Detection for Rare Actionable Genetic Alterations

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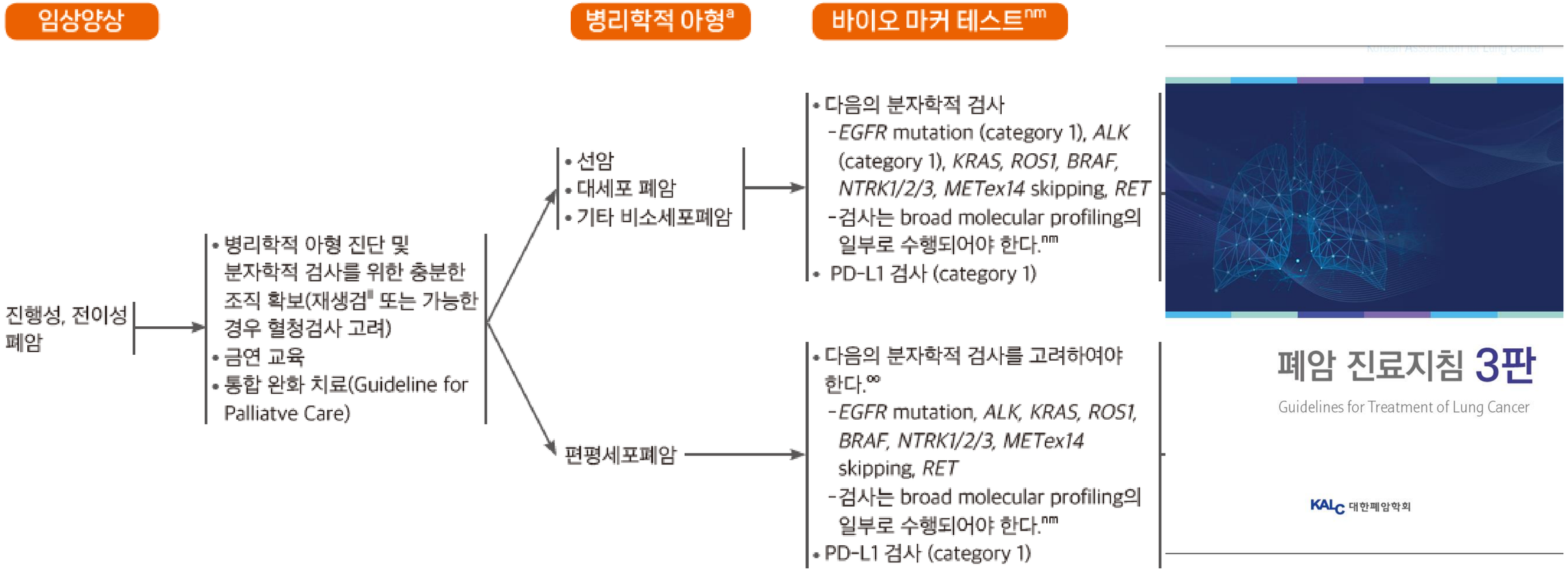
INDEX

Importance of Molecular Diagnostics in NSCLC

- Non–small cell lung cancer (NSCLC) often harbors actionable driver mutations (e.g. *EGFR*, *ALK*, *ROS1*, *BRAF*, *MET exon 14*, *RET*, *NTRK*).
- Identifying these alterations is critical, as targeted therapies significantly improve patient outcomes.

General Guidelines for Biomarker Testing
- According to the Korean Association for Lung Cancer

Following CAP/ IASLC/ AMP, ASCO, and NCCN



2023 Guidelines for Treatment of Lung Cancer 3rd Ed. KALC

General Guidelines for Biomarker Testing
– According to the Korean Association for Lung Cancer

The key principles include:

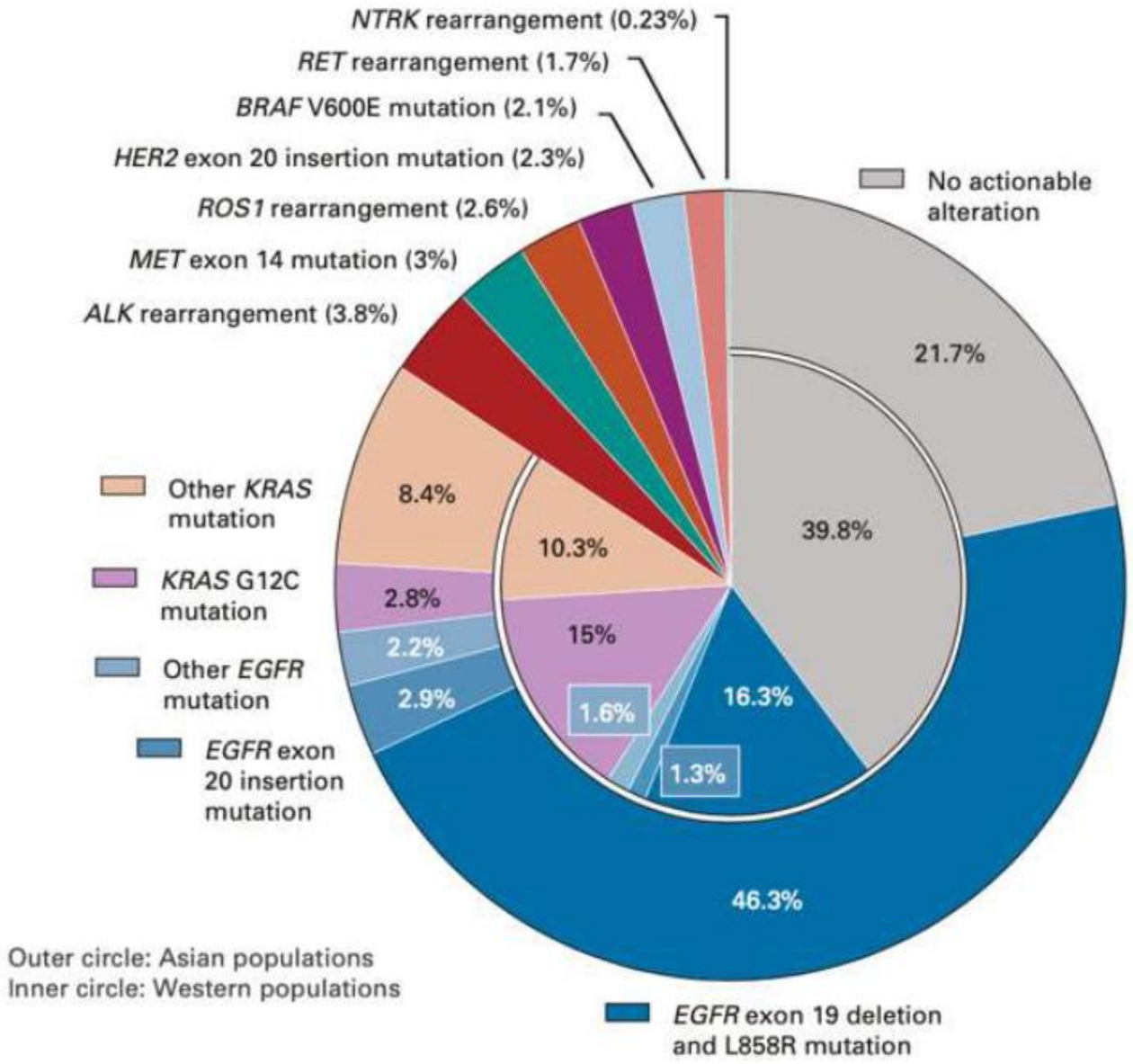
1. Molecular testing for actionable genetic alterations (AGAs) and PD-L1 expression **should be performed for all patients with NSCLC**.

2. Actionable gene alterations

   - Essential genetic tests for NSCLC include EGFR, ALK, ROS1, and BRAF.

   - Tests for NTRK, MET, RET, HER2, and KRAS are recommended if EGFR, ALK, ROS1, and BRAF are negative, or as part of a comprehensive panel.

Introduction



ADC

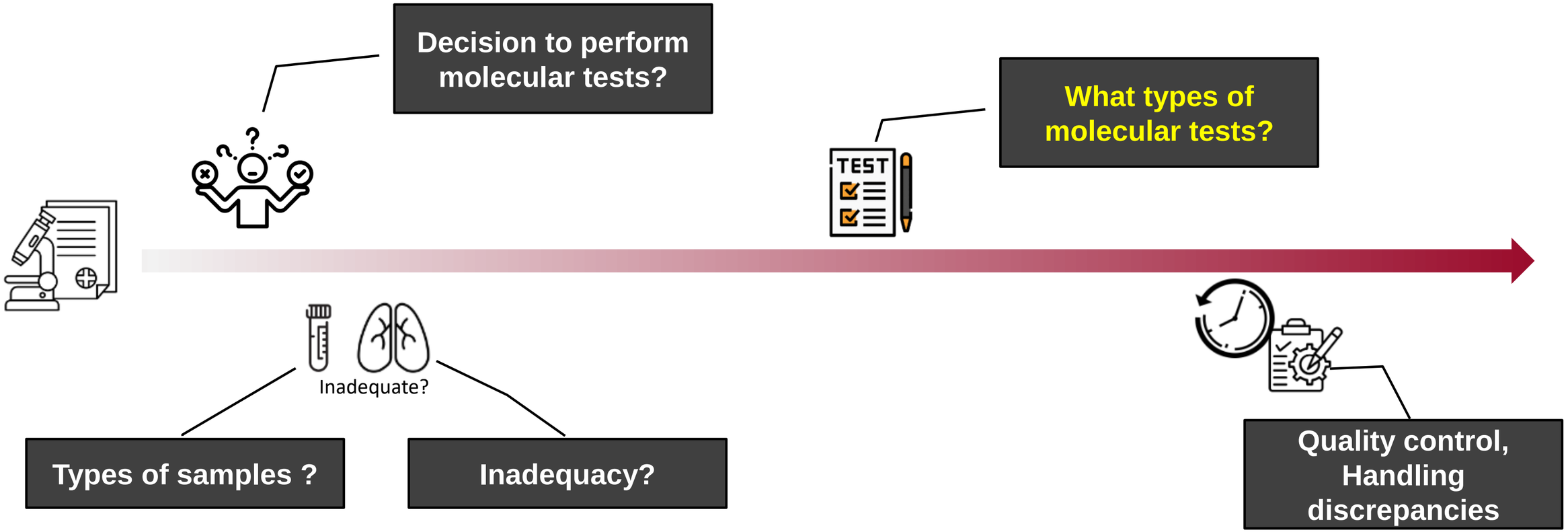
Tan et al. J Clin Oncol 2022

Why Do Rare AGAs Matter?

1. Rare AGAs now have approved therapies that improve outcomes.
2. Some are available as first-line treatments.

Alteration	Targeted Drug
EGFR Ex20ins	Amivantamab, Mobocertinib…
KRAS G12C	Sotorasib…
BRAF V600E	Dabrafenib/trametinib, Encorafenib/binimetinib…
HER2 mutation	Trastuzumab deruxtecan…
ROS1 fusion	Crizotinib, Entrectinib…
RET fusion	Selpercatinib, Pralsetinib…
NTRK fusion	Larotrectinib…
MET Ex14 skipping	Tepotinib, Capmatinib...

Workflow Overview



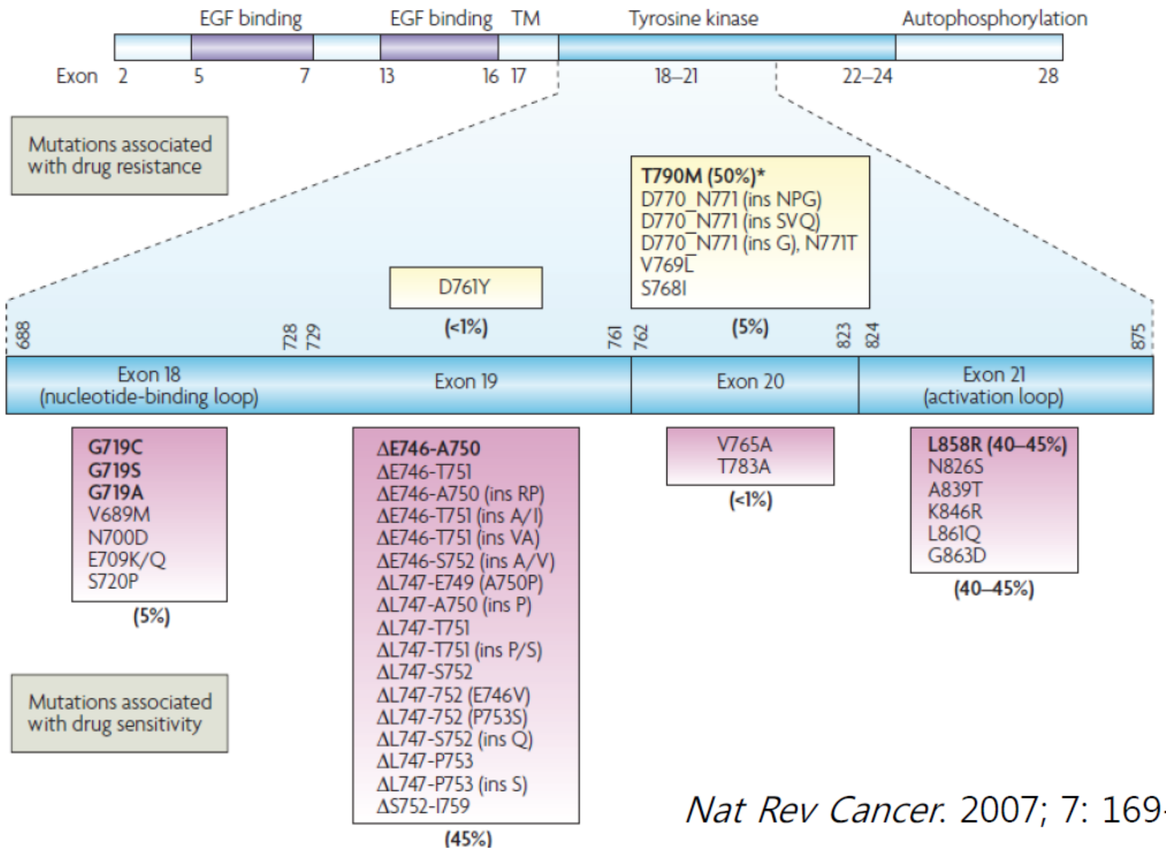
How to screen and detect the targetable oncogenes?

	SNV, Indel	Fusion	CNV (Amp)	Others
DNA	Sanger- or Pyrosequencing PCR-based method DNA-NGS	FISH DNA-NGS	FISH/CISH qPCR NGS	TMB, MSI (DNA based NGS)
RNA		RT-PCR (known partner) RNA-NGS (preferred)		
Protein	IHC* (mutation-specific Ab: BRAF VE1)	IHC* (ALK, ROS1, pan-TRK)	IHC* (e.g., HER2)	IHC (ex. PD-L1)

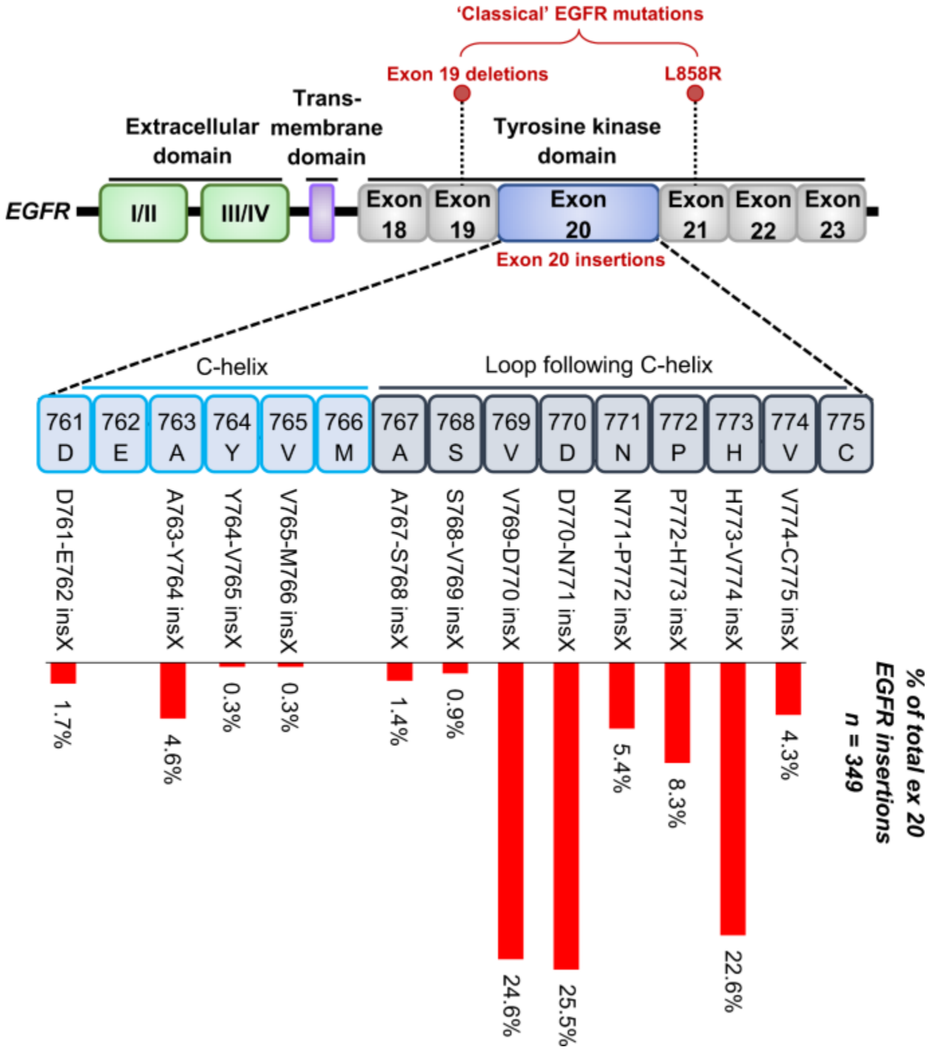
* As a screening tool, except ALK & HER2

Single Gene Tests

EGFR Exon 20 Insertion

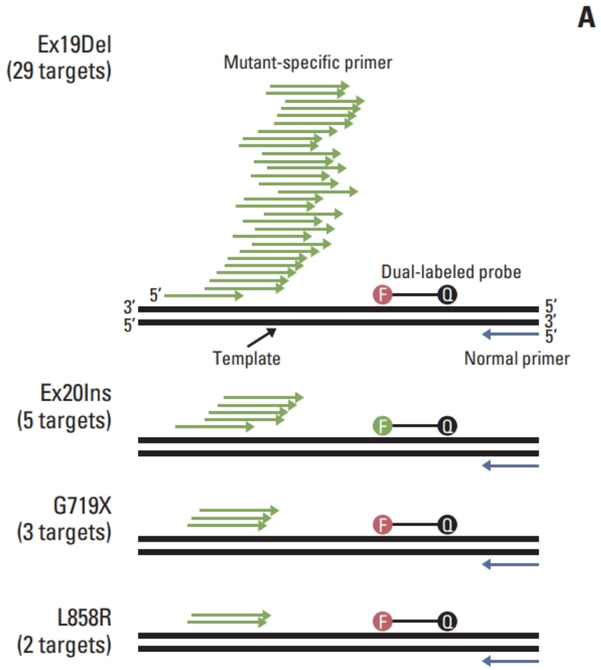


Nat Rev Cancer. 2007; 7: 169-81.



Signal Transduct Target Ther. 2019 Mar 8:4:5

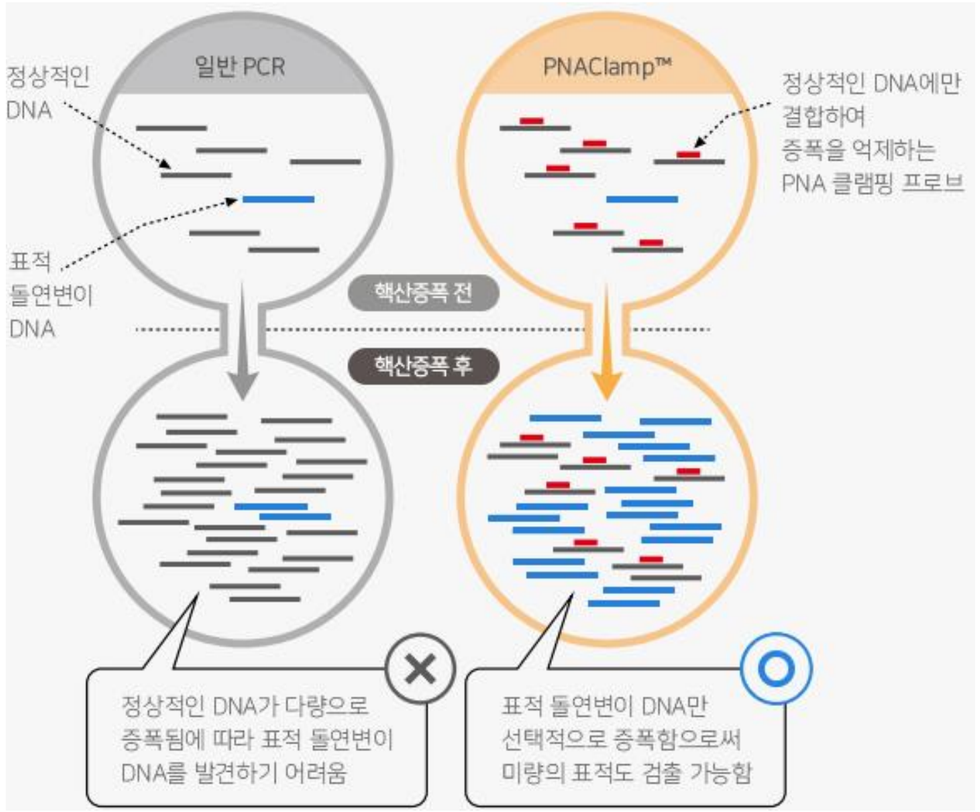
PCR is limited to pre-designed mutations.
It's ideal for confirming known variants, not for broad discovery.



B

	FAM	CFO 560	QS 670
MMX1	S768I	Ex19Del	IC
MMX2	T790M		IC
MMX3	L861Q	G719X	IC
MMX4	Ex20Ins	L858R	IC

Allele-Specific Real-Time PCR



PNA Clamp PCR

EGFR exon 20 insertions are heterogeneous !!

False negative >30%

	Sample	Detection limit	Detection				
			Exon18	Exon19	Exon20	Exon21	Total
Cobas EGFR mutation test	FFPE; plasma	1.3~13.4% (1~5%)	G719X (3)	Ex19Del (29)	S768I T790M Ex20Ins (5)	L858R (2) L861	42
PANAGene PANAMutyper	FFPE; plasma	<1%	G719X (3)	Ex19Del (29)	S768I T790M Ex20Ins (10)	L858R (2) L861Q	47
GenesWell ddPCR	FFPE; plasma	<1%	G719X (3)	Ex19Del (59)	S768I T790M Ex20Ins (33) C797X (4)	L858R (2) L861Q	107

EGFR Exon 20 Insertion

- **Test method: PCR** (widely used); DNA-based NGS
- **Tissue requirement:** Small biopsy samples acceptable (≥10% tumor cellularity)
- **Note:** Coverage of insertion variants may vary depending on the PCR kit
- **TAT consideration:** PCR can be prioritized; NGS should be ordered concurrently

→ NGS: all novel EGFR mutation can be detected

PCR kit	%
PANAGene PANAMutyper	25/30 (83.3)
GenesWell ddPCR	4/30 (9.1)
Cobas EGFR mutation test	3/30 (4.5)

KRAS G12C

- **Test method: PCR** (widely used); DNA-based NGS
- **Tissue requirement:** Small biopsy samples acceptable (≥10% tumor cellularity)

PCR kit	%
PANAGENE (Oncotector Mutation Detection Kit)	100
QIAGEN (Therascreen KRAS Kit)	0

Detection limit: ~2%

G12A
G12D
G12R
G12C
G12S
G12V
G13D

BRAF V600E

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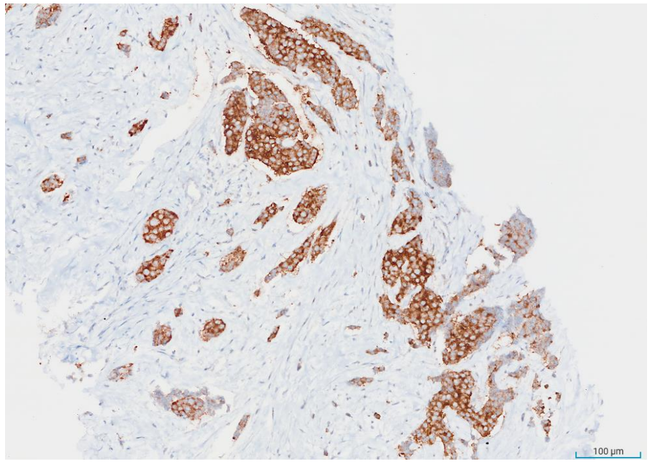
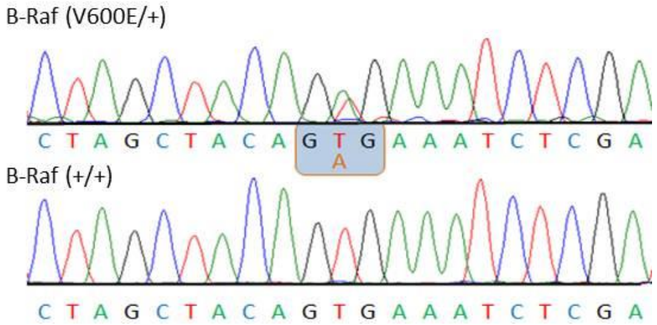
Screening

BRAF (VE1) IHC
- Sensitivity: 97.2%
- Specificity: 100%
- False negative rate: 2.9%



Confirmation

PCR
PNAClamp BRAF mutation kit
Detection limit: 1%



IHC Ab	%
VE1	100

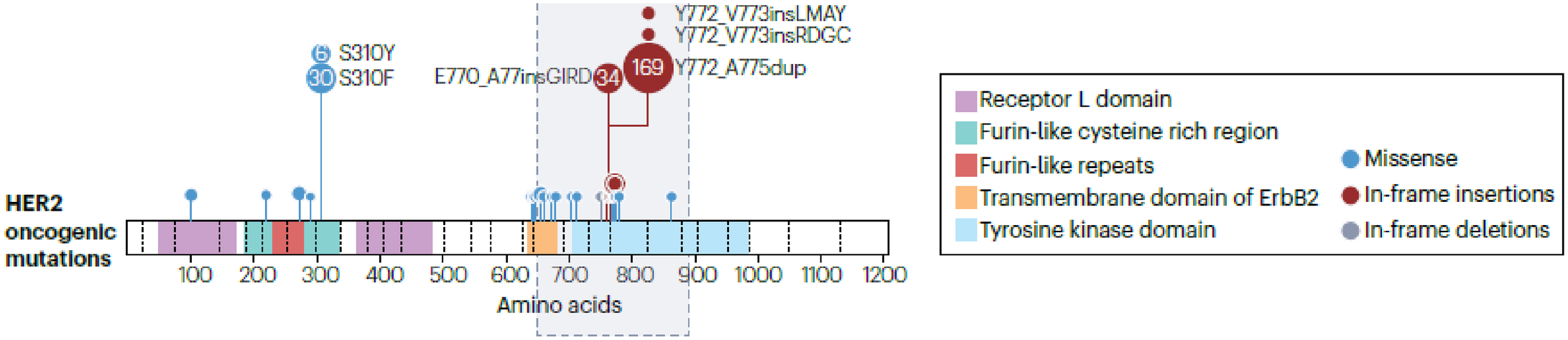
PCR kit	%
PNAClamp BRAF Mutation Detection Kit	78.6
Sanger sequencing	14.3
Biosewoom Real-Q BRAF V600E Detection Kit	7.1

33% start with IHC as a reflex test (screening) and confirm with PCR or sequencing

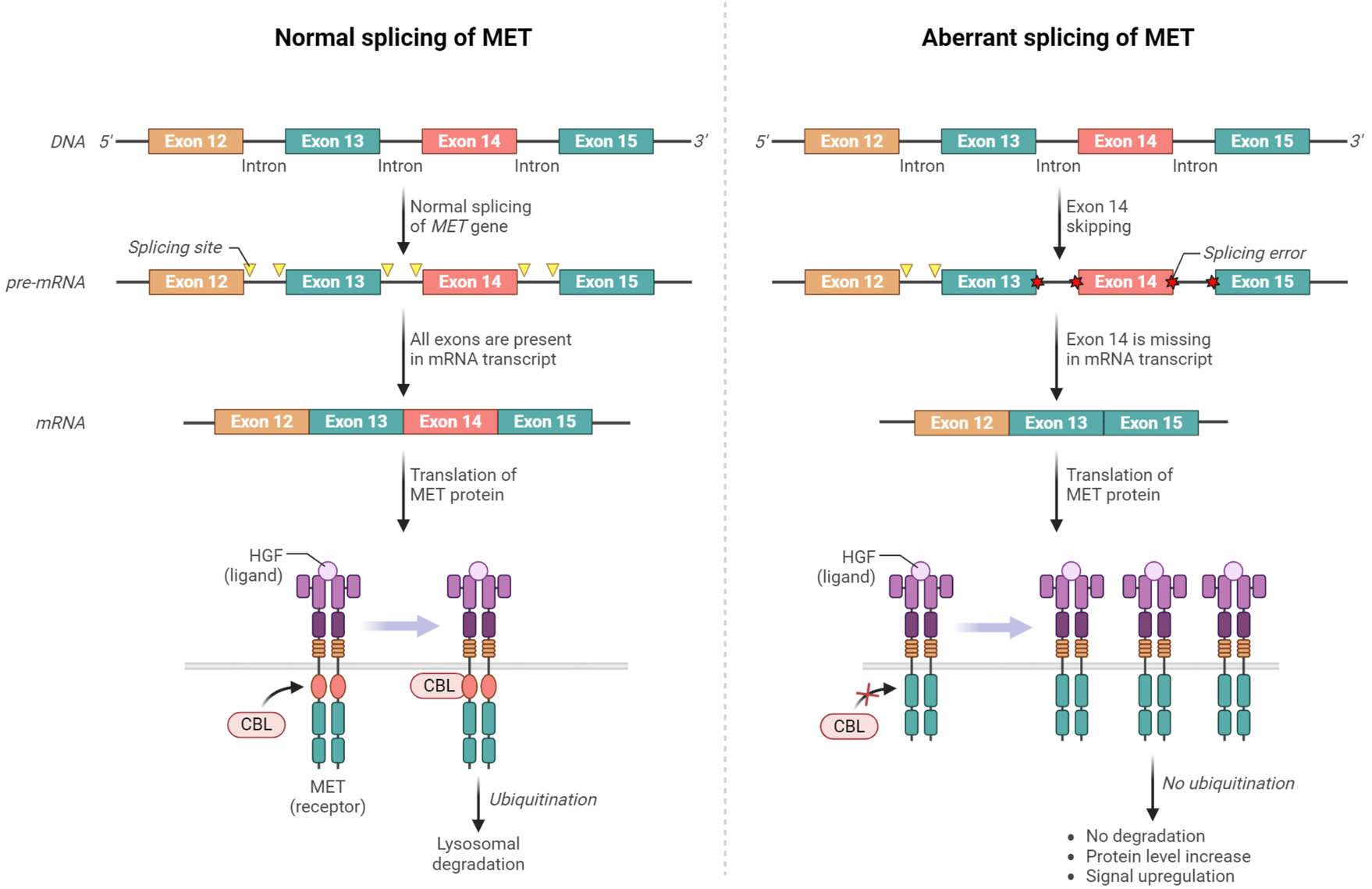
EP06.03-21 Usefulness of Immunohistochemistry for the Detection of BRAFV600E in Lung Cancer: A Multicenter Retrospective Study in China, Yuan, P. et al. JTO 2023, 18;11, S491 - S492

HER2 (ERBB2) mutation

- **Test method:** **DNA-based NGS** (PCR insufficient)
- **Tissue requirement:** Small biopsy samples acceptable (≥10% tumor cellularity)
- **Note:** HER2 mutation ≠ HER2 amplification (IHC/FISH results do not reflect HER2 mutations)



MET Exon 14 Skipping



MET Exon 14 Skipping

- **Test method: RNA-based NGS** (preferred)
  - RT-PCR (LDT) methods are still limited in Korea
- **Tissue requirement:** Ensure good RNA preservation (Recent fixation / high-quality FFPE block)
- **Note:** DNA-based NGS may miss splice variants

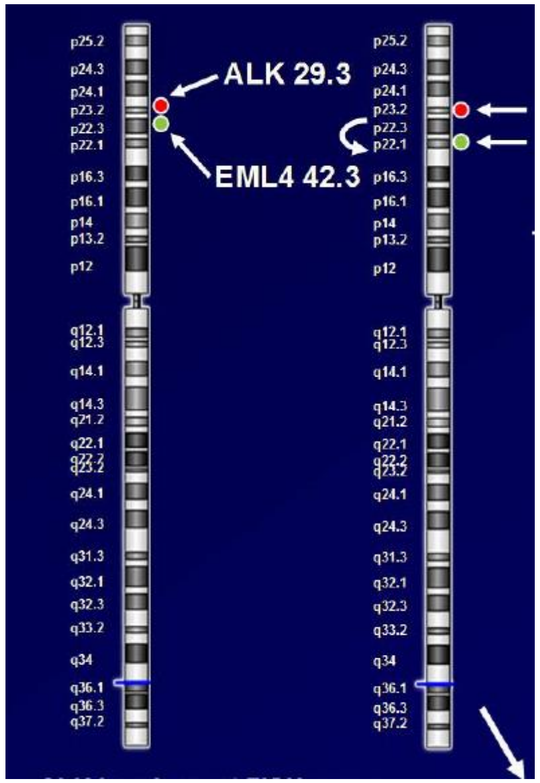
- **DNA-based NGS may miss splice variants**
  - Only captures **exonic or limited intronic** regions
- **Splice site mutations ≠ actual splicing event**
  - Functional effect is not always predictable
  - Cannot confirm **functional exon skipping**
- **RNA-based NGS detects actual exon skipping**

RT-PCR based test (AmoyDx Pan Lung PCR Panel): not commonly used in Korea

RET & NTRK fusion

Platform / Method	Fusion Detection Capability	Sample Type	Target Genes	Turnaround Time (TAT)	Remarks
IHC (for NTRK)	Low to moderate	FFPE tissue	TRK protein (pan-TRK)	1–2 days	**Screening**, not confirmatory
FISH (break-apart)	Moderate	FFPE tissue	RET, NTRK	2–4 working days	Useful for confirmation
RNA-based NGS	Excellent (targeted RNA)	FFPE RNA	RET, NTRK	7–10 working days	**Best**
DNA-based NGS	Limited (intronic coverage)	FFPE DNA	RET, NTRK	10–14 working days	May miss novel fusions

- RT-PCR based test (AmoyDx Pan Lung PCR Panel): not commonly used in Korea



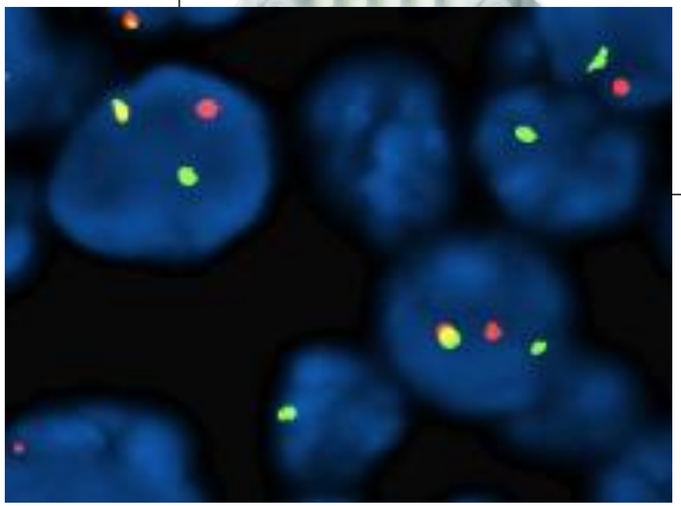
Product Description

Intended Use

The Vysis ALK Break Apart FISH Probe Kit is intended to detect rearrangements involving the ALK gene via fluorescence in situ hybridization (FISH) in formalin-fixed paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) tissue specimens.

Reduce variability with ready-to-use components

- Premixed, optimized probes
- ALK positive control slides
- ALK negative control slides
- Ready-to-use slide preparation reagents

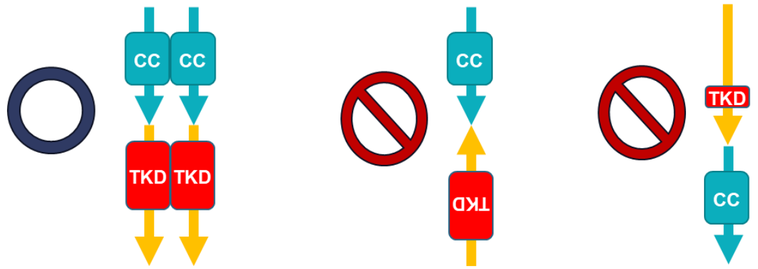


Fusion partner?
In-frame?

Labor-intensive
Requires a high level of expertise.
→ **Shift to NGS**
→ (In case of, ALK: IHC)

J Clin Oncol 2009;27:4247-4253

The Requirements for Fusion to be Functional



1. **In-frame fusion**
   - The orientation of two gene should be the same (sense to sense)
   - The fusion should not induce frameshift
2. **Retention of key functional domains**
   - e.g., kinase domain, DNA-binding domain must remain intact.
3. Promoter activation or enhancer hijacking
   - The fusion leads to overexpression of the oncogenic partner
4. Dimerization or oligomerization ability
   - Partner gene often provides domains like **coiled-coil** or **leucine zipper** to activate signaling.
5. Correct subcellular localization
   - Localization signals (e.g., NLS, membrane anchors) must direct the protein to its proper cellular compartment.

☐ **Structural changes detected by FISH ≠ Functional gene fusion**
RNA-based NGS is essential to confirm transcript-level events.

Why RET & NTRK Fusions Are Often Missed by DNA-Based Testing

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1. Intronic Breakpoints Are Unpredictable

- Most fusions occur in **large, variable introns**.
- Difficult to capture all possible breakpoints using DNA panels.

2. Intronic Regions Are Technically Challenging

- Introns are often **GC-rich or repetitive**, lowering sequencing and mapping efficiency.

3. Transcripts Matter, Not Just Genomic Structure

- **Only functional transcripts** produce proteins.
- DNA may capture structural rearrangements, but not confirm expression.

RNA-based NGS is the preferred method for detecting clinically actionable gene fusions.

RET & NTRK fusion

- **Test method: RNA-based NGS** (preferred)
  - Direct detection of transcripts
  - FISH can be used as a complementary tool
- **Tissue requirement:** Ensure good RNA preservation (Recent fixation / high-quality FFPE block)
- **Note:** DNA-based NGS may may have low sensitivity, especially for NTRK fusions

ROS1

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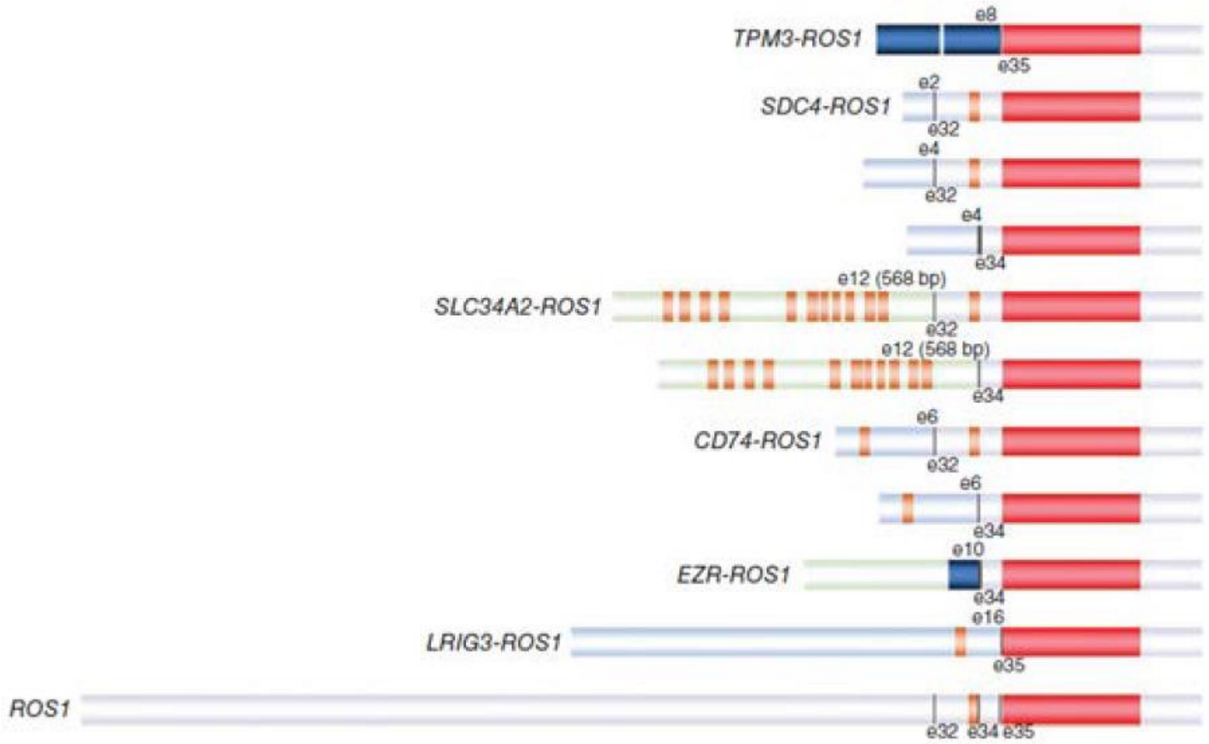
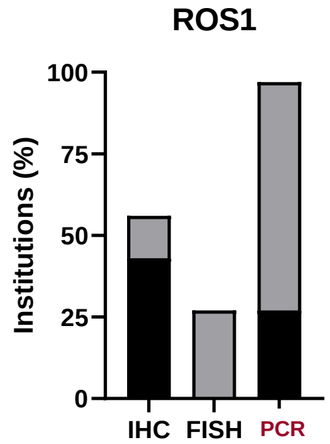
Variants of ROS1 **fusions**



Nat Med 2012;18:378-81.

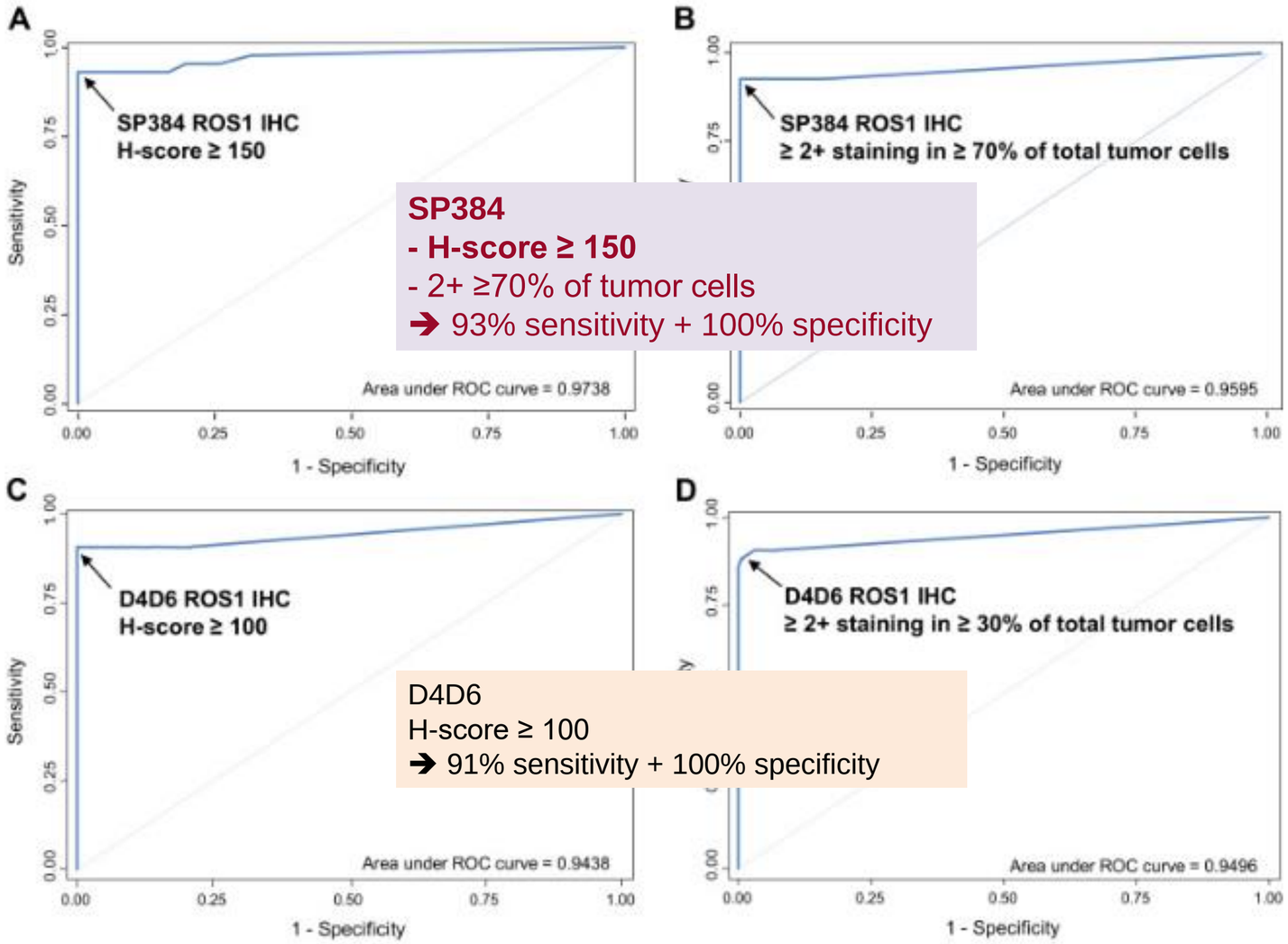


IHC Ab	%
SP384	94.1
D4D6	5.9

PCR kit	%
AmoyDx	100

About 43% screen with IHC first, then confirm with PCR

The ROSING Study



SP384
- H-score ≥ 150
- 2+ ≥70% of tumor cells
➔ 93% sensitivity + 100% specificity

D4D6
H-score ≥ 100
➔ 91% sensitivity + 100% specificity

EGFR, ALK, KRAS 등 key alteration negative시, PCR 고려

Assessment of a New ROS1 Immunohistochemistry Clone (SP384) for the Identification of ROS1 Rearrangements in Patients with Non-Small Cell Lung Carcinoma: the ROSING Study. J Thorac Oncol. 2019 Dec;14(12):2120-2132.

AmoyDx ® ROS1 Gene Fusions Detection Kit

***ROS1* Gene Fusions Detected by the Kit**

Reagent	Spliced Gene & Exon			ROS1 Spliced Exon
ROS1 Reaction Mix ①	*SLC34A2* exon4	*SLC34A2* exon13del	*CD74* exon6	32
	SDC4 exon2	*SDC4* exon4		
ROS1 Reaction Mix ②	*SLC34A2* exon4	*SLC34A2* exon13del	*CD74* exon6	34
	SDC4 exon4	*EZR* exon10		
ROS1 Reaction Mix ③	*TPM3* exon8	*LRIG3* exon16	*GOPC* exon8	35
ROS1 Reaction Mix ④	*GOPC* exon4			36

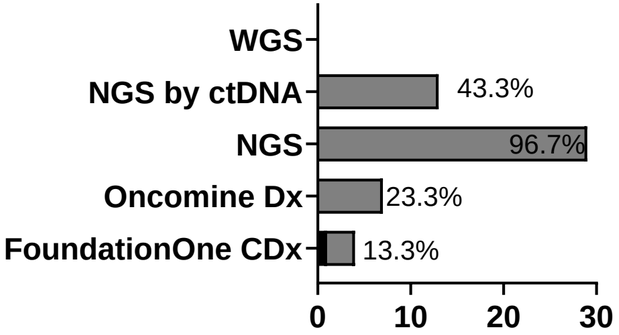
ROS1

- **Test method: RT-PCR based test** (widely used)**; RNA-based NGS** (preferred)
  - Direct detection of transcripts
  - FISH or IHC can be used as complementary screening tools
- **Tissue requirement:** Ensure good RNA preservation (Recent fixation / high-quality FFPE block)
- **Note:**
  - DNA-based NGS may miss ROS1 fusions due to large or complex intronic regions.
  - IHC may show false positives; confirmation by NGS or FISH is recommended.

Comprehensive genomic test
(NGS by tissue)

Types of molecular tests

For Broad molecular testing,



NGS

- 96.7% (29/30) of institutions
- In-house (72.4%) + Outsourced (27.6%)

NGS by ctDNA

- 43.3% (13/30) of institutions
- In-house (38.5%) + Outsourced (61.5%)

Custom panel (7.4% of institute)

Types	No of Genes	n
Lung cancer	75	1
Solid tumor	50-546	4

Commercial panel (92.6% of institute)

Types	No of Genes	n
Lung cancer	23	2
Solid tumor	323-550	29

Commercial panel provider,

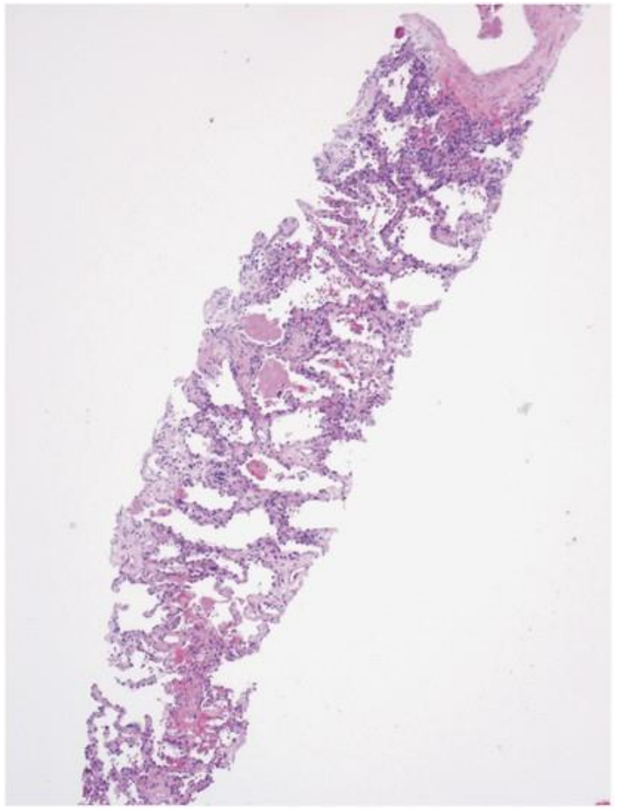
Illumina (53.3%) | Thermo Fisher (46.7%)

NGS

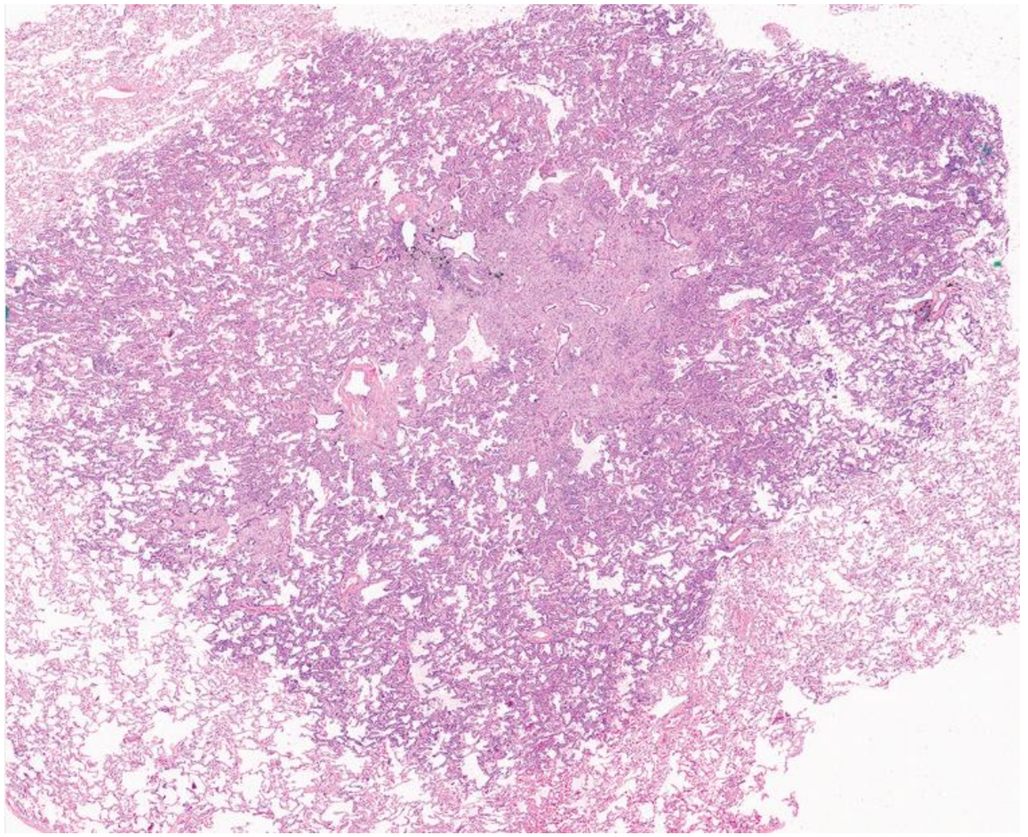
- 96.7% (29/30) of institutions
- Upon clinical request (100%)
- Through Pathology (86.2%), Clinical Laboratory department (3.4%) and Joint management with Clinical Laboratory department (10.3%)
- In-house (72.4%) + Outsourced (27.6%)

Single gene test vs NGS

	Single-Gene Testing	NGS
Comprehensiveness	Single-gene test; predefined targets	multi-gene coverage + multiple mutation types uncover rare or unexpected variants (for resistance work-ups, NGS provides a broader view of tumor evolution)
Analytical Sensitivity	Sanger (10-20%); PCR based (1-5% → <1%)	Comparable or even more sensitive
TAT	Shorter TAT	Longer (2-3wks)
Cost/Reimbursement	ALK (IHC), EGFR, ROS1, BRAF, KRAS (PCR) +NTRK1/2/3, MET, RET, HER2 Cumulative test cost: less expensive Patient's final burden: much lower	–
Tissue requirement	Cumulative, more	Less
Interpretation	A binary result for known targets.	Challenge of interpreting complex results Labor intensive



10μm
8-10 cut
DNA 1250~1750ng/50 μl
RNA 300-900ng/30 μl



10μm
4-6 cut
DNA 7500-10000ng/50 μl
RNA 450-1050ng/30 μl

Category	Thermo Fisher OCA Plus	Illumina TruSight Oncology 500
Sequencing Method	Amplicon-based target sequencing (DNA + RNA)	Hybrid capture-based target sequencing (DNA + RNA)
Number of Genes	517 genes (DNA + RNA) –fusion 49	523 genes (DNA), 55 genes (RNA fusion)
Detectable Variant Types	SNV, Indel, CNV, Fusion, Splice variants	SNV, Indel, CNV, Fusion, Splice variants
Fusion Detection Method	RNA-based AmpliSeq FusionSync	RNA-based hybrid capture; detects known and novel fusions
TMB Analysis	Yes (≥1Mb coverage; strong correlation with WES-based TMB)	Yes (mut/Mb based on ~1.94 Mb coding region)
MSI Analysis	Yes (76 markers; MSI score provided; proven high sensitivity)	Yes (Tumor-only MSI-H detection from microsatellite markers)
HRD Analysis	Yes; detects BRCA1/2 + 47 HRR genes and provides Genomic Instability Metric (GIM)	Not available (HRR genes present, but no HRD scoring)
Input Material (FFPE)	20 ng DNA + 20 ng RNA (as low as 10 ng each)	40 ng DNA + 40 ng RNA
Turnaround Time	~3 days (faster with Genexus:1-2day?)	~5-7 days (라이브러리 준비 2일 + Illumina 시퀀싱 1~2일 + 분석 1일)
Analytical Sensitivity	SNV: 99.6%; Fusion: 100%; CNV (≥8 copies): 97.6%	>96% (validated at 5% VAF); Fusion: ~5 copies/ng RNA; CNV: 2.2FC
Analytical Specificity	High (SNV specificity ~97-8%; Fusion ~97.5%)	> 99.99% (with UMI correction)
Bioinformatics Platform	Torrent Suite + Ion Reporter; Oncomine Reporter for clinical reporting	TSO500 Local App or DRAGEN pipeline
Panel Cost (per sample)	상대적 저렴; 장기 초기 비용 상대적으로 저렴	시퀀싱 장비 고가

Current Best-Practice Paradigm: Finding the Balance

- NGS panel testing has emerged as the preferred strategy for comprehensive mutation analysis.

- The **optimal strategy** often combines both approaches.
  - Rapid single-gene tests may be used for certain critical markers (to expedite treatment decisions), while broad NGS panels provide a comprehensive profile.
  - Histologic Dx + PD-L1 + EGFR/ALK/KRAS/ROS1/BRAF
  - NGS

감 사 합 니 다