AmoyDx® *C-KIT* Mutation Detection Kit

Detection of D816V mutation in the *C-KIT* gene

Instruction for Use

For Research Use Only

REF 8.01.20701X024E 24 tests For Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96

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Background

C-KIT, a 145-kD transmembrane glycoprotein, is the normal cellular homolog of the viral oncogene *v-kit* and a member of the receptor tyrosine kinase subclass III family that includes receptors for platelet-derived growth factor (PDGF), macrophage colony-stimulating factor (MCSF), and FLT3 ligand. It plays a critical role in activation and growth of hematopoietic stem cells, mast cells, melanocytes and germ cells. Cell growth factors and cytokines can regulate gene transcription through *C-KIT*-dependent activation of signal transducer and activator of transcription (STAT). The *C-KIT*-STAT signal transduction pathway regulates signaling cascades promoting cell growth and proliferation.

Activation of *C-KIT* by mutation of the amino acid at position 816 (D816V) is associated with systemic mastocytosis and a variety of cancers including acute myeloid leukemia, gastrointestinal stromal tumors and germ cell tumors.

Intended Use

The AmoyDx® *C-KIT* Mutation Detection Kit is a real-time PCR assay for qualitative detection of D816V mutation in *C-KIT* gene in human genomic DNA extracted from tumor tissue or whole blood samples. The kit is intended to be used by trained professionals in a laboratory environment. The kit is for research use only.

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 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 **D816V Reaction Mix**, ***C-KIT* Positive Control** and ***C-KIT* Enzyme Mix**.

1) The **D816V Reaction Mix** includes a mutation detection system and an internal control system. The mutation detection system is used to detect the mutation status of *C-KIT* 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 ***C-KIT* 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.
3) The ***C-KIT* Positive Control** contains recombinant *C-KIT* DNA with the D816V mutation.

Kit Contents

This kit contains the following materials (Table 1).

Table 1 Kit Contents

Content	Main Component	Quantity	Fluorescent Signal
D816V Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	1250 μL/tube×1	FAM, HEX/VIC
***C-KIT* Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	15 μL/tube×1	/
***C-KIT* Positive Control**	Plasmid DNA	150 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 recommend maximum freeze-thaw cycle is five cycles.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments are:
   Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, or Bio-Rad CFX96.
2) DNA extraction kit. We recommend use of AmoyDx® FFPE DNA Kit (Cat No.: 8.02.23501X036G) for FFPE tissues, AmoyDx® Blood DNA kit (Cat No.: 8.02.24201X036G) for whole blood sample.

3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Nuclease-free centrifuge tubes.
7) Nuclease-free PCR tubs and caps.
8) Adjustable pipettors and filtered pipette tips for handling DNA.
9) Tube racks.
10) Disposable powder-free gloves.
11) Sterile, nuclease-free water.
12) 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, 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 skin, eyes and mucous membranes contact 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.
- 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.
- Using 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 50 μL.
- For Stratagene Mx3000P™, if there's low net fluorescence signal (dR) but high background signal (R), please reduce the signal gain setting of 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 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 real-time PCR instrument operator's manual for detailed instructions.
- We recommend that all PCR instruments in use should be conducted fluorescence calibration once a year.

Assay Procedure

1. DNA Extraction

The specimen material must be human genomic DNA extracted from FFPE tissue or whole blood samples. DNA extraction reagents are not included in the kit. Before DNA extraction, it's essential to use standard pathology methodology to ensure tumor sample quality. Carry out the DNA extraction according to the instructions of 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 *C-KIT* mutation. 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 DNA concentration of extracted DNA used for PCR amplification should be 2~3 ng/μL.

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.*
- *Avoid using heparin anti-coagulated whole blood.*
- *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 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) Thaw **D816V Reaction Mix** and ***C-KIT* Positive Control** at room temperature. When the reagents completely thawed, invert each tube for 10 times and briefly centrifuge to collect all liquid at the bottom of the tube.
2) Briefly centrifuge ***C-KIT* Enzyme Mix** prior to use.
3) Prepare sufficient *C-KIT* Master Mix containing *C-KIT* Enzyme Mix and D816V Reaction Mix in a sterile tube according to the ratio in Table 2. Mix the solution thoroughly by gently pipeting it up and down more than 10 times. Centrifuge briefly.

Table 2 C-KIT Master Mix

Content	Volume per test
C-KIT Enzyme Mix	0.25 μL
D816V Reaction Mix	45 μL
Total	**45.25 μL**

Note:

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *Do not vortex enzyme mix or any mixture with enzyme mix.*
- *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.*

4) Prepare sufficient PCR tubes for PC, NTC and samples.
5) Take out the sample DNA and nuclease-free water for NTC.
6) Dispense 45 μL *C-KIT* Master Mix into the appropriate PCR tubes.

7) Add 5 μL nuclease-free water into NTC tube, add 5 μL each of sample DNA into each sample tube, add 5 μL *C-KIT* Positive Control to the PC tube. 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 3.

Table 3 Plate Layout

Well	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 4.

Table 4 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5min	/
2	15	95℃	25s	/
		60℃	20s	/
		72℃	20s	/
3	31	93℃	25s	/
		56℃	35s	FAM and HEX/VIC
		72℃	20s	/

11) Start the PCR run immediately.

12) After the PCR run is finished, analyze the data according to the "Results Interpretation" procedures.

3. Results Interpretation

Before mutation data analysis, 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 Positive Control: The FAM and HEX/VIC Ct values should be **< 22**, if not, the data is *INVALID*. The sample should be retested.

3) For the internal control HEX/VIC signal for each sample:
   a) The HEX/VIC Ct value should be between 13~31.
   b) If HEX/VIC Ct value is < 13, this indicates overloading of DNA. The DNA needs to be reduced and retested.
   c) If HEX/VIC Ct value is ≥ 31, this indicates the DNA degradation or the presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA.

Analyze the mutation assay for each sample:

4) Check the FAM Ct value for each sample.
   a) If the mutant FAM Ct value is **≥ 29** or there is no amplification, the sample is determined as negative (no mutation detected) or under the LOD of the kit.
   b) If the mutant FAM Ct value is < **26**, the sample is determined as positive (mutation detected).
   c) If the mutant FAM Ct value falls in **26~29**, calculate the ΔCt value for each mutation showing positive amplification.
      i. **ΔCt value = Mutant FAM Ct value – Internal control HEX/VIC Ct value**.
      ii. If the ΔCt value is < **10** (cut-off ΔCt value), the sample is determined as positive (mutation detected).
      iii. If the ΔCt value is ≥ **10**, the sample is determined as negative (no mutation detected) or under the LOD of the kit.

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 can only detect the D816Vmutation in *C-KIT* gene.

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 D816Vmutation in *C-KIT* gene.

6) Samples with negative result (No mutation detected) may harbor *C-KIT* mutations not detected by this assay.

References

1) Gommerman JL, *et al*. 1997. *J. Biol. Chem*. 272:30519–25.

2) Hirota S, *et al*. 1998. *Science*. 279: 577-80.

3) Schumacher JA, *et al*. 2008. *J Clin Pathol*. 61:109-14.

4) Tan A, *et al*.2006. *Clin Chem.* 52: 2250-7.

5) Tefferi A and Vardiman JW. 2008. *Leukemia*. 22:14-22.

Symbols

	Manufacturer		Catalogue Number
	Batch Code		Use By
	Contains Sufficient for <n> Tests		Temperature Limitation
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care