

# Detection of Cervical High-Grade Squamous Intraepithelial Lesions from Cytologic Samples Using a Novel Immunocytochemical Assay (ProEx<sup>TM</sup> C)

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**BACKGROUND.** Routine liquid-based cytology (LBC) provides excellent sensitivity for the detection of cervical high-grade squamous intraepithelial lesion (HSIL); however, its specificity is low. Consequently, many women who have atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) cytology undergo unnecessary colposcopy. The authors hypothesized that a novel immunocytochemical assay (ProEx<sup>TM</sup> C) that can be performed on LBC slides had a significantly higher positive predictive value (PPV) for biopsy-proven HSIL compared with routine LBC.

**METHODS.** The ProEx<sup>TM</sup> C immunocytochemical assay utilizes a cocktail of monoclonal antibodies directed against proteins associated with aberrant S-phase cell cycle induction (topoisomerase IIa, minichromosome maintenance protein 2). The ProEx<sup>TM</sup> C reagents were validated in the authors' laboratory for staining and scoring reproducibility, open-vial stability, and accuracy before a retrospective analysis using these reagents was performed on 317 residual cytology samples. Sensitivity, specificity, PPV, and negative predictive value (NPV) for the detection of biopsy-proven HSIL were determined.

**RESULTS.** The ProEx<sup>TM</sup> C assay was validated successfully in the authors' cytology laboratory. Using biopsy-proven HSIL as an endpoint, the ProEx<sup>TM</sup> C assay yielded a sensitivity of 85.3%, specificity of 71.7%, PPV of 44.6%, and NPV of 94.8%. Compared with the routine LBC results in the same cohort, the ProEx<sup>TM</sup> C sensitivity for biopsy-proven HSIL was 70.6% greater than HSIL-positive cytology (50% vs. 85.3%). ProEx<sup>TM</sup> C also showed a 114% increase in PPV relative to ASC-US cytology (21.1% vs. 44.6%).

**CONCLUSIONS.** The ProEx<sup>TM</sup> C immunocytochemical assay can be integrated into a clinical cytology laboratory and may increase the PPV of LBC for biopsy-proven HSIL. *Cancer (Cancer Cytopathol)* 2006;000:000-000. © 2006 American Cancer Society.

**KEYWORDS:** cervical cytology, liquid-based cytology, immunocytochemistry, high-grade squamous intraepithelial lesion.

The implementation of cervical cancer screening programs centered around the Papanicolaou (Pap) test have led to a marked reduction in the incidence of cervical cancer and mortality caused by invasive cervical cancer.<sup>1</sup> This is a result of the successful identification and ablation of the precursor of cervical cancer, the high-grade squamous intraepithelial lesion (HSIL). Despite improved screening programs, an estimated 9710 women in the United States will develop cervical cancer in 2006.<sup>2</sup> Although a significant percentage of those women will have not been screened, approximately 30% may have had at least 1 false-negative Pap test because of errors in either

sampling or cytologic interpretation prior to developing invasive cervical cancer.<sup>3</sup> Because the identification of HSIL on a Pap test does not offer great enough sensitivity, women with milder cytologic atypia must undergo colposcopy and cervical biopsy to achieve acceptable test sensitivity. In the Atypical Squamous Cells of Undetermined Significance (ASC-US)-Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS) trial, 12% of women with ASC-US cytology had an underlying, biopsy-proven HSIL.<sup>4,5</sup> Conservative estimates predict that approximately 3 million American women receive abnormal cervical cytology results that require colposcopic evaluation to exclude HSIL.<sup>6</sup>

Ancillary molecular testing of cervical samples has started to address some of these limitations. For example, high-risk human papillomavirus (HPV) DNA testing can segregate women with ASC-US cytology into 2 groups with significantly different risk of HSIL. Women who are negative for HPV DNA have a low risk of HSIL that is similar to the risk among women who have a cytologic diagnosis of no intraepithelial lesion or malignancy (NILM).<sup>7</sup> The combination of NILM cytology and a negative HPV DNA test may confer such a low risk of HSIL that screening intervals for these women may be increased.<sup>8-10</sup> Unfortunately, the specificity of a positive HPV DNA test for HSIL is very low.<sup>11</sup> The ideal molecular marker for HSIL would retain the negative predictive value (NPV) of a negative HPV DNA test but would have greater positive predictive value (PPV). Such a marker also would identify cells that have undergone neoplastic transformation and not simply transient viral cytopathic effects.

Recent gene expression profiling studies have identified a set of candidate molecular markers that are associated with HSIL.<sup>12</sup> These molecules fall into several categories, including those associated with the extracellular matrix and those involved in cell replication and proliferation. The induction of cell proliferation (S-phase) in HSIL cells is initiated, in part, by the activity of the E6 and E7 proteins of HPV.<sup>13</sup> It has been established that HPV E7 binds the RB protein and displaces the transcriptional activator, E2F, from its complex, thus inducing the aberrant transcription of S-phase proteins, such as minichromosome maintenance protein-2 (MCM2) and topoisomerase-IIA (TOP2A), that are responsible for DNA synthesis and cell proliferation. The MCM proteins function during DNA replication by loading the prereplication complex onto DNA and by unwinding DNA through helicase activity to permit DNA synthesis.<sup>14</sup> TOP2A is responsible for the enzymatic unlinking of DNA strands during replication.<sup>15</sup>

In an extension of these exploratory studies, a biomarker cocktail containing antibodies against TOP2A and MCM2 has been developed that may be applied to

cytologic specimens by using immunocytochemical techniques to detect aberrant S-phase induction (ProEx<sup>TM</sup> C).<sup>13</sup> We validated this reagent in our cytology laboratory and performed a retrospective study to test the hypothesis that ProEx<sup>TM</sup> C will yield a greater PPV and sensitivity for biopsy-proven HSIL when applied to cervical cytology compared with routine, liquid-based cytologic analysis.

## MATERIALS AND METHODS

### Specimen Selection

Prior to beginning this study, the protocol was reviewed and approved by the Johns Hopkins Institutional Review Board. The 344 specimens that were used in this study were residual cervical cytology samples in SurePath<sup>®</sup> preservative that were obtained from the Johns Hopkins Cytopathology Laboratory in 2004 after clinical evaluation was completed. The original clinical cytology diagnoses were rendered by the 5 board-certified cytopathologists on staff at the Johns Hopkins Hospital. All specimens had been stored at room temperature for less than 1 year. Priority was given to specimens with abnormal cytology and a follow-up biopsy within 6 months of the cytology specimen procurement. An effort was made to include cytology samples with a subsequent HSIL (Grade 2 cervical intraepithelial neoplasia [CIN-2]-positive) biopsy. All cytology samples were annotated with the original clinical cytology diagnosis, the follow-up biopsy diagnosis, and basic patient demographics (age, race); then, the samples were deidentified and assigned a research protocol number in the data base. Clinical high-risk HPV DNA testing was performed on SurePath<sup>®</sup> samples using Hybrid Capture 2 (Digene Inc., Gaithersburg, MD) based on in-house validation according to the manufacturer's instructions. The SiHa cell line used as a control for the immunocytochemical assay was derived originally from an invasive squamous cell carcinoma of the cervix and reportedly contains 1 or 2 copies of integrated HPV type 16. It was shown previously that SiHa cells over express MCM2 and TOP2A.<sup>16</sup> The NILM and HSIL pooled samples that were used in the validation studies were generated from residual liquid-based samples from approximately 15 individuals who had cytologically confirmed NILM or HSIL, respectively.

### Immunocytochemistry

ProEx<sup>TM</sup> C (TriPath Imaging Inc., Burlington, NC) is a Class I, *in vitro*, immunohistochemical diagnostic that is used with standard immunocytochemistry techniques to detect the presence of aberrant S-phase induction in liquid-based cervical cytology specimens. ProEx<sup>TM</sup> C

contains antibodies to MCM2 and TOP2A proteins. Thin-layer slides were prepared from liquid-based cervical cytology specimens in SurePath preservative vials using the PrepStain® processor and were treated with a pretreatment buffer for target retrieval (SureDetect™ slide-preparation buffer).

Immunocytochemistry was performed with ProEx™ C, a detection reagent that includes a 3,3'-diaminobenzidine tetrahydrochloride-based chromogen and hematoxylin-based counterstains (SureDetect™ detection reagents and SureDetect™ counterstains), using an automated staining platform (Dako Autostainer; DakoCytomation). With this reagent, brown nuclear staining is indicative of aberrant S-phase induction.

### Scoring Algorithm

Each slide was scored by manual microscopic evaluation, first by 1 certified cytotechnologist (D.K. or E.K.) and then by 1 board-certified cytopathologist (D.P.C. or D.L.R.) who was aware of the cytotechnologist's diagnosis. The following scoring algorithm was developed: 1) A specimen was determined to be adequate using Bethesda System 2001 criteria.<sup>17</sup> 2) Slides were screened for moderate-to-intense, brown nuclear staining in epithelial cells. 3) Stained cells were determined to be squamous or glandular. 4) Using Bethesda System 2001 criteria, the stained cell was evaluated for criteria of ASC-US or a more severe lesion, including atypical squamous cells, cannot rule out HSIL (ASC-H), LSIL, HSIL, and invasive carcinoma. If the cell was glandular, then criteria for atypical glandular cells (AGC) were applied. Positive responses to each of these steps, even in only 1 cell, resulted in a positive test result. A lack of stained cells or the identification of stained cells that did not satisfy the criteria for at least ASC-US or AGC resulted in a negative test result.

### Statistical Analysis

The data analysis was performed using SAS software (version 9; SAS Inc., Cary, NC). The estimated sensitivity, specificity, PPV, and NPV of ProEx™ C for the detection of HSIL (CIN-2-positive) in an ASC-US-positive population were calculated and compared with the original liquid-based cytology diagnoses. In addition, 95% confidence intervals were calculated and summarized in a comparison of ProEx™ C results with cytology diagnoses.

## RESULTS

### Analytic Validation

To validate the ProEx™ C reagents in our cytology laboratory, analytic testing was performed to assess staining reproducibility, scoring reproducibility, open-vial

reagent stability, and accuracy for rare positive cell detection. To assess the reproducibility of the immunocytochemical assay in the clinical cytopathology laboratory setting, the ProEx™ C antibody cocktail and general laboratory use reagents were tested in 2 lots on 5 consecutive days with 2 runs completed each day. For these studies, a panel of 3 specimens (NILM pooled specimen, HSIL pooled specimen, positive SiHa control cell line) was tested in duplicate (Fig. 1). SiHa control slides were considered positive if 30% of the cells on the slides showed moderate nuclear staining. Table 1 shows that all SiHa control slides were positive (40 of 40 slides). All NILM pooled specimens were negative (0 of 40 specimens), and all HSIL pooled specimens were positive (40 of 40 specimens). These results suggested that there was no difference in performance between the 2 lots of ProEx™ C antibody cocktail, the detection reagents, or the counterstains and that the staining reproducibility between runs over time was excellent.

To test scoring reproducibility, 2 pathologists independently scored the same set of blinded slides, including 10 NILM specimens and 10 HSIL specimens. Prior to testing the scoring reproducibility, pathologists and cytotechnologists were trained by using sets of standardized teaching slides. Slides were interpreted according to the scoring guide described above (see Materials and Methods). Table 1 shows that 95% concordance (19 of 20 specimens) was achieved between the 2 pathologists. These results suggest that, with minimal training in the use of a scoring guide, pathologists can achieve high concordance in their interpretation of ProEx™ C-stained slides.

Open-vial reagent stability was tested on 2 lots of reagents at 8 time points (Days 0 [freshly opened vial], 3, 6, 9, 12, 19, 26, and 35) using NILM pooled samples, HSIL pooled samples, and SiHa control cells. Table 1 shows that all of the NILM specimens were scored correctly as negative (0 of 32 specimens) and that all HSIL and SiHa specimens were scored correctly as positive in a blinded review (64 of 64 specimens for both). Consequently, there was no detectable difference in the performance of ProEx™ C reagents over a 35-day period.

Accuracy testing for rare positive cell detection was performed by using HSIL pooled specimens and NILM pooled specimens that were spiked with rare SiHa cells (approximately 20 SiHa cells per slide). All of the HSIL slides and all of the SiHa spiked samples were scored correctly as positive (10 of 10 slides for both) (Table 1).

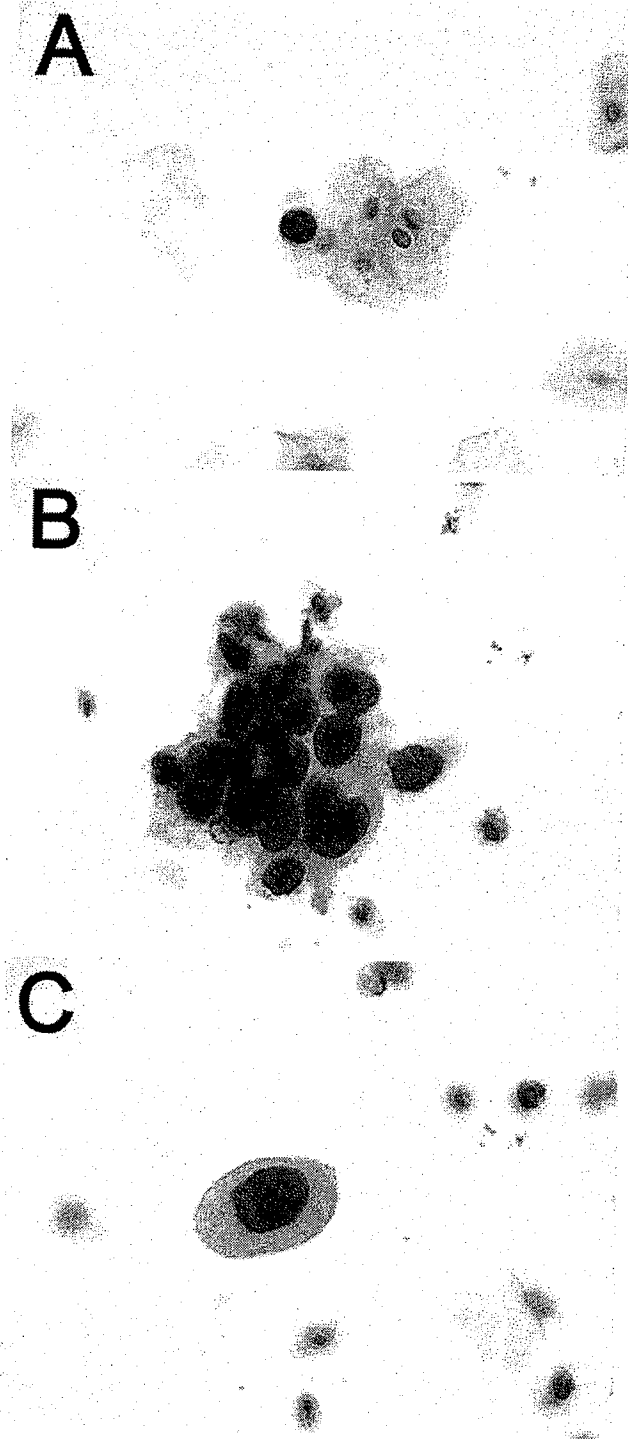
### Retrospective Clinical Testing Results

To test the ability of ProEx™ C to detect HSIL in cervical cytology samples, residual liquid-based cervical

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**FIGURE 1.** These photomicrographs show positive ProEx™ C immunocytochemical staining in (A) SiHa cells, (B) a syncytial cluster of high-grade squamous intraepithelial lesion cells, and in (C) a single atypical squamous cell (original magnification,  $\times 600$ ).

**TABLE 1**  
Summary of ProEx™ C Analytic Testing

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Analytic test	No. of women with positive ProEx™ results		
	NILM pool	HSIL pool	SiHa cell line
Scoring reproducibility			
Pathologist 1	1/10	10/10	NA
Pathologist 2	0/10	10/10	NA
Staining reproducibility	0/40	40/40	40/40
Stability			
Lot 1	0/16	32/32	32/32
Lot 2	0/16	32/32	32/32
Accuracy	NA	10/10	10/10

NILM indicates no intraepithelial lesion or malignancy; HSIL, high-grade squamous intraepithelial lesion; NA, not available.

cytology samples were identified for testing from a specimen repository at our institution. These samples were deidentified but annotated with the original cytology diagnoses and follow-up biopsy diagnoses. Three hundred forty-four samples were used that contained a spectrum of original cytologic diagnoses, and of these, 27 samples ultimately were considered unsatisfactory because of inadequate cellularity. The final analysis was limited to samples from 317 women (156 NILM samples, 55 ASC-US samples, 1 AGUS sample, 24 ASC-H samples, 53 LSIL samples, and 28 HSIL samples) (Table 2). The inadequate cellularity probably was associated with the use of residual specimens rather than original samples. This cohort contained a greater prevalence of atypia than the overall screening population at our institution. In selecting patients, preference was given to those who had biopsy follow-up within 6 months of procurement of the cytology samples. The prevalence of biopsy-proven HSIL within each cytologic category was designed to be similar to that observed in the ALTS trial, although the underlying HSIL rate in our ASC-H group (ASC-US, 11%; ASC-H, 17%; LSIL, 13%; HSIL, 61%) was somewhat lower than the prevalence reported in the ALTS trial. A thin-layer slide was prepared from a sample of each residual specimen using PrepStain® technology, stained with ProEx™ C reagents, screened by a cytotechnologist, and reviewed by a cytopathologist. A sample was considered positive if it met 3 criteria: 1) adequate cellularity, 2) moderate or strong nuclear immunocytochemical staining, and 3) atypical cytomorphology in the stained epithelial cells. The results of the immunocytochemical assays are summarized in Table 2. Overall, the immunocytochemical staining was very robust in positive samples; and the distinction rarely was problematic between faintly stained versus moderately stained nuclei. In a small number of specimens, there was

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**TABLE 2**  
Cytology and Histology Correlation in a Retrospective Study of the ProEx™ C Assay

Cytology	Histology (No. with positive ProEx™ C results)*				
	NILM	LSIL	HSIL	No biopsy	Total
NILM	2 (1)	0 (0)	0 (0)	154 (17)	156 (18)
ASC-US	43 (8)	6 (3)	6 (5)	0	55 (16)
AGUS	1 (0)	0 (0)	0 (0)	0	1 (0)
ASC-H	15 (6)	5 (2)	4 (4)	0	24 (12)
LSIL	26 (4)	20 (6)	7 (3)	0	53 (13)
HSIL	7 (4)	4 (3)	17 (17)	0	28 (24)
Total	94 (23)	35 (14)	34 (29)	154 (17)	317 (83)

NILM indicates no intraepithelial lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; AGUS, atypical granular cells of undetermined significance; ASC-H, atypical squamous cells, cannot rule out HSIL.

\* The number of women in each category is indicated along with the number (in parentheses) of women in each category who had positive ProEx C results.

staining of normal endocervical cell nuclei; however, these nuclei usually could be distinguished from atypical squamous or glandular cells. The total number of ProEx™ C-positive samples within the entire cohort was 83 (26%). A small percentage of the NILM cytology samples were positive (12%); because of the lack of follow-up biopsies in these patients, the prevalence of underlying HSIL could not be determined. The ASC-US-positive cytology category contained a greater overall prevalence of ProEx™ C-positive samples (40%) than the NILM cytology category (ASC-US, 29%; LSIL, 25%; HSIL, 86%). Within the ASC-US group, the prevalence of positive ProEx™ C results was low in patients who had NILM on follow-up biopsies (19%), similar to the NILM cytology group. The prevalence of ProEx™ C positivity was greater among the patients who had ASC-US samples with LSIL and HSIL on follow-up biopsies (50% and 83%, respectively). Likewise, within the LSIL cytology group, the prevalence of ProEx™ C positivity was low in patients who had NILM on follow-up biopsies (15%) but higher among the patients with LSIL samples who had LSIL and HSIL on follow-up biopsies (30% and 43%, respectively). The prevalence of ProEx™ C positivity was greater in the ASC-H cytologic category, ranging from 40% in patients who had NILM and LSIL on follow-up biopsies to 100% in patients who had HSIL on follow-up biopsies. The prevalence of ProEx™ C positivity was greatest among the patients with HSIL cytology samples, regardless of their follow-up biopsy results (NILM, 57%; LSIL, 75%; HSIL, 100%), possibly as a result of inadequately sampled or regressed HSIL lesions in those patients who had biopsies that showed NILM or LSIL.

**TABLE 3**  
Comparison of High-Risk Human Papillomavirus DNA Testing, ProEx™ C Score, and Biopsy Results in Women with Atypical Squamous Cells of Undetermined Significance\*

Variable	ProEx™ C positive	ProEx™ C negative	Total
HPV Positive	12 (3)	25 (1)	37
HPV Negative	0 (1)	14 (0)	14
Totals	12	39	51

HPV indicates human papillomavirus.

\* The number of women in each category who had biopsy-proven high-grade squamous intraepithelial lesions is shown in parentheses.

The results of clinical high-risk HPV DNA testing were available on a subset of women who had ASC-US cytology results (n = 51 women). Comparison of these data with ProEx™ C scores and follow-up biopsy results revealed that, whereas ProEx™ C was negative in 1 patient and HPV DNA testing was negative in another patient with biopsy-proven HSIL, the PPV of ProEx™ C for biopsy-proven HSIL was significantly greater than the PPV of HPV DNA testing (31% vs. 11%) (Table 3).

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### Discrepant Analyses

There were 5 cytologic samples within the HSIL biopsy follow-up group that were negative for ProEx™ C (false-negative results). These ProEx™ C slides were reviewed according to the data analysis described above. Upon review of these slides after data analysis, 3 samples contained rare atypical cells that were not stained, whereas 2 samples contained rare atypical cells with mild nuclear staining.

Within the NILM biopsy follow-up group, there were 23 specimens (24%) that were positive for ProEx™ C (false-positive results). Because virtually all of those specimens were from atypical cytologic categories, it is possible that colposcopy failed to identify an underlying HSIL in some of the patients. The inaccuracy of colposcopic biopsies has been described previously.<sup>18-20</sup> Because of the blinded nature of this study, long-term follow-up for subsequent biopsy results was not possible.

Within the NILM cytology group, 18 samples (12%) were positive for ProEx™ C. Only 2 patients in that group had corresponding biopsy results. Again, the blinded nature of the current study prevented both a review of the original Papanicolaou-stained, liquid-based cytology slide and long-term follow-up.

### Statistical Analysis

In an attempt to compare the performance of routine liquid-based cytology with the ProEx™ C assay for the detection of biopsy-proven HSIL, we calculated the

