# Onclarity Performance in Human Papillomavirus DNA Detection in Formalin-Fixed Paraffin-Embedded Cervical Samples

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**Objectives:** Diagnosis of HPV infection is usually performed from cervical liquid-based cytology specimens (LBC), but these often contain a large amount of human papillomavirus (HPV) genotypes, most of which might cause transient infections. The aim of the study was to evaluate the performance of BD Onclarity HPV test genotyping method on formalin-fixed, paraffin-embedded (FFPE) cervical specimens compared with genotyping results from LBC.

**Materials and Methods:** Formalin-fixed, paraffin-embedded specimens from women surgically treated for cervical intraepithelial lesions (CINs) at the European Institute of Oncology, Milan, from September 2012 to June 2013 were retrieved from the archives of the Department of Pathology of the European Institute of Oncology. The FFPE and LBC specimens were genotyped using the same extended genotyping Onclarity assay.

**Results:** We collected 99 samples (26 CIN 1, 30 CIN 2, and 43 CIN 3+), but 15 were excluded from the analysis: these 84 samples show an overall agreement of 89% for HPV status between FFPE Onclarity samples versus LBC samples. The FFPE and LBC samples showed identical genotype in 75% samples, compatible genotype (at least 1 of the genotypes detected in LBC sample was found in the tissue sample) in 14% specimens, and discrepant genotype in 11% samples.

**Conclusions:** Our data demonstrate a very good concordance between HPV genotypes found in cytological and tissue samples, suggesting that the Onclarity method could also be used to detect HPV in tissue samples and that the HPV genotype detected in FFPE samples is one of the HPV detected in cytological samples, supporting the thesis that one lesion is caused by one HPV genotype.

**Key Words:** HPV, CIN, cervical cancer, FFPE, liquid-based cytology, genotyping

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Persistent infection with oncogenic types of high-risk human papillomavirus (HR HPV) is a necessary cause of cervical intraepithelial lesion (CIN) that could develop in cervical cancer.[1,2] Screening programs are now based on molecular detection of HPV infection, but a single test does not discriminate between transient or persistent infections. Cervical liquid-based cytology specimens (LBC) positive for HPV genotypes are often transient infections and related to sexual activity.[3] For this reason, CIN 2+ detection has been designated as the clinical cutoff for HPV tests than have been developed and validated for cytological samples.[4] Fields of application of HPV assays also include clinical samples with degraded nucleic acids, vaccine development or monitoring of vaccination programs, epidemiological studies, and various other research purposes. In these cases, HPV tests require high sensitivity and specificity as well as genotype-specific accuracy of high- and low-risk HPV genotypes without cross-reactivity, in contrast to tests with clinically validated cutoffs, for which the research of low-risk HPV genotypes and/or transient infections is not useful.

Human papillomavirus genotyping assays allow identification of persistent infections, by detecting how many and which HPVs are present in cytological samples at present and at follow-up.[5] Only HPV infections persisting more than 2 years could lead to the development of CIN 2+. Furthermore, the identification of HPV DNA in cervical tissue could be important for understanding cervical carcinogenesis and for evaluating cervical cancer management, by detecting the specific genotype that has persisted and integrated into host DNA cell.

Standard HPV genotyping methods cannot be easily applied to tissue specimens, because formalin fixation may lead to extensive DNA damage, including cross-linking and fragmentation.

The aim of the study is to evaluate the performance of BD Onclarity HPV test (BD Diagnostics, Sparks, MD) genotyping method[6] in formalin-fixed, paraffin-embedded (FFPE) cervical specimens compared with genotyping results from LBC.

Although Onclarity is an automated Food and Drug Administration– and CE-approved method only for cervical samples and clinically validated test with a cutoff set for CIN 2+, we wanted to apply this assay in HPV detection and genotyping in FFPE samples to investigate its performance in samples other than LBC.

As secondary aim, we compared results of Onclarity from LBC and FFPE with results of Hybrid Capture 2 (HC2) and Linear Array from LBC.

## MATERIAL AND METHODS

### Population

Formalin-fixed, paraffin-embedded specimens from women surgically treated (including excisional procedures, such as loop electrosurgical excision procedure and laser conization, and ablative procedure, like laser vaporization) for histologically confirmed CIN at the European Institute of Oncology, Milan, from September 2012 to June 2013 were retrieved from the archives of the Department of Pathology of the European Institute of Oncology.

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The study was approved by the institutional ethical committee (R596/17-IEO630 study), and informed consent was obtained from all women at the entry of the study.

F.B., A.D.I., and M.T.S. designed the study. F.B., G.R., R.P., M.E.G., A.O., and A.D.I. performed the clinical and laboratory work. A.L. performed the data analysis. F.B., A.D.I., and A.L. assisted the interpretation of the results. All authors wrote, read, and approved the final version of the article.



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**TABLE 1.** Summary of the Population Enrolled Data

| | *n* | % (*N* = 99) |
|---|---|---|
| Cytology | | |
| HSIL | 58 | 58.6 |
| LSIL | 24 | 24.2 |
| ASCUS | 12 | 12.1 |
| AGC-neoplastic | 1 | 1.0 |
| AGC-NOS | 1 | 1.0 |
| NILM | 3 | 3.0 |
| Histology | | |
| Carcinoma | 4 | 4.0 |
| CIN 3 | 39 | 39.4 |
| CIN 2 | 30 | 30.3 |
| CIN 1 | 26 | 26.3 |
| HC2 LBC | | |
| Positive | 89 | 89.9 |
| Negative | 10 | 10.1 |
| Linear Array HR HPV LBC | | |
| Positive | 86 | 86.9 |
| Negative | 13 | 13.1 |
| Onclarity HR HPV LBC | | |
| Positive | 85 | 85.9 |
| Negative | 14 | 14.1 |

AGC-neoplastic indicates atypical glandular cells-neoplastic; AGC-NOS, atypical glandular cells-not otherwise specified; ASCUS, atypical squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

The study was approved by the designated institutional review board and the institutional ethical committee (R596/17-IEO630 study), and informed consent was obtained from all women at enrollment.

## Methods

A series of 4-μm-thick tissue sections was cut from each paraffin block. The first and last sections were stained with hematoxylin and eosin and microscope observed by an experienced pathologist to confirm the presence of the lesion in the sections and the histological diagnosis. During tissue processing and sectioning, standard measures to avoid HPV genotype cross-contamination were taken.

Formalin-fixed, paraffin-embedded specimens were genotyped using the extended genotyping Onclarity assay. Each FFPE tissue sample was extracted using the automated workflow on the BD Viper LT instrument. The tissue section was combined only with 0.5 mL of distilled water and added directly to a Viper dedicated tube. The sample was then lysed directly using the Viper LT prewarm station before being transferred onto the instrument where it underwent automated sample processing and polymerase chain reaction detection. No other pretreatment was required; in particular, deparaffination is not necessary.

HC2 (Qiagen) HPV detection results, Linear Array (LA; Roche) genotyping results, and LBC Onclarity genotyping data of women enrolled and performed before or at time of surgery were recovered from the laboratory archive.

The BD Onclarity HPV Assay detects 14 HPV genotypes and coamplifies a β-globin internal control (IC), which acts as processing control. The primers for the 14 HPV genotypes are designed to target a region of 79–137 bases in the E6/E7 genome, whereas the IC primers amplify a 75–base pair (bp) region in the human β-globin gene. The DNA was extracted from the samples (FFPE or LBC) using BD FOX magnetic particles, and the eluate containing DNA was used to set up 3 polymerase chain reaction genotyping reactions (G1, G2, and G3) and to detect all 14 HR HPV genotypes with 4 optical channels: HPV 16, 18, 31, 45, 51, and 52 as single infections and the remaining 8 genotypes in 3 groups (HPV 33/58, 56/59/66, 35/39/68) and the IC.

Qiagen HC2 test is a signal amplification detection method based on chemiluminescence that detects 13 HR HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) all together.

The Roche Diagnostics Linear Array test uses biotinylated PGMY09/11 consensus primers to amplify a 450-bp region of the L1 gene to detect 37 HPV genotypes: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108.

## Statistical Analysis

Hierarchical analysis of outcome measurements included positive percent agreement, negative percent agreement, and overall percent agreement; CIs were calculated using the Wilson score method.[7] Hierarchical order was performed as follows: HPV 16, else 31, else 18, else 33/58, else 52, else 45, else 35/39/68, else 51, and else 56/59/66. McNemar test with continuity correction[8] was used to examine the marginal homogeneity of LBC versus FFPE Onclarity results.

## Role of the Funding Source

The funding for this study was provided by BD Europe. The funder had the right to read and comment upon the manuscript, but without editorial rights.

## RESULTS

Overall, 99 women were enrolled with a median age of 34.1 years (range = 22.1–65.9 years). More than 50% of the patients had a high-grade cytology, and 74% had histopathology of CIN 2 or greater. The HPV tests performed on LBC samples showed a high percentage of positives as shown in Table 1. Fifteen FFPE samples were excluded from the analysis because 8 samples testing internal control failure results (not enough human β-globin detected) and 7 samples decreed unsuitable by the pathologist's microscope analysis, as the lesion was not taken in the tissue sample. All HR HPVs were detected in the CIN lesions tested: the most frequently detected genotypes in FFPE and LBC were HPV 16 and HPV 31 as shown in Table 2. The percent agreement (positive, negative, and overall) between LBC and FFPE Onclarity tests is shown in Table 3: hierarchical percentage of agreement for

**TABLE 2.** Summary of HPV Results (Hierarchical)

| | Onclarity FFPE | | Onclarity LBC | | Linear Array LBC | |
|---|---|---|---|---|---|---|
| HPV | *n* | % (*N* = 84) | *n* | % (*N* = 99) | *n* | % (*N* = 99) |
| 16 | 35 | 41.7 | 47 | 47.5 | 48 | 48.5 |
| 31 | 13 | 15.5 | 15 | 15.2 | 17 | 17.2 |
| 18 | 3 | 3.6 | 3 | 3.0 | 4 | 4.0 |
| 33/58 | 4 | 4.8 | 6 | 6.1 | 11 | 11.1 |
| 52 | 1 | 1.2 | 3 | 3.0 | 5 | 5.1 |
| 45 | 1 | 1.2 | 3 | 3.0 | 5 | 5.1 |
| 39/68/35 | 2 | 2.4 | 2 | 2.0 | 4 | 4.0 |
| 51 | 2 | 2.4 | 3 | 3.0 | 8 | 8.1 |
| 59/56/66 | 4 | 4.8 | 3 | 3.0 | 10 | 10.1 |
| NEG | 19 | 22.6 | 14 | 14.1 | 13 | 13.1 |

**TABLE 3.** Percent Agreement—Onclarity LBC Versus FFPE (Hierarchical)

| HPV | PPA, % | PPA, 95% CI | NPA, % | NPA, 95% CI | OPA, % | OPA, 95% CI | *p* |
|---|---|---|---|---|---|---|---|
| HR HPV | 98.5 | 91.79–99.73 | 57.9 | 36.28–76.86 | 89.3 | 80.88–94.26 | .046 |
| 16 | 100.0 | 90.11–100 | 87.8 | 75.76–94.27 | 92.9 | 85.28–96.69 | .041 |
| 18 | 100.0 | 43.85–100 | 100.0 | 95.47–100 | 100.0 | 95.63–100 | NA |
| 31 | 92.3 | 66.69–98.63 | 98.6 | 92.44–99.75 | 97.6 | 91.73–99.34 | 1 |
| 33/58 | 100.0 | 51.01–100 | 100.0 | 95.42–100 | 100.0 | 95.63–100 | NA |
| 39/68/35 | 50.0 | 9.45–90.55 | 100.0 | 95.52–100 | 98.8 | 93.56–99.79 | 1 |
| 45 | 100.0 | 20.65–100 | 98.8 | 93.49–99.79 | 98.8 | 93.56–99.79 | 1 |
| 51 | 100.0 | 34.24–100 | 98.8 | 93.41–99.78 | 98.8 | 93.56–99.79 | 1 |
| 52 | 100.0 | 20.65–100 | 98.8 | 93.49–99.79 | 98.8 | 93.56–99.79 | 1 |
| 59/56/66 | 75.0 | 30.06–95.44 | 100.0 | 95.42–100 | 98.8 | 93.56–99.79 | 1 |

*p* values are from the McNemar test.

NA indicates not available; NPA, negative percent agreement; OPA, overall percent agreement; PPA, positive percent agreement.

the 2 most detected HPV genotypes (16 and 31) ranged from 92.9% to 97.6% considering FFPE results as reference standards. The FFPE and LBC samples showed identical genotype in 63 (75%) of 84 samples, compatible genotype (at least 1 of the genotypes detected in LBC sample also found in FFPE) in 12 (14%) of the 84 specimens, and discrepant genotype in 9 (11%) of the 84 samples.

Discordant samples are shown in Table 4: 1 sample tested positive in FFPE and negative in LBC corresponding sample, whereas 8 tested negative in FFPE and positive in LBC. Most of HPV-negative FFPE samples were low-grade CIN (62.5%), with only 1 CIN 3 tested HPV negative in FFPE. In case of concordance, at least 1 of the genotypes detected in LBC sample was found in the tissue sample. Figure 1 summarizes the imbalance between multiple and single genotype infections detected in LBC and FFPE samples, respectively.

## DISCUSSION

There are many HPV tests on the market: only a small portion of these (18%) received performance studies in peer-reviewed journals,[9] and very few of these have been validated on extracervical materials. In this study, we evaluated the performance of BD Onclarity in HPV detection and genotyping of FFPE samples. Our findings are in agreement with previous reports indicating a good performance of Onclarity assay in tissue specimens.[10,11] Onclarity detected all HR HPV genotypes, and the overall agreement between LBC and FFPE was very good, ranging from 92.9% to 100%.

In addition, the percentages of identical, compatible, and discrepant are in line with data obtained by other authors.[10–12] Discordant results were found in low-grade lesions, generally with FFPE-negative and LBC-positive results. The HPV DNA detection accuracy in FFPE could be invalidated because formalin fixation may cause DNA damage, including cross-linking and fragmentation.[10] Moreover, in low-grade lesions, the amount of HPV DNA could be less than in high-grade lesion.[13] In addition, different performances of genotyping methods for tissue specimens depend on nucleic acid degradation, because of specimen age, preservation method, sample processing, and DNA extraction of FFPE materials. Therefore, it could be interesting to evaluate the performance of Onclarity on fresh tissue samples, not yet embedded in paraffin.

Castro et al.[10] already tested the Onclarity assay using FFPE specimens, with an overall agreement of HPV status between exfoliated cell and FFPE specimens of 90%, in agreement with our findings. Moreover, Castro et al.[10] compared Onclarity results for tissue samples with other genotyping methods, i.e., SPF10-PCR DEIA LiPA25 (Version 1), Inno-LiPA, and Linear Array. Human papillomavirus genotyping methods vary by target sequence and amplicon size. The principal targets are the L1 gene and the viral oncogenes E6/E7, whereas amplicon sizes range from 65 to 450 bp. Amplification of HPV sequences from FFPE specimens is inversely correlated to the size of the amplicon.[14] Despite larger amplicon size, Onclarity showed an overall agreement of 81.7%, 86.7%, and 91.7% versus Inno-LiPA, Linear Array, and SPF-LiPA2 with respect to carcinogenic HPV status for FFPE samples (9).

Assays, such as Onclarity, that target E6/E7 versus L1 DNA may also be advantageous for FFPE analysis because they avoid the issue of L1 target deletion after integration: Arroyo Mühr et al.[15] recently demonstrated that up to 60% of HPV-negative cancers lacked an intact L1 region with E6/E7 improving sensitivity of detection.

A very interesting finding of this work is the proportion of multiple infections detected in LBC samples as opposed to single infections in FFPE samples as shown in Figure 1. These data offer a biological explanation for the development of cervical preneoplastic and neoplastic lesions. Even in the presence of multiple HPV genotypes, there is only 1 genotype that integrates into the human cell and generates the imbalance of cellular genes that leads to carcinogenesis.[16,17]

Clinical implication of use of Onclarity assay in biopsies samples may be to search for the same genotype present in the cytological sample to establish whether the HPV infection is present and persistent in cervical cells, or in episomal form, and therefore not integrated into the cells. Indeed, HPV infection can occur in

**TABLE 4.** Discrepancies (Nonhierarchical)

| Histo | Onclarity FFPE | Onclarity LBC |
|---|---|---|
| CIN 2 | 39/68/35 | NEG |
| CIN 1 | NEG | 45 |
| CIN 1 | NEG | 52 |
| CIN 2 | NEG | 16 |
| CIN 2 | NEG | 16 |
| CIN 1 | NEG | 51 |
| CIN 1 | NEG | 16, 33/58 |
| CIN 1 | NEG | 31 |
| CIN 3 | NEG | 16 |

Histo indicates histology; NEG, no HPV genotypes detected.

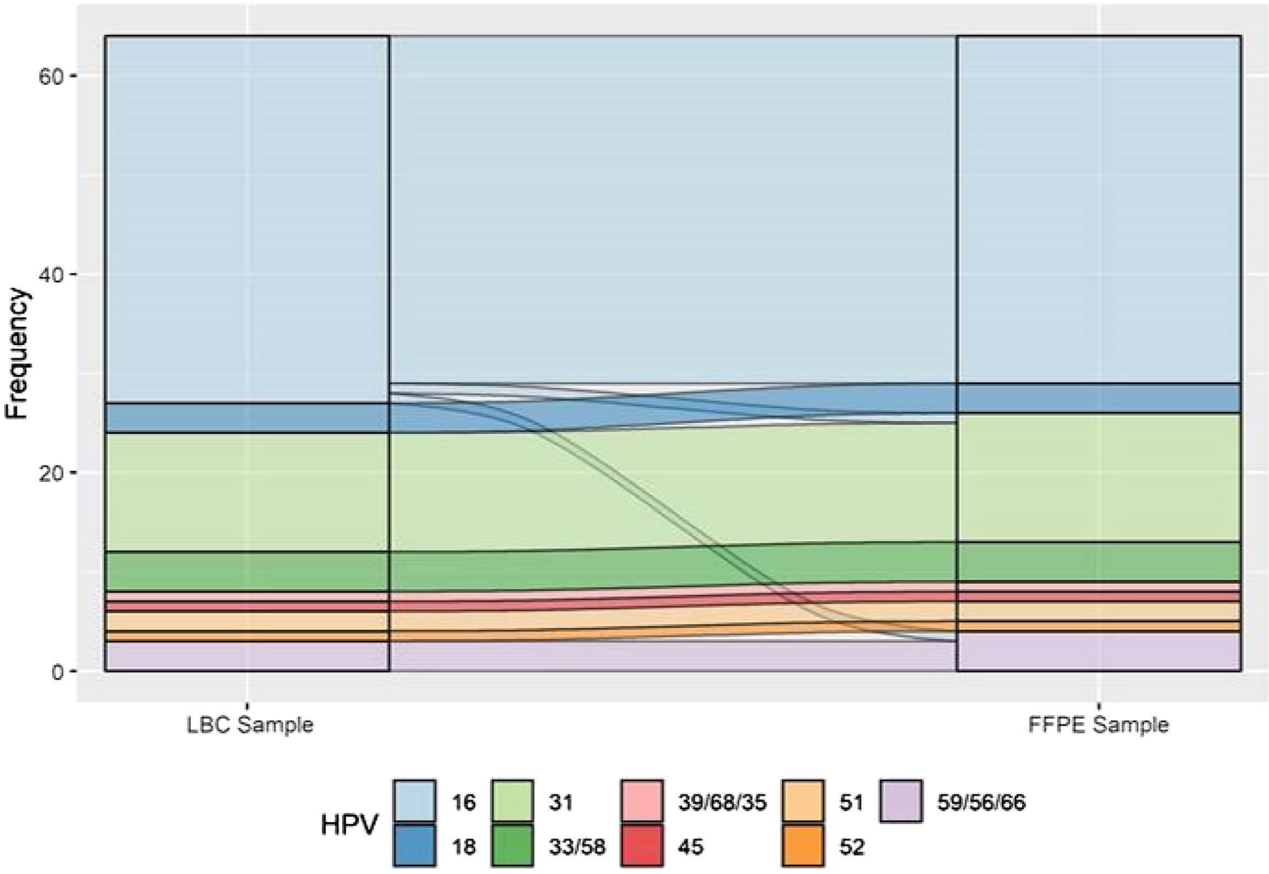


**FIGURE 1.** Reassignment of positive HPV genotypes from the LBC samples to the FFPE samples based on hierarchical order. Flow between genotypes is indicated by line, and frequency is indicated by thickness of the line.

acute, latent, and chronic forms, characterized by different virus activity, viral genes expression, cellular genes deregulation, and ability to induce local immunosuppression, cell proliferation, and oncogenicity.[18] The discrepancy between the viral DNA detected in the cytological liquid compared with that present in the tissue could clarify the transient nature of the infection (episomal) or the persistent nature of the infection (integration). Up to now, second level tests on cytological samples have investigated for the methylation status of the HPV genes or the HPV mRNA expression. Differential HPV genotypes detection in tissue and cytological samples had never been considered for this purpose and could be a new tool for the clinician who may obtain additional information, without the need for additional methods or instruments. In addition, after surgical treatment, the physician attention should therefore be focused on patients with biopsy infection with a closer follow-up than patients without HPV infection in FFPE sample.

Currently, clinical practice decision making for postexcision treatment is HPV-based testing in 6 months, with additional considerations for repeat excision in the event of positive margins. The application of a change in management based on the episomal versus integrated HPV could lead to a more tailored and personalized follow-up after surgical treatment. Only detection of the same HPV genotype that was present in tissue, at the 6-month follow-up, should be considered as a risk factor for treatment failure. On the contrary, detection of genotypes that were present only in LBC but not in FFPE samples may not require immediate colposcopy during follow-up.

Moreover, the 2 most detected HPV genotypes were 16 and 31 in our population, as previously outlined by other authors,[19,20] and showed a significant hierarchical percentage of agreement from 92.9% to 97.6%, considering FFPE results as reference standards.

Detection rate of HPV DNA observed in our study demonstrates that Onclarity assay is an attractive automated alternative for HPV genotyping from FFPE tumor samples. Each FFPE tissue sample was extracted using the automated workflow on the Viper LT system. No specific pretreatment is required, in particular deparaffination, thus saving work and time.

Limits of the present study are related to the small sample size and the retrospective design of our analysis. In particular, another limitation is the fact that only 85% of the samples were interpretable, because 15 of 99 samples were excluded from the analysis. Nevertheless, an overall agreement of 89% is relatively good for a diagnostic test using archived samples. Strengths of our analysis include the confirmed histological diagnosis of the selected specimens and the short interval between LBC and tissue samples collection.

## CONCLUSIONS

Our data showed a very good performance of Onclarity in HPV detection and genotyping of FFPE samples. Revealing a very good concordance between HPV genotypes found in cytological and tissue samples and that the HPV genotype detected in FFPE samples is one of genotypes detected in cytological samples, our findings support the thesis that one lesion is caused by one virus genotype.[16]

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