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Protocol for bacterial depletion of Aiptasia anemones - Towards the generation of gnotobiotic/germ-free cnidarian host animals V.1

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We use this protocol and it's working

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Abstract

This protocol aims to obtain bacteria-depleted Aiptasia polyps. It is divided into 2 sections: (1) a rearing protocol composed of sterile food preparation and anemone priming and (2) a bacteria-depletion protocol for Aiptasia.

Attachments

DOCX

Protocol for bacteri...

15.7MB

Guidelines

This protocol aims to obtain bacteria-depleted Aiptasia polyps. Given that the Aiptasia life cycle is not closed yet under laboratory settings, we start with xenic adult anemones. The here-described protocol is divided into 2 sections: (1) a rearing protocol composed of sterile food preparation and anemone priming and (2) a bacteria-depletion protocol for Aiptasia. Therefore, it is vital to start a "priming batch" of adult anemones and to ensure that all sources of potential contamination (seawater and food) are devoid of bacteria during the priming step. The priming step will reduce the bacterial cargo in order to increase the success of the antibiotic treatment.

Sterile food is obtained through decapsulation of *Artemia* cysts by hypochlorination and antibiotic treatment, adapted from (Sorgeloos et al., 1977). This process removes the outer layer of the cyst and decreases the hatching time, while also sterilizing it. Cysts are then de-hydrated for storage and re-hydrated for hatching at convenience. Primed anemones to be used for the depletion protocol must be exclusively fed with this food source for an extended period of time and only artificial seawater (ASW) may be used. The primed animals will then undergo an antibiotic treatment with daily media replacement for 7 days, and any manipulation from this point onwards must be done under aseptic conditions.

Bacterial contamination should be assessed after treatment by culture-dependent and -independent techniques. It is recommended that Aiptasia animals are not fed during and post-treatment to limit any potential contamination. A recovery of at 24 hours after antibiotic treatment is recommended to ensure full removal of antibiotics. Such obtained/treated animals are heavily depleted and ready for any microbial manipulation experiment. Treated animals should be used in experiments as soon as the recovery phase finishes to avoid potential bacterial re-growth/re-colonization.

In our current experience, treated Aiptasia animals may not remain depleted if reared for periods of more than 7 days after recovery with no selective pressure (i.e., antibiotics) applied.

NOTE: The antibiotic treatment can be prolonged for up to 1 month with no mortality. If working with bigger animals (>1cm oral disk) than prolonging the treatment is highly recommended. Increasing the dosage of an antibiotic was not tested *in vivo*, but was tested on bacterial isolates from Aiptasia with low to moderate success on bacterial growth inhibition (up to 200µl/ml of single antibiotics); nevertheless, can be considered in case of low success rates.

Materials

Reagents

For Animal rearing:

Artificial seawater (ASW):

- 420 mM Sodium chloride – CAS: 7647-14-5
- 10.5 mM Calcium chloride dihydrate – CAS: 10035-04-8
- 840 µM Potassium bromide – CAS: 7758-02-3
- 71.5 µM Sodium fluoride – CAS: 7681-49-4
- 9.4 mM Potassium chloride – CAS: 7447-40-7
- 485.2 µM Boric acid – CAS: 10043-35-3
- 28.8 mM Sodium sulfate – CAS: 7757-82-6
- 2.4 mM Sodium bicarbonate – CAS: 144-55-8
- 63.8 µM Strontium chloride hexahydrate – CAS: 10025-70-4
- 58.3 mM Magnesium chloride hexahydrate – CAS: 7791-18-6
- For pH adjustment: Sodium hydroxide – CAS: 1310-73-2
- Ethanol absolute – CAS: 64-17-5
- Clorox bleach (or any household bleach, unscented, with ~5,25% active chlorine)

For Antibiotic treatment (antibiotic 'cocktail'/solution, ABS):

- 50 µg/ml Rifampicin - CAS: 13292-46-1; Stock solution as 50 mg/ ml in 100% DMSO
- 50 µg/ml Nalidixic acid – CAS: 389-08-2; Stock solution as 50 mg/ml in MilliQ H2O with pH adjusted to 11 using NaOH
- 50 µg/ml Carbenicillin – CAS: 4800-94-6; Stock solution as 100 mg/ml in MilliQ H2O
- 50 µg/ml Chloramphenicol – CAS: 56-75-7; Stock solution as 50 mg/ml in 100% Ethanol
- Artificial seawater

For bacterial assessment:

- Difco™ Marine Agar 2216
- Qiagen DNeasy Blood & Tissue Kit
- Taq-polymerase or any PCR-ready mix (we use Qiagen Multiplex PCR kit Cat No: 206145)
- 16S rRNA gene primers: we use the universal primer pair
  27F 5'-AGAGTTTGATCCTGGCTCAG-3'
  1492R 5'-GGTTACCTTGTTACGACTT-3'
  or
  67F 5'-CAGGCCTAACACATGCAAGTC-3'

1542R 5′-AAGGAGGTGATCCAGCCGCA-3'

The latter primer pair allows a better separation of bacterial and (non-specific) eukaryotic amplifications on an agarose gel (Galkiewicz and Kellogg, 2008)).

Consumables

- 1 L plastic rearing containers, with lid
- Sterile 24-well tissue culture plates
- Sterile plastic 100 × 15 mm Petri dishes
- Cotton swabs, sterile (Cat: 89031-272)
- 0.2 µm pore size filter with disposable bottles (we use Corning® bottle-top vacuum filter system)
- Sterile polypropylene pellet pestles
- 1.5 ml polypropylene tubes
- 0.2 ml PCR tubes
- Sterile serological pipettes, 5, 10, 25 ml.
- Sterile Plastic Pasteur pipette
- 50 ml polypropylene tubes

Equipment

- Autoclave
- Biosafety cabinet
- Inverted microscope with 40x objective
- Incubator with controlled light and temperature
- *Artemia* hatching system or aerated closed bottles
- Thermocycler

Biological material

- Aiptasia (*Exaiptasia pallida*) anemones; here: strain CC7 in symbiotic and aposymbiotic state was used (see (**Baumgarten et al., 2015**))
- *Artemia* cysts (Brine Shrimp cysts)

Rearing Protocol

1 Prepare Artificial seawater (ASW) by mixing reagents in MilliQ water and adjusting the pH of the final volume to 8.0-8.2. Filter through 0.22 µm pore size filter.

Preparation of decapsulated Artemia cysts

2 To prepare the decapsulated *Artemia* cysts:

2.1 Weight 2g of cysts and mix with 150 ml of tap water. Let hydrate with aeration for 1h;

2.2 Prepare a saturated brine solution by mixing at least 35% w/v (better >40%) of NaCl in MilliQ water and autoclave it (80 ml). The solution must have a substantial amount of precipitated salts to account for the water that will come out of the hydrated cysts;

2.3 After 1h of hydration, add 150 ml of bleach to the cysts and mix with aeration between 4-6 minutes.

Note

The mixture will become foamy (don't aerate vigorously!) and the cysts will change color, first from brown to white, then orange. It is more important to watch the color change than to follow an exact time, so the cysts do not become inviable due to overtime in bleach. Still, as a first approximation, don't leave it longer than 5-6 min.

2.4 When the majority of the cysts look orange, strain them in a 63 µm strainer and rinse the cysts extensively under tap water for at least 1 min; or until they don't smell like bleach.

2.5 Rinse the cysts for an extra 30 sec in MilliQ water.

2.6 Pour the cysts in a 50 ml polypropylene tube and, in a biosafety cabinet, add the antibiotic solution (ABS) until covered. Incubate in a tilting lab mixer (or belly dancer) for 30 min.

2.7 In the biosafety cabinet, strain the cysts and scoop them to the cool autoclaved saturated brine solution and place the cysts in the fridge. This will de-hydrate them and prevent the hatching process.

2.8 Cysts should last up to 4 months at 4°C.

Note

If salt is not precipitated in the brine solution, add more as the cysts will only preserve while they are de-hydrated.

2.9 To hatch the cysts, in a biosafety cabinet, shake the brine to place the cysts in suspension, let the salts settle for a couple of seconds, take 1 ml of the suspension and mix with 35 ml of ASW in a 50 ml falcon tube (or a bigger amount onto a sterile hatching system) and incubate at RT with agitation. Cysts will hatch in 24-48h.

Note

In the first hatching, and before feeding Aiptasia animals, take 200 µl of the hatching water after 48 h and plate it in Marine Agar or other rich marine media and incubate for 5 days @ 25°C. Absence of colonies indicates successful treatment. In case of bacterial growth, hatch the cysts in ABS instead of ASW.

Note

The amount of decapsulated cysts can be scaled up using the same protocol in a higher proportion of cysts and solutions, but up to a limit (see (Sorgeloos et al., 1977)).

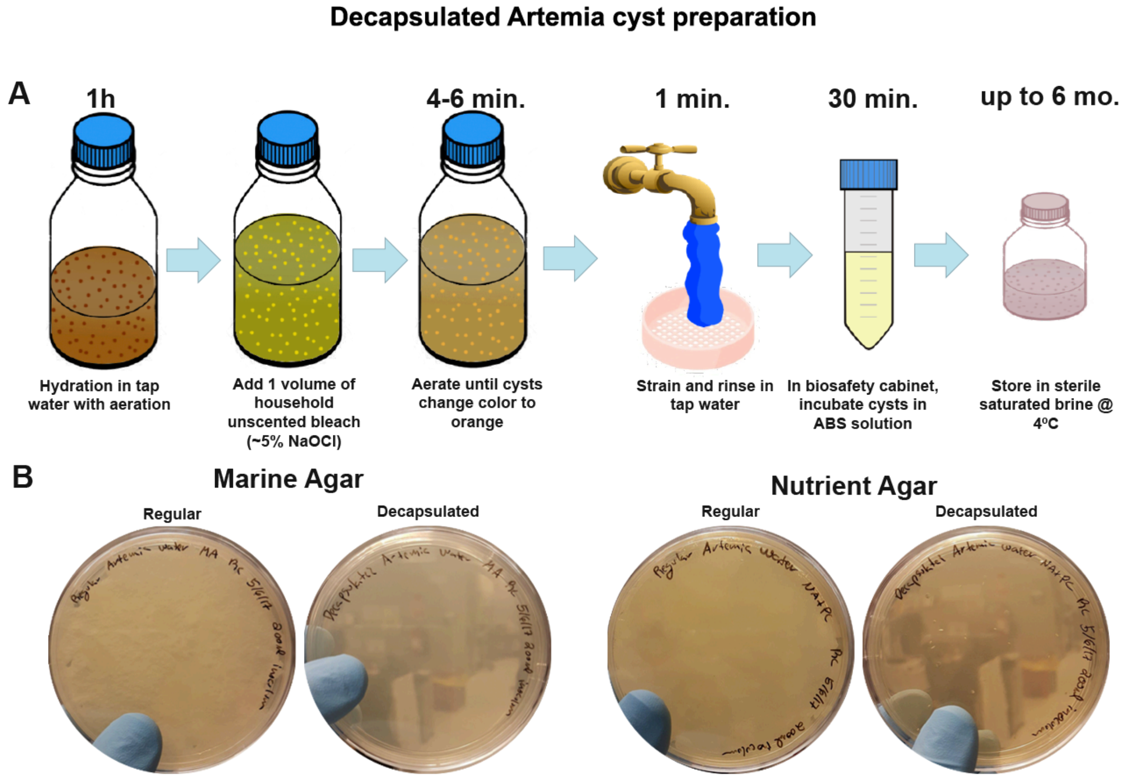

Figure 1. Overview of brine shrimp decapsulating protocol/procedure. (A) Decapsulation steps and (B) bacterial growth assessment before and after decapsulation using hatching water as inoculum.

Rearing Protocol

3 Prepare the antibiotic solution (ABS) by mixing antibiotics with ASW and sterile filter the final solution through 0.22 µm pore size filters. Cover the bottle with aluminum foil to protect from light.

Priming of Aiptasia animals targeted for bacterial depletion

4

1. Remove aposymbiotic (APO) or symbiotic (SYM) Aiptasia polyps (oral disk of approximately 5mm) from rearing containers and extensively wash them in ASW individually and sequentially in 2 petri dishes, by pipetting up and down with a plastic Pasteur pipette.

> Note
>
> Make sure the animals release mucus. This step depletes the bacteria on the surface mucus layer and also washes away parasitic eukaryotes (Figure 3).

5 1. Transfer the anemones destined for bacterial depletion to a clean container and rear them in a 12 h light: 12 h dark incubator (20–40 µmol photons m−2 s−1 of photosynthetically active radiation) at 25ºC, using only ASW and feeding them twice a week with the decapsulated *Artemia nauplii* for at least a month, changing the water on the day after each feeding.

> Note
>
> Always open the stock of decapsulated cysts under sterile conditions.

Aiptasia bacterial depletion protocol

6 Repeat step 4 from the Priming section;

7 After the polyps released mucus, transfer the polyps to a petri dish with the antibiotic solution (ABS).

> Note
>
> All steps after step 7 must be done in a biosafety cabinet, in a sterile environment applying proper sterility practices.

> Note
>
> Each antibiotic targets a different mechanism of bacterial replication to ensure different killing mechanisms. The antibiotic solution can be kept at 4°C protected from light during the course of the treatment (1 week).

8 Wash the Aiptasia animals once more in ABS and leave them in the plate for ~15 min.

9 Transfer each Aiptasia polyp to a 24-well plate, 1 polyp per well and fill with 1 ml of ABS.

10 Let the polyps adhere to the wells for a few hours and check in the inverted microscope for the presence of parasites (Figure 3). In case you still see something, wash the well again with ABS.

11 Treat the polyps for 7 days, with daily media exchange, and incubate @ 25°C under normal light cycle conditions.

12 In between exchanges, clean the bottom of the wells with sterile cotton tip swabs lightly wet with 70% Ethanol in MilliQ water and rinse 1 time with ASW.

Note

Treatment should be initiated and ASW exchanged at the beginning of the incubator’s dark cycle to prevent early photodegradation of light-sensitive antibiotics (e.g., Rifampicin).

Note

If incubating plates in incubators with high ventilation, put the plates inside a zip lock bag with a wet piece of paper inside to prevent evaporation.

13 After 7 days of treatment, recover the animals in ASW for 1 day, washing and exchanging the ASW twice.

Note

It is possible that you’ll see some round structures, between 6-12 µm in size, that burst and release tiny spore-like cells (~2-3 µm). These are Thraustochytrids, and are currently the only protist we cannot eliminate, although, if polyps are kept clean with daily ASW exchange, they should be minimally present.

14 To check the efficiency of the treatment, rinse and lysate individual polyps after recovery in ASW using a pestle and plate 50-100 µl of the lysates in Marine Agar plates or another rich marine medium, as well as a drop of water of each well. Absence of bacterial growth after 5 days at 25°C indicates successful treatment.

Note

Thraustochytrids can also grow in the agar plates when animal lysates are plated, although, colonies will only show up after 2/3 weeks of incubation.

15 Use the remaining lysate to perform DNA extraction and to perform a PCR of the 16S rRNA gene for 30 cycles using 10 ng of DNA template. Absence of amplification or

amplification comparable to the PCR negative control in gel electrophoresis indicates successful treatment.

Note

The PCR thermal profile was as follows: 95°C for 15 min, followed by 30 cycles of each: 30 s at 95°C, 90 s at 55°C, and 90 s at 72°C. A final extension step was set at 72°C for 10 min.

Note

One may consider doing PCRs using cDNA to circumvent amplification of DNA from dead bacteria. Thraustochytrids mitochondrial DNA can also amplify with 16S universal primers.

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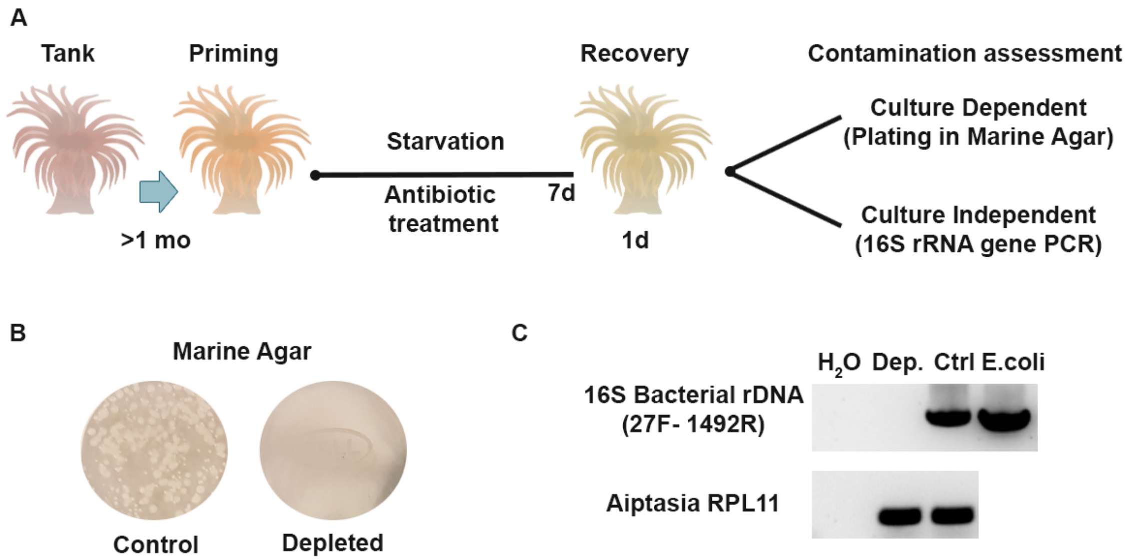

Figure 2. Overview of (A) the bacteria depletion protocol and (B, C) contamination assessment by means of culture-dependent (B) and -independent (C) methods.

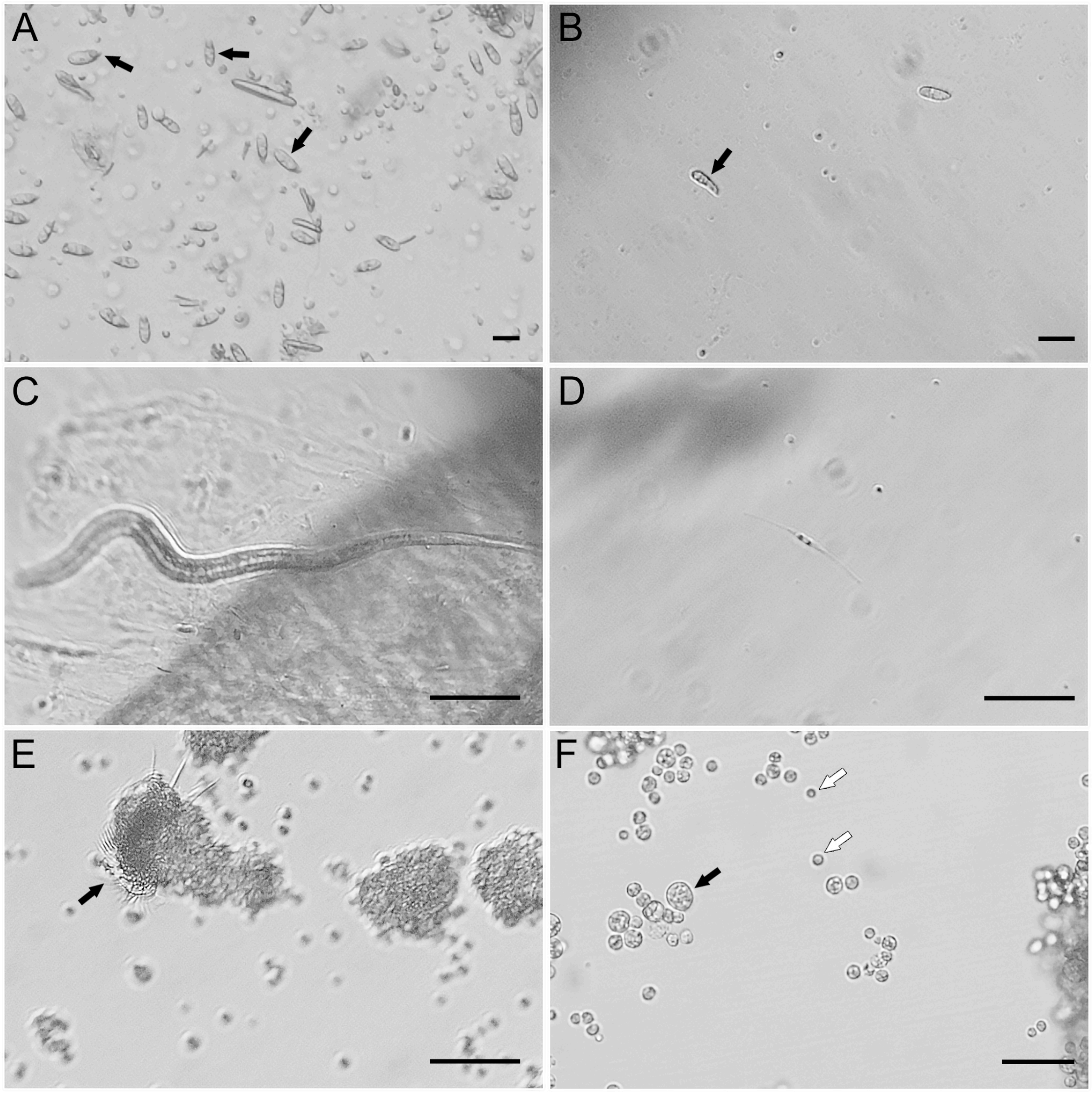

Figure 3. Micrographs of eukaryotes occasionally detected in rearing tanks before the priming step. (A) Ciliate protist resembling *Paramecium*; (B) Unknown protist, with contractile locomotion; (C) Marine Nematode; (D) Photosynthetic *Nitzschia longissima*, a common pennate marine diatom; (E) Ciliate of the genus *Euplotes*; (F) Thraustochytrids, fungal-like marine protists. The bigger cells (black arrow) burst to release smaller spore-like cells (white arrows) Scale bars: A/B/F: 20 µm, remaining:50µm.

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