CHAPTER 1

General introduction and thesis outline
Chapter 1
1.1 BACKGROUND

1.1.1 Short introduction
Infection of cervical epithelium with a human papillomavirus (HPV) can lead to the development of cervical intraepithelial neoplasia (CIN) and ultimately cervical cancer. Technologies that can detect and distinguish different human papillomaviruses are important to determine their involvement in disease, to evaluate the efficacy of HPV vaccines, to monitor prevalence of HPV genotypes in immunized cohorts, and to identify women at risk for having high-grade CIN within cervical cancer screening programs. Over 125 commercial HPV tests have been developed, with differences in design and performance. In this thesis we evaluated the performance of a number of established and novel technologies for the identification of HPVs in relation to their intended use.

1.1.2 Cervical cancer
Cervical cancer is the fourth most common malignancy among women worldwide, with 528,000 new cases and 266,000 deaths occurring each year (1). Squamous cell carcinoma (SCC; 80%) and adenocarcinoma (ADC; 15%) are the main histological types (2), while adenosquamous and neuroendocrine carcinomas account for less than 5% of cervical cancers (3). ADC develops from adenocarcinoma in-situ (AIS). Precursors of SCC are classified as cervical intraepithelial neoplasia (CIN) grade 1, 2, and 3 (including carcinoma in-situ). All precursor lesions can regress, persist or progress. However, the possibility of regression decreases with increasing CIN grade. The risk of progression from CIN3 to invasive cervical cancer (ICC) is estimated between 30-50% (4-7). The concept of cervical carcinogenesis is summarized in Figure 1.

CIN2 and CIN3 together are referred to as high-grade CIN (HG-CIN). In the USA, women with HG-CIN and AIS are treated to prevent cervical cancer. This approach results in a considerable degree of overtreatment, since currently progressive and regressive HG-CIN cannot be distinguished morphologically. In Europe, small CIN2 localized in a well-visible transformation zone are often followed by periodic colposcopy (one year). CIN1 lesions are considered low-grade CIN (LG-CIN) for which a wait-and-see policy is advised.

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Figure 1: The concept of HPV-mediated cervical carcinogenesis and the according morphological appearance. HPV gains access to the basal cells of squamous epithelium, followed by an ordered expression pattern of viral genes leading to the production and release of new virions. Deregulated expression of E6 and E7 oncoproteins and viral genome integration are associated with malignant transformation, leading to genomic instability, (epi)genetic changes, and ultimately invasive cervical cancer. The outcomes of HPV exposure are represented as a transient infection (no pathology), a productive infection (productive CIN; CIN1 and a subset of CIN2), and a transforming infection (transforming CIN; remaining subset of CIN2 and CIN3). The majority of transforming CIN and cervical cancers are suggested to arise from an hrHPV infection of embryonic squamo-columnar junction (SCJ) cells (adapted from (8) and (9)).

1.1.3 Human papillomaviruses
PVs are naked viruses with a circular, double-stranded DNA genome of about 7600-8000 base-pairs (bp) contained in a protein capsid. The genome is divided into three regions, i.e., the long control region (LCR), the early (E) coding region, and the late (L) coding region. The LCR regulates viral gene expression and replication. The E region contains six or more early open reading frames (ORFs), i.e., E1, E2, E4, E5, E6, E7, which encode proteins required for viral gene expression, replication and survival. The late (L) region encodes the two viral structural proteins, i.e., L1 and L2.

By convention, the similarity across the highly conserved L1 ORF was adapted as the basis for taxonomic classification of PVs (10). A PV "type" is defined as a complete PV genome, whose L1 ORF
The family of *papillomaviridae* currently consists of 189 classified PVs distributed over 29 genera (12). Presently, 170 HPV types have been described and are located in the α, β, γ, μ, and ν genera (13). HPVs are strictly epitheliotropic and can infect epithelium and/or mucosal areas of skin, conjunctiva, oropharynx, anus, penis, vulva, vagina and cervix (11, 14). The β, γ, μ, and ν genera comprise only cutaneous HPVs, while α genus contains both cutaneous and mucosal types divided over 13 species. HPVs within these species branch into three ancestral clades (15). The phylogenetic relationship between the different human papillomaviruses is shown in Figure 2.

nucleotide sequence is at least 10% different from that of any other known PV type (11). *PV subtypes* share 90 to 98% and *variants* more than 98% nucleotide sequence identity in L1.

At higher taxonomy levels, PVs have been grouped together into *species*, and species into *genera*. PV types within the same genus show less than 60% sequence identity to types of other genera (12). Different species within a genus share between 60% and 70% nucleotide identity, while PVs in the same species share between 71% and 89% identity in L1 ORF nucleotide sequence (10). Genera are denominated by a Greek letter and species by addition of a number to the letter, e.g., *species Alphapapillomavirus 9* (α9) (11).

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Figure 2: Phylogenetic relationship between human papillomaviruses (from (16)).
The nomenclature for variant lineages of an HPV type was initially based on the observed association between the variant and the continent of origin, e.g., European, Asian, African 1&2, North-American, and Asian-American 1&2 variants of HPV16 (17). Recently, a novel classification and nomenclature system has been proposed (18). This system is based on full-genome sequence analysis, and uses an approximate cut-off of 1.0% difference between complete genomes to define major lineages. Major lineages are named using an alphanumeric, with the reference genome of each type always located in the “A” clade. Differences between 0.5–1% are used to designate sublineages (e.g., A1, A2). The conversion between the novel and initial nomenclature for HPV16 is shown in Table 1.

### Table 1: Novel (18) and initial (17) nomenclature of HPV16 variant (sub)lineages.

<table>
<thead>
<tr>
<th>Novel (Burk, 2013)</th>
<th>Initial (Ho, 1993)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main lineage</strong></td>
<td><strong>Sublineage</strong></td>
</tr>
<tr>
<td>A</td>
<td>European (EUR)</td>
</tr>
<tr>
<td></td>
<td>A1</td>
</tr>
<tr>
<td></td>
<td>A2</td>
</tr>
<tr>
<td></td>
<td>A3</td>
</tr>
<tr>
<td>B</td>
<td>African 1a (AF1a)</td>
</tr>
<tr>
<td></td>
<td>B1</td>
</tr>
<tr>
<td></td>
<td>B2</td>
</tr>
<tr>
<td>C</td>
<td>African 2a (AF2a)</td>
</tr>
<tr>
<td></td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td>C2</td>
</tr>
<tr>
<td>D</td>
<td>North-American (NA)</td>
</tr>
<tr>
<td></td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>D2</td>
</tr>
<tr>
<td></td>
<td>D3</td>
</tr>
</tbody>
</table>

1.1.4 HPV and cervical cancer development

The large majority of infections with HPVs are rapidly cleared by a cell-mediated immune response, and do not give rise to lesions. About 20% of the remaining persisting HPV infections cause lesions that are considered productive infections. These infections lead to the generation of new virions in the upper epithelial layers using the host replication machinery under tightly controlled expression of E6 and E7. Morphologically, productive infections can appear as CIN1 and CIN2, but generally do not persist to progress to advanced precursor lesions (CIN3) and cancer (Figure 1).

Only a minority of HPV infections become transforming. Transforming infections are characterized by deregulated expression of E6 and E7 in the (para)basal cells, i.e., cells with proliferating capacity. Transforming infections are associated with CIN2, CIN3 and ultimately cervical cancer (Figure 1) (8). The exact mechanism for deregulation of E6 and E7 is not well understood. The majority of cervical carcinomas contain one or more copies of HPV integrated into the host genome. Integration occurs more or less randomly, but viral integration sites often lie within the ORF of E2, the transcriptional regulator of E6 and E7 (19, 20). The continuous overexpression of viral oncoproteins E6 and E7 in proliferating basal cells of the epithelium leads to genomic instability and (epi)genetic changes, and ultimately malignant transformation. Precursor lesions can develop within 2-3 years after HPV infection (21, 22), while progression from HG-CIN to cancer may take 10-30 years (21, 23).
1.2 IMPORTANCE OF HPV GENOTYPING TESTS

1.2.1 HPV types and disease association

HPV genotyping tests have been very important in epidemiologic studies towards HPVs and their association with disease. In a global cervical cancer case-control study, Munoz et al classified fifteen types as high-risk (hr) (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), three as probably high-risk (pr-hr) (26, 53, and 66), and twelve types as low-risk (lr) (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) (Table 2) (24). This observation correlated with the three ancestral phylogenetic clades of alpha HPVs (15, 25). The hrHPVs and pr-hrHPVs were located in α9, α11, α7, α5, and α6 ("high-risk clade"), while most lrHPVs were found in α10, α8, α1, and α13 ("low-risk clade 1") and α2, α4, α14/15, and α3 ("low-risk clade 2") (Figure 2).

The most recent carcinogenic classification of mucosal HPV genotypes by the WHO IARC was based on the evidence of the epidemiologic risk classification, supplemented with phylogenetic and biological evidence when available (26). IARC has classified mucosal types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 as carcinogenic (Class 1), type 68 as probably carcinogenic (Class 2A), and types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85 and 97 as possibly carcinogenic (Class 2B). HPV6 and HPV11 were not classifiable as to its carcinogenicity to humans (Class 3), and the remaining mucosal HPV types were considered probably not carcinogenic (Class 4) (Table 2).

Table 2: The epidemiologic risk classification (24) and the WHO IARC carcinogenic classification (26) of mucosal HPV genotypes.

<table>
<thead>
<tr>
<th>WHO IARC, 2009</th>
<th>Munoz, 2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-risk types</td>
<td>Probably high-risk types</td>
</tr>
<tr>
<td>Class 1 (carcinogenic)</td>
<td>16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59</td>
</tr>
<tr>
<td>Class 2A (probably carcinogenic)</td>
<td>68</td>
</tr>
<tr>
<td>Class 2B (possibly carcinogenic)</td>
<td>73, 82</td>
</tr>
<tr>
<td>Class 3 (not classifiable)</td>
<td>-</td>
</tr>
<tr>
<td>Class 4 (probably not carcinogenic)</td>
<td>-</td>
</tr>
</tbody>
</table>

The classification of HPVs that pose a high risk for causing cervical cancer is an evolving process, particularly for the HPV types in Class 2A/B. These genotypes have been classified as probably/possibly carcinogenic mainly based on their close phylogenetic relation with established carcinogenic HPVs, but epidemiological and biological evidence is virtually lacking. Most of these types are not targeted by commonly used HPV genotyping assays. Additional research is required to clarify their causal role and clinical importance. A specific definition of the HPVs considered (probably/possibly) high-risk is provided in each individual chapter of this thesis.
Even within the group of established hrHPVs (Class 1), there appears to be a difference in carcinogenic potential. Longitudinal studies have shown an elevated risk of HG-CIN for HPV16 in particular and to a lesser extent for HPV18, compared to other high-risk HPV types (27-32). In contrast to other hrHPV genotypes, these two types show a proportional increase in prevalence in women having normal cytology, precancer, and ICC (33, 34).

Technologies that have the resolution to distinguish intratypic variants were used to investigate differences in risk associations between variants, in particular for those of HPV16, the most carcinogenic HPV. Lineages B, C, and D (Non-European) of HPV16 appear to be more pathogenic in comparison to isolates from the A lineage (European) (18). HPV16 lineages B, C and D persist more frequently (35, 36), are more associated with precancer and specifically CIN3 (35-40), and have elevated risks for cancer compared to the HPV16 A variant lineage. In particular, this increased risk of cervical cancer is mostly related to the D (Asian-American) lineage (40-42).

1.2.2 HPV vaccine efficacy trials
HPV genotyping tests targeting a broad range of HPVs were used to demonstrate the efficacy of two HPV vaccines. Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) and Gardasil® (Merck & Co., Whitehouse Station, NJ, USA) are two prophylactic HPV L1 virus-like particle (VLP) vaccines that have been licensed to prevent anogenital HPV infections and their associated neoplasia. Cervarix is a bivalent vaccine protecting primarily against HPV16 and 18, and Gardasil is a quadrivalent vaccine that targets besides HPV16 and 18 also the lrHPV types 6 and 11. Both are three-dose vaccines composed of HPV L1 proteins self-assembled into virus-like particles (VLPs), but produced in different expression systems and administered with different adjuvants. In contrast to natural HPV infection, both vaccines induce high titers of neutralizing anti-L1 VLP antibodies, with virtually 100% seroconversion in vaccinees (43). The AS04 adjuvant system of the bivalent vaccine appears to induce a stronger, more sustained antibody response than the conventional adjuvant of the quadrivalent vaccine (44), although its effect on duration of protection is unknown.

For both vaccines, HPV genotyping tests targeting a broad range of HPVs were used to demonstrate high efficacy against several surrogate endpoints, from persistent HPV infection to high-grade CIN lesions (CIN2+) associated with vaccine-targeted HPVs (45). The quadrivalent vaccine has demonstrated strong protection against genital warts, of which 90% in men and women are caused by vaccine targets HPV6 and 11 (46-48). Long-term follow-up of vaccinated cohorts will determine the duration of protection against vaccine-targeted types (45).

Cross-neutralization of HPV types related to HPV16 and 18 confers cross-protection. Both vaccines provide partial cross-protective efficacy against HPV31 and 33 (49, 50). The bivalent vaccine also offers some cross-protection against HPV45 (51, 52).

1.2.3 Surveillance of HPV type prevalence
The bivalent and quadrivalent vaccines have been implemented in vaccination programs in various countries. Several aspects should be monitored to evaluate the effects of an HPV vaccination program, i.e., 1) the occurrence of vaccine-preventable disease, 2) the concentration of naturally occurring HPV type-specific antibodies and the prevalence of HPV vaccine-associated genotypes, 3) the titers of HPV vaccine type-associated antibody response, 4) vaccine uptake, and 5) vaccine safety. HPV genotyping tests can be used to determine the prevalence of HPV genotypes pre- and post-vaccination. This allows monitoring the effects of nation-wide vaccination on prevalence of HPV genotypes (and possibly type replacement) within an immunized cohort.
1.2.4 HPV-based screening

In the near future, HPV tests that can detect (and partially identify) a range of hrHPVs (hrHPV DNA testing) will be implemented in cervical cancer screening programs. Currently, cervical cancer screening programs rely on cytology performed on cells scraped from the transformation zone of the cervix and collected in a liquid-based medium, e.g., BD SurePath (BD, Burlington, NC, USA) or ThinPrep PreservCyt (Hologic, Marlborough, MA, USA). Morphological changes of cells are graded according to the degree of abnormality. Different cytology classification systems are used, e.g., the Bethesda 2001 (USA), BSSC (United Kingdom), CISOE-A (The Netherlands) and Papanicolaou (PAP). The different cytology classification systems are shown in Table 3.

**Table 3: Classification systems for cervical cytology (adapted from (53, 54)).**

<table>
<thead>
<tr>
<th>BETHESDA 2001</th>
<th>Unsatisfactory for evaluation</th>
<th>Negative (NILM)</th>
<th>Atrophy</th>
<th>ASC-H</th>
<th>HSIL</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AGC</td>
<td>AGC favor neoplastic</td>
<td>AIS</td>
</tr>
<tr>
<td>BSCC</td>
<td>Inadequate</td>
<td>Negative</td>
<td></td>
<td>Borderline nuclear change</td>
<td>Mild dyskaryosis</td>
<td>Moderate dyskaryosis</td>
</tr>
<tr>
<td>CISOE-A</td>
<td>C0</td>
<td>S1, E1-2, O1-2</td>
<td>S2-3, O3, E3</td>
<td>S4, E4-5</td>
<td>S5, O4-5</td>
<td>S6, O6, E6</td>
</tr>
<tr>
<td>PAP</td>
<td>PAP0</td>
<td>PAP1</td>
<td>PAP2</td>
<td>PAP3a1</td>
<td>PAP3a2</td>
<td>PAP3b</td>
</tr>
</tbody>
</table>

NILM, Negative for intraepithelial lesion or malignancy; ASC-H, atypical squamous cells cannot exclude HSIL; ASC-US, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; AIS, endocervical adenocarcinoma in situ; SCC, squamous cell carcinoma; AC, adenocarcinoma; CISOE-A, C composition, I inflammation, S squamous epithelium, O other abnormalities and endometrium, and E endocervical columnar epithelium

HrHPV DNA tests are an improved primary cervical cancer screening tool compared to cytology. Randomized controlled trials have shown that hrHPV DNA testing detects 30% more CIN2+ and 20% more CIN3+ in women aged 30 years and older (55-62). Secondly, trials with longitudinal data on CIN3+ in subsequent screening rounds (at 3-5 year intervals) have shown ~50% lower incidence rates of CIN3+ among women with a negative HPV test at baseline compared to those having normal cytology (56, 60-62). Moreover, a pooled analysis of these studies demonstrated that hrHPV-based screening provides 60–70% greater protection against invasive cervical carcinomas compared to cytology (63).

In general, these hrHPV DNA tests target a range of 13-14 hrHPVs but do not permit individual identification, or limited only to HPV16 and 18 (partial genotyping). The clinical use of hrHPV genotyping is currently unknown, but the separate identification of HPV16 and 18 might be of use in clinical management, due to their increased risk for cervical cancer compared to other hrHPVs.
1.3 **DESIGN AND PERFORMANCE ASSESSMENT OF HPV TESTS**

### 1.3.1 Intended use

The design of an HPV DNA test should match its intended use. HPV tests designed for epidemiologic purposes, e.g., disease association studies, vaccine efficacy trials, and surveillance of HPV prevalence, require a high analytical sensitivity and specificity and the capacity to individually identify a range of HPVs.

In a clinical setting, HPV tests should detect hrHPV infections mainly associated with clinically meaningful disease, i.e., CIN2+ lesions (clinical sensitivity), while limiting the detection of transient HPV infections not associated with CIN2+ (clinical specificity). In addition to primary cervical cancer screening, two other potential clinical applications of hrHPV testing have been defined. HrHPV testing can be used as a **test-of-cure** for women treated for HG-CIN, and as a **triage test** for women with borderline and mild abnormal cytology (ASC-US/LSIL) (64).

### 1.3.2 Clinical specimen, collection and processing

A range of clinical specimens have been used for HPV analysis, e.g., cervical cells collected by brush and stored in liquid-based medium (cervical swabs) (64), whole tissue sections (WTS) of freshly frozen or formalin-fixed paraffin-embedded (FFPE) cervical biopsies (65, 66), micro-dissected regions of tissue by laser-capture microscopy (LCM) (67), and cervicovaginal specimens self-collected by lavage or brush and stored in liquid-based medium, solid carrier cartridge or dryly (68, 69).

It is important to realize that the yield and quality of the specimen for HPV testing is influenced by the methodology used for collection, storage and processing. Thus, all elements of the diagnostic chain determine the outcome of the HPV test used and should be taken into account (68).

### 1.3.3 Target selection and amplification

Most commercial and in-house tests that have been developed for HPV detection and genotyping are based on PCR amplification of HPV DNA. DNA PCR-based methods will be the focus of this thesis, although some non-PCR based methods will also be briefly described.

HPV DNA PCR tests do not characterize the complete HPV genome or the L1 ORF, as is required for classification of PVs (10). Instead, these assays target one or more regions within the genome that are sufficient for accurate viral recognition of one or more previously classified HPVs. Misrecognition due to presence of unclassified types or genetic variants is inherently possible when using any HPV DNA assay.

The size of viral regions that are amplified vary across HPV tests, but smaller target regions allow more efficient amplification in specimens with poorly preserved DNA, e.g., archival FFPE cervical biopsies. The regions in the viral genome targeted by a selection of HPV DNA PCR-based assays are shown in Figure 3.
The degree of heterogeneity of the viral genome enables two approaches for amplification of viral DNA by PCR primers, i.e., broad-spectrum (consensus) primers or type-specific (TS) primers. Broad-spectrum primers target relatively well-conserved genomic sequences, enabling the simultaneous amplification of a broad-spectrum of HPV genotypes in a single test using only a limited number of primers. By default, these primer sets are not specifically designed for the amplification of only a single HPV type. Most broad-spectrum PCR genotyping assays target well-conserved regions in the L1 ORF. The likelihood that sequence variations occur at these positions causing false-negativity is relatively low. Broad-spectrum amplification can be accomplished by different approaches: 1) low-stringency PCR conditions to allow some degree of mismatch acceptance between primers and target sequence, 2) degenerate primers with nucleotide variations at variable base positions, 3) primers with the non-specific base-analogue inosine at ambiguous base positions, and 4) sets of multiple, overlapping primers.

A technical limitation of all broad-spectrum PCR-based assays is the underestimation of the prevalence of genotypes present in low concentrations within multiple infections, also known as masking. Broad-spectrum PCR primers do not necessarily have the same analytical sensitivity and specificity for each genotype and amplification efficiency might differ among individual genotypes. Spiking experiments with plasmid mixtures of different HPV genotypes have shown that a competitive effect occurs in mixed infections when one genotype is present in a much lower concentration than another (PCR competition).

As opposed to broad-spectrum PCR, type-specific (TS) primers target viral sequences that are specific for a single genotype. These primers permit highly sensitive and specific identification of HPVs. Although TS PCRs can be designed to target any region in the HPV genome, many TS PCR assays amplify regions in the E6 or E7 ORF. These PCR target regions are not interrupted by viral integration, since over-expression of E6 and E7 is required for transforming HPV infections.

An important advantage of TS PCRs is that they are less prone to underestimate the prevalence of HPV types that have low viral loads in multiple infections.
However, TS PCRs also have some limitations. Unknown variations in the primer target sequences could cause false-negative results (72). In addition, the performance of a separate type-specific PCR for each HPV is highly laborious and requires substantial quantity of clinical specimen. This issue can be addressed by the development of multiplex type-specific (MPTS) PCRs, where multiple TS primer sets are combined in a single PCR reaction for simultaneous amplification of a defined set of HPVs (72).

1.3.4  Read-out method

Amplification products generated by broad-spectrum and by type-specific PCRs can be detected by hybridization to a mix of oligonucleotide probes in a DNA enzyme immune-assay (DEIA/EIA) in a microtiter well plate. In addition, reverse hybridization (RH) can be done by separate genotype-specific probes immobilized on carriers such as nylon membrane, nitrocellulose strips, microsphere beads or DNA chips. Read-out can also be performed real-time using fluorochrome-labelled Taqman probes in a quantitative (q)PCR format as opposed to a conventional end-point PCR. Read-out systems of PCR products can be designed to recognize a range of HPV types simultaneously (detection), individually (full genotyping), or as a combination of detection and genotyping (partial genotyping).

The range of HPVs targeted by an HPV test should be in accordance with its design. Clinically relevant HPV tests generally target 13 hrHPVs (Class 1 and 2A), and have often incorporated HPV66 (Class 2B) in addition. These assays are therefore also referred to as hrHPV tests. HPV tests designed for epidemiologic purposes are not necessarily restricted to these hrHPVs and may also include possibly hr- and lrHPVs.

1.3.5  Assessing HPV test performance and quality assurance

The non-clinical performance assessment of HPV tests comprises analytical sensitivity (limit-of-detection), analytical specificity (interference), precision (reproducibility) and accuracy (comparison-of-methods). The analytical accuracy of a novel HPV test can be evaluated by comparison with an established "gold standard" or comparator test, using panels of artificial samples, e.g., plasmids cloned with HPV target sequences, and/or clinical specimens for which the HPV test was designed.

However, an analytically validated HPV test should not be used in clinical practice without prior clinical validation, since analytical and clinical accuracy are non-synonymous. The clinical relevance of a novel hrHPV test for primary cervical cancer screening has to be supported by data from longitudinal randomized controlled trials (RCT). Alternatively, a candidate assay can also be clinically assessed if it shows a clinical sensitivity and specificity that is non-inferior to a reference test that has already been validated in longitudinal RCT. International guidelines for panel composition and criteria for clinical accuracy and test reproducibility have been formulated (73).

The laboratories performing hrHPV testing should comply with quality assurance (QA) measures, such as a specific infrastructure for nucleic acid amplification, participation in external quality assessments (proficiency panels), and accreditation for clinical molecular testing (73). Some HPV tests have been equipped with an internal control (IC) to aid laboratories in assessing specimen quality and/or monitoring of sample processing, in order to prevent false-negative HPV results. An IC usually comprises a spiked exogenous nucleotide sequence or an endogenous target (e.g., the beta-globin housekeeping gene) that is simultaneously extracted and amplified with HPV DNA.
1.4 TECHNOLOGIES FOR HPV DETECTION AND GENOTYPING

1.4.1 Tests for pooled detection of HPVs

HPV DNA detection assays provide a qualitative test result for a group of HPVs simultaneously (pooled or grouped detection). These assays generally target 13 hrHPVs (Class1/2A) or 14 hrHPVs (Class1/2A and HPV66 in addition). Tests not intended for a clinical setting can detect additional genotypes, including lrHPVs (e.g., SPF10 DEIA).

Signal amplification

The Hybrid Capture 2 HPV DNA Test (HC2; Qiagen, Hilden, Germany) is the most frequently used HPV test in the world. HC2 uses liquid-based chemiluminescent signal amplification of hybridized target DNA for the simultaneous detection of 13 hrHPVs. This assay has no internal control for a human DNA target. The RNA probes can cross-hybridize with several other (lr)HPVs (74). The HC2 is intended for primary cervical cancer screening, management of women with equivocal cytology results, and test-of-cure. HC2 has been evaluated in longitudinal randomized, controlled studies, e.g., NTCC, ARTISTIC, and VUSASCREEN (59-61, 75). HC2 is therefore considered a reference test for validation of novel hrHPV assays (73).

The Cervista HPV HR Test (Cervista; Hologic, Madison, WI, USA) offers simultaneous detection of DNA from 14 hrHPVs using signal amplification by Invader chemistry. In a first isothermal reaction, a probe and an Invader oligonucleotide specifically anneal to HPV DNA to generate an overlapping structure. Enzymes specifically cleave and release the overlapping primary probes. In a second, simultaneous isothermal reaction, cleaved flaps combine with a fluorescence resonance energy transfer (FRET) probe, which generates a fluorescent signal. A region from the HIST2H2BE gene is also amplified as an internal control target. The Cervista is indicated for cervical cancer risk screening combined with cytology and for management of women with equivocal cytology results.

Cervista has been evaluated in the SHENCCAST II study (76) and clinically validated by Boers and colleagues (77). However, concerns have been raised about the relatively low clinical specificity of this assay (78), which might be resolved by increasing the assay threshold for positivity (79, 80).

PCR amplification and enzyme immuno assays

Most endpoint PCR-based methods utilize consensus primer sets targeting a conserved region of a broad-spectrum of viral genomes. Detection of amplimers is performed in a DNA enzyme immuno assay (designated DEIA or EIA) using a cocktail of specific probes for a defined set of hrHPVs (e.g., GP5+/6+ EIA) or a mix of universal probes for a very broad range of HPVs (e.g., SPF10 DEIA). Presence of HPV is determined by optical density measurement of labeled probes hybridized to single-stranded amplimers in a microtiter plate.

The SPF10-PCR-DEIA (Labo Bio-medical Products, Rijswijk, The Netherlands) was developed around 15 years ago (81) and is usually combined in a test algorithm with the LiPA25 reverse hybridization strip version 1 (Labo Bio-medical Products; described later) (82). This algorithm was designed to have high analytical sensitivity and specificity. The SPF10 primers are non-degenerated and amplify a 65-bp fragment in the L1 ORF of a broad-spectrum of at least 69 mucosal and cutaneous HPV types. Qualitative detection of HPV is performed in a DEIA with conservative, universal HPV probes. A control target for the housekeeping gene beta-globin can be separately amplified and detected. The SPF10 PCR system has been used in various epidemiologic studies and vaccine trials for the bivalent vaccine, e.g., PATRICIA and CVT (66, 83-87). The short region of only 65 bp targeted by SPF10 primers is particularly sensitive for amplification in formalin-fixed paraffin-embedded (FFPE) cervical biopsy specimens, in which DNA is often poorly preserved. The sensitivity of the SPF10-PCR-DEIA is too high for application in a clinical setting (88).
The GP5+/6+-PCR-EIA has been originally developed as an in-house test and is now commercially available (EIA kit GP HR; Diassay, Rijswijk, The Netherlands). The GP5+/6+ primers amplify a region of approximately 150 bp in the L1 ORF. Amplification of a broad-spectrum of HPV genotypes using only two primers is achieved by a relatively low annealing temperature. A cocktail of probes specific for 14 hrHPVs hybridizes with the GP5+/6+ amplification products in an EIA format, providing a qualitative result (89). A 313-bp fragment of human DNA is intrinsically co-amplified by the GP5+/6+ primers and can function as an internal control (90). A specifically designed probe can detect this fragment in a separate EIA format. The GP5+/6+-PCR-EIA is intended for primary cervical cancer screening, management of women with equivocal cytology results, and test-of-cure. Similarly to HC2, the GP5+/6+-PCR-EIA has been evaluated in longitudinal randomized, controlled studies, e.g., POBASCAM and SWEDESCREEN (56, 62). The GP5+/6+-PCR-EIA is therefore considered a reference assay for HPV testing in cervical cancer screening (73).

The AMPLICOR Human Papillomavirus Test (Amplicor; Roche Diagnostics, Almere, The Netherlands) has broad-spectrum primers that amplify a region of 165 bp from L1. Complementary probes can detect presence of amplification products of 13 hrHPVs by measuring the optical density in a microtiter plate. A beta-globin internal control target is amplified in the same PCR reaction and detected in a separate microtiter plate. Amplicor has not been clinically validated according to the international criteria (73). In a triage population, the clinical sensitivity of Amplicor was only marginally higher compared to HC2, but its clinical specificity was significantly lower (91). Two other studies reported equivalent clinical sensitivity and specificity for Amplicor and HC2 in triage populations (92, 93). Roche developed and introduced the Cobas 4800 HPV Test (Cobas; Roche Diagnostics) as an alternative for Amplicor because it has an improved clinical specificity.

1.4.2 HPV DNA detection tests with partial genotyping

A recent generation of HPV tests offers concurrent identification of a restricted number of HPV genotypes in addition to pooled hrHPV detection (designated as partial genotyping). Partial genotyping is usually limited to HPV16 and 18, with the remaining hrHPVs detected as a group. Separate identification of HPV16 and 18 might be of use in clinical management, due to their increased risk for cervical cancer compared to other hrHPVs.

Quantitative PCR amplification

In HPV tests with quantitative PCR (qPCR) technology, Taqman probes labeled with a fluorescent dye can hybridize with the amplimer during every annealing step. The increase in fluorescence can be monitored real-time during the exponential increase of amplified viral target DNA, until limiting reagents, accumulation of inhibitors or inactivation of the polymerase affect the PCR efficiency. The first cycle where generated fluorescence can be distinguished from the background signal is called the threshold cycle (Ct) or quantification cycle (Cq). The initial concentration of the measured target is correlated to the Cq value, and can be quantified by including a standard curve dilution series.

The Cobas 4800 HPV Test (Cobas) enables the qualitative, simultaneous detection of 14 hrHPVs using qPCR amplification of a region in L1. This test provides individual genotyping of HPV16 and 18, if desired, and a beta-globin internal control target in the same PCR reaction. The Cobas is intended for cervical cancer risk screening in combination with cytology (co-testing) and for management of women with equivocal cytology results. In recent years, its clinical value has been supported by the ATHENA trial (94) and by comparison with a reference test (95). Similar to Cobas, the Abbott RealTime High Risk HPV test (Abbott RealTime HR HPV test; Abbott,
Wiesbaden, Germany) provides qPCR-based detection of 14 hrHPVs, concurrent HPV16/18 genotyping, and a beta-globin internal control. A modified GP5+/6+ primer mix is used for L1-based viral amplification (96). The Abbott RealTime HR HPV test has been clinically validated for HPV-based cervical cancer screening in women aged 30 years and older in several studies (96-98).

The HPV-Risk assay (Self-Screen BV, Amsterdam, The Netherlands) is an E7-based broad-spectrum qPCR, which offers detection of 14 hrHPVs and HPV67, with concurrent HPV16/18 genotyping and sample quality assessment using an endogenous human beta-globin internal control target. This assay meets the clinical and reproducibility criteria of the international guidelines (73) and is also compatible with specimens that were self-collected using lavage- and brush-based devices (99).

The BD Onclarity HPV Assay (Onclarity; Becton Dickinson, Sparks, MD, USA) is an E6/E7-based qPCR, enabling detection of 14 hrHPVs in three separate reactions. Concurrent genotyping is offered for six types (i.e., HPV16, 18, 31, 45, 51 and 52), while the remaining hrHPVs are detected as three separate groups (i.e., HPV33/58, HPV56/59/66, and HPV35/39/68). The test also detects human beta-globin as an endogenous internal control. The Onclarity has been clinically validated according to the international guidelines (100).

Signal amplification

The Cervista HPV 16/18 Test (Cervista HPV16/18; Hologic) is a reflex test for women that tested positive by the Cervista HPV HR Test. The Cervista HPV16/18 can determine presence of HPV16 and/or 18 using the same Invader chemistry as the Cervista.

1.4.3 HPV DNA full genotyping tests using broad-spectrum PCR

HPV tests with a full genotyping capability for a wide range of HPV genotypes have great value in estimating the epidemiological burden of HPV infections and the efficacy of vaccines, while evidence for the clinical utility of full genotyping is limited.

Broad-spectrum PCR amplification and reverse hybridization

The LiPA25 version 1 (Labo Bio-medical Products) is used for identification of 25 individual HPV genotypes by reverse hybridization of generated SPF10 amplimers with genotype-specific probes immobilized on a reverse hybridization strip. In a combined test algorithm, SPF10 amplimers that are positive by the DEIA (Labo Bio-medical Products) can be used directly for LiPA25, eliminating the need for a separate PCR reaction (81, 82). This algorithm has been used in the aforementioned epidemiological and vaccine efficacy studies.

The INNO-LiPA HPV Genotyping Extra (INNO-LiPA; Innogenetics, Gent, Belgium) is another SPF10-based reverse hybridization strip with similar technology as the LiPA25 version 1 algorithm, but there are significant differences. The INNO-LiPA uses different SPF10 PCR primers and reverse hybridization probes than LiPA25, does not have a separate DEIA for detection of a broad-spectrum of HPV types, and offers a concurrent internal control (HLA-DPB1 gene). The intended use of INNO-LiPA is currently unclear. INNO-LiPA has been used in a clinical evaluation (101), but has not (yet) been fully clinically validated in a randomized controlled trial or by non-inferiority to a reference test according to defined guidelines (73). In addition, it has been used in epidemiologic studies (102-104), but the analytical sensitivity of INNO-LiPA remains to be investigated.

The LINEAR ARRAY HPV Genotyping Test (LA; Roche Diagnostics) is a reverse hybridization strip with immobilized probes for the recognition of 37 anogenital HPV (sub)types and an internal control target (beta-globin) (105). Degenerated PGMY primers are used for broad-spectrum amplification of a viral DNA fragment of 450 bp in the L1 ORF (105). LA is intended for the qualitative in vitro test for detection of HPV in clinical
specimens. LA has been applied in epidemiological studies (32, 106), but has not been clinically validated.

The Reverse Line Blot (RLB) is an in-house assay for the identification of 37 HPV s using the same GP5+/6+ amplification products as the EIA (89, 107). Oligonucleotide probes are attached to a nylon membrane in parallel lines, and PCR products are hybridized perpendicularly using a miniblotter. Hybrids are visualized by a conjugate-substrate reaction. The RLB has been used in a case-control study by WHO IARC to define the risk-classification of individual genotypes (24), but also in a screening setting, to identify the respective HPV type(s) in women testing positive for hrHPV by EIA (108).

Two commercially available alternative assays for the genotyping of GP5+/6+ amplimers have been recently developed, using the same probes as the RLB with minor modifications. The Genotyping Kit HPV GP, version 1 (GP5+/6+ strip, Diassay, Rijswijk, The Netherlands; previously marketed as digene HPV Genotyping RH Test) is a strip-based reverse hybridization assay for identification of 14 hrHPVs and 4 probably hrHPVs. This assay was designed as an easy-to-use alternative for RLB and could be used for reflex genotyping following hrHPV positivity by HC2 or EIA. The LMNX Genotyping Kit HPV GP (GP5+/6+ LMNX, Diassay; previously marketed as digene HPV Genotyping LQ Test) provides identification of the same HPVs, but read-out is performed using bead-based xMAP technology on a Luminex platform. This platform is more suitable for high-throughput testing, enabling the GP5+/6+ LMNX to be used as a reflex genotyping test but also as a stand-alone test for hrHPV detection and concurrent genotyping, if desired.

The PapilloCheck HPV-Screening (PapilloCheck; Greiner Bio-One GmbH, Frickenhausen, Germany) is a PCR-microarray and allows identification of 24 types, including 14 hrHPVs. Broad-spectrum primers amplify a 350 bp region from the E1 ORF. A region in the human ADAT1 gene is amplified to confirm presence of human DNA and a control-template is present in the PCR master mix to rule out inhibition of amplification. Fluorescently labeled amplification products hybridize with specific DNA probes fixed on a DNA chip and are measured. PapilloCheck has been clinically validated for simultaneous detection of 14 hrHPVs according to the requirements for clinical sensitivity and specificity (109).

1.4.4 HPV DNA full genotyping tests using type-specific (q)PCR

Type-specific PCR amplification

Separate type-specific (TS) PCRs for HPV16 and 18 have been developed (71) and incorporated into the HPV testing algorithm that was used in the two largest efficacy trials of the bivalent vaccine, PATRICIA and CVT. These assays utilize TS primers targeting short regions in the E6/E7 ORF (HPV16; 92 bp) and in the L1 ORF (HPV18; 126 bp) for endpoint PCR amplification, followed by read-out using DEIA technology, as described before (71). The combination of the L1-based SPF10 broad-spectrum PCR algorithm followed by TS HPV16/18 PCRs provided a higher analytical accuracy than both assays alone (71).

Recently, two novel assays for multiplex type-specific (MPTS) amplification of regions in E6, were introduced, i.e., MPTS12 and MPTS123 (Labo Biomedical Products). In MPTS12 amplification is performed in two separate TS PCRs, and pooled amplification products are genotyped by strip-based reverse hybridization targeting 9 hrHPVs, i.e., HPV16, 18, 31, 33, 35, 45, 52, 58, and 59 (110). MPTS123 requires performance and pooling of three separate PCRs, and utilizes bead-based xMAP technology for identification of 14 hrHPVs and 2 lrHPVs (HPV6 and 11) on a Luminex platform (72). These assays were designed to be used in conjunction with the SPF10 LiPA25 system and improved the analytical accuracy for a broader range of HPV s, similar to the TS16/18 assays. This is particularly important when investigating the cross-protection of the bivalent vaccine against other types than HPV16/18 (86).
Type-specific quantitative PCR amplification

TS HPV qPCR assays were internally developed at Merck Research Laboratories (MRL) and used for efficacy determination in the Phase III trials of the quadrivalent vaccine, e.g., the FUTURE I and II (111, 112). Merck TS HPV qPCR assays are a set of type-specific PCRs for amplification of multiple sequences simultaneously, i.e., in the L1, E6, and E7 ORFs (of HPV6, 11, 16, 18, 31, 45, 52, and 58) or in the E6 and E7 ORFs (of HPV33, 35, 39, 51, 56, and 59) (113-115). The triplex or duplex qPCR is performed for each HPV type individually. These assays appeared to have a higher analytical sensitivity than INNO-LiPA HPV Genotyping Extra in a direct analytical comparison, with a limit of detection (LOD) that was below 50 copies/test for all HPVs targeted (114).

An in-house method comprising a set of E6/E7-based type-specific qPCRs for 13 hrHPVs and 4 additional HPVs was developed at the RIATOL laboratory in Antwerpen, Belgium. A separate beta-globin qPCR is performed as an endogenous internal control to assess specimen quality. A density sedimentation method has been implemented in the processing of clinical specimens (116). The clinical validation of the RIATOL qPCRs was reported by Depuydt et al (117).

1.4.5 HPV mRNA detection tests

The detection of hrHPV mRNA encoding viral oncoproteins E6 and E7 instead of DNA might allow better distinction between productive (low expression of E6/E7) and transforming (high expression of E6/E7) infections (118). Overexpression of E6 and E7 is required for malignant transformation in HPV-related cancers.

The Aptima HPV assay (Aptima; Hologic Gen-Probe, San Diego, CA, USA) is an HPV assay designed for pooled detection of E6/E7 mRNA from 14 hrHPVs. Aptima is based on target capture after cell lysis, with subsequent transcription-mediated amplification (TMA) and probe hybridization protection for detection of E6/E7 mRNA expression (119, 120). Aptima met the cross-sectional clinical and reproducibility criteria of the international guidelines for HPV test requirements for cervical screening (121). It was noted that these requirements for cross-sectional equivalence were formulated for HPV DNA tests and may not necessarily be valid for other molecular markers, e.g., E6/E7 mRNA. Longitudinal data are needed to ensure that the long-term negative predictive value of this mRNA assay is similar to those of validated HPV DNA tests and allows for the same screening intervals (121).

1.4.6 HPV16 intratypic variant analysis

Sanger sequence analysis of parts of the HPV16 genome, e.g., E6, L1 and the long control region (LCR) (17, 122) was used to identify different HPV16 variants, which were associated with human population migrations and continent of origin (18) (Table 1). More recently, a novel HPV16 variant reverse hybridization assay (RHA) was developed and evaluated for simple and accurate recognition of these HPV16 variant lineages (123). This assay uses E6-based PCR amplification of a large, single region (570 bp) for clinical specimens of sufficient quality, or uses primer sets that generate four smaller, overlapping regions for samples in which DNA is poorly preserved. The generated amplification products encompass variant-specific single nucleotide polymorphisms (SNPs) that are targeted by oligonucleotide probes in a strip-based RHA. This assay can differentiate between the four main lineages A (European&Asian), B (African 1), C (African 2) and D (North-American&Asian-American), and has the resolution to distinguish some sublineages, i.e., A1&A2 (European) from A3 (Asian) within the main lineage A, and D1 (North-American) from D2&D3 (Asian-American) within the main lineage D.

The characteristics of HPV tests described in this chapter are summarized in Table 4.
Table 4: Brief overview of a selection of HPV tests, summarizing their technical characteristics (HPVs targeted, concurrent genotyping capability, technology, target, and internal control), main indicated use (based on kit manual or literature), supporting trials and/or studies, and the chapters of this thesis in which the assay was used (if applicable).

<table>
<thead>
<tr>
<th>HPV test</th>
<th>HPV(s) detected and Concurrent genotyping</th>
<th>Technology</th>
<th>Target</th>
<th>Internal control: endogenous/ exogenous</th>
<th>Main indicated use</th>
<th>Supporting trials and/or studies</th>
<th>This thesis, chapter:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC2</td>
<td>13 hrHPVs None</td>
<td>Signal amplification by hybrid capture</td>
<td>DNA</td>
<td>No/n.a.</td>
<td>Screening</td>
<td>NTCC ARTISTIC VUSASCREEN</td>
<td>3, 4</td>
</tr>
<tr>
<td>Gervista</td>
<td>14 hrHPVs None</td>
<td>Signal amplification by invader chemistry</td>
<td>DNA</td>
<td>Yes/n.a.</td>
<td>Screening (with cytology) Triage after cytology</td>
<td>Belinson, 2011 (76)</td>
<td>Boers, 2014 (77)</td>
</tr>
<tr>
<td>Aptima</td>
<td>14 hrHPVs None</td>
<td>Transcription-mediated amplification and probe hybridization</td>
<td>mRNA, E6/E7</td>
<td>No/yes</td>
<td>Screening (with cytology) Triage after cytology</td>
<td>Wu, 2010 (119)</td>
<td>Monsonego, 2012 (120)</td>
</tr>
<tr>
<td>Cobas</td>
<td>14 hrHPVs HPV16&amp;18</td>
<td>BS-qPCR</td>
<td>DNA, L1, ~200 bp</td>
<td>Yes/no</td>
<td>Screening (with cytology) Triage after cytology</td>
<td>ATHENA Heideman, 2011 (95)</td>
<td>-</td>
</tr>
<tr>
<td>Abbott RealTime HR HPV</td>
<td>14 hrHPVs HPV16&amp;18</td>
<td>BS-qPCR</td>
<td>DNA, L1, ~150 bp</td>
<td>Yes/no</td>
<td>Not specified. Clinically validated</td>
<td>Carozzi, 2011 (96)</td>
<td>Poljak, 2011 (97)</td>
</tr>
<tr>
<td>HPV-Risk assay</td>
<td>15 hrHPVs HPV16&amp;18</td>
<td>BS-qPCR</td>
<td>DNA, E7, ~150 bp</td>
<td>Yes/no</td>
<td>Screening</td>
<td>Hesselink, 2014 (99)</td>
<td>-</td>
</tr>
<tr>
<td>Oncclarity</td>
<td>14 hrHPVs HPV16&amp;18 and 3 other HPVs</td>
<td>Three separate BS-qPCRs</td>
<td>DNA, E6/E7</td>
<td>Yes/no</td>
<td>Not specified. Clinically validated</td>
<td>Ejegod, 2013 (100)</td>
<td>-</td>
</tr>
<tr>
<td>Miatol (in-house)</td>
<td>13 hrHPVs and 4 other HPVs</td>
<td>qPCR for each type separately</td>
<td>DNA, E6/E7</td>
<td>Yes/no</td>
<td>Not specified. Clinically validated</td>
<td>Depuydt, 2012 (117)</td>
<td>-</td>
</tr>
<tr>
<td>PapilloCheck</td>
<td>14 hrHPVs and 10 other HPVs</td>
<td>BS-PCR and probe hybridization</td>
<td>DNA, E1, ~350 bp</td>
<td>Yes/yes</td>
<td>Screening</td>
<td>Hesselink, 2010 (109)</td>
<td>-</td>
</tr>
<tr>
<td>GP5+/6+/EIA</td>
<td>14 hrHPVs None</td>
<td>BS-PCR and probe hybridization</td>
<td>DNA, L1, ~150 bp</td>
<td>Yes/no</td>
<td>Screening Triage after cytology Test-of-cure</td>
<td>POB ASCA M SWEDSCREEN</td>
<td>10, 11</td>
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<tr>
<td>HPV test</td>
<td>HPV s detected* Concurrent genotyping</td>
<td>Technology</td>
<td>Target</td>
<td>Internal control: endogenous/ exogenous</td>
<td>Main indicated use</td>
<td>Supporting trials and/or studies*</td>
<td>This thesis, chapter:</td>
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<tr>
<td>GP5+/6+ LMNX (LQ Test)</td>
<td>14 hrHPVs and 4 other HPVs</td>
<td>Full BS-PCR and probe hybridization</td>
<td>DNA, L1, ~150 bp Yes/no</td>
<td>Screening Triage after cytology Test-of-cure</td>
<td>-</td>
<td>4, 5, 10, 11</td>
<td></td>
</tr>
<tr>
<td>GP5+/6+ RLB (in-house)</td>
<td>14 hrHPVs and 23 other HPVs</td>
<td>Full BS-PCR and probe hybridization</td>
<td>DNA, L1, ~150 bp No/no</td>
<td>Epidemiology and reflex genotyping after hrHPV positivity by EIA</td>
<td>Van den Brule, 2002 (107) 3, 4 Munoz, 2003 (24) Rijkaart, 2012 (108)</td>
<td></td>
<td></td>
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<tr>
<td>GP5+/6+ strip (v1) (RH Test)</td>
<td>14 hrHPVs and 4 other HPVs</td>
<td>Full BS-PCR and probe hybridization</td>
<td>DNA, L1, ~150 bp No/no</td>
<td>Reflex genotyping after hrHPV positivity by EIA</td>
<td>-</td>
<td>3, 5</td>
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<td>PGMY Linear Array</td>
<td>14 hrHPVs and 23 other HPVs</td>
<td>Full BS-PCR and probe hybridization</td>
<td>DNA, L1, ~450 bp Yes/no</td>
<td>Not specified. Used in epidemiological and surveillance studies. Not clinically validated</td>
<td>Coutlee, 2006 (105) Wheeler, 2014 (32) Trabzini, 2014 (106)</td>
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<tr>
<td>Amplicor</td>
<td>13 hrHPVs</td>
<td>None BS-PCR and probe hybridization</td>
<td>DNA, L1, ~165 bp Yes/no</td>
<td>Not specified. Not clinically validated (replaced by Cobas)</td>
<td>Carozzi, 2007 (92) Mo, 2008 (93) Wentzensen, 2009 (91)</td>
<td>5</td>
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<tr>
<td>SPF10 DEIA (version 1)</td>
<td>14 hrHPVs and at least 55 other HPVs</td>
<td>None BS-PCR and probe hybridization</td>
<td>DNA, L1, 65 bp No/no</td>
<td>Vaccine trials, surveillance and epidemiology</td>
<td>Kletter, 1998 (81) PATRICIA CVT Tjalma, 2012 (66)</td>
<td>2, 6, 7, 8, 9, 11</td>
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<tr>
<td>SPF10 LiPA25 (version 1)</td>
<td>14 hrHPVs and 11 other HPVs</td>
<td>Full BS-PCR and probe hybridization</td>
<td>DNA, L1, 65 bp No/no</td>
<td>Vaccine trials, surveillance and epidemiology</td>
<td>Kletter, 1999 (82) PATRICIA CVT Tjalma, 2012 (66)</td>
<td>2, 6, 7, 8, 9, 11</td>
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<tr>
<td>Merck TS qPCRs</td>
<td>13 hrHPVs and 2 other HPVs</td>
<td>TS-qPCRs for each type separately</td>
<td>DNA, L1&amp;E6&amp;E7 or E6&amp;E7 or E7</td>
<td>Vaccine trials</td>
<td>Else, 2011 (114) FUTURE I FUTURE II</td>
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<tr>
<td>HPV test</td>
<td>HPVs detected</td>
<td>Concurrent genotyping</td>
<td>Technology</td>
<td>Target</td>
<td>Internal control: endogenous/exogenous</td>
<td>Main indicated use</td>
<td>Supporting trials and/or studies</td>
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<tr>
<td>TS16/18</td>
<td>HPV16 and 18</td>
<td>Full</td>
<td>TS-PCRs and probe hybridization separately</td>
<td>DNA, E6/E7, 92 bp and L1, 126 bp</td>
<td>No/no</td>
<td>Vaccine trials</td>
<td>Van Doorn, 2006 (71)</td>
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<tr>
<td>MPTS12</td>
<td>HPV16, 18, 31, 33, 35, 45, 52, 58, and 59</td>
<td>Full</td>
<td>Two separate TS-PCRs and one probe hybridization</td>
<td>DNA, E6, 55-139 bp</td>
<td>No/no</td>
<td>Vaccine trials</td>
<td>Van Alewijk, 2013 (72)</td>
</tr>
<tr>
<td>MPTS123</td>
<td>14 hrHPVs and 2 other HPVs</td>
<td>Full</td>
<td>Three separate TS-PCRs and one probe hybridization</td>
<td>DNA, E6, 55-139 bp</td>
<td>No/no</td>
<td>Vaccine trials</td>
<td>Van Alewijk, 2013 (72)</td>
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<tr>
<td>HPV16 variant RHA</td>
<td>HPV16 main variant lineages</td>
<td>n.a</td>
<td>TS-PCR and probe hybridization</td>
<td>DNA, E6, 570 bp</td>
<td>No/no</td>
<td>Natural history and epidemiological studies of HPV16 variant infections</td>
<td>Sanchez, 2011 (123)</td>
</tr>
</tbody>
</table>

*Unless otherwise specified, 13 hrHPVs refers to types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; 14 hrHPVs refers to aforementioned types plus HPV66; 15 hrHPVs refers to aforementioned types plus HPV66 and 67.

*bThe clinical trials are: NTCC (61); ARTISTIC (60); VUSASCREEN (75); POBASCAM (56); SWEDESCREEN (62); ATHENA (94); PATRICIA (85); CVT (83); FUTURE I (111); FUTURE II (112).

*Cervista offers no concurrent genotyping. Partial genotyping for HPV16 and 18 is possible through reflex testing using a separate assay (Cervista HPV 16/18 Test).

*dSize of region(s) targeted by primers not specified in kit manual or literature.

*The LMNX Genotyping kit HPV GP (GP5+/6+ LMNX; Diassay BV) was previously marketed as the digene HPV Genotyping LQ Test (LQ Test, Qiagen).

*iA triplex qPCR targets sequences in L1, E6, and E7 for HPV6, 11, 16, 18, 31, 45, 52, and 58. A duplex qPCR targets sequences in E6 and E7 for HPV33, 35, 39, 51, 56, and 59.

*jIn case of specimens with poorly preserved DNA, four smaller, overlapping fragments of 104, 109, 65, and 69 bp, respectively, can be amplified.
1.5 THESIS OUTLINE
In this thesis we aimed to evaluate a number of established and novel PCR-based technologies for the identification of HPVs, and apply these in epidemiologic and clinical studies in accordance with their intended use. These novel technologies comprise additions and improvements to two established PCR-based (hr)HPV test algorithms, which are current “gold standards” for epidemiologic and for clinical purposes, i.e., the SPF10 LiPA 25 (version 1) (Figure 5) and the GP5+/6+ EIA (Figure 6), respectively.

This thesis is divided into three main parts: 1) the assessment of analytical accuracy of several recently developed assays for HPV genotyping (Chapters 2-5), 2) the application of (novel) techniques for HPV characterization in several avenues of research (Chapters 6-9), and 3) the utility of novel methodologies for hrHPV detection and self-sample collection within a clinical setting (Chapters 10-11).

Part 1: Chapters 2 to 5 are technical chapters. These focus on evaluating the analytical accuracy of novel HPV genotyping assays.

Recently, three novel tests for HPV genotyping have been introduced, i.e., INNO-LiPA HPV Genotyping Extra (INNO-LiPA), the GP5+/6+ strip (previously marketed as digene HPV Genotyping RH Test) and the GP5+/6+ LMNX (previously marketed as digene HPV Genotyping LQ Test). Each test was evaluated against an established genotyping assay that is relevant for its intended use (“gold standard”), i.e., analytical or clinical. The INNO-LiPA was compared to the original SPF10 LiPA25 strip (version 1), the analytically sensitive gold standard assay for epidemiologic studies and vaccine trials (Chapter 2). Both assays were evaluated on a selected panel of cervical swabs and biopsies, the two types of specimens mostly used in epidemiologic HPV research.

In contrast, the GP5+/6+ strip and GP5+/6+ LMNX were evaluated against the Reverse Line Blot assay, an established in-house method for reflex genotyping following the clinically validated GP5+/6+ PCR, in Chapter 3 and Chapter 4, respectively. Accurate identification of individual hrHPVs by these assays might be valuable for further risk stratification of women that tested hr HPV positive in a clinical setting.

In Chapter 5, we investigated if the GP5+/6+ strip and GP5+/6+ LMNX can be used for direct genotyping of PCR products generated by another hrHPV test, i.e., Amplicor, in addition to the GP5+/6+ amplicons.

Part 2: Chapters 6 to 9 describe the applications of genotyping assays in a research setting, e.g., surveillance of prevalence, epidemiology (disease association), and natural history of HPV infections.

The SPF10 LiPA25 algorithm was used to determine the prevalence of and determinants for hrHPV genotypes in Paramaribo, Suriname, providing baseline data prior to implementation of an HPV vaccination program in 2013 (Chapter 6). Pre- and post-vaccination surveillance of HPV prevalence is important for short-term evaluation of the effects of a local HPV vaccination program.

Chapter 7 describes the HPV genotype distribution established by the SPF10 LiPA25 algorithm in a worldwide collection of 10,375 cases of invasive cervical carcinoma (ICC). The estimated attribution of individual HPVs to ICC can provide insight into which types should be given priority for assessment of cross-protective effects of current vaccines and for development of second-generation polyvalent vaccines.

In the same collection of ICC, we investigated the presence of HPV types rarely targeted by current HPV genotyping tests, using a novel sequence methodology (Chapter 8). The contribution of rare HPVs classified as possibly high-risk (Class 2B) due to their phylogenetic relation with established hrHPVs (Class 1/2A), is of particular interest. Class 2B HPVs are
currently not targets of hrHPV tests intended for cervical cancer screening, but their occurrence as single infections in ICC could strengthen the circumstantial evidence of a carcinogenic role.

In Chapter 9, we performed a longitudinal study towards the natural history of infections with different variant lineages of HPV16, the most carcinogenic HPV type. European, African, Asian, North American and Asian-American HPV16 variants were studied in cervical swabs, whole-tissue sections and laser-capture micro-dissected regions from biopsies, using a recently developed HPV16 variant reverse hybridization assay. We aimed to determine the prevalence of the different variant lineages, and the dynamics of these infections over time.

Part 3: Chapters 10 and 11 describe the applications of genotyping assays in a clinical setting, e.g., for primary cervical cancer screening and combined with a self-sampling device.

Chapter 10 describes a clinical evaluation of the GP5+/6+ LMNX using the GP5+/6+ EIA as a clinically validated comparator hrHPV test. The utilized sample panel was collected from a cervical cancer screening setting by an international consortium (VALGENT) and provides a cross-sectional clinical equivalence comparison. A non-inferiority analysis of sensitivity and specificity for detecting women with CIN2+ and CIN3+ allows clinical validation of the GP5+/6+ LMNX for screening purposes.

HrHPV analysis performed on cervicovaginal self-collected specimens seems a suitable alternative for liquid-based cervical specimens collected by a physician, particularly for non-responders in cervical cancer screening and in low-resource settings. The FTA solid-state carrier cartridge is a novel self-sampling device and was compared to physician-collected specimens using two different HPV assays, i.e., GP5+/6+ LMNX and the SPF10 LiPA25 algorithm, in Chapter 11.
Figure 5: Schematic overview of the SPF10 LiPA25 (version 1) test algorithm that is currently used as the gold standard for HPV detection and genotyping in a research setting. Additional or alternative technologies for HPV characterization that have been recently developed are also shown. The different technologies were evaluated and/or applied in the indicated chapters of this thesis.
Figure 6: Schematic overview of the GP5+/6+ EIA test algorithm that is currently regarded as one of the clinically validated reference assays for hrHPV detection in a clinical setting. Alternative technologies for hrHPV detection and genotyping as well as a novel method for sample collection are also shown. These novel technologies were evaluated in the indicated chapters of this thesis.
Chapter 1

1.6 REFERENCES


Chapter 1


General introduction and thesis outline
Chapter I


General introduction and thesis outline


Chapter 1