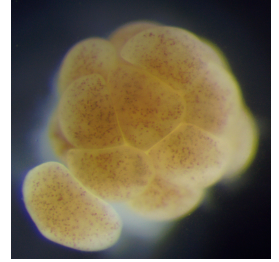


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🌐 Isolation of vertically-transmitted Symbiodiniaceae from coral eggs using centrifugation

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Sophie Centurino Renton^{1,2}

¹James Cook University; ²Australian Institute of Marine Science



Sophie SCR Centurino Renton

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We use this protocol and it's working

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Abstract

This protocol can be used to isolate Symbiodiniaceae from the eggs of corals. These symbionts are vertically transmitted from coral host to coral egg and are difficult to isolate through traditional methods of dinoflagellate culturing. This protocol produces aliquots of known counts of Symbiodiniaceae cells into tubes in preparation for high-molecular-weight DNA extraction. This protocol was developed on the coral species *Montipora aequituberculata* in November 2024.

This protocol is intentionally designed to suit the dynamics of coral spawning and can be adapted to suit various downstream DNA extractions. It is up to the discretion of the scientist on the night of spawning, whether one or multiple genotypes will be processed. Sufficient collection from one genotype alone, with no risk of contamination, is better than collection of multiple genotypes with risk of contamination.

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Materials

Personal equipment

- Head torch with red function capability
- Thin tip permanent marker *for labelling throughout the protocol and for the prevention of contamination between genotypes*
- White tape *for labelling spawning and gamete collection equipment with genotype identification*

Tools/perishables

- Pipettes (3 sizes: 2-20ul, 2-200ul, 200 -1000ul)
- Disposable pipette tips for each size
- Eppendorf tube 1.5ml or 2ml *This choice depends on which tube is used in the downstream DNA extraction as Symbiodiniaceae cells will be snap-frozen directly into 1.5ml or 2ml tubes ready for extraction*
- Regular spawning and gamete washing equipment (e.g bowls, cups, tape, marker, 100um or 50um filter)
- Plastic transfer pipettes
- 15ml or 10ml falcon tubes (approx. 10 tubes for each colony (genotype) that spawns) *Ideally, the number of falcon tubes is kept even at this stage for ease of immediate centrifugation*
- Electric pipette (e.g: Eppendorf Easypet 3) *These are necessary for pipette mixing to resuspend pellet in the large volume 10ml or 15ml falcon tubes*
- Disposable serological pipette tips for electric pipette (tip size: 10ml or 15ml *both sizes will fit into the electric pipette*)
- Haemocytometer
- Counting machine/click counter
- Styrofoam 2ml tube rack *for snap-freezing directly in tube on shallow bath (small Esky lid) of liquid nitrogen*
- Liquid nitrogen

Equipment/instruments

- Falcon tube rack *helpful for moving around at night with samples from tanks to lab and within the lab between centrifugation rounds and washes*
- Benchtop centrifuge *any benchtop centrifuge capable $\leq 3,500 \times g$ will work*
- Microcentrifuge *must fit 1.5 or 2ml Eppendorf for the final spin down to a pellet of known cell count prior to snap-freezing*
- High-power microscope

The ideal benchtop centrifuge is the Allegra V-15R with a maximum speed/RCF of 13,500 RPM /20,412 x g. However, if this is not available, a benchtop centrifuge with a lower maximum speed can be used, such as the Allegra X-12R with a maximum speed/RCF of 3,750 RPM / 3,270 x g. In the case of using a centrifuge which does not match the maximum speed of the protocol, simply use the maximum speed possible of the available benchtop centrifuge.

Section 1: Collect coral gametes (as per regular coral spawning protocol)

- 1 Isolate coral colonies at signs of setting.
- 2 Collect gamete bundles (hermaphroditic corals only) as they rise to the surface using cups and pipettes.
N.B Label all collections with coral ID and time of spawning.

Section 2: Wash Gamete bundles and prepare falcon tubes for centrifugation

- 3 Separate and wash gamete bundles (hermaphrodites) as per steps 3.1 to 3.4.

Wash station equipment for one genotype:

- 2 x bowls
- Access to running seawater
- Transfer pipettes
- 1 x filter *mesh must be finer than the egg size of target species*
- 10ml or 15ml falcon tubes

- 3.1 Fill both bowls with seawater and place the filter inside the first bowl.

N.B. Ensure the mesh side of the filter is submerged.

- 3.2 Pour the collected bundles into the filter and agitate the filter gently to break up the bundles.

N.B. Eggs will remain inside the filter and the sperm will enter the bowl turning the water cloudy.



Figure 1. *Montipora aequituberculata* gamete bundles inside 60µm filter, submerged in seawater.

- 3.3 Pick up the filter containing the eggs and move it into the second bowl of seawater, submerging the eggs once again.
N.B. Use the running seawater to wash down any eggs that get stuck on the inside of the filter. As sperm is not needed for fertilisation in this protocol, ensure thorough disposal of the (sperm) water before refreshing the bowls with seawater.
- 3.4 Continue to gently agitate and transfer the gametes between bowls, refreshing the seawater, until all bundles are broken up and the water is no longer cloudy.
N.B. Once all bundles are broken the individual eggs will form a mono-film on the surface of the water. Sperm cells interfere with the differential separation during centrifugation and must be completely removed in this step.

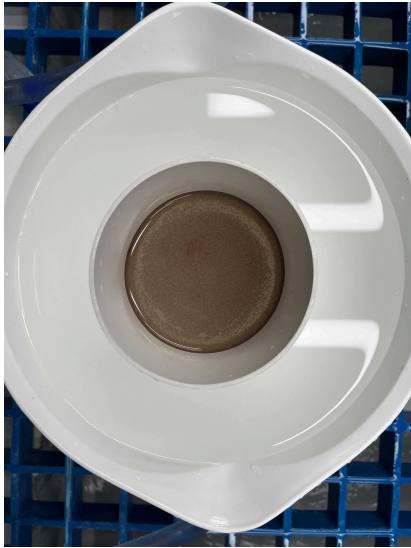


Figure 2. *Montipora aequituberculata* eggs washed and separated from sperm.

- 4 Use a transfer pipette to transfer the eggs into labelled falcon tubes ready for immediate centrifugation. Keep the eggs inside the filter during this step to minimise surface area. Use the positive buoyancy of eggs to reduce seawater in the pipette before depositing eggs into the falcon tube. Ensure that all falcon tubes have an equal total volume. The volume of eggs does not need to be strictly identical as all tubes will be combined shortly.



Figure 3. Utilise the positive buoyancy of the eggs to transfer desired amounts into falcon tubes.

Recommended egg-to-seawater ratios (see Figure 4 and Figure 5 for clarification):

- 15ml falcon tubes: 0.8ml eggs to 13ml seawater
- 10ml falcon tubes: 0.5ml eggs to 8ml seawater

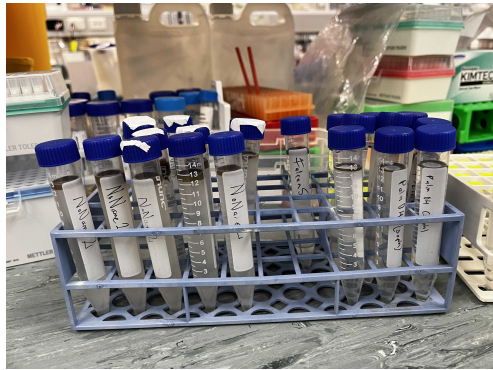


Figure 4. Correctly filled falcon tubes (15ml) containing *M.aequituberculata* eggs packed with Symbiodiniaceae (positively buoyant) and seawater.

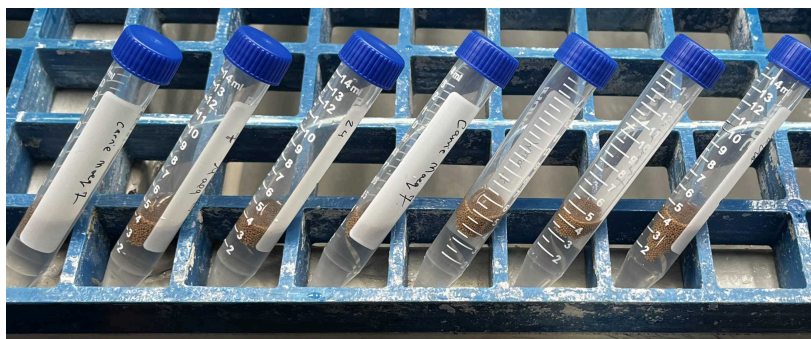


Figure 5. Incorrectly filled falcon tubes (15ml) containing too many eggs and too little seawater resulting in poor differential separation.

N.B. Do not overload falcon tubes with eggs. Excessive numbers of eggs can result in the production of lipids from the death of the gametes. The lipids interfere with successful centrifugation.

Section 3: Centrifuge, remove supernatant, resuspend and repeat

- 5 Centrifuge all falcon tubes in a benchtop centrifuge at the below specifications:

A	B
RCF	3500 x g
TIME	20 minutes
TEMP	23 degrees celsius

Figure 6. Centrifuge specifications for round 1 of separation.



N.B. The first spin should have the most force. If there is limited access to high power centrifuge, use a low-power centrifuge at the maximum speed possible. The centrifugal force will break the egg walls and pellet the Symbiodiniaceae cells to the bottom of the falcon tube.

- 6 Remove the supernatant by disposing of the seawater and the surface lipid layer (see Figure 7) into a liquid waste container.

Tip: Firmly tap the falcon tube on the side of a liquid waste container to dislodge the lipid "pancake". Do this in one swift motion to dispose of both the lipid "pancake" and the seawater at the same time. The Symbiodiniaceae cell pellet will remain at the bottom of the falcon tube.



Figure 7. The surface lipid layer ("lipid pancake") is shown in the black box

- 6.1 Remove the contact lipids (see Figure 8) with either of these techniques:
 1. Load a 1ml pipette with seawater and rest the tip of the pipette on the inside wall of the falcon tube just above the pellet. Expel the seawater from the pipette using a steady force. The stream of seawater will lift the contact lipids from the pellet in one piece.

N.B. The contact lipid layer is flaky and, in the best case, will lift in one piece. However, if the contact lipid comes off in multiple flakes, take the opportunity to remove each flake before trying to dislodge more.
 2. Gently scrape away the lipid layer with a pipette and disposable tips.
 3. If there are no or very little contact lipids, continue directly to step 7.



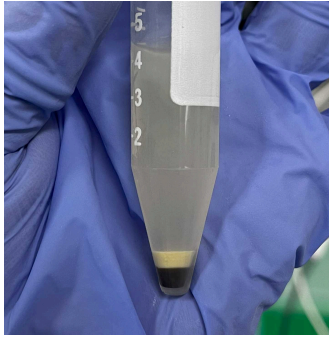


Figure 8. Contact lipids are those which are in direct contact with the Symbiodiniaceae pellet. Careful removal of this layer is required. See the result of successful removal in figure 9.

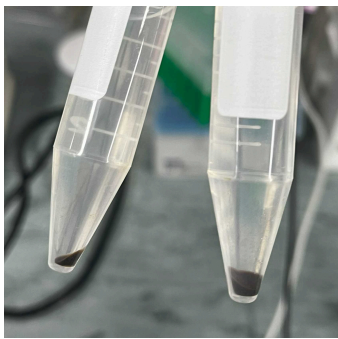


Figure 9. Successful removal of the contact lipid layer resulting in isolated Symbiodiniaceae cells.

- 7 Once the lipids are removed, resuspend the pellet in 1ml of seawater by pipette mixing with a 1000ul pipette.

N.B pipette mix until no clumps remain, and the entire pellet is resuspended.



Figure 10. Falcon tubes at the end of step 7 containing resuspended Symbiodiniaceae cells in 1 ml of seawater.

- 8 Optional step: Combine samples (of the same genotype) to minimise the number of tubes to handle in the next round.
N.B Do not combine separated cells with tubes that still contain lipids. No further centrifugation is needed once cells are isolated and the lipids have been removed.
- 9 For samples that require another round of separation, top up all tubes with seawater to equal volumes of 10mL and pipette mix (3 times).
- 10 Repeat steps 5, 6 and 7 until no lipids remain using the centrifuge specifications in Figure 11 and 12. Once no lipids remain and all samples are cleaned of lipids and resemble figure 10, continue to step 11.

N.B There are different centrifuge specifications for rounds 2 and 3, see figures 11 and 12.

Record any deviations from these specifications and only conduct these rounds if necessary to remove lipids.

	A	B
	RCF	3000 x g
	TIME	20 minutes
	TEMP	23 degrees celsius

Figure 11. Centrifuge specifications for round 2.

	A	B
	RCF	2500 x g
	TIME	12 minutes
	TEMP	23 degrees

Figure 12. Centrifuge specifications for round 3.

Section 4: Quantify cell count for each genotype

- 11 According to their respective genotypes, combine falcon tubes into a 250 mL flask, do not mix or contaminate genotypes. Label the flask with the appropriate genotypic

identification.

N.B. The flask containing the sample is called "master culture genotype x".

If two genotypes, A and B, are being processed in this protocol, they will be separate and labelled master culture genotype A and master culture genotype B.

- 11.1 Top up the master cultures with seawater to a known volume (e.g 100mL or until they match the colour of the sample in the black circle in Figure 13).

N.B. This step is required for:

(1) Total cell count calculations (steps 11.2 and 11.3)

(2) Dilution of Symbiodiniaceae cell abundance per millilitre of seawater to a workable density



Figure 13. Colour chart for cell density dilutions, the sample in the black circle shows the colour for the correct dilution.

- 11.2 Determine the **number of cells per 1mL**, this number is deemed "the Calculated Cell Count" (**CCC**).

Tip: Use a haemocytometer that uses only 10ul of the sample. Given the microscopic nature of the Symbiodiniaceae cells, 10ul of a correctly diluted sample will have approximately 60 cells per grid. Larger quantities are likely to contain a much greater number of Symbiodiniaceae cells and large counts become unreliable.

N.B. Homogenise the sample thoroughly each time before sub-sampling. Achieve this by pipette mixing 10 times around the flask.

*Example: **CCC** = 587,500 cells / 1mL*

- 11.3 Calculate the required volume for the Desired Cell Count (**DCC**).



Example:

- The desired cell count is **how many cells a given DNA extraction protocol requires to not overload the chemistries**, for this example, the DNA extraction protocol requires 5 million cells. Therefore:
- $DCC = 5,000,000 \text{ cells}$
- $CCC = 587,500 \text{ cells/mL}$

To calculate the required volume, divide the desired cell count by the calculated cell count:

$$\begin{aligned} & DCC / CCC \\ & 5,000,000 \text{ cells} / 587,500 \text{ cells/mL} \\ & = 8.5 \text{ mL} \\ & = 8.5 \text{ mL of the master culture contains 5 million cells.} \end{aligned}$$

If the master culture was made up to a total of 100mL in step 11.1, it will be possible to aliquot a total of 11 samples for snap-freezing.

$$\begin{aligned} & 100 \text{ mL (total volume)} / 8.5 \text{ mL (required volume for DCC)} \\ & = 11.76 \\ & = 11 \text{ complete samples of 5 million cells can be aliquoted from the master culture.} \end{aligned}$$

- 11.4 Label the necessary amount of 1.5mL LoBind Eppendorf tubes with genotypic identification and quantity of cells (*i.e Genotype M.aeq04, 3 million*).

Section 5: Aliquot and re-pellet into final tubes

- 12 If the required volume (calculated in step 11.3) **exceeds** the volume of the final tubes (in this case 1.5mL Eppendorf tubes), follow steps **12.1, 12.2, 12.3** and proceed to step 12.5.

If the required volume (calculated in step 11.3) **is less than** the volume of the final tubes (in this case 1.5mL Eppendorf tubes), go directly to step **12.4** and proceed to step 12.5.

- 12.1 For required volumes which **exceed 1.5mL**, aliquot the required volume into clean 15mL falcon tubes (*see Figure 14*) and centrifuge in a benchtop centrifuge to the specifications outlined in Figure 15.



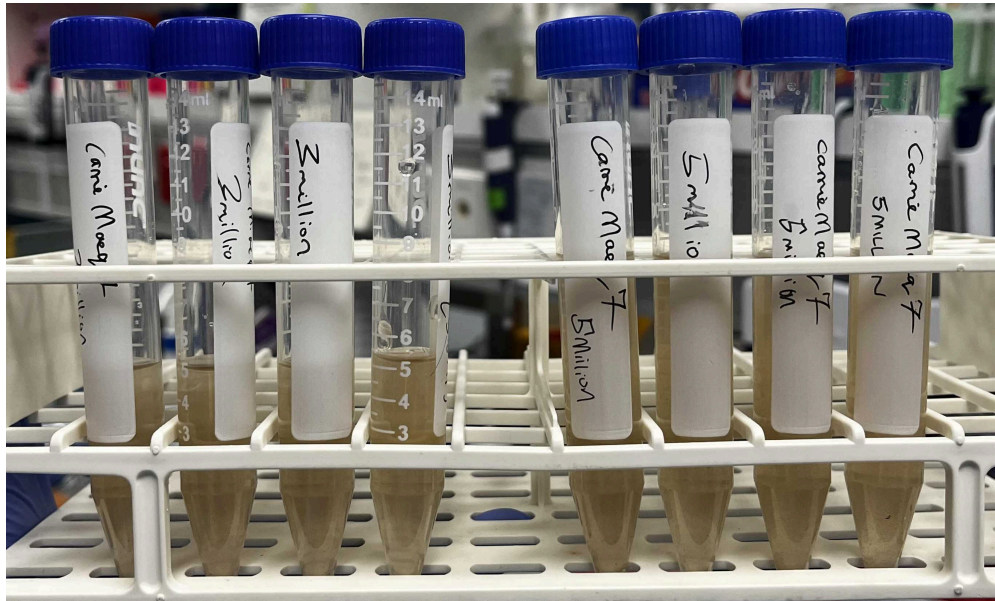


Figure 14. This example shows aliquots of two desired cell count (DCC) amounts, left: 3 million cells and right: 5 million cells.

A	B
RCF	2500 x g
TIME	10 minutes
TEMP	23 degrees celsius

Figure 15. Centrifuge specifications for benchtop centrifuge for required volumes greater than 1.5mL.

- 12.2 Discard the supernatant and resuspend the pellet (pipette mix) in 1mL of seawater. 
- 12.3 Pipette the resuspended Symbiodiniaceae cells into the labelled 1.5mL LoBind Eppendorf tube. 
- 12.4 For required volumes **less than 1.5mL**, aliquot the required volume from the master culture directly into the 1.5mL LoBind Eppendorf tubes.
N.B. Ensure the Symbiodiniaceae cells in the master culture are continually homogenised by pipette mixing.
- 12.5 Centrifuge all 1.5mL tubes in a micro-centrifuge at the following specifications:

	A	B
	RCF	Maximum RCF
	TIME	10 minutes
	TEMP	23 degrees celsius

Figure 16. Centrifuge specifications for micro-centrifugation of 1.5mL tubes.

- 12.6 Pour supernatant (seawater) into a liquid waste bin and carefully pipette any excess water from around the pellets.
N.B seawater can affect the effectiveness of snap freezing.



Figure 17. Drying the pellet and ensuring the removal of excess seawater prior to snap-freezing.

Section 6: Snap freeze samples with liquid nitrogen and store in -80 C

- 13 Use a Styrofoam raft to snap-freeze the tubes on a shallow bath of liquid nitrogen.
- 13.1 Remove the samples from the raft and place into a sample box.
- 13.2 Store in a -80C freezer until extraction.