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DISSERTATION

Analytical and clinical evaluation of the HPV DNA Array E1-based genotyping assay

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ABBREVIATIONS AND ACRONYMS

ASCUS	Atypical squamous cells of undetermined significance
BS	Broad spectrum
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
EIA	Enzyme Immunoassay
FDA	Food and Drug Administration
GP	Gravitt Patty
HPV	Human papillomavirus
HR	High risk
HGSIL	High-grade squamous intraepithelial lesion
LR	Low risk
LGSIL	Low-grade squamous intraepithelial lesion
MPG	Multiplexed genotyping
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPV	Positive predictive value
STM	Storage transport media
WHO	World Health Organization

SUMMARY

Background: HPV infection has shown to be mandatory for development of cervical dysplasia. Consequently, molecular HPV detection is used for cervical cancer screening, especially for genotype-specific persistence. Aim of this study was to evaluate the analytical and clinical performance of HPV DNA Array, an E1-based genotyping test for identification of 29 HPV types: 6,11,16,18,26,31,33,35,39,40,42,44,45,51,52,53,54,56, 58,59,66,67,68,69,70,73,82,85 and 97.

Methods: HPV DNA Array is based on a multiplexed PCR followed by reverse hybridization in a 96-well format with automated visual readout, capable of high-throughput in a time-effective manner. Technical performance of the assay was assessed with cervical cancer cell lines with known HPV status, and preselected clinical cervical scrapings genotyped by multiplexed genotyping (MPG) with Luminex readout. Intra- and inter-laboratory reproducibility experiments were performed to ensure reliability of the assay.

The clinical evaluation was performed against the reference assays, BS-GP5+/6+ MPG-Luminex, with 600 cervical smear samples of a referral population, and the FDA-approved Cobas 4800 HPV test on a study population of 500 cervical samples.

Results: HPV DNA Array identified the intrinsic HPV genotype in all cervical cancer cell lines and demonstrated a high sensitivity for the HPV16 probe (1 cell/PCR reaction), HPV18 and 45 probes (100 cells/PCR reaction). When compared with MPG within the analytical study, HPV DNA Array showed good agreement of 92.2% for HPV detection irrespective of type (κ=0.601), and demonstrated high agreement for HPV16 (80.7%, κ=0.836), and HPV18 (86.7%, κ=0.925). Furthermore, high intra-/inter-laboratory reproducibility was observed (90.9%-100%).

HPV DNA Array detected CIN2+ lesions with a sensitivity of 90.2%, identical to that of MPG-Luminex. Sensitivity for detection of CIN3+ lesions was 90.3%, as compared with 88.7% of MPG-Luminex. HPV DNA Array demonstrated very good agreement for HPV detection, irrespective of type, of 91.5% (κ=0.832) within the clinical evaluation study.

HPV DNA Array demonstrated a very high sensitivity of 100% for CIN2+/CIN3+ detection same as Cobas 4800. HPV DNA Array showed greater sensitivity for CIN2+, than cytology (100% vs. 13.6%). The agreement with Cobas 4800 for HPV detection, irrespective of type, was 81.4% (κ=0.613). The agreement for HPV16 was 92.8% (κ=0.929), and for HPV18 54.2% (κ=0.681).

Conclusion: HPV DNA Array has demonstrated a good performance in HPV and CIN2+ detection with high reproducibility and it is capable of extended HPV genotyping by a technically simple method. HPV DNA Array could be considered for extended HPV genotyping of cervical smears and in organized screening programs and potentially in low resource settings.

ZUSAMMENFASSUNG

Hintergrund: Eine HPV-Infektion ist obligatorisch für die Entwicklung von zervikalen Dysplasien. Dabei wird der molekulare HPV-Nachweis zur Früherkennung von Gebärmutterhalskrebs und genotypspezifischer Persistenz eingesetzt, insbesondere für letztere. Ziel dieser Studie war es, die analytische und klinische Leistungsfähigkeit des HPV-DNA-Arrays, eines E1-basierten multiplexen PCR-Tests zur Identifizierung von 29 HPV-Typen: 6,11,16,18,26,31,33,35,39,40,42,44,45,51,52,53,54,56,58,59,66,67,68,69, 70,73,82,85 und 97, zu bewerten.

Methoden: HPV DNA Array basiert auf einer Multiplex-PCR mit anschließender Rück-Hybridisierung im 96-Well-Format mit automatischer visueller Auslesung, die einen hohen Durchsatz in zeitsparender Weise ermöglicht. Die technische Leistung des Arrays wurde mit Zervixkarzinom-Zelllinien mit bekanntem HPV-Status und vorselektierten klinischen Zervixabstrichen, die durch Multiplex-Genotypisierung (MPG) mit Luminex-Auslesung genotypisiert wurden, bewertet. Intra- und interlaboratorische Reproduzierbarkeit wurde durchgeführt, um die Zuverlässigkeit des Arrays zu gewährleisten. Die klinische Auswertung erfolgte gegenüber den Referenz-Assays BS-GP5+/6+ MPG-Luminex mit 600 Zervixabstrichen von zur Abklärung überwiesenen Patienten und Cobas 4800 HPV-Test an einer Studienpopulation von 500 Zervixproben.

Ergebnisse: Das HPV DNA Array identifizierte den intrinsischen HPV-Genotyp in allen zervikalen Krebszelllinien und zeigte eine hohe Sensitivität für HPV16 Sonden (1 Zelle/PCR-Reaktion) sowie HPV18 und 45 Sonden (100 Zellen/PCR-Reaktion). Im Vergleich zu MPG in der analytischen Studie zeigte HPV DNA Array in der analytischen Studie eine gute Übereinstimmung von 92,2% für den HPV-Nachweis unabhängig vom Typ (κ=0,601) und eine hohe Übereinstimmung für HPV16 (80,7%, κ=0,836) und HPV18 (86,7%, κ=0,925). Darüber hinaus wurde eine hohe intra- bzw. interlaboratorische Reproduzierbarkeit beobachtet (90,9%-100%).

HPV DNA Array detektierte CIN2+ Läsionen mit einer Sensitivität von 90,2%, identisch mit der von MPG-Luminex. Der Nachweis von CIN3+ Läsionen erfolgte mit einer

Sensitivität von 90,3%, verglichen mit 88,7% bei MPG-Luminex. Es zeigte sich eine sehr gute Übereinstimmung für den HPV-Nachweis, unabhängig vom Typ, von 91,5% (κ=0,832) innerhalb der klinischen Evaluationsstudie.

HPV DNA Array zeigte eine sehr hohe Sensitivität von 100% für den CIN2+/CIN3+ Nachweis so wie der Cobas 4800. HPV DNA Array zeigte eine höhere Sensitivität für CIN2+, als die Zytologie (100% vs. 13.6%). Die Übereinstimmung mit Cobas 4800 zur HPV-Erkennung, unabhängig vom Typ, betrug 81,4% (κ=0,613). Die Übereinstimmung für HPV16 betrug 92,8% (κ=0,929) und für HPV18 54,2% (κ=0,681).

Schlussfolgerung: HPV DNA Array hat eine gute Zuverlässigkeit bei der HPV- und CIN-Detektion mit hoher Reproduzierbarkeit gezeigt und ist in der Lage, die HPV-Genotypisierung durch eine technisch einfache Methode zu erweitern. HPV DNA Array könnte für die erweiterte HPV-Genotypisierung von Zervixabstrichen und in organisierten Screening-Programmen sowie potentiell bei geringer Ressourcenverfügbarkeit in Betracht gezogen werden.

1. Introduction

The extraordinary research of Prof. Dr. Harald zur Hausen, a Nobel Prize laureate, and his team on the human papillomavirus (HPV) [1-3] has marked a significant change in our understanding of the development of cervical cancer. It is well-established that the main cause of cervical cancer is infection by human papillomavirus [4], a small double stranded DNA virus, with a high tropism for epithelial cells [5, 6]. There are more than 40 HPV types identified to have high tropism specifically for ano-genital mucosal epithelia. HPV types causing genital warts and benign lesions are labeled low-risk (LR) types, among which HPV6 and 11 are most commonly found [7]. HPV types associated with cervical cancer are grouped as high-risk (HR) HPV types [4]. The most clinically significant HR-HPVs are HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Cumulatively, they have been found in 94.5% of all squamous cell carcinomas of the cervix worldwide [8].

HPV infection is one of the most common sexually transmitted infections among sexually active persons [9, 10]. Apart from sexual transmission, other possible ways of HPV transmission are oral, digital, or perinatal [11]. The main risks of acquiring HPV are early first intercourse and number of sexual partners, the risk increasing with every new sexual partner [12]. Other potential factors associated with HPV persistence and development of cervical cancer include immunosuppression, HIV infection, cigarette smoking, Chlamydia infection, multiparity, and long-term use of oral contraceptives [13-17]. However, the most important factor associated with cervical cancer prevalence is lack of screening [18].

As mentioned previously, HPV has a high tropism for genital mucosal epithelia, with special affinity for the basal cells of the squamocolumnar junction. The basal cells are accessed through epithelial micro-abrasions. HPV infection that persists may cause histological transformation of the normal epithelial cells to abnormal cells, which form the pre-malignant pre-invasive state of cervical intraepithelial neoplasia (CIN) (Fig. 1). Histologically, cervical intraepithelial neoplasia could develop in three grades: CIN1, which consists of abnormal cells involving the lower third of the epithelium covered with

differentiated epithelium; in CIN2, the abnormal cells involve more than one third; and in CIN3 they involve the full thickness of the epithelium [19, 20]. When abnormal cells invade the dermis by breaking through the basal lamina of the epithelium, the condition is labeled as invasive cervical cancer.

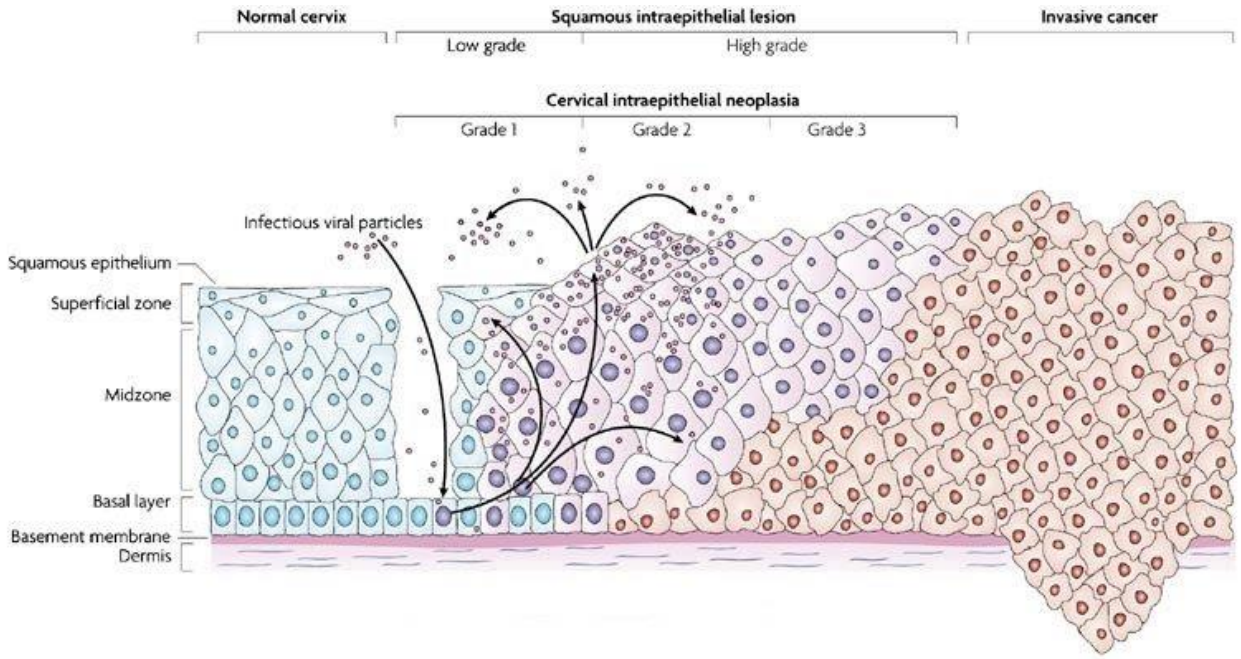

Figure 1: Schematic representation of CIN development

HPV has tropism for the basal cells of the cervical epithelium, which is accessed through micro-abrasions in the cervical epithelium. Present on the left are the cells of the normal cervical squamous epithelium, and towards the right are represented the progressively bigger layers of new abnormal small cells involving the epithelium. As the layer of abnormal cells thickens towards the right, it is considered to be CIN1, CIN2, and CIN3, which leads to invasive cancer if the abnormal cells break through the basal lamina (taken from Fig. 1 in Woodman et al. Nat Rev Cancer. 2007;7(1):13).

Although most sexually active women will become infected with HPV at least once in their life, only 10% of these HPV infections will become persistent. Most genital HPV infections are asymptomatic and in the majority of women they will clear within 2 years [9]. It is important to mention, that even if a low-grade intraepithelial neoplastic lesion develops, in most women, it will regress within 3 years [21]. However, if genotype-specific HPV

infections and lesions persist, women are at higher risk of developing cervical cancer [22]. Time needed for progression from initial HPV infection to cervical cancer is 10 to 20 years [23], which enables early detection of neoplastic changes by screening and allows early treatment.

Yet, cervical cancer is the second most common female cancer in the world. In 2014, the World Health Organization (WHO) reported an incidence of more than half a million new cases each year and mortality of approx. 266,000 registered deaths worldwide. The highest disease burden was observed in developing countries [24]. However, cervical cancer is an easily preventable disease. With the introduction of mass cervical cancer screening, a significant 75% decline in cancer incidence has been observed in developed countries, achieved through regular cytological screening [25, 26]. The most often used screening methods for cervical cancer are the Papanicolaou test (PAP) or liquid-based cytology [27], where the cervical cells are collected and investigated under the microscope for premalignant and malignant morphological changes.

In recent years, cytology-based screening has been strongly questioned. It is hard to implement cytology in developing countries, it is examiner-subjective, and has a variable sensitivity for disease detection (44-78%) [28-30]. There is evidence that HPV-based screening is more sensitive in detecting high-grade lesions [31-33]. HPV-based screening could give prognostic information e.g. positivity for HR-HPV at 6 months after lesion treatment can predict lesion recurrence [34]. Also, women with genotype-specific HPV persistence of more than 7 years have a higher risk of lesion development and progression to invasive cancer [35]. Specifically, genotyping for certain HPV types could provide valuable clinical information, as not all HPV types bear the same risk for cancer development e.g. HPV16+ lesions are significantly less likely to regress, than lesions positive for other HR-HPV types [36]. Any information on type shifting and persistence after treatment may have clinical impact [35]. An additional advantage of genotyping may be the identification of multiple HPV infections. Women infected with multiple types of HPV have an increased risk of developing cervical cancer [37-40]. Identification of the specific genotypes is also important to evaluate their frequency in epidemiological studies.

Therefore, the recent advance made in development of detection methods, and the established causal link between HPV and cervical cancer, has led to a change in the paradigm. HPV testing advanced from usage as a triaging method to a method for primary cervical cancer screening. In 2013, WHO recommended HR-HPV screening in settings where cytology is difficult to implement [18]. A year later, in 2014, FDA has approved the first HPV assay (Cobas, Roche), with partial HPV genotyping of HPV16 and 18, for primary cervical cancer screening [41]. The following year, the American Society for Colposcopy and Cervical Pathology included the HPV genotyping tests as primary tests (without cytology) for cervical cancer screening in its guidelines [42].

With the recognized importance of HPV-based screening, and its superiority to cytology, a growing number of new HPV detection methods have been emerging on the global market. Currently, there are more than 200 HPV assays available with various technical characteristics [43]. Although most assays detect HPV DNA, some also target mRNA (e.g. APTIMA HPV assay). Furthermore, various assays target various DNA genes or the whole genome (e.g. Hybrid Capture 2, CareHPV Test). The genes detected are: E1 (e.g. PapilloCheck HPV-screening test), E6/E7 (e.g. BD Onclarity HPV assay, Cervista HPV HR Test), or L1 (e.g. GP5+/6+ PCR-EIA, GP5+/6+-LMNX, Abbott RealTime, Cobas 4800 HPV test). Assays also use different nucleic acid hybridization methods to detect HPV such as direct nucleic acid probe methods, hybridization signal amplification, and target amplification methods [44].

In addition, HPV assays could be distinguished in relation to their HPV genotyping capabilities. HPV assays are full-genotyping assays, when giving information on detection of specific HPV types (e.g. GP5+/6+ PCR-EIA, GP5+/6+-LMNX); they are partial-genotyping assays, when genotyping only for few HPV types e.g. HPV16 or 18, and grouping other HPVs into a pool (e.g. Cobas 4800 HPV Test, Cervista HPV16/18 Test, BD Onclarity HPV); and can be non-genotyping but HPV-testing assays, that only give information on HR-HPV positivity or negativity, not specifying which HPV types are present in the infection (e.g. Hybrid Capture 2, Cervista HPV HR Test).

Due to the variety of HPV assay available and the lack of standardized assay formats, international guidelines for HPV test requirements for primary cervical cancer screening

have been established [45]. Meijer et al. recommend that validation studies should be conducted in comparison with internationally recognized and well-established assays such as Hybrid Capture 2 and GP5+/6+ EIA; they should demonstrate a CIN2+ sensitivity and specificity of 90% and 98%, respectively; as well as have a high inter- and intra-laboratory reproducibility. In addition, WHO has established a proficiency panel (HPV LabNet) with the purpose of continuously evaluating different HPV tests in different laboratories through organized studies [46-51].

The aim of this research was to evaluate and document the performance of HPV DNA Array, a full genotyping assay developed by AID Diagnostika GmbH (Strassberg, Germany), which is CE-marked for in vitro diagnostic use in the European Union. The HPV DNA Array is an E1-based DNA multiplex PCR assay, with potential for full HPV genotyping of 29 HPV types: 18 HR-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 11 LR-HPV types (6, 11, 40, 42, 44, 54, 67, 69, 70, 85, 97), as well as 3 internal controls. HPV is detected by multiplex polymerase chain reaction (PCR) and subsequent reverse dot blot hybridization with type-specific oligonucleotide probes. These probes are spotted together into one single well of a 96 well microtiter plate. Plates are evaluated and results computed by an ELISPOT reader and proprietary AiDot software.

The performance of HPV DNA Array was evaluated by using cultured cell lines with known HPV types, intra-/inter-laboratory reproducibility, and by comparison with the internationally recognized reference assays BSGP5+/6+ Multiplex Genotyping (MPG) with Luminex-based readout and Cobas 4800 HPV test. Both reference assays used in this study are well validated against Hybrid Capture 2 and GP5+/6+ EIA, as recommended by Meijer et al. [52, 53].

2. Material and Methods

2.1. Study design

To fully evaluate the performance of the HPV DNA Array, analytical and clinical validation studies were organized (Fig. 2). Analytical validation was focused on the technical performance of the assay in detection of HPV, and its reproducibility. For analytical purposes, 3 sets of samples were used: i) cultured cell lines with known HPV status; ii) preselected clinical cervical scrapings with known HPV status, genotyped by MPG; and iii) preselected clinical cervical scrapings with known HPV status for the intra- and inter-laboratory reproducibility experiments.

The clinical validation highlighted the performance in detecting high-grade pre-neoplastic lesions by comparing with two internationally recognized assays, histology and cytology. Two sets of samples were used: a) clinical cervical scrapings tested by MPG and with known diagnosis by biopsy; and b) clinical cervical scrapings tested with Cobas 4800 HPV test, with known diagnosis biopsy and cytological smear results.

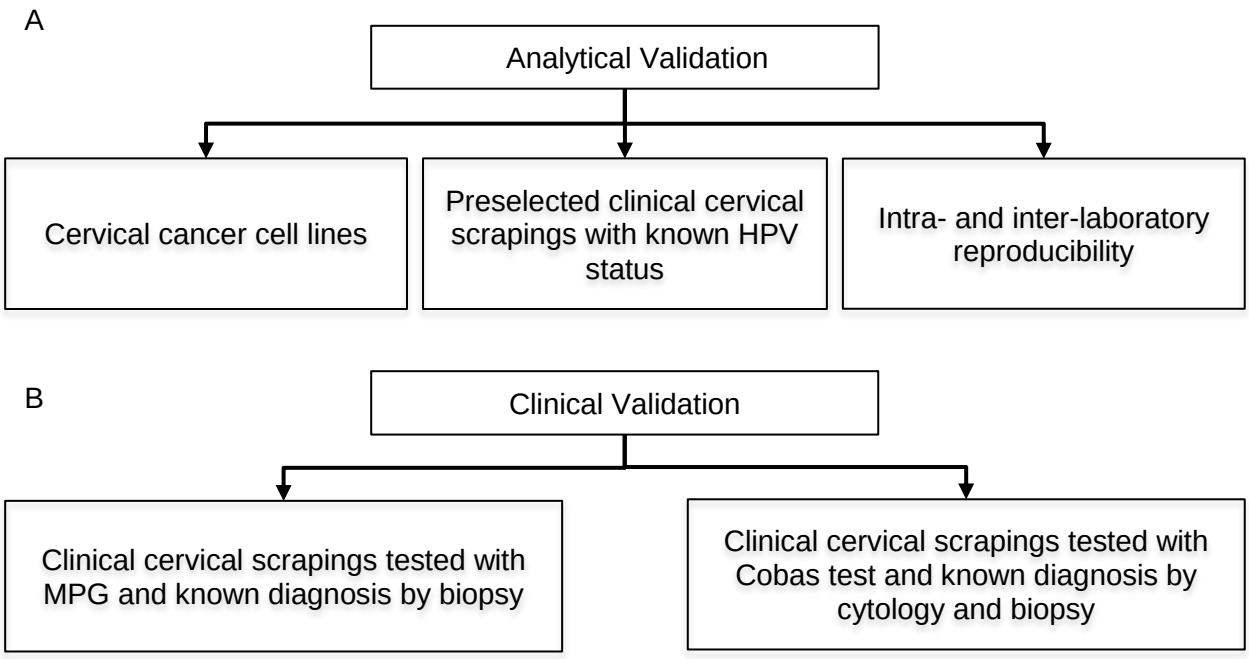

Figure 2: Study design overview
A: Analytical validation study design; B: Clinical validation study design

2.1.1. Cell Culture

Seven cervical cancer cell lines were obtained and cultured according to ATCC instructions, i.e. HeLa (HPV18+, ATCC® CCL-2™), CaSki (HPV16+, ATCC® CRL-1550™), SiHa (HPV16+, ATCC® HTB-35™), CERV (HPV45+, ATCC® HTB-34™), MS751 (HPV45+, ATCC® HTB-34™), ME180 (HPV68+, ATCC® HTB-33™), C33A (HPV-, ATCC® HTB-31™). HeLa cells were maintained in MEM medium (supplemented with 10% heat-inactivated fetal bovine serum (FCS) and 1% penicillin/streptomycin). SiHa cells were maintained in DMEM (supplemented with 10% FCS and 1% penicillin/streptomycin). CaSki cells were maintained in RPMI (supplemented with 10% FCS, 1% penicillin/streptomycin and 1% sodium pyruvate). CERV cells were maintained in MEM (supplemented with 10% FCS, 1% penicillin/streptomycin and 1% sodium pyruvate). MS751 cells were maintained in DMEM (supplemented with 10% FCS and 1% penicillin/streptomycin). ME180 were maintained in McCoy's medium (supplemented with 10% FCS, 1% penicillin/streptomycin and 1% sodium pyruvate). C33A cells were maintained in MEM (supplemented 10% FCS, 1% penicillin/streptomycin and 1% sodium pyruvate). The cells were plated in 75 cm^2 flasks and kept at 37°C in a humidified atmosphere of 5% CO_2. They were passaged at 70-80% confluence by trypsinisation. When growth reached 80-90%, confluent cells were deemed ready for experiments. The cells were trypsinised, counted with Countess® cell counting chamber (Thermo Fisher Scientific Inc. MA, USA) and suspended in two different storage transport media (STM): phosphate-buffered saline (PBS) and in PreservCyte (Hologic, MA, USA), at a concentration of 10^6 cells/ml, and aliquoted as 1 ml samples. DNA was extracted by QIAamp DNA Mini Kit (Qiagen, Hilgen, Germany) following manufacturer's instructions. Nucleic acid was eluted into a final volume of 160 µl. All samples were tested under identical conditions, and PCR amplification was performed and tested in duplicates. HPV genotyping was performed with HPV DNA Array using 4.8 µl DNA extract per PCR reaction.

To determine the sensitivity for HPV16, HPV18, HPV45 probes, and for DNA content control (Gap-DH), a titration of SiHa, HeLa, and MS751 cell lines was performed. Two different passage samples of each cell line were suspended in PBS at a concentration of 10^6 cells/ml and stored at -20°C until DNA extraction with QIAamp DNA Mini Kit. Nucleic acid was eluted to a final volume of 160 µl. Dilution series of the isolated DNA were made

in sterile water to obtain concentrations from 10^4 cells/PCR reaction to 10^{-2} cells/PCR reaction for each cell line and passage. Dilutions were tested under same conditions with HPV DNA Array.

2.1.2. Preselected clinical cervical scrapings with known HPV status by MPG

From the laboratory sample repository 244 HPV positive DNA samples were selected: 157 samples with single HR-HPV infection, 27 with single LR-HPV infection, and 60 samples with multiple HPV infections. At least one sample to represent any type included in the HPV DNA Array spectrum was selected, however, HPV40, 44, 67, 69, 85, 97 were not available. In addition, as control, 28 HPV negative samples were included. Samples were obtained from women undergoing colposcopy examination at the outpatient referral Gynecology Clinic, Charité-Universitätsmedizin Berlin, Germany. Patients consented to use residual material for research (IRB no. EA1/168/13). Cervical scrapings were taken by cytobrush rinsed in PreservCyte and stored at +4°C until processing. DNA was extracted by QIAamp DNA Mini Kit from 2 ml of 20 ml total sample volume. Nucleic acid was eluted to a final volume of 160 µl. HPV genotyping was performed with MPG using 5 µl DNA extract per PCR reaction, and with HPV DNA Array 4.8 µl of DNA of each sample.

2.1.3. Intra- and inter-laboratory reproducibility

Twenty-two cervical samples were selected: 3 HPV negative and 19 HPV positive, 8 with a single, and 11 with multiple HPV infections. Within the intra-laboratory reproducibility testing, an intra- and inter-assay comparison was performed. For the intra-assay experiment, the same PCR product of the sample set was tested as quadruplicates on the same assay plate in one run by one performer using the same assay lot. In the inter-assay setting, the sample set was tested independently by 3 performers using different assay lots. For the inter-laboratory reproducibility testing, DNA aliquots of the sample set were sent to two external laboratories (Lab2: GenID/AID, Strassberg, Germany; Lab3: Microbiology Laboratory, University of Zurich, Switzerland). Different assay lots were used in different laboratory settings.

2.1.4. Comparison with MPG and histology

To assess the clinical performance of HPV DNA Array, 600 samples were consecutively collected from women undergoing colposcopy at the outpatient referral dysplasia clinic of the Clinic for Gynecology, Charité-Universitätsmedizin Berlin, Germany. Patients consented to use residual material for research (IRB no. EA1/168/13). Cervical scrapings were taken by cytobrush rinsed in PreservCyte, and stored at +4°C until processing. DNA was extracted by QIAamp DNA Mini Kit of 2 ml from 20 ml total volume of sample in accordance with the manufacturer's instructions. Nucleic acid was eluted to a final volume of 160 µl. HPV genotyping was performed with MPG using 5 µl per PCR reaction, and with HPV DNA Array 4.8 µl of DNA of each sample. The person performing HPV DNA Array was blinded to the MPG genotyping results, cytology or histology status of the samples collected.

2.1.5. Comparison with Cobas 4800 HPV test, histology, and cytology

We used 500 of approx. 4000 cervical scrapings collected for the "HEllenic Real life Multicentric cErvical Screening" (HERMES) study that compared cytology and HPV-based screening [54]. Samples were collected from women undergoing routine cervical screening at 9 different outpatient Clinics in Greece (Athens, Thessaloniki, Larisa, Patras, and Alexandropoulis). Cervical scrapings were taken with Cervex Brush (Rovers Medical Devices, Oss, Netherlands) and rinsed in PreservCyte. One part of the sample was used for cytology, one part for HPV testing with Cobas HPV test, and the leftover volume of 500 samples was stored at +4°C and sent to Charité-Universitätsmedizin Berlin, Germany. One sample was not included in the shipment, hence, 499 samples were analyzed.

For this study, 2 ml of each sample was used for DNA extraction by QIAamp DNA Mini Kit, in accordance with manufacturer's instructions. Nucleic acid was eluted in a final volume of 160 µl. The cytological smear examination was performed at the corresponding pathology laboratory of the participating hospital where the sample was taken. Bethesda 2001 cytology classification guideline was followed [55]. The cytologists were blinded to the HPV test results.

2.2. HPV DNA Array

HPV DNA Array (AID Diagnostika GmbH, Strassberg, Germany) genotypes 18 high-risk (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 11 low-risk (6, 11, 40, 42, 44, 54, 67, 69, 70, 85, 97) HPV types (Tab. 1).

The assay detects HPV by amplifying E1-gene sequences of approx. 180 base pairs in length by multiplex PCR with specific biotin-labeled primers. Duration of the PCR program is 55 minutes, continuing one cycle for 3 min at 95°C, 10 cycles of 10 sec at 96°C and 20 sec at 60°, 26 cycles of 10 sec at 95°C, 15 sec at 55°C and 15 sec at 72°C, and one final cycle for 3 min at 72°C. Per PCR reaction 4.8 µl DNA and 20.2 µl of Master Mix, in a total volume of 25 µl, were used. For each PCR run negative and positive control (Gap-DH) were included to control the PCR performance. The amplified gene fragments were detected by a hybridization reaction with sequence-specific oligonucleotide probes, specific for each HPV type. All probes were spotted as triplets and immobilized at the bottom of each well of a 96 well microtiter plate (Fig. 3). Twenty-five µl of PCR amplicons were denatured by using 25 µl of proprietary Denaturation Reagent to allow binding to immobilized oligonucleotide probes. Ten µl of this mix was placed into a well for hybridization to the spotted HPV genotype-specific probes. Stringent washing procedure ensured binding only when 100% sequence homology was given. Streptavidin-coupled alkaline phosphatase was used to detect biotin-labeled amplified DNA hybrids by color reaction with BCIP/NBT. Spots were evaluated by ELISpot reader and reading software AiDot (AID Diagnostika GmbH, Strassberg, Germany). To be counted positive spots had to be stronger than 10% conjugate control probe color strength (Fig. 4). In addition, in each well 3 internal controls were spotted: Gap-DH control for verification of adequate DNA content, a conjugate control for correct test execution, and a specificity control to detect any potential unspecific binding. Information obtained by ELISpot reader and reading software AiDot can be exported (e.g. Microsoft Word) and pictures of each well stored.

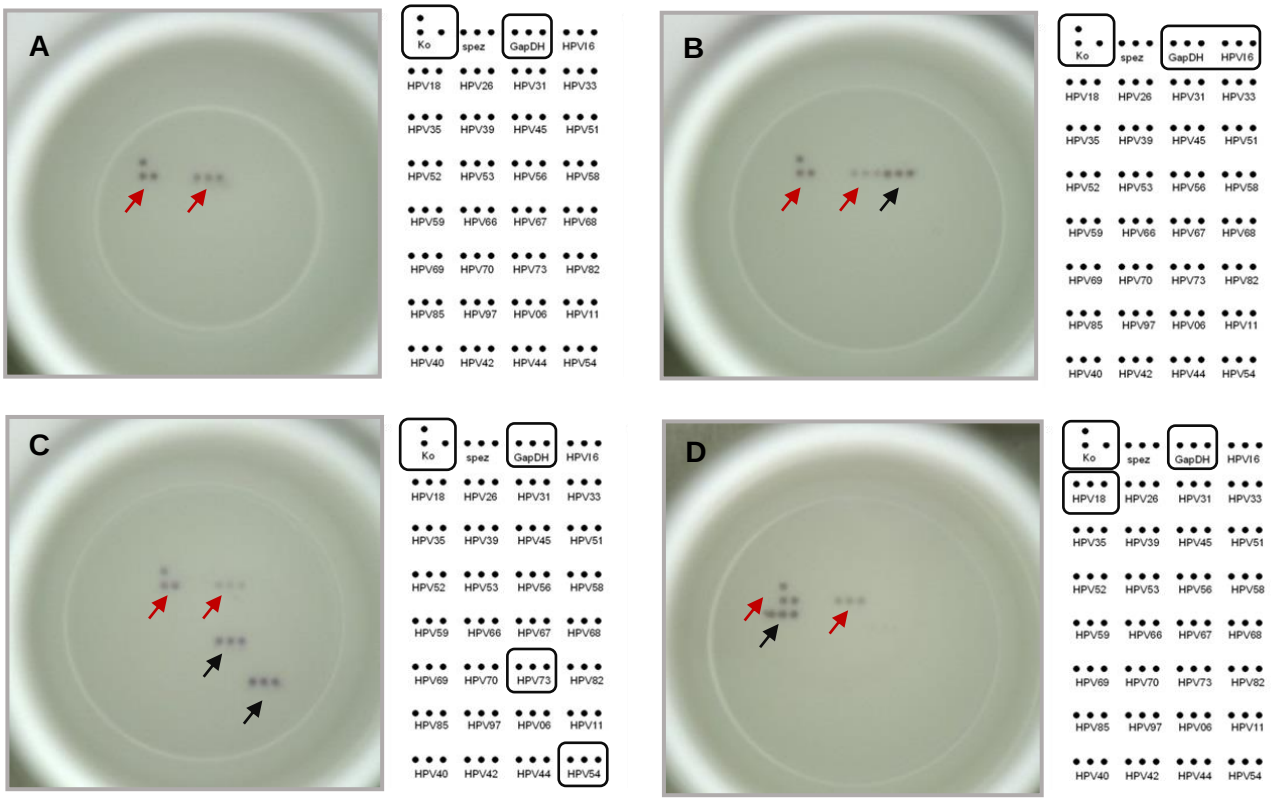

Figure 3: HPV DNA Array probe organization

Close-up pictures of four wells in a 96-well plate with spotted probes for detection of 29 HPV types and 3 controls. Red arrows point to controls; black arrows to HPV-specific signal. Next to well pictures are the probe spotting patterns with highlighted positions.
A: Example of HPV negative well (Conjugate and Gap-DH controls appear positive), B: HPV16 positive well with signals at position of HPV16 and controls, C: HPV54 and 73 positive wells with controls, D: HPV18 positive well with controls.

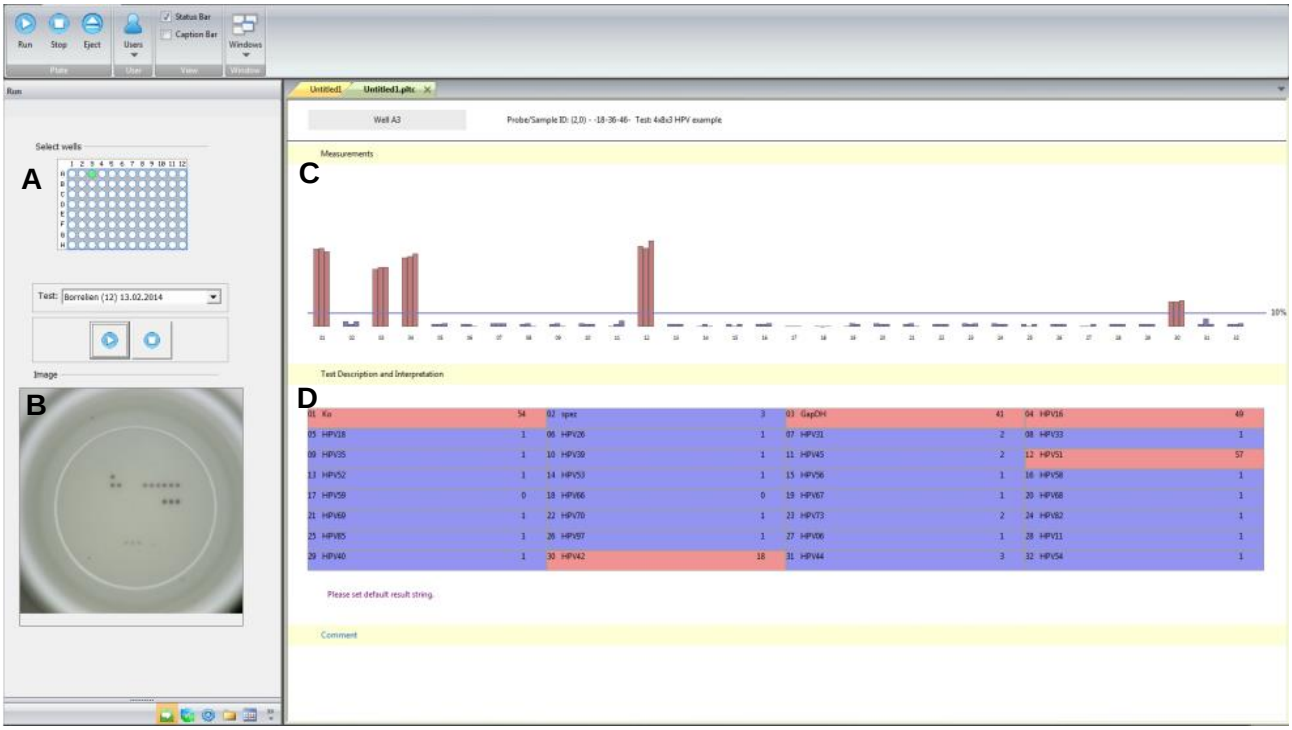

Figure 4: AiDOT software interface for evaluation of the HPV DNA Array plate
Icons and menu present on the top of the picture. A: Plate under evaluation. The well currently under evaluation is highlighted green. B: Image of the respective well. C: Strength of coloring of the three corresponding probe spots for each HPV probe (blue line: cut off for positivity pre-set at 10% of coloring strength of the Conjugate probe in first position on the left). D: Table representing each HPV type and the average coloring strength of all three probe spots for each probe in percentage. The HPV probes that are positive have their table cells highlighted with red. The table cells of negative HPV probes are colored blue.

2.3. Multiplex genotyping with Luminex-based hybridization following BS-GP 5+/6+ PCR (MPG)

MPG is an L1-based polymerase chain reaction (PCR) DNA test which is used routinely in the Laboratory for Gynecologic Tumorimmunology for HPV detection of following HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 68, 70, 72, 73, 82, and 90 (Tab. 1). Additionally, the assay measures the cellular beta-globin of each sample, as a control for adequate DNA amount. The MPG genotyping was carried out generally as described by Schmitt et al. [56, 57], however, in our Laboratory a final PCR volume of 25 µl was used. BS GP5+/6+ Multiplex Genotyping (MPG) assay with Luminex-based readout is a well-established assay proficient for HPV genotyping with high analytical sensitivity [52]. In our laboratory MPG assay performance was validated by participation in EQUALIS proficiency panel testing [47].

2.4. Cobas 4800 HPV Test

The Cobas 4800 HPV is an L1-based PCR test with capability of separately genotyping HPV 16 and 18, and grouping other 12 HR-HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in a single signal (Tab. 1). HPV testing was performed as described by Castle et al. [58, 59] at the Laboratory of Microbiology, Democritus University of Thrace, Alexandroupolis, Greece. The performer was blinded to cytology and histology results.

Table 1: Characteristics of HPV assays used in the validation studies

	Nucleic acid target	Target gene	HPV types genotyped	Other HPV types detected in a pool	Internal control
HPV DNA Array	DNA	E1	HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, 97		Gap-DH
MPG	DNA	L1	HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 68, 70, 72, 73, 82, 90		β-globin
Cobas	DNA	L1	HPV16, 18	HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	β-globin

2.5. Statistical Data Analysis

Main outcomes were agreement, sensitivity, and specificity, positive (PPV) and negative predictive values (NPV). The agreement between assays was evaluated using Cohen's Kappa (*k*). The *k* value was interpreted as follows [60]: poor (< 0.20), fair (0.21–0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00). The values of the McNemar's test were used to determine the significance of discordant cases between the assays. Statistical analysis was performed with IBM SPSS Statistics for Windows (Version 21.0. IBM Corp. Armonk, NY) and MedCalc 15.8 (MedCalc Software, Ostend, Belgium).

For determining the agreement of HPV detection between the HPV DNA Array and MPG only 23 HPV types covered by both assays were included in the analysis (6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 73, and 82).

Cobas 4800 HPV test gives information on HPV positivity by genotyping separately HPV16 and HPV18, and reporting the results for 12 other HR-HPV types in a pool (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), hence, the HPV DNA Array detected HPV types were grouped accordingly for comparative analytical purposes.

3. Results

3.1. Analytical performance in detection of HPV in cervical cancer cell lines

To test the stability of cellular DNA and suitability of two different storage transport media (STM), samples from 7 cervical cancer cell lines (HeLa, SiHa, CaSki, CERV, MS751, ME-180, and C33A) were re-suspended in PBS and in PreservCyte. The expected HPV type of each cell line was identified in both media, i.e. CaSki-HPV16, SiHa-HPV16, HeLa-HPV18, CERV-HPV45, MS751-HPV45, ME-180-HPV68, and C33A-HPV DNA negative. Sample adequacy was controlled by positive Gap-DH (Tab. 2).

Table 2: HPV genotype detection from cervical cell lines stored in PBS or PreservCyte

	HeLa (HPV18+)	CaSki (HPV16+)	SiHa (HPV16+)	CERV (HPV45+)	MS751 (HPV45+)	ME180 (HPV68+)	C33A[1] (HPV-)
PBS	✓	✓	✓	✓	✓	✓	✓
PreservCyte	✓	✓	✓	✓	✓	✓	✓

✓ symbol represents 100% of samples correctly identified HPV type;

[1] HPV negative cell line with HPV negative result

PCR and probe sensitivity for detection of HPV16, 18, 45 and Gap-DH was tested with titration series of SiHa, HeLa and MS751 cells in concentrations from 10^4 cells per PCR reaction to 10^{-2} cells per PCR reaction (Tab. 3). In two independent determinations, the detection limit for HPV identification was 1 cell per PCR reaction for HPV16 and 10^2 cells per PCR reaction for HPV18 and 45. The detection limit for Gap-DH control was 10^2 cells per PCR reaction for all three cell lines. No difference between different passages of each cervical cancer cell line was observed.

Table 3: HPV DNA Array HPV16, 18, 45 and Gap-DH probe sensitivity in cell per PCR reaction[a]

	SiHa (HPV16+)	HeLa (HPV18+)	MS751 (HPV45+)
HPV Type	1	100	100
Gap-DH Control	100	100	100

[a] Tested in two independent determinations.

3.2. Analytical performance in HPV detection with clinical cervical scrapings

The sample population comprised of 272 samples collected and selected with known MPG result: 184 with single HPV infection (27 with LR-HPV and 157 with HR-HPV), 60 with multiple HPV infections and 28 HPV negative. All samples were tested with the HPV DNA Array. Nine samples demonstrated positive HPV types with a negative Gap-DH control with HPV DNA Array. These samples were included in the analysis. No case of a HPV negative sample with negative Gap-DH control with HPV DNA Array was found.

219 samples showed concordant results, and in 54 samples discordant results were observed: either HPV DNA Array+/MPG-, HPV DNA Array-/MPG+, or both assays positive but not for same HPV genotypes.

3.2.1. Sample re-testing

To avoid operational mistakes, the discordant samples were re-tested with HPV DNA Array, along with 10 concordant samples as control. All control samples had the same concordant result. We found that in 32/54 samples, results stayed the same, still discordant to MPG, and in 22/54 samples result changed. To confirm which result was true, a third testing was performed on these 22 samples. HPV type that was found in 2 or more tests was counted as a true positive type. In 14/22 samples, the result now matched MPG for at least 1 HPV type, and for the residual 8 samples, the result changed but was still discordant to MPG. The results mostly changed by losing an HPV type that was positive the first time in multiple infections.

In 32 samples that were still discordant and 8 samples where result changed but was still discordant to MPG, a re-test with MPG was performed. Fourteen concordant samples were included as a control, and remained concordant. We found that in 29/40 samples, the result was the same, hence, a discordance was concluded. In 11/40 samples, the result changed. The samples were then tested a second time to confirm which result was true. Only HPV types found in 2 tests were counted as a true positive type. All 11 samples now matched the HPV DNA Array results.

3.2.2. Agreement between HPV DNA Array and MPG

After re-testing of discrepant samples, the results of all samples were analyzed and are shown in Tab. 4. MPG genotyping results were taken as reference.

In the MPG HPV negative population, 26 samples were also negative with the HPV DNA Array (26/28, 92.9%). Two, however, were positive for HPV16, and 31, respectively, with the HPV DNA Array.

Among the MPG HPV positive population, HPV DNA Array was positive in 225/244 samples, 92.2%. This group was stratified according to HPV single/multiple infection status. Agreement for HPV detection was 90.8% in the MPG single type infection group (167/184), and 96.7% in the multiple infections group (58/60). When stratifying the MPG HPV positive population according to HPV risk group, agreement was higher within the HR-HPV group, 90.4% (197/218). Agreement was 81.3% (39/48) within the LR-HPV group. The HR-HPV agreement became greater focusing on the 14 most important HR types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) with 169/184 (91.84%) detected.

Including all samples (HPV positive and negative) the agreement for HPV-positivity detection (irrespective of type) was 92.3% by HPV DNA Array, with kappa 0.671 (95% CI, 0.542 to 0.799), demonstrating good agreement between the assays, and with a specificity of 92.86%.

Table 4: Agreement between HPV DNA Array and MPG, stratified by MPG HPV status

		MPG	HPV DNA Array	%
HPV detection	MPG HPV-	28	26	92.9
	MPG HPV+	244	225	92.2
	Single HPV+	184	167	90.8
	Multiple HPV+	60	58	96.7
	HR-HPV+	218	197	90.4
	LR-HPV+	48	39	81.3
	14 HR-HPV[a]+	184	168	91.8

[a] HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68

3.2.3. Type-specific agreement between HPV DNA Array and MPG

Sensitivity for detection of individual HPV types varied from 28.6% for HPV 56 to 100% for HPV33, 35, 45, and 58 (Tab. 5). On average sensitivity was 73.6% over all genotypes. HPV DNA Array had a very high specificity for each HPV type with an average value of 98.0% (from 92.2% for HPV26 to 100% for HPV6, 18, 39, 66, 70, and 73). Kappa values were varying from 0.194 for HPV26 to 0.958 for HPV33. Average kappa of 0.67 demonstrated a good agreement between the assays by HPV-type. We observed a very good agreement for HPV16 and 18, with high kappa of 0.836 and 0.925, and sensitivity of 80.7% and 86.7%, respectively. The agreement among HR-HPV types by kappa value was considered to be very good/good ($\kappa>0.6$) for HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 66, 73 and 82, although the difference for detection in both assays was deemed statistically significant by McNemar test for HPV31, 51, and 66. Moderate agreement (κ 0.4 to 0.6) was found for HPV53, and 59, with significant difference for HPV53. Poor/fair agreement ($\kappa<0.4$) was found for HPV26, 56, and 68, with a significant difference.

Pearson correlation analysis discovered a cross-reactivity between HPV26 and HPV35 probes. A higher number of HPV26 HPV DNA Array positive samples (21) was observed, which were negative by MPG, explaining the low kappa agreement and specificity for this rare genotype.

Table 5: Analytical comparison in HPV detection between HPV DNA Array and MPG

HPV Genotyping		MPG	HPV DNA Array	Sensitivity (%)	Kappa (95% CI)	Interpretation[a]	McNemar's p value
HR-HPV	HPV16	57	46	80.7	0.836 (0.753 to 0.919)	very good	0.570
	HPV18	15	13	86.7	0.925 (0.821 to 1)	very good	0.500
	HPV26	4	3	75	0.194 (-0.001 to 0.389)	poor	0.000
	HPV31	16	15	93.8	0.656 (0.491 to 0.822)	good	0.002
	HPV33	12	12	100	0.958 (0.876 to 1)	very good	1.000
	HPV35	14	14	100	0.929 (0.832 to 1)	very good	0.500
	HPV39	18	13	72.2	0.829 (0.683 to 0.975)	very good	0.063
	HPV45	15	15	100	0.903 (0.795 to 1)	very good	0.250
	HPV51	23	14	60.9	0.718 (0.553 to 0.883)	good	0.021
	HPV52	18	15	83.3	0.730 (0.571 to 0.89)	good	0.344
	HPV53	16	12	75	0.553 (0.366 to 0.741)	moderate	0.049
	HPV56	21	6	28.6	0.355 (0.133 to 0.577)	fair	0.019
	HPV58	10	10	100	0.791 (0.613 to 0.968)	good	0.063
	HPV59	11	6	54.5	0.526 (0.268 to 0.785)	moderate	1.000
	HPV66	11	5	45.5	0.615 (0.334 to 0.896)	good	0.031
	HPV68	8	6	75	0.346 (0.133 to 0.559)	fair	0.000
	HPV73	10	6	60	0.743 (0.501 to 0.985)	good	0.125
	HPV82	12	8	66.7	0.753 (0.544 to 0.961)	good	0.375
LR-HPV	HPV6	11	10	90.9	0.950 (0.854 to 1)	very good	1.000
	HPV11	2	1	50	0.496 (-0.107 to 1)	moderate	1.000
	HPV42	16	13	81.3	0.606 (0.425 to 0.787)	good	0.035
	HPV54	8	5	62.5	0.375 (0.129 to 0.621)	fair	0.035
	HPV70	12	6	50	0.657 (0.401 to 0.912)	good	0.031

[a] Interpretation of kappa values: poor (< 0.20), fair (0.21–0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00)

3.2.4. Distribution of HPV DNA Array negative samples in single and multiple infections compared with MPG

To further understand the discrepancy in HPV-type detection, the samples, in which a respective HPV type was HPV DNA Array-/MPG+, were stratified by presence in MPG single or multiple infections (Tab. 6). In total, it was found that in 22 cases a respective HPV type was missed when present in MPG-detected single HPV infection, and in 63 cases when present in MPG-detected multiple infections. It was observed that in multiple infections in 57/63 cases, other types present in the infection were detected instead by HPV DNA Array. This corresponds with our previous finding that the agreement for general HPV detection was higher in multiple (96.7%, 58/60) vs. single infection (90.8%, 167/184) (Tab.4.).

Table 6: Distribution of HPV genotypes in single and multiple infections among discordant HPV DNA Array negative/MPG positive results[a]

	MPG single infection		MPG multiple infections		
	Concordant	Discordant	Concordant	Discordant	Other HPV types detected in multiple infection[b]
HPV16	22	1	24	10	10/10
HPV18	9	1	4	1	1/1
HPV26	3	0	0	1	0/1
HPV31	10	1	6	0	0/0
HPV39	7	0	6	5	5/5
HPV51	8	0	6	9	9/9
HPV52	9	1	6	2	2/2
HPV53	8	1	4	3	2/3
HPV56	2	6	4	9	7/9
HPV59	3	2	3	3	2/3
HPV66	3	2	2	4	3/4
HPV68	6	0	0	2	2/2
HPV73	5	2	1	2	2/2
HPV82	7	1	1	3	3/3
HPV6	9	0	1	1	1/1
HPV11	0	1	1	0	0/0
HPV42	10	0	3	3	3/3
HPV54	2	0	3	3	3/3
HPV70	3	2	3	4	4/4

[a] No missed samples for HPV33, 35, 45; [b] Number of samples in which HPV DNA Array matched MPG for ≥1 HPV genotype, but negative for respective HPV type

3.3. Intra- and inter-laboratory reproducibility

The reproducibility was determined with HPV positive vs. negative agreement sample set consisting of 3 HPV negative and 19 HPV positive samples (8 with a single, and 11 with multiple HPV infections). Within the intra-assay/intra-laboratory experiments, where the same PCR product of the sample set was tested as quadruplicates on the same assay plate, the agreement for HPV detection was 100% (22/22, κ=1), between all four sets in one plate run. Within the inter-assay/intra-laboratory reproducibility the agreement for HPV detection of the sample set tested independently by 3 performers using different assay lots, was 100% (22/22, κ=1), For the inter-laboratory reproducibility, DNA aliquots of the sample set were sent to two external laboratories, and agreement was 100% (22/22, κ=1) for the second and 90.9% (20/22, κ=0.69) for the third laboratory, where two HPV positive samples were marked HPV negative (Tab. 7).

In conclusion to the analytical evaluation, it should be highlighted that the study panel used was selected to contain different HPV genotypes, with intent to introduce the protocol and demonstrate HPV DNA Array performance for each HPV type. HPV DNA Array showed good agreement of 92.2% for HPV detection irrespective of type (κ=0.601), and demonstrated high agreement for HPV16 (80.7%, κ=0.836), and HPV18 (86.7%, κ=0.925), with various sensitivities for detection of other HPV types.

Although the detection of 29 HPV types is of benefit for epidemiological studies, the main purpose of HPV testing should be the detection of clinically important cases. Therefore, an additional study was organized to investigate the clinical sensitivity for high-grade lesions and cervical cancer of HPV DNA Array and MPG.

Table 7: Intra-/Inter-Laboratory Reproducibility

Intra-assay / Intra-laboratory experiment			
I set	**II set**	**III set**	**IV set**
HPV -	HPV -	HPV -	HPV -
HPV -	HPV -	HPV -	HPV -
HPV -	HPV -	HPV -	HPV -
HPV 6	HPV 6	HPV 6	HPV 6
HPV 16	HPV 16	HPV 16	HPV 16
HPV 16	HPV 16, 53	HPV 16	HPV 16
HPV 33	HPV 33	HPV 33	HPV 33
HPV 45	HPV 45	HPV 45	HPV 45
HPV 51	HPV 51	HPV 51	HPV 51
HPV 58	HPV 58	HPV 58	HPV 58
HPV 68	HPV 68	HPV 68	HPV 68
HPV 16, 51	HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 31, 52	HPV 31, 52	HPV 31, 52	HPV 31, 52
HPV 16, 51	HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 51, 82	HPV 51, 82	HPV 51, 82	HPV 51, 82
HPV 31, 51	HPV 31, 51	HPV 31, 51	HPV 31, 51
HPV 42, 53	HPV 42	HPV 42, 53	HPV 42, 53
HPV 16, 51	HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 16, 18, 53	HPV 16, 18, 53	HPV 16, 18, 53	HPV 16, 18, 53
HPV 6, 52, 58	HPV 6, 52, 58	HPV 6, 52	HPV 6, 52
HPV 33, 52, 58	HPV 33, 52, 58	HPV 33, 52, 58	HPV 33, 52, 58
HPV 6, 16, 18, 45	HPV 6, 16, 18, 45	HPV 6, 16, 18, 45	HPV 6, 16, 18, 45

Inter-observer / Intra-laboratory experiment		
I performer	**II performer**	**III performer**
HPV -	HPV -	HPV -
HPV -	HPV -	HPV -
HPV -	HPV -	HPV -
HPV 6	HPV 6	HPV 6
HPV 16	HPV 16	HPV 16
HPV 16	HPV 16	HPV 16
HPV 33	HPV 33	HPV 33
HPV 45	HPV 45	HPV 45
HPV 51	HPV 51	HPV 51
HPV 58	HPV 58	HPV 58
HPV 68	HPV 68	HPV 68
HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 31, 52	HPV 31, 52	HPV 31, 52
HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 51, 82	HPV 51, 82	HPV 51, 82
HPV 31, 51	HPV 31, 51	HPV 31, 51
HPV 42, 53	HPV 42, 53	HPV 42, 53
HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 16, 18, 53	HPV 16, 18, 53	HPV 16, 18, 53
HPV 6, 52	HPV 6, 52	HPV 6, 52, 58
HPV 33, 52, 58	HPV 33, 52, 58	HPV 33, 52, 58
HPV 6, 16, 18, 45	HPV 6, 16, 18, 39, 45, 51	HPV 6, 16, 18, 45

Inter-laboratory experiment		
I Laboratory	**II Laboratory**	**III Laboratory**
HPV -	HPV -	HPV -
HPV -	HPV -	HPV -
HPV -	HPV -	HPV -
HPV 6	HPV 6	HPV 6
HPV 16	HPV 16	HPV 16
HPV 16	HPV 16	HPV 16
HPV 33	HPV 33	HPV 33
HPV 45	HPV 45	HPV 45
HPV 51	HPV 51	HPV -
HPV 58	HPV 58	HPV 58
HPV 68	HPV 68	HPV 68
HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 31, 52	HPV 31, 52	HPV 31, 52
HPV 16, 51	HPV 16, 51	HPV 16
HPV 51, 82	HPV 51, 82	HPV -
HPV 31, 51	HPV 31, 33, 51	HPV 31, 51
HPV 42, 53	HPV 42, 53	HPV 42, 53
HPV 16, 51	HPV 16, 51	HPV 16, 51
HPV 16, 18, 53	HPV 16, 18	HPV 16, 18
HPV 6, 52	HPV 6, 52	HPV 6, 52, 58
HPV 33, 52, 58	HPV 33, 52, 58	HPV 33, 52, 58
HPV 6, 16, 18, 45	HPV 6, 16, 18, 45	HPV 6, 16, 18, 45

3.4. Clinical validation by comparing with MPG and histology

3.4.1. Characteristics of the study population

For this study, 600 consecutive samples were collected from women aged 18 to 94 years, with an average age of 39 years. 151 woman were younger than 30 years, and 449 were 30 years or older. Histology result was available for 348 patients: 195 woman had normal histology, 30 were positive for CIN1, 61 for CIN2, 47 CIN3, and 15 had cervical cancer.

Routinely MPG was performed: 262 women were HPV negative, 161 women had a single HPV infection, and 177 women had multiple HPV infections. 322 women had at least one HR-HPV type positive, 193 were HPV16 positive, and 26 were HPV18 positive. Two women had a single infection with HPV90, a HPV type not detected by HPV DNA Array, and they were additionally put into the HPV negative group for analytical purposes.

3.4.2. HPV DNA Array initial results and sample re-testing by both assays

After initial comparison, in 135 samples, a HPV DNA Array discordant result for any HPV type in single or multiple infections to MPG was discovered. To exclude operational mistakes, the discrepant samples were re-tested with both assays. Re-testing was always performed with 10 concordant samples as a control.

In 115/135 samples re-tested with HPV DNA Array, results stayed the same, still discordant to MPG. For 20/135 samples, the result changed. Eighteen samples now matched MPG for at least 1 HPV type, and two samples were discordant to MPG, the result changed by losing an HPV type that was positive the first time in multiple infection. These 20 samples were tested for the third time to confirm which result was true. HPV types that were found in 2 tests were counted as a true positive type.

We then re-tested 115 samples that were still discordant after HPV DNA Array re-testing, and 2 samples with different but still discordant result, with MPG. We found that in 58 samples, the result was the same, so we concluded a discordance in these samples. In 59 samples, the result changed. In those 59 samples where MPG-Luminex result changed, in 45 samples it matched the HPV DNA Array result, but in 11 samples it changed but was still discordant. Samples were tested for the third time to confirm which

result was true. Only a HPV type that was found in 2 tests concordantly was counted as a true positive HPV type.
The re-evaluated results were used for the following analyses.

3.4.3. HPV detection by HPV DNA Array in comparison with MPG

The HPV positivity rate, irrespective of genotype, of HPV DNA Array was 54.3% (326/600), compared with 55.6% (334/600) of MPG. Agreement between assays was 91.5% with kappa 0.832 (95% CI, 78.7% to 87.6%) showing very good agreement (Tab. 8). HPV DNA Array demonstrated a sensitivity for HPV detection of 88.7% (95% CI, 84.8% to 91.8%), a specificity of 92.1% (95% CI, 88.2% to 95.1%), with PPV of 93.7% (95% CI, 90.3% to 96%) and NPV of 86.3% (95% CI, 81.7% to 90.1%) in comparison with MPG.

Stratifying according to age, sensitivity for HPV detection within <30 years of age group was 93.9% (κ=0.805, 95% CI 69.5% to 91.5%), and within ≥30 years of age group 90% (κ=0.831, 95% CI 77.9% to 88.2%) demonstrating very good agreement between the assays. Further, when focusing on agreement among CIN2+ and CIN3+ lesions, a sensitivity of 96.4% (κ=0.631, 95% CI 39.5% to 86.6%) for CIN2+ and 98.2% (κ=0.742, 95% CI 46.5% to 100%) for CIN3+ was observed, demonstrating good agreement. McNemar's p values showed that the differences between assays were not statistically significant.

Table 8: Agreement for HPV detection between HPV DNA Array and MPG, stratified by age and histology

	HPV DNA Array	MPG					
		Positive	Negative	Sensitivity	Specificity	Kappa (95% CI)	McNemar's p
Overall population (n=600)	Positive	305	21	91.3%	92.1%	0.832 (78.7% to 87.6%)	0.322
	Negative	29	245				
<30 (n=151)	Positive	108	4	93.9%	88.9%	0.805 (69.5% to 91.5%)	0.549
	Negative	7	32				
≥30 (n=449)	Positive	198	16	90.0%	93.0%	0.831 (77.9% to 88.2%)	0.418
	Negative	22	213				
CIN2+ (n=123)	Positive	107	4	96.4%	66.7%	0.631 (39.5% to 86.6%)	1.000
	Negative	4	8				
CIN3+ (n=62)	Positive	54	2	98.2%	71.4%	0.742 (46.5% to 100%)	1.000
	Negative	1	5				

3.4.4. CIN2+ and CIN3+ lesion detection by HPV DNA Array in comparison with MPG

Both MPG and HPV DNA Array demonstrated high sensitivity for detection of CIN2+ lesions of 90.2% (95% CI, 83.58% to 94.86%). The specificity was 44% (95% CI, 37.41% to 50.75%) for MPG and 47.5% (95% CI, 40.88% to 54.30%) for HPV DNA Array. The PPV was 46.8% (95% CI, 95% 40.35% to 53.40%) and 48.5% (95% CI, 41.84% to 55.15%), and the NPV was 89.2% (95% CI, 81.88% to 94.29%) and 89.9% (95% CI, 83.05% to 94.68%) for MPG and HPV DNA Array, respectively (Tab. 9).

Sensitivity remained similar for CIN3+ lesion detection, however, HPV DNA Array detected one case more than MPG, resulting in a sensitivity of 90.3% (95% CI, 80.12% to 96.37%) vs. 88.7% (95% CI, 78.11% to 95.34%) of MPG. Specificity, PPV, NPV for MPG were 38.4% (95% CI, 32.56% to 44.45%), 24.8% (95% CI, 19.24% to 30.99%), and 93.7% (95% CI, 87.44% to 97.43%). HPV DNA Array had a specificity of 39.9% (95% CI, 34.23% to 45.84%), PPV 24.4% (95% CI, 19.03% to 30.55%) and NPV 95% (95% CI, 89.52% to 98.16%).

The CIN2+ and CIN3+ detection was stratified according to age and the results are represented in Tab. 9. A difference in the younger than 30 years age group for detection of CIN3+ can be observed, where HPV DNA Array had a sensitivity of 100% compared with 94.1% of MPG, due to a one missed case by MPG. Surprisingly, four cases with histology-confirmed cervical cancer were HPV negative by both assays. Further investigation (post treatment biopsy) showed that one woman had an adenocarcinoma and one woman had a relapsed vaginal cancer, for which she received radiation therapy two years prior sampling.

Table 9: Analytical comparison for HPV detection stratified by histology

			HPV DNA Array		MPG	
		Total	n	%	n	%
	Normal	195	96	49.23%	103	52.82%
	CIN1	30	22	73.33%	23	76.66%
	CIN2	61	55	90.16%	56	91.80%
	CIN3	47	45	95.74%	44	93.62%
	CxCa[a]	15	11	73.33%	11	73.33%
Clinical sensitivity	CIN2+	123	111	90.24%	111	90.24%
	<30	38	37	97.37%	37	97.37%
	≥30	85	74	88.23%	74	88.23%
Clinical sensitivity	CIN3+	62	56	90.32%	55	88.71%
	<30	17	17	100.00%	16	94.11%
	≥30	45	39	86.66%	39	86.66%

[a] Of the four cases missed: two were histologically confirmed squamous cell carcinoma, one was adenocarcinoma, and one was vaginal carcinoma post radiation therapy

In conclusion, HPV DNA Array has demonstrated, in comparison with MPG, a high clinical sensitivity for CIN2+ detection (90.2%) and a very good agreement with MPG for HPV detection, irrespective of type, of 91.5% (κ=0.832).

With a unique opportunity to obtain samples fully characterized by Cobas HPV results, cytology, and histology; the next validation study was organized to investigate if similar high clinical sensitivity can be demonstrated in comparison with Cobas HPV test, an assay FDA approved for primary cervical cancer screening.

3.5. Clinical validation by comparing with Cobas 4800 HPV test, histology, and cytology

3.5.1. Characteristics of the study population

499 samples were received from the HERMES study from women aged 19 to 66 years, with an average age of 33 years. 217 women were younger than 30 years, 276 were 30 years or older, and for 6 samples age information was unavailable. Ninety-five samples were HPV negative by Cobas test, and 404 were HPV positive. HPV16 was detected in 97 samples, HPV18 in 48 samples, and 321 samples were positive for one or more of 12 other HR-HPV types. Cytology results were obtained from 360 women: 274 had normal cytology, 43 had ASCUS, 35 LGSIL and 8 had HGSIL. Biopsy was taken from 74 women: 23 had normal histology, 29 had CIN1, 17 had CIN2, and 5 had CIN3 lesions. No cases of cervical cancer were reported in this population.

3.5.2. HPV DNA Array initial results and sample re-testing

All 499 samples were tested with HPV DNA Array. An HPV negative result was found in 146 samples, and 353 samples were HPV positive.

A disagreement with Cobas was observed in 90 samples (HPV DNA Array+/Cobas-, HPV DNA Array-/Cobas+ or both assays positive with different HPV types). To exclude test execution mistakes, the 90 samples were re-tested with HPV DNA Array, along with 10 concordant samples, as control. All control samples had the same concordant results. In 82 samples, the result stayed the same, still discordant to Cobas, and in 8 samples the result changed. To confirm which result was true, a third testing was performed, and only HPV types found in 2 or more tests were counted as truly type-specific positive. Four samples were initially HPV negative and now became HPV positive, matching the Cobas result; three samples were LR-HPV positive and now showed a co-infection with HR-HPV, and one sample was initially HPV positive for HPV6, but after re-testing it was twice HPV negative.

In summary, after re-evaluation, HPV DNA Array deemed 143 samples to be HPV negative and 356 samples to be HPV positive, among which 25 samples only for types not detected by Cobas, e.g. HPV42, 53, 54, and 67, hence these samples were additionally placed in the HPV negative group for analysis.

The following analyses are from the re-evaluated results.

3.5.3. HPV detection by HPV DNA Array in comparison with Cobas

HPV DNA Array was positive in 66.3% (331/499) cases, as compared with 81% (404/499) of Cobas HPV test (Tab. 10). The agreement between the assays was 81.4% (95% CI, 80.8% to 87.5%) with κappa 0.613 (95% CI, 53.9% to 68.7%). The results were stratified according to age; sensitivity, agreement for HPV detection and specificity. Within the <30 age group these results were 86.2% (95% CI, 80.5% to 90.8%), k 0.618 (95% CI, 49.1% to 74.5%), 100% (95% CI, 87.6% to 100%), and within ≥30 age group 77.4% (95% CI, 84.6% to 100%), k 0.593 (95% CI, 49.9% to 68.8%) and 96.87% (95% CI, 89.2% to 99.6%), respectively. Values of the McNemar's test deemed the differences statistically significant (p<0.001).

Further on, when focusing on agreement among CIN2+/HGSIL lesions, an agreement of 100% was observed.

Table 10: HPV detection between HPV DNA Array and Cobas, stratified by age, histology, and cytology

	HPV DNA Array	Cobas					
		Positive	Negative	Agreement	Kappa	Interpretation[a]	McNemar's p
Overall population (499)	Positive	329	2	81.4%	0.613	good	0.001
	Negative	75	93				
<30 (217)	Positive	163	0	86.2%	0.618	good	0.001
	Negative	26	28				
≥30 (276)	Positive	164	2	77.4%	0.593	moderate	0.001
	Negative	48	62				
CIN2+ (22)	Positive	22	0	100%			
	Negative	0	0				
HGSIL (8)	Positive	8	0	100%			
	Negative	0	0				

[a]Kappa interpretation values: poor (< 0.20), fair (0.21–0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00)

3.5.4. HPV partial genotyping by HPV DNA Array in comparison with Cobas

Cobas test genotypes HPV16 and 18 separately, and groups the results of 12 HR-HPV types in a pool (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), hence, the HPV DNA Array results were adjusted accordingly.

HPV16 was detected by HPV DNA Array in 90 samples, as compared with 97 detected by Cobas (Tab. 11), demonstrating a sensitivity of 92.8% (95% CI, 85.7% to 97%) and kappa agreement of 0.929 (95% CI, 88.7% to 97%), specificity of 99.00% (95% CI, 97.5% to 99.7%), PPV of 95.74% (95% CI, 89.5% to 98.8%) and NPV of 98.27% (95% CI, 96.5% to 99.3%). McNemar's test showed no statistical difference ($p>0.05$). HPV DNA Array detected HPV18 in 26 samples vs. 48 detected by Cobas, demonstrating a sensitivity of 54.2% (95% CI, 39.2% to 68.6%) with moderate agreement ($\kappa=0.681$, 95% CI, 55.8% to 80.5%), a specificity of 100% (95% CI, 99.2% to 100%), PPV of 100% (95% CI, 86.% to 100%) and NPV of 95.3% (95% CI, 93% to 97%). McNemar's test graded the difference significant ($p<0.05$).

Twenty-two HPV18 Cobas-positive samples were not detected by HPV DNA Array, 13 of which were single infections by Cobas, and 9 samples were co-infections with HPV16 and/or other HR-HPV, all of which were detected by HPV DNA Array and only HPV18 was missed by HPV DNA Array in the Cobas multiple infections. Histology was available for 6 samples of the 22 missed samples, and only one sample had a histologically confirmed high-grade lesion (CIN2). In that case, HPV DNA Array failed to detect HPV18, but other HR-HPV types present in the infection were found.

Sensitivity for detecting 12 other HR-HPV types was 75.4% (95% CI, 70.3% to 80%) with 242 samples detected as compared with 321 by Cobas. Correlation analyses showed good agreement of k 0.677 (95% CI, 61.6% to 73.9%). Specificity, PPV and NPV were 98.88% (95% CI, 96% to 99.9%), 99.18% (95% CI, 97% to 99.9%), and 69.02% (95% CI, 62.9% to 74.6%). McNemar's test value ($p<0.05$) graded the detection difference statistically significant.

HPV DNA Array was negative for other HR-types in 75 cases, whereas Cobas was positive. Sixty-five were Cobas single infections, and ten samples were co-infections with HPV16. In all cases HPV DNA Array detected HPV 16. Histology was available for only

7 of the 75 samples. Six samples had a low grade lesion (<CIN2), and one case with high-grade lesion (CIN3). In that case Cobas showed a co-infection with one or more of 12 other HR types with HPV16. HPV DNA Array did detect HPV16, missing to detect other HR-HPV types.

Table 11: HPV genotype detection of HPV DNA Array compared with Cobas

	Cobas	HPV DNA Array	Agreement	Kappa	Interpretation[a]	McNemar's p
HPV 16	97	90	92.8%	0.929	very good	0.549
HPV 18	48	26	54.2%	0.681	good	0.001
12 other HR-HPV[b]	321	242	75.4%	0.677	good	0.001

[a] Interpretation values: poor (< 0.20), fair (0.21–0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00); [b] HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68

3.5.5. CIN2+ lesion detection by HPV DNA Array in comparison with Cobas

HPV DNA Array showed a sensitivity for detection of CIN2+ lesions of 100% (95% CI, 84.6% - 100%), with a specificity of 9.43% (95% CI, 3.1% to 20.7%), PPV 31.43% (95% CI, 20.8% to 43.6%), and NPV 100% (95% CI, 47.8% to 100%) (Tab. 12). Similarly we observed a sensitivity of 100% for CIN3+ detection with all 5 lesions detected by HPV DNA Array.

Cobas test had a sensitivity for detection of CIN2+ lesions of 100% (95% CI, 84.6% - 100%), with a specificity of 0% (95% CI, 0% to 6.7%).

Difference between the assays was observed only for detection of low-grade lesions, 93.1% of HPV DNA Array vs. 100% of Cobas, with a lower specificity of Cobas test.

Table 12: HPV detection stratified to histology

		HPV DNA Array		Cobas	
Histology	Total	HPV+	%	HPV+	%
Normal	23	18	91.3%	23	100%
CIN1	29	24	93.1%	29	100%
CIN2	17	17	100%	17	100%
CIN3	5	5	100%	5	100%
CIN2+	22	22	100%	22	100%

When compared with cytology (Tab. 13), HPV DNA Array demonstrated a much better sensitivity for CIN2+ detection, 100% of HPV DNA Array vs. 13.64% of cytology. Only 3 of 22 CIN2+ lesions were classified as HGSIL**.** Conversely, specificity was much higher with cytology 100% (95% CI, 2.5% to 100%) than with HPV DNA Array 9.43% (95% CI, 3.1% to 20.7%).

Table 13: Cytology vs. HPV DNA Array testing for CIN2+ detection

		HPV DNA Array	Cobas	Cytology			
Histology	Total	HPV+	HPV+	Normal	**ASCUS**	**LGSIL**	**HGSIL**
Normal	23	18	23	17	3	2	1
CIN1	29	24	29	19	4	6	0
CIN2	17	17	17	11	0	3	3
CIN3	5	5	5	3	1	1	0
CIN2+	22	100% (22/22)	100% (22/22)				13.6% (3/22)

ASCUS, Atypical squamous cells of undetermined significance; LGSIL, Low-grade squamous intraepithelial lesion; HGSIL, High-grade squamous intraepithelial lesion

In conclusion, HPV DNA Array demonstrated a very high sensitivity of 100% for CIN2+/CIN3+ detection same as Cobas 4800. HPV DNA Array showed greater sensitivity for CIN2+, than cytology (100% vs. 13.6%). The agreement to Cobas 4800 for HPV detection, irrespective of type, was 81.4% (κ=0.613). The agreement for HPV16 was 92.8% (κ=0.929), and for HPV18 54.2% (κ=0.681).

4. Discussion

The main objective of this dissertation was to investigate the analytical and clinical performance of the newly developed HPV DNA Array, an E1-based multiplexed PCR assay for full HPV genotyping. HPV DNA Array has demonstrated to be a simple and robust assay, with a short four-hour protocol, with a hands-on time of approx. two hours, a reverse hybridization step, and an ELISA-like staining for assay development. The readout uses automated ELISPOT reader software AiDOT that evaluates the full 96 well plate in approx. three minutes, permitting high throughput and time efficacy. Automated plate read-out and analysis by AiDOT software avoids subjective variability. The data can be exported in various formats (e.g. Microsoft Word), as well as stored for documentation and re-evaluation.

Experiments performed on different cervical cancer cell lines established the high sensitivity for detection from cellular material. Importantly, HPV DNA Array proved it can be run from native PBS non-fixed material or PreservCyte samples. Dry brushes, swabs or liquid cytology, which are routinely used for cervical sampling, use PBS for wash-out or PreservCyte as standard transport media. In addition, a high sensitivity for specific probes was observed, e.g. only 1 cell/PCR for HPV16 and 100 cells/PCR reaction for HPV18 and -45. It should be pointed out that different cell lines have different HPV numbers integrated into the DNA, e.g. SiHa, one to two HPV16 copies per cell; HeLa, 10-50 HPV18 copies per cell; and MS751, more than one HPV45 copy per cell [61-63]. Therefore, this is not a sensitivity for a viral copy number.

Reproducibility experiments demonstrated highly reproducible agreement for general HPV detection (100%, $\kappa=1$), with exception within the inter-laboratory reproducibility where the laboratory 3 showed lower agreement (20/22, 90%, $\kappa=0.69$). Laboratory 3 obtained an HPV negative result for two HPV positive samples. This could possibly be due to pipetting mistakes or limited experience of the person performing the test, only recently acquainted with the assay protocol, as compared with a longer experience of the first two laboratories.

The reproducibility experiments highlighted the reliability and reproducibility of the assay, however, when testing a much larger number of samples, some inconsistencies were observed. When re-testing the discordant samples with HPV DNA Array, in a number of samples, the HPV results changed (22/54 re-tested samples within the analytical study, 20/135 re-tested samples within the clinical study with MPG, and 8/90 re-tested samples within the clinical study with Cobas). Similarly, the results changed with MPG after re-testing of discordant samples (11/40 re-tested samples within the analytical study, and 59/115 samples within the clinical validation). It was observed that the change of results is specific not only for HPV DNA Array, but also for MPG, and perhaps even for Cobas, however, re-testing the samples with Cobas was not possible.

This peculiar phenomenon could be caused by pipetting errors, reading errors or contamination, all of which could accompany the PCR diagnostics and hybridization. We could theorize that the HPV types missed were present in low copy numbers, hence missed during pipetting for the first time, but not the second time. Or the sequences of the missed HPV types could be more difficult to amplify within PCR due to competition with other HPV types.

In an effort to get the truest HPV results, especially for validation purposes, the re-testing of discordant samples was performed and included in the analysis. However, such re-testing would not be feasible in a screening setting or as part of routine diagnostics.

Overall, when focusing on HPV detection within all studies, against MPG and Cobas tests, HPV DNA Array demonstrated good agreement for HPV detection with $\kappa>0.6$ and sensitivity >80% (Tab. 14).

Table 14: Overview of HPV detection, irrespective of type, within analytical and clinical validation studies

	Analytical Validation (with MPG, n=295)	Clinical Validation (with MPG, n=600)	Clinical Validation (with Cobas, n=499)
Agreement	92.3%	91.3%	81.4%
Kappa agreement (interpretation[a])	0.671 (good)	0.832 (very good)	0.613 (good)
Specificity for HPV detection	92.9%	92.1%	97.9%

[a] Cohen's Kappa values were interpreted as follows [16]: poor (< 0.20), fair (0.21–0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00).

Within the analytical evaluation, HPV DNA Array has shown good agreement in HPV detection irrespective of type (κ=0.671, 95% CI, 54.2% to 79.9%). High agreement of >90% was documented when stratifying for single/multiple infection status and type-specific carcinogenic risk, with slightly lower agreement among LR-HPV types of 88%. It is worth mentioning that while evaluation and type-specific detection of LR-HPV types is important for epidemiological purposes, it is not recommended for cervical cancer screening.

Very good agreement for the most frequent cancer causing types HPV16 and 18, which account for more than 70% of cervical cancers [8], was found with kappa values of 0.836 (95% CI, 75.3% to 91.9%) and 0.925 (95% CI, 82.1% to 100%), respectively, however, differences were graded statistically significant by McNemar's test.

Agreement for HPV31, 33, 35, 52, and 58, which together with HPV16 and 18 account for 89% of cervical cancers [8], was found to be good to very good with κappa values higher than 0.6, but with McNemar's statistically significant difference for HPV31, 51, 53 and 66. Poor to fair agreement (κ<0.4) was found for 56, 68 among HR-HPV types, which are the 10th and the 12th ranking cancer causing types in the world [8].

Pearson correlation analysis discovered a cross-reactivity between HPV26 and HPV35 probes, as a higher number of samples HPV26 positive with HPV DNA Array, but negative with MPG (21 samples) was observed, explaining the low kappa agreement and specificity for this rare genotype. It was noted for future genotyping analysis that such cross reactions may occur. However, HPV26 is not one of the 14 types recommended by WHO for cervical cancer screening. Therefore, results for HPV26 for epidemiology studies seem not reliable and should be not be included for screening at all.

To further investigate the HPV DNA Array-missed genotypes, which resulted in lower agreement between the two genotyping assays, a stratification in relation to single vs. multiple infections was performed. It was observed that when an HPV type was missed by the HPV DNA Array, it was more frequently in single infections. Additionally, it was found that in multiple infections, when a respective HPV type was missed, other HPV types were detected by HPV DNA Array, e.g. in 10 cases, HPV16 was missed but other HR-HPVs were detected, similarly in 9 cases HPV51 was missed but other HPVs were detected, etc. We could hypothesize that this is possibly the result of competition among HPV type sequences during amplification. Or that HPV types not detected had a lower viral copy number, although, this could not be confirmed.

Evidently, this is not a unique phenomenon. As found within the LabNet Proficiency studies [46, 48, 51], for many assays on the market, HPV genotypes are more difficult to detect when present in multiple infections. Eklund et al. reported that correct genotyping within multiple infections was present in 61 - 79% of the data sets, compared with 90% when HPV type was present in a single infection [46].

Similarly, in this study, the sensitivity for HPV genotype detection was lower for types present in multiple infections. However, importantly, overall sensitivity for detection of multiple infections by at least one or more of the types present was found to be high at 96.6% (58/60). As multiple infections have a higher risk of developing lesions [37-40], it is clinically important not to miss those.

Furthermore, in the same LabNet Proficiency study [46], Eklund et al. reported that the sensitivity for detection of different HPV genotypes among many assays varied between 41% and 97%, which is similar to results reported within this study. Eklund et al. found that assays tended to be more sensitive in detection of HPV16, HPV11, and HPV18, due

to their epidemiological and clinical significance, but were, however, less sensitive for e.g. HPV31, HPV59, and HPV39.

Although, HPV DNA Array is capable of detecting 29 HPV types, due to differences in genotype detecting spectrum, the performance for detection of HPV40, 44, 67, 69, 85, and 97, could not be assessed, since they are missing in our version of the MPG assay setup, and are not detected by Cobas.

It must be considered that MPG has a very high analytical sensitivity [56, 57], which is of advantage for epidemiology, however it may be a disadvantage to achieve an adequate level of clinical sensitivity. Therefore, an additional study was organized to investigate the clinical performance of the HPV DNA Array for CIN2+ lesion detection. The overview of CIN2+ performance is presented in Table 15.

Table 15: Overview of CIN2+ detection performance within the clinical validation studies

	Clinical Validation with MPG-Luminex				Clinical Validation with Cobas			
	HPV DNA Array +		MPG-Luminex +		HPV DNA Array +		Cobas +	
	n/N	%	n/N	%	n/N	%	n/N	%
CIN2	55/61	90.1%	56/61	91.8%	17/17	100%	17/17	100%
CIN3	45/47	95.7%	44/47	93.6%	5/5	100%	5/5	100%
Cervical cancer	11/15	73.3%	11/15	73.3%	-	-	-	-
CIN2+	111/123	90.24%	111/123	90.24%	22/22	100%	22/22	100%

Within the clinical study that included samples from a referral population tested with MPG, HPV DNA Array demonstrated a good clinical sensitivity by detecting >90% of CIN2 and higher lesions, identical to clinical sensitivity of MPG. And it demonstrated a slightly higher sensitivity for detection of CIN3+ lesions (90.3% vs. 88.7%) than MPG, due to one additional case detected.

Surprisingly, four cervical cancer cases were missed by both assays, for which further investigation and search for pathology results post-treatment were conducted. It was

found that one patient had an adenocarcinoma, which are less likely to be HPV positive than squamous cervical carcinomas [64]. One patient had advanced vaginal cancer, treated with radiation therapy. The potential causal connection between HPV and vaginal cancer has been investigated in the literature, however, not all vaginal cancers are HPV+. Studies found that in approx. 60-80% of vaginal cancers, an HPV can be found [65]. Also, it has been discovered that viral load decreases significantly post treatment, which could explain the HPV negative result by both assays, if it was an HPV+ cancer [66]. The other two missed cervical cancers were squamous cell carcinomas with no prior therapy. It is possible that they were missed due to deletion of L1 and E1 genes, although such deletions are very seldom [67, 68]; or these were cancers with etiology other than HPV infection.

When compared with Cobas test, HPV DNA Array demonstrated a very good clinical performance with sensitivity for detection of CIN2+/3+ lesions of 100%, identical to the clinical sensitivity of Cobas HPV test, and higher than cytology (13.6%) in this population. The Cobas HPV test has been well investigated and its performance is well documented in the literature. Other researchers reported a similar performance [53, 69, 70]

Looking at agreement for HPV detection, irrespective of type, HPV DNA Array showed a good agreement of 81.4% (k=0.613) as compared with Cobas. This difference, however, was rated statistically significant by McNemar's test ($p<0.05$). Since all CIN2+ cases were detected, the difference had not clinical importance.

Analyzing the agreement with Cobas for specific HPV types, a high sensitivity for detection of HPV16 was observed (>90%, k= 0.929). A lower sensitivity for HPV18 detection (54.2%, $\kappa=0.681$, $p<0.05$), and for detection of other 12 HR-types (75.4%, $\kappa=0.677$, $p<0.05$) was observed. Unfortunately, histology information was not available for all HPV DNA Array missed cases. It was observed that although the HPV18 and/or other HR types were missed, this was the case only in multiple infections. Other HPV types present in the infection were detected, and HPV DNA Array did detect the high-risk lesions, so that this analytical difference had little clinical impact. One could suspect that the HPV type missed was not a driver of the infection, however, little information is available in the literature.

The tendency of HPV DNA Array to have a lower agreement for HPV detection when compared with MPG and Cobas, but a very good agreement for detection of cervical intraepithelial neoplasia to the reference assays, could be explained by the higher number of viral copies in such lesions. Cervical intraepithelial neoplasia tends to have larger viral amounts and is, therefore, easier to detect [45]. It is then important that HPV assays which are meant to be used as primary screening tests have the right balance of clinical sensitivity and specificity.

The analytical differences between the assays could possibly exist due to differences in assay design; the PCR target HPV gene (E1 gene for HPV DNA Array, and L1 gene for MPG and Cobas), the use of a multiplexed PCR approach versus a generic primer-based PCR, as well as genotyping ability (full vs. partial genotyping).

When comparing HPV assays, an ideal high agreement is difficult to reach, as shown by Rebolj et al. [71]. In their paper on disagreement between HPV screening tests, Rebolj et al. found 41% concordance for HPV positivity, irrespective of type, among 4 different fully validated FDA approved HPV assays (Hybrid Capture 2, Cobas, CLART and APTIMA). The agreement among assays was even lower in the 30–65-year-old screening population, 29%. When focusing specifically on the Cobas test, which we also used in our study, it was observed that the agreement for HPV positivity, irrespective of type, to HC2, CLART, and APTIMA, varied between 50 and 70%. Cobas was concordant with HC2 in 62% of cases; to CLART in 70%; and to APTIMA in 53% of cases.

Rebolj et al. did not analyze genotype-specific agreement, as in this study, and did not include HPV DNA Array in their comparison. However, agreement of HPV DNA Array with MPG of approx. 90%, and to Cobas of approx. 80%, underlines the good performance of HPV DNA Array for HPV detection.

Samples received from the HERMES study also included cytology results, which allowed a comparison of HPV DNA Array with cytology. This was especially important, as cytology is a well-established and long used screening method. Surprisingly, a low sensitivity of cytology, 13.6%, with only 3 of 22 CIN2+ lesions classified as HGSIL, was found. In comparison, HPV DNA Array had a CIN2+ detection sensitivity of 100%. Although it has been documented that HPV assays are more sensitive for disease detection in

comparison with cytology, [72] such difference was not expected. In previous publications, cytology demonstrated a sensitivity of approx. 60%, however, in this study, it was only 13.6%.

One of the main limitations of the validation studies organized, was the use of historic sample collections, with the advantage that clinical data on the underlying disease were mostly available. Furthermore, the study population consisted of samples collected from women attending the referral clinic, and thus, is not representative of a screening population. This explains the higher number of HPV positive and lesion positive samples than expected, approx. 60-90% within this study vs. approx. 10% within screening studies [70]. Also, samples for the study comparing HPV DNA Array to Cobas test received from the HERMES study panel were selected for validation purposes, explaining the overall higher positivity rate of Cobas (81%) and low specificity in this sample set, than found by Agorastos et al. in a screening population. Within the HERMES study, 12.7% of samples were HPV positive [54].

In addition, the clinical specificities reported for HPV DNA Array, MPG and Cobas, were found to be lower than expected (HPV DNA Array and MPG <40%, and HPV DNA Array and Cobas <10%). As mentioned previously, these were samples obtained from women attending the referral clinic, hence the HPV-/disease- population was underrepresented. Additionally, the samples received from the HERMES study panel were pre-selected for validation purposes and consisted more of HPV positive samples. Therefore, the low specificity, due to a high number of HPV positive, but histologically normal samples, was expected. For further specificity investigation, studies conducted on samples from the screening population are warranted.

An additional study limitation is that the guidelines set by Meijer et al. [45] could not be fully complied with due to the lack of samples from women attending the regular screening. According to Meijer et al., to validate an HPV assay, samples used must be from screening population of women older than 30 years of age; HPV assays should demonstrate a CIN2+ sensitivity and specificity of 90% and 98%, respectively; as well as have a high inter- and intra- laboratory reproducibility.

Setting aside the background of samples used, the sensitivity of HPV DNA Array for detection of CIN2+ in women 30 years of age and older was >90% of the reference assays, as required by the Meijer guidelines. The specificity is below the required 98%, but as mentioned previously, samples from a referral population tend to be more HPV+. Similarly, the 22 number of samples used for the intra- and inter-reproducibility studies is far smaller than the needed 500 samples, but the required kappa agreement value of at least 0.5 is fulfilled. Future studies that will investigate specificity, in addition with the intra- and inter-reproducibility, required by the Meijer guidelines, are warranted.

Despite the limitations, this research work has demonstrated the high potential of HPV DNA Array, and future studies are warranted to complete the validation and investigate the performance of HPV DNA Array in mass screening and potentially in low resource settings.

5. Conclusion

HPV DNA Array demonstrated excellent clinical performance for CIN2+ and CIN3+ lesion detection. It showed good agreement for HPV detection irrespective of type with the competitor assays. Most importantly, it was concordant with MPG and Cobas, with high sensitivity and agreement for the HPV 16, and -18. HPV DNA Array is a sensitive PCR-based assay, with a simple workflow for individual genotype detection with a possibility for automation. It is a full genotyping assay that can be performed as high throughput assay capable of testing up to 96 samples in one run, with automated read-out within three minutes per plate. HPV DNA Array could be considered for extended HPV genotyping of cervical smears and in organized screening programs and potentially in low resource settings.

6. List of Figures

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9. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

10. Affidavit

“I, [Pešić, Aleksandra] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Analytical and clinical evaluation of the HPV DNA Array E1-based genotyping assay]. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

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