LightMix® Kit *Legionella spp. / pn.* 16S RNA (EC)

Cat.-No. 40-0638-32

Kit with reagents for the detection of *Legionella* genomic DNA using 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 genus *Legionella*, family *Legionellaceae*, includes more than 40 different species of fastidious gram-negative bacilli, with over 60 described serogroups (2, 21, 41, 60). While these organisms represent normal environmental flora, many have been shown to cause human disease, most commonly opportunistic pneumonia especially in immunocompromised patients. The vast majority of such cases (approximately 85%) are due to *L. pneumophila*, with a substantial minority due to other species, in particular *L. micdadei, L. bozemanii, L. dumoffii*, and *L. longbeachae.*

L. pneumophila can be nosocomial or community-acquired and occurs sporadic as well as epidemic. Pulmonary infection may be subclinical or can be severe and life threatening; in immunocompromised patients the fatality rate can approach 50% [1].

Preferred targets for PCR detection of *Legionella* is the ribosomal 16S RNA gene. For a specific detection of *L. pneumophila* we recommend to use LightMix® Kit 40-0207 targeting the MIP gene.

2. Description

This kit provides a fast and accurate system to detect and identify different *Legionella* species in a nucleic acid extract A 391 bp long fragment of the 16S RNA gene is amplified with specific primers. The resulting PCR fragment is analyzed with two sets of LightCycler® Red 640 labeled hybridization probes specifc for the genus and for a variable region, allowing to identify *L. pneumophila*.

The control reaction generates a 123 bp fragment from the PhHV target, detected with LightCycler® Red 690 labeled hybridization probes. This 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).
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 DNA target even gets lost; the amount of ECT might has to be adapted to the extraction method.

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 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 **green** cap containing premixed primers and probes for each 32 PCR reactions

3 Vials with **white** cap containing premixed primers and probes for each 32 control reactions

1 Standard row with 6 lyophilized standards *Legionella pn.* with 10^1-10^6 target equivalents per rxn
1 Sealing foil for the standard row

1 Vial with **colorless** cap Positive Control *L. dumoffii.* DNA with 1,000 target equivalents / rxn

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 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 (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 4.10 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
Reagents detect 10 copies/reaction 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 *Legionella* 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℃). 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 of *Legionella.*
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 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; the amount may has to be adapted to the extraction method to get a Cp value in the range of 28-32. 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 Positive Control DNA

Add 160 µl of **NTC** to dissolve the vial with the **colorless** cap to run up to 32 pos. control reactions. For the IC procedure use water instead. Mix the DNA by pipetting the solution up and down 10 times.

►Use 5 µl Positive Control DNA for a 20 µl PCR reaction.

Please note that opening of these vials may cause contaminations of the work-space (aerosol).

6.7. 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	Roche FastStart HybProbe Master	Option: IC
1 reaction	**Component**	**1 reaction**
7.4 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.9 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.6 µl
2.0 µl	**PSR** mix (parameter specific reagents, see 6.1)	2.0 µl
2.0 µl	Primers and probe mix for the **EC/IC**	2.0 µl
---	**ECT** (white cap,DNA control target see 6.2)	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

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	00:00:10	00:00:10	00:00:15	00:00:30	00:00:30	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

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 *Legionella* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

The provided Positive Control of cloned and purified DNA with a concentration of 10^3 copies/rxn of *Legionella dumoffii* should have a Cp value between cycles 28 and 30 (Cp values calculated with Second Derivative Maximum method).

For the Control Reaction view channel 705 data, Quantification mode. The negative control and the low-concentrated *Legionella* DNA samples (10 to 1,000 copies) should show an amplification curve for the control reaction with a Cp value approximately at cycle 32.

The provided standard row of cloned DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Legionella* should have Cp values between cycles 18 and 37.

7.3. Sample Data – Typical results

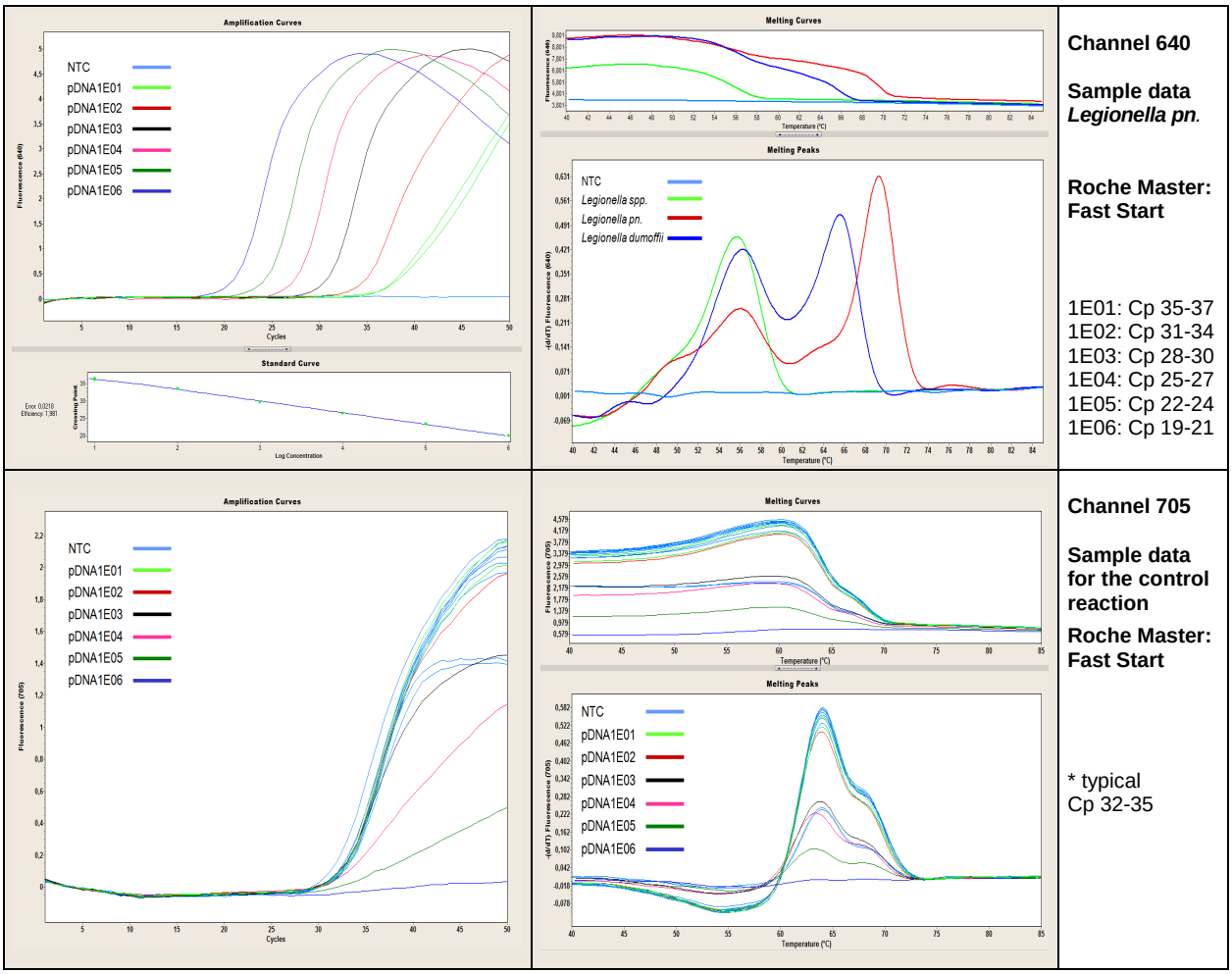

Fig.1. LightCycler® 2.0 sample data for the *Legionella* .detection system.

Upper panels: Left panel channel 640 quantification mode (Sec. Derivative Maximum) with amplification curves for *Legionella.* Right panel channel 640 melting analysis. The melting peak at about 69.7℃ is a proof for the presence of *L. pneumophila*. Other *Legionella* species show no peak or display a peak at 65℃ or lower but the temperature cannot be used for identification. The lower peak at about 56℃ is present for all *Legionellae*.
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, trend of fluorescence levels) should be similar to the curve shown. The fluorescent curves over cycles (quantification mode) must be smooth and not zig-zag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps is relative constant (delta Cp). Cp values described in this manual (chart text) have been obtained with the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of Data

Sample 640 *Legionella*	Melting Tm	Sample 705 *Ctrl Reaction*	Channel 640 *Pos. Control*	Channel 640 *NTC*	*Result (warnings)*
no amplification	**-**	detectable	amplification	negative	**Negative (not detectable)**
Cp < 38[+]	≈ 70℃ + 56℃	not relevant	amplification	negative	**Pos. for *Legionella pn.***
Cp < 38[+]	< 67℃ + 56℃	not relevant	amplification	negative	**Pos. for other *Legionella***
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

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: 565-510, 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	00:00:10	00:00:10	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***	**-**

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.

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

If the control reaction is used view data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Legionella* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 32.

The provided Positive Control of cloned DNA with an amount of 10^4 copies/rxn of *Legionella* should have a Cp value between cycles 26 and 28 (calculated with Second Derivative Maximum method).

The provided standard row of cloned DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Legionella* should have Cp values between cycles 18 and 37.

8.3. Sample Data – Typical Results

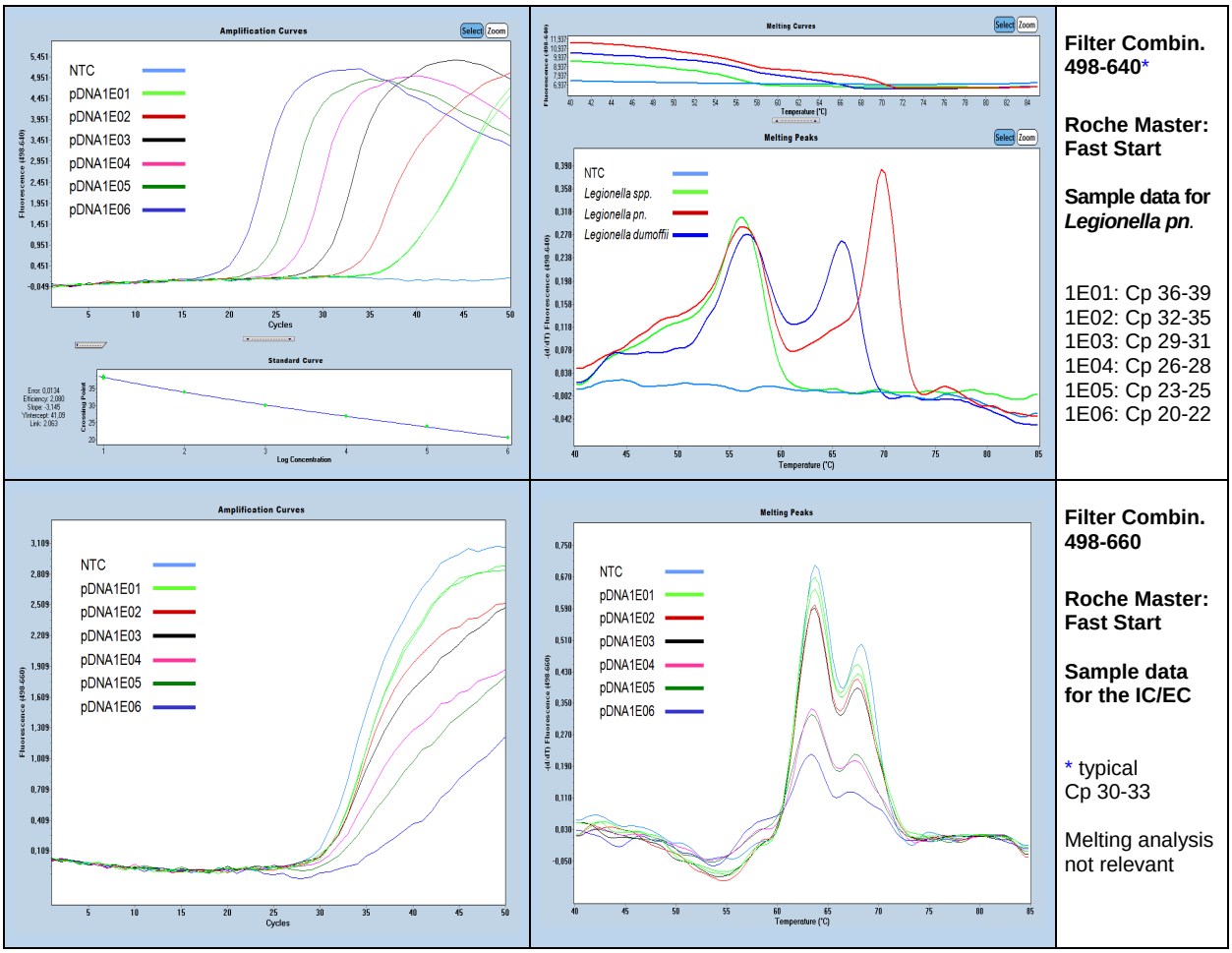

Fig.2. LightCycler® 480 II Sample data for the *Legionella* .detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Sec. Der. Max.) with amplification curves *Legionella.* Right panel channel 640 melting analysis. The melting peak at about 70℃ is a proof for the presence o f *L. pneumophila*. Other *Legionella* species show no peak or display a peak at 65℃ or lower; the respective Tm values cannot be used for identification of the species. The lower peak at about 56℃ is pre sent for all *Legionellae*.
Lower panels: Left panel Filter Combination 498-660 quantification mode (Second 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 zig-zag 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 *Legionella*	Melting Tm	Sample 660 *Crtl. Reaction*	Channel 640 *Pos. Control*	Channel 640 *NTC*	*Result (warnings)*
no amplification	**-**	detectable	amplification	negative	**Negative (not detectable)**
Cp < 40⁺	≈ 70℃ + 56℃	not relevant	amplification	negative	**Pos. for *Legionella pn.***
Cp < 40⁺	< 67℃ + 56℃	not relevant	amplification	negative	**Pos. for other *Legionella***
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

Table 5. Typical analysis results (LightCycler® 480 II 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.

10. Evaluation Data

Specificity: This kit has been tested running 40 different *Legionella* isolates in duplicate; all species except *L. maceachernii*, *L. micdadei* and *L. feeleii* showed a channel 640 melting peak of 56.2-56.8°C.

L. pneumophila serogroup 1 - 14 and 15 (subspecies *fraseri*) had a second peak at about 69.3°C, *L.anisa, L. bozemanii* sg 1 and 2, *L.cherii*, *L. cincinnatiensis*, *L. dumoffii*, *L. gormani*, *L. longbeachae* sg 1 and 2, *L. longbeachae*, and *L. parisiensis* had the second peak at about 65.5°C, *L. oakridgensis* at 63°C, *L. wadsworthii* at 62.5°C, *L. lansingensis*, *L. sainthelensi* sg 1/2 and *L. tucsonensis* at 60.8°C while *L. birminghamensis* and *L. erythra* sg 2 display the second peak at lower temperature of 51.5°C. *L. hackeliae* sg 1 and 2, and *L. jordanis* miss a second melting peak and have only the 56.5°C peak. *L. micdadei* has a single peak at 54.1°C, *L. feeleii* sg1 and 2 at 53.3°C, and *L. maceachernii* at 52.4°C.

Average Cp values for 430 fg of genomic DNA was 29-30, for some species up to 39.

11. References

[1] 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 et al., JCM, (2001)

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

Events requiring changes in procedures red, mod. sequences blue

V130813	Release Version Changes compared to kit version 40-0189 or 40-0271 : Internal control reaction changed to extraction control
V140606	Editorial changes, section 10 inserted
V140714	Correction in 6.7 Preparation of Reaction Mix, 2 µl
V150505	Change to universal ^{n}ECT target containing Lambda and PhHV DNA

Roche SAP order n° 07192550001

4260159330588