

**SS100 Testing for Human Papillomavirus:  
Methodology, Test Validation, and Quality Assurance**

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September 30, 2007 8:30 – 10:00 AM

## Course Content

- 1) Basic concepts of HPV biology
- 2) Indications for HPV testing
- 3) HPV testing methods
- 4) Quality control/assurance issues
- 5) HPV test validation

## Course Objectives

You will learn to:

- 1) Know the basics of HPV biology
- 2) Recognize appropriate indications for HPV testing
- 3) Describe the operating characteristics of the testing modalities available
- 4) Select appropriate testing methodologies for the detection of HPV
- 5) Perform appropriate quality control/assurance procedures for HPV testing

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### Introduction

During the past 10 years, testing for human papillomavirus (HPV) has gradually gone from a research tool to an integral part of the screening and management programs for cervical neoplastic disease. In the future it has the potential to become even more important, as testing methodologies and strategies are further refined. The purposes of this short course are multiple and include bringing the participant up to speed with background information on the virus and the role it plays in cervical carcinogenesis, and hence, why testing for its presence has become so useful. However, the testing process itself has become complicated, with much discussion about the proper test to perform in conjunction with Pap testing, and about the methods by which the laboratory can insure the quality, relevance, and usefulness of the results obtained. This latter topic forms the latter portion of the presentation.

### **Basic Concepts of HPV Biology**

The Human papillomavirus (HPV) is one of the most heavily studied infectious organisms in medicine. It is important because some members of this large family of viral types play an important role in one of the most common neoplastic conditions – that being cervical cancer and its precursor lesions. In order to understand the newest management guidelines for the triage of women with cervical abnormalities, and the evolving methods of cancer screening, it is important to know the basic facts about the virus, its mode of transmission, and the molecular mechanisms underlying neoplastic transformation.

#### **1) What is HPV?**

HPV is a member of the papovavirus family. It is an 8 Kbase single stranded DNA virus. It requires a host (human in this case) cell in which to replicate. There is no known reservoir outside of humans.

#### **2) How is HPV transmitted?**

HPV is almost exclusively considered to be sexually transmitted. There is some evidence to suggest that a small number of infections may be due to non-sexual contact via fomites.

#### **3) How many women are infected with high risk HPV?**

This figure depends on the age and population studied. Generally younger women have higher prevalence rates. These rates may be as high as 30% in the late teens and early twenties, fall through the reproductive years and then may rise again in the late 40's, sometimes to as high as 15-20%. The reason for this rise is uncertain but 2 possibilities have been suggested: 1) increased sexually activity in this population, 2) decrease in immune surveillance status allowing subclinical infections to become detectable. A typical screening population is generally thought to have a prevalence of about 5%.

#### **4) Does an HPV infection imply the presence of clinical disease?**

Not always – most women with detectable HPV will have either no manifestation of cervical disease (dysplasia or cancer) or will manifest only a low grade lesion (LSIL) which generally will clear after several months. Studies have shown that about 95% of infected women will clear their infections spontaneously within a 3 year period.

#### **5) What is the difference between “low risk” and “high risk” HPV types?**

Low risk types are virtually never associated with high grade disease. High risk types are the only viral types that cause CIN III or cancer. Simplistically the difference has to do with how effective the oncogenic portions of the different viral types' genomes are at transforming normal cells into malignant ones. This biologic difference is why clinical testing for low risk types is considered to be of no management value and why all guidelines include testing for high risk types only. It is important to remember that most low grade lesions are associated with high risk viral types – therefore low grade disease does not imply the presence of low risk virus.

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**6) What is the most prevalent HPV type?**

HPV type 16 is most prevalent and can comprise as many as 40% of the infections in studied populations. Its associated family members, types 18, 31, 33, 35, and 45 are the next most common types. Together with 16 these types account for about 80% of all infections.

**7) What is the biologic difference between low grade and high grade cervical lesions?**

LSIL (CIN 1) lesions are generally associated with replicative infections of virus which is present as an episome outside the host cell DNA. These infections allow completion of the viral lifecycle and the production of complete infectious virions. The koilocyte is the cell filled with complete virions ready to spill out to infect other cells. Low grade lesions typically run a benign course and resolve – similar to cutaneous HPV associated common warts of the skin. High grade preneoplastic lesions and cancer are typically associated with only oncogenic pieces of the HPV genome. These genes are integrated into the host's DNA in specific ways that allow uncontrolled expression. Because the entire genome is not present, these are not replicative infections and no intact virions are produced, hence no koilocytosis is noted.

**8) What are the oncogenic genes of the HPV and what do they do?**

In all high grade neoplasias and cancer, 2 HPV genes are always conserved. These are the E6 and E7 genes. The E6 acts by interfering with the host p53 tumor suppressor gene function and the E7 acts by interfering with the host pRb (retinoblastoma) tumor suppressor gene function. Hence both host tumor suppressor genes are “disabled” leading to cellular neoplastic transformation. The breakage of the HPV circular DNA during insertion occurs in the region of the E2 gene which disrupts the regulation of expression of the E6 and E7 genes leading to uncontrolled transcription. These tumor suppressor genes work as key “controllers” in the process of cell cycle DNA repair. Normally mutations generated during DNA replication are repaired at so-called cell cycle “checkpoints” prior to moving to the next phase. With loss of this “checkpoint” function, mutations accumulate abnormally, eventually leading to cellular transformation.

**9) What are HPV type “variants?”**

In order to qualify as a new “type” of HPV there must be at least a 10% difference in base pair sequence with every other type. However, within each type there are subtypes (2-10% sequence difference) and variants <2% sequence difference. The “story” of HPV variants is an interesting one as variant forms of the major types can be tracked geographically as populations of humans have migrated over time. For instance, European HPV type 16 variants are distinct from North American, African, and Asian HPV type 16 variants. Interestingly, different geographic variants have differing capabilities in transforming cells in different populations – meaning that a European variant might be a more “potent” transformer of cells in European populations than it might be in Asian populations, etc. This has been postulated to be caused by the longstanding interactions that have taken place between virus and population over time – and may be closely tied to host factors such as population HLA types and other unique population characteristics. In other work, investigators have also noted that some variants are more prone to cause adenocarcinomas, while others are prone more to cause squamous lesions. Glandular differentiation has been potentially tied to sequence differences that may be associated with estrogen response elements; ultimately causing tumors more akin to endometrial carcinomas in terms of pathogenesis.

**Indications and Support for HPV Testing**

Because hrHPV is associated with virtually all true cervical high grade preneoplasias and carcinomas, it is logical that testing for the virus with a highly sensitive test would be useful in ruling out disease (high negative predictive value (NPV)), and for triaging patients for appropriate diagnostic interventions, such as colposcopic examination, in the event of an equivocal Pap test. Data from the National Cancer Institute's ASCUS/LSIL Triage Study (ALTS) shows clearly the utility of this triage function. In women having equivocal (atypical) Pap tests, triage to colposcopy using positive HPV tests identified similar levels of high grade disease with about 50% of the colposcopic examinations, than did sending all such patients on to colposcopy (hitherto a standard for ASC-US management). It did so with more efficiency

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that did repeat cytology using any threshold of abnormality on the repeat Pap (meaning with triage to colposcopy for Pap results on repeat of either ASC-US, LSIL, or HSIL).

Hence the first accepted application of hrHPV testing has been for triage of ASC-US cases. In the ASCCP management guidelines, HPV testing is the preferred method for the initial assessment of patients with ASC-US when the specimens are taken in liquid-based fixatives or co-collection in a transport medium (along with a conventional Pap test) is performed.

The ALTS study also showed that HPV triage was NOT useful in the management of patients having an interpretation of LSIL. Approximately 85% of such patients were found to have positive tests for hrHPV. Hence HPV testing did not provide any significant utility as most would already be triaged to colposcopy using this test (colposcopy already being the management option of choice for Pap interpretations of LSIL).

Interestingly, hrHPV positive ASC-US was found to be the functional equivalent of LSIL, with followup rates for CIN II+ being virtually identical (27%) in the two groups.

For patients testing negative for hrHPV, the NPV of obtaining a result of CIN II+ was approximately 99%, making this test highly reliable in sorting out cases with little risk of significant abnormality. (Stoler 2003)

The second indication for hrHPV testing is a follow on to the last point; that indication being the use of hrHPV testing in conjunction with Pap testing, in a primary screening adjunctive role. The high NPV could be exploited when the test is negative in order to allow for increased screening intervals in women testing double negative - for both Pap and hrHPV test. The NPV for CIN II+ using this approach is 100%, and hence the latest recommendation is for increasing the screening interval to 3 years for such double negative tests. However, this indication is for use only in women over 30 years of age. Women in younger age groups are known to have higher hrHPV infection rates (as high as 20-30%), while in women over 30, the prevalence of hrHPV in the population falls significantly. Hence, cost effectiveness of this strategy is met only in the lower prevalence older population.

As of the present time there is no recommendation for the triage of ASC-H Pap specimens utilizing hrHPV testing. That being said, some laboratories (including our own) utilize reflex testing for ASC-H cases. Although all such patients do require a colposcopic examination, the ALTS study showed that colposcopic examinations were made more sensitive (for smaller or obscured lesions presumably) when it was known that the hrHPV test was positive. Second, in the event of a negative colposcopic examination, the hrHPV result can be useful in terms of "downstream" management - positive results might prompt more aggressive management, and vs. vs. If the test is not performed at the time that the initial Pap is read, the specimen will most likely be discarded and a new one will have to be obtained if further analysis is required.

Also at the present time there is no recommendation for the use of hrHPV testing for the triage of AGC cases. However, some have argued for its use based on the fact that, just as is squamous neoplasia, virtually all endocervical neoplasias and preneoplasias are caused by hrHPV. Studies have shown that specificity of triage with hrHPV in AGC is much higher than it is for ASC-US cases. However, the sensitivity for high grade disease is lower (mid-80% level) primary due to the mix of HPV-negative endometrial lesions in the AGC category. (Krane et al 2004) The newest management guidelines do take notice of the potential utility for use of hrHPV testing in the ongoing management of AGC cases (much in the same way as described above for ASC-H), in that they state that hrHPV test results (if obtained) should be utilized in management planning.

Regarding low risk HPV (LrHPV) testing; at the present time there is no association noted between true high grade neoplastic disease and LrHPV. Therefore, no management algorithm indicates testing for these viral types. In our laboratory at Massachusetts General Hospital we do not offer any LrHPV testing.

## **HPV Testing Methods**

There are a number of ways to test for the presence of HPV. Each of the tests has unique features and therefore limitations and strengths of its own. Knowledge of the operating characteristics (sensitivity, specificity, NPV, PPV) are important in selecting the proper test to perform for a given clinical purpose.

### 1) Immunochemistry

Antibodies have long been available to test for the presence of viral antigens (capsid antigens). These antibodies can be useful for the detection of whole virions in productive infections. However, for the 2 indications discussed above, immunochemical viral detection has limited or no utility. Because viral

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capsid antigen is only expressed in intact whole virions, immunochemistry will be positive only in the cells of low grade infections, not in cells representative of high grade neoplastic processes. In the latter, intact virions are not present, and viral DNA needs to be identified for any clinical significance.

2) DNA hybridization procedures

Hybrid Capture - the most commonly utilized test for the detection of hrHPV DNA is the Digene Hybrid Capture II method. It is FDA approved for use with ThinPrep Preservcyt samples and with Digene Standard Transport Media (STM). It was utilized in the ALTS study for the detection of hrHPV and as such, is the method whose operating characteristics have been "figured" into the current ASCCP management guidelines. The test utilizes a technique of DNA denaturation, followed by hybridization to whole genomic probes targeting 13 of the most common types of hrHPV. The "hybrids" are "captured" and stabilized by antibodies directed against these hybrid structures and then labeled for detection with additional tagged specific antibodies. A chemiluminescent reaction then ensues which is semi-quantitative for the amount of hrHPV DNA present in the sample. ALTS data has shown that HC II is 94% sensitive for CIN II+ lesions with a specificity of 41%, when utilized in the ASC-US triage mode, with a NPV of 99% and PPV of 20%.

In situ hybridization - ISH uses a similar denaturation/hybridization process, but performs the analysis directly on tissue or cytologic samples. In this manner, positive signal generated can be directly visualized and localized to the cell via microscopic examination. A number of commercial kits (Ventana INFORM and DakoCytomation GenPoint) have been evaluated. Data generated has shown better specificity for high grade disease using these assays, however the sensitivity of the assay for high grade disease is significantly lower, particularly when using the assay for ASC-US triage, than is HC II. In one study comparing HC II with ISH, a sensitivity for HC II of 97% was obtained, while ISH on the same specimens showed only 61% sensitivity with 50% of CIN II and 70% of CIN III being positive. (Hesselink et al 2003) Other studies have shown ISH to be 100% specific, and of comparable sensitivity with known dysplastic lesions, however, again, when utilized in the triage of equivocal specimens, sensitivities for high grade disease detection were lower than other modalities. (Kong et al 2007) ISH techniques have long been utilized in specialized research settings and have been shown to be nearly as sensitive as PCR procedures detailed below. In this modality they have been utilized to look at specific presence and transcription of specific genes in HPV. Such procedures have routinely utilized radioisotopic labels with long incubations ( $H^3$  or  $S^{35}$ ) thereby limiting their use as clinical assays.

3) Polymerase Chain Reaction

Because it represents an amplification procedure, PCR is theoretically the most sensitive method for the detection of hrHPV. It is a highly automated procedure, and has the advantage over HC II in that it allows specific viral typing in a sample. In the PCR process, specific primers are hybridized to denatured DNA, and then a polymerase is utilized, that through many cycles, continues to add specific segments of hybridization product to the reaction. This amplification process leads to large amounts of signal, and hence to detection sensitivity. Interestingly, data from ALTS shows slightly lower sensitivity when compared to HC II in paired samples (89 vs. 94%), however a variety of more recent studies using the commercially available kit from Roche (Amplicor HPV) have shown comparable (and sometime slightly higher) sensitivity compared to HC II. As one might expect, high sensitivities lead to high NPV, but with this, lower specificities have been reported, with concomitantly decreased PPV. (Wahlstrom et al 2007, Stevens et al 2007, Carozzi et al 2007, Sandri et al 2006)

There has been much debate in the pathology literature in recent years about the characteristics that must be present in hrHPV DNA tests performed. (see Titus K, CAP Today 2005;19(9):1) Because the number of tests performed is great, and because algorithmic management decisions are based on tests of known characteristics, the common position has been that any test utilized should be vetted for similar operating characteristics with either FDA approval or rigorous analytic and clinical validation procedures (to be discussed below). In a recent editorial on this subject, Stoler et al (2007) put forth a proposed "validation checklist" for any hrHPV test to be utilized in the current management scheme. Their criteria can be summarized as follows:

1) The test should be capable of detecting at least the 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). This takes into account >95% of cervical squamous carcinomas and precursors, and 90-95% of true endocervical adenocarcinomas and AIS.

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2) The test should have a sensitivity of at least 92% +/- 3% for the detection of CIN III+. These operating characteristics should be able to meet FDA scrutiny via its approval process or be rigorously documented in independent validations performed in a statistically valid manner and submitted publically to the peer review literature.

3) The test should have a clinical specificity of at least 85% for CIN III+.

4) Testing should be subject to reproducibility studies with high Kappa values to support test robustness.

5) An ideal test should have an internal standard for cellularity to insure that negative results are not merely the result of inadequate specimens (no current approved test meets this standard and this criterion is meant as a future improvement).

### **Quality Control/Assurance Issues**

#### 1) Quality control

**Training** - in any laboratory test, insuring that proper training of involved personnel takes place is critical. If the test performed is a commercially available assay, the training should be according to the manufacturer's protocol and is often under its direct guidance. For instance, our technologists performing HC II have undergone training at the Digene training center which provided basic training, verification of training, and now provides ongoing educational support and system troubleshooting.

**Internal standards** - each testing procedure should include known positive and negative control specimens.

**Active review of results prior to signout** - for HC II, supervisors and/or pathologists should review each run looking for particular artifacts that might indicate test problems such as splatter patterns or dilutional effects (indicating patterns of specimen cross contamination). Monitoring of positive rates compared to populations being tested should occur (comparing results in ASC-US triage vs. >30 primary screening is a useful monitor).

**Retesting of "equivocal" results** - in the HC II system, an equivocal result is one that falls between 1 and 2.5 RLU (1 being the +/- cutoff). For ThinPrep specimens, the FDA requires a retest up to 2 times with reporting of the result following the retesting procedure. For STM specimens retesting is not required. In our own clinical validation process, we determined that SurePath specimens with HC II do not require retesting of equivocal specimens. (Knoepp et al 2007)

#### 2) Quality assurance

**Interlaboratory comparison of test results** - Interlaboratory comparison is an integral part of clinical lab quality assurance. At present CAP provides a "viral detection" program that includes HPV detection as a small subset. While acceptable in the microbiology laboratory setting this is inadequate for anatomic (or other) laboratories performing only HPV testing. Interlaboratory comparison programs can be easily set up where commercial programs do not exist. In our own laboratory, we developed a system whereby we exchange unknown samples with a laboratory out of our geographic area, that utilizes the same system as we are using. We exchange 4 samples each quarter. Over a 3 year time period we have shown 97% correlation of results. We also set up an active system to investigate the reasons for non-correlations. (Kuebler et al 2007) Data from ALTS shows that HC II with ThinPrep had a 92% agreement between the clinical centers and the HPV QC Group with kappa values of about .8. (Castle et al 2004)

At present the CAP Cytopathology Resource Committee is developing a peer comparison program for laboratories performing only hrHPV testing. This program is currently being piloted and should be available during 2008. It will provide samples in either STM, ThinPrep, or SurePath media, or in mixed sets for those laboratories performing tests in more than one format. There will be 3 mailings/year, each consisting of 5 challenges. HPV testing will be a "reportable" analyte for both CMS and CAP LAP.

**Quality assurance of ASC-US interpretation** - while not explicitly QA of the HPV testing process, the HPV test can be used as a quality assurance tool for the control of ASC-US interpretation amongst cytologists. HPV positive rates and ASC-US/SIL ratios have been shown to correlate closely. (Ko et al 2007) However, the HPV positive rate provides a more objective measure to guard against morphologic errors in both of these categories that might not adversely affect their ratio. What should an ideal HPV + rate be? Theoretically, because ASC-US is an "equivocal" category, one might expect there to be a 50-50 chance of it representing an HPV-associated or non-associated case. In addition, there is a background rate of 5% HPV positivity in the general, cytologically-negative population. Therefore the

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ideal HPV+ rate should be  $50 + 5 = 55\%$ . In practice HPV+ rates tend to be less than this because of the natural tendency to overcall, particularly in the circumstance of a known objective test (HPV) that will be run with every ASC-US interpretation. This principle drives down the overall positive rate. In ALTS, the pathology QC group showed HPV+ rates in the 50% range. CAP Q-probes showed rates in the mid-40's, and in our laboratory at MGH, our HPV+ ASC-US rate runs at just about 40%. Therefore we believe that "acceptable" HPV + rates should be in the range of high 30's to 50's. Any deviation from this range could indicate either overcalling of ASC-US (lower rate), or undercalling of LSIL (higher rate). Use of this procedure as feedback to cytologists on a routine basis provides constant feedback on their morphologic performance.

### **HPV Test Validation**

As mentioned above, validation of the hrHPV test utilized in the laboratory is essential to insure comparability to tests from which algorithmic guidelines have been generated. The FDA approval process provides evidence of test validation, but at present only HC II with STM or ThinPrep have received FDA clearance. If a different technique, such as ISH, PCR, or HC II with another transport medium (e.g. SurePath), independent validation processes must be performed, according to the regulations of CLIA '88.

Test validations consist of 2 components: 1) analytic validation; and 2) clinical validation.

Analytic validation basically answers the question - "does the test perform with known cases as expected?" Methods to achieve analytic validation might include performance on known cases of HSIL, or comparing the results obtained in samples tested in another lab with your own results. These 2 exercises insure that the test is positive or negative on samples in which it should be, and that the results are reproducible. However, as mentioned previously, analytic validation is not sufficient for use with clinical management algorithms, because performance may appear acceptable here, but for its stated clinical use - ASC-US triage or adjunctive primary screening - it may not have the proper range of performance characteristics.

Clinical validation is required to insure that the test is operating within the parameters of its "clinical" use. This is a more extensive procedure and must be performed longitudinally with followup obtained on HPV + and - cases. For use in ASC-US triage application, the sensitivity of detection of CIN II+ and CIN III+ lesions must be determined by following tests to colposcopic (or other mode) of followup. This procedure is easy to do with HPV+ patients (who normally go to colposcopy), but is not so easy in HPV - patients who by algorithm would be conservatively managed. Therefore within the practical limits of such a clinical validation, long term cytologic followup of the HPV - population must be performed. Such followup will ultimately allow an assessment (or at least a modeling) of the NPV of the test. In our own laboratory we performed a 2 year clinical validation of the Surepath - HC II method with over 2000 ASC-US patients and determined that the operation of the test was in line with the test validation parameters as proposed by Stoler et al above. (Ko et al 2006)

### **Summation and Future**

There will be changes in the methods of testing for HPV in the future. In Europe, movement toward HPV primary screening (the more sensitive test) triaging to cervical cytology (the more specific test) is underway. In addition, type specific HPV testing has been proposed as a better way to assess the risk of cervical precancer progression. In a study of a large cohort of women in the Kaiser Permanente system, Khan et al showed that women with HPV types 16 and 18 were far more likely to develop high grade lesions than were those with other high risk viral types. (Khan et al 2004) Commercial manufacturers are reportedly developing type specific HPV tests that should become available in the near future.

Markers more specific for high grade disease (as opposed to the total HPV positive population) are being sought. Promising markers such as the p16/Ki67 combination, aberrant cell cycle markers, and chromosomal amplifications may provide more specific triage methods in the future.

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