Clinical and analytical performance of the BD Onclarity HPV Assay with SurePath screening samples from the Danish cervical screening program using the VALGENT framework

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Running title: Accuracy of Onclarity HPV assay using SurePath samples

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Abstract

The VALidation of HPV GENotyping Tests (VALGENT) framework is an international cooperation designed to evaluate HPV assays with genotyping capabilities. Here we assessed the performance of the BD Onclarity assay using Danish SurePath cervical screening samples collected under the fourth VALGENT instalment, constituting 998 consecutive samples from a screening population and 297 enriched samples with abnormal cytology (100 ASCUS, 100 LSIL, 97 HSIL). The Onclarity assay individually detects six HPV genotypes (16, 18, 31, 45, 51, 52) and eight genotypes in three bulks (33/58, 56/59/66, 35/39/68). The clinical performance of the Onclarity assay for detection of \( \geq \text{CIN2} \) and 2xNILM was assessed relative to that of the GP5+/6+ PCR Enzyme ImmunoAssay (GP-EIA) by a non-inferiority test. The relative sensitivity for \( \geq \text{CIN2} \) was 1.00 (95% CI: 0.97-1.04) and relative specificity for 2xNILM was 1.04 (95% CI: 1.02-1.06). The Onclarity assay was found to be non-inferior to GP-EIA for both sensitivity (p=0.0006) and specificity (p<0.0001). The type specific performance of the Onclarity was also assessed, using the GP5+/6+ PCR with Luminex genotyping (GP-LMNX) as comparator. Onclarity showed good concordance for almost all HPV genotypes groups. A stability analysis of SurePath samples was also performed, where a SurePath aliquot was stored for 7 months refrigerated and the internal control of the Onclarity assay was used as a marker for cellularity. The Ct value was the same (24.8) in the first and second Onclarity run (24.8), showing that SurePath samples can be stored refrigerated for 7 months and still remain a valid test specimen.
Introduction

Careful clinical validation of Human papillomavirus (HPV) assays is increasingly important as primary HPV screening is replacing cytology-based cervical cancer screening. The clinical performance of any HPV test in cervical screening relies on its ability to detect infections associated with cervical intraepithelial neoplasia of grade 2 or worse (≥CIN2) without over-diagnosing clinically irrelevant HPV infections. In 2009, Meijer et al. established guidelines and requirements which novel HPV tests must comply with for clinical sensitivity, specificity and reproducibility in order to be used for primary clinical screening (1). Compared to the number of commercially available assays today, a surprisingly small number of HPV assays are clinically validated with the International criterion (2-4). HPV assay development over the last decade has led to a host of PCR based assays reporting high-risk HPV (hrHPV) findings with various degrees of data resolution. To this end, HPV assays can be stratified into four categories. 1) Consensus assays which only report positive or negative outcome measuring presence of 13 or 14 most common oncogenic HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, & 68). 2) Consensus assays with limited genotyping reporting, often for HPV16, HPV18. 3) HPV assays with extended genotyping typically HPV16, 18 combined with more, but not all the oncogenic genotypes. 4) Full genotyping assays with individual reporting of 14 oncogenic HPV genotypes. Hybrid Capture 2 assay (HC2; Qiagen, Hilden, Germany) and GP5+/6+-PCR enzyme immunoassay (GP-EIA; DDL Diagnostic Laboratory, Rijswijk, the Netherlands) were considered as standard comparator assays since both have demonstrated longitudinal evidence of protection against cervical precancer and cancer through randomized trials (5, 6). The international validation criterion evaluates assay performance against one of these two standard comparator assays (1) for combined detection of 13 or 14 hrHPV genotypes but does not allow for more advanced performance evaluation at the
level of individual assay detected hrHPV genotypes, and given the technology development since 2009 in the field of HPV screening and diagnostics, this represents a limitation of the original International guidelines. Given the increasing amount of commercially available HPV assays with various degrees of genotype detection capabilities (1), it is imperative that assay performance is assessed robustly with well annotated cervical samples that are representative for a screening population.

The VALGENT (VALidation of HPV GENotyping Tests) framework represents an international collaboration designed to evaluate the comparative performance of HPV assays with genotyping capacity for use in primary cervical cancer screening [7–13]. The VALGENT validation panels furthermore take into account different sample collection media and include samples from women attending routine screening enriched with cytological defined abnormal samples (7, 8). In order to allow comparison with other HPV assays, each VALGENT panel includes a comparator assay that is clinically validated for cervical screening. Detailed objectives and study design of VALGENT4 and previous VALGENT panels are published elsewhere (7, 8).

Up till now four VALGENT panels have been collected from the Belgian (VALGENT1) (9-11), Scottish (VALGENT2) (12-15), Slovenian (VALGENT3) (16-20), and Danish (VALGENT4) cervical cancer screening programs (8). The fourth instalment of the VALGENT framework (8), VALGENT-4, specifically comprises a panel of SurePath collected samples as to date the majority of clinical validated HPV assays for use in screening had been undertaken on ThinPrep collected samples.

Here we present the clinical validation of the BD Onclarity HPV (Onclarity, BD Diagnostics, Sparks, MD, US) assay using Danish SurePath cervical screening samples from the fourth installment of the VALGENT panel. The Onclarity assay is an extended genotyping assay providing individual...
detection of six HPV genotypes (16, 18, 31, 45, 51, 52) and identification of additional eight
genotypes in three bulks (33/58, 56/59/66, 35/39/68). The validation is performed using the
International guidelines, using the GP-EIA assay as a comparator for clinical evaluation. In addition,
the stability of stored SurePath cervical samples for HPV analysis were assessed over a 7 months
period.

**Material and Methods**

**Sample collection and histological follow-up**

The sample collection process has been described in detail elsewhere (8). In short, the VALGENT4
panel was collected from women participating in the Danish cervical cancer screening program at
the Department of Pathology, Hvidovre Hospital, Denmark (The Parent laboratory). The VALGENT
panel is standardized (7), here comprising 998 consecutive screening samples from the routinely
screened Danish population (The Screening population) as well as a disease-enriched component
with cytological abnormalities (100 atypical squamous cells of undetermined significance (ASCUS),
100 low-grade squamous intraepithelial lesion (LSIL), 97 High-SIL (HSIL), The Enriched population).
All samples were collected in SurePath medium. All cytology and histology procedures were
performed at the Parent laboratory as described before (8) and all clinical follow-up was managed
according to the Danish guidelines, and the outcome of the VALGENT4 HPV testing did not affect
the clinical outcome assessment. Subsequent histological follow-up, if any, on women included in
the VALGENT4 study was retrieved from the Danish PatoBank.

**Comparator assay testing**
DNA was extracted from the VALGENT4 panel samples as described previously (8) and shipped to DDL Diagnostics Laboratory (Rijswijk, the Netherlands), where all GP5+/6+-PCR testing was performed. The clinically validated hrHPV GP-EIA assay for pooled detection of 14 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) oncogenic types was used as comparator for clinical performance of the Onclarity assay. For genotype concordance analysis, a Luminex-based readout was used (GP-LMNX) for individual genotyping of the 14 HPV types (14). Mean number of days from sample reception date to DNA extraction was 27 days (range 11-71). GP-LMNX testing on biobanked DNA aliquots was completed 685 days after sample reception. The GP-EIA testing was subsequently performed and completed on the GP5+/6+ amplicons 1008 days after sample reception. The samples were stored refrigerated.

The BD Onclarity HPV assay

The Onclarity HPV assay is a real time PCR DNA assay targeting the E6/E7 genome, which detects 14 oncogenic HPV genotypes in nine genotype readouts (16, 18, 31, 45, 51, 52, 33/58, 56/59/66, 35/39/68). The assay harbors an internal Human Beta globin control (HBB) for sample adequacy and assay performance. A specimen was considered adequate when the HBB CT value of <34.2 or any hrHPV could be identified. The full Onclarity assay workflow on the automated Viper Lt has been described in detail previously (21). In short, 0.5 ml of original resuspended SurePath material was transferred to a BD tube containing 1.7 ml CBD medium. The samples were pre-warmed at 120°C for 30 min, prior to being transferred to the fully automated Viper Lt platform where the samples were tested with the Onclarity HPV Assay according to manufacturer’s recommendations. All Onclarity testing was performed at the Parent Laboratory. Mean number of days from the
samples was received at the laboratory to Onclarity testing was 28 days (range 2-70 days). The samples were stored refrigerated prior to testing.

Stability of SurePath collected cervical samples for analysis of HPV

In addition to the baseline Onclarity analysis, 0.5 ml of SurePath material from 1212 samples was aliquoted into a separate Eppendorf tube and refrigerated (4°C) for 7 months until testing with the Onclarity assay on the Viper Lt platform. Eighty-five samples did not have enough material for the stability aliquot. The stability and cellularity of the VALGENT4 panel was assessed using values from the individual sample’s internal control of the Onclarity assay as a marker for cellularity, and analytical stability over time. The Onclarity has a three-well design with nine genotype readouts, the internal HBB control is included in each well.

Data analysis

A sample was considered Onclarity positive for one of the nine genotype groups if the Ct value was below 34.2 according to manufacturer’s recommendations. For GP-EIA and GP-LMNX, a sample was reported positive if at least one of the 14 genotypes reported by Onclarity was detected. The level of genotype agreement between Onclarity and GP-LMNX was determined by using kappa statistics. In addition, if a sample was GP-LMNX positive for HPV33 and HPV58, the infection counted only once in the 33/58 pool, the same was true the HPV56/59/66 and HPV35/39/68 pools.

For clinical validation, women with confirmed CIN grade 2 or worse (≥CIN2), CIN grade 3 or worse (≥CIN3), or cervical cancer within 33 months (range 32-35) after sample collection were classified as having high-grade disease (the diseased population). Women with two consecutive Negative for
Intraepithelial Lesion or Malignancy (NILM) cytology outcomes at enrolment and at 12-24 months prior were classified as having no evidence of disease (2xNILM Control population). The accuracy of the Onclarity assay for detection of ≥CIN2 and ≥CIN3 was assessed and compared to the GP-EIA assay as comparator for clinical performance. Non-inferiority of the Onclarity assay compared to the GP-EIA assay was assessed statistically with a score test for matched proportions, using 0.90 and 0.98 as benchmarks for relative sensitivity and specificity, respectively (1, 22).

Testing result for Onclarity, GP-EIA and GP-LMNX was sent to the Unit of Cancer Epidemiology, Sciensano (Brussels) for statistical analysis, which were performed using STATA version 14 (College Station, TX, USA).

For stability analysis, the Ct-value of the HBB was calculated as an average of the three individual HBB Ct values for baseline test versus 7 months end-point test.

**Ethical approval**

Sample collection and data retrieval for VALGENT4 is approved by the Danish Data Inspection Agency J. No. AHH-2017-024, I-Suite: 05356. EU-GDPR compliant data handler agreement was established between the principle site Copenhagen University Hospital, Denmark, and Sciensano, Belgium, for the data analysis. All collected samples were cross-referenced and found eligible with the Danish register relating to the collection, storage and use of human biological material in health research projects (Vævsanvendelsesregistret).
Results

Demographic, pathology and HPV characteristics of the VALGENT4 panel

When the entire VALGENT4 cohort was considered (the Screening plus Enriched populations together), the average age was 42.2 (range 30-59). Cytological stratification of the Screening population was; 947 NILM, 6 ASCUS, 21 LSIL, 24 HSIL/atypical glandular cells (AGC)/atypical squamous cells- cannot exclude HSIL (ASCH)/Adenocarcinoma in situ (AIS) (see Table 1, average age: 42.8, range 30-59). In the enriched population the average age was 40.4, range 30-59.

Histological follow-up retrieval revealed 122 women with ≥CIN2 histological follow-up, the majority of the ≥CIN2 cases was derived from the Enriched population (N=109). A total of 897 women had two consecutive cytology NILM smears and was used for the specificity calculations (2xNILM).

In the Screening and Enriched population, the Onclarity assay had a prevalence of 11.3% and 85.9%, respectively (Total population: 28.4%, Table 1). For comparison the GP-EIA assay had a prevalence of 14.3% and 85.2% in the Screening and Enriched population, respectively (Total population: 31.7%). The HPV positivity for Onclarity in the screening population was highest in women age 30-39 (16.4%) and lower in women age 40-49 (7.6%) and 50-59 (9.2%). The Prevalence of HPV genotypes in the screening population for HPV16, 52, 31, 18, 45, and 51 was 1.8%, 1.7%, 1.2%, 1.1%, 1.1%, and 0.8% respectively (decreasing order, Table 2). The prevalence of the three bulk genotype groups was: 2.3, 2.3, and 1.7 % for 56/59/66, 35/39/68, and 33/58, respectively (Table 2). The GP-LMNX assay had the highest prevalence for seven out of nine HPV genotype groups in the normal cytology samples, whereas the Onclarity had the highest overall HPV prevalence for ASCUS, LSIL and HSIL samples for most genotype groups. (Table 3).
Clinical performance of the Onclarity assay

Table 4 presents the cross tabulations of the Onclarity and comparator assay GP-EIA result for the ≥CIN2 cases, ≥CIN3 cases for the sensitivity and for 2xNILM subjects for specificity calculations.

Onclarity reported 113 out of the 122 ≥CIN2 cases positive, (sensitivity: 92.6%, CI: 86.5-96.6) for comparisons, the GP-EIA assay detected also 113/122 (sensitivity: 92.6%, CI: 86.5-96.6). The relative ≥CIN2 sensitivity was 1.00 (0.97-1.04). Onclarity detected 80 out of 83 ≥CIN3 cases (sensitivity: 96.4%, CI: 89.9-99.2) and GP-EIA 78/83 (sensitivity: 94.0%, CI: 86.5-98.0). The relative sensitivity for ≥CIN3 was 1.03 (0.99-1.06). The Onclarity was found to be non-inferior to that of GP-EIA for ≥CIN2 (p=0.0006) and ≥CIN3 (p<0.0001) sensitivity. A total of 897 2xNILM cases were found, Onclarity called 831 of them negative (specificity 92.6%, CI: 90.7-94.3) while GP-EIA found 800/897 negative (specificity: 89.2%, CI: 87.0-91.1). The relative specificity was 1.04 (1.02-1.06).

The Onclarity assay was found to be non-inferior to GP-EIA for specificity (p<0.0001).

HPV genotyping concordance between Onclarity and GP-LMNX

The hrHPV agreement between Onclarity and GP-LMNX was 93.6% (Kappa: 0.85) for the total VALGENT4 population, for the Screening population the agreement was 93.1% (Kappa: 0.71) and for the Enriched population 95.3% (kappa: 0.81), Table 5. The genotype concordance in the whole VALGENT population was good (k between 0.80-0.73) to excellent (k >0.80), with Kappas ranging from 0.73 to 0.90). When stratifying the genotype detection by Onclarity and GP-LMNX by VALGENT4 subsets the concordance was better for the Enriched population with kappa range 0.79 to 0.98) compared to the Screening population (Kappa range 0.56 to 0.86). The kappa for almost all genotype groups were higher in the Enriched population compared to the Screening population and only for HPV18 the concordance was below 0.6 and was limited to the Screening population.
Analytical stability of SurePath screening samples

Of the 1295 samples, a total of 1212 had sufficient material for the stability testing. Aliquots for this were stored refrigerated for 7 months after baseline testing prior to a 2nd test. Cytology for the 1212 included samples were 894 NILM and 318 cytology ≥ASCUS. In total, 1188 out of 1212 (98.0%, CI: 97.0-98.7) samples were reproducible after retesting at 7 months. Out of 24 discordant samples, 13 went from HPV positive to HPV negative whereas 11 samples went from negative to HPV positive after the second test. Furthermore, mean Ct values of the internal control HBB were similar between the baseline test (mean Ct= 24.8, CI: 24.71-24.89) and 2nd test at 7 months (Ct=24.8, CI: 24.70-24.91, p=0.96). No differences in Ct values detecting individual or bulk genotypes were observed between first and second test (data not shown).

Discussion

In the present analysis, the clinical and type specific performance of the Onclarity HPV assay was assessed. The Onclarity assay has the capacity to detect six individual oncogenic HPV genotypes, whereas the remaining eight oncogenic HPV genotypes are detected in three groups. The Onclarity assay showed similar clinical sensitivity (relative sensitivity: 1.00, CI: 0.97-1.04) and slightly better specificity (relative specificity: 1.04, CI:1.02-1.06,) than that of the standard comparator assay GP-EIA for the detection of ≥CIN2. The Onclarity assay was shown to be non-inferior to the comparator assay GP-EIA for both sensitivity (p=0.0006) and specificity (p<0.0001). The hrHPV concordance between Onclarity and GP-LMNX was high when assessed at the level of 14 oncogenic HPV genotypes combined as well as for all nine genotype groups (Table 5). The concordance was higher when considering only the disease enriched population compared to the screening population subset, for both overall oncogenic HPV detection and at individual genotype
level (Table 4). This is similar to previous studies looking at concordance between HPV assays, showing that HPV assays have better agreement in samples from women with disease than in screening samples (23, 24).

Some of the discordance observed, can be explained based on the assay technologies. The Onclarity HPV is a DNA assay targeting the E6/E7 gene and has an amplification target range from 79 to 137 base pairs whereas the GP-EIA and GP-LMNX targets the L1 gene and has a target amplification of 150 base pairs. Furthermore, the Onclarity assay is a real time PCR assay, whereas the GP-LMNX assay is a PCR-based assay with subsequent Luminex detection. Together, these assay specification differences can to some extent impact detection, especially in samples with a viral load close to the individual assay cut off between positive and negative.

The Onclarity assay has previously been validated using the VALGENT2 panel, which consisted of ThinPrep screening samples from the Scottish cervical cancer screening program (7, 13); here the GP-EIA assay was also used as a comparator (13). In VALGENT2 the Onclarity assay was shown to be non-inferior to that of GP-EIA for sensitivity (p=0.001) but not for specificity (p=0.186). Cuschieri et al. (13) found that the Onclarity assay detected more infections than GP-EIA but not significantly more ≥CIN2 cases. This was not observed in our study where Onclarity detected fewer HPV infections than GP-EIA and equal amount of ≥CIN2 cases. Cuschieri et al. noted that VALGENT2 panel included samples from women below the age of 30 and speculated that the high HPV prevalence of the Scottish population (18%) could negatively impact specificity calculations. For comparison, we only included samples from women ≥30 years of age, and the hrHPV prevalence of the screening population subset of the Danish VALGENT4 panel was 11%.
The Onclarity assay has also been validated using the international guidelines (1) in both ThinPrep (25) and SurePath (21) collected screening samples. In both studies the Onclarity assay fulfilled the international guidelines for both specificity and sensitivity and included only samples from women 30 years and above (21, 25).

In this study, a more detailed analysis of the individual genotype detection by Onclarity and GP-LMNX, showed good concordance for all genotype groups (Table 5) except for HPV18, 45 and 51 infections found in the screening population subset where the GP-LMNX assay detected more infections than the Onclarity assay. For the enriched population subset, the concordance was higher for all genotypes, with the lowest concordance observed for HPV52, HPV56/66/68 and HPV35/39/68. Here the Onclarity detected more HPV52 and HPV56/59/66 infections and less HPV35/39/68 infections compared to GP-LMNX (Table 5). In addition, the GP-LMNX detected more HPV16 and 18 infections in NILM cytology samples compared to the Onclarity assay (Table 3).

The clinical impact of these observations goes to detail the ability of the Onclarity assay to precisely detect clinically relevant HPV infections at the individual genotype level. This information can be used with advanced screening algorithms where individual follow-up recommendations are issued for women with HPV 16 and 18 found in screening samples, or as recently proposed by us (26) and shortly to be adopted in the Danish screening program, individual screening recommendations for samples positive for HPV16, 18, 31, 33 and 51 which has an overall higher risk of ≥CIN2 than the remaining HR genotypes (27, 28).

In this study we also evaluated the analytical stability of SurePath cervical screening samples for HPV testing and the reproducibility of sample adequacy of Onclarity after storage. This information is relevant as cervical screening samples are typically collected in one site and shipped...
for analysis at a laboratory; the timeframe between those events are defined by geography and logistic infrastructure. Moreover, the ability to reproduce a baseline test outcome also has relevance for quality procedures, where samples can be tested after a period of time i.e. for quality control or audit purposes. The adequacy of SurePath LBC for molecular HPV analysis has in the past been questioned on the premise that it contains a low concentration of formaldehyde in addition to the alcohol fixative to ensure adequate preservation of the cells for cytology evaluation. The cause of concern is the formalin that induces cross links between DNA and protein (29-31). The analytical stability of SurePath and Onclarity reproducibility was tested on an aliquot of the VALGENT4 samples stored at 4°C for 7 months after baseline testing. The 7 months’ time period chosen, greatly exceeds the period most routine laboratories will retain a sample for quality assurance purposes. Our argument is if stability can be proven for both human and HPV viral genomic material at such an extended period, discussions regarding the stability of SurePath collected samples intended for molecular HPV screening can cease. Here, data shows clearly that the stored SurePath samples were analytically stable and clinically reproducible by Onclarity HPV testing after 7 months storage at 4°C. Using the internal HBB control as a marker for analytical stability and cellularity the mean Ct at baseline was 24.8 (Std: 1.6) versus 24.8 (Std: 1.8) after storage. The overall reproducibility of baseline test results was 98.0 % with 1188 out of 1212 samples returning the same test result. Equally important, looking at the genotype outcomes reported, the baseline and post-storage showed no statistical differences either (Data not shown). The international validation of Onclarity on SurePath and ThinPrep previously reported hrHPV intra laboratory reproducibility of the Onclarity assay as 97.4% (21) and 98.6% (25), respectively. Supported further by the conclusions from Agreda et al. (32), we conclude that long time storage
of SurePath screening samples poses no analytical issue for HPV testing for at least 7 months storage in combination with the Onclarity HPV assay.

The strength of this study was that the samples were freshly collected cervical cancer screening samples from women age 30-59 years participating in the organized Danish national screening program. Onclarity HPV testing was done within weeks of samples collection (Mean: 4 weeks, range 2-70 days). A weakness discussed in the published protocol of Valgent4 (8) is the sample preparation protocol and aliquoting procedure which is off label for the Onclarity, GP-EIA and GP-LMNX assays. However, extensive quality assurance analysis showed that the resuspended samples contained sufficient material for testing which is reinforced by the observation that few samples were found assay invalid. The number of invalids by GP-LMNX (rate=0.54%) was not higher than observed in the previous VALGENT panels with invalid rates from 0.25% to 1.9% (13, 17, 18, 20). The relatively short follow-up period in VALGENT-4 of a maximum of 3 year is a limitation for interpretation of long-term safety of a negative screening result. However, since hrHPV GP-EIA originally was validated through randomized trials with follow-up currently reported at 14 years, the cross-sectional accuracy of this comparator test is well acknowledged for validation studies. The origin of the VALGENT-4 panels allows for a retrieval of longer follow up data from the Danish PatoBank at a later point in time, to provide information on long term safety.

In conclusion, the Onclarity assay has a high sensitivity for detection of ≥CIN2 and ≥CIN3 and a high specificity to exclude ≥CIN2 in SurePath screening samples and has demonstrated non-inferiority compared to standard comparator test GP-EIA in this LBC medium. The extended genotyping design allows for detailed information on the presence of HPV types including but not limited to HPV16 and 18; the precision of the genotyping detection was found to be at par with
Furthermore, the Onclarity test can safely be repeated for quality control or assurance purposes even after prolonged storage of sample. The results from this study confirm that the Onclarity test can be applied in primary cervical cancer screening when using SurePath collection medium.

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**Contributions**

M.A. coordinates the VALGENT frameworks. J.B and M.A. designed the study, H.P., D.M.E, performed the laboratory work, J.B, D.M.E. L.X and M.A. analyzed the data. J.B and D.M.E drafted the manuscript. All authors contributed to revisions of the manuscript, participated in the decision to submit, and had full access to all of the data in the study.

**Competing interest**

J.B. is the PI of studies funded in part by BD Diagnostics, Agena Bioscience, Genomica SAU, LifeRiver Biotech and QIAGEN. He has received honoraria for lectures from BD Diagnostics, Hologic, Roche Molecular Systems, QIAGEN and Genomica SAU. JB is an appointed member of the National Danish Cervical Screening Committee by the Danish Health Authority, and member of the Regional cervical screening steering committee of the Capital Region of Denmark.
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D.M.E. and H.P. attended meetings with various HPV test’ manufacturers

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Table 1: Characteristics of the study population of VALGENT4 panel, and prevalence of HR HPV assessed by Onclarity and GP-EIA

|                     | Total   | Onclarity assay | GP-EIA assay |
|---------------------|---------|-----------------|--------------|
|                     |         | HrHPV pos       | HrHPV pos    |
| All                 | 1295    | 368 (28.4%)     | 396 (30.6%)  |
| **Group**           |         |                 |              |
| Screening           | 998     | 113 (11.3%)     | 143 (14.3%)  |
| Enriched            | 297     | 255 (85.9%)     | 253 (85.2%)  |
| **Age**             |         |                 |              |
| 30-39               | 531     | 192 (36.2 %)    | 202 (38.0 %) |
| 40-49               | 519     | 126 (24.3 %)    | 136 (26.2 %) |
| 50-59               | 245     | 50 (20.4 %)     | 58 (23.7 %)  |
| **Cytology**        |         |                 |              |
| Normal              | 947     | 73 (7.7 %)      | 105 (11.1 %) |
| ASC-US              | 106     | 103 (97.2 %)    | 97 (91.5 %)  |
| LSIL                | 121     | 88 (72.7 %)     | 88 (72.7 %)  |
| HSIL                | 106     | 93 (87.7 %)     | 96 (90.6 %)  |
| AGC/ASC-H/AIS       | 15      | 1 (73.3 %)      | 0 (66.7 %)   |
| **Histological follow-up** |     |                 |              |
| No follow-up        | 946     | 106 (11.2%)     | 139 (14.7%)  |
| CIN0                | 154     | 82 (53.2%)      | 78 (50.6%)   |
| CIN1                | 73      | 67 (91.8%)      | 66 (90.4%)   |
| CIN2                | 39      | 33 (84.6 %)     | 35 (89.7%)   |
| CIN3                | 75      | 72 (96.0%)      | 70 (93.3%)   |
| Cancer              | 8       | 8 (100%)        | 8 (100%)     |
| ≥CIN2               | 122     | 113 (92.6%)     | 113 (92.6%)  |
| 2xNILM\(^1\)       | 897     | 66 (7.4%)       | 97 (10.8%)   |

\(^1\)Women with NILM at the prior screening round and the index screening

ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion. AGC: Atypical glandular cells. ASC-H: Atypical squamous cells -cannot exclude HSIL. AIS: Adenocarcinoma in situ. CIN: Cervical intraepithelial neoplasia. 

≥CIN2: CIN2 or more. NILM: Negative for intraepithelial lesions or malignancies.
Table 2: HPV genotyping prevalence assessed by Onclarity by age in the screening population

| Assay and HPV type | 30-39 (383) | 40-49 (408) | 50-59 (207) | Total (998) |
|--------------------|-------------|-------------|-------------|-------------|
| HrHPV              |             |             |             |             |
| 16                 | 10 (2.6%)   | 5 (1.2%)    | 3 (1.4%)    | 18 (1.8%)   |
| 18                 | 7 (1.8%)    | 4 (1.0%)    | 0 (0.0%)    | 11 (1.1%)   |
| 31                 | 6 (1.6%)    | 5 (1.2%)    | 1 (0.5%)    | 12 (1.2%)   |
| 45                 | 6 (1.6%)    | 3 (0.7%)    | 2 (1.0%)    | 11 (1.1%)   |
| 51                 | 4 (1.0%)    | 2 (0.5%)    | 2 (1.0%)    | 8 (0.8%)    |
| 52                 | 12 (3.1%)   | 1 (0.2%)    | 4 (1.9%)    | 17 (1.7%)   |
| 33/58              | 8 (2.1%)    | 4 (1.0%)    | 5 (2.4%)    | 17 (1.7%)   |
| 56/59/66           | 14 (3.7%)   | 6 (1.5%)    | 3 (1.4%)    | 23 (2.3%)   |
| 35/39/68           | 13 (3.4%)   | 7 (1.7%)    | 3 (1.4%)    | 23 (2.3%)   |

All infections observed are counted regardless of whether they are observed as single infections or multiple infections.
Table 3: HPV genotyping prevalence for Onclarity and GP-LMNX stratified by cytology result

| Cytology | Normal (947) | ASC-US (106) | LSIL (121) | HSIL (106) | AGC/ASC-H/AIS (15) | Total (1295) |
|----------|-------------|--------------|------------|------------|-------------------|--------------|
| Onclarity Assay                                      |             |              |            |             |                   |              |
| HrHPV     | 73 (7.7%)   | 103 (97.2%)  | 88 (72.7%) | 93 (87.7%) | 11 (73.3%)        | 368 (28.4%)  |
| 16        | 12 (1.3%)   | 26 (24.5%)   | 12 (9.9%)  | 36 (34.0%) | 2 (13.3%)         | 88 (6.8%)    |
| 18        | 6 (0.6%)    | 7 (6.6%)     | 6 (5.0%)   | 10 (9.4%)  | 2 (13.3%)         | 31 (2.4%)    |
| 31        | 7 (0.7%)    | 13 (12.3%)   | 13 (10.7%) | 16 (15.1%) | 1 (6.7%)          | 50 (3.9%)    |
| 45        | 7 (0.7%)    | 13 (12.3%)   | 5 (4.1%)   | 8 (7.5%)   | 2 (13.3%)         | 35 (2.7%)    |
| 51        | 3 (0.3%)    | 7 (6.6%)     | 11 (9.1%)  | 8 (7.5%)   | 1 (6.7%)          | 30 (2.3%)    |
| 52        | 13 (1.4%)   | 12 (11.3%)   | 12 (9.9%)  | 11 (10.4%) | 0 (0%)            | 48 (3.7%)    |
| 33/58     | 10 (1.1%)   | 13 (12.3%)   | 7 (5.8%)   | 14 (13.2%) | 2 (13.3%)         | 46 (3.6%)    |
| 56/59/66  | 12 (1.3%)   | 20 (18.9%)   | 37 (30.6%) | 10 (9.4%)  | 3 (20.0%)         | 82 (6.3%)    |
| 35/39/68  | 18 (1.9%)   | 19 (17.9%)   | 18 (14.9%) | 5 (4.7%)   | 1 (6.7%)          | 61 (4.7%)    |
| GP-LMNX                                           |             |              |            |             |                   |              |
| Assay and HPV type                                      |             |              |            |             |                   |              |
| HrHPV     | 122 (13.0%) | 97 (91.5%)   | 86 (71.1%) | 97 (91.5%) | 10 (66.7%)        | 412 (32.0%)  |
| 16        | 25 (2.7%)   | 26 (24.5%)   | 11 (9.1%)  | 38 (35.8%) | 2 (13.3%)         | 102 (7.9%)   |
| 18        | 23 (2.4%)   | 8 (7.5%)     | 7 (5.8%)   | 10 (9.4%)  | 2 (13.3%)         | 50 (3.9%)    |
| 31        | 11 (1.2%)   | 12 (11.3%)   | 12 (9.9%)  | 16 (15.1%) | 1 (6.7%)          | 52 (4.0%)    |
| 45        | 15 (1.6%)   | 14 (13.2%)   | 5 (4.1%)   | 7 (6.6%)   | 2 (13.3%)         | 43 (3.3%)    |
| 51        | 9 (1.0%)    | 8 (7.5%)     | 12 (9.9%)  | 8 (7.5%)   | 0 (0%)            | 37 (2.9%)    |
| 52        | 9 (1.0%)    | 9 (8.5%)     | 8 (6.6%)   | 6 (5.7%)   | 0 (0%)            | 32 (2.5%)    |
| 33/58     | 21 (2.2%)   | 13 (12.3%)   | 8 (6.6%)   | 13 (12.3%) | 3 (20.0%)         | 58 (4.5%)    |
| 56/59/66  | 23 (2.4%)   | 22 (20.8%)   | 37 (30.6%) | 13 (12.3%) | 3 (20.0%)         | 98 (7.6%)    |
| 35/39/68  | 17 (1.8%)   | 15 (14.2%)   | 13 (10.7%) | 3 (2.8%)   | 1 (6.7%)          | 49 (3.8%)    |

1For HPV genotypes have been pooled for comparison with Onclarity. HPV33+ therefore is counted in a combined HPV33/58 outcome. Multiple infection of e.g. HPV33 and HPV58 is only counted once as an HPV33/58 infection. The same for HPV56/59/66 and HPV35/39/68

27 samples were invalid for GP-LMNX
ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion. AGC: Atypical glandular cells. ASC-H: Atypical squamous cells-cannot exclude HSIL. AIS: Adenocarcinoma in situ.
### Table 4. Clinical accuracy of Onclarity and GP-EIA for ≥CIN2, ≥CIN3 and <CIN1 outcomes.

| Study population | GP-EIA results | Onclarity | GP-EIA | Non-inferiority test<sup>1</sup> |
|------------------|----------------|-----------|--------|----------------------------------|
|                  | Onclarity results | Pos | Neg | Total | Sensitivity | Sensitivity | Sensitivity |
| ≥CIN2 (N=122)    | Pos             | 111 | 2   | 113   | 92.6% (CI: 86.5-96.6) | 92.6% (CI: 86.5-96.6) | 0.0006 |
|                  | Neg             | 2   | 7   | 9     |             |             |             |
|                  | Total           | 113 | 9   | 122   |             |             |             |
|                  | Relative Sensitivity ≥CIN2 1.00 (0.97-1.04)<sup>2</sup> | | | | | | |
| ≥CIN3 (N=83)     | Pos             | 78  | 2   | 80    | 96.4% (89.9-99.2) | 94.0% (86.5-98.0) | <0.0001 |
|                  | Neg             | 0   | 3   | 3     |             |             |             |
|                  | Total           | 78  | 5   | 83    |             |             |             |
|                  | Relative Sensitivity ≥CIN3 1.03 (0.99-1.06) | | | | | | |
| 2xNILM<sup>3</sup> (N=897) | Pos            | 60  | 6   | 66    | 92.6% (90.7-94.3) | 89.2% (87.0-91.1) | <0.0001 |
|                  | Neg             | 37  | 794 | 831   |             |             |             |
|                  | Total           | 97  | 800 | 897   |             |             |             |
|                  | Relative specificity 2x NILM 1.04 (1.02-1.06) | | | | | | |

<sup>1</sup> P for the test for non-inferiority, P<sub>n.inf.</sub> <0.05 P<sub>n.inf.</sub> <0.05 means that the sensitivity or specificity of Onclarity is not significantly lower than that of GP-EIA, using the bench marks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

<sup>2</sup> Relative sensitivity for Onclarity with GP-EIA as comparator test

<sup>3</sup> Women with NILM at the prior screening round and the index screening round

CIN: Cervical intraepithelial neoplasia. ≥CIN2: CIN2 or more. ≥CIN3: CIN3 or more. NILM: Negative for intraepithelial lesions or malignancies.
Table 5: Detection of Individual oncogenic genotypes by Onclarity and GP-LMNX in Screening and Enriched VALGENT4 panel populations

| HPV Genotypes | Onc + | GP-LMNX+ | Onc+/GP-LMNX- | Onc-/GP-LMNX- | Onc +/GP-LMNX- | Agree | Kappa |
|---------------|-------|----------|--------------|--------------|---------------|-------|-------|
| Screening population (N=991) |       |          |              |              |               |       |       |
| 16            | 16    | 31       | 18           | 0            | 13            | 940   | 98.7  | 0.73  |
| 18            | 18    | 38       | 14           | 0            | 17            | 935   | 98.3  | 0.56  |
| 31            | 31    | 56       | 12           | 0            | 4             | 775   | 99.6  | 0.86  |
| 45            | 45    | 58       | 5            | 2            | 0             | 474   | 98.9  | 0.83  |
| 51            | 51    | 33       | 7            | 1            | 6             | 577   | 95.3  | 0.66  |
| 52            | 52    | 11       | 11           | 6            | 0             | 974   | 99.4  | 0.78  |
| 56/59/66      | 56    | 23       | 16           | 1            | 13            | 461   | 96.6  | 0.69  |
| 35/39/68      | 35    | 33       | 21           | 2            | 12            | 456   | 98.4  | 0.74  |
| 14 HPV        | 14    | 22       | 102          | 11           | 57            | 421   | 99.1  | 0.71  |

| Enriched Population (N=297) |       |          |              |              |               |       |       |
| 16            | 6     | 2         | 2             | 3            | 224           | 96.3  | 0.95  |
| 18            | 19    | 1         | 3             | 274          | 96.7          | 0.90  |
| 31            | 25    | 3         | 1             | 258          | 96.7          | 0.94  |
| 45            | 24    | 0         | 1             | 272          | 96.7          | 0.98  |
| 51            | 21    | 1         | 3             | 272          | 96.7          | 0.91  |
| 52            | 21    | 10        | 0             | 266          | 96.6          | 0.79  |
| 56/59/66      | 27    | 2         | 2             | 266          | 96.7          | 0.92  |
| 35/39/68      | 25    | 10        | 1             | 258          | 96.1          | 0.82  |
| 14 HPV        | 24    | 6         | 6             | 266          | 96.7          | 0.92  |

| VALGENT4 panel |       |          |              |              |               |       |       |
| 16            | 6     | 2         | 2             | 3            | 224           | 96.3  | 0.95  |
| 18            | 19    | 1         | 3             | 274          | 96.7          | 0.90  |
| 31            | 25    | 3         | 1             | 258          | 96.7          | 0.94  |
| 45            | 24    | 0         | 1             | 272          | 96.7          | 0.98  |
| 51            | 21    | 1         | 3             | 272          | 96.7          | 0.91  |
| 52            | 21    | 10        | 0             | 266          | 96.6          | 0.79  |
| 56/59/66      | 27    | 2         | 2             | 266          | 96.7          | 0.92  |
| 35/39/68      | 25    | 10        | 1             | 258          | 96.1          | 0.82  |
| 14 HPV        | 24    | 6         | 6             | 266          | 96.7          | 0.92  |

Adequate agreement Kappa

| HPV Genotypes | Onc + | GP-LMNX+ | Onc+/GP-LMNX- | Onc-/GP-LMNX- | Onc +/GP-LMNX- | Agree | Kappa |
|---------------|-------|----------|--------------|--------------|---------------|-------|-------|
| Screening population (N=991) |       |          |              |              |               |       |       |
| 16            | 16    | 31       | 18           | 0            | 13            | 940   | 98.7  | 0.73  |
| 18            | 18    | 38       | 14           | 0            | 17            | 935   | 98.3  | 0.56  |
| 31            | 31    | 56       | 12           | 0            | 4             | 775   | 99.6  | 0.86  |
| 45            | 45    | 58       | 5            | 2            | 0             | 474   | 98.9  | 0.83  |
| 51            | 51    | 33       | 7            | 1            | 6             | 577   | 95.3  | 0.66  |
| 52            | 52    | 11       | 11           | 6            | 0             | 974   | 99.4  | 0.78  |
| 56/59/66      | 56    | 23       | 16           | 1            | 13            | 461   | 96.6  | 0.69  |
| 35/39/68      | 35    | 33       | 21           | 2            | 12            | 456   | 98.4  | 0.74  |
| 14 HPV        | 14    | 22       | 102          | 11           | 57            | 421   | 99.1  | 0.71  |

| Enriched Population (N=297) |       |          |              |              |               |       |       |
| 16            | 6     | 2         | 2             | 3            | 224           | 96.3  | 0.95  |
| 18            | 19    | 1         | 3             | 274          | 96.7          | 0.90  |
| 31            | 25    | 3         | 1             | 258          | 96.7          | 0.94  |
| 45            | 24    | 0         | 1             | 272          | 96.7          | 0.98  |
| 51            | 21    | 1         | 3             | 272          | 96.7          | 0.91  |
| 52            | 21    | 10        | 0             | 266          | 96.6          | 0.79  |
| 56/59/66      | 27    | 2         | 2             | 266          | 96.7          | 0.92  |
| 35/39/68      | 25    | 10        | 1             | 258          | 96.1          | 0.82  |
| 14 HPV        | 24    | 6         | 6             | 266          | 96.7          | 0.92  |

| VALGENT4 panel |       |          |              |              |               |       |       |
| 16            | 6     | 2         | 2             | 3            | 224           | 96.3  | 0.95  |
| 18            | 19    | 1         | 3             | 274          | 96.7          | 0.90  |
| 31            | 25    | 3         | 1             | 258          | 96.7          | 0.94  |
| 45            | 24    | 0         | 1             | 272          | 96.7          | 0.98  |
| 51            | 21    | 1         | 3             | 272          | 96.7          | 0.91  |
| 52            | 21    | 10        | 0             | 266          | 96.6          | 0.79  |
| 56/59/66      | 27    | 2         | 2             | 266          | 96.7          | 0.92  |
| 35/39/68      | 25    | 10        | 1             | 258          | 96.1          | 0.82  |
| 14 HPV        | 24    | 6         | 6             | 266          | 96.7          | 0.92  |
Figure 1: Flowchart for collection of the Valgent4 panel and HPV testing.

ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion.
Figure 2: Stability of SurePath cervical screening samples tested on the Onclarity system.

Top: Scatter-plot over average HBB for baseline 1st testing and 2nd testing at 7 months. Bottom: Overall concordance between baseline 1st testing and 2nd testing on 1212 samples.
VALGENT-4 Panel

- 998 consecutive screening samples
- 297 enriched screening samples (100 ASCUS, 100 LSIL, 97 HSIL)

- 1295 samples
  - 0.5 ml original resuspended material
  - 25 µl extracted DNA

- 1295 Onclarity results (0 invalids)
  - Hvidovre Hospital
- 1288 LMNX results (7 samples invalid)
  - DDL laboratory
- 1295 EIA results (0 invalids)
  - DDL laboratory
Onclarity 2nd test at 7 months

| Onclarity baseline test | Pos  | Neg  | Total |
|------------------------|------|------|-------|
| Pos                    | 325  | 13   | 338   |
| Neg                    | 11   | 863  | 874   |
| Total                  | 335  | 877  | 1212  |

$y = 0.9856x + 0.3402$