AmoyDx® *PIK3CA* Five Mutations Detection Kit

Detection of five mutations in *PIK3CA* gene

Instructions for Use

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

REF 8.01.0057 24 tests/kit For Mx3000P™, ABI7500, LightCycler480, Bio-Rad CFX96, SLAN-96S

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Background

The phosphoinositide-3-kinase catalytic alpha (*PIK3CA*) gene produces the p110 alpha (p110α) protein, which is one subunit of an enzyme called phosphatidylinositol 3-kinase (PI3K). PI3K plays a key role of PI3K/Akt signaling pathway in numerous cellular processes critical for cancer progression, including metabolism, growth, survival, and motility. Somatic mutations in the *PIK3CA* gene are found in many types of cancer, including approximately 40% of patients with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer. *PIK3CA* mutations in which lead to Activation of the PI3K pathway in breast cancer have been typically associated with resistance to endocrine therapy and poor prognosis. The clinical studies demonstrate that PI3K inhibitors has shown significantly high response rate in patients with *PIK3CA*-mutated breast cancer.

Intended Use

The AmoyDx® *PIK3CA* Five Mutations Detection Kit is a real-time PCR assay for qualitative detection of five mutations in the *PIK3CA* gene in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue.
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 occurring.
The kit is composed of five Reaction Mixes, *PIK3CA* External Control Reaction Mix, *PIK3CA* Positive Control and *PIK3CA* Enzyme Mix.

1) The **Reaction Mix in Tubes** ①~⑤ include mutation detection and internal control systems. The mutation detection system includes primers and FAM-labeled probes specific for designated *PIK3CA* mutations, is used to detect the *PIK3CA* mutation status. The internal control system contains primers and HEX-labeled probe for a region of genomic DNA without known mutations and polymorphism, to detect the presence of inhibitors and monitor the accuracy of the experimental operation.
2) The ***PIK3CA* External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA without known mutations and polymorphism, used to assess the quality of DNA.
3) The ***PIK3CA* Positive Control (PC)** contains a recombinant gene with *PIK3CA* mutations.
4) The ***PIK3CA* Enzyme Mix** contains Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works 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	Channel
①	H1047R Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
②	H1047L Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
③	E542K Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
④	E545K Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
⑤	E545D Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
⑥	*PIK3CA* External Control Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM
/	*PIK3CA* Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	50 μL/tube ×1	/
/	*PIK3CA* Positive Control	Plasmid DNA	250 μL/tube ×1	/

Storage and Stability

The kit requires shipment on frozen ice packs. All contents 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) Compatible Real-time PCR instrument:
   Stratagene Mx3000P™, ABI7500, LightCycler480, Bio-Rad CFX96, or SLAN-96S.
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 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 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 25 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 instrument, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- 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.
- For SLAN-96S instrument, please set up as follows: Fluorophores/Dyes: FAM, VIC. During the result interpretation, select "Selected Wells" for "Y-Axis Scaling Auto-adjust By" and "Absolute fluorescence Method" for "Normalization algorithm".
- 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 FFPE tumor tissue. DNA extraction reagents are not included in the kit. Carry out the DNA extraction according to the instructions of DNA extraction kit.Before DNA extraction, it is essential to use a standard pathology methodology to ensure tumor sample quality.

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 *PIK3CA* 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 months	1.5 ng/μL	7.5 ng
	> 3 months & ≤ 1 year	2 ng/μL	10 ng
	> 1 year & ≤ 3 years	2.5~3 ng/μL	12.5~15 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 the **Reaction Mixes**, ***PIK3CA* PC** and ***PIK3CA* Enzyme Mix** out of the kit from the freezer, and other reagents remained in freezer at -20±5℃.
2) Thaw **Reaction Mixes** and ***PIK3CA* PC** 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 ***PIK3CA* Enzyme Mix** for 5~10 seconds prior to use.
4) Prepare sufficient *PIK3CA* Master Mix containing each **Reaction Mix** and ***PIK3CA* Enzyme Mix** in a separate sterile centrifuge tube according to the ratio in Table 3. Mix each Master Mix thoroughly by vortexing and centrifuge for 5~10 seconds.

Table 3 *PIK3CA* Master Mix

Master Mix	Volume per test	
	Reaction Mix (μL)	*PIK3CA* Enzyme Mix (μL)
H1047R Master Mix	20	0.16
H1047L Master Mix	20	0.16
E542K Master Mix	20	0.2
E545K Master Mix	20	0.2
E545D Master Mix	20	0.16
PIK3CA External Control Master Mix	20	0.16

Note:

- *Each run must contain one PC (Positive control) and one No Template Control (NTC).*
- *The prepared master mix 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 6 PCR tubes for NTC, transfer 20 μL of each of the 6 PIK3CA Master Mixes to the corresponding tubes. Then add 5 μL of nuclease-free water to each PCR tube, and cap the PCR tubes.

7) Prepare 6 PCR tubes for each sample, transfer 20 μL of each of the 6 PIK3CA Master Mixes to the corresponding tubes. Then add 5 μL of sample DNA to each PCR tube, and cap the PCR tubes.

8) Prepare 6 PCR tubes for PC, transfer 20 μL of each of the 6 PIK3CA Master Mixes to the corresponding tubes. Then add 5 μL of Positive Control to each PCR tube, and 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 Plate Layout

Well	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC
C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC
D	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC
E	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC
F	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample10	PC	NTC

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

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5 min	/
2	15	95℃	25 s	/
		64℃	20 s	/
		72℃	20 s	/
3	31	93℃	25 s	/
		60℃	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 ①~⑤ should be ≥31. If not, the data is *INVALID*. The sample should be retested.

2) For PC: The FAM Ct values of Tubes ①~⑥ and HEX/VIC Ct values of Tubes ①~⑤ should be < 20. If not, the data is *INVALID*. The sample should be retested.

3) For the internal control assay in Tubes ①~⑤ for each sample: The HEX/VIC Ct values of Tubes ①~⑤ should be < 31. If not, check the mutant FAM signals of Tubes ①~⑤ :
   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 in Tube ⑥ for each sample:
   a) The Ct value should be between 15 ~ 21.
   b) If Ct <15, the DNA is overloaded. The test should be repeated with reduced DNA.
   c) If Ct >21, this indicates the DNA degradation or the presence of PCR inhibitors, or any error in experimental operation. The sample should be retested with increased or re-extracted DNA.

Analyze the mutation assay for each sample:

5) Record the mutant FAM Ct values of Tubes ①~⑤ for each sample.

6) Check the mutant FAM Ct values of Tubes ①~⑤ according to Table 6:

Table 6 Results Determination

Mutation assay	H1047R	H1047L	E542K	E545K	E545D	Results
Optimal Ct range	Ct<25	Ct<26	Ct<25	Ct<26	Ct<26	Positive.
Acceptable Ct range	25≤Ct <28	26≤Ct <29	25≤Ct <29	26≤Ct <29	26≤Ct <29	Interpret the results according to the ΔCt value
Cut-off ΔCt value	11	12	12	12	12	
Negative Ct range	Ct≥28	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Negative or under the LOD*

* LOD: limit of detection

   a) If any FAM Ct value of Tube ①~⑤ is < 26, the sample is determined as positive (*PIK3CA* mutation detected).
   b) If any FAM Ct value of Tube ①~⑤ is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.
      i. **ΔCt value = Mutant FAM Ct value - External control FAM Ct value**.
      ii. If the ΔCt value is < cut-off ΔCt value, the sample is determined as positive (Mutation detected).
      iii. If the ΔCt value is ≥ 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 ①~⑤ are in Negative Ct range or there is no amplification, the sample is determined as negative (No mutation detected) or under the LOD (Limit of Detection) of the kit.

Performance Characteristics

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

1) Limit of Detection: for H1047R, H1047L, E545D gene, the kit can detect 1% mutation in 10 ng DNA sample, for E542K, E545K gene, the kit can detect 2% mutation in 10 ng DNA sample.

2) Accuracy: accuracy of the kit was established by testing positive references control, the test gave positive results and positive concordance rate was 100%.

3) Specificity: specificity of the kit was established by testing negative reference controls, the test gave negative results and negative concordance rate was 100%.

4) Precision: precision of the kit was established by performing a certain mutant positive reference control for 10 repeats; the test gave positive results, analyzed the FAM and HEX Ct, CV (%) ≤ 5%.

5) Interfering substance: two common potential interfering substances hemoglobin and triglyceride were evaluated in this study. It is confirmed that the potential maximum concentrations: 2 g/L 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 kit has been validated for use with FFPE tumor tissue DNA.
3) The kit can only detect the 5 *PIK3CA* 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 *PIK3CA* mutation.
6) Samples with negative result (No mutation detected) may harbor *PIK3CA* mutations not detected by this assay.
7) Circulating DNA extracted from plasma or serum with negative results (No Mutation detected) may harbor *PIK3CA* mutation, which could be confirmed with matched tissue DNA detection.

References

1) Bunney TD & Katan M, 2010. *Nature Rev. Cancer.* 10: 342-352.
2) Yap TA, *et al.* 2008.*Curr Opin Pharmacol.* 8: 393-412.
3) Sartore-Bianchi A, *et al.* 2009.*Cancer Res.* 69: 1851-57.
4) Serra V, *et al.* 2008. *Cancer Res.* 68: 8022-30

Symbols

	Manufacturer	REF	Catalogue Number
LOT	Batch Code		Use-by Date
Σ	Contains Sufficient for <n> Tests		Temperature Limit
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care
	Keep Away from Sunlight		

Appendix

PIK3CA Mutation Information

Name	Mutation	Base Change	Cosmic ID
PI-M1	H1047R	CAT > CGT	775
PI-M2	H1047L	CAT > CTT	776
PI-M3	E542K	GAA >AAA	760
PI-M4	E545K	GAG> AAG	763
PI-M5	E545D	GAG > GAT	765