CLART® CMA
ALK·ROS.1

DETECTION AND GENETIC IDENTIFICATION OF THE MAIN CHROMOSOMAL TRANSLOCATIONS IN THE ALK AND ROS.1 GENES IN PATIENTS WITH LUNG CANCER.

FOR IN VITRO DIAGNOSTIC USE

CLART® CMA ALK·ROS.1

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

CE marked

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
October 2016

Table of contents

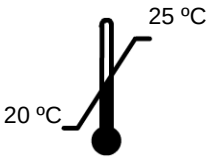
1. GLOSSARY

Attention, see instructions for use

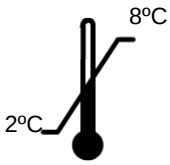
Expiration date

In vitro diagnostic medical device

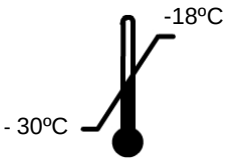
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® CMA ALK ·ROS.1 detects the main chromosomal translocations of ALK gene (Anaplastic lymphoma kinase) with EML.4 (Echinoderm microtubule-associated protein-like 4) and ROS.1 gene (Receptor tyrosine kinase) with SDC4 (syndercan 4), CD74 (Cluster of differentiation 74) y SLC34A2 (type II sodium/Phosphate cotransporter) in patients with lung cancer.

Published data demonstrate that lung cancer patients with translocations in ALK or ROS.1 genes respond favorably to specific therapy (the ALK inhibitors). Testing for the presence of ALK and ROS.1 mutations (especially in the advanced or metastatic NSCLC patients) is essential for identifying the patients that have higher probability to benefit from the ALK targeted therapy.

CLART® CMA ALK·ROS.1 detects the main translocations in these genes, detecting the total of 7 translocations between genes ALK and EML.4 and 5 translocations in ROS.1 gene:

- ***Traslocations EML.4-ALK:***

  - ✓ Varian V1: E13;A20 V1
  - ✓ Variant V6: E13; ins 69;A20
  - ✓ Variant V2: E20;A20
  - ✓ Variant V3a: E6:A20
  - ✓ Variant V3b: E6; ins 33 A20
  - ✓ Variant V5a: E2;A20
  - ✓ Variant V5b: E2; ins 117 A20

- ***Traslocations ROS.1:***

  - ✓ Traslocations SDC4-ROS.1
    - Variant SDC4-ROS1 exon 32 (S2;R32)
    - Variant SDC4-ROS1 exon 34 (S2;R34)
  - ✓ Traslocations CD74-ROS1 exon 34 (C6;R34)
  - ✓ Traslocations SLC34A2-ROS1
    - Variant SLC34A2-ROS1 exon 32 (S4;R32)
    - Variant SLC34A2-ROS1 exon 34 (S4;R34)

Starting material for is extracted RNA from lung biopsies in the form of formalin-fixed paraffin-embedded tumor tissue.

Detection is based on our CLART® technology: End-point Multiplex PCR amplification, followed by visualization in low-density microarray.

Displayed in Figure 1 is a CLART-Strip® (CS), each well including all specific probes for testing one sample.

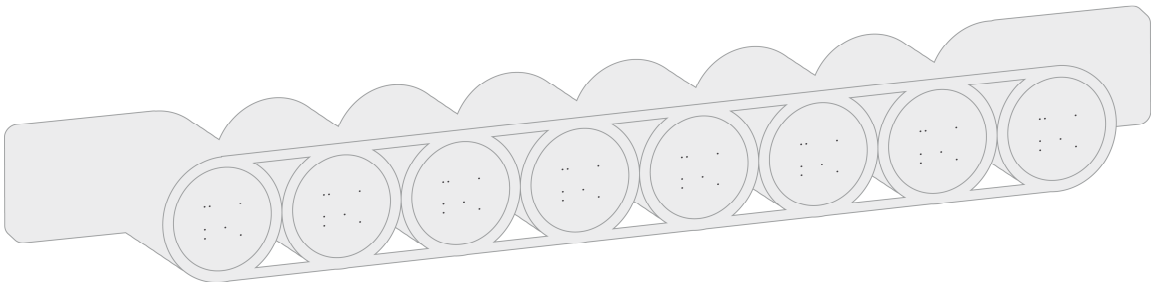


Figure 1. CLART-Strip® in the form of an 8-well strip.

A scheme of the detection system is displayed in Figure 2. Basically, RT-PCR amplified products labelled with biotin, hybridize with their specific complementary probes immobilised in well-defined areas of the microarray. Subsequent incubation steps take place thereon: first, with a streptavidin-peroxidase conjugate, and second, with an o-dianisidine substrate.
A non-soluble product precipitates thereafter in regions of the microarray where specific hybridization between amplified products and their specific probes has taken place.

Thereafter, analysis and interpretation of results are automatically performed by GENOMICA's reader (CAR® or CLINICAL ARRAY READER), running tailor-made software.

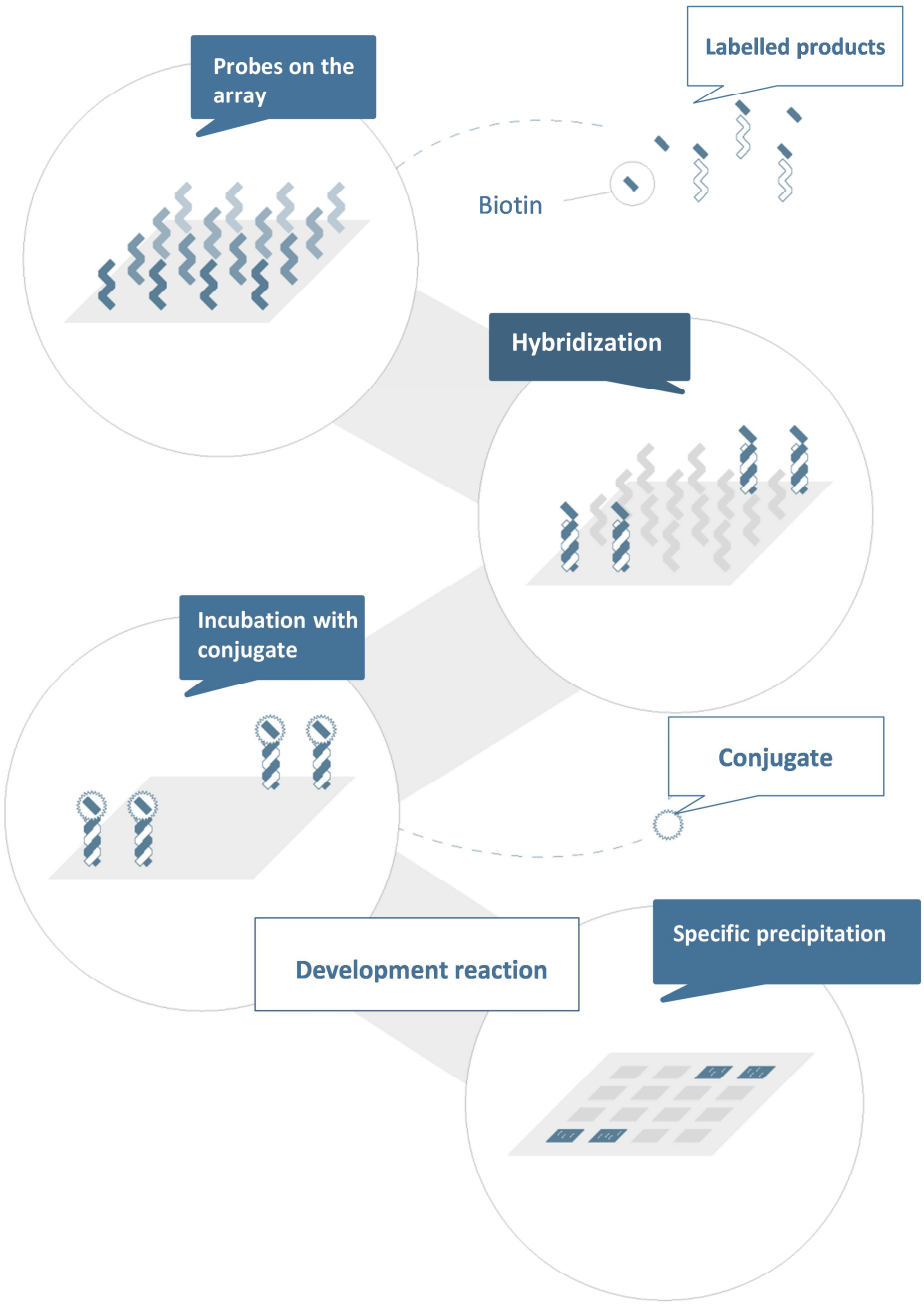


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields a precipitate in the area where hybridization has occurred.

3.-KIT COMPONENTS AND STORAGE

Components of the kit are provided at their optimal storage temperatures, and remain stable until the expiration date is reached, upon observance of recommended storage conditions.

Components of both formats are displayed herein:

3.1. Amplification reagents

Shipped and stored at -20ºC.

Amplification tubes are provided ready-to-use. Each amplification tube contains 45 µL of master mix. Only the exact number of required tubes should be thawed on ice. Remaining ones should be kept at -20ºC.

Mix 1: Blue tube. Detecting the translocations ALK-EML.4:

- Variant V1: E13;A20 V1
- Variant V6: E13; ins 69
- Variant V2: E20;A20
- Variant V3a: E6:A20
- Variant V3b: E6; ins 33 A20
- Variant V5a: E2;A20
- Variant V5b: E2; ins 117 A20
- Internal amplification control
- Genomic DNA extraction control

Mix 2: White tube. Detecting the translocations de ROS.1:

- Translocations SDC4-ROS.1
- Variant SDC4-ROS1 exon 32 (S2;R32)
- Variant SDC4-ROS1 exon 34 (S2;R34)
- Translocations CD74-ROS1 exon 34 (C6;R34)
- Translocations SLC34A2-ROS1
- Variant SLC34A2-ROS1 exon 32 (S4;R32)
- Variant SLC34A2-ROS1 exon 34 (S4;R34)
- Internal amplification control
- Genomic DNA extraction control

WARNING: 2 µl of enzyme mix must be added to each tube before the genetic material has been introduced.

Enzyme Mix: Ready-to-use mixture of **RT** (reverse transcriptase) and **DNA Polymerase**. Store at -20ºC.

Note: Boxes containing amplification tubes include a self-adhesive and irreversible temperature indicator; Red colour displayed on the visualization window of the indicator means that the package has exceeded at some time the storage temperature of –20°C and reagents should be discarded.

3.2. Visualization components

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

- GENOMICA's Reader **CAR®** or **CLINICAL ARRAY READER** (Figure 3).
  CAR® grants automatic reading, analysis and interpretation of up to 12 **CS** units (i.e., to a maximum of 96 samples) *per* run. It displays a user-friendly and intuitive graphical interface (CLEIS), and includes updates of GENOMICA's proprietary image processing software SAICLART® as well as Kit-specific Software.

Note: CAR® is to be used exclusively with GENOMICA's diagnostic kits.

Figure 3. CAR® (CLINICAL ARRAY READER)

4. ITEMS REQUIRED BUT NOT PROVIDED

A list of all items required but not provided is displayed below:

4.1. Reagents and materials

- Distilled water.
- Disposable gloves.
- Filter tips or positive displacement pipettes.
- Crushed ice container or cool tube-holder.
- 1.5 mL autoclaved Eppendorf tubes.
- 1.5 mL tube grids.
- 0.5 mL/0.2 mL tube holder.
- Qiagen's RNeasy FFPE Kit (recommended).

4.2. Equipment

- Microcentrifuge.
- UV-visible spectrophotometer (Nanodrop).
- Thermal cycler. Use of the conventional thermal cycler *Applied Biosystems 2720 Thermal Cycler* is recommended. In the unusual case of using a high speed ramp thermal cycler, *Eppendorf Mastercycler Nexus Gradient* thermal cycler is recommended. In any case, thermal cycler verification as explained in chapter 7.3.1 of the present manual is mandatory.
- Biosafety cabinet 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.
- Thermomixer compatible with 96-well skirted plates and adjustable shaking at 20°C, 25°C and 59ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

1. *CLART® CMA ALK·ROS.1* technique should be performed in two physically separated areas in order to minimise sample contamination:

Pre-PCR area: DNA extraction and sample preparation are performed in this area. Sample manipulation must be carried out within a biosafety cabinet.

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 material of the Pre-PCR area, thus the recommendation to avoid entering the Pre-PCR area after having worked in the Post-PCR area.

Independent working material should be available in each area (pipettes, tips, tubes, grids, gloves, etc.), never being used outside them.

2. Always use gloves. It is advisable to change gloves frequently, and mandatory to change gloves (i) before starting to work in each of the previously mentioned areas, and (ii) before DNA addition to the amplification tubes.

3. Xylene should always be used inside a chemical fume hood. Use of personal protective equipment as gloves and mask is mandatory during its manipulation. Common precautions for

flammable substance storage should be followed. Any xylene residue should be treated as a Non-Halogenated waste material.

4. Clean working areas (work bench, hoods, grids, pipettes) thoroughly with a 10% diluted bleach solution **after processing each sample batch;** it is mandatory to disinfect all working areas in the case of contamination. It is recommended to clean thermal cyclers and thermomixers before and after use, following the same procedure.

5. Use filter tips and positive displacement pipettes to avoid contamination due to micropipettes. Different sets of pipettes should be used in each area. Discard the micropipette tip after each pipetting.

6. Use disposable and autoclaved laboratory material.

7. Never mix reagents from different vials, even if belonging to the same lot.

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

6. SAMPLES

CLART® CMA ALK·ROS.1 has been designed and validated for the analysis of RNA extracted from lung cancer biopsies: surgically removed piece of tissue, endoscopic biopsy, endobronchial ultrasound (EBUS) or endoscopic ultrasound (EUS) biopsy, fine-needle aspiration biopsy (FNAB), mediastinoscopy and thoracotomy.

GENOMICA cannot warrant accuracy of results obtained if processing a different type of sample.

7. WORKING PROTOCOL

CLART® CMA ALK·ROS.1 has been validated using the below mentioned protocol , which constitutes the Working Protocol.

Attention is drawn to recommendations of Section 5 above, and Sections *7.2.1*, *7.3.1.* and *7.4.1* below, for a successful outcome of the technique.

7.1. Sample pre-treatment

Pre-analytical step

The tissue should be fixed in 10% neutral buffered formalin, within 1h maximum after the sample is obtained. The sample must be kept at room temperature, and no alcohol or mercury based fixatives should be used. Optimal fixation time is of 8-24 hours for large surgical samples and 6-12 hours for small surgical samples.

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

Analysis of each sample by the pathologist comprises hematoxylin and eosin staining. Staining should be performed immediately prior to obtaining the cuts that will be used in the molecular study. Staining will help to define and verify the tumor area, which will be defined as a percentage (%) of tumor cells. In order to obtain reliable results, it is recommended that tumor cells represent at least 5-10% of the sample.

The number of paraffin cuts used for extraction will depend on the size of the biopsy and the number of tumoral cells *per* section, fluctuating between 1 and 4 cuts per sample. It is highly recommended to select fragments with high levels of cellularity avoiding necrosal and *wild-type* cells areas. The cuts can be on glass slides or rolled. For more information about the procedure please refer to the national medical oncology guidelines.

In continuation are listed recommended number of paraffin cuts required for extraction dependint on the sample type, the size of tumoral zone and the percentage of tumoral cells:

- *Endoscopic biopsy:*

  As long as the percentage of the tumor is more than 10%, 1-4 paraffin cuts of 5 µm thickness is needed.

  If there is not sufficient sample in paraffin block, a sample could be obtained from the previously stained slide as long as there is more than 10% of tumoral cells: remove the coverglass and soak the glass slide in acetone for 10 minutes, re-hydrate the sample with 96% ethanol during 24-48h.

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

  As long as the percentage of the tumor is more than 10%, the samples should be processed using one of the following methods:

  Macrodissection : withdraw the coverglasses and scratch all the surface of the glass with blade. 1-4 paraffin cuts are required.

  Microdissection (with laser or needle): mark the tumoral zones on the sample with permanent marker first and then with a diamond pencil. Under microscope collect the cells with a 25G-needle. A minimum of 500 cells is required.

- *Surgically obtained tissue:*

  Mark the area with the mayor proportion of the tumor, avoiding the necrotic parts. 1-4 paraffin cuts thickness 5-10 µm are needed. Separate the area of interest.

Deparaffinization

Once the tumor zone on the sample has been defined, deparaffinization should start. This process may be performed either by placing the paraffin slices on a glass slide, or inside a tube. Corresponding protocols are displayed hereunder.

Paraffin slices on a glass slide:

1. Immerse the slide into a cuvette filled up with xylene during 5 minutes.
2. Immerse the slide into a cuvette filled up with Ethanol 96% during 5 minutes.
3. Immerse the slide into a cuvette filled up with fresh Ethanol 96%, withdraw the coverglasses and scrape the slide surface with a scalpel, while the slide is wet. Then introduce the collected sample into an Eppendorf tube.
4. Centrifuge for 8 minutes at maximum speed. Discard the supernatant.
5. Centrifuge for 4 minutes at maximum speed. Discard the supernatant. Allow the ethanol to air-dry: 5 minutes at 56ºC or at the room temperature until there are no traces of ethanol present.
6. Proceed with the DNA extraction.

Paraffin slices placed inside a tube:

1. Discard as much paraffin as possible from the block before preparing the slices.
2. Place the paraffin slices required for DNA extraction into a 1.5 ml tube.
3. Add 500 µl of mineral oil pipetting it directly on the sample. Make sure that the slices are completely covered by the oil.
4. Heat the sample at 95º C during 2 minutes with shaking (550 rpm) using a thermomixer.
5. Centrifuge for 2 minutes at 8000rpm.
6. Aspirate all the oil carefully, avoiding to touch the sample.
7. Repeat steps 3 to 6.
8. Add the lysis buffer ensuring that the sample is completely covered, and continue with the DNA extraction.

7.2. RNA Extraction

7.2.1. Extraction-specific recommendations

1. Clean working surfaces of the biosafety cabinet with a 10% diluted bleach solution.
2. Turn on the laminar flow at least 20 minutes before extraction step.
3. Sample preparation before extraction must be carried out inside the biosafety

cabinet.

7.2.2. Extraction protocol

The use of Qiagen's RNeasy FFPE Kit is recommended for RNA extraction. Following the Manufacturer's instructions is required, except for the incubation step with Proteinase K where it should be done 3-5h at 56ºC, with shaking. The final elution volume should be 30 µl. Another kit that provides good results is Recover All Total Nucleic Acid Isolation Ambion, AM 1975.

Notwithstanding, alternative DNA extraction methods might be used, in as far as they yield equivalent concentration and purity parameters (see below).

1. **To assure the 100% of sensitivity, the amount of genetic material to be added to each RT-PCR tube should be 100ng.** The total amount of RNA required per sample is 200ng. Use of lower RNA concentrations can result in decreased sensitivity, as indicated in the table below:

ng RNA /tube RT-PCR	Sensitivity (%)
100 ng	100
99-50 ng	88.23 %
< 50 ng	66.7 %

   Likewise adding RNA in excess can lead to erroneus diagnosis.
   Do not exceed the amount of 100ng of RNA per RT-PCR tube, nor the volume of 10 µl added to each RT-PCR tube (between 5 and 10µl of extracted RNA should be added to each tubedepending on the concentration).

   It is recommended to dilute the samples to 20ng/µl in order to add 5µl to each RT-PCR tube (100ng).

   If the concentration is inferior to 10 ng/µl, add 10µl to RT-PCR tube, regardless to te concentration, but taking into account that sensitivity of the kit might be affected, as indicated in the table above.

2. Extracted RNA should comply with basic purity requirements to avoid misdiagnosis. The ratio between the absorbance at 260 nm and the absorbance at 280 nm should be as close to 2 as possible. If purity is inadequate, the sample should be re-extracted.

3. The extracted material need to be conserved at -80ºC.

4. It is essential to include a negative control in every run to check whether the samples have been contaminated during any of the extraction, amplification or visualization processes, in which a false positive result might be obtained.

7.3. Amplification reaction

7.3.1. Amplification-specific recommendations

- Work in a pre-PCR area, always using a laminar flow chamber and following the recommendations of Section 5.
- **Amplification tubes should be kept at 4ºC during the whole preparation process** (In particular, during mixing with extracted RNA, and until being placed at the thermal cycler for PCR amplification).
- Use only the conventional thermal cyclers that have the cooling/warming speed ramp at most 2-3ºC per second and aluminum block. Some models of thermal cyclers with fast ramps permit lowering the ramp to 3ºC per second. Two thermal cyclers that fulfill these requirements have been validated for the use with this kit: *Applied Biosystems 2720 and Eppendorf Mastercycler Nexus Gradient.*
- Periodic thermal cycler verification is recommended.

7.3.2. Amplification protocol

1. Thaw on ice the required number of amplification tubes according to the number of samples and gene/s to be analysed. Keep them at 4 ºC.

2. Briefly centrifuge the amplification tubes to bring down all the liquid to the bottom of the tube (in case of non-availability of microcentrifuge tube adaptors, larger tubes having their caps cut off might also be used).

3. Add 2 µl of the enzyme mix to each amplification tube and resuspend several times with micropipette.

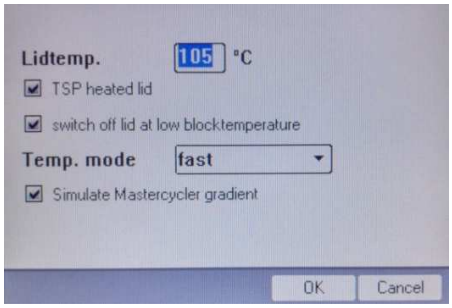
4. After having the RNA concentration and purity checked, add 5-10µl of the extracted RNA (the necessary volume according to indications in Section 7.2.2) to each amplification tube. Mix several times with the micropipette. Keep tubes at 4ºC.

5. Program the following temperature cycles on the thermal cycler:

   A. *Model Eppendorf Mastercycler® nexus or models with warming ramp of 3ºC/sec and cooling ramp of 2ºC/sec and aluminium block:*

1 cycle	45ºC 45 min
	95ºC 15 min
42 cycles	95ºC 60 sec
	62ºC 60 sec
	72ºC 60 sec
1 cycle	72ºC 7 min
4ºC until tube removal	

NOTE: Many of these thermal cyclers offer different options for PCR temperature mode, if this possibility exists choose option “fast”, as it is shown below for the *Mastercycler® nexus:*



B. Model Applied Biosystems 2720 o models with warming and cooling ramp of 2.7 ºC/sec and aluminium block:

1 cycle	45ºC 45 min
	95ºC 15 min
40 cycles	95ºC 60 sec
	62ºC 60 sec
	72ºC 60 sec
1 cycle	72ºC 7 min
4ºC until de tube removal	

6. Start the program and place the tubes in the thermal cycler once the block temeperature has been stabilized, in the meantime maintain the tubes at 4ºC.

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

7.4. Visualization of the amplified product

7.4.1. Visualization-specific recommendations

1. Visualization should always take place in the post-PCR area. Do not introduce the amplified product back into the pre-PCR area.

2. Turn on the CAR® before starting the whole procedure. Self-calibration of the equipment may last for a few minutes. The device should be ready at the time of reading to avoid unnecessary waiting and an excessive exposure to developer.

3. Make sure that the thermomixer temperature has been 59ºC for at least 60 minutes before the hybridization step.

4. SH forms crystals at room temperature, so it must be warmed up and maintained at 59ºC during at least half an hour, but no longer than one hour before use. SH should be homegeneous.

5. Do not to add SH to the **CS** wells until the amplified products are denatured.

6. Prepare diluted TL (Washing Buffer) immediately before use; do not reuse previously prepared solutions.

7. Clean the thermal cycler with a 10% diluted bleach solution before starting the denaturation program. Denaturing time should be of exactly 10 minutes.

8. When preparing samples for visualization, use a different tip for each well and change it every time a reagent is added, even if it is TL.

9. For visualization, the amplified product can only be denatured once. If you need to repeat the visualization process, aliquot the amplified product before the denaturalization step.

10. Always use filtered tips during the addition of amplified products to the **CS** wells.

11. Use vacuum pumps for aspirating solutions, and decontaminate with a 10% diluted bleach solution after every assay. Make sure the pump aspirates properly and no residual liquid is left at the bottom of the well after aspiration.

12. Following incubation with diluted CJ Solution, thorough and fast washing of the **CS** well is essential to avoid residues that might yield non-specific precipitation upon reaction with RE.

13. Aspirate the different solutions completely without touching the **CS** well. Touching the bottom of the well with the vacuum suction tip, might damage the probes of the bottom of the well.

14. Do not allow arrays to dry completely.

15. Dispense all solutions to the wall of the **CS** well; never directly to the bottom of the well.

16. Avoid foaming when adding reagents.

17. When visualizing the image on the CAR®, make sure that position markers appear correctly and that there are no bubbles, fibres or spots interfering with the read-out. Otherwise, clean the outer face of the well with a cellulose paper.

7.4.2. Visualization protocol

1. Denaturation of amplified products: Place the amplification tubes in the thermal cycler and incubate at 99ºC **for exactly 8 minutes**. After that, remove the tubes from the 99ºC incubation and immediately place on ice or at 4ºC.

2. Preparation of Washing Buffer: For each **CS** to be processed, prepare 10 mL of Washing Buffer by adding 1 mL of TL to 9 mL of distilled water.

3. Prewash of the **CS**: Place the necessary **CS** units on the Microtiter plate adaptor. Add 200 µL of Washing Buffer to each **CS** well before use. Mix the solution up and down with a multichannel pipette 10-15 times, without touching the array surface. It is advisable to carry out this wash during the Step of Denaturation of amplified products. Leave the Washing Buffer in the wells until Hybridization Step.

4. Hybridization Step:

   WARNING: SH must not be added until after denaturation of the tubes has finished. If added in advance, SH temperature might undergo a decrease, resulting in also probe intensity decrease and false negatives appearance.
   Once the amplified products have been denatured, discard the Washing Buffer from the **CS** wells with a vacuum pump. Immediately after that, add 100 µL of 59ºC-pre-warmed SH to each **CS** well, avoiding foaming.

   Note: Wells must be totally free of Washing Buffer residues, although they must never get dry. Thus the importance of immediate addition of SH upon removal of Washing Buffer.

   Add 5 µL of denatured amplified product from each Mix corresponding to a single sample/ patient, to **the same CS well.** Volumes to be added:

   Mix1:5 µl
   Mix2:5 µl

   Mix the solution up and down several times, being careful not to touch the bottom of the well. Cover the Microtiter plate adaptor and the **CSs** with the plastic lid and incubate in the thermomixer for 1 hour at 59ºC and 550 rpm. Follow recommendation 3 of Section 7.4.1. above.

   After incubation, remove the CSs from thermomixer and aspirate incubation solution from the **CS** wells with a vacuum pump.

   Set the thermomixer at 20ºC with 550 rpm shaking, for its further use in step 5 below.

   Double Wash: Add 200 µL of Washing Buffer to each **CS** well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the Washing Buffer with a vacuum pump without leaving any residues. Repeat the procedure. **This step must be carried out with different tips for each well in both washes.** Keep the samples on Washing Buffer until the thermomixer cools down and reaches 20ºC.

5. Blocking and conjugate incubation: Centrifuge CJ for 10 seconds before use. Prepare diluted

CJ Solution by adding **15 µL of CJ** to **1 mL of DC** (amounts calculated for 1 **CS** unit), at least 5 minutes before the end of the hybridization step.

Aspirate the Washing Buffer from **CS** wells without leaving any residues, and add **100 µL** of diluted CJ Solution *per* well. Incubate for **exactly 30 minutes** in the thermomixer at **20ºC and 550 rpm.** After this incubation, remove the plate and discard the solution rapidly with a vacuum pump. Once the incubation has finished, set the thermomixer at 25ºC with 550 rpm shaking, for its use on step 8.

6. Triple Wash: Immediately after Step 5 above, add 200 µL of Washing Buffer to each **CS** well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the Washing Buffer with a vacuum pump without leaving any residues. Repeat the procedure **two more times.**

   It is essential to thoroughly clean **CS** wells from any residue of diluted CJ Solution, otherwise reaction with RE might generate a non-specific signal.

7. Development: Thoroughly remove Washing Buffer from the **CS** wells; Next, add **100 µL** of RE to each well and incubate in the thermomixer for **10 minutes at 25ºC without shaking**.

8. Completely discard RE using a vacuum system. **CS** wells must be completely dry for reading.

9. Reading: Place the microtiter plate adaptor with the CS to be analysed on the CAR® tray. The CAR® will read and report the results automatically.

8. RESULTS

This kit has been designed to offer the possibility to analyse ALK translocations (Mix 1, blue tube) and ROS.1 translocations (Mix 2, white tube) independently. Notwithstanding, if the sample is analysed for both genes, both Mixes should be visualized on the same array by using the same CS well simultaneously.

The negative control should be included in order to confirm that sample did not suffer any contaminations during the extraction, amplification or visualization step that could generate false positive results.

The amplification tubes contain their own amplification and extraction control, which serves to confirm that genomic material used is sufficient to perform the test and at the same time permits the independent testing of each gene.

The control of extraction of genomic RNA is necessary for the confirmation of a true negative result, as it informs us of the presence of the patient's RNA 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 RNA was found in the sample.

Analysis of results and issuance of corresponding report are automatically performed by CAR®.

The software can report "NO RNA" result:

- Non-valid extraction: The presence of inhibitors or a 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.

The software can report "INHIBITED PCR/Not analyzed" result:

- Inhibited PCR or a non-valid amplification: The absence of amplification in the analyzed tubes indicates there has been a failure in the amplification step; to solve this, the corresponding tube must be amplified again.

The software can report "UNCERTAIN" result:

- The reason for this result to appear is when the readings of absorbance of the replicate probes of an array are very different from each other.

9. TECHNICAL SPECIFICATIONS OF THE KIT

9.1. Processing parameters

Analytical sensitivity

Analytical sensitivity was determined through amplification of dilution series of DNA recombinant plasmids for each mutation that kit detects.

Table 1 below displays results obtained after visualization in CS:

MUTATION	Copies/5µl
ALK-ELM4 Variant V1	10
ALK-EML4 Variant V6	10
ALK-EML4 Variant V3a	10
ALK-EML4 Variant V3b	10
ALK-EML4 Variant V5a	10
ALK-EML4 Variant V5b	10
ALK-EML4 Variant V2	10e2
SDC4-ROS1 exon 32	10
SDC4-ROS1 exon 34	10
CD74-ROS exon 34	10
SLC34A2-ROS exon 32	10
SLC34A2-ROS exon 34	10

Table 1. Number of copies of the recombinant plasmid required to obtain the 100% sensitivity for the detection of each translocation.

Analytical specificity

Specificity was determined through amplification of dilution series of recombinant plasmids, and cell lines. Non-specific detection was never observed. Thereby an analytical specificity of 100% is considered at limit of detection.

9.2. Diagnostic utility parameters

9.2.1 Diagnostic sensitivity and specificity

In order to determine diagnostic parameters of the kit, a comparative assessment of the ***CLART® CMA ALK·ROS.1 technique*** *versus* the reference technique (HI/FISH) was carried out. The totals of 115 samples were tested, out of which 101 samples were analyzed at GENOMICA in collaboration with following collaborating centers:

- 52 samples from University Hospital 12 de Octubre
- 24 muestras del University Hospital de Santiago
- 7 muestras del University Hospital Vall d´Hebron
- 13 samples from EQUA 2015/2016 panel

In addition the laboratory of Anatomy Pathology Department of the University Hospital 12 de Octubre participated in the validation by analyzing 19 samples (5 of which were previously analyzed in GENOMICA which is why they have not been included in the final count, resulting with 14 samples in the final count).

In 91.3% of samples (105/115) the RNA extraction yield was >10ng/µl, therefore the 100ng per PCR tube were added, as recommended. In 8.7% of the samples (10/115) the RNA extraction yield was <10ng/µl, therefore less than recommended 100ng per PCR tube was added.

Table 2 below shows the results obtained in the analysis of 115 samples:

N: 115	TP	FP	TN	FN	SENSITIVITY (%)	SPECIFICITY (%)
ALK V1 (n:11)	10	0	104	1*	90.9	100
ALK V6 (n:0)	0	0	115	0	ND	100
ALK V3a (n:2)	2	1**	113	0	100	99.1
ALK V3b (n:0)	0	0	115	0	ND	100
ALK V3a+V3b (n:11)	11***	1**	103	0	100	99
ALK V5a (n:2)	2	0	113	0	100	100
ALK V5b (n:0)	0	0	115	0	ND	100
ALK V2 (n:3)	3	0	112	0	100	100
ROS SDC4.1 + SDC4.2 (n:1)	1****	0	114	0	100	100
ROS SDC4.1 + CD74 (n:1)	0	0	114	1*	ND	100
ROS CD74 (n:1)	1	0	114	0	100	100
ROS SLC.1 (n:0)	0	0	115	0	ND	100
ROS SLC.2 (n:0)	0	0	115	0	ND	100
ALK V3b + ROS SDC4.1 (n:1)	1	0	114	0	100	100

Samples with < 100 ng/tube. The recommended amount is 100ng /tube

****Samples analyzed in reproducibility/repeatability where in 1 out of 5 repetitions FP result was obtained.***

*****In one samples in 1 out of 12 repetitions V3a was not detected. In the same samples in 3 out of 11 repetitions V3b was not detected.***

******In 1out of 5 repetitions SDC4.1 was not detected.***

Table 2. Diagnostic sensitivity and specificity of the CLART® CMA ALK·ROS.1 for each mutation detected. Sensitivity: TP/TP+FN. Specificity: TN/TN+FP. TP: True positive. FN: False negative. FP: False positive. TN: True negative. ND: Not detected, no sufficient data.

For each sample the result is considered valid when there is concordance between the reference technique and ***CLART® CMA ALK·ROS.1***. In case of discordant results between the two techniques, the valid result is considered the one obtained by Next Generation Sequencing (NGS):Oncomine Solid Tumor Fusion Panel, analysis with AmpliSeq RNA Lung Fusion single sample v5.2 (Ion Torrent, ThermoFisher).

9.2.2 Diagnostic repeatability and reproducibility

The diagnostic repeatability and reproducibility parameters of the kit were obtained by processing the samples from the extraction step up to visualization step of the protocol.

Repeatability and reproducibility parameters of ***CLART® CMA ALK·ROS.1*** **kit** are displayed in Table 3:

	% homology
Repeatability (n=32)	98.43
Reproducibility (n=28)	97.67

Table 3. ***CLART® CMA ALK·ROS.1 r***epeatability and reproducibility. n:Number of analyzed samples.

10. Control of known interferences

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

11. REFERENCES

Naidoo J. and Drilon A. Molecular Diagnostic Testing in Non-Small Cell Lung Cancer. *Am J Hematol Oncol*. 2014; 10:4-11.

Libro Blanco de la Anatomía Patológica en España 2015. Recomendaciones del Club de Patología Pulmonar de la SEAP. 433-456. SEAP-IAP.

Neal I. Lindeman, MD, Philip T. Cagle, MD, Mary Beth Beasley, MD, Dhananjay Arun Chitale, MD, Sanja Dacic, MD, PhD, Giuseppe Giaccone, MD, PhD, Robert Brian Jenkins, MD, PhD, David J. Kwiatkowski, MD, PhD, Juan-Sebastian Saldivar, MD, Jeremy Squire, PhD, Erik Thunnissen, MD, PhD, and Marc Ladanyi, MD. Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors. Journal of Thoracic Oncology®. Volume 8, Number 7, July 2013.

Kengo Takeuchi,Young Lim Choi, Manabu Soda, Kentaro Inamura,YukiTogashi,Satoko Hatano, Munehiro Enomoto, Shuji Takada, YoshihiroYamashita, Yukitoshi Satoh, Sakae Okumura, Ken Nakagawa, Yuichi Ishikawa and HiroyukiMano. Multiplex Reverse Transcription-PCR Screening for EML4-ALK Fusion Transcripts. Clin Cancer Res 2008;14(20) October 15, 2008.

Young Lim Choi, Kengo Takeuchi, Manabu Soda, Kentaro Inamura, Yuki Togashi, Satoko Hatano, Munehiro Enomoto, Toru Hamada, Hidenori Haruta, Hideki Watanabe, Kentaro Kurashina, Hisashi Hatanaka, Toshihide Ueno, Shuji Takada, Yoshihiro Yamashita, Yukihiko Sugiyama, Yuichi Ishikawa and Hiroyuki Mano. Identification of Novel Isoforms of the EML4-ALK Transforming Gene in Non–Small Cell Lung Cancer. Cancer Res 2008; 68: (13). July 1, 2008.

Kristin Bergethon, Alice T. Shaw, Sai-Hong Ignatius Ou, Ryohei Katayama, Christine M. Lovly, Nerina T. McDonald, Pierre P. Massion, Christina Siwak-Tapp, Adriana Gonzalez, Rong Fang, Eugene J. Mark, Julie M. Batten, Haiquan Chen, Keith D. Wilner, Eunice L. Kwak, Jeffrey W. Clark, David P. Carbone, Hongbin Ji, Jeffrey A. Engelman, Mari Mino-Kenudson, William Pao, and A. John Iafrate. ROS1 Rearrangements Define a Unique Molecular Class of Lung Cancers. Journal of Clinical Oncology, November 20, 2014.

Jinghui Wang, Yiran Cai, Yujie Dong, Jingying Nong, Lijuan Zhou, Guimei Liu, Dan Su2, Xi Li, Shafei Wu, Xuejing Chen, Na Qin, Xuan Zeng, Haiqing Zhang, Zongde Zhang, Shucai Zhang. Clinical Characteristics and Outcomes of Patients with Primary Lung Adenocarcinoma Harboring ALK Rearrangements Detected by FISH, IHC, and RT-PCR. PLOS ONE, July 2014, Volume 9.

Kengo Takeuchi, Manabu Soda, Yuki Togashi, Ritsuro Suzuki, Seiji Sakata, Satoko Hatano, Reimi Asaka, Wakako Hamanaka, Hironori Ninomiya, Hirofumi Uehara, Young Lim Choi, Yukitoshi Satoh, Sakae Okumura, Ken Nakagawa, Hiroyuki Mano andYuichi Ishikawa. RET, ROS1 and ALK fusions in lung cancer. Nature Medicine, volume 18, number 3, march 2012.