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CE IVD

Super-ARMS® *EGFR* Mutation Detection Kit

Instructions for Use

REF 8.01.0148 12 tests/kit For Stratagene Mx3000P™, ABI7500, LightCycler480, cobas® z480, SLAN-96S

Amoy Diagnostics Co., Ltd.
No. 39, Dingshan Road, Haicang District,
361027 Xiamen, P. R. China
Tel: +86 592 6806835
Fax: +86 592 6806839
E-mail: sales@amoydx.com
Website: www.amoydx.com

EC REP **Qarad EC-REP BV**
Pas 257,
2440 Geel, Belgium

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Background

The epidermal growth factor receptor (*EGFR*) is a transmembrane tyrosine kinase receptor that plays an important role in regulating cell division and death, Mutations in the gene encoding EGFR that lead to overexpression of the protein have been associated with a number of different cancers. These cancers have become the target of an expanding class of anticancer therapies, the 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 treatment with TKIs have been found to harbor somatic mutations in the *EGFR* gene. In these patients an impressive 60% response rate could be observed, 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. Therefore, clinical guidelines recommend routine testing and identification of EGFR mutations in all patients with NSCLC of non-squamous cell carcinoma histology to identify who may benefit of TKIs. As almost all patients will eventually develop resistance to therapy, either in the primary tumor or acquired after TKI treatment, testing is often also recommended later in the medical history of the patients.

Both tumor tissue and peripheral blood samples can be used for *EGFR* mutation detection. Currently, tumor tissue is the most frequently used specimen for *EGFR* mutation testing. Meanwhile, it could also be demonstrated that there is cell-free DNA (cfDNA) of the apoptotic and necrotic tumor cell existing in peripheral blood requiring a high sensitivity from the test kit. Non-invasive detection of *EGFR* mutation in circulating tumor DNA (ctDNA) extracted from plasma has been proved to be feasible as re-biopsy of tumor tissue is often not possible.

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 *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) and real-time PCR technology, which comprises specific primers and fluorescent probes to detect *EGFR* mutations in human genomic DNA and ctDNA. 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 **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 the experimental operation.
2) The **P-EGFR Positive Control (PC)** 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 cycles is five.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:

   Stratagene Mx3000P™, ABI7500, LightCycler480, cobas® z480, or SLAN-96S.

2) DNA Extraction kit. We recommend to use AmoyDx® Circulating DNA kit or QIAamp® Circulating Nucleic Acid Kit (Qiagen, Cat. No. 55114) for plasma samples.
3) Spectrophotometer for measuring FFPE 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

For *in vitro* diagnostic use.

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 a 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 80 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. We recommend to set up the Filter Set Gain Settings of FAM, HEX, ROX, CY5 as 4, 4, 1, 4, respectively.
- For ABI 7500, please set up as follows: Reporter Dye: FAM, VIC, ROX, CY5; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 and cobas® z480 instrument, it's necessary to conduct Color Compensation prior to the first use according to Color Compensation instructions. Please contact AmoyDx Technical Support or Account Manager to get the color compensation kit and Color Compensation instructions.
- For SLAN-96S, please set up as follows: Probe mode: FAM, VIC, ROX, CY5. 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". We recommend to set up the Gain of FAM, HEX, ROX, CY5 as 1, 2.5, 2, 7, respectively.
- Refer to the operations 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 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 PC**, **P-EGFR Reaction Mix (A, B1~B4)** and **P-EGFR Enzyme Mix** out of the kit from the freezer.
2) Thaw the **P-EGFR PC** 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 tubes 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	0.48
Total	**65.48**

Note:

- *Every PCR run must contain one PC and one No Template Control (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.*

5) Take out the sample DNA (see Table 3 for FFPE DNA concentration) and nuclease-free water for NTC.
6) Prepare four PCR tubes for NTC: Dispense 65.48 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 65.48 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 65.48 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) Place the PCR strip tubes into the real-time PCR instrument. A recommended plate layout is shown in Table 5.

Table 5 PCR Plate Layout

Well	1	2	3	4
A	Sample 1	Sample 3	Sample 5	NTC
B	Sample 1	Sample 3	Sample 5	NTC
C	Sample 1	Sample 3	Sample 5	NTC

D	Sample 1	Sample 3	Sample 5	NTC
E	Sample 2	Sample 4	Sample 6	PC
F	Sample 2	Sample 4	Sample 6	PC
G	Sample 2	Sample 4	Sample 6	PC
H	Sample 2	Sample 4	Sample 6	PC

11) Setup the PCR Protocol using the cycling parameters in Table 6.

Table 6 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	10min	/
2	15	95℃	40s	/
		64℃	40s	/
		72℃	30s	/
3	28	93℃	40s	/
		60℃	45s	FAM/ROX/CY5 and HEX/VIC
		72℃	30s	/

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 the mutation data, the following items should be checked:

1) For NTC: The FAM/ROX/CY5 signal of Reaction Mix 1~4 should show 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 PC: 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/VIC Ct value.**

6) Result interpretation for each tube according to the Cut-off ΔCt value in Table 7.

   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 7 Result Determination

Tube No.			FAM	ROX	CY5
Cut-off ΔCt value	1	19-Del / L858R	11	11	/
	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 Stratagene Mx3000P™, ABI7500, LightCycler480, cobas® z480, and SLAN-96S.

1) Limit of Detection

The LOD of the kit for each mutation is shown in Table 8.

Table 8 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) Specificity

Specificity of the kit was established by testing negative reference controls. The test gave negative results and negative concordance rate was 100%.

3) Accuracy:

The accuracy of the kit was established by testing positive reference controls. The test gave positive results and positive concordance rate was 100%.

4) Precision:

Three precision controls: negative control, weak PC (with 1% mutant content) and strong PC (with 50% mutant content) were used in the validation. Three lots of the kits were tested with the precision controls by two operators twice a day for 20 days on different PCR instruments. The Ct values were calculated, the CV values were all within 5%.

5) Interfering substance:

12 common potential interfering substances: endogenous Hemoglobin, Ferritin, Albumin and Triglyceride, exogenous pathogenic microorganism such as *Mycobacterium Tuberculosis* and *Atreptococcus Pneumoniae, therapeutic drugs such as* paclitaxel, carboplatin and erlotiniba, common anticoagulants such as heparin sodium, sodium citrate and EDTA were evaluated in this study. It is confirmed that the potential maximum concentrations of 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*Streptococcus Pneumoniae,* 90 μg/mL paclitaxel, 90 μg/mL carboplatin, 90 μg/mL erlotinib, 0.645 mol/L sodium citrate and 27 μmol/L EDTA would not interfere with the test result, while 150 U/mL heparin sodium may inhibit the test performance. It is stated in DNA Extraction section in the Instructions to avoid using *heparin anticoagulant.*

6) Cross-reactivity:

The cross reaction among the mutant sequences targeted by this kit, the cross reaction with other homologous mutant nucleotide sequences (*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 have not shown any cross-reactions.

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

References

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Symbols

EC REP	Authorized Representative in the European Community	IVD	In Vitro Diagnostic Medical Device
	Manufacturer	REF	Catalogue Number
LOT	Batch Code		Use By
Σ	Contains Sufficient for <n> Tests		Temperature Limitation
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care
	Keep Away from Sunlight		