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

Handbook

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

for use with 4 channels Real Time PCR instruments, like RotorGene™ 6000/Q (Corbett Research, Qiagen), SmartCycler® (Cepheid), iQ5, CFX™ (Biorad), MX3005P® (Stratagene), Applied Biosystems® 7500 Time PCR Systems (Applera), Eco Real Time PCR System® (Illumina)

REF B75-100FRT
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. 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 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, QS 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

<table>
<thead>
<tr>
<th>Contents</th>
<th>100 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-mix-1</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>PCR-buffer-FRT</td>
<td>2 x 0.3 ml</td>
</tr>
<tr>
<td>TaqF Polymerase</td>
<td>2 x 0.03 ml</td>
</tr>
<tr>
<td>TE-buffer</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Negative Control C-*</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>• Standard</td>
<td></td>
</tr>
<tr>
<td>o QSG1</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>o QSG2</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

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

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA isolation kit
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
3. Do not use a kit after its expiration date.
4. Do not mix reagents from different kits.
5. Dispose all specimens and unused reagents in accordance with local regulations.
6. Heparin has been shown to inhibit reaction. The use of heparinized specimens is not recommended.
7. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
8. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
9. Prepare quickly the Reaction mix.
10. Specimens may be infectious. Use Universal Precautions when performing the assay.
11. Specimens and controls should be prepared in a laminar flow hood.
12. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
13. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant. Follow by wiping down the surface with 70% ethanol.
14. 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.
15. Material Safety Data Sheets (MSDS) are available on request.
16. Use of this product should be limited to personnel trained in the techniques of amplification.
17. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification Area. Do not return samples, equipment and reagents in the area where you performed previous step. Personnel should be using proper anti-contamination safeguards when moving between areas.

STORAGE INSTRUCTIONS

U.parvum/U.urealyticum/M.hominis Real-TM Quant must be stored at -20°C. The kit can be shipped at 2-8°C but should be stored at -20°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.
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

The following isolation kit is recommended:
⇒ DNA-Sorb-A (Sacace, REF K-1-1/A).

Please carry out the DNA extraction according to the manufacturer’s instructions.

**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 buffer and 0,5*N 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 QS2 to the tube labeled Cpos;
   - add 10 µl of TE-buffer to the tube labeled Cneg;
6. For quantitative analysis prepare 4 tubes and perform QS1 and QS2* standards twice.
   - QS1 and QS2 values are specific for each lot and are reported in the Quant Data Card provided in the kit.

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp, °C</th>
<th>Time</th>
<th>Fluorescence detection</th>
<th>Cycle repeats</th>
<th>Temp, °C</th>
<th>Time</th>
<th>Fluorescence detection</th>
<th>Cycle repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95</td>
<td>15 min</td>
<td>–</td>
<td>1</td>
<td>95</td>
<td>15 min</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Cycling</td>
<td>95</td>
<td>5 s</td>
<td>–</td>
<td>5</td>
<td>95</td>
<td>5 s</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>20 s</td>
<td>–</td>
<td></td>
<td>60</td>
<td>20 s</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>15 s</td>
<td>–</td>
<td></td>
<td>72</td>
<td>15 s</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5 s</td>
<td>–</td>
<td></td>
<td>95</td>
<td>10 s</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cycling 2</td>
<td>60</td>
<td>20 s</td>
<td>FAM(Green), JOE(Yellow), Rox(Orange)</td>
<td>40</td>
<td>60</td>
<td>40 s</td>
<td>FAM, JOE/HEX/Cy3, Rox/TexasRed, Cy5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>15 s</td>
<td>–</td>
<td></td>
<td>72</td>
<td>15 s</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qagen)
*2 For example, iQ5™/iCycler™ (BioRad); Mx3000P/Mx3005P™ (Stratagene), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

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

<table>
<thead>
<tr>
<th>Channel</th>
<th>Threshold</th>
<th>More Settings/Outlier Removal</th>
<th>Slope Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM/Green</td>
<td>0.1</td>
<td>10 %</td>
<td>on</td>
</tr>
<tr>
<td>JOE/Yellow</td>
<td>0.1</td>
<td>10 %</td>
<td>on</td>
</tr>
<tr>
<td>Rox/Orange</td>
<td>0.1</td>
<td>10 %</td>
<td>on</td>
</tr>
<tr>
<td>Cy5/Red</td>
<td>0.1</td>
<td>10-30 %</td>
<td>on</td>
</tr>
</tbody>
</table>

**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 QS1 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 parvum* is detected on the FAM (Green) channel, *Ureaplasma urealyticum* on the JOE (Yellow)/Cy5/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**

<table>
<thead>
<tr>
<th>Control</th>
<th>Stage for control</th>
<th>Ct FAM (Green)</th>
<th>Ct JOE(Yellow)/HEX/Cy3</th>
<th>Ct ROX (Orange)/TexasRed</th>
<th>Ct Cy5 (Red)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C- DNA isolation</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>OK</td>
</tr>
<tr>
<td>TE-buffer PCR</td>
<td></td>
<td>–</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>OK</td>
</tr>
<tr>
<td>QS2 PCR</td>
<td>Pos</td>
<td>Pos</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>OK</td>
</tr>
</tbody>
</table>

- 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:

\[
(U. parvum, U.urealyticum, M.hominis \text{ reaction} \times 200^* = \text{copies DNA/ml})
\]

**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 EBV DNA in \(10^5\) cells using the following formula:

\[
\frac{Up\ (Uu, Mh)\ DNA\ copies/reaction}{IC\ Glob\ DNA\ copies/reaction} \times 2 \times 10^5 = Up\ (Uu, Mh)\ DNA/10^5\ cells
\]
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

- REF: List Number
- LOT: Lot Number
- IC: Internal Control
- NCA: Negative Control of Amplification
- C−: Negative control of Extraction
- C+: Positive Control of Amplification
- !: Caution!
- Σ: Contains sufficient for <n> tests
- VER: Version
- ⌚: Expiration Date

Consult instructions for use