Human KRAS/NRAS/PIK3CA/BRAF Gene Mutation Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

For Research Use Only

Product Name

Human KRAS/NRAS/PIK3CA/BRAF Gene Mutation Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Packing Specification

6 Tests/Kit, 12 Tests/Kit

Intended Use

This kit uses multiplex fluorescence PCR amplification technology to qualitatively detect 18 somatic mutations in exons 2, 3 and 4 of KRAS gene, 3 somatic mutations in exons 2 and 3 of NRAS gene, H1047R mutation in exon 20 of PIK3CA gene and V600E mutation in exon 15 of BRAF gene (Table 1) from DNA isolated from paraffin-embedded (FFPE) tumor tissue samples from patients with colorectal cancer. The test results are for research use only.

KRAS, NRAS, PIK3CA and BRAF genes are four important genes in the EGFR-dependent downstream signal transduction pathway RAS-RAF-MAPK pathway and PI3K-AKT pathway. When these genes are mutated by various internal and external inducements, they will not be regulated by the superior EGFR signal and maintain the continuous activation of the signal pathway, resulting in excessive cell proliferation, angiogenesis, and finally lead to tumor occurrence and metastasis. Clinical studies have found that colorectal cancer patients with KRAS, NRAS, PIK3CA and BRAF gene mutations are not sensitive to anti-EGFR therapy.

Table 1. Mutations Site Detected by the Kit

Tube Number	Gene Name	Mutation Name	Changes of Bases	Cosmic ID
1	KRAS	G12D	c.35G>A	521
2		G12A	c.35G>C	522
		G12V	c.35G>T	520
		G12S	c.34G>A	517
3		G12C	c.34G>T	516
4		G13D	c.38G>A	532
5		G13C	c.37G>T	527
		G13S	c.37G>A	528
		G13R	c.37G>C	529
		K117N	c.351A>C	19940
		K117N	c.351A>T	28519
6		Q61L	c.182A>T	553
		Q61R	c.182A>G	552
		Q61H	c.183A>C	554
		Q61H	c.183A>T	555
7		Al46T	c.436G>A	19404
		A146V	c.437C>T	19900
		A146P	c.436G>C	19905
8	NRAS	G12D	c.35G>A	564
9		Q61R	c.182A>G	584
		Q61K	c.181C>A	580
10	PIK3CA	H1047R	c.3140A>G	775
11	BRAF	V600E	c.1799T>A	476

Technological Principles

This kit uses multiplex fluorescence PCR technology to detect multiple somatic mutations of KRAS/NRAS/PIK3CA/BRAF gene in the sample of colorectal cancer. The kit uses the sequence of mutation site as the template to design ARMS primers and fluorescent probes, and the target gene sequences of each mutant are controlled within 150 bp. The target gene sequences of internal control is conserved sequences in human genome, and the length is 100 bp. For product analysis, the use of fluorescently labeled probe real-time tracking analysis makes the detection method automatic. The mutations in the sample DNA can be detected by the kit with high specificity and high sensitivity on the real-time PCR platform. When analyzing the results, the FAM signal indicates the genetic mutation and the external control, the HEX (VIC) signal indicates the internal control.

Kit Contents

Reaction reagents were pre-loaded in 12-tube strips; each strip detects one sample. Tube 1-11 contains 23 kinds of KRAS/NRAS/PIK3CA/BRAF gene mutation detection and internal control reagents, the FAM signal indicates the mutation, and the HEX (VIC) signal indicates the internal control; tube 12 contains external control detection reagents for DNA extraction quality, which is indicated by FAM signal. Internal control and external control as quality control of the reagents, DNA quality and operation, the selected detection region are the relatively conservative region of human genes.

Table 2. Kit Contents

Content Name	Components	6 Tests/Kit		12 Tests/Kit	
		Volume	Quantity	Volume	Quantity
KNPB 12-Tube Strips	Primers, probes, Mg^{2+}, dNTPs	35 μL	8 strips	35 μL	16 strips
KNPB Taq Polymerase	Taq DNA polymerase	35 μL	1 tube	35 μL	2 tubes
KNPB Positive Control	Positive plasmid DNA, wild type DNA	200 μL	1 tube	200 μL	1 tube

Note: The contents of different batches cannot be mixed.

Figure 1. Tube Sequence of 12-Tube Strip

Note: The reaction solutions are pre-loaded in 12-tube strips, as shown in Figure 1. From left to right are tubes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12.

Additional required Equipment and Materials

1. Commercialized nucleic acid extraction kit.
2. Nuclease-Free water (NTC).
3. Aerosol-barrier pipette tips.

Transportation, Stability and Storage

1. Storage Condition. Store the kit away from light at -15℃ to -25℃, valid for 9 months. Once opened, reagents can be stored in their original packaging at -15℃ to -25℃ until the stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 5 freeze-thaw cycles.
2. Transportation Condition. The kit should be transported at low temperature, 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

Stratagene Mx3000P™, ABI7500, SLAN-48P/96S, ABI StepOne Plus, etc.

1．For Stratagene Mx3000P™, FAM and HEX channel signal gain multiple is adjusted to 1.

2．For ABI instruments, the probe mode setting as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.

Specimen Material

1. Recommended sample types: FFPE tissues stored for no more than 2 years. The biopsies should be fixed with formalin and embedded in paraffin. For resection or surgical biopsies, the recommended tissue input is at least 2×5-micron sections. For coreneedle biopsies, the recommended tissue input is at least 10×5-micron sections.The tissue sample should contain at least 20% tumor cells, otherwise, the tissue samples should be macrodissected and enriched for tumor content.
2. Commercialized kit is recommended to extract DNA from the samples. Assess the quality of sample DNA with an microvolume ultraviolet-visible spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.7-2.2, the concentration is not less than 10 ng/μL. Once the DNA quality or concentration was not in conformity with the above requirements, re-extract DNA with new and/or larger input.
3. Proceed to sample detection or store the DNA at -15℃ to -25℃ for no more than 12 months. Freeze-thaw samples no more than 5 times.

Experimental Procedure

1. Reagent Preparation

   Prepare KNPB 12-tube strips and KNPB Taq polymerase according to samples; briefly centrifuge the strips and Taq polymerase; place them on ice and transfer to the sample processing area; detection of KNPB Positive Control (PC) and Negative Control (NTC, Nuclease-Free water) in each reaction/run is recommended.

2. Samples Processing

   (1) Sample preparation: Commercialized kit is recommended to extract genomic DNA. Then dilute sample DNA to 2 ng/μL, the dilution volume is for a minimum of 65 μL, which is so called tested DNA.

   (2) Template preparation: Respectively add 3.25 μL KNPB Taq polymerase to 65 μL of the tested DNA, PC and NTC, vortex slightly to mix, then pulse centrifuge.

   (3) Gently remove the cap of 12-tube strip, sequentially add 5 μL of the templates into tubes of each strip, cover the cap carefully.

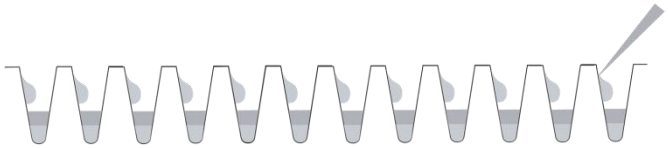

Figure 2. The 12-Tube Strip Sampling Diagram

3. Amplification

   (1) Centrifuge the 12-tube strips for 10 seconds to collect templates.

   (2) Load the 12-tube strips into the real-time PCR instrument; refer to Table 3 for overall arrangement if necessary.

Table 3. Suggested Overall Arrangement

No.	KNPB-1	KNPB-2	KNPB-3	KNPB-4	KNPB-5	KNPB-6	KNPB-7	KNPB-8	KNPB-9	KNPB-10	KNPB-11	External
1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1	Sample1
2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2	Sample2
3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3	Sample3
4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4	Sample4
5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5	Sample5
6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6	Sample6
7	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
8	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

   (3) Set and run the amplification program as shown in Figure 3.

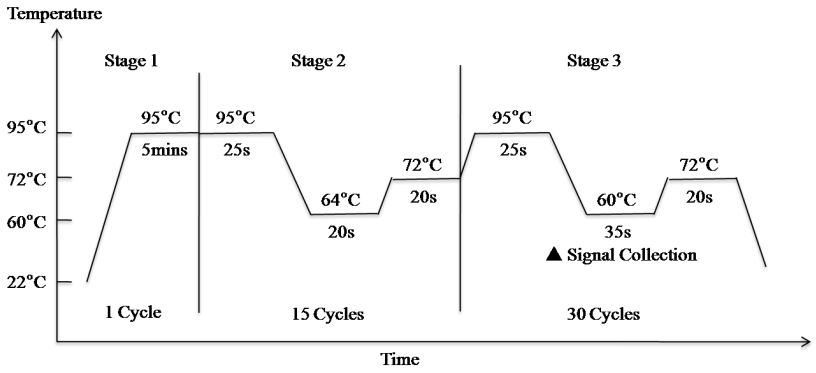

Figure 3. PCR Amplification Procedure

(4) Handle the strips properly after experiment; do not remove the caps in case contamination.

Data Analysis

1. The ΔCt Cut-off value of this kit was determined as 11, 11, 11, 10, 13, 13, 13, 13, 13, 13, 13 respectively with the assist of ROC curve method.
2. Result Judgment
   (1) Ct value: provided by the instrument software or by determining the threshold fluorescence of actual amplification curve.
   (2) Mutation Result (refer to Table 4):
   a) When the FAM Ct is greater than or equals to the stated negative Ct value, a negative call, or lower than the detection limit of the kit is returned.
   b) When the FAM Ct is less than the stated negative Ct value, calculated the ΔCt Cut-off value per the equation below. If the derived ΔCt Cut-off value is less than or equals to the stated, a positive call is returned; if the derived ΔCt Cut-off value is greater than the stated, a negative call is returned:

   Equation: ΔCt Cut-off = Ct (Mutation) – Ct (External)

   Ct (Mutation): The FAM Ct of tube 1-11 for each sample.

   Ct (External): The FAM Ct of tube 12 for each sample.

Table 4. Result Judgment

12-Tube Strip Number		1	2	3	4	5	6	7	8	9	10	11
Positive	Stated Threshold Ct Value	Ct <28	Ct <28	Ct <28	Ct <29	Ct <29	Ct <29	Ct <29	Ct <29	Ct <29	Ct <29	Ct <29
	Stated ΔCt Cut-off value	11	11	11	10	13	13	13	13	13	13	13
Negative	Stated Negative Ct Value	Ct ≥28	Ct ≥28	Ct ≥28	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29

Interpretation of Results

1. NTC: There should be no amplification curves of FAM in NTC reaction tube 1-11, or else, call the result invalid. Occasionally, amplification curve of HEX (VIC) generates in NTC tube 1-11 or FAM generates in NTC reaction tube 12, which has no influence on result interpretation.
2. PC: There should be amplification curves of FAM and HEX (VIC) in PC reaction tube 1-11, and FAM signal in PC reaction tube 12, with the value of Ct is less than 20. If the Ct value of FAM or HEX (VIC) in any one tube is greater than 20, the value is invalid and retest is recommended.
3. External Control: The FAM Ct of every sample in tube of external control (tube 12) should be 13-18, which must be qualified before proceeding to further analysis; If the FAM Ct is less than 13, that indicates excessive DNA amount, dilute sample DNA for a new detection; If the FAM Ct is greater than 18, that indicates insufficient DNA amount or that sample DNA was contaminated by PCR inhibitor, in this case, it is recommended to re-extract sample DNA for a new detection.
4. Internal Control: There should be amplification curves of internal control HEX (VIC) in tube 1-11 of each sample, if the internal control analysis is negative or part of the tube analysis is negative, the value is invalid and retest is recommended.

Limitations of the Kit

1. Negative results could not exclude the existence of KRAS/NRAS/PIK3CA/BRAF gene mutation; Cases like inadequate tumor cells, DNA degradation, or insufficient DNA 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 23 specific mutations of KRAS/NRAS/PIK3CA/BRAF gene.
5. The kit is only applicable with the stated sample types and detection system, including specified instruments, DNA extraction kit and analytical assay.

Performance Characteristics

1. The kit should be of neat appearance, clear labels, and of no leakage. When unfrozen, the reagents shall be clear, without precipitate.
2. The consistency rates of both positive and negative reference materials are 100%.
3. In 10 ng DNA samples, the lower detection limit of mutations detected by reaction tube 5 is 5%, and the lower detection limit of other reaction tubes are 1%.
4. Repeat the test 10 times for the same precision reference material, the Ct values of both FAM and HEX (VIC) are less than 25 (except for HEX (VIC) of external control), and the coefficient of variation (CV, %) of the Ct value should be less than 10%.
5. There's no nonspecific product with up to 200 ng wild-type DNA sample.
6. There was no cross reaction with Escherichia coli DNA, Yeast DNA, Mycobacterium tuberculosis DNA and Streptococcus pneumoniae DNA.

Warnings and Precautions

1. Please read the instruction carefully in prior to the use of the kit.
2. Avoid repeatedly freezing and thawing the reagents in the kit.
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 DNA, 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. The quality of DNA is crucial, and the quality control of DNA should be performed after extraction; proceed to sample detection immediately or store sample DNA properly at -15℃ to -25℃.
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 or resulting in false positive results.
7. Be cautious of contamination from external DNA; when sampling, always add NTC and sample DNA before positive control; segregate areas for 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. It is recommended wearing proper protective suit 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 which should be handled carefully.

Symbols

Symbol	Symbol definition
	Indicates the need for the user to consult the instructions for use.

	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.

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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