AmoyDx® *PIK3CA* Mutation Detection Kit

Detection of eleven mutations in *PIK3CA* gene

Instruction for Use

For Research Use Only

REF 8.0121602X024E 24 tests For Mx3000P™, ABI7500, LightCycler480, BioRad-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* Mutation Detection Kit is a real-time PCR assay for qualitative detection of eleven 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 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 eleven Reaction Mixes, *PIK3CA* External Control Reaction Mix, *PIK3CA* Positive Control and *PIK3CA* Enzyme Mix.

1) The **Reaction Mix in Tubes ①~⑪** 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 confirm the validity of each experiment.
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** 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	H1047Y Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 µL/tube ×1	FAM, HEX/VIC
⑦	E545A Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 µL/tube ×1	FAM, HEX/VIC
⑧	E545G Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 µL/tube ×1	FAM, HEX/VIC
⑨	Q546R Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 µL/tube ×1	FAM, HEX/VIC

⑩	Q546E Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
⑪	C420R Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM, HEX/VIC
⑫	*PIK3CA* External Control Reaction Mix	Primers, Probes, Mg^{2+}, dNTPs	650 μL/tube ×1	FAM
/	*PIK3CA* Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	100 μL/tube ×1	/
/	*PIK3CA* Positive Control	Plasmid DNA	500 μL/tube ×2	/

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 recommend maximum freeze-thaw cycle is five cycles.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible Real-time PCR instrument:
   Stratagene Mx3000P™, ABI7500, LightCycler480, BioRad-CFX96, or SLAN-96S.
2) DNA extraction kit. We recommend use of AmoyDx® FFPE DNA Kit (Cat No.: 8.02.23501X036G) for FFPE tissues.
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 tubes 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 25 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 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, please set up as follows: Probe mode: FAM. 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 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 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's essential to use standard pathology methodology to ensure tumor sample quality.

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 *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 1.7 ~ 2.2 (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* Positive Control** 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* Positive Control (PC)** at room temperature. When the reagent completely thawed, invert the tube for 10 times and briefly centrifuge to collect all liquid at the bottom of the tube.
3) Briefly centrifuge ***PIK3CA* Enzyme Mix** 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 gently pipetting up and down more than 10 times, and centrifuge briefly.

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
H1047Y Master Mix	20	0.16
E545A Master Mix	20	0.16
E545G Master Mix	20	0.16
Q546R Master Mix	20	0.16
Q546E Master Mix	20	0.16
C420R Master Mix	20	0.16
PIK3CA External Control Master Mix	20	0.16

Note:

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

5) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.
6) Prepare 12 PCR tubes for NTC, transfer 20 μL of each of the 12 *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 12 PCR tubes for each sample, transfer 20 μL of each of the 12 *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 12 PCR tubes for PC, transfer 20 μL of each of the 12 *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 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
C	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
D	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4

E	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5
F	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6
G	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	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℃	5min	/
2	15	95℃	25s	/
		64℃	20s	/
		72℃	20s	/
3	31	93℃	25s	/
		60℃	35s	FAM and HEX/VIC
		72℃	20s	/

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 mutation data analysis, the following items should be checked:

1) For NTC: The FAM Ct values of Tubes ①~⑪ should be ≥31. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values of Tubes ①~⑫ and HEX/VIC Ct values of Tubes ①~⑪ should be < 20. If not, the data is *INVALID*. The sample should be retested.

3) For the internal control assay in Tubes ①~⑪ for each sample: The HEX/VIC Ct values of Tubes ①~⑪ should be < 31. If not, check the mutant FAM signals of Tubes ①~⑪ :
   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 ⑫ 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 ①~⑪ for each sample.

6) Check the mutant FAM Ct values of Tubes ①~⑪ according to Table 6:
   a) If any FAM Ct value of Tube ①~⑪ is in the optimal Ct range, the sample is determined as positive (*PIK3CA* mutation detected).
   b) If any FAM Ct value of Tube ①~⑪ 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 ①~⑪ 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.

Table 6 Results Determination

Mutation assay	H1047R	H1047L	E542K	E545K	E545D	H1047Y	E545A	E545G	Q546R	Q546E	C420R	**Results**
Optimal Ct range	Ct <25	Ct <26	Ct <25	Ct <26	Ct <26	Ct <26	Ct <25	Ct <25	Ct <25	Ct <26	Ct <24	Positive.
Acceptable Ct range	25≤Ct<28	26≤Ct<29	25≤Ct<29	26≤Ct<29	26≤Ct<29	26≤Ct<28	25≤Ct<27	25≤Ct<27	25≤Ct<27	26≤Ct<28	24≤Ct<26	Interpret the results according to the ΔCt value
Cut-off ΔCt value	11	12	12	12	12	9	8	8	8	9	8	
Negative Ct range	Ct≥28	Ct≥29	Ct≥29	Ct≥29	Ct≥29	Ct≥28	Ct≥27	Ct≥27	Ct≥27	Ct≥28	Ct≥26	Negative or under the LOD*

* LOD: limit of detection

Performance Characteristics

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

1) Concordance rate with References: the positive concordance rate was 100%, and the negative concordance rate was 100%.
2) Wild-type DNA tolerability: the study showed that the kit can tolerate 10 ng wild-type DNA without non-specificity.
3) Limit of Detection: for H1047R, H1047L, E545D, H1047Y and Q546E mutations, the kit can detect 1% mutation in 10 ng DNA sample, for E542K, E545K, E545A, E545G, Q546R, and C420R mutations, the kit can detect 2% mutation in 10 ng DNA sample.
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/VIC Ct, CV (%) ≤ 5%.

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 11 *PIK3CA* 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 *PIK3CA* mutation.
7) Samples with negative result (No mutation detected) may harbor *PIK3CA* mutation not detected by this assay.

References

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3) Yap TA, Garrett MD, Walton MI, *et al.* Workman P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol.* 2008;8(4):393-412.
4) Serra V, Markman B, Scaltriti M, *et al.* NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res.* 2008;68:8022-30.
5) Juric D, Janku F, Rodón J, *et al.* Alpelisib Plus Fulvestrant in PIK3CA-Altered and PIK3CA-Wild-Type Estrogen Receptor–Positive Advanced Breast Cancer: A Phase 1b Clinical Trial. *JAMA Oncol*. 2019;5(2):e184475.
6) Vasan N, Razavi P, Johnson JL, *et al.* Double PIK3CA mutations in cis increase oncogenicity and sensitivity to PI3Kα inhibitors. *Science.* 2019;366(6466):714-723.
7) Andre, F. *et al.* Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N. Engl. J. Med.* 2019;380:1929-1940.
8) NCCN Clinical Practice Guidelines in Oncology, Breast Cancer, Version 4. 2020- May 8, 2020.

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

Appendix

PIK3CA Mutation Information

Mutation	Base Change	Cosmic ID	AmoyDx Name
H1047R	CAT > CGT	775	PI-M1
H1047L	CAT > CTT	776	PI-M2
E542K	GAA >AAA	760	PI-M3
E545K	GAG> AAG	763	PI-M4
E545D	GAG > GAT	765	PI-M5
H1047Y	CAT>TAT	774	P1-M6
E545A	GAG>GCG	12458	P1-M11
E545G	GAG>GGG	764	P1-M12
Q546R	CAG>CGG	12459	P1-M15
Q546E	CAG>GAG	6147	P1-M16
C420R	TGT>CGT	757	P1-M17