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

TIB MOLBIOL

LightMix® Kit for the detection of *Varicella-Zoster Virus (VZV)*

Cat.-No. 40-0211-32 **Internal Control (IC) changed to spiked Extraction Control (sEC)**

Kit with reagents for the detection of *Varicella-Zoster Virus* 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 / cobas z 480 analyzer see pages 6-7

1. Introduction

Varicella-Zoster Virus (VZV) is a member of the human herpesvirus family (HHV-3) causing chicken pox (varicella) and shingles (herpes zoster). VZV infections are medically significant, causing approximately 4 million cases of varicella and 500,000 cases of zoster each year.

More than 90% of the population develops a varicella infection by the time they reach adolescence, and virtually 100% of the population will develop it until the age of 60. After the primary infection the virus remains dormant - upon reactivation patients develop shingles, with an increasing risk with age. In immunosuppressed individuals VZV can cause severe and even fatal infections.

Common targets for PCR based tests are the thymidin kinase gene (orf26), glycoprotein gB (orf27), polymerase (orf28)[1], the DNA binding protein (orf29), and the glycoprotein E gene (orf67). The virus can be found not only in blood [2] but also in saliva and other body fluids [3].

[1] Diagnosis of VZV Infections in the Clin. Laboratory by LightCycler PCR. Espy, et al., Clin. Microbiol 38 (2000) 3187-9

[2] Diagnosis of infection with herpes viruses in routine laboratory practice. Mostafaie et al., Clin Chem Lab Med 2009

[3] Varicella DNA in blood, nasopharyngeal swab and saliva of patients with Herpes Zoster. Droppelmann et al. 2014 American academy meeting Chicago www.aad.org/eposters/Submissions/getFile.aspx?id=88&type=sub

2. Description

This kit [2,3] provides a fast and accurate system to determine *VZV* genomic DNA in a nucleic acid extract. A 290 bp long fragment from the orf28 gene is amplified with specific primers and detected with LightCycler® Red 640 labeled probe which can be identified by a melting analysis with a Tm of 63°C.

The control reaction generates an additional product of 300 bp using hybridization probes labeled with LightCycler® Red 690 (recorded in channel 705). This second PCR has no visible impact on the *VZV*- 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 a successful extraction and demonstrate the ability to run the PCR (absence of inhibition).
We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. Target and control primer/probe sequences remained unchanged.
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 TIB MOLBIOL 'LightMix® Kit - Color Compensation 530/640/690' 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.

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

3. Set Contents

3 Vials with blue cap containing premixed primers and probes for 32 PCR reactions of *VZV*
3 Vials with **white** cap containing premixed primers and probes for 32 control reactions

1 Vial with **white** cap containing Extraction Control Target (ECT): 2.4 x 10^6 copies (total)
1 Vial with **black** cap containing the stabilizer for preparation of the 'No Template Control' (NTC)

1 Standard row with 6 dried standards *Varicella-Zoster Virus* DNA 10-10^6 copies per rxn
1 Sealing foil for the standard row

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

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
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 692 001

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 or higher.

4.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit Cat.-No. 11 796 828 001

5. Product characteristics

PCR results (activation, 45 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 *VZV* positive control target DNA using FastStart DNA Master HybProbe.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of target genomic 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℃). Please see the ex piration 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℃).
- Dissolved reagents can be long-term stored frozen at -20℃. 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 :

One reagent vial with a blue cap contains primers and probes to run 32 reactions *VZV*.
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:

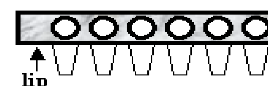
Before extraction add **10 µl** of ECT (vial white cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

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. After adding the target DNA to reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the LightCycler® 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**
7.2 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.7 µl
1.8 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.8 µl
2.0 µl	**PSR** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**Control Reaction** (see **6.1.**)	2.0 µl
---- µl	ECT Control Target (vials white cap)	0.5 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 0.5 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

Program:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Segment	1	1	2	3	1	2	3	1
Target [°C]	95	95	56	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:20	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
Acquisition Mode	None	None	Single	None	None	None	Continu.	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 ‘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 *Varicella-Zoster Virus* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Varicella-Zoster Virus* data in channel 640, Melting Curves mode.

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

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

For use in LightCycler® 1.x / 2.0 Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection.

7.3. Sample Data – Typical Results

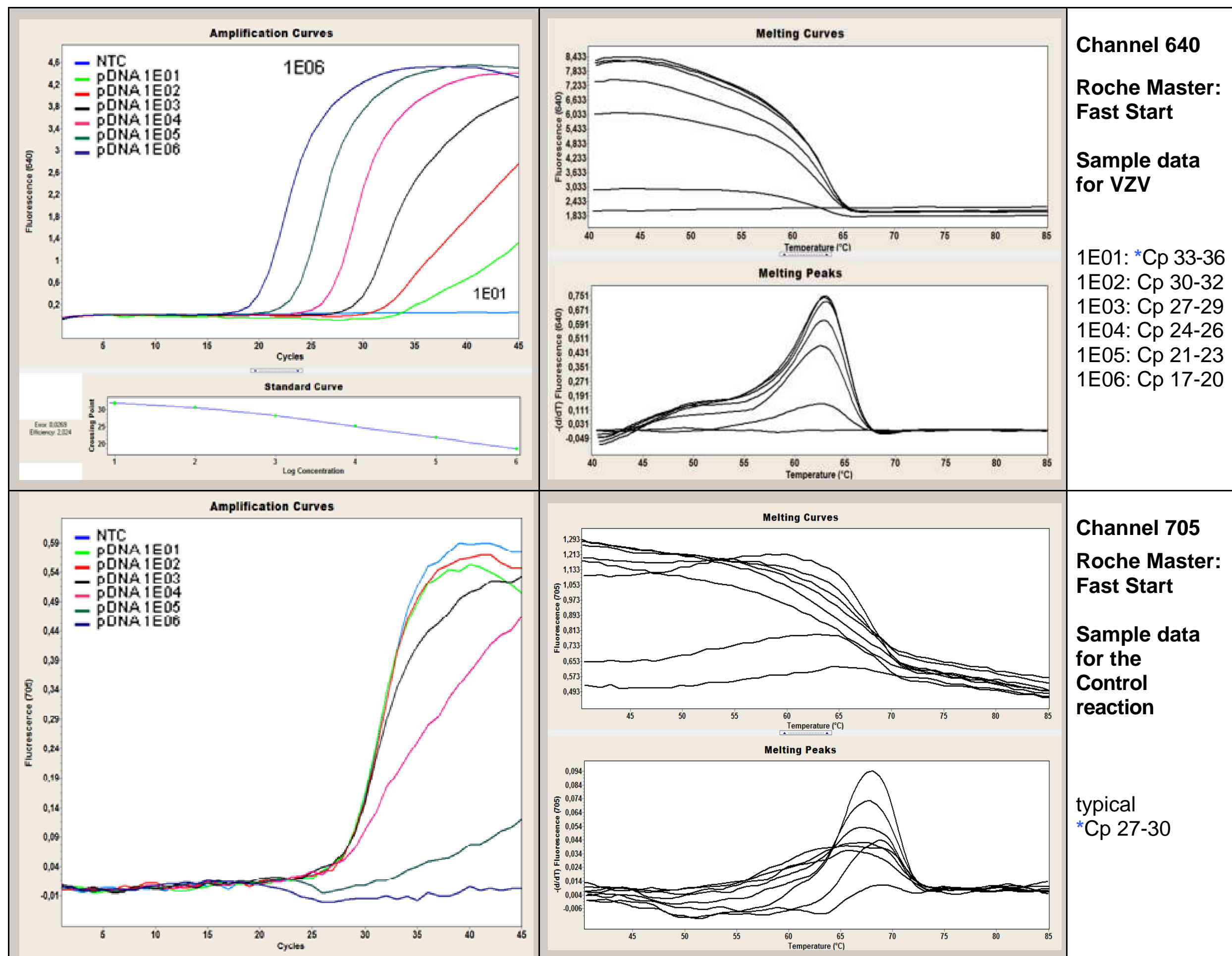


Fig.1. LightCycler® 2.0 sample data for the *Varicella-Zoster Virus* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Varicella-Zoster Virus*. Right panel channel 640 melting analysis for *Varicella-Zoster 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 *VZV*	**Sample 705** Control Reaction	**Channel 640 PositiveControl**	Negative Control (NTC)	**Result (warnings)**
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39[+]	not relevant	amplification	negative	**Positive for *Variazella Virus zella***
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: 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 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	45			1			1
Target [℃]	95	95	56	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:20	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 [℃]	-	-	-	-	-	-	-	-
Step Size [℃]	-	-	-	-	-	-	-	-
Step Delay (Cycles)	-	-	-	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per ℃]	-	-	-	-	-	-	1	-

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 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 *Varicella-Zoster Virus* data with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Varicella-Zoster Virus* data with Filter Combination 498-640, Melting Curves mode.

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

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

8.3. Sample Data – Typical Results

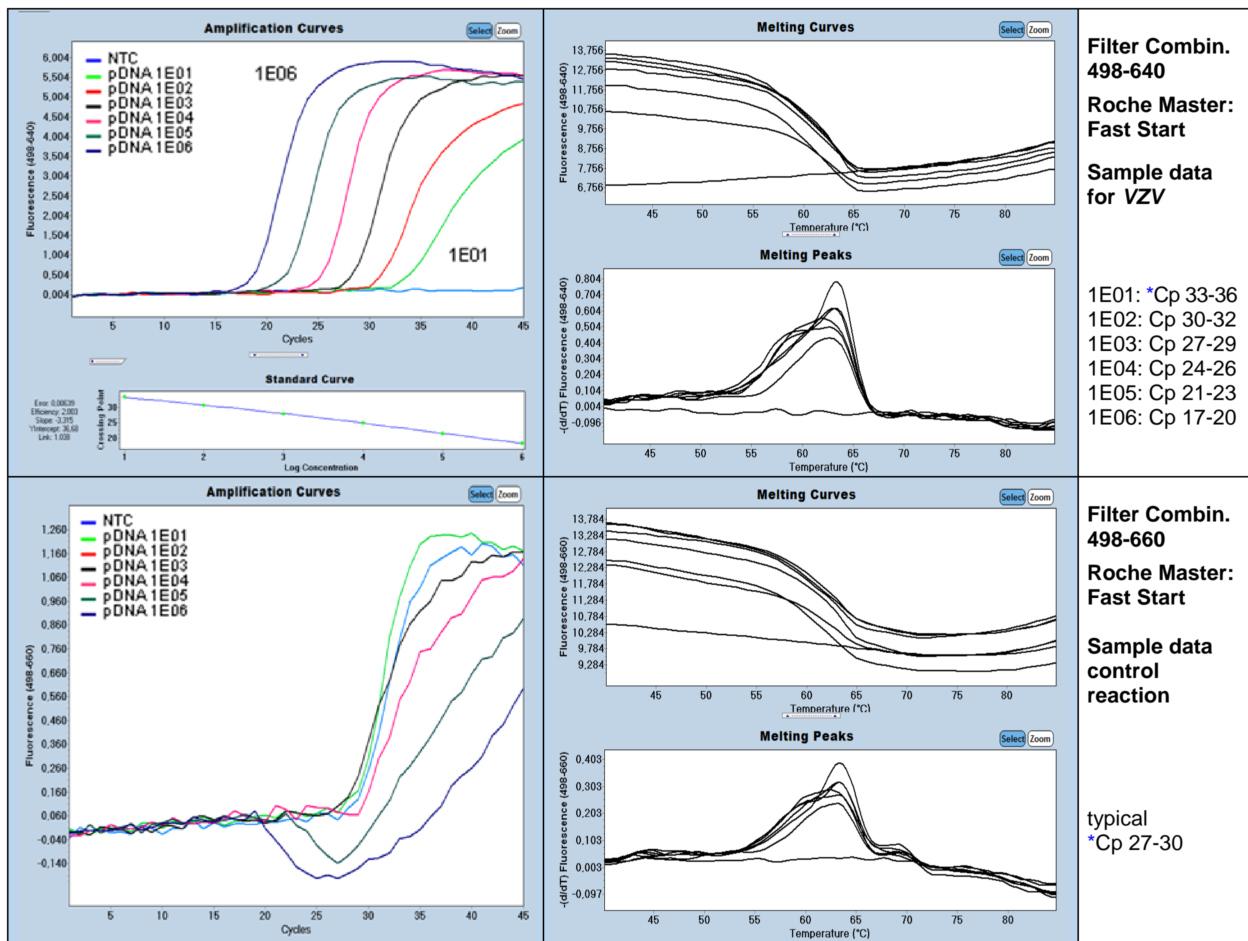


Fig.2. LightCycler® 480 II sample data for the *Varicella-Zoster Virus* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Varicella-Zoster Virus*. Right panel Filter Combination 498-640 melting analysis/peaks for *Varicella-Zoster Virus* (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 quantification mode (Sec. Derivative Maximum) for the control reaction. Right panel Filter Combination 498-660 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 640 *VZV*	**Sample 705** Control Reaction	**Channel 640 PositiveControl**	**Negative Control (NTC)**	**Result (warnings)**
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39[+]	not relevant	amplification	negative	**Positive for *Variazella Virus***
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 II Instrument, Roche 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 than observed for 10 copies coresponds to ~5 copies.

9. Conversion Factor

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

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] = 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, 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).

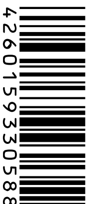
Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

11. Version History

Notes in red mark events require changing procedures

V100425	Data for LC480 Instruments
V111010	32 rxns per vial
V130725	Editorial changes
V141111	Chapter 9: Conversion Factor included. Chapter 10: MSDS included.
V150303	Internal Control changed to spiked Extraction Control (sEC)

Roche SAP order n° 05943655001



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