IVD For *in Vitro* Diagnostic Use

CE

Ureaplasma parvum/urealyticum Real-TM

Handbook

Real Time PCR Kit for qualitative detection and differentiation of Ureaplasma parvum and urealyticum

REF B19-100FRT

REF TB19-100FRT

Σ 100

NAME

Ur. parvum/U.urealyticum 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.

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 **Ur.parvum/urealyticum Real-TM** is a multiplex Real Time PCR test for the qualitative detection of *Ureaplasma parvum and Ureaplasma urealyticum* in the urogenital swabs, urine, prostatic liquid and other biological materials.

PRINCIPLE OF ASSAY

kit **Ur. parvum/urealyticum Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Ureaplasma parvum/urealyticum* DNA is extracted from the specimens, amplified using Real-Time amplification. and detected using fluorescent reporter dye probes specific for *Ureaplasma* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction

inhibition. *Ureaplasma parvum* is detected on the FAM (Green) channel, *Ureaplasma urealyticum* on the JOE (Yellow)/Cy3/HEX channel *and IC DNA* on the ROX (Orange)/Texas Red channel.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B19-100FRT)

Part N° 2 – **"Ureaplasma parvum/urealyticum Real-TM":** Real Time amplification

- **PCR-mix-1-FRT**, 1,2 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **TaqF Polymerase**, 2 x 0,03 ml;
- **Pos Control Complex C+**, 0,2 ml;
- **Negative Control C-**, 1,2ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 110 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB19-100FRT)

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

- **Lysis Solution**, 30 ml;
- **Sorbent**, 2 x 1,0 ml;
- **Washing Solution**, 100 ml;
- **DNA-eluent**, 2 x 5 ml;
- **Transport medium** , 30 ml.

Contains reagents for 100 tests.

Part N° 2 – **"Ureaplasma parvum/urealyticum Real-TM":** Real Time amplification

- **PCR-mix-1-FRT**, 1,2 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **TaqF Polymerase**, 2 x 0,03 ml;
- **Pos Control Complex C+**, 0,2 ml;
- **Negative Control C-**, 1,2ml;*
- **Internal Control 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:

- 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

Ur. parvum/urealyticum Real-TM 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

Ur. parvum/urealyticum 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

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

Ur. parvum/urealyticum Real-TM can analyze DNA extracted from:

- *cervical, urethral 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* (use the first part of the stream);
- *prostatic liquid* stored in "Eppendorf" tube;
- *seminal liquid: t*ransfer about 30 µl of seminal liquid to a polypropylene tube (1,5 ml) and add 70 µl of sterile saline solution;

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 DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

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 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–** (**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 for samples (N) and controls (N+2).
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1-FRT**, **5,0*(N+1)** of **PCR-mix-2** and **0,5*(N+1)** 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 panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Control C+** to the tube labeled Amplification Positive Control;
5. Insert the tubes in the thermalcycler.

Amplification

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

	Rotor type instruments[1]				Plate type or modular instruments[2]			
Stage	***Temp,℃***	***Time***	***Fluorescence detection***	***Cycle repeats***	***Temp,℃***	***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)		**60**	30 s	FAM, JOE/HEX/Cy3, Rox/TexasRed	
	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® 7300/7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)

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

Ureaplasma parvum is detected on the FAM (Green) channel, *Ureaplasma* urealyticum on the JOE (Yellow)/Cy3/HEX channel, IC on the ROX (Orange) channel

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	0.1	10-20 %	On
JOE/Yellow	*from 4 Fl to 8 Fl*	0.1	10-20 %	On
Rox/Orange	*from 4 Fl to 8 Fl*	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.

Ct Boundary values

Sample	Channel	Ct for rotor type instrument	Ct for plate type instrument
C+	FAM	33	36
	JOE	33	36
	ROX	33	36
Samples, C-	ROX	33	36

DATA ANALYSIS

The fluorescent signal intensity is detected in three channels:

– The signal from the *Ureaplasma* parvum DNA amplification product is detected in the FAM/Green channel;

– The signal from the *Ureaplasma* urealyticum DNA amplification product is detected in the JOE (Yellow)/Cy5/HEX channel;

– The signal from the IC amplification product is detected in the ROX (Orange) channel.

Interpretation of results

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.

The result of analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (Table 1).

Table 1. Results for controls

Control	Stage for control	Ct FAM (Green)	Ct JOE(Yellow) / HEX/Cy3	Ct Rox(Orange) / TexasRed	Interpretation
NCE	DNA isolation	NEG	NEG	POS	OK
NCA	PCR	NEG	NEG	NEG	OK
C+	PCR	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 result is **invalid** if the Ct value of a sample in the FAM (Green) / JOE(Yellow)/ channel is absent while the Ct value in the Rox(Orange) channel is either absent or greater than the specified boundary value (Ct > 33 for rotor type instrument – Ct > 36 for plate type instrument). It is necessary to repeat the PCR analysis of such samples.

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.

SPECIFICATIONS

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* primers and probes.

The specificity of the kit **Ur. parvum/urealyticum Real-TM** was 100%.

The potential cross-reactivity of the kit **Ur. parvum/urealyticum Real-TM** 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, Mycoplasma hominis, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV, CMV, HPV*). It was not observed any cross-reactivity with other pathogens.

Sensitivity

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

TROUBLESHOOTING

1. Weak or no signal of the IC (Rox(Orange) 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
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - 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. Fam (Green) 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 (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

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