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STREAM Benthic Metabarcoding Lab Protocol V.2

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We use this protocol and it's working

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Disclaimer

Abstract

STREAM (Sequencing the Rivers for Environmental Assessment and Monitoring; www.stream-dna.org) is a Canada-wide community-based science program established in 2018 which is led by the research laboratory of Dr. Mehrdad Hajibabaei at the University of Guelph' Centre for Biodiversity Genomics (Guelph, Canada), in collaboration with Environment and Climate Change Canada, and Living Lakes Canada. Using standardized training, field, laboratory, and bioinformatic protocols, communities can send freshwater benthic kick-net samples for DNA metabarcoding analysis, which allows for the rapid identification of benthic and diatom taxa within the sample. Through the duration of this program, over 2000 samples have been contributed and analysed to date.

This protocol details the DNA metabarcoding lab methods developed for use by the Hajibabaei lab on STREAM. Also linked within the protocol are the STREAM Field Sampling Protocol, and details of bioinformatic processing through the MetaWorks (Porter and Hajibabaei, 2022) pipeline.

Materials

General:
Note : Separate pre- and post- PCR reagents, consumables, equipment, and work areas are strongly recommended.
Eliminase - Decon Labs Cat#1102
De-ionized water
Ethanol (70% & Absolute)
Kimwipes
Nitrile Gloves
Parafilm
HyClone Water, Molecular Biology Grade - Cytiva Cat#SH3053801
PCR 96-well plates
Plate Foil/Sealer
1.5mL microcentrifuge tubes
Pipette tips filtered (10µL, 20µL, 200µL, 1000µL)
Pipette (10µL, 20µL, 200µL, 1000µL)
Multi-channel pipettes (10µL, 200µL)
Vortex
Microcentrifuge (24-sample, up to 17000xg)
Microcentrifuge (6-sample)
Centrifuge (rotors for PCR plates and 50mL tubes)

Homogenization:
50mL Centrifuge tubes (3 per sample) -
Black & Decker 10-speed Blender (1 per sample, multiple blenders helps) - Black & Decker Cat#043-2192-8
Absolute Zero Propylene Glycol Antifreeze (if samples stored in PG) - Recochem Cat#70069

Subsampling:
Forceps or scoopula
Bead Tubes (from PowerSoil Pro Kit below)

DNA Extraction:
DNEasy PowerSoil Pro Kit - Qiagen; Cat#47014
MP FastPrep Tissue Homogenizer (24 sample)

PCR & Imaging:
Platinum Taq DNA Polymerase kit - Invitrogen Cat #10966018
KAPA dNTPs (10mM) - Roche Cat#KK1017
(Primers below)
100bp DNA ladder
Agarose
Gel Dock

UV Transilluminator/Gel Imaging station

PCR Purification:
MinElute PCR Purification Kit - Qiagen Cat#28004
QIAcube (optional) - Qiagen
200µL tips (if using QIAcube) - Qiagen Cat#990332
1000µL tips (if using QIAcube) - Qiagen Cat#990352
2.0mL tubs (if using QIAcube) - Qiagen Cat#990381

Quantification:
Fluorometer ie. Qubit Flex - Invitrogen Cat#Q33327
dsDNA assay ie. Qubit dsDNA HS (if using Qubit) - Invitrogen Cat#Q32854
Assay tubes ie. Qubit Flex Assay Tube Strips (if using Qubit Flex) - Invitrogen Cat#Q33252

Library Prep & Sequencing:
Nextera XT v2 Index Kit (Min. one set - - two sets preferred) - Illumina, Cat #FC-131-2001, #FC-131-2002
AMPure XP magnetic beads - Beckman Coulter Cat#A63880
Magnetic Stand
Agilent Bioanalyzer 2100 - Agilent Cat#G2939BA
7500 DNA Chip Kit - Agilent cat#5067-1506
MiSeq v3 reagent kit (600 cycle) - Illumina Cat#MS-102-3003
Illumina MiSeq
PhiX v3 Control - Illumina Cat# FC-110-3001
Sodium Hydroxide (NaOH) 0.2N
Tris
Tween20 - MilliporeSigma Cat#P1379
Heat Block
Shaved Ice

Primers (1st PCR)

	Primer Name	Primer Sequence (5' - 3')
	B_F	CCIGAYATRGCITTYCCICG
	ArR5_R	GTRATIGCICCIGCIARIACIGG
	LCO1490_F	GGTCAACAAATCATAAAGATATTGG
	230_R	CTTATRTTRTTTATICGIGGRAAIGC
	mlCOIintF_F	GGWACWGGWTGAACWGTWTAYCCYCC
	jgHCO2198_R	TAIACYTCIGGRTGICCRAARAAYCA
	Diat_rbcL_708F_1	AGGTGAAGTAAAAGGTTCWTACTTAAA
	Diat_rbcL_708F_2	AGGTGAAGTTAAAGGTTCWTAYTTAAA

	Primer Name	Primer Sequence (5' - 3')
	Diat_rbcL_708F_3	AGGTGAAACTAAAGGTTCWTACTTAAA
	R3_1	CCTTCTAATTTACCWACWACTG
	R3_2	CCTTCTAATTTACCWACAACAG

Illumina Tagged Primers (2nd PCR)

	Primer Name	Primer Sequence (5' - 3')
	B_F_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCIGAYATRGCITTYCCICG
	ArR5_R_IL	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTRATIGCICCIGCIARIACIGG
	LCO1490_F_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCAACAAATCATAAAGATATTGG
	230_R_IL	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTATRTTRTTTATICGIGGRAAIGC
	mlCOIintF_F_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC
	jgHCO2198_R_IL	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAIACYTCIGGRTGICCRAARAAYCA
	Diat_rbcL_708F_1_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGTGAAGTAAAAGGTTCWTACTTAAA
	Diat_rbcL_708F_2_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGTGAAGTTAAAGGTTCWTAYTTAAA
	Diat_rbcL_708F_3_IL	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGTGAAACTAAAGGTTCWTACTTAAA
	R3_1_IL	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTCTAATTTACCWACWACTG
	R3_2_IL	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTCTAATTTACCWACAACAG

Field Methods

1 Benthic samples collected through STREAM are collected according to the STREAM Field Protocol, available here: STREAM Field Manual 2023.pdf 4.3MB

Homogenizing

25m

2 Blenders should be decontaminated ahead of homogenization. Clean by fully disassembling blender components and rinsing with water. Visually inspect to ensure all debris has been removed before scrubbing all surfaces with ELIMINase ™ and rinsing with deionized (DI) water. Treat with UV light for 00:30:00 before reassembling for use. 30m

3 Decontaminate lab bench by wiping with 70% ethanol, followed by ELIMINase ™ and finishing with DI water. 1m

NOTE: If the benthic samples contain medium/large rocks that would damage the blender, lab technicians can remove these using forceps that are decontaminated by cleaning with ELIMINase ™, followed by DI water, and 100% ethanol.

4 Remove bulk benthic sample from -20°C freezer storage and remove all parafilm or plastic sealing the sample bottle. Decontaminate the outside of the bottle with ELIMINase ™ and DI water. 2m

5 Pour entirety of bulk benthic sample into the blender. Pieces of sample tissue left on the inside of bottle can be directed into the blender using a wash bottle filled with the preservative used in the sample (100% ethanol or propylene-glycol antifreeze). Remove possible obstructions, such as rocks, using decontaminated forceps if necessary. 3m

6 Blend at maximum speed for 00:00:45, or as much time needed until bulk benthic sample is a completely homogenized slurry. 45s

7 Subsample into 50mL voucher tubes using serological pipette and bulb. One tube is needed for downstream extraction. Take additional vouchers depending on research needs. 2m

Preservative Removal

8h

8 Decontaminate lab bench by wiping with 70% ethanol, followed by ELIMINase ™ and finishing with DI water. 1m

9 Clean the outside of voucher tubes with ELIMINase ™ and DI water. 5m

10 00:02:00 00:02:00 Centrifuge vouchers for 24:00:00 at 2400 rpm. 1d 0h 4m

11 Avoiding tissue pellet, remove preservative with 50mL serological pipette and bulb. Discard preservative into waste container for appropriate disposal. 5m

12 Remove lid from voucher and set aside. Place small KimWipe ™ over the opening of the voucher tube and secure sides with tape. 5m

13 Place KimWipe ™ covered vouchers in an incubator set to 70°C to evaporate preservative. Check on samples every 2-3 hours and remove from incubator when dry. 1d

NOTE: Samples preserved in propylene glycol antifreeze will not completely dry. They can be incubated for 1 day to remove some preservative but can then be set aside for subsampling. Samples preserved in ethanol will need to be completely dried before extraction because of PCR inhibitors found in ethanol. Drying process for ethanol samples can take several days. Do not leave samples in the incubator overnight. Store sample vouchers in -20°C freezer between incubator sessions and while awaiting subsampling step.

Subsampling 25m

14 Decontaminate lab bench by wiping with 70% ethanol, followed by ELIMINase™ and finishing with DI water. 1m

15 Remove sample vouchers from freezer and bring to room temperature. 20m

16 Decontaminate subsample tools (either scoopulas or forceps) with ELIMINase™, DI water, and 100% ethanol. Set aside on clean Kimwipe™. 2m

17 Clean the outside of voucher tubes with ELIMINase™ and DI water. 5m

18 Label top and sides of bead tubes from DNeasy Powersoil Pro Kit (Qiagen Catalog #47014) to correspond with sample voucher tubes information. 5m

19 Using decontaminated subsample tools, transfer up to 250mg of sample tissue into each bead tube.

20 Move to extraction step immediately after subsampling, or store bead tubes in -20°C freezer until ready to proceed with extraction.

Extraction

2h

20.1 *Note: Extraction protocol uses reagents from DNeasy Powersoil Pro Kit* (Qiagen Catalog #47014) HB-2495-006_HB_DNY_PowerSoil_… 465KB .

Note: If kept in -20°C freezer for storage, bring bead tubes to room temperate before beginning extraction.

Note: A typical extraction involves 23 samples and 1 negative control that receives the same treatment as sample bead tubes but does not contain any benthic tissue.

21 Decontaminate lab bench by wiping with 70% ethanol, followed by ELIMINase™ and finishing with DI water.

22 Clean outside of bead tubes with ELIMINase™ and DI water and place on clean rack.

23 Add 800 µl of Solution CD1 to bead tube changing pipette tips between each sample. Vortex briefly to mix.

24 Load samples into FastPrep-24™ (MP Biomedicals SKU: 116004500) at speed of 6m/s for 60 seconds.

25 Centrifuge lysed bead tubes at 15,000 X g for 60 seconds.

26 Dispense 200 µl of Solution CD2 into labelled 2mL microcentrifuge tubes.

27 When centrifuge finishes running, transfer all supernatant from beads tube to corresponding microcentrifuge tubes. Vortex at max speed for 5 seconds.

28 Centrifuge samples at 15,000 X g for 60 seconds.

29 Dispense 600 µl of Solution CD3 into clean, labelled 2mL microcentrifuge tubes.

30 When centrifuge finishes running, transfer up to 700 µl supernatant into corresponding 2mL microcentrifuge tubes filled with Solution CD3. Carefully avoid pellet. Vortex at max speed for 5 seconds.

31 Label MB Spin Columns and transfer 650 µl of lysate from previous step on to filter. Centrifuge at 15,000 x g for 60 seconds.

32 Discard the flow-through and repeat step 4.11to ensure that all the lysate passed through the MB Spin Column.

33 Carefully remove Spin Column filter to avoid splashing flow-through. Place in a clean collection tube.

34 Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 X g for 60 seconds.

35 Discard the flow-through and place the MB Spin Column back into the same collection tube.

36 Dispense 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 X g for 60 seconds.

37 Discard the flow-through and place the MB Spin Column into a new collection tube. Centrifuge at 16,000 X g for 2 minutes to dry filter.

38 Transfer the MB Spin Column filter into a labelled elution tube. Avoid splashing as residual flow-through on the filter.

39 Add 50 µl of Solution C6 to the centre of the filter membrane. Centrifuge at 15,000 X g for 60 seconds.

40 Remove MB Spin Column from elution tube and discard. Store the eluted DNA in -20°C freezer until further downstream processing.

Polymerase Chain Reaction (PCR)

3h

41 The same DNA extract can be used to target macroinvertebrates or diatoms by using different primer sets, as shown in Robinson et al. (2022).

The macroinvertebrate PCR Protocol uses three primer sets, each targeting the barcode region of the mitochondrial cytochrome c oxidase *I* (COI) gene. Primer sequences and citations are outlined in the table below.

PCRs are performed in separate, single reactions (ie. one reaction each for F230R, BR5, and MLJG)

The Diatom PCR protocol uses one an equimolar pool of three forward and two reverse primers targeting the chloroplast Ribulose-Bisphosphate Carboxylase (rbcL) gene. Primer sequences and citations can be found below.

	Marker	Amplicon	Primer	Primer Sequence (5' - 3')	Fragment Length (w/o primer)	Reference
	COI	BR5	B_F	CCIGAYATRGCITTYCCICG	310	Hajibabaei et al. 2012
	COI	BR5	ArR5_R	GTRATIGCICCIGCIARIACIGG	310	Gibson et al. 2014
	COI	F230R	LCO1490_F	GGTCAACAAATCATAAAGATATTGG	230	Folmer et al. 1994
	COI	F230R	230_R	CTTATRTTRTTTATICGIGGRAAIGC	230	Gibson et al. 2015
	COI	MLJG	mlCOIintF_F	GGWACWGGWTGAACWGTWTAYCCYCC	313	Leray et al. 2013
	COI	MLJG	jgHCO2198_R	TAIACYTCIGGRTGICCRAARAAYCA	313	Geller et al. 2013
	rbcL	diatrbcL	Diat_rbcL_708F_1	AGGTGAAGTAAAAGGTTCWTACTTAAA	312	Vasselon et al. 2017
	rbcL	diatrbcL	Diat_rbcL_708F_2	AGGTGAAGTTAAAGGTTCWTAYTTAAA	312	Vasselon et al. 2017
	rbcL	diatrbcL	Diat_rbcL_708F_3	AGGTGAAACTAAAGGTTCWTACTTAAA	312	Vasselon et al. 2017
	rbcL	diatrbcL	R3_1	CCTTCTAATTTACCWACWACTG	312	Vasselon et al. 201

	Marker	Amplicon	Primer	Primer Sequence (5' - 3')	Fragment Length (w/o primer)	Reference
						7
	rbcL	diatrbcL	R3_2	CCTTCTAATTTACCWACAACAG	312	Vasselon et al. 2017

42 Remove PCR reagents from the freezer and allow them to thaw for approximately 20min.

Note: If you are running the rbcL primerset ensure that you have an equimolar pool of the three forward primers, and 2 reverse primers prepared beforehand.

43 PCRs should take place under a laminar flow hood (PCR hood) that has been decontaminated by wiping with 70% ethanol, followed by ELIMINase ™ and finishing with DI water. Treat hood surfaces with UV light for 30 minutes.

44 Prepare your master mix for each primer set using the formula detailed below. When calculating the volumes required for your mastermix, scale the volumes depending on desired number of samples and include 10% surplus reactions for insurance in case of pipetting errors or other mishaps.
A negative control with no DNA template should also be included for each primer set to ensure clean reagents. Platinum Taq, buffer, and MgCl2 are included in Platinum Taq DNA polymerase kit (Invitrogen; Cat #10966018), KAPA dNTPs (Roche; Cat #KK1017), Molecular Grade Water, and primers, must be ordered separately.

45

	Reagent	Volume per reaction (µl)
	Molecular Grade Water	17.5
	10X reaction buffer (200 mM Tris- HCl, 500 mM KCl, pH 8.4)	2.5
	MgCl2(50 mM)	1.0
	dNTPs mix (10 mM)	0.5
	Forward primer (10µM)	0.5
	Reverse primer (10µM)	0.5
	Platinum Taq (1U)	0.5

46 Once each mastermix has been prepared, transfer 23µL of the mastermix to a new PCR plate or PCR strip, up to the number of samples (and negative controls) needed. If the space on the plate allows for it, F230R, BR5 and MLJG can all be run on the same plate since they have the same thermocycler conditions. An example Plate Map can be found below.

47 Once the mastermix has been dispensed into each well, gently lift the plate or strip and visually inspect that the plate matches your intended layout (eg. there are no missed wells, or wells that have accidentally been filled twice)

48 Add 2µL of molecular grade water to your negative controls, then remove all reagents from the hood to minimize the risk for contamination from DNA.

49 Add 2µL of DNA from each sample to the corresponding well, pipetting up and down three times to mix the solutions.

50 Once DNA template has been added to all wells, seal the plate/strip with foil, lids or sealing tape, and ensure there are no bubbles connecting wells.

51 Once sealed, gently tap plate/strip against the bench to remove some bubbles, then centrifuge the plate for 1min at 1000xg. Visually inspect the plate to ensure there are no bubbles after centrifugation.

52 Place your plate in a thermocycler, and run the following program depending on the fragment:

COI - BR5, F230R, MLJG:
Initial denaturation: 95°C for 5min
25 cycles of:
Denaturation: 94°C for 45s
Annealing: 46°C for 45s
Extension: 72°C for 45s
With a final extension of 72°C for 5min, before an indefinite hold at 10°C.

rbcL - diatrbcL
Initial denaturation: 95°C for 15min
35 cycles of:
Denaturation: 95°C for 45s
Annealing: 55°C for 45s
Extension: 72°C for 45s
With a final extension of 72°C for 5min, before an indefinite hold at 10°C.

53 Once the cycler program has finished, the plate can be removed and stored for up to three weeks at 4°C (refrigerator), or 3mo at -20°C (freezer). To avoid freeze/thaw cycles, try and plan to proceed with the next steps within two weeks.

54 Once ready to verify the reaction success through gel electrophoresis, allow the PCR plate to come to room temperature - roughly 15min. Individual labs will have different methods for running/visualizing gels.

Typically the first PCR will have some weak bands, but some bands may not show up until after the 2nd PCR.

54.1 Centrifuge the plate at 1000xg for 1min to consolidate any droplets inside the wells.

54.2 Under a clean PCR hood, dispense 3µL loading dye for each sample

54.3 Dispense 5µL amplicon, pipetting up and down to mix with the loading dye. Seal the plate with new film/caps when finished dispensing all the samples.

54.4 Once all samples have been separately mixed, load into the wells of agarose gel. Include a 100bp DNA ladder for reference. Be sure to include any negative controls you may have (including extraction negative). If space allows for it, leave a gap between the negative controls and your samples.

54.5 Run the 1.5% agarose gel at 100V and 150mAMPs for 30min.

54.6 Visualize the gel.

54.7 Store the plate at 4°C until you're ready to move to the next step.

2nd PCR

2h

55 Using the 1st PCR as template, repeat steps 42 - 54, with primers that include the Illumina adapter overhang required for sequences to attach to the flowcell.

F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

56 If some samples did not amplify successfully after the 2nd PCR, you can try troubleshooting by doubling or diluting the initial DNA, and restarting from the 1st PCR using the doubled/diluted DNA as template.

PCR Purification

2d

57 PCR products will be individually purified using the MinElute PCR Purification kit (Qiagen, Catalog # 28004): FILE HB-2069-002_HB_MinElute_0120_W… 611KB

Purifications can be performed manually in batches of up to 24 samples, but Qiagen has several robotics options that could be used instead. This protocol outlines the process using a QIAcube (QIAGEN, catalog #9001292) which allows for a more passive processing of 12 samples at once.

Purifications are eluted with a final volume of 15µL of molecular biology grade water regardless of which method you use.

58 Label the required number of 1.5mL microfuge tubes, and 2.0mL tubes for the QIAcube. Ensure the labels include unique sample information (site, sample, replicate, amplicon)

59 Wipe the bench of a decontaminated PCR hood with 70% ethanol, ELIMINase, and deionized water.

60 Under the clean PCR hood, transfer the remaining 20µL of each successfully amplified PCR well to separate, previously labeled 2mL sample tubes.

61 In batches of 12 when possible, place the MinElute spin column, and previously labeled 1.5mL microfuge tube into the QIAcube rotor adapter in the appropriate position outlined in the QIAcube manual.

Briefly leave these to the side while you proceed with the next steps.

62 Place a full rack of 1000µL and 200µL QIAcube tips into the proper location in the QIAcube.

63 Fill each QIAcube reagent bottle with the appropriate reagent, and ensure they are placed in the proper position of the QIAcube's reagent tray. Unscrew the lid, but keep the unscrewed lid on the reagent bottles until just before starting the instrument.

64 Place the rotor adapters into the QIAcube centrifuge rotors.

65 Carefully place the 2mL tubes containing the reactions to be purified into the proper position of the QIAcube shaker.

66 Remove the lids from the reagent bottles and close the QIAcube lid. Leave the lids outside the QIAcube covered by a KimWipe.

67 Confirm that the fill volume on the QIAcube is set to 80µL, which is 100µL less the 20µL in our 2.0mL tubes. Start the protocol.

68 The QIAcube will run checks to ensure everything is balanced properly, there is enough of each reagent, and there are enough of each tip size. Wait until it has run these checks, in case there is an error. Once the checks are complete, you can walk away.

69 Once the protocol is complete, verify each of the spin columns transferred, all elution tubes contain eluate, and that the labels on your 1.5mL tubes match the labels on your 2.0mL tube. The 2mL tubes can be thrown out once you have confirmed everything is as expected.

Due to the low volume of the eluate, it is best to proceed with the next sections within 2 weeks. This minimizes the risk of the sample evaporating.

Quantification and Normalization

3h

70 Remove any purified samples from the fridge and allow them to come to room temperature. 15m

71 Centrifuge the 1.5mL tubes containing purified amplicon for 10s to consolidate the eluate.

72 Use a fluorometer to quantify the concentration of 1µL eluate. This protocol uses a Qubit Flex (Invitrogen, Category #Q33327), and High-Sensitivity dsDNA kit (Invitrogen, Category #Q33231) which allows for the simultaneous quantification of samples in an 8-well strip.

FILE TFS-Assets_LSG_manuals_Qubit_d… 229KB

73 Calculate the required amount of reaction mix for your number of samples, allow a 3-sample buffer to account for pipetting error. The Qubit Flex has a built in calculator for this.

198µL Qubit dsDNA HS Buffer (Component B)
1µL Qubit dsDNA HS Reagent (Component A)

To avoid excess light exposure, work in batches up to 48 samples (6 strips). It is most practical to process samples in the order outlined in the Plate Map.

74 Pour the reaction mix into a reservoir. Using an 8-channel pipette, dispense 199µL into each strip and well that will be quantified.

75 Add 1µL of the purified amplicon to the corresponding well in the strip. Pipette up and down to mix.

Close the lid. Briefly vortex the strips, and visually check if there are any bubbles.

If there are bubbles, gently flick the strip, then gently tap the strip on the lab bench to consolidate the solution to the bottom of the well.

76 Place the strips in a dark place (ie. drawer) to incubate at room temperature for 5 minutes.

77 Quantify one strip at a time by placing them in the Qubit Flex and closing the lid. Ensure that the strips are placed in the correct orientation, or the values will be reversed.

78 Qubit Flex will save the quantification values, which can be exported to a USB drive in batches.

79 Once all samples have been quantified, transfer the results to your computer and calculate the volumes required to have equimolar samples and amplicons. You need to calculate the amount of water to dilute 5µL of your eluate to 5nM. If a sample is less than 5nM, proceed without diluting that sample.

80 Once all values have been calculated, first ensure all samples are at room temperature. Clean the outside of each tube by wetting a Kimwipe with ELIMINase and wiping the outside the tube, followed by wiping with a Kimwipe wet with DI water. Briefly centrifuge all samples to collect any droplets.

81 With a new pair of gloves, and under a clean PCR hood, begin dispensing molecular grade water into each well in accordance with your plate map and calculations. If normalizing multiple plates, it is most efficient to dispense water to all plates first - covering each plate in foil once complete.

82 Confirm that all samples are in the correct order in accordance with the Plate Map, then while still under the PCR hood and working one plate at a time, transfer 5µL of purified amplicon to the corresponding well, pipetting up and down to mix. Once complete, seal the plate with foil.

Clean the PCR hood surface with 70% ethanol, eliminase, and DI water in between plates.

83 Once all samples have been normalized, and the plates have been sealed with foil, decontaminate the PCR hood surface, and run the UV for 15min.

84 Briefly centrifuge the normalized plates to consolidate any droplets.

85 Under the clean PCR hood, use an 8- or 12-channel pipette to transfer 5µL of each normalized amplicon and sample into a new plate, checking to make sure sure that the plates are all in the proper orientation. The result of this will be an equimolar pool of all three COI amplicons for each sample. These pooled samples will be used as template for the final indexing PCR.

Dual-Indexing PCR

1h

86 Dual-Indexing adds unique tags to each sample which allows us to pool all samples into one library, then bioinformatically separate out what sample each sequence corresponds.

Illumina produces the Nextera XT v2 indexes (Cat #FC-131-200X), which come in 4 sets and allows for the multiplexing of 384 samples.

We have found that sequencing 48 STREAM benthic samples on an Illumina MiSeq with a v3 600bp kit provides adequate coverage per sample. It is best practice to use different tag combinations in back-to-back runs, which minimizes the potential for carry-over from any residual library that may be left in the fluidics lines from the previous sequencing run. For this reason, it is helpful to have at least two sets of Nextera indexes.

Nextera XT Index Kit v2 Set A (Illumina, Cat #FC-131-2001)
Nextera XT Index Kit v2 Set B (Illumina, Cat #FC-131-2002)

87 Allow the Nextera Indexes, PCR reagents, and pooled amplicon plate to come to room temperature - approximately 30min.

Clean the exterior of each reagent tube by first wiping them with a Kimwipe wet with ELIMINase, then a Kimwipe wet with DI water.

88 Briefly vortex and centrifuge each index tube and PCR reagent, with the exception of Platinum Taq, which should only be centrifuged.

89 Centrifuge the pooled amplicon plate at 1000xg for 1min to collect any droplets.

90 Under a decontaminated hood, and with a fresh pair of gloves, make your master mix for indexing, with the following volumes.

	Reagent	Volume (1 rxn)
	Water	15.75 µL
	Buffer	2.5 µL
	MgCl2	1.0 µL
	dNTPs	0.5 µL
	Taq	0.25 µL
	Total MM Volume	20 µL
	N-index	1 µL
	S-index	1 µL
	Library Template	3 µL
	Total Reaction Volume	25 µL

Note: Unlike previous PCRs, this master mix will not contain the index (primer equivalent). These will be added after dispensing the master mix.

91 Dispense the mastermix into each well that will contain a sample to be indexed.

Loosely cover the plate with foil.

92 In a new plate, dispense the indexes into the bottom column left-most row, dispensing a volume equal to the number of wells in the column/row with an additional 2µL as a buffer. ie. if a column contained 4 samples, dispense 6µL to the bottom well of column.

As the indexes are being dispensed, confirm that they are in the expected order.

93 Briefly centrifuge the index plate to consolidate the liquid to the bottom.

94 Use a multi-channel pipette to transfer 1µL from the index plate to each well of the mastermix plate, while trying to minimize the amount of time the pipette is over the open plate. Pipette up and down to mix.

Dispense all column indexes then all row indexes (or visa versa).

It is critical that you minimize any risk of indexes transferring between wells.

95 Using a multichannel pipette, dispense 3µL of the amplicon plate to the mastermix plate, taking the same care to minimize any risk of the sample transferring between wells. Pipette up and down to mix.

Seal the plate, visually inspecting the seals to ensure there is no connection between wells, then briefly gently vortex the plate.

Centrifuge the plate at 1000 xg for 1 minute.

96 Place the plate in a thermalcycler and run the program with the following conditions:

72°C for 3:00min
95°C for 0:30min
12 cycles of:
95°C for 0:10min
55°C for 0:30min
72°C for 0:30min
with a final annealing of 72°C for 5:00min
and an indefinate hold at 10°C

97 Once the program is complete, allow the plate to come to room temperature, then centrifuge it at 1000xg for 1min.

98 Under a decontaminated hood, transfer 5µL of each indexed sample to a 1.5µL microfuge tube. For consistency, do not pipette up and down to mix.

This is your pooled, indexed library.

Store the index plate in the refrigerator until the library has been sequenced, then transfer it to -80°C for indefinite storage/archiving.

Magnetic Bead Purification

40m

99 Remove AMPure XP magnetic beads from the refrigerator and allow them to come to room temperature. You will prepare two different ratios (library:bead), the best of which will be used as our library input for sequencing.

100 Prepare one tube of fresh 80% ethanol in a 1.5mL.

200µL Molecular Grade Water
800µL Absolute ethanol

101 Vortex AMPure beads for 30 seconds to ensure that the beads are evenly dispersed

102 Dispense 50µL of the pooled library to each of two fresh 1.5mL (labelled 1:1 and 1:0.6)

103 Add 50µL (1:1) and 30µL (1:0.6) of magnetic beads to the corresponding tube, pipetting up and down 10 times to mix. Close and briefly vortex your stock of bead tubes in between tubes to ensure the beads are still evenly dispersed.

104 Once both tubes have had the beads added, briefly vortex and centrifuge them. Then allow the tubes to incubate on the bench at room temperature for 5min.

105 Move the 1.5mL tubes to a magnetic stand and wait 2min for the beads attract to the rack. The supernatant should be clear. If it is not, wait an additional 30s, or until the supernatant is clear.

106 While the tubes are still on the magnetic stand, slowly remove the supernatant. To avoid disturbing the beads, the pipette tip should be on the opposite side of the tube from the beads.

Change tips between samples.

107 While on the magnetic stand, wash the beads with 200µL of 80% ethanol. Make sure the ethanol washes over all surfaces of the tube.

108 Wait 30s, then remove the supernatant from the tubes.

109 Wash the beads with ethanol again by repeating steps 107 and 108.

110 While on the magnetic stand, leave the tubes open and place a Kimwipe loosely over the open tubes. Allow the tubes to air dry for 15 minutes to remove any residual ethanol.

Allow more time if the tubes are not dry after 15min.

111 Resuspend the DNA in 23µL molecular grade water or 1X buffer TE. To do this, rinse the beads with the 23µL of solution once while the tubes are still on the magnetic stand, then take the tubes off the stand and pipette the same 23µL up and down, focusing on the bead to dislodge the pellet.

By doing the first rinse while the beads are still on the stand, it minimizes the risk of dry beads accidentally flinging off the side of the tube when they are first removed from the magnetic stand.

112 Gently vortex the tubes to redistribute the beads. Centrifuge briefly to collect the liquid.

113 Place the tubes back on the magnetic stand, and wait 2min for the beads to collect.

114 Using a 10µL pipette to avoid disturbing the beads, transfer as much of the supernatant as possible without taking any beads to a fresh 1.5mL tube. Typically you can retrieve up to 20µL of the supernatant, after which beads start to be pulled with the supernatant.

This is your purified library for each ratio.

115

Library Qualification and Quantification

1h

116 Remove the Agilent 7500 DNA kit from the refrigerator and allow for it to come to room temperature in a drawer, or otherwise dark area.

117 Run the DNA 7500 chip following manufacturer's protocol, running each ratio library in two wells of the chip for redundancy. ie. since this protocol describes purifying a 1:1 and 1:0.6 library, having two wells for each will mean the chip uses four wells.

118 While the DNA chip is being analyzed, quantify the libraries through fluorometry (Qubit) as in Steps 75-82 above. Quantify with two separate assays for each ratio. The average of the values for your chosen ratio will be used to calculate the dilution.

119 Once the chip has finished, record all fragment lengths (peaks).

120 Based on the amount of primer-dimer, and overall results of the gel chip, decide which ratio to use. Typically the 1:0.6 library is most appropriate, due to the reduced primer-dimer.

Final Sequencing Preparation

1h

121 Remove the MiSeq v3 600 cycle kit (Illumina; Catalogue # MS-102-3003) from the freezer, open the package, and place the reagent tray in a room temperature water bath to thaw for 1.5 - 2h. Allow Buffer HT1 to that on the counter at room temperature (no water bath necessary). Once HT1 thaws (approx. 30min) stored it on ice (4-8°C).

Follow the best practices for preparing the MiSeq instrument for a sequencing run (ie. maintenance wash, data storage, etc.)

122 Prepare your Sample Sheet using the appropriate reagent tray barcode, PlateMap, and indexes.

123 STREAM uses a standard 40-character file naming system that allows for easy and consistent sample comparison & labelling. The basis of the site and sample names are from the Chain of Custody submitted by contributing groups, but may be modified from the site name originally submitted to fit our file format. Full site details are stored in an associated metadata file.
This is:
STREAM-CONTRIBUTINGGROUP[4 characters]-B (indicating BENTHOS) - SITE [4] - SAMPLE [4] - REPLICATE [1] - DATE (YEARMODA) [8] - MARKER [3]

eg. STREAM-UOGU-B-SPED-0001-A-20230720-COI

124 Copy your SampleSheet to the MiSeq, and perform a power cycle on the MiSeq.

125 Calculate the amount of molecular grade water required to dilute 5µL of the library to 4nM. This is the starting input of the MiSeq Denature & Dilution. Illumina document # 15039740 v10 outlines the full protocol for the 4nM library, and PhiX denature and dilution.

126 30min prior to beginning the Ilumina Denature and Dilution protocol:

Remove PhiX stock (Illumina; Catalogue #FC-110-3001) from the freezer and allow it to thaw at room temperature.

Set heat block to 96°C and allow it to reach temperature.

127 Prepare your 4nM library based on value calculated in Step 129.
Prepare a fresh 0.2N NaOH solution.

128 Proceed with MiSeq Denature & Dilution protocol - document # 15039740 v10

FILE miseq-denature-dilute-libraries-guide-… 569KB

129 There are two main judgement calls within the Denaturing and Dilution protocol:
Loading concentration, and PhiX spike-in

Most often, an 8pM loading concentration, and 10% PhiX spike-in will be used, but depending on the expected diversity of the samples, these may be higher or lower. If a user is new to using these protocols, it is a good idea to start with more PhiX (15%) and optimize the proportion on subsequent runs. Since these are low diversity libraries, the loss of data to PhiX is generally seen as better than the loss of read quality that can occur during unbalanced runs.

130 In cases with high GC content libraries, Illumina recommends an additional heat denaturation step before pooling the PhiX and Library. Perform this extra step with every run.

130.1 Prepare a 10°C ice bath.

130.2 Briefly vortex and spin down the diluted library and PhiX.

130.3 Place the library and PhiX on the heatblock preheated to 96°C. Incubate for 2min.

130.4 Remove tubes from the heat block and invert 3 times to mix. Take care that the tube lids do not accidentally come open.

130.5 Place the tubes on your ice bath and allow a minimum incubation time of 5min. Keep the tubes on ice until you are ready to pool the PhiX and Library, but you must start the sequencing protocol on the MiSeq within 20min of the heat denaturation.

131 Once the sequencing has begun on the MiSeq, allow approximately 56h for it to complete.

Bioinformatics

2d

132 When the run is complete, perform any on-instrument QA/QC you may need, then transfer the run data to the appropriate storage location following established standards and best practices.

133 Sample files and negative controls are processed using the MetaWorks pipeline (Porter and Hajibabaei, 2022).

For a walkthrough on installation and set-up for MetaWorks and the associated classifiers, visit https://github.com/terrimporter/MetaWorks.

134 Currently, all COI samples are processed using the v4 eukaryote COI classifier, available at https://github.com/terrimporter/CO1Classifier. Though, it should be noted that a v5.1 COI classifier has recently been released (May 2023)

And diatom rbcL samples are processed using the v1 eukaryote rbcL classifier, available at https://github.com/terrimporter/rbcLClassifier

135 Samples are initially processed through MetaWorks in smaller batches (50-100 samples) that contain
a. the output of the recently sequenced samples
b. any sequences that may have been processed in past years for the groups who have samples within the recent run.
This allows for a contributing group to have multi-year results that have been processed consistently.

136 Downstream analysis using the MetaWorks results output will vary, but the bootstrap cutoffs used to consider 'Accurate' identifications are outlined in the documentation for each classifier. Based on the fragment length of the COI amplicons used in this protocol, our standard bootstrap cutoffs are:

Phylum, Class, Order: 0.0 [99% accurate]
Family: 0.2 [99% accurate]
Genus: 0.3 [99% accurate]
Species: 0.7 [95% accurate]

Due to the factors including the marker biology, and database completion, the rbcL classifier does not produce accurate higher-level identifications. We have found that it's most effective to filter out any non-diatom identifications, and work at the ESV level.

137 Preliminary Data Reports are sent to the participants who contribute samples to STREAM, which contain the macroinvertebrate taxonomic results (presence-absence) at the Genus and Species rank for each samples they contribute. Since each contributing group has their own goals, which would require different analyses, the Preliminary Data Reports contain the simple metrics of Taxon Richness, and a summary of EPT taxa, with the expectation that the group will do their own, more extensive analysis if it is desired.

138 The complete STREAM dataset is annually re-analysed through MetaWorks for a globally consistent dataset. A summary of the 2018-2021 data can be found in the STREAM web portal at https://stream-dna.org/data-portal/.

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