LightMix® Kit *Human Herpesvirus 6* (*HHV-6) EC*

Cat.-No. 40-0282-32 Universal Extraction Control Target (^{n}ECT)

Kit with reagents for the detection of *Human Herpes Virus 6* (*HHV-6*) DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer (UDF open channel).

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 4-5
Instructions for use with the LightCycler® 480 II Instrument / cobas z 480 Analyzer see pages 6-7

1. Introduction

The Human Herpesvirus 6 *(HHV-6)* infects white blood cells and found specifically in T-lymphocytes. Roseola (three-days fever or sixths disease) is the main disease caused by *HHV-6* and *HHV-7*. More than 95% of the population is positive for antibodies to *HHV-6*. Most infections occur in infants and often without or mild symptoms. A small number of infants develop serious disease including bone marrow and brain infection. The factors that lead to reactivation in people with intact and functioning immune systems are unclear and probably include genetic and environmental causes. Most instances of reactivation will not result in chronic, active infection as the normal immune system will suppress the reactivated virus and return it to a latent state. Reactivation of *HHV-6* in adults has been associated with a mononucleosis syndrome, autoimmune disorders, and nervous system diseases (e.g. Multiple Sclerosis).

Typical clinical specimen are EDTA blood, serum, or liquor, but can be also feces or tissue.

2. Description

This kit provides a fast and accurate system to identify this target in a nucleic acid extract; a Control Reaction allows to monitor extraction and eventual PCR inhibition.

A 272 bp fragment of the antigenic virion protein 101K (U11) gene of the *HHV-6* genome is amplified with specific primers. The resulting PCR fragment is analyzed with LightCycler® Red 640 labeled hybridization probes (detected in channel 640).

The Control Reaction is based on an additional 858 bp long fragment amplified from Lambda DNA, detected with LightCycler® Red 690 labeled hybridization probes (channel 705). This second PCR has no visible impact on the *HHV-6* -specific reaction and will even fail in the presence of higher amounts of target (1,000 copies and more) while displaying an amplification signal in negative and low-concentrated samples.

The former Internal Control (IC) has been changed to a spiked Extraction Control (sEC) in order to monitor successful extraction and to demonstrate the ability to run a PCR reaction (no inhibition).
We recommend to use the 'Extraction Control' procedure; in case that the former procedure shall be maintained the usage as IC is described. Target and control primer/probe sequences remained unchanged. The novel extraction control target ^{n}ECT (no. 30-0259) contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).
Note: With a MagNA Pure Compact extraction the recovery rate for the EC target can be very low or the DNA target even gets lost; the amount of ECT might has to be adapted to the extraction method.

The use of a color compensation file generated with the LightMix® Color Compensation HybProbe 530/640/690 kit (order no. 40-0318-00) is a prerequisite to detect the Control 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.

The kit is tested with 'LightCycler® FastStart DNA Master HybProbe' only.

3. Set Contents

3 Vials with **blue** cap containing premixed lyophilized primers and probes for 32 reactions *HHV-6*
3 Vials with **white** cap containing premixed primers and probes for 32 Control Reactions

1 Standard row with 6 lyophilized standards of *HHV-6* from 10^1 to 10^6 target equivalents per rxn
1 Sealing foil for the standard row

1 Vial with **white** cap containing Extraction Control Target (^{n}ECT) 4.8 x 10^6 copies (total)
1 Vial with **black** cap containing the stabilizer for preparation of the 'No Template Control' (NTC)

1 Certicate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics
Color Compensation HybProbe order n°40-0318-00	Cat .-No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (480 Instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of 640 and F3 instead of channel 705. We recommend to upgrade to software version 4.10 or higher.

4.1. Optional Additional Reagents

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
Extraction Target nECT	TIB Cat.-No. 30-0259-96

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect 10 copies of *HHV-6* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (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 *HHV-6* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

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

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific Reagents (PSR) and Control Reaction:

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions *HHV-6*.
One reagent vial with a **white** cap contains primers and probes to run 32 reactions Control Reaction.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

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

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

6.2. Preparation of Extraction Control Target (ECT):

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target

6.3. Sample Preparation:

Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Control (NTC) including the Extraction Control Target

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and
add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. 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** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected. 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 only. After adding the target DNA to the reaction mix use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Reaction Mix

Include Positive Controls and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions plus one reserve :

sEC Procedure	For use with the Roche FastStart Master	IC Procedure
Single reaction	**Component**	**Single reaction**
7.0 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.75 µl
2.0 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.0 µl
2.0 µl	**Reagent** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**EC** mix (EC reagents containing primers, probes, see **6.1.**)	2.0 µl
---- µl	ECT Control Target (vial **white** cap)	0.25 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Table 1

To run the assay without the Control Reaction substitute ECT with 0.25 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

Start run.

7. LightCycler® 2.0 / 1.x Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: samples 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 Not relevant for detection			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

Table 2

7.2. Data Analysis

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 HybProbes'.

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 *HHV-6* data in channel 640, Quantification mode.
The negative control (NTC) must show no signal.

For the Control Reaction view channel 705 data, Quantification mode. The negative control and the low-concentrated *HHV-6* DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 30.

The provided standard row with 10^6 copies/rxn to 10^1 copies/rxn of *HHV-6* should have Cp values between cycles 19 and 36 (Cp values calculated with Second Derivative Maximum method).

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

7.3. Sample Data – Typical Results

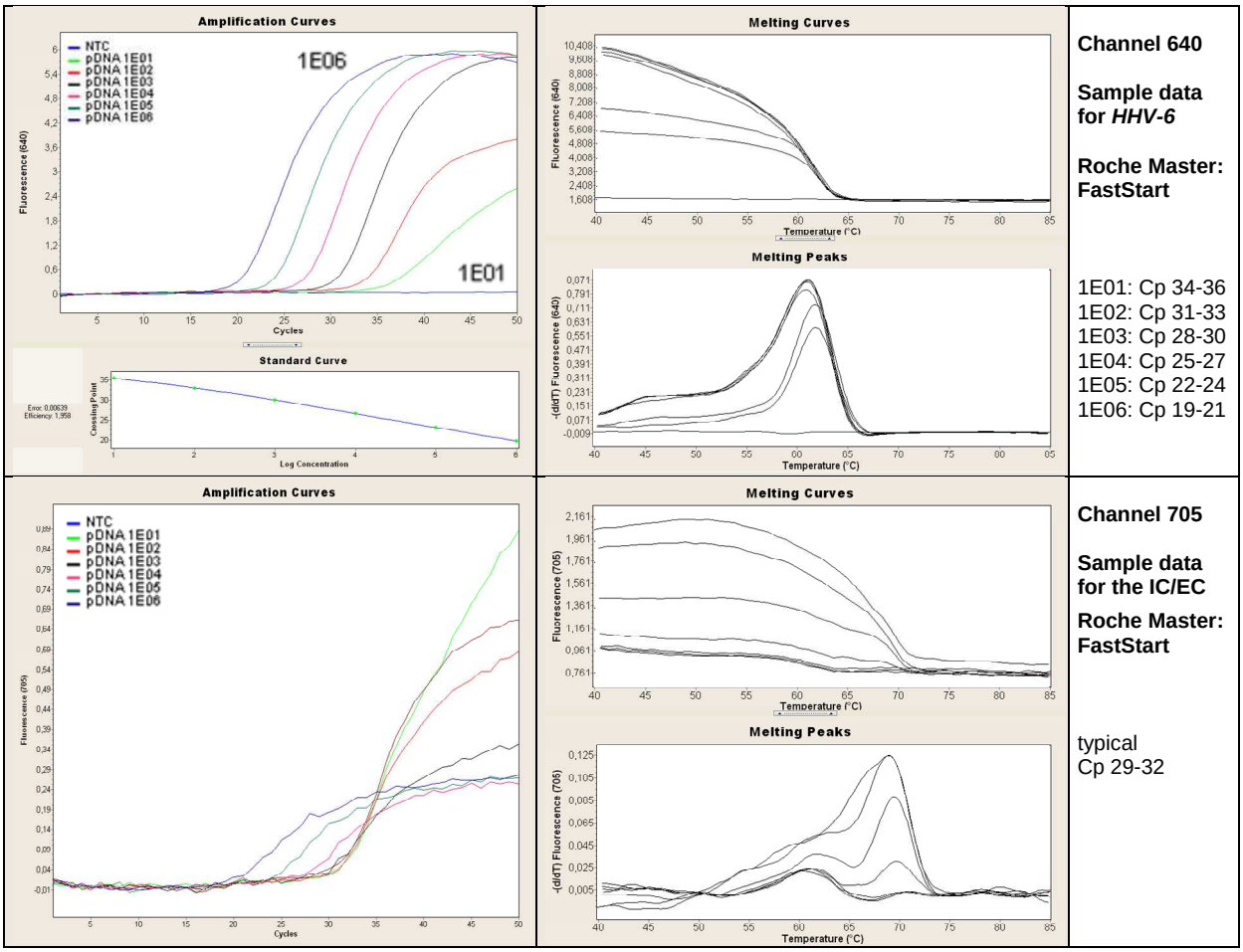

Fig.1. LightCycler® 2.0 Sample data for the *HHV-6* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with calibration curve for *HHV-6*. Right panel channel 640 melting analysis for *HHV-6* (not relevant for detection, shape may vary with concentration).
Lower panels:. Left panel channel 705 quantification mode (Second Derivative Maximum) for the control reaction.
Right panel channel 705 melting analysis for the Control Reaction (not relevant for detection).

7.4. Interpretation of Data

Sample 640 *HHV-6*	**Sample 660** *Control Reaction*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	***Result*** ***(warnings)***
No amplification	Cp 28-31	amplification	negative	**Negative (not detectable)**
Cp < 38*	not relevant	amplification	negative	**Positive for *HHV-6***
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 with LightCycler® 2.0 Instrument
* The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

8. LightCycler® 480 II Instrument and cobas z 480 Analyzer

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 z 480 Analyzer: 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	00:01:00	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]	-	-	-	-	-	-	1	-

(Melting not relevant for detection)

Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer 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 TIB ColorCompensation 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 *HHV6* data with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal.

For the Control Reaction view Filter Combination 498-660 (498-700) data, Quantification mode. The negative control and the low-concentrated *HHV-6* DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 30.

The provided standard row with 10^6 copies/rxn to 10^1 copies/rxn of *HHV-6* should have Cp values between cycles 19 and 37, Cp values calculated with Second Derivative Maximum method (see figure 2).

8.3. Sample Data – Typical Results

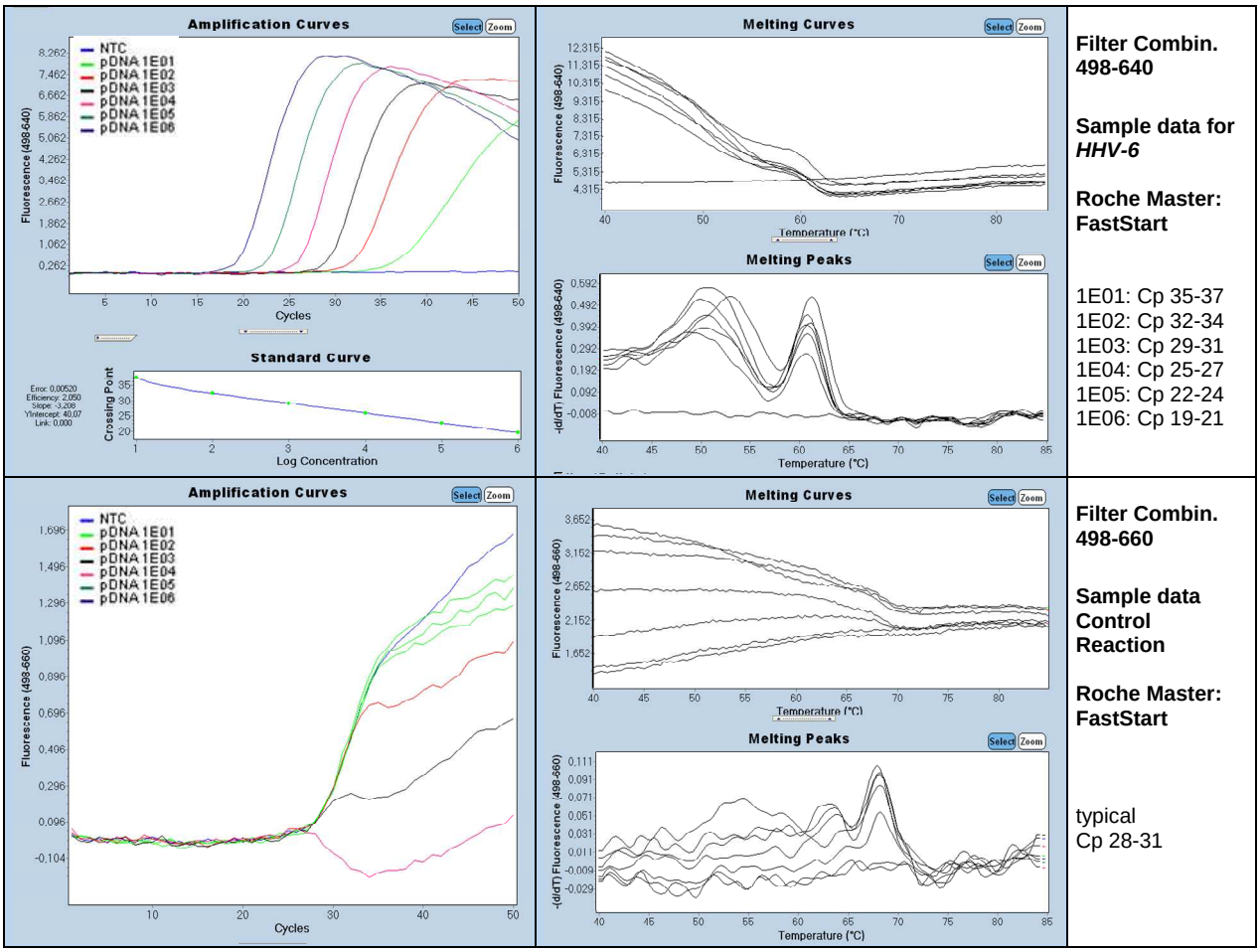

Fig.2. LightCycler® 480 II sample data for the *HHV-6* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) obtained with the standard row for *HHV-6*. Right panel Filter Combination 498-640 melting analysis for *HHV-6* (not relevant for detection, shape may vary with concentration).
Lower panels: Left panel filter combination 498-660 quantification mode (Second Derivative Maximum) for the conrol reaction. Right panel channel filter combination 498-660 melting analysis for the Control Reaction (not relevant for detection).

8.4. Interpretation of Data

Sample 640 *HHV-6*	**Sample 660** *Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	***Result*** **(warnings)**
No amplification	Cp 28-31	amplification	negative	**Negative (not detectable)**
Cp < 39*	not relevant	amplification	negative	**Positive for *HHV-6***
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 with LightCycler® 480 II Instrument
* The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

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 (Measured Value) 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]

Example: Extracting 200 µl clinical sample results in a correction factor of 5. Using 5 µl from a total eluation volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of x100:

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

10. Material Safety Data

According to OSHA 29CFR1910.1200, 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 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

Red notes mark events require changed procedures, blue mod. sequences

V090305	For LC 1.x/2.0/480II
V100819	02/2011 32 rxn per vial and
V111025	Editorial changes
V120212	Figures replaced with results from lot 7841201. Internal Control shifted by 1-2 Cp units.
V130813	cobas z480 included, Conversion Factor and MSDS included
V140312	Internal Control changed to spiked Extraction Control
V140414	Volume ECT and NTC changed (Section 6). Editorial changes
V150808	Change to universal ECT target containing Lambda and PhHV DNA

Roche SAP order n° 05997879001

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Notice to Purchaser

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.