IVD For *in Vitro* Diagnostic Use

CE

HPV 16/18 Real-TM Quant

Handbook

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

REF V12-100FRT

REF TV12-100FRT

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 displasia 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 epitelial cells (< 10^3-10^5) the Internal Control will not be detected or come very low.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V12-100FRT)

Part N° 2 – **"HPV 16/18 Real-TM Quant":** Real Time amplification kit

- **PCR-mix-1 "16-18"**, 4 x 0,3 ml
- **PCR- buffer-FRT**, 2 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.

Module No.2: Complete Real Time PCR test with DNA purification kit (TV12-100FRT)

Part N° 1 – **"DNA-sorb-A":** Sample preparation kit

- **Lysis Solution**, 2 x 15 ml;
- **Sorbent**, 2 x 1,0 ml;
- **Washing Solution**, 2 x 50 ml;
- **DNA-eluent**, 2 x 5 ml;
- **Transport medium** , 2 x 15 ml.

Contains reagents for 100 test.

Part N° 2 – **"HPV 16/18 Real-TM Quant":** Real Time amplification kit

- **PCR-mix-1 "16-18"**, 4 x 0,3 ml
- **PCR- buffer-FRT**, 2 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

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks

Zone 2: Real Time amplification:

- Real Time Thermalcycler
- Tubes
- Workstation
- Pipettes (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

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.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

HPV 16/18 Real-TM Quant can analyze DNA extracted 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 or 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 0,3 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

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A);
- ⇨ **SaMag STD DNA Extraction kit** (Sacace, REF SM007).

Please carry out DNA extraction according to the manufacture’s instruction.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **300 µl** of **Lysis Solution**.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Neg Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **20 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Add **500 µl** of **Washing Solution** to each tube. Vortex very vigorously and centrifuge for 30 sec at 10000g. Remove and discard supernatant from each tube.
10. Repeat step 9 and incubate all tubes with open cap for 5-10 min at 65°C.
11. Resuspend the pellet in **100 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
12. Centrifuge the tubes for 1 min at 12000g.
13. The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

PROTOCOL:

Reaction volume = 25 µl

1. Prepare required quantity of tubes (N + 4 controls (3 standards and 1 negative control).
2. Prepare **Mix** of **PCR- buffer-FRT** and **Hot Start DNA Polymerase**: add into the tube with **PCR- buffer-FRT** whole content of the tube with of **Hot Start DNA Polymerase (30 µl).** Carefully vortex the tube**.** This mix is stable for 3 months at +4°C.
3. Prepare reaction mix (see table 1). Add for each sample in the new sterile tube **10*(N+1) µl** of **PCR-mix-1 "16-18"** and **5*(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 1. 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"	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210
Mix (PCR-buffer-FRT + Hot Start DNA Polymerase)	35	40	45	50	55	60	65	70	75	80	85	90	95	100	105
Samples	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
PCR-mix-1 "16-18"	220	230	240	250	260	270	280	290	300	310	320	330	340	350	360
Mix (PCR-buffer-FRT + Hot Start DNA Polymerase)	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180

7. Close tubes and transfer them into the Real Time ThermalCycler.
8. Program the instrument.

Amplification

1. Create a temperature profile on your instrument as follows:

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s *fluorescent signal detection**		**60**	30 s *fluorescent signal detection**	
	72	15 s		**72**	15 s	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems), SmartCycler™ (Cepheid)*
** detection on Fam (Green), Joe (Yellow)/Hex, Rox (Orange)*

The following programs can also be used:

Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Repeats***
Hold	95	15 min	–	1
Cycling	95	15 sec	–	45
	60	30 sec*	FAM/Green, JOE/Yellow, ROX/Orange	

SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad), Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems)

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Repeats***
Segment 1	95	15 min	–	1
Segment 2	95	20 sec	–	45
	60	60 sec*	FAM, HEX, ROX	

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	

** detection on Fam, Joe/Hex, Rox*

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 4 Fl to 8 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.03*	*10 %*	*On*
Rox (Orange)	*from 4 Fl to 8 Fl*	*0.03*	*10 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

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 (copies HPV DNA (16 or 18)/copies human DNA x 200000) = lg (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

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

TROUBLESHOOTING

1. 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.
2. Human DNA concentration in the sample is less than 1000 copies: swab is not correctly prepared. Repeat the test.
3. The Coefficient correlation value R in the *"Standard Curve"* window is < 0,9: a retesting of all samples is required.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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