CLART® CMA
ALK·ROS1

GENETIC DETECTION AND IDENTIFICATION OF THE MAIN CHROMOSOMAL TRANSLOCATIONS IN THE ALK AND ROS1 GENES, IN PATIENTS WITH LUNG CANCER FOR *IN VITRO* DIAGNOSIS

CLART® CMA ALK· ROS1

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

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Contenido

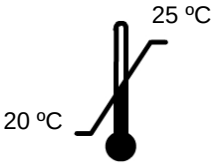
1. GLOSSARY

Attention, see instructions for use

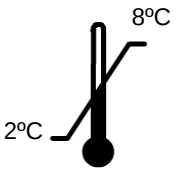
Expiration date

In vitro diagnostic medical device

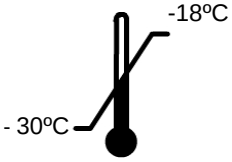
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® CMA ALK·ROS1 detects presence of the main chromosomal translocations between genes:

- ALK (Anaplastic lymphoma kinase) with EML.4 (Echinoderm microtubule-associated protein-like 4), and
- ROS.1 (Receptor tyrosine kinase) with SDC4 (syndercan 4), CD74 (Cluster of differentiation 74) and SLC34A2 (type II sodium/Phosphate cotransporter),

in patients with lung cancer.

Lung cancer patients with translocations in gene ALK or ROS1 have shown to respond favourably to specific therapy (the ALK inhibitors). Determination of ALK-positive and ROS.1-positive patients (especially in the advanced o metastatic non-small cell lung cancer patients) is essential for identifying the patients that have highest probability to benefit from this specific therapy.

Translocations detected with ***CLART® CMA ALK·ROS1*** kit are as follows:

- ***7 EML.4-ALK Translocations:***

  - Variant 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

- ***5 ROS1 Translocations:***

  ✓ SDC4-ROS.1 Translocations:

  - 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)

Starting material: RNA extracted from Formalin-fixed, paraffin-embedded (FFPE) lung biopsies (See Section 6 below).

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

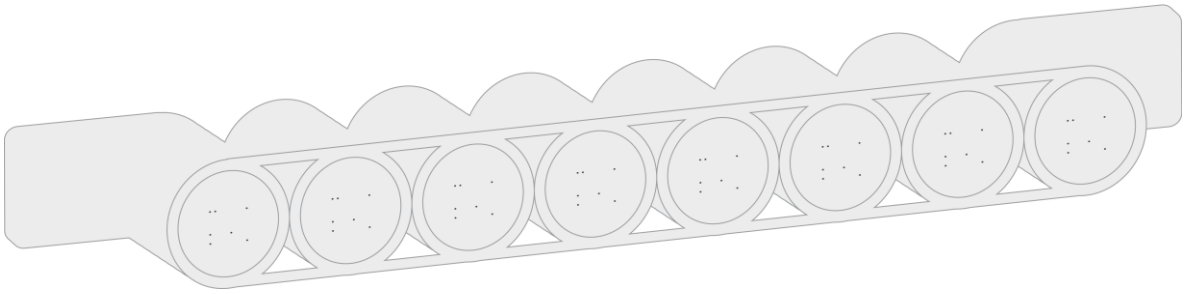


Figure 1. CLART-Strip® in the form of an 8-well strip.

A scheme of the detection system is displayed in Figure 2. Basically, 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.

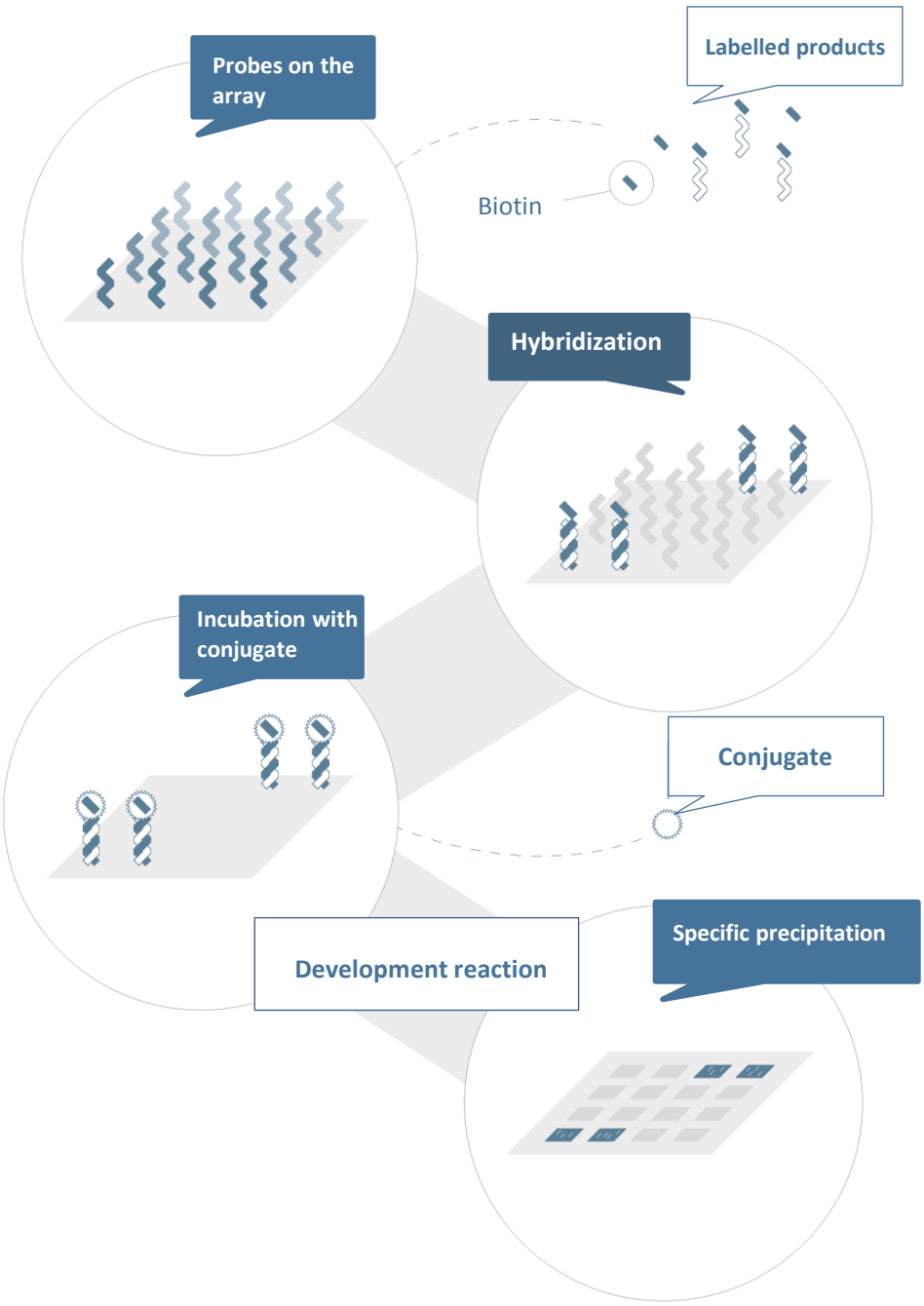


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3.1. Amplification reagents

Shipped and stored at -20ºC.

Amplification tubes are provided ready-to-use. Each amplification tube contains 43 µL of reaction mixture. Only the exact number of required tubes should be thawed on ice. Remaining ones should be kept at -20ºC.

2 amplification tubes and 1 tube of Enzyme mix are provided. Amplification tubes:

Mix 1: Blue tube. Allows detect the 7 ALK-EML.4 translocations:

ALK-EML.4 translocations
• Variant V1: E13;A20 V1 • Variant V6: E13; ins 69 • Variant V2: E20;A20 • Variant V3a: E6:A20 • Variant V3b: E6; ins 33 A20 • Varint V5a: E2;A20 • Variant V5b: E2; ins 117 A20

Mix 2: White tube. Allows detect the 5 ROS.1 translocations:

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

Both amplification tubes Mix 1 and Mix 2 contain in addition respective amplification and endogenous controls.

Enzyme mix: Ready-to-use mixture of RT (reverse transcriptase) and DNA Polymerase.

WARNING: 2 µl of Enzyme mix must be added to each Mix 1 and Mix 2 tubes before introduction of the genetic material.

Note: Boxes containing amplification tubes include a self-adhesive and irreversible temperature indicator; Red color displayed on the visualization window of the indicator means that the package has exceeded at some time the storage temperature of –20ºC 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 for detection of all translocations to be detected.

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). **Keep at Room Temperature.**

- Shipped and stored at 4ºC:

  - **DC** (Conjugate Diluent).
  - **CJ** (Conjugate Solution). Briefly centrifuge before use.
  - **RE** (Development Solution). Keep away from light.
  - **TL** (Wash Buffer).
  - **Microtiter plate adaptor and plastic lid.**

3.3 Other components

- GENOMICA's Reader **CAR®** or **CLINICAL ARRAY READER**.

  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)

- GENOMICA's **autoclart®**.
  autoclart® allows automatic processing of up to 12 CSs strips (96 samples) during the visualization step.

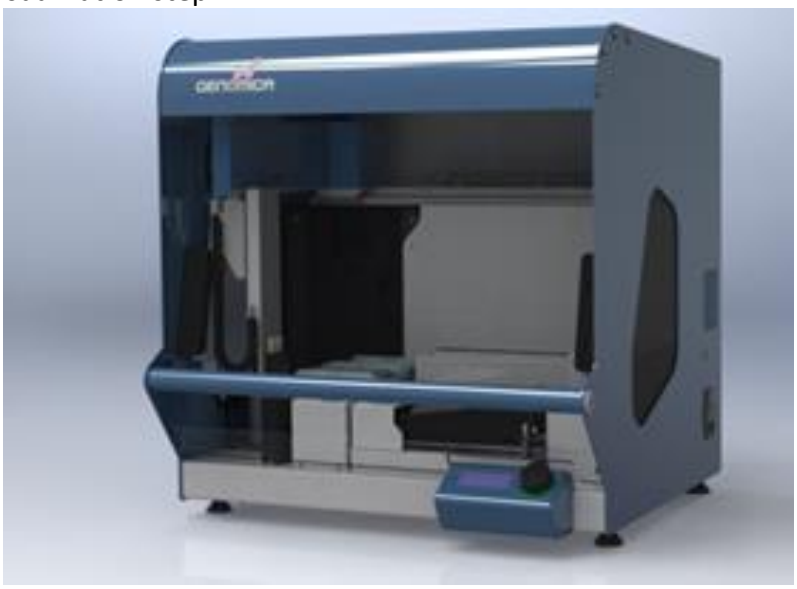


Figure 4. autoclart®

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.
- 0.2 mL tube holder.
- QIAGEN's RNeasy FFPE Kit (recommended).

4.2. Equipment

- Microcentrifuge.
- UV-visible spectrophotometer(Nanodrop).
- Thermal cycler. Exclusive use of standard "Applied Biosystems 2720 Thermal Cycler" is recommended. If making use of fast ramp thermal cyclers, use of Eppendorf's model "Mastercycler Nexus" is recommended. In all cases it is mandatory to program the thermal cycler according to instructions of Section 7.3.2. of the present manual.
- Biosafety cabinet for the pre-PCR area.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the pre-PCR area.
- One adjustable micropipette ranging from 1-20 µl, for adding genetic material to the amplification tubes.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the post-PCR area.
- Thermomixer compatible with 96-well skirted plates and adjustable shaking at 20°C, 25°C and 50ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination.

1. *CLART® CMA CMA ALK·ROS1* technique should be performed in two physically separated areas, in order to minimize sample contamination:

Pre-PCR area: Sample preparation, RNA extraction, and addition of the extracted material to the amplification tubes, are performed in this area. Always work within a biosafety cabinet, and under the strictest sterility conditions to avoid contamination.

Post-PCR area: Amplification and visualization of the amplified product are carried out in this area. The material of this Post-PCR 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 RNA 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.

9. GENOMICA cannot warrant accuracy of results obtained with the present kit under working conditions different to these.

6. MUESTRAS

CLART® CMA ALK·ROS1 kit has been designed and validated to be used with 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

Sample pre-treatment with one of the protocols described herewith is recommended, as they have been used in part of the external validations of the ***CLART® CMA ALK·ROS1*** kit.

7.1. Sample pre-treatment

Preprocessing

Once the sample is obtained, it should be kept at room temperature and the tissue fixed as soon as possible (within 1h maximum after the sample is obtained). Tissue fixation should be performed in 10% neutral buffered formalin, no alcohol or mercury based fixatives ever to be used. Optimal fixation time is of 8-24 hours for large surgical samples and 6-12 hours for small surgical samples. Cytology samples immediately fixed with standard techniques might also be used.

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

Prior to sample RNA extraction, the pathologist should perform an analysis of each cut by means of hematoxylin and eosin (H&E) 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 contained within the tumor area under study.

In order to obtain reliable results, it is recommended that tumor cells represent at least 5-10% of the sample. Lower percentages might not be assessable.

The number of cuts to be extracted will depend on the type of sample, size of the tumor tissue and the number of cells in the section, and might range between 1 and 4 cuts/ sample. It is highly recommend selecting the fragments with high levels of cellularity and less necrosis. The sample cuts might be placed on a slice or rolls. For more information about the procedure please follow the guidelines from SEAP/ SEOM.

SEAP/SEOM recommendations regarding number of cuts to be extracted depending on the sample type, size of the sample tumor area and percentage of tumor cells in the tumor area, are provided below:

- *Endoscopic biopsies:*

• 1-4 cuts 5 µm thick will be needed, taking into account that the tumor percentage should be higher than 10%.

Should the tumor sample within the paraffin block be exhausted or scarce, the slide of an already stained preparation might be removed with acetone for 10 minutes, and hydrated with 96º alcohol for 24-48 hours, should a minimum cellularity of 10% be recommended.

- *EBUS, EUS or PAAF cytology:*

Tumor percentage in the samples should be higher than 10%, and the sample processing mean selected from any of the methods below:

• Macrodissection: Withdraw the coverglass from the cytologic extension and scrape the whole surface of the slide with a blade; 1-4 slides will be needed;

• Microdissection (with laser or needle): Mark off the tumor groups at the slide, initially with a marking pen and later on with a diamond pencil. Cells are obtained with a 25G needle under microscopy control, a minimum cellularity of 500 cells being required.

- Surgical pieces:

• Mark the tumor area with highest tumor percentage, avoiding necrotic regions. Perform 1-4 cuts of 5-10 µm, and pick up regions of interest.

Deparaffinization of paraffin-embedded samples

Once the tumor area on the sample has been defined, deparaffinization of the sample, followed by extraction, should take place.

- Paraffin slices on a glass slide:

  - Immerse the slides into a cuvette filled up with xylene during 5 minutes.
  - Immerse the slide into a cuvette filled up with Ethanol 96% during 5 minutes.
  - Immerse the slide into a cuvette filled up with fresh Ethanol 96% and disrupt the tissue: Directly scrape the slide surface with a scalpel, preferably while the slide is still wet. Then introduce the collected sample into an Eppendorf tube with the assistance of Ethanol 96%.
  - Centrifuge for 8 minutes at maximum speed. Discard the supernatant.
  - Centrifuge for 4 minutes at maximum speed. Discard the supernatant. Allow the ethanol dry: Incubate 5 minutes at 56ºC or at room temperature until there are no traces of ethanol present.
  - Proceed with the RNA extraction.

- Paraffin slices placed inside a tube (slices are introduced within an Eppendorf tube without placing them on a glass slide):

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

7.2. RNA extraction

7.2.1. Specific recommendations for the steps of extraction and addition of extracted material to the amplification tube.

1. Always use gloves.
2. Clean working areas of the biosafety cabinet with a 10% diluted bleach solution.
3. Switch on the flow and UV light of the biosafety cabinet at least 20 minutes before the extraction step. Switch off the UV light when working within the cabinet.

4. Sample preparation before extraction must be carried out within the biosafety cabinet.

7.2.2. Extraction method

The use of Qiagen's RNeasy FFPE Kit following the Manufacturer's instructions is strongly recommended for RNA extraction, with the exception of incubation with Proteinase K, which should be performed during 3-5 h at 56ºC with shaking. The final elution volume should be 30 µl. An alternative kit yielding good results is Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE. Notwithstanding, alternative extraction methods might be used, in as far as they yield equivalent concentration and purity parameters as indicated below:

1. In order to achieve 100% sensitivity, the amount of RNA to be added to each RT-PCR tube should be of 100 ng total per tube (a total 200 ng are required per sample for this kit).
Lower RNA concentrations might lead to decreased sensitivity values as displayed below:

Sensitivity values obtainable depending on the ng of RNA added to each amplification tube:

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

In addition, an excess RNA might result in misdiagnosis.
Thereby, do not exceed 100 ng per RT-PCR tube, nor add more than 10 µl per tube either (5 to 10 µl of extracted RNA, depending on the RNA concentration, should be added to each amplification tube).
It is recommended to dilute samples to 20 ng/µl, in order to add 5 µl to each RT-PCR tube (for a total 100 ng).

If concentration is lower than 10 ng/µl, add 10 µl to the tube, irrespective of the final concentration that will be achieved, taking into account that the sensitivity results might be affected (see table above).

2. Extracted RNA must comply with some minimal purity levels to avoid misdiagnosis, as follows: The ratio between absorbance at 260 nm and absorbance at 280 nm should be as close to 2 as possible. If the purity level is different to this, sample should be re-extracted.

3. Extracted material must be stored at -80ºC.

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 case a false positive result might be obtained.

7.3. Amplification reaction

7.3.1. Amplification-specific recommendations

- Work in the **Pre-PCR area**, always using a laminar flow chamber and following the recommendations of Section 5.

- Keep the tubes separate and on ice during the whole process.

- Exclusive use of standard thermal cyclers only, with speed ramp of cooling/ heating of up to 2-3ºC per second, and aluminium block. Do not use fast ramp thermal cyclers. In some models of fast ramp thermal cyclers, however, the ramp speed can be reduced to 3ºC per second. This kit has been validated in two thermal cyclers compliant with these requirements: Eppendorf's "Mastercycler Nexus" and "Applied Biosystems 2720 Thermal Cycler".

- Place the amplification tubes in the thermal cycler when the block temperature is stable. In the meantime, they should be kept on ice. This is a way to prevent non-specific amplification due to incubation at temperatures below the hybridization temperature.

7.3.2. Amplification protocol

1. Thaw at 4ºC the required number of amplification tubes according to the number of samples and gene or genes to be analyzed.

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 mix several times with the micropipette.

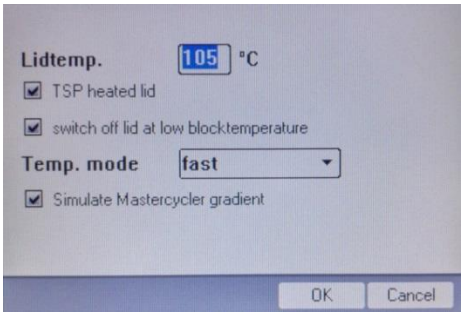
4. Add 5-10 µL of the extracted RNA to each amplification tube, according to indications of Section 7.2.2. Previously check its concentration and purity. Mix several times with the micropipette. Keep tubes at 4ºC.

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

A. *Mastercycler® Nexus Gadient (Eppendorf) model or models with heating ramp of 3ºC/sec. and cooling ramp of 2ºC/sec. in an 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 continuously until tube collection	

NOTE: Many of these Thermal cyclers provide different PCR options. If available, select the "fast" option, as displayed below for the *Mastercycler® Nexus* model:



B. *Applied Biosystems 2720 Thermal Cycler model, or models with a heating and cooling ramp of 2,7 ºC/sec. in an 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 continuously until tube collection	

6. Start the program and place the tubes in the thermal cycler when the temperature of the block has reached a stable value. In the meantime keep the tubes on ice.

The amplified product must be visualized within five days maximum to avoid its degradation. Store at 4ºC until use.

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. Clean the thermal cycler with a 10% diluted bleach solution before starting the denaturation program. Place the amplification tubes separate in the thermal cycler during denaturation and do not exceed the denaturation time of 10 minutes.

3. Dispense all solutions to the CS wall, never to the bottom of it.

4. Avoid touching the bottom of the well with the pipette or vacuum suction tip, as this might damage the probes of the bottom of the well.

5. Do not allow arrays to dry completely, any time before reading.

6. SH forms crystals at room temperature, so it must be warmed up at 59ºC before use until homogeneity. SH should not be added until just before the time of addition of the denatured PCR products, and it should be kept at 59ºC until then.

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

8. 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.

9. Avoid foaming when adding reagents.

10. 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.

11. Prepare diluted TL immediately before use; do not reuse previously prepared solutions.

12. During visualization it is not necessary to use filtered tips, but different tips should be used for different wells, and for adding different reagents, even TL.

13. It is, however, necessary to use filtered tips for adding the amplified products to the CS well.

14. 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.

15. 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.

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

7.4.2. Manual visualization protocol

1. Denaturation of amplified products: Place the amplification tubes in the thermal cycler when it has reached 99ºC, and incubate at 99ºC **for exactly 8 minutes**. **Under no circumstance the denaturation time might exceed 10 minutes.** After that, remove the tubes from the 99ºC incubation and immediately place on ice or at 4ºC.

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

3. Prewash of the **CS**: Place the necessary **CS** units on the Microtiter plate adaptor. Add 200 µL of diluted TL to each CS well before use. Mix the solution up and down with a multichannel pipette 10-15 times. It is advisable to carry out this wash during the step of denaturation of amplified products, and to leave the washing solution in the **CS** wells until addition of the above-mentioned products. Discard the washing solution with a pipette or preferably with a vacuum pump.

   Wells must be totally free of residues, although they must never remain dry for too long. Thus the importance of immediate addition of the next solution.

4. Hybridization Step: Once the amplified products have been denatured, discard the washing solution from the wells with a vacuum pump. Immediately after that, add 100 µL of 59ºC-pre-warmed SH to each CS well, avoiding foaming.

   Add, **to the same CS well**, the following volumes of denatured PCR product from the respective amplification tubes that have been used for a same sample:

   Mix 1: 5 µl

Mix 2: 5 µl

For the correct interpretation of the results, it is mandatory to visualize all the tubes of the same sample in the same CS well.

Mix the solution up and down several times, being careful not to touch the bottom of the well. It is advisable that each strip should be handled apart from the rest, to avoid contamination. Cover the Microtiter plate adaptor and the CSs with the plastic lid and incubate in the thermomixer for 60 min at 59ºC and 550 rpm.

After incubation, remove the plate from thermomixer and aspirate incubation solution from the CS wells with a pipette or a vacuum pump. The CS must be totally free of residues. Add the next solution immediately.

Set the thermomixer at 20ºC with shaking, for its further use in step 6 below. For a faster Temperature decrease, the lid may be removed.

5. Double Wash: Add 200 µL of diluted TL to each well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the Washing solution with a pipette or preferably with a multichannel vacuum pump. Repeat the procedure. Use different tips for each well in both washes. Keep the samples on the Washing solution until the thermomixer reaches 20ºC.

6. Blocking and conjugate incubation: Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ Solution by adding **15 µL of CJ** to **1 mL of DC** per CS. This solution should be prepared 5 minutes before the end of the hybridization step.

   Aspirate the diluted TL from the wells without leaving any residue, 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 pipette or multichannel vacuum pump. Set the thermomixer at 25ºC without shaking for its use on step 8. For a faster temperature decrease, the lid may be removed.

7. Triple wash: Immediately after step 6, add 200 µL of diluted TL to each well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the diluted TL with a pipette or vacuum pump trying to remove as much liquid as possible. Repeat the procedure **two more times**. It is **essential** to thoroughly clean the wells from any residue of diluted CJ Solution.

8. Development: Thoroughly remove the diluted TL from the wells; Next, add **100 µL** of RE to each well and incubate in the thermomixer for **10 minutes at 25ºC without shakin**g (Make sure that the thermomixer has reached 25ºC).

Completely discard RE using a pipette or vacuum system. 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.

7.4.3. autoclart® visualization protocol®

1. Switch on the autoclart® unit and follow the instructions on the screen.
2. Close the door and press the button.
3. Select “Run” at the main “Menu” screen.
4. Select the assay: ***CLART® ALK·ROS1***.
5. Select the well of the strip in which to start: A1 or E1, the latter in case of using CSs where the first 4 wells have previously been used.
6. Select the number of samples. autoclart® allows to process from 4 to 96 samples. The number of samples must be a multiple of 4.
7. Check that both the indicated number of samples and the initial well (A1 or E1) are correct.
8. Place the complete rack of tips in its corresponding position.
9. Check that the tip and waste through containers are empty.
10. Fill the water bottle with 250 ml distilled water.
11. Add the reagent volumes corresponding to the number of samples to be processed, as requested by autoclart®:

    TL. Volume showed in the display indicates the required diluted TL. Diluted TL is a 1:10 dilution of TL into distilled water.

    CJ. Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ solution as displayed in the screen. This is done by adding **15 µL of CJ** to **1 mL of DC** (amounts suitable for one CS). Vortex the diluted solution in order to mix it up properly.

    RE. Add the RE volume indicated on the display.

12. Denaturation of amplified products: Place the amplification tubes in the thermal cycler after it has reached **99ºC**, and incubate at **99ºC for exactly 10 minutes**. After that, remove the tubes from the 99ºC incubation and immediately place on ice or at 4ºC. Important: Denature PCR products before preparing the visualization reagents in the autoclart®.

13. **SH.** Add the volume of 59ºC tempered SH displayed in the screen.

    WARNING: It is essential to add SH at this point. If added in a previous step, SH's temperature might decrease, affecting probe intensity and leading to false negative results.

14. Close the door and press "Play" to start. The device will perform pre-wash of the CSs and the addition of SH. A beeping signal will indicate the moment of sample addition. The beeping will stop when user opens the device door.

15. For sample addition, place the CSs out of the autoclart® and add to the same CS well the volumes of denatured amplification products of each tube corresponding to a same sample, as follows:

    Mix 1: 5 µL
    Mix 2: 5 µL

    Resuspend several times in order to thoroughly mix with SH, making sure the bottom of the well is not touched. Put back the plate into autoclart® and press the arrow on the screen to continue with the visualization process.

16. Once the visualization process is over, the autoclart® will beep until the device door is opened by the user to retrieve the CSs and read them with the CAR®.

    WARNING: Once autoclart® visualization is complete, immediate reading of the results in the CAR® is required. Otherwise, false negative results due to loss of probe intensity might occur.

17. Place the plate in the CAR® for the read-out of all wells. The CAR® will read and report the results automatically.

8. RESULTS

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

This kit allows independent analysis of ALK (Mix 1, blue tube) and ROS1 (Mix 2, White tube) translocations, issuing independent results for each Mix. Notwithstanding, in case of analysis of a same sample with both tubes, results of both the amplification tubes are to be visualized within the same CS well.

A negative extraction control should be included to verify that samples have not undergone contamination during any of the extraction, amplification or visualization processes, that might lead to a false positive.

Each amplification tube contains its own amplification and extraction control to make sure that there is enough genomic material to carry out the test, and for an independent analysis.

The genomic RNA extraction control is necessary for confirming true negative results, as it reports patient RNA presence in the sample, even when no mutation has been amplified.
The internal amplification control will allow distinguish between cases of inhibition of the PCR reaction or sample not analyzed, and those of RNA absence in the sample.

Table 1, below, displays obtainable results, corresponding explanations and solutions:

Result	**Explanation**	**Solution:** Repeat…
NO RNA	• Non-valid extraction: Presence of inhibitors or a mechanic default in sample extraction did not allow amplification of translocations and/ or of the internal amplification and DNA extraction controls.	• …all the process.
PCR INHIBITED/ NOT ANALYZED	• Non-valid amplification: Absence of amplification in one of the tubes and presence in the other, reports a correct extraction but a default in amplification of the former.	• … amplification of the corresponding tube, and subsequent steps.

NON-CONCLUSIVE	• Very different results obtained with replicas of the same probe.	• …amplification and subsequent steps.

Table 1.

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. 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 DNA 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.

9.2. Technical specifications

9.2.1. Analytical parameters

Analytical sensitivity.

Analytical sensitivity was determined through amplification of serial dilutions of DNA recombinant plasmids corresponding to each of the mutations detectable with the kit. Visualization was performed in CS. Results are displayed below:

TRANSLOCATION	Copies/5 µl
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 2. Relation of the number of copies of recombinant plasmid required for obtaining 100% sensitivity in the detection of each translocation.

Analytical specificity.

Specificity tests were performed for recombinant plasmids and cell lines without translocation, no non-specific detection ever been observed. An analytical specificity value of 100% is thereby considered at limit of detection.

9.2.2. Diagnostic utility parameters

In order to determine the diagnostic utility parameters of the kit, comparative studies were performed between ***CLART® CMA ALK·ROS1*** kit *versus* the reference technique (HI/FISH). A total 115 samples were analyzed, 101 of which were analyzed at GENOMICA with samples provided by different collaborating centers:

- Hospital Universitario 12 de Octubre de Madrid, Spain: 12 samples.
- Hospital Universitario de Santiago, Spain: 24 samples.
- EQUA 2015/2016 Panel: 13 samples.
- Hospital Universitario Vall d'Hebron, Barcelona, Spain: 7 samples.

In addition, a collaboration was established with the Department of Pathological Anatomy of the Hospital Universitario 12 de Octubre de Madrid, within which 19 samples were analysed. 5 of them had already been analysed at GENOMICA, this being the reason why they were not taken into account in the final number of analysed samples, which turned out to be 14.

In 91.3% of the samples (105/115) the RNA extraction yield was > 10 ng/µl, the recommended 100 ng/RT-PCR tube being added. In 8.7 % of the samples (10/115) the RNA extraction yield was < 10 ng/µl, a lower amount than the recommended 100 ng/ RT-PCR tube being added.

Results of the analysis of the 115 samples are displayed in Table 3.

N: 115	VP	FP	VN	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

Sample with < 10 0 ng/tube. Below the recommended 100 ng/tube
***** Samples analyzed in reproducibility/repeatability where in 1 out of 5 repetitions a FP result was obtained.***
******* V3a is not detected in 1 out of 12 samples. V3b is not detected in 3 out of 11 samples.***
******** SDC4.1 is not detected in 1 out of 5 samples.***

Table 3: Diagnostic Sensitivity and Specificity of the *CLART® CMA ALK·ROS1* technique for each translocation. VPP: Positive predictive value (VP/VP+FP). VPN: Negative predictive value (VN/VN+FN). Sensitivity: VP/VP+FN. Specificity: VN/VN+FP.VP: True positive. FN: False negative. FP: False positive. VN: True negative. ND: Not detected, non-sufficient data.

Identical results obtained with the reference technic and ***CLART® CMA ALK·ROS1***, are considered valid results. In case of discordant results between the two techniques, the result obtained by sample sequencing through Next Generation Sequencing (NGS), Panel Oncomide Solid Tumor Fusion, análisis con AmpliSeq RNA Lung Fusion single sample v5.2 (Ion Torrent, ThermoFisher), was considered as the valid one.

Diagnostic repeatability and reproducibility.

Diagnostic repeatability and reproducibility have been tested from the step of biopsy extraction until CS visualization of the amplified material.

Obtained data are displayed in the following table:

	% homology
Repeatability (n=22)	92.5
Reproducibility (n=23)	94.3

Table 4: Diagnostic repeatability and reproducibility of the *CLART® CMA ALK·ROS1* kit.

10. REFERENCES

"PLX4032, a selective BRAF (V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells".
Halaban R, Zhang W, Bacchiocchi A, Cheng E, Parisi F, Ariyan S, Krauthammer M, McCusker JP, Kluger Y, Sznol M.
Pigment Cell Melanoma Res. 2010 Apr;23(2):190-200. doi: 10.1111/j.1755-148X.2010.00685.x. Epub 2010 Feb 10.

"Advances in personalized targeted treatment of metastatic melanoma and non-invasive tumor monitoring".
Klinac D, Gray ES, Millward M, Ziman M.
Front Oncol. 2013 Mar 19;3:54. doi: 10.3389/fonc.2013.00054. eCollection 2013.

"*Effects of AKT inhibitor therapy in response and resistance to BRAF inhibition in melanoma*".
Lassen A, Atefi M, Robert L, Wong DJ, Cerniglia M, Comin-Anduix B, Ribas A.
Mol Cancer. 2014 Apr 16;13:83. doi: 10.1186/1476-4598-13-83.

"*Detection of BRAF V600 mutations in melanoma: evaluation of concordance between the Cobas® 4800 BRAF V600 mutation test and the methods used in French National Cancer Institute (INCa) platforms in a real-life setting*".
Mourah S, Denis MG, Narducci FE, Solassol J, Merlin JL, Sabourin JC, Scoazec JY, Ouafik L, Emile JF, Heller R, Souvignet C, Bergougnoux L, Merlio JP.
PLoS One. 2015 Mar 19;10(3):e0120232. doi: 10.1371/journal.pone.0120232. eCollection 2015.

"Recomendaciones para la determinación de biomarcadores en el melanoma metastásico. Consenso Nacional de la Sociedad Española de Anatomía Patológica y de la Sociedad Española de Oncología Médica".
José Luis Rodríguez-Peralto, Enrique Espinosa, Juan José Ríos-Martín, Alfonso Berrocal, María Dolores Lozano, Ana Arance, Angel Santos-Briz, José Antonio López-Martín, María Teresa Fernández-Figueras y Salvador Martín-Algarra.
Rev Esp Patol. 2014;47(1):9-21.

"Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma".
Trunzer K, Pavlick AC, Schuchter L, Gonzalez R, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Kim KB, Weber JS, Hersey P, Long GV, Lawrence D, Ott PA, Amaravadi RK, Lewis KD, Puzanov I, Lo RS, Koehler A, Kockx M, Spleiss O, Schell-Steven A, Gilbert HN, Cockey L, Bollag G, Lee RJ, Joe AK, Sosman JA, Ribas A.
J Clin Oncol. 2013 May 10;31(14):1767-74. doi: 10.1200/JCO.2012.44.7888. Epub 2013 Apr 8.