PAP-ARMS®

EGFR Gene Mutations Detection Kit

Multiplex Fluorescence Polymerase Chain Reaction

Instruction for Use

Instruction Version: S2.1 Revision Date: September 2023

Product Name

EGFR Gene Mutations Detection Kit (Multiplex Fluorescence Polymerase Chain Reaction)

Packing Specification

10 Tests/Kit

Intended Use

The kit is intended for the detection of 29 somatic mutations (Attached List 1) of EGFR gene in FFPE pathological tissue collected from patients with non-small cell lung cancer (NSCLC) or colorectal cancer. The assay is indicated only for use as an aid in the identification of patients who may benefit from the EGFR-TKIs treatment. The results shall not be regarded as the only evidence whether a patient suits individualized therapy; clinically, determinants such as, but not limited to patients' condition, drug indications, therapeutic response, and other laboratory detection indexes should also be considered before making comprehensive judgments. The multiplex fluorescence Polymerase Chain Reaction (PCR) tech applied in this kit is intended for use on real-time PCR systems.

Epidermal growth factor receptor (EGFR), a member of receptor tyrosine kinase family, is the product of proto-oncogene c-erbB1. EGFR locates mainly on the surface of cell membrane. It will cause auto-phosphorylation after EGFR ligates to ligands, which further activates downstream pathways like MAPK, PI3K, and JNK, induces cell proliferation or differentiation, which is closely associated with tumor formation and development.

It is shown that EGFR mutation happens mainly in exons 18-21; 45% of which are of deletions in exon 19 encompass the amino acids from codons L747 to A750, the most frequent (74%) deletions among these are 2235-2249del15 and 2236-2250del15. Besides deletion mutation, point mutations in exon 21 (L858R), exon 18 (G719X), insertion mutation in exon 20 each account for ~40%, 5%, and 1% of all mutations found in EGFR of NSCLC. Patients with activating EGFR mutations, exon 19 deletion and exon 21 L858R point mutation, would be sensitive to the treatment of EGFR-TKI Gefitinib, while those with exon 20 T790M mutation or insertion mutation would be of no response to EGFR-TKIs treatment. Therefore, the sensitivity and accuracy of EGFR mutation detection has become a significant factor in aiding the treatment with TKI.

Technological Principle

This kit uses multiplex fluorescent PCR technology to detect a total of 29 types of EGFR gene mutations in the sample (Attached List 1), of which 18-G719X can detect three-point mutations in exon 18, G719C, G719S, and G719A; 19 -Del can detect 19 deletion mutations in exon 19; 20-Ins can detect three insertion mutations in exon 20, Ins-1, Ins-2, and Ins-3. This kit uses the sequence of the EGFR gene mutation site as a template to design ARMS primers and fluorescent probes. The length of the target gene sequence for each mutant type is controlled within 150 bp; The target gene sequences of the internal standard and external control are the conserved sequences on the human gene EGFR, with a length of 100 bp. In product analysis, the real-time tracking analysis technology of fluorescently labeled probes is used to automate the detection method. Fluorescent probe is a fluorescently labeled oligonucleotide probe.

The fluorescent group is attached to the 5' end of the probe, which is called a reporter group. The quencher whose absorption spectrum coincides with the emission spectrum of the reporter group is labeled on the probe. The 3' end of the probe is called the quenching group. When the probe is intact, due to the proximity of the fluorescent group and the quenching group, the excited fluorescence is absorbed by the quenching group through resonance energy transfer, showing fluorescence quenching. In the process of gene amplification during the product extension reaction, the 5' exonuclease activity of the polymerase cleaves and hydrolyzes the fluorescently labeled probe, so that the fluorescent group and the quenching group are separated and released, which shows fluorescent characteristics, that is, each DNA chain is amplified, a fluorescent molecule is formed, and the accumulation of fluorescent signals is completely synchronized with the formation of PCR products. This kit realizes the detection of mutations in the sample DNA on the real-time PCR platform, achieving high specificity and high sensitivity for the detection of rare mutations, and at the same time has high selectivity. When analyzing the results, the gene mutation is indicated by the FAM signal, the external control is indicated by the FAM signal, the internal control is indicated by the HEX (or VIC) signal.

Kit Contents

Reaction reagents are pre-loaded in 8-tube strips; each strip detects one sample, and each PCR reaction tube contains specific primers, fluorescent probes, dNTPs, $MgCl_2$, etc. Tube 1-7 are intended for the detection of 29 EGFR mutations, which contains internal control as well; tube 8 contains external control detection reagent. The FAM signal indicates the genetic mutation and the HEX (VIC) signal indicates the internal control. Internal control and external control are used as quality control of the reagents, DNA quality and operation.

Table 1. Kit Contents

Content Name	Components	Volume	Quantity
EGFR 8-Tube Strips	Primers, probes, Mg^{2+}, dNTPs	35 μL	12 strips
EGFR Taq Polymerase	Taq DNA polymerase	35 μL	1 tube
EGFR Positive Control	Positive plasmid DNA, wild type DNA	250 μL	1 tube

Table 2. Mutation Information of Each Strip

Tube No.	Mutation Name	Volume	Reporter Dyes
1	19-Del	35 μL	FAM, HEX/VIC
2	L858R	35 μL	FAM, HEX/VIC
3	T790M	35 μL	FAM, HEX/VIC
4	20-Ins	35 μL	FAM, HEX/VIC
5	G719X	35 μL	FAM, HEX/VIC
6	S768I	35 μL	FAM, HEX/VIC
7	L861Q	35 μL	FAM, HEX/VIC
8	External Control	35 μL	FAM

Note: The contents of different batches cannot be mixed.

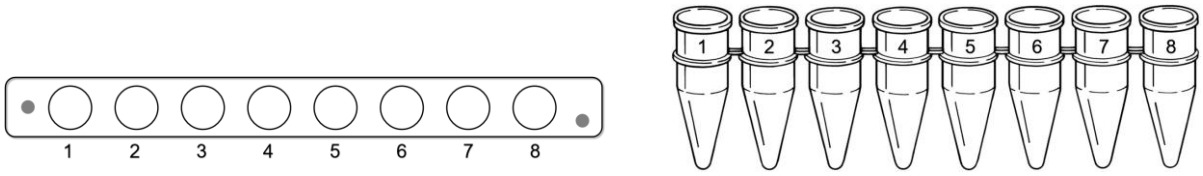

Figure 1. Tube Sequence of 8-Tube Strip

Note: The reaction solutions are pre-loaded in 8-tube strips, as shown in Figure 1. There are two different models on the left and right, which are 1, 2, 3, 4, 5, 6, 7 and 8 tubes from left to right.

Additional required Equipment and Materials

1. Commercialized nucleic acid extraction kit
2. Nuclease-Free water
3. Aerosol-barrier pipette tips

Transportation, Stability and Storage

1. Storage Condition. Store the kit away from light at -15℃ to -25℃, valid for 9 months. Once opened, reagents can be stored in their original packaging at -15℃ to -25℃ until the stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 5 freeze-thaw cycles.
2. Transportation Condition. The kit should be transported at low temperature, with transporting time less than one week and transporting temperature lower than 25℃.
3. Check labels for production date and expiration date of the kit.

Compatible PCR Instruments

Stratagene Mx3000P™, ABI7500, SLAN-48P/96S, ABI StepOne Plus, etc.

1. For Stratagene Mx3000P™, FAM and HEX channel signal gain multiple is adjusted to 1.
2. For ABI instruments, the probe mode setting as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.

Specimen Material

1. Recommended sample types: FFPE tissues stored for no more than 2 years. The biopsies should be fixed with formalin and embedded in paraffin. For resection or surgical biopsies, the recommended tissue input is at least 2×5-micron sections. For coreneedle biopsies, the recommended tissue input is at least 10×5-micron sections.The tissue sample should contain at least 20% tumor cells, otherwise, the tissue samples should be macrodissected and enriched for tumor content.
2. Commercialized kit is recommended to extract DNA from the samples. Assess the quality of sample DNA with an microvolume ultraviolet-visible spectrophotometer, the ratio of OD_{260}/OD_{280} should be within the range of 1.7-2.2. Once the DNA quality or concentration was not in conformity with the above requirements, re-extract DNA with new and/or larger input.
3. Proceed to sample detection or store the DNA at -15℃ to -25℃ for no more than 12 months. Freeze-thaw samples no more than 5 times.

Experimental Procedure

1. Reagent Preparation

   Prepare **EGFR 8-tube Strips** and **EGFR Taq Polymerase** according to samples; briefly centrifuge the strips and Taq polymerase; place them on ice and transfer to the sample processing area; detection of **EGFR Positive Control** (PC) and Negative Control (NTC, Nuclease-Free water) in each reaction/run is recommended.
2. Sample Processing
   (1) Sample preparation: Commercialized kit is recommended to extract genomic DNA. Then, dilute sample DNA to 2 ng/μL with TE buffer solution (pH 8.0), the dilution volume is for a minimum of 50 μL, which is so called tested DNA.
   (2) Template preparation: Respectively add 2.25 μL EGFR Taq polymerase to 45 μL of the tested DNA, PC, and NTC, vortex slightly to mix, then pulse centrifuge.
   (3) Gently remove the cap of 8-tube strip, sequentially add 5 μL of the templates into tubes of each strip, cover the cap carefully.

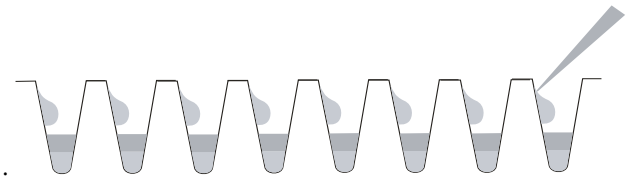

Figure 2. The 8-Tube Strip Sampling Diagram

3. Amplification
   (1) Centrifuge the 8-tube strips for 10 seconds to collect templates.
   (2) Load the 8-tube strips into the real-time PCR instrument; refer to Table 3 for overall arrangement if necessary.

Table 3. Suggested Overall Arrangement

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
20-Ins	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

   (3) Set and run the amplification program as shown in Figure 3.

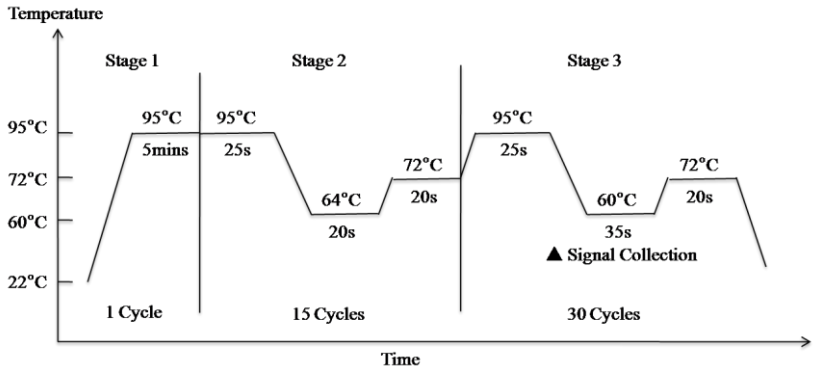

Figure 3. PCR Amplification Procedure

(4) Handle the strips properly after experiment; do not remove the caps in case contamination.

Data Analysis

1. The positive value of 19-Del, L858R, T790M, 20-Ins, G719X, S768I, L861Q assay in this kit is determined as 13, 13, 10, 11, 9, 11, 11 respectively with the assist of ROC curve method.
2. Result Judgment
   (1) Ct value: Provided by the instrument software or by determining the threshold fluorescence of actual amplification curve.
   (2) Mutation Result (refer to Table 4):
      a) When the FAM Ct is greater than or equals to the stated negative Ct value, a negative call or lower than the detection limit of the kit is returned.
      b) When the FAM Ct is less than the stated negative Ct value, calculate the ΔCt Cut-off value per the equation below. If the derived ΔCt Cut-off value is less than or equals to the stated, a positive call is returned; if the derived ΔCt Cut-off value is greater than the stated, a negative call is returned:

      Formula: ΔCt Cut-off=Ct (Mutation)–Ct (External)

      Ct (Mutation): The FAM Ct of tube 1-7 for each sample.

      Ct (External): The FAM Ct of tube 8 for each sample.

Table 4. Result Judgment

Mutation		19-Del	L858R	T790M	20-Ins	G719X	S768I	L861Q
Positive	Stated Threshold Ct Value	Ct <29	Ct <29	Ct <28	Ct <29	Ct <28	Ct <29	Ct <29
	Stated ΔCt Cut-off value	13	13	10	11	9	11	11
Negative	Stated Negative Ct Value	Ct≥29	Ct ≥29	Ct ≥28	Ct ≥29	Ct ≥28	Ct ≥29	Ct ≥29

Interpretation of Results

1. NTC: There should be no amplification curves of FAM in tube 1-7, or else, call the result invalid. Occasionally, amplification curve of HEX (VIC) generates in tube 1-7 or, of FAM generates in tube 8, either of which has no influence on result interpretation.
2. PC: There should be amplification curves of FAM and HEX (VIC), with the value of Ct is less than 20. If the Ct value of FAM or HEX (VIC) in any one tube is greater than 20, the value is invalid and retest is recommended.
3. External Control: The FAM Ct of External Control should be 13-18, which must be qualified before proceeding to further analysis; if the FAM Ct is less than 13, that indicates excessive DNA amount, dilute sample DNA for a new detection; if the FAM Ct is greater than 18, that indicates insufficient DNA amount or that sample DNA was contaminated by PCR inhibitor, in this case, it is recommended to re-extract sample DNA for a new detection.
4. Internal Control: Amplification curves of HEX (VIC) should generate in every tube from 1 to 7, otherwise, consider that DNA amount was insufficient, or DNA was contaminated by PCR inhibitor, in that case, re-extract sample DNA for a new detection; yet one situation that in a same tube, no amplification curve of HEX (VIC) generates but FAM generates, the result is considered reliable.

Limitations of the Kit

1. Negative results could not exclude the existence of EGFR gene mutation; cases like inadequate tumor cells, DNA degradation, or insufficient DNA amount may lead to negative results as well.
2. Different sampling locations may lead to diverse outcomes due to the heterogeneity of tumor tissues/cells.
3. Situations that may result in false negative or false positive result include but not limit to unreasonable sample collection, transportation, improper experimental operations or environment.
4. The kit is only intended for the qualitative detection of 29 mutation sites of EGFR gene.
5. The kit is only applicable with the stated sample types and detection system, including specified instruments, DNA extraction kit and analytical assay.

Performance Characteristics

1. The kit should be of neat appearance, clear labels, and of no leakage; when unfrozen, the reagents shall be clear, without precipitate.
2. The consistency rates of both positive and negative reference materials are 100%.
3. The kit allows the detection of 1% of specific gene mutations in 10 ng DNA sample.
4. The coefficient of variation (CV, %) of 10 Ct values by detecting designated sample for 10 repetitive times should be less than 10%.
5. There's no nonspecific product with up to 200 ng wild-type DNA sample.

Warnings and Precautions

1. Please read the instruction carefully in prior to the use of the kit.
2. Avoid repetitively freezing and thawing the reagents in the kit.
3. The results of this kit will be affected by the source, the process of collection, quality, condition of transport, pre-treatment of the sample, as well as the quality of the extracted DNA, model of fluorescence quantitative PCR instrument, operation environment, and the current technological limitation of molecular biology. The factors and variables mentioned above would lead to false positive or false negative test results. Users must be aware of the potential errors and accuracy limitations that may exist during the process of detection.
4. The quality of DNA is crucial, and the quality control of DNA should be performed after extraction; proceed to sample detection immediately or store sample DNA properly at -15℃ to -25℃.
5. Do not substitute any content of the kit; do not mix contents of different batches.
6. Pay special attention to the use of positive control to prevent contamination of reagents or resulting in false positive results.
7. Be cautious of contamination from external DNA; when sampling, always add NTC and sample DNA before positive control; segregate areas for reagent preparation and sample processing; use dedicated pipettes and tips for reagent preparation and template addition, respectively.
8. Sterilize the environment and pipettes with 10% hypochlorous acid, 75% ethyl alcohol, or UV radiation.
9. All the reagents in use have potential hazard. It is recommended wearing proper protective suit and gloves. For first-use of this kit, you may receive training by our technical supports.
10. All samples including positive control in the kit should be considered as potential infectious substances which should be handled carefully.

Symbols

Symbol	Symbol definition
	Indicates the need for the user to consult the instructions for use.
	Indicates the date when the medical device was manufactured.
	Indicates the manufacturer's batch code so that the batch or lot can be identified.
	Indicates the invitro diagnostic medical device.

	Indicates the temperature limits to which the medical device can be safely exposed.
	Indicates the date after which the medical device is not to be used.
	This is the correct upright position of the distribution packages for transport or storage.
	Indicates a medical device that needs to be protected from moisture.
	Indicates a medical device that needs protection from light sources.
	Indicates the medical device manufacturer.
EC REP	Indicate the authorized representative in the European Community
CE	The product meets the basic requirements of European in vitro diagnostic medical devices directive 98/79/EC.

References

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7. NCCN Clinical Practice Guidelines in Oncology for NSCLC.2013.

EC REP

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Attached List 1：

No.	Mutation	Exon	Base alteration	Cosmic ID
1	G719A	18	2156G>C	6239
2	G719S	18	2155G>A	6252
3	G719C	18	2155G>T	6253
4	E746_A750del (1)	19	2235_2249del15	6223
5	E746_A750del (2)	19	2236_2250del15	6225
6	L747_P753>S	19	2240_2257del18	12370
7	E746_T751>I	19	2235_2252>AAT(complex)	13551
8	E746_T751del	19	2236_2253del18	12728
9	E746_T751>A	19	2237_2251del15	12678
10	E746_S752>A	19	2237_2254del18	12367
11	E746_S752>V	19	2237_2255>T(complex)	12384
12	E746_S752>D	19	2238_2255del18	6220
13	L747_A750>P	19	2238_2248>GC(complex)	12422
14	L747_T751>Q	19	2238_2252>GCA(complex)	12419
15	L747_E749del	19	2239_2247del9	6218
16	L747_T751del	19	2239_2253del15	6254
17	L747_S752del	19	2239_2256del18	6255
18	L747_A750>P	19	2239_2248TTAAGAGAAG>C(complex)	12382
19	L747_P753>Q	19	2239_2258>CA(complex)	12387
20	L747_T751>S	19	2240_2251del12	6210
21	L747_T751del	19	2240_2254del15	12369
22	L747_T751>P	19	2239_2251>C(complex)	12383
23	T790M	20	2369C>T	6240
24	S768I	20	2303G>T	6241
25	H773_V774insH	20	2319_2320insCAC	12377
26	D770_N771insG	20	2310_2311insGGT	12378
27	V769_D770insASV	20	2307_2308insgccagcgtg	12376
28	L858R	21	2573T>G	6224
29	L861Q	21	2582T>A	6213