AmoyDx® *BRAF* Mutation Detection Kit (V2)

Instructions for Use

For Research Use Only. Not for use in diagnostic procedures.

REF	8.01.0104	24 tests/kit	For LightCycler480
REF	8.01.0105	24 tests/kit	For Bio-Rad CFX96

Amoy Diagnostics Co., Ltd.
No. 39, Dingshan Road, Haicang District,
361027 Xiamen, P. R. China
Tel: +86 592 6806835
Fax: +86 592 6806839
E-mail: sales@amoydx.com
Website: www.amoydx.com

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Background

BRAF is a serine/threonine kinase that functions within the Ras-Raf-MEK-MAPK pathway. This pathway normally regulates cell proliferation and survival under the control of growth factors and hormones. Mutations in the *BRAF* gene have been associated with the development of cancer. The most common alteration in the *BRAF* gene is a mutation called V600E, which alters the valine at position 600 in the protein to a glutamic acid. Other mutations observed at the V600 position are V600D, V600K, V600R and V600A. *BRAF* mutations occur in ~50% of melanoma, ~40% of papillary thyroid tumors, 1~4% of non-small cell lung cancer (NSCLC), ~30% of ovarian tumors, ~10% of colorectal tumors, ~10% of prostate tumors. In the clinical studies, BRAF targeted therapy is associated with clinical benefit in the majority of patients with BRAF V600 mutations. BRAF inhibitors and MEK inhibitors have been approved for treatment of patients with BRAF V600-mutated melanoma, NSCLC and thyroid cancer.

Intended Use

The AmoyDx® *BRAF* Mutation Detection Kit (V2) is a real-time PCR assay for qualitative detection of V600E, V600E2, V600K, V600D, V600D2, V600A, V600R mutations in *BRAF* gene in human genomic DNA extracted from tumor tissue sample.
The kit is for research use only, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The kit adopts amplification refractory mutation system (ARMS) technology which comprises specific primers and fluorescent probes to detect gene mutations in real-time PCR assay. During the nucleic acid amplification, the targeted mutant DNA is matched with the bases at the 3' end of the primer, amplified selectively and efficiently, then the mutant amplicon is detected by fluorescent probes labeled with FAM. While the wild-type DNA cannot be matched with specific primers, there is no amplification occurs.
The kit is composed of ***BRAF*** **Reaction Mix**, ***BRAF*** **Positive Control** and ***BRAF*** **Enzyme Mix**.

1) The ***BRAF*** **Reaction Mix 1~3** includes a mutation detection system and an internal control system. The mutation detection system includes primers and FAM-labeled probes specific for designated *BRAF* mutations, which is used to detect the *BRAF* mutation status. The internal control system contains primers and HEX-labeled probe for a region of a region of genomic DNA without known mutations and polymorphism, which is used to detect the presence of inhibitors and confirm the validity of each experiment.
2) The ***BRAF*** **External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA without known mutations and polymorphism, which is used to assess the quality of DNA.
3) The ***BRAF*** **Positive Control (PC)** contains recombinant gene with *BRAF* V600 mutations.
4) The ***BRAF*** **Enzyme Mix** contains Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which is working at room temperature to prevent PCR amplicon carryover contamination.

Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

Tube No.	Content	Main Ingredients	Quantity	Fluorescent Signal
①	***BRAF*** **Reaction Mix 1**	Primers, Probes, Mg^{2+}, dNTPs	1150 μL/tube ×1	FAM, HEX/VIC
②	***BRAF*** **Reaction Mix 2**	Primers, Probes, Mg^{2+}, dNTPs	1150 μL/tube ×1	FAM, HEX/VIC
③	***BRAF*** **Reaction Mix 3**	Primers, Probes, Mg^{2+}, dNTPs	1150 μL/tube ×1	FAM, HEX/VIC
④	***BRAF*** **External Control Reaction Mix**	Primers, Probes, Mg^{2+}, dNTPs	1150 μL/tube ×1	FAM
⑤	***BRAF*** **Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	100 μL/tube ×1	/
⑥	***BRAF*** **Positive Control**	Plasmid DNA	250 μL/tube ×1	/

Storage and Stability

The kit requires shipment on frozen ice packs. All components of the kit should be stored immediately upon receipt at -20±5℃ and protected from light.

The shelf-life of the kit is eight months. The maximal number of freeze-thaw cycle is five.

Materials Required But Not Supplied

1) The compatible PCR instrument:
   LightCycler480, or Bio-Rad CFX96.
2) DNA Extraction Kit. We recommend to use AmoyDx® FFPE DNA Kit for FFPE tissue specimens.
3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Vortexer.
7) Nuclease-free centrifuge tubes.
8) Nuclease-free PCR tubes and caps.
9) Adjustable pipettors and filtered pipette tips for handling DNA.
10) Tube racks.
11) Disposable powder-free gloves.
12) Sterile, nuclease-free water.
13) 1×TE buffer (pH 8.0).

Precautions and Handling Requirements

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use. Strictly follow the instruction during operation.
- Please check the compatible real-time PCR instruments prior to use.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT use any other reagents from different lots in the tests.
- DO NOT use any other reagent in the other test kits.

Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- Only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid contact of the skin, eyes and mucous membranes with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipet by mouth.

Decontamination and Disposal

- The kit contains positive control; strictly distinguish the positive control from other reagents to avoid contamination which may cause false positive results.
- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Use separate, dedicated pipettes and filtered pipette tips when handling samples and reagents to prevent exogenous DNA contamination to the reagents.
- Please pack the post-amplification tubes with two disposable gloves and discard properly. DO NOT open the post- amplification PCR tubes.
- All disposable materials are for one-time use. DO NOT reuse.
- The unused reagents, used kit, and waste must be disposed of properly.

Cleaning

- After the experiment, wipe down the work area, spray down the pipettes and equipment with 75% ethanol or 10% hypochlorous acid solution.

Instrument Setup

- Set up the reaction volume as 40 µL.
- For LightCycler480 I instrument, it's necessary to conduct fluorescence calibration prior to use. If there is fluorescence crossover on LightCycler480 II instrument, fluorescence calibration is also required. To run the assays on a LightCycler machine, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480.
- Refer to the operation manual of the real-time PCR instrument for detailed instructions.
- We recommend that for all PCR instruments in use, a fluorescence calibration should be conducted once a year.

Assay Procedure

1. DNA Extraction

The specimen material must be human genomic DNA extracted from tumor tissue. DNA extraction kit is not included in the kit. Before DNA extraction, it is essential to use a standard pathology methodology to ensure tumor sample quality. Carry out the DNA extraction according to the instructions of the DNA extraction kit.

Tumor samples are non-homogeneous, may also contain non-tumor tissue. Data from different tissue sections of the same tumor may be inconsistent. DNA from non-tumor tissue would not be detected with *BRAF* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The $OD_{260/280}$ value of extracted DNA should be between 1.8 ~ 2.0 (measured using the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended).

The amount of extracted DNA from tissue used for PCR amplification is shown in Table 2.

Table 2 Recommended DNA concentration

Tissue	Storage time	DNA concentration	DNA amount per reaction
FFPE tissue	≤ 6 months	1.5 ng/µL	7.5 ng
	> 6 months & ≤ 1 year	2 ng/µL	10 ng
	> 1 year & ≤ 3 years	2~3 ng/µL	10~15 ng
Non-FFPE tissue	/	0.4~1 ng/µL	2~5 ng

Note:

- *The extracted DNA should be used immediately. If not, it should be stored at -20±5 ℃ for no more than 6 months.*
- *Before detection, dilute the extracted tissue DNA with 1×TE buffer (pH 8.0) to designated concentration. We recommend using at least 5 µL DNA for 10 times dilution, to ensure the validity of final concentration.*

2. Mutation Detection

1) Take ***BRAF*** **Reaction Mix 1~3,** ***BRAF*** **External Control Reaction Mix,** ***BRAF*** **Enzyme Mix** and ***BRAF*** **PC** out of the kit from the freezer.
2) Thaw the *BRAF* Reaction Mix 1~3, *BRAF* External Control Reaction Mix, and BRAF Positive Control at room temperature. When the reagents are completely thawed, mix each reagent thoroughly by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.
3) Centrifuge ***BRAF*** **Enzyme Mix** for 5~10 seconds prior to use.
4) Prepare sufficient *BRAF* Master Mix 1~4 containing *BRAF* Enzyme Mix and each *BRAF* Reaction Mix (*BRAF* Reaction Mix 1/2/3, or *BRAF* External Control Reaction Mix, respectively) in a separate sterile centrifuge tube according to the ratio in Table 3. Mix *BRAF* Master Mix thoroughly by vortexing and centrifuge for 5~10 seconds.

Table 3 *BRAF* Master Mix

Content	Volume per test
Each *BRAF* Reaction Mix	35 µL
BRAF **Enzyme Mix**	0.72 µL
Total	**35.72 µL**

Note:

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *The prepared mixtures should be used immediately, avoid prolonged storage.*
- *Due to the viscosity of the enzyme mix, pipet slowly to ensure all mix is completely dispensed from the tip.*
- *Pipet enzyme mix by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.*

5) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.
6) Prepare 4 PCR tubes for NTC: Dispense 35.72 μL of *BRAF* Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of nuclease-free water to each PCR tube. Cap the PCR tubes.
7) Prepare 4 PCR tubes for each sample: Dispense 35.72 μL of *BRAF* Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of sample DNA to each PCR tube. Cap the PCR tubes.
8) Prepare 4 PCR tubes for PC: Dispense 35.72 μL of *BRAF* Master Mix 1~4 to each PCR tube respectively. Then add 5 μL of BRAF Positive Control to each PCR tube. Cap the PCR tubes.
9) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
10) Place the PCR tubes into the appropriate positions of the real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 PCR Plate Layout

Well	1	2	3	4
A	Sample 1	Sample 3	Sample 5	NTC
B	Sample 1	Sample 3	Sample 5	NTC
C	Sample 1	Sample 3	Sample 5	NTC
D	Sample 1	Sample 3	Sample 5	NTC
E	Sample 2	Sample 4	Sample 6	PC
F	Sample 2	Sample 4	Sample 6	PC
G	Sample 2	Sample 4	Sample 6	PC
H	Sample 2	Sample 4	Sample 6	PC

11) Set up the PCR Protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	10 min	/
2	15	95℃	25 s	/
		62℃	20 s	/
		72℃	20 s	/
3	31	93℃	25 s	/
		58℃	35 s	FAM and HEX/VIC
		72℃	20 s	/

12) Start the PCR run immediately.
13) When the PCR run is finished, analyze the data according to the "Results Interpretation" procedures.

3. Results Interpretation

Before analysis of mutation data, the following items should be checked:

1) For NTC: The FAM Ct values of Tubes ①~③ should be ≥**31**. If not, the data is *INVALID*. The sample should be retested.
2) For Positive Control: The FAM Ct values of Tubes ①~④ and the HEX/VIC Ct values of Tubes ①~③ should be **< 20**. If not, the data is *INVALID*. The sample should be retested.
3) For the Internal Control assay in Tubes ①~③ for each sample: The HEX/VIC Ct values should be < **31**. If not, check the mutant FAM signals in Tubes ①~③ :
    a) If mutant FAM Ct value is < 31, continue with the analysis.
    b) If mutant FAM Ct value is ≥31, the data is *INVALID*. The sample should be retested.

4) For the External Control assay, check the FAM signal in Tubes ④ for each sample:
    a) The FAM Ct value should be between **10~18** for paraffin embedded specimens, and between **8~16** for non-paraffin embedded specimens.
    b) If FAM Ct value is greater than the indicated range, this indicates the DNA degradation or the presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA. But if any FAM Ct value of Tube ①~③ is < 26, the sample is determined as positive.
    c) If FAM Ct value is less than the indicated range, this indicates overloading of DNA. The DNA needs to be reduced and retested. But if the FAM Ct values of Tubes ①~③ are > 28, the sample is determined as negative.

Analyze the mutation assay for each sample:

5) Record the mutant FAM Ct values of Tubes ①~③ for each sample.
6) Check the mutant FAM Ct values of Tubes ①~③ according to Table 6:

Table 6 Result Determination

Mutation assay	①	②	③	Results
Mutation detected	V600E/ V600E2/ V600D/ V600D2	V600K	V600R/ V600D/ V600D2/ V600A	
Optimal Ct range	Ct <26	Ct <26	Ct <26	Positive
Acceptable Ct range	26≤Ct<28	26≤Ct<28	26≤Ct<28	Interpret the results according to the ΔCt value.
Cut-off ΔCt value	10	10	10	
Negative Ct range	Ct≥28	Ct≥28	Ct≥28	Negative or under the LOD*.

* LOD: limit of detection

a) If any FAM Ct value of Tube ①~③ is < 26, the sample is determined as positive (*BRAF* mutation detected).
b) If any FAM Ct value of Tube ①~③ is in Acceptable Ct range, calculate the ΔCt value for each reaction mix showing positive amplification.
    i. **ΔCt value = Mutant FAM Ct value – External control FAM Ct value**.
    ii. If the ΔCt value is less than the corresponding cut-off ΔCt value, the sample is determined as positive (Mutation detected).
    iii. If the ΔCt value is equal or more than the corresponding cut-off ΔCt value, the sample is determined as negative (No mutation detected) or under the LOD of the kit.
c) If all the FAM Ct values of Tubes ①~③ are in Negative Ct range or there is no amplification, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

7) Some cross-reactivity may occur between *BRAF* mutation reactions. If a strong positive mutation is detected, some of the other mutation reactions may also give a positive curve. If two or more of reaction mixes are detected as positive, the one with the smallest Ct value is determined as true positive. Check the ΔCt value of other mutations to determine the result (see Table 7).
    a) If the ΔCt value is less than the cross-reactivity cut-off value, the positive curve is determined as true positive. A sample may be detected with one or more *BRAF* mutations.
    b) If the ΔCt value is greater than or equal to the cross-reactivity cut-off value, the result is determined as negative.

Table 7 Cross-reactivity Cut-off ΔCt value

Mutation Name \ Tube No.	①	②	③
V600E		11.24	12.43
V600K	10.9		9.07
V600E2		10.33	-
V600R	13.3	10.78	
V600D		10.78	
V600D2		13.94	
V600A	-	-	

Note: *"-"symbol of no cross-reactivity.*

8) Distinguish *BRAF* Mutation according to the mutation assay result in Tubes ①~③ (Table 8):

a) If the mutation assay in Tube ② is positive, the sample is determined as *BRAF* V600K mutation positive.

b) If the mutation assays in both Tubes ①/③ are positive, the sample is determined as *BRAF* V600D/V600D2 mutation positive.

c) If the mutation assay in Tube ① is positive, but negative in Tubes ③, the sample is determined as *BRAF* V600E/V600E2 mutation positive.

d) If the mutation assay in Tube ① is negative, but positive in Tubes ③, the sample is determined as *BRAF* V600A/V600R mutation positive.

Table 8 Mutation Distinguish

Mutation assay	①	②	③	**Mutation type**
Mutation detected	V600E/ V600E2/ V600D/ V600D2	V600K	V600D/ V600D2/ V600A/V600R	
Result	Negative	Positive	Negative	V600K
	Positive	Negative	Negative	V600E/ V600E2
	Positive	Negative	Positive	V600D/ V600D2
	Negative	Negative	Positive	V600A/ V600R

Performance Characteristics

1) Limit of detection:

The kit allows detection of 1% mutant DNA in a background of 99% normal DNA.

2) Specificity:

The specificity of the kit was established by testing negative reference controls. The test gave negative results and negative concordance rate was 100%.

3) Accuracy:

Accuracy of the kit was established by testing positive reference controls. The test gave positive results and positive concordance rate was 100%.

4) Precision:

Precision of the kit was established by testing of the precision reference control for 10 repeats; the test gave positive results, analyzed the FAM Ct values, CV (%) ≤ 10%.

Limitations

1) The kit is to be used only by personnel specially trained with PCR techniques.
2) The kit has been validated for use with FFPE tumor tissue DNA.
3) The kit can only detect the *BRAF* mutations listed in the appendix.
4) Reliable results are dependent on proper sample processing, transport, and storage.
5) The sample containing degraded DNA may affect the ability of the test to detect *BRAF* mutation.
6) Samples with negative result (No mutation detected) may harbor *BRAF* mutations not detected by this assay.

References

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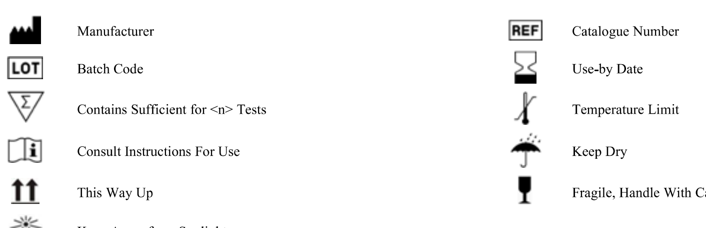
2) Dhomen, N., Marais, R., New insight into *BRAF* mutations in cancer. Curr Opin Genet Dev. 2007; 17(1):31-9.
3) Karreth FA, DeNicola GM, Winter SP, Tuveson DA. C-Raf inhibits MAPK activation and transformation by *BRAF*(V600E). Mol Cell 2009;36(3):477-86.
4) Cui Y, Guadagno TM. *BRAF*(V600E) signaling deregulates the mitotic spindle checkpoint through stabilizing Mps1 levels in melanoma cells. Oncogene 2008;27(22):3122-33.
5) Kondo T, Nakazawa T, Murata S, et al. Enhanced *BRAF* protein expression is independent of V600E mutant status in thyroid carcinomas. Hum Pathol 2007;38(12):1810-8.

6) Bentz BG, Miller BT, Holden JA, et al. *BRAF* V600E mutational analysis of fine needle aspirates correlates with diagnosis of thyroid nodules. Otolaryngol Head Neck Surg 2009;140(5):709-14.

Symbols



Appendix

***BRAF* Mutations Detected by the Kit**

Mutation	Base Changes	Cosmic ID	Name	LOD (%)
V600E	1799T>A	476	BRAF-M1	1%
V600K	1798_1799GT>AA(complex)	473	BRAF-M2	1%
V600E2	1799_1800TG>AA (complex)	475	BRAF-M3	1%
V600R	1798_1799GT>AG(complex)	474	BRAF-M4	1%
V600D	1799_1800TG>AC(complex)	308550	BRAF-M5	1%
V600D2	1799_1800TG>AT(complex)	477	BRAF-M6	1%
V600A	1799T>C	18443	BRAF-M8	1%