Feb 28, 2020

Aquatic eDNA sampling and plant community metabarcoding with portable Nanopore Flongle sequencing (v0.0.2) V.2

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In Development dx.doi.org/10.17504/protocols.io.bce5itg6

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ABSTRACT

Aquatic eDNA sampling, extraction, and plant metabarcoding on Oxford Nanopore's Flongle platform.

Sampling method adapted from:
Laramie, M.B., Pilliod, D.S., Goldberg, C.S. and Strickler, K.M., 2015. Environmental DNA sampling protocol-filtering water to capture DNA from aquatic organisms (No. 2-A13). US Geological Survey. https://pubs.er.usgs.gov/publication/tm2A13

Extraction protocol nearly identical to Qiagen DNEasy PowerWater kit manual:
https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=2ahUKEwj4jpSKkqfnAhUjnOAKHdAbDQ8QFjABegQIBBAB&url=https%3A%2F%2Fwww.qiagen.com%2Fus%2Fresources%2Fdownload.aspx%3Fid%3Dbb731482-874b-4241-8cf4-c15054e3a4bf%26lang%3Den&usg=AOvVaw32l8w94T6GAZUI4TAyUyT1

Amplicon sequencing is a modified version of the PCR barcoding protocol from Oxford Nanopore for kit SQK-PBK004.

ATTACHMENTS

nanopore_amplicon_barcoding_library.plf

nanopore_amplicon_barcoding_library.plfx

MATERIALS TEXT

eDNA Sampling Materials:

Filter funnels (catalog #: 09 740 30K) -- Thermo Scientific™ 0.45 µm pore filter, 250 mL capacity.
Cordless Drill-- Milwaukee M12 FUEL Hammer Drill Driver (2019) and 6Ah battery (+2Ah backup)
Hose ends (2 female garden hose ends ~1m each)
Hose clamp (1x) 1/2-1 inch
Drill pump (Pars2O FPDMP21HC)
GPS (Garmin eTrex 20x) *Or cell phone app
Thermometer (LaserGrip774 Infrared Thermometer)
Forceps (x2)
Falcon™ 15mL Conical Centrifuge Tubes (1 per sample planned)
Write-in-Rain field notebook + Pencil
Permanent Marker
Mineral Oil (for pump before packing)
Flagging Tape
Zip Ties (12 in)

DNA Extraction

Qiagen DNeasy PowerWater Kit (Cat No./ID: 14900-50-NF)
Shaker or vortex adapter capable of >500RPM
Centrifuge for 5mL tubes (~4000 x g)
Centrifuge for 1.5mL tubes (13000 x g)

DNA Quantification

Qubit™ dsDNA HS Assay Kit, Catalog #: Q32851
Qubit 3.0 or 4.0 FLuorometer

Clean-Up
AMPure XP beads (Fisher Cat # NC995933) Or equivalent
Nuclease Free Water

Amplicon Sequencing
MiniPCR mini16 thermal cycler
Oxford Nanopore Technologies PCR Barcoding Kit (SQK-PBK004) for library preparation
OneTaq Hot Start Master Mix with Standard Buffer (NEB Cat # : M0484S)
1M Tris-HCl pH 8.0
5M NaCl (molecular grade)

Target Primers: (diluted in Nuclease Free Water to 10mM)
rbcLa (~550 bp) [Fahner et al., 2016]
Forward -- 5' TTTCTGTTGGTGCTGATATTGCATGTCACCACAAACAGAGACTAAAGC 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCGTAAAATCAAGTCCACCRCG 3'

ITS2-S2 to ITS4 (ITS2 ~300-450bp) [Fahner et al., 2016]
Forward -- 5' TTTCTGTTGGTGCTGATATTGCATGCGATACTTGGTGTGAAT 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCTCCTCCGCTTATTGATATGC 3'

trnL (~550bp) [Pornon et al., 2016]
Forward -- 5' TTTCTGTTGGTGCTGATATTGCCGAAATCGGTAGACGCTACG 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCGGGGATAGAGGGACTTGAAC 3'

MATK-1RKIM (~840bp) [Fahner et al., 2016]
Forward -- 5' TTTCTGTTGGTGCTGATATTGCACCCAGTCCATCTGGAAATCTTGGTTC 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCCGTACAGTACTTTTGTGTTTACGAG 3

psbA3 to trnHf_05 (psbA ~450bp) [Sang et al., 1997; via http://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_PrimerSets-Plants.pdf]
Forward -- 5' TTTCTGTTGGTGCTGATATTGCGTTATGCATGAACGTAATGCTC 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCCGCGCATGGTGGATTCACAATCC 3'

18S to 28S (18S ~1kb)
Forward -- 5' TTTCTGTTGGTGCTGATATTGCCACACCGCCCGTCGCTACTACCGATTG 3'
Reverse -- 5' ACTTGCCTGTCGCTCTATCTTCAGACTCCTTGGTCCGTGTTTCAAGAC 3'

Note: New primers can be designed to target amplicons >300bp for use with this protocol by attaching standard sequence adapters to target specific primers as --

Forward primer: 5' – TTTCTGTTGGTGCTGATATTGC – your target primer sequence – 3' GC ratio: 9/22

Reverse Primer:
5' – ACTTGCCTGTCGCTCTATCTTC – your target primer sequence – 3' GC ratio: 11/22

Primer Citations

Fahner, N.A., Shokralla, S., Baird, D.J. and Hajibabaei, M., 2016. Large-scale monitoring of plants through environmental DNA metabarcoding of soil: recovery, resolution, and annotation of four DNA markers. *PloS one*, *11*(6).

Pornon, A., Escaravage, N., Burrus, M., Holota, H., Khimoun, A., Mariette, J., Pellizzari, C., Iribar, A., Etienne, R., Taberlet, P. and Vidal, M., 2016. Using metabarcoding to reveal and quantify plant-pollinator interactions. *Scientific Reports*, *6*(1), pp.1-12.

Sang, T., Crawford, D.J. and Stuessy, T.F., 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). *American journal of Botany*, *84*(8), pp.1120-1136.

Aquatic eDNA sampling

1h

1 Assemble hose ends and drill pump and attach to wooden bracket with zip ties. Lock drill driver to pump spindle.

2 Attach fresh, sterile 0.045 µm filter funnel assembly (Fisher Sci catalog #09 740 30K) to hose on the “In” side of the pump and tighten hose clamp. Be sure to leave the filter chamber cover on.

3 Filter water samples with the drill at ¾ to maximum drive (max = 1,700RPM) for **00:15:00** . STOP if the filter becomes clogged and no water is observed leaving the outlet hose. Refill the filter reservoir as needed. The volume of water filtered will depend on the microbial load and turbidity of the water sample.

4 When filtering is complete, switch to clean gloves *and change gloves* if they contact anything other than the filter assembly or the water being sampled.

5 Remove the upper portion of the filter assembly.

6 Using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inwards.

7 Place in fresh 15mL Falcon tube and freeze at -20C (or in 200 proof molecular grade Ethanol) until ready to extract DNA.

8 Label Tubes with location, date, time, and duration of sampling.

9 Record data (Assign locality name, GPS location, water temperature, sampling duration, and any other observations) in the field notebook.

10 Mark sample site with flagging tape if you plan to resample the same location. Label flagging tape with sample date, locality name, and email contact information.

DNA Extraction

11 Place all pipettes and tools needed inside of the UV box. Turn on the UV light source to treat tools and workspace. After this time ONLY open tubes with eDNA inside of this box.

12 Next steps use materials from the DNeasy PowerWater kit.

Check for:
Solutions -- PW1, IRS, PW3, PW4, Ethanol, and EB.
Collection tubes (2mL)
Bead tubes (5mL)
MB Spin columns + tubes

Wipe down all closed reagent bottles with 50% bleach solution and place in a designated area of UV box.

13 Using bleach cleaned forceps (spray with 50% bleach and leave for **00:02:00**), insert the filter membrane into a 5mL PowerWater DNA bead tube. (included in kit).

14 Add **1 ml** of Solution PW1 to the PowerWater DNA bead tube

15 Secure tubes to a rack with tape. Secure rack to shaker platform with tubes horizontal.

16 Shake at >=500rpm for **00:05:00**

17 Centrifuge the tubes ≤ 4000 x g for **00:01:00** at room temperature. (According to the manufacturer: "This centrifugation step is optional if a centrifuge with a 15 ml tube rotor is not available, but will result in minor loss of supernatant").

18 Transfer the supernatant to a clean 2 ml collection tube (included in kit).

Note: placing the pipette tip down into the beads is required. Pipette until you have removed all the supernatant. Expect to recover 600-650 µL of supernatant (DNA is in supernatant).

19 Centrifuge at 13,000 x g for **00:01:00** at room temperature.

20 Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube (included in kit). (DNA is in supernatant).

21 Add **200 µl** of Solution IRS (15 ml included in kit) and vortex briefly to mix.

22 Incubate at **On ice** for **00:05:00**

23 Centrifuge the tubes at 13,000 x g for **00:01:00**

24 Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube (included). (DNA is in supernatant).

25 Add **650 µl** of Solution PW3 and vortex briefly to mix.

NOTE: If solution PW3 has precipitated, heat at 55 °C for 5-10 minutes to dissolve precipitate.

26 Load **650 µl** of supernatant onto a MB Spin Column (included in kit). Centrifuge at 13,000 x g for **00:01:00** . Discard the flow-through. (DNA is in Column). Repeat until all the supernatant has been processed.

27 Place the MB Spin Column Filter into a clean 2 ml collection tube (included) (DNA is on filter at this point).

28 Shake Solution PW4.

Add **650 µl** of Solution PW4 (shake before use) to the MB Spin Column. Centrifuge at 13,000 x g for **00:01:00** .

29 Discard the flow-through and add **650 µl** of ethanol (included) and centrifuge at 13,000 x g for **00:01:00** . (DNA is on filter).

30 Discard the flow through and centrifuge again at 13,000 x g for **00:02:00** . (DNA is on the filter still, simply washed by ethanol and PW4).

31 Place the MB Spin Column into a clean 2 ml collection tube (included).

32 Add **100 µl** of Solution EB (10mM Tris-HCl pH 8.5) to the center of the white filter membrane. (DNA is released from the filter).

33 Centrifuge at 13,000 x g for **00:01:00** . (DNA is in eluate).

34 Discard the MB Spin Column.

Cap and label collection tubes.

The DNA is now ready for downstream applications.

DNA Quantification (OPTIONAL but recommended)

35 Qubit Quantification using Qubit Fluorometer and dsDNA HS Assay Kit.

Prepare:
n = # samples + 2
Working solution -- **199 µl per n** dsDNA HS Buffer + **1 µl per n** dsDNA HS Reagent

Assay:
Mix **198 µl working solution** and **2 µl** each DNA sample in separate Qubit tubes

Mix **190 µl working solution** and **10 µl** Standard #1 in 0.5mL Tube

Mix **190 µl working solution** and **10 µl** Standard #2 in 0.5mL Tube

Wait: Incubate assay reactions **00:02:00 at room temperature**

Set Standards: Follow Qubit Fluorometer on-screen instructions to calibrate using Standard 1 and then 2

Read: Place each sample in Qubit and press "Read", record concentration.

PCR Step 1: Target Amplification 2h

36 Prepare on ice:
Template DNA
Forward and Reverse Primers
OneTaq Hot Start 2x Master Mix w/Standard buffer
Nuclease free water

37 Set Up Reactions in 0.5mL PCR LoBind tubes. Add Template DNA Last.

Forward Primer	**0.5 µl** of **10 Milimolar (mM)** stock
Reverse Primer	**0.5 µl** of **10 Milimolar (mM)** stock
OneTaq Hot Start Master Mix	**12.5 µl**
Nuclease Free Water	**9.5 µl**
Template DNA	**2 µl**
Total:	**25 µl**

38 PCR Cycling conditions:

If using MiniPCR use library: nanopore_amplicon_barcoding_library.plf
nanopore_amplicon_barcoding_library.plfx and protocol "NanoporePCR ampl target"

Cycle Step	Temp.	Time	# of Cycles
Initial Denaturation	94 °C	00:01:00	1
Step 1			5
Denaturation	94 °C	00:00:30	--
Annealing	60 °C	00:00:30	--
Extension	65 °C	00:00:50 per kb	--
Step 2			30
Denaturation	94 °C	00:00:30	--
Annealing	62 °C	00:00:30	--
Extension	65 °C	00:00:50 per kb	--
Final Extension	65 °C	00:05:00	1

*Cool to Room temperature

39 Store at 4 °C until ready for PCR Step 2

PCR Step 2: Barcode Addition

40 Prepare on ice:
Template DNA (PCR Product from Step 1)
Nanopore PCR Barcoding Kit (SQK-PBK004) primers LWB01-LWB12
OneTaq Hot Start 2x Master Mix w/Standard buffer
Nuclease free water

41 Set Up Reactions in 0.5mL PCR LoBind tubes. Add Template DNA Last.

LWB# Primer mix	0.75 µl
OneTaq Hot Start Master Mix	12.5 µl
Nuclease Free Water	9.75 µl
Template DNA	2 µl
Total:	25 µl

42 PCR Cycling conditions:

If using MiniPCR use library: nanopore_amplicon_barcoding_library.plf
nanopore_amplicon_barcoding_library.plfx and protocol "NanoporePCR add barcodes"

Cycle Step	Temp.	Time	# of Cycles
Initial Denaturation	94 °C	00:01:00	1
Amplification			35
Denaturation	94 °C	00:00:30	--
Annealing	60 °C	00:00:30	--
Extension	65 °C	00:00:50 per kb	--
Final Extension	65 °C	00:05:00	1

*Cool to Room temperature

43 Store at 4 °C until ready to proceed to clean-up section.

Clean-Up: Ampure XP Beads

44 Prepare 5 ml of fresh [M]80 % volume ethanol in nuclease-free water per ~10 samples. Keep On ice .

45 Prepare the AMPure XP beads for use; resuspend by vortexing. Keep On ice

46 Prepare Elution Buffer -- 1500 µl [M]10 Nanomolar (nM) Tris-HCl pH 8.0 w/ [M]50 Nanomolar (nM) NaCl as:

15 µl [M]1 Molarity (M) Tris-HCl pH 8.0
15 µl 5M NaCl

into a 1.5ml tube and add 1470 µl of nuclease-free water.

47 Transfer the PCR products to 1.5mL Eppendorf LoBind tubes

48 Add **41 µl** of resuspended AMPure XP beads to the PCR products and mix by flicking the tube.

If using PCR reaction volumes other than **25 µl** use this equation to determine how much of the beads to dispense: (volume of beads pr rxn) = 1.8 x (reaction volume). See below --

Common Sample:Bead volume ratios for 200bp+ size selection:

Volume of PCR product	25ul	22ul	20ul	50ul
AMPure XP Beads	41ul	39.6ul	36ul	90ul

49 Incubate for **00:15:00** at **Room temperature**

50 Place samples on magnetic rack for **00:05:00** or until solution becomes clear.

51 Keep the tubes in the magnetic rack and pipette off the supernatant. (DNA of length > ~200bp is bound to beads)

52 Keep on magnet, wash beads with **200 µl** of cold **80 % volume** ethanol without disturbing the pellet and incubate for **00:00:30**

Remove the ethanol using a pipette and discard.

53 Repeat the previous step for a total of two washes.

54 Dry tubes (open) while still on the magnetic rack for **00:05:00**

55 Remove the tube from the magnetic rack and resuspend pellet in **40 µl** of Elution Buffer.

Flick tube to mix and incubated for **00:05:00** at room temperature.

56 Place tubes back in magnetic rack until the eluate is clear and colorless, or about **00:05:00**

57 Remove and retain **40 µl** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

58 Quantify concentration of eluted sample using a Qubit fluorometer. **go to step #35**

59 Prepare on ice:
Amplified and barcoded DNA samples (up to 12)
From PCR Barcoding Kit--
RAP
FLT
SQB
FB
LB
Nuclease Free Water

60 Transfer **0.5 µl** of each sample into ONE 0.5ml PCR tube (if final concentrations are < **30 ng/ul**). [OR: Pool in equimolar ratios ~25-50 fmol of total DNA into **5 µl**]

61 Add **0.5 µl** RAP. Incubate at **Room temperature** for **00:05:00** .

62 Place library **On ice**

63 Prepare Flush Buffer.

Mix **117 µl** FB with **3 µl** FLT

Keep **On ice**

64 Open MinKNOW software, install flowcell in MinION, and run flowcell check per manufacturer's instructions. Ensure that the flowcell has sufficient pores for sequencing and all other checks pass.

65 Prepare Library.

To the **5 µl** pooled, RAP treated DNA add:

13.5 µl SQB

11 µl LB (Mix loading beads by pipetting up and down just before measuring this volume)

Total library volume should be **29.5 µl**

66 Prime Flowcell.

Open the access to the Flongle loading port by peeling back the tape seal. Stick the tape to the lid of the MinION.

Measure **100 µl** of the priming buffer (FB/FLT mix) in a P200 pipette.

Position the pipette tip perpendicular to the flowcell and firmly in contact with the open port directly above the window into the pore array.

Visually inspect the pipette tip and port for bubbles. Start over with a new tip if bubbles are observed.

Slowly add the priming buffer over 5-10 seconds.

67 Loading the sample

Mix the library by gently flicking the tube.

Measure **29 µl** of the library using a P200 pipette.

Slowly add the library via the loading port just as with the priming buffer. Loading should take 3-5 seconds.

68 Replace the tape to cover the loading port and seal. Close the MinION lid.

69 Start Sequencing Run