



F-HPV typing™

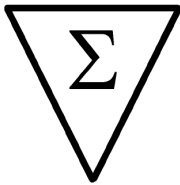
Multiplex Fluorescent-PCR Kit

**For Human Papilloma Virus (HPV)
Genotyping**

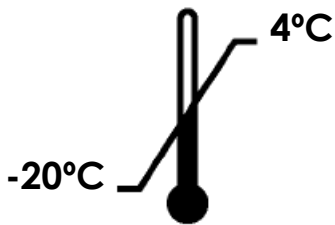
User's Manual



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For professional *in vitro* diagnostic use only



Keep Tubes Away from Direct Light

Read the User's Manual Carefully

Do not use if primary packaging is damaged

REF mlg.hpv.50

Product Overview

Purpose

Cervical cancer is the second most common cancer causing death in women with an annual incidence of around half a million and a mortality of almost 50%.

The most important risk factor for the development of cervical cancer is genital infection with human papillomavirus (HPV). More than 100 types are known, of which at least 30 usually infect the anogenital tract. HPVs are classified into low and high-risk categories based on their association with malignant lesions. The low-risk types include HPV-6, -11, -42 and -44, and mostly cause the development of genital condylomata (warts). The high-risk types HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68, induce cervical squamous intraepithelial lesions (SIL), which in turn are classified in low (LSIL) and high grade (HSIL) in severity, and which may progress to cervical cancer. In fact these 13 high-risk HPV types have been shown to be present in up to 99.7% of cervical cancer samples, the most frequently detected being HPV-16, followed by HPV-18, -31, -51, and -45.

Several studies have shown that a persistent HPV infection with the same high-risk type is a risk factor for the development and progression of lesions. If no high-risk specific HPV is present in a cervical sample, the patient has a very low risk to develop cancer for some years. However, if a patient treated of CIN has another positive HPV test within six months after treatment, it will be more predictive of a risk of recurrence for the patient.

On the other hand, women with more than one high-risk type HPV present at the cervix are more likely to have a persistent infection, and are at a strongly increased risk of developing cervical cancer.

Therefore, distinguishing between low or high-risk HPV genotypes and especially knowing the specific type is important in the prognostic and follow-up because this permits pinpointing patients with an increased risk for the disease.

F-HPV typing™ uses 15 primers amplifying within E6 and E7 regions of the HPV genome, the most likely to be retained after viral integration. Extracted DNA is amplified using a multiplex PCR with a set of 16 fluorescently labelled primers recognising HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and a human STR used as internal control. This sequence is added to check DNA integrity and PCR inhibitors; it is also helpful in detecting DNA mixtures due to sample mishandling. Different labelling of primers allows to generate amplicons of similar size in the same PCR reaction, the **F-HPV**

typing™ kit uses 5 dyes detection technology enabling easy single tube amplification of all sequences.

The kit has been developed to be used in conjunction with any Applied Biosystems Genetic Analyser compatibles with 5 dyes detection. After electrophoresis, PCR products are detected with high sensitivity and recognised by size and colour so that automated HPV genotyping is computer generated with great accuracy.

HPV simultaneous detection and genotyping by **F-HPV typing™** single tube multiplex PCR amplification also allows:

- Identifying type-specific HPV
- Differentiating between persistent and new infection, in patient follow up
- Detecting and identifying multiple HPV infection
- Analysing different samples such as fresh or paraffin embedded tissues (biopsies), ano-genital and oral samples

F-HPV typing™ is a sensitive, specific and rapid method for the identification of HPV genotypes, the automation involved in part of the procedure also allows high throughput of samples.

Five-Dye DNA Fragment Analysis

The **F-HPV typing™** kit uses a five-dye fluorescent system for automated DNA fragment analysis. This allows multiplex amplification and electrophoresis of over fifteen loci simultaneously. The kit is intended for use on Applied Biosystems ABI PRISM® genetic analysis instrumentation. Fluorochromes include 6-FAM™, VIC™, NED™ and PET™ to be used in conjunction with GS 500 LIZ™ size standard (Applied Biosystems PN° 4322682)

Hot Start Taq Polymerase and Optimised PCR Buffer

In order to maximise specificity of Multiplex PCR, Hot Start Taq Polymerase is included in the optimised PCR reaction buffer. The enzyme is completely inactive at room temperature. This prevents mis-priming during PCR set-up. Activation is obtained during the 15 min. step at 95°C before PCR cycling. This simplifies PCR set-up and handling, which can easily be done at room temperature.

Warming and precautions

- Specimens should always be handled as potentially infectious. Test should only be performed by adequately trained personnel.
- Waste should be handled according to the institution's waste disposal guidelines. Also observe federal state and local environmental regulations.

About this User's Manual

This user's manual describes the following:

- 1- Materials and equipment required to use the F-HPV Typing™ kit**
- 2- How to use the kit to amplify DNA samples**
- 3- How to perform automated detection**
- 4- How to analyze results**

Kit Storage

Fluorescent primers should be stored away from light.

The **F-HPV typing™** box is internally coated with aluminium in order to achieve maximum light protection.

F-HPV typing™ is stable for up to one year if stored at -20°C. The kit can be stored for up to six months at 4°C. Kit performance remains intact up to 48 hours at room temperature (20-25°C).

PCR mix can be stored as ready-to-use aliquots in PCR tubes at -20°C; thus reducing the risk of contamination.

1 Materials and Equipment Required to Use the F-HPV typing™ Kit

1.1 Laboratory Design

PCR amplification using fluorescently labelled primers is sensitive enough to amplify single target sequences. Thus particular care must be taken to avoid contamination. It is important to organise separate areas for DNA extraction, PCR and analysis in the lab. The main potential source of contamination is amplicons generated in previous runs. The PCR area should be dedicated to DNA extraction, kit handling and PCR set-up only.

PCR Set-Up Area

IMPORTANT: The following items should never leave the PCR set-up work area:

- ◆ Calculator
- ◆ Gloves, disposable
- ◆ Marker pen, permanent
- ◆ Microcentrifuge
- ◆ Microcentrifuge tubes, 1.5-mL, or 2.0-mL, or other appropriate clean tube
- ◆ Microcentrifuge tube rack
- ◆ Heated blocks or water baths
- ◆ Pipette tips, sterile, disposable hydrophobic filter-plugged
- ◆ Pipettes
- ◆ Vortexer
- ◆ Thermalcycler

Work area for Amplified DNA

- ◆ ABI Generic Analyser compatible with Five-Dye Detection
- ◆ Heated block
- ◆ Sequencer disposables and consumables
- ◆ Pipette tips, disposable hydrophobic
- ◆ Pipettes
- ◆ Vortexer

2 How to Use F-HPV typing™ Kit to Amplify DNA Clinical Samples by Multiplex-Fluorescent-PCR

2.1 DNA Extraction

F-HPV typing™ is based on PCR amplification, using a multiplex PCR with 15 sets of fluorescently labelled primers recognising HPV types 6, 11, 16, 18, 31,

33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and also primers recognising a human gene as internal control of DNA integrity and PCR inhibitors from extracted DNA of cervical samples collected in dry swab. However, it can also be used to analyse DNA extracted from PreservCyt Solution (ThinPrep® liquid Pap vial) from Cytoc Corporation (Marlborough, Massachusetts), biopsies, ano-genital and oral samples.

The Multiplex F-PCR Set allows simultaneous analysis of 15 different HPV types and a human polymorphic sequence (STR) in a single tube per sample. The Multiplex PCR Mix is ready to use for 50 reactions.

2 Suggested DNA extraction procedure for Dry Swab samples

2.1 Add 1 ml of sterile phosphate buffered saline (pH 8.0) to the collection tube with the dry swab.

2.2 Vortex 1 min.

2.3 Transfer the cell suspension into Eppendorf or Nunc tube.

This cell suspension can be kept at -20°C until needed. If frozen, allow it to equilibrate at room temperature and homogenise by vortex before using

2.4 Use 200 μL of cell suspension for DNA extraction.

2.6 Spin the sample at 13,000 r.p.m. for 5 min.

2.7 Remove and discard the supernatant.

2.8 Add 100 μL of 10 mM Tris (pH 8.0), 1mM EDTA and a final concentration of 200 $\mu\text{g}/\text{mL}$ of Proteinase K (PK) to the cellular pellet.

2.9 Gently vortex.

2.10 Incubate tubes at 56°C for 2 hours.

2.11 Pulse spin the sample at 13,000 r.p.m.

2.12 Incubate at 95°C - 100°C for 10 min.

2.13 Spin the sample at 13,000 r.p.m. for 5 min

2.14 Transfer the supernatant (ready to use DNA) to a clean Eppendorf tube taking care in not transfer cell pellet.

Extracted DNA can be stored at 4°C for up to one week or until completion of the tests. Longer storage should be at -20°C .

2.2 PCR Set-up Protocol

Thaw **F-HPV typing**[™] vial and mix thoroughly by vortexing a few seconds. Aliquot PCR mix in each PCR tube in accordance with the table below:

PCR Mix	20 μl
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Extracted DNA	5µl
Final PCR Volume	25 µl

F-HPV typing™ mix can be stored as ready-to-use 20 µl aliquots in PCR tubes.

Warning: In order to avoid possible contamination, **F-HPV typing™** mix must be aliquoted in the PCR area with dedicated pipettes and filtered tips. One drop of mineral oil on each PCR tube will also reduce the risk of contamination by amplicons generated in the previous PCR.

2.3 Performing PCR

Warning: According to good laboratory practice internal quality control samples of known genotype should be processed in each assay to assess the effectiveness of the procedure

Hot Start Taq Polymerase

In order to increase the PCR specificity, Hot Start Taq Polymerase is included in the reaction buffer. The enzyme is totally inactive at room temperature. This allows easy set-up of PCR reaction without ice. Activation is achieved with 15 min. hold at 95°C.

1 Program the Thermalcycler according to the following parameters:

Taq Activation	Denaturation	Annealing	Extension	Final extension	Storage
Hold	35 Cycles			Hold	Hold
95°C 15 min.	95°C 30 sec.	64°C 30 sec.	72°C 30 sec.	60°C 10 min.	4-20°C

2 Place tubes in Thermalcycler and close the lid.

3 Start the PCR.

4 PCR products are stable at room temperature overnight, longer storage before electrophoresis should be at 4°C.

Warning: After PCR is complete, tubes should never be opened in the PCR set up area. This is essential in order to avoid contamination at any future PCR amplification.

Particular care should be taken in disposing amplified products according to good laboratory practice and local legislation.

3 How to Perform Automated Electrophoresis and Detection

F-HPV typing™ is designed to be used in conjunction with Applied Biosystems Genetic Analysers supporting Five-Dye Data Collection.

Warning: Use the DNA sequencer in accordance with the manufacturer's instructions.

3.1 Software Requirements for Five-Dye Data Collection

ABI Collection™

Make sure your Applied Biosystems Data Collection™ Software supports Five-Dye data for DNA fragment analysis applications. Refer to the Genetic Analyser User's Manual.

Additionally, a matrix file or spectral calibration should be generated using the 6-FAM™, VIC™, NED™, PET™ and LIZ™ matrix standards (DS-33) according to the Genetic Analyser instructions.

3.2 Running Samples

Warning: Amplified products should be handled in the analysis area with dedicated pipettes and tips to avoid contamination in successive PCR amplifications.

3.2.1 Preparing samples for Electrophoresis

GeneScan™-500 LIZ™ Size Standard (ABI P/N 4322682) should be used with **F-HPV typing™**.

1 In a 1.5ml tube, prepare the necessary amount of size standard for all the samples to be analysed by combining:

- 20µl Hi-Di™ Formamide (ABI P/N 4311320)

- 0.3 µl GeneScan™-500 LIZ™

This mix can be prepared in excess and kept stored at 4°C for up to one week.

2 Use 20 µl of this mix to inject 2 µL of PCR product.

3 Denature the sample tube/plate with Formamide and Size Standard for 2 minutes at 95°C.

4 Load samples on the Genetic Analyser according to the User's Manual.

3.2.2 Capillary Electrophoresis

The **F-HPV typing™** kit generates amplicons between 156 and 489 bp, which are efficiently separated by electrophoresis through 36cm capillaries, using standard microsatellite modules.

1 Create a Five-Dye sample sheet using the Data Collection Software.

2 Select the appropriate run module.

3 Start the run.

Note: Injection time and/or voltage can be adjusted to the amount of PCR product. Increasing/decreasing of the injection time/voltage will allow more or less products to run through the capillary. Amplified products can be reinjected and re-analysed several times.

4 How to Analyse Results

4.1. Detection of HPV Types.

F-HPV typing™ detects 15 HPV types: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -6 and -11. Each HPV-type is identified by the size and colour of the corresponding amplicons. Size range is shown below; types with similar size are labelled with different fluorochromes. FAM, VIC, NED and PET dyes are used to label primers. These fluorochromes are detectable respectively as Blue, Green, Yellow-Black and Red on the electrophoretograms. LIZ dye (Orange) is only used for the Size Standard, which undergoes electrophoresis together with the F-PCR products.

HPV F-PCR products are detected on the electrophoretogram as peaks with a specific colour and size. Note that, depending on the Genetic Analyser used, sizes may differ of up to 6 b.p. Sizes shown in the tables are detected on the ABI 3130 instrument.

HPV Type	Colour of PCR Product	Size
HPV-58	PET	156
HPV-33	6-FAM	196
HPV-6	PET	256
HPV-16	NED	296
HPV-35	VIC	327
HPV-18	6-FAM	386
HPV-56	6-FAM	428
HPV-31	6-FAM	489
HPV-68	NED	189
HPV-39	VIC	244
HPV-59	6-FAM	283
HPV-52	PET	308
HPV-51	VIC	389
HPV-45	NED	414
HPV-11	PET	424
D18S391	VIC	140-180

A human polymorphic DNA sequence (the D18S391 Short Tandem Repeat) is amplified as internal control; it can generate two different patterns: a single PCR product (Homozygous) or two allele peaks of different sizes (Heterozygous). The presence of this product reflects DNA integrity and absence of PCR inhibitors in HPV negative samples. It also allows assessing eventual cross contamination between different samples, as in these cases extra STR alleles can be detected.

Co amplification of the human internal control has been set up in order to not compete with HPV amplification, as this could reduce detection sensitivity for samples with low viral load. For the same reason, in cases with high viral load, this control may fail to amplify as HPV sequences will be preferentially amplified.

HPV Positive: The presence of HPV is detected as fluorescent peaks in the electrophoretogram. The Type is assessed by size and colour of the peak, corresponding to one or more of the fifteen genotypes shown in the table. Detection of a single peak means sample with single infection; two or more peaks indicate simultaneous infection with multiple HPV types.

For samples with high viral load (i.e. very high HPV specific peaks), the internal control may be absent. The result is valid as PCR efficiency and absence of inhibitors is already confirmed by the presence of HPV specific products.

HPV Negative: The absence of HPV specific peaks and the presence of an heterozygous / homozygous pattern for the internal control, is characteristic of samples negative for all tested HPV types.

If the internal control results is poor amplification it is necessary to repeat the whole procedure (including DNA extraction) either because of inappropriate specimen collection, processing errors or for the possible presence of PCR inhibitors.

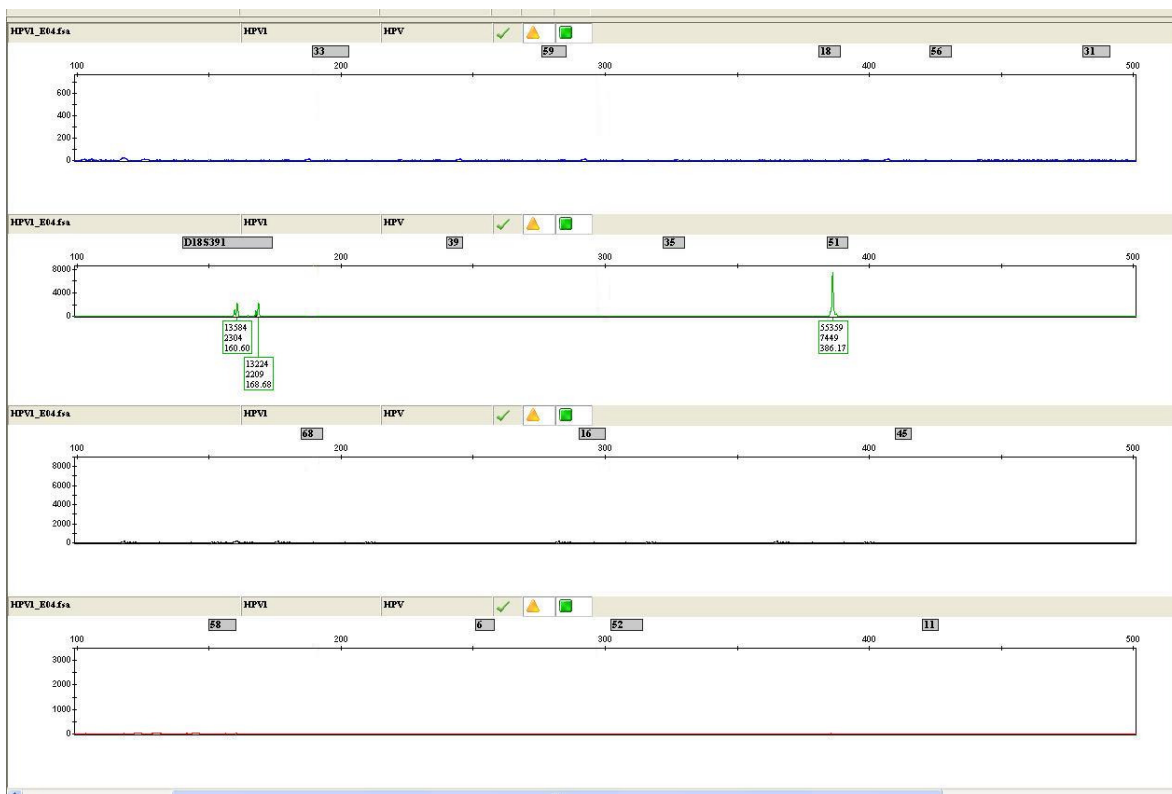
Invalid Result: No peaks are present in the electrophoretogram. The concomitant absence of products for both HPV and human DNA may be due to an inadequate specimen collection or sample type, errors during sample processing or the presence of PCR inhibitors. The whole procedure have to be repeated eventually also considering the collection of a second sample.

One of the most common causes of PCR inhibition is the presence of blood in the original sample. In these cases extra DNA purification using spin columns may increase the efficiency of PCR amplification.

4.2 Analysis Examples

Example 1

Electrophoretogram showing HPV detection and typing. Single HPV DNA is detected as a fluorescent peak; the HPV-51 genotype is determined by the size and colour of the amplicon. The D18S391 microsatellite, used as human DNA internal PCR control, is heterozygous.



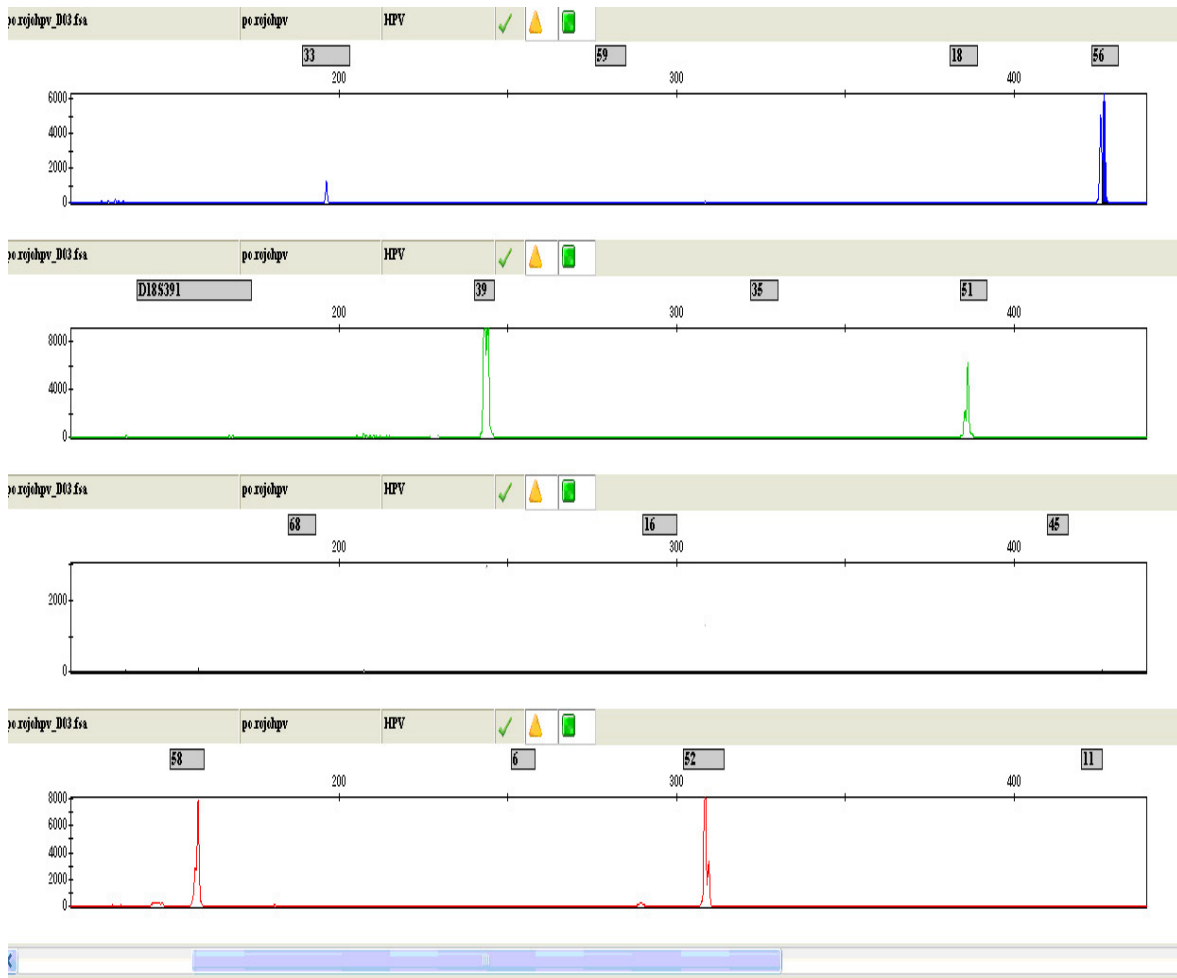
Example 2

Electrophoretogram showing the detection of multiple HPV infection. Five different peaks are present with sizes and colours corresponding to HPV-59, HPV-16, HPV-45, HPV-58 and HPV-11. D18S391 amplification is deliberately set up to not compete with HPV detection thus, the simultaneous amplification of 5 HPV types, reduces its efficiency.



Example 3

Simultaneous detection and typing of multiple infections with HPV-33, HPV-56, HPV-39, HPV-51, HPV-58 and HPV-52. The 5 different types are detected as peaks of the corresponding sizes and colours. Note that, in this example, the internal control is not detectable as HPV sequences are deliberately preferentially amplified.



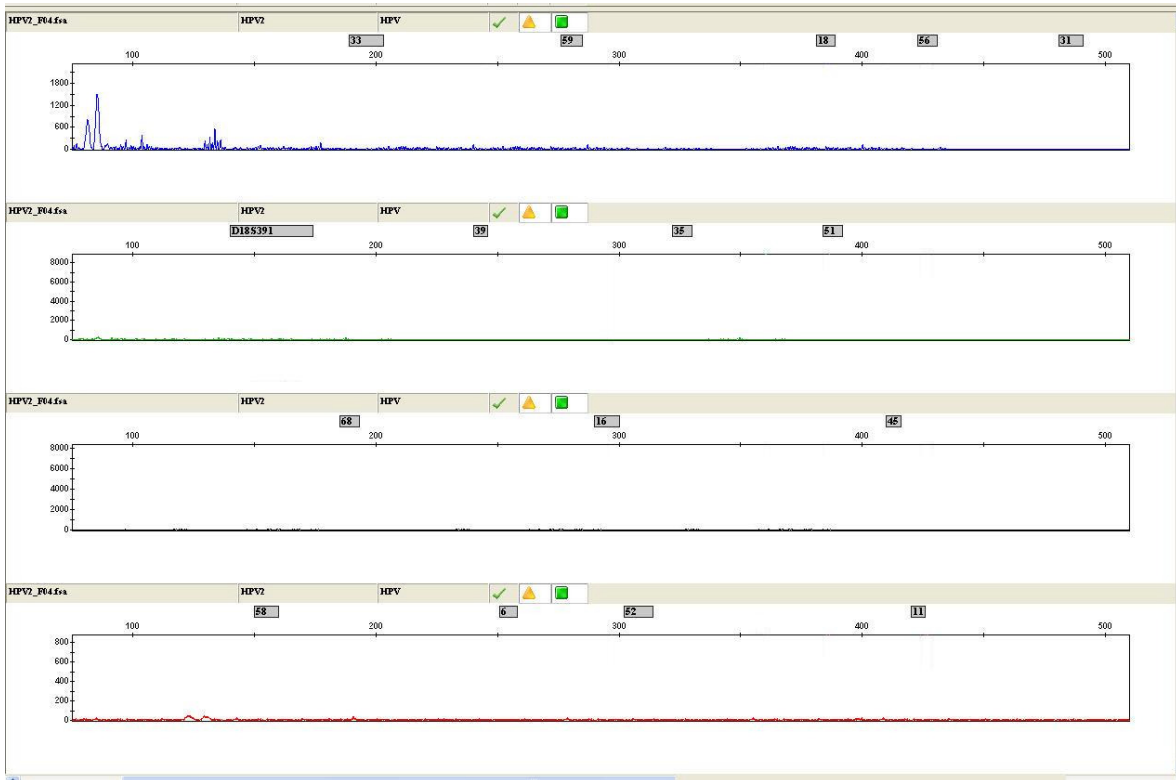
Example 4

HPV negative sample. No peaks are present other than the internal PCR control. The sample is homozygous (single peak) and the amplification of this sequence indicates the correct performance of PCR amplification and also confirming that sampling procedure was correctly performed.



Example 5

Electrophoretogram showing an invalid **F-HPV typing™** result. No HPV specific peaks are detected and the result is not reliable because of complete internal control amplification failure. This may be due to PCR inhibition or the absence of any DNA in the sample maybe due to inappropriate collection procedure.



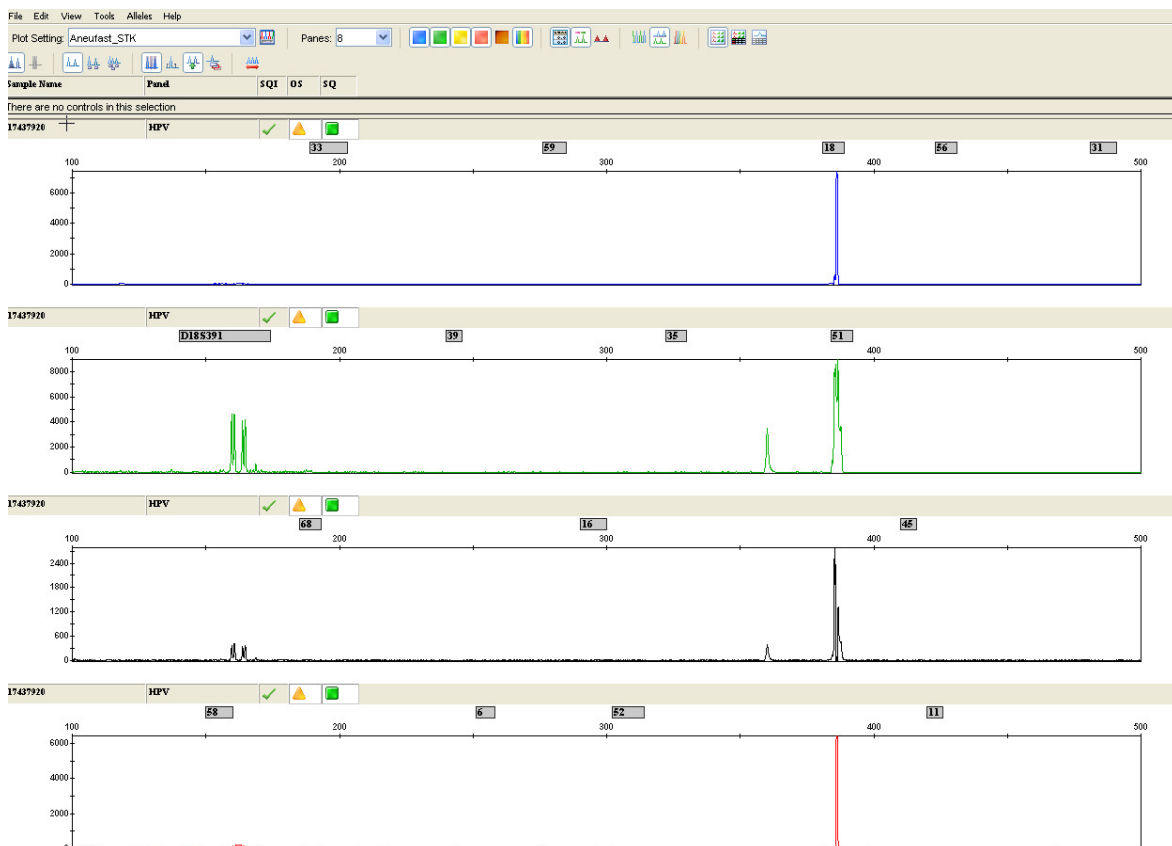
Example 6

Electrophoretogram showing results of the D18S391 human polymorphic microsatellite used as internal control of **F-HPV typing™**. All 4 samples are negative as assessed by lack of type specific HPV products. Allele sizes in all four samples are different and no extra alleles are detected thus excluding cross contamination between different samples.



Example 7

Electrophoretogram showing **F-HPV typing™** result on a sample with high viral load. Single HPV-51 genotype is detected according to size and colour of the amplicon. Over amplification generates a PCR product about 30b.p. shorter than the main amplicon which is detected as a green fluorescent peak of very high fluorescence. Note how the large amount of fluorescence generated by the over amplification hampers correct separation of colours by the analysis software; this results in peaks of same sizes to be observed in all other colours. However while this does not cause difficulties for red and green as no HPV types are present within the same size, in this particular case a simultaneous co-infection with HPV-18 cannot be ruled out. In these cases, dilution of PCR products (1/1000) and a second electrophoresis are recommended to obtain the correct result.



Sensitivity and Specificity for HPV typing from clinical Specimens.

The sensitivity and specificity of the **F-HPV typing**[™] toward HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -6 and -11 types were assessed by testing 164 cervical specimens in duplicate with the Hybrid Capture® 2 High-risk (Digene). Fifty of these specimens were positives for **F-HPV typing**[™] and 33 with the Hybrid Capture® 2 High-risk. Negative results were obtained for the **F-HPV typing**[™] and Hybrid Capture® 2 High-risk in 114 and 129 samples respectively.

Nineteen discordant results were verified using a third assay, the Linear-Array HPV genotyping test (Roche).

Concordance between 2 tests was used as criteria to confirm presence or absence of high-risk HPV DNA; samples positives for both **F-HPV typing**[™] and Linear-Array HPV, but HC2 negatives, were considered as true positives, negatives samples for both **F-HPV typing**[™] and Linear-Array HPV, but HC2 positives were considered true negatives. Thus overall results for the **F-HPV typing**[™] were 43 true positives and 112 true negatives with 96% sensitivity and 98% specificity.

Limitations

A negative **F-HPV typing**[™] result does not necessarily exclude the presence of HPV infection; it only refers to the particular sample analysed and to the 15 HPV types tested. The correct performance of the test relies upon several factors such as adequate sampling of the region of interest, correct processing, absence of inhibitors and viral load of HPV DNA to be detected by the assay.

Disclaimer

Results obtained with any IVD Kit should only be employed and interpreted within the whole clinical picture.

This product does not provide a licence to perform PCR under any patent that may be owned by any third party including Hoffman-La Roche (F. Hoffman-La Roche Ltd, Diagnostic, CH-4070 Basel, Switzerland) and Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501).

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