Jul 14, 2025

18S+16S library preparation for Illumina MiSeq eDNA metabarcoding - rocky intertidal seawater samples

DOI
dx.doi.org/10.17504/protocols.io.6qpvr6r1bvmk/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.6qpvr6r1bvmk/v1

External link: https://www.ncbi.nlm.nih.gov/sra/PRJNA1289101

Protocol Citation: Mary McElroy 2025. 18S+16S library preparation for Illumina MiSeq eDNA metabarcoding - rocky intertidal seawater samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvr6r1bvmk/v1

Manuscript citation:
In prep

Protocol status: Working
We use this protocol and it's working

Created: May 17, 2022

Last Modified: July 14, 2025

Protocol Integer ID: 62782

Keywords: environmental DNA, metabarcoding, marine, rocky intertidal, Illumina MiSeq, library preparation, amplicon sequencing, seawater sampling, rocky intertidal seawater samples this protocol, rocky intertidal seawater sample, seawater sample, north atlantic macroalgae with edna, amplicon metabarcoding with the illumina miseq system, library preparation for illumina miseq, genetic signatures of ecological diversity, rna, north atlantic macroalgae, environmental dna, studying protistan diversity, subunit ribosomal rna gene, protistan diversity, amplicon metabarcoding, ecological diversity, parallel sequencing of v9, rocky intertidal habitat, parallel sequencing, california rocky intertidal habitat, illumina miseq system

Funders Acknowledgements:
Zegar Family Foundation
Grant ID: SB200094

Abstract

This protocol describes library preparation for combined 18S+16S amplicon metabarcoding with the Illumina MiSeq system. Seawater samples were collected from California rocky intertidal habitats, filtered using Sterivex capsules, and extracted with Qiagen DNeasy Blood & Tissue kits (see related protocols). This protocol is modified from:

Illumina 16S Sample Preparation Guide
CALeDNA Metabarcoding Library Preparation
Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W. and Huse, S.M., 2009. **A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes**. *PloS one*,*4*(7), p.e6372.
Ørberg, S.B., Krause-Jensen, D., Geraldi, N.R., Ortega, A., Díaz-Rúa, R. and Duarte, C.M., 2022. **Fingerprinting Arctic and North Atlantic Macroalgae with eDNA–Application and perspectives.** *Environmental DNA*,*4*(2), pp.385-401.
Kelly, R.P., O'Donnell, J.L., Lowell, N.C., Shelton, A.O., Samhouri, J.F., Hennessey, S.M., Feist, B.E. and Williams, G.D., 2016. **Genetic signatures of ecological diversity along an urbanization gradient.** *PeerJ*,*4*, p.e2444.

Materials

This protocol requires a laboratory set up for standard PCR, gel electrophoresis and imaging, DNA quantitation, and cold storage.

See CALeDNA **Library Preparation** protocols and Illumina **16S Sample Prep Guide** for detailed lists of instruments and consumables. See **UCSB Genetics Core** for an example sequencing facility with appropriate equipment.

Before start

Order **18S-V9** primer set (Amaral-Zettler et al. 2009, Orberg et al. 2022) with Illumina overhangs:

Forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse overhang: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Forward primer (1380F): 5′-CCCTGCCHTTTGTACACAC
Reverse primer (1510R): 5′-CCTTCYGCAGGTTCACCTAC

Order **16S** primer set (Kelly et al. 2016) with Illumina overhangs:

Forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse overhang: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Forward primer (16s_Metazoa_fwd): 5′-AGTTACYYTAGGGATAACAGCG
Reverse primer (16s_Metazoa_rev): 5′-CCGGTCTGAACTCAGATCAYGT

https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos/custom-dna-oligos

18S Amplicon PCR

1 Dilute primers to 10 uM in PCR water. Dilute BSA to 4 mg/ml in PCR water. Set up triplicate PCRs for each sample.

2 Perform PCRs in 25 ul volumes with the following reaction chemistry:

12.5 ul NEBNext Ultra II Q5 Master Mix (#M0544)
0.5 ul 4 mg/ml bovine serum albumin (BSA)
1.25 ul 10 uM fwd primer
1.25 ul 10 uM rvs primer
1 ul undiluted DNA template
8.5 ul PCR grade water

Thermocycling conditions:

Initial denaturation, 98C for 30 sec

30 cycles of denaturation, 98C for 10 sec; annealing, 57C for 30 sec; extension, 72C for 30 sec

Final extension, 72C for 2 min, then hold at 4C

3 Pipet 25 ul PCR product from each replicate into a single tube/well for a total of 75 ul per sample.

See CALeDNA **Replicate Pooling** protocol

16S Amplicon PCR

4 Dilute primers to 1 uM in PCR water. Set up triplicate PCRs for each sample.

5 Perform PCRs in 25 ul volumes with the following reaction chemistry:

12.5 ul NEBNext Ultra II Q5 Master Mix (#M0544)
5 ul 1 uM fwd primer
5 ul 1 uM rvs primer
2.5 ul undiluted DNA template

Thermocycling conditions:

Initial denaturation, 98C for 30 sec

35 cycles of denaturation, 98C for 10 sec; annealing, 62C for 30 sec; extension, 72C for 30 sec

Final extension, 72C for 2 min, then hold at 4C

6 Pipet 25 ul PCR product from each replicate into a single tube/well for a total of 75 ul per sample.

See CALeDNA **Replicate Pooling** protocol

Check PCR products

7 Run gels for all samples to check for successful amplification.

See CALeDNA **Gel Electrophoresis** protocol

Amplicon PCR clean-up

8 Clean PCR products using 1.8X ratio and AMPure XP beads (Beckman Coulter). Use fresh 70% ethanol and elute DNA in 30 ul ultra pure PCR water.

Bead prep: x samples * (1.8)(45 ul sample) * 1.1(10% error) = x ul beads

See CALeDNA **PCR Purification** protocol

Product QC and quantification

9 Check PCR product sizes for a random mix of samples and controls.

Expected fragment size: 18S-V9, ~130 bp
16S, 114-140 bp

Quantify all pooled sample yields.

Normalize

10 Using product yields, dilute samples in a new plate to 10 ng in up to 11.25 ul PCR water per sample. If yield is less than 10 ng, then use 11.25 ul product for indexing reactions.

Index PCR

11 Use IDT for Illumina DNA/RNA Unique Dual Indexes (Sets A-D, 20027213-6).

For each sample, perform PCR in 25 ul reactions with the following reaction chemistry:

12.5 ul NEBNext Ultra II Q5 Master Mix (#M0544)
1.25 ul Illumina UD indexing primer mix
11.25 ul normalized product

Thermocycling conditions:
Initial denaturation, 95C for 5 min

8 cycles of denaturation at 98C for 20 sec; annealing at 56C for 30 sec; extension at 72C for 3 min.

Final extension, 72C for 5 min, then hold at 4C.

See CALeDNA **Indexing PCR** protocol

Index PCR clean-up

12 Check product sizes again (see Step 9). Expected fragment size (including index, adapters): 16S, 285-300 bp

18S, 295-320 bp

Determine bead ratio using observed fragment sizes after index PCR. Use bead prep calculation from Step 8, adjusted for 25 ul product and appropriate bead ratio from product size check. We used 1.8X.

Clean PCR products with AMPure XP beads (Beckman Coulter). Use fresh 70% ethanol and elute DNA in 30 ul ultra pure PCR water.

See CALeDNA **PCR Purification** protocol

13 Quantify all indexed sample yields.

Pool libraries

14 Pool cleaned, indexed samples by equal mass/number of molecules per sample, target final library concentration of 10-15 nM in at least 200 ul. Use fragment size to calculate

nM from ng/ul.

See CALeDNA **Library Pooling** protocol

15 Quantify pooled library and check fragment size (same methods as in previous steps).

Sequencing

16 Follow **16S Sample Preparation Guide** starting at page 17.

Prep PhiX Control Kit v3 for 15% spike-in.

Sequence with MiSeq v3 600-cycle kit, 2 × 100 PE reads.

Protocol references

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CALeDNA Metabarcoding Library Preparation

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