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Allplex HPV HR Detection assay fulfils all clinical performance and reproducibility validation requirements for primary cervical cancer screening

Anja Oštrbenk Valenčak [a], Kate Cuschieri [b], Linzi Connor [b], Andrej Zore [c,d], Špela Smrkolj [c,d], Mario Poljak [c,*]

[a] Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[b] Scottish HPV Reference Laboratory, Royal Infirmary of Edinburgh, Edinburgh, Scotland, United Kingdom
[c] Division of Gynaecology and Obstetrics, University Medical Centre Ljubljana, Ljubljana, Slovenia
[d] Department of Gynaecology and Obstetrics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

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ABSTRACT

Human papillomavirus (HPV)-based screening offers better protection against cervical cancer compared to cytology, but HPV screening assays must adhere to validation requirements of the international guidelines to ensure optimal performance. Allplex HPV HR Detection (Allplex) assay, launched in the late 2022, is a fully automated real-time PCR-based assay utilizing innovative technology that enables quantification and concurrent distinction of 14 high-risk HPV genotypes (HPV16,18,31,33,35,39,45,51,52,56,58,59,66 and 68). We assessed the validity of the Allplex for cervical cancer screening purposes, via comparison to a clinically validated comparator assay (Hybrid Capture 2; HC2), and through assessment of intra-laboratory reproducibility and inter-laboratory agreement. A clinical validation panel comprised of 973 residual ThinPrep samples was obtained from women aged 30-64 years participating in the organized Slovenian screening program, of these 863 were from women undergoing their regular screening visit after a previous negative screen test while 110 were from women with underlying cervical intraepithelial neoplasia grade 2 or worse (CIN2+) lesions. The Allplex's relative clinical sensitivity for detection of CIN2+ and CIN3+ were 1.01 (95%CI;0.98-1.04) and 0.98 (95%CI;0.95-1.02), compared to that of HC2. At recommended thresholds of ≥98% and ≥90%, the Allplex's clinical sensitivity and specificity (p=0.0004 and p=0.02, respectively) were non-inferior to HC2. High intra-laboratory reproducibility and inter-laboratory agreement, both overall (98.1% and 97.9%, respectively) and at genotype level (>98.7%) was observed. In addition, analytical genotype-specific performance of Allplex was compared to that of its predecessor Anyplex HPV HR; high overall agreement was observed (96.3%; kappa value 0.88), with some variations in performance. In conclusion, Allplex met all validation criteria described in the international guidelines on sensitivity, specificity and laboratory reproducibility and can be considered clinically validated for primary cervical cancer screening.

1. Introduction

Persistent infections with high-risk (hr) human papillomaviruses (HPV) are the causative agents of cervical cancer, the fourth most common cancer in women worldwide[1]. Testing for HPV DNA provides a greater protection against cervical cancer and its immediate precursors, i.e., high-grade (grade 2 or worse) cervical intraepithelial neoplasia (CIN2+), compared to cervical cytology (Pap test), as evidenced by several large-scale randomized clinical trials[2–6] as well as real-world HPV-based cervical cancer screening programs[7–9]. For HPV-based primary cervical cancer screening it is crucial that the HPV assays applied are clinically validated to ensure the best possible distinction between clinically relevant HPV infections associated with CIN2+ and transient HPV infections to prevent unnecessary referral and overtreatment of women. Therefore, HPV screening assays must adhere to validation requirements set by international guidelines for HPV DNA tests[10]. These guidelines warrant a cross-sectional clinical equivalence validation study, which compares the head-to-head clinical

* Corresponding author.
E-mail address: mario.poljak@mf.uni-lj.si (M. Poljak).

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performance of the candidate HPV assay with a reference, comparator HPV assay[10]. Unfortunately, a great majority of commercially available HPV assays on the market lack such validation[11]. In the last published update of HPV assays that are clinically validated for cervical cancer screening, only a few hrHPV DNA assays consistently fulfilled all validation criteria across multiple studies[12], compared to either of the two standard comparator HPV tests: Hybrid Capture 2 HPV DNA Test (HC2; Qiagen, Gaithersburg, MD, USA) and GP5+/6+ PCR EIA. HC2 and GP5+/6+ PCR EIA are accepted as standard comparator HPV tests since randomized trials showed that screening using one of these tests provides superior protection against cervical cancer compared to good quality cytology for at least 5 years[10].

The Allplex HPV HR Detection (Allplex; Seegene, Seoul, South Korea) assay, launched in late 2022, is a fully automated real-time PCR-based assay that surpasses its predecessor Anyplex II HPV HR Detection Test (Anyplex; Seegene) by utilizing multiple detection temperature technology (MuDT), combined with TOCE system that enables detection of multiple targets generating individual cycle threshold (Ct) values in a single channel. Allplex detects 14 hrHPV genotypes (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68) individually and concurrently. It includes an internal control, a human housekeeping gene (beta-globin) which is co-amplified simultaneously with the L1 gene of the targeted HPV genotypes.

The present study aimed to determine if the Allplex fulfilled internationally accepted validation guidelines for cervical cancer screening purposes[10]. Clinical performance of the Allplex was measure through assessment of its clinical sensitivity and specificity, in relation to a clinically validated comparator HPV assay (HC2). Intra-laboratory reproducibility and inter-laboratory agreement were also assessed. To the best of our knowledge, the present study represents the first evaluation of the clinical performance of Allplex as well as its reproducibility. In addition, genotype specific concordance analysis was performed to compare the analytical performance of Allplex versus its predecessor, Anyplex at the individual genotype level.

2. Materials and methods

2.1. Sample selection – clinical specificity

From women aged 30-64 years (median age of 40) attending for national organized cytology-based cervical cancer screening in Slovenia (target population: 20-64 years; three-year screening intervals; 71.4% three-year screening coverage) between January 2016 and May 2017, a total of 863 consecutive residual ThinPrep (Hologic, Marlborough, MA, USA) samples were selected for assessment of the Allplex's clinical specificity (referred here as controls). All women representing controls in this study were screened using both cytology (current standard-of-care in Slovenia) and an HPV test (additionally offered as part of the study; 97% acceptance rate) and had negative screen tests (cytology) three-years before (study inclusion criteria). In this control population with considerable screening history, 840 (97.3%) women had normal cytology (NILM), 16 (1.9%) women had atypical squamous cervical cells of undetermined significance (ASC-US), two women (0.2%) had atypical squamous cervical cells-cannot exclude high-grade lesion (ASC-H), and five women (0.6%) had low-grade squamous intraepithelial lesion (LSIL).

2.2. Sample selection – clinical sensitivity

A total of 110 residual ThinPrep samples obtained from women who had histologically confirmed CIN2+ (52 with underlying CIN2, 55 with underlying CIN3, and 3 with squamous cell cervical cancer) were selected from screening population for assessment of the Allplex's clinical sensitivity (referred here as cases). Women were referred to colposcopy on the basis of either (i) national guidelines where the benchmark is set at ASC-H or worse or (ii) based on HPV16 and/or HPV18 positivity irrespective of cytology findings. The median age of cases was 35 years (range, 30 to 63). Of the 110 cases, 86 (78.2%) had borderline or abnormal cytology: eight (7.3%) of these women had ASC-US, 14 (12.7%) had ASC-H, 11 (10.0%) had LSIL, 50 (45.5%) had high-grade squamous intraepithelial lesion (HSIL) and three (2.7%) women had atypical glandular cells. The remaining 24 (21.8%) cases had NILM.

2.3. Sample selection – reproducibility

To assess Allplex's intra-laboratory reproducibility, a total of 526 original ThinPrep aliquots (158 randomly selected HC2 HPV-positive samples and 368 randomly selected HC2 HPV-negative samples) were retested 60 to 136 days after initial testing at Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia (referred here as UL). For inter-laboratory agreement, the same panel was retested 144 to 231 days after initial testing at the Scottish HPV Reference Laboratory, Royal Infirmary of Edinburgh, Scotland, United Kingdom (referred here as RIE).

2.4. HPV testing

All samples included in the study (total n=973) were originally aliquoted upon arrival at the laboratory within 21 days of collection and stored at -70°C until further testing using three HPV assays.

(i) HC2 testing

HC2 is one of two standard comparators proposed in international guidelines for DNA test requirements for primary cervical cancer screening in women 30 years and older[10] and is currently used as a triage test for borderline and low grade cytology in the Slovenian organized cervical cancer screening program. It is hybridization-based assay and enables aggregate detection of 13 hrHPV genotypes (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68). HC2 was performed within two weeks after sample arrival in the laboratory and digene HC2 sample conversion kit (Qiagen) was used in sample pre-processing following the manufacturer's instructions.

(ii) Anyplex testing

Anyplex is a clinically validated, semi-quantitative PCR-based assay utilizing TOCE technology, that enables individual detection of 14 hrHPV genotypes (13 genotypes targeted by HC2 in addition to HPV66), where L1 region of targeted HPV genotypes is co-amplified simultaneously with a human housekeeping gene (beta-globin) that serves as internal processing control[13–16]. Anyplex testing was performed between November 2022 and January 2023 following the manufacturer's instructions. Briefly, up to 48 samples were processed in one run using Microlab STARlet IVD instrument for DNA extraction and PCR set-up and CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA) for PCR amplification and detection, with turnaround time (TAT) of approximately six hours. Anyplex's catcher melting temperature analysis (CMTA) provides three different readouts; a positive signal in the first CMTA point corresponds to a crossing point (Cq) range of 31 or fewer cycles and is interpreted as "+++" indicating high viral load, a positive signal in the second CMTA point corresponds to a range between 31 to 39 cycles and is interpreted as "++" indicating a medium viral load, whereas a positive signal in the third CMTA point corresponds to more than 40 cycles and is interpreted as "+" indicating a low viral load.

(iii) Allplex testing

Allplex is a quantitative PCR-based assay targeting the same 14

hrHPV genotypes as Anyplex but utilizing different chemistry and results analysis - instead of Anyplex's cyclic CMTA which provides only semi-quantitative readouts. Allplex is based on MuDT technology and generates individual quantitative readouts (Cycle threshold, Ct) for each of the 14 targeted hrHPV genotypes. Allplex's manufacturer performed extensive experiments to determine the limit of detection (LoD) for all 14 targeted HPV genotypes. According to the manufacturer's instructions, LoDs for all targeted HPV genotypes were determined by testing serial dilutions of HPV plasmids into pooled HPV negative cervical specimens collected in ThinPrep solution. LoD for each targeted HPV genotype was estimated by probit analysis. When applying absolute cut-off of Ct≤43 for all 14 targeted HPV genotypes LoDs for HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58 ranged from 1,217 to 5,643 IU/mL and for HPV35, HPV39, HPV51, HPV56, HPV59, HPV66, and HPV68 from 2,515 to 3,941 copies/mL). Similar to Anyplex, up to 48 samples can be processed using the same instruments and pipeline but TAT is significantly shorter (approximately four hours compared with Anyplex). Allplex testing was performed between November 2022 and January 2023 following the manufacturer's instructions (version 09/2022 V1.03_EN), except the use of updated cut-offs (see details below).

2.5. Interpretation of HPV results

For HC2, a sample was considered hrHPV positive if the relative light unit per cut-off (RLU/CO) ratio was higher than 2.50 and hrHPV negative if RLU/CO was lower than 1.00. Samples with RLU/CO ratio between 1.00 and 2.50 were retested and results were interpreted according to manufacturer's instructions. Evaluation of the Anyplex and Allplex HPV results was done based on the interpretation of the assay's software (Seegene Viewer). For Anyplex, no cut-off values were used and all samples exhibiting low (+), medium (++) or high (+++) viral load were considered as hrHPV positive. For Allplex, instead of absolute cut-off of Ct≤43, updated and clinically validated cut-offs provided and recommended by manufacturer were applied for each individual HPV genotype according to Ct values as follows: for HPV16 and HPV18 sample was considered as hrHPV positive at Ct≤40, for HPV31, HPV33, HPV45, HPV52 and HPV58 at Ct≤37 and for HPV35, HPV39, HPV51, HPV56, HPV59, HPV66 and HPV68 at Ct≤35.

2.6. Statistical analysis

(i) Clinical performance & reproducibility assessment

Allplex's clinical performance was assessed against the standard comparator (HC2) by determining whether the performance metrics of relative clinical sensitivity for cases reached at least 90% compared to HC2 and relative clinical specificity for controls reached at least 98% compared to HC2 using non-inferiority score test[10,17]. To assess Allplex's intra-laboratory reproducibility and inter-laboratory agreement, a lower confidence bound of ≥87% and a kappa value of at least 0.5 were used as a threshold[10]. All samples with observed discordant Allplex/HC2 results were also tested with Anyplex.

(ii) Genotype specific concordance analysis

All 973 samples from the clinical validation part were additionally tested with Anyplex to assess genotype specific concordance between two assays by the percent agreement, Cohen's kappa statistic[18] as well as McNemar exact $\chi 2$ test.

All statistical analyses were carried out using Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and R software version 4.2.2 (Free Software Foundation, Boston, MA, USA) and a *p* value below 0.05 was considered significant.

2.7. Ethical aspects

This study was conducted in accordance with the Helsinki Declaration and approved by Medical Ethics Committee of the Republic of Slovenia (consent numbers: 109/08/12).

3. Results

3.1. Clinical performance

A summary of clinical performance of Allplex compared to HC2 is shown in Table 1. Allplex correctly identified 104/110 women with underlying CIN2+, resulting in an absolute clinical sensitivity for CIN2+ of 94.5% (95% confidence interval [CI]; 88.5-98.0%) and correctly identified 55/58 women with underlying CIN3+, resulting in an absolute clinical sensitivity for CIN3+ of 94.8% (95% CI; 85.6-98.9%). These figures were 93.6% (103/110; 95% CI; 87.3-97.4%) for CIN2+ and 96.6% (56/58; 95% CI; 88.1-99.6%) for CIN3+, respectively, for HC2. Compared to HC2, the relative clinical sensitivity of Allplex were 1.01 (95% CI; 0.98-1.04) for CIN2+ and 0.98 (95% CI; 0.95-1.02) for CIN3+ and were non-inferior to that of HC2 (p=0.0004 and p=0.02, respectively). Out of 863 controls, Allplex tested hrHPV negative in 800 samples, resulting in absolute clinical specificity for CIN2+ of 92.7% (95% CI; 90.8-94.3%). These figures were 90.8% (784/863; 95% CI; 88.7-92.7%) for HC2. Allplex's performance was non-inferior to that of HC2 (p<0.00001) with the relative clinical specificity of Allplex versus HC2 of 1.02 (95% CI; 1.01-1.03).

3.2. Intra-laboratory reproducibility and inter-laboratory agreement

Of the 526 samples tested, all had a valid HPV test result in both testing rounds at UL and 3 samples were invalid in initial testing at RIE, but yielded valid results after re-testing. The intra-laboratory reproducibility at UL was 98.1% (516/526; 95% CI; 96.5-99.0%) with the majority of discordant samples (9/10) exhibiting HPV genotype-specific positive amplification signal(s) but exceeding the clinically validated cut-off recommended by the manufacturer. Similarly, inter-laboratory agreement between UL and RIE was 97.9% (515/526; 95% CI; 96.3-98.8%) with 7/11 discordant samples exhibiting signals near the assay's

Table 1
Comparison of Allplex and HC2 results among controls (women without underlying CIN2+ lesions; women with ≤CIN1) and cases (women with histologically confirmed CIN2+ lesions) in 973 samples selected from population-based cervical cancer screening cohort.

Study group and Allplex results	Samples tested by HC2, n (%)		
	Negative	Positive	Total
Controls*			
Negative	779 (90.3%)	21 (2.4%)[a]	800 (92.7%)
Positive	5 (0.6%)[b]	58 (6.7%)	63 (7.3%)
Total	784 (90.8%)	79 (9.2%)	863 (100.0%)
Cases#			
Negative	5 (4.5%)	1 (0.9%)[c]	6 (5.5%)
Positive	2 (1.8%)[d]	102 (92.7%)	104 (94.5%)
Total	7 (6.4%)	103 (93.6%)	110 (100.0%)

* Defined as women without underlying CIN2+ lesions, women with ≤CIN1.
\# Defined as women with histologically confirmed CIN2+ lesions.
[a] Samples which were HC2 positive/Allplex negative; 14 defined as Anyplex true positive [HPV31 (n=2), HPV39 (n=1), HPV51 (n=3), HPV52 (n=1), HPV58 (n=1), HPV59 (n=2), HPV68 (n=2), HPV18 and HPV39 (n=1), HPV51 and HPV56 (n=1)]. Seven samples defined as Anyplex true negative.
[b] Samples which were HC2 negative/Allplex positive; all 5 defined as Anyplex true positive [HPV16 (n=3), HPV18 (n=1), HPV51 (n=1)].
[c] Sample which was HC2 positive/Allplex negative; sample defined as Anyplex true positive [HPV31 (n=1)].
[d] Samples which were HC2 negative/Allplex positive; both defined as Anyplex true positive [HPV16 (n=1), HPV18 (n=1)].

pre-defined cut-off. With kappa values of 0.95 (95% CI; 0.93-0.98) and 0.95 (95% CI; 0.92-0.98), respectively, both validation metrics (i.e. lower confidence bound and kappa value) were above the requirements set out in the international guidelines[10].

3.3. Allplex reproducibility at the HPV genotype level

Allplex intra-laboratory and inter-laboratory reproducibility at the HPV genotype level was also assessed and results are summarized in Table 2 and Table 3.

Two rounds of intra-laboratory reproducibility testing performed at UL showed high overall agreement at the genotype level, ranging from 98.7-100.0% with kappa values consistently above 0.84 for 13/14 targeted HPV genotypes, indicating almost perfect agreement, except for HPV68 which showed a kappa value of 0.58, indicating moderate agreement for this particular genotype. The majority of discordant results were observed in samples with detectable HPV DNA but with Ct values exceeding the clinically validated cut-off recommended by the manufacturer (21/24) and no statistically significant difference was noted at the genotype level by McNemar test (all p values > 0.05).

Two rounds of inter-laboratory reproducibility testing performed at UL and RIE also showed high overall genotyping agreement for all 14 HPV targeted genotypes ranging from 99.0-100% as shown in Table 3. Apart from HPV68, where the kappa value was 0.66 indicating substantial agreement, kappa values for other 13 HPV genotypes were all above 0.88, indicating almost perfect agreement. The great majority of discordant results were noted in samples with detectable HPV but with Ct values exceeding the assay cut-off (17/23), and there was no statistically significant difference found at the genotype level according to the McNemar test (all p values > 0.05).

Table 4 shows the genotype specific concordance between Allplex and Anyplex at the individual HPV level assessed at UL on 973 samples originating from clinical validation part of the present study. The overall genotype agreement between two assays was high (96.3%; 95% CI; 94.9-97.3%) with kappa value of 0.88; however, McNemar test indicated a statistically significant difference in overall genotype detection ($p<0.0001$). The same applies for detection of three specific genotypes: HPV31, HPV51, and HPV68 (p values 0.002, 0.02, and 0.008, respectively). Observed agreement across all individual genotypes was consistently high (all above 98.5%), with kappa values indicating substantial agreement for HPV56, HPV59, HPV66, and HPV68 (range 0.66-0.75) and almost perfect agreement for the remaining HPV genotypes: HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, and HPV58) (range 0.88-1.00).

4. Discussion

Allplex, launched in late 2022, is the first commercial HPV assay which allows quantitative simultaneous individual detection of 14 hrHPV genotypes in a single reaction, utilizing improved MuDT technology and with a significantly shorter TAT than its predecessor, Anyplex. In this study, we evaluated the Allplex assay following guidelines for HPV DNA test requirements for primary cervical cancer screening in women 30 years and older[10]. To the best of our knowledge, we are the first to show evidence of the clinical performance and reproducibility of Allplex and based on study findings, Allplex can be considered suitable for primary cervical cancer screening purposes.

In this study, we compared the Allplex's clinical performance to one of the accepted standard comparator HPV tests – HC2. Although HC2 has lower analytical sensitivity than PCR-based assays, several studies have shown that the clinical sensitivity of HC2 is indistinguishable from clinically validated PCR-based assays[12]. However, HC2 has a slightly lower clinical specificity, mainly due to its cross-reactivity with certain non-targeted low-risk HPV genotypes[12,19]. When compared to the HC2 in this study, Allplex demonstrated non-inferior clinical sensitivity for CIN2+ ($p=0.0004$) and correctly identified 104 out of 110 women with underlying CIN2+ lesion. Allplex's non-inferior clinical sensitivity was also confirmed for CIN3+ ($p=0.02$), with Allplex correctly identifying 55 out of 58 women with an underlying CIN3+ lesion. Furthermore, Allplex demonstrated high clinical specificity in a screening population, which was non-inferior to the HC2 ($p<0.00001$).

Allplex also fulfilled the guideline requirements concerning the intra-laboratory and inter-laboratory reproducibility, with overall agreements of 98.1% and 97.9%, respectively and kappa values of 0.95, suggesting robust and reliable test performance. Although reproducibility of detection of the individual targeted HPV genotypes is neither required in the validation guidelines nor usually reported[20], we assessed it in the present study. This aspect of the assay might become crucial if HPV genotype information beyond HPV16 and HPV18 is incorporated into screening or clinical practice to inform management, as suggested in some recent studies[21,22]. High analytical concordance for all targeted individual HPV genotypes except HPV68, further

Table 2
Intra-laboratory reproducibility of Allplex at the HPV genotype level assessed at UL on 526 samples.

HPV genotype	Intra-laboratory reproducibility					Kappa value (95% CI)	McNemar
	+/+[a]	+/- [b]	-/+ [c]	-/- [d]	Overall agreement (95% CI)		
HPV16	56	2	0	468	99.6 (98.6-99.9)	0.98 (0.95-1.00)	0.48
HPV18	9	1	0	516	99.8 (98.9-100.0)	0.95 (0.84-1.00)	1.00
HPV31	32	0	3	491	99.4 (98.3-99.8)	0.95 (0.90-1.00)	0.25
HPV33	15	0	1	510	99.8 (98.9-100.0)	0.97 (0.90-1.00)	1.00
HPV35	5	0	0	521	100.0 (99.3-100.0)	1.00	NA
HPV39	12	2	0	512	99.6 (98.6-99.9)	0.92 (0.81-1.00)	0.48
HPV45	7	0	0	519	100.0 (99.3-100.0)	1.00	NA
HPV51	14	1	4	507	99.0 (97.8-99.6)	0.84 (0.71-0.98)	0.37
HPV52	10	0	1	515	99.8 (98.9-100.0)	0.95 (0.86-1.00)	1.00
HPV56	6	0	0	520	100.0 (99.3-100.0)	1.00	NA
HPV58	8	1	0	517	99.8 (98.9-100.0)	0.94 (0.82-1.00)	1.00
HPV59	5	0	1	520	99.8 (98.9-100.0)	0.91 (0.73-1.00)	1.00
HPV66	9	0	0	517	100.0 (99.3-100.0)	1.00	NA
HPV68	5	2	5	514	98.7 (97.3-99.4)	0.58 (0.27-0.89)	0.45
hrHPV	153	5	5	363	98.1 (96.5-99.0)	0.95 (0.93-0.98)	1.00

[a] sample positive for particular HPV genotype in both round of intra-laboratory reproducibility testing;
[b] sample positive for particular HPV genotype in the first round of intra-laboratory reproducibility testing but negative in the second round;
[c] sample negative for particular HPV genotype in the first round of intra-laboratory reproducibility testing but positive in the second round;
[d] sample negative for particular HPV genotype in both round of intra-laboratory reproducibility testing;
CI, confidence interval; NA, not applicable.

Table 3
Inter-laboratory agreement of Allplex at the HPV genotype level assessed at UL and RIE on 526 samples.

HPV genotype	Inter-laboratory agreement					Kappa value (95% CI)	McNemar
	+/+[a]	+/- [b]	-/+ [c]	-/- [d]	Overall agreement (95% CI)		
HPV16	56	2	1	467	99.4 (98.3-99.8)	0.97 (0.94-1.00)	1.00
HPV18	9	1	0	516	99.8 (98.9-100.0)	0.95 (0.84-1.00)	1.00
HPV31	32	0	2	492	99.6 (98.6-99.9)	0.97 (0.92-1.00)	0.48
HPV33	15	0	1	510	99.8 (98.9-100.0)	0.97 (0.90-1.00)	1.00
HPV35	5	0	0	521	100.0 (99.3-100.0)	1.00	NA
HPV39	12	0	2	512	99.6 (98.6-99.9)	0.92 (0.81-1.00)	0.48
HPV45	7	0	1	518	99.8 (98.9-100.0)	0.93 (0.80-1.00)	1.00
HPV51	15	0	4	507	99.2 (98.1-99.7)	0.88 (0.76-1.00)	0.13
HPV52	10	0	1	515	99.8 (98.9-100.0)	0.95 (0.86-1.00)	1.00
HPV56	6	0	0	520	100.0 (99.3-100.0)	1.00	NA
HPV58	8	1	1	516	99.6 (98.6-99.9)	0.89 (0.73-1.00)	1.00
HPV59	5	0	0	521	100.0 (99.3-100.0)	1.00	NA
HPV66	9	0	1	516	99.8 (98.9-100.0)	0.95 (0.84-1.00)	1.00
HPV68	5	2	3	516	99.0 (97.8-99.6)	0.66 (0.37-0.96)	1.00
hrHPV	154	4	7	361	97.9 (96.3-98.8)	0.95 (0.92-0.98)	0.55

[a] sample positive for particular HPV genotype at UL and RIE in inter-laboratory agreement testing;
[b] sample positive for particular HPV genotype at UL in inter-laboratory agreement testing but negative at RIE;
[c] sample negative for particular HPV genotype at UL in inter-laboratory agreement testing but positive at RIE;
[d] sample negative for particular HPV genotype at UL and RIE in inter-laboratory agreement testing;
CI, confidence interval; NA, not applicable.

Table 4
Genotype specific concordance between Allplex and Anyplex at the individual HPV level assessed at UL on 973 samples originating from clinical validation part of the present study.

HPV genotype	Genotype agreement (Allplex/Anyplex)					Kappa value (95% CI)	McNemar
	+/+[a]	+/- [b]	-/+ [c]	-/- [d]	Overall agreement (95% CI)		
HPV16	59	0	4	910	99.6 (98.9-99.8)	0.97 (0.93-0.99)	0.13
HPV18	10	0	2	961	99.8 (99.3-99.9)	0.91 (0.78-1.00)	0.48
HPV31	33	1	14	925	98.5 (97.5-99.1)	0.81 (0.71-0.90)	0.002
HPV33	16	0	1	956	99.9 (99.4-100.0)	0.97 (0.91-1.00)	1.00
HPV35	6	0	0	967	100.0 (99.6-100.0)	1.00	NA
HPV39	14	0	2	957	99.8 (99.3-99.9)	0.93 (0.84-1.00)	0.48
HPV45	6	0	1	966	99.9 (99.4-100.0)	0.92 (0.77-1.00)	1.00
HPV51	17	0	7	949	99.3 (98.5-99.7)	0.83 (0.70-0.95)	0.02
HPV52	14	0	2	957	99.8 (99.3-99.9)	0.93 (0.84-1.00)	0.48
HPV56	6	0	4	963	99.6 (98.9-99.8)	0.75 (0.50-0.99)	0.13
HPV58	8	0	3	962	99.7 (99.1-99.9)	0.84 (0.66-1.00)	0.25
HPV59	5	0	5	963	99.5 (98.8-99.8)	0.66 (0.37-0.96)	0.07
HPV66	8	1	6	958	99.3 (98.5-99.7)	0.69 (0.46-0.92)	0.13
HPV68	9	0	9	955	99.1 (98.3-99.5)	0.66 (0.44-0.88)	0.008
hrHPV	166	1	35	771	96.3 (94.9-97.3)	0.88 (0.84-0.92)	<0.0001

[a] sample positive for particular HPV genotype with Allplex and Anyplex;
[b] sample positive for particular HPV genotype with Allplex but negative with Anyplex;
[c] sample negative for particular HPV genotype with Allplex but positive with Anyplex;
[d] sample negative for particular HPV genotype with Allplex and Anyplex;
CI, confidence interval; NA, not applicable.

confirmed the Allplex's robustness. Notably, the great majority of the discordant genotyping results were observed in samples with a positive HPV result but with Ct values near assay cut-off, where greater variability of the test result is expected. Nonetheless, high reproducibility of Allplex at the level of individual HPV genotype was observed in the present study, representing important additional value of this full-range genotyping assay with clinically validated cut-offs for risk-based stratification of women within cervical cancer screening programs.

Genotype specific concordance analysis of Allplex and its predecessor Anyplex revealed some differences in detection capability of individual HPV genotypes. Although high overall agreements between two assays were observed for majority of HPV genotypes, statistically significant differences were observed in assays performance for HPV31, HPV51, and HPV68. To clarify reasons behind significant HPV genotype discordance between Allplex and Anyplex observed for HPV31, HPV51 and HPV68, analytical sensitivity of both assays was determined by testing octuplicates of 5-fold dilutions of the World Health Organization (WHO) International Standards for 12 HPV genotypes classified as high-risk HPV genotypes according to International Agency for Research on Cancer (IARC): HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58, HPV35, HPV39, HPV51, HPV56, and HPV59, starting from 250 IU per reaction. This head-to-head comparison showed similar analytical sensitivity of Allplex and Anyplex across all 12 IARC high-risk HPV genotypes when absolute cut-offs of Ct≤43 and (+), respectively, were applied for interpretation of results. However, when Allplex's clinically validated cut-offs recommended by manufacturer were applied (Ct≤40 for HPV16 and HPV18, Ct≤37 for HPV31, HPV33, HPV45, HPV52 and HPV58 and Ct≤35 for HPV35, HPV39, HPV51, HPV56, and HPV59) analytical sensitivity of Allplex and Anyplex remained similar only for HPV16 and HPV18, but evidently different for the other 10 IARC high-risk HPV genotypes, resulting in statistically significant differences in analytical sensitivity between the two assays for HPV31 and HPV51

(IARC high-risk genotypes) as well as for HPV68 in this study (Table 4). Among 14 HPV31 Allplex negative/Anyplex positive samples Allplex's Ct values for HPV31 ranged from 37.62 to 41.72 and in seven HPV51 Allplex negative/Anyplex positive samples Allplex's Ct values for HPV51 ranged from 36.10 to 41.03 (Table 4). A similar observation of higher analytical sensitivity of Anyplex over Allplex when Allplex's clinically validated cut-offs recommended by manufacturer were applied for interpretation of results was observed also for HPV33, HPV39, HPV45, HPV52, HPV56, HPV58 and HPV59, although these differences did not reach statistical significance in our study (Table 4).

To the best of our knowledge, we are the first to provide evidence on the genotype-specific performance of Allplex, an assay primarily intended for cervical cancer screening purposes. In a recent study, Bell et al. evaluated a companion assay (Seegene Allplex HPV28 assay) with three other established HPV assays on 114 mocked self-collected semicervical samples and showed comparable analytical performance of all four evaluated assays: Roche Cobas 4800 HPV assay, Abbott RealTi*m*e HR HPV, Seegene Anyplex II HPV28 and Allplex HPV28 assay[23]. However, although Allplex and Allplex HPV28 assays share same MuDT technology, Allplex HPV28 is not intended for cervical cancer screening due to unadjusted analytical sensitivity and specificity (Allplex has artificially reduced sensitivity when applying clinically validated cut-offs) and a HPV genotype coverage that is not suitable for screening purposes. Also for Anyplex, Allplex's predecessor, only scarce published data on genotype-specific performance is available. Anyplex's intra-laboratory reproducibility and inter-laboratory agreement at the HPV genotype level were assessed in 2016 when moderate to perfect agreement was observed with discrepant results most commonly found for HPV39 (kappa values 0.64 and 0.68, respectively) and for HPV45 (kappa values 0.50 and 0.54, respectively)[15]. For the majority of targeted HPV genotypes almost perfect agreement was observed, with an overall kappa of 0.87 and 0.89, respectively. Most discordant results were associated with samples from women with multiple HPV infections (simultaneous infection with different HPV genotypes) and those with low HPV loads[15].

In conclusion, a new to market HPV assay, which provides genotype specific resolution of all established high-risk genotypes, the Allplex, met all validation criteria set forth in the international guidelines for HPV DNA test requirements for primary cervical cancer screening in women 30 years and older[10]. It can thus be considered clinically validated for primary cervical cancer screening.

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CRediT authorship contribution statement

Anja Oštrbenk Valenčak: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Kate Cuschieri:** Conceptualization, Methodology, Writing – review & editing. **Linzi Connor:** Investigation, Methodology, Writing – review & editing. **Andrej Zore:** Conceptualization, Investigation, Writing – review & editing. **Špela Smrkolj:** Conceptualization, Investigation, Writing – review & editing. **Mario Poljak:** Conceptualization, Methodology, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

AOV has received reimbursement of travel expenses for attending conferences and honoraria for speaking from Abbott Molecular, Qiagen and Seegene. MP's and AOV's institution received research funding, free-of-charge reagents, and consumables to support research in the last 3 years from Qiagen, Seegene, Abbott, and Roche, all paid to their employer. KC & LC's institution received research funding, free-of-charge reagents, and consumables to support research in the last three years from Cepheid, Euroimmun, GeneFirst, Self-screen, Hiantis, Seegene, Roche, Abbott, Hologic, Vaccitech and Daye, all paid to their employer. AZ and ŠS declare no conflicts of interest.

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