

# **Anatomic Viral Detection Is Automated: The Application of a Robotic Molecular Pathology System for the Detection of DNA Viruses in Anatomic Pathology Substrates, Using Immunocytochemical and Nucleic Acid Hybridization Techniques**

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This paper presents the first automated system for simultaneously detecting human papilloma, herpes simplex, adenovirus, or cytomegalovirus viral antigens and gene sequences in standard formalin-fixed, paraffin-embedded tissue substrates and tissue culture. These viruses can be detected by colorimetric in situ nucleic acid hybridization, using biotinylated DNA probes, or by indirect immunoperoxidase techniques, using polyclonal or monoclonal antibodies, in a 2.0-hour assay performed at a single automated robotic workstation.

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## INTRODUCTION

The accurate diagnosis of viral pathogens in cells, cellular extracts, and standard histopathology specimens is one of the leading research goals in cell biology and investigative anatomic pathology. The application of molecular probes to the problem of viral diagnosis in formalin-fixed, paraffin-embedded tissues began in 1983, when Brigati et al. [1] described the first successful detection of viral genomic information in these substrates by in situ DNA hybridization using biotin-labeled deoxyribonucleic acid (DNA) probes. Several papers have since compared and contrasted the use of molecular probes and antibody-directed techniques for their specificity and sensitivity in detecting viruses in formalin-fixed, paraffin-embedded tissues [2,3,4,5], but none of these papers has shown a single method by which both of these techniques can be employed simultaneously. Recently, our development of the first automated system for

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*Abbreviations:* AEC: 3-amino, 9-ethyl carbazole BCIP: 5-bromo-4-chloro-3-indolyl phosphate p-toluidine CMV: cytomegalovirus DNA: deoxyribonucleic acid HPV: human papillomavirus HSV: herpes simplex virus INT: p-iodonitrotetrazolium violet

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tissue antigen [6] and gene [7] detection in formalin-fixed tissue sections provided the opportunity for our laboratory finally to unify and streamline these previously separate approaches into a single system for the accurate diagnosis of viral pathogens in 2.0 hours or less, by either molecular hybridization or immunologic techniques. For the first time, the cell biologist, clinical virologist, or surgical pathologist can combine the accuracy of genetic and immunologic diagnostic approaches into a single automated tissue-detection system or can use them separately.

## MATERIALS AND METHODS

### *Tissue Substrates*

Formalin-fixed, paraffin-embedded tissue blocks from various surgical and autopsy specimens of cases known to be infected with herpes simplex virus types 1 and 2, adenovirus types 2 and 5 [1], papillomavirus, or cytomegalovirus (CMV) were obtained from the paraffin archives of the Department of Pathology at Penn State's Milton S. Hershey Medical Center or the Memorial Sloan-Kettering Cancer Center. Serial five-micron sections were cut from these blocks and were used for either immunocytochemistry or in situ DNA hybridization. Adenovirus-infected A549 cells were grown on cover slips as previously reported [1].

Standard hematoxylin- and eosin-stained slides from all cases were reviewed prior to the study. The control cases of herpes simplex virus, adenovirus, and cytomegalovirus studied were backed by positive isolation of the pathogen in tissue culture. Other cases were used based upon a positive clinical history of exposure, the presence of significant positive serology, and the observation of morphologically characteristic viral inclusions. The cases of papillomavirus were all vulvar condylomas, and the diagnosis was made by the presence of koilocytotic atypia. One of these cases contained dysplasia and in situ squamous cell carcinoma in the adjacent squamous epithelium.

### *Reagents*

The reagents used were those previously described [6,7], with the following significant modifications. The distilled water with 2.5 ml BRIJ per liter in station 2 (Fig. 2) contained 25 percent methanol. The automation buffer in station 10 had 25 percent acetone added to it. Both of these changes appeared to improve capillary action as well as to decrease background staining. We found that eliminating the blocking protein before the primary antibody or after the biotin-labeled DNA probe reduced nonspecific staining.

The following were purchased as products of Fisher Scientific Company: reagent alcohol (Catalog No. A-962-4), acetone (Catalog No. A18-500), methanol (Catalog No. A-412-500), Hemo-De (Catalog No. 15-182-507), 30 percent hydrogen peroxide (Catalog No. A-325-500), BRIJ 35 (Catalog No. BP345-500), and a 50-slide holder (Catalog No. 15-185-293). The following items were purchased from Fisher Scientific Company as products of Biomeda Corporation: aqueous hematoxylin (Catalog No. BM-M10), Probe Wash (Catalog No. BM-M38), 10× automation buffer (Catalog No. BM-M30), primary antibody diluent (Catalog No. BM-M35), peroxidase reagent (Catalog No. BM-M32), peroxide/chromogen kit (Catalog No. BM-S01), Auto/Zyme (Catalog No. BM-M34), Crystal Mount (Catalog No. BM-M02), and prediluted rabbit anti-herpes simplex types 1 and 2 antibody (Catalog No. BM-304M). The following products were obtained from Sigma: pepsin (Catalog No. P 7012) and p-iodonitrotetrazolium violet (Catalog No. I-8377). Pronase was obtained from

Calbiochem Inc. (Catalog No. 53702). The following items were purchased as products of DAKO Corporation: rabbit anti-bovine papilloma virus antibody (Catalog No. B580), monoclonal mouse anti-cytomegalovirus antibody (Catalog No. M-757), and alkaline phosphatase-conjugated avidin (Catalog No. D365). Rabbit anti-adenovirus antibodies against common hexon antigen were kindly provided by Dr. Goran Wadell, University of Umea, Sweden. Biotinylated probes for herpes simplex 1 and 2, cytomegalovirus, and adenovirus were purchased from ENZO Biochem, Inc. (Catalog Nos. EBP-838, EBP-835, and EBP-834, respectively). Probes for papillomavirus types 6, 11, 16, and 18 were obtained from Life Technologies, Inc. (Catalog No. 42061020). The following items were purchased as products from AMRESCO: 45 percent formamide hybridization cocktail (Catalog No. PO652), 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (Catalog No. 0885), and dimethylformamide (Catalog No. P0046440).

#### *Automated Instrumentation*

Viral detection was performed with the Fisher Code-On Automated Molecular Pathology system, according to significant modifications of our previously described methods [6,7]. The system consists of an IBM System II-directed triaxial robotic unit, which is contained under a portable fume hood (Fig. 1). The robotic unit transports slides with tissue sections immobilized on them among 20 different stations. Figure 2 shows the basic reagent format for the unit. The most important aspect of this format is that these computer-assisted, triaxial robotic units can simultaneously perform viral localization with antibody or probe detection without any changes in the reagent set-up.

The Code-On molecular pathology system utilizes capillary action to perform automated immunocytochemistry or in situ DNA hybridization [6,7]. A 150-micron capillary gap is formed by apposing the painted surfaces of two specially made Probe-On glass slides (Fisher Scientific Co. Catalog No. 15-187M). The pairs of slides forming the capillary gaps are mounted in a specialized holder, which can hold up to 60 slides, and the robotic arm of the unit moves the slide holder through a programmed series of reagents which are taken up and expelled by capillary action (Fig. 3) [6].

As Fig. 2 shows, the stations are labeled 0 to 19 and include a low-temperature incubation chamber which can achieve temperatures as high as 55° Centigrade, a high-temperature oven which can achieve temperatures as high as 110° Centigrade, and a cool-down chamber. In stations 1 through 7, as well as in station 10, all of the slides in the holder are treated identically. This portion is the batch analysis part of the assay, and it includes dewaxing, hydrating, and buffer washing. In these stations, a single vessel containing a common reagent is employed; this one reagent fills all the capillary gaps simultaneously. In stations 13 through 15, the discrete analysis part of the assay is performed. The tissues in individual capillary gaps can be treated with different enzymes, probes, antibodies, or detection systems in these stations by employing a reagent isolon [6], which divides the station into 30 separate caplet-shaped wells, which can be filled with about 150 to 200 microliters of the particular reagent in question. The hydrophobicity of the isolon allows the reagent to bead up, and therefore a specific capillary gap will easily pick up a separate reagent. Since the robotic slide holder has a capacity of 60 slides, arranged in 30 capillary gaps, the machine can process 30 different probes or antibodies simultaneously in one run at the rate of one

TABLE 1  
The Virocode Program

PROGRAM: Virocode						
TECH: DJB			DATE: 07/22/88			
Event	Station No.	Hold	Time (minutes)	Mix	Temp (°C)	Solution
001	06	Y	1.0	N		Clear
002	19	N	10.0	N	80	Denature oven
003	12	N	0.5	N		Pad
004	06	N	0.1	N		Clear
005	12	N	0.5	N		Pad
006	06	N	0.1	N		Clear
007	12	N	0.5	N		Pad
008	05	N	1.0	N		Reagent alcohol
009	12	N	0.3	N		Pad
010	05	N	1.0	N		Reagent alcohol
011	12	N	0.3	N		Pad
012	04	N	0.1	N		Methanol peroxide
013	12	N	0.3	N		Pad
014	04	N	8.0	N		Methanol peroxide
015	12	N	0.3	N		Pad
016	03	N	0.1	N		95% alcohol
017	12	N	0.3	N		Pad
018	10	N	0.1	N		Buffer
019	08	N	0.1	N		Pad
020	13	N	1.0	Y		Enzyme
021	18	N	10.0	N	40	Chamber
022	09	N	1.0	N		Pad
023	03	N	0.1	N		95% alcohol
024	08	N	0.3	N		Pad
025	10	N	0.1	N		Buffer
026	11	N	0.5	N		Pad
027	14	N	0.5	Y		Primary
028	18	N	15.0	N	48	Chamber
029	09	N	1.0	N		Pad
030	10	N	0.1	N		Buffer
031	09	N	0.3	Y		Pad
032	15	N	1.0	Y		Detection system
033	18	N	15.0	N	48	Chamber
034	09	N	1.0	N		Pad
035	10	N	0.1	N		Buffer
036	09	N	0.5	N		Pad
037	02	N	0.1	N		Distilled water
038	09	N	1.0	N		Pad
039	16	N	0.3	Y		Chromogen
040	18	N	5.0	N	46	Chamber
041	09	N	1.0	N		Pad
042	02	N	0.1	N		Distilled water
043	09	N	1.0	N		Pad
044	16	N	0.5	Y		Chromogen
045	18	N	5.0	N	46	Chamber
046	09	N	1.0	N		Pad
047	02	N	0.1	N		Distilled water
048	09	N	0.5	N		Pad
049	01	N	1.5	N		Hematoxylin

TABLE 1—Continued

PROGRAM: Virocode		TECH: DJB			DATE: 07/22/88	
Event	Station No.	Hold	Time (minutes)	Mix	Temp (°C)	Solution
050	09	N	0.5	N		Pad
051	02	N	0.1	N		Distilled water
052	09	N	1.0	N		Pad
053	02	N	0.3	N		Distilled water
054	11	N	0.5	N		Pad
055	10	N	0.6	N		Buffer
056	11	N	0.5	N		Pad
057	02	N	0.1	N		Distilled water
058	11	N	0.5	N		Pad

Total time for completion = 92.4 minutes = 1.6 hours

probe or antibody per capillary gap. Special glass reagent isolons without depressions were developed in order to limit the amount of probe used for in situ hybridization.

#### *Viral Antigen Detection*

When immunocytochemistry was performed alone, we used methods described by Brigati et al. [6] with modification. Basically, five-micron sections from formalin-fixed, paraffin-embedded tissues were cut, floated on a 42° Centigrade water bath containing no protein or subbing solution, and placed on specially treated silanized Probe-On glass slides [8]. The slides were air dried at room temperature and then heated vertically in a 50-slide holder in an 80° Centigrade convection oven for ten minutes. Heating the slides in the oven immobilized the tissue sections and initiated the dewaxing process. After the slides had been cooled back to room temperature, they were placed in the specialized Code-On slide holder in capillary gap formation (Fig. 3).

The basic Virocode program for viral antigen detection required slightly more than one and a half hours to run (Table 1). One of the most crucial steps for the antibody method was the initial dewaxing step. Antigens from tissues that were not adequately deparaffinized did not stain well. The tissues were cleared with Hemo-De in the high-temperature oven at 80° Centigrade for ten minutes; this use of Hemo-De in the high-temperature oven allowed safe and complete dewaxing because of the high boiling point of the reagent. After hydration with reagent alcohol, endogenous peroxidase was blocked with a mixture of methanol and 3 percent hydrogen peroxide [6]. Enzyme digestion was necessary to demonstrate CMV in formalin-fixed tissue sections. Freshly prepared pronase was used at 10 mg/ml in 1× automation buffer for ten minutes at 40° Centigrade to produce the most intense nuclear localization of CMV. Decreasing the amount of pronase produced a smaller number of antigen-positive cells. The pronase was stable and worked in temperatures as high as 55° Centigrade. 1× automation buffer alone was used on the tissues infected with herpes and papillomavirus during the enzyme step, as the localization of these viral antigens was not improved by trypsin, pepsin, or pronase digestion. The Auto/Zyme system [6] was employed to localize the adenovirus hexon antigen.

Rabbit anti-bovine papilloma virus antibody and monoclonal mouse anti-cytomega-

TABLE 2  
The Virogene Program: Simultaneous Viral DNA and Antigen Detection

PROGRAM: Virogene		TECH: DJB			DATE: 07/22/88	
Event	Station No.	Hold	Time (minutes)	Mix	Temp (°C)	Solution
001	06	N	1.0	N		Clear
002	19	N	10.0	N	110	Denature oven
003	12	N	0.3	N		Pad
004	06	N	0.1	N		Clear
005	12	N	0.1	N		Pad
006	06	N	0.1	N		Clear
007	12	N	0.3	N		Pad
008	05	N	1.0	N		Reagent alcohol
009	12	N	0.1	N		Pad
010	05	N	1.0	N		Reagent alcohol
011	12	N	1.0	N		Pad
012	13	N	0.5	N		Enzyme
013	18	N	10.0	N	40	Chamber
014	11	N	0.6	N		Pad
015	10	N	0.1	N		Buffer
016	11	N	0.6	N		Pad
017	03	N	0.3	N		95% ethanol
018	11	N	0.6	N		Pad
019	05	N	0.3	N		Reagent alcohol
020	11	N	0.6	N		Pad
021	05	N	0.1	N		Reagent alcohol
022	11	N	0.5	N		Pad
023	14	N	2.0	N		Probe or Primary
024	19	N	8.0	N	110	Denature oven
025	00	N	3.0	N		Cool down
026	18	N	20.0	N	37	Chamber
027	07	N	0.1	N		Probe wash
028	11	N	1.0	N		Pad
029	07	N	1.0	N		Probe wash
030	08	N	0.6	N		Pad
031	07	N	0.1	N		Probe wash
032	08	N	0.6	N		Pad
033	07	N	2.0	N		Probe wash
034	18	N	3.0	N		Chamber
035	08	N	0.6	N		Pad
036	10	N	0.1	N		Buffer
037	09	N	1.0	N		Pad
038	15	N	1.0	N		Detection system
039	18	N	20.0	N	37	Chamber
040	11	N	0.6	N		Pad
041	10	N	0.2	N		Buffer
042	08	N	0.6	N		Pad
043	02	N	0.2	N		Distilled water
044	11	N	1.0	N		Pad
045	16	N	0.5	N		Chromogen
046	18	N	5.0	N	45	Chamber
047	11	N	0.6	N		Pad
048	02	N	0.1	N		Distilled water
049	09	N	1.0	N		Pad
050	16	N	1.0	N		Chromogen

TABLE 2—Continued

PROGRAM: Virogene		TECH: DJB			DATE: 07/22/88	
Event	Station No.	Hold	Time (minutes)	Mix	Temp (°C)	Solution
051	18	N	5.0	N	45	Chamber
052	09	N	0.6	N		Pad
053	02	N	0.1	N		Distilled water
054	09	N	0.6	N		Pad
055	02	N	0.1	N		Distilled water
056	09	N	1.0	N		Pad
057	01	N	1.5	N		Hematoxylin
058	09	N	1.0	N		Pad
059	02	N	0.1	N		Distilled water
060	09	N	0.6	N		Pad
061	02	N	0.1	N		Distilled water
062	09	N	1.0	N		Pad
063	10	N	0.6	N		Buffer
064	09	N	0.6	N		Pad
065	02	N	0.1	N		Distilled water
066	09	N	0.6	N		Pad
067	02	N	0.1	N		Distilled water
068	09	N	1.0	N		Pad

Total time for completion = 118.8 minutes = 2 hours.

lovirus antibody to the early nuclear antigen were both diluted 1:50 in the primary antibody diluent. Rabbit anti-herpes simplex virus types 1 and 2 antibodies were obtained already prediluted. Rabbit anti-adenovirus antibody to the hexon common antigen was diluted 1:200 in the primary antibody diluent. Primary antibody incubations were carried out for 15 minutes at 48° Centigrade. We detected these primary antibodies with a universal peroxidase reagent, which is a mixture of horseradish peroxidase conjugated anti-mouse and anti-rabbit antibodies [6]. The secondary antibody reagent was also reacted for 15 minutes at 48° Centigrade. The chromogen used was 3-amino, 9-ethyl carbazole (AEC), as previously described [6]. Nuclear counterstaining was achieved with aqueous hematoxylin for 1.5 minutes. After the program was complete, slides were dried, cover slipped with Crystal Mount, and heated in an 80° Centigrade oven for ten minutes. Immunostaining on cover slips containing adenovirus-infected tissue culture cells was performed by the procedure described above, with the exception that the cover slips were not put through the dewaxing steps.

#### *Viral Gene Detection*

When colorimetric in situ DNA hybridization was carried out alone, we employed a modification of our previously described method [7,9]. The basic Virogene program for our hybridization technique takes about two hours to run (Table 2). The probe for the HSV genome consisted of two sequences of the HSV 1 (3.0 and 8.0 kb) and one sequence of the HSV 2 (16.0 kb) genome. The CMV probe was made from a mixture of two sequences of the CMV genome, one 17.2 kb and the other, 25.2 kb. Fragment size ranged from 150 to 300 base pairs for both the herpes simplex virus (HSV) and CMV probes, as estimated by gel electrophoresis. Adenovirus probe was from genomic

adenovirus 5 DNA. Fragment size ranged from 150 to 300 base pairs as estimated by gel electrophoresis. Cloned human papillomavirus types 6, 11, 16, and 18 DNA probes ranged from 100 to 300 bases in length. The HSV, CMV, adenovirus, and papilloma probes were diluted to 0.5  $\mu\text{g}/\text{ml}$  in 45 percent formamide hybridization cocktail.

Slides with five-micron tissue sections were prepared identically to those described above for immunocytochemistry. Tissues were digested with freshly prepared pepsin, 2 mg/ml in 0.12 N HCl with 2.5 ml BRIJ/liter, at 40° Centigrade for 10.0 minutes. Following this step, the tissues were washed in 1 $\times$  automation buffer with 25 percent acetone and then with 95 percent ethanol.

Because of the viscosity of the hybridization cocktail, the capillary gaps had some difficulty in taking up the probe. This problem was overcome by increasing the time in station 14 to 2.0 minutes in order to assure adequate filling of the gaps. With this increase in time, only 50 microliters of probe per slide were needed to cover the sections immobilized on both sides of a capillary gap. Tissue and probe DNA melting was achieved in 8.0 minutes with the high-temperature oven set at 110° Centigrade.

Following denaturation, the slides were cooled down for 3.0 minutes at room temperature and then incubated at 37.0° Centigrade for 20.0 minutes. This interval was an adequate amount of time for significant hybridization. To remove the hybridization cocktail and wash the slides, a 1 $\times$  Probe Wash solution containing 25 percent acetone was used. It was observed that contacting the capillary gaps with the probe wash before removing the cocktail helped the capillary gaps to drain better. Heating the probe wash in the wet chamber for a few minutes at 37° Centigrade also helped the capillary gaps to clear the probe.

The detection system used for this study was alkaline phosphatase-avidin diluted 1:200 in 1 $\times$  automation buffer with 0.1 percent BSA, 1 mg/ml sodium azide, and 3 micromolar magnesium chloride. The slides were incubated in the detection system for 20 minutes at 37° Centigrade. The chromogen used (McGadey reagent) was made by adding 200 microliters of a 25 mg/ml solution of p-iodonitrotetrazolium violet (INT) in a 50/50 solution of dimethylformamide and distilled water, and 100 microliters of a 50 mg/ml solution of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) in dimethylformamide, to 30 ml of Tris saline, pH 9.5. Total time of incubation with the chromogen was usually for 10.0 minutes at 45.0° Centigrade. The amount of positive staining was dependent on the amount of time the slides were left in the chromogen; more staining was observed with a longer time of incubation (up to 2.0 hours), but background also increased. Slides were washed in distilled water containing 2.5 ml of 30 percent BRIJ 35 solution and 25 percent methanol. Nuclei were counterstained with aqueous hematoxylin for 1.5 minutes. After completion of a run, the slides were dried, cover slipped with Crystal Mount, and heated in an 80° Centigrade convection oven for ten minutes. DNA hybridization was performed on tissue culture cells infected with adenovirus in a way similar to that described above, with the exception that the tissues did not need to be dewaxed.

#### *Simultaneous Viral Gene and Antigen Detection*

We also found that the Virogene program (Table 2) and machine set-up for in situ DNA hybridization could also be used for automated viral antigen detection. Endogenous peroxidase was blocked by adding 3 percent hydrogen peroxide to the reagent alcohol in station 5; this addition did not affect the DNA probe signal. Pronase and Auto/Zyme were still used for digestion in station 13 to detect the CMV and adenovirus





FIG. 1. Color photograph of the Fisher Code-On Molecular Pathology system.

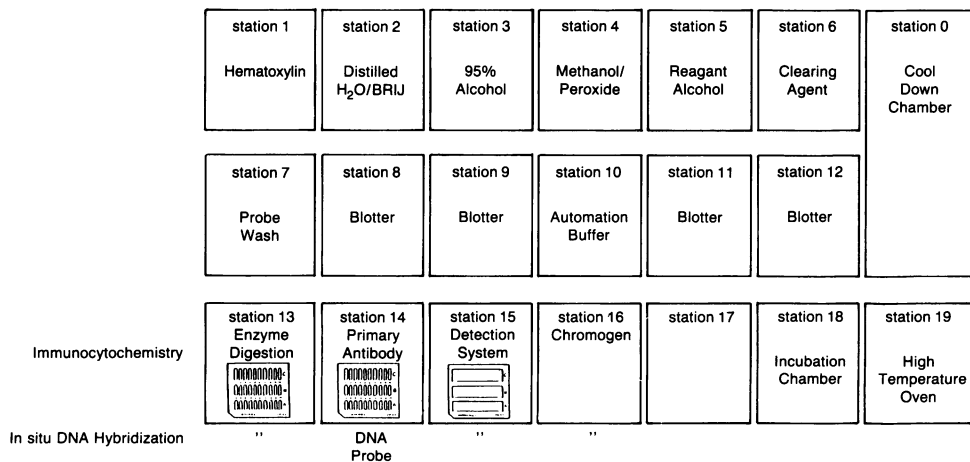


FIG. 2. Basic reagent format used to set up the Fisher Code-On system, showing that the machines can be arranged to do both immunocytochemistry and DNA hybridization.

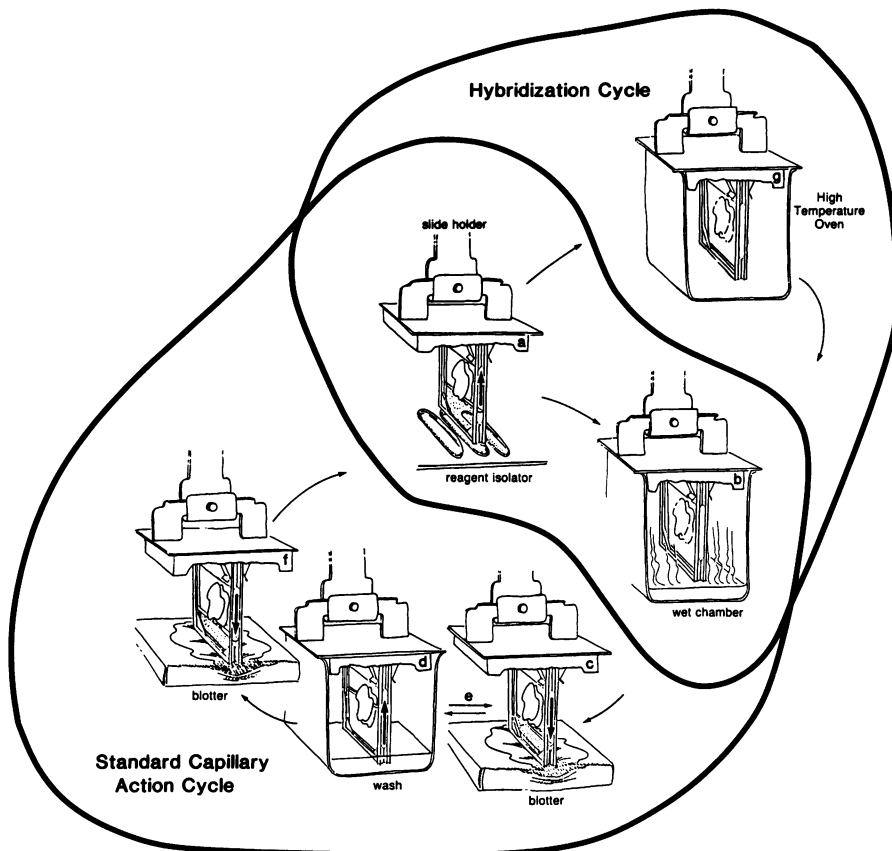


FIG. 3. Diagram of the capillary action cycle and its relationship to the hybridization cycle.

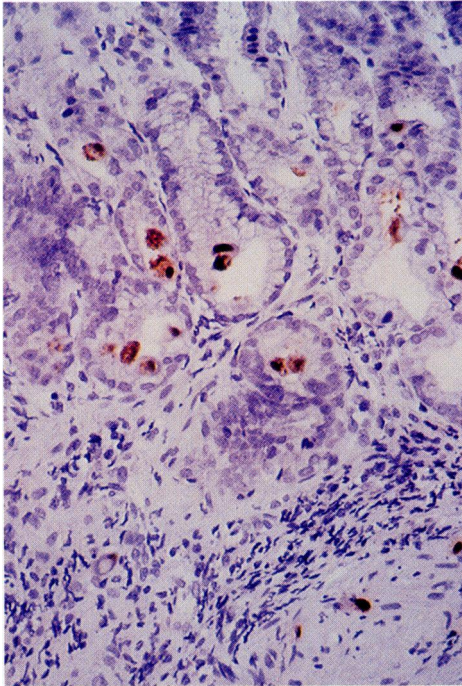


FIG. 4. Photomicrograph of CMV-infected gastric mucosal cells detected by DNA hybridization, using the Virogene program (160  $\times$ ).

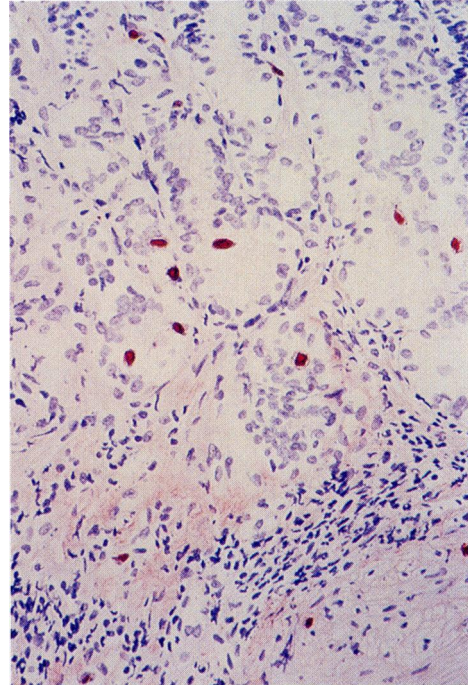


FIG. 5. Photomicrograph of CMV-infected gastric mucosal cells from section adjacent to those in Fig. 4, as demonstrated by immunocytochemistry employing the Virocode program (160  $\times$ ).

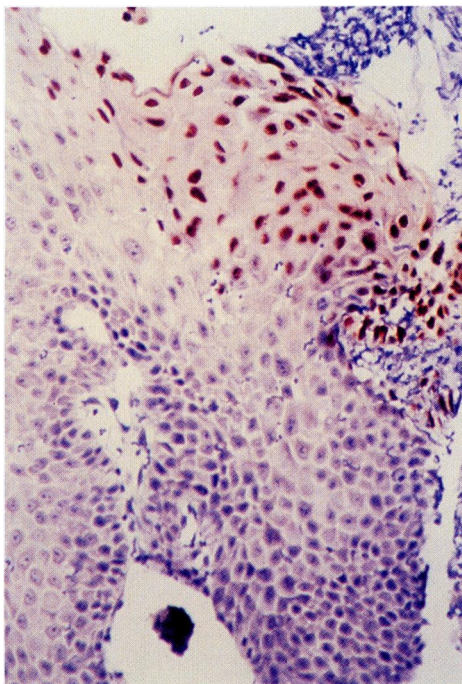


FIG. 6. Photomicrograph demonstrating DNA hybridization for a herpes simplex viral infection of the esophagus, using the Virogene program (160  $\times$ ).

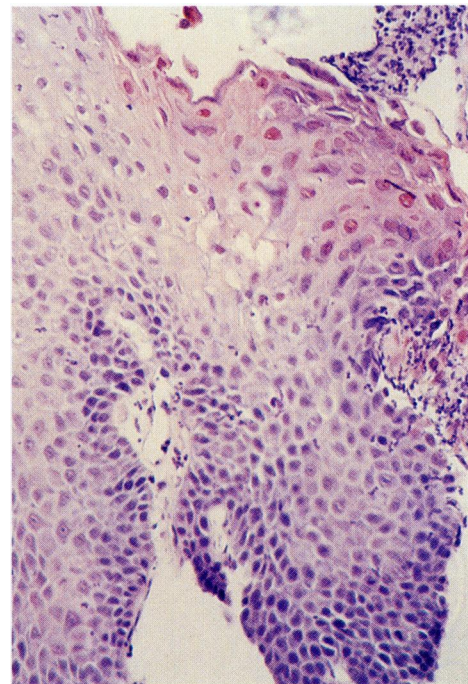


FIG. 7. Photomicrograph of a section adjacent to that of Fig. 6, illustrating HSV antigen detection with the Virogene program (160  $\times$ ). Figures 6 and 7 were taken from slides produced simultaneously in the same Virogene program run.

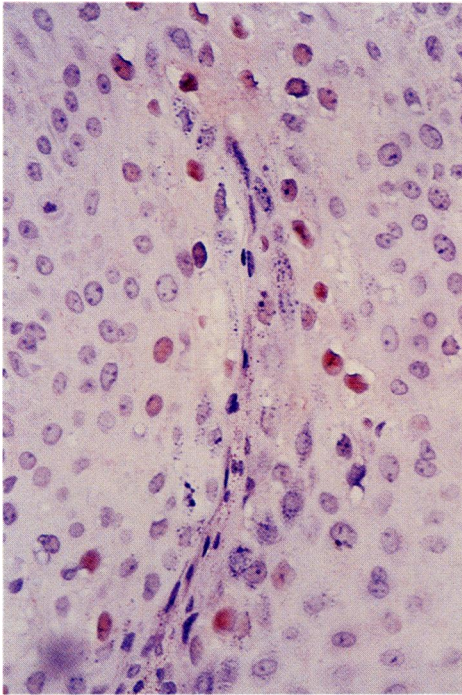


FIG. 8. Photomicrograph of a benign vulvar condyloma, demonstrating the HPV types 6 or 11 DNA, using the Virogene program (250  $\times$ ).

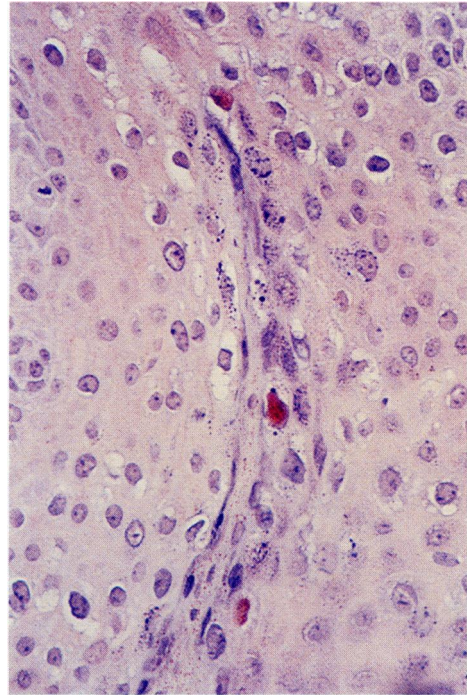


FIG. 9. Photomicrograph of a tissue section taken adjacent to that of Fig. 8, illustrating the HPV antigen by the Virocode program (250  $\times$ ).

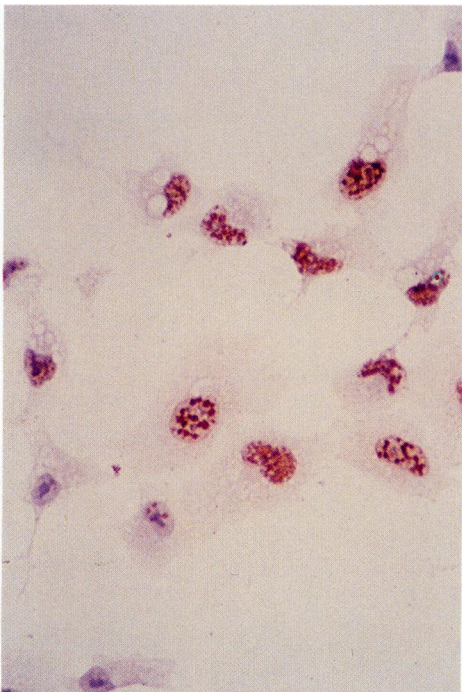


FIG. 10. Photomicrograph of adenovirus-infected tissue culture cells as demonstrated with a DNA probe, using the Virogene program (250  $\times$ ).

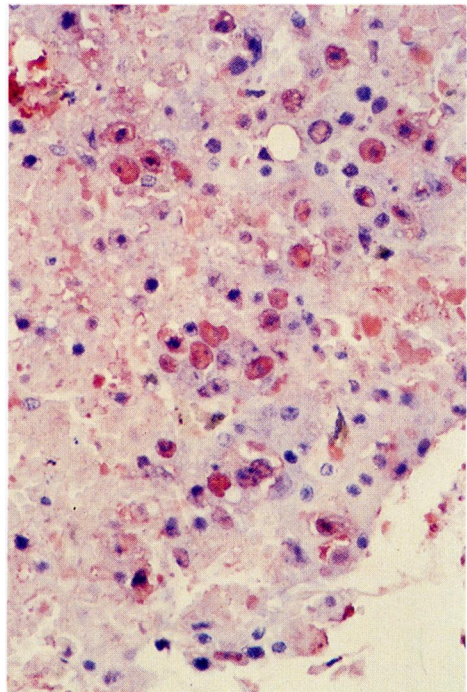


FIG. 11. Photomicrograph showing immunostaining for the hexon antigen in an adenovirus hepatitis with the Virocode program (160  $\times$ ).

antigens, respectively. Primary antibody was placed in the reagent isolon in station 14. Use of the isolons allowed us to place primary antibodies and DNA probes in station 14 but in separate wells; this placement would allow simultaneous staining of viral antigens or DNA in separate capillary gaps. The holder containing separate antibody and DNA probe-filled gaps was then robotically moved to the high-temperature oven in station 19. The primary antibody and DNA probes were heated in the high-temperature oven set at 110° Centigrade. Because of this high temperature, about 40–50 percent of the volume was lost in the antibody-filled gaps, but little was lost in the probe-filled gaps because of the high viscosity of the probe cocktail. Tissue sections were mounted on the bottom of the Probe-On glass slides near the painted triangles, so that this loss in volume did not uncover the tissue. An isolon placed in station 15 allowed the antibody-filled gaps to be treated with the universal peroxidase reagent, while the probe-treated gaps received avidin-alkaline phosphatase. AEC was still used as the chromogen for antigen detection and BCIP/INT for gene probe localization. The use of a three-laned reagent isolon allowed us to have separate compartments for the two chromogens and to detect tissue antigens with AEC and viral genes with BCIP/INT simultaneously. The Virogene program could therefore be run so that viral DNA and antigens could be detected in different tissues located in separate capillary gaps in the same assay.

#### *Controls for Automated Viral Antigen and Gene Detection*

For antigen detection, negative controls consisted of incubating with the primary antibody diluent alone. We also used antibodies against a certain virus on tissues known to be infected with a different virus; this procedure provided us with information about the specificity of our antisera. The negative controls for viral DNA detection included incubation of the slides with the probe cocktail alone. We also incubated probes against the DNA of one virus with tissue known to be infected with another virus. These negative controls provided us with information about whether or not our DNA detection system was specific for the different viruses.

#### *Microscopic Examination and Photography*

Slides were examined and photographs were taken by means of a standard Zeiss photomicroscope.

## RESULTS

### *Cytomegalovirus*

Nine cases of tissues known to be infected with cytomegalovirus were reviewed and studied. These cases included autopsy and surgical cases of patients with CMV colitis, gastritis, pleuritis, and pneumonitis. In all nine cases, cytomegalovirus was detected by both immunocytochemistry and in situ DNA hybridization techniques (Table 3). The CMV early antigen was detected by automation in cells with and without cytopathic changes, in agreement with results previously reported by manual methods [2]. Staining was almost always intranuclear, although some intracytoplasmic inclusions were detected by this method. Hybridization was also positive in the same cells and tissues that were positive for antigen. With molecular hybridization, both intranuclear and intracytoplasmic inclusions were found to stain clearly and consistently [2,3,4]. Cells with and without cytopathic changes consistent with CMV were detected with the CMV DNA probe [4]. Intracytoplasmic inclusions were found in some cells

TABLE 3  
Number of Cases Positive with Both in Situ Hybridization and Immunocytochemistry

Virus	No. of Cases	No. Positive with	
		Immunocytochemistry	DNA Hybridization
Cytomegalovirus	9	9	9
Herpes Simplex	4	4	4
Adenovirus	3	3	3
Papillomavirus	7	3	7

without any intranuclear staining; some cells with just one or two cytoplasmic inclusions could be detected by this method. In situ hybridization produced a slightly higher number of positive cells than the immunochemical stain. Because of the amount of pronase digestion used to detect the antigen, the morphology of the CMV-infected cells could not be studied as well as with the molecular probes, which required milder pepsin pretreatment. The results were identical when the rapid one-hour and 30-minute previously described Virocode assay was compared to the two-hour immunocytochemical method run under the high-temperature conditions of the Virogene DNA hybridization program (Table 2). Figures 4 and 5 show CMV-positive cells detected by both techniques.

#### *Herpesvirus*

Four cases of tissues known to be infected with herpesvirus were used for the study. These cases included herpes pneumonitis, adrenalitis, hepatitis, esophagitis, and splenitis. Herpesvirus was detected by both methods (Table 3). For some of the cases used, there was no significant difference between the methods, while for other cases the probe did much better at detecting the virus. Cytoplasmic inclusions were better visualized with in situ hybridization. Herpesvirus was observed in both epithelial as well as mesenchymal tissue compartments with either of the methods. Figures 6 and 7 show herpes simplex virus staining by these techniques. Antibodies against herpesvirus used in the two-hour Virogene program produced slightly less inclusion staining than they did in the 1.6-hour Virocode program. It is likely that some denaturation of the antibody may have taken place during the high-temperature step.

#### *Papillomavirus*

Seven cases of vulvar tissue known to contain koilocytosis, condylomatous growth, and carcinoma in situ (one case) were studied. Human papillomavirus (HPV) was detected by either of the methods employed in this study; however, only three cases were positive with the antibodies used (Table 3). The antibody used mainly stained rare cells in the stratum granulosum, although some cells in the deeper parts of the epidermis stained, as was previously described [5,10,11,12,13]. Staining was focal and rare, and many cells demonstrating clear koilocytosis failed to stain positively. Some antigen-positive areas were also seen in the stratum corneum. In situ DNA hybridization provided many more positive cells and was much more general in distribution; HPV DNA was detected in the superficial as well as the deep layers of the epidermis, indicating that the viral genome but not the antigen is more abundant throughout the layers. This finding is consistent with results described previously by manual methods [14,15]. Type 6 and type 11 DNA were observed in histologically diagnosed condylo-  
ma, while DNA for types 16 and 18 were observed in the one specimen with combined

koilocytosis, dysplasia, and carcinoma in situ; the probe-positive nuclei were observed in the condylomatous area but not in the dysplastic or carcinomatous zones. Figures 8 and 9 show results of both methods for papillomavirus detection. In situ hybridization was observed to be far better for the identification of papilloma virus by automation in standard surgical specimens. Viral inclusion staining was lost when the primary antibodies were subjected to 110° Centigrade preheating in the Virogene program. It is probable that not all antibodies will be capable of withstanding high temperatures in the media in which we diluted them.

#### *Adenovirus Automated Tissue Culture Analysis*

A549 cells infected for 24 hours with adenovirus type 2 showed strong nuclear localization with the probe for the adenovirus genome (Fig. 10). Much less signal was detected with the anti-hexon antiserum; this finding is related to the six-year period of refrigeration of this antibody. Two cases of formalin-fixed tissue with adenovirus pneumonitis and hepatitis, culture-positive for types 2 and 5 adenovirus, were studied. The two cases were positive with both gene probe and immunoassay technology (Table 3). The probe showed strong nuclear localization of the viral genome as previously reported [1], and the antibody showed comparable nuclear and cytoplasmic localization. Figures 10 and 11 show examples of the in situ hybridization on tissue culture cells and immunostaining for adenovirus hepatitis, respectively. No differences in staining were seen when these anti-hexon antibodies were used in the Virocode or the Virogene program. The advantage of the longer program is that viral DNA could simultaneously be localized in a separate capillary gap, using a single protocol. This single advantage overcame the inconvenience of the extra half hour in processing time.

## DISCUSSION

This paper describes the first combined automated immunocytochemical and in situ hybridization viral detection system, whose procedures are completed in two hours or less on the same run. This streamlined use of automation provides significant diagnostic advantages to the research virologist and the clinical morphologist alike. Viral detection in cells and tissue substrates can now be completed rapidly, accurately, and in large quantities. The Code-On Molecular Pathology system is capable of simultaneously performing 30 different immunoassays or gene probe detections in any combination. Many different tissue specimens as well as viral types can be studied at the same time and on the same machine. This capacity allows batteries of gene probes or antibodies to be completed on individual unknown tissue specimens, and the results are ready for comparative evaluation in one diagnostic session.

Both immunocytochemistry and in situ DNA hybridization have proved to be effective ways of detecting DNA viruses in surgical and autopsy specimens. In some cases, DNA hybridization produces far better results than immunocytochemistry. Herpesvirus, for instance, is detected very well by both methods but is consistently detected with stronger staining when biotinylated gene probe technology is used. In situ DNA hybridization for cytomegalovirus DNA detects intracytoplasmic inclusions better than immunocytochemistry. This result is expected, since the monoclonal antibody used in this study is directed against an early antigen whose localization predominates in the nucleus. DNA probe technology is by far the better method to study papillomavirus in tissue fixed in formalin, as the antigens detected by the

antibody were found mostly in the superficial areas of the lesions, while papilloma viral genetic information could be detected throughout most of the layers of the epidermis; less than one-half of the cases used stained positive for the antigen. Our conclusion is that immunocytochemically identifiable antisera against early papillomavirus antigens will have to be developed before antigen-based detection technology can approach the sensitivity of presently available gene probe methodology.

This study shows that four DNA viruses can be detected easily and rapidly by the methods of *in situ* DNA hybridization and immunocytochemistry. The majority of infected cells were accurately and rapidly detected with our programs, although increasing the primary antibody incubation or the DNA hybridization times could further improve the intensity of the signal. Both techniques are capable of providing the anatomist, microbiologist, or pathologist with two sets of unique biologic information, and therefore both are helpful in the diagnosis and study of viral pathogens and their association with disease states. It may be economically advisable to use antigen detection as a screening test for the virus and then to use a confirmatory DNA probe analysis if any uncertainty remains. This procedure may not always be practical, because antigen may not always be detected, as in the case of papillomavirus. Gene probe technology was easier to perform because a single combination of enzyme predigestion, cellular DNA denaturation, probe hybridization, and detection produced equivalent high-quality staining in all four viruses. Immunocytochemistry required more specialized conditions for enzyme predigestion and a prolonged search for primary antisera that worked in formalin-fixed tissues.

In order for molecular pathology to advance into the age of automation, it is ideal to have one instrument, capable of performing both *in situ* hybridization and immunocytochemistry, which employs a single programmed series of events. We have found that this breakthrough can be accomplished because selected primary antibodies can survive the 110° Centigrade temperatures used during DNA denaturation. This high-temperature survival is evidenced by the fact that horseradish peroxidase-conjugated secondary antibodies can specifically recognize these primary antibodies forming tissue immune complexes despite those drastic conditions. Polyclonal secondary antibodies can be selected to recognize extremely heat-insensitive epitopes on the primary antibody, thereby allowing simultaneous tissue gene and antigen detection. Antibodies are not as fragile as has been thought in the past. We have been able to run immunocytochemical studies using the temperature conditions of our two-hour DNA hybridization (Virogene) program with some loss in antigenic signal but no increase in background noise if the primary antibody was properly diluted. The only drawback of the present system is that 40 percent of the primary antibody volume is lost during the high-temperature phase of the program. This volume loss can be significantly diminished by shortening the denaturing time without losing hybridization signal.

The molecular basis for the surprising high-temperature survival of the antibodies used in this study will require further investigation. We do not yet know how general or restricted this property of immunoglobulins is. We know that primary rabbit IgG, guinea pig IgG, and monoclonal IgG1 antibodies are capable of working under the conditions of this study. Preliminary results from our laboratory indicate that standard primary rabbit antibodies, raised against CEA or S100, and monoclonal antibodies against muscle-specific actin are capable of surviving the hybridization program, although some diminished staining can be noted. Further work is needed to determine if the primary antibody diluent imparts this survivability or if it represents an inherent



property of immune molecules. What we have observed is that, after heating in a 110° Centigrade oven for eight minutes and cooling to 37° Centigrade for 20 minutes, significant numbers of primary immune complexes remain intact at the site of their tissue antigen. After eight minutes at 110° Centigrade, the air temperature between the oven walls and the capillary gaps achieved maximum temperatures of 95–100° Centigrade, which were sufficiently high to denature probe and tissue-bound target viral DNA molecules in adjacent capillary gaps. Whether all the signal detected in the final result was solely the product of immune complexes formed in the first ten minutes as the slides heated or whether immunoglobulins actually survive 95–100° Centigrade temperatures to react during the subsequent 20 minutes at 37° Centigrade is presently unknown. Preliminary evidence indicates that the latter is true, since no signal is detected in the areas of tissue uncovered as the volume of the primary antibody is lost in the high-temperature-oven step.

In the future, improved automated methods for the anatomic diagnosis of viral pathogens may revolutionize clinical virology. At the present time, tissue culture is often the primary screening method used for viral diagnostics. This system is often fruitful, as in the case of herpes simplex virus identification; time-consuming, especially when cytomegalovirus is cultured; or consistently negative when the pathogen is a papillomavirus. Present viral screening techniques can be revolutionized if a single automated screening method is developed for probing cells, extracts, or tissue sections, immobilized on planar microscope slides, with gene or immune probes. These substrates can then be rapidly examined with a standard light microscope or image analyzer in two hours or less to yield permanent, enzyme-generated, qualitative or quantitative information, which then directs the choice of selective media to obtain the highest percentage yield of viral growth. In the future, the “gold standard” of viral tissue culture can then become a confirmatory rather than a front-line screening test.

This study pioneers the identification of an initial set of important DNA viruses in surgical and autopsy material and is intended to demonstrate the first unified system for combined viral gene and antigen detection. Through a process of continued primary antibody and gene probe selection, we foresee the eventual development of the Code-On Molecular Pathology system into a unified approach for the anatomic diagnosis of all DNA and RNA viruses. With this paper, the once separate diagnostic fields of automated immunocytochemistry and DNA hybridization are now united into a single molecular discovery system.

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