HPV Direct Flow Chip Kit

Screening and genotyping of human papillomavirus based on amplification and specific hybridization

For all hybriSpot platforms

Compatible with the version 2.2.0R00 of hybriSoft HSHS.
For compatibility with other versions, please contact the manufacturer / supplier.

REF		Σ	
	Ref. MAD-003930MU-HS12-24		**24 tests**
	Ref. MAD-003930MU-HS12-48		**48 tests**
	Ref. MAD-003930MU-HS24-24		**24 tests**
	Ref. MAD-003930MU-HS24-48		**48 tests**

For in vitro diagnostic use only

Directive 98/79/EC and ISO 18113-2

2023/06/22

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1 INTENDED USE

HPV Direct Flow CHIP is an *in vitro* diagnostic kit for the detection of human papillomavirus (HPV). The infection with HPV is an essential factor in cervical and anogenital carcinogenesis (zur Hausen *et al,* 1974; Walboomer et al, 1999; zur Hausen, 1996; zur Hausen 2002).

Based onthe association with different degrees of cervical lesions, the different HPV genotypes have been classified (Muñoz 2003) as high-risk or oncogenic genotypes, which can induce carcinogenesis; and low-risk HPVs, which cause genital warts and collaborate with high-risk HPVs.

The **HPV Direct Flow CHIP** allows the qualitative detection of the HPV and the genotyping of 35 types of HPV (high-risk HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82, and low-risk HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81 and 84) by PCR (polymerase chain reaction), followed by reverse hybridization on a membrane containing specific probes. This system accepts both clinical samples that can be amplified directly, without the need for prior DNA extraction, and the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M).

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The **HPV Direct Flow CHIP** kit methodology is based on the amplification of a fragment in the viral region L1 of papillomavirus by PCR, followed by hybridization onto a membrane with DNA-specific probes using DNA-Flow technology on hybriSpot platforms, both automated and manual. The biotinylated amplicons obtained in the PCR step are hybridized in membranes containing an array of target-specific probes as well as amplification and hybridization control probes. The DNA-Flow technology allows a very fast binding of the PCR product and its specific probe in a three-dimensional porous environment, as compared to the hybridization in a conventional surface. Once the binding between the specific amplicons and their corresponding probes has occurred, the signal is visualized by an immunoenzymatic colorimetric reaction with Streptavidin−Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization. The results are analyzed automatically with the hybriSoft™ software.

The HPV Direct Flow CHIP kit does not require a prior extraction of DNA from the samples although it can be done if preferred. PCR amplification can be performed directly from cell suspensions, fixed cells or paraffin-embedded tissue sections, with the consequent reduction in time for the sample handling and results. This kit also works with the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M).

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3 COMPONENTS

The **HPV Direct Flow Chip** kit is commercialized in two main lyophilized formats depending on the type of hybridization platform to be used for the analysis of clinical samples. Both formats include all the necessary reagents for the amplification by multiplex PCR and subsequent hybridization of 24/48 clinical samples. Each kit format contains the following components and references:

3.1 Reagents for multiplex PCR

- 24 tests

MAD-003930MU-P-HS12-24		
HPV PCR mix	3 strips x 8 tubes	MAD-003930MU-MIX

Table 1: PCR reagents provided in the kit MAD-003930MU-HS12-24, compatible with the hybriSpot 12 platform.

MAD-003930MU-P-HS24-24		
HPV PCR mix	3 strips x 8 tubes	MAD-003930MU-MIX

Table 2: PCR reagents provided in the kit MAD-003930MU-HS24-24, compatible with the hybriSpot 24 and hybriSpot 12 PCR AUTO and hybriSpot 24 PCR AUTO platforms.

- 48 tests

MAD-003930MU-P-HS12-48		
HPV PCR mix	6 strips x 8 tubes	MAD-003930MU-MIX

Table 3: PCR reagents provided in the kit MAD-003930MU-HS12-48, compatible with the hybriSpot 12 platform.

MAD-003930MU-P-HS24-48		
HPV PCR mix	6 strips x 8 tubes	MAD-003930MU-MIX

Table 4: PCR reagents provided in the kit MAD-003930MU-HS24-48, compatible with the hybriSpot 24, hybriSpot 12 PCR AUTO and hybriSpot 24 PCR AUTO platforms.

Both presentations include DNase/RNase-free double distilled water for the handling of clinical samples: RNASE/DNASE-FREE DISTILLED WATER; Ref: MAD-DDW; Vol 60 mL. One vial is included for the presentation of 24 tests and two vials are included for the presentation of 48 tests.

The lyophilized PCR mix of HPV contains the PCR buffer, dNTPs (U/T), DNase/RNase-free water, biotinylated primers, DNA polymerase and UNG. The primers included are specific for the amplification of a fragment of the region L1 of the HPV, and they can detect at least 35 HPV genotypes. Furthermore, primers for the amplification of a human genomic DNA fragment (β-globin gene) are included and used as an internal control for the PCR reaction.

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3.2 Reagents for reverse hybridization

- 24 tests

MAD-003930M-H-HS12-24		
Name	Format	Reference
Hybridization Solution (Reagent A)	40ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10ml	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35ml	MAD-003930MD-HS12-24
Reagent E	10ml	MAD-003930ME
Washing Buffer II (Reagent F)	18ml	MAD-003930MF-HS12-24
HPV Chip (HS)	1x 24 units	MAD-003930M-CH-HS-24

Table 5: Hybridization reagents supplied in the kits MAD-003930MU-HS12-24, compatible with the hybriSpot 12 platform.

MAD-003930M-H-HS24-24		
Name	Format	Reference
Hybridization Solution (Reagent A)	60ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10ml	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35ml	MAD-003930MD-HS24-24
Reagent E	10ml	MAD-003930ME- HS24
HPV Chip (HS)	1x 24 units	MAD-003930M-CH-HS-24

Table 6: Hybridization reagents supplied in the kits MAD-003930MU-HS24-24, compatible with the hybriSpot 24, hybriSpot 12 PCR AUTO platforms and hybriSpot 24 PCR AUTO platforms.

- 48 tests

MAD-003930M-H-HS12-48		
Name	Format	Reference
Hybridization Solution (Reagent A)	80ml	MAD-003930MA-HS12-48
Blocking Solution (Reagent B)	18ml	MAD-003930MB-HS12-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18ml	MAD-003930MC-HS12-48
Washing Buffer I (Reagent D)	70ml	MAD-003930MD-HS12-48
Reagent E	18ml	MAD-003930ME-HS12-48
Washing Buffer II (Reagent F)	35ml	MAD-003930MF-HS12-48
HPV Chip (HS)	2x 24 units	MAD-003930M-CH-HS-24

Table 7: Hybridization reagents supplied in the kits MAD-003930MU-HS12-48, compatible with the hybriSpot 12 platform.

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MAD-003930M-H-HS24-48		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	115ml	MAD-003930MA-HS24-48
Blocking Solution (Reagent B)	18ml	MAD-003930MB-HS24-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18ml	MAD-003930MC-HS24-48
Washing Buffer I (Reagent D)	70ml	MAD-003930MD-HS24-48
Reagent E	18ml	MAD-003930ME-HS24-48
HPV Chip (HS)	2x 24 units	MAD-003930M-CH-HS-24

Table 8: Hybridization reagents supplied in the kits MAD-003930MU-HS24-48, compatible with the hybriSpot 24, hybriSpot 12 PCR AUTO platforms and hybriSpot 24 PCR AUTO platforms.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and Materials

A. Common reagents to platforms HS12, HS12a, HS24 and HS24a:

- Disposable gloves
- Pipette tips with DNase/RNase-free filters.
- DNase/RNase-free 0.2/0.5 ml tubes
- Paraffin Tissue Processing Kit, Ref: MAD-003952M (30 tests)

A. Specific reagents to platforms HS12a, HS24 and HS24a:

- Washing Reagent (Ref: MAD-003930WSH).

4.2 Equipment

A. Common equipment to platforms HS12, HS12a, HS24 and HS24a:

- Microcentrifuge
- Automatic micropipettes: P1000, P200, P20 and P2
- HybriSoft software.

B. Specific equipment:

- With HPV Direct Flow Chip kit (Ref: MAD-003930MU-HS12-24 and MAD-003930MU-HS12-48)
    - Manual equipment for hybridization hybriSpot 12 (VIT-HS12).
    - Thermal cycler
    - Thermal block to heat PCR tubes (can be substituted by a thermal cycler)
    - Cold plate (4°C)
    - Thermostatic bath / heater.
- With HPV Direct Flow Chip kit (Ref: MAD-003930MU-HS24-24 and MAD-003930MU-HS24-48)
    - Automatic equipment for hybridization hybriSpot 24 (VIT-HS24) or hybriSpot 12 PCR AUTO (VIT-HS12a) or hybriSpot 24 AUTO (VIT-HS24a).
    - Thermal cycler (not necessary for hybriSpot 12 PCR AUTO and hybriSpot 24 PCR AUTO).

- Thermal block to heat PCR tubes (not necessary for hybriSpot 12 PCR AUTO and hybriSpot 24 PCR AUTO).
- Cold plate (4°C).

5 STORAGE AND STABILITY CONDITIONS

HPV Direct Flow Chip consists of two components that are supplied in separate boxes:

PCR reagents: Shipment at 2-8 °C. Upon receipt, they must be stored at 2-8 °C. They will be stable until the specified expiration date. The PCR reagents must be stored in areas free of DNA or PCR products to avoid cross-contamination. **After opening the package containing the 8-strip PCR tubes with the lyophilized mixture, store unused tubes for up to one week at 2-8 °C in the original package.**
Hybridization reagents. Shipment at 2-8 °C. Upon receipt, they must be stored at 2-8 °C. The reagents as well as the HPV Chips are stable until the specified expiration date. **Do not freeze**.

Previous considerations about the hybridization reagents:

- The hybridization reagent A must be pre-heated in a thermostatic bath or heater to 41ºC before use (only in the manual equipment HS12).
- All other hybridization reagents should be used at room temperature (20-25°C).

Previous considerations about the chips:

- After opening the chips package, keep the sponge and desiccant inside until the end of use to ensure proper preservation of the chips.

6 WARNINGS AND PRECAUTIONS

- **Read the instructions for use before using this product.**
- **The safety and waste disposal information is described in the Safety Data Sheet of this product.** The product is only intended for professional laboratory purposes, and not for pharmacological use, domestic use or any other purposes. The current version of the Safety Data Sheet of this product can be downloaded from the web page www.vitro.bio or requested at regulatory@vitro.bio.
- **HPV Direct Flow Chip** does not require the prior extraction of DNA from the samples although it can be done if preferred, the PCR amplification can be performed directly from cell suspensions, fixed cells or paraffin-embedded tissue sections. If an extraction is used, It is the customer's responsibility to include the necessary controls to verify that the system functions correctly.
- **General considerations to avoid the contamination with pre-amplified PCR products:**
  The main source of contamination is pre-amplified PCR products. Therefore, it is recommended to handle the amplified products in a different area from where the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas, handling of DNA and preparation of the PCR tubes should take place in a pre-PCR area, and the manipulation and hybridization of the amplified products in a post-PCR area. 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 results. The workflow must always go in one direction only, from the pre-PCR area to the post-PCR area and never the opposite direction. The material and

personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid possible contamination with previous PCR products, the enzyme Cod-UNG, which degrades dUTP-containing PCR products, is included in the mix.

It is recommended to include negative amplification controls of all the reagents handled in the process, from the extraction to the amplification steps, except for the sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The result from the hybridization of this control must be negative, marking only the hybridization control and the amplification exogenous control. Consequently, it is verified that there is no contamination of patient DNA and/or amplified DNA in the pre and/or post-PCR area.

- **Waste disposal:** The handling of waste generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable the legislation in the country where these products are being used. As reference, the following table indicates the classification of waste generated by this kit according to the European Legislation, specifically according to the *European Commission Decision of December 18, 2014* amending decision 2000/532/CE on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council of the European Union:

POTENTIAL WASTE GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW
1. Rubbish/Waste generated from hybridization reagents. 2. Disposal of Liquid Waste ("Waste" in manual and automatic platforms)	161001	"Aqueous liquid waste 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"
3. Used chips 4. Consumables (tubes, tips, aluminum foil, etc.) 5. Any element that has been in contact with DNA	180103	"Waste whose collection and disposal is subject to special requirements in order to prevent infection"
6. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers containing waste or contaminated by dangerous substances"

Table 9: Classification of waste generated by "HPV Direct Flow Chip" 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 to comply with all the local, regional and national regulations on the disposal of this type of materials.

7 SAMPLE PREPARATION

HPV Direct Flow CHIP is optimized for direct use of clinical specimens such as cervical and anal swabs, liquid cytology, cervico-vaginal specimens obtained with the Cervico-Vaginal Self Collection Kit (Ref. MAD-VVC, Vitro S.A.), cytology in Digene specimen transport medium (STM) and paraffin-embedded tissue sections, without prior DNA extraction.

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The system has also been validated with purified DNA from clinical samples using the following extraction methods:

- Maxwell® 16 FFPE Tissue LEV DNA Purification Kit (Promega): for DNA purification from both fresh and paraffin-embedded samples.
- MagNa Pure (Roche): for DNA extraction from fresh samples.
- Nextractor NX-48 (Genolution):
    - FFPE DNA Kit: for DNA purification from both fresh and paraffin-embedded samples.
    - Viral DNA/RNA/NA/NA Kit: for DNA, RNA or AN isolation from fresh samples of different types.
    - Urine/Swab DNA Kit: for purification of DNA from fresh samples related to STD and cervical cancer.
    - "RNA/DNA Pathogen Extraction Kit (Robot Nextractor NX-48S)" (MAD-003955M-EX)
- "RNA/DNA Pathogen Extraction Kit (Robot Opentrons)" (MAD-003955M): automatic extraction system for isolation of high-quality DNA/RNA from fresh samples.

The system has not been validated with other DNA extraction systems, therefore, if a different purification system is employed, it should be previously verified.

Samples preparation protocols for direct PCR:

CERVICAL AND ANAL SWABS				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Shake the swab in 400 µl **DNase/RNase-free double distilled water** in a tube of 1.5-2 ml.	Collection of starting material.	Very high	Insufficient material, “blank” result.
	Use only DNase-RNase-free double distilled water to collect the cells and add to the PCR tube.	Remove chemicals that may inhibit the PCR.	Very high	If you use another buffer such as PBS to collect cells, the PCR may be inhibited.
2nd	- Mix the sample with vortex at low-medium speed. - Take 30µl of the homogenized suspension as DNA template for the PCR reaction. **Mix the cell suspension well before adding it to the PCR tube**	- Obtaining a homogeneous cell sample. - Avoid cell clusters.	Very high	Heterogeneous sample: - If only supernatant is taken -> Insufficient material, “blank” result. - If only sedimented cells are taken-> excess of material -> Possible PCR inhibition, “blank” result.
3rd	Once the samples have been added to the PCR tubes, amplify immediately.	Prevent proteases from degrading polymerases.	Very high	The cells begin to lyse and release proteases that can destroy the polymerases, “blank” results.

Table 10: Preparation protocol for direct PCR from cervical and anal swabs.

LIQUID-BASED CYTOLOGIES / CERVICAL-VAGINAL SAMPLES OBTAINED WITH THE CERVICAL-VAGINAL SELF COLLECTION KIT (Ref. MAD-VVC, Vitro S.A.)				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Take 400 µl of the homogenized sample with vortex and put in a tube of 1.5-2 ml.	Collection of starting material.	Very high	Insufficient material, “blank” result.
2nd	Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Concentration of starting material	Very high	Insufficient material, very diluted, "blank" result.
3rd	Wash the pellet with 400 µl **DNase/RNase-free double distilled water**. Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Remove chemicals that may inhibit the PCR.	Very high	PCR inhibition, “blank” result.
4th	Resuspend the pellet in 300 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.	Obtaining a homogeneous cell sample in liquid media.	High	Excess of material: Possible PCR inhibition, “blank” result.
	⚠ Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube.	Remove chemicals that may inhibit the PCR.	Very high	If you use another buffer such as PBS to collect cells, the PCR may be inhibited.
5th	Mix sample with vortex. Take 30µl of the homogeneous suspension as DNA template for the PCR reaction. ⚠ **Mix the cell suspension properly before adding it to the PCR tube**	- Obtain homogeneous cell sample. - Avoid cell clusters.	Very high	Heterogeneous sample: - If only supernatant is taken -> Insufficient material, “blank” result. - If only sedimented cells are taken-> excess of material -> Possible PCR inhibition, “blank” result.
6th	Once the samples have been added to the PCR tubes, amplify immediately.	Prevent proteases from degrading polymerases	Very high	Lysed cells release proteases that can destroy polymerases, “blank” results.

Table 11-a: Sample preparation protocol for direct PCR from liquid cytology or cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection Kit (Ref. MAD-VVC, Vitro S.A.).

If after following this protocol "Liquid Cytology / Cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection Kit (Ref. MAD-VVC, Vitro S.A.)", debris on the membrane is observed, it is recommended to follow the alternative protocol indicated below: Table 11-b.

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Step	Brief description of the procedure	Use	Importance	Consequence of not performing it correctly
1st	Take 50 µl from the bottom of the sample WITHOUT vortexing, and place in a 1.5-2 ml tube.	Collection of starting material.	Very high	Insufficient material, "blank" result.
3rd	Add 350 µl of DNase/RNase-free double distilled water. Centrifuge 2 min at 2000 rpm and remove the supernatant.	Remove chemicals that may inhibit the PCR.	Very high	PCR inhibition, "blank" result.
4th	Resuspend the pellet in 400 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.	Obtaining a homogeneous cell sample in liquid media	High	Excess of material: Possible PCR inhibition, "blank" result.
	Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube.	Remove chemicals that may inhibit the PCR.	Very high	If you use another buffer such as PBS to collect cells, the PCR may be inhibited.
5th	Mix sample with vortex. Take 30µl of the homogeneous suspension as DNA template for the PCR reaction. **Mix the cell suspension properly before adding it to the PCR tube**	- Obtain homogeneous cell sample. - Avoid cell clusters.	Very high	Heterogeneous sample: - If only supernatant is taken -> Insufficient material, "blank" result. - If only sedimented cells are taken-> excess of material -> Possible PCR inhibition, "blank" result.
6th	Once the samples have been added to the PCR tubes, amplify immediately.	Prevent proteases from degrading polymerases	Very high	Lysed cells release proteases that can destroy polymerases, "blank" results.

Table 11-b: Alternative sample preparation protocol for direct PCR from liquid cytology or cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection Kit (Ref. MAD-VVC, Vitro S.A.).

The system has been validated for direct PCR (without the need for previous DNA extraction) with the following transport media for liquid cytology:

- *Thinprep (Hologic)*
- *Surepath (Becton Dickinson)*
- *Novaprep (Novacyt)*
- *CellPrep (Biodyne)*
- *CY-PREP™ Pap Test (FJORD Diagnostics)*
- *HURO PATH® Cell-Preserve Solution (CelltraZone)*

CYTOLOGIES IN DIGENE TRANSPORT MEDIUM (STM)				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Take 500-1000 µl of cell suspension and put in a tube of 1.5-2 ml.	Collection of starting material.	Very high	Insufficient material, “blank” result.
2nd	Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Concentration of starting material.	Very high	Insufficient material, very diluted, “blank” result.
3rd	Wash the pellet with 400 µl **DNase/RNase-free double distilled of water**. Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Remove inhibiting agents for PCR reaction.	Very high	PCR inhibition, “blank” result.
	⚠ Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube.	Remove inhibiting agents for PCR reaction.	Very high	If you use another buffer such as PBS to collect cells, the PCR may be inhibited.
4th	Resuspend the pellet in 300 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.	Obtaining a homogeneous cell sample in liquid media	High	Excess material may lead to possible PCR inhibition, “blank” result.
5th	Mix sample with vortex. Take 30µl of the homogeneous suspension as DNA template for the PCR reaction. ⚠ **Mix the cell suspension properly before adding it to the PCR tube**	- Obtain homogeneous cell sample. - Avoid cell clusters.	**Very high**	Heterogeneous sample: - If only supernatant is taken -> Insufficient material, “blank” result. - If only sedimented cells are taken -> excess of material -> Possible PCR inhibition, “blank” result.
6th	Once the samples have been added to the PCR tubes, amplify immediately.	Prevent proteases from degrading polymerases	Very high	Lysed cells release proteases that can destroy polymerases, “blank” results.

Table 12: Preparation protocol for direct PCR from cytologies in a Digene liquid medium.

PARAFFIN-EMBEDDED TISSUE SECTIONS				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Take 1-3·paraffin-embedded tissue sections (depending on the size of the tissue section) of 10 µm thick. Put in an Eppendorf tube of 1.5 ml. *Note: it is recommended to remove as much paraffin as possible from the edges of the tissue sections.*	Collection of starting material.	Very high	Insufficient material, "blank" result.
2nd	Add 400 µl of mineral oil (Ref kit: MAD-003952M). Heat at 95°C for 2 min. Centrifuge for 1 min at 2000 rpm. Remove any mineral oil remains.	Remove paraffin.	Very high	Paraffin remains that interfere with the lysis of the posterior tissue (3rd step).
3rd	Add to the pellet: - 60 µl of extraction buffer - 1.5µl of DNA Release (Ref Kit: MAD-003952M). Note: for > 1 cm^2 tissue sections: - Increase extraction buffer volume and DNA Release proportionally. - Be sure that the tissue is completely submerged.	Guarantee correct performance of the lysis agents.	Very high	Insufficient material due to insufficient lysis of the tissue, "blank" result.
4th	Incubate in two steps: (a) 30 min at 60°C (b) 10 min at 98°C	a. Enzymatic digestion by proteases. b. Inactivation of proteases.	Very high	**Without step a**. -> Insufficient lysis of the cells and tissue material that prevent the DNA from being in suspension, "blank" result. **Without step b**. -> high risk of degrading DNA polymerase in the PCR, "blank" result.
	If after the incubation it is observed that the tissue has not been fully digested, it is recommended to add the proportional volume of Extraction buffer and DNA Release again and repeat the incubation for a further 30 min at 60 °C and 10 min at 98 °C. It is recommended to check that the section used for HPV determination contains lesion. For this purpose, it is recommended to perform H&E on the first and last section and to use the intermediate sections to extract DNA. If both the first and last sections are found to have lesions, this indicates that the middle sections to be used for PCR-HPV are valid. If there is no lesion in the two extremes sections, there is a risk that the sections used for PCR have lost the lesion.	Boost cell and tissue lysis to release DNA.	High	Insufficient lysis of the cells and tissue material that prevent the DNA from being in suspension, "blank" result.

	The direct PCR protocol has not been tested for other types of starting clinical samples (cytological extensions or stained tissue sections) on which HPV testing is also possible, so it is recommended to follow a DNA purification procedure on these samples.			
5th	- Centrifuge for 1 min at 2000 rpm -> decant tissue remains. - Add **27 µl DNase/RNase-free double distilled water** and **3 µl from the cell suspension** as template DNA for PCR to a reaction tube, avoiding taking remnants of tissue from the bottom of the tube ("debris"). *Note: Traces of mineral oil may remain in the upper part of the supernatant (Phase 1), these remnants do not interfere with the subsequent PCR, but it must be ensured that the aqueous supernatant (Phase 2) which is the one containing the DNA is taken.*	Starting sample suitable to be amplified.	Very high	Possible problems of PCR inhibition for suction of tissue debris, or taking mineral oil instead of aqueous supernatant, the test result would be "blank".
6th	Once the samples have been added to the PCR tubes, amplify immediately.	Prevent proteases from degrading polymerases	Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.

Table 13: Sample preparation protocols for direct PCR from paraffin-embedded tissue sections.

The analysis **procedure** to be used when the starting material is amplified product obtained with the **Vitro HPV Screening kit** (Vitro, S.A. Ref. MAD-003949M) is described in section 8.2.

8 ANALYSIS PROCEDURE for platforms HS12 and HS24

8.1 Direct clinical sample and DNA purified from clinical sample.

8.1.1 Reaction of amplification by multiplex PCR

The following thermal cyclers have been validated with HPV Direct Flow Chip:

- Veriti 96 (Life Technologies)
- GeneAmp PCR System 7900 (Applied Biosystems)
- TProfessional Thermocycler (Biometra)
- MJ Mini Personal Thermal Cycler (Bio Rad)
- Mastercycler Personal (Eppendorf)
- 2720 Thermal Cycler (Applied Byosystems)
- SimpliAmp Thermal Cycler (ThermoFischer)
- LifeECO Thermal Cycler (Bioer Technology)

The PCR reaction is carried out in a final volume of 30 µl in tubes containing the lyophilized PCR reaction mix.

Procedure:

- Take a tube containing the lyophilized PCR mix per sample to be analyzed.
- Volume to add to the PCR tube depending on the sample:
    - Direct PCR: Add up to 30 µl of direct sample in each tube following the recommended protocol in section 7.
    - **Paraffin-embedded tissue**: add **27 µl DNase/RNase-free double distilled water** and **3 µl** of DNA to PCR tube.
    - Purified DNA: Add first **18µl DNase/RNase-free double distilled water** and next **12 µl of DNA to the PCR tube.**
- Homogenize the mix by pipetting and centrifuge for a few seconds.
- If the number of samples to be analyzed is lower or higher than 8, the necessary tubes can be separated from the strip. The rest of the tube strip with the lyophilized PCR mix that is not going to be used immediately must be stored for maximum of 1 week at 4°C in its original package.
- Place the tubes in the thermal cycler and set the following amplification conditions:

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1 cycle	25°C	10 min
1 cycle	94°C	3 min
15 cycles	94°C	30 s
	47°C	30 s
	72°C	30 s
35 cycles	94°C	30 s
	65°C	30 s
	72°C	30 s
1 cycle	72°C	5 min
	8°C	∞

Table 14: PCR Program

Keep the tubes refrigerated at 8-10 °C when the reaction is finished. If the samples are not going to be processed immediately, they can be stored in the post-PCR zone at 8-10°C for 1-2 days. To store them for a longer period, it is recommended to do so at -20°C.

8.1.2 Flow-through reverse hybridization

All the reagents are provided in a "ready-to-use" format.
The membranes are single-use and must be handled with gloves.

8.1.2.1 Semi-automated reverse hybridization, ref. MAD-003930MU-HS12

The full hybridization process is performed semi-automatically in hybriSpot (HS12) following the instructions provided by the wizard of the system. The management of the samples, the capture of images and the analysis and report of the results are performed by the hybriSoft software.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process:

1. Denature the PCR products by heating at **95 °C during 10 min** in a thermal cycler and **cool quickly on ice** during at least **2 min**.

2. Preheat the **Reagent A** at 41 °C.

3. Place every **HPV Chip** in the position indicated in the platform (HS12).

Manual hybridization protocol:

a) Set the temperature of the equipment at 41 °C. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated at 41 °C for every Chip and incubate for at least **2 min at 41 °C**.
b) Remove the **reagent A (Hybridization Solution)** by activating the vacuum pump.
c) Mix **30 µl** of each PCR sample (previously denatured and kept in ice) with **270 µl** of **Reagent A (Hybridization Solution)** (41 °C) and dispense the mix on the corresponding **HVP Chip**.
   Note: When working with direct PCR samples, some cell debris may be deposited at the bottom of the PCR tubes; avoid taking this debris.

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d) Incubate at **41 °C** for **8 min**.
e) Activate the pump for at least 30 s to remove the PCR products.
f) Wash **3** times with **300 µl** with **Reagent A (Hybridization Solution)** (41 ºC).
g) **Set the temperature at 29°C.**
h) Add **300 µl** of **Reagent B (Blocking Solution)** and incubate for 5 min.
i) Activate the pump to remove the Reagent B.
j) When the temperature has reached **29 °C**, add **300 µl** of **Reagent C (Streptavidin-Alkaline Phosphatase)** to each Chip.
k) Incubate for **5 min at 29 °C**.
l) Activate the pump to remove the reagent.
m) Set the temperature at **36°C**.
n) Wash the membranes **4** times with **300 µl** with **Reagent D (Washing buffer I).**
o) When the temperature has reached **36 °C**, add **300 µl** of **Reagent E** to every Chip. Incubate for **10 min at 36 °C**.
p) Activate the pump to remove the reagent.
q) Wash the membranes **2** times with **300 µl** of **Reagent F (Washing buffer II).**
r) Activate the pump to remove the reagent.
s) Perform the image capture, analysis and results report following the instructions of the HS12 user manual.

8.1.2.2 Automated reverse hybridization, ref. MAD-003930MU-HS24

The whole hybridization process is performed automatically on hybriSpot 24 (HS24). The management of the samples, the capture of images and the analysis and report of the results are performed through the hybriSoft software.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process:

1. Denature the PCR products by heating at **95 °C during 10 min** in a thermal cycler and **cool quickly on ice** during at least **2 min**.
2. Place the PCR tubes, the HPV Chips and the reagents in their corresponding positions of hybriSpot 24.
3. Select the corresponding protocol in the equipment (HPV Direct Flow Chip) to start the automatic process.

8.2 PCR amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M).

Once the amplification product has been obtained with the Vitro HPV Screening kit, we proceed directly to the **Flow-through reverse hybridization process**.

Note: All the reagents are provided in a "ready-to-use" format. The membranes are single-use and must be handled with gloves.

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8.2.1 Semi-automated reverse hybridization, ref. MAD-003930MU-HS12

The full hybridization process is performed semi-automatically in hybriSpot (HS12) following the instructions provided by the wizard of the system. The management of the samples, the capture of images and the analysis and report of the results are performed by the hybriSoft software.
Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process:

1. **Denature** the wholes amplified product of the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. ref. MAD-003949M) by heating at **95 °C for 10 min** in a thermal cycler and **rapidly cooling on ice** for at least **2 min**.
2. Preheat the **Reagent A** at 41 °C.
3. Place every **HPV Chip** in the position indicated in the platform (HS12).

Manual hybridization protocol:

a) Set the temperature of the equipment at **60 °C**. Add **300 µl** of **Reagent A** (Hybridization Solution) preheated at 41 °C for each Chip and incubate for at least **2 min at 60 °C**.
b) Remove the **reagent A (**Hybridization Solution**)** by activating the vacuum pump.
c) Mix **the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. ref. MAD-003949M)** (previously denatured and kept on ice) with 270 of **Reagent A** (Hybridization Solution) pre-warmed to 41 °C and dispense the mixture onto the corresponding **HPV Chip**.
d) Incubate at **60 °C** for **1 min**.
e) Set the temperature of the equipment to 41°C and when it reaches this temperature, incubate the sample for 6 minutes.
f) Activate the pump for at least 30 s to remove the PCR products.
g) Set the temperature of the instrument to 29°C and wash **3** times with **Reagent A** (Hybridization Solution) preheated to 41°C **while the instrument is turned down to 29°C.**
h) Add **300 µl** of **Reagent B (Blocking Solution)** and incubate for 5 min.
i) Activate the pump to remove the Reagent B.
j) Add **300 µl** of **Reagent C (Streptavidin-Alkaline Phosphatase)** to each Chip.
k) Incubate for **5 min at 29 °C**.
l) Activate the pump to remove the reagent.
m) Set the temperature of the equipment to **36°C** and wash the membranes **4** times with **300 µl** with **Reagent D (Washing buffer I)** while the equipment reaches the temperature of 36°C.
n) When the temperature has reached **36 °C**, add **300 µl** of **Reagent E** to every Chip.
o) Incubate for **10 min at 36 °C**.
p) Activate the pump to remove the reagent.
q) Wash the membranes **2** times with **300 µl** of **Reagent F (Washing buffer II).**
r) Activate the pump to remove the reagent.
s) Perform the image capture, analysis and results report following the instructions of the HS12 user manual.

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8.2.2 Automated reverse hybridization, ref. MAD-003930MU-HS24

The whole hybridization process is performed automatically on hybriSpot 24 (HS24). The management of the samples, the capture of images and the analysis and report of the results are performed through the hybriSoft software.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Please note that the maximum number of samples to be hybridized in HS24 platform with the "Vitro HPV Screening kit" product is 12 samples.

Before starting the hybridization process:

1. **Denature** 20 µl of the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. ref. MAD-003949M) by heating at **95 °C for 10 min** in a thermal cycler and **rapidly cooling on ice** for at least **2 min**.
2. Place the PCR tubes, the HPV Chips and the reagents in their corresponding positions of hybriSpot 24.
3. Select the corresponding protocol in (HPV Direct Flow Chip (RT Genotyping)) the equipment to start the automatic process.

9 ANALYSIS PROCEDURE for platform HS12a y HS24a

The amplification through PCR and hybridization processes are performed automatically in the platform HS12a y HS24a.

The processing of the samples, the capture of images and the results analysis are performed by the hybriSoft software.

Before starting the process, it is recommended to carefully read the user manual (included in the HS12a and HS24a equipment) and follow the instructions to place the tube strips, chips and hybridization reagents in the instrument.

9.1 Direct clinical sample and DNA purified from clinical sample

- Take a tube containing the lyophilized PCR mix for each sample to be analyzed.
- Add the DNA samples to a PCR tube following the instructions described in section 8.1.1.
- Homogenize the mix by pipetting and centrifuge for a few seconds.
- If the number of samples to be analyzed is less than or more than 8, the necessary tubes can be separated from the strip without having to use the complete strips. The rest of the lyophilized tubes from the strip not used at that time should be stored for maximum of 1 week at 4 °C in the original packaging.
- Follow the instructions in the manual to place the tube strips, chips and hybridization reagents in the instrument and start the process.

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9.2 Amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M).

- Place the amplification product (in tube or plate) in the adapter provided in the equipment for this type of sample.
- Follow the instructions in the manual to place the hybridization chips and reagents in the instrument and start the process.

10 QUALITY CONTROL PROCEDURE

HPV Flow Chip Kit contains several controls to evaluate the quality of the results.

Probe	Control
B	Hybridization control
C	Endogenous amplification control

Table 15: Control probes included in HPV Flow Chip.

Hybridization control: After the development of the membranes, an intense signal must appear in all five hybridization control positions (B), which serve as a quality control. This signal indicates that the hybridization and development reagents have worked correctly. If no signal is detected, it indicates that an error has occurred during the hybridization process or that a reagent has not been used properly. Furthermore, this signal allows the software to correctly orientate the probe panel to perform the subsequent analysis.

Endogenous amplification control (C): It is a probe designed for the detection of the human β-globin gene contained in the test sample and is co-amplified during PCR. All the samples where the DNA sample has been amplified correctly will have a positive signal in the endogenous amplification Control (C). This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of starting DNA has been optimal. The absence of signal for this control indicates failures during the amplification, low quality/quantity of the DNA used in the amplification or the absence of human DNA in the sample. This last case is possible when the number of human cells present in the test sample is under the limit of detection. If also no positive signals are detected for any HPV genotype, the hybriSoft software will include the following message in the report: “BLANK: Inappropriate material. Insufficient material. PCR inhibited”.

When the sample is positive for any of the HPVs included in the kit, but there is no signal for the endogenous amplification control, the hybriSoft software will include the following message in the report: “Insufficient material”. The user must check the process and the quality of the samples before validating the results.

The user is responsible for determining the appropriate quality control procedures for their laboratory.

11 INTERPRETATION OF RESULTS

The following tables show the positions of the probes in the Chip and the interpretation of the results.

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	1	2	3	4	5	6	7	8	9
A	B	33	58	42	71	16	52	B	
B	B	35	59	43	72	18	53	6	69
C	C	39	66	44/55		26	56	11	70
D	U	45	68	54	84	31	58	40	71
E	16	51	73	61	B	33	59	44/55	72
F	18	52	82	62/81	C	35	66	54	
G	26	53	6	67	U	39	68	61	84
H	31	56	11	69	42	45	73	62/81	
I		B	40	70	43	51	82	67	

Table 16a: Position of the probes included in the HPV Direct Flow Chip

"B": Hybridization control
"C": Endogenous amplification control (human ß-Globin gene)
"U": HPV Universal Probe
"X": Specific probes for each HPV genotypes

All the probes are duplicated to guarantee the reliability in the automatic analysis of the results. The hybridization control (B) is repeated in 5 positions and allows the software to correctly orientate the probe panel for its subsequent analysis.

Expected results	Probe/positions (column-row)			
	HPV genotype probe	B (Hybridization control)	C (Endogenous amplification control)	U* (HPV Universal Probe)
HPV 16	1E-6A	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 18	1F-6B	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 26	1G-6C	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 31	1H-6D	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 33	2A-6E	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 35	2B-6F	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV39	2C-6G	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 45	2D-6H	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 51	2E-6I	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 52	2F-7A	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 53	2G-7B	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 56	2H-7C	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 58	3A-7D	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 59	3B-7E	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 66	3C-7F	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 68	3D-7G	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 73	3E-7H	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 82	3F-7I	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 6	3G-8B	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 11	3H-8C	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G

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Expected results	Probe/positions (column-row)			
	HPV genotype probe	B (Hybridization control)	C (Endogenous amplification control)	U* (HPV Universal Probe)
HPV 40	3I-8D	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 42	4A-5H	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 43	4B-5I	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 44/55	4C-8E	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 54	4D-8F	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 61	4E-8G	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 62/81	4F-8H	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 67	4G-8I	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 69	4H-9B	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 70	4I-9C	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 71	5A-9D	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 72	5B-9E	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV 84	5D-9G	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
HPV POSITIVE GENOTYPE NOT DETERMINED	--	1A-1B-2I-5E-8A	1C-5F	1D-5G
NEGATIVE RESULT	--	1A-1B-2I-5E-8A	1C-5F	--
BLANK. Inappropriate material. Insufficient material. PCR inhibited.	--	1A-1B-2I-5E-8A	--	--
Hybridization error	--	--	--	--

Table 16b: Position of the probes included in the HPV Direct Flow Chip and interpretation of the results.

*The HPV universal probe (U), includes a pool of probes designed inside the L1 region of the virus. Its sequence is shared by all the genotypes of the panel and by other HPV genotypes related with mucosa lesions that are not included in this kit. It should be considered that the sensitivity for each genotype with this probe is different from the sensitivity with each of the specific probes. For this reason, there may be positive results with a genotype-specific probe and not with the U-probe; in these cases, the absence of positivity with the U-probe does not invalidate the analysis or the positive result for a specific genotype. When only the HPV signal (U) not associated with specific probe positivity appears, the software interprets the sample as "HPV POSITIVE, GENOTYPE NOT DETERMINED". This result would indicate that the sample is positive but that the specific genotype has not been identified and could be a genotype other than those included in the panel.

An example of a report in which the analyzed case was positive for HPV 31, HPV66, HPV73 and HPV61 is shown below.

HPV Direct Flow Chip Kit

LOTS

PCR:	HPV001	1/27/2024
Chips:	HPV-001	1/17/2024
Reagent:	RG-001	1/17/2024

SAMPLE DETAILS

ID SAMPLE: sampleID-1 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: **BIRTHDATE:** **AGE:**

REPORT

HPV POSITIVE

Positive sample for:

High-Risk:

31, 66, 73

Low-Risk:

61

The sample is negative for the rest of genotypes included in the HPV direct flow chip test.

PROTOCOL

Detection and genotyping of HPV viral DNA by PCR and reverse dot blot hybridization:

- High risk genotypes: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.
- Low risk genotypes: 6, 11, 40, 42, 43, 44/55, 54, 61, 62/81, 67, 69, 70, 71, 72, 84.

Sample preparation/DNA purification

Add cell suspension/purified DNA for PCR amplification:

- PCR protocol (standard) HPV Direct Flow Chip: 1x 25°C 10 min, 1x 94°C 3min; 15x94-42-72°C (30"-30"-30"), 35x 94-60-72°C (30"-30"-30"), 1x 72°C 5 min.
- PCR protocol (lyophilized) HPV Direct Flow Chip: 1x 25°C 10 min, 1x 94°C 3min; 15x94-47-72°C (30"-30"-30"), 35x 94-65-72°C (30"-30"-30"), 1x 72°C 5 min.

REVERSE-DOT BLOT protocol:

- Hybridization of the biotinilated PCR products to the HPV CHIP.
- Post-hybridization washes.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.

Automatic analysis of results

NOTES

FACULTATIVE:	Default Doctor, doctor	**Validated:**	5/11/2023
Performed by:	Default Tech, tech	**Processed:**	5/11/2023

Instr. : Mock **Serial Nº:** MOCK-01 **hybriSoft:** HSHS 3.00.00 R05 / HSHS IPL 1.1.0.R1000

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HPV Direct Flow Chip Kit

LOTS

PCR:	HPV001	1/27/2024
Chips:	HPV-001	1/17/2024
Reagent:	RG-001	1/17/2024

SAMPLE DETAILS

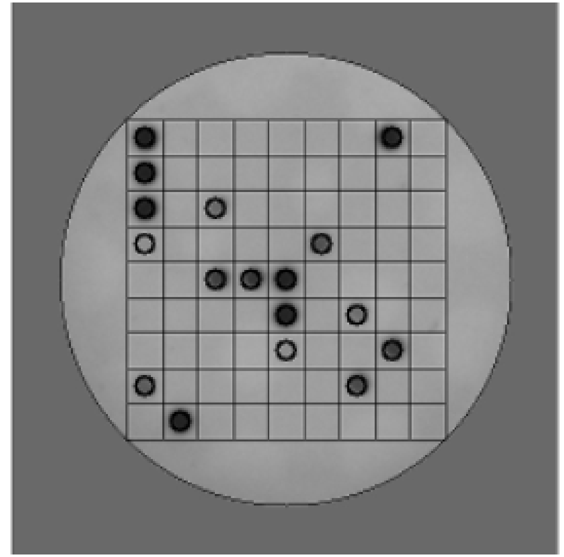
ID SAMPLE: sampleID-1 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: **BIRTHDATE:** **AGE:**

REPORT

B	33	58	42	71	16	52	B	
B	35	59	43	72	18	53	6	69
C	39	66	44/55		26	56	11	70
U	45	68	54	84	31	58	40	71
16	51	73	61	B	33	59	44/55	72
18	52	82	62/81	C	35	66	54	
26	53	6	67	U	39	68	61	84
31	56	11	69	42	45	73	62/81	
	B	40	70	43	51	82	67	



ANALYSIS INFORMATION

Threshold: 6

FACULTATIVE:	Default Doctor, doctor	**Validated:**	5/11/2023
Performed by:	Default Tech, tech	**Processed:**	5/11/2023

Instr. : Mock **Serial Nº:** MOCK-01 **hybriSoft:** HSHS 3.00.00 R05 / HSHS IPL 1.1.0.R1000

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12 PERFORMANCE CHARACTERISTICS

12.1 Analytical performance

12.1.1 Repeatability

The repeatability of the method was performed by testing at least four times genotypes included in the panel. The test was performed by the same operator, at the same location, on the same day, and using the same batch of reagents. Hybridization was performed on the hybriSpot platform, using hybriSoft software for the analysis.

HPV genotype	Genome equivalents (GE)/reaction	Positive/tested	% positive
HPV 16	5	2/4	50%
	50	4/4	100%
HPV 18	5	2/4	50%
	50	4/4	100%
HPV 26	50	2/4	50%
	500	4/4	100%
HPV 31	50	4/4	100%
HPV 33	50	4/4	100%
	500	4/4	100%
HPV 35	50	4/4	100%
HPV 39	50	4/4	100%
HPV 45	50	4/4	100%
HPV 51	50	4/4	100%
HPV 52	50	4/4	100%
HPV 53	50	0/4	0%
	500	4/4	100%
HPV 56	50	2/4	50%
	500	4/4	100%
HPV 58	50	4/4	100%
HPV 59	50	4/4	100%
HPV 66	500	4/4	100%
HPV 68	50	4/4	100%
HPV 73	50	4/4	100%
HPV 82	50	4/4	100%
	500	4/4	100%
HPV 6	50	4/4	100%
HPV 11	50	4/4	100%
HPV 40	50	4/4	100%
HPV 42	50	4/4	100%
HPV 43	50	4/4	100%
HPV 55	50	4/4	100%
HPV 54	50	4/4	100%
HPV 61	50	2/4	50%
	500	4/4	100%
HPV 62	50	4/4	100%
HPV 67	50	4/4	100%
HPV 69	NT		
HPV 70	50	4/4	100%
HPV 81	50	4/4	100%
HPV 71	50	4/4	100%
HPV 72	50	4/4	100%
HPV 84	50	4/4	100%

Table 17: Repeatability test for each genotype included in the panel. NT: not tested

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12.1.2 Reproducibility

The reproducibility of the method was tested by processing 10 HPV-positive samples, mimicking both single and multiple infections, at two different GE concentrations, in addition to 20 HPV-negative samples. All samples contained 10 ng of human genomic DNA. These samples were processed in two different laboratories, using different batches of reagents, and different equipment and operators. Each sample was tested three times on different days using the hybriSpot platform for the hybridization and the software for analysis of results hybriSoft. No false positives were obtained (100% of the negative samples for HPV gave expected results).

Sample	GE /reaction	Laboratory 1		Laboratory 2	
		Positive/Valid	% positive	Positive/Valid	% positive
HPV 16	50	3/3	100%	3/3	100%
	5	2/3	66%	1/3	33%
HPV 18	50	3/3	100%	3/3	100%
	5	2/3	66%	1/3	33%
HPV 31	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 35	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 6	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 11	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 16 + HPV 18	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 31 + HPV 6	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 16 + HPV 45 + HPV 6	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%
HPV 18 + HPV 31 + HPV 42	500	3/3	100%	3/3	100%
	50	3/3	100%	3/3	100%

Table 18: Inter-laboratory reproducibility for the HPV Direct Flow Chip kit.

12.1.3 Analytical specificity

The specificity of each HPV genotype from the panel was analyzed by using $5x10^6$ GE/reaction as starting material for each PCR reaction. The samples were hybridized on hybriSpot platform supported with hybriSoft software for the analysis of results. No cross-reactions were observed between the HPV genotypes in the panel, with the exception of genotypes 44 and 55 and genotypes 62 and 81. For this reason, the probes 62 and 81 and the probes 44 and 55 are located in the same position in the Chip, and the analysis software cannot discriminate between the genotypes 44-55 and 62-81.

No cross-reactions were observed with other bacteria and viruses tested: Herpesvirus simplex 1 and 2, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*.

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12.1.4 Analytical sensitivity

The sensitivity limit of each HPV genotype was calculated using serial dilutions of plasmids or synthetic DNA sequences of each genotype together with 10 ng of human genomic DNA per reaction. Each sample was repeated at least 5 times in order to calculate the sensitivity, specificity and confidence intervals of the kit. All PCRs products were hybridized on the hybriSpot platform and analyzed with hybriSoft software. The cut-off point (grey value) for positivity was set to 6.

Genotype	GE/ PCR reaction	Positive/tested	Sensitivity %	95% confidence interval	Specificity %	95% confidence interval
16	5	4/10	40	16.8-68.8	100	98.5-100
	50	10/10	100	72.3-100	100	98.5-100
18	5	5/10	50	29.9-70.1	100	98.5-100
	50	10/10	100	72.3-100	100	98.5-100
26	50	5/10	50	29.9-70.1	100	98.5-100
	500	10/10	100	72.3-100	100	98.6-100
31	50	10/10	100	72.3-100	100	98.6-100
33	50	10/10	100	72.3-100	100	98.6-100
35	50	10/10	100	72.3-100	100	98.5-100
39	50	10/10	100	72.3-100	100	98.5-100
45	50	10/10	100	72.3-100	100	98.5-100
51	50	10/10	100	72.3-100	100	98.5-100
52	50	10/10	100	72.3-100	100	98.5-100
53	50	0/10	0	0-27.8	100	98.5-100
	500	10/10	100	72.3-100	100	98.6-100
56	50	5/10	50	29.9-70.1	100	98.5-100
	500	10/10	100	72.3-100	100	98.5-100
58	50	10/10	100	72.3-100	100	98.5-100
59	50	10/10	100	72.3-100	100	98.5-100
66	50	4/10	40	16.8-68.8	100	98.5-100
	500	10/10	100	72.3-100	100	98.5-100
68	50	10/10	100	72.3-100	100	98.5-100
73	50	10/10	100	72.3-100	100	98.6-100
82	50	10/10	100	72.3-100	100	98.6-100
6	50	10/10	100	72.3-100	100	98.6-100
11	50	10/10	100	72.3-100	100	98.6-100
40	50	10/10	100	72.3-100	100	98.6-100
42	50	10/10	100	72.3-100	100	98.6-100
43	50	10/10	100	72.3-100	100	98.6-100
44/55	50	10/10	100	72.3-100	100	98.6-100
54	50	10/10	100	72.3-100	100	98.6-100
61	50	5/10	50	29.9-70.1	100	98.5-100
	500	10/10	100	72.3-100	100	98.6-100
62/81	50	10/10	100	72.3-100	100	98.6-100
67	50	10/10	100	72.3-100	100	98.6-100
69	NT					
70	50	10/10	100	72.3-100	100	98.6-100
71	50	10/10	100	72.3-100	100	98.6-100
72	50	10/10	100	72.3-100	100	98.6-100
84	50	10/10	100	72.3-100	100	98.6-100

Table 19: Analytical sensitivity (LoD): number of genomic equivalents of each genotype per PCR reaction with which 100% of positive results are obtained when analyzed with hybriSoft software, establishing a threshold value of 6. NT: not tested

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12.1.5 Evaluation of the direct protocol performance

We compared the performance of HPV Direct Flow Chip with the direct protocol (without prior DNA extraction) *vs.* use of purified DNA. 225 clinical cases of three different types of samples (cytological swab, liquid cytology, and paraffin sections) were tested simultaneously with the two types of protocols. The results obtained, are summarized in the following table:

HPV + (positive cases/total cases)			
Type of samples (total=225)	HPV Direct-Flow Chip test		concordance direct sample *vs* purified DNA
	Purified DNA	Direct sample	
Cytological swab (n=94)	45.7% (43/94)	43.6% (41/94)	98 % *(Kappa=0.957)*
Liquid based cytology (n=78)	69.2% (54/78)	70.5% (55/78)	99 % *(Kappa=0.97)*
Paraffin-embedded biopsies (n=53)	71.7% (38/53)	71.7% (38/53)	100 % *(Kappa=1)*

Table 20: Performance of HPV Direct Flow Chip Kit with direct samples in comparison with purified DNA.

12.1.6 Performance evaluation with the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M).

The performance of the HPV Direct Flow Chip was compared using the amplified product from clinical samples obtained with the Vitro HPV Screening kit (Vitro S.A. Ref. MAD-003949M) (Method 1) versus using purified DNA obtained with the RNA/DNA Viral Extraction Kit (Robot Opentrons) (Vitro, S.A. Ref. MAD-003955M) (Method 2).
A total of 196 clinical samples were analyzed in parallel with both protocols, obtaining a very good strength of agreement in all cases. The results are shown in the table below:

Genotype HPV	+/+	-/-	+/-	-/+	Kappa Index	Standard error	IC 95%	Strength of agreement
HPV 16	40	156	0	0	1	0	1-1	Very good
HPV 18	14	182	0	0	1	0	1-1	Very good
HPV 26	1	195	0	0	1	0	1-1	Very good
HPV 31	18	178	0	0	1	0	1-1	Very good
HPV 33	10	186	0	0	1	0	1-1	Very good
HPV 35	11	184	0	1	0.954	0.046	0.864 - 1.044	Very good
HPV 39	11	185	0	0	1	0	1-1	Very good
HPV 45	13	182	0	1	0.960	0.04	0.882 - 1.038	Very good
HPV 51	14	182	0	0	1	0	1-1	Very good
HPV 52	23	173	0	0	1	0	1-1	Very good
HPV 53	6	190	0	0	1	0	1-1	Very good

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Genotype HPV	+/+	-/-	+/-	-/+	Kappa Index	Standard error	IC 95%	Strength of agreement
HPV 56	15	180	0	1	0.965	0.035	0.897 - 1.033	Very good
HPV 58	13	183	0	0	1	0	1-1	Very good
HPV 59	8	186	0	2	0.884	0.082	0.723 - 1.044	Very good
HPV 66	9	187	0	0	1	0	1-1	Very good
HPV 68	16	180	0	0	1	0	1-1	Very good
HPV 73	5	191	0	0	1	0	1-1	Very good
HPV 82	0	196	0	0	ND	ND	ND	ND
HPV 6	8	187	0	1	0.939	0.061	0.818 - 1.059	Very good
HPV 11	5	190	0	1	0.906	0.093	0.724 - 1.089	Very good
HPV 40	4	192	0	0	1	0	1-1	Very good
HPV 42	7	188	1	0	0.931	0.069	0.795 - 1.066	Very good
HPV 43	5	191	0	0	1	0	1-1	Very good
HPV 44/55	6	190	0	0	1	0	1-1	Very good
HPV 54	7	188	0	1	0.931	0.069	0.795 - 1.066	Very good
HPV 61	6	190	0	0	1	0	1-1	Very good
HPV 62/81	7	189	0	0	1	0	1-1	Very good
HPV 67	2	194	0	0	1	0	1-1	Very good
HPV 69	0	196	0	0	ND	ND	ND	ND
HPV 70	2	194	0	0	1	0	1-1	Very good
HPV 71	0	196	0	0	ND	ND	ND	ND
HPV 72	2	194	0	0	1	0	1 - 1	Very good
HPV 84	5	191	0	0	1	0	1 - 1	Very good

Table 21. Operation of the HPV Direct Flow Chip Kit with amplification product from the Vitro HPV Screening kit and purified DNA. ND: not determined due to an insufficient number of samples. +/+: Nº of positive samples with both methods. -/-: Nº of negative samples with both methods. +/-: Nº of Samples that where positive with method 1 and negative with method 2, and -/+: Nº of Samples that were negative with method 1 and positive with method 2.

12.1.7 Analytical functioning in hybriSpot 24

The functioning and robustness of HPV Direct Flow Chip was validated in the automatic equipment HS24 by analyzing limiting concentrations of synthetic fragments of DNA of all the genotypes included in the panel (5 copies for the HPV 16 and 18, 50-500 copies for all other genotypes). This validation demonstrates the reproducibility of the results between positions 1 and 24 of the HS24 equipment and the reproducibility of the results with different programs for different numbers of samples.

- **Reproducibility of results using different programs depending on the number of samples**

Replicates of a positive sample containing several genotypes were run at a limiting concentration of 50 GE. These replicas were placed in different positions of the reaction chamber of the HS24 system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)

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- Protocol for 15 samples (4 replicas)
- Protocol for 24 samples (6 replicas)

The results were automatically analyzed with hybriSoft and no differences between the different positions of the reaction chamber nor the used protocol were detected.

- **Reproducibility of results by varying sample position in HS24**

Four replicas for each genotype were performed, placed in different positions of the two reaction chambers of the HS24 and the protocol for 24 samples was used. The results were automatically analyzed with hybriSoft, proving a 100% of reproducibility for all the analyzed genotypes in different positions.

HPV genotype	No. GE/reaction	Positive/samples	Difference between positions
16	5	4/4	No
16	50	4/4	No
18	5	4/4	No
18	50	4/4	No
26	500	4/4	No
31	50	4/4	No
33	500	4/4	No
35	500	4/4	No
39	50	4/4	No
45	500	4/4	No
51	50	4/4	No
52	50	4/4	No
53	500	4/4	No
56	500	4/4	No
58	50	4/4	No
59	500	4/4	No
66	50	4/4	No
66	500	4/4	No
68	500	4/4	No
73	50	4/4	No
82	50	4/4	No
82	500	4/4	No
6	50	4/4	No
11	50	4/4	No
40	50	4/4	No
42	50	4/4	No
43	50	4/4	No
44/55	50	4/4	No
54	50	4/4	No
61	500	4/4	No
62	50	4/4	No
67	50	4/4	No
69	NT		
70	50	4/4	No
81	50	4/4	No
71	50	4/4	No
72	50	4/4	No
84	50	4/4	No

Table 22: Reproducibility of HPV Direct Flow Chip in HS24. The positivity was analyzed with the hybriSoft software setting the cut-off point at 6. NT: not tested.

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12.1.8 Analytical functioning in hybriSpot 12 PCR AUTO

The functioning and the robustness of the HPV Direct Flow Chip was validated in the automatic equipment HS12a by analyzing limiting concentrations of synthetic DNA fragments of all the genotypes included in the panel. This validation also proves the reproducibility of the results with different programs for different number of samples.

- **Reproducibility of results using different programs depending on the number of samples**

Replicas of a positive sample that contained several genotypes at a limiting concentration were made. These replicas were placed in different positions of the reaction chamber of the HS12a system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)

The results were automatically analyzed with hybriSoft and no differences between the different positions of the reaction chamber nor the used protocol were detected.

- **Verification of the detection limit**

The functioning and the robustness of the HPV Direct Flow Chip was as validated on the automated HS12a by analyzing limiting concentrations of synthetic DNA fragments that mimic a region of the HPV genome. Three replicates of each synthetic fragment were run at the corresponding limit of detection. The whole process was performed automatically on two different HS12a equipment. The results were analyzed with hybriSoft.

HPV genotype	No. GE/reaction	Positive/replicates
16	10	3/3
18	10	3/3
26	500	3/3
31	50	3/3
33	50	3/3
35	50	3/3
39	50	3/3
45	50	3/3
51	50	3/3
52	50	3/3
53	500	3/3
56	500	3/3
58	50	3/3
59	50	3/3
66	500	3/3
68	50	3/3
73	50	3/3
82	50	3/3
6	50	3/3
11	50	3/3
42	50	3/3
54	50	3/3
67	50	3/3
72	50	3/3

Table 23: Verification of the detection limit of HPV Direct Flow Chip in HS12a. The positivity was analyzed with the hybriSoft software setting the cut-off point at 6.

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12.1.9 Analytical functioning in hybriSpot 24 PCR AUTO

The functioning and the robustness of the HPV Direct Flow Chip was validated in the automatic equipment HS24a by analyzing limiting concentrations of synthetic DNA fragments of all the genotypes included in the panel. This validation also proves the reproducibility of the results with different programs for different number of samples.

- **Reproducibility of results using different programs depending on the number of samples**

Replicas of a positive sample that contained several genotypes at a limiting concentration were made. These replicas were placed in different positions of the reaction chamber of the HS24a system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)
- Protocol for 15 samples (3 replicas)
- Protocol for 24 samples (4 replicas)

The results were automatically analyzed with hybriSoft and no differences between the different positions of the reaction chamber nor the used protocol were detected.

- **Verification of the detection limit**

The functioning and the robustness of the HPV Direct Flow Chip was as validated on the automated HS24a by analyzing limiting concentrations of synthetic DNA fragments that mimic a region of the HPV genome.

Three replicates of each synthetic fragment were run at the corresponding limit of detection. The whole process was performed automatically on two different HS24a equipment. The results were analyzed with hybriSoft.

Genotype HPV	No. GE/reaction	Positives/Replicas
16	30	3/3
18	30	3/3
26	500	3/3
31	30	3/3
33	30	3/3
35	30	3/3
39	30	3/3
45	30	3/3
51	30	3/3
52	50	3/3
53	50	3/3
56	30	6/6
58	30	3/3
59	30	6/6
66	500	3/3
68	30	9/9
73	50	3/3
82	50	3/3
6	50	3/3
11	30	3/3

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Genotype HPV	No. GE/reaction	Positives/Replicas
40	50	3/3
42	50	3/3
43	50	3/3
44/55	50	3/3
54	50	3/3
61	50	3/3
62	50	3/3
67	50	3/3
70	50	3/3
81	50	3/3
71	50	3/3
72	50	3/3
84	NT	

Table 24: Verification of the detection limit of HPV Direct Flow Chip on HS24a. Positivity was analyzed with hybriSoft software with a cut-off point of 6. NT: not tested.

12.2 Clinical Performance

552 routine cervical samples were analyzed to evaluate the clinical performance of the test. These samples included cytological swabs (n=440), liquid-based cytologies (n=76) and paraffin-embedded tissue sections (n=36). 249 positive HPV samples were detected, of which 232 were genotyped correctly, while 17 were positive for the HPV universal probe and negative for the genotype-specific probes.

Samples	HPV+	HR HPV+
Total (n=552)	45%	29.3%
NILM (n= 388)	33.7%	22.3%
ASCUS (n=71)	59.1%	33.8%
LSIL (n= 59)	84.7%	61%
ASC-H (n= 5)	40%	40%
HSIL/CIN II (n=8)	100%	100%
CIN I (n=21)	76.2%	23.8%

Table 25: Distribution of the diagnostic groups an positivity for HPV.HR: high risk.

13 LIMITATIONS

HPV Direct Flow Chip Kit has been validated with cytological and rectal swabs, liquid based-cytology samples, cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection kit (Ref. MAD-VVC, Vitro S.A.), cytology in Digene sample transport medium (STM) and paraffin-embedded tissue sections (see section 7). Moreover, the system has been validated using as starting material the amplification product obtained with the Vitro HPV Screening kit (Vitro, S.A. Ref. MAD-003949M). The use of any other type of sample can generate erroneous results and its operation must be previously verified.

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14 TROUBLESHOOTING

Problem	Potential	Solutions
No signal is observed/ there is no hybridization signal	Failure in the hybridization protocol.	Check that all the reagents have been correctly added during the hybridization process. Check the correct functioning of hybriSpot 12/12a/24/24a. Repeat the test.
	PCR reagents and/or expired or not stored properly.	Check the expiration date and the storage conditions of the reagents and the Chips. Repeat the test.
	Chip probes destroyed by remanents of decontamination reagents (e.g. Bleach) in the wells.	Clean with plenty of distilled water and repeat the experiment.
Detection of HPV in the negative control.	Contamination in pre-PCR and/or post-PCR areas.	Clean well the working areas and repeat the experiment.
No signals in the endogenous amplification control.	Not enough amount of human DNA in the clinical sample.	Repeat the PCR by increasing the amount of starting sample. Repeat the test.
	Presence of PCR inhibitors.	Purify the DNA of the sample and repeat the test.
Presence of chromogen precipitates in the Chip after finishing the hybridization protocol.	High cell and/or blood content of the sample.	Repeat the PCR by diluting the starting sample.
Weak hybridization signals.	PCR reagents and/or expired or stored improperly.	Check the expiration date of all the reagents and the storage conditions. Repeat the test.
	Erroneous sample volume used to re-suspend the lyophilized product.	Repeat the test using the correct sample volume
	Failure in the hybridization protocol.	Check the correct functioning of hybriSpot HS12/12a/24/24a and the hybridization protocol. Repeat the test.
	Low quality/quantity of the DNA in the sample.	Concentrate the sample during its processing by adding less water volume.

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16 LABEL AND BOX SYMBOLS

Explanation of the symbols of the product label and box:

Symbol	Meaning	Symbol	Meaning
IVD	In vitro diagnostic medical device		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions for use	Σ	Sufficient content for <n> assays
	Safety data sheet		

17 CHANGELOG

Date	Description
31/03/2021	• Inclusion of the changelog section. • Inclusion of the explanation of the pictogram of the Safety Sheet • A new liquid cytology transport medium is included in item 7.
16/07/2021	• A new extraction method with which the kit has been validated is included in section 7.

05/05/2023	• Two new types of starting samples are included: cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection kit (Ref. MAD-VVC, Vitro S.A.) as well as the amplification product obtained with the Vitro HPV Screening kit (Ref. MAD-003949M, Vitro, S.A.) in section 7. • The analysis procedure and evaluation of the performance of the kit using the amplification product obtained with the Vitro HPV Screening kit (Ref. MAD-003949M, Vitro, S.A.) as the starting sample is included in sections 8, 9 and 12. • A new extraction method with which the kit has been validated is included in section 7.
22/06/2023	• The analytical performance in hybriSpot 24 PCR AUTO is described. • The alternative protocol for sample preparation for direct PCR from liquid cytology or cervico-vaginal samples obtained with the Cervico-Vaginal Self Collection Kit (Ref. MAD-VVC, Vitro S.A.) is added.

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