LightMix® Kit Mycoplasma gen/hom and Ureaplasma

Cat.-No. 40-0460-32

Kit with reagents for the detection of *Mycoplasma genitalium, M. hominis* and *Ureaplasma* genomic DNA 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℃), do NOT freeze!**

1. Introduction

Mycoplasma are the smallest bacteria and have the smallest genome known. Since they lack a cell wall they are unassailable for the common beta-lactam antibiotics targeting cell wall synthesis. Infection with Mycoplasma is suspected to cause pelvic inflammatory disease and even infertility.

M. genitalium 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, and vaginosis in women.

M. hominis is part of the commensal gastrointestinal microbiota, infections of the urogenital tract in adults are common and mostly symptomless.

Ureaplasma urealyticum (*U. urealyticum* and *parvum)* are bacteria of the family Mycoplasmataceae. They are part of the normal genital flora and transmitted horizontally, but also during birth. *Ureaplasma* has been associated with urethritis, infertility, premature birth, chorioamnionitis, and stillbirth.

Infection of premature infants and newborns with *Mycoplasma* or *Ureaplasma* can cause pneumonia, sepsis, meningitis, and branchopulmonary dysplasia.

Mycoplasma and *Ureaplasma* are difficult to culture and PCR is a primary method for detection. Preferred specimens are genital swabs; urine has been reported to be insufficient.

2. Description

The present kit contains three analyte-specific primer and probe sets for *Ureaplasma urealyticum*, *Mycoplasma hominis* and *M. genitalium*; plus target DNA, primer and probe for a control reaction.

M. genitalium 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, identified by melting curve analysis with a specific melting point of about 67-69℃.

M. hominis A 129 bp long fragment of the gap gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 640, identified by melting point of about 59-61℃.

Ureaplasma A 187 bp long fragment of *16S RNA* gene is amplified with specific primers and detected with probes labeled with LightCycler® Red 610, identified by a melting point of about 64-67℃.
This kit detects also *U. urealyticum* Biotype 1 (*U. parvum*).

Roche Instrument	**Pathogen / Melting Temperature / Instrument Channel**				
		Ureaplasma	***Mycoplasma hominis***	***genitalium***	**Control**
Melting curve Tm		**64-67℃**	**59-61℃/ 56℃**	**67-69℃**	not relevant
LightCycler® 2.0		610	640		705
LightCycler® 480 II		498-610	498-640		498-660
Cobas® Z480		498-610	498-645		498-700

Table 1

The PCR reaction is monitored by a 125 bp long fragment detected with LightCycler® Red 690 labeled probes, amplified from the PhHV control target DNA, which is added before extraction (spiked Extraction Control, sEC); alternatively the control target DNA can be added during amplification (Internal Control, IC). Only one of the two procedures must be selected. The control reaction does not interfere with the analytical reaction. The control reaction must be visible in negative and low-concentrated samples while it will fail in the presence of higher amounts of target DNA (1,000 copies/PCR or higher). The extraction control target **nECT** contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the **nECT** provided with the other kit(s).

3. 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 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 target genomic DNA.

Storage and Stability - Do not freeze lyophilized Reagents !
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25℃). Please see the ex piration date on the product label.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4℃).
- Dissolved reagents can be long-term stored frozen at -20℃. Avoid multiple thaw-freeze cycles.

4. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (see **8.1. Additional reagents**).
Remember to spike samples prior extraction with the **nECT** (see **4.1**).

Negative Control: Always include at least one no-template control
- replace the sample extract by the provided **NTC**, as described in **4.3.**

Positive Control: Always include at least one positive control
- replace the sample extract with the provided **Positive Control**, see **4.5.**

4.1. Preparation of the Extraction Control Target (ECT)

Dissolve the **nECT** (white cap) in 1,200 µl PBS (not supplied), mix (vortex) and spin down.

4.1.1 Spiked Extraction Control (sEC) - Recommended Procedure -

Add 10 µl of **nECT** per 200 µl of biological sample and extract into 100 µl to have approximately 1,000 copies control target per reaction.

Remember to adapt the amount of control target according to different sample and extraction volumes.

For the use with the cobas® x 480 Instrument (cobas® 4800) we recommend ot use 20 µl of **nEC** .
For the use with the MagNA Pure compact use the IC procedure (see below).

4.1.2 Use as a Internal Control (IC) - Optional Procedure -

Use 0.5 µl of **nECT** (1,000 copies per reaction) per reaction mix (see **4.6. Reaction mix)**.

4.2. Preparation of PSR and IC mix (PSR):

One reagent vial with the **green** clip cap contains primers / probes for 32 reactions.

One reagent vial with the **white** clip 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** each reagent for a 20 µl PCR reaction.

| This solution is stable at least ten days when stored refrigerated at 4℃. Avoid prolonged exposure to light. See section 3.

4.3. Preparation of the Negative Control

Fill the empty vial with the **black** cap **NTC** with 950 µl of PCR-grade water.
Only when the spiked Extraction Control **(sEC)** procedure is selected (see **4.1**), add 50 µl of **nECT**.

►Use 5 µl **NTC** control DNA for a 20 µl PCR reaction.

4.4. Preparation of the Positive Controls Diluents (DIL)

Fill a clean empty vial with 950 µl of PCR-grade water
Only when the spiked Extraction Control **(sEC)** procedure is selected (see **4.1**), add 50 µl of **nECT**.

4.5. Preparation of the Positive Controls

Add 160 µl of **DIL** to dissolve the **blue** and **yellow** cap **Pos. Ctrl** tubes to run up to 32 positive control reactions. Mix the target DNA by pipetting the solution up and down 10 times.

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

Note: **DIL** is unstable and must be disposed immediately after use;
to avoid any possible contamination do not use **NTC** for this procedure.

4.6. Reaction Mix for using *M. hominis, M. genitalium* and *U. urealyticum*.

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 three additional reaction.

The assay can be run also without the External Control Reaction (see last column).

Use with the spiked Extraction Control (sEC)	**Roche FastStart HybProbe Master**	**Optional nECT used as Internal Control**	**Use without External Control reaction**
Single reaction	**Component**	**Single reaction**	**Single reaction**
6.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.1 µl	8.6 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	**PSR** mix (parameter specific reagents, see 4.2)	2.0 µl	2.0 µl
2.0 µl	**CTR** mix (Control amplification reagents, see 4.2)	2.0 µl	---
---	**nECT** (DNA control target see 4.1)	0.5 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**	**15.0 µl**

Table 2

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

Add 5 µl of sample, **NTC** or **Pos. Ctrl** to each capillary or well for a final reaction volume of 20 µl.

Start run.

5. LightCycler® 2.0 Instrument

5.1. 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 3

5.2. 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 HybProbes".

Perform data analysis, as described in the 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 *U. urealyticum* data in channel 610, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *U urealyticum* data in channel 610, Melting Curves mode, specific melting points at 64-67℃

View data for *M. hominis* and *M. genitalium* in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *M. hominis* and *M. genitalium* data in channel 640, Melting Curves mode: specific melting points at 67-69℃ for *M. genitalium* and 59-61℃ or 56℃ for *M. hominis*

If the spiked Extraction Control or the Internal Control is used, view sEC/IC data in channel 705, Quantification mode. The negative control and the low-concentrated DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 30-32.

Notes:

* 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 relatively 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.

§ The values of the respective melting temperatures (T_M) may vary ±2.5℃ between different experiments. Th e ΔT between the melting peaks may vary ±1.5℃. Samples with deviati ng melting curves should be subjected to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de)

5.3. Sample Data – Typical Results

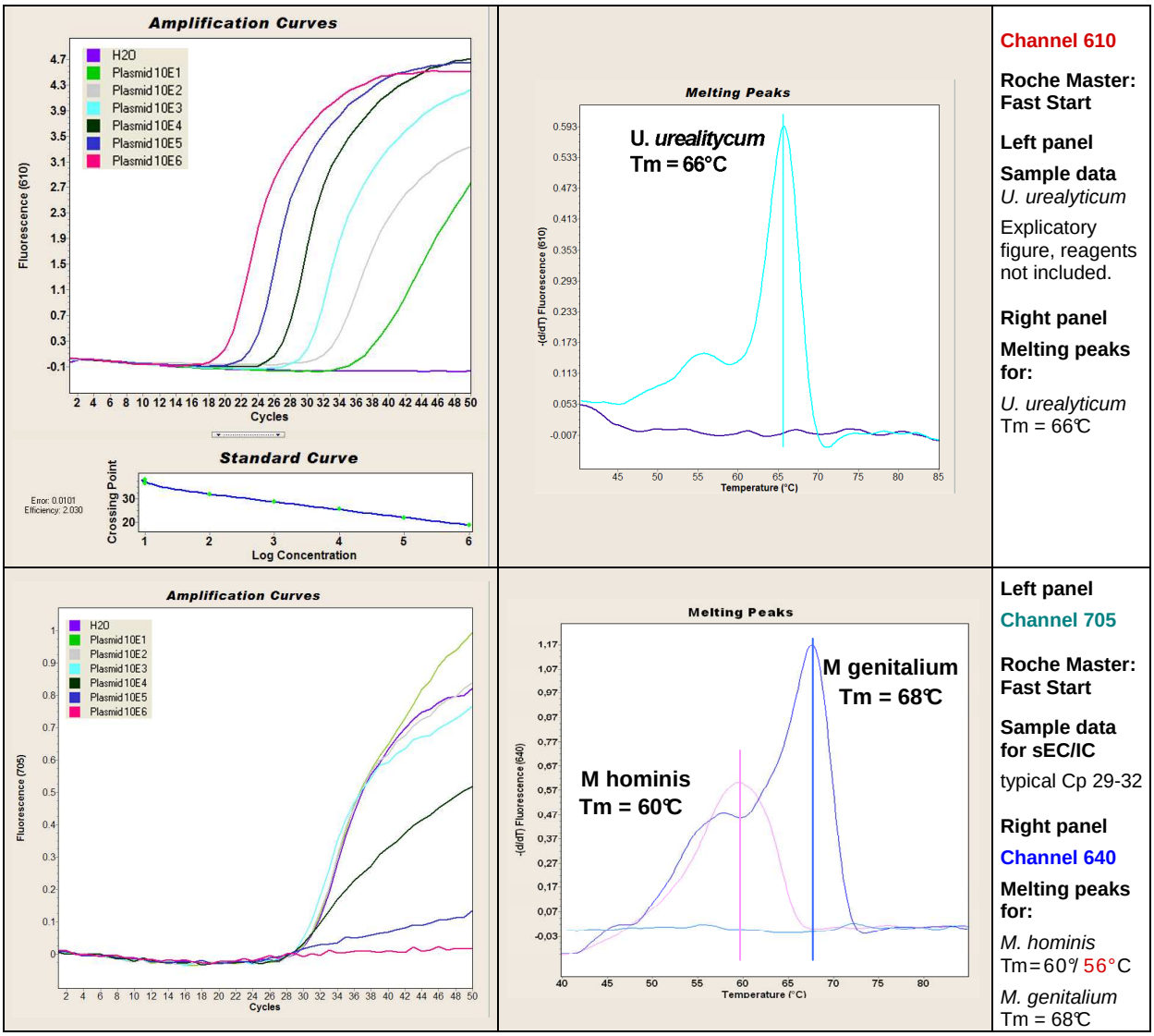

Fig.1. LightCycler® 2.0 sample data for the Mycoplasma / *Ureaplasma* detection system.

Upper panels: Channel 610: Left panel quantification mode (Second Derivative Maximum) with amplification curves for *U urealyticum*. Right panel melting analysis for *U. urealyticum*.

Lower panels: Left panel Channel 705 quantification mode (Second Derivative Maximum) for the sEC/IC.
Right panel Channel 640 melting analysis for *M. hominis and M. genitalium* .

5.4. Interpretation of Data

Sample				NTC	Result
U. urealyticum **610 TM=66℃ §**	***M. genitalium*** **640 TM=68℃ §**	***M. hominis*** **640 60℃ (or 56℃)§**	**IC 705**	**610 or 640**	
no amplification			detectable	negative	Negative (not detectable)
Cp < 38			not relevant	negative	Positive *U. urealyticum*
	Cp < 38		not relevant	negative	Positive *M. genitalium*
		Cp < 38	not relevant	negative	Positive *M. hominis*
no amplification signal			not	not relevant	PCR failure, repeat experiment
amplification signal			not relevant	positive	Contamination, repeat

Table 4. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

Notes: * and § see page 4

6. LightCycler® 480 II Instrument / cobas z 480 Analyzer

6.1. 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: MultiColor
LightCycler® 480 II Instrument: 465-510, 498-610, 498-640, 498-660
cobas z 480 Analyzer: 465-510, 498-610, 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 [℃]	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 [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [℃/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [℃]	-	-	55	-	-	-	-	-
Step Size [℃]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuou	None
Acquisitions [per ℃]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**

Table 5

6.2. 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 'LightMix® Kit – Color Compensation HybProbes.

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 *U. urealyticum* data with Filter Combination 498-610, Quantification. The negative control (NTC) must show no signal. For the identification of the PCR product view *U urealyticum* data with Filter Combination 498-610, Melting Curves mode, specific melting points of about 64-67℃

View data for *M. hominis* and *M. genitalium* with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *M. hominis, and M. genitalium* data with Filter Combination 498-640, Melting Curves mode: specific melting points at 67-69℃ for *M. genitalium* and 59-61℃ or 56℃ for *M. hominis*

If the spiked Extraction Control or the Internal Control is used, view sEC/IC data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated DNA samples (10 to 1,000 copies) should show an amplification curve with a Cp at approximately cycle 28-31.

Notes:

* 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 relatively 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.

§ The values of the respective melting temperatures (T_M) may vary ±2.5℃ between different experiments. Th e ΔT between the melting peaks may vary ±1.5℃. Samples with deviati ng melting curves should be subjected to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de)

6.3. Sample Data – Typical Results

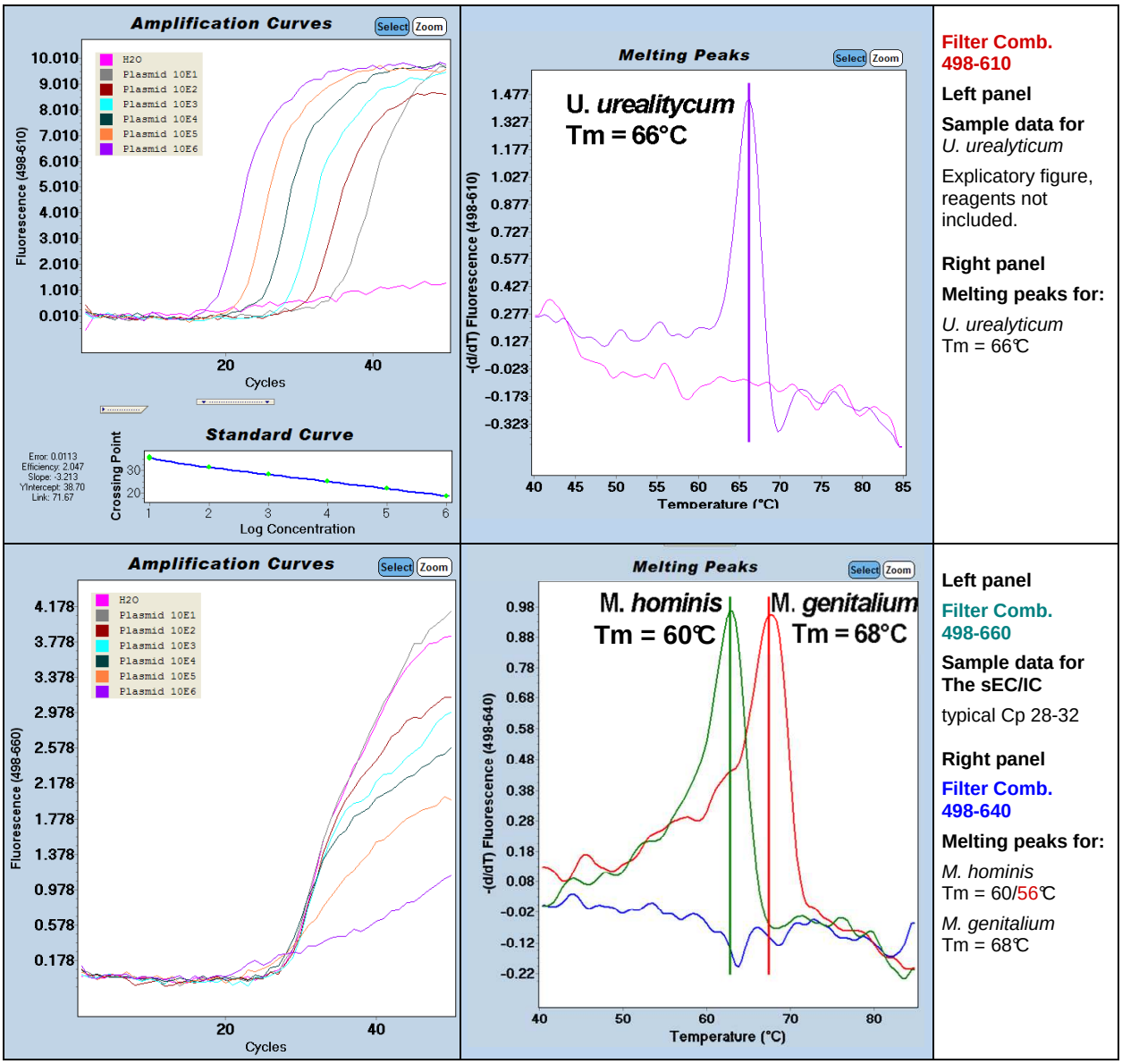

Fig.2. LightCycler® 480 sample data for the *Ureaplasma urealyticum* detection system.

Upper panels: Filter Combination 498-610: Left panel quantification mode (Second Derivative Maximum) with amplification curves for *U urealyticum*. Right panel melting analysis for *U. urealyticum*.

Lower panels: Left panel Filter Combination 4498-660 quantification mode (Second Derivative Maximum) for the IC. Right panel Filter Combination 498-640 melting analysis for *M. hominis and M. genitalium.*

6.4. Interpretation of Data

Sample				NTC	Result
U. urealyticum **498-610 TM=66℃ §**	***M. genitalium*** **498-640 TM=68℃ §**	***M. hominis*** **498-640 60℃ (or 56℃)§**	**IC 498-660**	**498-610 or 498-640**	
no amplification			detectable	negative	Negative (not detectable)
Cp < 38			not relevant	negative	Positive *U. urealyticum*
	Cp < 38		not relevant	negative	Positive *M. genitalium*
		Cp < 38	not relevant	negative	Positive *M. hominis*
no amplification signal			not	not relevant	PCR failure, repeat experiment
amplification signal			not relevant	positive	Contamination, repeat

Table 6. Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start) Notes: * and § see page 6

7. Set Contents

3 Vials with a **green** clips (**PSR**) containing lyophilized primers / probes for 32 PCR reactions.

1 Vial with a **blue** cap (**Pos. Ctrl Mg-Uu**) Positive Control for up to 32 reactions containing: 3.2 x 10^5 target equivalents *M. genitalium* and 3.2 x 10^5 target equivalents *U. urealyticum.*

1 Vial with a **yellow** cap (**Pos. Ctrl Mh**) Positive Control for up to 32 reactions containing: 3.2 x 10^4 target equivalents *M. hominis.*

3 Vials with a **white** clip (**CTR)** containing lyophilized primers / probes for 32 PCR control reactions.

1 Vial with a **black** cap (**NTC**) containing stabilizer for No-Template Control preparation.

1 Vial with a **white** cap (**nECT**) containing Extraction Control Target: 4.8 x 10^6 copies (total).

1 Certicate of Analysis (CoA) with lot-specific data.

8. Additional Reagents and Items Required

	Roche Diagnostics
Color Compensation HybProbe order n°40-0318-00	Cat .-No. 05 997 704 001

Note: Use of a color compensation file generated with the 'LightMix® Kit - Color Compensation HybProbes' is a prerequisite to run the reaction; analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 2.0 Instruments)	Cat.-No. 04 929 292 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001
Phosphat-Buffer-Saline (PBS)	Any vendor
PCR-grade water	Any vendor

8.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
or MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001
cobas® 4800 System Sample Preparation Kit	Cat.-No. 05 235 782 190
in combination with cobas® 4800 System Wash Buffer Kit	Cat.-No. 05 235 863 190

9. 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.

10. Version History

Events requiring changes in procedures red, mod. components blue

V130412	Release version	08.05.2013
V130813	Some isolates M.hominis exhibit a Tm of about 56℃	24.11.2013
V140513	Primer for M.hom adapted to detect previously missed variants	13.05.2014
V150505	Universal **nECT** target containing Lambda and PhHV DNA	25.06.2015
V160118	MagNA Pure 96 and x 480 extractors included. Table 6 corrected.	25.01.2016

Roche SAP order n° 07103913001

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.