16S Amplicon Library Generation for Reef Corals

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Abstract

Stony corals associate with a broad array of microbial partners that can influence the health, performance and survival of tropical reef ecosystems. While current research is largely focused on the nutritional benefit of microbial Eukaryotes (i.e., *Symbiodinium sp.*), less is known about the role of bacteria and Archaea partners in this system. This protocol describes the methods used to extract coral-holobiont gDNA and generate 16S amplicon libraries in preparation for MiSeq Illumina sequencing.

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Guidelines

This protocol contains the necessary steps to generate 16S V4 MiSeq amplicon libraries from reef-buildling corals.

Work Flow

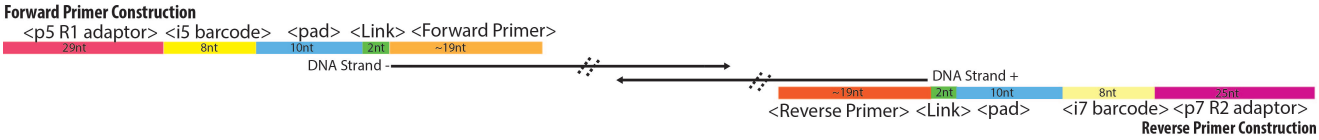
1. Isolate DNA using MoBio PowerSoil DNA Extraction Kit (Cat # 12888)
2. 16S V4 library generation
3. 16S library clean up and normalization using CharmBiotech Just-A-Plate normalization plates, and product pooling
4. Size select library

This protocol takes advantage of duel index sequencing (following Kozich et al. 2013 Appl Environ Microbiol. 79:5112-20) to allow multiplexing of many samples at lower costs. We recommend multiplexing up to 384 samples, which would generate approximately 26,000 paired-end sequences/sample.

The duel index primers are constructed as follows:

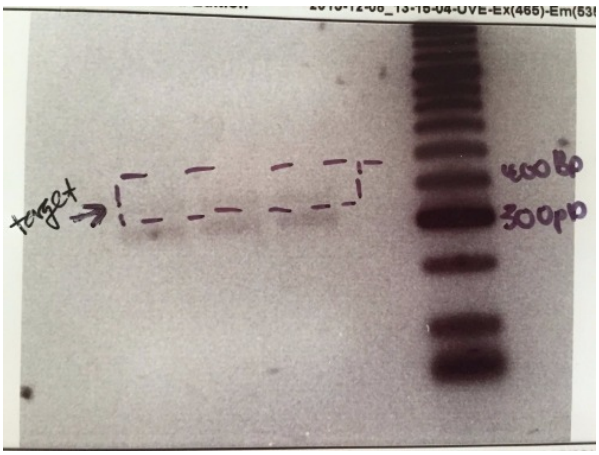
The Illumina R1 adaptor is attached to a barcode pad and linker sequence, which then attaches to the forward primer. All forward primers use the barcodes 501-508. The reverse primers are attached to links, primer pad, reverse barcodes and the reverse i7 illumina adaptor. The reverse primers use the barcodes 701-712.

Figure 1. Primer construction adapted from Kozich et al. 2013.



At the end of the protocol, a last section is added to size select the PCR library. This was necessary because the V4 primers used also amplify coral DNA. Consequently, to maximize the characterization of the microbial community, it is necessary to select the PCR band of interest. See figure 2 for a gel image of duel band PCR library.

Figure 2. Gel image showing the bacteria 16S sequence band labeled *target* and the coral mitochondrial band.



Before start

While extracting coral DNA and preparing sequencing libraries, always use clean filter tips and gloves. Clean all surfaces before beginning to ensure removal of contaminants. To do this wipe down all surfaces (e.g., bench, pipettes, etc) sequentially with a combination of (1) simple green, (2) bleach, (3) ethanol and (4) DNAse Away.

Materials

Ethyl alcohol, Pure 200 proof, for molecular biology E7023 by Sigma Aldrich

PowerSoil DNA Isolation Kit 12888-100 by Mobio

Q5 Hot Start High-Fidelity 2X Master Mix - 500 rxns M0494L by New England Biolabs

Just-a-Plate JN-120-2 by Charm Biotech

AccuClear Ultra high Sensitivity dsDNA quantification 31029 by Biotium

Hyclone Water by Contributed by users

Duel Index PCR Primers by Fisher Scientific

Qiaquick gel extraction kit 28704 by Qiagen

Protocol

Step 1.
DNA EXTRACTION
(modified from the MoBio **PowerSoil Kit)**

For each sample label (1) BeadBeating tube, (4) 2 mL collection tubes and (1) SpinFilter

Extract up to 24 samples at once, randomize the samples extracted across the experiment design. Finally, extract sample blanks to test for contamination.

Step 2.
Add coral samples (< 1 cm^2 biopsies) to PowerBead Tubes

The PowerBead tubes are beating the coral samples to help extract the DNA from the tissues and break apart cell walls. Furthermore, the bead tubes have a solution that is protecting the DNA from degradation.

Step 3.
Make sure C1 solution is dissolved (heat solution if not dissolved), add 60 µl of Solution C1

(This solution contains SDS needed for cell lysis)

Step 4.
Secure PowerSoil tubes in FastPrep Bead Beater. Beat bead tubes at setting 6 for 30 s

(Modified from MoBio protocol, helps to facilitate bead beating and lysis tissues)

Step 5.
Secure PowerBead tubes to MoBio Vortex Adaptor, vortex at max speed for 10 minutes

DURATION
00:10:00

Step 6.
Centrifuge tubes at 10,000 g (no more or tubes may break) for 30 seconds at room temperature. Make sure tubes can spin freely in the centrifuge.

Step 7.
Transfer supernatant to a clean pre-labeled 2 mL collection tube (expect bewteen 400 µL and 500 µL of supernatant)

Step 8.
Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes

(Solution C2 is an inhibitor removal reagent)

DURATION

00:05:00

Step 9.

Centrifuge tubes at room temperature for 1 minute at 10,000 g

Step 10.

Avoiding the pellet, transfer up to 600 µL of supernatant to a clean, pre-labeled 2 ml collection tube

(the pellet does not contain DNA)

Step 11.

Add 200 µL of solution C3 and vortex briefly. Incubate at 4°C for 5 minutes

(Solution C3 is an inhibitor removal reagent)

DURATION

00:05:00

Step 12.

Centrigue the tubes at room temperature for 1 minute at 10,000 g

Step 13.

Avoiding the pellet, transfer up to 600 µL of supernatant to a clean, pre-labled 2 ml collection tube.

(Pellet does not contain DNA)

Step 14.

Centrifuge tubes at room temperature for 1 minute at 10,000 g

Step 15.

Transfer 750 µL of supernatant to a clean, pre-labeled 2 ml collection tube

(Pellet does not contain DNA)

Step 16.

Shake to mix the Solution C4, add 1.2 mL of solution C4 to supernatant and vortex for 5 seconds.

(C4 is a high salt concentrated solution to help bind the DNA to the spin filter)

Step 17.

Load approximately 675 µL of the supernatant and solution onto a spin filter and centrifuge at 10,000 g for 1 minute at room temperature.

Discard flow through and add an additional 675 µL of supernatant to the spin filter, centrifuge at 10,000 g for 1 minute.

Load remaining supernatant on to the spin filter and centrifuge at 10,000 g for 1 minute, discard the flow through.

The DNA should now be bound to the spin filter and the contaminants should be passed through the filter and discarded.

Step 18.

Add 500 µL of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 g. Discard the flow through into an appropriate waste container (for ethanol)

This is a wash step to clean the DNA that is bound to the membrane.

Step 19.
Centrifuge the filter and 2 ml collection tube for 1 minute at 10,000 g.

This spin removes residual Solution C5, which is important because ethanol can inhibit PCR and other downstream applications

Step 20.
Carefully place spin filter into a new pre-labeled clean 2 ml collection tube.

Do not splash Solution C5 onto the filter!!!

Step 21.
Add 100 µl of Solution C6 to the center of the filter and centrifuge at room temperature for 30 seconds at 10,000 g.

Solution C6 is a sterile elution buffer that will release the DNA from the spin filter membrane. Therefore, after the spin the DNA is now in solution in the 2 ml tube. You can also elute your DNA using water or TE buffer

Step 22.
Discard the spin filter and store DNA at -20°C until further processing.

When ready proceed to next step. This is a potential stopping point in the protocol.

Step 23.
PCR AMPLIFICATION
for generation of 16s libraries

This protocol assumes 96 or more samples are being processed at the same time using PCR plates. Adjust accordingly if this is not the case.

Step 24.
Array extracted DNA into 96 well plates according to a detailed plate map design. The plate map should indicate the sample ID and barcodes being used. This is essential because it is needed to bioinformatically determine which sequences belongs to which samples after receiving data from the facility. Make sure to randomize the DNA samples across the plates.

Example Plate Map (only showing 1/4 of entire plate)

	1	2	3	4	5	6
A	SA501/SA701 Sample 010	SA501/SA702 Sample 020	SA501/SA703 Sample 045	SA501/SA704 Sample 007	SA501/SA705 Sample 087	SA501/SA706 Sample 055
B	SA502/SA701 Sample 013	SA502/SA702 Sample 100	SA502/SA703 Sample 067	SA502/SA704 Sample 037	SA502/SA705 Sample 068	SA502/SA706 Sample 027
C	SA503/SA701 Sample 001	SA503/SA702 Sample 080	SA503/SA703 Sample 087	SA503/SA704 Sample 051	SA503/SA705 Sample 062	SA503/SA706 Sample 005
D	SA504/SA701 Sample 002	SA504/SA702 Sample 081	SA504/SA703 Sample 090	SA504/SA704 Sample 056	SA504/SA705 Sample 073	SA541/SA706 Sample 059

Note this map has the forward barcodes SA501 to SA508 and the reverse barcodes SA701-SA712

Store DNA template plates at -20°C.

Step 25.
Reconstitute primers to 100 µM in original primer tubes in Hyclone water. After allowing primers to sit overnight at 4°C, store at -20°C.

Step 26.
Dilute a working stock of the 100 µM primers to 10 mM using HyClone water in a 2 mL collection tube. Make sure to dilute enough primer to array across primer plates. Depending how many samples are going to be multiplex, you may need to make several plates. It is important each primer combo is only used once across the entire study, therefore primer plates should be designed carefully. For instance the barcodes of each plate should be used as follows:

Plate 1: i5 Forward codes -- SA 501- SA508; i7 Reverse codes -- SA 701-SA712
Plate 2: i5 Forward codes -- SA 501- SA508; i7 Reverse codes -- SB 701-SA712
Plate 3: i5 Forward codes -- SB 501- SA508; i7 Reverse codes -- SB 701-SA712
Plate 4: i5 Forward codes -- SB 501- SA508; i7 Reverse codes -- SA 701-SA712

The forwad codes should be arrayed across the ROWS (i.e., A-H) of the primer plates, while the reverse codes should be arrayed across the COLUMNS (i.e., 1-12) of the primer plates.

Using the working stock and working carefully to avoid cross-contaminating PCR primers, array 20 µL of each 10 mM primer into a 96 well PCR plate based on a detailed primer map that corresponds to the DNA template map (see example above). After adding the forward and reverse primers, mix the contents of each well using a pipet tip. The primer plates should be stored at -20°C

Warning: it is essential to avoid contaminating the primer stocks. Make sure to use individual pipet tips when arraying and mixing the primers.

Step 27.

PCR Amplifications

Defrost PCR reagents (NEB Q5 HiFidelity Master Mix), primers and DNA samples and label PCR plate (e.g., PCR plate I).

To minimize basis introduced during PCR reactions, run PCR reactions in triplicate, so for each PCR plate run three plates using the same chemistry. If able, run PCR plates at the same time.

Step 28.
In a 2 ml collection tubes, mix together 12.5 µL of the NEB Q5 HiFidelity Master Mix and 8.5 µL of HyClone water for each 25 µl PCR reaction. Vortex.

Step 29.
Using a multi-channel pipet, array 1.5 µl of each primer pair into the PCR plate according to the plate map

Step 30.
Using a multi-channel pipet, array 21 µl of the master mix into each well of the PCR plate

Step 31.
Using a multi-channel pipett, array 2.5 µl of each sample DNA into the PCR plate according to the plate map. Make sure one sample corresponds to one well of the plate.

**Note: DNA amount can be altered, but you will need to adjsut the amount of water added in step 28 and the amount of master mix added in step 30 to ensure each PCR reaction is 25 µl in volume.

Step 32.
Seal PCR plate using a PCR plate cover and vortex PCR plate to mix contents. Spin PCR plate to ensure solution is at the bottom of each well.

Step 33.
Plate PCR plate into thrermocycler. Set up and run following protocol:
Select HotStart for Taq at 98°C
Step 1: (initial denaturation) 98°C, 30 seconds
Step 2: 30 Cycles of (denaturation) 98°C 10 seconds,
(primer annealing) 55°C 30 seconds,
(extentsion) 72°C 30 seconds
Step 3: (extension) 72°C 2 minutes
Step 4: 4°C Hold

Step 34.

Check random sub-set of PCR products by running them out on a 1.5% Agarose gel and visualizing the gel in a gel box to check for PCR bands. Store PCR products at 4°C

Briefly, to make a 1.5 % agarose gel, dissolve 1.5 g of aragose in 100 ml of TBE buffer by heating solution in microwave. Add 10 µl of sybrsafe dye to solution and poor into gel tray with comb. On a piece of parafilm, mix 2 µl of 10X loading buffer with 5-10 µl of PCR product. Load mixture onto gel. Load 5-10 µl of a 50 bp ladder onto gel alongside PCR products. Run gel at 100 V for 30-40 minutes. Visualize using a gel imaging system to check for PCR bands between 300 and 400 bp.

This is a potential stopping point in protocol. After all PCR reactions have been completed you can move forward to cleaning and normalizing your PCR libraries.

If you ran your PCR libraries in triplicate, pool replicate sample PCR products

Step 35.
PCR PURIFICATION AND NORMALIZATION
Using Charm Biotech, Just-a-Plate 96 PCR purification and normalization plates
Following the manufacture's protocol

Before starting, to prepare washing buffer (WB2), add 19.2 ml of 100 % Ethanol and mix well. Store at room temperature.

Make sure to have plenty of pipet tips on hand, kimwipes or absorbent paper, multichannel pipet and sterile multichannel liquid well.

Step 36.
Bind PCR products to plate wells

Using a multichannel pipet, transfer 20 µl of each PCR product to normalization plate and place into corresponding wells.

1. Mix binding buffer in bottle, using a multichannel pipet add 20 µl of the binding buffer into each well.
2. Mix with PCR products by pipetting up and down 5-6 times.
3. Use separate pipet tip for each well. Seal the plate and incubate at room temperature for 30-60 minutes.
4. Remove the plate cover
5. Aspirate the liquid from the plate by quickly flipping the plate upside down over a waster container and shaking vigorously. Then flip the plate onto a clean absorbent paper (i.e., kimwipe) and tap three or four times to remove liquid.
6. Move onto the washing step

Your PCR products should now be bound to the walls of the normalization plate

Step 37.
Wash PCR Products

1. Add 50 µl of wash buffer to each well. Mix solution by pipetting up and down 1-2 times. incubate plate for 30 seconds at room temperature
2. Aspirate wash buffer from the binding plate as done above for the binding buffer
3. Repeat these stesp once for a total of two washes
4. Air dry normalization plate in an open thermocycler set at 65°C until dry (2-4 minutes)
5. Proceed to elution step

Step 38.
Eluting PCR Products

1. Add 20-40 µl of an elution buffer (such as the PowerSoil elution buffer Solution C6 used during DNA extraction) into each well of the plate using a multichannel pipet.
2. Seal plate with a new adhesive cover and cortex for 30 seconds. Centrifuge the plate to collect all liquid at the bottom of the wells.
3. Store cleaned and normalized products at 4°C for short term storage or -20°C for longer storage. This is a potential stopping point.

Step 39.
OPTIONAL: Quantify cleaned and normalized PCR products following the AccuClear dsDNA quantification kit manufacture instructions.

Step 40.
Pool PCR Products
Using a multichannel pipet, pool PCR products by adding equal volumes of each pcr plate well to a sterile multichannel well. Transfer pooled library to a 2 mL tube.

Step 41.
Purify pooled library using AMPure XP PCR purification beads

This step is added to help remove primer dimers from pooled library

Step 42.
Make sure beads are at room temperature, shake beads to re-suspend any settled particles

Step 43.
Add beads at a 0.8 ratio (0.8 X) to pooled library. For instance, add 500 µL of the library to 400 µL of the beads.

Step 44.
Incubate at room temperature for 5 minutes

DURATION
00:05:00

Step 45.
Place tube on magnetic stand for 2 minutes, wait until the solution is clear

DURATION
00:02:00

Step 46.
Aspirate the clear solution with care to not scrape the beads.

Step 47.
Add 900 µL of 70% ethanol to the tube

Step 48.
Incubate at room temperature for 30 seconds

DURATION
00:00:30

Step 49.
Aspirate the ethanol

Step 50.
Preform a second ethanol wash by repeating steps 47-49.

Step 51.
Dry at room temperature for 15 mins or until ethanol is no longer visible

Step 52.
Remove tube from the magnet holder

Step 53.
Add 200 µl of TE buffer to elute the DNA from the beads. If a higher concentration is desired, add less TE buffer. Pipet buffer up and down ten times to thoroughly mix.

Step 54.
Place tube on magnet for 1 minute

Step 55.
Transfer eluent to a new pre-labled tube and submit to sequencing facility for QC and analysis.

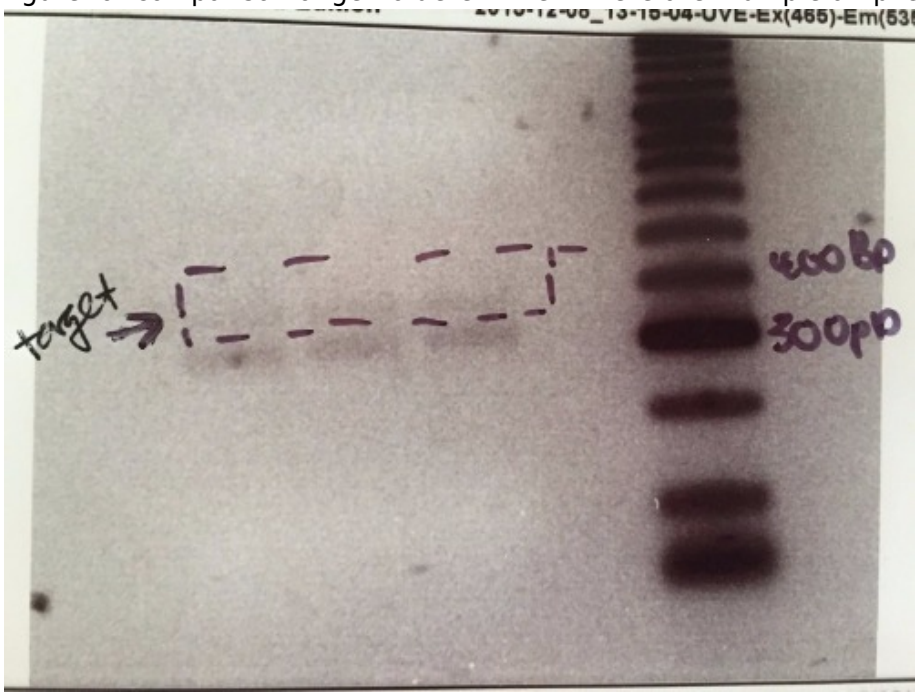
Step 56.
Size select PCR library
Using the QIAquick gel extraction kit, cat # 28704

If the bioanalizer trace from the sequencing facility indicates there have multiple peaks not corresponding to the target amplicon sequence, it may be necessary to size select the library. This is often the case with obtaining 16s amplicon sequences from corals.

Step 57.
Run 75 µL of the pooled library on a 2% agarose gel for appoximately 1 hour. Make sure to include a ladder.

DURATION
01:00:00

Step 58.
Excise the target amplicon band from gel with a razor blade and place into a clean 15 mL tube. See figure for comparison of gel to determine if there are multiple amplicons in library.



Step 59.
Obtain gel weight. 1 g of gel is equal to 1 mL in volume. Add 3 volumes of buffer QG for 1 volumn of gel.

Step 60.
Incubate at 50°C for 10 minutes or until the gel is fully dissolved. Mix by vortexing every 2-3 minutes to help dissolve the gel.

Step 61.
After the gel has dissolved, check the color of the gel. If it is orange or violet, at 10 µL of the 3 M sodium acetate and mix.

Step 62.
Add 1 gel volume of isopropanol to the sample and mix

Step 63.
Place spin column in the provided 2 mL collection tube.

Step 64.
To bind the DNA, apply the sample to the column and centrifuge for 1 min at 10,000 rpm. Discard the flow through. The volume of the collection tube only holds 800 µL, so if the sample is more then 800 µL spin the sample through the volumn in 800 µL batches to bind all of the DNA to the column.

Step 65.
Add 0.5 mL of buffer QG to the column and centrifuge for 1 min at 10,000 rpm.

Step 66.
To wash the sample, add 0.75 mL of Buffer PE to the column and centrifuge for 1 min at 10,000 rpm.

Discard the flow through.

Step 67.
Centrifuge the column for 1 additional minute at 13,000 rpm to ensure there is no residual Buffer PE.

Step 68.
Place the column in a new 2 mL micro-centrifuge tube.

Step 69.
To elute the DNA, add 25 µL of buffer EB to the center of the QIAquick membrane and centrifuge for 1 min at 13,000 rpm.

Step 70.
Submit the library to the sequencing facility.