

Description of HPV tests, typology of HPV tests (T. Iftner, R. Tachezy, M. Pawlita, P. Snijders)

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5.1 Introduction

Numerous professional organizations recommend testing for the mucosal “high risk” HPV types to increase the efficacy of cervical cancer screening, including The American College of Obstetricians and Gynecologists (ACOG), the American Cancer Society (ACS), the American Society for Colposcopy and Cervical Pathology (ASCCP), and the Association of Reproductive Health Professionals (ARHP). Under the recommended

guidelines, high-risk HPV DNA testing is typically conducted in two instances: first as an adjunct to cervical cytology analysis as primary screening tool in all women over 30, and second, for the triage of patients with ASC-US cytology results. Other potential indications for high-risk HPV testing involve the monitoring of women who have been treated for high-grade cervical intraepithelial neoplasia (CIN) lesions and the follow up of women negative in colposcopy but positive for ASCUS, LSIL & ASC-H cytology.

The diagnosis of an HPV infection nowadays is almost exclusively made by identification of the viral nucleic acids by molecular techniques using complementary probes hybridizing to the DNA/RNA followed by either signal amplification or the nucleic acid is amplified before hybridization to specific complementary probes (target amplification) by methods such as the Polymerase Chain Reaction (PCR) of a subgenomic region. Newer methods include isothermal target amplification of E6/E7 mRNA progression markers by transcription-mediated amplification (TMA) or nucleic acid sequence based amplification (NASBA).

In the following paragraphs and tables you will find only descriptive data about the test principles and their performance characteristics. We also abstained to provide information about the costs of the different tests, as they are different for each country and depend always on the quantity of test kits that are required. List prices should not be taken for given by anyone.

5.2. Categorisation of HPV assays

I. Signal amplification assays:

a. Liquid phase DNA detection using RNA probes and labelled antibodies recognizing DNA-RNA hybrids

b. Liquid phase DNA detection with labelled DNA probes that form a triplex DNA structure that is recognized and cleaved by a specific enzyme

II. DNA Target amplification assays:

a) broad-spectrum PCR

b) consensus primer PCR

III. Isothermal nucleic acid amplification methods (typically for mRNA)

a) Nucleic Acid Sequence Based Amplification (NASBA)

b) Transcription-mediated amplification (TMA)

IV. Read-out assays for target amplification methods

a) Methods that detect the HPV types as a pool (group detection)

- i) Enzyme immunoassays following oligonucleotide probe hybridizations to PCR products captured in microtiter plate wells
- ii) Real time readout using mixtures of type-specific probes

b) Methods that allow genotyping

- i) Reverse hybridization assays with labelled PCR products hybridized to oligonucleotides immobilized to various solid supports, such as:
 - Strips and filters
 - Microsphere (Luminex) beads
 - Medium and low density Microarrays

V) Real time readout assays with type-specific probes

5.3 Description of tests

Preamble: The following data are based mainly on published literature if available, otherwise taken from data sheets of specific tests or other informations from the respective companies that were publicly available. Further details and figures on the different test systems can be found in Appendix 4

5.3.1 Signal amplification assays

The Hybrid Capture 2 HPV Test

The best known example in the category liquid-phase signal amplification methods is the Digene Hybrid Capture 2 (HC2) assay (Qiagen). This assay is based on hybridization in solution of long synthetic RNA probes complementary to the genomic sequence of 13 high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low risk (6, 11, 42,

43, 44) HPV types, which are used to prepare a high (B) and low (A) probe mix that are used in two separate reactions. After degradation of the endogenous RNA, the denatured HPV DNA present in the biological specimen is hybridized with the probe mixes allowing the formation of specific HPV DNA-RNA hybrids. These hybrids are captured by antibodies bound to the wells of a microtiter plate that recognize specifically RNA-DNA hybrids. The immobilized hybrids are then reacted with alkaline phosphatase-labelled anti-DNA-RNA monoclonal antibodies and the plate is subsequently washed. This is followed by an incubation of the bound enzyme-conjugated antibodies with the chemiluminescent compound CDP-Star® (Tropix PE, Bedford, Mass, USA). Dephosphorylation of this substrate produces light in a glow reaction that is measured by a luminometer. Readings are transferred directly into a software program where the results are analyzed. The intensity of emitted light, expressed as relative light units (RLU), is proportional to the amount of target DNA present in the specimen, providing a semi-quantitative measure of the viral load. The Food and Drug Administration (FDA) recommended cut-off value for test-positive results is 1.0 RLU/Co (equiv. to 1pg HPV DNA per 1ml of sampling buffer).

See Figure 1: Principle of the HC2 test, (Appendix 5)

The *care*HPV Test

Because no current HPV-DNA test is appropriate for use in low-resource settings in developing countries, PATH (Seattle, WA, USA) was funded in 2003 by the Bill & Melinda Gates Foundation to lead the research and development of a new HPV-DNA screening test that was rapid, simple, and which can be used remotely. The new test, designated *care*HPV Test, is a signal-amplification assay that detects target HPV-DNA from 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). The assay needs only a small footprint of bench-top work space (about 25x50 cm), no mains electricity or running water, and can be done by technical support staff in roughly 25h. The short assay time would allow testing and clinical follow-up in the same day. This is only a test to be used in underdeveloped countries as it is specifically developed for low resource settings with portable equipment to be used under field conditions.

The Cervista Test

A more recent liquid-phase signal amplification method involves the Third Wave's Invader HPV test of Cervista (Hologics; Day et al., 2009). The Invader chemistry for the detection of HPV high risk types is composed of two simultaneous isothermal reactions. A primary reaction specifically detects the presence of high risk HPV DNA sequences after denaturation by hybridization with two oligonucleotides: the invader oligo and the probe oligo, which are DNA oligonucleotides without having a biotin molecule or an enzyme attached (invader assay). This is similar to the HC2 assay, where RNA probes hybridize to denatured HPV DNA. In the invader assay both oligonucleotides have to hybridize adjacent to each other with the target HPV DNA sequence. Because the 3' terminus of the Invader oligonucleotide invades one base (non-complementary to template) into the DNA–DNA duplex formed by hybridization between the probe oligo and the HPV DNA target an overlapping DNA–DNA triplex structure is formed.

See Figures 2 to 6: Principles of the Cervista test (Appendix 5)

This unusual structure that normally is not present in patient material is recognized by the cleavase enzyme, which possesses 5' nuclease activity, and recognizes and cleaves this specific structure, releasing the reporter DNA called "5' flap". Subsequently, probe oligos that are present in the reaction in large excess, rapidly undergo again association with the target HPV DNA replacing cleaved probe oligos. Therefore multiple probe oligos are cleaved per target molecule, and the label, detected in a secondary reaction, generated from the cleaved 5' flaps is amplified.

In situ hybridization and Immunohistochemistry methods

Besides liquid-phase target amplification methods also morphological target amplification methods are available that rely on DNA in situ hybridization (ISH) to cytological slides or histological preparations.

5.3.2 Target amplification-based techniques

Polymerase Chain Reaction (PCR) - based assays

PCR allows the in vitro replication of specific DNA target sequences in order to generate sufficient copies for subsequent detection and analysis. The first step in this process requires the separation of the double-stranded target DNA into single strands (denaturation), which is accomplished by heating the sample to about 95°C. At this

temperature, the hydrogen bonds between the complementary DNA bases break and the strands separate. The next step (annealing) involves cooling the reaction to 40-60°C, at which temperature short synthetic single stranded DNA molecules, called oligonucleotides will hybridise with their complementary sequences on the target strands. These oligonucleotides then act as primers for the last step in the reaction (extension) in which a thermostable DNA polymerase enzyme catalyses the formation of two new double-stranded DNA molecules (amplicon) using each of the original target DNA single strands as templates.

By repeating this cycle of denaturation, annealing and extension, each newly synthesised double-stranded DNA molecule can serve as a template for the next cycle, and the number of molecules increases in an exponential fashion. PCR can theoretically produce 10^6 identical copies from a single double stranded DNA molecule after 30 cycles of amplification and thereby achieve its exceptionally high sensitivity.

See Figure 7: Principle of a PCR reaction, (Appendix 5)

It should be evident from the above summary that any amplicon can contaminate either reagents or new samples entering the laboratory, thereby acting as a template for amplification and the result would be indistinguishable from that obtained with a sample that is truly infected with HPV. As such, the execution of PCR protocols requires carefully controlled conditions to eliminate the risk of contamination.

Broad spectrum PCR assays

In order to ensure detection of many, if not all, relevant mucosal HPV types in one reaction most widely used PCR assays for broad spectrum HPV detection employ so-called consensus primers. Such primers are directed to a highly conserved region of the HPV genome, mostly but not exclusively within the L1 gene, and are potentially capable of detecting all mucosal HPV types (Bernard et al., 1994). Nevertheless, despite sequence conservation, some degree of inter-type heterogeneity at the nucleotide level precludes the selection of single primer pairs that fully match corresponding sequences of a broad spectrum of HPVs. Therefore, to allow broad spectrum HPV detection, consensus primer assays either use low-stringency PCR conditions to allow some degree of mismatch acceptance between primers and target sequence, degenerate primers with nucleotide variations at variable base positions, primers with the non-specific base-analogue inosine at ambiguous base positions, or sets of overlapping primers (multiplex). More recently,

also multiplex assays have been described that use primers targeting different viral regions of the different HPV types, rather than a conserved region.

Consensus primer PCR systems

Amongst the first consensus PCR systems that have been described are the GP5/6-PCR (Snijders et al., 1990; van Den Brule et al., 1990) with its second generation, extended version GP5+/6+-PCR (de Roda Husman et al., 1995; Jacobs et al., 1997), and the MY09/11 PCR (Manos et al., 1989) with its modified version, PGMY09/11 (Gravitt et al., 2000). Both PCR systems target a conservative region within L1, generating 150bp (GP5/6- and GP5+/6+-PCR) and 450bp (MY09/11 and PGMY09/11) PCR fragments, respectively. GP5/6-PCR and GP5+/6+-PCR assays use a pair of single, unmodified, primers that are applied under low stringency PCR conditions. MY09/11 uses degenerate primers, and PGMY09/11 is based on the use of overlapping primers targeting the same regions as MY09/11. More recently, a short fragment PCR system, the SPF10 assay, was developed targeting a small (i.e. 65 bp) region within L1, thereby potentially increasing the sensitivity of the assay (Kleter et al., 1998). A more detailed description of some PCR-based HPV testing systems is given in Appendix 4 detailing examples of already long-time established test systems that form the basis for a variety of different commercial available HPV tests today.

5.3.3 Read-out systems

Analysis of the amplified products can be done in several ways. Current read-out systems include enzyme immunoassays (EIA) following probe hybridizations to PCR products captured in microplate wells, and reverse hybridization assays with labeled PCR products hybridized to oligonucleotides immobilized to various solid supports. As solid support in these reverse hybridization assays, strips, filters, microarrays and microsphere (Luminex) beads are used. Nowadays, read-out systems coupled to real time PCR formats using (mixtures of) type-specific probes labelled with fluorescent dyes for the fluorescent 5' exonuclease assay (TaqMan) or FRET (LightCycler), or CYBR green are increasingly used. Reverse hybridization assays have the advantage that full genotyping for a broad spectrum of types can be performed in a single analysis. Conversely, EIA and real time assays are generally limited to the detection of HPV types as a pool (i.e. group detection) per reaction, although the latter has the option to include genotyping for up to three types

per reaction. EIA and real time assays are more suited for high throughput analyses than reverse hybridization assays.

Enzyme immunoassays using microplates (EIA)

Microplate hybridisation requires that PCR amplification is undertaken using labelled primers usually with a biotin molecule, which subsequently becomes incorporated into the amplicon and allows its capture onto the walls of streptavidin coated microplates. The immobilized amplicon is hybridised with e.g. digoxigenin (DIG)-labelled DNA probes that are complementary to specific HPV sequences. The final step in detection uses an anti-DIG/alkaline phosphatase conjugate that binds to the DIG-label on the hybridisation probe, effectively immobilizing the alkaline phosphatase within the microplate well, but only when the probe has hybridised with the complementary HPV DNA sequence. The alkaline phosphatase catalyses colour formation upon the addition of a substrate, colour development is measured in a microplate reader and the sample is deemed positive if it exceeds a pre-established threshold.

See Figure 8: Microplate hybridization for the detection of PCR amplification products (DIG=digoxigenin), (Appendix 5)

Reverse hybridization assays using strips or filters

Reverse hybridisation line blot assays are based on the hybridisation of the amplicon to specific DNA probes that have been immobilized on nitrocellulose or nylon strips or filters. In order to provide type discrimination, probes for specific HPV types are bound to the strip/filter in individual parallel lines at defined positions and amplicon hybridisation at a particular position thereby identifying the type. In order to detect hybridisation, PCR amplification must again be performed with primers having an attached biotin molecule, which becomes incorporated into the amplicon. The amplicon is applied to the strips under conditions that allow for specific hybridisation with the immobilized probe. Once this has occurred, the presence of the amplicon is detected with an alkaline phosphatase-labelled streptavidin conjugate, whereby the streptavidin binds to the biotin on the amplicon and immobilises the alkaline phosphatase in the specific region where hybridisation has occurred. The alkaline phosphatase catalyses colour formation upon addition of a substrate and a coloured line develops where the amplicon has hybridised to a specific probe. Measuring the position of the coloured line relative to an established base line allows the type to be identified.

See Figure 9: Line blot hybridization for the detection of specific HPV types after PCR reaction, (Appendix 5)

Reverse hybridization assays using microsphere beads

Luminex colour-coded microsphere beads as solid support for HPV type-specific probes, for a subsequent flow cytometry-based method for genotyping was first described by Schmitt et al. (2006; and later by Söderlund-Strand et al., 2009) for genotyping of GP5+/6+-PCR products. In this system the different microsphere beads contain two spectrally distinct fluorochromes present in various concentrations. Thus, a spectral array is created encompassing up to 100 different microsphere sets with specific spectral addresses. For genotyping, each type-specific oligonucleotide probe is covalently attached to a specifically coloured microsphere bead set. During analysis, individual microsphere beads are interrogated by two lasers. The first laser excites the fluorochromes within the microsphere and allows identification of the microsphere set, and consequently the specificity of the attached type-specific probe. The second laser excites a reporter fluorochrome coupled to the hybridized PCR product and allows detection/quantification of the PCR product.

Reverse hybridization assays using microarrays

Papillocheck (Greiner Bio-One)

The PapilloCheck[®] HPV genotyping assay (Greiner Bio-One) is a low-density microarray based system for the detection of a fragment of the E1 gene of the HPV genome. The PapilloCheck[®] assay allows the simultaneous genotyping of 24 different HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44/55, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82 (Schopp et al., 2010; Hesselink et al., 2010). The 350 base pairs fragment of E1 amplified by PCR is hybridized to specific DNA probes fixed on the PapilloCheck[®] low density microarray Chip. During the hybridization reaction also the labeling of the PCR products takes place. Following few washing steps the PapilloCheck[®] Chip is scanned with the CheckScanner[™] (Greiner Bio-One). Due to the fluorescence signal the presence of HPV DNA is visualized with the CheckScanner[™] and analyzed with the corresponding CheckReport[™] Software (Greiner Bio-One) that creates a report. On one PapilloCheck[®] Chip 12 specimens can be processed at once. The quality of each specimen, PCR

performance as well as hybridization efficiency is automatically monitored based on integrated controls.

ProDect® Chip HPV Typing kit

The test uses the L1 and the E6/E7 regions as a target for PCR reactions and is able to type 19 HPV types (6,11,16,18,31,33,35,39, 42, 43, 44, 45,51,52,56,58,59,68,73) using 5 probes Using a multiplex PCR with Biotin-labelled primers, the denatured products are hybridized on the chip, followed by a wash step and the addition of alkaline phosphatase (AP) conjugated Streptavidin. After another wash step, a chromogen substrate (NBT-BCIP), mediates the colorimetric reaction required for detection by naked eye.

CLART HPV2 Test (Genomica)

The CLART HPV 2 Test Kit detects up to 35 (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42,43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83,84, 85 y 89) HPV types. Detection of the different HPV genotypes is achieved by PCR amplification of a 450 bp fragment within the highly conserved L1 region of the virus. The test combines a PCR with a technological platform (CLART® Clinical Array Technology), which is based on a low-density microarray fixed at the bottom of a classical 2 ml tube (Array Tube-AT) or in the bottom of an 8-well strip (Array Strip-AS).

5.3.4 Real time read-out assays

In contrast to end-point PCRs with defined amplification cycles before measurement, real time PCR measures the amplification products during synthesis and need no manual handling for detection, which enhances the contamination risk (see Appendix 4).

Abbott RealTime HR HPV Assay

The Abbott RealTime HR HPV Assay can use the automated Abbott m2000sp or Abbott m24sp instrument,for DNA extraction, and then the Abbott m2000rt instrument for real-time PCR. The PCR reaction consists of a mixture of primers that amplifies part of the L1 region. Detection is done using fluorescence labelled probes for 14 HPV-genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). An internal control (IC) signal is measured by amplifying the endogenous human beta-globin-sequence and it is used to evaluate cell adequacy in sample input, quality and efficiency of DNA extraction process

and efficiency of the amplification reaction. The probes for HPV 16, HPV 18, non-HPV 16/18-genotypes and IC are labelled with four different fluorochromes. This multi-color design allows both HR HPV detection and partial genotyping of HPV 16 and 18, within a single reaction. The assay cut-off was established based on clinical performance for detecting high-grade cervical disease.

The Cobas 4800 system

The cobas® 4800 system (Roche) allows for automated HPV and CT/NG testing. It is a two component system consisting of the cobas x480 sample processing instrument and the cobas z480 amplification and detection instrument. The system needs minimal manual intervention. A pool of HPV primers amplifies DNA of 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). An additional primer pair targets the human β -Globin gene to provide an internal process control. Specific fluorescence dyes allow differentiated detection of the internal control, of HPV 16, HPV 18 and/or the panel of high-risk HPV types. The assay cut-off was established based on clinical performance for detecting high-grade cervical disease.

5.3.5 Isothermal nucleic acid amplification methods

NASBA (Nucleic acid sequence based amplification) technology

NASBA (Compton et al., 1991) is a sensitive transcription-based amplification system (TAS) for the specific replication of nucleic acids in vitro. The main advantage of this technique is that the complete amplification reaction is performed at 41°C. Three enzymes are involved in this homogeneous isothermal reaction: avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 DNA dependent RNA polymerase (DdRp). Because of the integration of RT into the amplification process, the method is especially suited for mRNA analyses. The target RNA is denatured at 65°C and reverse transcribed with a primer not only possessing a sequence complementary to the target RNA, but also including a 5' sequence corresponding to the promoter of the T7 RNA polymerase promoter sequence. The RNA strand of the RNA-cDNA hybrid formed with AMV-Reverse transcriptase, will subsequently be degraded by RNaseH. With the help of a reverse primer complementary to the cDNA, a double-stranded DNA encompassing a T7 promoter sequence is been formed that can be used by the T7 RNA polymerase to synthesize new

RNA molecules complementary to the target RNA. After this initial reaction, NASBA now enters the amplification (cyclic) phase and new RNA will be produced by the activity of the RT and T7 RNA polymerase enzyme. The reaction continues in a self-sustained manner under isothermal conditions, thus achieving large amplification of the target (10^6 to 10^9 -fold). The detection of the amplified RNA is by the use of Molecular Beacon probes that are single-stranded oligonucleotides having a stem-loop structure. One arm of the stem is labelled with a fluorescent dye and the other arm is labelled with a non-fluorescent quencher, which inhibits fluorescence by energy transfer from the fluorescence dye to the quencher. After hybridisation of the Molecular Beacon to its specific target and unfolding of the stem structure the energy transfer is interrupted and fluorescence takes place which is related in intensity to the amplicon concentration if the Molecular Beacon is provided in large excess. Two oligonucleotide primers that are specific for the RNA target of interest determine the type-specificity of the reaction.

See Figure 10: Principles of the NASBA technology, (Appendix 5)

Transcription-mediated amplification (TMA)

The APTIMA HPV Assay is a target amplification assay utilizing TMA for the detection of E6/E7 viral mRNA from 14 HPV types (16/18/31/33/35/39/45/51/52/56/58/59/66/68). The assay involves three main steps, which take place in a single tube: target capture specimen processing; target amplification by Transcription-Mediated Amplification (TMA) (Hill, 2001) and detection of the amplification products (amplicon) by the Hybridization Protection Assay (HPA) (Nelson, & Kacian, 1990).

The assay incorporates an internal control to control for nucleic acid capture, amplification, and detection, as well as operator or instrument error. The transport solution in the tube lyses the cells, releases the mRNA, and protects them from degradation during storage. The target mRNA is isolated from the specimen by use of capture oligomers via target capture that utilizes magnetic microparticles. The capture oligomers contain sequences complementary to specific regions of the HPV mRNA target molecules as well as a string of deoxyadenosine residues. During the hybridization step, the sequence-specific regions of the capture oligomers bind to specific regions of the HPV mRNA target molecule. The oligomer:target complex is then captured out of solution by decreasing the temperature of the reaction to room temperature. This allows hybridization to occur between the deoxyadenosine region on the capture oligomer and the poly-deoxythymidine molecules that are covalently attached to the magnetic particles. The magnetic particles,

including the captured HPV mRNA target molecules bound to them, are pulled to the side of the reaction tube using magnets and the supernatant is aspirated.

See Figure 11: Target Capture specimen processing technology, (Appendix 5)

After target capture is complete, the HPV mRNA is amplified via TMA, which is a transcription-based nucleic acid amplification method similar to NASBA that utilizes two enzymes, MMLV reverse transcriptase and T7 RNA polymerase. The reverse transcriptase is used to generate a DNA copy of the target mRNA sequence containing a promoter sequence for T7 RNA polymerase. T7 RNA polymerase produces multiple copies of RNA amplicon from the DNA copy template.

See Figure 12: TMA technology (Appendix 5)

Detection of the amplicon is achieved by the Hybridization Protection Assay (HPA) using single-stranded nucleic acid probes with chemiluminescent labels that are complementary to the amplicon. The labelled nucleic acid probes hybridize specifically to the amplicon. The Selection Reagent differentiates between hybridized and unhybridized probes by inactivating the label on the unhybridized probes. During the detection step, light emitted from the labelled RNA:DNA hybrids is measured as photon signals in a luminometer and are reported as Relative Light Units (RLU). The internal control (IC) is added to each reaction via the Target Capture Reagent that contains the Internal Control.

5.4 Overview of analytical characteristics

The tables in Appendix 1 shall demonstrate based on the limit of detection for HPV16 the variability in the analytical sensitivities between either Group-Tests, or mixed Group-and/or Genotyping Tests.

5.4.1 Analytical characteristics of individual HPV assays

5.4.1.1. Analytical Sensitivities of the HPV tests

Table 1: Characteristics of Nucleic acid detection tests of class 1/2a carcinogenic HPV types, see appendix 1.

5.4.1.2 Analytical Sensitivities of the HPV tests¹

See appendix 2.

5.4.1.3 Analytical Specificities of the HPV tests

See appendix 3.

5.4. Conditions that influence reliability of the HPV tests

The basic prerequisite for reliable performance of HPV DNA detection and typing is the good quality of a sample. To obtain a good quality the sampling has to be done with the recommended device (see 5.5.), from the transformation zone and the endocervix or from a clinically visible lesion (ChXX). Very important for keeping the viral nucleic acids intact is the temperature of short and long term storage. These conditions vary according to the collection and transport medium and one should comply with the conditions specified on the respective leaflet of the HPV test. Another major factor influencing the performance of the HPV test is the method and accuracy of DNA extraction, which is not dealt with in this chapter.

5.5 Specimen sampling and transport media

Sampling for HPV detection is usually done together with collection of cells for cytology analyses. As an alternative for collection of material for HPV detection a large variety of approaches for self-sampling has been explored. For collection of cells done by physician there are basically two possibilities. In the settings where liquid-based cytological systems (LBC) are used HPV detection is being done from the residual material and therefore the collection of material is performed in the same way as sampling for cytology. In settings where classical cytology is being used the samples for HPV detection are taken usually after the collection of cytology sample. The order of sample collection does not seem to influence the number of HPV positive samples however the quality of the cytology result (Taha et al., 2006). When colposcopy is performed the sampling for HPV detection has to be done prior to the application of the acetic acid or iodine on the cervical epithelium.

¹ The values given are taken or recalculated from information published by the respective manufacturers

See Fig. 13-15 Cervical cytology collections devices (left to right): cytobrush, spatula, cervibroom, (Appendix 4)

5.5.1. Transport media

Media formulated for the preservation of viral nucleic acids

Currently there is only one medium **Specimen Transport Medium (STM, QIAGEN GmbH, Hilden, Germany)** formulated for the preservation of viral nucleic acids which has FDA approval to be used for samples processed by *digene* Hybrid Capture® 2 (HC2, QIAGEN GmbH, Hilden, Germany) tests.

Few other sampling and storage media are provided by manufactures of commercial HPV tests, e.g. PapilloCheck Collection Kit (Greiner Bio-One GmbH, Frickenhausen, Germany) and APTIMA Cervical Specimen Collection and Transport medium (Gen-Probe Incorporated, San Diego, CA, USA). Cobas® PCR Cell Collection Medium is a new transport medium of Roche Diagnostics GmbH preserving cellular samples for HPV-DNA tests.

Media formulated for cellular preservation

PreservCyt solution (PC, Cytoc, Boxborough, MA, USA), Surepath Preservative Fluid (SP, TriPath Imaging inc., Burlington, NC, USA), Thermo Fisher Papsin (TFP, Thermo Fisher Scientific, Runcorn, Cheshire, UK) and Universal Collection Medium (UCM, Digene Brasil, Sao Paulo, Brasil) are optimized for cellular preservation and are used for liquid-based cytology. PC and SP is FDA-approved for HPV testing directly from the same vial with HPV DNA testing with the *digene* Hybrid Capture® 2 (*digene*® HPV Test, QIAGEN GmbH, Hilden, Germany) and Cervista™ HPV HR, Cervista™ HPV 16/18 (Third Wave Technologies, Madison, WI, USA) tests.

Cells preserved by both widely used media - PC and SP - are suitable for HPV DNA detection, even though it has been shown that DNA is less stable in SP medium in comparison to PC. RNA extraction is only possible from PC medium (Castle et al., 2003; Depuydt et al., 2003; Powell et al., 2006) PC as well as SP specimen for cytological analyses may be stored for weeks at room temperature or at refrigerated temperature (2-8°C) but because of the high alcohol content cannot be frozen.

Another option for the collection of samples for HPV detection is **self-sampling** which has the advantage of being cheaper and suitable especially for populations difficult to

reach. Many approaches were examined – Dacron or cotton swab, tampon, cervicovaginal lavage, cytobrush, urine specimens.

It can be summarized that so far there is insufficient evidence to conclude that self-sampling is an alternative option to conventional Pap test. But the concordance in the HPV prevalence in the self- and clinician samples is high enough throughout studies to justify the use of self-sampling in the under-resourced areas, for accession of women in the remote areas and those reluctant to participate in the screening programs. Future research is needed to clarify the optimal type of self-sampling technique and to show if this approach will help to increase the participation rate in the screening programs.

See Figure 16: Examples of some self-sampling devices for collection of material for HPV detection, A/ Qvintip, B/ device for Mermaid cervicovaginal method, C/ FTA elute cartridge D/ Fournier device (Appendix 4.)

Table 2. Validated media for commercially available tests, see appendix 4

5.6 Special precautions for target amplification assays

See Appendix 4

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Appendix 1

Group Tests				
Test	Copy number per reaction for HPV 16	Copies/ml	FDA	CE
DNA				
HC2/Qiagen	6216	124324		
Cervista HR HPV/Hologic	1250-2500			
AMPLICOR HPV Test/Roche Molecular Systems	10	100		
AID HPV screening kit/GenID GmbH	1000			
RNA				
NucliSENS EasyQ HPV/BioMérieux		260		
APTIMA HPV Test/Gen-Probe	106			

Mixed Group and Genotyping Tests				
Test	Copy number per reaction For HPV 16	Copies/ml	FDA	CE
cobas®4800 HPV Test / Roche Molecular Systems	40	600		
RealTime High Risk HPV test / Abbott	500*			
AID HPV typing kit / Autolmmun Diagnostika GmbH	1000			

*Greater than 95% probability of detection.

Genotyping Tests				
Test	Copy number per reaction for HPV 16	Copies/ml	FDA	CE
Cervista™ HPV 16/18 /Hologic	625-1250			
<i>digene</i> HPV Genotyping RH Test/ Qiagen	4			
<i>digene</i> HPV Genotyping LQ! Test / Qiagen	5			
INNO LiPA Genotyping Extra/Innogenetics	2-5000			
HPV-DNA Chip/ Biomedlab	~30.000			
LINEAR ARRAY HPV Genotyping Kit/Roche Molecular Systems	21	200		
Papillocheck/Greiner Bio-One	50			
PCR HPV Typing Set/ Takara Bio INC.	1000-10000			
PCR HPV Detection Set/Takara Bio INC.	1000-10000			
CLART HPV2/ Genomica	10			
PapType/ Genera Biosystems	500			
ProDect® Chip HPV Typing	600			CE

Table 1: Characteristics of Nucleic acid detection tests of class 1/2a carcinogenic HPV types

Please use control plus left mouse click to activate the hyper links

Test name/ Company or reference (state 2009)	Types detected	Test classific ation A)Signal - Amplific ation B)Target - Amplific ation	Nucleic acid target, size	Test principle and detection method	Level of control: A)Sample adequacy B)Assay control C)PCR product contamina tion D)Neither	Performance <u>A) Analytical Sensitivity</u> (Copy number per reaction depending on HPV type) <u>B) Analytical Specificity</u> CR) cross- reacts with other HPV types NCR) no cross-reaction with other HPV types described Further details in Appendix 2	Certifica tion A) CE B) FDA C) None
At least Class 1/2a group detection							
HC2/ Qiagen	16,18,31,33, 35,39,45,51, 52,56,58,59, 68	A	DNA	Hybrid capture/ Enzymatic luminescen ce	B	A) 4500-8000 B) CR at least with 12 types	A, B
Cervista HR HPV/ Hologic	16,18,31,33, 35,39,45,51, 52,56,58,59, 66, 68 3 probe sets: <u>A5/A6</u> : 51, 56, 66 <u>A7</u> : 18, 39, 45, 59, 68 <u>A9</u> : 16, 31, 33, 35, 52, 58	A	DNA	Invader method/ Fluorescen ce resonance energy transfer (FRET)	A B	A) 1250-7500 B) CR with HPV67 and HPV70	A, B
AMPLICOR HPV Test/ Roche Molecular Systems	16,18,31, 33, 35,39,45, 51, 52,56,58, 59, 68	B	DNA L1 : 165bp	PCR/ Multiwell Plate hybridizatio n, enzymatic	A B C	A) 10-25 B) NCR with low risk HPV types and 74 microorganism	A

				colour reaction		s	
APTIMA HPV Test/ Gen-Probe	16,18,31,33, 35,39,45,51, 52,56,58,59, 66, 68	B	mRNA E6 E7	TMA/ chemoluminescent labels	A B	A)20-500 B) NCR with low risk HPV types and bacteria, yeast and fungi	A
AID HPV screening kit/ GenID GmbH	hrHPV's: 16,18,31,33, 35,39,45,51, 52,56,58,59,66, 68,73,82 lrHPV's: 6,11,40,42, 43,44	B	DNA E1	PCR/ reverse hybridization on filter strips, colorimetric detection	A B	A) at least <u>1000</u> B) NCR with 17 HPV types and C. trachomatis, HSV1/2, Trep. Pallidum and Neiss. gonorrhoea	A
cobas® 4800 HPV Test / Roche Molecular Systems (for automated HPV testing)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 Itemization of HPV 16 and 18	B	DNA L1: 200bp	PCR/ The detection of the amplicon is performed during thermal cycling using oligonucleotide probes labeled with four different fluorescent dyes	A B C	A) 5-160 B) NCR with 22 HPV types and 83 microorganisms	A
RealTime High Risk HPV test / Abbott	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 differentiation between 16,18 and non 16/18	B	DNA L1	real time PCR / multi color detection system during the last 38 cycles	A,B	A) 500-2000 B) NCR with 15 HPV types	A
partially class 1/2a group detection							
PreTect HPV-Proofer/ Norchip	16, 18, 31, 33, 45	B	mRNA E6 E7	NASBA/Molecular Beacons real-time detection technology		A) 150-250 B) NCR with HPV6/11, 35 and 51	A
NucliSENS EasyQ HPV/ bioMérieux	16,18,31,33, 45	B	mRNA E6 E7	NASBA real-time nucleic acid	A B	A) 230-30.000 B) not	A

				amplification/ molecular beacon probes for real-time detection		determined for HPV cross reactivity ,	
HPV OncoTect/ inCellDx	n.a	A	mRNA E6 E7	in situ in intact human cells/ flow cytometry or image analysis.	A B	A) $5 \cdot 10^6$, E7mRNA copies per cell B) n.a.	A
Type- specific detection							
Cervista™ HPV 16/18 / Hologic	16, 18	A	DNA	Invader method/ Fluorescence resonance energy transfer (FRET)	A B	A) HPV 16-18 625-1250 B) NCR with 19 HPV types	A?
<i>digene</i> HPV Genotyping RH Test/ Qiagen	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	B	DNA	PCR (GP5+/6+ primer) based reverse hybridization assay	A B	A) 4-100.000 B) NCR	A
<i>digene</i> HPV Genotyping LQ! Test / Qiagen	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	B	DNA	PCR (GP5+/6+ primer) based assay which uses xMAP technology on the LiquiChip System	A B	A) 1-10.000 B) NCR	A
INNO LiPA HPV Genotyping Extra/ Innogenetics	6,11,16,18, 26,31,33,3 5,39,40,43, 44,45,51,5 2,53, 54,56,58,5 9, 66,68/73,6 9, 70;71,74,8 2	B	DNA L1 : 65bp	PCR / reverse hybridization on membrane strips, enzymatic colour reaction	A B C	A) 20-700 B) NCR	A
SPF10- LiPA25/ Labo Bio- medical Products	6,11,16,18, 31,33, 34 ,35 ,39,40,42,4 3 ,44 ,45, 51,52 ,53 ,54, 56,58	B	DNA L1 SPF10 Primer	PCR/ reverse hybridization on membrane strips, enzymatic	A B	A) B) NCR	A

	,59,66,68, 70, 74			colour reaction			
HPV-DNA Chip/ Biomedlab	6,11,16,18, 31,33,34,3 5,39,40,42, 43,44, 45,51,52,5 6, 58,59,66,6 8, 69	B	DNA L1	PCR/ Microarray with specific oligoprobes	A B (?)	A) Appr. 30.000B) n/a	C
LINEAR ARRAY HPV Genotyping Kit/ Roche Molecular Systems	6,11,16, 18,26,31,3 3,35,39,40, 42,45,51,5 2,53,54,55, 56,58,59,6 1,62,64,66, 67,68,69,7 0,71,72,73(MM9),81, 82(MM4),8 3(MM7),84 (MM8),IS3 9 and CP6108	B	DNA L1 : 450bp	PCR (consensus PGMY Primers) / reverse line blot hybridizatio n enzymatic colour reaction	A B C	A) 6-31.000 B) specificity NCR to other HPV types and 62 microorganism s	A
Papillocheck / Greiner Bio- One	6,11,16,18, 31,33,35,3 9,40,42,44, 45,51,52, 53,55,56,5 8,59,66,68, 70,73,82 (44 and 55 coupled)	B	DNA E1 : 350bp	PCR (consensus primers) / oligoprobes immobilize d on a DNA chip	A B C	A) _20-750 B) CR HPV55 cross-reacts with HPV44 probe; HPV13 cross-reacts with HPV11 probe	A
PCR HPV Typing Set/ Takara Bio INC.	16,18,33,5 2b, 58 lr HPV's 6,11	B	DNA E6 E7 : 300 bp	PCR (consensus primers) / digestion of PCR products by restriction and subsequent agarose gel electrophor esis		A) $10^3 - 10^4$ B) n/a	C
PCR HPV Detection Set/ Takara Bio INC	16,18,33	B	DNA E6 :140 bp	PCR/ detection by agarose gel electrophor esis or dot blot	B	A) 1000 B) n/a	C
HPV HR Genotyping Assay/ GenoID	16,18,31,3 3,35,39,45, 51,52,56,5 8,59,66,68	B	DNA L1	PCR/ solid phased hybridizatio n.		A) not specified in the instructions	A

				enzymatic colour reaction		B) n/a	
Full Spectrum HPV Assay/ GenID	lr HPV's 6, 11, 42, 43, 44/55 hr HPV's 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68 other HPV's 2, 3, 7, 10,13, 26, 27, 28, 29, 30, 34, 40, 53, 54, 57, 61, 67,70, 72, 73, 74, 81, 82, 83, 84, 85, 89, 90, 91	B	DNA L1	PCR/ solid phased hybridization. enzymatic colour reaction		A) not specified in the instructions B) n/a	A
AID HPV typing kit/ GenID GmbH	6,11,16,18, 45 qualitative detection of 30ies and 50ies hrHPV types (31,33,35,39,51,52,53, 56,58,59) unspecific detection: 66,68,73,82,40,42,43, 44	B	DNA E1	PCR/ reverse hybridization on filter strips, colorimetric detection	A B	A) 1000 B) n/a	A
CLART HPV2/ Genomica	6,11,16,18, 26,31,33,35,39,40,42, 43,44,45,51,52,53,54, 56,58,59,61,62,66,68, 70,71,72,73,81,82,83, 84,85,89	B	DNA L1 :450bp	PCR/ Biotinylated amplicons hybridise to specific probes attached to low density microarray surface,enzymatic colour reaction	A B	A) 10-10.000 B) NA	A
ProDect [®] Chip HPV Typing/ Bcs Biotech S.p.A	6,11,16,18, 31,33,35,39,42,43,44, 45,51,52,56,58,59,68, 73	B	DNA E6/E7 L1 β Globin	PCR/ reverse hybridization on pre-coated 8-well strip	A B	A) 600-6.000 B) NA	A
Chipron	6,11,16,18,	B	NA	PCR/revers	NA	NA	NA

	31,33,35,39,42,44,45,51,52,53,54,56,58,59,61,62,66,67,68,70,72,73,81,82,83,84,90,91			e hybridization on a macro-array			
PapType/ Genera Biosystems	6,11,16,18,31,33,35,39,45,51,52,56,58,59,66,68	B	DNA	PCR/ hybridization on silica beads and detection by flow cytometry	A B C	A) 500 B) n/a	C
F-HPV typing/ molGENTIX	6,11,16,18,31,33,35,39,45,51,52,56,58,59,68	B	DNA E6 E7	multiplex PCR with a set of 16 fluorescently labelled primers/ identification by colour and size (electrophoresis)	A B	A) n.a B) n.a	A

Appendix 2

Analytical Sensitivities of the HPV tests (The values given are taken or recalculated from information published by the respective manufacturers)

Hybrid Capture 2 - Qiagen:

HPV DNA Type	Copies/ml	Copy Number per reaction
6	101132	7585
11	85924	6444
16	82882	6216
18	79841	5988
31	76799	5760
33	102652	7699
35	84403	6330
39	105694	7927
42	91246	6844
43	64633	4847
44	88965	6672
45	86684	6501
51	59310	4448
52	104173	7813
56	47144	3536
58	62352	4676
59	83643	6273
68	90486	6786

The *digene* HPV eHC Test

The Digene HPV eHC Test has an approximative sensitivity of 950 copies/assay (mean of all HPV types included)

The *careHPV* Test

The assay analytical sensitivity with a signal to noise ≥ 2 can detect 1,500 copies of HPV as the lowest limit of detection.

Cervista HR HPV - Hologic:

HPV DNA Type	Copy Number per reaction
16	1250-2500
18	1250-2500
31	1250-2500
33	2500-5000

35	5000-7500
39	2500-5000
45	1250-2500
51	2500-5000
52	1250-2500
56	1250-2500
58	2500-5000
59	2500-5000
66	2500-5000
68	2500-5000

AMPLICOR HPV Test -Roche:

HPV DNA Type	Copies/ml	Copy Number per reaction
16	100	10
18	100	10
31	240	25
33	100	10
35	100	10
39	100	10
45	100	10
51	100	10
52	240	25
56	100	10
58	240	25
59	240	25
68	100	10

APTIMA HPV Test - Gen-Probe:

HPV DNA Type	Copy Number per Reaction (DTS System)	Copy Number per Reaction (TIGRIS DTS System)
16	106	45
18	116	123
31	38	24
33	39	47
35	43	56
39	41	17
45	54	69
51	488	195
52	357	275
56	127	83
58	301	93
59	98	73
66	64	145
68	143	46

PreTect HPV-Proofer - Norchip:

The test detects at least 150-200 copies of HPV16 genomes and at least 250 copies of HPV18 genomes per reaction.

AID HPV screening kit - GenID GmbH:

The test detects at least 10^3 copies of HPV genomes per reaction not further specified.

cobas®4800 HPV Test - Roche:

cobas 4800		
PreservCyt-Medium		
HPV-DNA-Typ	Kopien/mL	Kopien/PCR
16	600	40
18	600	40
31	300	20
33	190	13
35	480	32
39	80	5
45	190	13
51	100	7
52	2.400	160
56	1.400	93
58	480	32
59	190	13
66	640	43
68	450	30

SurePath-Medium		
HPV-DNA-Typ	Kopien/mL	Kopien/PCR
16	300	20
18	600	40
31	150	10
33	480	32
35	1.400	93
39	190	13
45	480	32
51	100	7
52	7.200	480
56	1.400	93
58	1.400	93
59	480	32
66	640	43
68	1.100	73

RealTime High Risk HPV test – Abbott:

HPV DNA Type	Copy Number per Reaction*
16	500
18	500
31	2000
33	2000
35	500
39	500
45	500
51	500
52	2000
56	2000
58	5000
59	500
66	500
68	500

*Corresponding to >95% probability of detection.

Cervista™ HPV 16/18 – Hologic:

HPV DNA Type	Copy Number per reaction
16	625-1250
18	625-1250

***digene* HPV Genotyping RH Test – Qiagen:**

HPV DNA Type	Copy number per reaction
16	4
18	8
26	1000
31	10
33	10
35	10
39	1000
45	23
51	1000
52	1000
53	100000
56	10
58	100
59	100
66	100
68a	10000
68	100000
73	10000
82 MM4	100000
82 IS39	10000

***digene* HPV Genotyping LQ Test – Qiagen:**

HPV DNA Type	copy number per reaction
16	5
18	8
26	100
31	10
33	1
35	10
39	100-1000
45	20
51	100
52	100-1000
53	10000
56	10
58	100
59	100
66	10
68a	1000
68	10000
73	100
82 MM4	1000

INNO-LiPA HP Genotyping Extra – Innogenetics:

The limit of detection for HPV16,18, 31, 45 and 52 ranged from 20 to 700 copies per reaction.

NucliSENS EasyQ HPV – bioMérieux :

HPV DNA Type	HPV RNA (copies/ml)
16	260
18	6400
31	30000
33	3000
45	230

HPV-DNA Chip – Biomedlab:

The analytical sensitivity is for HPV16 and HPV18 5pg/ml, which is equivalent to ~30.000 copies/reaction.

LINEAR ARRAY HPV Genotyping Kit – Roche:

LINEAR ARRAY Assay		
PreservCyt-Medium		
HPV-DNA-Typ	Copies/mL	Copies/PCR
6	2.319	242
11	900	94
16	195	21
18	580	60
26	2.935	306
31	1.863	194
33	4.000	417
35	466	49
39	1.367	142
40	70.000	7.292
42	30.000	3.125
45	401	42
51	181	19
53	256	27
54	900	94
55	900	94
56	6.915	720
58	185	19
59	53	6
61	900	94
62	900	94
64	300.000	31.250
66	250	26
67	30.000	3.125
68	848	88
69	900	94
70	900	94
71	900	94
72	900	94
73	165	17
81	900	94
82	8.089	843
83	900	94
84	900	94
CP6108	900	94
IS39	1.500	156

Papillocheck - Greiner Bio-One:

HPV DNA Type	copy number per reaction
6	30
11	150
16	50
18	300
31	300
33	300
35	750
39	30
40	30
42	30
43	100
44	20
45	50
51	30
52	100
53	30
56	30
58	150
59	50
66	100
68	30
70	30
73	100
82	30

AID HPV typing kit - GenID GmbH:

The assay detects at least 10^3 copies of HPV genomes not further specified.

CLART HPV2 – Genomica:

HPV DNA Type	Copies per reaction
6	100
11	100
16	10
18	10000
31	100
33	100
35	10
39	10
45	100
51	100
52	100
56	10
58	10
59	1000
68	100
73	10000
82	10

ProDect CHIP HPV TYPYNG – bcs BIOTECH S.p.A.

Has an analytical sensitivity to detect $6 \cdot 10^2$ - $6 \cdot 10^3$ HPV DNA equivalent copies. .

Appendix 3

Analytical Specificities of the HPV tests

Hybrid Capture 2 - Qiagen:

- Certain amount of cross-hybridization between low risk HPV types 6 and 42 and the high risk probe.
- Cross reaction with HPV types 40, 53 and 66
- It has also been reported that there could be cross-hybridization with HPV types 11, 53, 54, 55, 66, MM4, MM7, MM8 or MM9
(HC2 instructions p. 40)

digene HPV eHC test

The *digene* HPV eHC Test shows no cross reactivity to other HR HPV types and LR HPV types at 10^7 copies/assay

The *careHPV* Test

Studies indicate that the *careHPV* Test does not cross-react with 250ng of human genomic DNA. The assay does not cross-react with the following micro-organisms: *Acinetobacter lwoffii*, *Acinetobacter* sp., *Chlamydia trachomatis*, type G, *Escherichia coli*, *Escherichia coli* (HB101), *Haemophilus ducreyi*, Human herpesvirus 2, *Lactobacillus acidophilus*, *Mobiluncus curtisii*, *Mobiluncus mulieris*, *Mycoplasma hominis*, *Neisseria lactamica*, *Neisseria sicca*, *Prevotella melaninogenica*, *Staphylococcus aureus*, *Treponema phagedenis*, *Ureaplasma urealyticum*.

Additionally, sequence analyses (blast method) were completed for the following to ensure there were no overlapping sequences that could be cross-reactive:

HIV, HBV, EBV, CMV, Adenovirus 2 and *Neisseria meningitidis*.

There is no interference with any of the following substances tested at levels greater than or equal to clinical relevance or routine product use: 10% Blood, Spermicide, Douche Vaginal Lubricant.

Cervista HR HPV - Hologic:

Human papillomavirus types 67 and 70 yielded positive results with the Cervista™ HPV HR test at 1×10^5 and 1×10^7 copies/reaction.

(Cervista HR HPV instructions p. 30)

AMPLICOR HPV Test - Roche Molecular Systems:

No cross reaction with low risk HPV types (6, 11, 26, 40, 42, 43S, 44, 53, 54, 55B, 57, 64, 66, 67, 70), and 64 other microorganisms (bacteria, fungi, virus), that can be present in cervix specimens.

(Instructions p.18)

APTIMA HPV Test - Gen-Probe:

No effect on APTIMA HPV Assay specificity or sensitivity was observed with low risk HPV's 6, 11, 42, 43, 44, 53, 61, 71, 81. or bacteria, yeast and fungi. (Introduction p. 22, 23)

AID HPV screening kit - GenID GmbH:

No cross-reactivity was observed when evaluated with cloned target DNA of 17 HPV genotypes as well as with human genomic DNA and genomic DNA of the following tested microorganisms: Chlamydia trachomatis, human Herpes Virus 1 and 2, Treponema pallidum and Neisseria gonorrhoea.

cobas®4800 HPV Test - Roche Molecular Systems:

No cross reaction observed with HPV types 6, 11, 26, 40, 42, 54, 55B, 61, 62, 64, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89 (CP6108) and 83 microorganisms (bacteria, fungi, virus), that can be present in cervix specimens.

(Instructions p. 11)

RealTime High Risk HPV Test – Abbott:

No cross reactivity observed with HPV types 6, 11, 13, 26, 30, 32, 40, 42, 43, 44, 53, 54, 55, 57, 61.

(Instructions p. 44, 45)

PreTect HPV-Proofer - Norchip:

No cross-reactivity detected with HPV6/11, 35 and 51

Cervista™ HPV 16/18 - Hologic:

Cloned DNA or PCR amplicons for the following HPV types were tested using the Cervista™ HPV 16/18 test. (HPV 1a, 6, 11, 31, 35, 39, 42, 43, 44, 45, 51, 52, 53, 58, 59, 66, 67, 68, 70) All samples yielded negative results.

(Instructions p.30)

***digene* HPV Genotyping RH Test – Qiagen:**

None of the probes showed any reaction with an amplicon from a non-targeted HPV type.

***digene* HPV Genotyping LQ! Test – Qiagen:**

None of the probes showed any reaction with an amplicon from a non-targeted HPV type.

INNO-LiPA HPV Genotyping Extra – Innogenetics:

No cross-reactivity observed.

LINEAR ARRAY HPV Genotyping Kit - Roche Molecular Systems:

HPV genotype specificity was positive only to the expected probe line for the 37 low and high risk HPV genotypes (evaluated with plasmid DNA or in case of HPV genotype 52 a clinical specimen). Cross-reactivity to other HPV types was not tested. There was no cross-reaction with 62 tested microorganisms (bacteria, fungi, virus), that can be present in cervix specimens.

(Instructions p. 23)

Papillocheck - Greiner Bio-One:

HPV55 gives a signal on the HPV44 probe. Because of this, the CheckReport™ software displays HPV44/HPV55 as one result.

HPV13 can cross-hybridize on HPV11 but there is no report in the scientific literature that HPV13 appears in in cervical specimens.

(Instructions p.26)

CLART HPV2 - Genomica:

The analytical specificity is 100%. **CLART[®] Human Papillomavirus 2** kit detects no other pathogens that might be found in cervicovaginal samples, such as herpesvirus.

ProDect CHIP HPV TYPYNG – bcs BIOTECH S.p.A.:

Specificity is equal to 100% when validated with confirmed negative samples or positive samples for different pathogens which can be interfering markers. Average specificity of 98% when validated among different HPV types (96% when using byotinilated synthetic controls).

Appendix 4: Additional information on the different HPV tests from Chapter 5.3

The Hybrid Capture 2 HPV Test

The HC2 is currently available in a 96-well microplate format, is easy to perform in clinical settings and is suitable for automation. Furthermore, HC2 does not require special facilities to avoid cross-contamination, since it does not rely on target amplification to achieve high sensitivity, as do PCR protocols. For clinically useful purposes, only the high risk probe mix is used; this reduces time and cost of the test. More recently, based on this Hybrid Capture technology an assay (*i.e. digene* HPV Genotyping PS Test) has been developed allowing genotyping for HPV 16, 18 and 45. For high volume laboratory testing Qiagen has developed a fully automated device for HC2 testing called the Rapid Capture System (RCS) that allows robotic handling of 96 well microplates. This robot station performs specimen transfer, all pipetting operations, incubations, shakings and washings. However, the denaturation of specimens in the sample device tubes still has to be performed by hand. This automatic station increases the accuracy of the test and allows a single user to test 352 specimens within 6 hours.

Several studies have noted that the HC2 high risk probe mix to a certain extent cross-reacts with HPV types that are not represented in the probe mix (Peyton et al., 1998; Peyton et al., 2001; Vernon S.D. et al., 2000). Peyton and colleagues (Peyton et al., 1998) found that HC2 using the high risk probe at a 1.0 pg/ml cut-off detected HPV types 53, 66, 67, 73, as well as other undefined types, and raising the cut-off to 10.0 pg/ml did not completely eliminate the cross reactivity to types 53 and 67. Cross-reactivity of HC2 high risk probe to HPV types that have a significant risk for cervical cancer may be considered beneficial, but cross-reaction with low risk types causes false positive results and may decrease the specificity of the test (Castle et al., 2002).

The *digene* HPV eHC Test

The test allows the qualitative detection of 15 HR-HPV types (16/18/31/33/35/39/45/51/52/56/56/59/66/68/82) using a probe cocktail. Nucleic acid

hybridization assay with signal amplification is performed on a fully automated platform called QIAensemble (enhanced Hybrid Capture technology).

The *care*HPV Test

The test is broadly based on the HC2 test with some important differences. The assay time is 2.5 h, compared with up to 6 h for HC2. The *care*HPV Collection Medium, unlike other collection media, contains no toxic chaotropic salts, but rather contains non-toxic surfactants and is formulated for solubilisation of cervical specimens from the collection brush without any requirement for extended mechanical shaking. The capture microplates in HC2 are replaced by magnetic beads coated by a monoclonal antibody with high affinity to RNA-DNA hybrids. Furthermore, the temperatures of some steps in the *care*HPV assay are increased to decrease the overall assay time by more than 2 h. The principle of the assay is as follows: target HPV DNA from lysed cells is denatured and hybridised to full-length complementary RNA, then captured by monoclonal antibodies coated on paramagnetic beads. The captured hybrids on the beads are detected by antihybrid monoclonal antibody conjugated to calf intestine alkaline phosphatase, which reacts with an added chemiluminescent substrate to produce light in proportion to the number of bound alkaline phosphatase molecules along the hundreds of antigenic binding sites per target molecule. Specimen test findings are expressed in relative light units (RLU) and compared with the mean RLU from a minimum positive control set at 1 pg/mL of HPV-16 DNA (expressed numerically as the cutoff) resulting in a ratio, the RLU/cut-off , the proportion of which is indicative of clinical positivity. Because the output signals of both HC2 and the new *care*HPV test are quite linear over a broad range around the cut-off –point ratio (RLU/cutoff) of 1.0, there is the possibility to vary the cut-point by adjusting the calculations to indicate specimen positives at lower or higher values than the value of positive controls, thus a cut-point of 0.5 reflects an assay that can score 0.5 pg/ml of HPV-16 DNA as positive. The testing itself is conducted on easily portable equipment and will run on batteries.

The Cervista Test

The probe oligos cycle rapidly on and off the target; each time an intact probe molecule binds to the specific target in the presence of the Invader oligo, the invasive triple structure is formed and cleavage can occur. The number of reporter DNAs (flaps) released is relative to the amount of target DNA in the sample, allowing for quantitative detection of HPV DNA. In a secondary reaction the released reporter DNAs from the primary reaction serve as Invader oligos by hybridizing to a labelled, synthetic oligo, the fluorescence resonance energy transfer (FRET) probe that carries a fluorescent dye in close proximity to a quencher. Cleavage of this FRET probe by the cleavase enzyme results in the release of the dye that is no longer in proximity to the quencher and can emit light after excitation with an appropriate wavelength and generate a fluorescent signal. Again each released reporter DNA (5' flap) from the primary reaction cycles on and off the cleaved and uncleaved FRET probes, enabling cleavage of many FRET probes in the secondary reaction to further amplify the target-specific signal. Because these two cleavage reactions occur simultaneously, they can produce 1 million to 10 million labelled cleavage products per target sequence. A standard 4-hour reaction produces over 10 million-fold signal amplification. Primary and secondary reactions are carried out simultaneously at a constant temperature in a single tube.

DNA in situ hybridisation or immunohistochemical detection of HPV proteins

Staining of hybrids can be achieved by fluorescent detection or coloured substrate deposition and bright field microscopy. The relatively small size of the HPV genome (7.8 kb) and consequently the probe generally precludes direct detection of hybrids and therefore some type of signal amplification is necessary. A commercially available HPV ISH system (Ventana Inform HPV Davis-Devine et al., 2005) makes use of an indirect biotin-streptavidin method, which, however, lacks sufficient sensitivity for high-grade cervical lesions. Alternatively, tyramide signal amplification (TSA), also known as catalysed reporter deposition (CARD) can be used, both in fluorescent and bright field applications. Also for the CARD method, a commercially available system exists (DAKO-Oxoid GenPoint™ HPV test; Birner et al., 2001). Despite the fact that CARD enhances sensitivity, HPV ISH assays generally suffer

from low sensitivity and from the fact that either only a single type or a group of types without further differentiation can be detected per sample.

Examples of PCR-based HPV testing systems

GP5+/6+-PCR system

De Roda Husman and colleagues (1995) developed the GP5+/6+ PCR system as a refinement of the original GP5/6 system (Snijders et al., 1990) through an elongation of the 3' end of the primers to enhance their affinity for highly conserved HPV DNA sequences. Analysis of the new primers on purified HPV DNA demonstrated that they were 10-100 times more sensitive than the original GP5/6 primers, having an analytical sensitivity at the femtogram (fg) level for highly complementary HPV types, and at the picogram level for less complementary types when using a radioactive Southern blotting procedure for amplicon detection. GP5+/6+-PCR followed by an EIA-based detection subsequently revealed analytical sensitivities for cloned HPVs ranging from 0.5fg to 10fg, depending on the type (Jacobs et al., 1997). Ever since several modified versions have been deduced from this assay aiming at better targeting HPV types that do not react that efficiently with GP5+/6+ primers. These include the BSGP5+/6+-PCR (Schmitt et al., 2008), the Abbott RealTime High Risk HPV test, and MGP PCR (Söderlund-Strand et al., 2009). In addition, various read-out systems have been described for GP5+/6+-PCR and its derivatives, as indicated below.

PGMY09/11 system

The PGMY09/11 primer system was developed by Gravitt and colleagues (2000) to address some limitations of the traditional MY09/11 degenerate primer system. The demonstrated sensitivity for the PGMY09/11 primer system is 10 HPV genomes per PCR amplification for all representative genotypes. PGMY09/11 is comprised of two non-degenerate pools of oligonucleotide primers designed to amplify the same 450 bp region of the L1 gene as the original MY09/11 primers. Members of the primer pools were chosen using sequence alignments of all known genital HPV types and minimising any potential mismatches while simultaneously minimising the number of oligonucleotides in each pool. The upstream PGMY11 primer pool is

comprised of 5 oligonucleotides, while the PGMY09 pool contains thirteen. In a comparison of PGMY09/11 with the MY09/11 primer system, there was an overall agreement of 91.5% but the PGMY system picked up significantly more HPV positives. There was also a significant increase in the detection of multiple infections using the PGMY09/11 primers, and the amplification of certain HPV types that are inefficiently detected using MY09/11 primers.

SPF10-PCR system

Kleter and colleagues (1998) developed the first generation SPF-PCR (SPF1/2) amplification and detection system, with the currently used modified version thereof referred to as SPF10. This short fragment PCR system targets only a small, 65 bp, conserved region within L1. Given that PCR amplification efficiency is, in general, inversely related to the size of the region amplified, the SPF system displays a high sensitivity for HPV.

Read-out-systems

Enzyme immunoassays using microplates (EIA)

This method is nowadays used as prime read-out method for GP5+/6+-PCR (i.e. GP5+/6+-PCR-EIA) using separate probe cocktails for high-risk HPVs and low-risk HPVs. The probe cocktail for high-risk HPVs, containing oligoprobes specific for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, has been used in various clinical trials (Naucler et al., 2007; Bulkman et al., 2007; Zielinski et al., 2001.). An enzyme immunoassay read-out, referred to as DEIA, has also been developed for SPF amplicons (Kleter et al., 1998).

Another colorimetric method is the Roche AMPLICOR assay, employing an oligonucleotide set which amplifies a 165 bp fragment of the L1 gene of high-risk HPV types. This amplicon is immobilized using a pool of capture molecules bound to the wells of a microtitre well plate (MWP) and visualized by colorimetric detection with Roche AMPLICOR chemistry. Moreover, this test employs the the AmpliTaq® Gold DNA polymerase, which minimizes the amount of non-specific amplification and

increases the analytical sensitivity of the test. It has been reported that these primers detect about 13% more HPV in cervical smears than the PGMY primers.

Reverse hybridization assays using strips or filters

The PGMY primer system was evaluated using a reverse line-blot assay (Gravitt et al., 1998), which includes probes for 27 different HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, MM4, MM7, MM9 and 6, 11, 40, 42, 53, 54, 57, 66, MM8) along with two human beta-globin control lines. This reverse line-blot system was expanded to include 12 additional genital HPV genotypes (61, 62, 64, 67, 69, 70, 71, 72, CP6108, CP8304, IS39) described by Coutlée et al., (2006).

The short PCR fragment (SPF-PCR) (Kleter et al., 1998; Kleter et al., 1999) is designed to discriminate a broad spectrum of HPV types by reverse line blot hybridisation, which allows the identification of 24 different HPV genotypes, in the original LiPA assay (Kleter et al., 1998) or 28 in the new LiPA Genotyping Extra format (Ref for new lipa to add). A reverse line blot typing assay for the GP5+/6+ system capable of typing 37 mucosotropic HPVs has been developed, allowing for high-throughput testing both in epidemiological and clinical research (Jacobs et al., 1997).

Reverse hybridization assays using microsphere beads

The *digene* HPV Genotyping LQ Test consists of 2 kits: the *digene* HPV Genotyping LQ Test, Amplification Kit and the *digene* HPV Genotyping LQ Test, Detection Kit. The *digene* HPV Genotyping LQ Test, Amplification Kit provides the reagents needed for the HPV PCR amplification. The *digene* HPV Genotyping LQ Test, Detection Kit enables identification of high-risk (HR) human papillomavirus (HPV) genotypes using reverse hybridization.

A highly conserved L1 sequence is amplified using the GP5+/6+ PCR primers. Amplification is performed using HotStarTaq Plus DNA Polymerase. The GP6+ primer is biotinylated, enabling detection and analysis of amplified sequences using the *digene* HPV Genotyping LQ Test, Detection Kit with the LiquiChip system. Beta-globin primers allow co-amplification of human genomic DNA present in the clinical

samples and function as an internal control for PCR inhibition and adequate sample and DNA purification

The QIAGEN LiquiChip System is a flexible system for suspension arrays that uses bead-based xMAP technology. A wide variety of assay types, such as immunoassays, kinase enzyme assays, and interaction assays are performed in an aqueous, homogeneous format, both quickly and efficiently. Multiplexing of assays offers the potential for the simultaneous detection of up to 100 different analytes within a single sample. With xMAP technology, molecular reactions take place on the surface of colour-coded beads. For each pathogen, target-specific capture probes are covalently linked to a specific set of colour-coded beads. Labelled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A microfluidics system delivers the suspension hybridization reaction mixture to a dual laser detection device. A red laser identifies each bead (or HPV probe) by its colour-coding, while a green laser detects the hybridization signal associated with each bead (indicating the presence or absence of a particular amplimer). Identification of HPV genotypes is based on a reverse hybridization procedure, using xMAP technology. Denatured biotinylated amplimers, resulting from amplification of part of the L1 region with the GP5+/6+ primer set, are hybridized with specific oligonucleotide probes, which are immobilized on specific types of beads. After hybridization and stringent washing, streptavidin-conjugated R-Phycoerythrin is added, which binds to any biotinylated hybrid present. After incubation and additional stringent washing, the samples can be analyzed on the LiquiChip System. As an internal control for the presence of amplifiable DNA after isolation, a fragment from the human beta-globin gene is co-amplified with the HPV DNA in the form of a multiplex PCR. Bead type 44 contains a probe capable of detecting the beta-globin amplimer.

The Papillocheck low density Microarray test

For control reactions a fragment of the human ADAT1 (human tRNA-specific adenosine deaminase1) gene as well as an internal control testing for PCR quality are amplified in the PCR reaction. Hybridization efficiency is controlled through the addition of a labeled probe in the Hybridization Buffer hybridizing to specific DNA probes on the PapilloCheck® Chip. Moreover, the utilization of Uracil-DNA

Glycosylase further enhances the quality of the system in eliminating carry-over contaminations from previous PCR reactions.

Isothermal nucleic acid amplification methods

NASBA (Nucleic acid sequence based amplification) technology

Norchip and bioMérieux used this RT-NASBA technology together with Molecular Beacon probes and developed a real time assay, referred to as Prelect HPV Proofer, and NucliSENS EasyQ® HPV v1 assay, capable of the qualitative detection of transcripts of viral oncogenes E6/E7 (Jeantet et al., 2009). These assays enable to **directly detect** the expression of the HPV oncogenic (E6 and E7 mRNA).

Transcription-mediated amplification (TMA) in the Aptima Test

The Internal Control measures target capture, amplification, and detection. Internal Control signal in each reaction is discriminated from the HPV signal by the differential kinetics of light emission from probes with different labels. Internal Control specific amplicon is detected using a probe with a rapid emission of light (flasher). Amplicon specific to HPV is detected using probes with relatively slower kinetics of light emission (glower). The Dual Kinetic Assay (DKA) is a method used to differentiate between the signals from the flasher and glower labels.

The APTIMA HPV assay can be run on the TIGRIS DTS instrumentation system. The TIGRIS fully automates the target capture specimen processing, TMA and DKA detection steps. Approximately 500 samples can be processed in 8 hours and multiple specimen types including PreservCyt liquid based media and cervical swab samples can be processed within the same work list. A DTS semi-automated instrumentation system is also available for smaller laboratories.

Specimen sampling and transport media

Most common collection devices (Fig. 13) for cervical smears are a cervibroom, or a spatula and cytobrush (preferably used in combination) and the choice depends on practitioner preference or valid national guidelines. The advantage of cervibroom is that only one specimen needs to be collected. Recently also an improved version of

cervibroom - Cervex-Brush Combi is available and it has been shown that while giving the same amount of squamous cells, it increases the volume of endocervical cells in the sample two- to threefold (Fig. 14). Importantly, sampling with Cervex-Brush Combi yields significantly higher HPV viral load than sampling with Cervex-Brush and is also more sensitive in detecting HR HPV in samples with cytologically abnormal findings. (Depuydt et al., 2006) For the use with HC 2 test, the cervical brush is provided in DNAPAP Cervical Sampler (QIAGEN GmbH, Hilden, Germany) (Fig. 15). The most widely used LBC systems use similar but not interchangeable collection vials and devices. The major difference is if the device is being snapped off and placed in the fixative (e.g. Surepath) or if it is only swirled, then removed and discarded (e.g. ThinPrep). Dacron swab is not recommended for sampling for cytology analyses and it has been shown that the detection rate of HPV is lower when Dacron swab is used in comparison to cervical brush from DNAPAP cervical Samples (Qiagen) (Nindl et al., 1998; Peyton et al., 1998).

Fig. 13 Cervical cytology collections devices (left to right): cytobrush, spatula, cervibroom.



Figure 14: Cervex-Brush Combi

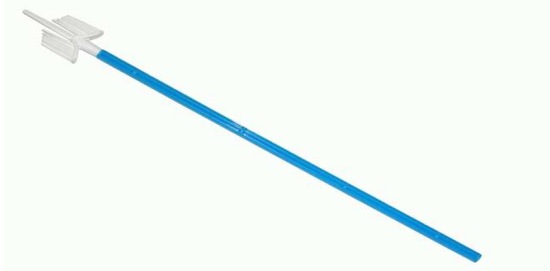


Figure 15: DNAPAP Cervical Sampler with cervical brush.



Transport media

Media formulated for the preservation of viral nucleic acids

Samples can be stored in STM at room temperature for up to 2 weeks and sent to the laboratory without refrigeration. If not processed immediately samples should be stored at 2-8°C for one week or -20°C for up to 3 months. It has been shown that samples at STM can be stored at -20°C for longer time even in the alkaline conditions. (LaMere et al., 2008; Sailors et al., 2005) Unpublished data show that the half life of DNA stored under denaturing conditions in STM is 6.4 months (Iftner et al., unpublished)

The performance of HPV DNA detection by HC2 on specimens collected at STM and PC has been explored in the interlaboratory and intralaboratory control of a quality study inserted within a multicentric randomized trial. The agreement in detection of positivity vs. negativity was excellent (kappa value 0.95 for PC and 0.96 for STM samples). However, samples collected in STM showed better reproducibility in comparison to PC collected specimens (kappa value 0.90 vs. 0.74). The lower reproducibility was noticed mostly for samples with low value of RLU/PC (1 to 11 RLU/PC; relative light units/positive control cutoff value) (Carozzi et al., 2005).

For specimens in TFP medium comparable sensitivity and specificity for HPV detection in the cytologically abnormal samples has been shown when used either in connection with HC2 or PCR (Weynand et al., 2009).

HPV DNA detection by HC2 in samples collected in UCM and STM has been shown to be identical. (Taha et al., 2006) Furthermore, UCM seems to preserve well samples also for PCR based methods (Nonogaki et al., 2004).

Media formulated for cellular preservation

PreservCyt solution is also recommended by other producers of HPV commercially available sets which are not FDA approved such as Aptima HPV Assay (Gen-Probe Incorporated, San Diego, CA, USA), Papillocheck (Greiner Bio-One GmbH, Frickenhausen, Germany), cobas® 4800 HPV test, AMPLICOR HPV test, LINEAR ARRAY HPV Genotyping test (Roche Diagnostics GmbH), and PreTec HPV Proofer (Norchip, Klokkarstua, Norway) and NucliSENS EasyQ HPV (bioMérieux, Marcy L'Etoile, France).

SurePath Preservative Fluid is validated for some commercial tests, e.g. AMPLICOR HPV test, LINEAR ARRAY HPV Genotyping test, cobas® 4800 HPV test (Roche Diagnostics GmbH), INNOLipa (Innogenetics Biologicals) (**Table 2**).

Few studies have addressed the issue of the use of media for liquid-based cytology for HPV RNA preservation. It has been shown that the integrity of RNA in the PC medium is very good while in the SP medium is severely compromised (Cuschieri et al., 2005; Horvath et al., 2008; Powell et al., 2006; Tarkowski et al., 2001). The APTIMA HPV Assay is CE marked for use with PreservCyt media and the APTIMA Cervical Specimen Collection and transport (CSCT) kit. The CSCT kit utilizes a Specimen Transport Medium (STM) for the stabilization of nucleic acids including the E6/E7 mRNA transcripts detected by the APTIMA HPV Assay. CSCT samples are stable at 2-30°C up to 60 days. PreservCyt specimens are stable up to 30 days at 2-30°C and a further 60 days once they are transferred to an APTIMA transfer tube. Both sample types can be stored frozen up to a year.

For HPV DNA detection it has been shown that besides specimens at STM (see above) also samples at UCM can be stored frozen (Campos et al., 2008) even under alkaline conditions and be consequently successfully analyzed by both PCR and HC2 method. For long-term storage of specimens for HPV DNA detection at ambient temperature the data are less conclusive. Some have shown that long term storage of samples in PC solution (Castle et al., 2003; Negri et al., 2004; Sailors et al., 2005) or SP medium (Hardie et al., 2009) at ambient temperature does not affect the HPV DNA detection by HC2 and/or PCR based methods while others have shown that DNA degradation after long-term storage can lead to false-negative results. The influence of the longest storage time on HPV DNA detection by both HC2 and PCR was analyzed by Castle et al. (2003). No effect on consequent HPV DNA detection by HC2 has been observed in samples stored for as long as 8 years while the storage time has influenced detection of internal control gene used for PCR. In about

15% of samples the internal control could not be amplified after 5 years of storage (P Trend <0.0001 for decline with storage time).

A simple approach for collection, transport and storage of cervical smears on a Whatman 3MM filter paper has been shown to give comparable results regardless of the storage temperature (ambient vs. refrigerated) even after longer period of time (Kailash et al., 2002).

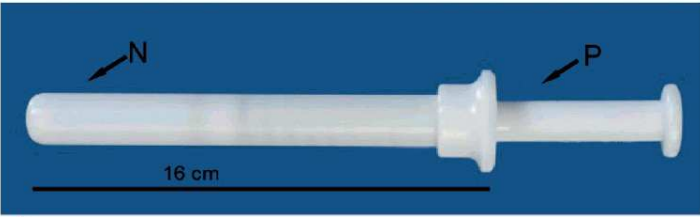
Self sampling devices

Two reviews summarize the results of numerous studies using self-sampling devices. (Petignat et al., 2007; Stewart et al., 2007) Additionally, reports on use of Fournier device (Castle et al., 2006), FTA elute cartridges (GE Healthcare,UK) (Leselink et al., 2009), pads (Kim et al., 2007), Mermaid cervicovaginal method (Brink et al., 2006), Qvintip (Aprovix AB, Uppsala, Sweden) (Stenvall et al., 2006) for self-sampling for HPV detection had been published recently (Figure 16). Even though the studies differ in many parameters (self-sampling method, clinicians sampling device, population studied, number of subjects enrolled) some general outcomes can be summarized.

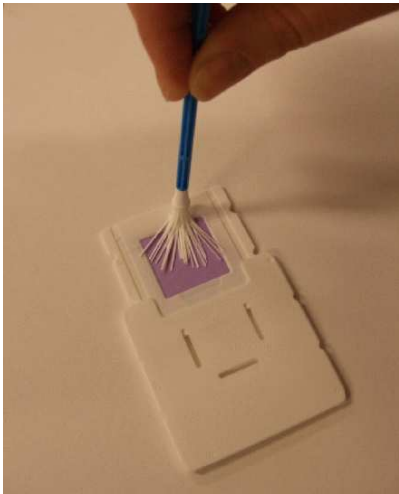
Figure 16: Examples of some self-sampling devices for collection of material for HPV detection, A/ Qvintip, B/ device for Mermaid cervicovaginal method, C/ FTA elute cartridge D/ Fournier device.



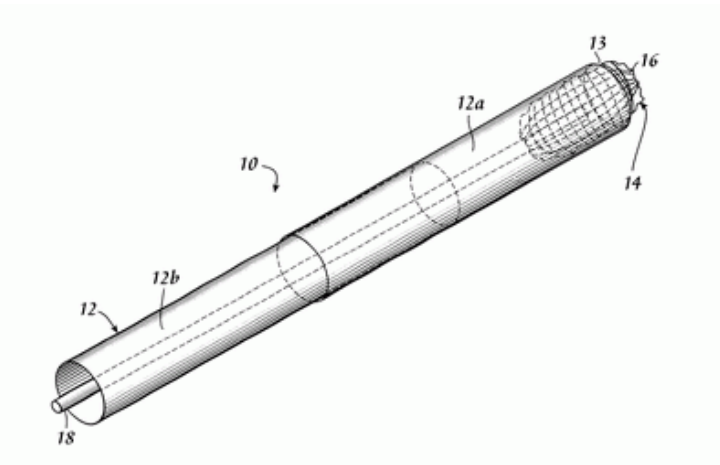
A



B



C



D

In the majority of published studies, regardless of the self-testing method used, women were able to collect samples and did not report on difficulties in performing the self-sampling. Several studies reported stronger preference of women for self-sampling method in comparison to the examination by clinician. Two studies reported that women with higher income and/or education prefer self-sampling but in another study performed in the minority groups of British women no difference was found. Furthermore, a study from China on women in rural areas reported potential barriers

in self-sampling in only 0.4-2.4% of participants. Self-sampling proved to provide representative samples for HPV DNA testing, yielding enough cells. The concordance between self- and clinician sample in regard of HPV or HR HPV detection was quite variable in studies. No difference in HPV detection in self-collected samples and clinician taken smears was observed in regard to the age of the screened population or disease prevalence in the tested population. The concordance in the prevalence of HR HPV was very similar while in several studies LR HPV prevalence was higher in the material obtained by self-sampling in comparison to the material collected by clinician. The accuracy of self-sampling for HPV DNA for screening for cellular abnormalities was dependent on the population tested. PPV of HPV DNA detection from self-collected samples was low in the screening population (9-35%) while it was much higher in patients referred for colposcopy (38-100%). (for review see (Petignat et al., 2007) and (Stewart et al., 2007))

Special precautions for target amplification assays

As indicated above both amplicons of previous target enrichment reactions and carry-over from other samples poses a major risk of contamination and consequently false positive results. Therefore, a special infrastructure and a strict working discipline are necessary to minimise contamination risk. Reaction product carryover can be avoided by using three physically separated laboratories: one for reagent preparation, one for sample preparation and setting up the reaction, and the third for amplification and detection of amplicons, each with its own set of lab coats, supplies and pipettes. Lab coats and equipment should be kept in the room where it is used and not be transferred between rooms. Cross-contamination between samples should be further avoided by using only pipettes fitted with disposable filter tips. In addition, the use of disposable powder-free examination gloves should be encouraged as well as the habit to wash hands before leaving a room. In addition, many diagnostic laboratories that rely on amplification techniques make it a practice for their staff not to re-enter the reagent preparation and reaction setup room for the rest of the day after having worked in the amplification and detection room.

Moreover, it is self-evident that reaction product carryover is less likely to occur when reaction vials do not need to be opened upon completion of the reaction. This is a great additional advantage of the abovementioned real-time detection assays. Finally, as indicated above the risk of amplicon carryover can be minimized by use of the enzyme uracil DNA glycosylase (UDG). UDG cleaves the uracil from the phosphodiester backbone of uracil containing DNA. The resulting apyrimidinic sites block replication by DNA polymerases, and in addition are very labile to acid/base hydrolysis. UDG does not react with free dUTP and is inactivated by heat denaturation. These properties can be utilized to prevent reaction product carryover by incorporating dUTP in all PCR products (either by substituting dUTP for dTTP, or by incorporating uracil during primer synthesis), and treating all subsequent preassembled reactions with UDG, followed by heat-denaturation of UDG. UDG is commercially available (e.g. AmpErase from Applied Biosystems) and is used in many diagnostic laboratories.

Table 2. Validated media for commercially available tests

Medium	Validated tests									
	<i>digene</i> Hybrid Capture® 2 *	INFINITI HPV- QUAD Assay (Erali et al., 2009)	ProDect® Chip HPV Typing/ Bcs Biotech S.p.A							
PreservCyt (FDA-approved)	<i>digene</i> Hybrid Capture® 2 *	Cervista™ HPV HR *	Cervista ™ HPV 16/18 *			ProDect® Chip HPV Typing/ Bcs Biotech S.p.A		ProDect® Chip HPV Typing/ Bcs Biotech S.p.A		
PreservCyt solution	Aptima HPVassay *	Papillocheck *	Pre Tec HPV Proofer * NucliSEN S EasyQ HPV	ProDect® Chip HPV Typing/ Bcs Biotech S.p.A	cobas 4800 HPV test *	LINEAR ARRAY HPV Test * (Jamison et al. 2009)	AMPLICOR HPV Test * (van Ham et al., 2005)	INFINITI HPV-QUAD Assay (Erali et al., 2009)	Cervista™ HPV HR *(Day et al. 2009)	Abbott RealTime HR HPV
SurePath preservative fluid	INNOLiPA *	LINEAR ARRAY HPV Test (Jamison et al., 2009)	AMPLICO R HPV Test * (Hardie et al., 2009)		cobas 4800 HPV test *	<i>digene</i> Hybrid Capture® 2 *(Ko et al., 2006; Kuebler et al., 2007; Siddiqi et al., 2009)	INFINITI HPV-QUAD Assay (Erali et al. 2009)	Cervista™ HPV HR *(Harvey et al., 2009)	Cervista™ HPV 16/18 *(Harvey et al., 2009)	Papillochec k
cobas® PCR Cell Collection Medium	LINEAR ARRAY HPV Test *	AMPLICOR HPV Test *	cobas 4800 HPV test *							
PapilloCheck Collection Kit	Papillochec k *									
Universal collection medium	<i>digene</i> Hybrid Capture® 2 (Taha et al., 2006)									
APTIMA cervical specimen collection and transport medium	Aptima HPVassay *									
Thermo Fisher Papsin medium	<i>digene</i> Hybrid Capture® 2 (Weynand et al., 2009)									

*Media mentioned in the product information leaflet

Figure 4: Principles of the Cervista test

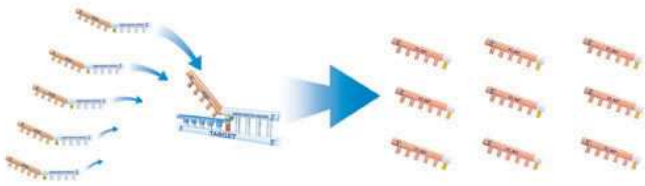


Figure 5: Principles of the Cervista test

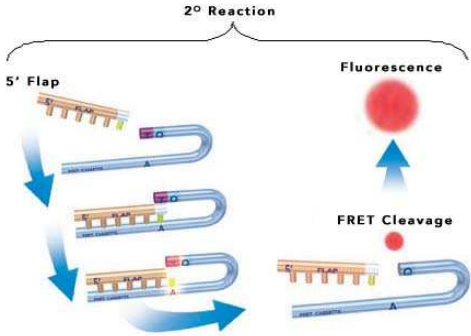


Figure 6: Principles of the Cervista test

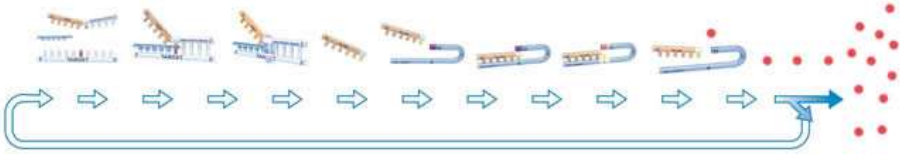


Figure 7: Principle of a PCR reaction

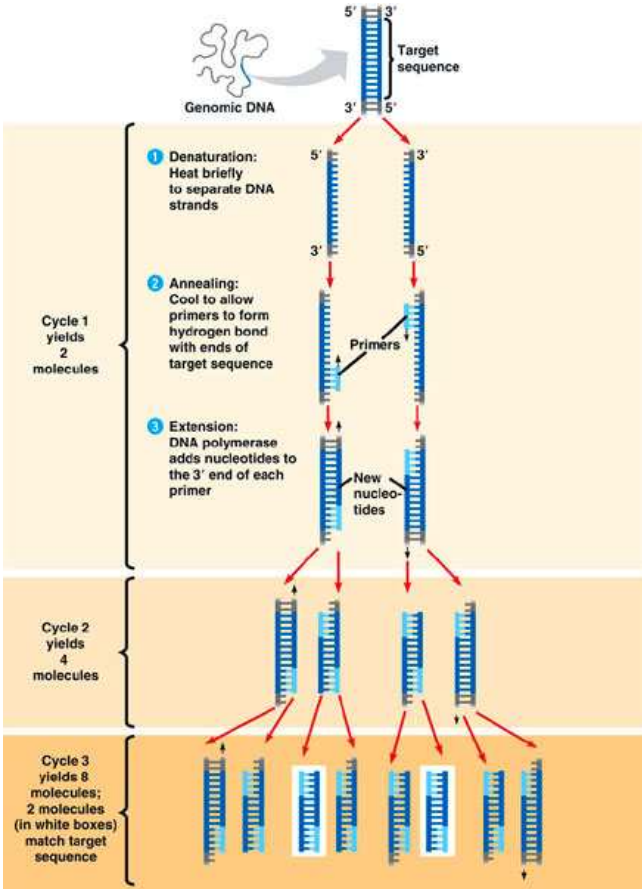


Figure 8: Microplate hybridization for the detection of PCR amplification products (DIG=digoxigenin)

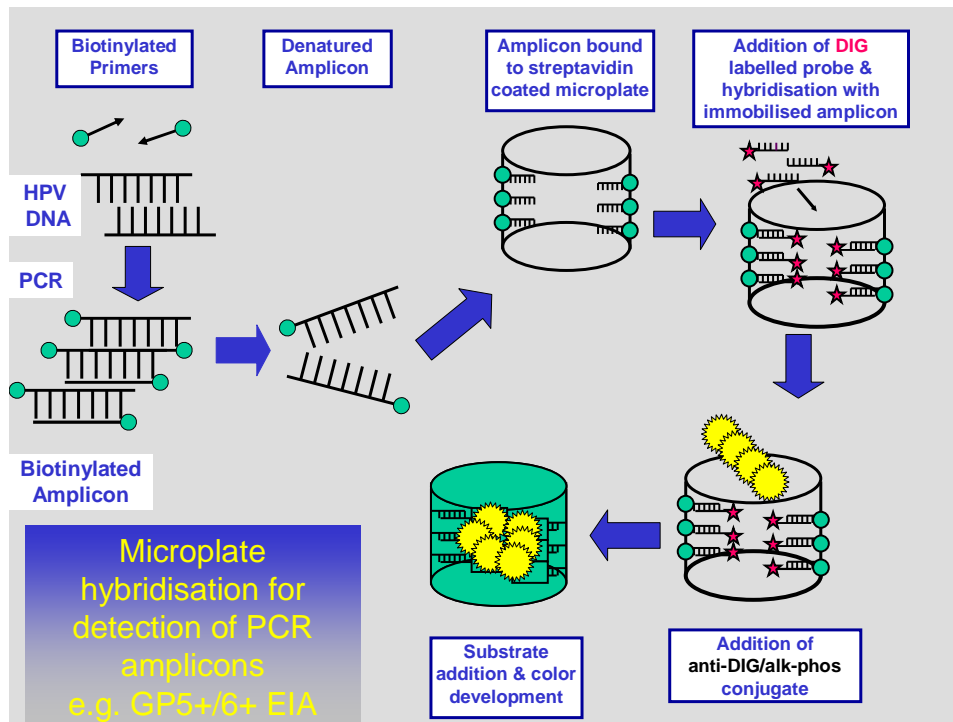


Figure 9: Line blot hybridization for the detection of specific HPV types after PCR reaction

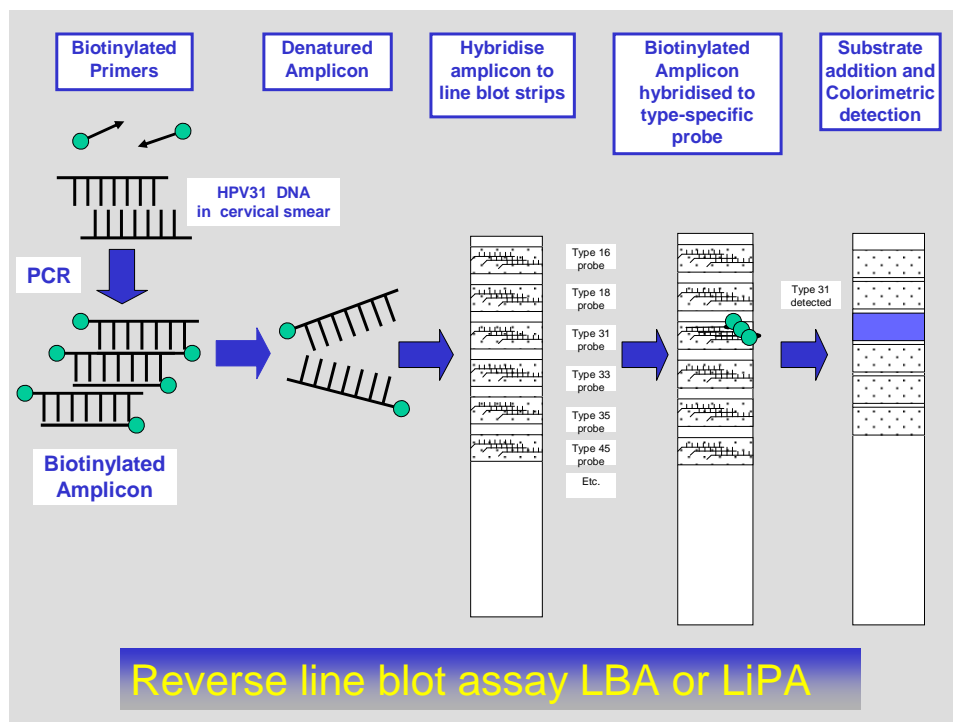


Figure 10: Principles of the NASBA technology

The formation of the newly generated RNA molecules is therefore determined in real time by continuous monitoring of fluorescence in a dedicated reader: the NucliSENS EasyQ® System. For a typical assay, the reaction time for real-time amplification and detection is just one hour.

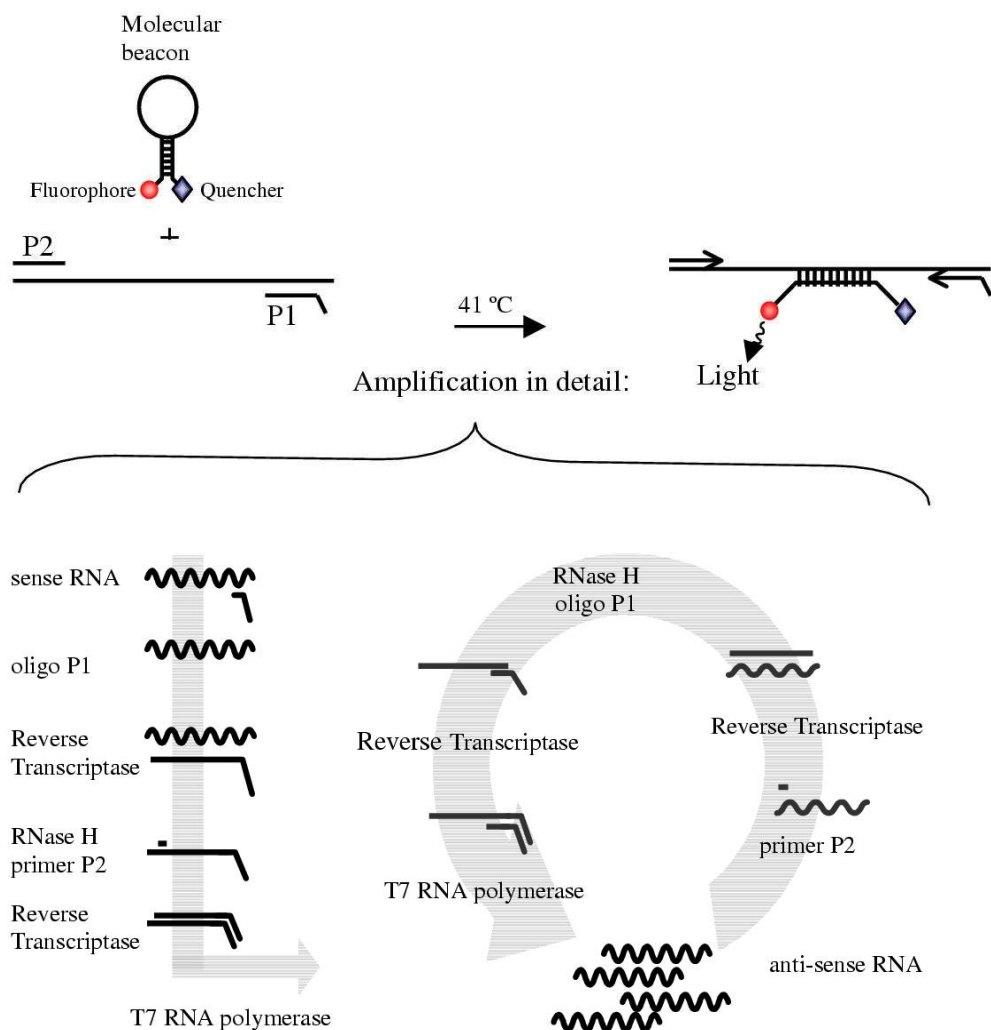


Figure 11: Target Capture specimen processing technology

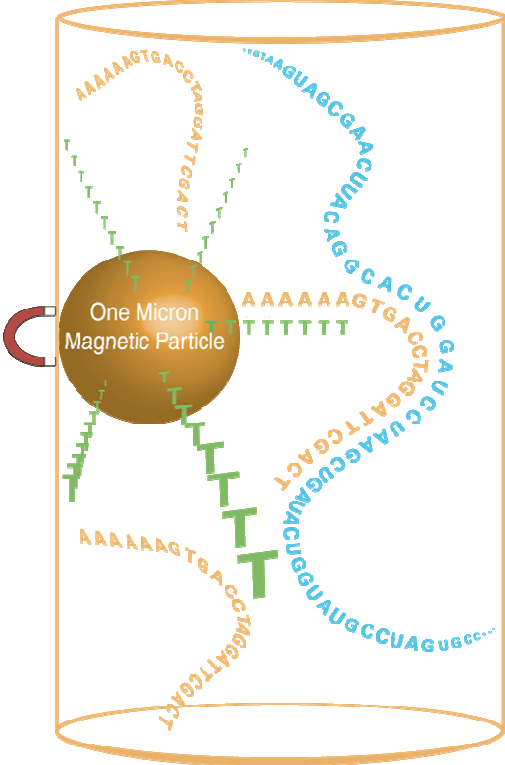


Figure 12: TMA technology

