Human Papillomavirus 23 Subtypes Diagnostic Kit Manual (PCR-RDB)

【Product Name】

Human Papillomavirus 23 subtypes diagnostic kit

(PCR reverse dot blot hybridization method)

【Packaging specification】

10 tests/box

【Intended Use】

This kit is used for the qualitative detection of human papillomavirus (HPV) subtypes including 5 low risk subtypes HPV 6, 11, 42,43, 44 and 18 medium & high risk subtypes HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, CP8304 in the samples of urethral secreta, cervical exfoliated cells and verruca cells. Genotyping for subtype detection is available.

The results are only for clinical reference, and should not be taken as the only basis for diagnosis or exclusion of disease cases. Because the relevant clinical test has not been completed, this kit should not be for the intended use of cervical cancer screening.

【Detection Principle】

This kit adopts specific PCR primer with its 5' end labeled with biotin to amplify the HPV target gene fragment which contains the decisive fragment (L1 region) for detection. According to the principle of complementary base pairing, specific oligonucleotide probes are designed with average length 15-25 bp, which makes use of the base differences at the detection sites for differentiation. The 5 'end of probes is labeled with amino group and fixed on the specific location of the nylon membrane by chemical bond to form the probe array membrane.

The PCR amplification products labeled with biotin are hybridized with the probes on the membrane strip under certain temperature and salt ion concentration. Streptomyces and peroxidase (POD) binds to the biotin, together with hydrogen peroxide (H_2O_2), to catalyze the tetramethyl benzidine (TMB) color change reaction. The presence/absence of blue dot at certain spot on the membrane determines the presence/absence of corresponding HPV subtype. Test results are identified automatically by software.

【Kit Contents】

Contents			Quantity	Ingredients
Kit 1	Extraction Buffer	HPV DNA Extraction Buffer	1mL x 1 vial	chelex 100、Tris HCl、NaOH、Triton×100、NP-40、EDTA
	PCR Reaction Reagent	HPV23 PCR Reaction Reagent	45μL x 10 vial	Primers, dN(U)TPs、At-Taq DNA polymerase、UGD enzyme、buffer system
		HPV23 Positive Control	30μL x 1 vial	Inactivated HPV16 subtype positive specimen
		HPV23 Negative Control	30μL x 1 vial	Inactivated HPV23 subtype negative specimen
Kit 2	Hybridization Reagent	A Reagent (5x)	20mL x 1 bottle	SSC、SDS
		B Reagent (5x)	40mL x 1 bottle	SSC、SDS
		C Reagent	5mL x 1 bottle	POD
		D Reagent (5x)	40mL x 1 bottle	Sodium Citrate
		E Reagent	5mL x 1 bottle	TMB、absolute ethyl alcohol
		F Reagent	5mL x bottle	H_2O_2
		HPV23 Membrane Strip	10 pcs/pack	nylon membrane、probes

Required reagents and consumables: sterilized 1.5mL, 15mL and 50mL centrifuge tubes.

Quality control instruction: HPV positive control and negative control are both collected from the clinical HPV16 positive and negative cervical specimens or secreta specimens that were treated through normal saline, centrifugation and suspension. Both quality controls were inactivated already.

Different contents from different batches are not interchangeable.

【Storage and validity】

1. **Storage:** Kit 1 (Nucleic acid extraction reagent and PCR reagent) should be kept at -20±5℃ away from lights, avoid repeated freezing and thawing. Keep kit 2 (Hybridization reagent) at 2-8° C.
2. **Validity:** Both kits are valid for 6 months under the above storage condition. Production date and valid date are labeled on the packing box.

Notes:

1. Bottle opening and melting stability: Kits are still valid after opening for 8 hours, but kept opened for a long time should be avoided due to evaporation.
2. Transportation stability: Kits are inspected qualified after 7 days' sealed storage in ice packs. It can meet the performance requirements of domestic long-distance transport
3. Repeated freezing thawing stability: Kits are still valid after repeated freezing and thawing for 6 times. Unnecessary freezing and thawing should be avoided in clinical use.

【Compatible Instruments】

Ordinary PCR instruments such as Takara TP600, TIB 3000 automatic hybridization instrument, manual hybridization instrument, ordinary thermostatic oscillating tank, ordinary office scanner.

【Sample Collection, Storage and Transport】

1. Collection

(1) For the urethral secreta, insert the cotton swab into the urethra under sterilized environment and gently rotate for a few cycles, stay for a few seconds, and take it out.

(2) For cervical exfoliated cell, insert the cervical brush into the cervix 2cm under sterilized environment and gently rotate for a few cycles. , stay for a few seconds, and take it out..

(3) For verrucous cell, use a sterile cotton swab to scrape the epithelium from the verruca.

2. Storage

Keep the cervical brush specimen or cotton swab specimen in the sterilized tube with 1mL normal saline. Stir and squeeze the brush or swab, then keep the remaining solution for detection. Preserve the solution in -20° C for detection. Valid for 6 months.

3. Transportation: Specimens should be transported carried out at around 0° C.

(Interference test proves that, a small amount of urine, blood and drugs for external use have no effects on the kit's performance. Nevertheless, avoid these interfered factors as much as possible during clinical specimen collection)

【Procedure】

1. Specimen Treatment

(1) Spin down the specimen solution tube and pipet 200μL into 1.5mL centrifuge tube. Centrifuge for 5 min at 10,000rpm and discard the supernatant. (Rinse the specimen one or two times with normal saline if there is any impurities)

(2) Thaw the positive control and negative control and then spin down briefly, pipet 10μL of each into two 1.5 mL centrifuge tubes respectively.

(3) Pipet 50μL DNA extraction buffer into the specimen tube, positive control tube and negative control tube respectively. Boil for 10 min and centrifuge for 5 min at 10,000rpm. Keep the supernatant for detection.

2. PCR amplification

(1) Pipet 5μL specimen solution, positive control and negative control into the PCR reaction tubes respectively.

(2) Spin down the PCR reaction tubes at 6,000rpm then put into the PCR instrument and amplify as per below sequence.

Cycle Parameters:

50° C ---- 3 min
93° C ---- 5 min

40 cycles of
93° C ---- 40 sec
55° C ----40 sec
72° C ----40 sec

72° C ---- 7 min

95° C ---- 5 min
4° C ---- 5 min

Note: PCR products denaturation occurs during the above underlined procedure. After the denaturation procedure, PCR products should be placed on ice for immediate hybridization. Otherwise, repeat the above underlined procedure before hybridization.

3. Hybridization Reaction

Prepare the hybridization reagents according to the below table:

Item	Specification	Preparation	Remarks
Reagent (5x) A	20mL x 1 vial	dilute with 80mL purified water	preheat before dilution if any ice crystals occur under low temperature situation
Reagent (5x) B	40mL x 1 vial	dilute with 160mL purified water	
Reagent (5x) D	40mL x 1 vial	dilute with 160mL purified water	

Label the specimen and positive, negative control numbers on the HPV23 membrane strip.

(1) Automatic Hybridization Procedure (Using TIB's automatic hybridization instrument)

a. Pour the A, B and D diluent into the corresponding labeled bottles. Put the Reagent C, E and F bottles at the corresponding spots labeled in the instrument.

b. Switch on the instrument and select "Open". Put one membrane strip (face up) into one hybridization trough. Pipet 40μL PCR products into one end of hybridization trough and select "Close". Then select "HPV genotype" hybridization procedure and select the sample number and start. After the hybridization, select "Open" and take out the membrane strips and the hybridization trough then power off the instrument.

(2) Manual Hybridization Procedure

a. Hybridization

Take 15 mL centrifugal tube, put a labeled strip, add 5-8 mL preheated solution A, the corresponding 40 mu L PCR products, tighten the cap, put in the oscillating water tank shake for 60 min at 46° C （100-150rpm）then discard the liquid.

b. Coupling (5 membranes simultaneously at one test)

Pipet 20mL preheated Reagent B, 1mL C reagent with one membrane into the 50mL centrifuge tube. Seal tight the tube and keep the tube into the vibration tank for 10 min at 46° C （40-50rpm）then discard the liquid. Pipet preheated Reagent B 40mL and shaked for 3 min at 46° C. Discard the liquid. Repeat this process one more time. Pipet 40mL Reagent D and vibrate for 3 min at 46° C then discard the liquid.

c. Color developing (5-10 membranes in one batch)

Pipet 1mL Reagent E, 1mL F reagent and 20mL Reagent D into a 50mL centrifuge tube, mix to be used as color developing liquid. Insert the membrane strip and keep away from lights for 8 min at room temperature, then discard the liquid.

(Note: Prepare the color developing liquid as per the clinical needs. If the liquid turns blue immediately after preparation, prepare again. If the color of color control is light, extend the color developing time appropriately 3-5 min for full color developing)

Add 40mL Reagent D and shake for 3 min then discard the liquid. Add 40mL water and shake for 3 min then discard the liquid. Take out the membrane strips and the hybridization is completed.

d. Membrane strip scanning

Use absorbent paper to absorb the liquid of membrane strips and scan them as soon as possible. (If immediate scan is unavailable, seal tight and store at -20° C)

e. Result analysis

Import the scanning picture as per the operating manual of auto hybridization analysis software (developed by TIB) and select "HPV 23 genotypes" procedure. Set up the parameters and proceed with data analysis and export the result and reports. More details refer to the operating manual of hybridization software.

f. Quality control standard

Color developing reaction control (CC) : signal value ⩾ cutoff value, hybridization and color developing processes are valid.

PCR reaction control (PC): signal value ⩾ cutoff value, PCR system is valid.

HPV 23 positive control: PC, CC and A3 on the membrane are positive, other positions are negative.

HPV 23 negative control：PC and CC on the membrane are positive, other positions are negative.

The test is considered valid only when all the above requirements are met simultaneously. Otherwise, retest is required.

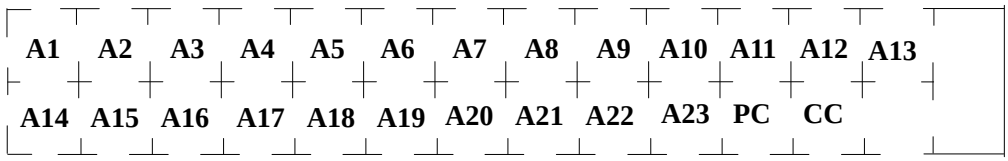
【Positive result】

Results are determined by membrane strip analysis software report or the reading of color developing areas by naked eyes. Hybridization spot signal value reflects the integral optical density (IOD).CC IOD value ≥ cutoff value means valid color developing. "ERROR" will occur when IOD value < cutoff value. The reason might be wrong spot zone selection or abnormal hybridization procedure.

Cutoff values of PC, CC and other subtypes detection spots are calculated by statistical method and preinstalled in the analysis software.

【Results interpretation】

There are 25 probe spots on HPV genotypes hybridization membrane strip including CC, PC and A1-A23 subtype detection spots. The probes array is listed as per below figure.



1. results interpretation:

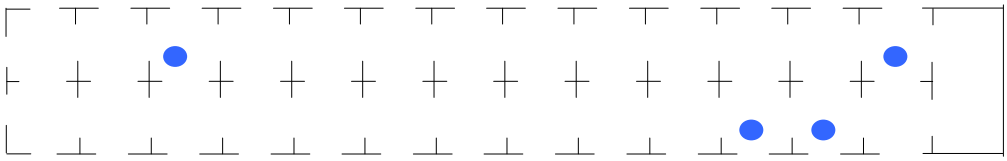
   Cutoff values of CC, PC and other subtypes detection spots are preinstalled in the analysis software. Software will come up with the info of every spot including testing credibility and HPV genotypes automatically.

2. Correspondences between valid colored probe spotsand subtype detection result interpretation is listed in below table:

No.	Control and Indication Points	Subtype Detection Spots	HPV Genotypes Result
1	CC、PC	A1	6
2	CC、PC	A2	11
3	CC、PC	A3	16
4	CC、PC	A4	18
5	CC、PC	A5	31
6	CC、PC	A6	33
7	CC、PC	A7	35
8	CC、PC	A8	39
9	CC、PC	A9	42
10	CC、PC	A10	43
11	CC、PC	A11	44
12	CC、PC	A12	45
13	CC、PC	A13	51

14	CC、PC	A14	52
15	CC、PC	A15	53
16	CC、PC	A16	56
17	CC、PC	A17	58
18	CC、PC	A18	59
19	CC、PC	A19	66
20	CC、PC	A20	68
21	CC、PC	A21	CP8304
22	CC、PC	A22	MM4(82)
23	CC、PC	A23	73
24	CC、PC	Multi spots positive result	Mixed infection
25	CC、PC	A1-A23 are all negative	1、specimen without HPV nucleic acid or lower than the LOD of this kit 2、other subtypes present besides these 23 types 3、possible random nucleotide sequence mutation

3. Example of membrane strip color developing sketch map after hybridization, a HPV16、51 subtypes mixed infection specimen:



【Detection Limitation】

1. Specimen concentration that is lower than the LOD of this kit cannot be detected.
2. This kit can detect 18 high risk subtypes of HPV including HPV16，18，31，33，35，39，45，51，52，53，56，58，59，66，68，73，82，CP8304 and 5 low risk subtypes including HPV6，11，42，43，44. Other HPV subtypes cannot be detected.
3. Some random nucleotide sequence mutations that are not taken into consideration in the design might lead to failure of detection.

【Kit Performance】

1. LOD (limit of detection) of this kit is 1×10^{3}copies/mL for the HPV 23 subtypes.
2. Specificity
   Interference test verifies that, small amount of urine, blood, vagina external used mycostatin or miconazole nitrate in the specimen has no effects on the test.

Cross specificity and genotypes cross validation show that, this kit has no cross reaction with urogenital tract pathogen such as treponema pallidum (TP), human mycoplasma (MH), Neisseria gonorrhoeae (NG), chlamydia trachomatis (CT), ureaplasma urealyticum (UU) and other HPV subtypes including HPV 3, 4, 10, 27, 28, 41, 49 etc.

3. Accuracy: this kit is highly consistent with the results of repeated tests on the same sample.

【Warnings and Precaution】

1. This product is for in vitro diagnosis purpose only.
2. Please read the full text of the instruction carefully before the experiment.
3. In order to avoid any potential biological hazards in the sample, samples should be regarded as contagious and avoid contact with skin and mucous membrane. Sample handling is recommended to be in the biological safety cabinet which can prevent aerosol outflow, and the used test tubes and pipet-tips for the operation should be sterilized before being discarded. The handling and disposal of specimens should meet the requirements of relevant laws and regulations: "The General Guidelines for Biosafety of Microbial Biomedical Laboratories" and "The Regulations on the Management of Medical Wastes" issued by the Ministry of Health.
4. Although the negative and positive quality control samples have been proved that the virus has been inactivated, no known test method can completely ensure that human-derived substances do not contain infectious substances after inactivation. All human-derived substances may be potentially contagious and should be treated as contagious during operation.
5. Lab personnel must be professionally trained. PCR experiment room should be separated from hybridization room. PCR experiments should be carried out in the sample processing room, PCR sampling room, PCR amplification room and hybridization room respectively, and each room should be relatively separated. Human and experimental materials should be moved unidirectionally from sample processing room to PCR sampling room to PCR amplification room to hybridization room. PCR kits should not be stored in hybridization room.
6. Provide negative pressure ultra-clean biosafety cabinet for reagent and sample preparation.. During experiments, please wear lab coats and disposable gloves and use self-unloading pipettes. Lab coats from PCR processing room and hybridization room should be separated.
7. Carry out quality control for each experiment.
8. Specimen treatment: Be careful when discarding the supernatant after adding the concentration solution. Discard the concentration solution as much as possible without touching the sediments. Then pipet 50μL DNA extraction solution into the sediments. Mix well before pipetting since there are some water insoluble granular materials in the DNA extraction solution. Use the sterilized scissor to cut a small part off from the pipet filter if there is any jam in the filter causing pipetting problems.
9. The 1.5mL centrifuge tubes and pipet-tips used for PCR reaction preparation should be autoclaved and used as disposable. Ensure the DNA amplification tubes numbers are conformed to the membrane numbers during hybridization.
10. Avoid touching the membrane strips by hands during hybridization procedure. Use sterilized tweezers to operate the membrane strips. Avoid scratching the membrane strips and label them on the corners by pencils. Oil-based pen will affect the accuracy of signal analysis.
11. Carry out the test at 20-30° C. Testing results might be inaccurate when the operating temperature is too low.
12. Warm bath the A reagent and B reagent at 40-50° C before using since they easily separate out crystals.
13. Use absorbent paper to absorb the liquid on membrane strips then levelly place the membrane strips on the screening instrument. During picture previewing, ensure no water stain or corrugations on the membrane strips to affect the accuracy of signal analysis.

14. The pipet-tips used in the experiment should be dismounted directly into the waste tank containing sanitizer and discarded after sterilized together with other waste materials.
15. After the experiment, sterile the workbench and pipette with 10% chloric acid or 70% alcohol or ultraviolet light.

【References】

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4 Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is anecessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12–9.

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【Manufacturer】

Name of registered manufacturer: Triplex International Biosciences (China) Co., Ltd.
Residence: Unit 407, 408, and 409, 4th floor of Siming photoelectric building, 55 QianPu Industrial Park, Siming District, Xiamen.
Production address: Unit 101, 201, and 301, 2041 Xizhou Road, Xike Town, Tongan District, Xiamen.
Name of after sales service: Triplex International Biosciences (China) Co., Ltd.
Tel: 0592-3737666 Fax: 0592-3788599
Enquiry Hotline: 400-7032032
Website: www.tibchina.com