

**Developmental validation of**  
**SPERM HY-LITER™**  
**A Specific, Sensitive and Confirmatory**  
**Microscopic Screening Method for the**  
**Detection of Human Sperm**  
**from**  
**Sexual Assault Evidence**

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## **Introduction**

The identification of sperm in sexual assault evidence (SAE) is a labor intensive, time consuming, and as presently performed, insensitive technique. Crime laboratories devote a great deal of effort, time, and resources to identifying sperm in SAE in order to satisfy legal requirements for prosecution and criminalistic requirements for proceeding with DNA-based evidence testing. Current methods used to locate and identify sperm in SAE are based on non-specific microscopic staining techniques (i.e., KPIC or 'Christmas Stain') and are not amenable to automation or computer-aided searches. As approximately half of all crime laboratory case work is related to SAE, the effort and expense devoted to screening for sperm is considerable. These issues are exacerbated with SAE that has been stored for long periods (e.g., backlogged rape kits) or for samples that have minimal amounts of biological material.

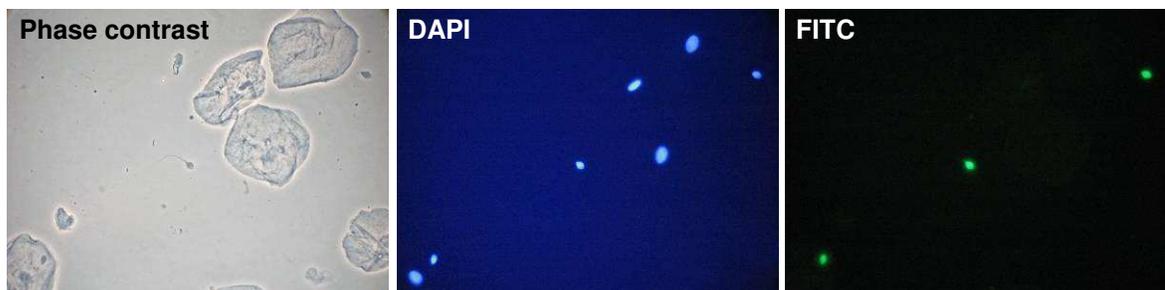
Here we present results using a reagent that has the potential to (1) provide the first scientifically justifiable and defensible method for the forensic identification of human sperm, and (2) greatly increase the efficiency of microscopic sperm searches for laboratory analysts.

## **SPERM HY-LITER™ Uses a Fluorescently Labeled, Human Specific, Anti-Sperm Head Antibody to Identify Human Sperm.**

**Experimental Rationale:** To demonstrate that the fluorescently labeled antibody supplied in the **SPERM HY-LITER™** kit identifies human sperm heads, a mixture of human epithelial cells (buccal swab extract) and sperm cells (50 µl human semen swab extract) was analyzed.

**Methods:** Samples were prepared from sterile cotton swabs where either buccal cells or semen extract (semen was obtained from a local sperm bank or from healthy volunteers under IRB supervision) were deposited on the swab and air dried in a protective environment. Semen and buccal swabs were extracted in 1 ml PBS for 1 hour at room temperature. The swab heads/cotton batting were removed and cells pelleted by brief centrifugation at 13,000 RPM in a table top centrifuge. Pelleted cells were re-suspended in 100 µl of PBS and subsequently combined (usually at 1:1, volume to volume). A portion of the mixed extracts (10 µl) was then added to the sample window of a **SPERM HY-LITER™** masked slide and processed as per manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** Phase contrast visualization demonstrated well preserved epithelial cells and sperm. Under DAPI fluorescence all cell nuclei are labeled and easily observed. Under FITC fluorescence only sperm heads are observed. Identical results were obtained from both male and female epithelial cells (data not shown).



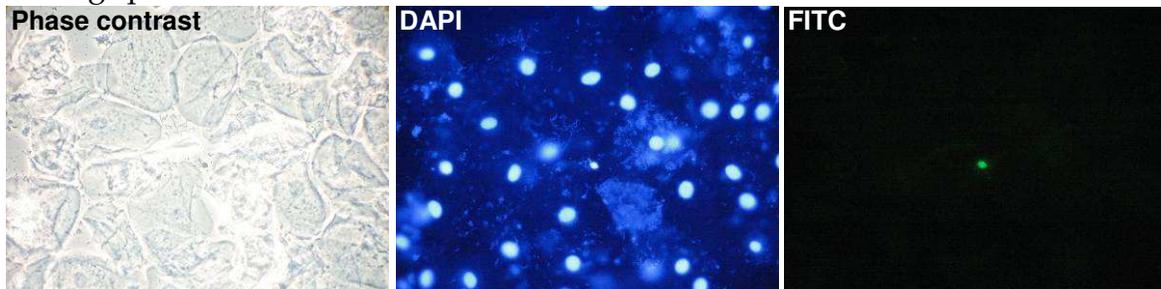
**Conclusions:** **SPERM HY-LITER™** fluorescently labels only human sperm heads; no FITC signal can be observed from epithelial cells. Unlike KPIC or H&E staining, **SPERM HY-LITER™** is cell type specific as only sperm cells are labeled. Fluorescent sperm heads are easily visible with negligible background staining.

## **SPERM HY-LITER™ Can Easily Distinguish a Single, Individual Sperm in a Confluent Background of Epithelial Cells**

**Experimental Rationale:** To test the sensitivity of **SPERM HY-LITER™** detection of human sperm, a mixed sample of a buccal swab extract and a very dilute sample of human semen extract was prepared and analyzed.

**Methods:** Individually prepared swabs of semen and buccal samples were extracted in 1 ml PBS for 1 hour at room temperature. The swab heads/batting were removed and cells pelleted by brief centrifugation at 13,000 RPM in a table top centrifuge. Epithelial cells were re-suspended in 100 µl, sperm cells were re-suspended in 1 ml. Decreasing amounts of semen extract was mixed with the buccal extract and the mixture applied to the sample window of a **SPERM HY-LITER™** slide and processed in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** After scanning multiple fields under FITC fluorescence filter, **SPERM HY-LITER™** stained preparations clearly demonstrated a strong signal from a single sperm. The observed FITC signal could be related, post analysis to visible DAPI staining providing additional confirmation that the fluorescence was related to a DNA-containing sperm head.



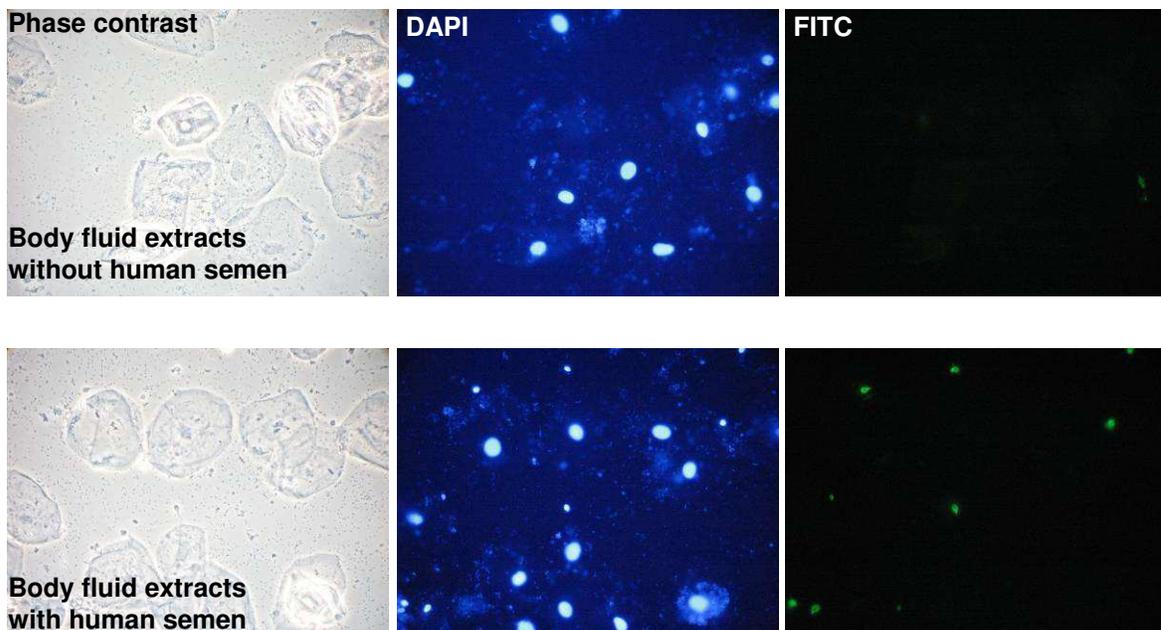
**Conclusions:** The **SPERM HY-LITER™** sexual assault evidence staining kit has sufficient sensitivity to identify a single sperm head present in an overwhelming background of epithelial cells.

## **SPERM HY-LITER™ Does Not Cross-React with other Human Body Fluids.**

**Experimental Rationale:** To determine whether **SPERM HY-LITER™** will label or cross-react with other human body fluids, specifically buccal (saliva), urine, and blood.

**Methods:** Individual blood, urine, buccal (saliva), and semen swabs were extracted in 1 ml PBS for 1 hour at room temperature. The swab heads were removed and cells pelleted by brief centrifugation at 13,000 RPM in a table top centrifuge. Pelleted cells were re-suspended in 100  $\mu$ l PBS. Aliquots of each extract were combined, with or without sperm extract and applied to the sample window of a **SPERM HY-LITER™** slide and processed in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** After scanning multiple fields of multiple slides of the mixed body fluid extracts *without* semen, no labeled cells were observed. However, scanning of the mixed body fluid extracts with semen, revealed clearly labeled sperm heads.



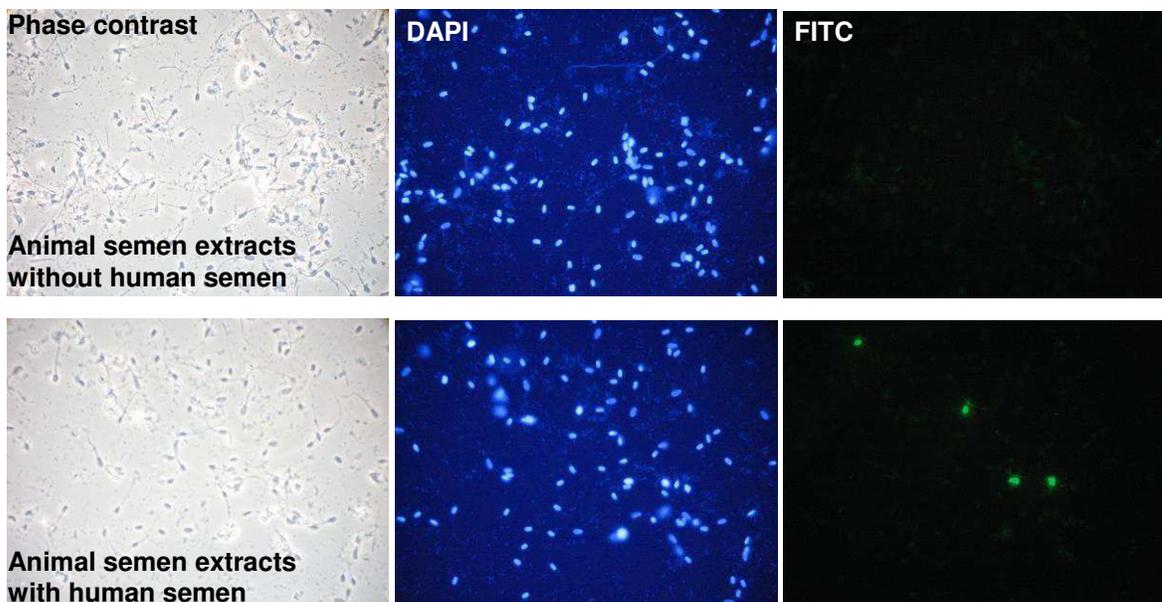
**Conclusions:** **SPERM HY-LITER™** does not cross react with other human body fluids specifically cell types found in blood, urine, or saliva including epithelial cells. Furthermore, the presence of other human body fluids does not interfere with the ability of **SPERM HY-LITER™** to specifically detect human sperm.

## SPERM HY-LITER™ Does Not Cross-React with Animal Sperm

**Experimental Rationale:** To determine whether **SPERM HY-LITER™** cross-reacts with sperm from other animal species, various animal semen extracts (dog, cat, cow, horse, goat, sheep, pig, and mouse) were analyzed.

**Methods:** Individual swabs with human and animal semen were extracted in 1 ml PBS for 1 hour at room temperature. The swab heads were removed and sperm cells pelleted by brief centrifugation at 13,000 RPM in a table top centrifuge. Pelleted sperm were re-suspended in 100 µl PBS. Aliquots of each extract were combined with or without human sperm and applied to the sample window of a **SPERM HY-LITER™** slide and processed in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** After scanning multiple fields of the mixed animal semen extracts without human semen, no labeled cells were identified. However, human sperm in the presence of a vast excess of animal sperm were easily seen.



**Conclusions:** **SPERM HY-LITER™** does not label or cross-react with animal sperm from the tested species: dog, cat, cow, horse, goat, sheep, pig, and mouse. In addition the presence of non-human animal sperm does not interfere with the ability **SPERM HY-LITER™** to specifically detect human sperm. **SPERM HY-LITER™** is specific for human sperm heads.

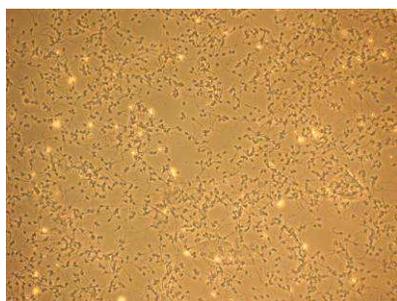
## SPERM HY-LITER™ Is Human Specific

**Experimental Rationale:** Previous experiments using **SPERM HY-LITER™** had demonstrated specificity against companion and many domesticated animal species. Additional tests of species specificity were conducted in order to more precisely define the specificity of the staining method. SWGDAM guidelines for DNA-STR analysis specify a requirement to quantify the amount of human DNA that is added to DNA-STR PCR reactions; counting human sperm prior to PCR would satisfy this requirement if the method could be demonstrated to be human specific as the DNA content of sperm cells is constant. Therefore various primate species were tested using **SPERM HY-LITER™**.

**Methods:** Raw semen obtained from human, cynomolgus, rhesus, and chimpanzee were obtained from volunteers and various primate centers (respectively) and stained with **SPERM HY-LITER™** using the manufacturer's suggested protocol. Both single species and non-human mixed with human sperm preparations were stained in order to provide internal controls for the specificity of the staining.

A second round of experiments using sperm extracted from swabs was also performed, again using single and mixed samples in order to verify that the observed specificity was not changed or altered due to the effects of air drying the samples or from extracting from swabs.

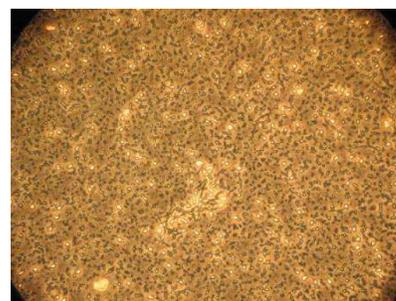
**Results:** Microscopical examination of sample preparations clearly demonstrated the presence of sperm:



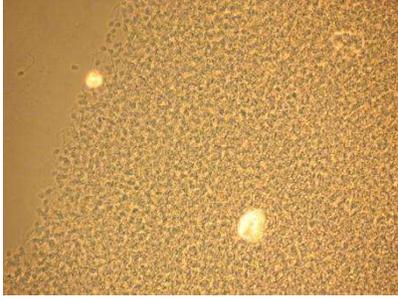
Chimpanzee  
Phase



Human  
Phase



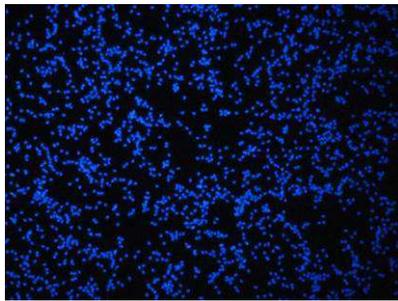
Cynomolgus  
Phase



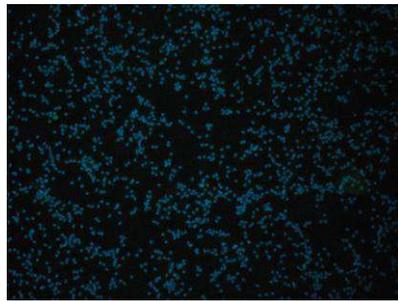
Rhesus  
Phase

Both single source and mixed (human and each of the non-human primate species) were stained using **SPERM HY-LITER™** according to manufacturer's instructions.

Chimpanzee (*Pan troglodytes*)



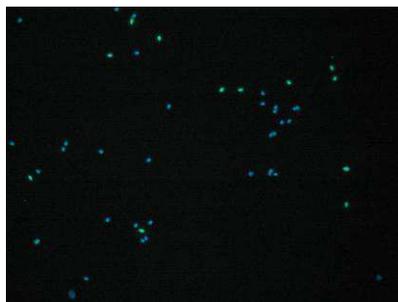
Chimpanzee, raw,  
DAPI filter



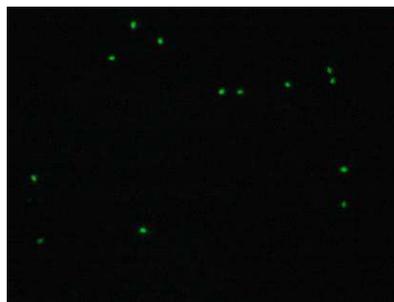
Chimpanzee, raw,  
DUAL filter



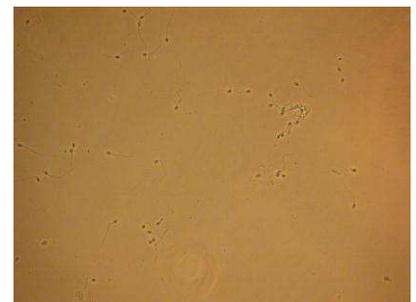
Chimpanzee, raw,  
FITC filter



Chimpanzee + Human  
Dual Filter

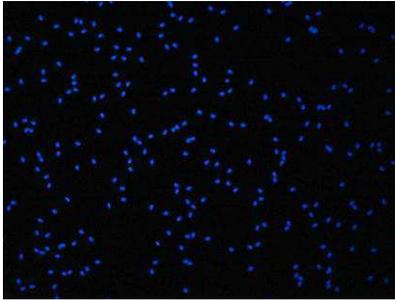


Chimpanzee + Human  
FITC Filter

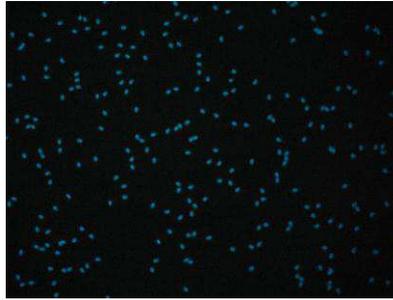


Chimpanzee + Human  
Phase

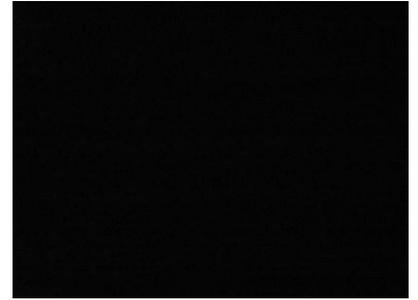
Macaque [Cynomolgus] (*Macaca fascicularis*)



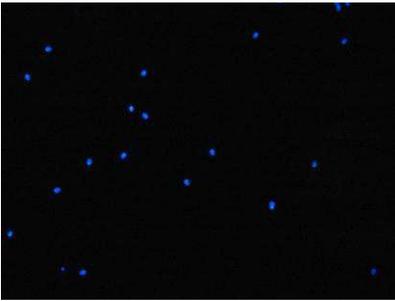
Macaque [Cynomolgus], Raw  
DAPI Filter



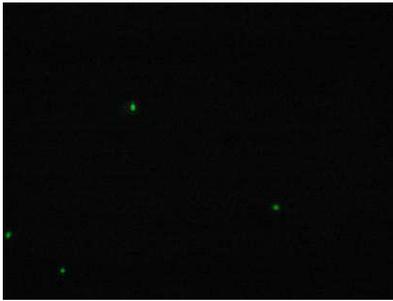
Macaque [Cynomolgus], Raw  
DUAL Filter



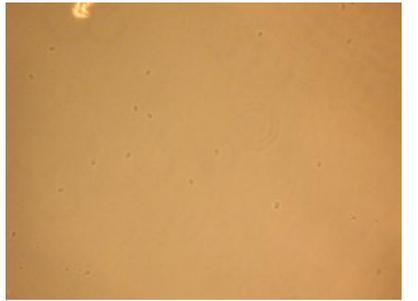
Macaque [Cynomolgus], Raw  
FITC Filter



Macaque + Human  
DAPI filter

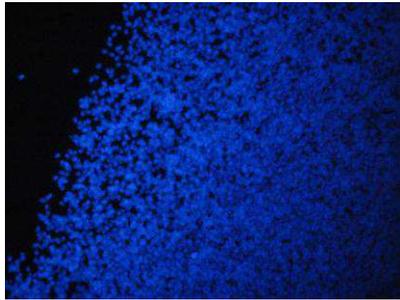


Macaque + Human  
FITC filter

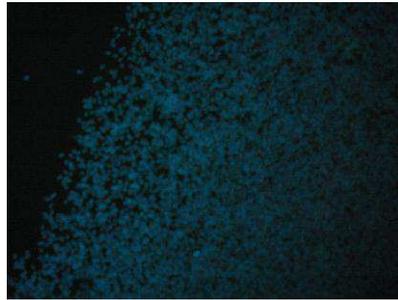


Macaque + Human  
Phase filter

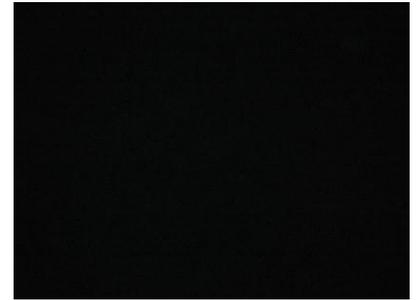
Rhesus (*Macaca mulatta*)



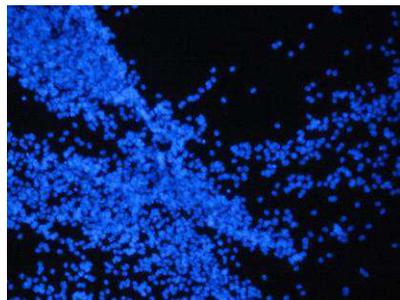
Rhesus, Raw  
DAPI filter



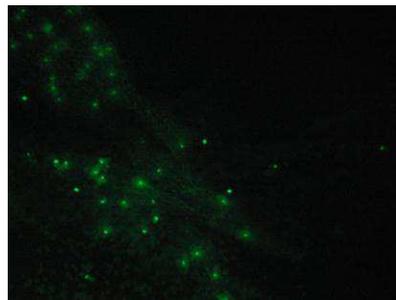
Rhesus, Raw  
DUAL filter



Rhesus, Raw  
FITC filter



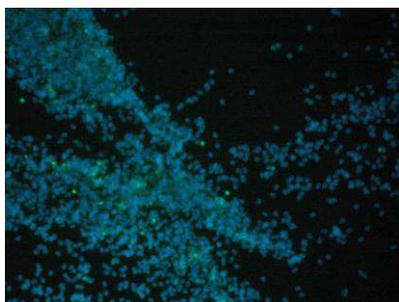
Rhesus + Human  
DAPI filter



Rhesus + Human  
FITC filter



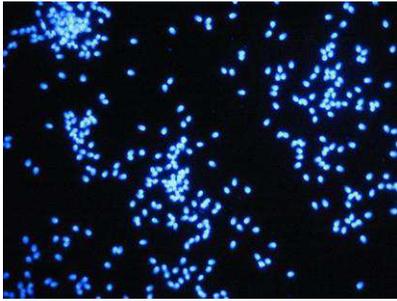
Rhesus + Human  
Phase



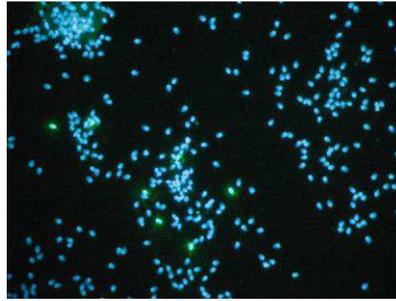
Rhesus + Human  
DUAL filter

Identical results were obtained from semen extracts prepared from air-dried swabs.

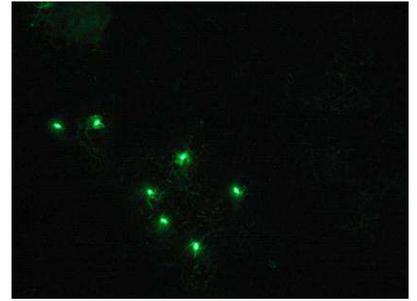
Extracts from semen prepared swabs.



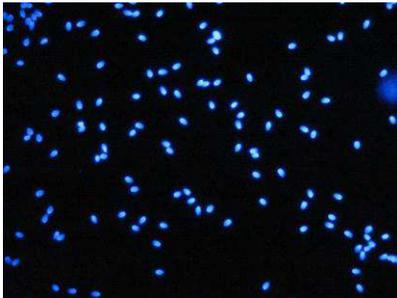
Chimpanzee + Human  
Swab Extract  
DAPI Filter



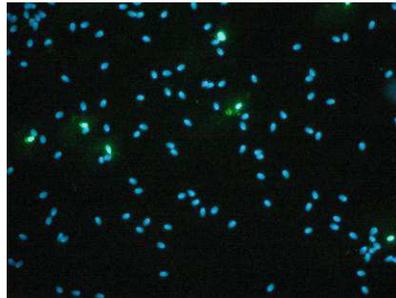
Chimpanzee + Human  
Swab Extract  
DUAL Filter



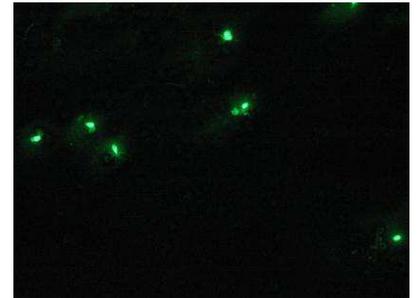
Chimpanzee + Human  
Swab Extract  
FITC Filter



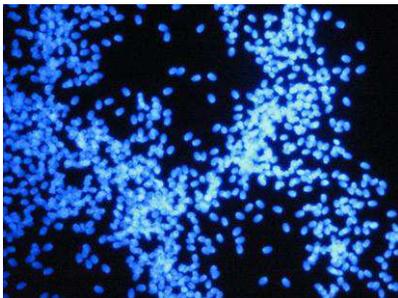
Macaque + Human  
Swab Extract  
DAPI Filter



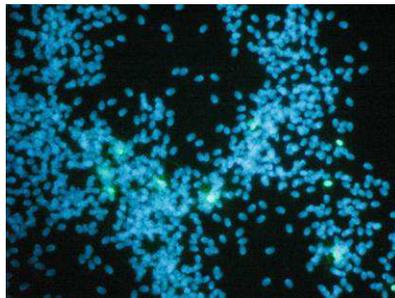
Macaque + Human  
Swab Extract  
DUAL Filter



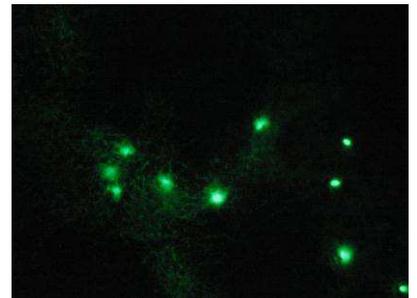
Macaque + Human  
Swab Extract  
FITC Filter



Rhesus + Human  
Swab Extract  
DAPI filter



Rhesus + Human  
Swab Extract  
DUAL filter



Rhesus + Human  
Swab Extract  
FITC filter

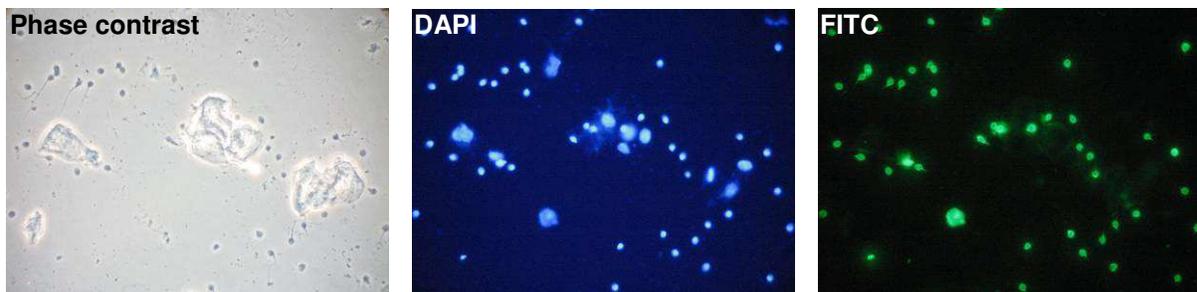
**Conclusions:** **SPERM HY-LITER™** does not stain non-human primate sperm samples and is specific for human sperm heads. The specificity of the method is not altered using swab extracts of non-human primate sperm.

## **SPERM HY-LITER™ Labels Human Sperm from Post-Coital Vaginal Swab Extracts.**

**Experimental Rationale:** Previous experiments using **SPERM HY-LITER™** were performed on mixed buccal and semen extracts: it is important to test the procedure from samples that would more approximate sexual assault evidence. Therefore, post-coital vaginal swabs were analyzed using **SPERM HY-LITER™**.

**Methods:** Post coital swabs, collected from healthy volunteers were extracted in 1 ml of PBS for 1 hour at room temperature. The swab head was removed and the extracted cells were pelleted and re-suspended in 100 µl of PBS. An aliquot of the extract (20 µl) was applied to the sample window of a **SPERM HY-LITER™** slide and processed in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** Scanning of the post-coital extract slide, clearly revealed labeled human sperm heads.



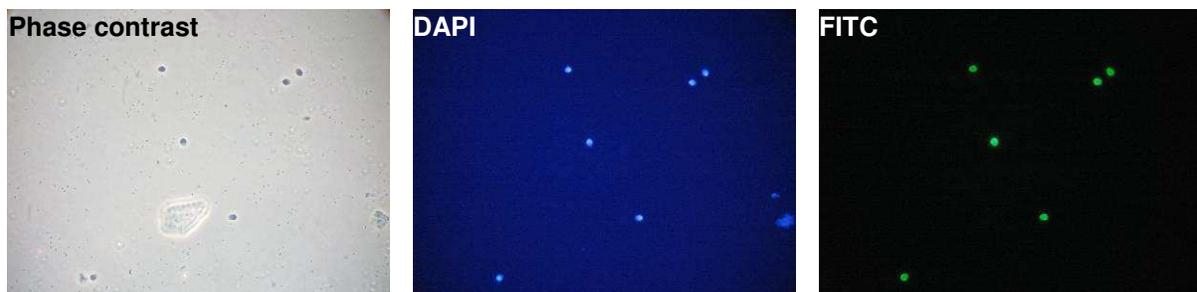
**Conclusions:** Post-coital vaginal swabs can be successfully analyzed using **SPERM HY-LITER™**. Importantly, **SPERM HY-LITER™** specifically and sensitively labels human sperm from post-coital vaginal swab extracts: no cellular cross reaction or inhibition was observed.

## **SPERM HY-LITER™ Labels Human Sperm from Simulated Sexual Assault Kit Smear Slides.**

**Experimental Rationale:** Forensic laboratories often have to examine smear slides, prepared at the time of sexual assault evidence collection, for sperm. Previous experiments demonstrating the specificity and sensitivity of **SPERM HY-LITER™** used extracts from swabs. Here we test **SPERM HY-LITER™** for its ability to specifically and sensitively identify sperm from post-coital vaginal smear slides.

**Methods:** Smear slides were prepared by moistening an air dried post-coital vaginal swab with PBS and gently smearing the swab onto a clean glass microscope slide. This sample type was then stained using **SPERM HY-LITER™** in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** Post-coital vaginal smear slides were easily, reliably and specifically stained with **SPERM HY-LITER™**: only sperm were seen in the FITC fluorescent channel.



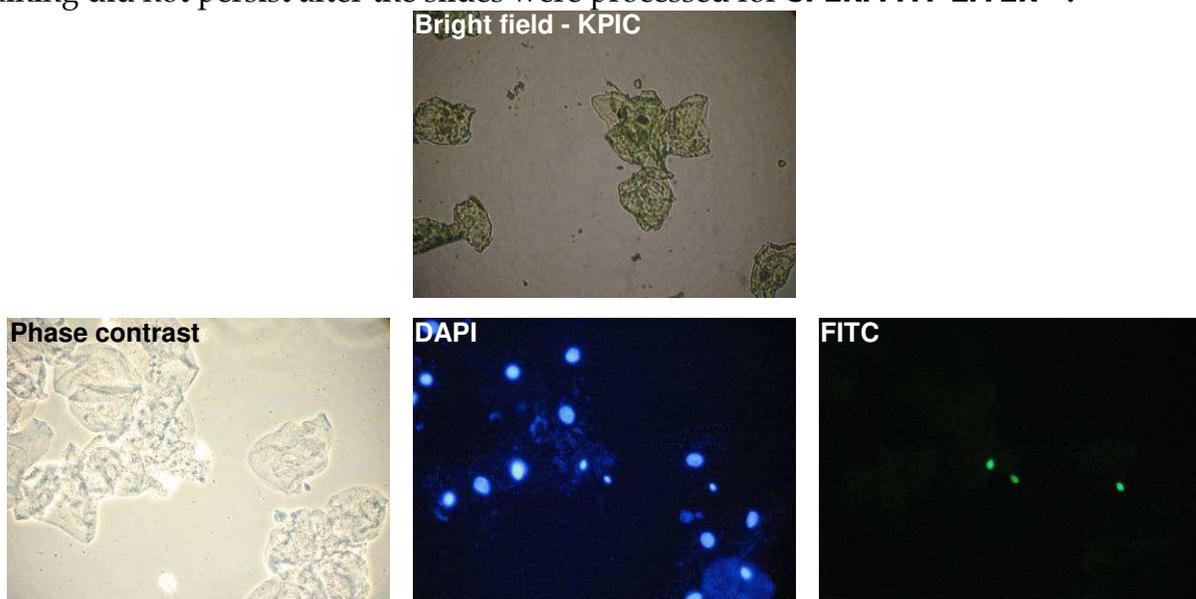
**Conclusions:** Post-coital vaginal smear slides, a common form of sexual assault evidence, are easily processed using **SPERM HY-LITER™**. Importantly, **SPERM HY-LITER™** specifically and sensitively labels human sperm present on post-coital vaginal smear slides.

## **SPERM HY-LITER™ Labels Human Sperm from KPIC Stained Slides.**

**Experimental Rationale:** As KPIC staining is the predominant staining method for the microscopic screening of sperm, we tested if a previously KPIC stained smear slide could be re-analyzed using **SPERM HY-LITER™**.

**Methods:** Individual semen and buccal swabs were extracted in 1 ml PBS for 1 hour at room temperature. Swab heads were removed and cells pelleted by brief centrifugation at 13,000 RPM in a table top centrifuge. Cells were re-suspended in 100 µl and aliquots combined 1:1 (volume to volume). This mixed extract was applied to both a clean microscope slide and to a **SPERM HY-LITER™** slide. Both slides were then stained using KPIC using commercially available reagents (SERI, California). Slides were examined for the presence of sperm and epithelial cells. KPIC stained slides were then processed using **SPERM HY-LITER™** in accordance with the manufacturer's suggested protocol. Processed slides were visualized using phase contrast and fluorescence microscopy: sequential photomicrographs of the identical microscope field with appropriate filters for DAPI and FITC fluorescence were recorded.

**Results:** Scanning of the KPIC/ **SPERM HY-LITER™** stained slides quickly demonstrated highly fluorescent labeled human sperm heads. However, the KPIC staining did not persist after the slides were processed for **SPERM HY-LITER™**.



**Conclusions:** Sexual assault smear slides that have been previously analyzed with KPIC staining protocols can be re-analyzed using **SPERM HY-LITER™**. This observation may make testing and validation of **SPERM HY-LITER™** a simpler and more straightforward process for forensic laboratories.