TIB MOLBIOL

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

LightMix® Kit *Legionella pneumophila (MIP)*

Cat.-No. 40-0207-32 Universal Extraction Control Target (^{n}ECT)

Kit with reagents for the detection of *Legionella pneumophila* 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

Legionella are thin, faintly staining gram-negative non-spore forming bacteria which can be found everywhere in the non-marine aquatic or moist environment. *Legionella pneumophila*, the most common species of the family Legionellaceae is responsible for the vast majority of human infections.

Many *Legionella* infections stay subclinical. Pneumonia is the most frequent clinical manifestation and *Legionella* is responsible for about 5% of all pneumonia cases. The onset is usually abrupt, with high fever, malaise, myalgias, headache, and a non productive cough. *Legionella* affects preferentially the lung and pericarditis, myocarditis, endocarditis, pancreatitis and hepatic abscesses are very rare.

Legionella are slowly growing and cultural detection with BCYE agar requires about five days while rapid immunological assays and PCR provide faster results. Preferred Real-Time-PCR target are the 5S rRNA, the 16S rRNA[1] or the macrophage infectivity potentiator (MIP) genes [2].

The region of the MIP gene targeted by this kit is specific for *L. pneumophila*; this kit has been tested to show no cross reactivity with *L. longbeachae*.

The alternative LightMix® Kit 40-0460 targets the 16S rRNA gene to detect all members of the genus followed by a melting curve to identify specifically *L. pneumophila.*

2. Description

This kit provides a fast, easy and accurate system to identify *L. pneumophila* in a nucleic acid extract. A 183 bp fragment of the MIP gene is amplified with specific primers. The resulting PCR fragment is analyzed with LightCycler® Red 640 labeled hybridization probes. The PCR product is identified by running a melting curve with a melting point of about 64°C.

The Control Reaction is based on an additional 139 bp long PCR product of a PhHV sequence, detected with hybridization probes labeled with LightCycler® Red 690 (channel 705). This second PCR has no visible impact on the *Legionella*-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; 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® Color Compensation HybProbe 530/640/690 kit (order no. 40-0318-00) is a prerequisite to detect the Control Reaction.

The supplied standard row DNA 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 **green** cap containing premixed primers and probes for 32 reactions of *Leg. pneu.*
3 Vials with **white** cap containing premixed primers and probes for 32 Control Reactions

1 Standard row with 6 dried standards *L. pneumophila* DNA 10^1-10^6 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
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 (480 Instruments)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (480 Instruments)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x / 2.0 Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection. We recommend to upgrade to software 4.10 or later.

4.1. Optional Additional Reagents

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 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 green cap contains primers and probes to run 32 reactions *Legionella pneu..*
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 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 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.

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**
6.2 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	5.7 µl
2.8 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.8 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 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 '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 *L. pneumophila* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For identification of the PCR product view *L. pneumophila* 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 *L. pneumophila* DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 31.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *L. pneumophila* should have Cp values between cycles 18 and 35.

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

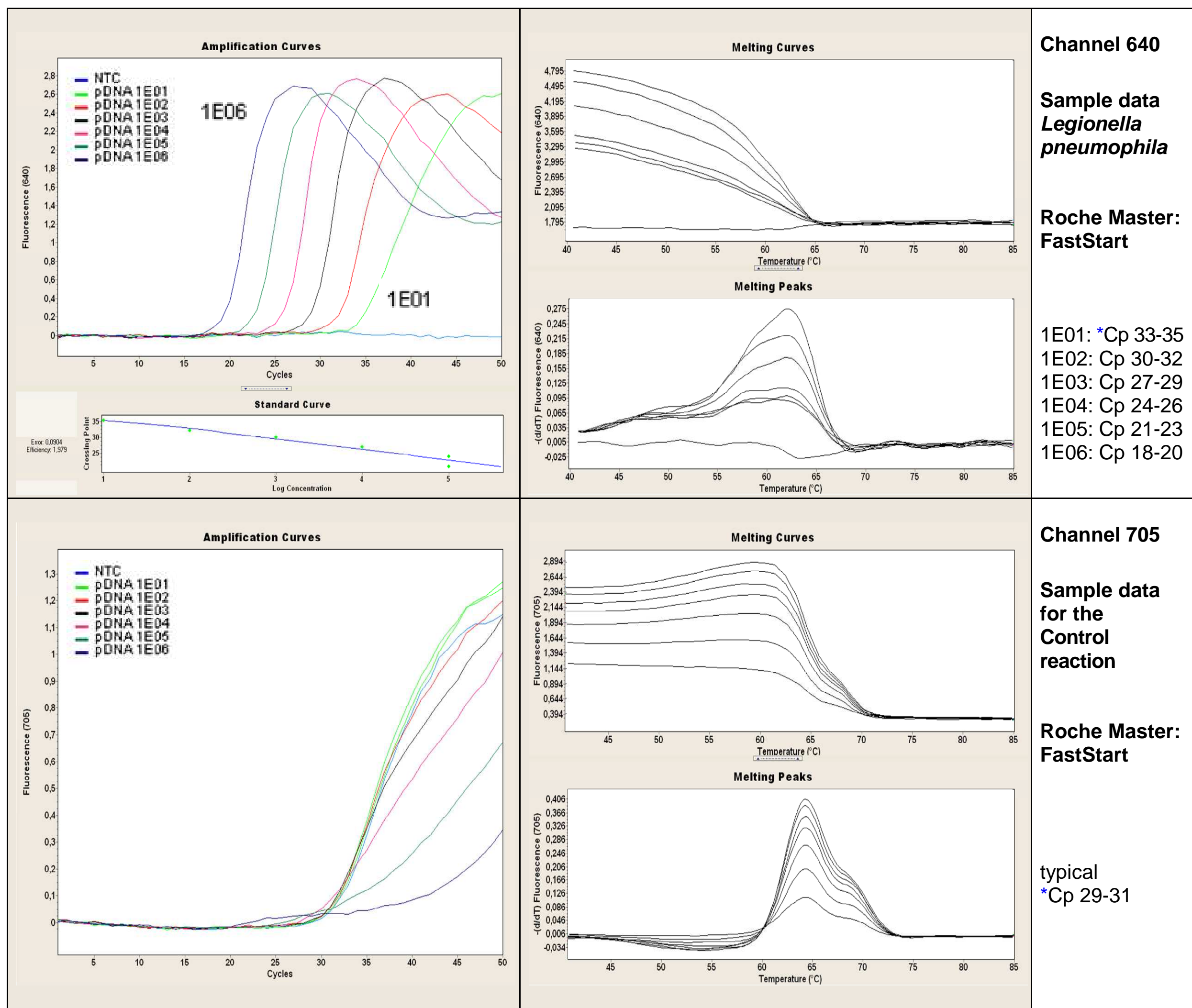


Fig.1. LightCycler® 2.0 sample data for the *Legionella pneumophila* detection system.

Upper panels: Left panel channel 640 quantification mode (Sec. Der. Maximum) with amplification curves for *L. pneumophila.* Right panel channel 640 melting analysis for *L. pneumophila* (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 space 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 shifted horizontally.

7.4. Interpretation of Data

Sample 640 *L. pneumophila*	Sample 705 Ctrl. Reaction	Channel 640 PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37 [+]	not relevant	amplification	negative	**Positive for *L. pneumophila***
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)

[+] 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	00:02:00	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 '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 *L. pneumophila* data with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *L. pneumophila* 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 *L. pneumophila* DNA samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycle 30.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *L. pneumophila* should have Cp values between cycles 18 and 35.

8.3. Sample Data – Typical Results

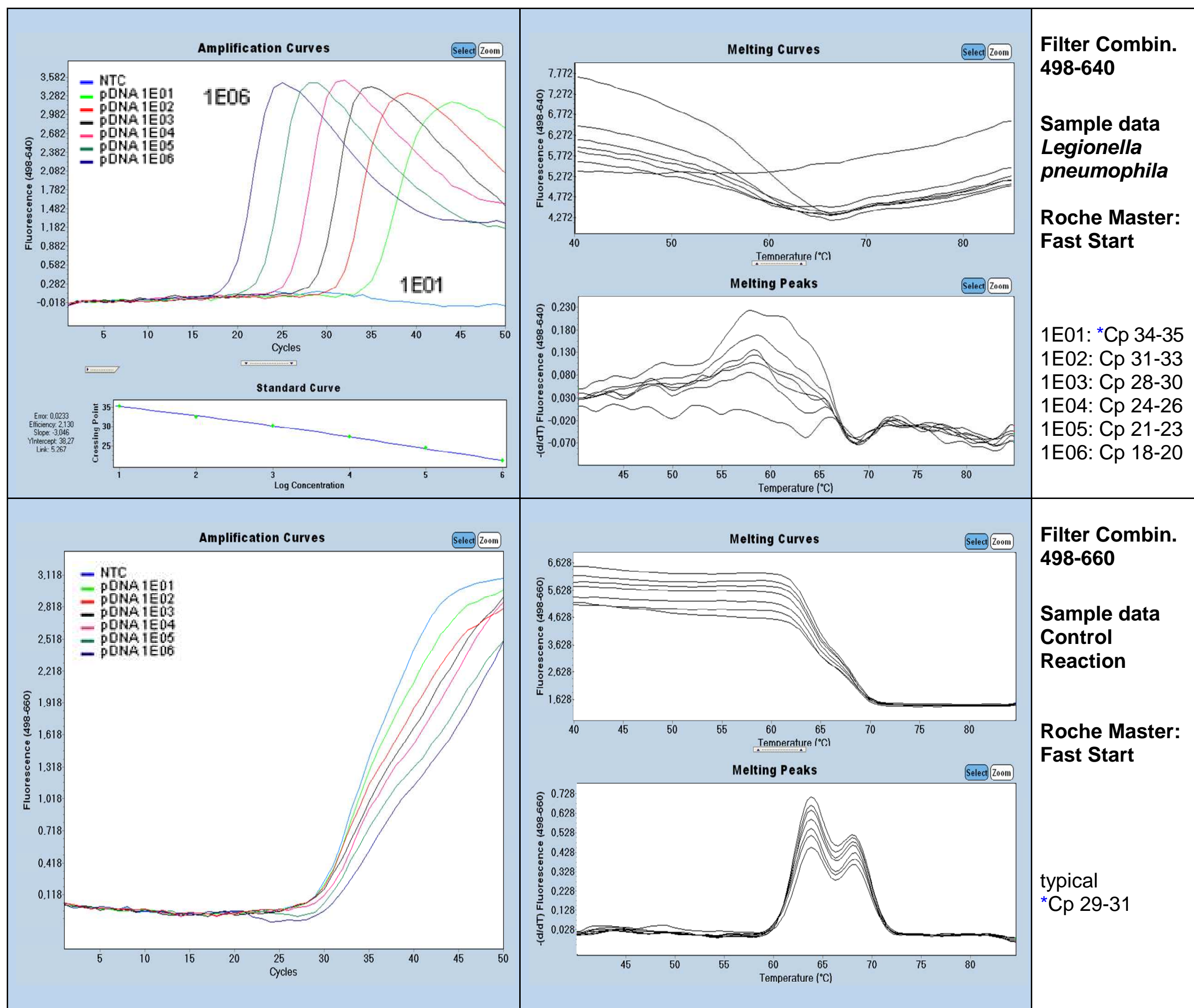


Fig.2. LightCycler® 480 II sample data for the *Legionella pneumophila* detection system.

Upper panels: Left: Filter Comb. 498-640 quantification mode (Sec. Der. Max.) with amplification curves for *L. pneumophila.* Right panel Filter Combination 498-640 melting analysis/peaks for *L. pneumophila* (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 space 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 shifted horizontally.

8.4. Interpretation of Data

Sample 640 *L. pneumophila*	Sample 705 Ctrl. Reaction	Channel 640 PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37 +	not relevant	amplification	negative	**Positive for *L. pneumophila***
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® 2.0 Instrument, Roche Master: Fast Start)

+ 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. Evaluation Study Results - Specificity (Inclusivity and Exclusivity)

Legionella pneumophila serogroups 1-15 have been verified to be detectable, using DNA obtained from defined ATCC *L. pneumophila* strains; the detection limit is 10 genome copies.

L.anisa, *L.birminhamensis, L.bozmanii* sg1+2, *L.cherri, L.cinncinnatiensis, L.dumoffii, L.erythra, L.gormani, L.hackeliae, L.hordanis, L.lansingensis, L.longbeachae* sg1+2, *L.maceachernii, L.micadei, L.oakridgensis, L.parisiensis, L.sainthelenis* sg1+2, *L.tusconensis,* and *L.wadsworthii* were tested to be PCR-negative, using about 10,000 genome copies.

Also *Aeromonas hydrophila, Alcaligenes faecalis, Bacillus subtitlis, Burkholderia cepacia, Clostridium perfringens, Enterobacter aerogenes, E.coli, Flavobacterium ceti, Klebsiella oxytoca, K. pneumoniae, Mucor mucedo, Proeteo vulgaris, P. hauersi, Pseudomonas aeroginosa, P. putida, Serratia marcescens, Stenotrophomonas maltophilia* and *Xanthomonas campestris* were tested negative.

This study was performed in accordance to the norm: AFNOR NF T 90-471:2010.

10. References

[1] Direct detection and differentiation of Legionella spp. and L. pneumophila in clinical specimens by dual-color real-time PCR and melting curve analysis. Reischl U, Linde HJ, Lehn N, Landt O, Barratt K, Wellinghausen N. JCM 40 (2002) 3814-7

[2] Direct detection of Legionella species from bronchoalveolar lavage and open lung biopsy specimens: comparison of LightCycler PCR, in situ hybridization, direct fluorescence antigen detection, and culture. Hayden RT, Uhl JR, Qian X, Hopkins MK, Aubry MC, Limper AH, Lloyd RV, Cockerill FR. JCM 39 (2001) 2618-2626

11. 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.

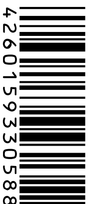
12. Version History

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

V060707	Release version
V080903	Change to the Universal PCR Program
V081020	Insertion of section: Interpretation of data
V091019	Instructions for the use of LC480 II Instruments
V100818	Editorial changes
V121010	Improved IC (primer/probe and target changed)
V140707	cobas z 480 analyzer included, Section 9 MSD included
V150202	Internal Control (IC) changed to Extraction Control (EC) Kit changed from 6 x 16 rxns to 3 x 32 rxns (remains 96 reactions total) Section 9 with Evaluation data inserted.Section 10 References added Note: MagNA pure Compact may fail to recover the sEC extraction target
V150808	Change to universal ^{n}ECT target containing Lambda and PhHV DNA

Roche SAP order n° 05997763001

Notice to Purchaser



LightCycler® hybridization probes and kits produced under license agreements with Roche. The purchase price of this product includes a limited, nontransferable license under US patent claims and corresponding patent claims outside the United States, licensed from BioFire Defense, to use only this amount of the product for HybProbe assays and related processes described in said patents solely for the research and development activities of the purchaser. Diagnostic uses require a separate license from Roche.

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