CLART® EnteroBac

DETECTION AND GENETIC IDENTIFICATION OF ENTERIC BACTERIA CAUSATIVE OF DIARRHEA FOR *IN VITRO* DIAGNOSIS

CLART® EnteroBac

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

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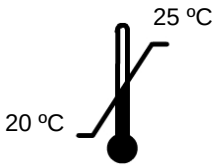
1. GLOSSARY

Attention, see instructions for use

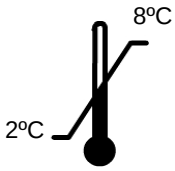
Expiration date

In vitro diagnostic medical device

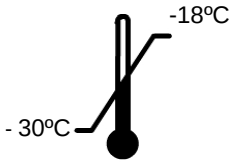
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

Manténgase fuera de la luz del sol.
Protect from sunlight

Warning! p-tertiary-octylphenoxy polyethyl alcohol

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® EnteroBac allows detect the presence of the following enterotoxin-producing bacteria types causative of diarrhea, in stool samples:

- *Salmonella spp. (all the described species)*
- *Shigella spp. (S. dysenteriae, S. Sonnei, S. boydii and S. flexneri)*
- *Yersinia enterocolitica*
- *Yersinia spp. (Y. pestis, Y.pseudotuberculosis, Y.enterocolitica)*
- *Campylobacter spp. (C.lari, C.laridis, C.upsaliensis, C.jejuni, C.coli)*
- *Campylobacter jejuni*
- *Campylobacter coli*
- *Escherichia coli enteropathogenic EPEC: (E. coli enterohemoragic, E. coli enteroinvasive, E. coli enterotoxigenic and E. coli enteropathogenic)*
- *Clostridium difficile B*
- *Aeromonas spp, producers of aerolisin*

Starting material: Stool samples (See Section 6 below).

Detection is based on our CLART® technology: End-point Multiplex PCR amplification, of genes coding for enterotoxins and virulence factors, and constitutive genes for *Campylobacter spp*. and *Salmonella spp*., 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.

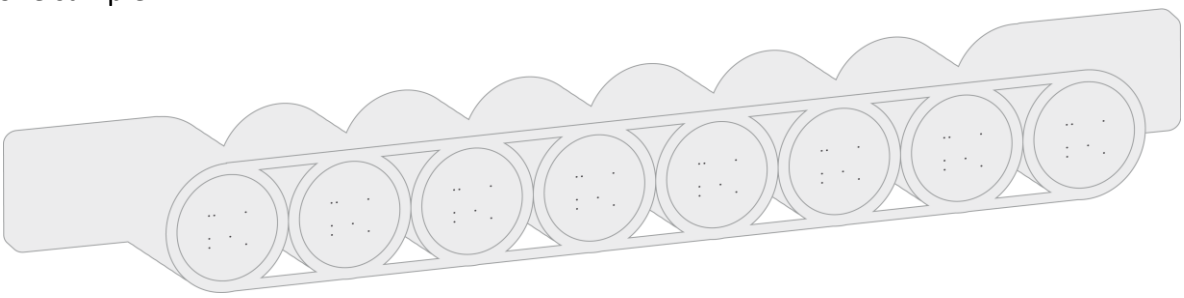


Figure 1. CLART-Strip® in the form of an 8-well strip.

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.

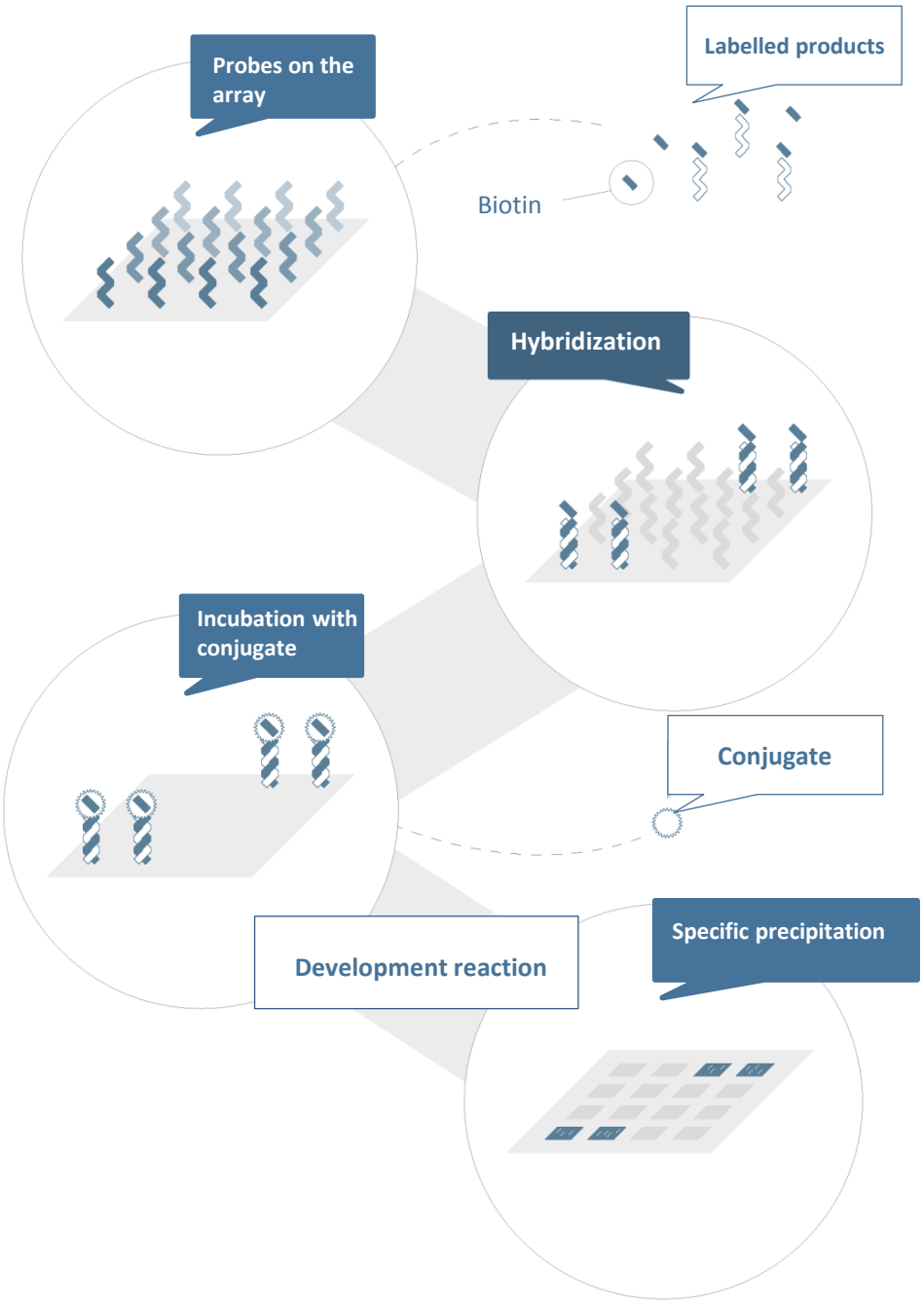


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3. KIT COMPONENTS AND STORAGE

CLART® EnteroBac kit contains enough reagents for the analysis of 16 and 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.

- 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 amplification tubes are one use only

2 types of amplification tubes are provided:

- Mix 1, White tubes, enabling amplification of *Shigella spp, Yersinia enterocolitica, Yersinia spp., Campylobacter coli, Campylobacter jejuni, Escherichia coli enteropatógena, Clostridium difficile B* and *Aeromonas spp, producers of aerolisin*.
- Mix 2, Green tubes, enabling amplification of *Salmonella spp*, and *Campylobacter spp.*

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 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** (Figure 3).

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

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

Figure 3. CAR® (CLINICAL ARRAY READER)

- GENOMICA's **autoclart®**.

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

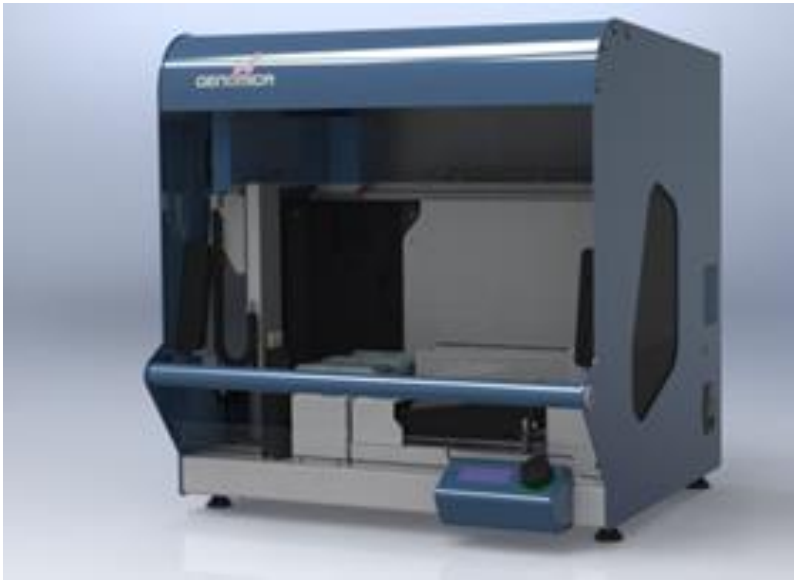


Figure 4. autoclart®

- GENOMICA's **autoclart® plus**.

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

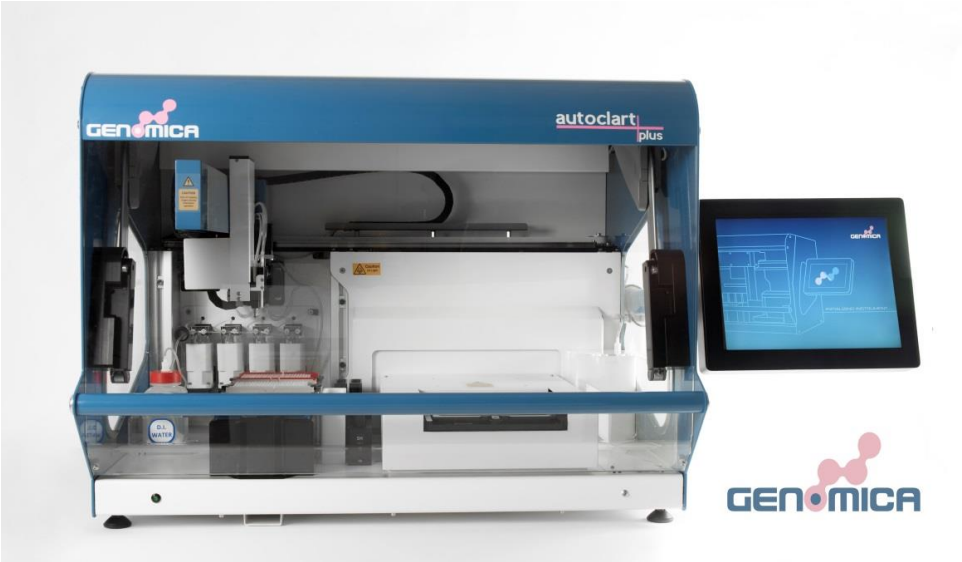


Figure 5. autoclart® plus

4. ITEMS REQUIRED BUT NOT PROVIDED

A list of all items required but not provided is displayed below:

4.1. Reagents and materials

- Distilled water.
- Disposable gloves.
- Filter tips or positive displacement pipettes.
- Crushed ice container or cool tube-holder.
- 1.5 mL autoclaved Eppendorf tubes.
- 1.5 mL tube grids.
- 0.5 mL/0.2 mL tube holder.
- Saline solution (0.9% NaCl).

4.2. Equipment

- Microcentrifuge.
- Thermal cycler.
- 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 the 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 plates and adjustable at 25°C, 30°C and 59ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

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

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

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. Discard the micropipette tip after each pipetting.

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

10. Prior to first use confirm the integrity of every container. Do not use the reactives if any container is damaged.

6. SAMPLES

***CLART® EnteroBac* kit** has been designed and validated for the analysis of genetic material extracted from STOOL samples.
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 48 hr following collection. Otherwise, they should be stored at -20ºC.

7. WORKING PROTOCOL

***CLART® EnteroBac* kit** has been validated by means of the protocol displayed below, which constitutes the Working Protocol.

7.1. Automatic extraction of genetic material from Enterobacteria.

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

- Most bacteria detectable with ***CLART® EnteroBac* kit** inhabit the normal flora of the human digestive tract. In order to avoid contamination by these microorganisms, the following recommendations should be followed: Always use gloves; Gloves should be put on without touching anything but the rim of them with the fingers, in particular avoiding touching their palms and fingers.
- Clean working areas of the biosafety cabinet with a 10% diluted bleach solution.
- Sterilize the cabinet with UV light before extraction.
- Switch on the flow of the biosafety cabinet at least 20 minutes before the extraction step.
- Sample preparation before extraction must be carried out within the biosafety cabinet. Do not work outside the laminar flow, nor open tip boxes outside it.

7.1.2. Protocol for the automatic extraction of Enterobacteria genetic material.

It is recommended that the following method should be followed:

1. Sample preparation prior to extraction (recommended for all automatic equipments):

- Transfer 1 g of stool sample and resuspend in 2 ml of saline solution (sodium chloride 0.9%). Centrifuge for 2 min at 1500 rpm.

Note: Stool sample taking should be performed inside an appropriate cabinet for the extraction and with the aid of sterile material.

- Discard pellet and place the supernatant into a sterile 1.5 ml tube, in a volume of 1.5 ml maximum.

Note: In this step, the majority of stool sample is discarded.

- Centrifuge at 13000 rpm for 5 min to concentrate the bacterial pellet.

- Discard supernatant and resuspend in 1.5 ml of saline solution (sodium chloride 0.9%). Resuspend vigorously.

- Transfer 300 µl to the automatic extractor cuvettes.

2. Internal lysis and extraction in the NucliSENS™ EasyMag DNA Biomerieux device: Follow the user's guide of the device for the "Specific B" protocol.
In case of making use of systems with several programs of sample-wash, select the most stringent one.
It is mandatory to identify in the program the elution volume, 110 µl.
This volume may vary depending on the automatic extractor being used.

3. Once the extraction step is finished, place 110 µl of the eluted DNA within a 1.5 ml Eppendorf tube. Pick up 5 µl for each amplification tube and keep the rest at –20°C.

The minimum yield of extracted DNA from stool samples is estimated to be of 5 ng/µl for yielding accurate amplification levels.

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.

- Place the amplification tubes in the thermal cycler when 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 be processed. For each sample to be processed, one Mix 1 tube and one Mix 2 tube shall be used. Avoid using temperatures higher than 37ºC for thawing the tubes.

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 from each sample to each Mix 1 and Mix 2 amplification tubes. Pipette up and down serveral times. Keep tubes on ice.

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

1 cycle	95ºC 5 min
45 cycles	95ºC 30 sec 53ºC 30 sec 72ºC 60 sec
1 cycle	72ºC 10 min
4ºC continuously until tube collection	

5. Start the program and place the amplification tubes in the thermal cycler when the temperature of the block is above 90ºC. Amplification duration is of about 3 and 1/2 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. The device should be ready at the time of reading to avoid unnecessary waiting and an excessive exposure to developer.

3. Make sure that the thermomixer temperature has been 56ºC for at least 30 minutes **before the hybridization step starts.**

4. SH should be warmed up at room temperature before use.

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

6. Clean the thermal cycler with a 10% diluted bleach solution before starting the denaturation program. Place the amplification tubes separate in the thermal cycler during denaturation and do not exceed the denaturation time of 10 minutes.

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

8. During visualization it is not necessary to use filtered tips, but different tips should be used for different wells, and for adding different reagents, even TL. It is, however, necessary to use filtered tips for adding the amplified products to the CS well.

9. 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.

10. 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.

11. Aspirate the different solutions completely from the CS well without touching the bottom of the well; Otherwise, the microarray might get damaged.

12. Do not allow arrays to dry completely.

13. Avoid foaming when adding reagents.

14. When visualizing the image on the CAR®, make sure that position markers appear correctly and that there are no bubbles, fibers 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. 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 Washing solution from the CS wells with a pipette or preferably with a vacuum pump. Immediately after that, add 100 µL of pre-warmed SH to each CS well, avoiding foaming.

   Add to each CS well, **5 µL** of denatured amplified product of Mix 1, and **5 µL** of denatured amplified product of Mix 2. 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.

   For the correct interpretation of the results, it is mandatory to visualize both tubes of the same sample in the same CS well.

   Cover the Microtiter plate adaptor and the **CS**s with the plastic lid and incubate in the plate thermomixer for **30 min 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 with shaking, 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: Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ Solution by adding **9 µL of CJ** to **1 mL of DC** per 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 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 after it has reached 95ºC, and incubate at 95ºC **for 10 minutes**. Time set 15

min in the thermal cycler so that after the 10 minutes incubation the amplified products remain at 95ºC. Once the 10 minutes incubation is over, remove the tubes from the 95ºC incubation and immediately place on ice.

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

4. Close the door and press the button.

5. Select "Run" at the main "Menu" screen.

6. Select the assay: ***CLART® EnteroBac.***

7. Select the well of the strip in which to start: A1 or E1, the latter in case of using CSs where the first 4 wells have previously been used.

8. Select the number of samples. autoclart® allows to process from 4 to 96 samples. The number of samples must be a multiple of 4.

9. Check that both the indicated number of samples and the initial well (A1 or E1) are correct.

10. Place the complete rack of tips in its corresponding position.

11. Check that the tip and waste through containers are empty.

12. Fill the water bottle with 250 ml distilled water.

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

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

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

    CJ. Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ solution as displayed in the screen. This is done by adding **9 µL of CJ to 1 mL of DC** (amounts suitable for one CS). Vortex the diluted solution in order to mix it up properly.

    RE. Add the RE volume indicated on the display.

14. Denaturation of amplified products: Place the amplification tubes in the thermal cycler after 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.

15. 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.

16. For sample addition, place the CSs out of the autoclart® and add, to the same CS well, **5 µl** of denatured amplification products of Mix 1, and **5 µl** of denatured amplification products of Mix 2, corresponding to a same sample. 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.

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

18. 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 “***EnteroBac***” 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 **9.5µl** 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 "***EnteroBac***" 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 9.5 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 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 “***EnteroBac***” 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 three columns: The column at the left shows the species detectable with the kit; The central column displays the positive/ negative result corresponding to each species; And the column at the right displays the conformity of the DNA extraction and amplification controls.

False negatives are one of the drawbacks of detection through genomic amplification, due to either an inadequate quality of the DNA of the sample (insufficient sample quantity, DNA degradation due to an inadequate storage or loss of DNA of the sample during extraction), or to the presence of DNA polymerase inhibitors in the stool samples.

CLART® EnteroBac kit avoids these false negatives through addition of respective different internal amplification controls to the Mix 1 and Mix 2 amplification tubes, for amplification efficiency checking.

For a correct result interpretation, it is essential that each sample must be processed both with the Mix 1 and Mix 2 amplification tubes, and corresponding results visualized within a same CS well.

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:

Mix 1

- 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.

Mix 2:

- 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. The plasmid is different to the one included within Mix 1.

In addition, both mixes contain target-specific oligonucleotides corresponding to the pathogens to be detected.
The amplification tubes have been designed to boost amplification of the targets corresponding to the pathogens to be detected *versus* that of the controls.

The reason for this design is:

- The internal amplification control allows distinguish between cases of inhibition of the PCR reaction and cases of absence of DNA in the sample.

Under certain circumstances (eg. When there is a high amount of microbial DNA) absence of amplification of all or any of the controls may happen, a "NO SIGNAL" read-out thereby being issued.

The different results obtainable with the Kit are as follows: **Positive, Negative, Non-valid and Non-conclusive.**

Table 1, below, displays possible explanations and corresponding solutions to Non-valid and Non-conclusive results:

Result	**Explanation**	**Solution:** Repeat…
NON-VALID	Non-valid amplification: Absence of amplification in one of the tubes and presence of bacterial and/ or human DNA in the other tube, reports a correct extraction but a default in amplification of the former.	…amplification of both tubes
NON-CONCLUSIVE	3 cases: • Very different results obtained with identical probes. • Coinfections of more than 3 bacteria. • Signal intensity in the range established as Non-conclusive by the software.	…visualization

Table 1. Keys for the interpretation of results

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. Control of known interferences

The main interferences of the ***CLART® EnteroBac*** kit are:

- Presence of substances that inhibit the amplification reaction.
- Use of non-suitable samples. The analysis of a different type of clinical sample to that of the present manual, or an incorrect sample taking, might lead to a non-valid or non-conclusive result due to lack of amplification due to sample shortage or inhibited reaction.
- Non-adequate storage of samples might influence in the result of the analysis. Should samples be exposed to conditions that result in degradation of the DNA they contain, the result of the analysis might be erroneous.

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	**Nº copies of recombinant plasmid per PCR reaction**
Yersinia enterocolitica	10^2
Escherichia coli enteropathogenic	
Campylobacter jejuni	
Shigella	10^3
Salmonella	
Clostridium difficile	
Campylobacter coli	
Aeromonas aerolisin +	

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

Analytic specificity

Analytical specificity tests were performed for all recombinant plasmids, no non-specific detection of different microorganisms 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 ***CLART® EnteroBac*** technic *versus* the stool culture characterization reference technique was carried out. Above-mentioned evaluation was carried out in collaboration with the following centers:

- Microbiology Service of the "Hospital Universitario Ramón y Cajal", Madrid.
- Microbiology Service of the "Hospital Universitari Germans Trías i Pujol", Badalona.

Starting from 334 respiratory samples, genetic material was extracted and presence of the bacteria of table 3 below was assessed.

Identical results obtained with the reference technic and ***CLART® EnteroBac***, are considered valid results. In case of discordant results between the two techniques, the result obtained by sequencing was considered as the valid one. In those specific cases, where this information was not available, the discrepancies were solved by means of specific Nested-PCR, followed by sequencing[2].

Results are displayed below:

N = 334	**Sensitivity**	**Specificity**	**VPP**	**VPN**
Campylobacter	90,0	100,0	100,0	94,0
Salmonella	83,2	99,0	98,3	89,4
Clostridium difficile	83,0	100,0	100,0	97,7
Escherichia coli enteropathogenic	100,0	100,0	100,0	100,0
Shigella	94.4	100,0	100,0	99,7

Table 3. Diagnostic Sensitivity and Specificity of the *CLART® EnteroBac* kit.

VPP: Positive predictive value. VPN: Negative predictive value.

NOTE: 15 *Aeromonas* samples were analysed, none of them being aerolisin positive (toxin targeted by this kit). Thereby, it was not possible to determine the diagnostic parameters for this microorganism.
The N=5 for *Yersinia* did not allow establish reliable diagnostic parameters.

Diagnostic specificity

This technique has been validated with stool samples, either negative or positive for microorganisms included in the kit. The results display a specificity value of 99%, there being no non-specific cross-reaction with other bacteria different to those present in the sample.

Diagnostic repeatability and reproducibility

Obtained data are as follows:

	% homology
Repeatability (n=48)	97.2
Reproducibility (n=49)	91.8

Diagnostic repeatability and reproducibility parameters have been established with samples tested from the step of the stool sample extraction, until CS visualization.

Detection of multiple infections

CLART® EnteroBac kit allows detect the presence of multiple infections (two or more bacteria detectable with the kit) in 15,9% of the stool samples analysed, *versus* 2,4% detected by means of stool culture characterization. This reports a higher sensitivity of the ***CLART® EnteroBac*** kit in detection of multiple infections, *versus* classical methods of culture identification.

10. REFERENCES

1. Alvarez, M., Buesa, J., Castillo. J. y J. Vila. ”Diagnóstico microbiológico de las infecciones gastrointestinales”. 2007. 2ª Edicion (24). Eds. Cercenado E. y Cantón, R. Procedimientos en microbiología clínica. Sociedad Española de Microbiología Clínica, (SEIMC).

2. Manjón, N., Moscoso, J., Margolles, Y., García, M., Morosini, M. I., Salazar, O., Cospedal R., Cantón, R., y M. L. Villahermosa. 2011. “Diseño y optimización de un sistema de detección molecular por hibridación en microarrays para la identificación rápida de patógenos bacterianos intestinales presentes en pacientes con diarrea”. 2011. Enfermedades infecciosas y Microbiología Clínica. Vol 29, 33-34. XV Congreso de de la Sociedad Española de Microbiología Clínica (SEIMC). Málaga, 1-4 Junio 2011.

COMPOSITION:

Amplification reagents;

Nº test	Manual		Autoclart	
16	16x	*45 µl*	16x	*45 µl*
48	48x	*45 µl*	48x	*45 µl*

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

Visualization reagents;

- **SH (Hybridization solution)**

Nº test	Manual		Autoclart	
16	1x	6ml	1x	11.5ml
48	1x	6ml	1x	11.5ml

COMPONENTS	%	CLASSIFICATION according to regulation 1272/2008:		
Sodium phosphate	25	*This product is a mixture of substances* ***Does not have dangerous components***		
SSC	5	*This product is a mixture of substances* ***Does not have dangerous components***		
EDTA	0.2	*This product is a mixture of substances* ***Does not have dangerous components***		
Triton X-100	2.25	*This product is a mixture of substances* **The concentration of the possible dangerous components according to the regulation is below the allowed limits**		
		DANGEROUS COMPONENT	**ICON**	**SENTENCE**
		p-tertiary-Octylphenoxy polyethyl alcohol CAS-No. : 9002-93-1 - **concentration <0.05**	Warning! 	H302,H319 H411,P273 P280, P305+P351 + P338

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

Nº test	Manual		Autoclart	
16	1x	3ml	-	-
48	1x	5.5 ml	1x	11.5ml

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

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

Nº test	Manual		Autoclart	
16	1x	7ml	1x	11.5ml
48	1x	7ml	1x	11.5ml

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

- ***CJ (Conjugate)***

Nº test	Manual		Autoclart	
16	1x	100 µl	1x	100 µl
48	1x	100 µl	1x	100 µl

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

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

Nº test	Manual		Autoclart	
16	1x	7ml	1x	11.5ml
48	1x	7ml	1x	11.5ml

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

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

RELATIVE CONSIDERATIONS TO ELIMINATION

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

Contaminated packaging
Remove as unused products.