Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

HPV High Risk Screen Real-TM Quant

HANDBOOK

Real Time PCR kit for quantitative detection of *Human Papillomavirus* (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59*)*

REF **V31-100/2FRT**

REF **TV31-100/2FRT**

Σ **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 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 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.

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 epitelial 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

Module No.1: Real Time PCR kit (V31-100/2FRT)

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)*

Module No.2: Complete Real Time PCR test with DNA purification kit (TV31-100/2FRT)

Part N° 1 – **"DNA-sorb-A/100":**

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

Contains reagents for 100 tests.

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

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- 65°C ± 2°C dry heat block
- Vortex mixer
- Pipettes with sterile, RNase-free filters tips
- 1,5 ml polypropylene sterile tubes
- Disposable gloves, powderless
- Tube racks

Zone 2: Real Time amplification:

- Real Time Thermalcycler
- PCR Tubes
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

DNA-sorb-A/100 must be stored at 2-8°C. **HPV High Risk Screen Real-TM Quant** must be stored at -20°C. The kit can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -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.

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***

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 0,3 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.
- *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 (reagents supplied with the Module No.2)

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 7-10 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 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:

1. Prepare required quantity of tubes (N+3(Standards)+1(Neg.Control)
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.

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)*
** detection on Fam (Green), Joe (Yellow)/Hex, Rox (Orange) and Cy5 (Red)*

The following programs can also be used:

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

	t,˚C	Time	Fluorescence detection	Cycles
Hold	95˚	15 min	–	1
Hold 2	65˚	2 min	–	1
Cycling	95˚	20 sec	–	5
	64˚ Touchdown: *1 deg. per cycle*	25 sec	–	
	65˚	55 sec	–	
Cycling 2	95˚	15 sec	–	40
	60˚	25 sec	–	
	65˚	40 sec	*fluorescent signal detection**	

+ fluorescence detection on the channels Fam (Green), Joe (Yellow), Rox (Orange) and Cy5 (Red) for 4x Rotor-Gene on the 2-nd Cycling (65°C)

CFX/iQ5™ (BioRad)

Cycle	Temperature, °C	Time	Fluoresc.detection	Repeats
Cycle 1	95	15 min	–	1
Cycle 3	95	15 s	–	6
	65 Touchdown: *1 deg. per cycle*	55 s	–	
	65	25 s	–	
Cycle 4	95	15 s	–	41
	60	55 s	*fluorescent signal detection*	
	65	25 s	–	

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*	*15 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.03*	*15 %*	*On*
Rox (Orange)	*from 4 Fl to 8 Fl*	*0.03*	*15 %*	*On*
Cy5 (Red)	*from 4 Fl to 8 Fl*	*0.03*	*15 %*	*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.

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 > 5×10^3;
   - *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:

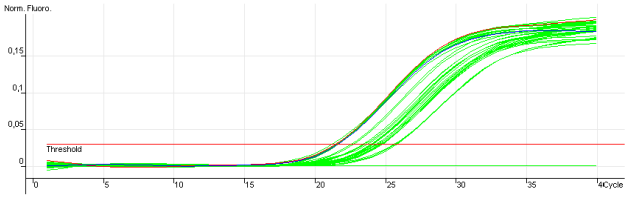
$$\log\left(\frac{HPV\ DNA\ copies/reaction}{genomic\ DNA\ copies/reaction} \times 200000\right) = \log\left(HPV\ DNA\ \text{in}\ 100000\ cells\right)$$

RESULTS INTERPRETATION:

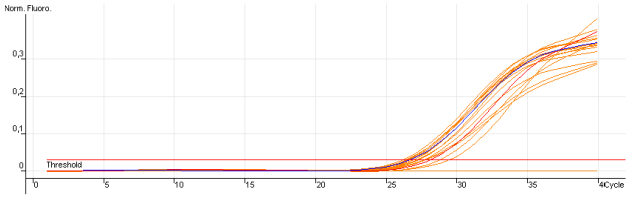
Result lg (HPV in 100.000 cells)	Interpretation
< 3	Clinically insignificant
3-5	Clinically important. Present risk of cervical dysplasia
> 5	Clinically very important. High risk of cervical dysplasia

The results can be calculated automatically using the program in Microsoft ® Excel format supplied with the kit.

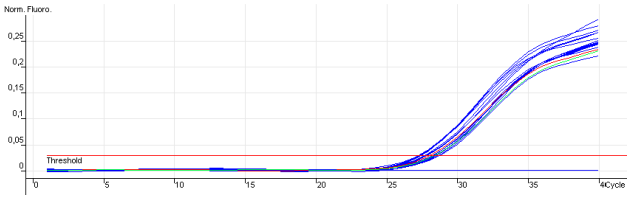
1. Open the program “HPV High Risk Screen Quant 4x” and in the window “Security Warning” click on the button “Enable Macros” (Security level of the Microsoft ® Excel must be selected as Medium (Tools→Macro→Security→Medium).
2. Copy with the right button of the mouse the names of the samples from the 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.



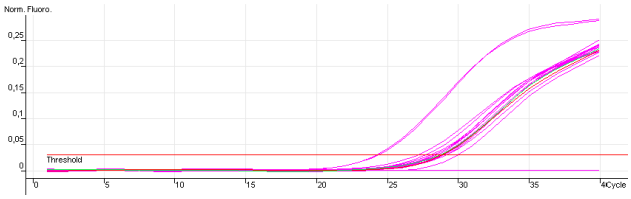
Fam channel – Internal Control (β-globine gene)



Cy5 channel - *HPV A5-A6 group*



Joe channel – *HPV A9 group*



Rox channel – *HPV A7 group*

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

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	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems

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