

1 **Clinical accuracy of Alinity m HR HPV assay on self- versus clinician-taken samples using the**  
2 **VALHUDES protocol**

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28 **Running title: Accuracy of Alinity m HR HPV assay on self-samples**

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31 **Keywords:** Cervical cancer screening, HPV, VALHUDES, self-sampling, diagnostic test  
32 accuracy

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41 **Conflicts of Interest**

42 The VALHUDES project is a researcher-induced study, designed by Sciensano (Principal  
43 Investigator; Brussels, Belgium), CEV (University of Antwerp, Antwerp, Belgium), and AML  
44 (Antwerp, Belgium). Manufacturers of HPV assays and devices can participate in the  
45 VALHUDES framework contributing equipment for laboratory testing and financial support for

46 statistical analysis under the condition of accepting independent publication of results. This  
47 research was supported by a grant from Abbott Laboratories (Abbott Molecular Diagnostics, Des  
48 Plaines, IL, USA), Novosanis NV (Wijnegem, Belgium), and University of Antwerp (Antwerp,  
49 Belgium). The study group received sample collection devices from Rovers Medical Devices B.V.  
50 (Oss, The Netherlands) and Aprovix AB (Uppsala, Sweden).

51 The funders had no role in study design; in the collection, analysis, and interpretation of data; in  
52 the writing of the report; and in the decision to submit the paper for publication. A Vorsters is co-  
53 founder and former board member of Novosanis (Belgium), a spin-off company of the University  
54 of Antwerp, and was minority shareholder until January 2019. D Vanden Broeck is employed by  
55 AML (Antwerp, Belgium), part of the National Reference Centre HPV, a private lab performing  
56 routine cervical cytology and HPV testing.

57

58 **Abstract**

59 The VALHUDES was established to evaluate clinical accuracy of HPV assays to detect cervical  
60 precancer on first-void urine (FVU) and vaginal self-samples (VSS) versus matched clinician-  
61 collected cervical samples (CCS). In this study, we evaluated clinical performance of Alinity m  
62 HR HPV assay (Alinity) in a colposcopy referral population. Home-collected FVU (Colli-Pee FV  
63 5020) one day before colposcopy (n=492), at-clinic collected dry VSS (*multi-Collect Swab [mC*;  
64 n=493], followed by Evalyn Brush [EB; n=233] or Qvintip [QT; n=260]) and matched CCS were  
65 available for the study. At the laboratory, *mC* swabs were resuspended in 2.5 mL Cervi-Collect  
66 buffer, EB and QT were transferred in 20 mL PreservCyt.

67 Sensitivity to detect CIN2+ of Alinity testing on FVU (ratio=0.94 [95%CI 0.85-1.03]), *mC*  
68 (ratio=1.00 [95%CI 0.94-1.06]) and EB/QT (ratio=0.92 [95%CI 0.85-1.00]) was not different to  
69 CCS. Specificity on FVU was similar to CCS (ratio=1.02 [95%CI 0.95-1.10]), whereas specificity  
70 on *mC* was lower (ratio=0.83 [95%CI 0.76-0.90]), but on EB/QT was higher (ratio=1.08 [95%CI  
71 1.01-1.15]) than on CCS. Accuracy on EB (sensitivity ratio=0.96 [95%CI 0.87-1.05]; specificity  
72 ratio=1.18 [95%CI 1.06-1.31]) was slightly better than on QT (sensitivity ratio=0.88 [95%CI 0.75-  
73 1.03]; specificity ratio=1.00 [95%CI 0.92-1.09]). In conclusion, clinical sensitivity of Alinity assay  
74 on all self-sample types was similar to cervical specimens in a colposcopy referral population.  
75 Adjustment of signal-thresholds improved assay's accuracy to detect CIN2+ in all self-sample  
76 types.

77

78

79 **Introduction**

80 Cervical cancer (CC) remains a global public health issue, particularly, in countries with limited  
81 resources <sup>1</sup>. CC can be largely prevented with screening and HPV vaccination. In western  
82 countries, CC screening programs were introduced in late 1950s and resulted in substantial  
83 decrease in CC incidence <sup>2</sup>. Evidence that HPV-based cervical cancer screening has superior  
84 sensitivity to cytology, triggered several western countries to change their recommendations and  
85 switch from cytological to primary HPV-based screening programs <sup>3</sup>. Moreover, HPV testing can  
86 be performed on self-samples which offers opportunities to reach out to women who do not attend  
87 screening regularly. Few countries had introduced HPV-based CC screening with self-sampling  
88 policies by 2022 <sup>4</sup>.

89 Meta-analyses have shown that polymerase chain reaction (PCR)-based HPV DNA tests are  
90 similarly sensitive to detect cervical intraepithelial neoplasia grade two or higher (CIN2+) on  
91 vaginal self-samples compared to clinician-collected cervical samples (CCS) <sup>5, 6</sup>. Another meta-  
92 analysis reported pooled sensitivity on urine-self samples lower than on cervical, although some  
93 PCR-based tests were similarly sensitive compared to CCS <sup>7</sup>. The meta-analyses were pivotal in  
94 triggering a high level of acceptance of self-sampling among women and policymakers, however  
95 optimisation of pre- and post-analytical workflows are still lacking <sup>8-11</sup>. In order to tackle these  
96 challenges, the validation of HPV assays and collection devices for HPV testing on vaginal self-  
97 samples and urine samples (VALHUDES) protocol was established <sup>12</sup>. Five VALHUDES studies  
98 were published showing similar sensitivity and specificity on first-void urine (FVU) <sup>13, 14</sup> and  
99 vaginal self-samples versus CCS <sup>15-17</sup>.

100 The current report aims to evaluate the accuracy of Alinity m HR HPV assay (Alinity; [Abbott  
101 Molecular Diagnostics, Des Plaines, IL, USA]) to detect cervical precancer on FVU and vaginal  
102 self-samples collected either with *multi-Collect* swab (*mC*; Abbott Molecular Diagnostics, Des  
103 Plaines, IL, USA), Evalyn Brush (EB; Rovers Medical Devices, Oss, The Netherlands) or Qvintip  
104 (QT; Aprovix AB, Stockholm, Sweden). In addition, we compared signal strength, expressed by  
105 the fraction cycle number (FCN) across the all specimen types.

106

## 107 **Materials and Methods**

### 108 **Study design**

109 The general design of the VALHUDES study (NCT03064087) has been described previously <sup>12</sup>.  
110 In total, 523 women were invited to one of five Belgian colposcopy clinics because of previous  
111 HPV infection or cervical abnormality <sup>13,15</sup>. The day before the colposcopy appointment, enrolled  
112 women used the Colli-Pee device (Novosanis, Wijnegem, Belgium), which collects ~13 mL of  
113 FVU in a collector tube prefilled with 7 mL of urine conservation medium (UCM). Women were  
114 instructed to store the FVU specimen at room temperature. At the colposcopy clinic, women  
115 presented the FVU sample to the study nurse and subsequently collected two vaginal self-samples.  
116 The first vaginal self-sample was taken with the *mC* swab, followed by the second self-sample  
117 with either EB or QT. Self-samples were collected according to manufacturer's instructions at the  
118 time of sample collection. The EB was first offered to women in the colposcopy clinics of Antwerp  
119 and Ghent, whereas QT was first offered in Brussels, Liège, and Tienen. When about half of the  
120 sample size was reached sampling devices were switched across the colposcopy clinics.  
121 Subsequently, a cervical sample was taken by a gynaecologist using a Cervex-Brush (Rovers  
122 Medical Devices, Oss, The Netherlands) after visualisation of cervix and prior to colposcopy,

123 according to the standards as recommended by European guidelines <sup>18</sup>. The cervical specimen was  
124 resuspended into a vial containing 20 mL ThinPrep PreservCyt (Hologic, Inc, Marlborough, MA,  
125 USA). After all specimens had been collected, colposcopy was performed followed by biopsy if  
126 indicated.

127 All self- and clinician-collected specimens were stored at room temperature (20-22 °C) in the  
128 colposcopy clinic for a maximum of six days (median =2 days). Subsequently, samples were  
129 transferred at room temperature to Algemeen Medisch Laboratorium (AML [Antwerp, Belgium])  
130 for further pre-processing and storage.

131 The FVU samples were transferred at 4°C to the Centre for the Evaluation of Vaccination ([CEV],  
132 University of Antwerp) where they were vortexed for 15-20 seconds, divided into secondary  
133 aliquots and stored at -80°C (Biobank Antwerp, Antwerp, Belgium; ID: BE 71030031000) <sup>19</sup>.  
134 Aliquots were frozen for 889 days on average before HPV testing (range: 491-1217 days).

135 In the AML laboratory, immediately upon arrival, 2.5 mL of Abbott Cervi-Collect buffer was  
136 added to dry *mC* swabs, whereas EB and QT brush heads were transferred in 20 mL ThinPrep  
137 PreservCyt solution. Vaginal and CCS were stored at 4°C for a maximum of up to three months,  
138 then vortexed for 15-20 seconds, aliquoted and frozen at -80°C (Biobank, BB190002). Aliquots  
139 were frozen for 885 days on average before HPV testing (range: 559-1183 days).

140

141

## 142 **Ethical approval**

143 The VALHUDES (NCT03064087) study was approved by the central Ethics Committee of the  
144 University Hospital of Antwerp/University of Antwerp (B300201733869) and the local Ethics

145 Committees of all other centres involved in the study. The study was conducted in accordance with  
146 the Helsinki declaration of 1964. All participants signed informed consent before enrolment.

147

#### 148 **HPV testing**

149 Of 523 study participants, HPV testing with the Alinity assay was performed on 499 quadruplets.  
150 Twenty-four sample quadruplets were excluded due to major protocol violations as described  
151 elsewhere <sup>13,15</sup>. HPV testing was performed on all quadruplets side by side by transferring 550 µl  
152 aliquots (400 µl of input volume is processed by the system) into the Alinity m System for fully  
153 automated integrated DNA extraction, amplification and result interpretation according to the  
154 manufacturer's instructions. The Alinity assay is a qualitative multiplex real-time PCR assay  
155 targeting a conserved sequence within the L1 gene of 14 hrHPV genotypes. The assay separately  
156 reports the presence of HPV16, 18, 45, and other genotypes in two groups using genotype specific  
157 probes in five channels (Group A: HPV31/33/52/58 and Group B: 35/39/51/56/59/66/68) at  
158 clinically relevant infection levels. The assay is clinically validated for use in cervical cancer  
159 screening on clinician-taken cervical samples <sup>20</sup>. DNA extraction was automatically performed by  
160 the Alinity m HR HPV system using the sample preparation kit, including Alinity m Lysis  
161 Solution, Alinity m Ethanol Solution and Alinity m Diluent Solution. The Alinity m System  
162 employs magnetic microparticle technology to facilitate nucleic acid capture, wash and elution.  
163 Purified DNA was mixed with liquid activation and lyophilized amplification/detection reagents  
164 and transferred into a reaction vessel. Subsequently, Alinity m Vapor Barrier Solution was added  
165 to the reaction vessel followed by a transfer to an amplification/detection unit for PCR  
166 amplification, and real-time fluorescence detection of HPV targets, The amplification/detection  
167 reagents of the Alinity assay include primers and probes that amplify and detect an endogenous

168 human  $\beta$ -globin sequence as sample validity control for cell adequacy, sample extraction and  
169 amplification efficiency<sup>21,22</sup>. Amplification signal strength is reported with FCN (fraction cycle  
170 numbers) which is inversely correlated with of  $\beta$ -globin DNA concentration and viral load in the  
171 sample. The reagents also contain Uracil-DNA Glycosylase as a contamination control, negative  
172 and positive controls.

173 Amount

#### 174 **Statistical analysis**

175 To estimate the relative clinical accuracy, HPV testing on self-samples was considered as the index  
176 test and HPV testing on CCS as the comparator. Colposcopy and histology outcomes were used  
177 as a reference test for disease verification. Histological outcomes were classified as normal, CIN1,  
178 CIN2 and CIN3 (no patients with cancer were diagnosed in the study). Participants without biopsy  
179 outcome were categorised as <CIN2 when colposcopy was satisfactory and yielded a normal  
180 impression. The disease thresholds were <CIN2 for specificity and  $\geq$ CIN2+ and CIN3 for  
181 sensitivity. HPV positivity was defined according to the confidential channel specific cut-offs  
182 established by the manufacturer for CCS. Alternative cut-off thresholds were applied for the self-  
183 samples a posteriori using an iterative statistical procedure to improve the relative clinical accuracy  
184 of Alinity testing on self- versus CCS. After increasing/decreasing cut-off thresholds relative  
185 accuracy was reviewed. Alternative cut-offs were defined when an optimal balance was reached  
186 between specificity and sensitivity. Common new cut-offs were applied on FVU and vaginal self-  
187 samples collected with EB/QT for HPV16 (FCN $\leq$ 32), Group A (FCN $\leq$ 31) and Group B  
188 (FCN $\leq$ 31.6), no changes were applied to HPV18 and 45 positivity thresholds. On *mC* samples  
189 new cut-offs were established for HPV16 (FCN  $\leq$ 28), HPV18/45 (FCN $\leq$ 27), Group A  
190 (FCN $\leq$ 25.3) and Group B (FCN $\leq$ 26).



191 Five quadruplets were excluded due to invalid test results on the CCS. Two urine and three vaginal  
192 self-samples were excluded due to invalid test results on respective specimen (Figure 1). Fourteen  
193 quadruplets were retested as follows: a) four quadruplets required retesting due to system error on  
194 all specimen types and deemed valid after retesting; b) four quadruplets were retested due to  $\beta$ -  
195 globin inadequacy and one due to max ratio abnormal response on CCS, all specimens remained  
196 invalid after retesting; c) a single quadruplet underwent retesting due to  $\beta$ -globin inadequacy on  
197 FVU and remained invalid upon retesting; d) three quadruplets were retested due to  $\beta$ -globin  
198 inadequacy on vaginal sample collected with EB or QT devices, retesting was successful for two  
199 samples, while one sample remained invalid; e) three quadruplets underwent retesting due to  
200 failure on *mC* swabs and were found to be valid after the retesting. Two *mC* and one FVU samples  
201 were invalid but were not retested and were excluded from the analysis. The exclusion was limited  
202 to a specific sample type. The dataset used for statistical analysis contained 492 matched CCS,  
203 FVU and vaginal samples collected with *mC* swab, and 493 matched CCS and vaginal samples  
204 taken with EB/QT. Clinical accuracy of the Alinity assay was evaluated for the whole study  
205 population and stratified for women of 30 years and older. Characteristics of the study population  
206 were reported previously <sup>13, 16</sup>.

207 McNemar test and matched 95% confidence intervals (CI) were used to assess the difference in  
208 clinical accuracy between cervical and all other sample types. Statistical significance was accepted  
209 when p-values <0.05 or 95% confidence intervals around relative accuracy measures excluded  
210 unity. Overall and type-specific HPV test concordance between different specimen types was  
211 assessed using Cohen's kappa values, which were categorized as follows: 0.00–0.19 as poor, 0.20–  
212 0.39 as fair, 0.40–0.59 as moderate, 0.60–0.79 as good and 0.80–1.00 as excellent concordance.  
213 Concordance was estimated for the total study population and stratified by disease outcome. The

214 differences in median signal level between matched samples was evaluated using Mann-Whitney  
215 test. For non-matched comparison (EB versus QT devices), Wilcoxon signed-rank test was used.  
216 Statistical analyses were performed using Stata 16.1 (College Station, Texas, USA).

217

## 218 **Results**

### 219 **Clinical accuracy**

220 Relative sensitivity of the Alinity assay on FVU versus CCS was 0.94 (95% CI 0.85-1.03) and  
221 0.90 (95% CI 0.783-1.04) for CIN2+ and CIN3, respectively. Specificity for <CIN2 on FVU and  
222 CCS was similar (ratio=1.02; 95%CI 0.95-1.10]). hrHPV testing with the Alinity assay on *mC*  
223 swab self-samples was similarly sensitive for the detection of CIN2+ (ratio=1.00; 95% CI 0.94-  
224 1.06) and CIN3 (ratio=0.98; 95% CI 0.88-1.09) compared to CCS. The specificity for <CIN2 was  
225 significantly lower than on CCS (ratio=0.83; 95% CI 0.76-0.90).

226 On vaginal self-samples collected with EB or QT, sensitivity relative to CCS was 0.92 (95% CI  
227 0.85-1.00) and 0.93 (95% CI 0.83-1.04) for CIN2+ and CIN3, respectively. The relative specificity  
228 for <CIN2 was significantly higher in vaginal self-samples compared to CCS (ratio=1.08; 95% CI  
229 1.01-1.15,  $p=0.02$ ). Relative sensitivity on samples collected with the EB (ratio=0.96; 95% CI  
230 0.87-1.05) was slightly higher than on QT samples (ratio=0.88; 95%CI 0.75-1.03) (Table 1). The  
231 relative sensitivity of independent non-matched comparison between EB versus QT was 1.11 (95%  
232 CI 0.93-1.33).

233

234 Adjusting the cut-off values on FVU (higher FCN cut-off than established by the manufacturer for  
235 CCS), resulted in sensitivity improvement for CIN2+ (ratio=1.00; 95% CI 0.93-1.07) and CIN3

236 (ratio=1.00; 95% CI 0.91-1.10) with slight decrease in specificity (ratio=0.95; 95% CI 0.88-1.03)  
237 (Table 1).

238 After adjusting cut-off values on *mC* swab samples (lower FCN cut-off than established by the  
239 manufacturer for CCS), relative specificity improved to 1.01 (95% CI 0.95-1.08] with a slight  
240 decrease in sensitivity (ratio=0.97; 95% CI 0.90-1.05) (Table 1).

241

242 The sensitivity on vaginal samples collected with EB or QT combined compared to CCS improved  
243 as a result of cut-off adjustment (higher FCN cut-off than established by the manufacturer for  
244 CCS): ratio=0.96 (95% CI 0.90-1.03) for CIN2+ and 0.95 (95% CI 0.86-1.05) for CIN3. Sensitivity  
245 for CIN2+ also improved on EB (ratio=1.00; 95% CI 0.90-1.06) and QT samples (0.91; 95% CI  
246 0.79-1.05) separately. The relative specificity on vaginal EB/QT samples decreased to 1.01 (95%  
247 CI 0.95-1.08). On EB samples, the specificity was still higher than on CCS after cut-off  
248 optimisation (ratio=1.11; 95% CI 1.01-1.22), but not on QT samples (ratio=0.94; 95% CI 0.86-  
249 1.02) (Table 1). Absolute sensitivity for the total population is reported in Supplementary Table  
250 S1 and for women 30 years and older in Supplementary Table S2. Relative accuracy for women  
251 30 years or older is shown in Supplementary Table S3.

252 An a posteriori cut-off optimisation yielded detection of five additional CIN2+ cases on FVU, and  
253 three on vaginal EB/QT self-samples, whereas 14 FVU and 13 vaginal EB/QT <CIN2 cases  
254 became HPV positive. On *mC* swab samples 37 additional <CIN2 subjects became HPV-negative,  
255 while two additional CIN2+ cases were missed (Table 1). Optimized cut-offs resulted in a  
256 sensitivity improvement of the Alinity assay on FVU and EB/QT samples and in a specificity  
257 improvement on *mC* swab samples, in the sense that the 95% CI around the relative sensitivity and

258 relative specificity of testing on the respective self-sample versus on CCS included unity or  
259 exceeded unity.

260

### 261 **Test concordance**

262 Eighty four percent of FVU and CCS were concordantly hrHPV positive or negative  
263 ([232+181]/492, kappa=0.68). The hrHPV test concordance between vaginal *mC* swab and vaginal  
264 EB/QT self-samples versus cervical samples was 86% ([258+163]/492, kappa=0.70) and 88%  
265 ([236+197]/493, kappa=0.76), respectively (Table 2).

266 After cut-off adjustments, the number of concordantly positive and negative samples between FVU  
267 and CCS increased to 86% ([247+177]/492, kappa=0.72) (Table 3). Similarly, between vaginal  
268 *mC* swab and vaginal EB/QT versus CCS, number of concordant samples increased to 87%  
269 ([246+190]/492; kappa=0.77) and 90% ([248+193]/493, kappa=0.79), respectively (Table 3).  
270 Type-specific agreement between the specimens according to the disease status is shown in  
271 Supplementary Table S5 and Supplementary Table S6.

272

### 273 **Signal strength**

274 Overall hrHPV, HPV16, other hrHPV Group A, other hrHPV Group B, and *β-globin* median FCN  
275 values were significantly higher in FVU compared to CCS. Similarly, hrHPV, HPV16 and other  
276 hrHPV Group A median viral FCN values were significantly higher in the vaginal samples  
277 collected with EB or QT than in CCS. In *mC* specimens, hrHPV, other hrHPV Group A, other  
278 hrHPV Group B, and *β-globin* FCN values were significantly lower than in CCS (Supplementary  
279 Table S7). When stratified by disease status, viral hrHPV and *β-globin* FCN values were

280 significantly lower in CIN2+ cases compared to <CIN2 for all sample types except *mC* swab  
281 (Figure 2 and Supplementary Table S8).

282

283

## 284 **Discussion**

285 In this VALHUDES study, the accuracy of the Alinity assay to detect high-grade cervical disease  
286 was assessed in diverse self-collected sample types compared to CCS. Four self-sampling devices  
287 were evaluated: FVU collected with the Colli-Pee device, and a vaginal self-sample taken with *mC*  
288 swab, EB or QT. All enrolled women were asked to collect a self-sample using Colli-Pee and *mC*,  
289 whereas only half of the women used either EB or QT. No significant difference in clinical  
290 sensitivity for CIN2+ and CIN3 was observed for Alinity assay on FVU and vaginal self-samples  
291 collected with EB/QT compared to CCS. Clinical specificity (<CIN2) on FVU was similar to CCS,  
292 whereas on vaginal self-samples collected with EB/QT, the clinical specificity was significantly  
293 higher than on CCS. The clinical sensitivity for CIN2+ and CIN3 on vaginal self-samples collected  
294 with *mC* swab was similar to CCS, while its clinical specificity was significantly lower. Following  
295 post-hoc optimisation of signal cut-off values an optimal balance was achieved between relative  
296 specificity and sensitivity for all self-sample types. Cut-off optimisation of signal thresholds  
297 improved the relative accuracy estimates with 95% CIs including unity or tending to include unity  
298 for CIN2+ for all self-sample types. Common cut-offs which were higher than the initial values  
299 were applied on FVU and vaginal self-samples collected with EB/QT, whereas lower cut-offs were  
300 used for the *mC* device.

301 Prior to cut-off optimisation, sensitivity of the Alinity assay on vaginal self-samples collected with  
302 EB or QT was somewhat lower in comparison to the *mC* swab, whereas specificity was higher.

303 This contrast in accuracy may be explained by the difference in resuspension volumes, transport  
304 media and sampling order <sup>9,10</sup>. EB and QT devices were placed in 20 mL of ThinPrep PreservCyt  
305 solution, whereas 2.5 mL Abbott Cervi-Collect buffer was added to *mC* swab transport tube with  
306 *mC* swab in it. As Abbott Cervi-Collect Buffer was added to the *mC* swab transport tube, *mC*  
307 sample could have a higher concentration of cell material than EB or QT. These pre-analytical  
308 differences resulted in ~3FCN stronger signal in *mC* compared to EB/QT vaginal samples, which  
309 translated into lower specificity for <CIN2 but only a slightly higher sensitivity. On the other hand,  
310 Colli-Pee allows standardised volumetric collection of ~13 mL FVU prefilled with 7 mL UCM.  
311 However, identical alternative cut-offs could be applied to FVU and vaginal self-samples collected  
312 with EV or QT. Three HPV tests have already been evaluated within VALHUDES <sup>13-17</sup>. RealTime  
313 HPV and BD Onclarity showed similar accuracy on self-collected vaginal and FVU samples  
314 compared to CCS. To improve accuracy on self-samples and CCS with RealTime HPV, signal cut-  
315 off adjustment on FVU and vaginal samples was performed <sup>13-16</sup>. Clinical accuracy of Xpert HPV  
316 was likewise not different on self-collected vaginal versus CCS <sup>17</sup>. No urine samples were run on  
317 Xpert HPV. In agreement with the present report, clinical performance of hrHPV testing on EB  
318 was similarly sensitive and specific compared to the CCS, whereas sensitivity and specificity on  
319 vaginal QT samples tended to be lower.

320 Two previously published studies exploring the performance of self- versus CCS in colposcopy  
321 referral populations observed a lower accuracy when vaginal self-samples were taken with QT,  
322 which is in agreement with our findings <sup>23,24</sup>. Cadman *et al.* assessed accuracy of four vaginal (dry  
323 flocked swab, Dacron swab, HerSwab, and QT) and one FVU device (Colli-Pee) in 600 women  
324 with the BD Onclarity HPV assay. Dry flocked and Dacron swabs performed better in detection  
325 of CIN2+ than QT and HerSwab <sup>23</sup>. FVU collected with 20 mL version of Colli-Pee device also

326 demonstrated high sensitivity, however at lower specificity. Jentschke *et al.* performed direct  
327 comparison of QT and EB in a small study including 136 women <sup>24</sup>. Although, the sample size  
328 was limited Jentschke's findings suggested somewhat better performance of EB compared to QT.  
329 Both studies resuspended vaginal brushes in 20 mL PreservCyt medium.

330 Another study by Ørnkov and colleagues evaluated the cobas 4800 HPV assay on EB vaginal  
331 samples and FVU recruiting 359 participants in colposcopy settings. Dry vaginal sample was  
332 resuspended in 20 mL PreservCyt, while FVU (minimum of 8 mL collected in a urine cup) was  
333 poured into 8 mL EDTA solution. The study showed that the clinical sensitivity on both self-  
334 collected vaginal samples with EB, and urine samples was not different compared to CCS <sup>25</sup>. In  
335 the Dutch IMPROVE trial accuracy of hrHPV testing using GP5+/6+ PCR enzyme immunoassay  
336 on EB vaginal self-samples was compared to CCS in women attending cervical cancer screening.  
337 In total, 7,643 women were included in a self-sampling arm, and another 6,282 in a clinician-  
338 collected arm. Here, EB resuspended in 1.5 mL of PreservCyt medium, was similarly accurate to  
339 detect CIN2+ and CIN3+ versus CCS resuspended in 10 mL PreservCyt medium <sup>26</sup>. Moreover,  
340 several meta-analyses have demonstrated that cross-sectional accuracy of HPV testing on vaginal  
341 self- is similar to CCS under the condition of using validated PCR-based HPV DNA assays <sup>5,7</sup>. A  
342 meta-analysis on urine samples showed that only some PCR-based hrHPV tests are similarly  
343 sensitive compared to CCS. From 21 included studies, four did not report which urine fraction was  
344 used for hrHPV testing and six did not report information on the collection device or use of  
345 preservative buffer. It has been demonstrated that when no preservation or first-void fraction is  
346 used accuracy is worse in urine compared to CCS <sup>8, 11, 27</sup>. A meta-analysis on vaginal samples  
347 found that out of 56 studies, only one did not report collection device and six did not document  
348 storage medium, while volume of resuspension was not well documented <sup>5, 10</sup>. Recent work

349 underlined the challenge to optimize pre-analytical and post-analytical laboratory workflows  
350 which could play a determinant role in identification of cervical precancer using self-samples. For  
351 instance, sample collection device, transport medium and volume, DNA extraction methods, PCR  
352 input volume or HPV positivity criteria could influence accuracy and require thorough  
353 understanding and optimisation on both urine and vaginal self-samples<sup>9-11, 28</sup>.

354 The Alinity assay has been clinically validated for cervical cancer screening using CCS<sup>20, 22</sup>. The  
355 assay has a broader extended genotyping ability than the Abbott RealTime assay, which might be  
356 useful for triage and risk management of HPV positive women<sup>29</sup>. Since HPV genotypes have  
357 different oncogenic potential, risk stratification might be an important clinical application for  
358 cervical cancer screening<sup>30, 31</sup>. Additionally, the Alinity assay's extended genotyping profile to  
359 separately identify all hrHPV types targeted by the nonavalent HPV vaccine (HPV16, HPV18,  
360 HPV45 and the combination of HPV31/33/52/58) could be useful in future screening and triage  
361 algorithms<sup>32</sup>.

362 Our study had several notable strengths, including its large sample size which, given the  
363 colposcopy setting, enabled finding sufficient number of CIN2+ cases and power to address  
364 sensitivity hypotheses. The study was conducted in accordance with STARD guidelines for good  
365 diagnostic test accuracy research<sup>33</sup> with disease outcome for all enrolled women avoiding partial  
366 verification bias. It was not expected at the time of designing VALHUDES, that HPV testing on  
367 EB and QT devices would impact clinical accuracy. Pooling of the VALHUDES results from  
368 multiple tests suggested somewhat lower relative sensitivity and specificity on QT self-samples,  
369 but not with EB. To address this limitation, we presented results both jointly for both devices and  
370 separately for each device. Recruitment from colposcopy clinics may be object of criticism since  
371 not representative for a screening population where HPV testing on self-samples is typically used.



372 As a result, the absolute specificity was lower than what would be observed in a screening  
373 population. However, our conclusions are based on relative accuracy, which is a robust parameter  
374 over diverse settings as demonstrated empirically in meta-analyses (6). Another limitation was the  
375 fact that women aged 19 to 70 years were enrolled in our study, while the age recommended in  
376 most (not all) countries for HPV-based screening is 30 years and above. Nevertheless, the  
377 sensitivity analysis showed that accuracy in the age group recommended for screening closely  
378 resembled that of the entire study population. Another notable drawback was the categorization of  
379 women with normal colposcopy findings and without biopsy results as non CIN2+. This  
380 classification might have influenced the overall accuracy assessments, potentially leading to a  
381 slight overestimation of absolute sensitivity and an underestimation of absolute specificity for both  
382 sample types. Nonetheless, potential bias from such misclassification would affect both sample  
383 types equally, given that colposcopists were unaware of HPV test outcomes for either group. As a  
384 result, the impact on relative accuracy would likely be limited. Finally, order of the sample  
385 collection could have an impact on cell yield and influence the accuracy on vaginal self-samples.  
386 However, previous studies have reported no impact of the sample order on HPV positivity or  
387 agreement rates <sup>23, 24</sup>. In our study, contrast between first and second collection could not be  
388 assessed, since two different devices were used and different pre-analytical laboratory workflow  
389 were applied. Nevertheless, these variations in the pre-analytical workflow could be corrected  
390 through cut-off optimization, as our study successfully achieved.

391 In conclusion, sensitivity of Alinity m HPV assay was not different between self- and clinician-  
392 collected cervical samples, although post-hoc adjustment of signal threshold values resulted in  
393 accuracy improvement and a satisfactory balance was achieved between sensitivity and specificity

394 for all self-sampling specimens. Further research is required to finetune and standardise laboratory  
395 workflows on self-samples.

### 396 **Acknowledgements**

397 We thank the team members of the Gynaecology departments of Antwerp University Hospital,  
398 Ghent University Hospital, General Regional Hospital Heilig Hart Tienen – Femicare vzw, UZ  
399 Brussel – VUB and University Hospital Liège for the study recruitment as well as the enrolled  
400 women. We thank Lies De Baere (AML), Dolores Maes (AML), and Annemie De Smet  
401 (UAntwerp) for the laboratory work.

402

### 403 **Author contributions**

404 Principal investigator and conceptualization of VALHUDES: MA.

405 Protocol development: MA, SVK, DVB, AV.

406 Funding acquisition: MA and AV.

407 Project administration: AL and MA.

408 Enrolment of patients: SW, GD, PS, WT, JD

409 Data Curation and formal analysis: AL, and MA.

410 Sample handling: DVB, SVK.

411 Drafting original manuscript: AL.

412 Critical review and editing of manuscript: all authors.

413

### 414 **Fundings**

415 MA and AL were supported by the RISCC Network [grant no. 8478459] funded by the Horizon  
416 2020 Program for Research and Innovation of the European Commission (Brussels, Belgium).

417 SVK is supported by a junior postdoctoral fellowship of the Research Foundation – Flanders  
418 (1240220N).

419

#### 420 **Conflicts of Interest**

421 The VALHUDES project is a researcher-induced study, designed by Sciensano (Principal  
422 Investigator; Brussels, Belgium), CEV (University of Antwerp, Antwerp, Belgium), and AML  
423 (Antwerp, Belgium). Manufacturers of HPV assays and devices can participate in the  
424 VALHUDES framework contributing equipment for laboratory testing and financial support for  
425 statistical analysis under the condition of accepting independent publication of results. This  
426 research was supported by a grant from Abbott Laboratories (Abbott GmbH, Wiesbaden,  
427 Germany), Novosanis NV (Wijnegem, Belgium), and University of Antwerp (Antwerp, Belgium).  
428 The study group received sample collection devices from Rovers Medical Devices B.V. (Oss, The  
429 Netherlands) and Aprovix AB (Uppsala, Sweden).

430 The funders had no role in study design; in the collection, analysis, and interpretation of data; in  
431 the writing of the report; and in the decision to submit the paper for publication. A Vorsters is co-  
432 founder and former board member of Novosanis (Belgium), a spin-off company of the University  
433 of Antwerp, and was minority shareholder until January 2019. D Vanden Broeck is employed by  
434 AML (Antwerp, Belgium), part of the National Reference Centre HPV, a private lab performing  
435 routine cervical cytology and HPV testing.

#### 436 **Ethical Approval**

437 VALHUDES trial (NCT03064087) was approved by the central Ethics Committee of the  
438 University Hospital of Antwerp/University of Antwerp (B300201733869) and the local Ethics  
439 Committees of all the other involved centres. The study was conducted in accordance with the

440 Declaration of Helsinki. Written informed consent was obtained from all study participants prior  
441 to enrolment.

442

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- 559

560 **Table 1.** Relative accuracy of the Alinity m HR HPV assay on self-samples compared to  
 561 clinician-collected cervical samples.

	Relative sensitivity [95% CI] CIN2+	Relative sensitivity [95% CI] CIN3	Relative specificity [95% CI] <CIN2
<i>Manufacturer cut-offs*</i>			
<b>Urine</b>	0.94 [0.85-1.03]	0.90 [0.78-1.04]	1.02 [0.95-1.10]
<b>mC swab</b>	1.00 [0.94-1.06]	0.98 [0.88-1.09]	0.83 [0.76-0.90]
<b>EB/QT<sup>†</sup></b>	0.92 [0.85-1.00]	0.93 [0.83-1.04]	1.08 [1.01-1.15]
<b>EB</b>	0.96 [0.87-1.05]	0.95 [0.87-1.05]	1.18 [1.06-1.31]
<b>QT</b>	0.88 [0.75-1.03]	0.90 [0.73-1.11]	1.00 [0.92-1.09]
<i>Alternative cut-offs<sup>‡</sup></i>			
<b>Urine</b>	1.00 [0.93-1.07]	1.00 [0.91-1.10]	0.95 [0.88-1.03]
<b>mC swab</b>	0.97 [0.90-1.05]	0.98 [0.88-1.09]	1.01 [0.95-1.08]
<b>EB/QT</b>	0.96 [0.90-1.03]	0.95 [0.86-1.05]	1.01 [0.95-1.08]
<b>EB</b>	1.00 [0.94-1.06]	1.00 [1.00-1.00]	1.11 [1.01-1.22]
<b>QT</b>	0.91 [0.79-1.04]	0.90 [0.73-1.11]	0.94 [0.86-1.02]

562 CI, confidence interval; CIN, cervical intraepithelial neoplasia; EB, Evalyn Brush; mC, multi-Collect; N, number; QT, Qvintip.

563 \*Confidential manufacturer's fraction cycle number (FCN) cut-off established for cervical samples.

564 <sup>†</sup>Samples collected with EB or QT combined.

565 <sup>‡</sup>New a posterior defined cut-offs for urine and vaginal self-samples collected with EB or QT devices: **HPV16 FCN<=32, Group A FCN<=31,**  
 566 **Group B FCN<31.6.** For mC swab samples following new cut-offs were defined: **HPV16 FCN <=28, HPV18/45 FCN<=27, Group A**  
 567 **FCN<=25.3, Group B FCN <=26.**

568 Relative sensitivity and specificity for women ≥ 30 years old are shown in Supplementary Table S3. Matched numbers of cases used to estimate  
 569 relative accuracy are present in Supplementary Table S4.  
 570  
 571

572 **Table 2.** Type-specific agreement and test concordance between cervical and self-samples based  
 573 on manufacturer cut-offs.

<i>Urine vs cervical</i>							
	HPV type	+/+*	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI] <sup>†</sup>
<b>Total population (n=492)</b>	hrHPV <sup>‡</sup>	232	44	35	181	83.9	0.675 (0.610 - 0.741)
	HPV16	62	4	8	418	97.6	0.898 (0.841 - 0.955)
	HPV18	19	2	5	466	98.6	0.837 (0.719 - 0.956)
	HPV45	12	4	8	468	97.6	0.654 (0.472 - 0.837)
	Group A <sup>§</sup>	79	32	27	354	88.0	0.651 (0.570 - 0.733)
	Group B <sup>¶</sup>	105	25	35	327	87.8	0.694 (0.622 - 0.766)
<i>mC swab vs cervical</i>							
	HPV type	+/+	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]
<b>Total population (n=492)</b>	hrHPV	258	18	53	163	85.6	0.702 (0.638 - 0.765)
	HPV16	64	2	16	410	96.4	0.855 (0.790 - 0.920)
	HPV18	18	2	9	463	97.8	0.754 (0.615 - 0.894)
	HPV45	11	4	7	470	97.8	0.655 (0.465 - 0.846)
	Group A	101	12	26	353	92.3	0.791 (0.727 - 0.854)
	Group B	117	13	44	318	88.4	0.723 (0.657 - 0.790)
<i>EB/QT vs cervical</i>							
	HPV type	+/+	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]
<b>Total population (n=493)</b>	hrHPV	236	41	19	197	87.8	0.756 (0.698 - 0.813)
	HPV16	61	5	4	423	98.2	0.921 (0.870 - 0.972)
	HPV18	17	4	2	470	98.8	0.844 (0.721 - 0.967)
	HPV45	12	4	2	475	98.8	0.794 (0.633 - 0.955)
	Group A	88	25	10	370	92.9	0.789 (0.723 - 0.856)
	Group B	103	26	18	346	91.1	0.764 (0.698 - 0.830)
<i>EB vs cervical</i>							
	HPV type	+/+	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]
<b>Total population (n=233)</b>	hrHPV	115	24	6	88	87.1	0.741 (0.655 - 0.826)
	HPV16	36	4	1	192	97.9	0.922 (0.855 - 0.990)
	HPV18	12	4	1	216	97.6	0.816 (0.659 - 0.973)
	HPV45	3	1	1	228	99.1	0.746 (0.406 - 1.000)
	Group A	41	14	1	177	93.6	0.806 (0.712 - 0.899)
	Group B	44	17	7	165	89.7	0.719 (0.614 - 0.823)
<i>QT vs cervical</i>							
	HPV type	+/+	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]
<b>Total population (n=260)</b>	hrHPV	121	17	13	109	88.5	0.769 (0.691 - 0.847)
	HPV16	25	1	3	231	98.5	0.917 (0.837 - 0.998)
	HPV18	5	0	1	254	99.6	0.907 (0.726 - 1.000)
	HPV45	9	3	1	247	98.5	0.810 (0.629 - 0.992)
	Group A	47	11	9	193	92.3	0.775 (0.682 - 0.869)
	Group B	59	9	11	181	92.3	0.803 (0.720 - 0.885)

574 CI, confidence interval; EB, Evalyn Brush; hr, high risk; HPV, human papillomavirus; mC, multi-Collect; N, number; QT, Qvintip.

575 \*+/+ positive on self- and cervical samples, +/- positive only on cervical samples, -/+ positive only on self-samples, -/- negative on both sample  
 576 types.

577 †Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: 0.00 – 0.20 Poor; 0.21 – 0.40 Fair; 0.41 –  
 578 0.60 Moderate; 0.61 – 0.80 Good; 0.81 – 1.00 Excellent.

579 ‡14 carcinogenic HPV genotypes.

580 §Group A: HPV31/33/52/58

581 ¶Group B : HPV35/39/51/56/59/66/68

582 Concordance by disease status is shown in Supplementary Table S5.  
 583  
 584

585 **Table 3.** Type-specific agreement and test concordance between cervical and self-samples with  
 586 alternative cut-offs.

<i>Urine vs cervical</i>							
	<b>HPV type</b>	<b>+/+*</b>	<b>+/-</b>	<b>-/+</b>	<b>-/-</b>	<b>Concordance [%]</b>	<b>Kappa [95% CI]†</b>
<b>Total population (n=492)</b>	hrHPV‡	247	29	39	177	86.2	0.718 (0.656 - 0.780)
	HPV16	63	3	10	416	97.4	0.891 (0.833 - 0.949)
	HPV18	19	2	5	466	98.6	0.837 (0.719 - 0.956)
	HPV45	12	4	8	468	97.6	0.654 (0.472 - 0.837)
	Group A§	91	20	28	353	90.0	0.728 (0.655 - 0.800)
	Group B¶	110	20	39	323	88.0	0.705 (0.636 - 0.775)
<i>mC swab vs cervical</i>							
	<b>HPV type</b>	<b>+/+</b>	<b>+/-</b>	<b>-/+</b>	<b>-/-</b>	<b>Concordance [%]</b>	<b>Kappa [95% CI]</b>
<b>Total population (n=492)</b>	hrHPV	246	30	26	190	86.6	0.769 (0.713 - 0.826)
	HPV16	64	2	11	415	97.4	0.892 (0.835 - 0.950)
	HPV18	17	3	4	468	98.6	0.822 (0.693 - 0.951)
	HPV45	11	4	5	472	98.2	0.700 (0.514 - 0.886)
	Group A	95	18	21	358	92.1	0.778 (0.712 - 0.844)
	Group B	109	21	22	340	92.3	0.776 (0.712 - 0.839)
<i>EB/QT vs cervical</i>							
	<b>HPV type</b>	<b>+/+</b>	<b>+/-</b>	<b>-/+</b>	<b>-/-</b>	<b>Concordance [%]</b>	<b>Kappa [95% CI]</b>
<b>Total population (n=493)</b>	hrHPV	248	29	23	193	89.5	0.786 (0.732 - 0.841)
	HPV16	62	4	5	422	98.2	0.922 (0.871 - 0.972)
	HPV18	17	4	2	470	98.8	0.844 (0.721 - 0.967)
	HPV45	12	4	2	475	98.8	0.794 (0.633 - 0.955)
	Group A	96	17	14	366	93.7	0.820 (0.759 - 0.881)
	Group B	106	23	23	341	90.7	0.759 (0.693 - 0.824)
<i>EB vs cervical</i>							
	<b>HPV type</b>	<b>+/+</b>	<b>+/-</b>	<b>-/+</b>	<b>-/-</b>	<b>Concordance [%]</b>	<b>Kappa [95% CI]</b>
<b>Total population (n=233)</b>	hrHPV	123	16	6	88	90.6	0.807 (0.731 - 0.884)
	HPV16	37	3	2	191	97.9	0.924 (0.858 - 0.990)
	HPV18	12	4	1	216	97.6	0.816 (0.659 - 0.973)
	HPV45	3	1	1	228	99.1	0.746 (0.406 - 1.000)
	Group A	46	9	2	176	95.3	0.863 (0.785 - 0.942)
	Group B	47	14	9	163	90.1	0.738 (0.637 - 0.838)
<i>QT vs cervical</i>							
	<b>HPV type</b>	<b>+/+</b>	<b>+/-</b>	<b>-/+</b>	<b>-/-</b>	<b>Concordance [%]</b>	<b>Kappa [95% CI]</b>
<b>Total population (n=260)</b>	hrHPV	125	13	17	105	88.5	0.768 (0.690 - 0.846)
	HPV16	25	1	3	231	98.5	0.917 (0.837 - 0.998)
	HPV18	5	0	1	254	99.6	0.907 (0.726 - 1.000)
	HPV45	9	3	1	247	98.5	0.810 (0.629 - 0.992)
	Group A	50	8	12	190	92.3	0.783 (0.693 - 0.874)
	Group B	59	9	14	178	92.3	0.776 (0.690 - 0.863)

587 CI, confidence interval; EB, Evalyn Brush; hr, high risk; HPV, human papillomavirus; mC, multi-Collect; N, number; QT, Qvintip.  
 588 \*+/+ positive on self- and cervical samples, +/- positive only on cervical samples, -/+ positive only on self-samples, -/- negative on both sample  
 589 types.  
 590 †Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: 0.00 – 0.20 Poor; 0.21 – 0.40 Fair; 0.41 –  
 591 0.60 Moderate; 0.61 – 0.80 Good; 0.81 – 1.00 Excellent.  
 592 ‡14 carcinogenic HPV genotypes.  
 593 §Group A: HPV31/33/52/58  
 594 ¶Group B : HPV35/39/51/56/59/66/68

595 New post-hoc fraction cycle number (FCN) cut-offs defined for urine and vaginal samples collected with EB or QT: *HPV16 FCN* ≤ 32, *Group A*  
 596 *FCN* ≤ 31, *Group B FCN* ≤ 31.6. For *mC* swab samples following new cut-offs were defined: *HPV16 FCN* ≤ 28, *HPV18/45 FCN* ≤ 27, *Group*  
 597 *A FCN* ≤ 25.3, *Group B FCN* ≤ 26  
 598 Concordance by disease status is shown in Supplementary Table S6.  
 599

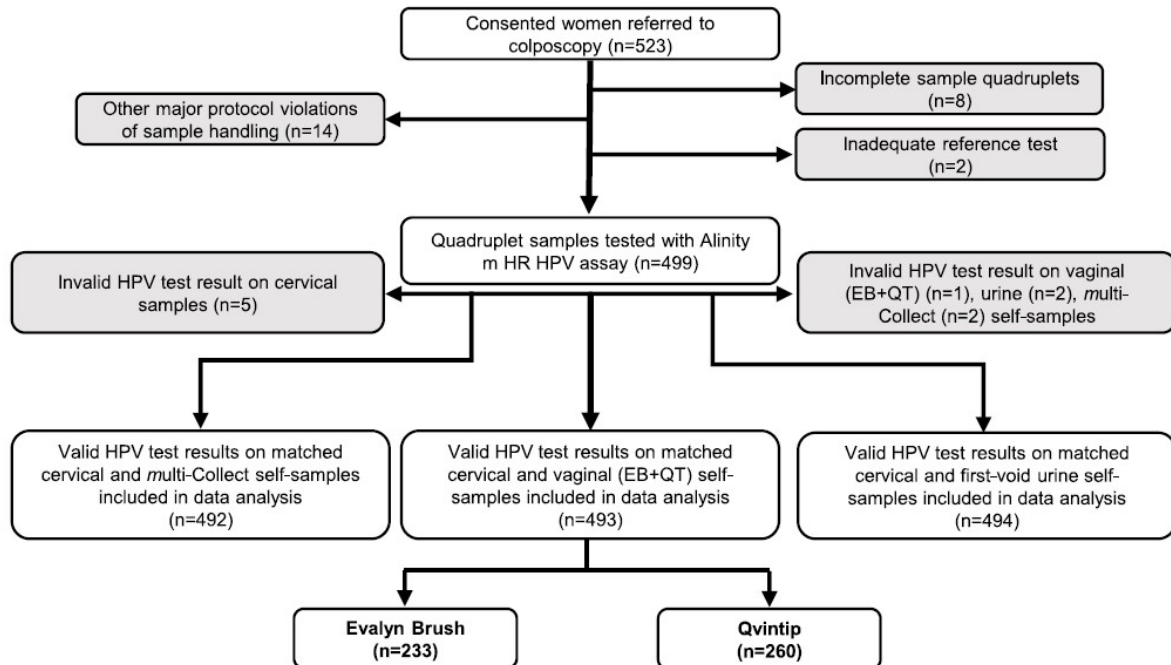
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602 **Figure Legends:**

603 **Figure 1.** Flow chart of samples included in the VALHUDES trial tested with the Alinity m HR  
 604 HPV assay. Grey boxes represent excluded samples. Detailed exclusions are reported elsewhere  
 605 13, 15.

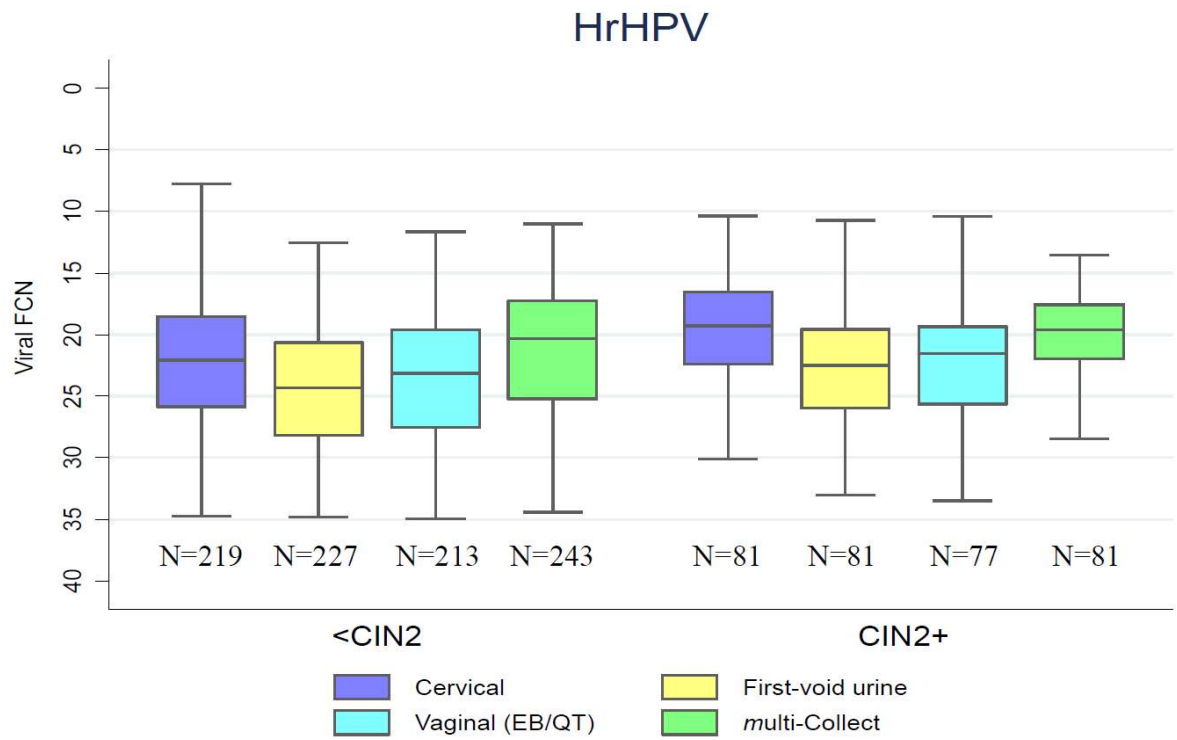
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608 **Figure 2.** Alinity m HR HPV assay signal strength expressed by the fraction cycle number (FCN)  
 609 stratified by CIN2+ and <CIN2 between specimen types.

610 Boxplots indicate median FCN values and interquartile ranges for HPV16, 18, 45, other hrHPV  
 611 Group A, other hrHPV Group B. N indicates number of samples with FCN value >0 in each category  
 612 of matched 489 quadruplets.



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