

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 semen.

2. Background

- 2.1. To establish the practices for documenting the examination of evidence to conform to the requirements of the Department of Forensic Sciences (DFS) Forensic Science Laboratory (FSL) *Quality Assurance Manual*, the accreditation standards under ISO/IEC 17025:2005, and any supplemental standards.
- 2.2. 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, midpiece and tail, or sperm heads showing an acrosomal cap.
- 2.3. 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 with the acrosome staining significantly less densely 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. diH₂O
- 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

4.6.1. NOTE: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions. Solutions should be stored in amber bottles.

5. Standards and Controls

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

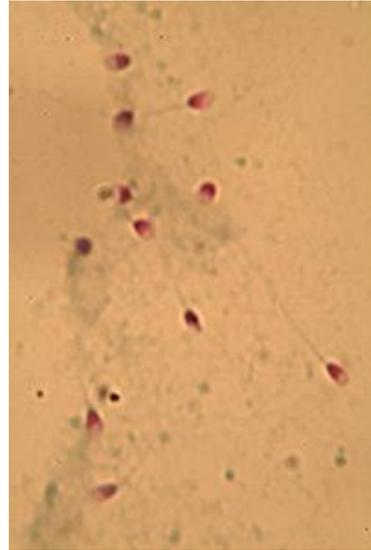
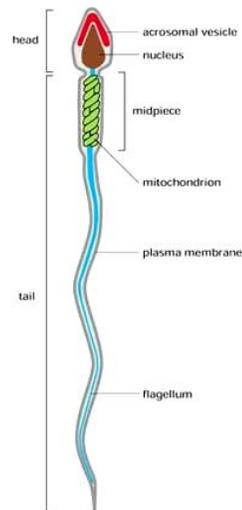
6. Calibration

- 6.1. Not applicable

7. Procedures

- 7.1. Follow all the steps if preparing a slide from a p30 extract or a differential extraction sample. For smear slides, begin with step **7.6**.
- 7.2. Microcentrifuge the sample tube(s) for 3 minutes.

- 7.3. Remove the supernatant until approximately 50µl is left in the tube, taking care to not disturb the pellet.
- 7.4. Properly label the slide with sample identifier. A circle may then be drawn in the center 0.5-1.0 cm in diameter with permanent pen to indicate location of sample area.
- 7.5. Resuspend sperm pellet. Pipet 3 – 5 µL 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 incubating on a hot plate (at high for 20-30 minutes) or by 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 in a room temperature humidity chamber or sit 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. Rinse the slide after 5 seconds with 95% ethanol until it washes clear.
- 7.12. Allow slide to air dry.
- 7.13. Mount slide using diH₂O and a coverslip. 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).



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

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

- +4 Many sperm in every field of view
- +3 Many sperm in some fields of view
- +2 Some sperm in some fields of view, easy to find
- +1 Hard to find, very few sperm (two or more) over the entire slide
- 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. When quantitative results are obtained, and the significance of the value may impact the report, the uncertainty of measurement must be determined. The method used to determine the estimation of uncertainty can be found in the *FSL Quality Assurance Manual – Estimation of Uncertainty of Measurement (Section 5.4.6)*.

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 analyst should have another qualified analyst verify the spermatozoon. The verification will be noted in the casework documentation. In this situation, the coordinates of the spermatozoon location and slide orientation should be documented in case the spermatozoon needs to be relocated.

12. Documentation

- 12.1. FBU Serology Examination Worksheets
- 12.2. Differential Organic Extraction Worksheet
- 12.3. FBU Report of Results

13. References

- 13.1. Allery, JP., Telman, N., Mieusset, 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. *Forensic Science Laboratory Quality Assurance Manual* (Current Version)
- 13.6. *FSL Departmental Operations Manuals* (Current Versions)
- 13.7. *FSL Laboratory Operations Manuals* (Current Versions)
- 13.8. FBS06 - P30 Antigen Test for the Presence of Semen (Current Version)
- 13.9. FBS09 - Differential Organic DNA Extraction (Current Version)
- 13.10. FBQ21 - Quality Control of Christmas Tree Stain Reagents (Current Version)