CLART®
PneumoVir

DETECTION OF VIRUSES CAUSATIVE OF HUMAN RESPIRATORY INFECTIONS VIA GENOMIC IDENTIFICATION FOR *IN VITRO* DIAGNOSIS

CLART® PneumoVir

CLART® PneumoVir is under protection of the patent family of the international PCT Patent Application WO2009144497, which comprises national and regional members granted in different territories, including Spain, Denmark, France, Germany, Italy, Sweeden, Switzerland, United Kingdom, Russia and Mexico, and under examination in Canada and Brazil.

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

GENOMICA, S.A.U.
Parque Empresarial Alvento, Edificio B
Calle Vía de los Poblados, 1 – 1ª planta
28033 Madrid, Spain
www.genomica.com

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Table of contents

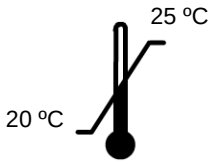
1. GLOSSARY

Attention, see instructions for use

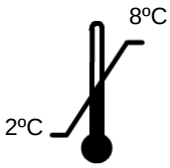
Expiration date

In vitro diagnostic medical device

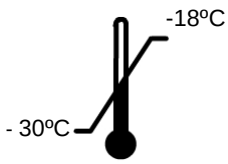
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® PneumoVir **allows detect the 19 most frequent types and subtypes of viruses causative of human respiratory infections, in the most common clinical samples:** Adenovirus; Bocavirus; Coronavirus; Enterovirus (Echovirus); Influenza virus A (H3N2, H1N1 y H1N1/2009, the latter causative of Influenza A (H1N1/2009); Influenza virus B; Influenza virus C; Metapneumovirus (subtypes A and B); Parainfluenza virus 1, 2, 3 y 4 (subtypes A and B); Rhinovirus; Respiratory syncytial virus type (RSV-A) and Respiratory syncytial virus type B (RSA-B).

Detection is based on our CLART® technology: End-point Multiplex RT-PCR (reverse transcriptase PCR) amplification, of a of a virus fragment of 120-330 bp, followed by visualization in low-density microarray.

Starting material for both formats is extracted DNA/ RNA from respiratory samples (See Section 6 below).

In order to avoid false negative results, each PCR tube includes an Internal control of amplification, which detection ensures the proper performance of the amplification process.

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

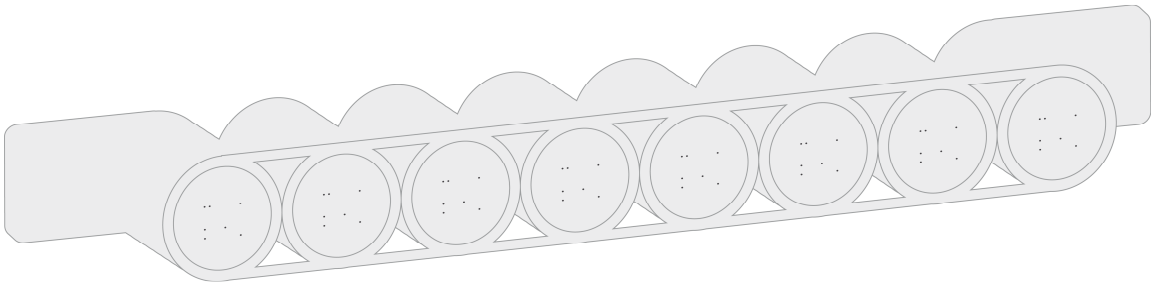


Figure 1. CLART-Strip® in the form of an 8-well strip.

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 3.3).

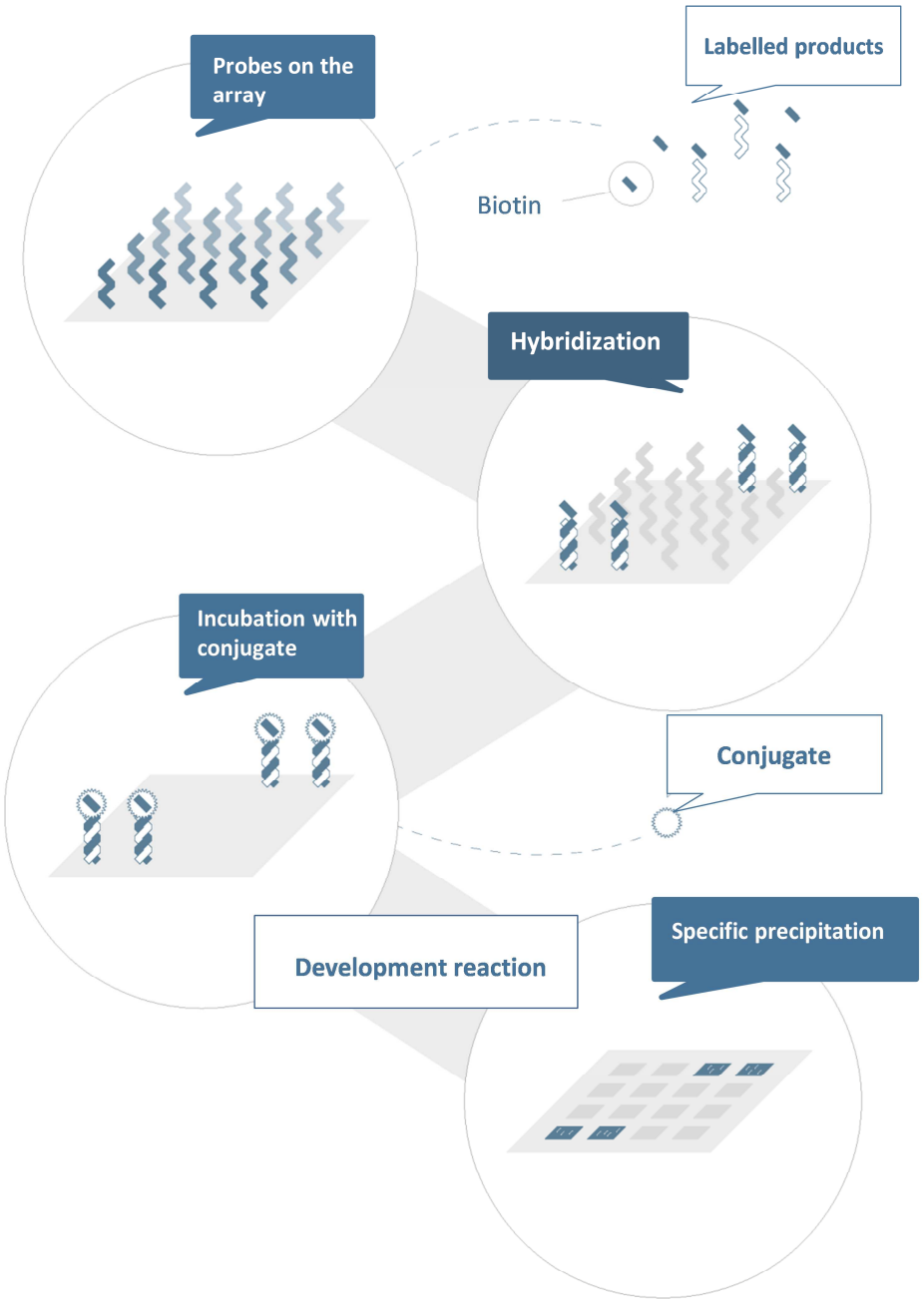


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3. KIT COMPONENTS AND STORAGE

CLART® PneumoVir Kit 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. Extraction reagents

Shipped at -20ºC. Stored according to the instructions below.

Components:

- SEML (Extraction solution of Liquid samples). **Once thawed, it must be stored at 4ºC and made use of within the next 8 days.**
- SD (Dilution solution). Store at -20ºC or at 4ºC.
- IP (Isopropanol). Store at -20ºC.
- DE (70% Ethanol). Store at -20ºC.

3.2. Amplification reagents

Shipped and stored at -20ºC.

- **Amplification tubes.** They are provided ready-to-use. Each amplification tube contains 43 µL of master mix. Only the exact number of required tubes should be thawed on ice. Remaining ones should be kept at -20ºC.

  Two types of amplification tubes are shipped:

  - Mix 1 (White tube), for amplification of Coronavirus; Metapneumovirus (subtypes A and B); Parainfluenza virus 1, 2, 3 and 4 (subtypes A and B) and Respiratory syncytial virus type A (RSV-A);

  - Mix 2 (Green tube), for amplification of Adenovirus; Bocavirus; Enterovirus; Influenza virus A (H3N2, H1N1 and H1N1/2009), Influenza virus B, Influenza virus C; Metapneumovirus, Rhinovirus and Respiratory syncytial virus type B (RSV-B).

- **Enzyme Mix.** It is a mixture of RT (retrotranscriptase) and DNA polymerase enzymes. It is provided ready to use. Store at -20ºC.

At the amplification step, Enzyme Mix is to be added to each amplification tube before addition of the extracted genetic material.

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 viruses 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®** Reader**.**

CAR® grants automatic reading, analysis and interpretation of up to 12 **CS** units (i.e., to a maximum of 96 samples) *per* run. It displays a user-friendly and intuitive graphical interface (CLEIS), and includes updates of GENOMICA's proprietary image processing software SAICLART® as well as Kit-specific Software.

Note: CAR® is to be used exclusively with GENOMICA's diagnostic kits.

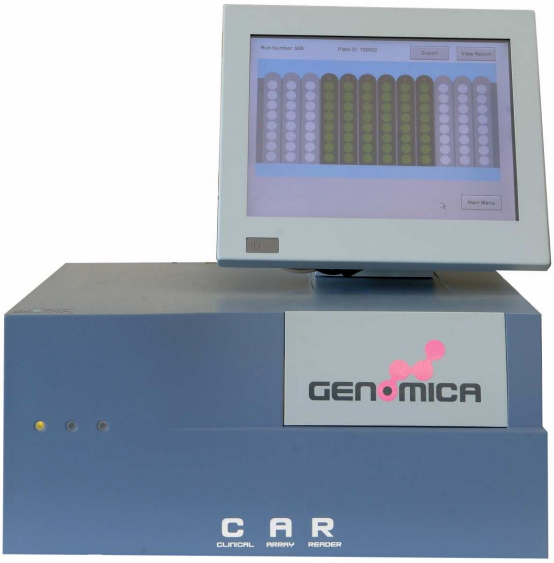


Figure 3. CAR® (CLINICAL ARRAY READER)

- GENOMICA's **autoclart®**.

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

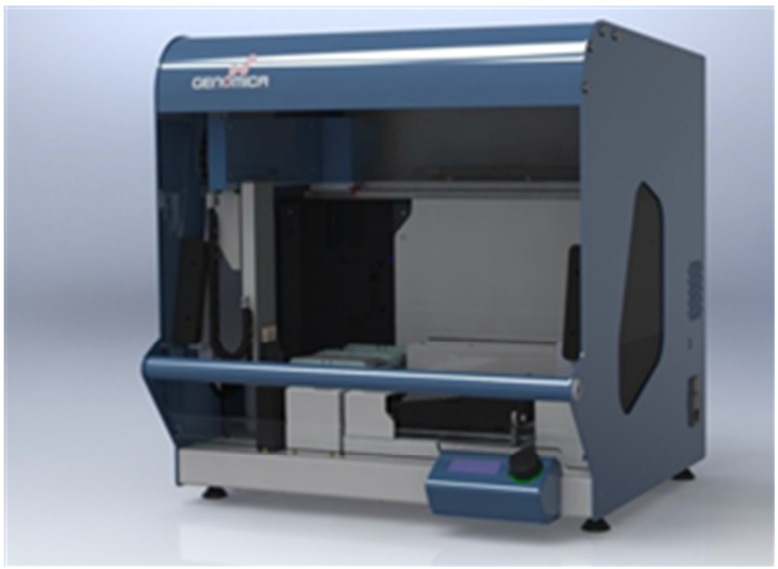


Figure 4. autoclart®

- **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.
- 1.5 mL autoclaved Eppendorf tubes.
- 1.5 mL tube grids.
- 0.5 mL/0.2 mL tube holder.

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.
- One adjustable micropipette ranging from 1-20 µL for adding genetic material to the Amplification tubes.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL, and 200-1000 µL for the post-PCR area.
- Thermomixer) compatible with 96-well skirted plates and adjustable shaking at 25°C, 30°C and 59ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

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

Pre-PCR extraction area. In this area extraction of DNA/ RNA from the sample is performed, always within a biosafety cabinet.
Pre-PCR area for preparation of the amplification tubes. In this area, the Enzyme Mix is added to the amplification tubes. It is recommended that this step is carried out within a biosafety cabinet.
Pre-PCR area for addition of the extracted material. In this area, the extracted DNA/RNA is added to the amplification tubes, to which the Enzyme Mix has already been added. It is mandatory that this step is 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 the Post-PCR area should never come into contact with material of the Pre-PCR area, thus the recommendation to avoid entering the Pre-PCR areas 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 addition of the genetic material 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® PneumoVir kit has been designed and validated for the analysis of genetic material extracted from the following types of respiratory samples: nasopharyngeal lavage and exudate, pharyngeal exudate and bronco-alveolar lavage (BAL).
GENOMICA cannot warrant accuracy of results obtained if processing a different type of sample.
Samples should be kept at 4ºC, if processed within the first 12 hr following collection. Otherwise, they should be stored at -20ºC or -80ºC.

7. WORKING PROTOCOL

CLART® PneumoVir kit has been validated using the below mentioned protocol, which constitutes the Working Protocol.

7.1. Extraction of genetic material.

Extraction might be performed either manually or automatically. For an optimal outcome, it is recommended that the minimum DNA/ RNA extraction yield should be in the range of 5-10 ng/µl, both for a manual or automatic extraction.

Should DNA/ RNA extraction be performed incorrectly, a false negative result will occur. It is thereby strongly recommended to take special care during this process.

7.1.1. Manual extraction-specific recommendations

- Work in the Pre-PCR extraction area, always using a laminar flow chamber and following the recommendations of Section 5.
- Before and after work, the area should carefully be cleaned with 10% diluted bleach.
- Switch on the flow and the UV light of the biosafety cabinet at least 20 minutes before the extraction step. Switch off the UV light when working within the cabinet.
- Sample preparation before extraction must take place within the biosafety cabinet.
- Keep the tubes as separate as possible in the tube grid to avoid contamination.

7.1.2. Manual extraction protocol

1. Place 200 µl of the clinical sample within a 1.5ml tube. In case of swabs carrying conservation medium, vortex for 30 sec. and take 200 µl. Process in parallel 200 µl of SD (Dilution solution), which will work as negative control.

2. Add 600 µl of SEML (Extraction solution of Liquid samples) to each tube. Wait until the solution thaws and turns completely transparent. Invert the tubes several times to mix, and incubate at Room Temperature for 15 min.

3. Add 600 µl of Isopropanol (stored at -20º C, and kept on ice until use). Invert the tubes several times to mix, and centrifuge, preferably at 4º C, at 13000 rpm for 20 min.

4. Aspirate supernatant with a micropipette. The micropipette in the 200-1000 µL range may be used to discard supernatant as long as a micropipette in a lower range is used to drain remains of the bottom of the tube. Avoid removing pellet by mistake.

5 Add 1000 µl of 70% Ethanol (stored at -20º C and kept on ice until use). Gently shake to clean the pellet at the bottom, and centrifuge for 15 min at 13000 rpm, preferably at 4ºC.

6. Discard supernatant carefully, as in step 4. Let pellets dry by leaving the tubes open within the cabinet during 15 to 20 min. Before resuspension, make sure that there are no ethanol remains left, which might inhibit PCR.

7. Resuspend in 20 µl of SD (Dilution Solution). Extracted DNA/RNA may be directly analysed (keep on ice until the moment it is added to the amplification tube) or may be kept at **-20º C.**

7.1.3. Automatic DNA extraction protocol

The extraction of the samples should be performed according to the manufacturer's instructions of the selected Extraction kit, always ensuring that the material extracted is suitable to *CLART® PneumoVir* requirements.

7.2. RT-PCR Amplification Reaction

7.2.1. Amplification-specific recommendations

- Work in the **Pre-PCR area for preparation of the amplification tubes**, always using a laminar flow chamber and following the recommendations of Section 5.

- Special care should be taken when adding the Enzyme Mix to the amplification tubes, as the former has high glycerol content. Thereby, if the tip of the pipette deepens too much into the Enzyme Mix, an excess amount of Mix might get stuck to the tip walls, and would then wrongly be added to the amplification tube; another consequence of this would be a shortage of Enzyme Mix for the remaining amplification tubes.
- Add the DNA/ RNA within the **Pre-PCR area for addition of the extracted material,** always within a biosafety cabinet. Keep the tubes separate and on ice during the whole process.

7.2.2. Amplification Protocol

1. Thaw on ice the required number of tubes according to the number of samples to be processed. Two tubes are required for each sample to be analysed, a white and a green one. 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. Add 2 µl of the Enzyme Mix to each amplification tube.

4. Add 5 µl of the extracted DNA/ RNA and pipette up and down several times to resuspend. Keep the tubes at 4ºC.

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

1 cycle	45ºC 45 min 95ºC 15 min
45 cycles	95ºC 30 sec 50ºC 90 sec 68ºC 60 sec
1 cycle	68ºC 10 min
4ºC Until tube removal	

6. Start the program and place the tubes in the thermal cycler. Amplification duration is of about 5 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. Turn on the CAR® before starting the whole procedure. Self-calibration of the equipment may last for a few minutes.

3. Make sure that the thermomixer temperature has been 59ºC for at least 60 minutes before the hybridization step starts.

4. SH forms crystals at room temperature, so it must be warmed up at 59ºC before use until crystals disappear.

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. 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 wells 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.

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

7.3.2. Manual visualization protocol

1. 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 at 95ºC and immediately place on ice or at 4ºC.

2. Preparation of Washing solution: For each **CS** to be processed, prepare 10 mL of diluted TL by adding 1 mL of TL to 9 mL of distilled water. Gently shake.

3. Prewash of the **CS**: Place the necessary **CS** units on the Microtiter plate adaptor. Add 200 µL of diluted TL to each 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. Discard the washing solution with a pipette or preferably with a vacuum pump.

   Wells must be totally free of residues, although they must never remain dry for too long. Thus the importance of immediate addition of the next solution.

4. Hybridization Step: Once the amplified products have been denatured, discard the diluted washing solution from the CS wells with a vacuum pump. Immediately after that, add 100 µL of 59ºC-pre-warmed SH to each CS well, avoiding foaming.

   Add, **to the same CS well**, 3 µL of denatured amplified product from the White tube and 3 µL from the Green tube.

   Mix the solution up and down several times, being careful not to touch the bottom of the well. It is advisable that each strip should be handled apart from the rest, to avoid contamination. Cover the Microtiter plate adaptor and the **CS**s with the plastic lid and incubate in the thermomixer for **1 hr at 59ºC 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 for its further use in step 6 below. For a faster Temperature decrease, the lid may be removed.

5. Double Wash: Add 200 µL of diluted TL to each well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the Washing solution with a pipette or preferably with a multichannel vacuum pump. Repeat the procedure. Use different tips for each well in both washes. Keep the samples on the Washing solution until the thermomixer reaches 30ºC.

6. Blocking and conjugate incubation: Centrifuge CJ for 10 seconds before use, and add **7.5 µL of CJ** to **1 mL of DC** (amount suitable per one **CS**).

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

7. Triple wash: Immediately after step 6, add 200 µL of diluted TL to each well, mixing it up and down 10-15 times with a multichannel pipette. Aspirate the diluted TL with a pipette or vacuum pump trying to remove as much liquid as possible. Repeat the procedure **two more times**. It is **essential** to thoroughly clean the wells from any residue of diluted CJ Solution.

8. Development: Thoroughly remove the diluted TL from the 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.

9. Reading: Place the microtiter plate adaptor with the CS to be analysed on the CAR® tray. The CAR® will read and report the results automatically.

7.3.3. autoclart® visualization protocol

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

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

3. Switch on the autoclart® unit and follow the instructions on the screen.

4. Close the door and press the bottom.

5. Select “Run” at the main “Menu” screen.

6. Select the assay: ***Pneumovir/ENTHERPEX.***

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 **5 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 **CS**s out of the autoclart® and add **3 µl** of denatured amplification product of the white tube, and **3 µl** of the green tube, to each CS well. Resuspend several times in order to thoroughly mix with SH, making sure the

bottom of the well is not touched. Put back the plate into autoclart® and press the arrow on the screen to continue with the visualization process.

16. Once the visualization process is over, the autoclart® will beep until the device door is opened by the user to retrieve the CSs and read them with the CAR®.

    WARNING: Once autoclart® visualization is complete, immediate reading of the results in the CAR® is required. Otherwise, false negative results due to loss of probe intensity might occur.

17. Place the plate in the CAR® for the read-out of all wells. The CAR® will read and report the results automatically.

7.3.4. 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 "***Pneumovir"*** 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.

<u>B) With manual addition of the sample</u>

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 "***Pneumovir"*** 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 **3 µl** of denatured amplification product of the white tube, and **3 µl** of the green tube, to each 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 "***Pneumovir"*** 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

Analysis of results and issuance of the corresponding report are automatically performed by CAR® or autoclart® plus.

The screen of the device will display a table with two columns: The column at the left shows the species and subtypes of viruses detectable with the kit; the right column displays the analytic results.

False negatives are one of the drawbacks of detection through genomic amplification, due to either an inadequate quality of the extracted DNA/RNA (insufficient sample quantity, DNA/RNA degradation due to inadequate storage or loss of DNA/RNA during extraction), or to the presence of DNA polymerase inhibitors in the samples where virus presence is to be checked (hemoglobin, salts, etc.).

CLART® PneumoVir Kit avoids these false negatives through addition of an internal control for amplification efficiency checking, within one of the amplification tubes.

An incorrect performance during the DNA/RNA extraction procedure may lead to false negative results. Special attention at this step is thereby strongly recommended.

In every run a negative extraction control should be included to verify that samples have not undergone contamination during any of the extraction, amplification or visualization processes that might lead to a false positive.

The amplification tubes have been designed to boost viral amplification *versus* amplification control. Thus, under certain conditions (e.g. when there is a high number of viral copies, or upon samples co-infected with several viruses), there may be no amplification of the control, and notwithstanding this, a valid result reading will be provided.

Displayed below are the different results obtainable with the Kit, together with the corresponding interpretations:

VALID RESULT:

RESULT for some virus	CONTROL	INTERPRETATION
√. POSITIVE	**√. POSITIVE**	**POSITIVE**
√. POSITIVE	**x. NEGATIVE**	**POSITIVE**
x. NEGATIVE	**√. POSITIVE**	**NEGATIVE**

NON- VALID RESULT:

RESULT for some virus	CONTROL	INTERPRETATION
x. NEGATIVE	x. NEGATIVE	PCR Inhibition

NON-CONCLUSIVE RESULT

CAUSES:

- Very different results obtained with replicas of the same probe.
- Co-infections of more than 5 viruses.
- Signal intensity in the range established as Non-conclusive by the software.

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. Control of known interferences

False negatives are one of the drawbacks of detection through genomic amplification, due to either an inadequate quality of the extracted genetic material (insufficient sample quantity, DNA/RNA degradation, inadequate storage or loss of genetic material during extraction), or to the presence of DNA polymerase inhibitors in the samples to be processed (alcohol, salts, etc.). To avoid such interference, please follow the instructions in sections 5, 6 and 7 of this Manual.

9.2. Technical specifications

9.2.1. Analytical parameters

Analytical sensitivity

Analytical sensitivity was determined through amplification of dilution series of DNA recombinant plasmids. Each of the plasmids had as an insert one of the amplification fragments corresponding to one of the viruses detected by the kit. The amplification fragment included the sequence complementary to the corresponding detection probe.

Results are displayed in Table 1:

Viruses associated to Respiratory Infections	Number of copies of recombinant plasmid per PCR reaction
Metapneumovirus	100
Coronavirus	
Influenza virus A (human H1N1, human H3N2, Influenza A H1N1/2009)	
Influenza virus B	
Influenza virus C	
Parainfluenza virus 4	
RSV-A	
RSV-B	
Adenovirus	
Bocavirus	
Enterovirus (Echovirus)	1000
Parainfluenza virus 1	
Parainfluenza virus 2	
Parainfluenza virus 3	
Rhinovirus	

Table 1: Relation of the number of copies of recombinant plasmid (specific of each viral type) required for obtaining 100% sensitivity in the detection of each virus.

Analytic specificity.

Analytical specificity was determined through amplification of dilution series of DNA of 17 recombinant plasmids, each bearing as an insert one of the amplification fragments corresponding to one of the 17 viruses detected by the kit. Non-specific detection was never observed. An analytical specificity value of 100% is thereby considered.

9.2.2. Diagnostic utility parameters

In order to determine diagnostic parameters of the ***CLART® PneumoVir*** kit, a comparative assessment of the ***CLART® PneumoVir*** technic *versus* reference techniques, Immunofluorescense, Immunochromatography and Quantitative PCR, was carried out.

Collaboration with the following centers was established for this evaluation:

- Microbiology Service of the “Hospital Universitari Germans Trías i Pujol” of Badalona, Spain.
- Virology Unit of the “Hospital Universitario Virgen de la Arrixaca” of Murcia, Spain .
- Virology Laboratory of the “Hospital Clínico Universitario de Reims”, France.

Genetic material was extracted from 296 samples of nasopharyngeal lavage, and presence of each of the viruses contained within Table 2 was checked. Corresponding results are displayed within the same Table.

Table 2.

Virus	**CLART® PneumoVir**	
	Sensitivity	**Specificity**
RSV-A	100,00	100,00
Parainfluenza 1	88.24	100
Parainfluenza 2	100,00	100,00
Parainfluenza 3	100,00	100,00
Parainfluenza 4	100,00	100,00
Coronavirus	100,00	100,00
Metapneumovirus	86.67	100
RSV-B	100,00	100,00
Adenovirus	98,15	99,55
Enterovirus	83.33	100
Influenza A	83.33	99.63
Influenza B	87,50	99,63
Influenza C	100,00	100,00
Rhinovirus	96.55	100
Bocavirus	95	100
Porcine H1N1	100,00	100,00
RSV	100,00	100,00

Identical results obtained with the reference technique and *CLART® PneumoVir*, are considered valid results. In case of discrepancies between both methods, sequencing result was considered as valid; should sequencing result not be available, discrepancies were analyzed with an “in house” Nested PCR followed by sequencing.

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