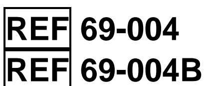
69-004CE_EN_v2_bMx_120413

IVD

HSV1 HSV2 VZV R-gene®



REF 69-004

REF 69-004B

COMPOSITION

REF 69-004	Extraction Kit, DNA EXTRACTION KIT	Ref.: 67-000
	HSV1 HSV2 VZV R-gene® real-time detection and quantification kit	Ref.: 69-004B

1. Presentation of the kit....2
2. Intended use....2
3. Principle of the test....3
4. Content of the kit and storage....4
5. Material and reagents required but not supplied....5
6. Reagents reconstitution....5
7. Warnings and precautions....6
8. Internal quantification standards and controls....7
9. Sample treatment and transport....8
10. Sample extraction protocol....8
11. Detection and Real Time Quantification protocol....10
12. Data Analysis....12
13. Validation and interpretation of results....14
14. Troubleshooting....16
15. Performance of the assays....18
16. References....23
17. Related products....24
18. Index of symbols....24

1. Presentation of the kit

The *Herpesviridae* are a family of DNA viruses which are responsible for a wide spectrum of infections in humans. The primary infection is generally limited to the mucous membranes and the skin. After the primary infection, the virus persists in its host in a latent state and, assisted by chronic or transient immunosuppression, can be reactivated to give recurrent infections.

There are eight human *Herpesviridae*, of which Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and Varicella Zoster Virus (VZV) are the most common in immunocompetent patients. Usually benign, the infections linked with these viruses can nevertheless develop severe clinical forms such as encephalitis, meningitis, retinitis and neonatal infections. These severe and often atypical infections do not allow the implicated virus to be identified.

For many years various antivirals have proved their worth in efficiently treating these pathologies if prescribed early and at appropriate doses. In severe infections, it is therefore essential to obtain an early and rapid diagnosis of the infection. Among the severe forms of HSV-1, HSV-2 or VZV infections in adults, HSV-1-induced encephalitis – of which there are 1/250,000 to 1/1,000,000 cases per year – can still be fatal if untreated. Encephalitis caused by HSV-2 or VZV usually has a more favorable prognosis. Neonatal encephalitis in new-born babies can be traced to both HSV-1 and HSV-2 viruses, but it is HSV-2 virus which is responsible for the most serious neurological disorders. VZV virus had been associated with encephalitis in immunodepressed subjects for a long time; thanks to viral genome detection techniques, it has been revealed increasingly in meningo-encephalitis type infections in immunocompetent subjects.

Although conventional immunological culture and detection techniques are suitable for the benign skin infections for which these viruses are responsible, they are unsuitable for severe infections of the central nervous system (CNS) and congenital infections. The time to obtain a result and the sensitivity of these immunological techniques are inadequate.

The HSV1 HSV2 VZV R-gene® kit enables the quantification of HSV-1, HSV-2 and VZV viruses in real time PCR after viral DNA extraction. User friendly and complete, the HSV1 HSV2 VZV R-gene® kit is suitable for any laboratory.

Several types of specimen and DNA purification systems (automatic and manual) have been validated with the kit. Extracted DNA is then amplified and detected by real time PCR on the common available platforms.

Thanks to a general amplification program within the DNA R-gene® range of products, the sample analysis can be simultaneously run for the other viruses : BKV with BK Virus R-gene® kit (ref.: 69-013B), Adenovirus with Adenovirus R-gene® kit (ref.: 69-010B), CMV with CMV R-gene® kit (ref.: 69-003B), CMV, HHV-6, HHV-7 and HHV-8 with CMV HHV6,7,8 R-gene® kit (ref.: 69-100B), and EBV with EBV R-gene® kit (ref.: 69-002B).

Results are validated with different controls, including an extraction control, all provided in the kit.

2. Intended use

The HSV1 HSV2 VZV R-gene® kit is used to detect and/or measure the viral load of viruses HSV-1, HSV-2 et VZV in cerebrospinal fluid (CSF), gynaecological smears, ENT and ophthalmological samples, skin and mucosal smears, and bronchoalveolar fluid (BAF).
This kit cannot be used for screening donors.

Combined with other methods of biological investigation (medical imaging, biochemical and immunological analysis, etc.), the results obtained with the kit HSV1 HSV2 VZV R gene® enable one to diagnose the first infections or reactivations of these viruses, and consequently to follow the progress of and to improve the therapeutic efficacy.

The quantification of HSV-1, HSV-2, and VZV viruses with HSV1 HSV2 VZV R-gene® kit can be carried out simultaneously with viral load measurement using the EBV R-gene® kit (ref.: 69-002), Adenovirus viral load measurement using the Adenovirus R-gene® (ref. : 69-010) and CMV, HHV-6, HHV-7, HHV-8 viral load measurement using the CMV HHV6,7,8 R-gene® kit (ref. : 69-100) and BKV viral load measurement using the BK Virus R-gene® kit (ref. : 69-013). Indeed, the protocols and amplification techniques are identical for all DNA viruses detected and/or quantified using R-GENE real-time PCR kits.

The validation of a common extraction and a common amplification programme (see Chapter "Performance") for the detection of the HSV-1, HSV-2 and VZV viruses and/or enteroviruses allows all of these pathogens to be analysed simultaneously using one CSF extract.

The amplification premixes of HSV1, HSV2 and VZV included in the HSV1 HSV2 VZV R-gene® kit (ref: 69-004B) are also available separately under reference 71-015 HSV1 r-gene® for amplification of HSV-1, 71-016 HSV2 r-gene® for amplification of HSV-2 and 71-017 VZV HSV2 r-gene® for amplification of VZV.

These products can be used in combination with the HSV1 HSV2 VZV R-gene® kit (ref.: 69-004B) to complete the number of tests to be performed, if necessary.

3. Principle of the test

3.1 Sample type

- The HSV1 HSV2 VZV R-gene® kit is used to detect and measure the viral load of viruses HSV-1, HSV-2 et VZV in cerebrospinal fluid (CSF), gynaecological smears, ENT and ophthalmological samples, skin and mucosal smears, and bronchoalveolar fluid (BAF). The viral load is measured using a quantification sequence common to the three viruses, provided in the kit.
- The quantification range for HSV-1, HSV-2, and VZV is linear with 500 copies/mL to 10^7 copies/mL i.e. 10 copies/PCR to 200 000 copies/PCR. The results are reported in copies/mL of sample. The results are validated by the extraction control, sensitivity control, inhibition control and negative controls provided with the HSV1 HSV2 VZV R-gene® kit.
- HSV1 HSV2 VZV R-gene® kit provides all reagents to analyze 60 samples.

3.2 Viral DNA purification

- The following DNA extraction methods are validated with the HSV1 HSV2 VZV R-gene® kit ref. : 69-004B:
  - MagNA Pure Compact® Instrument
  - MagNA Pure LC System® Instrument
  - NucliSENS® easy MAG®
  - Versant® kPCR Molecular System SP
  - QIAcube
  - QIAamp® DNA Blood Mini kit
  - DNA EXTRACTION KIT
  - QIAamp® MinElute® Virus Spin Kit
  - DNA/RNA EXTRACTION kit

- The target DNA contained in the sample and in the extraction + inhibition control (**IC2**) are extracted using one of the extraction methods above.
- The technique used by the DNA EXTRACTION KIT (Ref.: 67-000) associates the selective binding properties of silica gels with a microcentrifugation speed. The sample and internal control (**IC2**) are first lysed with protease in order to optimize the DNA binding capacities on the membrane. The use of the silica column allows, after DNA coating, efficient washing of the sample to eliminate contaminants. After elution, DNA is suitable for direct use in amplification techniques.

3.3 Real time amplification and quantification

- The principle of the real-time amplification utilizes the 5' nuclease technology TaqMan® (Patents N°5210015, 5487972). Amplification premix is provided ready-to-use with dNTP, amplification buffer, Taq polymerase, probes specific to each virus and primers and probes for an internal control (**IC2**) which goes through the extraction step (before lysis).
- The following range of real time PCR platforms are validated with the HSV1 HSV2 VZV R-gene® kit ref. : 69-004B:
  - LightCycler®
  - Applied Biosystems
  - SmartCycler® 2.0
  - Rotor-Gene®
  - Stratagene®, Versant® kPCR Molecular System AD or Agilent
  - Bio-Rad Dx Real-Time System

- Extracted samples are amplified and quantified at the same time.
  – The amplified fragment for **HSV-1** is located in the US7 gene. Size of amplified fragment : 142 base pairs.
  – The amplified fragment for **HSV-2** is located in the US2 gene. Size of amplified fragment : 177 base pairs.
  – The amplified fragment for **VZV** is located in the gp19 gene. Size of amplified fragment : 114 base pairs.
- A range of 4 quantification standards is provided with the HSV1 HSV2 VZV R-gene® kit (**QS1, QS2, QS3, QS4**). The quantification standards range from 2 000 copies/µL to 2 copies/µL of standard DNA corresponding to 20 000 copies to 20 copies of plasmid per PCR. The quantification standards are used to generate a new standard curve in the software provided with the thermocycler. The quantification of HSV-1, HSV-2 or VZV genome in unknown samples is extrapolated from this standard curve. Each quantification standard contains 3 plasmids specific for each virus.
- **QS3** is a quantification standard that contains 20 copies/µL of standard DNA corresponding to 200 copies of plasmid per PCR. **QS3** can be used to import a previously created standard curve (under the conditions defined in the "standard range and tests", "quantification standard (QS3)" chapter). It is also used as a positive control for qualitative detection of HSV-1, HSV-2 and VZV.
- **SC** is a sensitivity control that contains 3 plasmids specific for each virus. The concentration is 0.5 copy/µL of standard DNA corresponding to 5 copies/PCR. This control (**SC**) enables you to validate the kit's performance over time.
- An extraction and inhibition control (**IC2**) is included in the HSV1 HSV2 VZV R-gene®kit in order to check from the lysis step if the sample has been well extracted and to verify the presence of amplification inhibitors in the sample.

WARNING : **The performances described have been validated and are guaranteed using the technical extraction/amplification unit combinations recommended in the technical information sheet.**

Throughout the patient follow-up, it is imperative to use the same protocol and to use the same extraction and amplification instrument.

4. Content of the kit and storage

Package Insert: instructions are provided in the kit or can be downloaded from www.biomerieux.com/techlib

4.1 DNA EXTRACTION kit 67-000

- Number of extractions per kit: 50

A	QIAamp® mini column	5 x 10
B	Collection tubes (2 mL)	2 x 50
C	AL buffer Xn - HARMFUL	12 mL
D	AW1 buffer (concentrate) Xn - HARMFUL	19 mL
E	AW2 buffer (concentrate)	13 mL
F	AE buffer	12 mL
G	QIAGEN protease Xn - HARMFUL	24 mg
H	Protease solvent	1.2 mL

- The kit can be stored before and after first opening at +2°C/+8°C until expiration date written on the box. Storage at higher temperatures should be avoided. The reconstituted QIAGEN protease can be stored in aliquots at -18°C/-22°C to avoid successive freezing.

4.2 HSV1 HSV2 VZV R-gene ® DETECTION AND QUANTIFICATION KIT 69-004B

- Number of tests : 180 (60/virus)

W0	Water for extraction (molecular grade)	2 x 1.8 mL
IC2	Internal control 2	1 mL
R0	Water for amplification (molecular grade)	0.3 mL
QS1	Quantification standard **1** HSV1+HSV2+VZV	0.3 mL
QS2	Quantification standard **2** HSV1+HSV2+VZV	0.3 mL
QS3	Quantification standard **3** HSV1+HSV2+VZV	0.3 mL
QS4	Quantification standard **4** HSV1+HSV2+VZV	0.3 mL
SC	Sensitivity control HSV1+HSV2+VZV	0.3 mL
R1	HSV-1 and IC2 Amplification premix	2 x 0.450 mL
R2	HSV-2 and IC2 Amplification premix	2 x 0.450 mL
R3	VZV and IC2 Amplification premix	2 x 0.450 mL

- Keep the kit, ref. : 69-004B before and after first opening, frozen at -18°C/-22°C in the dark until the expiration date written on the box.
- The reagents **QS1**, **QS2**, **QS3**, **QS4**, **SC**, **IC2** and **W0** must be stored in the extraction room at -18°C/-22°C before and after first opening. The reagents **R0, R1, R2, R3** must be stored in the room reserved for the preparation of premix at -18°C/-22°C.

5. Material and reagents required but not supplied

5.1 For sample extraction

5.1.1 With DNA EXTRACTION KIT (Ref.: 67-000)

- Ethanol 96-100%.
- Centrifuge (6 000xg. – 12 000xg.)
- Vortex
- Test tubes (1.5 mL, 2 mL).
- Water bath +56°C.
- Sterile micropipets with plugged (aerosol barrier) tips or positive displacement tips.
- Single use latex or similar gloves

5.1.2 Other extraction methods validated

- Follow the manufacturer's instructions.

5.2 For Quantification Kit 69-004B

- Micropipets with plugged (aerosol barrier) tips or positive displacement tips.
- LightCycler®, Applied Biosystems, SmartCycler®, Rotor-Gene® or Stratagene®, Versant® kPCR Molecular System AD or Agilent, Bio-Rad Dx Real-Time System.
- LC Carrousel Centrifuge for LightCycler® or bench-top microcentrifuge convenient for 2mL reaction tubes, or plate centrifuge for Applied Biosystems and Stratagene®, Versant® kPCR Molecular System AD or Agilent.
- Single use latex or similar gloves.
- Capillaries, tubes, microplates for real time PCR platforms validated for HSV1 HSV2 VZV R-gene®.
- Cooling block suitable for the thermocycler of choice.
- U.V Light.
- Workstation or plexiglass screen for samples and premix distribution.
- Colour Compensation r-gene® (ref. : 71-103) for result interpretation on LightCycler® 2.0.
- **DP2** premix of DICO Extra r-gene® (ref.: 71-101) to obtain an extraction + inhibition control on LightCycler® 1.0

6. Reagents reconstitution

ONLY reconstitute reagents supplied with the extraction kit, DNA EXTRACTION KIT ref. : 67-000

6.1 Protease stock solution preparation

- Add 1.2 mL of protease solvent **(H)** to the 24 mg of lyophilized protease **(G)**.
- Store aliquots at -18°C/-22°C (repeated freezing and thawing must be avoided).

6.2 AL buffer (C) preparation

- Store at +2°C/+8°C.
- Mix AL buffer **(C)** thoroughly by shaking before use.
- Do not store protease mixed with AL buffer **(C)**.
- If precipitate is observed in AL buffer **(C)**, warm at +70°C to dissolve it.

6.3 AW1 buffer (D) preparation

- Store at +2°C/+8°C.
- AW1 buffer **(D)** is supplied as a concentrate. Prior to first time use, add a volume of 25 mL of ethanol (96-100%) to the 19 mL of concentrated buffer.

6.4 AW2 buffer (E) preparation

- Store at +2°C/+8°C.
- AW2 buffer **(E)** is supplied as a concentrate. Prior to first time use, add a volume of 30 mL of ethanol (96-100%) to the 13 mL of concentrated buffer.

7. Warnings and precautions

- This kit is intented for in vitro use only. The kit must be handled by a qualified staff member, in accordance with Good Laboratory Practice and handling instructions for molecular biology.
- Read all the instructions before starting the manipulation.

7.1 GENERAL WARNINGS AND PRECAUTIONS:

- Wear protective clothing, i.e: Disposable gloves, lab coat, safety goggles, mask.
- Avoid contact between the reagents and the skin. Wash immediately with copious amounts of water if contact occurs.
- Samples must be prepared under a biological safety hood.
- Never pipet by mouth.
- Do not smoke, eat or drink in dedicated work areas.
- Handle and dispose all specimens and materials as potentially infectious. After use: material, reagents and waste must be handled as potentially infectious.

7.2 WARNINGS AND PRECAUTIONS FOR MOLECULAR BIOLOGY:

- Amplification procedures require highly skilled techniques to avoid risk of sample contamination:
  - Use separate working places for sample preparation and amplification reactions. Movement in the laboratory must be in one direction only from the reagent preparation area to the amplification area. Allocate a set of lab coats and pipettes to each area. Never introduce an amplified product in reagent and/or sample preparation areas.
  - Pipettes used to handle samples are reserved for this purpose only. These pipettes must be positive displacement pipettes or pipettes equipped with filter tips. All tips must be sterile.
  - The pipettes used to aliquot reagents must be reserved only for this purpose. The necessary reagents for amplification are aliquoted in order to be used during one single experiment.
  - Tubes from different specimens and amplification premix must never be opened at the same time.
  - Used samples must be exclusively reserved for this analysis.
- Do not use reagents after expiration date printed on the labels.
- Do not mix reagents from kits originating from other manufacturers.
- The reagents must be fully defrosted to room temperature before testing.
- The use of a metal cold block (+2/+8°C) is recommended for the manipulation of the reagents and the samples.
- Always perform preventive maintenance for workstations, for automated extraction, amplification, and centrifuge systems, according to the manufacturer's recommendations.

For more detailed information, see the product safety data sheet which can be downloaded from www.biomerieux.com/techlib

7.3 Reagent specific warnings and precautions

- AL **(C)** buffer and AW1 buffer **(D)** contain guanidinium chloride (chaotropic salt).
  R22: Harmful if swallowed.
  R36/38: Irritating for eyes and skin.
  S13: Keep away from food, drink and animal feeding-stuffs.
  S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  S36: Wear suitable protective clothing.
  S46: If swallowed, seek medical advice immediately and show this container or label.
  This component must not be used with disinfecting agents that contain bleach.

- AW2 buffer **(E)** and protease solvent **(H)** contain 0.04% sodium azide as preservative.

- Protease (**G**) contains subtilisin
  R37/38: Irritating to respiratory system and skin.
  R41 : Risk of serious damage to eyes.
  R42 : May cause sensitization by inhalation.
  S22 : Do not breathe dust.
  S24 : Avoid contact with skin.
  S26 : In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  S36/37/39 : Wear suitable protective clothing, gloves and eye/face protection.
  S46 : If swallowed, seek medical advice immediately and show this container or label.

- Avoid contact between the reagents and the skin. Wash immediately with copious amounts of water if contact occurs. Wear gloves when handling the reagents.

8. Internal quantification standards and controls

GLOSSARY:

***530 nm** = the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "**530 nm**" is used.*

***560 nm** = the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

***CT** = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.*

WARNING: The order for adding samples and controls must be followed. (see: Chapter "Amplification preparation").

8.1 The internal quantification standards (QS1, QS2, QS3, QS4)

- The use of the internal quantification standard range is imperative for the sample quantification.
- The quantification standards are used to produce a standard curve in the software provided with the thermocycler. The standard curve allows the calculation of HSV-1or HSV-2 or VZV DNA load in clinical samples by means of extrapolation.
- Each quantification standard contains 3 plasmids : one plasmid specific to HSV-1, one plasmid specific to HSV-2 and one plasmid specific to VZV.
- The quantification standards are ranged from 2000 copies/ µL (**QS1**) to 2 copies/µL (**QS4**).
- The quantification standards must be designated as « standard » and their values must be entered when samples are defined in the table in the data analysis software.

8.2 The quantification standard (QS3)

- The quantification standard **QS3** allows the importation of the standard curve created in the first run. Thus, you can produce a new standard curve just by using the quantification standard (**QS3**) each time you perform a new run to measure the viral load of HSV-1 and/or HSV-2 and/or VZV.
- The importation of the standard curve is a quantification method that must only be used from run to run with reagents of the SAME batch. The period between the run defining the standard curve with all four quantification standards and the run using the imported standard curve should not be longer than 3 months.
- Applied Biosystems, Stratagene® and Versant® kPCR Molecular System AD or Agilent, Bio-Rad Dx Real-Time System real time PCR instrument does not allow the importation of the standard curve.

Note: In the event of purely qualitative detection, the QS3 serves as a positive control that verifies that the amplification step has been carried out correctly.

8.3 Sensitivity control (SC)

- The sensitivity control (**SC**) enables you to validate the kit's performance over time
- The sensitivity control (**SC**) contains 3 plasmids specific to each of the three viruses.
- Sensitivity control is amplified with amplification premix (**R1**, or **R2**, or **R3**) according to the virus targeted in the experiment.
- Systematically tested, the sensitivity control (**SC**) is the equivalent of a weak positive sample. For this reason, it may occasionally turn out to be negative.
- Signal reading at 530 nm.

8.4 Extraction + Inhibition controls

8.4.1 The sample extraction + inhibition control (IC2sample)

- This control consists of an internal control (**IC2**) to be added to patient samples, extracted, and amplified in order to control the effectiveness of the extraction and detect the possible presence of inhibitors.
- Signal reading is **560 nm**.

8.4.2 The reference extraction + inhibition control (IC2W0)

- This control consists of the internal control (**IC2**) to be added to the negative extraction control (**W0**), extracted and amplified at the same time as the patient samples to obtain a reference (**IC2W0**). It must be compared with the extraction+inhibition control of patient samples (**IC2sample**).
- Signal reading is **560 nm**.

⇒ The Comparison of CT values of both **IC2W0** and **IC2sample** controls at **560 nm** is used to evaluate the efficacy of extraction and detect the presence of any inhibitors.

8.5 Negative controls

8.5.1 Negative extraction+amplification control (IC2W0)

- This is the same tube as was described in the "Reference extraction+inhibition control" chapter. However, when analysing at 530 nm, this control demonstrates the absence of contamination during the extraction and amplification steps.
- Signal reading is **530 nm**.

8.5.2 Negative amplification control

- This control must be prepared by mixing molecular grade water (**R0**) with the amplification premix (**R1** or **R2**, or **R3**).
- This control shows any possible contamination during the amplification step.
- The use of this control is optional.

⇒ The comparison of the CT value at **530 nm** of the negative amplification control and **IC2W0** helps to identify the experiment step responsible for possible contamination.

9. Sample treatment and transport

Samples must be collected and transported following instructions of the laboratory.

9.1 SAMPLE TRANSPORT

- For samples to be transported, check your local legislation for hazardous and infectious material transport.
- Samples must be transported and treated by the laboratory in the shortest possible time (preferably within 24 hours).

9.2 SAMPLE COLLECTION

9.2.1 CSF samples

- CSF is collected following classical conditions of lumbar puncture.
- CSF sent in dry ice must be stored at -18°C/-22°C or preferentially -78°C/-82°C.

10. Sample extraction protocol

WARNING : **Before starting the extraction procedure, make sure samples, reagents IC2 and W0 have been homogenized**.

In the room reserved for sample extraction

10.1 DNA EXTRACTION KIT (ref. : 67-000 + IC2 + W0)

- Equilibrate samples, **IC2** and **W0** to room temperature +18°C/+25°C.
- Equilibrate AE buffer (**F**) to room temperature +18°C/+25°C.
- Make sure that AW1 buffer (**D**), AW2 buffer (**E**), and reconstituted protease solution have been prepared according the instructions given in section "Reagents reconstitution".
- Redissolve any precipitate in AL buffer (**C**) by heating it at +70°C if necessary, and cool to room temperature before use.
- All centrifugation steps must be carried out at room temperature.

10.1.1 Lysis

- Prepare and identify (on the lid) an equal number of 1.5 mL microcentrifuge tubes to samples being analyzed. Add ONE tube dedicated to the extraction of the mix **W0 + IC2**.
- Heat the water bath.
- Pipet 200 µL of AL buffer (**C**) into each 1.5 mL microcentrifuge tube previously identified.
- Add 20 µL of protease.
- Add 10µL of internal control (**IC2**).
- Add 200 µL of **W0** in the tube identified for the mix **W0 + IC2**.
- Add 200 µL of sample in the tubes identified for sample extraction. PBS may be added to the sample if the sample volume is less than 200µL. In this case the result obtained will be only qualitative.
- Mix by pulse-vortexing for 15 seconds.
- To ensure efficient lysis, it is essential that the sample is mixed thoroughly to yield a homogeneous solution.
- Incubate at +56°C for 10 minutes. Lysis is completed after 10 minutes incubation. Longer incubation time has no effect on the yield or quality of the purified DNA. Potentially infectious agents can be inactivated by incubating the sample at +95°C for 15 minutes after the lysis step. However, extending this incubation time gives rise to a degradation of DNA.
- Briefly centrifuge the 1.5 mL microcentrifuge tube to remove any droplets from the inside of the lid.

10.1.2 Column Loading

- Add 200 µL of 96-100% ethanol to the sample, and mix by pulse-vortexing for 15 seconds.
- Briefly centrifuge
- Prepare and identify the same number of spin columns as the samples to be tested. Carefully apply the above mixture to the spin column (in a 2 mL collection tube) without wetting the rim.
- Close each spin column in order to avoid aerosol formation during centrifugation and centrifuge at 6 000xg for 1 minute. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the spin column is empty.
- Place the spin column into a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.

10.1.3 Washing

- Carefully open the spin column and add 500 µL of AW1 buffer (**D**) without wetting the rim. Close the cap and centrifuge at 6 000xg for 1 minute.
- Place the spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.
- Carefully open the spin column and add 500 µL of AW2 buffer (**E**) without wetting the rim.
- Close the cap and centrifuge at full speed (12 000xg) for 3 minutes.
- Place the columns on a clean 2 mL tube (not provided) and eliminate tubes containing filtrates.
- Centrifuge for 1 min at full speed (12 000xg) prior to elution. This step eliminates any trace of AW2 buffer **(E)**.
- Place each column on a clean, identified 1.5 mL tube (not provided) and eliminate tubes containing filtrates.

10.1.4 Elution

- Carefully open the spin column and add **50 µL** of AE buffer (**F**).
- Close the cap and incubate at room temperature for 5 minutes.
- Centrifuge at 6 000xg for 1 minute.
- The DNA extracted is in the eluate.

Extracted DNA is stable for up to one year when stored at -18°C/-22°C.

10.2 WITH EXTRACTION INSTRUMENTS and/or KITS VALIDATED WITH HSV1 HSV2 VZV R-GENE® AMPLIFICATION KIT

These extraction instruments must be regularly maintained as recommended by the manufacturer by a qualified and trained staff.

INSTRUMENT	Kit	Sample + IC2 Volumes	Sample Type	Protocole	Elution volume
	QIAamp® DNA Blood Mini kit	200 µL of sample + 10 µL IC2	CSF, BAL, ENT and ophthalmologic specimens, gynecological and cutaneous smears		50 µL
	QIAamp® MinElute® Virus Spin Kit **		CSF		50 µL
QIAcube	QIAamp® DNA Blood Mini kit		CSF, BAL, ENT and ophthalmologic specimens, gynecological and cutaneous smears	Blood and body fluid spin protocol V3	50 µL
	QIAamp® MinElute® Virus Spin Kit **		CSF		
MagNA Pure Compact®	MagNA Pure Compact Nucleic Acid Isolation Kit I		CSF	Total_NA_Plasma_100_400	50 µL
MagNA Pure LC System®	MagNA Pure DNA Isolation Kit I		CSF	DNA I Blood Cell High Performance	50 µL
NucliSENS® easyMAG®**	NucliSENS® ** easyMAG® Reagents		CSF	Generic	50 µL
Versant® kPCR Molecular System SP	VERSANT® Sample Preparation 1.0 Reagents	400 µL of sample + 10 µL IC2* **(*extract 250 µL*)**	CSF	Sample Preparation Protocol 5	65 µL (***eluate 50µL***)

* To extract samples of an initial volume higher than 400µL, add the following amounts of IC2 reagent:
For 1 initial sample amount, add 1/40th the amount of IC2 reagent (for example: to extract a 600µL sample, add 15µL of IC2 reagent).

** To use HSV1, HSV2, VZV R-gene® and Enterovirus R-gene® kits simultaneously, add 10 µL IC1+ 10 µL IC2 to the sample.

69-004CE_EN_v2_bMx_120413

11. Detection and Real Time Quantification protocol

Note : To simplify this manual, the device containing the amplification reaction mix will be referred to as a "tube".

WARNING: Throughout the patient follow-up, it is imperative to use the same protocol and to use the same extraction and amplification instrument.

To determine the number of tubes, check:
- if the experiment requires the creation of a standard curve (see section "The quantification standard QS3 ").
- the number of viruses to be detected.

Plan :

1	tube	Per sample and targeted virus.
1 or 4	tube(s)	For the quantification standard range to be created (or imported) of each targeted virus.
1	tube	For QS3 as positive control in the event of qualitative detection
1	tube	For the sensitivity control (**SC**) of each targeted virus.
1	tube	For the reference extraction + inhibition control also used as a negative extraction+ amplification control (IC2W0).

NOTE: *When UNG is used, please refer to the protocols and programs described in the technical document of the product ref.:65-001.*

11.1 PROGRAM

Regardless which real time PCR platform is used the amplification program remains the same. The amplification program is described in the table below.

On Stratagene®/Versant® kPCR Molecular system AD or Agilent, parameter to 20 sec. the denaturation step.

Steps		Time	Temperature	Cycles	Fluorescence acquisition						
					LC1	LC2, LC480	SC2	Applied Biosystems	Rotor-Gene®	Stratagene® / Versant® kPCR Molecular System AD or Agilent	Bio-Rad Dx Real-Time System
Taq Polymerase Activation		15 min.	95°C	1	-	-	-	-	-	-	-
Amplification	Denaturation	10 sec. **20 sec.** for Stratagene	95°C	45	-	-	-	-	-	-	-
	Annealing Elongation	40 sec.	60°C		**530**	**530-560**	**FAM - Cy3**	**FAM-VIC**	**Green-Yellow**	**FAM-HEX**	**FAM-HEX**
					end of the elongation						

NOTE: To simultaneously detect the HSV-1, HSV-2, VZV viruses and/or enteroviruses, use the amplification program described in Enterovirus R-gene® (ref. 69-005) datasheet.

Note 1: Temperature transition rate/slope is pre-defined until 20°C/sec or 100%.

Note 2: On LightCycler®, add a cooling step: 30 sec / 40°C / 1 cycle at the end of the PCR.

Note 3: On LightCycler®, adjust the "seek temperature" parameter to 60°C when programming.

Note 4: On LightCycler® 2.0, the use of a colour compensation file is ESSENTIAL to interpret the results. Make sure that this is still valid (see appropriate technical information sheet) and has been created and recorded in the LightCycler® 2.0 management software, using the r-gene® Colour Compensation reagent (ref.: 71-103).

Note 5: On LightCycler® 480, there are two optical systems: only "System II" is compatible with the HSV1 HSV2 VZV R-gene® kit. "System II" includes automatic colour compensation in its software.

Note 6: On Applied Biosystems select "none" in "passive reference".

Note 7: On Rotor-Gene®, calibrate the signal by clicking on "gain optimisation".

Note 8: On Stratagene® or Versant® kPCR Molecular System AD or Agilent select "none" in "reference Dye".

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

11.2 AMPLIFICATION PREPARATION

Amplification room

Before starting the experiment:
- The reagents must be fully defrosted to room temperature before testing.
- Mix each reagent (to a vortex for 2 seconds or through successive pipetting) and centrifuge briefly.
- Make sure the cooling block was decontaminated by exposure under U.V. light for 30 min.
- Make sure the cooling block was correctly pre-cooled at +2°C/ +8°C.

WARNING: **To avoid contamination as much as possible, close the tubes as soon as distribution is completed. Replace the amplification premix (R1, R2, R3), quantification standards (QS) and sensitivity test (SC) at -18°C/-22°C immediately after use.**

- Collect 15 µL of the amplification premix by gently homogenizing with the pipet in order to distribute the same volume in all tubes.
- Distribute 15 µL volume of amplification premix in all tubes.

WARNING: **The order for adding samples/reagents as shown below must be followed.**

- Add 10 µL of each extracted sample in 1 tube containing the amplification premix corresponding to the targeted virus.
- Add 10 µL of the sensitivity control (**SC**) per tube containing the amplification premix corresponding to the targeted virus (see chapter "Controls").
- Add 10 µL of each standard (from **QS4** to **QS1**), per tube containing the amplification premix corresponding to the targeted virus (see chapter "Controls").
- Add 10 µL of extracted mix **IC2+W0** in 1 tube containing the amplification premix corresponding to the targeted virus for reference extraction + inhibition control and negative extraction + amplification control **IC2W0** (see chapter "Controls").

- Centrifuge the tubes in the relevant device and then transfer them into the thermocycler.

11.3 RUNNING THE HSV1 HSV2 VZV R-GENE® PROGRAM

- Run the amplification program (stored according to the instructions described in section "Amplification program")
- Designate the samples and controls.
- For HSV-1 and/or HSV-2 and/or VZV quantification enter the following values for the quantification standards (copies/mL):

	Quantification Copies/mL
QS1	500 000
QS2	50 000
QS3	5 000
QS4	500

12. Data Analysis

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

*Note : **530 nm**= the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "530 nm" is used.*

***560 nm** = the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

12.1 WITH LIGHTCYCLER® 1.0

- Use the Fit Points method in Arithmetic mode at 2 measuring points.
- Move the threshold line (red), so that it crosses each fluorescence curve in its linear part, above the background noise.

NOTE: If a straight line does not cross all curves in their linear part at the same time, repeat the operation as frequently as necessary to obtain a CP for each sample.

- For each sample, a CP Crossing Point is calculated at **530 nm**.
- To quantify the samples, use the "Second Derivative maximum" method in Arithmetic mode.
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the Calculated column (Copies/mL).

12.2 WITH LIGHTCYCLER® 2.0

- Viral target analysis must be performed in Absolute Quantification mode at **530 nm**.
- The extraction + inhibition test must be analysed in Absolute Quantification mode at **560 nm** after activating (Colour Compensation tab) then select the suitable file previously created with colour compensation file (Colour Compensation r-gene® ref: 71-103).
- Use the Fit Points method.
- Move the threshold line so that it crosses each fluorescence curve in its linear part, above the background noise.

NOTE: If a straight line does not cross all curves in their linear part at the same time, repeat the operation as frequently as necessary to obtain a CP for each sample.

- For each sample, a CP Crossing Point is calculated at **530 nm**.
- To quantify the samples, select Automated F'' max mode (second derivative method).
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the Conc column (Copies/mL).
- The extraction+inhibition controls are analysed by comparing the calculated CP value for each extraction + inhibition control (**IC2sample**) with the CP value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.3 WITH LIGHTCYCLER® 480 (System II)

- Switch on the LC480 (System II) FAM - HEX automatic compensation
- The viral target is analysed in Absolute Quantification mode at **530 nm** (FAM).
- The extraction + inhibition control is analysed in Absolute Quantification mode at **560 nm** (HEX).
- For each positive sample, a Crossing Point (CP) is calculated at **530 nm**.
- The extraction+inhibition controls are analysed by comparing the calculated CP value for each extraction + inhibition control (**IC2sample**) with the CP value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.4 WITH SMARTCYCLER® 2.0

- The viral target is analysed in FAM mode at **530 nm**.
- The extraction + inhibition test is analysed in Cy3 mode at **560 nm**.
- For each positive sample, a Crossing threshold (CT) is calculated at **530 nm** (FAM Ct).
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the FAM Std/Res Green column.
- The extraction+inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) with the CT value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.5 WITH Applied Biosystems

- Make sure that none is selected in the Passive reference field because the HSV1 HSV2 VZV R-gene® premix do not contain any passive reference fluorochrome.
- The samples are analysed in the same way after selecting the FAM R-gene detector in the Detector/target field.
- Adjust the Treshold Line manually to cut each amplification curve in its linear part. This stage is used to determine the positive samples for which a CT is calculated. The negative samples display the word "Undetermined" in the CT column.
- The inhibition controls (**IC2sample** and **IC2W0**) are analysed in the same way after selecting the VIC R-gene in the Detector/target field.
- To quantify the samples, return to linear mode.
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the report drafted and printed at the end of each experiment.
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) with the CT value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.6 WITH ROTOR-GENE®

- The viral target is analysed in Cycling A Green mode at **530 nm**.
- The extraction + inhibition test is analysed in Cycling A Yellow mode at **560 nm**.
- The threshold line must be adjusted in Linear Scale mode after selecting Dynamic tubes and Slope Correct.
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the Calc Conc column (copies/ml) in the Quant. Results Cycling A Green window.
- The extraction+inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) with the CT value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.7 WITH STRATAGENE®, Versant® kPCR Molecular System AD or Agilent

- Make sure that none is selected in the Dye reference field because the HSV1 HSV2 VZV R-gene® kit premix do not contain any passive reference fluorochrome.
- The viral target is analysed by deselecting the Hex button.
- The extraction + inhibition control is analysed by deselecting the Fam button.
- The threshold line must be adjusted in Linear scale mode.
- The concentration calculated for HSV-1 and/or HSV-2 and/or VZV appears in the Quantity column (copies) in the summary table Quant window.
- The extraction+inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) with the CT value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.8 WITH Dx Real-Time System

- Analysis of the viral target: select the QUANTITATION tab leaving the FAM button checked.
- If necessary, manually adjust the threshold line so as to cross each amplification curve at the end of the exponential phase.
  This step aims to identify positive samples for which a CT is calculated. Negative samples are indicated by N/A in the CT column. For each positive sample concentration calculated in column STARTING QUANTITY (SQ) tab QUANTITATION and QUANTITATION DATA.
- The analysis of the extraction + inhibition controls (**IC2sample** and **IC2W0**) are performed in the same way after selecting the HEX detector.

13. Validation and interpretation of results

13.1 TEST VALIDATION

WARNING : **The test is only valid if all following conditions are fulfilled.**
If this is not the case, all samples and controls must be tested again.

Note: *CT = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.*

1st **CONDITION: IC2W0** should not give a detectable signal at **530 nm**.
2nd **CONDITION: IC2W0** must be equal or lower than **32** cycles at **560 nm**.
3d **CONDITION:** - Quantitative interpretation: The CT value of QS3, and the slope or efficacy required for the standard range must be within the values listed in the following table.
- Qualitative Interpretation: CT value of the QS3, which serve as a positive control, must be between **31** and **35** cycles at **530** nm.

QUANTITATIVE DETECTION

Real Time PCR Platform	CT QS3	Valuable Slope/ Efficiency	
		The standard curve is created with all 4 quantification standards for each experiment.	The standard curve is created with all 4 quantification standards for subsequent experiments.
LightCycler® 1.0	31 – 35 cycles	4.339< Slope <-3.103	
LightCycler® 2.0 / LightCycler® 480		1.7 < Efficiency < 2.1	
SmartCycler® 2.0		-0.322<Slope<-0.230	
		-4.339< Slope* <-3.103	
Rotor-Gene®		0.7 < Efficiency < 1.1	
Applied Biosystems		-4,339< Slope <-3,103	*not applicable*
Stratagene®, Agilent, Versant® kPCR Molecular System AD		0.7 <Efficiency< 1.1	
Dx Real-Time System		0.7 <E< 1.1	

*With SmartCycler®, the slope of the regression line CT = f(Log (Concentration)) is obtained by right-clicking on the graph and by selecting "Transpose X and Y axes".

QUALITATIVE DETECTION

CT QS3
31 – 35 cycles

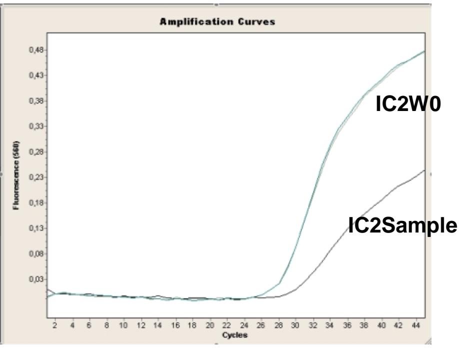
=> **If ALL of these conditions are fulfilled, the results obtained with the sample can be analysed.**

13.2 INTERPRETATION OF RESULTS

- Each sample must be analysed one by one.
- A positive sample displays a CT value.
- If a CT value can NOT be calculated, the sample is considered as negative or inhibited and/or poorly extracted.

Extraction+Inhibition control (IC2sample)	CT [IC2sample]≤ CT [IC2W0] + **3** cycles **NON INHIBITED SAMPLE and correctly extracted**		CT [IC2sample]> CT [IC2W0] + **3** cycles **INHIBITED SAMPLE and/or poorly extracted**	
Sample	Calculated CT	Non calculated CT	Calculated CT	Non calculated CT
HSV-1, HSV-2, VZV **quantitative** Interpretation	Sample validated as positive Validated quantification	Sample validated as negative	Sample validated as positive Perform quantification again	**Not Valid**
HSV-1, HSV-2, VZV **qualitative** Interpretation	Sample validated as positive	Sample validated as negative	Sample validated as positive	**Not Valid**

WARNING: In case of a negative sample:
If the slope of the curve generates a fall of the final fluorescence (≥ 50%) compared to final fluorescence of **IC2W0** (see figure opposite) a weak inhibition is possible. We suggest to extract and to test the sample again.



IMPORTANT NOTES:

It is strongly recommended to use the log_{10} reporting format.

Two quantification results are considered as different if the difference between both values is at least higher than 0.5 log_{10}, taking into account these results were obtained by using the same instruments and the same methods for extraction and amplification.

It is absolutely necessary to compare results obtained with HSV1 HSV2 VZV R-gene® kit to other diagnostic investigation methods in order to define patient viral status

The purchase of this product grants the purchaser rights under certain Roche patents to use it solely for providing human in vitro diagnostic services. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby by bioMérieux.

14. Troubleshooting

14.1 NO SIGNAL OR UNDERESTIMATED QUANTIFICATION IN POSITIVE SAMPLES

POSSIBLE CAUSES	RECOMMENDATIONS
The amplification premix has been defrosted too many times. The amplification premix has remained at room temperature for too long or has been defrosted at too high a temperature.	• Please refer to the "Content of the kit and storage" section. The premixes must not be defrosted more than 7 times. • Check that the amplification premixes, the quantification standards and the sensitivity control have been returned to -18/-22°C immediately after use. • Check that the amplification premixes, the quantification standards and the sensitivity control have been defrosted at room temperature. • Use a cooling block when preparing and distributing the premixes.
Incorrect blood collection , transport and storage in the laboratory.	• See chapter 9 that defines the optimal conditions (temperature, time) for transport and storage.
Transport and storage conditions were not met	• Follow instructions in section 4 regarding the storage of HSV1 HSV2 VZV R-gene® kit 69-004B at -18°C/-22°C and preferably in the dark.
Problem in extraction step	• Check if you carefully homogenized the samples before performing extraction. • Perform all washing steps and respect the incubation time when using extraction DNA EXTRACTION KIT extraction kit 67-000. (See section 10.1). • Check if material and protocol used to extract samples correspond to material and protocol recommended section 10.2. • Always perform preventive maintenance of workstations for automated extraction, and centrifuge systems, according to the manufacturer's recommendations.
Pipetting error	• Check the calibration of your pipets. • Check the distributed volume of reagents and samples. • Carefully homogenize reagents and samples before their distribution in tubes.
Programming error	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards.
Problem in amplification step	• Check the performances of the real time PCR platform as recommended by the manufacturer. • Always perform preventive maintenance of real time PCR platform, and centrifuge, systems according to the manufacturer's recommendations. • Check the attachment of the locking ring of the Rotor-Gene® carrousel.
Error in data analysis	• Check the baseline adjustement. • In the case of an analysis based on the import of scale, check that the imported scale is valid.
Error in interpretation results	• Check the validity of the results obtained in the experiments (check all the validation conditions as described in section 13). • With LightCycler® 2.0: Make sure the results obtained were corrected using a valid colour compensation file created in advance using kit ref.: 71-103 • With Applied Biosystems: check if None is selected in passive reference field. • Always compare the result of the inhibition control of the sample with the initial inhibition control (see section 13.2). Dilute the sample if necessary. • Compare the result of the extraction+inhibition control (**IC2sample**) of the suspected sample with the result of the reference extraction + inhibition control (**IC2W0**) (see chapter 13.2). Dilute the extracted sample if necessary.

14.2 FLUORESCENT SIGNAL ON NEGATIVE SAMPLES OR OVERESTIMATED QUANTIFICATION OF CLINICAL SAMPLE.

POSSIBLE CAUSES	RECOMMENDATIONS
Contamination during experiment	• Follow all recommendations in section 7. • Decontaminate the cooling block for capillaries with U.V. light. • Repect the manufacturer's recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • The HSV1 HSV2 VZV R-gene® kit must be handled only by a trained staff. • Use the **R0** reagent provided in the kit, in parallel with the samples extracted, to identify the contaminated stage.
Pipetting error	• Check the calibration of your pipettes. • Check the distributed volumes of reagents and samples. • Carefully homogenize reagents and samples before their distribution in tubes.
Programming error	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards.
Error in data analysis	• Check the baseline adjustement. • In the case of an analysis based on the import of scale, check that the imported scale is valid.
Error in results interpretation	• Check the validity of the results obtained in the experiments (check all the validation conditions described in section 13). • With LightCycler® 2.0: Make sure the results obtained were corrected using a valid colour compensation file created in advance using kit ref.: 71-103 • With Applied Biosystems: check if None is selected in passive reference field. • Compare the result of the extraction+inhibition control (**IC2sample**) of the suspected sample with the result of the reference extraction + inhibition control (**IC2W0**) (see chapter 13.2). Dilute the extracted sample if necessary.

14.3 THE SAMPLES ALL SEEM INHIBITED.

POSSIBLES CAUSES	RECOMMENDATIONS
Inadequate extraction stage.	• Check that the samples have been properly homogenised before being extracted. • In the case of manual extraction using the DNA EXTRACTION KIT R-gene®. ref. : 67-000, perform the number of washes and incubation time stated in the "DNA Extraction Kit" section. • Check the materials and protocols used for extracting samples. • Kit performances are only validated for the extractions described in the "Sample extraction protocol" section. • Carefully monitor to ensure that the extraction devices are maintained in line with the manufacturer's recommendations.
The IC2W0 does not result from the same extraction run	• Make sure that every sample tested includes the same batch of IC2 as IC2W0. • Each extraction run should have its own IC2W0.

15. Performance of the assays

WARNING : **The performances described have been validated and are guaranteed using the technical extraction/amplification unit combinations recommended in the technical information sheet.**

15.1 INTRA-ASSAY AND INTER-ASSAY REPRODUCIBILITY OF HSV1 HSV2 VZV R-GENE® KIT

15.1.1 Intra-assay reproducibility

The intra-assay reproducibility of HSV1 HSV2 VZV R-gene® assay has been tested in a study performed on different samples from HSV-1 or HSV-2 or VZV positive cell cultures. The experiment was repeated 8 times on the LightCycler® 2.0 after manual extraction (Qiagen DNA Blood Mini Kit or DNA EXTRACTION Kit ref.67-000).

The table shows the average CT obtained for each sample.

The coefficient of variation varied from 0.35 % to 1.15% for HSV-1, from 0.26% to 0.78% for HSV-2, from 0.33% to 0.70% for VZV.

These coefficients of variations show a good intra-assay reproducibility of the kit.

		Average CT	Standard deviation	Coefficient of variation
HSV-1 concentration in the sample	1 500 cp/mL	35.1	0.405	1.15%
	15 000 cp/mL	31.18	0.189	0.60%
	150 000 cp/mL	27.64	0.098	0.35%
HSV-2 concentration in the sample	10 000 cp/mL	32.4	0.121	0.37%
	100 000 cp/mL	29.03	0.076	0.26%
	1 000 000 cp/mL	25.52	0.2	0.78%
VZV concentration in the sample	150 000 cp/mL	32.9	0.206	0.63%
	3 000 000 cp/mL	26.36	0.186	0.70%
	50 000 000 cp/mL	22.54	0.075	0.33%

The same experiment was performed using the MagNaPure Compact automatic extraction on HSV-1.

The table shows the average CT obtained for each sample.

The coefficient of variation varied from 0.71 % to 1.63%.

These coefficients of variations show a good intra-assay reproducibility of the kit .

		Average CT	Standard deviation	Coefficient of variation
HSV-1 concentration in the sample	1 500 cp/mL	34.92	0.569	2.00%
	15 000 cp/mL	31.84	0.294	0.94%
	150 000 cp/mL	27.82	0.197	0.71%

15.1.2 Inter-assay reproducibility

The inter-assay reproducibility of HSV1 HSV2 VZV R-gene® assay is tested thanks to a study performed on different samples from HSV-1 or HSV-2 positive cell cultures. The experiment is repeated 7 times on LightCycler® 2.0 after manual extraction (Qiagen DNA Blood Mini Kit or DNA EXTRACTION Kit ref.67-000).

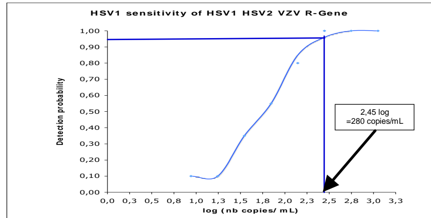
The coefficient of variation varies from 1.49 % to 2% for HSV-1 and from 3.5% to 3.9% for HSV-2.

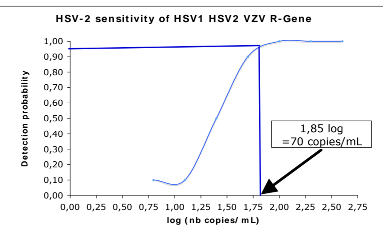
These coefficients of variations show a good inter-assay reproducibility of the kit .

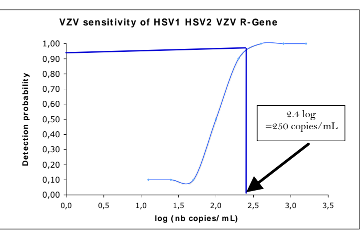
		Average CT	Standard deviation	Coefficient of variation
HSV-1 concentration in the sample	1 500 cp/mL	34.48	0.69	2.00%
	15 000 cp/mL	30.55	0.45	1.49%
	150 000 cp/mL	27.31	0.42	1.52%
HSV-2 concentration in the sample	10 000 cp/mL	31.7	1.11	3.50%
	100 000 cp/mL	28.2	1.1	3.90%

15.2 ANALYTICAL SENSITIVITY OF HSV1 HSV2 VZV R-GENE® KIT

Analytical sensitivity of the HSV1 HSV2 VZV R-gene® kit, has been evaluated on dilution ranges of extracted samples from HSV-1 and VZV QCMD panels and from HSV-2 (strain : ATCC,VR-734) positive cell cultures. Samples contained 10 to 1200 copies/ml for HSV-1 ; 10 to 400 copies/ml for HSV-2, and 20 to 1 600 copies/ml for VZV. The experiment was repeated 20 times.







The curves above indicate a 95% probability to detect a sample containing 280 HSV-1 copies/ml (11 copies/PCR), 70 HSV-2 copies/ml (3 copies/PCR) and 250 VZV copies/ml (10 copies/PCR).

Detection limit for the three viruses is 50 copies/mL.

15.3 ANALYTICAL SPECIFICITY OF HSV1 HSV2 VZV R-GENE® KIT

The recognition specificity of the primers and probes selected for the HSV1 HSV2 VZV R-gene® kit was determined after the sequence analysis (of viruses, bacteria and human pathogens) found in the banks. The specificity of the HSV1 HSV2 VZV R-gene® primers and probes were also checked by real time PCR on cell cultures of the following viruses:

- Human Herpesvirus: infected cells with HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8 ;
- Polyomavirus: plasmids containing the total genome of JCV and BKV;
- Adenovirus 5, 8;
- Enterovirus, poliovirus S1, Echovirus 9.

No cross-reactions were observed with HSV-1 ; HSV-2 and VZV viruses and no viral amplification or hybridization was observed with other pathogens.

15.4 STUDY ON 20 SAMPLES FROM QCMD PANELS 2012

During the European HSV and VZV test campaigns proposed by the QCMD in 2012, 10 samples for the HSV panel (both types 1 and 2) and 10 for the VZV panel, were tested blind with the HSV1 HSV2 VZV R-gene® kit. 10µl of each sample extracted using the NucliSENS® easyMAG® extractor was amplifed on the Dx Real Time (Bio-Rad) with the specific amplification premixes corresponding to the targets tested by each panel.

⇒ 90% (18/20) of the samples tested in all the panels agreed with the expected results.

HSV QCMD Panel

QCMD Panel Composition and Expected Results							HSV1 and HSV2 detection with HSV1 HSV2 VZV R-gene® 69-004B	
Panel Code	Sample Content	Sample Type	Sample Statut	Sample Matrix	Sample Concentration		NucliSENS® easyMAG® – Dx Real Time System	
					Copies/mL	Log10 copies/mL	Log Copies/mL	Delta Log copies/mL
HSVDNA 12-01	HSV negative	Core	Negative	VTM	Negative	Negative	Negative	Na
HSVDNA 12-02	HSV-1 (Macintyre)	Core	Frequently detected	VTM	9,441	3.98	4.22	0.25
HSVDNA 12-03	HSV-2 (MS)		detected	VTM	148	2.17	Negative	Na
HSVDNA 12-04	HSV-1 (Macintyre)	Core	Frequently detected	VTM	6,577	3.82	4.25	0.43
HSVDNA 12-05	HSV-2 (09-015681)		Infrequently detected	VTM	50	1.70	Negative	Na
HSVDNA 12-06	HSV-1 (95/1906)		detected	VTM	289	2.46	2.78	0.32
HSVDNA 12-07	HSV-1 (Macintyre)		Infrequently detected	VTM	143	2.16	2.10	0.06
HSVDNA 12-08	HSV-2 (09-015681)	Core	Frequently detected	VTM	5,000	3.70	3.24	0.46
HSVDNA 12-09	VZV	Core	Negative	VTM	Negative	Negative	Negative	na
HSVDNA 12-10	HSV-2 (MS)	Core	Frequently detected	VTM	3,972	3.60	2.80	0.80

In the HSV panel, 8 out of 10 samples were HSV positive: 4 samples were HSV-1 positive and 4 were HSV-2 positive.
The two positive “Core” HSV-1 samples were detected using the HSV1 HSV2 VZV R-gene® kit.
The two positive “Core” HSV-2 samples were detected using the HSV1 HSV2 VZV R-gene® kit.
The two positive “No Core” HSV-1 samples containing viral loads of 143 and 289 copies/mL were detected using the HSV1 HSV2 VZV R-gene® kit.
The two positive “No Core” HSV-2 samples containing viral loads of 50 and 148 copies/mL were not detected using the HSV1 HSV2 VZV R-gene® kit.
The two negative "Core" samples, including a positive sample in VZV identified with the premix VZV (R3), are not detected with the HSV1 (R1) and HSV2 (R2) premixes of the kit HSV1 HSV2 VZV R-gene®.
The absence of a cross-reaction between VZV and HSV1 or HSV2 shows the specificity of the HSV probes and initiators selected for the kit.

VZV QCMD Panel

QCMD Panel Composition and Expected Results							VZV detection with HSV1 HSV2 VZV R-gene® 69-004B	
Panel Code	Sample Content	Sample Type	Sample Statut	Sample Matrix	Sample Concentration		NucliSENS® easyMAG® – Dx Real Time System	
					Copies/mL	Log10 copies/mL	Log Copies/mL	Delta Log copies/mL
VZVDNA12 -01	VZV negative	Core	Negative	VTM	Negative	Negative	Negative	Na
VZVDNA12 -02	VZV (63/1444)	Core	Frequently detected	VTM	2,014	3.30	3.71	0.41
VZVDNA12 -03	VZV (OKA)	Core	Frequently detected	VTM	16,672	4.22	4.93	0.70
VZVDNA12 -04	VZV (Ellen)		Detected	VTM	282	2.45	3.21	0.76
VZVDNA12 -05	VZV (9/84)	Core	Frequently detected	VTM	542	2.73	3.18	0.45
VZVDNA12 -06	VZV (Ellen)	Core	Frequently detected	VTM	1,828	3.26	3.79	0.53
VZVDNA12 -07	VZV (OKA)	Core	Detected	VTM	923	2.97	3.56	0.59
VZVDNA12 -08	VZV (9/84)		Infrequently detected	VTM	98	1.99	2.08	0.09
VZVDNA12 -09	VZV (63/1444)		Detected	VTM	295	2.47	2.98	0.51
VZVDNA 12-10	HSV Type 1	Core	Negative	VTM	Negative	Negative	Negative	na

In the VZV panel, 8 out of 10 samples were VZV positive. These samples were all detected using the HSV1 HSV2 VZV R-gene® kit.
The five positive "Core" VZV samples were detected using the HSV1 HSV2 VZV R-gene® kit.
The two negative "Core" VZV samples, including one HSV1 positive sample, were not detected using the VZV (R3) premix in the HSV1 HSV2 VZV R-gene® kit.
The two positive "No Core" VZV samples containing viral loads of 98 and 295 copies/mL were detected using the HSV1 HSV2 VZV R-gene® kit..
These results demonstrate the sensitivity and specificity of the VZV premix (R3) in the HSV1 HSV2 VZV R-gene® kit for detecting the VZV parameter.

15.5 CLINICAL STUDY ON HSV1 HSV2 VZV R-gene® KIT

1- Retrospective clinical study on 131 samples collected at the department of Virology in St Vincent de Paul Hospital (Paris - France) .

131 samples (75 CSF and 56 genital specimens) were analysed with HSV1 HSV2 VZV R-gene® kit.

All samples have been previously characterized in this laboratory with a routine PCR assay for CSF samples (PCR consensus, Rozenberg et al., JCM Nov 1991) as well as a qualitative real time PCR assay for genital specimens.

The test with HSV1 HSV2 VZV R-gene® kit was performed on ABI Prism© 7500 platform after a manual extraction (DNA Extraction Kit ref: 67-000). Qualitative results are displayed on the following table.

Analysis of results in accordance with the virus:

- 24/24 HSV-1 samples were identified with HSV1 HSV2 VZV R-gene®.
- 33/35 HSV-2 samples were identified with HSV1 HSV2 VZV R-gene®.
- 20/26 VZV samples were identified with HSV1 HSV2 VZV R-gene®.

		Reference	
		+	**-**
Argene	**+**	**75**	**2**
	-	**9**	**45**

Analysis of discordant results:

Out of 11 discordant results, 7 results corresponded to samples that were identified as weak positive (>40 cycles) with the routine PCR. Moreover, positive samples were stored for several weeks and then thawed once again to be tested with HSV1 HSV2 VZV R-gene® kit. This condition of storage may explain the different result. The lack of material (specimen) did not allow an additional testing with both techniques.

Conclusion:

The first qualitative analysis shows an agreement of 91.6 % between both real time PCR techniques.

2- Retrospective clinical study on 105 samples at the department of Virology in the University Hospital of Strasbourg (France).

105 samples (25 CSF, 8 E.N.T and ophtalmologic samples, 22 BAL, 23 gynaecological samples and 27 cutaneous or mucous smears) were analysed with HSV1 HSV2 VZV R-gene® kit.

All samples have been previously characterized in this laboratory with a routine PCR assay (end point PCR Herpes Consensus and Herpes Identification Argene-France)

The test with HSV1 HSV2 VZV R-gene® kit was simultaneously performed on LightCycler® 2.0 and RotorGene® platforms after a manual extraction (DNA EXTRACTION Kit ref. 67-000).

The discordant results were simultaneously tested with both techniques (provided that remaining volume was sufficient).

Qualitative results displayed on the following table correspond to results obtained with both real time PCR platforms (LightCycler® 2.0 and RotorGene®).

		Reference	
		+	**-**
Argene	**+**	**64**	**1**
	-	**4**	**36**

The first qualitative analysis shows an agreement of 95.2 % between both real time PCR techniques.

Result analysis in accordance with the specimen:

Specimen	Expected positive result	Expected negative result
Gynaecological smears	18 /20*	3 / 3
Cutaneous and mucous smears	26 / 26	1 / 1
ENT (ear nose throat) and ophtalmological samples	6 / 6	2 / 2
CSF (cerebrospinal fluid)	3 / 3	21 /22**
BAL (bonchoalveolar fluid) and other fluids	11 /13***	9 / 9

Analysis of discordant results:

* The two gynaecological samples correspond to weak positive samples in cell culture. These samples were stored over 3 months at -20°C before being tested with HSV1 HSV2 VZV r-gene® kit.

** An additional CSF sample was detected as positive with HSV1 HSV2 VZV R-gene® kit.

*** The two discordant samples correspond to :
- one weak positive sample subsequently gave a negative result with the second analysis (irrespective of the used platform).
- one sample was finally confirmed in the second analysis with both techniques.

Result analysis in accordance with the virus:

37/37 HSV-1 samples were identified with HSV1 HSV2 VZV R-gene®.
12/12 HSV-2 samples were identified with HSV1 HSV2 VZV R-gene®.
0/4 Herpes simplex sample (no typing) was identified with HSV1 HSV2 VZV R-gene®.
16/16 VZV samples were identified with HSV1 HSV2 VZV R-gene®

Conclusion:

After an evaluation of the discordant results, a second qualitative analysis shows an agreement of 97 % between both real time PCR techniques. Two discordant results remain unsolved because of the lack of material required for a supplementary test.

The HSV1 HSV2 VZV R-gene® technique enables the viral quantification of HSV-1, HSV-2 and VZV. This feature can not be provided by the routine PCR assay of the laboratory.

3- Retrospective study of CSF specimens for the validation of a single Herpes/Enterovirus programme

This study was carried out on 29 specimens at the Clermont Ferrand University Hospital Virology Laboratory in France

The study was carried out in order to validate the Enterovirus R-gene® amplification program to simultaneously detect the presence of the HSV-1, HSV-2 and VZV viruses and/or enteroviruses using a single cerebrospinal fluid (CSF) specimen in patients admitted to hospital with suspected encephalitis or meningitis.

29 specimens were characterised using the Herpes Consensus Generic kit (ref. 67-090).

- 1 CSF specimen tested positive for HSV-1
- 2 dilutions of an HSV-1 strain in a genital specimen titrated at 105.6 TCID50/ml.
- 1 gynaecological specimen tested positive for HSV-1.
- 2 CSF specimens tested positive for HSV-2
- 2 dilutions of an HSV-2 strain in a skin vesicle titrated at 104.8TCID50/ml.
- 1 gynaecological specimen tested positive for HDV-2.
- 8 CSF specimens tested positive for VZV.
- 2 dilutions of a VZV strain in a skin vesicle titrated at 104.9TCID50/ml
- 10 CSF specimens tested negative for HSV-1, HSV-2 and VZV

Each specimen was extracted with internal RNA (IC1) and DNA (IC2) controls in place using the manual DNA/RNA extraction kit or the QIAamp® MinElute® Virus Spin Kit.

Each specimen was then amplified using the Rotor-Gene® device, using either the Enterovirus R-gene® amplification program or the HSV1, HSV2, VZV R-gene® amplification program.

Qualitative analysis:

Of the 29 specimens tested:

- 100% (19/19) of the specimens characterised as positive were found to be positive and the results were in line with the 2 amplification programs.
- 100% (9/9) of the specimens characterised as negative were found to be negative and the results were in line with the 2 amplification programs (1 of the 10 negative specimens was shown to be inhibited with the Enterovirus R-gene® PCR).

		Enterovirus R-gene® amplification program	
		+	-
HSV1, HV2, VZV R-gene® amplification program	+	19	0
	-	0	9

Quantitative analysis:

Quantitative analysis was carried out on the 19 positive specimens.

	Matrix	Concentration	Amplification program		Delta log[log10]
			HSV1 HSV2 VZV r-gene™	Enterovirus R-gene™	
			Results Copies/mL [log10]	Results Copies/mL [log10]	
HSV1	pool of CSF	3.9TCID50/ml	3,38	3,32	0,06
HSV1	pool of CSF	0.39TCID50/ml	2,49	2,25	0,24
HSV1	CSF		2,9	2,9	0
HSV1	gynecologic sampl		7,2	7,3	-0,1
HSV2	pool de LCR	63TCID50/ml	2,86	2,75	0,11
HSV2	pool de LCR	6.3TCID50/ml	1,63	1,8	-0,17
HSV2	CSF		3,5	3,6	-0,1
HSV2	CSF		1	0,6	0,4
HSV2	gynecologic sampl		5,71	5,67	0,04
VZV	pool of CSF	7.9TCID50/ml	4,4	4,38	0,02
VZV	pool of CSF	0.79TCID50/ml	3,15	3,67	-0,52
VZV	CSF		5,8	5,7	0,1
VZV	CSF		4,1	4	0,1
VZV	CSF		7	6,8	0,2
VZV	CSF		6,5	6,3	0,2
VZV	CSF		6,9	6,7	0,2
VZV	CSF		4	4,2	-0,2
VZV	CSF		6,5	6,3	0,2
VZV	CSF		4,4	4,2	0,2

Conclusion:

The quantitative analysis of the results obtained through the Enterovirus R-gene® amplification program and the HSV1, HSV2, VZV R-gene® amplification program shows excellent correlation: the delta logs between the two techniques are between 0 and 0,52 log.

Consequently, the use of the Enterovirus R-gene® amplification program is validated for the detection and quantification of the HSV-1, HSV-2 and VZV viruses.

16. References

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

1) Anami Patel, Swati Patel, Barbara Mason, Noel Lenny and Aaron Pacheco.
Evaluation of Argene's Research Use Only, Real-Time PCR Reagents for HSV1 and HSV2 R-GENE. CVS, 2008.

2) Vignoles M., Bes J., Magro S., Raoux N., Barranger C., Joannes. M.
Development of a new diagnostic tool for the quantitative detection of HSV-1, HSV-2 and VZV DNA. CVS, 2008.

3) Schvoerer E., Fafi-Kremer S, Obringer C., Schramm F., Vignoles M., Barranger C., Joannes M., Gut J.P., Stoll- Keller F.
Evaluation of a real-time PCR for HSV-VZV on various samples in the conditions of a clinical virology laboratory. ESCV Finland, March 2008.

4) Vignoles M., Bes J., Magro S., Raoux N., Barranger C., Joannes. M.
Development of a new diagnostic tool for the quantitative detection of HSV-1, HSV-2 and VZV DNA. ESCV, Nüremberg, Germany September 2007.

5) G. Billaud, E. Frobert, H. Janin, V. Escuret, P.L. Cornut, B. Lina.
Rétinite nécrosante à virus varicelle zona : apport du monitoring de la charge virale dans les ponctions de chambre antérieure (humeur oculaire). RICAI, 2010.

6) Germi R, Beyls N, Quetant S, Aribert S, P Bourgeois, Seigneurin JM, Morand P.
Mesure de la charge virale des herpesvirus HSV1, CMV, HHV6 et EBV dans 86 liquides broncho-alvéolaires (LBA) prélevés chez des patients transplantés pulmonaires. RICAI, 2010.

17. Related products

- DICO Extra r-gene® ref. : 71-101
- Colour Compensation r-gene® ref. : 71-103
- CELL Control r-gene® ref. : 71-106
- HSV1 r-gene® ref. : 71-015
- HSV2 r-gene® ref. : 71-016
- VZV r-gene® ref. : 71-017

18. Index of symbols

Symbol	Meaning
REF	Catalogue number
IVD	In Vitro Diagnostic Medical Device
	Manufacturer
	Temperature limitation
	Use by
LOT	Batch code
	Consult Instructions for Use
Σ	Contains sufficient for <n> tests
	Protect from light
	Keep dry
CE	Identification of notified body

BIOMERIEUX, the blue logo, ARGENE, R-gene, easyMAG and NucliSENS are used, pending and/or registered trademarks belonging to bioMérieux, or one of its subsidiaries, or one of its companies.
Any other name or trademark is the property of its respective owner.

bioMérieux SA
Chemin de l'Orme
69280 Marcy-l'Etoile - France

RCS LYON 673 620 399
Tel. 33 (0)4 78 87 20 00
Fax 33 (0)4 78 87 20 90
www.biomerieux.com