Symbiodinium cp23S RFLP and sequence analysis

Pringle Lab,Grossman Lab

Abstract

This protocol describes how to perform PCR on extracted *Symbiodinium* DNA to amplify a fragment of the chloroplast 23S rRNA gene. It is based on the method describes in Pochon *et al.*, 2006. The protocol then explains how to perform an RFLP (restriction fragment length polymorphism) analysis on the PCR products as well as eitherusing them directly for sequence analysis by Sanger sequencing or cloning them into a plasmid back bone to separate genes from mixed *Symbiodinium* populations and perform the sequence analysis on several clones per original sample.

The attached file contains reference sequences of the following Symbiodinium strains: Clade A strains SSA01, SSA02, SSA03; SSB01 (clade B), SSB01del6, SSB01del136, SSE01 (clade E) and A001 (Clade D).

Citation: Pringle Lab,Grossman Lab Symbiodinium cp23S RFLP and sequence analysis. **protocols.io** dx.doi.org/10.17504/protocols.io.qyjdxun
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Guidelines

20x Borax running buffer for agarose gels:

- Dissolve 38.18 g Sodium Tetraborate Decahydrate (Borax) in 900 ml distilled water (you might need to heat up the solution to dissolve everything)
- Bring to 1 L with distilled water
- DO NOT try adjusting the pH, it takes a looooot of boric acid, even with directly adding pure powder and the gels are running well without doing it.

This results in a 100 mM solution, adjust the amount accordingly, when using other forms of Tetraborate (anhydrous etc.).

Add 50 ml of the 20× buffer to 950 ml distilled water to obtain a 1× solution.

Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis
Jonathan R. Brody and Scott E. Kern
BioTechniques 2004 36:2, 214-216

Before start

Have/order the primers for the *Symbiodinium* cp23S rDNA locus:

Primer sequences:
23S4F (forward): 5’-GAC GGC TGT AAC TAT AAC GG-3’
23S7R (reverse): 5’-CCA TCG TAT TGA ACC CAG C-3’

Materials

- HphI - 1,000 units R0158S by New England Biolabs
- Microcentrifuge Tubes by Contributed by users
- Orange DNA loading dye R0631 by Thermo Fisher Scientific
- PCR tubes by Contributed by users
- Sodium Tetraborate Decahydrate B9876 by Sigma – Aldrich
- Agarose by Contributed by users
- PCR Thermocycler by Contributed by users
- DNA polymerase and buffers for PCR by Contributed by users
- 37°C chamber, heat block or water bath by Contributed by users
- GeneRuler 50 bp DNA Ladder SM0371 by Thermo Fisher Scientific
- GeneRuler DNA Ladder Mix SM0331 by Thermo Fisher Scientific

Protocol

PCR amplification of the cp23S rDNA Locus of Symbiodinium

Step 1.
Amplify the cp23S locus by PCR with primers 23S4F and 23S7R.

Primer sequences:
23S4F (forward): 5'-GAC GGC TGT AAC TAT AAC GG-3'
23S7R (reverse): 5'-CCA TCG TAT TGA ACC CAG C-3'

25 µl reactions are sufficient to obtain enough amplicon for RFLP analysis and sequencing.
It might be a good idea to include a negative control reaction without template added.

PCR amplification of the cp23S rDNA Locus of Symbiodinium

Step 2.
Example 25 µl PCR setup using Promega GoTaq® 2× master mix:

Component	Volume [µl]
GoTaq® 2× Master Mix	12.5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
Symbiodinium genomic DNA (10-100 ng/µl)	5
Nuclease-free water	5.5
Total volume	25

Thermocycler setup:

Step	Temperature	Time
1	95°C	5 min
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2	95°C	30 s
3	50 C	45 s
4	72°C	1 min
35 cycles of 2-4	----------	----------
6	72°C	5 min

REAGENTS

GoTaq Green Master Mix M7122
by Promega

PCR amplification of the cp23S rDNA Locus of Symbiodinium

Step 3.

Mix 3 µl of the PCR reactions with 2 µl water and 1 µl 6× DNA loading dye and run on a 1% agarose TAE gel in TAE buffer together with a standard DNA ladder.

This is just to check successful amplification. The amplified fragments should be between roughly 500 and 600 bp.

REAGENTS

GeneRuler DNA Ladder Mix
SM0331 by Thermo Fisher Scientific

PCR amplification of the cp23S rDNA Locus of Symbiodinium

Step 4.

Purify the remaining volumes of the PCRs using a PCR purification kit according to the manufacturer's instructions. Elute in 30-50 µl elution buffer (10 mM Tris, pH 8.5) or water.

Restriction fragment length analysis

Step 5.

Set up 30 µl restriction enzyme reactions using *Hph*I for the RFLP: 10 µl PCR Product; 3 µl CutSmart 10× Buffer (NEB); 16 µl nuclease-free water; 1 µl *Hph*I (NEB).

Using the NEB enzyme and buffer is just an example, enzymes from other manufacturers like Thermo Fisher Scientific (previously Fermentas) will work as well.

Restriction fragment length analysis

Step 6.

Incubate the reactions for 3 h at 37°C.

Alternatively, you can digest your DNA with 0.3 µl HphI over night (≥8 h) at 37°C.

Restriction fragment length analysis

Step 7.

Mix 10 µl of the digests with 2 µl 6× DNA loading dye and run on a 3% agarose gel, don't forget to load an appropriate (low range) DNA ladder as well (I like the GeneRuler 50bp Ladder).

You want to achieve a good separation, so run the gel over the whole length of the gel (at least 5 cm). We are using Borax gels and buffer for gels with these high agarose concentrations (see Guidelines section for recipe). TBE will work probably as well.

REAGENTS

GeneRuler 50 bp DNA Ladder

SM0371 by Thermo Fisher Scientific

Restriction fragment length analysis

Step 8.

Expected fragments for different strains:

Strain	DNA fragments
SSA01	343 bp 194 bp 86 bp
SSA02	194 bp 192 bp 152 bp 86 bp
SSA03	194 bp 186 bp 152 bp 86 bp
SSB01	335 bp 296 bp
SSB01 with large deletion	335 bp 160 bp
A001 (clade D1)	356 bp 280 bp 86 bp
SSE01	292 bp 221 bp 131 bp

(Sanger-)Sequencing of the PCR products

Step 9.

Follow the instructions of your sequencing service provider to sequence the purified PCR products with primers 23S4F and 23S7F.

You can find a collection of some reference sequences in the 'Reference Sequences Symbiodinium cp23S.doc' file attached to this protocol.

ATTENTION: If you expect mixes of different strains, you should consider subcloning the PCR fragments into plasmids and sequence a number of clones per original sample.
Sanger sequencing of mixed strains will give otherwise hard or impossible to interpret results.
For best results use a high-fidelity polymerase to minimize PCR-introduced sequence variations.

Warnings

Depending on the dyes you use for staining DNA gels, follow your institution's guidelines for safety measures (wear gloves/avoid direct contact to the dyes and don't drink them).

If you are using UV-light to detect the bands, take care to not directly expose your eyes or skin to the light.