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# Molecular Diagnosis of Human Papillomavirus

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## 1. Introduction

The Papillomaviruses are small double-stranded DNA viruses that infect squamous epithelia. They display absolute species-specificity: human papillomavirus (HPV) only infect humans, rabbit papillomaviruses only infect rabbits and so forth. They are also exquisitely tissue tropic, undergoing a complete infectious cycle only in fully differentiating squamous epithelium (Stanley, 2010). Nearly 100 HPVs were described based on the isolation of complete genomes (de Villiers et al., 2004). The circular genome, whose size is close to 8.0 kilobases (kb), is enclosed in a protein shell made from the major (L1) and minor (L2) capsid proteins resulting in virions of approximately 55nm in diameter. It can be divided into 3 domains: a noncoding upstream regulatory region (URR), an early region with 6 genes (E6, E7, E1, E2, E4 and E5), and a late region encoding two genes (L1 and L2). The early genes are involved in DNA replication, transcriptional regulation and cellular transformation. The late genes encode capsid proteins and facilitate the entry of viral DNA into the cell (Tjalma et al., 2005). Approximately 35 HPV types are known to infect the human genital mucosa. They can be grouped as “high risk” (HR) or “low risk” (LR) based on their epidemiologic association with cancer. HR types such as HPV16 and HPV18 are associated with low and high-grade intraepithelial lesions and invasive cancer. The LR types such as HPV6 and HPV11 are associated with genital warts or condyloma acuminata, recurrent respiratory papillomatosis, and low-grade cervical intraepithelial lesions (LSIL) (Trottier and Franco, 2006). The recognition that infection with HPV is essential for the development of cervical cancer led to the development of cervical cancer prevention strategies which incorporate HPV testing.

There are a variety of HPV tests in use worldwide and several of these may be adequate to use for cervical cancer screening and HPV vaccination. An ideal assay should have (Organization, 2009):

1. good sensitivity and specificity as evaluated in internal proficiency testing;
2. ease of transfer to laboratories with varying levels of experience and resources;
3. affordable cost, to allow use in low-resource settings.

It is important that quality assurance programs are established, both in HPV serology and DNA typing, in order to evaluate laboratory performance as well as assay characteristics. The WHO global reference laboratory recommends proficiency panels composed of a series of validated samples, traceable to International Standards, sent to unknown challenges to HPV LabNet members. Results allow an evaluation of individual laboratory performances and an evaluation of whether assays platforms used by multiple laboratories are robust in terms of the generation of acceptable results. HPV cannot be easily propagated by standard *in vitro* culture systems, and in malignant tissue there are little or no infectious HPV particles (Organization, 2009). Other classical direct virological diagnosis techniques, such as electron microscopy and immunohistochemistry, lack the sensitivity and specificity for the routine detection of HPV (Poljak and Kocjan, 2010). For these reasons, the preferred assay formats are based on the detection of HPV nucleic acids.

Molecular methods for HPV DNA detection can be grouped in two main categories: those that rely on signal amplification to detect the targets, and those that rely on target amplification; most of them are based on polymerase chain reaction (PCR). Results of HPV detection are strongly influenced by the technique, and comparison between assays is not always possible. This is why all HPV testing steps need careful standardization including sample collection, extraction and testing. Stability during transport and storage is very important for a good quality DNA. The viral nucleic acid must be preserved to avoid false negative results caused by degradation by endogenous endonucleases. To assess the integrity of genomic DNA in the specimen and its stability for molecular techniques it is crucial to use internal controls such as  $\beta$ -globin. There are several commercially available kits (PreservCyt, Cytoc Corp.) for sample transportation that adequately preserve nucleic acids for molecular diagnosis even after long periods of ambient temperature storage (Molijn et al., 2005, Chan et al., 2006). The choice of which DNA extraction method to use depends on the quality of the clinical material and the diagnostic test that will be executed.

In this chapter, the paramount methods for screening and typing high risk HPV types will be described (Table 2). In particular, we describe molecular methods for the identification of HPVs based on signal amplified hybridization, polymerase chain reaction (PCR), DNA sequencing, type-specific probes, reverse line-blot hybridization, *in situ* hybridization, southern blot hybridization and immunological techniques, including ELISA and western-blot. In addition, we give examples of commercially available kits, for each assay, and point out advantages and limitations of its use.

## 2. Signal amplified hybridization assays

Hybrid Capture to HPV detection was first introduced by Digene Corporation (Gaithersburg, MD, USA) in 1995. This assay is a non radioactive amplification method, based on the hybridization of the target HPV-DNA to labeled RNA probes in solution. It captures the resulting hybrids detecting them by a specific monoclonal antibody and a chemiluminescent substrate, providing a semi-quantitative measurement of HPV-DNA. The second generation of hybrid capture assay - hc2 - uses microtiter plate instead of tubes for the detection of 13 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, on liquid-based cervical specimens (Clavel et al., 1999). Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA:DNA hybrids are captured

onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids, and detected with a chemiluminiscent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted being measured as relative light units (RLUs) on a luminometer (Figure 1). The intensity of the light emitted denotes the presence or absence of target DNA in the specimen. An RLU measurement equal to or greater than the Cutoff Value (CO) indicates the presence of high risk HPV DNA sequences in the specimen, whereas an RLU measurement less than the CO indicates the absence of the specific high risk HPV DNA sequences tested or HPV DNA levels below the detection limit of the assay (Digene, 2004). This assay has an excellent sensitivity (93% according to referral Kaiser Study population) since it can detect HPV16 DNA of a concentration down to 1pg/ml. It is considered the most reliable signal amplified hybridization assay and is a CE-IVD test. On the other hand, it does not detect all high risk HPV types or very low levels of infection. Cross hybridizations with low risk types 6 and 11 may happen (Castle et al., 2002, Seme et al., 2006). Despite the negative aspects, it is still the gold standard technique for HPV detection, highly recommended for comparative evaluations. The hc2 assay has been used in the majority of key randomized controlled and other clinical trials that have proved the clinical value of HPV testing (Cox, 2009, Meijer et al., 2009). For this reason it has been recommended that new HPV assays should show that they possess similar characteristics as hc2 in the process of clinical validation of the test, before it can be used for cervical cancer screening purposes (Meijer et al., 2009).

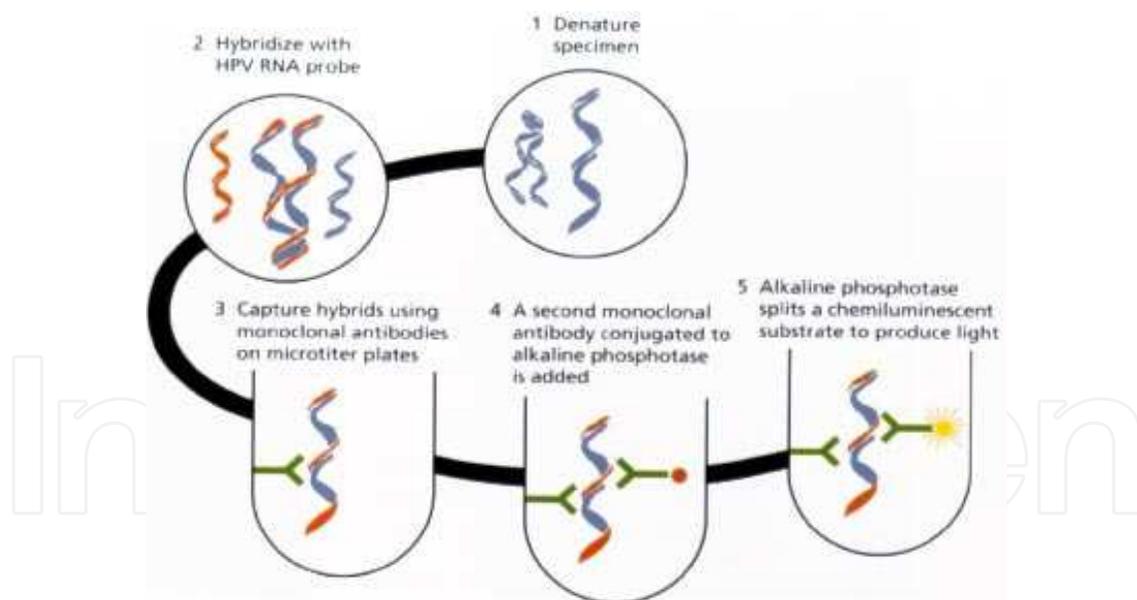


Fig. 1. Hybrid Capture2 test principle, adapted from [www.papillomavirus.cz/eng/diagnosis\\_kits\\_hybrid.html](http://www.papillomavirus.cz/eng/diagnosis_kits_hybrid.html)

The Cervista™ is another FDA approved qualitative test that detects the DNA from 14 high risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The detection of DNA from targeted HPV types, with similar characteristics, is performed with three different probe mixtures. It uses the Invader® chemistry, a signal amplification method for the detection of specific nucleic acid sequences. This method uses two types of isothermal

reactions: a primary reaction that occurs on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal. In the primary reaction, two types of sequence specific oligonucleotides – a probe oligonucleotide and an Invader oligonucleotide – bind to the DNA target sequence. When these oligonucleotides overlap by at least one base pair on the target sequence, an invasive structure forms that acts as a substrate for the Cleavase® enzyme. The enzyme cleaves the 5' portion (flap) of the probe at the position of the overlap. The probes are present in large molar excess and cycle rapidly on and off the target sequence so that many cleaved 5' flaps are generated per target sequence. The cleaved flaps then bind to a universal hairpin fluorescence resonance energy transfer (FRET) oligonucleotide creating another invasive structure that the Cleavase enzyme recognizes as a substrate. The enzyme cleaves the FRET oligonucleotides between the fluorophore and quencher and produces a fluorescence signal as the cleaved flaps cycle on and off. For each copy of target, the combined primary and secondary reactions result in  $10^6$  -  $10^7$  fold signal amplification per hour. The flap sequences and FRET oligonucleotides are universal since they are not complementary to the targeted sequence (Technologies, 2009). Oligonucleotides that bind to the human histone 2 gene (*HIST2H2BE*) are also present in all three oligonucleotides mixtures. This serves as an internal control, producing a semi-quantitative signal from cellular DNA present in the sample (Day et al., 2009). The Cervista™ HPV 16/18 uses the same chemistry for the identification of the two most highly oncogenic and persistent HPV types. It does not allow the exact determination of HPV type(s) present in the clinical specimen, but rather express the result as negative or positive (Day et al., 2009, Poljak and Kocjan, 2010). Sensitivity of the Cervista HPV HR test for detection of  $\geq$ CIN2 among women with ASCUS cytology was 92.8% (84.1-96.9) and the negative predictive value (NPV) was 99.1% (98.1-99.6). Sensitivity for detection of  $\geq$ CIN3 among women with ASCUS cytology was 100% (85.1-100) and the NPV was 100% (99.4-100). HPV 16/18 genotyping test sensitivity of  $\geq$ CIN2 was 68.8 (56.6-78.8) and NPV was 96.0% (93.9-97.4). HPV 16/18 genotyping test sensitivity of  $\geq$ CIN3 was 77.3 (56.6-89.9) and NPV was 99.0% (97.7-99.6) (Einstein et al., 2010). The reproducibility, tested at three different testing centers, resulted in an overall inter-run reproducibility agreement of 98.8% [1-sided 95% Confidence Lower Limit = 96.9%] and an overall inter-site reproducibility agreement of 98.7% [1-sided 95% Confidence Lower Limit = 97.9%] (Day et al., 2009). The Cervista HPV HR test showed no cross-reactivity with DNA from seven non-oncogenic HPV types or 17 different infectious agents at up to 10 copies per reaction (Day et al., 2009). This assay uses standard laboratory equipment and instrumentation, such as thermal cyclers and fluorescence plate readers reducing the need for additional equipment. It has technology proficiency, due to common reaction conditions for all DNA targets, and the simple analysis yields objective results producing straightforward interpretation. It should be used in conjunction with clinical information derived from other diagnostics and screening tests, physical examinations, and full medical history in accordance with appropriate patient management procedures (Day et al., 2009). The limitations of Cervista includes: 1) Cervista HPV HR only detects 14 high-risk HPV types and the Cervista 16/18 only detects 2 high risk types; 2) the Cervista HPV HR test shows cross-reactivity to two HPV types of unknown risk – HPV types 67 and 70 – while the Cervista 16/18 test shows cross-reactivity to high levels of high risk HPV 31 (an HPV16 positive result was observed with  $10^7$  copies/reaction of HPV31); 3) very low levels of infection may cause false negative results; 4) false-negatives can also take

place with cervical specimens contaminated with high-levels of contraceptive jelly and/or anti-fungal creams, when DNA was isolated with the Genfind™ DNA extraction kit; 5) the Cervista HPV HR and 16/18 have been validated only for use with cervical cytology specimens collected in Preserv Cyt® solution using a Rovers Cervex® Brush, Wallach Papette® or endocervical Brush/Spatula; 6) performance of Cervista HPV HR and HPV 16/18 were established exclusively using DNA extracted with the Genfind™ DNA extraction kit and using cervical cytology PreserveCyt® specimen processed on the ThinPrep 2000 processor; 7) the performance of the Cervista HPV HR and HPV 16/18 test has not been established for HPV vaccinated individuals (Technologies, 2009).

AMPLICOR HPV is a CE-IVD PCR based test launched in 2003, by Roche, in America, and in 2004 in Europe. It is capable of detecting 13 HR HPV types with simultaneous assessment of the presence of the human  $\beta$ -globin gene as a positive control. It expresses the result of the test HR HPV in either negative or positive. The method involves the isolation of nucleic acids, PCR amplification, hybridization and absorbance detection. It makes use of a pool of PCR primers designed to amplify HPV DNA from 13 high risk genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Capture probe sequences are located in polymorphic regions of L1 bound by these primers. One additional primer set is used to amplify the human  $\beta$ -globin gene to provide control for cell adequacy, extraction and amplification. After PCR amplification, the HPV amplicon and the  $\beta$ -globin amplicon are hybridized to the oligonucleotide probes bound to the well of the microwell plate. This hybridization of amplicon to the probes increases the overall specificity of the test. Following the hybridization reaction the plate is washed to remove unbound material and avidin-horseradish peroxidase conjugate binds to the biotin-labeled amplicon hybridized to the oligonucleotide probes bound to the microwell plate. The absorbance of the reaction mixture is measured at 450nm (Monsonogo et al., 2005, van Ham et al., 2005). This test has shown high analytical sensitivity with an specificity (Poljak et al., 2005) and overall specificity of 96.5% and sensitivity of 96.1% (Sun et al., 2005). It is comparable to hc2 assay in detecting high grade CIN among women examined for abnormal PAP test. This test sensitivity in detecting both CIN1 and CIN2-3 is practically identical with the sensitivity of colposcopy (with minor abnormality cutoff) but, such as hc2, AMPLICOR assay cannot compete with the specificity of cervical (LBC) cytology (Monsonogo et al., 2005). The current version of Amplicor is not well suited for high throughput testing since it includes many manual steps. The recommended extraction of DNA using the AmpliLute liquid media extraction kit is especially time-consuming, labor intensive and prone to potential cross-contamination, especially when large numbers of samples are being processed. AMPLICOR HPV test does not detect HPV 26, 53, 66, 73 and 82, HPV types that have been classified as probably high risk types (26, 53, 66) or high risk (73 and 82) types in a large epidemiological study (Munoz et al., 2003). Although this test is sensitive, specific, feasible, and easy to handle in routine it does not provide specific genotype information.

A new rapid screening test - careHPV (QIAGEN, Gaithersburg, MD, USA) - based on the simplified hc2 technology - has been developed to detect 14 high-risk types of carcinogenic human Papillomavirus: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68, in about 2.5 h (Qiao et al., 2008). This test is very promising as a primary "screen and treat strategy" due to the ability to obtain accurate HPV results in a few hours. This strategy is especially important for remote and low resource regions of the world where follow-up is difficult (Qiagen, 2009). The advantages of the use of this test are: 1) it can be run in any setting (no

special needs of humidity, temperature, mains electricity or water are required) by a healthcare worker with minimum laboratory training; 2) cervical samples can be collected by a healthcare worker or can be self-collected by the patient herself; 3) the results are easily interpreted and are available in approximately 3 hours (Qiagen, 2009). Limitations include the low throughput with only 24-90 specimens processed manually. However, it is very promising for developing countries HPV screening strategies such as China (Levin et al., 2010, Canfell et al., 2011, Shi et al., 2011) and Thailand (Trope et al., 2009). A cross sectional study assessed the clinical accuracy of careHPV, comparing it with the hc2, in two county hospitals in rural China. A sensitivity and specificity for CIN2+ of 90.0 and 84.2% while standard hc2 parameters were 97.1 and 85.6%, respectively (Qiao et al., 2008).

### 3. Polymerase chain reaction (PCR) assays

The polymerase chain reaction (PCR) is a molecular technique capable of amplifying selectively a particular DNA sequence several million-fold. It consists of repeated cycles of heating and cooling in which a heat stable enzyme (Taq polymerase) uses a denatured double strand DNA to synthesize new molecules of DNA. The starting point for DNA synthesis are the primers (forward and reverse), short strands of nucleic acid, which selectively recognise flanking regions of the DNA template. The sensitivity of this methodology is about 10-100 HPV viral genomes in a background of 100ng cellular DNA (Zaravinos et al., 2009). The sensitivity and specificity of PCR-based methods vary, depending on several aspects such as: DNA extraction procedures, site and type of clinical sample, sample transport and storage, primer sets, size of the PCR product, reaction conditions and performance of the DNA polymerase used in the reaction and ability to detect multiple types (Garland and Tabrizi, 2006).

#### 3.1 Type-specific and consensus primer PCR methods

HPV detection by PCR can be performed using either type-specific primers, which amplify a single HPV genotype, or consensus/general primers, which are usually directed to a highly conserved region of the L1 gene. Type-specific primers are designed to amplify exclusively a single HPV genotype and in order to detect the presence of HPV-DNA in a single sample, multiple type-specific PCR reactions must be performed separately (Molijn et al., 2005). The use of multiple type-specific PCR reactions has the disadvantages of being labor-intensive, costly, time consuming and requiring the evaluation of the type-specificity of each PCR primer set (Molijn et al., 2005, Zaravinos et al., 2009).

The use of consensus primers (Table 1) is more convenient because they can amplify a broad-spectrum of HPV genotypes. Most laboratories use consensus primers targeting the L1 region, since it is the most conserved part of the genome, referring to the assay as L1 consensus PCR. Amplification of each of the primer sets will result in different size amplicons and consequently can result in a variation in sensitivity for detection of certain HPV types, particularly when samples contain multiple genotypes (Garland and Tabrizi, 2006). There are numerous L1 consensus PCR primers that can be used. The first was described in 1989 by Manos and colleagues (Manos et al., 1989). This assay is a set of degenerated primers, MY09/MY11, designed with the intent of amplifying in a single

reaction all the included genotypes. It generates a 450pb amplicon ready for typing determination by subsequent molecular techniques such as restriction fragment length polymorphism and DNA sequencing (Manos et al., 1989). At the time that MY09/11 was described, only 5 of the 20 or more known HPV genotype sequences had been reported: HPV 6, 11, 16, 18, and 33 and the primer set was not entirely homologous even among these 5 genotypes. The intertypic sequence variation was compensated by introducing one or more degeneracies at the priming sites, resulting in a mixture of 24 unique oligonucleotide sequences (Gravitt et al., 2000). The disadvantage of this design is that the synthesis of oligonucleotides containing degeneracies is not highly reproducible and results in high batch-to-batch variation (Molijn et al., 2005). The degenerate MY09/11 primers were afterwards replaced by a set of 18 defined primers, PGMY09/11, to improve specificity and sensitivity. This amplification system combines a number of distinct forward and reverse primers, aimed at the same position of the viral genome. They do not contain random degeneracy but may contain inosine, which matches any nucleotide. Gravitt (Gravitt et al., 2000) and colleagues grouped the virus types by sequence homology in each of the two primer binding regions: a set of 5 upstream oligonucleotides comprising the PGMY11 primer pool and a set of 13 downstream oligonucleotides comprising the PGMY09 (Gravitt et al., 2000) primer set. Using a defined mixture of non-degenerate primers has the advantages that the oligonucleotides can be synthesized with high reproducibility and PCR is performed at optimal annealing temperatures (Molijn et al., 2005).

Other example of consensus primers is the GP5/6, incorporating one forward and one reverse primer aimed at short regions of homology conserved amongst HPV types 1a, 6, 8, 11, 13, 16, 18, 30, 31, 32 and 33 (Snijders et al., 1990). To improve efficiency, part of these sequences were used to elongate GP5 and GP6 at their 3' ends to generate the primers GP5+/6+ (de Roda Husman et al., 1995). The GP5+/6+ primer set generates a 160pb amplicon and reveals an improved HPV detection, reflected by a 10 to 100 fold higher sensitivity, compared with the GP5/6. The mismatching between the primers and the non complementary HPV types (it was only 100% complementary to just a few HPV genotypes) is overcome by setting a low annealing temperature at the PCR reaction (Zaravinos et al., 2009). Qu and colleagues (Qu et al., 1997) compared the MY09/11 and GP5+/6+ primer sets in a group of cervicovaginal samples and concluded that despite good agreement in the detection of HPV DNA -positive and -negative status, there is a significant difference in the detection of multiple types within samples. The MY-PCR primer set was more robust than the GP+-PCR method. The second was relatively inefficient in the amplification of HPV types 53 and 61 compared with the MY-PCR, which was inefficient in the amplification of HPV35. Chan and colleagues (Chan et al., 2006) compared the 3 methods: PGMY09/11, MY09/11, GP5+/6+ in 2006 and concluded that PGMY09/11 showed higher sensitivity with a positive rate of 95.8% compared to 84.2% of the MY09/11 and GP5+/6+ methods. Regarding samples with multiple infection, PGMY09/11 primer set detected most of them (9/11, 81.8%), MY09/11 detected 2/11 (18.2%), whereas GP5+/6+ failed to detect any of these.

General primers in the E1 region have also been described, and several other broad-spectrum PCR primers were reported, but have not been extensively used in clinical situations (Molijn et al., 2005) and therefore will not be mentioned in this chapter.

Primer	Sequence (5'-3') <sup>a</sup>	Amplimer length (bp)	Target <sup>b</sup>
MY09/11	(MY09)CGT CCM ARR GGA WAC TGA TC (MY11)GCM CAG GGW CAT AAY AAT GG	450	L1
PGMY09/11	(PGMY11)A GCA CAG GGA CAT AAC AAT GG (PGMY11B)GCG CAG GGC CAC AAT AAT GG (PGMY11C)GCA CAG GGA CAT AAT AAT GG (PGMY11D)GCC CAG GGC CAC AAC AAT GG (PGMY11E)GCT CAG GGT TTA AAC AAT GG (PGMY09F)CGT CCC AAA GGA AAC TGA TC (PGMY09G)CGA CCT AAA GGA AAC TGA TC (PGMY09H)CGT CCA AAA GGA AAC TGA TC (PGMY09Ia)G CCA AGG GGA AAC TGA TC (PGMY09J)CGT CCC AAA GGA TAC TGA TC (PGMY09K)CGT CCA AGG GGA TAC TGA TC (PGMY09L)CGA CCT AAA GGG AAT TGA TC (PGMY09M)CGA CCT AGT GGA AAT TGA TC (PGMY09N)CGA CCA AGG GGA TAT TGA TC (PGMY09Pa)G CCC AAC GGA AAC TGA TC (PGMY09Q)CGA CCC AAG GGA AAC TGG TC (PGMY09R)CGT CCT AAA GGA AAC TGG TC (HMB01b)GCG ACC CAA TGC AAA TTG GT	450	L1
GP5/6	(GP5)TTT GTT ACT GTG GTA GAT AC (GP6) TGA TTT ACA GTT TAT TTT TC	140-150	L1
GP5+/6+	(GP5+)TTT GTT ACT GTG GTA GAT ACT AC (GP6+)GAA AAA TAA ACT GTA AAT CAT ATT C	160	L1

<sup>a</sup> M, A1C; R, A1G; S, G1C; W, A1T; Y, C1T.

<sup>b</sup> L1, HPV late structural protein 1.

Table 1. Specifications of oligonucleotides used as primers for general HPV detection by PCR

### 3.2 Polymerase chain reaction-restriction fragment length polymorphisms assays (PCR-RFLP)

PCR-RFLP assay is an alternative technique developed to identify and characterize species detecting variations at the DNA sequence level. It involves the digestion of the PCR product with specific restriction endonucleases to generate DNA profiles. Appropriate software such as Webcutter v2.0 (<http://rna.lundberg.gu.se/cutter2/>) is used in order to find the most suitable restriction site. The restriction enzymes will generate several fragments of variable lengths which can be resolved by gel electrophoresis, producing DNA fingerprinting patterns (Kado et al., 2001). This method depends on the availability of restriction enzymes capable of detecting specific mutations. In addition to several in-house genotyping protocols based on PCR-RFLP, there is one commercially available kit. The BIOTYPAP kit (Biotools,

Nave, Spain) allows the detection and identification of 31 HPV types: 6, 11, 13, 16, 18, 30-35, 39, 40, 42-44, 51-54, 56-59, 61, 62, 64 and 66-69, and one subtype (subHPV-44 or subHPV-55). The kit consists of a multiplex amplification reaction, using two pairs of primers. One pair of primers (Pair1- GEN1 and GEN2) hybridizes with sequences common to all tested HPV genotypes (L1 and L2 genes), and therefore, indicates HPV presence. The second pair of primers (Pair 2 - ONC1 and ONC2) hybridizes with specific sequences for oncogenic HPV genotypes (E6 and E7 genes). The first pair of primers renders a band of approximately 450 bp, while Pair 2 renders a product of approximately 250 bp. Amplification products are then subjected to a restriction fragment analysis, on which different restriction patterns will indicate presence of a given HPV genotype (Figure 2) (BIOTYPAP Kit, 2009). Mammas and colleagues (Mammas et al., 2010) used the kit for genotyping of HPV in four children with respiratory papillomatosis. The use of PCR-RFLP has been shown to be useful in the identification of multiple HPV infections as well as the detection of novel HPV types (Kay et al., 2002).

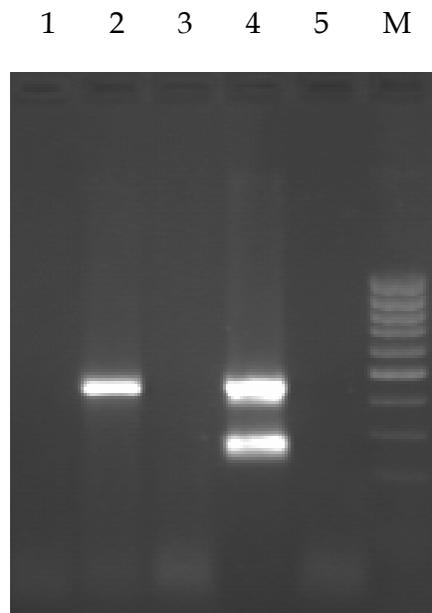


Fig. 2. Detection of HPV with BIOTYPAP Kit. Lane 1 and Lane 3: samples from healthy patients; Lane 2: patient infected with a generic HPV; Lane 4: patient infected with an oncogenic HPV; Lane 5: negative control; M: 100 bp Ladder, adapted from (BIOTYPAP Kit, 2009).

### 3.3 Polymerase chain reaction-restriction fragment mass polymorphism assay (PCR-RFMP)

The PCR-RFMP assay is based on PCR amplification, restriction enzyme digestion, and analysis by MALDI-TOF mass spectrometry. The precise sizing of oligonucleotides after genotype-specific base variation is detected by fragment scission using type IIS restriction enzymes (Lee et al., 2011). The use of a type IIS restriction enzyme makes the RFMP assay sequence independent and applicable to a wide variety of HPV genotypes, because these enzymes have cleavage sites at a fixed distance from their recognition sites. It is a simple and rapid protocol (4-4,5 h) and accurately detects and identifies at least 74 different HPV

genotypes (Hong et al., 2008). When Lee (Lee et al., 2011) and colleagues compared the assay with the well established automated direct sequencing, the PCR-RFMP had a higher analytical sensitivity - 92% and 84% respectively.

### 3.4 Real-time polymerase chain reaction based assays (rtq-PCR)

Real-time PCR reaction, also called quantitative real time PCR, is a technique capable of amplifying and simultaneously quantifying the target DNA molecules. At each PCR cycle it is possible to measure the amount of amplified product. The detection is performed using non-specific fluorescent dyes that intercalate with any double-stranded DNA or using sequence-specific DNA probes. The reaction is performed in a Real-time PCR thermocycler. After each cycle, to estimate the DNA concentration, the fluorescence is measured with a detector and is compared with a control used as reference. Given its capacity to detect the presence and abundance of a specific DNA sequence, rtq-PCR techniques have been developed to quantify HPV-DNA in clinical samples. Type-specific probes can be combined with fluorescence probes although multiplexing several type-specific primers within one reaction is technically difficult. Consensus primers are used in this technique but are less amenable to quantification than a type-specific system (Molijn et al., 2005). This methodology has the following advantages: 1) it is capable of detecting the viral load; 2) the reaction can be performed on multiplex, with the use of different fluorochromes which emit fluorescence as the PCR reaction proceeds; 3) using a 7-log dynamic range to extrapolate viral load/concentration in the standard curve, it is possible to detect nucleic acids at very small concentrations which would not be detectable by conventional PCR; 4) it is extremely reproducible, rapid and pertinent in a clinical setting (Zaravinos et al., 2009).

Novel RT-PCR methods have been released and are capable of being used as high-throughput screening tools. The GenoID (GenoID Ltd., Budapest, Hungary) is a molecular beacon-based one-step multiplex real-time PCR system which detects 15 high-risk HPV types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and 5 low-risk HPV types: 6, 11, 42, 43 and 44. The optimized PCR reaction mixture contains 16 forward, 16 reverse primers and 20 type-specific molecular beacon probes targeted to a special sequence of the L1 gene, where a highly variable sequence is flanked by two conserved sequences. Molecular beacons detecting high-risk types are 5-FAM-3-DABCYL-labeled, molecular beacons for low-risk detection are 5-TET-3-DABCYL-labelled, while the internal control added before sample DNA extraction is detected by a 5-FAMTexasRed-3-DABCYL wavelength-shifting molecular beacon. Accordingly, fluorescent data for HPV detection are collected at 530 nm for high-risk types, 560 nm in case of low-risk types and the reaction internal control is detected at 610 nm on a Roche LightCycler 2.0 instrument (Takacs et al., 2008). The sensitivity for detected types varies between 22 and 700 copies/reaction; the assay shows some cross-reactions, however without comprising the overall clinical applicability of the system.

The Abbott Real Time High Risk HPV test (Abbott Molecular Inc., Des Plaines, IL, USA) is performed on the *m2000rt* real-time PCR instrument (Abbott Molecular) and is designed to individually genotype HPV16 and HPV18 and detect other 12 HPVs: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 using a modified GP5+/6+ primer mix consisting of three forward and

two reverse primers. The assay uses four channels for detecting fluorescent probes: one for detecting the internal control (human beta-globin), a second one for detecting HPV16, a third one for detecting HPV-18, and a fourth one for detecting the remaining 12 hr-HPV genotypes. The assay turnaround time is 6 to 8 hours for 96 samples and depends on the method used for DNA extraction. The fully automated high throughput *m2000sp* instrument (Abbott Molecular Inc.) or smaller *m24sp* instrument (Abbott Molecular Inc.) can be used for DNA extraction, or alternatively DNA can be prepared manually (Poljak et al., 2009). Halfon and colleagues (Halfon et al., 2010b) evaluated the clinical performance of the Abbott Real time HR HPV test, in comparison with biopsy, hybrid capture II and Linear Array, for detection of high-grade disease (CIN2+) in women with abnormal cytology who referred to undergo colposcopic evaluation. All tests had high sensitivity in detecting CIN2+ lesions: Abbott RT HR HPV had 90%, LA had 92% and HCII had 95% and similar specificities: 50%, 47% and 50% for Abbott RT HR HPV, LA, HCII respectively. The authors concluded that the Abbott RT HR HPV assay is good and closely correlated with the other two assays with the advantages of automation and ability to identify HPV16 and HPV18. Another study compared the performance of Abbott RT HR HPV test with that of Hybrid Capture II in 250 liquid-based cervical cytology samples diagnosed with ASCUS, collected from Asian Screening Population. The two tests showed high concordance (absolute agreement: 90%; 95% CI = 0.726–0.874). For detecting cases with underlying HSIL/CIN2+, both tests achieved 100% sensitivity and 100% NPV but Abbott provided higher specificity (20.83% vs 12.50%). The RT-PCR assay is found to provide enhanced sensitivity and specificity for triage of ASCUS (Wong et al., 2011). This assay is also a reliable, sensitive and specific diagnostic tool for the detection and partial genotyping of targeted HPV genotypes in formalin-fixation and subsequent paraffin/paraplast embedding cervical cancer tissue specimens (Kocjan et al., 2011).

The cobas 4800 HPV Test (Roche Molecular Diagnostics, Pleasanton, CA, USA) is a real-time PCR assay for the detection of 14 high-risk HPV types in a single analysis in patient specimens. The test specifically identifies HPV16 and HPV18 while concurrently detecting the rest of the high risk types: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The cobas 4800 System consists of the cobas x 480 instrument for fully automated sample preparation directly from primary sample tubes and the cobas z 480 analyser for real-time PCR based amplification and detection. The cobas x 480 instrument is an automated multi-channel pipetting instrument used to extract, purify, and prepare target nucleic acid for subsequent PCR testing on the cobas z 480 analyser. After completion of sample preparation the cobas x 480 instrument automatically sets up the PCR in a microwell plate. The microwell plate with the PCR-ready samples is then manually unloaded, sealed, and transferred to the cobas z 480 analyser for amplification and detection using real-time PCR (Figure 3) (cobas 4800, 2010). When Castle and colleagues (Castle et al., 2009) described the cobas 4800 HPV DNA Test and compared its performance with the LA test, the percentage of total agreement was 94.7% (95%CI, 92.5%-96.5%), in 531 tested samples. A study enrolling 47,208 women from 61 clinical centers across USA (ATHENA study) evaluated the clinical performance of the cobas 4800 HPV DNA test. The clinical validation of the assay was achieved by determining its performance characteristics for the detection of CIN2+ or worse and CIN3 or worse and by comparing with hc2 test. Sensitivity rates for CIN 2 and CIN 3 were 90% (95%CI, 81.5%-94.8%) and 93.5% (95%CI, 82.5%-97.8%), respectively. The specificity for high grade disease

detected by cobas 4800 HPV Test was also comparable with hc2: 70.5% (95%CI, 68.1%-72.7%) vs 71.1% (95%CI, 68.8%-73.4%), respectively. The main advantage of the assay over hc2 is that it provides information on HPV16 and HPV18 separately (Stoler et al., 2011).

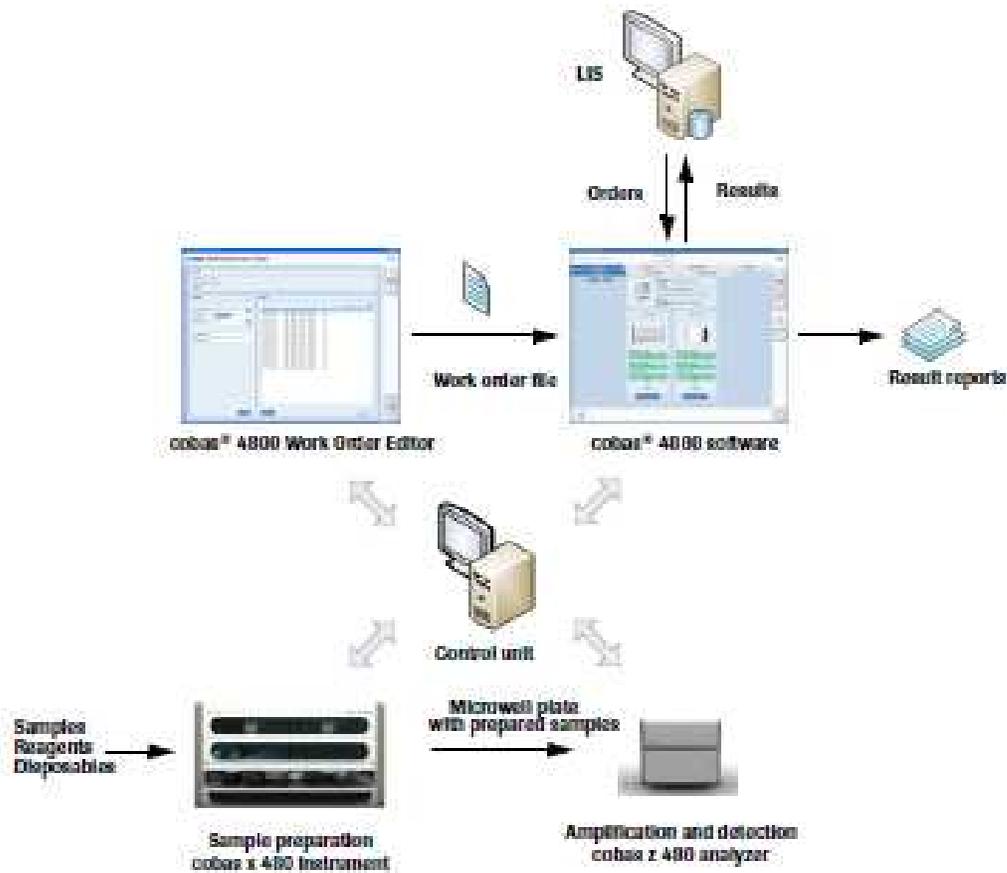


Fig. 3. cobas 4800 HPV Test system overview, adapted from (cobas 4800, 2010)

### 3.5 Reverse-transcription polymerase chain reaction assays (RT-PCR)

Although the majority of HPV detection strategies are DNA based, it is possible to look for specific HPV viral RNA by incorporating a reverse transcriptase (RT) step before PCR amplification (Molijn et al., 2005). Reverse transcriptase is a DNA polymerase enzyme that transcribes single-stranded RNA into double-stranded DNA. In RT-PCR a specific mRNA is first 'reverse transcribed' into its DNA complement (cDNA) and then amplified using traditional PCR or rtq-PCR methods. The most relevant transcripts for diagnostic purposes are those encoding viral oncoproteins E6 and E7. The detection of viral mRNA can be done by reverse transcriptase PCR or by nucleic acid sequence-based amplification (NASBA) (Poljak and Kocjan, 2010).

The Pretect HPV-Proofer (HPV-Proofer; NorChip, Klokkestua, Norway) is based on real-time multiplex NASBA and has the advantage of detection type-specific E6/E7 mRNA from the carcinogenic HPV types: 16, 18, 31, 33, and 45. Two primer and probe-sets are included in each reaction vessel. This assay can therefore be used in monitoring a persistent viral infection. Also, it is possible to detect the transforming potential of the infection, due to the oncogenic capacity of E6/E7 genes (Molden et al., 2007). The protocol of this assay is based

on isothermal RNA amplification, accomplished by the simultaneous enzymatic activity of avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase, and RNase H. For detection, two different types of probes are used, an ECL probe and a molecular beacon probe direct against E6/E7 mRNA (Figure 4). As performance control, to avoid false negatives due to degradation of nucleic acid, a primer set and probe directed against the human U1 small nuclear ribonucleoprotein-specific A protein (U1A mRNA) is included. Two differently labeled molecular beacon probes are used in each multiplex reaction. Fluorescein (FAM) is used as a fluorophore for the detection of HPV16, 31 and 33; Texas Red (TxR) is used for the detection of U1A, HPV 18 and 45. All the molecular beacons contain the non-fluorescent quencher Dabsyl CPG (1-dimethoxytrityloxy-3-[O-(N-4'-sulfonyl-4-(dimethylamino)-azobenzene)-3-aminopropyl]-propyl-2-O-succinoyl-long chain alkylamino-CPG) (Kraus et al., 2004, Molden et al., 2007). The performance of HPV-Proofer requires standard laboratory equipment, the lambda FL600 fluorescence reader- NASBA platform (Bio-Tek, Wnooski, VT, USA) and PreTect Analysis software (NorChip) for analysis of the experimental data. The analytical sensitivity of the assay is less than 10 SiHA cells, equivalent to 20 copies of HPV16 and less than 1 HeLa cell equivalent to 25 copies of HPV18 (Lie et al., 2005). All studies that have compared HPV-Proofer with hc2 or different in-house or commercial PCR-based HPV DNA assays, showed that HPV-Proofer has a lower clinical sensitivity for the detection of CIN2+ lesions than DNA-based assays, but a significantly higher clinical specificity (Kraus et al., 2004, Lie et al., 2005, Molden et al., 2007, Szarewski et al., 2008).

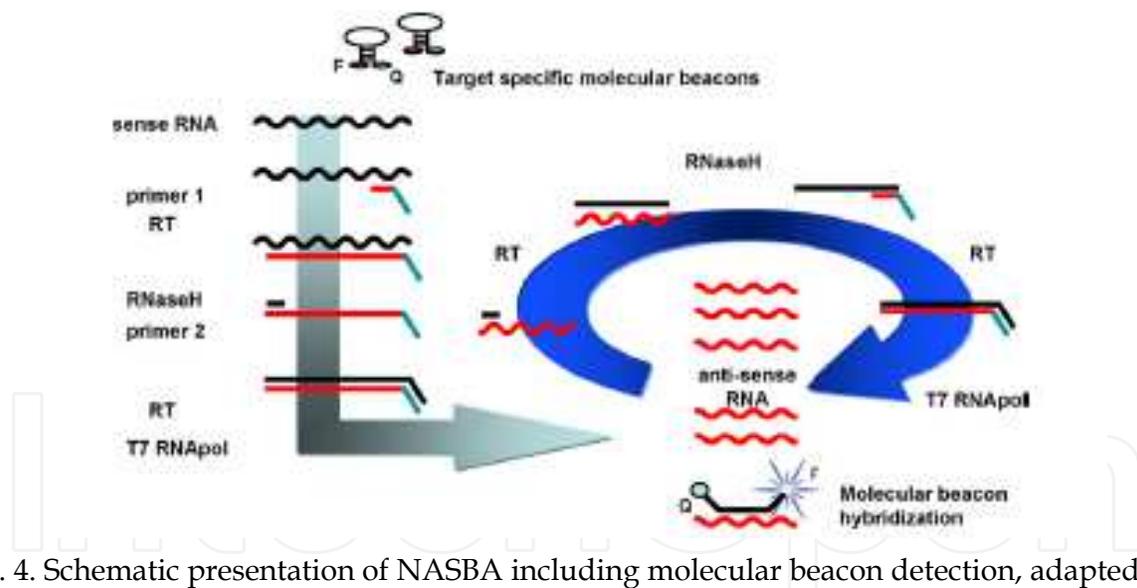


Fig. 4. Schematic presentation of NASBA including molecular beacon detection, adapted from (Molden et al., 2007)

The NucliSENS EasyQ HPV v1 (NucliSENS; bioMÉRIEUX) is a real-time nucleic acid amplification and multiplex detection assay for the qualitative determination of E6/E7 mRNAs of the HPVs 16, 18, 31, 33 and 45. The assay utilizes the nucleic acid sequence-based amplification (NASBA) technology, detecting both viral and bacterial nucleic acids. NucliSENS EasyQ HPV version 1 was launched in 2007 and was based on the original PreTect Proofer assay, except for the NucliSENS hardware platform and the software for NASBA measurements and data analysis (NucliSENS Director software). The latest NucliSENS EasyQ HPV assay version 1 has recently been improved in the following ways:

taking into account the RNA nature of the HPV-specific targets, the five positive controls for the different subtypes and the U1A internal control were changed from single stranded DNA to RNA; also, in order to facilitate handling and to increase reproducibility of the assay, the positive controls are no longer provided in liquid phase but in freeze-dried accospheres; aiming to improve and harmonize the sensitivity for the five HPV types covered by the assay, the concentrations of the primers and beacons for HPV16 and the U1A internal control were re-adjusted; the extraction protocol on NucliSENS EasyMAG® system was optimized; the HPV assay protocols were updated and the NucliSENtral™ HPV software V1.1 introduced to further improve the convenience of operator use for results reporting (Jeantet et al., 2009). The calculated analytical sensitivity ranges from  $2.3 \times 10^2$  to  $3.0 \times 10^4$  copies/mL, showing the highest sensitivity for HPV45 and the lowest for HPV31 (Jeantet et al., 2009). Halfon and colleagues (Halfon et al., 2010a) compared this mRNA assay with the hc2 assay in a population of atypical cells of undetermined significance/low-grade squamous intraepithelial lesion/high-grade squamous intraepithelial lesion (ASCUS/LSIL/HSIL) patients. The clinical sensitivity of NucliSENS EasyQ HPV was 63% for the detection of CIN2+ or HSIL, significantly higher than the sensitivity of hc2 and LA (49% and 45%, respectively). The sensitivity of the assay is significantly lower than that of hc2 and LA (76% vs 95% and 76% vs 92% respectively).

The APTIMA HPV Assay (Gen-Probe Incorporated, San Diego, CA) is a multiplex nucleic acid test that detects HPV E6/E7 mRNA from 14 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The assay provides a qualitative result (positive/negative) for the presence/absence of these high-risk HPV types, but does not determine the specific HPV type present in the specimen. The APTIMA HPV Assay involves three main steps, which take place in a single tube: capture of the target mRNA using HPV-specific capture oligomers and magnetic microparticles; target mRNA amplification using transcription-mediated amplification, and detect the amplification products (amplicons) using the Hybridization Protection Assay (Dockter et al., 2009a). An internal control transcript is added to each reaction to verify the performance of each step of the assay: capture, amplification and detection. One positive calibrator and one negative calibrator are used to determine the validity of the run and to establish the assay cutoff values for the internal control and analyse signals. A positive control and a negative control are also processed as separate samples and used to determine run validity. The assay can be performed on the semi-automated Direct TubeSampling (DTS) systems, or on the fully automated TIGRIS DTS system (Gen-Probe Incorporated, San Diego, CA). The throughput is approximately 180 specimens for 1 operator in about 5 to 6 hours for the DTS systems and approximately 1000 specimens in about 14 hours for the TIGRIS DTS system (Dockter et al., 2009a). The analytical sensitivity of the assay in the semi-automated DTS systems was between 38 and 488 HPV mRNA copies and in fully automated TIGRIS DTS system was between 17 and 275 HPV mRNA copies. The overall analytical specificity of the assay was equal to or greater than 99% in both systems. Dockter and colleagues (Dockter et al., 2009b) evaluated APTIMA HPV Assay performance for detection of high risk HPV and high-grade cervical intraepithelial neoplasia (CIN) compared to hc2. The mRNA based assay was found to be highly sensitive and specific for the detection of high-risk HPV in clinical samples, with a sensitivity of 92% and a specificity of 98%. The results obtained in the DTS and TIGRIS DTS systems were equivalent. Another study compared the sensitivity and specificity of several tests, including APTIMA HPV Assay, for the detection of high-grade CIN in a population

referred to colposcopy because of abnormal cytology (Szarewski et al., 2008). Four adjuvant tests had sensitivities greater than 95% for high-grade disease (CIN2+ and CIN3+ respectively): Amplicor (98.9% and 99.5%), Hybrid Capture II (99.6% and 99.5%), Linear Array (98.2% and 99.0%) and APTIMA (95.2% and 97.4%). Of these, APTIMA showed the highest specificity (42.2% and 38.8%).

#### **4. Sequencing reaction (GP6+) – big dye terminator cycle sequencing kit**

As previously mentioned, sequencing methods of PCR products are also now available for high throughput allowing its application to routine clinical analysis (Ekstrom et al., 2011). Nested PCR amplification of a conserved region of the HPV *L1* gene, with the consensus GP5+/GP6+ primers, followed by genotyping with direct DNA sequencing (BigDye Terminators, Applied Biosystems) and alignment of the hypervariable region of the L1 gene against known HPV genotype sequences, stored in the GenBank database, usually determines the genotype of the HPV isolates detected (Molijn et al., 2005). LoTemp™ HiFi® DNA polymerase has shown to be 10 times more efficient, than other Taq DNA polymerases, in amplifying HPV plasmid DNA by MY09/MY11 PCR and about 100 to 1000 times more efficient when the first amplification was followed by a GP5+/GP6+ nested PCR in tandem (Lee et al., 2007). The nested PCR technology described by Molijn *et al.* proved to be a sensitive method for the detection of 1-10 copies of purified genomic DNA of HPV types 16, 18 or 6B. However,  $10^4$  -  $10^5$  copies of genomic DNA were needed as PCR templates for UV visualization of a positive primer amplicon after electrophoresis. Reproducibility of this nested PCR assay was confirmed (Molijn et al., 2005). The exploitation of this method allows the identification of new HPV types given that it is not intended for the identification of specific types. Sequencing protocols, in particular the requirement for template purification, are too labor-intensive for routine applications. In the presence of samples infected with more than one genotype of HPV are unsuitable for HPV determination. Sequences which represent a minority species, in the total of the PCR product, may remain undetected underestimating the prevalence of infections with multiple HPV genotypes. After detecting a sample with multiple HPV infection the genotype can be deduced by two methods: 1) database homology search (BLAST) and 2) phylogenetic analyses (Kleter et al., 1999). In any case, it is time consuming and not totally reliable. Thus, this method is not readily adapted to routine diagnostic labs.

### **5. Type-specific probe assays**

#### **5.1 DNA Microarray genotyping assays**

The DNA microarray system has been largely used in HPV typing (Jacobs et al., 1997). A DNA microarray is a collection of microscopic DNA probes attached to a solid surface by a covalent bond. Each probe contains picomoles of a specific DNA sequence like a short section of a gene. The cDNA targets are fluorescently labelled and under high-stringency conditions hybridize with the probes on the surface of a chip. In each spot the fluorescent light is detected by excitation with monochromatic light and transformed in image by software. This technology can screen a high number of markers per individual and it is particularly suited for strategy using large populations. An example of this methodology is HPV DNA chip (Biomedlab, Seoul, South Korea) which contains 22 type-specific probes,

allowing identification of 15 high-risk types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69 and 7 low-risk types: 6, 11, 34, 40, 42, 43 and 44. In summary, target HPV DNA is amplified by PCR with hvp1/hpv2 primers and labeled utilizing Cy5-dCTP or Cy5-dUTP, with primers and conditions described by manufacturer. Secondly, the PCR product is hybridized onto the chip and, after washing, the hybridized signals are visualized with a DNA chip scanner (Seo et al., 2006). The HPVDNACHip test was successfully implemented in a population based study in Korea with 2.470 women (Hwang et al., 2004) and in a study that compared the performance of four HPV genotyping assays on a panel of 824 samples; the analytical sensitivity of the test was around 80% and specificity for the individual HPV types was above 94% (Klug et al., 2008).

The GG HPVCHIP (GoodGene, Seoul, South Korea) allows the identification of 42 alpha-HPV types; 15 high-risk types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69 and 70; Three probable high-risk types: 26, 53 and 66; 20 low-risk types: 6, 7, 10, 11, 27, 32, 40, 42, 44, 54, 55, 57, 61, 62, 72, 73, 91, CP8304/81, and MM7/83, MM8/84; and four undetermined-risk types: 30, 34, 67 and MM4/82. The DNA chips collect multiple oligonucleotide probes of the 42 HPV types and human  $\beta$ -globin gene. The DNA is amplified with Cy5-labelled primer for E6, E7 and L1 gene and the produced amplicons are applied onto the DNA chip, followed by hybridization and the genotypes of HPV within sample are identified by a fluorescent scanner (Kim et al., 2006). The performance of the GG DNACHIP was compared with PCR sequencing on 100 cervical cancer specimens and both methods detected 98% (98/100) high-risk types of the samples tested (Kim et al., 2006).

The HPV GenoArray Test Kit (GenoArray, HybriBio Limited, Hong Kong) uses both DNA amplification and HybriBio's proprietary flowthrough hybridization technique to simultaneously identify 21 alpha-HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66, 68 and 81. The test employs a microarray format with a nylon membrane onto which HPV genotype-specific oligonucleotide probes have been immobilized (Grisaru et al., 2008). Liu and colleagues (Liu et al., 2010) compared the HPV GenoArray test with Linear Array, revealing concordant or compatible results for 97,5% of the samples and discordant results for only eight samples (2,5%). The assay appears to be highly sensitive and specific for the genotyping of HPV and has the advantage that specifically detects HPV52, which overcomes a limitation of the Linear Array.

Less frequent in peer-reviewed literature are the kits GeneTrack HPV DNA Chip (GeneTrack, Genomic Tree, Daejeon, South Korea) and GeneSQUARE HPV Microarray (Kurabo Industries, Osaka, Japan). The first identifies 28 HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42-45, 51, 52, 54, 56, 58, 59, 62, 66-72 and consists in the amplification of a 450 bp region of the L1 gene using MY09/11/HMB01 primers and of an internal control (portion of the human interferon-2 gene). To determine the HPV genotype, the amplicons are hybridized on a DNA chip with eight microarrays chambers, each containing a duplicate of the 28 type-specific probes and a replicate of a human cellular target-specific probe (Oh et al., 2004, Poljak and Kocjan, 2010). The GeneSQUARE HPV is a microarray system in development that utilizes a multiplex PCR to amplify and identify 23 alpha-HPVs: 6, 11, 16, 18, 30, 31, 33-35, 39, 40, 42, 45, 51-54, 56, 58, 59, 61, 66 and 68. Type-specific primer pairs are used in the assay and the upstream primer in each pair is labeled with biotin at the 5' end. After hybridization, the microarray is washed, dried and scanned (Figure 5) (Ermel et al., 2010, Matsushita et al., 2011). Ermel and colleagues (Ermel et al., 2010) compared the hc2,

Linear Array and GeneSQUARE methods and the sample proportion agreement was very good, particularly between Linear Array and GeneSQUARE.

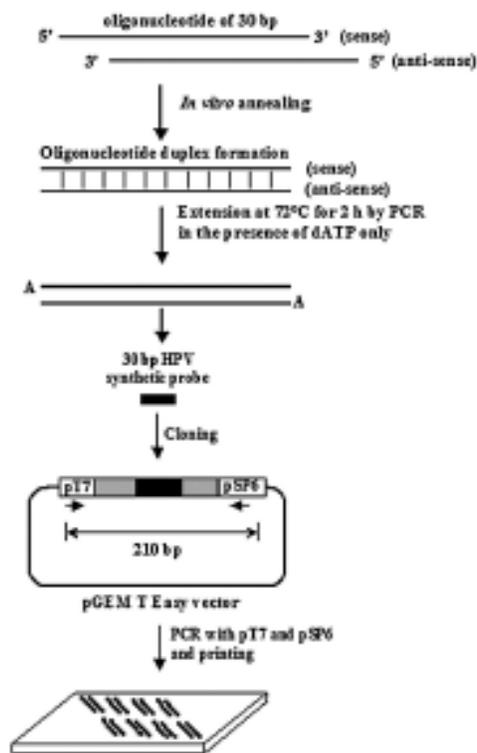


Fig. 5. Protocol for HPV-specific probe synthesis by oligonucleotide shuffling used in GeneTrack, adapted from (Oh et al., 2004)

The Infiniti HPV assays (AutoGenomics, Carlsbad, CA, USA) are commercially available genotyping platforms for HPV genotyping that uses multiplex PCR, followed by automated processing for primer extension, hybridization and detection (Erali et al., 2009). It groups three assays: Infiniti HPV Genotyping Assay, Infiniti HPV-HR Quad assay and Infiniti HPV-Quad Assay. The Infiniti HPV-Quad assay targets the E1 gene of the HPV genome and identifies five individual HPV types: 16, 18, 31, 33, and 45, five combinations of HPV types: 35/68, 39/56, 58/52, 59/51, 6/11 and a  $\beta$ -globin internal control. When Erali and colleagues (Erali et al., 2009) compared this assay with Hybrid Capture II, the overall concordance of positive and negative results was 83% among the 197 specimens tested.

The PANArray HPV Genotyping Chip (PANArray, PANAGENE, Daejeon, Korea) uses peptide nucleic acid probes for the identification of 31 HPV types: 6, 11, 16, 18, 26, 31-35, 39, 40, 42-45, 51-54, 56, 58, 59, 62, 66, 68-70, 73, 81, 83 and one subtype, subHPV-44 or HPV-55. MY09/11 PCR products nested with GP5+/biotinylated-GP6+ primers are used for the genotyping (Poljak and Kocjan, 2010). A study comparing the genotyping results of PANArray to sequencing with MY09/11 PCR products showed excellent agreement except for samples reflecting multiple infections (Choi et al., 2009).

Another illustration of this methodology is Clart HPV 2 assay (Genomica, Madrid, Spain), developed for the detection and genotyping of 35 HPV types: 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 and 89. It uses biotinylated primers that amplify a 450bp fragment within L1 region. Co-amplification of an 892 bp region of the CFTR gene and 1,202 bp fragment of a transformed plasmid provides a control to ensure DNA extraction and PCR efficiency (Pista et al., 2011). The performance of Clart HPV 2 requires standard laboratory equipment and the Clinical Arrays Processor (Genomica) for fully automated microarray processing and genotyping analysis (Poljak and Kocjan, 2010). A comparative evaluation of Clart HPV 2 with Hybrid Capture 2 Test on samples with cervical intraepithelial neoplasia grade 2 or worse showed a clinical sensitivity of 96.9% and specificity of 71.9% (Pista et al., 2011). When Galan-Sanchez and colleagues (Galan-Sanchez and Rodriguez-Iglesias, 2009) compared INNO-LIPA HPV Genotyping v2, Linear Arrays HPV Genotyping Test and Clart HPV 2 the results were absolutely concordant in 31 samples, compatible (some but not all genotypes) in 44 samples and discordant in only 3 samples; concluding that all the 3 methods are highly comparable and suitable for clinical and epidemiological studies. The advantages of this methodology, made a successful use in large-population-based study in Spain, where 2362 women from three different regions were investigated (Gomez-Roman et al., 2009).

The PapilloCheck HPV-Screening Test (Greiner Bio-One GmbH, Frickenhausen, Germany) is one of the two most frequently used PCR-microarrays-based assays (Poljak and Kocjan, 2010). It is a PCR-based test using a consensus primer set targeting the E1 HPV gene. HPV oligoprobes immobilized on a DNA chip allow for the identification of 24 HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42-45, 51-53, 56, 58, 59, 66, 68, 70, 73 and 82. Each PapilloCheck chip contains 12 wells defined by elevated rims, each of which contains one PapilloCheck array with 28 probes, each in 5 replicate spots (Dalstein et al., 2009). This test utilizes the amplification of 350 pb HPV DNA fragment as a new consensus primer set: each reverse primer was extended at 5' end with a universal tag. Additionally, a region within the human ADAT1 gene is simultaneously amplified using fluorescent-labeled primers with Cy5-dUTP. The amplicons hybridize to HPV specific oligoprobes immobilized on the DNA chip the readings are performed on a CheckScanner instrument (Dalstein et al., 2009). Several comparative evaluations of PapilloCheck with other HPV assays have been published (Halfon et al., 2010c, Hesselink et al., 2010, Iftner et al., 2010, Schopp et al., 2010, Kitchener et al., 2011). This genotyping test was compared with SPF10PCRLiPav and PGMY09/11 on Hybrid Capture2 pretested samples (Schopp et al., 2010). From results of 826 cervical samples, the PapilloCheck found 100% sensitivity in HPV types 53, 56 and 33. The lowest detection rate was observed for type 35 (72,2%) and the detection rates for the identification of high-grade intraepithelial neoplasia (CIN2+) ranged from 93,7% (PGMY09/11 PCR) to 98,4% (PapilloCheck, SPF10 PCR, HC2), leading to the conclusion that the PapilloCheck gives comparable results on established PCR methods (Schopp et al., 2010). Halfon and colleagues (Halfon et al., 2010c) compared PapilloCheck, Linear Array and hc2 in 239 women referred for colposcopy and histology, concluding that all tests showed a good sensitivity (greater than 90%) without statistically relevant differences between them.

The application of microarray technology as a diagnostic tool shows great advantages, since microarray can discriminate HPV genotypes and identify multiple infections (Kim et al., 2003). Ideally, a larger number of HPV type-specific probes could be spotted on a chip, augmenting the quantity of HPVs genotyped by assay. Unfortunately this method

requires the presence of expensive equipment and therefore is not suitable for all the laboratories.

## 5.2 Suspension array genotyping assays (HPV-SA)

The HPV-SA provides a rapid and cost-effective method to simultaneously detect different HPV genotypes. This technology uses microspheres as array elements that carry appropriate receptor molecules such as DNA oligonucleotide probes, antibodies, or other proteins. Microspheres are readily suspendable in solution and possess distinct optical properties. Each bead can easily be differentiated based on variations and intensity of colour. The most common detection systems are light scatter or fluorescence from an internal dye. The Luminex xMAP system is a flexible analyzer based on the principle of flow cytometry. It incorporates a proprietary process to internally dye polystyrene beads with two spectrally distinct fluorochromes. Each bead set is usually coupled to a single oligonucleotide probe specific for one HPV type. Genotyping is done by reverse hybridization using biotinylated PCR amplicons. After denaturing and hybridization of target HPV sequences to the bead-bound probes, labeling of the hybridized biotinylated amplicons is done using R-phycoerythrin-labelled streptavidin, serving as a reporter fluorophore. The bead sets are then read and analysed on a Luminex analyser (Poljak and Kocjan, 2010).

There are several in-house genotyping protocols based on xMAP technology. Jiang and colleagues (Jiang et al., 2006) developed a rapid high-throughput DNA suspension assay, capable of simultaneously typing 26 HPVs: 6, 11, 16, 18, 26, 31, 33-35, 39, 40, 42-45, 51-54, 56, 58, 59, 66, 68, 73 and 82. The HPV-SA consists of 26 different microsphere sets with spectral addresses. Each microsphere set shows an HPV type-specific probe on its surface, and they can be combined allowing up to 26 different HPV targets to be measured simultaneously in a single reaction vessel (Figure 6). Another in-house SA protocol was developed by Oh and colleagues (Oh et al., 2007) combining PCR amplification with Luminex hybridization. The use of HPV type-specific probes and YBT L1/GP6-1 primers detects HPVs 6, 16, 18, 31, 35, 42, 51, 52, 55, 56, 58, 59, 66, 67 and 68. The authors compared the SA to the established HPV DNA microarray chip for PCR products derived from 53 clinical samples. The evaluation showed excellent agreement as the SA was a very sensitive and reproducible technique for simultaneously genotyping of all clinically relevant genital HPV types (Oh et al., 2007).

In addition, at least two commercial assays based on this technology are available at present. The Multiplex HPV Genotyping kit v1.0 (Progen/Multimetrix, Heidelberg, Germany) is a research use only (RUO) assay that allows detection and identification of 24 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66, 68, 70, 73 and 82. The sample is subjected to PCR amplification, using sets of biotinylated primers contained in the kit. The amplicons are added to bead mix, containing 26 distinct bead populations coupled to 24 HPV, one  $\beta$ -globin and one hybridization control specific oligonucleotide probe. The  $\beta$ -globin control serves as quality control for genomic DNA in the PCR. After thermal denaturing and hybridization of target sequences to the bead-bound probes, labeling of the hybridized biotinylated PCR products is achieved by R-phycoerythrin labeled streptavidin. Schmitt and colleagues (Schmitt et al., 2006) compared the performance of Multiplex Human

Papillomavirus Genotyping assay to an established reverse line blot assay on GP5+/6+ PCR products derived from 94 clinical samples. The evaluation showed an excellent agreement but also a higher sensitivity of the Multiplex assay.

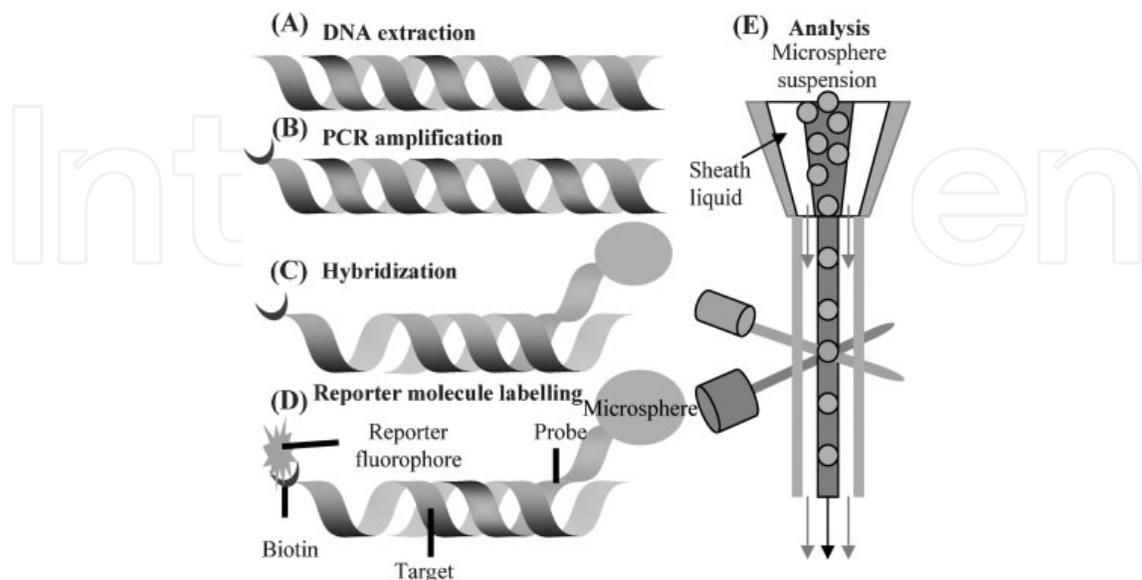


Fig. 6. Schematic representation of the HPV-SA. After HPV DNA extraction (A), the template was amplified with the MY09/11 primer set (B). PCR products are transferred to a PCR tube containing the 26-plex HPV-SA and hybridized (C). After washing, the hybridized microspheres were incubated with streptavidin-R-phycoerythrin at room temperature for 30 min (D). Finally, the mixture is analysed on the Luminex100 analyser (E), adapted from (Jiang et al., 2006).

The digene HPV genotyping LQ Test RUO (Digene LQ Test; Qiagen) uses multiplex, bead-based xMAP technology and an automated, high-throughput read-out by either the LiquidChip 200 workstation (Qiagen, Hilden, Germany) or Luminex 100 IS System (Luminex Corporation, Austin, TX, USA). The test was developed for identification of 18 high-risk HPVs: 16, 18, 26, 31, 33, 35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73 and 82 using GP5+/6+ PCR products. Read-out of this assay is expressed as the medium fluorescent intensity of the reporter fluorescence for each genotype (Geraets et al., 2009b). When compared with in-house GP5+/6+, the assay demonstrated high agreement for overall detection and type-specific identification (Geraets et al., 2009b).

## 6. Reverse line-blot hybridization-based HPV genotyping assays

It is the most common method used for HPV genotyping nowadays. In these types of assays a fragment of the HPV genome is first amplified by PCR, using biotinylated HPV-specific primers, and the resulting amplicons are then denatured and hybridized with HPV-specific oligonucleotide probes immobilized as parallel lines on nylon or a nitrocellulose membrane strip. After hybridization, streptavidin-conjugated alkaline phosphatase or horseradish peroxidase is added, which binds to any biotinylated hybrid formerly produced (Figure 7). Incubation with chromogenic substrates yields a colored precipitate at the probe positions where hybridization occurs. The genotyping strip is then visually interpreted by comparison with the test reference guide for each of the targeted HPV types. The principle of INNO-

LiPA HPV Genotyping assay is based on the amplification of a 65bp region of the HPV *L1* gene, using biotinylated SPF10 primers, followed by the hybridization of the resulting amplicons with HPV specific oligonucleotide probes immobilized on a nitrocellulose strip. This is one of the most widely used HPV genotyping kits and there are several versions developed of which the first version, the INNO-LiPA<sub>25</sub> HPV genotyping V1.0 (Labo Biomedical Products, Rijswijk, The Netherlands) allowed the identification of 26 HPVs: 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 73 and 74 (Kleter et al., 1999). More recent versions of this assay, the INNO-LiPA HPV Genotyping v2 and INNO-LiPA HPV Genotyping CE Assays (Innogenetics) allowed the identification of 24 and 17 individual types, respectively. The latest version, INNO-LiPA HPV Genotyping *Extra*, allows the simultaneous identification of 28 different HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69/71, 70, 73, 74 and 82. The former assay contains an additional primer pair, in the amplification mix, for the amplification of the *HLA-DPB1* gene to check for PCR inhibition, sample quality and DNA extraction, as well as an anticontamination system based on uracil-N-glycosylase (Poljak and Kocjan, 2010). Several evaluation studies have been performed in recent years. A recent study compared real-time multiplex HPV PCR assays with INNO-LiPA Genotyping *Extra* suggesting increased sensitivity of DNA HPV detection by type-specific multiplex HPV PCR assays (Else et al., 2011). Another interesting and important assessment was the comparative evaluation of different extraction methods for genotyping with INNO-LiPA and Linear array genotyping tests. This study suggests that the use of consistent protocols for DNA purification is a priority to guarantee intra-assay reproducibility over time (Dona et al., 2011). INNO-LiPA HPV Genotyping *Extra* is also recommended for the use with paraffin-embedded tissue. After comparison with Linear Array HPV Genotyping Test the INNO-LiPA genotyping *Extra* revealed greater sensitivity for HPV genotyping from archival tissue (Tan et al., 2010). INNO-LiPA *Extra* has a sensitivity of 20-70 viral copies per assay (estimated for HPV-16, 18, 31, 45 and 52) (Seme et al., 2009).

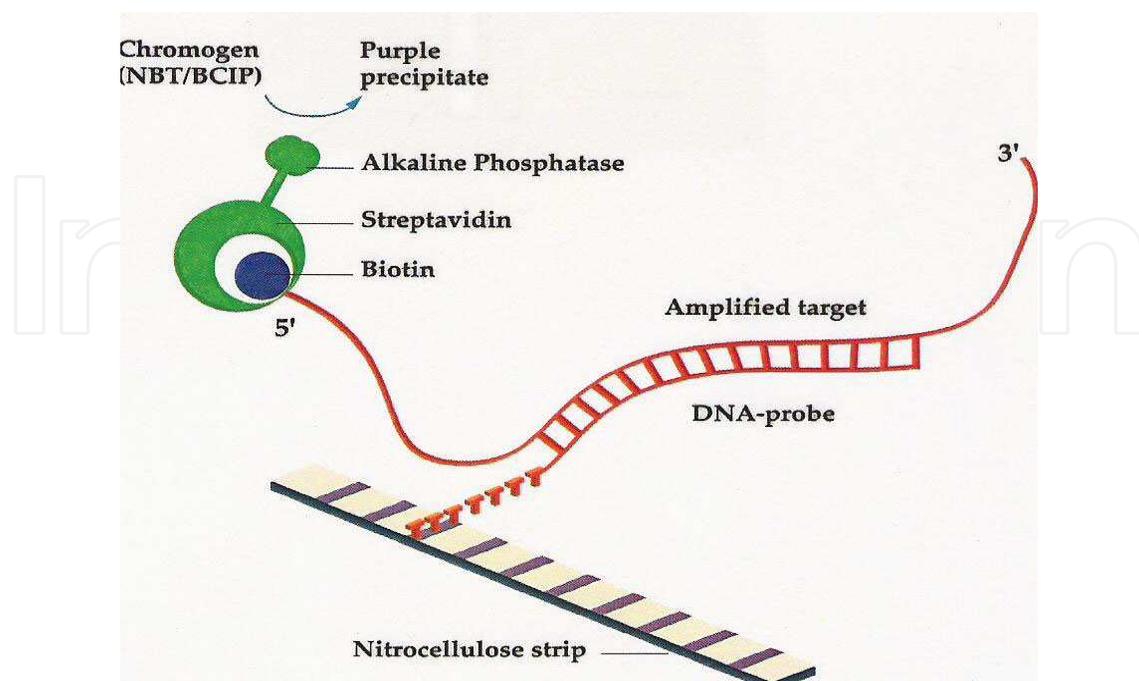


Fig. 7. INNO-LiPA HPV Genotyping test principle, adapted from INNO-LiPA brochure.

As with INNO-LiPA, Linear Array HPV Genotyping assay is based on the amplification of a region of the HPV *L1* gene (450 bp). This genotyping test also amplifies a 268bp region of the human  $\beta$ -globin gene, using biotinylated primer sets PGM09/PGMY11 and PC04/GH20, respectively. Subsequent hybridization of the resulting amplicons is made onto a single-typing nylon strip coated with HPV type specific and  $\beta$ -globin specific oligonucleotide probes. This test is one of the most commonly used HPV genotyping assays which combines PCR amplification and reverse line-blot hybridization for the identification of 36 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84 and 89 and one subtype (subHPV-82 or IS39) (Poljak and Kocjan, 2010). It is registered for use in the European Union (CE-IVD) but not available for use in the United States. Numerous comparative evaluations of Linear Array with other HPV genotyping assays have been performed. All studies reported high concordance amongst hybridization genotyping methods and suitability for clinical and epidemiological studies (Galan-Sanchez and Rodriguez-Iglesias, 2009). Linear Array has revealed good correlation and perfect agreement with hc2, with high sensitivity; 100% detection of cervical intraepithelial neoplasia (CIN) III was shown by both Linear array and hc2 (Paolini et al., 2011). Comparison of this genotyping assay with real-time multiplex HPV PCR assays, holding as constant the DNA extraction method (Qiagen Spin blood kit), revealed that multiplex PCR assays detect more HPV-positive specimens for the 14 HPV types common to both than Linear Array HPV genotyping assay. Type-specific agreements between the assays were good but were often driven by negative agreement in HPV types with low prevalence, as evidenced by reduced proportions of positive agreement. An alternate DNA extraction technique (Qiagen MinElute kit) impacted subsequent HPV detection in both the multiplex PCR and Linear Array (Roberts et al., 2011). A comparative evaluation with INNO-LiPA has shown high concordance amongst both methods; however, when comparing individual HPV types, Linear Array was able to detect significantly more HPV-16, 18, 39, 40, 42, 54, 58, 59, 66, 70 and 68/73, and fewer HPV-11, 31 and 52 than INNO-LiPA (Castle et al., 2008). Furthermore, INNO-LiPA was able to detect more multiple HPV infections and a greater number of HPV types per multiple infection (Castle et al., 2008). Linear array has shown a sensitivity of 98.2% and a specificity of 32.8% for the detection of CIN II+ lesions (Szarewski et al., 2008).

The Digene HPV Genotyping RH Test RUO is a reverse line-blot assay designed for the detection and identification of 18 HPV types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. As the previous genotyping tests it is based on the PCR amplification of a 150bp region of the HPV *L1* gene using biotinylated GP5+ and GP6+ primers, followed by the hybridization of the resulting amplicons onto a single-typing nitrocellulose strip containing 18 HPV type-specific probe lines (Geraets et al., 2009a). Digene HPV genotyping RH Test RUO was compared to INNO-LiPA genotyping test *Extra* showing comparable genotyping results. However, INNO-LiPA identified significantly more samples with multiple HPV types (Seme et al., 2009). Another evaluation study compared the Digene genotyping RH test with the in-house Reverse Line Blot (RLB) genotyping assay. Both genotyping assays demonstrated high concordance, in 493 HC2-positive samples, for overall HR HPV detection and type-specific identification of the 18 HR types. The Digene RH Test revealed positivity for one or more HR HPV type(s) in 86.6% of the HC2-positive women, and negativity was confirmed in 97.9% of the HC2-negative women. The Digene HPV

Genotyping RH Test revealed a high genotyping agreement with the established RLB assay on GP5+/6+ amplicons (Geraets et al., 2009a). It was reported recently another study encompassing the Digene HPV Genotyping RH test. The objective was to test whether the RH Test and LQ Test (XMap based) can be used as an universal hrHPV genotyping test. Self-collected 416 cervico-vaginal specimens from an epidemiologic study were analyzed with Amplicor. The amplicons obtained were also tested with the RH Test and LQ Test for identification of 18 HPV types, including the 13 hrHPVs targeted by Amplicor. 197 specimens were positive by Amplicor, in which the RH Test and LQ Test identified one of the 13 hrHPVs in 94.4% and 98.0%, respectively. In 219 specimens remaining negative by Amplicor, the RH Test and LQ Test, performed on the Amplicor amplification products, still detected one of the 13 hrHPVs in 3.7% and 5.5% respectively, and include identification of HPV53, 66, and 82. Overall, the RH and LQ Tests demonstrated high concordance with Amplicor for hrHPV detection ( $\kappa=0.908$  and  $\kappa=0.923$ , respectively). The authors suggest that the digene HPV Genotyping RH and LQ Tests can be directly used for amplicons generated by the Amplicor HPV Test (Geraets et al., 2011). The latest version of Digene RH test (v1.0) includes an additional primer pair for human  $\beta$ -globin gene amplification, serving as an internal control for PCR inhibition and adequate sample taking and DNA purification (Poljak and Kocjan, 2010).

The EasyChip HPB Blot kit (HPV Blot kit; King Car, Taiwan) is a reverse dot-blot assay designed for the identification, in the latest version, of 39 types of HPV: 6, 11, 16, 18, 26, 31, 32, 33, 35, 37, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 72, 74, 82, CP8061, CP8304, L1AE5, MM4, MM7 and MM8. The biotinylated PCR amplicons of several HPV general primer sets or their combinations can be used for genotyping, including MY11/MY09, PGMY11/PGMY09, GP5+/GP6+, MY11/GP6+, MY11/GP6+ nested with GP5+/GP6+ and SPF1/GP6+. The latest version of this kit provides two standardized PCR amplification mixes: in the first, a modified MY11/GP6+ primers set is used to amplify a 190bp region of the HPV *L1* gene; in the second, the quality of extracted DNA is validated by amplification of a 136bp region of the human *GAPDH* gene. The resulting amplicons from an individual DNA sample are genotyped together on a single-typing nylon membrane containing the 39 HPV type-specific and one *GAPDH*-specific oligonucleotide probe marked in duplicate (Poljak and Kocjan, 2010). The evaluation of the sensitivity, specificity, reliability and reproducibility of the EasyChip HPV blot for HPV genotyping was performed in two studies. In the first, type-specific sensitivity and specificity for the 39 types of HPV were examined. The operating environment, reliability, reproducibility and blot interpretation were assessed by a quality assurance system. Each batch experiment contained samples from 89 cervical specimens and 7 extrinsic controls. Caski, HeLa and Jurkat cells, male human blood cell DNA and sterile water were used to assess reliability. Furthermore, pairs of sibling controls were used to assess reproducibility. The overall sensitivity of HPV detection was 1-50 copies of HPV genome equivalent. There was no cross-reactivity with amplicons of other HPV genotypes. One hundred batch experiments demonstrated that the reliability was excellent. The intra-batch and inter-batch reproducibility was 98 and 97%, respectively. The authors concluded that the EasyChip HPV blot is a highly sensitive, reliable and reproducible tool for detection and identification of HPV genotypes (Lin et al., 2007). The second study compared the efficacy of HPV detection and typing with a general PCR-based genotyping array with EasyChip HPV Blot.

The concordance of the two tests in determining HPV positivity was 96.8% (419/433), with a Cohen's kappa=0.93 (95% CI: 0.90-0.97) and McNemar's test of P=1.0, which indicates excellent agreement. The overall concordance of the two tests in the identification of type-specific HPV was 91.0%. Sensitivity (90-100%), specificity (99.2-100%), and accuracy (98.6-100%) rates of HPV Blot against the gold standard were satisfactory for HPV-16, 18, 58, 33, 52, 39, 45, 31, 51, 70 while HPV-71 (63.6%) had suboptimal sensitivity. The authors conclude that the modified MY11/GP6+ PCR-based HPV Blot assay is accurate and sensitive for detection and genotyping of HPV in cervical swab samples (Lin et al., 2008).

M&D REBA HPV-ID® is a molecular diagnostic kit for genotyping HPV; it detects 15 high-risk genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 53, 66 and 10 low-risk genotypes: 6, 11, 42, 43, 44, 70, 72, 81, 84, 87, simultaneously. Genetic target of REBA HPV-ID® is the HPV L1 gene, which is expressed at the late stage of infection cycles in the host cells. The kit is based on one-step nested PCR with biotinylated MY09/11 and GP5+/6+ primers and subsequent genotyping of the resulting amplicons with a single typing strip containing 23 HPV type-specific probes and one HPVV universal probe. There are no evaluation studies about this kit in peer-reviewed literature (Inc, M&D, Poljak and Kocjan, 2010).

The reverse hybridization methodology uses standard laboratory equipment and the methodology is both reproducible and rapid. The price per sample can limit its use.

## 7. *In situ* hybridization assays (ISH)

*In situ* hybridization allows the detection of intact viral DNA directly in tissues sections or cell preparations (*in situ*). The method is based on the recognition and following bond of the labelled probe with the complementary viral target. The sample is fixed in fixative solution in order to preserve the tissue morphology and to prevent loss of genetic material (Carlson and Hu). The result of the hybridization reaction is evaluated microscopically and the appearance of a specific precipitate is indicative of infection (Huang et al., 1998, Poljak and Kocjan, 2010). Generally the assay is based on four steps: tissue fixation, permeabilization of tissue samples, hybridization of the sample with probes, and detection of the HPV positive cells. Critical points for a successful ISH test are the hybridization and post-hybridization treatment. Hybridization is performed by incubating the fixed tissue with the solution containing the hybridization probe and washing the tissue to remove the unbound probes. During these steps, many parameters play a crucial role. Different lengths of probes and different labelling conditions are an example (Montgomery, 2002). The ISH probe cocktails available in the market for HPV detection are several. The latest generation probes utilize stacked antibodies to enhance the sensitivities and probes containing viral genomic DNA in double strand form (INFORM HPV III test). The target of the probes can be the viral DNA and mRNA sequences or viral mRNA transcript in human cells. ISH assay showed a low sensitivity (50%) but a significantly higher specificity (Caussy et al., 1988). The values can vary, depending on the commercially method utilized.

INFORM HPV III test (Ventana, Tucson, AZ, USA) represents the last generation commercial kit for detecting HPV DNA in cytological and histological specimens. It utilizes stacked antibodies to enhance the sensitivity of the technique (Poljak and Kocjan, 2010). The antibody stacks consist of a primary antibody that binds the dinitro-phenol hapten linked to

the probe, a secondary antibody, a biotinylated tertiary antibody, and a streptavidin-alkaline phosphatase conjugate that generate the final colour precipitates. Several are the probe cocktails available for low and high HPV risk detection. The probe B and C target 12 HPVs: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66.

GenPoint HPV Biotinylated DNA probe is the last generation ISH kit using the GenPoint Tyramide Signal Amplification System (Dako) (Glostrup, Denmark). It can be used in both cytological and histological specimens. The probe is biotin-labeled and consists of a viral genomic double-stranded DNA of 500 bp (Gebeyehu et al., 1987). Biotin can be detected by using enzyme conjugates of streptavidin to produce covalent bounds with tyramide substrates and chromogenic indicator dye diaminobenzidine (DAB) (Bobrow et al., 1989). DAB is oxidized by peroxidase enzymes to produce a dark brown precipitate that can be visualized on Dako Hybridizer and Autostainer Plus (Poljak and Kocjan, 2010). The probe cocktail recognises 13 HPVs: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. In a comparative study of INFORM III, GenPoint, and HPV PCR evaluated on 58 tissue specimens, GenPoint showed the same specificity (100%) then INFORM III but lower sensitivity (Kong et al., 2007).

ZytoFast HPV Probes (ZytoVision, Bremerhaven, Germany) are specific probes for the detection of viral DNA and mRNA of E6, E7, and L1 proteins. It can be used in both cytological and histological specimens. The probes are biotin or digoxigenin labelled and consist of HPV-type-specific oligonucleotides (Poljak and Kocjan, 2010). The target is indirectly detected using an antidigoxigenin or antibiotin enzyme conjugated antibody. The use of a chromogenic substrate leads to the formation of a colour precipitate that is visualised by light microscopy. The ZytoFast HPV Kit is designed for the detection of low risk HPV types 6 and 11 and high risk types 16, 18, 31, and 33.

HPV OncoTect Test Kit (OncoTect; InCellDx, El Camino Real Menlo Park, CA, USA; Invirion Diagnostics, Oak Brook, IL, USA) measures the number of transforming cells and the quantity of E6, E7 mRNA in each intact human cell. The kit works as indicator of disease activity. The percentage of tested cells that overexpress the viral mRNAs provides a specific indication about the risk of an HPV infection can turn into cervical cancer (InCellDx, 2010)(InCellDx, 2010)(InCellDx, 2010)(InCellDx, 2010)(InCellDx, 2010, Bhat et al., 2007). Generally, HPV OncoTect Test is used in parallel with other HPV protocols (Poljak and Kocjan, 2010). The sensitivity and the specificity of the test are high (83.3% and 91.3% respectively) and is significantly increased (Narimatsu and Patterson, 2005). It is possible to detect 95% of infected cells in moderate dysplasia (HPV OncoTect™, 2010, Liu et al., 2007).

The advantages and disadvantages of this technique depends on the methodologies and the kind of probe used (Feldman et al., 1997): double stranded DNA probes are easy to use but need reannealing during the hybridization (decreasing the probe availability); single stranded DNA probes does not need probe denaturation and no reannealing during hybridization but it is technically complex. RNA probes have high specific activity, no probe denaturation needed, no reannealing, but less tissue penetration. Finally, oligonucleotide probes have stable good tissue penetration (small size), no self-hybridization, but lower specific activity, so less sensitive, less stable hybrids, and access to DNA synthesizer needed. The ISH based tests for HPV detection are insufficiently clinically validated. They are not

used in routine screening because the methodology is too laborious and not sufficiently sensible (Poljak and Kocjan, 2010).

Test name	Test principle	Nucleic acid target	Types		Performance	
			Detected	Genotyped	Sensitivity	Specificity
hc2	Signal amplified hybr.	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.	-	93%	
Cervista™	Signal amplified hybr.	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.	-	92,8%	
Amplicor	Signal amplified hybr.	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.	-	96,1%	96,5%
careHPV	Signal amplified hybr.	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.	-	90,0%	84,2%
GenoID	rtq-PCR	DNA	HPVs16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 6, 11, 42, 43 and 44.	-		
Abbott Real Time HR HPV test	rtq-PCR	DNA	HPVs31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.	HPV16 and HPV18.	90,0%	50%
Cobas4800	rtq-PCR	DNA	HPVs31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.	HPV16 and HPV18.	90,0%	70,5%
Prepect HPV-Proofer	RT-PCR	mRNA	HPVs16, 18, 31, 33 and 45.	-	78,1%	75,5%
NucliSENS EasyQ HPV v1	RT-PCR	mRNA	HPVs16, 18, 31, 33 and 45.	-	63%	
APTIMA HPV Assay	RT-PCR	mRNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.	-	92%	98%

Test name	Test principle	Nucleic acid target	Types		Performance	
			Detected	Genotyped	Sensitivity	Specificity
HPVDNA chip	DNA Microarray	DNA	-	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69, 6, 11, 34, 40, 42, 43 and 44.	80%	94%
GG HPVCHIP	DNA Microarray	DNA	-	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, 70, 26, 53, 66, 6, 7, 10, 11, 27, 32, 40, 42, 44, 54, 55, 57, 61, 62, 72, 73, 91, CP8304/81, MM7/83, MM8/84, 30, 34, 67 and MM4/82.		
HPV GenoArray Test Kit	DNA Microarray	DNA		HPVs6, 11, 16, 18, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66, 68 and 81.		
GeneTrack HPV DNA Chip	DNA Microarray	DNA		HPVs6, 11, 16, 18, 31, 33, 35, 39, 40, 42-45, 51, 52, 54, 56, 58, 59, 62 and 66-72.		
Clart HPV2	DNA Microarray	DNA	-	HPVs6, 11, 16, 18, 26,	96,9%	71,9%

Test name	Test principle	Nucleic acid target	Types		Performance	
			Detected	Genotyped	Sensitivity	Specificity
				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 and 89.		
Multiplex HPV genotyping kit v1.0	Suspension array	DNA	-	HPVs6, 11, 16, 18, 26, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66, 68, 70, 73 and 82.		
digene HPV genotyping LQ Test	Suspension array	DNA	-	HPVs16, 18, 26, 31, 33, 35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73 and 82.		
INNO-LiPA v2	Reverse line-blot	DNA	-	HPVs6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69/71, 70, 73, 74 and 82.		
Linear Array HPV	Reverse line-blot	DNA	-	HPVs6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51,	98,2%	32,8%

Test name	Test principle	Nucleic acid target	Types		Performance	
			Detected	Genotyped	Sensitivity	Specificity
				52, 53, 54, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84 and 89.		
Digene HPV Genotyping	Reverse line-blot	DNA	-	HPVs16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82.		
EasyChip HPB Blot	Reverse line-blot	DNA	-	HPVs6, 11, 16, 18, 26, 31, 32, 33, 35, 37, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 72, 74, 82, CP8061, CP8304, L1AE5, MM4, MM7 and MM8.	90,0%	99,2%
INFORM HPV III	<i>In situ</i> hybridization	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66.	-	~50%	
GenPoint HPV Biotinylated DNA	<i>In situ</i> hybridization	DNA	HPVs16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.	-	-	100%

Table 2. Most important commercially available assays for HPV detection and/or genotyping.

## 8. Southern blot hybridization assays

Southern blot hybridization was the first assay used to detect integrated HPV (Cooper et al., 1991) and is generally used to classify new viral types (Lorincz, 1996). In Southern blot hybridization, the genome is extracted and the DNA chain is broken using restriction enzymes. The products are integrated into a gel and separated by electrophoresis. The fragments are denatured in situ, transferred to a nitrocellulose membrane, and hybridized with cloned HPV genomic probes. The probes are then labelled, often using radioisotopes. The detection of the labelled DNA hybrids is visualized by autoradiography. The sensitivity and the specificity of the assay are high, ranging between 70% and 80% the first and 90% the second (Caussy et al., 1988). There are no commercial kits available; the method is entirely laboratory-based, with existing reagents and methodologies. Despite high sensibility, specificity and application to cells and biopsies, Southern Blot is not suitable for routine application: it is time consuming, labourious, expensive and requires large amount of purifying DNA and radio labeled probes.

## 9. Immunological techniques

### 9.1 Enzyme-linked immunosorbent (ELISA) assays

ELISA, also known as an enzyme immunoassay (EIA), is a technique used to detect the presence of antigens or antibodies in wide variety biological samples. The basic steps of this technique are the immobilization of the antigen and its detection by a labeled antibody. The immobilization can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been already attached to the solid phase. The detection can be performed either directly by a labeled primary antibody or indirectly by a labeled secondary antibody. The labeled antibody reacts with a colourless substrate to produce a coloured product. The most widely used ELISA methods for detecting antigens is the Sandwich system (Figure 8) (Baker et al., 2009). In this format, the antigen is sandwiched between two similar or different antibodies: a capture antibody, already bond to the solid phase, and an enzyme-conjugated secondary antibody. The enzyme reacted with the substrate producing a colour reaction which can be observed visually or measured with a spectrophotometer. The enzyme activity is directly proportional with the amount of the test antigen. Sandwich ELISA is especially used when the concentration of antigens is low or in presence of contaminating protein, as its sensitivity is 2-5 times more than other Elisa assays (Baker et al., 2009). The assay is very specific and simple (Biosupply, 2010, Chung et al., 2010) and there is no need to purify the antigen prior to use. On the other hand, only monoclonal antibodies can be used as matched pairs. They detect different epitopes on the antigen without impeding each other's binding (Biosupply, 2010).

To detect specific antibodies, the most widely used ELISA systems are the "indirect" and "competitive" systems (Figure 8). In the indirect system the antigen is directly adsorbed onto a solid phase and the primary and the conjugate antibodies are added to the plate successively. The intensity of the colour development between substrate-conjugate enzyme indicates the amount of antigen present. In the indirect ELISA there are many binding sites for the antibody, consequently more enzyme can be detected. A large variety of enzyme-linked secondary antibodies are commercially available. The method is sensitive and

versatile. The same enzyme-linked secondary antibody can detect many primary antibodies in one species. A non-specific signal might occur if cross-reactivity happens with the secondary antibody and an extra incubation step is required in the procedure. "Competitive" system is useful for identification and quantification of either antigen or antibody. The antigen is first incubated in presence of the unlabeled antibody, forming antigen-antibody complex, and after add to an antigen-coated plate. The production of colour elicited by antibody-antigen-substrate complex will be inversely proportional to the amount of antigen in the sample. In competitive ELISA, the secondary antibody competes with the sample antigen which is associated with the primary antibody. The more antigen in the sample the less labelled antibody is retained in the plate and the weaker the signal. It is possible to use impure samples (Biomaterials, 2007)(Biomaterials, 2007)(Biomaterials, 2007)(Biomaterials, 2007) and the assay is precise, rapid, accurate, and reproducible (Zhou et al., 2002). The use of monoclonal antibodies is not so adequate because they are more difficult to find and cost more. The sensitivity and specificity are lower than the sandwich assay (Chung et al., 2010).

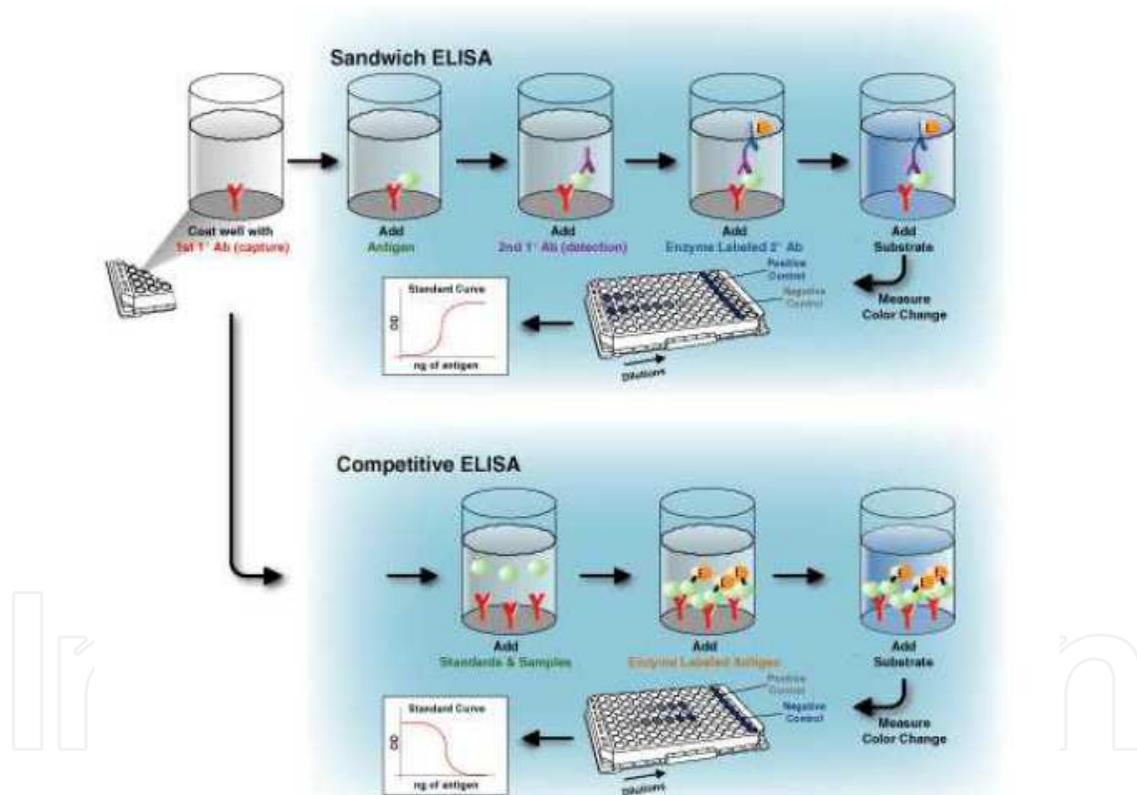


Fig. 8. Schematic representation of Sandwich and competitive ELISA.

The ELISA methodology is widely used to detect HPV antigens (Gutierrez-Xicotencatl et al., 2009). Several works are focused on antibodies against the viral HPV16L1 capsid protein and the viral HPV16E6 and HPV16E7 oncoproteins. L1 is the major capsid protein and it represents 80%-90% of the total viral proteins (Favre et al., 1975). The most of the antibody-mediated immune responses are directed against L1 antigen (Carter et al., 2000, Rocha-Zavaleta et al., 2003). A high frequency of anti-16L1 antibodies was found in patients with

high-risk HPV-associated LSIL and with cervical cancer. While a low antibody response was found from women without evidence of HPV infection and with low-risk HPV-associated LSIL (Cason et al., 1992, Ghosh et al., 1993, Di Lonardo et al., 1994, Rocha-Zavaleta et al., 2004, Leon et al., 2009). Furthermore, it is possible to discriminate between high-risk HPV and low-risk HPV types by using the epitopes of the L1 capsid protein. The viral oncogenes E6 and E7 are associated with cancer; they modify the cell cycle in order to favour the viral genome replication and consequent late gene expression (Munger and Howley, 2002). Most HPV-positive cancer cells maintain the expression of E6 and E7 (Baker et al., 1987, Shirasawa et al., 1987). A higher HPV16E6 and HPV16E7 seropositivity was observed in patients with cervical cancer compared to healthy or with subjects displaying early lesions (Kanda et al., 1992, Ghosh et al., 1993, Viscidi et al., 1993, Di Lonardo et al., 1994, Dillner et al., 1994, Fujii et al., 1995, Fisher et al., 1996). Hence, E6 and E7 oncoproteins might be used to monitor infected woman at the very late stage of the infection.

Several studies were focused on biomarker identification for early stages of cervical cancer in order to perform an early diagnosis of woman at risk of infection. For this reason the detection of viral HPV16E2, HPV16E4, and HPV16E5 proteins were studied. The expression of the HPV E4 protein is linked to cellular differentiation status (Breitburd et al., 1987, Chow et al., 1987a, Chow et al., 1987b, Doorbar et al., 1989, Palefsky et al., 1991) playing important role in the viral life cycle (Doorbar et al., 1986, Jareborg and Burnett, 1991). HPV E2 is the first protein to be expressed. High E2 levels repress expression of the E6 and E7 oncoproteins. When the HPV genome is integrated in its host, E2 function are disrupted, preventing repression of E6/E7 (Munger and Howley, 2002). In relation to the expression of HPV16E2 and HPV16E4 there are conflicting opinions. Some authors found elevated responses for E2 antigen in patients with cervical cancer. Others reported anti-E2 antibodies in normal subjects and in women with CIN lesions (Dillner, 1990, Dillner et al., 1994, Veress et al., 1994, Marais et al., 1997). A higher E4 seropositivity was observed in pre-cancerous and cancerous lesions as well as in healthy individuals (Jochmus-Kudielka et al., 1989, Crum et al., 1990, Kochel et al., 1991, Snyder et al., 1991, Kanda et al., 1992, Dillner et al., 1994, Muller et al., 1995), while other studies found a frequency of E4 antibodies in cancer patients higher than in normal subjects (Suchankova et al., 1991, Ghosh et al., 1993, Gaarenstroom et al., 1994). Moreover, an expression of the E4 protein was observed in patients with high-risk and low-risk HPV-associated but not in patients with cervical cancer (Crum et al., 1990, Vazquez-Corzo et al., 2003). HPV16E5 is a weakly oncogenic protein which potentiates the transforming activity of E7 (Bouvard et al., 1994, Valle and Banks, 1995), but its biological activity is mostly unknown. Chang et al (Chang et al., 2001) studied the HPV-16 E5 protein and found its expression in the lower third of the epithelium in LSIL.

ELISA assays are quick, convenient, and very accurate. The method can achieve high sensitivities and specificities, favorably comparable with other radioimmunoassay (Moore et al., 1999, Leng et al., 2008). It can be performed in a small laboratory and it has the advantage of automation and multiplexing (Leng et al., 2008, Adler et al., 2009). Moreover, ELISA assays need small volume of blood and do not use radioisotopes or a costly radiation system. It is suitable for large numbers of specimens. False positive results might happen if the blocking solution is ineffective and the enzyme/substrate reaction is short term and needs a rapid reading. It is a time-consuming methodology (>1.5h), uses refrigerated

reagents and probe design is difficult, but many kits are available. Considering PCR as a reference, ELISA methods are less sensitive than PCR (81.8%, 53%, 72.2% vs. 100%) but more specific (100%, 99%, 92.2% vs. 78%) for invasive cervical cancer (Meschede et al., 1998, Lack et al., 2005, Zaghoul, 2011) .

## 9.2 Detection of HPV antibodies - western blot

Western blot (alternatively, protein immunoblot) is an analytical technique used to identify proteins based on their ability to bind a specific antibody. The analysis can detect the target from a mixture of a great number of proteins from tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide or by the 3-D structure of the protein (Towbin et al., 1979). The proteins are transferred to a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF) to make the proteins accessible to antibody detection (Khan et al., 2005). The membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme. When the enzyme is exposed to an appropriate substrate drives a colorimetric reaction (Renart et al., 1979).

Several works based on Western Blot technique have studied the high correlation between the oncogene proteins and cervical cancer (Jochmus-Kudielka et al., 1989, Kochel et al., 1991, Suchankova et al., 1991, Fujii et al., 1995) and the possible role of the protein E4 in cancer disease (Jochmus-Kudielka et al., 1989, Ghosh et al., 1993, Pedroza-Saavedra et al., 2000, Middleton et al., 2003) but the results were controversial. The sensitivity of the Western blot assay is 67%. The specificity increases to 78% (Pedroza-Saavedra et al., 2000).

There are kits based on detection of antigens using horseradish peroxidase (HRP) conjugated antibodies and the chemiluminescent substrate luminol. Chemiluminescent system detects the protein target using the light produced by the catalysis of an enzyme substrate. It is rapid and allows accurate identification of samples. With this assay it is possible to detect the target protein from a mixture of antigens (Molijn et al., 2005); it is, nevertheless, expensive, demanding and complex and it is impossible to analyze a large number of samples simultaneously (Molijn et al., 2005).

## 10. Conclusions

There are two major purposes for the use of molecular HPV diagnosis. The first is to identify women at cervical cancer risk; these can be detected through community-based screening programs or through clinical counseling. In this scenario, the use of a highly sensitive assay will overestimate some HPV infections that would be cleared by the immune system in less than 24 months (Moscicki, 2007). If a less sensitive or even a quantitative assay is chosen, the efficacy in identifying women at risk of progression might be more effective. The other purpose includes vaccination trials, epidemiological and natural history studies (Molijn et al., 2005). In this case, contrarily to the clinical application, a highly sensitive and reproducible assay is required, and it should include the broadest spectrum of HPV types.

The ideal HPV testing for both goals should be a single assay with adjustable cut-off for detection, linked to a genotyping method (Molijn et al., 2005). Unfortunately, such method does not exist and combined detection systems have to be used. The molecular assays are

the gold standard for HPV testing and there are specific characteristics that positively distinguish each method. For instance, the extremely high sensitivity and specificity of PCR assays and the need of only small amounts of DNA template, makes it the support of several other high-quality assays. Within the signal amplification assays, hc2 is considered the most reliable assay and is a CE-IVD test. On the other hand, Southern blot technique is more expensive, requires large amount of purifying DNA and it is difficult to apply in routine practice due to time consumption; *in situ* hybridization is not as sensitive as the PCR and hc2 methods. The sampling and HPV DNA detection assays bearing different sensitivity and specificity, as shown above, are the most accountable aspects for the heterogeneity of the results. To overcome such problems, a combined detection system, to detect the presence of the HPV, and an antibody profile against different viral antigens, to stage the infection, will improve the signaling of persistent infections and prevent cervical cancer.

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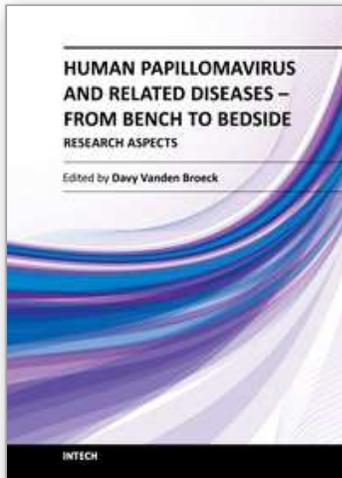
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## **Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects**

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on epidemiological and fundamental research aspects in the area of HPV, and it will update those working in this fast-progressing field with the latest information.

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