

## **ECCG Chapter 5: Description of HPV tests, typology of HPV tests (T. Iftner, R. Tachezy, M. Pawlita, P. Snijders)**

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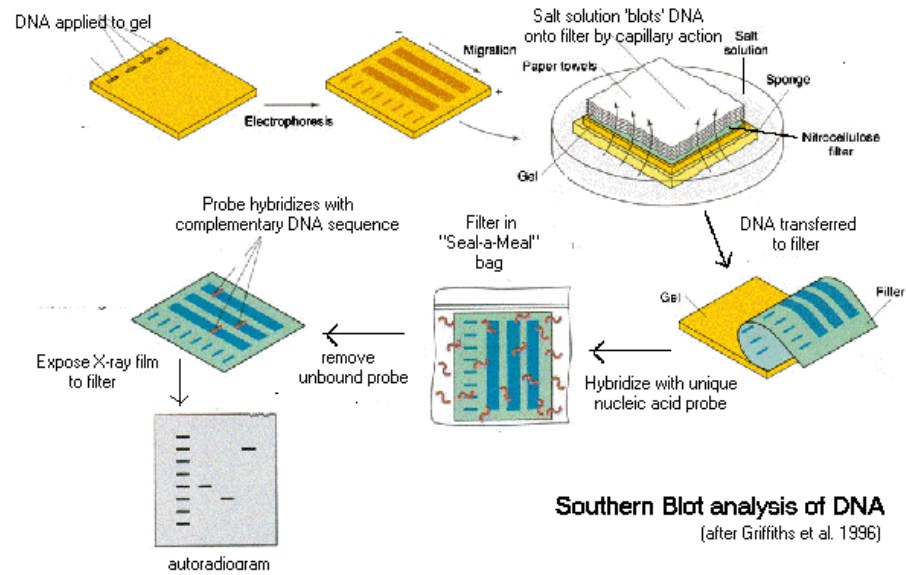
## 5.1 Introduction

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 those who have been vaccinated with prophylactic, L1 virus like particle-based vaccines.

The diagnosis of an HPV infection nowadays is almost exclusively made by identification of the viral nucleic acids, mostly DNA, 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 .

Before PCR became available for the detection of specific HPV types, the gold standard was the Southern blot technique using radioactively labelled hybridization probes. Southern blotting combines agarose gel electrophoresis for size separation of total cellular DNA cleaved with restriction enzymes isolated from patient material with methods to transfer the size-separated DNA to a filter membrane for probe hybridization.

The hybridization probe was a fragment of DNA from a known HPV type and usually labeled radioactively (commonly with  $P^{32}$ ). The labeled probe was denatured (by heating) into single DNA strands and hybridized to target DNA (Southern blotting) usually immobilized on a nitrocellulose membrane. DNA sequences that had moderate to high (depending on the stringency in the hybridization) sequence similarity to the probe, were detected by visualizing the hybridized probe via autoradiography or other imaging techniques. However, the time and material consuming nature of this assay as well as its relatively low sensitivity has ruled out its use for routine analysis of cervical specimens.



Since most of the currently applied HPV detection methods rely on either target amplification or signal amplification the technical principles of these methodologies are detailed below on the basis of various examples

## 5.2. Categorisation of HPV assays

Signal amplification assays:

- a. HC2
- b. Cervista Invader assay

PCR-based DNA amplification assays:

- a) broad-spectrum PCR

b) consensus primers

Isothermal nucleic acid amplification methods (typically for mRNA)

a) NASBA: PreTect HPV Proofer (and the related NucliSENS EasyQ HPV v1 assay of Biomerieux)

b) Transcription-mediated amplification: Aptima HPV assay

Read-out assays for target amplification methods

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

1) Enzyme immunoassays following oligoprobe hybridizations to PCR products captured in microtiter plate wells (examples: GP5+/6+-PCR EIA, SPF10-DEIA, Amplicor).

B) methods that allow genotyping:

1) Real time readout using:

- Mixtures of type-specific probes (examples: Abbott RealTime High Risk HPV test, Roche Cobas 4800 HPV test)

- CYBR green (example: GP5+/6+ SYBR Green; de Araujo et al., 2009)

- Reverse hybridization assays with labeled PCR products hybridized to oligonucleotides immobilized to various solid supports, such as:

- Strips (examples: Linear array, LiPa, for PGMY and SPF10 products, respectively, and

Digene HPV genotyping RH test for GP5+6+ products)

-Filters (example: reverse line blot, for GP5+/6+-PCR products)

-Microsphere (Luminex) beads (examples: MPG (Schmitt et al., 2008) and Digene

HPV genotyping LQ test for GP5+6+ products)

2) Microarrays (examples: Genomica and PapilloCheck)

3) Real time readout assays with type-specific probes (examples: PreTect HPV Proofer (5 types), Abbott RealTime High Risk HPV test (2 types), Roche Cobas 4800 HPV test (2 types))

### 5.3 Description of tests

#### 5.3.1 Definition of terms

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#### 5.3.2 Signal amplification assays

The HC2 test

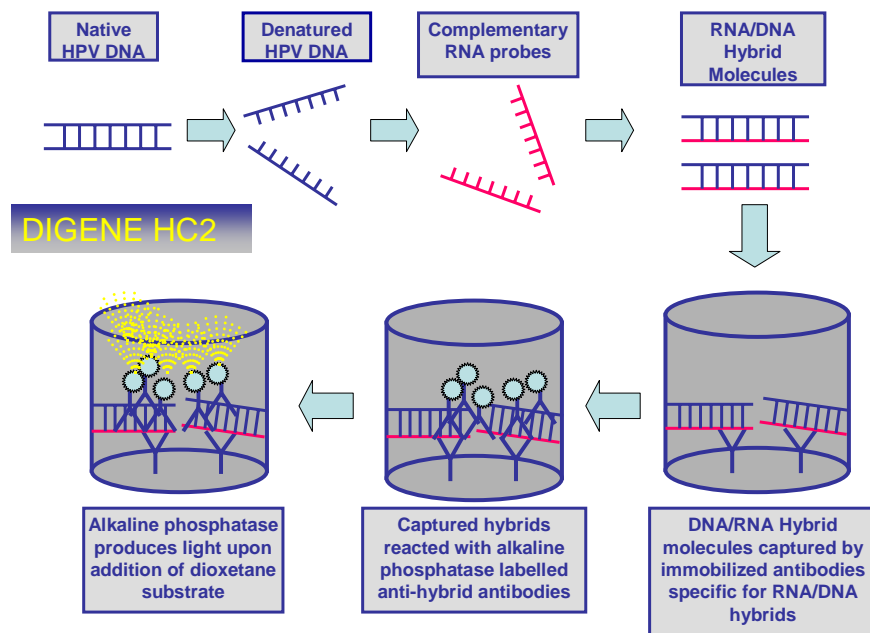
The best known example in the category liquid-phase signal amplification methods is the Hybrid Capture 2 (HC2) assay (Qiagen). This assay is based on hybridization in solution of long synthetic RNA probes complementary to the genomic sequence of 13 high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 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. Denatured HPV DNA present in the biological specimen is hybridized in solution with each of 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-labeled 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 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. The Food and Drug Administration (FDA) recommended cut-off value for test-positive results is 1.0 RLU (equiv. to 1pg HPV DNA per 1ml of sampling buffer)..

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.

Figure X: Principle of the HC2 test

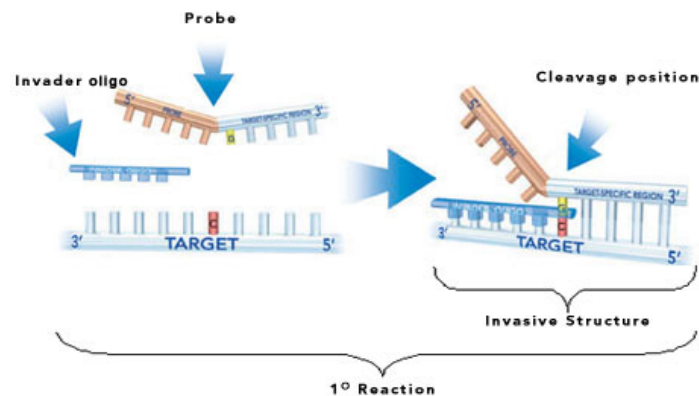


Several studies have noted that the HC-2 high risk probe mix to a certain extent cross-reacts with HPV types that are not represented in the probe mix (Peyton et al., 1998; Peyton et al., 2001; Vernon S.D. et al., 2000). Peyton and colleagues (Peyton et al., 1998) found that HC2 using the high risk probe 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., 2002a).

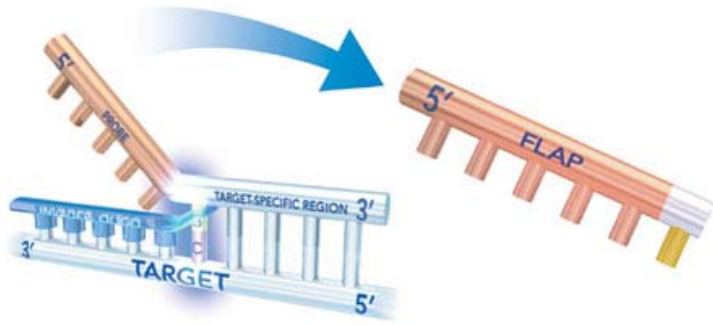
#### The Cervista Test

A more recent liquid-phase signal amplification method involves the Third Wave's Invader HPV test of Cervista. 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

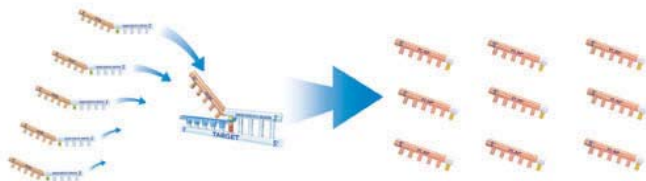
after denaturation by hybridization with two oligonucleotides: the invader oligo and the probe oligo, which are pure DNA oligonucleotides without having a biotin molecule or an enzyme attached. This is similar to the HC2 assay, where pure RNA probes hybridize to denatured HPV DNA. In the invader assay 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 as shown below.



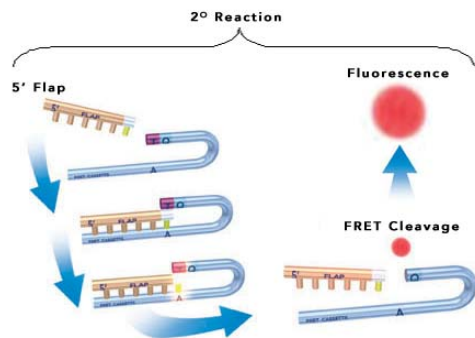
This unusual structure that normally is not present in patient material is recognized by the cleavase enzyme, which possesses 5' nuclease activity, and recognizes and cleaves this specific structure, releasing the reporter DNA called "5' flap" .



In the absence of the specific target, no flap is released.

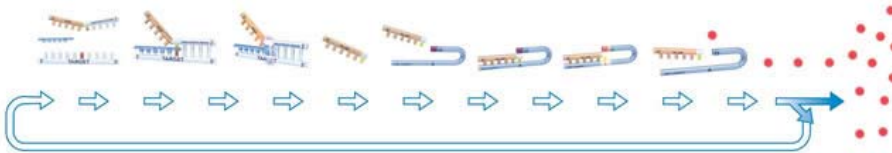


Subsequently, probe oligos that are present in the reaction in large excess, rapidly undergo again association with the target HPV DNA replacing cleaved probe oligos. Therefore multiple probe oligos are cleaved per target molecule, and the label, detected in a secondary reaction, generated from the cleaved 5' flaps is amplified. 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 labeled, 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.

The test principle is similar to the HC2 assay where RNA/DNA hybrids, normally not present in patient material, that are formed in the HC2 reaction are captured by a specific antibody recognizing the unusual structure bound to a surface.



Because these two cleavage reactions occur simultaneously, they can produce 1 million to 10 million labeled 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.

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. Staining of hybrids can be achieved by fluorescent detection or colored 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) 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). 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.

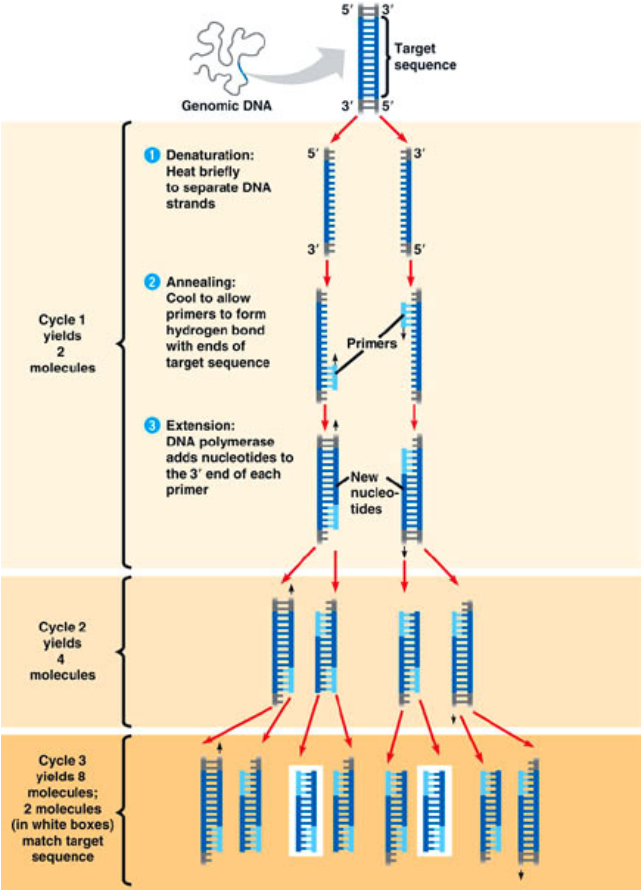
### **5.3.2 Target amplification-based techniques**

#### **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 target double-stranded 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 (Fig. XX).

Figure XX: Principle of a PCR reaction



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, as detailed below.

## **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 (de Roda Husman et al., 1995; van Den Brule et al., 1990) with its second generation, extended version GP5+/6+-PCR (Jacobs et al., 1997), and the MY09/11 PCR (Manos et al., 1989) with its modified version, PGMY09/11 (Gravitt et al., 1998). 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. Further details of these commonly used consensus PCR assays are given below.

Ever since, many PCR-based systems have been described, including those that target other conserved regions within viral L1 (such as Roche Amplicor) or E1 (such as PapilloCheck) regions, and assays that are largely based on modifications of existing (e.g. GP5+/6+-PCR) methods. Primer locations of some consensus primer assays targeting E1 or L1 regions are depicted in Figure Y. The following description of some PCR-based HPV testing systems are examples of already longtime established test systems that form the basis for a variety of different commercial available HPV tests.

### **GP5+/6+-PCR system (CLART HPV2, Digene LQ and RH, HPV DNA Chio etc....)**

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 (Söderland-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 (Linear Array HPV Genotyping)**

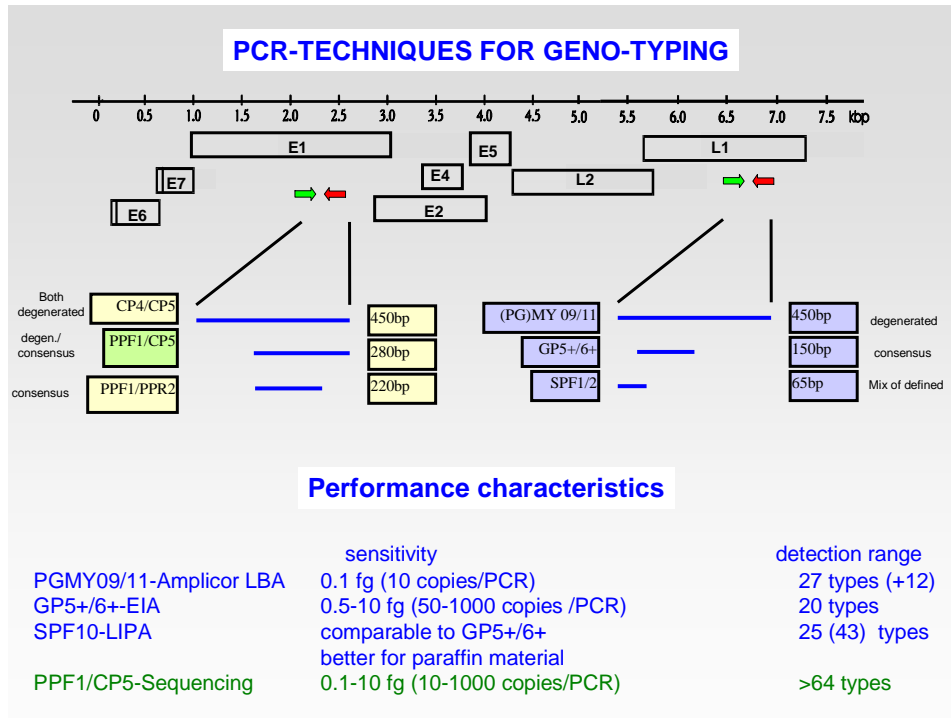
The PGMY09/11 primer system was developed by Gravitt et al. (2000) to address some limitations of the traditional MY09/11 degenerate primer system. The demonstrated sensitivity for the PGMY09/11 primer system is 10 HPV genomes per PCR amplification for all representative genotypes. PGMY09/11 is comprised 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. Among the 247 samples examined, there was an HPV prevalence of 62.8% using the PGMY09/11 compared to a prevalence of 55.1% with the MY09/11 primer pair. 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. This phenomenon has been observed in other studies where certain HPV genotypes are amplified with higher efficiency using PGMY09/11 compared to the MY09/11. Due to this technical phenomenon, it is expected that the prevalence of certain HPV genotypes has been underreported and preliminary data from studies around the world in which the PGMY primer system is being used are showing unexpected type distributions (Davies et al., 2001).

### **SPF-10-PCR system (INNO LiPa Genotyping Extra)**

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 target 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.

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Figure Y: Genomic regions used for PCR-based HPV tests



### 5.3.3 Read-out systems .

Analysis of the amplified products can be done in several ways. Originally, gel electrophoresis, either or not preceded by restriction enzyme treatment, Southern, dot blot and sequence analyses were used as read-out system for PCR products. Subsequent read-out systems include enzyme immunoassays (EIA) following oligoprobe 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 labeled 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 also genotype for up to about three types per reaction. EIA and real time assays are more suited for high throughput analyses than reverse hybridization assays..

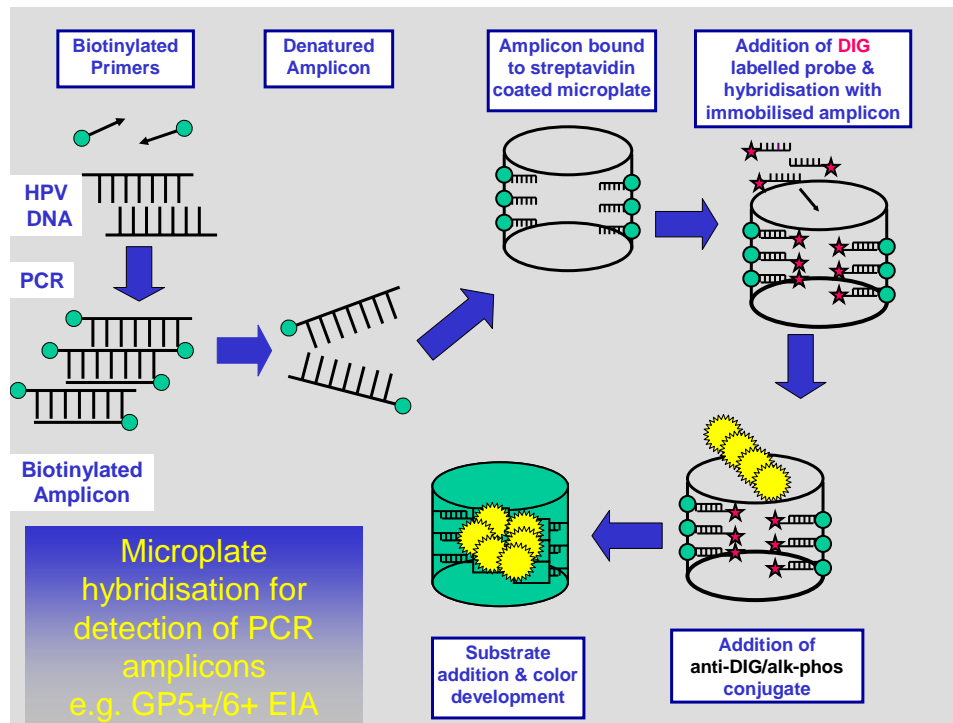
### **Enzyme immunoassays using microplates (EIA)**

Microplate hybridisation requires that PCR amplification be undertaken using primers labelled 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 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.

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 (Zielinski et al., Bulkman et al., Bulk et al.,). An enzyme immunoassay read-out, referred to as DEIA, has also been developed for SPF amplicons (Kleter et al.)

Another method that uses an EIA read-out is the Roche Amplicor assay, employing an oligonucleotide set which amplifies a 170 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 TaqGold 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 (Iftner and Villa 2003). Because these primers were designed for high risk types only (HPV16,18,31,33,35,39,45,51,52,56,58,59,and 68) this test is not truly generic.

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

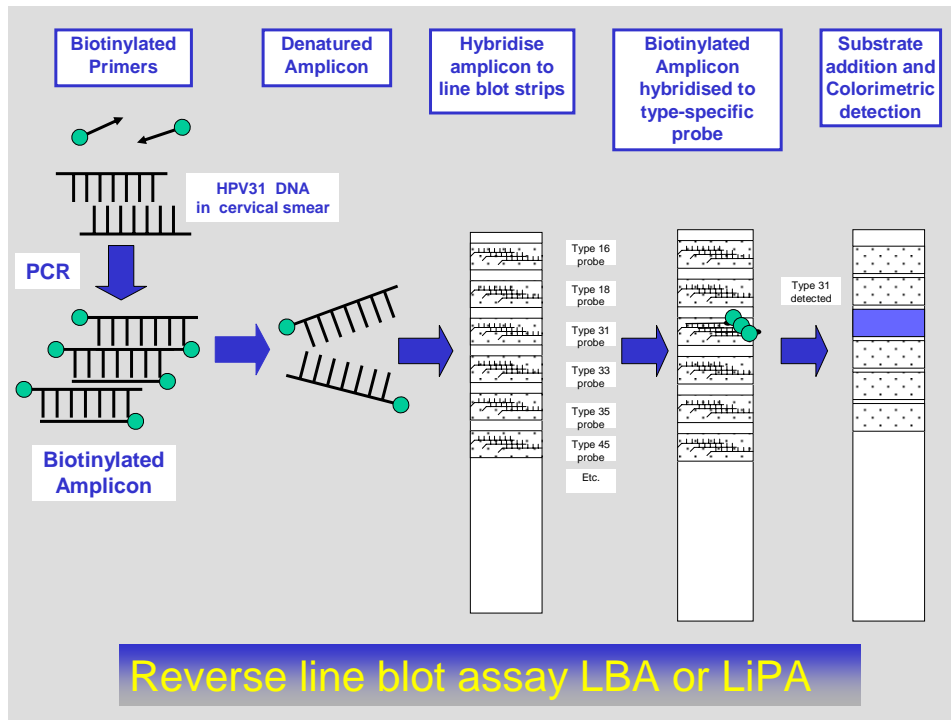


### Reverse hybridization assays using strips or filters

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 a defined positions and amplicon hybridisation at a particular position thereby identifies the type. In order to detect hybridisation, PCR amplification must again be undertaken 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 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 XX).

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



The PGMY primer system was evaluated using a reverse line-blot assay [25], 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 is currently being expanded to include 12 additional genital HPV genotypes (61, 62, 64, 67, 69, 70, 71, 72, CP6108, CP8304, IS39) described by Peyton et al (1998). The reverse line-blot genotyping system that includes 39 genotypes combined with these new primers offers an excellent tool for the detection of multiple infections and the genotyping of HPV DNA in clinical samples.

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 43 in the new LiPAv2 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)

The SPF1/2 primers were subsequently paired with a reverse hybridisation line blot assay (LiPA) for determination of 25 different HPV types (Kleter et al., 1999). This system was first evaluated with amplicon from cloned HPVs of known type, all of which were correctly identified and no cross-reactivity was observed.

### **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 easy and reliable 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 LiquiChip (LQ) Test, Detection Kit. 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 color-coded beads. For each pathogen, target-specific capture probes are covalently linked to a specific set of color-coded beads. Labeled 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 color-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 (Table 1, page 11). 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 (see flowchart on the next

page). As an internal control for the presence of amplifiable DNA after isolation, a fragment from the human beta-globin gene is coamplified with the HPV DNA in the form of a multiplex PCR. Bead type 44 contains a probe capable of detecting the beta-globin amplimer.

### **Reverse hybridization assays using microarrays**

Papillocheck (Greiner BioOne)

The PapilloCheck1 HPV genotyping assay (Greiner Bio-One GmbH) is based on the detection of a fragment of the E1 gene 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. At first, viral and human DNA are extracted

from a cervical smear specimen. Subsequently, a PCR fragment of about 350 bp of the E1 gene is amplified in the presence of a small subset of HPV specific primers by polymerase chain reaction (PCR). Additionally the human ADAT1 (t-RNAspecific adenosine desaminase1) gene is amplified and used as a control for the integrity of the purified DNA. The PCR product is hybridized to a low-density

microarray plastic HTA12 chip. Twelve hybridization reactions can be performed simultaneously on

one DNA chip. During hybridisation, the bound DNA is fluorescently labelled as well. The chip can be automatically scanned and analyzed using the CheckScanner™ and the CheckReport™ software (Greiner Bio-One), respectively. The DNA-array design of the PapilloCheck® DNA-chip permits the control of all critical steps during processing of the chip (e.g. spot homogeneity of the DNA-array, sample preparation, DNA hybridisation and PCR).

#### CLART HPV2 Test (Genomica)

The CLART HPV 2 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).

### 5.3.5 Real time read-out assays

#### Abbott RealTime HR HPV Assay

The Abbott RealTime HR HPV Assay uses the Abbott m2000sp machine, the Abbott m24sp sample preparation device or manually prepared samples and the Abbott m2000rt real time PCR machine for amplification and detection. The PCR reaction consists out of three forward- and two reverse-primers, that amplify a conserved part of the L1-region. Detection is done using fluorescence labelled probes for 14 HR-HPV-genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 und 68). As internal control (IC) serve primers which amplify the endogeneous human beta-globin-sequence and the IC-specific probe to measure the quality of DNA extraction and the performance of the amplification reaction. The probes for HPV 16-, HPV 18-, and the other non-HPV 16/18-genotypes (other HR HPV) and the IC-probe are labelled with different fluorochromes to be able to distinguish between the respective signals within one single reaction.

## The Cobas 4800 system

The cobas® 4800 system is a new analyzer from Roche to allow 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 provides full automation of the HPV assay with minimal manual intervention. The assay technology uniquely allows to adjust the cut off to the clinical situation. The cobas 4800 HPV Test is based on two processes:

- (1) automated specimen preparation to simultaneously extract HPV and cellular DNA
- (2) PCR amplification and real-time detection of target DNA sequences using both HPV and  $\beta$ -globin specific complementary primer pairs and probes



### 5.3.6 Isothermal nucleic acid amplification methods

#### NASBA technology

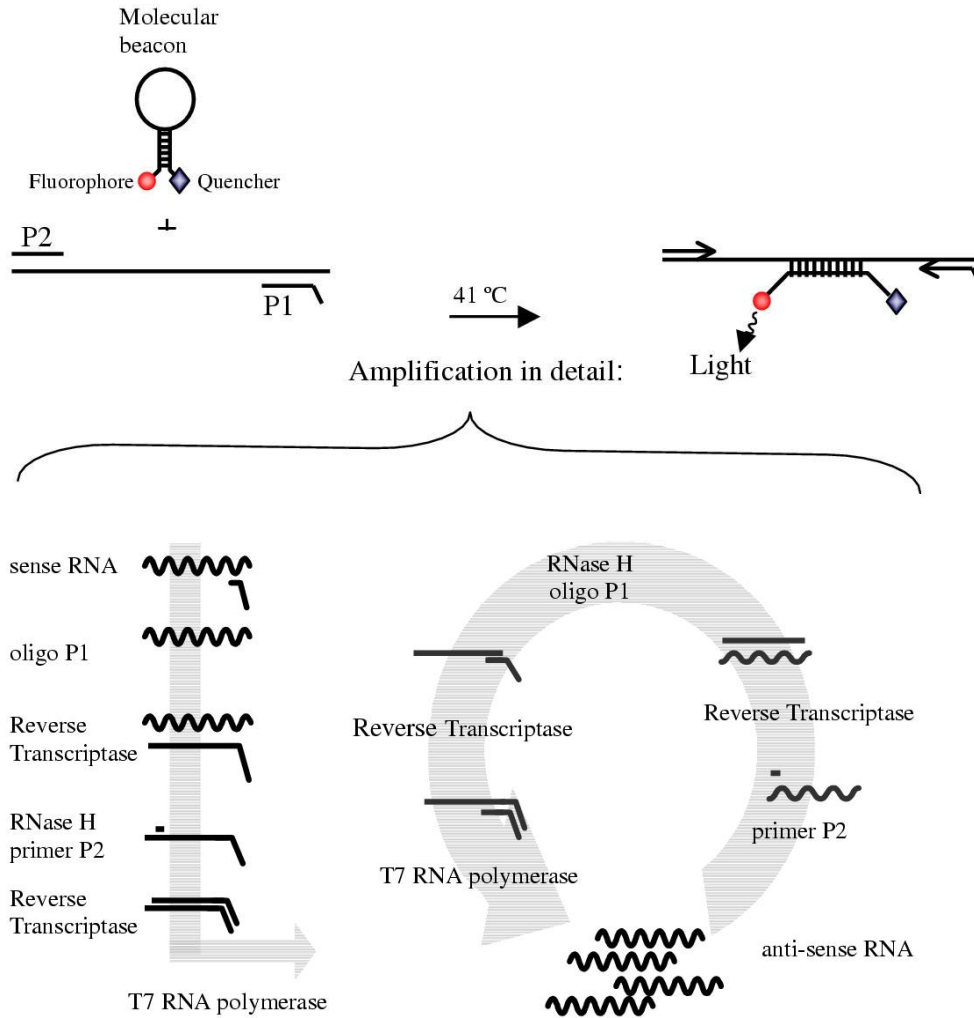
NASBA is a sensitive transcription-based amplification system (TAS) for the specific replication of nucleic acids in vitro. The main advantage of this technique is that the complete amplification reaction is performed at 41°C. Three enzymes are involved in this homogeneous isothermal reaction: avian 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 Norwegian company Norchip used this RT-NASBA technology together with Molecular Beacon probes and developed a real time assay, referred to as Prelect HPV Proofer, capable of the qualitative detection of transcripts of viral oncogenes E6/E7. A similar assay of Biomerieux (NucliSENS EasyQ HPV v1 assay) is on the market. These assays are limited to the detection of mRNA of five HR HPV types – 16, 18, 31, 33 and 45.

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.

Figure XX: Principles of the NASBA technology



### **Transcription-mediated amplification**

The APTIMA HPV Assay involves three main steps, which take place in a single tube: target capture; target amplification by Transcription- Mediated Amplification (TMA) (7); and detection of the amplification products (amplicon) by the Hybridization Protection Assay (HPA) (1). 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. When the APTIMA HPV Assay is performed, 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 capture oligomer:target complex is then captured out of solution by decreasing the temperature of the reaction to room temperature. This temperature reduction 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 microparticles, 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. After target capture is complete, the HPV mRNA is amplified via TMA, which is a transcription-based nucleic acid amplification method 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. Detection of the amplicon is achieved by Hybridization Protection Assay (HPA) using single-stranded nucleic acid probes with chemiluminescent labels that are complementary to the amplicon. The labeled 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 labeled 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. The Internal Control controls for 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 (12). 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 (12)

### 5.3.7. 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 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.

## 5.4 Overview of analytical characteristics

The following tables 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.

<b>Group Tests</b>				
Test	copy number per reaction for HPV 16	copies/mL	FDA	CE
<b>DNA</b>				
HC2/Qiagen	6216	124324		
Cervista HR HPV/ Hologic	1250-2500			
AMPLICOR HPV Test/ Roche Molecular Systems		100		
AID HPV screening kit/ Autolmmun Diagnostika GmbH	1000			
<b>RNA</b>				
NucliSENS EasyQ HPV/BioMérieux		260		
APTIMA HPV Test/ Gen-Probe	30-300			

<b>Mixed Group and Genotyping Tests</b>				
Test	copy number per reaction for HPV 16	copies/mL	FDA	CE
<b>cobas</b> @4800 HPV Test / Roche Molecular Systems		600		
RealTime High Risk HPV test / Abbott	500			
AID HPV typing kit/ Autolmmun Diagnostika GmbH	1000			

Genotyping Tests				
Test	copy number per reaction for HPV 16	copies/mL	FDA	CE
Cervista™ HPV 16/18 /Hologic	625-1250			
digene HPV Genotyping RH Test/ Qiagen	4			
digene HPV Genotyping LQ! Test / Qiagen	5			
INNO LiPa Genotyping Extra/Innogenetics	2-5000			
HPV-DNA Chip/ Biomedlab		5 pg/mL		
Linear Array HPV Genotyping Kit/Roche Molecular Systems		200		
Papillocheck/Greiner Bio-One	50			
PCR HPV Typing Set/ Takara Bio INC.	1000-10000			
PCR HPV Detection Set/Takara Bio INC	1000-10000			
HPV HR Genotyping Assay/GenoID	-			
Full Spectrum HPV Assay/ GenoID	-			
CLART HPV2/ Genomica	10			
PreDect HPV Typing/ Bcs Biotech s.p.A	-			
PapType/ Genera Biosystems	500			

### 5.4.1 Analytical characteristics of individual HPV assays

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 classificati on A)Signal- Amplificati on B)Target- Amplificati on	Nucleic acid target, size	Test principle and detection method	Level of control: A)Sample adequacy B)Assay control C)PCR product contamination D)Neither	Performance A) <a href="#">Analytical Sensitivity</a> B) <a href="#">Analytical Specificity</a> (not yet completed)	Certification A) CE B) FDA C) None	Costs (state 2009) Rather insecure data so far
<b>At least Class 1/2a group detection</b>								
HC2/ Qiagen	<a href="#">16,18,31,33, 35,39,45,51, 52,56,58,59, 68</a>	A	DNA	Hybrid capture/ Enzymatic luminescence	B	A) <a href="#">HC2 analytical sensitivity</a>  B) <a href="#">HC2 analytical specificity</a>	A, B	8,50-20 €/ Test
Cervista HR HPV/ Hologic	<a href="#">16,18,31,33, 35,39,45,51, 52,56,58,59, 66, 68</a>  3 probe sets: <a href="#">A5/A6</a> : 51, 56, 66 <a href="#">A7</a> : 18, 39, 45, 59, 68 <a href="#">A9</a> : 16, 31, 33, 35, 52, 58	A	DNA	Invader method/ Fluorescence resonance energy transfer (FRET)	A B	A) <a href="#">Cervista analytical sensitivity</a>  B) <a href="#">Cervista analytical specificity</a>	A, B	
AMPLICOR HPV Test/ Roche Molecular Systems	<a href="#">16,18,31,33, 35,39,45,51, 52,56,58,59, 68</a>	B	DNA L1 : 165bp	PCR/ Multiwell Plate hybridization, enzymatic colour reaction	A B	A) <a href="#">AMPLICOR HPV Test analytical sensitivity</a>  Problem: Unit c/ml	A	13,52€/ Test

						B) <a href="#">AMPLICOR HPV Test analytical specificity</a>		
APTIMA HPV Test/ Gen-Probe	<a href="#">16,18,31,33,35,39,45,51,52,56,58,59,66,68</a>	B	mRNA E6 E7	NASBA/ cheluminent labels	A B	A) <a href="#">Aptima HPV Assay analytical sensitivity</a>  B) <a href="#">APTIMA HPV Test analytical specificity</a>	A	
AID HPV screening kit/ Autolmmun Diagnostika GmbH	hrHPV's: <a href="#">16,18,31,33,35,39,45,51,52,56,58,59,66,68,73,82</a> lrHPV's: 6,11,40,42,43,44 Itemization of HPV 16 and 18	B	DNA L1	PCR/ reverse hybridization on filter strips, colorimetric detection	A B	A) <a href="#">The assay reaches a sensitivity of at least 1000 copies.</a>  B) n/a <a href="#">AID HPV screening kit analytical specificity</a>	A	
<b>cobas</b> ®4800 HPV Test / Roche Molecular Systems (for automated HPV testing)	<a href="#">16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68</a>  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	A) <a href="#">cobas®4800 HPV Test analytical sensitivity</a>  B) <a href="#">cobas®4800 HPV Test analytical specificity</a>	A	
RealTime High Risk HPV test / Abbott	<a href="#">16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68</a>  differentiation between 16/18 non 16/18	B	DNA L1	real time PCR / multi color detection system during the last 38 cycles	A,B	A) <a href="#">RealTime High Risk HPV test analytical sensitivity</a>  B) <a href="#">RealTime High Risk HPV test analytical specificity</a>	A	
<b>Only partially</b>								

<b>class 1/2a group detection</b>								
PreTect HPV-Proofer/ Norchip	16, 18, 31, 33, 45	B	mRNA E6 E7	NASBA/Molecular Beacons real-time detection technology		A) <a href="#">PreTect HPV-Proofer analytical sensitivity</a> B) <a href="#">PreTect HPV-Proofer analytical specificity</a>	A	
NucliSENS EasyQ HPV/ BioMérieux based on PreTect HPV Proofer	16,18,31,33, 45	B	mRNA E6 E7	NASBA/ molecular beacon probes for real-time detection	A B	A) <a href="#">Nucli SENS Easy Q analytical sensitivity</a>  Problem: Unit c/ml  B) not determined for HPV cross reactivity (introductions p.25)	A	
<b>Type-specific detection</b>								
Cervista™ HPV 16/18 / Hologic	16, 18	A	DNA	Invader method/ Fluorescence resonance energy transfer (FRET)	A B	A) <a href="#">Cervista HPV 16-18 analytical sensitivity</a> B) <a href="#">Cervista HPV 16/18 analytical specificity</a>	A?	
digene 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) <a href="#">digene HPV Genotyping RH Test analytical sensitivity</a> B) <a href="#">digene HPV Genotyping RH Test analytical specificity</a>	A	
digene HPV Genotyping	16, 18, 26, 31, 33, 35, 39, 45,	B	DNA	PCR (GP5+/6+ primer) based	A B	A) <a href="#">digene HPV Genotyping LQ! Test</a>	A	

LQ! Test / Qiagen	51, 52, 53, 56, 58, 59, 66, 68, 73, 82			assay which uses xMAP technology on the LiquiChip System		<a href="#">analytical sensitivity</a> B) <a href="#">digene HPV Genotyping LQ! Test analytical specificity</a>		
INNO LiPa Genotyping Extra/ Innogenetics	6,11,16,18,31,33,34,35,39,40,42,43,44,45,51,52,53,54,56,58,59,66,68/73,70, 74	B	DNA L1 : 65bp	PCR / reverse hybridization on membrane strips, enzymatic colour reaction	A B C	A) <a href="#">INNO LiPa Genotypink Extra analytical sensitivity</a> B) <a href="#">INNO LiPa Genotyping Extra analytical specificity</a>	A	86,30 €/ Test
HPV-DNA Chip/ Biomedlab	6,11,16,18,31,33,34,35,39,40,42,43,44,45,51,52,56,58,59,66,68, 69	B	DNA L1	PCR/ Microarray with specific oligoprobes	A B (?)	A) Analytical Sensitivity of HPVNAChip ~ 1x10 <sup>-8</sup> µg/ml HPVDNAChip's detection limit was about 10 fg/ml HPV DNA  B) n/a	C	7,93€/ Test
Linear Array HPV Genotyping Kit/ Roche Molecular Systems	6,11,16,18,26,31,33,35,39,40,42,45,51,52,53,54,55,56,58,59,61,62,64,66,67,68,69,70,71,72,73(MM9),81,82(MM4),83(MM7),84 (MM8),IS39 and CP6108	B	DNA L1 : 450bp	PCR (consensus PGMV Primers) / reverse line blot hybridization enzymatic colour reaction	A B	A) <a href="#">Linear Array HPV Genotyping Kit analytical sensitivity</a>  <b>Problem: Unit c/ml</b>  B) <a href="#">Linear Array HPV Genotyping Kit analytical specificity</a>	A	54,13€/ Test
Papillocheck/ Greiner Bio-	6,11,16,18,31,33,35,39,40,42,4	B	DNA E1 : 350bp	PCR (consensus primers) /	A B	A) <a href="#">PapilloCheck analytical sensitivity</a>	A	

One	4,45,51,52, 53,55,56,58,59, 66,68,70,73,82 (44 and 55 coupled)			oligoprobes immobilized on a DNA chip	C (optional)	B) <a href="#">PapilloCheck analytical specificity</a>		
PCR HPV Typing Set/ Takara Bio INC.	16,18,33,52b, 58 1r HPV's 6,11	B	DNA E6 E7 : 300 bp	PCR (consensus primers) / digestion of PCR products by restriction and subsequent agarose gel electrophoresis		A) 10 <sup>3</sup> – 10 <sup>4</sup> copies / reaction  B) n/a	C	
PCR HPV Detection Set/ Takara Bio INC	16,18,33	B	DNA E6 :140 bp	PCR/ detection by agarose gel electrophoresis or dot blot	B	A) 10 <sup>3</sup> copies / reaction  B) n/a	C	4,16€/ Test
HPV HR Genotyping Assay/ GenID	16,18,31,33,35, 39,45,51,52,56, 58,59,66,68	B	DNA L1	PCR/ solid phased hybridization. enzymatic colour reaction		A) not specified in the instructions  B) n/a	A	
Full Spectrum HPV Assay/ GenID	1r 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	6,11,16,18,45	B	DNA	PCR/ reverse	A	A) <a href="#">The assay</a>	A	

typing kit/ AutoImmun Diagnostika GmbH	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		E1	hybridization on filter strips, colorimetric detection	B	<a href="#">reaches a sensitivity of at least 1000 copies.</a>  B) n/a		
CLART HPV2/ Genomica	6,11,16,18,26,3 1,33,35,39,40,4 2,43,44,45,51,5 2,53,54,56,58,5 9,61,62,66,68,7 0,71,72,73,81,8 2,83,84,85,89	B	DNA L1 :450bp	PCR/ Biotinylated amplicons hybridise to specific probes attached to low density microarray surface,enzymatic colour reaction	A B	A) <a href="#">CLART analytical sensitivity</a>  B) <a href="#">CLART analytical specificity</a>	A	
PreDect HPV Typing/ Bcs Biotech s.p.A	6,11,16,18,31,3 3,35,39,42,43,4 4,45,51,52,56,5 8,59,68,73	B	DNA E6 E7	PCR/ reverse hybridization on pre-coated 8-well strip	A B	A) n/a  B) n/a	?	
PapType/ Genera Biosystems	6,11,16,18,31,3 3,35,39,45,51,5 2,56,58,59,66,6 8	B	DNA	PCR/ hybridization on silica beads and detection by flow cytometry	A B C	A) "the test is designed to have an analytic sensitivity of 500 HPV copies per reaction."  B) n/a	C	

# Analytical Sensitivities:

## Hybrid Capture 2 - Qiagen:

HPV DNA Type	Detectable HPV DNA Concentration (pg/ml)	Detectable HPV DNA per reaction (pg)	Copy Number per reaction
6	1,33	0,067	7585
11	1,13	0,057	6444
16	1,09	0,055	6216
18	1,05	0,053	5988
31	1,01	0,051	5760
33	1,35	0,068	7699
35	1,11	0,056	6330
39	1,39	0,070	7927
42	1,20	0,060	6844
43	0,85	0,043	4847
44	1,17	0,059	6672
45	1,14	0,057	6501
51	0,78	0,039	4448
52	1,37	0,069	7813
56	0,62	0,031	3536
58	0,82	0,041	4676
59	1,10	0,055	6273
68	1,19	0,060	6786

## 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	Detection threshold copies/ml
16	100
18	100
31	240
33	100
35	100
39	100
45	100
51	100
52	240
56	100
58	240
59	240
68	100

## APTIMA HPV Test - Gen-Probe:

HPV DNA Type	Copy Number per Reaction (DTS System)	Copy Number per Reaction (TIGRIS DTS System)
16	100-300	30-100
18	100-300	100-300
31	30-100	10-30
33	30-100	30-100
35	30-100	30-100
39	30-100	10-30
45	30-100	30-100
51	300-1000	100-300
52	300-1000	100-300
56	100-300	30-100
58	100-300	30-100
59	100-300	30-100
66	100-300	100-300
68	30-100	30-100

## PreTect HPV-Proofer - Norchip:

## AID HPV screening kit - Autolmmun Diagnostika GmbH:

The assay detects at least 10<sup>3</sup> copies

## cobas®4800 HPV Test - Roche:

HPV DNA Type	Detection Threshold (copies/ml)
31	>300
16	600
18	600

## 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

## 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 (PCR)
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 (PCR)
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
82 IS39	100

## INNO LiPa 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 (~30.000 copies/reaction)

## Linear Array HPV Genotyping Kit – Roche:

HPV DNA Type	Detection Threshold LOD concentration rate (copies/ml)	Observed 95% positive hit rate
6	2000	
16	200	
18	1300	
26	6000	
31	6600	
33	20000	
35	600	
39	1500	
45	900	
51	260	
53	400	
56	12000	
58	250	
59	76	
66	300	
68	900	
73	300	
82	20000	

## Papillocheck - Greiner Bio-One:

HPV DNA Type	Detection Threshold copies / reaction	Detection Threshold pg / ml
6	30	0,052
11	150	0,26
16	50	0,086
18	300	0,516
31	300	0,522
33	300	0,519
35	750	1,29
39	30	0,052
40	30	0,052
42	30	0,052
43	100	0,175
44	20	0,052
45	50	0,087
51	30	0,051
52	100	0,174
53	30	0,051
56	30	0,051
58	150	0,255
59	50	0,087
66	100	0,171
68	30	0,052
70	30	0,052
73	100	0,169
82	30	0,052

## AID HPV typing kit - AutoImmune Diagnostika GmbH:

The assay detects at least  $10^3$  copies

## CLART HPV2 – Genomica:

HPV DNA Type	Detection 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

## Analytical Specificities:

### Hybrid Capture 2 - Qiagen:

- Certain amount of cross-hybridization between HPV types 6 and 42 and the high risk probe group.
- 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)

### Cervista HR HPV - Hologic:

Human papillomavirus types 67 and 70 yielded positive results with the Cervista™ HPV HR test at  $1 \times 10^5$  and  $1 \times 10^7$  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), 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.  
(Introduction p. 21, 22)

### **AID HPV screening kit - Autolmmun Diagnostika GmbH:**

Not applicable

### **cobas®4800 HPV Test - Roche Molecular Systems:**

No cross reaction observed with HPV types 6, 11, 26, 40, 42, 54, 55B, 61, 62.  
(instructions p. 15)

### **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, 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 Genotyping Extra – Innogenetics:**

Not applicable.

### **Linear Array HPV Genotyping Kit - Roche Molecular Systems:**

The HPV genotype specificity of each HPV probe line on the HPV Strip was evaluated by testing high concentrations of HPV plasmid DNA for 36 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108). Plasmid DNA for HPV genotype 52 was not available for testing. The available HPV plasmid DNA stocks were quantitated by measuring the 260 nm absorbance on a spectrophotometer and diluted to 500,000 copies/mL in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Each HPV plasmid genotype was amplified and detected in duplicate using the LINEAR ARRAY HPV Genotyping Test. The results demonstrated HPV genotype specificity only to the expected probe line for the 36 HPV genotypes evaluated with plasmid DNA. In addition, specificity of HPV genotype 52 to the expected probe line was demonstrated in testing of a clinical specimen (genotype confirmed by sequencing) at an unknown HPV titer. (Instructions p. 22)

### **Papillocheck - Greiner Bio-One:**

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

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

(Instructions p.26)

### **CLART HPV2 - Genomica:**

The analytical specificity was 100%. CLART® HPV 2 kit detects no other pathogens that might be found in cervicovaginal samples, such as herpesvirus.

(Instructions p. 30)

## **5.7 Recommendations for laboratory design and procedures using PCR-based assays**

The following sequence of operations is recommended:

1. Preparation and aliquoting of PCR mixes
2. Preparation of samples (DNA isolation)
3. Amplification
4. Analysis of the PCR products :Personnel involved in steps 3 and 4 should not participate in subsequent work for steps 1 and 2 on the same day. Similarly, after being involved in step 2, personnel should not participate in subsequent work for step 1 on the same day.

To prevent contamination (for example, with amplification products) of specimens and to avoid false-positive results, the procedure should be performed in three physically separated rooms, each with its own set of supplies and pipettes. One room is needed for reagent preparation, another for sample preparation, and a third room for amplification and PCR product detection. All equipment should be kept in the room where it is used and should not be transferred between rooms. Filter tips should be used for pipetting to minimize cross-contamination between specimens. In addition, wear disposable gloves and change them frequently.

Room 1: This room should be used only for storage and preparation of PCR reagents. This room and its equipment must be kept free of amplification products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room. Disposable gloves should be worn at all times.

Room 2: This room is used for sample preparation and must be kept free of amplification products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room. During sample preparation, disposable gloves should be changed frequently. Carefully uncap vials containing processed sample to avoid cross-contamination. Avoid opening more than one reaction vial containing sample at the same time.

Room 3: This room is used for amplification and detection of PCR products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room and must be changed daily. When working with amplification products, disposable gloves should be worn.

## 5.6 Specimen Sampling and 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 (HC 2, QIAGEN GmbH, Hilden, Germany) tests. STM is a part of DNAPAP Cervical Sampler which additionally contains also cervical brush (Fig.1).



Fig. 1 DNAPAP Cervical Sampler with cervical brush.

STM is provided in plastic tubes in a volume of 1 ml and contains preservative (azid) to retard bacterial growth. This medium is not intended to be used for taking samples for cytological analyses. Samples can be stored in STM at room temperature for up to 2 weeks and send 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.

Some other sampling and storage media are provided by manufactures of commercial HPV tests, e.g. Papillocheck collection medium (Greiner Bio-One) and APTIMA Specimen Transfer Kit (Gene-Probe). This kit is used upon arrival of the sample to the laboratory. Once the PreservCyt Solution liquid Pap specimen is transferred to the APTIMA Specimen Transfer tube, the specimen must be tested within 30 days when stored at 2°C to 8°C or 14 days at 15°C to 30°C.

Media formulated for cellular preservation

PreservCyt solution (Cytoc, Boxborough, MA, USA) and Surepath preservative fluid (TriPath Imaging inc., Burlington, NC, USA) are optimised for cellular preservation and are used for liquid-based cytology. PreservCyt solution is also FDA-approved for HPV testing directly from the same vial with Cervista™ HPV HR, Cervista™ HPV 16/18 (Third Wave Technologies, Madison, WI, USA) and HPV DNA testing with the Digene Hybrid Capture® 2 (Digene® HPV Test, QIAGEN GmbH, Hilden, Germany) tests.

Cells preserved by both widely used media are suitable for HPV DNA detection, even though it has been shown that DNA is less stable in SurePath medium in comparison to PreservCyt (1;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 PreserCyt medium is very good while in the SurePath medium is severely compromised (2;3).

PreservCyt solution is used for making ThinPrep Pap test slides. Collection tubes contain 20 ml of the medium which consists of buffered preservative with 30-60% methanol (Fig. 2).

To use the specimen collected in this medium at least 4 ml has to be left for HC2 test after performing of the Pap test in order to avoid false negative results. PreservCyt solution specimen may be stored for up to 3 months at 2-30° and cannot be frozen.

For Cervista™ HPV HR testing, cervical specimens can be stored at room temperature (20-30°C) in PreservCyt solution for up to 18 weeks prior to performing the test. PreservCyt solution specimens cannot be frozen.

PreservCyt solution is also recommended by other producers of HPV commercially available sets which are not FDA approved such as Aptima HPVassay (Gene-Probe, Inc., Atlanta, GA, USA), Papillocheck (Greiner Bio-One GmbH, Frickenhausen, Germany) and PreTec HPV Proofer (Norchip, Klokkarstua, Norway).

SurePath preservative fluid is used for preparing slides for TriPath Imaging PrepStain Slide Processor (Fig. 2). SurePath Preservative Fluid contains an aqueous solution of <24 ethanol and also small amounts of methanol and isopropanol in a volume of XXX.

This medium is validated for some commercial tests, e.g. AMPLICOR HPV test, LINEAR ARRAY HPV test (Roche, ), INNOLipa (Innogenetics Biologicals, Gent, Belgium).

Fig. 2 Collection vials and devices: a/ SurePath b/ ThinPrep with PreservCyt medium.

Table 1. Validated media for commercially available tests

Medium	Validated tests					
Sample Transport Medium	Digene Hybrid Capture® 2 *					
PreservCyt (FDA-approved)	Digene Hybrid Capture® 2 *	Cervista™ HPV HR *	Cervista™ HPV 16/18 *			
PreservCyt solution	Aptima HPVassay *	Papillocheck *	Pre Tec HPV Proofer *	LINEAR ARRAY HPV Test * (4)	AMPLICOR HPV Test *	
SurePath preservative fluid	INNOLipa *	LINEAR ARRAY HPV Test	AMPLICOR HPV Test (5)	Cervista™ HPV HR (6)	Cervista™ HPV 16/18 (6)	Digene Hybrid Capture® 2 *(7-9)
Papillocheck transport medium	Papillocheck *					
Universal collection medium	Digene Hybrid Capture® 2 (10)					

### Sampling of cervical cells for HPV detection

Sampling for HPV detection is usually done together with collection of cells for cytology analyses. 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 sample for HPV should be taken first, followed by collection of cytology sample. If the sampling for HPV is performed under the colposcop it has to be done prior to application of the acetic acid on the cervical epithelium.

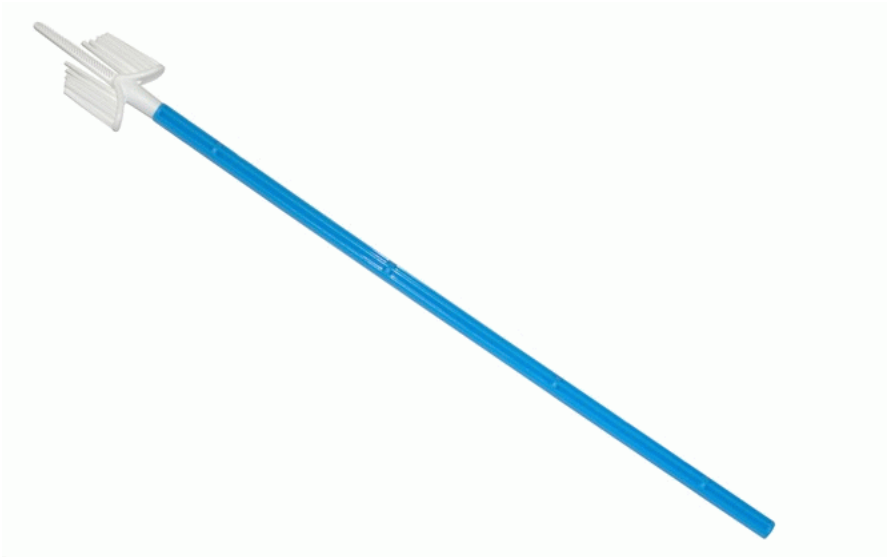
Most common collection devices (Figure 3) for cervical smears are a cervibroom, or a spatula and cytobrush (preferably used in combination). Both have similar efficacy and so the choice usually depends on practitioner preference. The advantage of cervibroom is that only one specimen needs to be collected. For the use with HC 2 test cervical brush from DNAPAP Cervical Sampler (QIAGEN GmbH, Hilden, Germany) can be used (Figure 1). Recently also improved version of cytobrush - Cervex-Brush Combi is available and it has been shown that while giving the same amount of squamous cells, increases the volume of endocervical cells in the sample (11) (Figure 4). The most widely used LBC systems use similar but not interchangeable collection vials and devices. For SurePath the tips of the spatula and cytobrush are snapped off and placed in the fixative. The head of the cervibroom slides off and is placed in the fixative. For ThinPrep the collection device is swirled in the fixative and the collection device is then removed and discarded. When using a combination of collection devices e.g. spatula with cytobrush, they should both be placed together into the same vial of fixative (for SurePath) or rinsed in the same vial of fixative (for ThinPrep).

Fig. 3 Cervical cytology collections devices (left to right) cytobrush, spatula, cervibroom.





Fig.4 Cervex-Brush Combi.



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