

Determination of Liquid-Based Cervical Cytology Specimen Adequacy Using Cellular Light Scatter and Flow Cytometry

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Background: The majority of cervical cytology specimens are being collected in liquid-based preservatives (LBP). However, the assessment of specimen adequacy, as mandated by The Bethesda System (TBS), is still being performed at the time of slide review. We present a rapid, flow cytometric method for assessing specimen adequacy. **Methods:** Three LBPs were compared for cell-surface antigen preservation. A three-color antibody panel was used to confirm the light scatter profile of specific cells in a liquid-based cervical cytology specimen. Using forward and orthogonal light scatter alone, we were able to assess the adequacy of liquid-based cytology specimens in all LBPs tested. **Results:** The number of polymorphonuclear neutrophils (PMNs), endocervical (columnar) cells, ectocervical (squamous) cells, and debris in 120 liquid-based cervical cytology samples was quantified in less than 10 min. Using cutoffs of >20% PMNs, <1.0% endocervical cells, <1.0% ectocervical cells, and <500 total cells per milliliter, light scatter correlated with microscopic determination of adequacy with a correlation coefficient of 0.99. **Conclusions:** This rapid method allows the quantitative determination of cervical cytology adequacy in liquid-based cytology preparations prior to the preparation of slides for morphologic assessment. *Cytometry (Comm. Clin. Cytometry)* 46:340–344, 2001. © 2001 Wiley-Liss, Inc.

Key terms: Papanicolaou smear; liquid-based preservatives; specimen adequacy; flow cytometry; cervix, cancer

Cervical cancer affects more than 12,000 women in the United States, with 6,500 deaths in 2000 (1). Millions of women are screened annually for cervical cancer. The Bethesda System (TBS) was created to provide uniformity for the description of cervical abnormalities seen in Pap smears. TBS also provided criteria for the adequacy of specimens. Cervical cytology specimens are evaluated for the total number of cells, number of ectocervical (squamous cells), endocervical (columnar cells), and the amount of inflammation (2). Based on the assessment of the sample, TBS categorizes specimen adequacy as unsatisfactory (UNS), satisfactory but limited by (SBLB), and satisfactory (2). UNS samples account for up to 2.5% of conventional smears and SBLB account for up to 30% of conventional smears (3,4). The increasing use of liquid-based cervical cytology preparations has decreased the number of UNS and SBLB samples up to 50% in some studies but not at all in others (4,5).

Cervical cancer is a process that involves the transformation of cells by human papillomavirus leading to a continuum of morphologic changes that result in cervical cancer (6,7). These changes are identified predominantly in squamous cells of the ectocervix, although morpho-

logic changes in the columnar cells of the endocervix are also assessed as a screen for adenocarcinoma (8,9). Cervical cancer screening involves the morphologic assessment of a heterogeneous cell population including ectocervical cells, endocervical cells, polymorphonuclear leukocytes (PMNs), and lymphocytes. The adequacy of a cervical cytology specimen is defined as containing both ectocervical and endocervical cells, thus providing evidence that the transformation zone has been sampled. Lack of endocervical cells and obscuring inflammation are leading reasons for an SBLB designation (10).

In the present study, we used a panel of antibodies to detect PMNs, endocervical cells, and ectocervical cells in liquid-based cervical cytology preparations. Using this antibody panel, we established instrument settings that allowed us to identify unequivocally these cellular subpopulations by forward and orthogonal light scatter. Using

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Received 26 June 2001; Accepted 6 September 2001

Table 1
SNRs of Common Antibodies Bound to PBMCs Fixed in Three LBPs and PBS as a Normal Control*

LBP	FI-1 (FITC)	FI-2 (PE)	FI-3 (PE-Cy5)
CYTORICH	32.8	49.6	61.9
CytoSpray	18.2	31.8	27.4
PreservCyt	5.6	8.5	No staining
PBS	48.5	39.8	112.9

*Values = median fluorescence intensity (MFI) (+cells)/MFI (−cells).

these instrument settings, 120 liquid-based preservatives (LBPs) were resolved into debris, ectocervical cells, endocervical cells, and PMNs by light scatter alone. The number of cells in each light scatter gate allowed for the assessment of specimen adequacy in 10 min in all LBP preservatives tested. These data support the utility of this method for a rapid assessment of liquid-based specimen adequacy.

MATERIALS AND METHODS

Specimens

Liquid-based cytology specimens were obtained in CYTORICH solution (Tripath Imaging, NC) for antibody staining and in either PreservCyt (Cytoc, MA), CYTORICH, or Streck Cytospray (Streck Laboratories, Omaha, NE) solutions for adequacy assessment by light scatter. All specimens were stored in preservative less than 3 days at room temperature.

Antigen Preservation in LBPs

Peripheral blood mononuclear cells (PBMCs) were prepared from blood drawn in citrate CPT tubes (BD Pharmingen, San Diego, CA). PBMCs (1×10^6) from the same sample were washed in phosphate buffered saline (PBS), pH 7.4, pelleted by centrifugation at $600 \times g$, and resuspended in 1 mL of either CYTORICH solution, PreservCyt, or Streck Cytospray. The cells were incubated for 2 h at room temperature. The PBMCs were washed in PBS and resuspended in an optimized cocktail of CD8-FITC, CD14-PE, and CD4-CyChrome (BD Pharmingen). The PBMCs

were stained for 20 min at 4°C , washed in PBS, and run on a Beckman-Coulter four-color XL flow cytometer (Beckman-Coulter, Miami, FL).

Antibodies

CyChrome-labeled CAM 5.2 (BD Pharmingen) was used at a 1:10 dilution to selectively stain endocervical cells. CAM 5.2 reacts with human keratin proteins of 48 and 52 kDa including those expressed on secretory epithelia but not on stratified squamous epithelia (11,12). Phycoerythrin (PE)-labeled CD16 was used at a 1:10 dilution to stain PMNs.

Flow Cytometry

A 1-mL aliquot of a 10-mL LBC sample was centrifuged at $1,000 \times g$ and resuspended in 300 L PBS with 2% fetal calf serum (FCS). Samples were run on either a FACScan or FACScalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer at high flow rates. A total of 10,000 events were counted. The instrument settings for forward scatter were E-1 for voltage, 6.00 for amplifier gain, and log for mode. The instrument settings for side scatter were 225 for voltage, 1.00 for amplifier gain, and log for mode.

RESULTS

Antigen Preservation in Liquid-Based Cervical Cytology Preparations

To determine the ability of common liquid-based cytology preservatives to maintain antigenicity, we fixed Ficoll-separated PBMCs for 24 h in CYTORICH, PreservCyt, and Streck Cytospray and compared the signal intensities of the common immunophenotypic markers CD4, CD8, and CD14. As a positive control, the same cells were stained immediately in PBS. As demonstrated in Table 1 and Figure 1A, the signal-to-noise ratio (SNR) in the FITC (CD8), PE (CD4), and PE-Cy5 (CD14) fluorescence channels varied significantly based on the method of preservation. The SNR in the FITC channel was highest in CYTORICH solution and decreased by 68% in PreservCyt and by only 26% in Streck Cytospray, which had the same SNR as the PBS control. The SNR in the PE channel again was brightest in

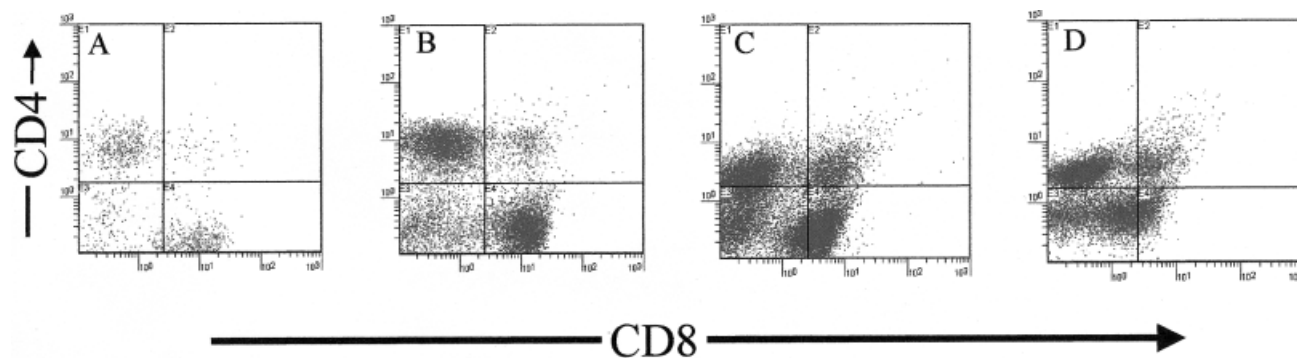


Fig. 1. Representative dot-plots demonstrating the staining pattern of common antibodies bound to cells fixed in **A**: PBS, **B**: CYTORICH, **C**: CytoSpray, **D**: PreservCyt. These dot-plots were used to calculate the SNRs in Table 1.

CYTORICH, although it decreased by 45%, 92%, and 30% compared with the PBS control, PreservCyt, and Streck Cytospray. In the PE-Cy5 channel, the SNR was greatest in cells fixed in CYTORICH solution, although it decreased by 30% compared with the PBS control and by 27% compared with Streck Cytospray. Staining by the CD8 antibody in cells fixed in PreservCyt was undetectable, suggesting antigen loss. These data are critical for the antibody confirmation of cervical cell-specific light scatter characteristics in LBP.

Light Scatter Characteristics of Specific Cell Populations in Liquid-Based Cervical Cytology Preparations

To determine the light scatter characteristics of PMNs, ectocervical cells, endocervical cells, and debris, we simultaneously stained cells fixed in either Streck cytospray or CYTORICH with CD16 to identify PMNs and CAM 5.2 to distinguish between ectocervical cells and endocervical cells. Using normal ectocervical and endocervical cells grown in culture, we determined that CAM 5.2 selectively stained endocervical cells (data not shown). As demonstrated in Figure 2, cervical cells in LBP resolved into four populations using log side scatter (orthogonal) and log forward scatter. Using these populations as gates, we determined that the population with the greatest side scatter and forward scatter (population A) was CD16 positive, consistent with PMNs. Population B was CD16 negative and CAM 5.2 positive, consistent with endocervical cells. Population C was CD16 negative and CAM 5.2 negative, consistent with ectocervical cells. Because of low side and forward scatter, population D was consistent with debris.

Assessment of Specimen Adequacy in LBP Specimens

To test the utility of this method for the assessment of specimen adequacy, we analyzed 120 samples including samples designated UNS, satisfactory but limited by endocervical cells (endocervical component [ECC]), satisfactory but limited by inflammation, and satisfactory but limited by ectocervical cells. Each sample required about 10 min to perform the analysis. Representative dot-plots of each TBS adequacy category are found in Figure 3. Using numeric cutoffs of >20% PMNs, <1.0% ectocervical cells, and <1.0% endocervical cells, light scatter assessment of specimen adequacy yielded a 0.99 correlation coefficient with the standard microscopic assessment. The discrepancy in 120 samples was a sample with 0.7% ectocervical cells that was called borderline inadequate, no ectocervical component by light scatter, but was found to be microscopically adequate.

DISCUSSION

Cervical cytology specimen adequacy is critical because it contributes significantly to false negative determinations. Our current system of determining specimen adequacy is ineffective because it is determined well after the specimen has been obtained and the woman has left the physician's office. The ideal assessment of specimen adequacy should be performed at the point of care where the

opportunity to acquire an adequate sample during the same office visit still exists. We describe a method using light scatter to assess specimen adequacy in less than 20 min, potentially allowing a point of care determination and immediate resampling if necessary.

TBS created categories of specimen adequacy in an attempt to standardize cervical cytology assessments. Since the establishment of the TBS guidelines, numerous studies have been performed that identify the relationship between UNS or SBLB samples and cytologic abnormalities. A computer search of over 70,000 Pap smears revealed that 16% had an eventual diagnosis of SIL or neoplasia on follow-up examinations (13). Other smaller studies have also emphasized the role of specimen adequacy in accurate diagnosis.

The shift toward liquid-based preparations for cervical cytology has improved specimen adequacy in some studies but not in others. The distinct advantage of LBP specimens, however, is that the cells are in suspension. This allows the use of high-throughput automatable analysis of cells by instruments such as the flow cytometer.

A study to assess the adequacy of cervical cytology on ThinPrep slides described a SBLB:no ECC rate of 9.36% compared with 9.4% for conventional smears in the feasibility component of the study. In the clinical trial, the reported rate of SBLB:no ECC was 4.96% compared with 4.4% for ThinPrep and conventional smears, respectively (5). In a study of similar size, the CYTORICH LBP and thin-layer system demonstrated a satisfactory rate of 82.5% for CYTORICH monolayers compared with 66.4% for matched conventional smears (4). Although these studies suggest that specimen adequacy in LBP specimens is the same or better compared with conventional smears, a significantly high UNS or SBLB rate still exists. Fortunately, technologies as presented here are readily applicable to cells in suspension and should contribute significantly to improved patient care.

We demonstrate in this study that flow cytometric assessment of LBP samples is easy, rapid, and addresses the specific categories of TBS. We analyzed over 100 samples and set arbitrary cutoffs for the adequacy of each cell type including ectocervical and endocervical cells. Further study and discussions within the field might allow the establishment of the most clinically useful parameters.

Further, the use of light scatter alone obviates problems that may exist if antibody staining were necessary for the determination of specimen adequacy as demonstrated in this study. All four major cell populations were distinctly separated in all preservatives tested. Last, this method may also facilitate the use of immunophenotypic/molecular methods and flow cytometry for cervical cancer screening (14). For example, several methodologies are currently being used to identify the expression of certain human papillomavirus (HPV) genes and cellular proteins associated with cervical dysplasia and cervical cancer (14). The popularity and increased utilization of liquid-based cervical cytology is the ideal setting for future solution-based assays that may augment or even replace slide-based morphologic screening.

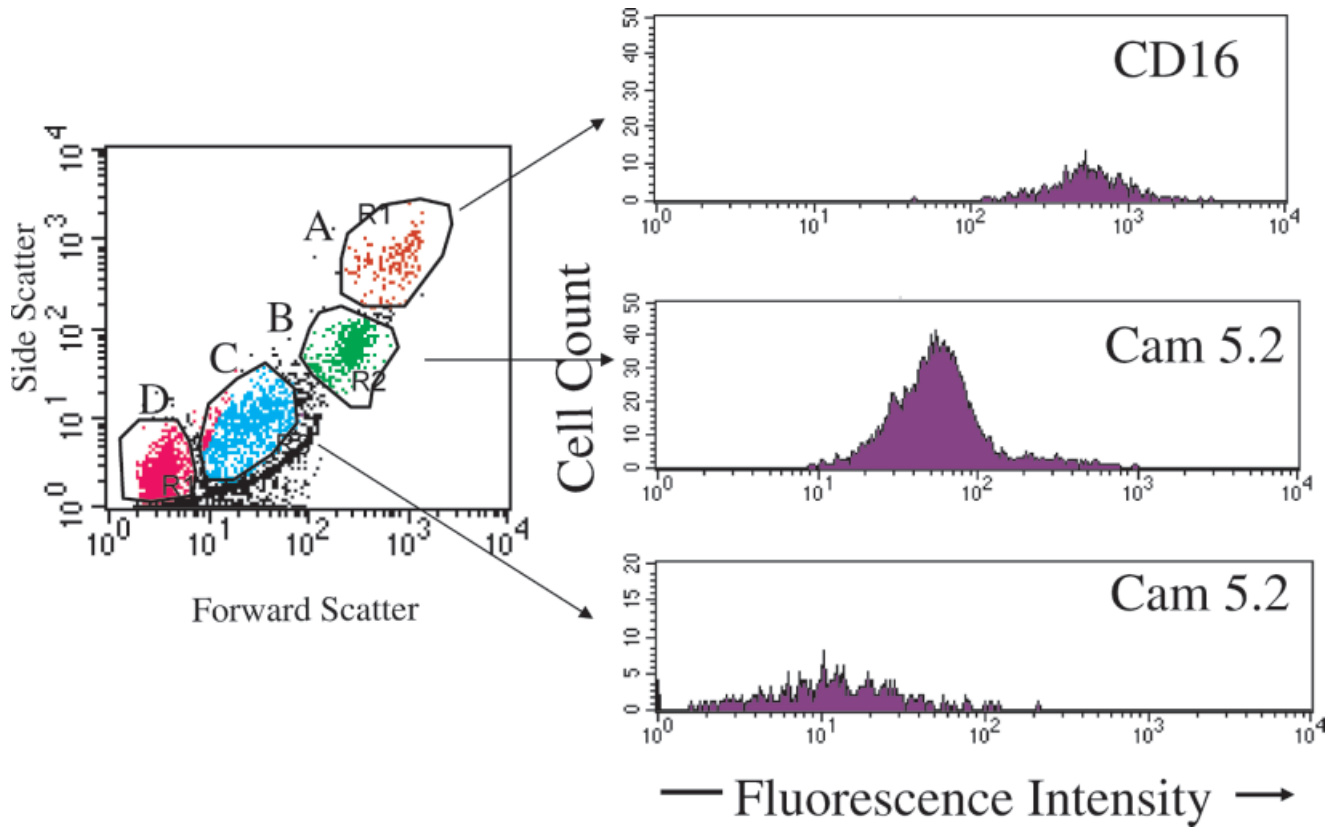


Fig. 2. Confirmation of cells present in cervical LBPs by cellular phenotype. Cells from cervical cytology specimens form four populations using forward and side light scatter (left). Region A was identified as PMNs using CD16 staining, region B was identified as endocervical cells using CAM 5.2 staining, region C was identified as ectocervical cells by the lack of CAM 5.2 staining, and region D was identified as debris by typical light scatter.

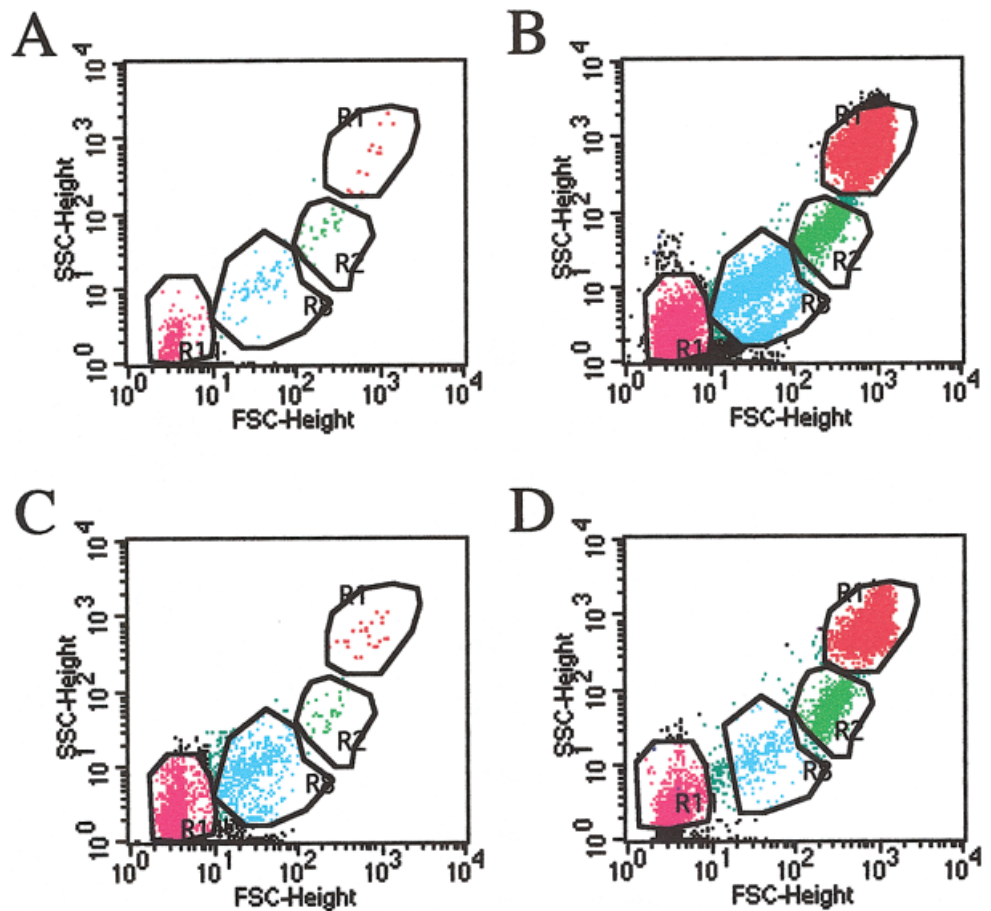


Fig. 3. Representative assessment of specimen adequacy using light scatter. Dot-plots represent **A**: unsatisfactory, **B**: satisfactory but limited by inflammation, **C**: satisfactory but limited by endocervical cells, and **D**: satisfactory but limited by ectocervical cells. Using >20% PMNs (inflammation), <1.0% ectocervical cells, <1.0% endocervical cells, and <500 cells/mL (A) as flow cytometric criteria, light scatter compared with microscopic assessment of specimen adequacy yielded a correlation coefficient of 0.99.

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