MOLECULAR BIOLOGY

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EXTRACTION: AA1001 NOT INCLUDED

HPV Genotypes 14 Real-TM Quant

Real Time (RT) detection

CE

IVD

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IVD For *in Vitro* Diagnostic Use

CE

HPV Genotypes 14 Real-TM Quant
HANDBOOK

Real Time PCR Kit
for quantitative detection and genotyping of Human Papillomavirus
(16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68)

Σ **100**

NAME

HPV Genotypes 14 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 Genotypes 14 Real-TM Quant** is an *in vitro* Real Time amplification test for **quantitative or qualitative** detection and **genotyping** of *Human Papillomavirus* (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68*)* in the urogenital swabs and biopsies.

PRINCIPLE OF ASSAY

kit **HPV Genotypes 14 Real-TM Quant** is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification of 4 PCR tubes for each sample. **HPV Genotypes 14 Real-TM Quant** detects the most widespread and oncogenic 14 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 Genotypes 14 Real-TM Quant** kit contains the internal control (human beta-globin gene), which allows to control the presence of cellular material in the sample. If the swab is not correctly prepared (high quantity of mucous or insufficient quantity of epitelial cells) the Internal Control will not be detected.

It is 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.

MATERIALS PROVIDED

“HPV Genotypes 14 Real-TM Quant ”:

- **PCR-mix-1 “16-18-31-IC”**, 4 x 0,3 ml (blue cap)
- **PCR-mix-1 “39-45-59-IC”,** 4 x 0,3 ml (pink cap)
- **PCR-mix-1 “33-35-56-68”,** 4 x 0,3 ml (green cap)
- **PCR-mix-1 “51-52-58-66”,** 4 x 0,3 ml (orange cap)
- **PCR- buffer-FRT**, 4 x 0,6 ml
- **Hot Start DNA Polymerase**, 4 x 0,06 ml
- **Negative Control***, 1,2 ml;

Standards:

- **K1 “16-18-31-IC”**, 0,2 ml (mix HPV DNA C+ 16, 18, 31 and human DNA) – 1.000.000 GE/ml
- **K2 “16-18-31-IC”**, 0,2 ml (mix HPV DNA C+ 16, 18, 31 and human DNA) – 1.000 GE/ml
- **K1 “39-45-59-IC”**, 0,2 ml (mix HPV DNA C+ 39, 45, 59 and human DNA) – 1.000.000 GE/ml
- **K2 “39-45-59-IC”**, 0,2 ml (mix HPV DNA C+ 39, 45, 59 and human DNA) – 1.000 GE/ml
- **K1 “33-35-56-68”**, 0,2 ml (mix HPV DNA C+ 33, 35, 56, 68) – 1.000.000 GE/ml
- **K2 “33-35-56-68”**, 0,2 ml (mix HPV DNA C+ 33, 35, 56, 68) - 1.000 GE/ml
- **K1 “51-52-58-66”**, 0,2 ml (mix HPV DNA C+ 51, 52, 58, 66) – 1.000.000 GE/ml
- **K2 “51-52-58-66”**, 0,2 ml (mix HPV DNA C+ 51, 52, 58, 66) – 1.000 GE/ml

Contains reagents for 110 tests.

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

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

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**
For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- 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.

STORAGE INSTRUCTIONS

HPV Genotypes 14 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 Genotypes 14 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

Clinical material:

For women:

- *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 with mucolytic agent. Vigorously agitate brush in medium for 15-20 sec.
  - Snap off shaft at scored line, leaving brush end inside tube.
- *Fluid Cytology (*Thinprep PreservCyt®, CytoScreen™, *BD SurePath*™):
  - Place cervical epithelial swab (endocervix) and superficial cervical swab (exocervix) into the tube with transport-fixation media. Working part of the probe is to be broken off and left in the tube with transport media.
- *Biopsy material from mucosa*

For men: Place the urethral epithelial swab obtained by universal probe, into the 2.0 ml volume tube with 0.5 ml of Transport media with mucolytic agent.

It is recommended to process samples immediately after collection. 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.

Please carry out DNA extraction according to the manufacturer's instruction.

Protocol:

1. Prepare required quantity of PCR tubes according to assay type:
   - ✓ **Quantitative analysis:** 4 tubes for each clinical sample (half strip), 4 tubes for K1 standards and 4 tubes for K2 standards (total 1 strip), 4 tubes for Neg.Control (half strip) – see table 1.

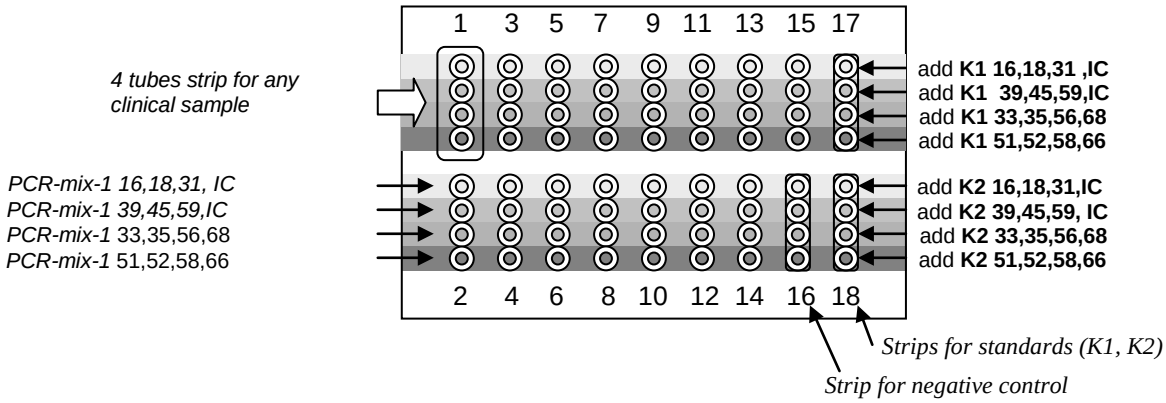


Table 1. Sample distribution for plate type instruments :SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems). For rotor type instruments like Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen) 0,1 ml strip tubes & caps are recommended.

   - ✓ **Qualitative analysis:** 4 tubes for any clinical sample (half strip), 4 tubes for standard K2 (half strip) and 4 tubes for Neg.Control (half strip)

2. Prepare **Mix** for 132 reactions: add into the tube with **PCR-buffer-FRT 60 µl** of **Hot Start DNA Polymerase** Carefully vortex the tube**.** This mix is stable for 3 months at +4°C.
3. Prepare for each PCR-mix-1 one new tube and add for each sample **10*N+3** (+2 for qualitative analysis) **µl** of **PCR-mix-1** and **5*N+3** (+2 for qualitative analysis) of **Mix** (PCR-buffer-FRT with Hot Start DNA Polymerase**).** For example, for quantitative analysis of 8 clinical samples prepare 110 µl of every PCR-mix-1 (10*[8+3]) and add 55 µl of Mix (see table 2 and 3).
4. Add **15 µl** of **Reaction Mix** into each tube. Dispense Reagents and Samples as shown in table 1 (every sample has to be tested in 4 tubes): add in the first line of tubes 15 µl of mix *"16,18,31,IC",* in the second line 15 µl of mix *"39,45,59,IC",* in the third 15 µl of mix "*33,35,56,68",* in the fourth 15 µl of mix *"51,52,58,66".*
5. Add 10 **µl** of **extracted DNA** sample to appropriate tube.
6. Prepare for each panel controls and standards:
   - add **10 µl** of **DNA extracted from Negative Control** to the tubes of strip N°16;
   - add **10 µl** of **each K1** to the tubes of strip N°17 (**required for quantitative analysis only**);
   - add **10 µl** of **each K2** to the tubes of strip N°18;

Table 2. Reaction Mix preparation for qualitative analysis

Clinical samples	Reactions	PCR-mix-1	Mix PCR-buffer-FRT and Polymerase
3	5	50	25
4	6	60	30
5	7	70	35
6	8	80	40
7	9	90	45
8	10	100	50
9	11	110	55
10	12	120	60
11	13	130	65
12	14	140	70
13	15	150	75
14	16	160	80
15	17	170	85
16	18	180	90
17	19	190	95
18	20	200	100
19	21	210	105
20	22	220	110
21	23	230	115
22	24	240	120

Table 3. Reaction Mix preparation for quantitative analysis

Clinical samples	Reactions	PCR-mix-1	Mix PCR-buffer-FRT and Polymerase
2	5	50	25
3	6	60	30
4	7	70	35
5	8	80	40
6	9	90	45
7	10	100	50
8	11	110	55
9	12	120	60
10	13	130	65
11	14	140	70
12	15	150	75
13	16	160	80
14	17	170	85
15	18	180	90
16	19	190	95
17	20	200	100
18	21	210	105
19	22	220	110
20	23	230	115
21	24	240	120

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)*

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*15 %*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*15 %*	*On*
Rox (Orange)	*from 5 Fl to 10 Fl*	*0.03*	*15 %*	*On*
Cy5 (Red)	*from 5 Fl to 10 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.

Data Analysis & Results Interpretation:

Signal in a tube in the channel is considered to be positive, if corresponding fluorescence accumulation curves cross the threshold line. The signal is characterized by the cycle (threshold Ct) corresponding to the intersection of the fluorescence curve with the threshold line. The softeare of analysis determines the Ct value. For quantitative analysis, the calibration curve is automatically plotted on the basis of these values, and human DNA and *HPV* DNA concentrations are calculated. To obtain the final result, *HPV* DNA concentration is normalized to the number of human genome equivalents according to the formula (see program Microsoft® Excel "*HPV Genotype 14 Real-TM.xls*"):

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

To calculate such values of copies please refer to the provided file Microsoft Excel "*HPV Genotype 14 Real-TM.xls*" and enclosed instructions. Clinical explanation of final values obtained is explained as follows:

Result log (HPV DNA in 100.000 cells)	Interpretation
< 3	Clinically insignificant *(Insignificance)*
3-5	Clinically important. Present risk of cervical dysplasia *(Significance)*
> 5	Clinically very important. High risk of cervical dysplasia *(Heightened)*

The reaction is ***valid*** if

- Negative control have no signal in all channels (FAM/Green, JOE/Yellow/HEX, ROX/Orange, Cy5/Red);
- All calibrators have signals in all channels (FAM/Green, JOE/Yellow/HEX, ROX/Orange, Cy5/Red);
- The correlation coefficient for calibration curves for all channels is not less than 0.98.

The result of *HPV* DNA detection of a given sample is considered to be:

- **Negative**, if the signal of the Internal Control (IC; FAM/Green channel) is detected in 2 first tubes for the sample and the quantity of human DNA genome equivalents per reaction exceeds 10^3.
- **Positive**, if a signal in the FAM/Green, JOE/Yellow/HEX, ROX/Orange, Cy5/Red channel is detected at least in one of the 4 tubes (except channel Cy5/Red in the mixes *16,18,31,IC and 39,45,59,IC which is reserved for* human beta-globine gene detection).

Genotyping of HPV :

- On the FAM/Green channel are detected 16,39,33,58 HPV genotypes,
- On the JOE/Yellow/HEX channel are detected 31,45,35,52 genotypes,
- On the ROX/Orange channel are detected 18,59,68,66 genotypes,
- On the Cy5/Red channel genotypes 56 and 51 (in the tubes with **PCR-mix-1 "33-35-56-68"** and **PCR-mix-1 "51-52-58-66"**).

Table 4. HPV genotypes detection

FAM	JOE	ROX	Cy5
16	31	18	IC
39	45	59	IC
33	35	68	56
58	52	66	51

⚠ Note:

The sample is considered to be *weak*, if IC signal (**Cy5/Red channel in the first 2 tubes**) is present in all tubes and Ct value is **less than 30** and there is a positive signal in **any other channel** that **exceed 33** (an *equivocal* result for this *HPV* type).

For this sample PCR run has to be repeated. If in the second run the result is *positive*, the sample is considered to be *positive*. If in the second run the result is *weak* or *negative*, the sample is considered to be *negative.*

The **absence** of the IC signal (**Cy5/Red channel** in the tubes with **PCR-mix-1 "16-18-31-IC"** and **PCR-mix-1 "39-45-59-IC"**) in a strip tube is acceptable if signal/signals in **FAM/Green**, **JOE/Yellow**, or **ROX/Orange** channels is/are detected and Ct values **do not exceed 33**.

Example

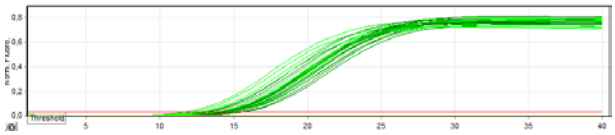


Fig. 1 Channel Cy5 – internal control (**PCR-mix-1 "16-31-18-IC"** and **PCR-mix-1 "39-45-59-IC"**)

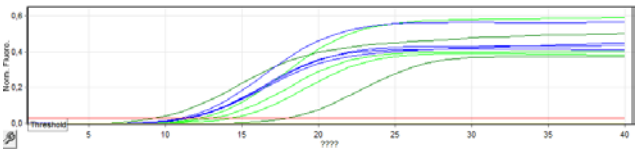


Fig. 2 Channel Fam

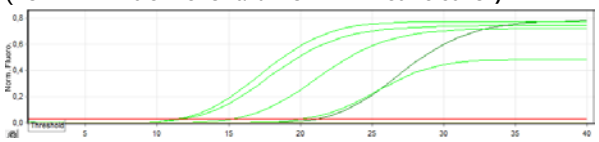


Fig. 3 Channel Joe

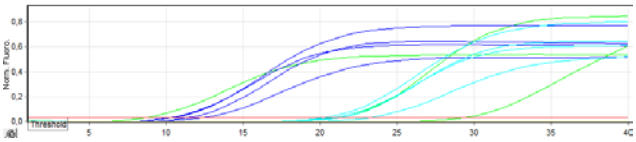
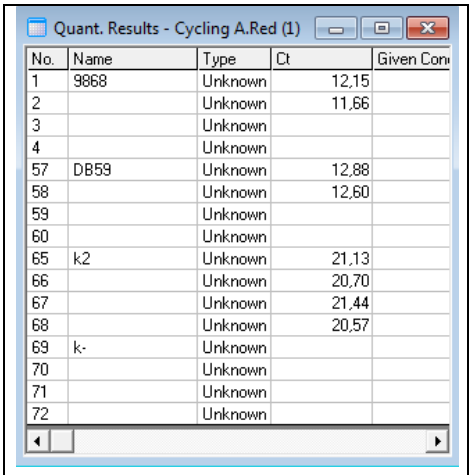


Fig. 4 Channel Rox



Quant. Results - Cycling A.Red (1)

No.	Name	Type	Ct	Given Con
1	9868	Unknown	12,15	
2		Unknown	11,66	
3		Unknown		
4		Unknown		
57	DB59	Unknown	12,88	
58		Unknown	12,60	
59		Unknown		
60		Unknown		
65	k2	Unknown	21,13	
66		Unknown	20,70	
67		Unknown	21,44	
68		Unknown	20,57	
69	k-	Unknown		
70		Unknown		
71		Unknown		
72		Unknown		

Fig. 5 The table results *Quant.Results*

date		Matrix for comparison				Reaction Ct-Results				Results
NN	Sample Name	Fam	Joe	Rox	Cy5	Fam Ct	Joe Ct	Rox Ct	Cy5 Ct	
1	9868	16	31	18	IC				12	
	9868	39	45	59	IC		12		12	45
	9868	33	35	68	56					
	9868	58	52	66	51					
2	DB59	16	31	18	IC	13			13	16
	DB59	39	45	59	IC	12	15	29	13	39,45,59
	DB59	33	35	68	56		20	21		35,68
	DB59	58	52	66	51		12			52
3	K+	16	31	18	IC	21	21	21	21	OK
	K+	39	45	59	IC	21	21	21	21	OK
	K+	33	35	68	56	22	20	20	21	OK
	K+	58	52	66	51	22	21	23	20	OK
4	k-	16	31	18	IC					OK
	k-	39	45	59	IC					OK
	k-	33	35	68	56					OK
	k-	58	52	66	51					OK

Fig.6 Program Microsoft ® Excel "*HPV Genotype 14 Real-TM.xls*"

NOTE: For **quantitative analysis** please refer to the provided file Microsoft Excel "*HPV Genotype 14 Real-TM.xls*" and enclosed instructions.

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 Genotypes 14 Real-TM Quant** was 100%. The potential cross-reactivity of the kit **HPV Genotypes 14 Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **HPV Genotypes 14 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: E6, E7

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