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RESEARCH ARTICLE

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Intra- and interlaboratory reproducibility of the RIATOL qPCR HPV genotyping assay

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Abstract

The implementation of cervical screening based on human papillomavirus (HPV) continues to progress rapidly across countries. Evidence has shown that assays detecting high-risk human papillomavirus (hrHPV) deoxyribonucleic acid (DNA) are more effective than cytology-based screening. Validation of new hrHPV DNA assays requires both noninferior clinical accuracy compared to a standard comparator for cervical precancer and good reproducibility. This study builds upon previous diagnostic accuracy assessments of the RIATOL HPV genotyping qPCR assay and aims to evaluate the international validation criteria for reproducibility. The intra- and interreproducibility of the RIATOL-qPCR assay were assessed using 550 remnant cervical cell material from the cytology archive of the National Reference Center for HPV in Belgium. Specimens were collected in the context of cervical cancer screening and tested in two different laboratories. The international reproducibility criteria include the lower bound of 95% confidence interval of the intra- and interlaboratory agreement regarding the detection of hrHPV DNA exceeding 87% with kappa ≥0.50. The RIATOL-qPCR assay demonstrated excellent intralaboratory reproducibility, achieving an overall agreement of 98.2 (95% CI

Abbreviations: AML, Algemeen Medisch Laboratorium; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; DNA, deoxyribonucleic acid; HC2, Hybrid Capture 2; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; PCR, polymerase chain reaction; VALGENT, validation of HPV genotyping tests.

Sharonjit K. Dhillon and Pui Yan Jenny Chung are co-first authors.

96.6–99.1%) and a kappa of 0.96. Interlaboratory testing showed an overall agreement of 98.5 (95% CI 97.1–99.4%) with a kappa of 0.97. The RIATOL-qPCR assay fulfills the third criterion for HPV test reproducibility requirement for use in cervical cancer screening.

KEYWORDS
cervical cancer, HPV genotyping, human papillomavirus, RIATOL, test validation

1 | BACKGROUND

Cervical cancer remains a public health problem with over 604 000 new cases and 341 000 deaths globally in 2020.[1] Mass population-level screening is essential as early detection and effective treatment offer better prognosis and outcomes than advanced-stage cancer. Robust evidence shows that high-risk human papillomavirus (hrHPV) based screening is more effective in reducing the incidence of cervical precancer and invasive carcinoma compared to cytology-based screening.[2,3] These findings have led to the rapid development of diagnostics focused on the molecular detection of human papillomavirus (HPV) deoxyribonucleic acid (DNA) and the proliferation of HPV assays in the market. A recent review published in 2020 found more than 250 distinct HPV assays on the global market with over 90% of assays lacking evaluation and proper validation but only a few of them are clinically validated.[4,5]

Clinical validation of HPV assays based on established international guidelines is essential to ensure that only validated assays be used in cervical cancer screening and diverse clinical settings. The guidelines require that novel HPV assays demonstrate noninferior accuracy (i.e., sensitivity and specificity) against a standard comparator test to detect cervical intraepithelial neoplasia grade 2 or worse (CIN2+). Additionally, the assay should be robust and have a highly reliable performance by displaying high intra- and interlaboratory reproducibility.[6]

The RIATOL HPV genotyping qPCR assay (herein referred to as "RIATOL-qPCR") is a multiplex real-time polymerase chain reaction (PCR) that detects viral HPV DNA using multiplex probe amplification technology and has been extensively used in opportunistic screening in combination of cytology for more than 15 years. The RIATOL-qPCR assay is capable of detecting 14 hrHPV types separately, distributed in eight distinct PCR reactions covering three different fluorescence channels.[7]

The accuracy of RIATOL-qPCR assay (Algemeen Medisch Laboratorium [AML]) for CIN2+ compared to the standard comparator test, Hybrid Capture 2 (HC2) has been demonstrated in two validation studies: one in an in-house study following the Meijer protocol suggested by the international validation guidelines.[8] and another in the VALGENT-3 framework.[9] The first validation study also assessed the intralaboratory reproducibility.[8] However, the interlaboratory reproducibility performance of the assay is yet to be evaluated. Building upon previous evaluations of diagnostic accuracy assessments, this study aims to assess both the intra- and interlaboratory reproducibility of the RIATOL-qPCR assay using relevant clinical samples from a screening population.

2 | METHODS

2.1 | Study population and protocol

Remnant material of cervical cells collected between October 2022 and December 2022 within the context of opportunistic cervical cancer screening in Belgium was prospectively used to assess the intra- and interreproducibility of the RIATOL-qPCR assay. Archived samples were stored in a liquid-based cytology medium (PreservCy, Hologic) according to the manufacturer's recommendations in agreement with European guidelines.[10] All samples were anonymised before processing and only patients who had not opted out for the use of samples for scientific research were included. This opt-out procedure was ethically approved by the Ethical Review Board of Ghent University and Ghent University Hospital (Belgium, ONZ-2022-0171). To assure the comparability of the data set according to the international guidelines,[6] a total of 550 samples were initially tested with the Abbott RealTime hrHPV m2000 assay (Abbot abbreviated RealTime HPV) The RealTime HPV assay is a validated high-risk HPV DNA assay proposed in a recent review as an acceptable new standard comparator test (data set here named: AML repro 1). Since validation guidelines determine that reproducibility panels should contain 30% of hrHPV-positive samples determined with a standard comparator test,[6] the study population contained 163 RealTime HPV-positive specimens. Three aliquots of the primary samples were separately subjected to the RIATOL-qPCR assay, that is, two aliquots were tested within the same laboratory (AML), while the third aliquot was tested in a different laboratory (Ghent University Hospital). Figure 1 provides an overview of the study setup implemented.

2.2 | Sample processing and HPV detection

Processing of the samples was performed in batches of 91 samples, as previously described.[7,9] Briefly, DNA extraction was performed using the GenFind DNA Extraction kit (Hologic) either by the

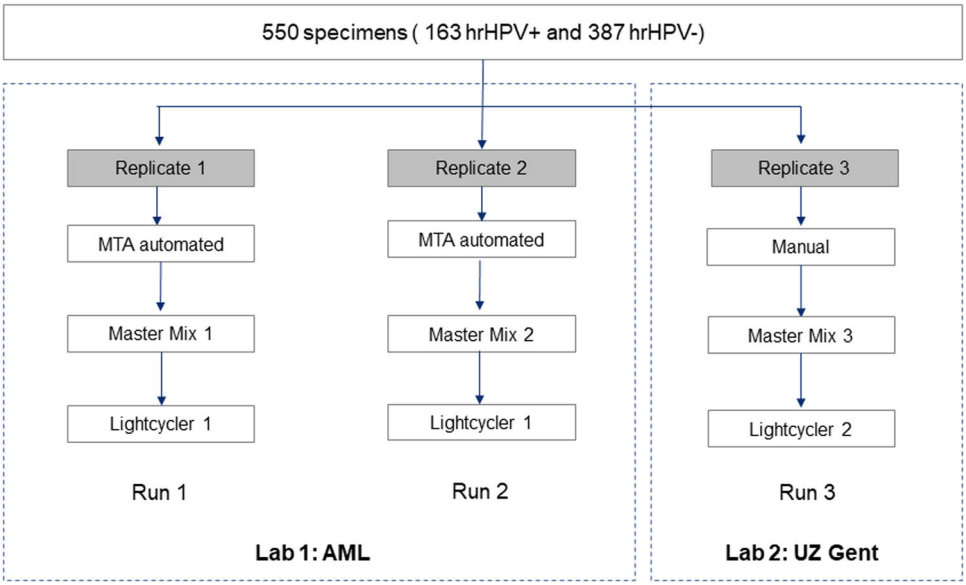

FIGURE 1 Reproducibility study experimental flowchart. 550 PreservCyt cervical samples were extracted in triplicate using the GenFind deoxyribonucleic acid Extraction kit (Hologic) either by the automated Cervista Medium Throughput Automation system (in laboratory 1, Algemeen Medisch Laboratorium [AML]) or manually (in laboratory 2, University Hospital [UZ] Gent). Intralaboratory reproducibility was evaluated by comparison of real-time polymerase chain reaction (RT-PCR) results between run 1 and run 2. Likewise, interlaboratory reproducibility was evaluated by comparison of PCR results between run 1 and run 3.

automated Cervista (Medium Throughput Automation) system (in AML laboratory) or manually (in Ghent University Hospital laboratory), according to the manufacturer's instructions. The extracted DNA was amplified in a total volume of 6 µL using a TaqMan-based real-time PCR targeting type-specific sequences of viral genes (HPV16 E7, HPV18 E7, HPV31 E6, HPV33 E6, HPV35 E6, HPV39 E7, HPV45 E7, HPV51 E6, HPV52 E7, HPV56 E7, HPV58 E6, HPV59 E7, HPV66 E6, HPV68 E7) on the LightCycler 480 (Roche Molecular Systems). In addition, human beta-globin gene detection was used as endogenous cellular quality control for each sample. Human DNA and virus concentrations were calculated based on standard curves generated by serial dilutions of type-specific synthetic gene constructs (gBlocks, Integrated DNA Technologies). Results were reported as hrHPV negative, hrHPV positive or invalid. A sample was considered analytically HPV negative if none of the 14 hrHPV tests showed a positive signal and the beta-globin DNA concentration was above 0.12 ng/µL. HPV positivity was defined using clinically optimized cut-offs (i.e., log10 of the viral concentration is above 6.493 copies/mL).[9] Invalid samples with a human DNA concentration below 0.12 ng/µL were excluded from the statistical analysis. Virologists performing each run were blinded to the results of the other runs.

2.3 | Statistical analysis

The statistical analysis of the reproducibility was restricted to the dichotomous results (HPV positive or negative). Reproducibility was assessed for positivity of overall hrHPV, HPV16, HPV18, and the aggregate of 12 other hrHPV types. The level of detail of HPV genotyping was restricted to the genotyping capacity of the Realtime comparator test. Samples that showed inadequate sample quality in at least one of the experiments were excluded from further analysis. The threshold of acceptable reproducibility was defined as a lower bound of the 95% confidence interval (CI) of ≥87% and a kappa value of >0.5 as recommended in international guidelines.[6] Ninety-five percent exact CIs were calculated for all proportions. The level of statistical significance was set at 0.05. Statistical analysis was performed using STATA version 16.

3 | RESULTS

From the 550 samples, one sample had a Ct value > 35 for the internal gene control for the Abbott m2000 assay resulting in a total of 549 adequate samples that were used for the RIATOL-qPCR reproducibility assessment. Of the 549 samples, an additional five samples (<1% of total samples) had a value of <0.12 ng/µL for the beta-globin gene assessed on the RIATOL-qPCR (one sample invalid during testing run 1 and run 2 at AML [sample 177] and five samples [samples 37, 44, 58, 145, and sample 177] were invalid during testing at UZ Ghent). In total, there were 544 matched samples with valid RIATOL-qPCR results in all runs. Table 1 summarizes findings regarding DNA concentration, expressed in ng/µL, of the samples that were invalid in at least one run and in either of the two laboratories.

Assessment of the intralaboratory at the AML laboratory resulted in 167 and 367 samples that were concordantly hrHPV

TABLE 1 Total DNA concentration (ng/μL) and presence of hrHPV (neg/pos) in five samples that were invalid in at least one run.

ID sample	AML run 1	AML run 2	UZ Ghent run
AML Repro 1/37	573, neg	192, neg	0.00
AML Repro 1/44	583, neg	169, neg	0.00
AML Repro 1/58	424, neg	141, neg	0.00
AML Repro 1/145	179, neg	67.9, neg	0.00
AML Repro 1/177	0.00	0.00	0.00

Abbreviations: AML, Algemeen Medisch Laboratorium; DNA, deoxyribonucleic acid; hrHPV, high-risk human papillomavirus; UZ, University Hospital.

TABLE 2 Intralaboratory and interlaboratory reproducibility of RIATOL-qPCR.

Intralaboratory analysis[a]			
	AML run 1		
AML run 2	Positive	Negative	Total
Positive	167	2	169
Negative	8	367	375
Total	175	369	544
Interlaboratory analysis[b]			
	AML run 1		
UZ Ghent run	Positive	Negative	Total
Positive	169	2	171
Negative	6	367	373
Total	175	369	544

Abbreviations: AML, Algemeen Medisch Laboratorium; CI, confidence interval; HPV, human papillomavirus; UZ, University Hospital.

[a]Overall HPV test agreement: 98.2 (95% CI 96.6%–99.1%); Kappa: 0.958 (95% CI 0.931–0.984).

[b]Overall HPV test agreement: 98.5 (95% CI 97.1%–99.4%); Kappa: 0.966 (95% CI 0.943–0.989).

positive and negative, respectively. Discordant results were noted in 10 specimens (8+/− and 2−/+). The overall agreement was 98.2% (95% CI = 96.6–99.1%) with a kappa value of 0.96 (95% CI = 0.93–0.98). Results from the interlaboratory assessment showed 169 and 367 samples that were concordantly hrHPV positive and negative, respectively. Discordant results were noted in eight specimens (6+/− and 2−/+) yielding an overall agreement of 98.5% (95% CI = 97.1%–99.4%) and a kappa value of 0.966 (95% CI = 0.94–0.99). Results of the reproducibility assessment for the RIATOL-qPCR are shown in Table 2.

At the genotype-specific level, the agreement in genotyping for HPV 16, HPV 18, and 12 other hrHPV was excellent for the interlaboratory run. Similar results were seen for the intralaboratory run. Results are summarized in Tables 3 and 4.

4 | DISCUSSION AND CONCLUSION

The RIATOL-qPCR assay showed an excellent hrHPV test result reproducibility of 98.2 (95% CI 96.6–99.1%) and a kappa of 0.958 for the intralaboratory assessment. The hrHPV interlaboratory reproducibility was 98.5 (95% CI 97.1–99.4%) with a kappa of 0.966. The results for both intra and interlaboratory assessments were clearly above the acceptance criteria (with lower confidence bound around the reproducibility not <87% and a kappa >0.5.

A recent publication by Cusheiri et al.[11,12] highlighted the importance of sample adequacy in HPV-based screening. Findings concerning invalid samples in our study showed the number of invalid samples was higher in the second laboratory UZ Ghent (0.91% compared to 0.18% in AML) where manual extraction was performed. These findings may suggest better performance of automated extraction although other explanatory factors such as sample degradation during transportation cannot be excluded. While a proportion of 1% may appear low, when considering the screening of 100 000 women, it would necessitate 1000 women to undergo retesting with potential dropout. This could result in lower valid participation rates and higher healthcare costs. Therefore, it is essential that steps are taken to minimize the risk of invalid results through quality control measures, proper sample collection and processing and adherence to established validation protocols.[13]

As the global shift toward using HPV testing as the primary cervical cancer screening tool continues, there is a need to increase awareness of the importance of proper validation of HPV assays. To achieve the World Health Organization's proposed goal of eliminating cervical cancer by 2030, only high-quality and clinically validated HPV assays should be used for cervical cancer screening efforts.[5] The accuracy of RIATOL-qPCR genotyping assay has been previously evaluated in two validation studies where one study also assessed the intralaboratory reproducibility. In the first in-house validation study using the Meijer protocol, the relative sensitivity and specificity for CIN2+ of RIATOL-qPCR compared to HC2 was 1.12 ([95% CI = 1.01–1.23] [pn. inf< 0.0001]) and 1.01 ([95% CI = 0.99–1.03] [pn. inf< 0.0001]), respectively. The intralaboratory reproducibility showed an overall agreement of 98.7% and a kappa value of 0.96. In the second validation study using the VALGENT-3 framework, a clinical cut-off was identified as a priori purpose. In the study, the application of a cut-off of 6.493 copies/mL (as clinically assessed in Benoy et al.[9]) resulted in noninferior sensitivity for CIN2+ (1.00 [95% CI = 0.95-1.05 pn. inf=0.0006]) and relative a specificity of 1.00 (95% CI = 0.98–1.01) (pn. inf=0.0069) for the detection of ≤CIN1. Both studies showed that the RIATOL-qPCR assay fulfilled the international accuracy criteria for primary cervical cancer screening but lacked interreproducibility assessment. This study addresses the reproducibility criteria requirement and hence completes all necessary clinical validation steps for HPV assay implementation in a routine setting. Hereby, the RIATOL qPCR assay may be added to the

TABLE 3 Intralaboratory genotype-specific level agreement of RIATOL-qPCR.

HPV type	−/−[a]	+/+[a]	−/+[a]	+/−[a]	General agreement (95% CI)	Kappa (95% CI)
Intralaboratory analysis						
HPV 16	512	31	1	0	99.8% (99.0%–100.0%)	0.98 (0.95–1.00)
HPV 18	537	7	0	0	100.0% (99.3%–100.0%)	1.00 (1.00–1.00)
Other hrHPV[b]	394	139	3	8	98.0% (96.4%–99.0%)	0.95 (0.92–0.98)

Abbreviations: AML, Algemeen Medisch Laboratorium; CI, confidence interval; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus.

[a]−/− both runs are concordantly negative; +/+ both runs are concordantly positive; −/+ Run 1 negative AML, Run 2 positive AML; +/− Run 1 positive AML, Run 2 negative AML.

[b]Other hrHPV includes the aggregate of HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

TABLE 4 Interlaboratory genotype-specific level agreement of RIATOL-qPCR.

HPV type	−/−[a]	+/+[a]	−/+[a]	+/−[a]	General agreement (95% CI)	Kappa (95% CI)
Interlaboratory analysis						
HPV 16	510	30	3	1	99.3% (98.1%–99.8%)	0.93 (0.87–1.00)
HPV 18	537	7	0	0	100.0% (99.3%–100.0%)	1.00 (1.00–1.00)
Other hrHPV[b]	394	141	3	6	98.3% (96.9%–99.2%)	0.96 (0.93–0.99)

Abbreviations: AML, Algemeen Medisch Laboratorium; CI, confidence interval; HPV, human papillomavirus; hrHPV, high-risk human papillomavirus; UZ, University Hospital.

[a]−/− both runs are concordantly negative; +/+ both runs are concordantly positive; −/+ Run 1 negative AML, Run 2 positive UZ Ghent; +/− Run 1 positive AML, Run 2 negative UZ Ghent.

[b]Other hrHPV includes the aggregate of HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

list of hrHPV assays that is considered as fully validated and suitable for primary cervical cancer screening.[5]

In conclusion, the RIATOL-qPCR assay shows excellent intra- and interlaboratory reproducibility and given earlier published evidence on noninferior accuracy for precancer compared to established comparator tests, fulfills the international reproducibility criteria for primary cervical cancer screening.

AUTHOR CONTRIBUTIONS

Marc Arbyn: Principal investigator; protocol development. **Sharonjit K. Dhillon and Pui Yan Jenny Chung**: Statistical analysis; protocol development; writing of the manuscript. **Ana Rita Pereira, Nina Redzic, and Davy Vanden Broeck**: Coordination of samples; coordination of intralaboratory analysis and sample testing. **Marleen Praet and Elizaveta Padalko**: Coordination of interlaboratory analysis and sample testing. **Pui Yan Jenny Chung and Sharonjit K. Dhillon**: Practical coordination of the study. All authors reviewed and/or edited the manuscript. All co-authors approved the final manuscript and its submission to this journal.

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CONFLICT OF INTEREST STATEMENT

This is a researcher-induced study protocol where manufacturers can participate under the condition of providing test kits and covering research costs. Researchers do not receive any financial advantage by collaborating with the validation study. This study was fully supported by AML. All study samples and reagents were provided at no cost by AML. The funding body had no influence on the design and analysis of the results.

DATA AVAILABILITY STATEMENT

Data sets generated by validation studies are stored locally and securely at Sciensano. Anonymized data can be made available by request to the corresponding author on a case-by-case basis pending approval from the information security coordinator at Sciensano.

ETHICS STATEMENT

Ethical approval for the study was obtained from the medical ethics committee, Commissie Medische Ethiek UZ Ghent, affiliated with Ghent University and Ghent University Hospital (reference number ONZ-2022-0171). This manuscript contains no identifiable individual personal data.

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