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DNA isolation from *Daphnia ehippia*

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We are still developing and optimizing this protocol

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Abstract

A DNA isolation protocol designed for recovering DNA from individual *Daphnia ehippia*

Guidelines

This protocol has been developed largely to isolate DNA from *Daphnia ehippia*. This protocol is for very low biomass samples, and thus it is important to try and ensure that you have all the appropriate equipment first. All steps should be conducted in a laminar flow or biosafety cabinet, with the exception of separating the ehippia from mud.

The quartz bottles are typically the hardest to obtain, and they are often very expensive. I personally bought some on alibaba - with great success. Quartz crucibles off aliexpress work fine as well - just make sure if you buy from those sorts of suppliers you have access to a UV-C meter to verify that they are UV-C transparent.



Materials

Essential Equipment

- Laminar flow hood
- UV cross-linker (UV-C capability, minimum 7,500 mJ/cm² total dose)
- 450nm blue light source (high intensity LED)
- Magnetic bead separation rack
- Centrifuge
- Incubators (37°C, 55°C, 70°C)
- Dissection microscope
- Geological sieves (1mm, 800µm, 200µm mesh sizes)
- Quartz bottles (UV-transparent for reagent sterilization)
- Vacuum filtration system
- Vortex mixer
- Pipettes (P20, P200, P1000)

Additional Equipment

- Bogorov tray or petri dishes
- White sorting tray
- Timer
- Ice bucket
- Pasteur pipettes

Consumables

- 1.5mL microcentrifuge tubes (low-binding recommended)
- 15mL falcon tubes
- 50mL falcon tubes or large centrifuge bottles
- Pipette tips (10µL, 200µL, 1000µL)
- Bottle-top 0.1µM vacuum filters
- 0.22µM syringe filters
- 0.45µM syringe filters
- GL45 bottle caps (sterile)
- Parafilm
- Aluminum foil (heavy duty)

Reagents List

- **Sucrose Lysis Buffer:** 0.75M sucrose, 40mM EDTA, 50mM Tris base, pH 8.5
- **SDS Solution:** 25% (w/v) sodium dodecyl sulfate
- **Ethanol:** 80% (prepare fresh from absolute ethanol)
- **Sodium Hypochlorite:** 2% solution (dilute from household bleach)
- **Sucrose Syrup:** 1:1 (w/v) sucrose to ADaM media (table sugar acceptable)
- **Ultra-pure water** (nuclease-free, molecular biology grade)



- **Lysozyme:** 100 mg/mL stock solution
- **Proteinase K:** 20 mg/mL stock solution
- **Ethidium Monoazide (EMA):** 23.8mM stock solution
- **AMPure XP beads** OR **SeraMag SPRI beads** (prepared following [dx.doi.org/10.17504/protocols.io.x54v9p7b1g3e/v2](https://doi.org/10.17504/protocols.io.x54v9p7b1g3e/v2))
- **ADaM media** (or filtered tap water)

Safety warnings



- UV-C radiation is harmful to eyes and skin - use only in proper cross-linkers with safety interlocks
- Sodium hypochlorite (bleach) is corrosive - use in ventilated area with appropriate PPE
- Ethidium monoazide (EMA) is a potential mutagen - handle with gloves in dark conditions
- Follow institutional biosafety guidelines for all procedures
- Read MSDS' for all chemicals before using

Sterilizing reagents

- 1 Once you have made reagents, following the composition outlined under materials, it is crucial to sterilize these as best as possible to minimize the risk of DNA contamination. To do this, I typically sterile filter and UV sterilize the reagents. To do this, you will need a quartz bottle (quartz is important, as it is UV-C transparent).

Note: I do not treat my enzymes this way, see below for guidance on this.

- 2 To sterilize reagents, I first attach a bottle top 0.1uM filter onto the top my quartz bottle. I then vacuum filter my reagents into my quartz bottles.
- 2.1 *Sometimes if the fit of the filter is not tight, the vacuum will leak - so to avoid this, wrap the connection between the filter and bottle in parafilm.*

- 3 Place a sheet of tin/aluminium foil on the base of your UV cross linker.



Once the reagents are filtered, remove the filter (once the vacuum has naturally broken), and place a clean and sterile GL45 cap on the bottle. Transfer the entire bottle into a UV cross-linker, and then remove the cap (while still working in the cross-linker), and place it face up. Close the cross-linker, and then UV irradiate for at least 1 hour.

You should calculate the total of dose of UV-C that your reagents will receive. It should be greater than $>7,500\text{mJ/cm}^2$, in order to break down as much free DNA as possible (theoretically, there shouldn't be much anyway, and it should be 'free' as the filtering should remove any cells).

- 3.1 *Note that UV-C is harmful, make sure you use an appropriate cross-linker to avoid inadvertent exposure to UV-C.*

- 4 After the UV-C treatment, open the cross-linker, carefully replace the lid onto the bottle - taking care not to contaminate anything. Transfer the bottle to a laminar flow hood, and aliquot your reagents into new, sterile, bottles/falcon tubes.

Sterilizing enzymes (within laminar flow hood)

- 5 Enzymes are not, typically, robust enough for the treatment we give our other reagents. Before you commit to sterilizing this, I recommend you extract DNA from them (i.e., just a 'blank' extraction) - and then do PCR on them with 16S primers to test for contamination. If you can avoid enzyme sterilizing, that is ideal.



- 6 Assuming you want to sterilize your enzymes, you will need an extremely bright ~460nm blue light source (this is most blue LEDs) and propidium or ethidium monoazide (PMA/EMA) alongside some 0.45 or 0.22µM syringe filters.
- 7 Sterile filter your enzymes into individual tubes using the filters above.
- 8 Working in a dark room, to each tube, add EMA/PMA to a concentration of 14µM EMA. This is a 1700x dilution of the typical EMA 23.8mM stock solution. e.g. to treat 100mL, add 58.8µL of stock EMA.
- 9 Keep the tubes on ice for 5 minutes, in the dark, wrapped in tin foil.
- 10 Now keeping the tubes on ice, place them under the blue light, and expose them to the light for 5 minutes. This should cross-link and deactivate DNA within the enzymes.

Daphnia separation from sediments

- 11 Place your sediment, up to 200g, into the top of a stack of geological sieves with 1mm, 800µm, and 200µm stacked together.
- 12 Wash the sediment through the stacks of sieve using filtered tap water, AdAM media, or similar, and retain the 800µm-200µm fraction
- 13 Transfer the 800µm-200µm fraction to a beaker, and add 500mL of 1:1 sucrose:water/AdAM and mix thoroughly.
- 14 Transfer the mixture into either 50mL falcon tubes, or large centrifuge bottles, and centrifuge at 250g/rcf for 5 minutes.
- 15 Pour the supernatant into a white tray, and carefully start picking out the ephippia one by one and moving them to a second beaker.
- 16 Once you've isolated as many ephippia as possible, you can either try to isolate any remaining ones from the pellet from the sediment, or move to the next step.
- 17 Wash the ephippia with more AdAM media to remove excess sugar syrup, and then transfer the samples to a bogorov tray or petri dish.



- 18 Under a microscope, start manually picking through the ephippia - discarding empty ephippia or things that are not ephippia. Transfer the good ephippia to a 15mL falcon tube.
- 19 You can now transfer each ephippia to individual 1.7mL tubes, and prepare for DNA isolation

Ephippia sterilization

- 20 We need to make sure that the DNA we are isolating comes from within the ephippia, not things on the surface of it. To do this, we gently bleach the surface and then treat it with EMA or PMA to crosslink the external DNA. This ensures that our DNA is from within the ephippia. The principle here is that the bleach will kill all cells on the surface of the ephippia or in your samples, while not penetrating into the ephippia. The EMA/PMA can then access the DNA within these cells to deactivate them.

I have done microscopy to confirm that the EMA does not typically penetrate into an intact ephippia.

- 21 Prepare your EMA. In a dark room, and in a tube wrapped in tinfoil add 110uL of water to a tube for every ephippia you are processing. You then add 0.065uL of EMA for every 110uL of water - so i typically process at least 10 ephippia at a time. Keep this tube in the dark, and on ice.
- 22 To each ephippia, add 200uL of 2% sodium hypochlorite, and let it sit for 2 minutes.
- 23 Remove the bleach, and replace with 1mL of ultra pure water.
- 24 Remove water, and then add 1ml of water.
- 25 Repeat the wash three times, to remove residual bleach.
- 26 Remove any remaining liquid, and add 100uL of your previously prepared EMA, in a dark room. Keep the ephippia in the dark, and incubate them for 2 minutes on ice.
- 27 After 5 minutes, transfer to your blue light source, and again keep on ice but exposed to light for 5 minutes. Remove any liquid, and add 100uL of H₂O.



At this point, you are ready to do your DNA extraction. You can freeze and pause the work here if you want.

DNA isolation - all in a laminar flow hood.

- 28 You should now have tubes containing ephippia and 100uL of water. To each of these tubes, you should add 60uL of sucrose lysis buffer.
- 29 At this point, you need to physically disrupt the ephippia by manually poking it with a pipette tip. Until it breaks up a bit. This can be tricky, and sometimes the ephippia can get stuck in the pipette tip. See the notes at the bottom for alternative approaches under development.
- 30 Once the ephippia is broken, add 30uL of lysozyme, flick to combine, and spin down briefly.
- 31 Incubate for 45 minutes at 37°C
- 32 Add 20uL of 25% SDS. Flick gently to combine, and spin down briefly.
- 33 Incubate at 70°C for 10 minutes.
- 34 Allow to cool slightly, then add 8uL of proteinase K.
- 35 Incubate at 55°C for 45 minutes.
- 36 Allow to cool to room temperature
- 37 Add 500uL of AMPure beads (2 volumes), and flick to combine. Spin down briefly.
- 38 Incubate at room temperature for 10 minutes.



- 39 Place tube on magnetic rack, and allow beads to settle on the side for 10 minutes.
- 40 Gently remove the liquid, being careful not to disrupt the beads.
- 41 Add 1mL of fresh 80% ethanol. Invert tube a few times, rotate the tube within the rack - so the bead 'fly' through the liquid onto the other side of the tube.
- 42 Spin down, and remagnetize the beads - then gently discard the supernatant again.
- 43 Repeat the wash step again, so the beads are washed twice.
- 44 Remove any remaining ethanol by spinning down, and repelleting the beads - then removing ethanol with a 20 or 100uL pipette tip.
- 45 Allow beads to dry at room temperature, with the lids open, for 1 minute.
- 46 Add 45uL of ultra pure water and close the tubes, remove the beads from the rack, and flick to resuspend the beads, then spin down briefly (not enough to repellet the beads though).
- 47 Incubate the tubes at 37°C for 10 minutes.
- 48 Spin down once more, then remagnetize the beads on the rack for 5 minutes.
- 49 Carefully transfer the liquid (up to 40uL), now containing your DNA, to a new tube - being careful not to take any beads with you.

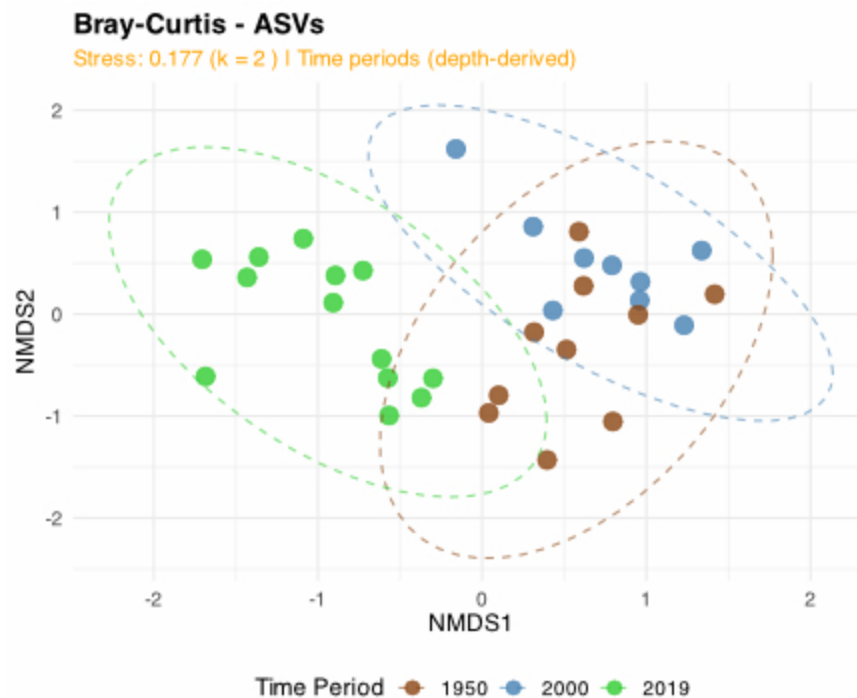
General notes

- 50 I am currently exploring the use of a laser cutter to aid in lysis. Once this method has been developed, I will add it to the protocol

- 51 I am also exploring replacing the SeraMag beads with silica coated magnetic beads. The benefits of this are two fold - first, silica coated beads are cheap to manufacture yourself, which would make the most expensive part of this extraction the enzymes. Secondly, the guanidine hydrochloride binding buffer, is also likely to aid in cell lysis - improving DNA extractions.

Preliminary Results

- 52 We have used this method to successfully isolate DNA from *Daphnia ephippia* in New Zealand, and used this DNA within 16S rRNA gene sequencing. Initial results demonstrate that the microbiome of the *Daphnia ephippia* has changed over the last ~70 years.



Acknowledgements

This work is an extension and combination of a few protocols:

First - EMA based sterilization is an extension of <https://doi.org/10.1016/j.mimet.2006.11.006>

Second - the DNA isolation method itself is a modification of <https://doi.org/10.1038/s43705-021-00079-z>

Third - the AMPure bead replacement is a from - [dx.doi.org/10.17504/protocols.io.x54v9p7b1g3e/v2](https://doi.org/10.17504/protocols.io.x54v9p7b1g3e/v2)