HPV Direct Flow CHIP Kit

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

30 determinations

Ref. MAD-003930M

Produced under UNE-EN 375 regulations

For in vitro diagnostic use

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TABLE OF CONTENTS

1. INTENTED USE 3
2. TEST PRINCIPLE 3
3. REAGENTS INCLUDED IN THE KIT 4
4. STORAGE AND STABILITY 4
5. EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED IN THE KIT 5
6. REMARKS AND PRECAUTIONS 5
7. OPERATING PROCEDURE 6
7.1. REAGENTS PREPARATION 6
7.2. SAMPLE PREPARATION FOR DIRECT PCR 6
7.3. PCR REACTION 8
7.4. AUTOMATIC FLOW-THROUGH REVERSE HYBRIDIZATION 8
8. INTERPRETATION OF THE RESULTS 10
9. REPRODUCIBILITY OF HPV Direct Flow CHIP 11
10. ANALITICAL SENSIBILITY AND SPECIFICITY OF HPV Direct Flow CHIP 11
11. TROUBLESHOOTING 11
12. BIBLIOGRAPHY 12

1. INTENTED USE

HPV Direct Flow CHIP is a diagnostic kit for *in vitro* detection of human papillomaviruses (HPV). HPV detection has become a very important tool in diagnostic because infection with these viruses constitutes an essential factor for cervical and anogenital carcinogenesis (zur Hausen *et al*, 1974; Walboomer *et al*, 1999; zur Hausen, 1996; zur Hausen 2002).

2. TEST PRINCIPLE

Based on their association with different lesion grades, HPV have been classified as (Muñoz 2003) high risk HPVs or oncogenic, that can induce carcinogenesis; and low risk HPVs, that cause genital warts and collaborate with high risk HPVs.

HPV Direct Flow CHIP is intended for simultaneous screening and genotyping of 36 HPV types (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 dot blot automatic hybridization, based on DNA-Flow Technology (e-BRID System®). Clinical samples (fresh and paraffin embedded) are amplified directly, without the need of DNA extraction.

This kit is based on the amplification of a human papillomavirus L1 consensus region by PCR and hybridization to specific DNA probes immobilized onto a nylon membrane. The DNA-Flow based automatic hybridization platform allows the binding of the amplified DNA to the complementary capture probes in a three-dimensional porous environment, which enables a very fast coupling between the PCR product and its specific probe. Biotinilated PCR products are hybridized with specific probes and the hybridization signal is developed by a colorimetric immunoenzimatic reaction (Streptavidin-Alkaline Phosphatase and NBT-BCIP chromogen). The substrate-chromogen reaction generates a dark-purple precipitate in the position where the specific probe has hybridized with the PCR amplicon and this signal is automatically captured and analyzed (e-BRID System®). This technology has a very high sensitivity for HPV detection and it can be performed in a very short time comparing to other systems, reducing total processing time from hours to minutes.

3. REAGENTS INCLUDED IN THE KIT

This kit includes all necessary reagents for direct PCR amplification and hybridization of 30 clinical samples.

- **Reagents for direct PCR:**
  - **PCR Mix (1x 1700 µl)**
  - **Phire® Hot Start II DNA polymerase* (1 x 30 µl).**

- **Reagents for Reverse dot blot hybridization:**

Reagent A:	***Hybridization solution***	***90 ml***
Reagent B:	***Blocking solution***	***35 ml***
Reagent C:	***Streptavidin-Alkaline Phosphatase***	***18 ml***
Reagent D:	***Washing buffer I***	***65 ml***
Reagent E-1:	***Substrate[a]***	***10 ml***
Reagent E-2:	***Chromogen[a]***	***10 ml***
Reagent F:	***Washing buffer II***	***50 ml***
HPV CHIP	***Spotted membranes***	***30 units***

[a]The developing solution must be prepared just before use by mixing 1:1 the reagents E-1 and E-2.

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4. STORAGE AND STABILITY

PCR reagents: Shipped at 2-8 ºC and then stored at -20ºC after reception. Thaw on ice just before use. Reagents are stable until expiration date. These reagents must be stored isolated from any source of contaminating DNA (e.g. PCR products).

Hybridization reagents: Shipped and stored at 2-8 ºC. Do not freeze. Reagents and HPV CHIPs are stable until expiration date. Developing solution must be prepared just before use. Hybridization reagent should be brought to 41ºC before use and all other hybridization reagents should be used at room temperature (20-25ºC).

5. EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED IN THE KIT

Equipment:

- Thermocycler.
- Microcentrifuge.
- Thermostatic bath or heating block.
- e-BRID System®.

Consumables:

- Disposable gloves.
- Disposable sterile pipette tips.
- Sterile PBS buffer (DNase/RNase-free).
- PCR DNase/RNase-free Eppendorf tubes 0.2/ 0.5/1.5 ml.
- Distilled water.
- PARAFFIN TISSUE PROCESSING KIT, Ref: MAD-003952M (30 tests).

6. REMARKS AND PRECAUTIONS

During the amplification and hybridization of the samples it is recommended to use disposable gloves. The most common source of contamination is the PCR product created in the laboratory. In order to avoid this, it is very important to keep two different working areas: pre- and post-PCR. In the pre-PCR area, clinical samples are manipulated and added after adding the Taq polymerase to the PCR tubes. Amplified products will be manipulated and hybridized in the post-PCR area. These two zones must be physically separated and it is very important not to share any material between both working stations (laboratory coats, pipettes, etc.). The working flow must be in one way direction, from pre-PCR to post-PCR and never in the other direction. This is important to avoid false positive cases due to contamination with PCR products.

It is recommended to include negative controls (containing all the PCR components except the DNA) during amplification.

7. OPERATING PROCEDURE

HPV Direct Flow CHIP kit has been optimized for direct use from clinical samples, without the need of previous DNA extraction. Although not required, the kit can be used with purified DNA.

The membranes are intended to be used only once. Do not touch the membranes with bare hands and keep them away from any source of contamination.

7.1. REAGENTS PREPARATION

All the reagents are supplied "ready to use".

PCR MIX:

Thaw PCR Mix and Phire® Hot Start II DNA Polymerase before the first use and proceed as follows:

- Spin down the vial of Phire® Hot Start II DNA Polymerase for a few seconds.
- Add **30 µl** of Phire® Hot Start II DNA Polymerase to the PCR Mix.
- Mix well by inverting the vial several times and centrifuge for a few seconds.
- Dispense **54 µl** aliquots of the mix into 30 0.2 ml PCR tubes.

The PCR tubes can be stored at 2-8ºC for one week or at -20ºC for 3 months.

7.2. SAMPLE PREPARATION FOR DIRECT PCR

- **Cytological swabs.**
  - Prepare one **1.5-2 ml** eppendorf tube containing **400 µl** of PBS buffer (repeat for every sample). Dip the tip of the swab or brush into the PBS solution and squeeze gently against the tube´s wall to detach the cells
  - Centrifuge at **2000 rpm for 1 min**. Remove the supernatant carefully.
  - Resuspend the cell pellet in **25-50 µl** of PBS buffer (depending on the size of the pellet).
  - Use **6 µl** of this cell suspension as DNA template for the PCR reaction. The remaining volume can be stored at 4ºC for one week or at –20ºC for 2 months.

- **Liquid-based cytology**.
  - Let the cells settle at the bottom of the vial. Place **150-200 µl** of this cell suspension in a 1.5- 2 ml tube.
  - Centrifuge at **2000 rpm for 1 min**. Remove the supernatant carefully.
  - Wash the cell pellet by resuspending the cells in **400 µl of** PBS buffer. Centrifuge at **2000 rpm for 1 min**. Remove the supernatant carefully.
  - Resuspend the cell pellet in **25-50 µl** PBS buffer.
  - Use **6 µl** of this cell suspension as DNA template for the PCR reaction. The remaining volume can be stored at 4ºC for one week or at –20ºC for 2 months.

- **Paraffin embedded sections**.

Although the system works properly with Direct PCR from paraffin tissue samples, sometimes, it is difficult to guarantee the correct handling of the paraffin block and therefore it is recommended to start from purified DNA to avoid any contamination.

- For DNA extraction from paraffin-embedded sections: please follow standard purification methods. Use 6 µl of purified DNA as template for PCR.

- For direct PCR from paraffin samples: it is recommended to use the reagents included in the PARAFFIN TISSUE PROCESSING KIT (Ref: MAD-003952M) and follow the protocol:
  - Take 1-3 sections (depending on the tissue size) of 10 µm thickness and place them in a 0.5 ml Eppendorf tube using a needle or dissection forceps. Note: remove as much rests of paraffin as possible from the tissue sections.
  - Add **400 µl** of mineral oil.
  - Heat the sample at **95ºC for 2 min.**
  - Centrifuge at **2000 rpm for 1 min** and remove the remaining mineral oil.
  - Add **60 µl** of **Extraction Buffer** and **1.5 µl** of **DNA Release** to the tube.
  - Heat the sample at **60ºC for 30 min** and **98 ºC for 10 min** (this procedure can be performed in a thermocycler or a heat-block).
  - Centrifuge at **2000 rpm for 1 min**.
  - The top of the liquid will contain the rests of paraffin and mineral oil from the samples. Cell-debris will be pelleted at the bottom of the tube and the DNA will be in the suspension under the paraffin layer. Use **6 µl** of this liquid suspension as DNA template. The remaining sample can be stored at 4ºC for one week or at –20ºC for 2 months.

Notes:

- *For tissue sections bigger than 1 cm^2, it is recommended to increase the volume of Extraction Buffer and DNA Release proportionally to assure the complete dipping of the tissue.*
- *If after incubation the tissue has not been digested completely, it is recommended to add additional volume of Extraction Buffer and DNA Release and repeat incubation for 30 min.*
- *Direct PCR has not been assayed with other kind of clinical samples (i.e. stained samples or cytological extensions). For these cases, DNA purification is recommended.*

7.3. PCR REACTION

Add **6 µl** of DNA template to a PCR mix tube (prepared as indicated in step 7.1). Place all the tubes in the thermocycler and amplify the DNA under these conditions (PCR program):

1 cycle	98ºC	5 min*
5 cycles	98ºC	5 s
	42ºC	5 s
	72ºC	10 s
45 cycles	98ºC	5 s
	60ºC	5 s
	72ºC	10 s
1 cycle	72ºC	1 min
	8ºC	∞

** When purified DNA is used, this initial denaturation step can be reduced from 5 min to 30 s.*

Keep PCR products at 8-10ºC after the reaction is completed. Samples can be hybridized immediately or stored in a post-PCR fridge at 8-10ºC for 1-2 days.

7.4. AUTOMATIC FLOW-THROUGH REVERSE HYBRIDIZATION

The hybridization procedure, images capture and analysis of results are performed automatically by the **e-BRID System®,** supported by **hybriSoft™**.

Set up the instrument following the instructions in the user manual (provided with the instrument).

Before starting the automatic procedure:

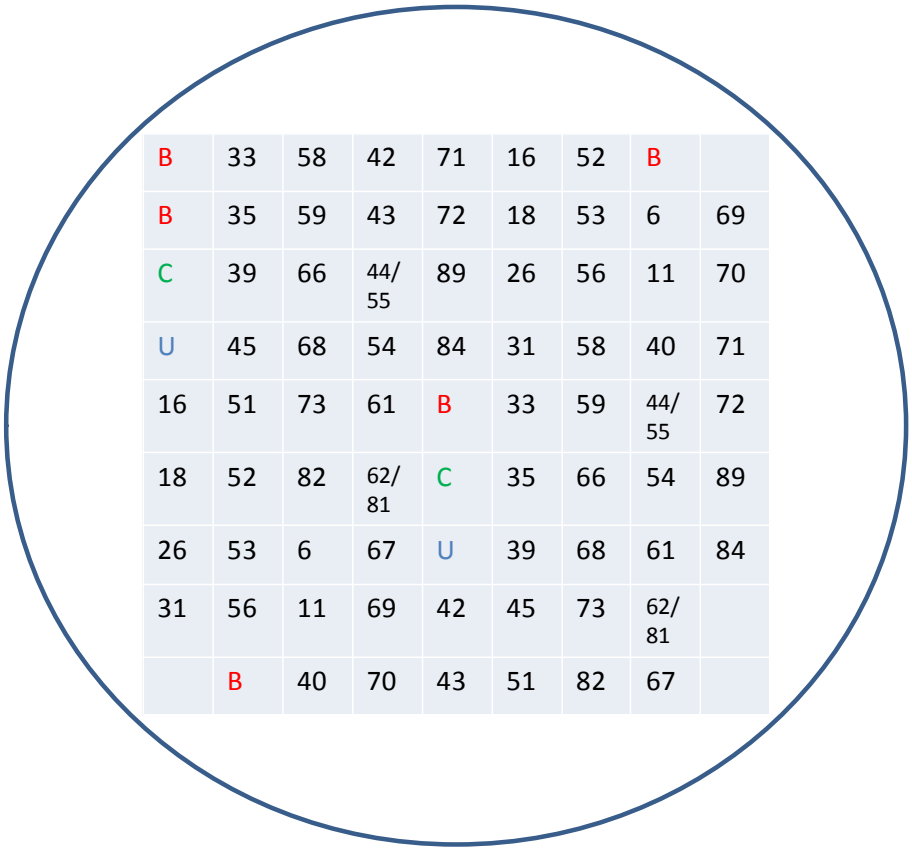
1. Denature the PCR products by heating them at **95 ºC for 5 min** in a thermocycler and then cooling them on ice for at least **2 min**.
2. Open the tubes caps and place the PCR tubes in the rack container of the **e-BRID System®**.
3. Select the hybridization program and the number of tests to be assayed.
4. Input the samples identification
5. Verify the required volumes for all the reagents and place them at room temperature in their corresponding sites.
6. Pre-warm **Reagent A (Hybridization solution)** at 41 ºC.
7. Place the **HPV CHIPs** in the indicated position of the chamber.
8. Prepare the required volume of developing solution by mixing reagents E1-E2 (1:1). Place it in the corresponding position in the reagents´ rack.

Automatic procedure:

a) Set the chamber temperature at 41ºC. Dispense **500 µl** of pre-heated **Reagent A (Hybridization solution)** into each HPV CHIP, incubate at **41 ºC** for **3 min**.
b) Remove the reagent by vacuum.
c) Mix **500 µl** of preheated **Reagent A (Hybridization solution)** and **40 µl** of each PCR sample. Dispense both together into the HPV CHIP.
d) Incubate at **41 ºC for 5 min**.
e) Remove the reagent by vacuum.
f) Perform **3** washes with **500 µl** of pre-heated **Reagent A (Hybridization solution)**.
g) **Set the chamber temperature at 30 ºC.**
h) Perform 2 incubations with **500 µl** of **Reagent B (Blocking solution)** into each HPV CHIP and incubate each time for **2 min 30 s** at **30 ºC**.
i) Remove the reagent by vacuum.
j) Dispense **500 µl** of **Reagent C (Streptavidin-Alkaline Phosphatase)** into each HPV CHIP. Incubate for **3 min at 30 ºC**.
k) Remove the reagent by vacuum.
l) **Set the chamber temperature at 36 ºC.**
m) Perform **4** Washes with **500 µl** of **Reagent D (Washing buffer I)**.
n) When the chamber reaches 36 ºC, dispense **500 µl** of **Reagent E (developing solution)** into each one of the HPV CHIPs. Incubate at **36 ºC for 4 min**.
o) Remove the reagent by vacuum.
p) Perform 3 washes with **500 µl of Reagent F (Washing buffer II)** into each CHIP.
q) Image capture and analysis.

8. INTERPRETATION OF THE RESULTS

The following figure shows the distribution of spots in the membrane:



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	

The HPV CHIP contains a quality control that ensures the correct interpretation of the results. All the membranes must be positive for the hybridization control (spots “B” in the figure above). This signal indicates that the hybridization has worked correctly.

A housekeeping human gene fragment is co-amplified during the PCR as internal amplification control (Spot “C”). A positive signal in it shows that the amplification worked efficiently and that there was enough DNA template in the clinical sample. No signal detected in spot “C” might indicate failures during amplification, low yield of DNA template or insufficient clinical material.

Clinical samples positive for HPV infection will have a signal in the spot “B” (hybridization control), spot “C” (PCR control), spot “U” (HPV Universal probe) and in the corresponding HPV genotype probe, whereas clinical samples that are negative for HPV infection will have signals only in spots “B” and “C”.

Some samples can show positivity for the hybridization control (“B”), PCR control (“C”) and HPV Universal control (“U”) but not for any specific HPV probe, indicating that the sample is positive for a HPV genotype not included in the CHIP detection panel.

9. REPRODUCIBILITY OF HPV Direct Flow CHIP

Negativity of the samples. Clinical samples that were negative for HPV infection when tested by **HPV Direct Flow CHIP** were re-analyzed by the same method demonstrating 100% reproducibility in all the cases. Moreover, when re-analysis was performed starting from purified DNA samples, negative results were confirmed.

Positivity of the samples. Reproducibility for detection of single and multiple HPV infection was determined, obtaining 100% agreement in all the assays. Single infection reproducibility was independently assayed by analysis of synthetic samples containing either HPV 16 or 18 (NIBSC standards). For multiple infections, a panel of HPV 16, 18, 31, 52, 45, 59, 73, 6, 40 and 42 was tested in 135 independent experiments, obtaining a 100% agreement between results.

10. ANALYTICAL SENSITIVITY AND SPECIFICITY OF THE HPV Direct Flow CHIP

Analytical sensitivity: Serial dilutions of HPV 16 and 18 WHO standard DNAs (NIBSC Institute) were tested under a background of 20 ng genomic DNA. The detection limit was 1-10 copies of viral genome equivalents.

Analytical specificity: No cross-reactivity was found between HPV genotypes included in the test. In addition, reactivity with genital pathogens Herpes, *Neiserria gonorrhoeae* and *Chlamydia trachomatis* was also excluded.

11. TROUBLESHOOTING

Observation	Approach
No signal in the hybridization control	Make sure all the reagents have been properly added and repeat the assay.
No signal in the amplification control	Insufficient amount of DNA/clinical specimen or inhibitors present in the sample. Repeat PCR increasing input sample and/or use purified DNA template.
HPV detection in the negative control	Contamination problems in pre-PCR area or in the hybridization reagents. Clean the working areas, repeat the PCR and the hybridization.
Weak hybridization signals	Check the expiry date of the solutions and storage conditions. Repeat the PCR increasing the input DNA. Confirm that the denaturation step is correctly performed.

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