Ureaplasma parvum/urealyticum Real-TM

for use with RotorGene™ 3000/6000 (Corbett Research), SmartCycler® (Cepheid), iQ iCycler™ and iQ5™ (Biorad), Applied Biosystems® 7300/7500 Real Time PCR Systems (Applera)

Key to symbols used

<table>
<thead>
<tr>
<th>REF</th>
<th>IVD</th>
<th>LOT</th>
<th>VER</th>
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</thead>
<tbody>
<tr>
<td>List Number</td>
<td>For in Vitro Diagnostic Use</td>
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<td>Consult instructions for use</td>
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<td>Manufacturer</td>
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</table>

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, ureaplasma, 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 Ur. parvum/urealyticum Real-TM is a test for the qualitative detection of Ureaplasma parvum/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
Part N° 1 – “DNA-Sorb-A”: sample preparation;
Part N° 2 – “Ureaplasma parvum/urealyticum TM”: Real Time amplification.

Part N° 1 – “DNA-Sorb-A”:
- 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 TM”:
- PCR-mix-l-FRT, 1,2 ml;
- PCR-Buffer-FRT, 2 x 0,35 ml;
- TaqF Polymerase, 0,06 ml;
- Pos Control Complex C+, 0,2 ml;
- Negative Control C-, 1,6 ml;*
- 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:
• Biological cabinet
• Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
• Vortex mixer
• Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
• 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
• Disposable gloves, powderless
• Biohazard waste container
• Refrigerator
• Tube racks
Zone 2: Real Time amplification:
• Real Time Thermalcycler
• Workstation
• Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
• Tube racks

WARNINGS AND PRECAUTIONS
1. 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).
2. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
3. Do not pipette by mouth.
4. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
5. Do not use a kit after its expiration date.
6. Dispose of all specimens and unused reagents in accordance with local regulations.
7. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. This kit is designed for use with “DNA-Sorb” extraction kit. It is the user’s responsibility if other kits than “DNA-Sorb” are used to perform this DNA extraction.
11. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

STORAGE INSTRUCTIONS
Ur. parvum/urealyticum Real-TM must be stored at 2-8°C. TaqF Polymerase must be stored at -20°C. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT
Ur. parvum/urealyticum Real-TM can analyze DNA extracted with DNA-Sorb-A (REF K-1-1/A) 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: transfer 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.

QUALITY CONTROL
The complete kit has been tested on an RotorGene 6000 (Corbett Research).
Certificates of Analyses are available on request at info@sacace.com.
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– (Neg Control provided with the amplification kit) to the tube labeled Cneg.**
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 **10*N µl of PCR-mix-1-FRT, 5*N µl of PCR-Buffer-FRT and 0.5*N µl of TaqF DNA Polymerase.** Vortex and centrifuge briefly.
3. Add to each tube **15 µl of Reaction Mix and 10 µl of extracted DNA. 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 Complex C+** to the tube labeled Amplification Positive Control;
5. Insert the tubes in the thermalcycler. The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.
Real Time Amplification with Rotor-Gene™ 3000/6000

1. Create a template for “Urogenital Assays” by activating in the window New Run the programming regime Advanced. Choose Dual Labeled Probe/Hydrolysis probes and click the button New.

2. Select in the new window the carousel type 26-Well Rotor or 72-Well Rotor and Reaction Volume (μL) 25.

3. Set in the window Edit Profile program “STD 65-60-45 RG-TaqF” (this program is universal for all Sacace™ Urogenital Assays):

   1. Hold                                       95 deg – 15 min
   2. Cycling                                  95 deg - 20 secs
                                               65 deg - 20 secs
                                               72 deg - 20 secs
                                               Cycle repeats – 10 times.
   3. Cycling 2                                 95 deg - 20 secs
                                               60 deg - 30 secs – Acquiring on Fam (Green), Joe (Yellow) *
                                               72 deg -15 secs
                                               Cycle repeats – 35 times

   fluorescence detection on the channels Fam (Green), Joe (Yellow) and Rox (Orange) for 4x Rotor-Gene on the 2-nd pass (60°C)

4. Make the adjustment of the fluorescence channel sensitivity: Channel Setup → Calibrate (Gain Optimisation for RG6000) → Auto Gain Calibration (Optimisation) Setup → Calibrate Acquiring (Optimise Acquiring) and select Perform Calibration (Optimisation) Before 1-st Acquisition. For Fam/Syb (Green) channel indicate Min Reading 5. Max Reading 10, for Joe (Yellow) channel Min Reading 4. Max Reading 8 and for 4x Rotor-Gene Rox (Orange) Min Reading 1, Max Reading 5. In the column Tube position program position of the tubes in the carousel of the Rotor-Gene 2000/3000/6000 (the 1-st position must contain reaction tube with reagents). Close the window Auto Gain Calibration Setup.

RESULTS ANALYSIS:

1. The results are interpreted with the software of Rotor-Gene 3000/6000 through the presence of crossing of fluorescence curve with the threshold line. Ur.parvum is detected on the FAM (Green) channel, Ur.urealyticum on the JOE channel (Yellow) and IC DNA on the ROX (Orange) channel.
2. Press Analysis then select button Quantitation. Perform the operation for the channel Fam (Cycling A FAM), then for the channels Joe (Cycling A JOE), ROX (Cycling A ROX).
3. For the channel Fam select Dynamic Tube, More Setting (Outlier Removal) 0%, Threshold: 0,1.
4. For the channel Joe select Dynamic Tube, Slope Correct, More Setting (Outlier Removal) 5%, Threshold: 0,1
5. For the channel Rox select Dynamic Tube, Slope Correct, More Setting (Outlier Removal) 5%, Threshold: 0,1
6. Specimens with Ct ≥ 32 in the channel Fam (Quant. Results – Cycling A FAM) are interpreted as positive for Ureaplasma parvum.
7. Specimens with Ct ≥ 32 in the channel Joe (Quant. Results – Cycling A JOE) are interpreted as positive for Ureaplasma urealyticum
8. The sample is considered to be negative if in the channels Fam and Joe the Ct value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Rox the Ct value is lower than 30.

Table 2. Results for controls

<table>
<thead>
<tr>
<th>Control</th>
<th>Stage for control</th>
<th>Ct channel Fam (Green)</th>
<th>Ct channel Joe (Yellow)</th>
<th>Ct channel Rox (Orange)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS DNA isolation</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos (&lt;30)</td>
<td></td>
<td>Valid result</td>
</tr>
<tr>
<td>DNA-buffer</td>
<td>Amplification</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos (&lt;30)</td>
<td>Valid result</td>
</tr>
<tr>
<td>Pos Complex C+</td>
<td>Amplification</td>
<td>Pos (&lt;30)</td>
<td>Pos (&lt;30)</td>
<td></td>
<td>Valid result</td>
</tr>
</tbody>
</table>

Example

632, 648, 668, 690, 701, 716, 716, 721, 724 – Positive Samples for U.parvum.
672, 678, 698, 708, 718 – Positive Samples for U.urealyticum.
706 Positive Sample for U.parvum and U.urealyticum.
Real Time Amplification with SmartCycler® (Cepheid)

Program SmartCycler as follows:

1. Transfer the SmartCycler tubes into the rotor of the minicentrifuge and centrifuge briefly (5-7 sec).
2. Select in the main menu Define Protocols and in the lower left corner select option New Protocol. Assign a name to the protocol and set the following parameters:
4. Press the button Define Graphs. In the opened window click New Graph. In the new window enter the name of graphic – Ureaplasma parvum. Choose Automatically added to new Runs, in the window Graph Type select Optics and choose Channels: Ch1, Ch2, Ch3. Click Save Graph.
5. Select in the main menu option Create Run and in the window Run Name give a name to the experiment.
6. Click button Dye Set and select FCTC25.
7. Choose Add/Remove Sites and select in the new window the Protocol and Sites for analysis. Click OK.
8. Transfer reaction tubes into the SmartCycler and start the experiment by pressing Start Run button.
9. In the menu View Results press Results Table and insert in the column Sample ID the name of the samples.

Results Analysis

1. Press Analysis settings and in the column Manual Thresh Fluor Units set value of the threshold line to 30 for the channels Fam, Cy3 and Texas Red. Click Update Analysis.
2. Click Save Run in the menu Results Table.
3. The sample is considered to be Positive for Ureaplasma parvum if in the column FAM Std/Res the result is indicated as POS (value of FAM Ct is different from zero). If the Ct value of the IC is higher than 35 a retesting of the sample is required.
4. The sample is considered to be Positive for Ureaplasma urealyticum if in the column Cy3 Std/Res the result is indicated as POS (value of Cy3 Ct is different from zero). If the Ct value of the IC is higher than 35 a retesting of the sample is required.
5. The sample is considered to be Negative if in the column FAM Std/Res and Cy3 Std/Res the result is indicated as NEG (value of FAM and Cy3 Ct = zero) and in the column TxR Std/Res the result is indicated as POS (if Ct > 40 retesting of sample is required).
6. The result is invalid if in the column FAM Std/Res, Cy3 Std/Res and TxR Std/Res the result is indicated as NEG (Ct = 0). Repeat the entire test including sample preparation and amplification.
7. Result is accepted as significant only when Positive and Negative Controls of amplification and DNA isolation are valid.

### Table 2. Results for controls

<table>
<thead>
<tr>
<th>Control</th>
<th>Stage for control</th>
<th>Ct channel Fam</th>
<th>Ct channel Cy3</th>
<th>Ct channel TxR</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>DNA isolation</td>
<td>NEG</td>
<td>NEG</td>
<td>POS</td>
<td>Valid result</td>
</tr>
<tr>
<td>DNA-buffer</td>
<td>Amplification</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td>Valid result</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>Valid result</td>
</tr>
</tbody>
</table>
Real Time Amplification with iQ iCycler™ and iQ5 (Bio-Rad)

1. Schedule in the window Edit Plate Setup of Workshop module the tube positions and the fluorescence signal detection in all tubes on the channels Fam and Hex. Save it and use this scheme by activating the button Run with selected protocol.
   - For iQ5 instrument edit the scheme in the regime Whole Plate loading; Select Sample Volume 25 µl, Seal Type: Domed Cap, Vessel Type: Tubes. Click the button Save & Exit Plate Editing.
2. Start on the iQiCycler or iQ5 the program “STD 65-60-45 iQ-TaqF”, choose or create it in the module View Protocols and start by activating the button Run with selected plate setup.
   95°C – 13 min 30 sec  
   10 cycles: 95°C – 10 sec, 65°C – 20 sec, 72°C – 20 sec  
   35 cycles: 95°C – 10 sec, 60°C – 30 sec, 72°C – 20 sec  
   fluorescence detection on the channels Fam, HEX and ROX on the 2-nd step (60°C)
3. Make sure that the following iQ iCycler settings for dynamicwf are selected:
4. Transfer tubes in the thermalcycler in accordance with the previously created model.
5. Select Experimental Plate under the line Select well factor source and choose the reaction volume 25 µl (for iQ iCycler).
6. Click Run button.

DATA ANALYSIS

The results are interpreted with the software of “iQ iCycler” or “iQ5” through the presence of crossing of fluorescence curve with the threshold line.

*Ureaplasma parvum* is detected on the FAM channel, *Ureaplasma urealyticum* on the HEX channel, *IC DNA* on the ROX channel.

Put the threshold line (with the left button of the mouse) at such level where curves of fluorescence are linear.
- The sample is considered to be positive for *Ureaplasma parvum* if in the channel Fam (FAM-490 in the window Select a Reporter) the value of Ct is different from zero (Ct < 33).
- The sample is considered to be positive for *Ureaplasma urealyticum* if in the channel HEX (HEX-530 in the window Select a Reporter) the value of Ct is different from zero (Ct < 33)
- Specimens with Ct ≤ 33 in the channel ROX (ROX-575 in the window Select a Reporter) and absent fluorescence signal (N/A value) in the channels FAM and HEX are interpreted as negative.
Program Applied Biosystems® 7300/7500 Real Time PCR Systems as follows:

1. Select in the main menu option “New” and set the data of new document: select in the window Assay the option Absolute Quantitation, in the window Template the option Blank Document. Press OK.

2. In the new window in the Tools menu click button Detector Manager.
3. In the lower left corner of the window click File and select New. Set in the window New detector probes features:
   a) Detection of “U.parvum”: in the lines Name and Description indicate POS; in the line Reporter Dye – Fam and in Quencher Dye – None. Select the Color (for example, red). Click button Create Another.
   b) Detection of “U.urealyticum”: in the lines Name and Description indicate POS; in the line Reporter Dye – Joe and in Quencher Dye – None. Select the Color (for example, green). Click button Create Another.
   c) The window New detector is opened against. Set the following parameters for Internal Control: in the lines Name and Description indicate IC; in the line Reporter Dye – Rox and in Quencher Dye – None. Select the Color (for example, blue). Click OK.
4. Close the window Detector manager with probes information.
5. Select window Instrument.
6. Activate Thermal profile and set the following amplification program:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Profile</th>
<th>Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C – 15:00</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95°C – 0:20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>65°C – 0:20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C – 0:20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>95°C – 0:25</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>60°C – 0:50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C – 0:15</td>
<td></td>
</tr>
</tbody>
</table>

*a*Fluorescence detection on the Fam, Joe, Rox channels

8. Save created document: in the menu File select Save as..., in the line File type select SDS Templates (*.sdt) and click Save.
9. In the upper right corner of the window choose Setup. In the opened window Plate, with the mouse select the cells in which the amplification has been planned. In the menu View click button Well inspector.
10. Click button Add Detector and select probes from the window Detector manager. To do this, select lines with mouse and click button Add to Plate Document and Done.
11. In the column Use of the window Well inspector select probes POS and IC.
12. In the lower right corner of the window in the line Passive Reference set none.
13. In the field Sample Name insert name of the samples.

Results Analysis

The results are interpreted with the software of Applied Biosystems® 7300/7500 Real Time PCR Systems through the presence of crossing of fluorescence curve with the threshold line. U.parvum is detected on the FAM channel, U.urealyticum on the JOE channel and IC DNA on the ROX channel:

a. Specimens with Ct < 35 in the FAM channel are interpreted as Positive Ureaplasma parvum
b. Specimens with Ct < 35 in the JOE channel are interpreted as Positive Ureaplasma urealyticum
c. Specimens with absent Ct (“Undet.”) in the ROX channel and Ct < 40 in the JOE channel are interpreted as Negative

Result is accepted as significant only when Positive and Negative Controls of amplification and DNA isolation are valid.
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/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. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity
The kit *Ur. parvum/urealyticum Real-TM* allows to detect *Ureaplasma parvum/urealyticum* DNA in 100% of the tests with a sensitivity of not less than 1000 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: urease complex component gene

*PCR: The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-La Roche and applicable in certain countries. Sacace does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this kit is recommended for persons that either have a license to perform PCR or are not required to obtain a license.