



**CLART® CMA
NRAS·i·KRAS**

**GENETIC DETECTION AND IDENTIFICATION OF POINT MUTATIONS
IN TWO GENES OF THE EGFR PATHWAY ASSOCIATED TO COLORECTAL
CANCER- –KRAS and NRAS-
FOR *IN VITRO* DIAGNOSIS**

CLART® CMA NRAS-iKRAS

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

CE Mark



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1. GLOSSARY



Attention, see instructions for use



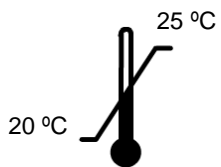
Expiration date



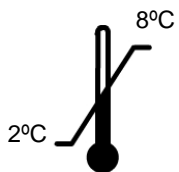
In vitro diagnostic medical device



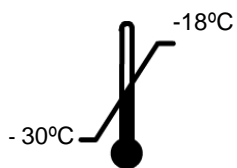
Lot



Store at room temperature



Store at 2°C to 8°C



Store at -30°C to -18°C

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® CMA NRAS-iKRAS detects the most prevalent point mutations of gene NRAS, as well as some some infrequent mutations of KRAS gene, all these mutations associated to colorectal cancer. Both genes belong to the Epidermal Growth Factor Receptor (EGFR) pathway. The point mutations detected by the kit are as follows:

NRAS: G12D
Q61H (183 A>T)
Q61R
Q61K
Q61L

KRAS: Q61H (183 A>C)
K117N (351 A>C)
K117N (351 A>T)
A146V
A146T

Starting material: Formalin-fixed, paraffin-embedded (FFPE) colorectal biopsies (See Section 6 below).

Detection is based on our CLART® technology: End-point Multiplex PCR amplification, mutation specific and that yields a variable fragment for each mutation of between 100-200 base pairs, followed by visualization in low-density microarray.

4 amplification tubes are provided, distinguished by their colour: (i) white tube with green-coloured master mix, (ii) white tube with colourless master mix, (iii) red tube, and (iv) yellow tube.

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

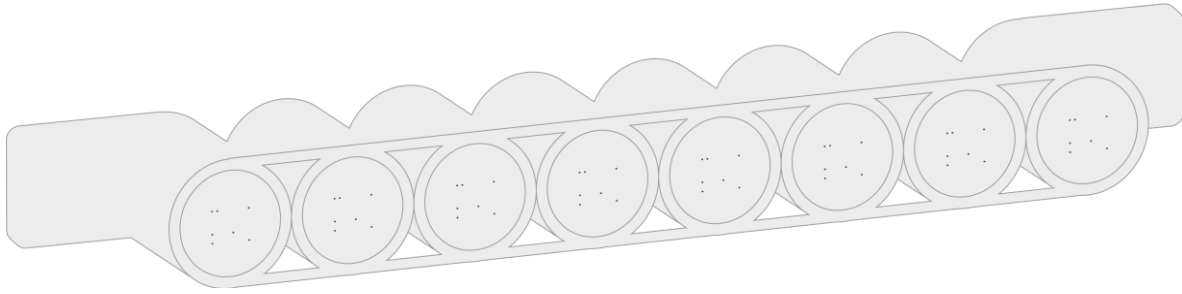


Figure 1. CLART-Strip® in the form of an 8-well strip.

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.

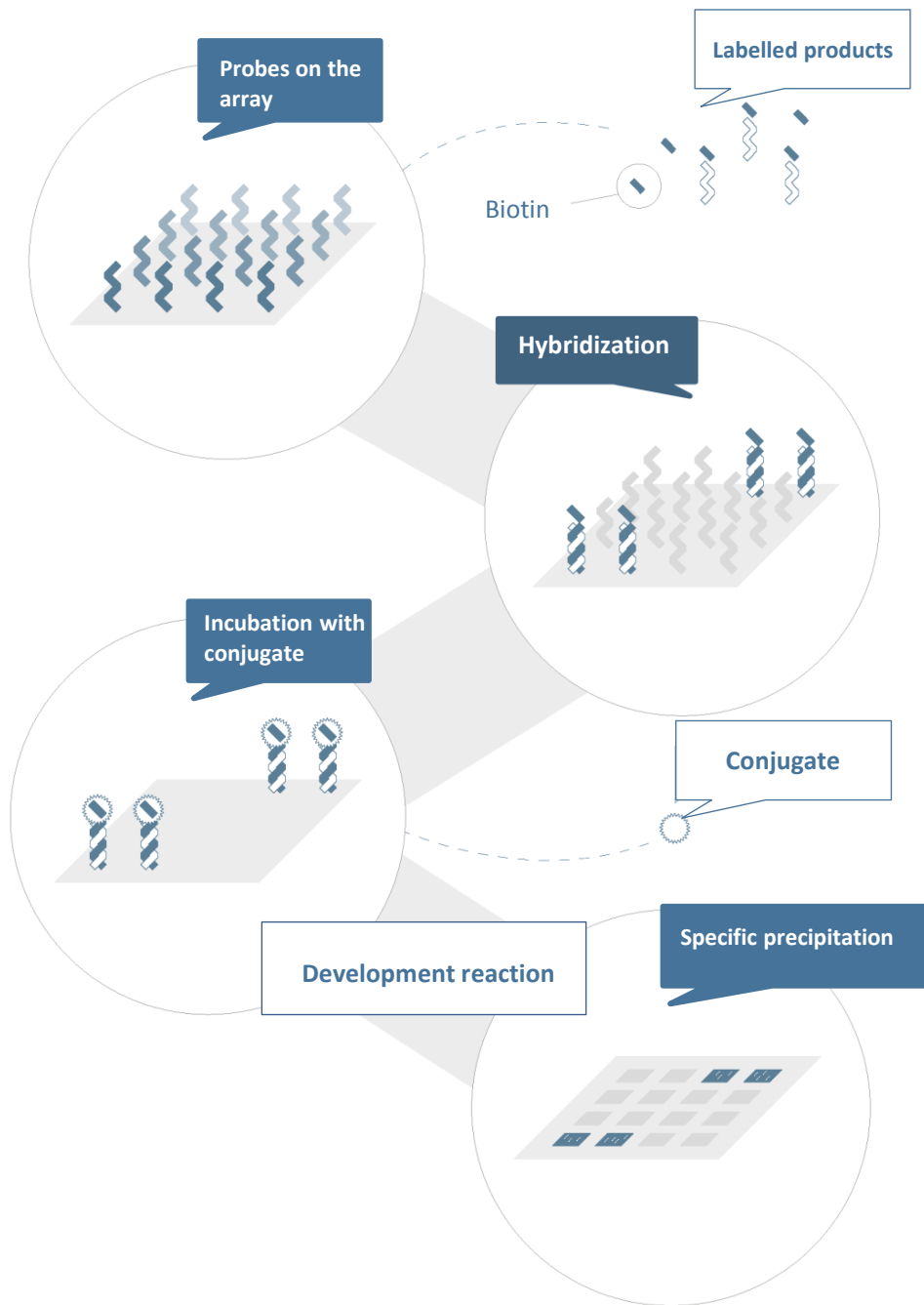


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3. KIT COMPONENTS AND STORAGE

CLART® CMA NRAS-iKRAS kit contains enough reagents for the analysis of 8 or 24 clinical samples. 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 -20°C.

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

4 amplification tubes are provided for analysis of the *NRAS* and *KRAS* genes: Mix 1: White tube with green-coloured master mix; Mix 2: White tube with colourless master mix; Mix 3: Red tube; and Mix 4: Yellow tube.

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 -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 for detection of all mutations 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. 25°C maximum) and protected from direct light and high temperatures.

For small numbers of samples, a pre-cut format of the 8-well CS might be provided, which enables cut the strip into two identical halves and visualize samples in multiples of four. In such a case, a reusable Microtiter plate adaptor and a set of magnetic adapters will be provided to allow immobilization of the four-wells strips (see figure 3).

- **SH** (Hybridization Solution). **Keep at Room Temperature.**

- Shipped and stored at 4°C:

- **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

- Microtiter plate and set of magnetic adapters to insert already cut four-well CSs (Figure 3).

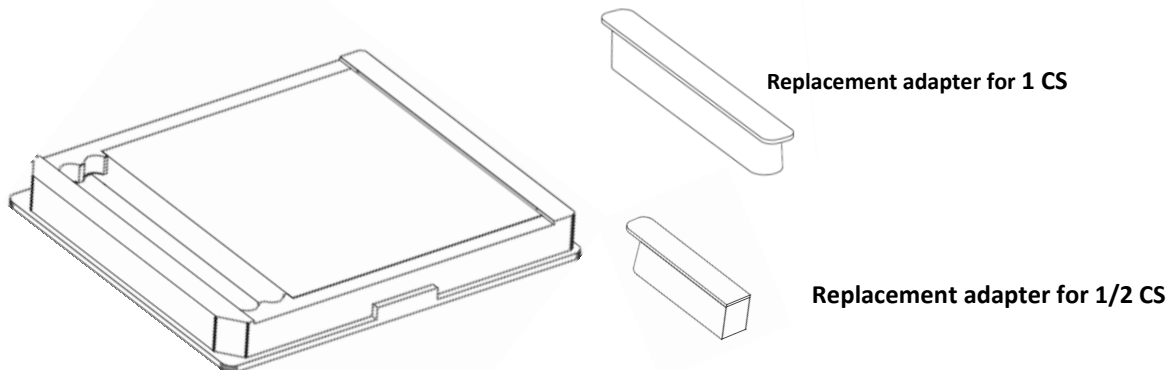


Figure 3. Microtiter plate and set of magnetic adapters for visualization of a number of samples multiple of four.

- 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 4. CAR® (CLINICAL ARRAY READER)

- GENOMICA's **autoclart®**.

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

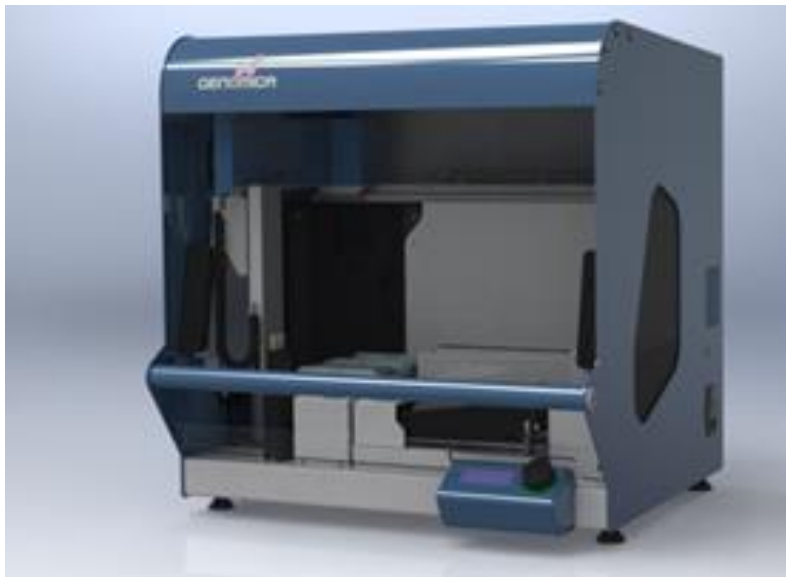


Figure 5. autoclart®

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.

4.2. Equipment

- Microcentrifuge.
- UV-visible spectrophotometer.
- Thermal cycler.
- Biosafety cabinet.
- Three adjustable micropipettes ranging from 1-20 μ L, 20-200 μ L, and 200-1000 μ L for the pre-PCR area.
- One adjustable micropipette ranging from 1-20 μ l, for adding genetic material to the amplification tubes.
- Three adjustable micropipettes ranging from 1-20 μ L, 20-200 μ L, and 200-1000 μ L for the post-PCR area.
- Thermomixer compatible with 96-well skirted plates and adjustable shaking at 20°C, 25°C and 50°C.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

1. CLART® CMA NRAS-iKRAS technique should be performed in two physically separated areas, in order to minimize sample contamination:

Pre-PCR area: Sample preparation, DNA 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 DNA addition to the amplification tubes.

3. 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.

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

5. Use disposable and autoclaved laboratory material.

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

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

8. Discard the micropipette tip after each pipetting.

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

6. SAMPLES

CLART® CMA NRAS-iKRAS kit has been designed and validated to be used with genetic material extracted from colorectal cancer biopsies.

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

7. WORKING PROTOCOL

CLART® CMA NRAS-iKRAS kit has been validated making use of two different protocols of sample pre-treatment.

7.1. Sample pre-treatment

Preprocessing

FFPE sections should be placed on a glass slide for examination by the pathologist. Each sample should be processed with new sterile scalpel.

The pathologist will perform a study of each cut and make use of hematoxylin and eosin (H&E) staining to define and verify the tumor area as percentage (%) of tumor cells.

Next, the following instructions should be followed:

- For tumor cell percentages <50% and a small area: take 6 cuts of 10 µm each.
- For tumor cell percentages >50% and a large area: take 2-4 cuts of 10 µm each.
- For large tissue amounts, regardless of tumor percentage: take 1 cut of 10 µm.
- For endoscopic biopsies (very small): take 10 cuts of 10 µm each.

Place all the cuts within a 1.5 mL tube.

Next, choose one of the following two protocols:

PROTOCOL 1.

1. Add 1 ml xylene to the tube containing the sample and vortex it for 5 sec.
2. Incubate at Room Temperature for 10 min.
3. Centrifuge the tube at 13.200 rpm for 5 min.
4. Discard supernatant.
5. Add 1ml 96-99% Ethanol and vortex it for 5 sec.
6. Centrifuge the tube at 13.200 rpm for 2 min.
7. Discard supernatant.

8. Incubate at 56°C for 15 min. in order to allow any remaining supernatant evaporate (dry pellet).
9. From this step on, please follow the protocol outlined in Qiagen's QiAmp DNA FFPE Tissue Kit. The final elution volume should be 50 µl.

PROTOCOL 2.

Day 1.

1. Centrifuge the tube containing the sample for 1 minute at 13.200 rpm, in order to get all FFPE cuts at the bottom of the tube.
2. Heat the sample in the thermomixer for 5 min at 75°C without shaking.
3. Add 190 µl of G2 lysis buffer of Qiagen's EZ1 DNA Tissue Kit to each sample.
4. Incubate at 75°C for 5 min, shaking at 1400 rpm.
5. Set the thermomixer temperature at 56°C.
6. Add 25 µl of Proteinase K, previously prepared according to the user's manual of above-mentioned kit (20 mg/ml).
7. Incubate overnight at 56°C and 1.400 rpm with shaking.

Day 2.

1. Add 10µl of Proteinase K and vortex briefly.
2. Incubate overnight at 56°C and 1.400 rpm with shaking.

Day 3.

1. Centrifuge the tube for 1 minute at 13.200 rpm.
2. Add 2 µl of glycogen at 20 mg/ml (optional, suitable for samples with scarce prior extraction material).
3. Collect supernatant.
4. Should there be any remaining tissue, homogenize with the pipette tip.
5. Decant supernatant within the specific extraction tube of Qiagen's QIAamp DNA FFPE Tissue Kit.
6. Place the specific tube at the BIO-ROBOT EZ1, in order to proceed with DNA extraction. Card must be "paraffin DNA", and elution volume must be set at 50 µl.

7.2. Genetic material 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

Any extraction method yielding the concentration and purity parameters of below might be used:

1. The total amount of genetic material to be added to the PCR tube must be of 150 ng. Excess or default DNA might lead to misdiagnosis.

Never exceed the 150 ng amount nor a 10 μ l volume per each PCR tube (5-10 μ l to be added to each amplification tube depending on the extracted DNA concentration).

The following table displays all the possible situations:

Extracted DNA (ng/μl)	Volume to be added to each PCR tube (μl)	Total amount of DNA added to each PCR tube (ng)
30	5	150
25	6	150
21.5	7	150
19	8	150
17	9	150
15	10	150

If concentration is lower than 15 ng/ μ l the sample should be re-extracted.

2. Extracted DNA 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. The extracted material should be kept at 4°C, if processed immediately. Otherwise, it should be stored at -20°C.

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.

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.
- Add the DNA always within a biosafety cabinet. 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 2-3°C per second, and aluminum block. Do not use fast ramp thermal cyclers. This kit has been validated in two thermal cycler models which fulfil these requirements: Mastercycler® Nexus (Eppendorf), and Applied Biosystems 2720. In some models of fast ramp thermal cyclers, however, the required conditions might be accomplished.
- Place the amplification tubes in the thermal cycler when the block has reached 95°C. 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.

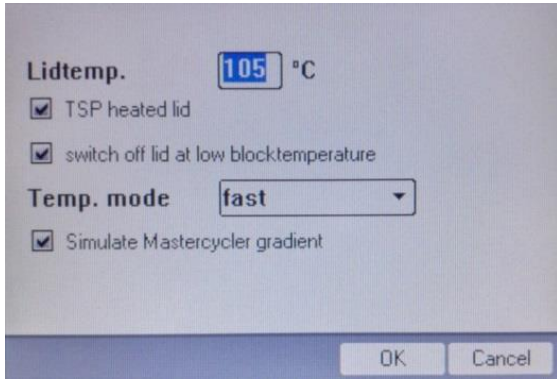
7.3.2. Amplification protocol

1. Thaw at 4°C the required number of amplification tubes according to the number of samples 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 5-10 µL of the extracted DNA according to indications of Section 7.3.1, and mix several times with the micropipette. Keep tubes at 4°C.
4. Program the following temperature cycles on the thermal cycler. Proceed according to the instructions below, depending on the Thermal cycler model being used:

A. Mastercycler® Nexus (Eppendorf) model or models with heating ramp of 3°C/sec. and cooling ramp of 2°C/sec. in an aluminium block:

1 cycle	99°C 10 min
40 cycles	94°C 60 sec 62°C 60 sec
1 cycle	72°C 10 min
4°C continuously until tube collection	

Many of these Thermal cyclers provide different PCR options. If available, select the “fast” option, as displayed below for the Mastercycler® Nexus model and Simulate Mastercycler gradient.



B. Applied Biosystems 2720 model, or models with a heating and cooling ramp of 2,7 °C/sec. in an aluminium block:

1 cycle	99°C 10 min
40 cycles	94°C 60 sec 62°C 60 sec
1 cycle	72°C 10 min
4°C continuously until tube collection	

5. Start the program and place the amplification tubes in the thermal cycler when the temperature of the block is above 90°C. In this way non-specific amplifications that might result from incubation below the hybridization temperature, are avoided.

The amplified product must be visualized within five days maximum to avoid its degradation. Store at 4°C 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. It is advisable to check accuracy of the thermomixer before starting the assay, by means of a thermocouple in direct contact with the thermomixer plate at temperatures of 20 °C, 25 °C and 50 °C.
3. 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.

4. The PCR product should only be denatured once for hybridization. Do not make use of any PCR product denatured more than once for visualization. If necessary, aliquote prior to the denaturation step.
5. In case of interest in visualizing samples in multiples of four, the CS strip should be cut in half by pressing in the direction of arrows as indicated in Figure 6. The CS halves should be placed within the Microtiter plate with the aid of the system of magnetic adapter, as displayed in figure 7. Additional ways to immobilize the CSs within the Microtiter plate, are displayed on the Microtiter plate surface.

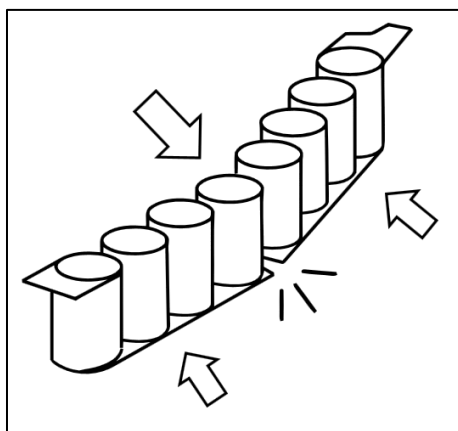


Figure 6: CS cutting scheme

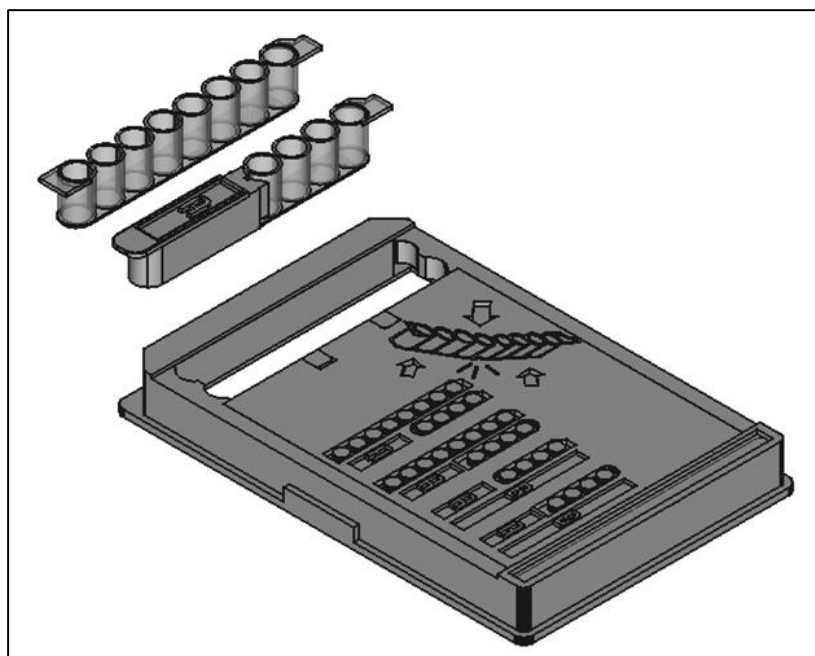


Figure 7: CS immobilization scheme to the Microtiter plate

6. Do not allow arrays to dry completely, any time before reading.
7. 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.
8. Dispense all solutions to the CS wall, never to the bottom of it.
9. SH forms crystals at room temperature, so it must be warmed up at 50°C until homogeneity before use. Warming should last at least 30 minutes and no more than 60 minutes. SH should not be added until just before the time of addition of the denatured PCR products, and it should be kept at 50°C until then.
10. Make sure that the thermomixer temperature has been 50°C for at least 60 minutes before the hybridization step starts.
11. 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.
12. Avoid foaming when adding reagents.
13. Turn on the CAR® before starting the whole procedure. Self-calibration of the equipment may last for a few minutes.
14. Prepare diluted TL immediately before use; do not reuse previously prepared solutions.
15. 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.
16. It is, however, necessary to use filtered tips for adding the amplified products to the CS well.
17. 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.
18. 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.
19. 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 95°C, and incubate at 95°C **for exactly 8 minutes**. After that, remove the tubes from the 95°C incubation and immediately place on ice or at 4°C. **Under no circumstance the denaturation time might exceed 10 minutes.**
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 50°C-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 NRAS-iKRAS: 5 µL

Mix 2 NRAS-iKRAS: 5 µL

Mix 3 NRAS-iKRAS: 5 µL

Mix 4 NRAS-iKRAS: 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, even if corresponding to different genes.

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 50°C and 550 rpm. The thermomixer should have previously been pre-heated to 50°C. Follow recommendations of Section 7.4.1.

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 20°C 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 20°C.
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 20°C 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 25°C without shaking for its use on step 8. For a faster temperature decrease, the lid may be removed.

7. Triple wash: Immediately after, remove the diluted CJ solution and 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 CS wells; Next, add **100 µL** of RE to each well and incubate in the thermomixer for **10 minutes at 25°C without shaking** (Make sure that the thermomixer has reached 25°C and that it is used without shaking).
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. Turn on the CAR[®] before starting the whole procedure. Self-calibration of the equipment may last for a few minutes.
2. Denaturation of amplified products: Place the amplification tubes in the thermal cycler after it has reached 95°C, and incubate at 95°C for exactly 8 minutes. After that, remove the tubes from the 95°C incubation and immediately place on ice.

This step should precede the reagent preparation step in the autoclart®

3. Switch on the autoclart® unit and follow the instructions on the screen.
4. Close the door and press the button.
5. Select “Run” at the main “Menu” screen.
6. Select the assay: **CMA**.
7. 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.
8. Select the number of samples. autoclart® allows to process from 4 to 96 samples. The number of samples must be a multiple of 4.
9. Check that both the indicated number of samples and the initial well (A1 or E1) are correct.
10. Place the complete rack of tips in its corresponding position.
11. Check that the tip and waste through containers are empty.
12. Fill the water bottle with 250 ml distilled water.
13. 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.

SH. Add the volume of 50°C 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 5 µL of denatured amplification product of each and all tubes corresponding to a same sample to the same CS well.
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®.

For a correct result interpretation, the sample must be processed with all the amplification tubes corresponding to a same sample, and visualized within a 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 PCR tube contains its own amplification and extraction control to make sure that there is enough genomic material to carry out the test.

The genomic DNA extraction control is necessary for confirming true negative results, as it reports patient DNA 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, and those of DNA absence in the sample.

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

Result	Explanation	Solution: Repeat...
NOT ANALYZED	<ul style="list-style-type: none"> Non-valid extraction: Presence of inhibitors or a mechanic default in sample extraction did not allow amplification of mutations and/ or of the internal amplification and DNA extraction controls. 	<ul style="list-style-type: none"> ...all the process.
	<ul style="list-style-type: none"> Non-valid amplification: Absence of amplification in one of the tubes and presence in the others, reports a correct extraction but a default in amplification of the former. 	<ul style="list-style-type: none"> ...amplification of the corresponding tube, and subsequent steps.
NON-CONCLUSIVE	<ul style="list-style-type: none"> Very different results obtained with replicas of the same probe. 	<ul style="list-style-type: none"> ...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 genetic material (insufficient sample quantity, DNA degradation, inadequate storage or loss of genetic material during extraction), or to the presence of DNA polymerase inhibitors in the samples to be processed (alcohol, salts, etc.). **CLART® CMA NRAS-iKRAS** kit avoids these false negatives through addition of internal amplification controls to the tubes, for amplification efficiency checking.

In addition, 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. Each plasmid had as an insert one amplification product (including the sequence complementary to the corresponding detection probes).

Above-mentioned sensitivity was also determined through amplification of serial dilutions of commercial cell lines bearing the mutation to be determined. Visualization was performed in CS. Results are displayed in Table 2 below:

NRAS-iKRAS Point Mutations	Number of copies/5 µl of clone per PCR reaction	Amount of cell line DNA
KRAS		
Q61H2	10000	
K117N1	1000	
K117N2	10000	
A146T	10000	5 ng
A146V	1000	

NRAS		
Q61R	1000	1 ng
G12D	1000	
Q61H1	10000	1 ng
Q61L	10000	1 ng
Q61K	1000	1 ng
AS146T	10000	
K117N1	1000	

Table 2. Relation of the number of copies of recombinant plasmid or nanograms of cell line required for obtaining 100% sensitivity in the detection of each mutation.

Analytic specificity.

Analytical specificity tests were performed for 15 recombinant plasmids and cell lines, no non-specific detection of different mutations to the targeted one ever been observed. An analytical specificity value of 100% is thereby considered.

9.2.2. Diagnostic utility parameters

Diagnostic sensitivity.

In order to determine the diagnostic utility parameters of the kit, comparative studies were performed between **CLART® CMA NRAS-iKRAS** kit *versus* the reference technique (pyrosequencing). Above-mentioned evaluation was carried out in collaboration with the following centers:

- Department of Pathological Anatomy of the Hospital Universitario Vall d’Hebron, Barcelona, Spain.
- Department of Pathological Anatomy of the Hospital Universitario 12 de Octubre de Madrid, Spain.
- Department of Pathological Anatomy of the Hospital Universitario Marqués de Valdecilla de Santander, Spain.

Results obtained are as follows:

Results (N=172) Clinical samples (N=89)	VP	VN	FP	FN	Sensitivity (%)	Specificity (%)	VPP (%)	VPN (%)
KRAS Q61H2	2	170	0	1	66.7	100	100	99.4
KRAS K117N1	0	172	0	0	ND	100	ND	100
KRAS K117N2	1	171	0	0	100	100	100	100
KRAS A146T	29	141	2	0	100	98.6	93.5	100
KRAS A146V	6	166	0	0	100	100	100	100
NRAS Q61R	18	154	0	0	100	100	100	100
NRAS G12D	15	157	0	0	100	100	100	100
NRAS Q61H1	1	171	0	0	100	100	100	100
NRAS Q61L	10	162	0	0	100	100	100	100
NRAS Q61K	20	152	0	0	100	100	100	100
EXON 4: Results (N=51); Clinical samples (N=29)								
NRAS A146T	0	51	0	0	ND	100	ND	100
NRAS K117N1	0	51	0	0	ND	100	ND	100

Table 3. Diagnostic Sensitivity and Specificity of the *CLART*[®] *CMA NRAS-i KRAS* technique for each mutation. VPP: Positive Predictive Value. VPN: Negative Predictive Value.

Sensitivity value for all mutations was of >98%, after testing of 89 clinical samples. Number of results tested reached 172 data.

Identical results obtained with the reference technic and *CLART*[®] *CMA NRAS-iKRAS*, are considered valid results. In case of discordant results between the two techniques, the result obtained by sequencing was considered as the valid one.

Diagnostic specificity.

The technique has been validated with a number of 36 negative samples, giving rise to a specificity value of 100% for all point mutations.

Diagnostic repeatability and reproducibility.

Obtained data are as follows:

	% homology
Reproducibility (n=22)	98.5
Repeatability (n=19)	100

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

10. REFERENCES

André T., Blons H., Mabro M., Chibaudel B., Bachet J-B., Tournigand C., Bennamoun M., Artru P., Nguyen S., Ebenezer C., Aissat N., Cayre A., Penault-Llorca F. P., Laurent-Puig P., de Gramont A. Panitumumab combined with irinotecan for patients with KRAS wild-type metastatic colorectal cancer refractory to standard chemotherapy: a GERCOR efficacy, tolerance, and translational molecular study. *Annals of Oncology*, 2012, 00: 1-8.

Irahara N., Baba Y., Nosho K., Shima K., Yan L., Dias-Santagata D., Lafrate A.J., Fuchs C.S., Haigis K.M., Ogino S. NRAS mutations are rare in colorectal cancer. *Diagn Mol Pathol*, 2012, 19(3): 157-163.

Janakiraman M., Vakiani E., Zeng Z., Pratilas C.A., Taylor B.S., Chitale D., Halilovic E., Wilson M., Huberman K., Ricarte Filho J.C., Persaud Y., Levine D.A., Fagin J.A., Jhanwar S.C., Mariadason J.M., Lash A., Ladanyi M., Saltz L.B., Heguy A., Paty P.B., Solit D.B. Genomic and biological characterization of exon 4 KRAS mutations in human cancer. *Cancer Res.* 2010, 15;70(14):5901-11.

Janku F., Wheler J.J., Hong D.S., Kurzrock R. Bevacizumab-based treatment in colorectal cancer with a NRAS Q61K mutation , 2013, 8(3):183-8.

Lurkin I., Stoehr R., Hurst C.D., van Tilborg A.A.G., Knowles M.A., Hartmann A., Zwarthoff E.C. Two multiplex assay that simultaneously identify 22 possible mutation sites in the KRAS, BRAF, NRAS and PI3KCA genes. *Plos One*, 2010, 5(1).

Pentheroudakis G., Kotoula V., De Roock W., Kouvatseas G., Papakostas P., Makatsoris T., Papamichael D., Xanthakis I., Sgouros J., Televantou D., Kafiri G., Tsamandas A.C., Razis E., Galani E., Bafaloukos D., Efstratiou I., Bompolaki I., Pectasides D., Pavlidis N., Tejpar S. and Fountzilas G. Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes. *Pentheroudakis et al. BMC Cancer* 2013, 13:49.