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PCR amplification of 16S rRNA, 18S rRNA, and *nifH* genes in coral samples

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bi9ukh6w

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MATERIALS TEXT

MATERIALS

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

Thermocycler **Contributed by users**

Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #BP2818500**

70% Ethanol **Contributed by users**

BSA, molecular biology grade, 20 mg/ml **New England Biolabs Catalog # B9000S**

DMSO **Sigma Catalog #D8418**

nuclease free water **Contributed by users**

MgCl₂ (magnesium chloride) (25 mM) **Thermo Fisher Catalog #R0971**

10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach) **Contributed by users**

HotStar Taq Plus Master Mix **Qiagen Catalog #203643**

Magnetic Stand **Contributed by users**

sterile filter tips **Contributed by users**

FrameStar-96 96-WELL SEMI-SKIRTED PCR **4titude Catalog #4ti-0770/C**

STRONG TRANSPARENT ADHESIVE SEALING FILM FOR PCR **4titude Catalog #4ti-0500**

Corning reagent reservoirs sterile **Contributed by users Catalog #4870**

2mL PCR clean microcentrifuge tubes **Contributed by users**

Spin down or centrifuge adapter for 96 well plate;
UV Hood;
Pipettes & multichannel pipette (for 96 well plate)

Primers

1

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A	B	C	D
Gene	**Primer**	**Sequence**	**Reference**
16S rRNA	B969F	5'-ACGCGHNRAACCTTACC-3'	Comeau 2011
16S rRNA	BA1406R	5'-ACGGGCRGTGWGTRCAA-3'	Comeau 2011
18S rRNA	UNonMetF	5'-GTGCCAGCAGCCGCG-3'	Bower 2004
18S rRNA	UNonMetR	5'-TTTAAGTTTCAGCCTTGCG-3'	Bower 2004
18S rRNA	V4 18S For	5'-CCAGCASCYGCGGTAATTCC-3'	Piredda 2017
18S rRNA	V4 18S Rev	5'-ACTTTCGTTCTTGATYRATGA-3'	Piredda 2017
nifH	IGK3-F	5'-GCIWTHTAYGGIAARGGIGGIATHGGIAA-3'	Gaby & Buckley 2012
nifH	DVV-R	5'-ATIGCRAAICCICCRCAIACIACRTC-3'	Gaby & Buckley 2012

Primer sequences used in this protocol. Overhang sequences were specified by the sequencing facility and appended to the 5' end of respective primers. Note that overhang adapters are not needed for the UNonMet primer set.

References:

Comeau AM, Li WK, Tremblay JÉ, Carmack EC, Lovejoy C. Arctic Ocean microbial community structure before and after the 2007 record sea ice minimum. *PLoS ONE*. 2011;6(11):e27492.

Bower SM, Carnegie RB, Goh B, Jones SR, Lowe GJ, Mak MW. Preferential PCR amplification of parasitic protistan small subunit rDNA from metazoan tissues. *J Eukaryot Microbiol.* 2004;51(3):325-332.

Piredda R, Tomasino MP, D'Erchia AM, Manzari C, Pesole G, Montresor M, Kooistra WHCF, Sarno D, Zingone A. Diversity and temporal patterns of planktonic protist assemblages at a Mediterranean Long Term Ecological Research site, *FEMS Microbiology Ecology*, 2017; 93(1):1-14.

Gaby JC, Buckley DH. A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase. *PLoS ONE*, 2012;7(7): e42149.

Set up and tips

2 Clean all surfaces and pipettes with 10% bleach and 70% ethanol.

Use a separate set of pipettes for preparing the master mix and for loading the template. Ideally, one set stays in UV hood and the other set on the bench.

3 Use filter pipette tips to avoid contamination of your reagents and contamination between samples.

4 Place PCR clean microcentrifuge tubes (for making master mix), PCR plate or tubes, and PCR lids or seals in UV hood, along with pipettes and pipette tips to be used in preparing the master mix.

Turn UV light on for 15 minutes.

5 While UV hood light is on, take out reagents from freezer and thaw. Mix and spin down after thawed.

6 ⚠

Only open PCR reagents (Polymerase, BSA, $MgCl_2$, DMSO, nuclease free water, primers, etc.) in clean UV hood. Always use filter tips when working with these reagents. If possible, work with aliquots.

7 ⚠

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NEVER bring DNA template into UV hood used to make master mix.

8 If primers have not been diluted, aliquot and dilute to 10µM, in clean & sterilized UV hood.

For primers at 100µM, add 10µl of primer to 90µl of nuclease free water.

16S

9 In UV hood, prepare the following master mix in a PCR clean microcentrifuge tube.

For each sample, perform PCR in duplicate reactions (20µl volume). Include *at least* 1x negative control and 1x positive control.

Always prepare several extra reactions, as some solution will be lost during pipetting. If using 96-well plate and multichannel pipette reservoir, prepare at least 5 extra reactions.

For N samples, multiply the following volumes by X, where

X = 2xN + (# negative controls) + (# positive controls) + (# spare reactions)

- **10 µl HotStarTaq Master Mix**
- **0.8 µl 10mM B696F** (0.4mM final concentration)
- **0.8 µl 10mM BA1406R** (0.4mM final concentration)
- **0.4 µl 25mM MgCl2** (2mM final concentration)
- **1 µl DMSO** (5% v/v final concentration)

+ DNA template (10-15ng in 20µl reaction)
+ nuclease free water to a **final volume of 20µl**

10 Dispense master mix from Step 9 into PCR wells inside UV hood.

Add nuclease free water to negative controls inside UV hood.

Outside of UV hood, add DNA template and positive controls to PCR wells.

Spin down PCR plate/tubes after all wells have been loaded and sealed.

11 Perform PCR in thermocycler using the following profile:

A	B	C	D
Temperature (°C)	**Time**	**Cycles**	
95	5 min	1x	Initial Denaturation
94	1 min	30x	Denaturation
52	1 min	30x	Primer Annealing
72	1 min	30x	Extension
72	10 min	1x	Final Extension
4	∞	1x	Hold

Thermocycler settings for 16S rRNA (B969 & BA1406) primers.

12 Pool duplicate samples. Perform gel electrophoresis to check results of PCR.

Clean PCR products (if required by sequencing facility). See clean-up protocol below.

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UNonMet & 18S

13 For coral (or other metazoan) samples, perform **nested PCR** with UNonMet primers followed by V4 18S primers. A clean-up step is necessary between the two PCR reactions.

14 In UV hood, prepare the following master mix in a PCR clean microcentrifuge tube.

For each sample, perform PCR in duplicate 10µl reactions. Include *at least* 1x negative control and 1x positive control.

Always prepare several extra reactions, as some solution will be lost during pipetting. If using 96-well plate and multichannel pipette reservoir, prepare at least 5 extra reactions.

For N samples, multiply the following volumes by X, where

X = 2xN + (# negative controls) + (# positive controls) + (# spare reactions)

5 µl HotStarTaq Master Mix

0.4 µl 10mM UNonMet F (0.4mM final concentration)

0.4 µl 10mM UNonMet R (0.4mM final concentration)

+ DNA template (5-10ng)
+ nuclease free water to a **final volume of 10µl**

15 Dispense master mix from Step 14 into PCR wells inside UV hood.

Add nuclease free water to negative controls inside UV hood.

Outside of UV hood, add DNA template and positive controls to PCR wells.

Spin down PCR plate/tubes after all wells have been loaded and sealed.

16 Perform PCR in thermocycler using the following profile:

Temperature (°C)	Time	Cycles	
95	5 min	1x	Initial Denaturation
94	1 min	35x	Denaturation
51.1	1 min	35x	Primer Annealing
72	1 min	35x	Extension
72	10 min	1x	Final Extension
4	∞	1x	Hold

Thermocycler settings for UNonMet primers (UNonMet-F & UNonMet-R).

17 ⚠

Clean UNonMet PCR products with AMPure XP beads to remove primers.

If the sample is not cleaned, primers from the first round will continue to amplify in the second PCR and there will be two bands (~600bp & ~400 bp). See AMPure XP beads clean-up below.

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18 In UV hood, prepare the following master mix in a PCR clean microcentrifuge tube.

Include *at least* 1x 18S negative control, 1x 18S positive control, 1x negative control from UNonMet PCR, and 1x positive control from UNonMet PCR. (Do not pool duplicates from UNonMet PCR, keep them as duplicates for 18S PCR).

For N reactions from the UNonMet PCR, multiply the following volumes by X, where

X = N + (# 18S negative controls) + (# 18S positive controls) + (# spare reactions)

10 µl HotStarTaq Master Mix

0.8 µl 10mM V4 18S For (0.4mM final concentration)

0.8 µl 10mM V4 18S Rev (0.4mM final concentration)

2ul of cleaned UNonMet PCR product
+ nuclease free water to a **final volume of 20µl**

19 Dispense master mix from Step 18 into PCR wells inside UV hood.

Add nuclease free water to 18S negative controls inside UV hood.

Outside of UV hood, add cleaned PCR products from UNonMet PCR (including UNonMet positive & negative controls), and 18S positive controls.

Spin down PCR plate/tubes after all wells have been loaded and sealed.

20 Perform PCR in thermocycler using the following profile:

Temperature (°C)	Time	Cycles	
95	5 min	1x	Initial Denaturation
94	1 min	25x	Denaturation
52	1 min	25x	Primer Annealing
72	1 min	25x	Extension
72	10 min	1x	Final Extension
4	∞	1x	Hold

Thermocycler settings for 18S rRNA primers (V4 18S For & V4 18S Rev).

21 Pool duplicate samples. Perform gel electrophoresis to check results of PCR.

Clean PCR products (if required by sequencing facility). See clean-up protocol below.

nifH

22 In UV hood, prepare the following master mix in a PCR clean microcentrifuge tube.

For each sample, perform PCR in duplicate 20µl reactions. Include *at least* 1x negative control and 1x positive control.

Always prepare several extra reactions, as some solution will be lost during pipetting. If using 96-well plate and multichannel pipette reservoir, prepare at least 5 extra reactions.

For N samples, multiply the following volumes by X, where

X = 2xN + (# negative controls) + (# positive controls) + (# spare reactions)

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10 µl HotStarTaq Master Mix

0.8 µl 10mM IGK3 (0.4mM final concentration)

0.8 µl 10mM DVV (0.4mM final concentration)

2 µl 25mM MgCl2 (4mM final concentration)

1 µl BSA (2ug/ul)

+ DNA template (aim for ~20ng in 20µl reaction)
+ nuclease free water to a **final volume of 20µl**

Note: Higher template concentration improved amplification of the nifH gene in coral samples. If working with seawater, sediment, etc. template concentration may need to be reduced.

23 Dispense master mix from Step 22 into PCR wells inside UV hood.

Add nuclease free water to negative controls inside UV hood.

Outside of UV hood, add DNA template and positive controls to PCR wells.

Spin down PCR plate/tubes after all wells have been loaded and sealed.

24 Perform PCR in thermocycler using the following profile:

Temperature (°C)	Time	Cycles	
95	5 min	1x	Initial Denaturation
94	1 min	35x	Denaturation
55	1 min	35x	Primer Annealing
72	1 min	35x	Extension
72	10 min	1x	Final Extension
4	∞	1x	Hold

Thermocycler settings for *nifH* primers (IGK3 & DVV) .

25 Pool duplicate samples. Perform gel electrophoresis to check results of PCR.

Clean PCR products (if required by sequencing facility). See clean-up protocol below.

Note: IGK3/DVV primers + the overhang adapters may form dimers. This was observed when using one specific overhang adapter sequence, but not in another. Clean-up with 0.8-0.9x Ampure XP beads can remove these adapter/primer dimers.

AMPure XP Bead Clean Up

26 Bring AMPure XP beads to room temperature.

27 Prepare [M]**70 % (v/v) ethanol** using molecular biology grade ethanol. (Each sample will need **400 µl** of 70% ethanol).

28 Vortex beads for **00:00:30** before use.

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Add AMPure XP beads in desired bead/sample ratio. When adding beads to sample, mix by gently pipetting up and down 10x.

Recommended for nested UNonMet clean-up after 1st PCR: 1.2x bead/sample.

Recommended for nifH IGK3/DVV: 0.9x bead/sample.

29 Incubate for **00:05:00** at **Room temperature** .

30 Place samples on magnetic stand and incubate for **00:02:00** at **Room temperature** or until the supernatant is clear.

31 Leaving samples on the magnetic stand, carefully remove and discard the supernatant without disturbing the beads. Leave some of supernatant behind to avoid disturbing sample.

32 Leaving samples on the magnetic stand, wash beads **2x** with ethanol:

32.1 Add **200 µl 70% Ethanol** to each sample.

32.2 Incubate on magnetic stand for **00:00:30** .

32.3 Remove and discard the supernatant without disturbing beads. Repeat **go to step #32.1**

33 After second wash, use a P20 or P10 pipette to completely remove any remaining ethanol.

34 **Keep samples on the magnetic stand** and allow beads to air dry for 5-10 minutes. The beads will go from shiny to matte. Do not over dry. If left for too long, the beads will appear to crack.

35 Remove samples from the magnetic stand and add **40 µl nuclease free water** to each sample. Resuspend beads by gently pipetting up and down 10x.

Note: Adding less than 40µl will require extra mixing to insure liquid comes into contact with beads.

36 Incubate at **Room temperature** for **00:02:00** .

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37 Place the samples on the magnetic stand for **00:02:00** or until the supernatant has completely cleared.

38 Carefully transfer **40 µl** of the supernatant to a new tube/plate, taking care not to transfer any of the beads.

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