EGFR T790M RealFast™ Assay

REF 8-110 / 8-113 Σ 100 / 32 reactions

-20°C 2-8°C CE IVD

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1. Intended Use

The EGFR T790M RealFast™ Assay is a real-time PCR test for the qualitative detection of the somatic c.2369C>T (p.T790M) mutation in exon 20 of the human *Epidermal Growth Factor Receptor (EGFR)* gene. The kit is designed to identify the resistance mutation in human plasma-derived, circulating cell-free DNA (cfDNA) from non-small cell lung cancer (NSCLC) patients. The test is intended as companion diagnostic to support the selection of NSCLC patients eligible for EGFR tyrosine kinase inhibitor (TKI) therapy with osimertinib (TAGRISSO™). The assessment of the EGFR T790M status in cfDNA from plasma is suggested for patients not amenable to tumor biopsy.
Reference sequence: NM_005228.3; HGVS: c.2369C>T (p.T790M); Cosmic ID: 6240.

2. Introduction

EGFR plays a crucial role in cell proliferation and growth by activation of the Ras-Raf-MAPK pathway. EGFR mutations in exon 18, 19, 20 and 21 are found with a prevalence of 10% to 30% in NSCLC patients. The most prominent activating EGFR mutations are deletions in exon 19 and L858R in exon 21. NSCLC patients with activating EGFR mutations may benefit from EGFR TKI therapy. However, approximately 50% of NSCLC patients that acquire resistance to these TKIs (e.g. erlotinib) exhibit the T790M mutation. Based on clinical studies osimertinib treatment is an option/alternative therapy for T790M positive patients.

3. Kit Contents

		100 / 32 Rxn
RealFast™ 2x **Probe Mix**	1 vial □ white cap	1000 / 320 µl
EGFR T790M **Assay Mix**	1 vial ■ purple cap	550 / 550 µl
EGFR T790M **Positive Control**	1 vial ■ green cap	75 / 75 µl

The kit contains reagents for 100 / 32 reactions in a final volume of 20 µl each.

The RealFast™ 2x Probe Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The EGFR T790M Assay Mix consists of gene-specific primers, dual-labeled hydrolysis probes for *EGFR* and the endogenous control (EC) gene, an EGFR wild-type suppressor and uracil-N-glycosylase to minimize the risk of cross-contaminating PCR products. A synthetic EGFR T790M Positive Control representing the mutated *EGFR* and the EC gene is supplied with the kit.

4. Storage and Stability

The EGFR T790M RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -20°C. Alternatively, store at 2 to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid prolonged exposure to intense light. If stored correctly, the kit will retain full activity until the expiration date indicated on the label.

5. Product Description

5.1. Principle of the Test

The test is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primer pairs for amplification of a 92 bp *EGFR* exon 20 fragment and a 147 bp EC gene fragment. Further components are two dual-labeled, gene-specific hydrolysis probes, the **FAM-labeled EGFR probe** and the **HEX-labeled EC probe,** which hybridize to an internal sequence of the amplified fragments. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5' - 3' exonuclease activity of Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product.
The EC serves as a PCR positive control and as a parameter to evaluate EGFR c.2369C>T (p.T790M) positivity within the range of/by determination of a ΔC_q ($C_{q\ EGFR} - C_{q\ EC}$) value ≤ 8.

5.2. Real-time PCR Instrument Compatibility

The EGFR T790M RealFast™ Assay is validated for use with the AB 7500 Fast instrument.
The kit is compatible with various common real-time PCR instruments capable of recording FAM and HEX fluorescence:

- ✓ AB 7500 Fast (Applied Biosystems®)
- ✓ AB StepOne™ (Applied Biosystems®)
- ✓ CFX96™ (Bio-Rad)
- ✓ LightCycler® 480 (Roche)
- ✓ MIC qPCR Cycler (bms)
- ✓ Mx3005P (Agilent Technologies)
- ✓ Rotor-Gene® 6000 (Qiagen)

» ***Note:*** *RealFast™ Variant Detection QuickGuides for setting up and analyzing experiments on different types of instruments can be downloaded from www.viennalab.com.*

When using AB 7500 Fast, StepOne™ or Mx3005P set passive reference dye to "ROX" ! «

The kit is supplied with **low ROX**. For use with real-time PCR instruments requiring high ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT), add ROX to a final concentration of 1 µM to the 2x Probe Mix.

5.3. Assay Performance Specifications

Determination of **sensitivity** was performed on 24 samples testing positive for the EGFR T790M mutation with a reference method. The EGFR T790M RealFast™ Assay determined 22 samples as positive, which equaled a true positive rate of 91,7%.

Determination of **specificity** was performed on 32 samples testing negative for EGFR T790M mutation with a reference method. The EGFR T790M RealFast™ Assay determined 31 samples as negative, which equaled a true negative rate of 96,9%.

Limit of detection: 8 mutant copies of EGFR T790M in a background of 2.5 ng wild-type cfDNA per reaction.
Recommended DNA concentration: 0.5 ng/µl to 10 ng/µl cfDNA.

6. Materials Required but not Supplied

Real-time PCR instrument with FAM (520 nm) and HEX (556 nm) filters, instrument-compatible reaction vessels, disposable powder-free gloves, vortexer, mini-centrifuge for 2.0 ml tubes, tube racks, set of calibrated micropipettes (0.5 – 1000 µl), sterile tips with aerosol-barrier filter, molecular grade water, DNA extraction system, freezer, biohazard waste container. Equipment for blood drawing, plasma preparation and cfDNA isolation.

7. Specimen handling and cfDNA extraction:

Sample material must be cfDNA isolated from **fresh or frozen** human plasma.

7.1. Plasma preparation

Collect 9 ml peripheral blood in EDTA tubes according to the manufacturer's instruction. Avoid unnecessary agitation to reduce cfDNA degradation and leukocyte lysis. Proceed with EDTA plasma preparation **within one hour** of blood drawing according to the following protocol:

- Centrifuge for 10 minutes at 1.600 x g in a swinging bucket rotor centrifuge.
- Transfer the plasma fraction (supernatant) to tubes suitable for high-speed centrifugation.

» **Note**: *Avoid carry-over of cells as contaminating leukocytes may increase the genomic DNA background and thus dilute the target cfDNA. «*

- Centrifuge the plasma for 10 minutes at 16.000 x g in a fixed angle rotor centrifuge.
- Transfer the supernatant to fresh tubes avoiding contact with and potential transfer of the pellet.
- Freeze the plasma at -40°C to -80°C or proceed immediately with cfDNA extraction.

» **Note**: *If blood collection tubes other than EDTA tubes e.g. PAXgene® Blood ccfDNA tubes or STRECK Cell-Free DNA BCT® tubes are used, follow the respective instructions of the supplier for blood drawing, storage conditions and plasma preparation. «*

7.2.DNA extraction

DNA extraction reagents are **not supplied** with the kit.
For cfDNA extraction from 4 ml double-centrifuged plasma the ViennaLab Plasma cfDNA Extraction Kit [REF 2-040] is recommended. The EGFR T790M RealFast™ Assay requires 2.5 – 50 ng cfDNA per reaction. The cfDNA concentration in human plasma is usually very low. Therefore, it is recommended to use a highly sensitive method (e.g. Qubit dsDNA HS Assay) for cfDNA quantification.

Ensure extracted DNA is suitable for amplification in terms of concentration, purity and integrity. Accidental carry-over of residual amounts of washing buffers during DNA extraction may interfere with cfDNA analysis. For reliable analysis the cfDNA concentration of the sample should be at least 0.5 ng/µl.

» ***Note**: UV-VIS or fluorescence-based quantification of cfDNA below 0.2 ng/µl is error-prone and hence may be inaccurate. For the EGFR T790M RealFast™ Assay however, samples showing a $C_{q\ EC\ [HEX]} \leq 32$ are eligible for analysis. «*

8. Experimental Protocol

8.1. PCR Controls

Always include a **No Template Control** (NTC) in each experiment to confirm absence of potential contaminations. Use PCR-grade water instead of DNA and run the NTC in duplicate.

Always include the EGFR T790M **Positive Control** as positive reference signal for your unknown samples.

» ***Note**: The Positive Control is a potential source of contamination. Make sure to handle it carefully «.*

8.2. Preparation of EGFR T790M RealFast™ **Master Mix**

Gently vortex and briefly centrifuge all solutions after thawing. Set up PCR at room temperature. Prepare sufficient **Master Mix** for all your reactions (N samples + positive control + negative controls) plus at least one additional reaction to compensate for pipetting inaccuracies:

Component	per reaction	e.g. 24+1 reactions
RealFast™ 2x Probe Mix	10 µl	250 µl
EGFR T790M Assay Mix	5 µl	125 µl
Master Mix	**15 µl**	375 µl

Dispense **15 µl Master Mix** into each well. Add **5 µl** purified **DNA** or **Control** template to reach a final reaction volume of 20 µl.
To minimize risk of contamination, always pipette templates in the following order: first NTC, then samples, last positive control. Immediately close reaction vessels.

» ***Note:** Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed. «*

8.3. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for quantitation experiments with two targets / reporter dyes. Place the samples into the thermal cycler and run the following program:

AB 7500 Fast, StepOne™, **CFX96™, LightCycler® 480, Mx3005P** and **other** Peltier heating block-based **instruments:**

Cycles	Temp	Time	Steps
1	37°C	10 min	Degradation of uracil-containing DNA
1	95°C	2 min	Initial denaturation
45	95°C	5 sec	Denaturation
	60°C	30 sec	Annealing/Extension – **Data acquisition** on FAM- and HEX-channel

MIC qPCR Cycler, Rotor-Gene® 6000*):

Cycles	Temp	Time	Steps
1	37°C	10 min	Degradation of uracil-containing DNA
1	95°C	2 min	Initial denaturation
45	95°C	5 sec	Denaturation
	60°C **)for 36-well rotor: **56°C***	30 sec	Annealing/Extension – **Data acquisition** on Green and Yellow channel

9. Data Analysis / Interpretation of Results

Successful amplification of a sample can be verified by a positive signal of the EC in the HEX channel. The presence or absence of an EGFR c.2369C>T (p.T790M) mutation in the sample is defined by the occurrence of a signal in the FAM channel within certain limits (see Table Data analysis).

EGFR c.2369C>T **negative** cfDNA samples exhibit amplification in the HEX channel only.

EGFR c.2369C>T **positive** cfDNA samples exhibit amplification in the FAM and HEX channel, they show a calculated **ΔC_q ($C_{q\ EGFR\ [FAM]} - C_{q\ EC\ [HEX]}$) ≤ 8.**

Fluorescent levels and corresponding amplification curves are automatically displayed in amplification plots in the real-time PCR software.

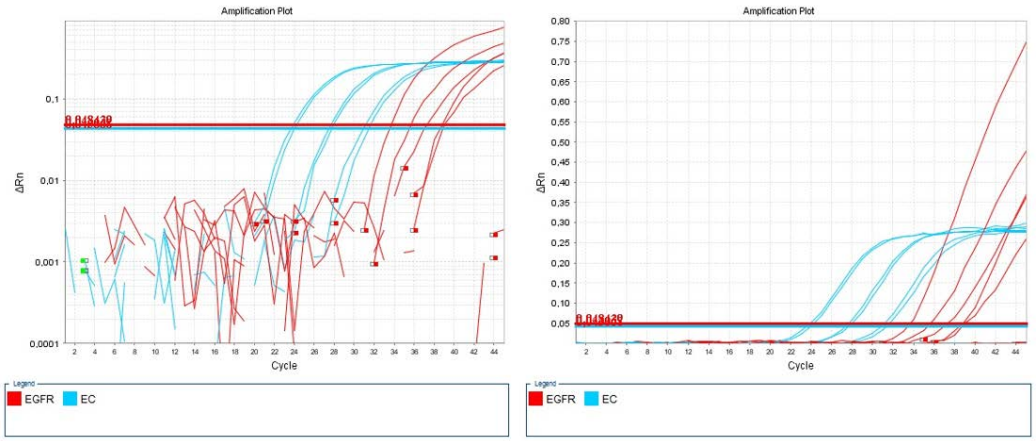
Data analysis		
Data analysis is performed automatically by the real-time PCR instrument software selecting **automatic threshold** and **base-line settings**. However, the user must verify the correct setting of the threshold and base-line → see section 10. Important Information.		
Target	**C_q**	**Interpretation**
EC [HEX]	22 - 32	Sample is within range of analysis → sufficient template DNA (> 0.5ng / reaction)
	< 22	Excess of template DNA → dilute sample and repeat testing
	> 32	Insufficient amounts of cfDNA or presence of PCR inhibitor → column-based purification and concentration of sample DNA recommended
	N.A.	Sample contains no template DNA
EGFR c.2369C>T (p.T790M) [FAM]	20 - 37	Sample is within range of analysis → perform ΔC_q calculation
	35 - 37	Sample is within range of analysis. However, to confirm the presence of mutated cfDNA at low level it is recommended to repeat the testing in duplicate
	< 20	Excess of template DNA → dilute the sample and repeat testing
	> 37 or N.A.	Sample is EGFR c.2369C>T (p.T790M) negative. FAM signals C_q > 37 represent co-amplified EGFR wild-type fragments.
Samples fulfilling the above requirements **AND** the condition **ΔC_q** ($C_{q\ EGFR} - C_{q\ EC}$) **≤ 8** are EGFR c.2369C>T (p.T790M) **positive**. A **ΔC_q > 8** indicates co-amplification of EGFR wild-type, sample has to be considered as EGFR c.2369C>T (p.T790M) **negative**.		

Examples for interpretation of results		
C_q Values	Evaluation	Interpretation
HEX C_q = 24 and FAM C_q = 30	Both signals within range and ΔC_q = 6	The sample is **positive** for EGFR c.2369C>T.
HEX C_q = 24 and FAM C_q = 23	Both signals within range and ΔC_q = -1	
HEX C_q = 24 and FAM C_q = 32	Both signals within range and ΔC_q = 8	
HEX C_q = 29 and FAM C_q = 36	HEX signal within range. FAM signal > C_q 35. ΔC_q = 7	**Retest** sample to confirm low-level EGFR c.2369C>T mutation.
HEX C_q = 30 and FAM C_q = 37.5	HEX signal within range. FAM signal out of range.	The sample is EGFR c.2369C>T **negative**. FAM C_q > 37 suggests EGFR wild-type co-amplification.
HEX C_q = 24 and FAM C_q = 33	Both signals within range and ΔC_q = 9	The sample is **negative** for EGFR c.2369C>T (ΔC_q > 8).
HEX C_q = 29 and FAM C_q = N.A.	HEX signal within range. No FAM signal.	The sample is **negative** for EGFR c.2369C>T.
HEX C_q = 32.5 and FAM C_q = N.A.	HEX signal out of range. No FAM signal.	**Repeat** the test with more DNA.

10. Important Information

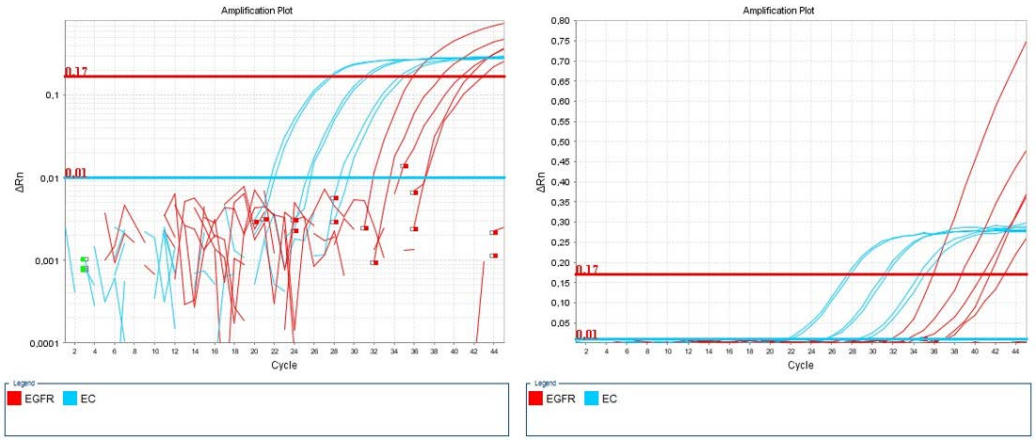
- The data analysis is valid for cfDNA testing only. The parameters cannot be used to evaluate DNA extracted from formalin fixed and paraffin embedded (FFPE) samples.
- C_q values < 22 for EC (HEX) or < 20 for EGFR (FAM) may result from excess DNA in the reaction or contaminating PCR products.
- Deviations from the protocol, e.g. variation of reaction volumes or modification of the PCR program, may affect automatic threshold setting and/or C_q values and thus lead to false-positive or false-negative results.
- The user is advised to visually inspect the software-generated curves and to verify the correctness of the automated threshold setting. However, manual adjustment of the thresholds and baselines must only be performed, if an intervention is necessary (see Correct Threshold Setting).

***Correct** Threshold Setting (logarithmic and linear view)*



The automatic threshold is set correctly in the exponential phase of the amplification curves.

***Wrong** Threshold Setting (logarithmic and linear view)*



The automatic threshold of the Control (blue line) is set too low. The threshold of EGFR (red line) is set too high.
→ Both thresholds must be individually placed in the exponential phase of the amplification curve by the user.

11. Warnings and Precautions

- For *in vitro* diagnostics use only.
- Always use disposable powder-free gloves and wear suitable lab coat when handling specimens and reagents.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use pipettes dedicated for PCR setup only, use aerosol-guarded pipette tips.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.
- Do not use expired kits or kit components.