IVD For *in Vitro* Diagnostic Use

CE

HPV 6/11 Real-TM

HANDBOOK

Real Time PCR kit for qualitative detection of
Human Papillomavirus 6 and 11

REF V11-100FRT

REF TV11-100FRT

Σ 100

NAME

HPV 6/11 Real-TM

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). Genital warts (technically known as condylomata acuminata) are most commonly associated with two HPV types, HPV 6 and HPV 11.

Several methods have been used to diagnose clinical or subclinical infections 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 values. 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.

Recently many countries introduced quadrivalent HPV 6/11/16/18 Vaccine program therefore the detection of HPV 6 and 11 types can be used for the evaluation of efficiency of the profilaxis.

INTENDED USE

The kit **HPV 6/11 Real-TM** is an *in vitro* Real Time amplification test for qualitative detection of *Human Papillomavirus* 6 and 11 in the urogenital swabs.

PRINCIPLE OF ASSAY

The kit **HPV 6/11 Real-TM** 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* types 6, 11 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 or will be low (the quantity of epithelial cells lower than 10^3 cells/reaction). *HPV 6* is detected on the FAM (Green) channel, *HPV 11* on the JOE (Yellow)/Cy3/HEX channel *and Human β-globine gene* on the ROX (Orange)/Texas Red channel.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V11-100FRT)

- PCR-mix-1-FRT, 4 x 0,3 ml;
- PCR-Buffer-FRT, 2 x 0,3 ml;
- TaqF Polymerase, 2 x 0,03 ml;
- Pos Control Complex C+ (HPV 6, 11, human DNA), 0,2 ml;
- Negative Control C-, 1,2 ml;*
- DNA-buffer, 0,5 ml;

Contains reagents for 120 tests.

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

Module No.2: Complete Real Time PCR test with DNA purification kit (TV11-100FRT)

"DNA-sorb-A":

- 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 tests.

"HPV 6/11 TM":

- PCR-mix-1-FRT, 2 x 0,6 ml;
- PCR-Buffer-FRT, 2 x 0,3 ml;
- TaqF Polymerase, 2 x 0,03 ml;
- Pos Control Complex C+ (HPV 6, 11, human DNA), 0,2 ml;
- Negative Control C-, 1,2 ml;*
- DNA-buffer, 0,5 ml;

Contains reagents for 120 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Vortex
- 65°C ± 2°C dry heat block
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Tube racks
- Microcentrifuge tubes, 1,5 - 2,0 ml
- Pipettes with sterile, RNase-free filters tips
- Biohazard waste container
- Disposable gloves, powderless
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters

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***

STORAGE INSTRUCTIONS

HPV 6/11 Real-TM must be stored at -20°C. **"DNA-sorb-A"** must be stored at +2-25°C°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

HPV 6/11 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.

QUALITY CONTROL

The complete kit has been tested on an RotorGene Q (Qiagen).
Certificates of Analyses are available on request at info@sacace.com.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

HPV 6/11 Real-TM 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 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.
- *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 (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 the 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 the required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare **Mix** for 60 samples: add into the tube with **PCR- buffer-FRT 30 µl** of **TaqF DNA Polymerase.** Carefully vortex the tube**.** This mix is stable for 3 months at +4°C.
3. Prepare **Reaction Mix** by adding for each sample into the new sterile tube **10 µl** of **PCR-mix-1-FRT** and **5 µl** of mix **PCR- buffer-FRT/ TaqF DNA Polymerase** (see table 2).
4. Add to each reaction tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA**. Mix by pipetting.
5. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Control Complex C+** to the tube labeled Amplification Positive Control;
6. Insert the tubes in the thermalcycler.

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

Table 2. Pipetting scheme for the quantity of reagents for N samples

Samples:	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
PCR-mix-1-FRT	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240
PCR- buffer-FRT/ TaqF DNA Polymerase	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120
Samples:	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
PCR-mix-1-FRT	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400	410
PCR- buffer-FRT/ TaqF DNA Polymerase	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205
Samples:	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**
PCR-mix-1-FRT	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580
PCR- buffer-FRT/ TaqF DNA Polymerase	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290

Note: the calculation of the quantity of mixes was made in consideration of reagents for 2 controls and 1 extra sample

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® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)

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˚	25 sec	Fam (Green), Joe (Yellow) and Rox (Orange)	

fluorescence detection on the channels Fam (Green), Joe (Yellow) and Rox (Orange) 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	Real-time	
	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*	*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.

Boundary value of the cycle threshold, Ct

Sample	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	28	29
	JOE/Yellow/Hex/Cy3	30	31
	Rox/Orange	27	28
Samples	Rox/Orange	< 30	< 30

DATA ANALYSIS

The results are interpreted with the software of Real Time PCR instrument through the presence of crossing of fluorescence curve with the threshold line.
HPV 6 is detected on the FAM/Green channel, *HPV 11* on the JOE/Yellow/HEX *and IC DNA* on the ROX /Orange channel.

The run result is considered to be **valid** if:

– The signal is absent on all channels (FAM/Green, JOE/Yellow, ROX/Orange) for negative controls ;

– The signals are present on all channels (FAM/Green, JOE/Yellow, ROX/Orange) for positive control.

If the run result is invalid, all obtained data are considered to be invalid, and the reaction must be repeated

The result of HPV DNA detection is considered to be:

- **negative**, if the fluorescence signal is registered only on ROX/Orange channel and the threshold cycle value doesn't exceed 30.
- **positive**, if

- the signal is registered on FAM/Green channel (positive for HPV type 6).
- the signal is registered on JOE/HEX/Yellow channel (positive for HPV type 11).

- **invalid** if

- the positive signals are not registered on FAM/Green and JOE/HEX/Yellow channels (HPV types 6 and 11) and the IC signal (ROX/Orange) is not registered or the threshold cycle value exceeds 30 ;

- the doubtful signal/signals is registered on FAM/Green and JOE/HEX/Yellow channels (HPV types 6 and 11) and the IC signal (ROX/Orange) is not or the threshold cycle value exceeds 30.

The invalid result requires to repeat sample analysis from the beginning DNA isolation or sampling.

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 6,11* primers and probes. The specificity of the kit **HPV 6/11 Real-TM** was 100%. The potential cross-reactivity of the kit **HPV 6/11 Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Table 1: Testing the specificity of the kit with other pathogens:

Control group	Results
Adenovirus type 2	-
Adenovirus type 3	-
Adenovirus type 7	-
Cytomegalovirus	-
Epstein Barr virus	-
Human immunodeficiency virus 1	-
Hepatitis B virus	-
Hepatitis C virus	-
Herpes simplex virus 1	-
Herpes simplex virus 2	-
Human herpes virus 6	-
Human herpes virus 8	-
HPV groups β, γ, μ (1,3,4,5,8,37,38,65,20,24,49,50,15)	-
HPV group α (7, 10, 16, 18, 26, 27, 31, 33, 35, 39, 45, 52 53, 58, 59)	-

Analytical sensitivity

The kit **HPV 6/11 Real-TM** allows to detect *HPV 6 & 11* 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: L1 gene

TROUBLESHOOTING

1. Weak or no signal of the IC (Rox/Texas Red channel) for the clinical samples.
    - Not correct swab preparation: high quantity of mucous or insufficient quantity of epithelial cells
        ⇒ Repeat the sample collection procedure
    - 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.
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. Any 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.

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

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