



For *in Vitro* Diagnostic Use



HPV 16/18 Real-TM Quant

Real Time Kit for quantitative detection and genotyping
of Human Papillomavirus (16, 18)

for use with RotorGene™ 3000/6000/Q (Corbett Research, Qiagen),
iQ iCycler™ and iQ5™ (Biorad), MX3000P® and MX3005P® (Stratagene),
Applied Biosystems® 7500 Real Time PCR Systems (Applied)

REF V12-100FRT



100

NAME

HPV 16/18 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 16/18 Real-TM Quant is an *in vitro* Real Time amplification test for quantitative detection and genotyping of *Human Papillomavirus* (16, 18) in the urogenital swabs and biopsies.

PRINCIPLE OF ASSAY

kit HPV 16/18 Real-TM Quant is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification. Amplification results of HPV 16 DNA are detected on the Fam/Green channel, amplification results of HPV 18 DNA are detected on the Rox/Orange channel and β -globine gene used as Internal Control is detected on the Joe/HEX/Yellow channel. If the swab is not correctly prepared (high quality of mucous or insufficient quantity of epithelial cells ($< 10^3$ - 10^5)) the Internal Control will not be detected or come very low.

MATERIALS PROVIDED

- PCR-mix-1 "16-18", 3 x 0,28 ml
 - PCR- buffer-FRT, 3 x 0,3 ml
 - Hot Start DNA Polymerase, 3 x 0,02 ml
 - Negative Control, 1,2 ml;
 - DNA-buffer (C-), 0,5 ml
 - Quantitation Standard HPV (QS1 HPV, QS2 HPV, QS3 HPV), 3 x 0,04 ml;
- Contains reagents for 108 samples.

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA isolation kit (see DNA isolation)
- 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

HPV 16/18 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 16/18 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 16/18 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.
- *Tissue* homogenized with mechanical homogenizer and dissolved in PBS sterile (recommended DNA-Sorb-C (REF) K-1-6/50 not included in this kit, but can be ordered separately)
- *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:

Reaction volume = 25 µl

1. Prepare required quantity of tubes (N + 4 controls (3 standards and 1 negative control)).
2. Prepare Mix for 40 samples: add into the tube with PCR- buffer-FRT 20 µl of Hot Start DNA Polymerase. Carefully vortex the tube. This mix is stable for 3 months at +4°C.
3. Prepare reaction mix (see table 2). Add for each sample in the new sterile tube 7*(N+1) µl of PCR-mix-1 "16-18" and 8*(N+1) µl of Mix (PCR- buffer-FRT + Hot Start DNA Polymerase)
4. Add 15 µl of Reaction Mix into each tube with samples and controls.
5. Add 10 µl of extracted DNA sample to appropriate tube.
6. Prepare for each panel 4 controls:
 - add 10 µl of Quantitation Standards HPV (QS1 HPV, QS2 HPV, QS3 HPV) into 3 labeled tubes;
 - add 10 µl of DNA-buffer to the tube labeled Negative Control;

Table 2. Mixes preparation x sample (with calculation of reagents for controls)

Samples	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
PCR-mix-1 "16-18"	56	63	70	77	84	91	98	105	112	119	126	133	140	147	154
Mix (PCR- buffer-FRT + Hot Start DNA Polymerase)	64	72	80	88	96	104	112	120	128	136	144	152	160	168	176
Samples	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
PCR-mix-1"16-18"	161	168	175	182	189	196	203	210	217	224	231	238	245	252	259
Mix (PCR- buffer-FRT + Hot Start DNA Polymerase)	184	192	200	208	216	224	232	240	248	256	264	272	280	288	296

7. Close tubes and transfer them into the Real Time ThermalCycler.
8. Program the instruments as follows:

RotorGene 3000/6000/Q (Corbett Research, Qiagen)

Step	t, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	-	1
Cycling	95	15 secs	-	45
	60	35 secs	FAM, JOE, ROX (Green, Yellow, Orange)	

- Perform the calibration for the first tube on the channels Fam, Joe, Rox (impost 3FI – 5FI) and select *Perform Calibration Before 1-st Acquisition*

iQ iCycler/ iQ5 (Bio-Rad), Mx3005P (Stratagene)

Step	t, °C	Time	Fluorescence detection	Cycles
1	95	15 min	-	1
2	95	20 secs	-	45
	60	1 min	FAM, HEX, ROX	

Applied Biosystems 7300/7500 (Applied)

Step	t, °C	Time	Fluorescence detection	Cycles
1	95	15 min	-	1
2	95	20 secs	-	45
	60	1 min	FAM, JOE, ROX	

- Select Quencher – *None* for all targets. Select *None* as passive reference dye.

SmartCycler (Cepheid)

Stage	Temp	Secs	Optics	Repeat
Stage 1. Hold	95°C	900	Off	1
Stage 2	95°C	20	Off	45
2-Temperature Cycle	60°C	60	On (FAM, JOE/TET/Cy3, ROX)	

- Program position of the tubes and enter the concentrations of the Quantitative Standards (reported on the HPV 16/18 Quant Data Card) in the Joe (Human DNA), Fam (HPV 16) and Rox (HPV 18) channels in order to generate Standard curves.

RESULTS ANALYSIS:

The results are interpreted with the software of instrument through the presence of crossing of fluorescence curve with the threshold line. *Internal Control* (Human DNA) is detected on the Joe/HEX/Yellow channel, *HPV 16* on the FAM/Green channel and *HPV 18* on ROX/Orange channel.

Calculate the concentration of HPV 16 and/or 18 DNA in 100.000 human cells using the following formula:

$$\lg (\text{copies HPV DNA (16 or 18)/copies human DNA} \times 200000) = \lg (\text{HPV in 100.000 cells})$$

Results interpretation:

Result lg (HPV in 100.000 cells)	Interpretation
< 3	Clinically insignificant
3-5	Clinically important. Present risk of cervical dysplasia
> 5	Clinically very important. High risk of cervical dysplasia

Troubleshooting

- Occurrence of any value Ct in the table of results for the negative control sample and for negative control of amplification testifies contamination of reagents or samples. In this case results of the analysis for all tests are considered invalid. It is required to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.
- Human DNA concentration in the sample is less than 1000 copies; swab is not correctly prepared. Repeat the test.
- The Coefficient correlation value R in the "Standard Curve" window is < 0,9: a retesting of all samples is required.

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 *HPV* primers and probes. The specificity of the kit **HPV 16/18 Real-TM Quant** was 100%. The potential cross-reactivity of the kit **HPV 16/18 Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **HPV 16/18 Real-TM Quant** allows to detect *HPV* 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: E7

GEL