Ancient DNA Extraction Protocol from Malhi Laboratory at the Carl R. Woese Institute for Genomic Biology at the University of Illinois at Urbana-Champaign

Note: the following should be conducted in an ancient DNA laboratory if possible, in an area isolated from other molecular work involving modern DNA.

DAY 1

Check to make sure you have the following in stock:

- Dental burs
- Weigh boats
- Isopropanol
- Bleach
- DNA-Off
- 15 mL polypropylene tubes
- 0.5M EDTA
- 3.33mg/mL Proteinase K
- 10% N-lauryl sarcosine

1. **Cleaning**
    a. Label weigh boats with sample names
    b. Soak up to 7 teeth or bone fragments in bleach for 3 minutes
    c. Rinse three times with molecular grad DNA-free H20
    d. Rinse once with isopropanol
    e. Dry samples under UV in DNA Crosslinker for 10-15min
2. Alternate cleaning method (for non-human samples): Wipe down with kimwipe soaked in pure bleach. Try to remove all dirt on tooth surface. If sample is caked with dirt, more thorough cleaning is recommended (see above)

3. **Drilling**
    a. Clean the inside of the drilling hood using bleach or DNA-Off
    b. Attach a dental bur to the Dremel tool.
    c. Place sheet of aluminum foil (exposed to UV) inside hood
    d. Drill a hole in the root of the tooth (or bone) until around 0.20g of sample is obtained
    e. Place powdered sample in 15ml polypropylene tubes and record the weight. Save excess sample in another 15ml tube.
    f. Soak the dental bur in bleach or DNA-Off and wipe down the Dremel tool and drilling hood
    g. Discard trash and repeat until ~0.20g powder of each sample is obtained

4. **Digestion**
    a. Prepare 1 tube without sample for use as a negative control
    b. Add 4ml of 0.5M EDTA to the 15ml tubes with powdered sample

c. Add 100ul of 33.3mg/ml proteinase K
d. Add 300ul of 10% N-lauryl sarcosine
    i. *Proper Dilution:* 1g N-lauryl sarcosyl powder to 10ml H2O
e. Seal the lids of the tubes with parafilm
f. Place 15ml tubes in the incubator, turn on the rotor and incubate 20-24 hrs at 37°C or until entire sample is digested (make sure incubator is at an angle so the sample doesn't leak out)

DAY 2

Check to make sure you have the following in stock:

- 15mL centrifugal filter units (30K molecular weight filter)
- Qiaquick PCR Purification Kit:
    - Qiaquick minicolumn/collection tube
    - PB Buffer
    - PE Buffer
    - Elution Buffer
- *Taq*
- dNTPs
- PCR Primers
- $MgCl_2$ (Mg^{++})
- PCR Buffer

5. Centrifugal Concentration

a. Label centrifugal filters with sample names
b. Spin 15 mL tubes down at 3500 rpm for 5 minutes to concentrate bone powder at the bottom
c. Add the digested sample to the centrifugal filter (try to avoid getting bone fragments in sample to speed centrifugation)
d. Spin at 3000-4000 rpm until concentrated to 250ul (start with 25 mins)
    i. 8 mins more if at ~500uL, 5 min more if at ~350uL
e. Pipette concentrated sample into a new 1.5ml tube

6. Qiagen Extraction (based on Yang et al. 1998)

a. Preheat incubator to 37ºC
b. Label 2 sets of standard 1.5mL tubes and 1 set of Qiagen 1.5mL (permanent storage) tubes with sample names
c. Turn on UV light in PCR prep area if doing PCR prep today (optional)
d. Add 5 volumes of PB buffer (1250 uL) to the amount of concentrated sample and vortex for 30s
e. Label Qiaquick minicolumn and collection tube and add 750ul PB buffer mix to center of Qiaquick membrane
f. Let sit 5 minutes at room temperature

g. Spin @ 13,000 rpm for 1 min and discard the flow through
h. Repeat until all PB buffer mix has been filtered
i. Add 750ul PE buffer* to the center of the minicolumn
    * make PE buffer fresh using 1ul PE to 5ul 100% ethanol; make double the amount because you will need to run through this twice Example: For 4 samples, 4x2x750=6000 (1000ul PE & 5000ul ethanol)
j. Spin @ 13,000 rpm for 1 min
k. Discard solution and centrifuge for an additional minute
l. Discard collection tube and place minicolumn into a clean, labeled 1.5ml tube.
m. Add 30ul Elution buffer to the **center** of the QIAquick membrane
n. Incubate @ 37°C in the heat block for 5min
o. Centrifuge @ 13,000rpm for 1 min
p. Add 30ul Elution buffer to the **center** of the QIAquick membrane
q. Incubate @ 37°C in the heat block for 5min
r. Centrifuge @ 13,000rpm for 1 min
s. Discard the minicolumn and save the 1.5ml tube.

7. Repeating Extraction

a. Either check for PCR inhibition or repeat extraction depending on color of extract.
b. Repeat from 5a to the end. The second time through, you are starting out with 30 +30 elution buffer, so the amount of PB buffer to add is always 5 x 60 = 300uL.
c. The only thing that changes is that at Step 5o reduce the amount of Elution buffer to 20uL, and pipette to mix samples rather than vortexing
d. For the second extraction you can pipette the PB buffer directly into the 1.5 ml tube from step s, mix by pipetting up and down, and then pipette it onto the center of Qiaquick membrane.

8. Storage

a. Let samples sit at 4°C overnight before PCR amplification.
b. Store DNA at -20°C for long term storage or 4°C for short term storage.

9. Inhibition Test

a. If PCR fails to amplify visible DNA on gel electrophoresis, run an additional Qiagen extraction as detailed above and perform another PCR.
b. If PCR still fails to amplify DNA, run an inhibition test PCR, adding 3ul of positive control to the same PCR tube as the ancient sample. Run a polyacrylamide gel and compare the ancient/positive combination sample to the same positive control on its own.

c. If the ancient sample is inhibited the ancient/positive combination will be expressed on the gel as the absence of a band or presence of a lighter band than that of the positive control alone.
d. If no inhibition is detected, the sample may not have amplifiable DNA and an extraction of a new digest may be necessary.
e. If inhibition is detected, perform another Qiagen extraction as detailed above and repeat until the sample amplifies or no inhibition is detected.

10. PCR Preparation

a. After two times through Extraction Steps, prepare the PCR
b. The primers are in the green tray in left fridge. The Taq is in the right fridge in a little box.
c. Prepare the Mastermix following these steps:
Sample size (N) = number of samples (including E_o) + 3

Buffer	2ul * N
$MgCl_2$ (Mg^{++})	1.2 to 2.0ul * N
dNTP	0.8ul * N
Primer Pair	0.3/0.3ul * N
Taq(ase)	0.15ul * N
H2O	12.45 to 13.25ul * N
Template	2.0ul
TOTAL:	20ul per sample

References:

Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. 1998. Technical note: improved DNA extraction from ancient bones using silica-based spin columns. *Am J Phys Anthropol.* 105(4):539-43. PMID: 9584894 DOI: 10.1002/(SICI)1096-8644(199804)105:4<539::AID-AJPA10>3.0.CO;2-1

Cui Y, Lindo J, Hughes CE, Johnson JW, Hernandez AG, Kemp BM, Ma J, Cunningham R, Petzelt B, Mitchell J, Archer D, Cybulski JS, Malhi RS. 2013. Ancient DNA analysis of mid-Holocene individuals from the Northwest Coast of North America reveals different evolutionary paths for mitogenomes. *PLoS One* 8(7):e66948. PMID: 23843972 PMCID: PMC3700925 DOI: 10.1371/journal.pone.0066948