Pneumo CLART bacteria®

GENETIC DETECTION AND IDENTIFICATION OF BACTERIA CAUSATIVE OF HUMAN RESPIRATORY INFECTIONS FOR *IN VITRO* DIAGNOSTICS

Pneumo CLART bacteria®

Pneumo CLART bacteria® is under protection of the patent family of the international PCT Patent Application PCT WO2015055768. This patent family comprises national and regional members under examination in Europe, Brazil and China.

CLART®, CLART-Strip®, CAR®, SAICLART®, AUTOCLART® and Pneumo CLART bacteria®, 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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Contenido

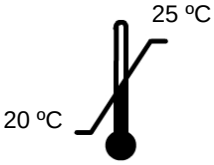
1. GLOSSARY

Attention, see instructions for use

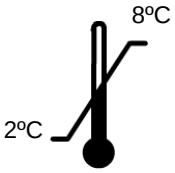
Expiration date

In vitro diagnostic medical device

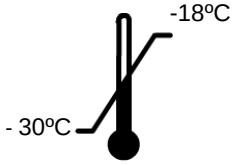
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

Pneumo CLART bacteria® enables to detect the presence of the main bacteria causative of human respiratory infections, in the following clinical samples: Sputum, nasopharyngeal lavage/exudate/aspirate, bronco-alveolar lavage (BAL) and bronchial suction.

Bacteria detectable with ***Pneumo CLART bacteria®*** are:

- *Staphylococcus aureus*[1, 2]
- *Streptococcus pneumoniae*[2]
- *Haemophilus influenzae*[2]
- *Haemophilus spp*[2]
- *Moraxella catarrhalis*[2]
- *Mycoplasma pneumoniae*
- *Bordetella pertussis*
- *Bordetella parapertusis*
- *Bordetella bronchiseptica*
- *Bordetella holmesii*
- *Bordetella spp.*[3]

1 Including detection of the transposon mecA, responsible of appearance of methicillin resistance. MecA detection might be due to the presence in the sample of *S. aureus*, but also of any other Coagulase-Negative Staphylococci (CNS). However, the present kit only reports mecA positive in the presence of *S. aureus*.

2 These microorganisms may be present in the commensal flora of the patient on a regular basis; therefore, a positive result provided by the kit for those bacteria, should be assessed taking into account the previously mentioned circumstance.

3 The *Bordetella spp*. positive result will exclusively be reported in cases when the software does not allow distinguish between the above mentioned *Bordetella* species.

Detection is based on our CLART® technology: End-point Multiplex PCR amplification, of a fragment of each bacteria of between 150-550 bp, followed by visualization in low-density microarray.

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

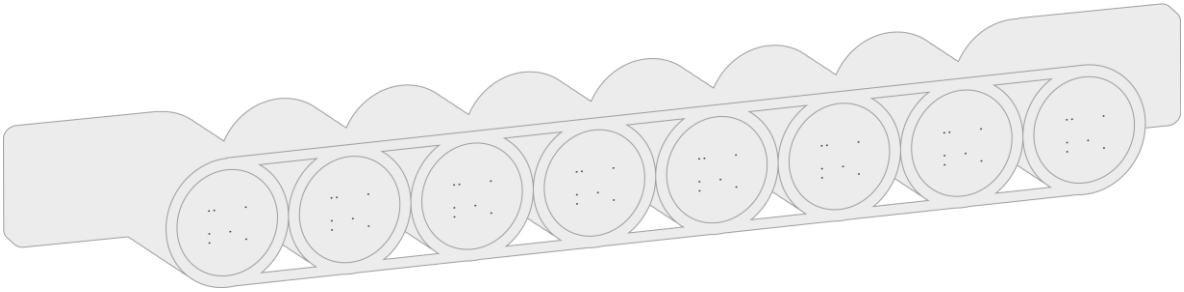


Figure 1. CLART-Strip® in the form of an 8-well strip.

A scheme of the detection system is displayed in Figure 2. Basically, PCR amplified products labelled with biotin, hybridize with their specific complementary probes immobilised in well-defined areas of the microarray. Subsequent incubation steps take place thereon: first, with a streptavidin-peroxidase conjugate, and second, with an o-dianisidine substrate. A non-soluble product precipitates thereafter in regions of the microarray where specific hybridization between amplified products and their specific probes has taken place.

Thereafter, analysis and interpretation of results are automatically performed by GENOMICA's reader (CAR® or CLINICAL ARRAY READER), running tailor-made software. autoclart® plus may alternatively be used (see Section 7).

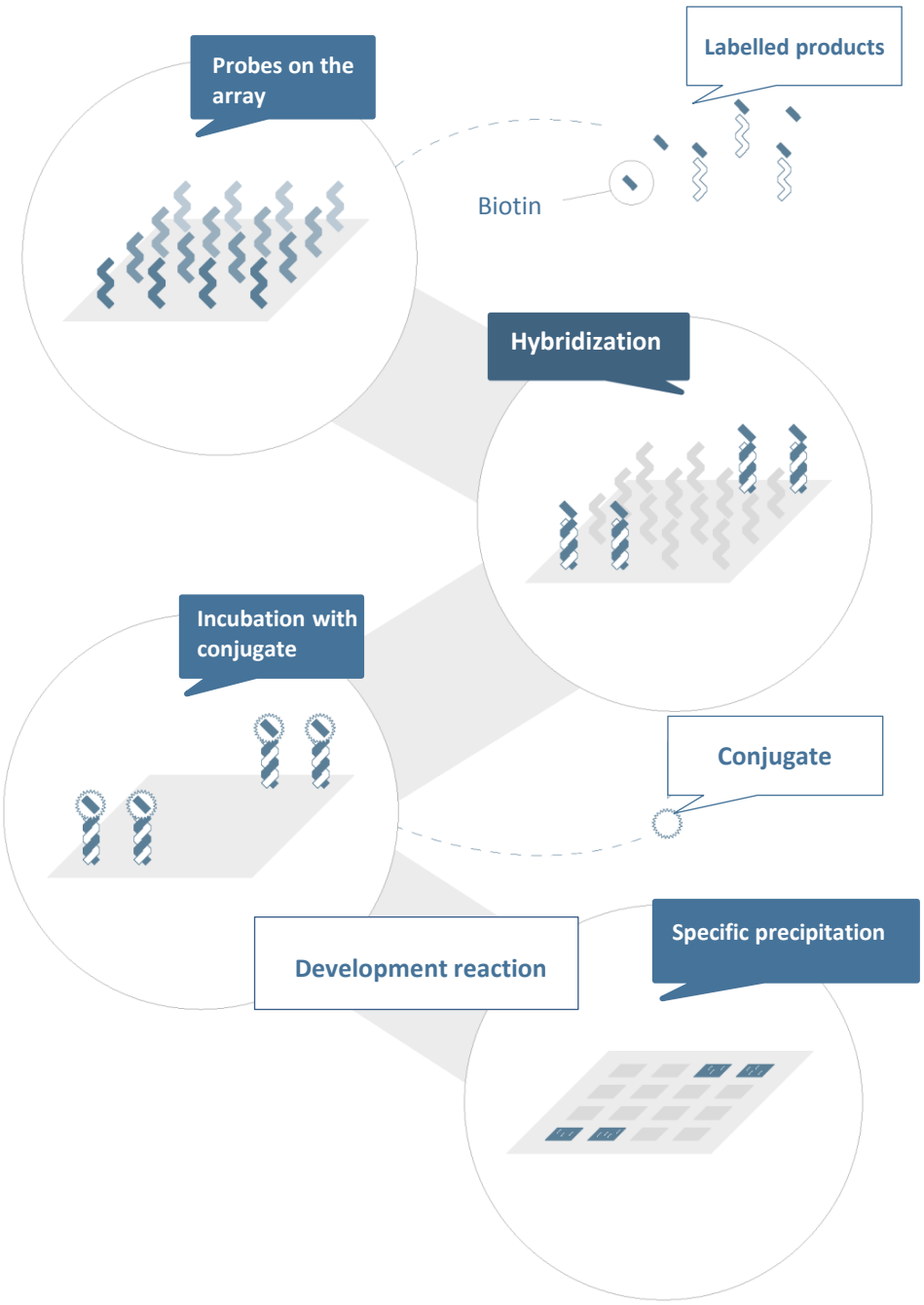


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3. KIT COMPONENTS AND STORAGE

Pneumo CLART bacteria®kit contains enough reagents for the analysis of 16 or 48 clinical samples. Components of the kit are provided at their optimal storage temperatures, and remain stable until the expiration date is reached, upon observance of recommended storage conditions.

Kit components are displayed herein:

3.1. Amplification reagents

Shipped and stored at -20ºC.

- PK (10X Proteinase K). Once thawed, store at 4ºC. Do not make use of it later than one month after being thawed.

- Amplification tubes. Amplification tubes are provided ready-to-use. Each amplification tube contains 45 µL of reaction mixture. Only the exact number of required tubes should be thawed on ice. Remaining ones should be kept at -20ºC.

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

3.2. 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 the bacteria 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 Reader **CAR®** or **CLINICAL ARRAY 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.

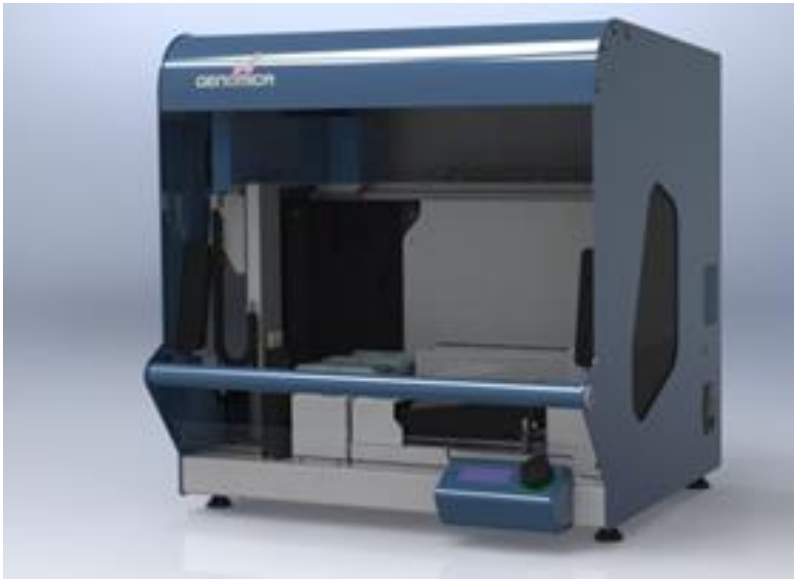


Figure 4. autoclart®

- **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 or cool tube-holder.
- 1.5 mL autoclaved Eppendorf tubes.
- 1.5 mL tube grids.
- 0.2 mL tube holder.
- Saline solution (0.9% NaCl).

4.2. Equipment

- Microcentrifuge.
- Thermal cycler.
- Biosafety cabinet.
- Three adjustable micropipettes ranging from 1-20 µL, 20-200 µL and 200-1000 µL for the pre-PCR area.
- 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.
- Termobloque (Thermomixer) compatible with 96-well skirted plates and adjustable shaking at 25°C, 30°C and 56ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

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

Pre-PCR area: Sample preparation, extraction of DNA, and addition of the extracted material to the amplification tubes, are performed in this area. Always work within a biosafety cabinet.

Post-PCR area: Amplification and visualization of the amplified product are carried out in this area. The material of this Post-PCR area should never come into contact with material of the Pre-PCR area, thus the recommendation to avoid entering the Pre-PCR area after having worked in the Post-PCR area.

Independent working material should be available in each area (pipettes, tips, tubes, grids, gloves, etc.), never being used outside them.

2. Always use gloves. It is advisable to change gloves frequently, and mandatory to change gloves (i) before starting to work in each of the previously mentioned areas, and (ii) before DNA addition to the amplification tubes.

3. Clean working areas (work bench, hoods, grids, pipettes) thoroughly with a 10% diluted bleach solution **after processing each sample batch.** It is mandatory to disinfect all working areas in the case of contamination. It is recommended to clean thermal cyclers and thermomixers before and after use, following the same procedure.

4. Use filter tips or positive displacement pipettes to avoid contamination. Different sets of pipettes should be used in each area. Discard the micropipette tip after each pipetting.

5. Use disposable and autoclaved laboratory material.

6. Never mix reagents from different vials, even if belonging to the same lot.

7. Close reagent tubes immediately after use in order to avoid contamination.

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

6. MUESTRAS

Pneumo CLART bacteria® kit has been designed and validated for the analysis of genetic material extracted from the following types of respiratory samples: Sputum, nasopharyngeal lavage/exudate/aspirate, bronco-alveolar lavage (BAL) and bronchial suction.

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.

7. WORKING PROTOCOL

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

7.1. Automatic DNA extraction, with NucliSENS easyMag of BioMérieux.

As extraction method, NucliSENS easyMag of BioMérieux was used.
Should a different extraction system be used, slight differences in the results obtained might be achieved. Notwithstanding, the starting sample volume should still be the same, and the elution volume should be in the range of 20-30 µl.
GENOMICA has just validated the automatic extraction protocol of below. Should different methods be used, minor yield differences may be achieved with them.

7.1.1. Specific recommendations for the steps of extraction and addition of extracted material to the amplification tube.

- Most bacteria detectable with ***Pneumo CLART bacteria®*** kit, inhabit the skin surface. In order to avoid contamination by these microorganisms, the following recommendations should be followed: Use a disinfectant gel or 70% ethanol to disinfect the skin of hands and forearms. Cover the disinfected area with long-cuff globes. Minimize the contact with the external surface of the globes while putting them on. Avoid touching either skin or hair, during sample manipulation. In case of accidental touch, proceed to change globes.
- Clean working areas of the biosafety cabinet with a 10% diluted bleach solution.
- Switch on the flow and 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 be carried out within the biosafety cabinet.

7.1.2. DNA automatic extraction Protocol with NucliSENS easyMag of BioMérieux

- In every run a negative extraction control (saline solution 0.9%) 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.
- For liquid samples, transfer 200µl of the sample to a 1.5 ml tube. For denser samples, add 1 ml of saline solution, vortex, and transfer 200µl of the sample to a 1.5 ml tube.
- Add 50µl of PK.
- Incubate during 30 min. at 56ºC with shaking, otherwise vortexing it every 10 min.

- Transfer 250 µl of sample to the extractor. Perform an internal lysis and extraction at the NucliSENS BioMérieux easyMag extraction unit, selecting the "GENERIC" protocol according to the manufacturer's User Manual. Set the elution volume at 25 µl.

7.2. Amplification reaction

7.2.1. Amplification-specific recommendations

- Work in the **Pre-PCR area**, always using a laminar flow chamber and following the recommendations of Section 5.
- Add the DNA always within a biosafety cabinet. Keep the tubes separate and on ice during the whole process.
- Do not place amplification tubes in the thermal cycler until the block has reached 90ºC. In the meantime, keep on ice. This is a caution measure to prevent non-specific amplification resulting from incubation at temperatures lower than the hybridization temperature

7.2.2. Amplification protocol

1. Thaw on ice the required number of amplification tubes according to the number of samples to analyse. Avoid using temperatures higher than 37ºC for thawing the tubes. 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 5 µl of extracted DNA to each amplification tube. Pipette up and down several times. Keep tubes at 4ºC.

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

1 cycle	95ºC 15 min
45 cycles	95ºC 30 sec 59ºC 60 sec 72ºC 60 sec
1 cycle	72ºC 10 min
4ºC Until tube removal	

5. Start the program and place the amplification tubes in the thermal cycler, once the block temperature has exceeded 90ºC.

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 area.

2. Make sure that the thermomixer temperature has been 56ºC for at least 1 hour before the hybridization step starts.

3. SH forms crystals at room temperature, so it must be warmed up at 56ºC before use until crystals disappear.

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

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

6. 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.

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

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

9. Do not allow arrays to dry completely.

10. Avoid foaming when adding reagents.

11. When visualizing the image on the CAR®, make sure that position markers appear correctly and that there are no bubbles, fibres or spots interfering with the read-out. Otherwise, clean the outer face of the well with a cellulose paper impregnated with alcohol.

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

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

4. Prewash of the **CS**: Place the necessary **CS** units on the Microtiter plate adaptor. Add 200 µL of diluted TL to each **CS** 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 56ºC-pre-warmed SH to each CS well, avoiding foaming.

   Add to each CS well, **5 µL** of denatured amplified product. Mix the solution up and down several times, being careful not to touch the bottom of the well.

   Cover the Microtiter plate adaptor and the **CS**s with the plastic lid and incubate in the plate thermomixer for **1 hour at 56ºC 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 7 below. For a faster temperature decrease, the lid may be removed.

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

7. Blocking and conjugate incubation: Diluted CJ Solution should be prepared 15 minutes before the end of the hybridization step, and be kept on ice. With this purpose, centrifuge CJ for 10 seconds before use, and add **7.5 µL of CJ** to **1 mL of DC** (amount suitable per one CS). Next, vortex to homogenize the solution.

Aspirate the diluted TL from the wells without leaving any residue, and add **100 µL** of diluted CJ Solution per well. Incubate for exactly **15 minutes in the thermomixer at 30ºC and 550 rpm**. After this incubation, remove the plate and discard the solution rapidly with a pipette or multichannel vacuum pump. Set the thermomixer at 25ºC and with shaking for its use on step 8. For a faster temperature decrease, the lid may be removed.

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

9. Development: Thoroughly remove the diluted TL from the CS wells; Next, add **100 µL** of RE to each well and incubate in the thermomixer for **10 minutes at 25ºC without shaking**.

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

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

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 when it has reached 95ºC, 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. 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: ***Pneumo CLART bacteria***

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.

    CJ. Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ solution as displayed in the screen. This is done by adding 7.5 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. Denaturation: Use the thermocycler for denaturing of PCR products. Place the amplification tubes in the thermocycler and incubate at 95ºC for 8 min. After this time, remove the tubes from the 95ºC incubation and place them immediately on ice. It is important that this denaturation step of the PCR products takes place before preparation of the visualization reagents in the autoclart®.

15. **SH.** Add the volume of tempered SH (at 56ºC for at least 60 minutes) displayed in the screen.

WARNING: It is essential to add SH at this point. If added in a previous step, SH's temperature might decrease, affecting probe intensity and leading to false negative results.

16. 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.

17. For sample addition, place the **CS**s out of the autoclart® and add **5 µl** of denatured amplification product to a 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.

18. 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 of the results should take place.

19. 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 assay "***Pneumo CLART bacteria***" 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 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 mL of diluted CJ solution indicated on the display should be prepared by adding 1 ml of DC and **7.5µl** 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 “***Pneumo CLART bacteria***” 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: Place the amplification tubes in the thermocycler once it has reached 95ºC, and incubate at 95ºC **exactly for 10 min**. Do not exceed this time. Remove the tubes from the 95ºC incubation and place them 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 1 mL of diluted CJ solution indicated on the display should be prepared by adding 1 ml of DC and **7.5 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 **7.5 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 “***Pneumo CLART bacteria***” from the list. To perform the same assay for all the **CS**s, 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 corresponding report are automatically performed by CAR® or autoclart® plus.

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

False negatives are one of the drawbacks of detection through genomic amplification, due to either an inadequate quality of the sample DNA (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 microorganism presence is to be checked (hemoglobin, salts, etc.). ***Pneumo CLART bacteria®*** kit avoids these false negatives through addition of two internal controls in each amplification tube:

- A genomic DNA internal control, witness of the efficiency of the amplification reaction.
- An extraction control which enables detect false negatives due to due to problems during the extraction step.

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

Each reaction tube contains the following oligonucleotides:

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

The amplification tubes have been designed to boost amplification of the pathogen targets *versus* that of the controls. Further, amplification of the extraction control leads over that of the amplification reaction control.

Obtainable results with the kit are: **Positive, Negative and Non-conclusive.**

Table 1 below display the possible explanations and corresponding solutions of Non-conclusive results:

Result	**Explanation**	**Solution:** Repeat…
NON-CONCLUSIVE	If No-DNA control. Failure in the extraction step.	• …extraction and subsequent steps.
	If PCR control inhibited. Failure in the amplification and/ or extraction step.	• …extraction and subsequent steps.
	If control OK. 2 possibilities: • Very different results obtained with replicas of the same probe. • Signal intensity in the range established as Non-conclusive by the software.	• …visualization.

Table 1.

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 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.). ***Pneumo CLART bacteria®*** kit avoids these false negatives through addition of internal amplification controls to the tubes, for amplification efficiency checking.
In addition, 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 corresponding to each of the microorganisms detectable with the kit. Each plasmid had as an insert one amplification product (including the sequence complementary to the corresponding detection probes), corresponding to one of the microorganisms detectable with the kit.
Visualization was performed in CS. Results are displayed in Table 2 below:

MICROORGANISM	**Copies/ 5 µL**
Moraxella catharralis	10^2
Mycoplasma pneumoniae	10
Streptococcus pneumoniae	10^2
Haemophilus influenzae	10^2
mec A	10^2
Staphylococcus aureus	10^2
Bordetella pertussis *Borderella parapertussis*	10^2 10^2
Bordetella holmesii	10^2
Bordetella bronchiseptica	10^2

Table 2. Relation of the number of copies of recombinant plasmid required for obtaining 100% sensitivity in the detection of each microorganism.

Analytical specificity.

Analytical specificity tests were performed for all recombinant plasmids, no non-specific detection of microorganism different to the targeted one ever been observed. An analytical specificity value of 100% is thereby considered.

9.2.2. Diagnostic utility parameters.

Diagnostic sensitivity.

In order to determine diagnostic parameters of the kit, a comparative assessment of the ***Pneumo CLART bacteria®*** technique versus culture and/ or PCR reference techniques was carried out. Above-mentioned evaluation was carried out in collaboration with the following centers:

- Microbiology Service of the "Hospital Universitari Germans Trias i Pujol" of Badalona, Spain.

- Microbiology Departamen of the "Hospital Universitario Ramón y Cajal" of Madrid, Spain.

Starting from 267 respiratory samples, genetic material was extracted and presence of the different bacteria detectable with the kit was assessed.

Identical results obtained with the reference technique and ***Pneumo CLART bacteria®***, are considered valid results. Discrepancies between the two techniques were solved as follows:

- In case of obtaining positive results with the reference technique and negative results with *Pneumo CLART bacteria®*, result of the former technique and not *Pneumo CLART bacteria®* was considered to be the accurate one.
- In case of obtaining negative results with the reference technique and positive results with *Pneumo CLART bacteria®*, the discrepancy was solved by means of specific Nested-PCR and sequencing. The result that was obtained was considered to be the valid one.

Results are displayed below:

(N= 267)[1]	**Gold standard**	**Sensitivity (%)**	**Specificity (%)**	**VPP (%)**	**VPN (%)**
Staphylococcus aureus	63	89,7±2,4	99,75±0,25	99,1±0,9	96,9±0,7
Streptococcus pneumoniae	89	89,3±1,7	98,6±0,3	96,9±0,6	94,9±0,8
Haemophilus sp./H. influenzae	187	96,8±0,5	100	100	93,1±1,1
Moraxella catarrhalis	77	98,7±1,3	100	100	99,5±0,5
Bordetella pertussis	10	100	100	100	100
Bordetella parapertussis	1	100	100	100	100

[1]175 Sputum, 12 Nasopharyngeal lavage, 6 Bronco-alveolar lavage (BAL), 69 Bronchial suction, 4 Nasopharyngeal exudate, 1 Nasopharyngeal aspirate.

Table 3. Diagnostic Sensitivity and Specificity of the *Pneumo CLART bacteria®* kit. VPP: Positive predictive value. VPN: Negative predictive value.

NOTE: Due to the low prevalence of the microorganisms *Mycoplasma pneumoniae, Bordetella holmesii* and *Bordetella bronchiseptica,* no positive samples containing them were to be found. Thereby, it was not possible to assess the diagnostic sensitivity of the kit for these pathogens. In all cases, analytic sensitivity parameters were determined by means of recombinant plasmids and DNA of strains of collection (DSMZ collection).

Diagnostic specificity.

This technique has been validated with samples, either negative or positive for other microorganisms not included in the kit, and the results show no cross-reaction with them.

Diagnostic repeatability and reproducibility for each sample type.

Diagnostic repeatability and reproducibility have been tested from the step of sample extraction until CS visualization.

Obtained data are as follows:

	% homology
Repeatability (n=58)	93.7
Reproducibility (n=53)	88.7

10. REFERENCES

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