Christmas Tree Stain to Identify Sperm Cells

Table of Contents

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1 INTRODUCTION

These procedures describe the process for creating slides from stains on evidentiary items or from samples undergoing differential extraction and the process of staining slides using Kernechtrot-picroindigocarmine (i.e., Christmas Tree Stain) to aid in the microscopic identification of sperm cells.

2 SCOPE

These procedures apply to DNA personnel who create and/or stain slides using Christmas Tree Stain and microscopically view slides to identify sperm cells.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., pipettes, scalpel, tubes)
- Masked glass microscope slides, 3" x 1" (Tekdon Slide #516-051-120, or equivalent)
- Coverslips (vWR, 48366 205, or equivalent)
- Hot plate (Corning Model PC-220, or equivalent)
- Microscope (Olympus CX31, or equivalent, with at least 20x and 40x objectives)
- Costar® spin baskets, or equivalent

3.2 Reagents

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

Refer to the appropriate DNA QA procedure (i.e., BIO-103) for reagent and control preparation information.

4 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.

5 SAMPLING

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.

6 PROCEDURE

Refer to DNA Procedures Introduction (i.e., BIO-100) for applicable laboratory quality assurance and cleaning instructions.

A slide may be prepared independent of the staining and microscopy and may be performed by different individuals. The case notes will reflect the identity of personnel responsible for each task.

Ensure the appropriate fields (i.e., reagents, KP) in STACS are completed, as necessary.

6.1 Slide Preparation

6.1.1 From Male Fraction of Differential Extraction

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

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

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.
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 and Spin baskets must NOT be used.

3.	Pipette 10 µL from the area of potential pelleting onto a labeled microscope slide.
4.	Allow the slide(s) to dry (generally 10 minutes) and then proceed to staining or store the slide for future staining.

6.2 Staining

1.	Fix the slides by placing on a hot plate set at 3 for approximately 15 seconds.
2.	• Remove slide from hot plate. • Add Christmas Tree Stain A (red reagent) to cover the specimen area being stained (generally 2-3 drops per masked slide). • Incubate on a level surface at room temperature for 10-15 minutes.
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.

4.	• Add Christmas Tree Stain B (green reagent) to cover the specimen area being stained (generally 2-3 drops per masked 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.	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.

6.	• In a chemical fume hood, add Permount (generally 1-2 drops for a masked slide) and an appropriately-sized coverslip to the stained area.

Permount will dry in ~10 minutes but slides may be viewed before completely dry.

6.3 Microscopy

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.

1A.	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.
1B.	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 case notes. If an examiner views a negative slide, this will also be recorded in the case notes.

The language an examiner should use to report the results from this testing and others is contained within the procedure for reporting serological testing results (i.e., BIO-400).

7 LIMITATIONS

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

8 SAFETY

- 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.
- Refer to the FBI Laboratory Safety Manual for information on personal protection, the proper disposal of the chemicals used in these procedures, as well as the biohazardous wastes generated.
- 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.

9 REFERENCES

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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.

10 REVISION HISTORY

Revision	Issued	Changes
00	09/30/2022	Reformatted DNA 112-3 into new template and assigned new Doc ID. Removed guidance for full slides. Added requirement for recording when FE views negative slides. Added appendix for QC of known semen slides.

11 APPENDIX A: REAGENT QUALITY CONTROL

Each new batch of Known Semen Slides will be tested using the above procedures for sections 6.2 through 6.3

A. A positive test result (i.e., presence of sperm cells) establishes that the new batch of Known Semen Slides are yielding the expected positive result.
B. A new batch of Known Semen Slides that do not yield a positive result must not be used for casework. If no sperm cells are present, a new batch of Known Semen Slides will be prepared with a different semen sample and the process will be repeated.