LightMix® Kit for the detection of *Epstein-Barr Virus (EBV) EC*

Cat.-No. 40-0186-32 PCR protocol harmonized (optional change)

Kit with reagents for the detection of *Epstein-Barr Virus (EBV)* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer.

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 and cobas z 480 Analyzer see pages 6-7

1. Introduction

The *Epstein-Barr Virus* (*EBV,* or *Human Herpesvirus 4, HHV-4*) is a DNA virus belonging to the family of *Herpesvirus*. Most people become infected sometime during their lives but only a small fraction of infected people develop a disease. Children usually show no symptoms or indistinguishable from other mild illness. In the Northern hemisphere infections in adults can cause Infectious Mononucleosis (IM) known as 'Pfeiffer's disease' with several symptoms (fever, sore throat, swollen lymph glands, rarely heart problems or involvement of the central nervous system) which last for some weeks but is seldom fatal. In Africa the infection is associated with Burkitt's lymphoma and in Southeast Asia with nasopharyngeal cancer; the different outcome is claimed to be related to virus types (DKFZ, 2013). Reactivation of the virus due to other illness usually occurs without symptoms.

There is no specific treatment for infectious mononucleosis**,** other than treating the symptoms but nevertheless identification of *EBV* may be needed for verification and to differentiate *EBV* infections from a mononucleosis-like illness induced by cytomegalovirus, adenovirus, or *Toxoplasma gondii*.

2. Description

This kit provides a fast and accurate system to detect *EBV* genomic DNA from a nucleic acid extract, including a spiked control working as extraction and amplification control. A 166 bp long fragment from the EBNA gene is amplified with specific primers. The resulting PCR fragment is detected with LightCycler® Red 640 labeled hybridization probes. The PCR product is identified by running a melting curve with a specific melting point (Tm) of 69°C (n ot relevant for detection).

The Control Reaction is based on a 278 bp long PCR fragment generated from Lambda DNA, detected with LightCycler® Red 690 labeled hybridization probes (channel 705). This Control Reaction PCR has no visible impact on the EBV specific reaction and will even fail in the presence of higher amounts of EBV 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) to monitor successful extraction and demonstrate the ability to run an amplification reaction (no PCR inhibition). We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. The Extraction Control Target ^{n}ECT 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 target DNA can even get lost.

The use of a color compensation file generated with the *TIB MOLBIOL* 'Light Mix® ColorCompensation HybProbe' kit (order no. 40-0318-00) is a prerequisite to detect the Control Reaction.

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

The kit must be used with 'LightCycler® FastStart DNA Master HybProbe' only (capillary and plate based LightCycler® Instruments).

3. Set Contents

3 Vials with **blue** cap containing premixed lyophilized primers and probes for 32 PCR rxns of *EBV*
3 Vials with **white** cap containing premixed primers and probes for each 32 Control Reactions

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

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

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

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 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 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 4.1 or higher.

4.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Extraction Control Target (^{n}ECT)	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 *EBV* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 and plate based 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 *EBV* DNA.

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 on 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).
- Dissolved reagents can be long-term stored frozen at -20°C. Avoid multiple thaw-freeze cycles.

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 (EC):

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions of *EBV.*
One reagent vial with a **white** cap contains primers and probes to run 32 Control Reactions.

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

►**Use 2µl** 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:

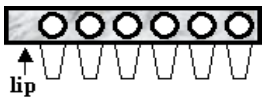
Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Extraction (NTC):

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 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** 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. 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

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.

sEC Procedure	For use with the Roche FastStart Master	IC Procedure
Single reaction	**Component**	**Single reaction**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.35 µl
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0 µl	**PSR** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**Primers and probe mix for the IC/EC**	2.0 µl
---- µl	ECT Control Target (vials **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® 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

The cycling protocol has been changed to the standard cycling protocol with a step-down annealing temperature from 62℃ to 55℃. The kit recipe has b een not changed and it is allowed to continue to work with the old cycling protocol, thus changing to the new protocol is not mandatory.

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

(Melting not relevant for detection) 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 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 *EBV* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *EBV* data in channel 640, Melting Curves mode.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/ reaction to 10^1 copies/ reaction of *EBV* should have Cp values between cycles 17 and 35 (see figure 1).

For the Control Reaction, view data in channel 705. The negative control and the low-concentrated *EBV* DNA samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycle 27-30.

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 to upgrade LightCycler® 1.x Instruments to software version 4.1 or higher.

7.3. Sample Data – Typical Results

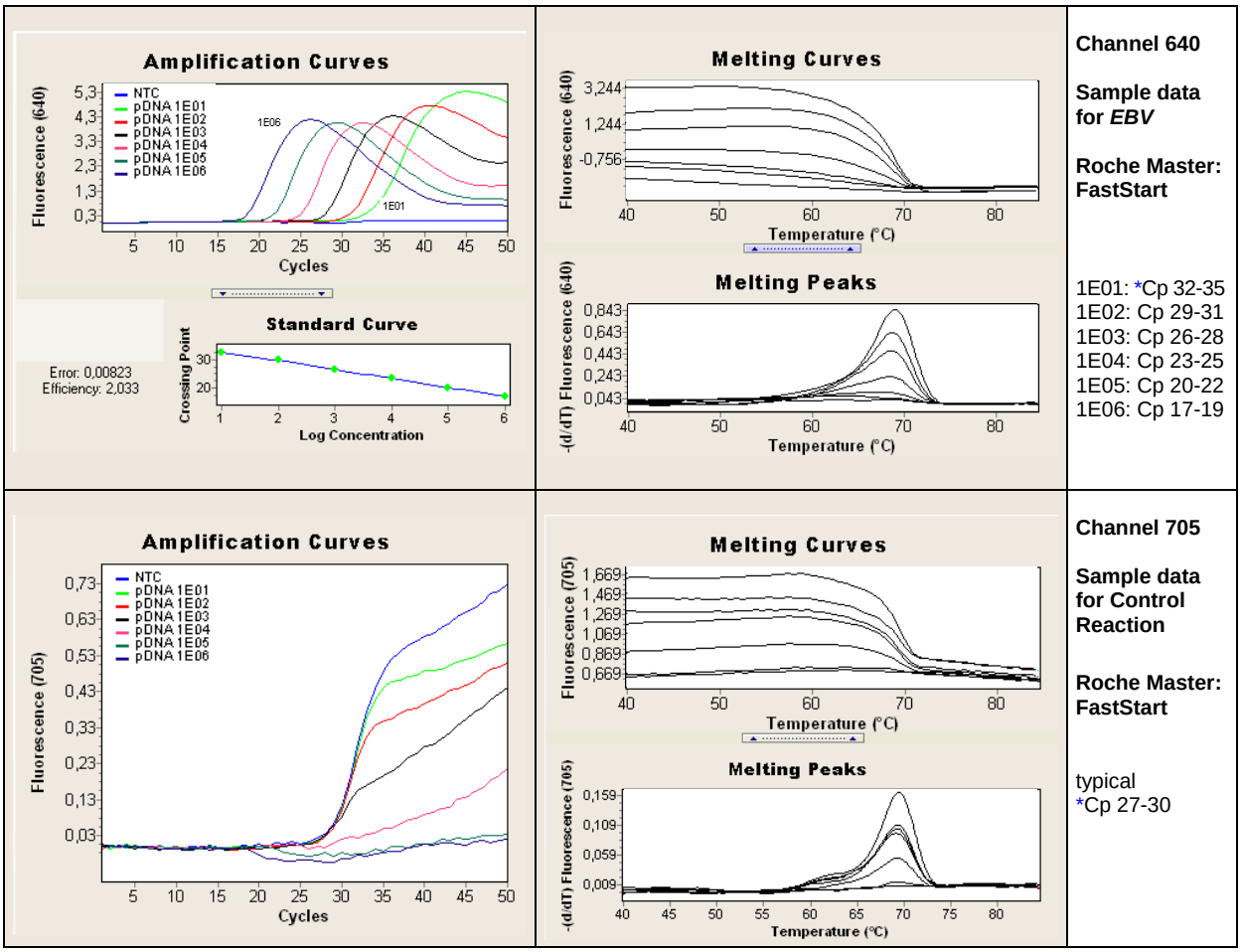


Fig.1. LightCycler® 2.0 sample data for the *EBV* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) amplification with standard curve for *Epstein-Barr virus.* Right panel channel 640 melting analysis/peaks for *Epstein-Barr virus* (not relevant for detection).
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).

* **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.

7.4. Interpretation of Data

Sample 640 *EBV*	**Sample 705** Control Reaction	**Channel 640** Positive Control	**Channel 640** Negative Control	**Result** (warnings)
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37*	not relevant	amplification	negative	**Positive for *EBV***
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® 1.x / 2.0 Instruments, Roche Diagnostics Master: FastStart)

* 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 corresponds to ~5 copies per reaction.

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

Using the kit with the LC 480 (Version I) will yield no or very weak signals for the control reaction while the signals in channel 640 for *EBV* are not affected.

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

The cycling protocol has been changed to the standard cycling protocol with a step-down annealing temperature from 62℃ to 55℃. The kit recipe has b een not changed and it is allowed to continue to work with the old cycling protocol, thus changing to the new protocol is not mandatory.

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
(Melting not relevant for detection)

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 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 *EBV* data with Filter Combination 498-640 (498-645), Quantification mode. The negative control (NTC) must show no signal.

The provided standard row of cloned and purified *EBV* DNA with concentrations in the range from 10^6 copies/ reaction to 10^1 copies/ reaction of *EBV* should have Cp values between cycles 17 and 35 (see figure 2).

If the Control Reaction is used, view data with Filter Combination 498-660 (498-700), Quantification mode. The negative control and the low-concentrated *EBV* DNA samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycle 27-30.

8.3. Sample Data – Typical Results

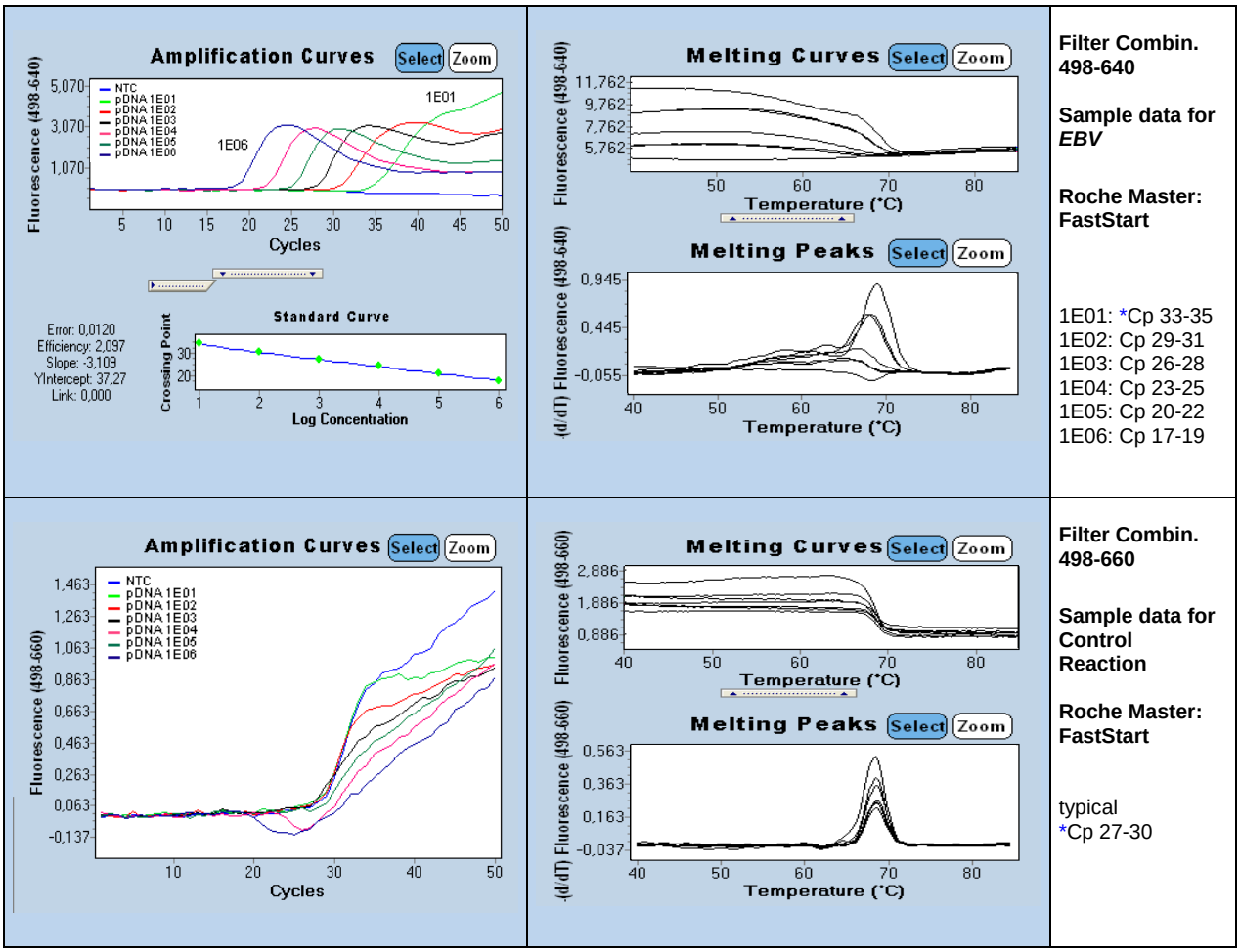


Fig.2. LightCycler® 480 II sample data for the *Epstein-Barr virus* detection system.

Upper panels: Left panel Filter Combination 498-640 (645) quantification mode (Second Derivative Maximum) amplification with standard curve for *EBV*. Right panel Filter Combination 498-640 (645) melting analysis/peaks for *EBV* (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 (700) quantification mode (Second Derivative Maximum) for the Control Reaction. Right panel Filter Combination 498-660 (700) melting analysis for the Control Reaction (not relevant for detection).

* **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.

8.4. Interpretation of Data

Sample 498-640 *EBV*	**Sample 498-660** Control Reaction	**Filter 498-640** Positive Control	Filter 498-640 Negative Control	**Result** (warnings)
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37*	not relevant	amplification	negative	**Positive for *EBV***
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® '480' plate based Instrument, Roche Diagnostics Master: FastStart)

* 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 corresponds to ~5 copies per reaction.

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**

Note: For clinical samples different from body fluids the amount of sample is dependent from the collecting technique (swab, tissue, FFPE) and the viral load cannot be related to the volume.

10. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU 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

Red notes mark changes in procedures, blue modification of components

V060331	Release version
V080328	Including figures LC 2.0
V081020	Editorial changes
V091008	Data for LC 480
V100816	Editorial changes
V130717	Including Version History
V130813	Cut-off values (recommendation), z480 included, Conversion Factor, MSDS included
V140303	Editorial changes
V150505	Change from 16 to 32 rxn/vial and change from IC to EC Kit equipped with the universal ^{n}ECT extraction control target
V160226	Standardized PCR cycle conditions. Kit may be used with with old cycles.

Roche SAP order n° 05943612001