HPV Direct Flow CHIP Kit

Screening and genotyping of human papillomavirus based on PCR amplification and reverse dot blot hybridization

For hybriSpot 12 (HS12) and hybriSpot 24 (HS24) platforms

Please refer to the correct Instructions for use according to the software version used:

1. Software version **2.2.0 HybriSoft HSHS and lyophilized format of the kit.**
2. Software version **2.1.0 HybriSoft HSHS.**

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HPV Direct Flow Chip Kit

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

For all hybriSpot platforms

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

REF	**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/CE and ISO 18113-2

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1 INTENDED USE

HPV Direct Flow CHIP is an *in vitro* diagnostic kit for the 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 on its association with different degrees of lesions, HPV has 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 genotyping of 36 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. In this protocol, the clinical samples can also be amplified directly without the need for DNA extraction previously.

Microbiological status: Product not sterile

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 by using the DNA-Flow technology both for automatic and manual hybriSpot platforms. The biotinylated amplicons generated after the PCR are hybridized in membranes containing an array of specific probes for each target 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.

HPV Direct Flow CHIP does not require the prior extraction of DNA from the samples, but the 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.

3 COMPONENTS

The **HPV Direct Flow Chip** kit is retailed 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 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 and hybriSpot 12 PCR AUTO platforms.

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 (beta-globin gene) are included and used as an internal control for the PCR reaction.

3.2 Reagents for reverse hybridization

- 24 tests

MAD-003930M-H-HS12-24		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	40 ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10 mL	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS12-24
Reagent E	10 ml	MAD-003930ME
Washing Buffer II (Reagent F)	18 ml	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)	60 ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10 mL	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS24-24
Reagent E	10 ml	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 and hybriSpot 12 PCR AUTO platforms.

- 48 tests

MAD-003930M-H-HS12-48		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	80 ml	MAD-003930MA-HS12-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS12-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS12-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS12-48
Reagent E	18 ml	MAD-003930ME-HS12-48
Washing Buffer II (Reagent F)	35 ml	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.

MAD-003930M-H-HS24-48		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	115 ml	MAD-003930MA-HS24-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS24-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS24-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS24-48
Reagent E	18 ml	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 and hybriSpot 12 PCR AUTO platforms.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and Materials

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

- Disposable gloves
- Pipette tips with DNase/RNase-free filters.
- DNase/RNase-free double distilled water
- Paraffin Tissue Processing Kit, Ref: MAD-003952M (30 tests)

A. Specific reagents to platforms HS12a and HS24:

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

4.2 Equipment

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

- 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).
  - Thermocycler
  - Thermal block to heat PCR tubes (can be substituted by a thermocycler)
  - Cold plate (4ºC)
  - 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).
  - Thermocycler (not necessary for hybriSpot 12 PCR AUTO).
  - Thermal block to heat PCR tubes (not necessary for hybriSpot 12 PCR AUTO).
  - Cold plate (4ºC).

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 -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 contamination. **Once the package containing the tubes strip with the lyophilized PCR mix is opened, store the remaining tubes up to a maximum of one week at 2-8°C in the original package.**
Hybridization reagents. Shipment at 2-8 °C. Upon receipt, they must be stored at -8 °C. They will be stable until the specified expiration date. The reagents as well as the HPV Chips are stable until the specified expiration date. Do not freeze. Previous recommendations on the hybridization reagents:

- The hybridization reagent A must be pre-heated in a thermostatic bath or heater (only before using it in the manual equipment HS12) at 41°C before its use.
- The rest of the hybridization reagents must be used at room temperature (20-25°C).

6 WARNINGS AND PRECAUTIONS

- **Read the instructions of use before using this product.**
- **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.md@vitro.bio.

- **HPV Direct Flow Chip** does not require the prior extraction of DNA from the samples, but the PCR amplification can be performed directly from cell suspensions, fixed cells or paraffin-embedded tissue sections. It is the client's responsibility to include the necessary controls to verify that the system works properly.
- **General considerations to avoid the contamination with PCR product:**
  The greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and hybridization 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 diagnosis. 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 Cod-UNG, which degrades the PCR products containing dUTP, is included in the kit.
  It is recommended to include negative amplification controls containing all the reagents handled in the kit, from the extraction to the amplification, except for the sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The hybridization in membrane of this control must be negative, marking only the hybridization control and the amplification exogenous control. This way, it is verified that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by Master Diagnóstica, S.L. 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 wastes generated by this kit according to the European Law, 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:
-

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW
1. Rubbish/Waste generated from hybridization reagents 2. Disposal of Liquid Wastes ("Wastes" in the equipments HS12 and HS24)	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, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"

3. Chips used 4. Consumables (tubes, tips, aluminum foil, etc.) 5. Any element that has been in contact with DNA	180103	"Wastes 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 having residues of or contaminated by dangerous substances"

Table 9: Classification of wastes generated by this kit according to the European Legislation. *ELW: English acronym for *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 SAMPLE PREPARATION

HPV Direct Flow CHIP is optimized to the direct use of clinical sample without the need for previous DNA extraction.

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 or paraffin-embedded samples.
- MagNa Pure (Roche): for DNA purification from fresh samples.
- NX-48 FFPE DNA Kit (Genolution): for DNA purification from both fresh or paraffin-embedded samples.

Note: 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 PELLETS				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Shake pellet 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.
2nd	- Mix the sample with vortex at low-medium speed. - Take 30µl of the homogeneous suspension as template DNA for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
3rd	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warning:	**Mix the cell suspension well before adding it to the PCR tube**		**Very high**	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
Warning:	Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube		Very high	If you use another buffer as PBS to collect cells, the PCR may be left inhibited.

Table 10: Preparation protocols for direct PCR from cervical and anal pellets.

CYTOLOGIES IN A LIQUID MEDIUM				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1st	Take 400 µl of 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 pellet with 400 µl **DNase/RNase-free double distilled water**. Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Remove inhibiting agents from PCR.	Very high	PCR inhibition, "blank" result.
4th	Resuspend the cell button in 300 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.	Suspend the cells in a liquid medium.	High	Excess of material Possible PCR inhibition, "blank" result.
5th	Mix sample with vortex. Take 30µl of the homogeneous suspension as DNA template for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warning:	**Mix the cell suspension well before adding it to the PCR tube**		**Very high**	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
Warning:	Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube.		Very high	If you use another buffer as PBS to collect cells, the PCR may be left inhibited.

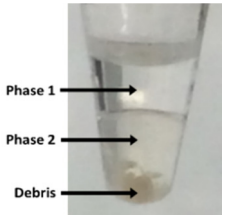
Table 11: Preparation protocols for direct PCR from cytologies in a liquid medium.

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)*

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 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 from PCR.	Very high	PCR inhibition, "blank" result.
4th	Resuspend the cell button in 300 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.	Suspend the cells in a liquid medium.	High	Excess of material Possible PCR inhibition, "blank" result.
5th	Mix sample with vortex. Take 30µl of the homogeneous suspension as DNA template for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warning:	**Mix the cell suspension well before adding it to the PCR tube**		**Very high**	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
Warning:	Use **DNase-RNase-free double distilled water** only to collect the cells and add to the PCR tube.		Very high	If you use another buffer as PBS to collect cells, the PCR may be left inhibited.

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-· paraffin-embedded tissue sections (depending on the size of the tissue section) of 10 µm thick. Put in an Eppendorf tube of 0.5 ml. *Note: it is advisable 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 DNArelease (Ref Kit: MAD-003952M). Note: for > 1 cm2 tissue sections: - Increase extraction buffer volume and DNArelease proportionally. - Make 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 a**. -> Insufficient lysis of the cells and tissue material that prevent the DNA from being in suspension, "blank" result. **Without b**. -> high risk of degrading DNA polymerase in the PCR, "blank" result.
5th	- Centrifuge for 1 min at 2000 rpm -> decant tissue remains. - Add **27 µl DNase/RNase-free double distilled water** by means of a reaction tube, and **3 µl from the cell suspension** as template DNA for PCR, avoiding taking tissue remains from the tube's bottom ("debris"). *Note: remains of mineral oil may remain in the upper part of the supernatant (Phase 1), these remains 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 supernatant, the test result would be "blank".
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warnings	If after the incubation it is observed that the tissue has not		High	Insufficient lysis of the cells and tissue material

	been fully digested, it is recommended to add the proportional volume of Extraction buffer and DNArelease 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 extreme sections there is a risk that the sections used for PCR have lost the lesion. 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.			that prevent the DNA from being in suspension, "blank" result.

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

8 ANALYSIS PROCEDURE for platforms HS12 and HS24

8.1 Reaction of amplification by multiplex PCR

The following thermocyclers 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.
- Add up to 30 µl of direct sample in each tube following the recommended protocol in section 7.
- If it involves **paraffin-embedded tissue**, add **27 µl DNase/RNase-free double distilled water** and **3 µl** DNA by means of a reaction 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 with no need for using complete strips. The rest of the tube strip with the lyophilized PCR mix that is not going to be used at that moment must be stored for maximum of 1 week at 4°C in its original package.
- Place the tubes in the thermocycler and set the following amplification conditions:

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 in that moment, they can be stored in the post-PCR zone at 8-10°C for 1-2 days. To store them for a longer period of time, it is recommended to do so at -20°C.

Important note: If purified DNA is used for PCR, 30 µl of this DNA can be added directly to the lyophilized PCR tube.

8.2 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.

8.2.1 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 thermocycler and **cool quickly in ice** during at least **2 min**.
2. Preheat the **Reagent A** (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 for 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.
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 every Chip
k) Incubate for **5 min at 29 ºC**.
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 ºC**.
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.

8.2.2 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 thermocycler and **cool quickly in 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 to start the automatic process.

9 ANALYSIS PROCEDURE for platform HS12a

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

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 equipment) and follow the instructions to place the tube strips, chips and hybridization reagents in the instrument.

Procedure:

- Take a tube containing the lyophilized PCR mix per sample to be analyzed.
- Add the DNA samples to a PCR tube following the instructions described in section 8.1.
- 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 with no need for using complete strips. The rest of the lyophilized tube strip that is not going to be used at that moment must be stored for maximum of 1 week at 4°C in its original package.
- Follow the instructions in the manual to place the tube strips, chips and hybridization 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 reagents and developing have worked properly. If the signal does not appear, 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 orientate correctly the probe panel to perform the subsequent analysis.

Endogenous amplification control (C): Probe to detect the gen of the human beta-globin in the test sample, that is co-amplified during the PCR. All the samples where the test DNA 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 shows 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 means that it has been an error during the amplification, a low quality/amount 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 and comply with the applicable legislation.

11 INTERPRETATION OF THE RESULTS

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

	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 orientate correctly the probe panel for its subsequent analysis.

Expected results	Probe/positions (column-row)			
	HPV genotype probe	B	C	U*
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
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-2K-6F-10A	1C-5F	--
BLANK. Inappropriate material. Insufficient material. PCR inhibited.	--	1A-1B-2K-6F-10A	--	--
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 inside the amplified region L1 of the virus. Its sequence is shared by all the genotypes of the panel and by other genotypes of mucosa not included in this kit. It should be taken into account 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 positivity results with a genotype-specific probe and not with the U probe; in these cases, the absence of positivity in 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 may be a different genotype from the ones included in the panel.

An example of a report in which the analyzed case has been positive for HPV 56 is shown below.

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HPV Direct Flow Chip Kit

LOTES

PCR:	HPV0014U	30/12/2020
Chips:	HPVE-56	30/12/2019
Reactivo:	H057-5	30/12/2020

DETALLES DE LA MUESTRA

ID MUESTRA: Muestra-22 **TIPO DE MUESTRA:**

ID PACIENTE: **PACIENTE:**

SEXO: - **FECHA NAC.:** **EDAD:**

INFORME

HPV POSITIVO

Muestra positiva para:
Alto Riesgo:
56
Muestra negativa para el resto de genotipos incluidos en el test HPV direct flow chip.

PROTOCOLO

Detección y genotipado del virus HPV mediante PCR y reverse dot blot, genotipos:
- Alto riesgo: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.
- Bajo riesgo: 6, 11, 40, 42, 43, 44/55, 54, 61, 62/81, 67, 69, 70, 71, 72, 84.

Preparación de la muestra/extracción del ADN
- Usar la suspensión celular/DNA para amplificar por PCR.

Protocolo PCR 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.

Protocolo REVERSE-DOT BLOT:
- Hibridación del producto de PCR biotinilado con HPV CHIP
- Lavados post-hibridación
- Incubación con enzima Estreptavidina-Fosfatasa
- Revelado con NBT-BCIP

Análisis automático de resultados

NOTAS

FACULTATIVO:	Default Doctor, doctor	**Validado:**	20/03/2019
Realizado por:	Default Tech, tech	**Procesado:**	20/03/2019

Instr. : Mock **Serial Nº:** 100001 **hybriSoft:** HSHS 2.2.0.R00 / HSHS IPL 1.0.0.R05

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HPV Direct Flow Chip Kit

LOTES

PCR:	HPV0014U	30/12/2020
Chips:	HPVE-56	30/12/2019
Reactivo:	H057-5	30/12/2020

DETALLES DE LA MUESTRA

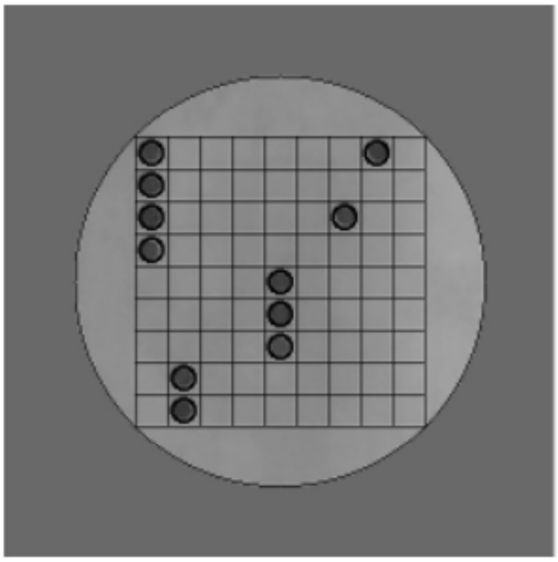
ID MUESTRA: Muestra-22 **TIPO DE MUESTRA:**

ID PACIENTE: **PACIENTE:**

SEXO: - **FECHA NAC.:** **EDAD:**

INFORME

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	



- Spot B: Control de hibridación (5 puntos para orientar correctamente el CHIP)
- Spot C: Control interno de DNA (Sonda de DNA genómino humano)
- Spot U: Sonda universal para HPV
- Spot #: Sondas específicas para cada genotipo HPV

Todos los puntos están impresos por duplicado.

INFORMACIÓN DEL ANÁLISIS

Umbral: 6

FACULTATIVO: Default Doctor, doctor **Validado:** 20/03/2019

Realizado por: Default Tech, tech **Procesado:** 20/03/2019

Instr. : Mock **Serial Nº:** 100001 **hybriSoft:** HSHS 2.2.0.R00 / HSHS IPL 1.0.0.R05

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical

12.1.1 Repeatability

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

HPV genotype	Genome equivalents/reaction	Positives/tested	% positives
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

12.1.2 Reproducibility

The reproducibility of the method was tested by processing 10 HPV positive samples, both from single and multiple infections, at two different GE concentrations, as well as 20 HPV negative samples, containing each of them 10 ng of human genomic DNA. These samples were processed in two different laboratories, using different batches of reagents, and different equipments and operators. Each of the samples was tested three times in different days using the hybriSpot platform for the hybridization and the software for analysis of results hybriSoft. No false positive was obtained (100% of the negative samples for HPV gave expected results. Concordance of the test (detection of the genotype vs negatives): *Kappa=0.96*.

Sample	GE /reaction	Laboratory 1		Laboratory 2	
		Positives/Valids	% positives	Positives/Valids	% positives
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 among the HPV genotypes included of the panel were observed, except for the 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.

Cross-reactivities were not observed with other analyzed viruses and bacteria: *Herpes simplex virus 1 and 2*, *Neisseria gonorrhoeae,* and *Chlamydia trachomatis*.

12.1.4 Analytical sensitivity

The limit of sensitivity for each HPV genotype was calculated using serial dilutions of plasmid or synthetic genes from each genotype with 10 ng of human genomic DNA per reaction. Each sample was repeated at least 5 times, in order to calculate sensitivity, specificity, and confidence intervals All PCRs were hybridized in the hybriSpot platform and analyzed with the hybriSoft software. A threshold value of 6 (gray intensity) was established for positivity.

Genotype	GE/ PCR reaction	Positives/tested	Sensitivity %	Confidence interval 95%	Specificity %	Confidence interval 95%
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

12.1.5 Evaluation of the direct protocol performance

The performance of HPV Direct Flow Chip was compared with the two types of protocols described, direct protocol (without DNA extraction) *vs* use of purified DNA. 225 clinical cases were tested simultaneously with the two types of protocols. 100 % agreement (*Kappa=0.99*) for positivity was obtained with both methods. The results obtained in the three kinds of samples, cytological swab, liquid-based cytology and paraffin sections are summarized in the table below:

	HPV + (positive cases/total cases)		
	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)	95,4 % *(Kappa=0.957)*
Liquid based cytology	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 Analytical functioning in hybriSpot 24

The functioning and sturdiness of HPV Direct Flow Chip was validated in the automatic equipment HS24 by analyzing limit 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 the rest of genotypes). This validation proves the reproducibility of the results between the positions 1 and 24 of the HS24 equipment and the reproducibility of the results with different programs for a different number of samples.

- **Reproducibility of results in program for a different number of samples**

Replicas of a positive sample that contained several genotypes at a limit concentration (50 GE) were made. 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)
- Protocol for 15 samples (4 replicas)
- Protocol for 24 samples (6 replicas)

The results were automatically analyzed with hybriSoft and differences between the different positions of the reaction chamber nor the used protocol weren't detected.

- **Reproducibility of results in different hybridization positions in HS24**

Four replicas for each genotype were made, placed in different positions of the two reaction chambers of the HS24 and using the protocol for 24 samples. 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	Positives/tested	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 21: Reproducibility of HPV Direct Flow Chip in HS24. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 6. NT: not tested

12.1.7 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 limit 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 in programs for a different number of samples**

Replicas of a positive sample that contained several genotypes at a limit 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 differences between the different positions of the reaction chamber nor the used protocol weren't detected.

- **Verification of sensitivity limit**

The functioning and the robustness of the HPV Direct Flow Chip was validated in the automatic equipment HS12a by analyzing limit concentrations of synthetic DNA fragments of all the high-risk genotypes included in the panel, as well as some low-risk ones.
3 replicas of each positive sample containing a simple genotype at a limit concentration were made. The whole process was performed automatically in two different HS12a equipments, and the results were analyzed with hybriSoft.

HPV genotype	No. GE/reaction	Positives/tested
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 22: Reproducibility of HPV Direct Flow Chip in HS12a. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 6. NT: not tested

12.2 Clinical

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 23: Distribution of the diagnostic groups and 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 and paraffin-embedded tissue sections (see section 7). The use of any other type of sample can generate erroneous results and its operation must be previously verified.

14 TROUBLESHOOTING

Problem	Causes	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. 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 rests of decontaminant reagents (e.g. Bleach) in the wells.	Clean with plenty of distilled water and repeat the experiment.
Presence of HPV in the negative control.	Contamination problems in pre-PCR 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. Presence of PCR inhibitors.	Repeat the PCR by increasing the amount of starting sample. Repeat the test. 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. Sample volume used to re-suspend the erroneous lyophilized product. Failure in the hybridization protocol. Low quality/quantity of the DNA in the sample.	Check the expiration date of all the reagents and the storage conditions. Repeat the test. Repeat the test by using the correct sample volume Check the correct functioning of hybriSpot HS12/12a/24 and the hybridization protocol. Repeat the test. Concentrate the sample during its processing by adding less water volume.

15 BIBLIOGRAPHY

- zur Hausen H, Meinhof W, Scheiber W, Bornkamm GW (1974). Attempts to detect virus specific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. *Int J Cancer* 13: 650-6.
- Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV *et al* (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 189: 12-9.
- zur Hausen H (1996). Papillomavirus infections-a major cause of human cancers. *Biochim Biophys Acta* 1288: F55-78.
- zur Hausen H (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2: 342-50.
- Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 348 (6): 518-27
- Elsa Herraez-Hernandez, Martina Alvarez-Perez, Gloria Navarro-Bustos, Javier Esquivias, Sonia Alonso, Jose Aneiros-Fernandez, Cesar Lacruz-Pelea, Magdalena Sanchez-Aguera, Javier Saenz Santamaria, Jesus Chacon de Antonio, Jose Luis Rodriguez-Peralto. HPV Direct Flow CHIP: A new human papillomavirus genotyping method based on direct PCR from crude-cell extracts. Journal of Virological Methods 193 (2013) 9–17.
- Herraez-Hernandez E, Preda O, Alonso S, Pardo RS, Olmo A. Detection and Genotyping of Human Papillomavirus DNA in Formalin-Fixed Paraffin-Embedded Specimens with the HPV Direct Flow CHIP System. Open Virol J. 2013 Oct 18;7:91-5.

16 LABEL SYMBOLS

Explanation of the symbols of the product label:

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

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HPV Direct Flow Chip Kit

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

For hybriSpot 12 (HS12) and hybriSpot 24 (HS24) platforms

Compatible with the version 2.1.0R05 of hybriSoft HSHS.
For compatibility with other versions, please contact the manufacturer / supplier.

REF	**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/CE and ISO 18113-2

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1 INTENDED USE

HPV Direct Flow Chip is an *in vitro* diagnostic kit for the 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 on its association with different degrees of lesions, HPV has 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 genotyping of 36 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, 84 and 89 (=CP6108)) by PCR (polymerase chain reaction), followed by reverse hybridization on a membrane containing specific probes. In this protocol, the clinical samples can also be amplified directly without the need for DNA extraction previously.

Microbiological status: Product not sterile

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 by using the DNA-Flow technology both for automatic and manual hybriSpot platforms. The biotinylated amplicons generated after the PCR are hybridized in membranes containing an array of specific probes for each target 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.

HPV Direct Flow Chip does not require the prior extraction of DNA from the samples, but the 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.

3 COMPONENTS

The **HPV Direct Flow Chip** kit is retailed in two main 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		
Description	**Format**	**Reference**
HPV PCR mix	1 vial x 950 µl	MAD-003930MU-MIX-HS12
Hot Start DNA Polymerase	1 vial x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	1 vial x 24 µl	MAD-UNG-4

Table 1: PCR reagents provided in the kit MAD-003930MU-HS12-24

MAD-003930MU-P-HS24-24		
Description	**Format**	**Reference**
HPV PCR mix	1 vial x 1200 µl	MAD-003930MU-MIX-HS24
Hot Start DNA Polymerase	1 vial x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	1 vial x 28 µl	MAD-UNG-3

Table 2: PCR reagents provided in the kit MAD-003930MU-HS24-24

- 48 tests

MAD-003930MU-P-HS12-48		
Description	**Format**	**Reference**
HPV PCR mix	2 vials x 950 µl	MAD-003930MU-MIX-HS12
Hot Start DNA Polymerase	2 vials x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	2 vials x 24 µl	MAD-UNG-4

Table 3: PCR reagents provided in the kit MAD-003930MU-HS12-48

MAD-003930MU-P-HS24-48		
Description	**Format**	**Reference**
HPV PCR mix	2 vials x 1200 µl	MAD-003930MU-MIX-HS24
Hot Start DNA Polymerase	2 vials x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	2 vials x 28 µl	MAD-UNG-3

Table 4: PCR reagents provided in the kit MAD-003930MU-HS24-48

The PCR mix of HPV contains the PCR buffer, dNTPs (U/T), Dnase/Rnase-free water and biotinylated primers. The primers included are specific for the amplification of a fragment of the region L1 of the HPV, and they can detect at least 36 HPV genotypes. Furthermore, primers for the amplification of a human genomic DNA fragment (beta-globin gene) are included and used as an internal control for the PCR reaction.

3.2 Reagents for reverse hybridization

- 24 tests

MAD-003930M-H-HS12-24		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	40 ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10 mL	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS12-24
Substrate (Reagent E1)	14 ml	MAD-003930ME1-HS12-24

Chromogen (Reagent E2)	14 ml	MAD-003930ME2-HS12-24
Reagent E	--	MAD-003930ME
Washing Buffer II (Reagent F)	18 ml	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

MAD-003930M-H-HS24-24		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	60 ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10 mL	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS24-24
Substrate (Reagent E1)	14 ml	MAD-003930ME1-HS24-24
Chromogen (Reagent E2)	14 ml	MAD-003930ME2-HS24-24
Reagent E	--	MAD-003930ME- HS24
Washing Buffer II (Reagent F)	18 ml	MAD-003930MF-HS24-24
HPV Chip (HS)	1x 24 units	MAD-003930M-CH-HS-24

Table 6: Hybridization reagents supplied in the kits MAD-003930MU-HS24-24

- 48 tests

MAD-003930M-H-HS12-48		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	80 ml	MAD-003930MA-HS12-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS12-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS12-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS12-48
Substrate (Reagent E1)	20 ml	MAD-003930ME1-HS12-48
Chromogen (Reagent E2)	20 ml	MAD-003930ME2-HS12-48
Reagent E	--	MAD-003930ME
Washing Buffer II (Reagent F)	35 ml	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

MAD-003930M-H-HS24-48		
Name	**Format**	**Reference**
Hybridization Solution (Reagent A)	115 ml	MAD-003930MA-HS24-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS24-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS24-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS24-48
Substrate (Reagent E1)	20 ml	MAD-003930ME1-HS24-48
Chromogen (Reagent E2)	20 ml	MAD-003930ME2-HS24-48
Reagent E	--	MAD-003930ME-HS24-48
Washing Buffer II (Reagent F)	30 ml	MAD-003930MF-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

IMPORTANT: All the reagents are supplied in a ready-to-use format, except reagents E1 and E2, which must be mixed 1:1 right before use in the empty vial supplied labelled as "Reagent E". The volume of the reagents E1 and E2 provided with the reference MAD-003934M-H-HS24-24 for the platform HS24 allows the preparation of the reagent E to perform a maximum of ten runs.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and Materials

A. Common reagents to platforms HS12 and HS24:

- Disposable gloves
- Eppendorf DNase/RNase-free tubes of 0.2/0.5/1.5 ml.
- Pipette tips with DNase/RNase-free filters.
- Saline solution (PBS buffer) 1X (DNase/RNase-free).
- Paraffin Tissue Processing Kit, Ref: MAD-003952M (30 tests)

B. specific reagents (Ref.: MAD-003930M-HS24):

- PCR Encoded Plates with Flat Cap Strips and Adhesive Film (Ref: MAD-003900P-HS24).
- HS24 PCR Tube Strips and Flat Cap Strips (Ref: MAD-003900ST-HS24).
- Washing Reagent (Ref: MAD-003930WSH).
- Plastic Applicator (Ref: MAD-00PA-2).

4.2 Equipment

A. Common equipment to platforms HS12 and HS24:

- Microcentrifuge
- Automatic micropipettes: P1000, P200, P20 and P2
- Thermocycler
- Thermal block to heat PCR tubes (can be substituted by a thermocycler).
- Cold plate (4°C).
- 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).
  - 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).

5 STORAGE AND STABILITY CONDITIONS

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

PCR reagents: Shipment between 2 and 8 °C*. Upon receipt, they must be stored at -20 °C. They will be stable until the specified expiration date. The PCR reagents must be stored in areas free of DNA or PCR products contamination. Avoid multiple cycles of freezing and thawing.

Hybridization reagents. Shipment and storage at 2-8°C*. The reagents as well as the HPV Chips are stable until the specified expiration date. Do not freeze. Previous recommendations on the hybridization reagents:

- The hybridization reagent A must be pre-heated in a thermostatic bath or heater (only before using in manual equipment) at 41°C before its use.
- The reagent E must be prepared (E1+E2) right before its use.
- The rest of the hybridization reagents must be used at room temperature (20-25°C).

***Note: Inside each box, there is a band indicating the time and temperature to control the conditions during shipment. It is recommended to contact the manufacturer before using the reagents included in the box if the cold chain has been interrupted.**

6 WARNINGS AND PRECAUTIONS

- **Read the instructions of use before using this product.**
- **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 Direct Flow Chip** does not require the prior extraction of DNA from the samples, but the PCR amplification can be performed directly from cell suspensions, fixed cells or paraffin-embedded tissue sections. It is the client's responsibility to include the necessary controls to verify that the system works properly.
- **General considerations to avoid the contamination with PCR product:**
  The greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and hybridization 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 diagnosis. 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 Cod-UNG, which degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls containing all the reagents handled in the kit, from the extraction to the amplification, except for the sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The hybridization in membrane of this control must be negative, marking only the hybridization control and the amplification exogenous control. This way, it is verified that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by Vitro S.A, S.L. 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 wastes generated by this kit according to the European Law, 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:

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW
1. Rubbish/Waste generated from hybridization reagents 2. Disposal of Liquid Wastes ("Wastes" in the equipments HS12 and HS24)	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, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"
3. Chips used 4. Perishable material (tubes, tips, aluminum foil, etc.) 5. Any element that has been in contact with DNA	180103	"Wastes 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 having residues of or contaminated by dangerous substances"

Table 9: Classification of wastes generated by this kit according to the European Legislation. *ELW: English acronym for *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 SAMPLE PREPARATION

HPV Direct Flow CHIP is optimized to the direct use of clinical sample without the need for previous DNA extraction.

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 or paraffin-embedded samples.
- MagNa Pure (Roche): for DNA purification from fresh samples.

Note: 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 swab in 400 µl PBS 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, "blank" result.
3rd	Re-suspend the resulting cell pellet in 25 ul-50 µl (depending on the size of the cell pellet) of PBS 1x to obtain a homogeneous suspension of cells.	Suspend the cells in a liquid medium.	High	Excess of material. Possible PCR inhibition, "blank" result.
4th	- Mix sample with pipette (several suctions). - Take 4-5µl of the homogeneous suspension (depending on the format of the kit) as template DNA for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, "blank" result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, "blank" result.
5th	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warning:	Use samples suspended in at the moment of obtaining them. The remaining sample volume can be stored at 4 ° C or frozen at -20° C, being stable at 4 °C for a week and at -20 ° C for 2 months.		High	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.

Table 10: Preparation protocols for direct PCR from cervical and anal pellets.

CYTOLOGIES IN A LIQUID MEDIUM				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1º	Take 150-200 µl of sample, sucking decanted cells from the bottom of the tube and place them in a tube of 1.5-2 ml.	Extraction of starting material.	Very high	Insufficient material, “blank” result.
2º	Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Concentration of starting material.	Very high	Insufficient material, very diluted, “blank” result.
3º	Wash pellet with 400 µl PBS 1X. Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Remove inhibiting agents from PCR.	Very high	PCR inhibition, “blank” result.
4º	Re-suspend the resulting cell button in 25 ul-50 µl (depending on the size of the cell pellet) of PBS 1x to obtain a homogeneous suspension of cells.	Suspend the cells in a liquid medium.	High	Excess of material Possible PCR inhibition, “blank” result.
5º	- Mix sample with pipette (several suctions). - Take 4-5µl of the homogeneous suspension (depending on the format of the kit) as template DNA for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, “blank” result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, “blank” result.
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, “blank” results.
Warning:	Use samples suspended in at the moment of obtaining them. The remaining sample volume can be stored at 4 ° C or frozen at -20° C, being stable at 4 ° C for a week and at -20 ° C for 2 months.		High	The cells begin to lyse and release proteases that can destroy the polymerase, “blank” results.

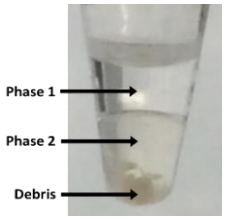
Table 11: Preparation protocols for direct PCR from cytologies in a liquid medium.

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)*

CYTOLOGIES IN DIGENE TRANSPORT MEDIUM (STM)				
Step	**Brief description of the procedure**	**Use**	**Importance**	**Consequence of not performing it correctly**
1º	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.
2º	Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Concentration of starting material.	Very high	Insufficient material, very diluted, “blank” result.
3º	Wash pellet with 400 µl PBS 1X. Centrifuge for 1 min at 2000 rpm and remove the supernatant.	Remove inhibiting agents from PCR.	Very high	PCR inhibition, “blank” result.
4º	Re-suspend the resulting cell button in 25 ul-50 µl (depending on the size of the cell pellet) of PBS 1x to obtain a homogeneous suspension of cells.	Suspend the cells in a liquid medium.	High	Excess of material Possible PCR inhibition, “blank” result.
5º	- Mix sample with pipette (several suctions). - Take 4-5µl of the homogeneous suspension (depending on the format of the kit) as template DNA for the PCR reaction.	- Obtain homogeneous cell sample. - Avoid cell clusters.	High	Sedimented cells: - If only supernatant is sucked -> Insufficient material, “blank” result. -If it sucks from the pellet -> cluster of excess of material -> Possible PCR inhibition, “blank” result.
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, “blank” results.
Warning:	Use samples suspended in at the moment of obtaining them. The remaining sample volume can be stored at 4 ° C or frozen at -20° C, being stable at 4 ° C for a week and at -20 ° C for 2 months.		High	The cells begin to lyse and release proteases that can destroy the polymerase, “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**
1º	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 0.5 ml. *Note: it is advisable to remove as much paraffin as possible from the edges of the tissue sections.*	Collection of starting material.	Very high	Insufficient material, "blank" result.
2º	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).
3º	Add to the pellet: - 60 µl of extraction buffer - 1.5µl of DNArelease (Ref Kit: MAD-003952M). Note: for > 1 cm2 tissue sections: - Increase extraction buffer volume and DNArelease proportionally. - Make 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.
4º	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 a. -> Insufficient lysis of the cells and tissue material that prevent the DNA from being in suspension, "blank" result. Without b. -> high risk of degrading DNA polymerase in the PCR, "blank" result.
5º	- Centrifuge for 1 min at 2000 rpm -> decant tissue remains. - Take 4-5 µl of the homogeneous suspension (Phase 2) as template DNA for PCR, avoiding taking tissue remains from the bottom of the tube ("debris"). *Note: remains of mineral oil may remain in the upper part of the supernatant (Phase 1), these remains 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 supernatant, the test result would be "blank".
6º	Once the samples have been added to the PCR tubes, amplify immediately.		Very high	The cells begin to lyse and release proteases that can destroy the polymerase, "blank" results.
Warnings	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 DNArelease again and repeat the incubation for a further 30 min at 60 °C		High	Insufficient lysis of the cells and tissue material that prevent the DNA from being in suspension, "blank" result.

	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 extreme sections there is a risk that the sections used for PCR have lost the lesion. 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.			

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

8 ANALYSIS PROCEDURE

8.1 Reaction of amplification by multiplex PCR

The following thermocyclers 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)

Important notes before starting:

- **It is important that the whole process is performed on an ice sheet to avoid the degradation of the enzymes in the kit and to avoid unspecific bindings between the primers.**
- **It is important that, while working with the HS24, you must only use the tube strips or plates provided by Vitro S.A., PCR plates with bar code, cover strips and adhesive paper (Ref: MAD-003900P-HS24) and tube strips and PCR cover for HS24 (Ref: MAD-003900ST-HS24).**

The PCR reaction is performed in a final volume of 40 µl in PCR tubes of 0.2 mL (MAD-003930MU-HS12), or in a final volume of 50 µl (MAD-003930MU-HS24) in bar-coded PCR plates (ref: MAD-003900P-HS24) or in tube strips (ref: MAD-003900ST-HS24), depending on the platform used.

It is recommended to aliquot the PCR mix when used for the first time to avoid repeated freezing and thawing cycles.

- Thaw HPV PCR Mix on ice.
- Add to the PCR mix tube all the full volume of Hot Start DNA Polymerase and Uracil-DNA Glycosylase, mix thoroughly by turning it upside down several times and centrifuge briefly.
- Aliquot **36 µl** in 24 PCR tubes, or aliquot of **45 µl** in bar-coded PCR plates (ref: MAD-003900P-HS24) or in tube strips (ref: MAD-003900ST-HS24) and store at -20°C (stable for at least 6 months).

Thaw a PCR tube and add **4-5 µl** of the test sample, prepared according to the protocol detailed in section 7.
If the PCR mix is prepared right before using it, follow these instructions:

Component	Volume per reaction (MAD-003930MU-HS12)	Volume per reaction (MAD-003930MU-HS24)
HPV PCR Mix	34.8 µl	43.5 µl
Hot Start DNA Polymerase	0.4 µl	0.5 µl
Uracil-DNA Glycosylase	0.8 µl	1 µl
Test sample	**4 µl**	**5 µl**

Table 14: Necessary reagents and volumes to prepare a PCR reaction.

Note: To avoid the degradation of the enzymes contained in the PCR mix, it is recommended to perform the full protocol on ice.

Place the PCR tubes in the thermocycler and program the amplification conditions listed below:

1 cycle	25°C	10 min
1 cycle	94°C	3 min
15 cycles	94°C	30 s
	42°C	30 s
	72°C	30 s
35 cycles	94°C	30 s
	60°C	30 s
	72°C	30 s
1 cycle	72°C	5 min
	8°C	∞

Table 15: PCR Program

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

8.2 Flow-through reverse hybridization

All the reagents are provided in a "ready-to-use" format.

The developer solution of the hybridization is provided as two reagents (Reagents E1 and E2) that must be mixed in a 1:1 proportion right before its use in the vial of the "Reagent E" with a volume depending on the nº of samples to be processed (see tables 16 and 17). **After every use, you need to clean the vial with distilled water to avoid the accumulation of precipitates due to consecutive uses.**

The membranes are single-use and must be handled with gloves.

8.2.1 Flow-through 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 thermocycler and **cool quickly in ice** during at least **2 min**.
2. Preheat the **Reagent A** (Reagent A) at 41 °C.
3. Place every **HPV Chip** in the position indicated by the platform (HS12)
4. Prepare the **Reagent E** at the moment of use, mixing in a 1:1 proportion the components 1 and 2 provided in the kit. In the following table, the required volumes of the reagent E1 and E2 are indicated depending on the number of tests:

	vol (µl)/1 test	vol (µl)/4 tests	vol (µl)/8 tests	vol (µl)/12 tests
E1	200	700	1400	2200
E2	200	700	1400	2200

Table 16: Volumes of reagents E1-E2 that must be mixed in the vial E depending on the nº of tests to be processed.

Manual hybridization protocol:

a) Set the temperature of the equipment at 41 °C. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated for 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.
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 every Chip
k) Incubate for **5 min at 29 ºC**.
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 **8 min** at **36 ºC**.
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.

8.2.2 Flow-through reverse hybridization, ref. MAD-003930MU-HS24

The whole hybridization process is performed automatically in 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 thermocycler and **cool quickly in ice** during at least **2 min**.
2. Prepare the **Reagent E** at the moment of use, mixing in a 1:1 proportion the components 1 and 2 provided in the kit. In the following table, the required volumes of the reagent E1 and E2 are indicated depending on the number of tests:

	vol (µl)/1 test	vol (µl)/4 tests	vol (µl)/8 tests	vol (µl)/12 tests	vol (µl)/16 tests	vol (µl)/20 tests	vol (µl)/24 tests
E1	1200	1800	2300	3000	3800	4200	5000
E2	1200	1800	2300	3000	3800	4200	5000

Table 17: Volumes of reagents E1-E2 that must be mixed in the vial E depending on the nº of tests to be processed.

3. Place the PCR tubes, the HPV Chips and the reagents in their corresponding positions of hybriSpot 24.
4. Select the corresponding protocol in the equipment to start the automatic process.

9 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 18: 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 reagents and developing have worked properly. If the signal does not appear, 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 orientate correctly the probe panel to perform the subsequent analysis.

Endogenous amplification control (C): Probe to detect the gene of the human beta-globin in the test sample, that is co-amplified during the PCR. All the samples where the test DNA 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 shows 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 means that it has been an error during the amplification, a low quality/amount 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 and comply with the applicable legislation.

10 INTERPRETATION OF THE RESULTS

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

	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	89	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	89
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 19a: 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 orientate correctly the probe panel for its subsequent analysis.

Expected results	Probe/positions (column-row)			
	HPV genotype probe	B	C	U*
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

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 89	5C-9F	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-2K-6F-10A	1C-5F	--
BLANK. Inappropriate material. Insufficient material. PCR inhibited.	--	1A-1B-2K-6F-10A	--	--
Hybridization error	--	--	--	--

Table 19b: 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 inside the amplified region L1 of the virus. Its sequence is shared by all the genotypes of the panel and by other genotypes of mucosa not included in this kit. It should be taken into account 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 positivity results with a genotype-specific probe and not with the U probe; in these cases, the absence of positivity in 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 may be a different genotype from the ones included in the panel.

An example of a report in which the analyzed case has been positive for HPV 56 is shown below.

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HPV Direct Flow Chip Kit

LOTS

PCR:	HPVP034-B	01/05/2019
Chips:	HPVH-034	01/05/2019
Reagent:	HPVH-034	01/05/2019

SAMPLE DETAILS

ID SAMPLE: M18-001

ID PATIENT:

PATIENT:

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

SAMPLE TYPE:

REPORT

HPV POSITIVE

GENOTYPES DETECTED:

High-Risk:

56

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, 89 (CP6108).

Sample preparation/DNA purification

Add cell suspension/purified DNA for PCR amplification:

- PCR protocol: 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.

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	**Processed:**	18/06/2018
Performed by:	Default Tech, tech	**Validated:**	18/06/2018

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HPV Direct Flow Chip Kit

LOTS

PCR:	HPVP034-B	01/05/2019
Chips:	HPVH-034	01/05/2019
Reagent:	HPVH-034	01/05/2019

SAMPLE DETAILS

ID SAMPLE: M18-001

ID PATIENT:

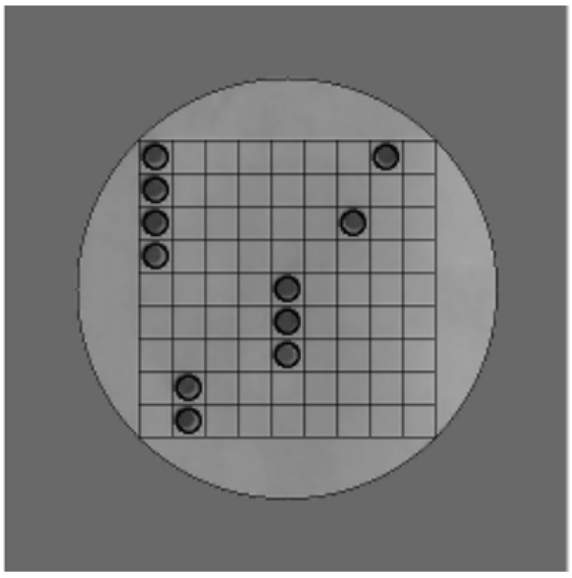
PATIENT:

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

SAMPLE TYPE:

REPORT

B	33	58	42	71	16	52	B	
B	35	59	43	72	18	53	6	69
C	39	66	44/55	89	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	89
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	



- Spot B: Hybridization control (5 signals to orientate the CHIP)
- Spot C: Internal DNA control (Genomic human DNA probe)
- Spot U: HPV Universal probe
- Spot #: Genotype specific probes

All the spots are printed in duplicate.

ANALYSIS INFORMATION

Threshold: 6

FACULTATIVE:	Default Doctor, doctor	**Processed:**	18/06/2018
Performed by:	Default Tech, tech	**Validated:**	18/06/2018

11 PERFORMANCE CHARACTERISTICS

11.1 Analytical

11.1.1 Repeatability

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

HPV genotype	Genome equivalents/reaction	Positives/tested	% positives
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%
	500	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 89	50	0/4	0%
	500	4/4	100%
HPV 84	50	4/4	100%

Table 20: Repeatability test for each genotype included in the panel. NT: not tested

11.1.2 Reproducibility

The reproducibility of the method was tested by processing 10 HPV positive samples, both from single and multiple infections, at two different GE concentrations, as well as 20 HPV negative samples, containing each of them 10 ng of human genomic DNA. These samples were processed in two different laboratories, using different batches of reagents, and different equipments and operators. Each of the samples was tested three times in different days using the hybriSpot platform for the hybridization and the software for analysis of results hybriSoft. No false positive was obtained (100% of the negative samples for HPV gave expected results). The percentage of the positive results are indicated in Table 21. Only in the sample containing 5 GE of HPV 18/reaction, less than 100% of positivity was obtained. Concordance of the test (genotype detection vs negative): *Kappa=0.96*.

Sample	GE /reaction	Laboratory 1		Laboratory 2	
		Positives/Valids	% positives	Positives/Valids	% positives
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 21: Inter-laboratory reproducibility for the HPV Direct Flow Chip kit.

11.1.3 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 among the HPV genotypes included of the panel were observed, except for the 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.

Cross-reactivities were not observed with other analyzed viruses and bacteria: *Herpes simplex virus 1 and 2*, *Neisseria gonorrhoeae,* and *Chlamydia trachomatis*.

11.1.4 Analytical sensitivity

The limit of sensitivity for each HPV genotype was calculated using serial dilutions of plasmid or synthetic genes from each genotype with 10 ng of human genomic DNA per reaction. Each sample was repeated at least 5 times, in order to calculate sensitivity, specificity, and confidence intervals All PCRs were hybridized in the hybriSpot platform and analyzed with the hybriSoft software. A threshold value of 6 (gray intensity) was established for positivity.

Genotype	GE/ PCR reaction	Positives/tested	Sensitivity %	Confidence interval 95%	Specificity %	Confidence interval 95%
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
89	50	0/10	0	0-27.8	100	98.5-100
	500	10/10	100	72.3-100	100	98.6-100
84	50	10/10	100	72.3-100	100	98.6-100

Table 22: 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

11.1.5 Evaluation of the direct protocol performance

The performance of HPV Direct Flow Chip was compared with the two types of protocols described, direct protocol (without DNA extraction) *vs* use of purified DNA. 225 clinical cases were tested simultaneously with the two types of protocols. 100 % agreement (*Kappa=0.99*) for positivity was obtained with both methods. The results obtained in the three kinds of samples, cytological swab, liquid-based cytology and paraffin sections are summarized in the table below:

	HPV + (positive cases/total cases)		
	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)	95,4 % *(Kappa=0.957)*
Liquid based cytology	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 23: Performance of HPV Direct Flow Chip Kit with direct samples in comparison with purified DNA.

11.1.6 Analytical functioning in hybriSpot 24

The functioning and sturdiness of HPV Direct Flow Chip was validated in the automatic equipment HS24 by analyzing limit 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 the rest of genotypes). This validation proves the reproducibility of the results between the positions 1 and 24 of the HS24 equipment and the reproducibility of the results with different programs for a different number of samples.

- **Reproducibility of results in program for a different number of samples**

Replicas of a positive sample that contained several genotypes at a limit concentration (50 GE) were made. 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)
- Protocol for 15 samples (4 replicas)
- Protocol for 24 samples (6 replicas)

The results were automatically analyzed with hybriSoft and differences between the different positions of the reaction chamber nor the used protocol weren't detected.

- **Reproducibility of results in different hybridization positions in HS24**

Four replicas for each genotype were made, placed in different positions of the two reaction chambers of the HS24 and using the protocol for 24 samples. 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	Positives/tested	Difference between positions
16	50	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
89	500	4/4	No

Table 24: Reproducibility of HPV Direct Flow Chip in HS24. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 6. NT: not tested

11.2 Clinical

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 a positivity for HPV.HR: high risk.

12 LIMITATIONS

HPV Direct Flow Chip Kit has been validated with cytological swabs, liquid based-cytology samples and paraffin-embedded tissue sections (see section 7). The use of any other type of sample can generate erroneous results and its operation must be previously verified.

13 TROUBLESHOOTING

Problem	Causes	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 12/24. 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 rests of decontaminant reagents (e.g. Bleach) in the wells.	Clean with plenty of distilled water and repeat the experiment.
Presence of HPV in the negative control.	Contamination problems in pre-PCR 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.	The reagent E has not been newly prepared.	Prepare the new substrate mix by mixing the reagents E1 and E2 (1:1) right before its use. Repeat the test.
	The preparation vial for Reagent E contains residual reagent E from previous uses.	Clean well the vial for the reagent E with distilled water before each use to avoid the accumulation of precipitates in consecutive uses. Repeat the test.
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.
	Failure in the hybridization protocol.	Check the correct functioning of hybriSpot HS12/24 and the hybridization protocol. Repeat the test.
	The PCR product was not denatured properly before the hybridization.	Check that the denaturation step of the PCR product before the hybridization has been performed properly. Repeat the test.
	Low quality/quantity of the DNA in the sample.	Increase the amount of sample or starting DNA in the PCR reaction. Repeat the test.

14 BIBLIOGRAPHY

- zur Hausen H, Meinhof W, Scheiber W, Bornkamm GW (1974). Attempts to detect virus specific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. *Int J Cancer* 13: 650-6.
- Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV *et al* (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 189: 12-9.
- zur Hausen H (1996). Papillomavirus infections-a major cause of human cancers. *Biochim Biophys Acta* 1288: F55-78.
- zur Hausen H (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2: 342-50.
- Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 348 (6): 518-27
- Elsa Herraez-Hernandez, Martina Alvarez-Perez, Gloria Navarro-Bustos, Javier Esquivias, Sonia Alonso, Jose Aneiros-Fernandez, Cesar Lacruz-Pelea, Magdalena Sanchez-Aguera, Javier Saenz Santamaria, Jesus Chacon de Antonio, Jose Luis Rodriguez-Peralto. HPV Direct Flow CHIP: A new human papillomavirus genotyping method based on direct PCR from crude-cell extracts. Journal of Virological Methods 193 (2013) 9–17.
- Herraez-Hernandez E, Preda O, Alonso S, Pardo RS, Olmo A. Detection and Genotyping of Human Papillomavirus DNA in Formalin-Fixed Paraffin-Embedded Specimens with the HPV Direct Flow CHIP System. Open Virol J. 2013 Oct 18;7:91-5.

15 LABEL SYMBOLS

Explanation of the symbols of the product label:

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