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Using genomic SCAR markers for genotyping Aiptasia strains

In 1 collection

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Aiptasia Symbiodiniaceae Model System

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ABSTRACT

This protocol describes (1) how to amplify the genomic markers SCAR3, SCAR4, SCAR5 and SCAR7 (developed by Thornhill et al., 2013) from genomic DNA of Aiptasia, (2) how to use Gibson Assembly® to clone them into a (pCR™ II-TOPO®-derived) plasmid and (3) performing colony PCRs to screen for clones to sequence by Sanger sequencing.

The subcloning is highly recommended since strains might be heterozygous for some or all of the markers.

SCAR: Aiptasia-specific inter-simple sequence repeat (ISSR)-derived sequence characterized amplified region markers.

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The PCR conditions are optimized for KOD (Hot Start) polymerase and work for SCAR3, SCAR4, SCAR5 and SCAR7 primers.

If you plan to subclone your SCAR marker fragments into plasmids before sequencing (and you should!), an effective way to do this is Gibson Assembly[®]. This method requires the fragments you want to assemble to have ≥ 20 bp homolog ends to each other.

To create linear plasmid fragments with flanks homolog to the respective SCAR primers do PCRs with the SCAR_x_in_TOPOII primer pairs from Table 1 on pCR[™]II-TOPO[®] plasmid DNA (or any plasmid derived thereof which has matching annealing sites for the primers).

I decided to keep the SCAR primers as they are and to add the homolog flanks to the plasmid backbone because the SCAR primers were already pretty sensitive to PCR conditions and PCRs on genomic DNA usually need more tweaking than on plasmid DNA.

You can stop at any step of this protocol and store the samples at -20°C to continue later.

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

MATERIALS

MATERIALS

Gibson Assembly Master Mix - 10 rxns New England Biolabs Catalog #E2611S
DpnI - 1,000 units New England Biolabs Catalog #R0176S
TAE Buffer (Tris-acetate-EDTA) Contributed by users Catalog #B49
Agarose Contributed by users Catalog #A5304
QIAquick PCR Purification Kit Qiagen Catalog #28104
GoTaq Green Master Mix Promega Catalog #M7122
Orange DNA loading dye Thermo Fisher Scientific Catalog #R0631
PCR Thermocycler Contributed by users
GeneRuler 50 bp DNA Ladder Thermo Fisher Scientific Catalog #SM0371
GeneRuler DNA Ladder Mix Thermo Fisher Scientific Catalog #SM0331
KOD Hot Start DNA Polymerase Millipore Sigma Catalog #71086-3
Ampicillin sodium salt Millipore Sigma Catalog #A0166
Liquid LB medium Contributed by users
LB plates with 100 µg/ml ampicillin Contributed by users
microcentrifuge tubes Contributed by users
PCR tubes, strips or plates Contributed by users
Chemocompetent E. coli cells Contributed by users

BEFORE START INSTRUCTIONS

This protocol uses Gibson Assembly[®] for cloning the amplified SCAR markers into a PCR-amplified pCR[™]11-TOPO[®] back bone.
You need the following primers:

Table1

	Forward primer	Sequence	Reverse primer	Sequence	Function
	ApSCAR3F	GAC CGT TAA AAT CTA AAG GTT TAC ATT AAG TAC AAG	ApSCAR3R	GGA TAA TGG TAG AGG GAA AGA AAG TGT CCG TAC	Amplification of the <i>Aiptasia</i> SCAR3 marker.
	ApSCAR4F	CTC ATC AGC CCA ATC AAA TTA GAC	ApSCAR4R	GTC CAA ATA TGA AAT AAT TAG ACT G	Amplification of the <i>Aiptasia</i> SCAR 4 marker.
	ApSCAR5F	GCA AGT GTT TGT CTC AGC TTG TGT GAA TCT G	ApSCAR5R	CAC AAA GTT TCA TGC TCT TAG TAA GTT CAG	Amplification of the <i>Aiptasia</i> SCAR 5 marker.
	ApSCAR7F	CCA TTC ATG ACG AAA TAG CTA TCC ACG TCC	ApSCAR7R	GAT CTT AAA TAA TTA ATA ATA TAG CCA CAT ATT AAT CC	Amplification of the <i>Aiptasia</i> SCAR 7 marker.
	SCAR3R_in_TOP OIL_fw	GTA CGG ACA CTT TCT TTC CCT CTA CCA TTA TCC GGC GAA TTC TGC AGA TAT CC	SCAR3R_in- TOP_OIL_rev	CTT GTA CTT AAT GTA AAC CTT TAG ATT TTA ACG GTC AAG CTT GAT GCA TAG CTT GAG	Amplification of a pCRII-TOPO backbone with flanks homologous to the SCAR3 amplicon allowing Gibson Assembly.

SCAR4R_in_TOP OII_fw	CAG TCT AAT TAT TTC ATA TTT GGA CGG CGA ATT CTG CAG ATA TCC	SCAR 4F _i n- T O P OII _r ev	GTC TAA TTT GAT TGG GCT GAT GAG AAG CTT GAT GCA TAG CTT GAG	Amplification of a pCRII-TOPO back bone with flanks homologous to the SCAR4 amplicon allowing Gibson Assembly.
SCAR5R_in_TOP OII_fw	CTG AAC TTA CTA AGA GCA TGA AAC TTT GTG GGC GAA TTC TGC AGA TAT CC	SCAR 5F _i n- T O P OII _r ev	CAG ATT CAC ACA AGC TGA GAC AAA CAC TTG CAA GCT TGA TGC ATA GCT TGA G	Amplification of a pCRII-TOPO back bone with flanks homologous to the SCAR5 amplicon allowing Gibson Assembly.
SCAR7R2_in_TO POII_fw	GGA TTA ATA TGT GGC TAT ATT ATT AAT TAT TTA AGA TCG GCG AAT TCT GCA GAT ATC C	SCAR 7F _i n- T O P OII _r ev	GGA CGT GGA TAG CTA TTT CGT CAT GAA TGG AAG CTT GAT GCA TAG CTT GAG	Amplification of a pCRII-TOPO back bone with flanks homologous to the SCAR6 amplicon allowing Gibson Assembly.

PCR amplification of pCR™ II-TOPO® back bones compatible...

1

Setup the PCR reactions to create the linear plasmid fragments for Gibson Assembly®.

The setup is the same for all primer pairs.

You can run several of these PCRs in parallel to have a good stock which you can store at -20°C and use whenever you are doing genotyping in the future.

Example PCR setup for amplification of the pCR™ II-TOPO® back bone with SCAR3-flanks using KOD Hot Start Polymerase:

Component	Volume [μl]
pCR™ II-TOPO® DNA (50 ng/μl → 50 ng total)	1
SCAR3R_in_TOPOII_fw (10 μM → 0.3 μM end concentration)	1.5
SCAR3F_in_TOPOII_rev (10 μM → 0.3 μM end concentration)	1.5

10× KOD polymerase buffer	5
MgSO ₄ (25 mM)	3
dNTPs (2 mM each)	5
Nuclease-free water	32
KOD Hot Start Polymerase	1
Total volume	50

Thermocycler setup:

Step	Temperature	Time
1	95°C	2 min
-----	-----	-----
2	95°C	20 s
3	60°C	10 s
4	70°C	2 min
30 cycles of 2-4	-----	-----
6	70°C	2 min

- 2 After the PCRs are finished add 1 µl (20 units) *DpnI* directly to the reactions. You do not need to add any buffer, *DpnI* is fully active in the KOD buffer.

This step ensures that the original pCR™II-TOPO® plasmid is removed (DpnI cleaves only dam-methylated DNA from dam⁺ E. coli strains, but not the unmethylated amplicons produced by PCR).

- 3 Incubate at 37°C for between 1 and 14 h.

- 4 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 to check successful amplification.

The amplified fragments should be ~4000 bp in size.

If you see strong unspecific bands (more than 10% of the specific ones), run a 0.8% TAE agarose gel with the whole PCR product and perform a gel extraction of the specific band. Follow manufacturer's instructions to do so.

- 5 Purify the PCRs using a PCR purification kit according to the manufacturer's manual.

This step is to remove any remaining PCR primers, salts and the DpnI enzyme from your

samples. If you did a gel extraction of the specific PCR products, skip this step.

6 Determine the DNA concentration in your samples.

PCR amplification of the *Aiptasia* SCAR markers.

7 Setup the PCR reactions to amplify the SCAR markers from your genomic *Aiptasia* DNA.

The following settings work well for SCAR3, SCAR4, SCAR5 and SCAR7 markers.

You need 100 ng of genomic DNA per 25 μ l PCR.

25 μ l reactions will produce more than enough DNA for subsequent cloning.

Adjusting all your samples to the same DNA concentration (e.g. 20 ng/ μ l) allows you to use one master mix per primer pair for all samples.

Example PCR setup for amplification of the SCAR3 marker using KOD Hot Start Polymerase:

Component	Volume [μ l]
<i>Aiptasia</i> genomic DNA (20 ng/ μ l $\rightarrow$ 100 ng total)	5
SCAR3F (10 μ M $\rightarrow$ 0.3 μ M end concentration)	0.75
SCAR3R (10 μ M $\rightarrow$ 0.3 μ M end concentration)	0.75
10 $\times$ KOD polymerase buffer	2.5
MgSO ₄ (25 mM)	1.5
dNTPs (2 mM each)	2.5
Nuclease-free water	11.5
KOD Hot Start Polymerase	0.5
Total volume	25

Thermocycler setup:

Step	Temperature	Time
1	95°C	2 min
-----	-----	-----
2	95°C	20 s
3	56°C	10 s
4	70°C	15 s
30 cycles of 2-4	-----	-----
6	70°C	2 min

8 Mix 3 μ l of the PCRs with 2 μ l water and 1 μ l 6 $\times$ DNA loading dye and run on a 3% agarose gel,

don't forget to load an appropriate (low range) DNA ladder as well (I prefer the Thermo Fisher Scientific GeneRuler 50bp Ladder).

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.

This step is for quality control of the PCR fragments, if you see a lot of unspecific bands, run a 3% Borax agarose gel with the whole PCR product and perform a gel extraction of the specific band. Follow manufacturer's manual to do so. You probably must adjust the pH with 3 M sodium acetate (pH 5.0-pH 5.3) after solubilization of the gel fragment.

Expected fragment sizes are:

ApSCAR3: ~298 bp

ApSCAR4: ~730 bp

ApSCAR5: ~732 bp

ApSCAR7: ~550 bp

Note

The SCAR7 fragment in the strains used in the original publication is only 266 bp long. We consistently found for all of our strains that it had a size between 500 and 600 bp.

9 Purify the PCRs using a PCR purification kit according to the manufacturer's manual.

This step is to remove any remaining PCR primers, salts and genomic DNA from your samples. If you did a gel extraction of the specific PCR products, skip this step.

10 Quantify the DNA concentration in your samples.

Gibson Assembly® of SCAR marker fragments into pCR™ II-...

11 New England Biolabs (NEB) has a very nice collection of material about the molecular principles of Gibson Assembly®: [NEB Gibson Assembly](#)

NEB also sells the Gibson Assembly® 2× Master Mix. The small size is meant for ten 20 µl reactions, but you can reduce the total volume up to 2 µl without any negative effect, so it probably outperforms all kinds of TOPO®-cloning in regards to costs, efficiency and robustness.

Calculate the DNA amounts for a 20 µl Gibson Assembly® reaction as recommended in the NEB manual.

NEB also has a tool on their homepage called NEBuilder where you can specify the kit you are using and upload the fragments you want to assemble and it calculates the amounts you need:

In brief:

Use 100 ng of your PCR-amplified plasmid back bone and calculate how many picomoles this is.

- *Using 100 ng of the modified pCR™II-TOPO® back bones results in 0.041 pmol per 10 µl DNA mix.*

You should use a roughly threefold higher number of molecules of the inserts (the SCAR fragments).

This means you need ~0.123 pmol of the respective SCAR PCR fragment if using the pCR™II-TOPO® back bone.

- *1 µg SCAR3 fragment is about 5.5 pmol. This means you need $0.123 \text{ pmol} / 5.5 \text{ pmol} \times 1000 \text{ ng} = \sim 22 \text{ ng}$ of SCAR3 fragment.*
- *1 µg SCAR4 fragment is about 2.2 pmol. $\rightarrow 0.123 \text{ pmol} / 2.2 \text{ pmol} \times 1000 \text{ ng} = \sim 56 \text{ ng}$ SCAR4 fragment.*
- *1 µg SCAR5 fragment is about 2.6 pmol. $\rightarrow 0.123 \text{ pmol} / 2.6 \text{ pmol} \times 1000 \text{ ng} = \sim 47 \text{ ng}$ SCAR5 fragment.*
- *1 µg SCAR7 fragment is about 2.9 pmol. $\rightarrow 0.123 \text{ pmol} / 2.9 \text{ pmol} \times 1000 \text{ ng} = \sim 42 \text{ ng}$ SCAR7 fragment.*

Fill the remaining volume of each reaction with nuclease-free water to 10 µl and mix well.

Example calculations for SCAR3:

The pCR™II-TOPO® fragment with SCAR3-compatible flanks has a concentration of 46 ng/µl. 1 µg of this fragment equals 0.41 pmol.

The SCAR3 PCR fragment has a concentration of 25 ng/µl. 1 µg of this fragment equals 5.5 pmol.

1. For 100 ng vector: $100 \text{ ng} / 46 \text{ ng} \times 1 \text{ µl} = 2.2 \text{ µl}$ of pCR™II-TOPO® back bone. This results in $0.1 \text{ µg} \times (0.41 \text{ pmol} / 1 \text{ µg}) = 0.041 \text{ pmol}$.
2. For a threefold excess of insert: $3 \times 0.041 \text{ pmol} = 0.123 \text{ pmol}$.
3. For 0.123 pmol SCAR3 PCR fragment you need: $0.123 \text{ pmol} \div (5.5 \text{ pmol} / 1000 \text{ ng}) \div (25 \text{ ng} / 1 \text{ µl}) = 0.123 \text{ pmol} \times (1000 \text{ ng} / 5.5 \text{ pmol}) \times (1 \text{ µl} / 25 \text{ ng}) = (0.123 \text{ pmol} \times 1000 \text{ ng} \times 1 \text{ µl}) / (5.5 \text{ pmol} \times 25 \text{ ng}) = \sim 0.9 \text{ µl}$

12 Mix 1 µl of the respective DNA mix with 1 µl 2× Gibson Assembly® Master Mix in a 0.2 ml PCR tube.

Continue directly with step 12 or keep the reaction on ice. Otherwise, the T5 exonuclease will chew down your whole PCR fragments...

13 Incubate the reaction for 30 min at 50°C in a PCR cycler with heated lid.

You can shorten this step to 10 min without a big impact on clone number after transformation. You can extend the incubation to up to 60 min. After that you might get weird or no clones.

14 Let the mix cool down on ice and transform the 2 µl into 50-100 µl chemocompetent *E. coli* cells

according to your (or the manufacturer's) standard transformation protocol. Use ampicillin-containing plates for selection if using the pCR™II-TOPO® back bone.

- 15 Incubate the plates over night ≤ 14 h at 37°C or until you see colonies by bare eye.

Colony PCR to check for positive clones

- 16 **Mark the colonies you want to test on the bottom of the plate and number them.**
Because the animals might be heterozygous for one or more loci, sequencing a minimum of at least 4 clones is recommended. Test one or two more clones to account for putative false positive colonies without insert.
- 17 **Prepare and label sterile glass test tubes and fill them with 3-5 ml LB with 100 µg/ml ampicillin.**

- 18 **Set up 20 µl PCR reactions. It is highly recommended to prepare a master mix for all of your reactions and then distribute it into the PCR tubes/strips/plate.**
20 µl volume is very robust to different amounts of colony material. Smaller volumes work as well but need more care to not use too much colony material.
We use GoTaq® Green Master Mix (Promega) for such PCRs where fidelity of the polymerase is not important. Polymerases from other vendors and different origin will work as well but might require some adjustments on the PCR conditions.
If you followed this protocol and cloned the SCAR markers into pCR™II-TOPO®, you can use the generic M13 Forward (-20) and M13 Reverse (-27) primers. They flank the insert and will yield a PCR product of roughly 180 bp + the size of the respective SCAR marker.

Primer sequences (5' to 3'):

M13 Forward (-20): GTA AAA CGA CGG CCA GT

M13 Reverse (-27): CAG GAA ACA GCT ATG AC

Example colony PCR setup for checking presence of SCAR marker inserts in pCR™II-TOPO®:

Component	Volume [µl]
GoTaq® Green Master Mix (2×)	10

M13 Forward (-20) (10 µM)	0.8
M13 Reverse (-27) (10 µM)	0.8
Nuclease-free water	8.4
Total volume	20

- 19 With a sterile 10 µl or 200 µl tip on a clean pipette adjusted to 2 µl, pick a small piece of a colony (half of a 1 mm colony maximum, less from larger colonies).**

It is critical not to introduce too much cell material into the PCR otherwise it will not work. Aim for a piece of the colony corresponding to a volume between 0.1 µl and 1 µl. Instead of a plastic tip, a wooden toothpick should work as well with this relatively large volume. Just be aware that it will soak in a good amount of liquid.

Move the tip into the tube with the PCR mix and resuspend the cells by pipetting up and down while moving the tip around at the walls.

This should not take more than a couple of seconds.

Expell the liquid from the tip and eject the tip into the glass test tube with LB_{amp} medium.

This is the fastest way to get to the pure plasmids for sequencing (one day). Alternatively, you can do a short streak on an LB_{amp} plate, incubate it over night at 37°C and only inoculate cultures for minipreps of clones you really will sequence. This method may save some medium and equipment but takes one day longer than the suggested one (two days).

- 20 Run the PCRs.**

Thermocycler setup:

Step	Temperature	Time
1	95°C	5 min
-----	-----	-----
2	95°C	30 s
3	58°C	45 s
4	72°C	1 min
35 cycles of 2-4	-----	-----
6	72°C	5 min

- 21 Load and run 10 µl of the PCR reactions on a 1% agarose TAE gel in TAE buffer together with a standard DNA ladder.**

If you cloned the SCAR markers into pCRII™-TOPO® and used the generic M13 Forward (-20) and M13 Reverse (-27) primers the fragments should be roughly 180 bp + the size of the respective SCAR marker sequence.

- 22 Extract the plasmid DNA (by whatever method or kit you prefer) from 4 positive clones per SCAR marker per strain and send to sequencing with primers M13 Forward (-20) and M13 Reverse (-27) or any other appropriate primers.**