CLART® CMA
BRAF·MEK1·AKT1

DETECTION OF POINT MUTATIONS IN GENES BRAF, MEK1 AND AKT1
—BELONGING TO THE MAPK PATHWAY AND ASSOCIATED TO MELANOMA–
FOR *IN VITRO* DIAGNOSIS

CLART® CMA BRAF·MEK1·AKT1

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

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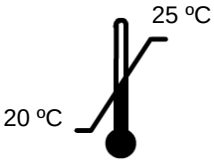
1. GLOSSARY

Attention, see instructions for use

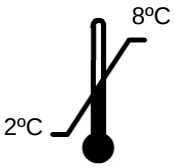
Expiration date

In vitro diagnostic medical device

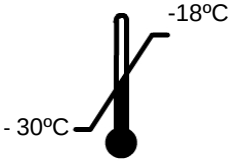
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® CMA BRAF·MEK1·AKT1 detects point mutations in genes *BRAF*, *MEK1* and *AKT1*. Products of these genes are members of the MAPK pathway (BRAF/MEK/ERK), involved in cellular proliferation and inhibition of apoptosis, associated with melanoma.

CLART® CMA BRAF·MEK1·AKT1, is available in two analysis formats, *CLART® BRAF* and *CLART® BRAF, MEK1 & AKT1*, each allowing detection of mutations as follows:

- ***CLART® BRAF***:

GENE	EXON	CODON	NUCLEOTIDE
BRAF	Exon 15	V600E	c.1799T>A
	Exon 15	V600K	c.1799delGT>insAA

Table 1

- ***CLART® BRAF, MEK1 & AKT1***:

GENE	EXON	CODON	NUCLEOTIDE
BRAF	Exon 15	V600E	c.1799T>A
	Exon 15	V600K	c.1799delGT>insAA
MEK1	Exon 3	I111S	c.332T>G
	Exon 3	P124S	c.370C>T
	Exon 6	E203K	c.607G>A
AKT1	Exon 3	Q79K	c.235C>A

Table 2

Starting material for both formats is extracted DNA from melanoma 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.

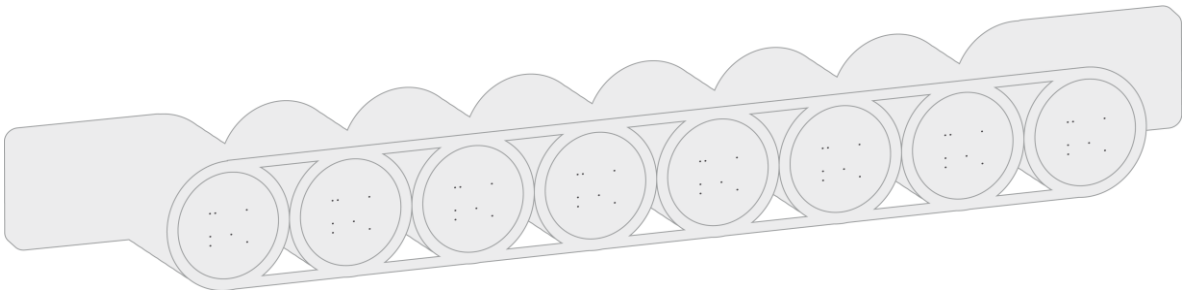


Figure 1. CLART-Strip® in the form of an 8-well strip.

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

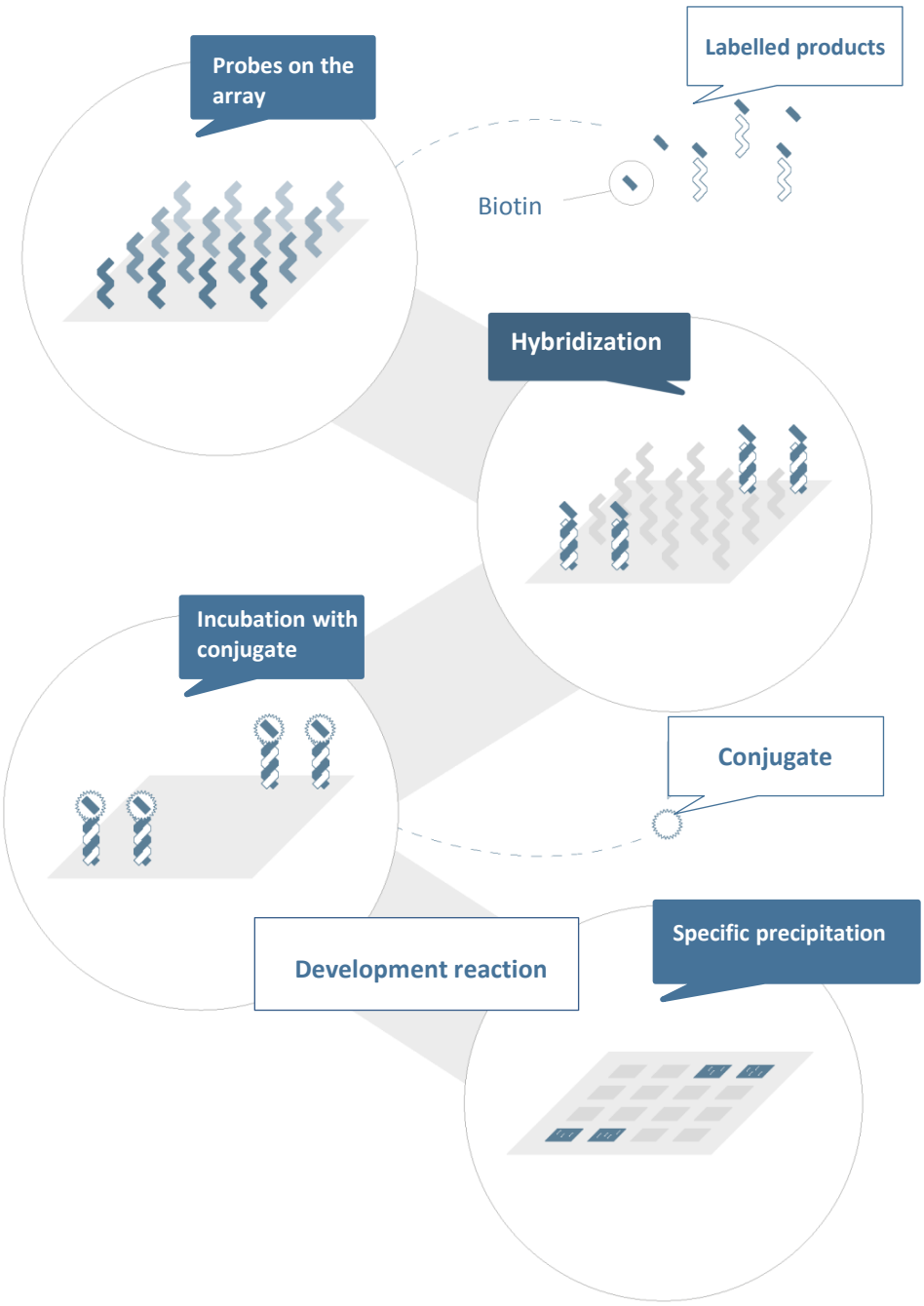


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

CLART® BRAF format includes 2 types of amplification tubes:
Mix 1: White tube
Mix 2: Blue tube

CLART® BRAF, MEK1 & AKT1 format includes 3 types of amplification tubes:
Mix 1: White tube
Mix 2: Blue tube
Mix 3: Red tube

It is mandatory that each sample is subjected to amplification with all the amplification tubes provided in the kit (white and blue tubes in the CLART® BRAF format; white, blue and red tubes in the CLART® BRAF, MEK1 & AKT1 format). This is essential for issuance of an accurate report.

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 QiAmp DNA FFPE Tissue Kit.

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 6.3.1 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 50ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

1. *CLART® CMA BRAF·MEK1·AKT1* 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 BRAF·MEK1·AKT1 has been designed and validated for the analysis of DNA extracted from melanoma biopsies in the form of formalin-fixed paraffin-embedded tumor tissue.
GENOMICA cannot warrant accuracy of results obtained if processing a different type of sample.

7. WORKING PROTOCOL

CLART® CMA BRAF·MEK1·AKT1 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 20% 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. It is highly recommended to select fragments with high levels of cellularity avoiding necrosal and *wild-type* cells areas. For more information about the procedure please refer to the national medical oncology guidelines.

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 5 minutes at maximum speed. Discard the supernatant.
5. Centrifuge for 2 minutes at maximum speed. Discard the supernatant. Allow the ethanol to air-dry.
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. DNA 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 QiAmp DNA FFPE Tissue Kit following the Manufacturer's instructions is strongly recommended for DNA extraction. The final elution volume should be 50 µl.
Notwithstanding, alternative DNA extraction methods might be used, in as far as they yield equivalent concentration and purity parameters (see below).

1. **The amount of genetic material to be added to each PCR tube should be 150 ng.** The total amount of DNA required per test will thus vary depending on the format used:

- ***CLART® BRAF*** format (2 tubes): At least 300 ng of total DNA;
- ***CLART® BRAF, MEK1 & AKT1*** format (3 tubes): At least 450 ng of total DNA.

Use of an excess or shortage of defect DNA amount might result in misdiagnosis.

2. It is recommended to work with a DNA concentration of 30 ng/µl (5 µl to each PCR tube for the total 150 ng). Different DNA concentrations might also be used, taking into account that the maximum volume of DNA that can be added to each PCR tube is 10 µl. Thereby, if the DNA concentration is lower than 15 ng/µl, the DNA extraction step should be repeated.
Conditions of points 1 and 2 above are summarized in Table 3:

***CLART® BRAF* format**		
TUBES	**Total DNA (ng) per tube**	**Maximum volume (µl) per tube**
Mix1: white	150 ng	10 µl
Mix2: blue	150 ng	10 µl
***CLART® BRAF, MEK1 & AKT1* format**		
TUBES	**Total DNA (ng) per tube**	**Maximum volume (µl) per tube**
Mix1: white	150 ng	10 µl
Mix2: blue	150 ng	10 µl
Mix3: red	150 ng	10 µl

Table 3

3. Extracted DNA 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.

4. Store extracted DNA at 4ºC for immediate processing, or at -20ºC for a later use.

5. 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 DNA, and until being placed at the thermal cycler for PCR amplification).

- **Do not place amplification tubes in the thermal cycler until the block has reached 95ºC.** This is a caution measure to prevent non-specific amplification resulting from incubation at temperatures lower than the hybridization temperature.
- Periodic thermal cycler verification is recommended.

- Use of the conventional thermal cycler is recommended. The cooling/ heating times should be checked, to make sure they are in accordance with Table 4:

Temperatures (ºC)	Time (Seconds)
95°C → 94°C	0.1
94°C → 66°C	23.7
66°C → 94°C	27.8
66°C → 72°C	10.8

Table 4. Temperature shifts and corresponding most adequate times for a conventional thermal cycler.

- In case of using the high-speed ramp thermal cycler, ramps should be adjusted to comply with parameters of Table 5:

Temperatures (ºC)	Time (Seconds)	ºC/sec
95°C → 94°C	0.1	NA*
94°C → 66°C	23.7	1.18
66°C → 94°C	27.8	1
66°C → 72°C	10.8	NA

*NA: not applicable.

Table 5. Temperature shifts and most adequate corresponding times for a speed ramp thermal cycler. The third column displays the ºC/second for each phase of a PCR program.

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

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

1 cycle	95ºC	15'
40 cycles	94ºC	60''
	66ºC	60''
1 cycle	72ºC	10'
1 cycle	4ºC	Until tube removal

5. Start the program and place the tubes in the thermal cycler.

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. 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 ºC, 25 ºC and 50 ºC.

4. Make sure that the thermomixer temperature has been 50ºC for at least 60 minutes before the hybridization step.

5. SH forms crystals at room temperature, so it must be warmed up and maintained at 50ºC during at least half an hour, but no longer than one hour before use. SH should be homegeneous.

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

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

8. Clean the thermal cycler with a 10% diluted bleach solution before starting the denaturation program. Denaturing time should be of exactly 8 minutes.

9. 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.

10. 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.

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

12. 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.

13. 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.

14. 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.

15. Do not allow arrays to dry completely.

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

17. Avoid foaming when adding reagents.

18. 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 95ºC **for exactly 8 minutes**. After that, remove the tubes from the 95ºC 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 50ºC-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 depending on the format used are detailed below:

***CLART® BRAF* format:** 5 µL of Mix 1 (White tube) + 5 µL of Mix 2 (Blue tube)

***CLART® BRAF, MEK1 & AKT1* format:** 5 µL of Mix 1 (White tube) + 5 µL of Mix 2 (Blue tube) + 5 µL of Mix 3 (Red tube)
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 50ºC 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, without shaking.** 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

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

Controls included in both kit formats:

- **Genomic DNA extraction control.** It reports the presence of patient's DNA in the sample, thereby endorsing validity of negative results.

- **Internal amplification control.** It checks efficiency of the PCR amplification reaction, thus allowing distinguish between real negative results, and those resulting from inhibition of the PCR reaction.

The report will show the result of the assay, together with the name of the gene or genes that were analysed:

- *BRAF* format: "BRAF analyzed"
- *BRAF, MEK1 & AKT1* format: "BRAF-MEK1-AKT1 analyzed"

Table 6 below displays obtainable results, corresponding explanations and solutions:

Result	**Explanation**	**Solution:** Repeat…
NO DNA	Non-valid extraction due to presence of inhibitors, failure during DNA extraction, or sample degradation	…entire process
PCR INHIBITED	Valid extraction step, but non-valid amplification	…amplification and subsequent steps
UNCERTAINTY	• Very different results obtained with identical probes of the same **CS** well, or • More than two mutations of the same gene reported positive, due to failure in the PCR reaction	…amplification and subsequent steps
NOT-VALID	Low signal of the development reaction	…visualization step

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. Each of the plasmids had as an insert one of the amplification fragments corresponding to one of the 6 mutations detected by the kit. The amplification fragment included the sequence complementary to the corresponding detection probe at the **CS** well.

  Additionally, analytical sensitivity of the BRAF mutations was determined through amplification of dilution series of commercial cell lines, containing the mutations detected by

the kit.

Tables 7 and 8 below display results obtained after visualization:

Point mutations	Number of Clone copies *per* PCR reaction	Sensitivity (clones) (%)	DNA Amount (Cell line)	Sensitivity (cell line) (%)
BRAF				
V600K	10000	100%	5 ng	100%
	1000	60%	2 ng	20%
V600E	1000	100%	0.5 ng	100%
	100	80%	0.2 ng	80%

Table 7

Point mutations	Number of Clone copies *per* PCR reaction	Sensitivity (clones) (%)
MEK1		
I111S	10000	100%
	1000	40%
P124S	10000	100%
	1000	20%
E203K	10000	100%
	1000	80%
AKT1		
Q79K	10000	100%
	1000	60%

Table 8

- **Analytical specificity.** Specificity was determined through amplification of dilution series of DNA recombinant plasmids, each bearing as an insert one of the amplification fragments corresponding to the mutations detected by the kit, or the corresponding *wild-type* gene. Non-specific detection was never observed. An analytical specificity value of 100% is thereby considered.

9.2. Diagnostic utility parameters

In order to determine diagnostic parameters of the kit, a comparative assessment of the ***CLART® CMA BRAF·MEK1·AKT1*** technic *versus* the reference technique (Sanger sequencing) was carried out. The referral hospital "Pathological Anatomy Service, 12 de Octubre University Hospital, Madrid, Spain" collaborated in the study.

Identical results obtained with both technics, are considered valid results. In case of discordant results between the two techniques, the result obtained by Sanger sequencing was considered as the valid one.

9.2.1 Diagnostic sensitivity

A total 155 samples were tested for the diagnostic sensitivity study of *CLART® CMA BRAF·MEK1·AKT1*. Results are displayed in Table 9 below.

N:155	Sensitivity (%)	PPV (%)
BRAF V600E (94)	100%	98,94%
BRAF V600K (15)	93,33%	100%
MEK I111S (0)	NA	NA
MEK P124S (1)	NA	NA
MEK E203K (1)	NA	NA
AKT Q79K (0)	NA	NA

Table 9

N: Number of analyzed samples
Positive samples for each mutation are enclosed between brackets
NA: Non-assessed (not significant results)
PPV: Positive predictive value

Sensitivity obtained with ***CLART® BRAF*** format was higher than 93% for all mutations.

Sensitivity obtained with ***CLART® BRAF-MEK1-AKT1*** format was also higher than 93% for all BRAF gene mutations. Regarding MEK1 and AKT1 mutations, results are displayed as "NA, Non-assessed", due the low number of samples tested (representative of the low prevalence of these mutations).
Both a positive sample for MEK1 mutation E203K, and a positive one for *MEK1* mutation P124S, were analyzed using the ***CLART® CMA BRAF·MEK1·AKT1*** format. The former was accurately detected with the kit, while the latter was at the limit of detection and was not detected.

9.2.2 Diagnostic specificity

Diagnostic specificity of *CLART® CMA BRAF·MEK1·AKT1* was determined through analysis of the samples displayed in Table 10 below.

N:155	Specificity (%)	NPV (%)
BRAF V600E (60)	98,33%	100%
BRAF V600K (139)	100%	99%
MEK I111S (151)	100%	100%
MEK P124S (151)	100%	99%
MEK E203K (150)	100%	100%
AKT Q79K (153)	100%	100%

Table 10

N: Number of analyzed samples
Positive samples for each mutation are enclosed between brackets
NPV: Negative predictive value.

Diagnostic specificity values of 100% and 98.33% were obtained for *BRAF* mutations V600K and V600E, respectively. The latter decrease in specificity was due to detection of a low prevalent *BRAF* V600D mutation present in one of the samples.

The diagnostic specificity values corresponding to all the *MEK1* and *AKT1* mutations were of 100%.

9.2.2 Diagnostic repeatability and reproducibility

The diagnostic repeatability and reproducibility parameters of the kit were obtained by subjecting DNA extracted from the samples of Table 11 below, to all the steps of the protocol up to visualization step.

Repeatability and reproducibility parameters of ***CLART® CMA BRAF·MEK1·AKT1*** **kit** are displayed in Table 11:

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

Table 11

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 DNA (insufficient sample quantity, DNA degradation, inadequate storage or loss of DNA 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 6 and 7 of this Manual.

11. REFERENCES

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