Dec 03, 2025

A standardized protocol for extracting and quantifying PLFA and NLFA biomarkers from soil

DOI
https://dx.doi.org/10.17504/protocols.io.rm7vz9zj8gx1/v1

Simone Cesarz[1,2], Alfred Lochner[1,2], Olga Ferlian[1,2]

[1]German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig; [2]Leipzig University

Simone Cesarz
German Centre for Integrative Biodiversity Research (iDiv) H...

DOI: https://dx.doi.org/10.17504/protocols.io.rm7vz9zj8gx1/v1

Protocol Citation: Simone Cesarz, Alfred Lochner, Olga Ferlian 2025. A standardized protocol for extracting and quantifying PLFA and NLFA biomarkers from soil. **protocols.io https://dx.doi.org/10.17504/protocols.io.rm7vz9zj8gx1/v1**

Protocol status: Working
We use this protocol and it's working

Created: November 25, 2025

Last Modified: December 03, 2025

Protocol Integer ID: 233450

Keywords: nlfa biomarkers from soil, microbial lipids in soil, phospholipid fatty acid, neutral lipid fatty acid, nlfa biomarker, fatty acid, microbial lipid, quantifying phospholipid, separate neutral lipid, lipid yield, microbial biomass, soil sample, lipid fraction, neutral lipid, phospholipid, organic phase, extraction, acid methyl ester, storage lipid pool, soil, chromatographic analysis, practical details on solvent preparation

Disclaimer

Abstract

This protocol describes a robust and standardized workflow for extracting, fractionating, and quantifying phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFAs) from soil to assess microbial biomass, community structure, and storage lipid pools. Soil samples (5 g fresh weight) are extracted using a Bligh–Dyer chloroform:methanol:citrate-buffer solvent system that efficiently recovers both polar and non-polar lipids. After extraction and phase separation with additional chloroform and citrate buffer, the organic phase is dried and fractionated on conditioned silica columns to sequentially separate neutral lipids (NLFAs; chloroform eluate), glycolipids (acetone wash), and phospholipids (PLFAs; methanol eluate). Lipid fractions are converted to fatty-acid methyl esters (FAMEs) via alkaline methylation using freshly prepared 0.2 M methanolic KOH in the presence of a C19:0 internal standard (methyl nonadecanoate). FAMEs are recovered in hexane, transferred to GC vials, and stored at low temperature for chromatographic analysis. Multiple cold “stop points” are included to enable sample batching, and dry soil mass is determined from post-extraction residues to normalize lipid yields to soil dry weight. Adapted from Frostegård et al. (1991), this protocol provides practical details on solvent preparation, silica column conditioning, equipment requirements, and quality safeguards (e.g., Na_2SO_4 dehydration, nitrogen evaporation), offering a standardized, scalable approach for quantifying both structural (PLFA) and storage (NLFA) microbial lipids in soil across ecological or experimental contexts.

Materials

Material/equipment

- Centrifuge glasses tubes (10 ml, 50 ml)
  - Ensure the tubes **fit your centrifuge rotor**. Check that the **lids are solvent-stable and tight**. **Glass is required**, as organic solvents can dissolve or extract fatty acids from plastic.

- Glass wool and NaSO4
  - Used to **remove particulates** (glass wool) and to **bind residual water** (Na2SO4) during organic phase cleanup. Ensure Na2SO4 is completely dry.

- Pasteur pipettes
  - Must be **made of glass** to prevent contamination from plastics when handling organic solvents. Use short and long pipettes as required for accessing the lower organic phase.

- Eppendorf tubes as adapters
  - Needed to ensure that Na2SO4 columns rest on the rim of centrifuge tubes rather than inside them. See detailed comment in SOP for correct assembly.

- Vacuum manifold system
  - Used to **pull solvents through the silica columns** for lipid fractionation. Ensure compatibility with organic solvents and proper waste collection.

- Vacuum pump
  - Must deliver **low, controllable pressure**. Always operate inside a fume hood and direct exhaust away from the user.

- Si-columns
  - Contain polar silica gel. They function by retaining lipids of different polarity to enable stepwise elution: neutral lipids → glycolipids → PLFAs. Ensure columns never run dry during use.

- Tube racks
  - Multiple racks are needed to organize samples and fraction tubes during extraction, separation, and fractionation steps.

- Multi-vortexer
  - Essential for efficient mixing of soil–solvent suspensions and reagents. Must accommodate 50 ml glass tubes.

- Overhead shaker
  - Provides continuous low-speed mixing to improve lipid extraction efficiency. Ensure speed calibration (e.g., 30 rpm).

- Drying oven (105 °C)
  - Used for drying soil residues to determine dry mass for normalization. Must be free of solvent vapors.

- Incubator (37 °C)
  - Required for alkaline methylation during conversion of lipids to fatty acid methyl esters (FAMEs). Ensure stable temperature.

- Evaporation block rack
  - Used for gentle evaporation under nitrogen to avoid oxidation of unsaturated fatty acids. Adjust needle height to prevent splashing.

- Vials, caps, micro inserts 6 septa
  - GC-compatible vials with solvent-resistant caps and glass micro-inserts. Ensure septa are PTFE-lined and solvent-safe.

Chemicals

- Bligh 6 Dyer-reagent (extraction solution)
  - Mix 300 ml chloroform + 600 ml methanol + 240 ml citrate buffer.

- Citrate buffer (to be stored in the fridge!)
  - Dissolve 31.52 g citric acid in 1000 ml water, adjust to pH 4 using ~7.7 g of NaOH *(Store in fridge!)*.

- Acetic acid (1 M)
  - Mix 5.72 ml 100% acetic acid with deionized water until you have 100 ml.

- Hexane-chloroform solution
  - Mix hexane and chloroform 4:1 (v/v).

- Methanol-toluene solution
  - Mix methanol and toluene 1:1 (v/v).

- Methanolic KOH 0.2 M (prepare always fresh one for each sample batch)
  - Dissolve 13.2 mg KOH (85%) per ml methanol. *Prepare freshly for each batch to ensure complete methylation.*

- 19:0 internal standard (methyl nonadecanoate)

Troubleshooting

Safety warnings

This is the first point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 14.

This is the second point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 20.

This is the third point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 28.

This is the last point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 47.

Before start

Samples should be sieved at 2mm to remove stones, animals, and roots. Samples also need to be processed as soon as possible, as PLFA content decreases with time, even when stored at -20°C.

Preparation

1 Label 2 × 12 50 ml centrifuge tubes (glass!). Include two blanks (no soil, only reagents): Order the blanks that there is one at the beginning and one at the end of your sequence.

2 Weigh in 5 ± 0.1g (fresh weight) of your sample into the tubes. Record the exact fresh weight and the empty tube weight. This allows later calculation of lipid content per g dry soil.

Extraction

3 Add 18.5 ml Bligh 6 Dyer (extracts both polar and non-polar lipids).

4 Vortex for 1 min (use multi-vortexer). Ensures full contact of solvent with soil particles.

5 Shake for 1 h at 30 rpm on overhead shaker. Facilitates lipid dissolution.

6 Vortex for 1 min (use multi-vortexer).

7 Shake it for 1 h (overhead shaker, speed 30 rpm).

8 Centrifuge the samples at 1500 g for 10 min (8°C). Separates soil debris from lipid extract.

9 Transfer the supernatant to new 50 ml glass tubes (label 12 glass tubes). Use Pasteur pipettes (glass).

10 Add another 5 ml Bligh 6 Dyer to the soil residue, vortex 5 min.

11 Centrifuge as described above.

12 Transfer the supernatant to the one in the new 50 ml tube.

13 Dry the remaining soil residue (overnight under fume hood, then 105 °C) to determine dry weight. This is used to normalize lipid concentrations per dry mass.

Phase separation

14 Add 6.2 ml chloroform and 6.2 ml citrate buffer to the pooled supernatant (This separates the extract into aqueous and organic phases).

15 Vortex for 5 min (use multi-vortexer).

16 Centrifuge (see above). Beginners centrifuge the first six 50 ml only, the second six later to prevent mixing of phases when being slow.

17 During centrifugation, prepare filtration columns: Fill Pasteur pipettes with glass wool. Add ca. 1 cm of Na2SO4 (water free) to Pasteur pipettes to remove residual water. The Na2SO4 columns should not sit inside the centrifuge tubes but rest on the rim. Therefore, build adapters from 2 ml reaction tubes and pipette tips.

18 Transfer ≥ 5 ml from the lower phase on the filtration columns with a Pasteur pipette. For this purpose, push a short Pasteur pipette through both phases while you slowly emit some air from the pipette. Then, use a long Pasteur pipette to draw in the lower phase through the short Pasteur pipette. Avoid cross-phase contamination.

19 Release the liquid into the Na2SO4 columns. Liquid will seep from the column into the new 10 ml centrifuge tube. The fill level in the new tube must not reach the adapters.

20 Transfer and count only full milliliters to the previously prepared 10 ml tubes (usually 7 ml). Write down the volume for each sample (important for later calculations).

21 This is the second point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 22.

Lipid fractionation

22 Evaporate the organic phase under a N2-atmosphere at 35°C. This prevents oxidation of unsaturated lipids. Check the level from time to time and lower the height of the jets when needed. Stop the process as soon as all liquids evaporated. Close the tubes immediately since the extract is sensitive against oxygen. Don’t forget to turn off the N2 at the end.

22.1 During Evaporation:
Set up the vacuum manifold system under the fume hood and connect it with the vacuum pump

22.2 Mount Si-columns on the vacuum manifold system and condition the columns (never let them dry):
Close the valves. Put the solvent waste beakers under the needles. Condition the Si-columns by adding 2 ml chloroform to each and opening the valves. NEVER let columns get dry! This takes a while. You can prepare the next steps during conditioning. Close the valves before the chloroform level reaches the Si-layer in the column.

23 Label two sets of 10 ml centrifuge tubes. Use the label “Chl” (Chloroform) for the NLFA fraction and “Me” (Methanol) for the PLFA fraction (i.e. Chl1, Chl2, ...; Me1, Me2, ...).

24 Once the evaporation is complete: Replace the waste beakers with the previously labeled centrifuge tubes. Start with the NLFA-tubes, i.e. “Chl1, Chl2, ...” etc..

25 Redissolve the evaporated extract with 300µl chloroform. Shake gently. Load the extract onto the columns with a pipette and let the column material absorb the liquid. Add 2.0 ml chloroform to each column. Pay attention to the order!

26 The solvent will be sucked out of the columns by the use of the vacuum pump (only use low pressure). Use the pump only under the hood and let the exhaust valve direct into the hood (not in your direction).

27 Open the column valves. Pay attention: You have to observe all columns simultaneously. Close them before they get dry, i.e., the solvent meniscus reaches the column material.

28 Repeat this step with further 2.5 ml chloroform.

29 Replace the rack with tubes (Chl1 etc.) with the waste containers. Close the Chl tubes. Flush the columns with 6 × 2.0 ml acetone. You can discard this.

30 Replace the waste containers with the rack with new tubes labeled with “Me1” etc. Add 2 x 2.5 ml methanol to each column to collect the PLFA fraction.

Alkaline methylation

31 Adjust the drying oven to 37°C. Take a metal evaporation block rack and place it in the drying oven.

32 Evaporate the Chl- or Me-phase at 35°C under N2 atmosphere (see above, beginners start with the evaporation/methylation of only one phase). During evaporation freshly prepare methanolic KOH (for step 33) for both phases.

33 Add 30 µl internal standard (C19:0, methylnonadecanoate) to your dry extract at the bottom of the tube. Use a pipette with preferably high accuracy.

34 Add 1.0 ml methanol-toluene-solution.

35 Vortex for 1 min.

36 Add 1 ml freshly prepared methanolic KOH and close the tubes carefully.

37 Vortex for 1 min.

38 Place the tubes in the drying oven for 15 min.

39 After removal, add 2.0 ml hexane-chloroform solution.

40 Add 0.3 ml acetic acid.

41 Add 2 ml deionized water.

42 Vortex for 5 min.

43 Centrifuge samples (see above).

44 Transfer the upper phase to new 10 ml centrifuge tubes.

45 Add again 2.0 ml hexane-chloroform solution to the remaining lower phase.

46 Vortex for 5 min.

47 Centrifuge samples. During centrifugation, you can start with the evaporation of the second phase (NLFA or PLFA).

48 Transfer the upper phase to the tube.

49 Repeat steps 30-46 with the second phase.

50 This is the last point where you can stop overnight. Samples are still solved in liquid and can be frozen at -20°C. Otherwise, go on with step 51.

51 Evaporate samples at 35°C under N2 atmosphere (see above).
During evaporation: Add micro inserts to sample vials and label the vials: 1. fraction, 2. clear sample name, 3. running number

52 Add 100 µl hexane to samples one after the other. Gently and shortly shake the tube and dilute the extract by carefully repeated pipetting. Try to solve the whole extract.

53 Transfer the extract into vials with micro inserts inside.

54 Close the vials with plastic caps and septa.

55 Freeze the vials at -20 °C upon measurement.

Protocol references

Frostegård, A., Tunlid, A., Bååth, E., 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14, 151-163.