Chlamydia trachomatis/Ureaplasma/ M.genitalium Real-TM

Handbook

Multiplex Real Time PCR kit for qualitative detection of Chlamydia trachomatis, Ureaplasma species and Mycoplasma genitalium

REF TB46-100FRT

Σ 100

NAME

Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM

INTRODUCTION

STDs (sexually transmitted diseases) refer to a variety of bacterial, viral and parasitic infections that are acquired through sexual activity. Some STDs, such as syphilis and gonorrhea, have been known for centuries — while others, such as HIV, have been identified only in the past few decades. STDs are caused by more than 25 infectious organisms. As more organisms are identified, the number of STDs continues to expand. Common STDs include: chlamydia, gonorrhea, herpes, HIV, HPV, syphilis, gardnerella and trichomoniasis.

The Chlamydia trachomatis is nonmotile, gram-negative bacterial pathogen and is the most common sexually transmitted bacterial agent. The prevalence of C. trachomatis infection in sexually active adolescent women, the population considered most at risk, generally exceeds 10%, and in some adolescent and STD clinic populations of women, the prevalence can reach 40%. The prevalence of C. trachomatis infection ranges from 4 to 10% in asymptomatic men and from 15 to 20% in men attending STD clinics. Chlamydial infections in newborns occur as a result of perinatal exposure; approximately 65% of babies born from infected mothers become infected during vaginal delivery.

The development of tests based on nucleic acid amplification technology has been the most important advance in the field of STD diagnosis. Because nucleic acid amplification is exquisitely sensitive and highly specific, it offers the opportunity to use noninvasive sampling techniques to screen for infections in asymptomatic individuals who would not ordinarily seek clinical care.

INTENDED USE

Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM PCR kit is an *in vitro* nucleic acid amplification test for multiplex detection of *Chlamydia trachomatis, Ureaplasma* (*parvum* and *urealyticum*) and *Mycoplasma genitalium* DNA in clinical materials (urogenital, rectal and pharyngeal swabs; conjunctival discharge; prostate gland secretion; and urine samples) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF ASSAY

C.trachomatis / Ureaplasma / M.genitalium detection by the multiplex polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *C.trachomatis / Ureaplasma / M.genitalium* primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM** PCR kit uses "hot-start", which greatly reduces frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by chemically modified polymerase (TaqF), which is activated by heating at 95 ºC for 15 min.

MATERIALS PROVIDED

Part N° 1 – **"DNA-Sorb-A":** sample preparation

- **Lysis Solution**, 2 x 15 ml;
- **Sorbent**, 2 x 1,0 ml;
- **Washing Solution**, 2 x 50 ml;
- **DNA-eluent**, 2 x 5 ml;
- **Transport medium** , 2 x 15 ml.

Contains reagents for 100 tests.

Part N° 2 – **"Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM":** Real Time amplification

- **PCR-mix-1-FRT *C.trachomatis/Ureaplasma/M.genitalium***, 1,2 ml;
- **PCR-mix-2-FRT**, 2 x 0,3 ml;
- **Polymerase (TaqF)**, 2 x 0,03 ml;
- **Positive control complex (C+)**, 0,2 ml;
- **Negative Control (C-)**, 1,2 ml;*
- **Internal Control-FL (IC)**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 110 tests.

**must be used in the isolation procedure as Negative Control of Extraction.*
***add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA-Sorb-A REF K-1-1/A protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator
- Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

All components of the **Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM** PCR kit are to be stored at 2–8 ºC when not in use. All components of the **Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM** PCR kit are stable until the expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FL *C.trachomatis / Ureaplasma / M.genitalium* is to be stored in the place protected from light.

Polymerase (TaqF) and PCR-mix-2-FRT are to be stored at the temperature no more than minus 16 °C.

DNA-sorb-A must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -16°C immediately on receipt.

STABILITY

Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (EN375). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM can analyze DNA extracted from:

- *cervical, urethral, conjunctival swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium (can be ordered separately, Sacace REF K12-Stab). Vigorously agitate swabs for 15-20 sec.
- *urine sediment* : collect 10-20 ml of first-catch urine in a sterile container. Centrifuge for 30 min at 3000 x g, carefully discard the supernatant and leave about 200 µl of solution. Resuspend the sediment. Use the suspension for the DNA extraction.
- *prostatic liquid* stored in “Eppendorf” tube;
- *seminal liquid:* maintain semen for 40 min in darkness until liquefaction. Use 100 µl for the DNA extraction.

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following isolation kits are recommended:

⇒ **DNA-Sorb-A** (Sacace, REF K-1-1/A);

⇒ **SaMag STD DNA Extraction kit** (Sacace, REF SM007).

Please carry out the DNA extraction according to the manufacturer’s instructions. Add 10 µl of Internal Control-FL (IC) during the DNA isolation procedure directly to the sample/lysis mixture.

(Note: the Sacace Internal Control is the same for all urogenital infectious kits)

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **10 µl** of **Internal Control** and **300 µl** of **Lysis Solution**.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **20 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Add **500 µl** of **Washing Solution** to each tube. Vortex very vigorously and centrifuge for 30 sec at 10000g. Remove and discard supernatant from each tube.
10. Repeat step 9 and incubate all tubes with open cap for 5-10 min at 65°C.
11. Resuspend the pellet in **100 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
12. Centrifuge the tubes for 1 min at 12000g.
13. The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

The total reaction volume is **25 µl**, the volume of DNA sample is **10 µl**.

1. Prepare the required number of the tubes for amplification of DNA from clinical and control samples.
2. For carrying out N reactions (including 2 controls), mix in a new tube: **10*(N+1) µl of PCR-mix-1-FRT *C.trachomatis* / *Ureaplasma* / *M.genitalium*, 5.0*(N+1) µl of PCR-mix-2-FRT** and **0.5*(N+1) µl of polymerase (TaqF).** Vortex the tube, then centrifuge shortly. Transfer **15 µl** of the prepared mixture to each tube.

Unfreeze PCR-mix-2-FRT before mixing.

3. Using tips with aerosol barrier, add **10 µl** of **DNA** obtained from clinical or control samples at the DNA extraction stage to the prepared tubes.

4. Carry out the control amplification reactions:

NCA -Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).
C+ -Add **10 µl** of **Positive Control complex** to the tube labeled C+ (Positive Control of Amplification).

Amplification

Create a temperature profile on your instrument as follows:

Step	**Rotor type instruments**			**Plate or modular type instruments**		
	Temperature, °C	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
Hold	95	15 min	1	95	15 min	1
Cycling	95	5 s	5	95	5 s	5
	60	20 s		60	20 s	
	72	15 s		72	15 s	
Cycling 2	95	5 s	40	95	5 s	40
	60	**20 s fluorescence detection**		60	**30 s fluorescence detection**	
	72	15 s		72	15 s	

[1] *For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied), SmartCycler® (Cepheid)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green, JOE/Yellow/HEX/Cy3, ROX/Orange/Texas Red, and Cy5/Red fluorescence channels.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*0.1*	*0 %*	*Off*
JOE/Yellow	*0.1*	*5 %*	*Off*
Rox/Orange	*0.1*	*5 %*	*Off*
Cy5/Red	*0.1*	*5 %*	*Off*

Plate-type instruments

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for Pos C+ sample during the last amplification cycle.

DATA ANALYSIS

- ***Chlamydia trachomatis*** **DNA** amplification product is detected in the **FAM/Green** fluorescence channel,
- ***Ureaplasma*** **spp.** *(**U.parvum*** and ***U.urealyticum**)* **DNA** is detected in the **JOE/Yellow/HEX** channel,
- ***Mycoplasma genitalium*** **DNA** is detected in the **ROX/Orange** channel,
- **Internal Control** is detected in the **Cy5/Red** channel.

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

RESULTS INTERPRETATION

The results are interpreted by the device software by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The results of the analysis are considered reliable only if the results obtained for both Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct.

Table 2. Results for controls

Control	Stage for control	Ct channel FAM/Green, JOE/Yellow/HEX, ROX/Orange	Ct channel Cy5/Red	Interpretation
NCE	DNA extraction	Neg	Pos (< 33)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< 33)	Pos (< 33)	OK

1. The sample is considered to be positive for *Chlamydia trachomatis* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the FAM/Green channel.
2. The sample is considered to be positive for *Ureaplasma* spp. if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the JOE/Yellow/HEX/Cy3 channel.
3. The sample is considered to be positive for *Mycoplasma genitalium* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the ROX/Orange channel.
4. The sample is considered to be negative for *Chlamydia trachomatis, Ureaplasma* spp. and *Mycoplasma genitalium* if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in FAM/Green, JOE/Yellow/HEX and ROX/Orange channels and in the results grid in the Cy5/Red channel the Ct value doesn't exceed boundary value (Ct < 33).

SPECIFICATIONS

Sensitivity

Clinical material	Nucleic acid extraction kit	Microorganism	Sensitivity, copies/ml
Urogenital swabs	DNA-sorb-A	*Chlamydia trachomatis*	$5x10^2$
		Ureaplasma spp.	10^3
		Mycoplasma genitalium	10^3
Urine	DNA-sorb-A	*Chlamydia trachomatis*	10^3
		Ureaplasma spp.	$2x10^3$
		Mycoplasma genitalium	$2x10^3$

The analytical sensitivity of each microorganism does not change even if two other microorganisms are present at high concentrations.

Specificity

The analytical specificity of **Chlamydia trachomatis/Ureaplasma/M.genitalium Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

Nonspecific responses were absent in tests of human DNA samples and DNA of the following microorganisms: *Gardnerella vaginalis, Lactobacillus* spp., *Escherichia coli, Staphylococcus* spp., *Streptococcus* spp., *Candida albicans, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis, Chlamydia trachomatis, Mycoplasma genitalium, Neisseria* spp., *Neisseria gonorrhoeae, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV* types 1 and 2, *CMV,* and *HPV*.

TROUBLESHOOTING

1. Weak or no signal of the IC (Cy5 channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Any signal on Fam(Green), Joe (Yellow)/Hex/Cy3, Rox (Orange)/TexasRed channels with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Store at	VER	Version
	Manufacturer	**NCA**	Negative Control of Amplification
	Consult instructions for use	**NCE**	Negative control of Extraction
	Expiration Date	**C+**	Positive Control of Amplification
		IC	Internal Control

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