

## Detection of Human Papillomavirus DNA in Genital Lesions by Using a Modified Commercially Available In Situ Hybridization Assay

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**A modified, commercially available DNA-DNA in situ hybridization test that uses biotinylated probes for the identification of human papillomavirus (HPV) DNA types 6/11, 16/18, and 31/33/35 was evaluated. HPV DNA was detected in 314 of 787 (40%) histologically abnormal genital biopsy specimens by using the ViraType in situ assay (Life Technologies, Gaithersburg, Md.), in which the hybridization time was increased from 2 to 16 h. Ninety percent of positive condyloma acuminata specimens contained HPV type 6/11 DNA. The prevalences of HPV DNA for cervical intraepithelial neoplasia I, II, and III lesions by this in situ hybridization test were 42, 54, and 55%, respectively. The combined prevalence of HPV type 16/18 and 31/33/35 DNAs increased with the severity of the lesion, while the prevalence of type 6/11 DNA decreased. HPV type 6/11 DNA was found only in 1 of 16 (6%) positive cervical intraepithelial neoplasia III specimens. HPV type 16/18 and 31/33/35 DNA was detected in 11 of 16 (69%) and 4 of 16 (25%) in situ hybridization-positive cervical intraepithelial neoplasia III specimens, respectively. Thus, the observation that certain "higher-risk" HPV genotypes are associated with upper-grade cervical precancer lesions was confirmed by this commercial hybridization system. In general, the assay was found to be well suited for use in the clinical laboratory. The ViraType in situ procedure modified for a longer hybridization time may be helpful in identifying lesions containing higher-risk HPV strains.**

Numerous studies have implicated certain genotypes of human papillomavirus (HPV) in the development of cervical dysplasia and carcinoma (9, 11, 12). HPV type 16, 18, and/or 31 DNA has been found in both upper-grade precursor cervical lesions and frank cervical carcinomas, while HPV type 6 and 11 DNA has rarely been detected in such specimens. Rather, these latter HPV genotypes have been identified mainly in overt condylomas and lower-grade cervical intraepithelial neoplasia (CIN) lesions (17). Therefore, separation of CIN lesions containing HPV type 16, 18, and/or 31 DNAs from those with type 6 and 11 DNAs may help to identify those patients at the greatest risk of developing cervical carcinoma.

Various hybridization techniques (3-5, 8, 10, 14, 18, 20) have been used to detect different HPV genotypes, including Southern blotting, dot blotting, and in situ hybridization (ISH). A simple, rapid, sensitive, and inexpensive assay to identify different HPV DNA genotypes in cervical lesions would facilitate the investigation of the role of HPV in cervical carcinoma and would help to determine the usefulness of HPV typing in patient management. The ISH technique is more readily suited than the Southern blot method for use in a clinical laboratory. We recently reported our HPV genotyping results for a large series of cervical lesions in which we used an ISH system developed in our laboratory (1). Several commercial ISH test kits are now available for use on Formalin-fixed, paraffin-embedded material. The present investigation was undertaken to evaluate the Life Technologies (Gaithersburg, Md.) ISH assay for the detec-

tion of HPV DNA in the entire spectrum of genital lesions and to determine its applicability in the clinical laboratory.

### MATERIALS AND METHODS

**Specimens tested.** A total of 806 Formalin-fixed, paraffin-embedded genital biopsy specimens submitted for routine histological examination during 1989 were tested for HPV DNA by ISH by using the Life Technologies ViraType in situ kit. The 806 specimens were selected on the basis of an original diagnostic report that indicated that they were histologically abnormal. These specimens were considered by one of the nine regular staff pathologists to have some evidence of HPV infection (presence of koilocytic atypia, dysplasia, or both), as determined from examination of hematoxylin-and-eosin-stained sections. All hematoxylin-and-eosin-stained slides were reviewed by one of the authors (A.J.A.) without knowledge of the original histological diagnosis or the ISH results, and the reviewed diagnoses are reported here. No major discrepancies between the original and reviewed diagnoses were found. If there were two or more histologically abnormal biopsy specimens from the same patient on the same day, the specimen with the worst histological diagnosis was tested.

**ISH assay.** Sections of 6  $\mu$ m in thickness were cut and placed on the 3-aminopropyltriethoxysilane slides supplied with the kit. The sections were baked at 80°C overnight and then deparaffinized in two changes of xylene followed by two changes of absolute ethanol. The slides were air-dried. Specimens were tested for HPV DNA according to the directions of the manufacturer, with several modifications. Slides were treated with prewarmed digestion solution (200 mg of reagent B proteolytic enzyme per ml in 0.1 N HCl) for 15 min at 37°C and dehydrated in 95% ethanol and then

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absolute ethanol each for 1 min. The slides were then air-dried.

One drop of the appropriate probe reagent (type 6/11, 16/18, or 31/33/35) was added to the treated tissue section, a coverslip was placed on the tissue-probe, and then the slides were placed on a 100°C hot plate for 8 min to denature both the target and probe DNA. The hot plate was covered to maintain a more uniform temperature. Following denaturation, the slides were sealed with rubber cement and incubated at 39°C in a moist chamber for 16 h.

Following hybridization, the rubber cement was removed and the coverslips were soaked off in a Tris-saline solution consisting of 50 mM Tris and 150 mM NaCl. The slides were then rinsed in Tris-saline several times; this was followed by a wash in the same buffer containing 3% bovine serum albumin. The slides were again rinsed in Tris-saline. The slides were washed three times for 3 min each time in prewarmed buffer 2 solution (a buffered saline solution containing bovine serum albumin) from the kit at 37°C. Visualization of the hybridization was done exactly as specified by the manufacturer by using the alkaline phosphatase supplied in the kit. The substrates used in this system were 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

Slides were counterstained in 1% fast green in phosphate-buffered saline instead of the nuclear fast red stain supplied in the kit. After washing off the excess fast green in tap water and distilled water, the slides were dehydrated for 1 min each in 95% ethanol and then 100% ethanol, followed by a rinse in xylene. Coverslips were placed on the slides by using Permount.

Therefore, the following major modifications of the test kit were made: (i) baking of the slides at 80°C instead of 58°C, (ii) increasing the denaturation time from 5 to 8 min, and (iii) increasing the hybridization time from 2 to 16 h.

All slides were read by one of the authors (M.P.M.) without knowledge of the histological results. Cases were considered positive for HPV DNA whenever specific dark purple nuclear staining occurred in cervical epithelial cells in the absence of any staining in the underlining connective tissue. The specific type of HPV DNA present was determined by comparing the number of positive cells and the intensity of staining among the individual slides hybridized with the three different HPV probe groups used in each case. Assignment of the specific viral type was made on the basis of which slide contained the greatest number of positive cells. Criteria for multiple or mixed infections included staining with two different probe types in different foci of epithelial cells.

## RESULTS

A total of 806 biopsy specimens with adequate tissue for testing by ISH were identified and evaluated. Of the 806 biopsy specimens tested, insufficient tissue for proper evaluation remained on the slides following ISH for 19 specimens (2%). Therefore, ISH results were available for the remaining 787 specimens, indicating that the coating was very effective in maintaining adherence of the tissue to the slides throughout the harsh ISH procedure. Positive ISH results were obtained from 314 of 787 (40%) specimens. HPV type 6/11 DNA was detected in 101 of 314 (32%) positive specimens, while type 16/18 DNA was found in 137 of 314 (44%) positive specimens. Twenty-four percent (76 of 314) of samples with HPV, as demonstrated by ISH, contained HPV type 31/33/35 DNA.

TABLE 1. Detection of HPV in genital lesions by ISH by using biotinylated probes

Histologic diagnosis	No. positive/total no. (%)	No. (%) of the following HPV DNA types in positive ISH specimens:		
		6/11	16/18	31/33/35
Condyloma	49/64 (77)	44 (90)	1 (2)	4 (8)
Normal/minimal dysplasia	27/182 (15)	8 (30)	13 (48)	6 (22)
CIN I	185/444 (42)	43 (23)	96 (52)	46 (25)
CIN II	37/68 (54)	5 (14)	16 (43)	16 (43)
CIN III	16/29 (55)	1 (6)	11 (69)	4 (25)
Total	314/787 (40)	101 (32)	137 (44)	76 (24)

The correlation of HPV types with the histological diagnosis is shown in Table 1. Histologically, 8% (64 of 787) and 22% (182 of 787) of the specimens were considered condyloma acuminata and "normal/minimal dysplasia," respectively. The remaining 70% of the specimens had evidence of CIN. Of 64 condyloma acuminata specimens, 49 (77%) were positive by ISH, with 44 of 49 (90%) positive condyloma specimens being infected with HPV type 6/11 DNA. Only 27 of 182 (15%) specimens considered to be normal/minimal dysplasia (inflammatory or reactive changes with or without koilocytic atypia) were positive for HPV DNA by ISH. The prevalences of HPV DNA demonstrated by ISH for CIN I, II, and III lesions were 42, 54, and 55%, respectively. If the specimens with minimal dysplasia were eliminated from the analysis, then the detection rate for HPV for lesions clearly considered to be CIN was 44% (238 of 541 specimens). Similarly, the overall HPV detection rate would increase to 47% (287 of 605 specimens) by elimination of the normal/minimal dysplasia samples.

The combined prevalence of HPV types 16/18 and 31/33/35 increased with the severity of the lesions, with 15 of 16 (94%) ISH-positive CIN III lesions containing these viral types. Only 1 of 16 (6%) ISH-positive CIN III specimens contained HPV type 6/11 DNA.

While cross-hybridization between the three different probes often occurred, especially for types 16/18 and 31/33/35, one slide usually contained significantly more positive cells and greater intensity. No multiple or mixed infections were detected on the basis of staining by different probes in different foci of cells.

**Effect of hybridization time.** The 806 specimens were hybridized for 16 h instead of 2 h, as specified in the instructions of the manufacturer. In order to evaluate the effect of increasing the hybridization time, two experiments were performed. In the first experiment, extra tissue slides, which were prepared, deparaffinized, treated with proteinase, and dehydrated at the time of the original testing, were identified for 59 ISH-positive specimens. These extra slides were then denatured, hybridized, and stained as specified above, except that a 2-h hybridization time was used. Of these 59 specimens which were positive after 16 h of hybridization, only 39 (66.1%) were also positive with 2 h of hybridization. The dysplastic lesion in all 59 specimens was present on the extra slides tested with the 2-h hybridization.

A second experiment was then performed to determine whether the results were reproducible on repeated examination. Eighteen positive and 20 negative specimens were selected to obtain a distribution of specimens similar to that of the original 806 specimens in terms of histological status

TABLE 2. Comparison of different hybridization times for detection of HPV by ISH

Specimen no.	Histology	HPV type found with:			Lesion present on repeat sample
		Original results at 16 h of hybridization	Repeat result at the following hybridization times:		
			16 h	2 h	
1	Normal/minimal dysplasia	16/18	16/18	16/18	Yes
2	Normal/minimal dysplasia	16/18	31/33/35	31/33/35	Yes
3	Condyloma	6/11	6/11	— <sup>a</sup>	Yes
4	Condyloma	6/11	6/11	—	Yes
5	Condyloma	6/11	6/11	6/11	Yes
6	CIN I	6/11	6/11	6/11	Yes
7	CIN I	6/11	6/11	6/11	Yes
8	CIN I	6/11	31/33/35	31/33/35	Yes
9	CIN I	16/18	16/18	16/18	Yes
10	CIN I	16/18	16/18	31/33/35	Yes
11	CIN I	16/18	31/33/35	—	Yes
12	CIN I	16/18	31/33/35	—	Yes
13	CIN I	16/18	—	—	No
14	CIN I	31/33/35	31/33/35	31/33/35	Yes
15	CIN I	31/33/35	6/11	—	Yes
16	CIN II	16/18	16/18	16/18	Yes
17	CIN II	16/18	—	—	No
18	CIN II	16/18	—	—	No

<sup>a</sup> —, negative for HPV 6/11, 16/18, or 31/33/35.

and viral types. The paraffin blocks from these 38 specimens were recut and new tissue slides were prepared. One set of new recut slides was tested with a 16-h hybridization, while another set of new recut slides was tested with only a 2-h hybridization for the specimens that were originally positive with the 16-h hybridization. Upon retesting, positive ISH results after 16 h of hybridization were obtained from 15 of 18 (83.3%) specimens that were initially positive (Table 2). The dysplastic lesion was not present on the recut tissue slides for the three specimens in which negative retest results were obtained. Of the 15 specimens in which positive retest results were obtained with the 16-h hybridization time, only 10 (66.7%) were positive upon retesting with the 2-h hybridization. The same viral type was identified in 9 of 10 (90%) specimens in which positive retest results were found after both hybridization periods.

Upon retesting of the 20 specimens that were originally negative, the new recut tissue slides were all still negative with the 16-h hybridization. The dysplastic lesion was present on the recut tissue slides in 17 of 20 (85%) specimens.

## DISCUSSION

The present study was undertaken to determine the usefulness of a commercially available ISH assay for the detection of HPV DNA in cervical lesions. This assay allows the identification of three HPV DNA types (6/11, 16/18, 31/33/35) in Formalin-fixed, paraffin-embedded material. We previously identified HPV DNA in 266 of 584 (45.5%) histologically abnormal cervical biopsy specimens using a ISH system developed in our laboratory (1). Both our system and the Life Technologies assay use biotinylated DNA probes. However, in the previous study (1), specimens were hybridized for 5 days and hybridization was visualized by using a six-step avidin-biotin immunoperoxidase system, while in the present study, hybridization was conducted for 16 h and was visualized by using an alkaline phosphatase procedure. In addition, separate probes against DNA types 6, 11, 16, 18,

and 31 were used in the former study, while combined probes (types 6/11, 16/18, 31/33/35) were used in the Life Technologies test. The exact stringency of hybridization was not specified for the ViraType HPV in situ system; however, it appeared to be low because of the increased cross-reactivity of the different probes, especially HPV types 16/18 and 31/33/35. Nuovo and Richart (7) reported the stringency of this commercial system to be low ( $T_m - 40^\circ\text{C}$ ). This cross-reactivity is shown in Table 2.

The results obtained with the commercial system were very similar to our previous results (1), since 314 of the 787 (40%) biopsy specimens tested contained HPV DNA. Similar to our earlier study and results from other investigators (16, 17), most condyloma acuminata specimens were positive for HPV DNA by the commercial assay. Likewise, the prevalence of the "higher-risk" viral strains (types 16/18 and 31/33/35) increased with the severity of the lesions, while the prevalence of type 6/11 decreased. The Life Technologies system appears to be somewhat more sensitive than our old system for the detection of HPV in lesions with very minimal dysplasia, since 15% of such lesions were positive compared with 9% of such cases in our earlier investigation (1). This may be due to the inclusion of type 33 and 35 DNA probes in the Life Technologies kit. Similar to our results, Czegledy et al. (2) detected HPV DNA in 15% of normal cervical samples using Southern blotting. Wilbur and colleagues (17) found HPV mRNA sequences with a tritiated RNA ISH system in 26% of lesions that were histologically suggestive but not diagnostic for HPV. The lesions that are histologically suggestive but not diagnostic of HPV infection present difficult diagnostic and therapeutic challenges. Of the 787 biopsy specimens for which ISH results were obtained in the present study, 182 (22.5%) specimens were considered to be in this borderline category. HPV typing technology may prove to be useful for such lesions in order to differentiate truly HPV-associated from non-HPV-related lesions. Further studies with more sensitive typing procedures are needed to determine the actual prevalence of HPV in these questionable lesions.

The detection rate of HPV type 31/33/35 DNA found in the present investigation was higher than expected. However, high prevalence rates for these viral types have been reported by other investigators by using both radioactive and biotin-labeled ISH systems (7, 19). There appears to be some geographic variation in the distribution of these viral types (19).

Our results with the Life Technologies system are almost identical to the experience of Nuovo and Richart (7) with this commercial assay. These investigators detected HPV in 24, 47, and 29% of CIN I, II, III lesions, respectively. Nuovo and Richart (7) found the ViraType HPV in situ test to be as sensitive as filter and Southern blot hybridization. In another study, those same researchers found the Life Technologies in situ assay to be as equivalent to ISH when <sup>35</sup>S-labeled probes were used (6). Syrjanen (15) reported that the ViraType ISH kit is as sensitive as dot blot hybridization and radioactive ISH. Other biotin-based ISH systems have been compared with other hybridization methods, with favorable results (13, 16).

Overall, HPV DNA was detected in 44% of our CIN lesions. It has been assumed that virtually all CIN lesions are HPV associated. Use of polymerase chain reaction technology and other techniques more sensitive than ISH or Southern blotting is needed to confirm this assumption. Alternatively, it is possible that CIN lesions negative for HPV DNA by ISH may be infected with other HPV DNA types.

Only a few studies that have compared the Life Technologies in situ system with the Enzo Diagnostics (New York, N.Y.) Pathogene HPV ISH assay have been reported (6, 15). Results of these initial studies indicate that the former system is more sensitive than the latter one for HPV detection. More in-depth comparisons will be needed.

In general, we found the Life Technologies in situ HPV detection assay to be well suited for use in the clinical laboratory and easier to use than our former, more cumbersome method. We increased the sensitivity of the test by increasing the hybridization time from 2 to 16 h. In addition, we increased the denaturation time from 5 to 8 min. Provided that the dysplastic lesion is present upon recutting of the tissue, the assay is generally reproducible, although differences in type-specific interpretation may occur between the type 16/18 and 31/33/35 probes.

While the Viratype ISH assay can be used to identify those lesions containing higher-risk viral types, the usefulness of HPV DNA typing remains controversial. At present, treatment of patients with CIN is based primarily upon the histological diagnosis and not HPV typing results. Some gynecologists reexamine or follow patients with HPV DNA types 16/18 at shorter intervals. Further follow-up prospective studies are needed to determine whether treatments should be modified because of the presence of higher-risk HPV types.

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