CLART® HPV4

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

CLART® HPV4

CLART®, CLART-Strip®, CAR®, SAICLART® and *AUTOCLART® are registered Trademarks of GENOMICA.*

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

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CE

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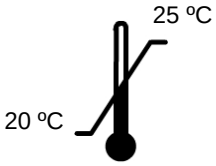
1. GLOSSARY

Attention, see instructions for use

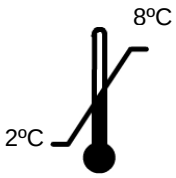
Expiration date

In vitro diagnostic medical device

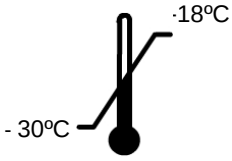
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® HPV4 is available in two analysis formats:

- ***CLART® HPV4***, which enables to detect the 35 most clinically relevant types of Human Papillomavirus (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68a and b, 70, 71, 72, 73, 81, 82, 83, 84, 85 and 89); and
- ***CLART® HPV4S***, which enables to detect the following 16 HPV types: 14 high oncogenic risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and 2 low oncogenic risk types (6 y 11).

Starting material for both formats may be both swabs as well as cell suspensions (See Section 6).

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.

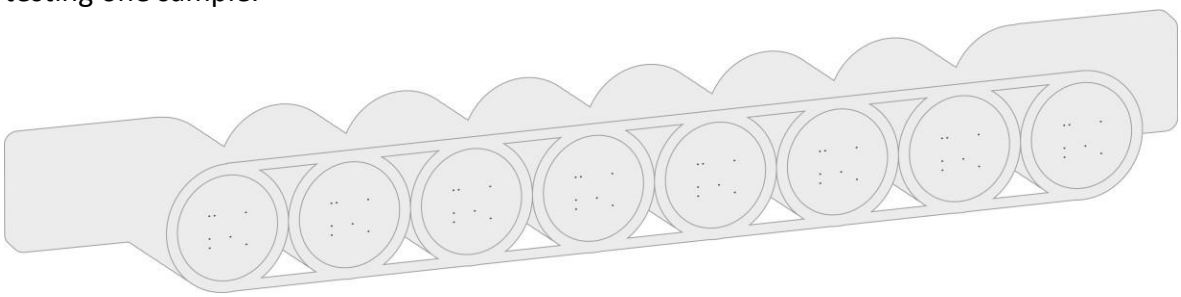


Figure 1. CLART-Strip® in the form of an 8 -well strip.

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. autoclart® plus may alternatively be used (see Section 8).

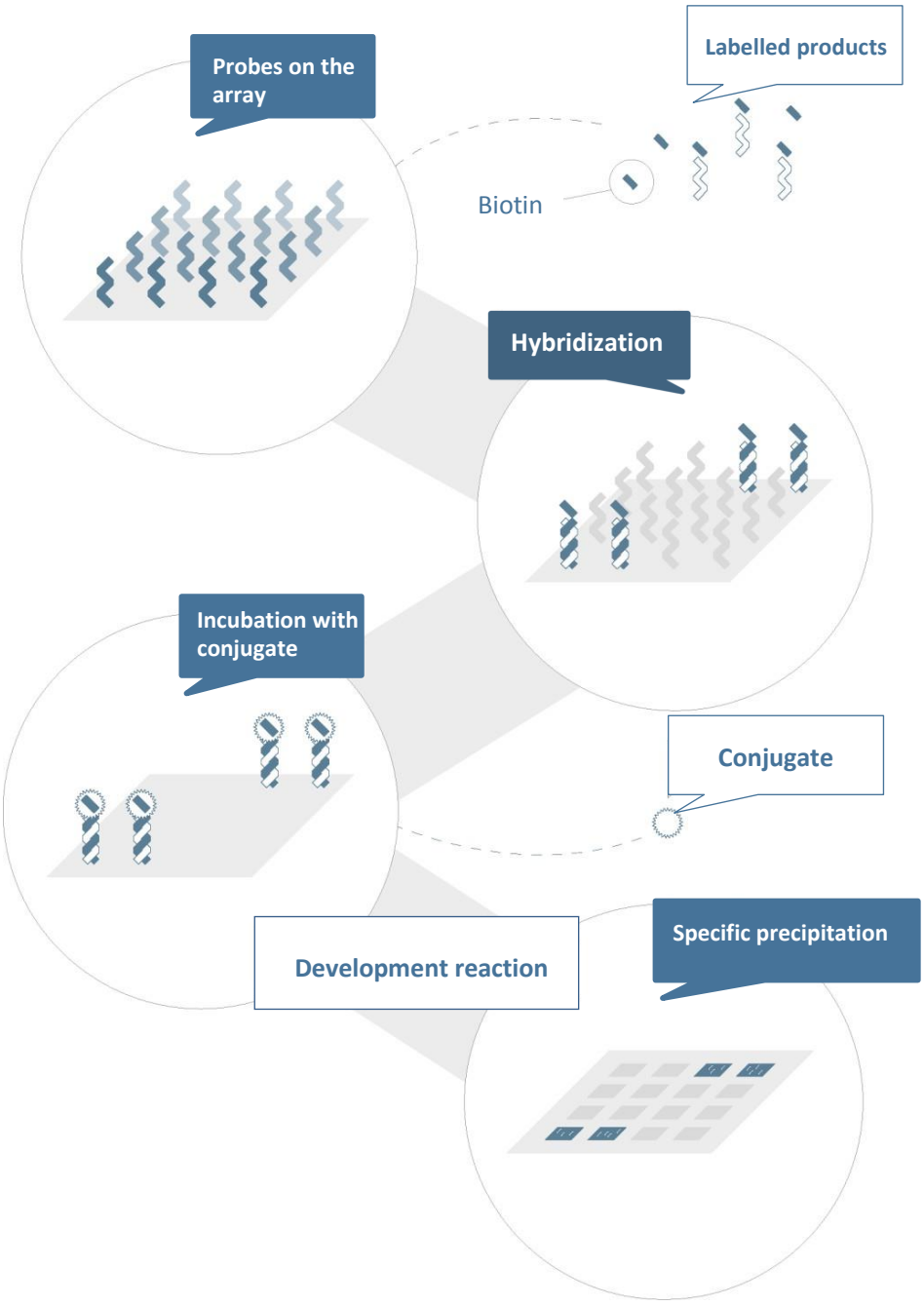


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3.-KIT COMPONENTS AND STORAGE

CLART® HPV4 kit, in both its formats, contains enough reagents for the analysis of 16, 48 or 96 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. Amplification reagents

Shipped and stored at -20ºC.

Two possible formats available:

- Amplification tubes.
- Amplification plate.

Both the Amplification tubes as well as the wells of the Amplification plate are provided ready-to-use. Each of them 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.

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

3.2. Visualization components

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

- Shipped 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.**

3.3 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)

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

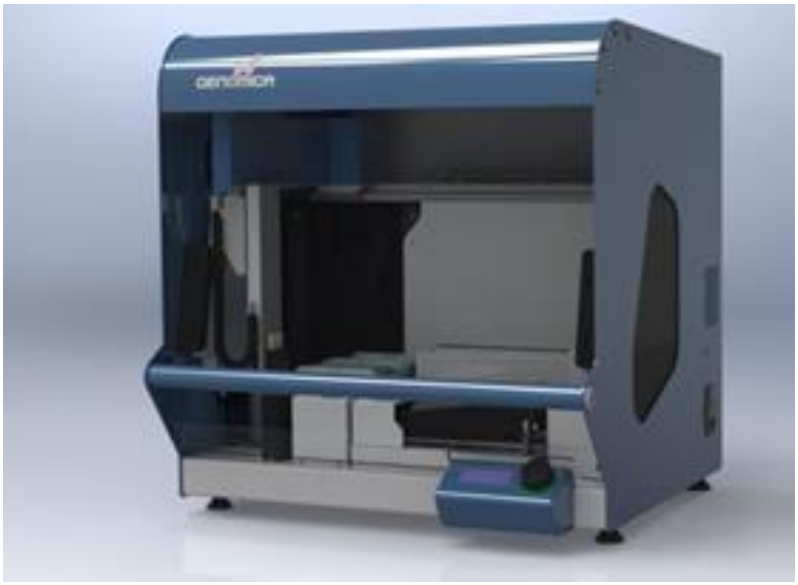


Figure 4. autoclart®

- GENOMICA's **autoclart® plus**.

  autoclart® plus is a fully automated electromedical device capable of processing up to 96 samples per run, starting from the denatured amplification product, and ending with issuance of the corresponding diagnostic report.

Figure 5. autoclart® plus

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.
- Saline solution (0.9% NaCl).

4.2. Equipment

- Microcentrifuge.
- Thermal cycler. This technique has been validated for exclusive use in thermal cyclers with standard ramps, not with fast ramps.
- 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 25°C, 30°C and 65ºC.
- Vortex.
- Vacuum pump (optional).

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

1. ***CLART® HPV4*** **technique should be performed in two physically separated areas,** in order to minimize sample contamination:

Pre-PCR area: Sample preparation and sample addition to the amplification tubes or plate 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 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/ plate.

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® HPV4 kit allows analyze the 2 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. 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 (FFPE) tissue

Fix the samples in the neutral buffered formalin for the shortest possible time (never exceeding 24 hours). Using unbuffered formalin or fixation longer than 24 hours may degrade the DNA. Trim excess paraffin around the tissue with a knife. It is important to carefully clean the blade with xylene, before and after cutting the sample, to avoid dragging the remains of the previously cut sample. Make with the microtome 2-3 cuts of 5µm and place them in a sterile 1.5 ml tube.

7. WORKING PROTOCOL

CLART® HPV4 kit has been designed and validated for the following uses:

<table>
<tr><td></td><td>Direct sample Amplification.</td><td>Extracted DNA Amplification</td></tr>
<tr><td>Manual visualization</td><td>Dry Swab
Hybrid capture
ThinPrep®
SurePath®</td><td rowspan="2">All visualization systems</td></tr>
<tr><td>autoclart® visualization</td><td>Dry Swab
Hybrid capture
ThinPrep®
SurePath®</td></tr>
</table>

autoclart® plus visualization	Dry Swab	

Select the protocol that best suits your needs, and proceed according to the corresponding instructions of use.

- Direct sample Amplification. Please follow the corresponding protocols below.
- Extracted DNA Amplification. In this case it is advisable to check that the chosen extraction system is suitable for the *CLART®* technique.

7.1. Sample processing without DNA extraction.

7.1.1. Dry swab

- Add 1-1.5 ml of Saline solution just immediately before sample processing, never earlier. Vortex thoroughly for at least 30 seconds. Sample should be ready now for amplification. The second use of saline solution mixture with cells is not recommended.

7.1.2. Specimen Transport Medium™ Digene, Hybrid Capture medium

- Place 200 µl of sample within a 1.5 ml tube.
- Centrifuge for 1 min at 4.000rpm. Discard supernatant.
- Add 1 ml of Saline solution, Vortex properly, centrifuge 1 minute at 4.000 rpm. Discard supernatant.
- Resuspend the pellet in 50 µl of DNAse free water.

7.1.3. Cell suspension_ ThinPrep®

- Place 400 µl of cell suspension within a 1.5 ml tube.
- Centrifuge for 1 min at 13.000 rpm. Discard supernatant.
- Resuspend pellet in 400 µl Saline solution.
- Centrifuge for 1 min at 13.000 rpm. Discard supernatant.
- Resuspend pellet in 25 µl of DNAsa free water.

7.1.4. Cell suspension_SurePath®

- Place 800 µl of cell suspension within a 1.5 ml tube.
- Centrifuge for 1 min at 13.000 rpm. Discard supernatant.
- Resuspend pellet in 1000 µl Saline solution.
- Centrifuge for 1 min at 13.000 rpm. Discard supernatant.
- Resuspend pellet in 1000 µl Saline solution.
- Centrifuge for 1 min at 13.000 rpm. Discard supernatant.
- Resuspend pellet in 25 µl of DNAsa free water.

7.2. Sample processing including DNA extraction.

7.2.1. Formalin-fixed paraffin-embedded tissue

1. Introduce 4-5 tissue cuts of around 5 µm/ each within a 1.5ml tube, and add 180 µl of Buffer T1.

2. After mashing the tissue using the pipette tip, vortex vigorously to facilitate cell lysis.

3. 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

4. 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.

5. 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.*

6. 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

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

Add 500 µl B5 1 min, 12,000 rpm

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

Add
600 µl B5
1 min,
12,000
rpm

9. Place the column again within the collection tube. Centrifuge for 1 minute at 12000 rpm. to eliminate any remaining Buffer B5.

1 min
12,000
rpm

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

10. 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 rpm.

Add 100 µl
BE
1min,
12,000
rpm

11. 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.

7.3. 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.

- Avoid using temperatures higher than 37ºC for thawing the Amplification tubes and plates.

7.3.2. Amplification protocol

1. Thaw on ice the required number of amplification tubes/ plates according to the number of samples to be processed. Keep them at 4 ºC.

2. Briefly centrifuge the amplification tubes/ plates 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 µl of direct sample or of extracted DNA to each amplification tube or amplification plate well. Keep tubes/ plate at 4ºC.

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

1 cycle	98ºC 5 min
45 cycles	98ºC 15 sec 55ºC 15 sec 72ºC 30 sec
1 cycle	72ºC 1,0 min
4ºC Until tube/ plate removal	

5. Start the program and place the amplification tubes/ plate in the thermal cycler. Amplification duration is of about 2 hr, the exact time depending on the thermal cycler used. This technique has been validated for exclusive use in thermal cyclers with standard ramps, not with fast ramps.

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 areas.

2. Make sure that the thermomixer temperature has been 65ºC for at least 30 minutes before the hybridization step starts.

3. Warm up SH at room temperature until crystals disappear. Should crystals not clear up completely, the bottle may be placed within the thermomixer while it warms up for the hybridization step.

4. Avoid adding SH to the CS wells before denaturation has concluded.

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

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

7. 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.

8. 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.

9. 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.

10. Do not allow the well to dry completely.

11. Avoid foaming when adding reagents.

12. 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.

Manual (Section 7.3.2), "autoclart®" (Section 7.3.3) and "autoclart® plus" (Section 7.3.4) visualization protocols are provided.

7.4.2. Manual visualization protocol

1. Turn on the CAR® before starting the whole procedure. Self-calibration of the equipment may last for a few minutes.

2. <u>Denaturation of amplified products:</u> Place the amplification tubes / plate in the thermal cycler and incubate at 95ºC **for exactly 10 minutes**. After that, remove the tubes / plate and immediately place on ice or at 4ºC.

3. <u>Preparation of Washing solution:</u> 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 TL 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 TL 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, **10 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 CSs with the plastic lid and incubate in the thermomixer for **30 minutes at 65ºC and 550 rpm.**

   After incubation, remove the CSs from thermomixer and aspirate incubation solution from the CS wells with 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 **9 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 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.

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 / plate in the thermal cycler and incubate at 95ºC **for exactly 10 minutes**. After that, remove the tubes / plate from the 95ºC incubation and immediately place on ice or at 4ºC.

3. Swith 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 ***CLART® HPV 4*** or ***CLART® HPV 4S.***

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.

    SH. Add the volume of tempered SH displayed in the screen.

    CJ. Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ solution as displayed in the screen. This is done by adding 9 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.

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 **10 µl** of denatured amplification product to the 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 with CSs 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®. Immediate reading is required.

17. Place the plate in the CAR®. The CAR® will read and report the results automatically.

7.4.4. autoclart® plus visualization protocol

The device might be used in three distinct ways depending on the user needs. For further information see below, and also the "autoclart® plus" user manual.

A) With automatic addition of the sample. Exclusive for plates.

1. Switch on the autoclart® plus unit and follow the instructions on the screen.

2. Select "New Analysis" at the main "Menu" screen.

3. Press on the crosses at the top row to select the number of required CSs.

4. Select the type of assay "HPV4" or "HPV4S" from the list. To perform the same assay for all the CSs, press "Auto Select", followed by the right arrow key to proceed ahead.

5. Select the "automatic sample addition" option at the Analysis Setting screen. Press the right arrow key to continue.

6. The instrument will initiate the temperature preconditioning step. A series of checklists will be displayed onscreen; Press the arrow key to accept and proceed.

7. Place the full rack of 10 µl and 1000 µl tips for reagent dispensation.

Note: At this moment cooling of the reagents begins. A beeping signal will acknowledge end of the cooling step.

8. Denaturation: Use the thermocycler for denaturing of amplification products. Place the plate in the thermocycler once it has reached 95ºC and incubate the tubes for **10 minutes**. Remove the plate from the 95ºC incubation and place it immediately on ice.

9. Place the adaptor in the equipment with the required number of **CS**s.

10. Check that the tip and waste through containers are empty. Go to next step.

11. Fill the water bottle with 250 ml distilled water, go to next step.

12. Add the reagent volumes corresponding to the number of samples to be processed, as requested by autoclart® plus.

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

SH. Add the volume of tempered SH displayed in the screen.

CJ. It is recommended to centrifuge CJ for 10 seconds before use. Each mL of diluted CJ solution indicated on the display should be prepared by adding 1 ml of DC and 5 µl of CJ reagent. Vortex the diluted solution in order to mix it up properly.

RE. Add the RE volume indicated on the display.

13. Place the amplification plate on the autoclart® plus.

14. Close the door and press "Play" to start.

15. Once the visualization and reading process is over, the autoclart® plus will beep indicating the end of the run.

B) With manual addition of the sample

1. Switch on the autoclart® plus unit and follow the instructions on the screen.

2. Select "New Analysis" at the main "Menu" screen.

3. Press on the crosses at the top row to select the number of required CSs.

4. Select the type of assay "HPV-4" or "HPV-4S" from the list. To perform the same assay for all the CSs, press "Auto Select", followed by the right arrow key to proceed ahead.
5. Select the "Manual sample addition" option at the Analysis Setting screen. Press the right arrow key to continue.

6. The instrument will initiate the temperature preconditioning step. A series of checklists will be displayed onscreen; Press the arrow key to accept and proceed.

7. Place the full rack of 1000 µl tips for reagent dispensation.

WARNING: The 10 µl tips for sample addition ARE NOT NECESSARY.

Note: At this moment cooling of the reagents begins. A beeping signal will acknowledge end of the cooling step.

8. Denaturation: Use the thermocycler for denaturing of amplified products. Place the amplification tubes/ plate in the thermocycler, and incubate at 95ºC for 10 min. Do not exceed this time. Remove the tubes from the 95ºC incubation and place them immediately on ice. Remove the tubes/ plate from the 95ºC incubation

and place them immediately on ice.

9. Place the adaptor in the equipment with the required number of CSs.

10. Check that the tip and waste through containers are empty. Go to next step.

11. Fill the water bottle with 250 ml distilled water, go to next step.

12. Add the reagent volumes corresponding to the number of samples to be processed, as requested by autoclart® plus.

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

    SH. Add the volume of tempered SH displayed in the screen.

    CJ. It is recommended to centrifuge CJ for 10 seconds before use. Each 1 mL of diluted CJ solution indicated on the display should be prepared by adding 1 ml of DC and 5 µl of CJ reagent. Vortex the diluted solution in order to mix it up properly.

    RE. Add the RE volume indicated on the display.

13. 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 the autoclart® plus door is closed or the user clicks on the "Mute" symbol on the screen.

14. For sample addition, place the CSs out of the autoclart® plus and add **10 µl** of denatured amplification product to the 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® plus and press the arrow on the screen to continue with the visualization process.

15. Once the visualization process is over, the autoclart® plus will beep indicating the end of the run.

C) Reading of samples

1. Switch on the autoclart® plus unit.

2. Place the adaptor and **CS** units to be used within the device, and follow the instructions on the screen.

3. Select “New Analysis” at the main “Menu” screen.

4. Press on the cross at the top row to select the number of required CSs.

5. Select the assay “HPV4” or “HPV4S” from the list. To perform the same assay for all the CSs, press “Auto Select”, followed by the **right arrow key to proceed ahead**.

6. **Select the “Reading” option at the “Analysis Setting” screen. Press the right arrow key to continue.**

7. Once the reading process is over, the autoclart® plus will beep indicating the end of the run.

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® or autoclart® plus.

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 4*** kit avoids these false negatives through addition of two internal controls within each reaction tube or plate well:

- 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 or plate well 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/ well, and that is used as amplification control of the PCR reaction.
- HPV-specific oligonucleotides.

The amplification tubes/ plate wells 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	GENOMICA/AMPLIFICATION CONTROLS	INTERPRETATION
√. POSITIVE	√ RIGHT	POSITIVE
x. NEGATIVE	√ RIGHT	NEGATIVE

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 the types:

INTERPRETATION	CAUSE	SOLUTION
NO DNA	No DNA present in the sample	Repeat the technique from the extraction step, or obtain a new sample from the patient.
PCR INHIBITED	This is due to the fact that some substances may inhibit the PCR reaction through impairment of the DNA polymerase activity.	Check the presence of any of these substances in the samples or extracted genetic material. In most cases it is recommended to repeat extraction or obtain a new sample from the patient.

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. Control of known interferences

Some substances might interfere with the ***CLART® HPV4*** system. These are mainly substances that inhibit the DNA polymerase, and thereby, the amplification reaction. The most known interferences are:

1 **Presence of hemoglobin and excess of cellular debris.** Due to the fact that no purification of the sample is performed, some inhibitions might result from the addition of an excess of hemoglobin or cell debris to the amplification tube/ plate well.

2 **Presence of acetic acid or iodine** in the sample to be analysed. If a sample for analysis with ***CLART® HPV 4*** 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 4*** 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, the analysis result will be of inhibition due to lack of amplification of the sample's control DNA.

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 (2015 WHO HPV)	10 copies
6		100%	100%
11	100%	100%	80%
16	100%	100%	80%
18	100%	100%	80%
26			100%
31		100%	100%
33	100%	100%	80%
35	100%	100%	60%
39	100%	100%	80%
45	100%	100%	80%
51	100%	100%	80%
52		100%	100%
53			100%
56	100%	100%	80%
58	100%	100%	60%
59	100%	100%	80%
66		100%	100%
68a	100%	100%	
68b	100%	100%	80%
82			100%

N=110

Table 1. Analytic sensitivity of *CLART® HPV4* format.

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. In addition, Table 1 displays sensitivity values corresponding to other HPV types included within the above-mentioned study panel. 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® HPV4* format.

9.2.2. Diagnostic utility parameters

In order to determine the diagnostic parameters of the ***CLART® HPV4*** system, the following comparative studies were performed:

In order to validate the ***CLART® HPV4*** format, a comparative study between *CLART® HPV4* and *CLART® HPV2* was performed, in collaboration with four Spanish hospitals:

- Microbiology Service of the “Hospital Universitari Germans Trias i Pujol” of Badalona.
- Virology Department of the “Hospital Universitario Virgen de la Arrixaca”.
- Department of Anatomic Pathology of the “Hospital Universitario Clínico San Carlos”.
- Department of Anatomic Pathology of the “Hospital Universitario Marqués de Valdecilla”.

421 samples were analysed, of which 88 were dry swabs, 101 samples in Hybrid Capture medium and 232 ThinPrep®-like liquid citologies.

Table 2 displays the diagnostic sensitivity and specificity values for the HPV types detected by the HPV4 format, directly from sample and without DNA extraction:

Type	Sensitivity	Specificity	Type	Sensitivity	Specificity
Type 6	100,0	100,0	Type 56	100,0	99,7
Type 11	83,3	100,0	Type 58	93,3	100,0
Type 16	100,0	100,0	Type 59	95,0	99,7
Type 18	100,0	100,0	Type 61	100,0	100,0
Type 31	93,8	100,0	Type 62	87,0	100,0
Type 33	100,0	100,0	Type 66	100,0	99,8
Type 35	100,0	100,0	Type 68	100,0	99,8
Type 39	100,0	100,0	Type 70	81,8	100,0
Type 40	100,0	100,0	Type 71	100,0	100,0
Type 42	100,0	100,0	Type 72	100,0	100,0
Type 43	100,0	100,0	Type 73	100,0	100,0
Type 44	100,0	99,5	Type 81	100,0	100,0
Type 45	100,0	100,0	Type 82	90,0	100,0
Type 51	100,0	100,0	Type 83	100,0	100,0
Type 52	92,6	100,0	Type 84	100,0	100,0
Type 53	97,1	100,0	Type 89	100,0	99,5
Type 54	100,0	100,0			

Table 2. Diagnostic parameters of *CLART® HPV4* format.

In order to validate the ***CLART® HPV4S*** format, a concordance study between the results obtained with *CLART® HPV4* and *CLART® HPV4S* was devised.
The objective of the study was to obtain a concordance percentage of 95% or higher, for the 16 types included within the *CLART® HPV4S* format.

495 samples were studied in parallel. The results obtained show that the objective of the study was achieved; thereby the conclusion is drawn that the diagnostic results obtained with *CLART® HPV4S* are equivalent to those of *CLART® HPV4.*

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Anex I: Table displaying the oncogenic risk* of the HPV types detectable with ***CLART® HPV4*** Kit.

The following table indicates the oncogenic risk of the HPV types detected with *CLART® HPV4* kit:

HIGH RISK TYPES
HPV 16
HPV 18
HPV 31
HPV 33
HPV 35
HPV 39
HPV 45
HPV 51
HPV 52
HPV 56
HPV 58
HPV 59
HPV 66
HPV 68

POTENTIALLY HIGH RISK TYPES
HPV 26
HPV 53
HPV70
HPV 73
HPV 82

LOW RISK TYPES
HPV 6
HPV 11
HPV 40
HPV 42
HPV 43
HPV 44
HPV 54

* Oncogenic risk classification determined within:

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

COMPOSITION:

Amplification reagents;

Nº test	Manual		autoclart® plus	
48	48x	*15 µl*	48x	*15 µl*
96	96x	*15 µl*	96x	*15 µl*

COMPONENTS	%	CLASSIFICATION according to regulation *1272/2008:*
PCR Master Mix	89	*This product is a mixture of substances* **Does not have dangerous components**
Amplification oligonucleotides	11	*This product is a mixture of substances* **Does not have dangerous components**

Visualization reagents;

- **SH (Hybridization solution)**

Nº test	Manual		autoclart® plus	
16	1x	6ml	1x	11.5ml
48	1x	6ml	1x	11.5ml
96	2x	6ml	2x	11.5ml

COMPONENTS	%	CLASSIFICATION according to regulation 1272/2008:
Sodium phosphate	25	*This product is a mixture of substances* ***Does not have dangerous components***
SSC	5	*This product is a mixture of substances* ***Does not have dangerous components***
EDTA	0.2	*This product is a mixture of substances* ***Does not have dangerous components***
Triton X-100	2.25	*This product is a mixture of substances* **The concentration of the possible dangerous components according to the regulation is below the allowed limits**

	DANGEROUS COMPONENT	ICON	SENTENCE
	p-tertiary-Octylphenoxy polyethyl alcohol CAS-No.: 9002-93-1 - **concentration <0.05**	Warning! 	H319, H412 P280, P305+P351+338

- ***RE (Development solution)***

Nº test	Manual	autoclart® plus

16	1x	3ml	-	-
48	1x	5.5 ml	1x	11.5ml
96	2x	5.5 ml	1x	11.5ml

COMPONENTS	**%**	**CLASSIFICATION according to regulation 1272/2008**
Aqueous solution of o-dionisine in a citrate buffer with hydrogen peroxide	100	*This product is a mixture of substances* ***The concentration of the possible dangerous components according to the regulation is below the allowed limits*** **It can be dangerous for aquatic organisms**

- ***DC (Conjugate solvent)***

Nº test	**Manual**		**autoclart® plus**	
16	1x	7ml	1x	11.5ml
48	1x	7ml	1x	11.5ml
96	2x	7ml	2x	11.5ml

COMPONENTS	**%**	**CLASSIFICATION according to regulation 1272/2008**
BSA saline phosphate buffer	100	*This product is a mixture of substances* ***Does not have dangerous components***

- ***CJ (Conjugate)***

Nº test	**Manual**		**autoclart® plus**	
16	1x	100 µl	1x	100 µl
48	1x	100 µl	1x	100 µl
96	2x	100 µl	2x	100 µl

COMPONENTS	**%**	**CLASSIFICATION according to regulation 1272/2008**
Streptavidin peroxidase	1	*This product is a mixture of substances* ***Does not have dangerous components***
Coonjugate solvent	99	

- ***TL (Wash buffer)***

Nº test	**Manual**		**autoclart® plus**	
16	1x	7ml	1x	11.5ml
48	1x	7ml	1x	11.5ml
96	2x	7ml	2x	11.5ml

COMPONENTS	**%**	**CLASSIFICATION according to regulation 1272/2008**
Saline phosphate buffer with Tween	100	*This product is a mixture of substances* ***Does not have dangerous components***

*** Fully text to the H-Sentences mentioned:**

H302- Harmful if swallowed.
H319 - Causes serious eye irritation.
H411- Very toxic to aquatic life with long lasting effects.
P280 – Wear gloves / garments / goggles / protective mask.
P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes.

RELATIVE CONSIDERATIONS TO ELIMINATION

Waste treatment methods
Handover the surplus and non-usable solutions to an accredited waste company.

Contaminated packaging
Remove as unused products.