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 recovered from evidentiary items for nuclear DNA typing.

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. 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 leaving the sperm cells intact. The remaining sperm cells are treated with dithiothreitol (DTT) which breaks the protein disulfide bonds that make up the cell membranes. This method should 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 (FBR36)
4.3. Phenol/Chloroform/Isoamyl Alcohol (PCI)
4.4. Note: This reagent and its waste must be handled in a fume hood.
4.5. 1.0 M DTT (FBR38)
4.6. TE Buffer (FBR14)
4.7. Phase Lock Gel Tubes
4.8. Sterile water
4.8.1. NOTE: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions.

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) 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).

5.3. To maintain a separation in time and space between questioned and known samples, all questioned samples must be processed through incubation and extraction before the known samples are processed. At no time should questioned and known samples be simultaneously incubating in the same heat block. At no time should questioned and known samples be simultaneously extracted in the organic fume hood. The RB should always be the last sample processed in a set. **Any RBs created must be matching the volume of the smallest sample.**

6. Calibration

6.1. Not applicable

7. Procedures

7.1. To each sample tube, pipette 400 µL Digest Buffer and 12 µL of Proteinase K (10 mg/mL) solution. Vortex and quick-spin in a microcentrifuge.

7.2. 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.

7.3. Incubate the samples either at 37ºC for 2 hours to overnight or at 56ºC for 1 hour.

7.4. After digestion, 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 "substrate remains."

7.5. Remove the supernatant from the samples, being careful not to disturb the sperm pellet (approximately 50µL remaining in the tube). Place the supernatant (non-sperm fraction [EF]) into a new appropriately labeled microcentrifuge tube.

7.6. Wash the sperm pellet by re-suspending in 500-1000 µL of TE Buffer or diH_2O. Vortex and spin the samples in a microcentrifuge for 3-5 minutes at maximum velocity. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet (up to 50µL may be left in the tube).

7.7. Repeat Steps 7.6 and 7.7 an additional 2 times for a total of 3 washes of the sperm pellet.

7.7.1. **NOTE**: The wash step can be repeated an additional 1 to 5 times depending upon the nature of the sample.

7.8. After the final spin, remove and discard all but approximately 50 µL of the supernatant.

7.9. OPTIONAL: Re-suspend the pellet within the remaining 50 µL of supernatant by gently mixing the sample with a pipette. Remove approximately 3-5 µL of the sample and spot it on a glass microscope slide. Perform a Christmas Tree Stain using the technique described in (FBS07). 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:

7.9.1. Add 400 µL of Digest Buffer to re-suspend the sperm pellet.

7.9.2. Add 12 µL of Proteinase K (10 mg/mL). Mix gently.

7.9.3. Incubate at 37°C for 1 hour.

7.9.4. Spin at a maximum velocity for 3-5 minutes in a microcentrifuge. Remove and discard all but approximately 50 µL of the supernatant.

7.9.5. Re-suspend the pellet in 500 µL of Digest Buffer and vortex. Spin the sample 3-5 minutes in the microcentrifuge at maximum velocity. Remove and discard all but approximately 50 µL of the supernatant. Proceed to Step 7.11.

7.10. To the sperm pellet add 400 µL Digest Buffer, 12 µL 1M DTT, and 15 µL Proteinase K (10mg/mL). The Digest Buffer and Proteinase K may be added to the sample and incubated at 37°C or 56ºC 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.

7.10.1. 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.

7.11. Mix the samples gently and incubate at 56ºC for 2 hours to overnight.

7.12. OPTIONAL: Obtain an appropriate number of phase lock gel tubes and label appropriately. Centrifuge at maximum speed for 20 to 30 seconds.

7.13. OPTIONAL: Add entire sample to phase lock gel tube.

7.14. In a fume hood add 500 µL of Phenol/Chloroform/Isoamyl Alcohol (PCI) solution to the sperm and non-sperm fractions. Thoroughly mix to form a transiently homogeneous suspension. Centrifuge the samples at room temperature for 5 minutes at maximum speed to separate the two phases. Proceed to Step 7.16 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 or phase lock gel tube. Repeat step 7.15 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 µL of TE Buffer to the upper reservoir to pre-wet the membrane.

7.17. Transfer the entire aqueous phase of the samples to the upper reservoir containing the TE Buffer. Centrifuge at 500 x g for at least 10 minutes. 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.

7.18. Add 200 µL of TE Buffer to the upper reservoir.

7.19. Centrifuge the samples at 500 x g for at least 10 minutes. If any liquid remains, additional spin(s) may be performed.

7.20. Label a new set of Microcon tubes with a case number and sample number (including “EF” or “SF” accordingly). RBs should be labeled with “RB#EF”” or “RB#SF.”

7.21. Add an appropriate volume (approximately 25 µL and 100 µL) of TE Buffer to the specimen reservoir.

7.22. Remove the specimen reservoir from the initial reservoir tube. Invert the specimen reservoir and place it into the new Microcon tube. Centrifuge at 500 x g for 3 to 5 minutes to elute the concentrated DNA. (Be certain that the caps of tubes are all facing inward in the microcentrifuge to avoid possible snapping of the cap).

7.23. Discard filter and close final Microcon tube. Final extract volume should be approximately 25-100 µL. The contents of the final Microcon tube may be transferred to an appropriately labeled sterile extract tube.

7.24. Store the samples frozen.

7.25. If needed, a sample may be re-concentrated following steps 7.16 - 7.25. If desired final volume is less than 20µL, then omit step 7.22.

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. 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 the female and male fractions is not always a complete division. It is not unusual for male DNA to be observed in the female 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 inhibit the use of the DNA typing results from that fraction.

11.3. The presence of nuclear DNA in the male fraction of a differential extraction is not a dependable method of determining whether semen or spermatozoa in a particular biological specimen is present.

11.4. All nuclear DNA isolation steps in which Phenol/Chloroform/Isoamyl Alcohol reagent is used must be performed in a fume hood.

11.5. To ensure a homogeneous solution, the Phenol/Chloroform/Isoamyl Alcohol reagent should be removed from the refrigerator and allowed to equilibrate to room temperature before beginning extractions.

11.6. Caution should be taken to prevent the filters from exceeding their specified limitations. Excessive g-force may result in leakage or damage to the centrifugal device.

12. Documentation

12.1. Differential Organic DNA Extraction Worksheet

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. *FSL Departmental Operations Manuals* (Current Versions)

13.5. *FSL Laboratory Operations Manuals* (Current Versions)

13.6. FBR35 - Digest Buffer (Current Version)

13.7. FBR36 - 10 mg/ml Proteinase K in 10mM Tris pH8.0 (Current Version)

13.8. FBR38 - 1.0 M Dithiothreitol (DTT) (Current Version)

13.9. FBR14 - TE Buffer (Current Version)

13.10. FBS07 - Microscopic Examination of Spermatozoa by Christmas Tree Stain (Current Version)