



AmoyDx[®] *EGFR* 29 Mutations Detection Kit

Detection of 29 mutations in exons 18-21

Instruction for Use

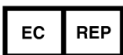
REF 8.01.20201X024E

24 tests

For Stratagene Mx3000P™, ABI7300, ABI7500, ABI7900HT,
ABI StepOnePlus, LightCycler 480, Bio-Rad CFX96, SLAN-96S



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Background

The epidermal growth factor receptor (EGFR) plays a central role in transmitting signals that promote cell growth and proliferation. Due to its association with malignancies, *EGFR* has become the target of an expanding class of anticancer therapies, such as gefitinib, erlotinib and afatinib, which are tyrosine kinase inhibitors (TKIs). The TKIs target the *EGFR* tyrosine kinase domain. These drugs work best on non-small cell lung cancer (NSCLC) patients whose cancer is driven by abnormal *EGFR* signaling. Lung cancer patients who experienced rapid, durable, complete or partial responses to TKI therapy have been found to harbor somatic mutations in *EGFR* gene. NSCLC patients with sensitizing *EGFR* mutations treated with TKI therapy have shown longer progression-free survival and higher response rate, compared with conventional chemotherapy. Resistance to TKI therapy, either in the primary tumor or acquired after TKI treatment, is associated with *EGFR* T790M mutation. Therefore, assessment of *EGFR* mutation status facilitates personalized treatment to lung cancer patients.

Intended Use

The AmoyDx[®] *EGFR* 29 Mutations Detection Kit is a real-time PCR assay for qualitative detection of 29 somatic mutations in exons 18, 19, 20 and 21 of *EGFR* gene in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue, or circulating DNA extracted from plasma/serum. The kit is intended to be used to assess *EGFR* mutation status in NSCLC patients and aid in identifying patients who may respond to the treatment with *EGFR*-TKI.

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 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 eight **Reaction Mixes**, ***EGFR* Enzyme Mix** and ***EGFR* Positive Control**.

- 1) The **Reaction Mix in Tubes ①-⑦** include mutation detection and internal control systems. The mutation detection system includes primers and FAM-labeled probes specific for designated *EGFR* mutations, which is used to detect the *EGFR* 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 **External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA without known mutations and polymorphism, which is used to assess the quality of DNA.
- 3) The ***EGFR* Positive Control** contains a recombinant gene with *EGFR* mutations.
- 4) The ***EGFR* 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	Fluorescent Signal
①	19-Del Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
②	L858R Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
③	T790M Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
④	Insertions Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
⑤	G719X Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
⑥	S768I Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
⑦	L861Q Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM, HEX/VIC
⑧	External Control Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	1000 μL/tube ×1	FAM
/	<i>EGFR</i> Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	85 μL/tube ×1	/
/	<i>EGFR</i> Positive Control	Plasmid DNA	500 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 $-20\pm 5^{\circ}\text{C}$ 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 Real-time PCR instrument:
Stratagene Mx3000P™, ABI7300, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96, or SLAN-96S.
- 2) DNA extraction kit. We recommend use of AmoyDx® FFPE DNA Kit (Cat No.: 8.02.23501X036G) for FFPE tissues, AmoyDx® Circulating DNA kit (Cat No.: 8.02.26201X024G) for plasma/serum 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 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

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 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 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 instrument, 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 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 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 plasma/serum 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 *EGFR* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The OD₂₆₀/OD₂₈₀ value of extracted DNA from FFPE tissue 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. And the circulating DNA isolated from plasma/serum should be used directly without dilution.

Table 2 Recommended DNA concentration

Tissue	Storage time	DNA concentration	DNA amount per reaction
FFPE tissue	≤ 3 months	1.5 ng/ μL	7.05 ng
	> 3 months & ≤ 1 year	2 ng/ μL	9.4 ng
	> 1 year & ≤ 3 years	2.5~3 ng/ μL	11.75~14.1 ng

Note:

- The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.
- The plasma/serum sample should be derived from EDTA anti-coagulated peripheral whole blood.
- The extracted DNA should be used immediately, if not, it should be stored at -20 ± 5 °C for no more than 3 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 eight **Reaction Mix**, **EGFR Positive Control**, and **EGFR Enzyme Mix** out of the kit from the freezer.
- 2) Thaw the eight **Reaction Mix** and **EGFR 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.
- 3) Briefly centrifuge **EGFR Enzyme Mix** prior to use.
- 4) Prepare sufficient **EGFR Master Mix** 1~8 containing **EGFR Enzyme Mix** and each **Reaction Mix** (19-Del / L858R / T790M / Insertions / G719X / S768I / L861Q / External Control Reaction Mix, respectively) in separate sterile centrifuge tube according to the ratio in Table 3. Mix each **EGFR Master Mix** thoroughly by gently pipetting up and down more than 10 times, and centrifuge briefly.

Table 3 **EGFR Master Mix**

Content	Volume per test
Reaction Mix	35 μ L
EGFR Enzyme Mix	0.3 μ L
Total volume	35.3 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.
- 5) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.
 - 6) Prepare 8 PCR tubes for NTC: Dispense 35.3 μ L of each **EGFR Master Mix** 1~8 to each PCR tube respectively, then add 4.7 μ L NTC to each PCR tube, and cap the PCR tubes.
 - 7) Prepare 8 PCR tubes for each sample: Dispense 35.3 μ L of each **EGFR Master Mix** 1~8 to each PCR tube respectively, then add 4.7 μ L each sample DNA to each PCR tube, and cap the PCR tubes.
 - 8) Prepare 8 PCR tubes for PC: Dispense 35.3 μ L of each **EGFR Master Mix** 1~8 to each PCR tube respectively, then add 4.7 μ L PC to each PCR tube of PC strip, 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 real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 PCR Plate Layout

96 well layout												
Assay	1	2	3	4	5	6	7	8	9	10	11	12
19-Del	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
L858R	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
T790M	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
Insertions	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
G719X	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
S768I	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
L861Q	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC
External Control	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	PC	NTC

- 11) Setup the PCR Protocol using the cycling parameters in Table 5.
- 12) Start the PCR run immediately.
- 13) When the PCR run is finished, analyze the data according to the “Results Interpretation” procedures.

Table 5 Cycling Parameters

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

3. Results Interpretation

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

- 1) For NTC: The FAM Ct values of Tubes ①~⑦ should be ≥ 31 . If not, the data is *INVALID*. The sample should be retested.
- 2) For Positive Control: The FAM Ct values of Tubes ①~⑧ and HEX/VIC Ct values of Tubes ①~⑦ should be < 20 . If not, the data is *INVALID*. The sample should be retested.
- 3) For the internal control assay in Tubes ①~⑦ for each sample: The HEX/VIC Ct values should be < 31 . If not, check the mutant FAM signals in Tubes ①~⑦ :
 - 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 ⑧ for each sample:
 - a) The FAM Ct value should be between 15~21 for DNA extracted from FFPE tissues, and between 13~19 for DNA extracted from plasma/serum.
 - b) If the FAM Ct value is < 15 for DNA extracted from FFPE tissues or < 13 for DNA extracted from plasma/serum, this indicates the DNA is overloaded. The DNA amount should be reduced and retested. But if the FAM Ct values of Tubes ①~⑦ are in Negative Ct range (see Table 6), the sample is determined as negative.
 - c) If the FAM Ct value is > 21 for DNA extracted from FFPE tissues or > 19 for DNA extracted from plasma/serum, 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. But if any FAM Ct value of tubes ①~⑦ is < 26 , the sample is determined as positive.

Analyze the mutation assay for each sample:

- 5) Record the FAM Ct values in Tubes ①~⑦ for each sample.
- 6) Check the mutant FAM Ct values in Tubes ①~⑦ according to Table 6:
 - a) If any FAM Ct value of Tube ①~⑦ is < 26 , the sample is determined as positive (*EGFR* mutation detected).
 - b) If any FAM Ct value of Tubes ①~⑦ is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.
 - i. $\Delta Ct \text{ value} = \text{Mutant FAM Ct value} - \text{External control FAM Ct value}$.
 - ii. If the ΔCt value is $<$ the Cut-off ΔCt value, the sample is determined as positive (Mutation detected).
 - iii. If the ΔCt value is \geq the 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 ①~⑦ are in Negative Ct range or there is no amplification, the sample is determined as negative or under the LOD of the kit.

Table 6 Result Determination

Mutation assay	19-Del	L858R	T790M	Insertions	G719X	S768I	L861Q	Results
Optimal Ct range	Ct $<$ 26	Ct $<$ 26	Ct $<$ 26	Ct $<$ 26	Ct $<$ 26	Ct $<$ 26	Ct $<$ 26	Positive.
Acceptable Ct range	26 \leq Ct $<$ 29	26 \leq Ct $<$ 29	26 \leq Ct $<$ 28	26 \leq Ct $<$ 29	26 \leq Ct $<$ 29	26 \leq Ct $<$ 29	26 \leq Ct $<$ 29	Interpret the results according to the ΔCt value.
Cut-off ΔCt value	12	11	7	9	7	8	8	
Negative Ct range	Ct \geq 29	Ct \geq 29	Ct \geq 28	Ct \geq 29	Ct \geq 29	Ct \geq 29	Ct \geq 29	Negative or under the LOD*.

* LOD: limit of detection

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7300, ABI7500, ABI7900HT, ABI StepOnePlus, LightCycler480, Bio-Rad CFX96 and SLAN-96S.

1. Analytical sensitivity:

Analytical sensitivity of the kit was established using plasmid DNA. 29 single *EGFR* mutant plasmid DNAs were diluted with 2 ng/μL wild-type DNA to prepare 29 plasmid DNAs of 1% mutant content. The 29 single *EGFR* mutant plasmid DNAs of 1% mutant content were tested for 20 repeats using three batches of AmoyDx® *EGFR* 29 Mutations Detection Kit, and come out a positive rate of at least 95%. Therefore, the kit allows detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng sample DNA amount.

2. Specificity:

Specificity of the kit was established by testing 10 negative reference controls, which were prepared from 30 cases of NSCLC FFPE tissue samples with wild-type DNA confirmed by Sanger Sequencing. The test gave negative results and with 100% concordance rate.

3. Accuracy:

Accuracy of the kit was established by testing 29 *EGFR* positive reference controls, which were prepared from 29 cases of NSCLC FFPE tissue samples with *EGFR* mutations confirmed by Sanger Sequencing. The test gave corresponding positive results and with 100% concordance rate.

4. Interference factor:

2 common interference substances: hemoglobin and triglyceride, were evaluated in this study. It is confirmed that the potential maximum concentrations: 15 mg/mL hemoglobin and 37mmol/L triglyceride would not interfere with the test result.

5. Precision:

3 precision controls: negative control, weak positive control (the mutant content is 5%) and strong positive control (the mutant content is 50%) were used in the validation. 3 batches of the kits were tested with the precision controls by 2 operators twice a day for 20 days on different PCR instruments. The Ct values were calculated, the CV values were all within 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 DNA extracted from FFPE tissue and plasma/serum samples.
- 4) The kit can only detect the 29 *EGFR* 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 *EGFR* mutation.
- 7) Samples with negative result (No mutation detected) may harbor *EGFR* mutations not detected by this assay.
- 8) Circulating DNA extracted from plasma or serum with negative results (No Mutation detected) may harbor *EGFR* mutation, which could be confirmed with matched tissue DNA detection.

References

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- 7) Yasuda H, S Kobayashi, Costa, D. B, et al. *EGFR* exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. *Lancet Oncol*, 2012, 13(1): e23-31.
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Symbols

	Authorized Representative in the European Community		In Vitro Diagnostic Medical Device
	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

EGFR Mutations Detected by the Kit

Tube No.	Reagent	Exon	Mutation	Base Change	Cosmic ID
①	19-Del Reaction Mix	19	E746_A750del (1)	2235_2249del15	6223
			E746_A750del (2)	2236_2250del15	6225
			L747_P753>S	2240_2257del18	12370
			E746_T751>I	2235_2252>AAT(complex)	13551
			E746_T751del	2236_2253del18	12728
			E746_T751>A	2237_2251del15	12678
			E746_S752>A	2237_2254del18	12367
			E746_S752>V	2237_2255>T(complex)	12384
			E746_S752>D	2238_2255del18	6220
			L747_A750>P	2238_2248>GC(complex)	12422
			L747_T751>Q	2238_2252>GCA(complex)	12419
			L747_E749del	2239_2247del19	6218
			L747_T751del	2239_2253del15	6254
			L747_S752del	2239_2256del18	6255
			L747_A750>P	2239_2248TTAAGAGAAG>C(complex)	12382
			L747_P753>Q	2239_2258>CA(complex)	12387
			L747_T751>S	2240_2251del12	6210
L747_T751del	2240_2254del15	12369			
L747_T751>P	2239_2251>C(complex)	12383			
②	L858R Reaction Mix	21	L858R	2573T>G	6224
③	T790M Reaction Mix	20	T790M	2369C>T	6240
④	Insertions Reaction Mix	20	H773_V774insH	2319_2320insCAC	12377
			D770_N771insG	2310_2311insGGT	12378
			V769_D770insASV	2307_2308insGCCAGCGTG	12376
⑤	G719X Reaction Mix	18	G719A	2156G>C	6239
			G719S	2155G>A	6252
			G719C	2155G>T	6253
⑥	S768I Reaction Mix	20	S768I	2303G>T	6241
⑦	L861Q Reaction Mix	21	L861Q	2582T>A	6213