HPV High Risk Screen Real-TM Quant
Real Time Kit for use with RotorGene™ 3000/6000 (Corbett Research), iQ iCycler™ and iQ5™ (Biorad), MX3000P® and MX3005P® (Stratagene)

REF V31-100/2FRT
VER 10.11.2009
100
NAME
HPV High Risk Screen Real-TM Quant

INTRODUCTION
Genital infection with HPV is one of the most common sexually transmitted diseases (STDs) of viral etiology worldwide (20% - 46% in different countries in sexually active young women). Cervical cancer is the second most common cancer in women worldwide, and a compelling body of clinical, epidemiological, molecular, and experimental evidence has established the etiological relationship between some sexually transmitted HPV genotypes and cervical neoplasia throughout the world. Based on the frequency of detection of HPV genotypes from different grades of Cervical Intraepithelial Neoplasia (CIN Grades I – III), HPV genotypes are subdivided into High-risk HPV types (16, 18, 31 and 45), Intermediate-risk types (33, 35, 39, 51, 52, 56, 58, 59, and 68), and Low-risk types (6, 11, 42-44).
Several methods have been used to diagnose clinical or subclinical infection with HPVs including clinical observation, cytological screening by Pap smear, electron microscopy, immunocytochemistry, but these methods have some disadvantages such as non-standardization and subjectivity, insufficient sensitivity and low predictable value. The most perspective way of HPV diagnosis is a direct detection of DNA of the human papilloma virus of high carcinogenic risk by the polymerase chain reaction. While the value of the Pap smear in routine screening for cervical dysplasia is undisputed, it is now known that 99% of cases of cervical carcinoma are caused by infection with twelve genotypes of the human papilloma virus (HPV). Identification of these high-risk genotypes is very valuable in the management of cervical carcinoma, both as a prognostic indicator and as a secondary screening test where results of a Pap smear are inconclusive. Results from the combination of the Pap smear and the HPV DNA test can aid in determining the intervals for screening.
The PCR-based methods have been used successfully for the detection and typing of genital HPV genotypes in clinical specimens such as cervical swabs or scrapes, cervicovaginal lavages, frozen biopsies and formalin-fixed paraffin-embedded tissues.

INTENDED USE
Kit HPV High Risk Screen Real-TM Quant is an in vitro Real Time amplification test for quantitative detection of Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) in the urogenital swabs. It being known, that the parameter of viral load has a prognostic value and the viral load less than $10^5$ HPV genomic equivalents in the swab or $10^3$ genomic equivalents for $10^5$ cells is considered as insignificant and indicates the presence of transitory infection, however such level of load may have a value only in cases of treatment monitoring. Viral load of more than $10^5$ genomic equivalents for $10^5$ cells is considered to be important with high significance and indicates the existence of dysplastic changes or high risk of their occurrence. Quantitative detection of viral load allows to evaluate the character of the infection and to make a forecast concerning the stage of the disease.
HPV High Risk Screen Quant detect the most widespread and oncogenic 12 genotypes of human papilloma virus with determination of clinical significance. Since the human papilloma virus is an intracellular agent, there is need to monitor the presence of cellular material in the sample, in order to avoid false-negative results. HPV High Risk Screen Quant kit contains the internal control (human beta-globine gene), which allows to control the presence of cellular material in the sample.
This kit is optimized for use with RotorGene™ 3000/6000 (Corbett Research), iQ iCycler™ and iQ5™ (Biorad), MX300P® and MX3005P® (Stratagene)
**PRINCIPLE OF ASSAY**

Kit HPV High Risk Screen Real-TM Quant is based on two major processes: isolation of DNA from specimens and Real Time amplification. PCR-mix-1 tube contains primers directed against regions of HPV A7, A9 groups (HPV types 16, 18, 31, 33, 35, 39, 45, 52, 58, 59), HPV A5 group (HPV type 51), HPV A6 group (HPV type 56) and β-globine gene used as Internal Control. If the swab is not correctly prepared (high quantity of mucous or insufficient quantity of epithelial cells) the Internal Control will not be detected. The kit contains the quantitative standards with known concentration of HPV DNA which allows to determinate the viral load. For the calculation of viral load it is used the relation between the obtained HPV DNA concentration and the quantity of genomic DNA which allows to eliminate the possible errors during the sample preparation.

**MATERIALS PROVIDED**

Part N° 1 – “HPV High Risk Screen Real-TM Quant”: Real Time amplification.

Part N° 2 – Program in Microsoft® Excel format for results interpretation

Part N° 2 – “HPV High Risk Screen Real-TM Quant”:
- **PCR-mix-1-FRT**, 3 x 0,28 ml
- **PCR-mix-2 buffer**, 3 x 0,30 ml
- **TaqF DNA Polymerase**, 3 x 0,02 ml
- **Negative Control**, 1,2 ml
- **DNA-buffer (C-)**, 0,5 ml
- **QS HPV K1**, 3 x 0,04 ml (mix HPV DNA C+ 16, 18, 51 and human DNA);
- **QS HPV K2**, 3 x 0,04 ml (mix HPV DNA C+ 16, 18, 51 and human DNA);
- **QS HPV K3**, 3 x 0,04 ml (mix HPV DNA C+ 16, 18, 51 and human DNA);

Contains reagents for 108 samples.

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

** Standards’ concentration is specific for every lot (reported on the HPV High Risk Screen Real-TM Quant Data Card)

**MATERIALS REQUIRED BUT NOT PROVIDED**

- Real Time Thermalycler
- Reaction Tubes (strips)
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks
- DNA extraction kit
WARNINGS AND PRECAUTIONS
1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
3. Do not use a kit after its expiration date.
4. Dispose of all specimens and unused reagents in accordance with local regulation.
5. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
6. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
7. Avoid contact of specimens and reagents with the skin, eyes and mucose 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. This kit is designed for use with “DNA-Sorb” extraction kit. It is the user’s responsibility if kits other than “DNA-Sorb” are used to perform this DNA extraction.
10. Use of this product should be limited to personnel trained in the techniques of PCR.
11. 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
HPV High Risk Screen 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
HPV High Risk Screen 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT
HPV High Risk Screen Real-TM Quant can analyze DNA extracted with DNA-Sorb-A (REF K-1-1/A) from:
- **Cervical swabs:**
  - Remove excess mucus from the cervical os and surrounding ectocervix using a cotton or polyester swab. Discard this swab.
  - Insert the Sampling Cervical Brush 1.0-1.5 centimeters into the cervical os until the largest bristles touch the ectocervix. Do not insert brush completely into the cervical canal. Rotate brush 3 full turns in a counterclockwise direction, remove from the canal.
  - Insert brush into the nuclease-free 2.0 ml tube with 0.5 mL of Transport medium (Sacace). Vigorously agitate brush in medium for 15-20 sec.
  - Snap off shaft at scored line, leaving brush end inside tube.
- **Liquid-based cytology samples** (Cytoscreen, PreservCyt) (recommended DNA-Sorb-D REF K-1-8/100 not included in this kit, but can be ordered separately)
  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.
Protocol:
1. Prepare required quantity of tubes (N+3(Standards)+1(Neg.Control)
   (for 0.2 ml tubes with RotorGene 6000 add 1 drop of mineral Oil to each tube)
2. Prepare Mix for 40 samples: add into the tube with PCR-mix-2 buffer 20 µl of TaqF DNA Polymerase. Mix by pipetting. This mix is stable for 3 month at +2-8°C.
3. Add for each sample tube 7,0 µl of PCR-mix-1-FRT and 8,0 µl of Mix (PCR-mix-2 buffer and TaqF DNA Polymerase).
4. Add 10 µl of extracted DNA sample to appropriate tube with Reaction Mix.
   (Re-centrifuge all the tubes with extracted DNA for 1 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don’t disturb the pellet, sorbent inhibit reaction!).
5. Prepare for each run 3 standards and 1 Neg Control:
   - add 10 µl of DNA-buffer for Negative PCR control;
   - add 10 µl of QS HPV K1 for tube labelled K1;
   - add 10 µl of QS HPV K2 for tube labelled K2;
   - add 10 µl of QS HPV K3 for tube labelled K3.
6. Close tubes and transfer them into the Real Time PCR instrument.
7. Program position of the samples and enter the concentrations of the Quantitative Standards (reported in the Quant Data Card) in the Joe (Yellow)/HEX, Fam (Green), Rox(Orange) and Cy5 (Red) channels in order to generate Standard curves. Use name “Unknown” for the wells that contain samples, K1, K2, K3 for “Standards” and “-” for Negative Controls.

Data Analysis:
1. The experiment may be considered valid if:
   - the Negative Controls haven’t any positive fluorescence signal;
   - the standards have positive signals in all channels (Fam, Joe/Hex, Rox, Cy5)
2. The result of the sample is considered:
   - Invalid in case of absence of any fluorescence signal (positive or internal);
   - Negative if signal is present only in the Fam (Green) channel with the concentration of genomic DNA > 5 x 10^3;
   - Positive:
     - for HPV A9 group (16, 31, 33, 35, 52, 58) if contains the positive signal in the Joe (Yellow)/Hex channel with Ct ≤ 33;
     - for HPV A7 group (18, 39, 45, 59) if contains the positive signal in the Rox (Orange) channel with Ct ≤ 33;
     - for HPV A5-A6 group (51, 56) if contains the positive signal in the Cy5 (Red) channel with Ct ≤ 33;

Calculate the concentration of HPV DNA using the following formula:
\[
\log\left(\frac{\text{HPV DNA copies/reaction}}{\text{genomic DNA copies/reaction}} \times 200000\right) = \log(\text{HPV DNA in 100000 cells})
\]

RESULTS INTERPRETATION:

<table>
<thead>
<tr>
<th>Result lg (HPV in 100.000 cells)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3</td>
<td>Clinically insignificant</td>
</tr>
<tr>
<td>3-5</td>
<td>Clinically important. Present risk of cervical dysplasia</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>Clinically very important. High risk of cervical dysplasia</td>
</tr>
</tbody>
</table>

The results can be calculated automatically using the program in Microsoft ® Excel format supplied with the kit.
1. Open the program “HPV High Risk Screen Quant 4x” and in the window “Security Warning” click on the button “Enable Macros” (Security level of the Microsoft ® Excel must be selected as Medium (Tools→Macro→Security→Medium).
2. Copy with the right button of the mouse the names of the samples from the column “Name” and paste them in the column “Name” of the program “HPV High Risk Screen Quant 4x”.
3. Copy in the same way the Ct values from the channel FAM (Green) and paste them in the correspond column of the program. Repeat the same procedure for all channels. Standards must be named as K1, K2, K3 and Negative controls must be marked as “-“.
4. Select the “Quantitative analysis” and choose “Internal Calibration…”
5. At the top right of the window insert in the table “Standards” the concentrations of the Quantitative standards reported in the Quant Data Card.
6. Click on the buttons “Sign unnamed” and “ Results”.
7. Save the file with a new name.
Real Time Amplification with Rotor-Gene 3000/6000

1. Close tubes and transfer them into the carousel of Rotor-Gene.
2. Reaction volume, 25 µl. Make sure that for RG6000 the window “15 µL oil layer volume” is selected.
   **Important:** For the Rotor-Gene 6000 must be used software 1.7 Build 67 or updated version (for software information contact info@sacace.com).

3. Program Rotor-Gene 3000/6000 as follows:

<table>
<thead>
<tr>
<th>°C</th>
<th>Time</th>
<th>Fluorescence detection</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95°</td>
<td>15 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold 2</td>
<td>65°</td>
<td>2 min</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>95°</td>
<td>20 sec</td>
<td>–</td>
</tr>
<tr>
<td>Cycling</td>
<td>64°</td>
<td>Touchdown: 1 deg. per cycle</td>
<td>25 sec</td>
</tr>
<tr>
<td></td>
<td>65°</td>
<td>55 sec</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>95°</td>
<td>15 sec</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>60°</td>
<td>25 sec</td>
<td>–</td>
</tr>
<tr>
<td>Cycling 2</td>
<td>65°</td>
<td>25 sec</td>
<td>Fam (Green), Joe (Yellow), Rox (Orange), Cy5 (Red)</td>
</tr>
</tbody>
</table>

4. Fluorescence detection at 65°C on the channels Fam (Green), Joe (Yellow), Rox (Orange), Cy5 (Red)
   **Note:** It is highly recommended to use “HPV high Risk Screen Real-TM Quant.rex” file supplied with kit to settle the instrument.

5. Select **Perform Calibration Before 1-st Acquisition** and set 4FI – 8FI for all channels (Auto gain calibration channel settings).

Results Analysis:

1. Press **Analysis** then select button **Quantitation**. Perform the operation for the channels Fam (Green), Joe (Yellow), Rox (Orange), Cy5 (Red)
2. Set **Threshold:** 0,03
3. Select **Dynamic Tube, Slope Correct**
4. Select **More settings (Outlier Removal… For RG6000)** and set **NTC Threshold** to 20%
5. Set **Eliminate cycles before:** 5

Fam channel – Internal Control (β-globine gene)

Cy5 channel - HPV A5-A6 group

Joe channel – HPV A9 group

Rox channel – HPV A7 group
Real Time Amplification with iQ iCycler and iQ5 (Bio-Rad)

Note: The instrument should be switched on at least 15 minutes before starting the experiment

1. In the “Workshop” insert and save the following amplification protocol:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature, °C</th>
<th>Time</th>
<th>Fluoresc. detection</th>
<th>Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>95</td>
<td>15 min</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Touchdown:</td>
<td>55 s</td>
<td>–</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>25 s</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 4</td>
<td>60</td>
<td>55 s</td>
<td>Real-time</td>
<td>41</td>
</tr>
<tr>
<td>65</td>
<td>25 s</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Select fluorescence detection on the channels FAM, HEX, ROX, Cy5.

I. Original file (Background subtracted)

II. Dates after setting (PCR Base Line Subtracted Curve Fit)
Real Time Amplification with MX3000P, MX3005P™ (Stratagene)

1. Start the program Stratagene
2. Select in the menu window “New Experiment Options” “Quantitative PCR (Multiple Standards)” and put “Turn lamp on for warm-up”

Note: The instrument should be switched on at least 15 minutes before starting the experiment

3. Insert the tubes (plate) in the thermalcycler and close the instrument.
4. In the menu “Options” choose “Optics Configuration” and insert Fam in “FAM filter set” and Joe in “HEX/JOE filter set”.
5. In the menu “Instrument” choose “Filter Set Gain Setting…” and in the new window set the following parameters:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5</td>
<td>x4</td>
</tr>
<tr>
<td>ROX</td>
<td>x1</td>
</tr>
<tr>
<td>HEX/JOE</td>
<td>x4</td>
</tr>
<tr>
<td>FAM</td>
<td>x4</td>
</tr>
</tbody>
</table>

6. In the window “Well type” set “Unknown” for the samples and in the field “Collect fluorescence data” select for all samples the channels Fam, Hex, Rox, Cy5
7. Click twice on all the cells and insert the samples informations (field “Well Information”), all positive samples as “+” and negative as “-”. Enter the concentrations of the Quantitative Standards (reported on the HPV Quant Data Card) in the “Standard Quantity” box

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

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Fluorescence detection</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment 1</td>
<td>95˚</td>
<td>15 min</td>
<td>1</td>
</tr>
<tr>
<td>Segment 2</td>
<td>65˚</td>
<td>2 min</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>95˚</td>
<td>20 sec</td>
<td>1</td>
</tr>
<tr>
<td>Segment 3</td>
<td>64˚</td>
<td>25 sec</td>
<td>–</td>
</tr>
<tr>
<td>(Cycling)</td>
<td>95˚</td>
<td>55 sec</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>65˚</td>
<td>20 sec</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>60˚</td>
<td>25 sec</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>65˚</td>
<td>55 sec</td>
<td>–</td>
</tr>
<tr>
<td>Segment 4</td>
<td>95˚</td>
<td>20 sec</td>
<td>40</td>
</tr>
<tr>
<td>(Cycling)</td>
<td>65˚</td>
<td>25 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fam, Joe, Rox, Cy5</td>
<td></td>
</tr>
</tbody>
</table>

Results Analysis:

1. Choose button “Analysis” at the top left of the window and then “Results”
2. In the menu “Dyes shown” put in the field “Threshold fluorescence” the following threshold:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5</td>
<td>150</td>
</tr>
<tr>
<td>ROX</td>
<td>150</td>
</tr>
<tr>
<td>HEX/JOE</td>
<td>150</td>
</tr>
<tr>
<td>FAM</td>
<td>150</td>
</tr>
</tbody>
</table>

3. Be sure that all four fluorescent channels are active (buttons Cy5, Rox, Hex, Fam in the field Dyes Shown are selected)

Example
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 Human Papillomavirus primers and probes. The specificity of the kit HPV High Risk Screen Real-TM Quant was 100%. The potential cross-reactivity of the kit HPV High Risk Screen Real-TM Quant was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity
The kit HPV High Risk Screen Real-TM Quant allows to detect Human Papillomavirus 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: E1, E2
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