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V600E Correlated Mutation in B-Raf Gene Test Kit

(Fluorescent Probe-Based Real-Time PCR Assay)

Instructions for Use

English VER 2.0

REF **T011T012B0C2** **12 Tests/Box** **Basic UDI 697089547BRAF00QC**

PRODUCT NAME

V600E Correlated Mutation in B-Raf Gene Test Kit (Fluorescent Probe-Based Real-Time PCR Assay)

INTENDED USE

This kit is intended for the qualitative detection of V600E mutation in *B-raf* gene in DNA extracted from formalin-fixed, paraffin-embedded tumor tissues from patients with papillary thyroid cancer or colorectal cancer.
The kit is specific for detecting V600E correlated mutation in *B-raf* gene. It can be used for the individualized molecular diagnosis of tumors and assists individualized therapy for tumor patients.

SUMMARY AND EXPLANATION

B-raf gene is a kind of oncogene and the coded protein is an important regulatory factor in the RAS-RAF-MEK-ERK signaling pathway. When this pathway is abnormally activated, it can promote cell proliferation and growth, and eventually leads to tumorigenesis. Studies have shown that there are different frequencies of *B-raf* oncogene mutations in a variety of human tumors, such as malignant melanoma, papillary thyroid cancer and colorectal cancer. Sequence analysis showed that mutations in *B-raf* gene mainly occurred in the two regions of exon11 and exon15, and the main mutation was located at position 1799 (from T to A) in exon 15 (V600E mutation). The V600E mutation in B-Raf protein causes tcontinuous activation, resulting in the changes of phosphokinase activity in MAPK kinase signaling pathway, which affects the progress of the tumor.

TEST PRINCIPLE

This kit uses Amplification Refractory Mutation System (ARMS) combined with TaqMan probe fluorescence quantitative PCR technology to detect V600E correlated mutation in *B-raf* gene in formalin-fixed, paraffin-embedded tumor tissues from patients with papillary thyroid cancer or colorectal cancer. The detection of each sample was carried out in two tubes. Primers were designed for wild-type *B-raf* gene in reference tube, ARMS primer was designed for V600E mutation in mutation tube, and the probe was labeled by FAM. The Housekeeper gene (GAPDH) was used as an internal control, and the probe targeted GAPDH was labeled with HEX. The difference Ct values (ΔCt) in FAM channel between reference and mutation tubes can be used to identify the existence of the mutation in *B-raf* gene. A ≤ 7 ΔCt value indicates a mutation. The closer the ΔCt to 7 (≤ 7), the lower the proportion of mutated DNA.

REAGENTS PROVIDED

Seq.	Labels	Main contents	No. (12 tests)
1	Reference tube	Wild type Primers and Probes/ Each deoxy-ribonucleotide triphosphates (dNTPs)/ magnesium *etc.*	1 tube (300 μL/tube)
2	Mutation tube	Mutated type Primers and Probes/ Each deoxy-ribonucleotide triphosphates (dNTPs)/ magnesium *etc.*	1 tube (300 μL/tube)
3	Enzyme	Contains DNA polymerase	1 tube (10 μL/tube)
4	Positive Control	B-raf gene plasmid, V600E mutant plasmid and Internal Control plasmid	1 tube (20 μL/tube)
5	Negative Control	TE Solution	1 tube (20 μL/tube)

OTHER MATERIALS REQUESTED BUT NOT PROVIDED

The following list includes the materials that are required for use but not included in this Kit:

- Nucleic acid extraction kit.
- Nuclease-free consumables: Filter tips, 1.5mL tubes, PCR-well strips or 96-well plate.
- Experimental equipment: Centrifuge for 1.5mL tubes and PCR-well strips or 96-well plate (if available), Vortex. Real-Time PCR instrument (thermocycler).

Others: Micropipettes (0.5-20μL, 10-100μL, 20-200μL,100-1000μL), Powder-free disposable gloves, Microplate sealing film.

STORAGE CONDITIONS AND SHELF LIFE

The shelf life of the kit is 12 months when stored in a freezer at -(20±5) ℃. It is suggested to transport the kits in a sealed foam box with dry ice and/or ice packs. The kit needs to be stored away from light. Never leave the kit for more than 7 days at 2-8℃. Never leave the kit for more than 1 day at 37℃. Never repeat freeze-thaw more than 3 times (the effects of more than 3 times were not verified). After opening, keep it at 2-8℃ and use it within 15 days.

APPLICABLE EQUIPMENT

Applicable to ABI 7500 Real-Time PCR thermocyclers. For other Real-Time PCR thermocyclers, please consult the manufacturer before use.

ACCEPTABLE SPECIMENS

The samples were formalin-fixed and paraffin-embedded tumor tissues from papillary thyroid cancer or colorectal cancer patients.

SPECIMENS COLLECTION AND STORAGE

Fresh samples should be immediately put into the specimen box or wrapped in a wet cloth, transported to the laboratory for segmentation and fixation. Then the samples should be paraffin-embedded in successive steps including fixation, sampling, dehydration, transparency, wax dipping, embedding, sectioning, and baking slices. Paraffin-embedded pathological tissue or section samples should be confirmed by experienced pathological doctors to contain tumor cells, and the requested parts should be in the middle of the wax block as much as possible, and non-tumor tissues should be eliminated as much as possible. Pay attention to aseptic operation during sample collection.

A label with a unique identification number is attached to the outside of the sample container. The necessary information should be attached during transportation and storage, such as sample number, date of onset, and date of sample collection. Formalin-fixed and paraffin-embedded tumor tissues and extracted DNA should not be stored for more than 9 days at 2-8℃ or 6 months at -(20±5) ℃.

TEST METHODOLOGY

1. Nucleic Acid Extraction (Pre-PCR)

Extract the nucleic acids from the clinical samples and negative control according to the instructions of the nucleic acid extraction kit:

1.1. Clinical samples: Clinical specimen to be tested. The concentration of nucleic acid after extraction should not be less than 5 ng/μL.

1.2. Negative control: Negative control is included in this kit.

Note: The proportion of tumor cells in tissue samples should be greater than 80%. Positive quality control does not require extraction.

*We have validated that the kit for nucleic acid extraction from paraffin-embedded tumor tissues is QIAamp DNA FFPE Tissue Kit from Qiagen (Cat. No.56404). If you use nucleic acid extraction kits from other suppliers, please verify first.

2. Amplification Processes (PCR)

2.1 Preparation of Amplification Reagent (PCR Room I)

To prepare the PCR reaction mixtures, take the Reference tube, Mutation tube and Enzyme from the kit. Thaw them on ice or at 2-8℃, shake well and centrifuge all reagent tubes at low speed shortly. Prepare the Amplification PCR Mixture according to the following ratios:

Regent	Enzyme (μL)	Reference Tube (μL)	Mutation Tube (μL)
Reference tube	0.3	22.7	—
Mutation tube	0.3	—	22.7

Calculate the amount of each reagent. Add the reagents into an appropriate volume centrifugal tube, mix well and centrifuge shortly. Total number of PCR reaction mixtures = number of clinical samples + 1 positive control +1 negative control.

Add 23.0 μL of the PCR reaction mixture into each PCR well/tube, and then transfer the plate/tubes to PCR room II.

2.2 Add the Templates (PCR Room II)

Add 2.0 μL of nucleic acid extracted from each sample (prepared in the first step: Pre-PCR) into each PCR well/tube which was added with PCR reaction mixtures. Vortex the sealed plate or tubes to mix well and then centrifuge at 2000-3000rpm for 1min.Refer to the table below for the sampling layout of a 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A Reference	Positive Control	Negative Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
B Mutation	Positive Control	Negative Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
C Reference	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22
D Mutation	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22
E Reference	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34
F Mutation	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34
G Reference	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46
H Mutation	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46

2.3 Amplification (Detection Area)

Put the reaction tubes/plates into the fluorescent real time PCR thermocycler and set the cycle program as follows:

Step	Cycles	Temperature (℃)	Time (min.: sec.)
1	1	95	10:00
2	40	95	00:05
		62*	00:40

Fluorescent dye signals assigning: FAM (target gene), HEX/VIC (internal control). *The signal data is collected at 62℃. When using the ABI7500, select the 'Quencher' and 'Passive reference' columns as "none". Set the reaction volume per tube/well to 25 μL.

EXPLANATION OF THE TEST RESULTS

After the reaction is completed, the instrument automatically saves the results, and adjusts the Start, End, and Threshold values of the Baseline after analyzing the image (It is user self-adjustable: Start values can be between 3 and 10. End values can be between 15 and 20). Adjust the amplification curve of the PCR-negative control to straight or below the threshold line. The difference Ct values (ΔCt) in FAM channel between reference and mutation tubes are calculated as the followed formula:

$$\Delta Ct = Ct_{\text{mutation}} - Ct_{\text{reference}}$$

1. The Ct value in HEX/VIC (Internal control) of all reaction wells except negative control should be Ct ≤31.00, with a typical S-type amplification curve, If not, there may be PCR inhibitors in the extracted DNA, the template quantity is insufficient or the extraction fails, in this case, re-extraction or sample usage increasing is needed.
2. The Ct value in FAM channel of the reference tube should in the range of 21≤Ct≤ 31. If Ct<21, the sample should be diluted, if >31, the amount of extracted DNA is too low, and increasing the sample size for re-extraction is recommended.
3. For Positive control, ΔCt should be in the range of 3 <ΔCt ≤7. For negative control, the Ct value in all channels of reference and mutation wells should be negative.

The above requirements must be met in the same test at the same time, otherwise, the PCR reaction is considered invalid and should be re-performed. The interpretation and judgment of test results are as follows when met all the above requirements:

No.	Reference Tube	Mutation Tube	Interpretation
1	Reference positive Internal control positive	Internal control positive, no amplification curve for mutation or ΔCt > 10	V600E mutation was not detectable.
2	Reference positive Internal control positive	Internal control positive, mutation positive and ΔCt ≤ 7	V600E mutation was detected.
3	Reference positive Internal control positive	Internal control positive, mutation positive and 7<ΔCt ≤ 10	Re-extract and then re-test, if ΔCt≤10, V600E mutation was detected, otherwise V600E mutation was not detectable.
4	-	Internal control negative	Re-extract and then re-test. Increasing the sample size used for re-extraction is recommended.
5	Reference Negative	-	
6	Internal control negative	-	

Notes:"-" means that the tube result is not considered.

LIMITATIONS OF THE TEST METHOD

1. The test results of this kit are for clinical reference only, not as the only basis for treatment or other clinical management, the clinical diagnosis and treatment of patients should be comprehensively considered in conjunction with their symptoms/signs, medical history, other laboratory tests and treatment reactions.
2. Negative results cannot completely exclude the presence of target gene mutations, too few tumor cells in the sample, excessive degradation or target gene concentration in the amplification reaction system below the detection limit can also cause negative results.
3. Tumor tissues (cells) may have great heterogeneity, sampling from different parts may result in different test results. Unreasonable sample collection, transportation and processing, as well as improper test operation and experimental environment may lead to false negative or false positive results.
4. The test is limited to the specified sample type and test system (including applicable equipment, nucleic acid extraction reagents, detection methods, *etc.*).

PRODUCT PERFORMANCE

1. The appearance of the kit should be clean and tidy, with clear marks and no leakage. After melting, the reagent should be clear and transparent without turbidity and precipitation.
2. LoD: when detecting a 1.0 % ratio of mutated DNA in a total 50 ng/μL genomic DNA, the result should be V600E positive.
3. Precision: the coefficient of variation of inter- and intra- batches are both not higher than 15%.
4. Accuracy：the coincidence rate of 14 mutation positive specimens is 14/14； of 6 mutation negative specimens is 6/6.
5. No false positive results were found when using 50 ng genomic DNA as a template. Other mutations in exon 15 in the human genome, such as D594G(A1781G), K601E(A1801G), S616F(C1847T), V600Ec (G1798A, T1799A), V600D (G1798T, T1799A), V600K (T1799A, G1800A) and V600R (T1799G, G1800A) showed negative results by this kit.
6. In the tested sample, when the concentrations of common interfering substances such as 4 mg/mL 5-fluorouracil, 0.8mg/mL oxaliplatin, 150μg/mL cytarabine, 7 mmol/L triglyceride, 800 mg/L hemoglobin, 700μmol/L bilirubin and 70μmol/L melanin have no influence on the detection.
7. Sequencing was used as a reference method for validation, and 1109 clinical samples were detected. The sensitivity and specificity of the kit were 100% and 97% respectively.

PRECAUTIONS

1. It is important to check the quality of all samples. Before extracting the genomic DNA of tissue samples, an experienced pathologist must confirm the samples contain tumor cells.
2. Avoid repeatedly freezing and thawing the reagents in the kit. The kit needs to be transported at a low temperature, and it should be frozen when it reaches its destination.
3. The test is manually operated. Experimental personnel who perform this test should have received professional training in gene amplification or molecular biology diagnostics and be qualified for relevant experimental operations. There should be reasonable biosecurity precautions and protective procedures in the laboratories. The test should only be performed in laboratories that follow safety practices according to the applicable Biosafety Regulations in Microbiological and Biomedical Laboratories.
4. The whole detection process should be carried out in three areas: the first area is for reagent preparation. The second area is for specimen processing and reaction system preparation. The third area is for amplification, fluorescence detection and results analysis. Instruments, equipment and lab coats should be used independently in each area to prevent contamination.
5. In the testing process, should always take care to avoid RNase contamination, wear disposable gloves without fluorescent substances (Frequent replacement is recommended), use the disposable thin-walled 200μL PCR tube (or 96-well PCR plate with optical film) and pipette tips with filter. Never touch the reaction tube directly with bare hands.
6. The handling of Clinical Specimens should be performed in the biosafety cabinet to ensure the safety of laboratory staff and prevent environmental pollution. Harmful and/or toxic specimens and reagents in the experiment should be properly placed and stored, and in charge by an assigned person. Waste should be disposed of properly in special containers. Lab bench, equipment such as operator's stations, pipettes, centrifuges, and PCR thermocyclers etc., should be regularly wiped and disinfected with 1.0% sodium hypochlorite and/or 70% ethanol. Laboratory room, ultra-clean bench should be treated with ultraviolet lamp regularly and after each experiment.
7. Prior to the experiment, reagents should be fully thawed, mixed well, and centrifuged for a few seconds to bring down all the liquid to the bottom of the centrifuge tubes. When preparing the reaction solution, attention should be paid to mixing all liquids on the vortex mixer, no blowing with the pipette to avoid bubbles, and centrifuging the reaction mixture solution for a few seconds. Use the kit before the expiration date and do not combinate the reagents with different batch numbers.

Manufacturing date and expiration date: view on label

INDEX OF SYMBOLS

	Consult Instructions for Use		Contain <n> tests	EC REP	Authorized Representative in the European Community
IVD	In vitro diagnostic medical device		Use-by date	-15℃ -25℃	Temperature limit -25 to -15℃
REF	Catalogue #	LOT	Lot Number	CE	CE conformity marking
	Manufacture Date		Manufacturer		

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