

PANAMutyper EML4-ALK Screening Kit

INTENDED USE

The PANAMutyper™ EML4-ALK Screening Kit is an in vitro diagnostic reagent for detecting 16 different EML4-ALK fusion genes of formalin-fixed paraffin-embedded (FFPE) tissue fresh, tissues and biopsy tissues from non-small cell lung cancer (NSCLC) patients.

This kit is an amplified RNA test for the qualitative detection using peptide nucleic acid (PNA) probes, Ct and melting temperature (Tm) analysis in a real-time PCR (polymerase chain reaction) system.

Table 1. Target genes detected by this kit

No.	EML4-ALK Fusion Types	EML4 Last observed exon	ALK First observed exon
1	E2;A20	Exon 2	Exon 20
2	E2;ins117A20		
3	E6;A20	Exon 6	
4	E6ins33;A20		
5	E6;ins18A20		
6	E6;A19		Exon 19
7	E13;ins69A20	Exon 13	Exon 20
8	E13;A20		
9	E14ins11;del49A20	Exon 14	
10	E14;del14A20		
11	E14ins2;ins56A20		
12	E17;ins30A20	Exon 17	
13	E17ins61;ins34A20	Exon 18	
14	E18;A20	Exon 20	
15	E20;A20		
16	E20;ins18A20		

The PANAMutyper™ EML4-ALK Screening Kit is a CE marked diagnostic device in accordance with the European Union *in vitro* Diagnostic Medical Device Directive 98/79/EC.

PANAMutyper EML4-ALK Screening Kit

PRINCIPLE AND OVERVIEW

PANAMutyper™ EML4-ALK Screening Kit uses PNA probe-based fluorescence melting curve analysis technology in a real-time PCR system. Each specific PNA probe, which is conjugated with a fluorescent dye and a quencher, is used as a reporter in a real-time PCR reaction. These PNA probes are designed to hybridize only to their specific target sequence during the annealing step of PCR reaction. This specific binding is interfered by even a single mismatch between a PNA probe and a target sequence. Therefore, this PNA probe system has high specific target detection property and can be used to detect multiple targets in single PCR reaction.

Furthermore, PANAMutyper™ EML4-ALK Screening Kit implement melting curve analysis, and then the target genes are determined by measuring a melting temperature. The specific PNA probes have each unique T_m value. The combination of unique T_m and pre-determined fluorescent dye of PNA probe is used for detecting amplified human genomic RNA in the PCR reaction. This kit allows detecting in a single PCR reaction.

The principle of the assay outlined in Figure 1. The kit is designed for detecting of a fragment in the unique gene from clinical specimens. Human genomic RNA is extracted from clinical specimens simultaneously. (Specimen collection and RNA extraction kits are not part of the kit.)

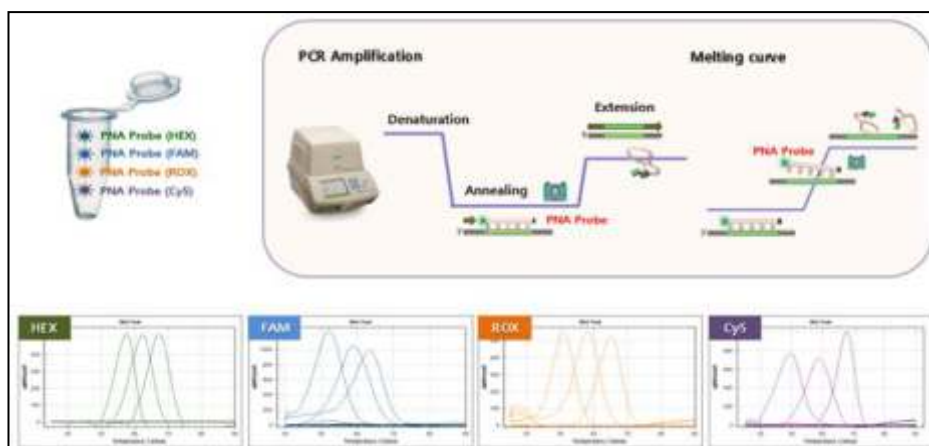


Figure 1. Principle of the PANAMutyper™ EML4-ALK Screening Kit

EML4-ALK Screening Mix tube contains a fluorescent dye (FAM, HEX (or VIC)) and a quencher conjugated specific PNA probes. The EML4-ALK fusion genes can be detected by analyzing the unique T_m value.

PANAMutyper EML4-ALK Screening Kit

EQUIPMENT AND MATERIALS SUPPLIED BY THE USER

- ✓ Reagents and equipment for RNA extraction
 - ✓ Pipettes (capacity 10 µl, 20 µl, and 200 µl)
 - ✓ Filter pipette tips
 - ✓ Bench top microcentrifuge
 - ✓ Vortex mixer
 - ✓ Disposable gloves, powder-free
- ◆ It is recommended to use below PCR system and plastic consumables for the best performance.

Table 2. Compatible real time PCR instruments and plastic consumables

Company	Model	Consumables
Bio-Rad	CFX96	➤ White PCR plate (Catalog No. BRMLL-9651) ➤ Adhesive seals (Catalog No. MSB-1001)
Applied Biosystems	QuantStudio® 5	➤ 96 well plate (Catalog No. N8010560) ➤ Adhesive film (Catalog No. 4311971)

PANAMutyper EML4-ALK Screening Kit

WARNINGS AND PRECUTIONS

- ✓ Please read carefully this instruction and become familiar with all components of the kit prior to use.
- ✓ PANAMutyper™ EML4-ALK Screening Kit is for *in vitro* diagnostic use.
- ✓ This kit should be used by trained laboratory professionals.
- ✓ All experiments should be performed under proper clean conditions in order to prevent contamination. It is recommended that a user has separate, dedicated pipettes and filter pipette tips to add RNA template and prepare PCR reagents.
- ✓ Always wear powder-free gloves when you handle the kit.
- ✓ To avoid repeated freezing and thawing, aliquot all reagents into appropriate volumes and store frozen until use. Thaw appropriate volumes of reagents before each experiment.
- ✓ All experimental procedures should be performed at room temperature. However, please minimize exposure time of RT-Taq Polymerase at room temperature for the optimal amplification.
- ✓ Dissolve reagents completely and mix them thoroughly by vortex.
- ✓ Tubes should be briefly centrifuged before use.
- ✓ Tubes containing PNA probe should be protected from prolonged exposure to light.
- ✓ Use only recommended instrument and consumables only. If not, it may cause loss of performance and increase the chance of false result.
- ✓ Additional validation testing by a user may necessary when non-recommended instrument is used.
- ✓ Do not use incorrect volume of reagent or target RNA; it may cause loss of performance and increase the chance of false result.
- ✓ Do not interchange or mix reagents from different lots or other manufacture's product.
- ✓ Do not re-use any remaining reagents after PCR amplification is completed.
- ✓ Do not use the reagents after their expiration date.

PANAMutyper EML4-ALK Screening Kit

STORAGE CONDITION AND STABILITY

The PANAMutyper™ EML4-ALK Screening Kit is shipped on ice packages and must still be frozen on arrival. If the kit is not frozen on arrival please contacts PANAGENE or the local distributor (see back cover).

The PANAMutyper™ EML4-ALK Screening Kit should be stored immediately upon receipt below - 20°C. When stored under the recommended storage conditions, the kit is stable until the labeled expiration date.

After open the kit, reagents can be stored in their original packaging below -20°C for 90 days or until the expiration date, whichever comes first.

KIT CONTENTS

◆ A total of 24 samples can be tested using a kit.

Table 3. Reagents provided in the PANAMutyper™ EML4-ALK Screening Kit

No.	Name of content	Description	Volume	Label & color of cap
1	EML4-ALK Screening Mix	PNA probes and primers	550 µl	EML4-ALK S
2	Negative Control	Negative Control	100 µl	NC
3	Positive Control	Positive Control	100 µl	PC
4	RT-Taq Polymerase	RT-Taq Polymerase	100 µl	RT-Taq

PANAMutyper EML4-ALK Screening Kit

PROCEDURES

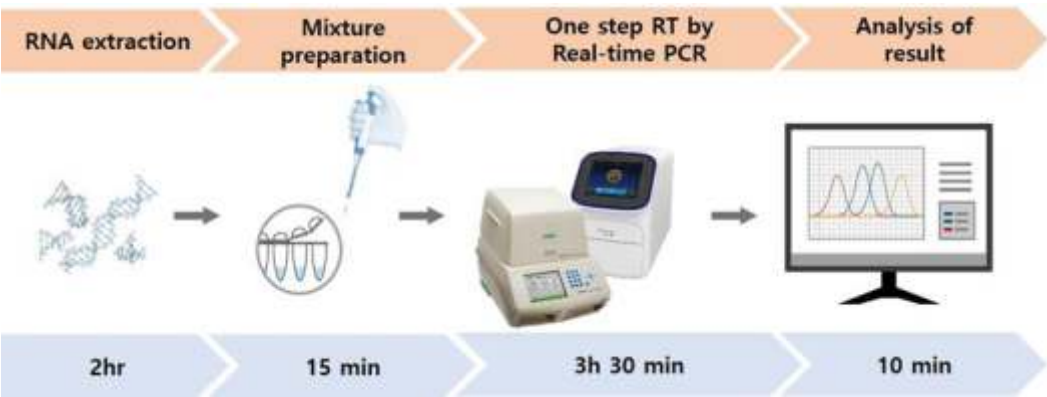


Figure 2. Workflow of the PANAMutyper™ EML4-ALK Screening Kit

1. Sample Preparation and Storage

Specimen collection and RNA extraction reagents are not included in the kit so they should be provided by the user.

- 1) Formalin fixed paraffin embedded (FFPE) tissues, fresh tissues and biopsy tissues can be used as specimens.
- 2) Specimen transport: Use standard pathology methods to ensure specimen quality.
- 3) For RNA extraction, it is recommended to use the following RNA extraction kits in Table 4.

Table 4. The list of recommended RNA isolation kit

Type	Model	Company
FFPE tissue	PureLink™ FFPE Total RNA isolation Kit	Invitrogen Corporation (USA)
Fresh tissues and biopsy tissues	easy-BLUE™ Total RNA Extraction Kit	iNtRON Biotechnology (Republic of Korea)

- 4) Extracted RNA can be stored at 4°C for up to 24 hours, or at -80°C for up to 3 months if it is over 24 hours after RNA extraction.

PANAMutyper EML4-ALK Screening Kit

2. Preparation of the Real-Time PCR Mixture

Table 5. Set up reaction mixture per one reaction

Reagent	Volume*
EML4-ALK Screening Mix	19 μ l
RT-Taq Polymerase	1 μ l
Extracted RNA, Positive Control(PC), or Negative Control(NC)	5 μ l
Total volume	25 μ l

* Prepare one extra volume for each component to compensate pipette error.

- 1) Prepare the reagent mix (EML4-ALK Screening Mix) after thaw, vortex and spin down at room temperature.
- 2) Prepare test sample (extracted RNA) and control samples (NC and PC).
- 3) Prepare the PCR plate. Label them as A1 if it is necessary.
- 4) Load 19 μ l of the reagent mix (EML4-ALK Screening Mix) into the PCR plate.
For example, A1 well will contain EML4-ALK Screening Mix.
- 5) Add 1 μ l of RT-Taq Polymerase to the PCR plate.
- 6) Add 5 μ l of prepared test sample into each well of the PCR plate to yield a total 25 μ l of final volume.
- 7) One set of PC and NC for the EML4-ALK Screening Mix should be included in each run.
Add 5 μ l of PC or NC into wells of the PCR plate to yield a total 25 μ l of final volume.
- 8) Immediately close the seal the PCR plate tightly and spin down. Otherwise, the PCR mixture can be evaporated and the result of the test may not accurate.

PANAMutyper EML4-ALK Screening Kit

3. Real-time PCR reaction

- 1) Place the prepared the PCR plate on the block of a real-time PCR instrument.
- 2) Please set the PCR protocol according to following Table 6.

Table 6. Real-time PCR protocol for PANAMutyper™ EML4-ALK Screening Kit

ONE CYCLE		
cDNA synthesis	55℃	15 min
	60℃	15 min
Pre-denaturation	95℃	15 min
3-STEP CYCLING (45 CYCLES)		
Denaturation	95℃	30 sec
Annealing and Detection*	60℃	45 sec
Extension	72℃	30 sec
MELTING CURVE ANALYSIS		
72℃		10 min
95℃		5 min
35℃		5 min
35℃ to 80℃ (increment 0.5℃)*		5 sec

- 3) Select four fluorescent dyes (FAM, HEX for CFX96 and FAM, VIC for QuantStudio® 5) for all reaction wells (*).

4. PCR result and data analysis

- 1) Set the baseline threshold of melting peak analysis according to Table 7.

Table 7. The baseline threshold values for Tm determination

CFX96		QuantStudio® 5	
Fluorescent Dye	Baseline threshold values	Fluorescent Dye	Baseline threshold values
FAM	80	FAM	6,000
HEX	80	VIC	6,000

PANAMutyper EML4-ALK
Screening Kit

- 2) For C(t) determination, set regression mode for CFX96 and set baseline of VIC amplification plot (linear graph type) as 1/20 value of the max ΔR_n .
- 3) Assess the results according to the fluorescent dyes, Ct and melting temperatures listed on Tables 8 and 9.

A. Negative Control and Positive Control

The melting temperatures and Ct for Negative Control (NC) and Positive Control (PC) must fall into the ranges that given in Table 8. The assay must be repeated if the values are not in these recommended ranges.

Table 8. The Acceptable ranges for NC and PC

Fluorescent Dye	Parameter	Negative Control	Positive Control
FAM	Melting Temperature (°C)	-	54.0 - 63.5
HEX (or VIC)	Melting Temperature (°C)	55.5 - 61.5	55.5 - 61.5
	Ct	Ct ≤33	Ct ≤33

B. Sample

If the melting temperatures and Ct of each fluorescent dye in EML4-ALK Screening Mix is in the criteria range (Table 9), please assess the result as ‘Positive’. If the Tm of each fluorescent dye is out of the criteria range, please assess the result as ‘Negative’.

Table 9. Criteria of EML4-ALK Detection

Fluorescent Dye	Parameter		Assessment
FAM	Melting Temperature (°C)	54.0 - 63.5	EML4-ALK positive
HEX (or VIC)	Melting Temperature (°C)	55.5 - 61.5	Internal Control Valid
	Ct	Ct ≤33	

* Tm values are rounded to second decimal places and applied to the criteria

PANAMutyper EML4-ALK Screening Kit

Determine the acceptability of RNA samples according to the values of Internal Control. Ct value of Internal Control indicates the purity and concentration of RNA. Thus, the validity of the test can be decided by Ct value and melting temperature as shown in Table 10.

Table 10. Acceptability of Samples

Fluorescent Dye	Ct	Acceptability		Descriptions and recommendations
HEX (or VIC)	$Ct \leq 28$	Valid	Optimal	The amplification and the amount of RNA sample are optimal
	$28 < Ct \leq 33$		Acceptable	The target gene was amplified with low efficiency. For more reliable result, it is suggested that repeat PCR reaction with a higher amount of RNA.
	$Ct > 33$	Invalid		The PCR amplification failed. Please check the RNA amount and purity. It might be required to extract RNA again.

C. Interpretation of results

Test results are interpreted as shown in Table 11.

Table 11. Interpretation of results

Internal Control	EML4-ALK Screening Mix	Interpretation
Valid	Positive	EML4-ALK positive*
Valid	Negative	EML4-ALK negative†
Invalid	Positive or Negative	Invalid‡

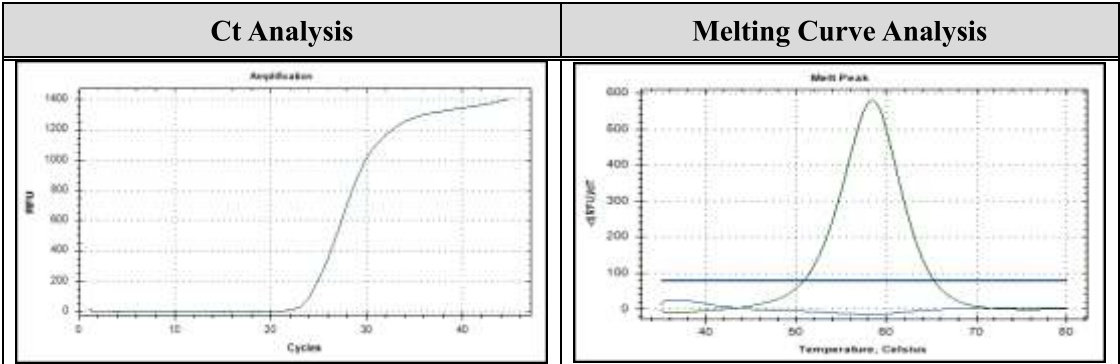
* If the sample is invalid, please refer to the description and recommendations for the sample as given in Table 10.

PANAMutyper EML4-ALK
Screening Kit

EXAMPLES OF ANALYSIS

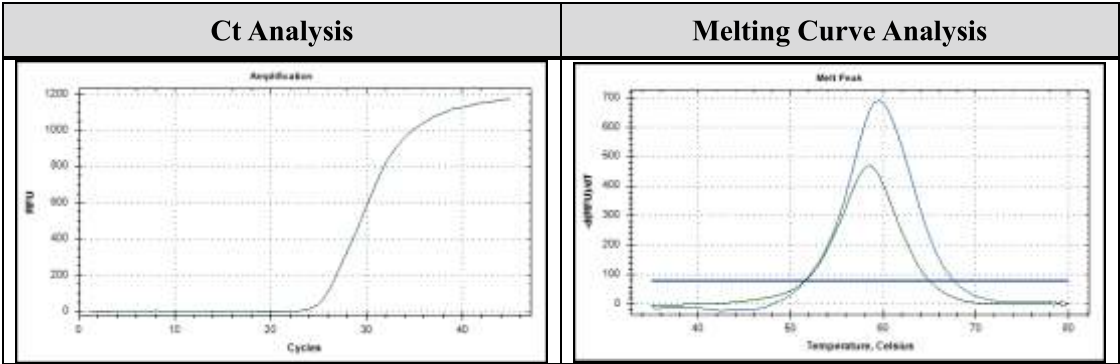
1. Using Bio-Rad CFX96

1) Negative Control (NC)



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Acceptable
HEX	58.5	23.23	I.C Valid	

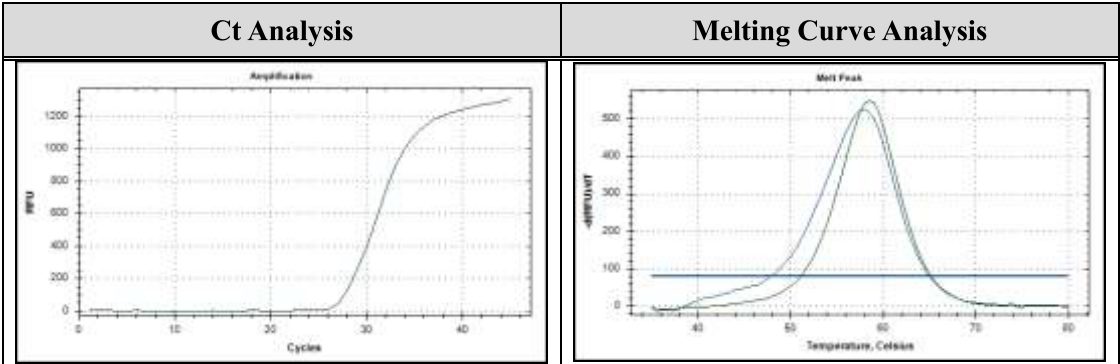
2) Positive Control (PC)



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	59.5	-	Positive	Acceptable
HEX	58.5	24.92	I.C Valid	

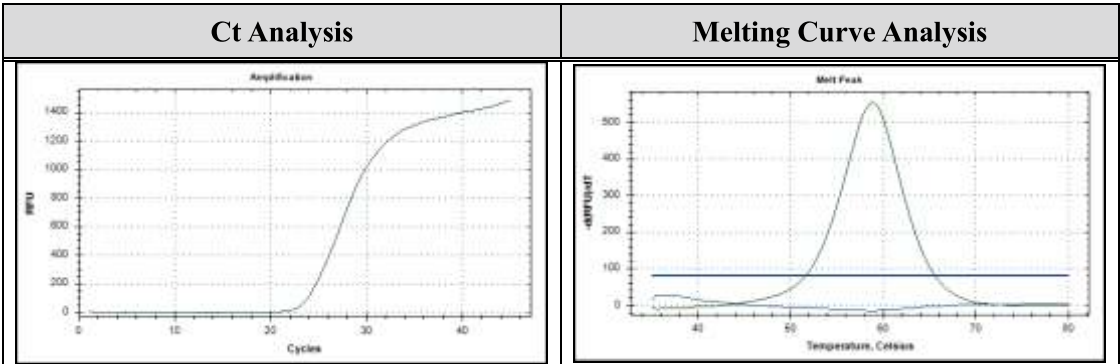
PANAMutyper EML4-ALK
Screening Kit

3) Sample 1



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	58.0	-	Positive	Positive
HEX	58.5	26.84	I.C Valid	

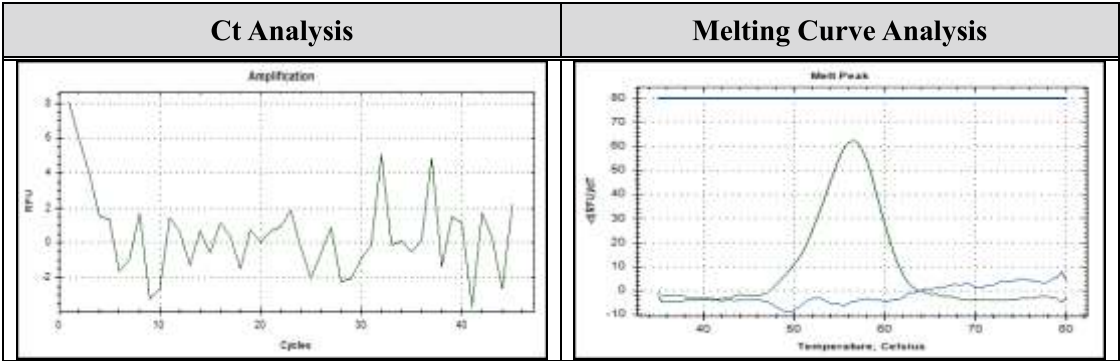
4) Sample 2



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Negative
HEX	59.0	23.06	I.C Valid	

PANAMutyper EML4-ALK
Screening Kit

5) Sample 3

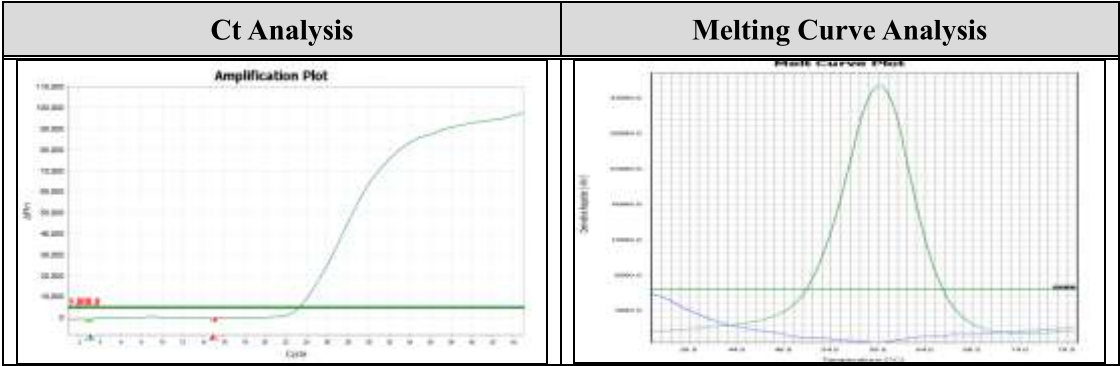


Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Invalid
HEX	-	N/A	I.C Invalid	

PANAMutyper EML4-ALK
Screening Kit

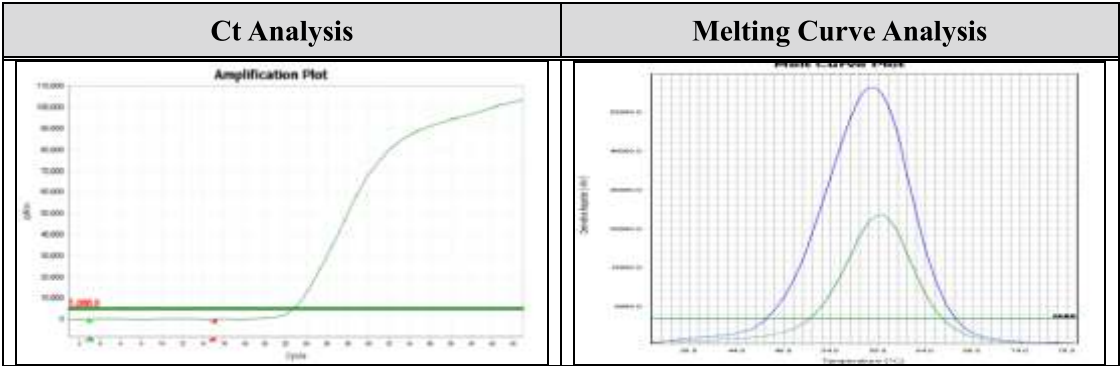
2. Using ABI QuantStudio® 5

1) Negative Control (NC)



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Acceptable
VIC	59.1	23.24	I.C Valid	

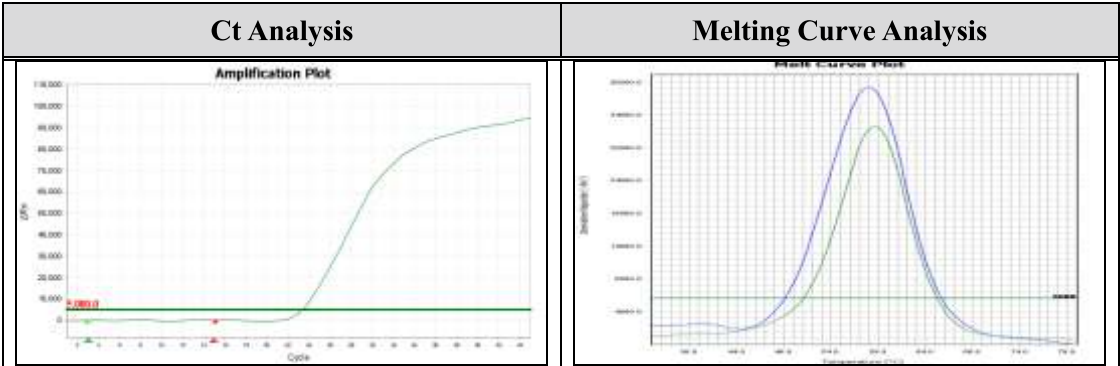
2) Positive Control (PC)



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	58.3	-	Positive	Acceptable
VIC	59.1	22.80	I.C Valid	

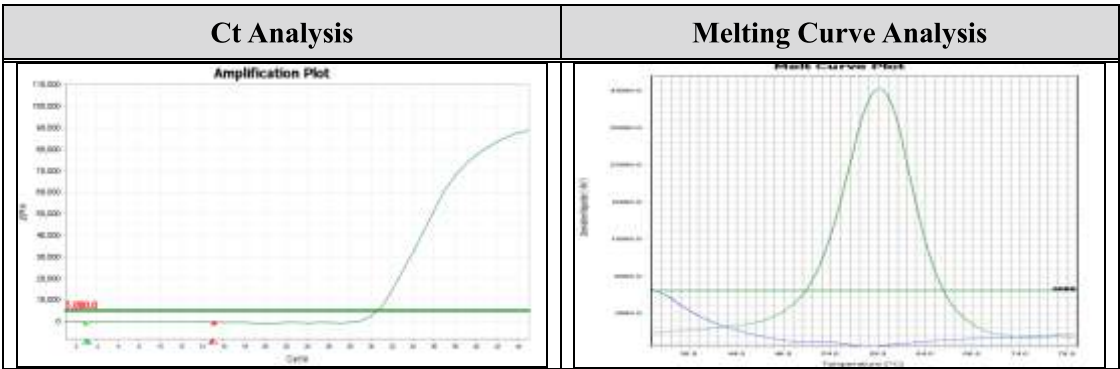
PANAMutyper EML4-ALK
Screening Kit

3) Sample 1



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	58.1	-	Positive	Positive
VIC	58.6	30.43	I.C Valid	

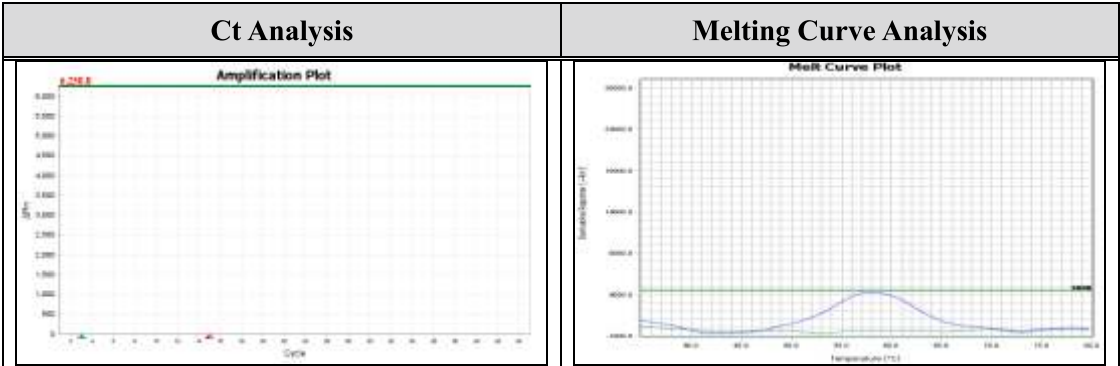
4) Sample 2



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Negative
VIC	59.1	23.32	I.C Valid	

PANAMutyper EML4-ALK
Screening Kit

5) Sample 3



Fluorescent Dye	Melting Temperature (°C)	Ct	Assessment	Result
FAM	-	-	Negative	Invalid
VIC	-	N/A	I.C Invalid	

PANAMutyper EML4-ALK Screening Kit

QUALITY CONTROL

Each lot of PANAMutyper™ EML4-ALK Screening Kit is tested against predetermined specifications to ensure consistent product quality in accordance with PANAGENE’s ISO 9001 & 13485-certified Quality Management System.

PERFORMANCE TEST

1. Analytical Sensitivity

The limit of detection study was evaluated using three batches of kits and the standard materials, plasmids and cell lines containing the target gene. The detection limit for each target gene is summarized in Table 12.

Table 12. Limit of detection for PANAMutyper™ EML4-ALK Screening Kit

Fusion Gene	Limit of detection (LOD)
E14ins2; ins56A20	10 ³ copies/rxn
E13;A20	10 ³ copies/rxn
E20;A20	10 ³ copies/rxn
E14ins11;del49A20	10 ³ copies/rxn
E2;A20	10 ⁴ copies/rxn
E2;ins117A20	10 ⁴ copies/rxn
E13;ins69A20	10 ³ copies/rxn
E14; del12A20	10 ³ copies/rxn
E17;ins30A20	10 ³ copies/rxn
E17ins61;ins34A20	10 ³ copies/rxn
E6-ins18A20	10 ³ copies/rxn
E6-A19	10 ³ copies/rxn
E18-A20	10 ³ copies/rxn
E20-ins18A20	10 ³ copies/rxn
E13;A20 (V1)	1 ng/rxn
E6;A20(V3a), E6ins33;A20 (V3b)	0.1 ng/rxn

PANAMutyper EML4-ALK Screening Kit

2. Analytical Specificity

The cross reactivity study was evaluated using one batch of kits and RNA samples isolated from wild cell line without EML4-ALK fusion gene. The results showed no cross reactivity.

3. Reproducibility







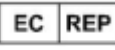



The reproducibility study was evaluated across testing sites, operators, runs, and days using two batches of kits and the standard materials, plasmids containing the target gene. All the results showed within 5% CV (coefficient of variation).

REFERENCES

1. Soda, M et al., Identification of the transforming EML4–ALK fusion gene in non-small-cell lung cancer. Nature, 448 (7153): 561-566, 2007.

2. Shaw, A. T et al., Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol, 27 (26):4247-4253, 2009.

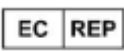
EXPLANATION OF SYMBOLS ON THE LABEL

	In Vitro Diagnostic Medical Device		Manufacturer
	Batch code		Contains Sufficient for <n> tests
	Catalogue number		Upper limit of storage temperature
	Authorized European representative		Use by
	Consult instructions for use		This product fulfills the requirements of the European Directive 98/79 EC for <i>in vitro</i> diagnostic medical devices.



PANAGENE Inc.

54, Techno 10-ro, Yuseong-gu, Daejeon, 34027, Korea



MT Promedt Consulting GmbH

Altenhofstrasse 80, 66386 St. Ingbert, Germany