IVD For *in Vitro* Diagnostic Use

CE

Ureaplasma species Real-TM Quant

Handbook

Real Time PCR Kit for quantitative detection of Ureaplasma species

REF B2-100FRT Q

REF TB2-100FRT Q

100

NAME

Ureaplasma species Real-TM Quant

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, mycoplasma, ureaplasma, herpes, HIV, HPV, syphilis, gardnerella and trichomoniasis.

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

Kit **Ureaplasma species Real-TM Quant** is a test for the quantitative detection of *Ureaplasma species* in the urogenital swabs, urine, prostatic liquid and other biological materials.

PRINCIPLE OF ASSAY

Kit **Ureaplasma species Real-TM Quant** is based on two major processes: isolation of DNA from specimens and Real Time amplification. In real-time PCR, the fluorescent signal is generated from the presence of an olygonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase cleaves the probe due to its 5'-3' nuclease activity. As a result, the fluorescent dye molecule becomes separated from the quencher, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample, and its value allows quantitative comparisons of analyzed and control samples.

In **Ureaplasma species Real-TM Quant** kit there are 2 independent reactions running in parallel in each tube: the first reaction allows to detect and to quantify the specific fragment of *Ureaplasma species* (Fam/Green channel) and the second reaction detects Internal Control (Joe/Yellow/Cy3/HEX channel) present in all samples obtained from cells and allows not only to control all analysis steps, but also to estimate sample handling and storage.

The result of Internal Control amplification is detected in the JOE/Yellow fluorescence channel. The DNA target selected as an endogenous internal control is a fragment of human genome (a β-globin gene fragment). It must be always present in the sample (urogenital swab) in sufficient quantities equivalent to the number of cells in the swab (10^3–10^5 genome equivalents). Thus, the use of an endogenous internal control makes it possible not only to monitor test stages (DNA extraction and amplification) but also to assess the adequacy of sampling and storage of clinical material. If epithelial swab was taken incorrectly (the number of epithelial cells is insufficient), the amplification signal of β-globin gene will be underestimated. The number of epithelial cells may be insufficient if prostate gland secretion and urine samples are used.

Quantitative DNA analysis is based on the linear dependence between the cycle threshold (Ct) and the initial concentration of DNA target. Quantitative analysis is performed in the presence of DNA calibrators (samples with a known concentration of *Ureaplasma species* DNA), which are added during amplification. The results of amplification of DNA calibrators are used to construct a calibration curve, on the basis of which the concentration of *Ureaplasma species* DNA in samples determined. To minimize the effect of variation during material sampling, the quantitative results (*Ureaplasma species* DNA concentrations) are normalized to the genomic DNA quantity.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B2-100FRT Q)

Part N° 2 – **"Ureaplasma species Real-TM Quant":** Real Time amplification

- **PCR-mix-1 FRT Ureaplasma spp. Q,** 1,2 ml;
- **PCR-mix-2,** 2 x 0,3 ml**;**
- **Taq Polymerase,** 2 x 0,03 ml;
- **DNA-buffer,** 0,5 ml**;**
- **QS1** (10^2 copies/sample Ureaplasma spp. + β-globin), 0,1 ml;
- **QS2** (10^4 copies/sample Ureaplasma spp. + β- globin), 0,1 ml;
- **Negative Control C-*,** 1,2 ml

Contains reagents for 110 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB2-100FRT Q)

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 – **"Ureaplasma species Real-TM Quant":** Real Time amplification

- **PCR-mix-1 FRT Ureaplasma spp. Q,** 1,2 ml;
- **PCR-mix-2,** 2 x 0,3 ml**;**
- **Taq Polymerase,** 2 x 0,03 ml;
- **DNA-buffer,** 0,5 ml**;**
- **QS1** (10^2 copies/sample Ureaplasma spp. + β-globin), 0,1 ml;
- **QS2** (10^4 copies/sample Ureaplasma spp. + β- globin), 0,1 ml;
- **Negative Control C-*,** 1,2 ml

Contains reagents for 110 tests.

**must be used in the isolation procedure as Negative Control of Extraction.*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 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
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Ureaplasma species Real-TM Quant must be stored at 2-8°C except for **PCR-mix-2** and **TaqF Polymerase** that must be stored at -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

Ureaplasma species Real-TM Quant 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

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.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). 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

Ureaplasma species Real-TM Quant 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. Vigorously agitate swabs in medium 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

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

⇒ **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.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

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 **300 µl** of **Lysis Solution** in each tube.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Neg 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.

PROTOCOL:

1. Prepare required quantity of reaction tubes (or PCR plate) for samples and controls.
2. Prepare in the new sterile tube for each sample **10*N µl** of **PCR-mix-1-FRT**, **5,0*N µl** of **PCR-mix-2** and **0,5*N µl** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add to each tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Prepare for each run 2 standards and 1 Neg Control:
   - add **10,0 µl** of **Quantitation Standards** (QS1, QS2) into 2 labeled tubes;
   - add **10,0 µl** of **DNA-buffer** to the tube labeled PCR Negative Control;
5. Insert the tubes in the thermalcycler.

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Ureaplasma sp. is detected on Fam/Green channel and β-globin gene on Joe/Yellow/Cy3/HEX.

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor-type instruments[1]			Plate- or modular type instruments[2]		
Step	***Temperature, °C***	***Time***	**Cycles**	***Temperature, °C***	***Time***	**Cycles**
Hold	**95**	15 min	1	**95**	15 min	1
Cycling 1	**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 *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)
[2] For example, *SaCycler-96™ (Sacace), CFX96™/* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	0.1	10-20 %	on
JOE/Yellow	0.1	10-20 %	on

Plate- or modular type instruments

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for UG1 sample during the last amplification cycle.

RESULTS INTERPRETATION

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line. To set threshold put the line at such level where curves of fluorescence are linear.

- *Ureaplasma sp.* DNA amplification is detected on FAM (Green) channel;
- β-globin gene DNA amplification is detected on JOE(Yellow)/HEX/Cy3 channel

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed.

Results for controls

Control	Stage for control	Ct FAM (Green)	Ct JOE(Yellow)/HEX/Cy3	Interpretation
C-	NCE	NEG	NEG	Valid result
QS1, QS2	PCR	POS	POS	Valid result
Neg Control	PCR	NEG	NEG	Valid result

1. *Ureaplasma species* DNA is **detected** in a sample if its Ct value is defined in the results grid in the FAM/Green channel.
2. *Ureaplasma species* DNA is **not detected** in a sample if its Ct value is not defined in the results grid in the FAM/Green channel (the fluorescence curve does not cross the threshold line) whereas the Ct value in the JOE(Yellow)/HEX/Cy3 channel in the results grid is defined and the quantity of human genome equivalents per reaction is greater than 10^3 for women and greater than $5x10^2$ for men.
3. The result of analysis is **invalid** if the Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in the JOE channel or the number of human genome equivalents per reaction is less than 10^3 for women and less than $5x10^2$ for men. In this case, PCR should be repeated starting from the DNA extraction.

The DNA concentration per human genome equivalents (GE) is calculated by the following formula:

$$\log\left(\frac{Ureaplasma\ spp\ DNA\ copies/reaction}{human\ DNA\ copies/reaction}\times 200000\right)=\log\left(\mathrm{GE}\ Ureaplasma\ spp\ per\ 10^5\ cells\right)$$

N.B. *In case of using urine samples and the prostate gland secretion, the number of human genome equivalents per reaction can be less than 10^3 for women and less than $5x10^2$ for men.*

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **Ureaplasma species Real-TM Quant** PCR kit is ensured by selection of specific primers and probes as well as strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis. Nonspecific reactions were absent while testing human DNA samples and DNA panel of the following microorganisms: *Gardnerella vaginalis, Lactobacillus spp., Escherichia coli, Staphylococcus* spp.*, Streptococcus* spp., *Candida albicans, Chlamydia trachomatis, Mycoplasma genitalium, Neisseria gonorrhoeae, Mycoplasma hominis, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV* of 1 and 2 types, *CMV* and *HPV.* The clinical specificity of **Ureaplasma species Real-TM Quant** PCR kit was confirmed in laboratory clinical trials.

Analytical sensitivity

The kit **Ureaplasma species Real-TM Quant** allows to detect *Ureaplasma sp.* DNA in 100% of the tests with a sensitivity of not less than 500 GE/ml. The detection was carried out on the control standard and its dilutions by negative sample.

TROUBLESHOOTING

1. 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.
2. Fam (Green) and Joe/Yellow/Hex signal 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.
3. 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
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX96™, iCycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of *Qiagen*
*MX3000P® and MX3005P® are trademarks of Stratagene
*Applied Biosystems® is trademarks of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid

Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
mail: ***info@sacace.com*** *web:* ***www.sacace.com***