



**SPERM HY-LITER™**

**INSTALLATION AND  
TRAINING MANUAL**







**SPERM HY-LITER™** was developed for the microscopic screening of sexual assault evidence. The method was designed for forensic DNA laboratories to solve one of the most time consuming and inefficient chores that DNA analysts face: finding sperm on diverse items of evidence.

The specificity of the method is obtained from the unique monoclonal antibody incorporated as a part of the staining; the sensitivity derives from the signal to noise advantage of fluorescent detection.

**SPERM HY-LITER™** finds its roots in a research and development contract to develop new body fluid tests – this work is the origin of the **RSID™** line of body fluid tests. These tests all derive their specificity and sensitivity from mouse monoclonal antibodies. During this research, scientists at IFI identified, through fluorescent microscopy, unique antibodies that recognized human sperm heads. These antibodies were the origin of **SPERM HY-LITER™**.

**SPERM HY-LITER™** incorporates two fluorescent dyes – the addition of the second, non-specific nuclear staining dye is no accident; it is included so that **SPERM HY-LITER™** can be used with newly emerging automated searching and cell capture techniques (e.g., laser capture microdissection).

**SPERM HY-LITER™** fits into your work flow to aid in both past and present cases. **SPERM HY-LITER™** will not damage DNA. Profiles can be obtained from post-**SPERM HY-LITER™** stained preparations. **SPERM HY-LITER™** can be performed on previously stained slides (KPIC and/or H&E) with no loss of specificity.

Visualization of **SPERM HY-LITER™** stained preparations requires a fluorescent microscope fitted with the correct filters - this manual is designed to provide some experience and background in observing fluorescent preparations under the microscope. Microscopic immunofluorescent detection is a long-established technique in both research and hospital laboratories - some background staining is always visible with fluorescent techniques.

The use of controls (both positive and negative) and mixing experiments will help analysts gain the required experience and confidence to quickly identify human sperm from sexual assault evidence.

--Karl Reich, Ph.D.





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# SPERM HY-LITER™

## Installation and Training Manual

The new SPERM HY-LITER™ and SPERM HY-LITER™ *PLUS* kits are designed for specific, sensitive, reliable and simple detection of human sperm from sexual assault evidence. The tests can detect a single human sperm head in an overwhelming background of epithelial cells. Both kits contain the same staining reagents; SPERM HY-LITER™ *PLUS* contains masked slides for use with user prepared extracts. SPERM HY-LITER™ includes a hydrophobic pen that is used to outline the area on the slide to be stained. The 'ink' from the hydrophobic pen creates a barrier such that staining reagents can be conveniently dispensed and retained on the slide. Unless otherwise noted SPERM HY-LITER™ and SPERM HY-LITER™ *PLUS* will be referred to only as SPERM HY-LITER™.

Both kits, SPERM HY-LITER™ incorporate an Alexa 488 derivatized mouse monoclonal antibody to human sperm heads to specifically identify human sperm from sexual assault evidence by fluorescence immuno-microscopy. In addition to a human sperm specific reagent, SPERM HY-LITER™ incorporates a second fluorescent dye (4',6-diamidino-2-phenylindole, DAPI) that stains all nuclei present in the sample.

This installation and training manual is designed to provide users of SPERM HY-LITER™ with a background in fluorescent immuno-detection; representative images of SPERM HY-LITER™ stained slides are presented under a variety of conditions, both optimal and challenging. Brief descriptions of the techniques used are described below.

**What is immuno-fluorescence?** Immuno-fluorescent detection is a technique to identify an antigen using a fluorophore-conjugated antibody that is visualized under fluorescent microscopy. The antibody can be visualized directly (as in SPERM HY-LITER) or indirectly with a secondary reagent (e.g., fluorescent anti-mouse antibody). Specific binding of the antibody to the antigen can be observed under a microscope by exciting the fluorophore and imaging the emitted light. The antibody used in SPERM HY-LITER™ has been covalently modified with a fluorescent compound (Alexa-488) – the fluorophore is stable and can be seen under the microscope as green fluorescence.

**What is phase contrast?** Phase contrast is an optical method of providing contrast (black and white) to specimens that are inherently transparent. Biological materials are

typically imaged using phase contrast as good three-dimensional information can be observed from unstained preparations. SPERM HY-LITER™ stained slides can be readily imaged under phase contrast – note that fluorescence is not typically seen in the visible light range. Both sperm and epithelial cells can be clearly visible using phase contrast; however the size and loss of cell morphology of sperm after extraction from sexual assault evidence makes distinguishing sperm from cellular debris difficult.

**What is FITC?** FITC is an acronym for fluorescein isothiocyanate, a common and much used fluorophore. FITC fluoresces green, and has long been used for immuno-fluorescent detection. However, FITC is quickly photo-bleached – the Alexa 488 dye used in SPERM HY-LITER™ has a fluorescent spectrum essentially identical to FITC, but is much more photo-bleach resistant and was specifically chosen to take advantage of the many FITC filters and light sources that have been developed. Note: due to the long standing history of FITC in immunofluorescent detection, we refer to the fluorescent filters used to visualize SPERM HY-LITER™ stained preparations as FITC – technically we are visualizing Alexa 488 fluorophores through a filter assembly designed for FITC.

**What is DAPI?** DAPI or 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to DNA in the nuclei of cells. This dye will bind to the nuclei of all cells (bacterial, fungal, plant, animal, etc.) in a sample. The nuclei will appear blue under fluorescent detection with appropriate filters.

**What is Dual?** This is an abbreviation for a specialized fluorescent filter assembly that permits the simultaneous visualization of FITC and DAPI fluorescence. Under appropriate illumination, SPERM HY-LITER™ stained preparations can be examined with a Dual filter: all non-sperm nuclei will appear blue and all sperm head will appear green (under these conditions the DAPI stained sperm heads are masked by the green Alexa 488 fluorescence).

**Interpretation of SPERM HY-LITER™ stained slides.** SPERM HY-LITER™ incorporates two fluorescent dyes – one non-specific stain for all nuclei (DAPI) and one specific for sperm heads (Alexa 488, here FITC). When possible we suggest that phase contrast, FITC, and DAPI images from the same microscope field be evaluated for the presence of sperm in a sample. Sperm heads will be stained green in the FITC filter – the same structure should be stained blue in the DAPI filter and some kind of phase contrast visible structure should be present. SPERM HY-LITER™ does not destroy the morphology of sperm – if tails, midsection or acrosomes are present, these subcellular organelles will be maintained and could be visible under appropriate magnification. Samples that contain large numbers of cells, or large amounts of debris (cellular or particulate) may not be amenable to phase contrast analysis – users should rely on the fluorescent stains (DAPI and Alexa488) for positive sperm identification.

**Work-flow Integration of SPERM HY-LITER™.** SPERM HY-LITER™ is designed to provide robust, scientifically defensible identification of sperm from sexual assault evidence. We strongly recommend documenting the results of SPERM HY-LITER™ staining with image analysis software. Copies of images may be stored in a case file or archived. Many laboratories perform an initial screening of sexual assault evidence under phase contrast – samples that contain readily identifiable sperm can be triaged as per SOP – samples where sperm are not identified can then be SPERM HY-LITER™ stained and screened under fluorescent detection. [Note: automated sperm detection requires SPERM HY-LITER™ stained preparations.]

**Preservation of SPERM HY-LITER™ stained slides.** SPERM HY-LITER™ stained slides can be stored in the dark at room temperature. SPERM HY-LITER™ slides stored in the dark may be viewed several months later, although the FITC and DAPI staining will become more diffuse as the slide ages. It is important to avoid viewing stained slide using fluorescent illumination for extended periods of time as it is possible to photo-bleach SPERM HY-LITER™ preparations. The provided mounting media contains a fluorescence stabilizing agent that will help preserve the fluorescent signal. The SPERM HY-LITER™ staining procedure has been optimized to provide the best image possible from a variety of sample types and to allow the sample to be successfully processed for DNA-STR analysis if required; therefore no post-fixation step has been included. Laboratories that wish to preserve SPERM HY-LITER™ stained slides for extended times may wish to incorporate a post-fixation step: incubation with 3.7% paraformaldehyde for 10 minutes at room temperature (after antibody staining and before the addition of mounting media). This step is *not* recommended if downstream DNA-STR amplification is contemplated.

Independent Forensics  
SPERM HY-LITER™  
Technical Information Sheet

## INTENDED USE

The new SPERM HY-LITER™ kit is designed for specific, sensitive, reliable and simple detection of human sperm from sexual assault evidence smear slides. The test can detect a single human sperm head in an overwhelming background of epithelial cells.

Sample processing and fluorescent detection of human sperm can be completely integrated into current forensic laboratory procedures for DNA-based analysis, prior to STR testing (see Provided Protocols).

SPERM HY-LITER™ is highly specific for human sperm heads such that if a fluorescent signal is observed, an analyst can conclude that human sperm has been detected and that male genetic material is present in the tested sample.

SPERM HY-LITER™ is the first commercially available, specific, confirmatory test for human sperm: morphological characteristics and non-specific staining methods are **NOT** used to identify human sperm heads. No other human body fluids cross-react. Unlike other commercially available sperm detection kits, SPERM HY-LITER™ only stains human sperm heads, providing a bright fluorescent signal from the only sperm structure remaining in most sexual assault evidence: the DNA-containing sperm head. SPERM HY-LITER™ utilizes a unique monoclonal antibody specific for human sperm heads in conjunction with a simple, defined protocol to provide a scientifically justifiable identification of human sperm by fluorescence microscopy.

*NOT FOR IN VITRO DIAGNOSTIC USE.*

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## Introduction

SPERM HY-LITER™ uses a fluorescently tagged anti-human sperm head monoclonal antibody to detect the presence of human sperm. The tests are confirmatory for human sperm and have numerous

advantages over other methods of sperm detection, including increased sensitivity and specificity. Current identification methods for semen lack discrimination and are by definition presumptive (provide a basis for continued analysis of the tested exhibit but are not specific for human sperm), and open to legal and scientific challenge.

## Principle of the Test

SPERM HY-LITER™ uses an Alexa 488 derivatized mouse monoclonal antibody to human sperm heads to specifically identify human sperm from sexual assault evidence by fluorescence microscopy. The method requires a fluorescence microscope: processed slides can be visualized on *any* commercial fluorescence microscope fitted with the correct excitation and emission filters. In addition to a human sperm specific reagent, SPERM HY-LITER™ incorporates a second fluorescent dye that stains all nuclei present in the sample (4',6-diamidino-2-phenylindole, DAPI). Visualization of fluorescent nuclei is not required for sperm detection, but is recommended for both manual and automated sperm searches.

SPERM HY-LITER™ staining requires simple, sequential sample processing using provided solutions to prepare, block and stain previously prepared sexual assault evidence smear slides. Subsequent fluorescence microscope visualization allows detection of human sperm. Processed slides may be visualized immediately with or without the addition of mounting media or a coverslip. Mounted slides are recommended for optimal visual quality. However, laboratories that intend to isolate sperm from stained preparations for DNA-STR analysis might consider leaving their preparations unmounted. Alternatively, mounted coverslips can be removed by soaking in water for several hours.

SPERM HY-LITER™ incorporates a fluorescent nucleic acid stain that can be used to locate all cells in the preparation: dual color analysis (DAPI and Fluorescein) can be used as an aid to visualizing crowded preparations and/or with image analysis software to electronically eliminate fluorescent background signals. This additional fluorescent stain is included in anticipation of the widespread use of automated sperm search software and the use of Laser Capture Microdissection methods. It is not required for the detection of human sperm.



## Reagents and Materials Provided

### i) Provided Solutions:

|                              |                |
|------------------------------|----------------|
| Fixative Solution            | store at 2-8°C |
| Sample Preparation Solution  | store at 2-8°C |
| Blocking Solution            | store at 2-8°C |
| Sperm Head Staining Solution | store at 2-8°C |
| Mounting Media               | store at 2-8°C |
| Wash Buffer 10X Stock        | store at RT    |

ii) Hydrophobic barrier pen for encircling sexual assault evidence smear. Store inverted at RT.

iii) 50, 18 x 18 glass cover slips. Store at RT.

iv) Staining Protocol and Technical Information Sheet

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## User-Prepared Solutions

### i) 1 X Wash Solution

Users must prepare a 1X wash solution from the provided 10X stock – dilute provided stock 1:10 with laboratory quality H<sub>2</sub>O into a convenient wash/squirt bottle. Store at RT.

### ii) Sample Preparation Solution + DTT

Prepare Sample Preparation Solution + DTT daily before use: for each sexual assault evidence smear slide to be stained, add 3 µl of freshly thawed 1 M DTT to six (6) drops of Sample Preparation Solution (yellow bottle cap) in a microcentrifuge tube, mix thoroughly. Laboratories that do not use 1 M DTT stock solutions should adjust DTT volumes accordingly; final concentration of DTT in Sample Preparation Solution should be ~12 mM.

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## Staining Protocol

1. Fixation: Cover entire sexual assault evidence smear with 6 drops of FIXATIVE Solution (white bottle cap). Incubate at room temperature for 10 min. Wash: Use a wash/ squirt bottle to *gently* rinse each slide with ~2-3 mL of 1X wash buffer. Vigorous or lengthy washing or rinsing is *not* required. After the wash step, use the corner of a lab wipe to wick away residual wash buffer.

2. Sample Preparation: Cover entire sexual assault evidence smear with user-prepared SAMPLE PREPARATION Solution + DTT (~240 µl). Incubate at room temperature for 30 min. Wash slide as described above.

3. Block: Cover entire sexual assault evidence smear with 6 drops of BLOCKING Solution (red bottle cap). Incubate at room temperature for 30 min. Wash slide as described above.

4. Stain: Cover entire sexual assault evidence smear with 6 drops of SPERM HEAD STAINING Solution

(green bottle cap). Incubate at room temperature for 30 min. Wash slide as described above.

Slides may be visualized immediately, or for better optical and photographic quality, mounted and coverslipped (see below). Processed slides should be stored at room temperature protected from light.

5. OPTIONAL - Mount: Add three drops of MOUNTING Solution (blue bottle cap) to encircled sexual assault evidence smear and place cover slip(s) over sample. Place slide between paper towels and gently press down to position coverslip and remove excess mounting media. Mounting media will semi-harden after 20 min at room temperature. Please note that warm and humid conditions can slow or prevent hardening of the mounting media. Coverslips may be stabilized by outlining with clear nail polish. Slides may be visualized immediately and are stable for several weeks stored in the dark at room temperature.

6. Archived slides previously stained with KPIC. See provided SPERM HY-LITER™ Staining Protocol Sheet.

7. To stain freshly prepared slides with KPIC followed by SPERM HY-LITER™. See provided SPERM HY-LITER™ Staining Protocol Sheet.

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## Visualization of Human Sperm Heads

Stained slides must be visualized using a fluorescence microscope fitted with appropriate filters. Cell nuclei, including epithelial and sperm, can be visualized using DAPI-compatible filters. Human sperm heads can be visualized using fluorescein or Alexa 488 compatible filters. Slides may be scanned at a final magnification of 100x, 200x, 400x or 1000x at the operator's discretion.

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## Specificity

SPERM HY-LITER™ PLUS is specific for human sperm heads. No cross-reactivity with epithelial cells, blood cells or animal semen from horse, bull, sheep, goat, pig, dog, cat and mouse has been observed. Sperm specific staining has been observed from gorilla and chimpanzee semen.

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## Test Sensitivity

When used as suggested, the detection limit for SPERM HY-LITER™ is one human sperm head.



Manufactured by:

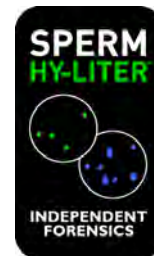
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SPERM HY-LITER™ - August 27, 2008

# SPERM HY-LITER™



## SPERM HY-LITER™ Staining Protocol

This protocol is designed for staining the entire 25 x 55 mm sample area of a standard 25 x 75 mm sexual assault evidence smear slide.

### Provided Materials:

|                              |  |
|------------------------------|--|
| Hydrophobic Barrier Pen      | white bottle cap   |
| FIXATIVE Solution            | yellow bottle cap ( <i>addition of DTT required before use</i> ) |
| SAMPLE PREPARATION Solution  | red bottle cap   |
| BLOCKING Solution            | green bottle cap   |
| SPERM HEAD STAINING Solution | blue bottle cap  |
| MOUNTING Media               | square 250 ml bottle ( <i>dilution required before use</i> )     |
| WASH Buffer 10X Stock        |  |

### User-prepared solutions:

#### 1X Wash Solution

Prepare 1X wash solution from provided 10X Stock: dilute 1:10 with H<sub>2</sub>O into a convenient wash/squirt bottle.

#### Sample Preparation Solution + DTT

Prepare Sample Preparation + DTT daily before use: for each smear slide to be stained, add 3 µl of 1 M DTT to six drops of Sample Preparation Solution (yellow bottle cap) in a microcentrifuge tube and mix thoroughly.

### Procedure:

1. Use hydrophobic barrier pen to encircle the entire sexual assault evidence smear.

**Note:** The volume of solutions provided in one kit is sufficient to stain **25** smear slides.

2. **Fixation:** Add 6 drops of **FIXATIVE Solution (white bottle cap)** into circled area. Incubate at room temperature for 10 minutes.

**Wash:** Use a wash bottle to **gently** rinse each sample window with approximately 2-3 mL of 1X wash buffer. Vigorous washing or rinsing is **not** required. After the wash step, use the corner of a paper towel or a lab wipe to wick away the residual wash buffer in the sample window.

3. **Sample Preparation:** *[DTT must be added to this solution prior to use! Please see User-Prepared Solutions above.]* Pipette user-prepared **SAMPLE PREPARATION Solution + DTT** (~ 240 µl) into circled area. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

4. **Block:** Add 6 drops of **BLOCKING Solution (red bottle cap)** into circled area. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

5. **Stain:** Add 6 drops of **SPERM HEAD STAINING Solution (green bottle cap)** into circled area. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

**6. OPTIONAL - Mount:** Slide mounting is recommended for optimal visual quality. Add three drops of **MOUNTING Media (blue bottle cap)** into circled area. Gently place provided cover slips over stained smear. Place slide between two small stacks of paper towels and gently press down to position coverslip and remove excess mounting media. Mounting media will semi-harden after 20 minutes at room temperature\*. Coverslips may be stabilized by outlining with clear nail polish. Slides may be visualized immediately and are stable for weeks.

**\*Please note that warm and humid conditions can slow or prevent hardening of the mounting media.** This will not affect the quality of the staining. To stabilize the coverslip, users can outline the edges of the coverslip with clear nail polish or Permount.

**7. Visualize:** Stained slides must be visualized using a microscope fitted with a fluorescence light source and appropriate filter cubes. Cell nuclei, including epithelial and sperm cells, can be visualized using DAPI-compatible filter cubes. Human sperm heads can be visualized using fluorescein or Alexa 488 compatible filter cubes. Slides may be scanned at a final magnification of 100x, 200x, 400x or 1000x at the operator's discretion

## Additional Suggested Protocols:

### Integration of KPIC staining and SPERM HY-LITER™:

**Archived slides previously stained with KPIC.** Mounting media, if present, must be removed before previously prepared slides can be stained with SPERM HY-LITER™. Permounted slides may be soaked in xylene to dissolve the media.

Proceed with SPERM HY-LITER™ staining as per protocol. Archived KPIC slides will demonstrate weaker SPERM HY-LITER™ sperm staining: more intense staining may be observed by increasing the DTT concentration in the Sample Preparation Solution by 10X (i.e., 10 µl of 1M DTT per two drops of Sample Preparation Solution – Yellow Capped bottle).

**To stain freshly prepared slides with KPIC followed by SPERM HY-LITER™.** We recommend that analysts slightly modify their existing procedure by substituting the final ethanol wash in the KPIC protocol with a gentle water rinse. The SPERM HY-LITER™ protocol may then be followed as described.

NOT FOR IN VITRO DIAGNOSTIC USE

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Independent Forensics  
**SPERM HY-LITER™ PLUS**  
**Technical Information Sheet**

#### **INTENDED USE**

The new SPERM HY-LITER™ PLUS kit is designed for specific, sensitive, reliable and simple detection of human sperm from sexual assault evidence extracts. The test can detect a single human sperm head in an overwhelming background of epithelial cells.

Sample processing and fluorescent detection of human sperm can be completely integrated into current forensic laboratory procedures for DNA-based analysis, prior to STR testing (see Provided Protocols).

SPERM HY-LITER™ PLUS is highly specific for human sperm heads such that if a fluorescent signal is observed, an analyst can conclude that human sperm has been detected and that male genetic material is present in the tested sample.

SPERM HY-LITER™ PLUS is the first commercially available, specific, confirmatory test for human sperm: morphological characteristics and non-specific staining methods are **NOT** used to identify human sperm heads. No other human body fluids cross-react. Unlike other commercially available sperm detection kits, SPERM HY-LITER™ PLUS only stains human sperm heads, providing a bright fluorescent signal from the only sperm structure remaining in most sexual assault evidence: the DNA-containing sperm head. SPERM HY-LITER™ PLUS utilizes a unique monoclonal antibody specific for human sperm heads in conjunction with a simple, defined protocol to provide a scientifically justifiable identification of human sperm by fluorescence microscopy.

*NOT FOR IN VITRO DIAGNOSTIC USE.*

#### **Introduction**

SPERM HY-LITER™ PLUS uses a fluorescently tagged anti-human sperm head monoclonal antibody to detect the presence of human sperm. The tests are confirmatory for human sperm and have numerous advantages over other methods of sperm detection, including increased sensitivity and specificity. Current identification methods for semen lack

discrimination and are by definition presumptive (provide a basis for continued analysis of the tested exhibit but are not specific for human sperm), and open to legal and scientific challenge.

#### **Principle of the Test**

SPERM HY-LITER™ PLUS uses an Alexa 488 derivatized mouse monoclonal antibody to human sperm heads to specifically identify human sperm from sexual assault evidence by fluorescence microscopy. The method requires a fluorescence microscope: processed slides can be visualized on *any* commercial fluorescence microscope fitted with the correct excitation and emission filters. In addition to a human sperm specific reagent, SPERM HY-LITER™ PLUS incorporates a second fluorescent dye that stains all nuclei present in the sample (4',6-diamidino-2-phenylindole, DAPI). Visualization of fluorescent nuclei is not required for sperm detection, but is recommended for both manual and automated sperm searches.

SPERM HY-LITER™ PLUS requires simple, sequential sample processing using provided solutions to attach, prepare, block and stain microscopic evidence for the detection of human sperm. Practitioners apply extracts to provided slides that are specially prepared for efficient attachment of biological material and have defined sample application areas such that consistent results can be achieved by all users. Processed slides may be visualized immediately with or without the addition of mounting media or a coverslip. Mounted slides are recommended for optimal visual quality. However, laboratories that intend to isolate sperm from stained preparations for DNA-STR analysis might consider leaving their preparations unmounted. Alternatively, mounted coverslips can be removed by soaking in water for several hours.

SPERM HY-LITER™ PLUS incorporates a fluorescent nucleic acid stain that can be used to locate all cells in the preparation: dual color analysis (DAPI and Fluorescein) can be used as an aid to visualizing crowded preparations and/or with image analysis software to electronically eliminate fluorescent background signals. This additional fluorescent stain is included in anticipation of the widespread use of automated sperm search software and the use of Laser Capture Microdissection methods. It is not required for the detection of human sperm.

## Reagents and Materials Provided

### i) Provided Solutions:

|                              |                |
|------------------------------|----------------|
| Fixative Solution            | store at 2-8°C |
| Sample Preparation Solution  | store at 2-8°C |
| Blocking Solution            | store at 2-8°C |
| Sperm Head Staining Solution | store at 2-8°C |
| Mounting Media               | store at 2-8°C |
| Wash Buffer 10X Stock        | store at RT    |

ii) 25, two (2) position masked slides (SPERM HY-LITER™ PLUS only). Store at RT.

iii) 50, 18 x 18 glass cover slips. Store at RT.

iv) Staining Protocol and Technical Information Sheet

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## User-Prepared Solutions

### i) 1 X Wash Solution

Users must prepare a 1X wash solution from the provided 10X stock – dilute provided stock 1:10 with laboratory quality H<sub>2</sub>O into a convenient wash/squirt bottle. Store at RT.

### ii) Sample Preparation Solution + DTT

Prepare Sample Preparation Solution + DTT daily before use: for each sample window to be stained, add 1 µl of freshly thawed 1 M DTT to two (2) drops of Sample Preparation Solution (yellow bottle cap) in a microcentrifuge tube, mix thoroughly. Laboratories that do not use 1 M DTT stock solutions should adjust DTT volumes accordingly; final concentration of DTT in Sample Preparation Solution should be ~12 mM.

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## Staining Protocol

1. Fixation: Add 2 drops of FIXATIVE Solution (white bottle cap) to each sample. Incubate at room temperature for 10 min. Wash: Use a wash/ squirt bottle to *gently* rinse each sample with ~2-3 mL of 1X wash buffer. Vigorous or lengthy washing or rinsing is *not* required. After the wash step, use the corner of a lab wipe to wick away residual wash buffer.

2. Sample Preparation: Add user-prepared SAMPLE PREPARATION Solution + DTT (~80 µl) to each sample. Incubate at room temperature for 30 min. Wash slide as described above.

3. Block: Add 2 drops of BLOCKING Solution (red bottle cap) to each sample. Incubate at room temperature for 30 min. Wash slide as described above.

4. Stain: Add 2 drops of SPERM HEAD STAINING Solution (green bottle cap) to each circular sample window. Incubate at room temperature for 30 min. Wash slide as described above.

Slides may be visualized immediately, or for better optical and photographic quality, mounted and coverslipped (see below). Processed slides should be stored at room temperature protected from light.

5. OPTIONAL - Mount: Add one drop of MOUNTING Solution (blue bottle cap) and place cover slip over each sample. Place slide between paper towels and gently press down to position coverslip and remove excess mounting media. Mounting media will semi-harden after 20 min at room temperature. Please note that warm and humid conditions can slow or prevent hardening of the mounting media. Coverslips may be stabilized by outlining with clear nail polish. Slides may be visualized immediately and are stable for several weeks stored in the dark at room temperature.

6. Archived slides previously stained with KPIC. See provided SPERM HY-LITER™ Staining Protocol Sheet.

7. To stain freshly prepared slides with KPIC followed by SPERM HY-LITER™. See provided SPERM HY-LITER™ Staining Protocol Sheet.

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## Visualization of Human Sperm Heads

Stained slides must be visualized using a fluorescence microscope fitted with appropriate filters. Cell nuclei, including epithelial and sperm, can be visualized using DAPI-compatible filters. Human sperm heads can be visualized using fluorescein or Alexa 488 compatible filters. Slides may be scanned at a final magnification of 100x, 200x, 400x or 1000x at the operator's discretion.

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## Specificity

SPERM HY-LITER™ PLUS is specific for human sperm heads. No cross-reactivity with epithelial cells, blood cells or animal semen from horse, bull, sheep, goat, pig, dog, cat and mouse has been observed. Sperm specific staining has been observed from gorilla and chimpanzee semen.

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## Test Sensitivity

When used as suggested the detection limit for SPERM HY-LITER™ PLUS is one human sperm head.

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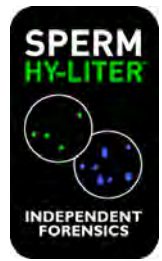


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# SPERM HY-LITER™

## SPERM HY-LITER PLUS™ Staining Protocol



### Kit Provided Solutions:

|                              |  |
|------------------------------|--|
| FIXATIVE Solution            | white bottle cap   |
| SAMPLE PREPARATION Solution  | yellow bottle cap ( <i>addition of DTT required before use</i> ) |
| BLOCKING Solution            | red bottle cap   |
| SPERM HEAD STAINING Solution | green bottle cap   |
| MOUNTING Media               | blue bottle cap  |
| WASH Buffer 10X Stock        | square 250 ml bottle ( <i>dilution required before use</i> )     |

### User-prepared solutions:

#### 1X Wash Solution

Prepare 1X wash solution from provided 10X Stock: dilute 1:10 with H<sub>2</sub>O into a convenient wash/squirt bottle.

#### Sample Preparation Solution + DTT

Prepare Sample Preparation + DTT daily before use: for each sample window to be stained, add

1 µl of 1 M DTT to two drops of Sample Preparation Solution (yellow bottle cap) in a microcentrifuge tube and mix thoroughly.

### Procedure:

- 1. Fixation:** Add 2 drops of **FIXATIVE Solution (white bottle cap)** to each circular sample window. Incubate at room temperature for 10 minutes.

**Wash:** Use a wash bottle to **gently** rinse each sample window with approximately 2-3 mL of 1X wash buffer. Vigorous washing or rinsing is **not** required. After the wash step, use a corner of a paper towel or a lab wipe to wick away the residual wash buffer in the sample window.

- 2. Sample Preparation:** *[DTT must be added to this solution prior to use! Please see User-Prepared Solutions above.]* Pipette user-prepared **SAMPLE PREPARATION Solution + DTT** (~ 80 µl) to each circular sample window. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

- 3. Block:** Add 2 drops of **BLOCKING Solution (red bottle cap)** to each circular sample window. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

- 4. Stain:** Add 2 drops of **SPERM HEAD STAINING Solution (green bottle cap)** to each circular sample window. Incubate at room temperature for 30 minutes.

**Wash:** Wash slide as described above.

- 5. OPTIONAL - Mount:** Add one drop of **MOUNTING Media (blue bottle cap)** to each circular sample window. Gently place provided cover slip over each sample window. Place slide between two small stacks of paper towels and gently press down to position coverslip and remove excess mounting media. Mounting media will semi-harden after 20 minutes at room temperature\*. Coverslips may be stabilized by outlining with clear nail polish. Slides may be visualized immediately and are stable for days.

**\*Please note that warm and humid conditions can slow or prevent hardening of the mounting media.** This will not affect the quality of the staining. To stabilize the coverslip, users can outline the edges of the coverslip with clear nail polish or Permount.

**6. Visualize:** Stained slides must be visualized using a fluorescence microscope fitted with appropriate filters. Cell nuclei, including epithelial and sperm, can be visualized using DAPI-compatible filters. Human sperm heads can be visualized using fluorescein or Alexa 488 compatible filters. Slides may be scanned at 10x, 20x, 40x or 100x at the operator's discretion.

## Additional Suggested Protocols:

### Extract Preparation:

Remove the fabric cutting, swab batting or the entire swab head using either a clean scalpel or a clean pair of scissors. Place cutting, batting or swab head in a microfuge tube.

Incubate the swab batting, swab head or cutting in PBS at room temperature for one hour. Laboratory personnel should use a volume of soak solution compatible with their own methods.

Remove swab batting, swab head or cutting from tube using Spin-Eze™, tweezers or similar, and pellet cells by centrifugation for 1 min at 13,000 X RPM.

Remove supernatant with fine-tipped pipette or similar.

Re-suspend pellet in 25-100 µl of PBS.

Remove ~10 µl of the re-suspended cells and place in a circular sample window of a **SPERM HY-LITER™** slide. Printed side of slide should be facing up.

Spread the sample evenly over the sample window using a pipette tip.

Allow the sample to air dry until no liquid remains in the sample window, approximately 15 minutes. Drying can be expedited by placing the slide in a 37°C incubator, under a desk light, or on a hot plate set to the **lowest** setting.

Dried slides may be stored for several weeks or processed immediately for **SPERM HY-LITER™** staining.

### Integration of KPIC staining and SPERM HY-LITER™:

**Archived slides previously stained with KPIC.** Mounting media, if present, must be removed before previously prepared slides can be stained with SPERM HY-LITER™. Permouted slides may be soaked in xylene to dissolve the media.

Proceed with SPERM HY-LITER™ staining as per protocol. Archived KPIC slides will demonstrate weaker SPERM HY-LITER™ sperm staining; more intense staining may be observed by increasing the DTT concentration in the Sample Preparation Solution by 10X (i.e., 10 µl of 1M DTT per two drops of Sample Preparation Solution – Yellow Capped bottle).

**To stain freshly prepared slides with KPIC followed by SPERM HY-LITER™.** We recommend that analysts slightly modify their existing procedure by substituting the final ethanol wash in the KPIC protocol with a gentle water rinse. The SPERM HY-LITER™ protocol may then be followed as described.

*NOT FOR IN VITRO DIAGNOSTIC USE*

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# **SPERM HY-LITER™**

## **Representative Images and Troubleshooting Guide**

# SPERM HY-LITER™ Staining - Normal

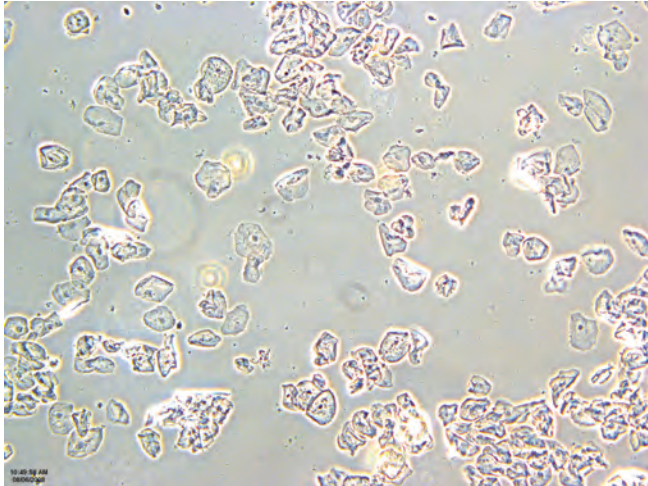
On this, and the following page, Phase, FITC, DAPI and DUAL staining using 10X, 20X and 40X objectives are shown. As the objectives used on the field increase, note the blue fluorescent detection of all nuclei with the DAPI filter, the green fluorescent detection of sperm heads with the FITC filter and the three-dimensional structure visible with phase contrast. Both epithelial and sperm can be seen in the same image with the Dual filter. Examples shown are from swab extracts of epithelial and sperm cells.

## PHASE CONTRAST

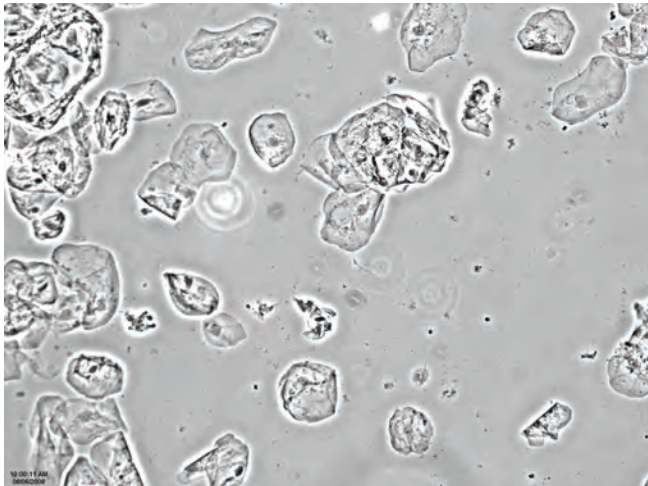
## FITC

SPERM HEADS ARE GREEN

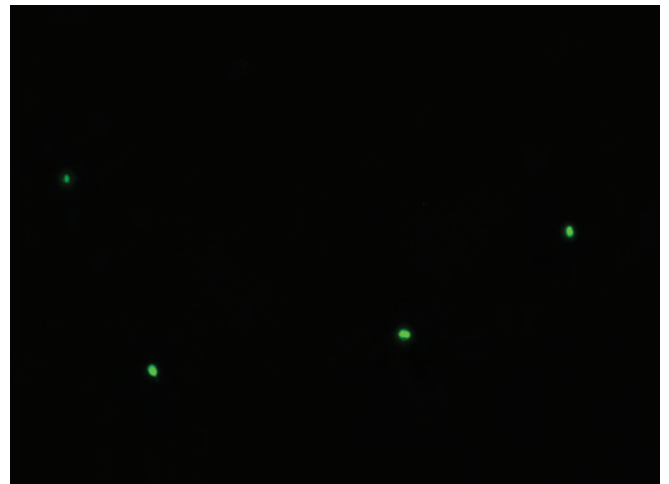
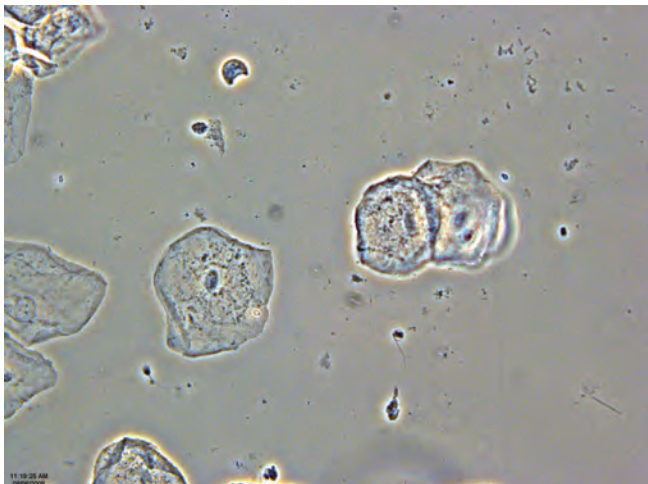
10X



20X



40X



# SPERM HY-LITER™ Staining - Normal

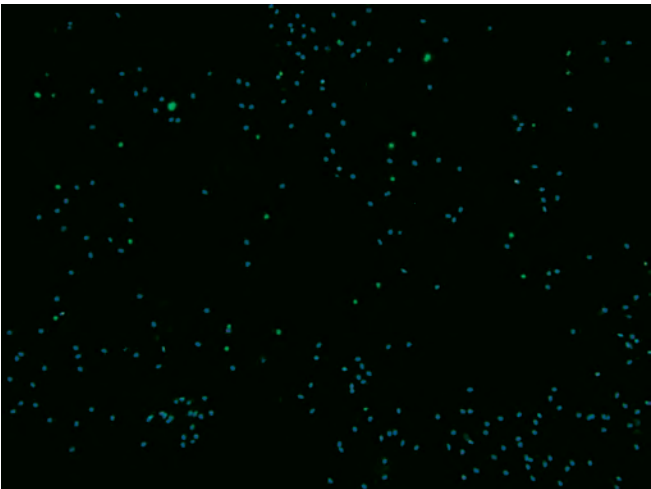
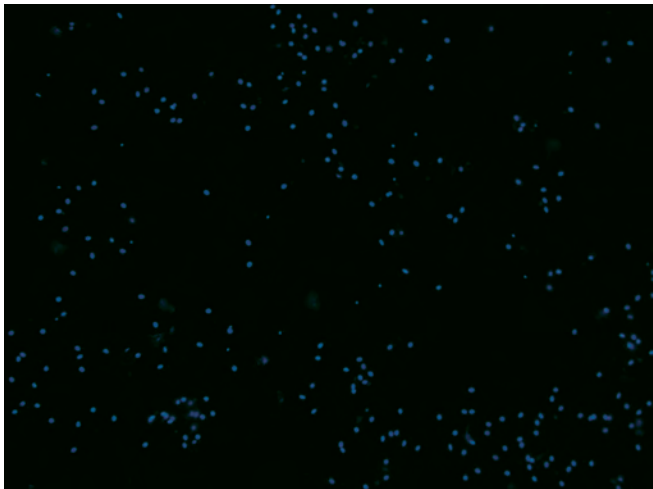
## DAPI

ALL CELL NUCLEI ARE BLUE

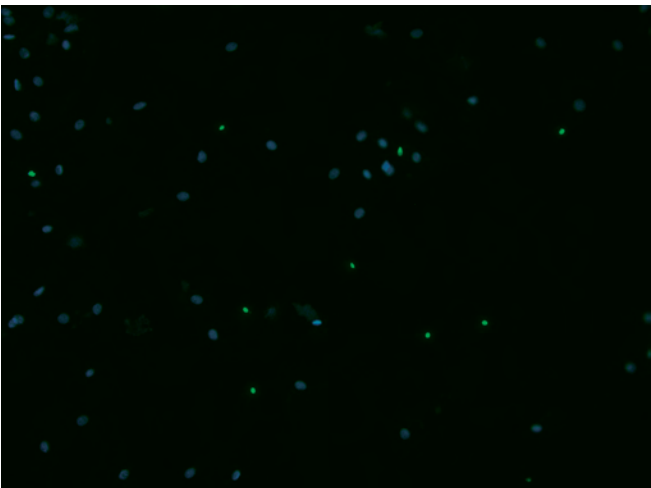
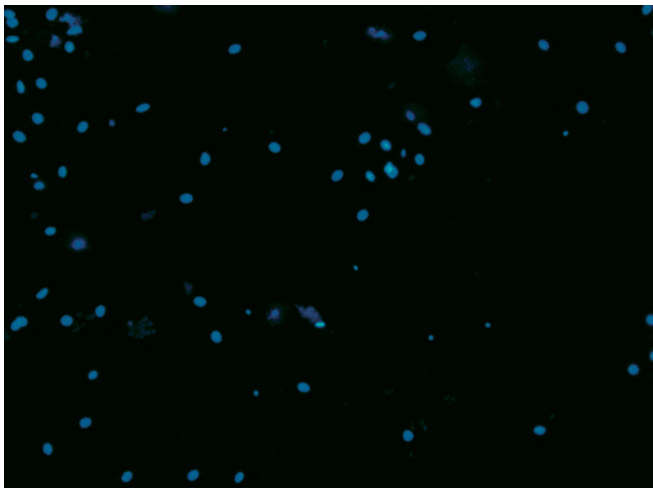
## DUAL

SPERM HEADS ARE GREEN  
REMAINING CELL NUCLEI ARE BLUE

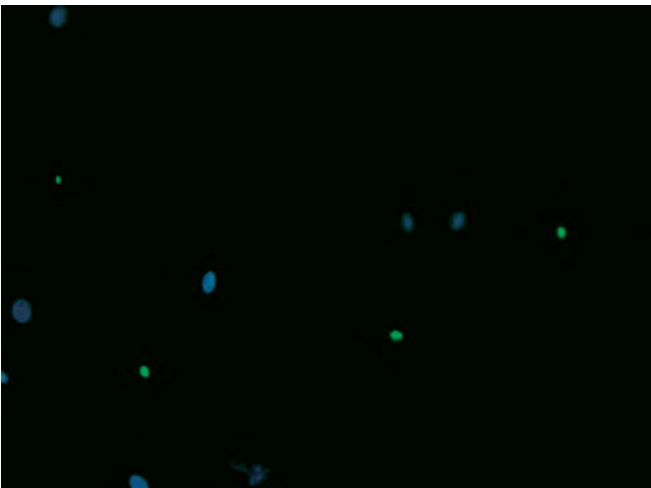
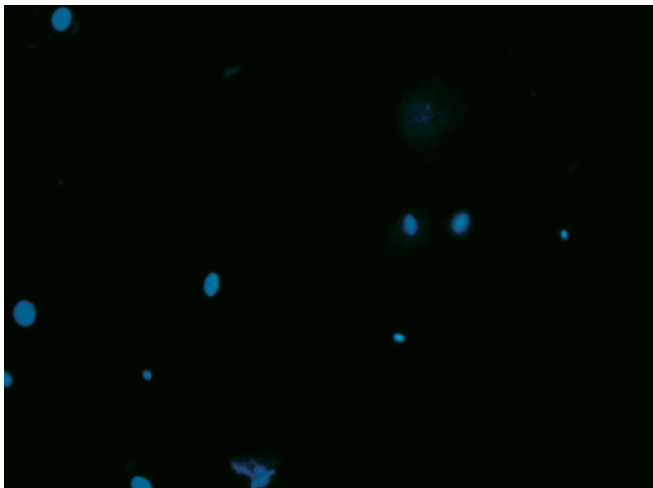
10X



20X



40X

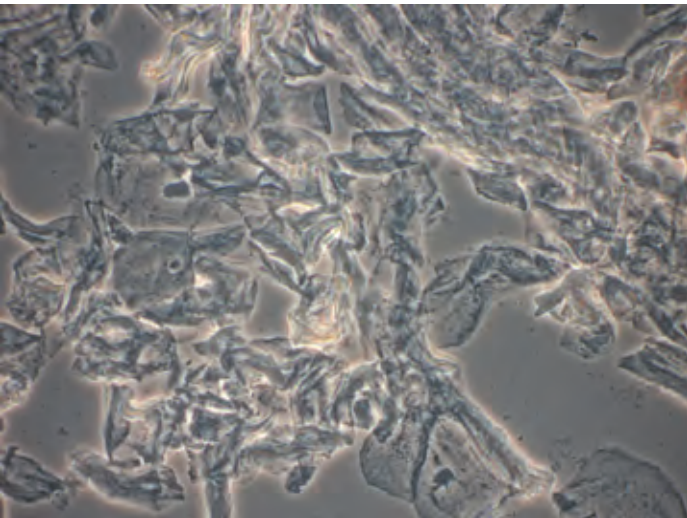


# SPERM HY-LITER™

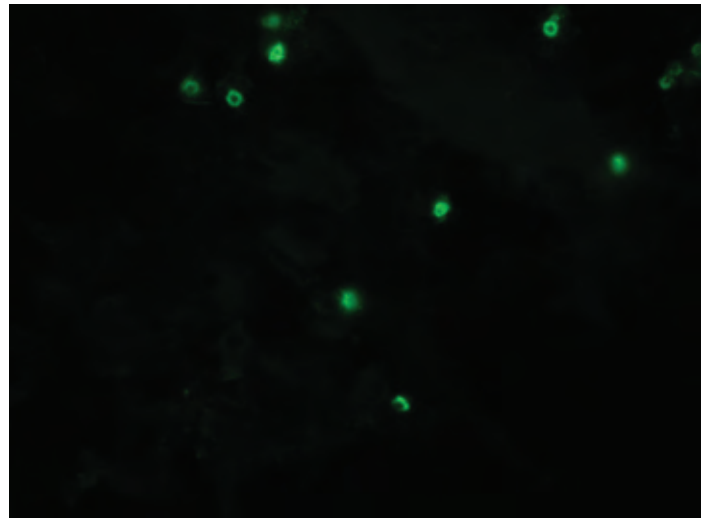
## Normal Staining - Post-Coital Sample High Density

Phase, FITC and DAPI staining (using a 40X objective) of two slides prepared from an extract of a post-coital swab (4 hours after intercourse) are shown. Note the cellular debris and morphologically indistinct sperm. Sperm were identified by SPERM HY-LITER™ staining.

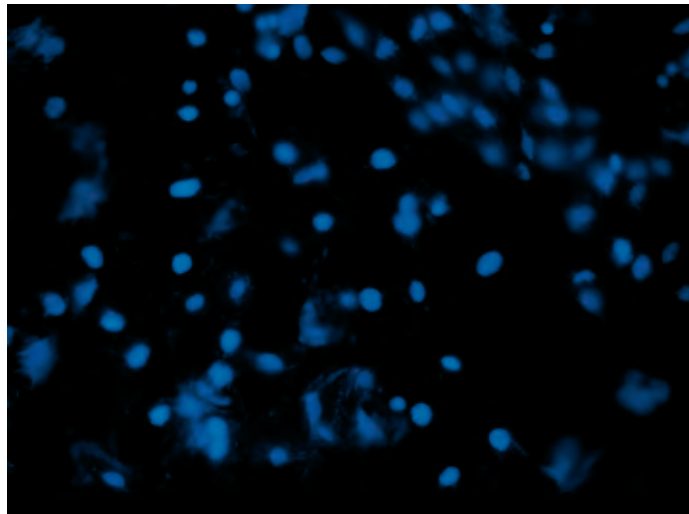
**PHASE**



**FITC**



**DAPI**

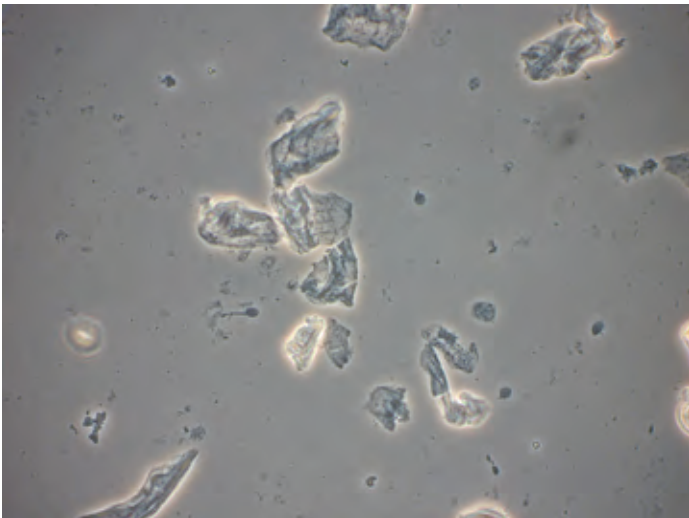


# SPERM HY-LITER™

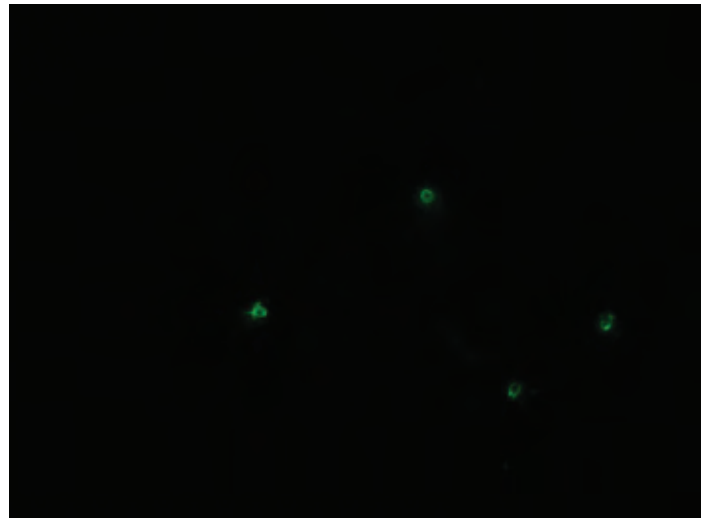
## Normal Staining - Post-Coital Sample Low Density

Phase, FITC and DAPI staining (using a 40X objective) of two slides prepared from an extract of a post-coital swab (4 hours after intercourse) are shown. Note the cellular debris and morphologically indistinct sperm. Sperm were identified by SPERM HY-LITER™ staining.

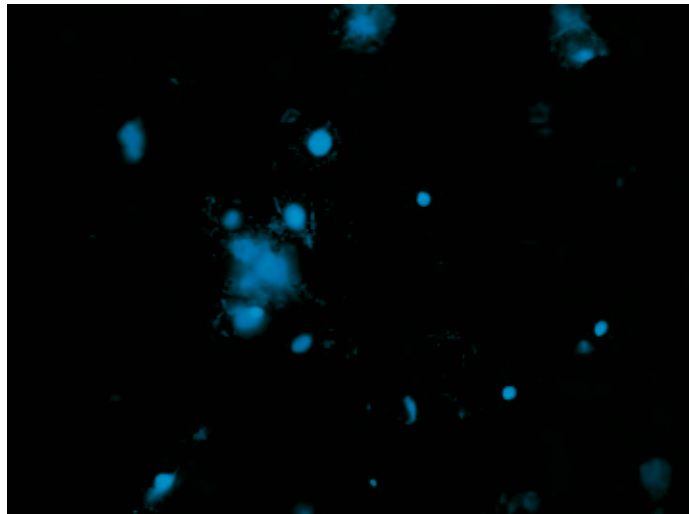
**PHASE**



**FITC**



**DAPI**



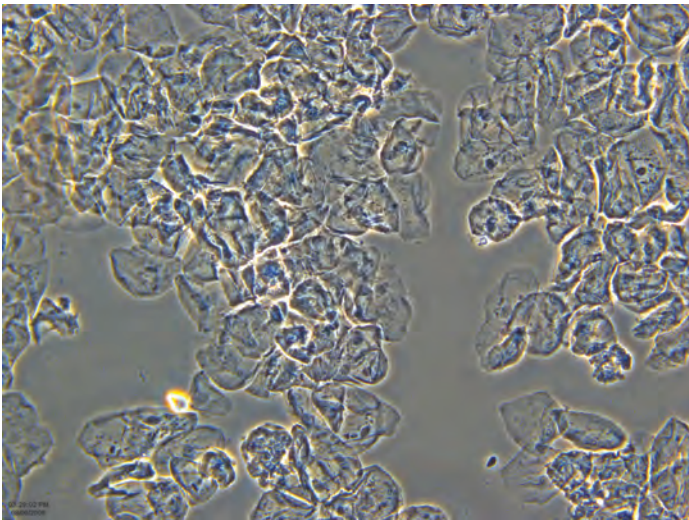


# SPERM HY-LITER™

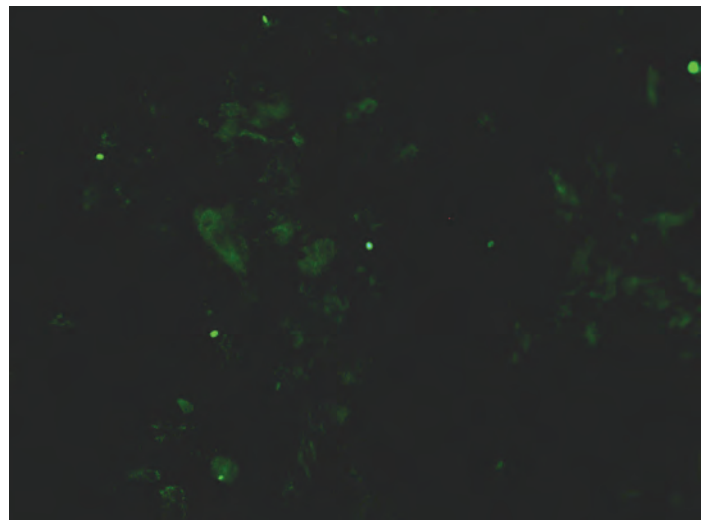
## Normal Staining – Increased Background Immunofluorescence

Phase, FITC, and DAPI staining (using a 20X objective) of a slide prepared from a post-coital swab. A characteristic of immunofluorescence is “background staining”; all fluorescently stained preparations will have some level of background signal. Background staining refers to non-specific interactions between the fluorophore and cellular structures. It can appear as a hazy cloud or a brightly fluorescent structure clinging to debris or ‘junk’. Background staining is a normal feature of immuno-fluorescent detection but can be reduced with a “blocking” step to inhibit non-specific interactions of the antibody or fluorophore. In practice, the fluorescence of sperm heads will be much brighter than background staining. We strongly recommend the use of controls (both positive and negative) and where necessary, mixing experiments, in order to correctly identify sperm from preparations with unacceptably high background. The experienced analyst will have no trouble identifying sperm from the vast majority of evidence samples.

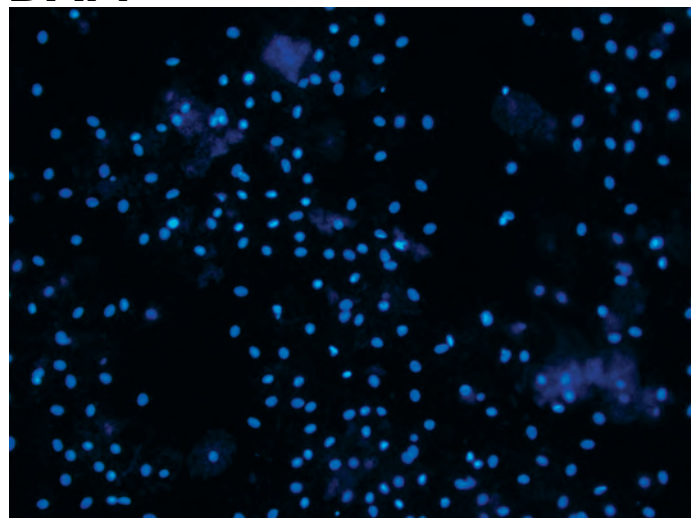
**PHASE**



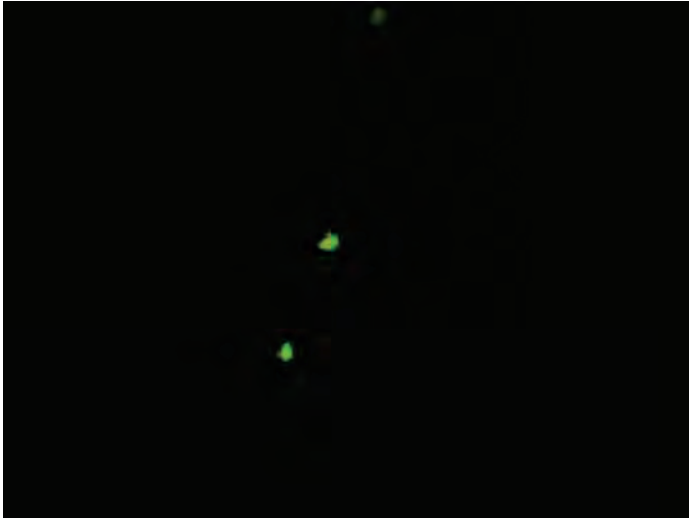
**FITC**



**DAPI**



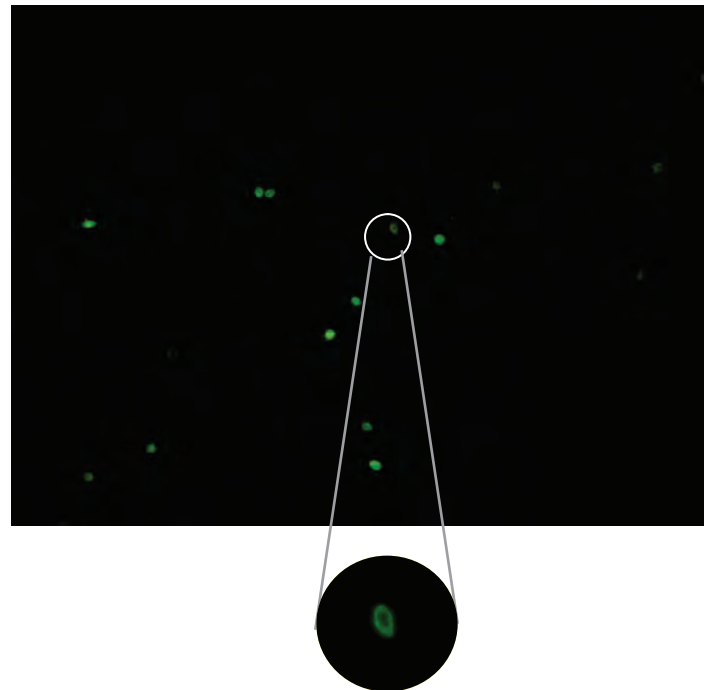
# SPERM HY-LITER™ Staining – Excess of DTT



SPERM HY-LITER™ staining was performed on extracts from semen and buccal swabs with an excess of DTT in the sample preparation solution in the SPERM HY-LITER™ protocol. The FITC fluorescence using a 40X objective is shown. Note the sperm heads are swollen and the fluorescence is throughout the sperm head.

# SPERM HY-LITER™ Staining – Insufficient DTT

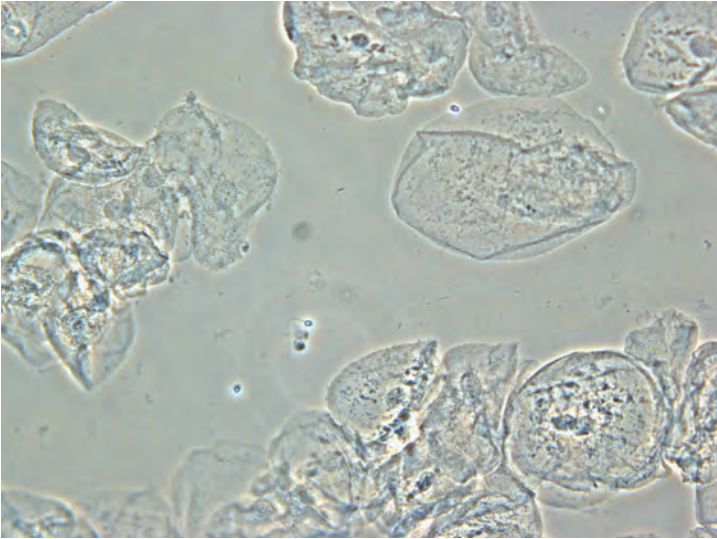
SPERM HY-LITER™ staining was performed on extracts from semen and buccal swabs with insufficient DTT in the sample preparation solution. The FITC fluorescence using a 40X objective is shown. Note the decreased fluorescence of the sperm heads overall and the ring-like staining around the edge of the sperm heads.



# SPERM HY-LITER™ Staining – Omission of Sample Preparation Solution

SPERM HY-LITER™ staining was performed on extracts from semen and buccal swabs with omission of the sample preparation solution in the SPERM HY-LITER™ protocol. Phase, FITC, DAPI and Dual fluorescence using a 40X objective are shown. Note the absence of FITC fluorescence of the sperm heads and the blue fluorescent detection of all nuclei with the DAPI filter. Note that only the blue fluorescence of the nuclei of the epithelial cells and sperm heads can be seen with the Dual filter. This effect can also be seen when the DTT has not been added or is not freshly thawed. DTT should have a strong pungent odor.

**PHASE**



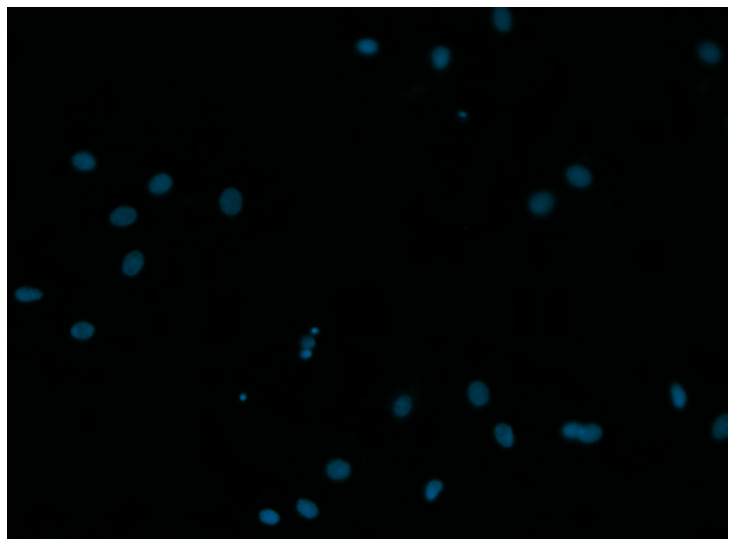
**FITC**



**DAPI**



**DUAL**



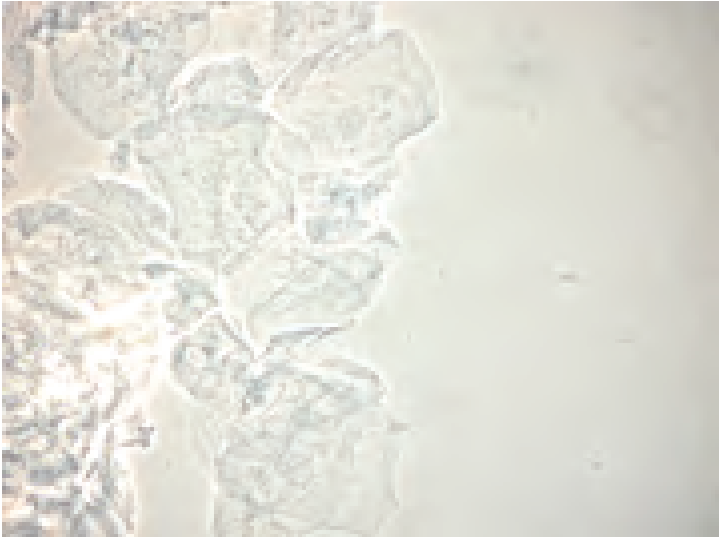


# SPERM HY-LITER™ Staining – Omission of Blocking Solution

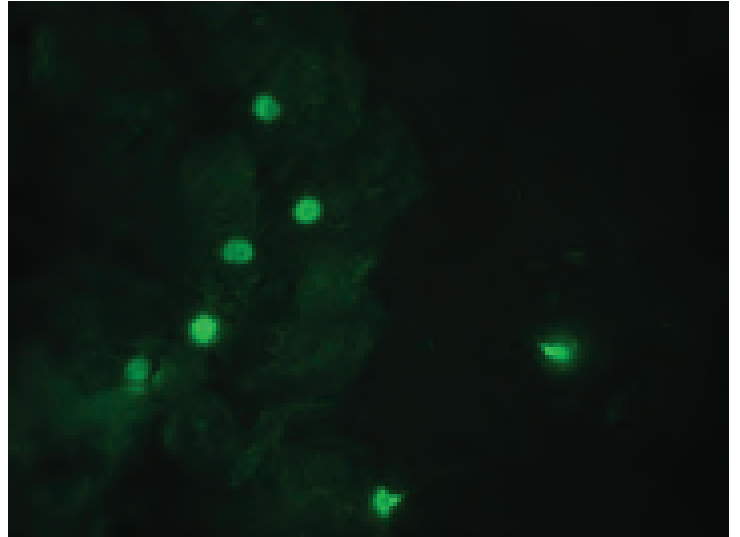
Note that DAPI is part of the blocking solution

SPERM HY-LITER™ staining was performed on extracts from semen and buccal swabs with omission of the blocking solution in the SPERM HY-LITER™ protocol. Phase, FITC, DAPI and Dual fluorescence using a 40X objective are shown. Note the absence of DAPI fluorescence of any cells in the sample and the presence of only green fluorescent sperm heads in the Dual filter. Also, the background in the FITC picture is noticeably higher.

**PHASE**



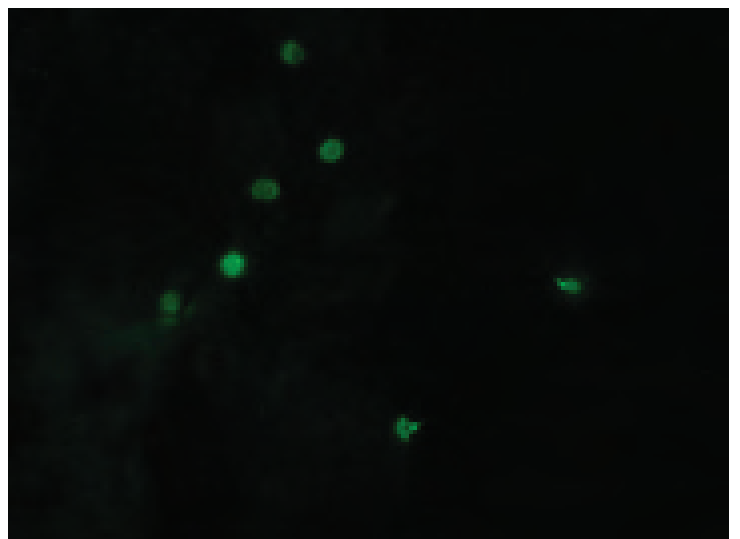
**FITC**



**DAPI**



**DUAL**



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

**Independent Forensics**  
**4600 West Roosevelt Road**  
**Suite 201**  
**Hillside, IL 60162**  
**USA**

**Telephone: (708) 234-1200**  
**Fax: (708) 978-5115**

**e-mail: [info@ifi-test.com](mailto:info@ifi-test.com)**  
**[www.IFI-test.com](http://www.IFI-test.com)**

## **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 Tree 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.

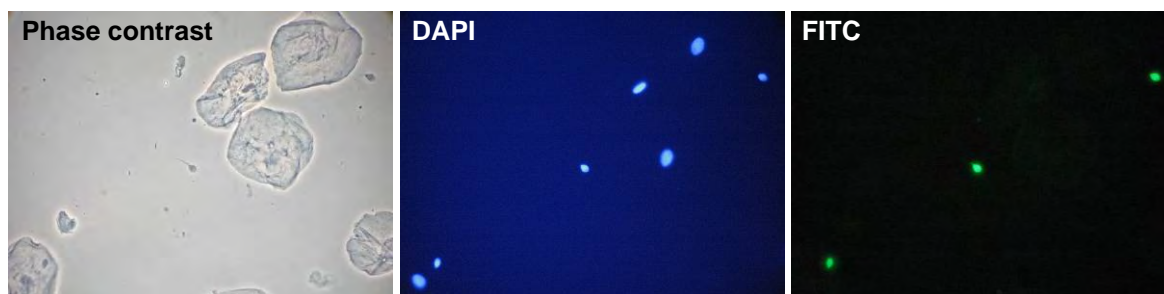
Here we present results using a reagent that has the potential to (1) provide the first scientifically justifiable and defensible method for the forensic identification of human sperm, and (2) greatly increase the efficiency of microscopic sperm searches for laboratory analysts.

## **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).



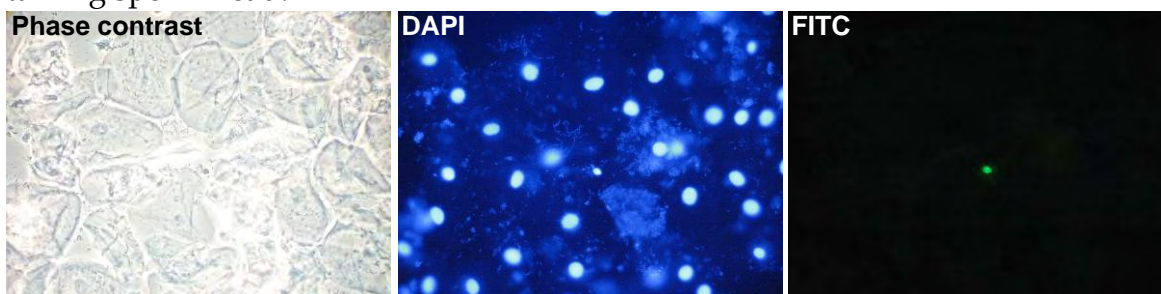
**Conclusions:** **SPERM HY-LITER™** fluorescently labels only human sperm heads; no FITC signal can be observed from epithelial cells. Unlike KPIC or H&E staining, **SPERM HY-LITER™** is cell type specific as only sperm cells are labeled. Fluorescent sperm heads are easily visible with negligible background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



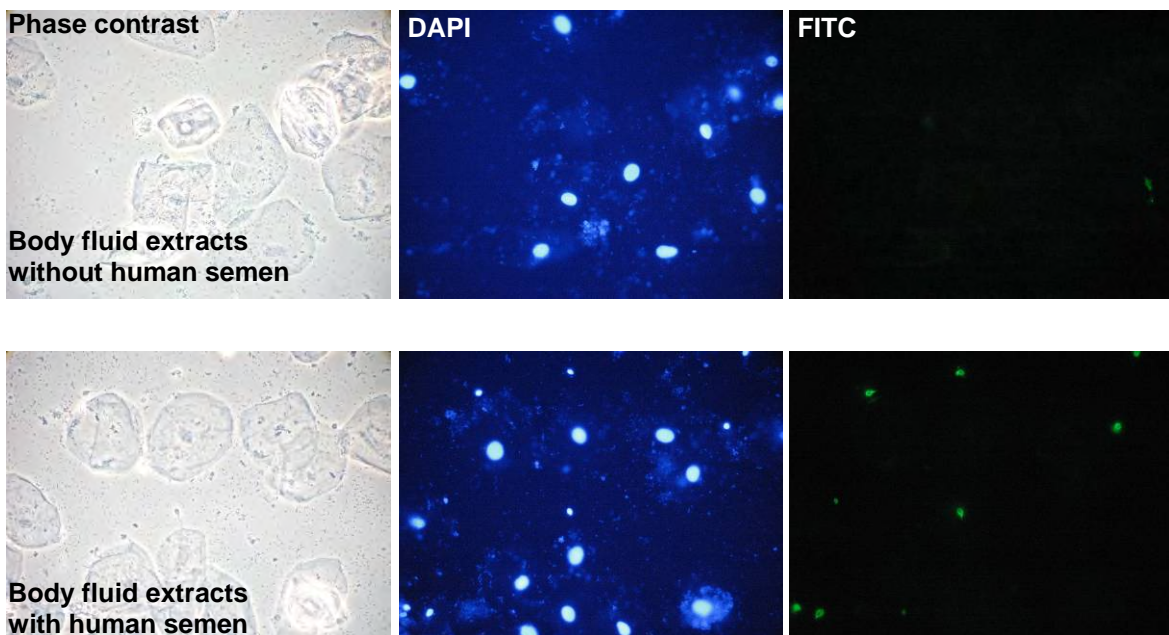
**Conclusions:** The **SPERM HY-LITER™** sexual assault evidence staining kit has sufficient sensitivity to identify a single sperm head present in an overwhelming background of epithelial cells.

## **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 µl 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.

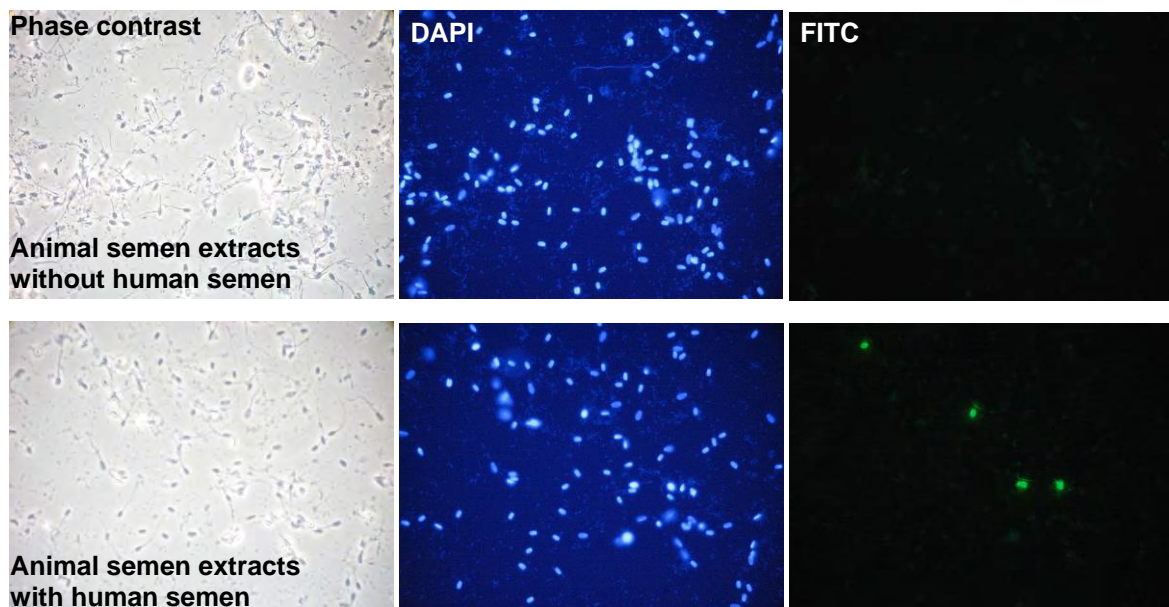


## SPERM HY-LITER™ Does Not Cross-React with Animal Sperm

**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.



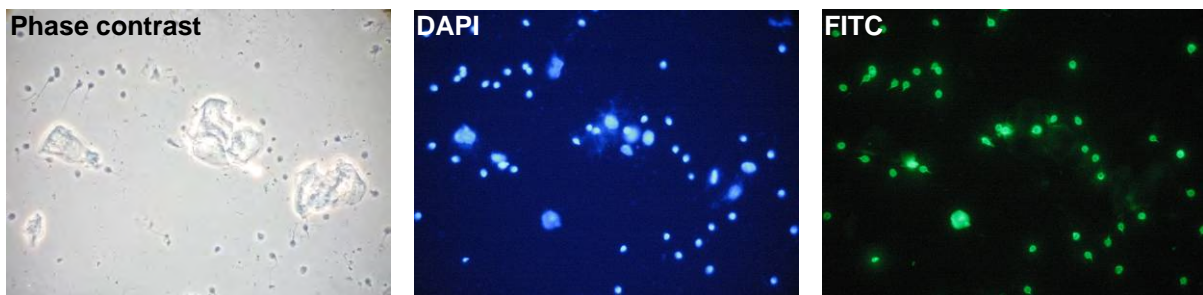
**Conclusions:** **SPERM HY-LITER™** does not label or cross-react with animal sperm from the tested species: dog, cat, cow, horse, goat, sheep, pig, and mouse. In addition the presence of non-human animal sperm does not interfere with the ability **SPERM HY-LITER™** to specifically detect human sperm.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

## **SPERM HY-LITER™ 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.



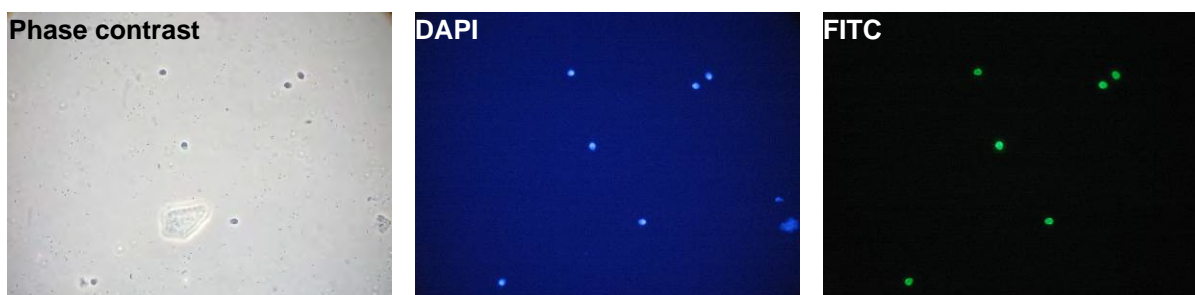
**Conclusions:** Post-coital vaginal swabs can be successfully analyzed using **SPERM HY-LITER™**. Importantly, **SPERM HY-LITER™** specifically and sensitively labels human sperm from post-coital vaginal swab extracts: no cellular cross reaction or inhibition was observed.

## **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.



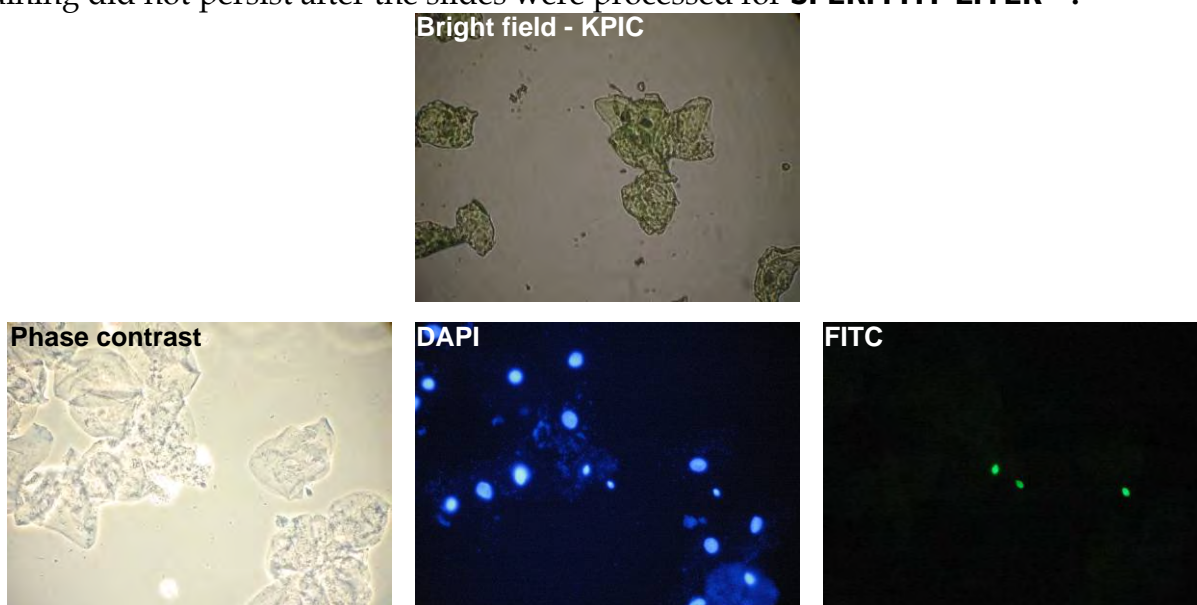
**Conclusions:** Post-coital vaginal smear slides, a common form of sexual assault evidence, are easily processed using **SPERM HY-LITER™**. Importantly, **SPERM HY-LITER™** specifically and sensitively labels human sperm present on post-coital vaginal smear slides.

## **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.

## **SPERM HY-LITER™**

### **Validation Protocol**

Independent Forensics  
Hillside, Illinois

Forensic laboratories must perform validation studies on the critical reagents and techniques used to process and analyze evidence. The requirement for validation derives from the scientific and legal obligation to demonstrate that a method or reagent provides robust, reproducible, precise and accurate results that can be relied upon by the courts. In addition, validation studies are required by external auditors of accreditation agencies in order to demonstrate compliance with published laboratory standards. Generally, validation studies do not break new scientific ground, but are designed to demonstrate compliance with accreditation standards, to record the thorough testing of a technique or reagent, and to provide an ongoing record of the reliability of the method or reagent to continue to provide results that can be relied upon. In broad terms validation reinforces an already accepted method, and sets the scientific boundaries of the technique for each individual laboratory.

Many forensic DNA laboratories perform microscopic sperm searches on sexual assault evidence using a general cell stain, Kernechtrot-Picroindigocarmine (KPIC, sometimes described as Christmas Tree Stain due to the red nuclei and green cytoplasm of the cells) and relying on the experience of the analyst to identify some morphological remnant of sperm to confirm the identification. Given the variety and condition of sexual assault evidence, and this non-specific staining method, identifying sperm to a reasonable degree of scientific certainty is neither straightforward, nor often possible. SPERM HY-LITER™, a new commercially available microscopic staining method for the identification of sperm from sexual assault evidence is specifically designed to overcome the inherent issues with current forensic sperm identification methods.

Labs who desire a more sensitive, specific, and efficient sperm search method, SPERM HY-LITER™ staining of sexual assault evidence should be integrated into the laboratory's work flow in place of histological staining (KPIC, H & E, etc.). SPERM HY-LITER™ slides can be visualized with phase microscopy prior to staining in order to verify the presence of biological material and in rare instances, when intact sperm are abundant, sperm may be identified. In most instances, where few sperm that lack morphological details are present, the slide can then be stained with SPERM HY-LITER™ in accordance with the manufacturer's suggested protocol. Subsequent to SPERM HY-LITER™ staining, slides can be visualized using fluorescence microscopy to reveal clearly labeled, fluorescent green, human sperm heads (when present).

SPERM HY-LITER derives its specificity from a unique monoclonal antibody that specifically identifies human sperm heads. Monoclonal antibodies are widely used for their ability to confer specificity and have a long history in forensic serology. The sensitivity of SPERM HY-LITER™ is a direct result of the signal-to-noise advantage of fluorescent microscopy; by coupling a highly fluorescent 'tag' (Alexa 488) to the monoclonal antibody, the microscopic screening of sperm is considerably enhanced. Fluorescent immunostaining is a widely used and robust technique that is in current use both in medical diagnostics and screening.

Laboratories that choose to upgrade their ability to identify sperm from sexual assault evidence case work must validate the procedure. Here we outline a series of experiments that will demonstrate the cell type specificity of the method, the species specificity of the method, the sensitivity of the method and its reproducibility. By performing the described experiments, documenting their results and concluding with a summary of this work, forensic laboratories will have a fully validated procedure that can be used to examine case work, reduce backlogs of sexual assault evidence and for the first time be able to confidently identify sperm to a reasonable degree of scientific certainty from any evidence sample.



### **Cell type specificity:**

Staining of various body fluid extracts with and without human sperm: Human semen may be acquired from sperm banks or volunteers (samples from un-vasectomized males). Samples of human blood, urine, saliva, etc. should be acquired from volunteers.

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| Samples to test: | Blood sample - no sperm<br>Saliva sample- no sperm<br>Urine sample - no sperm<br>Epithelial cell sample (buccal and/or vaginal) – no sperm<br><br>Blood sample – human sperm added<br>Saliva sample - human sperm added<br>Urine sample - human sperm added<br>Epithelial cell sample (buccal and/or vaginal)– human sperm added                 |
| Procedure:       | Biological material should be extracted from swabs in a minimal volume Phosphate Buffered Saline pH 7.4 (PBS). Five to twenty microliters of extract should then be added to SPERM HY-LITER™ masked slides and air dried prior to staining in accordance with the manufacturer's provided protocol. Results should be recorded and images saved. |
| Typical Results: | Cells present in blood, saliva, urine and epithelial extracts are not labeled, but when human sperm cells are present (alone or in body fluid extract mixtures) they are labeled fluorescent green.  |
| Conclusions:     | Cells present in blood, saliva, urine, and epithelial extracts are not labeled.<br>Sperm is detected from mixtures of blood, saliva, urine, epithelial cells.<br>Cells present in blood, saliva, urine, and epithelial extracts do not inhibit sperm labeling.   |

### **Sample type specificity:**

Staining of various swabs and stains with and without human sperm: Human semen may be acquired from sperm banks or volunteers (samples from un-vasectomized males). Oral, anal, and vaginal swabs should be acquired from volunteers. Moistened swabs may be used to, prepare sample smear slides and liquid semen can be used to prepare stains on a variety of common fabrics.

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| Samples to test: | Oral swab - no sperm<br>Vaginal swab – no sperm<br>Anal swab – no sperm<br>Fabric cutting (various common fabrics) - no sperm<br>Vaginal swab smear slide – no sperm<br><br>Oral swab sample – human sperm added<br>Vaginal swab sample – human sperm added<br>Anal swab – human sperm added<br>Fabric cutting (various common fabrics) – human sperm added<br>Vaginal swab smear slide – human sperm added |
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| Procedure:       | Biological material should be extracted from swabs and fabric cuttings in a minimal volume PBS. Five to twenty microliters of extract should then be added to SPERM HY-LITER™ masked slides and air dried prior to staining in accordance with the manufacturer's provided protocol. Biological material present on smear slides should be encircled with provided hydrophobic barrier pen prior to staining. Results should be recorded and images saved. |
| Typical Results: | Cells, bacteria, fibers, etc. present in various sample types are not labeled, but when human sperm cells are present (alone or in mixtures of various sample type extracts) they are labeled fluorescent green. Note that some fibers (cotton) may be fluorescent in all three filters.   |
| Conclusions:     | Material (cells, bacteria, dye, etc.) present in oral, vaginal, and fabric extracts are not labeled.<br><br>When present, sperm is detected from oral, vaginal, and fabric extracts. Material (cells, bacteria, dye, etc.) present in oral, vaginal, and fabric extracts do not inhibit sperm labeling.  |

**Species specificity:**

Staining of various animal sperm samples with and without human sperm: Human semen may be acquired from sperm banks or volunteers (samples from un-vasectomized males). Animal semen can be acquired from breeders and/or zoos. Outdated or otherwise expired semen samples can be requested at reduced prices or may be provided free of charge.

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| Samples to test: | Sperm from domestic pets and farm animals – no human sperm added<br><br>Sperm from domestic and barnyard animals – human sperm added   |
| Procedure:       | Animal and human sperm should be extracted from swabs in a minimal volume PBS. Five to twenty microliters of extract should then be added to SPERM HY-LITER™ masked slides and air dried prior to staining in accordance with the manufacturer's provided protocol. Results should be recorded and images saved. |
| Typical Results: | Animal sperm cells are not labeled, but when human sperm cells are present (alone or in animal semen extract mixtures) they are labeled fluorescent green.   |
| Conclusions:     | Animal sperm is not labeled.<br>Human sperm is labeled in animal/human sperm mixtures.<br>Animal sperm does not inhibit human sperm labeling.<br>Staining is specific for human sperm.   |

**Sensitivity:**

Staining of increasingly diluted sperm samples: Human semen may be acquired from sperm banks or volunteers (samples from un-vasectomized males). Due to the wide variation in sperm counts, dilution results may vary or need to be adjusted when semen from different samples are used.

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| Samples to test: | Undiluted human sperm<br>Human sperm diluted 1/10 with PBS<br>Human sperm diluted 1/100 with PBS<br>Human sperm diluted 1/1,000 with PBS   |
| Procedure:       | Undiluted and PBS diluted sperm should be added to cotton swabs, air dried, and extracted in one milliliter of PBS. The swab head should then be removed and the sperm pelleted by spinning briefly in a tabletop centrifuge. Next the pellet should be re-suspended in 20 microliters of extract and added to SPERM HY-LITER™ masked slides, air dried, and stained in accordance with the manufacturer's provided protocol. Results should be recorded and images saved. |
| Typical Results: | As the human semen extract is diluted to a greater degree, the number of sperm present on each slide is decreased. At certain dilutions, very few sperm will be present on the slide, but any sperm present will be labeled fluorescent green. The exact dilution factor that provides very few, but more than zero, sperm will vary from sample to sample due to sperm count variation.   |
| Conclusions:     | Single sperm heads can be visualized.  |

## **Additional Uses of SPERM HY-LITER™**

### **Staining of previously screened slides**

Staining of previously analyzed sexual assault evidence slides (KPIC, H & E, phase contrast)

**Samples to test:** Samples that have been previously subjected to laboratory's currently validated sperm search SOP – no sperm identified

Samples that have been previously subjected to laboratory's currently validated sperm search SOP – sperm identified

**Procedure:** Previously stained slides must be free of non-aqueous mounting media. PermMount or CytoSeal mounted slides must be soaked in xylene or toluene (respectively) to remove all traces of mounting media before SPERM HY-LITER™ staining. If required, removal of PermMount or CytoSeal can be incorporated into the Archived Slide SOP.

**Typical Results:** Human sperm cells that have been previously stained with histological stains can be successfully stained with SPERM HY-LITER™. A greater number of sperm may be visualized after SPERM HY-LITER™ staining than after histological staining. Some slides that were determined to be negative for human sperm may be found to contain human sperm after SPERM HY-LITER™ staining. Due to the increased number of slide manipulations (heat fixing, ethanol washes, SPERM HY-LITER™ staining) the final staining result (especially with respect to sperm morphology) may appear slightly different from slides that have not been previously stained histologically. However, if human sperm are present they will be labeled and identified.

**Conclusions:** Sperm from previously analyzed sexual assault evidence can/cannot be labeled.

### **Validation Summary Document:**

Summarize number and types of samples tested for each validation test and conclusion from data obtained. Laboratory auditors expect summary documents for all validation studies.



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# SPERM HY-LITER™

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- **SPECIFIC**
- **SENSITIVE**
- **FLEXIBLE**
- **AUTOMATION**

COMPLETE FLUORESCENT STAINING  
KIT FOR SEXUAL ASSAULT EVIDENCE

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