Symbiodinium CTAB DNA Extraction Protocol

Revised from Mieog 2009 and http://ccb.ucr.edu/lab_protocols.html (Updated 7.19.17 R.Eckert)

Supplies	Reagents	Equipment
48 2 mL tubes (2 sets of 24)	2X CTAB Extraction Buffer	4 ºC centrifuge
24 scalpel or razor blades	Proteinase K (20 mg/mL)	Bead homogenizer
Parafilm	Chloroform:Isoamyl Alcohol (24:1)	Thermomixer
0.5 mm glass beads	Isopropanol @ -20 ºC	Nanodrop
Kimwipes	70% Ethanol	
Zymo DCC Kit	1X TE Buffer pH 8.0	

1. Prepare CTAB extraction buffer just prior to use.
2. Scrape tissue from coral fragment and place into a 2 mL tube with 0.1 mL (~ 0.075 g) of 0.5 mm glass beads.
3. Add 800 μL CTAB extraction buffer.
4. Add 0.8 μL Proteinase K. Seal tubes with parafilm. Invert to mix.
5. Bead beat for 2–3 min (6M/s, three 45 sec intervals w/ 2 min cool down between).
6. Incubate at 60 ºC 1.5 hrs while mixing.
7. Add 800 μL Chloroform:Isoamyl Alcohol (24:1). Invert to mix.
8. Mix and centrifuge at 20,000 x g for 15 min at 4 ºC.
9. Transfer aqueous phase to new tube (600 μL then 150 μL), taking care not to disturb interphase layer.
10. Add 800 μL cold (-20 ºC) Isopropanol.
11. Mix and incubate for 20 min at -20 ºC
12. Centrifuge at 20,000 x g for 20 min at 4 ºC.
13. Carefully pour off supernatant.
14. Add 150 μL of 70% Ethanol at room temperature. Invert to mix.
15. Centrifuge at 20,000 x g for 5 min at 4 ºC.
16. Remove supernatant (pour off, quick spin, pipette off remaining avoiding pellet) and dry inverted on Kimwipe for 15 min at room temperature.
17. Elute in 100–200 μL of 1X TE pH 8.0.
18. Incubate at 55 ºC for 10 min.

2X CTAB Extraction Buffer	20 mL	Target
CTAB	0.4 g	2%
1M Tris-HCl (pH 8.0)	2 mL	100mM
0.5M EDTA (pH 8.0)	800 μL	20mM
5M NaCl (add after CTAB dissolves, but before DEPC Treated H_2O)	5.6 mL	1.4M
DEPC Treated H_2O	to 20 mL	
20 mg/mL Proteinase K (Do not add to buffer)	0.8 μL per sample	20 μg/mL

Stock Recipes	100 mL
5M NaCl	29.22 g NaCl + 80 mL DEPC Treated H_2O Add DEPC Treated H_2O to 100 mL
1M Tris-HCl (pH 8.0)	12.11 g Tris + 80 mL DEPC Treated H_2O Adjust pH with HCl and add DEPC Treated H_2O to 100 mL
0.5M EDTA (pH 8.0)	18.61 g EDTA + 80 mL DEPC Treated H_2O Adjust pH with NaOH and add DEPC Treated H_2O to 100 mL
1X TE Buffer (pH8.0)	1 mL 1M Tris-HCl pH 8.0 +200 μL 0.5M EDTA pH 8.0 Add DEPC Treated H_2O to 100 mL

Cleaning genomic DNA with Zymo DNA Clean & Concentrator™-5 Kit

After extracting genomic DNA, Zymo DNA Clean & Concentrator™-5 (D4014) is used to clean DNA and remove inhibitors prior to running a PCR.

1. Nanodrop DNA for concentration. Prepare 0.6 mL tubes with 5 μg (or less) DNA in 100 μL total volume 1X TE to be cleaned.
2. Set Elution Buffer for elution step in heat block at 60–70 °C
3. In a 2 mL tube add 2:1 volume of Binding Buffer:DNA (200 μL) to each volume of genomic DNA and vortex thoroughly.
4. Transfer the mixture to a provided Zymo-Spin Column in a collection tube.
5. Centrifuge 10,000 x g for 30 sec at room temperature. Discard flow through.
6. Add 200 µL DNA Wash Buffer to the column. Centrifuge at 10,000 x g for 1 min at room temperature. Repeat.
7. Transfer the column to a new labeled 1.5 mL tube. Elute by adding 20 μL of Elution Buffer directly to the column matrix and incubate at room temperature for 3–5 min. Centrifuge for 30 seconds to elute DNA.
8. Nanodrop cleaned DNA and prepare 10 ng/μL dilutions.

Symbiodinium ITS2 Amplification

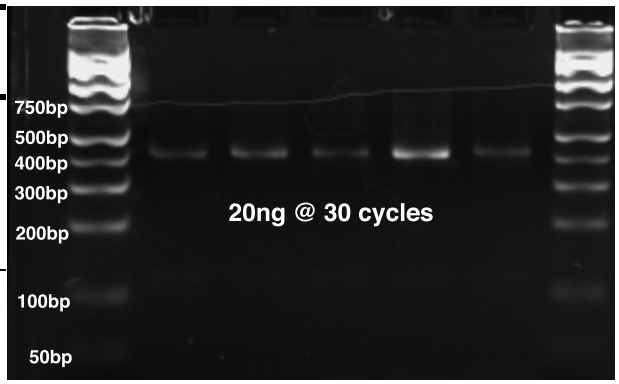
Protocol created by C. Kenkel, last modified by J. Polinski (6/19/2015)

Master Mix Recipe		
Reagent	1X	
RNase free H2O	23.1	μL
10X ExTaq Buffer	3	μL
10 mM dNTP mix	0.7	μL
ITS2-F primer (10μM)	0.5	μL
ITS2-R primer (10μM)	0.5	μL
TaKaRa ExTaq HS	0.2	μL
Total	**28**	**μL/rxn**
	+ 2	μL template (20ng total)

PCR Profile	
95 ºC for 5 min	
95 ºC for 40 sec 65 ºC for 2 min 72 ºC for 1 min	***x* cycles**
72 ºC for 10 min	

1. Amplify samples with the ITS2-linker forward and reverse primers (see ITS2-F-miseq and ITS2-R-miseq under “Primer Sequence Information”) using cycle checks to obtain a faint but distinct band. Avoid over-amplification. To add cycles, place samples back into thermocycler and run for the additional number of cycles (no initial heating or final elongation steps).
2. Visualize on a gel using 3 μL of PCR product. If band is still not visible after checked on 3 gels, redo reaction using appropriate number of cycles.
3. Clean PCR product with GeneJET PCR Purification Kit.

Sodium Borate/EtBr 1.5% Agarose Gel Recipe		
Reagent	300	mL Gel
DI H_2O	285	mL
20X SB Buffer	15	mL
Agarose	4.5	g
Ethidium Bromide	6	μL
Add EtBr just before pouring gel (55ºC)		
Use 3 μL PCR Product and 2 μL loading dye per well Use 3 μL Ladder and 2 μL loading dye		
Run gel at 150V for 15–20 min		



Cleaning PCR product with Thermo Scientific GeneJET PCR Purification Kit

Prior to first use add ethanol to Wash Buffer and label bottle.

1. Add 1:1 volume of Binding Buffer:PCR product (27 μL). Mix thoroughly. Check color of solution after adding Binding Buffer. Yellow indicates optimal pH for DNA binding. If orange or violet, add 10 μL of 3M sodium acetate (pH 5.2) and mix.
2. Transfer reaction mixture/binding buffer solution to GeneJET purification column.
3. Centrifuge at 12,000 x g for 1 min at room temperature. Discard flow-through.
4. Add 700 μL of Wash Buffer to purification column.
5. Centrifuge at 12,500 x g for 1 min at room temperature. Discard flow-through.
6. Centrifuge empty column for an additional 1 minute to completely remove Wash Buffer as residual ethanol may inhibit subsequent reactions.
7. Transfer purification column to 1.5 mL microcentrifuge tube. Add desired volume of Elution Buffer.
    a. 30 μL used for PCR products after adding universal ITS2 primers with linkers.
    b. 40 μL used for PCR products after adding barcode and MiSeq adapter primers.
8. Allow to sit at room temperature for 1 minute, then centrifuge at 13,000 x g for 1 min at room temperature.
9. Nanodrop cleaned sample and dilute with Elution Buffer for a final concentration of 10ng/ μL.

Symbiodinium ITS2 Barcoding Amplification for MiSeq platform NGS

Protocol created by C. Kenkel, last modified by J. Polinski (6/19/2015)

Master Mix Recipe

Reagent	1X	
RNase free H_2O	9.3	μL
10X ExTaq Buffer	2	μL
10 mM dNTP mix	0.5	μL
TaKaRa ExTaq HS	0.2	μL
Total	**12**	**μL/rxn**
	+ 3	μL1μM barcoded F primer
	+ 3	μL1μM barcoded R primer
	+ 2	template (20ng total)

PCR Profile

95 ºC for 5 min	
95 ºC for 40 sec	**6 cycles**
65 ºC for 2 min	
72 ºC for 1 min	
72 ºC for 10 min	

1. Use cleaned and diluted samples in a PCR to incorporate barcoded MiSeq-adapter primers (see “Primer Sequence Information” for information on barcoded primers)
2. Visualize on a gel. Once all samples are run on the same gel, “eyeball” product and decide how much of sample product to pool into final sample.
    a. Start with 5 μL of sample for the brightest bands, then use 10 μL for samples with bands that are half as bright, etc.
3. Clean pooled samples using Thermo Scientific GeneJET PCR Purification Kit. Elute with 40 μL Elution Buffer.
4. Run 20–40 μL of eluted sample on gel. Should get a single band approximately 500 bp.

Sodium Borate/SYBR Green 2% Agarose Gel Recipe		
Reagent	300	mL Gel
DEPC-treated H_2O	285	mL
20X SB Buffer	15	mL
Agarose	6	g
SYBR Green	30	μL
Add SYBR Green just before pouring gel (55 ºC) Use 20–40 μL PCR Product and 10 μL loading dye per well Use 6 μL Ladder and 10 μL loading dye Run gel at 150 V for 20–30 min		

Freeze and Squeeze

1. Excise bands, removing as much of the surrounding agarose as possible, and soak in 10–20 μL of DEPC-treated H_2O overnight at 4 ºC.
2. Remove liquid (containing DNA), perform ethanol precipitation to remove dye, and dissolve in 1xTE. Sample is ready for sequencing.

Be sure to follow preparation/labeling instructions of sequencing facility when sending sample to be analyzed.

Primer Sequence Information

This protocol was developed to reduce sequencing costs by giving each sample a unique "barcode" or "index" sequence so that samples can be pooled and run as a single sample on the MiSeq platform. The ITS2 forward and reverse primer sequences are universal ITS2 sequences (Pochon et al. 2001) that have been modified to include a linker and adapter that any of the barcode/index primers can then bind to, shown below:

Primer name	**Sequence: Adapter + Linker + Universal ITS2 F Primer**
ITS2-F-miseq	**TCG TCG GCA GCG TC**A GAT GTG TAT AAG AGA CAG **GTG AAT TGC AGA ACT CCG TG**
	Sequence: Adapter + Linker + Universal ITS2 R Primer
ITS2-R-miseq	**GTC TCG TGG GCT CGG** AGA TGT GTA TAA GAG ACA G**CC TCC GCT TAC TTA TAT GCT T**

Barcoded primers that also contain the Illumina adapter needed to bind to the flow cell of the MiSeq platform can then be to the amplified products. Dual indexing (placing unique barcode sequences on both the forward and reverse primers is a cost-efficient way to include more samples while purchasing less barcode primers (i.e. 20 forward and 20 reverse barcode primers can label up to 400 unique samples). See below for examples

Primer Name	**Sequence: Illumina Forward Primer + Linker + Barcode + Adapter**
Hyb_F1_i5	**AAT GAT ACG GCG ACC AC**C GAG ATC TAC AC**A GTC AA**T **CGT CGG CAG CGT C**
Hyb_R1_i7	**Sequence: Illumina Reverse Primer + Linker + Barcode + Adapter** **CAA GCA GAA GAC GGC ATA C**GA GAT **AAG CTA GTC TCG TGG GCT CGG**

For more primer and barcode examples:

https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors

https://wikis.utexas.edu/display/GSAF/rRNA+bacterial+gene+and+fungal+ITS+metagenomics+samples