18S Metabarcoding

Materials:

- FC-121-1031 (Illumina) Nextera DNA Sample Preparation Kit (96 samples)
- FC-121-1012 (Illumina) Nextera Index Kit (96 Indexes, 384 samples)
- Q32854 (Thermo Fisher) Invitrogen Qubit dsDNA HS Assay Kit (500 assays)
- MS-102-2003 (Illumina) MiSeq Reagent Kit v2 (500-cycle)
- AM9932 (Thermo Fisher) Ambion Nuclease-free water (not DEPC-treated)
    - ph 8.0, decanted in falcon tube under fume hood
- F530S/L Thermo Fisher Phusion High-Fidelity DNA polymerase (2 U/ul)
- KK8002 KAPA Pure Beads (60mL)
- Z5342 Promega MagneSphere Technology Magnetic Separation (twelve-position)
- V4_18SNext.For & V4_18SNext.Rev primers (see table below)
- V9_18SNext.For & V9_18SNext.Rev primers (see table below)
- dNTPs
- 80% ethanol (freshly prepared and kept on ice)
- 1.7 ml tubes
- 0.5 ml Qubit tubes
- 96-well PCR plates
- Filter tips
- Loading dye
- Gel red dye
- 1xSB Buffer
- SeaKem Agarose
- Hi-Lo DNA Marker

Ocean Sampling Day Procedure:

This protocol is based on the 2015 Ocean Sampling Day Protocol, which is available on the Ocean Sampling Day website: https://www.microb3.eu/osd

Amplification of V4 18S rRNA

1. Retrieve genomic material from freezer and put in fridge to thaw.
2. Get reagents for master mix from freezer.
3. Use V4_18SNext.For & V4_18SNext.Rev primers. Primer stocks should be diluted.

Primer Label	Primer Sequence(5'-3')
V4_18SNext.For	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG [CCAGCASCYGCGGTAATTCC]
V4_18SNext.Rev	GTCTCGTGGGCTCGGAGATCTGTATAAGAGACAG [ACTTTCGTTCTTGATYRATGA]

4. Let everything thaw on bench. Put dNTPs and primers back on ice after they have thawed.
5. Label 96 well plate. Make plate map of where each sample is placed in 96-well PCR plate.
6. Vortex & spin down everything. Put all components back on ice.
7. Set up PCR reaction:

PCR Reaction	Concentration	Volume	x28
ddH_2O	varies	13.5 ul	378 ul
V4_18SNext.For	0.5 uM	1.25 ul	35 ul
V4_18SNext.Rev	0.5 uM	1.25 ul	35 ul
dNTPs	200 uM (0.2mM)	2.5 ul	70 ul
5x HF DNA Buffer	1x	5 ul	140 ul
Phusion DNA Polymerase	1 U	0.5 ul	14 ul
DNA template	2.5 ng	1 ul	28 ul
Total	25 ul	25 ul	700 ul

8. Briefly centrifuge PCR plate.
9. Place PCR plate in Thermal Cycler. PCR should run for about one hour.

Cycle	1	10			15			1	1
Temp	98.0	98.0	44.0	72.0	98.0	62.0	72.0	72.0	10.0
Time	0:30	0:10	0:30	0:15	0:10	0:30	0:15	7:00	∞

Run Gel

1. Prepare 1.2% agarose gel and gel dye red.
2. Protocol: 8 minutes at 110V. Should yield 470 bp PCR product.

Bead Clean, Qubit & Dilute

1. Clean the samples with KAPA Pure Beads following the manufacturer's instructions.
2. Use Qubit protocol to record new concentrations of the samples.
3. Calculate ul of each sample required to reach 40 ng for next PCR.

Illumina Nextera Procedure:

This protocol is based on the Nextera DNA Library Prep Reference Guide.

Amplification of Index Adapters

1. Retrieve genomic material.
2. Prepare consumables:

Item	Instructions
Index adapters (i7 and i5)	Thaw on ice for ~20 mins. Gently invert 3-5 times and centrifuge briefly.
5x HF Buffer	Thaw and immediately place on ice.
Phusion DNA Polymerase	Thaw and immediately place on ice.

3. Use 1.7 ml tubes for adapters on the microcentrifuge. Index adapter sequences:

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	N502	CTCTCTAT
N702	CGTACTAG	N503	TATCCTCT
N703	AGGCAGAA	N504	AGAGTAGA
N704	TCCTGAGC	N505	GTAAGGAG
N705	GGACTCCT	N506	ACTGCATA
N706	TAGGCATG	N507	AAGGAGTA
N707	CTCTCTAC	N508	CTAAGCCT
N708	CAGAGAGG	N517	GCGTAAGA
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

4. Label 96 well plate. Make plate map of where each sample is placed in 96-well PCR plate.
5. Vortex & spin down everything except adapters. Put all components back on ice.
6. Indicate placement of dual-indexing adapters. Arrange Index 1 (i7) adapter along columns 1-6 and Index 2 (i5) adapters along rows A-D.

	1	2	3	4	5	6
A	N701 N502	N702 N502	N703 N502	N704 N502	N705 N502	N706 N502
B	N701 N503	N702 N503	N703 N503	N704 N503	N705 N503	N706 N503
C	N701 N504	N702 N504	N703 N504	N704 N504	N705 N504	N706 N504
D	N701 N505	N702 N505	N703 N505	N704 N505	N705 N505	N706 N505

7. Set up PCR reaction:

PCR Reaction	Concentration	Volume	x28
ddH_2O	varies	x ul	x ul
Index 1 (i7)	5 ul	5 ul	140 ul
Index 2 (i5)	5 ul	5 ul	140 ul
dNTPs	0.2mM	5 ul	140 ul
5x HF DNA Buffer	1x	10 ul	280 ul
Phusion DNA Polymerase	1 U	1 ul	28 ul
DNA template	40 ng	x ul	x ul
Total	50 ul	50 ul	1400 ul

8. Pipette ddH_2O, Index 1, Index 2, master mix, and DNA in each well.
9. When transferring 40 ng of cleaned PCR product from original 18S amplification, pipette up and down 3-5 times to mix.
10. Briefly centrifuge PCR plate.
11. Place PCR plate in Thermal Cycler. PCR should run for 25 minutes.

Cycle	1	5			1
Temp	98.0	98.0	65.0	72.0	10.0
Time	0:30	0:10	0:30	3:00	∞

12. The dual indexed amplicons should be 536 bp for V4.

Bead Clean & Qubit PCR-amplified DNA

1. Clean the amplicon pool with KAPA Pure Beads following the manufacturer's instructions.
2. Use Qubit protocol to record new concentrations of the samples.

Run Gel

1. Prepare gel with ladder.
2. Protocol: 40 minutes at 100V with DNA Hi-Lo Ladder.

Pool Samples

1. Based on Qubit concentrations, calculate the relative concentrations of each sample (nM).
2. Pool the samples so that equimolar ratios of each amplicon are contributed from each sample.
3. Qubit the pooled sample before evaporation/dilution.

MiSeq

1. Sample must be 2.0 nM to run on the MiSeq platform.
2. Use a V2 500 cycle kit.
3. 20-30% PhiX to increase diversity.