LightMix® for the detection of *Francisella tularensis*

Cat.-No. 40-0250-16

Reagents for the quantitative detection of *Francisella tularensis* DNA using the LightCycler® Instrument 1.x / 2.0.

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

Respiratory tularemia is a fulminant disease caused by *Francisella tularensis*, a virulent, facultative intracellular, gram-negative bacterium. From the two main species type A (*F. tularensis* biovar tularensis) is the more important one - the mortality rate is about 10% (without antibiotic treatment), compared to 1% for the type B (*F. tularensis* biovar palaearctica). The bacterium is widely distributed in nature and has been isolated from many wildlife species. Tularemia is acquired by exposure to infected animals, through contaminated water or food, and possibly through biting insects. Airborne infections occur especially during processing of agricultural products. *F. tularensis* has the potential of being used as a biological warfare agent.

The LightMix® for the detection of DNA from *Francisella tularensis* provides a fast, easy and accurate system to identify and quantify this microorganism.

This LightMix®-System 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

This LightMix® detects parts of the *Francisella tularensis* genome indicating the presence of *Francisella tularensis* DNA in a nucleic acid extract. A control amplification reaction acts as internal positive control (IPC).

A 185 bp fragment of the *Francisella tularensis* genome is 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 a specific melting point (Tm) of 61.5°C in channel 640.

An additional PCR product of 278 bp is formed from the internal positive control DNA. This control will not interfere with the *Francisella tularensis* specific reactions. The amplification will usually fail in the presence of higher concentrated *Francisella tularensis* 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 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® Instruments other than 2.0 use channel F2 instead of channel 640 and channel F3 instead of channel 705 for detection.

3. Set contents

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

4. Programming

The protocol consists of four program steps

• Program 1: Denaturation of sample and activation of the enzyme
• Program 2: PCR-amplification of the target DNA
• Program 3: Melting curve for identification of the *Francisella tularensis* DNA derived PCR product
• Program 4: Cooling the instrument

When using the Roche FastStart reagents run an initial heating for 10 min at 95°C.

Program:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification			Melting Curves			None
Cycles	1	55			1			1
Segment	1	1	2	3	1	2	3	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	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. 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 user's influences.

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

If the internal positive control is used, view *Francisella tularensis* data in channel 640, Quantification mode and the IPC in channel 705, quantification mode. The negative control and the low-concentrated *Francisella tularensis* DNA 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 18 and 35 (CPs calculated with Second Derivative Maximum method).

6. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 10 copies of *Francisella tularensis* 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 *Francisella tularensis* 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°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4°C).

7. Experimental protocol

The following procedure was developed for use with the LightCycler® Instrument 1.x / 2.0.
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).

(A) Preparation of parameter-specific reagents (16 reactions):

One reagent vial with a **green** clip contains all primers and probes to run 16 LightCycler® reactions for *Francisella tularensis*.
One reagent vial with a **white** clip 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°C. Avoid prolonged exposu re to light.

(B) 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 opening of these vials may cause contaminations of the work-space (aerosol).

(C) Preparation of the LightCycler® reaction mix

In a reaction tube cooled below 4°C, prepare the re action 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)	2.6 µl
--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **A**)	4.0 µl
4.0 µl	**IPC** mix (IPC reagents containing primers, probes and DNA, see **A**)	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 15µl each of the reaction mix to a LightCycler® capillary.
Add 5 µl of sample or standard (standard dilutions of control target, see instruction **B**) to each capillary to give a final reaction volume of **20 µl**, start run.

8. Sample data - typical results

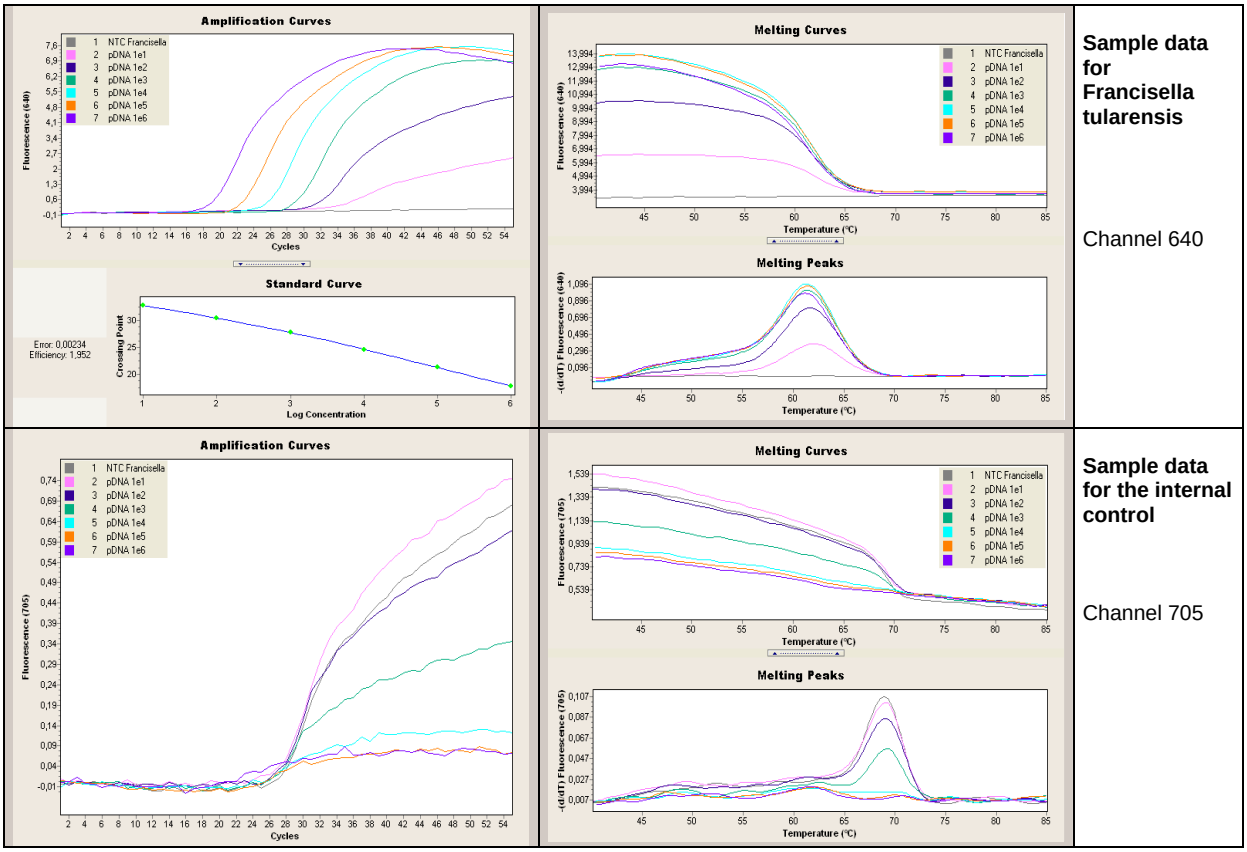

Fig.1. Sample data for the *Francisella tularensis* detection system.

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

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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.