LightMix® Kit *Chlamydophila pneumoniae* EC

Cat.-No. 40-0222-32 Internal Control (IC) changed to Extraction Control (EC), 32 rxns/vial

Kit with reagents for the detection of *Chlamydophila pneumoniae* 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 N OT 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

Chlamydophila pneumoniae has been established as an important human respiratory pathogen causing both endemic and epidemic disease, including pneumonia, bronchitis, pharyngitis and sinusitis. It is commonly spread by respiratory secretions, and has a high incidence rate with an estimated infestation rate of 50 -70%. Newer investigations show that *C. pneumoniae* is also linked to atherosclerosis which finally may cause heart attacks and strokes.

A long term antimicrobial treatment with common antibiotics is necessary for eradication of *C. pneumoniae* from macrophages and endothelial cells of infected arteries.
Several assays for the Real-Time-PCR detection *C. pneumoniae* have been published, targeting amongst others the 16S RNA[1], the J38 fragment [2], or the Pst fragment [3].

[1] Rapid and standardized detection of Chlamydia pneumoniae using LightCycler real-time fluorescence PCR. Reischl et al, J Clin Microbiol Infect Dis. 2003 Jan;22(1):54-7

[2] Evaluation of real-time quantitative PCR for identification and quantification of Chlamydia pneumoniae by comparison with immunohistochemistry. Mygind et al., . J Microbiol Methods 2001 Sep;46(3):241-51

[3] Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions, Welti et al, 2003, Diag. Microbiol. Inf. Disease 45, 85-95

This LightMix® kit provides a fast, easy and accurate system to identify this target in a nucleic acid extract. The internal control (IC) has been changed to a spiked extraction control (sEC) to monitor a successful extraction and demonstrate the ability to run an amplification reaction (thus absence of PCR inhibition). The kit has been tested with 'LightCycler® FastStart DNA Master HybProbe' only.

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.

2. Description

A 140 bp long fragment of the J38 genomic region of *C. pneumoniae* is amplified with specific primers. The resulting PCR fragment is analyzed with LightCycler® Red 640 labeled hybridization probes (detected in channel 640). The PCR product is identified by running a melting curve with a specific melting point (Tm) of approximately 67°C in channel 640.

The control reaction generates an additional product of 278 bases which is detected with LightCycler® Red 690 labeled hybridization probes. This second PCR has no visible impact on the *C.pn.* specific reaction and will even fail in the presence of higher amounts of *C.pn.* target (1,000 copies and more).

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run the duplex reaction *C. pneumoniae* and the control.

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.

3. Set Contents

3	Vials with **green** cap containing premixed primers and probes for each 32 PCR reactions
1	Standard row with 6 lyophilized standards of *C.pn* from 10^1 to 10^6 target equivalents per rxn
1	Sealing foil for the standard row
3	Vials with **white** cap containing premixed lyophilized primers / probes for 32 control reactions
1	Vial with **white** cap containing the Extraction Control Target (ECT) with 2 x 10^6 copies (total)
1	Vial with **black** cap containing the stabilizer for the No Template Control (NTC)
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 (plate based instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (plate based 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 PCR Template Preparation Kit	Cat.-No. 11 796 828 001

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 *C. pneumoniae* 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 *C. pneumoniae* 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℃). 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℃).

6. Experimental Protocol

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

6.1. Preparation of parameter-specific reagents and reagents for the EC (32 reactions):

One reagent vial with a **green** cap contains primers and probes to run 32 reactions *C.pn.*
One reagent vial with a **white** cap contains primers and probes to run 32 reactions control reaction.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (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 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 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. 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 at least one 'No Template Control' (NTC) and one Positive Control. In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions (samples plus controls) to be cycled, plus one additional reaction**.**

sEC Procedure	For use with the Roche FastStart Master	IC Procedure
Single reaction	**Component**	**Single reaction**
5.8 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	5.3 µl
3.2 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	3.2 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 (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 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

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 HybProbe 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.

View *C. pneumoniae* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *C. pneumoniae* data in channel 640, Melting Curves mode.

If the control reaction is used, view data in channel 705, Quantification mode. The negative control and the low-concentrated *C. pneumoniae* samples (10 to 1,000 copies) should show an amplification curve for the IC/EC with a Cp at approximately cycle 30.

The provided standard row with 10^6 to 10 copies/rxn of *C. pneumoniae* should have Cp values between cycles 18 and 35 (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

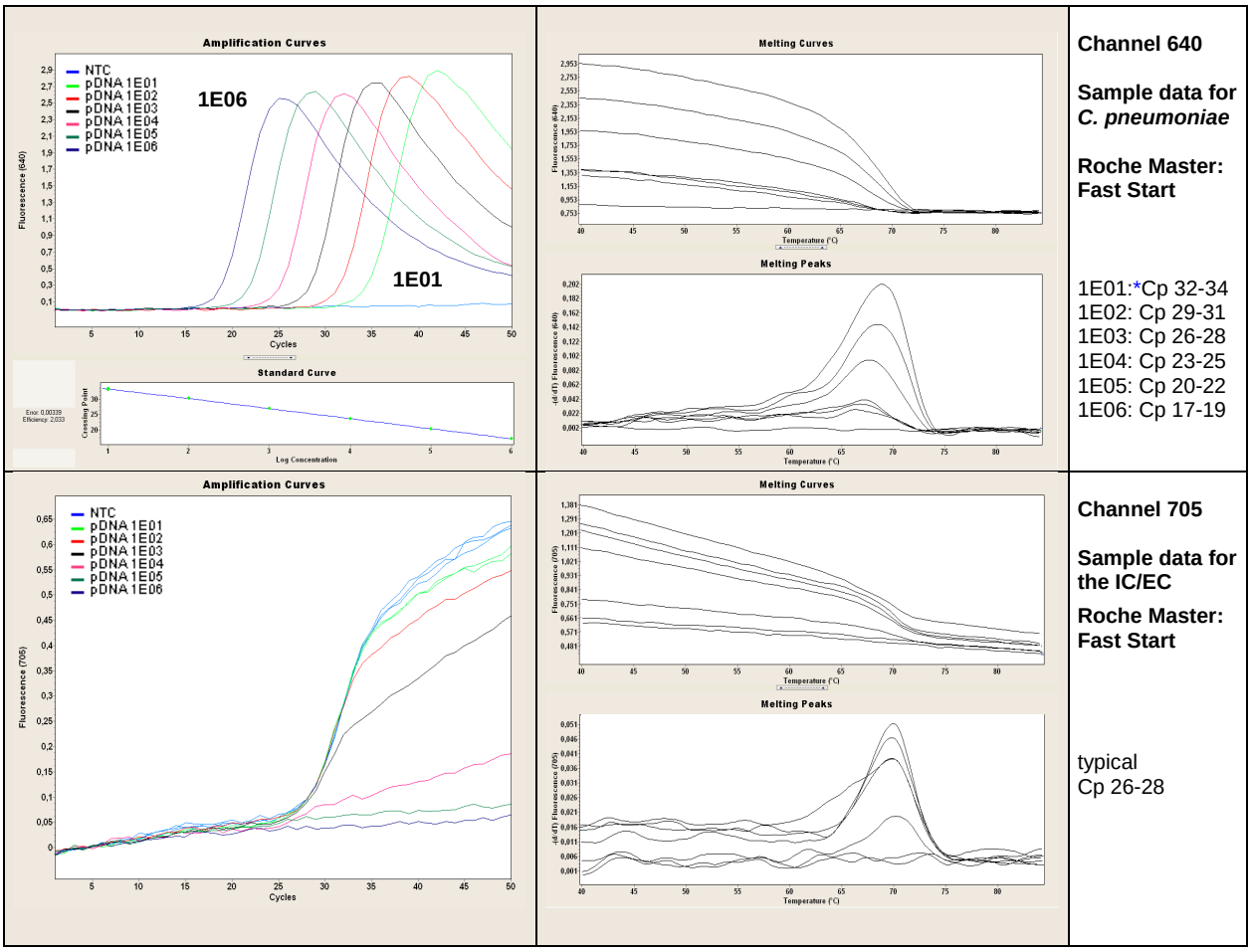

Fig.1. LightCycler® 2.0 sample data for the *C. pneumoniae* detection system.

Upper panels: Left panel. channel 640 quantification mode (Second Derivative Maximum) with the amplification curves for *C. pneumoniae*. Right panel channel 640 melting analysis/peaks for *C. pneumoniae* (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 *C. pneumoniae*	**Sample 705 IC**	**PositiveControl**	**Negative Control (NTC)**	**Result (warnings)**
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36*	not relevant	amplification	negative	**Positive for *C. pneumoniae***
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 / 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 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]	-	-	-	-	-	-	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 the Color Compensation HybProbe 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.

View *C. pneumoniae* data with Filter Combination 498-640. The negative control (NTC) must show no signal. For the identification of the PCR product view *C. pneumoniae* data with Filter Combination 498-640, Melting Curves mode (not relevant for detection).

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

The provided standard row with 10^6 copies/rxn to 10 copies/rxn of *C. pneumoniae* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – Typical Results

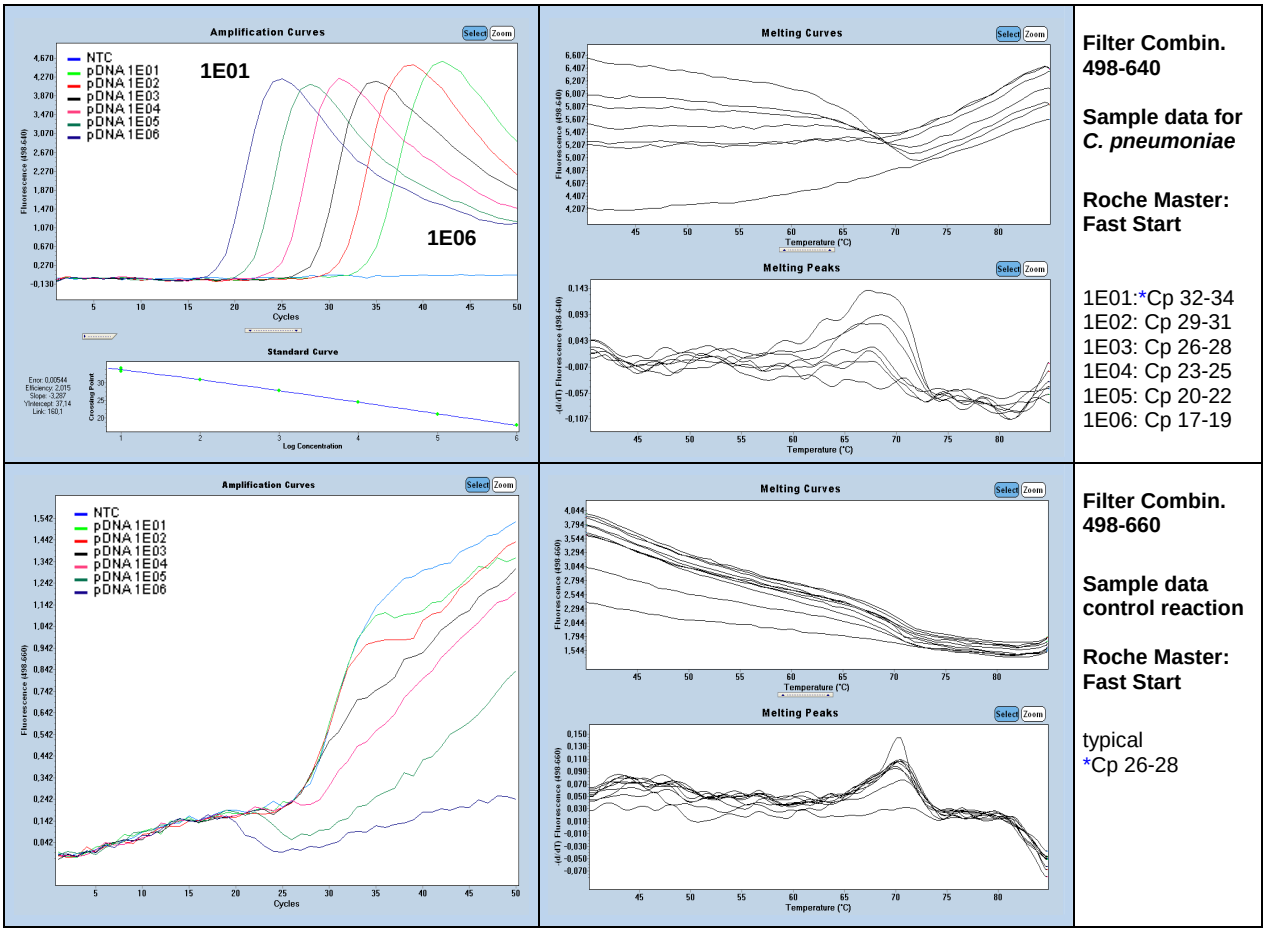

Fig.1. LightCycler® 480 II sample data for the *C. pneumoniae* detection system.

Upper panels: Left panel Filter Combination 498-640 (645) quantification mode (Second Derivative Maximum) with the standadrd row for *C. pneumoniae*.
Right panel Filter Combination 498-640 (645) melting analysis for *C. pneumoniae* (not relevant for detection).
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).

* **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 *C. pneumoniae*	Sample 660 IC	PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36*	not relevant	amplification	negative	**Positive for *C. pneumoniae***
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. 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.

10. Version History

Notes in red mark events require to change procedures

V110816	Released for 480 II Instruments also
V131211	MSDS included, z 480 Analyzer included, editorial changes
V140414	Change from 16 reactions / vial to 32 reactions / vial Change Internal Control (IC) to Extraction Control (EC)

Roche SAP order n° 05997798001

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