Organic Extraction

1 Sample Incubation

1.1 An extraction negative needs to be processed with every batch of extractions. Obtain two empty 1.5 mL screw cap tubes for the extraction negatives and manually label them as Extraction Negative 1, and Extraction Negative 2.

1.1.1 For bone samples, obtain two 50 mL conical tubes for extraction negatives.

1.2 Fill out the "Extraction Set Up" performed by tab in LIMS. This will add the date and time to the extraction negative for the batch.

1.3 Following the tables below, prepare the master mix in a microcentrifuge tube or conical tube and mix thoroughly by swirling or vortexing *very briefly*.

1.4 **Extraction WITNESS:** Have a witness verify the input sample labels and tube tops.

* NOTE: Mastermix preparation incorporates N+2 calculation to accommodate for the nature of bone samples.

For bone samples:

	Per bone (~2g dust)	**2 ENegs + 1 bone sample**	**2 ENegs + 3 bone samples**	**2 ENegs + 5 bone samples**
Organic Extraction Buffer	2370 µL	7.11 mL (7,110 µL)	11.85 mL (11,850 µL)	16.59 mL (16,590 µL)
20% SDS	300 µL	900 µL	1.5 mL (1,500 µL)	2.1 mL (2,100 µL)
1.0 M DTT	120 µL	360 µL	600 µL	840 µL
Proteinase K (20 mg/mL)	210 µL	630 µL	1.05 mL (1,050 µL)	1.47 mL (1,470 µL)
Total Incubation Volume *per Extraction Negative*				*1000 µL*
Total Incubation Volume *per bone sample*:				*3000 µL*

For liquid blood, dry blood, bone marrow and saliva samples:

	Per sample	**2 ENegs + 3 Samples**	**2 ENegs + 8 Samples**
Organic extraction buffer	400 µL	2000 µL	4000 µL
20% SDS	10µL	50 µL	100µL
Proteinase K (20 mg/mL)	13.6 µL	68 µL	136 µL
Total Incubation Volume *per sample*:			*400 µL*

* NOTE: Mastermix preparation incorporates N+1 calculation to accommodate for the nature of tissue, pseudo-exemplars and teeth samples.

**NOTE: The addition of DTT is optional in the incubation of pseudo-exemplars, depending on the nature of the particular sample

For tissues (POC/muscle), paraffin embedded tissue (e.g. microdissection), and pseudo-exemplars:

	Per tissue	**2 ENegs + 1 sample**	**2 ENegs + 3 samples**
Organic extraction buffer	395 µL	1580 µL	2370 µL
20% SDS	50 µL	200 µL	300 µL
1.0 M DTT	20 µL	80 µL	120 µL
Proteinase K (20 mg/mL)	35 µL	140 µL	210 µL
Total Incubation Volume *per sample*:			*500 µL*

For teeth samples:

	Per tooth	**2 ENegs + 1 tooth sample**	**2 ENegs + 3 tooth samples**	**2 ENegs + 5 tooth samples**
Organic Extraction Buffer	790 µL	3160 µL	4740 µL	6320 µL
20% SDS	100 µL	400 µL	600 µL	800 µL
1.0 M DTT	40 µL	160 µL	240 µL	320 µL
Proteinase K (20 mg/mL)	70 µL	280 µL	420 µL	560 µL
Total Incubation Volume *per sample*:				*1000 µL*

1.5 Add the appropriate incubation volume of master mix to each sample tube and Extraction Negative tube.

1.6 Vortex tubes briefly. Make certain the substrate, tissue, or swab is totally submerged.

1.6.1 **Note:** Reagent volumes may be adjusted in order to accommodate the size or nature of a particular sample.

1.7 Wrap each tube (Extraction Negatives and samples) in parafilm after the addition of mastermix.

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

1.8.1 For samples in 1.5mL screw cap tubes: shake at 1,400 rpm at 56°C

1.8.2 For samples in 50mL conical tubes: shake at 124 rpm at 56°C

1.9 Proceed to Section 2: Phenol Chloroform and Microcon Clean up® cleanup.

2 Phenol Chloroform and Microcon Clean up

****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.

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

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

2.3 Once overnight incubation is complete, remove samples from thermomixer or incubator-shaker.

2.3.1 For samples in 1.5mL screw cap tubes: Vortex samples and then centrifuge the samples at high speed for 1 minute.

2.3.2 For samples in 50mL conical tubes: Vortex samples and then centrifuge bone dust, for 5-10 minutes at 1,000 rpm.

2.4 Obtain and label 1.5mL microcentrifuge tubes for phenol-chloroform extraction step.

2.4.1 Extraction negatives and samples in 1.5mL screw cap tubes only require 1 1.5mL microcentrifuge tube each.

2.4.2 Bone samples will need multiple 1.5mL microcentrifuge tubes per sample to accommodate the total volume of lysate per sample (3mL). Every sample will require a different number of tubes. Approximately 3-6 1.5mL microcentrifuge tubes will be needed per bone sample.

2.5 Add an appropriate volume of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (PCIA) to the 1.5mL microcentrifuge tube which is equal to the volume of lysate (e.g. 400 µL) but this may be adjusted as needed.

2.5.1 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.

2.6 Obtain and label a sufficient amount of Microcon® DNA Fast Flow sample filters and collection tubes for each sample.

2.7 Prepare the Microcon® filters by adding 100 µL of TE^{-4} to the membrane located on the filter side (top) of each concentrator. Set aside until step 2.14.

2.8 Obtain and label a 1.5mL microcentrifuge tube for the final DNA extract of each sample.

2.9 **Purification WITNESS:** Have a witness verify your 1) input sample labels and tube tops, 2) tube top labels on 1.5mL microcentrifuge tubes for phenol-chloroform clean-up, 3) tube top labels on Microcon® filters and collection tubes, and 4) output sample labels and tube tops

2.10 Pipette and transfer the appropriate volume of lystate (e.g.400 µL) to the respective 1.5mL microcentrifuge tube(s) already containing PCIA.

2.10.1 For bone dust samples, pipette several aliquots of the supernatant into multiple 1.5mL microcentrifuge tubes. **Note: Do not disturb bone pellet.**

2.11 Shake the 1.5 microcentrifuge tubes vigorously by hand or by inversion until a milky colored emulsion is formed. **Note: Do NOT vortex the 1.5mL microcentrifuge tubes.**

2.12 Centrifuge samples for 5 minutes at 13,200 rpm to achieve phase separation.

2.13 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 1.5mL microcentrifuge tube containing PCIA and repeat Steps 2.10-2.12.

2.13.1 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 2.14.

2.14 Carefully transfer the aqueous phase (top layer) to the prepared Microcon® filter. To do so, slowly lower the pipette tip just beneath the surface of the aqueous phase and aspirate the top

layer. When transferring the aqueous layer, do not let the pipette tip touch the interphase (middle layer) or organic phase (lower layer). Leave behind ~10-20µL of the aqueous phase to avoid accidentally aspirating the interphase.

2.14.1 Note: When transferring the aqueous phase (top layer), position and hold the tube at eye level, being careful not to disturb the contents – even gentle agitation can disrupt the interphase.

2.14.1.1 Troubleshoot: if the interphase is accidentally disrupted and/or aspirated, deposit the aliquot back into the 1.5mL microcentrifuge tube and spin for 5 minutes at 13,200 rpm to achieve phase separation followed by collection of the aqueous phase again.

2.15 Discard the used 1.5mL microcentrifuge tubes, with the remaining PCIA, into the organic waste bottle.

2.16 Spin the Microcon® concentrators for 12-24 minutes at 2,600 rpm.

2.16.1 Ensure that most fluid has passed through filter. If the fluid does not appear to be moving through the filter, spin for additional time in 8-minute increments. If volume does not decrease after additional time and more than ~2µL remains, consider eluting and transferring sample to a new coated filter and continue centrifuging.

2.17 Discard the used collection tubes and place the filters into a new labeled collection tube.

2.18 Add 400 µL of TE^{-4} to the Microcon® filter of each sample.

2.19 Spin again for 12 minutes at 2,600 rpm.

2.19.1 Ensure that most fluid has passed through filter. If the fluid does not appear to be moving through the filter, spin for additional time in 8-minute increments. If volume does not decrease after additional time and more than ~2µL remains, consider eluting and transferring sample to a new coated filter and continue centrifuging.

2.20 When the sample is ready to elute, add TE^{-4} to each Microcon® filter:

2.20.1 For controls and non-bone samples: add 40 µL of TE^{-4} to each filter

2.20.2 For bone samples: add 10-20 µL of TE^{-4} to each filter to ensure smallest elution volume.

2.21 Separate the filter from the used collection tube and invert the sample filter for each sample and place into a new labeled collection tube.

2.22 Spin the inverted filters at 3,600 rpm for 3 minutes

2.23 Remove and discard the sample filter.

2.24 Transfer the sample elutants to the labeled microcentrifuge output tubes.

2.25 Measure the approximate volume recovered and record the value in LIMS.

2.25.1 Combine bone elutants before measuring volume.

2.26 Adjust sample volume depending on the starting amount and expected DNA content as follows using TE^{-4}.

2.26.1 Samples may be microcon'ed again to further concentrate low DNA content samples.

2.26.1.1 *The final volume of the sample depends on the condition of the submitted material for extraction. Please consult your supervisor or the reporting analyst for further guidance.

Sample type	**Final Volume**
High DNA content: Small amounts of blood, fresh tissue (product of conception), bone marrow, saliva samples, and dried bloodstains; differential lysis samples	200 µL
Medium DNA content: Formalin fixed tissue, dried bone, teeth, samples from decomposed or degraded remains, some reference samples	100 µL
Low DNA content: Teeth, samples from decomposed or degraded remains, pseudo-exemplars	25*-50 µL

2.27 Transfer samples to newly labeled 1.5mL microcentrifuge tubes for storage.

2.28 Record the resulting final volume of each sample in LIMS.

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

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