LightMix® Kit *Mycoplasma genitalium (EC)*

Cat.-No. 40-0169-32 2014: Internal Ctrl changed to spiked Extraction Ctrl, 32 rxsn/vial

Kit with reagents for the detection of *Mycoplasma genitalium* 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 Instrument / cobas z 480 Analyzer see pages 6-7

1. Introduction

Mycoplasma are the smallest bacteria known; since they lack a cell wall they are unassailable for the common beta-lactam antibiotics targeting cell wall synthesis. *Mycoplasma genitalium (M. genitalium)* has the smallest known genome that can create a cell. They are parasitic living and can be found on the ciliated epithelial cells of the genital and respiratory tracts, causing discharge, burning while urinating, urethritis in men, and vaginal itching, pain during intercourse and vaginosis in women. Long-term infection is suspected to cause pelvic inflammatory disease.

Preferred targets for the genome-based detection of *M. genitalium* are the 16S RNA [1], MgPa adhesin[2], P115 [3] or the gap genes [4].

2. Description

This kit provides a fast and accurate system to detect and identify *M. genitalium* in a nucleic acid extract. A 224 bp long fragment of the glyceraldehyde-3-phosphate dehydrogenase (gap) gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (channel 640).

The control reaction generates an additional product of 125 bases from the PhHV target, detected with LightCycler® Red 690 labeled hybridization probes. This second PCR has no visible impact on the *M. genitalium* 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 internal control (IC) has been changed to a spiked extraction control (sEC) to monitor a successful extraction and demonstrate the ability to run an amplification reaction (no PCR inhibition). We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. 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.

This kit can be combined with the LightMix® Kit *M. hominis* (40-0139-32); the *Mycoplasma* species in the samples can be identified through the melting points at 67-69°C for *M. genitalium* and 62-64°C for *M. hominis.* A protocol for combining both assays is described in this manual under **6.3 Preparation of the LightCycler® reaction mix**. The internal control used for both kits is identical. Alternatively use LightMix® Kit 40-0460-32 for simultanous detection of *M. genitalium*, *M. hominis* and *Ureaplasma.*

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run 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.

Performance testing has been made with the ‘FastStart DNA Master HybProbe’ only.

3. Set Contents

3	Vials with **green** cap containing lyophilized primers and probes for each 32 PCR reactions
1	Standard row with 6 lyophilized plasmid standards of *M. genitalium* 10^1 - 10^6 target equivalents / rxn
1	Sealing foil for the standard row
3	Vials with **white** cap containing premixed lyophilized primers and probes for 32 reactions EC
1	Vial with **white** cap containing Extraction Control Target (**ECT**): 4.8 x 10^6 copies (total amount)
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
Color Compensation 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 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.

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
These reagents detect 10 copies of *M. genitalium* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (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 *M. genitalium* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

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 (32 reactions PSR):

One reagent vial with a **green** cap contains primers and probes to run 32 reactions of *M. genitalium.*
One reagent vial with a **white** cap contains primers and probes to run 32 reactions of control reaction (EC/IC).

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 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 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 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	Optional: IC	
Single reaction	**Duplex reaction**	**Component**	**Single reaction**	**Duplex reaction**
6.6 µl	4.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.1 µl	4.1 µl
2.4 µl	2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl	2.4 µl
2.0 µl	2.0 µl	**PSR** mix (parameter specific reagents, see 6.1 for *M. genitalium*)	2.0 µl	2.0 µl
-	2.0 µl	**PSR** mix (parameter specific reagents, see 6.1 for *M. hominis*)	-	2.0 µl
2.0 µl	2.0 µl	Primers and probe mix for the **IC/ECT**	2.0 µl	2.0 µl
-	-	**ECT** (white cap, DNA control target, see 6.2.)	0.5 µl	0.5 µl
2.0 µl	2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl	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 [℃]	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

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 Color Compensation Kit HybProbe 530/640/690.

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 *M. genitalium* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view data in channel 640, Melting Curves mode: specific melting points at 67-69℃ for *M. genitalium* (62-64℃ for *M. hominis*, if combined).

If the control reaction is used view data in channel 705, Quantification mode. The negative control and the low-concentrated *M. genitalium* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a Cp at approximately cycles –29-31.

The provided standard row of DNA with concentrations in the range from 10^6 to 10^1 copies/rxn of *M. genitalium* should have Cp values between cycles 19 and 37.

7.3. Interpretation of Data

Sample 640 *M. genitalium*	Sample 705 Control reaction	PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39 [+]	not relevant	amplification	negative	**Positive for *M. genitalium***
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 for 10 copies corresponds to ~5 copies/reaction.

* **Note:** Fluorescence levels may vary also depending on instrument settings. 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 relativly 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 horizontally shifted.

7.4. Sample Data – Typical Results

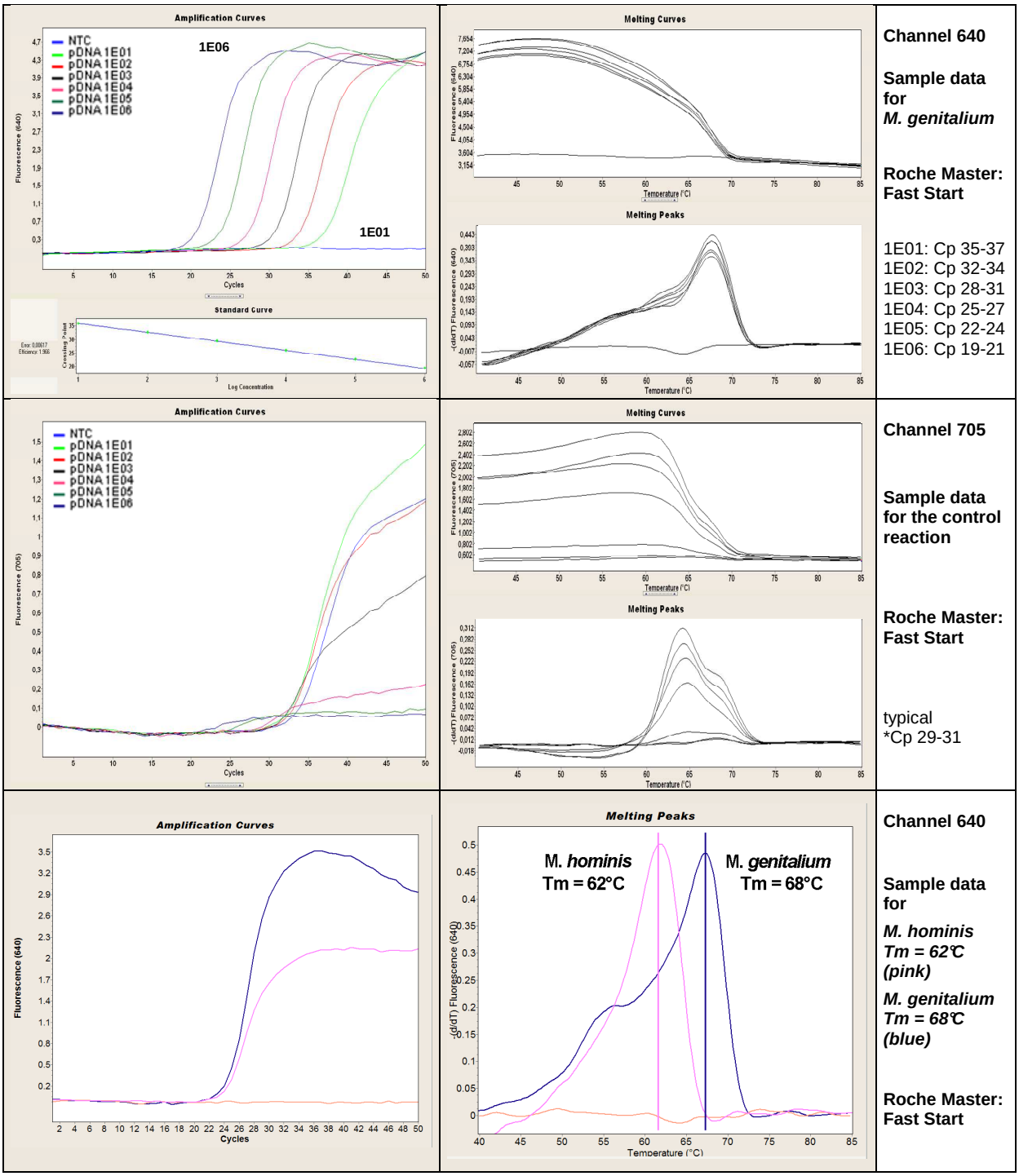

Fig.1. LightCycler® 2.0 sample data for the *M. genitalium* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curves for *M. genitalium.* Right panel channel 640 melting analysis for *M. genitalium* (not relevant for detection).
Central 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).
Lower panels: Left panel channel 640 quantification mode (Second Derivative Maximum) for *M. hominis* and *M. genitalium*. Right panel channel 640 melting analysis for *M. hominis* and *M. genitalium* - identification of the species if combined with LightMix® Kit 40-0139 *M. hominis*.

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	Continuou	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 Color Compensation Kit HybProbe 530/640/690.

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 *M. genitalium* data with Filter Comb. 498-640, Quantification mode. The negative control (NTC) must show no signal. For identification of the PCR product view data with Filter 498-640, Melting Curves mode - melting point at 67-69°C for *M. genitalium* (and 62-64°C for *M. hominis*, if combined).

If the control reaction is used, view data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *M. genitalium* DNA samples (10 to 1.000 copies) should show an amplification curve for the IC/EC with a Cp at approximately cycle 29 - 31.

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

8.3. Interpretation of Data

Sample 640 *M. genitalium*	Sample 660 IC	PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39[+]	not relevant	amplification	negative	**Positive for *M. genitalium***
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® 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 for 10 copies corresponds to ~5 copies/reaction.

8.4. Sample Data – Typical Results

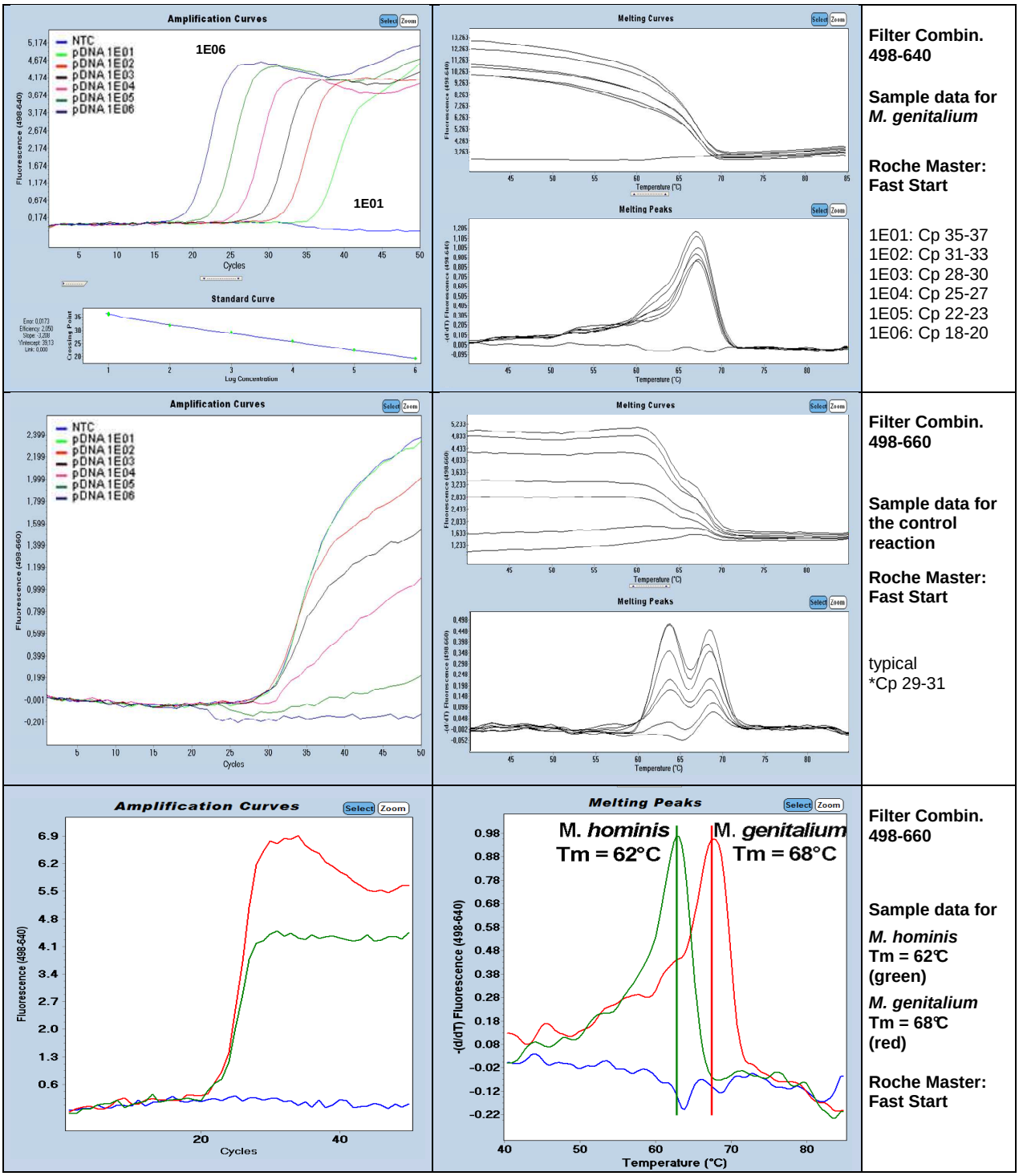

Fig.2. LightCycler® 480 II sample data for the *M. genitalium* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *M. genitalium.* Right panel Filter Combination 498-640 melting analysis for *M. genitalium* (not relevant for detection)
Central 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).
Lower panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) for *M. hominis* and *M. genitalium*. Right panel Filter Combination 498-640 melting analysis of *M. hominis* and *M. genitalium* – identification of the species if combined with LightMix® Kit 40-0139 *M. hominis*.
* **Note:** Fluorescence levels may vary also depending on instrument settings. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual.

9. Evaluation Study Results

The LightMix® kit was compared with the Diagenode M. genitalium RT-PCR kit (DIA-MG-050 vs2) and an in-house published TaqMan assay[2] targeting the MgPa gene using a cobas z 480 instrument, running 54 positive and 50 negative DNA extracts. The sensitivity compared to the in-house test was 92.6% (Diagenode 87.7%) while the specificity was 100% for both commercial kits. Three of the four discrepant samples were missed also by the Diagenode kit. Missed samples had Cp values of 38 and later, which is at the detection limit of the test and outside of the claimed detection limit of 10 copies [5].

10. References

[1] Quantitative detection of M. genitalium from first-pass urine of men with urethritis and asymptomatic men by real-time PCR. Yoshida et al., J Clin Microbiol.40 (2002) 1451-5

[2] Use of TaqMan RT-PCR for Quantitative Detection of M. genitalium DNA in males with and without urethritis who were attendees at a Sexually Transmitted Disease Clinic. Jensen et al., JCM 42 (2004) 683-692

[3] Detection and quantification of M. genitalium in male patients with urethritis. Dupin et al., Clin Infect Dis. 2003 Aug 15;37(4):602-5

[4] Development of a quantitative real-time PCR assay for detection of M. genitalium. Svenstrup et al. J Clin Microbiol. 2005 Jul;43(7):3121-8

[5] Evaluation of Two Commercial Real-Time PCR Assays for Detection of Mycoplasma genitalium in Urogenital Specimens. Le Roy et al., . J Clin Microbiol. 2014 (3) 971-973

11. 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.

12. Version History

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

V120309	Release version
V120313	Correction section 6.3 pipetting volumes
V121005	Data for the use in combination with LightMix Kit 40-0139 M. hominis
V130308	Update section 6.3 pipetting volumes. cobas z 480 analyzer included. Use in combination with LightMix Kit 40-0153-32 *Ureaplasma urealyticum*.
V130813	Editorial changes
V140814	Internal control (IC) reaction changed to spiked extraction control (sEC)
V150101	MagNA pure Compact may fail to recover the sEC extraction target Section 9 with Evaluation data inserted.Section 10 References added.
V150505	Universal Extraction Control target nECT with Lambda and PhHV

Roche SAP order n° 06295096001

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