Procedure for Semen and Sperm Analysis

1.0 Purpose - This procedure specifies the method for conducting analysis for semen and sperm in forensic casework.

2.0 Scope - This procedure applies to those Forensic Scientists who have been released to conduct semen and sperm analysis in forensic casework.

3.0 Definition - N/A

4.0 Equipment, Materials and Reagents

- Working solution (see Forensic Biology Section QC procedure)
- Disposable scissors or disposable scalpel blade
- Glass culture tube (10 x 75 mm)
- Whatman 55 mm filter papers
- Disposable transfer pipettes
- Known seminal stain
- Kernechtrot and Picroindigocarmine stain (see Forensic Biology Section QC Procedure)
- Microscope slides
- 22 x 50 cover slips
- Olympus BX41 microscope
- Hot plate
- Deionized water
- Methanol
- Permount
- Wooden applicator sticks
- RSID kits which contain the test cards and universal buffer
- 1.5 mL centrifuge tube

5.0 Procedure

5.1 Acid Phosphatase Test (Walker Test)

5.1.1 When examining clothing, a visual examination shall be conducted for semen-like stains. These stains shall be photographed (refer to Forensic Biology Section Procedure for Photographing Evidence), marked, and cut for examination. When multiple articles of clothing are contained together as one item, not all of the articles need to be examined if information is received that accounts for only one possible semen donor and a sperm quantitation greater than rare is observed on at least one article of clothing from that item or on a previously examined item.

5.1.2 Following the visual examination of the clothing, the Mini-CrimeScope shall be used to detect stains that fluoresce (refer to Forensic Biology Section Procedure for Mini-CrimeScope). Stains shall be photographed, marked, and cut for examination.

5.1.3 If the information received accounts for only one possible semen donor and the sperm quantitation is greater than rare on slides prepared from the vaginal, rectal or oral swabs, then the panties and any additional evidence do not need to be examined for semen. Additional evidence would be examined to support possible additional charges.

5.1.4 If the item tested is underwear worn during or immediately after the alleged assault, at least three cuttings from the crotch area (or drainage area) are required even if there are no stains visualized. If multiple pairs of underwear are submitted and the Forensic Scientist is unable to determine which pair meets this qualification, then the pair in the Sexual Assault Evidence Collection Kit shall have a minimum of three cuttings taken and the remaining pair(s) shall be treated as clothing (refer to Forensic Biology Section Procedure for Mini-CrimeScope). The only exception to this is covered in **5.1.3** of this procedure.

5.1.5 If information is received that multiple donors may exist, further analysis shall be conducted in accordance with the analyst's training and experience.

5.1.6 Using a disposable pair of scissors or a disposable scalpel blade, remove a cutting of the suspected stained area or the tip of each swab and place the sample in a separately labeled clean 10 x 75 mm glass tube or on a piece of filter paper.

5.1.7 Add enough working solution to cover each sample and agitate. The results shall be read within one minute from the addition of the working solution.

5.1.8 Results

5.1.8.1 A positive result occurs when a purple color develops quickly on the material or bleeds into the solution/test paper. This positive result is indicative for the presence of semen.

5.1.8.2 A negative result occurs if no purple color change is observed. This negative result fails to indicate the presence of semen.

5.1.8.3 If the substrate has a color that could affect the ability to see a potential color change when the reagents are applied and the test is recorded as inconclusive, the reason shall be documented in the notes. If there is enough material, prepare a microscopic slide from the area as provided in **5.2**, or conduct the RSID semen test as provided in **5.3**.

5.1.9 If the Forensic Scientist performs further testing due to the nature of the sample, he/she shall document the reason in the worksheet.

5.2 Sperm Identification

5.2.1 A microscopic examination is conducted to confirm the presence of spermatozoa. This shall be done on samples meeting any of the following criteria:

5.2.1.1 All vaginal and rectal smears, unless no penile penetration is indicated. If no smears, then a slide shall be prepared from the swabs in the sexual assault kit. If oral assault is indicated from the paperwork, and the sample is collected within 24 hours of the assault (in a living victim), the oral smear shall be examined. If no smear, then a slide shall be made from the oral swabs.

5.2.1.2 If rare or no spermatozoa are seen on the slide prepared from the vaginal swabs and swabbings are received from the external labia area, a slide shall be made from the external labia.

5.2.1.3 Any area which gives an AP result which is indicative for the presence of semen. If multiple areas tested from one item give an AP result indicative for the presence of semen, a slide needs to be prepared only from the area that gives the strongest color change. If information is received that more than one semen donor may be present on the item the analyst will, based on their training and experience, determine if multiple slides need to be prepared.

5.2.1.4 Condoms: Separately swab both the inside and the outside of a condom (if possible) and make a slide directly from each swabbing. An AP Test on these swabbings is not required prior to doing a sperm search.

5.2.1.5 If a liquid urine sample is requested to be examined for sperm, spin down the sample to pellet any cellular material. Return liquid to submitted container. Re-suspend the pellet in TE or original liquid (approximately 25-50 µL). Pipette 10 µL onto slide and continue with **5.2.5**. If the re-suspended sample will be transferred to another analyst for DNA examination, the remaining sample shall be placed on sterile swabs and air dried before it is transferred to the analyst for DNA testing.

5.2.2 If a slide has been prepared previously, proceed to **5.2.5**. If a slide is to be prepared by the Forensic Scientist, continue with **5.2.3**.

5.2.3 Using a sterile disposable utensil, cut a sample from the item of evidence which contains the suspected stain or the tip of each swab and place the sample on a clean microscope slide.

5.2.4 Add 1-2 drops of deionized water to the sample and tease the sample apart with wooden applicator sticks.

5.2.5 Heat fix the sample onto the slide by placing the slide on a hot plate.

5.2.6 Place the slide(s) on a rack and apply the Kernechtrot stain to the slides. Leave the stain on for a minimum of 15 minutes. The stain can be left on the slide longer; however, the stain should not be allowed to dry onto the slide.

5.2.7 Remove the Kernechtrot stain by pouring it into a biological waste container (see biohazard safety procedure) and immediately apply the Picroindigocarmine stain to each slide. Leave this stain on for no more than 15 seconds. Pour the stain into a biological waste container.

5.2.8 Wash off the stain with methanol. Let the slides dry.

5.2.9 Once dry, apply a small amount (a couple of drops) of Permount onto the slide and add a 22 x 50 mm cover slip over the slide.

5.2.10 Observe the slide under the microscope at 200X or 400X magnifications and confirm the microscopic characteristics of the sperm head at 400X. Spermatozoa have a clear acrosomal cap, a red head and a green tail. Spermatozoa may be identified without the presence of a tail, but the clear acrosomal cap must be present and clearly visible.

5.2.11 Sperm shall be quantitated in a microscopic field at 200X. The following ranges shall be noted for the quantitation of spermatozoa:

- Rare – one sperm up to 1 sperm per 3 Field of View (FOV)

- Occasional - >1 sperm per 3 FOV up to 5 sperm per FOV
- Moderate - 6 sperm per FOV up to 20 sperm per FOV
- Heavy - >20 sperm per FOV

5.2.11.1 Forensic Scientist who is doing body fluid identification only - If the sample has a rare sperm quantity on an intimate sample, an attempt should be made to locate an additional sample of evidence with a higher quantity of sperm for DNA testing.

5.2.12 If multiple slides are made from an item (with the exception of slides prepared from condoms) and some of the slides are positive for sperm and some are negative for sperm, RSID shall be run on those areas which were AP positive and the slides failed to reveal sperm. These results shall be documented in the case notes and Laboratory Report for both the positive sperm and positive or negative semen areas.

5.2.13 If more than one vaginal, rectal, or oral smear are collected and spermatozoa is identified on one of the smears, the other smear does not need to be examined.

5.3 RSID-Semen Test

5.3.1 A RSID-Semen test shall be run on all items which give an AP result indicative for the presence of semen and no spermatozoa are identified, unless there is not enough sample remaining to perform the test.

5.3.2 Cut a small sample, approximately 0.5 cm^2 (depending on the concentration of the stain), from the evidence sample using sterile disposable scissors or a sterile scalpel blade and place the cutting into a 1.5 mL centrifuge tube.

5.3.3 Add a minimum of 150 µL, up to 1 mL, of RSID universal buffer to each sample and mix well. (The amount of buffer added will depend on the sample size; buffer should cover the sample completely.)

5.3.4 Allow the sample to extract for a minimum of 2 hours. For weak or older samples, Forensic Scientists should use a larger quantity of material and/or an extended extraction time to include overnight (not to exceed 24 hours).

5.3.5 After completing the extraction process, pipette 100 µL of the extracted sample into the sample well on the RSID card.

5.3.6 Results

5.3.6.1 A positive reaction will have two lines appear in the test window. One line will appear in the area marked "C" for control and one line will appear in the area marked "T" for test. A positive result can be recorded as soon as both of these lines appear, but no later than 10 minutes. The lines must be reddish in color.

5.3.6.2 If a line does not appear in the “T” area within ten minutes, for both neat and diluted samples (if applicable) then the test is considered negative. A line must appear at the area marked “C” to ensure that the test is working properly.

5.3.6.3 If no line appears at the area marked "C," the test shall be repeated. If no line is seen in

the "C" window in the repeated test, the Body Fluid Technical Leader shall be notified as soon as possible. Refer to Forensic Biology Section Administrative Policy and Procedure.

5.3.6.4 If the analyst, through training and experience, believes a sample that was indicative for semen and gave a negative result with the RSID-Semen test was a result of high-dose hook effect then a 1:10 dilution of the sample shall be made using the RSID buffer and an additional test performed (refer to **5.3.5**) and results recorded.

5.3.6.5 The RSID reader shall be used to read the test cards immediately following the 10 minutes.

5.3.6.5.1 Turn on the RSID Reader using the power button. Note: The stylus must be used to make selection choices on the reader screen.

5.3.6.5.2 Select RSID icon on the Main Menu screen.

5.3.6.5.3 Select RSID-Semen on the Select Test Screen. Then select Run Tests.

5.3.6.5.4 Using the alpha or numeric keys, enter the name of the sample. Select Done. Review the sample name. If name is correct, select Proceed. If name is incorrect, select Back and enter the correct name.

5.3.6.5.5 Insert test card into the reader until the green status light is engaged. The Test Area of the cassette must enter the reader's port first and face out to ensure proper positioning. The message "Analyzing Cassette. Please Wait." will appear on the screen. Both the positive and negative controls shall be read first, followed by the unknown card(s).

Note: Take care to insert the card completely and do not remove it prior to results being obtained by the reader.

5.3.6.5.6 Read the results from the reader. These results are the results that shall be reported.

Note: The reader notes inconclusive as invalid. If the reader reports an invalid result, the test shall be repeated. If the repeated test is inconclusive (invalid), the Body Fluid Technical Leader shall be notified as soon as possible.

5.3.6.5.7 Select Finish. Continue with 5.3.6.5.3 to continue testing any additional cards. When all cards are read, select the home icon.

5.3.6.5.8 Turn the RSID Reader off using the power button.

5.4 Reporting Guidelines - The results statements shall reflect only the work that is performed. Portions of the statements may be omitted to address what testing is actually performed. This interpretation may include or build upon one (1) or more of the following responses depending on the circumstances of the case and the nature of the examination.

5.4.1 This phrase shall be used if the Acid Phosphatase Test is negative:

Examination of a sample(s) taken from_____ (Item(s) ___), using the Acid Phosphatase Test, failed to indicate the presence of semen.

5.4.2 This phrase shall be used if the Acid Phosphatase Test is positive:

Examination of a sample(s) taken from_____ (Item(s) ___), using the Acid Phosphatase Test indicates, but is not specific for, the presence of semen.

5.4.3 This phrase shall be used when an inconclusive test is indicated and there is possible interference of the substrate:

Examination of a sample(s) taken from _____ (Item(s) ___), using the Acid Phosphatase Test, failed to reveal conclusive results to indicate the presence of semen because of possible interference of the substrate.

5.4.4 This phrase shall be used if no confirmatory semen testing was done.

No confirmatory semen testing was performed.

5.4.5 This phrase shall be used if, due to limited sample, no confirmatory semen testing was done.

Due to the limited quantity of the sample, no confirmatory semen testing was done.

5.4.6 This phrase shall be used if spermatozoa are seen microscopically:

Microscopic examination of the ____smear/slide prepared from _____ (Item(s) ___) revealed the presence of spermatozoa.

5.4.7 This phrase shall be used if spermatozoa were not seen microscopically:

Microscopic examination of the ____smear/slide prepared from _____ (Item(s) ___) failed to reveal the presence of spermatozoa.

5.4.8 This phrase shall be used if the cellular material contained on the slides is not microscopically human in origin:

Microscopic examination of the ____smear/slide prepared from _____ (Item(s) ___) was conducted. The morphology of the cellular material is not consistent with human spermatozoa.

5.4.9 This phrase will be used if the RSID Semen test is positive:

Further examination of sample(s) taken from ____ (Item(s) ____), using the RSID Semen Test and the RSID reader, revealed the presence of human semen.

5.4.10 This phrase shall be used if the RSID Semen test is negative:

Further examination of sample(s) taken from ____ (Item(s) ____), using the RSID Semen

Test and the RSID reader failed to reveal the presence of human semen.

5.4.11 This phrase shall be used if the RSID Semen test reads invalid:

Examination of a sample(s) taken from ____(Item ___), using the RSID Semen Test and the RSID reader failed to give conclusive results for the presence of human semen.

5.5 Controls

5.5.1 Acid Phosphatase Controls: A known seminal stain is used as a positive control and the working solution is used as a reagent control. A substrate control, if available, is set up using a control cutting from an apparently "unstained" area of the same material from which the suspected stain has been cut. A positive and negative control shall be tested prior to analysis once each day the Acid Phosphatase Test is performed per each lot used and the results shall be recorded in the case notes as positive or negative for each case that was worked that day. The controls must react appropriately.

5.5.2 RSID-Semen Controls: A positive control (applicable body fluid standard), and a negative control (100 µL of universal buffer) shall be run with every case or every batch of cases and the results will be recorded in the case notes as a positive or negative. If a reddish line is seen in the negative control "T" area, the test shall be rerun. If a reddish line appears again in the negative control "T" area, the test shall be considered inconclusive. If this occurs, the Technical Leader shall be notified immediately.

6.0 Limitations – Limitations include, but are not limited to, the following: The Acid Phosphatase Test is a presumptive test for semen. It detects the enzyme acid phosphatase which is present in semen. If the enzymatic activity is low, it is possible for a seminal stain to give a negative Acid Phosphatase reaction. Enzyme activity used to screen for semen (Acid Phosphatase) is more easily degraded than sperm cells, can be affected by various disease states, and is extremely water soluble. For these reasons, it is not unexpected that with older samples one may find a sample which yields a negative Acid Phosphatase result, but is positive for sperm cells.

RSID-Semen- High dose hook effect can occur.

7.0 Safety – N/A

8.0 References

Forensic Biology Section Procedure for Mini Crimescope

Forensic Biology Section Procedure for Photographing Evidence

Forensic Biology Section Procedure for Aseptic Technique and Contamination Control

Forensic Biology Section Body Fluid training documents

Forensic Biology Section Procedure for Calibration and Maintenance

Forensic Biology Section Administrative Policy and Procedure

9.0 Records - N/A

10.0 Attachments - N/A

Revision History		
Effective Date	Version Number	Reason
10/26/2012	1	Original Document - Combined Procedure for Acid Phosphatase Test, Sperm Identification Procedure, and RSID-Semen portion of the RSID Procedure. Added reporting guidelines and allowed for changes to be made to by the Forensic Scientist to address the testing actually performed.
02/01/2013	2	5.1.1 – Added previously examined item to requirement for examination; 5.1.3 – Clarified wording; 5.1.5, 5.2.3 – Removed size of cutting; 5.1.6 – Addition of time limit to read results; 5.1.7 – reworded results statements, indicative of semen explained in 5.1.7.1, negative results explained in 5.1.7.2; 5.1.7.3, 5.2.12, 5.2.14 – Clarified wording; Added 5.1.8 to allow analyst to perform further testing due to the nature of samples; 5.2.1.4 – Added "separately" for swabbing; 5.2.1.5 – Added requirement for samples to being sent for DNA analysis; 5.2.1.6 – Reworded for clarification; 5.2.1.3, 5.3.1, Limitations – changed "positive" to "indicative for the presence of semen"; 5.3.5.2 – Added clarification for results of neat and diluted samples; Added new 5.3.5.4 – Moved requirement for performing test on diluted samples from limitation section; 5.4.1, 5.4.2, 5.4.4 – reworded reporting guidelines to "indicate the presence of"; 5.4.3 – reworded for consistency; 5.5.1 – Added requirement for QC testing each lot used
02/15/2013	3	5.4.4, 5.5.5 – changed "no further confirmatory" to "no confirmatory" 6.0 - clarified limitations
09/13/2013	4	5.1.5, 5.2.1.3 – Added to allow analyst to use training/experience to determine what additional evidence should be evaluated; 5.2.1.1 – added limitation for analysis of samples in oral assault; 5.2.1.5 – removed requirement for additional slide prep; 5.2.11 – added sperm quantitation ranges; 5.1.1, 5.1.3, 5.2.1.2, 5.2.11.1 – reworded to include sperm quantitation range; 5.2.13 – removed due to change in quantitation; 5.3.5.4 – clarified when dilution is needed for RSID-semen; 5.4.6 – removed requirement to create slide sub-item; 5.4.8 – add spermatozoon to statement; 5.4.9 – removed reporting for 1 sperm seen due to change in quantitation; 6.0 – removed dilution wording, language is in procedure (5.3.5.4)
12/18/2013	5	Header – added issuing authority

08/29/2014	6	5.1.1, 5.1.7 – clarified wording; 5.2.1.1 – clarified when smears shall be examined; 5.2.12 – clarified when RSID semen would be run; 5.3.4 – clarified maximum extraction time; 5.3.6.3 – clarified wording; 5.3.6.5 – added RSID reader; 5.4.10, 5.4.11, 5.4.12 – added reader to statements; 5.4.13 – added report wording for inconclusive RSID test
02/27/2015	7	5.3 – adjusted minimum extraction time to make all RSID tests consistent; 5.3.6.5 – required RSID reader for all samples; 5.4.7, 5.4.9 – removed to clarify reporting statements; 5.4.11, 5.4.12, 5.4.13 – removed parentheses and renumbered