ALK/ROS1/RET Gene Fusion Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

Product Name

ALK/ROS1/RET Gene Fusion Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Package Specification

6 Tests/Kit

Intended Use

This kit uses multiplex fluorescence PCR amplification technology to qualitatively detect ALK/ROS1/ RET gene fusion mutation in RNA samples extracted from paraffin embedded tissue sections of non-small cell lung cancer patients (see Annex for details).

Summary

The fusion of EML4 gene (Echinoderm microtubule-associated protein-like 4) and ALK gene (anaplastic lymphoma kinase) is a common fusion type in non-small cell lung cancer. EML4-ALK fusion leads to the continuous expression of ALK gene encoding transmembrane tyrosine kinase receptor, which activates ALK tyrosine kinase region and downstream RAS/ERK, PI3K/AKT, MAPK/JNK and other signal pathways, resulting in tumor occurrence. ALK kinase inhibitor blocks the signal transduction pathway downstream of ALK through competitive bidding to ALK kinase region, so as to achieve the therapeutic effect.

ROS1 is a receptor tyrosine kinase on the cell surface, which is closely related to cell growth and proliferation. When ROS1 gene is fused with SLC34A2, CD74 and other genes, it will continue to activate ROS1 tyrosine kinase region and downstream signal pathway, resulting in tumor occurrence. ROS1 kinase inhibitor blocks the signal transduction pathway downstream of ROS1 through competitive bidding to ROS1 kinase region, so as to achieve the therapeutic effect.

RET protein is a transmembrane protein encoded by proto-oncogene RET, belonging to tyrosine kinase receptor superfamily. It is a cell surface molecule that transmits signals for cell growth and differentiation. When RET gene is fused with KIF5B, CCDC6, NCOA4 and other genes, it will continue to activate RET tyrosine kinase region and downstream RAS/ERK, PI3K/AKT, MAPK/JNK and other signal pathways, resulting in tumor occurrence. RET kinase inhibitor blocks the signal transduction pathway downstream of RET through competitive bidding to RET kinase region, so as to achieve the therapeutic effect.

Technological Principles

The kit detects various kinds of gene fusion in RNA samples collected from FFPE pathological tissue of NSCLC. The RNA sample is reverse transcripted to cDNA by reverse transcriptase, then the PCR amplification technology is performed to detect the status of ALK/ROS1/RET gene fusion qualitatively. The kit designs primers and fluorescent probes based on the sequence of designated mutation sites. The length of target sequence and internal control (conserved sequence of human genome) is <150 bp. For product analysis, the use of fluorescently labeled probe real-time tracking analysis makes the detection method automatic. When analyzing the results, the FAM signal indicates the gene fusion and the HEX/VIC signal indicates the RNA quality.

Kit Contents

The kit contains the following materials (Table 1). ASR 12-tube reaction strip are intended for the detection of ALK/ROS1/RET gene fusion mutations of each sample (dye: FAM). Internal control is added into each reaction mix to indicates the control of RNA quality as well as reagents and operations (dye: HEX/VIC).

Table 1 Composition of The Kit

Content Name	Main Ingredients	Volume	Quantity
ASR 12-tube reaction strip	Primer, Probe, Buffer	25 μL	8 Strips
ASR enzyme mix	Reverse transcriptase, Taq DNA polymerase, Uracil-DNA Glycosylase	130 μL	1 Tube

ASR Positive Control	Positive plasmid, Wild-type cDNA	100 μL	1 Tube

Table 2 Composition of ASR 12-tube reaction strip

Component name	Main contents	Volume	Fluorescence signal
1	ALK-1	25 μL	FAM，HEX/VIC
2	ALK-2	25 μL	FAM，HEX/VIC
3	ALK-3	25 μL	FAM，HEX/VIC
4	ALK-4	25 μL	FAM，HEX/VIC
5	ROS1-1	25 μL	FAM，HEX/VIC
6	ROS1-2	25 μL	FAM，HEX/VIC
7	ROS1-3	25 μL	FAM，HEX/VIC
8	ROS1-4	25 μL	FAM，HEX/VIC
9	RET-1	25 μL	FAM，HEX/VIC
10	RET-2	25 μL	FAM，HEX/VIC
11	RET-3	25 μL	FAM，HEX/VIC
12	RET-4	25 μL	FAM，HEX/VIC

Note: The contents of different batchs cannot be mixed .

Equipment and Reagents Required

1. Commercialized nucleic acid extraction kit;
2. DNase-free and RNase-free purified water;
3. DNase-free and RNase-free pipettes and tips.

Transportation, Stability and Storage

1. Storage Condition. Store the kit away from light at -20±5°C, valid for 9 months. Once opened, the kit is stable at -20±5°C until the stated expiration date. Do not use the reagents after 5 freezing-thawing cycles.
2. Transportation Condition. The kit should be transported in foam cases with ice bags, with transporting time less than one week and transporting temperature lower than 25°C.
3. Check labels for production date and expiration date of the kit.

Compatible PCR Instruments

Stratagene Mx3000P™, ABI 7300Plus, ABI 7500, SLAN-96S.

1. For Stratagene Mx3000P™, please set up the Fliter Set Gain Settings of FAM and HEX as 1.
2. For ABI 7500, please set up as follows: Reporter Dye: FAM and VIC; Quencher Dye: NONE; Passive;Reference: NONE.
3. For SLAN-96S, please set up as follow: Probe mode: FAM and VIC. During the results analysis, open the "Preference" window, in "Chart Option" section, select "Selected Wells" for "Y-Axis Scaling Auto-adjust by "and "Absolute Fluorescence Value Normalization" for "Amplification Curve".

Specimen Material

1. Recommended sample types: FFPE sample. Ensure that at least 20% of the collected pathological tissue were tumor lesions.
2. Choose FFPE samples which have not been stored for more than 2 years.
3. Commercialized kit is recommended to extract RNA. Assess the quality of sample RNA with an microvolume UV-Vis spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.6-2.3; the concentration of sample RNA should be >10 ng/μL. Once the RNA quality or quantity was not in conformity with the above requirements, re-extract RNA with new and/or larger input.
4. If the concentration of sample RNA is between 10 and 100 ng/μL, it is recommended to detect the original concentration directly. If the concentration of sample RNA is greater than 100 ng/μL, dilute the RNA with DNase-free and RNase-free purified water to 100 ng/μL

before detection.

5. Proceed to sample detection or store the RNA at -20±5°C for no more than 3 months.

Experimental Procedure

1. Reagent preparation

   Take out the 12-tube reaction strip and ASR enzyme mix from the kit according to the number of samples, thaw them in ice box and move them to the sample processing area; It is recommended to analyze samples, positive control (PC) and negative control (NTC, purified water) at the same time in each PCR reaction.

2. Samples Processing

   a) It is suggested to use commercialized nucleic acid extraction kit to extract sample RNA. The concentration of sample RNA should be between 10 and 100 ng/μL. Prepare more than 20 μL of the sample RNA. Dispense 30 μL DNase-free and RNase-free purified water and 15μL ASR enzyme mix into PCR tube with 20μL sample RNA. Mix reagent thoroughly by vortexing and centrifuge for 5~10 seconds. This is sample RNA template awaiting for amplification.

   b) Dispense 15μL ASR enzyme mix into each PCR tubes with 50μL PC / NTC. Mix reagent thoroughly by vortexing and centrifuge for 5~10 seconds. These are PC / NTC templates awaiting for amplification.

   c) Gently remove the cap of the 12-tube reaction strip prepared in the ice box.Add 5.2 μL templates into tubes of each strip according to the Figure 1 (5.2 μL includes 4 μL sample and 1.2 μL mixed enzyme), cover the tubes tightly and move to the amplification detection area for PCR detection.

Fig. 1 Schematic diagram of 12-tube strips

3. Amplification

   a) Centrifuge the 12-tube stripes for 10 seconds to collect templates;

   b) Load the 12-tube stripes into the real-time PCR instrument;

Table 3 Recommended layout of 96 well PCR reaction plate

Tube number	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
C	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
D	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4
E	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5
F	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6
G	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

   c) Set and run the amplification program as shown in Figure 2;

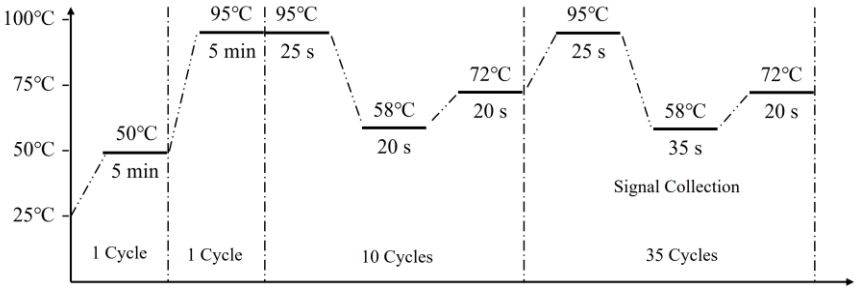

Fig. 2 PCR amplification procedure

d) Handle the stripes properly after experiment; do not remove the caps in case contamination.

Positive Judgment Value

1. Analyze single fluorescent signal each time, and select the PC amplification curve to adjust the threshold value. Choose the reaction wells of PC, NTC and sample simultaneously for analysis.
2. Result Judgment
   a) When no amplification curve of FAM generates in the 12 tubes, the sample is negative or lower than the detection limit of this kit.
   b) When the amplification curve of FAM generates in any one of the 12 tubes and the FAM Ct value is < 30, the sample is determined as positive.
   c) When the amplification curve of FAM generates in any one of the 12 tubes and the FAM Ct value is ≥ 30, increase the concentration of sample RNA for a new detection. If the FAM Ct value is < 30 in the new detection, the sample is determined as positive. Otherwise the sample is negative or lower than the detection limit of this kit.

Interpretation of Results

1. NTC: There should be no amplification curves of FAM and HEX/VIC; or else, call the result invalid.
2. PC: The FAM and HEX/VIC Ct value of PC is always < 24, if the value is ≥ 24, it is recommended to detect again.
3. Internal Control: The HEX/VIC Ct value of every sample reaction tube should be< 26, which must be qualified before proceeding to further analysis. If HEX/VIC Ct value is ≥26, consider that RNA amount was insufficient or RNA was contaminated by PCR inhibitor, in that case, re-extract sample RNA for a new detection.

Limitations of Detection Methods

1. The test results of this kit are only for clinical reference.
2. Negative results could not exclude the existence of ALK/ROS1/RET gene fusions; cases like inadequate tumor cells, RNA degradation or, insufficient RNA amount may lead to negative results as well.
3. Situations that may result in false negative or false positive result include but not limit to unreasonable sample collection, transportation, improper experimental operations or environment.
4. This kit is only used for qualitative detection of 19 fusion mutations of EML4-ALK gene, 18 fusion mutations of ROS1 gene and 16 fusion mutations of RET gene in patients with non-small cell lung cancer.
5. The kit is only applicable with the stated sample types and detection system, including specified instruments, RNA extraction kit and analytical assay.

Product Performance

1. The kit should be of neat appearance, clear labels, and of no leakage; when unfrozen, the reagents shall be clear, without sediments.
2. The consistency rates of both positive and negative control reference samples are 100%.
3. This kit can detect 10~100 ng/μL FFPE RNA sample containing fusion mutations as low as 100 copies.
4. The capacity of wild-type FFPE RNA sample of this kit is 100 ng/μL and below.
5. Precision of the kit was established by testing positive Precision Reference for 10 repeats; all the Ct values of FAM and VIC/HEX should be < 24 and the coefficient of variation (CV, %) of Ct values should be less than 5%.

Precautions

1. Please read the instruction carefully in prior to the use of the kit.
2. Avoid repetitive freezing and thawing reagents.
3. Perform quality control of RNA after extraction; proceed to sample detection immediately or store sample RNA properly.
4. Do not substitute any content of the kit; do not mix contents of different batches.
5. Pay special attention to the use of positive control to prevent contamination of reagents or resulting in false positive results.
6. Be cautious of contamination from external RNA; when sampling, always pipet NTC and sample RNAs before positive control; segregateareas for reagent preparation and sample processing; use dedicated pipettes and tips for reagent preparation and template addition, respectively.
7. Sterilize the environment and pipettes with 10% hypochlorous acid, or 75% ethyl alcohol, or UV radiation.
8. All the reagents in use have potential hazard. Only people who have work permit for PCR laboratories are allowed to use this kit. It is suggested to wear proper protective suit and gloves. For first-use of this kit, you may receive training by our technical supports.
9. All samples including positive control in the kit should be considered potential infectious substances. They should be handled carefully.

Notes

Symbol	Legend
	Indicates the need for the user to consult the instructions for use.
	Indicates a medical device that is intended to be used as an in vitro diagnostic medical device.
	Indicates the date when the medical device was manufactured.
	Indicates the manufacturer’s batch code so that the batch or lot can be identified.
	Indicates the temperature limits to which the medical device can be safely exposed.
	Indicates the date after which the medical device is not to be used.
	This is the correct upright position of the distribution packages for transport or storage.
	Indicates a medical device that needs to be protected from moisture.
	Indicates a medical device that needs protection from light sources.
	Indicates the medical device manufacturer.
	Indicates the authorized representative in the European Community/European Union.

CE	The product meets the basic requirements of European in vitro diagnostic medical devices directive 98/79/EC.

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EC REP

Lotus NL B.V.
Address:Koningin Julianaplein 10, 1e Verd, 2595AA, The Hague, Netherlands.
E-mail:peter@lotusnl.com

Manufacturer: XIAMEN SPACEGEN CO., LTD.
Address: 4th floor, No.2041 Xizhou Road, Xike Town, Tong'an District, Xiamen 361100, P. R. China
Tel: +86 592 7578317 Fax: +86 592 7578319
E-mail: spacegen@ispacegen.com
Website: http://www.sspacegen.com

Appendix Table 1

Types of Gene Fusion

Reaction Tube	Exon of Fusion Genes
1	EML4 exon6; ALK exon20
	EML4 exon6 ins33; ALK exon20
	EML4 exon6; ins18 ALK exon20
	EML4 exon13; ALK exon20
	EML4 exon13; ins69 ALK exon20
	EML4 exon18; ALK exon 20
	EML4 exon20; ALK exon20
	EML4 exon20; ins18 ALK exon20
2	EML4 exon2; ALK exon20
	EML4 exon2; ins117 ALK exon20
	EML4 exon15; ALK exon20
	EML4 exon17; ins68 ALK exon20
3	EML4 exon14; del12 ALK exon20
	EML4 exon14 ins11; del49 ALK exon20
	EML4 exon17 ins23; del46 ALK exon20
	EML4 exon17 ins6; del46 ALK exon20
	EML4 exon15 del60; del71 ALK exon20
4	EML4 exon14; ins123 ALK exon20
	EML4 exon6; ALK exon19
5	SLC34A2 exon4; ROS1 Exon32
	SLC34A2 exon13 del2046; ROS1 Exon32
	CD74 exon6; ROS1 Exon32
	SDC4 exon2; ROS1 Exon32
	SDC4 exon4; ROS1 Exon32
6	SLC34A2 exon4; ROS1 Exon34
	SLC34A2 exon13 del2046; ROS1 Exon34
	CD74 exon6; ROS1 Exon34
	SDC4 exon2; ROS1 Exon34
	SDC4 exon4; ROS1 Exon34
	EZR exon10; ROS1 Exon34
7	TPM3 exon8; ROS1 Exon35
	GOPC exon8; ROS1 Exon35
	LRIG3 exon16; ROS1 Exon35
	CCDC6 exon5; ROS1 Exon35
	CLTC exon31; ROS1 Exon35
8	GOPC exon4; ROS1 Exon36
	LIMA1 exon10; ROS1 Exon36
9	KIF5B exon15; RET Exon12
	KIF5B exon22; RET Exon12
	KIF5B exon23; RET Exon12
	KIF5B exon18; RET Exon12
	NCOA4(RFG) exon 6; RET Exon12

	TRIM33 (RFG7) exon11; RET Exon12
	KIAA1468 exon10; RET Exon12
	KIF13A exon18; RET Exon12
	CUX1 exon10; RET Exon12
10	KIF5B exon16; RET Exon12
	CCDC6 exon1; RET Exon12
	TRIM33 (RFG7) exon14; RET Exon12
	RUFY2 exon9; RET Exon12
11	KIF5B exon24; RET Exon8
	KIF5B exon24; RET Exon11
12	KIF5B exon15; RET partial Exon11