



CLART® EnteroBac

DETECTION AND MOLECULAR IDENTIFICATION OF ENTERIC BACTERIA CAUSING DIARRHEA

FOR *IN VITRO* DIAGNOSTICS.

CLART® EnteroBac

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

For further information and questions do not hesitate to visit 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



Version 4
July 2015

CONTENTS:

1. GLOSSARY OF TERMS

2. DESCRIPTION

3. KIT COMPONENTS AND STORAGE

3.1. Extraction, purification, and amplification reagents

3.2. Visualization reagents

3.3. Other components

4. ADDITIONAL MATERIAL

4.1. Reagents and material

4.2. Equipment

5. HANDLING PROCEEDINGS AND RECOMMENDATIONS

5.1. General recommendations

5.2. Extraction precautions

5.3. Visualization precautions

6. SAMPLES: STOOL SAMPLES

7. WORKING PROTOCOLS

7.1. Automatic extraction of DNA from microorganisms present in stool samples.

7.2. Amplification reaction.

7.3. Visualization of amplified product.

7.3.1. Manual visualization

7.3.2. autoclart® visualization

8. RESULTS READING

9. RESULTS INTERPRETING

10. TECHNICAL AND OPERATIONAL SPECIFICATIONS

11. BIBLIOGRAPHY

1. GLOSSARY OF TERMS



Please, check handling instructions



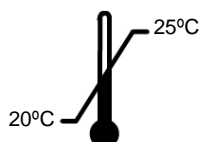
Expiry date



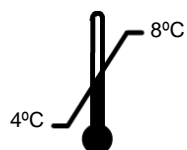
In vitro diagnostic device



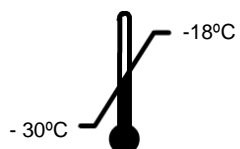
Batch



Store at room temperature



Store between 4 °C and 8 °C



Store between –30 °C and –18 °C

2.- DESCRIPTION

CLART[®] EnteroBac detects the presence of the following enteric bacteria causing diarrhea:

- *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* enterohemorrhagic, *E. coli* enteroinvasive, *E. coli* enterotoxigenic and *E. coli* enteropathogenic)
- *Clostridium difficile* B
- *Aeromonas* spp, producers of aerolisin

Detection of the different microorganisms is achieved by **PCR amplification** of a specific regions coding for enterotoxins and virulence factors for the microorganisms included in the kit, and for constitutive genes of *Salmonella* spp. and *Campylobacter* spp.

Amplification was performed in two different types of PCR tubes. Mix 1 tubes are white and allow the amplification and subsequent detection of *Shigella* spp, *Yersinia enterocolitica*, *Yersinia* spp., *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli* EPEC, *Clostridium difficile* B and *Aeromonas* aerolisin toxin positive. Mix 2 tubes are green and contain everything needed for amplification and detection of *Salmonella* spp. and *Campylobacter* spp.

The platform is based on a low-density microarray fixed at the bottom of an 8-well strip (CLART Strip[®] -CS) (Fig. 1), thus rendering a very efficient and easy to use system. This technology allows the simultaneous detection of multiple molecular markers for diagnostic use while providing the controls needed to ensure the reliability of the results. Moreover, this system considerably simplifies the processes of hybridization and visualization when compared with classic microarray systems.



Figure 1. CLART-Strip[®] (CS) platform in the form of an 8-well strip.

Hybridization of the amplified PCR product is detected by generation of an insoluble precipitate at the sites of the microarray where the amplified products have been captured by the probes. This fact is achieved using biotin-labelled oligonucleotides. Biotinylated amplification products hybridize to their specific probes attached to the microarray surface and become immobilised. These immobilised biotinylated products are recognized by the streptavidin of a streptavidin-peroxidase conjugate, thus providing with peroxidase activity to the hybridised products. Peroxidase activity will then metabolise o-Dianisidine and produce an insoluble product which will precipitate in those places where hybridisation occurred (Fig. 2).

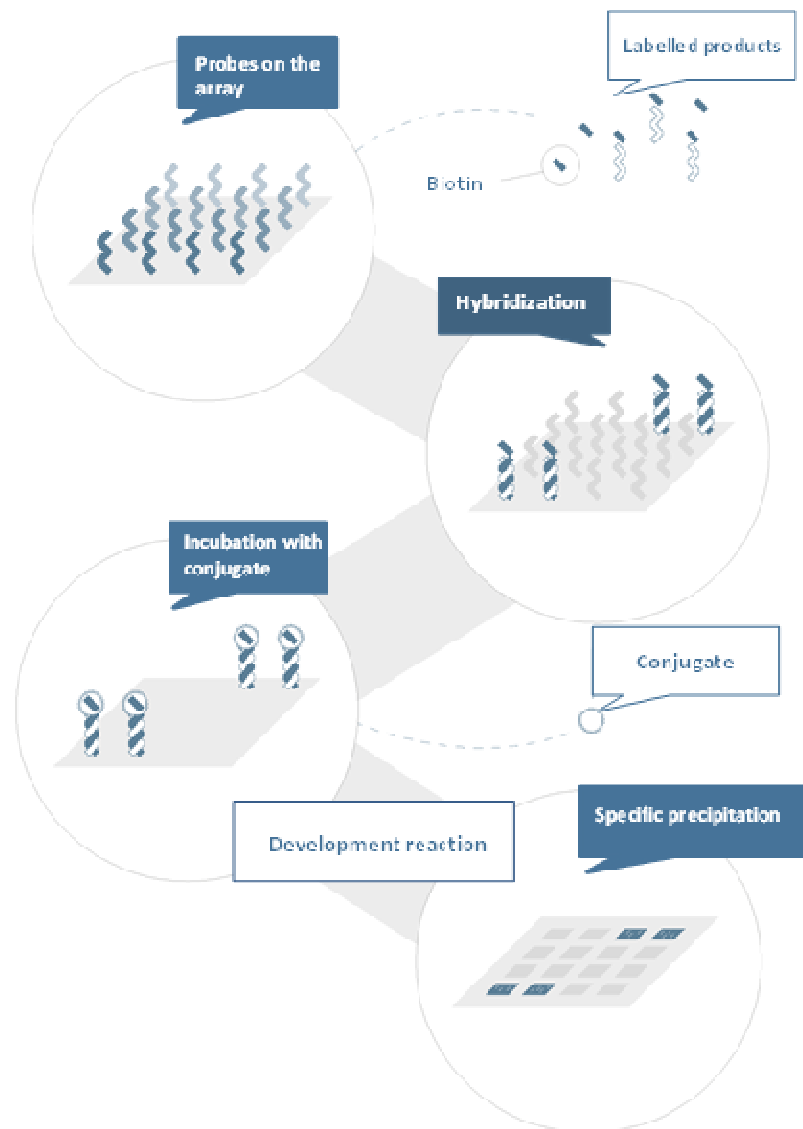


Figure 2: Diagram of the visualization method. Probes, immobilized on the surface, capture their complementary biotin-labeled amplified products. With the help of the biotin, the conjugate binds, in this case streptavidin-HRP (**HorseRadish Peroxidase**). Due to the HRP action, the o-dianisidine substrate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

3. KIT COMPONENTS AND STORAGE

CLART[®] Enterobac Kit contains sufficient reagents for the extraction and analysis of DNA from 48 clinical samples. These reagents are provided in two different boxes, depending on the temperature they should be stored at. All the reagents provided are stable under the appropriate conditions until the indicated expiration date.

3.1. Amplification tubes

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

Two different amplification tubes are included in the kit:

- **Mix 1: White tube.** Amplification of *Shigella spp*, *Yersinia spp.*, *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli EPEC*, *Clostridium difficile* and *Aeromonas aerolisin* toxin positive. Internal amplification control and extraction control are included in this mix.
- **Mix 2: Green tube.** Amplification of *Salmonella spp.* and *Campylobacter spp.* Internal amplification control different that internal control from mix 1 is included.
- **DNA EC** (Extraction control). Genomic human DNA extraction control.

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

3.2. Visualization reagents.

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

- **Microarrays: CS well strips** (including the specific probes). These are delivered in a sealed envelope. **After opening, the envelope should be closed and stored at room temperature, protected from light exposure.**
- **RE** (Developer). **Store at 4°C..**
- **SH** (Hybridization solution). **Store at 4°C.**

- **DC** (Conjugate solvent). **Store at 4°C.**
- **CJ** (Conjugate). **Store at 4°C. Centrifuge briefly before using.**
- **TL** (Washing buffer). **Store at 4°C.**
- **Support and lid for the 8-well strip.**

3.3 Other components

The following components are required for the capture and subsequent image processing:

- **CAR® (CLINICAL ARRAY READER):** which allows the reading and automatic interpretation up to 12 CS, that means, a total amount of 96 samples. This platform is manufactured exclusively for GENOMICA kits use only.
- **SAICLART®:** software developed by GENOMICA for image processing.
- **CLART® EnteroBac Software:** It is specific for *CLART® EnteroBac* designed and validated by GENOMICA. Installed and ready to use.

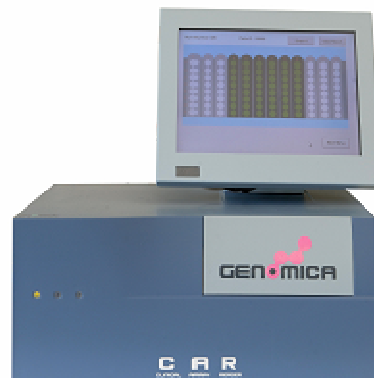


Figura 3. CAR® (CLINICAL ARRAY READER)

4. ADDITIONAL MATERIAL.

4.1. Reagents and materials.

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

4.2. Equipments.

- autoclart® (Figure 4). The following equipment is needed for the automatic visualization phase. It enables the automatic visualization of up to 12 CS, that means, a total amount of 96 samples.

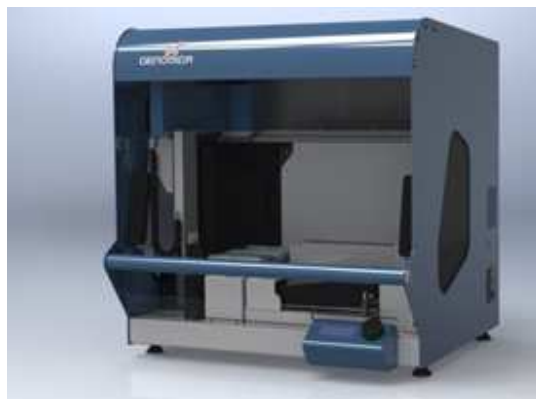


Figure 4. autoclart®

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

5. HANDLING PROCEEDINGS AND RECOMMENDATIONS.

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

5.1. General recommendations.

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

- **Pre-PCR area:** samples are prepared and DNA is extracted. Manipulations of stool samples must be done inside safety cabin.
- **Post-PCR area:** products are amplified and then visualized. Material in this area should never come into contact with material in the pre-PCR area.

AFTER WORKING IN THE POST-PCR AREA, DO NOT RETURN TO THE PRE-PCR AREA.

2. Use gloves at all times. It is recommended to change gloves quite frequently while it is mandatory to change them prior to start working in each of the above-mentioned areas. New gloves should be used for the preparation of the amplification tubes and every time DNA is added to them.

3. Clean working areas (laboratory benches, hoods, grids, pipettes, thermocycler) thoroughly with 10% diluted disinfectant **following every sample batch processing;** it is mandatory to disinfect all working areas preventing contaminations.

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

5. Use disposable and autoclaved laboratory materials.

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

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

8. Dispose of micropipette tips after use.

9. Separate the tubes from each other at all times during handling, with special caution during extraction steps.

10. GENOMICA does not assume any responsibility for those results obtained without following the directions in this manual.

5.2. Extraction precautions.

Many of the microorganisms in *CLART*[®] *EnteroBac* kit are part of the normal flora of the human digestive tract, so it must be extremely careful when processing the clinical sample.

- Wear gloves at all times.
- Put on gloves with fingers touching the edge of them.
- Clean work surfaces with 10% diluted bleach.
- Turn on laminar flow and UV light at least 20 minutes before extraction.
- The preparation of the samples before extraction should be inside the cabin.

5.3 Visualization precautions.

1. Prevent the pipette tip or vacuum system from touching the bottom of the tube where the microarray is located.
2. It is recommended to add all solutions to the side wall of the CS; never directly to the bottom.
3. Only add the hybridization solution immediately before adding the PCR products.
4. Using **CS** there will be a slight residual volume left avoiding the array to dry out.
5. After incubating with the conjugate, it is very important to thoroughly wash the CS to avoid any remaining conjugate from reacting with the developer.
6. Avoid bubbles on the surface of the microarray when adding the different solutions.
7. Keep the bottom of the CS clean in order to avoid possible interference when reading the results.
8. When visualizing the image in the reader, confirm that position markers appear and that there are no bubbles or spots interfering with the reading. If required, clean the bottom of the tube with cellulose paper or gently tap the tube with your finger.

6. SAMPLES: STOOL SAMPLES.

The *CLART*[®] *EnteroBac* kit has been designed and validated for use on stool samples⁽¹⁾. GENOMICA is not responsible for the results when using other samples.

7. WORKING PROTOCOL.

7.1. Automatic DNA extraction.

1. Sample preparation before extraction (performed out of the equipment). 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 1500rpm.

Note: Manipulations of stool samples must be performed in safety hood.

- Take the supernatant into a sterile 1.5 ml tube and discard the pellet
- Centrifuge at 13000rpm 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 275 μ to a new tube. Add 25 μ l of **DNA CE** (extraction control).
- Transfer the mix to the extractor.

2. Internal lysis and DNA extraction in automatic extractor equipment. Follow the computer's user guide. The elution volume selected is 110 μ l. This volume may vary depending on the automatic extractor instrumentation used. If the device has several washing programs select the most comprehensive option (e.g. Specific protocol B in Nuclisens easyMAG from Biomerieux).

3. Once the extraction is made with the micropipette 110 μ l of eluted DNA and placed in Eppendorf tubes of 1.5 ml. Use 5 μ l for every tube of amplification and store the rest at -20° C.

The estimated minimum yield of the extraction of DNA required for successful amplification is **5 ng/ μ l**.

7.2. Amplification reaction

Specific recommendations for amplification:

- Work in pre-PCR area, always using hood and following the recommendations described in 5.1.
- Add the DNA to the amplification tubes always in hood. During the process keep the tubes separated on ice.

1. Thaw one reaction tube from Mix1 and Mix2 for each sample and keep them on ice. Do not use temperatures above 37°C for thawing.
2. Centrifuge the reaction tubes in a microcentrifuge so that all the liquid goes to the bottom (if no adapters are available to hold the reaction tubes, they can be placed in larger tubes with their caps removed).
3. Add 5 µl of extracted DNA from each sample to Mix 1 and 5 µl of extracted DNA from each sample to Mix 2 tubes and resuspend several times with a micropipette. Leave the tubes on ice.
5. Program the thermocycler as follows:

1 cycle	95°C 5 min
45 cycles	95°C 30 sec 53°C 30 sec 72°C 1 min
1 cycle	72°C 10 min
4°C (maintained) until tube collection	

6. Start the program and place the Mix 1 and Mix 2 tubes in the thermocycler when the block is above 90°C. This minimises any non-specific amplifications due to hybridization occurring below the reaction temperature. The amplification process lasts about 3 and a half hours, although this can vary depending on the thermocycler used.

7.3. Visualization Protocol for CLART-Strip® (CS)

Specific recommendations before starting the visualisation process:

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

1. At the beginning of the assay:
 - Switch on the CAR® (CLINICAL ARRAY READER) in order to allow auto-calibration of the device.
 - Input the sample IDs. It is important to have the equipment ready to read the samples at the end of the assay so that they are not exposed to an excess of developing time.

2. Switch on the thermomixer at 56°C at least 30 min before starting the hybridization process.
3. Keep the SH (Hybridization Solution) at room temperature.
4. Prepare the washing solution before each assay. Do not use previous solutions or any remaining from previous assays.
5. Before starting the denaturing program, wash the thermocycler with bleach diluted at 10%. During the denaturing process, place the amplification tubes separated from each other into the thermocycler. Do not denature for more than 10 minutes.
6. It is not necessary to use filter tips during the visualization process, only when adding amplified products to every well. However, a different tip for each sample and for every reagent must be used. This precaution must also be undertaken for the TL buffer.
7. The 8-tip combs used with the aspiration pumps must be discarded after use or decontaminated with 10% bleach solution after each assay. Make sure that the vacuum pump works properly and do not let remaining liquid in the wells.
8. All the buffers must be thoroughly aspirated from the wells without touching the bottom.

7.3.1. Manual Visualization.

1. Denaturation: use the thermocycler to denature the samples.

Adjust the program for 15 minutes at 95°C. Place the amplified products into the thermocycler and incubate 10 minutes at 95°C. After 10 minutes, take off the tubes from the thermocycler (still at 95°C) and place them straight away on ice.

2. Prepare TL diluted Solution:

For 8 wells (one strip) add as follows:

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

3. Pre-washing of CS:

Add 200 µl of diluted TL Solution to every array and pipet up and down 10 to 15 times. Discard the diluted TL Solution using a pipette or preferably a vacuum system.

This step is necessary in order to wash already packaged CSs, before adding the sample. The CS should not contain washing solution residues. Under no circumstances, should CSs be allowed to dry out for a long period of time.

4. Hybridization: Hybridization solution (SH) must be heated at 56°C in order to dissolve crystallized salts. Add 100 µl of SH buffer (avoiding the appearance of foam) + 5 µl of denatured product from **Mix1 tube** and **Mix2 tube** to each well. Mix properly with the pipette avoiding touching the array and incubate the strip, covered with the transparent plastic lid in the thermomixer for **30 minutes at 56°C, shaking at 550 rpm**.

To obtain correct results, amplified products in Mix 1 and Mix 2 tubes must be visualized in the same array.

Remove the CSs and discard the SH Solution using a pipette or a vacuum system. Program the heating block at 30°C and leave it running so that it can be used later on step 6. You can remove the lid from the heating block to let it cool down quicker.

5. Double washing: use different tips for each well in both washes. Wash twice the CS with 200 µl of diluted TL Solution in every well mixing 10 to 15 times with the pipette. Discard the diluted TL Solution using a pipette or a vacuum system, leaving a remanent volume. In case the heating block has not reached a temperature of 30°C when you get to this step, leave the CSs filled with diluted TL Solution until the heating block reaches the necessary temperature.
6. Blocking and conjugate: Prepare the diluted CJ solution 15 minutes before hybridization time is over and keep it on ice until its use. It is recommended to spin the CJ buffer for 10 seconds before using.

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

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

Vortex the diluted CJ solution briefly before use.

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

After this incubation, take the strip and remove the diluted CJ buffer immediately with the pump.

(Set the thermomixer at 25°C and no shaking for step 8. Remove the lid to speed up the cooling).

7. Triple Washing: **Add straight away** 200 µl of TL diluted solution per well. Mix well 10 to 15 times with the pipette and remove the diluted TL buffer with the pump without drying the array out. Repeat this washing twice and leave the CS with 200 µl of TL buffer at RT for 5-10 minutes or until the thermomixer has reached 25°C.
It is very important that the diluted CJ buffer is completely washed off. Any remaining buffer could react with the RE buffer producing an unspecific signal.
8. Developing with RE buffer: remove the diluted TL buffer without drying the array out and add 100 µl of RE buffer per well. Incubate in the thermomixer **at 25°C for 10 minutes without agitation.**

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

9. Remove the RE buffer with the pump. The array must be dry at this time.
10. CAR® (CLINICAL ARRAY READER): Place the plate normally on the tray and the CAR® will take and analyse the arrays automatically.

7.3.2. autoclart® visualization

1. Denaturation: Use the thermocycler to denature the amplification tubes. Place the amplification tubes in the thermocycler when this has reached 95°C and incubate the tubes for 10 min. Program the thermocycler during 15 minutes in order to Remove the tubes when the 10 minutes are over. Place the tubes immediately on ice.
2. Switch on the autoclart® unit and follow the instructions described on the screen :
 1. Close the door and press the knob.
 2. Select “Run Program” at the main menu.
 3. Select the assay “**Enterobac test**” among those listed.
 4. Select the well of the strip where the run should start: A1 or E1 in case the first 4 wells have already been processed.
 5. Select the number of samples to be processed. With the autoclart®, the user can process from 4 up to 96 samples per run. In any case, samples must be multiples of four.
 6. Confirm that the number of samples and the start up-well (A1 or E1) are correct.
 7. Place the tip-rack (full) on its position.
 8. Load the array microplate in the holder. Make sure that the catch is fastened in order to clamp the plate down.
 9. Check that both, the tip waste and the liquid waste containers are empty and on its proper position.

10. Fill the DI bottle with 250 ml distilled water.
11. Add each of the reagents to its specific container. The autoclart® calculates the specific volumes required according to the amount of samples indicated:
 - **TL** (Washing buffer). The volume showed in the display indicates the diluted washing buffer required. In order to prepare the diluted washing buffer please dilute the TL reagent provided 1:10 into distilled water.
 - **SH** (Hybridization solution). Ready-to-use. Add the specified volume in the container once it has been tempered.
 - **CJ** (Conjugate). It's recommended to shortly spin the CJ before use. The display shows the final volume of diluted CJ to add, meaning that each mL indicated on the display should be prepared as follows: **1 ml of DC (Conjugate Diluent) and 9µl CJ reagent**. Vortex the diluted solution in order to mix it properly up.
 - **RE** (Developer). Add the RE volume indicated on the display.
12. Close the door and press the knob to start the program.
13. The device will start priming the system. Then it will perform the pre-washes of the CS and add the Hybridization Solution. Once finished these steps, the device will beep as a signal for the user to add the samples on the CS. The autoclart® will keep beeping until the user opens the door.
14. For the addition of the samples on the CSs, please carefully remove the plate from autoclart® unit and add 5 µl of the denatured products from the white tube and the green tube, both to the same well. Mix it up carefully in order to not touch the array and place the microplate again on the autoclart®. Press the knob to continue the visualization process.
15. Once the visualization process is finished, the autoclart® will beep indicating the end of the run. Please carefully remove the microplate and proceed with the reading step on the CAR®.

WARNING: Once the visualization phase is finished on the autoclart®, the arrays should immediately be read on the CAR®, otherwise false negatives might appear caused by intensity loss.

16. CAR® (CLINICAL ARRAY READER): Place the plate normally on the tray and the CAR® will take and analyse the arrays automatically.

8. RESULTS READING

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

On the screen, a table with three columns will appear. The left column shows the microbial taxa suitable to be detected, the central column will give either a positive or negative result for each microbial *taxa*, and the right column will show if the amplification and DNA extraction controls have been passed.

9. RESULTS INTERPRETATION

One of the main drawbacks of genomic amplification is the utilization of poor quality DNA samples (too short DNA fragments, degradation of DNA, or loss of DNA during extraction) or the presence of DNA polymerase inhibitors (e.g., haemoglobin, remains of paraffin wax, salts etc.) in the samples to be analyzed, thus interfering with the genomic amplification and resulting in false negatives. However, the *CLART[®] EnteroBac* eliminates false negatives via the use of internal controls within the same tubes (Mix1 and Mix2) in which the sample is analyzed, and that are amplified at the same time as the microbial DNA.

Included in the Mix 1 tube are specific oligonucleotides that amplify the extraction control (DNA EC), which is added to the sample before extraction. The presence in the array of genomic DNA indicates that the extraction process has been adequate, thus eliminating false negative results due to failures in the extraction.

To obtain correct results, sample must be amplified in Mix 1 and Mix 2 tubes, and amplified products must be visualized in the same array.

In every set of analysis a negative extraction control should be included to check that samples have not been contaminated during the extraction, amplification and visualization, thus giving rise to a false positive.

Each tube contains the following amplification primers:

Mix 1:

- A pair of primers that amplify the extraction control
- A pair of primers that amplifies a modified plasmid included in the amplification tube and used as a control for amplification of the PCR reaction.
- Target-specific oligonucleotides designed to detect microbial pathogens.

Mix 2:

- A pair of primers that amplify a modified plasmid included in the amplification tube and used as a control for amplification of the PCR reaction. This plasmid is different from that included in the mix 1 tube.
- Target-specific oligonucleotides designed to detect microbial pathogens.

The reaction tube has been designed in order to favour the amplification of microorganisms comparing to the other two controls. Among these two controls, the amplification of the genomic DNA will be performed preferentially comparing to the control of the amplification reaction.

The reason for this design is:

Genomic DNA control would only be essential for confirming a negative result, since it informs you that the DNA extraction from stool sample was conducted successfully.

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

Under certain conditions (e.g. high concentration of microbial DNA), it could happen a negative amplification of the two controls, or any of them, displaying a reading: NO SIGNAL.

There are two possibilities that may lead to a not valid result:

- Not valid extraction: The amplification of the microbial target and/or the amplification of the extraction and amplification controls may be disturbed due to the presence of amplification inhibitors or due to a mechanical mistake during extraction. The process has completely to be repeated.
- Not valid amplification: The microbial presence in one tube and the absence of amplification in the other indicates a valid extraction procedure but indicates the presence of a problem during amplification. The amplification with both tubes has to be repeated.

There are three possibilities that may lead to an inconclusive result:

- In those cases when replicates of an array probe are very different from each other.
- In multiple infections of more than 3 bacteria.
- When the signal intensity of non-normalized absorbance is found at the detection limit of the technique, which range is set by the software for each type of microorganism.

10. TECHNICAL AND OPERATIONAL SPECIFICATIONS

10.1. Known sources of interference

Certain substances can interfere with the *CLART[®] EnteroBac* kit. They are mainly substances inhibiting enzyme mixing and, thus, inhibiting the amplification reaction. The most common factors are as follows:

- **Use of unsuitable samples.** The analysis of any kind of clinical samples other than those specified in the manual of the *CLART[®] EnteroBac* kit, as well as incorrect sampling, can produce an inconclusive or invalid analysis result due to the lack of amplification as a consequence of sample shortage or inhibited reaction.
- **Inadequate storage of samples** can influence the result of the analysis. If samples are subject to conditions that can result in the degradation of their DNA, the result of the analysis might lead to a false negative.

10.2 Technical specifications:

Analytical parameters:

Analytical sensitivity. Analytical sensitivity of microorganisms presented in the kit⁽²⁾ was determined via amplification of a series of DNA dilutions of recombinant plasmids with the cloned specific amplification fragment product (including the complementary part of the detection-specific probe) for each microorganism. The visualization step was performed in CLARTStrip[®], obtaining the results which are summarized in the following Table 1:

Microorganism	Nº of copies of plasmid per PCR reaction
<i>Yersinia enterocolitica</i>	100
<i>Escherichia coli</i> (enteropathogenic)	
<i>Campylobacter jejuni</i>	
<i>Shigella</i>	1000
<i>Salmonella</i>	
<i>Clostridium difficile</i>	
<i>Campylobacter coli</i>	
<i>Aeromonas aerolisina</i> +	

Table 1. Number of copies of recombinant plasmid necessary to obtain 100% sensitivity for the detection of each of the microorganisms.

Analytical specificity. Specificity experiments were performed with 8 recombinant plasmids ⁽²⁾, showing that there is no nonspecific detection of other microorganisms. Therefore, it is considered that the analytical technique reaches a specificity of 100%.

Diagnostic parameters.

In order to determine the diagnostic parameters of the kit *CLART® EnteroBac* comparative studies against stool culture characterization were performed ^(1, 2).

These comparisons were performed in collaboration with two Spanish hospitals and one Portuguese hospital

- Microbiology Service of the University Hospital Germans Triás i Pujol, Badalona, Barcelona.
- Microbiology Service of the University Hospital Ramon y Cajal, Madrid.

A total amount of 334 stool samples were processed. The following table illustrates the data of diagnostic sensitivity and specificity for the microbial species detected in the kit *CLART® EnteroBac*

CLART® EnteroBac results were compared to the results of the reference technique (Stool culture + characterization). In case of discrepancies, the results of sequencing were considered as the valid results. In those specific cases, where this information was not available, the discrepancies were evaluated by Nested-PCR and further DNA sequencing.

N = 334	Sensitivity	Specificity	PPV	NPV
<i>Campylobacter</i>	90,0	100,0	100,0	94,0
<i>Salmonella</i>	83,2	99,0	98,3	89,4
<i>Clostridium difficile</i>	83,0	100,0	100,0	97,7
<i>Escherichia coli</i> <i>enteropathogenic</i>	100,0	100,0	100,0	100,0
<i>Shigella</i>	94,4	100,0	100,0	99,7

Table 2. Diagnostic sensitivity and specificity of *CLART® EnteroBac* for each microorganism. PPV: positive predictive value. NPV: negative predictive value.

- In this validation 15 samples of *Aeromonas spp.* have been processed, none of them positive for aerolisin toxin (targeted in this kit), so the diagnostic parameters for this microorganism have been not determined.
- Due the low prevalence of *Yersinia* in the samples used in this validation, only 5 samples with this microorganism have been processed, being not enough to calculate reliable diagnostic parameters

Diagnostic specificity.

The specificity was determined using negative stool samples and positive samples for the microorganisms included in the kit. A value of 99% of specificity has been obtained, which means no unspecific identification of other bacteria than those that are present in the sample.

Diagnostic reproducibility and repeatability

The obtained data are as follows:

- Reproducibility: 91,8 % (n=49 samples)
- Repeatability: 97.2 % (n= 48 samples)

Samples have been processed from the extraction step.

Detection of multiple infections

CLART® *EnteroBac* results shows the presence of multiple-infections (two or more microorganisms) in the 15,9% of the stool samples analyzed vs. 2,4% of multiple infections detected in the stool samples by culture characterization, supporting the higher sensitivity of CLART® *EnteroBac* for detection of multiple-infections vs classical methods of culture identification.

BIBLIOGRAPHY

1. Alvarez, M., Buesa, J., Castillo, J. and J. Vila. *"Microbiologic diagnostic of enteric diseases"*. 2007. 2nd Ed. (24). Eds. Cercenado E. and R. Cantón. Proceedings in clinical microbiology. Spanish Society of Clinical Microbiology (SEIMC).
2. Manjón, N., Moscoso, J., Margolles, Y., García, M., Morosini, M. I., Salazar, O., Cospedal R., Cantón, R., and M. L. Villahermosa. 2011. *"Design and optimization of microarray based in vitro assay, for the rapid identification of bacterial pathogens responsible of infectious diarrhea"*. 2011. Infectious diseases and Clínica Microbiology. Vol 29, 33-34. 15th Congress of the Spanish Society of Clinical Microbiology (SEIMC). Malaga, Spain. June 1st-4th 2011.