BK Virus R-gene®

REF 69-013

REF 69-013B

NEW EXTRACTION PROTOCOL NucliSENS easyMAG:

« Whole Blood Viral Extraction Protocol »

CE

IVD

COMPOSITION

REF 69-013	**Extraction Kit, DNA EXTRACTION KIT**	**Ref.: 67-000**
	BK Virus R-gene® real-time detection and quantification kit	**Ref.: 69-013B**

1. Presentation of the kit

BK virus, a polyomavirus belonging to the family of papovaviridae, particularly infects the human population (seroprevalence 60 to 100%).
Primary infection is asymptomatic and migrates through the respiratory tract during early childhood. Hereafter, the virus migrates to the primary sites of latency, such as the kidney and the urothelium.
In general, the prevalence of BK virus in urine lies between 0.3% and 6%, but this prevalence increases with the level of immunosuppression.

The reactivation of symptoms is associated with urinary tract infections and manifests itself as hemorrhagic cystitis (in bone marrow transplant patients), urethral stenosis, tubulo-interstitial nephropathy and interstitial nephritis (in kidney transplant patients). Interstitial nephritis is an opportunistic and emerging renal tropism infection and is the major cause of organ rejection. In this stage the virus can then be detected in both blood and urine samples.

A virological diagnosis is obtained by a search for the specific virus. Serological diagnosis only has a limited application due to the high seroprevalence of polyomavirus antibodies. Electronic microscopy and urine cytology remain the major techniques to put in place.

The BK Virus R-gene® kit enables the quantification of BK virus in Real Time PCR after viral DNA extraction. BK Virus R-gene® is a user-friendly and complete kit and therefore ideal for routine diagnostics.
The kit has been clinically validated on many types of specimen in combination with numerous DNA purification systems (automatic and manual) and amplification platforms.
Thanks to a general amplification program for the entire range of R-gene® kits, the sample analysis can be simultaneously run for all other DNA viruses, Adenovirus with Adenovirus R-gene® kit (ref.: 69-010B), CMV with CMV R-gene® kit (ref.: 69-003B), CMV, HHV-6, HHV-7 and HHV-8 with CMV HHV6,7,8 R-gene® kit (ref.: 69-100B), EBV with EBV R-gene® kit (ref.: 69-002B), HSV-1, HSV-2, and VZV with HSV1 HSV2 VZV R-gene® kit (ref.: 69-004B).
Results are validated with all necessary controls (including an internal extraction control) provided with the kit.

2. Intended use

BK Virus R-gene® measures the viral load of BK virus in whole blood, plasma and urine samples. 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.

This kit cannot be used for screening donors.

Combined with other methods of biological investigation (medical imaging, biochemical and immunological analysis, etc.), the results obtained with the kit BK Virus R gene® enable to follow the progress of and to improve the therapeutic efficacy.

The quantification of BK virus with the BK Virus R-gene® kit can be carried out simultaneously, with viral load measurement using the EBV R-gene® kit (ref.: 69-002), Adenovirus viral load measurement using the Adenovirus R-gene® (ref. : 69-010) and CMV, HHV-6, HHV-7, HHV-8 viral load measurement using the CMV HHV6,7,8 R-gene® kit (ref. : 69-100) and HSV-1, HSV-2 and VZV viral load with HSV1 HSV2 VZV R-gene®.
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

- BK virus R-gene® measures the viral load of BK virus in whole blood, plasma and urine samples.
- The quantitative results are reported in copies/mL. The quantification range for BK virus is linear with $2{,}5.10^3$ copies/mL to 10^{11} copies/mL.
- 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 BK Virus R-gene® kit.
- BK Virus R-gene® provides all reagents to analyze 87 samples.

3.2 VIRAL DNA PURIFICATION

- The following DNA extraction methods are validated with the BK Virus R-gene® kit ref. : 69-013B:
  - MagNA Pure Compact Instrument® and MagNA Pure LC System®.
  - MagNA Pure 96 System
  - NucliSENS® easyMAG®
  - QIAsymphony SP
  - Versant® kPCR Molecular System SP
  - QIAcube.
  - QIAamp® DNA Blood Mini kit.
  - DNA EXTRACTION KIT. (supplied under reference 67-000 of ref. 69-013).
- The target DNA contained in the sample and in the extraction + inhibition control (**IC2**) are extracted using one of the extraction methods above.
- The technique used by the DNA EXTRACTION KIT (Ref.: 67-000) associates the selective binding properties of silica gels with a microcentrifugation speed. The sample and internal control (**IC2**) are first lysed with protease in order to optimize the DNA binding capacities on the membrane. The use of the silica column allows, after DNA coating, efficient washing of the sample to eliminate contaminants. After elution, DNA is suitable for direct use in amplification techniques.

3.3 REAL TIME AMPLIFICATION AND QUANTIFICATION

- The principle of the real-time amplification utilizes the 5' nuclease technology TaqMan® (Patents N°5210015, 5487972). Amplification premix is provided ready-to-use with dNTP, amplification buffer, Taq polymerase , probes specific to each virus and primers and probes for an internal control (**IC2**) which goes through the extraction step (before lysis).
- The following range of real time PCR platforms are validated with the BK Virus R-gene® kit ref. : 69-013B:
  - All LightCycler®
  - All Applied Biosystems
  - SmartCycler® 2.0
  - 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.
- The size of the amplified fragment is 158 bp in size and is located in the StAg area.
- A range of 4 quantification standards is provided with the BK Virus R-gene® kit (**QS1, QS2, QS3, QS4**). The quantification standards range from 5 000 copies/µL to 5 copies/µL corresponding to 50 000 copies to 50 copies of plasmid per PCR. The quantification standards are used to generate a new standard curve in the software provided with the thermocycler. The quantification of BK virus 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 50 copies/µL of standard DNA corresponding to 500 copies plasmid per PCR. **QS3** can be used to import a previously created standard curve (provided that conditions described in chapter "The quantification standard (QS3) » are met). It's also used as a positive control for qualitative detection of BKV.
- **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 BK Virus 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 the 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 BK Virus R-gene® DETECTION AND QUANTIFICATION KIT 69-013B

- 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** BKV	0.3 mL
QS2	Quantification standard **2** BKV	0.3 mL
QS3	Quantification standard **3** BKV	0.3 mL
QS4	Quantification standard **4** BKV	0.3 mL
SC	Sensitivity control BKV	0.3 mL
R13	BKV and IC2 Amplification premix	3x0.450 mL

- Keep the kit, ref. : 69-013B before and after first opening, frozen at -18°C/-22°C in the dark until the expiration date written on the box.
- The reagents **QS1**, **QS2**, **QS3**, **QS4**, **SC**, **IC2** and **W0** must be stored in the extraction room at -18°C/-22°C before and after first opening. The reagents **R13** and **R0** must be stored in the room reserved for the preparation of the premix at -18°C/-22°C.
- Each premix cannot undergo more than 7 freezing/defrosting cycles.
- Return the amplification premix (**R13**) to -18°C/-22°C immediately after use.

5. Material and reagents required but not supplied

5.1 FOR SAMPLE EXTRACTION

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

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

5.1.2 Other extraction methods validated

- Follow the manufacturer's instructions.

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

- Micropipets with plugged (aerosol barrier) tips or positive displacement tips.
- Thermocyclers validated with BK Virus 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 or microplates for real time PCR platfroms validated for BK Virus 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

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

6.3 AW1 buffer (D) preparation

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

6.4 AW2 buffer (E) preparation

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

7. Warnings and precautions

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

7.1 GENERAL WARNINGS AND PRECAUTIONS:

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

7.2 WARNINGS AND PRECAUTIONS FOR MOLECULAR BIOLOGY:

- Amplification procedures require highly skilled techniques to avoid risk of sample contamination:
  - Use separate working places for sample preparation and amplification reactions. Movement in the laboratory must be in one direction only from the reagent preparation area to the amplification area. Allocate a set of lab coats and pipettes to each area. Never introduce an amplified product in reagent and/or sample preparation areas.
  - Pipettes used to handle samples are reserved for this purpose only. These pipettes must be positive displacement pipettes or pipettes equipped with filter tips. All tips must be sterile.
  - The pipettes used to aliquot reagents must be reserved only for this purpose. The necessary reagents for amplification are aliquoted in order to be used during one single experiment.
  - Tubes from different specimens and amplification premix must never be opened at the same time.
  - Used samples must be exclusively reserved for this analysis.

- Do not use reagents after expiration date printed on the labels.
- Do not 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**) 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.*

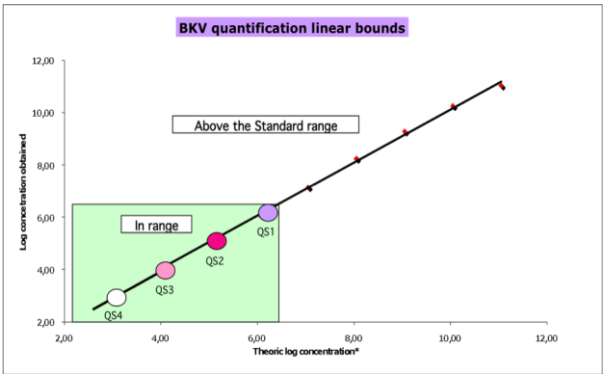
***560 nm** = the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

***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

- The use of the internal quantification standard range is imperative for sample quantification.
- The quantification standards 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 linearity of results is maintained beyond the standard quantification range. BK virus quantification is perfectly linear from $2.5.10^3$ to 1.10^{11} copies/mL (See graph opposite).
- The quantification standards must be designated as « standard » and their values must be entered when samples are defined in the table in the data analysis software.
- 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.
- 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 real time PCR instrument does not allow the importation of the standard curve.

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

8.3 SENSITIVITY CONTROL (SC)

- The sensitivity control (**SC**) validates the performance of the assay and should be considered as a run-control.
- The sensitivity control (**SC**) is amplified with the **R13** 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.
- Signal reading at 530 nm.

8.4 EXTRACTION + INHIBITION CONTROLS

8.4.1 The sample extraction + inhibition control (IC2sample)

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

8.4.2 The reference extraction + inhibition control (IC2W0)

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

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

8.5 NEGATIVE CONTROLS

8.5.1 Negative extraction + amplification control (IC2W0)

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

8.5.2 Negative amplification control

- The negative amplification control consists of reagent (**R0**) amplified in the amplification premix (**R13**).
- This control shows any possible contamination during the amplification step.
- The use of this control is optional.

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

9. Sample treatment and transport

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

9.1 SAMPLE TRANSPORT

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

9.2 SAMPLE COLLECTION

9.2.1 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.
- Period between blood collection and arrival in 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

- 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 (200 µL minimum) plasma is decanted under a 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 Urine samples

- Urine samples are collected in a sterile pot (e.g. urine cytology test pot).
- If the urine is not treated when it arrives in the laboratory, it should be stored at +2°C/+8°C for a maximum of one week. Beyond this timeframe, store urine at -18°C/-22°C.

10. Sample extraction protocol

WARNING : **Before starting the extraction procedure, make sure samples and 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 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. 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 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.
- 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 whole blood and urine samples, add **100 µL** of balanced elution buffer AE (**F**) at room temperature.
- For samples of plasma samples, 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 WITH EXTRACTION INSTRUMENTS and/or KITS VALIDATED WITH BK Virus R-GENE® EXTRACTION KIT

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

INSTRUMENT	Kit	Sample + IC2 Volumes	Sample Type	Protocole	Elution volume
	QIAamp® DNA Blood Mini kit Ref.: 51 104 / 51 106	200 µL of sample + 10 µL IC2	Whole blood, Urine		100 µL
			Plasma		50 µL
QIAcube QIAGEN ref. : 9001292 / 9001293			Whole blood, Urine	Blood and body fluid spin protocol V3	100 µL
			Plasma		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, Urine	DNA_Blood_100_400	100 µL
			Plasma	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	100µL
			Plasma Urine	Total NA Variable_elution_volume	50µL
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	Viral NA Universal SV	100 µL
NucliSENS® easyMAG® bioMerieux Ref.: 280110	NucliSens® easyMAG® Reagents		Whole Blood New	The Manufacturer's Specific B with 140 µL silica(1)	50 µL
			Whole Blood	Manufacturer's Specific B protocol with 140 µL silica and 2 ml lysis buffer.	50 µL
			Plasma	Specific B + 50 µL silica	50 µL
			Urine		100µL
QIAsymphony SP	QIAsymphony DNA Mini Kit	300 µL of sample + 10 µL IC2(2) (*extract 200 µL*)	Whole Blood	Virus Blood DefaultIC	90 µL (*eluate 60µL*)
Versant® kPCR Molecular System SP SIEMENS Ref.: 06635740	Versant® Sample Preparation 1.0	400 µL of sample + 10 µL IC2(3) (*extract 250 µL*)	Plasma	Sample Preparation Protocol 5	65 µL (*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 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 Quantification protocol

Note : To simplify this manual, the device containing the amplification reaction mix will be referred to 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 "The quantification standard QS3 ").
- Plan :

- 1 tube per tested sample;
- 1 or 4 tube(s) for BK virus quantification standard curve imported/created
- 1 tube for QS3 as positive control in the event of qualitative detection
- 1 tube for BK virus sensitivity control (**SC**)
- 1 tube for reference extraction + inhibition control (**IC2W0**) also used as negative control for extraction and amplification.

Notes: - *When UNG is used, please refer to the protocols and programs described in the technical document of the product ref.65-001.*
- *Use the transparent plates (ref.: HSP9601) with the optical stoppers (ref.: TCS0803) for the Dx Real-Time System amplification device.*

11.1 PROGRAM

- Regardless the real time PCR platform used, the amplification program remains the same. The amplification program is decribed in the table below.

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

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

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

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

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

Note 4: On LightCycler® 2.0, 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 BK Virus 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:
- Homogenize thawed reagents by vortexing or by pipetting, then briefly centrifuge.
- Mix each reagent (to a vortex for 2 seconds or through successive pipetting) and centrifuge briefly.
- Make sure the cooling block was decontaminated by exposure under U.V. light for 30 min.
- Make sure the cooling block was correctly pre-cooled at +2°C/ +8°C.

WARNING: **To avoid contamination as much as possible, close the tubes as soon as distribution is completed.**
Replace the amplification premixes (R13), quantification standards (QS) and sensitivity control (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 (**R13**) by gently homogenizing with the pipet in order to distribute the same volume in all tubes.

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

- Add 10 µL of each extracted sample in the corresponding tubes.
- Add 10 µL of the sensitivity control (**SC**) in the corresponding tube (see chapter "Internal quantification standards and controls").
- Add 10 µL of each standard (from **QS4** to **QS1**) in the corresponding tubes (see chapter "Internal quantification standards and controls").
- Add 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 in the relevant device and then transfer them to the thermocycler.

11.3 RUNNING THE BK Virus R-gene® PROGRAM

- Run the amplification program (stored according to the instructions described in section **"**Amplification program")
- Designate the samples and controls.
- Depending on the extraction protocol followed, enter the QS values (copies/mL) indicated in the table below:

	QS Values (copies/mL)			
	Extraction 200µL Elution in 50 µL	Extraction 200µL Elution in 100 µL	Extraction 250 µL Elution in 65µL	Extraction 300 µL Elution in 90µL
QS1	1 250 000	2 500 000	1 250 000	1 500 000
QS2	125 000	250 000	125 000	150 000
QS3	12 500	25 000	12 500	15 000
QS4	1 250	2 500	1 250	1 500

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 each fluorescence curve in its linear part, above the background noise.

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

- For each sample, a CP Crossing Point is calculated at **530 nm**.
- To quantify the samples, use the "Second Derivative maximum" method in Arithmetic mode.
- The concentration calculated for BKV 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 so that it crosses each fluorescence curve in its linear part, above the background noise.

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

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

12.3 WITH LIGHTCYCLER® 480 (System II)

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

12.4 WITH SMARTCYCLER® 2.0

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

12.5 WITH APPLIED BIOSYSTEMS

- Make sure that none is selected in the Passive reference field because the BK Virus 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 Manual Baseline manually to cross each amplification curve in its linear part. This stage is used to determine the positive samples for which a CT is calculated. The negative samples display the word "Undetermined" in the CT column.
- The inhibition controls (**IC2sample** and **IC2W0**) are analysed in the same way after selecting the VIC R-gene® in the Detector/target field.
- To quantify the samples, return to linear mode.
- The concentration calculated for BKV appears in the report drafted and printed at the end of each experiment.
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) with the CT value of the reference extraction + inhibition test (**IC2W0**) at **560 nm**.

12.6 WITH ROTOR-GENE®

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

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

- Make sure that none is selected in the Dye reference field because the BK Virus R-gene® 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 BKV 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.

- The analysis of the viral target, the QS, SC and the negative control is made in the tab Quantitation, leaving the FAM button ticked.
- 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.
- Analysis of the extraction controls + inhibition (**IC2sample** and **IC2W0**) is carried out in the same way after ticking the HEX detector.

13. Validation and interpretation of results

13.1 TEST VALIDATION

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

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

1st CONDITION: **IC2W0** should not give a detectable signal at **530 nm**.
2nd CONDITION: **IC2W0** must be equal to 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.

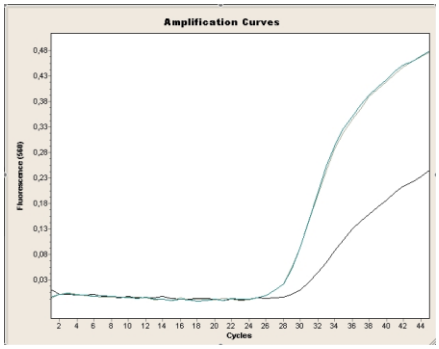
=> If ALL of these conditions are fulfilled, the results obtained with the sample can be analysed.

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

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



IMPORTANT NOTES:

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

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

It is absolutely necessary to compare results obtained with BK Virus 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
Change in conditions for using amplification premixes. 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 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 cold block to prepare and distribute premix.
Failure to adhere to conditions for transport and storage of samples 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.
Failure to adhere to storage conditions and expiry date of the BK Virus R-gene® kit.	• Follow instructions in section "Content of the kit and storage" regarding the storage of BK Virus R-gene® kit 69-013B 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 extraction DNA EXTRACTION KIT extraction kit 67-000. (See section "DNA EXTRACTION KIT"). • Check if material and protocol used to extract samples correspond to the material and protocol recommended section "With extraction instruments and/or kits validated with BK Virus r-gene® extraction kit". • Always perform preventive maintenance of workstations for automated extraction, and centrifuge systems, according to the manufacturer's recommendations.
Pipetting error	• Check the calibration of your pipets. • Check the distributed volume of reagents and samples. • Carefully homogenize reagents and samples before their distribution in tubes.
Programming error	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards.
Problem in amplification step	• Check the performances of the real time PCR platform as recommended by the manufacturer. • Always perform preventive maintenance of real time PCR platform, and centrifuge, systems according to the manufacturer's recommendations. • Check the attachment of the locking ring of the Rotor-Gene® carrousel.
Error in data analysis	• Check the baseline adjustement. • In the case of an analysis based on an imported standard curve, check that the imported curve 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"). • With LightCycler® 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 that None is selected in passive reference field. • Always compare the result of the inhibition control of the sample with the initial inhibition control (see section "Result interpretation"). Dilute the sample if necessary. • Compare the result of the extraction + inhibition control (**IC2sample**) of the suspected sample with the result of the reference extraction + inhibition control (**IC2W0**) (see chapter "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 capillary cooling block with U.V. light. • Repect the manufacturer's recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • The BK Virus R-gene® kit must be handled only by a trained staff. • Use the **R0** reagent provided in the kit, in parallel with the extracted samples, to identify the contaminated stage.
Pipetting error	• Check the calibration of your pipettes. • Check the distributed volumes of reagents and samples. • Carefully homogenize reagents and samples before their distribution in tubes.
Programming error	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards.
Error in data analysis	• Check the baseline 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 "Validation and interpretation of results"). • 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 that None is selected in passive reference field. • Compare the result of the extraction + inhibition control (**IC2sample**) of the suspected sample with the result of the reference extraction + inhibition control (**IC2W0**) (see chapter "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 BK Virus R-gene® kit can only be guaranteed for the recommended extraction systems and PCR instruments.**

15.1 INTRA-ASSAY AND INTER-ASSAY REPRODUCIBILITY OF BK Virus R-gene® KIT

15.1.1 Intra-assay reproducibility

a. On plasma

The intra-assay reproducibility study was conducted on different samples analysed ten times on the ABI Prism 7500 Fast device (Applied Biosystems), after extraction using the NucliSENS® easyMAG® (BioMérieux).

A sample of the QCMD 2009 proficiency panel was chosen in order to carry out three successive dilutions in BKV negative plasma in ratios of 10 allowing us to obtain viral loads between $1.6.10^4$ and $1.6.10^2$ copies/mL.

The table below shows the average CTs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 0.44% and 3.2%. These values demonstrate the good intra-assay reproducibility of the BK Virus R-gene® kit on plasma.

		Average CT (cycles)	Standard deviation	Coefficient of variation (%)
BKV	1,6E+04 copies/mL	30,44	0,135	0,44%
	1,6E+03 copies/mL	34,39	0,486	1,41%
	1,6E+02 copies/mL	38,76	1,241	3,20%
Reference extraction+inhibition control		26,17	0,284	1,08%

The table below shows the average quantification logs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 0.83% and 15.97%. However, it should be noticed that the coefficient of variation of 15.97% was obtained from a very low positive sample (160 copies/mL).

These values demonstrate the good intra-assay reproducibility of the BK Virus R-gene® kit on plasma.

		Average Log	Standard deviation	Coefficient of variation (%)
BKV	1,6E+04 copies/mL	4,63	0,039	0,83%
	1,6E+03 copies/mL	3,5	0,14	3,99%
	1,6E+02 copies/mL	2,24	0,358	15,97%

b. On urine

The intra-assay reproducibility study was conducted on different samples analysed ten times on the ABI Prism 7500 Fast device (Applied Biosystems), after extraction using the NucliSENS® easyMAG® (BioMérieux).

A sample of the QCMD 2009 proficiency panel was chosen in order to carry out three successive dilutions in BKV negative urine in ratios of 10 allowing us to obtain viral loads between $1.6.10^4$ and $1.6.10^2$ copies/mL.

The table below shows the average CTs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 0.55% and 3.12%. These values demonstrate the good intra-assay reproducibility of the BK Virus R-gene® kit on urine.

		Average CT (cycles)	Standard deviation	Coefficient of variation (%)
BKV	1,6E+04 copies/mL	31,96	0,177	0,55%
	1,6E+03 copies/mL	36,11	0,652	1,81%
	1,6E+02 copies/mL	40,12	1,253	3,12%
Reference extraction+inhibition control		27,39	0,309	1,13%

The table below shows the average quantification logs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 1.15% and 16.74%. However, it should be noticed that the coefficient of variation of 16.74% was obtained from a very low positive sample (160 copies/mL).

These values demonstrate the good repeatability of the BK Virus R-gene® kit on urine.

		Average Log	Standard deviation	Coefficient of variation (%)
BKV	1,6E+04 copies/mL	4,49	0,052	1,15%
	1,6E+03 copies/mL	3,3	0,187	5,68%
	1,6E+02 copies/mL	2,15	0,36	16,74%

15.1.2 Inter-assay reproducibility on plasma samples

The reproducibility study (inter-assay variation) was conducted on different samples analysed 10 times on the ABI StepOne® device (Applied Biosystems), after extraction using MagNA Pure Compact (Roche).

A concentrated BKV positive sample was chosen in order to carry out 3 successive dilutions in BKV negative plasma in ratios of 10 allowing us to obtain viral loads between 1.10^5 and 1.10^3 copies/mL.
The table below shows the average CTs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 0.61% and 1.14%. These values demonstrate the good reproducibility inter-assay of the BK Virus R-gene® kit on plasma.

		Moyenne CT (cycles)	Ecart Type	Coefficient de variation (%)
BKV	1E+05 copies/mL	26,51	0,162	0,61%
	1E+04 copies/mL	29,94	0,237	0,79%
	1E+03 copies/mL	33,95	0,352	1,04%
Contrôle d'extraction+inhibition référence		26,39	0,301	1,14%

The table below shows the average quantification logs obtained for each one of the samples. The reference deviation and the coefficient of variation were calculated. The coefficient of variation is between 1.04% and 3.61%. These values demonstrate the good reproducibility inter-assay of the BK Virus R-gene® kit on plasma.

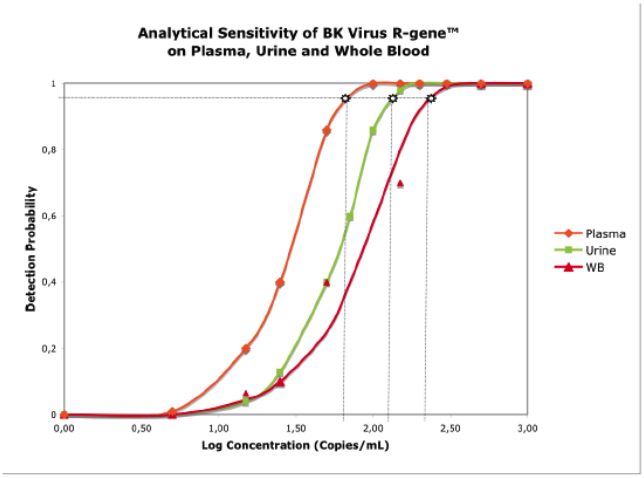
		Moyenne Log	Ecart Type	Coefficient de variation (%)
BKV	1E+05 copies/mL	5,06	0,052	1,04%
	1E+04 copies/mL	4,06	0,071	1,76%
	1E+03 copies/mL	2,89	0,104	3,61%

15.2 ANALYTICAL SENSITIVITY OF BK Virus R-gene® KIT

The analytical sensitivity of the kit has been determined on a range of dilutions of a BKV sample from the Accrometrix panel, quantified at 5.10^3 copies/mL.
Serial dilutions were carried out using plasma, urine or whole blood that has previously tested negative.

Each dilution was extracted 15 times using the Nuclisens® easyMAG® (bioMérieux) extraction machine, then amplified with the BK Virus R-gene® kit on the ABI 7500Fast device (Applied Biosystems) for the plasma and urine and on the Rotor-Gene® (Corbett) device for the whole blood.

The curves shown here represent the probability analysis.



These curves show that there is:
- 95% probability of detecting the BK virus in plasma at 65 copies/mL.
- 95% probability of detecting the BK virus in urine at 140 copies/mL.
- 95% probability of detecting the BK virus in whole blood at 260 copies/mL.

15.3 ANALYTICAL SPECIFICITY OF BK Virus R-gene® KIT

The specificity of the Adenovirus primers and probes was tested by means of sequence analysis (of virus, bacteria and human sequences) in the data banks, and the human polyomaviruses WUV, KIV, and Merkell Cell in particular. According to this analysis, no crossed reaction is expected.
It has been tested on the following viruses in experiments
- Human Herpesvirus : HSV-1, HSV-2, VZV, CMV, EBV, HHV6
- Human Adenovirus : AdV12, AdV3, AdV11, AdV5, AdV8, AdV4, AdV40
- Human Polyomavirus JCV.

⇒ None of these viruses were amplified with the BK Virus R-gene® kit, which clearly proves the specificity of the assay.

NOTE: Tests were performed on human DNA extracts and clinical samples negative for BK virus.. These tests proved BK Virus R-gene® assay does not amplify human sequences.

15.4 STUDY FROM QCMD PANELS

15.4.1 QCMD panels 2011

On the occasion of the European campaign to control polyomavirus proposed by the QCMD in 2011, 12 samples were tested blind using the BK Virus R-gene® kit.
200µL of each sample were extracted with NucliSENS® easyMAG® (BioMérieux) and eluted in 50 µL, according to the Specific B extraction protocol, then amplified on the ABI 7500 Fast.

	QCMD Expected Results			BK Virus R-gene™ easyMAG (bioMerieux)	
	Sample Content	Sample type	Concentration (Copies/mL)	*Copies/mL*	*Delta Log*
JC/BK11-01	JCV and BKV negative	Core	-	-	-
JC/BK11-02	JC Virus (1A)	Core (JCV)	1,51E+03	-	-
JC/BK11-03	BK Virus (1b-1)	Core (BKV)	2,16E+05	3,02E+05	-0,14
JC/BK11-04	JC Virus (1A)	Core (JCV)	1,48E+04	-	-
JC/BK11-05	JC Virus (1A)		1,32E+02	-	-
JC/BK11-06	BK Virus (1b-2)	Core (BKV)	5,82E+03	3,86E+03	-0,18
JC/BK11-07	BK Virus (1b-2)		8,50E+01	1,60E+01	-0,73
JC/BK11-08	BK Virus (1b-2)	Core (BKV)	5,74E+04	4,80E+04	-0,08
JC/BK11-09	BK Virus (1b-1)		1,96E+02	1,30E+02	-0,18
JC/BK11-10	JC Virus (1A)		2,02E+02	-	-
JC/BK11-11	JC Virus (1A)	Core (JCV)	3,98E+02	-	-
JC/BK11-12	JC Virus (3A)	Core (JCV)	9,73E+02	-	-

⇒ 12/12 (100%) of the samples tested by the QCMD 2011 panel for polyomavirus agreed with the expected results.
⇒ 5 samples of 12 were BKV positive.
⇒ 7 samples of 12 were BKV négative.

The results obtained on the “Core” BKV samples, selected by the QCMD due to the clinical relevance they represent, are 100% (3/3) concordant with the expected results.

The detection of samples of low viral load for BKV (JC/BK11-09 to 196 copies /mL and JC/BK11-07 to 85 copies /mL) attests to the high sensitivity of the BK Virus R-gene® kit.
Apart from the weakly positive sample JC/BK11-07, there is no significant difference between the expected quantification results and those obtained (<0.5log).

The absence of cross-reaction between polyomavirus BKV and JCV demontstrated the specificity of BKV primers and probes selected for the BK Virus R-gene® kit

15.5 CLINICAL STUDY

15.5.1 Prospective and retrospective clinical studies on plasma and urine –Saint Louis Virology Laboratory (AP-Paris)

a. Prospective study

A prospective clinical trial was conducted in the virology laboratory of the CHU de Saint Louis, on 175 plasma and 126 urine samples. These samples were analysed using the ABI 7500Fast (Applied Biosystems) after atomatic extraction using the MagNA Pure LC (Roche). For this prospective study, the samples were therefore extracted and then tested simultaneously with the same extract using the reference technique and using the BK Virus R-gene® kit.

Qualitative analysis of the results:

		RT-PCR St Louis		
		+	-	
BK Virus R-gene®	+	91	10	101
	-	17	183	200
		108	193	301

- There was 91% concordance between the two techniques (274/301).
  91 samples were positive and 183 were negative with both techniques.

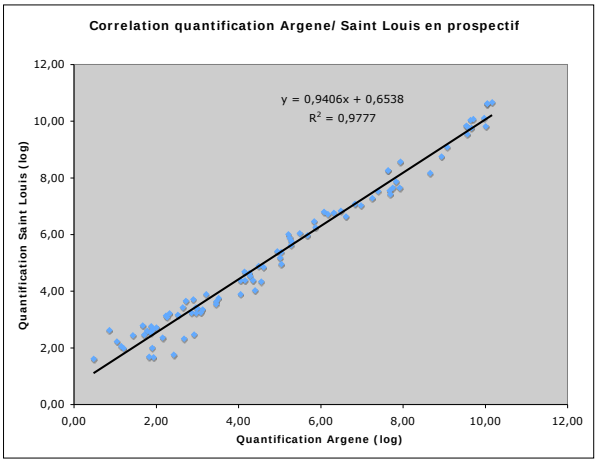
- 17 samples tested positive with the reference technique and negative with the Argene technique. These were 12 plasma and 5 urine samples taken from 15 kidney transplant patients and 2 bone marrow transplant patients.
  Of the 17 samples, 13 were measured below 100 copies BKV/mL and 4 had concentrations between 161 and 305 copies BKV /mL.

- 10 samples tested positive with the Argene technique and negative with the reference technique. These were 6 plasma and 4 urine samples taken from 9 kidney transplant patients and 1 bone marrow transplant patient.
  All 10 samples were below 100 copies BKV /mL.

Quantitative analysis of the results:

Analysis of the results between the laboratory's real-time PCR technique and the BK Virus R-gene® real-time PCR technique was carried out on all the samples (urine and plasma) measured by the two techniques.
This analysis shows a correlation coefficient between the two techniques of 0.97.
The slope of the regression line (0.94) shows good correlation between the quantification of both techniques.

The correlation coefficient of Spearman's rho was calculated to be 0.9574, thus demonstrating excellent correlation between the two techniques (p<0.00001).



b. Retrospective study

A retrospective study was conducted in the virology laboratory of the CHU de Saint Louis, on 51 plasma and 55 urine samples. These samples were analysed on the ABI 7500Fast (Applied Biosystems) after automatic extraction using the MagNA Pure LC (Roche). For this retrospective study, the samples were therefore extracted and then tested simultaneously with the same extract using the reference technique and using the BK Virus R-gene® kit.

Qualitative analysis of the results:

		RT-PCR St Louis		
		+	-	
BK Virus R-gene®	+	99	1	100
	-	1	5	6
		100	6	106

- There was 98% concordance between the two techniques.
  99 samples were positive and 5 were negative with both techniques.

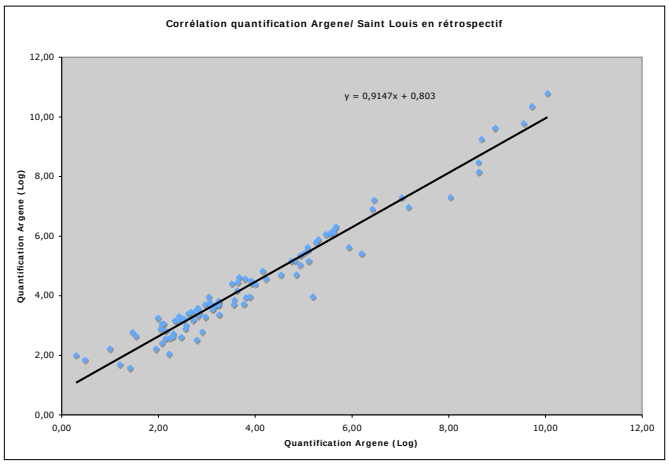
- 1 sample tested positive with the reference technique and negative with the Argene technique. This sample had 243 copies/mL.
  This was a plasma sample taken from a kidney transplant patient.

- 1 sample was positive with the Argene technique and negative with the reference technique. This sample had 22 copies/mL.
  This was a plasma sample taken from a kidney transplant patient.

Quantitative analysis of the results:

Quantitative analysis of the results between the laboratory's real-time PCR technique and the BK Virus R-gene® real-time PCR technique was carried out on all the samples (urine and plasma) measured by the two techniques.
This analysis shows a correlation coefficient between the two techniques of 0.95.
The slope of the regression line (0.91) shows good correlation between the quantification of both techniques.

The correlation coefficient of Spearman's rho was calculated to be 0.9574, thus demonstrating excellent correlation between the two techniques (p<0.00001).



15.5.2 Clinical trial on plasma and urine – Virology Laboratory of Saint Luc (Brussels)

A retrospective clinical trial was conducted in the virology laboratory of the CHU de Saint Luc on 132 plasma and 46 urine samples. These samples were analysed on the LC480 (Roche) after automatic extraction using the MagNA Pure Compact (Roche). For this retrospective trial, the samples were therefore extracted and then tested simultaneously with the same extract using the reference technique and using the BK Virus R-gene® kit.

Qualitative analysis of the results:

		RT-PCR St Luc		
		+	-	
BK Virus R-gene®	+	80	22	102
	-	4	72	76
		84	94	178

- There is 85% concordance between the two techniques.
  80 samples were positive and 72 were negative with both techniques.

- 4 samples tested positive with the reference technique and negative with the Argene technique.
  These 4 samples had low positives < 1200 copies/mL (quantification using the reference technique).
- 22 samples tested positive with the Argene technique and negative with the reference technique.
  These samples had weak positives between 60 and 4000 copies/mL (quantification using the Argene technique).

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.

Publications :

1) Noah G. Hoffman, Linda Cook, Ederlyn E. Atienza, Ajit P. Limaye and Keith R. Jerome
Marked Variability of BK Virus Load Measurement Using Quantitative Real-Time PCR among Commonly Used Assays. Journal of Clinical Microbiology, Aug. 2008, p. 2671–2680

2) Bialasiewick S, Whiley DM, Lambert SB, Nissen ND, Sloots TP
Detection of BK, JC, WU, or KI polyomavirus in faecal, urine, blood, cerebrospinal fluid and respiratory samples. Journal of Clinical Virology, jul. 2009, p. 249–254.

3) Mathur VS, Olson JL, Darragh TM, Yen TS.
Polyomavirus-induced interstitial nephritis in two renal transplant recipients: case reports and review of the literature. Am J Kidney Dis. 1997 May;29(5):754-8.

4) Randhawa PS, Finkelstein S, Scantlebury V, Shapiro R, Vivas C, Jordan M, Picken MM, Demetris AJ.
Human polyoma virus-associated interstitial nephritis in the allograft kidney. Transplantation. 1999 Jan 15;67(1):103-9.

5) Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Steiger J.
Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. N Engl J Med. 2002 Aug 15;347(7):488-96.

Posters :

1) Claeys L., Kabamba B., Goubau P., Padalko E.
Validation of BK virus R-gene® assay on iQ5 Real-Time PCR Detection System. 2011 ESCV Madeira

2) M. Vignoles , J. Bes , S. Magro, M. Bertrand, C. Barranger. and M. Joannes.
Development of a new diagnostic tool for the detection and quantification of BK virus by real time PCR. 2010 (Milan, Italy) and CVS 2010 (Daytona, US).

17. Related products

- JC Virus r-gene® Primers/Probe ref. : 71-004
- 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

18. Index of symbols

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

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