CLART® ENTHERPEX

GENOTYPING OF HERPES AND HUMAN ENTEROVIRUS
VIA GENOMIC IDENTIFICATION
FOR *IN VITRO* DIAGNOSIS

CLART® ENTHERPEX

CLART® ENTHERPEX is under protection of the patent family of the international PCT Patent Application WO2009122201, which comprises national and regional members granted in different territories, including Spain, Canada, France, Germany, Italy, Switzerland, United Kingdom, Russia and Mexico, and under examination in Brazil.

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

ONLY CMV DIAGNOSTIC HAS BEEN ASSENTED BY THE NB 0318.

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CE

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Contenido

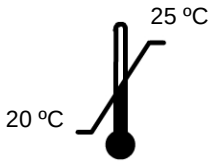
1. GLOSSARY

Attention, see instructions for use

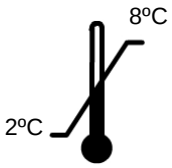
Expiration date

In vitro diagnostic medical device

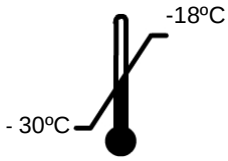
Lot



Store at room temperature



Store at 2ºC to 8ºC



Store at –30ºC to –18ºC

2. DESCRIPTION OF THE DETECTION SYSTEM

CLART® ENTHERPEX enables to detect infections of the 8 human herpes viruses HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7 and HHV-8, and of the 3 most relevant viruses of the Enterovirus family from a clinical point of view, Poliovirus, Echovirus y Coxsackievirus, without allowing distinguish between them.

As starting material, different types of human samples may be used: swabs, serum, plasma, cerebrospinal fluid and biopsy.

Detection is based on our CLART® technology: PCR amplification of a virus fragment of 106-328 bp, followed by visualization in low-density microarray. The chosen sequence for each virus is highly conserved, while at the same time being specific enough so as to distinguish each virus type by means of specific probes.

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

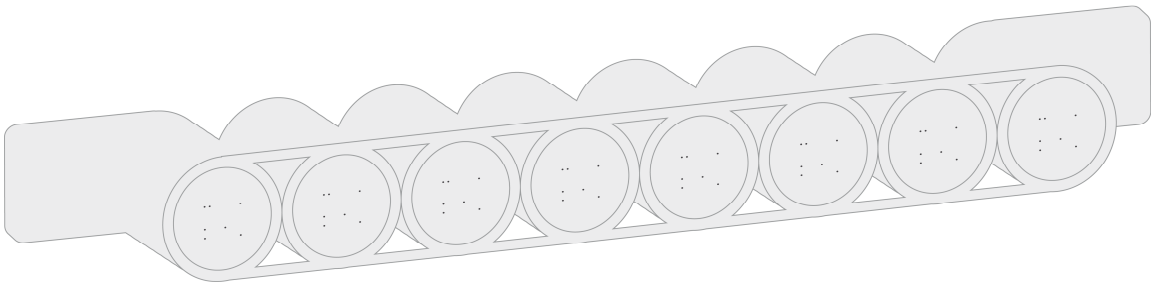


Figure 1. CLART-Strip® in the form of an 8-well strip.

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).

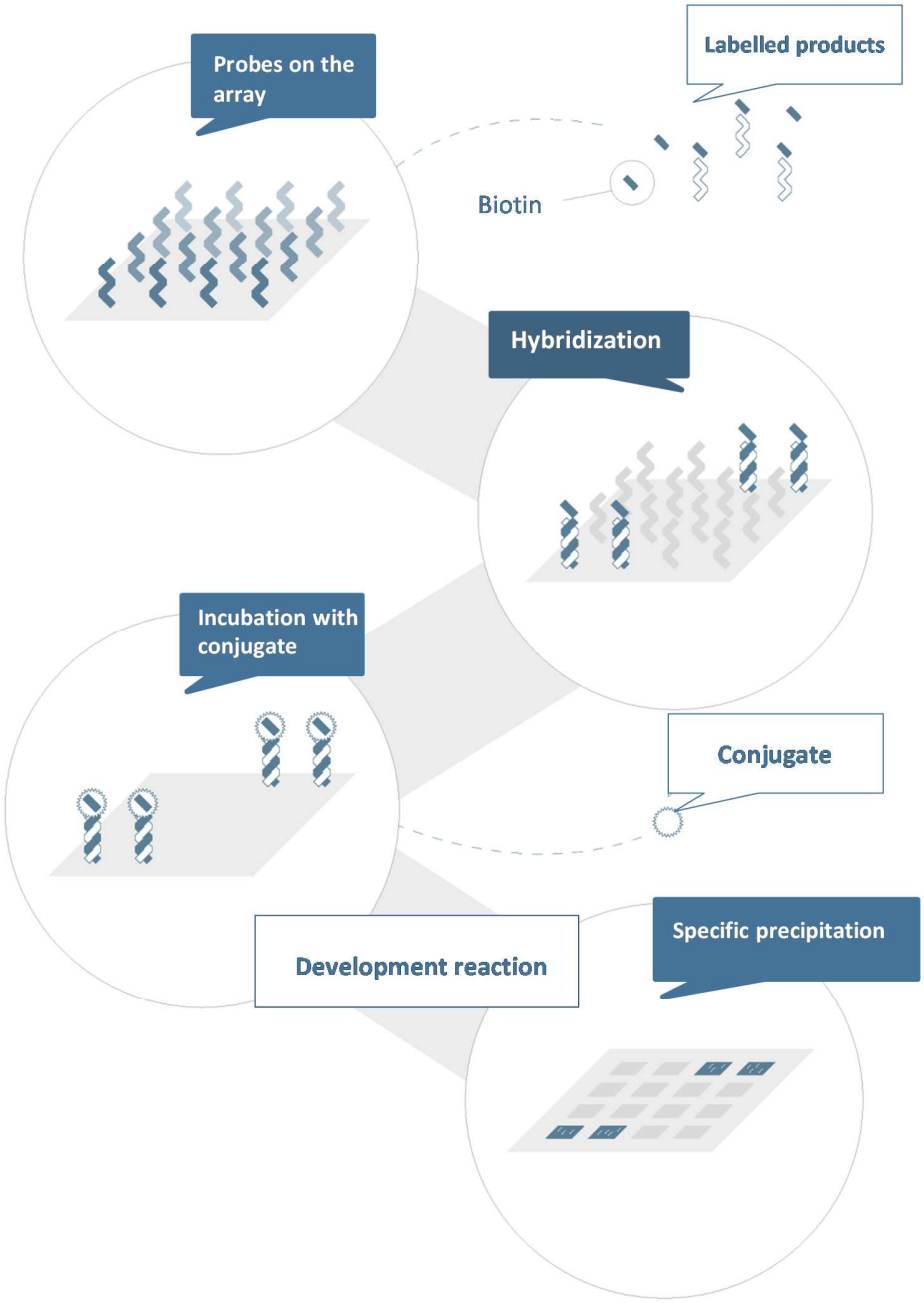


Figure 2. Scheme of the detection system. Probes immobilized on the microarray surface, capture complementary biotin-labelled amplified products. Subsequent binding of biotin to the streptavidin-peroxidase conjugate takes place. Finally, incubation with the peroxidase substrate o-dianisidine, yields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

3.-KIT COMPONENTS AND STORAGE

CLART® ENTHERPEX 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. Extraction and purification reagents

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

Components:

- SEML (Extraction solution of Liquid samples). Once thawed, it must be stored at 4ºC and made use of within the next 8 days.
- SD (Dilution solution). Store at -20ºC or at 4ºC.
- IP (Isopropanol). Store at -20ºC.
- DE (70% Ethanol). Store at -20ºC.
- DB (5X Digestion solution). Once thawed, store at 4ºC.
- PK (10X Proteinase K). Once thawed, store at 4ºC. Do not make use of it later than one month after being thawed.

3.2. Amplification reagents

Shipped and stored at -20ºC.

They consist of Amplification tubes and Enzyme Mix.

Two kinds of Amplification tubes are shipped:

- Colorless tube (Multiplex PCR 1 or Mix1) for the amplification of HSV-1, HSV-2 and VZV. It contains 45 µl of reaction mixture.
- Green tube (Multiplex-RT-PCR 2 or Mix2) for the amplification of CMV, EBV, HHV-6, HHV-7, HHV-8 and Enterovirus (Echovirus, Poliovirus and Coxsackivirus). It contains 43 µl of reaction mixture.

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

It is mandatory to add 2 µl of Enzyme Mix to each green tube before addition of the extracted genetic material. Enzymes are already present within each colourless tube.

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

3.3. Visualization components

Visualization components are divided into two groups, according to optimal shipping and storage temperatures:

- Shipped at 4ºC and stored at Room Temperature:

- **CLART-Strip® (CS)**, each well including all specific probes for detection of all viruses to be detected.

  Note: Required **CS** units are shipped in a sealed pouch. Each unit should be kept until use, in the unopened pouch, at room temperature (i.e. 25ºC maximum) and protected from direct light and high temperatures.

- Shipped and stored at 4ºC:

- **DC** (Conjugate Diluent).
- **SH** (Hybridization Solution).
- **CJ** (Conjugate Solution).
- **RE** (Development Solution). Keep away from light.
- **TL** (Wash Buffer).
- **Microtiter plate adaptor and plastic lid.**

3.4. Other components

- GENOMICA's **CAR®** 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.

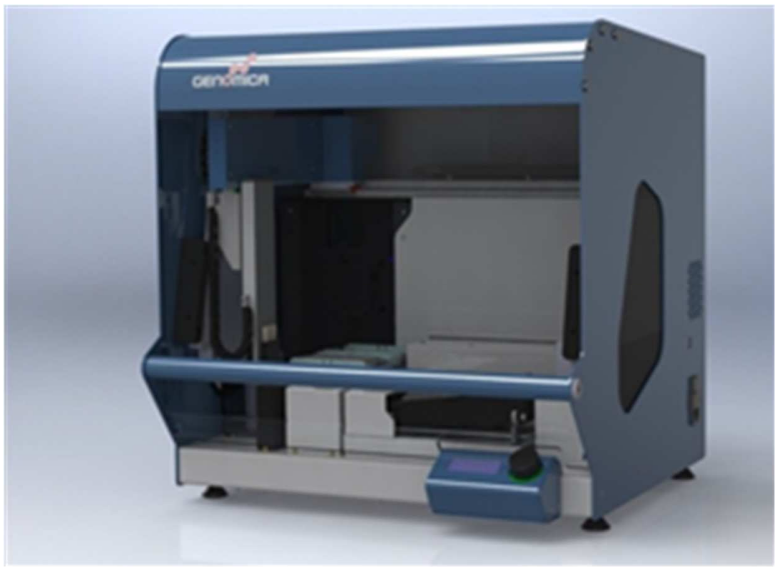


Figure 4. autoclart®

- 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 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 59ºC.
- Vortex.
- Vacuum pump.

5. GENERAL RECOMMENDATIONS AND HANDLING PROCEDURES

Read carefully to avoid contamination!

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

Pre-PCR area: Sample preparation, extraction of the genetic material, 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.

9. As a specific precaution for the steps of extraction and addition of the extracted material to the amplification tube, it is recommended that the flow and the UV light of the biosafety cabinet are switched on at least 20 minutes before the extraction step. Switch off the UV light when working within the cabinet. Sample preparation before extraction must take place within the biosafety cabinet.

6. SAMPLES

CLART® ENTHERPEX kit allows analyze the following types of samples:

6.1. Swabs

Sample should be taken with a dry and sterile, cotton or alginate swab, preferably of a urethral type (even for vaginal samples). Do not use devices that might cause bleeding. Place the swab back in its tube, in the absence of any preservation medium. Keep the swab at 4ºC, if processing is to take place within 7 days, or at –20ºC if processing is to take place later. Swabs are for a single use only, and must be discarded after being used. It is important to avoid freeze-thaw cycles.

6.2. Serum or plasma

Blood samples from which plasma is to be extracted should be collected in tubes containing citrate or EDTA as anticoagulant, never heparin.
In order to extract serum, allow the blood sample to coagulate for 30 min and then centrifuge at 1500 g for 20 min.
In some cases, following sampling it is possible to isolate the cellular fraction and to subject it to DNA/RNA extraction.
If the sample is to be processed within 12 hours, it must be stored at 4º C. If the analysis is to be performed later, the sample should be stored aliquoted at -70º C and thawed just before it is processed. It is important to avoid repeated freeze-thaw cycles.

6.3. Cerebrospinal fluid

If processing of the sample is going to take place within the first 12 hours, sample should be stored at 4º C. If processing is to be performed later, the sample should be stored aliquoted at -70º C and thawed just before processing starts. It is important to avoid repeated freeze-thaw cycles.

6.4. Paraffin-embedded biopsies fixed in formaldehyde or ethanol.

Samples should be fixed in buffered formaldehyde for the shortest possible time (never higher than 24 hr). Use of non-buffered formaldehyde or fixation times longer than 24 hr might lead to sample DNA degradation. It is important to carefully clean the blade with xylene or to use disposable blades, before and after sample cut, to avoid contamination with previously cut samples. Use the microtome to prepare 2 or 3 slices of 5 µm section, and place them in a clean 1.5 ml tube.

GENOMICA cannot warrant accuracy of results obtained if processing a different type of sample.

7. WORKING PROTOCOL

DNA/ RNA extraction may be manual or automatic. In any case, to achieve optimal results it is recommended that the extraction yield should be at least of 5-10 ng/µl DNA/RNA.

7.1. Manual DNA extraction.

7.1.1. Manual DNA extraction specific recommendations.

- Work in the Pre-PCR area, always using a laminar flow chamber and following the recommendations of Section 5.
- Before and after work, the area should carefully be cleaned with 10% diluted bleach.
- Clean pipettes before and after use with 10% diluted bleach.
- Keep samples on ice.
- Use 1.5 mL clean tubes. Keep them separate and on ice during the whole process to avoid contamination.
- Keep Proteinase K on ice while in use, and after that store at 4ºC. Never freeze it back again once thawed.

7.1.2. DNA/ RNA Manual Extraction Protocol

7.1.2.1 Swab

1. Add 1.5 ml of Saline solution (0.9% Sodium chloride) to the tube containing the swab. Vortex for 1 min. In every run a negative extraction control made of 1.5 Saline solution must be included and processed.
2. Place supernatant in a sterile 1.5 ml tube.
3. Centrifuge samples for 10 minutes at 12.000 r.p.m. in a microcentrifuge, and discard liquid remains with a micropipette, avoiding removing pellet.
4. Thaw one DB (5X Digestion solution) tube, one PK (10X Proteinase K) tube and one SD (Dilution Solution) tube. Prepare the Digestion Mix, by mixing the following amounts per sample to be analysed:

   70 µl x (tube number + 1) = __ µl SD (Dilution Solution).
   20 µl x (tube number + 1) = __ µl DB (5X Digestion solution).
   10 µl x (tube number + 1) = __ µl PK (10X Proteinase K).

5. Add 100 µl Digestion Mix to each sample. Gently resuspend pellet by pipetting up and down.
6. Incubate at 55-60ºC for2 h.
7. Inactivate Proteinase K through boiling during 10 min. Should the tube closing not be too tight, make a hole in the cap with a needle, so that they do not open during

incubation at 100ºC. Avoid entrance of bath water through the hole. Do put the bath lid.

8. Centrifuge at maximum speed for 10 min in a microcentrifuge. Immediately after, transfer the supernatant to a clean tube and make use of a 5 µl aliquote to carry out the amplification reaction. Keep the rest at -20 ºC.

7.1.2.2 Serum or plasma

1. Thaw the sample on ice.

2. Place 100 µl of the clinical sample within a 1.5ml tube. Process in parallel 100 µl of Saline solution, which will work as negative control of the amplification and visualization reactions.

3. Add 400 µl of SEML to each tube. Wait until the solution thaws and turns completely transparent. Invert the tubes several times to mix, and incubate at Room Temperature for 15 min.

4. Add 500 µl of Isopropanol (stored at -20º C, and kept on ice until use). Invert the tubes several times to mix, and centrifuge, preferably at 4º C, at 13000 rpm for 20 min.

5. Aspirate supernatant with a micropipette. The micropipette in the 20-200 µL range may be used to discard supernatant as long as a micropipette in a lower range is used to drain remains of the bottom of the tube. Avoid removing pellet by mistake.

6. Add 500 µl of 70% Ethanol (stored at -20º C and kept on ice until use). Gently shake to clean the pellet at the bottom, and centrifuge for 15 min at 13000 rpm.

7. Discard supernatant carefully, as in step 5. Let pellets dry by leaving the tubes open within the cabinet during 15 to 20 min. Before resuspension, make sure that there are no ethanol remains left, which might inhibit PCR.

8. Resuspend in 25 µl of SD (Dilution Solution). Extracted DNA/RNA may be directly analysed (keep on ice until the moment it is added to the amplification tube) or may be kept at **-20º C.**

7.1.2.3 Cerebrospinal fluid

1. Thaw the sample on ice.

2. Place 50 µl of the clinical sample within a 1.5ml tube. Process in parallel 50 µl of Saline solution, which will work as negative control of the amplification and visualization reactions.

3. Add 200 µl of SEML to each tube. Wait until the solution thaws and turns completely transparent. Invert the tubes several times to mix, and incubate at Room Temperature for 15 min.

4. Add 250 µl of Isopropanol (stored at -20º C, and kept on ice until use). Invert the tubes several times to mix, and centrifuge, preferably at 4º C, at 13000 rpm for 20 min.

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

6. Add 500 µl of 70% Ethanol (stored at -20º C and kept on ice until use). Gently shake to clean the pellet at the bottom, and centrifuge for 15 min at 13000 rpm.

7. Discard supernatant carefully, as in step 5. Let pellets dry by leaving the tubes open within the cabinet during 15 to 20 min. Before resuspension, make sure that there are no ethanol remains left, which might inhibit PCR.

8. Resuspend in 25 µl of SD (Dilution Solution). Extracted DNA/RNA may be directly analysed (keep on ice until the moment it is added to the amplification tube) or may be kept at **-20º C.**

7.1.2.4 Biopsies

1.a. **Paraffin-embedded:** Use the microtome to slice two 5 µm sections, and place them within an autoclaved 1,5 ml tube. Use different blades for different samples.

1.b. **Fixed in formaldehyde or etanol:** Cut/crush a 2-3 mL sample fragment with a clean blade on a clean slide and introduce it in a 1.5 mL clean tube. Crush the tissue with the pipette tip, and vortex to mix up and facilitate lysis.

2. Thaw one DB (5X Digestion solution) tube, one PK (10X Proteinase K) tube and one SD (Dilution Solution) tube at Room temperature. Proteinase K must be kept on ice while on use, and stored at 4ºC afterwards, never being frozen again after thawed. Prepare the Digestion Mix, by mixing the following amounts per sample to be analysed:

70 µl x (tube number + 1) = __ µl SD (Dilution Solution).
20 µl x (tube number + 1) = __ µl DB (5X Digestion solution).
10 µl x (tube number + 1) = __ µl PK (10X Proteinase K).

Add 100 µl of Digestion Mix to each tube. Push the sample with the micropipette tip to completely cover it with digestion mixture.

Add 100 µl of Digestion Mix to an empty 1.5 ml tube, which will be processed as negative control of the extraction and visualization reactions.

3. Incubate at 56º C for 3 hr in a Thermomixer or a bath.

4. Boil for 10 min to inactivate the Proteinase K. Should the tube closing not be too tight, make a hole in the cap with a needle, so that they do not open during incubation.

5. Centrifuge immediately for 10 min in a microcentrifuge. Transfer the supernatant to a clean 1.5 ml tube, reaching through the top layer of the solidified paraffin with a micropipette. The extracted DNA might be analysed immediately, or may be stored at -20ºC.

7.1.3. Automatic DNA extraction protocol

Follow manufacturer's instructions, taking into account that for an optimal outcome, it is recommended that the minimum DNA/ RNA extraction yield should be in the range of 5-10 ng/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.
- Special care should be taken when adding the Enzyme Mix to the amplification tubes, as the former has high glycerol content. Thereby, if the tip of the pipette deepens too much into the Enzyme Mix, an excess amount of Mix might get stuck to the tip walls, and would then wrongly be added to the amplification tube; Another consequence of this would be a shortage of Enzyme Mix for the remaining amplification tubes.
- During the DNA/ RNA addition process, keep the tubes separate and on ice.

7.2.2. Amplification protocol

1. Thaw on ice the required number of tubes according to the number of samples to be processed. Two tubes are required for each sample to be analysed, a colourless and a green one, corresponding respectively to Mix 1 and Mix 2. Avoid using temperatures

higher than 37ºC for thawing the Amplification 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 2 µl of the Enzyme Mix to the green amplification tubes (Mix 2). Keep the Enzyme Mix on ice during the process.

4. Add 5 µl of the extracted sample DNA/ RNA, at a minimum concentration of 3ng/µl, to each reaction tube. Pipette up and down several times. Keep the tubes at 4ºC.

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

1 cycle	45ºC 45min 95ºC 15min
45 cycles	95ºC 30 sec 56ºC 90 sec 72ºC 60 sec
1 cycle	72ºC 10 min
4ºC Until tube removal (optional)	

6. Start the program and place the amplification tubes in the thermal cycler. Amplification duration is of about 5 hr, the exact time depending on the thermal cycler used.

7.3. Visualization of the amplified product

7.3.1. Visualization-specific recommendations

1. Visualization should always take place in the post-PCR area. Do not introduce the amplified product back into the pre-PCR areas.

2. Make sure that the thermomixer temperature has been 59ºC for 30 min-1 hr before the hybridization step starts.

3. Warm up SH at room temperature. Make sure there are no crystals left at the time of use.

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

5. Clean the thermal cycler with a 10% diluted bleach solution before starting the denaturation program. Place the amplification tubes apart from each other in the thermal cycler, **for exactly 10 min.**

6. 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.

7. When using vacuum pumps for aspirating solutions, decontaminate with a 10% diluted bleach solution after every assay, and rinse the bleach solution aspirating distilled water. Make sure the pump aspirates properly and no residual liquid is left at the bottom of the well after aspiration.

8. When using CSs, place the CS in the Thermomixer **immediately** after addition of the amplified products, especially when there is a high number of CSs to be processed.

9. In case a high number of CSs is processed at the same time, it is advisable to add all reagents (except the amplified products) with the assistance of multichannel pipettes and separate reservoirs for the different reagent types. At the end of each assay the different reservoirs should be washed with 10% diluted bleach, followed by distilled water.

10. Upon aspiration of the different solutions within the CS wells, NO residual volume will be left (see specific recommendation of Section 7.3.2.).

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

12. 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.

13. Do not allow the well to dry completely.

14. Avoid foaming when adding reagents.

15. 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. The device should be ready at the time of reading to avoid unnecessary waiting and an excessive exposure to developer.

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

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 well before use. Mix the solution up and down with a multichannel pipette 10-15 times, making sure not to touch the microarray surface. It is advisable to carry out this wash during the step of denaturation of amplified products, and to leave the diluted washing solution in the CS wells until addition of the above-mentioned products.

   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 washing solution from the CS wells with a pipette or preferably with a vacuum pump. Immediately after that, add 100 µL of room-temperature 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.
   It is recommended to load each strip independently and apart from the rest, to avoid contamination. Once sample addition to each CS is complete, any such CS is to be introduced immediately into the plate thermomixer.

   Cover the Microtiter plate adaptor and the **CS**s with the plastic lid and incubate in the plate thermomixer for **1 hour at 59ºC and 550 rpm.**

   After incubation, remove the plate from thermomixer and aspirate incubation solution from the CS wells with a pipette or a vacuum pump. The CS must be totally

free of residues. Add the next solution immediately.

Set the thermomixer at 30ºC 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, 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 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 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 and incubate at 95ºC **for exactly 10 minutes**. After that, remove the tubes from the 95ºC incubation and immediately place on ice or at 4ºC.

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

4. Close the door and press the bottom.

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

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

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

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

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

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

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

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

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

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

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

    CJ. Centrifuge CJ for 10 seconds before use. Next, prepare diluted CJ solution as displayed in the screen. This is done by adding 5 µL of CJ to 1 mL of DC (amounts suitable for one CS). Vortex the diluted solution in order to mix it up properly.

    RE. Add the RE volume indicated on the display.

14. Close the door and press "Play" to start. The device will perform pre-wash of the CSs and the addition of SH. A beeping signal will indicate the moment of sample addition. The beeping will stop when user opens the device door.

15. For sample addition, place the **CS**s out of the autoclart® and add **5 µl** of denatured amplification product of the colourless tube, and **5 µl** of the green tube, to each CS well. Resuspend several times in order to thoroughly mix with SH, making sure the bottom of the well is not touched. Put back the plate into autoclart® and press the arrow on the screen to continue with the visualization process.

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

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

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

7.3.4. autoclart® plus visualization protocol

The device might be used in two distinct ways depending on the user needs. For further information see below, and also the "autoclart® plus" user manual.

A) With manual addition of the sample

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 **CS**s.

4. Select the assay "CLART_ENTHERPEX" 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.

5. Select the "Manual sample addition" option at the Analysis Setting screen. Press the right arrow key to continue.

6. The instrument will initiate the temperature preconditioning step. A series of checklists will be displayed onscreen; Press the arrow key to accept and proceed.

7. Place the full rack of 1000 µl tips for reagent dispensation.

WARNING! The 10 µl tips for sample addition ARE NOT NECESSARY.

Note: At this moment cooling of the reagents begins. A beeping signal will acknowledge end of the cooling step.

8. Denaturation: Use the thermocycler for denaturing of amplification products. Place the amplification tubes in the thermocycler and incubate at 95ºC **for 10 min**. 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 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. It is recommended to centrifuge CJ for 10 seconds before use. Display shows final volume of diluted CJ, meaning that each mL indicated on the display should be prepared as follows: 1 ml of DC and 5 µl CJ reagent. Vortex the diluted solution in order to mix it properly up.

    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 **CS**s 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 plate with **CS**s out of the autoclart® plus and add **5 µl** of the denatured amplification product of the colourless tube and **5 µl** of the green tube to the same 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 with CS 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.

B) 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 **CS**s.

5. Select the assay “CLART_ENTHERPEX” 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 arrow key to continue.**

7. Once the reading process is over, the autoclart® plus will beep indicating the end of the run.

8. RESULTS

Data processing of each analysis is automatically performed. Analysis of results and issuance of the corresponding report are automatically performed by CAR® or autoclart® plus.

The screen of the devices will display a table with three columns: The column at the left shows the viruses detectable with the microarray; The central column displays the positive or negative result for each virus; And the right column displays the amplification control conformity.

False negatives are one of the drawbacks of detection through genomic amplification, mainly due to the presence of DNA polymerase inhibitors in the samples where virus presence is to be checked (hemoglobin, paraffin remains, salts, etc.). ***CLART® ENTHERPEX*** Kit avoids these false negatives through addition of an internal control within both reaction tubes.

Each reaction tube contains the following oligonucleotides:

- Oligonucleotides that amplify a modified plasmid included within the amplification tube, and that is used as amplification control of the RT-PCR/PCR reaction.
- Human herpes viruses and Enterovirus-specific oligonucleotides.

The RT-PCR tube has been designed to boost viral amplification *versus* that of the amplification control. Thereby, under certain circumstances (eg. when there is a high number of viral copies, or upon coinfection with several viruses), it may happen that no amplification of the control takes place, and that a "VALID" read-out issues.

Displayed below are the different results obtainable with the Kit:

VALID RESULT:

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

NON- VALID RESULT:

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

It is recommended to repeat extraction or, if this is not possible, to request the facultative a new sample from the patient.

NON-CONCLUSIVE RESULT

CAUSE:

- Very different results obtained with replicas of the same probe.
- In coinfections of more than 5 viruses.

9. TECHNICAL AND OPERATIONAL SPECIFICATIONS

9.1. Control of known interferences

Some substances might interfere with the ***CLART® ENTHERPEX*** system. These are mainly substances that inhibit the Enzyme Mix, and thereby, the amplification reaction. The most known interferences are:

1 **Presence of hemoglobin or paraffin** after DNA/RNA extraction. Both DNA extracted from whole blood or other blood products, as well as that obtained from paraffin-embedded tissue samples, might contain hemoglobin and paraffin remains, respectively. This might be avoided by purifying DNA/RNA after extraction.

2 **Isopropanol residues in DNA/RNA.** During the DNA/RNA extraction process from serum, plasma and cerebrospinal fluid samples, DNA/RNA has to be precipitated with isopropanol. If the precipitate is not allowed to dry correctly, presence of isopropanol in the sample may inhibit the amplification reaction.

3 **Use of non-suitable samples**. Analysis of sample types other than those indicated in this manual of the ***CLART® ENTHERPEX*** kit, or a non-accurate sample taking, might lead to non-conclusive analysis results. For example, if a sampling swab is placed in an alternative medium, PCR inhibition might result.

4 Inadequate conservation of samples might affect the result of the analysis. If the samples are kept under conditions that might lead to DNA degradation, for instance, an excess formalin fixation time, the analysis result will be of inhibition due to lack of amplification of the sample's control DNA.

5 Residual Proteinase K activity. During DNA/RNA extraction starting from swabs and biopsies, Proteinase K has to be inactivated by means of incubation at 100ºC for 10 min. Under these conditions complete inactivation is achieved. Should this step be omitted, or should less stringent inactivation conditions be used, some residual activity of Proteinase K might remain and result in degradation of the DNA polymerase, and in PCR inhibition.

9.2. Technical specifications

9.2.1. Analytical parameters

Analytical sensitivity

Analytical sensitivity was determined through amplification of a dilution series of DNA recombinant plasmids corresponding to each of the viruses detected with the kit. Each plasmid had as an insert one amplification product, including the sequence complementary to the corresponding detection probes. Results are displayed below:

VIRUS	**Number of copies of recombinant plasmid per PCR reaction**
VZV	10
HHV-7	
HSV-1	
HSV-2	100
CMV	
EBV	
HHV-6	
HHV-8	
Coxsackivirus	
Echovirus	1000
Poliovirus	

Table 1. Relation of the number of copies of recombinant plasmid required for obtaining 100% sensitivity in the detection of each virus.

Analytic specificity.

Specificity experiments were performed with recombinant plasmids visualized in CS.

VIRUS	Analytic specificity
HSV-1	100 %
VZV	
CMV	
HHV-6	
HHV-7	
HHV-8	
HSV-2	99.4 %
EBV	
Enterovirus	92.2 %

Table 2: Analytic specificity per virus.

9.2.2. Diagnostic utility parameters

In order to determine the diagnostic utility parameters of the ***CLART® ENTHERPEX*** system, comparative studies were performed between the manual extraction technique and the following techniques:

- Herplex (GENOMICA's kit): for HSV-1, HSV-2, VZV, CMV, EBV and HHV-6 viruses
- Quantitative PCR: for CMV and EBV in some cases
- Real time PCR: for Enterovirus
- In-house PCR followed by gel detection: for HHV-7 and HHV-8 viruses
- Immunohistochemistry for HHV-8

Above-mentioned studies were carried out in collaboration with the following hospitals:

- "Hospital Universitario Marqués de Valdecilla" (Santander)
- "Hospital Clínico Universitario Lozano Blesa" (Zaragoza)
- "Hospital Universitario La Paz" (Madrid).

173 samples were analysed of which 30 were swabs, 56 serum/plasma samples, 19 cerebrospinal fluid (CSF) samples and 68 biopsies.

Table 3 displays the diagnostic parameters obtained for ***CLART® ENTHERPEX:***

VIRUS	**Positive samples analysed**	**Negative samples analysed**	**Total**	**Sensitivity (%)**	**Specificity (%)**
HSV-1					
Swabs	16	14	30	100	100
Serum/plasma	4	52	56	75	100
CSF	7	12	19	57.1	100
Biopsies	8	60	68	87.5	100
Total	*35*	*138*	*173*		
HSV-2					
Swabs	4	26	30	75	100
Serum/plasma	0	56	56	--	--
CSF	0	19	19	--	--
Biopsies	4	64	68	100	100
Total	*8*	*165*	*173*		
VZV					
Swabs	3	27	30	100	100
Serum/plasma	1	55	56	100	100
CSF	2	17	19	100	94.1
Biopsies	4	64	68	100	95.3
Total	*10*	*163*	*173*		

CMV					
Swabs	2	28	30	50	100
Serum/plasma	47	9	56	87.2	100
CSF	8	11	19	62.5	100
Biopsies	41	27	68	95.1	100
Total	*98*	*75*	*173*		
EBV					
Swabs	6	24	30	100	100
Serum/plasma	2	54	56	100	98.1
CSF	2	17	19	100	94.1
Biopsies	38	30	68	94.7	93.3
Total	*48*	*125*	*173*		
HHV-6					
Serum/plasma	9	47	56	77.8	100
Swabs	0	30	30	--	--
CSF	3	16	19	33.3	100
Biopsies	23	45	68	91.3	100
Total	*35*	*138*	*173*		
HHV-7					
Swabs	3	27	30	100	100
Serum/plasma	2	54	56	100	100
CSF	1	18	19	100	100
Biopsies	11	57	68	100	100
Total	*17*	*156*	*173*		
HHV-8					
Swabs	0	30	30	--	--
CSF	0	19	19	--	--
Serum/plasma	3	53	56	100	100
Biopsies	2	66	68	100	100
Total	*5*	*168*	*173*		
ENTEROVIRUS					
Serum/plasma	0	56	56	--	--
Biopsies	0	68	68	--	--
Swabs	0	30	30	--	--
CSF	10	9	19	80	100
Total	*10*	*163*	*173*		

Table 3. Diagnostic parameters of *CLART® ENTHERPEX*

10. REFERENCES

1. Tsan-Chi Chen, Guang-Wu Chen, Chao Agnes Hsiung, Jyh-Yuan Yang, Shin-Ru Shih, Yiu-Kay Lai, Jyh-Lyh Juang: "Combining Multiplex Reverse Transcription-PCR and a Diagnostic Microarray to Detect and Differentiate Enterovirus 71 and Coxsackievirus A16". Journal of Clinical Microbiology. 44(6), 2212-2219 (2006).

2. Jaaskelainen AJ, Piiparinen H, Lappalainen M, Koskiniemi M, Vaheri A.: "Multiplex-PCR and oligonucleotide microarray for detection of eigh different herpesviruses from clinical specimens". Journal of Clinical Virology, 37(2), 83-90 (2006)

3. Coiras MT, Aguilar JC, García ML, Casas I, Pérez-Breña P.: "Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays". Journal Medical Virology , 72(3), 484-495 (2004).

4. Zheng ZB, Wu YD, Yu XL, Shang SQ.: "DNA microarray technology for simultaneous detection and species identification of seven human herpes viruses". Journal Medical Virology, 80(6), 1042-1050 (2008).

5. Hudnall SD, Chen T, Allison P, Tyring SK, Heath A.: "Herpesvirus prevalence and viral load in healthy blood donors by quantitative real-time polymerase chain reaction. Trasnfusion, Apr 15 (2208).

6. Afenjar A, Rodriguez D, Rozenberg F, Dorison N, Guët A, Mignot C, Doummar D, Billette de Villemeur T, Ponsot G.: "Human herpes virus type 6, etiology of an acute encephalitis in childhood: case report". Arch Pediatric, 14(5), 472-475 (2007).

7. Zambrano Y, Chiarello A, Soca A, Villalobos I, Marreno M, Soler M, Laferte J, Alvarez M." Use of polymerase chain reaction for the diagnosis of central nervous system infections". Invest Clin., 47(4),337-347 (2206).

8. Huang C, Morse D, Slater B, Anand M, Tobin E, Smith P, Dupuis M, Hull R, Ferrera R, Rosen B, Grady L. " Multiple-year experience in the diagnosis of viral central nervous system infections with a panel of polymerase chain reaction assays for detection of 11 viruses.". Clin Infect Dis., 39(5), 630-635 (2004).

9. Casas I, Pozo F, Trallero G, Echevarría JM, Tenorio A. "Viral diagnosis of neurological infection by RT multiplex PCR: a search for entero- and herpesviruses in a prospective study". Journal Medical Virology 57(2), 145-151 (1999).

10. Kanerva M, Jääskeläinen AJ, Suvela M, Piiparinen H, Vaheri A, Pitkäranta A. "Human herpesvirus-6 and -7 DNA in cerebrospinal fluid of facial palsy patients". Acta Otolaryngol. 128(4), 460-464 (2008).

11. Ginanneschi F, Donati D, Moschettini D, Dominici F, Cermelli C, Rossi A. "Encephaloradiculomyelitis associated to HHV-7 and CMV co-infection in immunocompetent host". Clin. Neurol. Neurosurg., 109(3), 272-276 (2007).

12. Calvario A, Bozzi A, Scarasciulli M, Ventola C, Seccia R, Stomati D, Brancasi B.: "Herpes Consensus PCR test: a useful diagnostic approach to the screening of viral diseases of the central nervous system". Journal Clinic Virology, 25(suppl.), S71-S78 (2002).

13. Deback C, Agbalika F, Scieux C, Marcelin AG, Gautheret-Dejean A, Cherot J, Hermet L, Roger O, Agut H.: "Detection of human herpesviruses HHV-6, HHV-7 and HHV-8 in whole blood by real-time PCR using the new CMV, HHV-6, 7, 8 R-genetrade mark kit". Journal Virology Methods, 149(2), 285-291 (2008).

14. Hubacek P, Sedlacek P, Keslova P, Formankova R, Stary J, Kulich M, Cinek O.: "Incidence of HHV7 in donors and recipients of allogeneic hematopoietic stem cell transplantation". Pediatric Blood Cancer. 50(4), 935 (2008).

15. Holden SR, Vas AL.: "Severe encephalitis in a haematopoietic stem cell transplant recipient caused by reactivation of human herpesvirus 6 and 7". Journal Clinic Virology, 40(3), 245-247 (2007).

16. Berger C, Hug M, Gysin C, Molinari L, Frei M, Bossart W, Nadal D.: "Distribution patterns of beta- and gamma-herpesviruses within Waldeyer's ring organs". Journal Med. Virology, 79(8), 1147-1152 (2007).

17. Chen T, Hudnall SD.: "Anatomical mapping of human herpesvirus reservoirs of infection". Mod. Pathol.,19(5), 726-737 (2006).

18. Mendoza LP, Bronzoni RV, Takayanagui OM, Aquino VH, Figueiredo LT:: "Viral infections of the central nervous system in Brazil". Journal Infect., 54(6), 589-596 (2007).

19. Kaneko H, Kawana T, Ishioka K, Ohno S, Aoki K, Suzutani T. "Evaluation of mixed infection cases with both herpes simplex virus types 1 and 2". Journal Med. Virology, 80(5), 883-7 (2008).

20. Ulrich C, Hackethal M, meyer T, Geusau A, Nindl I, Ulrich M, Forschner T, Sterry W, Stockfleth E.: "Skin infections in organ transplant recipients". Journal Dtsch Dermatol Ges., 6(2), 98-105, (2008).

21. Anne de Pagter PJ, Schuurman R, Visscher H, de Vos M, Bierings M, van Loon AM, Uiterwaal CS, van Baarle D, Sanders EA, Boelens J.: "Human herpes virus 6 plasma DNA positivity after hematopoietic stem cell transplantation in children: an important risk factor for clinical outcome". Biol. Blood Marrow Trasplant, 14(7), 831-839 (2008).

22. Kim DH, Messner H, Minden M, Gupta V, Kuruvilla J, Wright J, Lipton J.: "Factors influencing varicella zoster virus infection after allogeneic peripheral blood stem cell transplantation: low-dose acyclovir prophylaxis and pre-transplant diagnosis of lymphoproliferative disorders". Transpl. Infect Dis., 10(2), 90-98 (2008).

23. Dolcetti R.: “B lymphocytes and Epstein-Barr virus: the lesson of post-transplant lymphoproliferative disorders”. Autoimmun Rev., 7(2):96-101 (2007).

24. Sugita S, Shimizu N, Watanabe K, Mizukami M, Morio T, Sugamoto Y, Mochizuki M.: “Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis”. Br. J. Ophthalmol., 11 (2008)

25. Karatas H, Gurer G, Pinar A, Soylemezoglu F, Tezel GG, Hascelik G, Akalan N, Tuncer S, Ciger A, Saygi S.: “Investigation of HSV-1, HSV-2, CMV, HHV-6 and HHV-8 DNA by real-time PCR in surgical resection materials of epilepsy patients with mesial temporal lobe sclerosis”. J. Neurol Sci., 164(1-2); 151-6 (2008).

26. Dierssen U, Rehren F, Henke-Gendo C, Harste G, Heim A.: “Rapid routine detection of enterovirus RNA in cerebrospinal fluid by a one-step real-time RT-PCR assay”. Journal Clin. Virology, 42(1): 58-64 (2008).

27. Read SJ, Mitchell JL, Fink CG.: “LightCycler multiplex PCR for the laboratory diagnosis of common viral infections of the central nervous system”. Journal Clin. Microbiology, 39(9): 3056-3059 (2001).

28. Mette Kusk Bøving, Lisbeth Nørum Pedersen, and Jens Kjølseth Møller
Eight-Plex PCR and Liquid-Array Detection of Bacterial and Viral Pathogens in Cerebrospinal Fluid from Patients with Suspected Meningitis . J. Clin. Microbiol. April 2009 47: 908-913