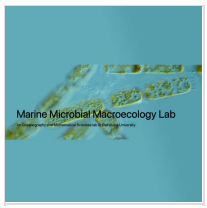

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Dec 16, 2021

Rapid extraction of total lipids from microalgae V.2

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protocol .

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In this protocol, total lipids from miroalgae is extracted with Folch solvent (2:1 chloroform-methanol v/v) and the addition of 5% water. Filter and cell debris is commonly removed by filtration, which is laborious and time consuming. It is also the main reason to either cause sample loss and therefore underestimation; or contamination from filtration system and therefore overestimation. We now use centrifugation to remove filter debris. The extract is then mixed with 0.88% potassium chloride solution to form a biphasic system, where in between the two phases is the thin distinct cell debris layer. The lower phase with extracted lipids is collected and dried under N_2 gas flow. The residue is stored under -80 ºC for further measurement.

FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem, 1957, 226, 497-509.

Axelsson M, Gentili F (2014). A single-step method for rapid extraction of total lipids from green microalgae.. PloS one. https://doi.org/10.1371/journal.pone.0089643

Yingyu Hu, Zoe V Finkel 2021. Rapid extraction of total lipids from microalgae. **protocols.io** https://protocols.io/view/rapid-extraction-of-total-lipids-from-microalgae-bx2zpqf6
Yingyu Hu

lipids, microalgae, Folch solvent

protocol ,

Sep 08, 2021

Dec 16, 2021

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Biomass requirement

Considering that

(1) lipids are approximately 10~30% of microalgal dry mass
(2) the linear range for colorimetric lipid analysis is 4.2 to 80 µg, the low limit of quantitation is 20 µg

The minimum requirement of sample volume for total lipids is calculated as following:
V_L=20/Chl-a/(17.3/1.1)

If both total lipids and phospholipids are expected to be measured, the minimum sample volume needs to be at least doubled.

Operate chloroform in fumehood.

Follow the disposal guidelines regarding the halogenated organic waste.

Collect microalgae samples

4h

1 Precombust GFF filter at **450 °C** for **04:00:00**

2 Rinse forceps with 95% ethanol, air-dry.

Filter forceps
blunt end, stainless steel
Millipore **XX6200006P**

Wipe-dry forceps can cause carbon contamination of samples.

3 Filter microalgae in liquid media onto precombusted GFF filters, using gentle vacuum pressure (5 inches Hg).

4 Rinse sample with filtered seawater

5 Place sample filters in cryogenic vials

6 Filter blank media (without cells) through precombusted GFF filter as blank.

7 Flash freeze filters and stored at **-80 °C**

8 Freeze dry before measurement.

FreeZone® 2.5 L Benchtop Freeze Dryers
Labconco® **700202000**

9 Follow <Total particulate carbohydrate from microalgae> protocol to hydrolyze the sample. Hydrolyzation treatment can improve the extraction efficiency:

1. Hydrolysis releases bound lipids into easily extractable forms.
2. Acidified water fraction can facilitate separation of the lipid fraction from extraneous protein and other material.
3. Acid can charge phospholipid to optimize extraction.

10 Freeze dry the residue for total lipids extraction.

Prepare glassware

11 Precombust the centrifuge tubes, scintillation vials and storage vials at **500 °C** for **06:00:00** 6h

12 Precombust pasteur pipets at **500 °C** for **02:00:00** 2h

Disposable Soda-Lime Glass Pasteur Pipets
5 3/4"
Fisherbrand **13-678-6A**

13 Rinse caps with 95% ethanol and air-dry prior to use

14 Rinse serological pipets and the reagent bottle for dichromate reagent with 95% ethanol until there is not stain and with chloroform for the final rinse. Air-dry.

VWR® Volumetric Pipets, Reusable, Color Coded, Class A
0.5 mL and 5 mL
VWR **10546-004 and 10546-014**

PYREX® Media Bottles
Corning® **1395-100**

15 Latex bulbs are required for Pasteur pipets

Prepare reagent

16 Folch solvent ($CHCl_3$: MeOH=2:1 v/v)

16.1 Mix two parts of chloroform and one part of methanol in a 1 L amber bottle. Log the volume of each solvent for double checking the ratio.

A	B
Chloroform (mL)	
Methanol (mL)	

Chloroform (HPLC grade) **Sigma Aldrich Catalog #439142-4L**

Methanol **Sigma**
Aldrich Catalog #34860

16.2 Attach dispensette to the bottle, mix well.

Bottle-top dispenser
BrandTech Dispensette® S 4731330

16.3 Label bottle with MSDS label.

17 KCl solution ([M] **0.88 %**)

17.1 Weigh the pyrex media bottle and tare.

PYREX® Media Bottles
Corning® **1395-100**

17.2 Directly weigh **0.44 g** KCl in the bottle.
Potassium chloride **Sigma**
Aldrich Catalog #P3911-500G

17.3 Top bottle with MilliQ water to **50 g**

A	B
KCl (g)	
Final (g)	

Extraction

18 Transfer freeze dried samples and blanks into muffled centrifuge tubes

It takes about 7 to 8 hours to process 16 samples.

Disposable Glass Screw-Cap Centrifuge Tubes
10 mL
Corning® **99502-10**

Polypropylene Screw Caps
Linerless, 15-415
Kimble Chase **73805-15415**

19 Add **100 µL** MilliQ directly onto the sample.

20 Freeze at **-80 °C** **00:10:00**

21 Remove vials from freezer.

22 Purge the dispensette, fill the tubing with solvent before dispensing solvent into sample tube.

23 Dispense **2.0 mL** Folch solvent into sample tube.

24 Vortex **00:28:00** by using a tube insert. 28m

VWR ANALOG VORTEX MIXER
VWR **10153-838**
With tube insert

25 Sonicate **00:02:00**

26 Vortex **00:30:00** by using a tube insert. 30m

27 Prepare one set of precombusted tubes (#T1), label the tubes, cap is not required.

Disposable Glass Screw-Cap Centrifuge Tubes
10 mL
Corning® **99502-10**

28 Place one pasteur pipet (#P1) into one tube

Disposable Pasteur Pipet
9 inch
VWR **14672-380**

29 Prepare another set of precombusted tubes (#T2) for supernatant. Cap the tube to avoid contamination.

Disposable Glass Screw-Cap Centrifuge Tubes
10 mL
Corning® **99502-10**

Polypropylene Screw Caps
Linerless, 15-415
Kimble Chase **73805-15415**

30 Work on eight samples first. Use the pasteur pipet (#P1) to gently lift the filter upwards and transfer liquid (as much as possible) to the centrifuge tube. Keep the pasteur pipet (#P1) in its corresponding empty tube (#T1).

31 Add **50 µL** MilliQ and **1 mL** Folch solvent to the residue.

32 Vortex the eight samples at the highest speed to loosen the filter.

33 Vortex the eight samples by using a tube insert while transferring supernatant from another eight samples to centrifuge tubes. 15m

34 Sonicate **00:02:00**

35 Vortex the second eight samples by using a tube insert while transferring supernatant from the first eight samples to centrifuge tubes.

Same alternate routine for the following steps.

36 Use the pasteur pipet (#P1) to gently lift the filter upwards and transfer all liquid to the centrifuge tube (#T1). Keep the pasteur pipet (#P1) in its corresponding empty tube (#T1).

37 Add **50 µL** MilliQ and **1 mL** Folch solvent to the residue.

38 Vortex while transferring supernatant from another set of samples to centrifuge tubes.

39 Sonicate **00:02:00**

40 Use the pasteur pipet (#P1) to gently lift the filter upwards and transfer all liquid to the centrifuge tube (#T1). Keep the pasteur pipet (#P1) in its corresponding empty tube (#T1).

41 Add **50 µL** MilliQ and **1 mL** Folch solvent to the residue.

42 Vortex while transferring supernatant from another set of samples to centrifuge tubes.

43 Sonicate **00:02:00**

44 Use the pasteur pipet (#P1) to gently lift the filter upwards and transfer all liquid to the centrifuge tube (#T1).

45 Dispose the pasteur pipet (#P1), place clean pasteur pipet (#P2) into the centrifuge tube (#T1)

46 Prepare a set of clean centrifuge tubes (#T3) for the next section.

Remove filter debris

47 Centrifuge at **3200 rpm, Room temperature, 00:05:00** 5m

General-purpose benchtop centrifuge
IEC CENTRA CL2
Thermo **00427 0F**

48 Use clean pasteur pipet (#P2) to transfer as much as possible supernatant to #T3. Do not disturb the debris.

49 Return pipet (#P2) back to #T1 tube.

50 Add **1 mL** Folch solvent and **50 µL** MilliQ into the centrifuge tube with debris (#T2).

51 Vortex the mixture.

52 Centrifuge at **3200 rpm, Room temperature, 00:05:00** 5m

53 Use pasteur pipet (#P2) to transfer as much as possible supernatant to #T3. Do not disturb the debris.

54 Add **1 mL** Folch solvent and **50 µL** MilliQ into the centrifuge tube.

55 Vortex the mixture.

56 Centrifuge at **3200 rpm, Room temperature, 00:05:00** 5m

57 Use pasteur pipet (#P2) to transfer as much as possible supernatant to #T3. Do not disturb the debris.

58 Add **1 mL** Folch solvent and **50 µL** MilliQ into the centrifuge tube.

59 Vortex the mixture.

60 Centrifuge at **3200 rpm, Room temperature, 00:05:00** 5m

61 Use pasteur pipet (#P2) to transfer as much as possible supernatant to #T1, do not disturb the debris. Keep the pipet (#P2).

62 Shake the tube (#T1), and then use pasteur pipet (#P2) to transfer all fluid to #T3.

Separation 1h 16m 10s

63 Generally, it yields a final volume of 7.5 mL for samples collected on 25 mm GFF filter; final

volume is 7 mL for sample collected on 47 mm GFF filter.

64 In order to obtain a biphasic system so that the extract can be separated from the water, the final composition of $CHCl_3$:MeOH:H_2O is 8:4:3 (v/v), i.e. 2.67:1.33:1.

A	B	C	D	E	F	G
GFF size (mm)	Final (mL)	KCl (mL)	CHCl3 (mL)	MeOH (mL)	H2O (mL)	Ratio
25	7.5	1.5	5	2.5	1.875	2.67:1.33:1
47	7	1.4	4.67	2.33	1.733	2.69:1.34:1

65 Depending on the size of filter, add corresponding volume of 0.88% KCl to #T3.
25 mm GFF: 1.5 mL KCl
47 mm GFF: 1.4 mL KCl

66 Vortex **00:01:00** by using a tube insert. 1m

67 Vortex each tube for about **00:00:10** individually. 10s

68 Centrifuge at **3200 rpm, Room temperature, 00:05:00** or until biphasic layers separate completely.

69 Remove most of upper aqueous phase with the pasteur pipet (#P2).

70 Use #P2 to transfer the lower organic phase to a 12 mL storage vial.
Do not disturb the cell debris in between the two phases.

If the organic layer is not clear, allow it to remain in the pasteur pipet for seconds, water mixed in the organic layer will separate on the top. Carefully transfer the lower layer in pipet to sample vial, avoid the water layer. The organic layer can become clear.

If it has already been the end of the day, keep samples at **-80 °C**

Glass Vials PTFE/SILiCone SEPTA Clear
16 mL
Thermo Scientific **B7990-4**

Screw Vial Convenience Kit, 12mL solid top PTFE cap
Thermo Scientific **B7800-12A**

71 Dry organic phase extract at **37 °C** under a stream of N_2 gas (<2 psi) for about **00:30:00** to **00:45:00** 1h 15m

A	B	C
	Time	Gas cylinder pressure
Start		
End		

Reacti-Vap Evaporator
Thermo Scientific **TS-18825**

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Troubleshooting for other components (protein) in lipids extract

(1) Lipids extract is hard to be dried if protein coexists. The residue has high viscosity.

(2) Redissolve the thick extract with chloroform, transfer chloroform dissolved solution for lipids measurement, avoid disturbing the protein layer isolated to the bottom layer. It won't affect phospholipids measurement. But this amount of protein can introduce overestimation of lipids.

73 The lipids extract might still have water residue (which can't be dried by nitrogen gas).

(1) If the extract is partially used for lipids measurement:

Redissolve it with chloroform by using glass serological pipet, transfer certain amount of chloroform dissolved solution for lipids measurement based on the estimation.

The rest will contain small amount of water, but it doesn't affect the estimation of phospholipids (see <Estimate phospholipids from microalgae> protocol).

Safetypette

Jencons **75856-442**

(2) If the extract is totally used for lipids measurement:

Freeze dry to remove water.

74 Freeze at **-80 °C** .