LightMix® in-vitro diagnostics kit Polyomaviruses JC and BK

Cat.-No.: 40-0206-32

Detection of JCV and BKV genomic DNA

for use with the

Roche Diagnostics LightCycler® Instruments

Reagents for 96 reactions

Upon arrival:

Store Premixed PCR reagents and Controls protected from light at room temperature or cooled (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20°C) (if included)

CE IVD

Manual version V160606

Summary

1. Product Information

1.1 Contents: LightMix® Kit Polyomaviruses JC and BK

Lyophilized premixed PCR reagents

⚠ **Store cooled or at room temperature (4-25°C) in the dark**

	Cap color	Label	Description content	Reaction / Tube status	Total
3 x	Blue	PSR	Parameter Specific Reagents (PSR) and Control Reaction containing premixed and lyophilized primers and hybridization probes for 32 reactions each. <0,01pg unlabeled oligonucleotides (BKV, JCV, Control Reaction specific forward and reverse primers); <0,01pg LightCycler Red 640 labeled oligonucleotides (BKV and Control Reaction specific probes); <0,01pg LightCycler Red 690 labeled oligonucleotides (JCV specific probe); <0,01pg Fluorescein-labeled oligonucleotides (BKV, JCV and Control Reaction specific probe);	32 reactions lyophilized	96 rxs

Control DNA

⚠ **Store cooled or at room temperature (4-25°C) in the dark**

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	Green	BK +	**Positive Control BKV** <0,01pg plasmid target (synthetic) [1E5 copies / reaction]	32 reactions lyophilized	32 rxs
1 x	Yellow	JC +	**Positive Control JCV** <0,01pg plasmid target (synthetic) [1E3 copies / reaction]	32 reactions lyophilized	32 rxs
2 x	White	nECT	**Extraction Control Target** <0,01pg plasmid target (synthetic) [4.8E6 copies: total amount PhHV and Lambda]	100 reactions lyophilized	96 rxs
1 x	Black	NTC	**No Template Control** Stabilizer (DNA)	---	---

Standard Dilution Row (Strip)

⚠ **Store cooled or at room temperature (4-25°C) in the dark**

	Strip	Description content	Status	Total
1 x	Clear	Synthetic **BKV** Plasmid **Standard** representing genomic equivalent targets: BKV-1 = 1E1 copies / reaction BKV-2 = 1E2 copies / reaction BKV-3 = 1E3 copies / reaction BKV-4 = 1E4 copies / reaction BKV-5 = 1E5 copies / reaction BKV-6 = 1E6 copies / reaction <0,01pg plasmid target (synthetic)	lyophilized	8 rxs
1 x	Clear	Synthetic **JCV** Plasmid **Standard** representing genomic equivalent targets: JCV-1 = 1E1 copies / reaction JCV-2 = 1E2 copies / reaction JCV-3 = 1E3 copies / reaction JCV-4 = 1E4 copies / reaction JCV-5 = 1E5 copies / reaction JCV-6 = 1E6 copies / reaction <0,01pg plasmid target (synthetic)	lyophilized	8 rxs
2 x	Strip cover	Adhesive strip cover		
1 x	Certificate of Analysis (CoA)	Expected crossing point for the plasmid strips included in the kit		

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ Store at -20°C upon arrival

The FastStart DNA Master HybProbe mastermix is included only in kits supplied directly by TIB MOLBIOL to customers in Central Europe (1).
The mastermix is not included in any kits supplied through Roche or its local distributor.

	Cap color	Label	Description Content	Reaction Tube storage	Total
3 x	**Red**	**1a**	LightCycler® FastStart Enzyme	32 reactions frozen	96 rxs
3 x	**White**	**1b**	LightCycler® FastStart Reaction Mix Hyb-Probe	32 reactions frozen	96 rxs
3x(2)	**Color-less**	**Water**	H_2O PCR grade	frozen	96 rxs
3 x(2)	**Blue**	**$MgCl_2$**	$MgCl_2$, 25 mM	32 reactions frozen	96 rxs

1) FastStart enzyme is shipped by TIB MOLBIOL at ambient temperature.
2) FastStart enzyme supplied through Roche Diagnostics contains only 2 tubes of H_2O and 1 tube of $MgCl_2$, nevertheless the quantity provided is sufficient for the use described for this kit.

Table 1

1.2 Intended Use

This device is an *in-vitro* nucleic acid amplification test for the detection and quantification of genomic DNA from Polyomaviruses JC and BK from nucleic acid extracts obtained from human plasma, serum, blood or urine; the kit does not detect Polyomaviruses KI or WU.

The amount of virus in these body fluids is an indication for reactivation of virus in immune incompetent patients, in particular in renal transplant patients.

This test is intended to be used to monitor polyoma virus amounts in blood or urine (viral load) in kidney transplant patients who are at risk of developing **Polyomavirus Nephropathy** (PVAN). The test result shall inform the clinician about an impending damage of the kidney.

This test is not intended to identify viral subtypes.

Results are reported as virus copies/ml of sample.

Polyomaviruses are widespread in the population and commonly asymptomatic in healthy individuals. Detection of virus in urine from healthy subjects is no indication for a disease requiring treatment.

The present diagnostic device must be used by qualified personnel only.

Note: The performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® Instruments (see 1.3.2 for details).

1.3 Specifications

1.3.1 Clinical Samples

Running the test requires 5 µl of purified DNA in aqueous solution. The test is performed on DNA extracted from urine, EDTA blood, plasma, or serum.

Collect at least 1 ml sample for extraction. Whole blood should be shipped on a cold pack. Plasma and urine samples should be shipped frozen.

Use 200 µl sample, add 10 µl of Extraction Control Target (**nECT**) to perform the spiked Extraction Control procedure, sEC, described throughout this manual and elute into 50 µl. Remember to complete No Target Control (NTC, water) by adding **nECT** too (see **7.1.3**).

Note: The viral load in urine can be very high; in case that urine is diluted prior extraction remember to include the dilution factor in the viral load calculation.

Clinically relevant is the amount of virus per sample volume, while PCR reports copies per reaction. The conversion factor between both numbers depends on the sample and extraction volumes. Extraction starts usually from less than one milliliter and PCR test does not use all extracted material. The viral load (VL) can be calculated using the following general formula :

VL [copies/ml] = MV x EVF x SF x EY x 1/CN

where :

VL = **Viral Load**
MV = **M**easured **V**alue [copy number per reaction]
EVF = **E**xtraction **V**olume **F**actor [Final extraction volume / PCR sample volume]
SF = **S**ample **F**actor [1,000 µl / extracted volume of clinical sample]
EY = **E**xtraction **Y**ield [according to the manufacturere's specification] (assumed to be 1)
CN = **C**opy **N**umber of the target region per organism (for Polyomavirus is 1)

For example, **200 µl** of clinical sample extracted in **50 µl** of final volume results in a conversion factor of 50, and **VL [copies/ml]** is calculated as:

$$\mathbf{MV} \times \frac{\text{50 µl Final volume}}{\text{5 µl PCR Sample Volume}} \times \frac{\text{1,000 µl}}{\text{200 µl Extracted Volume}} = \mathbf{MV \times 50\ [copies/mL]}$$

The effect of different volumes is demonstrated best in the following table:

Extracted Volume	Final Volume	PCR Sample Volume	Conversion Factor	LOD/ml for LOD 10/rxn(*)	Extraction Method
200 µl	100 µl	5 µl	x 100	1,000	MagnaPure Total NA Roche 03038505001
200 µl	**50 µl**	**5 µl**	**x 50**	**500**	
200 µl	35 µl	5 µl	x 35	350	
400 µl	60 µl	5 µl	x 30	300	QIAamp EZ1 Virus
1,000 µl	100 µl	5 µl	x 20	200	

* Limit of detection (LOD) per mL sample resulting from the calculated Conversion Factors assuming a technical LOD of 10 genome copies per PCR reaction.

Using a bigger sample volume and/or a reduced extraction volume increase naturally the achievable sensitivity (LOD), but remember that this is not too important for Polyomavirus detection, because the relevant and critical virus load is at rather high levels.

We recommend to insert the conversion factor derived from the extraction method into the standard curves of the instrument in order to report virus copies/ml sample, but this requires to run one standardized extraction protocol and to remember to change the values in case of changes in the extraction method.

1.3.2 Instruments, Software and Productivity

One kit contains reagents for 96 reactions performed in a 20 µl volume.
Each run requires including two standards and one negative control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Total PCR Run Time (approx.)	Maximum Samples per run (2)	Maximum Productivity of the kit (3)	Minimum Productivity of the kit (4)
LC 1.2	4.10 (1)	70 min	29 + 3 ctrl.	75	21
LC 1.5	4.10 (1)	70 min	29 + 3 ctrl.	75	21
LC 2.0	4.05	70 min	29 + 3 ctrl.	75	21
LC480 II(6) (96 wells)	1.5	100 min	93 + 3 ctrl.	81	21
LC480 II(6)(384 wells)	1.5	100 min	381(5) + 3 ctrl.	81	21
z 480 (open channel)	1.5	100 min	93 + 3 ctrl.	81	21

1 Running the test with the LightCycler® 1.2 or 1.5 Instruments with the software version 3.5 yields comparable results. Instruction for programming, data analysis and interpretation of results are not described in this manual. **Upgrade to version 4.10 or higher when possible**.
2 Each run must include two standards and one No-Target Control (NTC) for a total of 3 control reactions.
3 The first run of the kit requires including 15 controls (instead of 3) to teach the quantification module. The maximum number of samples that can be processed is reduced accordingly.
4 Calculated considering a single clinical sample analyzed in each run.
5 It requires using four kits.
6 LightCycler® 480 "first version" cannot be used with this kit.

Table 2

1.3.3 LightCycler® Instruments Channel (Filter) Settings

Instrument	Dye / channel name		
	530	**LC640**	**LC690**
LightCycler® 1.2 / 1.5(1)	F1	F2	F3
LightCycler® 2.0	530	640	705
LightCycler® 480 II	465-510	498-640	498-660
cobas z 480 Analyzer	465-510	498-645	498-700

(1)Instruments with the software version 3.5

Table 3

1.4 Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix!

Reagents and Controls:
Store the lyophilized reagents (PSR, Standard dilution row and Control DNA) protected from light and at room temperature or cooled (4°C - 25°C).
Do not freeze these dried reagents. Expiration date is printed on the kit label.

Polymerase mix :
Store the LightCycler® FastStart DNA Master HybProbe at -15°C to -25°C.
See expiration date on the polymerase tube label.

Shipping:
Products are shipped at ambient temperature. Transport stability of reagents and enzyme components have been tested under shipping conditions.

2. Additional Devices and Reagents

2.1 Required

	TIB Molbiol
LightMix® Kit –Color Compensation HybProbes	Cat.-No. 40-0318-00
LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Cat.-No. 12 011 468 001
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
or	
LightCycler® 480 II Instruments	**Roche Diagnostics**
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
cobas 4800 System (cobas z 480 Analyzer)	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
or	
LightCycler® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
General materials	
Phosphate Buffer Saline (PBS)	any supplier
Nuclease-free PCR grade water	any supplier

2.2 Optional

	TIB Molbiol
Extraction Control Target (**nECT**)	30-0259-96
	Roche Diagnostics
LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.3 Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit (#)	Cat.-No. 11 796 828 001
or High Pure Viral Nucleic Acid Kit (#)	Cat.-No. 11 858 874 001
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier
Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
MagNA Pure 96 IVD Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001
MagNA Pure Instrument (#)	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 2.0 Instrument (#)	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument (§)	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001

(#) Additional Extraction Control Target tube is required.

(§) The use of MagNA Pure Compact Instrument allows to perform only the **Internal Control** procedure (see **3.3.2**).

3. Background Information

3.1 Medical Background

Polyomavirus JC and BK are double stranded DNA viruses ubiquitous in the human population. Infections of healthy individuals are usually asymptomatic. JCV is more common than BKV.

JCV can cross the blood-brain barrier into the central nervous system and can destroy the oligodendrocytes, causing Progressive Multifocal Leukoencephalopathy (PML), a neurological disease found in particular in immunecompromised patients (immunosuppression after organ transplantation, AIDS).

BKV has been associated with various kidney diseases such as interstitial nephritis, hemorrhagic cystitis and ureteral stricture, however, most important is reactivation in immune incompetent individuals.

BKV infection takes probably an oral route. After the normally sub-clinical primary infection in early childhood, the virus reproduces in the epithelial cells of the kidney and establishes latency in renal tissues. Latent inf
ions of the brain are rare.

Reactivation of polyomaviruses can occur during immunosuppression after organ transplantation, AIDS, but also after pregnancy. The introduction of new immunosuppressive drugs led to emergence of Polyomavirus Nephropathy (PVAN) in up to 10% of all kidney transplant recipients, resulting in persistent graft dysfunction, or loss of the transplanted organ. About half of the patients might lose their grafts unless diagnosed at an early stage (Singh et al., 2006)[1].

In this case reduction of immunosuppression helps the patient to rise antiviral immune response, but also treatment with cidofovir has been applied.

Diagnosis

The presence of decoy cells infected by polyomavirus (microscopy) in urine and/or high levels of BKV and less extend JCV DNA in urine and plasma (or blood) detected by PCR are a warning sign for PVAN. Low levels of viruria are not clinically relevant (Randhawa et al., 2006)[2].

Quantitative detection of BKV in renal transplantation patients helps to predict which patients are at risk to develop PVAN, and can be used to follow response to therapy. Serial monitoring is reported be more useful than single time detection.

Real-Time quantitative PCR is used to detect polyomavirus since many years (Biel et al., 2000; Narayanan et al., 2007) [3,4], commonly targeting the conserved T-antigen gene region.

According to the literature patients with more than 10^4 BKV copies/mL plasma or $>10^7$ copies/mL urine have a risk to develop PVAN (Hirsch et al., 2002)[5].

Quantitative PCR for BKV may also be useful for monitoring bone marrow transplant patients who are at risk for hemorrhagic cystitis.

3.2 Methodology and Assay Principle

This device detects a fragment of the *Polyomaviruses JC* and/or *BK* genome in a nucleic acid extract obtained from blood or urine.

This device does not detect *Polyomavirus KI* (Allander et al., 2007)[6] or *WU* (Gaynor et al., 2007)[7]. Pathogenicity and prevalence are not known; *WU* virus has been found in particular in patients with acute respiratory tract infection.

A 172-175 bp long fragment of the Small t-Antigen gene is amplified with specific primers and detected with a universal fluorescein donor probe and virus specific probes labeled with LightCycler® Red 690 for JCV and 640 for BKV, similar to the assay described by Narayanan et al., 2007[4].
After every PCR cycle the probes bind during the annealing step to the PCR generated target and bring the fluorophores in close proximity. The donor fluorophore fluorescein is excited by light, part of the energy is transferred to the acceptor dye and detected. The fluorescence intensities are plotted against the cycle number, givin a picture of the amplification and to calculate a crossing point (Cp) which is related to the log of the concentration of target in the sample.
Hybridization Probes can be used to identify the PCR product in a melting curve analysis in order to verify that the right product has been formed. The melting point (Tm) is dependent on length and G+C content, but also upon the degree of homology to the sensor probe. In this device the Tm is not used for virus discrimination; slight temperature shifts are acceptable as the shape of curve depends also on the amount of product. However, a significant decrease in the Tm is an indication of a sequence variation, explaining an eventual lower signal level in the quantification plot and may give cause for a subsequent analysis of the sample by DNA sequencing (please contact us at: service@tib-molbiol.de).

The supplied control DNA allows comparison with unknown patient samples.

3.3 Control Reaction

An additional PCR product of 658 bp is amplified from the Extraction Control Target (**nECT**) and detected with a short LightCycler® Red 640 labeled probe, which is visible in the melting curve analysis only.
The control reaction does not interfere with the analytical reaction; it is designed to be more sensitive to the presence of inhibitors and it will fail to amplify in presence of high-positive samples. The control reaction must be visible in negative and low-concentrated samples while it will fail in the presence of higher amounts of target DNA (1,000 copies/PCR or higher).

The Extraction Control Target can be utilized in two different procedures: either as Spiked Extraction Control (3.3.1) or as Internal Control (3.3.2).

	Pathogen		
	JCV	**BKV**	**Control**
Channel	**LC690**	**LC640**	**LC640**
PCR Fragment	**172-175 bp**	**172-175 bp**	**658 bp**
Melting curve Tm	**63-73°C**	**56-66°C**	**49-50°C**

Table 4

3.3.1 Spiked Extraction Control (sEC)

The spiked Extraction Control (**sEC**) procedure monitors the extraction and evaluates the presence of amplification inhibitors (recommended procedure).

When sample extraction is performed with High Pure PCR Template Preparation Kit, High Pure Viral Nucleic Acid Kit or MagNA Pure LC DNA Isolation Kit I a double amount of **nECT** is required, due to the low recovery rate.

3.3.2 Internal Control (IC)

The Internal Control (**IC**) procedure evaluates the ability of a PCR amplification thus absence of inhibitors only.

This procedure is mandatory if extraction is performed with MagNA Pure Compact Instrument.

3.4 Performance Characteristics

Analytical Specificity
The specificity to the target gene and the suitability of the PCR amplification employed in the present test has been evaluated by direct DNA sequencing of the amplicon.

Analytical Sensitivity
Analytical sensitivity for BK virus from cell culture has been determined with 1.34 copies per reaction (Probit-95), corresponding to 67 copies / mL clinical sample, using the conversion factor 50x (see 1.3.1). The analytical sensitivity for JC virus from an urine sample has been determined with 1.45 copies per PCR reaction, or 73 copies / mL clinical sample, using the conversion factor 50x.
Estimating a maximum loss of 95% during extraction (depending on the kit), the LOD values are 1,340 copies and 1,460 copies per mL, respectively.
Rubio et al., 2010 [8] published for an in-house assay using the identical primer and probes an analytical sensitivity of 53 copies per reaction , corresponding to 2,650 copies / mL clinical sample.

Linearity
The Cp values were reported to be linear in the range down to 7,000 copies BKV/ mL and 15,000 copies JC/ mL for plasma and urine (Rubio et al., 2010)[8].

Inter-assay Variability
Inter-assay variability was obtained in 14 and 10 consecutive runs for BKV and JCV, respectively; the CV was less than 4% and the assays showed consistent repeatability and a high precision (Rubio et al., 2010)[8].

Diagnostic Specificity
Other common human-pathogen DNA viruses do not yield a positive result, including the related polyomaviruses WU and KI. No cross-reactivity between BKV and JCV was observed and no other DNA viruses such as CMV or EBV were detected (Rubio et al., 2010)[8]. [Diagnostic Specificity = 100%].

Diagnostic Sensitivity
The evaluation study was performed with one group of 76 spiked samples and a second set of 22 positive and 23 negative specimen (Rubio et al., 2010)[8] :

BKV from urine, sensitivity =	96.5%
BKV from plasma, sensitivity =	100.0%
JCV from urine, sensitivity =	96.4%
JCV from plasma, sensitivity =	100.0%
Average Diagnostic Sensitivity =	97.9%

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.

Use the manual version which is delivered with the kit (see kit label).

General Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual .

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "PCR protocol" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Sample Preparation

5.1 Preparation of the Extraction Control Target (nECT)

Spin the tube at 10,000 RPM for 1 minute, dissolve the **nECT** in 1,2 ml PBS (not supplied with the kit), mix and spin down.

5.1.1 Use of nECT as spiked Extraction Control (recommended)

When running the spiked Extraction Control procedure (**sEC**), add the following volumes of **Extraction Control Target** to 200 µl of sample to be extracted.

High Pure PCR Template Preparation Kit	**20 µl**	11 796 828 001
High Pure Viral Nucleic Acid Kit	**20 µl**	11 858 874 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	**10 µl**	05 467 497 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	**10 µl**	06 543 588 001
MagNA Pure LC DNA Isolation Kit I	**20 µl**	03 003 990 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	**(#)**	03 730 964 001

(#) Only the use of the **Internal Control procedure** is allowed (see **3.3.2**).

Perform nucleic acid purification with the appropriate extraction kit as described in the respective extraction protocols and elute into 50 µl.
Store purified DNA refrigerated (4°C - 8°C), if stored over a longer period of time keep frozen at -20°C.

Remember to complete No Target Control (**NTC**) and Diluents for Control DNAs (**DIL**) by adding **nECT** too (see **7.1.3 and 7.1.4**).

5.1.2 Use of nECT as Internal Control

If running the Internal Control Procedure, use the Extraction Control Target **only** in the preparation of the Reaction mix (see **7.2**).

5.2 Plasma

Perform nucleic acid purification using the Roche High Pure PCR Template Preparation Kit or a MagNA Pure Instrument with the appropriate extraction kit (Virus Total NA extraction kit , see section 2. Additional Devices and Reagents) and as described in the respective extraction protocols.

5.3 Urine

Urine can be extracted with kits designed for extraction of blood or serum.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments using the extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols. Avoid the spiked extraction control procedure if the extract is diluted afterward, use the IC procedure instead.

5.4 Whole Blood

For preparation of genomic DNA use human peripheral blood (EDTA).
Heparin is strongly discouraged it might interfere with the PCR.
Perform nucleic acid purification using the Roche High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments with the Virus Total NA extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

5.5 Converting Factors

The starting volume of the biological sample and the volume of the elution buffer used in the DNA purification have to be taken in account to calculate the viral copies number expressed in copies/ml (see section 1.3.1).

Controls	**Viral copies number expressed in copies/ml**			**Table 4**
Copies / reaction	**200 µl sample/ 100 µl elution**	**200 µl sample/ 50 µl elution**	**200 µl sample/ 35 µl elution**	**Elution vol. x sample vol. / 200**
1E1	1,000	500	350	x 10
1E2	10,000	5,000	3,500	x 100
1E3	100,000	50,000	35,000	x 1,000
1E4	1000,000	500,000	350,000	x 10,000
1E5	10,000,000	5,000,000	3,500,000	x 100,000
1E6	100,000,000	50,000,000	35,000,000	x 1,000,000
BK +	10,000,000	5,000,000	3,500,000	x 100,000
JC +	100,000	50,000	35,000	x 1,000

6. Programming

6.1 Color Compensation

Color Compensation is required
for the use of the *LightMix*® *Kit* Polyomaviruses JC and BK.
Analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

6.2 Capillary Based Instruments

For details see the LightCycler® Operator's Manual.
The protocol consists of four program steps (Tab.5):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:05**	**00:00:15**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate [°C/s]*	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [°C]	**0**	**0**	**55**	**0**	**0**	**0**	**0**	**0**
Step Size [°C]	**0**	**0**	**0.5**	**0**	**0**	**0**	**0**	**0**
Step Delay [cycles]	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

* For LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' [°C/s] instead of Ramp Rate.

Table 5:
Programming of capillary based Instruments

Note:
While programming maintain default software values: max. samples = 32 and capillary size = 20 µl. Set seek temperature = 40°C and channel = 640.
Store the program and the default values as '**RUN Template'** which can be loaded to start every Polyomaviruses JC and BK LightCycler® run.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.
For instructions see the Operator's Manual

6.3 Multiwell Based Instruments

LightCycler® 480 II Instrument and cobas z 480 Analyzer
For details see the Instrument Operator's Manual.

Detection Format: TIB MOLBIOL 640-690
Please refer to the manual of:
LightMix® Kit Color Compensation HybProbes Cat. No. 40-0318-00

Reaction Volume: 20 µl

Programming:
The protocol consists of four program steps (Tab.6):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:05**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate [C°/ s] **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.19**	**1.5**
Ramp Rate [C°/ s] **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.19**	**2.0**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**
Sec Target [°C]	**0**	**0**	**55**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0,5**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**1**	**0**	**-**	**-**	**-**	**-**

Table 6: Programming of '480' multiwell based instruments (96 well and 384 well formats)

Note:

a) Store the program and the default values as '**RUN Template'** which can be loaded to start every Polyomaviruses JC and BK LightCycler® run.
b) Ensure to program only 1 acquisition per second instead the default value 3; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.

7. Experimental Protocol

Program the Instrument before preparing the solutions (see **5. Programming** and read the LightCycler® Operator's Manual for details).

7.1 Reagents Preparation

7.1.1 LightCycler® FastStart DNA HybProbe Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Quickly spin tubes to collect drops.
4	The solution must be free of particles.
5	Add 60 µl of **1b** to the vial **1a**.
6 ⚠	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles.
7	Spin the tubes to collect drops.
8	Use reagent to prepare the Reaction Mix (6.3).
9	Store left over reagent at 4°C.

7.1.2 Preparation of Parameter-Specific Reagents (PSR)

►	Each **PSR** reagent tube is sufficient for 32 reactions.
1	Spin the premixed **PSR** tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom.
3	To each **PSR** tube add **66 µl** of PCR-grade **Water.**
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 2 µl of **PSR** reagent for a 20 µl PCR reaction.

7.1.3 No Template Control (NTC)

Fill the **NTC** vial with 950µl PCR-Grade water.

⚠ Only if running the spiked Extraction Control (**sEC**) procedure add 50 µl **nECT** (see chapters **3.3.1** and **5.1**).

NTC is sufficient for 32 reactions and for **standards** / **controls** preparation.

► Use **5 µl** of **NTC** for a 20 µl PCR reaction.

Do not manipulate **NTC** in the same location where Standard Strip and Controls are used.

Note: the extraction of an already purified plasmid is not efficient as the native DNA, the dilution factor compensate for the expected lost.

7.1.4 Diluents for Standard Strip and Controls (DIL)

Fill a clean vial with 950µl PCR-Grade water.

⚠ Only if running the spiked Extraction Control (**sEC**) procedure add 50 µl **nECT** (see chapters **3.3.1** and **5.1**).

DIL is sufficient for the preparation of all **standards** / **controls:**
it must be prepared fresh and disposed immediately after use.

DIL, used in close proximity of Standard Strip and Controls, may become contaminate.

7.1.5 Preparation of Standard Strips

The LightCycler® software can be calibrated with reference standards to perform an automated quantification of unknown clinical samples.

►	Plasmid DNA located at the bottom of the strips' well is blue colored to enhance visualization. The lower concentration (1E1 copies / reaction) is identified by the longer lip.
1	Without disturbing the pellets in the well's bottom, perforate aluminum cover with a pipette tip starting from well #1(1E1 copies / reaction).

Using a new tip for each well:

2	Add 40 µl **DIL**.
3	Pipette up and down 10 times.
4	Discard tip.

► Use **5 µl** of **Standards** for a 20 µl PCR reaction
► All standards must only used once to calibrate the quantification module (first run of the lot); strips must be immediately sealed and dispose after lot validation.

Please note: Opening the vials may cause contaminations of the work-space (aerosol).

7.1.6 Preparation of Positive Controls

►	Each **Control** reagent tube is sufficient for 32 reactions. Controls are used in later runs to recall the first run standard curve.
1	Spin the two tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 160 µl **DIL**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec. and spin the tubes to collect drops

► Use **5 µl** of each **Control** for a 20 µl PCR reaction.
► All two **Controls** must be used in each run.

Please note: Opening the vials may cause contaminations of the work-space (aerosol).

7.2 Preparation of the Reaction Mix

Use left column **sEC** for the spiked Extraction Control procedure (see **3.3.1** and **5.1**).
Use right column **IC** for the Internal Control procedure (see **3.3.2**).

7.2.1 Preparation of 32 x Reactions Mix

We recommend preparing 32 reactions to prevent storage of dissolved or activated reagents in varying volumes. For the preparation of reaction mix for fewer samples, please go to step 7.2.2 "Reaction mix for single reaction".

Prepare the reaction mix in the PSR reagent tube:		**sEC**	**IC**
	Components	**32 reactions**	
Add:	To the **PSR** tube (red cap) already containing	66.0 µl	66.0 µl
	H_2O, PCR-grade (colorless cap)	297.0 µl	280.5 µl
	Mg^{2+} solution 25 mM (blue cap)	66.0 µl	66.0 µl
	LightCycler® FastStart DNA Master HybProbe (red cap), see **7.1.1**	66.0 µl	66.0 µl
	nECT when used as IC Internal control (white cap), see **3.3.2**	---	16.5 µl
	Substitute of the "long neck cap" of the **PSR** tube with the red cap from FastStart		
	Total Volume	**495.0 µl**	**495.0 µl**

Table 7: Volumes of components for preparing 32 reaction mixture

7.2.2 Preparation of a Single Reaction Mix

Prepare the reaction mix by multiplying each volume by the number of biological samples to be analyzed plus four reactions (**NTC**, two **Controls**, one excess). In the first run add also the twelve **Standards**.

Prepare the reaction mix in a cooled vial:	**sEC**	**IC**
Components	**Single reaction**	
H_2O, PCR-grade (colorless cap)	9.0 µl	8.5 µl
Mg^{2+} solution 25 mM (blue cap)	2.0 µl	2.0 µl
PSR (blue cap), see **7.1.2**	2.0 µl	2.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	2.0 µl	2.0 µl
nECT when used as IC Internal control (white cap), see **3.3.2**	---	0.5 µl
Volume of reaction mix	**15.0 µl**	**15.0 µl**

Table 8: Volumes of components for preparing a single reaction mixture

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due to a non homogeneous reaction mix!

7.2.3 Capillary / Well Loading Procedure

Each run must include one Negative Control (**NTC**) to demonstrate the absence of contaminations with target DNA or PCR product and at least two **controls** to ensure the performance of the kit.

Capillary / Well Loading Procedure

▶	Remember to include the controls when setting up the run.
1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **15 µl** per capillary/well of reaction mix.
3	**Mandatory:** Add **5 µl** of **Negative Control** (**NTC**) Add **5 µl** of **JC+ Control** Add **5 µl** of **BK+ Control**
	In the first run only: Add **5 µl** of **JCV strip dilutions** in positions 4 to 9 (A4 to A9). Add **5 µl** of **BKV strip dilutions** in positions 10 to 15 (A10 to A15).
4	Add **5 µl** of **Sample** in the remaining capillaries / wells.
5 ⚠	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the LightCycler® Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment's name when instructed.
10	Save sample data in the samples' window.

* See section **7.4** for the Sample loading and Standards calibration.

7.3 Storage and Stability of Diluted Components

Reaction Mix
The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (4°C - 8°C) for 30 days. Avoid prolonged exposure to light.

Parameter Specific Reagents (PSR)
Once diluted, store PSR refrigerated at 4°C - 8°C for a maximum period of 30 days. Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe
The combined FastStart mastermix (1a+1b) can be stored refrigerated (4°C - 8°C) for 30 days.

Controls
The dissolved **Controls** are stable for 30 days when stored refrigerated (4°C - 8°C). When using them over a longer period of time, aliquot and store frozen at -20°C. Controls stored at -20°C are stable for 180 days.

Note:
More than five (5) freezing and thawing cycles of controls may result in shift in the Cp value compared to the freshly dissolved vial; avoid the use of the control when the Cp value changes more than 1 cycle.

Standards (Strip)
Seal strips immediately after use with the included sealer and discard after lot validation. Work-space contamination and evaporation might occur in dissolved and unsealed strips.
Dissolved Standards strips are stable for one day only.

7.4 Loading of Standards or Controls

Routine Runs:

Controls attributes are summarize in the table below:

Pos	Sample Name	Channel LC1.5 / 2.0	Channel LC480II	Channel z 480	Sample Type	Copies per reaction
1	NTC	640	498-640	498-645	Negative	---
1		705	498-660	498-670	Negative	---
2	JC + Control	640	498-640	498-645	Unknown	
2		705	498-660	498-670	Standard	1.00E3
3	BK + Control	640	498-640	498-645	Standard	1.00E5
3		705	498-660	498-670	Unknown	

Table 8

The values for concentration inserted in above table are copies per reaction. Correct numbers by the Conversion Factor (see sections 1.3.1 and 5.5).

First Run:

For the first run (repeat once per lot) use the dilution rows and the two controls. Standards attributes are summarize in the table below:

Pos	Sample Name	Channel LC1.5 / 2.0	Channel LC480II	Channel z 480	Sample Type	Copies per reaction
1	NTC	640	498-640	498-645	Negative	---
1		705	498-660	498-670	Negative	---
2	JC + Control	640	498-640	498-645	Unknown	
2		705	498-660	498-670	Unknown	
3	BK + Control	640	498-640	498-645	Unknown	
3		705	498-660	498-670	Unknown	
4	JCV-1	640	498-640	498-645	Unknown	
4		705	498-660	498-670	Standard	1.00E1
5	JCV-2	640	498-640	498-645	Unknown	
5		705	498-660	498-670	Standard	1.00E2
6	JCV-3	640	498-640	498-645	Unknown	
6		705	498-660	498-670	Standard	1.00E3
7	JCV-4	640	498-640	498-645	Unknown	
7		705	498-660	498-670	Standard	1.00E4
8	JCV-5	640	498-640	498-645	Unknown	
8		705	498-660	498-670	Standard	1.00E5
9	JCV-6	640	498-640	498-645	Unknown	
9		705	498-660	498-670	Standard	1.00E6
10	BKV-1	640	498-640	498-645	Standard	1.00E1
10		705	498-660	498-670	Unknown	
11	BKV-2	640	498-640	498-645	Standard	1.00E2
11		705	498-660	498-670	Unknown	
12	BKV-3	640	498-640	498-645	Standard	1.00E3
12		705	498-660	498-670	Unknown	
13	BKV-4	640	498-640	498-645	Standard	1.00E4
13		705	498-660	498-670	Unknown	
14	BKV-5	640	498-640	498-645	Standard	1.00E5
14		705	498-660	498-670	Unknown	
15	BKV-6	640	498-640	498-645	Standard	1.00E6
15		705	498-660	498-670	Unknown	

Table 9

The values for concentration inserted in above table are copies per reaction. Correct numbers by the Conversion Factor (see sections 1.3.1 and 5.5).

Example Run - adapted to copies / volume :
For a sample volume of 200 µl and a final extraction volume of 50 µl use the correction factor **x50** resulting in 50,000 copies/mL for 'JC + control' and 5,000,000 copies/mL for 'BK + control' :

Pos	Sample Name	Channel LC1.5 / 2.0	Channel LC480II	Channel z 480	Sample Type	Copies per mL sample
1	NTC	640	498-640	498-645	Negative	---
1		705	498-660	498-670	Negative	---
2	JC + Control	640	498-640	498-645	Unknown	
2		705	498-660	498-670	Standard	5.00E4
3	BK + Control	640	498-640	498-645	Standard	5.00E6
3		705	498-660	498-670	Unknown	

Table 10

7.4.1 Capillary Based Instruments

In “Samples data - Capillary View”, input Sample Name as described in the second column.
Select “Analysis Type – Abs Quant”. Select Channel 640 and 705 only !
From the pull down menu select “Sample Type” as above and input the corresponding concentration.

For LightCycler®1.x software versions 3.5 see the Operator’s Manual.

7.4.2 Plate Based Instruments

In the “Sample Editor” window, in “Step1: Select Workflow” section, select “Abs Quant”. Input the description in “Sample Name” column,
From the pull down menu select “Quantification Sample Type” as above and input the corresponding concentration.

8. Data Analysis and Interpretation

8.1 Limits and Interferences

The present test is specific for *Polyomaviruses JC* and *BK*.

Some viruses which appear to be positive for JCV and BKV will be BKV variants having an altered sequence in the probe binding region; see section 8.7.

Other Polyomavirus such as KI and WU are not detected.
Particular interferences for this assay are not known.

8.2 Calibration

Calibration has to be performed following the procedure described in, 7.1.5, 7.1.6, 7.2.3, 7.4, 8.3.2 and 8.3.3.

8.3 Quality Control – Acceptance Criteria

In order to perform a reliable quantification analysis, it is essential that Negative Control **NTC** and all **Controls** are included in each run.
In addition to the reagents needed to detect JCV and BKV, the PSR contains also primers and probes for the amplification of a foreign DNA target (**nECT**) added to the PCR following the **spiked Internal Control (sEC)** (see **3.3.1**) or the **Internal Control (IC)** (see **3.3.2**) procedures.
At the end of the run a melting-curve analysis will display a specific melting peak in LC640 at a temperature of 49ºC indicating **nECT** amplification.
The amplification, performed at an annealing temperature of 55ºC ensures that the **nECT** amplification is not visible, thus do not interfere with BKV quantification.

Activate Color Compensation.
Analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

8.3.1 Negative Control (NTC)

NTC Negative Control is mandatory - mimics negative clinical samples.
Amplification analysis of **NTC** must provide a **negative result**.

If any amplification is visible in either channel, a contamination or a pipetting error has occurred; the session is not valid and the whole procedure has to be repeated (amplification and detection).

If the problem sustains, change water and/or reagents and repeat.

Melting-curve analysis of **NTC** must provide a **positive result**:

One melting peak from **nECT** amplification must be detected (Tm = 49ºC).

If the **nECT** melting peak in channel 640 at 49ºC is absent or a melting peak at a different temperature or one peak in channel 690 are present, the session is not valid and the whole procedure must be repeated (amplification & detection).

8.3.2 BK+ Control or Standards

BK + Control or a **Standards** curve is mandatory - mimics positive samples.

Amplification analysis of **BK + Control** or **Standards** must provide a **positive amplification** in channel 640. Cp must conform with expected results (see 8.5)
If amplification is absent see instruction in 8.3.4.
If any amplification signal for the BK+ Control or Standards is seen in the 690 channel, the session is not valid and the whole procedure has to be repeated.
Melting-curve analysis of BK+ **Control** or **Standards** must provide a melting curve with a Tm of about 65ºC in channel 640; also the **nECT** melting peak at 49ºC will be detected in low copy number samples; **nECT** fails in high copy number samples. If a melting peak for BK+ Control and Standards is present in channel 690, the session is not valid and the entire procedure has to be repeated (amplification and detection).

8.3.3 JC+ Control or Standards

JC + Control or a complete **Standards** curve is mandatory.
JC + Control mimics JCV positive clinical samples.

Amplification analysis of J**C + Control** or **Standards** must provide a **positive amplification** in channel LC690. The Cp value must conform with expected results (see 8.5). If amplification is absent see instruction in 8.3.4
If any amplification signal for JC+ Control or Standards is recorded in the 640 channel, the session is not valid and the whole procedure has to be repeated (amplification and detection).

Melting-curve analysis of JC+ **Control** or **Standards** should provide a melting curve with Tm = 69ºC in channel 690.
In channel 640 the **nECT** melting peak (49ºC) will be detected in low copy number samples; **nECT** fails in high copy number samples; if any other melting peak for JC+ Control and Standards is present in channel 640, the session is not valid and the whole procedure has to be repeated (amplification and detection).

8.3.4 Failures in runs of Controls and Standards

Complete data from standard rows and controls are essential to get accurate results for later runs with patient samples.

Single missing data points or single aberrant values in the **standard row** (probably a pipetting error) within a run in which all other results comply with the expected Ct values described in the certificate of analysis can be 'disabled' by setting the Sample Type as 'unknown' (see 7.4). In any runs missing the lowest copy number, the curve must be **immediately** repeated with the lowest copy number in **triplicate**, because it is not possible to validate the kit performance nor to store an appropriate standard curve. The deviation must be recorded.
Runs without adequate results for the **controls** must be repeated - otherways it is not possible to recall the stored standard curve (see 8.4)

Before repeating a run consider common errors; check in particular the amplification program, use of the correct master-mix and $MgCl_2$ concentration; remember that also inadequate storage of reagents may cause a failure of the device.

8.3.5 Samples

Positive Clinical Samples:

Amplification analysis provides a **positive amplification** in the respective channel (640 for BKV and 690 for JCV). Samples positive in both channels have been observed.
Melting-curve analysis should yield a melting curve in the respective channel with Tm = 56°C-65ºC in channel 640 and / or Tm = 69ºC in channel 690.
The **nECT** derived melting peak at Tm = 49ºC in channel 640 will be visible only in samples with a low copy of virus.

Negative Clinical Samples:

Amplification analysis provides **no amplification** signal in either channel.
Melting-curve analysis must exhibit the **nECT** derived melting peak at Tm = 49ºC in channel 640.
Clinical samples displaying neither amplification nor **nECT** melting peaks are invalid and the whole procedure has to be repeated (sample preparation, amplification and detection).

8.3.6 Out of range Cp Amplification Values

The software will flag all biological samples with values outside the standard curves with an “out of range” message.
Report samples as:
“positive above 1E6 quantification limit” when the Cp is higher than 1E6 and “positive below 1E1 quantification limit” when the Cp is lower than 1E1.
The “out of range” messages are common.

8.3.7 Abnormal Melting Curves

Abnormal melting curves generated by viral variants are sometime reported; (See Fig 12 for a known example). In the latter case another method must be used for comparison / verification of the amplified product.
Submit the PCR fragment for DNA sequencing to confirm viral identification.
Report deviations to service@tib-molbiol.de.
Feel free to send deviant melting samples to the Berlin laboratories to confirm the obtained results by DNA sequencing.

8.4 Saving External Standards Curve

After the quantification analysis, check if Controls and Standards comply with the acceptance criteria (see **8.3 Quality Control – Acceptance Criteria**), and check if the Cp values are within the required range (see certificate of analysis), save the Standards curve as follows and recall curves in all successive runs.

8.4.1 Capillary Based Instruments

Absolute Quantification analysis – in the “Standard curve (In Run)” pool down menu select “Save standards as External…”. Repeat the procedure for each channel.

8.4.2 Plate Based Instruments

In the Abs Quant/2nd Derivative Max analysis window – “Calculate” - in the “Standard curve (In Run)” menu select “Save standards as External…”. Repeat procedure for each channel.

8.5 Reading the Results

Perform data analysis as described in the LightCycler® Operator's Manuals.

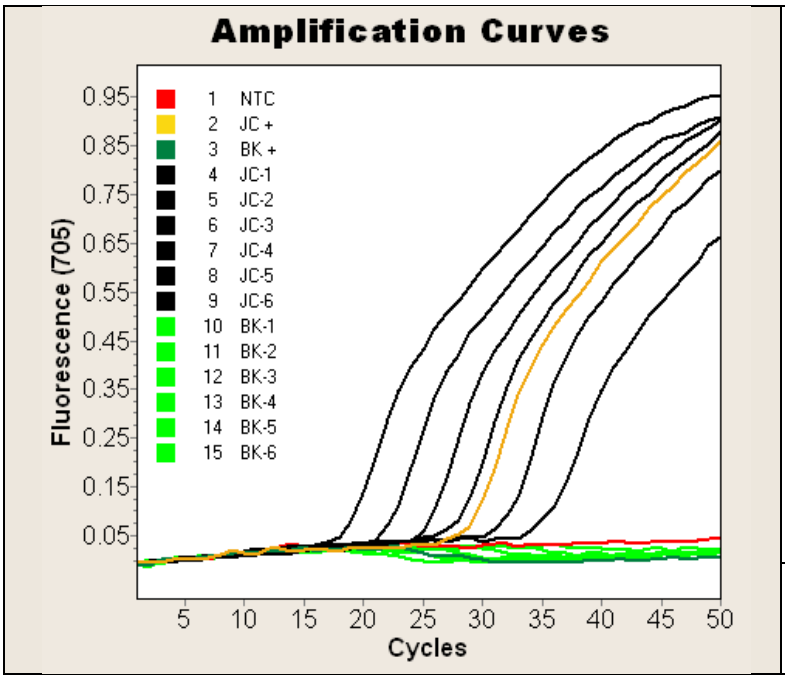
Activate Color Compensation! Analyzing data with deactivated 'Color Compensation'will generate invalid readouts of the results.

8.5.1 Amplification Analysis: Capillary Based Instruments

View data for JCV amplification in LC 690 and BKV amplification in LC 640.

View data as follows:

LightCycler® 2.0 System (software ver.4.0.5) select: "Absolute Quantification" analysis mode.
LightCycler® 1.x System (software ver.4.0.5) select: "Absolute Quantification" analysis mode.
LightCycler® 1.x System (3.5) select: "Quantification – Second Derivative Maximum" mode.

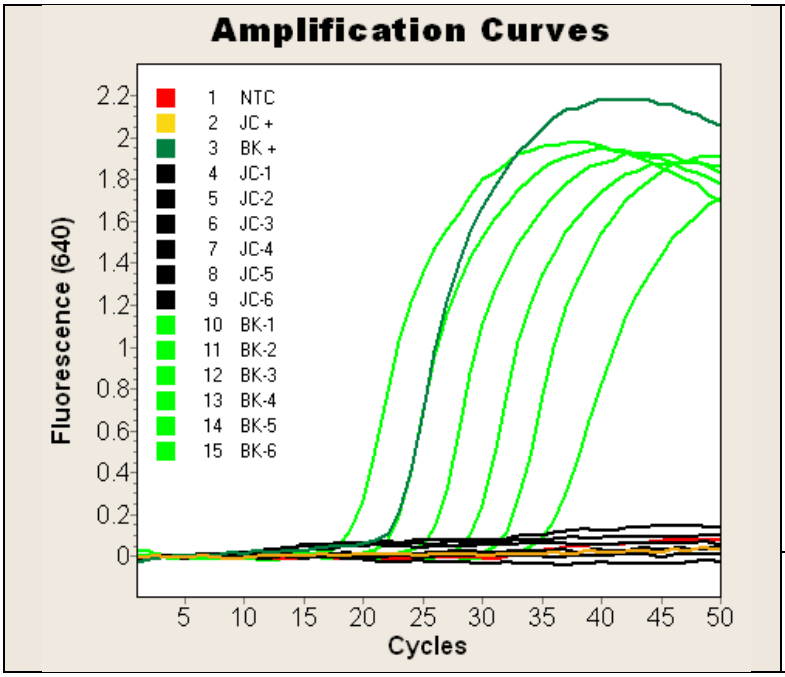


LC 690

1) **NTC** must show a flat line
2) **JC +**
4) JCV-1
5) JCV-2
6) JCV-3
7) JCV-4
8) JCV-5
9) JCV-6

Compare with expected Cp values described in the certificate of analysis. BKV Controls (green lines) must show a flat line.

Fig. 1: Amplification sample data for JCV.



LC 640

1) **NTC** must show a flat line
3) **BK +**
10) BKV-1
11) BKV-2
12) BKV-3
13) BKV-4
14) BKV-5
15) BKV-6

Compare with expected Cp values described in the certificate of analysis. JCV Controls (black and yellow lines) must show a flat line.

Fig. 2: Amplification sample data for BKV.

Note: The values of the crossing point (Cp) may vary ±1.5 cycles between different experiments.

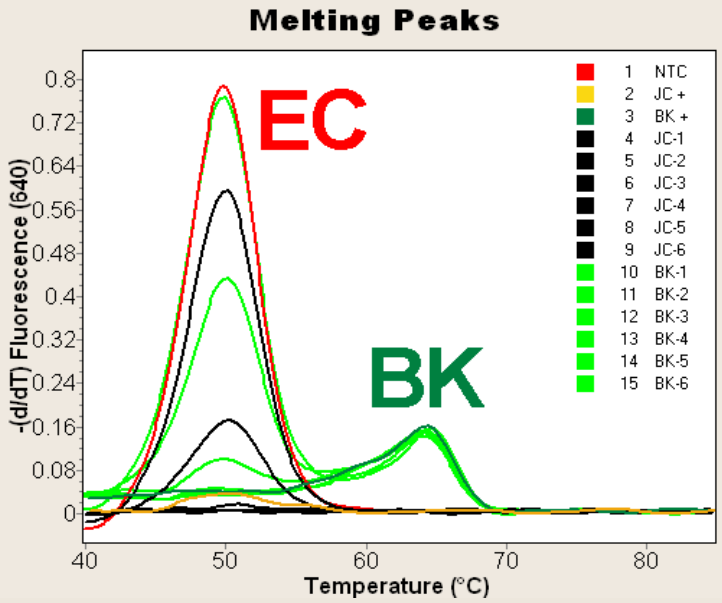
In case of variations see instructions: **8.3.4 Absent amplification in Controls or Standards.**

8.5.2 Melting Curve Analysis: Capillary Based Instruments

View Control Reaction and BKV melting curve data in LC640.

View data as follows:
LightCycler® 2.0 Instrument (software versions 4.0.5) select:
Melting curve: "Tm Calling" analysis mode.

LightCycler® 1.x, Instrument (software versions 3.5) select:
Melting curve: "Melting Curve – Manual Tm" analysis mode.



LC640

BK melting curve at 59-66°C

1) **NTC** must show NO melting peak
2) **JC +** must show NO melting peak
3) **BK +** a 64°C melting peak

Negative samples
show NO melting peak
Positive Sample
show a 56-65°C melting peak

nECT melting curve at 50°C

1) **NTC** must show a melting peak
2) **JC +** should show a melting peak
3) **BK +** not relevant

Negative samples
must show a melting peak
Positive Samples
not relevant

Fig. 3: Melting curve for nECT and BKV.

Note: The values of the melting temperatures (Tm) of the Control Reaction may vary ± 2.5°C between different experiments.

Tm of BKV positive samples varies between 56-65°C (see **8.7. Unusual Melting Peaks** for details)

JCV Melting curve in LC690 channel.

Melting curve for JC + controland JCV positive samples is not relevant.

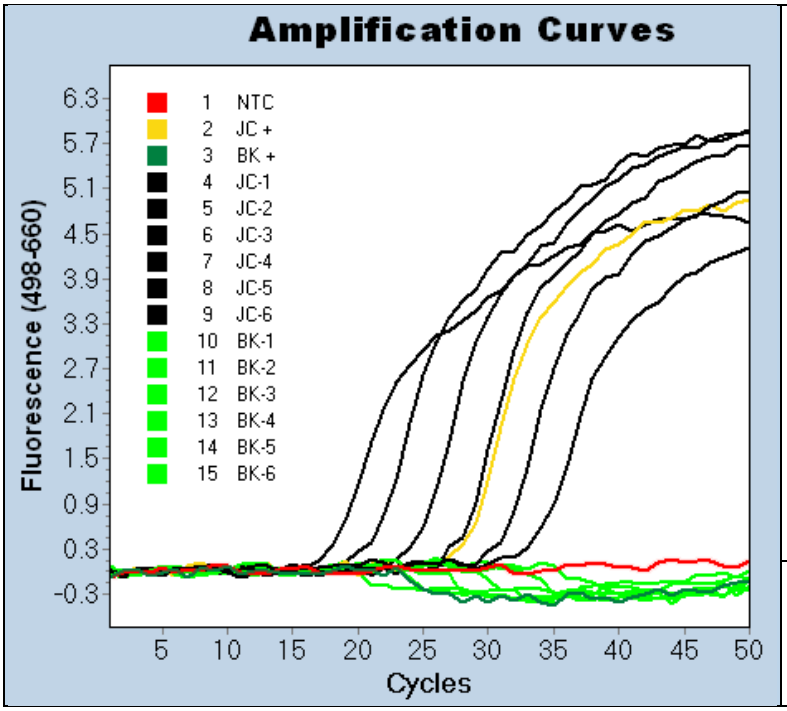
8.5.3 Amplification Analysis: Plate Based Instruments

View data for BKV amplification in LC640 and JCV amplification in LC690.

View amplification data as follows:

LightCycler® 480 II Instruments select:

Amplification: “Abs Quant/2nd Derivative Max ” analysis mode.

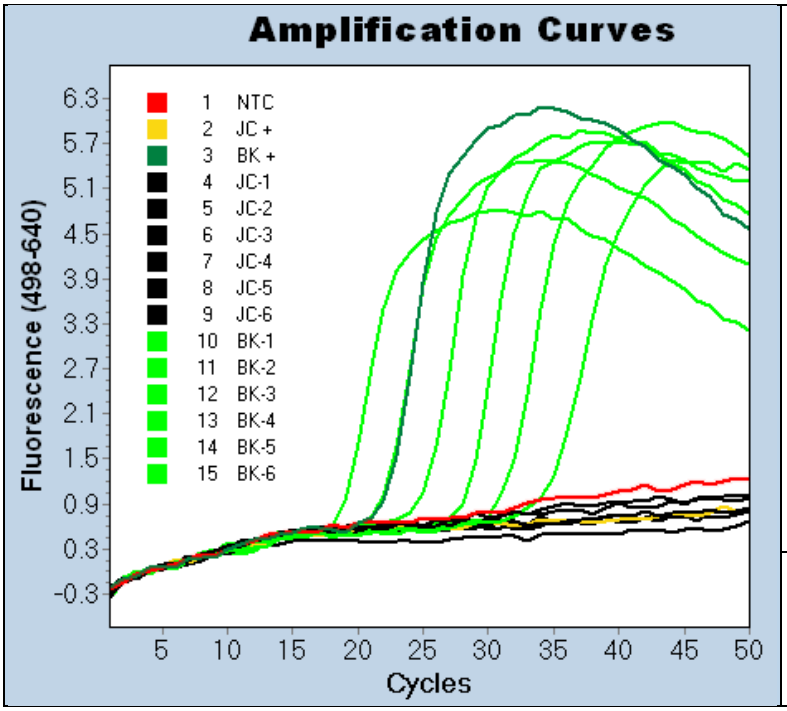


LC690

1) **NTC** must show a flat line
2) **JC +**
4) JCV-1
5) JCV-2
6) JCV-3
7) JCV-4
8) JCV-5
9) JCV-6

Compare with expected Cp values described in the certificate of analysis. BKV Controls (green lines) must show a flat line.

Fig. 4: Amplification sample data for JCV.



LC640

1) **NTC** must show a flat line
3) **BK +**
10) BKV-1
11) BKV-2
12) BKV-3
13) BKV-4
14) BKV-5
15) BKV-6

Compare with expected Cp values described in the certificate of analysis. JCV Controls (black and yellow lines) must show a flat line.

Fig. 5: Amplification sample data for BKV.

Note: The values of the crossing point (Cp) may vary ±1.5 cycles between different experiments.

In case of variations see instructions: **8.3.4 Absent amplification in Controls or Standards.**

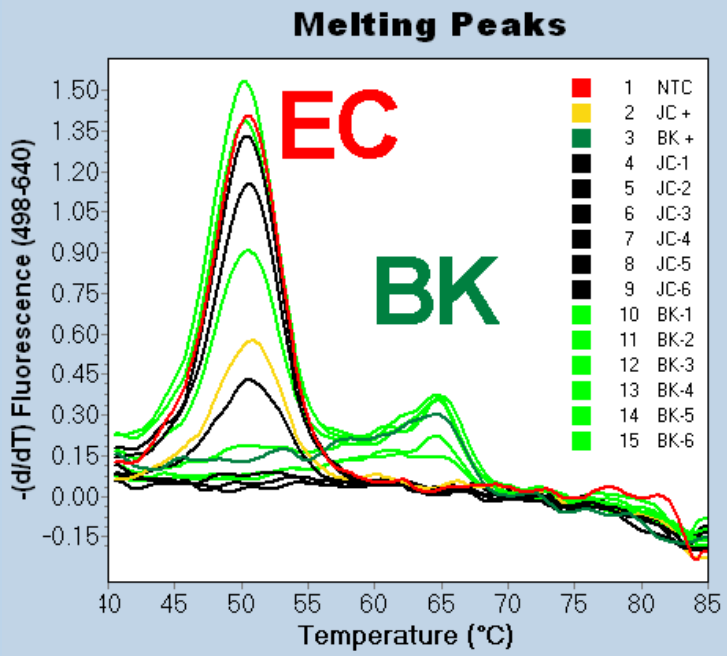
8.5.4 Melting Curve Analysis: Plate Based Instruments

View Control Reaction and BKV melting curve data in LC640.

View amplification data as follows:

LightCycler® 480 II Instruments select:

Melting curve: “Tm Calling” analysis mode.



LC640

BK melting curve at 59-66°C

1) **NTC** must show NO melting peak
2) **JC +** must show NO melting peak
3) **BK +** a 64°C melting peak

Negative samples
show NO melting peak

Positive Sample
show a 56-65°C melting peak

nECT melting curve at 50°C

1) **NTC** must show a melting peak
2) **JC +** could show a melting peak
3) **BK +** not relevant

Negative samples
must show a melting peak

Positive Samples
not relevant

Fig. 6: Melting curve for nECT and BKV.

Note: The values of the melting temperatures (Tm) of the Control Reaction may vary ± 2.5°C between different experiments.

Tm of BKV positive samples varies between 56-65°C (see **8.7. Unusual Melting Peaks** for details)

JCV Melting curve in LC690 channel.

The melting signals in the 690 channel are very low or might be even invisible. Melting analysis for JC + control and JCV positive samples is not relevant.

8.6 Interpretation Table

First check results obtained from the Controls.
For a valid run all controls must be passed :

Controls			Results
LC690 Amplification	**LC640 Amplification**	**LC640 Melting**	
NTC			
Negative	**Negative**	**49°C-50°C**	**Negative Control Passed**
Negative	Negative	**No peak**	**Negative Control Failed** Check if sEC/IC was used !
Amplification	**Amplification**	not relevant	**Negative Control Failed** (1) Contamination ! Repeat !
Positive Controls			
Amplification	Negative	**49°C-50°C** (2)	**JC+ Positive Control Passed**
Negative	Negative	not relevant	**JC+ Positive Control Failed**
Negative	**Amplification**	**56°C - 65°C** (2)	**BK+ Positive Control Passed**
Negative	**Negative**	not relevant	**BK+ Positive Control Failed**

Note: **(1)** The Negative Control is failed when amplification is present even only in one channel.
(2) The **nECT** peak could be absent.

Table 11

Then read results for the clinical samples :

Clinical Sample			Results
LC690 Amplification	**LC640 Amplification**	**LC640 Melting**	
Negative	**Negative**	**49°C-50°C**	**Negative for BKV / JCV (not detectable)**
Amplification	**Negative**	not relevant	**Positive for JCV Determine Viral Load**
Negative	**Amplification**	**56°C-66°C**	**Positive for BKV Determine Viral Load**
		49°C-50°C not relevant	
Amplification	**Amplification**	**56°C-66°C**	**Positive JCV and BKV** (1) **Determine Viral Load**
		49°C-50°C not relevant	
Negative	Negative	**No peak**	**Sample problem / inhibition:** Repeat from sample preparation

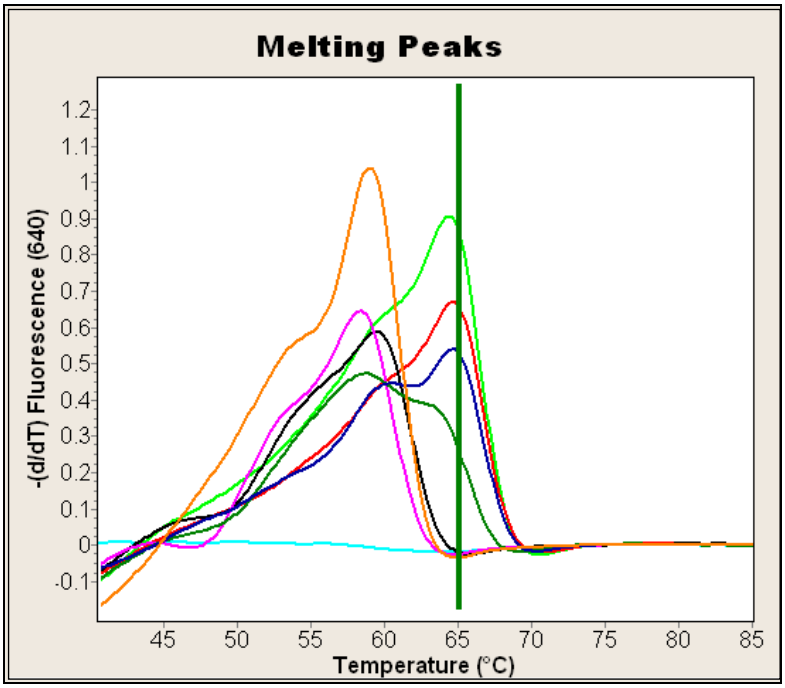
Report the viral load for all positive samples.

(1) If Cp is identical for BKV and JCV (+/- 0,5 Cp) this is BKV. See chapter 8.1 or 8.7

Table 12

8.7 Unusual Melting Peaks

Variation in the virus sequences produces different homology between PCR product and Hybridization Probes; the cartoons depict the expected variability.



LC640

BKV melting curve at 56-66°C

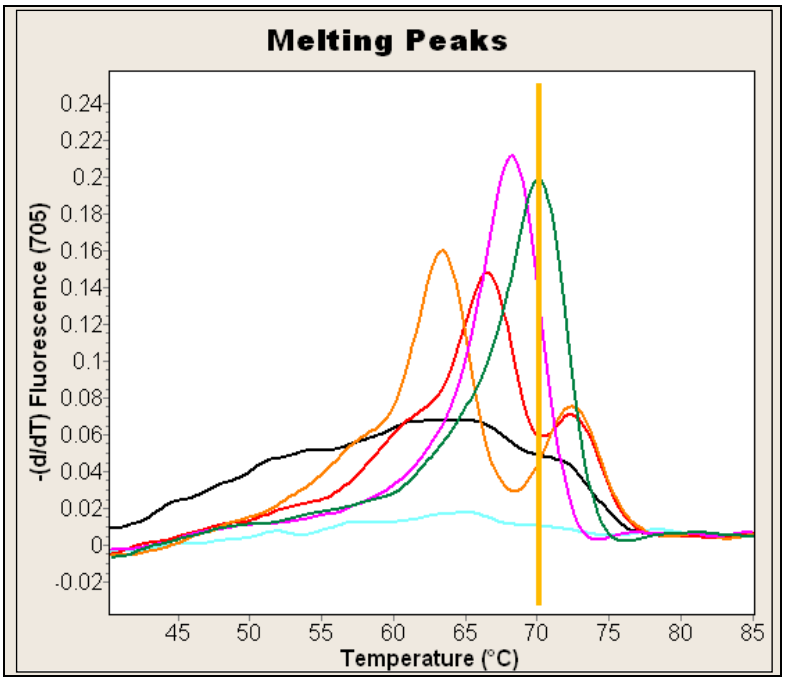
Different BKV subtypes produces variation of the melting curve.

The expected Tm for the most common subtypes I, V and VI is about 65°C;
the Tm for the subtypes III and IV is about 61°C.

See nomenclature described in ref 9.

Do not use melting peak for subtype identification.

Fig. 7: Melting curve range for BKV.



LC690

JCV melting curve at 63-73°C

Different JCV subtypes produces variation of the melting curve.

Note:
Many biological samples, as the included **JC +** standards and controls, do not produce a clear melting curve (Black line) or a baseline curve.
This event is expected.

Fig. 8: Melting curve for for JCV.

8.8 External Quality Asessment (EQA) Samples

For running EQA samples check the declared amount(s) per mL and compare with the detection limit of this device for the respective extraction volumes. Adapt - if necessary - sample and extraction volumes before running the EQA samples to detect low-concentrated samples.

9. Troubleshooting

Instrument specific codes:	Capillary based instruments	Multiwell based instruments
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline "Saw teeth like"	Non homogeneous mix	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in **NTC** Negative Control	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR- grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	Possible altered virus sequence	Verify by repeating, sequence the virus (if applicable). Report to: service@tib-molbiol.de and/or send samples for further analysis to the laboratory in Berlin.

10. References

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Rapid Quantification and Differentiation of Human Polyomavirus DNA in Urine from Patients after Bone Marrow Transplantation *J.Clinical Microbiology* 38, 3689-3695

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[7] Gaynor et al. 2007 **Identification of a Novel Polyomavirus from Patients with Acute Respiratory Tract Infections**. *PLoS Pathogens* 3 (5): e64

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[9] Hoffman NG, Cook L, Atienza EE, Limaye AP, Jerome KR, 2008 **Marked Variability of BK Virus Load Measurement Using Quantitative Real-Time PCR among Commonly Used Assay** *JCM* 46: 2671–2680

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Classification / References

Reference	Classification
EDMA	15.04.40.22 (BK virus) 15.04.40.23 (JC virus)
CPV	33694000-1
EAN	4260159332155
Roche SAP No.	06295100001

FastStart Enzyme

FastStart DNA Master HybProbe is included in the package for TIB MOLBIOL customers in Central Europe only.

When the present kit is distributed through Roche Diagnostics or their local distributors, FastStart DNA Master HybProbe is supplied as a separate product:

Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
Roche Diagnostics Cat.-No. 12 239 272 001 kit for 480 reactions

11. Material Safety Data Sheet

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

12. Version History

Notes: in red mark events that require to change laboratory procedures
Notes in blue mark improvements and changes in composition

Version	Event	Date
V140605	Release Version	04-07-2014
V150808	Corrected the quantity of **nECT** in table 7	10-08-2015
V160116	2 vials nECT, new study data results for LOD, JC no melting peaks	28-12-2015
V160606	Loading step 3 'water' replaced by 'NTC' (7.2.3), Storage (1.1, 1.4)	06-06-2016

Produced by:

TIB MOLBIOL Syntheselabor GmbH
Eresburgstrasse 22-23
12103 Berlin, Germany
www.tib-molbiol.com

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