



Short communication

Clinical validation of Anyplex™ II HPV HR Detection according to the guidelines for HPV test requirements for cervical cancer screening



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ABSTRACT

Background: Anyplex™ II HPV HR Detection (Seegene, Seoul, Korea) is a multiplex real-time PCR using tagging oligonucleotide cleavage and extension (TOCE) technology for simultaneous detection and genotyping of 14 high-risk (HR) HPV types, including HPV16 and HPV18.

Objectives: To evaluate whether the clinical performance and reproducibility of Anyplex™ II HPV HR Detection meet the international consensus guidelines for HPV test requirements for cervical cancer screening [1].

Study design: The clinical performance of Anyplex™ II HPV HR Detection for detecting cervical intraepithelial neoplasia grade 2 or worse (CIN2+) was determined relative to that of the reference assay, i.e., HR HPV GP5+/6+-PCR-EIA, by analysis of a total of 879 cervical liquid based cytology (LBC) specimens from a screening population, of which 60 were from women with CIN2+. The intra-laboratory reproducibility and inter-laboratory agreement were determined on 509 LBC samples, of which 172 were positive by the reference assay.

Results: Anyplex™ II HPV HR Detection showed a clinical sensitivity for CIN2+ of 98.3% (59/60; 95% CI: 89.1–99.8) and a clinical specificity for CIN2+ of 93.6% (764/816; 95% CI: 89.8–96.1). The clinical sensitivity and specificity were non-inferior to those of HR HPV GP5+/6+-PCR-EIA (non-inferiority score test: $P=0.005$ and $P=0.023$, respectively). Both intra-laboratory reproducibility (96.8%; 95% CI: 95.3–98.1; kappa value of 0.93) and inter-laboratory agreement (96.0%; 95% CI: 94.3–97.4; kappa value of 0.91) were high.

Conclusions: Anyplex™ II HPV HR Detection performs clinically non-inferior to HR HPV GP5+/6+-PCR-EIA. Anyplex™ II HPV HR Detection complies with international consensus validation metrics for HPV DNA tests for cervical cancer screening [1].

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1. Background

HPV testing is increasingly considered for primary cervical cancer screening. To ensure high-quality screening, clinical utility of HPV assays has to be demonstrated. In 2009, an international consortium proposed criteria for assay validation in primary

Abbreviations: TOCE, tagging oligonucleotide cleavage and extension; HR, high-risk; HPV, human papillomavirus; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; LBC, liquid based cytology; BMD, borderline or mild dyskaryosis.

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screening context based on minimal relative clinical accuracy of a given HPV assay compared to a clinically validated reference (i.e., Hybrid Capture 2 or high-risk (HR) HPV GP5+/6+-PCR-EIA), and minimal intra- and inter-laboratory reproducibility [1,2]. Several HPV assays have partially or completely been clinically validated using these criteria [3]. An assay not yet evaluated according to these international consensus validation metrics is Anyplex™ II HPV HR Detection (Seegene, Seoul, Korea). Anyplex™ II HPV HR Detection comprises an automated system from specimen handling to HPV result, including an automated instrument Microlab Nimbus IVD or STARlet (Hamilton) for DNA isolation and real-time PCR setup, subsequent HPV testing with CFX96 PCR instrument (Bio-Rad), and data analysis with Seegene Viewer (Seegene).

The multiplex real-time PCR design using tagging oligonucleotide cleavage and extension (TOCE) technology allows for simultaneous detection and genotyping of 14 high-risk (HR) HPV types, including HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 (L1 gene), and an internal control (human beta-globin) in a single reaction [4].

2. Objective

The current study was set out to determine whether Anyplex™ II HPV HR Detection meets the international guidelines for primary screening [1] by comparing its clinical sensitivity and specificity for cervical intraepithelial neoplasia grade 2 or worse (CIN2+) to that of the reference HR HPV GP5+/6+-PCR-EIA (further referred to as GP5+/6+-PCR), and assessing intra-laboratory reproducibility and inter-laboratory agreement.

3. Study design

3.1. Study population

For clinical sensitivity analysis, a representative set of 60 cervical liquid based cytology (LBC) samples (PreservCyt) from women participating in population-based screening in the Netherlands, who were diagnosed with histologically confirmed CIN2+, were used. These comprised 25 CIN2, 31 CIN3, and 4 squamous cell carcinomas. The median age of the women was 40 years (range 30–60 years). These clinical cases were detected on the basis of an abnormal cytology result and/or a positive result by GP5+/6+-PCR [5]. The samples of 43 (72%) of these women revealed abnormal cytology (5 borderline or mild dyskaryosis (BMD) and 38 >BMD), 17 (28%) had normal cytology, and all but one (98.3%) were positive by GP5+/6+-PCR.

For clinical specificity analysis, we used 819 consecutively collected LBC samples (PreservCyt) from the screening population of women (median age of 41 years, range 31–60 years) with normal cytology and without evidence of CIN2+ within 2 years of follow-up.

Intra-laboratory reproducibility and inter-laboratory agreement of Anyplex™ II HPV HR Detection were evaluated using three equal portions of in total 509 cervical PreservCyt samples, of which one-third was positive by GP5+/6+-PCR (172/509; 33.8%). Two portions were tested in a blinded manner within the same laboratory (Lab A; VU University Medical Center, Amsterdam, The Netherlands) by different technicians at different time points. The third set was analyzed blinded for the results of lab A, at another laboratory (Lab B; Institute of Microbiology, CHUV, University of Lausanne, Lausanne, Switzerland).

All LBC samples were stored in aliquots at -80°C until further use.

3.2. Anyplex™ II HPV HR Detection

Anyplex™ II HPV HR Detection comprising DNA extraction, HPV testing and data analysis, was performed according to the manufacturer's instructions (Seegene, Seoul, Korea). In short, up to 40 clinical samples per run were processed by the automated system with a laboratory working time from specimen handling to HPV result of approximately 6 h (i.e., 2 h for DNA extraction, 3 h 40 min for PCR amplification with three melting analyses, and hands-on time of 15–20 min for specimen handling and 10 min for PCR machine handling).

3.3. Statistical analyses

Anyplex™ II HPV HR Detection was performed blinded for earlier obtained GP5+/6+-PCR-EIA results [5] and cyto-/histopathology outcomes, and data were correlated afterwards. Clinical sensitivity and specificity values for CIN2+ of Anyplex™ II HPV HR Detection were compared to those of GP5+/6+-PCR using a non-inferiority score test, as described by Tang et al. [6], with a relative sensitivity threshold for CIN2+ of 90% and a relative specificity threshold for CIN2+ of 98% as proposed in the consensus guidelines [1]. For intra-laboratory and inter-laboratory analyses, the agreement and kappa values were determined. The 95% lower confidence bounds of the intra-laboratory reproducibility and inter-laboratory agreement values should be $\geq 87\%$, with kappa values of ≥ 0.5 . Only samples with valid test results were included in the analyses. The level of genotype agreement was determined by using kappa statistic. Association between semi-quantitative viral load values (based on signal intensity scores +, ++, or +++), and discordance was evaluated with chi-square test for trend. The level of statistical significance was set at 0.05.

4. Results

4.1. Clinical sensitivity and clinical specificity

Of the 879 cervical LBC samples for clinical sensitivity and specificity analyses, 876 (99.7%) revealed valid results with Anyplex™ II HPV HR Detection. Agreement between Anyplex™ II HPV HR Detection and GP5+/6+-PCR was high. All 60 samples of women with CIN2+ (100%; 95% CI: 94.0–100; cases) and 788 of 816 samples of the specificity set (96.6%; 95% CI: 93.3–98.3; controls) showed an identical outcome with both tests (Table 1).

Table 1
Anyplex™ II HPV HR Detection on 876 cervical LBC samples from population-based screening stratified by case-control status.

Controls (<CIN2)		GP5+/6+-PCR ^b		Total
		HR HPV negative	HR HPV positive	
Anyplex™ II HPV HR Detection ^a	HR HPV negative	752	12	764
	HR HPV positive	16	36	52
	Total	768	48	816
Cases (CIN2+)				
Anyplex™ II HPV HR Detection ^a	HR HPV negative	1	0	1
	HR HPV positive	0	59	59
	Total	1	59	60

^a Input in PCR comprising 1/650 fraction of the cervical scrape.

^b DNA was extracted from cervical scrapes using the Nucleo-Mag 96 Tissue kit (Macherey–Nagel, Germany) and Microlab Star robotic system (Hamilton, Germany) according to manufacturers' instructions. Ultimately, input in PCR comprised 1/400 fraction of the cervical scrape.

Table 2A
Intra-laboratory reproducibility over time of Anyplex™ II HPV HR Detection.

Lab A, result run 1	Lab A, result run 2		Total
	HR HPV negative	HR HPV positive	
HR HPV negative	334	9	343
HR HPV positive	11	151	162
Total	345	160	505

Table 2B
Inter-laboratory agreement of Anyplex™ II HPV HR Detection.

Lab B	Lab A, result run 1 ^a		Total
	HR HPV negative	HR HPV positive	
HR HPV negative	336	8	344
HR HPV positive	8	153	161
Total	344	161	505

^a Similar results were obtained when using results of run 2 from Lab A: agreement of 96.2% (95% CI: 94.6–97.6) with a kappa value of 0.91.

Anyplex™ II HPV HR Detection was positive for 59 women with CIN2+, resulting in a clinical sensitivity for CIN2+ of 98.3% (59/60; 95% CI: 89.1–99.8) (Table 1, cases). The clinical specificity for CIN2+ was 93.6% (764/816; 95% CI: 89.8–96.1) (Table 1, controls). By comparison, these figures were 98.3% (59/60; 95% CI: 89.1–99.8) and 94.1% (768/816; 95% CI: 90.3–96.5), respectively, for GP5+/6+-PCR (Table 1). Both clinical sensitivity and specificity for CIN2+ of Anyplex™ II HPV HR Detection were non-inferior to that of GP5+/6+-PCR, i.e., relative clinical sensitivity for CIN2+ of 1.00 ($P=0.005$) and relative clinical specificity for CIN2+ of 0.99 ($P=0.023$).

4.2. Intra-laboratory reproducibility and inter-laboratory agreement

Of the 509 samples, 506 (99.4%) had a valid test result in run 1 at lab A, 507 (99.6%) in run 2 at lab A, and one sample had insufficient material left for testing at lab B, leaving 505 samples with valid data for both intra- and inter-laboratory analyses. The intra-laboratory reproducibility over time was 96.0% (485/505; 95% CI: 94.3–97.4), with a kappa value of 0.91 (Table 2A).

The inter-laboratory agreement was 96.8% (489/505; 95% CI: 95.3–98.1), with a kappa value of 0.93 (Table 2B).

Both the intra-laboratory reproducibility over time and the inter-laboratory agreement fulfilled the validation metrics given a lower confidence bound of percentage of agreement that was higher than 87%, and a corresponding kappa value that was higher than 0.5 [1]. Most samples with discrepant findings (18/20 in intra-lab and 13/16 in inter-lab analysis) had low signal intensity (score +), suggesting association of discrepant results with low viral loads ($P=0.0008$ and $P=0.007$, respectively).

At the genotype level, moderate to perfect agreement was observed, with overall kappa of 0.87 (range: 0.50–1.00) and 0.89 (range: 0.54–1.00) for intra- and inter-laboratory genotyping agreement, respectively (Tables 3A and 3B). Discrepant results at the type-specific level, that were most commonly found for HPV39 and HPV45, mostly concerned multiple infections and low signal intensities for respective types.

5. Conclusions

In this study, we compared the clinical performance of Anyplex™ II HPV HR Detection with that of GP5+/6+-PCR in a cohort of screening participants. The clinical sensitivity and specificity for CIN2+ of Anyplex™ II HPV HR Detection were non-inferior to those

Table 3A
Intra-laboratory reproducibility of genotype findings of Anyplex™ II HPV HR Detection.

HR HPV type	Number of samples found positive by ^a			Kappa	95%CI
	Both runs	Run 1 only	Run 2 only		
16	49	6	2	0.92	0.86–0.97
18	20	1	3	0.91	0.82–1.00
31	17	2	4	0.84	0.72–0.97
33	12	0	0	1.00	–
35	6	1	0	0.92	0.77–1.00
39	11	6	6	0.64	0.44–0.83
45	3	1	5	0.50	0.15–0.84
51	15	2	1	0.91	0.80–1.00
52	17	1	1	0.94	0.86–1.00
56	14	1	4	0.84	0.71–0.98
58	7	0	3	0.82	0.62–1.00
59	5	1	1	0.83	0.60–1.00
66	12	1	0	0.96	0.88–1.00
68	12	0	3	0.89	0.76–1.00

^a Data are presented as the number of each genotype detected in each run (i.e., run 1 and/or run 2), and numbers do not count up to the total number of HR HPV positive samples due to multiple infections.

Table 3B
Inter-laboratory agreement of genotype findings of Anyplex™ II HPV HR detection.

HR HPV type	Number of samples found positive by ^a			Kappa	95% CI
	Both labs	Lab 1 only	Lab 2 only		
16	51	3	3	0.94	0.89–0.99
18	21	0	2	0.95	0.89–1.00
31	19	0	3	0.92	0.84–1.00
33	11	1	0	0.96	0.87–1.00
35	7	0	0	1.00	–
39	11	5	5	0.68	0.49–0.87
45	3	1	4	0.54	0.18–0.90
51	16	1	1	0.94	0.85–1.00
52	16	2	0	0.94	0.85–1.00
56	14	1	8	0.75	0.59–0.91
58	7	0	2	0.87	0.70–1.00
59	6	0	1	0.92	0.77–1.00
66	12	1	2	0.89	0.76–1.00
68	11	1	2	0.88	0.74–1.00

^a Data are presented as the number of each genotype detected at each location (i.e., laboratory 1 and/or 2), and numbers do not count up to the total number of HR HPV positive samples due to multiple infections.

of GP5+/6+-PCR using the predetermined thresholds of 90% and 98%, respectively, as set out by the international consortium [1]. Furthermore, the assay displayed sufficient intra-laboratory reproducibility and inter-laboratory agreement, both complying with the international consensus validation metrics for HPV DNA tests for cervical cancer screening [1]. Based on our findings, Anyplex™ II HPV HR Detection can be added to the list of HPV assays that fulfill the 2009 guidelines [3].

Anyplex™ II HPV HR Detection adds to some other validated assays [3] that it directly provides genotyping data of 14 HR HPV types. Genotyping data turned out to be highly reproducible in this study as well, with moderate to perfect agreement. Although at present genotyping of non-HPV16/18 types is in the current guidelines not recommended for primary cervical screening, it may be useful in the future for measuring persistence of a specific HR HPV type in certain setting (e.g., post-treatment monitoring of women treated for high-grade CIN).

In conclusion, this study demonstrates that Anyplex™ II HPV HR Detection is clinically non-inferior to GP5+/6+-PCR. The clinical performance and reproducibility of the assay meet the international

criteria for validation of an HPV test for cervical cancer screening purposes [1].

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Ethical approval

This study followed the local ethical guidelines of VU University Medical Center.

Competing interests

ATH and MLS are employees of Self-screen B.V. DAMH and PJFS have minority stake in Self-screen BV, a spin-off company of VU University Medical Center. DAMH has been on the speaker's bureau of Hologic/Gen-Probe and serves occasionally on the scientific advisory boards of AMGEN and Pfizer. JB has received consultancy or speaker fees from Qiagen, Roche, DDL, Merck and GSK. PJFS has

been on the speaker's bureau of Roche, Abbott, Gen-Probe, Qiagen and Seegene. He is consultant for Crucell Holland B.V. All other authors declare that they have no conflict of interest.

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