JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, July 2011, Vol. 56, No. 4
doi: 10.1111/j.1556-4029.2011.01796.x
Available online at: onlinelibrary.wiley.com

PAPER

CRIMINALISTICS

Kevin W. P. Miller,[1] Ph.D.; Jennifer Old,[2] Ph.D.; Brian R. Fischer,[1] B.S.; Brett Schweers,[2] Ph.D.; Simona Stipinaite,[2] B.S.; and Karl Reich,[2] Ph.D.

Developmental Validation of the SPERM HY-LITER™ Kit for the Identification of Human Spermatozoa in Forensic Samples*,†

ABSTRACT: With sexual assault evidence, the visualization of spermatozoa confirms that ejaculation has occurred. However, microscopic examination of spermatozoa is a laborious process and can sometimes result in sperm cells being overlooked. Here, we present the developmental validation of the SPERM HY-LITER™ kit, which contains a human sperm–specific mouse monoclonal antibody coupled to a fluorescent Alexa 488 dye. The kit was tested using samples of human semen, saliva, blood, and urine, various animal semen extracts, sexual lubricants, and a commercially available spermicidal film. Postcoital vaginal swabs, degraded semen samples, and samples prepared with sample fixation techniques that deviated from the kit-provided protocol were also tested. In each case, the SPERM HY-LITER™ kit was demonstrated to bind only to human sperm cell heads. Limitations to this fluorescent staining procedure include nonspecific staining and increased background fluorescence with extreme heat fixation in some samples.

KEYWORDS: forensic science, forensic serology, spermatozoa, SPERM HY-LITER™, sexual assault, developmental validation

Despite best efforts to reduce the forensic DNA backlog, a large number of unsolved sexual assault cases that contain biological evidence are still left unanalyzed (1). In cases involving sexual assault, spermatozoa are generally the biological material of importance (2,3). The microscopic detection of human spermatozoa in sexual assault evidence (SAE) has long been used as a confirmatory method for the presence of ejaculate (4–13). Confirming the presence of ejaculate assists in corroborating the victim's allegations (4,7,10,12,14) and provides material for generating the alleged suspect's genetic profile through additional DNA analysis (2,3). However, the visualization of spermatozoa can be inhibited by the presence of other cells in the sample (e.g., epithelial, bacterial, etc.; [2,3,10,15,16]), malformation or degradation of the sperm cells themselves (i.e., detachment of the tail from the head; [4,8,9,15–17]), or a low number of spermatozoa in the sample through dilution or azoospermia of the alleged suspect as a result of vasectomy or other related conditions (4,5,7–9,12,14,18–21). As a result, forensic analysts often devote a great deal of time and effort searching for spermatozoa in SAE (16).

Alternatively, examination of SAE for the presence of semen may involve the use of alternate light sources (ALS), and/or the detection of semen-specific markers such as prostatic acid phosphatase (AP), prostate-specific antigen (PSA), seminal vesicle-specific antigen (SVSA), or semenogelin (4,6–9,11,12,14,19–26). Each of these screening techniques, however, has its limitations. Use of ALS does not specifically identify semen, because other biological fluids and several other substances have been shown to fluoresce under these types of light (4,8,22,24,27). Similarly, AP can be detected in other body fluids, including vaginal secretions, and false positives can be obtained in the presence of commonly encountered household materials (4,6–8,11,14,19,21,23,24,28,29). In addition to being detected in seminal fluid, PSA has also been detected at low quantities in male and female urine and even in female tissues in certain disease states (9,11,14,17,18,20,26,30). SVSA can be detected by immunofluorescence using the MHS-5 polyclonal antibody. However, the use of this test has been limited in forensic laboratories because of the fact that the biological activity of SVSA remains little studied (9). Additionally, while SVSA has not been detected in semen from common domestic animals, it is found in semen from higher-order primates (i.e., chimpanzee, gorilla, and orangutan; [7,31]). Semenogelin has also been detected in several body tissues (11). Most recently, semenogelin has been detected in the serum of individuals diagnosed with certain types of cancer (15,32), and ejaculates from some new-world primates (33). For these reasons, seminal-specific markers are generally considered preliminary screening methods for the detection of semen in SAE. Confounding the use of semen-specific markers is their detection in

[1]Forensic Biotechnology Institute of California, California State University-Fresno, 2576 East San Ramon Avenue, M/S ST104, Fresno, CA 93740.
[2]Independent Forensics of Illinois, 500 Waters Edge, Lombard, IL 60148.

*Presented in part at the 19th International Symposium on Human Identification, October 13–16, 2008, in Hollywood, CA.

†The SPERM HY-LITER™ kit was developed and is manufactured by Independent Forensics of Illinois (IFI), a company engaged in the delivery of products and services to the forensic community. The Forensic Biotechnology Institute of California (FBIC) is a nonprofit ancillary unit of the College of Science and Mathematics at the California State University, Fresno. IRB permission was obtained for *all* samples reported in this study and taken by personnel at the California State University, Fresno, for studies conducted by University personnel at the University. Samples collected by personnel at Independent Forensics of Illinois (IFI) and tested at IFI by IFI personnel were not subject to IRB regulation.

Received 5 Feb. 2010; and in revised form 28 April 2010; accepted 22 May 2010.

postcoital samples stored for a long time. Spermatozoa, on the other hand, have been demonstrated to persist for greater periods of time within the vaginal vault and can be detected on postcoital sample swabs at longer time intervals than PSA, AP, and SVSA semen markers (6,18,31,34,35). Therefore, the detection of spermatozoa may be more likely when analyzing backlogged (c)old cases. Consequently, barring cases involving azoospermia, the visual identification of sperm cells themselves is generally accepted to be the most definitive method for the confirmation of the presence of ejaculate in SAE (4–13).

Current methods used to locate and identify spermatozoa in evidentiary samples are largely based on microscopic staining techniques that are not specific to human sperm cells, such as hemaoxylin–eosin (H&E), alkaline fuchsin, and Kernechtrot–Picroindigocarmine (KPIC or "Christmas Tree Stain") (10,16). These staining methods are also not amenable to automation or computer-aided searching because of the low levels of color contrast that are generally achieved between the cell types of interest in these preparations (10). Thus, the forensic analyst must still spend a great deal of time examining microscope slides for the presence of sperm cells after staining is complete. As a significant portion of backlogged case work in forensic biology is related to SAE (1), the effort and expense devoted to the visualization of sperm cells is considerable. SAE that has been stored for long periods (e.g., backlogged sexual assault kits) or collected evidence containing minimal amounts of biological material is particularly problematic (4,8,15,16).

The development of monoclonal antibody-based systems with predefined specificity to definite antigens has greatly advanced our ability to preferentially discriminate between specific markers of interest (36) and, thus, to attribute source to unknown biological stains in forensic casework (7,11,13,15,16,18,19,21,25,26,31,37). Several fluorescent dyes, each with their own ideal performance parameters, have been introduced for use in immunofluorescence (38). Among these dyes is the Alexa class of fluorochromes, which have been shown to be capable of covering the entire visible spectrum while being stable over a broad range of pH (39). In addition to these factors, the Alexa fluorochromes exhibit superior fluorescence capabilities, resistance to photobleaching, and convenient coupling chemistries, making them ideally suited for the detection of particular cell types when coupled to specific monoclonal antibodies (38,39). Previous attempts to employ antibody technologies to the forensic detection of individual sperm cells have shown promise in improving current microscopic staining procedures of SAE. The most recent, SpermPaint, is an immunofluorescent technique that uses two mouse monoclonal antibodies, each coupled to an Alexa 488 dye, to fluorescently label the equatorial segment of the sperm head and the sperm tails within a given sample (16). SpermPaint has not become widely used in the forensic community (16). This is most likely due to the facts that: (i) it is expensive, (ii) the technical procedure is complex, and (iii) the overall visibility of the sperm head is not dramatically improved over traditional staining methods, as only the equatorial segment is stained and the reagents are not commercially available.

SPERM HY-LITER™ (Independent Forensics of Illinois, Lombard, IL) is a novel kit that employs a fluorescently labeled human sperm–specific antibody for the routine microscopic detection of human spermatozoa in forensic SAE. Similar to SpermPaint, the SPERM HY-LITER™ kit contains a proprietary human sperm–specific mouse monoclonal antibody, which was produced *in vivo* using standard immunological methods (40). The antibody is chemically derivatized with a fluorescent Alexa 488 dye so that the entire head of the sperm cells (i.e., acrosome, equatorial segment, and postacrosomal region) will fluoresce when viewed under a microscope that is fitted with a fluorescein isothiocyanate (FITC) filter. Unlike SpermPaint, the SPERM HY-LITER™ kit also incorporates a second 4,6-diamidino-2-phenylindole (DAPI) fluorescent dye for simultaneous viewing of all cell nuclei, regardless of cell type, in the sample using a DAPI compatible fluorescent filter.

The Scientific Working Group on DNA Analysis Methods (SWGDAM) has recommended several guidelines for both developmental and internal validations of DNA analysis methods with the intention of establishing the reliability, robustness, and reproducibility of DNA typing procedures (41). However, SWGDAM has not yet established any guidelines for the validation of serological techniques. Developmental and internal validations of serological techniques are, nonetheless, warranted. In this paper, we attempt to adapt SWGDAM guidelines for the validation of DNA typing methods to serological methods to present a critical evaluation of the SPERM HY-LITER™ kit for use in forensic casework. The kit was tested against several scenarios that could potentially be encountered in SAE to demonstrate that the antibody binds reliably to its intended target.

Materials and Methods

Fluorescent Labeling Reaction Conditions

The protocol for fluorescently labeling sperm cells involved applying a series of four chemical solutions to the sample area of a microscope slide. These chemical solutions, namely *Fixative Solution*, *Sample Preparation Solution*, *Blocking Solution*, and *Staining Solution*, are provided in individual dropper bottles within the SPERM HY-LITER™ kit. Localization of the solution to the sample area was achieved by drawing a ~16-mm-diameter hydrophobic barrier around the sample. A proprietary hydrophobic pen is supplied within the kit for this purpose. The barrier was allowed to air dry completely before further processing. The *Fixative Solution* consists of an aqueous solution of cross-linking fixative agent combined with a detergent to allow permeabilization of the sample cells. Samples were fixed to the slide by applying two drops (~80 μL) of the *Fixative Solution* to the sample area within the hydrophobic barrier. The solution was applied for 10 min at room temperature and then rinsed off. All chemical solutions were removed by rinsing the sample area with 2–3 mL of 1× wash buffer. The 1× wash buffer was prepared by diluting the 10× wash buffer provided within the kit with autoclaved 18-MΩ filter-purified water. After each wash, excess wash buffer was carefully wicked away from the sample area with a clean Kimwipe® (Kimberly-Clark, Hoffman Estates, IL) before continuing on to the next step. The *Sample Preparation Solution* is a slightly alkaline proprietary salt-buffered solution that requires the addition of 1 M dithiothreitol (DTT; DL-Dithiothreitol, Amresco, Solon, OH) prior to its use. DTT is used to reduce disulfide bonds in the sample and is a required component of the staining procedure. For each sample, 1 μL of 1 M DTT was added per two drops of the *Sample Preparation Solution*. The total volume of the solution mixture was adjusted when multiple sample slides were processed together. The *Sample Preparation Solution/*DTT mixture was made fresh in a microcentrifuge tube just before application to the sample area. Approximately 80 μL of the *Sample Preparation Solution/*DTT mixture was applied to each sample area. The mixture was applied to the sample area and incubated for 30 min at room temperature before removing as previously described. After removal, two drops of the *Blocking Solution*, which contains an albumin and DAPI fluorescent dye that stains all nuclei in an appropriate buffer, were then applied to the sample area for 30 min at room temperature.

The solution was removed as previously described. In the final step in the procedure, *Staining Solution* was added to the preparation area for 30 min at room temperature. The *Staining Solution* contains an Alexa 488 antihuman sperm mouse monoclonal antibody complex. Removal of the *Staining Solution* after the allotted time concluded the staining procedure. Stained slides were allowed to air dry completely before microscopic visualization was performed.

Microscopic Visualization

Sample slides were mounted with a glass cover slip (18 mm^2; 0.13–0.16 mm) to prevent cross-contamination between samples. Both the dropper bottle of *Mounting Solution* and glass cover slips that are provided within the SPERM HY-LITER™ kit were used. One drop (~50 μL) of the aqueous-based, semi-hardening *Mounting Solution* was applied to the sample area. A cover slip was then placed over the sample, and gentle pressure was applied to remove air bubbles and excess *Mounting Solution*. The mounted slide was kept undisturbed on a level surface for 20 min to allow the *Mounting Solution* to harden.

Phase-contrast imaging of sample slides was performed using a compound microscope fitted with a reflective light source and a high numerical aperture objective. Absorption and emission wavelengths of the Alexa 488 and DAPI fluorochromes are 494 nm/519 nm (16) and 347 nm/448 nm (42), respectively. Fluorescent imaging was performed using a FITC or DAPI compatible filter to view the desired differentially stained cellular structures. Each sample slide was scanned back and forth in a grid pattern at a final magnification of 200× using the FITC filter until the entire area within the hydrophobic barrier was visualized. Spot checks were performed every 3–5 frames using both phase-contrast and DAPI filters to note what, if any, other cellular structures/types were present. Additionally, phase-contrast imaging of sample slides was performed at a final magnification of 400× when greater detail was needed to identify a cell type. Image capture was performed at 200× magnification using a CCD camera with image capture software. Sequential photomicrographs of identical image frames were taken using phase-contrast, DAPI, and FITC imaging.

Reproducibility

Numerous replicate samples from several different subjects were tested to ensure the reproducibility of our results. The numbers of individuals from which samples were collected, as well as the total number of replicate slide preparations made for each study are listed in Table 1. A notable limitation to the reproducibility study was the small number of animal samples tested in the Alternate Fixation Study. In this study, only one canine semen sample was obtained and tested in triplicate using the alcohol fixation protocol. Additionally, Macaque semen samples were obtained from three subjects but were each tested only once. This was because of the limited number of samples that could be obtained for these species.

Species Study

To determine whether the antibody would cross-react with the sperm of nonhuman species, bovine (*Bos taurus*), canine (*Canis lupus familiaris*), caprine (*Capra aegagrus hircus*), equine (*Equus ferus caballus*), feline (*Felis catus*), murine (*Mus musculus*), ovine (*Ovis aries*), porcine (*Sus domestica*), and simian (*Pan troglodytes*, The University of Texas-MD Anderson Cancer Center) sperm were analyzed. Semen samples were collected from two individuals of

TABLE 1—*The numbers of samples used in each validation study are listed here. Column 2 shows the number of individual subjects that samples were collected from for each portion of a study. The total number of replicate samples performed from those subjects is shown in column 3. Sample numbers were lower in the Alternate Fixation Study, because only one replicate from each of three nonhuman primates was performed for a total of three replicates; and canine samples were collected from only one individual, but replicated three times for a total of three replicate samples performed. Numbers in bold indicate total numbers of sample replicates for the respective study.*

	Number of Sample Subjects Collected	Total Number of Sample Replicates Performed
Species study	–	108
Animal semen	–	–
Bovine	2	12
Canine	2	12
Caprine	2	12
Equine	2	12
Feline	2	12
Murine	2	12
Ovine	2	12
Porcine	2	12
Simian (*Pan troglodytes*)	2	12
Sensitivity (semen/saliva)	–	300
1:1	25 semen samples	75
1:4	5 saliva samples	75
1:8		75
1:10		75
Case-type samples	–	–
Resuspended (SPERM HY-LITER™ slides)	–	42
Precoital vaginal swabs	3	21
Postcoital vaginal swabs	3	21
Smeared (plain glass slides)	–	42
Precoital vaginal swabs	3	21
Postcoital vaginal swabs	3	21
Specificity	–	270
Human	–	–
Blood	15	90
Buccal (saliva)	15	90
Urine	15	90
Mixtures (w/human semen)	–	–
Human semen samples	25	–
Human	–	390
Blood	15	80
Buccal (saliva)	15	230
Urine	15	80
Animal semen	–	108
Bovine	2	12
Canine	2	12
Caprine	2	12
Equine	2	12
Feline	2	12
Murine	2	12
Ovine	2	12
Porcine	2	12
Simian (*Pan troglodytes*)	2	12
KPIC stain interference	–	200
SPERM HY-LITER™ slides	25 semen samples	–
Human buccal/semen mixture	15 saliva samples	100
Plain glass slides		–
Human buccal/semen mixture		100
Alternate fixation	–	234
Alcohol fixation	–	–
Animal semen	–	–
Canine	1	3
Human	–	–
Blood	3	9
Buccal (saliva)	3	9
Urine	3	9
Aged semen	3	11

TABLE 1—*Continued.*

	Number of Sample Subjects Collected	Total Number of Sample Replicates Performed
Mixed w/human semen	–	–
Human semen	6	–
Sexual lubricants	–	–
Body Heat Cinnamon Flavored	1 tube	9
ID® Fresh Peach	1 tube	9
Fusion Body Glide	1 tube	9
Spermicide	1 package	11
Human	–	–
Blood	3	9
Buccal (saliva)	3	11
Urine	3	9
Heat fixation	–	–
Animal semen	–	–
Simian (*Macaca mulatta* and *Macaca fascicularis*)	3	3
Human	–	–
Blood	3	9
Buccal (saliva)	3	9
Urine	3	9
Aged semen	3	11
Mixed w/human semen	–	–
Human semen	6	–
Sexual lubricants	–	–
Body Heat Cinnamon Flavored	1 tube	9
ID® Fresh Peach	1 tube	9
Fusion Body Glide	1 tube	9
Spermicide	1 package	11
Human	–	–
Blood	3	9
Buccal (saliva)	3	11
Urine	3	9

KPIC, Kernechtrot–Picroindigocarmine.

each of the species listed earlier, and several replicate sample slides were prepared for microscopic analysis (see Table 1).

After each ejaculate was collected, replicate swabs were prepared by adding 150 μL of semen directly to the batting of individually wrapped sterile cotton swabs (Fisherbrand* Sterile Plastic Applicators with Cotton Tips; Fisher Scientific, Pittsburgh, PA) and allowing them to air dry in a protective environment. Once the swabs were dried, they were repackaged in their original paper wrappings and stored at room temperature until they could be analyzed. Sperm cells were collected from each swab by incubating the sample swab in 1.0 mL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) for 1 h at room temperature. The cotton battings from each swab were then removed, and the cells were pelleted by brief centrifugation at $18{,}000 \times g$ at room temperature in a tabletop centrifuge. After centrifugation, the supernatant was removed, and the pelleted cells were resuspended in 100 μL of PBS. Twenty microliters of each pellet resuspension was applied to the sample window of a proprietary positively charged SPERM HY-LITER™-masked slide (provided within the kit), and the slides were processed in accordance with the labeling protocol described earlier.

Sensitivity

Sensitivity studies in DNA validation are typically used to define the lowest concentration of DNA that is detectable in a solution of known concentration. This does not apply directly to the microscopic visualization of spermatozoa by immunofluorescence, because the ability of an antibody to attach to its intended target does not depend upon a minimum number of sperm cells within a given sample. For our purposes, it was important to note that a predominant number of nontarget cells in a given sample did not prevent the fluorescent labeling of spermatozoa through some act of physical barrier or greater affinity of the antibody–dye complex. Also, important to note is the fact that the number of sperm cells in a forensic sample will vary from individual to individual and even from one ejaculate to another within a single individual (43). Therefore, we did not feel that it was necessarily meaningful to measure individual sperm numbers within each sample for our studies of sensitivity. However, none of the subjects from whom semen was collected displayed azoospermia. Rather, we defined sensitivity as: (i) the ability of the antibody to always and only recognize human sperm heads in those microscopic fields examined and (ii) the ability of an analyst to consistently visualize a relatively low number of spermatozoa among a relatively high number of nontarget cells in a mixed sample, such as those typically encountered in forensic casework.

For the sensitivity study, semen samples from 25 individuals were obtained either from a local sperm bank or from healthy volunteers, and buccal epithelial cells were obtained from 15 healthy volunteers. Buccal epithelial cells were collected by having subjects gently chew on the insides of their cheeks to release saliva and then spit into sterile 50-mL conical tubes. One hundred and fifty microliters of either saliva or semen was added to individually wrapped sterile cotton swabs. Each swab was air dried, repackaged in its original packaging, and stored at room temperature until analyzed. Cells were eluted from the swabs and pelleted as previously described. Buccal epithelia were then resuspended in 100 μL of PBS, and sperm cells were resuspended in 1 mL of PBS. Sperm cells were resuspended in a larger volume of PBS to produce a dilute sperm cell solution. Mixtures of the human sperm and epithelial cell suspensions were then prepared at ratios of 1:1, 1:4, 1:8, and 1:10 (volume of sperm cell suspension to volume of epithelial cell suspension), respectively, for analysis. Twenty microliters of the mixed cell suspension was then applied to the sample window of a SPERM HY-LITER™-masked slide for each replicate as described in Table 1 and processed in accordance with the above labeling protocol.

Case-Type Samples

To test the kit under conditions commonly encountered with SAE, pre- and postcoital vaginal swabs were analyzed using the developed staining protocol. Pre- and postcoital vaginal swabs were collected from three healthy volunteers. Removal of cellular material from the swabs was performed by incubating the swabs in 1.0 mL of PBS for 1 h at room temperature. The swab heads were then removed, and the cells were pelleted by brief centrifugation at 13,000 rpm. The sample cells were resuspended in 100 μL of PBS. Twenty microliters of the resulting cell suspensions were then applied to the sample window of a SPERM HY-LITER™-masked slide (see Table 1) and processed in accordance with the above staining protocol.

Although proprietary charged microscope slides are provided with the SPERM HY-LITER™ kit, microscope slides contained within SAE kits are typically plain uncharged glass slides. The sample fixation protocol for the SPERM HY-LITER™ kit was optimized with the use of the charged microscope slides supplied within the kit. Therefore, it is important to know the extent to which standard glass microscope slides will retain sample cells

during the various preparation, wash, and staining steps of the labeling procedure. The SPERM HY-LITER™ protocol also includes steps for the removal of cells from the sample collection material (e.g., swab) and resuspension of cells in PBS prior to their application to a microscope slide. Microscope slides prepared as part of a SAE kit, however, are generally prepared as sample smears during the sexual assault examination. As a result, forensic analysts often have to examine smear slides for the presence of spermatozoa in which extraction of the prepared smear is not performed. Additionally, sexual assault examiners who prepare sample smears at the time of their examinations often only have access to standard glass microscope slides. It is important, therefore, to know whether standard glass microscope slides can retain a sample that has been fixed using the protocol described herein. Pre- and postcoital sample swabs were collected from three healthy volunteers and allowed to air dry prior to analysis. Smear slides were made by moistening a previously prepared vaginal swab with PBS and gently smearing the swab onto a clean plain glass microscope slide (Fisherbrand* Plain Glass Microslides; Fisher Scientific; see Table 1). The slides were then allowed to air dry completely, before fixing to the slide and staining with the fluorescently tagged antibody in accordance with the above labeling protocol.

Specificity Study

Human saliva, urine, and blood samples were tested with the SPERM HY-LITER™ kit to determine whether the antibody would label/cross-react with other human body fluids commonly encountered in forensic casework. Individual body fluids were collected from 15 healthy volunteers. Urine samples were collected midstream, and the first elimination from male subjects postejaculation was never collected. Sample swabs were prepared by depositing 150 μL of the collected sample onto a cotton swab. The swabs were allowed to air dry, stored, and pelleted as previously described. The pelleted sample cells were each resuspended in 100 μL of PBS. Twenty microliters of the resuspended sample was transferred to a SPERM HY-LITER™-masked slide (see Table 1) and processed according to the labeling protocol outlined earlier.

Mixture Study

Human sperm cell suspensions were mixed with cell suspensions of human saliva, urine, and blood to test for physical inhibition of the antibody–dye complex as a result of the presence of nontarget cells in the sample. Additionally, these samples were also tested to determine whether nontarget samples would cross-react with the antibody in the presence of human spermatozoa. Nonhuman mammalian sperm cell suspensions of several animal species, including bovine (*Bos taurus*), canine (*Canis lupus familiaris*), caprine (*Capra aegagrus hircus*), equine (*Equus ferus caballus*), feline (*Felis catus*), murine (*Mus musculus*), ovine (*Ovis aries*), porcine (*Sus domestica*), and simian (*Pan troglodytes*) were also tested to determine whether they would cross-react in the presence of human sperm cells. Animal semen samples were obtained from two subjects of each of the species listed. Human saliva, urine, and blood samples were obtained from 15 healthy volunteers. Human semen samples were obtained from 25 healthy volunteers or from a local sperm bank. Cell suspensions were prepared from sample swabs that were previously saturated with 150 μL of the respective sample fluid and allowed to air dry prior to analysis. Sample cells were removed and pelleted as previously described. The pelleted sample cells were each resuspended in 100 μL of PBS. Mixtures were prepared by combining 20 μL of human sperm cell suspension with 20 μL of the respective body fluid cell suspension being tested. Ten microliters of the mixed cell suspension was transferred to the sample window of a SPERM HY-LITER™-masked slide (see Table 1) and processed in accordance with the above labeling protocol.

KPIC Stain Interference Study

Kernechtrot–Picroindigocarmine is a staining method that is widely utilized by forensic laboratories for the microscopic identification of human spermatozoa in SAE. Therefore, smear slides that had been previously stained with KPIC were tested for their ability to be restained and reanalyzed using the SPERM HY-LITER™ protocol developed herein. Both plain glass microscope slides and microscope slides provided within the SPERM HY-LITER™ kit were used. Semen samples were obtained from 25 individuals from a local sperm bank or from healthy volunteers, and buccal swabs were obtained from 15 healthy volunteers. Swabs were prepared from all body fluids, and cells were isolated as previously described. The sample cells were each resuspended in 100 μL of PBS. Twenty-microliter aliquots of both the sperm and epithelial cell suspensions were combined to form a cell mixture, and 10 μL of the mixture was applied to both a clean glass microscope slide (Fisherbrand* Plain Glass Microslides; Fisher Scientific) and to a SPERM HY-LITER™-masked slide. Both slides were stained with KPIC using commercially available reagents (Xmas Tree Stain, SERI) according to the manufacturer's protocol (see Table 1). However, water was substituted for ethanol in the final wash step. Stained slides were examined for the presence of both sperm and epithelial cells using a standard compound microscope. The KPIC-stained slides were examined to confirm that the appropriate cell types stained differentially: red for sperm cells and green for epithelial cells. Once KPIC-staining was confirmed, each microscope slide was further processed using the SPERM HY-LITER™ kit according to the labeling protocol described earlier.

Alternate Fixation Study

Proper fixation of the sample to the microscope slide is an essential step in the SPERM HY-LITER™ procedure. This is because of the fact that the multiple wash steps involved in the staining protocol can wash away improperly fixed cells. The *Fixation Solution* provided in the kit was designed for use with the SPERM HY-LITER™-masked slides at room temperature. Some forensic laboratories, however, use a variety of alternate fixation protocols, some of which involve the addition of heat to the slide. Therefore, it was necessary to test if alternate slide fixation procedures, and in particular heat fixation, did not interfere with the SPERM HY-LITER™ protocol. To do this, several heat- or alcohol-fixed sample slides were prepared for analysis. Canine semen, simian (*Macaca mulatta* and *Macaca fascicularis*) semen, human blood, saliva, urine, and aged human semen samples (semen from three healthy volunteers stored for approximately 7–12 months postejaculate at 4 ± 1°C) were tested individually. Additionally, 200 μL-aliquots of human semen samples were mixed with 200-μL aliquots (100 μL of human blood) of human blood, saliva, or urine, and three commercially available sexual lubricants (Body Heat Cinnamon Flavored; ID® Fresh Peach; or Fusion Body Glide), or a commercially available spermicidal film (VCF® Vaginal Contraceptive Film®, Apothecus Pharmaceutical Corp., Oyster Bay, NY; nonoynol-9, 28%) made into a solution, for analysis. A solution of the spermicidal film was made by dissolving one film square in 25 mL of 18-MΩ filter-purified water that was warmed

to ~37 ± 1°C. Ten microliters of the individual samples or prepared sample mixtures were plated on ProbeOn™ Plus positively charged microscope slides (Fisherbrand ProbeOn™ Plus, Fisher Biotech, Pittsburgh, PA; see Table 1). It should be noted that the composition of charged microscope slides from both Independent Forensics and Fisher Scientific are proprietary, so neither firm would disclose formulation. However, both firms indicated that the action of the slide was much like that of poly-lysine coatings. ProbeOn™ Plus slides were used exclusively for this portion of the study. Samples were smeared onto the microscope slides and allowed to air dry completely before performing the fixation procedure. Human semen was collected from six healthy volunteers, and human blood, saliva, and urine samples were collected from three different volunteers. Sexual lubricant and spermicidal solutions were prepared from individual retail trial-size packages of each material described earlier. For this portion of the study, all canine semen samples were replicates of an ejaculate from a single subject. Owing to the small sample quantities, canine semen was only alcohol fixed. Macaque semen used in this study was donated by investigators at the California National Primate Research Center (CNPRC) at the University of California, Davis. The samples obtained came from the ejaculates of three separate subjects, but the quantity of sample prohibited the preparation of replicate slides. All macaque semen samples were obtained preheat fixed to plain glass microscope slides. Heat fixation of macaque slides was carried out at the CNPRC facility as a precautionary measure against the possible spread of hepatitis that may have been present in the primate semen samples at the time of sampling. While it is known that a heating block was used, the exact heat fixation protocol is unknown.

The alcohol fixation procedure consisted of first suspending the microscope slide in 95% methanol at −20°C for 25 min and then in reagent-grade acetone at −20°C for 15 min. This was carried out in separate, sterile 50-mL conical tubes to ensure that there was no cross-contamination from sample to sample. Samples were allowed to air dry completely after incubation in each solution. Once completely dried, a hydrophobic barrier was drawn around the sample area of each slide, and the fluorescent labeling procedure was performed. Heat fixation was achieved by gently waving the air-dried slide (sample side up) over a small Bunsen burner flame several times, with care given to prevent discoloration of the sample by excessive heating. As soon as each slide cooled, a hydrophobic barrier was applied, and the fluorescent staining procedure was performed. Processed slides were visualized using phase-contrast and FITC imaging.

Results

Species Study

The Alexa 488-antibody–dye complex showed no apparent affinity for the nonhuman mammalian sperm cells tested. All nonhuman SPERM HY-LITER™-stained ejaculate samples (i.e., bovine, canine, caprine, equine, feline, murine, ovine, porcine, and simian [*Pan troglodytes*]) consistently failed to reveal any fluorescent signal. Notably, the antibody complex did not cross-react with spermatozoa from the common chimpanzee (*Pan troglodytes*). Proper application of the SPERM HY-LITER™ reagents was confirmed through microscopic visualization of cells using a DAPI filter. All cells that contained nuclei properly stained with the DAPI fluorochromes, which consistently appeared bright fluorescent blue in color. Phase-contrast imaging of identical image frames confirmed that DAPI staining correlated with cell nuclei within the sample slide. Alexa 488 dye fluorescence was not observed in any of the replicate nonhuman mammalian sample slides.

Sensitivity Study

Using the FITC fluorescent filter, stained slide preparations of sperm and epithelial cell dilutions (i.e., 1:1, 1:4, 1:8, and 1:10, volume of sperm cell suspension to volume of epithelial cell suspension) visibly demonstrated strong Alexa 488 fluorescent signals from human spermatozoa in the sample. Clear fluorescent signals, bounded by each sperm head, were observed. The acrosome, equatorial segment, and the postacrosomal region of the sperm cell head each appeared fluorescent green in color as a result of being stained with the antibody–dye complex. Epithelial cells from both the oral and vaginal cavities are of the simple squamous type. Therefore, all human epithelium used in these studies was simple squamous epithelium. Buccal cells were often used instead of vaginal cells, because they were easier to obtain and more readily available than vaginal epithelium. Regardless of the origin of the simple squamous epithelium used, neither buccal nor vaginal cells ever fluoresced when following the developed SPERM HY-LITER™ protocol. Moreover, epithelial cells in the sample mixtures did not physically prevent the proper staining of sperm cells. The Alexa 488 fluorescent signal also allowed viewing of sperm cells that were visually obscured by epithelial cells when using phase-contrast or DAPI imaging. An example of this can be seen in Fig. 1, which shows side-by-side comparisons of the phase-contrast, DAPI, and FITC images of a 1:10 sperm-to-epithelial cell mixture. Affinity of the antibody complex was only observed toward human sperm cells within the sample mixture. Affinity of the antibody toward nontarget cells was not observed in any of the replicate samples analyzed. Figure 1*A* demonstrates that the visualization of the sperm cells using phase-contrast imaging was often difficult. DAPI fluorochromes successfully stained both epithelial and sperm cell nuclei within the sample mixtures, as can be seen in Fig. 1*B*, but individualization of sperm cells remained difficult. By contrast, visualization under FITC revealed brightly displayed sperm cells only without cross-reaction of the antibody with any epithelial cell components (see Fig. 1*C*).

Reproducibility Study

Numerous replicate samples were processed and analyzed using the SPERM HY-LITER™ kit for each of the sample types prepared for the various studies discussed herein (see Table 1). Unstained human spermatozoa were not observed in any of the SPERM HY-LITER™-stained replicate samples tested in the Sensitivity, Case-Type Samples, Mixture, KPIC Stain Interference, or Alternate Fixation studies. This includes the replicate samples prepared using aged semen samples, semen samples mixed with spermicide, and semen samples mixed with sexual lubricants performed in the Alternate Fixation study. An exception to this was seen along the border of the hydrophobic barrier. Human spermatozoa caught under the hydrophobic barrier or in close proximity (<1 mm) to it did not stain in any of the sample replicates. Spermatozoa from all of the nonhuman mammalian species tested (i.e., bovine, canine, caprine, equine, feline, murine, ovine, porcine, and simian [*Pan troglodytes, Macaca mulatta,* and *Macaca fascicularis*]) did not display an Alexa 488 fluorescent signal when observed in any of the replicate sample slides prepared in the Species, Mixture, and Alternate Fixation studies. Similarly, FITC fluorescent signals were not observed from nontarget cells found in human blood, urine, saliva, or vaginal secretions in the replicate sample

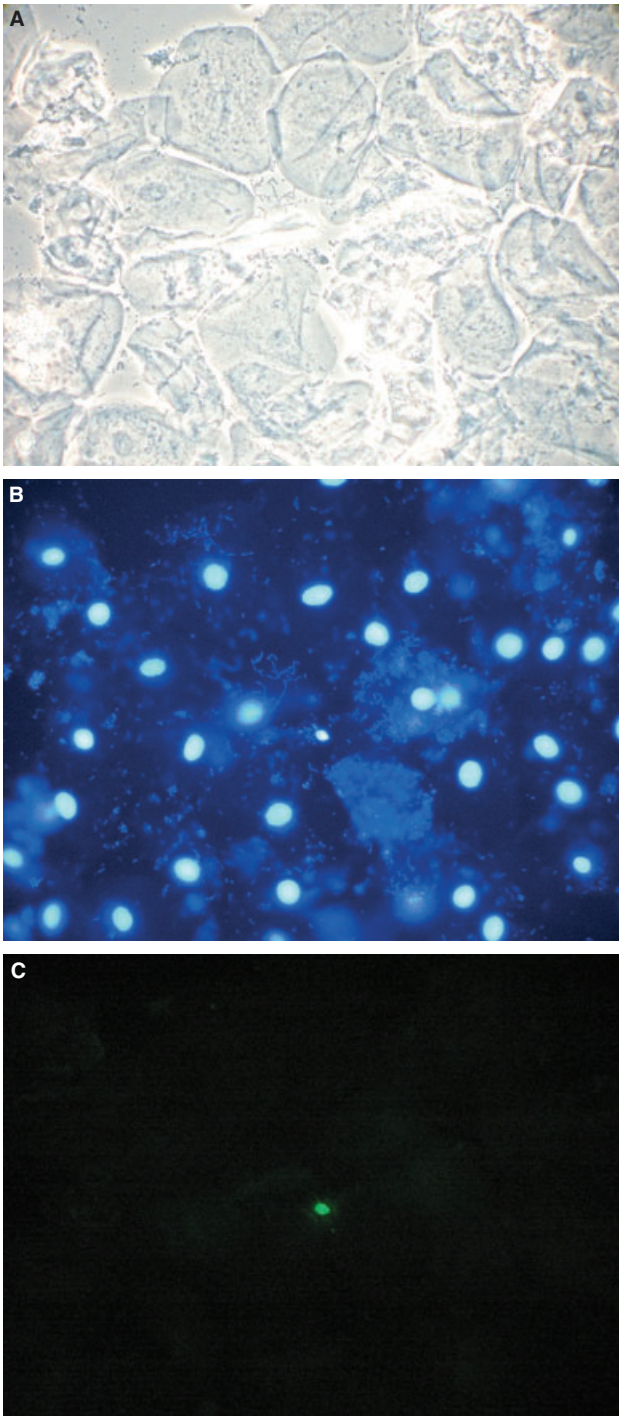

FIG. 1—*Single sperm cell (note center of photograph) among a vast excess of human epithelial cells can be extremely difficult to detect when visualized using phase-contrast imaging (A). When visualized under a DAPI filter, nuclei of both epithelial and sperm cells can be detected (B); however, sperm cells are still difficult to distinguish. However, only the single human sperm cell head is visualized under the FITC filter (C). Cross-reactivity or physical inhibition of the SPERM HY-LITER™ antibody was not seen with nontarget cell types. 200× magnification.*

slides prepared for the Sensitivity, Case-Type Samples, Specificity, Mixture, KPIC Stain Interference, or Alternate Fixation studies. The notable exception to this is that nontarget cells from human blood, urine, and saliva did cross-react when heat fixed in the Alternate Fixation study. In these heat-fixed samples, staining was completely nonspecific and was observed in all nontarget cells. Visualization of stained human sperm cells in the heat-fixed sexual lubricant and human sperm cell mixture samples performed in the Alternate Fixation study was uniformly difficult because of very high background fluorescence. However, sperm cells were completely obscured in only one of the replicate slides in the Alternate Fixation study. Partial visualization was still possible in the remaining replicates. Otherwise, background fluorescence was never an issue for any sample slide in any of the studies conducted.

Case-Type Samples Study

Human spermatozoa were not observed in any of the precoital vaginal swabs using phase-contrast, DAPI, or FITC visualization techniques. Visualization of the precoital vaginal swabs with a DAPI filter confirmed that the samples were properly stained using the SPERM HY-LITER™ kit, as epithelial cells were stained with the DAPI fluorochrome. Human sperm cells from the postcoital vaginal swabs were identified via phase-contrast and DAPI imaging and were routinely and easily identified using the FITC filter. In contrast, vaginal epithelial cells from the postcoital vaginal swabs did not fluoresce and were, therefore, not identifiable when viewed using the FITC filter. The Alexa 488-antibody complex showed no apparent affinity for the vaginal epithelial cells in either the pre- or postcoital vaginal swabs (images not shown). Additionally, the epithelial cells did not physically prevent staining of the spermatozoa.

Identical results were obtained when pre- and postcoital vaginal swabs were smeared on plain glass microscope slides without prior cell removal from the sample swab. Spermatozoa were not observed in any precoital vaginal smears using phase-contrast, DAPI, or FITC imaging techniques. However, proper staining of precoital sample slides was confirmed by the visualization of DAPI-stained vaginal epithelial cells. In contrast, spermatozoa were observed in all postcoital vaginal swab samples under phase-contrast, DAPI, and FITC filters. Correlation of Alexa 488 fluorescent signals to sperm heads was confirmed using the phase-contrast and DAPI filters. The use of plain glass microscope slides for the visualization of SAE-type samples required that extra care be used during the wash steps to avoid sample loss. This involved using a dropper rather than a standard wash bottle to apply a reduced volume of wash buffer (1–2 mL) with a less forceful flow between applications of the chemical solutions. In each case, the use of plain glass microscope slides did not interfere with or prevent proper staining of the spermatozoa. Additionally, prior cell removal from the cotton swab did not prevent sufficient sample fixation to the slide. The results described here were consistent for all the pre- and postcoital sample replicates (images not shown).

Specificity Study

The antibody used in the SPERM HY-LITER™ kit did not cross-react with the blood, saliva, or urine samples that were tested. No fluorescent signals were observed when the complete sample area of each of these body fluids was scanned using the FITC filter. However, the cell types appropriate to each body fluid (i.e., epithelial, erythrocytes, leukocytes) were observed in the sample slides using phase-contrast imaging. Likewise, those cells exhibiting chromatin material (i.e., epithelial, leukocytes, and rarely, an immature erythrocyte) fluoresced when DAPI filters were used. Spermatozoa were not observed in any of the tested body fluid samples, most notably urine, when viewed using phase-contrast, DAPI, or FITC filters. Sample results discussed here were observed in all replicate body fluid samples tested (images not shown).

Mixture Study

When mixed with human spermatozoa, cell suspensions from human saliva, urine, and blood did not physically prevent the SPERM HY-LITER™ antibody–dye complex from binding to human sperm cells. Additionally, cross-reaction of the antibody with the nontarget cells was not observed in the presence of human spermatozoa. Figure 2 shows the side-by-side comparison of the phase-contrast, DAPI, and FITC images of a human semen and saliva cell suspension mixture. In the figure, a single uncovered sperm cell can be clearly seen in the center of the phase-contrast, DAPI, and FITC images. The phase-contrast image of Fig. 2 also reveals a single sperm cell in the lower left of the frame that is completely covered by an epithelial cell. Despite being completely masked, the covered sperm cell is properly labeled with both the DAPI and FITC stains (Fig. 2*B,C*, respectively). Similar results were observed in each of the cell sample mixtures tested. Using the FITC filter, Alexa 488–stained human sperm cells were seen in the presence of unstained nontarget cells from human blood, urine, and saliva.

The nonhuman mammalian semen samples tested (i.e., bovine [*Bos Taurus*], canine [*Canis lupus familiaris*], caprine [*Capra aegagrus hircus*], equine [*Equus ferus caballus*], feline [*Felis catus*], murine [*Mus musculus*], ovine [*Ovis aries*], porcine [*Sus domestica*], and simian [*Pan troglodytes*]) did not cross-react with the SPERM HY-LITER™ antibody in the presence of human spermatozoa. Most notably, sperm cells from the common chimpanzee (*Pan troglodytes*) were not stained with the Alexa 488-antibody complex. This can be seen in Fig. 3, which demonstrates the side-by-side comparison of the phase-contrast, dual DAPI/FITC, and FITC images of a human semen and common chimpanzee semen mixture sample. Figure 3*A* shows the phase-contrast image of several sperm cells of similar morphology scattered throughout the frame. Comparison of the phase-contrast and dual DAPI/FITC images demonstrates that the sample slide was properly stained with the SPERM HY-LITER™ kit, as all sperm cell nuclei in the sample are labeled with the DAPI stain. Furthermore, it shows that human spermatozoa can be identified by both blue and green fluorescence, while chimpanzee spermatozoa can only be identified by blue fluorescence. Lastly, visualization of only the FITC image reveals the human sperm cells in the sample that were exclusively labeled with the Alexa 488-antibody complex. Identical sample results could be seen from replicate sample mixture slides of each of the mammalian species tested with each of the filters used.

KPIC Stain Interference Study

Semen and epithelial cell mixtures were successfully fixed and KPIC stained on both SPERM HY-LITER™-masked slides and plain glass microscope slides. The previously KPIC-stained sample slides were then stained using the SPERM HY-LITER™ kit. This was carried out to see whether previously KPIC-stained slides could be restained and reanalyzed using the dyes from the kit. The KPIC stain was consistently removed by the SPERM HY-LITER™ staining procedure. Consequently, KPIC staining did not result in a visible color change of the Alexa 488 and DAPI stains or prevent the dyes from properly staining the cells in the sample. Human sperm cells in the mixture samples were properly stained with the Alexa 488-antibody complex and identified using the FITC filter. Both the sperm and epithelial cells were labeled with the DAPI fluorochromes. Correlation of the cell type to the observed dye was confirmed by comparing the FITC and DAPI photomicrographs with phase-contrast imaging. Identical results were obtained whether plain glass or charged microscope slides were used. Additionally, samples were not lost from either the SPERM HY-LITER™-masked slides or plain glass microscope slides after the second staining procedure. These results were obtained for all the replicate sample slides tested (images not shown).

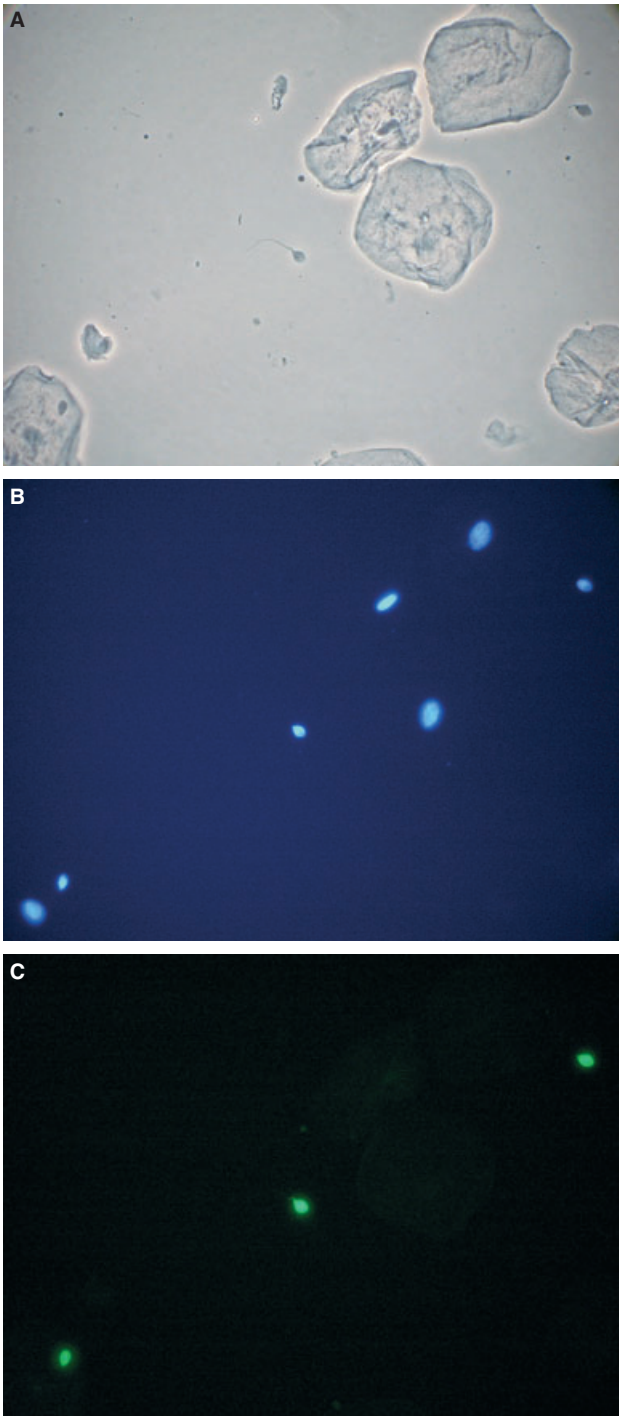

FIG. 2—*Mixed human epithelial and sperm cells, such as that observed in sexual assault evidence, may be visualized under phase-contrast imaging (A); however, sperm heads may be masked by the much larger epithelia (note lower left of photograph). When visualized under a DAPI filter, nuclei of both epithelial and sperm cells are visible (B). Note that sperm heads previously masked by epithelia are now visible using the DAPI filter (lower left of photograph). Only human sperm cell heads are able to be visualized under a FITC filter (C). Photographs taken under 200× magnification.*

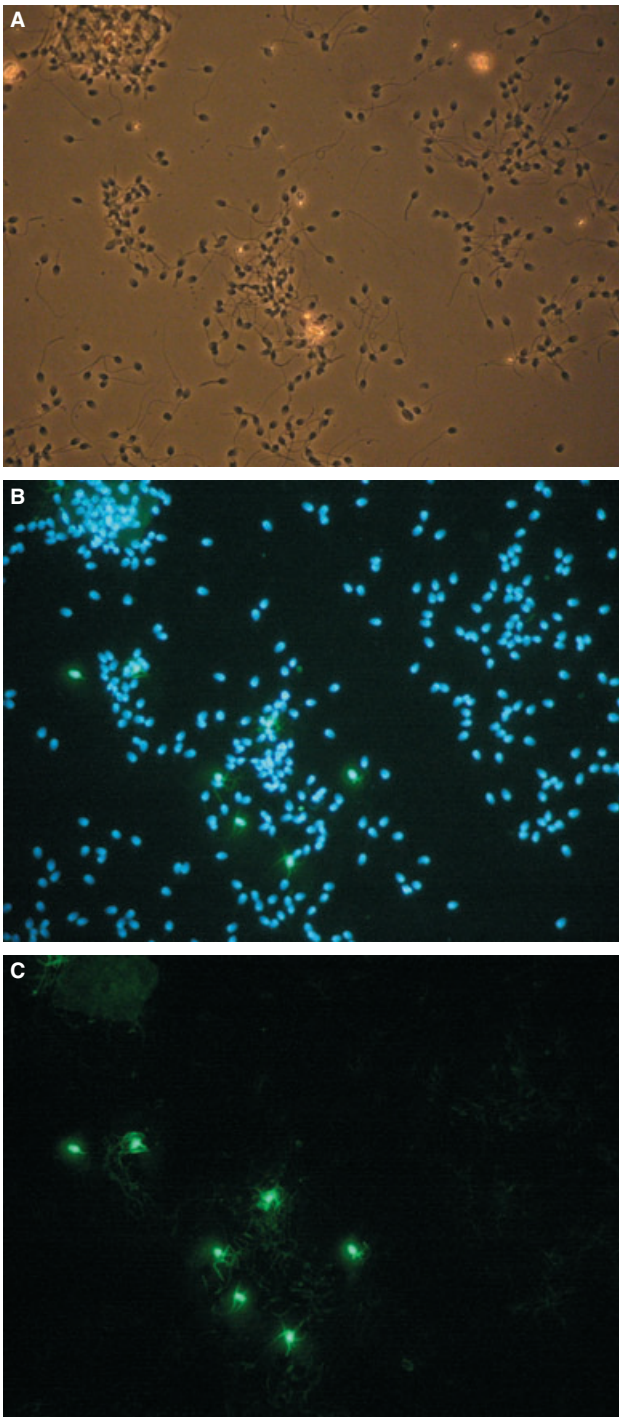

FIG. 3—*With human and common chimpanzee (*Pan troglodytes*) semen mixtures, individual sperm cells from each species are almost indistinguishable from one another under phase-contrast imaging because of their similar morphology (A). Nuclei of both human and chimpanzee sperm cells are visible under DAPI and FITC filters, but blue-green coloration is only visible with human sperm cells because the Alexa 488 dye only stains human cells (B). When visualized exclusively under a FITC filter, only human sperm cells are visible (C). SPERM HY-LITER™ did not label nonhuman primate spermatozoa. All images taken at 200× magnification.*

Alternate Fixation Study

The alcohol fixation procedure did not result in nontarget staining of the SPERM HY-LITER™ antibody toward canine sperm cells, or human saliva, urine, and blood cell suspensions. These results were consistent for both the neat samples and samples mixed with human semen. Alcohol-fixed aged human semen samples and human semen samples mixed with spermicidal solution displayed Alexa 488 fluorescent signals comparable to fresh ejaculate semen samples. Refrigeration of the aged semen samples was typical of standard SAE storage conditions encountered in forensic laboratories. Our results demonstrate that the SPERM HY-LITER™ kit is capable of detecting human sperm cells in semen samples subjected to standard storage conditions for up to 1 year. Alcohol-fixed semen samples that were mixed with sexual lubricants were also successfully stained with the antibody–dye complex. However, excess unbound Alexa 488 dye was observed in close proximity to the sperm heads of sample mixtures containing sexual lubricants (images not shown). This was likely due to excess dye that remained trapped in the lubricant on the slide as a result of incomplete washing during the rinse procedure. Regardless, the excess dye did not interfere with sperm cell staining or visualization. While the alcohol fixation procedure was capable of fixing the prepared sample smears to the ProbeOn™ Plus microscope slides, care was needed to ensure that cells were not lost from the slide as a result of the staining procedure. In our hands, this was primarily an issue with samples containing blood or sexual lubricants. To avoid sample loss, smaller volumes of wash buffer (1–2 mL) were rinsed over the sample using a dropper instead of a standard wash bottle. The sample results discussed here were identical for the replicate sample slides tested.

Conversely, nonspecific antibody staining was often observed using the SPERM HY-LITER™ kit on microscope slides that were heat fixed using a Bunsen burner. Nontarget cells (i.e., epithelial, erythrocytes, and leukocytes) from human blood, urine, and saliva cell suspensions were stained at similar intensities to human sperm cells that were also stained in the sample mixtures. These nontarget cells were also stained with the Alexa 488 dye in samples without human spermatozoa. In the presence of spermatozoa, visualization of sperm cells was inhibited because of the fluorescence of other nontarget cells in the sample. Complete inhibition of the detection of sperm cells was predominantly seen in human blood and semen mixture samples. An example of the antibody's nonspecific staining of human blood can be seen in the phase-contrast, FITC, and field overlay images shown in Fig. 4. The fluorescent signals from the nontarget cells in the sample mixture were of similar intensity to those observed from human sperm cells. Consequently, identification of sperm cells in these samples was difficult. An exception to this observation was seen in heat-fixed slides containing simian (*Macaca mulatta* and *Macaca fascicularis*) sperm cells. The heat-fixed nonhuman primate sperm cells did not fluoresce after being stained with the Alexa 488-antibody complex. This may be because of the use of a lower temperature heating block to fix the samples, whereas other samples in the study were heat fixed with a high temperature Bunsen burner flame. Effects of temperature variations on the SPERM HY-LITER™ kit were not further explored in this study. Heat-fixed samples containing sexual lubricants also showed an increased level of background fluorescence that significantly hindered the identification of spermatozoa. This apparent retention of excess dye in the background of the heat-fixed samples was comparable to that seen in the alcohol-fixed lubricant samples. However, in the alcohol-fixed samples, the effect was contained to the area immediately surrounding the sperm heads and did not interfere with sperm cell visualization. The excess dye throughout the slide field in the heat-fixed lubricant sample preparations significantly limited the viewer's ability to identify human sperm cells. This effect was the most pronounced in the ID® Fresh Peach lubricant samples,

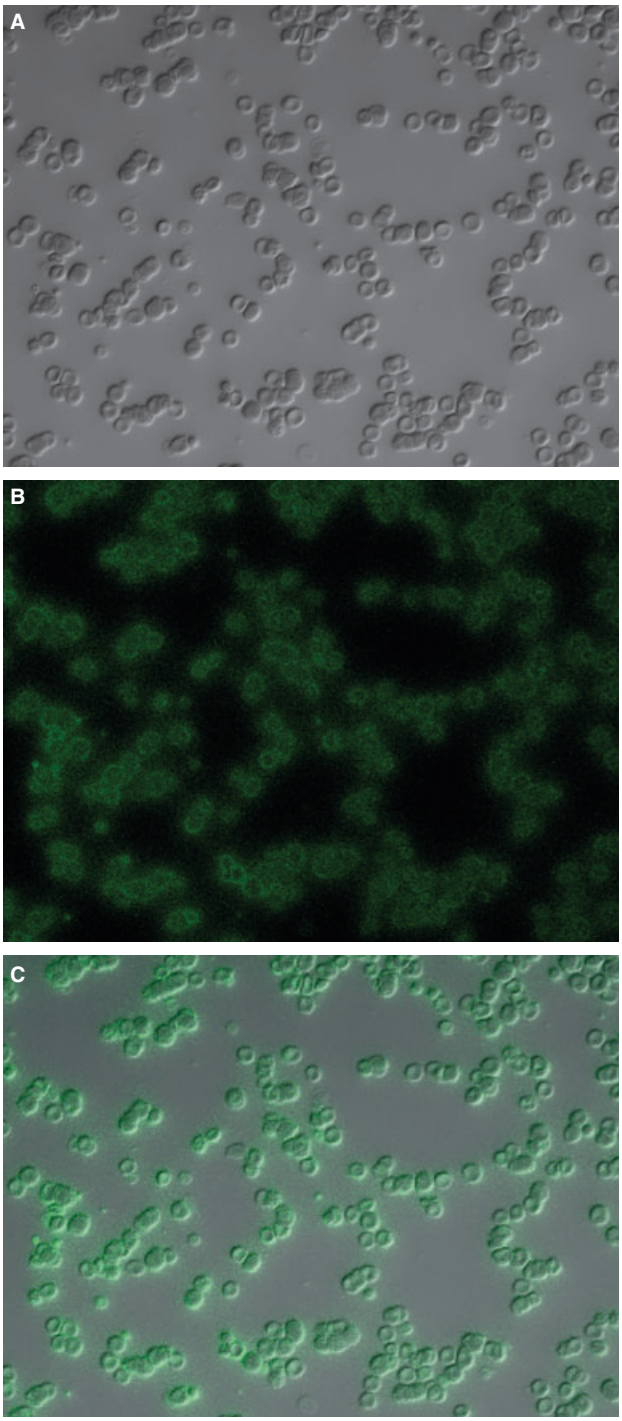

FIG. 4—*Heat fixation of slide samples with a Bunsen burner prior to the SPERM HY-LITER™ staining procedure resulted in the labeling of nontarget cell types. Visualization of phase-contrast images reveals blood cells in the sample (A). Using an FITC filter reveals the cells stained in the procedure with the antibody–dye complex (B). An overlay comparison of the phase-contrast and FITC images reveals the correlation of the antibody with the stained blood cells (C). The SPERM HY-LITER™ kit loses its specificity when applied to samples fixed with extreme heat. Photographs taken at 200× magnification.*

which was the most viscous of the sexual lubricants tested. Figure 5 shows the phase-contrast, FITC, and field overlay images of an ID® Fresh Peach lubricant sample mixed with human semen. Despite the limitations observed from heat fixation, fluorescent labeling of aged human semen samples and human semen samples mixed with spermicide was not inhibited. Both sample types were stained with similar intensity to fresh semen sample ejaculates that were fixed according to the SPERM HY-LITER™ protocol.

Discussion

The purpose of this study was to critically evaluate the performance of the SPERM HY-LITER™ kit for forensic use, particularly with SAE. As far as we are aware, this is the first commercially available confirmatory test for the visual identification of human spermatozoa that does not rely on morphological characteristics or nonspecific staining to positively identify human sperm heads. This statement is made with the understanding that SpermPaint is not commercially available for routine use in forensic laboratories.

Over the years, several studies have been performed in an attempt to estimate an optimal time frame for detection of seminal markers from postcoital vaginal swabs. From these studies, a number of different factors have been shown to affect time range estimates of detection (6). However, it is generally agreed that spermatozoa will, on average, persist longer than other seminal fluid markers (6,18,31,34,35). The detection of seminal fluid markers such as PSA and SVSA at high concentrations is generally considered indicative of the presence of semen in the absence of spermatozoa (11–14,31). However, results indicating low concentrations of the respective marker, such as in degraded or (c)old case samples, can confound the analyst's interpretation of the presence of semen on evidentiary items. This is because of the fact that the seminal markers most often tested are found at low concentrations in other sources (7,9,11,14,17,18,20,26,30,31). As a result, they are not completely specific to human semen. Conversely, the detection of spermatozoa in SAE is still widely considered confirmatory for the presence of semen (4–13). Therefore, the identification of spermatozoa, and in particular sperm heads, in unknown biological stains is the most direct method of confirming the presence of semen on the item in question. The results of this study show that microscopic visualization of human sperm heads in the presence of other body fluids and commonly encountered SAE scenarios is greatly improved using SPERM HY-LITER™ over traditional staining methods. Our studies also demonstrate that the Alexa-bound antibody supplied within the kit specifically stains human sperm heads when the staining protocol described herein is followed. Additionally, although slides in this study were viewed immediately, Alexa 488 fluorescent signal intensity was not observed to decrease when the same slides were viewed up to a week later. In fact, our ongoing work (not reported here) indicates that samples remain fluorescent for up to 2 years after staining.

While further sample testing of semen from greater and lesser nonhuman primates is desirable, our studies showed that the SPERM HY-LITER™ antibody does not cross-react with semen from the common chimpanzee (*Pan troglodytes),* two types of macaques (*Macaca mulatta* and *Macaca fascicularis*), and several other mammalian species (i.e., *Bos Taurus, Canis lupus familiaris*, *Capra aegagrus hircus*, *Equus ferus caballus*, *Felis catus*, *Mus musculus*, *Ovis aries*, and *Sus domestica*). This is in contrast to other seminal markers that show cross-reactivity (7,31,33). Additionally, other nontarget cells found in human blood, urine, saliva, or vaginal secretions were not stained with the Alexa 488-antibody complex. These results indicate that the antibody is specific for human sperm cells and does not cross-react with the semen from common species or with human tissues likely to be encountered in SAE. Perhaps the most beneficial characteristic of the SPERM

HY-LITER™ kit observed from our sample mixture experiments was the ability of the fluorescent antibody to stain sperm cells that were completely obscured by other nontarget cell types. The most practical advantage of this is that analysts can quickly detect sperm cells in the presence of other nontarget cell types, even when these cells greatly outnumber and/or obscure the numbers of spermatozoa that might otherwise remain undetected through the use of current nonspecific staining techniques. Therefore, the SPERM HY-LITER™ kit has the potential to greatly reduce the amount of time necessary to analyze a SAE sample slide, and thus increase overall sample throughput.

Another advantage of the SPERM HY-LITER™ kit is its ability to fluorescently label sperm cells in SAE that had been previously analyzed using more traditional staining methods such as KPIC. The KPIC stain did not prevent antibody staining or visually alter the color of the fluorescent dyes used in the SPERM HY-LITER™ kit. However, photodocumentation of KPIC-stained slides should be completed before the slide is processed with the SPERM HY-LITER™ protocol, because the KPIC stain is removed as a result of additional staining. The results of our studies show that the developed fluorescent antibody–dye complex is much more specific for human spermatozoa than traditional sperm-staining methods. Staining procedures such as KPIC or H&E staining only create a color contrast for slightly easier viewing (10). Fluorescence is a defining feature that allows for rapid, easy, and automatable identification of sperm cells. Fluorescent sperm heads are easily visible with negligible background staining under ''normal'' conditions and sufficient sensitivity to identify single sperm heads among much larger numbers of nontarget cells.

This work also demonstrated that the antibody-binding sites on the sperm head remained intact despite the fact that some sperm cells were subjected to prolonged storage for up to 1 year, alcohol fixation, and exposure to commercial spermicides such as nonoxynol-9. While all of these scenarios have the potential to damage the sperm cell, nonoxynol-9 is specifically designed to dissolve the lipid components of the cell membrane (44). Despite these damaging effects, the antibody was still able to attach successfully to the acrosome, equatorial segment, and postacrosomal region of the sperm heads without loss of specificity. Given the large backlog of unsolved SAE cases in storage (1), the ability of the SPERM HY-LITER™ kit to function on significantly aged samples of unknown condition should greatly benefit future analysis of (c)old cases.

The mechanism for the loss of antibody specificity with heat-fixed samples was not explored. However, it is reasonable to assume that proteins to which the antibody binds on the surface of the sperm head are denatured with increased temperatures. While the effective temperature range of the antibody in the SPERM HY-LITER™ kit was not tested, serological stains have been observed to remain immunologically reactive after exposure to temperatures as high as 68°C (5). Our studies showed that extreme temperatures will affect the specificity of the antibody used in the kit. Despite nontarget cell fluorescence, visualization of individual sperm cells in the sample mixtures was still possible. However, this visualization was largely because of morphological differences observed between sperm cells and other nontarget cell types (i.e., epithelial cells). Of the sample mixtures tested, blood mixtures displayed the most drastic interference because of the amount of nontarget cells present and similar morphology of red blood cells and sperm cell heads. The nonhuman primate sperm cells were the only heat-fixed cell type that did not fluoresce in a similar fashion to the labeled human sperm cells. The fixation protocol used by the CNPRC employed a heating block rather than a Bunsen burner, so fixation temperatures were likely much lower in the nonhuman primate samples. A Bunsen burner was used in this study to demonstrate the effects of over heat fixing. However, further testing of more controlled heat fixation protocols is warranted for those laboratories wishing to deviate from the fixation protocols that are

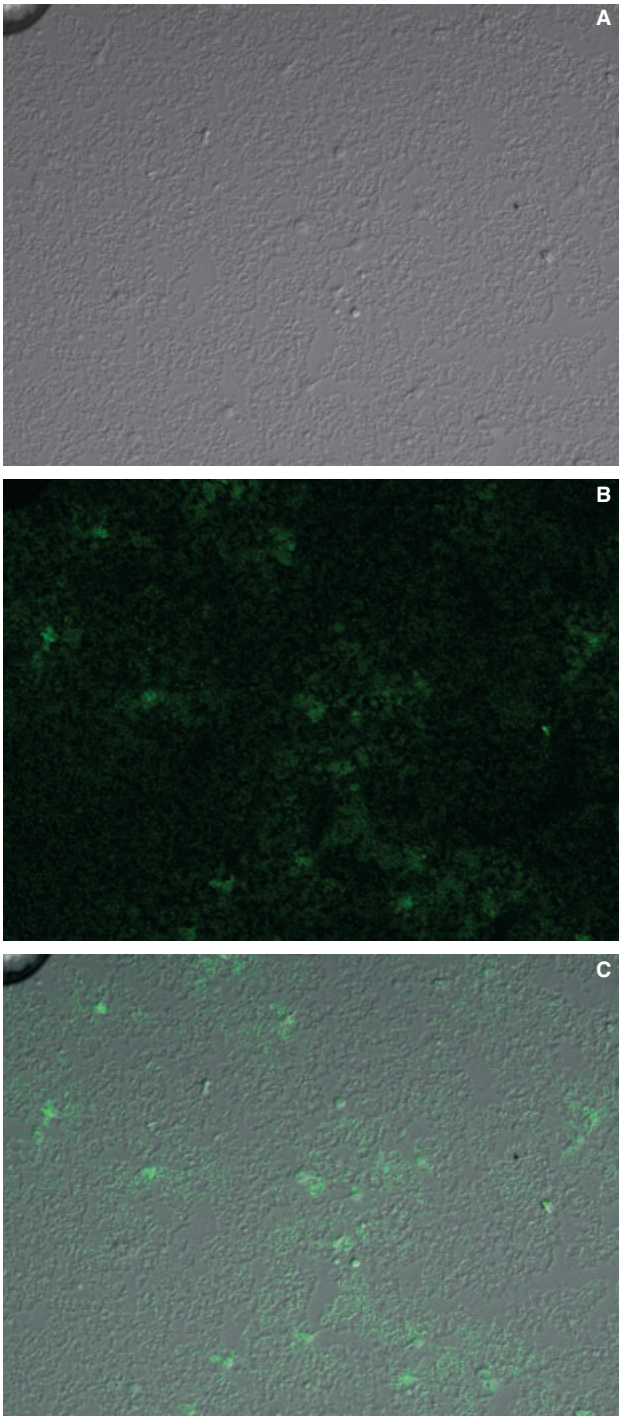

FIG. 5—*Heat-fixed samples of mixed human sperm cells and ID® Fresh Peach sexual lubricant may be visualized under phase-contrast imaging (A). Upon visualization using an FITC filter (B), the sperm cells are not as easily distinguishable as when the sexual lubricant is not present. An overlay comparison of the two images (C) reveals that much of the antibody–dye complex remains in the sexual lubricant remaining on the slide sample. This excess dye causes visual interference from the sample background, making identification of the sperm cells difficult. Photographs taken at 200× magnification.*

recommended here. The wash steps required by the kit's protocol dramatically increases the likelihood of loosing sample evidence on slides that are poorly fixed. Slide fixation parameters other than those described in the SPERM HY-LITER™ kit should be carefully tested for increased fluorescent background and sample loss. Masked slides and *Fixation Solution* are supplied in, and work optimally with, other kit components. It is highly recommended that charged slides rather than plain glass slides be used.

Among the unexpected effects seen as a result of heat fixation was the interference observed in the presence of sexual lubricants. While the spermatozoa were still labeled properly, excess antibody was retained on slides containing lubricants, with more pronounced background fluorescence associated with the more viscous lubricants. This retention of the antibody–dye complex to the slide often restricted visualization of individual sperm cells. In one instance, nonspecific staining was so great that no direct outline of the sperm cells could be seen. Nonspecific staining was not observed in lubricant samples that were alcohol fixed. Only a small amount of excess stain was seen in alcohol-fixed samples surrounding the sperm head, and visualization of the sperm heads themselves was not hindered. This was probably due to the fact that the alcohol fixation procedure dissolved a majority of the sexual lubricant on the slide. It is possible that the alcohol washed away more of the lubricant on the sample slides prior to labeling, which limited the amount of dye that could be retained on the slide. If the presence of sexual lubricants is suspected, it might be beneficial to rinse the slides for a longer period of time in between labeling solutions. However, vigorous washing will also increase the risk of sample loss.

The dramatic contrast produced by the Alexa 488 dye used in this kit, in concert with modern laser microdissection platforms, provides a readily automatable method for the detection and separation of human spermatozoa from female vaginal cells (2,3). Such automated platforms could save both analyst time and laboratory resources, significantly improving turn-around time in cases involving SAE, even in backlogged cases stored for a prolonged period of time.

References

1. Pratt TC, Gaffney MJ, Lovrich NP, Johnson CL. This isn't CSI: estimating the national backlog of forensic DNA cases and the barriers associated with case processing. Crim Justice Policy Rev 2006;17(1):32–47.
2. Sanders CT, Reisenbigler EK, Peterson DA. Laser microdissection separation of pure spermatozoa populations from mixed cell samples for forensic DNA analysis. Washington, DC: United States Department of Justice, 2007 Feb; Report No.: 217268.
3. Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. J Forensic Sci 2006;51(4):748–57.
4. Kaye S. Identification of seminal stains. J Crim L, Criminology Police Sci 1947;38:79–83.
5. Mischler TW, Reineke EP. Immunological identification of human seminal stains. J Crim L, Criminology Police Sci 1996;57(1):107–11.
6. Daves A, Wilson E. The persistence of seminal constituents in the human vagina. J Forensic Sci 1974;3:45–55.
7. Herr JC, Woodward MP. An enzyme-linked immunosorbent assay (ELISA) for human semen identification based on a biotinylated monoclonal antibody to a seminal vesicle-specific antigen. J Forensic Sci 1987;32(2):346–56.
8. Hooft PJ, Van De Voorde HP. The zinc test as an alternative for acid phosphatase spot tests in the primary identification of seminal traces. Forensic Sci Int 1990;47:269–75.
9. Sato I, Yoshiike M, Yamasaki T, Yoshida K, Takano S, Mukai T, et al. A dot-blot-immunoassay for semen identification using a polyclonal antibody against semenogelin, a powerful seminal marker. Forensic Sci Int 2001;122:27–34.
10. Allery JP, Telmon N, Mieusset R, Blanc A, Rougé D. Cytological detection of spermatozoa: comparison of three staining methods. J Forensic Sci 2001;46(2):349–51.
11. Pang BCM, Cheung BKK. Identification of human semenogelin in membrane strip test as an alternative method for the detection of semen. Forensic Sci Int 2007;169:27–31.
12. Talthip J, Chirachariyavej T, Peonim V, Atamasirikul K, Teerakamchai S. An autopsy report case of rape victim by the application of PSA test kit as a new innovation for sexual assault investigations in Thailand. J Med Assoc Thai 2007;90(2):348–51.
13. Peonim V, Chirachariyavej T, Atamasirikul K, Talthip J. Comparable between rapid one step immunochromatographic assay and ELISA in the detection of prostate specific antigen in vaginal specimens of raped women. J Med Assoc Thai 2007;90(12):2624–9.
14. Graves HCB, Sensabaugh GF, Blake ET. Postcoital detection of a male-specific semen protein: application to the investigation of rape. N Engl J Med 1985;312:338–43.
15. Sato I, Barni F, Yoshiike M, Rapone C, Berti A, Nakaki S, et al. Applicability of Nanotrap Sg as a semen detection kit before male-specific DNA profiling in sexual assaults. Int J Legal Med 2007;121:315–9.
16. Herr J. SpermPaint optimization and validation. Washington, DC: United States Department of Justice, 2007 Oct.; Report No.: 220289.
17. Sato I, Sagi M, Ishiwari A, Nishijima H, Ito E, Mukai T. Use of the ''SMITEST'' PSA card to identify the presence of prostate-specific antigen in semen and male urine. Forensic Sci Int 2002;127:71–4.
18. Kamenev L, Leclercq M, Francois-Gerard C. An enzyme immunoassay for prostate-specific p30 antigen detection in the postcoital vaginal tract. J Forensic Sci Soc 1989;29(4):233–41.
19. Kamenev L, Leclercq M, Francois-Gerard C. Detection of p30 antigen in sexual assault case material. J Forensic Sci Soc 1990;30(4):193–200.
20. Simich JP, Morris SL, Klick RL, Rittenhouse-Diakun K. Validation of the use of a commercially available kit for the identification of prostate specific antigen (PSA) in semen stains. J Forensic Sci 1999;44(6):1229–31.
21. Herr JC, Summers TA, McGee RS, Sutherland WM, Sigman M, Evans RJ. Characterization of a monoclonal antibody to a conserved epitope on human seminal vesicle-specific peptides: a novel probe/marker system for semen identification. Biol Reprod 1986;35:773–84.
22. Santucci KA, Nelson DG, McQuillen KK, Duffy SJ, Linakis JG. Wood's lamp utility in the identification of semen. Pediatrics 1999;104(6):1342–4.
23. Brackett JW Jr. The acid phosphatase test for seminal stains: the importance of proper control tests. J Crim L, Criminology Police Sci 1957;47:717–20.
24. Kind SS. The use of the acid phosphatase test in searching for seminal stains. J Crim L, Criminology Police Sci 1957;47:597–600.
25. Allen SM. An enzyme linked immunosorbent assay (ELISA) for detection of seminal fluid using a monoclonal antibody to prostatic acid phosphatase. J Immunoassay 1995;16(3):297–308.
26. Horchmeister MN, Budowle B, Rudin O, Gehrig C, Borer U, Thali M, et al. Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid. J Forensic Sci 1999;44:1057–60.
27. Auvdel MJ. Comparison of laser and ultraviolet techniques used in the detection of body secretions. J Forensic Sci 1987;32(2):326–45.
28. Hooft PJ, Van De Voorde HP. Interference of body products, food and products from daily life with the modified zinc test and the acid phosphatase test. Forensic Sci Int 1994;66(3):187–96.
29. Kloosterman AD, Pouw-Arnou M, Persijn JP. Comparison of enzyme assay and radioimmunoassay for the measurement of human acid phosphatase in cases of sexual assault. Forensic Sci Int 1984;25:45–55.
30. Diamandis EP, Yu H. Nonprostatic sources of prostate-specific antigen. Urol Clin North Am 1997;24(2):275–82.
31. Keil W, Bachus J, Tröger HD. Evaluation of MHS-5 in detecting seminal fluid in vaginal swabs. Int J Legal Med 1996;108:186–90.
32. Berti A, Virgili A, D'Errico G, Vespi G, Lago G, Cavazzana A. Expression of seminal vesicle-specific antigen in serum of lung tumor patients. J Forensic Sci 2005;50(5):1114–5.
33. Valtonen-André C, Olsson AY, Kullberg M, Nayudu PL, Lundwall Å. The common Marmoset (*Callithrix jacchus*) has two very similar semenogelin genes as a result of gene conversion. Biol Reprod 2007;76:604–10.
34. Khaldi N, Miras A, Botti K, Benali L, Gromb S. Evaluation of three rapid detection methods for the forensic identification of seminal fluid in rape cases. J Forensic Sci 2004;49(4):749–53.
35. Bryson CK, Garlo AM, Piner SC. Vaginal swabs: endogenous and postcoital components. J Forensic Sci Soc 1989;29(3):157–71.
36. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256(5517):495–7.

37. Kyurkcheiv SD, Kehayov IR, Tsankov Y, Dimitrova D. Application of an immunological method to detect trace amounts of dried human semen. Forensic Sci Int 1989;43:27–35.
38. Benchaib M, Delorme R, Pluvinage M, Bryon PA, Souchier C. Evaluation of five green fluorescence-emitting streptavidin-conjugated fluorochromes for use in immunofluorescence microscopy. Histochem Cell Biol 1996;106:253–6.
39. Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, et al. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. J Histochem Cytochem 1999;47(9):1179–88.
40. Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 1977;266:550–2.
41. Butler JM. Forensic DNA typing: biology, technology, and genetics of STR markers, 2nd edn. San Diego, CA: Elsevier Academic Press, 2005.
42. Kapuscinski J. Interactions of nucleic acids with fluorescent dyes: spectral properties of condensed complexes. J Histochem Cytochem 1990;38(9):1323–9.
43. Rehan N, Sobrero AJ, Fertig JW. The semen of fertile men: statistical analysis of 1300 men. Fertil Steril 1975;26(6):492–502.
44. Halpern V, Rountree W, Raymond EG, Law M. The effects of spermicides containing nonoynol-9 on cervical cytology. Contraception 2008;77:191–4.

Additional information and reprint requests:
Karl Reich, Ph.D.
Independent Forensics of Illinois
500 Waters Edge
Lombard, IL 60148
E-mail: info@ifi-test.com