

White Paper

Performance Validation of NeoLAB[®] Solid Tumor Liquid Biopsy

Detection of variants in plasma total nucleic acid

Wendy Wolfson, Scientific Writer



Key points

- The NeoLAB® Solid Tumor Liquid Biopsy pan-cancer 44-gene assay includes single nucleotide variants (SNVs), insertions and deletions (Indels) present in targeted genes in plasma cfDNA
- Covers actionable markers of all solid tumor indications supported by FDA/EMA labels, guidelines and clinical trials
- Non-invasively characterizes primary tumor drivers, resistance mutations and hotspots for real-time and repeated monitoring of tumor evolution and treatment response
- Uses whole blood, plasma, cfDNA and ctDNA extracted from plasma
- Assay validated for 0.1% LOD for SNV/indels
- Validated for MET Exon 14 Skipping

Introduction

While the gold standard for cancer diagnosis and monitoring, conventional tissue biopsy testing is not feasible in certain situations. Tissue biopsy can overlook clinically-relevant mutations for multiple reasons, mainly tumor heterogeneity, but obtaining good quality tissue and taking repeated biopsies can be barriers to effective cancer treatment:

- **Breast cancer:** issues in serial biopsies for monitoring disease recurrence can make it difficult to monitor for minimum residual disease or to know when adjuvant therapy is appropriate.¹
- **Colorectal cancer:** conducting serial biopsies to detect tumor resistance mechanisms is usually a health burden for the patient due to the procedure's invasiveness.²

Next-generation sequencing of cell-free circulating solid tumor nucleic acid from blood samples, known as “liquid biopsy,” is increasingly used in cancer care testing for an expanding number of indications because of the advantages it offers. Liquid biopsy can be used to non-invasively characterize primary tumors and metastases for diagnosis and real-time monitoring of tumor evolution and treatment response. It can supply essential information for cancer diagnosis and therapy decisions when tissue biopsies are difficult to conduct or tissue from biopsies is unavailable. This testing method is now being used to aid data collection for prospective clinical trials when tissue samples may be challenging to obtain.



Tissue biopsy vs. liquid biopsy

	Advantage	Disadvantage
Tissue biopsy	High diagnostic certainty	Lack of representativeness due to tumor heterogeneity
Liquid biopsy	• Can detect tumor heterogeneity • Representative analysis of multiple tumor foci	• Cannot detect location of mutations • No diagnostic value if no mutation detected

Liquid biopsy vs. tissue biopsy: Combining complimentary methods can provide greater precision and insight in tumor diagnosis.

Potential of liquid biopsies in precision medicine

- Mutations as biomarkers
- Monitoring tumors
- Tracking resistance
- Minimal residual disease
- Molecular remission
- Early detection

The NeoLAB® solid tumor liquid biopsy

The NeoLAB® Solid Tumor Liquid Biopsy interrogates tumor components released in the bloodstream. This gene-specific, primer-based panel is built for use in the enrichment of genes, hotspots, and construction of next-generation sequencing (NGS) libraries for patient testing and clinical trial research. The NeoLAB® Solid Tumor assay contains 44 of the most commonly mutated genes (44 DNA), which are involved in solid tumor development and progression, and which are implicated in a range of cancers, including: bladder, brain and CNS, breast, cervical, endometrial, esophageal, gastric, head and neck, kidney, liver, melanoma, ovarian, pancreatic, prostate, sarcoma, thyroid, and colorectal malignancies.

The NeoLAB® Solid Tumor Liquid Biopsy 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. The liquid biopsy panel comprises single nucleotide variants (SNVs), insertions and deletions (indels), present in targeted genes in plasma cell-free DNA (cfDNA). The assay's level of detection (LOD) for SNVs/indels achieves a 0.1% allele fraction. The NeoLAB® Solid Tumor Liquid Biopsy can be used with whole blood, plasma, cfDNA and cell-free total nucleic acid (cfTNA) extracted from plasma within a 7-day turnaround time. In addition to detecting clinically relevant genes for patient testing and monitoring for minimal residual disease, the NeoLAB® Solid Tumor Liquid Biopsy panel can be used as a laboratory test (LDT) to enrich genes, identify hotspots and construct NGS libraries for clinical trials.

Types of genes in the NeoLAB® Solid Tumor Liquid Biopsy

The NeoLAB® Solid Tumor Liquid Biopsy targets 44 unique cfDNA genes.

HOTSPOT GENES

• AKT1	• DDR2	• FGFR3	• IDH1	• MTOR	• RAF1
• ALK	• EGFR	• FGFR4	• IDH2	• NRAS	• RET
• AR	• ERBB2	• FLT3	• KIT	• NTRK1	• ROS1
• ARAF	• ERBB3	• GNA11	• KRAS	• NTRK3	• SF3B1
• BRAF	• ESR1	• GNAQ	• MAP2K1	• PDGFRA	• SMAD4
• CHEK2	• FGFR1	• GNAS	• MAP2K2	• PIK3CA	• SMO
• CTNNB1	• FGFR2	• HRAS	• MET	• PTEN	

SUPPRESSOR GENES

- APC
- FBXW7
- PTEN
- TP53

Liquid biopsy NGS testing specifications

- Single library from DNA
- 272 amplicons
- >900 hotspots SNVs and indel
- Extended coverage of TP53
- MET exon 14 skipping

NeoLAB® Solid Tumor Liquid Biopsy Panel interrogates 44 actionable biomarkers

The NeoLAB® Solid Tumor Liquid Biopsy panel includes all actionable DNA SNVs/indels, approved as cancer biomarkers supported by labels, guidelines and clinical trials.

This assay can detect pan-cancer genes that play multiple roles in oncogenesis and cancer progression across various tumor types. The NeoLAB® Solid Tumor Liquid Biopsy panel

can detect targetable alterations before first-line therapy for applications such as diagnosing breast cancer and colorectal cancer or monitoring disease status and response to therapy. The liquid biopsy panel can also be used to predict the likelihood of disease relapse.

APPROVED LABELS, HOTSPOTS (FDA AND EMA)

- BRAF
- EGFR
- KRAS
- NRAS

GUIDELINES, HOTSPOTS (NCCN, ESMO)

- BRAF
- EGFR
- ERBB2
- KIT
- KRAS
- NRAS
- PDGFRA

GLOBAL CLINICAL TRIALS

- | | | | |
|---------|---------|----------|----------|
| • AKT1 | • EGFR | • GNAS | • NTRK1 |
| • ALK | • ERBB2 | • HRAS | • NTRK3 |
| • AR | • ERBB3 | • IDH1 | • PDGFRA |
| • ARAF | • ESR1 | • IDH2 | • PIK3CA |
| • BRAF | • FBXW7 | • KIT | • PTEN |
| • CCND1 | • FGFR1 | • KRAS | • RAF1 |
| • CCND2 | • FGFR2 | • MAP2K1 | • RET |
| • CCND3 | • FGFR3 | • MAP2K2 | • ROS1 |
| • CDK4, | • FGFR4 | • MET | • SMO |
| • CDK6 | • FLT3 | • MTOR | • TP53 |
| • CHEK2 | • GNA11 | • MYC | |
| • DDR2 | • GNAQ | • NRAS | |

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

A deeper dive: lung, breast and colorectal cancer

Breast cancer		CRC		NSCLC	
Hotspot SNV/indel					
• AKT1	• KRAS	• CTNNB1	• NRAS	• CTNNB1	
• ERBB2	• PIK3CA	• GNAS	• PIK3CA	• KRAS	
• ERBB3	• SF3B1	• KRAS	• SMAD4	• NRAS	
• ESR1				• PIK3CA	
Tumor suppressor					
• PTEN		• APC	• FBXW7	• APC	• PTEN
• TP53		• BRAF	• TP53	• BRAF	• TP53
				• FBXW7	

Validation study design

This white paper presents NeoGenomics' analytical validation studies of the NeoLAB® Solid Tumor Liquid Biopsy. In assessing the accuracy, precision, analytical specificity and sensitivity of the NeoLAB® Solid Tumor Liquid Biopsy, this study documented that the liquid biopsy assay was fully analytically validated for SNVs/indels, and demonstrates superior assay performance across all criteria.

The study used a total of 115 unique patient samples; 13 pre-characterized reference samples and their dilutions, 25 retrospective patient plasma samples, 10 healthy donor blood specimens, and 67 prospective patient peripheral blood samples, in addition to samples of solid tumor tissue from patients with lung, breast, colon, head and neck, bladder, rectal, pancreatic, cervical, gastric, kidney, ovarian, prostate, stomach, and thyroid cancers. Each reference cfDNA (containing SNVs/indels), with annotated mutation status was tested on at least one variant position using the NeoLAB® Solid Tumor Liquid Biopsy. The 115 unique samples were processed into 179 libraries and sequenced on Ion S5 550 chips. Bioinformatics analysis was conducted using the dedicated Ion Report workflow. Each sequencing run generated a minimum of 20,000x depth of coverage for every sample.

Analytical accuracy

The 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 samples (10 for SNV/indel) with annotated mutation status on at least one variant position. Following variant calling, concordance was evaluated for each known variant position by comparing the annotated mutation status with each sample's called status per position. Accuracy was calculated as the percentage of concordant calls divided by the total number of evaluated calls. Accuracy rates of 100% for SNVs and 95% for indels achieved the acceptance criteria of ≥95% concordance with annotated mutations/wild-type samples.

How analytical accuracy results were calculated

Annotated variant	SNV	Indel
Libraries (n=20) prepared between 2 operators	10	10
# positions queried	SNVs (n=79)	Indels (n=6)
Concordance if expected variant or wildtype detected (VAF cutoff = 0.065% 1.2 ratio)	3 copies	3 copies
Accuracy acceptance criteria	≥95%	≥95%
Concordance achieved	100%	95w%

The NeoLAB® Solid Tumor Liquid Biopsy demonstrated greater than 95% accuracy.

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

Analytical specificity

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

Specificity

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

The NeoLAB® Solid Tumor Liquid Biopsy demonstrated 100% specificity.

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

Analytical 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 and indels, achieved the acceptance criteria for sensitivity and positive predictive value (PPV).

Sensitivity

Sensitivity	SNVs	Indels
Acceptance criteria	≥97%	≥90%
Variant sensitivity results	100%	90%
PPV acceptance criteria	≥99%	≥95%
Variant PPV results	100%	100%

The NeoLAB® Solid Tumor Liquid Biopsy demonstrated high sensitivity

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

Limit of detection

The lowest allele frequency that consistently identified the expected mutations with a concordance percentage of 80% or higher was defined as the limit of detection (LOD). The LOD for SNVs/indels ranged from 0.1%–0.25% (0.15, estimated by linear interpolation), was achieved at 0.1% for SNVs/indels, respectively.



How limit of detection (LOD) was determined

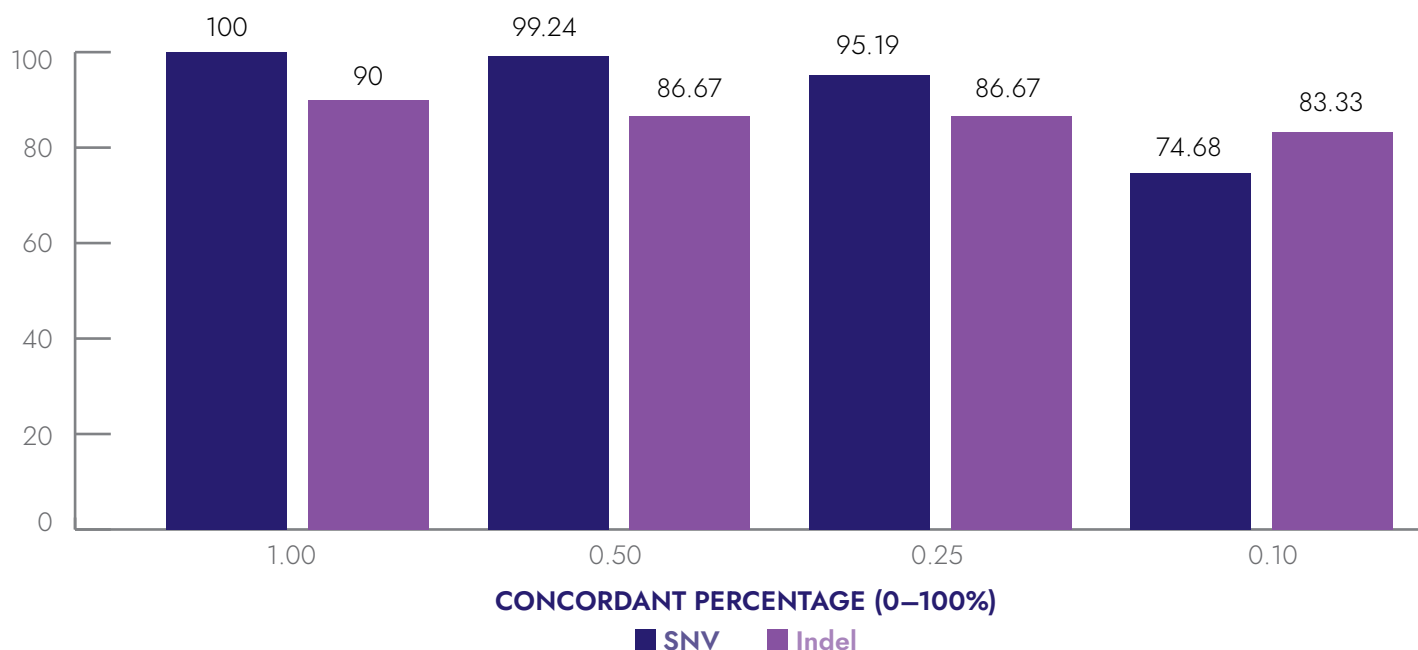
	SNV/Indel	Indels
Cohort of previously characterized samples	Annotated mutations at allele frequencies of 1%, 0.5%, 0.25%, and 0.1%	Annotated mutations at allele frequencies of 1%, 0.5%, 0.25%, and 0.1%
Concordance criteria with reference for each sample	Lowest variant allele frequency that consistently identified expected mutations with a concordance percentage of 80%	Lowest variant allele frequency that consistently identified expected mutations with a concordance percentage of 80%
LOD	0.1%-0.25% (0.15, estimated by linear interpolation), 0.1% indels	0.1%

NeoLAB® Solid Tumor Liquid Biopsy Assay achieved 0.1% limit of detection.

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

LOD: SNV/Indel

SNV LOD averaged 0.1%.





Analytical repeatability and reproducibility

Repeatability for the NeoLAB® Solid Tumor Liquid Biopsy panel was established by running 5 samples in triplicate for a total of 15 libraries, then sequencing the triplicate libraries together on S5 550 chip runs. Concordance was determined by comparing variant/wild-type calls for each sample against expectations and against their replicates; following the variant calling, the pairwise concordance rate among replicates was evaluated for each annotated mutation. Repeatability was calculated by averaging the concordance rate of all replicate sets. Repeatability results of 99.92% achieved the acceptance criteria of $\geq 95\%$.

Reproducibility for the NeoLAB® Solid Tumor Liquid Biopsy panel was determined using 5 samples processed through library preparation, sequenced repeatedly by the same operator, then by a second operator. Concordance was established after variant calling by comparing the annotated variants/wild-types with expectations and between times and operators. The reproducibility concordance of 99.92% across times and 99.94% across operators achieved the acceptance criteria of $\geq 95\%$.

How limit of detection (LOD) was determined

Repeatability and reproducibility	SNV/Indel
Repeatability acceptance criteria	$\geq 95\%$
Average repeatability 99.92%	99.6%
Average reproducibility (time to time) 99.92%	90.8%
Average reproducibility (operator to operator) 99.94%	99.8%

The NeoLAB® Solid Tumor Liquid Biopsy had greater than 99% precision.

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

Clinical accuracy, specificity, sensitivity, repeatability and reproducibility

Clinical accuracy, specificity, and sensitivity were calculated using the same formula and acceptance metrics as analytical validation. Testing was conducted using clinical samples from 92 patients with a stage III or IV solid tumor samples, including 25 retrospective plasma samples, 67 prospective blood samples, and 10 blood specimens from healthy donors.

Orthogonal testing using NGS or ddPCR was conducted on 45 found or wild-type variants and 9 healthy donor samples, and 14 mutation-negative patient samples using the ddPCR KRAS G12/G13 screening assay, which tests 7 KRAS mutations:

- p.G12A/c.35G>C
- p.G12S/c.34G>A
- p.G12C/c.34G>T
- p.G12V/c.35G>T
- p.G12D/c.35G>A
- p.G13G>c.38G>A
- p.G12R/c34G>C

To establish clinical repeatability and across-time reproducibility, 4 patient samples were processed twice and independently by the same operator: singly for the first time and in duplicate for the second iteration. The same sample set was then processed by the second operator to determine across-operator reproducibility. After which 16 libraries were prepared and sequenced between two operators; each library queried 969 positions for SNVs/indels, specificity, and sensitivity of the NeoLAB® Solid Tumor Liquid Biopsy achieved the acceptance criteria.

Clinical accuracy, specificity, sensitivity, repeatability and reproducibility

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%

Source: NeoLAB® Solid Tumor Liquid Biopsy Validation summary

About NeoGenomics Pharma Services

NeoGenomics' Pharma Services unifies several innovative companies' scientific and medical leadership under one leading brand, offering one of the most comprehensive laboratory services menus available for biomarker testing supporting oncology clinical trials globally. We provide our clients with an unparalleled level of expertise, service, flexibility, and scalability. Additionally, we offer alternative business models and solutions across the development continuum, from pre-clinical research and development through commercialization.

To learn more about NeoGenomics Pharma Services, visit us online at neogenomics.com/pharma-services.

NeoGenomics Pharma Services is your premier research partner with NGS and other innovative services. Please contact NeoGenomics Pharma Services at 800.720.4363 or email at pharmaservices@neogenomics.com.

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12701 Commonwealth Dr., Suite 9
Fort Myers, FL 33913
United States
Phone: 866.776.5907
Fax: 239.690.4237

31 Columbia
Aliso Viejo, CA 92656
United States
Phone: 800.720.4363
Fax: 949.425.5865

7256 S. Sam Houston Pkwy W. Suite 300
Houston, TX 77085
United States
Phone: 800.720.4363 / 713.528.4363
Fax: 713.668.3565

NeoGenomics Laboratories, Inc.
4570 Executive Drive, Suite 250
San Diego, CA 92121
Phone: 949.445.7300 5056
Fax: 713.668.3565

A-One Business Center
Bâtiment A5, 2nd Floor
Z.A. La Pièce / Route de l'Etraz 1
1180 Rolle, Switzerland
Phone: +41 (0)21 721 06 00
Fax: +41 (0)21 826 00 73

NeoGenomics Singapore Pte Ltd
61 Science Park Road, #02-11,
Singapore 117525
Phone: +65 6591 5200

Suzhou NeoGenomics Pharmaceutical
Research Co., Ltd Building 6, Block 19,
Yong'an Road Huguan Industrial Park,
Suzhou New District, China



neogenomics.com

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