MODIFICATION OF A COMMERCIALY AVAILABLE DOT BLOT HYBRIDIZATION ASSAY FOR THE DETECTION OF HUMAN PAPILLOMAVIRUS

by

Sally J. Ondiek

Approved by Committee

Neil Szuminsky, Committee Chairman  4/10/92
Rosemarie Matuscak, Committee Member  4/16/92
Mike Meyer, Committee Member  4/15/92
Dr. Amortegui, Committee Member  4/15/92
MODIFICATION OF A COMMERCIALY AVAILABLE DOT BLOT HYBRIDIZATION ASSAY FOR THE DETECTION OF HUMAN PAPILLOMAVIRUS

by

Sally J. Ondiek, B.S., Goshen College, 1983

Submitted to the Graduate Faculty of the School of Health and Rehabilitation Sciences in partial fulfillment of the requirements for the degree of Master of Science

University of Pittsburgh

March, 1992
FOREWORD

I would like to express my appreciation to all the members of my thesis committee for their guidance and support. I am especially grateful to Michael Meyer for the time he spent reviewing and editing this thesis.

I also wish to thank the staff of the Virology Laboratory at Magee-Womens Hospital for their technical assistance.

Lastly, I thank my parents, Dr. and Mrs. Patroba Ondiek, and my husband, Milton Ogot, for their continued support and encouragement throughout the course of this study, and in my future academic endeavors.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>FOREWORD</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. Background</td>
<td>3</td>
</tr>
<tr>
<td>B. Literature Review</td>
<td>9</td>
</tr>
<tr>
<td>II. STATEMENT OF PROBLEM</td>
<td>18</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>1. The Highest Dilution of The Low Positive Control Detectable With Standard Methodology</td>
<td>22</td>
</tr>
<tr>
<td>2. Hybridization Time Experiments</td>
<td>22</td>
</tr>
<tr>
<td>3. Quantitation of Hybridization Signals by Scintillation Counting versus Autoradiography</td>
<td>24</td>
</tr>
<tr>
<td>4. Doubling the Amount of Sample and Exposure Time</td>
<td>26</td>
</tr>
<tr>
<td>5. Preparation of a ViraPap Database</td>
<td>27</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>28</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>31</td>
</tr>
<tr>
<td>VI. SUMMARY AND CONCLUSIONS</td>
<td>35</td>
</tr>
<tr>
<td>VII. LITERATURE CITED</td>
<td>36</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Comparison of the Autoradiographic Signal using Different Hybridization Times</td>
<td>43</td>
</tr>
<tr>
<td>2.</td>
<td>Quantitation of Hybridization Signals by Scintillation Counting versus Autoradiography</td>
<td>44</td>
</tr>
<tr>
<td>3.</td>
<td>Reproducibility of 32 Hour Hybridization Experiment</td>
<td>45</td>
</tr>
<tr>
<td>Figure</td>
<td>Title</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Autoradiograph</td>
<td>46</td>
</tr>
<tr>
<td>2.</td>
<td>A Comparison of Scintillation Signal to Noise Ratio at Different Hybridization Times</td>
<td>47</td>
</tr>
<tr>
<td>3.</td>
<td>Single and Double Exposure Results for the 15 samples found Questionable or Positive as a Result of Double Sample ...</td>
<td>48</td>
</tr>
<tr>
<td>4.</td>
<td>Flowchart I</td>
<td>49</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

Over the past decade there has been increasing evidence indicating that certain Human Papillomaviruses (HPV) are important etiological agents involved in the development of cancer of the cervix (12, 13, 36). HPV types 6 and 11 tend to be associated with benign genital warts and/or low-grade cervical intraepithelial neoplasia (CIN); whereas, HPV types 16, 18, 31, 33 and 35 are more prevalent in high grade CIN (53, 63, 65). Based upon this association, it has been hypothesized that the presence of certain HPV genotypes may help predict the progression from various stages of CIN to invasive cervical cancer.

Detection of Papillomavirus infections present difficult diagnostic problems. HPV cannot be cultivated in vitro and viral antigens are only rarely present in high grade CIN lesions. HPV infection is suspected by the presence of typical morphological changes in the epithelial cells detectable by cytology or histopathology. Specific identification of HPV infection is possible by nucleic acid hybridization with type specific probes (55). Several DNA hybridization methods are available, including Southern blotting (SB), dot blotting (DB), filter in-situ hybridization (FISH) and in-situ hybridization (ISH) on tissue sections (58).
The purpose of the present study was to evaluate the effect of three modifications made to the Virapap HPV DNA filter hybridization assay (Digene Diagnostics; Gaithersburg, MD) in detecting HPV DNA from cervical scrape specimens. The three modifications included increasing the amount of specimen tested, increasing the hybridization time and increasing the exposure time.
A. BACKGROUND

As early as 1842, an Italian physician, Rigoni-Stern, reported on the statistical evaluation of death records from cancer in the city of Verona for the years 1760-1839. Rigoni-Stern found that deaths due to "cancer of the uterus" were rare among virgins and nuns, but quite common among married women and widows (54). Rigoni-Stern was the first epidemiologist to point out that "marital events" (sexual intercourse) played an important role in the development of this disease.

These observations were repeatedly confirmed in extensive epidemiological studies of carcinoma of the uterine cervix and its precursor lesions conducted over the past 50 years (26,31,56). The epidemiology of cervical carcinoma is similar to the epidemiology of venereally transmitted infections. The two most important high risk factors for cervical carcinoma include young age at the onset of sexual activity and multiplicity of sexual partners.

The concept of squamous carcinoma of the uterine cervix from abnormal cervical epithelium may be traced to a paper published by Schauenstien in 1908 (57). Based on the similarity of histological patterns, Schauenstien proposed that cancerous changes confined to the epithelium of the cervix represented a precursor stage of invasive cancer.
Subsequently, the term "carcinoma-in-situ" was proposed for such precancerous epithelial lesions (30).

Mass screening of women conducted for the past 40 years by means of vaginal and cervical smears as first proposed by Papanicolaou and Trant (47) has led to a large number of studies of the events leading up to cervical carcinoma. Extensive cytological and colposcopic studies have documented that most initial neoplastic events occur within a small segment of cervical squamous epithelium adjacent to the endocervical epithelium. This area is known today as the "transformation zone". It appears that during the process of transformation of columnar epithelium into squamous epithelium, a temporary state of genetic imbalance may occur that renders the young squamous epithelium susceptible to neoplastic events. The proliferation and maturation of HPV is related in an unknown fashion to maturation of squamous epithelium (47).

Morphological differences that have been observed among precancerous lesions, were thought to represent early stages in the development of cervical cancer. It has been proposed that the disease can progress from minor epithelial changes in the squamous epithelium to moderate and severe changes, usually located in the endocervical canal, to invasive cancer. The term "dysplasia" has been used to characterize the minor changes and "carcinoma-in-situ" the more severe changes. The term "cervical intraepithelial
neoplasia" as proposed by Richart (52) and encompassing all intraepithelial neoplastic lesions, regardless of the degree of histological or cytological abnormality, has been nearly universally accepted as a replacement for the "dysplasia/carcinoma-in-situ" system of nomenclature. Not all precancerous lesions would be expected to advance to invasive cancer. Many precancerous lesions may disappear either spontaneously or after minor diagnostic procedures (29). Nasiell et al (45) have estimated after 12 years of follow-up that not more than 30% of "moderate dysplasias" will persist or progress.

Infection of the genital tract with HPV may be asymptomatic or have manifold clinical manifestations, including flat lesions of the epithelium of the vulva, vagina and uterine cervix. The most common lesion associated with HPV infection is Condylomata Acuminata, which appears as grossly visible, usually multiple, wart-like lesions of the external genitalia and has been recognized as a venereally transmitted disease for many years (40). In 1976 and 1977, Meisels and Fortin (38) and Purola and Savia (48) simultaneously suggested that certain epithelial abnormalities of the uterine cervix, until then considered a form of intraepithelial neoplastic lesions (CIN Grade 1 or 2) were in fact flat mucosal equivalents of the wart-like condylomas. This suggestion was based on the similarities in the cytological picture between the obvious condylomas and
the flat lesions, mainly the presence of cells known as koilocytes. It has been shown that the "warty" intraepithelial lesions and the corresponding koilocytosis found in smears of the uterine cervix described in 1956 by Koss and Durfee (28) are associated with HPV infection.

In 1977, Hein et al (22) described the findings from cervical smears in 403 sexually active adolescent girls. Low-grade CIN was found in 14 patients. On review, 12 of the 14 smears contained koilocytes. With renewed interest in koilocytosis as an indicator of HPV infection, its relationship with other forms of CIN and invasive cancer became the subject of numerous studies. Various authors reported such associations in variable proportions of women with CIN, ranging from 25.6% to nearly 50% (39,60). This association has been shown more extensively in a paper by Reid et al (50) suggesting that in virtually every instance of CIN or invasive carcinoma, there is some morphological evidence of infection with HPV.

The circumstantial evidence associating HPV and genital cancer stems from their epidemiological similarities. There is a high prevalence of HPV in individuals who start sexual activity early in life and those who have multiple sexual partners as contrasted with those who remain celibate. Numerous studies have demonstrated the presence of HPV in cervical cancers (5,17,18,20) with the finding that HPV can be detected in 80%-90% of cervical carcinomas.
By using DNA hybridization techniques (58,68,70,72), numerous studies have shown an association between HPV infection and CIN. HPV DNA can be demonstrated throughout the epithelium (59), and synthesis of HPV transcripts has also been shown to occur in all layers of the epithelium in CIN lesions (14,44,46).

Transformation of cell lines to continuous ones has been accomplished with HPV types 6, 16 and 18 (24,43,69). In addition, HPV types 16 and 18 have been shown to immortalize primary keratinocytes (25). Cell lines derived from cervical cancers (such as HeLa, Caski and SiHa) have also been shown to contain and express HPV DNA (2,60,61). In contrast to the benign and dyplastic HPV-associated lesions, HPV in most malignant lesions has been found to be integrated into cellular DNA (10). It is well known that certain animal papillomavirus are oncogenic in their species (23) and tissue culture cell lines have been transformed into malignant tumors by these viruses.

The current theory for the role of HPV in cervical carcinoma can be summarized using the "seed, soil and nutrient analogy" (51). The soil in this theory is the cervical epithelium, with HPV genotypes acting as seeds and cofactors, such as cigarette smoking, acting as nutrients that initiate cervical carcinogenesis. Several studies have identified cigarette smoking as a risk factor for the development of cervical dysplasia and cancer (7,33). In
*vitro* studies have also supported a role for oncogene activation as a cofactor in cervical carcinogenesis (35).
B. LITERATURE REVIEW

Nucleic acid hybridization procedures are based on the structure of the DNA molecule (64). Duplex DNA is composed of two complementary polynucleotide chains. Each nucleotide base of one strand links specifically to a complementary nucleotide base on the opposite strand by hydrogen bonds. Thymidine base pairs with adenine in DNA, while guanine base pairs with cytosine. The hydrogen bonds between each pair of bases can be broken by heat or enzymatically and this is known as denaturation. In different hybridization methods, fragments of DNA or RNA of interest (probes) are labelled with radioactive isotopes, or nonradioactively with enzymes. These labelled probes then hydrogen-bond (hybridize) to cellular DNA or RNA containing complementary sequences. These hybrids are then visualized by various means depending on the label used.

In dot blot hybridization cells are digested with proteolytic enzymes and the DNA is denatured by heat or an alkali solution (64). The denatured target DNA strands are then filtered onto a nylon membrane and labelled probe is added. The labelled probe DNA strands then hybridize with complementary sequences in target DNA. Hybridization is followed by washes and autoradiography.

The success of these hybridization tests are dependent on the rate of hybrid formation and the stability of the hybrids (32). The rate of hybrid formation is influenced by
several factors. The optimal rate of duplex formation occurs at 25°C below the hybrid melting temperature. The rate of hybrid formation increases with increasing sodium concentration up to 1.2 molar. Hybridization rate increases with increasing DNA concentration and dextran sulfate also increases hybridization rate. However, hybridization rate decreases with increasing mismatches between probe and target.

Hybrid stability is important because unstable hybrids will dissociate spontaneously. Therefore, increasing the temperature to or above a critical level, called the melting temp (Tm), will denature the duplex. The Tm for perfectly matched DNA is usually in the vicinity of 100°C. Duplex stability increases with increases sodium concentration in the range 0.01 to 1M. Strongly alkaline conditions (pH >12) and increased concentrations of formamide will denature the duplex. Guanine-Cytosine base pairs are more stable than Adenine-Thymidine base pairs because G-C pairs have three hydrogen bonds while A-T pairs only have two. Duplex stability is significantly reduced by increasing mismatches between hybrids (32).

"Stringency" refers to the degree of discrimination attainable between imperfectly matched and highly matched hybrids through choice of hybridization and wash conditions. Hybridizations and washes in the range Tm -35°C to Tm -40°C are considered of low stringency. Hybridizations at Tm -
25°C, followed by washes at Tm -10°C are considered high stringency. HPV typing is generally done at high stringency in order to minimize cross-reactivity between different HPV types.

The kinetics of hybridization are different when the probe is in excess over the filter-bound sequences (21). The following equation shows the fraction of filter-bound sequence i actually hybridized at time t:

\[ E_i(t) = 1 - e^{-kt} \]

The ratio of the extent of hybridization of cross-hybridizing sequence i, to perfectly matched sequence f is:

\[ E_i(t) = 1 - e^{-kt} \]
\[ E_f(t) = 1 - e^{-kt} \]

The ratio here is not constant but varies with time. The rate of cross-hybridization compared to hybridization between perfectly matched sequences reaches its maximum point very early in the reaction but declines with increasing hybridization time. Although the homologous reaction is faster and reaches completion earlier, the heterologous reaction eventually catches up. This means that with increasing time of reaction, discrimination becomes poorer (21).

The current standard for HPV typing is generally considered to be SB analysis. Studies using both the SB technique and filter hybridization procedures have shown that HPV 16/18 DNA is often found in invasive cervical
carcinomas and in high-grade premalignant lesions, while HPV 6/11 is rarely found in these tissues (37,34). Zhang et al. (73) used both DB and SB hybridization to detect HPV DNA in 59 women with cervical lesions. A group of 33 women lacking evidence of cervical abnormalities served as controls. HPV DNA, predominantly type 16/18, was detected in the cervical biopsies of 57/59 (96%) of the cervical cancer patients, 47/59 (80%) of the CIN III patients, and 39/59 (66%) of the CIN I-II patients. In contrast, only 3/33 (9%) of the cervical biopsies from the control group contained detectable HPV 6,11,16, or 18 DNA.

A similar study conducted by Batholoma et al. (3) compared DB and SB for their ability to detect HPV DNA in 50 cervical swab specimens and 11 biopsy specimens. The overall agreement between the two methods was 78.7%. With SB analysis, HPV 6,11,16, or 18 was detected in 22 specimens. DB analysis detected HPV 6/11, 16/18, or 31/33/35 in those same 22 specimens and in nine additional specimens. Two of these nine specimens had HPV type 16/18 for which SB was either negative or gave indefinite results, and the remaining seven specimens contained HPV type 31/33/35.

In a study comparing SB analysis to Polymerase chain reaction (PCR), Tham et al. (66) found HPV DNA by SB in 74% of biopsy specimens (42 of 57 cases), with the predominant types being HPV 16 and HPV 18. In contrast, PCR analysis of the same 57 biopsy specimens revealed that all samples were
positive for at least one HPV type. These investigators concluded that the higher number of positive samples in the latter analysis was due to the increased sensitivity of PCR, which can detect as few as 10-100 HPV DNA molecules. The sensitivity of SB hybridization has been approximated to be $10^5$ molecules of HPV DNA (66).

FISH allows for the detection of DNA sequences present in smears or scrapes. A limited number of studies have been conducted comparing this method with others. Garuti et al. (19) have reported a sensitivity of 90.4% in 52 samples tested with the FISH method compared to SB analysis. In contrast, De Villiers et al. (15) have estimated that FISH is one-half to one-third as sensitive as the SB technique. Comparing the percentage of cervical smears scored as HPV positive by DB versus FISH, Cornelissen et al. (11) found FISH to be approximately one-third as sensitive as DB. Thus, the sensitivity of FISH as observed in the Garuti study (19) is much higher than those observed by other investigators.

In a study using tissue in-situ hybridization Amortegui et al. (1) showed that ISH with biotinylated probes appeared helpful in identifying lesions containing higher risk viral strains. The prevalence of HPV types 16, 18, and 31 increased with the severity of the lesions, with 20 out of 20 (100%) positive CIN III lesions containing those viral types as compared to 102 of 157 (65%) positive CIN I lesions.
Commercial hybridization kits for the detection of HPV have recently become available Digene Diagnostics (Gaithersburg, MD) has three kits available for the identification of HPV: one for the detection of HPV DNA present in cervical swabs (ViraPap), one allowing specific genotyping of the HPV DNA from cervical swabs (ViraType), and one for genotyping DNA within formalin-fixed paraffin-embedded tissue sections (ViraType in-situ Kit). The first two kits use radiolabeled RNA probes and a modification of the DB technique. The last kit uses a biotinylated probe to detect DNA by ISH. The literature accompanying the ViraPap and ViraType kits indicates that these assays are as sensitive and specific as SB hybridization.

In comparing the ViraType in-situ assay to a dot blot assay, Faulkner et al. (16) found the former to be less sensitive than their dot blot assay. Faulkner and his colleagues evaluated biopsy specimens from 46 women and found that 50% of them were positive by DB hybridization, while only 39% were positive by ViraType in-situ hybridization.

Researchers from the University of Washington have compared the ViraPap kit to SB (27). Four hundred and fifty females attending a sexually transmitted diseases clinic were tested for seven types of HPV. Among 47 patients with cytologic dysplasia, HPV DNA was detected in 44% (21/47) by ViraPap and in 35% (17/47) by SB. Although 26% of specimens
positive by ViraPap were not confirmed by SB, cervical
dysplasia was detected in 5/20 (25%) specimens with HPV DNA
detected by ViraPap alone as compared with 25/327 (8%) of
those with no definitive evidence of HPV by either method
and with 16/53 (30%) with HPV DNA detected by both methods.
The sensitivity and specificity of the ViraPap kit was found
to be 90% and 94% compared to SB, respectively.

These commercial kits has also been compared to PCR
analysis. Bauer et al. (4) determined the presence of HPV in
cervical and vulvar samples from 467 women using ViraPap and
PCR. Of the total 467 women, 213 (46%) showed infection with
HPV at one or both sites using the PCR method, while only 51
(11%) were positive by ViraPap. Of the PCR positive
subjects, 146 (69%) of the women were positive at both
sites. Of the ViraPap positive subjects, 23 (45%) of the
women were positive at both sites. Of the 51 women who were
positive by ViraPap, all were positive by PCR. Of the women
found positive by PCR but negative or indeterminant by
ViraPap (n = 162), 44 (27%) were infected with types
included in the ViraPap/ViraType systems, while the
remaining 118 (73%) women were infected with types not
tested by ViraPap.

The AffiProbe HPV test kit (Orion Corp, Orion
Pharmaceutica, Helsinki, Finland), which is a one day
solution hybridization test for HPV type 6/11, 16, or 18,
has been compared to the ViraPap and ViraType kits (49). For
this comparison, two simultaneously obtained cervical scrapes were available from 174 patients. A total of 18 specimens were positive and 129 specimens were negative for HPV types 6/11, 16, and 18 by both tests. The agreement between the two tests was 85%. Eleven AffiProbe positive specimens were negative by the ViraPap test. Seven additional AffiProbe positive specimens (five positive for HPV type 16 or 18 and two positive for HPV type 6/11), were considered to contain HPV type 31/33/35 by the ViraType test. Eighteen additional specimens that were negative by the AffiProbe test contained HPV type 31/33/35 by the ViraType test. Probes for HPV types 31, 33, and 35 are not included in the AffiProbe HPV test.

Besides comparison studies of current kits used in the detection of HPV, there have been studies comparing conventional morphological analysis to these kits. Meyer et al. (42) detected HPV DNA in 314 of 787 (40%) histologically abnormal genital biopsy specimens by using the ViraType in-situ assay. Ninety percent of condyloma acuminata specimens contained HPV type 6/11 DNA. The prevalence of HPV in CIN I, II, and III lesions was 42%, 54%, and 55%, respectively. The prevalence of type 16/18 and 31/33/35 DNA was found to increase with the severity of the lesion, while the prevalence of 6/11 DNA decreased.

Weintraub et al. (71) compared ViraType to standard morphological methods using 412 cervical samples taken from
381 patients referred to a colposcopy clinic. Simultaneous cytological smears were obtained in 289 patients, colposcopically directed biopsy samples in 284 patients and both smear and biopsy samples in 171 samples. HPV DNA was detected in 164 specimens (41%), of which 24 (15%) were type 6/11, 74 (45%) were type 16/18, 39 (24%) were type 31/33/35, and 27 (16%) were untyped due to the presence of multiple signals. Viral types 16/18 and 31/33/35 were found significantly more frequently in CIN II/CIN III lesions than in condyloma/CIN I cases. When the cytologic diagnosis was considered the standard of reference, the results of ViraType for the detection of HPV were concordant in 167 (56%) paired samples. The sensitivity and specificity of ViraType compared to cytologic abnormalities was 48% and 77% respectively. When the histologic diagnosis was considered the standard of reference, the sensitivity was 59%, and the specificity was 79%. The results demonstrated that there were patients positive by both ViraType and by morphologic methods as well as those positive by one method or the other. Therefore, it is important to consider all three categories when comparing the sensitivity of the two methods.
II. Statement of Problem

DNA hybridization techniques for the detection of HPV may be used for two reasons: (1) to confirm the presence of an oncogenic virus in histologically questionable/borderline lesions and (2) to identify women at greater risk of progression from CIN to cervical carcinoma. It is important to establish that a sample is indeed negative. Negative HPV results are usually due to three reasons:

1) No HPV DNA present,
2) HPV DNA present, but below detection levels or
3) other HPV types present

Since HPV may be present but below detectable levels negative HPV results, especially in lowgrade/borderline lesions, have limited usefulness to clinicians.

A variety of techniques currently are available to identify HPV sequences present in clinical specimens. These include analyses which require isolation of DNA from the specimen SB, DB, and Northern blot and those which can be performed directly on tissue sections, scrapes or smears (ISH and FISH). Each of these techniques has advantages and disadvantages. At present, SB is considered the gold standard for HPV detection, because it is both sensitive and specific. However, SB is labor intensive, time consuming and
difficult to interpret. DB hybridization is an easier but less sensitive technique than SB and false positives may occur because crude preparations of DNA are usually used. FISH has the same drawbacks as the DB. Although less sensitive than SB, ISH is a moderately easy technique that can be performed on routinely collected formalin-fixed paraffin-embedded biopsies. PCR is the most sensitive of all these techniques with the potential of detecting less than one copy of DNA per cell. However, PCR is a technically difficult technique with various contamination problems.

The purpose of this study was to increase the sensitivity of a commercially available DB hybridization system for detection of HPV DNA. Improving the sensitivity of an easy to perform assay to detect small amounts of DNA would help in confirming the diagnosis of low-grade/borderline cervical lesions and thus, facilitate the proper treatment of these patients.
III. MATERIALS AND METHODS

A. Description of ViraPap Kit:

The principle of the ViraPap kit is based on the specific binding of complementary $^{32}$P-labeled RNA probes to target HPV DNA. The assay involves several steps. First, the sample is collected and put into a buffer solution that lyses the cells. Then, the sample preparation reagent which contains proteases, is added to the cervical swab specimen to digest histones and other proteins surrounding the DNA and thus release the viral DNA from cervical cells. Next, the sample is transferred into a tube containing sample diluent (0.6% NAOH). The NAOH solution acts to destabilize the hydrogen bonds of the duplexes and the DNA strands are thus denatured chemically. Then, the DNA is filtered onto a nylon membrane. The filter is then incubated with a blocking solution which contains formamide and sodium dodecyl sulfate to prevent non-specific binding of the probe to the filter. This is referred to as the prehybridization step. A solution of radioactive labeled RNA probes are incubated with the filter, and hybrids are allowed to form. Non-specific bound and unbound probe are removed by extensive washing at carefully controlled stringency. The first wash is a high salt wash and is done at room temperature and is thus, a low stringency wash. It removes single stranded probe bound non-specifically to the filter and physically washes away
unhybridized single stranded probe. After the first wash RNase reagent is added which digests the remaining single stranded probe bound to the nylon filter and it also breaks up and destabilizes mismatched duplexes. The second wash which is a low salt, high temperature wash and is therefore considered a high stringency wash. During the high stringency wash perfectly matched hybrids stay intact while mismatched duplexes dissociate. The formation of a hybrid between the probe and target DNA is visualized by autoradiography as illustrated in Fig. 1.

B. Project Design

The study consisted of the following projects:

1) Determination of the highest dilution of the low positive control detectable with standard methodology
2) Hybridization time experiments
3) Quantitation of hybridization signals by scintillation counting versus autoradiography
4) Doubling the amount of sample and exposure time on previously negative samples
5) Preparation of a Virapap database
1. **The Highest Dilution of The Low Positive Control Detectable With Standard Methodology**

This experiment was performed to determine the highest dilution of the low positive control that could still be detected by autoradiography using the standard methodology. Three dilutions of the high positive control (undiluted, 1:10 and 1:100) and 10 serial two-fold dilutions of the low positive control were all processed on the same filter. The dilutions were made in specimen collection buffer. Two drops of the blue sample preparation protease reagent from the Virapap kit were added to all the tubes and the controls. The tubes were then incubated for an hour at 37°C. Afterwards, 0.25 ml from each sample was transferred to individual tubes each containing 0.75 ml of sample diluent. After adequate mixing, the entire mixture was filtered onto the nylon membrane and processed according to manufacturer's instructions. This experiment was done to determine the highest dilution of the low positive control to use as a standard in the other experiments.

2. **Hybridization Time Experiments**

The different hybridization times tested were 4, 8, 16 and 32 hours. The same experimental set-up was repeated for all the different hybridization times. Two sets of serial
two-fold dilutions of the low positive control from 1:2 to 1:128 were made by adding 0.5 ml of the specimen collection buffer to 0.5 ml of the low positive control and transferring 0.5 ml of this amount from one tube to the next. Two drops of the blue sample preparation protease reagent were added to six control tubes (two sets of high positive, low positive and negative controls) and the 14 dilutions tubes. All the tubes were incubated for an hour at 37°C and then 0.25 ml from each tube was transferred to another set of tubes each containing 0.75 ml of sample diluent.

One set of wells in rows one and two of the nylon membrane were occupied by the controls and the seven dilutions of the low positive control. Row three was left empty with the other set of wells in rows four and five containing the same controls and dilutions as those found in rows one and two. Following filtration, the filter was cut at row three and both halves placed in a single Virapap reaction tray. The prehybridization reagent was added and the tray incubated for 30 minutes. Afterwards, the hybridization reagent was added and the tray incubated at 60°C. After two hours of hybridization, the first half of the filter was removed and excess hybridization reagent blotted off and placed into another Virapap reaction tray and wash buffer 1 added. The other half of the filter was then left to hybridize until either 4, 8, 16 or 32 hours had
elapsed from initially adding the hybridization reagent probe. The assay was then completed as usual with both filter halves together. The autoradiographs of the different hybridization times were scored and compared to the standard two hour hybridization seen on the first half of the filter.

3. **Quantitation of Hybridization Signals by Scintillation Counting Versus Autoradiography**

Controls were pooled together from different kits in order to obtain sufficient amounts of low positive control needed for the experiment. Then, dilutions of the low positive control (1:2 and 1:4) were made in specimen collection buffer for each of the four different hybridization times. The blue sample preparation protease reagent was added to all the tubes containing either undiluted controls or dilutions of the low positive control. All tubes were then incubated at 37°C for 1 hour. After the one hour incubation, 0.25 ml from each tube was transferred to another set of tubes each containing 0.75 ml of sample diluent and the entire amount of the various tubes placed onto the two filters. Five columns and five rows had been drawn on the two filters with a special marker capable of drawing on nitrocellulose filters. One filter was developed as a routine autoradiograph and the other was cut up and counted in the scintillation counter. Each of the five
columns contained a well for the high positive control, low positive control, a negative control, a 1:2 dilution of the low positive control and a 1:4 dilution of the low positive control.

The filters were then processed as usual. After the standard two hour hybridization, the first column was cut from both filters and blotted dry and placed in separate reaction trays. After the two wash steps and the application of the RNase reagent, the first strip from the first filter was blotted dry and cut into squares and placed in scintillation vials containing 5 ml of scintillation fluid. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Company., Downers Grove, Illinois). Six counts each for 5 minutes for each sample were averaged. This process was repeated for the four different hybridization times. After blotting dry, each strip from the second filter was placed in a filter cassette to be developed as a regular autoradiograph. This process was repeated for all four different hybridization times. The 2, 4 and 8 hour hybridization strips were developed together in one cassette. The 32 hour strips were developed separately in another cassette in order to avoid excessive decay of $^{32}$P-labelled probe from the 2, 4 and 8 hour strips.
4. Doubling The Amount of Sample and Exposure Time

Approval from the Research Review and Human Experimentation Committee at Magee-Womens Hospital and the Institutional Review Board for Biomedical Research at the University of Pittsburgh was obtained for this part of the research. Of the 735 cervical swab specimens submitted to the Virology Laboratory at Magee-Womens Hospital for HPV DNA testing during 1991, 212 specimens were selected for this study. Most of the selected cases had originally been received by the Virology Laboratory between April and October of 1991. No histologic or cytologic reports were obtained on these cases. These 212 specimens, which had been found to be negative for HPV DNA using the standard ViraPap assay, were re-processed using a modified assay. These specimens were re-processed using twice the amount of sample (0.5 ml) as compared to 0.25 ml used for the standard assay. After thawing these specimens, 0.5 ml of sample was added to 1.5 ml of sample diluent and the whole amount (2.0 ml) placed on the extra spaces on the filters not occupied by the routine clinical samples. A second set of controls each containing 0.5 ml was processed along with the previously negative samples. After filtering, the ViraPap assay was conducted according to the manufacturer's instructions using the standard two hour hybridization period. After the routine autoradiograph was developed, the filter was re-set.
up for a double autoradiographic exposure accounting for the
$^{32}p$ decay that had occurred during the initial
autoradiographic exposure.

5. Preparation of a ViraPap Database

A ViraPap database using dBase III was created for the
ViraPap specimens received by the Virology Laboratory of
Magee Womens Hospital from 1989-1991. The following
information was entered for each patient:

1) Twelve digit code including the year, date and
   patients accession number
2) Name
3) Medical record number
4) Age
5) Name of Physician
6) The year and date the specimen was submitted
7) Numerical code to indicate the ViraPap and ViraType
   results.
IV. RESULTS

1. The Highest Dilution of the Low Positive Control Detectable With Standard Methodology

The highest dilution of the low positive control detectable with the conventional ViraPap assay was found to be 1:8 using either a single or double autoradiographic exposure.

2. Hybridization Time Experiments

Serial two-fold dilutions of the low positive control from 1:2 to 1:128 were hybridized at different times instead of the two hours specified by the manufacturer. In order to evaluate the effect of increasing the hybridization time, four different experiments were performed. The highest dilution of the low positive control detected at all the different hybridization times was found to be 1:8 as indicated in Table 1 using either a single or double autoradiographic exposure. However, the intensity of the autoradiographic signal increased as the hybridization time increased.

The double exposure results were more intense than the single exposure results.
3. **Quantitation of Hybridization Signals by Scintillation Counting Versus Autoradiography**

To confirm that the intensity of the autoradiographic signals increased as the hybridization time increased, the dot blots for the different hybridization times were counted in a scintillation counter. The scintillation count results were comparable to those of the autoradiographs in that they indicated an increase in the amount of radioactivity as the hybridization time was increased up to 8 hours (Table 2). Instead of increasing, the counts of the 32 hour experiment dropped. The autoradiographic results for the 32 hour experiment in keeping with the scintillation count results showed a decrease in signal intensity. The 32 hour experiment was conducted twice to demonstrate reproducibility (Table 3). The ratio of scintillation counts signal to noise increased as the hybridization time increased except for the 32 hour hybridization time as indicated in Fig 2.

4. **Doubling the Amount of Sample and Exposure Time**

Of the 212 previously negative specimens that were retested using twice the amount of sample, 197/212 (93%) were found to be negative at both single and double exposure. The remaining 15 specimens were found to be
questionable or positive as a result of doubling the amount of specimen as shown in Fig. 3. The double exposure results were concordant with the standard exposure results. Of these 15 specimens, eight were scored as positive at the regular exposure and at double exposure. Of the seven remaining specimens that were borderline positive with single exposure, four yielded strong positives at double exposure and the other three became definitively positive.

None of the experiments involving the Viratype procedure as outlined in flowchart I (see Fig. 4) of the original proposal were performed due to insufficient sample. No samples were saved for future PCR studies for the same reason. Testing previously negative samples from patients with cytologic evidence of reactive atypia could not be performed due to limited funds.

5. Preparation of a ViraPap Database

Of 2,792 samples tested during 1989-1991, 466 (16.7%) were positive by ViraPap. Of the positive ViraPap cases, 65 (14%) were assigned to HPV group 6/11, 224 (48.1%) to HPV group 16/18, 138 (29.6%) to HPV group 31/33/35 and 34 (7.3%) were untypeable by ViraType. These findings closely correlate to those reported by Chimera et al. (9) who used ViraPap/Viratype to assess 17,000 clinical specimens for HPV DNA.
V. DISCUSSION

The prevalence of HPV in our patient population using ViraPap was 16.6%, a rate similar to that found by other investigators (8). The present study was undertaken to determine if minor modifications to a commercial dot blot assay could increase the sensitivity of the kit in detecting HPV. The first experiments established that the low positive control could be detected at a dilution of 1:8. With this in mind, other experiments were then carried out to see the effects of these modifications using the 1:8 dilution of the low positive control as a cutoff standard for comparison to the standard assay.

The first parameter evaluated was the length of hybridization. Longer hybridization times (4 or 8 hours) seemed to increase the signal intensity. Increasing hybridization time as a means to increase sensitivity has been previously demonstrated by Meyer et al (41). These investigators demonstrated a significant increase in the detection of HPV DNA by ISH using a 16 hour hybridization compared to a 2 hour hybridization. Of the 59 specimens positive after 16 hours of hybridization, only 39% specimens were also positive with the recommended 2 hour hybridization. Although testing at two hours or 16 hours is more suitable for most clinical laboratories, a four hour hybridization is also reasonable because the assay can still be performed in a single eight-hour shift. In the present
study, there was a slight increase in the autoradiographic signal intensity as the hybridization time was changed from two hours to 32 hours. However, one drawback to a longer hybridization period is that greater background signals may be produced. We observed an increase in the background signal, which was probably due to the probe either binding to the nylon or non-specific binding (mismatched hybridization) of probe to cellular DNA.

Since the interpretation of signal intensity is made visually, quantitation by a scintillation counter was performed. In retrospect, the counting should have been performed for a longer period of time in order to obtain more counts for greater accuracy. Despite low absolute counts, a definite increase in the amount of radioactivity detected by scintillation counting up to the eight hour hybridization was demonstrated. The scintillation counts for the 32 hour hybridization time showed a marked decrease. The autoradiograph for the 32 hour hybridization time also showed a decrease in the signal intensity. It is possible that by 8 hours, a saturation point had been reached and there was no more binding of the probe to HPV DNA beyond that point, while radioactive decay of the bound probe continued from 8 to 32 hours. The discrepant results could also be due to consistent technical error in both the autoradiography and scintillation counts. The latter explanation seems more reasonable, since the results of the
32 hour hybridization using dilutions of the low positive control in part 2 showed increased signal intensity.

The second parameter evaluated was amount of sample used. Doubling the amount of specimen in 212 previously negative samples yielded 15 samples found to be positive with the modified ViraPap assay. In order to establish whether or not these 15 specimens were true positives, paraffin blocks were obtained from five of these 15 samples and evaluated using ISH. All five cases were found to be negative for the seven different HPV types normally detected by the ISH assay. It is possible these discrepant results can be attributed to the lower sensitivity of the in-situ hybridization procedure. A study by Faulkner et al (16) comparing a dot blot assay to the Viratype in-situ assay found the latter to be less sensitive than the dot blot assay.

The third parameter tested was the length of autoradiographic exposure. The intensity of the signal increased in all the autoradiographs re-set up for double exposure using either control samples or reprocessed clinical specimens. Although longer autoradiography seemed to increase the intensity of the signal it would also mean delaying reporting the results of the assay for a few more days. Since the ViraPap is not considered a stat procedure, waiting a few extra days would not be unreasonable.

Confirmation of positive results using the increased
sample size by the most sensitive method (PCR) was not feasible. A study done by researchers at the University of Washington compared ViraPap to PCR (8). These investigators analyzed 270 cervical samples for the presence of HPV types 6, 11, 16 and 18 by PCR. Samples from 154 of these 270 patients were concurrently analyzed by the ViraPap kit. These investigators found that the sensitivity of PCR was higher than that achieved by ViraPap alone. The prevalence of HPV in cytologically negative and borderline patients by PCR was 22% and 40% compared to only 7% and 10%, respectively by the ViraPap method. Meyer et al (42) using a similar patient population have reported finding ViraPap positive cases in 45% of their patients with CIN.

The 7% increase observed with our modified assay seems reasonable, given the probable maximum increase of 22% using PCR by the Seattle group. This suggests that our increased detection probably represents cases with low levels of HPV, rather than false positives by the modified system.
VI. SUMMARY AND CONCLUSIONS

Overall, increasing the hybridization time increased the intensity of the signal in the control samples. Doubling the amount of sample in previously negative specimens appeared to increase the detection rate of HPV DNA. Doubling the exposure time increased the intensity of the signal. Although all three factors were not tested together, doubling the amount sample seemed to be the most useful of the three variables tested in improving the sensitivity of the assay. Future studies should involve testing all three factors simultaneously to evaluate the effectiveness of combining all three parameters.

Other parameters that were not tested but could be investigated include increasing the time involved in the digestion/proteinase step and also increasing the concentration of the proteinase and the use of pre-flashed film for autoradiography. The use of PCR technology and histologic and cytologic follow-up are needed to validate the increased sensitivity of this modified ViraPap assay.


APPENDIX A
Tables
Table 1. Comparison of Autoradiographic Signals using Different Hybridization Times

<table>
<thead>
<tr>
<th>Dilution of LPC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hybridization Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>AR&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1:2</td>
<td>+</td>
</tr>
<tr>
<td>1:4</td>
<td>+</td>
</tr>
<tr>
<td>1:8</td>
<td>+</td>
</tr>
<tr>
<td>1:16</td>
<td>-</td>
</tr>
<tr>
<td>1:32</td>
<td>-</td>
</tr>
<tr>
<td>1:64</td>
<td>-</td>
</tr>
<tr>
<td>1:128</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> LPC: low positive control  
<sup>b</sup> AR: Autoradiography  
<sup>c</sup> Intensity of single exposure autoradiographic signal ranging from 1x-4x
Table 2. Quantitation of Hybridization Signals by Scintillation Counting versus Autoradiography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hybridization Time</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hours</td>
<td>4 hours</td>
<td>8 hours</td>
<td>32 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cpm d</td>
<td>intensity e</td>
<td>cpm</td>
<td>intensity</td>
<td>cpm</td>
</tr>
<tr>
<td>HPC a</td>
<td>472</td>
<td>1x</td>
<td>722</td>
<td>2x</td>
<td>1138</td>
</tr>
<tr>
<td>LPC b</td>
<td>287</td>
<td>1x</td>
<td>328</td>
<td>2x</td>
<td>454</td>
</tr>
<tr>
<td>1:2 LPC</td>
<td>266</td>
<td>1x</td>
<td>321</td>
<td>2x</td>
<td>368</td>
</tr>
<tr>
<td>1:4 LPC</td>
<td>258</td>
<td>1x</td>
<td>290</td>
<td>2x</td>
<td>295</td>
</tr>
<tr>
<td>NEG c</td>
<td>248</td>
<td>-</td>
<td>266</td>
<td>-</td>
<td>282</td>
</tr>
</tbody>
</table>

a HPC: High positive control from ViraPap kit
b LPC: Low positive control from ViraPap kit
c NEG: Negative control from ViraPap kit
d CPM: Average counts per minute using all six readings
e Intensity of single exposure autoradiographic signal ranging from 1x-3x
Table 3. Reproducibility of 32 Hour Hybridization Experiment

<table>
<thead>
<tr>
<th>Sample</th>
<th>First 32 hour experiment</th>
<th>Second 32 hour experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm&lt;sup&gt;d&lt;/sup&gt;</td>
<td>intensity&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>HPC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>382</td>
<td>1x</td>
</tr>
<tr>
<td>LPC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>328</td>
<td>1x</td>
</tr>
<tr>
<td>1:2 LPC</td>
<td>291</td>
<td>1x</td>
</tr>
<tr>
<td>1:4 LPC</td>
<td>315</td>
<td>1x</td>
</tr>
<tr>
<td>NEG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>285</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> HPC: High positive control from Virapap kit
<sup>b</sup> LPC: Low positive control from Virapap kit
<sup>c</sup> NEG: Negative control from Virapap kit
<sup>d</sup> Cpm: Average counts per minute using all six readings
<sup>e</sup> Intensity of autoradiographic signal
APPENDIX B
Figures
Fig 1. Autoradiograph
Lanes 1A-1C represent the positive controls and negative control
Lanes 1E, 2C, 2D and 5D represent clinical samples
FIGURE 2. A comparison of scintillation signal to noise ratio at different hybridization times
FIGURE 3. Single and double exposure results for the 15 samples found questionable or positive as a result of double sample.
Fig. 4 Flow Chart I

Routine VP
2 hour hybridization

1x exposure

(+)

1x exposure

(-)

Routine VT

1x exposure

1x exposure

2x exposure

Not enough sample to test by VP

Negative
6/11
16/18
31/33/35

Negative
6/11
16/18
31/33/35

2x exposure

2x exposure

Not enough sample to test by VP

Save 0.1 ml for PCR

Next routine VT with 1x sample

- Next routine VP with 2x sample

- 2 hr hybridization

- Save 0.1 ml aliquot for PCR