For *in Vitro* Diagnostic Use

Ureaplasma parvum/Ur.urealyticum/ M.hominis Real-TM Quant

Handbook

Real Time PCR Kit for quantitative detection of Ureaplasma parvum, Ureaplasma urealyticum, Mycoplasma hominis

REF B75-100FRT Q

REF TB75-100FRT Q

Σ **100**

NAME

U.parvum/U.urealyticum/M.hominis 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, herpes, HIV, HPV, syphilis, gardnerella and trichomoniasis.

Bacterial vaginosis (BV) is a lower genital tract infection characterized by the presence of thin, white, homogeneous, fishy-smelling vaginal discharge. This discharge is present in the absence of signs of vaginal irritation, such as pain, itching, burning, soreness, and dyspareunia. As such, in reference to the lack of demonstrable inflammatory response, the term 'vaginosis' was adopted instead of vaginitis. Bacterial vaginosis is characterized by a disruption of the normal vaginal equilibrium.The lactobacilli population decreases, which leads to an increase in vaginal pH (as high as 7.0) and overgrowth of and replacement by vaginosis-associated anaerobic microorganisms. Up to 90% of women with bacterial vaginosis harbor *Gardnerella vaginalis*. Other associated microbial populations identified include *Prevotella bivia*, *Mobiluncus* species, Gram-positive cocci, *Bacteroides*, *Mycoplasma hominis*, *Ureaplasma*, *Megasphaera*, and *Leptotrichia*. *Atopobium vaginae*, *Streptococcus*.

The development of tests based on nucleic acid amplification technology has been the most important advance in the field of STD and BV 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 **U.parvum/U.urealyticum/M.hominis Real-TM Quant** is a multiplex Real Time PCR test for the quantitative detection of *Ureaplasma parvum, Ureaplasma urealyticum and Mycoplasma hominis* in the urogenital swabs, urine, prostatic liquid and other biological materials.

PRINCIPLE OF ASSAY

U. parvum, U.urealyticum, M.hominis detection by the multiplex polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific regions using specific 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 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.

Kit **U.parvum/U.urealyticum/M.hominis Real-TM Quant** is based on two major processes: isolation of DNA from specimens and Real Time amplification.

U.parvum/U.urealyticum/M.hominis Real-TM Quant kit allows to make the quantitative detection in two ways:

1. *Absolute quantification* which gives absolute results of copies in 1 ml of clinical sample
2. *Relative quantification* which gives the results of the ratio between copies of U.parvum, U.urealyticum, M.hominis and the quantity of human cells. To obtain this result the primers and probes against specific regions of *U.parvum, U.urealyticum, M.hominis* and against human β-globin gene were added to the PCR mix. Furthermore to obtain a quantitative result of human cells, QSG standards contain human DNA calibrators. The obtained results of the ratio between the concentration of U.parvum, U.urealyticum, M.hominis and human DNA evaluate the level of the presence of these microorganisms among a population of human cells.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B75-100FRT Q)

Part N°2 - **U.parvum/U.urealyticum/M.hominis Real-TM Quant**: Real Time amplification

- **PCR-mix-1**, 1,2 ml
- **PCR-mix-2**, 2 x 0,3 ml
- **TaqF Polymerase**, 2 x 0,03 ml
- **TE-buffer**, 0,5 ml
- **Negative Control C-***, 1,2 ml
- **Standard**
  - **QSG1**, 0,1 ml
  - **QSG2**, 0,1 ml

Contains reagents for 110 tests

Module No.2: Complete Real Time PCR test with DNA purification kit (TB75-100FRT 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 - **U.parvum/U.urealyticum/M.hominis Real-TM Quant**: Real Time amplification

- **PCR-mix-1**, 1,2 ml
- **PCR-mix-2**, 2 x 0,3 ml
- **TaqF Polymerase**, 2 x 0,03 ml
- **TE-buffer**, 0,5 ml
- **Negative Control C-***, 1,2 ml
- **Standard**
  - **QSG1**, 0,1 ml
  - **QSG2**, 0,1 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

U.parvum/U.urealyticum/M.hominis 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

U.parvum/U.urealyticum/M.hominis 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

***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

U.parvum/U.urealyticum/M.hominis Real-TM Quant can analyze DNA extracted from:

- *cervical, urethral swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,5 ml of Transport medium (can be ordered separately, Sacace REF K12-Stab) . 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 500 µl of solution. Resuspend the sediment. Use the suspension 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 tubes or PCR plate.
2. Prepare for each sample in the new sterile tube **10*N µl** of **PCR-mix-1**, **5*N µl** of **PCR-mix-2** and **0,5*N µl** of **TaqF Polymerase.**
3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted DNA** sample to appropriate tube with Reaction Mix.
5. Prepare for qualitative run 1 positive control and 1 negative control:
   - add **10 µl** of **QSG2** to the tube labeled C*pos*;
   - add **10 µl** of **TE-buffer** to the tube labeled C*neg*;
6. For quantitative analysis prepare 4 tubes and perform QSG1 and QSG2* standards twice.

   **QSG1 and QSG2 values are specific for each lot and are reported in the Quant Data Card provided in the kit.*

Close tubes and transfer them into the instrument in this order: samples, negative controls, Standards.

Amplification:

Create a temperature profile on your Real-time instrument as follows:

	Rotor type instruments[1]				Plate type or modular instruments[2]			
Stage	**Temp, °C**	**Time**	**Fluorescence detection**	**Cycle repeats**	**Temp, °C**	**Time**	**Fluorescence detection**	**Cycle repeats**
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	FAM(Green), JOE(Yellow), Rox (Orange) Cy5 (Red)		60	30 s	FAM, JOE/HEX/Cy3, Rox/TexasRed, Cy5	
	72	15 s	–		72	15 s	–	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	0.1	10 %	on
JOE/Yellow	0.1	10 %	on
Rox/Orange	0.1	10 %	on
Cy5/Red	0.1	10-30 %	on

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

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* parvum is detected on the FAM (Green) channel, *Ureaplasma* urealyticum on the JOE (Yellow)/Cy3/HEX channel, *Mycoplasma hominis* on the ROX (Orange) channel and β-globin gene on the Cy5 (Red) channel

Qualitative analysis

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	Ct Rox (Orange)/ TexasRed	Ct Cy5 (Red)	Interpretation
NCE	DNA isolation	–	–	–	–	OK
NCA	PCR	–	–	–	–	OK
QSG2	PCR	Pos	Pos	Pos	Pos	OK

- The sample is considered to be positive for *Ureaplasma* parvum if in the channel FAM (Green) the value of **Ct** is different from zero;
- The sample is considered to be positive for *Ureaplasma* urealyticum if in the channel JOE(Yellow)/HEX the value of **Ct** is different from zero;
- The sample is considered to be positive for *Mycoplasma hominis* if in the channel ROX (Orange) the value of Ct is different from zero;
- Specimens with absent signal in the Cy5 (Red) channel are interpreted as invalid.

Quantitative analysis

Absolute quantification:

Absolute quantification gives absolute quantitative concentration of microorganisms in the clinical specimens put in 500 μl of transport medium.

For each patient specimen, calculate the concentration of *U. parvum, U.urealyticum, M.hominis* DNA in 1 ml of sample using the following formula:

U. parvum, U.urealyticum, M.hominis **reaction x 200*= copies DNA/ml**

Relative quantification:

Relative quantification which gives the results of the ratio between copies of *U.parvum, U.urealyticum, M.hominis* and the quantity of human cells. The obtained results of the ratio between the concentration of U.parvum, U.urealyticum, M.hominis and human DNA evaluate the level of the presence of these microorganisms among a population of human cells.

For each patient specimen, calculate the concentration of DNA in 10^5 cells using the following formula:

$$\frac{\textit{Up (Uu, Mh)}\ \textbf{DNA copies/reaction}}{\textbf{IC Glob DNA copies/reaction}} \times 2*10^5 = \textit{Up (Uu, Mh)}\ \textbf{DNA}/10^5\ \textbf{cells}$$

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Ureaplasma parvum, Ureaplasma urealyticum and Mycoplasma hominis* primers and probes.

The specificity of the kit **U.parvum/U.urealyticum/M.hominis Real-TM Quant** was 100%.

The potential cross-reactivity of the kit **U.parvum/U.urealyticum/M.hominis Real-TM Quant** was tested against the group control (*Gardnerella vaginalis, Lactobacillus spp., Escherichia coli, Staphylococcus spp., Streptococcus spp., Candida albicans, Chlamydia trachomatis, Neisseria gonorrhoeae, Neisseria spp., Mycoplasma genitalium, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV, CMV, HPV*). It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **U.parvum/U.urealyticum/M.hominis Real-TM Quant** allows to detect *Ureaplasma parvum, Ureaplasma urealyticum and Mycoplasma hominis* DNA in 100% of the tests with a sensitivity of not less than 500 copies/ml.

Linearity

U.parvum/U.urealyticum/M.hominis Real-TM Quant is linear from 10^3 to 10^7 copies/ml.

TROUBLESHOOTING

1. Weak or no signal of the IC (Cy5/Red channel) for the clinical samples.
    - Incorrect collection of clinical material. Repeat this step.
    - Problems during DNA extraction.
        - ⇒ 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 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.
2. Weak or no signal of Standards.
    - 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 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.
    - 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

Symbol	Meaning	Symbol	Meaning
REF	List Number		Caution!
LOT	Lot Number	Σ	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
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid

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