PAP-ARMS®

Thyroid Carcinoma RET Gene Fusions Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

Product Name

Thyroid Carcinoma RET Gene Fusions Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Packing Specification

20 Tests/Kit

Intended Use

The kit is intended for the qualitative detection of RET gene fusions (Attached Table 1) in RNA derived from FFPE pathological tissue of patients with thyroid carcinoma. The assay is indicated only for use as an aid in the identification of patients who may benefit from personalized treatment. The results shall not be regarded as the only evidence whether a patient suits individualized therapy; Clinically, determinants such as, but not limited to patients' condition, drug indications, therapeutic response, and other laboratory detection indexes should also be considered before making comprehensive judgments. The multiplex fluorescence Polymerase Chain Reaction (PCR) tech applied in this kit is intended for use on real-time PCR systems.

The proto-oncogene RET encodes a transmembrane protein, which is a member of tyrosine kinase receptor superfamily and plays important roles in cell growth and signal transduction. When RET gene fuses with KIF5B, CCDC6, or NCOA4, the mutated fusion protein will activate kinase domain of itself and downstream signaling of RAS/ERK, PI3K/AKT and MAPK/JNK pathways permanently, thus leads to cancer at last. The tyrosine kinase inhibitor of RET treats patients with cancer through competitive binding to the RET kinase domain to blocking RET kinase activity. Therefore, the sensitivity and accuracy of RET fusion mutation detection has become a significant factor in aiding the treatment of patients with thyroid cancer.

Table 1 Types of RET Gene Fusions

Reaction tube	COSMIC ID	Exon of fusion genes	Exon of RET
RET-1	COSF1272	CCDC6 E1	E12
	COSF1492	NCOA4 E8	E12
	COSF1512	PRKAR1A E7	E12
RET-2	COSF1233	KIF5B E15	E12
	COSF1610	KIF5B E16	E12
	COSF1254	KIF5B E22	E12
	COSF1235	KIF5B E23	E12
RET-3	COSF1482	PCM1 E29	E12
	COSF1504	GOLGA5 E7	E12
	COSF1510	HOOK3 E11	E12
	COSF1514	KTN1 E29	E12
RET-4	COSF1341	NCOA4 E6	E12

Technological Principles

This reagent uses thyroid carcinoma paraffin-embedded tissue RNA as the detection sample to detect various types of RET gene fusion mutations. The sample RNA is reverse transcribed into cDNA, then the multiple fluorescent PCR amplification technology is used to complete the qualitative detection of the RET gene fusion state on a real-time PCR instrument. This kit uses the fusion mutant sequence as a template to design primers and fluorescent probes. The length of the target gene sequence of each mutant type is controlled within 150 bp; The target gene sequence of the internal standard is the conserved sequence on the human housekeeping gene β-actin. In product analysis, the

real-time tracking analysis technology of fluorescently labeled probes is used to automate the detection method. The RET gene fusion state is indicated by the FAM signal, and the sample RNA quality detection is indicated by the HEX (VIC) signal.

Kit Contents

The kit is pre-loaded in 8-tube strip, and each PCR reaction tube contains specific primers, fluorescent probes, dNTPs, $MgCl_2$, $(NH_4)_2SO_4$, KCl and purified water. The reaction solution has been pre-loaded in 8-tube strips, from left to right are tubes 1, 2, 3, 4, 5, 6, 7, and 8 (Figure 1). Each 8-tube strip detects 2 samples, namely tubes 1 to 4 and tubes 5 to 8, were tested for one sample respectively. Primers and probes of internal control genes were added to reaction tubes 4 and 8 at the same time as the quality control of reagents, cDNA quality and operation.

Table 2. Kit Contents

Content Name	Volume	Quantity
RET 8-tube strips	45 μL	12 strips
RET Taq Polymerase	35 μL	1 tube
RET Positive Control	100 μL	1 tube
reverse transcription master mix	300 μL	1 tube
random primer	25 μL	1 tube
reverse transcriptase	25 μL	1 tube
Nuclease-Free Water	200 μL	1 tube

Note: The contents of different batches cannot be mixed

Equipment and Reagents Required

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

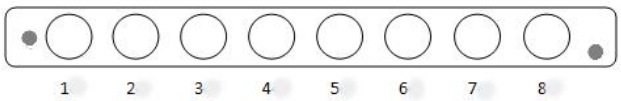

Figure 1. Tube Sequence of 8-Tube Strip

Note: Reaction reagents are pre-loaded to 8-tube strips, from left to right followed by No. 1, 2, 3, 4, 5, 6, 7, 8, respectively.

Transportation, Stability and Storage

1. Storage Condition. Store the kit away from light at - 20±5℃, valid for 9 months. Once opened, the kit is stable at - 20±5℃ 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℃.
3. Check labels for production date and expiration date of the kit.

Compatible PCR Instruments

ABI7500, ABI7300, ABI StepOne Plus, LightCycler480, Bio-Rad CFX96, etc.

1. For ABI instruments, define targets and passive reference as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.

Specimen Material

1. Recommended sample types: FFPE tissues, ensure that at least 30% of the collected pathological tissue were tumor lesions; Choose

FFPE samples which have not been stored for more than 3 years; Extract sample RNA with at least 8 slices of 5 μm section or at least 5 slices of 10 μm section.

2. Commercialized kit is recommended to extract tissue 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 1.8 - 2.3; Once the RNA quality or quantity was not in conformity with the above requirements, re-extract RNA with new and/or larger input.
3. Proceed to reverse-transcription or store the RNA at - 70℃ for no more than 6 months and avoidance of repeated freezing and thawing.

Experimental Procedure

1. Reagent Preparation
   a) Prepare the DNase / RNase-free PCR reaction tubes according to the number of samples. Pipet 1 μL random primer to each tube, place them on ice before transferring to the sample processing area;
   b) Prepare reaction buffer for reverse-transcription according to the sample number in another one DNase / RNase-free centrifuge tube. The reverse transcription reaction solution of each sample is composed of 1 μL reverse transcriptase + 14 μL reverse transcription master mix; After the preparation is completed, place in an ice box and move to the sample processing area for use;
   c) Prepare 8-tube stripes and RET Taq polymerase for the test samples; Place them on ice before transferring to the sample processing area; Detection of sample combined with Positive Control (PC) and NTC in each reaction / run is suggested.
2. Sample Processing
   a) Commercialized kit is recommended to extract sample RNA;
   b) Pipet 4 μL sample RNA (supplemented with Nuclease-Free Water if the volume of sample is less than 4 μL, the total amount of RNA is less than 5 μg) to PCR reaction tube pre-loaded with 1 μL random primer. Incubation at 70℃ for 5 min, then cooling at ice for 5 min, briefly centrifuge and pipet 15 μL reverse transcription solution prepared above. Mix well and perform the process of reverse transcription referring to reaction condition of table 3;

Table 3. Procedure of Reverse Transcription Reaction

Temperature	Time
25 ℃	5 min
42 ℃	60 min
70 ℃	15 min
4 ℃	∞

   c) Centrifuge the PCR tubes a few seconds followed with adding 1 μL RET Taq polymerse to each one after reaction of reverse transcription, then mix the solution by vortexing gently. The soultion in the PCR tube includes tested cDNA is called template for amplification;
   d) Gently remove the cap of RET 8-tube strip, sequentially pipet 5 μL of the templates into tubes of each strip, each strip detect 2 samples, cover the cap carefully and transfer to the amplification detection area.

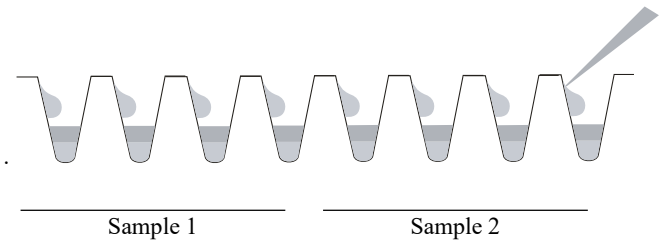

Figure 2. The 8-Tube Stripe Sampling Diagram

3. Amplification
   a) Centrifuge the 8-tube strips for 10 seconds to collect templates;

b) Load the 8-tube strips into the real-time PCR instrument; Refer to Table 4 for overall arrangement if necessary;

Table 4. Suggested Overall Arrangement for 96-Wells Plate

No.	Assay	1	2	3	4	5	6	7	8	9	10	11	12
1	RET-1	Sample1	Sample3	Sample5	Sample7	Sample9	Sample11	Sample13	Sample15	Sample17	Sample19	Sample21	PC
2	RET-2	Sample1	Sample3	Sample5	Sample7	Sample9	Sample11	Sample13	Sample15	Sample17	Sample19	Sample21	PC
3	RET-3	Sample1	Sample3	Sample5	Sample7	Sample9	Sample11	Sample13	Sample15	Sample17	Sample19	Sample21	PC
4	RET-4	Sample1	Sample3	Sample5	Sample7	Sample9	Sample11	Sample13	Sample15	Sample17	Sample19	Sample21	PC
5	RET-1	Sample2	Sample4	Sample6	Sample8	Sample10	Sample12	Sample14	Sample16	Sample18	Sample20	Sample22	NTC
6	RET-2	Sample2	Sample4	Sample6	Sample8	Sample10	Sample12	Sample14	Sample16	Sample18	Sample20	Sample22	NTC
7	RET-3	Sample2	Sample4	Sample6	Sample8	Sample10	Sample12	Sample14	Sample16	Sample18	Sample20	Sample22	NTC
8	RET-4	Sample2	Sample4	Sample6	Sample8	Sample10	Sample12	Sample14	Sample16	Sample18	Sample20	Sample22	NTC

c) Set and run the amplification program as shown in Figure 3;

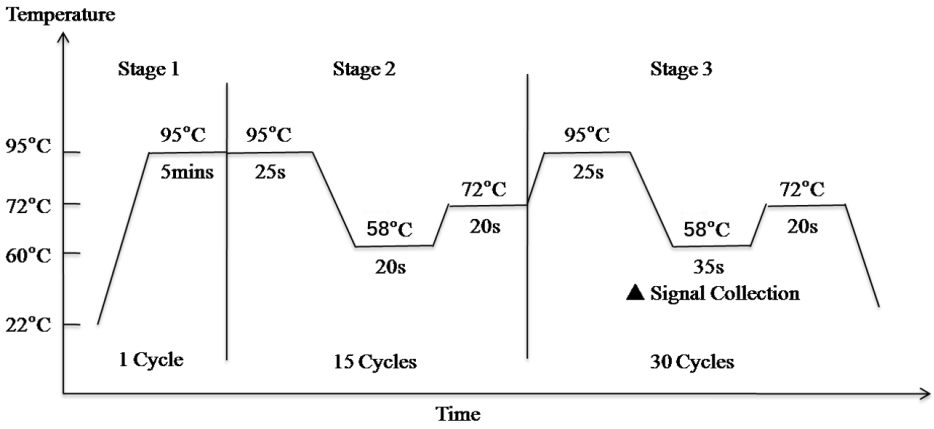

Figure 3. PCR Amplification Procedure

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

Positive Judgment Value

1. The positive judgment value of the RET gene fusion assay in this kit is 28; It was determined in clinical trials with the assist of ROC curve method.
2. Result Judgment
   a) Ct value: Provided by the instrument software or by determining the threshold fluorescence of actual amplification curve.
   b) Mutation Result: When any one of the four reaction tubes, the FAM Ct is less than 28, a positive call is returned. When no amplification curve of FAM generates in the well, a negative call or lower than the detection limit of the kit is returned. When 28 ≤ FAM Ct < 30, retest the sample with larger DNA amount; per the retest result, if 28 ≤ FAM Ct < 30, a negative call is returned, if FAM Ct < 28, a positive call is returned.

Interpretation of Results

1. NTC: There should be no amplification curves of FAM and HEX (VIC), or else, call the result invalid, recommend to testing again.
2. PC: There should be amplification curves of FAM and HEX (VIC), with the value of Ct is ≤ 24. If the Ct value of FAM or HEX (VIC) in any one tube is bigger than 24, the value is invalid and retest is recommended.
3. Internal Control: The HEX (VIC) Ct of Internal Control (tube 4 or tube 8) should be ≤ 20, which should be qualified before proceeding to further analysis; If the HEX (VIC) Ct is greater than 20, that indicates insufficient RNA amount or the sample RNA was contaminated with PCR inhibitor, in this case, re-extract sample RNA for a new detection is recommended.

Limitation of the Kit

1. Negative results could not exclude the existence of RET gene fusion completely; Cases like inadequate tumor cells, RNA degradation or, insufficient RNA amount may lead to negative results as well.
2. Different sampling locations may lead to diverse outcomes due to the heterogeneity of tumor tissues / cells.
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. The kit is only intended for the qualitative detection of 12specific gene fusions of RET gene in patients with thyroid cancer.
5. The kit is only applicable with the stated sample types and detection system, including specified instruments, RNA extraction kit and analytical assay.

Physical 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. The kit allows the detection of ≤ 100 copies of RET gene fusion in RNA sample.
4. For 10 repetitive times detection of the designated sample, the Ct values of FAM and HEX channel should be less than 24, and the coefficient of variation (CV, %) of Ct values should less than 10%.

Precautions and Warning

1. Please read the instruction carefully in prior to the use of the kit.
2. Avoid repetitively freezing and thawing reagents.
3. The results of this kit will be affected by the source, the process of collection, quality, condition of transport, pre-treatment of the sample, as well as the quality of the extracted RNA, model of fluorescence quantitative PCR instrument, operation environment, and the current technological limitation of molecular biology. The factors and variables mentioned above would lead to false positive or false negative test results. Users must be aware of the potential errors and accuracy limitations that may exist during the process of detection.
4. Since the quality of RNA is important, commercial RNA extraction kit is recommended. Perform quality control of RNA after extraction followed with proceeding to sample detection immediately or store sample RNA below - 70℃.
5. Do not substitute any content of the kit; Do not mix contents of different batches.
6. Pay special attention to the use of positive control to prevent contamination of reagents which would be resulting in false positive results.
7. Be cautious of contamination from external RNase. Segregate areas of reagent preparation and sample processing; Use dedicated pipettes and tips for reagent preparation and template addition, respectively.
8. Sterilize the environment and pipettes with 10% hypochlorous acid, 75% ethyl alcohol, or UV radiation.
9. 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 suits and gloves. For first-use of this kit, you may receive training by our technical supports.
10. All samples including positive control in the kit should be considered as 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.

LOT	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.
EC REP	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.

References

1. D Lipson, M Capelletti, R Yelensky, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies[J]. Nature medicine, 2012, 18(3): 382.
2. Corvi R, Berger N, Balczon R, et al. RET/PCM-1: a novel fusion gene in papillary thyroid carcinoma[J]. Oncogene, 2000, 19(37): 4236.
3. Grubbs E G, Ng P K, Bui J, et al. RET Fusion as a Novel Driver of Medullary Thyroid Carcinoma[J]. Journal of Clinical Endocrinology & Metabolism, 2015, 100(3): 788.
4. Cristofaro J D, Marcy M, Vasko V, et al. Molecular genetic study comparing follicular variant versus classic papillary thyroid carcinomas: association of N- ras, mutation in codon 61 with follicular variant[J]. Human Pathology, 2006, 37(7): 824-30.
5. Sheu SY, Schwertheim S, Worm K, et al. Diffuse sclerosing variant of papillary thyroid carcinoma: lack of BRAF mutation but occurrence of RET/PTC rearrangements[J]. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc, 2007, 20(7): 779.
6. Young Seok Ju, WonChul Lee, JongYeon Shin, et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing[J]. Genome Res. 2012, 22(3): 436-445.
7. Bongarzone I, Monzini N, Borrello M G, et al. Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of ret tyrosine kinase and the regulatory subunit RI alpha of cyclic AMP-dependent protein kinase A.[J]. Molecular & Cellular Biology, 1993, 13(1): 358-66.

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