BKV DNA QUANTITATION (QT)

Real-Time PCR for the BKV genome Quantification

4BShopLab

Rev. 6

BKV DNA

A. INTENDED USE

The **BKV DNA Quantitation** Real-Time PCR kit coded **BKVDNAQT.CE** is intended for the quantitative detection of the BK Virus DNA in human plasma and urine with a simultaneous control of the amplification/extraction reaction through an **Internal Control (IC).**

The kit has been adapted for the use on the Real-Time Thermacyclers ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) or MX3000P® (Software MxPro version 4.01, Stratagene™***) and CFX96 (Software CFX manager version 1.7, Biorad™**).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsisdiaries in the US and/or certain other countries.
** Biorad is a registered trademark.
***Stratagene is a registered trademark.

B. INTRODUCTION

Human Polyoma BK Virus (Polyomavirus hominis 1) belongs to the genus Polyomaviridae, which are non enveloped DNA viruses containing circle double-stranded DNAof about 5 Kb in size. It was first identified in 1970 from the urine of a renal allograft recipient named BK, who developed ureteric stenosis.

BK virus infection occurs during the early childhood years and is asymptomatic or associated with fever and mild upper respiratory symptoms. Up to 90% of adults are seropositive. Transmission is believed to occur via the respiratory route but this has not be formally proven.

After primary infection, BK virus enters a latent state and resides in uroepithelial cells and possibly lymphocytes. Other reservoirs of latent infection are possible but are currently not known. The virus remains quiescent unless a natural or iatrogenic state of immunosuppression is imposed. BKV shedding in the urine has been detected in pregnant women and in the elderly. The shedding of virus is also frequently detected in the HIV/AIDS population. The greatest incidence of shedding occurs in solid organ and bone marrow transplanted patients. Because of BK virus tropism for genitourinary epithelium, genitourinary tract disease is the most common manifestation. BKV may provoke hemorrhagic cystitis, urethral stenosis and tubulo-interstitial nephritis that in case of renal transplantation can lead to an irreversible graft failure.

While the use of urine cytology for the detection of decoy cells has been in use for decades, other diagnostic modalities to detect BKV have emerged, including tissue biopsy, viral culture, serology. In this last few years, molecular based assay such as real time PCR assays were also demonstrated to be a useful tool for BK virus detection/quantification because of high sensitivity, specificity and easy to use and quick method.

C. PRINCIPLE OF THE TEST

The BKVDNAQT.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes.

BKV DNA, recovered from the biological sample under investigation through an extraction step, is amplified using Real Time amplification system. The amplified product is detected and quantified, against the standard curve using a fluorescent reporter dye probe specific for a BKV unique genomic sequence.

Internal Control (IC) serves as an amplification/extraction control for each individually processed specimen aiming to the identification of reaction inhibitors.

An external standard curve is supplied allowing the determination of the viral load.

D. COMPONENTS

The standard format of the product code BKVDNAQT.CE contains reagents for 50 tests.

Component	Contents	BKVDNAQT.CE 50 Reactions
A **CODED: : ALL/MM-4** **COLOR CODE: CLEAR**	Master mix	N°1 vials / 0.825 ml
B **CODED: BKV/CB** **COLOR CODE: YELLOW**	Lyophilised Primers/Probes	N°2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: RED**	MG Water	N°4 vials /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N°1 vials /1.5 ml
STD Quantitation Standard ($2.5x10^5$ copies/µl) **CODED: BKV/STD** **COLOR CODE: RED**	Lyophilised Quantitative Standard	N°6 vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control **CODED: ALL/IC** **COLOR CODE: GREEN**	Lyophilised Internal Control	N° 2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below **:**

1. Component A	n°1 vial/0.4 ml	n°2 vials/0.825 ml	n°3 vials/0.825 ml
2. Component B	n°1 vial	n°4 vials	n°6 vials
3. Component C	n°2 vial/1.5 ml	n°4 vials/1.5 ml	n°6 vials/1.5 ml
4. NTC	n°1 vial/1.5 ml	n°1 vial/1.5 ml	n°1 vial/1.5 ml
5. IC	n°1 vial	n°4 vials	n°6 vials
6. STD	n°3 vial	n°4 vials	n°6 vials
7.Pack. insert	n° 1	n° 1	n° 1
Number of tests	**25**	**100**	**150**
Code	**BKVDNAQT.CE.25**	**BKVDNAQT.CE.100**	**BKVDNAQT.CE.150**

E.STORAGE AND STABILITY

The kit BKVDNAQT.CE must be stored at +2…8 °C .

Once dissolved **Component B** (coded BKV/CB) and **Component IC** (coded ALL/IC) are stable for 4 months at -20°C. Once dissolved **Component STD** (coded BKV/STD) is stable for 2 weeks at -20°C. If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided, Only one defreezing is allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. DNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tips with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible for the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents.
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human serum/blood/plasma specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount, stated in the labels, with Molecular Grade water (Component C Coded: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. Workflow in the laboratory must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Area. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.
22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H.SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venepuncture and plasma is prepared using standard techniques of preparation of samples for clinical laboratory analysis.
2. No influence has been observed in the preparation of the sample with citrate, EDTA.
   Attention: Heparin (≥10 IU/ml) affects the PCR reactions. Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.
3. Avoid any addition of preservatives to samples.
4. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
5. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
6. Plasma and urine, if not used immediately, must be aliquoted and stored at -20°..-80°C after collection. Samples can be stored frozen at -80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
7. The plasma samples for DNA extraction must be collected according to the common laboratory procedures, transported and stored at +2 / +8 °C for a maximum period of 4 hours. The plasma samples can be stored frozen at –20°C for a maximum period of 30 days or at -70 °C for longer periods.
8. We recommend you, for optimal storage of samples, to split them in several aliquots (minimum volume 300 µl) and store them frozen at -20°C for a maximum period of 30 day or -70°C for longer periods. Avoid repeated freezing / thawing cycles.
9. When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible cases of nucleic acid degradation.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:
Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: Component A is light sensitive. Protect it from strong light exposition.

Primers/Probes:
Component B.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water:
Component C. Ready to use.

Negative Control:
NTC. Ready to use.

Standard Curve:
STD.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized STD with the volume of Component C (Code: ALL/C) indicated on the vial label
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex
- Prepare 4 Nuclease Free tubes for the preparation of the Standard Curve
- Set up an STD 1:10 serial dilution in Component C (Code: ALL/C) to obtain the standard curve points as described in the table below:

	Standard curve preparation	
STD	**Calibrator 250000 copies/ µl**	Add the Volume of Component C (Code: ALL/C) as written on the vial label
STD 1	**25000 copies/ µl**	10 µl (STD) + 90 µl Component C (Code: ALL/C)
STD 2	**2500 copies/ µl**	10 µl (STD 1) + 90 µl Component C (Code: ALL/C)
STD 3	**250 copies/ µl**	10 µl (STD 2) + 90 µl Component C (Code: ALL/C)
STD 4	**25 copies/ µl**	10 µl (STD 3) + 90 µl Component C (Code: ALL/C)

Internal Control:
I.C.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT
1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The BKVDNAQT.CE Kit is intended for the use in combination only with QIAamp DNA Minikit Code.51306 (QIAGEN),Nucleospin Blood kit Code: 740951(Macherey-Nagel) and NA Body Fluid Kit Code: D-2021 (Chemagen distributed by Dia.Pro). The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers.** The BKVDNAQT.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500 (Software SDS version 1.3.1, Applied Biosystems), and MX3000P (Software MxPro version 4.01, Stratagene), and CFX96 RTS, software CFX manager version 1.7 (Biorad).
   The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS
1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Code: ALL/C) as described in the proper section (I).
4. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
5. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE
The assay has to be carried out according to what reported below.

N.1 DNA extraction

The extraction step of the BKV genomic DNA has to be carried out exclusively in combination with the following kits:

Manual Extraction tools

Material	Description	Kit code	Manufacturer
Plasma/urine	Nucleospin Blood	740951	MN™
Plasma/urine	QIAamp DNA mini kit®	51306	Qiagen™

Automatic Extraction tool in combination with DIA.FASTEX Instrument

Material	Description	Kit code	Manufacturer
Plasma/urine	NA Body Fluid Kit	D-2021	Chemagen distributed by Dia.Pro

The DNA isolation must be carried out only according to the Instruction Manual (QIAGEN™, MN™, Dia.Pro).

Important Note : The following volumes have to be strictly used in the extraction procedures :

Description	Sample volume µl	Elution volume µl
Nucleospin Blood	200	100
QIAamp DNA mini kit®	200	100
NA Body Fluid Kit	200	100

The DNA extracted from the samples, not used in the run , has to be stored frozen (-20°C….-80°C).

Important note: *The IC of the* BKVDNAQT.CE Kit *can be used in the isolation procedure as extraction control.*
The Internal Control Ct value is used to evaluate if the DNA extraction procedure has been performed correctly (see section Q).

For this application:

PLASMA SAMPLES
- Nucleospin Blood and QIAamp DNA mini kit : add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.
- NA Body Fluid Kit: add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied in the Extraction Kit by the manufacturer.

URINE SAMPLES
- Nucleospin Blood and QIAamp DNA mini kit : add 10 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.
- NA Body Fluid Kit: add 10 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied in the Extraction Kit by the manufacturer (Plasma Protocol).

N.2 Setting up of the reaction

BKVDNAQT.CE kit is intended to be used exclusively in combination with ABI 7500 standard (Software SDS version 1.3.1, Applied Biosystem) and MX3000P (Software MxPro version 4.01, Stratagene) and CFX96 software CFX manager version 1.7 (Biorad).

N.2.1 Preparing the PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting to read the instructions here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Standard curve (prepared as described in Section I).

Important note: *Use only optical tubes or microplates suggested by the Real-Time thermocyclers manufacturers.*

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and standard curve** as table below:

Preparation of the Amplification Mix
(I.C. as Amplification control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
I.C.	Internal Control	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

Important note: *If the Internal Control was added in the DNA isolation procedure, prepare the* ***Amplification Mix*** *for* ***Samples*** *as table below:*

Preparation of the Amplification Mix
(I.C. as Extraction/Amplification control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

N.2.2 Amplification procedure

- Dispense 15 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC and standard curve** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the tubes in the Real-Time Thermacycler Thermoblock Holder.
- After the setting operations described in the Sections N3 (Instrument Programming) start the Thermacycler run.

Important note: The Components Lyophilized after dissolution in Component C (MG water) are stable no more than 3 hours kept in ice or at 2°…8° °C.

At the end of the working day discard adequately the material leftover of the STD Dilution Points.

The not used volume of Component B, STD and I.C. can be freeze at -20°C and used as described in Section E.

N.3 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

Important Note: *For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x8, VIC/JOE = x1. (see MxPro™ QPCR Software Instruction Manual, p.41)*

N.3.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
2	1	95°C	10 min
3	50	95°C	15 sec
		60°C **(*)**	1 min

IMPORTANT NOTE: (*) step for the real time data collection

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct Thermal Profile following the Instruments Manual supplied by the manufacturer.

N.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermocyclers suggested (ABI 7500, MX3000P Stratagene and BioRad CFX96) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
BKV	FAM	TAMRA
Internal Control (I.C.)	JOE/VIC	Non Fluorescent
Passive Reference	ROX	Not Present

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct settings following the Instruments Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for Quantitative Analysis is reported below:

Microplate or tubes

	1	2	3
A	**STD 1** 25000 copies/ µl	Sample 4	
B	**STD 2** 2500 copies/ µl	Sample 5	
C	**STD 3** 250 copies/ µl	Sample 6	
D	**STD 4** 25 copies/ µl	Sample 7	
E	NTC	Sample 8	
F	Sample 1	Sample 9	
G	Sample 2	Sample 10	
H	Sample 3	Sample 11	

Legenda: NTC = Negative Control STD 1,2,3,4 = BKV DNA Standard Curve, Sample 1,2,etc. = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting

Before starting the analysis:

- Set the "Baseline" (the background fluorescence level) as reported below :

"Baseline"	
ABI™PRISM® 7500 SDS	**Auto Baseline**
STRATAGENE™ MX3000P®	**Adaptive Baseline** ***Do not use Mx4000 v1.00 to v3.00 algorithm***
BIORAD™ CFX96®	Auto calculated Baseline

- Set manually the FAM/JOE/VIC fluorescence "Threshold"

FAM fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.15**
STRATAGENE™ MX3000P®	**0.15**
BIORAD™ CFX96®	**400**

JOE/VIC fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.1**
STRATAGENE™ MX3000P®	**0.02**
BIORAD™ CFX96®	**350**

P.2 Data Analysis

A check is carried out on the STD calibrators any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

ABI™PRISM® 7500 SDS - BIORAD™ CFX96®	
Check FAM	**Requirements**
STD 1	20 < Ct (Threshold Cycle) < 23

STRATAGENE™ MX3000P®	
Check FAM	**Requirements**
STD 1	20.5 < Ct (Threshold Cycle) < 23.5

Moreover the Slope and R2 values are checked in order to verify the quality of the run. The following requirements must be fulfilled.

Check FAM	Requirements
Slope	-3.1 < Slope <-3.9

Check FAM	Requirements
Efficiency	R^2 > 0.98

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE fluorescence are assumed to validate BKV detection as described in the table below:

The following results are possible:

BKV FAM	Internal Control JOE/VIC	Assay Result
SAMPLE POSITIVE	20 < Ct < 40	CORRECT
	Ct > 40 or undetermined	CORRECT*
SAMPLE NEGATIVE	20 < Ct < 40	CORRECT
	Ct > 40 or undetermined	INVALID**

* Concentration of BKV DNA higher than 1000 copies/µl (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition

**In this case problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors or initial sample containing insufficient cells number) which may lead to incorrect results and false negatives. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

For each positive samples detected by kit code BKVDNAQT.CE a correct Quantitation of the viral load can be applied within the 2.5E+05 to 1.2E+00 copies/ul on ABI PRISM 7500 SDS and BIORAD CFX96 and within the 2.5E+05 to 2E+00 copies/ul on STRATAGENE Mx3000P.

BKV viral load must be expressed as reported in the table below:

ABI™PRISM® 7500 SDS - BIORAD™ CFX96® -	
Sample BKV run data (copies/µl)	**BKV viral load (copies/µl)**
Quantity > 2.5E+05	BKV viral load > 2.5E+05
1.2E00 ≤ Quantity ≤ 2.5E+05	***QUANTITATION***
Quantity < 1.2E+00	BKV viral load < 1.2E+00

STRATAGENE™ Mx3000P®	
Sample BKV run data (copies/µl)	**BKV viral load (copies/µl)**
Quantity > 2.5E+05	BKV viral load > 2.5E+05
2.0E00 ≤ Quantity ≤ 2.5E+05	***QUANTITATION***
Quantity < 2.0E+00	BKV viral load < 2.0E+00

Important note: *For samples quantitation refer to Section R*

The results obtained with the BKVDNA.CE Kit must be interpreted by the responsible of the laboratory keeping in consideration the clinical symptoms of the patients and the other laboratory markers of Infection.

The following results are possible:

Troubleshooting table

	FAM	JOE/ VIC	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: Concentration of BKV DNA higher than 1000 copies/µl (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or misfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the BKV detection and JOE/VIC for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. That the Extraction procedure have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
STD	+	+/-	CORRECT RESULT	IMPORTANT: 1.Concentration of BKV DNA higher than 1000 copies/µl (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition. 2.Negative JOE/VIC signal is correct only if I.C. was used as extraction control
STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected

			pipetting or in the procedure	FAM for the BKV detection and JOE/VIC for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
STD	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctely 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the BKV detection and JOE/VIC for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly;
NTC	-	+/-	CORRECT RESULT	Negative JOE/VIC signal is correct only if I.C. was used as extraction control
NTC	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.;that the kit has been stored correctly;

Important notes:

1. *Interpretation of results has to be done under the supervision of the laboratory Responsible to reduce the risk of judgment errors and misinterpretations.*
2. *When the test results are transmitted from the laboratory to an informatics centre, attention has to be paid to avoid erroneous data transfer.*

If the results of the test match the *CORRECT ASSAY RESULT* requirements stated above, proceed to the next section.
If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

R. QUANTITATION

The STD calibrators are treated as patient samples and the same volume,10µl, is used during the amplification step.
The STD calibrators concentration is expressed in copies/µl.
The **Viral Genome Concentration per mL** for each patient specimen is calculated applying the following formula:

$$\textbf{Results (copies/ml)} \equiv \frac{\textbf{copies/µl (run data)x Elution sample volume (µl)}}{\textbf{Sample Extraction volume (ml)}}$$

Example:

$$\textbf{Results (copies/ml)} \equiv \frac{\textbf{150 x 100}}{\textbf{0.2}}$$

Results (copies/ml) ≡ 7.5 E+04

S. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.

The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical labs.

S.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **Limit of Detection** and as **Limit of Quantitation**.
Limit of detection (LOD): it is the lowest amount of target that can be detected by the system with a stated probability.
For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive result.
The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.
The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in $\geq$ 95% of samples under routine laboratory conditions).
In the kit code BKVDNAQT.CE the **LOD** has been determined by analysis of 24 replicates, 8 replicates in three different runs, of the lowest dilution of the analyte that can be detected in 100% of them.

The results are the following:

Detection Limit	
ABI™PRISM® 7500 SDS	**1.2 copies/ µl**
STRATAGENE™ MX3000P®	**1.2 copies/ µl**
BIORAD™ CFX96®	**1.2 copies/ µl**

This means that there is the 100% probability that 1.2 copies/ µl will be detected on ABI PRISM 7500 SDS, STRATAGENE MX3000P and BIORAD CFX96 instrument.

S.1.1 Limit of quantitation

The **Limit of Quantitation** was determined by measuring the **linearity**, the **dynamic range** and the **reproducibility.**
The **Linearity** is the measure of the degree to which a curve approximates a straight line. It is expressed with the **SLOPE** value.
The **dynamic range** is the span of analyte concentrations for which the final output value (Ct threshold cycle) of the system is directly proportional to the analyte concentration, with acceptable trueness and precision.
The boundaries of the dynamic range are the lower and upper limits of quantitation **(Limit of quantitation).**
In the kit code BKVDNAQT.CE a limiting dilution curve with defined copies/ul of a plasmid carrying the specific target viral sequence were prepared. The dilution points were tested in the analytical system and their Ct (threshold cycle) determined.
The upper **limit of quantitation** is $5.39\log_{10}$ (2.5E+05 copies/ul) and the **lower limit of quantitation** is $0.08\log_{10}$ (1.2E00 copies/ul) on ABI PRISM 7500 SDS and BIORAD CFX96.
The upper **limit of quantitation** is $5.39\log_{10}$ (2.5E+05 copies/ul) and the **lower limit of quantitation** is $0.30\log_{10}$ (2.0E00 copies/ul) on STRATAGENE Mx3000P.

S.2 ANALITYCAL SPECIFICITY

Analytical specificity is the ability of a method to detect and quantify only the target marker.
The analytical specificity of BKV DNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (Lionsoft v.1.0 supplied by Biotools and Primer Express v.3.0" supplied by Applied Biosystems Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with BKV, and by the

"ClustalX" software, in order to compare the genome target sequences of the different genotypes of BKV.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Samples coming from patients suffering infections due to potential interfering organisms were obtained from a reference Clinical Centre.

The results are reported in the following table:

Organism	Result
JCV	negative
CMV	negative
EBV	negative
VZV	negative
HHV8	negative
HHV6	negative
HSV1	negative
HSV2	negative

S.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.3.1 Diagnostic Specificity:
The Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So the **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device
This parameter was studied by examining 10 BKV DNA negative samples extracts:

TRUE NEGATIVES	10
FALSE POSITIVES	0
TOTAL SAMPLES	10
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated in the 100%.

S.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So the **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.
In the kit code BKVDNAQT.CE this parameter was studied by examining 8 BKV DNA positive samples in duplicates in the same run. Also QCMD 2010 and QCMD 2011 JC Virus and BK Virus panel samples were tested. Then it was been calculated the percentage (%) of positive samples.

TRUE POSITIVES	8
FALSE NEGATIVES	0
TOTAL SAMPLES	8
SENSITIVITY %	**100**

On the basis of the results obtained Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	100 %

S.4 PRECISION
Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.
In the kit code BKVDNAQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. 4 dilution points in 8 replicates were tested In the same run (intra-assay) and in three different runs (inter-assay).
On the basis of the results obtained Intra and inter-assay variability were then calculated.

In absence of an established parameters in the European IVD Directive CTS we have identified the following value of acceptability for the BKV DNA:
Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

T. LIMITATIONS
The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting, application of a correct workflow along with careful programming of thermocycling step is essential for accurate and reproducible BKV DNA detection and quantitation.
The determination of the BKV DNA in a patient sample has extensive medical, social, Psychological and economic implications.
It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.

U. BIBLIOGRAPHY
1. Polyomavirus nephropathy: a current perspective and clinical considerations.Wiseman AC. Am J Kidney Dis. 2009 Jul;54(1):131-42.

2. Update on human polyomavirus BK nephropathy.Cimbaluk D, Pitelka L, Kluskens L, Gattuso P. Diagn Cytopathol. 2009 Oct;37(10):773-9.

3. From plasma BK viral load to allograft damage: rule of thumb for estimating the intrarenal cytopathic wear.Funk GA, Hirsch HH. Clin Infect Dis. 2009 Sep 15;49(6):989-90.

4. The decade of polyomavirus BK-associated nephropathy: state of affairs.Ramos E, Drachenberg CB, Wali R, Hirsch HH. Transplantation. 2009 Mar 15;87(5):621-30

5. Virological, epidemiological and pathogenic aspects of human polyomaviruses.Hurault de Ligny B, Godin M, Lobbedez T, El Haggan W, Pujo M, Etienne I, Ryckelynck JP. Presse Med. 2003 Apr 12;32(14):656-8.

6. Molecular and clinical perspectives of polyomaviruses: emerging evidence of importance in non-kidney transplant populations.Vilchez RA, Kusne S. Liver Transpl. 2006 Oct;12(10):1457-63.

5. Symbols

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

CE

DISTRIBUTOR

4BShop Lab Srls

info@4BShopLab.com

www.4BShopLab.com

+39.0371.18.56.643

MANUFACTURER

Dia.Pro - Diagnostic Bioprobes Srl

MADE IN ITALY

EN ISO 13485:2013 Certified