LightMix® Kit *Dengue Virus* EC

Cat.-No. 40-0439-32

Change to 3x32 rxns, change IC to Extraction Control

Kit with reagents for the detection of *Dengue Virus* (types 1-4) cDNA using Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer. Kit is compatible with the Chipron Dengue Array.

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

Dengue virus is a plus strand ssRNA virus of the Flavivirus family splitting into 4 serotypes (DENV1-4). Aedes mosquitoes are responsible for transmissions to humans and the disease affects therefore preferentially urban regions with tropical or subtropical climate. About 80% of all *Dengue* infections are asymptomatic; the others have usually only a few days or weeks of fever, headaches and skin rush. However, a second infection with a different type can be fatal, making the DENV *so* dangerous.

Most PCR detection and typing assays for DENV address the partially conserved 3-UTR region [1,2]. Due to the typical RNA-viral sequence variations it is difficult to identify one particular probe to be strictly related to one type, or use one probe for a melting curve based analysis. In contrast, array detection provides the possibility to employ different probes at the same time.

2. Description

This kit provides a fast and accurate system to detect the viral genome from cDNA generated from a NA extract. The PCR product can be applied on the DENV Chipron Array to determine the serotype.

In the first step the viral RNA is transcribed to cDNA, using a specific primer (now provided in the kit). A 164 to 187 bp (depending on the virus serotype) fragment of the 3-UTR region is amplified with specific primers and detected with a mixture of LightCycler® Red 640 labeled hybridization probes.

The Control Reaction is based on an additional 349 bp long PCR product from Lambda DNA, detected with hybridization probes labeled with LightCycler® Red 690 (channel 705). This second PCR has no visible impact on the *Dengue*-specific reaction and will even fail in the presence of higher amounts of target (1,000 copies or more) while displaying a 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; 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 use of a color compensation file generated with the LightMix® ColorCompensation HybProbe Kit 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.

The kit is tested with 'LightCycler® FastStart DNA Master HybProbe' only (including 480 instruments).

The detection limit (LOD) in a 1-step RT PCR procedure (not decribed here) is about one log less. Using the Roche Multiplex RNA Virus Master the LOD is about 50 copies, or, if omitting the Control Reaction, about 10 copies; the LOD with the Realtime Ready RNA master is 100 copies or higher.

3. Set Contents

3	Vials with **blue** cap containing lyophilized primers and probes for 32 PCR reactions of *Dengue*
3	Vials with **white** cap containing premixed primers and probes for 32 Control Reactions
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
1	Vial with **orange** cap containing lyophilized Dengue Primer for use in cDNA synthesis, 100 rxns
2	Vials 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
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
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

4.1. Optional Additional Reagents

High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
LightCycler® Multiplex RNA Virus Master	Cat.-No. 06 754 155 001
Extraction Target nECT	TIB Cat.-No. 30-0259-96

5. Product Characteristics

PCR results are obtained within 1 hour (55 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 90 minutes (55 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Dengue Virus* cDNA using FastStart DNA Master HybProbe.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Dengue Virus* cDNA.

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

6. Preparation of the cDNA Target from the Nucleic Acid Extract

Dissolve vial with the **orange** cap containing the Dengue primer in 100 µl and use 1 µl per reaction. Prepare the cDNA synthesis according to the manufacturer's instructions; the amount of Extraction Target ECT is adjusted to a two-fold dilution of the RNA.

7. Experimental Protocol

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

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

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions *of Dengue virus.*
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.

7.2. Preparation of Extraction Control Target (ECT):

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

7.3. Sample Preparation:

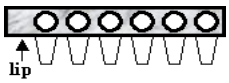
Before extraction add **20 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation and cDNA synthesis as described in the protocol of the kits used.

7.4. 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.

7.5. 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** (**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 DNA 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.

7.6. 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**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.1 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	**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.

8. LightCycler® 1.x / 2.0 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

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	55			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:10	00:00:10	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	Continuous	None

(Melting not relevant for detection) Table 2

8.2. 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 Color Compensation kit.

Perform data analysis, as described in the 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.

Check DENV data in channel 640, Quant. mode. The negative control (NTC) must show no signal.

If the Control Reaction is used, view DENV data in channel 640, Quantification mode, and the Control Reaction in channel 705, Quantification mode. The negative control and low-concentrated DENV samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycle 28.

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

The provided standard row of cloned DNA with concentrations in the range from 10^6 to 10^1 copies/rxn of the DENV target sequence should have Cp values between cycles 18 and 36 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – Typical Results

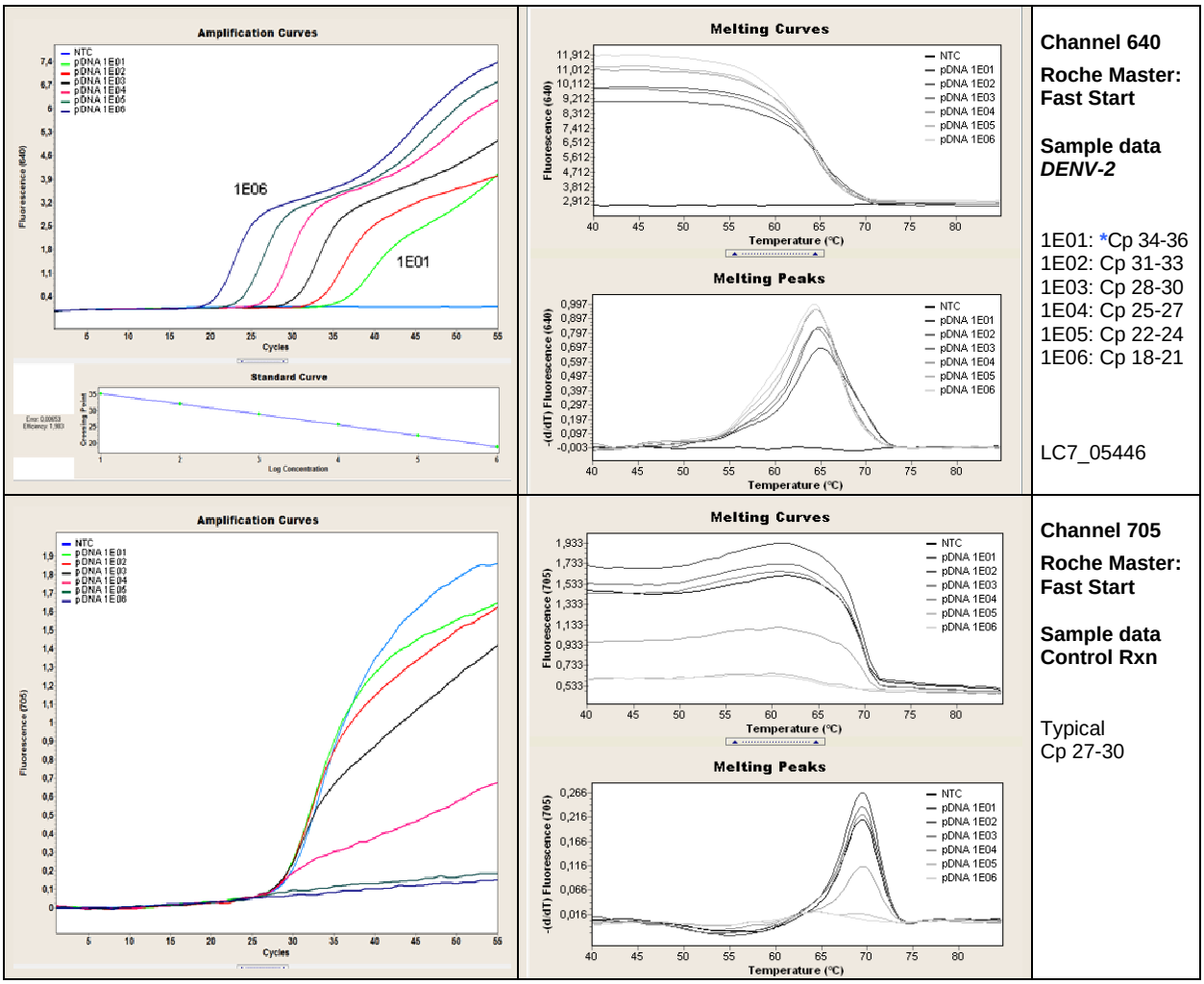


Fig.1. LightCycler® 2.0 sample data for the *Dengue Virus* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with calibration curve for DENV-2. Right panel channel 640 melting analysis (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 IC/EC (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 *Dengue*	**Sample 705** *Control Reaction*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39	not relevant	amplification	negative	**Positive for *Dengue 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 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

9. LightCycler® 480 II Instruments / cobas z 480 Analyzer

9.1. 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	55			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:10	00:00:10	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
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	1	-

(Melting not relevant for detection) Table 4

9.2. 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 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.

Check DENV data with Filter Combination 498-640. The negative control (NTC) must show no signal.

If the Control Reaction is used, view DENV data with Filter Comb. 498-640, Quantification mode, and the Control Reaction with Filter Comb. 498-660, Quantification mode. The negative control and low-concentrated DENV samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycle 27.

The provided standard row of cloned DNA with concentrations in the range from 10^6 to 10^1 copies/rxn of the DENV target sequence should have Cp values between cycles 19 and 37 (Cp values calculated with Second Derivative Maximum method).

9.3. Sample Data – Typical Results

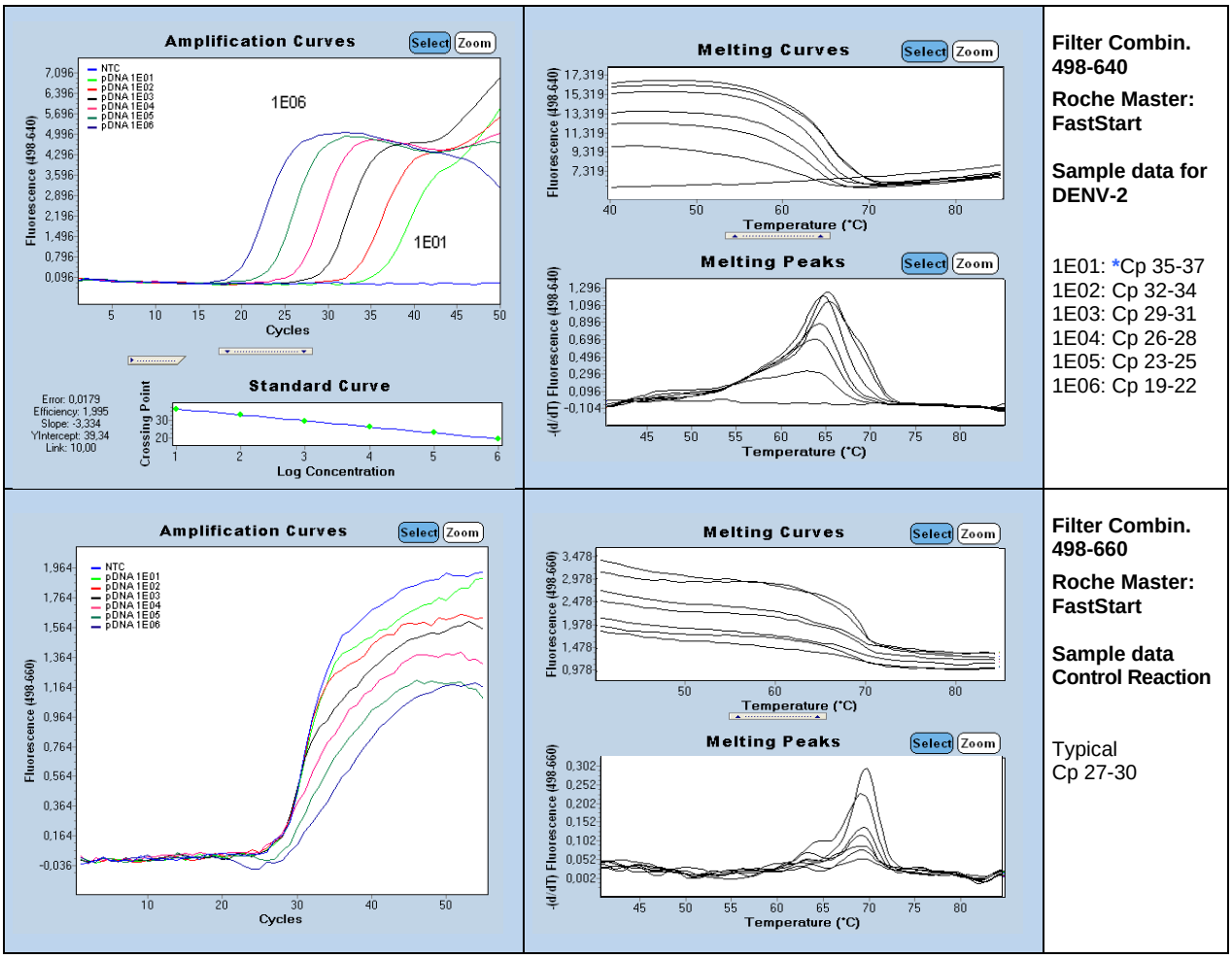


Fig.2. LightCycler® 480 II sample data for the *Dengue Virus* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for DENV-2**.** Right panel Filter 498-640 melting analysis (not relevant for detection).
Lower panels: Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) with amplification curves the Control Reaction**.** Right panel Filter 498-660 melting analysis/peaks (not relevant for detection).

9.4. 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/EC) which must be detectable for negative samples.

Sample 640 *Dengue*	**Sample 660** *Control Reaction*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	*Result (warnings)*
No amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39	not relevant	amplification	negative	**Positive for *Dengue 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: Fast Start)

* **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.

10. 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**

11. References

[1] Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1-4 using conserved and serotype-specific 3' noncoding sequences. Houng HS, Chung-Ming Chen R, Vaughn DW, Kanesa-thasan N. J Virol Methods. 95 (2001)19-32

[2] Rapid Detection and Quantification of RNA of Ebola and Marburg Viruses, Lassa Virus, Crimean-Congo Hemorrhagic Fever Virus, Rift Valley Fever Virus, Dengue Virus, and Yellow Fever Virus by Real-Time Reverse Transcription-PCR. Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S. JCM 40 (2002) 2323-2330

12. 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.

13. Version History

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

V081209	First version (2008)
V110215	Revised version (2011)
V130813	Conversion Factor, MSDS, Cut-off values and Version History included
V150808	Dengue primer for cDNA synthesis included (improves detection limit) Internal Control (IC) changed to Extraction Control (EC) Kit changed from 6 x 16 rxns to 3 x 32 rxns (remains 96 reactions total)

Roche SAP order n° 05940427001

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Notice to Purchaser

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