AmoyDx® *BRAF* V600 Mutations Detection Kit

Instructions for Use

8.01.0153 24 tests/kit For Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96, SLAN-96S

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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 and V600R. These mutations cause the BRAF protein to be permanently activated, even in the absence of growth factors, resulting in excessive cell proliferation and resistance to apoptosis. *BRAF* mutations occur in ~50% of melanoma tumors, ~40% of papillary thyroid tumors, ~30% of ovarian tumors, ~10% of colorectal tumors, ~10% of prostate tumors and 1~4% of non-small cell lung cancer. The search for drugs that block oncogenic *BRAF* signaling is an active area of pharmaceutical research and development.

Intended Use

The AmoyDx® *BRAF* V600 Mutations Detection Kit is an *in vitro* real-time PCR assay for qualitative detection of V600 mutations in *BRAF* gene in human genomic DNA extracted from cancer formalin-fixed paraffin-embedded (FFPE) tumor tissue.
The kit is for *in vitro* diagnostic use, 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 occurring.
The kit is composed of **V600 Reaction Mix**, ***BRAF* Positive Control** and ***BRAF* Enzyme Mix**.

1) The **V600 Reaction Mix** includes a mutation detection system and an internal control system. The mutation detection system is used to detect the mutation status of *BRAF* gene (positive or negative). The internal control system is designed to detect the presence of inhibitors and monitor the accuracy of experimental operation, which may lead to false negative results.
2) The ***BRAF* Positive Control** (PC) contains recombinant *BRAF* DNA with the V600 mutations.
3) 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

Content	Main Ingredients	Quantity
V600 Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	1150 μL/tube ×1
***BRAF* Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	20 μL/tube ×1
***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 twelve months. The maximal number of freeze-thaw cycles is five.

Additional Reagents and Equipment Required but Not Supplied

1) The compatible PCR instruments:
   Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96 or SLAN-96S.
2) DNA Extraction Kit. We recommend to use AmoyDx® FFPE DNA Kit.

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

For *in vitro* diagnostic use.

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use, and 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 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

- Setup the reaction volume as 40 μL.
- For Stratagene Mx3000P™, if there is a low net fluorescence signal (dR) but a high background signal (R), please reduce the signal gain setting of the instrument properly.
- For ABI instruments please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For ABI7900HT, please set up as follows: Instrument: Standard, Ramp speed: Standard, Reaction volume: 40 μL. It's necessary to use the ABI7900 adaptor, available from BIOplastics, Cat No. 7900RAN.
- For LightCycler480 instrument, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480.
- For SLAN-96S, please set up as follows: Probe mode: FAM, VIC. During the result analysis, open the "Preference" window, in "Chart Options" section; select "Selected Wells" for "Y-Axis Scaling Auto-adjust By" and "Absolute Fluorescence Value Normalization" for "Amplification Curve".
- Refer to the operations 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 FFPE 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 not homogeneous, they may also contain non-tumor tissue. Data from different tissue sections of the same tumor may be inconsistent. DNA from non-tumor tissue may not contain detectable *BRAF* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The OD_{260}/OD_{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 FFPE 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	< 3 years	2 ng/μL	10 ng
	≥ 3 years	3 ng/μL	15 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 **V600 Reaction Mix, *BRAF* Enzyme Mix** and ***BRAF* PC** out of the kit from the freezer.
2) Thaw the V600 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 containing V600 Reaction Mix and *BRAF* Enzyme Mix 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.

   Note:
   - *Every PCR run must contain one PC (Positive control) and one No Template Control (NTC).*
   - *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.*

Table 3 *BRAF* Master Mix

Content	Volume per test
V600 Reaction Mix	35 μL
***BRAF* Enzyme Mix**	0.4 μL
Total volume	**35.4 μL**

5) Dispensing 35 μL BRAF Master Mix to each PCR tube.
6) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.
7) Add 5 μL NTC, 5 μL each sample DNA, 5 μL PC to a PCR tube with 35 μL BRAF Master Mix respectively (refer to the PCR plate layout in Table 4), and cap the PCR tubes.
8) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
9) 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

Row	1	2	3	4	5	6	7	8
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
B	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16
C	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	PC	NTC

10) Setup the PCR Protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5min	/
2	15	95℃	25s	/
		64℃	20s	/
		72℃	20s	/
3	31	93℃	25s	/
		60℃	35s	FAM and HEX/VIC
		72℃	20s	/

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

3. Results Interpretation

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

1) For NTC: The FAM Ct value should be **≥31**. If not, the data is *INVALID*. The sample should be retested.
2) For PC: The FAM and HEX/VIC Ct values should be **< 20**. If not, the data is *INVALID*. The sample should be retested.
3) For the HEX/VIC signal for each sample, the Ct value should be between **13~21**.
   a) If Ct value > 21, this indicates the DNA degradation or the presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA. But if the FAM Ct is < 28, the result is determined as positive.
   b) If Ct value < 13, this indicates overloading of DNA. The DNA needs to be reduced and retested. But if the FAM Ct is > 28, the result will be determined as negative.

Analyze the mutation assay for each sample:

4) Check the FAM Ct value for each sample:
   a) If the sample FAM Ct value is **< 28**, the sample is determined as positive (Figure 1).
   b) If the sample FAM Ct value is **≥ 28**, the sample is determined as negative or below the detection limit of the kit (Figure 2).

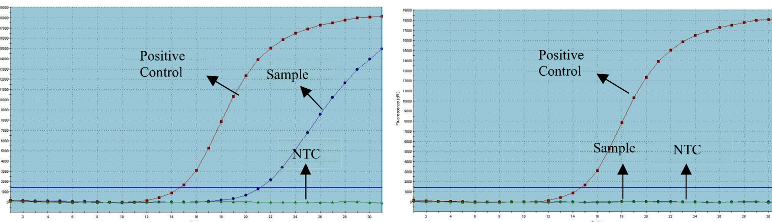


Figure 1 Curve of sample with mutant *BRAF* gene   Figure 2 Curve of sample with wild-type *BRAF* gene

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96 and SLAN-96S.

1) Analytical specificity:
   The kit was tested by 10 negative reference controls, which were prepared from 10 cases of FFPE tissue samples with wild-type *BRAF* gene confirmed by Sanger Sequencing. The test gave negative results and with 100% concordance rate.
2) Accuracy:
   The kit also was tested by 10 *BRAF* positive reference controls, which were prepared from 10 cases of lung cancer FFPE tissue samples with *BRAF* mutations confirmed by Sanger Sequencing. The test gave corresponding positive results and with 100% concordance rate.
3) Limit of Detection:
   The limit of detection of the kit was established by testing the diluted *BRAF* mutant plasmids. The kit allows detection of 10 copies/μL *BRAF* mutant plasmid DNA.
4) Precision:
   Precision of the kit was established by performing a certain mutant positive reference control for 10 repeats. The test gave positive results; the FAM and HEX Ct values were analyzed, and the CV value (%) was within 5%.
5) Interfering substance:
   Two common interfering substances hemoglobin and triglyceride were evaluated in this study. It is confirmed that the potential maximum concentrations: 15 mg/mL hemoglobin and 37 mmol/L triglyceride would not interfere with the test result.

Limitations

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

References

1) Shinozaki, M,O'Day, S, J.Kitago, M, et al. Utility of circulating *BRAF* DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. Clin Cancer Res. 2007; 13(7):2068-74.
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

EC REP — Authorized Representative in the European Community

Manufacturer

Batch Code

Contains Sufficient for <n> Tests

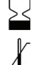
Consult Instructions For Use

This Way Up

Keep Away from Sunlight

In Vitro Diagnostic Medical Device

Catalogue Number


Use By

Temperature Limitation

Keep Dry

Fragile, Handle With Care

Appendix

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

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