

FBS09- Differential Organic DNA Extraction

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1. Scope

- 1.1. This procedure describes the isolation of deoxyribonucleic acid (DNA) from biological specimens presumed to have sperm, recovered from evidentiary items for nuclear DNA typing.

2. Background

- 2.1. This procedure, in which epithelial and sperm cells are separated, is a modified version of the Organic DNA Extraction method. The Differential Extraction procedure preferentially lyses the non-sperm cells (EF) leaving the sperm cells (SF) intact. The remaining sperm cells are treated with dithiothreitol (DTT) which breaks the protein disulfide bonds that make up the cell membranes. This method will be used on any sample where the presence of spermatozoa is suspected or has been confirmed.

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. Digest Buffer (FBR35)
- 4.2. Proteinase K (10 mg/ml) (FBR36)
- 4.3. Phenol/Chloroform/Isoamyl Alcohol (PCI)
NOTE: This reagent and its waste must be handled in a fume hood.

- 4.4. 1.0 M DTT (FBR38)
- 4.5. TE Buffer or diH₂O
- 4.6. Sterile water
NOTE: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions.

- 4.7. Microcentrifuge
- 4.8. 1.5mL and 2.0mL Microcentrifuge tubes
- 4.9. Microcon devices

5. Standards and Controls

- 5.1. At least one reagent blank (i.e., extraction control) must be prepared and processed in parallel with each set of evidentiary specimens processed for DNA typing purposes. The reagent blank(s) (RB) is comprised of all the reagents used in the analytical process and is carried through the same extraction, quantitation, amplification and electrophoretic typing procedures as the evidence samples. If more than one extraction method is used then at least one reagent blank must be processed for each type of procedure.
- 5.2. For differentials, the reagent blank created in conjunction with the isolation of the female fraction is designated as the non-sperm fraction (RB#EF). The reagent blank created in conjunction with the isolation of the male fraction is designated as the sperm fraction (RB#SF). The RB will always be the last sample processed in a set.
- 5.3. In order to maintain a separation in time and space between questioned and known samples:
 - 5.3.1. At no time will questioned and known samples be simultaneously incubating in the same heat block.
 - 5.3.2. At no time will questioned and known samples be simultaneously extracted in the organic fume hood.

6. Calibration

- 6.1. Not applicable

7. Procedures

- 7.1. Place each sample into a labeled, sterile 2.0 ml microcentrifuge tube.
- 7.2. To each sample tube, pipette 400 μ L Digest Buffer and 12 μ L of Proteinase K (10 mg/mL) solution. Vortex and quick-spin in a microcentrifuge.
- 7.3. Note: The Digest Buffer and Proteinase K may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the prepared date.
- 7.4. Incubate on the heat block the samples either at 37°C for 2 hours to overnight or at 56°C for 1 hour.
- 7.5. After incubation, vortex and spin-down the sample tubes. Transfer the substrate to a filterless basket in a 2.0mL microcentrifuge tube and spin for 3 – 5 minutes at maximum speed. Save the substrate in a new sterile tube labeled with the case number, sample number, and “SR.” Note: SR = substrate remains.
- 7.6. Remove the supernatant from the samples, being careful not to disturb the sperm pellet (approximately 50 μ L remaining in the tube). Place the supernatant (non-sperm fraction [EF]) into a new appropriately labeled microcentrifuge tube. Set non-sperm fraction [EF] extracts to the side and proceed to next step.
- 7.7. Wash the sperm pellet by re-suspending in 500-1000 μ L of TE Buffer or diH₂O. Vortex and spin the samples 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 μ L may be left in the tube).
- 7.8. Repeat step 7.7 an additional 2 times for a total of 3 washes of the sperm pellet.
 - 7.8.1. **NOTE:** The wash step can be repeated an additional 1 to 5 times depending upon the nature of the sample.
- 7.9. After the final spin, remove and discard all but approximately 50 μ L of the supernatant.
- 7.10. **OPTIONAL:** Resuspend the sperm pellet within the remaining 50 μ L of supernatant by gently mixing the sample with a pipette. Remove approximately 4 μ L of the sample and spot it on a glass microscope slide. Perform a Christmas

Tree Stain using the technique described in Steps 7.6 through 7.14 of FBS07-Microscopic Examination of Spermatozoa by Christmas Tree Stain (Document Control Number: 1577). Proceed to Step 7.11 if no epithelial cells are observed. However, if any intact epithelial cells remain, re-digest the sperm pellet by following these additional steps:

- 7.10.1. Add 400 μ L of Digest Buffer to re-suspend the sperm pellet.
 - 7.10.2. Add 12 μ L of Proteinase K (10 mg/mL). Mix gently.
 - 7.10.3. Incubate at 37°C for 1 hour.
 - 7.10.4. Spin at a maximum velocity for 3-5 minutes in a microcentrifuge. Remove and discard all but approximately 50 μ L of the supernatant.
 - 7.10.5. Re-suspend the pellet in 500 μ L of Digest Buffer and vortex. Spin the sample 3-5 minutes in the microcentrifuge at maximum velocity. Remove and discard all but approximately 50 μ L of the supernatant. Proceed to Step 7.11.
- 7.11. To the sperm pellet add 400 μ L Digest Buffer, 12 μ L 1M DTT, and 15 μ L Proteinase K (10mg/mL). The Digest Buffer and Proteinase K may be added to the sample and incubated at 37°C or 56°C while performing the Christmas Tree Stain. Once a slide is confirmed as containing no epithelial cells, the DTT may be added and the incubation continued.
- 7.11.1. **Note:** The Digest Buffer, Proteinase K and DTT may be aliquoted as a master mix. If the master mix method is used, this aliquot expires at the end of the day.
- 7.12. Mix the samples gently and incubate on the heat block at 56°C for 2 hours to overnight.
- 7.13. In a fume hood add 500 μ L of Phenol/Chloroform/Isoamyl Alcohol (PCI) solution to the sperm and non-sperm fractions. Thoroughly mix (vortex or invert by hand) to form a transiently homogeneous suspension.
- 7.13.1. All nuclear DNA isolation steps in which Phenol/Chloroform/Isoamyl Alcohol reagent is used must be performed in a fume hood.
 - 7.13.2. To ensure a homogeneous solution, the Phenol/Chloroform/Isoamyl Alcohol reagent will be removed from the refrigerator and allowed to equilibrate to room temperature before beginning extractions.
- 7.14. Microcentrifuge the samples at room temperature for 5 minutes at maximum speed to separate the two phases. Proceed to Step 7.15 if the upper aqueous phase is clear.

- 7.14.1. Note: If the aqueous phase is not clear (e.g., cloudy, dark in color or colored from dyes) due to incomplete phase separation, then transfer the upper aqueous phase to a new sterile 1.5 mL microcentrifuge tube. Repeat step 7.13 – 7.14 an additional 2 to 3 times, until the interface is clean and the aqueous phase is clear. (For additional extractions, the lower Phenol/Chloroform/Isoamyl Alcohol layer may be removed and discarded, eliminating the need for a new microcentrifuge tube).
- 7.15. Assemble the Microcon and label the specimen reservoir.
- 7.16. Add 100 μ L of TE Buffer to the upper reservoir to pre-wet the membrane.
- 7.17. Note: When adding sample and/or buffer to specimen reservoir be cautious to avoid touching the filter with the pipette tip.
- 7.18. Transfer the entire aqueous phase of the samples to the upper reservoir containing the TE Buffer. Microcentrifuge at 500 x g for at least 10 minutes.
- 7.18.1. Caution will be taken to prevent the filters from exceeding their specified limitations. Excessive g-force may result in leakage or damage to the centrifugal device.
- 7.19. Remove the upper specimen reservoir from the tube, discard the effluent in the lower reservoir, and re-insert the upper reservoir into the Microcon tube. Alternatively, the upper reservoir may be placed into a new appropriately labeled Microcon tube and the original tube containing the effluent discarded.
- 7.20. Add 200 μ L of TE Buffer to the upper reservoir.
- 7.21. Microcentrifuge the samples at 500 x g for at least 10 minutes. If any liquid remains, additional spin(s) may be performed.
- 7.22. Label a new set of Microcon recovery tubes.
- 7.23. When the fluid has been drawn completely through the filter, add approximately 10-25 μ L of TE buffer to reservoir, and invert the upper reservoirs into the recovery tubes. Microcentrifuge for 3 minutes at 500 x g. Be certain that the caps on the tubes are all facing inward in the microcentrifuge to avoid possible snapping of the caps.
- 7.24. Remove and discard filter and close final Microcon tube. The contents of the final Microcon tube will be transferred to an appropriately labeled sterile

microcentrifuged tube. Add additional TE buffer, if necessary, to bring the final sample volume to at least 25 μL . Note: A higher recovery volume of $\sim 50 \mu\text{L}$ is recommended for samples of expected high DNA concentration, e.g. reference samples.

- 7.25. Reagent Blanks must be less than or equal to the lowest volume (highest concentration) of an associated sample within an extraction set.
- 7.26. Store the samples frozen.
- 7.27. If needed, a sample may be re-concentrated following steps below:
 - 7.27.1. Assemble the Microcon and label the specimen reservoir.
 - 7.27.2. Add the DNA sample to the upper reservoir, being cautious to avoid touching the filter with the pipette tip.
 - 7.27.3. Repeat steps 7.21 – 7.25.
 - 7.27.4. Note: If a sample is to be re-concentrated, the associated reagent blank must also be re-concentrated as well.

8. Sampling

- 8.1. Not applicable

9. Calculations

- 9.1. Not applicable

10. Uncertainty of Measurement

- 10.1. Not applicable

11. Limitations

- 11.1. The quantity and quality of the DNA present within any biological material ultimately determines if a nuclear DNA isolation is successful.
- 11.2. The separation of non-sperm and sperm cell DNA into their respective fractions is not always complete. It is not unusual for sperm cell DNA to be observed in the non-sperm (or epithelial) cell fraction and vice versa. The number of intact cells recovered in a sample and their capacity to endure the abrasive conditions of the differential extraction method is dependent upon the quality of the biological material being tested and the environmental conditions to which it has

been subjected. The detection of residual DNA within a given fraction does not prohibit the use of the DNA typing results from that fraction.

- 11.3. The presence of nuclear DNA in the sperm fraction of a differential extraction is not always a dependable method of determining whether semen or spermatozoa in a particular biological specimen is present. Many factors may influence this determination such as differing profiles in the sperm fraction and non-sperm fraction of the sample and the associated semen screening results (AP, p30, microscopic sperm identification)

12. Documentation

- 12.1. Differential Organic DNA Extraction Worksheet (Document Control Number: 1581)

13. References

- 13.1. Comey, C.T., Koons, B.W., Presley, K.W., Smerick, J.B., Sobieralski, C.A., Stanley, D.M., and Baechtel, F.S. DNA extraction strategies for amplified fragment length polymorphism analysis. *Journal of Forensic Sciences* (1994) 39: 1254-1269.
- 13.2. Millipore Corporation. Microcon[®] Centrifugal Filter Devices User Guide. Millipore Corporation, Billerica, MA, 2000.
- 13.3. Forensic Science Laboratory Quality Assurance Manual (Current Version)
- 13.4. Forensic Biology Unit Quality Assurance Manual (Current Version)
- 13.5. DFS Departmental Operations Manuals (Current Versions)
- 13.6. FSL Laboratory Operations Manuals (Current Versions)
- 13.7. FBR35 - Digest Buffer (Current Version)
- 13.8. FBR38 - 1.0 M Dithiothreitol (DTT) (Current Version)
- 13.9. FBS07 - Microscopic Examination of Spermatozoa by Christmas Tree Stain (Current Version)