Interpretation Guide for Ventana INFORM®
HPV Probes In Situ Hybridization (ISH)
Staining of Cervical Tissue

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I. Introduction

A. General Description of INFORM® HPV Probes

The INFORM HPV III Family 16 Probe (B) contains a cocktail of HPV genomic probes in a formamide-based diluent. The intended targets are the common high-risk HPV genotypes found to be associated with cervical neoplasia. The probe cocktail has demonstrated affinity to the following genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66.

The INFORM HPV II Family 6 Probe (B) contains a cocktail of HPV genomic probes in a formamide-based diluent. The intended targets are the HPV genotypes found commonly in condyloma and some early cervical intraepithelial lesions, which are not commonly associated with cervical cancer. The probe cocktail has demonstrated affinity to the following genotypes: 6 and 11.

B. Purpose of Interpretive Guide

The following 53 cases illustrate the variety of staining patterns that may be present in cervical biopsies when stained with Ventana INFORM HPV Probes (Figures 1-19). The photomicrographs allow a new user to become familiar with the spectrum of staining patterns including episomal and integrative patterns of HPV positivity and patterns of artifactual staining that they may encounter in a variety of cervical tissues, including normal, cervical intraepithelial neoplasia (CIN I, II / III), carcinoma, and condyloma, when using a validated assay involving the Ventana INFORM HPV Probes. The intent is to provide pathologists with a tool to facilitate interpretation of INFORM HPV Probes staining results. Any staining performed in the end users lab should be interpreted within the context of the controls run with the clinical cases at the time of evaluation. See the package insert provided with these products for further information.

The images contained in this interpretive guide we obtained using an assay developed and validated at Ventana Medical Systems, Inc., employing the INFORM HPV Probes.
II. Identification of Appropriate Staining Pattern

A. Staining Patterns of Episomal and Integrated HPV

When utilizing the INFORM HPV III Family 16 Probe (B) in the Ventana-validated assay, the presence of HPV is demonstrated when either the episomal or integrated pattern is found within the nuclei of cervical epithelial cells (Figure 1).

The episomal pattern appears as a large, homogeneous, globular navy-blue precipitate within the epithelial cell nucleus (see Cases 1-3). The integrative pattern is a discrete, stippled navy blue nuclear pattern (see Cases 4-6). As shown in Cases 1-3, the episomal pattern is generally prominent in the superficial keratinized region of the epithelium often within koilocytotic cells. The discrete, dot-like nuclear integrative pattern is more often in groups of epithelial cells in a more basal location (Case 7, Figure 2). In some cases, the nuclear integrative pattern may be so discrete as to require microscopic examination at higher magnification (40x or 100x objective) to judge nuclear epithelial cell localization. In this context, it is specifically the finding of a distinct pattern (e.g. a field of epithelial cells with dot-like nuclear spots), which is pivotal to judging positivity. In contrast, the finding of a rare “speck” of navy-blue in a rare scattered cell could be artifactual as a pattern of cellular involvement is not evident.

These characteristic nuclear epithelial cell patterns of staining indicate positivity. In contrast, artifactual non-specific staining unrelated to HPV manifests distinctly separate features. As illustrated in Section VII, the non-specific, non-HPV associated artifacts may include focal non-cellular stromal precipitates, a slight diffuse low-level light blue staining, the periodic staining of the cytoplasm of PMNs and eosinophils, and non-specific staining of nucleoli and lymphocyte/endothelial cells. With some of these artifacts, it again may be necessary to examine borderline cases at higher magnification (40x or 100x objective) to judge nuclear localization.

Fig. 1 Staining Patterns With INFORM HPV III Family 16 Probe (B)

- Episomal Pattern: Cases 1-3

- Integrative Pattern: Cases 4-6
B. Definition of Positive and Negative Results

Case 7 (Figure 2) illustrates the quintessential features of judging INFORM HPV III Family 16 Probe (B) staining when using the Ventana-validated assay. The case is an example of Cervical Intraepithelial Neoplasia (CIN I) at the transition zone of the cervix. In this case, the CIN I lesional tissue (right side of photo) is seen in close contiguity with adjacent normal cervical epithelial tissue (left side of photo). In this example, there are two positive patterns: (1) the “episomal” staining within the superficial epithelial cells overlying the CIN I lesional tissue and (2) the discrete, dot-like nuclear, or “integrative”, pattern in the mid-and basal layer CIN lesional tissue. Importantly, a negative staining pattern is found within the non-lesional adjacent normal cervical epithelial nuclei, endocervical glands, and the stromal cells, including fibroblasts, endothelial cells, and lymphocytes. Of note is a very superficial layer of navy-blue India Ink used at colposcopy to mark lesional tissue.

As shown in Case 7, the determination of a true positive INFORM HPV III Family 16 Probe (B) ISH result requires finding either the episomal and/or integrative pattern of navy-blue staining in lesional cervical epithelium, in the context of adjacent negative staining within non-lesional cervical epithelium and endocervical glands and in non-lesional stromal cells, fibroblasts, lymphocytes, and endothelial cells, while discounting artifactual staining, such as found in the cytoplasm of PMN and India Ink margins. Substantial artifactual staining of non-lesional cells of epithelial or non-epithelial origin may preclude judging a positive result.

The determination of a true negative INFORM HPV III Family 16 Probe (B) ISH result requires finding no signal (neither globular “episomal” or discrete “integrative” pattern) within lesional epithelial cells, in the context of a nuclear DNA-positive control probe result and an appropriate cell line slide control (Section IV and V).

Fig. 2 Interpretive Example: Case 7
B. Definition of Positive and Negative Results (cont.)

In some instances, particularly with high-grade dysplastic cervical lesions (eg CIN II/III or Cervical Carcinoma), the staining may show a low copy number integrative pattern, which may be most noticeable at higher microscope magnification (eg 40x or 100x objective). Figure 3 illustrates two such cases, one with a HeLa-like (10-50 copy) integrated pattern and another SiHa-like very low copy number. In both cases, the pivotal observation is the finding of a discrete, speckled nuclear localization involving numerous contiguous cervical epithelial cells with the adjacent stroma, lymphocytes, submucosal glands, and normal cervical epithelial showing an absence of this pattern.

Fig. 3 Instructive Cases: Case 8 (a, b) and Case 9 (c, d)

C. HPV Pattern and Disease Progression

As illustrated in Figures 4A and 4B, the viral copy number and pattern of nuclear involvement varies with disease progression. Initially with CIN I and CIN II, the globular episomal pattern predominates consistent with the high viral copy number and high infectivity of the patient. Subsequently in CIN III and overt carcinoma the more basally oriented low copy number, speckled, integrative pattern prevails.

Fig. 4 HPV Copy Number and Disease Progression
III. Representative Clinical Case Materials

Following is a portfolio of cervical biopsies first diagnosed morphologically by Hematoxylin and Eosin (H&E) staining and then stained with INFORM HPV Probes for Family 16 and/or Family 6 HPV. Included are representative examples of normal cervical epithelial, CIN I, CIN II, CIN III, carcinoma and condyloma.

A positive HPV result is judged when either the episomal or integrated pattern is found. The episomal pattern appears as a large homogeneous navy-blue precipitate within the nucleus. The integrative pattern is a discrete, stippled navy blue nuclear pattern within a “field” of cervical cells. As shown, the episomal pattern is generally prominent in the superficial keratinized region of the epithelium often within koilocytotic cells. The “speckled” nuclear integrative pattern is more often in more basal epithelial cells.

Clinical Examples:

A. Normal

These cases are all cervical biopsies taken at colposcopy in the context of a preceding abnormal PAP or a visual colposcopic abnormality. As shown in Figure 5, these biopsies were all judged by H&E microscopy to be within normal limits without definitive pathologic alteration.

In each case, the INFORM HPV III Family 16 Probe (B) was judged as negative with no evidence of either an episomal or integrative pattern present. In comparison with the negative reagent control, a very slight bluish haze is found. This trace staining is unrelated to HPV.

In Cases 23 and 24, a common artifact is noted as a very superficial layer of navy-blue India Ink used at colposcopy to mark lesional tissue.

Fig. 5 Normal Cervical Tissue Stained With INFORM HPV III Family 16 Probe (B)

Case 22

Case 23

Case 24

Case 25
B. Cervical Intraepithelial Neoplasia (CIN) I

These 3 cases illustrate, in Figure 6, the histopathologic and INFORM HPV III Family 16 Probe (B) staining characteristics of CIN I lesions. As shown, a predominance of the episomal nuclear staining in the superficial layers of the epithelial are the usual finding.

Occasionally, a case of CIN I may have the mid-and basal-layer speckled, integrative pattern (see Case 7, Fig 2).

Numerous studies have documented the high level of intraobserver and interobserver variability associated with the histopathologic diagnosis of CIN I. Upon adjudication in the ALTS trial, 41% of cases diagnosed on H&E as CIN I were down-graded to normal and 13% were upgraded to CIN II and CIN III10. The biologic potential of CIN I lesions is characterized by high rates of spontaneous regression and low rates of progression to cancer. The poor reproducibility and uncertain biologic potential of CIN I have made the management of women with CIN I problematic. Recent evidence2,3,5,11 suggests the identification of integrated HPV in the basal epithelium by means of in situ hybridization may distinguish patients with histologic CIN I who are at greater risk for the development of high-grade lesions.

Fig. 6 CIN I Lesional Tissue Stained With INFORM HPV III Family 16 Probe (B)
C. Cervical Intraepithelial Neoplasia (CIN) II / III

In contrast with the predominance of the episomal pattern in CIN I, the majority of CIN II / III cases (Figure 7) illustrate two patterns of HPV positivity. These include the prominent globular “episomal” staining found notably in the superficial epithelial cells. These represent the “infected”, exfoliated form, which is involved in subsequent disease transmission. Further, below in the epithelium, are found the epithelial cells with an integrative pattern of staining. These include cells from the mid-level to basal-level epithelial cells, which are thought to represent the sites of HPV-clonal integration into host cells. The adjacent stromal cells, endothelial cells, and lymphocytes are appropriately negative.

As illustrated in Case 32, some CIN III lesions may contain only the integrative pattern with very low viral copy number. In this instance only one integration site per epithelial nucleus is observed.

Fig. 7 CIN II/III Lesional Tissue Stained With INFORM HPV II Family 16 Probe (B)
D. Squamous Cell Carcinoma

These cases of invasive cervical squamous carcinoma (Figure 8) demonstrate nuclear HPV integrative pattern positivity in the neoplastic epithelial cell nuclei. While invasive cervical carcinoma may show two patterns of staining, both episomal and integrative, the majority of cervical carcinoma cases have a very low viral copy number (as documented in the literature), which manifest in tissue section ISH as the prominence of the integrated pattern of nuclear staining.

As shown in Cases 35 and 36, the integrative pattern may often be the sole staining found in the neoplastic cells. Notice in the two cases illustrated the viral copy number and number of integration sites per nucleus is very similar to that observed within HeLa nuclei within the cell line control slide (see Figure 10). The adjacent stromal, lymphocyte, and endothelial cells are negative.

Fig. 8  Cervical Carcinoma Lesional Tissue Stained With INFORM HPV III Family 16 Probe (B)
E. Condyloma

Condyloma are superficial wart-like lesions found within the epithelium of the cervix and found in association with “specific” HPV genotypes (e.g. HPV 6 and 11). These HPV 6/11-associated lesions are expectedly negative for INFORM HPV III Family 16 Probe (B) (see Fig 9, Case 37 (B)) and positive for INFORM HPV II Family 6 Probe, as shown in Figure 10 (Case 37 and Case 38).

The staining patterns in Cases 37 and 38 speak to the specificity of the INFORM HPV Family 16 and Family 6 Probes.

Fig. 9 Condyloma Stained With INFORM HPV III Family 16 (B) and HPV II Family 6 Probes
A. Staining Patterns of Episomal and Integrated HPV

The determination of a true negative and/or positive HPV INFORM ISH result requires, in the same run, a positive control slide such as a known HPV-positive control slide (see Figure 11) or an index case with an integrative-only pattern (see Figure 10). The appropriate positive “integrative” nuclear pattern is found in both CaSki and HeLa cells and absent in HPV negative cells. The CaSki cells are HPV 16 genotype cells with abundant copy number (200-600 copies/cell). HeLa cells are HPV 18 genotype cells with lower viral copy number (10-50 copies/cell). The HeLa cell line in particular serves as a sensitivity control for the low level integrative staining pattern, which may be found in some CIN III (Figure 7) and in most invasive cervical carcinomas (Figure 8 and 10). The HPV-negative cells serve as an important indicator of inappropriate noise in the HPV assay.

The importance of using a patient sample “index case” is well illustrated by Case 40 (see Figure 10). This patient sample of cervical carcinoma has a HeLa-like level of HPV expression and the multiple blocks available allow the use of either a same-slide or same-run control from a constant source for extended time.

Fig. 10 Patient “Index Case” Control Samples

CaSki-like Case: Case 39

HeLa-like Case: Case 40

Fig. 11

A. HPV 3 in 1 System Control Slide

B. HPV Cell Lines Stained With HPV III Family 16 Probe (B)
V. Alu Positive Control Probe II for Tissue Qualification

Specifically, the Alu Positive Control Probe II detects Alu repeats. A positive nuclear signal within epithelial and non-epithelial cells confirms the tissue sample contains intact DNA (Figures 12 and 13). A negative Alu Positive Control Probe II indicates loss of DNA intactness probably related either to tissue handling and/or tissue fixation. The Alu Positive Control probe serves as the equivalent of the “housekeeping gene” control used in PCR assays. Accordingly, like PCR, if DNA intactness is lost, a negative result may not be true and study of additional lesional tissue is necessitated.

Fig. 12 DNA Positive Alu Control Slides

Case 41 (A): Alu, no CC  *  * CC = Cell Conditioning  
Case 41 (B): Alu with CC

Fig. 13 Genome Integrity and ISH Assay

Case 42 (A)  
Case 42 (B)

Case 43 (A)  
Case 43 (B)

Figures 12 and 13 illustrate two clinically relevant examples of this assay. In Case 41, the Alu or DNA positivity is stronger with cell conditioning than without cell conditioning, indicating the importance of the unmasking of DNA by heat, pH, or enzymes. In Cases 42 and 43 are shown the relationship between Alu or DNA abundance and Alu signal and HPV assay signal.
Notice in Case 43 the nearly absent Alu ISH is mirrored by a nearly-absent HPV result.

Thus, the Alu ISH probe may be used to optimize the conditions for detection of HPV DNA allowing selection of ideal DNA signal preservation relative to cell conditioning or denaturation conditions. Finally, the Alu ISH probe may prove especially useful when there is an unexpected HPV-negative result in the face of lesional tissue (eg CIN I, II, III). In this instance, the Alu ISH assay might be absent or nearly absent establishing the lack of DNA preservation as an explanation of the unexpected negative assay result (Figure 13). The finding of a positive Alu ISH result, while indicating the intactness of biopsy DNA, does not establish DNA accessibility or openness related to fixation method or assay sensitivity. Accordingly while DNA may be intact and accessible for hybridization with neutral buffered formalin, it may be intact but not accessible with certain fixatives (Figure 14).

VI. Fixation

The Ventana-validated assay was developed utilizing tissue fixed in 10% neutral buffered formalin. Artificial tumors generated using the SiHa characterized human cell line (1-2 copies HPV 16 per nucleus) were fixed in a variety of fixatives for eighteen hours, two days, or five days. Tissue was prepared in 5μm sections and stained using HPV III Family 16 Probe (B) with extended cell conditioning and ISH protease 3 for four minutes. Figure 14 illustrates the ability of the Ventana validated assay to detect HPV in differentially fixed tissue. Results show HPV staining is robust in Neutral Buffered Formalin and Zinc Formalin fixed tissues; no degradation of signal or loss of antigen unmasking is noted for fixation times from eighteen hours to five days. In contrast, Prefer and Bouin’s fixatives show different levels of detection among the times tested when compared to Neutral Buffered Formalin. Less than optimal tissue acquisition, fixation and storage are major factors in the ability to detect HPV signal for microscopic interpretation.

**Fig. 14 HPV In Situ Hybridization Performance in SiHa Cell Lines**
VII. Interpreting Artifacts

A. Overdigestion Artifacts

As illustrated in Figure 15, use of overly strong amounts of protease or prolonged protease treatment may result in a loss of tissue integrity making interpretation of lesional tissue difficult.

B. Leukocyte-associated Artifacts

Cytoplasmic staining of leukocytes, as shown in Figure 15, occurs frequently. This non-specific dense cytoplasm staining is unrelated to the DNA probes. It is associated with the secondary antibodies and/or bioconjugates reacting non-specifically with cytoplasmic components. The localization in the cytoplasm, and not the nuclei, of both polymorphonuclear (PMNs) leukocytes and eosinophils indicates a non-specific, non-viral-associated artifact.

Occasionally acute cervicitis with an abundance of PMNs is seen. The consequent artifactual staining is readily perceived as non-specific as the signal is found in the cytoplasm of PMNs and not in the nuclei of epithelial cells. Note that the “true positive” integrative pattern of HPV staining is also found in the epithelial cells of Case 46.

Fig. 15 Artifacts With INFORM HPV III Family 16 Probe (B)
C. Drying Artifacts

Rarely, liquid reactants (buffers and liquid coverslip) may wick off the slide resulting in focal drying artifact. This may result in focal deposition of chromogen (e.g., NBT-BCIP complex) resulting in either a pool or band of blue dye (see Figure 16).

Fig. 16 Artifacts With INFORM HPV III Family 16 Probe (B)
D. Chromogen Precipitate Artifact

Rarely, the NBT-BCIP chromogen may discretely precipitate resulting in irregular, “meteorite-like” dark blue foci of dye (see Figure 16). These “burr-like” objects are notably found above the plane of focus of the tissue surface, in contrast with the true “episomal” staining with smooth globular navy-blue staining, which is within the focal plane of the epithelial nucleus. Rarely the NBT-BCIP chromogen may precipitate in crystalline form (see Figure 17). This artifact is caused specifically by inadequate dehydration prior to cover-slipping.

Fig. 17 Chromogen Precipitate Artifact: Case 51
E. Nuclear Artifact

Two separate nuclear artifacts have been observed as rarities. First, a pale blue staining of nucleoli may be noted (see Figure 18). This phenomenon is not a virus-associated phenomenon but most probably reflects weak cross-hybridization with RNA under certain fixation and cell conditioning circumstances. As illustrated, this phenomenon characteristically occurs in all nucleoli, including both epithelial and non-epithelial cells. Second, stippled nuclear and/or cytoplasmic staining may occur in a blotchy pattern within lymphocytes, fibroblasts, and some endothelial cells (see Figure 19). This artifact has no viral association and, again, may reflect weak cross-hybridization with RNA under certain circumstances. The presence of this phenomena in lymphomal and stromal cells indicates non-specific staining and in the extreme may preclude diagnostic interpretation.

Fig. 18 Nuclear Artifact: Case 52

Fig. 19 Nuclear Artifact: Case 53
VIII. Bibliography


