ARGENE

CE 0459

IVD

CMV R-gene®

REF 69-003
REF 69-003B

NEW EXTRACTION PROTOCOL NucliSENS easyMAG :

« Whole Blood Viral Extraction Protocol »

COMPOSITION:

REF 69-003	**DNA EXTRACTION KIT**	**Ref.: 67-000**
	QUANTIFICATION Kit, CMV R-gene®	**Ref.: 69-003B**

1. Presentation of the kit

CMV is a Herpes virus responsible for a wide spectrum of diseases in man. The primary infection occurs during childhood. The virus spreads through the blood, provoking clinical manifestations that, when they exist, remain rare and benign. The virus then remains in its latent state in its host and can be reactivated during an immunosuppression. The severity of the resulting diseases is mainly determined by the immune status of the patient. The fragile subjects are basically patients receiving bone marrow or organ allografts and AIDS patients.

The CMV is known to be involved in neurological damage, pneumopathies and the aggravation of immunosuppression in these patients.

The CMV R-gene® kit enables the genome of the CMV to be identified in whole blood, plasma, serum, cerebrospinal fluid (CSF), urine, biopsies, broncho alveolar liquid (BAL) and amniotic fluid, provided that there is a specific 2-stage protocol for the latter. Indeed, high viral loads are frequently found in amniotic fluid when it is positive in relation to CMV. Following clinical studies, such concentrations prompted us to define a specific two-stage strategy for this type of sample in order to enable full interpretation of the results. Thus, the same sample of amniotic fluid must be tested twice: a tube containing the pure sample and another tube containing the same sample but diluted to 1/100th. At the end of this test, a positive or negative qualitative response may be given. On the other hand, if the sample is found to be positive and inhibited, the extracted DNA from the sample must be tested again with a greater dilution according to the first results.

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

The World Health Organisation's (WHO) first international standard for CMV is now available. It is intended for use as a reference material for the calibration of the quantification of viral loads. The results obtained will thus be standardised, no matter what sort of samples and combinations of extraction and amplification platforms are used. Use of this international standard will mean that the viral load of a patient followed successively with two different techniques, can be compared from one laboratory to the next.
The results obtained using the CMV R-gene® kit, expressed in cp/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.

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), EBV with EBV R-gene® kit (ref : 69-002B), BKV with BK Virus R-gene® kit (ref : 69-013) CMV, HHV-6, HHV-7 and HHV-8 with CMV HHV6,7,8 R-gene® kit (ref : 69-100B), and Adenovirus with Adenovirus R-gene® (ref : 69-010B).

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

2. Intended use

The human cytomegalovirus (HCMV) is a virus enveloped in double stranded DNA of the family of Herpesviridae. The poorer the socio-economic conditions, the higher the prevalence (50 to 100 %). After the primary infection, HCMV remains in its latent state in the host and may be the cause of recurrent secondary infections, during chronic or transient immunosuppression, by reactivation of the endogenous genome or by reinfection with a new strain.

The consequences of the HCMV infection mainly depend on the cell immunity of the subject involved. Most often asymptomatic in healthy subjects, it may lead to serious damage in immunosuppressed patients and in the foetus or newborn child after in utero transmission.

The infection with HCMV after organ or marrow allograft: HCMV is the main infectious agent after bone marrow allograft and organ transplant. Infection with HCMV is observed, on the average, in two third of all receivers, irregardless of the type of transplant. It occurs in the absence of prophylactic treatment between the 1st and 4th month after the graft. It is symptomatic in two-thirds of cases of primary infection, in 40 % of cases of reinfection and less than 20 % of the reactivations. Prolonged fever may be the only clinical manifestation of the infection or comprise complications of thrombo-leukopenia, cytolitic hepatitis, gastric disorders or cystitis. Chorioretinitis is rare. Interstitial pneumonitis is a major complication of a marrow graft. It occurs in about 20 % of all receivers and its evolution, without treatment, is dangerous (90 % mortality). In addition, infection with HCMV is a factor that triggers or accelerates the rejection or the GVH (reaction of the graft against the host). In addition, it aggravates the immunosuppression and favours superinfections.

Infection with HCMV during AIDS: The incidence of infections with HCMV has decreased by 80 % since the beginning of highly active anti-retroviral treatments that provide at least partial immune restoration. Clinical manifestations occur at a major stage of immunosuppression, characterised by an average number of CD4+ T lymphocytes under 50/mm3. Retinitis, observed before the era of the tri-therapies, in about 15

to 35 % of the patients, remains the most common clinical manifestation. Peptic ulcers are the second leading clinical manifestations. Multiple types of neurological damage have been described although the incidence has not been established. Pneumopathy is exceptional.

During pregnancy, the advent of a maternal primary infection gives rise to complications in 50% of the cases of foetal infection, with this being severe in approximately 10% of cases, with neurological impairment occurring in particular.

The techniques used in the diagnosis of the active infection with HCMV include: cell culture with search for a cytopathogenic effect (6 weeks, « benchmark method»), fast culture with detection by immunocytochemistry using monoclonal antibodies of the anti IEA type (24 to 48h), leukocyte antigenemia ppUL83 (2 to 3h). These methods attest to an active infection since there is a production of viral antigens.

PCR (traditional and in real time) is also used though methods specific for each laboratory. These various methods don't enable a fine comparison of the quantitative results as well as the elaboration of transposable threshold values.

Moreover, European and American regulations tend to limit the use of these «in- house » tests by requiring very constraining stages of control and validation in development and production. It is now very difficult to develop this type of diagnostic test while complying with all of the prevailing regulations, without counting the many necessary operating licences required for diagnostic use.

A highly standardised commercial kit developed in accordance with the strictest standards such as CMV R-gene® makes a test available for the majority of laboratories whose results are documented and transposable from one laboratory to another.

The CMV R-gene® kit is a rapid help in the diagnosis and monitoring of Cytomegalovirus infections by drawing together the values obtained with those using current methods of diagnosis. The goal is to let clinicians use their therapeutic reflexes elaborated through the methods of diagnosis (cell culture, antigenemia) used until now within each patient's clinical context.

The CMV R-gene® kit quickly determines the CMV viral load by means of a standard series, an internal control, an inhibition control, and a negative control, which are provided in the kit. The results are expressed in number of copies per milliltre of sample.

Measurement of the viral load enables the monitoring of the evolution of chronic viral infections, and thus to quickly decide whether to begin adapted treatment or to evaluate its efficacy.

The types of patient concerned are graft receivers (organs or marrow), AIDS patients and the immunosuppressed in general. The very high sensitivity and precision of this kit enable its use for the determination of the CMV in the receiver of a bone marrow allograft.

The positive and negative predictive values (probability of the advent or non-occurrence of a CMV disease upon completion of a positive or negative test) will be determined by the user within the context of the prophylactic and therapeutic strategies used in order to determine the clinical threshold values for the viral quantification. At the time of writing there is no possibility of a consensus regarding these values; they depend to a very large extent on the quantification kit used, the site's therapeutic strategy, the type of patient in question (marrow transplant, solid organ transplant, patient with an HIV infection), and on the patient's immune status.

The CMV R-gene® kit is used to detect and/or measure the viral load of CMV viruses in whole blood, plasma, serum, CSFs, urine, BALs, biopsies and amniotic liquid under absolute conditions of a specific protocol.
This kit cannot be used for screening donors.

The CMV R-gene® kit enables the viral load of CMV virus to be measured in the whole blood, plasma, serum, CSF, urine, BAL, biopsies and amniotic fluid subject to the conditions of a specific protocol.

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

The quantification of CMV with the CMV R-gene® kit can be carried out simultaneously with EBV viral load measurement using the EBV R-gene® kit (ref.: 69-002), HSV-1, HSV-2, VZV viral load measurement using the HSV1 HSV2 VZV R-gene® kit (ref.: 69-004), CMV and HHV-6 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. : 69-013). All R-gene® Real Time PCR kits follow the same amplification procedure and thus can be simultaneously used in the same experiment.

3. Principle of the test

3.1 SAMPLE TYPE

- CMV R-gene® is used to detect and measure the viral load on CMV virus in whole blood, cerebrospinal fluid (CSF), plasma, serum, bronchoalveolar liquid (BAL), urine, biopsies and amniotic fluid subject to the conditions of a specific protocol.
- The quantification range for CMV 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 with an extraction, inhibition, positive and negative control provided with the CMV R-gene®.
- CMV R-gene® kit provides all reagents to analyze 83 samples.

3.2 DNA PURIFICATION

- The following DNA extraction methods are validated with the CMV R-gene® kit ref. : 69-003B:
  - MagNA Pure Compact Instrument
  - MagNA Pure LC Instrument
  - MagNA Pure 96 System
  - NucliSENS® easyMAG®
  - QIAamp® DNA Blood Mini kit
  - DNA EXTRACTION KIT (supplied under reference 67-000 of ref. 69-003)
  - 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.

3.3 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 CMV 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 CMV R-gene® kit ref.: 69-003B:
  - LightCycler®
  - Applied Biosystems®
  - Rotor-Gene®
  - Stratagene®, Agilent or Versant® kPCR Molecular System AD
  - Dx Real-Time System (Bio-Rad)

- Extracted samples are amplified and quantified at the same time.
- CMV genome is quantified by real time amplification. The amplified gene for CMV is the gene coding for ppUL83 protein. Size of amplified fragment : 283 base pairs.
- A range of 4 quantification standards is provided in the CMV R-gene® kit. It consists in 4 points (**QS1, QS2, QS3, QS4**). Each quantification standard contains a plasmid specific to CMV. The quantification standards are ranged from 5000 copies/µL (**QS1**) to 5 copies/ µL (**QS4**) or from 50 000 copies to 50 copies of plasmid per PCR.
- **QS3** is a quantification standard that contains 500 copies of plasmid /PCR. It enables to import the standard curve created in the first run. This method avoids to test all 4 quantification standards each time a quantification experiment is launched (provided that conditions described in chapter : "The quantification standard QS3 »). It is also used as a positive control for qualitative detection of CMV.
- **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 CMV 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 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 the expiration date written on the box. Storage at higher temperature should be avoided. The reconstituted QIAGEN protease can be stored in aliquots at -18°C/-22°C to avoid freeze/thaw cycles.

4.2 CMV R-GENE DETECTION AND QUANTIFICATION KIT® 69-003B

- Number of tests: 90

W0	Water for extraction (molecular grade)	2x1.8 mL
IC2	Internal control 2	1 mL
R0	Water for amplification (molecular grade)	0.3 mL
QS1	Quantification standard **1** CMV	0.3 mL
QS2	Quantification standard **2** CMV	0,3 mL
QS3	Quantification standard **3** CMV	0.3 mL
QS4	Quantification standard **4** CMV	0.3 mL
SC	Sensitivity control CMV	0.3 mL
R5	CMV and IC2 Amplification premix	3x0.450 mL

- Keep the kit (ref. 69-003B) 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. : 69-003B), 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 **R5** must be stored in the room reserved for the preparation of premix at -18°C/-22°C.
- Each amplification premix (**R5**) cannot undergo more than 7 freezing/defrosting cycles.
- Replace the amplification premixes (**R5**), quantification standards (**QS**) and sensitivity control (**SC**) at -18°C/-22°C immediately after use.

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 other extraction methods validated:

- Follow the manufacturer's instructions.

5.2 FOR THE QUANTITATIVE/QUALITATIVE DETECTION KIT 69-003B

- Micropipets with plugged (aerosol barrier) tips or positive displacement tips.
- Thermocyclers validated with CMV R-gene®.
- LC Carrousel Centrifuge for LightCycler® or benchtop microcentrifuge convenient for 2mL reaction tubes, or plate centrifuge.
- Single use latex or similar gloves.
- Capillaries, tubes, microplates for real time PCR platforms validated for CMV 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 results 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

- 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)** 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)** 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 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 "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 (**QS1**-**4**) are used to produce a standard curve in the software provided with the thermocycler.
- The quantification standards range from 5 000 copies/µL (**QS1**) to 5 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.
- The QS signal is detected 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 CMV.
- 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®, Agilent or Versant® kPCR Molecular System AD, Dx Real-Time System real time PCR instrument do 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**) validates the performance of the assay and should be considered as a run-control.
- The sensitivity control (**SC**) is amplified with amplification premix (**R5**).
- 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 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 the same tube as was described in the "Reference extraction+inhibition control" chapter but, once the reading has been made at **530 nm**, it constituted a negative control used to check the absence of contamination on extraction and amplification.
- Signal reading of negative extraction+amplification sample at 530 nm.

8.5.2 The negative amplification control (R0)

- The negative amplification control consists of reagent (**R0**) amplified in the amplification premix (**R5**). 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 HCMV 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 CMV 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.

9.2.4 Urine samples

- Urine samples are collected in a sterile jar (e.g. CBUT jar)
- The urine samples are transported to the laboratory at ambient temperature (+18°C/25°C) or +2°C/+8°C.
- If the urine is not processed on arrival at the laboratory, it will be stored at +2°C/+8 °C for not more than one week. Beyond this period, store the urine at -18°C/-22°C.

10. Extraction protocol

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

The amniotic fluid sample must be extracted twice ; one extraction from the undiluted sample and one extraction from the sample diluted to 1:100 in water (molecular grade). Each of the 2 tubes is considered as a sample in the protocol of extraction and amplification.

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 instruction given in section "Reagents reconstitution".
- Dissolve 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 (2 tubes for each amniotic fluid sample) and add ONE tube in which the mix (**W0**+**IC2**) will be extracted.
- Heat the water bath or thermocycler at +56°C.
- Pipet 200 µL of AL buffer (**C**) into each 1.5 mL microcentrifuge tube previously identified.
- Pipet 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.
  WARNING : The elution volumes differ depending on the nature of the samples:
  - For whole blood and amniotic fluid samples, add **100 µL** of balanced elution buffer AE (**F**) at room temperature.
  - For serum, plasma or CSF samples, add **50 µL** of balanced elution buffer AE (**F**) at room temperature.
- Incubate at room temperature for **5 min.**
- 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 CMV 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 Ref: 51 104 / 51 106	200 µL of sample + 10 µL IC2	Whole blood, amniotic fluid		100 µL
			Plasma, serum, CSF		50 µL
QIAcube QIAGEN Ref. : 9001292 / 9001293			Whole blood, amniotic fluid	Blood and body fluid spin protocol V3	100 µL
			Plasma, serum, CSF		50 µL
MagNA Pure Compact® Roche Diagnostics Ref.: 03 731 146 001	MagNA Pure Compact Nucleic Acid Isolation Kit I Ref. 03 730 964 001 32 isolations		Whole blood	DNA_Blood_100_400	100 µL
			Plasma, serum, CSF	Total_NA_Plasma_100_400	50 µL
MagNA Pure LC System® Roche Diagnostics Ref.: 12 236 931 001	MagNA Pure DNA Isolation Kit I Ref. 03 003 990 001 192 isolations		Whole blood	DNA I Blood_Cell High Performance	100 µL
			Amniotic fluid	Total_NA_Serum_plasma_blood	100 µL
			Plasma, serum, CSF		50 µL
MagNA Pure 96 System Roche Diagnostics Ref.: 05 195 322 001	DNA and Viral NA Small Volume kit Ref. : 05 467 497 001		Whole blood, urine, amniotic fluid, CSF, BAL	Viral NA Universal SV	100 µL
NucliSENS® easyMAG® bioMerieux Ref. 280110	NucliSens® easyMAG Reagents		Whole blood (New)	The manufacturer's "**Whole Blood Viral Extraction** " specific B protocolwith 140 µL of silica [1]	50 µL
			Whole blood	The manufacturer's Specific B protocol with 140 µL of silica	50 µL
			Plasma, CSF, amniotic fluid [2], serum	Generic/Specific B	50 µL
QIAsymphony SP	QIAsymphony DNA Mini Kit	300 µL of sample + 15 µL IC2[3] (extraction de 200 µL)	Whole blood	Virus Blood DefaultIC_V4	90µL *(eluate 60µL)*
m2000sp® Abbott Ref. 9K1401	Sample Preparation System DNA Ref. 06K12-24	800 µL of sample + 10 µL IC2[4] (*extract 300 µL*)	Whole blood, plasma, BAL, urine, biopises, amniotic fluid	DNA-Blood-LL-300-150 v081507	250µL *(eluate 150µL)*
Versant® kPCR Molecular System SP SIEMENS Ref. 06635740	Versant ® Sample Preparation 1.0 Ref. : 06496457/06496465	400 µL of sample + 10 µL IC2[5] (*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] For amniotic liquids and in case of extraction with the NucliSENS® easyMAG® apparatus, a pre-treatment of the samples with proteinase K is required. In this case, add 10 µL of proteinase K at 20 mg/mL for 200 µL of sample and allow to incubate for 15 min. at 56°C.

[3] 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.

[4] 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).

[5] 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: With a view to simplifying the instructions, 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.

Each sample of amniotic fluid must be tested twice : 1 extract from the undiluted sample and 1 extract from the diluted sample at 1:100 in molecular grade water. (*See Chapter "Presentation of the kit"*)

To determine the number of tubes, check if the experiment requires the creation of a standard curve (see section "The quantification standard QS3 **"**).

Plan:

1 **2**	tube **/tubes**	Per sample. **Per sample of amniotic fluid.**
1 or 4	tube(s)	For CMV quantification standard curve imported/created.
1	tube	For QS3 as positive control in the event of qualitative detection
1	tube	For CMV sensitivity control (**SC**).
1	tube	For reference extraction+inhibition control and CMV negative extraction+ amplification control (**IC2W0**).

NOTE:

- Use the transparent plates (ref.: HSP9601) with the optical stoppers (ref.: TCS0803) for the Dx Real-Time System amplification device).

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

Example 1:
Quantitative search for CMV in 2 blood samples using LightCycler®2.0 or Rotor-Gene®. The standard curve for CMV is imported.
Amplification plan:
2 tubes for samples analysis:2 (samples + R5).
1 tube to import the quantification standard curve of CMV (QS3+R5).
1 tube for the sensitivity control of CMV (SC+R5).
1 tube for CMV reference extraction+inhibition control and CMV negative extraction+ amplification control (IC2W0+R5).

Example 2:
*Quantitative search for CMV in 2 **amniotic fluid** samples using LightCycler®2.0 or Rotor-Gene®. The standard curve for CMV is imported.*
Amplification plan:
***4** tubes for samples analysis: 2 tubes (**undiluted extracted** sample + R5) and 2 tubes (**1:100th extracted** sample + R5)*
1 tube to import the quantification standard curve of CMV (QS3+R5).
1 tube for the sensitivity control of CMV (SC+R5).
1 tube for CMV reference extraction+inhibition control and CMV negative extraction+ amplification control (IC2W0+R5).

11.1 AMPLIFICATION PROGRAM

Regardless which real time PCR platform is used the amplification program remains the same. The amplification program is described in the table below.
WARNING : On Stratagene®/Agilent or Versant® kPCR Molecular system AD, parameter to 20 sec. the denaturation step.

Steps		TIME	TempEratur e	Cycles	Fluorescence acquisition					
					LC1	LC2, LC480	Applied Biosystems	Rotor-Gene	Stratagene ou Agilent, Versant® kPCR Molecular system AD	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-VIC**	**Green-Yellow**	**FAM-HEX**	**FAM-HEX**
					end of the annealing					

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 the LightCycler® 2, 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 CMV 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®, Agilent or Versant® kPCR Molecular System AD 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 (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 soon as distribution is completed.**
Replace the amplification premix (R5), quantification standards (QS) and sensitivity test (SC) at -18°C/-22°C immediately after use.
Each premix cannot undergo more than 7 freezing/defrosting cycles.

- 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 and controls must be followed.

- For each amplification run, add :
  - 10 µL of each extracted sample in the corresponding tube.
  - 10 µL of the sensitivity control (**SC**) in the corresponding tubes (see chapter "Internal quantification standards and controls").
  - 10 µL of each standard (from **QS4** to **QS1**) in the corresponding tubes (see chapter "Internal quantification standards and controls").
  - 10 µL of extracted mix **IC2+W0** in the corresponding tube. This tube is the **IC2W0** control (see chapter "Internal quantification standards and controls").
  - Centrifuge the tubes with the corresponding device and transfer them to the thermocycler.

11.3 RUNNING THE PROGRAM

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

	Quantification (Whole blood, plasma, serum, CSF, BAL, urine, biopsies, amniotic fluid)				
	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	1 250 000	2 500 000	4 000 000	1 250 000	1 500 000
QS2	125 000	250 000	400 000	125 000	150 000
QS3	12 500	25 000	40 000	12 500	15 000
QS4	1 250	2 500	4 000	1 250	1 500

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 all the fluorescence curve of all the samples 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 CMV 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 the 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 the fluorescence curves of all 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 CMV 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 Applied Biosystems

- Make sure that none is selected in the Passive reference field because the CMV 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 CMV 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.

12.5 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 CMV 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.

12.6 WITH STRATAGENE® Agilent or Versant® kPCR Molecular System AD

- Make sure that none is selected in the reference Dye field because the CMV R-gene® **R5** 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 CMV 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.

12.7 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** and **R0** must not give any signal (CT) at 530 nm.

2nd CONDITION: **IC2W0** must be equal to 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 **30** and **35** cycles at **530** nm.

QUANTIFICATION				QUALITATIVE DETECTION
Real Time PCR Platform	CT QS3	Valuable Slope/ Efficiency		
	CMV	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	30 – 35 cycles	-3.917< Slope <-3.103	-3.587< Slope <-3.208	*Not aplicable*
LightCycler® 2.0 / LightCycler® 480		1.8 < Efficiency < 2.1	1.9 < Efficiency < 2.05	
Rotor-Gene®		0.8 <Efficiency< 1.1	0.9 < Efficiency < 1.05	
Applied Biosystems		-3.917< Slope <-3.103	*not aplicable*	
Stratagene®, Agilent, Versant® kPCR Molecular System AD		0.8< Efficiency < 1.1		
Dx Real-Time System		0.8< E < 1.1		

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

13.2 RESULT INTERPRETATION

- 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.

13.2.1 For amniotic fluid

First step: **Undiluted** sample analysis			Second step: Sample diluted to **1:100** analysis		
UNDILUTED SAMPLE			**SAMPLE DILUTED TO 1:100**		
CMV +/-	**IC2sample** conform/not conform*	**CONCLUSION 1**	**CMV +/-**	**IC2sample** conform/not conform*	**CONCLUSION 2**
+	conform	Sample validated as positive. Validated quantification.	**No complementary analysis needed**		
-	conform	Sample validated as negative.			
+	not conform	Sample validated as CMV positive. To obtain quantification results , see complementary analysis (analysis of the sample diluted to 1:100).	**+**	conform	Sample validated as positive. Quantification of the sample diluted to 1:100 is validated.
			+	not conform	Sample validated as positive. To obtain quantification results, dilute extracted DNA (extracted DNA from undiluted sample and extracted DNA from sample diluted to 1:100) to 1:10 and perform amplification step once again.
			-	conform	
			-	not conform	
-	not conform	See complementary analysis (analysis of the sample diluted to 1:100).	**+**	conform	Sample validated as positive. Quantification of the sample diluted to 1:100 is validated.
			+	not conform	Sample validated as positive. To obtain quantification results, dilute extracted DNA (extracted DNA from undiluted sample and extracted DNA from sample diluted to 1:100) to 1:10 and perform amplification step once again.
			-	conform	Clinical sample contains inihibitory agents. Perform once again extraction step from undiluted sample.
			-	not conform	Clinical sample contains inihibitory agents. Perform once again extraction step from undiluted sample and from sample diluted to 1:100.

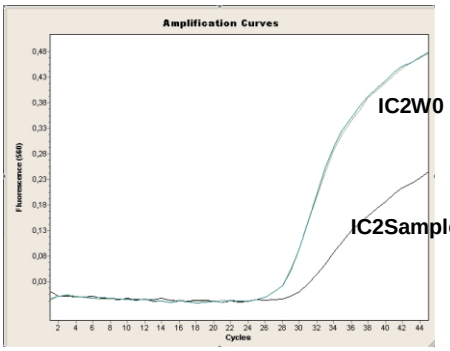
*__IC2sample conform__: CT [IC2sample]≤ CT [IC2W0] + **3** cycles
*__IC2sample not conform__: CT [IC2sample]> CT [IC2W0] + **3** cycles

WARNING: **If the valid quantification is that obtained starting from a dilution of the sample (1:10 or 1:100 or 1:1000) . the diluted sample must be restored to the dilution factor (x10, x100 or x1000).**

13.2.2 For the other specimens

Extraction+Inhibition control **(IC2sample)**	CT [IC2sample]≤ CT [IC2W0] + **3** cycles		CT [IC2sample]> CT [IC2W0] + **3** cycles	
	NON INHIBITED SAMPLE and correctly extracted		**INHIBITED SAMPLE and/or poorly extracted**	
Sample	Calculated CT	Non calculated CT	Calculated CT	Non calculated CT
CMV quantitative Interpretation	Sample validated as positive Validated quantification	Sample validated as negative	Sample validated as positive Perform quantification again	**Not Valid**
CMV 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 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 CMV R-gene® kit to other diagnostic investigation methods in order to define patient viral status**.**

13.3 Results expressed in International Units:

Note: The conversion factor has been determined on the basis of the 1st CMV International Standard (NIBSC 09/162).

- The results obtained in copies/mL can be converted into International Units (IU)/mL.
- For this purpose, a conversion factor must be defined. This conversion factor depends on the type of sample tested and the extraction and amplification instruments used.
- Studies carried out with the CMV R-gene® kit determined the various conversion factors for the following combinations of devices and samples:

	PLASMA					
Extraction / Plateformes PCR	LC2	LC480	ABI7500 Fast	RotorGene	Dx Real-Time System	Stratagene Agilent Versant kPCR Molecular System AD
NucliSENS easyMAG®	**0,169**	**0,308**				

Example: For a sample of plasma extracted with NucliSENS easyMAG® and amplified on a LightCycler 480, the conversion factor for copies/mL converted into IU/mL is **0.308**.

Number of IU/mL = Number of cp/mL x 0.308

i.e. for a sample determined at 10 000 cp/mL, the corresponding quantification is 3 080 IU/mL.

Nevertheless, each laboratory must establish its own conversion factor.

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 sample collection , transport and storage in the laboratory.	• See chapter "Sample treatment and transport" that defines the optimal conditions (temperature, time) for transport and storage. • Check the delay between sample collection and the beginning of its analysis.
Transport and storage of CMVR-gene® kit conditions were not met	• Follow instructions in section "Content of the kit and storage" regarding the storage of R-gene® kit 69-003B 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 DNA Extraction kit (ref.67-000). See section "DNA EXTRACTION KIT". • Check if material and protocol used to extract sample corresponds to material and protocol recommended for analysis with CMV R-gene® kit 69-003B (See section "Extraction protocol"). • 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 adjustment of the baseline noise line. • In the case of an analysis based on range importation, check that the range imported 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 "Validation and interpretation of results"). • 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. • On LightCycler® 2.0 : Make sure the results obtained were corrected using a valid colour compensation file created in advance using kit ref.: 71-103 • 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 "Result interpretation"). 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 "Warnings and precautions". • Decontaminate the cooling block for capillaries with U.V. light. • Respect the manufacturer's recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • The CMV 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 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 adjustment of the baseline noise line. • In the case of an analysis based on range importation, check that the range imported is valid.
Error in results interpretation	• Check the validity of the results obtained in the experiments (check all the validation conditions described in section "Validation and interpretation of results"). • 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. • On LightCycler® 2.0 : Make sure the results obtained were corrected using a valid colour compensation file created in advance using kit ref.: 71-103 • 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 "Result interpretation"). 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 assay

WARNING: **The described performances of the CMV R-gene® kit can only be guaranteed for use on the use recommended extraction systems and PCR instruments.**

15.1 INTRA-EXPERIMENTAL REPRODUCIBILITIES OF THE CMV R-gene ® KIT ref. : 69-003

The study of intra-experimental reproducibility of the CMV R-gene® kit was carried out on three whole blood samples reconstituted from the "CMV sample 1 WHO" (strain HCMV Merlin).). The CMV WHO sample was diluted in whole blood and tested negative to CMV at the following 3 concentrations: 750 000, 75 000 and 7 500 copies/mL. Each dilution was extracted 10 times during a single series of extraction by NucliSENS easyMAG using the Specific B protocol (140 µL of silica) with 200µL of sample and an elution in 50µL.
The 30 extracts obtained were amplified on the Dx Real Time System (Bio-Rad) during a single series.
The results obtained are shown in the following table:

		Extraction NucliSENS easyMAG whole blood V1			
	Quantification WHO (cps/mL)	Mean quantification (cps/mL)	Log10 Mean quantification	Standard Deviation (log 10 Mean quantification)	Coefficient of variation (°/°)
CMV sample 1 WHO	750 000 copies CMV /mL	375267,3	5,57	0,08	1,39%
	75 000 copies CMV /mL	41709,61	4,62	0,06	1,41%
	7 500 copies CMV /mL	6609,05	3,76	0,22	5,72%

The results show coefficients of variation between 1.39% and 5.72% for a NucliSENS easyMAG extraction using the Specific B protocol (140 µL of silica), demonstrating good repeatability of the CMV R-gene kit®.

15.2 INTRA-EXPERIMENTAL REPRODUCIBILITIES OF THE CMV R-gene ® KIT ref. : 69-003

The study of intra-experimental reproducibility of the CMV R-gene® kit was carried out on three whole blood samples reconstituted from the "CMV sample 1 WHO" (strain HCMV Merlin).). The CMV WHO sample was diluted in whole blood and tested negative to CMV at the following 3 concentrations: 750 000, 75 000 and 7 500 copies/mL. Each dilution was extracted 5 times during 5 independent extraction and amplification series. The extraction took place on the NucliSENS easyMAG using the Specific B protocol (140 µL of silica) with 200µL of sample and elution in 50µL. Amplification was carried out on the Dx Real Time System (Bio-Rad).
The results obtained are shown in the following table:

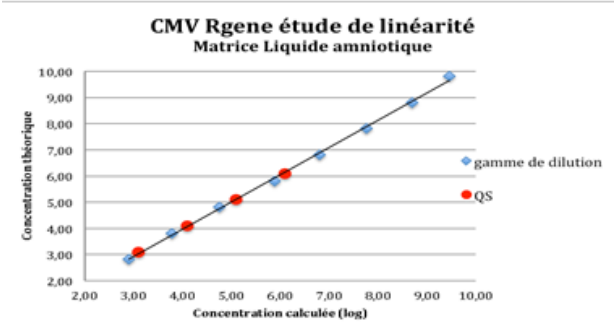
		Extraction NucliSENS easyMAG whole blood V1			
	Quantification WHO (cps/mL)	Mean quantification (cps/mL)	Log10 Mean quantification	Standard Deviation (log 10 Mean quantification)	Coefficient of variation (°/°)
CMV sample 1 WHO	750 000 copies CMV /mL	270153,2	5,43	0,03	0,61%
	75 000 copies CMV /mL	27231,6	4,43	0,08	1,70%
	7 500 copies CMV /mL	2584,8	3,4	0,1	3,09%

The results show coefficients of variation between 0.61% and 3.09% for NucliSENS easyMAG extraction using the Specific B protocol (140 µL of silica), demonstrating good reproducibility of the CMV R-gene kit®.

15.3 STUDY OF THE CMV LINEARITY RANGE:

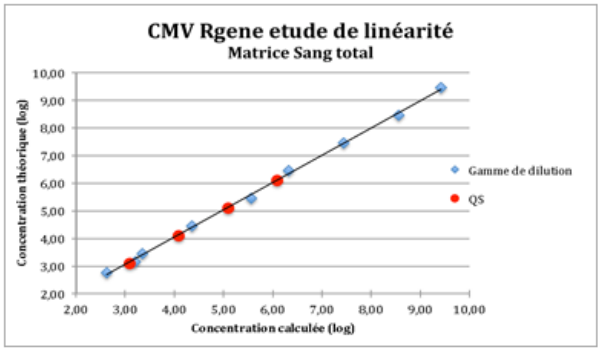
One of the problems posed by a CMV diagnosis is the wide difference in viral loads found in the sample, particularly for samples of amniotic liquid.
This broad range of viral load in the samples, particularly amniotic liquid, has led us to verify the linearity of our quantification range beyond the first point in the range, i.e. $2.5.10^6$ copies/mL for whole blood matrices (the most widely used matrix) and amniotic liquid.
For this purpose, a culture sample of MRC5 infected with positive strain CMV AD169 was diluted before extraction in amniotic fluid or negative whole blood. The dilution range covers 8 log (from 1.43 log to 9.43 log).
The extraction unit used was the easyMAG and amplification took place on the Dx Real-Time System.
The results are shown in the following graphs:

Whole blood linearity range :



Quantification of CMV using whole blood as the matrix, using the combination of Biorad Dx Real Time System/NucliSENS easyMAG devices, was linear from 9.43 log(Cp/mL) to 2.63 log(Cp/mL).

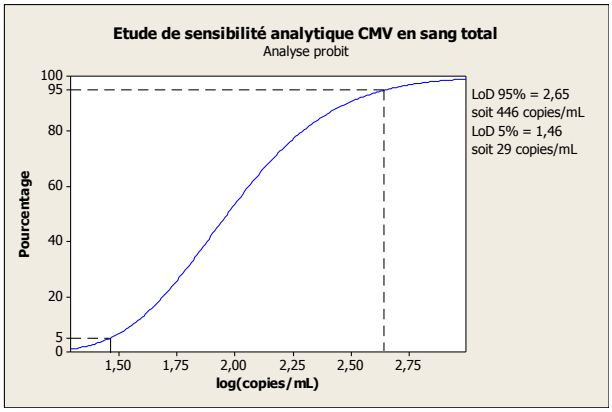
Linearity range in amniotic fluid :



Quantification of CMV using amniotic fluid as the matrix, with the combination of Biorad Dx Real Time System/NucliSENS easyMAG devices, was linear from 9.45 log(Cp/mL) to 2.91 log(Cp/mL).

15.4 ANALYTICAL SENSITIVITY OF CMV R-GENE®

The analytical sensitivity or detection limit of the CMV R-gene kit® was determined on a range of WHO panel dilutions (strain HCMV Merlin) in whole blood.The samples were diluted in EDTA whole blood previously tested negative for CMV.
These samples contained from 1 to 1.10^4 copies/mL.
Each dilution was extracted 15 times using the NucliSENS easyMAG automatic system in accordance with the Specific B 2.0.1 protocol (140 µL de silica), Sample vol.: 200µL, Elution vol.: 50µL and Silica vol.: 140µL.
The 15 extracts obtained for each dilution and extraction protocol were amplified on the Dx Real Time System (Bio-Rad) with the CMV R-gene® kit ® (69-003).



The curve opposite shows the probit analysis of the results obtained:

The results indicate there is:
- 95% probability of detecting the CMV virus in whole blood containing 446 copies/mL
- 5% probability of detecting the CMV virus in whole blood containing 29 copies/mL.

15.5 ANALYTICAL SPECIFICITY OF CMV R-GENE®

The specificity of the CMV R-gene® primers and probes were tested on the following viruses :
- Human Herpesvirus : HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, HHV-8.
- Human Polyomavirus : JCV and BKV.
- Adenovirus 12.

⇒ No viral amplification has been observed with any of these pathogens, except CMV. There was no cross-reaction.

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

15.6 2010 TEST REPORT ON QCMD PANELS

On the occasion of European CMV and HHV6 control campaigns conducted in 2010 by QCMD, 10 samples for the CMV panel and 10 for the HHV-6 panel were blind tested with the CMV HHV6, 7, 8 R-gene® kit.
200µL of each sample are extracted with the NucliSENS easyMAG then amplified on ABI Prism 7500 Fast with the specific premix amplification corresponding to the targeted searches for each of the panels.

⇒ 100% (10/10) of the samples tested over all panels were concordant with expected results.

	Sample Content	QCMD Results		CMV R-gene® Results		Delta Log
		cp/mL	Log	cp/mL	Log	
CMV 10-01	CMV (AD169)	1 879	3.27	2 692	3.43	0.3
CMV 10-02	CMV (AD169)	230	2.36	298	2.47	0.31
CMV 10-03	CMV (AD169)	275 423	5.44	552 077	5.74	0.3
CMV 10-04	CMV (AD169)	5 534	3.74	6 918	3.84	0.09
CMV 10-05	Négative	-	-	-	-	-
CMV 10-06	CMV (AD169)	2 552 701	6.40	5 116 818	6.7	0.3
CMV 10-07	CMV (AD169)	1 799	3.25	3 499	3.54	0.28
CMV 10-08	CMV (AD169)	23 988	4.38	25 706	4.41	0.03
CMV 10-09	CMV (AD169)	684	2.83	333	2.52	0.03
CMV 10-10	CMV (AD169)	16 904	4.22	24 717	4.39	0.16

With the CMV 2010 panel, 9 samples of 10 were CMV positive.

The negative sample is confirmed negative by the kit.

The detection of samples of low viral load of CMV (CMV10-02 at 230 copies/mL and CMV10-09 at 684 copies/mL) attests to the high sensitivity of the kit.

In terms of quantification, one observes an excellent correlation with the QCMD results with the delta log between 0.03 and 0.31.

15.7 CLINICAL STUDY

1- Retrospective clinical study on 117 whole blood samples tested in the University Hospital of Caen (France).

Major part of the samples were collected in patients with renal transplant. The samples were previously characterized with CMV ppUL83 antigenemia method (Cinakit).

CMV DNA samples were extracted using DNA EXTRACTION Kit (provided with ref. : 69-003) then simultaneously tested on LightCycler® 2.0 with routine laboratory real time PCR* and CMV R-gene® kit (ref. : 69-003).

*Quantitative analysis of HCMV DNA load in whole blood of renal transplant patients using real-time PCR assay_Gouarin S, et al. J Clin Virol. 2004 Mar;29(3):194-201

Comparison of the results obtained with CMV ppUL83 antigenemia assay and CMV R-gene®:

		Antigenemia ppUL83 Cinakit Argene		
		+	-	
CMV R-gene™	+	86	20	106
	-	4	7	11
Total		90	27	117

79 % of the results are in agreement.

This result confirms that CMV R-gene® kit enables an earlier CMV detection than CMV antigenemia assay . The analysis of a part of the discordant results was performed by comparing results obtained with the in-house real time PCR to those obtained with CMV R-gene® kit. The comparison of the results with both methods are displayed in the following table.

Comparison of the results obtained with in-house real time PCR and CMV R-gene®. (Gouarin S, et al. JCV 2004; 29 : 45-52)

		In House Real Time PCR		
		+	-	
CMV R-gene™	+	105	1	106
	-	7	4	11
Total		112	5	117

93% of the results are in agreement. This comparison allows to confirm :

-19 of 20 positive samples with CMV R-gene® were previously tested negative with CMV antigenemia Cinakit.

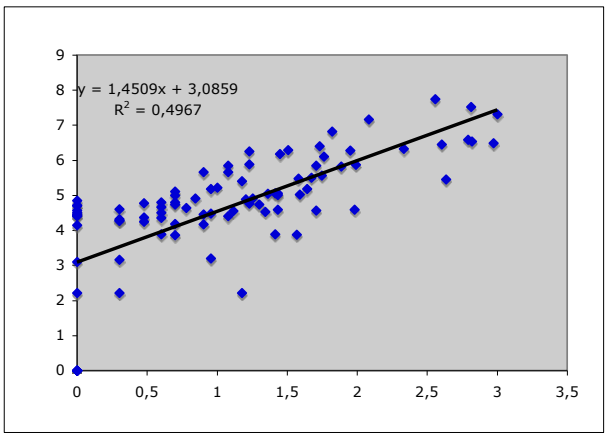
-1 positive sample with CMV antigenemia Cinakit (1 positive nucleus/200 000 cells)

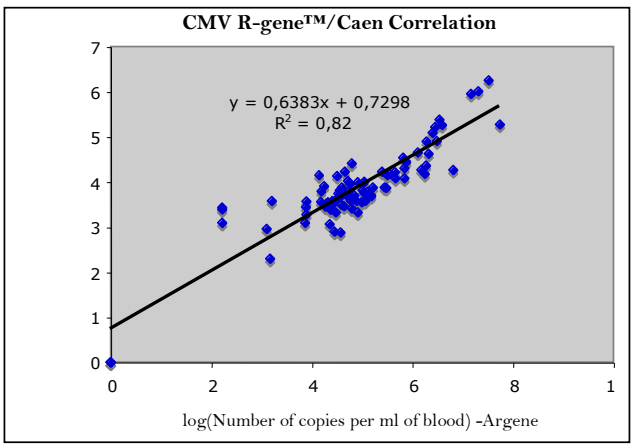
- 1 negative sample with both real time PCR assay.

The 4 other discordant results (2 samples < 5 nuclei/200 000 cells and 2 samples > 5 nuclei/200 000 cells) between Cinakit and CMV R-gene® were weak positive samples using in house real time PCR (< 3 100 copies/ml).

The latter 4 samples will be tested again using the 3 assays. Their corresponding inhibition controls will be analysed taking account the clinical background.

Quantitative analysis of positive samples obtained with the 3 assays

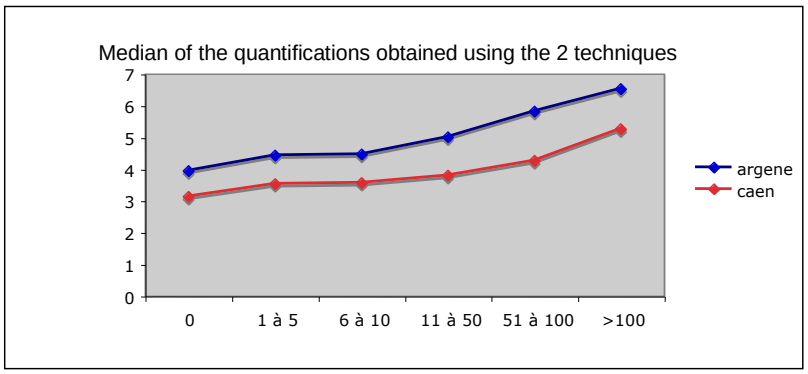




Comparison of the quantifications obtained with the Argene kit and the antigenemia ppUL83 assay shows a relative dispersion of the values. On the other hand, comparison of the quantifications obtained using the two real-time PCR techniques shows a much better correlation.

A quantification difference of about 0.7 log clearly appears between the Caen and Argene techniques, as shown in the figure below.

log(Nu



log(c

2- Clinical study on 218 whole blood samples tested in the Department of Virology in St Louis Hospital (Paris, France).

The samples were collected in patients with bone marrow transplant.

Viral DNA samples are extracted using MagnaPure® LC instrument then simultaneously tested on ABI PRISM 7500HT® with routine laboratory real time PCR and CMV R-gene® kit (Argene ref. : 69-003).

Comparison of the CMV results obtained with in-house real time PCR and CMV HHV6,7,8 R-gene®:

96% of qualitative results are in agreement.

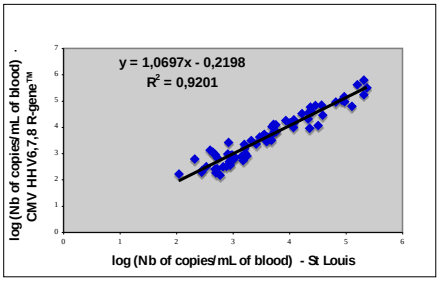
		In House Real Time PCR		
		+	-	
CMV HHV,6,7,8 R-gene™	+	73	1	74
	-	7	137	144

All the 7 negative results with CMV R-gene® but positive with in house real time PCR correspond to samples with low viral load (less than 413 copies/ml). Taking account that the sensitivity threshold of CMV R-gene® is 500 copies/ml, it's statically probable to obtain negative result on sample with CMV viral load which is under 500 copies/ml.

The same argument explains how a sample (289 copies/ml) is tested positive with CMV R-gene® but negative with in-house real time PCR.

Analysis of the CMV results using clusters of quantification.

In House Real Time PCR	CMV HHV6,7,8 R-gene™					
DISPATCHING OF THE RESULTS	Nb of samples	0 to 3000 copies/mL	3000 to 25 000 copies/mL	25 000 to 250 000 copies/mL	250 000 to 2 500 000 copies/mL	> 2 500 000 copies/mL
0 to 3000 copies/mL	180	178	2			
3000 to 25 000 copies/mL	25		21	4		
25 000 to 250 000 copies/mL	13			9	3	
250 000 to 2 500 000 copies/mL	0				0	
> 2 500 000 copies/mL	0					0
TOTAL	218	178	24	13	3	0



95.41% (208/218) of the CMV clusters of quantification are in agreement. The analysis of all discordant results shows a difference of quantification lower than 0.5 log between in house real time PCR and CMV R-gene®.
Taking account of the intra and inter assay reproductibility of the CMV R-gene® kit, a difference of 0.5log between 2 results cannot be considered as significative.

Difference of CMV quantification between CMV HHV6,7,8 R-gene® and in house real time PCR assay

Quantification				Delta Log
RT-PCR du Laboratoire		CMV HHV6,7,8 R-gene™		
Copies/ml	Log	Copies/ml	Log	
2100	3,32	3131	3,50	-0,17
2935	3,47	4212	3,62	-0,16
16 549	4,22	33 862	4,53	-0,31
21 700	4,34	35 602	4,55	-0,22
23 151	4,36	58 593	4,77	-0,40
24 629	4,39	38 474	4,59	-0,19
32 056	4,51	11 912	4,08	0,43
158 391	5,20	424 381	5,63	-0,43
206 004	5,31	630 596	5,80	-0,49
231 817	5,37	317 915	5,50	-0,14

Though the 2 assays ampiify 2 different regions of the CMV genome, the CMV results obtained with both assays are totally correlated.

3- Retrospective clinical study on 74 whole blood samples tested at the Department of Virology in Toulouse Hospital (France).

Major part of the samples were collected in patients with renal transplant. The samples were previously characterized with in house real time PCR*.

CMV DNA samples are extracted using DNA EXTRACTION KIT (provided with ref. : 69-003) then tested on LightCycler® 2.0 with CMV R-gene® kit (ref. : 69-003)**.**

*Automated Extraction and Quantification of Human Cytomegalovirus DNA in Whole Blood by Real-Time PCR Assay_ Mengelle et al. J Clin Microbiol 2003, 41, 3840-3845)

Comparison of the results obtained with in-house real time PCR and CMV R-gene®:

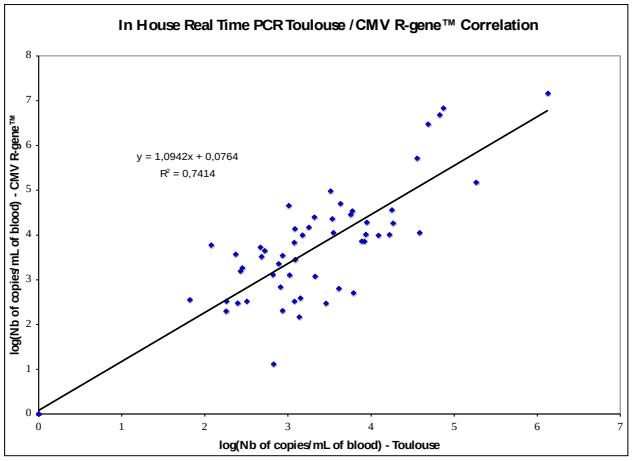
		In House Real Time PCR +	In House Real Time PCR -	
CMV R-gene™	+	54	8	62
	-	8	4	12
Total		62	12	74

78% of the results are in agreement.

Of 16 discordant results, 13 results corresponded to samples with weak viral load (less than 1000 copies/ mL of whole blood).

Quantitative analysis of positive samples obtained with the 2 assays (in-house real time PCR and CMV R-gene®) :

The slope of the straight regression line is 1,09. This value proves the good correlation between both real time PCR assays.



4 - Retrospective study on amniotic fluid at the Necker University Hospital Centre's Virology Laboratory – Associated laboratory: Cytomegalovirus National Reference Centre (Public Assistance – Paris Hospitals)

138 samples of amniotic fluid from pregnant women for whom CMV diagnostics had been requested were tested using the laboratory's real-time PCR technique (Leruez *et al.* J Clin Microbiol 2003 May; 41(5): 2040-6) and using the CMV HHV6,7,8 R-gene® kit. Of these samples, 38 were characterized as positive in terms of CMV and 100 as CMV negative (including 2 which were positive for parvovirus B19) in first line treatment with the reference technique.

200 µL of samples were extracted and eluted in 100µL with the MagNa Pure automatic device and then amplified on ABI Prism. Each sample was tested pure and diluted to 1/10th.

The results of the retrospective study show a 99% concordance between the 2 techniques.

The only aberrant sample had a viral load at the sensitivity limit and, moreover, was found to be negative using both techniques during a second test.

		RT – PCR Necker +	**RT – PCR Necker** -
R-gene® kit	**+**	**37**	**0**
	-	**1**	**100**

Out of 100 samples, being previously detected as negative, one sample appeared to be inhibited. Due to limited available equipment this sample could not be tested again.

Out of 37 samples, being previously detected as positive, an unexpected number of 24 samples appeared to be inhibited. In fact, the extreme high CMV viral load used up the amplifcation reagent entirely. As a result the inhibition control was either negative or outside the scope of the test specifications. This observation clearly proves that the CMV viral load in amniotic fluid samples can be extremely high.

Of these 24 samples, 19 extracts were in adequate volumes to allow further tests on 1/100th and 1/1000th dilutions. The 1/100th or 1/1000th dilutions of extracts were used to lift inhibitions due to excess CMV and quantify the samples.

Collecting the results allows us to define a specific protocol for the amniotic liquid, allowing in the first place to give a qualitative result sytematically and (sometimes quantitative as well) then in the second place a quantitative result, such as is explained in chapter "For amniotic fluid**"**.

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17. Related products

- DICO Ampli r-gene® ref. : 71-100
- DICO Extra r-gene® ref. : 71-101
- Colour Compensation r-gene® ref. : 71-103
- CELL Control r-gene® ref. : 71-106
- CMV Positive Control r-gene® ref. : 68-015

18. Table des symboles

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 0459	Identification of notified body

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