**eDNA Preservation Experiment II**

**Longmire’s RT -- PCI**

**Phenol-Chloroform-Isoamyl (PCI) Extraction Protocol**

Filters preserved in Longmire’s Buffer solution are stored at room temperature and are extracted using the phenol-chloroform-isoamyl (PCI) extraction protocol described by Renshaw et al. 2015. *Molecular Ecology Resources*; the protocol was modified to include an initial incubation step with Proteinase K (Longmire et al. 1997) and a second CI wash, which reduced inhibition effects in trial runs.

**Day 1**

1. Turn shaker to 55℃
2. Add 22.5 µL Proteinase K to each sample (final concentration 0.5 mg/mL [Longmire et al. 1997])
3. Incubate overnight at 55℃

**Day 2**

1. Turn heating block to 65℃
2. Remove PCI from refrigerator to acclimate to room temperature
3. Incubate microcentrifuge tubes containing filter and Longmire’s Buffer at 65℃ for 10 minutes
4. Add 900 µL of phenol:chloroform:isoamyl alcohol (25:24:1) to each tube; Remove filter paper using pipette tip
5. Vortex samples to thoroughly mix solution and filter for 10 seconds
6. Centrifuge tubes at 14,000 rpm for 5 minutes
7. Transfer 700 µL of aqueous layer to new 2-mL microcentrifuge tubes
8. Add 700 µL of chloroform:isoamyl alcohol (24:1) to each tube (1st wash)
9. Vortex samples for 5 seconds
10. Centrifuge tubes at 14,000 rpm for 5 minutes
11. Transfer 600 µL of aqueous layer to new 2-mL microcentrifuge tubes
12. Add 600 µL of chloroform:isoamyl alcohol (24:1) to each tube (2nd wash)
13. Vortex samples for 5 seconds
14. Centrifuge tubes at 14,000 rpm for 5 minutes
15. Transfer 500 µL of aqueous layer to new 2-mL microcentrifuge tubes
16. Add 1.3 mL of 100% (200 proof) **ice-cold** ethanol to each tube
17. Add 20 µL of 5 M NaCl to each tube
18. Precipitate samples at -20℃ overnight

**Day 2**

1. Centrifuge tubes at 14,000 rpm for 10 minutes
2. Decant liquid using a filtered pipette tip, **making sure not to disrupt the pellet**
3. Dry pellets in a vacuufuge at 45°C for 15 min
4. Air dry until no visible liquid remains
5. Rehydrate pellets in 100 µL of 1xTE Buffer