel manner. It has rapidly become an invaluable choice of testing technologies for cancer diagnosis and therapy decisions when invasive tissue biopsy is inaccessible and/or inadequate for molecular characterization. This testing method is now being used to aid data collection for prospective clinical trials when tissue samples may be challenging to obtain.

Methods: In this study, we present the NGS-based NeoLAB® Solid Tumor Liquid Biopsy assay that was designed and validated specifically for liquid biopsy characterization. The assay was designed to test cell free DNA and cell free RNA from blood plasma for detection of single nucleotide variants (SNV), insertion/deletion (Indel), copy number variants (CNV), and gene fusions present in 52 most commonly mutated genes in cancers. The NeoLAB® Solid Tumor Liquid Biopsy assay comprehensively covers all actionable markers currently supported by evidence from FDA and EMA-approved drug labels, National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO) Guidelines, and global clinical trials.

Results: To implement this assay for clinical utility, we performed an analytical and clinical assay validation to establish the assay accuracy, specificity, sensitivity, repeatability, and reproducibility, with including pre-characterized reference controls, retrospective plasma samples from cancer patients, and prospectively-collected clinic blood specimen. Triplicates were included and testing was performed at different times and by different operators to assess the assay precision. Over 100 clinic blood specimens were collected in Streck cfDNA tubes from multiple trial centers, including treatment naïve phase III-IV patients with various solid tumor types (lung cancer, breast cancer, brain cancer, prostate cancer, kidney cancer, melanoma, etc) as well as heathy donors, processed within 7 days for plasma separation and total nucleotide acid (TNA) extraction, and assessed by the NGS assay for mutation detection. The identified mutations and wild-types were verified with orthogonal ddPCR. Assay sensitivity as low as 0.1% for SNV and Indel, 1.3 fold change for CNV, and 10 copies for gene fusion were observed, with near-perfect assay specificity (>99.99%) at these sensitivity levels.

Conclusion: A targeted NGS liquid biopsy assay was analytically and clinically validated under medical oversight, with a demonstrated rigor of the test by its high sensitivity/specificity and robust reproducibility. The validated assay has been utilized for clinical diagnostics and to support research and development efforts in clinical trials.

Assay Gene Content & Workflow

	Hotspot Genes			Suppressor Genes	Copy Number Variants	Gene Fusions
AKT1	FGFR1	KRAS	RET	APC	CCND1	ALK
ALK	FGFR2	MAP2K1	ROS1	FBXW7	CCND2	BRAF
AR	FGFR3	MAP2K2	SF3B1	PTEN	CCND3	ERG
ARAF	FGFR4	MET	SMAD4	TP53	CDK4	ETV1
BRAF	FLT3	MTOR	SMO		CDK6	FGFR1
CHEK2	GNA11	NRAS			EGFR	FGFR2
CTNNB1	GNAQ	NTRK1			ERBB2	FGFR3
DDR2	GNAS	NTRK3			FGFR1	MET
EGFR	HRAS	PDGFRA			FGFR2	NTRK1
ERBB2	IDH1	PIK3CA			FGFR3	NTRK3
ERBB3	IDH2	PTEN			MET3	RET
ESR1	KIT	RAF1			MYC	ROS1
Liquid Biopsy NGS Testing Specifications						
52 genes		272 amplicons		Extended coverage of TP53		12 CNVs
Single library from DNA and RNA		>900 hotspots, SNVs and indels		96 fusions		MET exon 14 skipping

Table 1. Gene content of the NeoLAB® Solid Tumor Liquid Biopsy assay. It covers all the actionable markers supported by drug labels and clinical guidelines for Hotspot, CNV and gene fusion

Relevant evidence for genes on the panel:
- Approved labels (FDA, EMA)
- Guidelines (NCCN, ESMO)
- Clinical Trials (global)

Bladder	Esophageal	Melanoma
Brain and CNS	Gastric	Ovarian
Breast	Head and Neck	Pancreatic
Cervical	Kidney	Prostate
Colorectal	Liver	Sarcoma
Endometrial	Lung	Thyroid

Table 2. Sample cancer types covered by the NeoLAB® Solid Tumor Liquid Biopsy assay

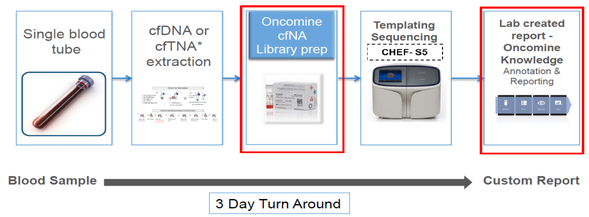

Figure 1. Workflow of the NeoLAB® Solid Tumor Liquid Biopsy assay. Human whole blood specimen went though plasma preparation, cfTNA extraction, library preparation using the Oncomine cfTNA library prep kit, and sequenced on Ion S5 with the 550 chip. Raw sequencing data was then analyzed for variants calling and reporting..

Analytical Assay Validation

Table 3. How Analytical Accuracy Results were calculated

Annotated variant	SNV/Indel		CNV		Gene Fusion
Libraries (n=25) prepared between 2 operators	10		5	5	10
# Positions queried	SNVs (n=79)	ndels (n=6)	CNV (n=12) CNV-positive (n=2), CNV-negative (n=10)	CNV (n=12) CNV-positive (n=2), CNV-negative (n=10)	Gene fusions (n=99) (gene fusion-positive (n=1) gene fusion-negative (n=98)
Concordance if expected variant or wildtype detected (VAF cutoff = 0.065% 1.2 ratio)	3 copies	3 copies		3 copies	3 copies
Accuracy acceptance criteria	≥95%		≥95%	≥95%	≥95%
Concordance achieved	100%		95%	100%	100%

Table 3. Analytical assay accuracy. The analytical accuracy of the NeoLAB® Solid Tumor Liquid Biopsy panel was assessed for all of the gene targets by testing a total of 25 libraries of pre-characterized reference cell-free DNA and cell-free RNA samples (10 for SNV/indel, 5 for CNV, and 10 for gene fusion) with annotated mutation status on at least one variant position.

Table 4. Analytical Assay Specificity

Specificity	SNV/Indel	CNV	Gene Fusion
Acceptance criteria	≥95%	≥95%	≥95%
Analytical specificity	100%	100%	100%
Clinical specificity (tumor patients and healthy donors)	100%	100%	100%

Table 4. Analytical assay specificity. The analytical specificity of the NeoLAB® Solid Tumor Liquid Biopsy panel was determined by testing both wild-type and annotated reference samples to determine concordance for each annotated position in every sample. The SNV, indel, CNV, and gene fusion specificity rate of 100% achieved the specificity acceptance rate of ≥95%.

Table 5. Analytical Assay Sensitivity

Sensitivity	Acceptance Criteria	Variant Sensitivity Results	PPV Acceptance Criteria	Variant PPV Results
SNVs	≥97%	100%	≥99%	100%
Indels	≥90%	90.00%	≥95%	100%
CNVs	≥90%	100%	≥95%	100%
Gene Fusions	≥90%	100%	≥95%	100%

Table 5. Analytical assay sensitivity. The analytical sensitivity of the NeoLAB® Solid Tumor Liquid Biopsy panel was determined by testing previously characterized samples, each containing annotated mutations at known allele frequencies. Concordance of reference genes with each annotated mutation in every sample, including SNVs, indels, CNVs and gene fusions, achieved the acceptance criteria for sensitivity and positive predictive value (PPV)

Table 6. How limit of detection (LOD) was determined

	SNV/Indel	Indels	CNVs	Gene Fusions
Cohort of previously characterized samples	Annotated mutations at allele frequencies of 1%, 0.5%, 0.25%, and 0.1%	Annotated mutations at allele frequencies of 1%, 0.5%, 0.25%, and 0.1%	Copies of 7.0, 6.4, 5.9, 3.25, 3.0, and 2.75	Copies of 40, 20, 10, and 0
Concordance criteria with reference for each sample	Lowest variant allele frequency that consistently identified expected mutations with a concordance percentage of 80%	Lowest variant allele frequency that consistently identified expected mutations with a concordance percentage of 80%	Lowest copy number that consistently identified expected mutations with a concordance percentage of 80%	Lowest gene fusion copy that consistently identified expected mutations with a concordance percentage of 80%
LOD	0.1%-0.25% (0.15, estimated by linear interpolation), 0.1% indels	0.1%	2.75 copies	10 copies

Clinical Assay Validation & Clinical Utility

Table 7. Clinical Assay Validation

Category	Acceptance Criteria	Percentage Concordance
Clinical Accuracy	≥95%	98.02%
Clinical Specificity	≥95%	98.76%
Clinical Sensitivity	≥95%	95.12%
Clinical Repeatability	≥95%	99.95%
Clinical Across-Time Reproducibility	≥95%	99.97%
Clinical Across-Operator Reproducibility	≥95%	99.94%

Figure 2. Applications in Clinical Diagnosis and Clinical Trials

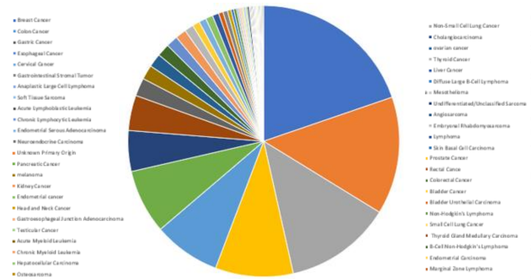

Table 7. Clinical assay validation. Clinical accuracy, specificity, and sensitivity were calculated using the same formula and acceptance metrics as analytical validation. Blood samples from late stage (III/IV) patients with various types of solid tumors were procured from 11 different clinical centers in USA and Europe and were included in the validation.

Figure 2. Applications in clinical diagnosis and clinical trials. Over **40** types of solid tumor were tested with NeoLAB® Solid Tumor Liquid Biopsy assay. Over **75%** of patients with actionable mutation detected by NeoLAB® Solid Tumor Liquid Biopsy assay.

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