Procedure for Organic DNA Extractions

1.0 Purpose - This procedure specifies the steps for performing organic DNA extractions.

2.0 Scope - This procedure applies to Forensic Scientists in the Forensic Biology Section who perform organic DNA extractions.

3.0 Definitions

- **Differential lysis:** Included in the procedure for the analysis of mixed stains. It includes what is referred to as sperm and non-sperm lysis. The non-sperm lysis is contained in the aqueous portion remaining after a gentle lysis treatment of the stain. A more rigorous treatment follows for the pellet material (generally sperm); this is referred to as the sperm lysis.
- **Dithiothreitol (DTT):** Present to reduce the disulfide bonds that maintain the integrity of the sperm head. Sperm heads do not readily lyse in the absence of DTT.
- **Ethylenediaminetetraacetic acid (EDTA):** A component of the reactions used in the lysis process, which inhibits nuclease activity.
- **Known:** Biological material whose identity is established. These types of samples are used in casework for comparison to evidence.
- **Organic DNA Extraction:** A method that uses organic chemicals (phenol/chloroform/isoamyl alcohol) to isolate genomic DNA.
- **Proteinase K (Pro K):** A proteolytic enzyme that reduces proteins to their constituent amino acids. In particular, ProK removes the histone groups that keep the DNA tightly bound within the cell. The enzymatic activity of ProK lasts for approximately two hours and eventually self-digests.
- **Sodium dodecyl sulfate (SDS):** Serves to rupture the cell nuclear membrane to expose the nucleic acids. It also assists in the denaturation of the nuclear proteins that are attached to the DNA.
- **Unknown:** Biological material whose identity has not been established. These types of samples are used in casework for comparison to any available known samples.

4.0 Equipment, Materials, and Reagents

- Stain Extraction Buffer (SEB)
- Tris/EDTA Solution (TE)
- Tris/EDTA/NaCl Solution (TEN)
- Proteinase K Solution (ProK)
- Dithiotheritol (DTT)
- Phenol/Chloroform/Isoamyl Alcohol (PCI)
- Sterile disposable scalpel blades/scissors/forceps
- Centrifuge
- Heat Block with calibrated thermometer
- Calibrated pipets (various sizes)
- ART Pipet Tips (or equivalent, various sizes)
- Autoclaved 1.5 mL microcentrifuge tubes
- Autoclaved Spin Ease Baskets (or equivalent)
- Microcon 100 Filters and corresponding centrifuge tubes (or equivalent)
- Vortex mixer
- Certified biosafety cabinet
- Various lab equipment (various disposable conical tubes, lab tape, lab coat, microcentrifuge tubes and

racks, wipes, etc)
- 10 % Bleach solution

5.0 Procedure

5.1 Overview

5.1.1 All known samples shall be extracted separately from unknowns.

5.1.2 For casework, only one case shall be extracted at a time.

5.1.3 For casework unknowns, equal amounts of each swab present shall be cut for analysis.

5.1.4 Negative extraction control

5.1.4.1 For each case, a reagent blank shall be prepared each time an extraction set is started (i.e., knowns and unknowns). This blank will consist of the reagents used in the extraction process and be treated the same as any other sample throughout the entire process. Also, the final volume of this control shall be the same as the forensic sample(s) brought up in the most minimal volume and amplified using the maximum volume.

5.1.4.2 If a dilution is made of a sample prior to amplification, a dilution of the corresponding negative control does not have to be made if the same lot of TE is used for the Negative Amplification Control.

5.1.4.3 It is acceptable to run more than one reagent blank in anticipation of having to re-run or dilute samples for amplification.

5.2 Organic Extractions of Blood, Epithelial Cells, Hair Roots, and Tissue

5.2.1 All tubes shall be labeled with a unique identifier.

5.2.2 Aseptically place the sample in a labeled microcentrifuge tube. Hair roots are cut and placed in a tube by the Trace Evidence Section and transferred to the Forensic Biology Section.

5.2.3 To the sample, add 500 µL SEB and 10 µL of Proteinase K solution. Vortex briefly on low speed and spin briefly in a microcentrifuge to force the cutting into the extraction fluid. (Note: Volumes may be increased if working with larger size samples.)

5.2.4 Incubate the samples from 2 hours to overnight at 56 °C. If samples cannot be extracted immediately after incubation, then freeze and heat back to 56 °C before proceeding.

5.2.5 Additional Proteinase K may be added to samples after the initial incubation if a high amount of protein is suspected of being in the sample. Add an additional 10 µL of Proteinase K solution. Vortex briefly on low speed, spin briefly in a microcentrifuge to force the cutting into the extraction fluid and incubate at least an additional hour at 56 °C.

5.2.6 Spin briefly in a microcentrifuge to force condensate into the bottom of the tube.

5.2.7 For unknown samples, aseptically transfer the cutting into a basket insert. Place the basket in the tube

containing the stain extract and cap the tube. Spin in a microcentrifuge at high speed for 5 minutes. Remove the basket and discard it into a biohazard waste container.

5.2.8 In a certified chemical fume hood or cabinet, add 500 µL phenol/chloroform/isoamyl alcohol (PCI) to the stain extract. Mix briefly with the pipettor by drawing the solution up and down slowly several times. Cap the tube and vortex or hand shake the mixture briefly to attain a milky emulsion. Spin the tube in a microcentrifuge at high speed for 3 minutes.

5.2.9 Label the Microcon 100 filter or equivalent (concentrator) and corresponding centrifuge tube. The label shall be a unique identifier (i.e., the item number). Wet the membrane of the concentrator with approximately 20 µL TE. Transfer the aqueous phase (top phase) from the tube into the concentrator. Avoid pipetting organic solvent (bottom phase) or protein interface from the tube into the concentrator.

5.2.10 Cap the centrifuge tube containing the concentrator and spin in a microcentrifuge for 10 minutes at no greater than 4000 rpm. If fluid remains on the concentrator, spin for an additional 10 minutes.

5.2.11 Remove the tube cap and add 200 µL TE to the concentrator. Replace the tube cap and spin the unit in a microcentrifuge at no greater than 4000 rpm for 10 minutes. If fluid remains in the concentrator, spin for an additional 10 minutes. **Note:** Residual protein can cause the concentrator filter to become clogged, resulting in some liquid remaining in the concentrator after centrifugation. Adjust for this volume when performing the next step.

5.2.12 Remove the spin cap and add a measured volume of TE. The TE cannot to be less than 20 µL and is dependent on the estimated concentration of the stain. If the concentration is low, bring to volume with lower amounts of TE. If sample could be processed for Y-STR analysis, bring it up in no less than 40 µL of TE unless there is original evidence remaining. If the concentration is heavy, bring to volume with a higher amount of TE. Remove the concentrator from the centrifuge tube and carefully invert the concentrator onto a labeled microcentrifuge tube. Discard the centrifuge tube.

5.2.13 Centrifuge the assembly in a microcentrifuge at approximately 4000 rpm for 5 minutes.

5.2.14 Discard the concentrator. Cap the microcentrifuge tube.

5.3 Samples with Identified Sperm

5.3.1 Cut the sample and place it into a labeled microcentrifuge tube.

5.3.2 To the sample add 500 µL of SEB and 10 µL proteinase K. **Note:** Volumes may be increased correspondingly due to sample size.

5.3.3 Vortex briefly and spin briefly in a microcentrifuge to force the material into the extraction fluid.

5.3.4 Incubate at 37 °C for at least 2 hours to overnight.

5.3.5 Aseptically transfer the material to a basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge at maximum speed for 5 minutes. Discard the basket with the material.

5.3.6 While being careful not to disturb the pelleted material, remove the supernatant fluid from the original

tube and place it into a new labeled tube. This supernatant is the non-sperm fraction. Analysis of the non-sperm fraction resumes at **5.3.12**. The pellet remaining in the tube is the sperm fraction.

5.3.7 Wash the sperm pellet by adding 1000 µL of TEN, vortex the suspension briefly, and spin the tube in a microcentrifuge at maximum speed for 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the sperm pellet.

5.3.8 Repeat the step above two to four (2-4) times for a maximum of five washes to the sperm pellet. The number of washes may depend on the estimated quantity of sperm by the body fluid analysis.

5.3.9 To the tube containing the washed pellet, add:

350 µL SEB
40 µL .39M DTT
10 µL proteinase K

5.3.10 Close the tube caps, vortex briefly, and spin briefly in a microcentrifuge to force all the fluid and material to the bottom of the tubes.

5.3.11 Incubate at 37 °C for at least 2 hours.

5.3.12 To the tube containing the sperm pellet and to the tube containing the non-sperm fraction, add 500 µL of PCI. Mix briefly with the pipettor by drawing the solution up and down slowly several times. Vortex (low speed) or shake by hand the mixture briefly to attain a milky emulsion. Spin the tube in a microcentrifuge at maximum speed for 3 minutes.

5.3.13 Assemble and label a concentrator and corresponding centrifuge tube unit for each of the above samples. Wet the membranes of the concentrator(s) with approximately 20 µL TE. Transfer the aqueous phase (top phase) from the appropriate tube to the concentrator. Avoid pipetting organic solvent (bottom phase) or the protein interface from the tube into the concentrator.

5.3.14 Cap the concentrator and spin in a microcentrifuge at no greater than 4000 rpm for 10 minutes.

5.3.15 Remove the spin cap and add 200 µL TE to the concentrator. Replace the spin cap and spin in a microcentrifuge at no greater than 4000 rpm for 10 minutes.

5.3.16 Remove the spin cap and add a measured volume of TE. The TE cannot be less than 20 µL and is dependent on the estimated concentration of the stain. If the concentration is low, bring to volume with lower amounts of TE. If sample could be processed for Y-STR analysis, bring it up in no less than 40 µL of TE unless there is original evidence remaining. If the concentration is heavy, bring to volume with a higher amount of TE. Remove the concentrator from the corresponding centrifuge tube and carefully invert the concentrator onto a labeled microcentrifuge tube. Discard the corresponding centrifuge tube.

5.3.17 Spin the assembly in a microcentrifuge at no greater than 4000 rpm for 5 minutes.

5.3.18 Discard the concentrator. Cap the microcentrifuge tube.

5.4 DNA Extraction from Bone and Teeth

5.4.1 Sample Preparation – shall be performed in a Biological Safety Hood.

5.4.1.1 Dried Bone (no marrow or associated tissue)

5.4.1.1.1 Cleaning the bone.

5.4.1.1.1.1 Small bones: If the bone is heavily soiled, it may be necessary to remove any debris or associated dirt from the bone or part of the bone prior to cutting/grinding. The bone shall be placed in a weigh boat and a new toothbrush and sterile dH_20 or SEB used to physically remove any excess dirt.

5.4.1.1.1.2 Large bones: The area of the bone to be cut and used in analysis shall be ground off mechanically using a Dremel tool (cleaned with 10 % bleach) and a new grinding bit to remove soil and dirt.

5.4.1.1.2 Remove a cross-sectional wedge or rectangle of bone using a cutting tool (e.g., a Dremel tool with a cut-off wheel). Do not cut the bone in half unless necessary due to size; this preserves the bone for further anthropological study.

5.4.1.2 Bone with Associated Tissue and Marrow

5.4.1.2.1 If the tissue and marrow are not too degraded, take a sample of tissue and marrow and place in separate labeled microcentrifuge tubes for DNA extraction.

5.4.1.2.2 Cleaning the bone:

Using a sterile scalpel blade or the Dremel tool (e.g., a Dremel tool with a cut-off wheel) remove any associated tissue on the bone to be processed. Note: It is helpful to remove the tough fibrous membrane, the periosteum, prior to processing because it aids in the organic extraction (the periosteum is a large source of protein and it is difficult to remove it all during the PCI portion of the extraction).

5.4.1.2.3 Removal of bone section for DNA extraction using one of the following methods.

- Remove a cross-sectional wedge or rectangle of bone using a cutting tool (e.g., a Dremel tool with a cut-off wheel).
- Use a decontaminated drill bit, drill 4 - 5 holes through the bone. Collect the powder for analysis.

5.4.1.3 Tooth

Wash the tooth. It may be necessary to remove debris and/or associated dirt from the tooth. The tooth should be placed in a weigh boat and thoroughly washed with SEB. A new toothbrush shall be used to remove any debris or dirt.

5.4.2 Produce a Fine Powder from the Bone or Tooth.

5.4.2.1 Obtain a new coffee grinder.

5.4.2.2 Wipe down inside and outside with a Kim-wipe wetted with fresh 10 % bleach. Wipe down second time with a Kim-wipe wetted with 100 % alcohol. Allow to dry.

5.4.2.3 Place the sample into the cleaned grinder and grind the sample to a fine powder. Note: for small bones that are too small for the coffee grinder, a mortar and pestle may be used to grind the bone to a powder for DNA extraction.

5.4.3 DNA Extraction

5.4.3.1 Aseptically transfer approximately 0.5 g - 3 g of powder to a sterile labeled microcentrifuge tube. Larger volumes of powder (approximately 1 g - 3 g) may be extracted in a 15 mL conical tube if the bone is highly degraded. Place any excess bone or tooth powder into a separately labeled tube and save.

5.4.3.2 To 0.5 g - 3 g of sample (depending upon the quality of the bone/tooth) add approximately 500 µL to 3 mL of SEB and approximately 20 µL - 75 µL Proteinase K solution to the sample. For every 500 µL SEB, 40 µL of DTT shall be added. Vortex at low speed for approximately 15 - 20 seconds and briefly centrifuge to force the liquid and bone into the bottom of the tube.

5.4.3.3 Incubate the tube containing the sample at 56 °C overnight (18 hour minimum/24 hour maximum). After incubation, 15 µL of Proteinase K solution and 40 µL DTT shall be added to every 500 µL SEB. If additional Proteinase K and/or DTT is added, incubate for an additional 2 hours at 56 °C.

5.4.3.4 Centrifuge the tube briefly to force condensate into the bottom of the tube.

5.4.3.5 Add equal volumes of PCI to the bone extract. Mix briefly with the pipettor drawing the solution up and down slowly several times. Recap and vortex (low speed) the mixture briefly to attain a milky emulsion. Centrifuge at high speed for 3 minutes. Repeated PCI extractions may be necessary if a large protein interface is present following the first extraction.

5.4.3.6 Prepare a Microcon or Centricon 100 concentrator (or equivalent) by labeling the concentrator and centrifuge tube and wet the membrane with 20 µL TE. Transfer the aqueous phase (top phase) from the tube to the concentrator. Avoid pipetting the organic solvent (bottom layer) into the concentrator.

5.4.3.7 Place a spin cap on the concentrator and spin in a centrifuge (no greater than 4000 rpm) for 15 minutes. If liquid remains in the concentrator, repeat spin step.

5.4.3.8 Carefully remove the concentrator unit from the assembly and discard the filtrate from the filter cup. Return the concentrator to the top of the corresponding centrifuge tube.

5.4.3.9 Add 500 µL TE to the concentrator, replace spin cap, and centrifuge (no greater than 4000 rpm) for 15 minutes. If liquid remains in the concentrator, repeat spin step.

5.4.3.10 Remove the spin cap and add 50 µL TE to the concentrator. Remove the concentrator from the filter cup and carefully invert the concentrator onto a new labeled microcentrifuge tube. Discard the used corresponding centrifuge tube.

5.4.3.11 Centrifuge the assembly at 4000 rpm for 5 minutes.

5.4.3.12 Remove and discard the concentrator. Transfer to a new labeled microcentrifuge tube and cap.

5.6 Clean-Up of Extracted DNA

NOTE: This procedure may be used if there are particulates in the extract that might affect the amplification, or inhibitors are suspected (as observed in the results from quantitation) in the extract. The associated negative extraction control must also be subjected to this procedure in the event that any reagents used in the initial extraction have changed in the interim.

5.6.1 Thoroughly vortex the extracted DNA and centrifuge briefly at high speed (5 seconds).

5.6.2 Bring the total volume of the DNA extract to 200 µL using TE.

5.6.3 Add an equal volume of PCI to the DNA extract. Mix briefly with the pipettor by drawing the solution up and down slowly several times. Vortex (low speed) or hand-shake the mixture briefly to attain a milky emulsion. Spin the tube in a microcentrifuge at high speed for 5 minutes.

5.6.4 Wet the membrane of a new, labeled Microcon 100 concentrator (or equivalent) with 20 µL TE. Transfer the aqueous phase (top phase) from the tube to the concentrator. Avoid pipetting organic solvent (bottom phase) from the tube into the concentrator.

5.6.5 Cap the concentrator and spin in a microcentrifuge at 4000 rpm for 10 minutes.

5.6.6 Remove the spin cap and add 200 µL of TE to the concentrator. Replace the spin cap and spin the assembly in a microcentrifuge at 4000 rpm for 10 minutes.

5.6.7 Remove the spin cap and add a measured volume of TE. The TE cannot be less than 20 µL and is dependent on the results of the previous quantitation. If sample could be processed for Y-STR analysis, bring it up in no less than 40 µL of TE unless there is original evidence remaining. Remove the concentrator from the corresponding centrifuge tube and carefully invert the concentrator onto a labeled microcentrifuge tube. Discard the corresponding centrifuge tube.

5.6.8 Spin the assembly in a microcentrifuge at 4000 rpm for 5 minutes.

5.6.9 Discard the concentrator. Cap the microcentrifuge tube.

5.7 Concentration of Extracted DNA

NOTE: This procedure may be used if the original final volume of the DNA extract leaves the extract too diluted to obtain a usable DNA profile. If the final volume used in **5.8.4** is less than the final volume of the associated negative extraction control, the control must also be concentrated using the steps below.

5.7.1 Thoroughly vortex the extracted DNA and centrifuge briefly at high speed (5 seconds).

5.7.2 Wet the membrane of a new, labeled Microcon 100 concentrator (or equivalent) with TE. Transfer the extracted DNA to the concentrator.

5.7.3 Cap the concentrator and spin in a microcentrifuge at 4000 rpm for approximately 10 minutes.

5.7.4 Remove the spin cap and add a measured volume of TE. The TE cannot be less than 20 µL and is dependent on the results of the previous quantitation. If sample could be processed for Y-STR analysis, bring it up in no less than 40 µL of TE unless there is original evidence remaining. Remove the concentrator from the corresponding microcentrifuge tube and carefully invert the concentrator onto a labeled microcentrifuge tube. Discard the corresponding centrifuge tube.

5.7.5 Spin the assembly in a microcentrifuge at 4000 rpm for 5 minutes.

5.7.6 Discard the concentrator. Cap the microcentrifuge tube.

5.8 Storage of DNA Extracts - Store the samples at 4 °C (short term) or frozen (long term). Prior to use of samples after storage, they shall be vortexed, and then centrifuged for 5 seconds.

6.0 Limitations- N/A

7.0 Safety

7.1 Phenol/Chloroform/Isoamyl alcohol is a known irritant, inhalation hazard and a suspect carcinogen. Nitrile gloves, fume hood and eye protection is required during use.

7.2 Pregnancy – those who are currently pregnant should avoid performing this procedure.

8.0 References

Forensic Biology Section Procedure for DNA Casework Training

Forensic Biology Section Procedure for Performance Check and Equipment Maintenance

Forensic Biology Section Procedure for Human DNA Quantitation with Quantifiler®

9.0 Records

- Forensic Biology Section Extraction worksheet (to be used QC and training)

10.0 Attachments – N/A

Revision History		
Effective Date	Version Number	Reason
09/17/2012	1	Original Document
12/7/2012	2	5.1.4 – added negative extraction control requirements and requirements for dilutions
09/13/2013	3	2.0 – removed DNA database analysts; 5.1.3 – clarified wording; 5.1.4.1 – added explanation for wording; 5.2.9 – removed Database reference; 5.4 – removed section for extraction of slides; 5.6 – removed section for extraction in paraffin; 8.0 – removed reference to database procedure
12/18/2013	4	Header – added issuing authority