LightMix® in-vitro diagnostics kit *Ureaplasma urealyticum / Ureaplasma parvum*

Cat.-No.: 40-0461-32

Detection of *Ureaplasma urealyticum* *and Ureaplasma parvum* genomic DNA

for use with the

Roche Diagnostics LightCycler® Instruments

Reagents for 96 reactions

Upon arrival:

Store Premixed PCR reagents and Controls protected from light at room temperature (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20°C) (if included)

CE IVD

Manual version 160116

Table of Contents

1. Product Information

1.1. Intended Use

This device is an *in-vitro* nucleic acid amplification test for the detection and discrimination of genomic DNA from *Ureaplasma urealyticum* (**U.u.**) *and Ureaplasma parvum* (**U.p.**) from nucleic acid extracts obtained from human urogenital swabs.

The present diagnostic device must be used by qualified personnel only.

Note: The performance of the assay can be guaranteed only when used with LightCycler® Instruments (see 1.2.2 for details).

1.2. Specifications

1.2.1. Clinical Samples

Running the test requires 5 µl of purified DNA in aqueous solution.

The test is performed on DNA extracted from human urogenital swabs.

Before performing samples extraction read **5. Sample Preparation**.

The quantity of biological sample obtained with swabs is highly dependent on the collection procedure therefore absolute quantification is impossible.

1.2.2. Instruments, Software and Productivity

One kit contains reagents for 96 reactions performed in a 20 µl volume.
Each run requires one positive control and one negative control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Run Time (approx.)	Max Samples per run (1)	Maximum Productivity of the kit(1)	Minimum Productivity of the kit (1,2)
LC1.X	3.5(5)	70 min	30 + 2 ctrl.	90	32
LC 2.0	4.05	70 min	30 + 2 ctrl.	90	32
LC480 II(4) (96 wells)	1.5	100 min	94 + 2 ctrl.	94	32
LC480 II(4)(384 wells)	1.5	100 min	382(3) + 2 ctrl.	94	32
z 480 (open channel)	1.5	100 min	94 + 2 ctrl.	94	32

1 Each run includes 2 control reactions: one positive control and one No-Target Controls (NTC).
2 Calculated considering a single clinical sample analyzed in each run.
3 It requires using four kits.
4 LightCycler® 480 “first version” cannot be used with this kit.
5 Upgrade to version 4.10 or higher when possible.

Table 1.

1.2.3. LightCycler® Instruments Channel (Filter) Settings

Instrument	Dye / channel name			
	530	**LC610**	**LC640**	**LC690**
LightCycler® 1.X	F1	---	F2	F3
LightCycler® 2.0	530	610	640	705
LightCycler® 480 II	465-510	498-610	498-640	498-660
cobas z 480 Analyzer	465-510	498-610	498-645	498-700

Table 2.

1.3. Contents

Lyophilized premixed PCR reagents

⚠ **Store at room temperature in the dark**

	Cap color	Label	Description content	Reaction Tube status	Total
3 x	Violet	PSR	Parameter Specific Reagents (**PSR**): Primers and hybridization probes specific for: ***U.urealyticum, U.parvum and control reaction*** <0,01pg unlabeled oligonucleotides; <0,01pg Fluorescein-labeled oligonucleotides <0,01pg LightCycler Red 640 labeled oligonucleotides (U. p.) <0,01pg LightCycler Red 690 labeled oligonucleotides (U. u.) <0,01pg LightCycler Red 640 labeled oligonucleotides (EC)	32 reactions Lyophilized blue-green pellet	96 rxs

Control DNA

⚠ **Store at room temperature**

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	Orange	Pos Ctr U.u.-U.p.	**Positive Control for *Ureaplasma urealyticum* / *Ureaplasma parvum*** <0,01pg plasmid target (synthetic) [1E4 copies / reaction each]	32 reactions Lyophilized blue pellet	32 rxs
1 x	White	nECT	**Extraction Control Target** <0,01pg plasmid target (synthetic) [4.8E6 copies: total amount]	100 reactions lyophilized blue pellet	96 rxs
1 x	Black	NTC	**No Template Control** Stabilizer (DNA)	blue pellet	---

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ **Store at -20°C upon arrival**

FastStart DNA Master HybProbe is included in kits supplied directly by TIB MOLBIOL for customers in Central Europe only (1).
The FastStart DNA Master HybProbe is not included in any kits supplied through Roche Diagnostics or its local distributor.

	Cap color	Label	Description Content	Reaction Tube storage	Total
3 x	Red	1a	LightCycler® FastStart Enzyme	32 reactions frozen	96 rxs
3 x	White	1b	LightCycler® FastStart Reaction Mix Hyb-Probe	32 reactions frozen	96 rxs
3 x(2)	Colorless	Water	H_2O PCR grade	frozen	96 rxs
3 x(2)	Blue	$MgCl_2$	$MgCl_2$, 25 mM	32 reactions frozen	96 rxs

1) FastStart enzyme is shipped by TIB MOLBIOL at ambient temperature.
2) FastStart enzyme supplied through Roche Diagnostics contains only 2 tubes of H_2O and 1 tube of $MgCl_2$, nevertheless the quantity provided is sufficient for the use described for this kit.

Table 3.

1.4. Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix!

Storage Conditions

Reagents and Controls

Store the lyophilized reagents (premixed PCR reagents and Control DNA) protected from light and at room temperature (18°C / 25°C).
Do not freeze these dried reagents. Expiration date is printed on the kit label.

Polymerase mix

Store the LightCycler® FastStart DNA Master HybProbe at -15°C to -25°C.
See expiration date on the polymerase tube label.

Shipping

Products are shipped at ambient temperature (no cooling). Transport stability of reagents and Roche FastStart enzyme components have been tested under common European shipping conditions (30°C and less). The dried reagents (PSR) have been tested to tolerate temporary incubation like two days at 60°C as to be expected to happen in Arabian countries.

2. Additional Devices and Reagents

2.1. Required

	TIB Molbiol
LightMix® Kit –Color Compensation HybProbes	Cat.-No. 40-0318-00
LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Cat.-No. 12 011 468 001
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
or	
LightCycler® 480 II Instruments	**Roche Diagnostics**
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
cobas 4800 System (cobas z 480 Analyzer)	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
General materials	
Phosphate Buffer Saline (PBS)	any supplier
Nuclease-free PCR grade water	any supplier

2.2. Optional

	TIB Molbiol
Extraction Control Target (**nECT**)	30-0259-96
	Roche Diagnostics
LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.3. Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit (#)	Cat.-No. 11 796 828 001
or High Pure Viral Nucleic Acid Kit (#)	Cat.-No. 11 858 874 001
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier
Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
MagNA Pure 96 IVD Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001
MagNA Pure Instrument (#)	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 2.0 Instrument (#)	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument (§)	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
X480 Instrument (#)	Cat.-No. 05 200 890 001
cobas® 4800 System Sample Preparation Kit **and**	Cat.-No. 05 235 782 190
cobas® 4800 System Wash Buffer Kit	Cat.-No. 05 235 863 190

(#) Additional Extraction Control Target tube is required.

(§) The use of MagNA Pure Compact Instrument allows to perform only the **Internal Control** procedure (see **3.3.2**).

3. Background Information

3.1. Medical Background

Ureaplasma are laborious to be identified by culture; NAT based tests such as PCR are becoming the primary detection methods.

Ureaplasma (*U. urealyticum* and *U. parvum*) are bacteria from the same 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.

Preferred specimens are genital swabs; urine has been reported to be insufficient.

3.2. Methodology and Assay Principle

This device amplifies and detects fragments from the genomes of *Ureaplasma* from a nucleic acid extract obtained from swabs.

A partialy conserved fragment of the urease gene is amplified with specific primers (182 bp long) and detected with a common fluorescine probe, a specific probe labeled with the dye LightCycler® Red 640 for *U. parvum* or a specific probe labeled with the dye LightCycler® Red 690 for *U. urealyticum.*
Pathogens are discriminated by dye colors.

After every PCR cycle the probes bind during the annealing step to the PCR generated target and bring the fluorophores in close proximity. The donor fluorophore fluorescein is excited by light, part of the energy is transferred to the acceptor dye and detected. The fluorescence intensities are plotted against the cycle number, giving a picture of the amplification and calculating a crossing point (Cp) which is related to the log of the concentration of target in the sample.
Hybridization Probes can be used to identify the PCR product in a melting curve analysis in order to verify that the right product has been formed. The melting point (Tm) is dependent on length and G+C content, but also upon the degree of homology to the sensor probe. In this device the Tm is used for discrimination; slight temperature shifts are acceptable as the shape of curve depends also on the amount of product.

Any significant decrease in the melting temperature is an indication of a sequence variation, explaining an eventual lower signal level in the quantification plot and may give cause for a subsequent analysis of the sample or PCR product by DNA sequencing (feel free to contact us at: service@tib-molbiol.de for assistance).

The supplied control DNAs allow comparison with unknown patient samples.

3.3. Control Reaction

An additional PCR product of 182 bp is amplified from the Extraction Control Target (**nECT**) and detected with a short probe labeled with LightCycler® Red 640 which is visible in the melting curve analysis only.

The control reaction does not interfere with the analytical reaction; it is designed to be more sensitive to the presence of inhibitors and it will fail to amplify in presence of high-positive samples. 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/rxn or higher).

The Extraction Control Target can be utilized with two different procedures: either as Spiked Extraction Control (3.3.1) or as Internal Control (3.3.2).

	Pathogen		
	U. Urealyticum	***U. parvum***	**Control**
Channel	**LC690**	**LC640**	**LC640**
PCR Fragment	**182 bp**	**182 bp**	**182 bp**
Melting curve Tm	**67-69°C**	**66-68°C**	**49-51°C**

Table 4.

3.3.1. Spiked Extraction Control (sEC)

The spiked Extraction Control (**sEC**) procedure monitors the extraction and evaluates the presence of amplification inhibitors (recommended procedure).

The recovery rate of the purified plasmid present in the nECT is dependent of the extraciton procedure used; the amount of nECT must be adjusted accordingly. See detailed instruction in **5.1**.

3.3.2. Internal Control (IC)

The Internal Control (**IC**) procedure evaluates the presence of amplification inhibitors only.

This procedure is mandatory if extraction is performed with MagNA Pure Compact Instrument.

3.4. Performance Characteristics

Analytical Specificity

The specificity to the target gene and the suitability of the PCR amplification employed in the present test has been evaluated by direct DNA sequencing of the amplicon.

Analytical Sensitivity

Analytical sensitivity for cloned standards is 10 copies per PCR corresponding to 500 copies / mL clinical sample.

3.4.1. Diagnostic Specificity, Sensitivity and Predictive Values

Cell culture is the reference gold-standard method for *Ureaplasma*. For a correct identification of false positive samples, all discordant samples were amplified also with other two independent PCR systems. Only samples positive to both PCR analyses were considered true positive and added to the Culture positive samples.

			Culture + 2 independent PCR				
						Prevalence	57.5%
TIB Molbiol	U.u./U.p.		Neg	Pos		Sensitivity	96.9%
		167	71	96		Specificity	95.8%
	Neg	71	68	3		PPV	96.9%
	Pos	96	3	93		NPV	95.8%

Diagnostic Specificity

A total number of 24 different single infected biological samples were analyzed in parallel by sequencing and with the present kit. DNA sequencing data has been obtained with ABI 3730xl by LGC Genomics GmbH, Berlin.

In particular, from the samples analyzed 11 were found to be *U. urealyticum* and 13 *U. parvum*.

Study results: Results for both analytical methods were in 100% concordance.

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.

Use the manual version which is delivered with the kit (see kit label).

General Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips with filter is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual .

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "PCR protocol" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Sample Preparation

The quantity of biological sample obtained with swabs is highly dependent on the collection procedure therefore absolute quantification is not possible.

5.1. Preparation of the Extraction Control Target (nECT)

Spin the tube at 10,000 RPM for 1 minute, dissolve the n**ECT** in a PBS volume that depends on the extraction procedure used, as described below; mix and spin down (PBS is not supplied with the kit).

Extraction Procedure	Cat. No.	PBS
Internal Control procedure	(see 3.3.2).	**1200 µl**
High Pure PCR Template Preparation Kit	11 796 828 001	**600 µl**
High Pure Viral Nucleic Acid Kit	11 858 874 001	**600 µl**
MagNA Pure 96 DNA and Viral NA Small Volume Kit	05 467 497 001	**1200 µl**
MagNA Pure 96 DNA and Viral NA Small Volume Kit	06 543 588 001	**1200 µl**
MagNA Pure LC DNA Isolation Kit I	03 003 990 001	**600 µl**
cobas® 4800 System Sample Preparation Kit **(§)**	05 235 863 190	**600 µl**
MagNA Pure Compact Nucleic Acid Isolation Kit I**(#)**	03 730 964 001	**1200 µl**
(§) See **5.3** for specific instructions. **(#)** Only the use of the **Internal Control procedure** is allowed (see **3.3.2**).		

Table 5.

5.1.1. Use of nECT as spiked Extraction Control (recommended)

When running the spiked Extraction Control procedure (**sEC**), add 10 µl of **Extraction Control Target** to the sample to be extracted.
Remember to complete No Target Control (**NTC**) and Diluents for Control DNAs (**DIL**) by adding n**ECT** too (see **7.1.3**).

5.1.2. Use of nECT as Internal Control

If running the Internal Control Procedure, use the Extraction Control Target **only** in the preparation of the Reaction mix (see **7.2**).

5.2. Swabs

5.2.1. Swabs in transport medium

Use 200 µl transport medium.
Perform nucleic acid purification with the appropriate extraction kit as described in the respective extraction protocols and extract into 50 µl.

5.2.2. Swabs in agarose

If vaginal or urethral swab arrives to the testing laboratory in agarose, disperse the swab in 250µl of PBS, let the particles of agarose to sediment for 3-5 minutes (agarose is a known PCR inhibitor), consider supernatant as transport medium (5.2.1).

5.3. X480 instrument

Spike the deep well plate with 10µl of n**ECT**; utilize 400 µl of transport medium for extraction and set the elution to 100 µl.

Store purified DNA refrigerated (4°C - 8°C),
if stored over a longer period of time keep frozen at -20°C.

6. Programming

6.1. Color Compensation

Color Compensation is required for the use of the *LightMix® Kit Ureaplasma urealyticum / Ureaplasma parvum*. Analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

6.2. Capillary Based Instruments

For details see the LightCycler® Operator's Manual.
The protocol consists of four program steps (Tab.5):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
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**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate [°C/s]*	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [°C]	**0**	**0**	**55**	**0**	**0**	**0**	**0**	**0**
Step Size [°C]	**0**	**0**	**0.5**	**0**	**0**	**0**	**0**	**0**
Step Delay [cycles]	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

Note:
While programming maintain default software values: max. samples = 32 and capillary size = 20 µl. Set seek temperature = 40°C and channel = 640.
Store the program and the default values as '**RUN Template'** which can be loaded to start every run.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.
For instructions see the Operator's Manual

Table 6. **Programming of capillary based Instruments.**

6.3. Multiwell Based Instruments

LightCycler® 480 II and cobas z 480 Analyzer.
For details see the LightCycler® Operator's Manual.

Detection Format: Multicolor
Please refer to the manual of:
LightMix® Kit Color Compensation HybProbes Cat. No. 40-0318-00

Reaction Volume: 20 µl

Programming:
The protocol consists of four program steps (Tab.6):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
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
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
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**	**0.14**	**1.5**
Ramp Rate [C°/ s] **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.14**	**1.5**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**
Sec Target [°C]	**0**	**0**	**55**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0.5**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**1**	**0**	**-**	**-**	**-**	**-**

Note:
Store the program and the default values as '**RUN Template'** which can be loaded to start every run.
Ensure to program only 1 acquisition per second instead the default value 3; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.

Table 7. **Programming of multiwell based instrument (96 well and 384 well formats)**

7. Experimental Protocol

Program the Instrument before preparing the solutions (see **6. Programming** and read the LightCycler® Operator's Manual for details).

7.1. Reagents Preparation

7.1.1. LightCycler® FastStart DNA HybProbe Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Quickly spin tubes to collect drops.
4	The solution must be free of particles.
5	Add 60 µl of **1b** to the vial **1a**.
6	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles. 
7	Spin the tubes to collect drops.
8	Use reagent to prepare the Reaction Mix (6.3).
9	Store left over reagent at 4°C.

7.1.2. Preparation of lyophilized PSR

►	Each reagent tube is sufficient for 32 reactions.
1	Spin the premixed tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom.
3	To each tube add **66 µl** of PCR-grade **Water.**
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 2 µl of reagent for a 20 µl PCR reaction.

7.1.3. No Template Control (NTC) and Diluents (DIL)

Label a clean vial as **DIL**.
Fill the **NTC** vial and the **DIL** vial with PCR-Grade water and n**ECT** as described:

Extraction Procedure	**H_2O**	**nECT**
Internal Control procedure	**1000 µl**	**---**
High Pure PCR Template Preparation Kit	**975 µl**	**25 µl**
High Pure Viral Nucleic Acid Kit	**975 µl**	**25 µl**
MagNA Pure 96 DNA and Viral NA Small Volume Kit	**950 µl**	**50 µl**
MagNA Pure 96 DNA and Viral NA Small Volume Kit	**950 µl**	**50 µl**
MagNA Pure LC DNA Isolation Kit I	**975 µl**	**25 µl**
cobas® 4800 System Sample Preparation Kit	**975 µl**	**25 µl**
MagNA Pure Compact Nucleic Acid Isolation Kit I	**1000 µl**	**---**

Table 8.

NTC is sufficient for 32 reactions.
► Use **5 µl** of **NTC** for a 20 µl PCR reaction.
Do not manipulate **NTC** in the same location where Positive Controls are used.

DIL is sufficient for the preparation of all **standards** / **controls:**
it must be prepared fresh and disposed immediately after use.
DIL, is used in close proximity of Positive Controls, may become contaminate.

7.1.4. Preparation of Control DNAs

►	Each **Control** reagent tube is sufficient for 32 reactions.
1	Spin the two tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 160 µl **DIL**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec. and spin the tubes to collect drops.

► Use **5 µl** of each **Control** for a 20 µl PCR reaction.
► All **Controls** must be used in each run.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

7.2. Preparation of the Reaction Mix

We recommend preparing 32 reactions to prevent storage of dissolved or activated reagents in varying volumes. See chapter 7.3 for storage and stability of dilute components. For the preparation of reaction mix for fewer samples, please go to step 7.2.2.

7.2.1. Preparation of 32 Reactions Mix

Use left column **"sEC"** for the spiked Extraction Control procedure (see **3.3.1** and **5.1**).
Use right column **"IC"** for the Internal Control procedure (see **3.3.2**).

Prepare the reaction mix in the PSR reagent tube:		**sEC**	**IC**
	Components	**32 reactions**	
	To the **PSR** tube (violet cap) already containing	66.0 µl	66.0 µl
Add:	H_2O, PCR-grade (colorless cap)	283.8 µl	267.3 µl
	Mg^{2+} solution 25 mM (blue cap)	79.2 µl	79.2 µl
	LightCycler® FastStart DNA Master HybProbe (red cap), see **7.1.1**	66.0 µl	66.0 µl
	nECT when used as Internal control (white cap), see **3.3.2**	---	16.5 µl
	Substitute the "long neck cap" of the **PSR** tube with the red cap from FastStart		
	Total Volume	**495.0 µl**	**495.0 µl**

Table 9. **Volumes of components for preparing 32 reaction mixture**

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due to a non homogeneous reaction mix!

7.2.2. Preparation of a Single Reaction Mix

Use left column **"sEC"** for the spiked Extraction Control procedure (see **3.3.1** and **5.1**).
Use right column **"IC"** for the Internal Control procedure (see **3.3.2**).

Prepare the reaction mix by multiplying each volume by the number of biological samples to be analyzed plus three reactions (**NTC**, one **Control**, one excess).

Prepare the reaction mix in a cooled vial:	**sEC**	**IC**
Components	**Single reaction**	
H_2O, PCR-grade (colorless cap)	8.6 µl	8.1µl
Mg^{2+} solution 25 mM (blue cap)	2.4 µl	2.4 µl
PSR (violet cap), see **7.1.2**	2.0 µl	2.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	2.0 µl	2.0 µl
nECT when used as Internal control (white cap), see **3.3.2**	---	0.5 µl
Volume of reaction mix	**15.0 µl**	**15.0 µl**

Table 10. **Volumes of components for preparing a single reaction mixture**

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due to a non homogeneous reaction mix!

7.3. Storage and Stability of Diluted Components

Reaction Mix
The complete reaction mix containing lyophilized premixed PCR reagents, LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (4°C - 8°C) for 30 days. Avoid prolonged exposure to light.

Lyophilized premixed PCR reagents
Once diluted, store the reagents refrigerated at 4°C - 8°C for a maximum period of 30 days. Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe
The combined FastStart mastermix (1a+1b) can be stored refrigerated (4°C - 8°C) for 30 days.

Controls
The dissolved **Controls** are stable for 30 days when stored refrigerated (4°C - 8°C). When using them over a longer period of time, aliquot and store frozen at -20°C. Controls stored at -20°C are stable for 180 days.

Note:
More than five (5) freezing and thawing cycles of controls may result in shift in the Cp value compared to the freshly dissolved vial; avoid the use of the control when the Cp value changes more than 1 cycle.

7.4. Loading of Controls and Samples

7.4.1. Samples attributes

Controls and samples attributes are summarized in the table below:

Number	Sample Name	LC1.X	LC2.0	LC480II	z 480	Quantification Sample Type
1	**NTC**	 F2 F3	610 640 705	498-610 498-640 498-660	498-610 498-645 498-700	Unknown Negative Negative
2	**Pos. Ctr. U.u. - U.p.**	 F2 F3	610 640 705	498-610 498-640 498-660	498-610 498-645 498-700	Unknown Positive for *U.parvum* Positive for *U.urealiticum*
3…	**Sample**	 F2 F3	610 640 705	498-610 498-640 498-660	498-610 498-645 498-700	Unknown Unknown Unknown
The suggested loading order simplifies the programming of the 'Sample List' in the LightCycler® Instruments.						

Table 11.

7.4.2. Capillary / Well Loading Procedure

Each run must include one Negative Control (**NTC**) to demonstrate the absence of contaminations with target DNA or PCR product and the **positive controls** to ensure the performance of the kit.

Capillary / Well Loading Procedure

1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **15 µl** per capillary/well.
3	**Remember to include the controls when setting up the run:** Add **5 µl** of **Negative Control** (**NTC**) (Black cap) Add **5 µl** of **U.u.-U.p. Positive Control** (Orange cap)
4	Add **5 µl** of **Sample** in the remaining capillaries / wells.
5	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the LightCycler® Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment's name when instructed.
10	Save sample data in the samples' window.

7.4.3. Capillary Based Instruments

In "Samples data - Capillary View", input Sample Name as described in the second column of Table 11. Select "Analysis Type – Abs Quant". Select Channel 610, 640 and 705 only !

7.4.4. Plate Based Instruments

In the "Sample Editor" window, in "Step1: Select Workflow" section, select "Abs Quant". Input sample Name as described in the second column of Table 11.

8. Data Analysis and Interpretation

8.1. Limits and Interferences

The present test is specific for *Ureaplasma urealyticum* and *Ureaplasma parvum*.

8.2. Calibration

Calibration has to be performed following the procedure described in 7.4, 8.3.1, 8.3.2 and 8.3.3.

8.3. Quality Control – Acceptance Criteria

In order to perform a reliable analysis, it is essential that Negative Control **NTC** and all **Controls** are included in each run.
In addition to the reagents needed to detect for *U. urealyticum* and *U parvum*, the PSR contains also primers and probes for the amplification of a foreign DNA target (**nECT**) added to the PCR following the **spiked Extraction Control (sEC)** (see **3.3.1**) or the **Internal Control (IC)** (see **3.3.2**) procedures.
At the end of the run a melting-curve analysis will display a specific melting peak in **LC640** at a temperature of 49-51°C indicating **nECT** amplification.
The amplification, performed at an annealing temperature of 55ºC ensures that the **nECT** signal is not visible, thus it does not interfere with quantification of the targets.
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).

Activate Color Compensation.
Analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

8.3.1. Negative Control (NTC)

NTC Negative Control is mandatory – it mimics negative clinical samples.

Amplification analysis of **NTC** must provide a **negative result** in channels **LC640** and **LC690**.
If amplification is visible in even one of these channels, a contamination or a pipetting error has occurred; the session is not valid and the whole procedure has to be repeated (amplification and detection).
If the problem sustains, change water and/or reagents and repeat.

Melting-curve analysis of **NTC** must provide a **negative result** in channel **LC690**.
NTC must provide a **positive result** with a Tm of 49-51°C in channel **LC640.**
If the **nECT** melting curve is absent, the session is not valid and the whole procedure must be repeated (amplification and detection).

8.3.2. Positive Control for *U. urealyticum - U. parvum*

Inclusion of the **U.u.-U.p. Pos Ctr** is **mandatory:** the positive control mimics positive samples.

Amplification analysis must provide a **positive amplification** in channel **LC690** and in **LC640**. The Cp value must conform with expected results (see 8.4). If amplification is absent see instruction in 8.3.3.

Melting-curve analysis must provide a **melting curve** with Tm = 67-69ºC in channel **LC690** and a **melting curve** with Tm = 66-68ºC in channel **LC640**; a third melting curve with Tm = 49-51ºC in channel **LC640** corresponding to the **nECT** might also be present.

8.3.3. Failures in runs of Controls

Complete data from controls are essential to get accurate results with patient samples.

Runs without adequate results for the **controls** must be repeated.

Before repeating a run consider common errors; check in particular the amplification program, use of the correct master-mix and $MgCl_2$ concentration; remember that also inadequate storage of reagents may cause a failure of the device.

8.3.4. Samples

Negative Clinical Samples

Amplification analysis provides **no amplification** signal in channels **LC690** and **LC640**.

Melting-curve analysis must provide melting curve with Tm = 49-51ºC in channel **LC640** corresponding to the **nECT**.

Clinical samples displaying neither target amplification nor nECT signal are invalid and the whole procedure has to be repeated (sample preparation, amplification and detection).

Positive Clinical Samples for *Ureaplasma urealyticum.*

Amplification analysis provides a **positive amplification** in channel **LC690**.

Melting-curve analysis must yield a melting curve in channel **LC690** with Tm = 67-69ºC.

A melting curve with Tm = 49-51ºC in channel **LC640** corresponding to the **nECT** might also be present.

Positive Clinical Samples for *Ureaplasma parvum.*

Amplification analysis provides a **positive amplification** in channel **LC640**.

Melting-curve analysis must yield a melting curve in channel **LC640** with Tm = 66-68ºC.

A melting curve with Tm = 49-51ºC in channel **LC640** corresponding to the **nECT** might also be present

Multiple infection have been frequently observed.

8.3.5. Abnormal Melting Curves

Abnormal melting curves generated by variants are sometime reported; in this case another method must be used for verification of the amplified product.

Report deviations to service@tib-molbiol.de.

Feel free to send deviant melting samples to TIB Molbiol Berlin laboratories to confirm the obtained results by DNA sequencing.

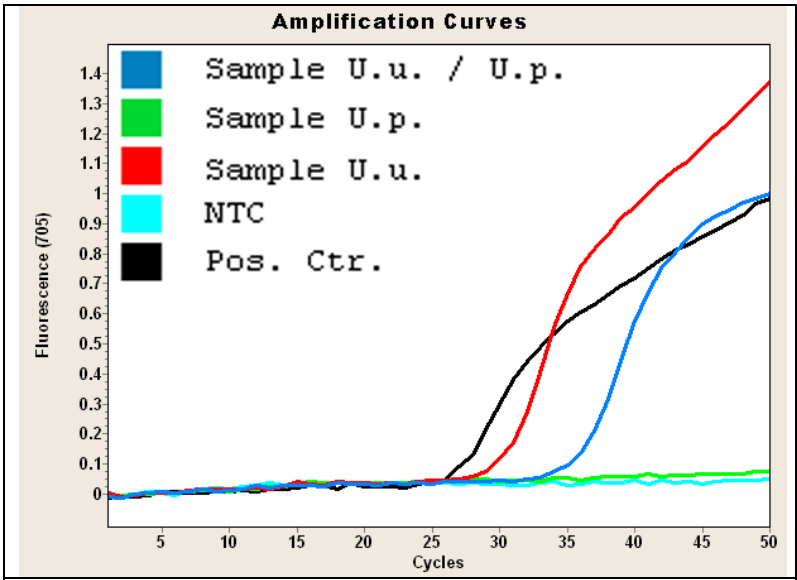
8.4. Reading the Results

Perform data analysis as described in the LightCycler® Operator's Manuals.

Activate Color Compensation!
Analyzing data with 'Color Compensation' deactivated will generate invalid readouts of the results.

8.4.1. Amplification Analysis: Capillary Based Instruments

View data for *Ureaplasma urealyticum* in channel LC690 and for *Ureaplasma parvum* in channel LC640, "Absolute Quantification" analysis mode.



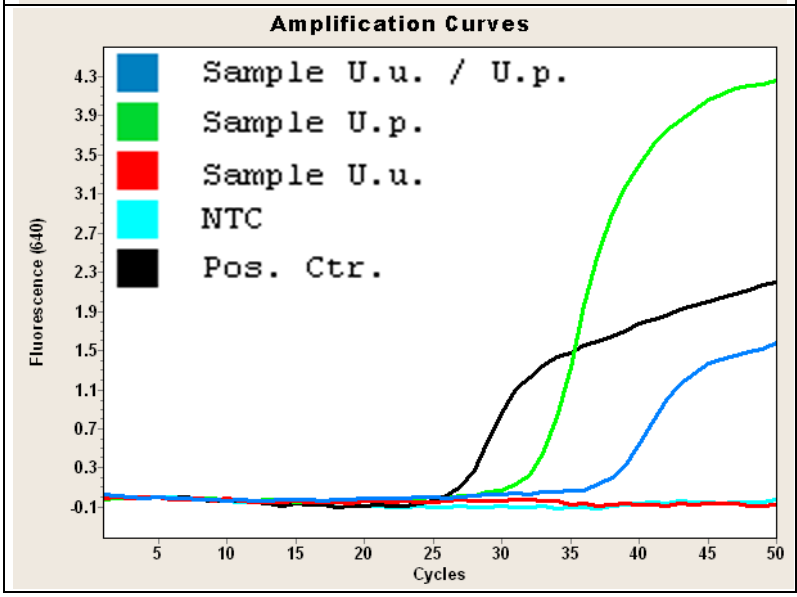
LC690

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show an amplification signal.
Typical Cp=25-27.

U.u. Positive samples show an amplification signal.

Fig. 1: Amplification data for *Ureaplasma urealyticum*.



LC640

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show an amplification signal.
Typical Cp=25-27.

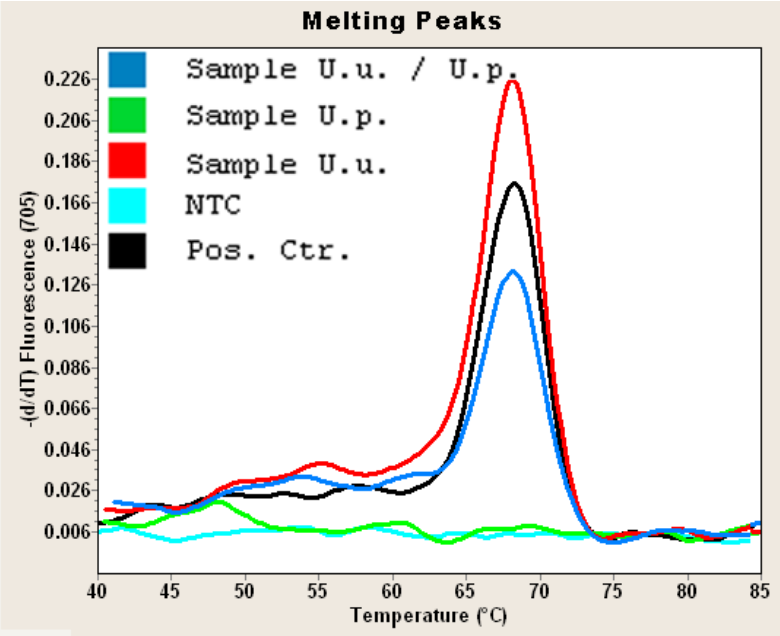
U.p. Positive samples show an amplification signal.

Fig. 2: Amplification data for *Ureaplasma parvum*.

Note: The values of the crossing point (Cp) may vary ±1.5 cycles between different experiments.
In case of variations see instructions: **8.3.3 Failures in runs of Controls.**

8.4.2. Melting Curve Analysis: Capillary Based Instruments

View data for *Ureaplasma urealyticum* in channel LC690 and for *Ureaplasma parvum* in channel LC640, “Tm Calling” analysis mode. **nECT** signal is also detected in LC640.



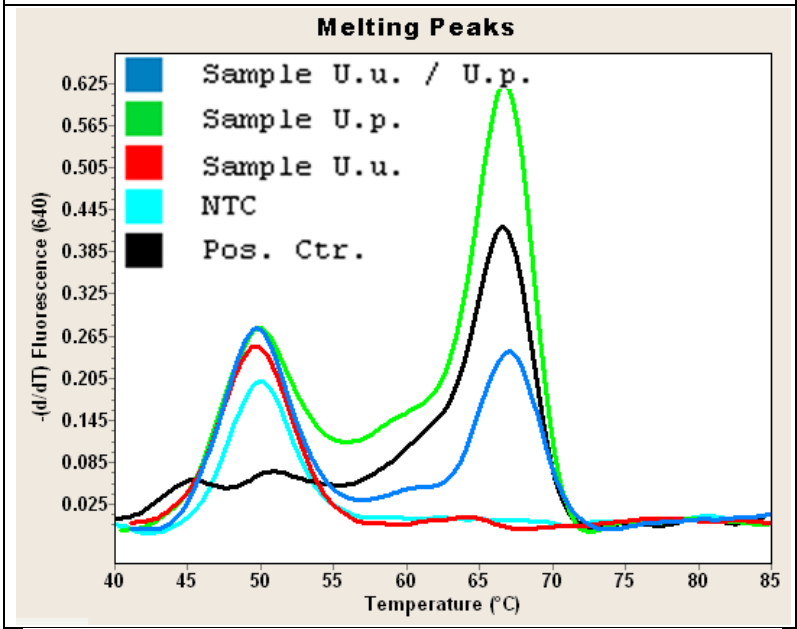
LC690

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show a melting peak.
Tm = 67-69°C.

U.u. Positive samples show melting peak.

Fig. 3: Melting curve for *Ureaplasma urealyticum*.



LC640

NTC Negative Control (light blue line) MUST show a specific melting peak corresponding to the **nECT** signal with a Tm = 49-51°C.

Pos. Ctr. Positive Control (black line) must show a melting peak.
Tm = 66-68°C.
nECT signal is usually absent.

U.p. Positive samples show melting peak with a Tm = 66-68°C.
nECT signal might be present.

Fig. 4: Melting curve for *Ureaplasma parvum.*

	EC	U.u.	U.p.
Sample U.u. / U.p.	49.84	68.16	67.22
Sample U.p.	49.86		66.68
Sample U.u.	49.60	68.00	
NTC	49.71		
Pos. Ctr.		68.08	66.70

Fig. 5: Details of melting curve temperature.

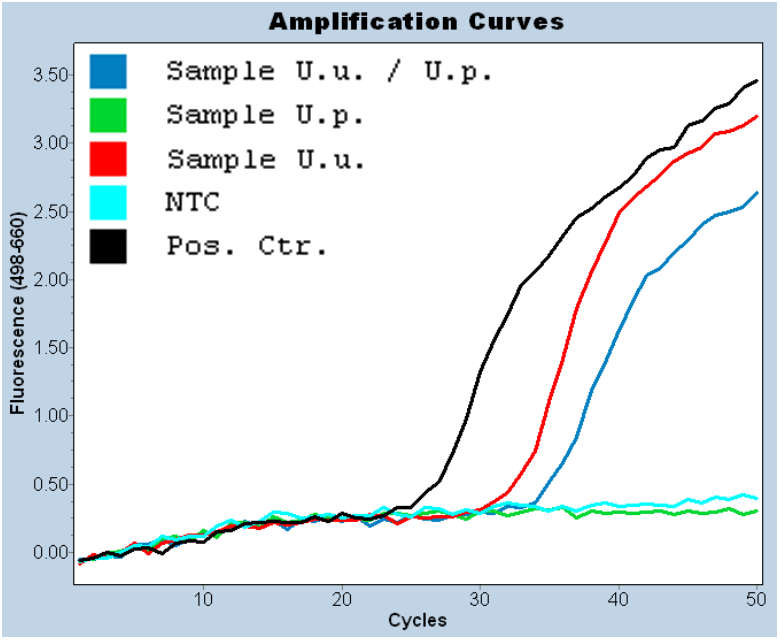
Note: The values of the melting temperatures (Tm) may vary ± 2.5°C between different experiments.

nECT Melting curve in LC640 channel Tm = 49-51°C.

nECT Melting curve for positive samples and **Pos. Ctr.** is not relevant and might be absent.

8.4.3. Amplification Analysis: Plate Based Instruments

View data for *Ureaplasma urealyticum* in channel LC690 and for *Ureaplasma parvum* in channel LC640, “Abs Quant/2nd Derivative Max ” analysis mode.



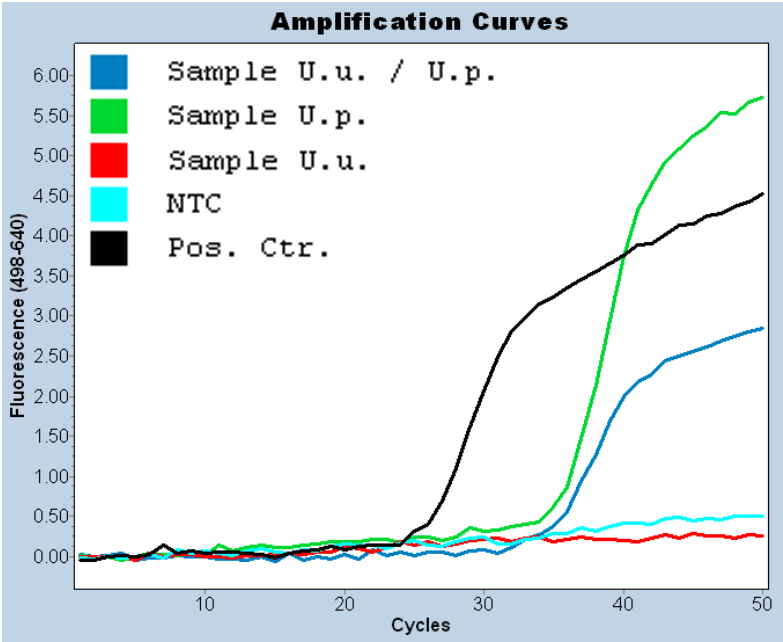
LC690

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show an amplification signal.
Typical Cp=25-27.

U.u. Positive samples show an amplification signal.

Fig. 6: Amplification data for *Ureaplasma urealyticum*.



LC640

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show an amplification signal.
Typical Cp=25-27.

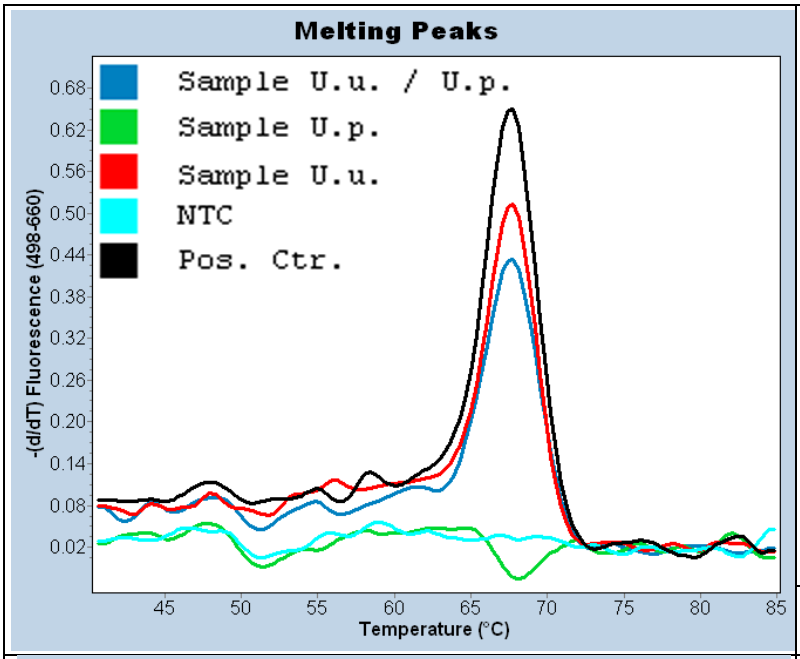
U.p. Positive samples show an amplification signal.

Fig. 7: Amplification data for *Ureaplasma parvum.*

Note: The values of the crossing point (Cp) may vary ±1.5 cycles between different experiments.
In case of variations see instructions: **8.3.3 Failures in runs of Controls.**

8.4.4. Melting Curve Analysis: Plate Based Instruments

View data for *Ureaplasma urealyticum* in channel LC690 and for *Ureaplasma parvum* in channel LC640, “Tm Calling” analysis mode. nECT signal is also detected in LC640.



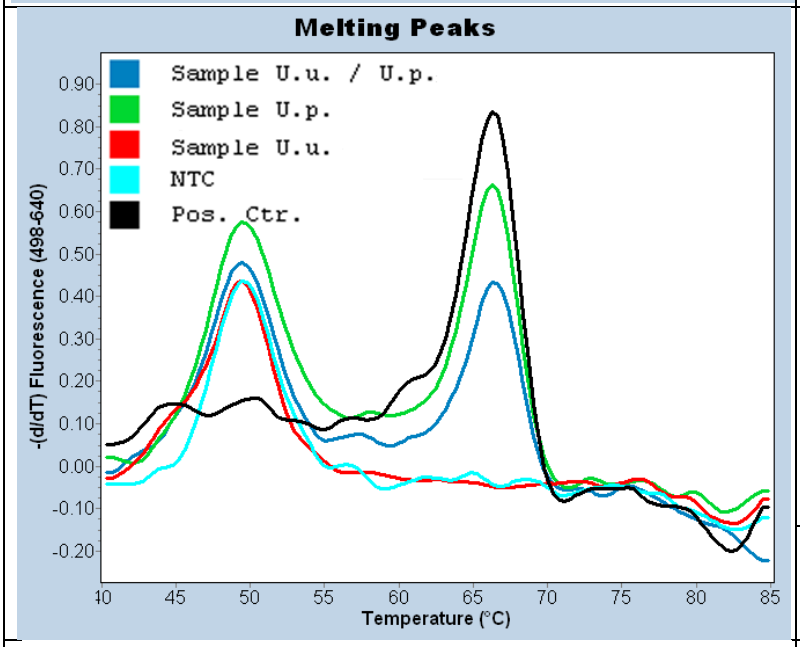
LC690

NTC (light blue line) must show a flat line.

Pos. Ctr. Positive Control (black line) must show a melting peak.
Tm = 67-69°C.

U.u. Positive samples show melting peak.

Fig. 8: Melting curve for *Ureaplasma urealyticum*.



LC640

NTC Negative Control (light blue line) MUST show a specific melting peaks corresponding to the **nECT** signal with a Tm = 49-51°C.

Pos. Ctr. Positive Control (black line) must show a melting peak.
Tm = 66-68°C.
nECT signal is usualy absent.

U.p. Positive samples show melting peak with a Tm = 66-68°C.
nECT signal might be present.

Fig. 9: Melting curve for *Ureaplasma parvum*.

	EC	U.u.	U.p.
Sample U.u. / U.p.	49.45	67.73	66.30
Sample U.p.	49.53		66.33
Sample U.u.	49.44	67.72	
NTC	49.45		
Pos. Ctr.		67.69	66.31

Fig. 10: Details of melting curve temperature.

Note: The values of the melting temperatures (Tm) may vary ± 2.5°C between different experiments.

nECT Melting curve in LC640 channel Tm = 49-51°C.

nECT Melting curve for positive samples and Pos. Ctr. is not relevant and might be absent.

8.5. Interpretation Table

First check results of Controls: for a valid run all controls must be passed.

Controls			Results
Amplification	Amplification	Melting curve 49-51°C	
LC690	LC640	LC640	
NTC			
Negative	Negative	Melting curve	Negative Control Passed
Negative	Negative	Negative	nECT Failed Check if sEC/IC was used !
Amplification	Amplification	not relevant	Negative Control Failed (1) Contamination ! Repeat !
U.u.-U.p. Pos Ctr			
Amplification	Amplification	not relevant	Positive Control Passed
Amplification	Negative	not relevant	*U. parvum* Positive Control Failed
Negative	Amplification	not relevant	*U.urealyticum* Positive Control Failed
Negative	Negative	not relevant	Positive Control Failed

Note: (1) If amplification is present in either channel (LC610 or LC640), Negative Control is failed.

Table 12.

Then read results for the clinical samples :

Clinical Samples			Results
Amplification	Amplification	Melting curve 49-51°C	
LC690	LC640	LC640	
Negative	Negative	Melting curve	Negative for U.u. and U.p. (not detectable)
Amplification	Negative	not relevant	Positive for *U. urealyticum*
Negative	Amplification	not relevant	Positive for *U. parvum*
Amplification	Amplification	not relevant	Positive for *U .urealyticum and U. parvum*
Negative	Negative	Negative	Amplification Control Failed Check if sEC/IC was used and repeat from extraction

Clinical samples with multiple infection will show corresponding amplification and melting data.

Table 13.

9. Troubleshooting

Instrument specific codes:	Capillary based instruments	Multiwell based instruments
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline "Saw teeth like"	Non homogeneous mix	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in **NTC** Negative Control	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR-grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	Possible altered target sequence	Verify by repeating, sequence the virus (if applicable). Report to: service@tib-molbiol.de and/or send samples for further analysis to the laboratory in Berlin.

10. References

[1] Ellen Vancutsem, Oriane Soetens,Maria Breugelmans, Walter Foulon,and Anne Naessens
Modified Real-Time PCR for Detecting, Differentiating, and Quantifying Ureaplasma urealyticum and Ureaplasma parvum.
The Journal of Molecular Diagnostics, Vol. 13, No. 2, March 2011

[2] Robertson JA, Vekris A, Bebear C, Stemke GW.
Polymerase chain reaction using 16S rRNA gene sequences distinguishes the two biovars of Ureaplasma urealyticum.
J Clin Microbiol. 1993 Apr;31(4):824-30

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Classification / References

Reference	Classification
EDMA	15.30.04.49 (15.01.08.40) (15.01.40.90)
CPV	33694000-1
EAN	4260159333794
Roche SAP No.	

FastStart Enzyme

FastStart DNA Master HybProbe is included in the package for TIB MOLBIOL customers in Central Europe only.

When the present kit is distributed through Roche Diagnostics or their local distributors,
FastStart DNA Master HybProbe is supplied as a separate product:

Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
Roche Diagnostics Cat.-No. 12 239 272 001 kit for 480 reactions

11. Material Safety Data Sheet

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union 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

Notes: in red mark events that require to change laboratory procedures
Notes in blue mark improvements and changes in composition

Version	Event	Date
150430	Release Version	30-04-2015
151126	Page 22, correction in fig. 5; page 24, correction in fig.10.	26-11-2015
160116	X480 instrument allowed (Sections 2. and 5.3); Changes in the preparation procedures of nECT (5.1), NCT and DIL (7.1.3);Internal Control procedure: changed nECT volume in the Master Mix preparation (7.2.1 and 7.2.2); editorial changes.	16-01-2016

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CE IVD