NYC/NJ Aquatic eDNA Project Protocols 4april2017

see also: Stoeckle MY, Soboleva L, Charlop-Powers Z. 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. PLOS ONE: e0175186 link

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Overview. From start to finish there are four laboratory components, each of which can potentially be completed in ½ to 1 day. Except for filtration, which we have tried to do within 1-3 days of collection, samples can be stored between steps at -20C. The procedures can also be stopped between the sub-headings shown. Cross-contamination is a hazard after PCR. We use a separate work area, reagents, and pipettors for post-PCR work.

I. COLLECT WATER SAMPLES

II. LABORATORY PROCEDURES

PRE-PCR----------

1. Filter water samples

2. Isolate DNA
    A. Isolate DNA from filter (MoBio)
    B. quantify DNA (QuBit)
    C. purify DNA(AMPure)

3. Amplify vertebrate 12S (GE Illustra)
    A. PCR vertebrate 12S

POST-PCR----------
    B. check PCR products by gel electrophoresis
    C. purify PCR products (AMPure)

4. Add Nextera tags, pool libraries
    A. PCR to add Nextera barcode tags
    B. check product size by gel electrophoresis
    C. purify PCR products (AMPure)
    D. quantify PCR products (QuBit)
    E. standardize library concentration, pool libraries
    F. deliver pooled library sample to sequencing facility

III. DATA ANALYSIS

IV. LABORATORY SUPPLIES

1. Filtration water sample

Summary: Filter 1 liter water sample to concentrate aquatic eDNA

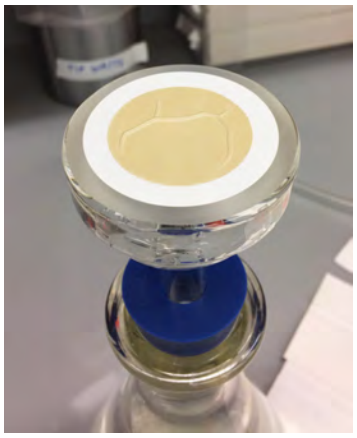
Time: About 2-3 h depending on how quickly filter clogs

Equipment: 1000 ml side arm flask
250 ml filtration manifold (Millipore)
Nylon filters: 47 mm diameter, 0.45 µM pore size (Millipore)
Flat forceps for handling filters
Paper coffee filters
Plastic funnel

1. Store samples at 4C until filtered. We have filtered water up to 72h after collection with good eDNA detection and up to 1 month with at least some recovery of eDNA.

2. Assemble filtration equipment listed above and attach to wall suction. Pour sample into manifold through 2 paper coffee filters placed in a funnel (this removes large debris). The volume that can be filtered before process slows drastically seems to correlate with the amount of DNA recovered per liter, so presumably slowly-filtering samples have a lot of planktonic organisms (bacteria, algae, other). It usually takes about 2-3 h to filter one liter from marine samples. For samples that filter very slowly, we usually stop the process after about 4 hours and record the volume filtered.

3. When filtration complete, disassemble apparatus, fold the filter over twice using forceps (sample side inward so it doesn't rub on side of tube), place in 15 mL tube, and store in -20C freezer. Filters have been stored up to 3 months before extracting DNA. After filtration we wash collection bottles and filtration equipment with tap water, taking particular care to remove any material adherent to glass manifold by rubbing under tap with gloves and paper towels.

2A. DNA Extraction from filter

Summary: Extract DNA using MoBio Powersoil Kit*

Time: About 2 h. We have processed up to 8 filters per person at a time.

Equipment: MoBio PowerSoil Kit (includes buffers C1-C6, PowerBead Tubes, Collection Tubes, Spin Filters)
Vortex platform with clips to hold PowerBead tubes
Single-edge razor
Forceps
Petri dish

*We had surplus PowerSoil kits; PowerWater Kit would likely be easier as they do not require cutting up filters. Other differences from manufacturer's protocol include the amounts of buffers C1, C2, C3, C4. Some modifications are from the PathoMap project. This modified protocol has worked well so far.

1. Using a single-edge razor and plastic petri dish as a cutting surface, first trim filter, discarding the outer "unused" portion that was not exposed to the filtrate (this makes it easier to fit filter into 1 PowerBead tube. Then cut filter into pieces approximately 2-3 mm x 10 mm, using single-edge razor and plastic petri dish as a cutting surface. Use forceps (or the 15 mL tube the filter was stored in, turned upside down with cap off) to hold the filter while cutting. Place cut up filter into a PowerBead tube.

2. Add 180 µl C1 solution.

3. Place on vortexer platform on high for 10 min. Watch that device does not fall off bench.

4. Centrifuge at 10,000*g* for 1 min.

5. Transfer ~465 µl supernatant to a 2 ml Collection Tube (1 Collection Tube per PowerBead Tube). We use a P200 set at 155 µl, and transfer 3 aliquots of supernatant from each PowerBead tube. You can stick pipette tip down deeply along the tube wall, beads and paper fragments are too large to go into pipette.

6. Add 100 µl of Solution C2 and 100 µl of Solution C3. Vortex 10 seconds.

7. Incubate at 4°C for 5 min (put rack in refrigerator/cold room).

8. Centrifuge at room temp 1 min at 10,000*g*.

9. Transfer 625 µl supernatant to a new 2 ml Collection Tube.

10. Shake Solution C4 before use. Add 1 ml Solution C4 to the supernatant. Invert 20 times to mix.

11. -Load 675 µl onto a Spin Filter and centrifuge at 10,000*g* for 1 min at room temp.
-Discard flow through.

-Add another 675 µl to Spin Filter and centrifuge at 10,000*g* for 1 min.
-Discard flow through.
-Load the remaining supernatant onto Spin Filter and centrifuge at 10,000*g* for 1 min.
-Discard flow through.

12. Add 500 µl Solution C5 to Spin Filter and centrifuge at room temp for 30 secs at 10,000*g*. Discard flow through.

13. Centrifuge at room temp for 1 min at 10,000*g*.

14. Place Spin Filter in a clean 2 ml Collection Tube.

15. Add 50 µl Solution C6 to the center of the white filter (don't touch filter with pipette tip).

16. Centrifuge at room temp for 30 secs at 10,000*g*. Usually recover ~45 µl (compare to 50 µl C6 input). Store at -20C.

17. Measure DNA yield with Qubit, DNA yield from marine samples so far averages ~2000 ng per liter water filtered (range 500 ng -10,000 ng).

2B. Quantify DNA: QuBit Protocol

Summary: Assay DNA concentration with QuBit (Qiagen)

Time: 15 min

Equipment: Qiagen Qubit machine
Qiagen High Sensitivity (HS) dilution buffer, HS dye
Qiagen HS DNA standards

1. Set out 0.25ml PCR tubes in tray (need n+2, where n = number of samples).

2. Add: Tube 1: 10 µl of HS Standard 1
Tube 2: 10 µl of HS Standard 2
Sample tubes: 2 µl per sample

3. Make buffer-dye mix
Qiagen HS buffer solution: [(n+3)*200 µl]. Add (n + 3) µl of HS dye, vortex to mix.

3. Standards Tubes 1,2: Add 190 µl buffer-dye mix
Sample tubes: Add 198 µl buffer-dye mix

4. Wait 1 min.

5. Measure DNA concentration with QuBit: sample DNA conc (ng/µl) = Qubit reading x 1/10

Derivation:
1. concentration1 x volume1 = concentration2 x volume2
2. sample DNA conc x 2 µl = Qubit conc x 200 µl
3. sample DNA conc (in ng/µl) = [Qubit reading (in ng/ml)/1000 to convert to ng/µl] x 200 µl/2 µl
4. sample DNA conc (ng/µl) = Qubit reading x 1/10

2C. DNA purification with AMPure beads

Summary: DNA purification, concentration using magnetic beads (this closely follows manufacturer's protocol)

Time: About 1-2 h, depending on number of samples.

Equipment: AMPure beads in solution per manufacturer's protocol
MagnaRack
Elution Buffer (Qiagen) or equivalent (10mM Tris, pH 8.3)
80% EtOH

1. Resuspend AMPure beads by inverting multiple times or vortexing.

2. Add 2:1 ratio by volume of AMPure beads to DNA sample. Place AMPure bead suspension into bottom of tubes, pipette 2-3 times to mix. If starting with PCR products, transfer rxn mixtures to bottom of 1.5 mL eppendorf tubes, then add AMPure beads.

3. Incubate at room temp 10 min.

4. Place tubes on MagnaRack for 5 min. Solution should become clear, with beads along back wall. Before placing in rack, open and then "toe-in" the caps to make it easier to open them without jarring beads or supernatant.

5. Carefully remove supernatant with pipette, avoid touching beads.

6. Add 500 µl 80% ETOH while tubes are on MagnaRack (gently pipet in ETOH along the side of the tube, do not re-suspend beads). Wait 30 secs. Per Illumina protocol, minimum wash volume is (DNA sample + bead) volume.

7. Carefully remove supernatant with pipette. Do not remove tubes from rack.

8. Repeat EtOH wash (steps 6 +7). Do not remove tubes from rack. After aspirating second wash, use a small pipette (P20 or P200) to remove any remaining ETOH.

9. Let tubes air dry in rack for 10 min. Beads are hard to re-suspend after a long drying time.

10. Disassemble MagnaRack so tubes are not adjacent to magnets. Add desired volume Elution Buffer (EB) to each tube (we re-suspend eDNAs in 54 µl and PCR products in 44 µl; as noted below, the extra 4 µl are to minimize bead transfer at final step), then mix by vortexing until beads are completely resuspended.

11. Incubate room temperature 5 min.

12. Assemble MagnaRack so tubes are adjacent to magnets. Before assembly, open and then “toe-in” the caps to make it easier to open them without jarring beads or supernatant. Wait 2 min. Solution should be clear.

13. Without removing tubes from rack, carefully pipette the supernatant (whatever volume of Elution Buffer was added in step 10 less 4 µl), transfer to a new tube, store at -20C. (Leaving behind 4 µl reduces chance of beads in final sample. [note: per Illumina protocol, carry-over of a small amount of beads usually does not interfere with subsequent reactions.]

3A. PCR amplification of vertebrate 12S fragment

Summary: Amplification of vertebrate 12S DNA from eDNA sample
12S primers from Riaz Nucl Acids Res 2011 39: e145)
Amplification strategy based on Illumina 16S protocol

Time: About 2.5 h to set up and run PCR, another 1 h to run gel to visualize results.

Equipment: GE Illustra beads
Thermal cycler
DNA primers (12S with Illumina tails)
Forward: 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG ACT GGG ATT AGA TAC CCC -3'
Reverse: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTA GAA CAG GCT CCT CTA G -3'
Agarose gel electrophoresis equipment
SyberSafe dye

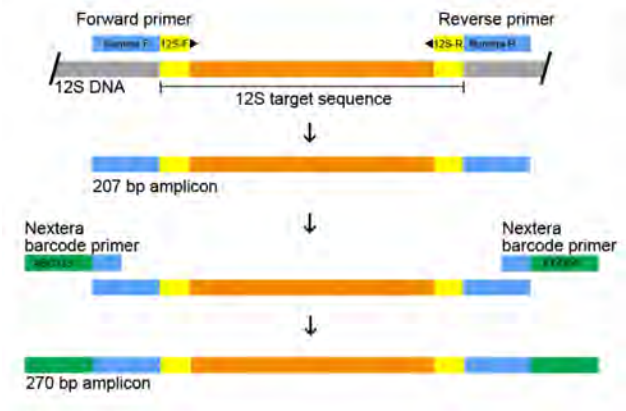
1. Prepare 2 µM primer mix in Elution Buffer (2 µM each primer)

2. Add following to GE Illustra PCR tubes
5 µl DNA (represents DNA from 100 mL of water) or 5 µl H2O for negative control
5 µl primer mix (final concentration each primer is 200 nM)
15 µl H20

Spin briefly if needed to make sure all reagents including bead are at bottom of tube.

3. Amplification protocol: 95C x 7m
40 cycles: 95C x 30s, 52C x 30s, 72C x 30s
72C x 10m, then hold at 4C

Schematic of amplification strategy



POST-PCR----------

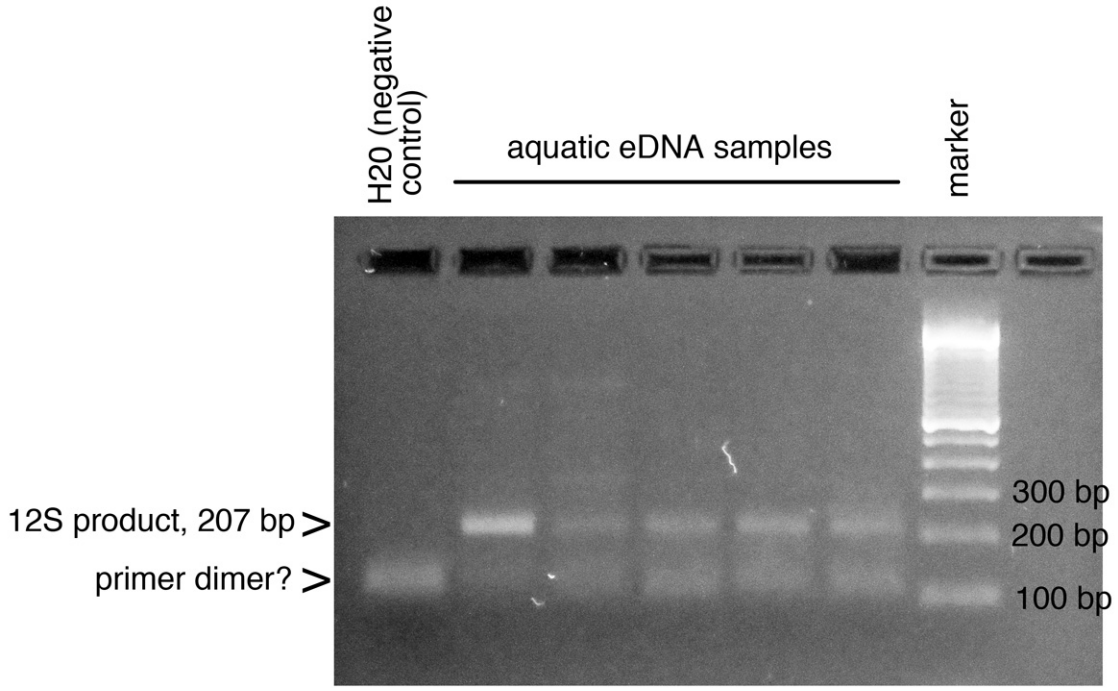
All post-PCR work done at separate bench with separate pipettors, reagents, etc.

3B. Check PCR yield

1. Run 5 µl each PCR tube on 2.5% agarose gel in 1X TBE with SyberSafe dye and DNA size markers. Expected product approximately 207 bp. We also usually get a band at about 100 bp including in negative control; this presumably is a primer dimer.

In some runs there is a noticeable 200 bp band in negative controls. So far these have turned out to be human or domestic animal (pig, cow) amplicons. Human and domestic animal DNA is reported as a common contaminant in PCR reagents.

Representative gel:



3C. AMPure purify PCR products

1. Follow AMPure protocol (2B), resuspend in 40 µl Elution Buffer.

4. Add Nextera tags, standardize and pool libraries

4A. Add Nextera tags by PCR amplification-Follow Illumina 16S protocol

GE Illustra tubes (25 µl rxn volume)

-Input DNA:	10 µl of AMPure-purified 12S PCR product
-Nextera tags:	2.5 µl each index (forward and reverse)
	10 µl H2O
PCR conditions	95C x 3m
	[95C x 30s, 55C x 30s, 72C x 30s] x 8 cycles
	72C x 5 min
	hold at 4C

4B. Check product size by gel electrophoresis

-Run 5 µl each sample on 2.0% agarose 1X TBE, with SyberSafe dye, markers

-Expected size about 270 bp

4C. Purify PCR products (AMPure)

-Resuspend in 40 µl Elution Buffer

4D. Quantify PCR products (QuBit)

4E. Prepare pooled library

-Prepare 15 nM pooled library in Elution Buffer

-If product ~270 bp, then 15 nM = 2.7 ng/µl

-Add 2.7 ng x 20 = 54 ng each library to 1.5 mL eppendorf

-Then add Elution buffer to final volume (#libraries x 20 µl)

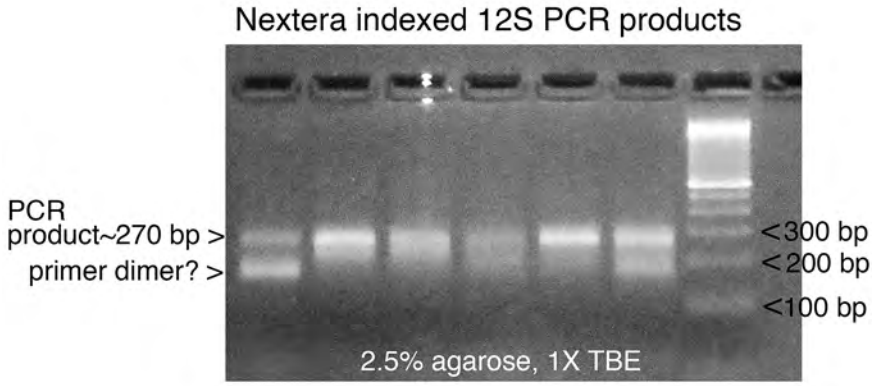
-e.g., if 10 libraries, then final volume is 200 µl

Alternatively, it may make sense to simply add the same volume of each library to the pool, and adjust the final pooled sample to the desired concentration before sequencing.

4F. Pool libraries, deliver to sequencing facility

-Deliver (5 µl x #libraries) to sequencing facility

-e.g., if 10 libraries, then deliver 50 µl



IV. LABORATORY SUPPLIES

Agencourt AMPure XP beads, Beckman Coulter, 60 mL, $1170.00
http://www.beckman.com/nucleic-acid-sample-prep/purification-clean-up/pcr-purification

Buffer EB, 250 mL, Qiagen, $33.90
https://www.qiagen.com/us/shop/lab-basics/buffers-and-reagents/buffer-eb/#orderinginformation

Hyclone water, molecular biology grade, GE Healthcare, 500 mL, $16.10
https://promo.gelifesciences.com/gl/hyclone/product/hyclone-water-molecular-biology-grade.html

Nylon membrane filters, 0.45 micron, 47 mm, 100/bx, Millipore HNWP04700, $150.00
http://www.emdmillipore.com/US/en/product/Nylon-Net-Filter,MM_NF-HNWP04700

Illustra PuReTaq Ready-To-Go PCR beads, 96 0.2mL tubes, GE Healthcare, 27-9559-01, $163.89
http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/products/AlternativeProductStructure_17007/27955901

PCR primers, Integrated DNA Technologies (IDT)
https://www.idtdna.com/site