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Symbiodinium spp. genotyping protocol
Last updated: 03/30/09, by Shini

A) SSU (small subunit) rRNA gene RFLP genotyping (after Rowan and Powers, 1991)

B) ITS2 sequencing (after LaJeunesse and Trench 2000)

Overview

Both protocols start with the amplification of the target region by PCR, for which the PCR protocols differ and are described in the following. In case of RFLP analysis, an enzymatic digestion of the amplified SSU rRNA gene (SSU rDNA) is performed. In case of ITS2 sequencing, the amplified ITS2 amplicons are subjected to sequencing.

A) PCR-protocol RFLP

The amount of DNA needed for the PCR reaction is ~20 ng and the PCR is done in a volume of 20 uL using 1 uL of template DNA.

Set up:

	1x (20 uL rxn)*
PCR-grade H_2O	15.6 uL
10 x PCR buffer	2 uL
dNTPs (10 mM)	0.4 uL
Primer SS3Z (10 uM)	0.4 uL
Primer SS5 (10 uM)	0.4 uL
Taq polymerase (5U/uL)**	0.2 uL
Template DNA (~20 ng/uL)***	1 uL

* scale up all reagents (except template DNA) for master mixes

** adjust Taq polymerase and PCR-grade H2O volumes if Taq concentration is different (e.g. use 1 uL of Taq polymerase if concentration is 1U/uL).

*** adjust template and PCR-grade H_2O volumes if needed

Note: if needed (i.e. no stocks are available), Symbiodinium clade standards can be obtained in the same way. Instead of the template DNA, use 1 uL of clade standard template DNA and scale up PCR reactions (e.g. to 100 uL).

Run the following program:

Hot-start: 94°C – 2'

35 cycles: 94°C – 1'

56°C – 1'

72°C – 2'

1 cycle: 72°C – 8'

For ever: 4°C

Check PCR amplicons:

Run out 5 uL of the PCR reaction on a 1% agarose gel. Check for a band at ~1,800 bp.

A) RFLP analysis

During the enzymatic digestion, the amplified SSU rDNA will be cut into fragments of distinct sizes and subsequently compared to digested clade standard amplicons. The total reaction volume is 10 uL.

1. Set up:

	1x (10 uL rxn)*
PCR-grade H_2O	2.5 uL
10 x PCR buffer	1 uL
Taq I restriction enzyme	0.5 uL
PCR product	4 uL

* scale up all reagents (except template DNA) for master mixes. Remember to include clade standards for your calculations.

2. Mix each tube by flicking. Collect liquid at bottom of tubes by a quick spin.

3. Incubate the samples at 65°C for 2 hours.

4. Run out the digested amplicons on a 2% agarose gel. Be sure to run clade standards and a MW ladder.

5. Compare banding pattern of your samples with those of clade standards.

B) Touchdown PCR-protocol ITS2

The amount of DNA needed for the PCR reaction is ~20 ng and the PCR is done in a volume of 20 uL using 1 uL of template DNA.

Set up:

	1x (20 uL rxn)*
PCR-grade H_2O	15.6 uL
10 x PCR buffer	2 uL
dNTPs (10 mM)	0.4 uL
Primer ITS2_fw (10 uM)	0.4 uL
Primer ITS2_rv (10 uM)	0.4 uL
Taq polymerase (5U/uL)**	0.2 uL
Template DNA (~20 ng/uL)***	1 uL

* scale up all reagents (except template DNA) for master mixes

** adjust Taq polymerase and PCR-grade H2O volumes if Taq concentration is different (e.g. use 1 uL of Taq polymerase if concentration is 1U/uL).

*** adjust template and PCR-grade H_2O volumes if needed

Run the following program:

Hot-start:	94°C – 1’
20 cycles:	94°C – 15”
	62-52°C – 30” (-0.5°C every cycle)
	72°C – 30”
20 cycles:	94°C – 15”
	52°C – 30”
	72°C – 30”
1 cycle:	72°C – 8’
For ever:	4°C

Check PCR amplicons:

Run out 5 uL of the PCR reaction on a 1% agarose gel. You should see a band at ~350 bp.

B) Cloning and sequencing

1. Perform a cloning reaction using the TOPO TA Cloning kit for sequencing.

Use manufacturer’s instruction with the exception of setting up 1/3 reactions.

Modifications

a. For the cloning reaction, set up a 2 uL reaction (instead of a 6 uL reaction):

PCR product: 1 uL

Salt: 0.33 uL

H_2O: 0.33 uL

Vector (pCR4): 0.33 uL

b. For the transformation, use 1 uL of the cloning reaction (instead of 2 uL).

c. Split a vial of chemically competent cells (50 uL) into 3 x 16 uL.

d. Add 83 uL of SOC to reach vial (instead of 250 uL)

2. Pick clones and perform a colony PCR.

a. Set up complete PCR reaction mixes

	1x (20 uL rxn)*
PCR-grade H_2O	16.6 uL
10 x PCR buffer	2 uL
dNTPs (10 mM)	0.4 uL
Primer M13_fw (10 uM)	0.4 uL
Primer M13_rv (10 uM)	0.4 uL
Taq polymerase (5U/uL)**	0.2 uL

* scale up all reagents (except template DNA) for master mixes

** adjust Taq polymerase and PCR-grade H2O volumes if Taq concentration is different (e.g. use 1 uL of Taq polymerase if concentration is 1U/uL).

b. Use a P10 pipettor and dip the tip into a colony (try to get as little material as possible). Transfer the tip into a PCR reaction mix, and pipet up and down 5 times to release the cells into the solution. Repeat for each colony you pick.

c. Run the following program:

Hot-start: 94°C – 10’

30 cycles: 94°C – 30”

55°C – 30”

72°C – 30”

1 cycle: 72°C – 10’

For ever: 4°C

d. Clean-up PCR products using PCR cleanup kit or MiniElute kit (or 96 well

plate cleanup plates)

e. Nanodrop to determine concentrations.

3. Set up sequencing reactions.

In-house sequencing (UCM Core facility):

In a 96 well plate, mix 50ng/1000bp of PCR product (note: 50 ng=minimum) with 0.4 pmol primer, and add H_2O to a total volume of 7 uL. Follow instructions at: https://campillos.ucmerced.edu/cgi-bin/genomics/register/login.pl

External sequencing: follow guidelines of provider

4. Send out for sequencing.

Materials

Primer sequences

SS3Z: AGCACTGCGTCAGTCCGAATAATTCACCGG

SS5: GGTTGATCCTGCCAGTAGTCATATGCTTG

ITS2_fw: GAATTGCAGAACTCCGTG

ITS2_rv: GGGATCCATATGCTTAAGTTCAGCGGGT

Cloning kit

Item: TOPO TA Cloning Kit for sequencing (TOPO pCR4 vector, with chemically competent cells)

Company: Invitrogen

Cat. #: K457501 (20 rxns), K457540 (40 rxns)

PCR-cleanup kit

Item: QIAquick PCR purification kit

Company: Qiagen

Cat. #: 28106 (250 rxns)

MiniElute kit

Item: MiniElute Reaction cleanup kit

Company: Qiagen

Cat. #: 28206 (250 rxns)