AmoyDx® *HER2* Mutation Detection Kit

Instructions for Use

For Research Use Only

REF	8.01.0232	24 tests/kit	For Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96, Rotor-Gene Q/6000 (72 wells), SLAN-96S

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Version: B1.0
May 2022

Background

Human epidermal growth factor receptor 2 (*HER2*), also known as Neu, ErbB-2, CD340 (cluster of differentiation 340) or p185, is encoded by a proto-oncogene ERBB2 which located at the long arm of human chromosome 17 (17q12). Activating mutations in the tyrosine kinase domain of *HER2* have been described in a subset of lung adenocarcinomas (ADCs) and are mutually exclusive with *EGFR*/*KRAS*/*ALK* driver mutations. *HER2* mutations, consisting of in-frame insertions in exon 20, have been identified in approximately 2~4% of non-small cell lung cancer (NSCLC) patients. These insertions cause activation of downstream *HER2*-pathway components such as AKT and MEK.

Studies have shown that NSCLC patients harboring a *HER2* exon 20 insertion are sensitive to trastuzumab (marketed as Herceptin) and the irreversible dual *EGFR* and *HER2* TKIs, lapatinib, neratinib, and afatinib. However, the presence of this mutation is associated with primary resistance to the first generation *EGFR* TKIs, erlotinib and gefitinib. These studies highlighted the importance of screening for *HER2* mutations in NSCLC and suggest that *HER2*-positive patients may be responsive to *HER2*-targeted therapy.

Intended Use

The AmoyDx® *HER2* Mutation Detection Kit is a real-time PCR assay for qualitative detection of 13 mutations in *HER2* 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.

This kit is composed of 6 reaction mixes, sufficient Enzyme Mix and Positive Control.

1) The ***HER2* Reaction Mix** ①~⑤ include mutation detection and internal control systems. The mutation detection system includes primers and FAM-labeled probes specific for designated *HER2* mutations, which is used to detect the *HER2* 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 ***HER2* External Control Reaction Mix** contains external control to assess the DNA quality, that is, to detect the presence of inhibitors, which may lead to false negative results.
3) The ***HER2* Enzyme Mix** contains the Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.
4) The ***HER2* Positive Control** contains a recombinant gene with *HER2* mutations.

Kit Contents

This kit contains the following materials (Table 1).

Table 1 Kit Contents

Tube No.	Content	Main Ingredient	Quantity	Fluorescent Signal
①	***HER2* Reaction Mix** ①	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
②	***HER2* Reaction Mix** ②	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
③	***HER2* Reaction Mix** ③	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
④	***HER2* Reaction Mix** ④	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
⑤	***HER2* Reaction Mix** ⑤	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
⑥	***HER2* External Control Reaction Mix**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM
/	***HER2* Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	60 μL/tube ×1	/
/	***HER2* 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 cycle is five.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments are:
   Stratagene Mx3000P™, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96, Rotor-Gene Q/6000 (72 wells), or SLAN-96S.
2) DNA extraction kit. We recommend use of DNA extraction kit (AmoyDx® FFPE DNA Kit for FFPE tissue sample).
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 tubs 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.
- As all the chemicals have potential hazard, 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

- Setup the reaction volume as 40 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.
- For SLAN-96S, please setup as follows: Probe mode: FAM, VIC. During the result analysis, select "Absolute Fluorescence Value Normalization" in "Amplification Curve" section.
- 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 tissue. DNA extraction reagents are not included in the 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 *HER2* mutation. It's better to use tumor tissue samples with more than 30% tumor cells.

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.

The OD_{260}/OD_{280} value of extracted DNA from FFPE tissue should be between 1.6~2.2 (measured using the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended). The DNA concentration of extracted DNA used for PCR amplification should be between 1~3 ng/μL (2 ng/μL is recommended).

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.*
- *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) Thaw ***HER2* Reaction Mix** ①~⑤, ***HER2* External Control Reaction Mix** and ***HER2* 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.
2) Centrifuge ***HER2* Enzyme Mix** for 5~10 seconds prior to use.
3) Prepare sufficient *HER2* Master Mix 1~6 containing *HER2* Enzyme Mix and each *HER2* Reaction Mix (*HER2* Reaction Mix ①~⑤ or *HER2* External Control Reaction Mix, respectively) in separate sterile centrifuge tube according to the ratio in Table 2. Mix each

HER2 Master Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.

Table 2 *HER2* Master Mix

Content	Volume per test
Reaction Mix	35 μL
***HER2* Enzyme Mix**	0.3 μL
Total volume	35.3 μL

Note:

- *Each PCR run must contain one PC and one 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.*

4) Take out the sample DNA and nuclease-free water for NTC.

5) Prepare 6 PCR tubes for NTC: Dispense 35.3 μL of *HER2* Master Mix 1~6 to each PCR tube respectively. Then add 4.7 μL of nuclease-free water to each PCR tube, and cap the PCR tubes.

6) Prepare 6 PCR tubes for each sample: Dispense 35.3 μL of *HER2* Master Mix 1~6 to each PCR tube respectively. Then add 4.7 μL of sample DNA (1~3 ng/μL) to each PCR tube, and cap the PCR tubes.

7) Prepare 6 PCR tubes for PC: Dispense 35.3 μL of *HER2* Master Mix 1~6 to each PCR tube respectively. Then add 4.7 μL of *HER2* Positive Control to each PCR tube, 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 real-time PCR instrument. A recommended plate layout is shown in Table 3.

Table 3 Recommended Plate Layout

Tube No.	①	②	③	④	⑤	⑥
A	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
C	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
E	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
G	PC	PC	PC	PC	PC	PC
H	NTC	NTC	NTC	NTC	NTC	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°C	5 min	/
2	15	95°C	25 s	/
		64°C	20 s	/
		72°C	20 s	/
3	31	93°C	25 s	/
		60°C	35 s	FAM and HEX/VIC
		72°C	20 s	/

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 mutation data analysis, 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 Positive Control: 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) Check the internal control assay in Tubes ①~⑤ for each sample: The HEX/VIC Ct values should be **< 31**. If not, check the mutant FAM signals in 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) Check the external control of Tube ⑥ for each sample:
   c) The FAM Ct value should be between **15~21**.
   d) If the FAM Ct value is < 15, that indicates the DNA is overloaded, the sample should be retested with reduced DNA. However, if any FAM Ct value of Tube ①~⑤ is > 29, the test result is determined as negative.
   e) If the FAM Ct value is > 21, which indicates the DNA sample contains PCR inhibitors or DNA amount is insufficient. The sample should be retested with re-exacted or sufficient DNA. However, if any FAM Ct value of Tube ①~⑤ is < 26, the test result is determined as positive.

Analyze the mutation assay for each sample:

5) Record the mutant FAM Ct values of Tubes ①~⑤ for each sample.
6) Check the FAM Ct values of Tubes ①~⑤ according to Table 5:
   a) If all the mutant FAM Ct values of Tubes ①~⑤ are **≥ 29** or there is no amplification, the sample is determined as negative (no mutation detected) or under the LOD of the kit.
   b) If any FAM Ct value of Tube ①~⑤ is **< 26**, the sample is determined as positive (*HER2* mutation detected).
   c) If any FAM Ct value of Tube ①~⑤ falls in 26~29, calculate the ΔCt value for each mutation, interpret result in accordance with Table 5.
      i. **ΔCt value = Mutant FAM Ct value –External control FAM Ct value**.
      ii. If the ΔCt value is < ΔCt Cut-off value, the sample is determined as positive (mutation detected).
      iii. If the ΔCt value is ≥ ΔCt Cut-off value, the sample is determined as negative (no mutation detected) or under the LOD of the kit.

Table 5 Result Determination

Mutation assay	①	②	③	④	⑤	**Results**
Optimal Ct range	Ct<26	Ct<26	Ct<26	Ct<26	Ct<26	Positive.
Acceptable Ct range	26≤Ct<29	26≤Ct<29	26≤Ct<29	26≤Ct<29	26≤Ct<29	Interpret the results according to the ΔCt value.
Cut-off ΔCt value	10	9	10	9	9	
Negative Ct range	Ct≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Negative or under the LOD*.

* LOD: limit of detection

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 tissue.
4) The kit can only detect the 13 mutations in *HER2* gene listed in 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 *HER2* mutation.

7) Samples with negative result (No mutation detected) may harbor *HER2* mutations not detected by this assay.

References

1. Arcila ME *et al.* 2012. *Clin Cancer Res.* 18: 4908-10.
2. Buttitta F *et al.* 2006. *Int J Cancer.* 119: 2586-91.
3. Shigematsu H *et al.* 2005. *Cancer Res.* 65: 1642-46.
4. Stephens P, *et al.* 2004. *Nature.* 431:525-6.

Symbols

	Manufacturer		Catalogue Number
	Batch Code		Use-by Date
	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

HER2 Mutations Detected by the Kit

Reagent	Exon	Mutation	Base Change	Cosmic ID
HER2 Reaction Mix ①	20	A775_G776insYVMA	2325_2326 ins12 (TACGTGATGGCT)	12558
		A775_G776insYVMA	2324_2325 ins12 (ATACGTGATGGC)	20959
		M774_A775insAYVM	2322_2323 ins12 (GCATACGTGATG)	/
HER2 Reaction Mix ②	20	G776>VC	2326_2327 ins3 (TGT)	12553
		G776>VC	2326_2327 ins3 (TTT)	12552
		G776R	2326G>C	/
		G776C	2326G>T	303938
HER2 Reaction Mix ③	20	P780_Y781insGSP	2339_2340 ins9 (TGGCTCCCC)	303948
		P780_Y781insGSP	2339_2340 ins9 (GGGCTCCCC)	12555
		P780_Y781insGSP	2340_2341 ins9 (GGCTCCCCA)	12556
		P780_Y781insGSP	2339_2340 ins9 (CGGCTCCCC)	/
HER2 Reaction Mix ④	20	V777L	2329G>T	14062
HER2 Reaction Mix ⑤	19	L755P	2263_2264 TT>CC	683