A. PURPOSE:

To identify spermatozoa in Forensic samples.

B. RESPONSIBILITY:

Forensic Science Examiners from the Connecticut State Forensic Science Laboratory who have been trained in the discipline of identifying spermatozoa according to SOP-FB-31 (Training Manual).

C. SAFETY:

Use appropriate measures for the proper handling of the Picroindigocarmine and DTT solutions according to SOP-GL-2 (Safety Manual).

D. DEFINITIONS:

1. DTT: Dithiothreitol solution
2. KS: Kernechtrot solution
3. PICS: Picroindigocarmine solution
4. OCME: Office of the Chief Medical Examiner
5. AP: Acid Phosphatase

E. PROCEDURE:

These staining procedures will be performed at the discretion of the examiner based on the submitting agency requests, case information and condition of the evidence.

1. Materials:
   - a. Hospital/OCME prepared smears
   - b. Samples
   - c. Positive AP swab
   - d. Micropipet and tips
   - e. Microcentrifuge/centrifuge tubes and spin baskets
   - f. Glass slides
   - g. Christmas Tree Stain
     - aa. KS stain*
     - bb. PICS stain*
     - cc. Distilled Water
     - dd. Ethanol
     - ee. Microscopes

       * provided by the manufacturer

E. 1. h. Sperm Hy-Liter Stain

aa. RSID™-Universal Buffer
bb. Swabs
cc. Aliquot of diluted spermic semen
dd. Known spermic semen/epithelial cell swab
ee. Known spermic semen swab
ff. Known epithelial cell swab
gg. Fixative Solution*
hh. Sample Preparation Solution*
ii. 1M DTT solution
jj. Blocking Solution*
kk. Sperm Head Staining Solution*
ll. Mounting Media*
mm. 1X Wash Buffer (from 10X Wash Buffer*)
nn. ImmEdge Pen*
oo. Sperm Hy-Liter masked slides*
pp. Cover slips*
qq. Clear nail polish
rr. Sperm Hy-Liter Microscope System

* provided by the manufacturer

2. Procedures:

Christmas Tree

a. For Hospital/OCME prepared smears, proceed with step 'd' below.

b. Sample preparation

aa. For extracted samples, transfer the sample into a spin basketand place back into tube. Centrifuge within the range of 10,000 – 14,000 rpm (13,000 rpm is recommended) for 10 minutes.

For a sample submitted in a liquid form (ie. urine or vomit suspected to contain semen) transfer the sample into microcentrifuge/centrifuge tube(s). Centrifuge within the range of 10,000 – 14,000 rpm (13,000 rpm is recommended) for 10 minutes. If necessary, decant excess liquid back into the original container.

bb. Remove 3µl of the pellet with a micropipet onto a glass slide and form a smear.

cc. For a liquid sample suspected to be semen, a portion of the sample may be transferred directly onto a glass slide to form a smear.

dd. If a positive acid phosphatase result was observed, the swab may be used to form a smear directly onto a glass slide.

c. Allow the slide to air dry or dry at 37°C.

E. 2. d. Cover the smear with KS stain and allow to stand for 20 minutes.

e. Gently rinse KS off slide with distilled water.

f. Cover the smear with PICS stain and allow to stand for 15-20 seconds.

g. Gently rinse PICS off slide with ethanol* and blot dry or air dry at room temperature.
*Wash off with dH_2O if Sperm Hy-Liter procedure may be needed.

h. Examine the smear for spermatozoa and other cells under the microscope at 200X, verify under 400X if needed.

<u>Sperm Hy-Liter</u>

a. Hospital/OCME prepared smears

aa. Prepare a sperm/epithelial cell control for Hospital/OCME prepared smear:

- Collect an epithelial cell sample on a swab and form a smear onto a glass slide.
- With a micropipet place approximately 3µl of thawed diluted spermic semen onto the smear. Re-freeze the remaining semen aliquot.
- Dry the positive control smear at room temperature or 37°C (do not apply open flame heat).
- Control smears may be made in advance. Store at room temperature and replace as needed. Record appropriate information on the Body Fluid Standard Reagent Log Sheet.

bb. Shake the ImmEdge Pen. Then depress the tip of the ImmEdge Pen onto the inside wall of a microcentrifuge tube to release reagent (need ~50-100µl per smear). Pipette the reagent around the positive control smear and questioned smear(s), then allow to dry at room temperature or 37°C.

cc. Stain the sperm/epithelial cell control smear concurrently with the questioned smears.

b Case Sample Extract smears made directly from the pellet

aa. Prepare the extracted sample smear:

- Transfer the sample into a spin basket and place back into the tube. Centrifuge within the range of 10,000 – 14,000 rpm (13,000 rpm is recommended) for 10 minutes.

E. 2. b.

- Remove 3µl of the pellet with a micropipet to a glass slide (standard or Sperm Hy-Liter) and form a smear.
- Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).

bb. Prepare the sperm/epithelial cell control for smears made directly from pellet:

- Extract a known spermic semen/epithelial cell swab in a microcentrifuge tube with 350µl of RSID-Universal Buffer.
- Incubate for 30 minutes in the ultrasonic bath.
- Follow steps from aa above.

cc. If made on a standard glass slide: Shake the ImmEdge Pen, then depress the tip of the ImmEdge Pen onto the inside wall of a microcentrifuge tube to release reagent (need ~50-100µl per smear). Pipette the reagent around the positive control smear and questioned smear(s), then allow to dry at room temperature or 37°C.

dd. Stain the sperm/epithelial cell control smear concurrently with the questioned smears.

ee. Record this type of extract smear was made on the appropriate Quality Record Worksheet.

c. Case Sample Extract smears made from re-suspended pellet

aa. Prepare extracted sample smear:

- Transfer the sample into a spin basket and place back into the tube. Centrifuge within the range of 10,000 – 14,000 rpm (13,000 rpm is recommended) for 10 minutes.
- Remove 300µl of the supernatant, place into a new microcentrifuge tube and set aside (do not discard).
- Re-suspend the pellet in the remaining supernatant.
- Place 10µl of the re-suspended pellet onto the well of the Sperm Hy-Liter slide.
- Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).

E. 2. c. bb. Prepare the sperm/epithelial cell control for smears made from re-suspended pellet:

- Place a known spermic semen swab into a microcentrifuge tube.
- Place a known epithelial cell swab into another microcentrifuge tube.
- Add 350µl of RSID-Universal Buffer to each tube.
- Incubate for 30 minutes in the ultrasonic bath.
- Place the samples into spin baskets and place back into the tubes. Centrifuge within the range of 10,000 – 14,000 rpm (13,000 rpm is recommended) for 10 minutes.
- Remove 300µl of the supernatant from each tube and discard.
- Re-suspend the pellets in the remaining supernatant.
- Place 8µl of the epithelial cell re-suspended pellet onto one (1) well of the slide and 2µl of the positive semen re-suspended pellet into the same well.

- Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).
- Any remaining portion of the re-suspended pellets may be re-used. Store in the refrigerator for one (1) week and then discard.

cc. Stain the sperm/epithelial cell control smear concurrently with the questioned smears.

dd. Record this type of extract smear was made on the appropriate Quality Record Worksheet.

d. Staining Procedure:

Type of smear	Extract smears	Hospital/OCME Prepared Smear
# of drops of reagent per smear	2-3 drops	6 – 12 drops

Use sufficient volume of reagent to cover sample.

aa. Add the Fixative Solution to each smear. Incubate at room temperature for five (5) minutes.

E. 2. d. bb. During Fixative incubation: Thaw an aliquot of 1M DTT solution. For each smear to be stained, add 5µl of 1M DTT solution per one (1) drop of Sample Preparation Solution into a microcentrifuge tube according to FBQR-14 (Sperm Hy-Liter Calculation Worksheet).
**For Anal Smears, see paragraph 2.e. below.

cc. Use 1X wash buffer to gently rinse each smear. Remove excess wash buffer with the corner of a paper towel.

dd. Add the Sample Preparation Solution + 1M DTT solution to each smear. Incubate at room temperature for fifteen (15) minutes.

Type of smear	Extract smear	Hospital/OCME Prepared Smear
Volume of Sample Preparation Solution + 1M DTT solution	75µl - 100µl	200µl – 320µl

Use sufficient volume of Sample Preparation Solution + 1M DTT solution to cover sample.

ee. Follow step cc.

ff. Add the Blocking Solution to each smear. Incubate at room temperature for fifteen (15) minutes.

gg. Follow step cc.

hh. Add Sperm Head Staining Solution to each smear. Incubate at room temperature for fifteen (15) minutes.

ii. Follow step cc.

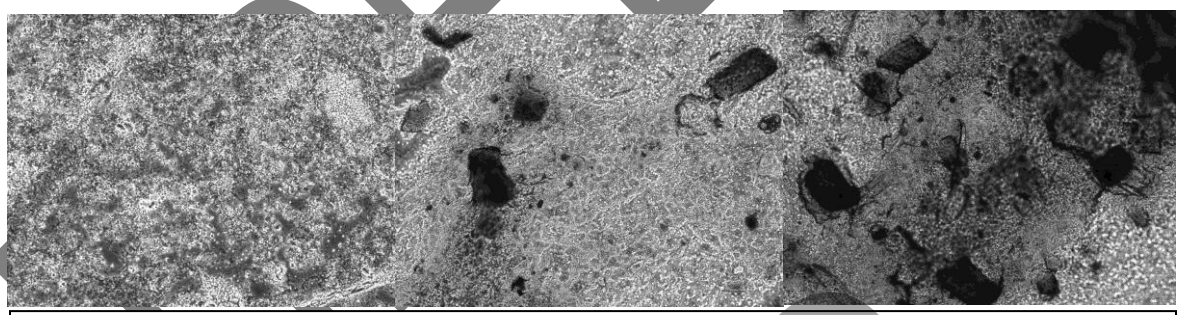
jj. Add one (1) to three (3) drops of Mounting Media to each smear and cover each smear with a cover slip. Remove excess Mounting Media by placing slide between two (2) paper towels and gently pressing down to blot. Outline edge of cover slips with clear nail polish to seal.

kk. Examine the smear for spermatozoa and other cells with the Sperm Hy-Liter microscope manual search instructions below, using Phase Contrast, FITC fluorescence and DAPI fluorescence.

E. 2. e. Anal smears (Fecal Material)

aa. The photos below may be used to determine the amount of fecal material on the anal smear to be examined.

No Fecal Material	Light Fecal Material	Medium Fecal Material	Heavy Fecal Material



Light Fecal Material Phase Contrast	Medium Fecal Material Phase Contrast	Heavy Fecal Material Phase Contrast

bb. For an anal smear with light fecal material, add 50µl of 1M DTT solution (10X concentration) per one (1) drop of Sample Preparation Solution*.

cc. For an anal smear with medium fecal material, add 100µl of 1M DTT solution (20X concentration) per one (1) drop of Sample Preparation Solution*.

dd. For an anal smear with heavy fecal material, add 150µl of 1M DTT solution (30X concentration) per one (1) drop of Sample Preparation Solution*.

*According to FBQR-14 (Sperm Hy-Liter Calculation Worksheet).

ee. If unable to determine which concentration of 1M DTT to use, it is suggested to use the higher concentration.

ff. If the Hospital/OCME prepared anal smear is very heavy/thick with fecal material, the examiner may choose to proceed to extracting an anal swab and staining the extract smear according to the protocol for anal smears.

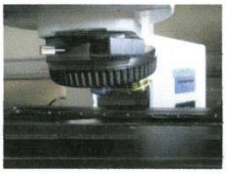
Instructions on how to use the Automated System for Manual Analysis

There are times when the user may want to quickly view a slide without performing an automated analysis. The SPERMTELLIGENCE System was designed to be used both automatically and manually.

1. Turn on Olympus BX51 microscope. Light source LED should be kept at 6-8V.

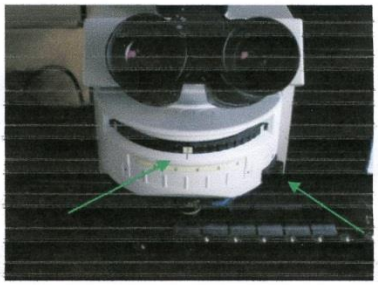
2. Turn on Proscan II controller.

3. Turn on Lumen Pro illuminator. Note: Illuminator must be on for a minimum of 15 minutes before turning off. This will save on bulb life and insure proper wavelength is optimized. Bulb is guaranteed for 2000 hours.



4. Load microscope slides. Using knurled ring on objective holder, rotate objectives toward back of microscope to allow more working room for loading slides. Slides can be loaded in any convenient position. Once slides are loaded, rotate 20X objective back into place.

5. Use joystick control to move stage and joystick focus to focus slides



6. Rotate fluorescence turret to cube #1 for phase contrast viewing.

7. Open/close shutter for fluorescence light as needed.

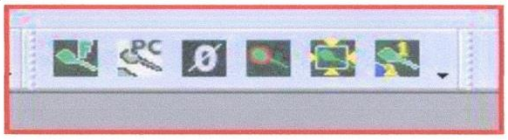
SPERMTELLIGENCE™
AUTOMATED SPERM HY-LITER™

8. Rotate bottom condenser to PH1 position which has bright fluorescent green dot for easier identification.

9. Turn on computer and monitor.

10. Double click on **SPERMTELLIGENCE** Automated Sperm Hy-liter icon to open up software.

Software Action Buttons



11. Turn on transmitted light shutter for phase contrast by clicking top button on joystick. This is the ideal way to turn on the light, because both phase contrast and fluorescence can be shuttered on at the same time. User can toggle this button on and off as needed. Clicking phase contrast icon will also shutter on the light.

12. Turn on fluorescence light by clicking fluorescence shutter icon. Verify fluorescence is working by rotating fluorescence turret to postion #2 for FITC, opening fluorescence shutter, and viewing blue fluorescence light. User can switch back to phase by rotating fluorescence turret to position #1. Fluorescence can be toggled on and off as needed using this icon. Note: this will open/close the shutter only. The Lumen Pro light source stays on until power is turned off.

13. Turn off shutters by clicking on Turn Off Light Sources icon.

14. Using joystick to move stage and joystick focus control, user can now use microscope to view slides.

15. View live images on the monitor and capture them by clicking on camera icon.

EASY 5 STEP SPERM HY-LITER Procedure for Quick Sperm Detection:

1. **Turn on microscope power/Turn on fluorescence power supply/Rotate to cube position 1.**
2. **Engage 10X or 20X phase contrast objective and PH1 position of condenser – focus on specimen.**
3. **Cover bottom transmitted light source with black dust cover (this saves on bulb life).**
4. **Rotate to cube position 2 or FITC, open shutter, refocus if necessary, scan slide to view if SPERM HEADS are present.**
5. **OPTIONAL: To confirm SPERM HEADS are present, double check by rotating to DUAL or DAPI cube, view fluorescence and phase at same time, or view specimen in phase contrast.**

E. 3. Results:

a. Christmas Tree: A stained, intact spermatozoon will have a red head and a greenish tail and neck. A stained, non-intact spermatozoon will have a red head and no tail. Epithelial cells will be green or bluish with purplish-red nuclei. Some bacteria or yeast may also stain red.

b. Sperm Hy-Liter: A stained spermatozoon will have a green head under FITC fluorescence. Epithelial cell nuclei and spermatozoon will be blue under DAPI fluorescence. Bacteria and yeast will also stain blue under DAPI fluorescence.

c. *Positive.* The identification of a stained, intact spermatozoon or the head portion of a non-intact spermatozoon. Sperm search results are rated as follows and recorded on the appropriate Quality Record Worksheet:

- 4+ - numerous sperm in every field
- 3+ - a few sperm in every field
- 2+ - sperm not in every field but easy to locate unstained
- 1+ - a few sperm (coordinates are needed to relocate)

d. *Negative.* The absence of identifiable stained spermatozoa.

Note: If an AP swab was used to make the smear and no spermatozoa were identified, then the sample will be extracted according to SOP-FB-12 (Extraction of Samples for Semen).

e. Record the results of the sperm/epithelial cell controls (Sperm Hy-Liter) and samples on the appropriate Quality Record Worksheet.

Note: If an AP swab was used to make the smear then record this and the result on the appropriate Quality Record Worksheet.

f. Record test(s) used on the appropriate Quality Record Worksheet.

4. Place a photocopy of FBQR-14 (Sperm Hy-Liter Calculation Worksheet) into the appropriate case jacket(s). File the original in the designated notebook.

5. Record reagent(s) and solutions used on FBQR-09 (General Reagent Sheet).

F. REFERENCES:

1. Independent Forensics, Sperm Hy-Liter™ Technical Information and Protocol sheets.

2. Independent Forensics, Sperm Hy-Liter™ *PLUS* Technical Information and Protocol sheets.

3. Independent Forensics, Sperm Hy-Liter Express Technical Information and Protocol sheets

F. 4. Independent Forensics, Sperm Hy-Liter™ Recommended Laboratory Recipes and Procedures, p 1-12.

5. Jennifer Old Ph.D., Brett A. Schweers Ph.D., Pravat Boonalangoor Ph.D. and Karl Reich Ph.D, Developemental Validation of Sperm Hy-Liter™ A Specific, Sensitive and Confirmatory Microscopic Sceening Method for the Detection of Human Sperm from Sexual Assault Evidence, p 34-51.

6. Jennifer Old Ph.D., Dina Mattes, Pravat Boonalangoor Ph.D. and Karl Reich Ph.D, Development Validation of Spermtelligence™, Software Aided Identification of Human Sperm using Sperm Hy-Liter™ Stained Slides, p 2-13.

7. Jennifer Old, Chris Martersteck and Anna Kalinina, Developmental Validation of Sperm Hy-Liter Express™, p 1-11.

8. Connecticut State Forensic Science Laboratory, Sperm Hy-Liter Internal Validation, 2010.

9. Connecticut State Forensic Science Laboratory, Sperm Hy-Liter Express and Fecal Material Internal Validation, 2012.

10. Gaensslen, R.E., Ph.D., Sourcebook in Forensic Serology, Immunology, and Biochemistry, Detection and Identification of Spermatozoa, 1983, p 150-152.

11. SOP-GL-2 (Safety Manual).