INNO-LiPA HPV Genotyping *Extra* II Amp

IVD CE

研究用

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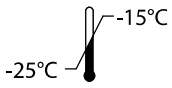
Symbols used

	Manufacturer
IVD	*In vitro* diagnostic medical device
LOT	Batch code
REF	Catalogue number
	Use by
	Consult instructions for use
	Temperature limitation
AMP KIT	Amplification kit
MASTER MIX	Master mix
CONTROL +	Positive control

Intended use

The INNO-LiPA HPV Genotyping *Extra* II Amp kit, for *in vitro* diagnostic use, is designed to amplify part of the L1 region of the human papillomavirus (HPV) by using the polymerase chain reaction (PCR).

Test principle

Amplification of a broad spectrum of HPV genotypes necessitates the use of consensus primers targeting a region in the HPV genome conserved between different genotypes. The most conserved region in the HPV genome is the L1 region, and several consensus PCR primer sets have been described in this region (Molijn et al. 2005). Examples are the GP5+/6+ (Jacobs et al. 1997), MY09/11 (Hildesheim et al.1994) and PGMY (Gravitt et al. 2000) primer sets.
The primer set used in the INNO-LiPA HPV Genotyping *Extra* II Amp amplifies a 65-bp region in the L1 open reading frame (Kleter et al. 1998) and has the potential to amplify at least 54 HPV types (Safaeian et al. 2007). This primer set is an upgrade version of the “SPF10” primer set. This application is protected by Fujirebio Europe N.V. EP patent 1012348B, US patent 6,482,588B and foreign equivalents.

PCR amplification is performed in a reagent mixture containing an excess of deoxynucleoside 5'-triphosphates (dNTPs) including deoxyuridine triphosphate, biotinylated primers, thermostable DNA polymerase and uracil-N-glycosylase (UNG). An incubation step prior to the amplification removes uracil bases from any contaminating amplification products present in the reaction mixture. The UNG enzyme is inactivated when the temperature is increased during the following denaturation step at 94°C. The sample mixture is heated in order to separate the two strands of the DNA helix (denaturation) and expose the target sequences to the primers. These primers are complementary to the regions flanking the target. In this way, two exact biotinylated copies of the template sequence are produced after one cycle of denaturation, annealing, and extension.
After 40 cycles, a multi-amplified biotinylated target sequence is obtained.

Reagents

Description, preparation for use, and recommended storage conditions

- If kept at -20°C, opened or unopened, and stored in the original vials, the reagents are stable until the expiry date of the kit. Do not use the reagents beyond the expiry date.
- The reagents should be stored isolated from any source of contaminating DNA, especially amplified products.
- To prevent contamination, store the Positive Control separately from amplification reagents and amplified material.
- Thaw the ready-to-use Master Mix by leaving it at room temperature. After thawing, put it immediately in a cooling bloc or on ice. Spin down this vial before use.
- Alterations in physical appearance of the kit reagents may indicate instability or deterioration.

Reagents supplied:

Component	Quantity	Ref.	Description
Master mix	1x 1 mL	60587	Contains biotinylated primers in buffer with dNTP/dUTP mix, $MgCl_2$, AmpliTaq Gold 360 DNA polymerase, uracil-N-glycosylase, and 0.05% NaN_3 as preservative.
Positive control	1x 0.05 mL	59732	PCR control contains HPV6 DNA and HLA-DPB1 DNA, and 0.05% NaN_3 as preservative.

Materials required but not provided

- QIAamp MinElute Media Kit, QIAGEN, cat. No. 57414
- Disposable gloves
- Disposable aerosol-resistant DNA/Dnase-free pipette tips
- DNA/DNase free microtubes
- DNA*Zap* (Ambion, Cat. No. 9890)
- Microtube racks
- Microtube centrifuge
- Vortex mixer or equivalent
- Heating block
- DNA thermal cycler and equipment
- Pipettes adjustable to deliver 1 - 20 µL, 20 - 200 µL, and 200 - 1000 µL
- Mineral oil, silicone grease (if required)
- Ethanol (96-100%)
- DNA/DNase-free deionized/distilled water (PCR grade)

Safety and environment

Please refer to the Safety Data Sheet (SDS) and product labeling for information on potentially hazardous components. The most recent SDS version is available on the website www.fujirebio-europe.com.

- Only adequately trained personnel should be permitted to perform the test procedure.
- Specimens should always be handled as potentially infectious.
- Use of personal protective equipment is necessary. Wear gloves and safety spectacles when manipulating dangerous or infectious agents.
- Waste should be handled according to the institution's waste disposal guidelines. Also observe (inter)national, regional and local environmental regulations.
- Master mix and positive control contain sodium azide as preservative.
  To prevent the formation of very toxic gas, avoid contact of sodium azide with acids.
  To prevent the formation of explosive lead or copper azide in plumbing, thoroughly flush drains with water after disposal of solutions containing sodium azide.

Specimen collection and DNA extraction

Collection of the sample, transport and subsequent DNA extraction are not part of the INNO-LiPA HPV Genotyping *Extra* II Amp kit.

The INNO-LiPA HPV Genotyping *Extra* II Amp kit has been validated using cervical cells collected in Surepath medium or PreserveCyt medium, followed by a QIAamp MinElute Media Kit (QIAGEN) adapted extraction procedure as described below. Other standard protocols for cervical cell sampling in collection media (e.g. alcohol-containing solutions) combined with HPV DNA extraction using any other commercially available kits can be used but require in-house validation.

Preparation of cervical cells

- Loosen the cells from the brush by vortexing or rigorous mixing for 15 seconds.
- Transfer 1 mL of cervical cell suspension to a microtube, avoiding cross-contamination between samples.

- Spin the vials at approximately 13000 rpm for 15 seconds.
- Discard the supernatant using a clean fine-tipped, disposable pipette for each reaction vial. Recap each vial.
- Add 1 mL of distilled water and vortex briefly to resuspend the cells.
  NOTE: resuspension of the cell pellet should be done in the same volume as the cervical cell suspension.

DNA isolation using QIAamp MinElute Media Kit (QIAGEN)

The protocol of the QIAamp MinElute Media Kit (QIAGEN) was modified in order to make it more user friendly. A ¾ ratio of the volumes used in the standard protocol of the QIAamp MinElute Media Kit, was applied in this protocol:

1. Pipet 50 µL of Buffer ATL into a 2 mL microcentrifuge tube (not provided).
2. Add 150 µL of sample into the 2 mL microcentrifuge tube.
3. Add 15 µL QIAGEN proteinase K. Close the lid and mix by pulse-vortexing for 10 s.
4. Incubate at 56ºC for 30 min. Shake the samples to ensure high nucleic acid yields. For optimal results, use a thermomixer at 900 rpm. If using a heating block, vortex the samples occasionally throughout the incubation period.
5. Briefly centrifuge the 2 mL tube to remove drops from the inside of the lid.
6. Add 150 µL of Buffer AL (containing 10 µg/mL of carrier RNA). Close the lid and mix by pulse-vortexing for 10 s.
   To ensure efficient lysis, it is essential that the sample, Buffer ATL, QIAGEN proteinase K, and Buffer AL are mixed thoroughly to yield a homogeneous solution.
   A white precipitate may form when Buffer AL is added to Buffer ATL.
   The precipitate does not interfere with the procedure and will dissolve during the incubation in step 7.
7. Incubate at 70ºC for 15 min. Shake the samples to ensure high nucleic acid yields. For optimal results, use a thermomixer at 900 rpm. If using a heating block, vortex the samples occasionally throughout the incubation period.
8. Briefly centrifuge the 2 mL tube to remove drops from the inside of the lid.
9. Add 200 µL of ethanol (96–100%) to the sample. Close the lid and mix thoroughly by pulse-vortexing for 15 s. Incubate the lysate with the ethanol for 5 min at room temperature (15–25ºC).
   NOTE: If ambient temperature exceeds 25ºC, ethanol should be cooled on ice before adding to the lysate.
10. Briefly centrifuge the 2 mL tube to remove drops from the inside of the lid.
11. Insert a QIAamp MinElute column into a clean 2 mL collection tube.
12. Carefully pipet all of the lysate from step 10 into the QIAamp MinElute column, close the lid, and centrifuge at 1 min at 8000 rpm. Place the QIAamp MinElute column in a clean 2 mL collection tube and discard the collection tube containing the filtrate.
13. Carefully open the QIAamp MinElute column and add 500 µL of Buffer AW2 into the QIAamp MinElute column without wetting the rim. Close the cap and centrifuge 1 min at 8000 rpm. Discard the collection tube containing the filtrate.
14. Apply 500 µL of ethanol (96–100%) into the QIAamp MinElute column. Close the cap and centrifuge 1 min at 8000 rpm.
15. Discard the collection tube containing the filtrate.

16. Place the QIAamp MinElute column in a clean 2 mL collection tube and centrifuge at full speed (20000 x *g*, 14000 rpm) for 3 min to dry the membrane completely.
17. Place the QIAamp MinElute column in a clean 1.5 mL collection tube (provided) and discard the 2 mL collection tube with the filtrate. Open the lid of the QIAamp MinElute column, and incubate the column at room temperature (15–25ºC) for 15 min.
    NOTE: Alternatively, for faster incubation, heat the opened QIAamp MinElute column at 56ºC for 3 min.
18. Apply 80 µL of Buffer AVE to the center of the membrane in the QIAamp MinElute column. Close the lid and incubate at room temperature (15–25ºC) for 1 min. Centrifuge at full speed (20000 x *g*, 14000 rpm) for 1 min. Important: Ensure that Buffer AVE is already equilibrated to room temperature. Incubating the QIAamp MinElute column loaded with Buffer AVE for 5 min at room temperature before centrifugation generally increases yield.

Table 1. Volumes of Buffer AL and Carrier RNA/Buffer AVE Mix Required for the QIAamp MinElute Media Procedure

N° samples	Vol. Buffer AL (mL)	Vol. Carrier RNA/AVE (µL)
1	0.2	2
2	0.4	4
3	0.6	6
4	0.8	8
5	1	10
6	1.2	12
7	1.4	14
8	1.6	16
9	1.8	18
10	2	20
11	2.2	22
12	2.4	24
13	2.6	26
14	2.8	28
15	3	30
16	3.2	32
17	3.4	34
18	3.6	36
19	3.8	38
20	4	40
21	4.2	42
22	4.4	44
23	4.6	46
24	4.8	48

Store DNA at -20°C or proceed with the amplification.
Automated test procedure is available. Contact Fujirebio Europe N.V. or your local distributor for further details.

Remarks and precautions

- In order to avoid DNA contamination, a maximum physical separation between the pre- and post-amplification steps is recommended: separate rooms, separate

pipettes and other lab material, separate lab coats and gloves (and their stock) are minimum precautions for prevention of contamination and part of good laboratory practice. The reagents should be isolated from any source of contaminating DNA, especially amplified DNA products. Also avoid microbial contamination of reagents.
- Avoid any return of materials from the post-amplification room to the pre-amplification room.
- All pipette tips and tubes used for the amplification process should be autoclaved. Aerosol-resistant pipette tips are recommended. Use a new DNA/DNase-free pipette tip for each aliquoted specimen.
- The reagents for amplification processes should be handled in a room free of DNA.
- After thawing, shake Master Mix by inverting the vial three times, vortex positive control, and spin down all reagents.

Test procedure

NOTE:
- This protocol was designed for optimal amplification in 0.2 mL PCR tubes in GeneAmp PCR System 9700 thermal cyclers.
- This protocol can be used for most commercial types of thermal cyclers, but may require some modifications indicated by the manufacturer of the cycler.
- Prior to use, determine whether the protocol is compatible with the thermal cycler in use at your laboratory.
- Ensure the thermal cycler is calibrated prior to use.

PCR mix preparation

It is very important to use the correct amount of each component. Too much or too little sample or reagents could result in aspecific amplification or even in no amplification at all.

IMPORTANT NOTE: Prepare the PCR mix on ice and avoid unnecessary delays in the setup of the run.

1. Determine the number of vials to be prepared (N) as:
   N = number of DNA samples + 1 (negative control; no DNA) + 1 (CONTROL +)
2. Volume of the master mix needed for 1 sample: 40.0 µL master mix
3. Aliquot 40 µL of this master mix into DNA/DNase-free amplification tubes. Cover the PCR mix with mineral oil if required.
4. Pipette 10 µL of the extracted material into the PCR mix. Add 10 µL CONTROL + to the positive control tube. Add 10 µL of DNA/DNase-free distilled water to the negative control tube.
5. Place the samples into the preheated and calibrated thermal block (see manufacturer's instructions).
   Start the amplification program designed for the INNO-LiPA HPV Genotyping *Extra* II amplification.

PCR cycling

The correct temperature profile for the INNO-LiPA HPV Genotyping *Extra* II amplification should be selected.

INNO-LiPA HPV Genotyping *Extra* II PCR profile (cycler type: GeneAmp PCR System 9700):

	Step	Temp	Time	
1	Decontaminate	37°C	10 min	Degradation of uracil containing DNA
2	Denature	94°C	9 min	Inactivation of UNG and activation of the AmpliTaq Gold 360 DNA polymerase
3	Denature	94°C	30 sec	*Repeat cycle*
4	Anneal primers	52°C	45 sec	*steps 3 to 5*
5	Extend primers	72°C	45 sec	*40 times*
6	Hold at 72°C			Duration < 2 hrs

Remove tubes from the thermal cycler, store the amplicon immediately at -20° ± 5°C or proceed immediately to the INNO-LiPA HPV Genotyping *Extra* II.
NOTE: Do not store the amplified DNA products together with amplification reagents.

Results

Validation

- Include at least one positive and one negative control each time an amplification is performed. As with any new laboratory procedure, the inclusion of additional positive and negative controls should be considered until a high degree of confidence is reached in the ability to correctly perform the test procedure.
- If the inclusion of an additional positive control is desirable, use a known positive sample.

Limitations of the procedure

- Use of this product should be limited only to personnel well trained in the techniques of amplification.
- Powder from disposable gloves and sodium hypochlorite have an inhibiting effect on amplification.
- Strongly hemolyzed samples may inhibit the PCR amplification and result in false-negative results.
- No experiments were performed to test the effect of possible interfering substances.
- Repeated freezing/thawing of the DNA samples might result in less efficient amplification.
- Specific amplification depends on good laboratory practice and careful performance of the procedures as specified under **Remarks and precautions** and under **Recommendations on laboratory design and procedures**.

Test performance

See INNO-LiPA HPV Genotyping *Extra* II insert.

Recommendations on laboratory design and procedures

The following sequence of operations is recommended:
1. Preparation and aliquoting of PCR mixes.
2. Preparation of samples (DNA isolation).
3. Polymerase chain reaction.
4. Analysis of the biotinylated PCR products by reverse hybridization.

Personnel involved in steps 3 and 4 should not subsequently participate in work for steps 1 and 2 on the same day. Similarly, after being involved in step 2, do not subsequently participate in work for step 1 on the same day.

To prevent contamination (e.g., with amplimers) of specimens and to avoid false-positive results, the procedure should be performed in three physically separated rooms, each with its own set of supplies and pipettes. One room is necessary for reagent preparation, another for sample preparation, and a third room for amplification and amplimer detection. All equipment should be kept in the room where it is used and not be transferred between rooms.

Aerosol-resistant pipette tips should be used to prevent cross-contamination between specimens. For the same reason, wear disposable examination gloves and change them frequently.

Room 1 - storage and preparation of reagents

This room and its equipment must be kept **free of DNA**. This room is only to be used for preparing PCR reagents. The positive control should not be brought into Room 1. The personnel involved should wear a clean laboratory coat, which must not be worn outside this room. Wear disposable gloves when handling reagents.

Room 2 - sample preparation

This room and its equipment must be kept **free of amplimers**. The personnel involved in specimen processing should wear a clean laboratory coat, which must not be worn outside this room. During sample preparation, disposable examination gloves should be worn and changed frequently. Carefully uncap vials containing (processed) sample. Avoid opening more than one reaction vial containing sample at the same time.

To avoid contamination or to clean contaminated surfaces, it is recommended to clean pipettes and work surfaces with DNA*Zap* (Ambion). Be aware that the use of DNA*Zap* is only an additional precautionary measure, and the described recommendations on laboratory design and procedures should be followed as strictly as possible.

Room 3 - amplification and amplimer detection

The personnel involved in amplification and amplimer detection should wear a clean laboratory coat, which must not be worn outside this room and must be changed daily. Wear disposable examination gloves when working with amplimers.

Licenses

The purchase of this product allows the purchaser to use it for amplification of nucleic acid sequences for human *in vitro* diagnostics in accordance with the patented method described in the package insert. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

This product is sold under licensing arrangements between Fujirebio Europe N.V. and Invitrogen IP Holdings, Inc. The purchase price of this product includes limited, nontransferable rights under European Patent Numbers 0401037; 0522884; and 0415755 and corresponding foreign patents, other than United States patents, owned by Invitrogen Corporation to use only this amount of the product to practice the claims in said patents solely for activities of the purchaser in detection of human papilloma virus (HPV) within the field of human diagnostics. No other rights are conveyed.

Further information on purchasing licenses under the above patents may be obtained by contacting the Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008. Email: outlicensing@invitrogen.com.

Trademarks

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- GeneAmp is a registered trademarks of Applied Biosystems, LLC.
- QIAamp is a registered trademark of the QIAGEN Group.
- DNA*Zap* is a trademark of Ambion Inc., USA.
- PreservCyt is a registered trademark of Hologic Inc., USA.
- Surepath is a trademark of BD Diagnostics, USA.
- AmpliTaq Gold is a registered trademark of Roche Molecular Systems Inc., USA.

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Symbols used

Manufacturer

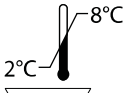
In Vitro Diagnostic Medical Device

Batch code

Catalogue number

Use By

Consult Instructions for Use



Temperature limitation

Contains sufficient for <n> tests

CONJ 100x	Conjugate 100x
CONJ DIL	Conjugate Diluent
DENAT SOLN	Denaturation Solution
HYBRIDIZ SOLN	Hybridization Solution
RINSE SOLN 5x	Rinse Solution 5x
STRIN WASH SOLN	Stringent Wash Solution
STRIPS	Strips
SUBS BCIP/NBT 100x	Substrate BCIP/NBT 100x
SUBS BUF	Substrate Buffer
DANGER	Danger
WARNING	Warning

Intended use

The INNO-LiPA HPV Genotyping *Extra* II is a line probe assay, for *in vitro* diagnostic use, designed for the qualitative detection and identification of genotypes of the human papillomavirus (HPV) by detection of specific sequences in the L1 region of the HPV genome. The following HPV genotypes are detected and identified:

- HPV HR* GT: 16, 18, 31,33, 35, 39, 45, 51, 52, 56, 58, 59, 68
- HPV pHR* GT: 26, 53, 66, 70, 73, 82
- HPV LR* or not classified GT: 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83, 89

(*) The designation of HR, pHR, or LR is for information use only, and is based on publications of Munoz *et*. *al*. (2003) and IARC Monographs Volume 100B (2012).

Test principle

The INNO-LiPA HPV Genotyping *Extra* II is based on the principle of reverse hybridization. Part of the L1 region (SPF10 region) of the human papillomavirus (HPV) genome is amplified by HPV specific primers, and the resulting biotinylated amplicons are then denatured and hybridized with specific oligonucleotide probes. An additional primer pair for the amplification of the human HLA-DPB1 gene is added to monitor sample quality and extraction. All probes are immobilized as parallel lines on membrane strips. After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added, which binds to any biotinylated hybrid previously formed.

Incubation with BCIP/NBT chromogen yields a purple precipitate and the results can be visually interpreted or by using the LiRAS for LiPA HPV software.

An amplification kit (INNO-LiPA HPV Genotyping *Extra* II Amp) is available for standardized preparation of biotinylated amplified material. This amplification kit is based on the polymerase chain reaction (PCR) using SPF10 based primers.
The application of SPF10 in the INNO-LiPA HPV Genotyping *Extra* II is protected by Fujirebio Europe N.V. EP patent 1012348B, US patent 6,482,588B and foreign equivalents.

Amplification products are subsequently hybridized using a single typing strip on which 32 sequence-specific DNA probe lines and 4 control lines are fixed (see Figure 1).

INNO-LiPA HPV Genotyping *Extra* II assay: steps involved

Step 1 Amplification of the extracted DNA.
Step 2 Hybridization of the amplified product on the strip, followed by stringent wash.
Step 3 Addition of conjugate and substrate, resulting in color development.
Step 4 Visual interpretation of the signal pattern or use of the LiRAS for LiPA HPV Software.

Reagents

Description, preparation for use, and recommended storage conditions

- If kept at 2 - 8°C, opened or unopened, and stored in the original vials, the reagents are stable until the expiry date of the kit. **Do not use the reagents beyond the expiry date. Do not freeze any of the reagents.**
- The reagents should be stored isolated from any source of contaminating DNA, especially amplified products.
- All reagents and the tube containing the strips should be brought to room temperature (20 - 25°C) approximately 60 minutes before use and should be returned to the refrigerator immediately after use.
- Alterations in physical appearance of the kit components may indicate instability or deterioration.
- To minimize the possibility that strips curl before use, it is recommended to store the tube horizontally.

Reagents supplied:

Component	Quantity	Ref.	Description
Strips	1x 20	60584	Containing 20 INNO-LiPA HPV Genotyping *Extra* II strips marked with a blue marker line.
Denaturation Solution	1x 1 mL	56718	Alkaline solution containing EDTA. This vial should be closed immediately after use; prolonged exposure of this solution to air leads to a rapid deterioration of the denaturing strength.
Hybridization Solution	1x 80 mL	57420	SSC buffer containing sodium lauryl sulfate (SLS) and 0.01% MIT/<0.1% CAA as preservative, to be prewarmed to a temperature of at least 37°C and not exceeding 49°C.
Stringent Wash Solution	1x 200 mL	57421	SSC buffer containing SLS and 0.01% MIT/ < 0.1% CAA as preservative, to be prewarmed to a temperature of at least 37°C and not exceeding 49°C.
Conjugate 100x	1x 0.8 mL	56952	Streptavidin labeled with alkaline phosphatase in Tris buffer containing protein stabilizers and 0.01% MIT/ <0.1% CAA as preservative. To be diluted 1/100 in Conjugate Diluent: prepare 2 mL Conjugate working solution for each test trough + 2 mL in excess for manual testing. The Conjugate working solution is stable for 8 hours at room temperature (20 -25°C) if stored in the dark.

Component	Quantity	Ref.	Description
Conjugate Diluent	1x 80 mL	56951	Phosphate buffer containing NaCl, Triton, protein stabilizers, and 0.01% MIT/<0.1% CAA as preservative.
Substrate BCIP/NBT 100x	1x 0.8 mL	56954	BCIP and NBT in DMF. To be diluted 1/100 in Substrate Buffer before use: prepare 2 mL Substrate working solution for each test trough + 2 mL in excess for manual testing. The Substrate working solution is stable for 8 hours at room temperature (20 - 25°C) if stored in the dark.
Substrate Buffer	1x 180 mL	56953	Tris buffer containing NaCl, $MgCl_2$, 0.01% MIT/ <0.1% CAA as preservative.
Rinse Solution 5x	1x 80 mL	56721	Phosphate buffer containing NaCl, Triton, and 0.05% MIT/0.48% CAA as preservative. To be diluted 1/5 in distilled or deionized water before use: prepare 8 mL Rinse working solution for each test trough + 10 mL in excess for manual testing. The Rinse working solution is stable for 2 weeks at 2 - 8°C.
Incubation trays	3	-	
Reading card	1	-	For identification of the positive probes
Data reporting sheet	1	-	For storage of developed strips.

Materials required but not provided

- INNO-LiPA HPV Genotyping *Extra* II Amp.
- Distilled or deionized water.
- Disposable gloves.
- Disposable DNA/Dnase-free pipette tips (aerosol resistant).
- Forceps for strip handling.
- Graduated cylinders (10, 25, 50, and 100 mL).
- Adjustable pipettes to deliver 1 - 20 µL, 20 - 200 µL, and 200 - 1000 µL.
- Vortex mixer or equivalent.
- Microcentrifuge.

Materials required for the manual procedure only:

- Water bath with shaking platform (80 rpm; with inclined lid; temperature adjustable to 49°C ± 0.5°C).
- Aspiration apparatus.
- Calibrated thermometer.
- Orbital, reciprocal, or rocking platform shaker.
- *Recommendations*

  For an orbital shaker:
  - The diameter of the circular motion should be equal to or greater than 13 mm.
  - Recommended speed for a 13 mm circular motion is 160 rpm.

  For a reciprocal shaker:
  - Recommended speed for the to-and-fro motion is 80 movements per minute.

  For a rocking platform shaker:
  - The shaking angle should not exceed 13° to avoid spilling of liquid.
  - Recommended speed is 50 rpm.

- Dispensing multipipette (Eppendorf, optional).
- Timer, 2 hours (± 1 minute).

Health and safety information

Please refer to the Safety Data Sheet (SDS) and product labeling for information on potentially hazardous components. The most recent SDS version is available on the website www.fujirebio-europe.com.

Danger DENAT SOLN

Contains sodium hydroxide

H314-P260-P280-P303+P361+P353-P305+P351+P338-P310-P363

Warning RINSE SOLN 5x

Contains 2-Chloroacetamide

H317-P280-P261-P362+P364-P333+P313-P302+P352

Danger SUBS BCIP/NBT 100x

Contains N,N-Dimethylformamide, 5,5'-diphenyl-3,3'-bis (4-nitrophenyl)-2,2'-(3,3'-dimethoxybiphenyl-4,4'-ylene)ditetrazolium dichloride and methanol

H312-H332-H319-H360D

P261-P280-P201-P303+P361+P353-P305+P351+P338-P308+P313

Hazard statements

H312	Harmful in contact with skin.
H314	Causes severe skin burns and eye damage.
H317	May cause an allergic skin reaction.
H319	Causes serious eye irritation.
H332	Harmful if inhaled.
H360D	May damage the unborn child.

Precautionary statements

P201	Obtain special instructions before use.
P260	Do not breathe mist/vapours/spray.
P261	Avoid breathing mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P303+P361+P353	IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P308+P313	If exposed or concerned: Get medical advice/attention.
P310	Immediately call a POISON CENTER/doctor/...
P333+P313	If skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before reuse.
P363	Wash contaminated clothing before reuse.

- Only adequately trained personnel should be permitted to perform the test procedure.
- Specimen should always be handled as potentially infectious. Therefore, all blood components and biological materials should be considered as being potentially infectious and should be handled as such. All blood components and biological materials should be disposed of in accordance with one of the following established safety procedures.
  - Autoclave for at least 15 minutes at 121°C.
  - Incinerate disposable material.
  - Mix liquid waste with sodium hypochlorite so that the final concentration is ± 1% sodium hypochlorite. Allow to stand overnight before disposal.
    Caution: Neutralize liquid waste that contains acid before adding sodium hypochlorite.
- Use of personal protective equipment is necessary: gloves and safety spectacles when manipulating dangerous or infectious agents.
- Waste should be handled according to the institution's waste disposal guidelines. Also observe all (inter)national, regional and local environmental regulations.

Specimens

Since the INNO-LiPA HPV Genotyping *Extra* II test utilizes biotinylated amplified DNA material as specimen, an amplification kit, INNO-LiPA HPV Genotyping *Extra* II Amp, is available as an accompanying tool.

Remarks and precautions

- For professional use only
- Do not mix reagents from different kits unless the components have identical lot numbers.
- Do not reuse disposable lab material.
- All vessels used to prepare conjugate and substrate solutions should be cleaned thoroughly and rinsed with distilled water.
- Avoid microbial contamination of reagents.
- Use a new DNA/DNase-free pipette tip for each aliquoted specimen. Sterile-packed, aerosol-resistant, disposable pipette tips are recommended.

Preparation and manipulation procedures

Strip handling

- The strips are designed to be used only once!
- Do not touch the strips with bare hands; use clean forceps.
- Use a **pencil** for identification of the test strips. Do not use ballpoints, etc. Write the ID above the marker line on the strips.
- Place the test strips in the troughs with their coated membrane side up (this side is marked).
- Test strips should always remain in the same trough throughout the different incubation steps.
- Unused or developed strips should be kept away from intense light and heat.
- Allow the developed strips to dry completely before interpretation, covering, and storing.

- Developed dry strips should be stored preferably in the dark at room temperature (20 - 25°C).
- Do not reuse the troughs.

Manual test procedure

Directions for incubation

- The hybridization and stringent wash incubations should be performed at **exactly 49°C** and are the most critical steps to avoid false-positive (temperature too low) or false-negative/very weak signals (temperature too high). A shaking water bath with **inclined** lid allows a good control of temperature variations. Strict temperature control (within 0.5°C from the set point of 49°C) with a calibrated thermometer is necessary.
- Always **close** the lid of the water bath during incubations in order to avoid false-positive signals.
- **Do not use a hot air shaker for the hybridization and stringent wash.**
- The amplitude of the motion generated by both the shaking water bath (hybridization and stringent wash procedure) and the orbital, reciprocal shaker or rocking platform (color development procedure) is critical in achieving maximum sensitivity and homogeneous staining. The amplitude should be as high as possible, so that both the liquid and the test strips move back and forth in the trough. However, spillage of liquid over the edges of the troughs must be avoided.
- For the hybridization and the stringent wash, the troughs should be placed on the shaking platform of the water bath. Adjust the water level to between 1/3 and 1/2 of the height of the trough. Make sure that the troughs do not float on the water. The water should be in direct contact with the troughs.
- Incubation steps for the color development should be performed at a temperature between 20 - 25°C. If the temperature is below 20°C, weaker results may be obtained. If the temperature is above 25°C, high background and/or false-positive signals may be obtained.
- The specified incubation times should be strictly respected in order to ensure correct performance of the assay.
- Do not cover the tray. During hybridization and stringent wash incubations, the troughs can be left uncovered in the water bath. Covering the troughs with microplate sealers may result in cross-contamination.

Directions for changing liquid in the troughs

- Aspirate the liquid from the trough with a pipette, preferably attached to a vacuum aspirator. Hold the tray at an angle to allow all liquid to flow to one end of the trough.
- Add 2 mL of the appropriate solution to each trough and follow the protocol.
- NOTE: A dispensing Multipette (Eppendorf) is useful for this purpose.
- Repeat this step as many times as indicated in the test protocol.
- NOTE:
  - Do not allow the strips to dry between the washing steps.
  - Make sure the surface of the strips is not damaged when aspirating. Preferably aspirate the liquid from the top of the strip above the marker line.

- Make sure the whole strip is thoroughly washed by complete submersion in the solution.
- Alter the speed of the shaker when necessary.

Hybridization

NOTE: Wear disposable gloves and use forceps.

1. Heat a shaking water bath to **exactly 49°C**. Check the temperature using a calibrated thermometer and adjust if necessary. Prewarm the Hybridization Solution and Stringent Wash solution to at least 37°C but do not exceed 49°C. Mix before use.
2. Using forceps, remove the required number of the test strips from the tube (1 strip per sample) and put an identification number above the marker line on the strip using a pencil.
3. Take the required number of test troughs (1 trough per strip) and place them in the tray.
4. Pipette 10 µL Denaturation Solution into the upper corner of each trough.
   NOTE: Close the vial immediately after use.
5. Add 10 µL amplified biotinylated product to the Denaturation Solution and carefully mix by pipetting up and down several times. Always use DNA/DNase-free aerosol-resistant pipette tips. Allow denaturation to proceed for 5 minutes at room temperature (20 - 25°C).
6. **Shake the prewarmed Hybridization Solution** and **gently** add 2 mL to the denatured amplified product in each trough. Take care not to contaminate neighbouring troughs during pipetting.
7. Immediately place the strip into the trough.
   The strips should be completely submerged in the solution.
8. Place the tray in the 49°C shaking water bath (approximately 80 rpm; see Directions for incubation), close the lid, and incubate for 60 minutes.
   NOTE: Avoid splashing water from the water bath into the trough. Adjust the water level to between 1/3 and 1/2 of the height of the trough.

Stringent wash

- After hybridization, remove the tray from the water bath.
- Hold the tray at a low angle and aspirate the liquid from the trough with a pipette, preferably attached to a vacuum aspirator. Add 2 mL prewarmed Stringent Wash solution to each trough and rinse by shaking the tray for 10 - 20 seconds at room temperature. Aspirate the solution from each trough.
- Repeat this washing step once (see also Directions for changing liquid in the troughs).
- Finally, aspirate the solution and incubate each strip in 2 mL prewarmed Stringent Wash solution in the shaking water bath at 49°C for 30 minutes. Close the lid of the water bath.
  NOTE: Prepare Rinse Solution and Conjugate during stringent wash incubation (see Reagents).

Color development

All subsequent incubations are carried out at **20 - 25°C on a shaker**.

1. Wash each strip twice for 1 minute using 2 mL diluted Rinse Solution (see Directions for changing liquid in the troughs). Aspirate.
2. Add 2 mL of Conjugate solution to each trough and incubate for 30 minutes while shaking. Aspirate.
   NOTE: Prepare Substrate solution about 10 minutes prior to the end of the conjugate incubation (see Reagents).
3. Wash each strip twice for 1 minute using 2 mL diluted Rinse Solution and wash once more using 2 mL Substrate Buffer. Aspirate.
4. Add 2 mL of Substrate solution to each trough and incubate for 30 minutes while shaking. Aspirate.
5. Stop the color development by washing the strips twice in 2 mL distilled water while shaking for at least 3 minutes.
6. Using forceps, remove the strips from the troughs and place them on absorbent paper. Let the strips dry completely and fix them to the data reporting sheet. The uppermost line is the marker line. The conjugate control line aids correct alignment of the strips on the data reporting sheet.

Automated test procedures

Instruments and associated protocols are available from Fujirebio Europe N.V. (see www.fujirebio-europe.com/automation).

Results

Reading

The strips should only be read when they are completely dry. All visible lines should be scored by using the INNO-LiPA HPV Genotyping *Extra* II Reading Card, by aligning the blue marker line of the strip towards the marker line indication on the Reading Card.

Figure 1 illustrates the position of the different oligonucleotide probes on the INNO-LiPA HPV Genotyping *Extra* II strip. A line is considered positive when a purple/brown band appears at the end of the test procedure.

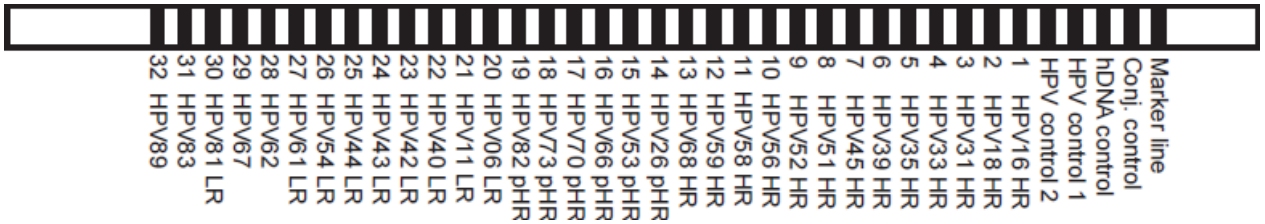


Figure 1: Location of the specific probes on the INNO-LiPA HPV Genotyping *Extra* II strip. A marker line is present at the top of the strip for orientation. This marker line is blue and can be differentiated from the INNO-LiPA HPV Genotyping *Extra* strip by this color difference.

Quality control

- The first line (immediately below the marker line) is the Conjugate Control line. This line controls for the addition of reactive Conjugate and Substrate solution during the detection procedure. It should always be positive and should have approximately the same intensity on each strip in the same test run. A negative result on the Conjugate Control line indicates an error in the color development of the assay. The test result should be considered as invalid and it is advised to repeat the LiPA assay.

- The second line is a human DNA control line. Primers amplifying a fragment of the human HLA-DPB1 gene are added to the HPV amplification kit to control the sample quality and extraction efficiency. This line should always be positive; however, if a high amount of HPV is present in the sample, it is possible that the human DNA control line is negative because of the competition between HPV and human DNA.
- Always include a positive run control: e.g., the positive amplification control included in the INNO-LiPA HPV Genotyping *Extra* II Amp kit. The positive control contains HPV6 and HLA-DPB1 and should react on the following lines: Conj Control, hDNA control, HPV control 1, and line 20 (HPV6). If a negative or deviating LiPA pattern is obtained for the positive control, the entire run should be discarded and the complete procedure should be repeated starting from PCR.
- Always include a negative run control: this should preferably be a negative sample that is processed simultaneously with the patient samples in the DNA extraction and PCR step. If a positive band is obtained on the LiPA for the negative control, (except for the hDNA control that must be positive in case of an HPV negative clinical sample) the entire run should be discarded and the complete procedure starting from the DNA extraction should be repeated.

Interpretation of the results

- A sample is considered HPV positive if at least one of the type-specific lines is positive, or if one of the HPV control lines is positive.
- Samples which don't generate any positive type-specific lines (lines 1 - 32) but have at least one HPV control line positive must be scored as HPV positive, but are untypeable ("HPVX").
- If more than one HPV genotype line is positive, a mixture of those HPV genotypes is present.
- A sample is considered HPV negative if the hDNA control line is positive and all HPV type-specific lines (including the HPV control lines) are negative.
- If none of the HPV type-specific lines are positive, and the hDNA control line is negative, the test result should be considered as invalid. This test result may indicate inadequate specimen collection, processing, or presence of inhibitors in the DNA extract. In the latter case, testing of a 1:10 dilution of the DNA extract can improve amplification performance. If not successful, start complete procedure from a new aliquot of the specimen.
- A positive signal on at least 7 out of 12 consecutive lines on strip should be interpreted as possibly an invalid result. It is advised to repeat the whole procedure starting from DNA extraction for this sample. Shall this occur a second time, this could be a true genotyping result for certain populations, such as immunocompromised individuals, with multiple infections*.
  (* As described by Wheeler *et. al.* 2006. Human Papillomavirus Genotypes and the Cumulative 2-Year Risk of Cervical Precancer. J Infect Dis. 2006;194:1291–1299).

Summarized strip interpretation results:

Table 1: Summarized strip interpretation results

cc line	hDNA Result	HPV Result	Interpretation
-	+ or -	+ or -	**Invalid Result** A negative result on the Conjugate Control line indicates an error in the color development of the assay. It is advised to repeat the LiPA assay.
+	–	–	**Valid result in case of a negative control sample** **Invalid result in case of a human sample** A negative result on the hDNA control line indicates inadequate specimen collection, processing, or presence of inhibitors in the DNA extract. In the latter case, testing of a 1:10 dilution of the DNA extract can improve amplification performance. If not successful, start procedure from a new aliquot of the specimen.
+	+	–	**HPV not detected** A negative result on both HPV control lines and type-specific lines indicates the absence of HPV DNA, but cannot preclude the presence of an HPV infection.
+	+ or -	+	**HPV detected** A positive result on at least one of the type-specific lines or HPV control lines indicates the presence of HPV DNA. The presence of additional HPV genotypes cannot be completely ruled out. If a high amount of HPV is present in the sample, it is possible that the human DNA control line is negative because of the competition between HPV and human DNA.
+	+ or -	≥ 7 out of 12 consecutive lines positive	**Not interpretable Result** A positive signal on at least 7 out of 12 consecutive lines on strip should be interpreted as possibly an invalid result. It is advised to repeat the whole procedure starting from DNA extraction for this sample. Shall this occur a second time, this could be a true genotyping result for certain populations, such as immunocompromised individuals, with multiple infections**.**

Interpretation software: LiRAS for LiPA HPV

The LiRAS for LiPA HPV software is designed to assist with the interpretation of the INNO-LiPA HPV Genotyping *Extra* II results. Please contact your local distributor to obtain the latest version. Always compare the strip pattern and patient information shown in the report with the original strip to ensure that the strip was identified, aligned, and scanned correctly.

Limitations of the procedure

- Mixed HPV genotype infections are common. The INNO-LiPA HPV Genotyping *Extra* II Amp kit uses a set of primers that amplify all genotypes simultaneously.
- Due to PCR competition and the absence of particular genotypes on strip, it is possible that certain genotypes present in the co-infected sample are not detected.
- If HPV52 is present, a weak non-specific reactivity on probe line HPV31 might be observed due to sequence homologies.

Recommendations on laboratory design and procedures

The following sequence of operations is recommended:
1. Preparation and aliquoting of PCR mixes.
2. Preparation of samples (DNA isolation).
3. Polymerase chain reaction.
4. Analysis of the biotinylated PCR products by reverse hybridization.

Personnel involved in steps 3 and 4 should not subsequently participate in work for steps 1 and 2 on the same day. Similarly, after being involved in step 2, do not subsequently participate in work for step 1 on the same day.

To prevent contamination (e.g., with amplimers) of specimens and to avoid false-positive results, the procedure should be performed in three physically separated rooms, each with its own set of supplies and pipettes. One room is necessary for reagent preparation, another for sample preparation, and a third room for amplification and amplimer detection. All equipment should be kept in the room where it is used and not be transferred between rooms.

Aerosol-resistant pipette tips should be used to prevent cross-contamination between specimens. For the same reason, wear disposable examination gloves and change them frequently.

Room 1 - storage and preparation of reagents

This room and its equipment must be kept **free of DNA**. This room is only to be used for preparing PCR reagents. The Control PCR should not be brought into Room 1. The personnel involved should wear a clean laboratory coat, which must not be worn outside this room. Wear disposable gloves when handling reagents.

Room 2 - sample preparation

This room and its equipment must be kept **free of amplimers**. The personnel involved in specimen processing should wear a clean laboratory coat, which must not be worn outside this room. During sample preparation, disposable examination gloves should be worn and changed frequently. Carefully uncap vials containing (processed) sample. Avoid opening more than one reaction vial containing sample at the same time.

To avoid contamination or to clean contaminated surfaces, it is recommended to clean pipettes and work surfaces with DNA*Zap* (Ambion). Be aware that the use of DNA*Zap* is only an additional precautionary measure, and the described recommendations on laboratory design and procedures should be followed as strictly as possible.

Room 3 - amplification and amplimer detection

The personnel involved in amplification and amplimer detection should wear a clean laboratory coat, which must not be worn outside this room and must be changed daily. Wear disposable examination gloves when working with amplimers.

Test performance

Inclusivity

The SPF10 fragments of all 32 HPV genotypes present on the strip were cloned into plasmids and were tested with the INNO-LiPA HPV Genotyping *Extra* II. All HPV genotypes were specifically detected by their respective probe.

Agreement

Agreement between the INNO-LiPA HPV Genotyping *Extra* II and the INNO-LiPA HPV Genotyping *Extra* was evaluated using a total of 150 *Digene* HC 2 High Risk HPV DNA positive cervical cell suspensions collected in either Surepath (BD- Tripath) or ThinPrep PreservCyt Solution preservative fluid. Tests were performed using the *Auto*-LiPA 48 and visual interpretation. All samples had valid results (150/150; 100%, 1-sided 95%CI 98.2%).

Agreement was defined as the percentage of valid and interpretable LiPA results using HC2+ samples in agreement with the reference method INNO-LiPA HPV Genotyping *Extra*, for assay-common (HC2, LiPA *Extra*, and LiPA *Extra* II) high risk genotypes (=HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). A concordant result was thus defined as having at least one of these high risk genotypes in agreement between the new assay and the reference method.

Out of all 150 samples tested, 135 had a result that was concordant with the reference method (135/150; 90%, 1-sided 95% CI 85.2%).
A total of 15 samples had discordant results.

- In 12 of these samples, high risk genotypes were detected with the new assay and not with reference method (samples were determined high risk positive on HC2). In 7 of these samples these types were HPV59 or 68. Such discordant results were expected, as improved sensitivity amongst others for these genotypes was aimed for during development of the new test.
- Two other samples were mixed infections, where probable high risk genotypes (HPV70 in one case, HPV53 and HPV82 in the other) were detected for both the new assay as well as the reference method.
- One remaining sample showed a HPV68 on the new assay and a HPV51 on the reference method.

LiRAS for LiPA HPV software

All 150 developed strips from the agreement study were interpreted both visually and using the LiRAS for LiPA HPV v3.00 software. On these samples, 143 samples showed an exactly matching genotype result (95.3%,1-sided 95% CI 91.6%). The discrepant genotypes in the remaining 7 samples, all had optical density values very close to the programmed cut-off, with one exception of a probe line for HPV31 with OD of 0.0305. All of the 7 samples were mixed infections where there was at least one high risk genotype detected with visual interpretation as well as with software.

Precision

A panel of 8 HPV-positive samples (5 single infections, 3 multiple infections) was tested on different days, on 3 different lots by 3 operators using different automates (*Auto*-LiPA 48 and PCR cycler). All variability introduced resulted in the same test outcome in all replicates of the samples, except for one sample for which in one replicate an extra HPV16 was detected. The precision therefore is 111/112 (99.1%, 1-sided 95% CI 96.1%).

Analytical sensitivity

The limit of detection was determined for HPV genotypes 16, 18, 31, 33, and 45 representing five of the most prevalent high risk genotypes. Plasmid DNA, in the presence of human DNA, was diluted (1/3) ranging from 5000 copies/PCR reaction to 0 copies/PCR reaction. Each dilution was tested 8 times.
Defining the limit of detection as a 95% probit value (point estimate) was not possible for HPV16, HPV18 and HPV31 due to quasi-complete separation of the observed data. In a sensitivity analysis where it was assumed that one observation was negative at the point where complete positivity was first obtained (8/8 replaced by 7/8), estimations of probit values could be calculated. A very low LoD was obtained for HPV16 and HPV18 (upper limit 95% CI was 6 copies/PCR) and higher LoDs for HPV31, 33 and 45 (upper limit 95% CI was ranging from 42 copies/PCR to 437 copies/PCR).

Licenses

The purchase of this product allows the purchaser to use it for amplification of nucleic acid sequences for human *in vitro* diagnostics in accordance with the patented method described in the package insert. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

Trademarks

- INNO-LiPA is a worldwide trademark™ such as in USA.
- **LiPA** is a registered trademark® in the Benelux and is a trademark™ in the rest of the world (including USA).
- LiRAS is a registered trademark® in Europe and is a trademark™ in the rest of the world (including USA).
- Auto-LiPA is a worldwide trademark™ such as in USA.