LightMix® Kit for the detection of *Coxiella burnetii*

Cat.-No. 40-0316-32 **New Version 05/2011 32rxn/vial**

Kit with reagents for the detection of *Coxiella burnetii* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments.

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 Instrument see pages 6-7

1. Introduction

Q fever is a disease caused by the bacteria *Coxiella burnetii*. Farm animals, predominantly cattle, sheep and goats are the primary reservoirs of *C. burnetii*. Infections of humans usually occur by inhalation of airborne bacteria during handling and processing of animals. Tick bites and human to human transmission of the disease are rare. As an agent of biological warfare (BW), Q fever is an incapacitating agent. Symptoms appear about 10-20 days after *Coxiella* are inhaled and normally last for 2 days to 2 weeks at which time the disease resolves without permanent effects on the individual. The symptoms resemble flu symptoms and include fever, chills, headache, fatigue and muscle aches.

Detection of *C. burnetii* infections is based on serology or PCR methods. Preferred PCR targets are the superoxide dismutase (SOD)[1], the outer membrane 27 kDa protein (com1)[2] or the multicopy transposase (IS1111a)[3] gene. The *C. burnetii* reference strain 'Ninemiles' contains 20-116 copies of this gene (according to different references).

The LightMix® Kit *Coxiella burnetii* detects two genes and provides a fast and accurate system to identify this target in a nucleic acid extract. A control amplification reaction acts as internal control (IC).

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'

[1] Detection of Coxiella burnetti by DNA amplification using polymerase chain reaction. Stein A, Raoult D. J Clin Microbiol. 1992 Sep;30(9):2462-6

[2] Clinical evaluation of a new PCR assay for detection of Coxiella burnetii in human serum samples. Zhang GQ, Nguyen SV, To H, Ogawa M, Hotta A, Yamaguchi T, Kim HJ, Fukushi H, Hirai K. J Clin Microbiol. 1998 Jan;36(1):77-80

[3] Rapid and Specific Detection of Coxiella burnetii by LightCycler PCR. Stemmler M. and Meyer H. Rapid Cycle Real-Time PCR (2001) 149-154

2. Description

A 124 bp fragment (com1) and a 290 bp fragment from the repetitive element IS1111a (Transposase) of the *Coxiella burnetii* genome are amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). The gene specific products are identified by running a melting curve with specific melting points (Tm) of 58.5°C (com1) and 66.0°C (Transposase).

An additional PCR product of 300 bp is formed from the internal positive control DNA (IC). This control will not interfere with the *C. burnetii* specific reactions. The amplification will usually fail in the presence of higher concentrated *C. burnetii* DNA samples (1,000 - 10,000 copies or higher) but it will display an amplification signal in negative and low-concentrated samples. The probes are labeled with the dye LC690. Detection is recorded in channel 705; the specific Tm is in the range of 67-69°C. The IC is supplied separately to allow running the assay with or without IC.

The use of a color compensation file generated with TIB MOLBIOL 'LightMix® Kit - Color Compensation 530/640/690 or the Roche Diagnostics 'LightCycler®-Color Compensation Set' or with the Roche Diagnostics 'LightCycler® Multicolor Demo Set' 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.

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.

3. Set contents

3 Vials with green caps containing premixed lyophilized primers and probes for 32 PCR reactions each of *Coxiella burnetii*
3 Vials with white caps containing the internal control (IC)
1 Standard row with 6 lyophilized cloned plasmid standards of *Coxiella burnetii* from 10^1 to 10^6 target equivalents per reaction
1 Sealing foil for the standard row

4. Additional reagents and items required

TIB MOLBIOL:

LightMix® Kit – Color Compensation 530/640/690	Cat.-No. 40-0318-00

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854 001
or LightCycler® Color Compensation Set (LightCycler® 1.x Instrument)	Cat.-No. 12 158 850 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
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

5. Product characteristics

PCR results are obtained within 50 minutes (55 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (55 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Coxiella burnetii* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments (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 *Coxiella burnetii* genomic DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 II Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment when stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days when stored protected from light and refrigerated (4°C).

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 II Instruments. Start programming before preparing the solutions. See the LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure PCR Template Preparation Kit').

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA. For quantification always use a fresh solution prepared from the provided standard row (single use only).

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

One reagent vial with a **green** cap contains primers and probes to run 32 LightCycler® reactions for *Coxiella burnetii.*
One reagent vial with a **white** cap contains primers, probes and DNA to run 32 reactions for the IC.

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 five days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. 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** PCR-grade water to each vial of the row. 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. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control). After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.3. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
7.4 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
2.0 µl	**IC** mix (IC reagents containing primers, probes and DNA, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

To include the internal control **add 2 µl** of the IC reagent per reaction to the reaction mix.
To run the assay without the internal control substitute the 2 µl of IC with 2 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instrument) or to a multiwell plate (LightCycler® 480 II Instrument).

Add 5 µl of sample or standard DNA to each capillary or well for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 1.x / 2.0 Instrument

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	55			1			1
Target [°C]	95	95	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	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
Acquisition Mode	None	None	Single	None	None	None	Continuous	None

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 TIB MOLBIOL 'LightMix® Kit – Color Compensation 530/640/690 or the Roche Diagnostics 'LightCycler® – Color Compensation Kit' (LightCycler® 1.x Instrument) / 'LightCycler® Multicolor Demo Set'(LightCycler® 2.0 Instrument).

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

If the internal control (IC) is used, view IC data in channel 705, Quantification mode. The negative control and the low-concentrated *Coxiella burnetii* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 31-33.

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 *Coxiella burnetii* should have CPs between cycles 17 and 35 (CPs calculated with Second Derivative Maximum method).

7.3. Sample Data – typical results

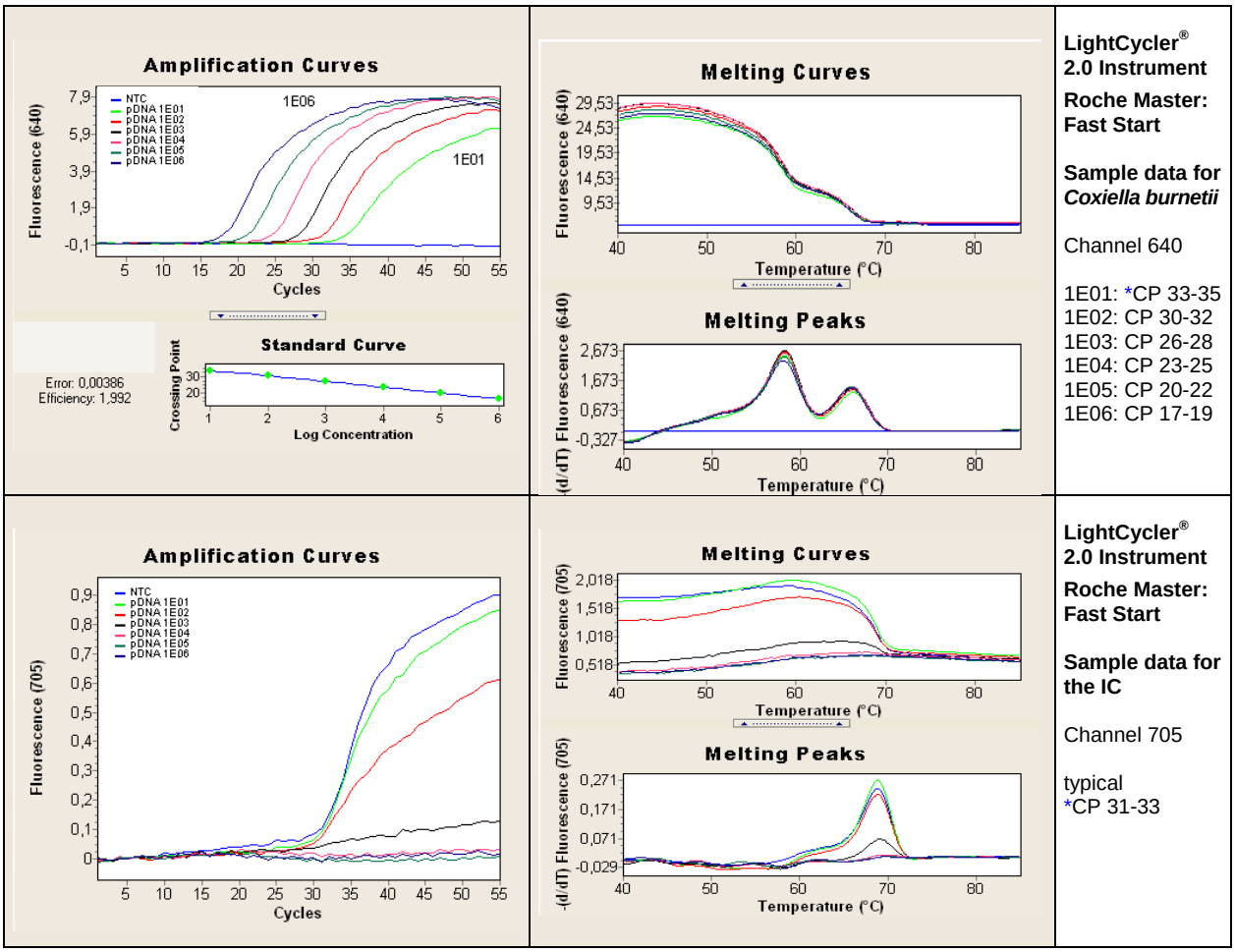

Fig.1. Sample data for the *Coxiella burnetii* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *Coxiella burnetii* . Right panel channel 640 melting analysis for *Coxiella burnetii* (not relevant for detection).
Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 705 quantification mode (Second Derivative Maximum) for the IC. Right panel channel 705 melting analysis for the IC.

* **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

Coxiella burnetii **(sample)**	**IC (sample)**	**NTC (non target sample)**	**Result**
no amplification	detectable	negative	Negative
amplification signal	not relevant	negative	Positive
no amplification	not detectable	not relevant	PCR failure, repeat experiment
amplification signal	not relevant	positive	Contamination, repeat experiment

Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

Notes*:* The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. Samples with deviating melting curves should be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

8. LightCycler® 480 II Instrument

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

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	55	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:08	00:00:15	00:00:20	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]	-	-	-	-	-	-	**3**	-

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 TIB MOLBIOL 'LightMix® Kit – Color Compensation 530/640/690 or the Roche Diagnostics 'LightCycler® Multicolor Demo Set'.

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

If the internal control is used, view data with Filter Combination 498-660, Quantification mode, and the IC with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *Coxiella burnetii* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 31-33.

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 *Coxiella burnetii* should have CPs between cycles 17 and 35 (CPs calculated with Second Derivative Maximum method).

8.3. Sample Data – typical results

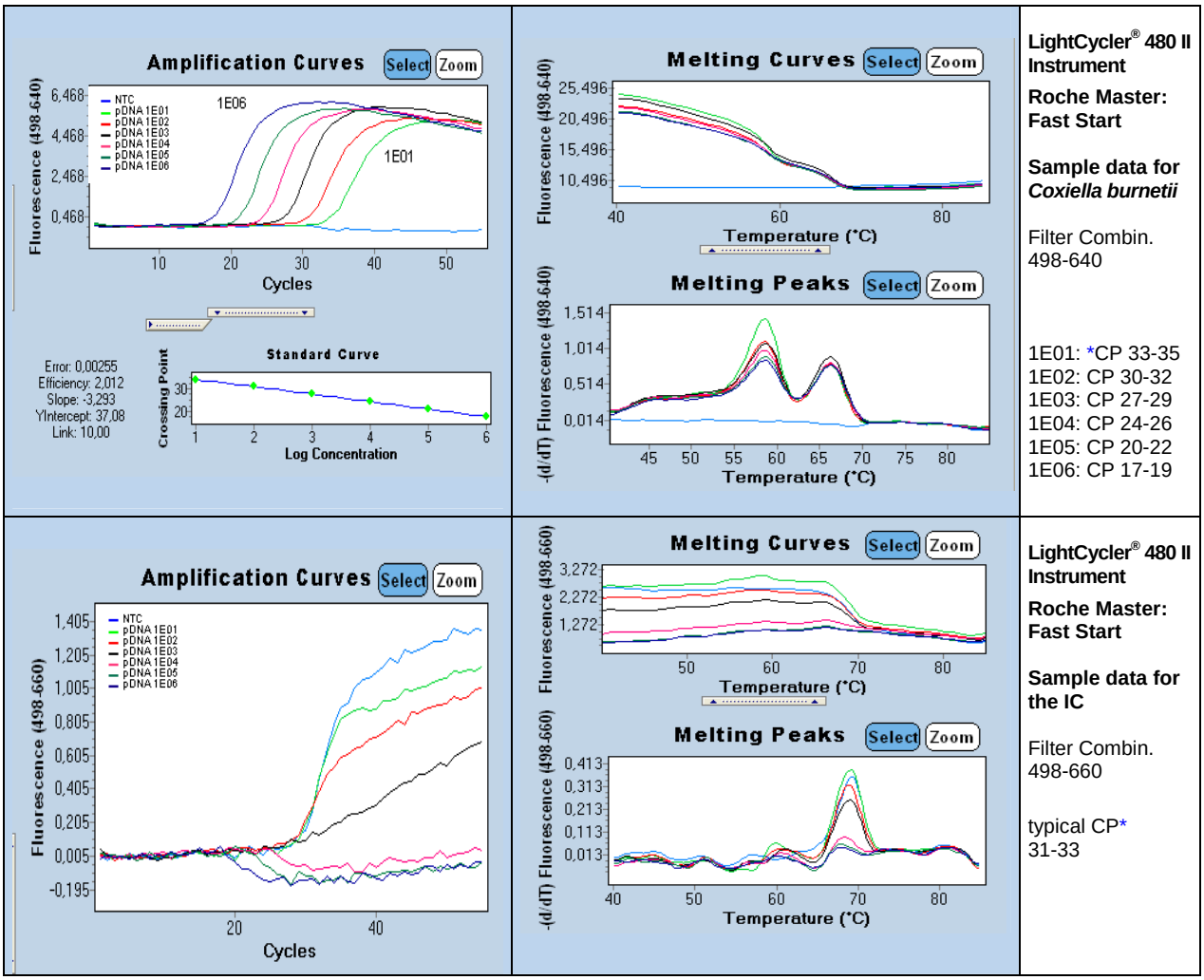

Fig.1. Sample data for the *Coxiella burnetii* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *Coxiella burnetii*. Right panel Filter Combination 483-640 melting analysis for *Coxiella burnetii* .
Lower panels: Data from LightCycler® 480 Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the IC. Right panel Filter Combination 498-660 melting analysis for the IC.

* **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

Coxiella burnetii (sample)	IC (sample)	NTC (non target sample)	Result
no amplification	detectable	negative	Negative
amplification signal	not relevant	negative	Positive
no amplification	not detectable	not relevant	PCR failure, repeat
amplification signal	not relevant	positive	Contamination, repeat

Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments. Samples with deviating melting curves should be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact: service@tib-molbiol.de)

9. Version history

V_110509	Change to 32rxn/vial

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
LightCycler® hybridization probes produced under license from Roche Diagnostics GmbH.