CE

SuperARMS® *EGFR* T790M Mutation Detection Kit

Detection of *EGFR* T790M mutation in plasma sample

Instruction for Use

Instruction Version: B1.0
Revision Date: May 2016

Store at -20±5℃

Background

Due to its association with malignancies, epidermal growth factor receptor (*EGFR*) has become the target of an expanding class of anti-cancer 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. Lung cancer patients who experienced rapid, durable, complete or partial responses to TKIs therapy have been found to harbor somatic mutations in the *EGFR* gene[1-2]. However, it is demonstrated that many of the patients who had received EGFR-TKI treatment for a period of time will appear EGFR-TKI resistance. *EGFR* T790M mutation (c.2369C>T) in exon 20 occurs in ~60% of *EGFR*-mutated lung cancers that have developed acquired resistance to EGFR-TKIs therapy [3]. Therefore, it is an important way to predict the curative effect by monitoring the T790M mutation status.

It is demonstrated that there is cell-free DNA of the apoptotic and necrotic tumor cell existing in peripheral blood. Noninvasive detection of EGFR-T790M in plasma circulating free DNA (cfDNA) has been proved to be feasible as re-biopsy of tumor tissue was challenging. Continuous monitoring *EGFR* T790M mutation dynamics in plasma cfDNA could predict the clinical outcome of EGFR-TKIs and guide further therapy for advanced NSCLC patients. The purpose of the kit is to aid clinician in identifying NSCLC patients whose tumor harbor *EGFR*-T790M mutation.

Intended Use

The SuperARMS® EGFR T790M Mutation Detection Kit is a highly sensitive real-time PCR-based test designed to accurately identify T790M mutation in exon 20. The used DNA is extracted from peripheral blood (plasma or serum). Our company's patented technology allows detection of 0.2% mutant DNA in a background of 99.8% normal DNA, while ensuring that false negatives are minimized.

Kit Contents

This kit contains sufficient reagents to carry out 24 tests (Table 1), and additional Positive Control DNA (genomic DNA plus T790M mutated plasmid DNA) for positive control reactions.

Table1 Kit Contents

Reagents Supplied	Volume (24T)
P-T790M Reaction Mix A	1740 μL
P-T790M Reaction Mix B	140 μL
P-T790M Enzyme Mix	13 μL
P-T790M Positive Control	200 μL

Equipment and Reagents Not Supplied With Kit

1. Compatible PCR instruments:

   Stratagene Mx3000P™/Mx3005P™, ABI7500, SLAN-96S

   (1) For Stratagene Mx3000P™/Mx3005P™, if there's low net fluorescence signal (dR) but high background signal (R), please reduce the signal gain setting of instrument properly.

   (2) For ABI instruments please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.

   (3) For SLAN-96S, please setup as follows: Probe mode: FAM, VIC. During the result analysis, select

"Absolute Fluorescence Value Normalization" in "Amplification Curve" section.

(4) We recommend that all PCR instruments in use should be conducted fluorescence calibration once a year.

2. Sterile, nuclease-free tubes and pipette tips.
3. Dedicated pipettes and filtered pipette tips for handling DNA template.
4. Sterile, nuclease-free H_2O.

Shipping and Storage

The kit requires cold-chain-transportation. All contents of the kit should be stored immediately upon receipt at -20±5℃ in the dark in a constant temperature freezer. Avoid unnecessary freezing and thawing of the kit contents. Do not use the reagent after five freeze-thaw cycles. Once opened, this reagent is stable at -20±5℃ until the expiry.

Stability

The shelf-life of the kit is eight months when stored under the recommended conditions and in the original packaging. Do not use the kit after the stated expiry date.

Specimen Material

Human genomic DNA must be extracted from peripheral blood (plasma or serum). Selection of high quality DNA extraction reagents is essential for the kit. We recommend using AmoyDx® Circulating DNA kit, Cat No. ADx-BL03-R.

Note:

a) The kit requires 10 mL whole blood and the recommended volume of plasma is no less than 4 mL.
b) The plasma should be separated from whole blood within 2 hours, if not, please store the blood sample at 2~8℃ for no more than 4 hours. We recommend using AmoyDx® Cell-free DNA Protection Vacuum Tube (Cat No. ADx-VT01-R) for blood collection. The collected blood samples are stable for 5~7 days at 4~25℃ for storage and transportation.
c) EDTA is recommended for anticoagulation, avoid using heparin anticoagulant.
d) The DNA should be extracted from plasma within 2 hours, if not, please store the plasma at -20±5℃ for no more than 2 years.
e) If the extracted DNA is not used immediately, it should be stored at -20±5℃ for no more than 3 months.

Technological Principles

The kit adopts ARMS (Amplification Refractory Mutation System) and real-time PCR technology, which uses novel, proprietary primers and probes to detect *EGFR* T790M mutation in human lung cancer plasma cfDNA samples. The *EGFR* T790M mutant sequence is amplified by the mutant-specific primers, and detected by the fluorescence-labeled probes.

Protocol

1. The **P-T790M Reaction Mix A** contains the reaction buffer and Mg^{2+}.
2. The **P-T790M Reaction Mix B** contains the primers, probes and dNTPs, which include a mutation detection system and an internal control system. The mutation detection system adopts FAM-labeled probes specific for *EGFR* T790M mutation. The internal control system uses HEX-labeled probes specific for conserved sequences of *EGFR* gene to monitor DNA quality and the accuracy of experimental operation.
3. The **P-T790M 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 **P-T790M Positive Control** contains a recombinant gene with *EGFR* T790M mutation and normal human

genomic DNA.

Experimental Procedure

1. Thaw the **P-T790M Reaction Mix A, P-T790M Reaction Mix B** and **P-T790M Positive Control** at room temperature. When the reagents are completely thawed, mix the reagents by inverting the tube 10 times and centrifuge briefly to collect the contents at the bottom of the tube.
2. Briefly centrifuge **P-T790M Enzyme Mix** prior to use.
3. According to the ratio of 0.4μL **P-T790M Enzyme Mix**, 60μL **P-T790M Reaction Mix A** and 5μL **P-T790M Reaction Mix B** per sample, transfer the appropriate amount of **P-T790M Enzyme Mix** and **P-T790M Reaction Mix A&B** into a sterile tube. The information is shown in table 2.

Table 2 Ratio of Reaction Mix and Enzyme Mix

Component	Volume (μL)
P-T790M Reaction Mix A	60
P-T790M Reaction Mix B	5
P-T790M Enzyme Mix	0.4
Total	**65.4**

Note:

- The volumes given for each reaction mix have been optimized and validated. Changing volumes of any reagent may result in a loss of performance.
- Do not store user-prepared mixes, use immediately.
- Since enzyme mix is viscous, please pay attention to the centrifugation and pipetting process.
- Minimize the contact interface between the pipette tip and enzyme mix to avoid adding excess enzyme.

4. Mix the solution thoroughly by gently pipeting it up and down more than 10 times.
   Note: avoid vortexing solutions with enzyme mix.
5. Centrifuge briefly.
6. Transfer 65.4μL of the above mixed solution into the appropriate PCR tubes.
7. Add 15μL sample DNA, 15μL **P-T790M Positive Control (PC)** or 15μL ddH_2O (no-template control, NTC) to the appropriate PCR tubes.
8. Seal the PCR tubes.
9. Spin down the PCR tubs gently or centrifuge them briefly to collect the reagents at the bottom of tubes.
   Note: this spin or centrifuge step is essential for proper mixing of the reagents.
10. Place the PCR tubes into the real-time PCR instrument.
    The layout for 22 samples, a positive control and a no-template control is shown in Table 3.

Table 3 Plate Layout (example for 24 tests)

Well	1	2	3
A	Sample 1	Sample 9	Sample 17
B	Sample 2	Sample 10	Sample 18
C	Sample 3	Sample 11	Sample 19
D	Sample 4	Sample 12	Sample 20
E	Sample 5	Sample 13	Sample 21

F	Sample 6	Sample 14	Sample 22
G	Sample 7	Sample 15	PC
H	Sample 8	Sample 16	NTC

11. Carry out real-time PCR using the cycling conditions described in Table 4.

Table 4 Cycling Parameters

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

Note:

- The reaction volume is 80.4 μL per well (65.4 μL reagents plus 15 μL template).
- Please pack the post-PCR tubes with two disposable gloves and discard properly. Do NOT open the post-PCR tubes to avoid contamination.

Sample Data Analysis

1. The threshold at which the signal is detected above background fluorescence is called the Cycle threshold (Ct). The Ct values used to determine if a sample is positive or negative are based on extensive validation. If the Ct value falls within the appointed range, the sample is classified as positive. If the Ct value is outside the appointed range, the sample is classified as negative or below the detection limit of the kit.
2. Assess NTC Ct value to ensure that there is no positive amplification, if the NTC has positive amplification, the data must be discarded as there is contamination. If the HEX/VIC signals occasionally rise, further analysis could be carried out.
3. For **P-T790M Positive Control**, the Ct value for both FAM and HEX/VIC signal should be **less than 20**, but variation may occur due to different threshold settings on different instruments.
4. Make sure that each sample gives a HEX/VIC signal and the Ct value should be **less than 19**. If the Ct value of HEX/VIC signal is ≥19, it shows that the DNA sample contains PCR inhibitors or the DNA amount is insufficient, indicating that the DNA needs to be re-extracted, the whole experiment should be carried out again.
5. Check the FAM Ct value for each sample.

   If the FAM signal has positive amplification, and the ΔCt value is **< 8**, the sample is classified as *EGFR* T790M positive. If the the ΔCt value is **≥ 8**, the sample is classified as negative or below the detection limit of the kit.

   The calculation of ΔCt: **ΔCt = FAM Ct value – HEX/VIC Ct value**. The FAM Ct value indicates the Ct value of the sample's FAM signal; the HEX/VIC Ct value indicates the Ct value of the sample's HEX/VIC signal.

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™/3005P™, ABI7500 and SLAN-96S.

1. Sensitivity: the kit allows detection of 0.2% *EGFR* T790M mutant DNA in a background of 99.8% normal DNA.
2. Productivity: the kit can be used to analysis 28 samples maximum. (see Table 5)

Table 5 Sample Qty detected with the Kit

PCR Run(s)	Control Qty	Total samples can be detected
1 run	2 (2 controls/run * 1)	28
2 runs	4 (2 controls/run * 2)	26
3 runs	6 (2 controls/run * 3)	24

3. Accuracy: Accuracy of the kit was established by testing strong positive reference controls, medium positive reference controls and weak positive reference controls, the detection concordance rate are 100%.
4. Specificity: specificity of the kit was established by testing negative reference controls, the detection concordance rate is 100%.
5. Precision: Precision of the kit was established by performing a certain mutant positive reference control for 10 repeats, the detection results all are positive.
6. Cross-reaction: the kit has no cross-reaction towards other oncogenic genes and common bacteria.

Warnings and Precautions

1. Please read the instruction carefully and become familiar with all components of the kit prior to use.
2. The product specified above does not contain any virus, reagent by-product of the same or metabolic by-product of Hepatitis A, B, C, D or HIV.
3. Do not exchange and mix up the kit contents with different batches.
4. The kit and its contents cannot be resold or modified for resale without the written approval of manufacturer.
5. Using other sources of reagents is not recommended. Strictly distinguish the reagents from Positive Control to avoid contamination. Otherwise, false positives may be produced.
6. Do the experiments with attention to prevent exogenous DNA contamination to reagents. It is recommended that users have separate, dedicated pipettes and filtered pipette tips to add DNA template during the preparation of reagents.
7. To optimize the activity and performance, mixtures should always be protected from light to avoid photo bleaching.
8. Only trained professionals could use this kit. Please wear suitable lab coat and disposable gloves. The used kit should be disposed of properly.

Notes

1. 

Symbol for "AUTHORISED REPRESENTATIVE IN THE EUROPEAN COMMUNITY"

2. 

Symbol for "IN VITRO DIAGNOSTIC MEDICAL DEVICE"

3. 
Symbol for "KEEP DRY"

4. 
Symbol for "THIS WAY UP"

5. 
Symbol for "FRAGILE，HANDLE WITH CARE"

Information of European Authorised Representative

Wellkang Ltd t/a Wellkang Tech Consulting Suite B, 29 Harley Street, London W1G 9QR United Kingdom

References

1. Han JY, Park K, Kim SW, et al. First-SIGNAL: first-line single-agent iressa versus gemcitabine and cisplatin trial in never-smokers with adenocarcinoma of the lung [J]. J Clin Oncol, 2012, 30 (10):1122-1128.

2. Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL,CTONG-0802): a multicentre, open-label, randomised, phase 3 study[J].Lancet Oncol, 2011,12 (8):735-742.

3. Helena A Y, Arcila M E, Rekhtman N, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers [J]. Clin Cancer Res, 2013, 19(8): 2240-2247.

Metachem Diagnostics Ltd.
29, Forest Road, Piddington, Northampton. NN7 2DA
Tel: 01604 870370. Fax: 01604 870194
e-mail: info@metachem.co.uk www.metachem.co.uk
Company Registration No: 1663672