For life science research use only. Not for use in diagnostic procedures. For *in vitro* use only.

TIB MOLBIOL

LightMix® Kit *Polyomaviruses JC and BK*
Cat.-No. 40-0203-32

Kit with reagents for the quantitative detection of *Polyomavirus JC and BK* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / Z480 / 480 II / Z480 Instruments (not for use with LC480 version I).

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do NOT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 5-7
Instructions for use with the LightCycler® 480 II / Cobas® Z480 Instrument see pages 9-11

1. Introduction

Polyomaviruses JC and *BK* are double stranded DNA viruses which are ubiquitous in the human population. Primary infections are usually asymptomatic. *JCV* is the causative agent of progressive multifocal leukoencephalopathy, a neurological disease, which occurs frequently in AIDS patients. *BKV* is associated with hemorrhagic cystitis, ureteral stenosis and other urinary tract disease[1] in transplant patients with immunosuppressive therapy. Polyomaviruses cause nephropathy in up to 10% of renal transplant patients. An effective management of the infection requires an early detection and further quantification of viral load in urine and blood samples [2].

The LightMix® Kit for the detection of *Polyomaviruses JC* and *BK* provides a fast, easy and accurate system to detect and quantify these viruses in a nucleic acid extract. The supplied standard row allows to perform an absolute quantification of genome equivalents per PCR reaction. The kit includes an internal control (IC). The BK single assay and the multiplex assay have been clinically evaluated [1,2].

This LightMix® Kit is tested on the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II (96 well and 384 well formats) Instruments with Roche Diagnostics "LightCycler® FastStart DNA Master HybProbe".

[1] BK virus associated renal cell carcinoma: case presentation with optimized PCR and other diagnostic tests. Narayanan M, Szymanski J, Slavcheva E, Rao A, Kelly A, Jones K, Jaffers G. *Am J Transplant* **7(6)**:1666-71 (2007).

[2] Evaluation and Validation of a Multiplex Real-Time PCR for BK/JC Virus Quantification. Rubio et al., submitted (2009)

2. Description

This LightMix® detects parts of the *Polyomaviruses JC* and *BK* genomes indicating the presence of *Polyomavirus JC* DNA and / or *Polyomavirus BK* DNA in a nucleic acid extract obtained from blood or urine. A control amplification reaction, acts as internal positive control (IC).

A 172 bp fragment (*JCV)* and/or 175 bp fragment (*BKV)* of the *Small t-Antigen* gene are amplified with specific primers and detected with probes labeled with LightCycler® Red 690 (detected in channel 705) for *JCV* or with LightCycler® Red 640 (channel 640) for *BKV*, respectively.

An additional PCR product of 658 bp is formed from the internal control DNA (IC). This internal control will not interfere with the *Polyoma* specific reactions but it will display a melting curve signal in negative and low-concentrated samples. The detection probe or the IC is labeled with the dye LC640 (previously LC705), resulting in a specific melting peak at 49.5°C (visible in melting analysis only).

The use of a color compensation file is a prerequisite to run the duplex reaction. Generate the color compensation file using the TIB MOLBIOL LightMix® Kit - Color Compensation 40-0318 HybProbe.

The supplied standard rows allow to determine the linear range of the reaction and quantification of unknown samples; this dilution row is intended for single use only. In addition we provide positive controls for JC and BK to be included in every single experiment.

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 and channel F1 instead of channel 530 for detection. However, we recommend to upgrade LightCycler® 1.x Instruments to software version 4.1.

The LightCycler® Instrument reports copies per PCR reaction. For a calculation of the copy number per volume please see section 9 'Conversion Factor'.

3. Set contents

3	Vials with **blue** cap containing premixed lyophilized primers, probes for *Polyomavirus* and Ithe nternal Control (including IC target) for 32 PCR reactions each
1	Standard row with 6 lyophilized cloned standard of *JC* from 10^1 to 10^6 target equivalents per rxn
1	Vials with **yellow** cap **(JC+)** containing plasmid **10E^3** copies / reaction: Positive Control JC
1	Standard row with 6 lyophilized cloned standard of *BK* from 10^1 to 10^6 target equivalents per rxn
1	Vials with **green** cap **(BK+)** containing plasmid **10E^5** copies / reaction: Positive Control BK
2	Sealing foils to close the standard rows

4. Additional reagents and items required

	Roche Diagnostics
ColorCompensation HybProbe order n°40-0318-00	Cat. -No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
or High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 II Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 II System)	Cat.-No. 04 729 692 001

5. Product characteristics

PCR results are obtained within 50 minutes (50 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (50 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity

These reagents detect 10 copies of *Polyomaviruses JC/BK* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments (in an exemplary system, using cloned targets as reference).

The detection limit for clinical specimen was determined to be 7,000 copies/ml from plasma and 15,000 copies/ml from urine. The test had a specificity of 100%, sensitivity of 97.9% and showed a high precision (coefficient of variation < 4%) [2].

Measuring range

The linear measuring range of the assay is 10^2 to 10^6 copies of *Polyomaviruses JC/BK* DNA per reactions, using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments and a cloned standard.

The clinical study stated a linear range of six logs.

Storage and Stability

- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry date o n the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 II Instruments.
Start programming before preparing the solutions. See the Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Prep. Kit'.

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.
For quantification always use a fresh solution prepared from the provided standard row (single use only).

6.1. Preparation of parameter-specific reagents and reagents for the IC (32 reactions):

One reagent vial with a blue cap contains primers, probes to run 32 rxns for *Polyomavirus* and IC.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

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

This solution is stable refrigerated at 4℃ /8℃ fo r a maximum period of 30 days. Avoid prolonged exposure to light.

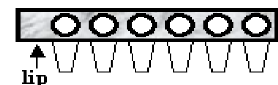
6.2. Preparation of the control DNA

Add 160 µl PCR-grade water to each control vial (green cap BK and yellow cap JC).
Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 10^5 target molecules in 5 µl for BK and 10^3 target molecules in 5 µl for JC).

►**Use 5 µl** control DNA for a 20 µl PCR reaction.

The dissolved Controls are stable for 30 days when stored at 4℃. When using them over a longer period of time, aliquot and store frozen at -20℃. Avoid repeated freezing thaw ing cycles.
Please note that opening these vials may cause contamination of the work-space (aerosol).

6.3. Preparation of the standard row



The target DNA is provided in 6 different quantities to yield from 10^1 to10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. **Add 40 µl** PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

►**Use 5 µl** standard for a 20 µl PCR reaction.

This standard solution is not long-term stable. Use only fresh prepared solutions as quantification references. After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.4. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
9.0 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.0 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers, probes and IC, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Table 1

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instrument) or to a multiwell plate (LightCycler® 480 II Instrument).

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1 Color Compensation

Switch the color compensation mode on, select the **CC 640-690** file. If this mode is not available run the color compensation program following the instructions in the manual of TIB MOLBIOL 'LightMix® Kit – Color Compensation.
Analyzing data without 'Color Compensation' (deactivated) will generate invalid readouts of the results.

7.2. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [℃]	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 [℃/s]	20	20	20	20	20	20	0.2	20
Sec Target [℃]	-	-	55	-	-	-	-	-
Step Size [℃]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

Table 2

7.3. Data Analysis

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Polyomavirus BK* data in channel 640 and *Polyomavirus JC* data in channel 705, Quantification mode. The negative control (NTC) must show no signal.

View IC data in channel 640 Melting Curve Mode. The negative control and the low-concentrated *Polyomaviruses JC/BK* DNA samples (10 to 1,000 copies) should show a melting curve for the IC with a Tm of approximately 49.5℃.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Polyomavirus JC and Polyomavirus BK* should have Cp values between cycles 17 and 36.

To recall saved standard curves, use the provided **JC+** and **BK+** controls; please see the LightCycler® Instrument operator's manual.

7.4. Sample Data – Typical Results

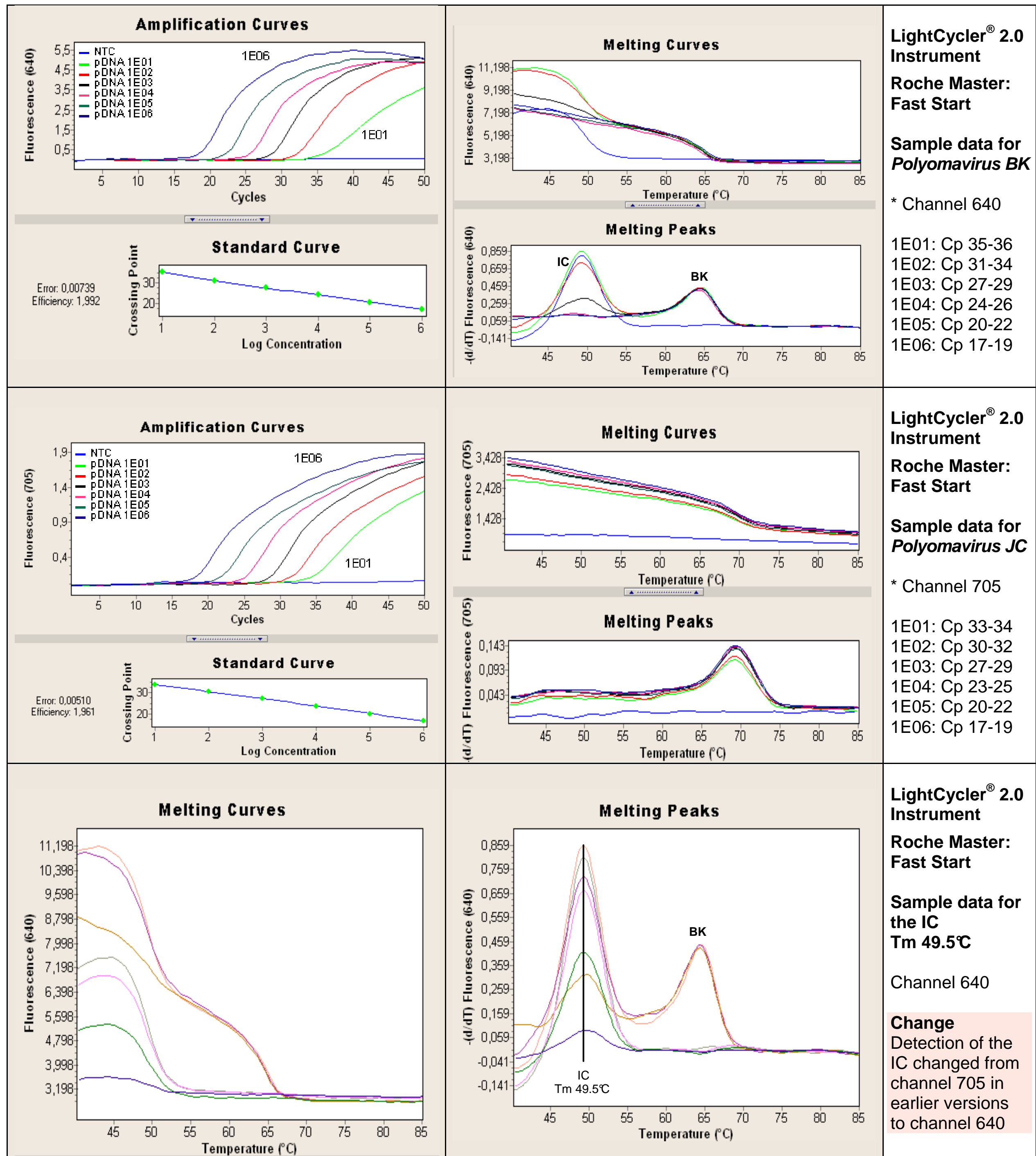


Fig.1. Sample data for the *Polyomaviruses JC/BK* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve *Polyomavirus BK*. Right panel channel 640 melting analysis for *Polyomavirus BK*.
Middle panels: Data from LightCycler® 2.0 Instrument. Left panel channel 705 quantification mode (Second Derivative Maximum) for *Polyomavirus JC*. Right panel channel 705 melting analysis *Polyomavirus JC*.
Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 with melting curves for the IC. Right panel channel 640 melting peaks for the IC.

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

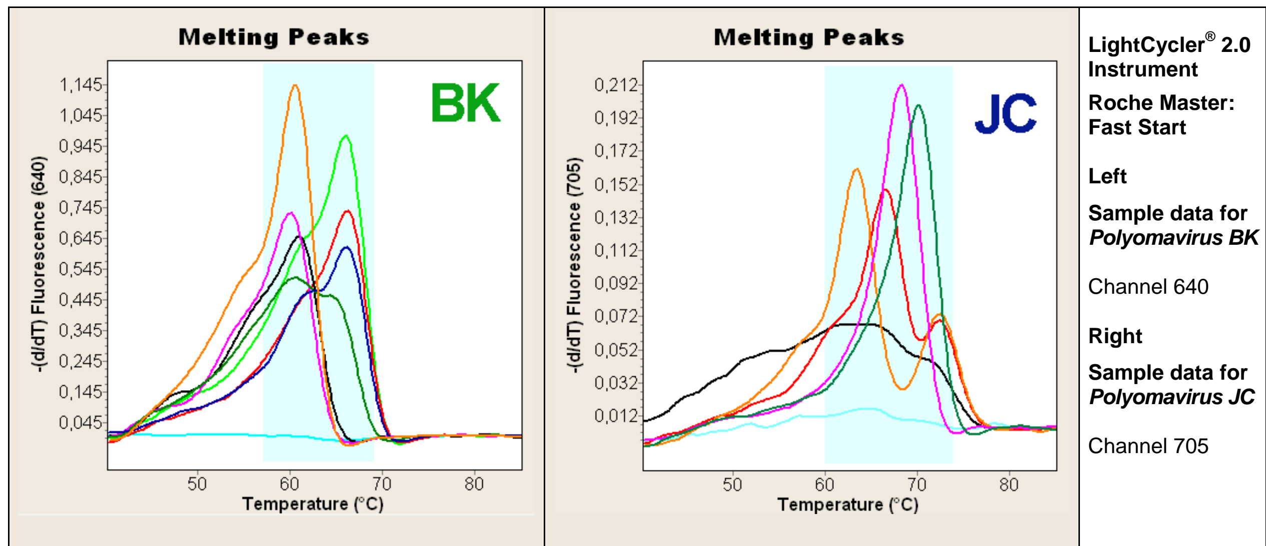
7.5. Interpretation of data

Polyomavirus JC/BK (sample)		IC (sample)	Positive Control		Negative Control (NTC)		Result (warnings)
BK 640 (F2)	JC 705 (F3)	640 (F2)	BK+ 640 (F2)	JC+ 705 (F3)	640 (F2)	705 (F3)	
no signal	**no signal**	melting peak	amplification	amplification	negative	negative	**negative (not detectable) for *Polyomavirus JC/BK***
amplification	**no signal**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus BK***
no signal	**amplification**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus JC***
amplification	**amplification**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus JC* and *Polyomavirus BK***
Irregular and non-valid results							
no signal	**no signal**	**no melting peak**	amplification	amplification	not relevant	not relevant	PCR failure, repeat experiment/ sample preparation
not relevant	not relevant	not relevant	**no signal**	**no signal**	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	not relevant	not relevant	**positive**	**positive**	Contamination, repeat experiment

Tab. 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

7.6. Unusual Melting Peaks

Variation in the viral sequence targeted by the hybridization probes cause melting curves with a lower melting temperature. The figure below shows examples for melting curves with lower Tm values:



LightCycler® 2.0 Instrument

Roche Master: Fast Start

Left

Sample data for *Polyomavirus BK*

Channel 640

Right

Sample data for *Polyomavirus JC*

Channel 705

BK virus. The expected Tm for the more prevalent subtypes I, V and VI is about 65°C as shown in the manual (sections 7.4 and 8.4) subtypes III and IV will exhibit a Tm of about 61°C.
The lowest reported Tm of about 58°C is probably related to African variants which are less prevalent in the Northern hemisphere.

Since there is no published correlation between Tm value and type do not use the Tm value for typing.

8. LightCycler® 480 II / Cobas® Z 480 Instruments

8.1. Programming Color Compensation

Switch the color compensation mode on, select the **CC 530-640-690** file. If this mode is not available run the color compensation program following the instructions in the manual of TIB MOLBIOL 'LightMix® Kit – Color Compensation.
Analyzing data without 'Color Compensation' (deactivated) will generate invalid readouts of the results.

8.2. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:

TIB Molbiol 640-690 detection format as described in the Color Compensation manual
or
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
Cobas® Z480 Instrument: 465-510, 498-645, 498-700
Filter 465-510 is not required to read results but must be included to calculate the color compensation.

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [℃]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [℃/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [℃]	-	-	55	-	-	-	-	-
Step Size [℃]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per ℃]	-	-	-	-	-	-	**1**	-

Table 4

8.3. Data Analysis

Note: Cobas® Z480 Instruments signal levels are about 50% compared to LightCycler® 480 II results.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Polyomavirus BK* data with Filter Combination 498-640 and *Polyomavirus JC* data with Filter Combination 498-660, Quantification. The negative control (NTC) must show no signal.

View IC data with Filter Combination 498-640. The negative control and the low-concentrated *Polyomaviruses JC/BK* DNA samples (10 to 1,000 copies) should show a melting curve for the IC with a Tm of approximately 49.5℃.

The provided standard rows of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Polyomavirus JC and Polyomavirus BK* should have Cp values between cycles 17 and 36.

To recall saved standard curves, use the provided **JC+** and **BK+** controls; please see the LightCycler® Instrument operator's manual.

8.4. Sample Data – typical results

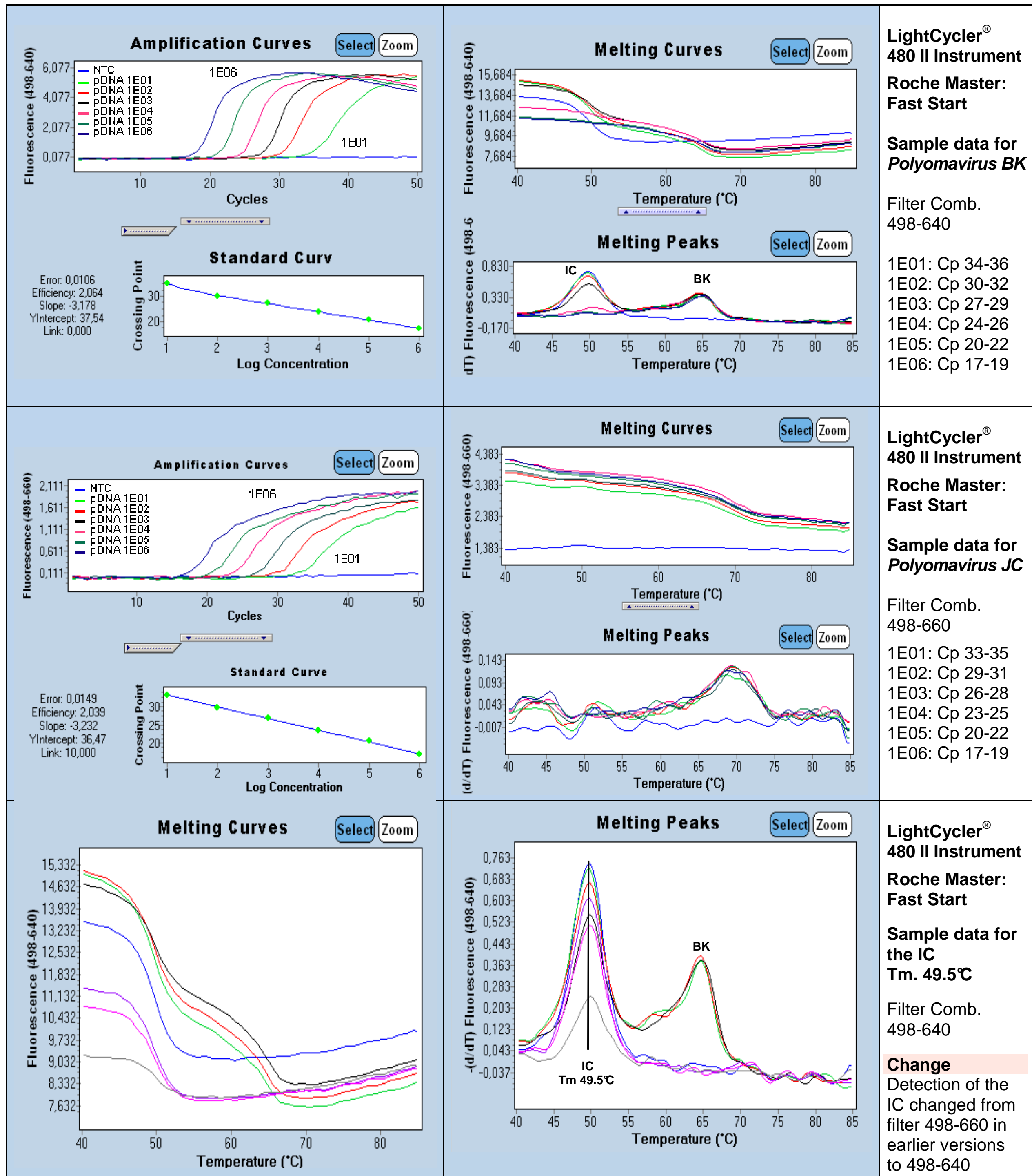


Fig.2. Sample data for the *Polyomaviruses JC/BK* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with standard curve. Right panel Filter Combination 498-640 melting analysis for *Polyomaviruses BK* .
Middle panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) with standard curve *Polyomaviruses JC.* Right panel Filter Combination 498-660 melting analysis for *Polyomaviruses JC.*
Lower panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 for the IC. Right panel Filter Combination 498-640 melting analysis for the IC.

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.5. Interpretation of data

Polyomavirus JC/BK (sample)		IC (sample)	Positive Control		Negative Control (NTC)		Result (warnings)
BK Filt. Comb. 498-640	JC Filt. Comb. 498-660	Filt. Comb. 498-640	BK+ Filt. Comb. 498-640	JC+ Filt. Comb. 498-660	Filt. Comb 498-640	Filt. Comb. 498-660	
no signal	**no signal**	melting peak	amplification	amplification	negative	negative	**negative (not detectable) for *Polyomavirus JC/BK***
amplification	**no signal**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus BK***
no signal	**amplification**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus JC***
amplification	**amplification**	not relevant	amplification	amplification	negative	negative	**positive for *Polyomavirus JC* and *Polyomavirus BK***
Irregular and non-valid results							
no signal	**no signal**	**no melting peak**	amplification	amplification	not relevant	not relevant	PCR failure, repeat experiment/ sample preparation
not relevant	not relevant	not relevant	**no signal**	**no signal**	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	not relevant	not relevant	**positive**	**positive**	Contamination, repeat experiment

Tab. 5. Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

8.6. Unusual Melting Peaks

Variation in the viral sequence targeted by the hybridization probes cause melting curves with a lower melting temperature. For details please see section 7.6

9. Conversion Factor

The amount of virus per sample (Viral Load) is commonly reported as copies / ml while PCR reports copies per reaction. The conversion factor between both depends on sample and extraction volume. Extraction usually starts 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

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]

For example, extracting from 200µl of clinical sample results in a correction factor of 5. Using 5 µl extract from a total elution volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of 100.

VL [copies/ml] (200 µl / 100 µl) **= M**easured **V**alue [copy number per reaction] **X 100**

10. Version History

Notes in red mark events that require to change laboratory procedures

V081002	Use for LightCycler® 1.x and 2.0
V090209	Universal PCR-Program and improved primers and probes
V100818	Editorial changes
V100826	Now working on LightCycler® 1.x/2.0 and 480 **II** Instruments Change of Internal Control from channel 705 to channel 640
V110401	Change to 32 reactions per vial
V130123	Positive controls included, Extended granted reagent stability, Explanation for lower Tm melting peaks (section 7.6 and 8.6) Conversion factor for copies per volume included (section 9) Filter setting for Cobas® Z480 listed (section 8.2)
V130813	Editorial changes

Sections containing major changes to the previous version are highlighted.

Roche SAP order n° 05879540001

Notice to Purchaser

A license under U.S. Patents 4,683,202, 4,683,195 and 4,965,188 or their foreign counterparts, owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd ("Roche"), has an up-front fee component and a running-royalty component. The purchase price of this product includes limited, nontransferable rights under the running-royalty component to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with a thermal cycler whose use is covered by the up-front fee component. Rights to the up-front fee component must be obtained by the end user in order to have a complete license. These rights under the upfront fee component may be purchased from Perkin-Elmer or obtained by purchasing an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process for research applications may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501. The purchase of this product does not convey any right for its use in clinical diagnostic applications. No rights for TaqMan technology under U.S. Patents 5,210,015 and 5,487,972 are hereby conveyed.

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
This Cycler® hybridization probes produced under license from Roche Diagnostics GmbH.