IDENTIFICATION OF SPERMATOZOA

14.1 PURPOSE

14.1.1: To identify spermatozoa in Forensic samples.

A. Theory

1. Christmas Tree Stain is used to confirm the presence of semen by microscopic identification of sperm cells (or spermatozoa).

    a. Two reagents are used in succession to stain questioned smears on glass microscope slides: Nuclear Fast Red (also known as Kernechtrot solution) and Picroindigocarmine.

    b. Nuclear Fast Red stains nuclear material red. This includes the nuclear material of spermatozoa, epithelial cells and yeast. Bacteria may also stain red.

    c. Picroindigocarmine stains cellular cytoplasm a greenish color. This includes the neck/tail portions of spermatozoa.

    d. Spermatozoa can be identified by their relative size, distinct morphology and staining pattern (red head and greenish neck/tail if present).

    e. Epithelial cells can be identified by their morphology and staining pattern. The epithelial cell nucleus generally stains pink to red and the cytoplasm stains greenish.

2. The Sperm Hy-Liter method of sperm identification utilizes a human sperm head specific monoclonal antibody tagged with a fluorophore dye referred to as fluorescein isothiocyanate (FITC). In addition, a fluorescent nucleic acid dye stain (4′,6-diamidino-2-phenylindole or DAPI) is utilized.

    a. The stained smear is examined under a fluorescence microscope.

    b. DAPI binds to all nuclei present in the sample, including nuclei in spermatozoa, epithelial cells and yeast. DAPI may also bind less specifically to bacteria. Under the proper microscope filter setting for DAPI, these nuclei and bacteria can be visualized by their emission of a blue fluorescent signal.

c. FITC binds specifically to the sperm heads using anti-human sperm head monoclonal antibody. Under the proper microscope filter setting for FITC, the sperm heads can be visualized and identified by their emission of a bright green fluorescent signal and distinctive morphology.

d. This method allows spermatozoa to be visualized even when they are located underneath epithelial cells.

B. Limitations

1. Christmas Tree Stain
   a. Heavy smears may inhibit the visualization of the sperm cells.
   b. Bacteria and yeast also stain red with nuclear fast red.

2. Sperm Hy-Liter Stain
   The visualization of sperm cells may be inhibited by high background fluorescence due to considerable bacterial presence, non-specific binding and/or cross reactivity.

14.1.2: To perform quality control on new stain lots.

14.2 RESPONSIBILITY

14.2.1: Test Procedure – Personnel qualified to perform Forensic Biology duties.

14.2.2: Preparation/QC Procedure – Personnel qualified to perform Forensic Biology duties. Ordering information is maintained in a logbook and/or electronically in the Forensic Biology Unit. New chemicals and reagents are purchased according to GL-6 (Purchasing). For additional information, refer to the Biological Inventory located in Appendix 3.

14.3 SAFETY

Use appropriate measures for the proper handling of biohazardous materials and Picroindigocarmine, potassium hydroxide, DTT according to GL-2 (Safety Manual) and the Safety Data Sheets.

14.4 DEFINITIONS/ABBREVIATIONS

A. KS: Kernechtrot solution
B. PICS: Picroindigocarmine solution
C. OCME: Office of the Chief Medical Examiner
D. AP: Acid Phosphatase
E. DTT: Dithiothreitol
F. RSID™: Rapid Stain Identification
G. PC: Phase Contrast
H. QRW(s): Quality Record Worksheet(s) (Appendix 1)

14.5 TEST PROCEDURES

These staining procedures will be performed at the discretion of the examiner, with input from the Unit Lead(s) when applicable, based on the submitting agency requests, case information and condition of the evidence.

A. When applicable, Sperm Hy-Liter staining is typically used for Hospital/OCME prepared smears and Christmas Tree staining is typically used for extract smears.

B. Sperm searches may be conducted unstained according to FB SOP-02 (Sexual Assault Evidence Collection Kit Examination, section 2.8.4.3).

14.5.1: Materials

A. Hospital/OCME prepared smears
B. Extracted Samples
C. Positive AP swab
D. Micropipette and tips
E. Centrifuge tubes and spin baskets
F. Centrifuge
G. Glass slides
H. Acetone
I. Swabs
J. dH_2O
K. Blades
L. Christmas Tree Stain
    1. KS stain* (Stain A)
    2. PICS stain* (Stain B)
    3. dH_2O
    4. Ethanol
M. Microscopes
N. Sperm Hy-Liter Stain
    1. RSID™-Universal Buffer
    2. Aliquot of diluted spermic semen
    3. Swabs
    4. Known spermic semen/epithelial cell swab
    5. Known spermic semen swab
    6. Known epithelial cell swab
    7. Fixative Solution*
    8. Sample Preparation Solution*
    9. 1M DTT solution
    10. Blocking Solution*
    11. Sperm Head Staining Solution*

12. Mounting Media*
13. 1X Wash Buffer (from 10X Wash Buffer*)
14. ImmEdge Pen*
15. Sperm Hy-Liter/masked slides*
16. Cover slips
17. Forceps
18. Clear nail polish

O. Sperm Hy-Liter Microscope System
P. Ultrasonic bath
* provided by the manufacturer

14.5.2: Christmas Tree Stain Smear Preparation Procedure

A. Hospital/OCME prepared smears, no additional preparation needed.

B. Directly from pellet:
1. Label a glass slide with case #, item #, examiners initials and date.
2. Remove 3µl of the pellet with a micropipette and deposit onto the glass slide to form a smear.
3. Allow the smear to dry at room temperature or 37°C.

C. Liquid sample suspected to contain semen:
1. Place the sample into centrifuge tube(s) and centrifuge for 10 minutes. The centrifuge will be set at approximately 13,000 rpm. If necessary, decant excess liquid (supernatant) back into the original container.
2. Remove 3µl of the pellet with a micropipette and deposit onto a glass slide to form a smear.
3. Allow the smear to dry at room temperature or 37°C.

D. Directly from a questioned sample that yields a positive acid phosphatase result:
1. The swab/cutting may be used to form a smear directly onto a glass slide.
2. Allow the smear to dry at room temperature or 37°C.

14.5.3: Christmas Tree Staining Procedure

A. Cover the smear with KS solution (Stain A) for 20 minutes.

B. Gently rinse KS off slide with dH_2O. Do not carry the smears/slides over other smears/slides staining during this step.

C. Cover the smear with PICS solution (Stain B) for 15-20 seconds.

D. Gently rinse PICS off slide with ethanol* and blot dry or air dry at room temperature. Do not carry the smears/slides over other smears/slides staining during this step.

*Rinse off with dH_2O if Sperm Hy-Liter procedure may be needed.

E. Examine the smear for spermatozoa and other cells (i.e. epithelial cells) under the microscope at 200X (verify under 400X as necessary). Rate identified spermatozoa according to section 14.5.9.
   1. Record the microscope(s) used on the appropriate QRW(s).
   2. It is not necessary to conduct a full search if spermatozoa are identified and rated prior to completing the search. If applicable, record 'partial search' on the appropriate QRW(s).
   3. Extract smears are generally discarded once examination has been completed and sperm identification has been confirmed. Therefore, coordinates are not recorded for extract smears as described in section 14.5.9.C.

F. Record the staining procedure used on the appropriate QRW(s).

G. Record the lot numbers of reagent(s) and solutions used on FBQR-09 (General Reagent Sheet).

H. Record the results of the samples on the appropriate QRW(s).
   1. If an AP positive sample was used to make the smear, then record on the appropriate QRW(s) along with the result ("positive" or "no sperm noted/ identified").
   2. If no spermatozoa were noted/identified, then the sample will be extracted according to FB SOP-13 (Extraction of Samples for Semen).

I. See 14.5.9.C.2 for confirmation of sperm identification.

14.5.4: Sperm Hy-Liter Smear Preparation Procedure

A. Hospital/OCME prepared smears
   1. Prepare a sperm/epithelial cell control for Hospital/OCME prepared smear:
      a. Collect an epithelial cell sample on a swab and form a smear onto a glass slide.
      b. Agitate dilute spermic semen (1:250 suggested) and using a micropipette place approximately 3µl onto the same smear.
      c. Dry the control smear at room temperature or 37°C (do not apply open flame heat).

d. 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.

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

3. Stain the sperm/epithelial cell control smear concurrently with the questioned smears as an intermediate check. Reagent QC is always conducted prior to use on case samples.

B. Smears made directly from the pellet

1. Prepare the extracted sample smear:
   a. Remove 3µl of the pellet with a micropipette and deposit onto a glass slide (standard or Sperm Hy-Liter/masked) to form a smear.

   b. Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).

2. Prepare the sperm/epithelial cell control for smears made directly from pellet:
   a. Extract a known spermic semen/epithelial cell swab in a centrifuge tube with 350µl of RSID™-Universal Buffer.

   b. Incubate for 30 minutes in the ultrasonic bath.

   c. Place the sample into a spin basket and place back into the tube. Centrifuge for 10 minutes. The centrifuge will be set at approximately 13,000 rpm.

   d. Remove 3µl of the pellet with a micropipette and deposit onto a glass slide (standard or Sperm Hy-Liter/masked) to form a smear.

   e. Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).

3. 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 centrifuge 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.

4. Stain the sperm/epithelial cell control smear concurrently with the questioned smears as an intermediate check. Reagent QC is always conducted prior to use on case samples.

5. Record the type of extract smear made on the appropriate QRW(s).

C. Smears made from re-suspended pellet

1. Prepare extracted sample smear:
   a. Remove 300µl of the supernatant, place into a new centrifuge tube and set aside (do not discard).
   b. Re-suspend the pellet in the remaining supernatant.
   c. Deposit 10µl of the re-suspended pellet onto the well of the Sperm Hy-Liter/ masked slide.
   d. Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).

2. Prepare the sperm/epithelial cell control for smears made from re-suspended pellet:
   a. Place a known spermic semen swab into a centrifuge tube.
   b. Place a known epithelial cell swab into another centrifuge tube.
   c. Add 350µl of RSID™-Universal Buffer to each tube.
   d. Incubate for 30 minutes in the ultrasonic bath.
   e. Place the samples into spin baskets and place back into the tubes. Centrifuge for 10 minutes. The centrifuge will be set at approximately 13,000 rpm.
   f. Remove 300µl of the supernatant from each tube and discard.
   g. Re-suspend the pellets in the remaining supernatant.
   h. Deposit 8µl of the epithelial cell re-suspended pellet onto the well of the Sperm Hy-Liter/masked slide and deposit 2µl of the positive semen re-suspended pellet into the same well.
   i. Allow the smear to dry at room temperature or 37°C (do not apply open flame heat).
   j. Any remaining portion of the re-suspended pellets may be re-used. Store in the refrigerator for one (1) week and then discard.

3. Stain the sperm/epithelial cell control smear concurrently with the questioned smears as an intermediate check. Reagent QC is always conducted prior to use on case samples.

4. Record the type of extract smear made on the appropriate QRW(s).

14.5.5: Sperm Hy-Liter Staining Procedure

A. Record the staining procedure used on the appropriate QRW(s).

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

C. Add the Fixative Solution to each smear. Use sufficient volume of reagent to cover sample:

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

1. Once the number of drops has been determined in the first step with the Fixative Solution, the same number of drops will be used for each subsequent solution (Sample Preparation Solution, Blocking Solution and Sperm Head Staining Solution).

2. Incubate at room temperature for five (5) minutes. For dense/thick smears the smear may be incubated for ten (10) minutes with the Fixative Solution.

D. During Fixative incubation prepare the working solution of DTT:
1. Thaw an aliquot of 1M DTT solution. If a precipitate is observed, agitate until it goes back into solution. If it does not go back into solution, discard/replace with new aliquot.
   a. Any unused portion may be re-frozen one (1) additional time.

   b. Discard any unused portion after being thawed a second time.

2. For each smear to be stained, add 5µl of 1M DTT solution per one (1) drop of Sample Preparation Solution into a centrifuge tube according to FBQR-14 (Sperm Hy-Liter Calculation Worksheet).

3. For Anal Smears, see section 14.5.6 below.

E. Use 1X wash buffer to gently rinse each smear.
1. Do not carry the smears/slides over other smears/slides staining during this step.
2. Remove excess wash buffer with the corner of a paper towel.

F. Add the Sample Preparation Solution + 1M DTT solution to each smear.

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

1. Use a sufficient volume of Sample Preparation Solution + 1M DTT solution to cover sample. The above calculations are used as an approximation.

2. Incubate at room temperature for fifteen (15) minutes.

G. Use 1X wash buffer to gently rinse each smear.

1. Do not carry the smears/slides over other smears/slides staining during this step.

2. Remove excess wash buffer with the corner of a paper towel.

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

I. Use 1X wash buffer to gently rinse each smear.
1. Do not carry the smears/slides over other smears/slides staining during this step.

2. Remove excess wash buffer with the corner of a paper towel.

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

K. Use 1X wash buffer to gently rinse each smear.
1. Do not carry the smears/slides over other smears/slides staining during this step.

2. Remove excess wash buffer with the corner of a paper towel.

L. 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 the slide between two (2) paper towels and gently pressing down to blot. Outline edge of cover slips with clear nail polish to seal.

M. Examine the control and questioned smears for spermatozoa and other cells (i.e. epithelial cells) according to section 14.5.7 below, under the microscope filter settings for Phase Contrast, FITC and DAPI. When necessary, capture images of the questioned smears according to section 14.5.8 below. Rate identified spermatozoa according to section 14.5.9.

1. Record the microscope(s) used on the appropriate QRW(s).

2. It is not necessary to conduct a full search if spermatozoa are identified and rated prior to completing the search. If applicable, record ‘partial search’ on the appropriate QRW(s).

3. Extract smears are typically discarded once examination has been completed and sperm identification has been confirmed, when applicable.

N. Record the results of the control and questioned smears on the appropriate QRW(s).

O. See 14.5.9.C.2 for confirmation of sperm identification.

P. Scan the original FBQR-14 (Sperm Hy-Liter Calculation Worksheet) and file electronically. Photocopy the original if more than one (1) case tested. Place the original or photocopy into each relevant case jacket.

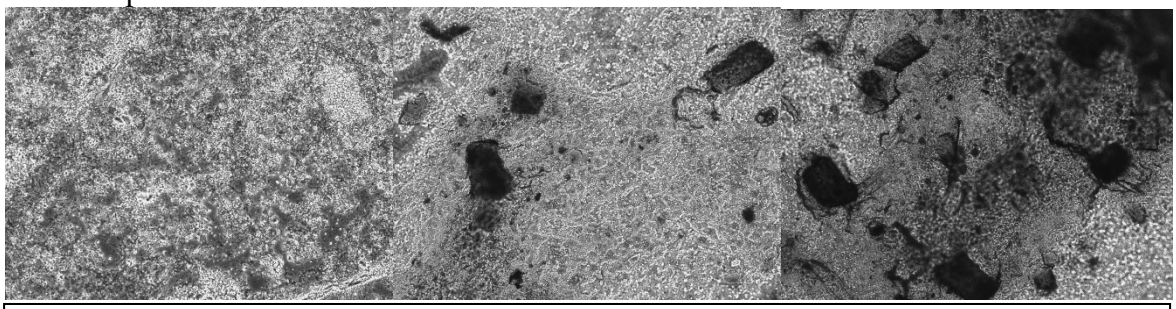
14.5.6: Sperm Hy-Liter Anal Smears (Fecal Material)

A. The photos below may be used to assess how light or heavy the fecal material appears on the anal smear to be stained.

Macroscopic:

No Fecal	Light Fecal	Medium Fecal	Heavy Fecal

Microscopic:



Light Fecal Material	Medium Fecal Material	Heavy Fecal Material

B. For an anal smear with light fecal material, add 50µl of 1M DTT solution (10X concentration) per one (1) drop of Sample Preparation Solution according to FBQR-14 (Sperm Hy-Liter Calculation Worksheet).

C. For an anal smear with medium fecal material, add 100µl of 1M DTT solution (20X concentration) per one (1) drop of Sample Preparation Solution according to FBQR-14 (Sperm Hy-Liter Calculation Worksheet).

D. 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).

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

F. 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.

14.5.7: Conducting Sperm Hy-Liter Sperm Searches (Zeiss Microscope System)

A. Turn on the light source, microscope, and computer (note that the light source should be left on for at least 5 minutes before turning off - this will conserve bulb life and ensure proper wavelength is optimized).

B. Load slide with smear onto stage.

C. Using the 20x objective and the phase contrast setting (fluorescent shutter closed, filter in #1 position, transmitted light source uncovered, setting 2 on the condenser) check that the smear is in focus.
   1. This may be done by focusing on epithelial cells present in the control/sample.
   2. If the smear has little cellular material and a masked slide was used, move to the edge of the masked area and use the defined boarder for the initial focus.

D. Search the smear using the fluorescent settings (transmitted light source covered, fluorescent shutter open, filter in the #2 position for FITC, #3 position for dual, and #4 position for DAPI) at 200x magnification (setting 2 on the condenser).

E. If a magnification of 400x is used, no adjustments to the condenser are needed.

F. For additional information, see Sperm Hy-Liter user manual.

14.5.8: Capturing Sperm Hy-Liter Images (Zeiss Microscope System)

A. Open the Sperm Capture Software.

B. On the microscope, slide the metal rod (beam splitter) fully out to the camera only setting.

C. On the computer, manually adjust the image settings, or a preset filter may be used to visualize the image (access the preset filter options by selecting the "file" menu, select "load camera settings" and select the appropriate filter setting (FITC, DAPI, Dual, PC) from the list). Refocus as needed. Prior to capturing the PC image, perform a white balance by clicking the global white balance (globe) icon.

1. Although there are preset capture settings for each filter choice, manual adjustments may still be necessary (including but not limited to brightness, contrast, exposure, gain, aperture size, etc.).

2. If the preset filters are not listed, they are saved to the following location where they may be recalled from: Windows 7_OS(c:)
Program files(x86)
Lumenera Corporation
Infinity Software

D. Capture the image by selecting the "capture and display image" (camera) icon. A new window will open on the desktop with the captured image. Once the image has been captured, close the fluorescence shutter to prevent photo-bleaching of smear.

E. Before closing the window containing the captured image, save it to the appropriate folder in the desktop "SH Casework Photos" folder (click "file", then "save as" to name and save image). If the "close" option is selected prior to saving, there will be no dialogue box warning that the image has not been saved.

F. Repeat steps C-E for each image being captured.

G. Create a document containing the images. Using Open Office on the desktop, select "text document". In the new document, select "table" then "insert", then "table" to create a table with

3 rows. Drag and drop the captured images to the table.

H. Resize the images as necessary and include the case #, item #, magnification, filter set, and coordinates on the document.

I. Save this document to the desktop folder labeled “SH case reports”.

J. A printed copy of this document should be maintained in the case jacket.

K. If the images are being captured from an extract smear it is not necessary to record the coordinates.

L. For additional information, see Sperm Hy-Liter user manual.

14.5.9: Results and Conclusions

A. Christmas Tree

The head portions of spermatozoa stain red. Some bacteria or yeast may also stain red. The neck/tail portions of intact spermatozoa stain greenish (non-intact spermatozoa have no neck/tail portions). Epithelial cells stain greenish with pink or red stained nuclei.

B. Sperm Hy-Liter

The head portions of spermatozoa fluoresce green under the filter setting for FITC. The head portions of spermatozoa and epithelial cell nuclei fluoresce blue under the filter setting for DAPI. Bacteria and yeast also fluoresce blue under the DAPI setting.

C. <u>Positive</u>

1. 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 QRW(s):
   4+ - numerous sperm in every field
   3+ - a few sperm in every field
   2+ - sperm not in every field but easy to locate
   1+ - a few sperm (coordinates are needed to relocate, excluding extract smears)
   1 spermatozoon - head portion or intact (coordinates are needed to relocate, excluding extract smears)

2. A second qualified examiner will observe and confirm a positive identification.
   a. They may observe the actual smear or may review the photo-documentation (i.e. during the Technical Review).
      i. The rating of the sperm search result will not be confirmed.
      ii. Control smears are not rated.

b. They will initial and date the appropriate QRW(s) documenting the confirmation.

3. Suggested Report Wording:

a.

Testing Performed	Result	Conclusion
Microscopic - Spermatozoa	Positive	Spermatozoa/One (1) spermatozoon identified
Microscopic - Red blood cells	Positive	Red blood cells identified

b. Kit/Extract Smears
Spermatozoa were identified upon microscopic examination of [ ].
The head portion of one (1) spermatozoon was identified upon microscopic examination of [ ].

c. AP smears
Spermatozoa were identified upon microscopic examination of a sample/portion of [ ].
The head portion of one (1) spermatozoon was identified upon microscopic examination of a sample/portion of [ ].

d. Red Blood Cells
Red blood cells were identified upon microscopic examination of [ ].

D. Negative

1. The absence of identifiable stained spermatozoa.

2. Suggested Report Wording:

a.

Testing Performed	Result	Conclusion
Microscopic - Spermatozoa	Negative	Spermatozoa not identified
Microscopic - Red blood cells	Negative	Red blood cells not identified

b. Kit/Extract Smears
Spermatozoa were not identified upon microscopic examination of [ ].

c. Red Blood Cells
Red blood cells were not identified upon preliminary microscopic examination of [ ].

E. Failed

1. The control fails to yield the appropriate result.

2. If there is not enough sample to repeat the test, then no conclusion is possible.

3. Suggested Report Wording:

a.

Testing Performed	Result	Conclusion
Microscopic - Spermatozoa	Failed	No conclusion possible

b. *A microscopic procedure for the identification of spermatozoa was performed on this/these smear(s). Due to failure of this procedure, no conclusion is possible.*

4. Record the reason the procedure failed on the appropriate QRW(s).

F. Unsuitable

1. See section 14.1.1.B.2 for additional information.

2. Suggested Report Wording:

a.

Testing Performed	Result	Conclusion
Microscopic - Spermatozoa[1]		
Comments: [1]This smear was determined to be unsuitable for further examination due to high background staining.		

b. *This smear was determined to be unsuitable for further examination due to high background staining.*

3. If a sample is determined to be unsuitable for further examination, it is not precluded from further serological testing or being forwarded for DNA analysis, as determined on a case-by-case basis.

4. Record the reason the sample is unsuitable on the appropriate QRW(s).

G. If a sample is determined to be unsuitable for further serological testing, it is not precluded, based on any result above, from being forwarded for DNA analysis, as determined on a case-by-case basis. See FB SOP-15 (RIA for Semen).

1. Record the reason the sample is unsuitable on the appropriate QRW(s).

14.6 QC Procedure

14.6.1: Christmas Tree Stain

This reagent is purchased from an approved outside vendor.

Materials

A. KS stain (provided)
B. PICS stain (provided)
C. Known diluted spermic semen aliquot (thawed)
D. Swab
E. Glass slide

Procedure

A. Collect a buccal sample on a swab and form a smear onto a glass slide.

B. Place approximately 3µl of the known diluted semen onto the smear.

C. Dry the control smear at 37°C or over an alcohol burner for staining.

D. Control smears may be made in advance. Store in the freezer and replace as needed. Record appropriate information on the Body Fluid Standard Reagent Log Sheet.

E. Test each new lot of reagent before use with the control smear according to the test procedure and the Christmas Tree Stain Reagent Log Sheet. Record the required information.

A second qualified examiner will observe/confirm a positive identification and initial/date the Christmas Tree Stain Reagent Log Sheet.

F. If the appropriate results are not obtained, review the procedure and repeat the test. If the reagent still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

G. If the lot is acceptable for use, record the date received, date opened and examiner's initials on each bottle.

The lot is acceptable for use when spermatozoa and epithelial cells are identified in a spermatozoa/epithelial cell control demonstrating that they have stained properly according

to section 14.5.9.A.

H. Store according to the manufacturer's instructions.

I. Discard/replace according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical Reagent QC).

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

14.6.2: Sperm Hy-Liter 1M Potassium Hydroxide Solution

Materials

	1ml 1M KOH	2ml 1M KOH	12ml 1M KOH
A. Potassium Hydroxide (KOH)	0.056g	0.112g	0.672g
B. dH_2O	1ml	2ml	12ml

Procedure

A. Mix all materials together and use immediately for 1M DTT solution.

B. Discard excess potassium hydroxide solution after preparing 1M DTT solution.

C. Discard/replace the potassium hydroxide according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical Reagent QC).

14.6.3: Sperm Hy-Liter 1M DTT Solution

Materials

	1ml 1M DTT	10ml 1M DTT	100ml 1M DTT
A. DTT	0.154g	1.54g	15.4g
B. 1M Potassium Hydroxide (KOH)	0.11ml	1.1ml	11ml
C. Total volume of dH_2O	0.89ml	8.9ml	89ml

D. pH paper

E. Centrifuge tubes

The control date will be one (1) year from the date of preparation.

Procedure

A. Combine DTT, 1M KOH and dH_2O (see chart below).

For 1ml 1M DTT	For 10ml 1M DTT	For 100ml 1M DTT
Add 0.77ml dH_2O	Add 7.7ml dH_2O	Add 77ml dH_2O

B. Test pH should be approximately pH 8.

C. Add dH_2O to final solution volume (see chart below).

For 1ml 1M DTT	For 10ml 1M DTT	For 100ml 1M DTT
Add 0.12ml dH_2O	Add 1.2ml dH_2O	Add 12ml dH_2O

D. Test each new lot before use according to the test procedure and the Sperm Hy-Liter Stain Reagent Log Sheet. Record the required information.

1. A second qualified examiner will observe/confirm a positive identification (spermatozoa/epithelial cells) and initial/date the Sperm Hy-Liter Stain Reagent Log Sheet and/or the DTT Reagent Log Sheet.

   i. The rating of the sperm search result will not be confirmed.
   ii. Control smears are not rated.

2. It is not necessary to photograph for QC.

E. If the appropriate results are not obtained, review the procedure and repeat the test. If the solution still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

F. If the lot is acceptable for use, aliquot into centrifuge tubes labeled with the reagent and quantity.

The lot is acceptable for use when spermatozoa and epithelial cells are identified in a spermatozoa/epithelial cell control demonstrating that they have stained properly according to section 14.5.9.B.

G. Store in the freezer labeled with the solution, lot # (date of preparation), control date and examiner's initials. Record the required information on the DTT Reagent Log Sheet.

H. Any unused portion may be re-frozen one (1) additional time.

I. Discard any unused portion after being thawed a second time.

J. Discard any frozen aliquots on the control date.

K. Discard/replace the DTT according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical Reagent QC).

14.6.4: Sperm Hy-Liter 1X Wash Buffer

Materials

A. 10X wash buffer (provided) 1 part
B. dH_2O 9 parts
C. Bottle (stock)

Procedure

A. Measure the quantity of the provided 10X wash buffer. Make a 1:10 stock dilution of the 10X wash buffer with dH_2O and place into a stock bottle.

B. Label the stock bottle with the buffer, lot # (date of preparation), expiration date and examiner's initials. Place diluted buffer into a wash bottle labeled with the buffer, lot # (date of preparation), fill date, expiration date and examiner's initials. Record the required information on the Sperm Hy-Liter Stain Reagent Log Sheet.

C. Store according to the manufacturer's instructions.

D. Discard/replace according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical Reagent QC).

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

14.6.5: Sperm Hy-Liter Stain Kits

A. Test each new lot before use according to the test procedure and the Sperm Hy-Liter Stain Reagent Log Sheet. Record the required information.

1. A second qualified examiner will observe/confirm a positive identification (spermatozoa/epithelial cells) and initial/date the Sperm Hy-Liter Stain Reagent Log Sheet.

2. It is not necessary to photograph for QC.

B. If the appropriate results are not obtained, review the procedure and repeat the test. If the reagent still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

C. If the lot is acceptable for use, record the date received, date opened and examiner's initials on each box.

The lot is acceptable for use when spermatozoa and epithelial cells are identified in a

spermatozoa/epithelial cell control demonstrating that they have stained properly according to section 14.5.9.B.

D. Store according to the manufacturer's instructions.

E. Discard/replace according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical Reagent QC).

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

14.7 REFERENCES

A. Independent Forensics, Sperm Hy-Liter™ Technical Information and Protocol sheets.
B. Independent Forensics, Sperm Hy-Liter™ PLUS Technical Information and Protocol sheets.
C. Independent Forensics, Sperm Hy-Liter™ Express Technical Information and Protocol sheets.
D. Independent Forensics, Sperm Hy-Liter™ Recommended Laboratory Recipes and Procedures, p 1-12.

E. 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 34-52.
F. 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.
G. Jennifer Old, Chris Martersteck and Anna Kalinina, Developmental Validation of Sperm Hy-Liter Express™, p 1-11.
H. Axio Scope.A1 Operating Manual
I. Li, Richard, Forensic Biology, Second Edition, CRC Press, FL, 2015, Chapter 14: "Identification of Semen", pp. 257 (14.1.1) - 259 (Figure 14-3) and pp. 264-265 and p. 266 (Figures 14.13 and 14.14).
J. Connecticut State Forensic Science Laboratory, Sperm Hy-Liter Internal Validation, 2010.
K. Connecticut State Forensic Science Laboratory, Sperm Hy-Liter Express and Fecal Material Internal Validation, 2012.
L. DNA Analyst Training, Laboratory Training Manual, Protocol 2.05, Semen Stain Identification: Kernechtrot Picroindigocarmine Stain (KPIC), President's DNA Initiative, Illinois State Police.
M. GL-2 (Safety Manual)
N. GL-6 (Purchasing)
O. Safety Data Sheets