*CLART® HPV3*

# GENOTYPING OF HUMAN PAPILLOMAVIRUS VIA GENOMIC IDENTIFICATION for *IN VITRO* DIAGNOSIS

# *CLART® HPV3*

*CE Marking: HPV Genotype 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85 and 89.*

*For research use only: HPV Genotype 34, 64, 67, 69 74, 86, 87, 97, 101, 102, 103, 106, 150 and 151.*



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CE

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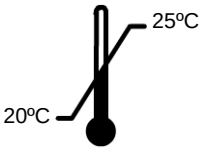
**1. GLOSSARY OF TERMS**

Please, check handling instructions

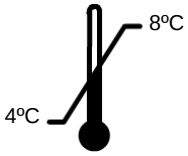
Expiry date


*In vitro* diagnostic device

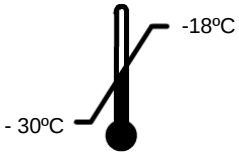

Batch




Store at room temperature




Store between 4 ºC and 8 ºC




Store between –30 ºC and –18 ºC

## 2. DESCRIPTION

***CLART® HPV3* Kit** detects up to 49 (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) of the most clinically relevant HPV types in a wide range of samples (swabs, cell suspensions, tissues fixed in FFPE).

Detection of the different HPV genotypes is achieved by **PCR amplification of a 450 bp fragment within the highly conserved L1 region of the virus**. This highly conserved sequence presents slight variations among each individual HPV type that allows its genomic identification by recognition of the viral DNA by specific probes. This slight variation guaranties the detection specificity.

The detection of the product amplified by PCR is carried out by means of a low-density microarray platform: CLART® (Clinical Arrays Technology). The platform is based on a very simple principle, but at the same time cost effective. It consists of a microarray printed at the bottom of a microtiter plate, which simplifies the entire hybridization and visualization process when compared to classic microarray systems. Figure 1 displays a CLART-Strip® or CS of 8 wells.

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

The ***CLART®*** *HPV3* detection system is based on the precipitation of an insoluble product in those areas of the microarray where hybridization of amplified products with specific probes occurs. During the PCR, the amplified products are labeled with biotin. After amplification, the products are hybridized to their respective specific probes that are immobilized at specific and known areas of the microarray. These immobilised biotinilated products are recognized by the streptavidin of a streptavidin-peroxidase conjugate, thus providing with peroxidase activity to the hybridised products. Peroxidase activity will then metabolise o-Dianisidine and produce an insoluble product which will precipitate in those places where hybridisation occurred (Fig. 2).

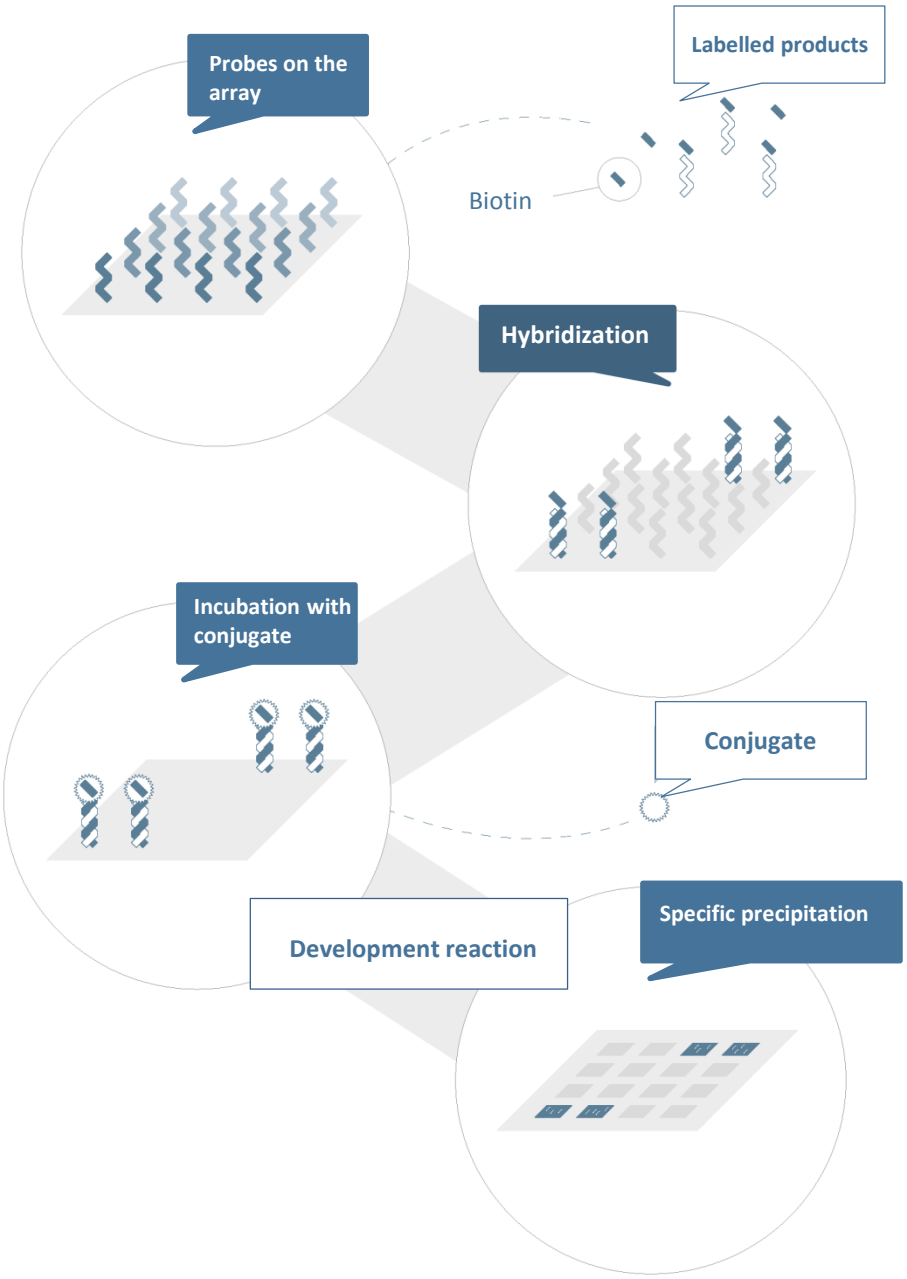



**Figure 2: Diagram of the visualization method.** Probes, immobilized on the surface, capture their complementary biotin-labeled amplified products. With the help of the biotin, the conjugate binds, in this case streptavidin-HRP (***H****orseR**adish* ***P****eroxidase*). Due to the HRP action, the o-dianisidine substrate produces a precipitation on the hybridization site.

## 3. KIT COMPONENTS AND CONSERVATION

***CLART® HPV3 Kit*** contains sufficient reagents for the extraction and analysis of DNA from 48 clinical samples. These reagents are provided in two different boxes, depending on the

temperature at which they should be kept. All the reagents provided are stable under the appropriate conditions until the indicated expiration date.

**3.1. Extraction, purification and amplification reagents.**

The ***CLART® HPV3*** Extraction and Purification Kit is delivered to users at 4ºC or room temperature.

Components:

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

Note: In Kits for processing of 16 samples, already resuspended Proteinase K is sent at -20ºC within the same box as the amplification tubes.

Amplification tubes are sent at -20 ºC:

**Amplification tubes contain 45** µl of reaction mix. They are sent ready to use and must be stored at -20ºC. Only the required number should be thawed on ice at any given time while the remainders should be kept at –20ºC.

***Note: the kit includes an adhesive temperature indicator strip. If a red colour appears in the viewing window of this temperature indicator strip, the cold chain may have been broken and the kit should not be used.***

**3.2. Visualization reagents.**

**WARNING:** Once received, the microarrays should be stored at room temperature.

- **CLART-Strip® (CS)** (including the specific probes). These are delivered in a sealed envelope. **After opening, the envelope should be closed and stored at room temperature,** provided that the temperature in the lab does not exceed 25 °C, and **protected from light exposure.**
- **SH** (Hybridization solution). **Store at 4ºC.**
- **DC** (Conjugate solvent). **Store at 4ºC.**
- **CJ** (Conjugate). **Store at 4ºC**. **Centrifuge briefly before using**.
- **TL** (Washing buffer). **Store at 4ºC.**
- **RE** (Developer). **Store at 4ºC and protected from light.**
- **Support and lid for the 8-well strip.**

**3.3. Other components.**

The technique requires a system to capture and process the picture obtained from the micro-array, automatically generating a single report for every analyzed sample. The following table shows the characteristics of the system marketed by GENOMICA. It has a specific **Software for *CLART® HPV3***, designed and validated by GENOMICA.

- **CAR®** (CLINICAL ARRAY READER) (figure 3): which allows the reading and automatic interpretation up to12 CS, that means, a total amount of 96 samples. This platform is manufactured exclusively for GENOMICA kits use only.
- **SAICLART®**: software developed by GENOMICA for image processing.
- *CLART® HPV3* **Software**: It is specific for *CLART® HPV3* designed and validated by GENOMICA. Installed and ready to use.


**Figure 3.** CAR® (CLINICAL ARRAY READER)

## 4. ADDITIONAL MATERIAL

### 4.1. Reagents and material.

- Distilled water
- Disposable gloves
- Positive displacement or filtered pipette tips
- Bowl of chipped ice
- Autoclaved Eppendorf type tubes (1.5 ml).
- Racks for 1.5 ml tubes.
- Racks for 0.5 ml/0.2 ml tubes.

### 4.2. Equipment.

- Microcentrifuge.

- Thermocycler.
- Three adjustable micropipettes (1-20 µl, 20-200 µl and 200-1000 µl) for use in the extraction laboratory.
- Three adjustable micropipettes (1-20 µl, 20-200 µl and 200-1000 µl) for use in the visualisation laboratory.
- Thermoblocks (at 37°C, 55°C and 100ºC) with an adjustable agitation source and compatible with Eppendorf like tubes.
- Vortex
- Vacuum system (optional)

## 5. HANDLING PROCEEDINGS AND RECOMMENDATIONS

***Very important: read this section carefully before beginning any work.***

### 5.1. General recommendations.

**1. The procedure should be performed in two physically separated areas.** This will avoid samples contamination with previously amplified products. Each area should have its own, identified working materials (pipettes, tips, tubes, racks, gloves etc.) which should never leave the assigned area.

- **Pre-PCR area**: This is the area where samples are prepared and DNA is extracted.
- **Post-PCR area**: This is the area where products are amplified and then visualized. Material in this area should never come into contact with material in the pre-PCR area. **AFTER WORKING IN THE POST-PCR AREA, DO NOT RETURN TO THE PRE-PCR AREA**.

**2. Use gloves at all times and change them frequently.**

**3. Clear working areas** (laboratory benches, hoods, grids, pipettes, Thermocycler) thoroughly with 10% diluted bleach **following every sample batch processing;** it is mandatory to disinfect all working areas in case of contamination.

**4. Always use pipette tips containing a filter or use positive displacement pipettes** to avoid contamination.

**5. Use disposable and autoclaved laboratory materials**.

**6. Never mix reagents from two different tubes, even though they belong to the same lot.**

**7. Close reagent tubes immediately after use, as this will avoid contamination.**

**8. Dispose of micropipette tips after use.**

**9. GENOMICA S.A.U. does not assume any responsibility for those results obtained without following the directions in this manual.**

### 5.2. Visualization precautions.

**1.** Prevent the pipette tip or vacuum system from touching the bottom of the tube where the microarray is located.

**2.** It is recommended to add all solutions to the wall side of the array; never directly on the bottom.

**3.** Only add the hybridization solution immediately before adding the PCR products.

**4.** There will be a slight residual volume left so that the array will not dry out at any time.

**5.** After incubating with the conjugate, it is very important to thoroughly wash the array to avoid any remaining conjugate from reacting with the developer.

**6.** Avoid bubbles formation on the surface of the microarray when adding the different solutions.

**7.** Keep the bottom of the array clean in order to avoid possible interference when reading your results.

**8.** When visualizing the image in the reader, confirm that position markers appear and that there are no bubbles or spots interfering with the reading. If required, clean the external part of the bottom of the tube with cellulose paper or gently tap the tube with your finger.

## 6. TAKING SAMPLES

### 6.1. Swabs.

Samples should be taken with a clean, dry, cotton or alginate swab large enough to obtain a good sized sample. Do not use devices that could cause any bleeding, as blood may interfere with the assay. Place the swab in its tube, which should not contain any kind of preservation medium. Maintain the swab at 4ºC if it is to be processed within seven days, or at –20ºC if processing is to occur later.

### 6.2. Cell suspensions.

Cervicovaginal cytology tests (membrane-filtered monolayers [ThinPrep®, Cytyc]) are refered in this section. After taking the sample with a brush or spatula, resuspend the sample in a vial containing the transport medium by agitating the sampling device. Dispose of the sampling device and keep the sample at 4ºC until use.

### 6.3. Formalin, ethanol and paraffin wax-embedded samples.

Fix the samples in **buffered formalin** for the shortest time possible (never more than 24 h). The use of non-buffered formalin or fixation for longer than 24 h could cause sample DNA degradation. Before cutting the sample, it is important to carefully clean the blade with xylene before and after cutting. This will help to avoid any cross contamination of the cut samples. Remove any excess wax from the sample with another blade. Use a microtome to make four or five 5 µm sections and store them in a 1.5 ml sterile tube.

## 7. WORKING PROTOCOL

## 7.1. HPV DNA extraction

It is important to include an extraction negative control to verify that the samples have not been contaminated during the extraction, amplification or visualization processes, thus giving rise to a false positive.

### 7.1.1. Manual extraction of HPV DNA.

*PREPARATION PROCEDURES*

1. Dissolve Proteinase K in BP before use, in order to reach a concentration of 20 mg/ml. The volume of BP required, is indicated on 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.

2. Buffer B5 preparation: Add 28 ml of 96-100% ethanol to the Buffer B5 bottle before use.

3. Heat solution BE to 70°C before use.

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

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

**EXTRACTION OF HPV DNA**

**1. Sample preparation**

***Cell suspensions:***

- Shake the sample (invert the sample container a few times) and take 1 ml to a sterile 1.5 ml microcentrifuge tube.

- Centrifuge the samples for 10 min in a microcentrifuge at 12,000 rpm and remove the liquid with a micropipette. Be careful not to remove the precipitate.

- Resuspend the precipitate with 1 ml sterile water.

- Centrifuge the samples for 10 min in a microcentrifuge at 12,000 rpm and remove the liquid with a micropipette. Be careful not to remove the precipitate.

- Resuspend the precipitate in 180 µl Solution T1.

- Transfer to a sterile 1.5 ml microcentrifuge tube, and proceed to step 2.

***Swabs:***

- Add 1.5 ml saline buffer (0.9% NaCl) to the tube containing the swab and agitate vigorously with a vortex for 1 min.
- Decant the supernatant into a sterile 1.5 ml tube.
- Centrifuge the samples for 10 min at 12,000 rpm and then remove all liquid with a micropipette. Be careful not to remove the precipitate.
- Resuspend the precipitate in 180 µl solution T1, and proceed to step 2.

***Tissues fixed in formalin or paraffin wax:***

Introduce the samples into a sterile 1.5 ml microcentrifuge tube and add 180 µl of Solution T1.

***SAMPLES WILL FOLLOW THE SAME TREATMENT FROM NOW ON***

**2.** Add 25 µl of proteinase K solution and incubate the samples at 56°C for 1-3 h (overnight in case of paraffin embedded tissues) in a water bath or Thermoblock (with agitation) until the sample is completely lysed. Vortexing samples every 15 minutes for a few seconds will accelerate lysis.

180 µl T1
+ 25 µl
proteinase K
1-3 h, 56°C

**3.** After lysis, add 200 µl of solution B3 to each sample. Mix the samples thoroughly by vortexing them and incubate them at 70°C for 10 min.

Add
200 µl B3
70°C, 10 min.

**4.** Add 210 µl of 96% ethanol to each sample and vortex them immediately.

***Note****: Do not discard any white precipitate that might form after adding the ethanol. Along with the rest of the solution, this precipitate should be added to the purifying column in the next step.*

**5.** After preparing a purifying column for each sample, add the samples and centrifuge them for 1 min at 12,000 rpm. Make sure that all the solution has completely crossed the membrane and discard the filtered solution and the 2 ml collection tube.

Add the
sample

1 min,
12,000 rpm

**6.** Add 500 µl of Solution BW to the column and centrifuge at 12,000 rpm for 1 min. Dispose the filtered solution and the 2 ml collection tube.

Add
500 µl B5

1 min,
12,000 rpm

**7.** Add 600 µl of Solution B5 to the column and centrifuge at 12,000 rpm for 1 min. Dispose the filtered solution.

Add
600 µl B5

1 min,
12,000 rpm

**8.** Centrifuge once more at 12,000 rpm for 1 min in order to eliminate any remaining Solution B5.

1 min
12,000 rpm

***Note:*** *Any residual ethanol from Solution B5 might inhibit the required enzymatic reactions; it must therefore be completely eliminated by centrifugation.*

**9.** Place the column in a clean 1.5 ml microcentrifuge tube. Incubate the DNA column with 100 µl of Solution BE (pre-heated at 70°C) for 1 min and then centrifuge at 12,000 rpm for 1 min.

Add 100 µl
BE 1 min

1min, 12,000
rpm

**10.** Recover the filtrate (approximately 100 µl) in the microcentrifuge tube and take 5 µl for the amplification reaction. Store the rest at –20°C.

**7.1.2. Automatic extraction of HPV DNA**

**NucliSENS™ Biomérieux easyMAG device**

The following protocol is recommended:

**1. Preparation of samples for the internal lysis (performed inside the device)**

***Swabs:***

- Add 1.5 ml of saline serum (0.9% sodium chloride) to the tube that contains the swab and vortex vigorously for 1 minute.
- Pour the supernatant into a sterile 1.5 ml tube.

- Transfer 1 ml into a well of the tray (each tray contains eight wells).

***Transportation medium (volume less than 3 ml):***

- Shake the sample by inverting the vial several times and transfer 0.5 ml into one well of the tray.

***Cell suspensions (volume less than 3 ml):***

- Shake the sample by inverting several times the vial and transfer 1 ml into one well of the tray.

**2**. Internal lysis and DNA extraction: follow the user's protocol. It is necessary to set the elution volume in the program to 110 µl.

**3**. Once the extraction is finished transfer 110 µl of eluted DNA into a 1.5 ml Eppendorf type tube. Use 5 µl for the amplification reaction and store the rest at -20ºC.

**Qiagen BioSprint 96 device.**

The following protocol is recommended:

*BUFFER PREPARATION*

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

1. Before using, **reconstitute the lyophilized protease** by adding 4.4 ml of the buffer indicated in the label. Once reconstituted, keep at 4ºC for up to 2 months.

2. **Preparing Buffer AW1**

| Volume concentrated AW1 (ml) | Volume of 96% ethanol to be added | Final Volume (ml) |
|---|---|---|
| 19 | 25 | 44 |
| 27 | 35 | 62 |
| 98 | 130 | 228 |

**Note**: keep at RT. Before using, shake the bottle five times.

3. **Preparing Buffer AW2**

| Volume concentrated AW2 (ml) | Volume of 96% ethanol to be added | Final Volume (ml) |
|---|---|---|
| 17 | 40 | 57 |
| 68 | 160 | 228 |

**Note**: keep at RT. Before using, shake the bottle five times.

4. **Preparing 0.0002% Tween 20**

| | | |
|---|---|---|
| RNAase free $H_2O$ | 30 ml | 250 ml |
| Tween 20 | 6 µl | 50 µl |

**Note**: Keep the mixture at 4ºC.

*SAMPLE PREPARATION*

**1.** Caution! Set the thermomixer at 70ºC to be ready for the protease lysis.

***Swabs:***

- Cut the swab and introduce it into a 1.5 ml tube. Add:
  - 400 µl of ATL buffer
  - 20 µl of Protease

***Cell suspensions (volume less 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 the incubation is performed in a plate, cover it with a transparent film and a pre-heated lid at 70ºC in order to avoid condensation of the sample which could lead to contamination.

**3**. Master-mix preparation. Pipetting errors can be reduced by preparing 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 the solutions into the plates.

It is necessary to use 6 S-Block and 2 Microplate MP.

The **table 1** shows the slot of each plate in the device and the volumes that must be added into the plates:

| Slot in the device | Plate | To add | Volume per well (µl) |
|---|---|---|---|
| 8 | Micro-plate MP | Place the support with the protective cover | ------ |
| 7 | Micro-plate MP | Buffer AE (Elution Buffer) | 100 or 200 |

| | | | |
|---|---|---|---|
| 6 | S-Block | RNAase free $H_2O$ + Tween 20 | 500 |
| 5 | S-Block | Buffer AW2(2) | 500 |
| 4 | S-Block | Buffer AW2(1) | 500 |
| 3 | S-Block | Buffer AW1(2) | 500 |
| 2 | S-Block | Buffer AW1(1) | 500 |
| 1 | S-Block | Lysed samples + Master-mix (*) | 200 + 420 |

(*) When using swabs, spin the samples before adding the lysates. First add 200 µl of the lysed sample and then, 420 µl of the master-mix into the well.
If the lysis has been performed in the plates, add 420 µl of master-mix directly into the well containing the lysed sample.

Always mix sample and master-mix by pipetting up and down several times.

**5**. Extraction.

- Once the plates are prepared, switch on the **BioSprint 96** at the power switch.
- Open the front door of the protective cover.
- Select the protocol DNA Swab using the up and down arrows. Press "Start" to start the protocol run.

The LCD displays a message asking you to load slot 8 of the worktable with the 96-rod cover. After loading slot 8, press "Start". The worktable rotates and a new message appears, asking you to load spot 7 with the elution plate. Load slot 7 and press "Start" again. Continue this process of pressing "Start" and loading a particular slot until all slots are loaded.

***Table 1*** shows in which slots the plates should be loaded. Load each plate so that well A1 is aligned with the slot's label (i.e., well A1 faces inward).

- Check that the protective cover is correctly installed: it should fit exactly into the body of the BioSprint 96. Slide the door shut to protect samples from contamination.
- Press "Start" to start sample processing. The process will take 20 minutes. The extracted DNA can be stored in the plate (elution plate in slot 7) at -20ºC, covering the wells with a transparent film. If a full plate has not been used, transfer the extracted DNA into a 1.5 ml tube by using a pipette and store it at -20ºC.

## 7.2. Amplification reaction.

**1.** Thaw one **reaction tube** for each sample and keep them on ice. Do not use temperatures above 37ºC for thawing.

**2.** Centrifuge the **reaction tubes** in a microcentrifuge so that all the liquid goes to the bottom (if no adapters are available to hold the **reaction tubes**, they can be placed in larger tubes with their caps removed).

**3**. Should your DNA sample be obtained from paraffin embedded tissues; add 1.5 µl 25mM Magnesium Chloride to the **reaction tubes**.

**4.** Add 5 µl of extracted DNA from each sample to the **reaction tubes** and resuspend several times with a micropipette. Leave the tubes on ice.

**5.** Program the thermocycler as follows:

- For 0.2 ml **reaction tubes**:

| | |
|---|---|
| 1 cycle | 95ºC 5 min |
| 40 cycles | 94ºC 30 seg<br>55ºC 60 seg<br>72ºC 90 seg |
| 1 cycle | 72ºC 8 min |
| 4ºC (maintained) until tube collection (optional) | |

**6.** Start the program and place the **reaction tubes** in the Thermocycler when the block is above 90ºC. This minimises any non-specific amplifications due to hybridization occuring below the reaction temperature. The amplification process lasts about 4 hours, although this can vary depending on the Thermocycler used.

**7.3. Visualization of amplified products on CLART-Strip® (CS)**

***Specific recommendations before starting the visualization process:***

**THE PROTOCOL DESCRIBED BELOW SHOULD BE FOLLOWED IN THE POST-PCR AREA. NEVER TAKE THE AMPLIFIED PRODUCT INTO THE PRE-PCR AREA.**

**1**. At the beginning of the assay:

- Switch on the CAR® (CLINICAL ARRAY READER) in order to allow auto-calibration of the device.
- Input the sample IDs. It is important to have the equipment ready to read the samples at the end of the assay in order to avoid a developing time overexposure.

**2.** One hour before starting the hybridization process, switch on the Thermomixer at 65ºC.

**3.** Allow the SH (Hybridization Solution) to reach room temperature.

**4.** PREPARE THE **WASHING SOLUTION BEFORE** EACH ASSAY. DO NOT USE PREVIOUS SOLUTIONS OR ANY REMAINING FROM PREVIOUS ASSAYS.

**5.** Before starting the denaturing program, wash the Thermocycler with 10% diluted bleach. .During the denaturing process, place the amplification tubes separated from each other into the Thermocycler. Do not denature more than 10 minutes.

6. It is not necessary to use filter tips during the visualization process, only when adding amplified products to every well. A different tip for each sample and for every reagent must be used. This precaution must also be undertaken for the TL buffer.

7. The 8-tip combs used with the aspiration pumps must be discarded after use or decontaminated with 10% bleach solution after each assay. Make sure that the vacuum pump works properly and do not let remaining liquid in the wells.

8. All the buffers must be thoroughly aspirated from the wells without touching the bottom.

**VISUALIZATION**

1. Denaturation: use the Thermocycler to denature the samples.
   Adjust the program for 15 minutes at 95º C. Place the amplified products into the Thermocycler and incubate 10 minutes at 95º C. After 10 minutes, take off the tubes from the Thermocycler (still at 95$^{o}$C) and place them straight away on ice.

2. Prepare TL diluted Solution:

   For 8 wells (one strip) add as follows:

   - 1 ml of TL solution + 9 ml of distilled water. This will make up 10 ml of diluted TL solution necessary for one strip.

3. Pre-washing of CS: Before starting the assay, it is necessary to pre-wash the CS by adding 200 µl of TL diluted solution per well. After addition, mix the TL diluted solution 10 to 15 times with the pipette avoiding touching the surface of the array. Aspirate the TL diluted solution with the pump, making sure that the well remains completely clean with no remaining liquid. Add the buffer straight away as follows:

4. Hybridization: Hybridization solution (SH) must be heated at 50ºC in order to dissolve crystallized salts. Add 100 µl of SH buffer (avoiding foam formation) + 5 µl of denatured product to each well. Mix well with the pipette avoiding touching the array and incubate the strip, covered with the transparent plastic lid in the thermomixer for 1 hour at 65 $^{o}$C, shaking at 550 rpm.

   After one hour, take the CS out and remove the SH buffer with the pump.
   (Set the Thermomixer at 30$^{o}$C and shaking at 550 rpm for step 6. Remove the lid to speed up the cooling).

5. Double washing: use different tips for each well in both washes. Add 200 µl of diluted TL buffer and mix 10 to 15 times with the pipette. Remove the TL diluted solution with the pump. Repeat this wash once and leave the CS with 200 µl of TL buffer until the Thermomixer has reached 30$^{o}$C.

6. Blocking and adding conjugate: Prepare the diluted CJ solution 15 minutes before hybridization time is over and keep it on ice until its use. It is recommended to spin the CJ buffer for 10 seconds before using.

   Prepare the diluted CJ buffer: For one strip (8 wells) add as follows:

- 1 ml of DC buffer
- 7.5 µl of CJ buffer

Vortex the diluted CJ solution briefly before use.

Remove the diluted TL buffer without drying out the array and add 100 µl of diluted CJ buffer to each well. Incubate in the Thermomixer at 30°C, 550 rpm, for 15 minutes exactly.

After this incubation, take the strip and remove the diluted CJ buffer immediately with the pump (see figure 4).
(Set the Thermomixer at 25°C and no shaking for step 8. Remove the lid to speed up the cooling).

**7.** Triple Washing: **Add straight away** 200 µl of TL diluted solution per well. Mix well 10 to 15 times with the pipette and remove the diluted TL buffer with the pump without drying out the array. Repeat this wash twice and leave the CS with 200 µl of TL buffer at RT for 5-10 minutes or until the Thermomixer has reached 25°C.

It is very important that the diluted CJ buffer is completely washed off. Any remaining buffer could react with the RE buffer producing an unspecific signal.

**8.** Developing with RE buffer: Remove the diluted TL buffer without drying out the array and add 100 µl of RE buffer per well. Incubate in the Thermomixer at 25°C for 10 minutes **without agitation**.

**Attention! It is very important to use the Thermomixer without agitation in this step.**

**9.** After 10 minutes, remove the RE buffer with the pump. The array must be dry at this time.

**10.** CAR® (CLINICAL ARRAY READER): place the plate normally on the tray and the CAR® will take and analyze the arrays automatically.

## 8 RESULTS READING

The processing of the obtained data in each analysis is completely automatic. The reading/analysis equipment will provide a report with the results.

On the screen, a table with three columns will appear. The left column shows the HPV genotypes that can be detected, the central column will give either a positive or negative result for each genotype, and the right column will show if the amplification and DNA controls are fulfilled.

## 9. RESULTS INTERPRETATION

One of the main drawbacks of genomic amplification is the utilization of poor quality DNA samples (too short DNA, degradation of the DNA, or loss of DNA during extraction) or the presence of DNA polymerase inhibitors (e.g., hemoglobin, remains of paraffin wax, salts etc.) in the samples to be analyzed, thus interfering with the genomic amplification and resulting in false negatives. However, ***CLART® HPV3*** eliminates false negatives using internal controls within the same tube where the sample is analyzed, and that are amplified at the same time as the viral DNA.

Every reaction (amplification) tube of the kit contains the following primers:

- A pair of primers that amplify a fragment of the human gene CFTR (genomic DNA or DNA from the patient). It is used as **genomic DNA control.**
- A pair of primers that amplify a modified plasmid that is included in the tube and which is used as a **amplification reaction control.**
- HPV primers.

The reaction tube has been designed in order to favour the amplification of the HPV against the other two controls. Among these two controls, the genomic DNA will amplify preferentially compared to the amplification reaction control.

The reason for this design is:

**Genomic DNA control** would only be essential for confirming a negative result, since it reports that DNA from the patient was present in the sample even if no HPVs were found.

**PCR control** would only be essential if no amplification in the tube is found, because it will help to distinguish between an inhibited PCR and a sample where no DNA has been found.

However, when HPVs are present in the sample, there is always a preference to amplify genotypes instead of amplifying the controls. Hence, under certain conditions (i.e. high copy numbers of a particular HPV genotype or several HPV genotypes present in one sample) **internal controls may not appear (NO SIGNAL).**

An uncertain result may appear due to one of these possibilities:

- If the three copies of the same probe are very different among each other.
- If there is a co-infection and one of the detected viruses is in the threshold between positive and negative.

## 10. TECHNICAL AND WORKING SPECIFICATIONS

*KNOWN SOURCES OF INTERFERENCE*

Certain substances can interfere with the *CLART® HPV3 k*it. These are mainly substances that inhibit DNA polymerase, and therefore the amplification reaction. For example:

- **Hemoglobin or paraffin.** DNA extracted from cervicovaginal swabs may contain small amounts of hemoglobin, while DNA extracted from samples in paraffin wax may be

contaminated with this medium. Although our Extraction-Purification kit minimises these effects there is still potential for interference.

- **Acetic acid and iodine.** If a sample for analysis is taken after a colposcopy, contamination of the sample with acetic acid or iodine is possible. Since both compounds can inhibit PCR, we strongly recommend taking the samples before performing any procedure containing these compounds.

- **Use of inadequate samples.** The use of any sample types other than those indicated in this manual, or the incorrect taking of samples, could lead to non-conclusive results. For example, if a sampling swab is placed in an alternative medium, PCR might be inhibited, or if samples are left in formalin for too long, the DNA may degrade.

- **Residual proteinase K activity.** During DNA extraction, the proteinase K has to be inhibited by incubation at 70ºC for 10 min. This leads to its complete inactivation. If this step is omitted, or the conditions not adhered to, some proteinase K could be left over and degrade the DNA polymerase needed for the PCR, thus inhibiting the reaction.

- **Inadequate conservation of samples.** If the samples are held under conditions that lead to the degradation of their DNA, the results may be unreliable.

*TECHNICAL SPECIFICATIONS*

**1. Analytical parameters:**

- **Analytical sensitivity.** The analytical sensitivity was determined by specific amplification of the different HPV genotypes cloned in recombinant plasmids. Sensitivity of HPV types 16 and 18 were also determined from samples from the 2010 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 1**. Analytical sensitivity of ***CLART® HPV2*** *kit*.

Due to the clinical significance of HPV types 16 & 18, we have included the sensitivity data from these types from the 2010 WHO HPV LabNet Proficiency Study of HPV DNA Typing in order to compare HPV DNA detection and typing methodologies for the evaluation of HPV vaccines and in effective implementation and monitoring of HPV vaccination programs. A data set was considered proficient when it was detecting at least 50 international units (genome equivalents) of HPV 16 and HPV 18, fact achieved within *CLART® HPV 3*.

For the 14 genotypes included in *CLART® HPV3*,other than those already included in *CLART® HPV2* (see Table 1), we evaluated the analytical sensitivity of the two most prevalent genotypes, 34 and 74. In both the analytical sensitivity is of $10^2$ copies.

**Analytical specificity.** The analytical specificity was 100%. ***CLART® HPV3*** kit detects no other pathogens that might be found in cervicovaginal samples, such as herpesvirus.

**2. Diagnostic parameters.**

In order to determine the diagnostic parameters of the kit ***CLART® HPV2***, comparative studies against the previous version of the kit were performed.

This comparison was performed in collaboration with two Spanish hospitals and a Portuguese one.

- Microbiology Service of the University Hospital *Germans Trías i Pujol of Badalona.*
- Virology Unit of University Hospital *Virgen de la Arrixaca, Murcia*.
- Infection Diseases Department of the National Health Institute *Ricardo Jorge*, I. P. Lisboa (Portugal).

A total amount of 386 samples were performed, including 9 swabs, 25 paraffin embedded tissues and 364 LBC.

The following table illustrates the diagnostic sensitivity and specificity data for HPV types detected in the kit ***CLART® HPV2***:

| HPV type | Sensitivity | Specificity | HPV type | Sensitivity | Specificity |
|---|---|---|---|---|---|
| 6 | 97,37 | 100,00 | 56 | 100,00 | 100,00 |
| 11 | 100,00 | 100,00 | 58 | 97,44 | 100,00 |
| 16 | 100,00 | 99,69 | 59 | 100,00 | 99,73 |
| 18 | 100,00 | 100,00 | 61 | 100,00 | 100,00 |
| 26 | 100,00 | 100,00 | 62 | 100,00 | 99,46 |
| 31 | 100,00 | 100,00 | 66 | 100,00 | 100,00 |
| 33 | 97,14 | 99,72 | 68 | 97,44 | 98,33 |
| 35 | 100,00 | 99,74 | 70 | 94,44 | 100,00 |
| 39 | 88,89 | 100,00 | 71 | 100,00 | 100,00 |
| 42 | 100,00 | 99,46 | 72 | 100,00 | 100,00 |
| 43 | 100,00 | 99,50 | 73 | 100,00 | 99,74 |
| 44 | 100,00 | 100,00 | 81 | 100,00 | 100,00 |

| | | | | | |
|---|---|---|---|---|---|
| 45 | 92,86 | 99,74 | 82 | 94,74 | 99,47 |
| 51 | 100,00 | 100,00 | 83 | 100,00 | 100,00 |
| 52 | 96,15 | 100,00 | 84 | 100,00 | 100,00 |
| 53 | 95,83 | 100,00 | 85 | 100,00 | 100,00 |
| 54 | 100,00 | 100,00 | | | |

**Table 2**. Diagnostic parameters of ***CLART® HPV2*.**

In the following study we evaluated the diagnostic parameters for the 14 genotypes included in *CLART® HPV3* and not included in *CLART® HPV2* (see Table 2):

A total amount of 678 samples were analyzed, 213 of them were positive for one of the genotypes detected in HPV2 (all genotypes are present), and 465 were negative.
Of the 678 samples analyzed, 10 samples have tested positive for any of the new genotypes, particularly for genotypes 34, 67, 87 and 102.

Positive samples were not available for the other genotypes.

The diagnostic specificity for the 14 new genotypes is 100%.

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**12. TABLE**

Oncogenic risk of the HPV types detectable with *CLART® HPV2*.

| TYPE | ONCOGENIC RISK * | TYPE | ONCOGENIC RISK * |
|---|---|---|---|
| PVH 6 | Low Risk | PVH 56 | High Risk |
| PVH 11 | Low Risk | PVH 58 | High Risk |
| PVH 16 | High Risk | PVH 59 | High Risk |
| PVH 18 | High Risk | PVH 61 | Low Risk |
| PVH 26 | Problable High Risk | PVH 62 | Low Risk |
| PVH 31 | High Risk | PVH 66 | High Risk |
| PVH 33 | High Risk | PVH 68 | High Risk |
| PVH 35 | High Risk | PVH 70 | Low Risk |
| PVH 39 | High Risk | PVH 71 | Low Risk |
| PVH 40 | Low Risk | PVH 72 | Low Risk |
| PVH 42 | Low Risk | PVH 73 | Probable High Risk |
| PVH 43 | Low Risk | PVH 81 | Low Risk |
| PVH 44 | Low Risk | PVH 82 | Probable High Risk |
| PVH 45 | High Risk | PVH 83 | Low Risk |
| PVH 51 | High Risk | PVH 84 | Low Risk |
| PVH 52 | High Risk | PVH 85 | Low Risk |
| PVH 53 | Probable High Risk | PVH 89 | Low Risk |
| PVH 54 | Low Risk | | |

*According to:

*Bouvar d V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F et al.*
*A review of human carcinogens -Part B: biological agents. Lancet Oncol 2009:10( 4):321 322*