LightMix® Kit *Human Herpesvirus 8 (HHV-8)*

Cat.-No. 40-0198-16

Kit with reagents for the detection of *Human Herpesvirus 8 (HHV-8)* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II / Cobas® Z480 (open channel) Instruments.

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

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II / Cobas® Z480 Instrument see pages 6-7

1. Introduction

Human Herpesvirus 8 (HHV-8), a member of Gammaherpesvirinae, is known in particular for causing Kaposi's sarcoma (KS), but is associated also with Castleman's disease and body cavity-based lymphomas. The virus enters lymphocytes where it remains in a latent state until it is reactivated like known for other herpes viruses. The virus was isolated first in 1994 from tissue from KS tissue. Before the AIDS epidemics Kaposi's sarcoma was a very rare disease. The seroprevalence in the Northern hemisphere is only about 2% but up to 35% in HIV-positive homosexual men; *HHV-8* antibodies can be found in at least 85% of Karposi patients. Standard diagnosis is based on serology. *HHV-8* has been detected by PCR in KS lesions, peripheral blood mononuclear cells, breast cancer and prostate tissue, but also in saliva and semen.

The gene which has been used for the first published TaqMan HHV-8 detection assay was the latent nuclear antigen (ORF73)[1] and has been also used for a similar design with hybridization probes [2]. There are only a few Real-Time-PCR publications referring to use the LightCycler® instruments [3,4].

The LightMix® Kit *Human Herpesvirus 8* provides a fast, easy and accurate system to identify and quantify this target in a nucleic acid extract. A control amplification reaction acts as internal control (IC).

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

[1] Quantitative analysis of HHV- 8 viral load using a real-time PCR assay. Lallemand et al. JCM 38 (2000) 1404-1408

[2] Epidemiological aspects of HHV-8 and association with diseases. PhD thesis S.A. Omar, University Homburg/Saar (2007)

[3] Quantification of HHV-8 by real-time PCR in blood fractions of AIDS patients with Kaposi's sarcoma and multicentric Castleman's disease. Boivin et al. J Med Virol. 2002 Nov;68(3):399-403.

[4] Absence of HHV-8 DNA in hobnail hemangiomas. Gutzmer et al.. J Cutan Pathol. 2002 Mar;29(3):154-8.

2. Description

A 142 bp fragment of the latent nuclear antigen ORF73 from the *Human Herpesvirus 8* genome is amplified with specific primers and the resulting PCR fragment is analyzed with LightCycler® Red 640 labeled hybridization probes (detected in channel 640).

The PCR reaction is monitored by an internal control with an additional PCR product of 318 bp. This control does not interfere with the *HHV-8* specific reactions. The amplification will usually fail in the presence of higher concentrated *HHV-8* DNA samples (1,000 copies or higher) while displaying an amplification signal in negative and low-concentrated samples. The hybridization probes are labeled with LightCycler® Red 690 (recorded in channel 705). The IC is supplied separately to allow running the assay in the presence or absence of the IC.

The use of a color compensation file generated with the ColorCompensation kit HybProbe 40-0318 is a prerequisite to run the duplex reaction.

The supplied standard row allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples.

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. We recommend upgrading LightCycler® 1.x Instruments to software version 4.1.

3. Set Contents

6 Vials with **blue** cap containing lyophilized primers and probes for 16 PCR reactions *HHV-8*
6 Vials with **white** cap containing the internal control (IC)
1 Standard row with 6 lyophilized plasmid standards from 10^1 to 10^6 target equivalents per rxn
1 Sealing foil for the standard row

4. Additional Reagents and items required

	Roche Diagnostics
LightMix® Kit ColorCompensation HybProbe 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
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 Instrument)	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 *Human Herpesvirus 8* 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).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Human Herpesvirus 8* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25℃). 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℃).

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 Viral Nucleic Acid 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 (16 reactions):

One reagent vial with a **blue** cap contains primers and probes to run 16 reactions *HHV-8*.
One reagent vial with a **white** cap contains primers, probes and DNA to run 16 reactions for the IC.

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

►**Use 4 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of the standard row

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 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 and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control). 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).

6.3. 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**
2.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
4.0 µl	**IC** mix (IC reagents containing primers, probes and DNA, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Table 1

To include the internal control **add 4 µl** of the IC reagent per reaction to the reaction mix.
To run the assay without the internal control substitute the 4 µl of IC with 4 µl PCR-grade water.

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. 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 [°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]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

(Melting not relevant for detection) Table 2

7.2. Data Analysis

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

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the TIB ColorCompensation HybProbe. Perform data analysis, as described in the LightCycler® Instrument operator's manual.

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 *Human Herpesvirus 8* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

If the internal control (IC) is used view IC data in channel 705. The negative control and the low-concentrated *HHV-8* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a Cp at approximately cycle 30.

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 *HHV-8* should have Cp values between cycles 17 and 34.

7.3. Sample Data – typical results

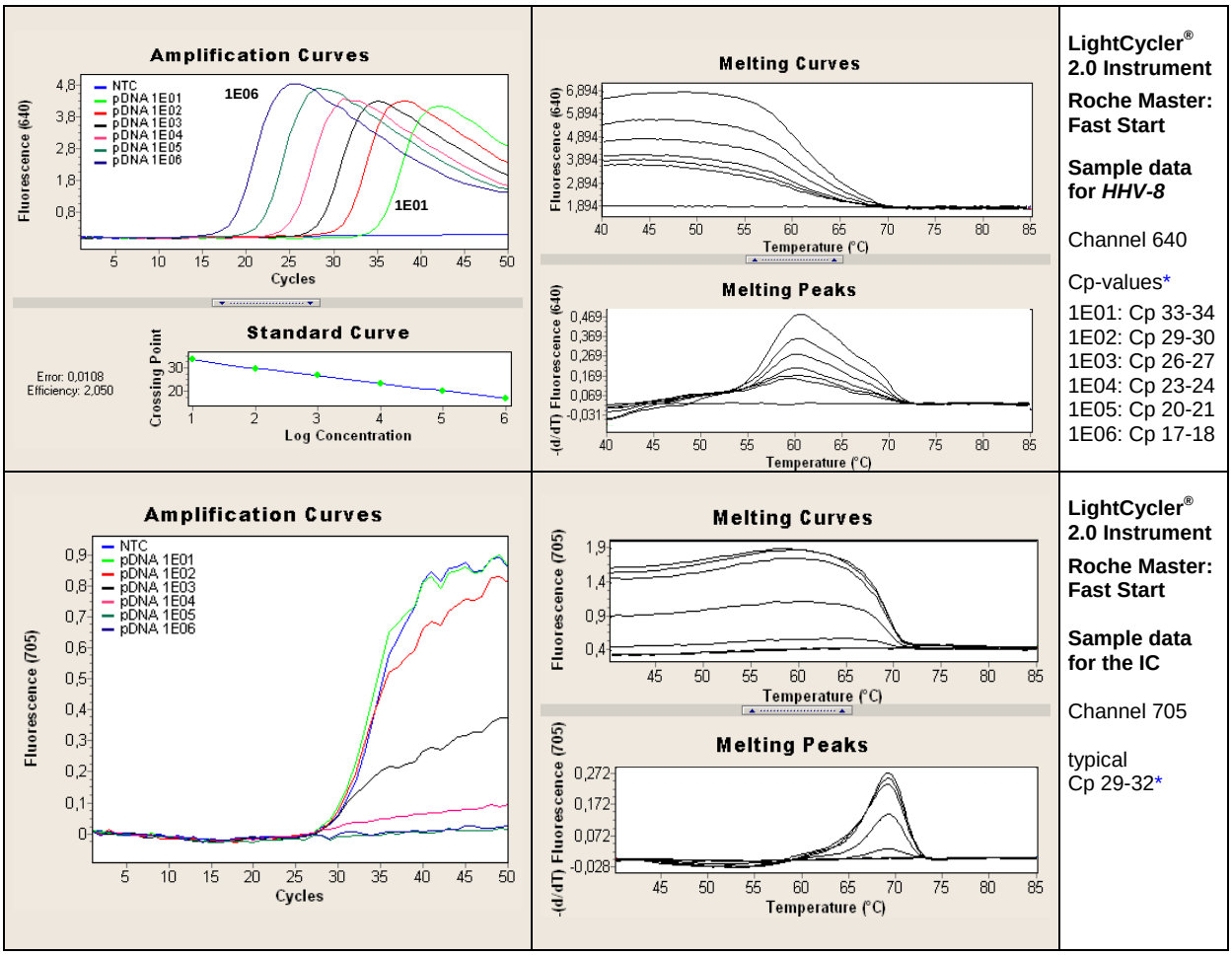

Fig.1. LightCycler® 2.0 sample data for the *Human Herpesvirus 8* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for *HHV- 8.* Right panel channel 640 melting analysis for *Human Herpesvirus 8* (not relevant for detection).
Lower panels:. Left panel channel 705 quantification mode (Second Derivative Maximum) for the IC. Right panel channel 705 melting analysis for the IC (not relevant for detection).

* Cp values listed were obtained with the supplied dilution row of DNA standard in water. Please note that Cp values may vary by 2-3 cycles depending on the instrument settings while the delta ct steps between two dilutions will remain rather constant. These values must not be used for absolute quantification purposes unless using an external reference or international standard. The values describe copy numbers in the PCR. Estimation of the virus copies per sample volume needs to calculate all dilution steps and to evaluate the extraction method for its efficiency.

7.4. Interpretation of data

Negative results mean that the virus is not detectable. The virus could be present in amounts lower than the detection limit or could be lost during the extraction process. Relevant PCR inhibition can be excluded by the inclusion of the control PCR (IC) which must be detectable for negative samples.

Sample 640 *HHV-8*	**Sample 705** *Int Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 35	not relevant	amplification	negative	**Positive for *HHV-8***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

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

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

8.1. 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:
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
Cobas® Z480 Instrument: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
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	00:00:30	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	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	**3**	-

(Melting not relevant for detection) Table 4

8.2. Data Analysis

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

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the 'LightMix® Kit Color Compensation HybProbe.

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 *Human Herpesvirus 8* data with Filter Combination 498-640 Quantification mode. The negative control (NTC) must show no signal.
If the internal control is used view IC data with Filter Combination 498-660. The negative control and the low-concentrated *Human Herpesvirus 8* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a Cp at approximately cycle 29-30.

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 *Human Herpesvirus 8* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – typical results

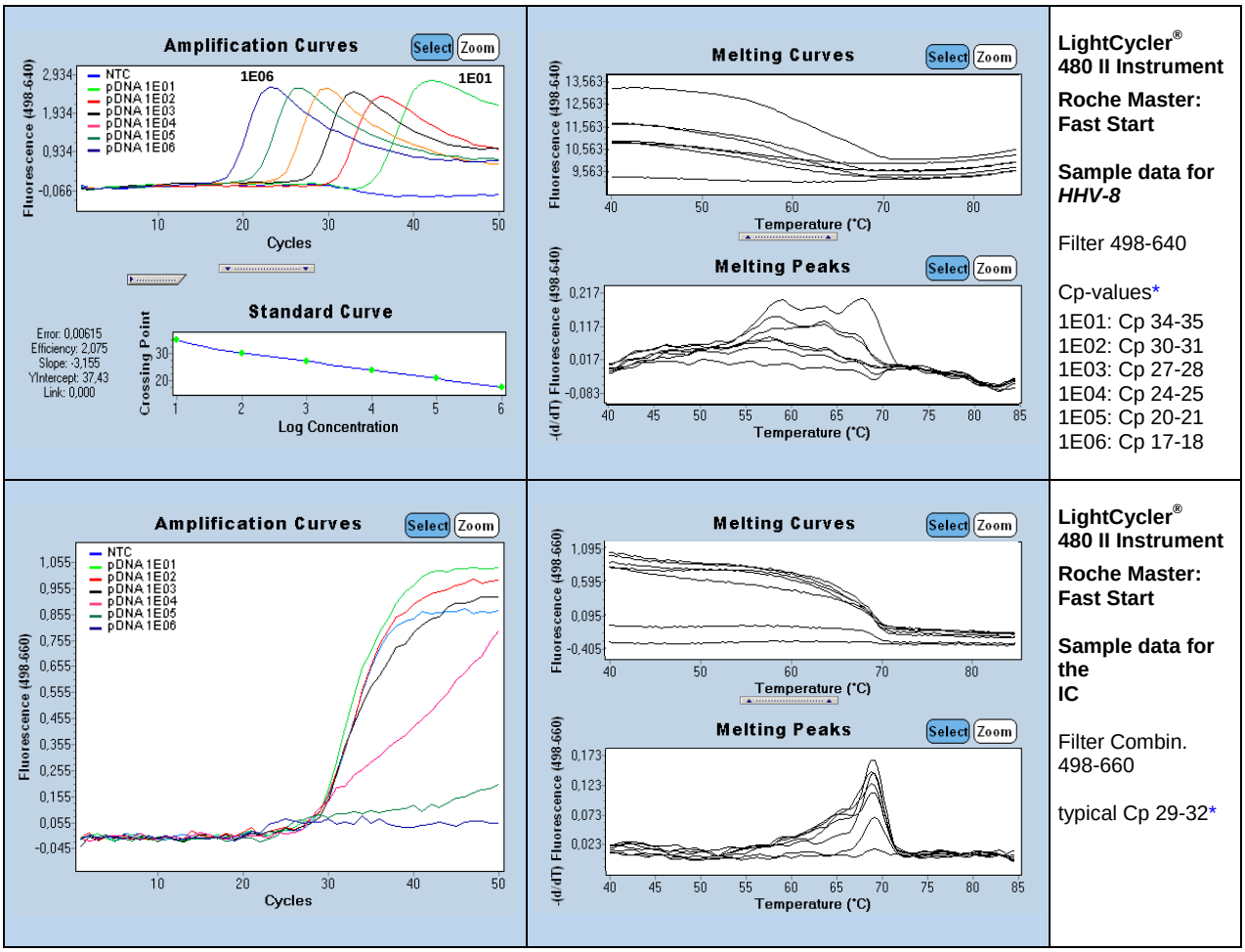

Fig.1. LightCycler® 480 II Sample data for the *Human Herpesvirus 8* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Sec. Der. Maximum) with standard curve for *HHV- 8.* Right panel Filter Combination 498-640 melting analysis for *Human Herpesvirus 8* (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the IC. Right Filter Combination channel 498-660 melting analysis for the IC (not relevant for detection).
Do not use the Roche Diagnostics default color compensation because signals from channel 640 may be visible in the 660 channel.

* Cp values listed were obtained with the supplied dilution row of DNA standard in water. Please note that Cp values may vary by 2-3 cycles depending on the instrument settings while the delta ct steps between two dilutions will remain rather constant. These values must not be used for absolute quantification purposes unless using an external reference or international standard. The values describe copy numbers in the PCR. Estimation of the virus copies per sample volume needs to calculate all dilution steps and to evaluate the extraction method for its efficiency.

8.4. Interpretation of data

Negative results mean that the virus is not detectable. The virus could be present in amounts lower than the detection limit or could be lost during the extraction process. Relevant PCR inhibition can be excluded by the inclusion of the control PCR (IC) which must be detectable for negative samples.

Sample 640 *HHV-8*	**Sample 705** *Int Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36	not relevant	amplification	negative	**Positive for *HHV-8***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 5. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

9. Conversion Factor

The amount of virus per sample (Viral Load) is usually reported in copies / ml while PCR reports copies per reaction. The conversion factor between both depends on sample and extraction volumes. Extraction starts usually from less than one milliliter and PCR does not use the total extracted volume.

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 eluation volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of 100.

VL [copies/ml] = Measured **V**alue [copy number per reaction] **X 100**

10. Material Safety Data

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.

11. Version History

Notes in red mark events require to change procedures

V050816	First version (2005)
V100826	Released for LightCycler 480 II Instruments
V130813	Conversion Factor, MSDS and Version History included

Roche SAP order n° 05945224001

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

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