**Pristine Seas eDNA extraction protocol**

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NOTES

* According to JHU-APL protocol, ATL+ProtK lysate should be incubated for at least two hours. If not incubated overnight, record the amount of time samples were incubated for.
* When using the syringe, eject liquid as slow as possible to avoid splashing
* If no sterivex caps are available, make sure to seal tight and thick with parafilm
* Dab rims and/or exteriors of spin columns and collection tubes onto dry kimwipes to remove liquid when discarding flow through

DAY 1

1. Follow all eDNA lab usage protocols including those for PPE, daily, and weekly cleaning
2. Sterilize the 200ul pipette, tips, 2mL and 5mL racks, one 2mL tube for extraction negative
3. Add 1.8mL of ATL buffer to a 2mL tube (plus steps below) for an extraction negative
4. Wipe the outside of the sterivex capsule with 10% bleach and allow to dry
5. Wipe the outside of the sterivex capsule with 70% EtOH and allow to dry
6. Ensure that the outlet cap (on the small end) is on tightly and parafilmed to prevent leakage
   1. If no outlet cap is present, remove the parafilm, add an outlet cap, and re-parafilm
7. Shake sterivex capsule to clear liquid in the inlet cap neck (likely replaced with bubbles)
8. Carefully remove the inlet cap up off the sterivex capsule (on the large end)
9. Add 100 μL of (~20μg/mL concentration) Proteinase K
10. Cap inlet port and seal with parafilm to prevent leakage
11. Invert five times to mix.
12. Incubate capsule overnight at 56°C in rotating/inverting incubator

Sanitize for Day 2:

* Vacuum manifold, VacConnectors, extension tubes
* Centrifuges (high speed and tabletops)
* Pipettors (10,000μl, 1,000μl, 200μl)
* Pipette tips (same as above) and waste beaker
* Tubes (0.2mL strip tubes, 2mL, 5mL)
* Tube racks (2mL, 5mL)
* 50mL tubes and rack (one to mix AL:EtOH and one with 100% EtOH working stock)
* Notebook, pen, calculator

DAY 2

1. Preheat Buffer AE to 56°C
2. Shake the sterivex capsule vigorously by hand five times
3. Remove all liquid from the capsule using a 3mL syringe (save inlet caps in separate beaker)
4. Record the volume of liquid from the capsule and transfer it to a 5mL LoBind tube
5. Add up the total volume of ATL+Prot K lysate across all samples
6. Mix a 1:1 ratio of Buffer AL and 100% EtOH (twice the total volume of lysate is needed)
7. Add two parts of AL:EtOH mixture to the one part of ATL+Prot K lysate in each tube and vigorously vortex the tube **immediately** for approximately 10 seconds
   1. If there is more than 1.5mL ATL+Prot K lysate, add only up to 3mL AL:EtOH mixture so there will be no more than 5 mL in the 5mL tube
8. Label a spin column for each sample (save the collection tube for step 24c)
9. Connect the pump to the manifold+valves, then load connectors, spin columns & ext. tubes
10. Open valve before transferring fluid to ext. tube+spin column via pipette or careful pouring
11. Turn pump on after first sample is loaded and leave it running until all are done
12. Once all fluid is through a particular column:
    1. Close the valve to maintain higher pressure and filter other samples quicker
    2. Remove its extension tube and cap the spin column
    3. Remove the spin column from the connector and place it into a collection tube
13. Discard the extension tubes and connecters and place the manifold by the sink for cleaning
14. Add 500 μL of Buffer AW1 to the spin column, centrifuge for 1 min @ 8,000 rpm
15. Place the spin column into a new collection tube, discarding the tube and flow through
16. Add 500 μL of Buffer AW2 to the spin column, centrifuge for 3 min @ 14,000 rpm (approx.)
17. Discard flow through & centrifuge again for 1 min @ 14,000 rpm (into same collection tube)
18. Transfer the spin column into a new (labelled) 2mL LoBind tube
19. Add 100 μL of 56°C Buffer AE directly to the filter membrane in each column
20. Incubate at room temperature for 10 min, centrifuge for 1 min @ 8,000 rpm
21. Add eluate (flow through) back to the spin column and repeat step 32
22. Discard the spin column
23. Transfer the eluate from the 2mL tube into a 0.2mL strip tube
24. Store at –20°C with other samples in clearly marked boxes

CLEANUP

In addition to lab protocols:

* Clean the vacuum manifold by:
  1. Removing the black end cap and draining liquid
  2. Repeatedly rinse the interior until soapy bubbles no longer form
  3. Rinse through the valves by tipping it upside down when full with water
* Empty saved caps into storage bag in bottom right drawer (optional)
* Rinse 2mL and multi-size tube racks with DI water before soaking them in bleach bucket
* Wipe down 5mL tube racks with 10% bleach

Adapted from:

* Protocol for eDNA Extraction of Marine Mammals – John Hopkins Applied Physics Laboratory (Nicole Brown, Hayley DeHart, Mark Gasser, Peter Thielen, Kristina Zudock) January 2022
* eDNA extraction protocol of the Laboratories of Analytical Biology of the Smithsonian Institution National Museum of Natural History. This protocol cites two publications:
  + Spens et al. (2017) – Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods Ecol Evol*
  + Wacker et al. (2019) – Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. *Environmental DNA*
* Sample collection and eDNA extraction from Sterivex filter units – Oscar E Chiang, Pedro Inostroza, Department of Biological & Environmental Sciences, University of Gothenburg, Sweden (dx.doi.org/10.17504/protocols.io.bwaspaee)