

FBS07- Microscopic Examination of Spermatozoa by Christmas Tree Stain

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1. Scope

- 1.1. This procedure is used to confirm the presence of spermatozoa/semen.

2. Background

- 2.1. The microscopic identification of spermatozoa is a method of confirming the presence of semen in an evidentiary stain. Spermatozoa are identified by either the presence of intact sperm cells displaying a head, mid-piece and tail, or sperm heads showing an acrosomal cap.
- 2.2. The microscopic examination can be enhanced by staining the slide using a differential stain known as "Christmas Tree Stain" which consists of two dyes: Nuclear Fast Red and picroindigocarmine. Sperm heads are usually well differentiated (red) with the acrosome staining significantly less densely (pink) than the distal region of the head. Nuclei inside epithelial cells appear pink to purple in color. Sperm tails and epithelial membranes are stained green by the picroindigocarmine.

3. Safety

- 3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures.
- 3.2. Read Material Safety Data Sheets to determine the safety hazards for chemicals and reagents used in the standard operating procedures.

4. Materials Required

- 4.1. Deionized Water (diH₂O) or TE Buffer
- 4.2. Nuclear Fast Red Dye or SERI R540 Christmas Tree Stain A
- 4.3. Picroindigocarmine Solution or SERI R540 Christmas Tree Stain B
- 4.4. 95% Ethanol
- 4.5. Slides
- 4.6. Coverslips
- 4.7. 2.0mL microcentrifuge tubes (optional)
- 4.8. Microscope

5. Standards and Controls

- 5.1. It is not necessary to prepare a Positive Control slide for this procedure. **Optional:** The slide(s) created by the quality control procedure (FBQ21) may be used as Positive reference slide(s).
- 5.2. It is not necessary to prepare a Negative Control slide for this procedure.

6. Calibration

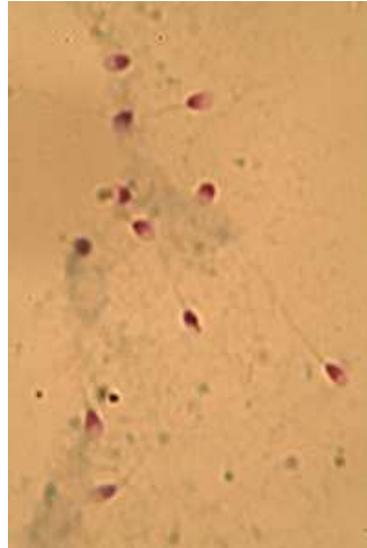
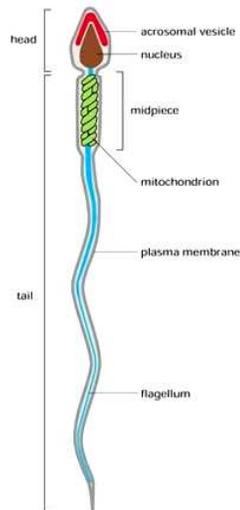
- 6.1. Not applicable

7. Procedures

- 7.1. Follow all the steps if preparing a slide from a p30 extract sample. For smear slides and differential extraction samples, begin with step 7.6.

- 7.2. Transfer the substrate to a filterless basket in a 2.0mL microcentrifuge tube and spin the sample tube(s) for 3 minutes at maximum speed.
- 7.3. Remove the supernatant until approximately 50µl is left in the tube, taking care to not disturb the pellet.
 - 7.3.1. **Optional:** Depending on the nature of the sample, the sperm pellet may be washed by re-suspending in 500-1000 µL of TE Buffer or diH₂O. Vortex and spin the sample(s) in a microcentrifuge for 3-5 minutes at maximum velocity. Remove and discard the supernatant, being careful not to disturb the sperm pellet (up to 50 µL may be left in the tube). Repeat this washing step an additional 2 times for a total of 3 washes of the sperm pellet. After the final spin, remove and discard all but approximately 50 µL of the supernatant, taking care not to disturb the sperm pellet.
- 7.4. Properly label the slide with sample identifier.
- 7.5. Re-suspend sperm pellet by agitating with a pipet tip. Pipet 4 µL of the re-suspended sperm pellet onto the slide. The remainder of the extract may be stored at -20°C to be recombined prior to DNA extraction if necessary or until ready to proceed with the next step in the differential extraction procedure.
- 7.6. Heat-fix cells to the microscope slide by conducting one of the following:
 - incubating in an oven (at 60°C for at least 1 hour)
 - incubating on a hot plate (at high for 20-30 minutes)
 - passing the slide (2-4 times) through the flame from a Bunsen Burner
- 7.7. Cover the stained area with Nuclear Fast Red Solution or Stain A.
- 7.8. Allow the slide to incubate at room temperature for at least 15 minutes.
- 7.9. Wash the slide gently with diH₂O until it washes clear.
- 7.10. Cover the stain area with Picroindigocarmine stain or Stain B.
- 7.11. Wash the slide after 5 seconds with 95% ethanol until it washes clear.
- 7.12. Allow slide to air dry or dry on a hot plate.
- 7.13. Wet mount slide using diH₂O and coverslip. Using a microscope, view the slide under 200-400x magnification. Epithelial cells will stain green with red nuclei.

Sperm cells will stain red with green tails. The sperm head will stain differentially with the acrosomal cap pink and the nuclear material red (see diagram below).



Note: Slides may be viewed with a light microscope with the option of using a phase contrast filter.

7.14. Observations of sperm cells, such as intact and/or sperm heads, will be noted in the appropriate casework documentation, as listed in the Documentation section. Additionally, the presence of spermatozoa will be documented as follows:

- +4 More than one sperm observed in every examined field
- +3 Sperm cells observed without difficulty in at least >50% to 90% of examined fields
- +2 Sperm cells observed in >10% to 50% of examined fields
- +1 Sperm cells observed in less than 10% of all fields
- 1 One sperm observed on entire slide
- 0 No sperm

8. Sampling

8.1. Not applicable

9. Calculations

9.1. Not applicable

10. Uncertainty of Measurement

10.1. Not applicable

11. Limitations

- 11.1. Insufficient sample quality and/or quantity could limit the detection of spermatozoa.
- 11.2. Yeast cells stain red and may resemble a sperm head. However, the stain is uniform throughout the cell and extends into polyp-like structures, which are occasionally observed with yeast cells.
- 11.3. If only a single spermatozoon is found on a slide, the coordinates of the spermatozoon location and slide orientation will be documented in case the spermatozoon needs to be relocated. A newly trained analyst (i.e. less than 3 months of sperm search experience) will have another qualified analyst verify the spermatozoon. The verification will be noted in the casework documentation.

12. Documentation

12.1. FBU Serology Examination Worksheets:

12.1.1. Serology Examination Worksheet – Document Control Number: 1569

12.1.2. PERK Worksheet – Document Control Number: 2154

12.2. FBU Extraction Worksheets:

12.2.1. Organic Extraction Sample Sheet (Differential) Worksheet – Document Control Number: 1581

12.2.2. EZ1 Extraction Sample Sheet (Differential) Worksheet – Document Control Number: 2110

12.3. FBU Report of Examination

13. References

- 13.1. *Allery, JP., Telman, N., Miesusset, R., Blanc, A., Rough, D. Cytological detection of spermatozoa: comparison of three staining methods. Journal of Forensic Sciences 2001; 46(2): 349-351.*
- 13.2. *Leubitz, S., Savage, R.A. Sensitivity of Picroindigocarmine/Nuclear Fast Red (PIC/NF) Stain for the Detection of Spermatozoa: A Serial Dilution Study of Human Ejaculate. American Journal of Clinical Pathology 1984: 81: 90-93.*
- 13.3. *Serological Research Institute. Christmas Tree Stain R540 Informational Flyer, February 1999.*
- 13.4. *Kamenev, L., Leclercq, M., and Francois-Gerard, C. Detection of p30 Antigen in Sexual Assault Case Material. Blood Group Laboratory and Forensic Institute, University of Lege, B-4000 Lefe, Belgium. Journal of the Forensic Science Society 1990;30:193-200.*
- 13.5. *P30 Antigen Test for the Presence of Semen (FBS06)*
- 13.6. *Differential Organic DNA Extraction (FBS09)*
- 13.7. *Quality Control of Christmas Tree Stain Reagents (FBQ21)*
- 13.8. *Forensic Science Laboratory Quality Assurance Manual (Current Version)*
- 13.9. *DFS Departmental Operations Manuals (Current Versions)*
- 13.10. *FSL Laboratory Operations Manuals (Current Versions)*