Organic Extraction

Sample Preparation

Liquid/dry blood, bone marrow, oral swab and tissue sample preparation

Stained substrates and oral swabs should be cut into small pieces (3 x 3 mm). Tissues should be minced into small pieces in a weigh boat using a sterile scalpel or razor blade. Place samples in 1.5mL microcentrifuge tubes or conical tubes when appropriate. See table below for various sample types.

Proceed to Section B: Sample Incubation

Sample type	Amount
Liquid blood	100 to 500 µL
Bone marrow	0.5 x 0.5 cm to 1.5 x 1.5cm
Oral swab	1/3 to a whole swab
Blood stain	0.5 x 0.5 cm to 1.5 x 1.5cm
Soft tissue	0.5 x 0.5 cm to 1.5 x 1.5cm
Paraffin embedded tissue	0.3 x 0.3 cm to 1.0 x 1.0 cm

Bone preparation

Before extraction, a bone or tooth specimen should be cleaned entirely of soft tissue and dirt using a range of methods, such as scraping, rinsing and sonication. A combination of sterile scalpels, sterile toothbrushes and running water should be used to clean the specimen. For a sonication bath, the sample is placed in a conical tube and covered with a 5% Terg-a-zyme solution. For additional cleaning, the sonication step may be repeated multiple times by decanting the liquid and replacing with fresh Terg-a-zyme solution. After cleaning, the sample is usually rinsed with distilled water and dried using a 56°C incubator (drying time may vary from a few hours to overnight).

Note: Terg-a-zyme is an enzyme-active powdered detergent. A 5% solution should be made fresh prior to bone preparation and cleaning. Refer to Appendix A in the Quality Assurance Manual. Once prepared, the reagent will only be effective for up to 16 hours.

Photograph bone or tooth sample after cleaning. Measure and weigh specimen prior to sampling.

1. If several bones are available, generally compact bone is preferred, such as humerus, femur, or tibia.

****WARNING****
Protective eyewear, lab coats, cut resistant gloves, sleeve protectors, and HEPA-filtered facial masks should be worn when cutting bone. Avoid breathing bone dust. All cutting of bone must be done under a biological hood.

2. Using an autopsy saw or a Dremel tool equipped with a 409 or 420 cutting wheel, cut the bone specimen into approximately 5x5x5mm size pieces. Take enough cuttings for an end weight of approximately 2g. For older or compromised bones, several aliquots of 2g can be extracted and combined during the Microcon step. For tooth samples, the whole root should be taken. **Note: The cutting wheel should be disposed of after each use and the Dremel and hood should be completely wiped down with bleach and ethanol**.

3. Place bone cuttings in 50mL conical tubes labeled with the FB case number, ME#, PM item #, initials, and date.

4. Cover bone cuttings with 5% Terg-a-zyme solution and sonicate samples for 30-45 minutes. **Note: Ensure water level in the sonicator is 1-2 inches from the top.**

5. Decant the Terg-a-zyme and wash with distilled water until no detergent bubbles remain.

6. If necessary, repeat with fresh changes of 5% Terg-a-zyme and water washes until the dirt has been removed.

7. Place the clean cuttings in a weigh boat on a small Kim Wipe. Cover with another weigh boat. Label the weight boat with the FB case number, ME#, PM item #, initials, and date.

8. Seal with evidence tape.

9. Dry in a 56°C incubator for a few hours or overnight. After sufficient drying, weigh bone cuttings. **The bone sample must be completely dry before milling.**

Sample milling with the SPEX Certiprep 6750 Freezer Mill

All freezer mill parts that come into contact with bone specimens, such as the cylinders, metal

end plugs and impactors, should be cleaned, dried and sterilized prior to use. See Step 22 for appropriate cleaning procedure.

1. Assemble specimen vials in the following order: metal bottom, plastic cylinder, impactor, and metal top.

2. Place under UV light for a minimum of 15 minutes.

3. Label metal bottoms with a case identifier using a blue ink Sharpie.

4. Add bone cuttings to specimen vial around impactor using decontaminated forceps. Cover with metal top. **Note: Shake specimen vial and ensure that the impactor can move back and forth.**

5. Wipe down inside of mill with a wet paper towel. **Do not use bleach or ethanol.**

6. Plug in mill and switch ON.

7. Obtain liquid nitrogen from tank by filling transfer container. Be aware that the liquid nitrogen tank may be empty when the detector level reads anywhere from "¼" to "empty".

   ****WARNING****
   Liquid Nitrogen can be hazardous. Use cryogenic gloves, protective eyewear/face shield and lab coats when handling. Avoid liquid nitrogen splashes to face and hands.

8. Open the freezer mill lid. Add liquid nitrogen slowly into the mill up to the **FILL LINE** to avoid splashing and boiling over.

9. Place the specimen vial into the round chamber. If processing more than one bone sample it is possible to save pre-cooling time by placing up to two vials in the mesh container inside the mill.

10. Change cycle number to match total number of samples plus two (n + 2).

11. Adjust mill settings as follows:

Cycle	**set to # of samples + 2**	
Time	**T1 (milling)** **T2 (pause)** **T3 (pre-cool)**	**2.0 min** **2.0 min** **15.0 min**
Rate	**Bones – 8-10** **Teeth – 6-8**	

12. Close cover slowly to avoid any liquid nitrogen splashes and press **RUN** to start the mill. Pre-cooling will begin followed by the milling cycle.

13. During the 2-minute pause phase, it is now possible to open the mill and remove the finished sample using cryogenic gloves.

14. Place one of the pre-cooled specimens waiting in the dock in the round chamber.

15. If liquid nitrogen level is below the **FILL LINE**, refill. A loud noise during milling means that the liquid nitrogen level is low. If liquid nitrogen is not refilled, damage to the mill, mill parts, and cylinder can occur.

16. Close the lid and press **RUN** again. Repeat from Step 11 until all samples are processed.

17. Inspect each sample after removal from the mill. If sample is sufficiently pulverized, remove the metal top using the Spex Certi-Prep opening device. **Note: Samples may be reinserted into the mill for additional grinding.**

18. Using decontaminated tweezers, remove impactor from vial and submerge in 10% bleach.

19. Empty bone dust into labeled 50mL Falcon tube. Ensure complete dust transfer by tapping bottom of cylinder. Weigh bone dust and document.

20. Soak metal end parts and plastic cylinder in 10% Bleach.

21. When milling is complete, switch mill to **OFF** and unplug. Leave cover open for liquid nitrogen to evaporate. The next day, lower cover and place in storage until next use.

22. **Mill Parts -Clean Up**: Mill parts must be cleaned immediately after processing. If this is not possible, steps a-b must be completed before leaving overnight.
    a. Rinse off with 10% bleach.
    b. Soak all parts in 0.1% SDS.
    c. Brush parts with a new toothbrush to remove any residual bone dust.
    d. Rinse with water.
    e. Soak parts in 10% bleach and brush each part in bleach individually.
    f. Rinse with water.
    g. Separate the plastic cylinders from the metal parts.
    h. Rinse in 100% ethanol. **ONLY** the metal top, metal bottom, and compactor can be rinsed in 100% ethanol. **DO NOT** rinse the plastic cylinder in ethanol as it will cause the plastic cylinder to break.
    i. Use isopropanol to remove any identifying marks made with a Sharpie on the tops or bottoms of the cylinders.
    j. Dry and expose the parts to UV light for a minimum of 2 hours. The UV light in a biological hood or a StrataLinker can be used.

23. Proceed to Section B: Sample Incubation.

Laser Microdissection of Products of Conception

1. Initial processing

The product of conception (POC) can be received in different stages of preparation:

a) POC scrapings in saline buffer:

Remove tissue from liquid either by filtration or centrifugation:

- Transfer liquid to 50mL falcon tube
- Spin sample in a bench top Eppendorf or IEC Centra CL3R at 1000 RPM for 5 minutes
- Discard liquid supernatant

Submit sample to the Histology department for tissue processing according to the OCME Histology Procedure Manual section E. Then proceed as for b).

b) POC fixated and embedded in paraffin blocks:

Contact histology department and ask them to prepare microscope slides from the paraffin block using the following precautions:

- Use disposable blades for the microtome and discard after each case.
- Clean working surface on microtome by wiping with 10% bleach and alcohol before and after each case.
- Use individual floating chambers for each case
- Use uncharged microscope slides

The slides then should be stained with hematoxylin and eosin-phloxine (H&E technique) as described in the OCME Histology Procedure Manual. But again during the staining procedure, separate sets of jars have to be used for each case.

c) Stained or unstained microscope slides from POC blocks:

If the slides are unstained, ask the histology department to stain them as described above. Otherwise proceed with the microdissection technique. **Attention:** for slides that were prepared by a histology laboratory outside of the OCME, foreign DNA not from the mother and the fetus might be present on the slide.

2. PixCell IIe Laser Capture Microdissection

A trained pathologist has to be present to distinguish decidual tissue from chorionic villi and operate the laser. After the slide has been placed on the microscope platform the pathologist will visually identify the area of interest, mark this area for the laser, and activate the laser. The laser setting is specified in the Arcturus instrument manual. The Forensic Biology Criminalist needs to be present during the complete procedure to maintain chain of custody of the evidence.

An area of chorionic villi and an area of maternal tissue should be collected on separate CapSure caps. The caps can be stored and transported in 50 ml Falcon tubes. A third unused CapSure cap should be extracted as an extraction negative control.

Use new scalpel and clean forceps to remove the film from the cap and transfer the film

to a fresh 1.5mL microcentrifuge tube containing 500µL of organic extraction buffer, DTT, SDS and Proteinase K as described below.

Sample Incubation

1. Process an extraction negative with every batch of extractions.

2. Prepare the master mix in microcentrifuge tube or conical tube and mix thoroughly by swirling or vortexing *very briefly*.

For liquid blood, dry blood and bone marrow samples:

	1 Sample	**5 Samples**	**10 Samples**	**15 Samples**
Organic extraction buffer	400 µL	2.0 mL	4.0 mL	6.0 mL
20% SDS	10µL	50 µL	100µL	150 µL
Proteinase K (20 mg/mL)	13.6 µL	68 µL	136 µL	204 µL
Total Incubation Volume *per sample*:				*400 µL*

For bone samples:

	Per bone (~2g dust)	**1 sample (N+ 2)**	**3 samples (N+ 2)**	**5 samples (N+ 2)**
Organic Extraction Buffer	2370 µL	7.11 mL	11.85 mL	16.59 mL
20% SDS	300 µL	900 µL	1.5 mL	2.1 mL
1.0 M DTT	120 µL	360 µL	600 µL	840 µL
Proteinase K (20 mg/mL)	210 µL	630 µL	1.05 mL	1.47 mL
Total Incubation Volume *per sample*:				*3000 µL*

For teeth samples:

	Per tooth	**1 sample (N+ 2)**	**3 samples (N+ 2)**	**5 samples (N+ 2)**
Organic Extraction Buffer	790 µL	2.37 mL	3.95 mL	5.53 mL
20% SDS	100 µL	300 µL	500 µL	700 µL
1.0 M DTT	40 µL	120 µL	200 µL	280 µL
Proteinase K (20 mg/mL)	70 µL	210 µL	350 µL	490 µL
Total Incubation Volume *per sample*:				*1000 µL*

For tissues and paraffin embedded tissue (e.g. microdissection) samples:

	Per tissue	**1 sample (N+ 2)**	**3 samples (N+ 2)**
Organic extraction buffer	395 µL	1185 µL	1975 µL
20% SDS	50 µL	150 µL	250 µL
1.0 M DTT	20 µL	60 µL	100 µL
Proteinase K (20 mg/mL)	35 µL	105 µL	175 µL
Total Incubation Volume *per sample*:			*500 µL*

3. Add the appropriate incubation volume of master mix to each sample tube and eneg tube. Vortex tubes briefly. Make certain the substrate, tissue, or swab is totally submerged. **Note: Reagent volumes may be adjusted in order to accommodate the size or nature of a particular sample.**

4. Place tubes in a shaking 56°C heat block and incubate overnight.

5. Proceed to Section C: Phenol Chloroform Extraction and Microcon® cleanup.

C. Phenol Chloroform and Microcon Clean up

Set Up

Remove the Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (PCIA) from the refrigerator.

Obtain organic waste jug for disposal of any tubes or pipette tips that come in contact with PCIA.

****WARNING****
Phenol Chloroform is toxic. Protective eyewear, mask, lab coat, and nitrile gloves should be worn when handling. All work must be conducted under a chemical fume hood.

For samples possibly needing mtDNA or High Sensitivity DNA testing: Place one Microcon® collection tube and one 1.5 mL microcentrifuge tube for each sample, including the extraction negative, in the StrataLinker for at least 15 minutes. **Note: Irradiate multiple tubes (4-6) per bone sample to accommodate the total volume of incubation buffer.**

1. Vortex and centrifuge the incubated microcentrifuge tube samples at high speed for 1 minute. Vortex and centrifuge bone dust, incubated in 50 mL conical tubes, for 5-10 minutes at 1000 RPM in Eppendorf Centrifuge Model 5810.

2. Obtain and label one prepared Eppendorf Phase Lock Gel (PLG) tube per sample, including the extraction negative. PLG tubes make phase separation easier and are optional.

   NOTE: For bone samples, label as many tubes to accommodate the total volume of incubation buffer per sample. For example, if you incubated 2g of bone dust with 3 mL of incubation buffer, you will need 6 PLG tubes.

   NOTE: See section D for PLG tube preparation instructions.

3. Centrifuge PLG tubes at maximum speed for 30 seconds.

4. Label Microcon® filters for each sample. Prepare the Microcon® concentrators by adding 100 µL of TE^{-4} to the filter side (top) of each concentrator. Set aside until step 11.

5. Add a volume of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (PCIA) to the PLG tube which is equal to the volume of incubation buffer (typically 400 µL) to be added from the sample. **Note: When pipetting PCIA, you must penetrate the top buffer layer and only aliquot the desired amount from the lower, clear organic layer. Place used pipette tips in the organic waste bottle.**

6. Have someone witness your sample tubes, PLG tubes, and Microcon® tubes.

7. Pipette the sample supernatant (typically 400 µL) to the PLG tube already containing PCIA. For bone dust samples, pipette several aliquots of the supernatant into multiple PLG tubes. **Note: Do not disturb bone pellet.**

8. Shake the PLG tube vigorously by hand or by inversion to form a milky colored emulsion. **Note: Do NOT vortex the PLG tube.**

9. Centrifuge samples for 2 minutes at maximum speed to achieve phase separation. (On Eppendorf Centrifuge Model 5415D, spin at 16.1 RCF or 13.2 RPM).

10. If the sample is discolored, contains particles in the aqueous phase, or contains a lot of fatty tissue, transfer the top layer (aqueous phase) to a new PLG tube and repeat Steps 7-9. Note: The aqueous layer from bone and teeth will usually be discolored. Only repeat the phenol-chloroform clean-up steps if any dust or particles are present in the aqueous layer. If it is not necessary to repeat the clean-up step, go to Step 11.

11. Carefully transfer the aqueous phase (top layer) to the prepared Microcon® concentrator. Be careful not to let the pipette tip touch the gel. **Note: Discard used PLG tubes into the organic waste bottle.**

12. Spin the Microcon® concentrators for 12-24 minutes at 500 x g, which is approximately 2500 RPM. (On Eppendorf Centrifuge Model 5415D, spin at 0.6 RCF or 2600 RPM). **Note: Ensure that all fluid has passed through filter. If it has not, spin for additional time, in 8-minute increments. If fluid still remains, transfer sample to a new filter and microcon again.**

13. Discard the wash tubes and place the concentrators into a new collection tube.

14. Add 400 µL of TE^{-4} to the filter side of each Microcon® concentrator.

15. Spin again for 12 minutes at 500 x g. (On Eppendorf Centrifuge Model 5415D, spin at 0.6 RCF or 2600 RPM). **Note: Ensure that all fluid has passed through filter. If it has not, spin for additional time, in 8-minute increments. If fluid still remains, transfer sample to a new filter and microcon again.**

16. Add 40 µL of TE^{-4} to the filter side of each Microcon® concentrator. **Note: For bone samples, add only 10-20 µL of TE^{-4} to each filter side to ensure smallest elution volume.**

17. Invert sample reservoir and place into a new labeled collection tube. *(For samples possibly needing mtDNA or High Sensitivity DNA testing, invert sample reservoirs into irradiated collection tubes).* Spin at 1000 x g, which is approximately 3500 RPM, for 3 minutes. (On Eppendorf Centrifuge Model 5415D, spin at 1.2 RCF or 3600 RPM).

18. Measure the approximate volume recovered and record the value. **Note: Combine bone elutants before measuring volume.**

19. Discard sample reservoir and adjust sample volume depending on the starting amount and expected DNA content as follows using TE^{-4}. **Note: Samples may be microcon'ed again to further concentrate low DNA content samples.**

Sample type	**Final Volume**
High DNA content (Large amounts of blood, fresh tissue, bone marrow, oral swabs, and dried bloodstains)	400 µL
Medium DNA content (Small amounts of blood, fresh tissue, bone marrow, oral swabs, and dried bloodstains); differential lysis samples	200 µL
Low DNA content (Formalin fixed tissue, dried bone, teeth, samples from decomposed or degraded remains, some reference samples)	100 µL

20. Transfer samples to newly labeled 1.5mL microcentrifuge tubes for storage. *(For samples possibly needing mtDNA or High Sensitivity DNA testing, transfer samples to irradiated 1.5 mL microcentrifuge tubes).* Record the approximate final volume.

21. As needed, pipette aliquots of neat and/or diluted extract (using TE^{-4}) into microcentrifuge tubes for real-time PCR analysis to determine human DNA concentration.

22. Store the extracts at 2 to 8°C or frozen.

23. In the LIMS system, navigate to the Data Entry page, assign the samples to a storage unit (cryobox), and indicate which samples are complete.

24. Have a supervisor review the assay.

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NOTE: See Microcon® troubleshooting (in the appropriate section of the STR manual) as needed.

D. Preparation of Phase Lock Gel (PLG) tubes

Make sure the plasticware being used is resistant to phenol and chloroform.

1. Without putting pressure on the plunger, twist off the **orange cap** and discard. Attach the **gray dispensing tip** (supplied) to the syringe and tighten securely. (NOTE: Use of gray tip is optional for a smoother application of PLG. Less force is necessary when gray tip is NOT used.)

2. Apply firm pressure on the plunger to dispense PLG until it reaches the end of gray tip. Add heavy PLG based on Table below. NOTE: 325µL = 3.25 cc corresponds to 3 lines on the syringe

Tube size	PLG heavy	Tube size	PLG heavy
0.5mL	100µL	15mL	3mL
1.5mL	325µL	50mL	5mL
2.0mL	325µL		

3. Pellet the PLG by spinning the tubes prior to use. See table below.

Tube size	Centrifuge model	Speed	Time
0.5 to 2.0mL	Eppendorf 5415C Eppendorf 5415D	14 x 1000 RPM 13.2 x 1000RPM/16.1 x 1000RCF	30s
15 and 50mL	Sigma 4-15 C	1500 RCF	2m