16S and 18S Metabarcoding analysis for Chilean coastal waters harmful algal blooms

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MATERIALS

■ 1st Step PCR

- Sterilized 1.5 mL tubes (Cat no. 2150N; ThermoFisher, Inc).
- Sterilized 8-strip 0.2 mL PCR tubes and flat cap (Cat no. AB0451 & 4323032; ThermoFisher, Inc).
- High performance Inhibitor-tolerant DNA polymerase (Terra PCR Direct Polymerase Mix, Cat no. 639271; Takara Bio USA, Inc.)
- Molecular grade nuclease-free water (Cat no. 11-05-01-04, Integrated DNA Technologies Inc).

■ Electrophoresis

- TBE buffer (Cat no. B52; ThermoFisher Inc.)
- Multi-Purpose Agarose (Cat no. CSL-AG500; Cleaver Scientific, Ltd.)
- DNA loading dye (Blue/Orange Loading Dye 6X, Cat no. G1881; Promega, Inc.)
- AccuRuler 100 bp DNA Ladder, Cat no. 02001-500; MaestroGen, Inc.)
- Agarose electrophoresis gel stain (GelRed® Nucleic Acid Gel Stain, Cat no. 41002; Biotium, Inc.)
- Transilluminator and image capture system (GelDoc-It^{TS2} Imager, Analytik Jena, AG)

■ PCR Clean-Up

- 96-well 0.2 mL PCR plate (Cat no. N8010560; ThermoFisher Inc).
- Microseal film (Cat no. 4311971; ThermoFisher Inc).
- Magnetic beads DNA cleanup system (ProNex® Size-Selective Purification System, Cat no. NG2002; Promega, Inc)
- Magnetic stand for 96 wells (Magnetic Stand-96; Cat no. AM10027; ThermoFisher Inc.)
- Ethanol Molecular Biology Grade (Cat no. E7023; Merck KGaA)

■ 2nd Step PCR

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- 96-well 0.2 mL PCR plate (Cat no. N8010560; ThermoFisher Inc).
- Microseal film (Cat no. 4311971; ThermoFisher Inc).
- High performance sequencing-grade DNA polymerase (KAPA HiFi Hotstart ReadyMix, Cat no. KK2602; Roche, Inc)
- Illumina Nextera XT v2 Index Kit set A, B, C and D (Cat no. FC-131-2001, 2002, 2003 and 2004; Illumina Inc.)

- **Library verification**
 - Fragment analysis verification matrix (D1000 ScreenTape, Cat no. 5067-5582; Agilent Technologies Inc)
 - Fluorescent 1000 bp DNA markers (D1000 Reagent, Cat no. 5067-5602; Agilent Technologies Inc)
 - Loading tips (Specific for Agilent TapeStation systems; Cat no. 5067-5598; Agilent Technologies Inc.)

- **DNA concentration measurement**
 - 0.5 mL single tube (Cat no. Q32856, ThermoFisher Inc.)
 - Fluorescent DNA concentration measurement kit (Qubit™ dsDNA HS Assay kit, Cat no. Q32851; Thermo Fisher Scientific,)
 - Generic bench vortexer (e.g. Vortex Genie 2, Cat no. SI-0236; Scientific Industries, Inc.)

- **Metagenomic Sequencing**
 - PhiX control Kit v3 (Cat no. FC-110-3001; Illumina Inc.)
 - MiSeq Reagent Kit v3 (Cat no. MS-102-3003; Illumina Inc.)
 - Molecular grade sodium hydroxide (NaOH) 1M (Cat no. 1091371000, Merck KGaA)

- **Equipment**
 - Tabletop centrifuge (Eppendorf 5427R Centrifuge; Eppendorf AG)
 - PCR cabinet (Biobase PCR-800 PCR Cabinet; Biobase Co. Ltd.)
 - ThermalCycler (MiniAMP™ Plus Thermal Cycler, Cat no. A37835; ThermoFisher, Inc.)
 - Agarose Gel electrophoresis system (Cat no. D2; ThermoFisher Inc.)
 - Fragment Analyzer (Agilent TapeStation 4150, Cat no. G2992AA; Agilent)
 - Fluorometer (Qubit4™ Fluorometer, Cat no. Q33238; ThermoFisher Inc.)

16S and 18S rRNA Metagenomic assay

- 1 The following protocol is based on 16S Metagenomic Sequencing Library Preparation (Illumina, Inc.) with modifications. We strongly recommend reading the entire sequencing protocol prior to start library preparation as there are several steps that must be done in parallel. The protocol has been standardized for 140 – 170 samples per run. Prior to the begin of the library preparation procedure, thaw all DNA samples at room temperature, centrifuge at 4000 rpm x 2 min, and transfer 100 µL of each sample supernatant to 8-tube strips for easy manipulation. Both pipettors and the inside surface of the PCR cabinet requires wiping with 70% ethanol and exposed to UV light for 30 min for each step. To avoid primer contamination, for all PCR reactions, primers in Table 1 are exactly diluted from stock to 1 µM. e.g.: 1 µL of 100 µM primer stock + 99 µL of PCR

grade water.

Figure 1. Summarized libraries preparation workflow, and required reagents and equipment

1st Step PCR

2 PCR reactions

This step consists of an endpoint PCR protocol to generate amplicons for sequencing. For easefulness, we suggest having all samples distributed in 8-tube strips as stated before.

- For 25 µL reactions, in 1.5 mL centrifuge tubes prepare a master mix containing the following amounts per sample:
- 2.5 µL of 1 µM 16S-341F (16S) or SSU-F1289 (18S) primer
- 2.5 µL of 1 µM 16S-805R (16S) or SSU-R1772 (18S) primer
- 12.5 µL of 2X Terra PCR Direct Buffer
- 0.5 µL of Terra PCR Direct Polymerase Mix (1.25 U/µl)
- 5 µL of PCR grade water

When making calculations for the mastermix, consider a surplus of 3-5 samples for negative control, and preemptive mistake avoidance.

- *Carefully distribute 22.5 µL of the master mix into factory sterilized 8-tube strips and mix it with 2.5 µL of DNA templates. For negative control, use 2.5 µL of PCR grade water. We recommend performing this for one strip, cap it and then sequentially proceed with the following strips as 16S and 18S genes are highly ubiquitous and prone to contamination.*

Put sample tubes into the thermal cycler with the following temperature program:

- Initial denaturation: 95°C x 3 min
- 35 cycles of: 95°C x 30 s, 55°C x 30 s, 72°C x 30 s
- Final elongation: 72°C x 5 min
- Hold at 4°C
- Store samples at -20°C until use

Gel electrophoresis and bands reveal

- Prepare 2% w/v Agarose-TBE gels. Once gel is melted, add 10 µL of 1X GelRed® Nucleic Acid Gel Stain per each 100 mL of Agarose-TBE mixture.
- Leave gels in dark for a minimum of 30 min.
- Mix 1.5 µL of 1X DNA loading dye with 4 µL of each PCR product and load the whole amount of mixture (5 µL) into the gel.
- Run electrophoresis at 100 v x 30 min.
- Put gels under UV light. For both amplified genes, observe for bands at 500-600 bp range. For documenting, use an Ethidium bromide filter.

Due to sample nature and prior DNA extraction, primer dimer bands can be observed at 80 bp. Those are removed in the next step.

NOTE: Make sure the negative control has no bands other than the primer dimer. If there is a band near the target (500-600 bp), retry the 1st step PCR.

Amplicon PCR clean-up

The following volumes have been adjusted for Pronex® Size-Selective Purification kit, not for other magnetic bead-based kits such as AMPure XPTM (A63882; Beckman-Coulter Inc.). This procedure can be performed outside a PCR cabinet and its purpose is to remove PCR reaction residues (dNTPs, primers, small size amplicons, buffer and others).

- Distribute 8-tube strips in a PCR tube racks. Carefully uncap with a tube opener to avoid spillage (if not available, lift caps from the side instead of pulling from the tip).
- Vortex Pronex® Chemistry Reagent flask for 15 s.
- Add 1.2 volumes of Pronex® Chemistry reagent to each PCR product (25.2 µL of reagent for 21 µL of PCR product), mix thoroughly (10 times), and incubate at room temperature for 10 min. NOTE: Time excess in this step can reduce cleanup yield.
- Distribute the 8-tube strips on a 0.2 mL 96-well plate and place it on the magnetic stand. Incubate for 2 min and carefully discard the supernatant avoiding the magnetic pellet with the tips.
- Without removing from the magnetic stand, add 80 µL of wash solution to the tubes without mixing, incubate for 30 – 40 s, remove carefully and discard. This process shall be done two times. Procure removing as much wash solution as possible.
- Leave tubes to dry at room temperature for 10 - 15 min, or until no wash solution is observed.
- Remove the strips from the magnetic stand, mix thoroughly with 22 µL of elution buffer (sterile TE Buffer pH 8.0) and incubate for 5 min.

- Place the strips on the 96-well plate, and place it on the magnetic stand. Incubate 2 min.
- Once pellet is separated, transfer 20 μ L of supernatant to a new sterile 96-well plate and seal it with a microseal film.
- Store at -20°C until required.

2nd Step PCR

3 PCR Reactions

For this process, all samples are amplified with different primer combinations. Primers designed for this process (Table 2) are specifically designed to bind to the 1st PCR reactions overhang adapter and for this reason, this can be done outside a PCR cabinet. However, primer cross contamination can be occurred, therefore tips must be replaced on each step of the process.

- Dilute all index primers up to 1 μ M in 8-tube PCR strips. Index 1 primers as well as Index 2 primers must be in separated strips.
- Arrange Index 2 primers in a vertical row, and Index 1 primers in a horizontal row (Figure 2).

Figure 2. Index primers distribution layout for 2nd Step PCR.

In a new sterile 96-well plate, add 12.5 μ L of 2x KAPA HiFi HotStart ReadyMix to each well.

- Add 2.5 μ L of each index primer (1 μ M) to each well of a new 96-well plate using a multichannel pipettor.
- Add 7.5 μ L of purified PCR product DNA, mix gently (10 times) and cover with a microseal film.
- Put the plate into the thermal cycler with the following program:
 - Initial denaturation 95°C x 3 min
 - 8 cycles of 95°C x 30 sec, 55°C x 30 s, 72°C x 30 s,
 - Final elongation: 72°C x 5 min
 - Hold at 4°C^a

^aLibraries shall be kept at -20°C in case of not being immediately validated in the next procedure.

Libraries verification

4 *Using the primer sets on this protocol, the expected size of the final library is ~ 613 bp for both 16S and 18S rRNA genes. This process can be performed in other fragment analyzers (e.g: 5200 Fragment Analyzer, Agilent Technologies Inc. with the DNF-473-0500 kit). The following is described for TapeStation 4000 series. For this particular library preparation and equipment, 35 – 1000 bp reagents and ScreenTape (D1000, Agilent Technologies Inc.) are used.*

- Allow D1000 Sample Buffer and ScreenTape to equilibrate at room temp for 30 min.
- Remove the cover, and place a ScreenTape and a loading tips box in the TapeStation machine.
- Vortex D1000 reagents and spin down before use.
- Carefully mix 2 µL of D1000 Sample Buffer and 3 µL of sample in new 8-tube strips. The formation of air bubbles must be avoided.
- Insert the 8-tube strips into the machine.
- After the run is finished, software will reveal the molecular size of each library. If electronic ladder is selected, calibration might be required manually for some samples. Library size shall be comprised between 600 - 630 bp.

2nd clean-up, quantification, normalization, and pooling

5 *After verification, samples must be clean-up a second time. For this, follow the prior instructions in the PCR clean-up section (Step 2). On the other hand, the concentration of DNA sample is measured with a fluorometer, in this case Qubit4TM, coupled with QubitTM HS dsDNA kit (ThermoFisher Inc.). As with the former processes, this can be replaced with another brand equipment and kits.*

- Equilibrate the assay components to room temp. Check for crystallization as fluorochromes are usually provided in DMSO solution.
- Prepare working solution mixing 1 µL of QubitTM HS dsDNA reagent with 199 µL of sterile PCR grade water per sample. Consider adding a surplus of 3 - 5 reactions for negative control, and unexpected sample measurement repetition.
- If the fluorometer requires calibration (10 uL of each standard with 190 uL of working solution), proceed with the Qubit4TM instructions.
- Combine 197 µL of working solution with 3 µL of purified DNA sample in new 0.5 mL tubes.
- Label and vortex all tubes for 2-3 sec at low speed (bubbles must be avoided).
- Incubate the tubes for 5 min at room temperature in dark.
- Insert and measure the samples in the Qubit4TM fluorometer.

For this protocol, the average size of the final library is ~630 bp for both 16S and 18S rRNA genes. Calculate DNA concentration in nM based on the size of DNA amplicons using the following formula:

$$\frac{\text{concentration in ng/}\mu\text{L}}{660 \frac{\text{g}}{\text{mol}} \times 630} \times 10^6 = \text{concentration in nM}$$

- After molarity determination, make calculations and dilute the libraries using sterile TE buffer (pH 8.0) to 4 nM in a new sterile 0.2 mL 96-well plate. That new plate can be stored at -20°C in case of halting the preparation process. **Warning: This is the last step where halting can occur, the further procedures must be performed all at once.**
- Aliquot 5 µL of diluted DNA from each library and mix aliquots for pooling libraries in a new sterile 1.5 mL tube. This tube must be kept at 4°C or ice bath at all times.
- For confirmation, measure the concentration of the pooled libraries and estimate concentration. If higher to 4 nM ($\sim 1.6 \text{ ng } \mu\text{L}^{-1}$ DNA), it requires adjustment.

Library denaturation and MiSeq sample loading

6 *One day before sequencing, Illumina MiSeqTM reagent cartridge must be removed from -20°C and stored at 4°C for thawing. All reagents must be completely thawed at sequencing time. Gently invert the cartridge 10 times to assure reagent mixing and store at 4°C until the end of the process. In addition, several steps must be performed prior to sequence in order to assure proper sequencing yield and quality. Pooled libraries must be diluted, combined with Illumina PhiXTM (Illumina Inc.) (minimum 5%, recommended 15-30%) and denatured prior to their loading into the MiSeqTM cartridge. The inclusion of PhiXTM is mandatory as it serves for internal calibration as the 16S and 18S rRNA libraries present low diversity. Its inclusion also serves as a tool for Illumina Inc. troubleshooting in case of sequencing errors. If no PhiXTM is available, it can be replaced with a draft genome, however the troubleshooting support of Illumina Inc. is not guaranteed. In this protocol, it is standardized that 8 pM libraries with 25% PhiXTM are optimal for proper cluster density and sequencing yield (Cluster density: 700 – 800; % $\geq$ Q30 = $\sim$ 90%). This protocol also assumes that the pre-run and maintenance washing of the MiSeqTM machine are already performed. If not, refer to the software instructions.*

Dilution and denaturation

- Set a heat block suitable for 1.5 mL microcentrifuge tubes to 96°C.
- Place HT1 Hybridization buffer on ice.
- Dilute molecular grade NaOH from 1N to 0.2 N in a new tube (20 µL NaOH + 80 µL PCR grade water).
- Dilute PhiXTM from 10 nM stock to 4 nM in a new tube (2 µL PhiXTM + 3 µL TE Buffer pH 8.5).
- Mix 16 µL of the pooled libraries with 4 µL of PhiXTM in a new tube labelled “1”.
- Mix 10 µL of tube “1” with 10 µL of 0.2 N NaOH in a new tube labelled “2”.
- Vortex tube “2” for 5 s, spin down it briefly and allow it to incubate at room temperature for 5 min. After incubation, add 980 µL of HT1 Hybridization buffer (at this point, the concentration of the Pool and PhiXTM mix will be 20 pM). **NOTE: strictly follow the timeframe and do not mix with the pipettor, mix by overturning.**
- In a new tube labelled “3”, mix 260 µL of tube “2” with 390 µL of HT1 Hybridization buffer (at this point, the concentration of the Pool and PhiXTM mix will be 8 pM). **NOTE: do not mix with the pipettor, mix by overturning.**
- Incubate tube “3” at 96°C for 2 min and immediately put on ice for a maximum of 2 min. **NOTE: strictly follow the timeframe. Over exposure to heat will degrade the tube “3” content.**
- Remove MiSeqTM v3 cartridge from 4°C refrigerator.

Tube No.	Ingredient
1	16 μ L of the pooled libraries with 4 μ L of PhiX
2	10 μ L of tube "1" with 10 μ L of 0.2 N NaOH +980 μ L of HT1 Hybridization buffer
3	260 μ L of tube "2" with 390 μ L of HT1 Hybridization buffer

Table 1. Tube distribution for libraries denaturation and dilution

Illumina MiSeq™ sequencing

- Set up the samplesheet with each corresponding index i5 and i7 adapters on Illumina Experiment manager software. For easefulness, the samplesheet file can also be generated manually in Microsoft Excel in .csv format. This is recommended only for users with proper knowledge of .csv table files and Illumina Inc. samplesheet file structure. If you do not have experience in doing the former, please use the Illumina Experiment Manager software in the MiSeq Computer. **NOTE: For MiSeq™ v3 kit, this protocol considers reading 301 cycles for both indexes. The inclusion of 1 cycle after the 300 cycles designed by the kit allows the machine to do sample processing, pre-clean, post-clean and assignation.**
- Remove the flowcell from the Illumina MiSeq™ v3 kit box. Discard the container flask and carefully clean the flowcell with sterile molecular grade PCR water using a gentle flow. WARNING: do not pour water on the capillary dots of the flowcell. Gently wipe with non-fibrous paper (e.g. lens paper, not Kimwipe) until the glass is translucent.
- Load all the content (650 μ L) of the tube "3" into the MiSeq™ cartridge.
- In the MiSeq™ software, select sequencing and follow the instructions for the samplesheet, flowcell, incorporation buffer and cartridge insertion.
- Once finished the preparation protocol, sequence can start. This process will take around 3 days.
- The data will be automatically uploaded to Illumina™ Basespace platform. In case of errors during the creation of the samplesheet, corrections and demultiplexing can be done. Avoid at all costs to delete runs with mistakes during indexing, as those can be fixed easily.
- Raw data (sequencing pictures + first multiplexing) will be contained in two folders in MiSeq™ computer. MiSeq_Analysis folder will contain fastq files without .bcl files and pictures. **MiSeq_output will contain .bcl files and .jpeg pictures that can be used for troubleshooting, thus, this folder must be copied for permanent backup.**