HPV Screening Real Time PCR Kit

Kit for the detection of 14 genotypes of high-risk HPV by Real-Time PCR

REF **Ref. MAD-003949M-W** Σ **100 determinations**

For in vitro diagnostic use only
Directive 98/79/EC

Rev.: 2022/03/08

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1 INTENDED USE

HPV Screening Real Time PCR kit is an *in vitro* diagnostic kit for the qualitative detection of DNA of 14 genotypes of the human papillomavirus (HPV) considered of high oncogenic risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) from purified DNA from human clinical specimens of different origin such as liquid-based cytology and vaginal and rectal swabs. It is based on the multiplex real-time PCR technique and uses fluorescent primers and probes for a conserved region of the target gene L1 of the HPV genomes. The test allows the specific identification of the genotypes HPV 16 and 18 and the simultaneous detection of the genotypes 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 at clinically relevant infection levels.

Specific primers and fluorescent probe are included for the simultaneous detection of the human beta globin gene as internal quality control of the starting material and amplification. The detection channels of the different targets are:

Target	Fluorophore
HPV 16	ROX
HPV 18	Cy5
HR	FAM
Beta globin	HEX/JOE/VIC

Table 1. Detection channels for the different targets of the HPV Screening Real Time PCR kit

This test must be carried out at hospital level in clinical microbiology laboratories to those patients who show symptoms compatible with HPV infection. The intended end use is as aid in the diagnosis of this infection in combination with clinical risk and epidemiological factors.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

HPV Screening Real Time PCR kit is a multiplex assay based on the real-time polymerase chain reaction. The Master mix contains a set of primers and probes that allow the detection of DNA in the genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 of the human papillomavirus. It also includes a set of primers and probe for the detection of the human beta globin gene in clinical or control specimens. The oligonucleotides used as primers and probes were selected in evolutionary conserved regions of the viral genome.

In the presence of any of these HPV genotypes in clinical specimens, viral DNA is amplified by polymerase chain reaction (PCR). The detection of the amplicons obtained is based on the TaqMan probe technology. These probes are modified single-stranded DNA oligonucleotides that have a fluorophore (reporter) covalently attached to the 5' end and a quencher attached to the 3' end. If the target nucleic acids are present, these are amplified and, during the PCR process, the probes will bind specifically in the complementary regions located between the forward and reverse primers.

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While the extension phase of the PCR occurs, the 5' nuclease activity of the DNA polymerase degrades the probes bound specifically to their targets, causing the split between the reporter and the quencher, and a fluorescent signal will be generated. The specific probes for HPV 16, HPV 18, the other high-risk genotypes and the internal control are labeled with different fluorophores, so that in each case a fluorescent signal will be generated at different wavelengths, allowing the Real Time PCR platform to simultaneously detect and differentiate the different signals in a single reaction. In each denaturation-extension cycle, the split of new molecules of reporter occurs, and, consequently, the intensity of the fluorescent signal increases. The intensity of the fluorescence is monitored on the real-time PCR instruments in each of the cycles and the data are analyzed with a specific analysis software for each platform.

The detection of viral DNA is of great usefulness in the diagnosis and follow-up of infections caused by these microorganisms.

3 COMPONENTS

HPV Screening Real Time PCR is commercialized as a ready-to-use Master Mix which includes all the necessary reagents to perform the real-time PCR.

Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme Uracil-DNA Glycosylase (Cod-UNG), which degrades PCR products containing dUTP.

A positive control (PC) and DNase/RNase-free DEPC-treated water to include in the negative controls (NTC) are supplied along with the real-time PCR-Mix.

Components of the kit for 100 tests:

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003949M-100-W (HPV Screening MMIX)	**MAD-003949-MIX-W (HPV Screening MMix)**	Hot Start Polymerase (125 U/mL), Uracil DNA glycosylase (50 U/mL), 0.1-0.4 µM primers, 0.1 - 0.4 µM fluorescent probes, 2x reaction buffer, 1 mM dUTP, 1.3 mM dNTPs (A, C, G, T)	**2 vials with 50 test/vial**
	MAD-DDW-DEPC (RNase/DNase-free water)	---	**1 vial (200 µl)**
MAD-HPV (HPV Screening PC)		Synthetic non-infectious DNA containing part of the genome of HPV 16, HPV 18, HPV 45 (12500 copies/µL) and human DNA (0.625 ng (µL)	**1 vial (100 µl)**

Table 2. Reagents and concentrations of the active substances supplied in the HPV Screening Real Time PCR kit.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- DNase/RNase-free filtering pipette tips.
- DNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.
- Microcentrifuges of PCR tube strips or 96-well plates.
- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real-Time PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

HPV Screening Real Time PCR kit must be transported and stored at -20 °C*. Nonetheless, besides the recommended transport at -20 °C, it is also possible to transport it at refrigeration temperature (2 °C - 8 °C), as long as the transit period does not exceed a maximum of ten days. In any case, the kit must be stored at a temperature of -20 °C upon receipt.

HPV Screening MMix is sensitive to physical state changes and it has been proven that it supports up to five freeze-thaw cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance.

The HPV Screening MMix is stable for 29 days at room temperature. If the Arrhenius equation is applied using a Q10 equal to 2.2 to estimate the shelf life of the product, it is equivalent to a time stability of 4 months stored at 4 °C, and of at least 2 years stored at -20 °C.

The time stability tests of the product are currently in progress. Therefore, the MMix is stored at -20 °C and its stability is determined after 2, 4, 6, 8, 10 and 12 months.

The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and repeated freeze-thaw cycles are to avoid. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain

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is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- The HPV Screening MMix must be thawed before use and handled on ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex, and centrifuge briefly.
- The positive control must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- HPV Screening Real Time PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.
- **General considerations to avoid the contamination with PCR product**
  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the RNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test RNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-DNA Glycosylase (Cod-UNG)*, which degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls replacing the DNA specimen with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.
- **Waste disposal**
  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of waste generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/EC on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

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POTENTIAL WASTE GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, this waste must be considered as "waste whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Waste whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers containing waste or contaminated by dangerous substances"

Table 3. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

7.1 Sample collection

HPV Screening Real Time PCR kit has been validated for its use from purified genetic material from different types of clinical samples, such as liquid-based cytologies and vaginal and rectal swabs.

This kit has been validated with starting genetic material obtained from the following DNA/RNA purification kits and extraction equipments* starting with 200 µl of clinical sample and eluting in 100 µl of elution buffer (for purification with Opentrons start with 92 µl of clinical specimen and elute in 60 µl of elution solution):

EXTRACTION KITS	EXTRACTION EQUIPMENT
Maxwell® 16 FFPE Tissue LEV ADN Purification Kit (Promega)	Maxwell® 16 (Promega)
NX48S – Urine/Swab DNA Kit (Genolution)	Nextractor NX-48S (Genolution)
RNA/DNA pathogen extraction kit (Robot Opentrons OT2) (Vitro, ref. MAD-003955M)	Opentrons OT-2

Table 4. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be verified beforehand.

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8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

The PCR is carried out in a final volume of 20 µl. Prepare the Master Mix as indicated below:

1. Thaw and homogenize HPV Screening MMix (do not use vortex). Once it is thawed, centrifuge briefly.
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
HPV Screening MMix	12 µl
Sample	8 µl

3. Include a negative control by adding 8 µl of the water included in the kit.
4. Include a positive control by adding 8 µl of the positive DNA control HPV Screening PC included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

It is recommended to keep the MMix on cold plate during the preparation of the samples and not thaw the vial more than five times.

8.2 Configuration of the instrument for real-time PCR

Enter the different targets and detection channels for each of them in the instrument's software. Create the samples, the positive control (PC), the non template control (NTC) and allocate the positions of the samples in the PCR plate.

Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25°C	5 min	1 cycle
95°C	5 min	1 cycle
95°C	15 sec	5 cycles
42°C	15 sec	
72°C	30 sec	
95°C	15 sec	45 cycles
60°C*	40 sec	

Table 5. PCR program of HPV Screening Real Time PCR kit

* Fluorescence data should be collected during the extension stage (*) through ROX (HPV 16), Cy5 (HPV 18), FAM (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and HEX, JOE or VIC (Internal Control) channels.

This kit has been validated with the platforms:

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- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- VitroCycler (Vitro S.A.)

For its use in other platforms, it is recommended to verify the compatibility of the fluorochromes with the detection channels of each instrument. Although the fluorochromes included in the kit are compatible with the majority of the most-used real-time instruments available on the market. In Applied Biosystems 7500 Fast Real-Time PCR System, QuantStudio™ 5 Real-Time PCR System thermal cyclers the ROX passive control option must be disabled.

In the thermal cyclers Applied Biosystems QuantStudio™ 5 Real-Time PCR System and Applied Biosystems 7500 Fast Real-Time PCR System, select Ramp Speed Standard in the menu "Select New Experiment/Advanced Setup/Experiment Properties".

9 INTERPRETATION OF RESULTS

Before interpreting the results of the clinical samples, it is necessary to follow the interpretation guide of the positive and negative controls as in the table below:

	RESULT	INTERPRETATION
Positive Control HPV	Signal for the channels FAM, ROX, Cy5 and JOE*	The control/reaction is correct
	No signal for FAM and/or ROX and/or Cy5 and/or JOE	Problem in the amplification: repeat analysis
Negative control:	Signal for the channels FAM and/or ROX and/or Cy5 and/or JOE	Contamination, repeat analysis
	No signal	The control/reaction is correct

Table 6. Interpretation guide for the positive and negative controls.

*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

The run is considered valid when adequate results have been obtained for all reaction controls and the Cts values obtained in the positive control for the different targets are within the range of expected values, being these:

- 20±2 for HPV 16 (ROX)
- 20±2 for HPV 18 (Cy5)
- 20±2 for HPV HR (FAM)
- 20±2 for Beta globin (JOE)

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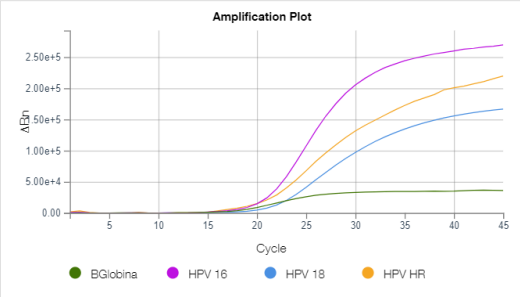

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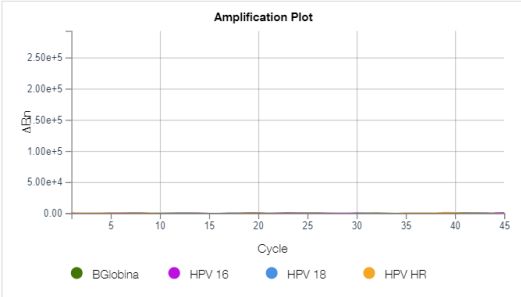

Figure 1: Graphs of amplification of the positive control PC (A) and of a negative control with water NTC (B). (Expected Cts values for PC: HPV 16 (ROX) 20±2; HPV 18 (Cy5) 20±2; HPV HR (FAM) 20±2, Beta globin (JOE) 20±2. Experiment performed in Applied Biosystems QuantStudio™ 5 Real-Time PCR System.

If the run has been validated, interpret the results of the clinical samples according to the following table:

HPC Screening Real Time PCR				INTERPRETATION
HPV 16 (ROX)	**HPV 18 (Cy5)**	**HPV HR (FAM)**	**Beta globin (JOE)**	
Signal	No signal	No signal	Signal	Positive sample for HPV 16
			No signal	
No signal	Signal	No signal	Signal	Positive sample for HPV 18
			No signal	
No signal	No signal	Signal	Signal	Positive sample for other high-risk HPV genotypes (Types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
			No signal	
Signal	Signal	No Signal	Signal	Positive sample for HPV 16 and HPV 18
			No signal	
Signal	No Signal	Signal	Signal	Positive sample for HPV 16 and other high-risk genotypes (Types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
			No signal	
No Signal	Signal	Signal	Signal	Positive sample for HPV 18 and other high-risk genotypes (Types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
			No signal	
Signal	Signal	Signal	Signal	Positive sample for HPV 16, HPV 18 and other high-risk genotypes (Types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
			No signal	
No signal	No signal	No signal	Signal	Negative result [1]
			No signal	Invalid [2]: Problems in the extraction or amplification

Table 7. Interpretation of results guide.

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[1] Negative or below the limit of detection of the kit.
[2] It is recommended to repeat the PCR or start from a new DNA extraction.

It is recommended to use the default threshold line stablished automatically by the instrument. If required, the threshold line can be manually adjusted until it lies within the exponential phase of the fluorescence curves and above any background signal.

A sample is positive if the Ct value obtained is ≤ 38, even if the internal control does not show an amplification graph. Sometimes, it might occur that the internal control is not amplified correctly due to the presence of a high initial number of copies of target bacterial nucleic acid, which can cause a preferential amplification of the latter.

A sample is negative if an amplification curve is not detected over the threshold value, and if the internal control does show it. The inhibition of the PCR reaction can be excluded by the amplification of the internal control.

10 PERFORMANCE CHARACTERISTICS

10.1 Analytical sensitivity

The analytical sensitivity of the **HPV Screening Real Time PCR** kit was determined by performing three replicates of serial dilutions of synthetic fragments of each of the targets from 10^6 copies/rxn to 10 copies/rxn. By adjusting the obtained Cts data to a line, the amplification efficiency, R^2 and the slope were determined for each of the genes.

It has been established that **HPV Screening Real Time PCR** kit has a limit of detection of 10 copies/reaction for the genotypes HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66 and HPV 68 (Figure 2).

The amplification efficiency of each target as well as the R^2 and the slope of the obtained line are shown in Table 8.

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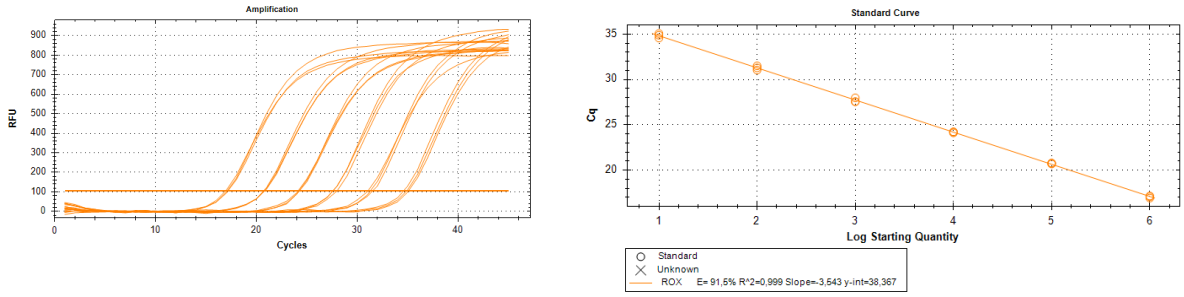

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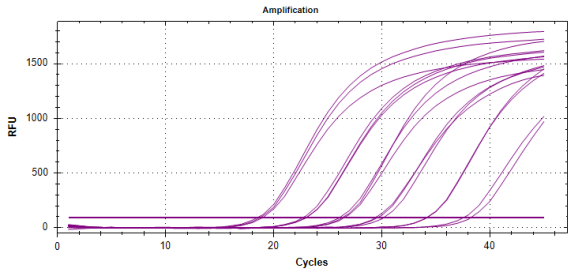

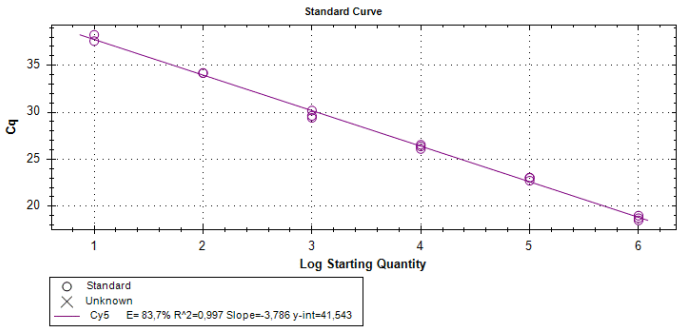

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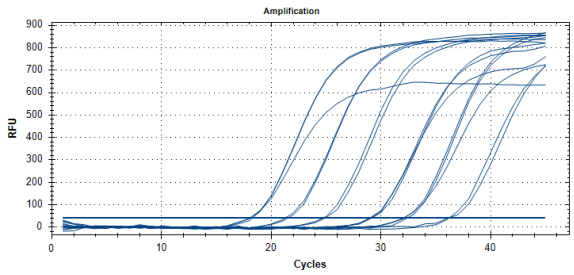

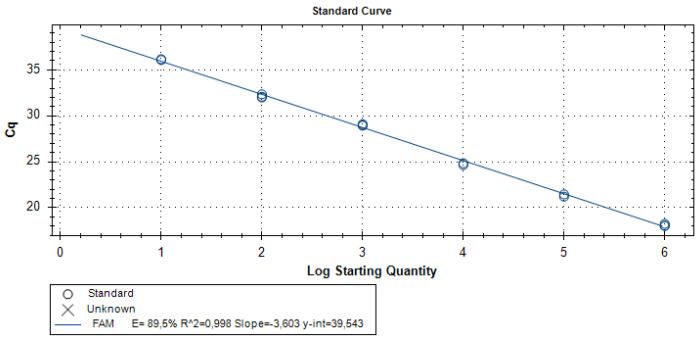

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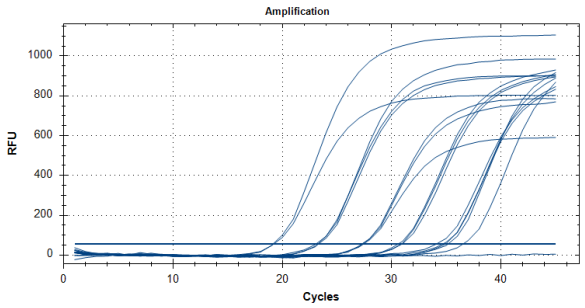

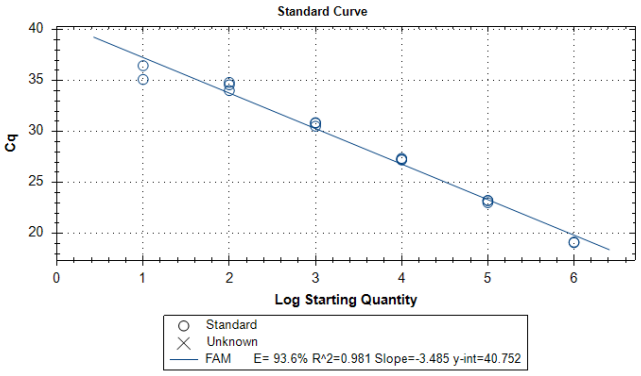

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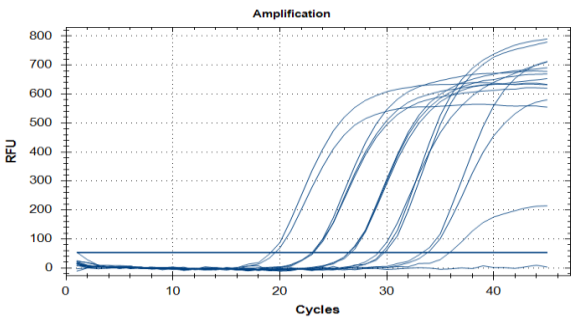

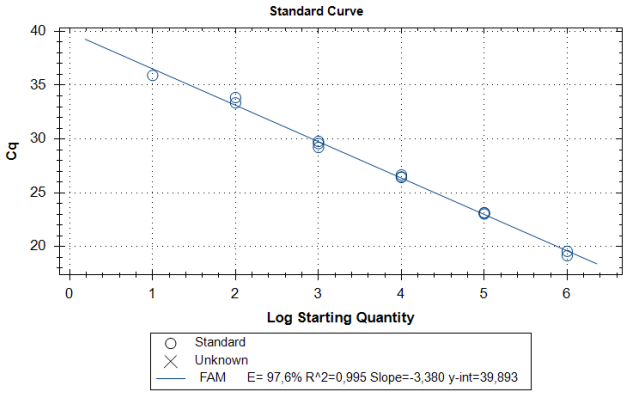

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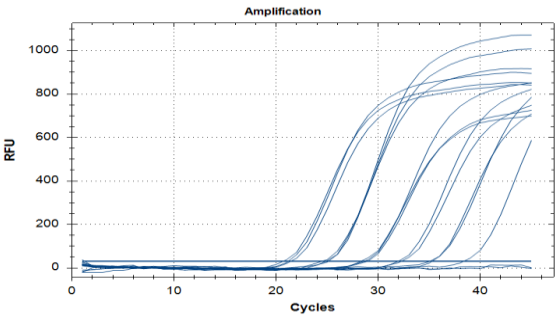

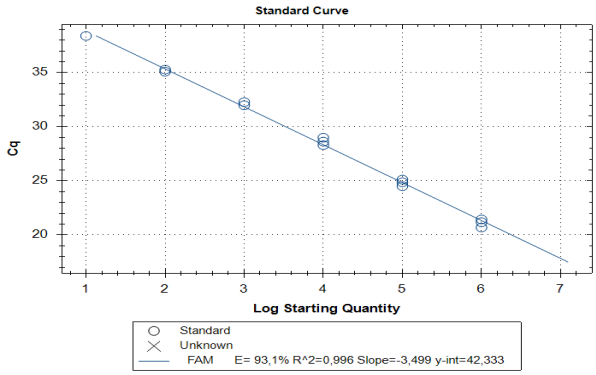

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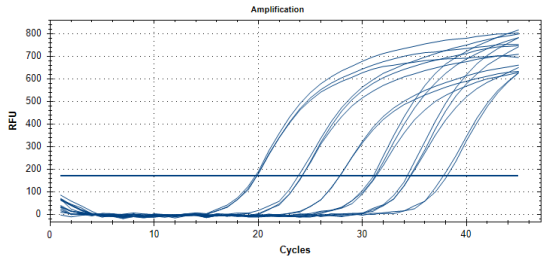

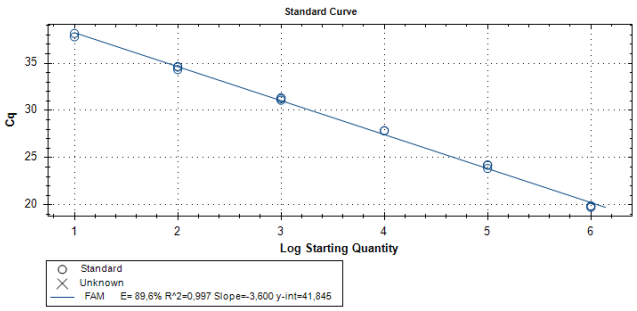

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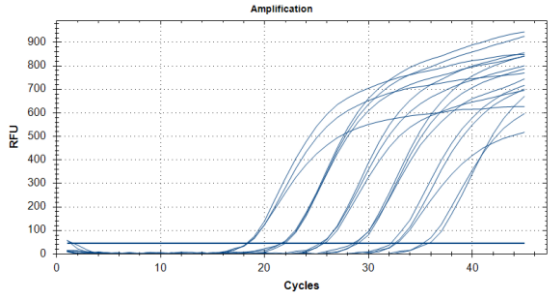

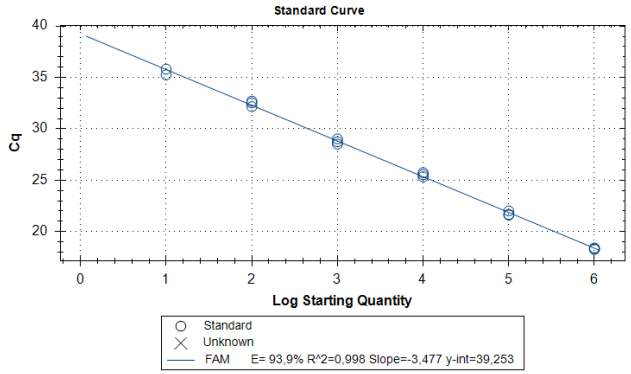

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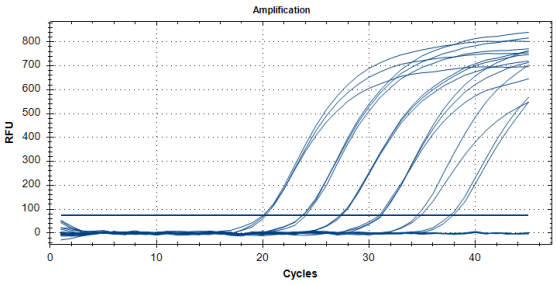

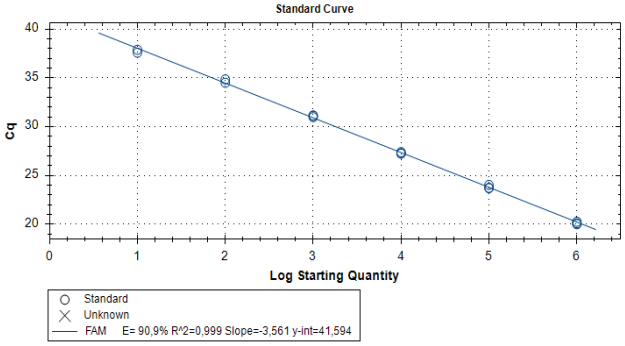

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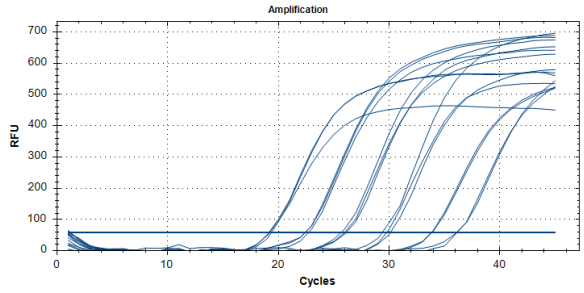

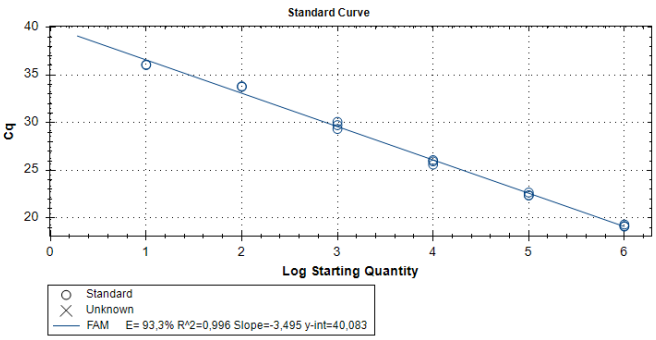

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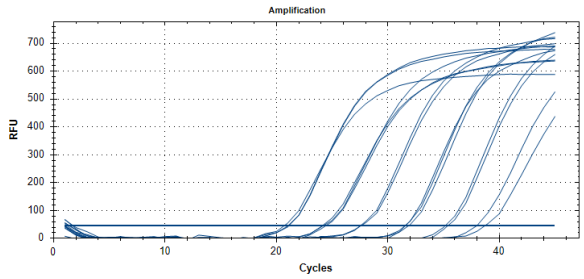

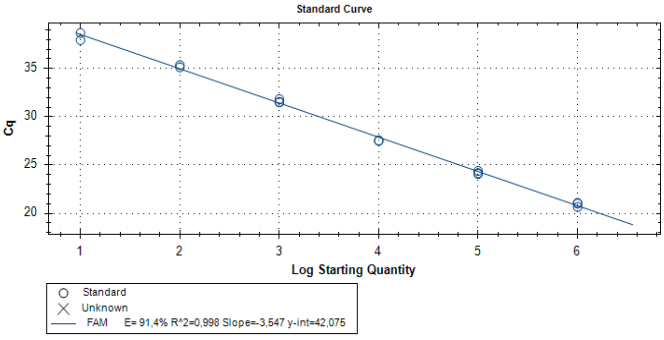

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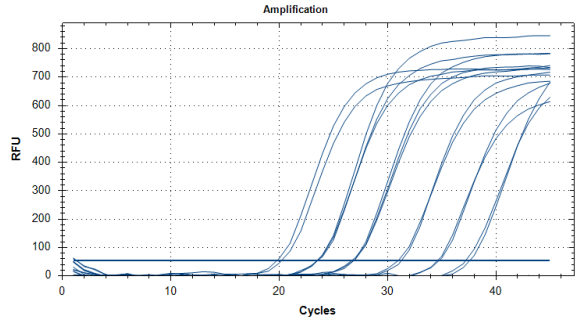

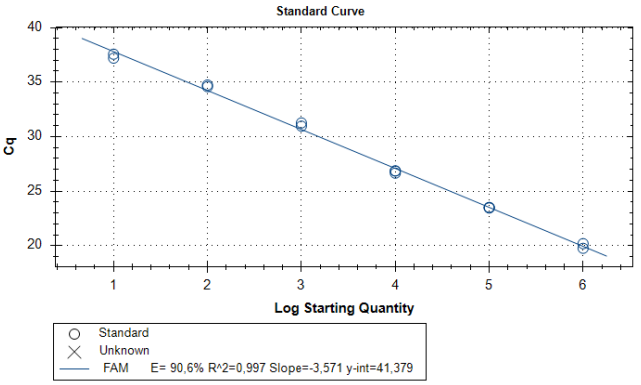

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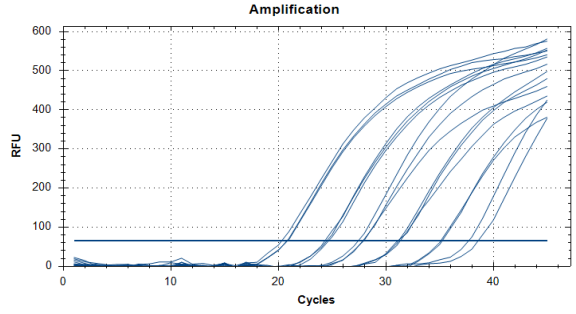

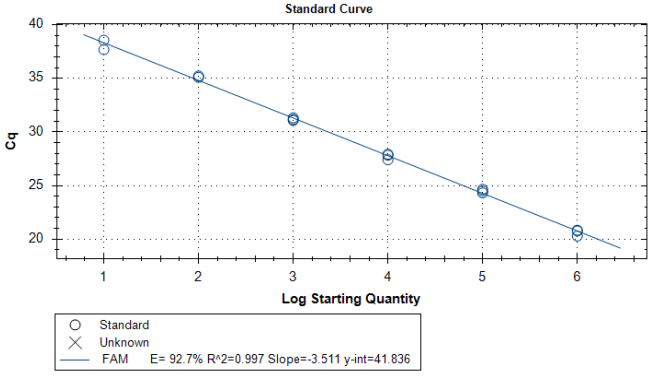

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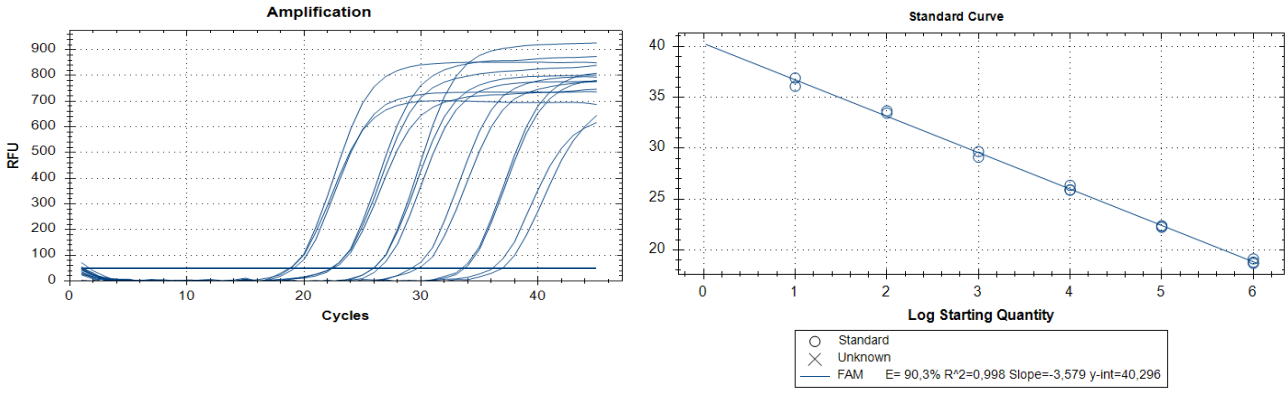

Figure 2: Left: Serial dilutions from 10^6 copies/reaction to 10 copies/reaction of synthetic fragments of HPV 16 in the ROX channel (A), HPV 18 in the Cy5 channel (B) and HPV 31 (C), HPV 33 (D), HPV 35 (E), HPV 39 (F), HPV 45 (G), HPV 51 (H), HPV 52 (I), HPV 56 (J), HPV 58 (K), HPV 59 (L), HPV 66 (M) and HPV 68 (N) in the FAM channel. Right: Calibration lines obtained for each target. Experiment performed on CFX96™ Real-Time PCR Detection System (Bio-Rad).

HPV genotype	Efficiency	R^2	Pending
HPV 16	91.5%	0.999	-3.543
HPV 18	83.7%	0.997	-3.786
HPV 31	89.5%	0.998	-3.063
HPV 33	93.6%	0.981	-3.485
HPV 35	97.6%	0.995	-3.380
HPV 39	93.1%	0.996	-3.499
HPV 45	89.6%	0.997	-3.600
HPV 51	93.9%	0.998	-3.477
HPV 52	90.9%	0.999	-3.561
HPV 56	93.3%	0.996	-3.495
HPV 58	91.4%	0.998	-3.547
HPV 59	90.6%	0.997	-3.571
HPV 66	92.7%	0.997	-3.511
HPV 68	90.3%	0.998	-3.579

Table 8. Amplification efficiency, R^2 and slope of the straight lines obtained with serial dilutions of synthetic fragments of each target.

10.2 Analytical specificity

The specificity of the HPV Screening Real Time PCR kit was confirmed by testing positive clinical samples for other pathogens related to sexually transmitted diseases. In addition, specificity tests were also performed against potential high-risk and low-risk genotypes of human papillomavirus and against other bacteria that share an ecological niche. The full list of organisms tested for cross-reactivity is shown in the following table.

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Cross-reactivity test	
Microorganism	**Results**
Candida albicans	Negative
Chlamydia trachomatis	Negative
Enterobacter cloacae	Negative
Enterococcus faecium	Negative
Enterococcus faecalis	Negative
Escherichia coli	Negative
Haemophilus ducrey	Negative
HR genotype HR 26	Negative
HR genotype HR 53	Negative
HR genotype HR 73	Negative
HR genotype HR 82	Negative
LR genotype HR 11	Negative
LR genotype HR 42	Negative
LR genotype HR 43	Negative
LR genotype HR 54	Negative
LR genotype HR 6	Negative
LR genotype HR 61	Negative
LR genotype HR 62	Negative
LR genotype HR 67	Negative
LR genotype HR 70	Negative
LR genotype HR 71	Negative
LR genotype HR 72	Negative
LR genotype HR 81	Negative
LR genotype HR 84	Negative
Klebsiella oxytoca	Negative
Klebsiella pneumoniae	Negative
Mycoplasma genitalium	Negative
Mycoplasma hominis	Negative
Neisseria gonorrhoeae	Negative
Proteus mirabilis	Negative
Pseudomonas aeruginosa	Negative
Staphylococcus aureus	Negative
Staphylococcus epidermidis	Negative
Staphylococcus aureus	Negative
Streptococcus pneumoniae	Negative
Streptococcus pyogenes	Negative
Treponema pallidum	Negative
Trichomonas vaginalis	Negative
Ureaplasma urealyticum	Negative

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Respiratory Syncytial Virus-1	Negative
Respiratory Syncytial Virus-2	Negative

Table 9. Microorganisms tested in the specificity test.

No cross-reactions were detected with any of the pathogens tested.

10.3 Repeatability

The repeatability was analyzed by testing the method 6 times for each of the targets included in the panel. For this purpose, a known concentration of synthetic DNA fragments mimicking each of the targets to be amplified was used. The test was performed by the same operator, in a single location and using the same reagent lot and the same platform. The platform used was Applied Biosystems QuantStudio™ 5 Real-Time PCR System and the results were analyzed with the version v. 2.4.3 of the software Design and Analysis (Applied Biosystems). The variability between trials was determined from the Cts values obtained from the repetitions and the coefficient of variation (CV) was calculated as standard deviation divided by the mean of the Cts, being 0.35 % for HPV 16, 0.93 % for HPV 18 and 1.43 % for HPV HR.

10.4 Reproducibility

The reproducibility of the method was analyzed by simulating the inter-laboratory variability, changing the operator, the equipments used in the process and the lots of PCR mix. 40 purified DNA samples were tested with the RNA/DNA pathogen extraction kit (Robot Opentrons OT2), (Vitro, ref. MAD-003955M) using the Opentrons OT2 automated extraction system. Of the 40 samples, 24 were positive for HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66 and/or HPV 68 and 16 samples were negative.
Concordance was calculated with a kappa index of 1.00, standard error of 0 and a 95% CI of 1,000-1,000 showing a very good concordance strength for the **HPV Screening Real Time PCR** kit.

	Laboratory 1		
Laboratory 2	positive	negative	Total
positive	**24**	**0**	24
negative	**0**	**16**	16
Total	24	16	40

Table 10. Reproducibility test for the targets included in HPV Screening Real Time PCR kit.

10.5 Measurement range

The measurement range of the assay was determined using synthetic DNA fragments for each of the targets that are included in the MMix of HPV Screening Real Time PCR kit.

HPV Screening Real Time PCR kit has been shown to work correctly in the presence of synthetic fragments of each of the targets from 10^6 copies/reaction to 10 copies/reaction. (See section 10.1 Analytical

sensitivity).

To determine the upper limit, serial dilutions from 10^9 copies/reaction to 10^7 copies/reaction of synthetic fragments of each target were tested. Three replicates were tested at each level.

All reactions were performed with the QuantStudio ™ 5 real-time PCR system and analyzed with QuantStudio ™ 2.4.3 design and analysis software.

It has been established that the HPV Screening Real Time PCR kit has a measurement range of 10^9 copies/reaction to 10 copies/reaction for HPV 16 and HPV 18; 10^6 copies/reaction to 10 copies/reaction for HPV 31, HPV 33, HPV 35, HPV 45, HPV 51, HPV 52, HPV 56, HPV 59, HPV 66 and HPV 68 and 10^7 copies/reaction to 10 copies/reaction for HPV 39 and HPV 58.

10.6 Clinical sensitivity and specificity

HPV Screening Real Time PCR kit was validated from DNA purified by any of the extraction methods afore mentioned. The diagnostic capability of HPV Screening Real Time PCR kit was evaluated by studying its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

- The **diagnostic specificity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided by the sum of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP).

- The **diagnostic sensitivity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/ (TP + FN).

A total of 266 clinical samples of different origin from different hospitals and laboratories were analyzed in a retrospective study: Costa de la Luz Hospital (Huelva), Clinical University San Cecilio Hospital (Granada), Laboratory Dr. Aneiros (Granada), Laboratory Dra. Lasso (Madrid), Jiménez Ayala Institute (Madrid), Laboratory Dra. Maestro, Laboratory Dr. Cueva S.L. (Jaén), Anatomic Pathology Laboratory Luresa (Lugo) and Bioportugal (Porto). Of these samples 101 were positive for one or more of the genotypes detected by the kit and 165 were negative. The comparative study was performed using the CE-IVD marked HPV Direct Flow Chip Kit (Vitro S.A.) as the reference method. DNA from the samples was extracted with the RNA/DNA pathogen extraction kit (Robot Opentrons OT2) (Vitro, ref. MAD-003955M).

Tables 11 and 12 show the diagnostic sensitivity and specificity of the HPV Screening Real Time PCR kit as well as its positive and negative predictive value.

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Organism	TN	FP	TP	FN	Diagnostic Specificity	95% CI	Diagnostic Sensitivity	95% CI
HPV 16	248	0	18	0	100%	98.09 – 100 %	100%	78.12 – 100 %
HPV 18	256	0	10	0	100%	98.16 – 100 %	100%	65.54 – 100 %
HPV HR	187	0	77	2	100%	97.49 – 100 %	97.5%	90.31 – 99.56 %

Table 11. Sensitivity and specificity results obtained with HPV Screening Real Time PCR kit.

Organism	TN	FP	TP	FN	PPV	95% CI	NPV	95% CI
HPV 16	248	0	18	0	100%	78.12 – 100 %	100%	98.09 – 100 %
HPV 18	256	0	10	0	100%	65.54 – 100 %	100%	98.16 – 100 %
HPV HR	187	0	77	2	100%	94.08 – 100 %	98.94%	95.83 – 99.82 %

Table 12. Positive predictive value and negative predictive value of HPV Screening Real Time PCR kit.

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. Use of inadequate samples: The types of clinical specimens that have been validated are liquid-based cytologies and vaginal and rectal swabs. The method has been validated on the basis of purified genetic material from them. The analysis of any other type of specimen not indicated can lead to wrong or inconclusive results due to PCR reaction inhibition by inhibiting chemical agents.

3. The correct functioning of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for HPV does not exclude the possibility that other pathogens are present in the clinical sample.

6. A negative result of the test does not exclude that there is an infection with HPV and it should not be used as the sole diagnostic method to establish a treatment or patient management regime .

7. A negative result of the test must be analyzed in the context of medical history of the patient and epidemiology.

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13 LABEL AND BOX SYMBOLS

IVD	Health product for in vitro diagnosis.		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions of use		Sufficient content for <n> assays
	Material safety data sheet		Keep away from sunlight

14 CHANGELOG

Date	Description
2021/07/22	Creation of the document.
2022/03/08	Inclusion of the explanation of the pictogram “Keep away from sunlight”.

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