IVD

EBV R-gene®

REF 69-002
REF 69-002B

NEW EXTRACTION PROTOCOL NucliSENS easyMAG:

« Whole Blood Viral Extraction Protocol »

COMPOSITION

REF 69-002	**DNA EXTRACTION KIT**	**Ref.: 67-000**
	Quantification kit, EBV R-gene®	**Ref.: 69-002B**

1. Presentation of the kit

Epstein-Barr virus (EBV) is an ubiquitous herpesvirus which infects the major part of human population (seroprevalence is greater than 95%). After a prime infection (transmission by saliva), EBV remains latent in B cells. Periodically, EBV can reactivate in healthy individuals. This reactivation occurs with a replication of EBV in the oropharynx and doesn't have any clinical signs.

EBV is a causative agent of infectious mononucleosis. EBV is also involved in several pathogenesis of cancers such as Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. In immunosuppressed individuals, EBV may lead to lymphoproliferation of B cells. Indeed, the main characteristic of EBV is to make the B lymphocytes multiply and to subsequently produce a maligne lymphoma.
In this context the measure of the EBV viral load is very significant.

The EBV R-gene® kit enables the quantification of EBV genome. After EBV DNA isolation, the quantification is performed using the EBV R-gene® real time PCR assay. User friendly and complete, the EBV R-gene® kit is suitable for any laboratory.

Many types of specimen and numerous 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 with the entire range of R-gene® products, the sample analysis can be simultaneously analyzed with the following other targets : HSV1, HSV2, VZV with HSV1 HSV2 VZV R-gene® kit (Ref: 69-004B), 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), Adenovirus with Adenovirus R-gene® (ref : 69-010B) and BKV with BK Virus R-gene® (ref : 69-013B).

Results are validated with various controls, including an extraction control, which are all provided with the kit.

2. Intended use

EBV R-gene® kit measures the viral load in whole blood and various other samples. The viral load represents the number of virions or the number of EBV-infected B lymphocytes in the replication phase. The viral load can be measured using a quantification range supplied with the kit. Viral DNA must be extracted prior to the Real Time PCR amplification.

<u>This kit cannot be used for screening donors.</u>

It is absolutely necessary to compare results obtained with EBV R-gene® kit with other diagnostic investigation methods (medical examination, biochemical and immunological analysis, etc.) in order to define patient viral status. Besides this indication, the EBV R-gene® kit is also useful to confirm an EBV primary infection, to precise the diagnostic of the Hodgkin's lymphoma or nasopharyngeal carcinoma.

The quantification of EBV with EBV R-gene® kit can be carried out simultaneously with HSV-1, HSV-2, VZV viral load measurement using the HSV1 HSV2 VZV R-gene® kit (ref.: 69-004), CMV, HHV-6, HHV-7, HHV-8 viral load measurement using the CMV HHV6,7,8 R-gene® kit (ref. : 69-100), Adenovirus viral load measurement using the Adenovirus R-gene® (ref. : 69-010) and BKV viral load measurement using the BK Virus R-gene® (ref : 69-013B). All R-gene® Real Time PCR kits follow the same amplification procedure and thus can be simultaniously used in the same experiment.

3. Principle of the test

3.1 Sample type

- EBV R-gene® measures the viral load of EBV in whole blood specimens, cerebrospinal fluid (CSF), plasma, bronchoalveolar liquid (BAL) and biopsies using the extraction systems shown in section 3.2.
  The quantification range for EBV is linear between 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 via the extraction test, inhibition test and negative controls supplied in the EBV R-gene® kit.
- DNA purification
- The following DNA extraction methods are validated with the EBV R-gene® kit ref.: 69-002B:
  - MagNA Pure Compact® Instrument,
  - MagNA Pure LC System® Instrument
  - NucliSENS®\ easy MAG®
  - QIAsymphony SP
  - QIAamp® DNA Blood Mini kit
  - DNA EXTRACTION KIT (supplied under reference 67-000 of ref. 69-002).
  - QIAcube
  - *m*2000sp®
  - Versant® kPCR Molecular System SP
- The target DNA present in the sample and in the extraction + inhibition control (**IC2**) is 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.

3.2 Real time amplification and quantification

- Amplification is performed using the 5' nuclease TaqMan® technology (patent n°: 5210015, 5487972) also called hydrolysis probes. The ready-to-use amplification mixture includes: primers, dNTPs, amplification buffer, Taq Polymerase, specific EBV primers and probe as well as the primers and probe specific for the internal control (**IC2**) which also must be subjected to the entire extraction procedure (including lysis).
- The following range of Real Time PCR platforms are validated with the EBV R-gene® kit ref.: 69-002B:
  - LightCycler®.
  - Applied Biosystems.
  - SmartCycler®2.0.
  - Rotor-Gene®.
  - Stratagene®, Versant® kPCR Molecular System AD or Agilent.
  - Dx Real-Time System (Bio-Rad).
- Extracted samples are amplified and quantified at the same time.
- The amplified gene is the BXLF1 gene coding for thymidine kinase. Size of amplified fragment : 169 base pairs.
- A range of 4 quantification standards is provided with the EBV R-gene® kit (**QS1, QS2, QS3, QS4**). The quantification standards are ranged from 10 000 copies/µL to 10 copies/µL corresponding to 100 000 copies to 100 copies per PCR. The quantification standards are used to generate a new standard curve in the software provided with the thermocycler. The quantification of EBV genome in unknown samples is extrapolated from this standard curve (the necessary calculation software is provided with the PCR instrument).
- **QS3** is a quantification standard that contains 1 000 copies of standard DNA per PCR. This standard can be used to import a previously created standard curve (provided that conditions described in chapter **8.2** are met). It's also used as a positive control for qualitative detection of EBV.
- **SC** is a sensitivity control that contains 1 copy plasmid DNA per µL, corresponding to 10 copies/PCR. This control (**SC**) validates the performance of the assay and should be considered as a run-control.
- An extraction and inhibition control (**IC2**) is included in the EBV R-gene® kit in order to check, starting 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. To monitor evolution of a patient's viral load test after test, it is vital for the successive sample analyses to take place using strictly the same protocol and with the same combinations of extraction/amplification units.**

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 Kit for purification of DNA, "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 temperature should be avoided. The reconstituted QIAGEN protease can be stored by aliquots at -18°C/-22°C to avoid successive freezing.

4.2 Quantification Kit EBV R-gene® 69-002B

- Number of tests : 90

W0	Water for extraction (molecular grade)	2 x 1,8 mL
IC2	Internal control 2	1 mL
R0	Water (molecular grade)	150µL
QS1	Quantification standard **1**	150µL
QS2	Quantification standard **2**	150µL
QS3	Quantification standard **3**	150µL
QS4	Quantification standard **4**	150µL
SC	Sensitivity control	150µL
R4	EBV and IC2 Amplification premix	3x0.450 mL

- Keep the kit (ref. 69-002B) in the dark before and after first opening frozen at -18°C/-22°C until the expiration date printed on the box.
- Before and after opening of the kit (ref. : 69-002B), Internal control 2 (**IC2**), reagent (**W0**), quantification standards (**QS1**, **QS2**, **QS3**, **QS4**) and sensitivity control (**SC**) must be stored in the extraction room at -18°C/-22°C. The reagents **R0,** and **R4** 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 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 With ither extraction methods validated:

- Follow the manufacturer's instructions.

5.2 For the 69-002B detection and quantification kit 69-002B

- 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, Dx Real-Time System.
- LC Carrousel Centrifuge for LightCycler® or benchtop 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 EBV 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. Reagent 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

- 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.
- AL buffer **(C)** is stable when stored tightly closed at +2°C/+8°C.

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 substitute reagents from kits with different batch numbers or 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)** of the kit 67-000 contain guanidinium chloride (chaotropic salt).
  R22: Harmful if swallowed.
  R36/38: Irritating for eyes and skin.
  S13: Keep away from food, drink and animal feedingstuffs.
  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)** of the kit 67-000 contain 0.04% sodium azide as preservative.

- Protease (**G**) of the kit 67-000 contains subtilisine.
  R37/38: Irritating to respiratory system and skin.
  R41 : Risk of serious damage to eyes.
  R42 : May cause sensitisation 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 11.2).

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 (**QS1**-**4**) are used to produce a standard curve in the software provided with the thermocycler.
- The quantification standards range from 10 000 copies/µL (**QS1**) to 10 copies/µL (**QS4**).
- The quantification standards must be designated as « standard »and their values must be entered when samples are defined in the table of data analysis software.
- Signal reading of QS at 530 nm.

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 EBV.
- 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, Dx Real-Time System, Stratagene® and Versant® kPCR Molecular System AD or Agilent real time PCR instrument does not allow the importation of the standard curve.

8.3 Sensitivity control (SC)

- The sensitivity control (**SC**) validates the performance of the assay and should be considered as a run-control.
- The sensitivity control (**SC**) is amplified with **R4** amplification premix.
- 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.

8.4 Extraction + inhibition control

8.4.1 The sample extraction + inhibition control (IC2sample)

- This control consists of an internal control (**IC2**) that must be added to the samples prior to extraction and checks both the efficacy of the extraction and detects the presence of possible inhibitors.
- Signal reading at 560 nm.

8.4.2 The reference extraction + inhibition control (IC2W0)

- This control consists of an internal control (**IC2**) that must be added to the negative extraction control (**W0**) prior to extraction and must be amplified at the same time as the patient samples in order to obtain a reference (**IC2W0**). The results must be compared with the extraction + inhibition control of patient samples (**IC2sample**).
- Signal reading at 560 nm.

⇒ Comparison of CT (Crossing Threshold) values of both **IC2W0** and **IC2sample** controls at 560 nm evaluates the efficacy of the extraction and detects the presence of possible inhibitors.

8.5 The negative controls

8.5.1 The negative extraction + amplification control (IC2W0)

- This is exactly the same tube as the one described in 8.4.2 (reference extraction + inhibition control). However, when analyzing at 530 nm this control demonstrates the absence of contamination during extraction and amplification.
- Signal reading of negative extraction + amplification sample at 530 nm.

8.5.2 The negative amplification control

- The negative amplification control consists of reagent (**R0**) amplified in the amplification premix (**R4**). This control shows a possible contamination during amplification.
- This control is optional.

⇒ The comparison of the CT value at 530 nm of the negative amplification control (**R0**) and **IC2W0** identifies a possible contamination.

8.6 Who EBV International standard (not supplied):

- The WHO expert committee has drafted references for the use of biological substances for the prevention, treatment and diagnosis of human diseases. WHO international standards are recognised as being the reference in this field and constitute the International Unit (IU).
  The results obtained with the EBV R-gene® kit, expressed in copies/mL using real-time PCR software, can then be converted to International Units by the user, using a conversion factor described in the paragraph on "Validation and interpretation of results" in this technical information sheet. This conversion factor takes into account the nature of the sample and the combination of extraction/amplification platforms used.

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 PREPARATION

9.2.1 Blood samples

WARNING : The use of heparinized tubes does not suit genic amplification analysis. Blood collection tubes contain citrate which may be responsible for decreasing signal during the detection of amplified products.

- Blood must be collected in EDTA tubes.
- Before the extraction step, homogenize blood sample by over turning the sample tubes for 10 minutes with an automatic shaker
- Aliquot each blood sample in small volumes under a biological safety cabinet.
- Period between blood collection and arrival at the laboratory must not exceed 24 hours.
- Blood samples must be sent to the laboratory at room temperature (+18°C/+25°C).

9.2.2 Plasma samples

- Blood must be collected in a dry tube or a tube containing EDTA.
- Centrifuge tube at 1200xg for 10 minutes at 20°C. A maximum of 2 mL (200µL minimum) plasma is decanted under biological safety cabinet in cryotubes.
- Plasma must be sent to the laboratory preferably at room temperature (+18°C/25°C) or at +2°C/+8°C. Plasma not directly treated upon arrival must be store at +2°C/+8°C for one week maximum. In case this delay exceeds one week, store plasma at -18°C/-22°C.
- If plasma samples are sent to the laboratory on dry-ice, they must be stored afterwards at -18°C/-22°C or preferably at-78°C/-82°C.

9.2.3 CSF samples

- CSF is obtained 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. 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 6 "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 or thermocycler to +56°C.
- 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 a 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 Loading step

- 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 minute 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.

WARNING: The elution volumes vary depending on the nature of the samples:

- For samples of whole blood add **100 µL** of balanced elution buffer AE(**F**) at room temperature.
- For samples of plasma and CSF, add **50 µL** of balanced elution buffer AE (**F**) at room temperature.

- Incubate at room temperature for **5 minutes**.
- Centrifuge at 6000xg for 1 min.
- 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 EXTRACTION INSTRUMENTS and/or KITS VALIDATED WITH EBV R-GENE®

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

INSTRUMENTS :	Kit	Sample + IC2 Volumes	Sample Type	Protocol	Elution volume
	QIAamp® DNA Blood Mini kit	200 µL of sample + 10 µL IC2	Whole blood		100 µL
			Plasma, CSF		50 µL
QIAcube			Whole blood	Blood and body fluid spin protocol V3	100 µL
			Plasma, CSF		50 µL
MagNA Pure Compact® Instrument	MagNA Pure Compact Nucleic Acid Isolation Kit I		Whole blood	DNA_Blood_100_400	100 µL
			Plasma, CSF	Total_NA_Plasma_100_400	50 µL
MagNA Pure LC System® Instrument	MagNA Pure DNA Isolation Kit I		Whole blood	DNA I Blood_Cell High Performance	100 µL
			Plasma, CSF	Total_NA_Serum_plasma_blood	50 µL
NucliSENS® easyMAG®	NucliSens® easyMAG® Reagents		Whole blood New	Manufacturer's Specific B protocol with 140 µL silica(1)	50 µL
			Whole Blood	Specific protocol B with 140 µL silica	50 µL
			Plasma, CSF	Generic/Specific B	50 µL
QIAsymphony SP	QIAsymphony DNA Mini Kit	300 µL of sample + 15 µL IC2(2) (*extract 200 µL*)	Whole Blood	Virus Blood DefaultIC_V4	90µL (*eluate 60µL*)
*m*2000sp®	Sample Preparation System DNA	800 µL of sample + 10 µL IC2(3) (*extract 300 µL*)	Whole blood, plasma, BAL, biopises	DNA-Blood-LL-300-150 v081507	250µL (*eluate 150µL*)
Versant® kPCR Molecular System SP	Versant® Sample Preparation 1.0	400 µL of sample + 10 µL IC2(4) (*extract 250 µL*)	Plasma	Sample Preparation Protocol 5	65µL (*eluate 50µL*)

(1) A premix consisting of IC2, Lysis buffer and Silica must be prepared extemporaneously and added to the samples previously distributed in shuttles containing 2 mL of Lysis buffer. For n samples, mix 600µL Lysis buffer x (n+1) + 10µL IC2 x (n+1) + 140µL Silica x (n+1). Add 740µL of the mixture to each sample. For greater precision, see the detailed protocol "Worksheet easyMAG Viral Whole Blood extraction protocol" by bioMérieux

(2) If using the QIAsymphony SP automatic system, it is possible to prepare a premix containing IC2 (Argene-bioMérieux) and ATE buffer (QIASymphony) extemporaneously. For 24 samples, mix 1 414µL of ATE buffer + 266µL IC2.

(3) To extract samples of an initial volume higher than 800µL, add the following amounts of IC2 reagent:
For 1 initial sample amount, add 1/80th the amount of IC2 reagent (for example: to extract a 1mL sample, add 12.5µL of IC2 reagent).

(4) 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).

11. Detection and Real Time PCR Quantification protocol

Note : *In order to simplify the protocol, the device dedicated to holding the amplification reaction mix is referred to as a "tube".*

WARNING: **The performances described have been validated and are guaranteed using the technical extraction/amplification unit combinations recommended in the technical information sheet. To monitor evolution of a patient's viral load test after test, it is vital for the successive sample analyses to take place using strictly the same protocol and with the same combinations of extraction/amplification units.**

To determine the number of tubes, check if the experiment requires the creation of a standard curve (see section **8.2**).

Plan:

1	**tube**	**Per sample.**
1 or 4	**tube(s)**	**For EBV quantification standard curve imported/created.**
1	**tube**	**For QS3 as positive control in the event of qualitative detection**
1	**tube**	**For EBV sensitivity control (SC).**
1	**tube**	**For reference extraction+inhibition control also used as EBV 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 Argene 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	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 1: The temperature increases and decreases are parametered by default, which means at 100% or at their maxima.

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, it is **ESSENTIAL** to use a colour compensation file 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 EBV 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:
- Homogenize thawed reagents by vortexing or by pipetting, then briefly centrifuge.
- Make sure the cooling block (LightCycler Centrifuge Adapters) was decontaminated by exposure under U.V. light for 30 min.
- Make sure the cooling block (LightCycler Centrifuge Adapters) was correctly pre-cooled at +2°C/+8°C.

WARNING: **To avoid contamination as much as possible, close the tubes as distribution is completed. Replace the amplification premixes (R4), quantification standards (QS) and sensitivity control (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.**

- 10 µL of each extracted sample in the corresponding tube.
- 10 µL of the sensitivity control (**SC**) in the corresponding tubes (see chapter 8).
- 10 µL of each standard (from **QS4** to **QS1**) in the corresponding tubes (see chapter 8).
- 10 µL of extracted mix **IC2+W0** in the corresponding tube. This tube is the **IC2W0** control (see chapter 8).

- Centrifuge the tubes with the relevant device and then transfer them to the thermocycler.

11.3 RUNNING THE EBV R-GENE® PROGRAM

- Run the amplification program (stored according to the instructions described in section Amplification program)
- Designate the samples and controls.

- For EBV quantification enter the following values for the quantification standards (copies/mL):

	Quantification (Whole blood, plasma, CSF, BAL, biopsies)				
	Extraction 200µL Elution in 50µL (copies/mL)	Extraction 200µL Elution in 100µL (copies/mL)	Extraction 300µL Elution in 250µL (copies/mL)	Extraction 250 µL Elution in 65µL (copies/mL)	Extraction 300 µL Elution in 90µL (copies/mL)
QS1	2 500 000	5 000 000	8 000 000	2 500 000	3 000 000
QS2	250 000	500 000	800 000	250 000	300 000
QS3	25 000	50 000	80 000	25 000	30 000
QS4	2 500	5 000	8 000	2 500	3 000

12. Data Analysis

The details of results analysis per type of device can be downloaded 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 all the fluorescence curves of the samples in their 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 EBV 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 *(red horizontal cursor line)* to a position where it crosses all the fluorescence curves of the samples in their linear part, above the baseline 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 EBV 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**) to 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**) to 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 EBV 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**) to 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 EBV R-gene® premix does 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 threshold line manually to a position where it crosses the fluorescence curves of all the samples in their linear part. This step is performed to identify the positive samples which correspond to a calculated CT value. Negative samples are defined as UNDETERMINED displayed in the CT column by the SDS software.
- 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 EBV 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**) to 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 each sample 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**) to the CT value of the reference extraction + inhibition test (**IC2W0**) at 560 nm.

12.7 WITH STRATAGENE® Mx3000, Versant® kPCR Molecular System AD or Agilent

- Make sure that NONE is selected in the REFERENCE DYE field because the EBV R-gene® **R4** kit premix does 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 each sample 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. Validations 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 the Applied Biosystems PRISM® range, Rotor-Gene®, Smartcycler® and Stratagene®, Versant kPCR Molecular System AD or Agilent, Dx Real-Time System. This term corresponds to CP (Crossing Point) for devices in the LightCycler® range. To simplify the instructions, only the term 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.
3thrd 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 **28** and **32**cycles at **530** nm.

.

QUANTIFICATION				QUALITATIVE 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	28-32 cycles	-3.917< Slope <-3.103	-3.587< Slope <-3.208	*Not applicable*
LightCycler® 2.0 / LightCycler® 480		1.8 < Efficiency < 2.1	1.9 < Efficiency < 2.05	
SmartCycler® 2.0		-0.322< Slope <-0.255	-0.311< Slope <-0.278	
		-3.917< Slope *<-3.103	-3,587< Slope *<-3.208	
Rotor-Gene®		0,8 < Efficiency < 1.1	0.9 < Efficiency < 1.05	
Applied Biosystems		-3.917< Slope <-3.103	*Not applicable*	
Stratagene®, Versant® kPCR Molecular System AD or Agilent		0.8< Efficiency <1,1		
Dx Real-Time System (Bio-Rad)		0.8< 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".

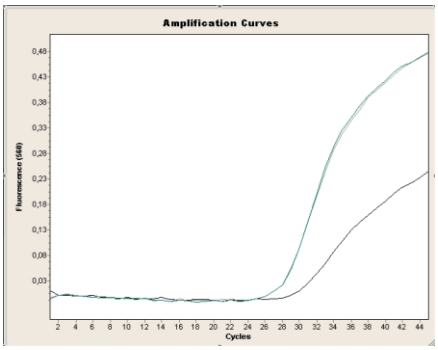
=> **If ALL of these conditions are fulfilled, the results obtained with the sample can be analysed.**

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**
EBV quantitative Interpretation	Sample validated as positive Validated quantification	Sample validated as negative	Sample validated as positive Perform quantification again	**Not Valid**
EBV qualitative Interpretation	Sample validated as positive	Sample validated as negative	Sample validated as positive	**Not Valid**

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



IMPORTANT NOTES:

- It is high 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 the EBV R-gene® kit to other diagnostic investigation methods in order to define patient viral status.

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. • Check the delay between the sample collection and its analysis. • Collect blood in EDTA tubes.
Transport and storage conditions were not met	• Follow instructions in section 4 regarding the storage of EBV R-gene® kit 69-002B 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 the DNA Extraction kit ref. 67-000. (See section 10.1). • Check if material and protocol used to extract sample correspond to material and protocol recommended for analysis with the EBV R-gene® kit 69-002B (See section 10). • 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 amplification 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 threshold line adjustment. • 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. • Compare the result of the extraction + Inhibition control (**IC2sample**) of the suspected sample to 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 with U.V. light. • Repect the manufacturer's recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • Only a trained staff must handle the EBV R-gene® kit. • 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 amplification 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 threshold line adjustment. • In the case of an analysis based on an imported standard curve, check that the imported curve 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 to 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. • For coloured extracts and inhibited samples obtained on the NucliSENS easyMAG for the whole blood matrix, use the "**Whole Blood Viral Extraction**" protocol by preference.
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

15.1 Intra-assay and inter-assay reproducibility of EBV R-gene® kit ref.: 69-002

The intra-assay and inter-assay reproductibility of EBV R-gene® assay is tested thanks to a study performed on different samples (dilution range of Namalwa cells). The experiment is repeated 7 times.

The tables below show : the average CT obtained for each sample as well as the standard deviation obtained for each kind of study. The standard deviation varies from 0.21% to 0.48% and from 0.28% to 1.83% respectively for intra-assay reproducibility and inter-assay reproducibility.

		Average CT	Standard deviation	Coefficient of variation
EBV (EBV culture on Namalwa cells)	100 000 cp/PCR	24.66	0.118	0.48%
	10 000 cp/PCR	28.20	0.082	0.29%
	1 000 cp/PCR	31.99	0.127	0.40%
Intra-assay variability : Inhibition control (different and not inhibited blood samples)		31.46	0.066	0.21%

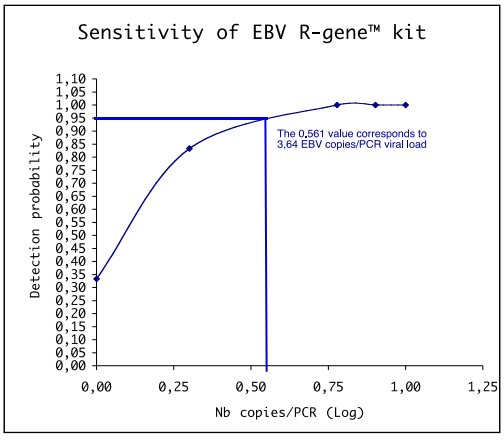
Table 1 : Intra-assay reproducibility of EBV R-gene® kit

		Average CT	Standard deviation	Coefficient of variation
EBV (culture on Namalwa cells)	100 000 cp/PCR	24.84	0.237	0.95%
	10 000 cp/PCR	28.59	0.300	1.05%
	1 000 cp/PCR	31.92	0.234	0.73%
EBV R-gene™ Quantification Standards (QS)	**QS1:** 100000 cp/PCR	23.35	0.351	1.51%
	QS2: 10000 cp/PCR	27.00	0.495	1.83%
	QS3: 1000 cp/PCR	30.36	0.288	0.95%
	QS4: 100 cp/PCR	33.87	0.547	1.62%
	SC :10 cp/PCR	36.25	0.556	1.53%
Inter-assay variability : Inhibition control (H2O)		31.80	0.091	0.28%

Table 2 : Inter-assay reproductibility of EBV R-gene® kit

15.2 Analytical sensitivity of EBV R-gene® kit

Analytical sensitivity of the EBV R-gene® kit has been evaluated through a limit dilution method with EBV culture on Namalwa cells containing 1 to 2 EBV copies/PCR. The experiment was repeated 6 times.The curve below shows the results of the probability analysis.



These experiments indicate a 95% probability to detect a sample containing 3.64 EBV copies/PCR when using EBV R-gene® assay (corresponding to 182 copies/mL of whole blood sample).

This value is 25 times lower than the risk threshold which is estimated at 5 000 copies/ mL of whole blood.

15.3 Analytical specificity of EBV R-gene® kit

The specificity of the EBV R-gene® primers and probes were tested on the following viruses :

- Human Herpesvirus : infected cells with HSV-1, HSV-2, VZV, CMV, HHV-6, HHV-7, HHV-8.
- Human Polyomavirus : plasmids containing the total genome of JCV and BKV.
- Adenovirus 12.

None of these viruses amplified with the EBV R-gene® kit, thus proving the specificity of the assay.

NOTE : In order to prove EBV R-gene® assay does not amplify human sequences, additional tests have been performed on EBV negative blood samples and human specimens.

15.4 Test Report

15.4.1 On 50 blood samples

EBV R-gene® assay was evaluated on 50 clinical blood samples with LightCycler 1.0® real time PCR platform.
EBV R-gene® assay was used in 2 different ways :
- with QiaAmp® DNA Mini Blood extraction kit
- with BioRobot EZ1® Workstation.

QiaAmp® DNA Blood Mini Kit 69-002A, PCR on LightCycler® 1.0		**BioRobot EZ1® extraction,** PCR on LightCycler® 1.0			
Samples dispatching	Nb of samples	0 to 1000 copies/mL	1000 to 10 000 copies/mL	10 000 to 100 000 copies/mL	> 100 000 copies/mL
0 to 1000 copies/mL	35	**32**	3		
1000 to 10 000 copies/mL	12	2	**8**	2	
10 000 to 100 000 copies/mL	2			**2**	
> 100 000 copies/mL	1				**1**
TOTAL	**50**	**34**	**11**	**4**	**1**

Table 3

43 samples gave the same quantification values with both extraction methods.
5 samples gave a higher quantification value with BioRobot EZ1® compared to QiaAmp® DNA Mini Blood extraction. Two samples gave alower quantification value with BioRobot EZ1® compared to QiaAmp® DNA Mini Blood extraction.
These differences indicate the importance to stay with the same extraction procedure (in the context of patient follow-up).

15.4.2 On samples from QCMD panel 2009

During the European EBV screening campaign in 2009, 10 specimens underwent blind testing with the EBV R-gene® kit.
200µL of each specimen was extracted using the QIAamp® DNA Blood Mini Kit and then eluted with 100 µL. Each specimen was extracted with the internal IC2 control in place, which is provided in the kit, in order to check the efficiency of the extraction stage and to detect the presence of agents which inhibit amplification.
10µL of each specimen extracted was amplified using the ABI Prism® 7500 with the EBV (**R4**) amplification premix.

	QCMD Results		EBV R-gene®		Delta Log
	Copies/mL	Log copies/mL	Copies/mL	Log copies/mL	
EBV09-1	-	-	-	-	-
EBV09-2	2.660×10^2	2.42	1.694×10^2	2.23	0.20
EBV09-3	5.240×10^2	2.72	5.348×10^2	2.73	-0.01
EBV09-4	8.299×10^3	3.92	6.248×10^3	3.80	0.12
EBV09-5	1.652×10^4	4.22	1.208×10^4	4.08	0.14
EBV09-6	2.698×10^4	4.43	2.727×10^4	4.44	0.00
EBV09-7	1.581×10^5	5.20	1.624×10^5	5.21	-0.01
EBV09-8	5.410×10^2	2.73	4.440×10^2	2.65	0.09
EBV09-9	2.582×10^3	3.41	2.821×10^3	3.45	-0.04
EBV09-10	2.065×10^3	3.31	1.368×10^3	3.14	0.18

The specimen which did not contain EBV was confirmed as negative with the EBV R-gene® kit.
The nine specimens which did contain EBV were confirmed as positive with the EBV R-gene® kit

⇒ 100% (10/10) of the EBV specimens gave the expected results.
⇒ The detection of low viral levels of EBV (EBV09-02: 2.660×10^2 copies/mL; EBV09-03: 5.240×10^2 copies/mL and EBV09-08: 5.410×10^2 copies/mL) demonstrates that the EBV R-gene® kit is highly sensitive.

15.5 Clinical Study on 150 blood samples

A clinical study on 150 blood samples was performed at the department of Virology in the University Hospital of Grenoble (France). The samples were collected and analyzed following routine laboratory procedures (automated extraction with MagNa Pure LC®, amplification with FRET probes in BXLF1 EBV gene).

The results were compared to those obtained with **EBV R-gene®** assay in two different ways :
- with MagNapure LC® extraction and SmartCycler® 2.0 real time amplification platform.
- with MagNapure LC® extraction and LightCycler® 1.0 real time amplification platform.

Two samples were considered as equivalent when the difference between their corresponding quantification values was lower than 0.5log. The number of samples presenting equivalent results are displayed in Table 4.

	EBV R-gene™ kit			
	MagNA Pure LC® / **SmartCycler® 2.0**		MagNA Pure LC® / **LightCycler® 1.0**	
In-House Assay	121		74	
	Average of difference	0.22	Average of difference	0.57
	Standard deviation	0.67	Standard deviation	0.82

Table 4

These results were analysed using clusters of quantification.

In-House Assay		Dispatchning and number of samples tested with EBV R-gene™ MagNAPure LC System® extraction, PCR on **LightCycler® 1.0**			
Samples dispatching	Nb of Samples	0 to 1000 copies/mL	1000 to 10 000 copies/mL	10 000 to 100 000 copies/mL	> 100 000 copies/mL
0 to 1000 copies/mL	41	**41**			
1000 to 10 000 copies/mL	69	47	**22**		
10 000 to 100 000 copies/mL	35	2	20	**13**	
> 100 000 copies/mL	5			1	**4**
TOTAL	**150**	**90**	**42**	**14**	**4**

Table 5

In-House Assay		Dispatching and number of samples tested with EBV R-gene™ MagNAPure LC System® extraction, PCR on **SmartCycler® 2.0**			
Samples dispatching	Nb of samples	0 to 1000 copies/mL	1000 to 10 000 copies/mL	10 000 to 100 000 copies/mL	> 100 000 copies/mL
0 to 1000 copies/mL	41	**36**	5		
1000 to 10 000 copies/mL	69	21	**43**	5	
10 000 to 100 000 copies/mL	35	1	6	**25**	3
> 100 000 copies/mL	5				**5**
TOTAL	**150**	**58**	**54**	**30**	**8**

Table 6

When using LightCycler® 1.0 as a platform (see table 5):
Out of 150 samples, 80 samples belong to the same quantification cluster with both PCR methods.
No sample gave a higher quantification value with EBV R-gene PCR compared to in-house PCR assay.
70 sample gave a lower quantification value with EBV R-gene PCR compared to in-house PCR assay.

When using SmartCycler® 2.0 as a platform (see table 6):
Out of 150 samples, 109 samples belong to the same quantification cluster with both PCR methods.
13 samples gave a higher quantification value with EBV R-gene® PCR compared to in-house PCR assay.
28 samples gave a lower quantification value with EBV R-gene® PCR compared to in-house PCR assay.

These results prove how imperative it is to stay with the same process (extraction and amplification) in order to perform a reliable patient follow-up.
In short: Respect the same protocols and the same instruments for extraction and real time amplification.

16. References

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

Detailed protocol "Worksheet easyMAG Viral Whole Blood extraction protocol" from bioMérieux.

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Comparative evaluation of the NucliSENS® easyMAG® automated system for the extraction of viral DNA from whole blood samples: application to the monitoring of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) loads. Poster ECCMID Barcelone 2008 (Spain).

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Poster, 6th International Symposium on Molecular Diagnostics 2006 (Austria).

7) Ruimei Li, Hongwei Sun, Apostolia M. Tsimberidou, Michael J. Keating, Razelle Kurzrock
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8) Germi R, Beyls , Quetant , Aribert , P Bourgeois, Seigneurin , Morand P. RICAI 2010
Mesure de la charge virale des herpesvirus HSV1, CMV, HHV6 et EBV dans 86 liquides broncho-alvéolaires (LBA) prélevés chez des patients transplantés pulmonaires

9) C Brunet, L Ovaguimian, S Burrel, H Agut, D Boutolleau. RICAI 2010
Evaluation de la trousse EBV R-gene® (Argène) : Mesure de la charge virale EBV dans des prélèvements de sang total et dans les échantillons du panel européen QCMD

17. Related products

- DICO Extra r-gene® ref. : 71-101
- DICO Ampli r-gene® ref. : 71-100
- Colour Compensation r-gene® ref. : 71-103
- CELL Control r-gene® ref. : 71-106

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
	Contains sufficient for <n> tests
	Protect from light
	Keep dry
CE	Identification of notified body

BIOMÉRIEUX

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