

# Ultra-Sensitive cfDNA EGFR Mutation Detection Kit

## User Manual

Catalog Number: EGFR0002-20 EGFR0002-50

Size: 20 tests/Kit 50 tests/Kit

Intended Use: For Research Use Only

Doc. No.: 100-EGFR0002

Revision: Rev. A

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## 1. PRODUCT INFORMATION

### 1.1 Background

Epidermal growth factor receptor (EGFR) is one of the most frequently amplified, mutated and/or overexpressed genes in human malignancies (1). Inactivation of EGFR kinase with tyrosine kinase inhibitors (TKIs), such as gefitinib (Iressa) and erlotinib (Tarceva), suppresses signal transduction pathways which control tumor cell growth, proliferation, and resistance to apoptosis (2). TKIs therapy has shown a significant symptom benefit and prolongs survival on the treatment of Non-Small Cell Lung Cancer (NSCLC) patients with somatic mutations in the EGFR gene. Therefore, detection of the somatic mutations in the EGFR gene may predict a more accurate response of the cancer patients to TKIs therapy.

### 1.2 Intended use

Medaysis Ultra-Sensitive cfDNA EGFR Mutation Detection Kit is a highly specific and sensitive PCR technique that is able to detect common somatic mutations in the EGFR gene. Used with Sanger sequencing, it can detect less than 1% (as little as 20 ng to 100 ng of) mutant genes mixed with the wild type (Table 1). It is designed to amplify EGFR gene in circulating cell-free DNA (cfDNA) from plasma samples.

Sample quality assurance for diagnostic tests has not been widely implemented in clinical laboratories.

Table 1. cfDNA EGFR mutations detected by the Kit:

No	Reagents	Exon	Mutation/Hot spot	Amino Acid Range
1	EGFR Exon 18 PCR primer mix	18	G719	688~728
2	EGFR Exon 19 PCR primer mix	19	Exon 19 deletion	739~760
3	EGFR Exon 20 PCR primer mix	20	S768/ Insertion mutation	761~823
4	EGFR Exon 21 PCR primer mix	21	L858 / L861	850~875
5	EGFR Exon 20 T790 PCR primer mix	20	T790	782~823

## 2. KIT CONTENT

List of components \*:

No.	Catalog Number	Name of Components	Volume (µl)	
			EGFR0002-20	EGFR0002-50
1	EGFR0021	EGFR Exon 18 PCR primer mix	100	250
2	EGFR0022	EGFR Exon 19 PCR primer mix	100	250
3	EGFR0023	EGFR Exon 20 PCR primer mix	100	250
4	EGFR0024	EGFR Exon 21 PCR primer mix	100	250
5	EGFR0025	EGFR Exon 20 T790 PCR primer mix	100	250
6	EGFR0041	EGFR Exon 18 sequencing primer	10	25
7	EGFR0042	EGFR Exon19 sequencing primer	10	25
8	EGFR0043	EGFR Exon 20 sequencing primer	10	25
9	EGFR0044	EGFR Exon 21 sequencing primer	10	25
10	EGFR0045	EGFR Exon 20 T790 sequencing primer	10	25
11	SQC0021	DNA Quality Control Primer Mix	100	250
12	OTH0001	2x PCR Master Mix	1400	3500
13**	EGFR0061	Exon 19 Wild-type Control	20	50
14**	EGFR0062	EGFR Exon 20 plasmid wild type	20	50
15**	EGFR0063	EGFR Exon 21 plasmid wild type	20	50

<b>16**</b>	EGFR0064	EGFR Exon 19 plasmid deletion	20	50
<b>17</b>	OTH0002	Nuclease-Free Water	1000	2500
<b>18</b>	OTH0003	Cell-Free DNA BCT®	20	50

\* Each component contains enough material to test 20 or 50 DNA samples

\*\* Plasmid controls contain high copy number of control templates. Please perform carefully to avoid cross contamination.

### 3. SHIPPING AND STORAGE

Medaysis cfDNA EGFR mutation detection kit is shipped at 4°C and recommends being store at -20°C for long-term storage. When stored under the recommended storage conditions in the original packing, the Kit is stable for one year from the date of shipment. Repeated thawing and freezing should be avoided. Non-hazardous. No MSDS required.

### 4. PRECAUTIONS FOR USE

- Please read the instruction carefully before use.
- The Kit is intended for research use only, not for diagnostics purpose.
- Experiments should be performed under proper sterile condition with aseptic techniques.
- All reagents should be thawed thoroughly, mix the components by inverting and centrifuge briefly before use.
- Medaysis Ultra-Sensitive cfDNA EGFR mutation detection Kit is a PCR-based test to be used by trained laboratory technicians with the appropriate laboratory facilities and equipment.
- Avoid inhalation and ingestion.

### 5. ADDITIONAL REAGENTS AND INSTRUMENTS REQUIRED

#### 5.1 Reagents

- DNA extraction
- 6 x sample loading buffer
- Agarose
- 1 x TAE buffer
- Novel juice or ethidium bromide
- 100bp DNA ladder standard (Range: 100 – 1000 bp)

#### 5.2 Materials

- Sterile, nuclease-free PCR tubes for preparing mastermixes
- Adjustable Pipettes for samples preparation
- Disposable sterile pipette tips with filter
- Disposable gloves

#### 5.3 Instruments

- Electrophoresis equipment and power supply
- Sanger Sequencer (our Kit is compatible with DNA analyzer ABI3730)
- The Kit has currently been optimized by using ABI Veriti Thermo Cycler. Table 2. List of compatible PCR instruments which has been tested:
- Optimization might be necessary for other instruments. For more information of instrument compatibility, please contact the technical service at Medaysis.

Table 2. PCR instruments

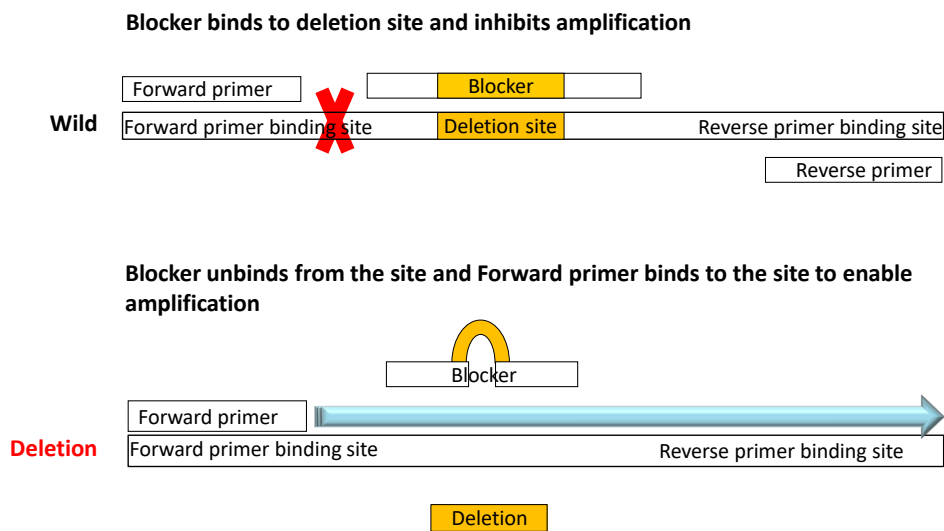
Company	Model
Applied Biosystems	Veriti
Bio-Rad	T100
Biometra	T-3000

**6. PRODUCT DESCRIPTION AND PRINCIPLE**

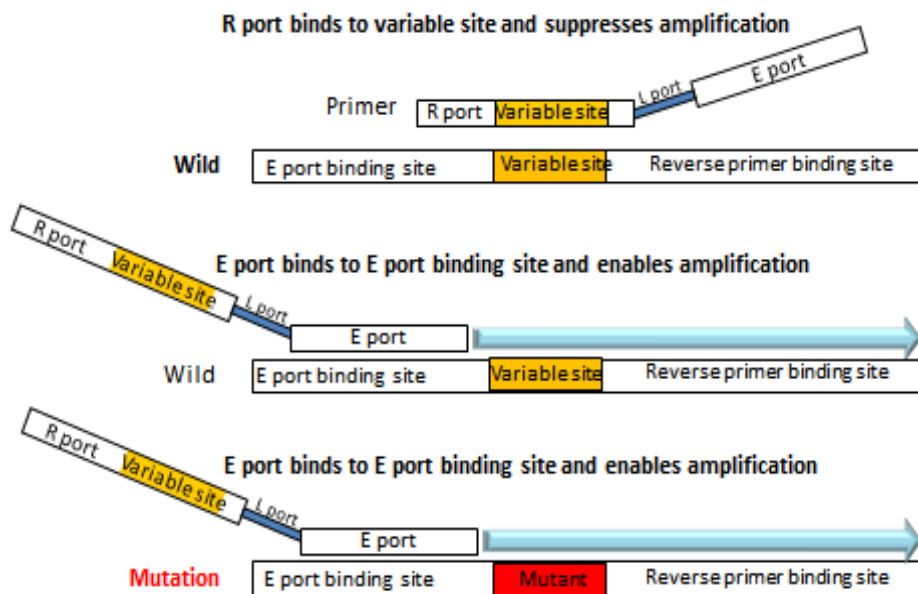
Ultra-Sensitive cfDNA EGFR Mutation Detection Kit provides CloDiA™ PCR using novel and proprietary mutation enrichment technology. CloDiA™ PCR has two types of technique involved - Unindel™ PCR and Stuntmer™ PCR. Unindel™ PCR is designed to detect a broad range of insertions/deletions (universal insertions/deletions) in the target region. The three-primer set comprising forward primer, reverse primer and blocker inhibits amplification of wild type gene but enables amplification of exonic insertions/ deletions. Stuntmer™ PCR is designed to detect a broad range of point mutations in the target region. Dual port primer of R Port and E Port is designed for self-competition to preferably amplify mutated gene but suppress wild type gene amplification. Combination of Unindel™ PCR and Stuntmer™ PCR detects a broad range of insertions/deletions and point mutations in exons 18, 19, 20 and 21 of human EGFR gene in cfDNA. Sanger sequencing can be used to analyze the sequence. Sanger sequencing can be used to analyze the sequence.

Figure 1. Principle of the Technology

**Unindel PCR: Detects a Broad Range of Insertions/Deletions**



**Stuntmer PCR: Detects a Broad Range of Point Mutations**



## 7. PROTOCOL

To minimize the risk of contamination with foreign DNA, it is recommended that the Kits should be conducted in a PCR workstation.

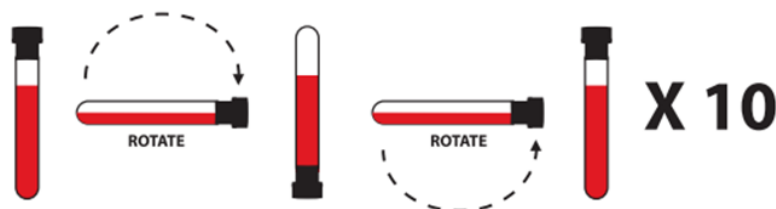
### 7.1 Plasma sample collection

Cell-Free DNA BCT is authorized from Streck and used as 10 mL blood collection tube for stabilization of cell-free plasma DNA. The formaldehyde free preservative in Cell-Free DNA BCT stabilizes white blood cells, preventing the release of cellular genomic DNA and degradation of cfDNA, allowing isolation of high quality cell-free DNA.

Plasma Sample Collection, Processing and Packaging:

1. Collect 10 ml blood samples into Cell-Free DNA BCT tubes until fill tube completely.
2. Mix the tube immediately by gentle inversion 8 to 10 times as Fig.2 below.
3. After collection, transport and store tubes within the recommended temperature range. Note: Blood samples collected in Cell-Free DNA BCT for cfDNA are stable at 6 °C-37 °C up to 14 days. For circulating epithelial cells (tumor cells) in whole blood are stable for up to 4 days at 6 °C-37 °C.
4. Cell-free DNA and cell genomic DNA can be extracted from plasma by using most commercially kits. For instructions, refer to the manufacturer's manuals.

Figure 2. Gently rotate the tube 8 to 10 times



### 7.2 DNA preparation

Human cell-free DNA and cell genomic DNA can be extracted from plasma by using most commercially manual/automated kits. For instructions, refer to the manufacturer's manuals.

The OD value of genomic DNA extractions should be measured using the spectrophotometer or similar approach. Make sure that OD 260/OD280 value of sample is between 1.8 and 2.0. Extracted genomic DNA specimens may be stored at -20°C for long-term storage or refer to the manufacturer's manuals.

For further information regarding the compatibility of the device with different extraction methods please contact [techsupport@medaysis.com](mailto:techsupport@medaysis.com).

### 7.3 DNA quality control preparation

Numerous molecular detection tests rely on the quality of the genomic DNA specimens. DNA quality control (DNA QC) primer mix is provided to ensure consistent DNA quality.

1. Thaw and centrifuge DNA QC Primer Mix, 2x PCR Master Mix and Sterile H<sub>2</sub>O at 4°C before use.
2. Prepare two PCR tubes and label it as QC and NC.
3. Prepare the PCR Reaction Mixture by adding 10 µl 2x PCR Master Mix, 4.8 µl DNA QC Primer Mix and 4.2 µl Sterile H<sub>2</sub>O with a total of 19 µl mixture per reaction.
4. Add 1 µl (20~100 ng) DNA specimen and 1 µl sterile H<sub>2</sub>O (negative control) into the PCR reaction mixture.
5. Pipette the mixture gently and centrifuge briefly.

Note: If more than one DNA specimens need to be tested, we suggest preparing a reaction mix containing appropriate amount of 2x PCR Master Mix, DNA QC Primer Mix and Sterile H<sub>2</sub>O. Then aliquot 19 µl mixture per reaction to the PCR tube and add 1 µl DNA specimen on each tube.

### 7.4 PCR reaction preparation

1. Thaw and centrifuge all tubes (Exon 18, Exon 19, Exon 20, Exon 21, T790 each Primer Mix, 2x PCR Master Mix and Sterile H<sub>2</sub>O) at 4°C before use.
2. Prepare PCR tubes and label them S1, S2, S3, S4, S5, S6 for each primer mix.
3. Prepare separately PCR Reaction Mixture by adding 10 µl 2x PCR Master Mix, 4.8 µl Primer Mix and 4.2 µl Sterile H<sub>2</sub>O with a total of 19 µl mixture per reaction for S1~S6.

4. Add 1µl (20~100 ng/µl) DNA specimen into the PCR reaction mixture S1~S5 and 1µl positive control into the PCR reaction mixture S6.
5. Pipette the mixture gently and centrifuge briefly.

Note: Same as the preparation of the DNA quality control, if more than one DNA specimens need to be tested, it is recommended to prepare a reaction mix of table 3 & 4 (No. 2~4) and aliquot 19µl to each PCR tube (S1-S6).

Table 3. PCR tube preparation per one reaction:

S1	S2	S3	ES	S5	S6	DNA QC	NC
Exon 18 mixture	Exon 19 mixture	Exon 20 mixture	Exon 21 mixture	T790 Mixture	Exon 19 positive control	DNA quality control	Negative control

Table 4. Prepare the reaction mixture per one reaction according to the table below:

No.	Component	Volume (µl)
1	DNA specimen (20~100 ng/µl) / positive control	1
2	Each primer Mix	4.8
3	2x PCR Master Mix	10
4	Nuclease-Free Water	4.2
	Final Volume	20

## 7.5 PCR thermal cycling condition

Table 5. Follow the PCR protocol exactly when operate PCR instrument

	Temperature (°C)	Time (min)	Cycle(s)
Stage 1			
Pre-denaturation	95	10	1
Stage 2			
Denaturation	94	1	45
Blocking Primer Annealing	65	3	
Primer Annealing	61	1	
Elongation	72	1	
Stage 3			
Extension	72	10	1
Preservation	10	∞	

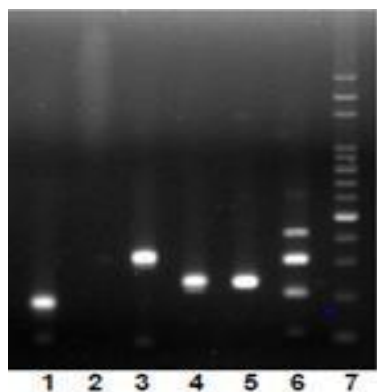
### 7.6 Run PCR gel electrophoresis (optional)

Before Sanger sequencing, the PCR products can be examined by the standard agarose gel electrophoresis (2% agarose in 100ml 1X TAE buffer). The DNA will be visualized by ethidium bromide or novel juice fluorescence.

1. Loading PCR products: mix 5µl of PCR products with 1 µl of 6X novel juice loading dye and load in the 2% agarose gel.
2. Check the results of each amplicon in Figure 3.
3. Perform Sanger sequencing referring to the manufacturer's manuals. Store the rest of PCR products at 4°C.

Note: For the instructions of DNA sequencer, refer to the manufacturer's manuals.

Figure 3. The amplicons of each PCR product on gel electrophoresis



- Lane 1 : Exon 18 PCR product (~140bp)
- Lane 2 : Exon 19 PCR product (blank or ~144bp)
- Lane 3 : Exon 20 PCR product (~274bp)
- Lane 4 : Exon 21 PCR product (~203bp)
- Lane 5 : Exon 20 T790 PCR product (~213bp)
- Lane 6 : Sample Quality Control PCR product (100, 200, 300, 400, 600bp)
- Lane 7 : 100bp DNA Marker

Note: It's possible that no amplicon shown at the lane of Exon 19 on agarose gel due to primer design which inhibits wild-type sequence replication during PCR steps. The results should be determined based on the sequencing data.

### 7.7 Recommended Sanger sequencing protocol

Our Kit is validated and compatible with DNA analyzer ABI3730. For the instructions of DNA sequencer, refer to the manufacturer's manuals.

For more information of instrument compatibility, please contact the technical service at [techsupport@medaysis.com](mailto:techsupport@medaysis.com).

Note: PCR products may need to be cleaned up before performing Sanger sequencing.



**8. DATA ANALYSIS**

PCR products must be sequenced for further analysis. For data analysis, please interpret results refer to the manufacturer’s manuals of the software.

Note: To get reasonable interpretation of your results, it is recommended to eliminate baseline “noise” of data. For the common mutation information, please refer to the following data.

Figure 4. Example of sequence data

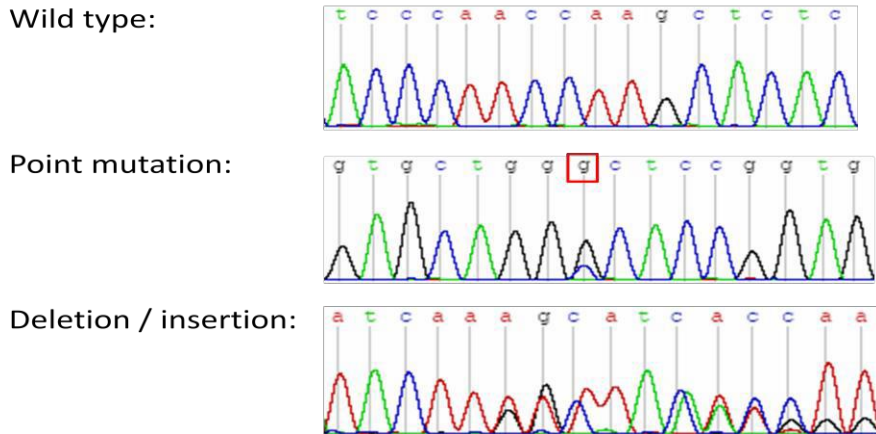


Figure 5. Example of sequence analysis

(A) Harbored a C-To-T transition; (B) Harbored a C-to-T transition and G-to-A transition; (C) Harbored a G-to-A transition.

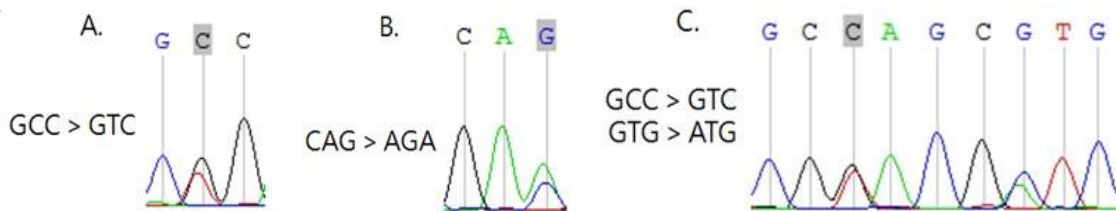
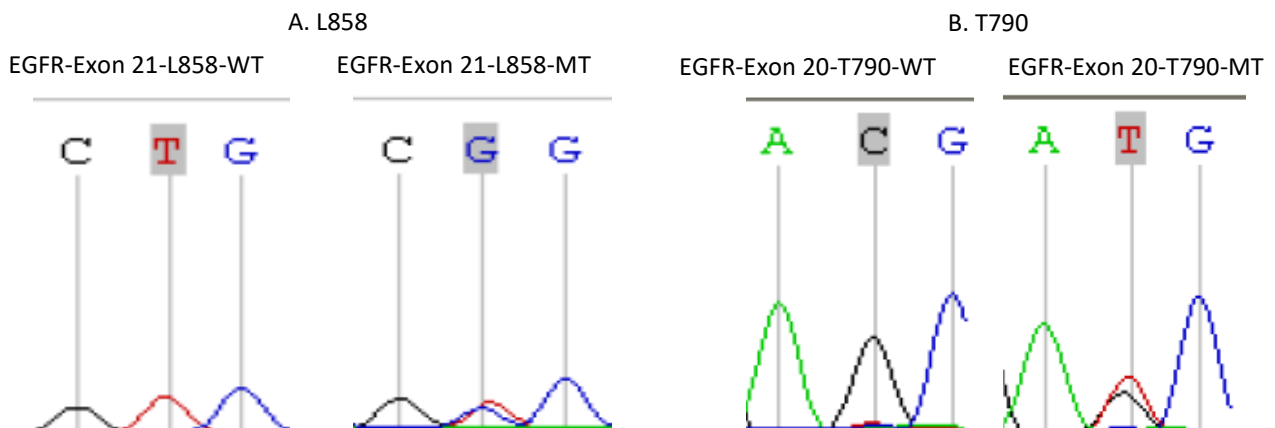


Figure 6. Example of sequence analysis of EGFR mutation.

(A) EGFR Exon21 Harbored a T-to-G transition refer to L858R. (B) EGFR Exon20 T790 Harbored a T-to-G transition refer to T790M.



## 9. TROUBLESHOOTING GUIDE

This troubleshooting guide may be helpful in solving similar problems that may arise. If there is any other question, please welcome to contact [techsupport@medaysis.com](mailto:techsupport@medaysis.com).

Problems	Questions	Suggestions
No Amplicon /No Band	No PCR products observed on gel electrophoresis.	<ol style="list-style-type: none"> <li>1. Check the results of sample quality control. If there is no amplicon shown at the size of 400bp, DNA may be fragmented during inadequate fixation steps. Please check your sample fixation process.</li> <li>2. Remove presence of inhibitor in reaction in case it exists, and then repeat SQC reaction.</li> <li>3. Inspect temperature calibration on PCR instrument.</li> <li>4. Check both the storage conditions and the expiration date on the label. Use a new kit if needed.</li> </ol>
Non-Specific Amplification/ Multiple Products/ Wrong Size Band Amplified	How to eliminate the multiple or non-specific PCR products?	<ol style="list-style-type: none"> <li>1. Conduct Kit in PCR workstation to minimize the risk of contamination with foreign DNA.</li> <li>2. Inspect temperature calibration on PCR instrument.</li> <li>3. Blocking primer annealing temperature is too low. Increase 2 to 3°C at annealing step to reduce non-specific binding and amplification.</li> </ol>
Equipment Variation	Have you checked the discrepancy among different PCR instruments?	Yes. We had done parallel tests on different instruments to make sure our Kit compatible with different instrument models including ABI, Biometra, BioRad (Table2).
Novel Mutation	How to verify the novel mutation and confirm the accuracy of the results?	Please check any existed mutations on COSMIC website or do parallel tests with the proven data.

## 10. REFERENCE

1. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Pai, R., et al., Nat. Med. 2002.
2. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Di Fiore, P.P., et al., Cell, 1987.