Using genomic SCAR markers for genotyping Aiptasia strains

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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.

Citation: Pringle Lab,Christian Renicke Using genomic SCAR markers for genotyping Aiptasia strains. **protocols.io** dx.doi.org/10.17504/protocols.io.qykdxuw
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Guidelines

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 effictive 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 SCARx_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

Before start

This protocol uses Gibson Assembly® for cloning the amplified SCAR markers into a PCR-amplified pCR™II-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 Aiptasia SCAR3 marker.
ApSCAR4F	CTC ATC AGC CCA ATC AAA TTA GAC	ApSCAR4R	GTC CAA ATA TGA AAT AAT TAG ACT G	Amplification of the Aiptasia SCAR4 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 Aiptasia SCAR5 marker.
ApSCAR7F	CCA TTC ATG ACG AAA TAG CTA TCC ACG TCC	ApSCAR7R2	GAT CTT AAA TAA TTA ATA ATA TAG CCA CAT ATT AAT CC	Amplification of the Aiptasia SCAR7 marker.
SCAR3R_in_TOPOII_fw	GTA CGG ACA CTT TCT TTC CCT CTA CCA TTA TCC GGC GAA TTC TGC AGA TAT CC	SCAR3F_in_TOPOII_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 back bone with flanks homologous to the SCAR3 amplicon allowing Gibson Assembly.
SCAR4R_in_TOPOII_fw	CAG TCT AAT TAT TTC ATA TTT GGA CGG CGA ATT CTG CAG ATA TCC	SCAR4F_in_TOPOII_rev	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_TOPOII_fw	CTG AAC TTA CTA AGA GCA TGA AAC TTT GTG GGC GAA TTC TGC AGA TAT CC	SCAR5F_in_TOPOII_rev	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_TOPOII_fw	GGA TTA ATA TGT GGC TAT ATT AAT TAT TTA AGA TCG GCG AAT TCT GCA GAT ATC C	SCAR7F_in_TOPOII_rev	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.

Materials

Gibson Assembly Master Mix - 10 rxns E2611S by New England Biolabs

DpnI - 1,000 units R0176S by New England Biolabs

TAE Buffer (Tris-acetate-EDTA) B49 by Contributed by users

Agarose A5304 by Contributed by users

QIAquick PCR Purification Kit 28104 by Qiagen

GoTaq Green Master Mix M7122 by Promega

Orange DNA loading dye R0631 by Thermo Fisher Scientific

PCR Thermocycler by Contributed by users

GeneRuler 50 bp DNA Ladder SM0371 by Thermo Fisher Scientific

GeneRuler DNA Ladder Mix SM0331 by Thermo Fisher Scientific

KOD Hot Start DNA Polymerase 71086-3 by Millipore Sigma

Ampicillin sodium salt A0166 by Millipore Sigma

Liquid LB medium by Contributed by users

LB plates with 100 µg/ml ampicillin by Contributed by users

microcentrifuge tubes by Contributed by users

PCR tubes, strips or plates by Contributed by users

Chemocompetent E. coli cells by Contributed by users

Protocol

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

Step 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
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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

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

Step 2.

After the PCRs are finished add 1 µl (20 units) *Dpn*I directly to the reactions. You do not need

to add any buffer, *Dpn*I 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).*

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

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

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

Step 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.

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

Step 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.*

PCR amplification of pCR™II-TOPO® back bones compatible for cloning of Aiptasia SCAR markers.

Step 6.
Determine the DNA concentration in your samples.

PCR amplification of the Aiptasia SCAR markers.

Step 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 µl PCR.
25 µl reactions will produce more than enough DNA for subsequent cloning.
Adjusting all your samples to the same DNA concentration (e.g. 20 ng/µl) 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 [µl]

Aiptasia genomic DNA (20 ng/µl → 100 ng total)	5
SCAR3F (10 µM → 0.3 µM end concentration)	0.75
SCAR3R (10 µM → 0.3 µM end concentration)	0.75
10× 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

PCR amplification of the Aiptasia SCAR markers.

Step 8.

Mix 3 µl of the PCRs with 2 µl water and 1 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 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

NOTES

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.

PCR amplification of the Aiptasia SCAR markers.

Step 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.

PCR amplification of the Aiptasia SCAR markers.

Step 10.

Quantify the DNA concentration in your samples.

Gibson Assembly® of SCAR marker fragments into pCR™II-TOPO® vector back bones.

Step 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: http://nebuilder.neb.com/.

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 0.125 pmol / 5.5 pmol × 1000 ng = 22 ng of SCAR3 fragment.*
- *1 µg SCAR4 fragment is about 2.2 pmol. → 0.123 pmol / 2.2 pmol × 1000 ng = 56 ng SCAR4 fragment.*
- *1 µg SCAR5 fragment is about 2.6 pmol. → 0.123 pmol / 2.6 pmol × 1000 ng = 47 ng SCAR5 fragment.*
- *1 µg SCAR7 fragment is about 2.9 pmol. → 0.123 pmol / 2.9 pmol × 1000 ng = 42 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 ng / 46 ng × 1 µl = 2.2 µl of pCR™II-TOPO® back bone. This results in 0.1 µg × (0.41 pmol / 1 µg) = 0.041 pmol.
2. For a threefold excess of insert: 3 × 0.041 pmol = 0.123 pmol.
3. For 0.123 pmol SCAR3 PCR fragment you need: 0.123 pmol ÷ (5.5 pmol / 1000 ng) ÷ (25 ng / 1 µl) = 0.123 pmol × (1000 ng / 5.5 pmol) × (1 µl / 25 ng) = (0.123 pmol × 1000 ng × 1 µl) / (5.5 pmol × 25 ng) = 0.9 µl

Gibson Assembly® of SCAR marker fragments into pCR™II-TOPO® vector back bones.

Step 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...

Gibson Assembly® of SCAR marker fragments into pCR™II-TOPO® vector back bones.

Step 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.

Gibson Assembly® of SCAR marker fragments into pCR™II-TOPO® vector back bones.

Step 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.

Gibson Assembly® of SCAR marker fragments into pCR™II-TOPO® vector back bones.

Step 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

Step 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.

Colony PCR to check for positive clones

Step 17.

Prepare and label sterile glass test tubes and fill them with 3-5 ml LB with 100 µg/ml ampicillin.

Colony PCR to check for positive clones

Step 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/stripes/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 pCRII™-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

Colony PCR to check for positive clones

Step 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 then 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).

Colony PCR to check for positive clones

Step 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

Colony PCR to check for positive clones

Step 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.

Colony PCR to check for positive clones

Step 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.