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Guide about the technical procedure, interpretation and incidence of the driver mutations - protocol for DNA extraction from FFPE tissue and from liquid biopsies and EGFR genotyping

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Content

1. Introduction

Real-time PCR analysis is a highly sensitive method based on Taq-Man technology for the detection of somatic mutations in exons 18, 19, 20 and 21 of the EGFR gene in human DNA samples. This method amplifies the mutated sequences in the mutated samples, as well as wild-type DNA, using fluorescent probes for detection.

Each amplification reaction contains a set of primers for the detection of EGFR mutations as well as primers for endogenous control. The primers for the endogenous control amplify a gene to check the quality of the reagents used and whether the reaction contains enough DNA for amplification.

Mutations that can be detected by real time PCR

1. • **T790M**
2. • **48 deletions of the 19th exon (without the possibility of distinction between them)**
3. • **L858R**
4. • **L861Q**
5. • **S768I**
6. • **G719X (G719A, G719S, G719D and G719C) (without the possibility of distinction between them)**
7. • **insertions in the 20th exon: 2319-2320 insCAC and 2310-2311 insGGT (without the possibility of distinction between them)**
8. • **insertions in the 20th exon: 2307-2308 insGCCAGCGTG (ins9)**

Principle of the method: TaqMan technology uses a fluorescent labeled probe that attaches specifically to the area of mutated DNA. A probe is fluorescently labeled at the 5 'end and at the 3' end has a fluorescence inhibiting compound. At each amplification cycle the allele-specific probe is attached to the DNA strand and the generation of amplicons by sense (FW) and antisense (RW) primers causes the emission of the fluorescent signal by degrading the probe (hydrolysis) and removing the reporter fluorochrome from the quencher, in the presence of HotStart polymerase. Thus, at each amplification cycle, fluorescent molecules are released that determine the exponential intensification of the fluorescent signal. If the specific probe does not attach to the DNA, the probe will not be hydrolyzed, as it does not emit fluorescence.

2. Terminology

Genotyping is the process of determining the DNA sequence, called a **genotype**, at specific positions within the genome of an individual. Sequence variations can be used as markers in linkage and association studies to determine genes relevant to specific traits or disease.

Sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine.

NGS is a massive parallel **sequencing** reaction that generates hundreds of megabases to gigabases of nucleotide **sequence** reads in a single instrument run. This has enabled a drastic increase in available **sequence** data and fundamentally changed genome **sequencing** approaches in the biomedical sciences.

NGS library is a collection of similarly sized **DNA** fragments with known adapter sequences added to the 5' and 3' ends. A **library** corresponds to a single sample and multiple **libraries**, each with their own unique adapter sequences, can be pooled and sequenced in the same sequencing run.

Real-time polymerase chain reaction (**real-time PCR**) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules) (semi-quantitative real-time PCR).

3. Quality score (Q score)

Determination of DNA concentration and quality

To determine the concentration and purity of a solution containing DNA, the absorbance of an undiluted aliquot of 2 µl is measured at 260 nm and 280 nm in a spectrophotometer (**Eppendorf, 1mm Helma Lp cuvette**).

Concentration (ng / µl) = absorbance (260nm) x 50 x dilution factor

A double-stranded DNA sample with a concentration of 50 ng / µl at a wavelength of 260 nm, has a specific absorbance of 1 for an optical path length of 10 mm.

An $A_{260/280}$ ratio of pure nucleic acids is 1.8-2.0.

DNA samples extracted from formalin-fixed tissue and embedded in paraffin cannot be correctly quantified by UV spectrometry. The values calculated from the optical density measurement at 260 nm will substantially overestimate the actual concentration of DNA present. Only the use of appropriate fluorometric quantization platforms (eg Invitrogen Qubit) will allow reliable quantification of DNA from FFPE tissue.

QUALITY CONTROL

Internal control

The quality of the mutation analysis of the EGFR gene is ensured by:

• Carrying out the DNA extraction control by simultaneously processing a paraffin block sample with the test samples.

• A negative control (Nuclease free extraction / water control) and a positive control provided by the kit are used to prepare the amplification mixes for the detection of EGFR mutations.

• The amplification mix of the RT52-EGFR kit contains an endogenous control that allows checking the quality of the DNA used in the reaction.

• A thorough understanding of the described procedure, as well as precise laboratory equipment and techniques are required to obtain reliable results. Only properly trained personnel can use RT52-EGFR for in vitro human diagnosis.

• RT52-EGFR components are not used after the expiration date printed on the outside of the kit box. Do not mix reagents from different batches.

• Avoid microbial contamination and cross-contamination of reagents or samples by using sterile disposable pipette tips throughout the procedure. Do not change the lids between bottles.

POST-EXAMINATION PROCESSES

Checking the results

The results are verified throughout the analytical process by the person in charge of the analysis.

Checking patient results includes:

- analysis of positive, negative and without template internal control results
- browsing parameter by parameter in terms of their concordance and compliance with existing medical information both on the referral note and in the laboratory database.

Access to the database is limited due to the use of identification passwords

4. Mutations

The Test is designed to detect the following mutations

Exon	EGFR Mutation Group	EGFR Nucleic Acid Sequence	COSMIC ID6
Exon 18	G719X	2156G>C	6239
		2155G>A	6252
		2155G>T	6253
Exon 19	Ex19Del	2240_2251del12	6210
		2239_2247del9	6218
		2238_2255del18	6220
		2235_2249del15	6223
		2236_2250del15	6225
		2239_2253del15	6254
		2239_2256del18	6255
		2237_2254del18	12367
		2240_2254del15	12369
		2240_2257del18	12370
		2239_2248TTAAGAGAAG>C	12382
		2239_2251>C	12383
		2237_2255>T	12384
		2235_2255>AAT	12385
		2237_2252>T	12386

		2239_2258>CA	12387
		2239_2256>CAA	12403
		2237_2253>TTGCT	12416
		2238_2252>GCA	12419
		2238_2248>GC	12422
		2237_2251del15	12678
		2236_2253del18	12728
		2235_2248>AATTC	13550
		2235_2252>AAT	13551
		2235_2251>AATTC	13552
		2253_2276del24	13556
		2237_2257>TCT	18427
		2238_2252del15	23571
		2233_2247del15	26038
Exon 20	S768I	2303G>T	6241
	T790M	2369C>T	6240
	Ex20Ins	2307_2308ins9GCCAGCGTG	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
		2311_2312ins9GCGTGGACA	13428
		2309_2310AC>CCAGCGTGGAT	13558
Exon 21	L858R	2573T>G	6224
		2573_2574TG>GT	12429
	L861Q	2582T>A	6213

5. Variant allele frequency (VAF) and limit of detection (LOD)

Sensitivity of the method

Real-time PCR genotyping has a detection limit of up to 1% mutant alleles in a wild-type DNA background.

Mutation	Limit of detection
T790M	1%
Exon 19 deletions	1 %
L858R	1 %

L861Q	1 %
S768I	1.5 %
G719X	3.2 %
Exon 20 insertions	2 %

6. Technical protocol

DNA extraction from FFPE

DNA extraction is performed from the tissue selected from the area indicated by the pathologist.

Procedure for selecting the tissue of interest:

This procedure is performed in an area outside the molecular biology unit (without strong air currents), in order to eliminate the risk of contamination with any biological product of the PCR amplification zone.

Procedure:

In the case of section samples made on slides, prepare the work table as follows: clean the work surface with 70% alcohol before processing each sample. Prepare: HE blades with the area of interest surrounded, the blades from which the sample will be collected and a properly labeled 1.5 ml tube.

Disposable sterile utensils will be used to scrape the tissue of interest: scalpel blades. Pipette 1 ml of xylene into a 1.5 ml sterile Eppendorf tube. Take a little of this xylene and pipette it into the open lid of the tube.

Take the hematoxylin-eosin-colored tissue section slide and place under a non-stained tissue section slide. Through transparency, the area designated by the pathologist as a tumor will be observed on the blade with uncolored tissue. With a thin-tipped marker, the tumor area is surrounded on the blade with uncolored tissue.

Take a scalpel and soak it in the xylene from the lid of the Eppendorf tube. Gently scrape the designated area using a scalpel. From time to time the scraped material is collected and introduced into the Eppendorf tube. Repeat the procedure for another 3-5 sections of uncolored tissue.

When all the tumor material has been collected, carefully close the Eppendorf tube without spilling the xylene from the lid and shake manually.

In the case of section samples in 1.5ml or 2ml tubes, add 1ml xylene over the section / sections and shake manually.

• The tissue fixed in paraffin and suspended in xylene is incubated at 60°C, for 15 minutes, with stirring at 450 rpm on the thermoblock, then centrifuged for 3 minutes at room temperature, at 13,500 rpm; the supernatant is removed with a semi-automatic pipette of 20- 200 µl. Repeat the washing with xylene.

• Rehydration with alcohol: add 1 ml of absolute ethanol and vortex, centrifuge for 3 minutes at 13,500 rpm at room temperature. Remove the supernatant with a 20-200 µl semi-automatic pipette. Repeat washing with alcohol.

• With the lid open, allow the tubes to dry on the thermoblock for 10 minutes at 60° C or centrifuge for 10 minutes at room temperature in a vacuum centrifuge.

NOTE: *Each patient will be treated individually.*

NOTE: *Like all procedures for handling biological samples and extracting nucleic acids, the procedure will be performed by an* ***executor*** *and confirmed by a* ***witness****, who will sign on the* ***processing and extraction sheet*** *the conformity of the annotation of the tubes and their handling.*

ReliaPrep™ FFPE gDNA Miniprep System extraction protocol

The ReliaPrep™ FFPE gDNA Miniprep System kit uses optimized incubation conditions to partially break the cross-links formed during the process of formalin fixation and paraffin embedding of the tissue, without the need for overnight digestion. This system also includes a method of deparaffining that is not based on xylene or other volatile solvents.

ReliaPrep™ FFPE gDNA Miniprep System - 100 reactions

Kit components:

• 50ml Mineral Oil
• 20ml Lysis Buffer (LBA)
• 2 × 1.1ml Proteinase K (PK)
• 32.5ml BL Buffer
• 30ml Wash Solution
• 1ml RNase A
• 15ml Elution Buffer
• 2 packages Collection Tubes (50 tubes/pack)
• 2 packages ReliaPrep™ FFPE Binding Columns (50 columns/pack)

Storage conditions: at room temperature.

Preparation before use

Preparing 1X Wash Solution

Materials not supplied in the kit: 95–100% ethanol

Add 120 ml of 95-100% ethanol to the flask containing 30 ml of concentrated Wash Solution and label the flask.

Deparaffining using mineral oil

• Centrifuge the tube in which the sample was collected to collect the tissue and to avoid spreading it when opening the tube in the extraction area.
• Add 300 µl of mineral oil over the sample.
• Incubate at 80° C for 1 minute.
• Vortex for mixing.

Lysis of the sample

• Add 200µl of Lysis Buffer over the sample.
• Centrifuge at 10,000 × g for 15 seconds. Two phases will form, a lower (aqueous) phase and an upper (oily) phase.
• Add 20µl of Proteinase K directly to the lower phase; mix the lower phase by pipetting.
• Incubate at 56° C for one hour.
• Incubate at 80° C for one hour.
• Cool the sample for 5 minutes at room temperature. Centrifuge briefly to collect any droplets formed under the tube cap.

RNase treatment

• Add 10µl of RNase A directly over the sample lowered. Mix the lower phase by pipetting.

• Incubate at room temperature (20–25° C) for 5 minutes.

Nucleic acid binding

• Add 220µl of BL Buffer over the lysed sample.

• Add 240µl of ethanol (95–100%).

• Swirl briefly for mixing.

• Centrifuge 10,000 × g for 15 seconds. Two phases will form, a lower (aqueous) phase and an upper (oily) phase.

• For each sample, place a column in the collecting tube and number it accordingly.

• Fully transfer the lower (aqueous) phase of the sample, including any precipitate that has formed, into the column and collection tube assembly and close the column cap. Discard the remaining mineral oil.

Note: The mineral oil is inert and will not interfere with the extraction procedure if some of it is transferred to the column.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collecting tube and reinsert the column into the collecting tube.

• Immediately proceed with washing the column and eluting.

Column washing and elution

• Add 500µl of 1X Wash Solution (with added ethanol) over the column and close the column lid.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collector tube and reinsert the column into the same collector tube.

• Add 500µl of 1X Wash Solution (with added ethanol) over the column and close the column lid.

• Centrifuge at 10,000 × g for 30 seconds.

• Discard the liquid from the collecting tube and reinsert the column into the same collecting tube.

• Open the column cover and centrifuge the column/tube assembly at 16,000 × g for 3 minutes to dry the column.

• Transfer the column to a 1.5 ml microcentrifuge tube (not supplied), and discard the collecting tube.

• Add 30–50µl of Elution Buffer over the column and close the column cover.

• Centrifuge at 16,000 × g for 1 minute. Remove and discard the column.

• Close the lid of the microcentrifuge tube, and keep the eluted DNA at -20° C.

NucleoSpin FFPE DNA extraction protocol

The NucleoSpin FFPE DNA kit is designed to purify total DNA from paraffin-fixed samples (FFPE). After deparaffining, the tissue is subjected to the action of proteinase K, so the DNA is released into solution. The release of DNA from different bonds is done by incubation at a high temperature in the presence of the decross-link buffer and precipitation is done with absolute alcohol. The entire lysate is loaded onto a NucleoSpin FFPE DNA Column and the DNA is attached to the silicon membrane. After two washes, the salts, metabolites, macromolecular cellular components are removed and the DNA is eluted under conditions of low ionic strength. The DNA obtained, with dimensions of 100-300 bp, is suitable for PCR and other enzymatic reactions.

Kit components:

Paraffin Dissolver	125ml
Lysis Buffer FL	8 ml
Decrosslink Buffer D-Link	30 ml
Wash Buffer B5 (concentrate)	50ml
Proteinase K (lyophilized)	75 mg
Proteinase K Buffer PB	8ml
Elution Buffer BE (5 mM Tris/HCl, pH 8.5)	13ml
NucleoSpin DNA FFPE Columns (green ring) and collection tubes	250
Collection tubes 2ml	500
User manual	1

Preparation before use:

• Proteinase K: add the volume of Proteinase Buffer PB, 3.35ml for the 250-reaction kit, to dissolve the lyophilized Proteinase K. Proteinase K is stable at -20°C for 6 months.

• Wash Buffer B5: add the indicated volume of 96-100% ethanol, 200ml, over Buffer B5 Concentrate. Wash Buffer 5 is stored at room temperature (18-25°C) for up to 1 year.

Non-supplied kit materials: xylene, ethanol (96-100%), 1.5-2ml tubes for elution

DNA extraction from the FFPE sample

1. Add 100 µl lysis Buffer FL over the dewaxed tissue and homogenize by pipetting.
2. Add 10 µl of Proteinase K, vortex vigorously (5 seconds), short spin.
3. Incubate overnight at room temperature with stirring 450 rpm in the thermoblock.
4. Vortex, spin down, add 100 µl Decrosslink Buffer D-Link.
5. Vortex vigorously (5 seconds) and incubate the tubes at 90° C for 30 minutes.
6. Vortex the tubes (5 seconds), spin down and allow to cool to room temperature for 2 minutes.
7. Add 200 µl of 96% ethanol and vortex the tubes vigorously (2x5 seconds).
8. For each sample, prepare a NucleoSpin FFPE DNA Column (green color) with a 2ml collecting tube.
9. Load the entire sample into the column and centrifuge for 30 seconds at 2000 x g.
10. Discard the collector tube. The column is inserted into a new collecting tube.
11. Add 400 µl Buffer B5 and centrifuge for 30 seconds at 11000 x g.
12. Discard the collecting tube. The column is inserted into a new collecting tube.
13. Add 400 µl Buffer B5 and centrifuge for 2 minutes at 11000 x g.
14. Discard the collecting tube and place the column in a 1.5 ml Eppendorf tube.

15. Pipette 50 µl of Buffer BE into the center of the column membrane, leave to incubate for 1 minute.
16. Centrifuge for 30 seconds at 11000 x g.
17. Pipette 50 µl of Buffer BE into the center of the column membrane, incubate for 1 minute.
18. Centrifuge for 30 seconds at 11000 x g.
19. The column is thrown. The extracted DNA is now in the Eppendorf tube. Transfer the DNA to a 1.5 ml Eppendorf tube.
20. Quantify the extracted DNA. The DNA tube is labeled and stored at 4° C, -20° C or -80° C.

LIQUID BIOPSY (LB) DNA EXTRACTION COBAS cfDNA SAMPLE PREPARATION KIT

Note: Only K2 EDTA Plasma samples are to be used with the cobas® cfDNA Sample Preparation Kit. **Reagent preparation and storage**

Prepare working reagents as shown in the table below prior to using the kit for the first time. Use a 5-mL serological pipette to dispense the water. Use 25-mL serological pipettes to dispense the ethanol. If the Proteinase K has already been reconstituted and frozen, thaw a sufficient number of aliquots to process the number of specimens to be run.

Reagents	Reconstitution / Preparation
Proteinase K (**PK**)	Reconstitute **PK** by adding 4.5 mL of sterile water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Aliquot 1.1 mL of reconstituted **PK** into 1.5-mL microcentrifuge tubes and store at -20°C for up to 30 days or until the expiration date, whichever comes first. If the **PK** has already been reconstituted and frozen, thaw sufficient number of aliquots to process the number of specimens to be run (250 µL of reconstituted **PK** is required for each specimen).

Wash Buffer I (**WB I**)	Prepare working **WB I** by adding 15 mL of absolute ethanol to the bottle of **WB I**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II (**WB II**)	Prepare working **WB II** by adding 50 mL of absolute ethanol to the bottle of **WB II**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB II** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.

All solutions stored at 15 - 30°C should be clear. If precipitate is present in any reagent, warm the solution to 37°C until the precipitate dissolves. Do not use until all precipitate has been dissolved.

1. Label a 15-mL conical tube for each plasma sample and a negative control. Sterile water can serve as a negative control and can be processed the same way as samples.
2. Vortex plasma then transfer 2 mL of each plasma sample or negative control (sterile water) to a separate 15-mL tube.
Note: A minimum of 2 mL of plasma is required to process a sample with the cobas® cfDNA Sample Preparation Kit.
3. Add 250 µL **PK** to each tube.
4. Add 2 mL of **DNA PBB** to each tube.
5. Mix the specimen tubes containing **DNA PBB/PK** by inverting 3 to 5 times.
6. Incubate each tube at room temperature (15°C to 30°C) for 30 minutes.
Note: During the incubation, prepare the required number of HPEA FT by labeling each HPEA FT with proper identification on the cap of each HPEA FT.
Note: Each specimen will need one HPEA FT, three collection tubes (CT) and one elution tube (1.5-mL microcentrifuge tube).

Note: During the incubation, label the required number of elution tubes (1.5-mL microcentrifuge tubes) with specimen identification information.

7. Add 500 µL isopropanol and mix lysate by inverting 3 to 5 times.
8. Transfer all of the lysate into the appropriately labeled **HPEA FT**.
9. Using table top centrifuge, centrifuge **HPEA FT** at 4,000 x g for 5 minutes.
10. After centrifugation, remove the **HPEA FT** from the 50-mL conical collection tube. Place the **HPEA FT** onto a **CT**. Remove the larger locking clip by twisting and pulling it away from the assembly.
11. Remove the smaller locking clip from underneath the filter tube (**FT**) cap by pushing it up so that the seal is broken on both sides of the cap and then pulling it away from the assembly.
12. Remove the **HPEA** from the **FT** by tilting the extender away from the cap side of the FT.
13. Discard the flow-through from the **HPEA FT** into chemical waste and properly dispose of the unit.
14. Label the filter cap appropriately.
15. Add 500 µL working **WB I** to each **FT**.

Note: Preparation of working WB I is described in the table in the Reagent preparation section.

16. Use benchtop microcentrifuge for the rest of the protocol.
17. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
18. Place each **FT** onto a new **CT**. Discard the flow-through in each **CT** into chemical waste and properly dispose of old **CT**.
19. Add 500 µL working **WB II** to each **FT**.

Note: Preparation of working WB II is described in the table in the Reagent preparation section.

20. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
21. Place each **FT** onto a new **CT**. Discard the flow-through from the old CT into chemical waste and properly dispose of the old **CT**.
22. Centrifuge **FT/CT** units at 16,000 x g – 20,000 x g for 1 minute to dry the filter membrane.
23. Place the **FT** onto an elution tube (1.5-mL RNase/DNase-free microcentrifuge tube) pre-labeled with specimen identification information. Discard any flow-through in each **CT** into chemical waste and properly dispose of the old **CT**.

24. Add 100 µL **DNA EB** to the center of the **FT** membrane without touching the **FT** membrane.
25. Incubate **FT** with elution tube at room temperature (RT: 15°C to 30°C) for 5 minutes.
26. Centrifuge FT with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube (pre-labeled 1.5-mL RNase/DNase-free microcentrifuge tube). The eluate is the DNA stock. Vortex eluate prior to use.
27. Discard the FT. Close the caps on the elution tubes.
28. DNA stock is ready for PCR tests after vortexing. Store DNA stock according to instructions in the **Processed sample storage and stability** section.

Determination of DNA concentration and quality

To determine the concentration and purity of a solution containing DNA, the absorbance of an undiluted aliquot of 2 µl is measured at 260 nm and 280 nm in a spectrophotometer (**Eppendorf, 1mm Helma Lp cuvette**).

Concentration (ng / µl) = absorbance (260nm) x 50 x dilution factor

A double-stranded DNA sample with a concentration of 50 ng / µl at a wavelength of 260 nm has a specific absorbance of 1 for an optical path length of 10 mm.

An $_{A260/280}$ ratio of pure nucleic acids is 1.8-2.0.

However, amplifiable DNA samples extracted from formalin-fixed tissue and embedded in paraffin cannot be correctly quantified by UV spectrometry. The values calculated from the optical density measurement at 260 nm will substantially overestimate the actual concentration of DNA present. Only the use of appropriate fluorometric quantization platforms (eg Invitrogen Qubit) will allow reliable quantification of DNA in FFPE tissue.

In Vitro Amplification (real time-PCR) kit-Roche Diagnostics

All real-time PCR reagents are stored at -20°C and DNA at 4°C / -20°C.

For RT52-EGFR use the DNA concentration must be adjusted to 5-10ng / µl.

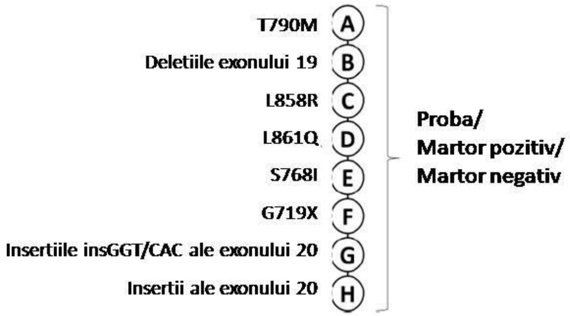
All steps until the start of the program in AB7500 are performed at 0-4°C (on ice or cold stand).

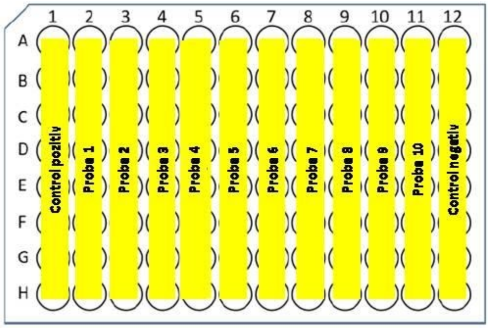
An ice PCR mix is prepared for each sample (calculated with a 5% surplus):

- 126µl Reaction Master Mix 2x
- 8.4 µl 10ng / µl DNA template
- 58.8 Nuclease-Free water.

The same mix is prepared for the positive control provided by the kit as well as for the negative control (Nuclease-Free water) to validate the amplification reaction.

The mixture is vortexed, centrifuged briefly and then distributed 23 µl per well in an A-H column for each sample.





Add 7 µl of each primer per sample mix, homogenize several times by pipetting.

After distributing the primers, the prepared plate is sealed with optical foil (in the case of strips it is covered with specific covers) and centrifuged briefly.

The amplifier board is inserted into the real time PCR AB7500.

Preparation of the amplification program:

1. To create the amplification program, create a new experiment: **Create a new experiment: File> New Experiment> Advanced Setup**

2. In the **Experiment Proprieties** section, name the amplification program, select the instrument type, **Quantitation-Comparative CT experiment**, **TaqMan® Reagents** and **Standard.**

3. Setting the parameters for amplification involves: defining the amplification targets (mutations) and the fluorescence channel on which the amplification is read: endogenous control is read on the VIC channel and mutations on the FAM, samples are defined, **Passive Refer Dye** is selected as "**None**".

Setting the amplification thermal conditions:

• denaturation: 95°C, 10 minutes,

• 40 cycles: 95°C for 15s, 58°C for 30s.

4. Select the reading on the FAM and VIC channels at the hybridization and extension step at 58°C.

7. Tumor type

Non–small-cell lung cancer (NSCLC) is a heterogeneous disease composed of unique molecular subsets with distinct clinical outcomes.

Multiple randomized studies have established the superiority of molecularly targeted therapies versus chemotherapy for the treatment of EGFR-mutant and ALK-positive NSCLC.

In other molecular subsets, single-arm studies confirm that treatment with targeted therapies can induce durable responses.

As drugs that target these molecular drivers are approved for first-line treatment, genotyping in newly diagnosed NSCLC is considered the standard of care.

Activating mutations in the gene encoding EGFR occur primarily in **NSCLC**, and result in constitutive activation of the kinase activity of the EGFR protein, thereby contributing to the oncogenic process.

The prevalence of these mutations in unselected cases of NSCLC is approximately 10% - 30%.

However, these mutations occur more frequently, but not exclusively, in non-smoking/light-smoking female patients of Asian ancestry with adenocarcinoma histology.

The most common EGFR mutations in NSCLC include a variety of deletions in exon 19 and the substitution mutation L858R in exon 21; these mutations collectively constitute approximately 85% of EGFR mutations observed in NSCLC.

The EGFR test is used as a companion diagnostic test for TKI, a compound that reversibly inhibits the kinase activity of EGFR, preventing autophosphorylation of tyrosine residues associated with the receptor and thereby inhibiting further downstream signaling that promotes cell survival and proliferation.

Erlotinib binding affinity for EGFR exon 19 deletion or exon 21 L858R mutations is higher than its affinity for the wild-type receptor.

Clinical trials have shown that patients with advanced NSCLC and with exon 19 deletion mutations or L858R substitution mutation in exon 21 that were treated with Tarceva as first-line treatment, are likely to experience clinical benefit compared to patients treated with chemotherapy.

Osimertinib, an irreversible inhibitor of both EGFR TKI-sensitizing and T790M resistance mutations in advanced NSCLC, inhibits the kinase activity of EGFR, which inhibits a cascade of intracellular downstream signaling events that promote cell proliferation, survival, and angiogenesis.

Clinical trials have shown that patients with advanced non-squamous NSCLC with an EGFR TKI-sensitizing mutation and have progressed following therapy with a first-generation EGFR TKI and who have developed a T790M resistance mutation in exon 20 that were treated with Osimertinib are likely to experience clinical benefit.

Iași Regional Institute of Oncology, as a beneficiary, launches the project "Network of excellence for diagnosis and research in lung cancer", acronym LUNGNEX-RD - Project funded under the ROMD Program 2014-2020, through the European Neighborhood Instrument (ENI) and co-financed by the project partners, based on the contract.

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“The European Union is made up of 28 Member States who have decided to gradually link together their knowhow, resources and destinies. Together, during a period of enlarge- ment of 50 years, they have built a zone of stability, democra- cy and sustainable development whilst maintaining cultural diversity, tolerance and individual freedoms. The European Union is committed to sharing its achievements and its values with countries and peoples beyond its borders”.