INTENDED USE

The PANAMutyper™ ROS1 Screening Kit is an in vitro diagnostic reagent for detecting 20 different ROS1 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.	ROS1 Fusion Types	Partner Last observed exon	ROS1 First observed exon
1	CD6-R32	CD74 exon 6	exon 32
2	CD6-R34		exon 34
3	EZ10-R34	EZR exon 10	exon 34
4	SL4-R32	SLC34A2 exon 4	exon 32
5	SL4-R34		exon 34
6	SL13del-R32	SLC34A2 exon 13del	exon 32
7	SL13del-R34		exon 34
8	SD2-R32	SDC4 exon 2	exon 32
9	SD2-R34		exon 34
10	SD4-R32	SDC4 exon 4	exon 32
11	SD4-R34		exon 34
12	TP2-R35	TPM3 exon 2	exon 35
13	TP2-R36		exon 36
14	TP8-R35	TPM3 exon 8	exon 35
15	TP8-R36		exon 36
16	GO4-R35	GOPC exon 4	exon 35
17	GO4-R36		exon 36
18	GO8-R35	GOPC exon 8	exon 35
19	GO8-R36		exon 36
20	LR16-R35	LRIG3 exon 16	exon 35

The PANAMutyper™ ROS1 Screening Kit is a CE marked diagnostic device in accordance with the European Union *in vitro* Diagnostic Medical Device Directive 98/79/EC.

PRINCIPLE AND OVERVIEW

PANAMutyper™ ROS1 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™ ROS1 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 Tm value. The combination of unique Tm 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.)

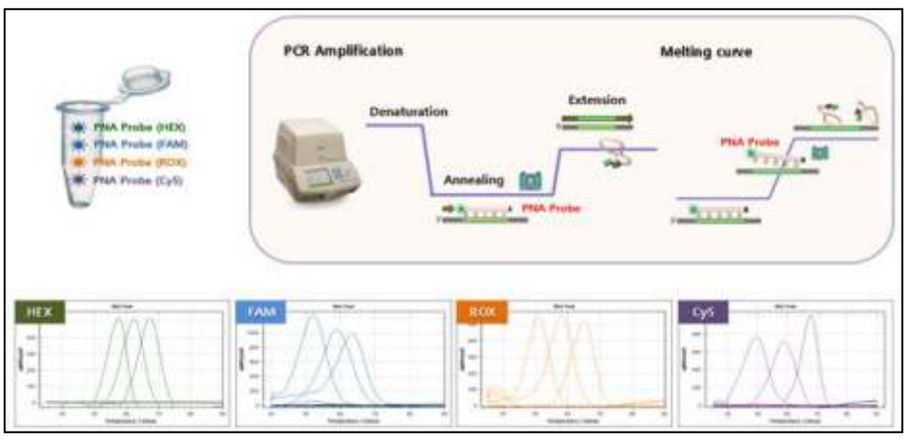


Figure 1. Principle of the PANAMutyper™ ROS1 Screening Kit

ROS1 Screening Mix tubes contain a fluorescent dye (FAM, HEX (or VIC)) and a quencher conjugated specific PNA probes. The ROS1 fusion genes can be detected by analyzing the unique Tm value.

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)
Thermo Fisher Scientific	QuantStudio® 5	➢ 96 well plate (Catalog No. N8010560) ➢ Adhesive film (Catalog No. 4311971)

WARNINGS AND PRECUATIONS

- ✓ **Please read carefully this instruction and become familiar with all components of the kit prior to use.**
- ✓ **PANAMutyper™ ROS1 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.

STORAGE CONDITION AND STABILITY

The PANAMutyper™ ROS1 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™ ROS1 Screening Kit should be stored immediately upon receipt below -20℃. 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℃ 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™ ROS1 Screening Kit

No.	Name of content	Description	Volume	Label & color of cap
1	ROS1 Screening Mix #1	PNA probes and primers	550 µl	**ROS1 S1**
2	ROS1 Screening Mix #2	PNA probes and primers	550 µl	**ROS1 S2**
3	Negative Control	Negative Control	200 µl	**NC**
4	Positive Control	Positive Control	200 µl	**PC**
5	RT-Taq Polymerase	RT-Taq Polymerase	100 µl	**RT-Taq**

PROCEDURES

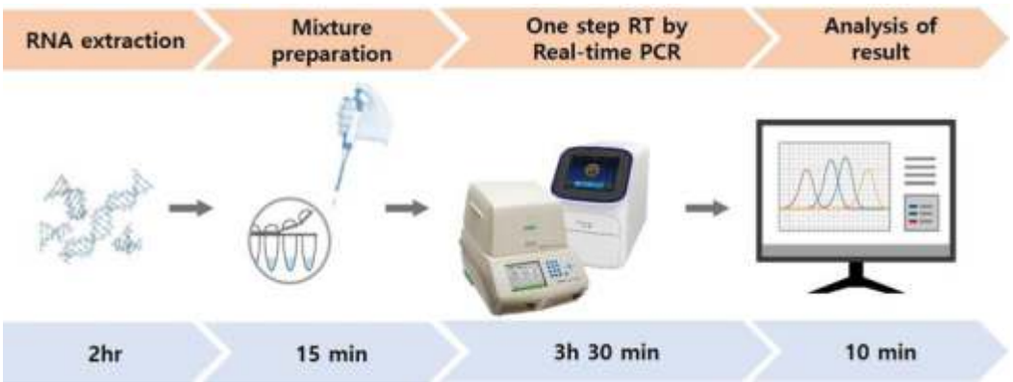


Figure 2. Workflow of the PANAMutyper™ ROS1 Screening Kit

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℃ for up to 24 hours, or at -80℃ for up to 3 months if it is over 24 hours after RNA extraction.

2. Preparation of the Real-Time PCR Mixture

Table 5. Set up reaction mixture per one reaction

Reagent	Volume*
ROS1 Screening Mix #1 and #2	19 µl
RT-Taq Polymerase	1 µl
Extracted RNA, Negative Control (NC), or Positive Control (PC)	5 µl
Total volume	25 µl

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

1) Prepare each reagent mix (ROS1 Screening Mix #1 and #2) 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 µl of each reagent mix (ROS1 Screening Mix #1 and #2) into the PCR plate.
   For example, A1 well will contain ROS1 Screening Mix #1 and A2 well will contain ROS1 Screening Mix #2.
5) Add 1 µl of RT-Taq Polymerase to the PCR plate.
6) Add 5 µl of prepared test sample into each well of the PCR plate to yield a total 25 µl of final volume.
7) One set of PC and NC for the ROS1 Screening Mix should be included in each run. Add 5 µl of PC or NC into each well of the PCR plate to yield a total 25 µl 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.

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™ ROS1 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 the 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

Reagent	CFX96		QuantStudio® 5	
	Fluorescent Dye	Baseline threshold values	Fluorescent Dye	Baseline threshold values
ROS1 Screening Mix #1	FAM	60	FAM	4,000
ROS1 Screening Mix #2	FAM	60	FAM	4,000
	HEX	80	VIC	6,000

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 ΔRn.

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

Reagent	Fluorescent Dye	Parameter	Negative Control	Positive Control
ROS1 Screening Mix #1	FAM	Tm (℃)	-	47.5 - 61.5
ROS1 Screening Mix #2	FAM	Tm (℃)	-	47.5 - 61.5
	HEX (or VIC)	Tm (℃)	54.0 - 62.0	54.0 - 62.0
		Ct	Ct ≤36	Ct ≤36

B. Sample

If the melting temperatures and Ct of each fluorescent dye in ROS1 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 ROS1 Detection

Reagent	Fluorescent Dye	Parameter		Assessment
ROS1 Screening Mix #1	FAM	Tm (℃)	47.5 - 61.5	ROS1 positive
ROS1 Screening Mix #2	FAM	Tm (℃)	47.5 - 61.5	ROS1 positive
	HEX (or VIC)	Tm (℃)	54.0 - 62.0	Internal Control Valid
		Ct	Ct ≤36	

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

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

Reagent	Fluorescent Dye	Ct	Acceptability		Descriptions and recommendations
ROS1 Screening Mix #2	HEX (or VIC)	Ct ≤33	Valid	Optimal	The amplification and the amount of RNA sample are optimal
		33< Ct ≤36		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 >36	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

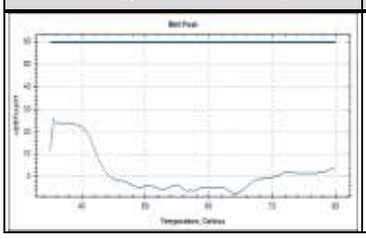
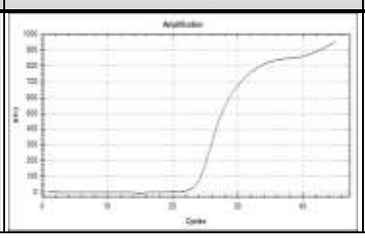
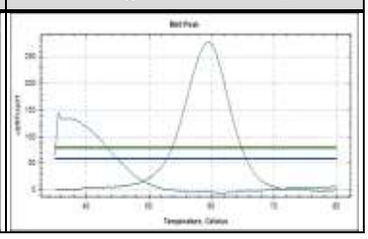
Internal Control	ROS1 Screening Mix #1 or #2	Interpretation
Valid	Positive	ROS1 positive*
Valid	Negative	ROS1 negative†
Invalid	Positive or Negative	Invalid#

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

EXAMPLES OF ANALYSIS

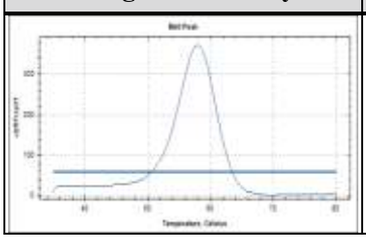
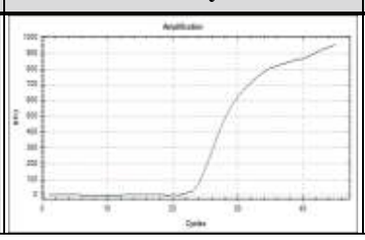
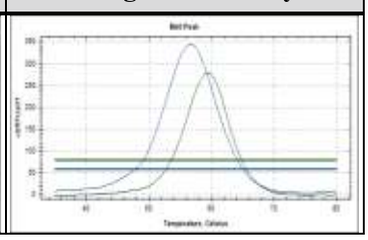
1. Using Bio-Rad CFX96

1) Negative Control (NC)

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	Ct Analysis	Melting Curve Analysis
		

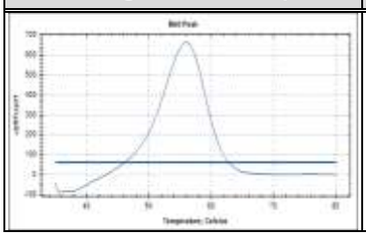
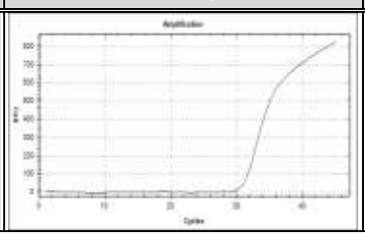
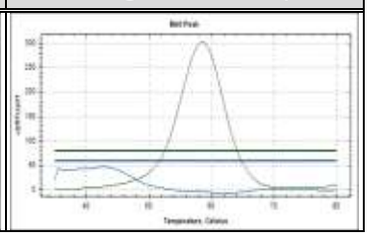
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	-	-	Negative	**Acceptable**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	HEX	59.5	22.52	I.C Valid	

2) Positive Control (PC)

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	Ct Analysis	Melting Curve Analysis
		

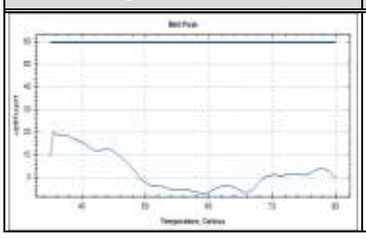
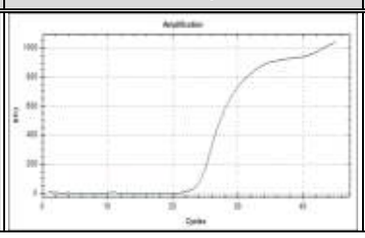
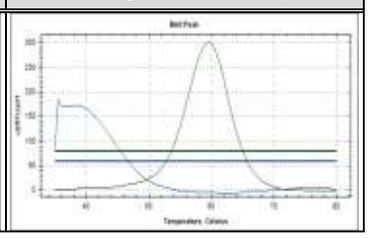
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	58.0	-	Positive	**Acceptable**
ROS1 Screening Mix #2	FAM	56.5	-	Positive	
	HEX	59.5	22.57	I.C Valid	

3) Sample 1

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

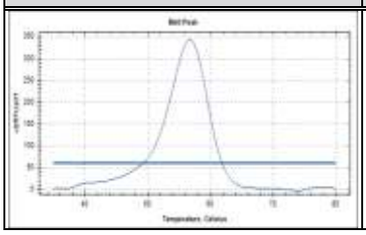
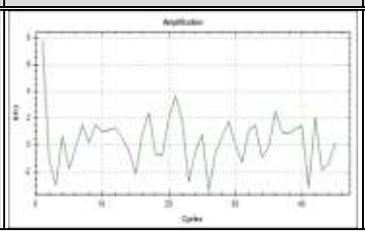
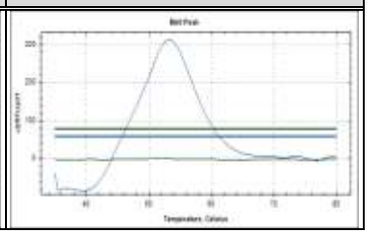
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	56.0	-	Positive	**Positive**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	HEX	58.5	29.56	I.C Valid	

4) Sample 2

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	-	-	Negative	**Negative**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	HEX	59.5	22.62	I.C Valid	

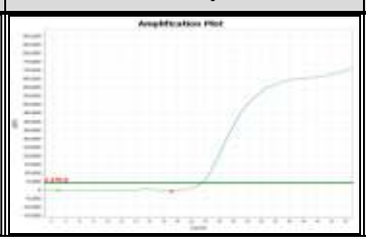
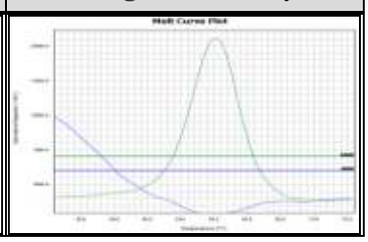
5) Sample 3

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	56.5	-	Positive	**Invalid**
ROS1 Screening Mix #2	FAM	53.5	-	Positive	
	HEX	-	-	I.C Invalid	

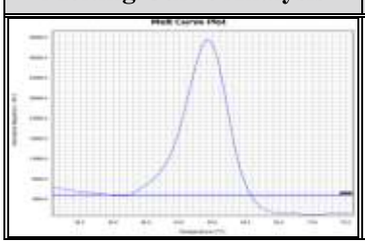
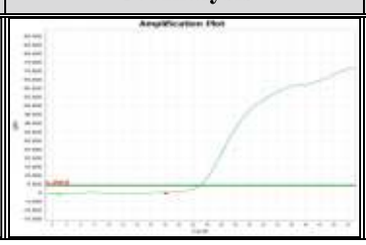
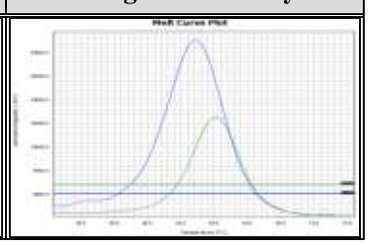
2. Using QuantStudio® 5

1) Negative Control (NC)

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	Ct Analysis	Melting Curve Analysis
		

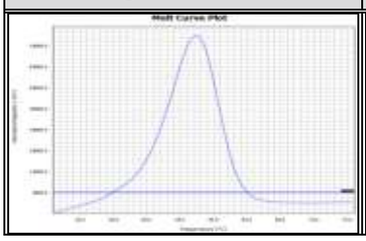
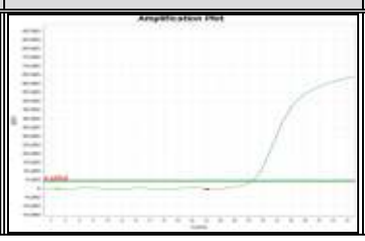
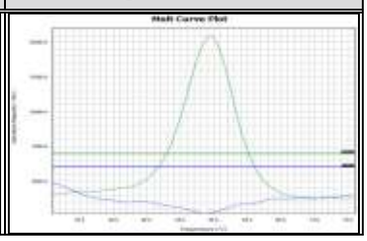
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	-	-	Negative	**Acceptable**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	VIC	59.3	23.49	I.C Valid	

2) Positive Control (PC)

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	Ct Analysis	Melting Curve Analysis
		

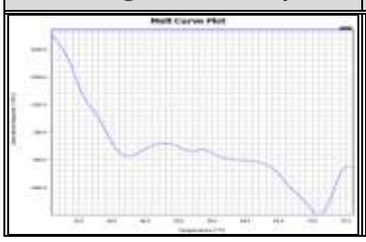
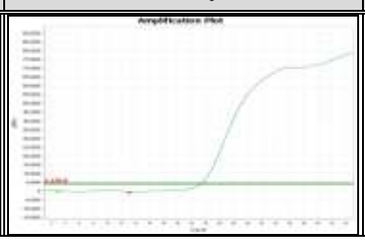
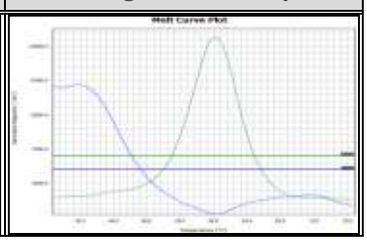
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	58.3	-	Positive	**Acceptable**
ROS1 Screening Mix #2	FAM	56.0	-	Positive	
	VIC	59.3	22.96	I.C Valid	

3) Sample 1

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

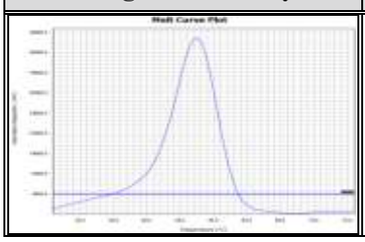
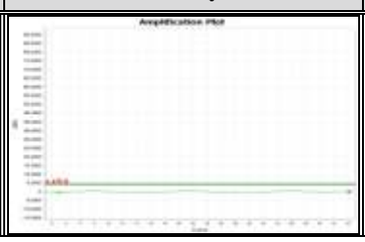
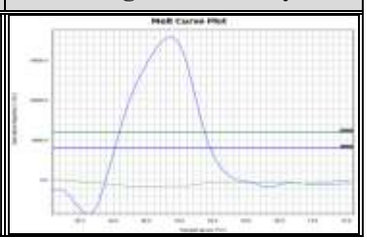
Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	56.3	-	Positive	**Positive**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	VIC	58.3	30.53	I.C Valid	

4) Sample 2

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	-	-	Negative	**Negative**
ROS1 Screening Mix #2	FAM	-	-	Negative	
	VIC	59.3	23.39	I.C Valid	

5) Sample 3

ROS1 Screening Mix #1	ROS1 Screening Mix #2	
Melting Curve Analysis	**Ct Analysis**	**Melting Curve Analysis**
		

Reagent	Fluorescent Dye	Tm (℃)	Ct	Assessment	Result
ROS1 Screening Mix #1	FAM	56.3	-	Positive	**Invalid**
ROS1 Screening Mix #2	FAM	52.6	-	Positive	
	VIC	-	-	I.C Invalid	

QUALITY CONTROL

Each lot of PANAMutyper™ ROS1 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™ ROS1 Screening Kit

Fusion Gene	Limit of detection (LOD)	Fusion Gene	Limit of detection (LOD)
CD74_exon6-ROS1_exon32	10^3 copies/rxn	TPM3_exon2-ROS1_exon35	10^3 copies/rxn
CD74_exon6-ROS1_exon34	10^3 copies/rxn	TPM3_exon2-ROS1_exon36	10^3 copies/rxn
EZR_exon10-ROS1_exon34	10^3 copies/rxn	TPM3_exon8-ROS1_exon35	10^4 copies/rxn
SLC34A2_exon4-ROS1_exon32	10^3 copies/rxn	TPM3_exon8-ROS1_exon36	10^5 copies/rxn
SLC34A2_exon4-ROS1_exon34	10^2 copies/rxn	GOPC(=FIG)_exon4-ROS1_exon35	10^3 copies/rxn
SLC34A2_exon13deletion-ROS1_exon32	10^3 copies/rxn	GOPC(=FIG)_exon4-ROS1_exon36	10^3 copies/rxn
SLC34A2_exon13deletion-ROS1_exon34	10^3 copies/rxn	GOPC(=FIG)_exon8-ROS1_exon35	10^3 copies/rxn
SDC4_exon2-ROS1_exon32	10^3 copies/rxn	GOPC(=FIG)_exon8-ROS1_exon36	10^3 copies/rxn
SDC4_exon2-ROS1_exon34	10^3 copies/rxn	LRIG3_exon16-ROS1_exon35	10^4 copies/rxn
SDC4_exon4-ROS1_exon32	10^3 copies/rxn	SLC34A2_exon4-ROS1_exon32, SLC34A2_exon4-ROS1_exon34	0.1 ng/rxn
SDC4_exon4-ROS1_exon34	10^3 copies/rxn		

2. Analytical Specificity

The cross reactivity study was evaluated using one batch of kits and RNA samples isolated from wild cell line without ROS1 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. Davies, K. D et al., Identifying and targeting ROS1 gene fusions in non–small cell lung cancer. Clin Cancer Res, 18 (17): 4570-5479, 2012.
2. Takeuchi, K et al., RET, ROS1 and ALK fusions in lung cancer. Nat Med, 18 (3):378-381, 2012.

EXPLANATION OF SYMBOLS ON THE LABEL

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

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