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TIB MOLBIOL

LightMix® for the detection of *Coxiella burnetii*

Cat.-No. 40-0316-16

Reagents for the quantitative detection of *Coxiella burnetii* DNA using the LightCycler® 1.x / 2.0 Instruments.

Lyophilized mix of primers and probes (6 tubes with 16 rxns each) 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!**

Additional reagents required

Roche Diagnostics:

LightCycler® FastStart DNA Master^{PLUS} HybProbe	Cat.-No. 03 515 575 001
or LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Color Compensation Set (LightCycler® 1.x Instrument)	Cat.-No. 12 158 850 001
or LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

1. Introduction

Q fever is a disease normally found in sheep, cattle goats and ticks. It is caused by Coxiella burnetii, which becomes airborne during handling and processing of animals and which can also infect humans. 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.

The LightMix® kit for the detection of DNA from *Coxiella burnetii* provides a fast, easy and accurate system to identify and quantify this target in a nucleic acid extract. A control amplification reaction acts as internal positive control (IPC).

This LightMix® kit is tested with the Roche Diagnostics “LightCycler® FastStart DNA Master Hybridization Probes” ready–to-use reaction mix in the LightCycler® Instrument 2.0.

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 PCR product is identified by running a melting curve with specific melting points (Tm) of 58,5°C (com1) and 66,0°C (Transposase) in channel 640.

An additional PCR product of 349 bp is formed from the internal positive control DNA. This control will not interfere with the *Coxiella burnetii* specific reactions. The amplification will usually fail in the presence of higher concentrated *Coxiella 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 LC705. Detection is recorded in channel 705; the specific Tm is about 67-69°C. The IPC DNA is supplied separately to allow running the assay with or without IPC.

The use of a color compensation file generated with the LightCycler®-Color Compensation Kit is a prerequisite to run the internal control.

The supplied standard row allows the absolute quantification of the unknown samples.

For use in LightCycler® 1.x Instruments use channel F2 instead of channel 640 and channel F3 instead of channel 705 for detection.

3. Set contents

6 Vials with green cap containing premixed and lyophilized primers and hybridization probes for 16 reactions each
6 Vials with white cap containing the internal positive control (IPC)
1 Row with 6 lyophilized standards from 10^1 to 10^6 target equivalents per reaction of *Coxiella burnetii* DNA
1 Sealing foil for the standard row

4. Programming

The protocol consists of four program steps

• 1: Denaturation: samples 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:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	55			1			1
Segment	1	1	2	3	1	2	3	1
Target [℃]	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 [℃/s]	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	Single	None	None	None	Continu.	None

5. 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 LightCycler® – Color Compensation Kit.

Perform data analysis, as described in the LightCycler® operator's manual.

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

If the internal positive control is used, view *Coxiella burnetii* data in channel 640, Quantification mode and the IPC in channel 705, Quantification mode. The negative control and the low-concentrated *Coxiella burnetii* samples (10 to 1,000 copies) should show an amplification curve for the IPC with a CP approximately at cycle 30.

Typical results (Software Version 4.0)
The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn should have CPs between cycles 17 and 33 (CPs calculated with Second Derivative Maximum method).

6. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 10 copies of *Coxiella burnetii* DNA (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* DNA.

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

7. Experimental protocol

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

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

Negative control: Always run at least one negative control - 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).

7.1 Preparation of parameter-specific reagents and reagents for the IPC (16 reactions):

One reagent vial with a **green** cap contains all primers and probes to run 16 LightCycler® reactions for *Coxiella burnetii.*
One reagent vial with a **white** cap contains all primers, probes and DNA to run 16 LightCycler® reactions for the IPC.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►Use 4 µl **reagent** for a 20 µl PCR reaction.

This solution is stable for three days or longer if stored refrigerated at 4℃. Avoid prolonged exposu re to light.

7.2 Preparation of the standard row (quantification)

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resuspended. 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 for a 20 µl PCR reaction.

This standard solution is not long-term stable and will loose 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 reopening of these vials may cause contaminations of the work-space (aerosol).

7.3 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^{PLUS} kit		**For use with the Roche FastStart kit**
Single reaction	**Component**	**Single reaction**
3.0 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart or FastStart^{PLUS} kit)	3.4 µl
--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.6 µl
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **7.1**)	4.0 µl
4.0 µl	**IPC** mix (IPC reagents containing primers, probes and DNA, see **7.1**)	4.0 µl
4.0 µl	FastStart mix (vial 1 (red cap), combined from vials 1a and 1b, see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

To include the internal positive control add 4 µl of the IPC reagent per reaction to the reaction mix.
To run the assay without the internal control add additional 4 µl PCR-grade water instead of the IPC reagent to the reaction mix.

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary.
Add 5 µl of sample or standard (standard dilutions of control target, see instruction **7.2**) to each capillary to give a final reaction volume of **20 µl.**

Start run.

8. Sample data - typical results

Sample data for *Coxiella burnetii*

Channel 640

Sample data for the internal positive control

Channel 705

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

Upper panels: Data from channel 640. Left panel quantification (Second Derivative Maximum) with calibration curve. Right panel melting curves for the target (com1 low Tm peak, Transposase high Tm peak).
Lower panels: Data from channel 705. Left panel quantification mode, right panel melting analysis for the IPC.

Note: The values of the respective melting temperatures (T_M) may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ± 1.5°C.

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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.