DETECTION AND GENETIC IDENTIFICATION OF POINT MUTATIONS, INSERTIONS AND DELETIONS IN THE GENE EGFR PATHWAY ASSOCIATED TO NON SMALL CELL LUNG CANCER FOR IN VITRO DIAGNOSIS
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For more information, please refer to the web site: www.genomica.com
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1. Glossary

Attention, see instructions for use

Expiration date

In vitro diagnostic medical device

Lot

Store at room temperature

Store at 4°C to 8°C

Store at –30°C to –18°C
2. PROTOCOL DESCRIPTION

**CLART® CMA EGFR** detects 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.

**CLART® CMA EGFR** kit detects a total of 40 high-prevalence mutations located in the following exons associated with sensitivity or resistance to the treatment.

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

<table>
<thead>
<tr>
<th>EXONs</th>
<th>3 Point Mutations</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXON 18</td>
<td>G719A (c.2156 G&gt;C)</td>
<td>0.3%-1.05%</td>
</tr>
<tr>
<td></td>
<td>G719C (c.2155 G&gt;T)</td>
<td>0.3%-1.05%</td>
</tr>
<tr>
<td></td>
<td>G719S (c.2155 G&gt;A)</td>
<td>0.3%-1.05%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EXONs</th>
<th>28 Deletions</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXON 19</td>
<td>6223* E746_A750del (c.2235_2249 del 15)</td>
<td>17.8%</td>
</tr>
<tr>
<td></td>
<td>12370* L747_P753&gt;S (c. 2240_2257del18)</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>12369* L747_T751del (c.2240_2254del15)</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>6255* L747_S752del (c. 2239_2256del18)</td>
<td>10.2%</td>
</tr>
<tr>
<td></td>
<td>12384* E746_S752&gt;V (c.2237_2255&gt;T)</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td>12382* E747_A750&gt;P (c.2239_2248TTAAGAGAAG&gt;C)</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EXONs</th>
<th>2 Point Mutations</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXON 20</td>
<td>S768I (c.2303 G&gt;T)</td>
<td>0.7%</td>
</tr>
<tr>
<td></td>
<td>T790M (c.2369 C&gt;T)</td>
<td>0.5-1.75%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EXONs</th>
<th>5 Insertions</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H773_V774insH (c.2319_2320insCAC)</td>
<td>0.4-3.22%</td>
</tr>
</tbody>
</table>
D770_N771insG (c.2310_2311insGGT)
V769_D770insASV (c.2307_2308ins9GCCAGCGTG)
V769_D770insASV (c.2309_2310AC>CCAGCGTGAG)
D770_N771insSVD (c.2311_2312ins9GCGTGGACA)

### EXON 21

<table>
<thead>
<tr>
<th>2 Point mutations</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L858R (c.2573 T&gt;G)</td>
<td>4.3-15.05%</td>
</tr>
<tr>
<td>L861Q (c.2582 T&gt;A)</td>
<td>0.2-0.7%</td>
</tr>
</tbody>
</table>

**SAMPLE:** formalin-fixed, paraffin-embedded Lung biopsies.

Detection is carried out by the specific amplification of the mutation, deletion or insertion in the sample, originating a variable fragment for each alteration of between 100-200 base pairs.

The amplification is performed in 3 PCR tubes of different colours: white, green and red.

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) (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.](image)

The **CLART®CMA EGFR** 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 labelled with biotin. After amplification, these products are hybridized with their respective specific complementary probes that are immobilised 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 (*Horseradish Peroxidase*). 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 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.

Ready-to-use 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.

Mix 1: (White tube) y Mix 2: (White tube with a black mark on the lid)
Detected:

<table>
<thead>
<tr>
<th>9.5% OF RESISTANCE MUTATIONS.</th>
<th>94.5% OF SENSIBILITY MUTATIONS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• EXON 21: L858R Y L861Q.</td>
<td></td>
</tr>
<tr>
<td>• EXON 19: Deletions.</td>
<td></td>
</tr>
<tr>
<td>• EXON 18: G719C, G719A, G719S.</td>
<td></td>
</tr>
</tbody>
</table>

Mix 3: (Red tube)
Detected:

<table>
<thead>
<tr>
<th>90.5% OF RESISTANCE MUTATIONS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• EXON 20: T790M, S768I, and Insertions.</td>
</tr>
</tbody>
</table>

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

The visualization kit is shipped at 4ºC. This visualization kit should be stored at 4ºC except Strips and hybridization solution which should be stored at room temperature.

**WARNING!** Upon arrival, the CLART-Strip® (CS), as well as the hybridization solution (SH) must be kept at room temperature.

- **CS strips** (including all specific probes). They are provided in a sealed thermal envelope. **Store it closed, at room temperature, protected from direct light and high temperatures.**
- **SH** (Hybridization Solution). **Store at room temperature.**
- **DC** (Conjugate Diluent). **Store at 4ºC.**
- **CJ** (Conjugate). **Store at 4ºC.** Centrifuge once before use.
- **RE** (Development Solution). **Store at 4ºC and protected from light.**
- **TL** (Wash Buffer). **Store at 4ºC.**
- **Adaptor and lid for 8-well strips (microtiter plate).**

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** designed and validated by GENOMICA. Installed and ready to use.
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.

4.2. Equipment.

- Microcentrifuge.
- UV-visible spectrophotometer.
- Standard thermal cycler, cooling / heating ramps up to 3°C/second. (Maximum cooling/heating block ramp rate is 3 °C).
- Laminar flow chamber for the extraction laboratory.
- 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 50°C.
- Vortex.
- Vacuum system (desiderable).

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).

   2. **Post-PCR area**: Amplification 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. **Used the Xylene always inside the flow chamber**: it should be used gloves and mask during its manipulation. The common precautions for flammable substance storage should be followed. The rest of xylene should be treated as a Non-halogenated waste.

3. **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.

4. **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.

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

6. Use disposable and autoclaved laboratory material.

7. Never mix reagents from two different vials, even if they belong to the same lot.
8. Close reagent tubes immediately after use in order to avoid contamination.

9. 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 hybridation temperature.

5.4 Precautions for visualization

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 50°C. 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.-SAMPLES

The CLART® CMA EGFR kit has been designed and validated to be used with DNA extracted from cancer biopsies. GENOMICA is not responsible for the results obtained if other types of samples are used.

7.-WORKING PROTOCOL

CLART® CMA EGFR has been validated using two different protocols for sample pre-treatment. Therefore it is highly recommended to use one of the following pre-treatment:

7.1. Sample pre-treatment:

Preprocessing.

The tissue should be fixed as soon as possible (maximum 1 h after the sample collection.) in 10% neutral buffered formalin. Do not use based on alcohol or mercury fixatives. The optimum fixation should last: from 8 to 24 hours (for large surgical samples) and from 6 to 12 hours (for small surgical samples) It also could be used cytological samples fixed by the usual techniques immediately.

Then the samples fixed should be embedded in blocks of paraffin. These blocks of paraffin should be cut into slices and placed on a glass slide for the examination by the pathologist. Each sample must be processed using a new sterile scalpel.

The pathologist will perform a study of each sample by staining it with hematoxylin and eosin (H&E). The staining should be performed in the immediately previous cuts to the ones used in the molecular study. The staining will help to define and verify the tumor area, being indicated as a percentage (%) of tumor cells. In order to obtain significant results with the technique, it is recommended that the tumor cell percentage be between 5-10% in the sample.

The number of cuts to be extracted will depend on the size of the tumor and the number of cell in the section. It is highly recommend selecting the fragments with high levels of cellularity and less necrosis. For more information about the procedure please follow the guidelines from SEAP / SEOM.
Please follow the following recommendations of the SEAP:

- **Endoscópic biopsy:**

  As long as the percentage of the tumor is more than 10%, it will be needed the following number of slices.

  - From a completed block of paraffin, it will be needed between 10 – 15 of 5 µm slices.
  
  *An spare sample could be obtain from the stained preparation If the paraffin block is not enough or runs out: please withdraw the cover-glasses during 10 minutes with acetone and hydrate the sample with 96% ethanol during 24-48h.*

  - From neoplastic fragments obtain by micro dissection, it will be needed between 20 -30 slices

- **Cytologies obtained by EBUS, EUS or PAAF:**

  As long as the percentage of the tumor is more than 10%, It should be developed the following processing:

  - Macrodisection : withdraw the coverglasses and scratch all the surface of the glass with blade

  - Microdissection (with laser or needle) : As long as the cellularity is more than 500 cells, mark the different zones of the tumor firstly with a permanent marker and then with a diamond pencil. After that, obtain the cells with a 25 G-needle under microscopy control.

- **Surgical Pieces :**

  - Mark the zone of the tumor with the mayor proportion of the tumor. Please avoid the necrotic parts. 5-10 cuts of 10 µm are needed. Then choose the cuts with the interesting zone.

**Dewaxing :**

Once you have selected the zone of the tumor, the dewaxing of the sample will be performed:

- When the slices of the sample are placed on a glass slice :

  - Immerse the glass slices into a cuvette filled up with xylene during 5 minutes.
  
  - Immerse the glass slices into a cuvette filled up with Ethanol 96% during 5 minutes.
• Immerse the glass slices into a cuvette filled up with fresh Ethanol 96%, withdraw the coverglasses and scratch all the surface of the glass with a blade (once the surface is wet). Then take the sample into an eppendorf.
• Centrifuge during 5 minutes (maximum speed). Discard the supernatant
• Centrifuge during 2 minutes (maximum speed). Discard the supernatant. Allow the ethanol to air-dry
• Continue with the DNA extraction

- When the slices of the samples are in an eppendorf:
  • Once it is discarded the largest possible quantity of paraffin, take all the slices needed to proper developed of the extraction into the 1.5 ml tube
  • In order to disintegrate the sample, please add 500 ul of mineral oil pipetting it on the sample. Be careful of having the entire sample embedded
  • Heat the theromixer to 95º during 2 minutes (with shaking)
  • Centrifuge at 8000rpm during 2 minutes.
  • Aspirate all the oil carefully of not withdrawing the sample.
  • Repeat steps from 2 to 5.
  • Add the lysis buffer making sure the sample is completely covered and continue with the extraction

7.2. Extracted Material.

The extraction method chosen should be one that ensures the following concentration and purity parameters:

The amount of genetic material added to the PCR tube should be 50 ng in each tubes of the mixes 1 and 2 (white and green) and a total of 100 ng in mix3 (red tube). Therefore 200ng of DNA is required. Never add more than 50 ng of extracted DNA in mix 1 tube, 50 ng in Mix 2 tube, and 100 ng in Mix3 tube. An excess or deficiency of DNA can lead to misdiagnosis.

Never add more than 10µl of volume to each PCR tube. It should be added between 5 - 10 µl of extracted DNA to each amplification tube, with the adequate concentration.

<table>
<thead>
<tr>
<th></th>
<th>DNA( ng)/ per tube.</th>
<th>Maximum volumen(µl) / per tube.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix1</td>
<td>50ng</td>
<td>10 µl</td>
</tr>
<tr>
<td>Mix2</td>
<td>50ng</td>
<td>10 µl</td>
</tr>
<tr>
<td>Mix3</td>
<td>100ng</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

It is recommended to dilute the samples to 10 ng/µl, in order to add 5 µl to the mix1 tube (50ng of DNA), 5 µl to the mix2 tube (50ng of DNA), and 10 µl to the mix3 (100ng of DNA).

If the purity is lower than 10 ng/µl, sample should be re-extracted.
The extracted DNA should have the following purity enough to avoid misdiagnosis. The ratio between the absorbance at 260nm and the absorbance at 280 nm should be as close to 2 as possible. If the purity is not adequate, sample should be re-extracted.

Extracted genomic DNA may be stored at 2-8ºC for 1 week post extraction, or at -15 to -25ºC for up to 8 weeks before use.

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.

### 7.3. Amplification reaction.

**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.
- Exclusive use of standard thermal cyclers only, with speed ramp of cooling / heating up to 3ºC per second. (The maximum cooling/heating block ramp rate is 3 °C). Do not use fast thermal cyclers. In some of those the ramp rate can be adequate according these needs.

1. Thaw the necessary number of amplification tubes according to the number of samples and gene/s to analyse. Thawing the tubes at 4ºC.

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).

1. Add 5-10 μL (see point 7.2) of the extracted DNA to each amplification tube, just after having check for DNA concentration and purity and mix several times with the micropipette. Keep the tubes refrigerated at any time.

   **Mix 1:** 50 ng /tubo (5-10 μL tubo).
   **Mix 2:** 50 ng /tubo (5-10 μL tubo).
   **Mix 3:** 100 ng /tubo (5-10 μL tubo).

4. Program the following temperature cycles on the thermocycler:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle</td>
<td>95ºC</td>
<td>15 min</td>
</tr>
<tr>
<td>40 cycles</td>
<td>94ºC 94ºC</td>
<td>15 sec. 60 sec.</td>
</tr>
</tbody>
</table>
5. Start the program and place the tubes in the thermocycler when the block has exceeded 90°C. This way, possible unspecified amplifications due to incubation below hybridization temperature are minimized. The duration of the amplification ranges between 90-150 minutes depending on the thermocycler.

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

7.4. 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 50°C for at least 60 minutes.

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

7.4.1 Manual 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

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 50ºC shaking at 550 rpm** (previously this termomixer has to be prewarm 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). For the correct interpretation of the results, it is mandatory to visualise 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 ºC** 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.

### 7.4.1 autoclart® visualization

1. **Switch on the autoclart® unit and follow the instructions described on the screen:**
2. Close the door and press the knob.
3. Select Run Program at the main menu.
4. Select the assay **CMA test** among those listed
5. Select the well of the strip where the run should start: A1 or E1 in case the first 4 wells have already been processed.
6 Select the number of samples to be processed. With the autoclart®, the user can process from 4 up to 96 samples per run. In any case, samples must be multiples of four.

7 Confirm that the number of samples and the start up-well (A1 or E1) are correct.

8 Place the tips rack (full) on its position.

9 Load the array microplate in the holder. Make sure that the catch is fastened in order to clamp the plate down.

10 Check that both, the tip waste and the liquid waste containers are empty and on its proper position.

11 Fill the DI bottle with 250 ml distilled water.

12 Add each of the reagents to its specific container. The autoclart® calculates the specific volumes required according to the amount of samples indicated:

- **TL** (Washing buffer). The volume showed in the display indicates the diluted washing buffer required. In order to prepare the diluted washing buffer please dilute the TL reagent provided 1:10 into distilled water.

- **CJ** (Conjugate). It's recommended to shortly spin the CJ before use. The display shows the final volume of diluted CJ to add, meaning that each mL indicated on the display should be prepared as follows: 1 ml of DC (Conjugate Diluent) and 15 µl CJ reagent. Vortex the diluted solution in order to mix it properly up.

- **RE** (Developer). Add the RE volume indicated on the display.

13 **Denaturation**: Use the thermocycler to denature the amplification tubes. Place the amplification tubes in the thermocycler when this has reached 95ºC and incubate the tubes for 8 min. Remove the tubes from the 95ºC incubation and place them immediately on ice. Denature the amplification product before setting the visualization reagents in the autoclart®.

14 **SH** (Hybridization solution). Add the volume indicated on the screen once it has been warmed to 50ºC (during at least 60 minutes)

**WARNING**: It is important to add the HS at this point; If the HS is added in a previous step, its temperature might decrease affecting to the intensity of the probes and it might lead to false negative results.

15 Close the door and press the knob to start the program.

The device will start priming the system. Then it will perform the pre-washes of the CS and add the Hybridization Solution. Once finished these steps, the device will beep as a signal for the user to add the samples on the CS.

The autoclart® will keep beeping until the user opens the door.
13 For the addition of the samples on the CSs, please carefully remove the plate from autoclart® unit and add 5 µl of the denatured products from the same sample to the same well. Mix it up carefully in order to not touch the array and place the microplate again on the Autoclart. Press the knob to continue the visualization process.

16 Once the visualization process is finished, the autoclart® will beep indicating the end of the run. Please carefully remove the microplate and proceed with the reading step on the CAR®.

   WARNING : Once the visualization phase is finished on the autoclart®, the arrays should immediately be read on the CAR®, otherwise false negatives might appear, caused by intensity lost.

17 CAR® (CLINICAL ARRAY READER): place the plate normally on the tray and the CAR® will take and analyse 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 analysed and visualised, 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 "NON-VALIDATED" 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 1). Sensitivity has also been determined by means of the amplification of serial dilutions of commercial cell lines that contain the mutation to be determined (Table 2). The visualisation was done in CS, giving rise to the following results:

<table>
<thead>
<tr>
<th>MUTATION</th>
<th>Cop/5 ul</th>
<th>SENSIBILITY (%)</th>
<th>REPRODUCIBILITY (%)</th>
<th>REPEATABILITY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L858R</td>
<td>10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DEL. 19 (82,8%)*</td>
<td>10e2-10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T790M</td>
<td>10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L861Q</td>
<td>10e4</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G719A</td>
<td>10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G719C</td>
<td>10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G719S</td>
<td>10e3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>INS. 20</td>
<td>10e4-10e5</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S768I</td>
<td>10e5</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1. Relationship of the number of copies of recombinant plasmid/cell line nanograms necessary to obtain a sensitivity of 100% in the detection of each one of the microorganisms. Lower prevalence deletions of the exon 19 (17,2% prevalent) show a sensibility between 10e2-15e5.

<table>
<thead>
<tr>
<th>MUTATION</th>
<th>ng</th>
<th>SENSIBILITY (%)</th>
<th>REPRODUCIBILITY (%)</th>
<th>REPEATABILITY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L861Q LINE</td>
<td>0.3ng</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L858R LINE</td>
<td>0.1ng</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T790M LINE</td>
<td>1 ng</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. nanograms of cell lines required for a 100% sensitivity in order to detect each of the mutations.

Analytical specificity.

Specificity experiments were carried out with 46 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 %, with the exception of the S768I mutation whose analytical specificity is greater than 98%.

Diagnostic utility parameters:
In order to determine the diagnostic parameters of the kit, a comparative assessment of the **CLART® CMA EGFR** kit was carried against the reference technique (Cobas, Roche; Therascreen, Qiagen). 363 samples were analyzed in total, 153 of these were analyzed in GENOMICA. For this study GENOMICA has collaborated with the following laboratories:

- 68 samples were analyzed in the Department of Pathological Anatomy of the Vall de Hebrón University Hospital, Barcelona, Spain.
- 32 samples were analyzed in the Department of pathological anatomy of the Clínico San Carlos hospital, Madrid, Spain.
- 63 samples were analyzed in the pathological anatomy service of the Universitario 12 de Octubre hospital, Madrid, Spain.
- 46 samples were analyzed in the pathological anatomy service of the Universitario Marqués de Valdecilla hospital, Santander, Spain.

363 samples were analyzed. The results are in the following table:

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sensibility (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>PNV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L858R N: 76</td>
<td>97.4</td>
<td>99</td>
<td>96.1</td>
<td>99.3</td>
</tr>
<tr>
<td>L861Q N: 5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Deleción exón 19</td>
<td>96.2</td>
<td>99.6</td>
<td>99</td>
<td>98.5</td>
</tr>
<tr>
<td>N: 104</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inserción exón 20</td>
<td>75</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>N: 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T790M N: 17</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S768I N: 8</td>
<td>62.5</td>
<td>100</td>
<td>100</td>
<td>99.2</td>
</tr>
<tr>
<td>G719C N: 1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G719A N: 6</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>G719S N: 4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. Diagnostic sensitivity and specificity of the **CLART® CMA EGFR** 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).

It is assumed as true result when the result obtained by the gold standard and **CLART® CMA EGFR** technique is the same.
In any discrepancies between the two techniques, it is considered as the true result, the result obtained from the sequencing of the sample.

**Reproducibility and diagnostic repeatability.**

The reproducibility and repeatability series have been set starting with the extraction of the biopsy up to visualization in the array of the amplified material.

The results shown in the following table:

<table>
<thead>
<tr>
<th></th>
<th>% homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability (n=22)</td>
<td>93.4</td>
</tr>
<tr>
<td>Reproducibility (n=44)</td>
<td>91.7</td>
</tr>
</tbody>
</table>

Table 4. Diagnostic repeatability and reproducibility of kit CLART® CMA EGFR.
11. BIBLIOGRAPHY:


