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 microscopical 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, bacteria and yeast.

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

    d. Spermatozoa can be identified by their relative size, distinct morphology and staining pattern.

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

2. The Sperm-Hyliter method of sperm identification employs a process whereby a fluorescently tagged, anti-human sperm head monoclonal antibody is used to detect the presence of human spermatozoa.

    a. Nuclei present in a questioned smear are fluorescently tagged during a staining process and the smear is examined on a fluorescence microscope.

    b. One fluorescent tag binds to all nuclei present in the sample, including spermatozoa, bacteria and yeast. Under the proper microscope filter (DAPI), these nuclei can be visualized by their emission of a blue fluorescent signal.

    c. The other fluorescent tag binds specifically to the sperm heads by using anti-human sperm head monoclonal antibody. Under the proper microscope filter (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: Forensic Science Examiners (however titled) from the Division of Scientific Services who have been trained in the discipline of identifying spermatozoa according to FB SOP-26 (Training Manual and Checklist).

14.2.2: Forensic Science Examiners in the Forensic Biology Unit. Ordering information is maintained in a log book in the Forensic Biology Unit. New chemicals, reagents and kits are purchased according to GL-6 (Purchasing). For additional information, refer to the Biological Inventory (Appendix 3) in the FB folder on the shared drive.

14.3 SAFETY

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

14.4 DEFINITIONS

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

14.5 TEST PROCEDURES

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

A. Typically, Sperm Hy-Liter staining is used for Hospital/OCME prepared smears and Christmas Tree staining is used for extract smears.
B. Sperm searches may be conducted unstained according to FB SOP-02 (Sexual Assault Evidence Collection Kit Examination, section 2.6.F).

14.5.1: Materials

A. Hospital/OCME prepared smears
B. Extracted Samples
C. Positive AP swab
D. Micropipet 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. 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. Remove 3µl of the pellet with a micropipet onto a glass slide and form a smear.
2. 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 micropipet onto a glass slide and form a smear.
3. Allow the smear to dry at room temperature or 37°C.

D. Directly from a positive acid phosphatase sample:
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 stain (Stain A) for 20 minutes.

B. Gently rinse KS off slide with dH_2O.

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

D. 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.

E. Examine the smear for spermatozoa and other cells (i.e. epithelial cells) under the microscope at 200X, verify under 400X if needed.
1. Record the microscope(s) used on the appropriate Quality Record Worksheet (Appendix 1).

2. It is not necessary to conduct a full search if spermatozoa are identified and rated prior to completing the search.

3. If applicable, record 'partial search' on the appropriate Quality Record Worksheet (Appendix 1).

F. Record the staining procedure used on the appropriate Quality Record Worksheet (Appendix 1).

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 Quality Record Worksheet (Appendix 1).
1. If an AP positive sample was used to make the smear, then record on the appropriate Quality Record Worksheet 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).

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 and using a micropipet place approximately 3µl onto the 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.

B. Smears made directly from the pellet
1. Prepare the extracted sample smear:
   a. Remove 3µl of the pellet with a micropipet to a glass slide (standard or Sperm Hy-Liter/masked) and 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 micropipet to a glass slide (standard or Sperm Hy-Liter/masked) and 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.

5. Record the type of extract smear made on the appropriate Quality Record Worksheet (Appendix 1).

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. Place 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. Place 8µl of the epithelial cell re-suspended pellet onto the well of the Sperm Hy-Liter/masked slide and 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.

4. Record the type of extract smear made on the appropriate Quality Record Worksheet (Appendix 1).

14.5.5: Sperm Hy-Liter Staining Procedure

A. Record test(s) used on the appropriate Quality Record Worksheet (Appendix 1).

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

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

1. Use sufficient volume of reagent to cover sample.
2. 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).

C. Add the Fixative Solution to each smear. 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: 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 centrifuge tube according to FBQR-14 (Sperm Hy-Liter Calculation Worksheet). For Anal Smears, see section 14.5.6 below.

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

F. 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. The above calculations are used as an approximation.

G. Use 1X wash buffer to gently rinse each smear. 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. 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. 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 smear for spermatozoa and other cells (i.e. epithial cells) according to section 14.5.7 below, using Phase Contrast, FITC fluorescence and DAPI fluorescence. When necessary, capture images according to section 14.5.8.

1. Record the microscope(s) used on the appropriate Quality Record Worksheet (Appendix 1).

2. It is not necessary to conduct a full search if spermatozoa are identified and rated prior to completing the search.

3. If applicable, record 'partial search' on the appropriate Quality Record Worksheet (Appendix 1).

N. Record the staining procedure used on the appropriate Quality Record Worksheet (Appendix 1).

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

P. Record the results of the sperm/epithelial cell controls (Sperm Hy-Liter) and samples on the appropriate Quality Record Worksheet (Appendix 1).

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

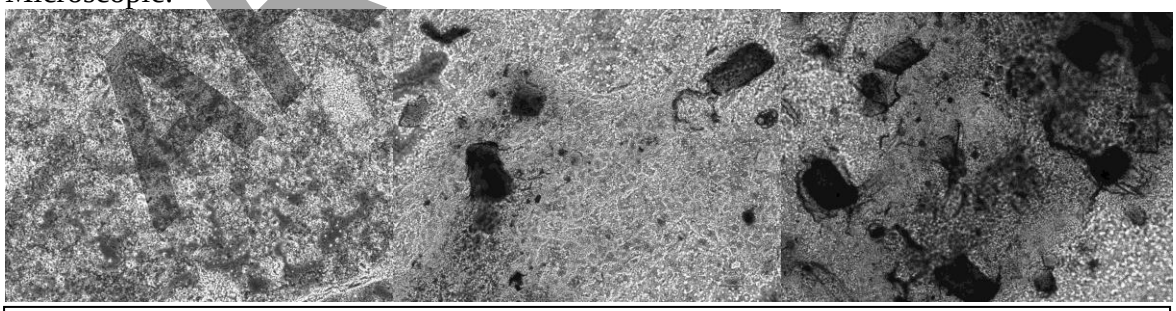
14.5.6: Sperm Hy-Liter Anal Smears (Fecal Material)

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

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 Searches Using the Sperm Hy-Liter Systems

A. Sperm searches using the Sperm Hy-Liter Olympus microscope

1. Turn on the light source, microscope, computer, and Proscan II controller (note that the light source should be on for 15 minutes before turning off - this will conserve bulb life and ensure proper wavelength is optimized).

2. Open the Spermtelligence software. Click on the Fluorescence and the Phase contrast icons as needed to switch between microscope settings (the transmitted light may also be turned on/off using the button on the top of the joystick - by this method both PC and fluorescence may be shuttered at the same time).

3. Load smear onto stage.

4. Using the 20x objective and the phase contrast setting (fluorescent shutter closed, filter in #1 position, transmitted light source uncovered, setting with 1 dot on the condenser) check that the smear is in focus.

5. 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 with 1 dot on the condenser).

6. If a magnification of 400x is used, the condenser setting with the 2 dots should be used.

B. Sperm searches using the Sperm Hy-Liter Zeiss microscope

1. 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).

2. Load smear onto stage.

3. 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.

4. 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).

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

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

14.5.8: Capturing Images Using the Sperm Hy-Liter Systems

A. Capturing images using the Sperm Hy-Liter Olympus microscope system

1. On the microscope, slide the metal rod (light path selector rod) fully out to the camera only setting.

2. On the computer, select the "setting" button. From the list, select the appropriate filter setting (FITC Full, DAPI, PC). Refocus as necessary. When capturing the image in phase contrast reset the white balance by selecting the "WB" button and lighten or darken as needed with the light adjustment knob.

3. Select the "capture" button to take photo.

4. Repeat steps 2 and 3 for each image being captured.

5. To save the images, select the "x" in the upper right hand corner of the image (this will close the image). In the window that appears, select the "pick folder" option, and in the next window select the "hard drive" option. The cabinet will appear, select the appropriate file cabinet with current cases, save the photos to the next available file folder by clicking "save" in the window that appears.

6. Go to the file cabinet, select the drawer and then the file folder with the case images. From this window you can label the file with the case # by selecting the image of the file folder with a pencil, and label the photos with the appropriate information (Case #, Sample/Item #, Investigator, Technician (if different from Investigator), Lot #, Optics, Coordinates).

7. Once finished, save by selecting the tan floppy disc icon.

8. A word document containing the images is automatically created as the images are captured.

9. Resize the images as necessary and include the case #, item #, magnification, filter set, and coordinates by each image.

10. Save this document to the desktop folder labeled "SH case photos", create a folder for the case, then save the document to that folder.

11. A printed copy of these images should be maintained in the case jacket.

B. Capturing images using the Sperm Hy-Liter Zeiss microscope system

1. Open the Sperm Capture Software.

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

3. 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.

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

4. 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.

5. 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.

6. Repeat steps 3-5 for each image being captured.

7. Create a document containing the images. Using Open Office, 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. Resize the images as necessary and include the case #, item #, magnification, filter set, and coordinates on the document. Save this document to the desktop folder labeled "SH case reports".

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

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

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

14.5.9: Results and Conclusions

A. Christmas Tree
A stained, intact spermatozoon will have a red head. A stained, non-intact spermatozoon will have a red head and no tail. Epithelial cells will be greenish with pink to 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. <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 Quality Record Worksheet (Appendix 1):

   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 (record coordinates unless extract smear)
   1 spermatozoon - head portion or intact (record coordinates unless extract smear)

2. 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 microscopical examination of [ ].
The head portion of one (1) spermatozoon was identified upon microscopical examination of [ ].

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

d. Red Blood Cells
Red blood cells were identified upon microscopical 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

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

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

E. 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.*

14.6 QC Procedure

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

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.

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 and discard according to the manufacturer's instructions.

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.

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 two (2) years 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 (see chart below) to final solution volume.

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.

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.

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 and discard according to the manufacturer's instructions.

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.

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 and discard according to the manufacturer's instructions.

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.
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