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# ACME-sorbitol (ACMEsorb) Fixation and Mechanical Dissociation of Whole Soft-Bodied Cnidarians

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**We use this protocol and it's working**

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## Abstract

**ACME (Acetic-Methanol) maceration** is a tissue dissociation and fixation method described by Jordi Solana's group that preserves both cell morphology and RNA integrity, and is compatible with single-cell transcriptomics (scRNA-seq) (García-Castro et al., 2021). ACME has been tested on various organisms, including freshwater planarians (*Dugesia japonica*), annelids (*Pristina leidyi*), snails (*Limnaea stagnalis*), vertebrate embryos (*Mus musculus* and *Danio rerio*), terrestrial arthropods (*Parasteatoda tepidariorum* embryos and *Drosophila melanogaster* larvae), and the starlet sea anemone (*Nematostella vectensis*), which inhabits brackish waters (García-Castro et al., 2021).

However, for marine organisms, this protocol proved suboptimal in our hands, as we observed cells bursting immediately upon contact with the fixative solution—presumably due to the hypo-osmolarity of the fixative relative to seawater. To compensate for the osmolarity and preserve cell integrity during fixation, we replaced the water (or 1× PBS) fraction in the original ACME formulation with a 0.8 M sorbitol solution (Najle et al., 2023). This modified version, which we named **ACMEsorb**, was successfully applied for single-cell atlasing of various marine species. ACMEsorb fixation of whole organisms, followed by cell dissociation, is the preferred method, as it minimises cellular stress and reduces viability biases observed during the dissociation of fresh samples.

Dissociation post-fixation can be performed either mechanically, as described here, or enzymatically (a protocol using cold-protease dissociation after ACMEsorb fixation is provided separately). In this protocol, we use the gentleMACS$^{TM}$ Octo Dissociator with a custom program (see below) to fix and dissociate whole specimens of the starlet sea anemone *N. vectensis*. This protocol has also been successfully applied to three other sea anemone species (*Exaiptasia diaphana*, *Diadumene lineata*, and *Metridium senile*), with similar results.

## Guidelines

- Use ultrapure reagents (free of nucleases) and keep your pipettes and workbench clean and free of RNases (clean surfaces with RNase Zap or similar) throughout the whole experiment.
- Use nuclease-free water (for example, Sigma-Aldrich #W4502-1L) for all the solutions and buffers. During sample prep, keep all the reagents and samples on ice unless otherwise indicated in the protocols.
- ACMEsorb-fixed cells are very resistant to mechanical stress. This is the reason for the high centrifugation speed (2500 x g) used after fixation, which is intended to maximize cell recovery. However, this speed can be optimized (reduced) to avoid cell clumping, provided this is observed after thoroughly resuspending the pellet.

## Materials

**Bill of Materials:**

- Vacuum filter system (e.g., Corning CLS430517)
- GentleMACS C tubes (Milteny #130-093-237)
- Sorbitol (Sigma-Aldrich #S1876)
- BSA Fraction V (Sigma-Aldrich #126609-10GM)
- $NaHCO_3$ (Sigma-Aldrich #S5671)
- KCl (Sigma-Aldrich #P9541)
- NaCl (Sigma-Aldrich #S7653)
- $Na_2SO_4$ (Sigma-Aldrich #239313)
- 1M Tris-HCl pH8 (Life Technologies #15568-025)
- RiboLock RNase inhibitor (Thermo Fisher #EO0382)
- Methanol (Sigma Aldrich #34860-1L-R)
- Glacial Acetic Acid (Merck # 1000631000)
- Glycerol (Sigma Aldrich # G7757-1GA)
- 10X Ca-Mg-free PBS (Thermo Fisher #AM9624)

**Equipment:**

- Refrigerated centrifuge with swing-bucket rotor
- GentleMACS Octo-dissociator
- Vortex

**Stock Solutions:**

**2.4 M Sorbitol**

- Weigh 218.6 g of sorbitol and place it in a 1 L container.
- Add 250 mL of nuclease-free water (NFW) and dissolve using a magnetic stirrer. Sorbitol dissolution is endothermic, so gentle heating may be required to aid dissolution.
- If some crystals remain undissolved, add a small amount of additional water, being careful not to exceed a total volume of 500 mL.
- Once fully dissolved, use a volumetric cylinder to bring the volume up to 500 mL with NFW.
- Filter the solution through a 0.22 µm membrane using a bottle-top vacuum filter system.
- Store at Room temperature .

**10% BSA (Nuclease-Free)**

- Add 10 mL of nuclease-free water to a bottle of BSA.
- Dissolve thoroughly by gentle agitation, minimizing foam formation.
- Dispense into 1 mL aliquots and store at -20 °C .

**Nematostella vectensis Calcium and magnesium-free artificial seawater (Nv-CMFSW)**

- Weigh the following reagents and place in a clean 1 L recipient:
  0.06 g of $NaHCO_3$
  0.26 g of KCl
  9 g of NaCl
  0.33 g of $Na_2SO_4$
  3.33 mL of 1M Tris-HCl pH8
- Add Milli-Q water to approximatelly 800 mL and stir using a magnetic stirrer until all salts are fully dissolved.
- Once fully dissolved, use a volumetric cylinder to bring the volume up to 1 L with Milli-Q water.
- Filter the solution through a 0.22 µm membrane using a bottle-top vacuum filter system (e.g., Corning #CLS430517).
- Store at r Room temperature .

**Resuspension Buffer 1 (RB1)**

- Prepare 1X calcium- and magnesium-free PBS (Ca-Mg-free PBS) as the buffer base.
- Add 10% BSA (Nuclease-free) to a final concentration of 0.1%. For example, add 100 µL per 1 mL of final volume.
- Add sorbitol to a final concentration of 0.8 M. For example, if using a 2.4 M sorbitol stock, add 333 µL per 1 mL of final volume.
- Add RiboLock RNase inhibitor to a final concentration of 0.2 U/µL (e.g., 5 µL of RiboLock per 1 mL of buffer).
- Mix gently by inversion or pipetting. Avoid vortexing to preserve enzyme activity.
- Prepare fresh or keep On ice until use.

## Safety warnings

Volumes of fixative solution and buffers are orientative and should be adjusted according to the amount of material. As a reference, the volumes specified here were used for 3 *N. vectensis* specimens of ~1cm each.

## Ethics statement

The protocols.io team notes that research involving animals and humans must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

## Before start

Prepare an appropriate volume of **ACMEsorb solution** and **Resuspension Buffer 1 (RB1)** based on the number of samples to be processed. Refer to the 'Materials' section for the composition of each solution.

## Procedure

1h 20m 30s

1 Transfer animal(s) to a small Petri dish or 6-well plate, in a minimum volume of artificial seawater (ASW) at the corresponding salinity depending on the species. Here, we use 14 ppm (1.4 g/L) Red Sea Salts for *N. vectensis*. 1m

2 Quickly rinse the animals twice with 2 mL Nv-Calcium and Magnesium-free seawater (Nv-CMFSW). 2m

3 Remove as much Nv-CMFSW as possible, and add 3 mL of freshly made ACMEsorb solution of the following composition: 1m

- 1950 µl 1.2M sorbitol
- 300 µl glycerol
- 300 µl glacial acetic acid
- 450 µl methanol

4 Immediately chop the animals into 3-4 mm pieces using a scalpel or a razor blade. 3m

5 Using a wide bore tip or a plastic Pasteur pipette, transfer the tissue pìeces in ACMEsorb to a gentleMACS C tube. 5m

6 Place the tube in the gentleMACS dissociator, and run the following pre-set program: 42m 30s

| | Step | Speed (rpm) | Time (hh:mm:ss) |
|---|---|---|---|
| | Initial incubation (fixation) | 40 | 00:20:00 |
| | Dissociation & fixation (repeat 2x) | 40 | 00:10:00 |
| | | 300 | 00:00:10 |
| | | -800 | 00:00:05 |
| | | 600 | 00:00:10 |
| | | -600 | 00:00:05 |
| | | 120 | 00:00:15 |

GentleMACS program 'BCA001'

The full fixation/dissociation program lasts for 42.5 min.

7 After GentleMACS program is finished, evaluate the dissociation efficiency. If big chunks or undissociated tissue are still visible by the end of the procedure, try to aid dissociation by pipetting with a P1000 set at 300 µl. 3m

8 Once fixation/dissociation is completed, transfer the sample to a 5 mL protein LoBind tube using a P1000 and filtering through a 70 µm strainer. 2m

9 Collet the cells by 2500 x g, 4°C, 00:05:00 , in a swing-bucket rotor. 5m

10 Remove ¾ ( 2 mL ) supernatant and add an equal volume of Resuspension Buffer 1 (RB1). 3m

11 Gently resuspend the cell pellet by pipetting up and down 5 times with a P1000 set at 300 µl. 3m

12 Collet the cells by 2500 x g, 4°C, 00:05:00 , in a swing-bucket rotor. 5m

13 Carefully remove as much supernatant as possible and thoroughly resuspend the pellet with 900 µL RB1. 3m

14 Add 100 µL DMSO, invert the tube three times to mix and freeze at -80 °C using a Nalgene® Mr Frosty freezing container following the recommendations of the manufacturer. 2m

## Protocol references

1: García-Castro H, Kenny NJ, Iglesias M, Álvarez-Campos P, Mason V, Elek A, Schönauer A, Sleight VA, Neiro J, Aboobaker A, Permanyer J, Irimia M, Sebé-Pedrós A, Solana J. ACME dissociation: a versatile cell fixation-dissociation method for single-cell transcriptomics. Genome Biol. 2021 Apr 8;22(1):89. doi:10.1186/s13059-021-02302-5. PMID: 33827654; PMCID: PMC8028764.

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