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Super-ARMS® *EGFR* Mutation Detection Kit

Instructions for Use

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

REF 8.01.0107 12 tests/kit For Rotor-Gene Q (36 wells)

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Background

Due to its association with malignancies, epidermal growth factor receptor (EGFR) has become the target of an expanding class of anticancer therapies, such as gefitinib (Iressa) and erlotinib (Tarceva), which are tyrosine kinase inhibitors (TKIs). These drugs work best on patients whose cancer is driven by abnormal EGFR signaling. Non-small cell lung cancer (NSCLC) patients who experienced rapid, durable, complete or partial responses to TKIs therapy have been found to harbor somatic mutations in the *EGFR* gene. Cancer patients with somatic *EGFR* mutations have shown an impressive 60% response rate, much higher than that for conventional chemotherapy. Therefore, detection of the *EGFR* mutation status in tumor tissue is key to offering tailored, personalized treatment to cancer patients. Resistance to therapy, either in the primary tumor or acquired after TKI treatment, is also associated with somatic mutations.

Both tumor tissue and peripheral blood samples can be used for *EGFR* mutation detection. Currently, tumor tissue is the most frequent specimen for *EGFR* mutation testing. In meanwhile, it is demonstrated that there is cell-free DNA of the apoptotic and necrotic tumor cell existing in peripheral blood. Noninvasive detection of *EGFR* mutation in circulating tumor DNA (ctDNA) extracted from plasma has been proved to be feasible as re-biopsy of tumor tissue was challenging.

Intended Use

The Super-ARMS® *EGFR* Mutation Detection Kit is a real-time PCR assay for qualitative detection of 31 somatic mutations in exons 18, 19, 20 and 21 of *EGFR* gene in circulating DNA extracted from plasma sample. The kit is intended to assess *EGFR* mutation status in NSCLC patients and aid in identifying patients who may respond to treatment with an *EGFR*-TKI.

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) and real-time PCR technology, which comprises specific primers and fluorescent probes to detect *EGFR* mutations in circulating DNA. 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 **P-*EGFR* Reaction Mix**, **P-*EGFR* Enzyme Mix** and **P-*EGFR* Positive Control**.

1) The contents in **P-*EGFR* Reaction Mix A** and **P-*EGFR* Reaction Mix B** formed a mutation detection system and an internal control system. The mutation detection system includes primers and FAM/ROX/CY5-labeled probes specific for designated *EGFR* mutations, to detect the *EGFR* mutation status. The internal control system contains the 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 experimental operation.
2) The P-EGFR Positive Control contains recombinant gene with EGFR mutations.
3) The P-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 (see Table 1):

Table 1 Kit Contents

Contents	Main Ingredient	Quantity
P-*EGFR* Reaction Mix A	Buffer, Mg^{2+}	1540 µL/tube ×2
P-*EGFR* Reaction Mix B1	Primers, Probes, dNTPs	140 µL/tube ×1
P-*EGFR* Reaction Mix B2	Primers, Probes, dNTPs	140 µL/tube ×1
P-*EGFR* Reaction Mix B3	Primers, Probes, dNTPs	140 µL/tube ×1
P-*EGFR* Reaction Mix B4	Primers, Probes, dNTPs	140 µL/tube ×1
P-*EGFR* Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	30 µL/tube ×1
P-*EGFR* Positive Control	Plasmid DNA	400 µL/tube ×1

The detailed detection information is listed in Table 2.

Table 2 Detection Information

Reagent	Mutation detected	Fluorescent Signal			
		FAM	HEX	ROX	CY5
P-*EGFR* Reaction Mix A **P-*EGFR* Reaction Mix B1**	19-Del/ L858R	19-Del	IC	L858R	/
P-*EGFR* Reaction Mix A **P-*EGFR* Reaction Mix B2**	T790M	T790M	IC	/	/
P-*EGFR* Reaction Mix A **P-*EGFR* Reaction Mix B3**	G719X/ L861Q/S768I	G719X	IC	L861Q	S768I
P-*EGFR* Reaction Mix A **P-*EGFR* Reaction Mix B4**	20-Ins	20-Ins	IC	/	/

* IC: Internal Control

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.

Materials Required But Not Supplied

1) PCR instruments: Rotor-Gene Q (36 wells).
2) DNA Extraction kit. We recommend use of AmoyDx® Circulating DNA kit or QIAamp® Circulating Nucleic Acid Kit (Qiagen, Cat. No. 55114) for plasma samples.
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, 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 80 μL.
- Prior to the operation, please set up the PCR program by the following steps: ①select "**Gain Optimization**", the "**Auto Gain Optimization Setup**" window will open (see Figure 1); ②Click "**Perform Calibration Before 1st Acquisition**" and "**Optimize Acquiring**" (see Figure 2). ③Click "**OK**", then click "**Close**" to continue (see Figure 3).

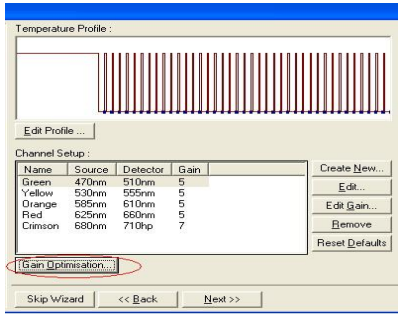

Figure 1

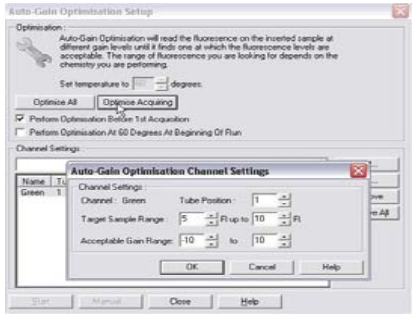

Figure 2

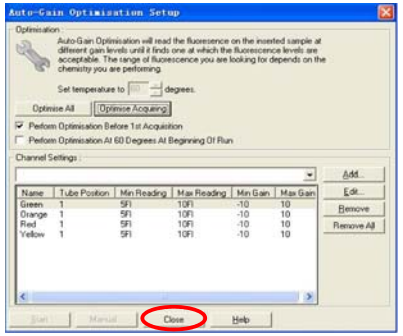

Figure 3

- If fluorescence crosstalk occurs between Orange and Red channels in Tube 1, continue with the analysis as that has no impact on the data analysis.
- 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 circulating DNA extracted from plasma samples of NSCLC patients. DNA extraction reagents are not included in the kit.

Note:

- *The plasma samples should be derived from EDTA anti-coagulated peripheral whole blood. The recommended volume of whole blood is no less than 10 mL.*
- *It is required to separate the plasma from the whole blood within 2 hours after collection. The recommended volume of plasma is no less than 4 mL.*
- *The extracted DNA should be used immediately. If not, it should be stored at -20±5℃ for no more than 3 months.*
- *It is recommended to test the extracted circulating DNA with original concentration.*

2. Mutation Detection

1) Take the **P-*EGFR* Positive Control**, **P-*EGFR* Reaction Mix (A, B1~B4)** and **P-*EGFR* Enzyme Mix** out of the kit from the freezer.
2) Thaw the **P-*EGFR* Positive Control** and **P-*EGFR* Reaction Mix (A, B1~B4)** 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 **P-*EGFR* Enzyme Mix** for 5~10 seconds prior to use.
4) Prepare sufficient P-*EGFR* Master Mix 1~4 containing P-EGFR Enzyme Mix, P-EGFR Reaction Mix A and P-EGFR Reaction Mix B (B1~B4 respectively) in separate sterile centrifuge tube respectively according to the ratio in Table 4. Mix each Master Mix thoroughly by vortexing and centrifuge for 5~10 seconds.

Table 4 P-*EGFR* Master Mix 1~4

Content	Volume per test (μL)
P-*EGFR* Reaction Mix A	55
P- *EGFR* Reaction Mix B (B1/B2/B3/B4)	10
P- *EGFR* Enzyme Mix	0.36
Total	**65.36**

Note:

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *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 and nuclease-free water for NTC.
6) Prepare four PCR tubes for NTC: Dispense 65.36 μL of P-*EGFR* Master Mix 1~4 to each PCR tube respectively. Then add 15 μL of nuclease-free water to each NTC tube and cap the PCR tubes.
7) Prepare four PCR tubes for each sample: Dispense 65.36 μL of P-*EGFR* Master Mix 1~4 to each PCR tube respectively. Then add 15 μL of each sample DNA to each sample tube and cap the PCR tubes.
8) Prepare four PCR tubes for PC: Dispense 65.36 μL of P-*EGFR* Master Mix 1~4 to each PCR tube respectively. Then add 15 μL of P-*EGFR* Positive Control to each PC tube and cap the PCR tubes.
9) Briefly centrifuge the PCR strips to collect all liquid at the bottom of each PCR tube.
10) Setup the PCR Protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	10min	/
2	15	95℃	40s	/
		64℃	40s	/
		72℃	30s	/
3	28	93℃	40s	/
		62℃	45s	Green/Yellow/Orange/Red*
		72℃	30s	/

* FAM: Green, HEX: Yellow, ROX: Orange, CY5: Red.

11) Start the PCR run immediately.
12) When the PCR run 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/ROX/CY5 signal of Reaction Mix 1~4 should be no amplification and Ct value should be ≥ 28, HEX/VIC Ct values of Reaction Mix 1~4 should be ≥ 22. If not, the data is *INVALID*. The sample should be retested.
2) For Positive control: for FAM and HEX/VIC signals, the Ct values of Reaction Mix 1~4 should be < 20; for ROX signal, the Ct values of Reaction Mix 1 and 3 should be < 20; for CY5 signal, the Ct value of Reaction Mix 3 should be < 20. If any of the above requirements is not met, the data is *INVALID*. The sample should be retested.
3) For the internal control assay for each sample: the HEX/VIC Ct values of Reaction Mix 1~4 should be < 19. If not, this indicates insufficient DNA or presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA.

Analyze the mutation assay for each sample:

4) Record the mutant FAM/ROX/CY5 Ct values of Reaction Mix 1~4.
5) Calculate the ΔCt value for each tube: ΔCt value = Mutant Ct value (FAM/ROX/CY5) – HEX Ct value
6) Result interpretation for each tube according to the Cut-off ΔCt value in Table 6.
   a) If the ΔCt value is < the Cut-off ΔCt value, the sample is determined as positive.
   b) If the ΔCt value is ≥ the Cut-off ΔCt value, the sample is determined as negative or under the LOD (limit of Detection) of the kit.
   c) Two or more *EGFR* mutations may be detected for a sample.

Table 6 Result Determination

Tube No.			FAM (Green)	ROX (Orange)	CY5 (Red)
Cut-off ΔCt value	1	19-Del / L858R	10	10	/
	2	T790M	8	/	/
	3	G719X / L861Q / S768I	12	12	12
	4	20-Ins	11	/	/

Performance Characteristics

The performance characteristics of this kit were validated on Rotor-Gene Q (36 wells).

1. Limit of Detection

The Limit of Detection (LOD) of the kit for each mutation is shown in Table 7.

Table 7 LOD for each EGFR mutation

Exon	Mutation	Base Change	Cosmic ID	Name	LOD (%)
Exon 18	G719A	2156G>C	6239	E-18-M1	0.20%
	G719C	2155G>T	6253	E-18-M3	0.40%
Exon 19	E746_A750del (1)	2235_2249del15	6223	E-19-M1	0.20%
	E746_A750del (2)	2236_2250del15	6225	E-19-M2	0.20%
	L747_P753>S	2240_2257del18	12370	E-19-M3	0.60%
	E746_T751>I	2235_2252>AAT(complex)	13551	E-19-M4	0.40%
	E746_T751del	2236_2253del18	12728	E-19-M5	0.40%
	E746_T751>A	2237_2251del15	12678	E-19-M6	0.20%
	E746_S752>A	2237_2254del18	12367	E-19-M7	0.20%
	E746_S752>V	2237_2255>T(complex)	12384	E-19-M8	0.20%
	E746_S752>D	2238_2255del18	6220	E-19-M9	0.40%
	L747_A750>P	2238_2248>GC(complex)	12422	E-19-M10	0.40%
	L747_T751>Q	2238_2252>GCA(complex)	12419	E-19-M11	0.20%
	L747_E749del	2239_2247delTTAAGAGAA	6218	E-19-M12	0.40%
	L747_T751del	2239_2253del15	6254	E-19-M13	0.40%
	L747_S752del	2239_2256del18	6255	E-19-M14	0.40%
	L747_A750>P	2239_2248TTAAGAGAAG>C(complex)	12382	E-19-M15	0.40%
	L747_P753>Q	2239_2258>CA(complex)	12387	E-19-M16	0.40%
	L747_T751>S	2240_2251del12	6210	E-19-M17	0.80%
	L747_T751del	2240_2254del15	12369	E-19-M18	0.40%
	L747_T751>P	2239_2251>C(complex)	12383	E-19-M19	0.40%
Exon 20	T790M	2369C>T	6240	E-20-M1	0.20%
	S768I	2303G>T	6241	E-20-M2	0.20%
	H773_V774insH	2319_2320insCAC	12377	E-20-M3	0.40%
	D770_N771insG	2310_2311insGGT	12378	E-20-M4	0.60%
	V769_D770insASV	2307_2308insGCCAGCGTG	12376	E-20-M5	0.60%
	D770_N771insSVD	2311_2312insGCGTGGACA	13428	E-20-M8	0.40%
	V769_D770insASV	2309_2310AC>CCAGCGTGGAT	13558	E-20-M9	0.40%
	H773_V774insNPH	2319_2320insAACCCCCAC	12381	E-20-M10	0.80%
Exon 21	L858R	2573T>G	6224	E-21-M1	0.20%
	L861Q	2582T>A	6213	E-21-M2	0.20%

2. Cross-reactivity

   The cross reaction among the mutant sequences targeted by this kit, the cross reaction with other homologous mutant nucleotide sequence (*HER2* gene, belongs to the same family as *EGFR* gene, the plasmids with five *HER2* hotspot mutations were selected in this study), the cross reaction with wild-type genomic DNA (DNA concentrations are 1~15 ng/reaction), and the cross reaction with non-human gene (the DNA was extracted from *Escherichia Coli, Yeast, Mycobacterium tuberculosis* and *Streptococcus pneumonia* which were common microorganism causing lung infection) were evaluated, the results shown no cross-reactions.

3. Interference factor

   12 common interference substances: endogenous Hemoglobin, Ferritin, Albumin and Triglyceride, exogenous pathogenic microorganism such as *Mycobacterium Tuberculosis* and *Atreptococcus Pneumoniae, therapeutic drugs such as* Taxol, Carboplatin and Tarceva, common anticoagulants such as Heparin sodium, Sodium citrate and EDTA were evaluated in this study. It is confirmed that the potential maximum concentrations: 2 g/L hemoglobin, 37 mmol/L triglyceride, 200 ng/mL Ferritin, 60 g/L Albumin, 10^6 CFU/mL *Mycobacterium Tuberculosis,* 10^6 CFU/mL *Atreptococcus Pneumoniae,* 90 μg/mL Taxol, 90 μg/mL Carboplatin, 90 μg/mL Tarceva, 0.645 mol/L Sodium citrate and 27 μmol/L EDTA would not interfere with the test result. While 150 U/mL Heparin sodium would inhibit the test performance. It is stated in DNA Extraction section in the Instructions to avoid using *heparin anticoagulant.*

4. Precision

   3 precision controls: negative control, weak positive control (with 1% mutant content) and strong positive control (with 50% mutant content) 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%.

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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 circulating DNA extracted from plasma samples.
3) Reliable results are dependent on proper specimen collection, processing, transport, and storage.
4) The sample containing degraded DNA may affect the ability of the test to detect *EGFR* mutation.
5) This kit can only assess the *EGFR* mutation status and detect 31 *EGFR* mutations indicated above.
6) Samples with negative result (No mutation detected) may harbor *EGFR* mutations not detected by this assay.

References

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Symbols