Document Title: Identification of Spermatozoa
Controlled: Yes, with red stamp present
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Prepared By: _____ Date: _____

Approved By: _____ Date: _____

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. 1:500 semen aliquot
- dd. Known spermic semen swab
- ee. Known buccal swab
- ff. Fixative Solution*
- gg. Sample Preparation Solution*
- hh. 1M DTT solution
- ii. Blocking Solution*
- jj. Sperm Head Staining Solution*
- kk. Mounting Media*
- ll. 1X Wash Buffer (from 10X Wash Buffer*)
- mm. ImmEdge Pen*
- nn. Sperm Hy-Liter 2 X 11 mm well slides*
- oo. Cover slips*
- pp. Clear nail polish
- qq. 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 basket, place back into tube and centrifuge at 13,000 rpm for 5-10 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) and centrifuge at 13,000 rpm for 5-10 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.

~~e~~dd. If a positive acid phosphatase result was observed, the swab may be used to form a smear directly onto a glass slide. Record AP swab used on the appropriate Quality Record Worksheet.

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

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

E. 2. 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.

Sperm Hy-Liter

a. Staining Hospital/OCME prepared smears

aa. Prepare a positive control:

- Collect a buccal sample on a swab and form a smear onto a glass slide.
- With a micropipet place approximately 3µl of thawed 1:500 diluted semen onto the smear. Re-freeze the remaining semen aliquot.
- Dry the positive control smear at room temperature or 37°C (do not apply 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. Stain the positive control smear concurrently with the questioned smears.

cc. 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 smear and allow to dry.

dd. Add six (6) drops of the Fixative Solution to each smear. Incubate at room temperature for 10 minutes.

ee. During Fixative incubation: Thaw an aliquot of 1M DTT. For each smear to be stained, add 3µl of 1M DTT to seven (7) drops of Sample Preparation Solution into a microcentrifuge tube.

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

gg. Add 200µl of Sample Preparation Solution + DTT to each smear. Incubate at room temperature for 30 minutes.

E. 2. a. hh. Follow step ff.

ii. Add six (6) drops of Blocking Solution to each smear. Incubate at room temperature for 30 minutes.

jj. Follow step ff.

kk. Add six (6) drops of Sperm Head Staining Solution to each smear. Incubate at room temperature for 30 minutes.

ll. Follow step ff.

mm. Add 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.

nn. 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.

b Case Sample Extract smears made directly from the pellet

aa. Prepare the extracted sample smear:

- Transfer the sample into a spin basket, place back into the tube and centrifuge at 13,000 rpm for 5-10 minutes.
- Then remove 3µl of the pellet with a micropipet to a glass slide and form a smear.
- Dry the smear at room temperature or 37°C (do not apply heat).

bb. Prepare the positive control:

- Extract a known buccal/spermic semen swab in a microcentrifuge tube with 300µl of RSID-Universal Buffer.
- Incubate for 30 minutes in the ultrasonic bath.
- Follow steps from aa above.

cc. Stain the positive control smear concurrently with the questioned smears.

E. 2. b. dd. Add two (2) drops of the Fixative Solution to each well. Incubate at room temperature for 10 minutes.

ee. During Fixative incubation: Thaw an aliquot of 1M DTT. For each smear to be stained, add 1µl of 1M DTT to three (3) drops of Sample Preparation Solution into a microcentrifuge tube.

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

gg. Add 75µl of Sample Preparation Solution + DTT to each smear. Incubate at room temperature for 30 minutes.

hh. Follow step ff.

ii. Add two (2) drops of Blocking Solution to each smear. Incubate at room temperature for 30 minutes.

jj. Follow step ff.

kk. Add two (2) drops of Sperm Head Staining Solution to each smear. Incubate at room temperature for 30 minutes.

ll. Follow step ff.

mm. Add one (1) drop 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. Outline cover slips with clear nail polish.

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

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

aa. Prepare extracted sample smear:

- aa. Remove 250µl of the supernatant, place into a new microcentrifuge tube and set aside (<u>do not discard</u>).
- bb. Re-suspend the pellet in the remaining 50µl of supernatant.
- cc. Place 10µl of the re-suspended pellet onto the well of the Sperm Hy-Liter slide.
- dd. Allow the smear to dry at room temperature or 37°C (do not apply heat).

E. 2. c. bb. Prepare the positive control:

- Place a known buccal/spermic semen swab into a microcentrifuge tube.

- Place a known buccal swab into another microcentrifuge tube.
- Add 300µl of RSID-Universal Buffer.
- Incubate for 30 minutes in the ultrasonic bath.
- Place the samples into spin baskets then back into the tubes and centrifuge at 13,000 rpm for five (5) minutes.
- Remove 250µl of the supernatant from each tube (spermic semen and epithelial cell extracts) and discard.
- Re-suspend the pellets in the remaining 50µl of supernatant.
- Positive control: 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 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 positive control smear concurrently with the questioned smears.

dd. Add two (2) drops of the Fixative Solution to each smear. Incubate at room temperature for 10 minutes.

ee. During Fixative incubation: Thaw an aliquot of 1M DTT. For each smear to be stained, add 1µl of 1M DTT to three (3) drops of Sample Preparation Solution into a microcentrifuge tube.

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

gg. Add 75µl of Sample Preparation Solution + DTT to each smear. Incubate at room temperature for 30 minutes.

hh. Follow step ff.

E. 2. c ii. Add two (2) drops of Blocking Solution to each smear. Incubate at room temperature for 30 minutes.

jj. Follow step ff.

kk. Add two (2) drops of Sperm Head Staining Solution to each smear. Incubate at room temperature for 30 minutes.

ll. Follow step ff.

mm. Add one (1) drop 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. Outline cover slips with clear nail polish.

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

SPERMTELLIGENCE™

AUTOMATED SPERM HY-LITER™

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.

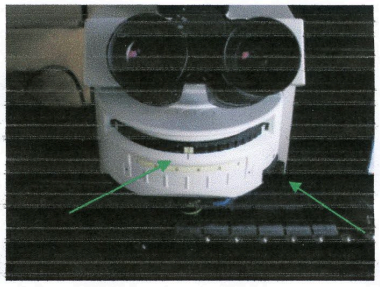
2. Turn on Proscan II controller.

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

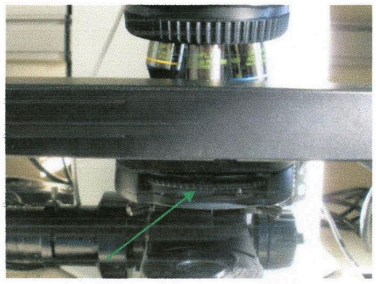
6. Rotate fluorescence turret to cube #1 for phase contrast viewing.

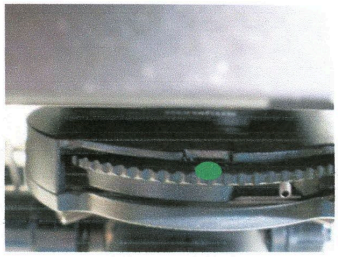


7. Open/close shutter for fluorescence light as needed.

SPERMTELLIGENCE™
AUTOMATED SPERM HY-LITER™

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

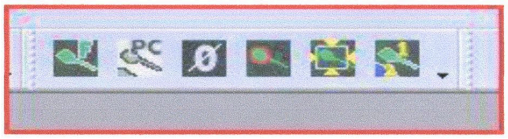




9. Turn on computer and monitor.

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.

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

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

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

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™ Recommended Laboratory Recipes and Procedures, p 1-12.

4. 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.

5. 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.

F. 6. Connecticut State Forensic Science Laboratory, Sperm Hy-Liter Internal Validation, 2010.

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

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