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Extraction of Total Soluble Lipid from Ground Coral Samples

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1 Works for me dx.doi.org/10.17504/protocols.io.bc4qiyvw

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ABSTRACT

This method is adapted and updated from methods originally published in Grottoli et al. (2004) and is based on the original methods of Folch & Stanley (1957), and Bligh & Dyer (1959). There are five parts to extracting lipids from ground corals: 1) grind and sub-sample the coral and store at -80 °C until ready to extract, 2) freeze-dry the sample, 3) extract the lipids from the freeze-dried samples, 4) standardize the lipid concentration to ash-free dry weight (AFDW), and 5) resuspend the extracted lipid for long-term storage and possible later analysis of lipid classes or isotopes. The lipid extraction procedure must be conducted in a fume hood with the sash as low as possible with the researcher wearing protective eyewear, gloves, and lab coat at all times.

Important considerations regarding lipid analysis were gained from reading Chapter 1.3 “Lipid extraction, storage, and sample handling” from the textbook *Lipid Analysis* by Christie (2003).

This method was originally developed by Andréa Grottoli and refined by Rowan McLachlan (06-11-18) with the guidance of Dr. Agus Muñoz-Garcia at The Ohio State University. This protocol was written by Rowan McLachlan (03-12-2020).

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KEYWORDS

lipid, coral, soluble lipid, lipid extraction, solvent extraction and partitioning, liquid–liquid extraction (LLE)

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GUIDELINES

Practical considerations

- Glassware: the solvents used in this procedure will also extract contaminants from any containers or apparatus containing plastic. Therefore, only Teflon-lined caps and clean glassware are used throughout this procedure.
- Antioxidant: to prevent inadvertent hydrolysis of lipids, an antioxidant such as butylated hydroxytoluene (BHT) should be added to all solvents in this procedure (about 50 mg per liter).
- Extraction: all solvent extractions must be conducted in sealed containers to prevent evaporation. Otherwise, the chloroform will evaporate quicker than the methanol, and your solvent system ratio will change throughout the duration of the extraction.
- Storage: Lipid extracts should not be stored in the dry state but resuspended in 2ml BHT-enhanced chloroform and stored in Teflon-lined screw cap amber vails e at -80°C. Oxidative degradation is slower in solutions even in the absence of added antioxidants. Air and light should be avoided
- Operators: This method can be conducted by one person; however, this protocol is most efficient if a second person conducts step #17 concurrently as the first person conducts steps 18 - 21.

MATERIALS TEXT

LEGEND

[1] for Grinding of Coral Fragments

[2] for Freeze-Drying Ground Coral Samples

[3] for Preparation on Day of Lipid Extraction

[4] for Timed Extraction Procedure

[5] for Re-suspending Lipids

[6] for AFDW Procedure

Reusable materials:

- Mortar and pestle [1]
- Plastic spatula [1]
- Metal spatula [1, 2, 4]
- 4 ml volume, amber pyrex, flat bottomed, Teflon-lined screw-cap bottles (two per sample) [1, 5]
- Wide-mouth flask attachment for freeze dryer [2]
- 18 ml volume, clear pyrex, round-bottomed, Teflon-lined screw-cap vials [2]
- Sharpie [3]
- 25 ml Erlenmeyer flasks [3]
- Mechanical pencil [3]
- 2 retort stands [3]
- 13 retort rings, covered with plastic tubing [3]
- 60 ml Pyrex separatory funnels with stopper and stopcock (Teflon or glass)[3,4]
- 250 ml Borosilicate funnel (47 mm diameter) [4]
- Borosilicate frit base (47 mm diameter) [3,4]

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- Rubber stopper with a hole for frit-support (part of vacuum rig) [4]
- Metal clamp for filtration assembly [3,4]
- 500 ml Erlenmeyer flask with side arm (part of vacuum rig) [4]
- 1000 ml measuring cylinder [3]
- Polyvinyl alcohol-coated (PVA) gloves [3]
- Large glass funnel for pouring chemicals from 5 L bottles into measuring cylinders [3]
- 1000 ml glass-stoppered volumetric flask (or alternative containers) for storing 2:1 Chloroform:Methanol solution [3]
- 200 ml glass-stoppered volumetric flasks (or alternative containers) for storing 100% chloroform, 100% methanol, and potassium chloride solution, respectively [3]
- 250 ml glass beakers [4]
- 10 ml glass syringes [4]
- Glass Petri dish, ~ 4 cm in diameter [4]
- 50 ml glass beaker [4]
- Zip-ties, connected into a loop that *slightly* greater diameter than the Erlenmeyer flasks [4]
- Plastic test tube rack, with holes wide enough for neck of 25 ml Erlenmeyer flasks to fit through (part of drying rig) [4]
- Plastic test tube rack, fitted with cut pipette tips, rigged to hold glass Pasteur pipettes (part of drying rig) [4]
- Aquarium airline tubing, connectors and taps (part of drying rig) [4]
- Large metal baking tray, at least 5 cm depth (water bath) [4]
- Thermometer [4]
- 1 ml Hamilton glass syringe (for re-suspending extracted lipids) [5]

Disposable materials:

- Aluminum pans (two per sample) [1,3]
- 50 ml polypropylene centrifuge tube [1]
- Disposable glass test tubes [3,4]
- GF/F glass fiber filters 42 mm diameter [3,4]
- Kim wipes [2,4]
- Glass Pasteur pipettes [4]
- Nitrile Gloves [1,2,3,4,5,6]
- Teflon Tape [5]

Equipment:

- -80 °C freezer [1,2,6]
- Weighing balance accurate to 4 decimal places [1,2,3,4]
- Benchtop Freeze Dryer [2]
- Fume hood [3,4,5]
- Vacuum pump [3,4]
- Digital clock [4]
- Vortex [4]
- Heat plate large enough to hold water bath (pancake griddle works well) [4]
- Muffle furnace [6]
- Drying oven [6]

Chemicals (please see Tables 1 and 2 for exact volumes required):

- Chloroform ACS grade [3]
- Methanol ACS grade [3]
- 0.88% Potassium chloride (KCl) [3]
- Butylated hydroxytoluene (BHT) [3]
- UHP grade 5.0 Nitrogen [4]

Software:

- Microsoft Excel[4]

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SAFETY WARNINGS

This procedure uses hazardous chemicals (chloroform and methanol)

1. Read the MSDS forms for each chemical in the procedure.
2. Use nitrile or PVA gloves throughout the procedure. If chloroform spills on the Nitrile gloves, remove gloves immediately and discard, wash hands, and use new gloves.
3. Wear a lab coat and safety glasses throughout the procedure.
4. Dispose of all chemical waste in appropriately labeled containers.

BEFORE STARTING

Washing glassware

- All glassware should be washed using phosphate-free soap (e.g. Liquinox) and scrubbed with clean, non-rusted bottle brushes. Glassware should then be rinsed in tap water, before being rinsed three times in three separate baths of milli-Q water.
- Note: glass syringes, mortars, and pestles should not be baked in a muffle furnace as they will crack/deform. Instead, these and any other equipment that cannot be baked should be rinsed in 100% chloroform and allowed to dry in the fume hood prior to use.

Baking glassware & other supplies

- Load glassware into a muffle furnace and bake overnight at 450 °C for approximately four hours. Allow to cool completely in the oven overnight before removing to prevent cracking of the glass.
- Upon removal from muffle furnace, all glassware must be handled using gloves to prevent lipid contamination from skin.
- Bake all GF/F filters (45mm diameter, 0.7 um pore size, one per sample), aluminum weighing pans (two per sample), Pasteur pipets in a muffle furnace at 450°C for two hours. These items do not need to fully cool overnight before removing but can be removed shortly after the baking cycle ends. **Caution as items may be very hot and may cause burns to skin.**

Preparation of ground coral samples

1 **Grind coral fragments.** Grind frozen coral fragments into a homogeneous paste using a chilled mortar and pestle, or within an ice-bath. Attempt to grind the paste into as fine a consistency as possible to get evenly fine skeletal grains.

2 **Weigh and sub-sample ground coral paste.** Place a pre-baked aluminum weighing pan on the balance and tear (i.e. zero). Remove the pan, and using a plastic spatula, transfer all the ground coral material from the mortar into the pan. Place the pan on the balance and record the *total wet weight of the ground coral fragment (grams).* Using a metal spatula, measure ~ 1 g of wet paste from the pan into a *freeze-drying vial* (i.e., pre-burned and labeled, 4 ml volume, amber pyrex, flat bottomed, Teflon-lined screw-cap bottle). Return vial to the -80 °C freezer as soon as possible for storage until you are ready to go to the next step. For best results, extract lipids as soon as possible, as it has been shown that some lipid parameters (cholesterol, triacylyglycerides) may be altered after one year of storage at -80 °C (Devanapalli et al. 2002). The remaining ground coral material in the pan not allocated for lipid extraction can be transferred to a separate 50 ml centrifuge tube and archived in a -80 °C freezer for other future analyses.

Devanapalli B., Bermingham M. A., Mahajan D. (2002). Effect of long-term storage at -80 degrees C on the various lipid parameters in stored plasma samples. Clinica Chimica Acta 322:179-181.

Freeze dry ground coral samples

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3 **Turn on freeze dryer.** Samples are freeze-dried prior to lipid extraction. Arrive early to turn on freeze dryer, as it takes ~40 minutes for appropriate temperature (-53 °C) and vacuum pressure (100 millitorr) to be reached (Fig. 1). Follow the protocol for operating your freeze dryer correctly.

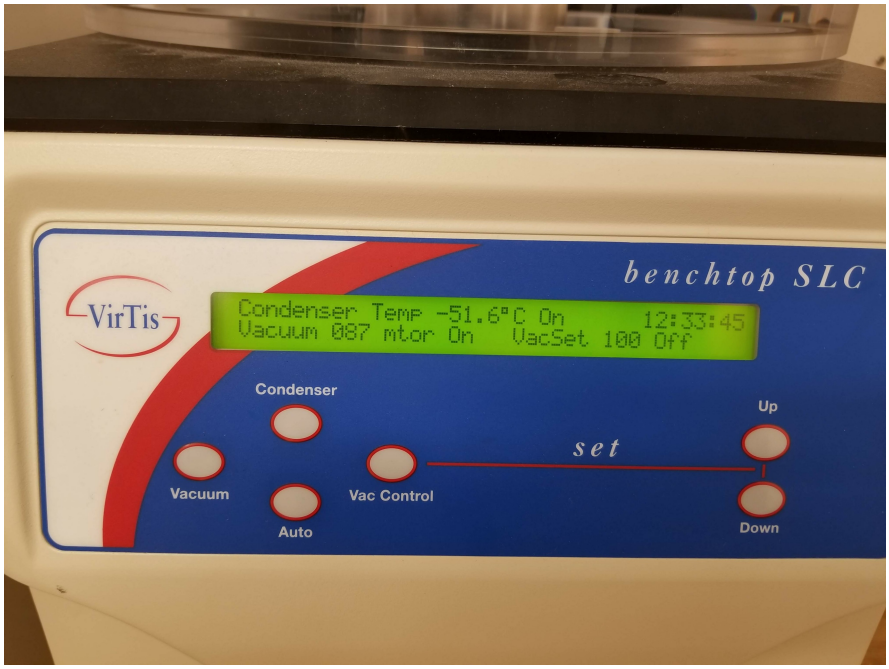

Fig 1. Correct temperature and pressure settings for VirTis Benchtop SLC freeze dryer.

4 **Load samples into the freeze dryer.** When freeze dryer is at the desired temperature and vacuum (Fig. 1), remove your samples (which are in the *Freeze-Drying Vials*) from the -80 °C freezer, and loosen the caps of each vial, but do not remove completely. A maximum of 60 samples can be dried in a 12 hour period (unless a different size of *Freeze-Drying Vial* is used). Assemble vials inside of the wide-mouth flask attachment, ensuring that all vials are upright, and that samples will not spill out (Fig. 2).

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Fig 2. By tilting the wide-mouth flask on its side, it is easier to assemble the *Freeze-Drying Vials* inside in two layers.

5 **Attach wide-mouth flask.** Snap the black rubber cover onto the top of the wide-mouth flask. Attach the tube part of the cover into a Quickseal Valve on the manifold (Fig. 3).

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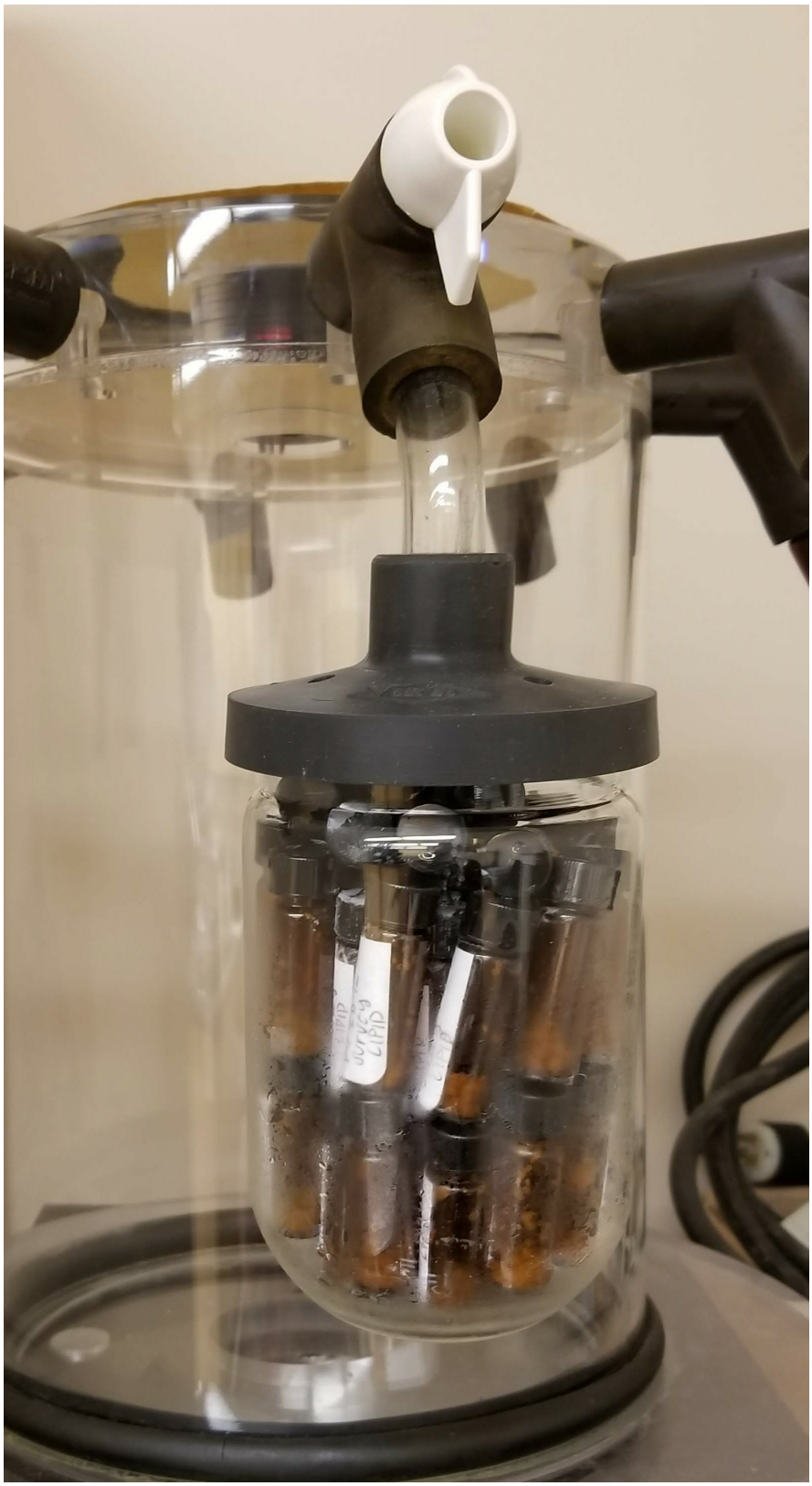

Fig 3. Wide mouth flask with black cap, and tube inserted into the Quickseal Valve on the VirTis Benchtop SLC freeze dryer manifold.

6 **Start vacuum.** Open the QuickSeal valve by turning the white selector knob counter-clockwise a half turn (Fig. 4). Note the vacuum level will be affected as air in introduced with the samples, however it shouldn't take more than 10

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mins for the correct vacuum level to return. Samples should be left in the freeze dryer for 12 hours (+ or – 1 hour, but try to be consistent).

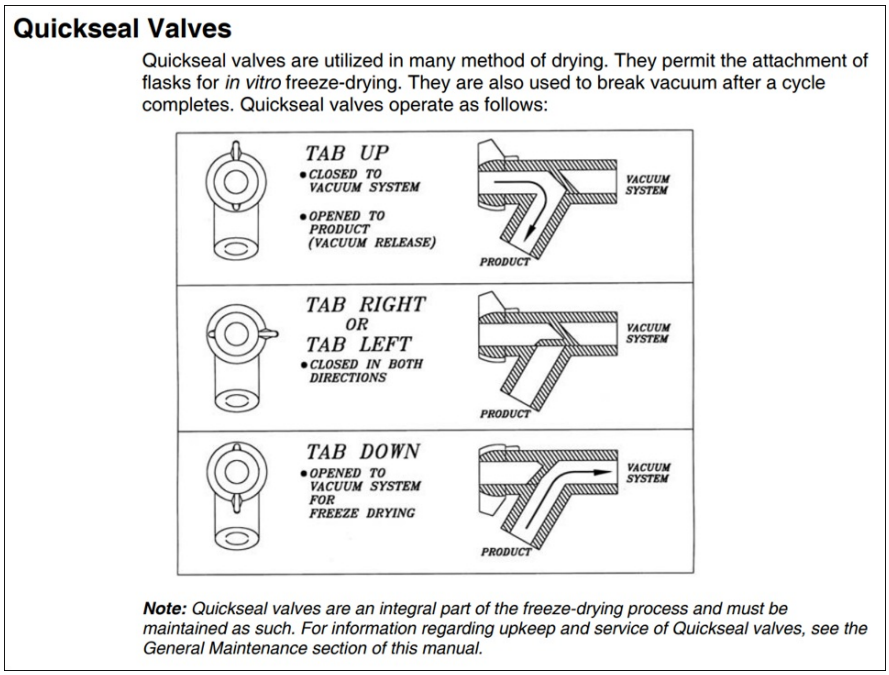

Fig 4. Diagram from user manual showing the open and closed positions of the QuickSeal valves.

7 **Remove samples from the freeze dryer.** When removing samples from the dryer, turn the Quickseal valve to the off position (Fig. 4), and detach the wide-mouth flask. Switch off the freeze dryer. Remove the black cover from the wide mouth flask. As quickly as possible, remove the *Freeze-Drying Vials,* and tighten their screw caps. This is to prevent moisture from entering the sample.

8 **Transfer samples into larger vials.** Unscrew the cap of a *Freeze-Drying Vial,* and using a metal spatula (with a bent end), scrape the freeze-dried power out of the vial and into the *Extraction Vial* (pre-burned and labeled, 18 ml volume, clear pyrex, round bottomed, Teflon-lined screw-cap vial) (Fig. 5). Attempt to remove as much of the powder as possible. Cap all *Extraction Vials* and transfer into the -80 °C freezer once again, awaiting lipid extraction.

Alternatively, the ground sample can be directly partitioned into the *Extraction Vials* in Step 2. However, as these vials are larger, you will not be able to fit as many into the freeze dryer in one batch.

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Fig. 5. Example of vial suitable as *Extraction Vial.* Must have at least 11 ml capacity.

Preparation on day of lipid extraction

9 Label each 25 ml glass Erlenmeyer flask (henceforth, referred to as *E-Flask*) with a sharpie.

Weigh each *E-flask* on a balance. All *E-Flasks* are weighted in triplicate and recorded on side 1 of the lipid datasheet (Fig. 6). Enter these values into excel and calculate the average of the three "*Empty Erlenmeyer Weights".*

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SPECIES-SITE										
Funnel #	DATE: Sample ID	Time Solvent Added	Time Sample Filtered	Empty Erlenmeyer Weight (g) #1	#2	#3	Dry Lipid + Erlenmeyer Weight (g) #1	#2	#3	NOTES
1		:	:							
2		:	:							
3		:	:							
4		:	:							
5		:	:							
6		:	:							
7		:	:							
8		:	:							
9		:	:							
10		:	:							
11		:	:							
12		:	:							
13		:	:							
NOTES										

Fig 6: Side 1 of the lipid datasheet. Record the weight of empty Erlenmeyer flasks in the designated columns. The flip-side (side 2) of this data sheet is shown in Fig 17.

10 Label/number all test tubes using a sharpie (Fig. 7).

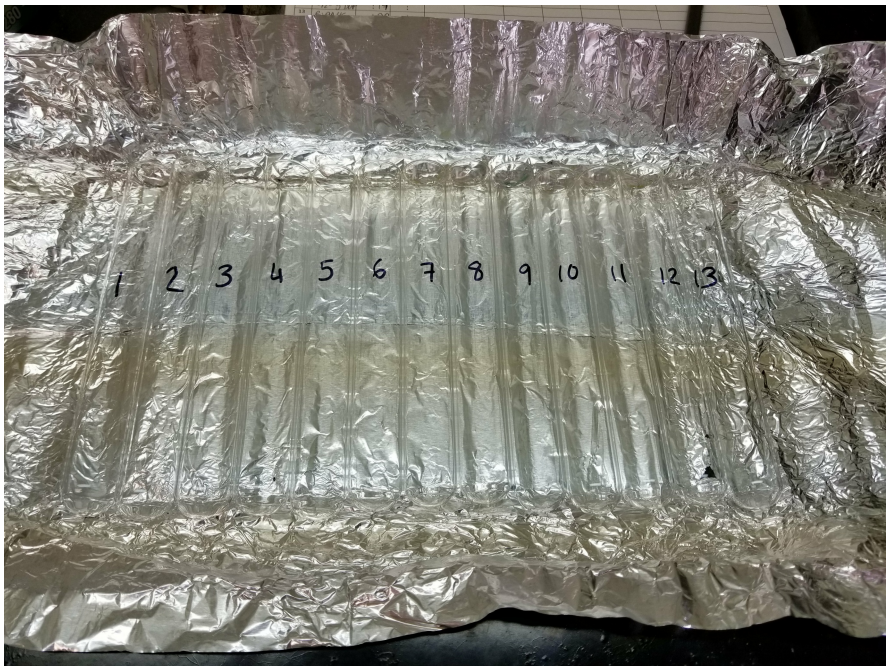

Fig. 7. Disposable test tubes for collecting filtered organic solvent labeled 1 through 13 for twelve coral samples, and one control.

11 Engrave the base of pre-baked aluminum pans with a sample ID using an un-leaded mechanical pencil.

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12 Set-up retort stands with rings to hold separatory funnels (60 ml capacity). The rings should be covered with tubing, so that the funnels do not fall through. Assemble the separatory funnels with their Teflon or glass stopcock. Place separatory funnels into retort stand rings (Fig. 8).

Fig. 8. Retort stand assembly for holding 13 separatory funnels within fume hood. Only 7 separatory funnels are shown above, each with a glass stopper and a Teflon or glass stopcock.

13 Assemble the vacuum-filtration system assembly: funnel (250 ml), glass frit base (47 mm diameter), GF/F filter, metal clamp, rubber stopper, disposable test tube, side-arm Erlenmeyer flask (Fig. 9). *Note: by placing a foam ring inside the side-arm Erlenmeyer flask, this supports the test tube, and prevents it from falling over or breaking.* The test tube will collect the organic phase after it passes through the GF/F filter, thus separating it from the particulate matter.

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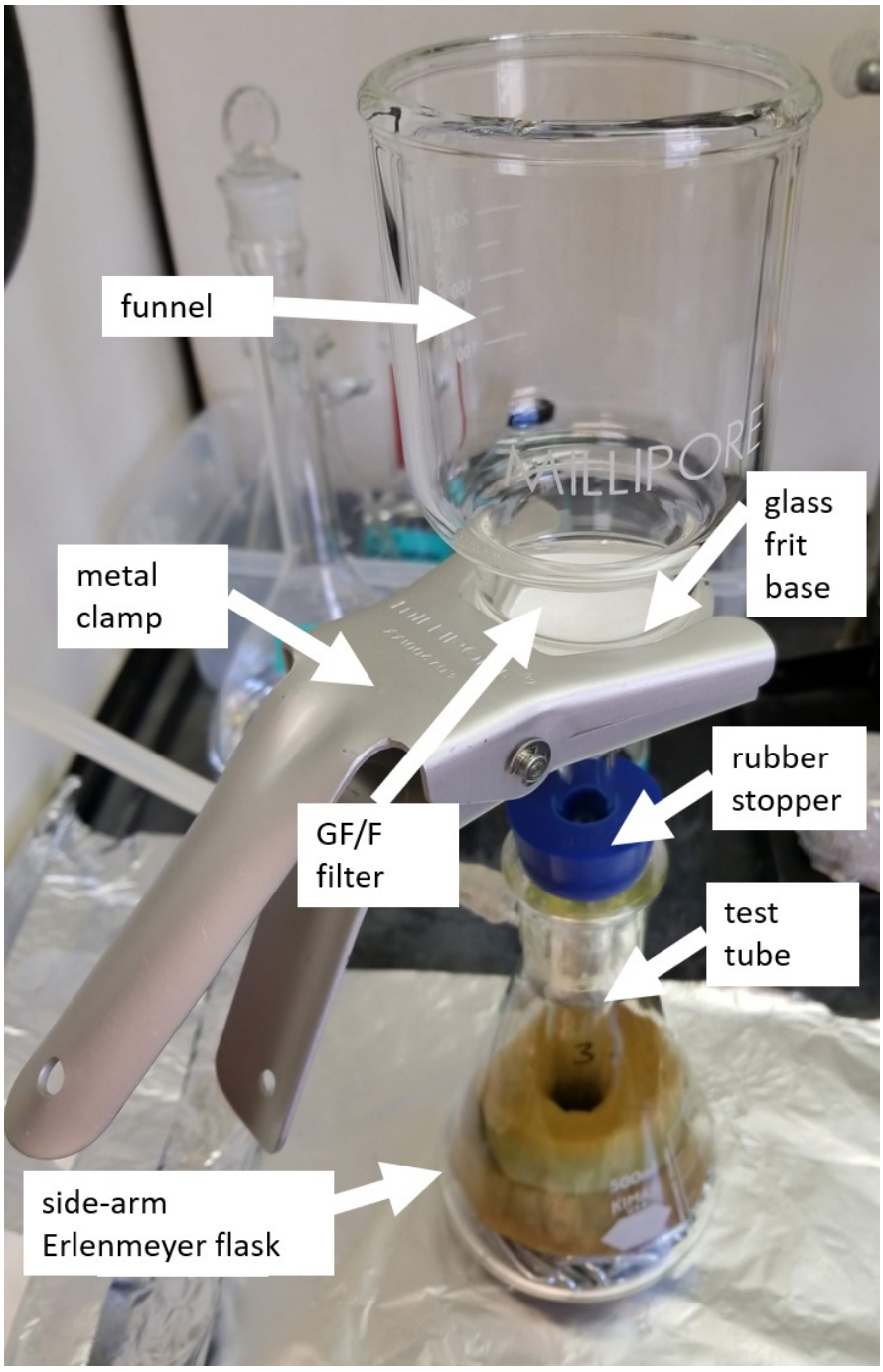

Fig. 9. Components of the vacuum assembly. The side-arm Erlenmeyer flask is connected to an electronic vacuum pump (not shown in the image above). The GF/F filter is placed between the funnel and the glass frit base, held together with the metal clamp. The glass base is connected to the side-arm Erlenmeyer flask using the blue rubber stopper. **MAKE SURE TO PLACE A TEST TUBE INSIDE THE FLASK BEFORE POURING SOLVENT THROUGH THE FILTRATION ASSEMBLY.**

14 **Prepare solvents.** Using a glass measuring cylinder and a glass funnel, dispense chlorofrom and methanol into a 1000 ml glass-stoppered volumetric flask (or alternative container) according to the volumes outlined in Table 1. This is the 2:1 (v/v) chloroform:methanol extraction solvent. The extraction solvent is clear in color and fully transparent. Wear protective polyvinyl alcohol-coated (PVA) gloves during this step. It is paramount that you do not spill chloroform on your skin. Ensure you add about 50 mg of Butylated hydroxytoluene (BHT) per liter of the solvent system prepared. The BHT prevents oxidation of the extracted lipids. Weigh out the BHT using an aluminum pan and spatula.

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Total Solvent Volume	# Samples *(remember to account for blank)*	Chloroform Volume	Methanol Volume	BHT weight
180 ml	7	120 ml	60 ml	0.009 g
360 ml	13	240 ml	120 ml	0.0180 g
720 ml	26	480 ml	240 ml	0.0360 g

Table 1: Guide to solvent volumes depending upon the number of samples/batches to be processed in a given day: 6 samples + 1 control, 12 samples + 1 control, or 24 samples + 2 controls. Make the solvent mixture fresh every day.

Using a measuring cylinder, dispense ~100 ml of 100% chloroform into a 200 ml volumetric flask, and label.

Using a measuring cylinder, dispense ~100 ml of 100% methanol into a different 200 ml volumetric flask, and label.

In total, there should be three separate containers of solvent:
1. *2:1 (v/v) chloroform:methanol*
2. *100% chloroform*
3. *100% methanol*

15 **Prepare KCl.** Using a glass measuring cylinder, prepare a 0.88% potassium chloride (KCl) solution as shown in Table 2. Weigh out the KCl using an aluminum pan and spatula. Combine Milli-Q and KCl powder in a 200 ml glass stoppered volumetric flask (or alternative container). This solution may be stored long-term, but it is preferred that it be made fresh daily.

Total Solution Volume	# Samples *(remember to account for blank)*	Milli-Q Water Volume	KCl weight
30 ml	7	30 ml	0.264 g
60 ml	13	60 ml	0.528 g
120 ml	26	120 ml	1.056 g

Table 2: Guide to potassium chloride solution volumes depending upon the number of samples/batches to be processed in a given day: 6 samples + 1 control, 12 samples + 1 control, or 24 samples + 2 controls.

Timed lipid extraction procedure

16 **Extract Lipids.** Pour ~ 12 ml of 2:1 (v:v) chloroform:methanol from the stock container (Fig. 10A) into a 250 ml beaker (Fig. 10B). Using a 10 ml glass syringe (Fig. 10B), add exactly 10 ml of this to the *Extraction Vial,* containing the freeze-dried sample (Fig 10C). Record time on your datasheet (Fig 6). Cap vial and vortex for 30 seconds. The solvent should now be yellowish in color (Fig. 10D). Using a digital clock for accuracy, delay solvent addition by 10 minutes* between samples to allow for sufficient time to filter each sample later. Store the *Extraction Vial* in a dark cupboard, out of direct sunlight for one hour. *Note: If lipid extracts are destined for lipid class analysis, we recommend that Extraction Vials be individually wrapped in aluminum foil, in addition to being placed in a dark cupboard to ensure that no photooxidation occurs.*

*This time can be reduced to 5 minutes if a second person conducts step #17 concurrently as the first person conducts steps 18 - 21.

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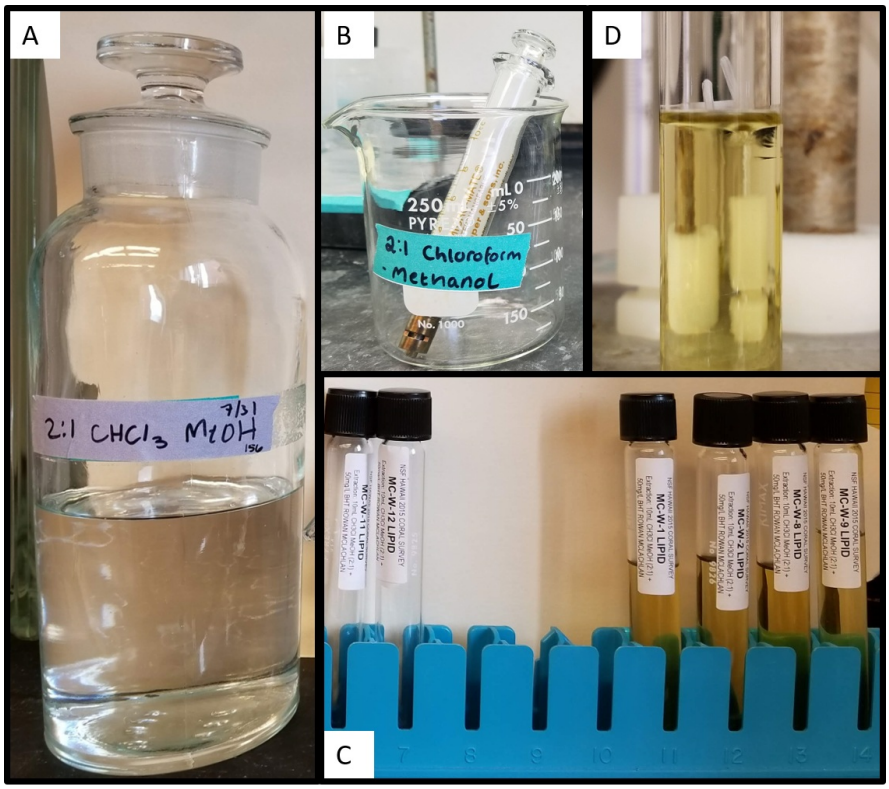

Fig. 10. A) Stock bottle of extraction solvent (2:1 chloroform: methanol + BHT), B) glass syringe and beaker for stock solvent, C) *Extraction Vials* before (left) and after (right) 10 ml of extraction solvent has been added, D) close up of *Extraction Vial* after the solvent has been added showing the instantaneous color change from clear and transparent to yellow and transparent.

17 **Filter samples.** After exactly one hour, pour the contents of the *Extraction Vial* through the glass vacuum filtration system fitted with a pre-burned GF/F filter, and a disposable glass test tube as shown in Figure 9 above. A successful method to ensure all particulate matter leaves the *Extraction Vial* when pouring is shown in the video link below. Basically, tip the vial to move all particulate matter near the mouth/cap end of the vial, then carefully tip the vial back so that only the solvent is at the base of the vial, uncap, then quickly tip the vial upsidedown into the vacuum funnel (Fig. 11). Ensure all drips enter the funnel. Try to consistently filter one sample every 10 minutes*, exactly 1 hour after solvent addition.

*This time can be reduced to 5 minutes if a second person conducts step #17 concurrently as the first person conducts steps 18 - 21.

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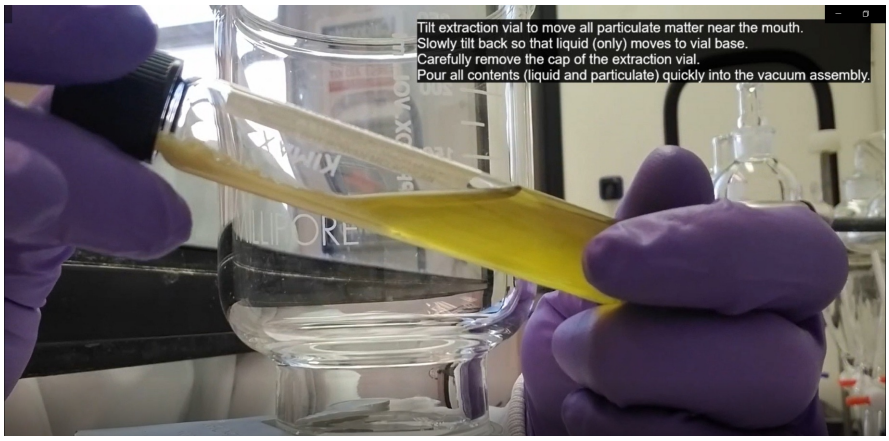

Fig. 11. Method of tipping the *Extraction Vial* in such a way that all particulate matter is left near the cap-end, while the solvent is at the base. This ensures that all material leaves the vial when pouring into the filtration assembly. Watch the video link below for a demonstration.

Then, using a glass syringe, dispense 2 ml of the 2:1 solvent into the *Extraction Vial,* cap, vortex, and pouring this into the same funnel. After pouring this first rinse through the filter assembly, add another 2 ml of 2:1 solvent to the *Extraction Vial,* cap, vortex, and pour into the funnel. The extraction vial can now be discarded for later washing.

Use a metal spatula to scrape the funnel walls, to ensure all particulate matter passes onto the GF/F filter. Turn off the vacuum. Carefully unclamp the filtration system. Lift the glass funnel, and hold over a pre-labeled aluminum weighing pan. Use the spatula and remove any material stuck on the funnel into the pan. Using forceps or a spatula, gently remove the GF/F filter from the glass-frit, and place this inside the same weighing pan. Set aluminum pans on a tray to the side. Later these will be placed in an oven to determine the ash-free dry weight (AFDW) of the sample.

A video of this step can be found here: https://youtu.be/W2KiGVzy5TU

Disassemble the filtration system, and remove the glass test tube containing the filtered lipid sample. Remove the stopper of a separatory funnel, and pour the contents of the disposable test tube into the separatory funnel (Fig. 12). **ENSURE THE STOPCOCKS OF THE SEPARATORY FUNNELS ARE CLOSED FIRST.**

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Fig. 12. Separatory funnel with filtered lipid extract inside, stopcocks in the closed position, and stoppers in place.

Rinse the test tube with exactly 2 ml of 2:1 (v:v) chloroform:methanol, to remove residual lipids from the inside walls of the test tube. After adding the solvent to the test tube, swirl, and then add the contents into the same separatory funnel. The test tube can now be discarded or set aside to be washed later. Stopper the separatory funnel to prevent oxidation of sample and volitalization of the solvents (Fig. 12).

Clean the metal spatula with 100% chloroform. Assemble the filtration system with a clean test tube, frit-support, GF/G and funnel. Continue and repeat for all samples.

18 **Wash extracted lipid with KCl.** When samples have been filtered and loaded into individual separatory funnels, wash samples with potassium chloride to remove salts and other contaminants. Using a clean 10 ml glass syringe, add exactly 4 ml of 0.88 % KCl gently along the inside wall of the separatory funnel. The KCl forms an aqueous white layer above the organic solvent layer (Fig. 13).

Citation: Rowan Mclachlan, Agus Munoz-Garcia, Andrea Grottoli (03/13/2020). Extraction of Total Soluble Lipid from Ground Coral Samples. https://dx.doi.org/10.17504/protocols.io.bc4qiyvw

Fig. 13. White aqueous phase sits above the yellow organic phase of the solvent mixture in the separatory funnel.

Replace the stopper. Gently invert funnel 3 times, remove stopper after each inversion to release pressure. Do not shake the separatory funnel as it will cause an emulsion and the upper and lower phases will not separate. Let funnel stand until a clear separation is observed (Fig 13).

19 **Drain lipid extract (#1).** Remove the stopper (to avoid creating a vacuum) and drain the lower organic phase (yellowish color) into the pre-labeled *E-flask* by slowly turning the stopcock to the open position (Fig. 14). Be careful to stop draining (i.e. close the stopcock) before all of the organic phase has all passed through the stopcock. You do not want any of the white aqueous phase to enter the *E-Flask*. Use a small glass Petri dish as a lid to the *E-flask* to prevent oxidation of the lipid sample (Fig. 14).

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Fig. 14. Left: *E-Flask* with organic phase from coral sample drained from the separatory funnel. Right: Example of how Petri dish can be used as a lid on each of the *E-Flasks*. Note, *E-Flask* #13 is the control, hence why the liquid has a clear color in this example.

20 **Wash lipid residue remaining in each separatory funnel with chloroform.** Dispense ~ 5 ml of 100% chloroform from the stock bottle into a 50 ml glass beaker. Using a 10 ml capacity glass syringe, add 1 ml of 100 % chloroform to the aqueous phase remaining in the separatory funnel, replace the stopper, gently invert funnel 3 times (with removing the stopper each time), and allow to separate (Fig. 15).

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Fig. 15. Add 1 ml of 100% chloroform to the separatory funnel to collect any remaining lipids.

21 **Drain lipid extract (#2).** Remove the stopper and drain the small remaining organic phase (bottom, yellow, Fig. 15) into same *E-flask* (Fig. 14) by turning the stopcock to the open position. Again, be careful not to let any of the aqueous phase pass through the stopcock. Discard the aqueous phase (left in the funnel) in an appropriate waste container. Replace the glass Petri dish as a lid to the *E-flask* to prevent oxidation of the lipid sample (Fig. 14).

22 **Add methanol.** After you have conducted the KCL-wash and chloroform rinse steps on all samples in the batch, dispense ~ 20 ml of 100% methanol from the stock bottle into a 50 ml glass beaker. Using a 10 ml capacity glass syringe, add 1 ml of 100% methanol to each *E-flask*. Swirl the contents of the *E-Flasks*. You will see a change in the opacity of the solvent within each vial, from translucent to transparent. This is because the methanol in the bottom of the vial "clings" to the traces of water and the sample turns clear. Make sure the solvent in each *E-Flask is* clear before proceeding. If not, add another 1 ml of 100% methanol.

A video of this step can be found here: https://youtu.be/9Lb1o8--cSA

Dry lipid extracts. Insert all *E-flask* into the drying rig (Fig. 16A). Take care not to spill any sample. Secure flasks in

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23 place using a zip-tie and place the drying-rig in the water bath, on top of the heating plate (Fig. 16). Make sure the water temperature is exactly 55 °C. Place the pipette-tubing rig on top of flasks (Fig. 16B), attach to the UHP grade 5.0 nitrogen tank, and turn on the gas. Set pressure to ~ 15 PSI. At this pressure and temperature, it will take between 20 – 25 minutes for all of the lipids in the *E-flasks* to dry down.

A video of this step can be found here: https://youtu.be/592SGVuS4CM

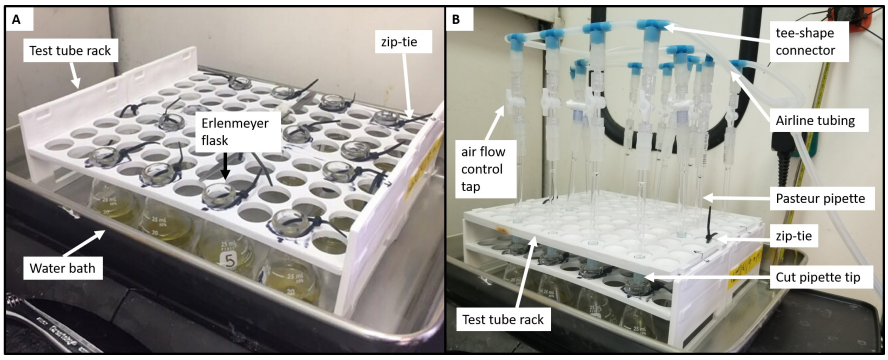

Fig. 16. Photographs of (A) Erlenmeyer flasks secured to test tube rack drying rig with zip-ties, and (B) pipette-tubing rig on top of Erlenmeyer flasks (one Pastaur pipette will direct blow nitrogen gas into each Erlenmeyer flask). Watch the video link above for a full demonstration of this step.

24 **Weigh lipid extracts.** When lipids have fully evaporated, turn-off N gas and disconnect from drying rig. Remove drying rig with flasks from the water bath. Remove zip ties from the neck of *E-Flasks* and remove flasks from the rack. Dry the outside of each *E-flask* using a Kim wipe. Wait 5 minutes until they have cooled.

Weigh each *E-flask* on a balance. All *E-Flasks* are weighted in triplicate and recorded on side 1 of the lipid datasheet (Fig. 6). Enter these values into excel and calculate the average of the three "*Dry Lipid + Erlenmeyer Weights*".

The weight of lipid extracted (in grams) is:
[(average CORAL *Dry Lipid + Erlenmeyer Weight*) - (average CORAL *Empty Erlenmeyer Weight*)] - [(average CONTROL *Dry Lipid + Erlenmeyer Weight*) - (average CONTROL *Empty Erlenmeyer Weight*)]

The energy content of lipid extracted (in Joules) is:
Weight of lipid extracted (mg) x 39.5 *

* Using the enthalpy of combustion for lipid as -39.5 J mg^{-1} from Gnaiger and Bitterlich (1984)

Gnaiger E, Bitterlich G (1984). Proximate biochemical composition and caloric content calculated from elemental CHN analysis: a stoichiometric concept. Oecologia 62:289–298.

Ash-free dry weight (AFDW) procedure

25 Place all the aluminum weighing pans containing the GF/F filter and the lipid-free ground coral sample material in a 60 °C drying oven overnight. The next day, remove pans from the oven and allow them to cool for 5 minutes before weighing.

Weigh each dried pan on a balance. All pans are weighted in triplicate and recorded on side 2 of the lipid datasheet (Fig.

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17). Enter these values into excel and calculate the average of the three "*Pan + Filter + Dry Residue Weights".*

After weighing, place pans into a muffle furnace. Burn the dried sample at 450 ºC for 5 hours. The next day, remove pans from the muffle furnace and allow them to cool for 5 minutes before weighing.

Weigh each burnt pan on a balance. All pans are weighted in triplicate and recorded on side 2 of the lipid datasheet (Fig. 17). Enter these values into excel and calculate the average of the three "*Pan + Filter + Burnt Residue Weights".*

Ash-free dry weight (AFDW) of the coral tissue is calculated using the following equations:

[1] *Δ Coral weight (g) = average weight dry coral pan (g) – average weight burnt coral pan (g)*

[2] *Δ Control weight (g) = average weight dry control pan (g) – average weight burnt control pan (g)*

[3] *AFDW (g) = Δ Coral weight (g) – Δ Control weight (g)*

SPECIES-SITE							
Sample #	Pan + Filter + Dry Residue Weight (g)			Pan + Filter + Burnt Residue Weight (g)			Date/Time Out of Oven: Date/Time Out of Muffle: NOTES
	#1	#2	#3	#1	#2	#3	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							

Fig 17: Side 2 of the lipid datasheet. Record the weight of dried and burned aluminum pans containing GF/F filter and particulate matter in the designated columns. The flip-side (side 1) of this data sheet is shown in Fig 6.

26 The total lipid energy content of the coral (in Joules), standardized to ash-free dry weight (AFDW) is:
[(energy content of lipid extracted (J))/ (AFDW + weight of lipid extracted)]

Resuspending and archiving extracted lipid residue

27 To preserve the lipid for additional downstream analyses (i.e., lipid classes or isotopes), re-suspend the dried lipid in 300 microliters of 2:1 chloroform using a 1 ml glass Hamilton syringe, and transfer sample to a *Storage Vial* (pre-burned and labeled, 4 ml volume, amber pyrex, flat bottomed, Teflon-lined screw-cap bottle). Seal vial with Teflon tape and store in -80 °C freezer until future analysis.

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