CLART® CMA
EGFR LB

LIQUID BIOPSY BASED TEST FOR DETECTION AND GENETIC IDENTIFICATION OF POINT MUTATIONS, INSERTIONS AND DELETIONS IN THE EGFR GENE PATHWAY ASSOCIATED TO NON SMALL CELL LUNG CANCER

FOR *IN VITRO* DIAGNOSIS

For more information, please refer to the web site: www.genomica.com

GENOMICA, S.A.U.
Parque Empresarial Alvento, Edificio B
Calle Vía de los Poblados, 1 – 1ª planta
28033 Madrid, Spain
www.genomica.com

Version 1
June 2016

TABLE OF CONTENTS:

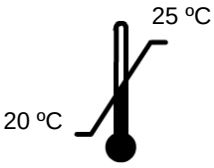
1. GLOSSARY

Attention, see instructions for use

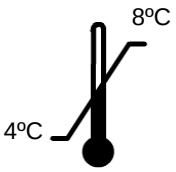
Expiration date

In vitro diagnostic medical device

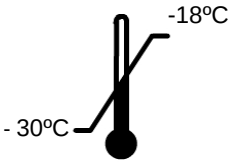
Lot



Store at room temperature



Store at 4ºC to 8ºC



Store at –30ºC to –18ºC

2. PROTOCOL DESCRIPTION

The mutational analysis of the tissue biopsy samples provides essential information for the diagnosis of the cancer patients as well as the response prediction for the possible treatments. For certain diseases, such as non-small-cell lung cancer, it is difficult to obtain tissue biopsy due to specific tumor localization and sometimes the patients are not healthy enough to undergo the invasive procedure.

The tumoral cells are shedding free circulating DNA into the bloodstream. Using a simple blood draw, the DNA shed both by healthy and tumoral cells can be extracted.

CLART® CMA EGFR detects in the plasma samples the presence of the most prevalent mutations in the Epidermal Growth Factor Receptor (EGFR) gene associated to non-small-cell lung cancer.

The study of the mutations in EGFR will allow to selects the most appropriate treatment of patients with non-small-cell lung cancer.

In the clinical environment, the EGFR mutational analysis done on the circulating tumoral DNA helps determine the appropriate treatment for the non-small-cell lung cancer patients. Furthermore it allows the monitoring of the appearance of the mutations associated with the drug resistance, such as the EGFR T790M resistance mutations in patients on tyrosine kinase inhibitor therapy (TKIs).

CLART® CMA EGFR kit detects a total of 39 high-prevalence mutations located in the 18, 19, 20 and 21 exons associated with sensitivity or resistance to the treatment (Table 1).

The mutations found in exon 20 provide resistance to treatment, while the rest of the mutations are activators and confer response to the treatment.

MUTATIONS DETECTED AND ITS PREVALENCE		
EXONs	**Types of mutations**	
EXON 18	**3 Point Mutations**	**Prevalence**
	G719A (c.2156 G>C)	0.3%-1.05%
	G719C (c.2155 G>T)	0.3%-1.05%
	G719S (c.2155 G>A)	0.3%-1.05%
EXON 19	**28 Deletions**	**Prevalence**
	6223* E746_A750del (c.2235_2249 del 15)	17.8%
	12370* L747_P753>S (c. 2240_2257del18)	3.4%
	12369* L747_T751del (c.2240_2254del15)	1.5%
	6255* L747_S752del (c. 2239_2256del18)	10.2%

	12384* E746_S752>V (c.2237_2255>T)	1.3%
	12382* E747_A750>P (c.2239_2248TTAAGAGAAG>C)	2.5%
	6225*, 12678*, 6218*, 12728*, 6220*, 12419*, 6210*, 13556*, 12386*, 12385*, 18427*, 12403*, 12383*, 6254*, 13551*, 12367*, 12422*, 12387*, 26038*, 13552*, 12416*, 23571*	< 1.5%
EXON 20	**1 Point Mutation**	**Prevalence**
	T790M (c.2369 C>T)	0.5-1.75%
	5 Insertions	**Prevalence**
	H773_V774insH (c.2319_2320insCAC)	0.4-3.22%
	D770_N771insG (c.2310_2311insGGT)	
	V769_D770insASV (c.2307_2308ins9GCCAGCGTG)	
	V769_D770insASV (c.2309_2310AC>CCAGCGTGGAT)	
	D770_N771insSVD (c.2311_2312ins9GCGTGGACA)	
EXON 21	**2 Point Mutations**	**Prevalence**
	L858R (c.2573 T>G)	4.3-15.05%
	L861Q (c.2582 T>A)	0.2-0.7%

Table 1: Mutations detected by ***CLART® CMA EGFR LB*** kit

SAMPLES: Plasma samples from the non-small-cell lung cancer patients.

After extraction of the circulating DNA, the mutational analysis is done in two steps:

1- Pre-amplification step

2- Amplification step, specific for the deletion and insertion mutations present in the samples obtained with pre-amplification. A fragment of variable size, between 100 and 200 base pairs is obtained in this step.

Finally, the detection of the product amplified by PCR is carried out by means of a low-density microarray platform: CLART® (Clinical Arrays Technology). The platform is based on a principle that is very simple, but at the same time economical and effective It consists in a microarray printed at the bottom of a microtiter plate well - CLART Strip® (CS), shown in Figure 1, which simplifies the entire hybridization and visualization process when compared to classic microarray systems.

Figure 1. **CLART-Strip®** (CS) *platform in the form of an 8-well strip.*

The ***CLART®CMA EGFR LB*** detection system is based on the precipitation of an insoluble product in those microarray areas in which hybridization of amplification products with specific probes takes place. During PCR, amplified products are labeled with biotin. After amplification, these products are hybridized with their respective specific complementary probes that are immobilized in specific and well-known microarray areas. Afterwards they are then incubated with a streptavidine-peroxidase conjugate. The conjugate is bound through streptavidine with the biotin present in the amplified products (which are bound to their specific probes) and the peroxidase activity prompts the appearance of a non-soluble product in the presence of the o-dianisidine substrate, which precipitates on the microarray areas where hybridization occurs (Figure 2).

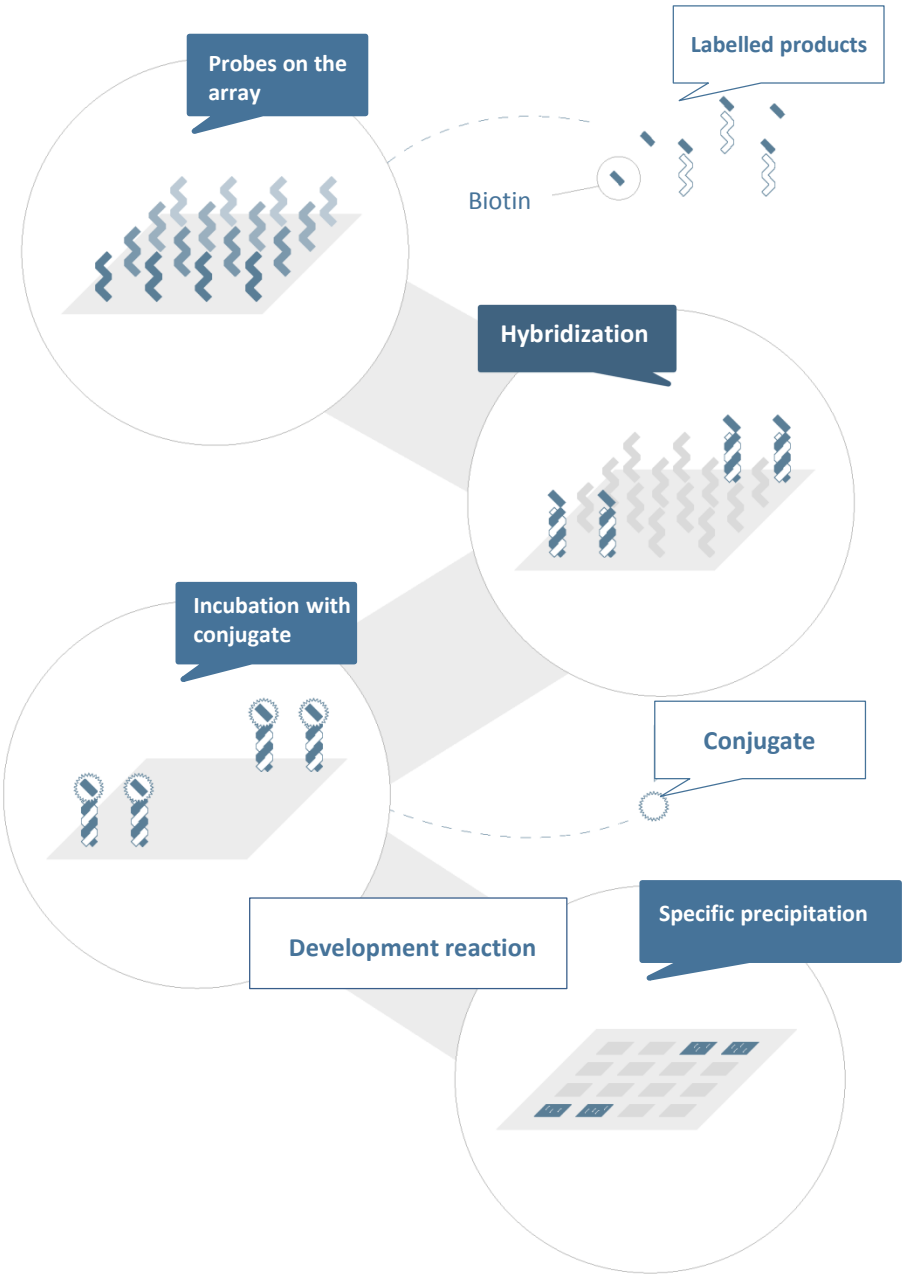


Figure 2: Diagram of the visualization method. Probes, immobilized on the surface, capture their complementary biotin-labelled amplified products. With the help of biotin, they bind to the conjugate, in this case streptavidine-HRP (***H****orseradish* ***P****eroxidase*). The o-dianisidine substrate, by the action of the HRP, produces a precipitate on the area where hybridization occurs.

3.-KIT COMPONENTS AND STORAGE

The ***CLART® CMA EGFR LB*** kit contains enough reagents for the analysis of 8 or 24 clinical samples. The reagents included in the kit have been grouped into various packages, depending on the temperature at which they should be stored. When storage recommendations are observed, all reagents should remain stable until kit's expiration date.

3.1. Amplification reagents

They are shipped and should be stored at -20ºC.

1- Ready-to-use **pre-amplification tubes**. Only thaw on ice the exact number of amplification tubes that will be used and keep the rest at -20ºC. For the EGFR gen analysis using ***CLART® CMA EGFR LB*** kit, 2 pre-amplification tubes are provided:

- ✓ **Yellow tube (Pre-amp Mix):** pre-amplification mix, contains 30 µl reaction mixture.
- ✓ **Yellow tube marked on the lid (CI Mix):** tube containing the internal controls, contains 45 µl reaction mixture.

2- Ready-to-use mutation-specific **amplification tubes**. They contain 45 µL of reaction mixture. Only thaw on ice the exact number of amplification tubes that will be used and keep the rest at -20ºC. For the EGFR gen analysis using ***CLART® CMA EGFR LB*** kit, 4 amplification tubes are provided:

- ✓ **Mix 1: White tube**
- ✓ **Mix 2: White tube marked on the lid**
- ✓ **Mix 3: Red tube**
- ✓ **Mix 4: Blue tube**

Mix 1 (White tube) and Mix 2 (White tube with a black mark on the lid) detect :

- EXON 21: L858R and L861Q
- EXON 19: DELETIONS
- EXON 18: G719C, G719A, G719S

Mix 3 (Red tube) and Mix4 (blue tube) detect:

- EXON 20: T790M and Insertions

Note: **The kit package includes a self-adhesive and irreversible temperature indicator; the appearance of a reddish color on the visualization window indicates that, at a certain moment, products have exceeded the storage temperature of –20°C and they should not be used.**

3.2. Visualization reagents

Visualization components are divided into two groups, according to optimal shipping and storage temperatures:

- Shipped and stored at room temperature:

  - **CLART-Strip® (CS)**, each well including all specific probes.

Note: Required **CS** units are shipped in a sealed pouch. Each unit should be kept until use, in the unopened pouch, at room temperature (i.e. 25ºC maximum) and protected from direct light and high temperatures.

- **SH** (Hybridization Solution).
- **Microtiter plate adaptor and plastic lid.**

- Shipped and stored at 4ºC:

  - **DC** (Conjugate Diluent).
  - **CJ** (Conjugate Solution).
  - **RE** (Development Solution).
  - **TL** (Wash Buffer).

3.3 Other components

For the capture and subsequent processing of the image, a reader unit, running a tailor made software and, the plate adaptor, are required:

- **CAR®** (CLINICAL ARRAY READER): it allows for the automatic reading and interpretation of up to 12 CS, i.e., up to a maximum of 96 samples. It is distributed by GENOMICA, to be used exclusively with its diagnostic kits.
- **SAICLART®:** software developed by GENOMICA for image processing.
- **Software**: It is specific for ***CLART® CMA EGFR LB*** designed and validated by GENOMICA. Installed and ready to use.

Figure 3: CAR® (CLINICAL ARRAY READER)

4.-MATERIALS REQUIRED BUT NOT PROVIDED

Below you can find a list of all materials required but not provided.

4.1. Reagents and materials.

- Distilled water.
- Disposable gloves.
- Filter tips or positive displacement pipettes.
- Crushed ice container.
- 1.5 mL autoclaved Eppendorf tubes.
- 1.5 mL tube grids.
- 0.5 mL/0.2 mL tube holder.
- Bleach
- QIAamp Circulating Nucleic Acid kit (QIAGEN)
- PBS (Phosphate buffer solution)
- *Qubit dsDNA HS Assay* kit

4.2. Equipment.

- Centrifuge with refrigeration (4ºC) and capacity to reach 16.000g
- Fluorometer
- Standard thermal cycler
- Laminar flow chamber for the extraction laboratory.
- Vacuum pump capable of producing a vacuum of –800 to –900 mbar, necessary for the DNA extraction from plasma.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the extraction laboratory.
- One adjustable micropipette ranging from 1-20 µL, to add the genetic material to the amplification tubes.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the visualization laboratory.
- Thermoblock (Thermomixer) compatible with 96-well plates and adjustable shaking at 20°C, 25°C and 50ºC.
- Vortex.
- Vacuum system (desirable).
- QIAvac 24 Plus
- Water bath or heating block capable of holding 50 ml centrifuge tubes
- Microcentrifuge.

5.-RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully before starting the assay in order to avoid contamination!

5.1. General recommendations

1. This assay should be performed in two physically separated areas, in order to avoid sample contamination with the previously amplified product. Separate working materials should be available in each area (pipettes, tips, tubes, grids, gloves, etc.) which should never be used outside these areas.

1. Pre-PCR area: DNA extraction and sample preparation are performed in this area. Sample manipulation must be carried out within a biosafety cabinet (BSC). The samples must be manipulated maintaining all the possible sterile measures in order to avoid contaminations. After DNA has been extracted from plasma, add it to the pre-amplification tube in the pre-PCR area.

2. Post-PCR area: Pre-amplification step, amplification mutation-specific and visualization of the amplified product are carried out in this area. The material of this area should never come into contact with the material of the extraction area. Avoid entering the pre-PCR area after having worked in the visualization area.

2. Always use gloves. It is recommended to change gloves quite frequently, and it is mandatory to change gloves before start working in each of the aforementioned areas. New gloves must always be used when DNA is added to the amplification tubes.

3. Clean working areas (laboratory cabinets, hoods, grids, pipettes) thoroughly with a 10% diluted bleach solution **after every sample batch processing;** it is mandatory to disinfect all working areas in case of contamination. For thermocyclers and thermomixers, it is advised to clean them before and after used, in these same conditions.

4. Always use filter tips and positive displacement pipettes to avoid contamination due to micropipettes. Different sets of pipettes should be used in each area.

5. Use disposable and autoclaved laboratory material.

6. Never mix reagents from two different vials, even if they belong to the same lot.

7. Close reagent tubes immediately after use in order to avoid contamination.

8. Discard the micropipette tip after pipetting.

5.2. Precautions for the extraction and addition of extracted material to the amplification tube

1. Always wear gloves.
2. Clean working surfaces of cabinets with a 10% diluted bleach solution.
3. Turn on the laminar flow and UV light at least 20 minutes before extraction. Turn off the UV light when it is working inside the cabinet.
4. The preparation of the samples before extraction must be made inside the cabinet.

5.3 Precautions for amplification

Place the amplification tubes in the thermocycler when the block has reached 90°C. Unspecific amplification will be minimized due to the incubation temperature being below the hybridization temperature.

The use of conventional thermocycler is recommended. Before use, validate the temperature ramps. The values should be in-between the limits listed in the tables below.

Pre-amplification:

Temperature (ºC)	Time (Seg)
95°C → 94°C	**0.1**
94°C → 66°C	**23.7**
66°C → 94°C	**27.8**
66°C → 72°C	**10.8**

Amplification:

Temperature (ºC)	Time (Seg)
95°C → 94°C	**0**
94°C → 62°C	**26.8**
62°C → 94°C	**30.3**
62°C → 72°C	**14.7**

5.4 <u>Precautions for visualization.</u>

1. Before starting the assay is recommended to verify the THERMOMIXER measuring the temperatures which will be used during the assay: 20°C, 25°C and 50ºC. For it using a thermocouple in direct contact to themomixer plate.

2. The amplification product must be denatured only one time. Don´t use for visualization a PCR product which has been denatured more than one time. If you have this necessity you must do aliquots previously to denaturalized step.

3. Avoid the pipette tip or the vacuum system touching the bottom of the well, since this could damage the probes printed at the well's bottom.

4. It is recommended to add all solutions to the wall of the CS well; never directly at the bottom.

5. At room temperature the SH solution (hybridization solution) forms crystals, so is need before using pre-warm up at 50ºC until becomes homogeneous. Not to add the SH solution until the denatured products of PCR are ready, therefore SH solution must be maintained at 50ºC until it is going to be added.

6. The array must not remain dry.

7. Following incubation with the CJ solution, it is very important to wash the microarray thoroughly in order to avoid any residues that could react with the RE solution, resulting in a non-specific precipitation that could lead to false interpretations of the result.

8. Avoid foaming when adding any reagent.

9. When visualizing the image in the reader, ensure that position markers appear and that there are no bubbles, fibers or spots interfering with the reading. Otherwise, clean the outer face of the well with cellulose paper.

6.-<u>SAMPLES</u>

CLART® CMA EGFR LB kit was designed and validated for the use with circulating tumoral DNA obtained from the plasma of non-small-cell lung cancer patients.
GENOMICA cannot be hold responsible for the results obtained using other type of samples.

7.-WORKING PROTOCOL

CLART® CMA EGFR LB was validated using the protocols described below for the blood sample processing and extraction of the free circulating DNA.

7.1. Plasma separation from whole blood sample

7.1.1. Obtain 5-10ml of blood from the patient and place the whole blood in the tubes containing EDTA as anti-coagulant. It is important to obtain the recommended volume of the blood samples as at least 2ml of plasma is required for the analysis.

7.1.2. Immediately after blood is recollected into the tube, invert the tube gently 10 times.

7.1.3. Collect the patient information and mark the tubes with identifying code. Mark the day and time when blood extraction was performed.

7.1.4. **The blood collection tubes must be stored at 4ºC after extraction.** The plasma separation must be done right away, performing two centrifugation steps without pausing in between.

7.1.5. Centrifuge blood samples for 10 min at 1900g (3000rpm) and 4ºC temperature setting.

7.1.6. Carefully aspirate plasma supernatant without disturbing the buffy coat layer, leaving approximately 5mm of plasma above this layer to avoid the contamination of plasma by cells. Transfer the aspirated plasma into the fresh 15ml centrifuge tubes with conical bottom, previously marked with patients identifying codes.

7.1.7. Centrifuge plasma samples for 10 min at 16 000g (in fixed-angle rotor) and 4ºC temperature setting.

7.1.8. Carefully aspirate supernatant to a new tube with a pipette, leaving approximately 7mm of plasma to avoid disturbing the pellet. Transfer the plasma into the 2ml vials, previously marked. For each 10ml of whole blood samples approximately 2 vials of 2ml of plasma are obtained. Mark the final volume of plasma obtained.

7.1.9. If plasma will be used for nucleic acid extraction on the same day, store at 4°C until further processing. Keep plasma frozen at –80°C if it will not be processed immediately. Frozen plasma can only be thawed once and for that reason storing 2ml aliquots is recommended.

7.2. Purification of circulating DNA from plasma

In order to purify the circuating DNA from plasma use of QIAamp Circulating Nucleic Acid Kit from QIAgen is recomended. Kit must be used according to manufacturer's instructions. Final elution volume must be 30 µl.

The QIAamp Circulating Nucleic Acid procedure takes approximately 2 hours and it comprises 4 steps:

- sample lysing

- binding to the column
- washing
- elution

The "QIAamp Mini" columns, included in the kit, are connected to the vacuum manifold, avoiding the successive centrifugation steps. QIAamp Mini columns can bind fragmented nucleic acids that are as short as 20 bases, however yield depends on the sample volume and the concentration of circulating nucleic acids in the simple.

The following steps are recommended:

7.2.1. Even though the starting volume of plasma can be up to 5ml, use only 2ml of plasma per column. Follow carefully the kit manufacturer's protocol for purification of 2ml of plasma.

7.2.2. Thaw 2ml of plasma at the room temperature and once thawed keep it at 4ºC until processing. Purification of circulating DNA must be done immediately after thawing, since the circulating DNA half-life is very short. In case that plasma volume is somewhat less than 2ml, add PBS up to 2ml. Do not use less than 2ml of plasma, because the sensitivity parameters of ***CLART® CMA EGFR LB*** might not be maintained.

7.2.3. If QIAvac 24 Plus is used, up to 24 columns can be processed in parallel.

7.2.4. After finishing the purification process, elute the circulating DNA using 30µl of AVE buffer (included in the QIAmp kit). DNA should be recollected in the 1,5ml tube, previously marked.

After elution the DNA concentration should be determined. If the quantification will be done the same day maintain the simple at 4ºC, if it will be done in the following days, store the simple at -20ºC.

7.3. DNA quantification and sample addition

7.3.1 DNA quantification

It is recommended to use Qubit® 3.0 Fluorometer for quantifying circulating DNA concentration. Use the instrument according to the manufacturer's instructions (ThermoFisher Scientific). Use 2 µl of the DNA to perform the quantification with Qubit® dsDNA HS Assay Kit. DNA samples should be store at -20ºC once the quantification is done. Do not thaw circulating DNA more than once.

In case Qubit® 3.0 Fluorometer is not available, use a similar quantifying method that permits detecting concentrations less than 0.5ng/µl. Do not use methods based on absorbance measurement, since they might not detect the low concentrations of double strand DNA present in this type of samples.

Obtained DNA concentration should be in the 0.05 ng/µl - 5 ng/µl range for 95% of the patients. For the patients with low proportion of circulating tumoral DNA, mutations might not be detected.

If obtained DNA concentration is below 0.05ng/µl, it is strongly recommended to repeat the purification whenever it is possible, in order to assure the results reliability.

7.3.2 Addition of circulating tumoral DNA

The next step, after circulating DNA quantification is addition of DNA into the pre-amplification tube (yellow tube). 10 ng of DNA has to be addd into the pre-amplification tube. Adding too much or not enough DNA can result in incorrect diagnosis. Below is described how to proceed:

- If the obtained DNA concentration is between 0.05 ng/µl and 0.5 ng/µl add 20 µl of DNA into the pre-amplification tube.
- If the obtained DNA concentration is higher than 0.5 ng/µl, dilute the sample to the concentration of 0.5 ng/µl by adding to the ore-amplification tubesthe volumes indicated in the Table 7.1.

Initial concentration of DNA (ng/µl)	Preparing DNA dilution at 0.5 ng/µl		ng of DNA per amplification tube
	DNA volume (µl)	H_2O volume (µl)	
0.5	20	-	10
0.6	20	4	10
0.7	20	8	10
0.8	15	9	10
0.9	15	12	10
1	15	15	10
2	10	30	10
3	5	25	10
4	5	35	10
5	5	45	10

Table 7.1. Sample and water volumes required for adding the 10ng of sample to the pre-amplification tube.

- If an assay is performed using concentration lower then required, the result can be considered valid only if it is positive.

It is important to include a negative control in every run, to verify that the samples have not been contaminated during the extraction, amplification and visualization processes, which might lead to a false positive result.

7.4. Pre-Amplification reaction

Pre-Amplification specific recommendations:

- Work in the **pre-PCR area**, always using a cabinet and following the recommendations mentioned in section 5.1.

- During the process, keep tubes separate and refrigerated.
- **Do not manipulate more than one pre-amplification tube open at the saame time.**

Pre-amplification reaction:

1. Thaw at 4ºC on pre-amplification tube per sample (yellow tube) and one control tube per two samples (yellow tube with marked lid).

2. Centrifuge the amplification tubes for a few seconds, so that all liquid can get to the bottom of the tubes (in case you don't have microcentrifuge adaptors available for the tubes, you can use larger tubes after having cut their cap off).

3. Add 20 µl of the sample to the pre-amplification tube (see paragraph 7.4) and resuspend several times with micropipet. Keep the tubes on ice at all times.

4. Keep the control tube (yellow tube with the marked lid) on ice without opening. The control tube is placed into the thermocycler without adding any sample in it.

5. Place in the thermocycler n number of tubes for pre-amplification (one tube per samples) and n/2 number of control tubes.

6. Program the thermocycler according to the following temperature cycles:

1 cycle	95°C 15 min
40 cycles	94°C 1min 66°C 1min
1 cycle	72°C 10 min
4°C continual until tubes are collected	

Start the program and place the tubes in the thermocycler when the block has exceeded 90ºC.

7.5. Mutation-specific amplification

Amplification-specific recommendations:

- Work in the **pre-PCR area**, always using a cabinet and following the recommendations mentioned in section 5.1.
- During the process, keep tubes separate and refrigerated.
- Do not manipulate more than one open amplification tube coresponding to different patients.

Mutation-specific amplification reaction

1. Once the pre-amplification reaction has finished, take the tubes out from the thermocycler and vortex for few seconds. Centrifuge the tubes in the microcentrifuge.

2. Add 2µL of the pre-amplified PCR product to each amplification tube. Mutation-specific amplification tubes are white, white with marked lid, red and blue.

3. Repeat for each sample that is being tested.

4. Program the thermocycler according to the following temperature cycles:

1 cycle	95°C 15 min
20 cycles	94°C 15 sec 62°C 60 sec
1 cycle	72°C 10 min
4°C continual until tubes are collected	

5. Start the program and place the tubes in the thermocycler when the block has exceeded 90ºC.

The amplified product must be visualized **within a maximum of 5 days** to avoid its degradation, and kept at 4ºC.

7.6. Visualization of the product amplified in CLART-Strip® (CS)

Specific recommendations before starting visualization:

THE PROTOCOL DESCRIBED BELOW SHOULD ALWAYS BE USED IN THE **POST-PCR AREA**. DO NOT TAKE THE AMPLIFIED PRODUCT IN THE PRE-PCR AREA.

1. Turn on the **CAR®** (CLINICAL ARRAY READER) before starting the whole procedure. The self-calibration of the equipment takes a few minutes, and it is also necessary to introduce the name of the sample in the program before the reading. The device must be ready at the moment of reading to avoid unnecessary waiting that would produce an excessive exposure to developer.

2. Make sure that, before the hybridization begins, the thermomixer temperature has been 50ºC for at least 60 minutes.

3. At room temperature the SH solution (hybridization solution) forms crystals, so is need before using pre-warm up at 50ºC until becomes homogeneous and it must be maintained at 50ºC until it is going to be added.

4. PREPARE THE WASH SOLUTION BEFORE EACH ASSAY; DO NOT REUSE PREVIOUSLY PREPARED SOLUTIONS OR RESIDUES.

5. Clean the thermocycler with a 10% diluted bleach solution before starting the denaturation program. Place the amplification tubes in the thermocycler during the process that should never exceed 10 min.

6. During the visualization, it is not necessary to use filtered tips, but it is necessary to use a different tip for each well and change it every time a reagent is added, even if it is TL. It is necessary, though, to use filtered tips during the addition of amplified products to the CS well.
7. In case of using vacuum pumps equipped with 8-tip comb for aspirating solutions, discard the combs after each use or decontaminate them with a 10% diluted bleach solution after every assay. Make sure the pump aspirates properly and does not leave traces at the bottom of the well.

8. Aspirate the different solutions completely without touching the array.

VISUALIZATION:

1. Denaturation: Use the thermocycler to denature the PCR products. For this step, place the amplification tubes in the thermocycler and incubate at 95ºC for 8 min. Remove the tubes from the 95ºC incubation and place them immediately in a container at 4ºC. **It is advised not to exceed 10 min time of denaturation.**

2. Diluted TL solution preparation: For each CS strip (a total of 8 wells); prepare 10 mL of diluted wash solution by adding 1 mL of TL solution to 9 mL of distilled water.

3. Prewash of the CS: Before beginning the assay, it is necessary to wash the strips by adding 200 µL of diluted TL solution to each well. Mix it with the multichannel pipette 10 to 15 times, taking into account that the surface of the array must not be touched. **It is advised to carry out this wash while the amplified samples are being denatured and maintain the wash solution in the well until samples are going to be added.** Discard the diluted TL solution with a pipette, or preferably with a vacuum pump. **The array must be free from solution residues,** although it must never remain dry. Add the next solution immediately.

4. Hybridization:

   Before using the SH solution, it must be heated at 50ºC until the complete dilution of the salts. Once the PCR products have been denatured, add 100 µL of SH solution (prevent foaming) to each CS well. Next, add the denatured PCR product from each mix to **the same array well according to the following volumes:**

   Mix 1: 5 µl
   Mix 2: 5 µl
   Mix 3: 5 µl
   Mix 4: 5 µl

   Use one array per sample/patient. Mix it several times, being careful not to touch the bottom of the well. It is recommended to load each strip independently and separately from the rest to avoid contaminations. Cover the microtiter plate with the plastic lid provided and incubate

in the thermomixer for **1 hour at 50º C shaking at 550 rpm** (previously this thermomixer has to be pre-warm at 50ºC at least for 60 minutes and make sure that the thermomixer reaches 50ºC correctly previously to start the assay, see point 5.3**).** F**or the correct interpretation of the results, it is mandatory to visualize all the tubes of the same sample in the same well, even if they are different genes.**

After this incubation, remove the plate from thermomixer and aspirate the SH solution of the CS with a pipette or, preferably, with a vacuum pump: **The array must be free from solution residues**, although it must never remain dry. Add the next solution immediately. After incubation set the thermomixer at 20ºC, and in motion, so it may be used later in step 6.

5. Double Wash: Add 200 µL of diluted TL solution to each CS well, resuspend 10 to 15 times with the multichannel pipette. Discard the diluted TL solution with a pipette, or preferably with a multichannel vacuum pump without leaving any residues. **Repeat the procedure. This step must be carried out with different tips for each well in both washes.** If having arrived at this step, the thermomixer has not reached 20ºC; the wells are left with TL solution until the thermomixer reaches the temperature.

6. Blocking and conjugate: It is recommended to centrifuge the high-affinity CJ solution for 10 seconds before use. Then, prepare the diluted CJ solution as follows: for each CS strip, mix **1 mL of DC solution** and **15 µL of high-affinity CJ solution**. Prepare this solution at least 5 minutes before ending the hybridization step.

   Discard the diluted TL Solution without leaving any residues of the solution and add 100 µL of diluted CJ solution to each CS well. Incubate for **30 exact minutes in the thermomixer at 20ºC, shaking at 550 rpm.** After this incubation, remove the plate and discard the solution rapidly with a pipette or a multichannel vacuum pump. Once finished the incubation, set the thermomixer at 25ºC, and in motion, so it may be used later in step 8.

7. Triple Wash: **Add immediately** 200 µL of diluted TL solution to each CS well, mixing it 10 to 15 times with the multichannel pipette and discard the solution completely with the pipette or vacuum pump. **Repeat the procedure two more times.**

   It is **very important** to avoid any residues of the CJ solution, since they could react with the RE Solution generating an unspecified signal.

8. Development with RE solution: Remove the diluted TL solution completely and add 100 µL of RE solution to each CS well and incubate for **10 minutes at 25 º C** in the thermomixer **without shaking** (previously make sure that the thermomixer reaches 25ºC**).**

   Warning! It is very important to use the thermomixer without shaking.

9. Discard the complete TL solution using a pipette or a vacuum system. The array must remain dry for the reading.

10. CAR® (CLINICAL ARRAY READER): place the plate normally on the tray and the CAR® will take and analyze the arrays automatically.

8. READING OF THE RESULTS

The processing of data obtained from each analysis is carried out automatically. The reading and analysis system (CAR®) will provide a report indicating the results.

9. INTERPRETATION OF THE RESULTS

Each gene has its own extraction control to make sure that there is enough genomic material to carry out the test.

For the correct interpretation of the results, the sample must be processed with all the amplification tubes corresponding to each gene to be analyzed and visualized, placed in the same array.

As described in point 7.1., a negative control of extraction must be included to verify that the samples have not suffered contaminations during the processes of extraction, amplification and visualization, which would give rise to a false positive result.

The control of extraction of genomic DNA is necessary for the confirmation of a true negative result, as it informs us of the presence of the patient's DNA in the sample, although there has been no amplification of any mutation.

The internal control of amplification it will allow us to distinguish between cases of PCR reaction inhibition and those in which no DNA was found in the sample.

There is a possibility that gives rise to an "**NO DNA"**: result:

- Non-valid extraction: The presence of inhibitors or a mechanic failure in the extraction of the sample does not allow the amplification of mutations and/or of the controls of amplification and extraction; to solve this problem the entire process must be repeated.

There is a possibility that gives rise to a "**PCR INHIBITED"** result:

- Non-valid amplification: The absence of amplification in one of the tubes, and the presence of amplification in other tubes will indicate that a correct extraction has been carried out, but that there has been a failure in the amplification of one of the tubes; to solve this, the corresponding tubes must be amplified again, before continuing with the process.

There are three possibilities that give rise to an **UNCERTAIN"**:

- In cases in which the readings of absorbance of the replicate probes of an array are very different from each other.

- In cases in which more than 3 mutations are positive due to failure in PCR tube. ; To solve this, the corresponding tubes must be amplified again, before continuing with the process.
- In cases in which the readings of absorbance of the L858R and T790M mutations are in the detection limit. The entire process should be repeated from a new sample.

There is a possibility that gives rise to a "**NOT VALID"** result:

- In cases where the software detects a low signal of the development reaction, the visualization phase should be repeated.

10. TECHNICAL AND OPERATIONAL SPECIFICATIONS

10.1 Control of known interferences

False negatives are one of the drawbacks in the detection by genomic amplification due to either, an inadequate quality of the extracted DNA (due to insufficient sample quantity, DNA degradation, inadequate storage or DNA loss during extraction) or to the presence of DNA polymerase inhibitors in the samples that are to be processed (alcohol, salts, etc.). To avoid such interference, please follow the instructions in sections 5, 6 and 7 of this manual.

10.2 Technical specifications

Processing parameters:

Analytical sensitivity

- Analytical sensitivity has been determined by the amplification of serial dilutions of recombinant plasmids for each one of the mutations detected by the kit (Table 6). Sensitivity has also been determined by means of the amplification of serial dilutions of the DNA extracted from cell lines, fragmented to approximately 160 base pairs and containing the mutations in different percentage: 5%, 3% and 2% (Table 7). The visualization was done in CS, giving rise to the following results:

Analytical sensitivity: PLASMIDS				
MUTATION	**Cop/5 ul**	**SENSIBILITY (%)**	**REPRODUCIBILITY (%)**	**REPEATABILITY (%)**
L858R	10 e1	100	100	100
DEL 19	10 e3 - 10 e1	100	100	100
T790M	10 e2	100	100	100
L861Q	10 e2	100	100	100
G719A	10 e2	100	100	100
G719C	10 e1	100	100	100

G719S	10 e3	100	100	100
INS. 20	10 e5 - 10 e2	100	100	100

Table 6. Relation of the number of copies of recombinant plasmid necessary to obtain a sensitivity of 100% in the detection of each one of mutations.

Analytical sensitivity: CELL LINES				
MUTACIÓN	**% TUMORAL**	**ng**	**SENSIBILITY (%)**	**REPEATABILITY (%)**
L858R	**5%**	2ng	100	100
	3%	2ng	100	100
	2%	5ng	100	100
DEL19	**5%**	2ng	100	100
	3%	5ng	100	100
	2%	10ng	100	100
T790M	**5%**	10ng	100	100
	3%	10ng	100	100
	2%	10ng	100	100

Table 7. Relation of nanograms of cell line with different tumoral percentage (5%, 3%, 2%) necessary to obtain a sensitivity of 100% in the detection of each one of mutations.

Analytical specificity

Specificity experiments were carried out with recombinant plasmids and cell lines, observing that an unspecific detection of other mutations different to what is sought to be determined is not produced. Therefore, it is considered that the technique reaches an analytical specificity of 100 %.

Diagnostic utility parameters

In order to determine the diagnostic parameters of the kit, a comparative assessment of the ***CLART® CMA EGFR LB*** kit was carried against the reference technique:

- Amplicon libraries from the EGFR exons 18, 19, 20 and 21 were generated using the ThermoFisher Scientific designed panels. The samples were loaded on the 316™ chip (ThermoFisher Scientific) and sequenced with Ion Personal Genome Machine (**Ion PGM™ System** from ThermoFisher Scientific) using the Ion Torrent technology. The average depth of 15.000 reads was chosen to assure the result robustness. For this technique, addition of 10ng of circulating DNA is recommended. For those samples where 10ng were not obtained by DNA purification, 6 µl were used, being the maximum volume permitted by the technique.

- A part of the samples were analyzed using digital PCR (dPCR) utilizing QuantStudio™ 3D Digital PCR System 20K (ThermoFisher Scientific). Chip kit V2 (ThermoFisher Scientific) was employed.

- The tissue biopsy samples were analyzed with Cobas® EGFR Mutation Test v2 kit (Roche), Therascreen® EGFR RGQ PCR Kit (Quiagen) y CLART® CMA EGFR kit (GENOMICA).

CLART® CMA EGFR LB sensitivity was established in the way that the proportion of the mutated DNA strands has to be above 2-3% of the total. Below this level the sensitivity of the kit is decreased, being capable to detect the mutations (positive samples) but not in the 100% of cases.

Total of 53 samples were analyzed, thanks to collaboration with following centers:

- Department of Pathological Anatomy of the Vall de Hebrón University Hospital, Barcelona, Spain.
- Department of Oncology, Hospital Clínico San Carlos, Madrid, Spain.
- Anatomy Pathology Service of the 12 de Octubre University Hospital, de Madrid, Spain.
- Department of Oncology of the University Hospital of Santiago, Santiago de Compostela, Spain.
- Department of Oncology of the Hospital Puerta de Hierro, Madrid, Spain.

53 samples were analyzed. The results are in the table 8:

N: 53	Sensibility (%)	Specificity(%)	PPV (%)	PNV (%)
L858R (13)	100%	100%	100%	100%
DEL. 19 (8)	100%	100%	100%	100%
T790M (5)	80%	100%	100%	98%
L861Q (0)	NV	100%	NV	100%
G719A (0)	NV	100%	NV	100%
G719C (0)	NV	100%	NV	100%
G719S (0)	NV	100%	NV	100%
INS. 20 (0)	NV	100%	NV	100%

Table 8. Diagnostic sensitivity and specificity of the ***CLART® CMA EGFR L****B* technique for each mutation. Sensibility (PV/PV+NF); Specificity (NV/NV+PF); Positive predictive value.(PPV= TP/TP+PF); Negative predictive value (NPV: =TN/TN+NF).TP(True positive) FN (False-Negative result) FP(False-Positive result) TN(True negative).

Considering that the limit of detection for ***CLART® CMA EGFR LB*** kit is when mutated DNA strands are present in 2-3% of total DNA, the diagnostic data was determined in the following manner:

- True positive: The result obtained with ***CLART® CMA EGFR LB*** is considered true positive if it is concordant with the positive result obtained by NGS (mutated strains presence

>2%) or by dPCR (mutated strains presence >2%) or by tissue biopsy positive for activating mutations.

- True negative: The result obtained with ***CLART® CMA EGFR LB*** is considered true negative if it is concordant with the negative result obtained by NGS or NGS positive result when mutated strains presence was <2%; or concordant with negative result obtained by dPCR or dPCR positive result obtained when mutated strains presence was <2%.

Diagnostic reproducibility

The diagnostic reproducibility was obtained by processing the pre-amplified material from circulating DNA till the visualization on the array. The results are presented in the Table 9.

	% homology
Reproducibility (n=45)	94.82

Table 9. Diagnostic reproducibility of *CLART® CMA EGFR LB* kit.

11. BIBLIOGRAPHY

Bettegowda C., Sausen M., Leary R.J. (2014). **Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies.** Sci Transl Med. 19; 6(224): 224ra24

Diaz Jr. L. A. and Bardelli A. (2014). **Liquid Biopsies: Genotyping Circulating Tumor DNA**. Clin Oncol 32:579-586.

Imamura F., Uchida J., Kukita Y., Kumagai T., Nishino K., Inoue T., Kimura M., Oba S., Kato K. (2016). **Monitoring of treatment responses and clonal evolution of tumor cells by circulating tumor DNA of heterogeneous mutant EGFR genes in lung cancer**. Lung Cancer. 94:68-73.

Karachaliou N., Mayo-de las Casas C., Queralt C., de Aguirre I., Melloni B., Cardenal F., Garcia-Gomez R., Massuti B, Sánchez J.M., Porta R., Ponce-Aix S., Moran T., Carcereny E., Felip E., Bover I., Insa A., Reguart N., Isla D., Vergnenegre A., de Marinis F., Gervais R., Corre R., Paz-Ares L., Morales-Espinosa D., Viteri S., Drozdowskyj A., Jordana-Ariza N., Ramirez-Serrano J.L., Molina-Vila M.A., Rosell R. (2015). **Association of EGFR L858R Mutation in Circulating Free DNA With Survival in the EURTAC Trial.** Spanish Lung Cancer Group. JAMA Oncol. May;1(2):149-57.

Luke J.J., Oxnard G.R., Paweletz C.P., Camidge D. R., Heymach J.V., Solit D.B., Johnson B.E. (2014) **Realizing the Potential of Plasma Genotyping in an Age of Genotype-Directed Therapies.** JNCI J Natl Cancer Inst .106(8).

Marchetti A., Palma J.F., Felicioni L., De Pas T.M., Chiari R., Del Grammastro M., Filice G., Ludovini V.,Brandes A. A., Chella A., Malorgio F., Guglielmi F., De Tursi M., Santoro A., Crinò L., and Buttitta F.(2015). **Early Prediction of Response to Tyrosine Kinase Inhibitors by Quantification of EGFR Mutations in Plasma of NSCLC Patients**. J.of Thoracic Oncology ®, 10 (10).

Mok T., Wu Y.-L., Lee J.S., Yu C-J, Sriuranpong V., Sandoval-Tan J., Ladrera G., Thongprasert S., Srimuninnimit V., Liao M., Zhu Y., Zhou C., Fuerte F., Margono B., Wen W., Tsai J., Truman M., Klughammer B., Shames D.S., and Wu L. (2015). **Detection and Dynamic Changes of EGFR Mutations from Circulating Tumor DNA as a Predictor of Survival Outcomes in NSCLC Patients Treated with First-line Intercalated Erlotinib and Chemotherapy**. Clin Cancer Res; 21(14).

Schwaederle M., Husain H., Fanta P.T., Piccioni D.E., Kesari S., Schwab R.B., Patel S.P., Harismendy O., Ikeda M., Parker B.A., Kurzrock R (2016). **Use of Liquid Biopsies in Clinical Oncology: Pilot Experience in 168 Patients**. Clin. Cancer Res. pii: clincanres.0318.2016.

Sorensen B.S., Wu L., Wei W., Tsai J., Weber B., MD, Nexo E., and Meldgaard P. (2014). **Monitoring of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor-Sensitizing and Resistance Mutations in the Plasma DNA of Patients With Advanced Non–Small Cell Lung Cancer During Treatment With Erlotinib**. Cancer, 120(24):3896-901.

Thress K.S., Brant R., Carr T.H., Dearden S, Jenkins S, Brown H., Hammett T., Cantarini M., Barrett J.C. (2015). **EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform**

comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer. 90(3):509-15.

Qiu M, Wang J, Xu Y, Ding X, Li M, Jiang F, Xu L, Yin R. (2015). **Circulating tumor DNA is effective for the detection of EGFR mutation in non-small cell lung cancer: a meta-analysis**. Cancer Epidemiol. Biomarkers Prev. 24(1):206-12.