# Coral Larvae Lipidomics/Metabolomics Sample Preparation for Symbiotic Larvae

This was originally written for extracting lipids from plasma. Before starting, you must run some trial samples as a loading study to determine the volume/mass of coral tissue to use. This is achieved by loading an increasing volume of the sample (1, 2.5, 5mL) and examining the Total Ion Chromatogram (TIC) and mass spectrums for sufficient ion intensity or detector saturation. The aim is to pick the injection volume that gives the highest signal without detector saturation.

Reference: Matyash, V., et al. (2008). "Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics." J Lipid Res **49**(5): 1137-1146.

This larval protocol is based on previous adult coral protocols and larval protocols for aposymbiotic larvae. This protocol includes modifications to separate host and symbiont fractions prior to extractions and performing protein quantifications prior to extractions.

## Equipment and Supplies

**Suggested Schedule**Day 1: Perform host and symbiont separations; prepare reagents  
Days 2-n: Perform extractions in batches until complete

Day n+1: Perform all protein assays

Store extracts at -80°C until LC-MS (lipid analysis) and sending samples for metabolomics analyses

After all extractions are done, plan for 1-2 days to prepare and dry samples for LCMS and sending for metabolomics analyses

**Reagents**

For fraction separation:

EDTA (20 mg/mL)

BHT (20 mg/mL)

LC-grade MilliQ water

For extractions:

MTBE - Methyl tert-butyl ether

LC-MS or HPLC hypergrade Methanol (stored at -20˚C)

LC grade water (MilliQ in LC-clean glassware\*\*, stored at 4˚C)

SPLASH internal Standard (<https://avantilipids.com/product/330707/>)  
BHT (Butylated hydroxy toluene)   
13C sorbitol (link here)

For LC-MS analysis

ACN - Acetonitrile

IPA - Isopropanol

Formic Acid

Ammonium Formate

For Bradford Protein assays  
Bradford reagent  
Bovine Serum Albumin at stock of 1 mg/mL in 1 mL aliquots stored at -20°C

**Equipment**

For extractions and separations

Vacuum pump

0.22 µm filter paper (Millepore hydrophilic paper)

Plastic eppendorf tube pestle

Sonicator

Rotating platform

Electric homogenizer

Centrifuge

1.5-2 mL eppendorf tubes

LC cleaned graduated cylinder

P1000 pipette and tips

P200 pipette and tips

Cooler and ice

Chattaway spatula

GF/F filters

Tweezers

70-80% ethanol in spray bottle

Paper towels

For Bradford Protein assays  
96-well plates  
Spectrophotometer  
1.5-2 mL eppendorf tubes

## Reagent preparation

Prepare the following reagents and store at -20°C.

**Mix 1:**

75 mL MTBE

25 mL 100% LC MS hypergrade methanol

500 µL SPLASH internal standard

50 uL (of a 1 mg/mL stock in water) 13C sorbitol (internal standard)

Store at – 20˚C

**Mix 2:**

75 mL BHT-spiked (10 µL/mL) LCMS water \*\*

25 mL 100% LC MS hypergrade methanol

Store at – 20˚C

\*\* Prepare the BHT-spiked water by preparing a stock solution of 20 mg dry BHT per mL of methanol. Add this stock solution at 10 uL per mL into the LCMS water in Mix 2 by mixing 750 uL into 75 mL of LCMS water. Store any remaining stock solution at room temperature.

Scale the volume of these mixes to meet needs depending on the number of samples.

## Cleaning and preparing LC clean glassware

Prepare the following reagents and store at -20°C.

Any glass container used to prepare or store mobile phase (e.g. methanol, water) must be thoroughly cleaned before use to be LC clean. To clean laboratory glassware:

a. First, rinse it with organic solvent (e.g. methanol) and then water.

b. Next, rinse it with the solvent that will be put into it (or MilliQ water).

c. If more aggressive cleaning is required (for example, when the container’s history is unknown), use the following procedure: Sonicate with 10% formic or nitric acid, then water, then methanol or acetonitrile, then water. Repeat two more times.

d. Wash and store glassware separately from other containers.

**CAUTION:** Do not wash glass bottles in detergent, with other glassware, or in washing facilities that may have detergent residue. Washing glassware in a common dishwashing facility can contaminate it with detergent residues, which may contain polyethyleneglycol (PEG) and other “sticky” substances. Vinyl-coated steel racks can be an additional source of contamination. Once glassware is cleaned, it can be used multiple times as long as there are not possible sources of contamination.

## Before starting extractions

1. Pre-chill centrifuge motors at 4°C
2. Take the Bradford assay out of fridge to warm to room temperature (only on days you are doing protein assays)
3. Make BSA stock solutions at 1 mg BSA / 1 mL MilliQ water and store at -20°C in 1 mL aliquots. Make protein standard dilutions from stock and store at 4°C (only on days you are doing protein assays). Note that stock BSA solutions should be made and stored at -20°C. Stock can NOT be thawed and refrozen.
4. Label tubes
5. Pre-chill LC-grade water at 4°C

## Host and symbiont fraction separation

**Throughout all portions of this protocol, samples should be kept on ice (4°C) at all times to ensure they are in liquid form whilst preventing on-going metabolism.**

1. Randomize your sample order! I use excel, with all my samples in one column, and a random number using “=RAND()” in the next column, copy and paste as values, and order by the random number.
2. Prepare three new 1.5 mL eppendorf tube with the sample ID with one of these tubes labeled with “Host”.
3. Remove samples from freezer and thaw on ice.
4. Add 100 µL of LC-grade water at 4°C with 1 µL EDTA (20 mg/mL) and 1 µL BHT (20 mg/mL) to one of the new labeled 1.5 mL eppendorf tubes. Prepare a blank tube that will not have larvae added.
5. Pipette larvae from original sample tube onto 0.22 µm Millepore hydrophilic filter paper on a vacuum pump. Allow water to be pulled through the filter, leaving the larvae on the filter. Scrape the larvae into the 1.5 mL eppendorf tube with water already added using the spatula.
6. Add 200 µL LC-grade MilliQ water into the original sample tube to remove any remaining larvae. Pipette onto the filter. This also helps to rinse any residual seawater from the larvae.
7. Homogenize with a plastic pestle in the tube and vortex for 30 sec.
8. Between samples, clean spatula with 70-80% ethanol. Use a new filter for each sample.
9. Separate the host and symbiont fractions.
   1. Centrifuge the homogenate at 3,000 g for 5 min at 4°C
   2. Collect supernatant as the host layer on top of the algal pellet, being careful not to disturb the pellet and place into a new labeled tube. Gently remove any lipid buildup in the tube on top of the pellet with a pipette tip. Scrape any lipid layers with the pipette, leaving behind as much of the symbiont cell pellet as possible.
   3. Vortex the supernatant briefly.
   4. Centrifuge the supernatant at 3,000 g for 5 min at 4°C
   5. Collect supernatant again and add to another new tube as described in step (b) above.
   6. Repeat a third time if necessary. Twice was sufficient in this protocol to generate a clean supernatant and remove a majority of the pellet.
   7. During this process, keep a subsample of homogenate and a subsample of cleaned host fraction to check on a scope for presence of symbiont cells. This can be done for 1-3 samples.
   8. After this process, check that the pellet is clean and there is not a lipid layer on top of the pellet. We want to have the lipids in our host fraction. If needed, centrifuge and separate the host fraction again.
   9. Discard the tubes with pellets.
10. If separation is not desired, use the holobiont homogenate.
11. Return the host samples to the freezer at -80°C.

## Extractions for Coral Larvae

1. Randomize your sample order! I use excel, with all my samples in one column, and a random number using “=RAND()” in the next column, copy and paste as values, and order by the random number.
2. Prepare a 2mL eppendorf tube for each sample with “extract” for total extract, “-L” for lipids and “-M” for metabolites.
3. Remove “host” tube samples from freezer and thaw on ice in the fume hood. **Keep all samples on ice.**
4. Gather all materials needed in the fume hood including Reagents Mix 1 and Mix 2, vortex, kim wipes, pipettes, and ethanol.
5. Include a blank sample for all extraction batches that includes reagents but without addition of a sample.
6. Add 1 mL of chilled Mix 1 (MTBE + MeOH) to a new 2 mL eppendorf tube labeled with the sample ID and “extract”.
7. Add all host fraction sample to the tube and record the volume added.
8. Vortex for 30 seconds once all samples are ready. Avoid touching the tube with the liquid to keep sample cold.
9. Take all samples on ice to the walk in fridge.
10. Shake for 45 min at 4°C (1000 rpm) on a rotating platform. Use the outer edges to provide the greatest degree of rotation. Move Mix 1 back to the -20°C freezer.
11. Sonicate at 4°C for 15 min on high in the walk in fridge.
12. Start the centrifuge to cool to 4°C if not done already.
13. Move the samples from the sonicator to ice and back to the fume hood.
14. Add 650 µL of Mix 2 chilled to 4°C or colder for phase separation.
15. Vortex on high for 1 min.
16. Separate phases by centrifuging at 20,000 g for 5 min at 4°C.

**To separate the lipids:**

1. Carefully remove samples from the centrifuge to ice and back to the fume hood.
2. Collect 500 µL \*\* of the organic top layer to a new 2 mL eppendorf tube marked with sample ID and “-L”. This is your lipid phase. Remove with a pipette by following the meniscus down the side of the tube. Do not disturb the lower phase. If so, return all liquid to the tube and centrifuge again.
   1. This volume \*\* is dependent on sample volume. Select the maximum value that can be consistent across all samples. I used 500 µL.
   2. Do this part quickly and be careful for drips from the pipette. Have the new tube ready close by and move quickly.
3. The samples (“L” tubes) can now be stored at -80°C until LCMS analysis.

**To separate the metabolites:**

1. Collect any remaining lipid phase (if present) and discard, or if there is a lot of material left it can be collected and stored at -80°C. This can be used for other analyses or if more material is needed.
2. Collect 800 µL \*\* of the polar bottom layer to a new 2 mL eppendorf tube marked with “-M” and the sample ID. This is your metabolite phase.
   1. \*\* As with lipids, use as much as possible of this layer that can be kept consistent across all samples. I used 800 µL.
3. Store the samples (“M” tubes) at -80°C until preparation for sending to metabolomics facility.
4. Return the sample extract tubes (“extract” tubes) to the freezer. Hold onto these until analyses are complete in case we need to go back to these for measurement of protein.

## Lipid preparation for LC-MS

1. Immediately prior to analysis, dry the lipid phase under a nitrogen stream (avoid over drying – depending on sample volume this should take < 1 hour). Make sure to clean manifold with methanol between samples.
2. Include your blanks from the separation and from each batch of the extractions.
3. Transport samples on ice to the proteomics lab, where we will resuspend samples in 100uL of 2:1 IPA/methanol (or acetonitrile) for LC/MS/MS.
4. Once resuspended, take 20 µL of each sample (and blanks) and combine in one tube – this is your Pooled Biological Quality Control (PBQC) lipid sample, which is important for the data analysis (if separated host and symbiont, do one for all host samples and one for all symbiont samples)

## Metabolite preparation to send to Metabolomics Australia

1. Thaw samples on ice.
2. Take 20 µL of each sample (and blanks) and combine in one tube – this is your Pooled Biological Quality Control (PBQC) metabolite sample, which is important for the data analysis (if separated host and symbiont, do one for all host samples and one for all symbiont samples)
3. Prior to sending, dry the metabolite phase in 6 x 50 µL aliquots in a 250 µL glass insert at 30˚C.
4. Send dried samples in tubes in a bag with desiccant to Metabolomics Australia for analysis.

## Protein Quantification of Cell Debris

1. Quantify total protein in the protein pellet using the Bradford assay.
2. Allow Bradford reagent to warm up to room temperature (do at the start of the day).
3. Prepare 6 serial dilutions of Bovine Serum Albumin (BSA). At mg/ml, make 1, 0.5, 0.25, 0.125, and 0.062.
   1. Weight out 10 mg/ml.
   2. Dilute to 1 mL aliquots of 1 mg/ml stock solution (100 ul stock in 900 ul of MilliQ). Freeze aliquots at -20˚C.
   3. Use 1 mL of 1 mg/mL stock to create serial dilutions
   4. e.g. add 500 µL MilliQ to 4 x 1.6 mL tubes, transfer 500 µL stock to first tube (this will be you 0.5 mg/mL), pipette mix, and transfer 500 µL to next tube (this will be your 0.25 mg/ML) and so on through all 4 tubes.
4. Prepare cell debris
   1. Centrifuge cell debris/metabolite extract at 10,000 g for 10 min at 4°C.
   2. Transfer liquid, including separated phased into a new 2mL eppendorf tube to carefully avoid disturbing the pellet.
   3. Resuspend the pellet with 1 mL of 0.2M Sodium Hydroxide.
   4. Incubate at 98°c for 20 min - use lid stoppers to prevent sample bursting open and spraying sodium hydroxide everywhere!
   5. Cool samples on ice.
   6. Centrifuge at 3,000 g for 5 min.
5. In a 96-well plate, put the 0.062 – 1 mg/ml in duplicates, a new blank (MilliQ water), and then the samples including the separation/host blank sample.
6. Record sample location in a plate map in your notebook.
7. Use 10 ul of each resuspended sample. Put 10 µL into each well first and THEN add 250 ul of Bradford reagent (at room temp). Use 3 replicates per sample.
8. Dilute samples if too concentrated, using the following steps:
   1. If samples have a higher concentration than the 1 mg/mL BSA they will need to be diluted.
   2. Check samples are not visually any darker than the darkest standard when pipetted into the 96-well plate after the Bradford reagent has been added
   3. If darker, dilute by taking 10 ul of sample and adding 10 ul of MilliQ to give you a 50% dilution
   4. Check that the new concentration isn’t any darker or lighter than the standards
   5. Analyze on a spectrophotometer if necessary
9. Pipette mix gently
10. Leave for 10 mins to rest on rotating platform at a speed of 70 at room temp, with plate wrapped in foil
11. Analyze on a spectrophotometer (absorbance 595 nm, 25 flashlets, 0) and download data.
12. After analysis, dispose of Bradford assay reagent from wells according to SDS (e.g. in translucent bottle via cytotoxic waste)
13. Next, calculate total protein concentration for each sample.
    1. <https://github.com/AHuffmyer/larval_symbiont_TPC/blob/main/scripts/protein_omic_extractions.Rmd> (use by downloading and running R project or cloning repository)
    2. Excel spreadsheet