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Analytical and clinical sample performance characteristics of the Onclarity Assay for the detection of Human Papillomavirus

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ABSTRACT (190 words)

Objective: To determine result reproducibility, and performance of the BD Onclarity™ HPV Assay (Onclarity) on the BD Viper™ LT platform using both contrived and clinical specimens.

Methods: Reproducibility was assessed in BD SurePath™ LBC media (SurePath) using contrived panels (HPV16+, 18+, or 45+) or clinical specimens (HPV16+, 18+, 31+, 33/58+, 45+, 52+, or HPV-). In addition, specimens from 3,879 individuals from the Onclarity trial were aliquoted prior to or following cytology processing and tested for HPV. Finally, specimens were collected using either the Cervex-brush® or Cytobrush® (or Cytobrush/spatula) for comparison of HPV results.

Results: Contrived specimens showed >95% concordance with expected results and pooled clinical specimens had standard deviation and coefficient of variation values ranging from 0.87 to 1.86 and 2.9% to 5.6%, respectively. For pre-cytology and post-cytology aliquot analyses, specimens showed >98.0% overall agreement and mean differences in Ct score for HPV ranging from -0.07 to 0.31. Positivity rates were close between the Cervex-brush and Cytobrush/spatula for all age groups tested.

Conclusions: Onclarity results are reproducible and reliable, regardless of sample collection before or after the cytology aliquot. Onclarity performs well regardless of the method of specimen collection (Cervex-brush or Cytobrush/spatula) for cervical cancer screening.
Key Words: Cervical Cancer Screening; Atypical squamous cells-undetermined significance; Human Papillomavirus; Genotype; Cervical Intraepithelial Neoplasia; Triage
INTRODUCTION (535 words)

Over 95% of cervical cancer cases are caused by persistent infection with 13-14 HPV genotypes,(1-4) which can be clinically detected using assays targeting nucleic acid sequences in the HPV genome.(5, 6) In the United States, the 2019 ASCCP guidelines recommend a risk-based approach for cervical cancer screening and patient management following a positive screening result (7) and HPV testing is a key component for risk-based management in this context. An HPV negative result provides excellent negative predictive value to extend a woman’s screening interval to five years.(8) In addition, extended genotyping has been shown to stratify risk in women with a negative cytology/positive HPV result to help determine those women at greatest risk for high-grade cervical disease or cancer; and nearly 30 countries in Europe utilize HPV testing in some capacity. Most programs currently involve HPV testing as part of cytology triage or cotesting. Countries including Australia, Netherlands, Italy, the United Kingdom, and Sweden have transitioned to HPV primary screening with cytology follow-up as necessary.(9, 10)

The BD Onclarity™ HPV assay (“Onclarity;” Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, Sparks, MD, USA) is clinically validated through USA- and non-USA-based studies for detection of fourteen high risk HPV genotypes.(11-13) Onclarity is FDA-approved for reporting genotypes HPV 16 and 18 during HPV primary screening (for women ≥25 years of age), and HPV 16, 18, and 45 during ASCUS triage (for women ≥21 years of age) and cotesting (for women ≥30 years of age); in addition it is CE-IVD Marked for detection of 14 high-risk HPV genotypes.(14-16) In addition, Onclarity is FDA-approved for reporting individual genotype results for 16, 18, 31, 45, 51, and 52, and groupd...
results for 33/58, 35/29/68, and 56/59/66 in order to facilitate risk-based screening for cervical cancer and precancer.

The Onclarity assay is performed on samples obtained from liquid-based cytology (LBC) specimens collected using a Cervex-brush or Cytobrush, (or Cytobrush/spatula) device. However, the order of aliquotting for the Onclarity assay can vary based on the respective screening strategy employed by each laboratory. For example, sites utilizing HPV primary screening with cytology triage will perform HPV testing from an initial LBC aliquot (pre-cytology), and use remaining vial/specimen for cytology. Conversely, sites employing a screening program with primary cytology testing and HPV triage testing will perform cytology testing first, followed by HPV testing from the specimen after cytology processing (post-cytology aliquot). Therefore, it is important to establish that the performance of the HPV assay is unaffected by the order in which the aliquot is taken.

Contrived specimens and pooled clinical specimens were utilized to test reproducibility within Onclarity assay runs, and between Onclarity assay runs, study sites, operators, reagent lots, and days of operation. In addition, data from pre- and post-cytology aliquot specimens were analyzed to determine whether Onclarity assay performance is impacted by the aliquot order in which the sample is (i.e., before or after cytology). Finally, from BD SurePath™ (“SurePath”; Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, Sparks, MD, USA ) vials obtained during the Onclarity trial, Onclarity assay results were compared in specimens obtained using two different collection devices in order to determine whether performance results are affected based on the method of sampling for endocervical specimens.
2. METHODS (1,159 words)

2.1 Clinical trial population

Women ≥21 years of age (women >65 years were included if they met USPSTF screening recommendations) were invited to join the Onclarity trial between 2013 and 2015. Initially, 33,858 subjects (across 31 collection sites) were enrolled; the trial population, criteria for inclusion/exclusion, and procedures involving LBC collection, cytology testing, colposcopy/biopsy procedures, and histology examination/diagnosis have been described previously. (14) By cytology, 30,489 women were identified with negative for intraepithelial lesions or malignancies (NILM) cytology, 1,960 women identified with atypical squamous cells-undetermined significance (ASCUS) cytology, and 1,122 women identified with >ASCUS cytology were included in the baseline data from the Onclarity trial. The study was approved by institutional review boards at each study site and written informed consent was obtained prior to any trial-related procedures; this study was conducted according to the principles set forth by the Declaration of Helsinki and Good Clinical Practice and this report was prepared according to STARD guidelines for reporting diagnostic accuracy.

2.2 Preparation for clinical reproducibility, pre- and post-cytology aliquots, and collection
device experiments

For reproducibility testing, contrived panel members were prepared using SiHa, HeLa, and MS751 transformed cell lines that express HPV 16, 18, and 45, respectively. Aliquots from each cell panel preparation were added to HPV negative SurePath clinical matrix to yield high-negative specimens (C5; specimens called positive approximately 5% and negative 95% of the time), low-positive specimens (C95; specimens called positive approximately 95% and negative
5% of the time), and moderate-positive specimens (3x C95; specimens approximately three times above the C95 level and expected to be positive 100% of the time). These determinations were made based on the assay Ct values relative to the clinical cut-point (C95) associated with the assay. Pooled clinical specimens, positive for HPV 16, 18, 45, 31, 33/58, or 52, were diluted (with HPV negative clinical specimen matrix) to a detection level close to C95 (the clinical cut-off). Negative panel members were created by pooling high-risk HPV negative clinical specimens. All panel members were stored at -20°C prior to Onclarity assay testing. Standard deviation and the coefficient of variability for PCR reaction mean cycle time within a run, between runs, between operators, between sites, between reagent lots, and between days (see Figure S1) were all factors used as outcome measures of reproducibility.

During the Onclarity trial, endocervical specimens were collected using a Rovers® Cervex-brush® (“Cervex-brush”; Rovers Medical Devices, The Netherlands), or the Cytobrush® GT and Pap Perfect® Plastic Spatula (“Cytobrush/spatula”; Cooper Surgical, Inc., Trumbull, CT, USA) and stored/transported in SurePath LBC specimen vials. Clinical specimens were processed (as described below) and utilized for HPV testing via the Onclarity assay on the BD Viper™ LT system (“Viper LT”; Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, Sparks, MD, USA).

For pre-cytology and post-cytology specimens, central laboratory personnel vortexed the SurePath LBC specimen and manually aliquoted 0.5mL of the specimen into an HPV LBC diluent tube (pre-cytology aliquot). Aliquoting from SurePath LBC specimen vials to diluent tubes was performed in the same order as the specimen vials were received. Following removal
of the 0.5 mL pre-cytology aliquot, 8.0 mL of specimen was removed from the SurePath LBC vial and a cytology slide was processed (using the BD PrepMate™/PrepStain™ System, Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, Sparks, MD, USA) per manufacturer’s instructions. A final, 0.5 mL aliquot from residual fluid in the SurePath LBC vial, was manually transferred into a second HPV LBC diluent tube (post-cytology aliquot). Thus, pre- and post-cytology aliquot diluent tubes were obtained from the same SurePath LBC specimen vials; both diluent tubes were sent to one of four laboratories that ran Viper LT testing (Figure S2). There was minimal delay for the post-quot specimens while cytology slides were prepared; this was within the validated room temperature storage time. Overall, 3,879 SurePath vials were utilized in the study to provide pre-and post-cytology aliquot pairs for HPV testing and analysis.

2.3 Sample processing for HPV testing

The details for HPV testing with the Oncclarity assay on Viper LT using LBC specimens have been described previously.(16, 17) Briefly, Oncclarity uses three processing steps: 1) aliquoted, collected specimen matrix in SurePath media is vortexed, and pre-warmed, 2) the nucleic acids are extracted using BD FOX™ Extraction (Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, Sparks, MD, USA) that involves automated matrix homogenization, cell lysis, binding and elution of DNA; and 3) real-time PCR amplification of both HPV E6/E7 and human β-globin target DNA sequences was performed on the Viper LT System. TaqMan® DNA probes (Thermo Fisher, Pittsburgh, PA, USA) include a fluorescent dye at the 5’ end and a quenching molecule at the 3’ end of the oligonucleotide. Three individual PCR tubes (G1, G2, and G3), collectively, detect 14 high risk HPV genotypes (six individual
genotypes: 16, 18, 31, 45, 51, 52; and three groups containing eight genotypes: 33/58, 35/39/68, and 56/59/66). The human beta globin gene serves as the internal control for each PCR reaction across all three PCR tubes.

2.4 Data collection and analysis

For reproducibility testing, three test sites analyzed panels testing one panel in duplicate (once per operator) daily, for nine days. Three different reagent lots were utilized: one lot per three days of testing. Panel members were randomized and technical staff were blinded to genotypes in each panel member. A total of 162 results (54 per testing site) were expected for each panel member. Percent agreement (with accompanying lower and upper 95% confidence intervals) analyses were performed for high-negative, low positive, and moderate positive contrived specimens. Acceptance criteria for HPV assay performance during testing of panel members was predetermined; for low positive it was 94% and for moderate positive it was 98% (Table S1). For clinical specimen analysis, specific mean cycle threshold (Ct) scores (between 34.2 and 38.3 for HPV 16 and 29.6 and 34.2 for the other 13 genotypes), were required to ensure that genotypes were being detected in proximity to the clinical cutoff. The limit of detection around the clinical cutoffs for HPV 16 (Ct value of 38.3) is around 1,500 viral genome copies/mL of undiluted SurePath media; for the other 13 genotypes (Ct value of 34.2) it ranges from 3,000 to 10,000. Additional information regarding this issue is available in the product’s Information For Use document.(18)

For pre- versus post-cytology aliquot comparison, positive, negative, and overall agreement were determined using the pre-cytology aliquot result to define positive and negative. Mean (with
lower and upper 95% confidence intervals) pre- and post-cytology aliquot Ct scores, including mean difference between the two, were calculated and statistical comparison was performed using a paired t-test. Linear regression was performed for high-risk HPV genotype detection between pre- and post-cytology aliquot specimens.

Data for comparison of collection devices was generated at four testing sites in the USA from a pre-cytology aliquot. Human beta-globin (HBB) Ct scores, HPV Ct scores, and high-risk HPV positivity rates were analyzed in three intended use populations (ASCUS ≥21 years, NILM ≥30 years, and primary screening ≥25 years) and age groups (21-24, 25-29, 30-39, 40-49, and ≥50 years). The mean HBB Ct score was calculated by averaging each specimen’s three internal control Ct score results. The HPV Ct score was calculated by selecting the strongest Ct score from nine channels, excluding subjects without HPV Ct score result. The HBB and HPV Ct scores were compared using a two sample t-test. The p-value that used the Satterthwaite approximation for degrees of freedom was reported. To test whether HPV positivity rates were different between the two collection devices, the Fisher’s two-sided exact test was performed.
3. RESULTS (865 words)

3.1 Onclarity assay reproducibility

For reproducibility testing, contrived specimens were created using cells expressing HPV 16 (SiHa), HPV 18 (HeLa), and HPV 45 (MS751) to spike HPV negative clinical specimen matrix at pre-specified low and moderate positive concentrations. As shown in Figure 1 (also Table S2), the Onclarity assay reported results for HPV 16, 18, and 45 that were all above 95% agreement within the low positive panels and near 100% for the moderate positive panels (both compared to expected results). For the pooled HPV high-negative clinical panels, 91.6% of the samples were negative for HPV 16, whereas, 100% of the HPV18 and HPV45 samples returned a correct result of negative. For pooled clinical specimens positive for HPV 16, 18, 45, 31, 33/58, or 52, the reproducibility for mean Ct score met the acceptance criteria; the overall standard deviation and percent coefficient of variation ranged from 0.87 to 1.86 and 2.9% to 5.6%, respectively, with the greatest variation being observed within replicates on the same instrument run (Table 1).

HPV negative samples (HPV negative clinical matrix or HPV negative cell line suspended in SurePath LBC media) were all reported as negative (100% were above 38.3 on the HPV16 channel and 34.2 for channels relative to the other eight HPV results) by Onclarity (Table 1).

3.2 Onclarity assay results from pre- and post-cytology aliquot specimens

Individual Onclarity results were compared from pre-cytology aliquot versus post-cytology aliquot specimens. The total number of individual results were as follows: n=77 for HPV 16, n=34 for HPV 18, n=65 for HPV 31, n=59 for HPV 33/58, n=42 for HPV 45, n=72 for HPV 51, n=80 for HPV 52, n=195 for HPV 35/39/66, and n=149 for HPV 56/59/66. Individual Onclarity assay Ct score results are plotted in Figure 2 with those from the pre-cytology aliquot on the x-
axis and those from the post-cytology aliquot on the y-axis. Although there was a slight difference in the distribution of Ct scores between pre- and post-cytology aliquot groups, the results corresponding to pre-cytology aliquots and post-cytology aliquots were linear and represented a one-to-one correlation.

Table 2 shows four categories (ASCUS, >ASCUS, NILM, any cytology), which correspond to cytology/HPV triage (ASCUS) for women ≥21 years of age, cotesting (NILM) for women ≥30 years of age, and the primary screening population (any cytology), for women ≥25 years of age. Positive and negative percent agreement were high for all cytology categories and the overall percent agreement between the pre- and post-cytology aliquot specimens was >98% for all cytology categories (Table 2). Comparisons for the post-cytology aliquot results relative to the pre-cytology aliquot results showed 96.1% (73/76), 100% (83/83), 89.9% (160/178), and 92.2% (353/383) concordant rates for positive results from the ASCUS, >ASCUS, NILM, and any cytology (≥25 years of age), respectively. In addition, concordant rates for the post-cytology aliquot specimens compared to the pre-cytology aliquot specimens were 100% (129/129), 90.5% (19/21), 98.9% (2431/2457), and 98.9% (3052/3087) for negative results from the ASCUS, >ASCUS, NILM, and any cytology (≥25 years of age), respectively. The majority of the discordant results were from women with NILM cytology and the discordant results also split across the two sample types (pre-quot [+] / post-quot [-] and pre-quot [-] / post-quot [+] ) (Table S3). Approximately 85% of the discordant results were close to the clinical cut-off of the assay (data not shown).
Mean Ct score were determined for Onclarity results from specimens that were positive for any high-risk HPV genotype (n=773), or individual genotypes HPV 16 (n=77), HPV 18 (n=34), or HPV 45 (n=42) (Table 3). Pre- and post-cytology aliquot mean Ct scores were close across all four test groups with the mean difference (post-cytology aliquot – pre-cytology aliquot) no greater than 0.31 cycle from zero. Statistical analyses revealed no significant difference between the mean Ct scores from pre- and post-cytology aliquot specimens for any of the genotype categories.

3.3 Onclarity results based on specimen collection device

Onclarity performance was compared following collection with either Cervex-brush or the Cytobrush (or Cytobrush/spatula) in three screening populations: ASCUS, ≥21 years of age (n=989 for Cervex-brush and n=964 for Cytobrush), NILM, ≥30 years of age (n=11,145 for Cervex-brush and n=11,139 for Cytobrush), the primary screening, ≥25 years of age (n=14,858 for Cervex-brush and n=14,654 for Cytobrush). To compare Onclarity performance for each collection device, the Ct values were averaged for all samples with a signal (Ct <40) on the Viper LT (n=427 and n=424 for Cervex-brush and Cytobrush, respectively, in the ASCUS population; n=1,427 and n=1,390 for Cervex-brush and Cytobrush, respectively, in the NILM population; and n=2,637 and n=2,586 for Cervex-brush and Cytobrush, respectively, in the primary screening population; Table S4). No significant difference was observed between the Cervex-brush and the Cytobrush (or Cytobrush/spatula) across the three screening populations or by age group (Figure 3a and Table S4). In addition, no significant difference was observed between the Cervex-brush and the Cytobrush (or Cytobrush/spatula) Ct scores related to detection of the internal control (HBB gene; Tables S4 and Figure S3). The HPV positivity rate (for those
samples with a signal $\leq 38.3$ for HPV16 channel and $\leq 34.2$ for the other eight HPV channels) for
specimens collected through Cervex-brush and Cytobrush (or Cytobrush/spatula) devices were
not significantly different across all three screening populations. In addition, positivity rates with
both collection devices tended to decrease with increasing age in the primary screening
population (Figure 3b and Table 4). Positivity rates were not significantly different between
devices when results were stratified by age (21-24; 25-29; 30-39; 40-49; and $\geq 50$ years of age)
(Table 4).
4. DISCUSSION (1,570 words)

The results presented here demonstrate the high reproducibility of Onclarity (within run, between run, between operator, between site, between reagent lots, and between days). Onclarity met reproducibility criteria for contrived specimens containing individual genotypes 16, 18, and 45, and for pooled, clinical specimens positive for either HPV 16, 18, 45, 31, 33/58, or 52. The overall agreement of the results from the Onclarity assay using pre-cytology aliquot and post-cytology aliquot samples for each of the subject populations was above 98% with a lower bound of the 95% confidence interval being ≥93%. Finally, HPV positivity rates and HPV mean Ct scores, both overall and when stratified by age groups, were not statistically different for the two collection devices (Cervex-brush or Cytobrush (or Cytobrush/spatula)) investigated here.

Results from the contrived HPV 16 specimens showed a slightly lower percent agreement to the expected result for HPV 16 high negative specimens. As shown in Table S2, the majority of the discordance involving the high negative HPV16 contrived specimens was based on a difference in one location (Site 3) and one lot (lot 2). It is not clear that these two instances represent a true depiction of Onclarity assay performance for differentiating HPV 16 negative specimens from HPV 16 positive specimens around the cut-off. The clinical cutoff Ct value for HPV16 (38.3) is approximately four cycles higher than that for the other eight Onclarity results (34.2), which may explain the low reproducibility of HPV16 high negative specimens relative to the other genotype results. However, the Onclarity assay has been clinically validated for HPV 16 detection and previous results for HPV 16 from screening populations have demonstrated good specificity and positive predictive values for detection of the individual HPV 16 genotype. In addition, the high reproducibility results observed here for the Onclarity assay regarding
contrived and pooled clinical specimens are consistent with previous work. Ejegod and colleagues demonstrated high reproducibility with good intra-laboratory agreement (98.6%) and kappa value (0.967) and good inter-laboratory agreement (98.4%) and kappa value (0.962) for Onclarity assay positive/negative results from specimens collected in PreservCyt® LBC media in a subset of an English screening populations. (12) Similarly, Ejegod and colleagues observed good intra- and inter-laboratory reproducibility with the Onclarity assay from specimens collected in SurePath LBC medium from a Danish population. (13) In this study, the greatest variation for pooled clinical specimen results was observed within Onclarity assay runs. This was not unexpected as LBC specimens are inherently non-homogeneous. They are composed of sheaths of sloughed-off, exfoliated cells that are stored in fixative, which can lead to clumping. For viral signal, this is further exacerbated by the focal nature of HPV infections, often representing just a small fraction of the total cell population in a specimen. All other factors, including between run, between operator, between site, between reagent lot, and between days showed relatively low variation in results compared to the within run results.

The Onclarity assay is an FDA-approved and CE-marked HPV test, for which, clinical validation has previously been established. (5, 11-15, 17, 20-32) In accordance with Meijer criteria (6) for a clinically validated HPV assay, the Onclarity assay has been shown to be non-inferior to HC2 (≥90% of HC2 sensitivity and ≥98% of HC2 specificity). (11, 12) In addition, since 2011, the Onclarity assay has been compared with and validated against other established HPV assays in large screening/opportunistic screening population studies, (12, 13, 15, 21, 28, 30, 31) clinical comparison studies, (11-13, 20) studies involving referral populations, (20, 22-24, 27, 29, 32, 33) and in studies involving large repositories of well-characterized specimens (5, 26) derived from
populations from numerous countries including the USA, Belgium, Denmark, Italy, England, Japan, and Mexico. Clinical and analytical performance of the Onclarity assay for extended genotyping has been previously established in studies using comparators, including sequencing-based assays.\(^{(5, 20, 24, 29, 31, 32, 34)}\)

The Onclarity assay is the only HPV assay approved for extended genotyping (reporting of individual genotypes 16, 18, 31, 45, 51, and 52, and reporting of grouped results for 33/58, 35/39/68, and 56/59/66) in the primary screening population (≥25 years of age). Importantly, Onclarity assay provides coverage of the three HPV genotypes (HPV16, 18, and 45) that represent approximately 77% of invasive cervical cancer\(^{(35)}\) and for adenocarcinoma, which has previously been difficult to detect by cytology-based screening alone.\(^{(36)}\) Wright et al. (2019) demonstrated comparable sensitivity and specificity for Onclarity and HC2 for ≥CIN2 and ≥CIN3 in the ASCUS triage population.\(^{(23)}\) In addition, Stoler et al. (2019) demonstrated comparable sensitivity and specificity for Onclarity and HC2 for ≥CIN2 and ≥CIN3 in the cotesting population.\(^{(15)}\) Here we observed a high concordance for HPV 16, 18 and 45 with the expected result for contrived specimens corresponding to low and medium positive, and high negative concentrations.

In addition to the >95% agreement with expected results for HPV 16, 18, and 45 positive contrived specimens, the mean Ct scores of individual results for HPV 16, 18, 45, 31, 33/58, and 52 from HPV positive, pooled clinical specimens also demonstrate the reproducibility of the Onclarity assay. In addition, 100% HPV negative specimens (clinical matrix only) were associated with Ct values above the cutoff for a positive result (38.3 on the HPV 16 channel and...
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356 34.2 for channels relative to the other eight HPV channels). This reproducibility is important as countries in North America, Europe, Australia, and Asia continue to consider extended and full genotyping as a triage approach to improve risk detection for high-grade cervical disease during HPV primary screening. Publications from both the Onclarity clinical trial and Kaiser Permanente Northern California have previously demonstrated the potential benefit of extended genotyping to identify either those with NILM cytology as high-enough at risk for a referral to colposcopy (e.g., those with NILM cytology and positive for HPV 16 or 31), or those with ASCUS/LSIL as low enough risk to return for follow up as opposed to a referral to immediate colposcopy (e.g., with ASCUS/LSIL cytology and positive for HPV 56). (21, 22, 32) A recent systematic review outlines further evidence for extended/full genotyping as an effective means for triage in both USA-based populations and populations outside the USA. (37)

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368 As many HPV assays are nucleic acid amplification based, pre-cytology aliquot specimens are typically preferred for HPV testing prior to LBC processing. However, current cervical cancer screening recommendations, both inside and outside the USA, vary based on the age of the screening population (among other factors including prior screening/treatment status).

373 Approaches to cervical cancer screening also vary from country to country. In Europe, for example, approximately 55% of countries utilize cytology as the primary screening modality, with 45% of countries utilizing some combination of cytology and HPV testing. (9) Depending on country or region, specimens for HPV testing could be aliquoted either before or after the specimen is processed for cytology. Therefore, it is important to understand how pre-cytology aliquot and post-cytology aliquot specimens may or may not vary for HPV assay performance.

377 The overall agreement of the results from the Onclarity assay using pre-cytology aliquot and
post-cytology aliquot specimens, representing three cervical cancer screening populations, was high. However, the discordant results that were observed are not unexpected, especially in samples close to the cut-off of the Onclarity assay. As discussed above, LBC specimens are inherently non-homogeneous, which warrants confirmation of pre- and post-cytology analysis to confirm within-specimen consistency. Although specimens are not routinely tested twice; laboratories may test either before or after cytology, depending on their preferred workflow and standard of care screening paradigm (e.g., co-testing versus HPV primary screening). In addition, lower agreement was observed in the pre- and post-cytology results in the NILM cytology group. NILM cytology positive for HPV *a priori* represents early or receding infections; thus, enrichment is likely occurring in this cytology group for HPV-positive results that are close to the clinical cut-off of the assay. Qualitative HPV assays do show more variability at low infection levels.

Here the collection device had no overall impact on the HPV result. In addition, there was no observed effect of collection device type across age groups and, therefore, either collection device should be effective for different screening populations (ASCUS triage, ≥21 years; cotesting ≥30 years; ≥25 years). The squamocolumnar junction is an anatomical area in which cellular transformation occurs at a high rate and is a common region in which abnormal cells develop. With age, the cervical transformation zone (including its distal edge—the squamocolumnar junction or SCJ) recedes into the cervical canal,(27) which renders LBC collection from the SCJ challenging. Here, the choice of collection device did not impact the ability to detect HPV for the ≥40 and ≥50 year age groups.
4.1 Limitations

Clinical specimens used in this study were obtained from the Onclarity Trial, a large cervical cancer screening trial conducted in the USA, which has been described previously. Therefore, some aspects of bias or imprecision associated with the experimental design or procedures related to the Onclarity Trial may apply to these analyses. These would include some types of partial verification bias when stratifying results by age or cytology result. This was addressed for results from the Onclarity trial previously through statistical methodology to adjust for verification bias, which was not conducted during stratification by cytology results for pre- and post-cytology and collection device analyses. In addition, classification bias, due to a lack of a true reference for pre- and post-cytology and collection device analyses on HPV detection may have led to some inaccuracies in our results here. Some form of analytic bias could have occurred here, especially between study sites, which has not been explained and may have impacted our results (for example, results for the high negative HPV 16 specimens). Finally, as discussed above, regarding the HPV 16 high negative results, bias could have affected the accuracy of our results for HPV 16 compared to the other eight HPV results as the difference in HPV signal/negative (38.3 < Ct < 40) and signal/positive (Ct ≤ 38.3) is smaller (the cutoff is closer than the limit of detection) than that for the other eight HPV results with a signal/negative of (34.2 < Ct < 40) and a signal/positive of (Ct ≤ 34.2). Finally, histological outcomes were not used here to determine whether how Onclarity assay results involving clinical specimens corresponded to performance as compared to histological outcomes. However, the objective of this study was to determine the analytical performance of the Onclarity assay using clinical and contrived specimens, irrespective of the ability of the Onclarity assay to detect cancer or pre-
cancer. The clinical performance of the Onclarity assays, compared to histology reference, has been described extensively, elsewhere. (11, 15, 20, 23)

5. Conclusion

Overall, the results here characterize the impact of pre-analytical activities on Onclarity assay reproducibility and provide evidence for the potential flexibility of the Onclarity assay within different workflows during cervical cancer screening. This includes sample collection devices, aliquoting order and other laboratory workflow practices. Regardless of each of these factors, the results obtained with the Onclarity assay on the Viper-LT were robust and reproducible.
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All authors provided final approval of the manuscript and agree to be accountable for the accuracy and integrity of this work.
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REFERENCES


Performance of Onclarity HPV Assay  Young et al., 2020  JCM


FIGURE LEGEND

Figure 1. Contrived specimens positive for HPV 16, 18, and 45 were tested with the Onclarity assay. The contrived specimens were prepared at concentrations categorized as “low positive”, “moderate positive”, and “high negative”; all three are characterized relative to the clinical cutoff. Results from the Onclarity assay for each of the three contrived sample groups were compared to the expected results. An HPV negative group was included with the high negative contrived sample run.

Figure 2. Pre- and post-cytology aliquot specimens obtained from the baseline screening phase of the Onclarity trial. The two sample types were tested by the Onclarity assay and the results (either HPV16, 18, 31, 33/35, 45, 51, 52, 35/39/68, or 56/59/66) were plotted by mean Ct score for pre-cytology aliquot on the x-axis and post-cytology aliquot on the y-axis. The solid line represents the regression line for best-fit across the data points.

Figure 3. Specimens were obtained from participants of the Onclarity trial with either the Cervex-brush or the Cytobrush (or Cytobrush/spatula). (a) Results (mean Ct score) are plotted for both types of collection devices and are stratified by age group. (b) Positivity rate from the specimens in (a) are shown for each collection device and stratified by age group.
Table 1. Mean cycle threshold scores for pooled clinical specimens

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group number</th>
<th>Mean Ct</th>
<th>Within Run SD</th>
<th>Within Run %CV</th>
<th>Between Runs SD</th>
<th>Between Runs %CV</th>
<th>Between Operators SD</th>
<th>Between Operators %CV</th>
<th>Between Sites SD</th>
<th>Between Sites %CV</th>
<th>Between Reagent Lots SD</th>
<th>Between Reagent Lots %CV</th>
<th>Between Days SD</th>
<th>Between Days %CV</th>
<th>Total SD</th>
<th>Total %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16</td>
<td>156</td>
<td>35.22</td>
<td>1.52</td>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.29</td>
<td>0.8</td>
<td>0.32</td>
<td>0.9</td>
<td>1.57</td>
<td>4.5</td>
</tr>
<tr>
<td>HPV 18</td>
<td>156</td>
<td>30.47</td>
<td>1.08</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>0.08</td>
<td>0.3</td>
<td>0.3</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.11</td>
<td>3.8</td>
</tr>
<tr>
<td>HPV 45</td>
<td>156</td>
<td>33.35</td>
<td>1.76</td>
<td>5.3</td>
<td>0.24</td>
<td>1.0</td>
<td>0</td>
<td>0.25</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.83</td>
<td>5.5</td>
</tr>
<tr>
<td>HPV 31</td>
<td>156</td>
<td>33.21</td>
<td>1.81</td>
<td>5.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.51</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.86</td>
<td>5.6</td>
</tr>
<tr>
<td>HPV 33/58</td>
<td>156</td>
<td>30.73</td>
<td>1.38</td>
<td>4.5</td>
<td>0.20</td>
<td>0.7</td>
<td>0</td>
<td>0.12</td>
<td>0.4</td>
<td>0.19</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.41</td>
<td>4.6</td>
</tr>
<tr>
<td>HPV 52</td>
<td>156</td>
<td>30.98</td>
<td>0.73</td>
<td>2.4</td>
<td>0.24</td>
<td>0.9</td>
<td>0</td>
<td>0.33</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.87</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Abbreviations: Ct, cycle threshold; SD, standard deviation; %CV, percent coefficient of variation; HPV, human papillomavirus

*100% HPV negative specimens (clinical matrix only) were associated with Ct values above the cutoff for a positive result (38.3 on the HPV16 channel and 34.2 for channels relative to the other eight HPV results.)
Table 2. Percent agreement for Onclarity assay results between pre- and post-cytology aliquot specimens.

<table>
<thead>
<tr>
<th>Population</th>
<th>Positive percent agreement (95% CI)</th>
<th>Negative percent agreement (95% CI)</th>
<th>Overall percent agreement (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NILM (≥30 years)</td>
<td>86.0% (80.3, 90.3)</td>
<td>99.3% (98.9, 99.5)</td>
<td>98.3% (97.8, 98.8)</td>
</tr>
<tr>
<td>ASCUS (≥21 years)</td>
<td>100% (95.5, 100)</td>
<td>97.7% (93.5, 99.2)</td>
<td>98.5% (96.8, 99.5)</td>
</tr>
<tr>
<td>&gt;ASCUS (≥21 years)</td>
<td>97.6% (91.8, 99.4)</td>
<td>100% (83.2, 100)</td>
<td>98.1% (93.3, 99.5)</td>
</tr>
<tr>
<td>Primary Screening (≥25 years)</td>
<td>91.0% (87.1, 93.4)</td>
<td>99.0% (98.6, 99.3)</td>
<td>98.1% (97.6, 98.5)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; ASCUS, atypical squamous cells-undetermined significance; NILM, negative for intraepithelial lesions or malignancies.
Table 3. Onclarity assay—mean cycle threshold scores for pre- and post-cytology aliquot specimens

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group number</th>
<th>Post-cytology mean Ct</th>
<th>Pre-cytology mean Ct</th>
<th>Mean difference (post-cytology – pre-cytology)</th>
<th>Lower 95%CI</th>
<th>Upper 95%CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled</td>
<td>773</td>
<td>30.31</td>
<td>30.25</td>
<td>0.06</td>
<td>-0.06</td>
<td>0.18</td>
<td>0.3298</td>
</tr>
<tr>
<td>HPV 16</td>
<td>77</td>
<td>30.19</td>
<td>30.33</td>
<td>-0.14</td>
<td>-0.51</td>
<td>0.22</td>
<td>0.4353</td>
</tr>
<tr>
<td>HPV 18</td>
<td>34</td>
<td>31.55</td>
<td>31.25</td>
<td>0.31</td>
<td>-0.32</td>
<td>0.94</td>
<td>0.3299</td>
</tr>
<tr>
<td>HPV 45</td>
<td>42</td>
<td>32.58</td>
<td>32.65</td>
<td>-0.07</td>
<td>-0.75</td>
<td>0.62</td>
<td>0.8462</td>
</tr>
</tbody>
</table>

**Abbreviations:** Ct, cycle threshold; CI, confidence interval; HPV, human papillomavirus

*a Mean Ct results for the total study population (>21 years of age) for all nine, combined HPV genotype channels (three channels for each of three wells)  
*b 100% HPV negative specimens (clinical matrix only) were associated with Ct values above the cutoff for a positive result (38.3 on the HPV16 channel and 34.2 for channels relative to the other eight HPV results.)*
### Table 4

Onclarity assay positivity rate following collection by either Cytobrush/spatula or Cervex-brush

<table>
<thead>
<tr>
<th>Population</th>
<th>Number positive / total</th>
<th>Percent</th>
<th>Number positive / total</th>
<th>Percent</th>
<th>Fisher Exact test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCUS (≥21 years)</td>
<td>381 / 989</td>
<td>38.5%</td>
<td>382 / 964</td>
<td>39.6%</td>
<td></td>
<td>0.6428</td>
</tr>
<tr>
<td>NILM (≥30 years)</td>
<td>895 / 11,145</td>
<td>8.0%</td>
<td>866 / 11,139</td>
<td>7.8%</td>
<td></td>
<td>0.4869</td>
</tr>
<tr>
<td>Primary Screening (≥25 years)</td>
<td>1,888 / 14,858</td>
<td>12.7%</td>
<td>1,860 / 14,654</td>
<td>12.7%</td>
<td></td>
<td>0.9721</td>
</tr>
<tr>
<td>Age 21-24 years</td>
<td>597 / 2,023</td>
<td>29.5%</td>
<td>567 / 1,897</td>
<td>29.9%</td>
<td></td>
<td>0.8066</td>
</tr>
<tr>
<td>Age 25-29 years</td>
<td>603 / 2,805</td>
<td>21.5%</td>
<td>613 / 2,626</td>
<td>23.3%</td>
<td></td>
<td>0.1036</td>
</tr>
<tr>
<td>Age 30-39 years</td>
<td>679 / 4,854</td>
<td>14.0%</td>
<td>631 / 4,643</td>
<td>13.6%</td>
<td></td>
<td>0.5319</td>
</tr>
<tr>
<td>Age 40-49 years</td>
<td>362 / 3,667</td>
<td>9.9%</td>
<td>358 / 3,741</td>
<td>9.6%</td>
<td></td>
<td>0.6663</td>
</tr>
<tr>
<td>Age ≥50 years</td>
<td>244 / 3,552</td>
<td>6.9%</td>
<td>258 / 3,644</td>
<td>7.1%</td>
<td></td>
<td>0.7461</td>
</tr>
</tbody>
</table>

**Abbreviations:** ASCUS, atypical squamous cells-undetermined significance; NILM, negative for intraepithelial lesions or malignancies.
Figure 1

Percent of results concordant with expected results

- Low positive
- Moderate positive
- High negative

Values: n=159, n=156, n=156, n=158
Figure 2

Parameter Estimates
Intercept = 1.5271
Slope = 0.9515

R-Square = 0.90