Neisseria gonorrhoeae Real-TM

HANDBOOK

for use with RotorGene™ 3000/6000/Q (Corbett Research, Qiagen), SmartCycler® (Cepheid), , iQ iCycler™ and iQ5™ (Biorad), MX3000P® and MX3005P® (Stratagene), Applied Biosystems® 7300/7500/StepOne Real Time PCR Systems (Applera), LightCycler 2.0® (Roche), LineGene K® (Bioer), Eco Real Time PCR System® (Illumina)

REF B5-100FRT
VER 06.05.2011
**NAME**
Neisseria gonorrhoeae 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: Neisseria gonorrhoeae, gonorrhea, herpes, HIV, HPV, syphilis, ureaplasma, mycoplasma, 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 Neisseria gonorrhoeae Real-TM is a test for the qualitative detection of *Neisseria gonorrhoeae* in the urogenital swabs, urine, prostatic liquid and other biological materials.

**PRINCIPLE OF ASSAY**
Kit Neisseria gonorrhoeae Real-TM is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Neisseria gonorrhoeae* DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *Neisseria gonorrhoeae* 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 *Neisseria gonorrhoeae*.

**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-, 1,2 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**
- 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
Neisseria gonorrhoeae 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
Neisseria gonorrhoeae 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
Neisseria gonorrhoeae Real-TM can analyze DNA extracted from:
• 
cervical, urethral, conjunctival 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.

DNA ISOLATION
The following kit is recommended:
⇒ DNA-Sorb-A (Sacace, REF K-1-1/A)
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 $10^*(N+1) \mu l$ 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 $15 \mu l$ of Reaction Mix and $10 \mu l$ of extracted DNA sample to appropriate tube. Mix by pipetting.
4. Prepare for each panel 2 controls:
   - add $10 \mu l$ of DNA-buffer to the tube labeled Amplification Negative Control;
   - add $10 \mu l$ of Positive Control C+ to the tube labeled Amplification Positive Control;
5. Insert the tubes in the thermalcycler.

Program the Real Time PCR instrument according to Table*.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature, °C</th>
<th>Time</th>
<th>Cycles</th>
<th>Rotor and plate type instruments1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>15 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5 s</td>
<td>5</td>
<td></td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>5 s</td>
<td>40</td>
<td>Fluorescence detection*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30 s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>15 s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen), iQ5™/iQ iCycler™ (BioRad); Mx3000P/ Mx3005P™ (Stratagene), Applied Biosystems® 7300/7500/StepOne Real Time PCR (Applera), LineGeneK™ (Bioer), Eco Real Time PCR System® (Illumina)

*for instruments mentioned in attachments follow the amplification program reported below

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

*Neisseria gonorrhoeae* is detected on the FAM (Green) channel, *IC DNA* on the JOE(Yellow)/HEX/Cy3 channel
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 Neisseria gonorrhoeae primers and probes. The specificity of the kit Neisseria gonorrhoeae Real-TM was 100%. The potential cross-reactivity of the kit Neisseria gonorrhoeae Real-TM was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity
The kit Neisseria gonorrhoeae Real-TM allows to detect Neisseria gonorrhoeae 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.
EXPLANATION OF SYMBOLS

REF  Catalogue Number

IVD  For In Vitro Diagnostic Use

LOT  Lot Number

Expiration Date

Σ  Contains reagents

Caution!

VER  Version

Manufacturer

Temperature limitation

* Cycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Corbett Research
* MX3000P® and MX3005P® are trademarks of Stratagene
* Applied Biosystems® is trademarks of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid
* LightCycler® is a registered trademark of Roche
* LineGene K® is a registered trademark of Bioer
* Eco PCR Real Time System® is a registered trademark of Illumina
Add 1. Programming of SmartCycler® (Cepheid)

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 – Neisseria gonorrhoeae DNA detection:

Cy3 Channel – Internal Control DNA detection:
Add 2. Programming of RotorGene™ 3000/6000/Q

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 36-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 1-st Acquistion. 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 Program 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/Q through the presence of crossing of fluorescence curve with the threshold line. Neisseria gonorrhoeae 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 FAM 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 (Neisseria gonorrhoeae) 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. FAM/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>
<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</td>
<td>DNA isolation</td>
<td>No signal</td>
<td>&lt; 30</td>
<td>Valid result</td>
</tr>
<tr>
<td>DNA-buffer</td>
<td>Amplification</td>
<td>No signal</td>
<td>No signal</td>
<td>Valid result</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>&lt; 30</td>
<td>&lt; 30</td>
<td>Valid result</td>
</tr>
</tbody>
</table>
Example

1, 2, 3, 5 – Positive Samples; 23, 24, 26, 31 – Negative Samples;
Add 3. Programming of iQ iCycler™ and iQ5

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 Joe 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. Neisseria gonorrhoeae 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 Neisseria gonorrhoeae 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 2. Results for controls

<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</td>
<td>DNA isolation</td>
<td>No signal</td>
<td>≤ 33</td>
<td>Valid result (Negative)</td>
</tr>
<tr>
<td>DNA-buffer</td>
<td>Amplification</td>
<td>No signal</td>
<td>No signal</td>
<td>Valid result (Negative)</td>
</tr>
<tr>
<td>C+</td>
<td>Amplification</td>
<td>≤ 33</td>
<td>≤ 33</td>
<td>Valid result (Positive)</td>
</tr>
</tbody>
</table>

FAM Channel – Neisseria gonorrhoeae DNA detection

HEX Channel – Internal Control DNA detection
Add 4. Programming of MX3000P® and MX3005P®:

1. Open the program, select “Quantitative PCR (Multiple Standsarts)” 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. *Neisseria gonorrhoeae* 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 *Neisseria gonorrhoeae*.

6. Specimens with Ct < 40 in the FAM channel are interpreted as positive for *Neisseria gonorrhoeae* regardless of the Joe channel (IC) results.

7. Specimens with Ct > 40 or absent in the FAM channel are interpreted as negative for *Neisseria gonorrhoeae*.

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>
Add 5. Programming of Applied Biosystems® 7300/7500/StepOne Real Time PCR Systems:
1. Select in the main menu the option “New Experiment” (Advanced Setup), insert experiment details. Select your Instrument Type (7500 / 7500 fast/StepOne/StepOne Plus), Quantitation – Standard curve, Taqman Reagents, Standard ramp speed (DO NOT select Fast RampSpeed).

Note: when using StepOne software, be sure to click New Experiment – Advanced Setup

2. Click , add two targets: one for Pathogen (Reporter FAM) and the other one for Internal Control (Reporter Joe); select Quencher – None for both targets.

3. Click Add new samples multiple times and enter name for all samples and controls.

4. Click Assign target and Samples tab, associate samples ( ) with the two targets and select None as passive reference dye as shown in the following pictures:

5. Click , insert Reaction volume (25 µl) and program thermalcycling as follows:

6. Click to begin PCR reaction.

7. After the run is complete, select all samples and standards and click .

8. Deselect Use default settings, Automatic threshold and automatic baseline; suggested values are*:

   * for StepOne instrument suggested Threshold values are: Joe (Internal Control) = 1,000, Fam (Pathogen) = 10,000. NOTE: please slightly adapt suggested values according to your result curves

9. Click .
10. The results are interpreted with the software of Applied Biosystems® 7500/7300/StepOne Real Time PCR Systems through the presence of crossing of fluorescence curve with the threshold line. Pathogen is detected on the FAM channel, Internal Control on the JOE channel.

![FAM – Pathogen](image1)

![Joe – Internal control](image2)

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>
Add 6. Programming of Line-Gene K:

Open LineGeneK Software. Click File -> New. In the Setup Programs tab, insert User Name and Test Name. Click Dyes button and select FAM, JOE. Insert the correct liquid quantity (25 µl).

Click the button 3 times and program the 3 thermalcycling steps. At the end it must look like this:

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Sample Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>None</td>
</tr>
<tr>
<td>60°C</td>
<td>Single</td>
</tr>
<tr>
<td>95°C</td>
<td>None</td>
</tr>
</tbody>
</table>

95°C – 5 min
10 cycles: 95°C – 20 sec, 65°C – 20 sec, 72°C – 20 sec
35 cycles: 95°C – 20 sec, 60°C * – 30 sec, 72°C – 15 sec

* On the second phase (60.0 °C) of step 3 select “Single” on the Sample Mode column to collect fluorescence data, as shown in this picture:

Click the menu System Parameters (P) -> Gain Setup (G). Select Auto Gain box and click OK (see picture below for Gain Setup details).

Click the menu Sample Information (S) -> Sample Group Setup…(R). Select at least 2 groups.

Click the menu Sample Information (S) -> Sample Data…(S). Insert Sample names.

Click or click File -> Run Programs (R) to Run the PCR program.

Results Analysis - FAM
In the Quantitative Analysis tab, select from the menu Dyes(D) -> FAM(F).
Then do in this order:
1) Under Analysis Method select *Fit Points*.
2) Under Analysis Step Click *Zero Adjust*, select *Auto* and then click *OK*. Then click *Baseline*
3) Select 2 points and set the base line (drag and drop the red baseline) *as low as possible but above the noise of each sample* (usually 1-4)
4) Under Analysis Step click *Analysis*. Ct Results of *Pathogen* should appear in the *Ct* column.

Results Analysis - Joe
In the *Quantitative Analysis* tab, select from the menu Dyes(D) -> Joe(F).

Then do in this order:
1) Under Analysis Method select *Fit Points*.
2) Under Analysis Step Click *Zero Adjust*, select *Auto* and then click *OK*. Then click *Baseline*
3) Select 2 points and set the base line (drag and drop the red baseline) *as low as possible but above the noise of each sample*.
4) Under Analysis Step click *Analysis*. Ct Results of Internal Control DNA should appear in the *Ct* column.

Pathogen – FAM

Internal control DNA – Joe
Add 6. Programming of Eco Real Time PCR System (Illumina):

1. Open Eco software, click and under Experiment Type select , select DNA as starting material.

2. Under Quantification Method select , and insert experiment name. Click .

3. Click , set up two assays: one for Internal Control (Reporter HEX) and the other one for Pathogen detection (Reporter Fam); select Quencher – Non fluorescent for both targets. Click .

4. Click and enter name for all samples and controls. Click .

5. Associate samples and standards with the two previously designed assays following this procedure: select the well, select Unknown for samples and controls and to assign the corresponding assay click the white circle of the Pathogen and Internal control assays (the circle will become colored): for each well both Assays must be assigned.

6. Click and program the instrument as follows (reaction volume is 20 µl**):

<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 – 0:10</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>60°C – 0:30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C – 0:20</td>
<td></td>
</tr>
</tbody>
</table>

** Add 12 µl of Reaction Mix into each tube. Add 8 µl of extracted DNA sample or control to the appropriate tube with Reaction Mix.

7. Click Start Run to begin PCR reaction.

8. After the run is complete click then click tab to interpret the results.
Add 6. Programming of LightCycler 2.0 (Roche)

1. Select “New”, “LightCycler Experiment”, Click and insert as follows:

   - Max. Seek Pos. indicates the number of samples tested in the experiment
   - Choose Instrument Type according to your instrument
   - Choose capillary Size according to your capillaries (we suggest to select 20 µl and use 30 µl polycarbonate capillaries)

2. Program the thermal cycling as follows:

3. Click and insert sample names in Capillary View tab; then select Qualitative Detection from Analysis Type and select 530, 560 in Selected Channels section:

4. Insert Target Name (insert pathogen name for the 530 Channel and “Internal Control” for the 560 channel):

5. Click to start PCR reaction.

6. After Run is completed click button and select Qualitative Detection, click OK. Click Advanced. Call values (Negative or Positive) and calculated Cts results will appear, for the selected channel, under the Call and CP column in the Qualitative detection table.
At first usage the operator must perform a color compensation experiment asking Sacace for the detailed instructions.