All you need to know about emerging NSCLC biomarker testing in precision oncology research

Nine target genes with mutation and fusion alterations, which can be tested simultaneously by a combined NGS method that utilizes both DNA and RNA

Despite a substantial increase in treatment options, lung cancer remains the most common cause of cancer death worldwide. This represents a large unmet need, and in order to improve the situation there is ongoing intense and large-scale translational and clinical research.

While some of the biomarkers and their relevance have been known for years now, and the testing is reasonably established, there are more new, emerging biomarkers coming into clinical research, and the testing landscape is becoming complex. The increasing number of biomarkers of different alterations, and notoriously small tissue samples means effective profiling can be done only by multiple-biomarker next-generation sequencing (NGS) panels.

	NSCLC targeted genes	Alterations	Testing methods					
			NGS		Single-gene tests			
			DNA-based	RNA-based	IHC	FISH	PCR	Sanger
Established	*EFGR*	Mutations	•				•	•
	ALK	Fusions	•	•	•	•	•	
	ROS1	Fusions	•	•	•	•	•	
	BRAF	V600E mutation	•				•	•
	MET	Exon 14 skip	•	• (preferred)			•	•
	RET	Fusions	•	• (preferred)		•	•	
	NTRK	Fusions	•	• (preferred)	•	•	•	
Emerging	*EGFR*	Exon 20 insertions	• (preferred)				•	•
	ERBB2	Mutations and exon 20 insertions	• (preferred)				•	•
	KRAS	G12C	•				•	•
	MET	Amplification	•			•		
	Immuno-oncology biomarkers							
Established	*PDL1*	Overexpression			•			
	MSI	Instability or Overexpression	•		•		•	
Emerging	TMB	Tumor mutation load	•					

ThermoFisher SCIENTIFIC

Methods for NSCLC biomarkers testing

A variety of methods are routinely used to detect non-small cell lung cancer (NSCLC) biomarker alterations. While NGS plays a major role in NSCLC molecular testing, other methods may still be necessary for comprehensive molecular profiling. The method that works best depends on the specific biomarker to be tested.

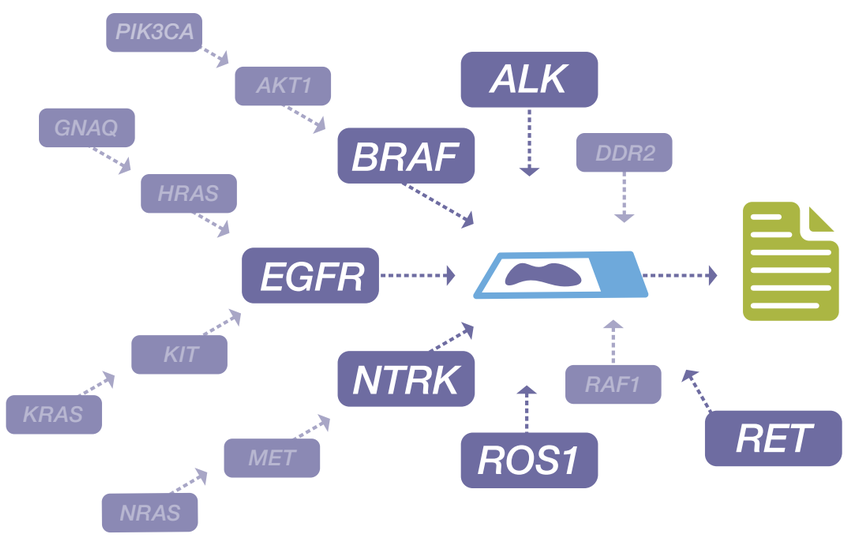
Immunohistochemistry (IHC): this method is mostly suited to test for PD-L1 in samples who are negative for other alterations (e.g., *EGFR* negative). However, IHC is not suitable for testing the majority of the other NSCLC biomarkers, except for MSI status. For example, pan-TRK IHC antibodies detect TRK proteins A, B, and C, which may be expressed in both the wild-type and fusion protein versions. However, protein overexpression may not necessarily be the result of a gene fusion event.[1] Overall, IHC lacks specificity to be a stand-alone assay for gene fusion testing and confirmatory NGS should be performed.

DNA fluorescence *in situ* hybridization (FISH): DNA is usually employed to test for gene rearrangements, such as fusions or copy number variants. While fluorescence signal associated with break-apart probes allows visualization of the gene translocation within the histological context of the sample, testing may have limited utility because it is not designed for multiplexing. In fact, multiple FISH tests would need to be run in order to detect *ALK, ROS,* and *NTRKs* alterations, making it extremely unpractical in the context of NSCLC where usually only limited specimen is available for testing. In addition, FISH is highly subjective, time consuming, and therefore not cost effective, as well as subject to false positives for fusions that are rearranged but do not lead to activating mutations.

Reverse transcription polymerase chain reaction (RT-PCR): this method is highly sensitive and routinely used by most laboratories. Nonetheless, while this method is easy to be implemented and analyzed, its limited multiplexing capability makes it unsuitable for comprehensive molecular NSCLC biomarkers profiling. For example, RT-PCR is challenging to use when large introns need to be amplified across genes and makes it difficult to identify to translocation. RT-PCR applications are mostly confined to testing of point mutations or small insertions and deletions. But even then RT-PCR is limited in the number of variants that can be detected. For example, there are many known variants of *EGFR* exon 20 insertions, while the commonly used *EGFR* RT-PCR tests can only detect up to 5.

NGS allows for testing of multiple relevant biomarkers and large numbers of variants. There are currently 11 genes with relevant biomarkers in targeted therapy clinical research, which should be tested at once, from one sample. Only NGS using a combined RNA and DNA panel can enable such comprehensive of assessment.

All relevant NSCLC biomarkers from one sample.



Choose the optimal NGS method for NSCLC biomarker testing in clinical research

While NGS is the ideal testing platform for multiple biomarkers while preserving precious sample tissue, not all NGS technologies are the same.

Four key factors you should consider when choosing the NGS technology for NSCLC biomarker testing:

- **DNA + RNA panel combination**—some of the important fusion biomarkers (e.g., *NTRK*, *RET*) and *MET* exon 14 skip mutations are more sensitively detected by RNA-based NGS, while other mutation biomarkers (e.g., *EGFR*, *ERBB2*, *KRAS*, *BRAF*) are comprehensively detected by DNA-based NGS. Choose an NGS assay that combines both DNA and RNA for one sample to deliver all biomarkers comprehensively and sensitively.
- **Minimal sample requirement**—tissue is still the issue, and often the amount available is very limited. Different NGS methods differ significantly in the amount they require, ranging from 10 to 500 ng of nucleic acid (RNA or DNA). Choose an NGS assay that maximizes your ability to successfully test all samples.
- **Turn around time (TAT)**–TAT to result can differ significantly depending if the testing is outsourced (>2 weeks) or performed in your own laboratory, but even then different NGS technologies vary in the TAT by 1 to 5 days. Choose an in-house NGS platform with fastest TAT.
- **Completeness and automation of the workflow and implementation support**—NGS workflows can be complex. Choose an easy-to-use, integrated workflow with verified protocols from sample to a clear and annotated final report that also simplifies lab operation and test implementation. High-touch consultation service and support from the vendor helps accelerate a lab's process to implement the test in a time-efficient manner and save costs.

		Ion Torrent™ Oncomine™ assays				
		FFPE tissue			Liquid biopsy	Dual-use (FFPE and liquid biopsy)
		Oncomine™ Comprehensive Assay v3	Oncomine™ Focus Assay	Oncomine™ Comprehensive Plus Assay	Oncomine™ Pan Cancer cfTNA	Oncomine™ Precision Assay
Biomarker coverage	(*EFGR, ALK, ROS1, BRAF, NTRK,* MET, *RET, ERBB2, KRAS*)	X	X	X	X	X
	(MSI, TMB)			X		
Panel details	Panel	DNA + RNA	DNA + RNA	DNA + RNA	DNA + RNA	DNA + RNA
	Number of genes	161	52	>500	52	50
	Sample input amount (ng) for DNA or RNA	20 ng	10 ng	20 ng	20 ng	10 ng
Instrument + TAT	Ion GeneStudio™ S5 System (4-day TAT)	X	X	X	X	
	Ion Torrent™ Genexus™ System (1 day TAT*)	X				X
Software and reporting	Oncomine™ Reporter Software	X	X	X	X	X

Figure 1: Oncomine™ Solutions for NSCLC biomarker testing.

* Specimen-to-report workflow will be available after the Ion Torrent™ Genexus™ Purification System and integrated reporting capabilities are added in 2020. Fully integrated specimen-to-report workflow will be available after the Ion Torrent™ Genexus™ Software 6.4 update.

Emerging biomarkers in NSCLC

EGFR exon 20 insertions

While the traditional *EGFR*-activating mutations, including exon 19 deletions and the exon 21 point mutation L858R, are commonly identified in 15 to 20% of non-squamous NSCLC (in the Caucasian population), exon 20 insertions are much less common and seen in approximately 2% of such cases, or 10% of all *EGFR* mutations.[1] *EGFR* exon 20 insertions are typically represented by in-frame insertion of 3 to 12 base pairs, or 1 to 4 amino acids, involving codons 763 to 775.

NSCLC with *EGFR* exon 20 insertions, with the exception of the A763_Y764insFQEA variant, do not typically respond to first- and second-generation tyrosine kinase inhibitors (TKIs) or anti–PD-L1 treatments.[2] Related to this lack of response of *EGFR* exon 20 insertions to TKIs is the affinity for ATP in the kinase domain that is encoded by exons 18–25. Over 60 unique variants of *EGFR* exon 20 insertions have been identified through comprehensive genomic profiling, the majority of which are rare variants.[2]

RT-PCR has proven to be a well-established method for detection of *EGFR* exon 19 deletions and SNVs. However, its coverage for exon 20 insertions is very limited to just a few common variants that account for less than 50% of exon 20 insertion prevalence.[1] A DNA-based NGS method can comprehensively detect a broad range of common and rare variants, and is therefore the preferred detection method for exon 20 insertions research.

RET fusions

RET (rearranged during transfection) encodes a transmembrane receptor tyrosine kinase with proto-oncogene properties that signals through multiple pathways, including the RAS/mitogen-activated protein kinase (MAPK), RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3K)/AKT, and c-Jun N-terminal kinase (JNK). Aberrant activation of RET in solid tumors might occur though different mechanisms, including genetic rearrangements. A variety of different RET fusion partners have been described in NSCLC, although the most frequent are *KIF5B-RET* (70–90%) and *CCDC6-RET* (10–25%).

RET gene rearrangements occur in up to 2% of advanced NSCLC. Studies have shown that IHC, while an effective screening tool to detect *ALK* and *ROS* fusions, has often produced false positive and false negative results for RET detection.[3] FISH and RT-PCR are sensitive single-gene techniques commonly used in screening and clinical trials. NGS provides accurate and sensitive profiling of *RET* fusions and is able to detect different and functional genomic *RET* variants by analysis of both DNA and RNA.

MET exon14 skipping mutations

MET is a proto-oncogene that encodes for the hepatocyte growth factor receptor and has a physiological role during embryogenesis. Dysregulation of the *MET* pathway can be found in several solid tumors, including NSCLC, through a variety of mechanisms: overexpression, amplification, mutations, and rearrangements. Notably, some specific *MET* mutations cause alternative splicing to occur in exon 14. These mutations have been observed in 3% of all NSCLCs.[4] MET exon 14 alterations exhibit a highly diverse sequence composition, posing a challenge for testing. Studies have shown NGS is the method of choice for detection of MET exon 14 skipping, followed by RT-PCR as single-gene test.[4] Sanger sequencing has reported high false-negative results. DNA-based NGS has shown lower sensitivity than RNA-based NGS because genomic alterations inducing exon 14 skipping are very diverse. A study has shown DNA-based NGS can potentially miss *MET* exon 14 skipping events when the panel primers do not target both the 3′ splice site of intron 13, and the 5′ splice site of intron 14.[5] A reflex workflow interrogating RNA fusions in cases without DNA-detected driver mutations can potentially capture such event.[5]

ERBB2 (HER2)

Activating mutations in *ERBB2 (HER2)* were first identified in non-squamous NSCLC,[2] and result in downstream activation of the PI3K-AKT and RAS-MAPK pathways. The relevance of tyrosine kinase domain mutations has been extensively studied in lung cancer where they are mutually exclusive of other common driver mutations such as *KRAS, EGFR,* and *ALK*, and represent up to 5% of this subtype of NSCLC.

Activating *HER2* mutations can be generally viewed as SNVs in the extracellular domain (ECD), transmembrane domain (TD), and both SNVs and in-frame insertions in the tyrosine kinase domain. The majority of activating variants are represented by SNVs in exon 19 and 21 and in-frame insertions or SNVs in exon 20. Given the diversity of variants in *ERBB2* SNVs and insertions, DNA-based NGS is an ideal method of detection.

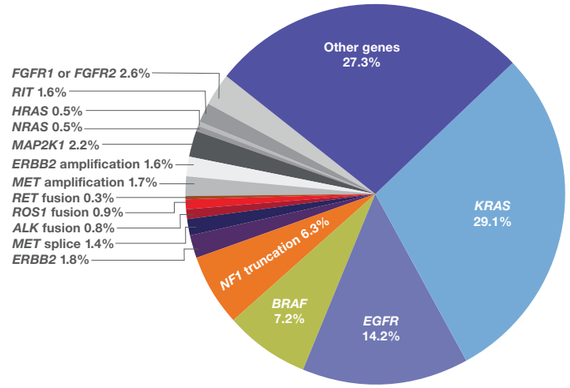
KRAS mutations

The RAS family genes encode for small guanosine triphosphatase (GTPase) proteins, with KRAS, HRAS, and NRAS being the best known and investigated. Their activation leads to multiple biochemical pathways such as RAF-MEK-ERK, PI3K-AKT-mTOR, and RALGDS-RA that regulate cell proliferation, differentiation, and apoptosis. Somatic activating mutations of the *KRAS* gene are very frequently observed in NSCLC, especially in lung adenocarcinoma (about 20–30%), while less frequently (about 5%) in the squamous cell subtype.

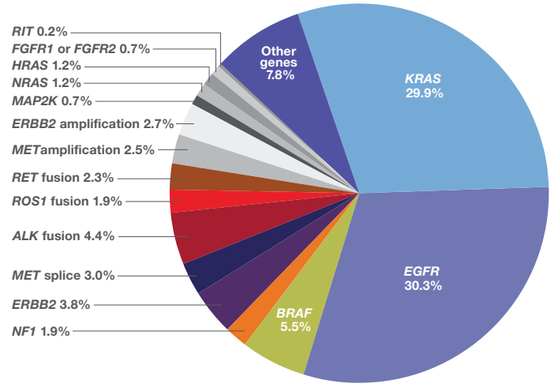
By far the most common mutations occur in codons 12 and 13. Notably, *KRAS* mutations are typically but not always mutually exclusive with *EGFR* mutations or *EML4-ALK* rearrangement. Despite RAS being the first oncogene discovered in human cancer and years of efforts dedicated to target RAS family proteins with small-molecule inhibitors, no significant results were achieved until recently with the discovery of $KRAS^{G12C}$ inhibitors. Various molecular methods are commonly used for *KRAS* testing, including single-gene techniques such as RT-PCR and Sanger sequencing, and multiplexing techniques such as NGS.

Most common alterations in NSCLC

a. Early stage



b. Metastatic

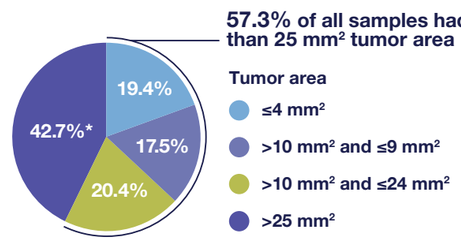


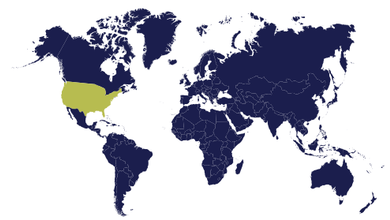
From: Skoulidis F & Heymach JV. (2019) Co-occurring genomic alterations in non-small-cell lung cancer biology and therapy. Nat Rev Cancer, 19:495-509.

Tissue is still an issue with NSCLC samples and not all NGS methods can handle small samples well

Based on the sample tumor area requirement, only one of two samples could be tested by hybrid capture-based NGS method, while >98% of samples could be successfully tested with a PCR amplicon–based method.

Multicentric feasibility study, US





21,722
NSCLC samples

*From: Scott, A, et al. (2020) Actionable CR-based comprehensive genomic profiling (PCR-CG P): Feasibility from >20,000 tissue specimens and predicted impact on actionable biomarker identification vs. hybrid capture (H)-CG P and plasma (P)-CGP. Presented at ASCO 2020.

Featured solution: the Oncomine Precision Assay on the Genexus System

Empower your lab to deliver NGS genomic profiles based on DNA and RNA analysis with the speed and simplicity of immunohistochemistry. Maximize your ability to deliver results even from small samples and low-level variants.

Curated pan-cancer content

- DNA- and RNA-based biomarkers across 50 key genes
- *EGFR, ALK, BRAF, ROS1, RET, NTRK, ERBB2, and MET,* among others

Molecular tagging

- Enhanced low-level variant detection
- Key for liquid biopsy testing

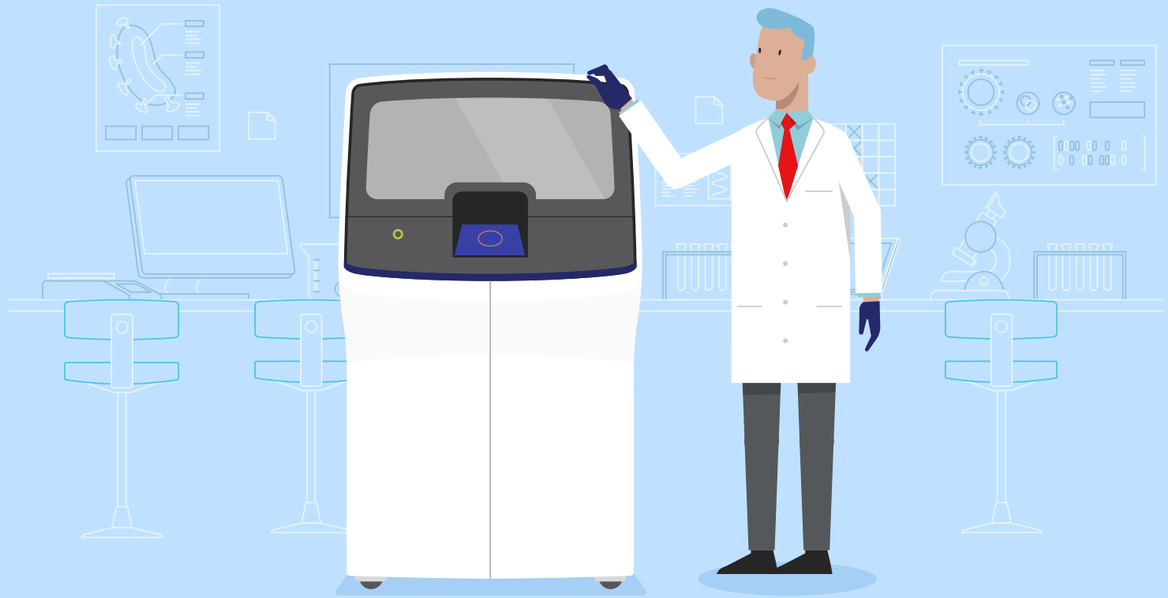
Tissue and plasma samples

- One test, one workflow, multiple sample types
- Maximizes the number of tumors that can be profiled

FusionSync™ Detection Technology

- Sensitive and specific—targeted isoform designs
- Novel fusion detection

The Oncomine Precision Assay on the Genexus System is NGS, but is just as fast and simple as immunohistochemistry or PCR. You need only 10 minutes of total hands-on time and 2 user touchpoints to get results on 50 key biomarkers from 1 sample in 1 day. The entire workflow is operated by a single software program and provides a full, easy-to-read annotated report the next day*.



* Specimen-to-report workflow will be available after the Genexus Purification System and integrated reporting capabilities are added in 2020. Fully integrated specimen-to-report workflow will be available after the Genexus Software 6.4 update.

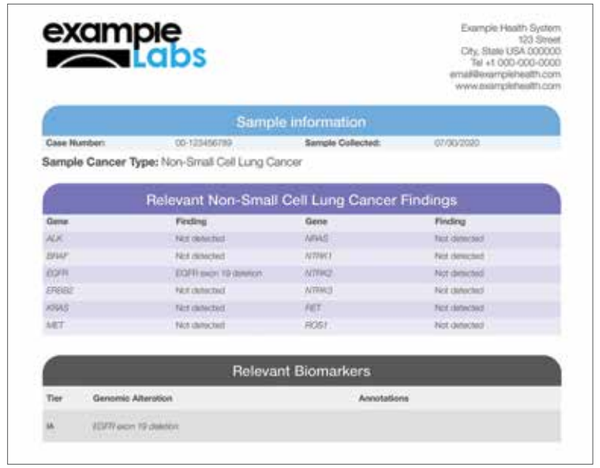
Oncomine Reporter Software

With Oncomine Solutions, you get a streamlined bioinformatics analysis pipeline optimized for each Oncomine assay—all packaged in a user-friendly experience.

The Oncomine Reporter Software delivers clear and concise reports that present all biomarker results fully annotated.

The report format is fully customizable, allowing you to easily make it your own.

To see content and an example of a report, please visit **thermofisher.com**.



Accelerate successful implementation of Oncomine assays in your lab with Analytical Validation consulting services

Analytical Validation (AV) consulting services provides technical project management of your lab's AV to help verify that the assay is tested for required parameters. We work with you to optimize and develop your validation workflow while providing data analysis support and template documentation as part of your end-to-end instrument and reagent investment. On average, we can help you complete the validation process 62–75% faster than on your own; and by supplying control samples, data analysis, and reporting, we can help you reduce costs up to 50% for your completed AV.

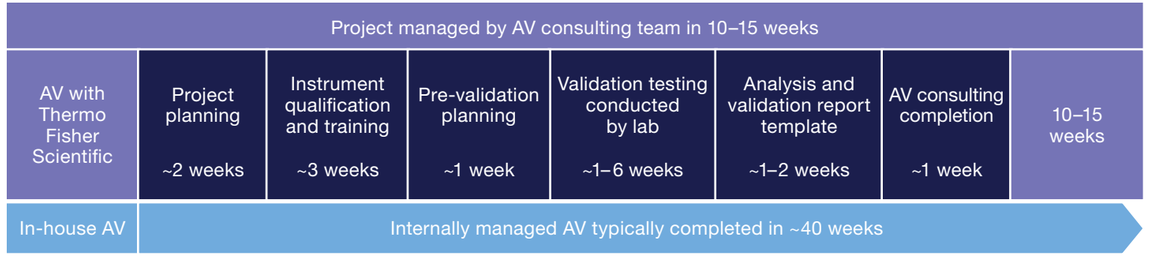


Figure 2. Analytical validation workflow completed 62–75% faster with AV consulting service.

References

1. Kumar-Sinha C, Kalyana-Sundaram S, Chinnaiyan AM. Landscape of gene fusions in epithelial cancers: seq and ye shall find. *Genome Med.* 2015;7:129. doi:10.1186/s13073-015-0252-1.
2. Jonathan W. Riess, et al. Diverse EGFR Exon 20 Insertions and Co-Occurring Molecular Alterations Identified by Comprehensive Genomic Profiling of NSCLC. Journal of Thoracic Oncology, 2018;13(10):1560-1568.
3. Roberto Ferrara, et al. Clinical and Translational Implications of RET Rearrangements in Non–Small Cell Lung Cancer. Journal of Thoracic Oncology, 2018;13(1):27-45.
4. Eun Kyung Kim, et al. Molecular Diagnostic Assays and Clinicopathologic Implications of MET Exon 14 Skipping Mutation in Non-small-cell Lung Cancer. Clinical Lung Cancer, 2018;20(1):123-32.
5. Magdalena Jurkiewicz, et al. Efficacy of RNA vs DNA based method in MET Exon 14 Skipping Mutation Detection. Journal of Clinical Oncology, 2020;38(15 suppl):9036-9036.

For more information about Oncomine NGS solutions, go to **oncomine.com**

ThermoFisher SCIENTIFIC