**Gravimetric Total Lipid Quantificaiton**

Adapted from Wall & Huffmeyer protocol by T. Lindsay (taylor\_lindsay@uri.edu)

Purpose & Notes

Protocol for lipid extraction and quantification in coral samples.

*This volume was optimized (12 mL of solvent for 500 µL of original sample volume) based on conducted trials to ensure saturation and full lipid extraction. This volume should be optimized for either smaller or larger sample volumes.*

Lyophilization is utilized in this protocol to increase the quality of lipid extraction, but this step is not required and instead liquid slurry or homogenized larvae could be used without lyophilization if not available.

Careful pipetting and reducing organic contamination is *crucial* for this protocol. Wear gloves, work in a fume hood, and do not rush through this protocol.

Materials

Disposables

* Glass scintillation vials – 20ml, 4 per sample
* Aluminum pans – 4 per sample, large enough to hold filters
* Fiberglass (GF/F) filters – 20mm, must fit on vacuum pump, 2 per sample

Equipment

* Vacuum pump setup – culture vial, funnel & filter apparatus
* Tube rack that will fit scintillation vials
* Pipettes & Tips – (10 or 5mL & 1mL)
* Muffle furnace (450˚)
* Drying oven (100˚C)
* Tweezers or forceps
* Freeze Dryer (optional)

Solvents

* 100% chloroform
* 100% methanol
* 88% KCl solution (8.8g KCl per 1 L DI/MilliQ water)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Number of samples (one replicate) | | 1 | 8 | 20 | 100 |
|  | **Total amount of each solvent needed (mL)** | | | |  |
| Chloroform | | 12 | 16 | 240 | 1200 |
| Methanol | | 7 | 56 | 140 | 700 |
| 88% KCl | | 12 | 96 | 240 | 1200 |

**Pre-burning & labeling**

1. Label aluminum pans with numbers by engraving the numbers using a dull pencil.
2. Pre burn the following at 450˚C for 4-6hrs to remove organic material: Aluminum pans, Pans + filters, vials (NOT the plastic lids)
3. Label vials with permanent marker on lid and 2 sides. This will rub off if solvents come into contact. *Note: I recommend writing the corresponding pan numbers on here too.*
4. Weigh all pans or pans + filters and record on data sheet.

**Aliquot**

1. Thaw samples (kept at -80°C) if required.
2. Aliquot two 500 µL replicates of each sample into two 20 mL glass scintillation vials.

**Lyophilize (optional)**

1. Turn on lyophilizer and ensure pressure and temperature reach targets as specified (-80°C and <1.0 atm pressure).
2. Arrange vials with frozen sample in the lyophilizer beakers. If samples begin to thaw at any point, it is critical to re-freeze at -80°C to ensure proper lyophilization.
3. Lyophilize samples for ~8 h until water fraction is completely removed (samples will appear to be a white powder or film). Ensure there is no leakage once lyophilization begins, if this happens, remove and refreeze samples.
4. Store lyophilized or aliquoted samples at -80°C.

**Lipid purification**

1. Prepare solvents.
2. Add 12ml 2:1 chloroform:methanol solution to each vial and gently swirl.
3. Allow to sit in a dark fridge for ~3 hours to allow for lipid extraction.

**Filtering**

1. Set up filter apparatus, pump, and tray of pans + filters.
2. Remove samples from fridge and pour the extract through the filter apparatus (with vacuum pump) such that particulates are captured on the GF/F filter and the liquid fraction passes through the filter to be captured by a culture vial.
3. Rinse any remaining material in the glass scintillation vial with 1ml 2:1 chloroform:methanol solvent, pour into filter setup.
4. Use 1ml 2:1 chloroform:methanol to rinse down the sides of the filter apparatus to capture all lipids.
5. Turn off the vacuum and slowly remove the filter stand. Using tweezers/forceps, gently remove the GF/F filter and place in a pre-burned, pre-labeled small aluminum pan.
6. Pour all filtered liquid extract in the culture vial back into the original glass scintillation vial.
7. Add 2 mL of 2:1 chloroform:methanol into the culture vial to rinse all material back into the glass scintillation vial.
8. Repeat filtration for all samples.

**Lipid Extraction**

1. Add 6 mL 88% KCl to the filtered lipid extract in the glass scintillation vial and GENTLY invert 4-5 times. *DO NOT vortex or mix vigorously. You are looking for gentle separation of liquid phases.*
2. Allow vials to sit for 20-30 minutes to allow for separation. After separation, the vial will now contain a *lower* phase (yellow color) that contains lipids and an *upper* phase (white/cream color) that contains water soluble products.
3. Remove the *lower* phase (containing lipids) into a new, labeled, pre-burned glass scintillation vial using a pipette. It is recommended to gently angle the vial and carefully pipette out as much of the lower phase as possible while avoiding taking any of the upper phase.
4. Add 1 mL of 100% chloroform to the *upper* phase, invert, and allow to separate for 10-15 minutes. Again remove the *lower* phase with a pipette and add to the new vial with the lipid phase.
5. Dispose of the remaining upper phase in the original vial. This is mostly KCL solution.
6. Rinse the *lower* phase of the new vial with 6 mL of 88% KCl again. Gently invert and allow to separate for 10-15 minutes.
7. Now remove the separated lower phase with a pipette and directly transfer into 1 large aluminum pan. This fraction is now your purified lipid!
8. Dispose of the remaining upper phase in the second vial. This is mostly KCL solution.
9. Squirt a little methanol in the pan with this purified lipid to remove any remaining water products. This will clear up cloudy liquid such that it is a clear, yellow color.
10. Proceed with this purification for all samples.
11. Allow samples to sit for ~20 minutes in the pans in a closed fume hood to allow some of the chloroform to evaporate. This makes transportation to the drying oven safer.

**Drying and Burning**

1. Dry lipid fraction in pans and filters in pans overnight (or at least 8 hrs) at 60-65˚C.
2. After drying, record the dry weight of lipid pans and pans + filters in the data sheet.
3. Dispose of the lipid pans.
4. Burn the pans + filters for 4-6 hrs at 450˚C in a muffle furnace. This burns away all organic carbon.
5. After cooling, record the burned weight of pans + filters.

**Calculations**

**Calculate mass of organic material on filter**

(dry weight of pan + filter) - (burned weight of pan + filter) = organic material on filter (g)

**Calculate mass of lipids**

(dry weight of pan + lipids) - (pre-weight of lipid pan) = lipids (g)

**Calculate AFDW that will be used for normalization for lipids**

AFDW = (g lipids + g organic material on filter) / original sample volume = AFDW in g/mL

This can be used to multiply to total slurry volume to give AFDW per sample, which can later be normalized to surface area or used for other calculations.

**Calculate percent lipids (lipids normalized to AFDW)**

(g lipids) / (g lipids + g organic material on filter)

This provides the percent of lipids in total organic biomass. This typically ranges from 20-35% in coral adults.

**Calculate total lipids in sample**

(g lipids) / sample volume (0.5 mL) = g lipids per mL

This can be multiplied by total slurry volume to give total lipids in a coral sample and later normalized to surface area or other responses.

Suggested data sheet columns

Date  
Sample ID  
Volume sample  
Filter pan pre-weight (small pan)  
Lipid pan pre-weight (large pan)  
Time drying starts and ends  
Dry weight of filter + pan  
Dry weight of lipid + pan  
Time burning starts and ends  
Burned weight of filter + pan

**Links to the equipment I used**

Vials: <https://www.amazon.com/ALWSCI-Scintillation-Borosilicate-Counting-Aluminum/dp/B0C3R3W24F/?_encoding=UTF8&pd_rd_w=ULFYq&content-id=amzn1.sym.d0ebfbb2-6761-494f-8e2f-95743b37c35c%3Aamzn1.symc.50e00d6c-ec8b-42ef-bb15-298531ab4497&pf_rd_p=d0ebfbb2-6761-494f-8e2f-95743b37c35c&pf_rd_r=G3WPYKTWHZ9RFPAB7JKN&pd_rd_wg=KqByH&pd_rd_r=24ab54f2-a45b-4539-97ff-42443b78baa0&ref_=pd_gw_ci_mcx_mr_hp_atf_m>

Filtration setup: <https://www.glasscolabs.com/product/all-glass-filter-assembly-with-funnel-fritted-base-cap-clamp-47mm-ground-joint-flask-1ltr/>

Chloroform: <https://www.fishersci.com/shop/products/chloroform-molecular-biology-reagent-3/AAJ67241K2#?keyword=chloroform>

Methanol: <https://www.fishersci.com/shop/products/methanol-uhplc-ms-thermo-scientific/A4581#?keyword=methanol>