Treponema pallidum 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), MX3000P® and MX3005P® (Stratagene)

**INTENDED USE**
Kit *Treponema pallidum Real-TM* is a test for the qualitative detection of *Treponema pallidum* in the plasma, liquor, amniotic liquid, tissue and other biological materials.

**PRINCIPLE OF ASSAY**
Kit *Treponema pallidum Real-TM* is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Treponema pallidum* DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *Treponema pallidum* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *Treponema pallidum*.  

**MATERIALS PROVIDED**
- PCR-mix-1-FRT, 1.2 ml;
- PCR-Buffer-FRT, 2 x 0.35 ml;
- TaqF Polymerase, 2 x 0.03 ml;
- Pos 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 lysates mixture (see DNA-Sorb-A [REF K.1-1/A protocol].

**NAME**
*Treponema pallidum Real-TM*
MATERIALS REQUIRED BUT NOT PROVIDED
• Real Time Thermal cycler
• Reaction tubes
• Workstation
• Pipettes (adjustable)
• Sterile pipette tips with filters
• Desktop centrifuge with rotor for 1,5/2,0 ml tubes
• Vortex mixer
• Freezer, refrigerator

WARNINGS AND PRECAUTIONS
1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. 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.
8. Material Safety Data Sheets (MSDS) are available on request.
9. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
10. 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
Treponema pallidum 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
Treponema pallidum 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
Treponema pallidum Real-TM can analyze DNA extracted from:
• plasma collected blood in ACD or EDTA tubes;
• liquor stored in “Eppendorf” tube;
• amniotic liquid stored in “Eppendorf” tube;
• tissue (≈1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
• serous exudation from primary syphilis hard chancre and other syphilitic lesions: insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium or sterile Saline solution. Vigorously agitate swabs in medium for 15-20 sec.

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 kit is recommended:
• DNA-Sorb-B (Sacace, K-1-1/B)

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.

(Note: the Sacace Internal Control is the same for all urogenital infection Real Time kits)

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-Buffer-FRT and 0,5*(N+1) of TaqF DNA 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.

Treponema pallidum is detected on the FAM (Green) channel, IC DNA on the JOE( Yellow)/HEX/Cy3 channel.
Program SmartCycler as follows:
1. 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:

2. Choose Save Protocol.
3. Select in the main menu option Create Run and in the window Run Name name the experiment.
4. Click button Dye Set and select FCTC25.
5. Choose Add/Remove Sites and select in the new window the Protocol and Sites for analysis. Click OK.
6. Transfer reaction tubes into the SmartCycler and start the experiment by pressing Start Run button.
7. In the menu View Results press Results Table and insert in the column Sample ID 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 and 30 for the channel Cy3. Click Update Analysis.
2. Click Save Run in the menu Results Table.
3. The sample is considered to be Positive 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 40 a retesting of the sample is required.
4. The sample is considered to be Negative if in the column FAM Std/Res the result is indicated as NEG (value of FAM Ct = zero) and in the column CY3 Std/Res the result is indicated as POS. Negative samples Ct values on the Cy3 channel in a range of 40-42 cycles testify an inefficient DNA extraction (if Ct > 40 retesting of sample is required).
5. The result is invalid if in the column FAM Std/Res and in the column CY3 Std/Res the result is indicated as NEG (Ct = 0). Repeat the entire test including sample preparation and amplification.
6. 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>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>DNA isolation</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>Valid result</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>POS</td>
<td>POS</td>
<td>Valid result</td>
</tr>
</tbody>
</table>

Example:

FAM Channel – Treponema pallidum DNA detection:

Cy3 Channel – Internal Control DNA detection:

Table 2. Results for controls
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

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

5. Save the protocol in the folder Programm files / RotorGene 6 / Templates (for RG6000 Programm files / RotorGene 6000 Software / Templates) and close the window New Run Wizard. The new template appears in the list of templates in the window New Run and can be used for all Sacace™ Real-TM Urogenital Assays.

START AMPLIFICATION AND DETECTION

1. Select Quick Start (Advanced Start for RG6000) in window New Run and choose from the templates “STD 65-60-45 RG-TaqF”.

2. Select carousel type and mark No Domed 0,2 Tubes for 36-Well Rotor or Locking Ring Attached for 72-Well Rotor. Click Next.

3. Program position of the tubes in the window Sample Setup.

4. Click Next and Start Run to begin the experiment.

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. Treponema pallidum is detected on the FAM (Green) channel, Internal Control on the JOE (Yellow) channel

2. Press Analysis then select button Quantitation. Perform the operation for the Fam (Green) channel (Cycling A FAG or Cycling A. Green for RG6000), then for the Joe (Yellow)channel (Cycling A JOE or Cycling A. Yellow)

3. For the Fam (Green) channel (Treponema pallidum) select Dynamic Tube, More Setting (Outlier Removal for RG6000) 0%, and Threshold: 0,05.

4. For the Joe (Yellow) channel (IC) select Dynamic Tube, More Setting (Outlier Removal for RG6000) 5%. Threshold: 0,1

5. Specimens with Ct < 33 in the channel Fam (Green) (Quant. Resultes – Cycling A. FAG/Green) are interpreted as positive.

6. Specimens with Ct < 30 in the channel Joe (Yellow) (Quant. Resultes – Cycling A.JOE/Yellow) and absent fluorescence signal in the channel Fam (Green) are interpreted as negative.

7. Specimens with absent signal in the FAM (Green) and JOE (Yellow) (or Ct > 30) are interpreted as invalid.

Table 2. Results for controls

<table>
<thead>
<tr>
<th>Control</th>
<th>Step control</th>
<th>Ct channel Fam (Green)</th>
<th>Ct channel Joe (Yellow)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS DNA isolation</td>
<td>No signal</td>
<td>No signal</td>
<td>&lt; 30</td>
<td>Valid result</td>
</tr>
<tr>
<td>DNA buffer Amplification</td>
<td>No signal</td>
<td>Amplification</td>
<td>No signal</td>
<td>Valid result</td>
</tr>
<tr>
<td>Ct Amplification</td>
<td>&lt; 30</td>
<td>&lt; 30</td>
<td>Valid result</td>
<td></td>
</tr>
</tbody>
</table>

Example:
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 and HEX 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.

*Treponema pallidum* is detected on the FAM channel, *IC DNA* on the HEX channel.

For Fam and Hex channels activate the button “Log View”. 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 *Treponema pallidum* if in the channel Fam (FAM-490 in the window Select a Reporter) the value of Ct is different from zero (Ct < 33).
- Specimens with Ct ≤ 33 in the channel HEX (HEX-530 in the window Select a Reporter) and absent fluorescence signal (N/A value) in the channel Fam are interpreted as negative.
- Specimens with Ct absent or > 33 in the FAM and HEX channels are interpreted as invalid.

<table>
<thead>
<tr>
<th>Control</th>
<th>Step control</th>
<th>Ct channel Fam</th>
<th>Ct channel HEX</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS DNA isolation</td>
<td>No signal</td>
<td>≤ 33</td>
<td>≤ 33</td>
<td>Valid result (Negative)</td>
</tr>
<tr>
<td>DNA-buffer Amplification</td>
<td>No signal</td>
<td>No signal</td>
<td>≤ 33</td>
<td>Valid result (Negative)</td>
</tr>
<tr>
<td>C+ Amplification</td>
<td>≤ 33</td>
<td>≤ 33</td>
<td></td>
<td>Valid result (Positive)</td>
</tr>
</tbody>
</table>

**FAM Channel – *Treponema pallidum* DNA detection**

**HEX Channel – Internal Control DNA detection**
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 “POS”: 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) 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 – Joe 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>

*Fluorescence detection on the Fam, Joe 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 POS and IC 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
1. 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. Pos result is detected on the FAM channel, Internal Control on the JOE channel
   a. Specimens with Ct < 40 in the Fam channel are interpreted as Positive regardless of the Joe channel (IC) results
   b. Specimens with absent Ct (“Undet.”) in the Fam channel and Ct < 40 in the Joe channel are interpreted as Negative
   c. The result is invalid if in the column Fam and in the column Joe the result is indicated as “Undet.” or with Ct >40. Repeat the entire test including sample preparation and amplification.
2. 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 Joe</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>DNA isolation</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>Valid result</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>POS</td>
<td>POS</td>
<td>Valid result</td>
</tr>
</tbody>
</table>
Program MX3000P® and MX3005P® (Stratagene) as follows:

1. Open the program, select “Quantitative PCR (Multiple Standards)” and click “OK”

2. At the top left of the window choose “Plate Setup”

3. In the window “Well type” set “Unknown” for the samples.

4. In the window “Collect fluorescence data” select for all samples the channels Fam and Joe.

5. At the top left of the window select button “Thermal Profile Setup”

6. Set the following parameters of amplification:
   - **Hold** 95°C – 15 min
   - **Cycling 1** 95°C – 20 sec
   - 65°C – 30 sec
   - 72°C – 20 sec
     - **Cycle Repeats** – 10 times
   - **Cycling 2** 95°C – 25 sec
   - 65°C – 50 sec*
   - 72°C – 20 sec
     - **Cycle Repeats** – 35 times
   *
   Fluorescence is measured at 65°C on the 2nd Cycling.

7. Click “Run” button, enter a name for the experiment and save it.

**Results Analysis**

1. Soon after amplification is over, choose button “Analysis” at the top left of the window.

2. Choose button “Results”

3. At the right angle of the window “Area to analyze” select “Amplification plots”.

4. The results are interpreted with the software of the instrument through the presence of crossing of fluorescence curve with the threshold line. *Treponema pallidum* is detected on the FAM channel, *Internal Control* on the JOE channel.

5. Inhibition of IC may occur in specimens with high initial concentration of *Treponema pallidum*.

6. Specimens with Ct < 40 in the Fam channel are interpreted as positive for *Treponema pallidum* regardless of the Joe channel (IC) results.

7. Specimens with Ct > 40 or absent in the Fam channel are interpreted as negative for *Treponema pallidum*.

8. Specimens with absent signal in the FAM and JOE are interpreted as invalid.

**Table 2. Results for controls**

<table>
<thead>
<tr>
<th>Control</th>
<th>Stage for control</th>
<th>Ct channel Fam</th>
<th>Ct channel Joe</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>DNA isolation</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>Valid result</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>POS</td>
<td>POS</td>
<td>Valid result</td>
</tr>
</tbody>
</table>
TROUBLESHOOTING

1. Weak or no signal of the IC (Joe/Hex/Cy3 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
   - 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.

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 Treponema pallidum primers and probes. The specificity of the kit Treponema pallidum Real-TM was 100%. The potential cross-reactivity of the kit Treponema pallidum Real-TM was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity
The kit Treponema pallidum Real-TM allows to detect Treponema pallidum DNA in 100% of the tests with a sensitivity of not less than 500 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: antigen gene

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