CLART® HPV3

GENOTYPING OF HUMAN PAPILLOMAVIRUS
VIA GENOMIC IDENTIFICATION
FOR *IN VITRO* DIAGNOSIS

CLART® HPV3

CLART® HPV3 is under protection of 2 patent families corresponding to International PCT Patent Applications PCT WO2007017699 and WO2011116797, which comprise national and regional members granted in different territories, including Spain, Germany, Denmark, France, Italy, Sweden, Russia, Mexico, China and Israel, and under examination in Brazil and Canada.

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

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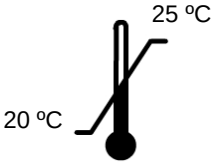
1. GLOSSARY

Attention, see instructions for use

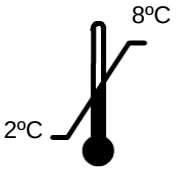
Expiration date

In vitro diagnostic medical device

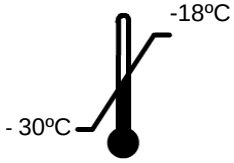
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® HPV3 enables to detect infections and coinfections of the 49 most clinically relevant types of Human Papillomavirus (HPV) (6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85, 86, 87, 89, 97, 101, 102, 103, 106, 150 and 151).
As starting material, the following different types of human samples may be used: swabs, cell suspensions and formalin-fixed paraffin-embedded tissue.

Detection is based on our CLART® technology: PCR amplification of a fragment of the viral region L1, followed by visualization in low-density microarray. The chosen sequence is highly conserved in all HPV types, while at the same time displays enough variations among HPV types so as to distinguish each type by means of specific probes.

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

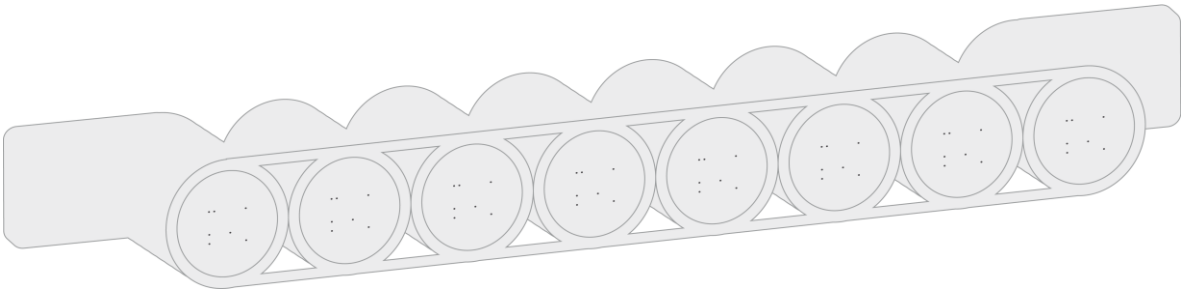


Figure 1. CLART-Strip® in the form of an 8-well strip.

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.

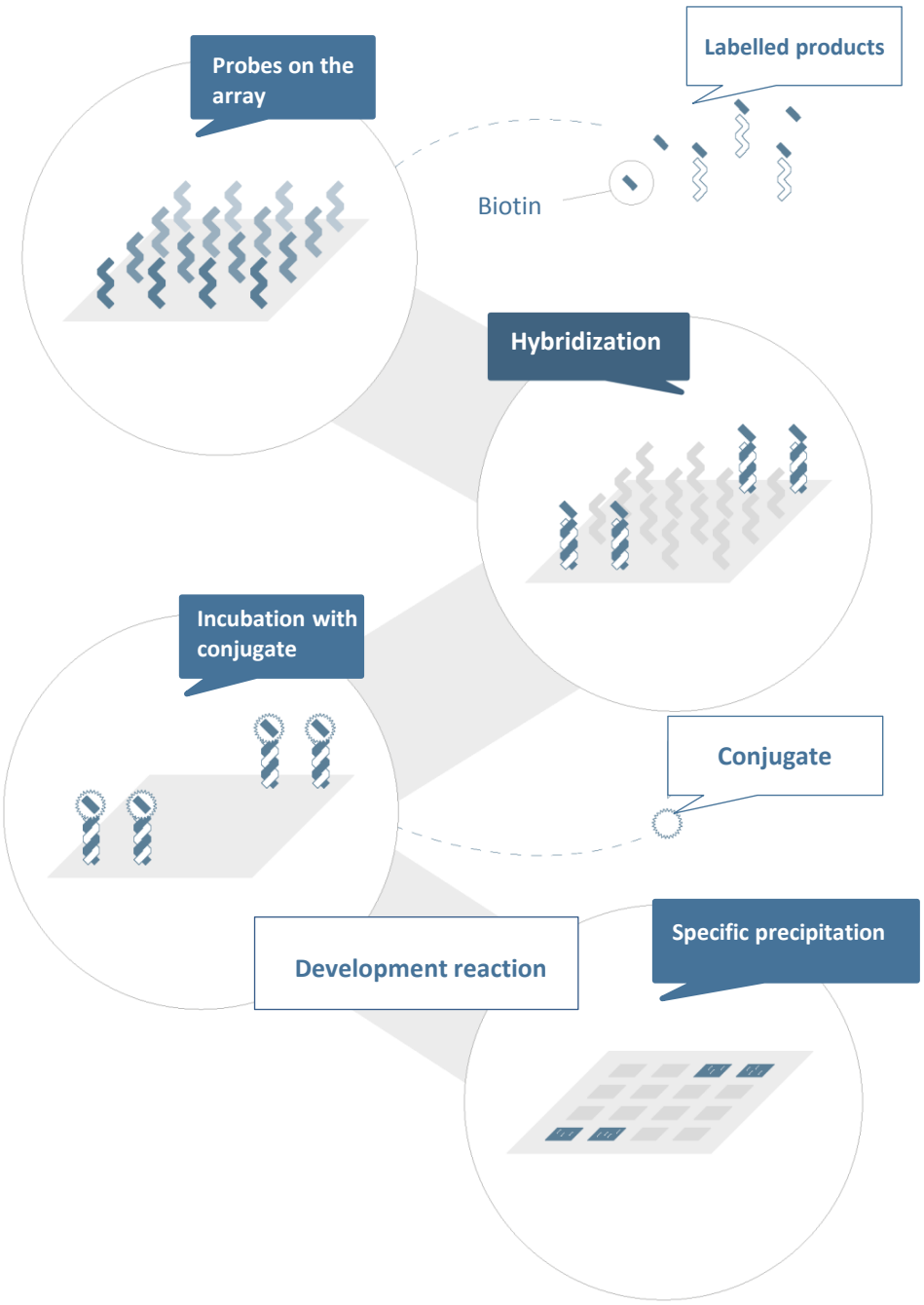


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3.-KIT COMPONENTS AND STORAGE

CLART® HPV3 kit contains enough reagents for the DNA extraction and analysis of 48 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.

Kit components are displayed herein:

3.1. Extraction and purification reagents

Shipped and stored at 4ºC or Room Temperature.

Components:

- Purification columns adapted to 2 ml tubes
- 2 ml tubes
- T1 Buffer
- B3 Buffer
- B5 Buffer
- BE Buffer
- BW Buffer
- Proteinase K, lyophilized (keep at -20ºC when resuspended)
- PB Buffer

3.2. Amplification reagents

Shipped and stored at -20ºC.

- **Amplification tubes:** They 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 -20ºC.

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.

3.3. Visualization components

Visualization components are divided into two groups, according to optimal shipping and storage temperatures:

- Shipped at 4ºC and stored at Room Temperature:

- **CLART-Strip® (CS)**, each well including all specific probes for detection of all HPV types 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.

- Shipped and stored at 4ºC:

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

3.4. Other components

- GENOMICA's **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.
- 96% Ethanol.
- 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.
- Saline solution (0.9% NaCl).

4.2. Equipment

- Microcentrifuge.
- Thermal cycler.
- Biosafety cabinet.

- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL and 200-1000 µL for the pre-PCR area.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the post-PCR area.
- Termobloque (Thermomixer) compatible with 96-well skirted plates and adjustable shaking at 37°C, 55°C, 60°C and 100ºC.
- Vortex.
- Vacuum pump (optional).

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

1. *CLART® HPV3* 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.

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

6. SAMPLES

CLART® HPV3 kit allows analyze the following types of samples:

6.1. Swabs

Sample should be taken with a dry and sterile, cotton or alginate swab, big enough to obtain a good sized sample. Do not use devices that might cause bleeding. Place the swab back in its tube, in the absence of any preservation medium. Keep the swab at 4ºC, if processing is to take place within 7 days, or at –20ºC if processing is to take place later.

6.2. Cell suspensions

Cell suspensions referred to herein belong to the kind that are used for cervicovaginal cytology tests based on membrane-filtered monolayers (ThinPrep®, Cytyc). The sample should be taken with a brush or spatula. The sample should then be resuspended by means of shaking the device within in a vial containing transport medium. Dispose of the sampling device and keep the sample at 4ºC until use.

6.3. Formalin-fixed paraffin-embedded tissue.

Samples should be fixed in buffered formalin during the shortest time possible (never exceeding 24 hr). Use of non-buffered formalin or a fixation time longer than 24 hr might result in degradation of DNA of the sample. It is important to carefully clean the scalpel with xylene, before and after cutting the sample, in order to prevent contamination with the previously cut sample. Discard as much paraffin as possible from the block before preparing 2-3 slices of 5 µm, after which they should be placed in a 1,5 ml sterile tube.

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

7. WORKING PROTOCOL

DNA extraction may be manual or automatic.

7.1. Manual DNA extraction.

7.1.1. Manual DNA extraction specific recommendations.

- Dissolve Proteinase K in Buffer PB before use, to reach a concentration of 20 mg/ml. The volume of Buffer PB required is indicated in the bottle of Proteinase K. Once the Proteinase K is dissolved, it must be stored at -20ºC, at which temperature it will be stable for at least 6 months.

- Add (96-100) % Ethanol to Buffer B5 before use. The volume of Ethanol to be added is indicated in the bottle of Buffer B5.

- Heat Buffer BE at 70°C before use.

- All centrifuging should be performed at room temperature unless otherwise stated.

- Solutions of Buffer B3 and BW contain guanidine hydrochloride. The use of gloves, glasses and laboratory clothing is recommended when handling.

7.1.2. Manual DNA extraction protocol

7.1.2.1. Sample preparation

7.1.2.1.1 Dry swab

- Add 1.5 ml of Saline solution (0.9% sodium chloride) to the tube containing the swab. Vortex for 1 minute.
- Place supernatant in a sterile 1.5 ml tube.
- Centrifuge samples for 10 minutes at 12.000 r.p.m. in a microcentrifuge, and discard liquid remains with a micropipette, avoiding removing pellet.
- Resuspend pellet in 180 µl Buffer T1. Proceed to step 7.1.2.2.

7.1.2.1.2 Cell suspensions

- Invert the sample container several times, and place 1 ml of the cell suspensión within a 1.5 ml tube.
- Centrifuge for 10 min at 12.000 rpm. Discard supernatant, avoiding removing pellet.
- Resuspend pellet in 1 ml sterile distilled water.

- Centrifuge for 10 min at 12.000 rpm. Discard supernatant, avoiding removing pellet.
- Resuspend pellet in 180 µl Buffer T1.
- Place in a 1.5ml tube. Proceed to step 7.1.2.2.

7.1.2.1.3 Formalin-fixed paraffin-embedded tissue

- Introduce 4-5 tissue cuts of around 5 µm/ each within a 1.5ml tube, and add 180 µl of Buffer T1.
- After mashing the tissue using the pipette tip, vortex vigorously to facilitate cell lysis. Proceed to step 7.1.2.2.

7.1.2.2 Common part of the protocol.

1. Add 25 µl of Proteinase K solution.

- Vortex to mix.
- Incubate at 56°C, for 1-3 hours, (overnight in the case of paraffin samples), in a bath or thermomixer with shaking, until complete lysis of the sample. Vortexing samples every 15 minutes will accelerate lysis.

180 µl T1 + 25 µl Proteinase K

1-3 h, 56Cº

2. After lysis, add 200 µl of Buffer B3 to each sample. Vortex to mix, and incubate at 70°C for 10 min.

Add 200µl B3 70°C, 10 min.

3. Add 210 µl of 96% Ethanol to each sample, and vortex immediately.

***Note**: Do not discard any white precipitate that might have formed after Ethanol addition. Any such precipitate should be added together with the rest of the solution to the purifying column in the next step.*

4. Place one purifying column per sample, within a 2 ml collection tube. Add sample and centrifuge for 1 minute at 12.000 rpm. Should there be any remaining solution left within the column, centrifuge again. Discard the filtered solution together with the 2 ml collection tube.

Add sample
1 min, 12,000 rpm

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5. Place the column within a different collection tube and add 500 µl of Buffer BW to the column. Centrifuge for 1 minute at 12000 r.p.m. Discard the filtered solution together with the collection tube.

Add 500 µl B5
1 min, 12,000 rpm

6. Place the column within a different collection tube and add 600 µl of Buffer B5 to the column. Centrifuge for 1 minute at 12000 r.p.m. Discard the filtered solution.

Add 600 µl B5
1 min, 12,000 rpm

7. Place the column again within the collection tube. Centrifuge for 1 minute at 12000 r.p.m. to eliminate any remaining Buffer B5.

1 min 12,000 rpm

Any residual ethanol from Buffer B5 might inhibit enzymatic reactions, thereby the need to completely eliminate it through this centrifugation step.

8. Place the column in a clean 1.5 ml microcentrifuge tube. Elute DNA with 100 µl of Buffer BE (pre-heated at 70°C): Incubate the pre-heated solution with the column for 1 minute at Room Temperature. Centrifuge for 1 minute at 12000 r.p.m.

Add 100 µl BE
1min, 12,000 rpm

9. Collect the filtered solution (approximately 100 µl) within the 1.5 ml microcentrifuge tube. Make use of 5 µl for the amplification reaction, and store the rest at –20°C.

7.1.3. Automatic DNA extraction protocol

Provided below are the protocols recommended for operating with the NucliSENS BioMérieux easyMag and BioSprint 96 de Qiagen devices.

7.1.3.1 NucliSENS BioMérieux easyMag device

1. Sample preparation for internal lysis (performed inside the device)

Dry swab:

- Add 1.5 ml of saline solution (0.9% sodium chloride) to the tube containing the swab and vortex vigorously for 1 minute.
- Place supernatant in a sterile 1.5 ml tube.
- Transfer 1 ml into a well of the tray (each tray contains eight wells).

Cell suspensions (volumes smaller than 3 ml):

- Shake the sample by inverting the vial containing it several times. Transfer 1 ml into one well of the tray.

Hybrid Capture medium:

- Shake the sample by inverting the vial containing it several times. Transfer 0.5 ml into one well of the tray.

2. Internal lysis and DNA extraction: follow the device's User's Guide. The elution volume in the program should be set to 110 µl.

3. Once the extraction step is finished, transfer 110 µl of eluted DNA to 1.5 ml Eppendorf tubes. Use 5 µl for the amplification reaction, and store the rest at –20°C.

7.1.3.2 Qiagen BioSprint 96 device

BUFFER PREPARATION

Make sure that buffers are prepared before starting the extraction process.

1. The protease is lyophilized. Add 4.4 ml of the buffer indicated in the label before use, and from that moment on, store at 4º C, up to 2 months.

2. Preparation of Buffer AW1

Volume of Concentrated AW1 (ml)	Volume of 96% Ethanol to add	Final volume (ml)
19	25	44
27	35	62
98	130	228

Note: Keep at Room Temperature. Shake the bottle about 5 times before use.

3. Preparation of Buffer AW2

Volume of Concentrated AW2 (ml)	Volume of 96% Ethanol to add	Final volume (ml)
17	40	57
68	160	228

Note: Keep at Room Temperature. Shake the bottle about 5 times before use.

4. Preparation of 0.0002% Tween 20.

RNase free H_2O	30 ml	250 ml
Tween 20	6µl	50 µl

Note: Once the mixture prepared, keep it at 4º C.

SAMPLE PREPARATION

1. IMPORTANT: Set the thermomixer at 70ºC to be ready for the protease lysis.

Dry swab:

Cut the swab and place it inside a 1.5 ml tube.
Add next:
- 400 µl ATL buffer
- 20 µl Protease

Cell suspensions (volumes smaller than 3 ml):

Liquid cytology: Vortex and add to the (S-Block) plate:
-200 µl of sample
-20 µl of Protease

2. Incubate in the thermomixer at 70ºC for 10 minutes. If incubation is performed in a plate, cover it with a transparent film and place a pre-heated lid at 70ºC on top. In this way sample condensation which might result in contamination might be avoided.

3. Master-mix preparation. Prepare volume for one extra tube per each group of 10.

Add the following volumes:

Components	Volume per sample (µl)
AL Buffer	200
Isopropanol	200
MagAttract Suspension G	20

4. Dispense solutions into the plates.

6 S-Block and 2 Microplate MP will be needed.

Table 1 displays the slot of each plate in the device and the volumes to be added to the plates:

Table 1.

Slot in the device	Plate	To add…	Volume per well (µl)
8	Microplate MP	Place the protective cover on top of the support.	------
7	Microplate MP	AE Buffer (Elution Buffer)	100 or 200
6	S-Block	RNase free H_2O + Tween 20	500
5	S-Block	AW2(2) Buffer	500
4	S-Block	AW2(1) Buffer	500
3	S-Block	AW1(2) Buffer	500
2	S-Block	AW1(1) Buffer	500
1	S-Block	Lysed samples + Master-mix (*)	200 + 420

(*) When using dry swabs, briefly spin the samples before adding the lysates. First, 200 µl of the lysed sample should be added to the well, followed by 420 µl of the Master-mix.
If the lysis has been performed in the plates, add 420 µl of Master-mix directly into each well containing the lysed sample.

Pipet up and down several times in both cases.

5. Extraction.

- Once the plates and corresponding volumes are prepared, switch on the power of the device.

- Open the front door of the protective cover.

- Select the "BS96_DNA_Swab" program using the up and down arrows, and press the START bottom.

The LCD displays a message requesting plate load in slot 8 (see Table 1). After loading slot 8, press "Start". The worktable rotates and a new message appears, requesting load of elution plate within slot 7. Load slot 7 and press "Start" again. Continue this process until all slots are loaded.
Table 1 displays the slots in which the different plates are to be loaded. Labels should face the inside.

- Once all the plates are loaded, close the front door of the protective cover of Biosprint 96.

- The extraction process will last about 20 minutes. Extracted DNA may be stored at -20ºC within the elution plate itself (corresponding to slot 7 position), the wells being covered with a transparent film. Should the whole plate not be used, extracted DNA should be pipetted into a 1.5 ml tube and stored at -20ºC.

7.2. Amplification reaction

7.2.1. Amplification-specific recommendations

- Work in the **Pre-PCR area**, always using a laminar flow chamber and following the recommendations of Section 5.
- Avoid using temperatures higher than 37ºC for thawing the Amplification tubes.
- During the DNA addition process, keep the tubes separate and on ice.

7.2.2. Amplification protocol

1. Thaw on ice the required number of amplification tubes according to the number of samples to be processed. 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. Should DNA be obtained from paraffin-embedded samples, 1,5 μl of 25mM Magnesium Chloride is to be added to the amplification tubes.

4. Add 5 μl of extracted DNA to each amplification tube. Pipette up and down several times. Keep tubes at 4ºC.

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

1 cycle	95ºC 5 min
40 cycles	94ºC 30 sec 55ºC 60 sec 72ºC 90 sec
1 cycle	72ºC 8 min
4ºC Until tube removal	

6. Start the program and place the amplification tubes in the thermal cycler, once the block temperature has exceeded 90ºC. Amplification duration is of about 4 hr, the exact time depending on the thermal cycler used.

7.3. Visualization of the amplified product

7.3.1. 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 areas.

2. Make sure that the thermomixer temperature has been 65ºC for at least 1 hour before the hybridization step starts.

3. Warm up SH at room temperature.

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

5. When preparing samples for visualization, use a different filtered tip for each well and change it every time a reagent is added.

6. When using vacuum pumps for aspirating solutions, 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.

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

8. Dispense all solutions to the CS wall, never to the bottom of it. Likewise, aspirate the different solutions completely from the CS well without touching the bottom of the well; Otherwise, the microarray might get damaged.

9. Do not allow the well to dry completely.

10. Avoid foaming when adding reagents.

11. 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 impregnated with alcohol.

7.3.2. 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 and incubate at 95ºC **for exactly 10 minutes**. After that, remove the tubes from the incubation and immediately place on ice.

3. 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.

4. Prewash of the **CS**: Place the necessary **CS** units on the Microtiter plate adaptor. Add 200 µL of diluted TL to each 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 diluted washing solution in the CS wells until addition of the above-mentioned products.

   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.

5. Hybridization Step: Once the amplified products have been denatured, discard the diluted washing solution from the CS wells with a pipette or preferably with a vacuum pump. Immediately after that, add 100 µL of room-temperature SH to each CS well, avoiding foaming.

   Add to each CS well, **5 µL** of denatured amplified product. 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 **CS**s with the plastic lid and incubate in the plate thermomixer for **1 hour at 65ºC 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 30ºC with shaking for its further use in step 6 below. For a faster temperature decrease, the lid may be removed.

6. 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 30ºC.

7. Blocking and conjugate incubation: Diluted CJ Solution should be prepared 15 minutes before the end of the hybridization step, and be kept on ice. With this purpose, centrifuge CJ for 10 seconds before use, and add **7.5 µL of CJ** to **1 mL of DC** (amount suitable per one CS). Next, vortex to homogenize the solution.
   Aspirate the diluted TL from the wells without leaving any residue, and add **100 µL** of diluted CJ Solution per well. Incubate for exactly **15 minutes in the thermomixer at 30ºC 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 and with shaking for its use on step 8. For a faster temperature decrease, the lid may be removed.

8. 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.

9. 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 25ºC without shaking**.

Completely discard RE using a pipette or vacuum system. Wells must be completely dry for reading. Reading must be performed immediately after removal of RE.

10. 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

Processing of data obtained from each analysis takes place automatically. Analysis of results and issuance of corresponding report are automatically performed by CAR®.

False negatives are one of the drawbacks of detection through genomic amplification, due to either an inadequate quality of the DNA from the sample (insufficient sample quantity, DNA degradation due to inadequate storage or loss of DNA during extraction), or to the presence of DNA polymerase inhibitors in the samples where virus presence is to be checked (hemoglobin, paraffin remains, salts, etc.). ***CLART® HPV 3*** kit avoids these false negatives through addition of two internal controls within each reaction tube:

- A genomic DNA internal control, necessary to endorse validity of negative results, as it reports the presence of patient's DNA in the sample, even when no amplification of any HPV type has been detected.

- An Internal amplification control, which will allow distinguish between cases of inhibition of the PCR reaction, and those of DNA absence in the sample.

Each reaction tube contains the following oligonucleotides:

- A pair of oligonucleotides that amplify a fragment of the human CFTR gene. This would be the genomic DNA extraction control, or the patient's DNA control.
- A pair of oligonucleotides that amplify a modified plasmid included within the amplification tube, and that is used as amplification control of the PCR reaction.
- HPV-specific oligonucleotides.

The amplification tubes have been designed to boost HPV amplification *versus* that of the controls. Further, amplification of the genomic DNA control leads over that of the amplification reaction control.

Under certain circumstances (eg. when there is a high number of copies of an HPV virus, or when the sample contains several HPV types), it may happen that no amplification of any of the amplification controls, or at least of one of them, take place, and that a "NO-SIGNAL" read-out issues.

Displayed below are the different results obtainable with the Kit:

VALID RESULT:

RESULT for some genotype	GENOMIC/AMPLIFICATION CONTROLS	INTERPRETATION
√. POSITIVE	**√ RIGHT**	**POSITIVE**
√. POSITIVE	**√ GENOM Ctrl, No signal AMPLIF Ctrl.**	**POSITIVE**
√. POSITIVE	**No signal GENOM Ctrl, √ AMPLIF Ctrl**	**POSITIVE**
x. NEGATIVE	**√ RIGHT**	**NEGATIVE**
x. NEGATIVE	**√ GENOM Ctrl, No signal AMPLIF Ctrl.**	**NEGATIVE**

INVALID RESULT:

RESULT for some genotype	GENOMIC/AMPLIFICATION CONTROLS	INTERPRETATION
x. NEGATIVE	**No signal GENOM Ctrl, No signal AMPLIF Ctrl.**	**PCR Inhibition**
x. NEGATIVE	**No signal GENOM Ctrl, √ AMPLIF Ctrl**	**Absence of DNA in the sample**

NON-CONCLUSIVE RESULT for one type.

CAUSE:

- Very different results obtained with three replicas of the same probe.
- In coinfections, for viruses in the limit of detection of the technique.

NON-CONCLUSIVE RESULT for all types:

INTERPRETATION	CAUSE	SOLUTION
NO DNA	No DNA present in the sample.	Repeat the technique from the extraction step, or request the facultative a sample from the patient.
PCR INHIBITED	This is due to the fact that some substances may inhibit	Check the presence of any of these substances in the samples or

	the PCR reaction through impairment of the DNA polymerase activity.	extracted genetic material. In most cases it is recommended to repeat extraction or, if this is not possible, to request the facultative a new sample from the patient.

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. Control of known interferences

Some substances might interfere with the ***CLART® HPV3*** system. These are mainly substances that inhibit the DNA polymerase, and thereby, the amplification reaction. The most known interferences are:

1 **Presence of hemoglobin or paraffin.** DNA extracted both from cervicovaginal samples as well as from paraffin-embedded tissue samples, may contain rests of hemoglobin. The latter may also contain rests of paraffin. Both kinds of rests may cause inhibition when added to the amplification tube. Notwithstanding this, following the DNA extraction protocols of Section 7.1, may minimize these effects.

2 **Presence of acetic acid or iodine** in the sample to be analysed. If a sample for analysis with ***CLART® HPV 3*** is taken after a colposcopy, contamination of the sample with acetic acid or iodine, both of which inhibit PCR, might occur. In order to avoid this, it is strongly recommended to take the sample before performing the colposcopy.

3 **Use of non-suitable samples**. Analysis of sample types other than those indicated in this manual for each format of the ***CLART® HPV 3*** system, or a non-accurate sample taking, might lead to non-conclusive analysis results. For example, if a sampling swab is placed in an alternative medium, PCR inhibition might result.

4 **Inadequate conservation of samples** might affect the result of the analysis. If the samples are kept under conditions that might lead to DNA degradation, for instance, and excess time in formalin fixation, the analysis result will be of inhibition due to lack of amplification of the sample's control DNA.

5 **Residual Proteinase K activity.** During DNA extraction, Proteinase K has to be inactivated by means of incubation at 70ºC for 10 min. Under these conditions complete inactivation is achieved. Should this step be omitted, or should less stringent inactivation conditions be used, some residual activity of Proteinase K might remain and result in degradation of the DNA polymerase, and in PCR inhibition.

9.2. Technical specifications

9.2.1. Analytical parameters

Analytical sensitivity

Analytical sensitivity was determined through specific amplification of the L1 region of the different HPV genotypes cloned within recombinant plasmids ("10^2 copies" and "10 copies" columns). Sensitivity of the types displayed within the column of the table corresponding to "50 copies" was also determined through detection of samples belonging to the WHO's Program of evaluation of laboratory tools for HPV typing (2015 WHO HPV LabNet Proficiency Study of HPV DNA Typing).

HPV GENOTYPE	**10^2 copies**	**50 copies***	**10 copies**
6	100%		40%
11	100%		60%
16	100%	100%	80%
18		100%	
26	100%		80%
31	100%		80%
33	100%		80%
35			100%
39			100%
45	100%		60%
51	100%		80%
52	100%		80%
53	100%		80%
56			100%
58			100%
59	100%		80%
66			100%
68	100%		80%
82			100%

N=95 ***** Data expressed in genome equivalents

Table 2. Analytic sensitivity of *CLART® HPV2/ HPV3* kit

Due to the clinical significance of HPV types 16 and 18, the sensitivity data corresponding to these types, as obtained from sample detection within the WHO's Program of evaluation of laboratory tools for HPV typing, have been included. This program compares and evaluates the different methodologies commercially available for HPV detection within the HPV vaccination programs. Based on this program, a diagnostic tool is considered proficient for diagnosis if allowing detection of at least 50 International Units (genome equivalents or copies) of HPV types 16 and 18, a proficiency achieved with *CLART® HPV2/ HPV3* kits.

For the 14 genotypes included in HPV3 that differ from those included in HPV2 (see Table 2), sensitivity of the two most prevailing genotypes, 34 and 74, has been evaluated. Both cases display a sensitivity of 10^2 copies.

Analytic specificity.

Analytic specificity value of *CLART® HPV3* kit is 100%. No non-specific detection of viruses different to those to be determined occurs.

9.2.2. Diagnostic utility parameters

In order to determine the diagnostic utility parameters of the ***CLART® HPV3*** system, comparative studies were performed *versus* the old version of the same product.

These studies were performed in collaboration with two Spanish hospitals and a Portuguese one.

- Microbiology Service of the "Hospital Universitari Germans Trias i Pujol" of Badalona.
- Virology Department of the "Hospital Universitario Virgen de la Arrixaca".
- Department of infectious diseases. National Health Institute Ricardo Jorge, I. P. Lisboa (Portugal).

386 samples were analysed, of which 9 were dry swabs, 25 paraffin-embedded tissues and 352 liquid citologies.

Table 3 displays the diagnostic sensitivity and specificity values for the HPV types detected with ***CLART® HPV2/ HPV3*** kit:

HPV Type	Sensitivity	Specificity	HPV Type	Sensitivity	Specificity
6	100,00	100,00	56	100,00	100,00
11	100,00	100,00	58	100,00	100,00
16	100,00	99,70	59	100,00	99,73
18	100,00	100,00	61	100,00	100,00
26	100,00	100,00	62	100,00	99,47
31	100,00	100,00	66	100,00	100,00
33	100,00	99,73	68	100,00	98,35
35	100,00	99,74	70	100,00	100,00
39	100,00	100,00	71	100,00	100,00
42	100,00	99,47	73	100,00	99,74
43	100,00	99,50	81	100,00	100,00
44	100,00	100,00	82	94,44	99,48
45	100,00	99,74	83	100,00	100,00
51	100,00	100,00	84	100,00	100,00
52	100,00	100,00	85	100,00	100,00
54	100,00	100,00			

Table 3. Diagnostic parameters of *CLART® HPV3* Kit.

For the 14 genotypes included in HPV3 that differ from those included in HPV2 (see Table 3), the diagnostic parameters have been evaluated with the following study:

678 samples were analyzed, of which 213 were positive for genotypes detectable with HPV2 (all genotypes were represented), and 465 were negative.

Of the 678 samples that were analyzed, 10 samples tested positive for some of the new genotypes, in particular for genotypes 34, 67, 87 and 102.

No positive samples were available for remaining genotypes.

The 14 new types had a diagnostic specificity of 100%.

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Anex I: Table displaying the oncogenic risk* of the HPV types detectable with ***CLART® HPV3*** Kit.

TYPE	ONCOGENIC RISK
HPV 6	Low Risk
HPV 11	Low Risk
HPV 16	High Risk
HPV 18	High Risk
HPV 26	Potentially High Risk
HPV 31	High Risk
HPV 33	High Risk
HPV 35	High Risk
HPV 39	High Risk
HPV 40	Low Risk
HPV 42	Low Risk
HPV 43	Low Risk
HPV 44	Low Risk
HPV 45	High Risk
HPV 51	High Risk
HPV 52	High Risk
HPV 53	Potentially High Risk
HPV 54	Low Risk

TYPE	ONCOGENIC RISK *
HPV 56	High Risk
HPV 58	High Risk
HPV 59	High Risk
HPV 61	Low Risk
HPV 62	Low Risk
HPV 66	High Risk
HPV 68	High Risk
HPV 70	Low Risk
HPV 71	Low Risk
HPV 72	Low Risk
HPV 73	Potentially High Risk
HPV 81	Low Risk
HPV 82	Potentially High Risk
HPV 83	Low Risk
HPV 84	Low Risk
HPV 85	Low Risk
HPV 89	Low Risk

* Oncogenic risk classification determined within: *Bouvar et al. A review of human carcinogens - Part B: biological agents. Lancet Oncol. 2009, 10(4):321 322.*