Frozen Tissue Homogenization

Application: Selection of a homogenization method for cnidarians is dependent on how the specimen is preserved and the type of cnidarians (containing calcium carbonate skeleton, gorgonian skeleton or soft body tissue only) as well as the intended assays. The homogenization protocol described here is appropriate for western blots, ELISA, total porphyrin determination and many DNA-based assays.

Equipment & Supplies:

- 2 ml cryovials
- Bench paper, e.g. Versi-Dry® lab soakers (VWR International, Sugarland TX)
- Ceramic mortar & pestle, e.g. catalogue # 60313 chemical-porcelain mortar with 80 mm diameter, 53 mm height, 65 ml capacity and 130 mm length pestle (Coorstek, Golden CO)
- Teflon® cutting board
- Freezer grip, e.g. rubber drawer liners cut into 5 in x 10 in swatches and folded over once (household goods store) or Kevlar w/Latex Coated Palm Gloves (e.g. Wells Lamont Fisher)
- Freezer mill for bulk grinding, e.g. Freezer/Mill 6850, (SPEX CertiPrep®, Metuchen NJ)
- Freezer mill grinding accessories i.e., polycarbonate grinding vial to hold sample, stainless steel impactor to mill sample into a fine powder, end plugs for vials to retain sample in vial, and other accessories required by the manufacturer of the freezer mill, e.g. extractor, tube racks (see manufacturer's manual for 6850 Freezer/Mill (SPEX CertiPrep®)(**LINK** below)
- Hammer & chisel, e.g. slot head screwdriver
- Hemostat or forceps
- Liquid nitrogen (LN_2)
- LN_2 Dewar, e.g. 4150 Dewar Flasks (Nalgene labware – Thermo Fisher Scientific, Rochester NY)
- Micropipettor with 20-200 μl range (P200), (e.g. Gilson Inc, Middleton WI)
- P200 micropipette tips
- Spatulas, e.g. Fisherbrand Cat. 2140115; 5.5 in, blade 19 x 4.8 mm (Fisher)
- Styrofoam ™ container (e.g., 6x8 inches with dividers) to chill spatulas and cryovials with LN_2
- Transfer pipette
- Weigh boats or other disposable containers for fragmenting coral tissue

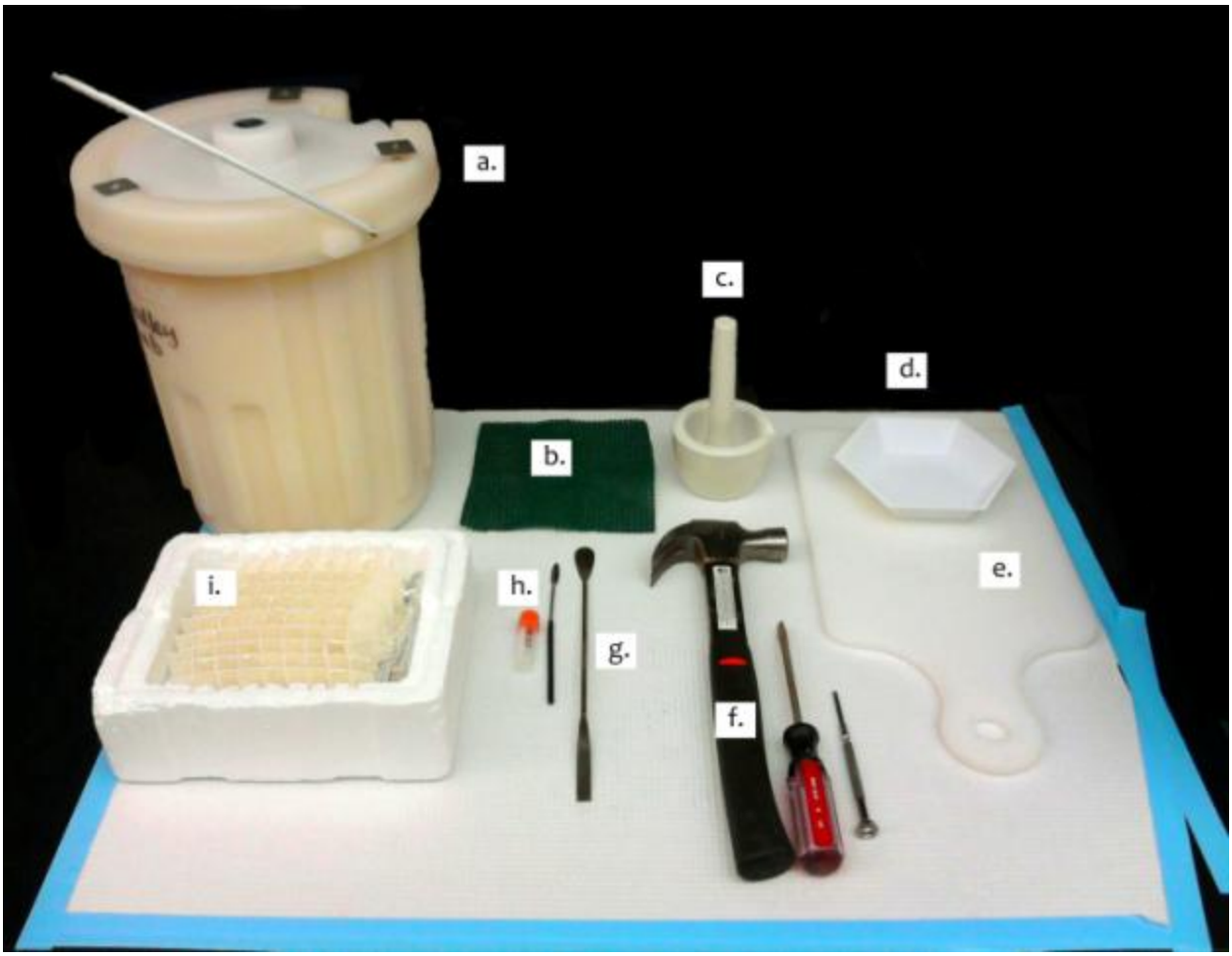

Figure 1. Grinding Materials: a) Dewar, b) freezer grip, c) mortar and pestle, d) weigh boat, e) cutting board, f) hammer and chisels, g) spatulas, h) cryovial, and i) Styrofoam® container with dividers.

Methods for Frozen Tissue Homogenization

A. Frozen Tissue Homogenization Using a Freezer Mill

1. Chill freezer mill with LN_2 according to the manufacturer's recommendation.
2. Label grinding vials numerically and keep a log of numbers in relation to sample information.
3. Insert a tube stand large enough to hold grinding vials in a Styrofoam ® container filled with liquid nitrogen (LN_2) sufficient to cover the lower ¾ of the vials.
4. Seal the bottom of the grinding vials with the stainless steel end plugs, insert steel impactors, and chill vials in LN_2. *Do not chill more vials than the mill holds at a time because frost build-up can interfere with the sample integrity.*
5. Use separate container (e.g., Styrofoam ®) with cryobox dividers to chill a few spatulas and pre-labeled 2 ml cryovials for ground tissues. Larger cryovials can be used if aliquotting tissue into multiple vials is not required.
6. Prior to grinding a sample, place it onto a clean surface (e.g. weigh boat) and

inspect each piece for endolithic algae in the skeleton or other contaminating epibionts. Remove these and excess skeleton using a hammer and chisel or micro-slotted screwdriver, ensuring the tissue remains frozen by adding LN_2 as needed. Larger coral pieces require fragmenting to easily fit into grinding vials. Photo document the samples at this point.

7. Place frozen tissue fragments into chilled grinding vials, making sure fragments fit loosely around impactor and do not exceed the amount of tissue recommended by the manufacturer.
8. Experimentally determine the parameters for milling tissue using the manufacturer's guidelines for grinding, duration, and impact frequency.
9. Follow recommendations by the manufacturer for retrieving samples from freezer mill.
10. Use the extractor to open each grinding vial according to manufacturer's instructions.
11. Use a clean pre-chilled spatula to scoop ground tissue into chilled cryovials and place into LN_2 prior to storage at -80°C.

*This homogenization can be used for all frozen tissue samples that are in large quantity and meet the size limit recommended by the manufacturer. Refer to the manufacturer's manual(**LINK is URL under Links below**) for specific guidelines and parameters to follow when using the freezer mill*

B. Frozen Tissue Homogenization Using a Mortar and Pestle (See Fig. 1 & 4)

1. Cover work area with absorbent pad (bench paper) and chill cleaned spatulas in LN_2.
2. Pre-chill clean mortar and pestle by filling with LN_2 and letting it evaporate twice before adding tissue sample. Set pestle upright in the mortar prior to adding LN_2.
3. A clean hammer and chisel rinsed with 70% ethanol can be used to remove visible algae on coral fragments before grinding.
4. Add tissue fragments to mortar and fill halfway with LN_2. Let the LN_2 evaporate before grinding with the pre-chilled pestle.
5. Add LN_2 slowly if sample begins to thaw. *The force exerted by pouring too quickly can cause the boiling tissue to slosh out of the mortar.* It is important to keep sample frozen throughout the grinding process. Use an insulated rubber grip (freezer grip) to hold mortar while grinding. Grind slowly initially and use the gloved-hand that holds the mortar to partially cover the top of the mortar while grinding.
6. Intermittently add LN_2 to your sample to prevent thawing and to allow ease of grinding tissue.
7. Use pre-chilled spatulas to dispense ground tissue from mortars into 2 ml cryovials. *Do not close tubes until all nitrogen vapors have evaporated to avoid pressure build up from the nitrogen gas.*

8. After grinding process is completed, store ground samples at -80°C. For long-term storage consider a liquid nitrogen freezer.

Considerations & Caveats:

A. Time

The freezer mill operation for 25 stony coral samples, will take 2 to 5 hr with a minimum of two people working together. Most of the time is spent removing extraneous endolithic algae and excess skeleton from coral samples prior to grinding. Grinding 20 samples with a mortar and pestle can take up to two 8 hr days depending on how big the samples are and how much endolithic algae is associated with the samples.

B. Samples

It is best to use the freezer mill for samples that are 2 cm or greater and with more than 20 samples. Refer to the manufacturer's recommendation for sample size limitations.

C. LN_2

*Review material safety data sheet (MSDS) before handling liquid nitrogen **(LINK to pdf).***

Expected Results

1. Removing algae: Shown in Figs. 2 & 3, forceps are used by one individual to hold the frozen sample in place while another individual chips away endolithic algae (within stony coral skeleton) using a hammer and slotted head screwdriver. If contaminating epibionts are not an issue, then breaking up the frozen tissue for grinding ease is recommended.

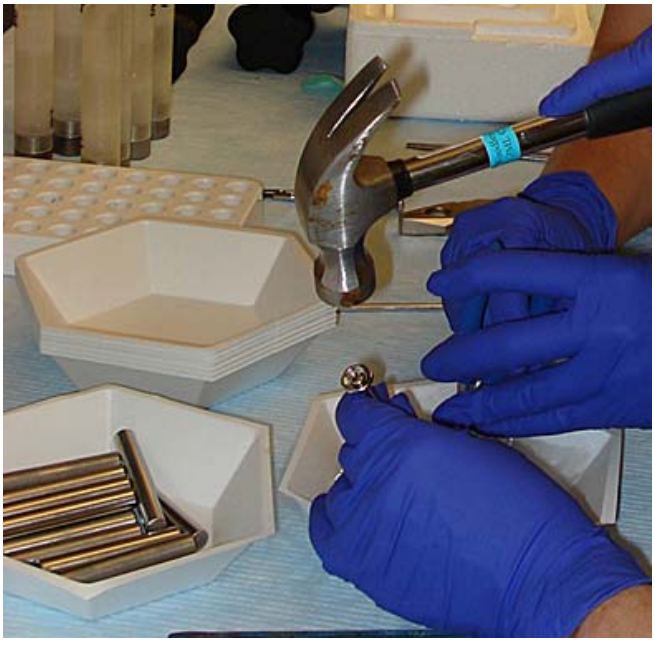

Figure 2. Removing endolithic algae with a hammer and slotted head screwdriver.

Figure 3. Breaking up frozen coral.

2. Ground Coral: A chilled mortar and pestle are used to grind small frozen tissue samples to a fine powder. The ground sample should be flour like and not the consistency of sand as shown In Fig. 4 & 5. In Fig. 5, the finely ground frozen tissue sample is carefully scooped into a chilled 2 ml cryovial using a chilled spatula.

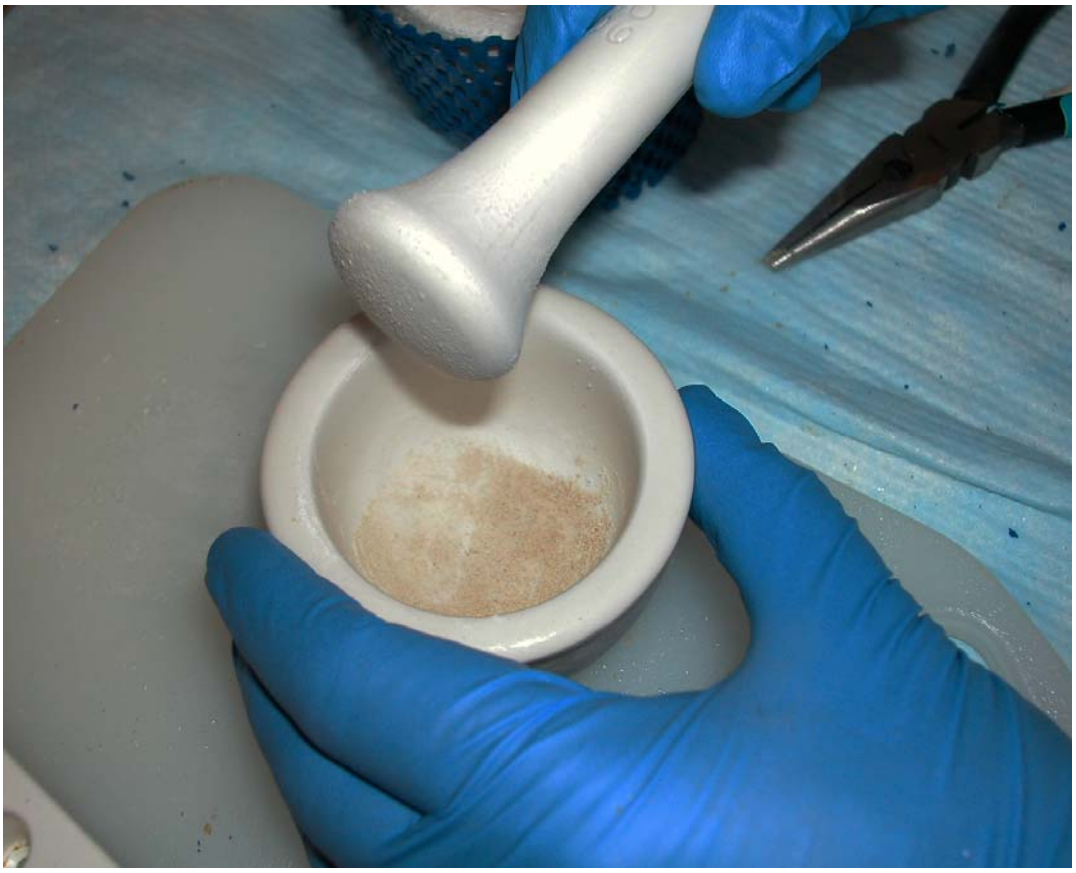

Figure 4. Grinding coral with a chilled mortar and pestle.

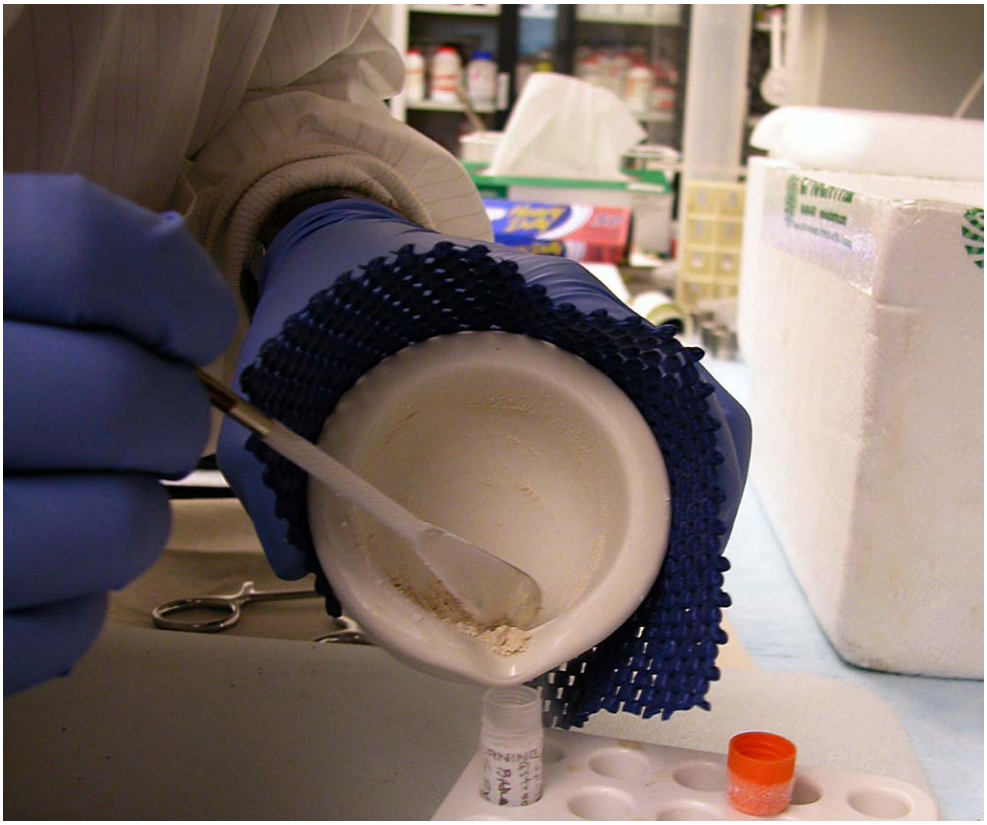

Figure 5. Transferring finely ground coral into a chilled cryovial for longterm storage at -80°C.

Links

SPEX CertiPrep® manual: http://www.gbcpolska.pl/ul/mlynki/pdfy/6850manual_en.pdf

(**LINK to TM pdf**)

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