Procedure for the Christmas Tree Stain to Identify Sperm Cells

1 Scope

These procedures apply to DNA personnel who create slides stains on evidentiary items or samples undergoing differential extraction, and stain slides using Kernechtrot-picroindigocarmine (i.e., Christmas Tree Stain) to aid in the microscopic identification of sperm cells.

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., pipettes, scalpel, tubes)
- Masked glass microscope slides, 3" x 1" (Tekdon Slide #516-051-120, or equivalent)
- Adhesive gaskets (Independent Forensics of Illinois, 8005, or equivalent)
- Coverslips (vWR, 48366 205, or equivalent)
- Hot plate (Corning Model PC-220, or equivalent)
- Microscope (Olympus CX31, or equivalent, 200x or 400x magnification)
- Costar® spin baskets, or equivalent

Reagents

- SERI Christmas Tree (Xmas Tree) Stains A and B (SERI, R540, or equivalent)
- Ethanol, 95%
- Water, Reagent Grade or equivalent
- Permount (Fisher Scientific, S70104, or equivalent)

3 Standards and Controls

A known positive (KP) control (dried human semen slide) must be processed in parallel with each staining batch, and should be the first slide examined prior to the examination of evidence slides. The KP control is used to demonstrate that the staining procedure was successful and is a reference for the expected staining and morphology of a sperm cell. If the KP control fails, no conclusions can be made for the samples in the staining batch. No control is required for slides that are prepared but are not stained or microscopically examined.

4 Sample Selection

Items with an indication that semen may be present (e.g., acid phosphatase positive, scenario information) are appropriate for slide preparation and/or microscopic examination.

5 Procedures

Refer to the appropriate DNA procedure (DNA QA 600) and follow applicable general precautions and cleaning instructions.

Slide may be prepared independent of staining and microscopy and may be performed by different individuals.

If necessary, apply a frame gasket to a submitted smear slide and proceed to staining. If a proper seal cannot be attained, the procedure may be continued without a gasket.

5.1 Slide Preparation

If another slide preparation method is used, record the details in the case notes and proceed to staining.

5.1.1 From Male Fraction of Differential Extraction

5.1.1.1	After the QIAcube "Separate and Lyse 12B Mod" protocol is run, the tubes containing the washed male fraction should be resuspended (generally by vortexing and a brief quick-spin).	

5.1.1.2	Remove a portion of the washed male fraction (generally 4 µL from each sample tube) and pipette it onto a labeled microscope slide (generally a masked slide is used).	

5.1.1.3	Allow the slide(s) to dry (generally 10 minutes) and then proceed to staining or store the slide for future staining.	

5.1.2 From a Possible Semen Stain (without a DNA extraction)

5.1.2.1	Place ~1.5 mm x 1.5 mm of a stain or equivalent cutting of a swab in a tube. Add 500 µL of water. (More or less may be used to cover cutting.) Incubate at room temperature for ~30 minutes.	

5.1.2.2	Vortex and pulse spin. Transfer the cutting to a spin basket*. Spin tubes (generally between 9,000 and 13,000 rpm for 5 minutes).	

*Lyse & Spin baskets must NOT be used.

5.1.2.3	Pipette 10 µL from the area of potential pelleting onto a labeled microscope slide.	

5.1.2.4	Allow the slide(s) to dry (generally 10 minutes) and then proceed to staining or store the slide for future staining.	

5.2 Staining

Ensure the appropriate fields (i.e., reagents, KP) in the Sample Tracking and Control System (STACS) are completed from any network computer, as necessary.

5.2.1	Fix the slides by placing on a hot plate set at 3 for approximately 15 seconds.	

5.2.2	Add Christmas Tree Stain A (red reagent) to cover the specimen area being stained (generally 2-3 drops per masked slide or 10-15 drops per full slide). Incubate on a level surface at room temperature for 10-15 minutes.	

5.2.3	Gently rinse slide with reagent grade water and appropriately discard waste.	

Generally ~1 mL is sufficient for rinsing a masked slide and ~1-3 mL for a full slide.

5.2.4	Add Christmas Tree Stain B (green reagent) to cover the specimen area being stained (generally 2-3 drops per masked slide or 10-15 drops per full slide). Incubate on a level surface at room temperature for ~15 seconds.	

Staining one slide at a time with Stain B will facilitate keeping the incubation time less than 15 seconds. Greatly exceeding the ~15 second incubation time may lessen the intensity of the red stain and should be avoided.

5.2.5	Gently rinse slide with 95% ethanol and collect rinsate in an appropriate waste container. Allow slide to dry at room temperature (generally ~5 minutes).	

Generally ~1 mL is sufficient for rinsing a masked slide and ~1-3 mL for a full slide.

5.2.6	In a chemical fume hood, add Permount (generally 1-2 drops for a masked slide or 10-15 drops for a full slide) and an appropriately-sized coverslip to the stained area. Permount will dry in ~10 minutes but slides may be viewed before completely dry.	

5.3 Microscopy

5.3.1	Scan the stained portion of the slide(s) at an appropriate magnification (generally 200x or 400x) and using appropriate condenser settings (for example, brightfield or “0”, phase 1 “Ph1”, and/or phase 2 “Ph2”) for any cellular material exhibiting suitable sperm head morphology. Sperm heads are generally dark pink/red with a pale pink/white acrosomal cap. If the	

	sperm cell has an intact tail, it may be stained a faint green color. Epithelial cells are typically blue/green with large red/purple nuclei.	

5.3.2	When an intact sperm cell(s) or an appropriately stained, morphologically suitable sperm head(s) is observed, record a positive result (or POS) in the casework notes.	

5.3.3	When no morphologically suitable sperm heads or intact sperm cells are observed, record a negative result (or NEG) in the casework notes.	

The confirmation of a sperm cell or sperm head must be made by a serology qualified Examiner. The confirming Examiner will be recorded in the casework notes.

The language an Examiner should use to report the test results from this examination and others is contained within the appropriate procedure (i.e., Sero 100) in the *DNA Procedures Manual.*

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 Insufficient stain quantity and/or quality may affect the number of sperm cells observed during a smear slide examination.

8.2 While the absence of sperm cells indicates that no semen was detected on an item, the failure to detect sperm cells in biological material is not the basis of a conclusive determination that semen is not present.

8.3 Because the cellular structure of a sperm cell is susceptible to damage from the uncontrolled conditions to which a semen stain may be exposed between the time of its deposition and the time of its collection, the sensitivity of microscopically based methods of semen detection may be reduced for a particular specimen.

8.4 Though this staining procedure is not human specific (i.e., other mammalian sperm cells will also be differentially stained), human sperm have distinct morphological characteristics that allow for their identification.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the "Safe Work Practices and Procedures," "Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)," "Personal Protective Equipment Policy," and "Chemical Hygiene Plan" sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the "Hazardous Waste Disposal" section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Ethanol can be hazardous. Wear appropriate protective clothing and eyewear when handling. Be careful not to expose face or hands to splashes.
- Stain A contains aluminum sulfate which can be harmful if inhaled, if comes into contact with skin, or if swallowed. Wear appropriate protective clothing and eyewear when handling.
- Stain B contains picric acid. This staining procedure uses a dye prepared in a saturated solution of picric acid. As a dry material, picric acid is an impact and/or shock sensitive explosive. Do not allow the liquid picric acid reagent to collect and dry between the cap and lid of the container. Do not open the container when dried material appears to be present, and if present contact the Laboratory Health and Safety Group.
- Permount Mounting Media should be used in a chemical fume hood. It is flammable and may cause central nervous system depression. It is an aspiration hazard, can enter lungs and cause damage, and may be absorbed through intact skin.

10 References

DNA Procedures Manual

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

Allery JP, Telman N, Mieusset R, Blanc A, Rough D. Cytological detection of spermatozoa: comparison of three staining methods. *J Forensic Sci* 2001; 46(2):349-351.

Leubitz SS, Savage RA. Sensitivity of Picroindigocarmine/Nuclear Fast red (PIC/NF) Stain for the Detection of Spermatozoa: A Serial Dilution Study of Human Ejaculate. *Am J Clin Pathol* 1984; 81: 90-93.

Oppitz E. A New Method of Dyeing Used to Detect Traces of Spermatozoa in Sexual Offenses. *Arch Kiminol* 1969; 144: 145-148.

Serological Research Institute. Christmas Tree Stain R540 Informational Flyer, November 2011.

Rev. #	Issue Date	History
1	09/10/14	Entire document revised to reflect new practice.
2	05/25/16	Updated references to DNA Procedures Manual 1: Added slide preparation. 2: Added Costar basket. 3: KP is processed with each staining batch. Added purpose of KP. No control needed for slides not stained. 4: Relocated and renamed to Sample Selection and added guidance. 5.1.2: Added slide prep from stain. 5.2: Changed to STACS. 5.2.3: Added appropriately. 5.2.6: Changed to 1-2 drops. 9.3: Added Stain A hazard info. Removed confirmation and reporting from procedural step boxes.
3	03/22/19	1: Updated scope 2: Removed no longer available Spray-Cyte™ Fixative, added hot plate. 3: Added if the KP fails 5.1.1.2: Added from each tube to encompass samples that will be combined. 5.2.1: Replaces Spray-Cyte™ Fixative instruction with heat fixing instruction. 9.3: Removed Spray-Cyte™ hazards

Redacted - Signatures on File

Approval

DNA Technical Leader Date: 03/21/2019

DCU Chief Date: 03/21/2019

QA Approval

Quality Manager: Date: 03/21/2019