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BIOTYPAP Kit

*Kit for Papillomavirus (HPV) DNA detection
and typing in human clinical samples*

Instructions for Use

Ref. 90.017

***PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT,
ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL.***

BIOTYPAP Kit

For research use only (RUO)
Not for use in diagnostic procedures
Test results may be used for preliminary analysis only

Some of the applications which may be performed with this product may be covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK KIT AND REAGENTS INTEGRITY BEFORE USE. USE OF DETERIORATED KITS MAY CAUSE LACK OF RESULTS AND/OR EQUIVOCAL RESULTS.

1. INTENDED USE

The BIOTYPAP Kit is a method for the determination, in a qualitative form, of human papillomavirus (HPV) by DNA amplification, followed by restriction digest (RFLP) in order to detect the HPV genotypes involved in the infection. The test detects thirty-two (32) HPV genotypes (6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68 and 69) in cervix samples. Identity of each genotype is then assessed by RFLP, followed by agarose gel electrophoresis.

The BIOTYPAP Kit is to be used with the following cervix samples:

- Samples taken with validated sterile cotton swabs. Use conservation solutions which do not contain denaturing substances or that may interfere with DNA polymerase activity¹.
- Standard cytological samples or after-colposcopy cytological samples, but before adding iodide or acetic acid. Use conservation solutions which do not contain denaturing substances or that may interfere with DNA polymerase activity.
- Samples taken with brush-type specific devices, such as Cervical Sampler™.

2. SUMMARY AND EXPLANATION OF THE TEST

Human Papilloma Virus (HPV) is a virus affecting mainly to cutaneous and anogenital tissues, and with average prevalence values around 15 %, but with a high degree of variation depending on age and other demographical values². HPV is a DNA virus, with a genome of approximately 8,000 nucleotides. Diseases caused by infection by HPV vary from condiloma to neoplastic transformations in cervix, vagina and vulva, as well as carcinoma. Some genotypes, of more than 100 genotypes described up to date, are associated to neoplastic transformation in cervix³. Neoplastic transformation is associated to the expression of E6 and E7 genes. Traditionally, presence of genotypes 16 and 18 is associated with high risk of neoplastic development⁴. Assignment of risk for other HPV genotypes is based on phylogenetic and/or epidemiological criteria. Thus, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 82 are classified as high risk based on both classification criteria⁵. Regarding HPV 67, which has been classified as high risk upon the phylogenetic point of view by some authors⁶, a cervix cancer cell line has been isolated, which presents integrated sequences from this type of HPV⁷. Also, types 26, 53 and 66, classified as high risk upon phylogenetic criteria, are now considered as probably carcinogenic based upon epidemiological criteria (see Muñoz *et al.*). Finally, types HPV 70 and 73 are of variable classification depending on the applied criteria (see Muñoz *et al.*).

HPV cannot be cultured *in vitro*, and there are no immunological techniques for its detection. Traditional techniques for indirect detection of HPV include physical examination to observe cellular changes associated with viral replication, followed by Papanicolau staining. Alternatively, hybridisation on biopsies can be performed for detection of the presence of viral DNA.

Viral DNA, in contrast to whole viral particles, is present in all epithelial layers. Presence of oncogenic HPV genotypes is directly associated with cervix cancer, and therefore, an early and trustworthy diagnosis is vital for correct patient management.

The BIOTYPAP Kit is an amplification test for the qualitative detection of HPV DNA, as well as for typing of HPV genotypes, and has been developed for use with cervix samples.

¹ Use of solutions similar to PreservCyt® may cause a decrease in the yield of amplification reactions.

² Parkin *et al.*; 1999. CA Cancer J Clin, 49: 33-64.

³ Schwartz *et al.*, 2001. J Clin Oncol, 19: 1906-15.

⁴ Jacobs *et al.*, 1995. J Clin Microbiol, 33: 901-5.

⁵ Muñoz *et al.*, 2003. New Engl J Med, 348: 518-27.

⁶ Chan *et al.*, 1995. J Virol, 69: 3074-83.

⁷ Koopman *et al.*, 1999. Cancer Res, 59: 5615-24.

3. PRINCIPLES OF THE PROCEDURE

BIOTYPAP Kit is a test for qualitative analysis, using a DNA amplification technique, followed by RFLP in order to determine the HPV genotype involved in the infection. Viral DNA present in positive samples is specifically amplified by using specific primers, which hybridise in homologous sequences in the viral genome. Amplification products are then digested using specific restriction endonucleases, rendering different patterns depending on the HPV genotype involved in the infection.

The kit consists of a multiplex amplification reaction, using two pair of primers. One pair of primers (Pair 1 – GEN1 and GEN2) hybridises with sequences common to all tested HPV genotypes (L1 and L2 genes), and therefore, indicates HPV presence. The second pair of primers (Pair 2 – ONC1 and ONC2) hybridises with specific sequences for oncogenic HPV genotypes (E6 and E7 genes).

The first pair of primers renders a band of approximately 450 bp, while Pair 2 renders a product of approximately 250 bp. Amplification products are then subjected to a restriction fragment analysis, on which different restriction patterns will indicate presence of a given HPV genotype.

The detection process using BIOTYPAP Kit consists of four main steps: sample preparation, viral DNA amplification, RFLP and detection by agarose electrophoresis and ethidium bromide staining.

A) Sample Preparation

The BIOTYPAP Kit is used with DNA purified from cervix samples.

NOTE

For viral DNA purification, use of the SPEEDTOOLS TISSUE DNA EXTRACTION KIT (Ref. 21.136, 21.137) is recommended. Use of other methods is also possible. However, the user must confirm that the purified DNA can be used with the BIOTYPAP Kit (concentration 50-100 ng/ μ l, $A_{260/280}=1.8 - 2.0$, absence of inhibitors that may affect the amplification, etc.). It is recommended to check the quality and suitability of the purified DNA for amplification reactions, for example, by performing control amplifications in parallel. For further information, please contact our Technical Dpt. (info@biotools.eu).

B) Viral DNA Amplification

Target selection

Selection of the target HPV sequences has been based on the study of highly conserved regions in the HPV genome for tested generic genotypes (GEN1 and GEN2 primers), and of minimally variable regions for tested oncogenic HPV genotypes (ONC1 and ONC2). Selected regions have a high degree of conservation between the tested genotypes. Pair 1 (GEN1 and GEN2) defines a segment of approximately 400 bp, while Pair 2 (ONC1 and ONC2) defines a sequence of approximately 250 bp in oncogenic HPV genotypes.

Amplification

DNA amplification is performed with a thermostable DNA polymerase from *Thermus*. In the presence of magnesium, and with the suitable salt and ionic strength conditions, the enzyme shows DNA polymerisation activity using as anchor a primer and a DNA molecule as template.

DNA purified from the sample to be analysed is added to the reaction mixture prepared with the reagents provided by the Kit, and containing all necessary reagents to perform the amplification reaction. Reaction mixture, already containing DNA, is incubated at different temperatures in order to allow the hybridisation of the primers pairs to the DNA from the sample. Once the hybridisation has taken place, and in the presence of triphosphate deoxynucleotides, the DNA polymerase extends the primer and forms a DNA strand complementary to the template DNA. Cyclic repetition of this process results in exponential amplification of the sequences originally present in the sample included between the primer pairs.

C) RFLP analysis

Amplification products are subjected to a restriction digestion using five different restriction endonucleases. The obtained patterns indicate presence of HPV genotypes and their identity.

D) Detection

Detection of digested products is performed by agarose gel electrophoresis followed by ethidium bromide staining.

NOTE

Ethidium bromide is a highly mutagenic intercalating agent. We recommend use of gloves and the maximum caution in handling.

For agarose electrophoresis performed prior to RFLP, the presence of HPV is indicated by a band of approximately 450 bp. If oncogenic HPV genotypes are present in the sample, an additional band of 250 bp will also appear.

For agarose electrophoresis performed after RFLP, please see the corresponding tables in the "Interpretation of results" chapter.

4. REAGENTS

The BIOTYPAP Kit contains amplification reagents in liquid format for performance of 96 amplification reactions (Ref. 90.017).

- HPV Mixture:** Two vials: 2 x 1980 µl
 A Tris-HCl solution, containing <10 % glycerol, KCl, <0.001 % dATP, dCTP, dGTP, dTTP and primers. HPV mixture includes all the amplification reagents for the HPVs detection and differentiation of oncogenic and generic genotypes, except MgCl₂ and DNA polymerase, in the optimal proportions to allow an effective amplification of the HPVs DNA.

Store at -15±8°C. Thaw and handle on ice. Do not freeze/thaw repeatedly. For frequent use, aliquot the contents in different vials.

- 50 mM MgCl₂ Solution:** Vial: 1.8 ml
 Store at -15±8°C. Thaw on ice. Mix well before use.
- Biotoools DNA Polymerase (1 U/µl):** Vial: 105 µl
 Store at -15±8°C. Add to the amplification mixture shortly before introduction of vials in thermal cycler.
- Restriction Enzymes and Buffers:**
 Contains different restriction enzymes and the corresponding reaction buffers to perform the typing.

- Enzyme A:** Vial: 160 µl
Restriction enzyme A

- Buffer A:** Vial: 112 µl
Reaction buffer for enzyme A

- Enzyme B:** Vial: 160 µl
Restriction enzyme B

- Buffer B:** Vial: 112 µl
Reaction buffer for enzyme B

- Enzyme C:** Vial: 160 µl
Restriction enzyme C

- Buffer C:** Vial: 112 µl
Reaction buffer for enzyme C

- Enzyme D:** Vial: 160 µl
Restriction enzyme D

- Buffer D/E:** Vial: 224 µl
Reaction buffer for enzymes D and E

- Enzyme E:** Vial: 160 µl
Restriction enzyme E

Store at -15±8°C. Thaw and handle on ice. Avoid repeated freeze/thaw cycles. For frequent use, aliquot the content of the vial.

- Positive Control:** Vial: 300 µl
 One single vial containing sequences of generic HPV and oncogenic HPV, both in a Tris-HCl EDTA solution. Both amplified DNA products are at a concentration of 10⁶ copies/µl. The positive controls included in the kit are non-infective, and can be used as intra-tube control to verify the the presence or absence of inhibitors in the analysed sample* (see the footnote at the section instructions for use).

Store at -15±8°C. Thaw and handle on ice. Avoid repeated freeze/thaw cycles. For frequent use, aliquot the content of the vial.

5. INSTRUCTIONS FOR USE

NOTE

Thaw and keep reagents on ice while in use. For optimal results keep reaction mixtures and vials in refrigerated conditions until their introduction into the thermal cycler.

Proceed to Reagent Preparation Area in a laminar flow cabinet

1.- Final reaction volume is 50 μ l. Prepare the **Reaction Mixture** following the table bellow (Table 1) in a 1.5 ml vial, according to the number of reactions to be performed (Reaction Mixture = HPV Mixture + MgCl₂ + Biotools DNA Polymerase). For each round of analysis include at least one positive control and one negative control. To ensure sufficient volume for all reactions, prepare the Reaction Mixture for n+1 reactions.

Table 1. Reaction Mixture

REAGENT	For 1 Reaction
HPV Mixture	37.5 μ l
50 mM MgCl ₂ Solution	1.5 μ l
Biotools DNA Polymerase (1U/ μ l)	1 μ l

2.- Aliquot **40 μ l** of the **Reaction Mixture** in each amplification vial.

Remove vials from laminar flow cabinet and proceed to DNA Purification Area. Never introduce DNA from samples or positive controls in the laminar flow cabinet at the reagent preparation area. The amplification reaction must start in the next 10 minutes after adding purified DNA from samples and controls to the reaction mixture.

3.- Add **50-100 ng DNA** from the purified samples to each reaction vial. **Complete up to 50 μ l final volume with sterile bidistilled water.**

NOTE

Clinical samples, depending on their nature and viraemia levels, may have different DNA concentrations, and therefore, amount of template to be added to the amplification reaction is expressed in ng rather than indicating sample volume. Quantity and purity of template must be calculated, e.g. by measuring A_{260/280} values.

4.- **Positive Controls** should be prepared by adding **5 μ l of Positive Control vial** (provided by the Kit) + 5 μ l of sterile bidistilled water to an amplification vial with 40 μ l of reaction mixture. **Negative Controls** should be prepared adding **10 μ l of sterile bidistilled water** to an amplification vial with 40 μ l of reaction mixture.

Proceed to the Amplification Area

5.- Close amplification vials. Place them in thermal cycler. Store remaining of Positive Control, HPV Mixture, MgCl₂ and Biotools DNA Polymerase at -15 \pm 8°C.

Perform the amplification according to the following program:

Amplification Program	
INITIAL DENATURING	94°C / 5 min
CYCLIC AMPLIFICATION	94°C / 30 sec
	55°C / 1 min
	72°C / 1 min
NUMBER OF CYCLES	35
FINAL ELONGATION	72°C / 10 min
	4 °C / ∞

NOTE

This protocol has been adapted for Eppendorf, MJ Research and Applied Biosystems GeneAmp™ thermal cyclers. For other thermal cyclers, optimisation of reaction parameters may be necessary. For any question, please contact our Technical Dpt. (info@biotools.eu).

6.- Once the amplification has taken place, the result may be observed by agarose electrophoresis. However, this will result in loss of sample for the following RFLP step. Store amplified reactions at 2-8 °C. Store at room temperature only if restriction analysis is performed within 1-2 hours.

Proceed to the Reagent Preparation Area

7.- For RFLP analysis, perform **five enzymatic digestions** for each amplification. Prepare each Digestion mixture following the indications below:

Digestion A: 1 µl Enzyme A + 1 µl Buffer A
Digestion B: 1 µl Enzyme B + 1 µl Buffer B
Digestion C: 1 µl Enzyme C + 1 µl Buffer C
Digestion D: 1 µl Enzyme D + 1 µl Buffer D/E
Digestion E: 1 µl Enzyme E + 1 µl Buffer D/E

Proceed to a DNA Area

8.- Add **4-5 µl amplification product** to each digestion mixture depending on the band intensity. Final digestion volume must not exceed 10 µl, **complete final volume with sterile bidistilled water. Digest for 1 hour at 37 °C.** Incubation period can be extended up to sixteen hours.

9.- Store the digested samples at room temperature if electrophoresis is to be performed in 1-2 hours. For further storage, store them at 2-8 °C.

10.- Store remaining of Enzymes and Buffers vials at -15±8°C.

* Optative: for the intra-tube control add to a reaction mixture (40 µl) containing purified DNA from the sample (5 µl) an aliquot of the positive control (5 µl). After amplification the analysed sample must render two bands of approximately 450 bp and 250 bp. Negative results in samples including the positive control indicate the presence of inhibitors, therefore DNA must be re-purified or another aliquot of the original sample must be processed. It is recommended to check the suitability of the purified DNA for amplification reactions.

6. INTERPRETATION OF RESULTS

A) Analysis of the Amplification Products

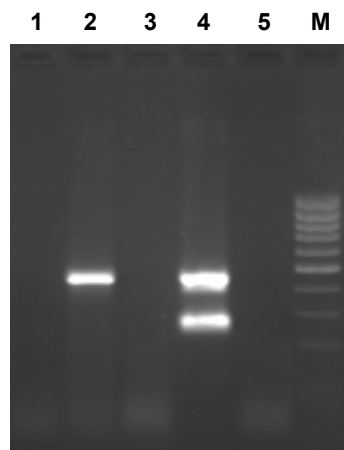
The analysis of amplification products is performed by horizontal electrophoresis in low EEO-agarose gels (e.g. MB Agarose, Ref. 20.011). Band visualisation is improved in 1.5-2 % gels using TAE 1X or TBE 0.5X as running buffers. It is recommended to add ethidium bromide in the agarose gel for a better resolution and visualisation.

NOTE

Ethidium bromide is a highly mutagenic intercalating agent. Use of gloves and maximum caution is recommended on handling this reagent.

After amplification, samples containing generic HPV sequences will render a band of approximately 450 bp, while samples containing oncogenic HPV genotypes will also render a band of approximately 250 bp (see Figure 1). In some cases, for oncogenic genotypes, only the 250 bp band will appear, with no 450 bp band. This does not interfere in the result. Nevertheless, should this occur, annealing temperature is to be decreased at 47 °C for obtaining of the 450 bp.

Figure 1: Detection of HPV with BIOTYPAP Kit. Lane 1 and Lane 3: samples from healthy patients; Lane 2: patient infected with a generic HPV; Lane 4: patient infected with an oncogenic HPV; Lane 5: negative control; M: 100 bp Ladder (Ref. 31.006).



B) Standard RFLP Analysis for Oncogenic Genotypes

For restriction analysis, the products of each digestion are analysed by gel electrophoresis in 2-2.5% (W:V) agarose gels stained with ethidium bromide. Restriction patterns for each genotype are shown on the following tables. In the case of coinfections or virus mutations the result interpretation can be difficult. Use Tables 2 and 3 for a standard RFLP analysis, only if a detailed analysis is desired see Tables 4, 5, 6 and 7.

Table 2. Restriction pattern for the ≈ 250 bp fragment of the oncogenic genotypes.

	HPV 16 238 bp	HPV 18 268 bp	HPV 31 232 bp	HPV 33 244 bp	HPV 35 232 bp	HPV 52 231 bp	HPV 58 244 bp	HPV 67 240 bp
Digestion A	NC	NC	NC	NC	NC	NC	126 , 118	NC
Digestion B	157 , 81	172 , 96	NC	136 , 138	NC	NC	NC	NC
Digestion C	NC	NC	NC	NC	NC	176 , 55	NC	NC
Digestion D	NC	NC	NC	NC	186 , 46	NC	NC	NC
Digestion E	NC	NC	117 , 115	NC	NC	NC	NC	NC

NC= No restriction sites for the specified enzymes

Table 3. Restriction pattern obtained from the ≈ 450 bp fragment after Digestion E. Oncogenic genotypes are marked in bold.

Genotype	Band Pattern (bp)	Genotype	Band Pattern (bp)
HPV 6	161, 149, 72, 67	HPV 51	380, 72
HPV 11	216, 135, 72, 26	HPV 52	449
HPV 13	175, 135, 73, 72	HPV 53	449
HPV 16	310, 72, 70	HPV 54	138, 125, 117, 72
HPV 18	135, 125, 85, 72, 38	HPV 55	165, 161, 72, 57
HPV 30	449	HPV 56	310, 72, 49, 18
HPV 31	380, 72	HPV 57	449
HPV 32	216, 161, 72	HPV 58	306, 111, 32
HPV 33	236, 102, 72, 39	HPV 59	452
HPV 34	186, 161, 96, 15	HPV 61	185, 180, 72, 18
HPV 35	177, 161, 72, 42	HPV 62	359, 72, 18
HPV 39	260, 123, 72	HPV 64	186, 161, 72, 39
HPV 40	365, 90	HPV 66	449
HPV 42	242, 135, 72	HPV 67	310, 72, 67
HPV 43	338, 72, 45	HPV 68	260, 85, 72, 38
HPV 44	222, 161, 72	HPV 69	365, 72, 18
HPV 45	338, 72, 45		

C) Detailed RFLP Analysis for the ≈ 450 bp Fragment

Table 4. Restriction pattern obtained from the ≈ 450 bp fragment after Digestion A. Oncogenic genotypes are marked in bold.

Genotype	Band Pattern (bp)	Genotype	Band Pattern (bp)
HPV 6	449	HPV 51	452
HPV 11	449	HPV 52	449
HPV 13	455	HPV 53	108, 341
HPV 16	183, 269	HPV 54	452
HPV 18	455	HPV 55	455
HPV 30	449	HPV 56	449
HPV 31	452	HPV 57	449
HPV 32	120, 329	HPV 58	449
HPV 33	449	HPV 59	118, 337
HPV 34	52, 406	HPV 61	455
HPV 35	452	HPV 62	32, 417
HPV 39	154, 301	HPV 64	52, 406
HPV 40	55, 400	HPV 66	449
HPV 42	449	HPV 67	55, 394
HPV 43	68, 387	HPV 68	455
HPV 44	114, 341	HPV 69	455
HPV 45	149, 306		

Table 5. Restriction pattern obtained from the \approx 450 bp fragment after Digestion B. Oncogenic genotypes are marked in bold.

Genotype	Band Pattern (bp)	Genotype	Band Pattern (bp)
HPV 6	449	HPV 51	452
HPV 11	449	HPV 52	449
HPV 13	455	HPV 53	83, 366
HPV 16	452	HPV 54	452
HPV 18	455	HPV 55	83, 372
HPV 30	83, 366	HPV 56	83, 366
HPV 31	452	HPV 57	20, 429
HPV 32	449	HPV 58	449
HPV 33	449	HPV 59	83, 372
HPV 34	83, 375	HPV 61	455
HPV 35	452	HPV 62	83, 126, 240
HPV 39	455	HPV 64	458
HPV 40	455	HPV 66	449
HPV 42	449	HPV 67	449
HPV 43	455	HPV 68	20, 435
HPV 44	83, 372	HPV 69	455
HPV 45	455		

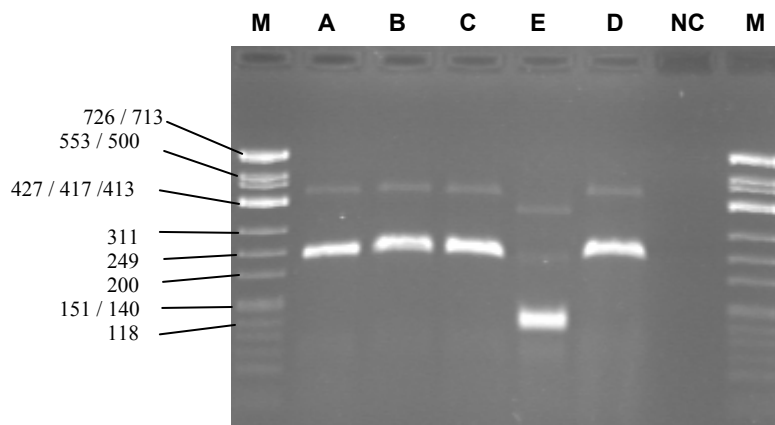
Table 6. Restriction pattern obtained from the \approx 450 bp fragment after Digestion C. Oncogenic genotypes are marked in bold.

Genotype	Band Pattern (bp)	Genotype	Band Pattern (bp)
HPV 6	449	HPV 51	452
HPV 11	449	HPV 52	449
HPV 13	455	HPV 53	449
HPV 16	452	HPV 54	452
HPV 18	455	HPV 55	50, 405
HPV 30	449	HPV 56	449
HPV 31	452	HPV 57	449
HPV 32	449	HPV 58	26, 423
HPV 33	449	HPV 59	24, 26, 405
HPV 34	458	HPV 61	455
HPV 35	452	HPV 62	449
HPV 39	455	HPV 64	458
HPV 40	455	HPV 66	449
HPV 42	449	HPV 67	449
HPV 43	455	HPV 68	455
HPV 44	455	HPV 69	455
HPV 45	455		

Table 7. Restriction pattern obtained from the ≈ 450 bp fragment after Digestion D. Oncogenic genotypes are marked in bold.

Genotype	Band Pattern (bp)	Genotype	Band Pattern (bp)
HPV 6	449	HPV 51	452
HPV 11	449	HPV 52	449
HPV 13	455	HPV 53	449
HPV 16	452	HPV 54	452
HPV 18	455	HPV 55	455
HPV 30	449	HPV 56	449
HPV 31	452	HPV 57	205, 244
HPV 32	449	HPV 58	449
HPV 33	449	HPV 59	455
HPV 34	458	HPV 61	455
HPV 35	452	HPV 62	129, 320
HPV 39	455	HPV 64	175, 283
HPV 40	455	HPV 66	449
HPV 42	449	HPV 67	449
HPV 43	455	HPV 68	455
HPV 44	455	HPV 69	455
HPV 45	455		

Figure 2. Restriction analysis of Lane 4 from Figure 1 (patient infected with an oncogenic HPV). Lane A: Digestion A pattern, Lane B: Digestion B pattern, Lane C: Digestion C pattern, Lane E: Digestion E pattern, Lane D: Digestion D pattern, Lane NC: Negative Control, Lane M: Molecular Marker ϕ X174/HinI (Ref. 31.001).



The infecting genotype in this case was HPV 31 since the ≈ 250 bp band in Digestion E renders two overlaying fragments of 117 bp and 115 bp. The ≈ 450 bp band was also digested rendering two fragments of 380 bp and 72 bp.

7. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipments, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, specially for thermal cyclers and pipettes. Maintenance and calibration of instruments allows its correct functioning, and helps detecting problems that may render an incorrect analysis result.

Pre-amplification area

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions)
- Timer
- Automatic pipettes⁸ (10, 20 and 200 µl), filter or positive displacement tips, RNase-free⁹
- Disposable examination gloves, powder-free
- Sterile bidistilled water
- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls.
- Racks for 1.5 ml vials
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor¹⁰ or equivalent, in order to remove DNA from working surfaces

Amplification area

- Thermal cycler: Eppendorf MasterCycler™ Personal, MJ Research MiniCycler™ or Applied Biosystems GeneAmp™ 2700. Use of this kit in other equipments has not been tested. For further information, contact our Technical Dpt. (info@biotools.eu).
- Laminar flow cabinet
- Racks for reaction vials
- Reaction vials (0.2 ml, thin-walled)
- Sterile bidistilled water (Ref. 20.033 or equivalent)
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor or equivalent, in order to remove DNA from working surfaces

Post-amplification area

- Water or dry bath for incubation of restriction digestion mixtures
- Electrophoresis power supplies and tanks
- Gel Documentation system
- UV transilluminator
- Ethidium bromide
- Low EEO agarose (Ref. 20.011) or equivalent
- TAE or TBE
- DNA Ladder ranging between 150 to 700 bp (Ref. 31.006 and Ref. 31.001) or equivalent
- Electrophoresis loading buffer
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Protective mask / goggles for UV
- Microwave

8. WARNINGS AND PRECAUTIONS

Following is a list of warning and precautions. For more information, we recommend to read the Material Safety Data Sheet (MSDS), available in our webpage (www.biotools.eu), or by request to our Technical Dpt. (info@biotools.eu).

A. Research Use Only.

⁸ Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, calibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.

⁹ It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification, post-amplification), in order to avoid contaminations that may render false positive results.

¹⁰ Available in Biotools' catalogue (Ref. 22.001).

- B. This test must be used with cervix samples collected, handled and stored as indicated in the corresponding chapter. Efficiency of the test in other samples has not been tested.
- C. The kit detects the following genotypes: 6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68 and 69. Detection of other genotypes (including those that may be considered as oncogenic now or in the future) has not been tested. The genotype identity is as indicated in the corresponding tables. Some of the indicated genotypes may not be differentiable by this kit, as per the information contained in the tables. For mixed infections, and due to the intrinsic nature of RFLP experiments, bands may overlap and therefore difficult interpretation of results.
- D. Handle all samples and discarded material as infectious or potentially infectious.
- E. Use powder-free examination gloves while handling reagents or samples, as well as lab coat. Wash hands thoroughly after performing the test.
- F. Open and close reagent vials carefully. Observe temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
- G. All materials used with the kit, including reagents and samples, must be discarded as to inactivate all possible infectious agents
 - 1. **Solids:** autoclave
 - 2. **Liquids:** add sodium hypochloride¹¹ at a final concentration of 1 %, and incubate 30 minutes at room temperature before discarding any material.
- H. Spills: wash spills with a 5 % solution of sodium hypochloride. Cover surface with absorbent material, saturated with a 5 % solution of sodium hypochloride. Let at least for 10 minutes. In order to avoid fume exposure, a plastic or glass cover can be used. All materials used for washing spills must be treated as infectious or potentially infectious material.
- I. Do not use product after expiry or best before date.
- J. Kit components have been tested as a whole. **Do not interchange components** with other kits, or components from different lots.
- K. Nucleic acids are very sensitive to degradation by nucleases. Nucleases are present in human skin and surfaces that have been in contact with humans. Wash with Termi-DNA-Tor and cover working surfaces with suitable paper. Use powder-free examination gloves throughout the whole process
- L. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes.
- M. Do not pipette by mouth.
- N. Packaging material included with the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of kit contents.
- O. Plastic material included with the kit is resistant in the normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, impossibility to use the kit.
- P. Kit reagents, once used, must be discarded. Reagents cannot be reused once they have been used for the analysis of clinical samples, as this may cause false positive or false negative results.
- Q. Laboratory workflow must be unidirectional, from pre-amplification area to amplification area. Specific equipment for each working area must be used, in order to avoid cross contaminations. Equipment used for amplification must remain in this area at all times.

9. STORAGE AND HANDLING INSTRUCTIONS

1. After reception, store the different reagents at the indicated temperatures (see previous chapter). Use non frost-free freezers. Also, for frequent use (more than 1 time a week), aliquot the contents of the Positive Control vial in different tubes, in order to avoid repeated freeze/thaw cycles.
2. Do not use the kit after expiry date. The closed kit is stable until the indicated date, if storage instructions are correctly followed. Do not mix reagents from other kits and/or other lots. If trace amounts of reagents remain, they must be discarded.

10. SAMPLE COLLECTION, TRANSPORT AND STORAGE

The different cervix samples recommended for use with the BIOTYPAP Kit are indicated at the beginning of this Manual. Samples taken with other methods or transported according to different specifications have not been tested for use with the kit. Cervix samples must be collected before applying acetic acid or iodinated if colposcopic examination is to be performed.

Cervical brushes and swabs

Samples may be stored up to three days at room temperature (maximum 25 °C), and shipped without refrigeration to the clinical analysis laboratory. For prolonged storages, we recommend temperatures of

¹¹ Commercial bleach usually contains sodium hypochlorite at a concentration of 5 %. Bleach can be used, after performing the necessary calculations in order to achieve the indicated concentration.

-15±8 °C. Once in the laboratory, they can be stored at 2-8 °C if analysis is to be performed in 48 hours. If the analysis is to be performed later, store samples at -15±8 °C. Samples must be kept away from heat sources and preserved of environmental humidity. Bacterial growth must be avoided and DNA integrity must be kept.

Cervical biopsies

Fresh biopsies of up to 5 mm must be used. Biopsy must be immediately placed in a preservative medium and stored at -15±8 °C. Biopsies can be shipped by express courier (24 hours maximum) at a maximum temperature of 25 °C, and stored at -15±8 °C in the laboratory until performance of the test. Biopsies with a diameter under 2 mm must not be used.

In order to avoid accidents or accidental opening of the sample container, it is recommended to seal its closure with Parafilm® or equivalent before freezing.

Sample shipment must comply with local, national and international regulations for transport of etiological agents.

11. QUALITY CONTROL

It is recommended that at least one (1) Positive Control and one (1) Negative Control be run each time the test is performed. As with any new laboratory procedure, novel users should consider performing additional controls (both positive and negative) until a high degree of confidence is reached.

The Positive Control must render two amplification bands (450 bp and 250 bp). Vials containing negative control (sterile bidistilled water) must render no bands. Any analysis not fulfilling any of these results must be completely invalidated and discarded. It is necessary to repeat the process from its beginning, including DNA purification, processing other aliquot of the original sample. A failure of instruments during the test, indicated by error messages, also means that the test has not been valid. Repeat all the procedure for each sample from the amplification step.

For laboratories requiring an external control, this must contain a defined number of copies of the target sequence for HPV, and the viraemia levels in this control must be a multiply of the limit value of the testing procedure.

12. PROCEDURAL PRECAUTIONS

1. Laboratory workflow must be unidirectional, from pre-amplification area to post-amplification area. Pre-amplification tasks must be initiated with the preparation of the reagents and sample purification. Equipments, materials and reagents must be dedicated and they must not be used for other activities or be transferred from one to another area. Gloves must be worn in each area, and must be discarded before proceeding to the next area. Equipments and materials used for setting-up of reactions must not be used for other activities, or for pipetting or processing amplified DNA or other DNA sources.
2. As with any analytical procedure, it is fundamental to use a good laboratory practice to obtain good results with this technique. Due to the high analytical sensitivity of the test, extreme care must be taken in order to keep the purity of all kit reagents and all reaction mixes. All reagents must be carefully checked in order to ascertain their purity. Discard all suspect reagents.

13. PROCEDURAL LIMITATIONS

1. Research Use Only.
2. Instructions must be followed in order to obtain correct results. Should the user have any questions, please contact our Technical Dpt. (info@biotools.eu).
3. This test has been validated for use with the reagents provided by the kit. The use of other amplification methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for validating the modifications for this test, in any of the indicated parameters.
4. This test has been validated with samples collected and shipped as per the "Sample Collection" chapter. Any modification has not been validated, and therefore, obtained results may not be correct.

5. Detection of HPV DNA depends on the number of viral particles present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.
6. False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives.
7. Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.
8. Use of this product is limited to qualified professional personnel, experienced in DNA purification and DNA amplification techniques.
9. It is important to pipette the indicated amounts, and mix well after each reagent addition. Check pipettes regularly.

14. WARRANTY

Products are guaranteed to conform to the quality and content indicated on each vial and external labels during their shelf life. BIOTOOLS obligation and purchaser's rights under this warranty are limited to the replacement by BIOTOOLS of any product that is shown defective in fabrication, and that must be returned to BIOTOOLS, freight prepaid, or at BIOTOOLS' option, replacement of the purchasing price.

Any complaint on damaged goods during transport must be directed to the handling or transport agent.

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