Phospholipid Fatty Acid Analysis (PLFA)

NEON Specific

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High Throughput Method

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Phospholipid Fatty Acid Analysis

Materials, Methods, and Instruments

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General Information

This manual describes in detail the complete process for handling soil samples submitted to the Soil Health Assessment Center (SHAC) for phospholipid fatty acid analysis (PLFA) by the National Ecological Observatory Network. This includes sample intake, APHIS considerations, sample prep, sample extraction (including the extraction of PLFAs from soil by solvents, separation of PLFAs from neutral lipids and glycolipids, fatty acid methylation, suspension, and analysis on a gas chromatograph), data export and reporting, and other topics that are part of the process such as instrument operation and reagent instructions. Overall laboratory quality assurance (QA) procedures are listed in a separate QA manual. These manuals should be referenced and utilized by SHAC employees for all parts of sample handling. After extraction of samples, the amount of each lipid found in the sample will be reported in nanomoles, where users can then access the data to draw conclusions about the abundance and composition of microbes in the soil.

Acronym Table	
PLFA	Phospholipid Fatty Acid
NEON	National Ecological Observatory Network
SPE	Solid Phase Extraction (96 well plate)
SHAC	Soil Health Assessment Center
STD 8	SHAC Soil Standard 8
QA/QC	Quality Assurance/Quality Control
APHIS	Animal & Plant Health Inspection Service
CoA	Certificate of Analysis
DI	Deionized (water) (Type I)
GC	Gas Chromatography

Notes

- Wear gloves for everything to protect hands from chemicals and protect samples from skin oils. Eye protection and lab coats should be used when running the analysis.

- Before you use a chemical, make sure to have read the corresponding safety data sheet and be aware of how to use and dispose of that chemical safely as well as the associated risks.
- Only use ultrapure DI water (≥ 18.20 MΩ·cm) for analysis, making reagents, and cleaning labware.
- When taking in new reagents, label with received date(rec'd) and highlight expiration date on label. If expiration date is not listed, find retest/expiration dates on the Certificate of Analysis (COA) and record with the COA. When opening new reagents, write opened date on label. Also, download the appropriate COA from websites (below) and add to the PLFA chem log file (PLFA Chemical CoA&Expiration Dates.docx) inside the General folder. When reagent bottles are emptied, remove their associated certificates, mark up the label with a sharpie, and place in normal trash.
- Before making new solutions/filling secondary chemical containers, check expiration dates to ensure chemicals are in date. Check to ensure current solutions are in date before use.
- Every six months (January and July), conduct a complete chemical inventory. Note any and replace any chemicals expiring before the next inventory.
- *If samples are **APHIS-regulated**, follow special protocol in APHIS Standard Operating Procedure. Label samples as APHIS regulated, store samples in APHIS permitted areas/freezers, store with double containment, process all samples in metal pans to prevent spillage of soil, clean all areas and scales after use with ethanol or disinfectant wipes, sterilize all utensils, trash, contaminated packing material and pans by oven baking at 125°C overnight or wiping with ethanol or disinfectant wipes if oven is unavailable. Collect all materials that have come into contact with chemicals for off site decontamination.*

Methods

New Sample In-take

- When a NEON PLFA sample package arrives, determine if samples are non-APHIS-regulated, APHIS-regulated, or 526-Permit-AHPIS-regulated samples, or unregulated NEON samples. If samples are APHIS regulated, **only** proceed if APHIS trained.
- If the samples are APHIS-regulated, open the package in the fume hood or the biosafety cabinet in a disposable aluminum tray.
    - Wear a lab coat and gloves. If samples are 526-regulated samples, assemble alcohol vials and forceps before opening box. Be ready to capture any plant/pests that may be contained in box.
    - Remove tubed samples from packaging. Remove all plant/pests and contain them in alcohol vials. Inspect all packaging materials. Heat-treat any shipping material that has been in contact with quarantined material according to APHIS Permit and SOP.
    - Place an APHIS Regulated sticker on to each sample.
- Find the electronic and hard-copy list of all samples included (e.g., a 'shipping inventory')
- Record all needed information into the APHIS sample log.
- Assign SHAC ID numbers sequentially.
- Numbering samples: First, obtain the sample manifest form and assign SHAC IDs to each sample, then, wearing gloves, lay out each sample in order following the sample manifest-SHAC ID sheet, and number each sample with its SHAC ID using a sharpie.
- Place samples in the appropriate freezer (-80°C APHIS, -80°C NEON).

Freeze Drying NEON Samples—Labconco Lyph-lock 12 Freeze Dryer

NOTE: this should be done within 15 days of receiving samples.

- Wipe out reservoir in cold trap of moisture with paper towels.
- Gather: Gloves, Styrofoam sample holders, rubber bands, paper towels, Kimwipes, and samples.
- Unscrew sample cap and place Kimwipe folded once over tube opening. Place rubber band to hold Kimwipe on sample vial.
- Mark the vial with sample id number for reference later if needed.
- Place entire sample in Styrofoam holder and lower into freeze dryer. Repeat until all samples are loaded.
- Replace the freeze dryer lid and turn "REFRIDGE" switch to ON.
- Wait for the temperature to drop below -50°C before turning on the vacuum. Keep samples in the vacuum dryer until the gauge reads below 1,000 microns (10 on the gauge).
- When the samples are dry, turn the vacuum off, turn "REFRIDGE" off, and slowly loosen a rubber stopper so that air is let into the system and the vacuum is released.
- Kimwipes on the samples should be thrown away and sample lids with matching lab ID's placed back onto the sample. Samples should then be placed into the appropriate freezer (-80°C APHIS, -20°C non-APHIS).
- Record all necessary information on the freezer log.

Transferring/grinding samples

- Gather: Gloves, small mortar, and pestle, empty lab tray, rubber bands, paper towels, ethanol, and samples. Work in small batches so that samples do not warm too much while grinding.
- If samples are APHIS regulated, **only** proceed if APHIS trained. If samples are decontaminated after freezing (for fire ants only) then samples can be treated normally. For others, all work done must be kept within the APHIS room and contained within large tins. All materials must be contained and decontaminated after use. Once done, wipe all work areas down with disinfectant wipes. Place back into -80°C APHIS freezer.
- Place a paper towel on the lab tray so that cleanup between samples is easier. Wipe out mortar and pestle with ethanol and a kim wipe. Make sure tools are completely dry between samples.
- Pour part of the sample from the tin into the mortar. Grind sample fine enough to pass through a 2mm sieve. Pick out rocks, large roots/plant matter that will not grind.
- Once ground, pour ground sample into sample bag. Repeat grinding with the rest of the sample.
- In between each sample, wipe off pestle and wipe out mortar with ethanol and a kim wipe. Make sure tools including gloves are cleaned and dried between samples to prevent cross contamination. If gloves get particularly dirty, replace them.
- Mark on **PLFA Sample Log** the samples that have been ground.
- When finished, thoroughly clean workspace by wiping off surface with disinfectant wipe, clean mortar and pestle and tray, and put all supplies back in Clean Room (do not leave PLFA tools in main lab). Place samples back into the freezer that they were originally stored in.

Prep-Work for PLFA Analysis

- All prep-work should be complete BEFORE Day 1 of PLFA Extraction.

Culture Tube Sets:

- PLFA analysis requires two sets of new glass culture tubes, numbered from 1 to 96.
- Gather: 4 empty tube racks (=2 racks per set of tubes), gloves, rotor, black caps.
- Fill the rotor with 96 tubes, starting in the second row and working inwards, using holes 1, 5, 9, 13 on the inner most.
- Starting with rotor hole 73 (always start a new row of tubes with the lowest rotor hole number), number the tubes with their tube number.
- Set 1 – fill two racks with tubes, write number on tubes (1 to 96), and cap with black caps.
- Obtain **Pre-weights** of each tube + cap on three-decimal place scale and save weights on the "Sample Weight Sheet" of the Sample Run Sheet Excel file.
    - **NOTE:** weight columns have conditional formatting to flag weights outside of normal range. If cells turn red, ensure that weight is correct.
- Set 2 – fill two racks with tubes the same as Set 1, (no caps); cover each rack with foil (to protect tubes from dust contamination).

Loading sample into culture tubes:

- Gather: Sample Run Sheet, gloves, Set-1 tubes, PLFA loading kit (wooden block, 2mm sieve, 2-gram scoop, and small metal funnel), kim wipes, ethanol wipe.
- **Note:** PLFA method uses approximately one 2-gram scoop volume of soil. Organic soils or compost will often require sample masses of less than 1-gram due to their greater microbial biomass per unit mass, whereas sandy soils typically need more than 2-grams due to a smaller microbial biomass. This volume-measure usually works well.

Use disposable black caps. If there is a supplier shortage, use reusable caps that have followed cleaning protocols found in the clean up section.

- Obtain the pre-weights (tube + cap) of tube Set 1.
- If samples are APHIS regulated, **only** proceed if APHIS trained. All APHIS material must be contained and decontaminated.
- Following along sample sheet to make sure Sample ID # and Tube # match, scoop out a soil sample from the shaken sample bag using 2-gram scoop, and add into sample tube.
- Wipe out funnel, sieve, and 2g scoop with ethanol wipe between each sample to prevent cross contamination; and set aside.
- Cap tube and return tube to rack.
- When finished loading the tubes with soil, wipe off all tools with ethanol wipes and store.
- If samples are not going to be immediately dried down, store racks in -20 °C freezer.
- Record that they have been loaded and rack letter on the top of the run sheet.

Drying down samples in culture tubes:

- Samples should be dried down to 500 microns to ensure uniform dryness at the time of sample weighing prior to Day 1 of PLFA Extraction. Samples have already been freeze-dried, but sample dryness may vary, and samples may have reabsorbed moisture through the sample processing procedure. This should be done before sample weights are taken to ensure dry weights are recorded.

- Before using the Cold Trap, it is cooled between 107 and 110°C to prevent moisture from getting into vacuum.
- Uncap tubes and place caps on clean foil or metal pan where the first cap goes into the bottom left and the last cap into the top right of the pan. Work in rows like in tube rack from left to right, bottom to top so that caps are in the same position as racks are arranged.
- Load centrifuge rotor with un-capped tubes by loading from the outside row and working inwards; the last four samples should be evenly spaced in the inner row (1,5,7,13)
- Before placing rotor in CentriVap, make sure the lid and rim of basin are clean of dirt and glass fragments to ensure a tight seal.
- Use CentriVap on **Program 5 (37°)** with cold trap and vacuum pump to dry down samples to **500 microns.**
- Once the CentriVap is up to speed, switch on the vacuum pump. And turn on vacuum gauge.
- When dry-down is finished (vacuum meter reaches 500 microns), stop CentriVap, and slowly pull out small red stopper in Cold Trap to allow air into CentriVap. Make sure that the stopper is pulled out while centrifuge is still spinning so soil is held in place. Pulling too fast will make soil spill. Once the vacuum is eliminated, open CentriVap lid.
- Remove samples and turn off CentriVap and Cold Trap if done using for the day.
- Transfer tubes from rotor back into Set 1 racks and replace all black caps onto appropriate tubes.
- **Obtain post-weights** (tubes+soil+caps) on the three decimal scale and record in the previously saved excel file with Pre-weights. The Excel file is already set up to calculate the soil weight in the fourth column using formula:
  [Post-weight (tube+cap+soil) – Pre-weight (tube+cap) = dry soil weight]
  - **NOTE:** weight columns have conditional formatting to flag weights that are outside of normal range. If cells turn red, ensure that weight is correct.
- If samples have been frozen before weighing, make sure samples are at room temperature and condensation is evaporated before weighing.
- Record that they have been dried and weighed on top of run sheet.
-

Making Run Sheets

NEON runs must be set up very specifically. A run needs 4 blanks and 2 standards with the order being blank1 in tube 1, standard in tube 2, 33 samples, blank2 in tube 36, 24 samples, blank3 in tube 61, 33 samples, standard in tube 95, blank4 in tube 96.

- When making run sheets, keep samples in ascending order unless there is a mix of APHIS and non-APHIS samples, in which APHIS samples go at the end of the sheet.
- Samples should be processed as soon as possible, so try to do the oldest samples first.
  - The target run date of NEON samples is 60 days from freeze-drying.

1. Map out run sheet following notes above.
2. Make a copy of the runsheet, rename to run number, and open copy in desktop excel.
3. Copy and paste SHAC ID and Sherlock Name columns. Make sure to not move cells in spreadsheet or it will not auto calculate further into the sheet. Samples need to be added in the order that they will appear on the run sheet.

a. The Sherlock name for NEON runs consists of NEONYear-Domain-Barcode (ex. NEON00-D00-B000000000).

4. Click into the "Run Sheet" tab of the excel document. Add the run number to the top of the sheet and enter a NEON number as well. Highlight tube number of APHIS samples **yellow** and SHAC ID text of NEON Reruns **green.**
5. Print Sheet.
6. Draw a line in between all breaks in ordered samples (if samples numbers change) so that it is easy for the loader to see. Highlight run number in **green.**
7. Make sure that the run sheet is uploaded online and place on the freezer.

8-Channel Pipette Tips

NOTE: 8-channel pipette tips are a known source of contamination and must be washed **Before** use. Each run takes 2.5 boxes of tips.

- Gather **new** pipette tips, pipette box marked "clean", methanol and methanol dish, chloroform and chloroform dish, acetone and acetone dish, and 8-channel pipettor set to wash setting (800µl).
- Attach first column of unwashed pipette tips to 8-channel pipettor. Wash pipette tips by taking up methanol, dispensing into waste beaker, taking up chloroform, dispensing into waste beaker, taking up acetone, and dispensing into waste beaker. Place now clean pipette tips into "clean" box. Continue washing pipette tips until box is finished. Place clean box onto pipette tip shelf and the original box onto the unmarked box shelf.

PLFA Extraction Method

Before Day 1 of PLFA Analysis:

- Obtain sample post-weights of tube dried samples and initial on run sheet.
- Make sure to have a 2nd set of numbered tubes.
- Fill all chemical bottles; make sure to have enough Dipotassium Phosphate (100mL), Acetic Acid(60mL), Internal Standard, Calibration Solution(1.25mL) for next run
- Make sure to have a clean set of Cap Mats.
- Empty chemical waste beakers into appropriate barrel for unwanted materials.
- Know that PLFA extraction can only be paused in a few places. PLFA can be paused after Day 1 is complete for 1-2 days, after drying down but before transesterification in day 2 for 1 day, and for a few weeks if necessary if suspended in hexane. Make sure to look at standard total responses once ran to ensure quality after storing.

Extraction (DAY 1)

NOTE: IF DOING 2 RUNS, make a double batch of Bligh Dyer (step 2) and stagger. Do steps 3-7 on first batch and while incubating on step 7, complete steps 3-6 on batch 2. Once batch 1 finishes incubating, start on steps 8-24 while batch 2 incubates during step 7.

1. Remove two racks of samples (in culture tubes) from the freezer so they warm to room temperature; Make sure the dried samples in the culture tubes have been weighed before adding Bligh-dyer solution; If post-dry-down weights haven't been obtained, take tubes to 3-place balance, recording weights in proper Excel file.
   ***IMPORTANT**: moisture will condense onto outside of cold tubes, so wipe off moisture from each tube with Kimwipe before weighing.*

2. Mix up the Bligh-Dyer extraction solution (with the Internal Standard) in the Bligh-Dyer bottle-top dispenser.
3. Add 4 mL of Bligh-Dyer extractant solution to each culture tube, replace black cap, and vortex tubes to mix the soil with the solution.
4. Make sure the water level is correct in the sonicator, and then set racks side-by-side in the Ultrasonic Bath, on top of the bottom spacers.
5. Sonicate samples for 10 minutes (set Timer) at room temperature.

While sample are sonicating:
Rinse out bottle and dispenser with ultrapure DI water (three times).
And lay out to dry on paper towels/Kim wipes in fume hood.

6. When finished sonicating, turn machine off. Place sample racks on paper towel lined metal tray.
7. Wipe the water off the culture tubes with paper towels, Vortex each tube, and load onto a Labquake Rotator; incubate for 2 hours on the Rotators at room temperature (set timer for 60 minutes – then gently push each rotator to rotate in the opposite direction for final 60 minutes); make sure tubes are mixing and are not leaking.
8. Vortex culture tubes to get the soil out of the caps and from upper part of tubes.
9. Load half of the tubes (with caps on) into the rotor.
10. Set the CentriVap to **Program 4 (30°)** without vacuum pump and start. centrifuge for 10 minutes. After 10 min, stop machine.
11. While the first half of samples are centrifuging, vortex the last half of samples and load into tube rack; When the first half of tubes are done centrifuging, unload tubes and replace with the last half (48 tubes) to centrifuge.
12. One sample at a time, remove the cap and discard into small waste container; Transfer the liquid phase of each tube into the 2nd set of new culture tubes; Don't cap the new set of culture tubes yet.
    NOTE: *Make sure to not hold tubes over sample racks while transferring so it won't contaminate other samples if it drips or spills.*
13. Gather: Chloroform bottle-top dispenser; HandyStep dispenser with the 'H2O' tip; 'H2O' beaker with ultrapure DI water; and new black caps.
14. Add 1 mL of Chloroform to each tube using the Chloroform bottle-top dispenser.
15. Add 1 mL of ultrapure DI water to each tube using a HandyStep dispenser with the 'H2O' tip.
16. Cap each tube with a clean, black (PTFE lined) cap.
17. Vortex each tube for 5 seconds on the Vortex Genie, loading first half the tubes (with caps on) into rotor; vortex last half while first half is centrifuging.
18. Set the CentriVap to **Program 4** without vacuum pump and start.

Centrifuge for 10 minutes; after 10 min stop machine; unload tubes and repeat centrifuge on the last half of samples (48 tubes). After centrifugation, the solution separates into 2 phases. The surface phase is mostly aqueous, and the bottom phase is organic solvents.

While samples are centrifuging, Set up in Fume Hood:
1L glass jug with cork/tube apparatus, small beaker of ultrapure DI water,
Pasteur pipets, Kimwipes, and metal pan for caps.
NOTE: Be careful not to contaminate the Pasteur pipette by touching the tip to any surfaces.

19. With tubes back into their racks: One tube at a time, remove the cap and place on metal pan; Aspirate (suck up) the top [aqueous] phase using a Pasteur pipette hooked up to a vacuum bottle for each of the culture tubes. If at any time the tip of the pipette slips into the lower phase in a culture tube, dispose of the pipette and use a new one. This is to avoid contaminating the lower solvent phase containing the lipids. ***NOTE:*** *If debris gets stuck in pipette tip, use a new pipette tip.*
20. Load ALL tubes (without caps) into the CentriVap rotor.
21. Wipe/clean around CentriVap lid and basin before placing rotor in the machine.
22. Set CentriVap to **Program 3 (30°)** and centrifuge with vacuum pump and cold trap until the vacuum reaches **500 microns**.

While samples are centrifuging:
Suck up remaining water in beaker to rinse out tube.
Dump jug waste into chloroform waste beaker and rinse out jug with water.
Put everything away and properly discard trash

23. When dry down is finished, remove rotor from CentriVap, place tubes back in tube rack, recap tubes and put into freezer (at -20 °C) for overnight storage.

Chromatography (Day 2)

NOTE: IF DOING 2 RUNS, all steps are the same (including amounts of 5:5:1 and Transesterification) except instead of splitting rack into even and odds in step 24, keep batch 1 in rack "1" and batch 2 in rack "2".

NOTE: Make sure to only use solvent washed 8-channel pipette tips.

1. Get a used/new [50 mg silica gel] 96-well SPE plate from drawer and mount it on the Vacuum Manifold with a disposable collection trough in the bottom of the apparatus; Note run number on 96-well plate bag.

NOTE: The SPE plate must be conditioned/washed with methanol and chloroform before using. It can be used up to 7 times. If it is slow to drain, replace.

DO NOT set the SPE plate on a non-sterile surface that might contaminate the 96 nozzles on the bottom of the SPE plate.

2. Gather: Methanol, Methanol beaker, HandyStep dispenser with the Methanol-tip.
3. Pour Methanol into beaker and wash each well with 1 mL of Methanol using a HandyStep dispenser with the Methanol-tip; allow to fully drain before next wash.
4. Repeat 2 more times for a total of 3 mL of methanol wash.

While waiting for plate to drain, in Fume Hood:
2. Add Methanol (75 mL) to the Transesterification dish (see Transesterification recipe) and cover with large watch glass (keep in fume hood).
3. Make 5:5:1 solution, cover with watch glass.
4. Weigh out the KOH pellets (0.561 g) and add to methanol in the Transesterification dish.

5. After the 3rd methanol wash, use a very low vacuum (-1 to -2 psi) to drain the last drops of methanol from the 96-well SPE plate before going on to the next step.
6. Switch to the solvent safe '96-well collection trough' for the following chloroform and acetone steps (chloroform and acetone will dissolve the disposable trough).
7. Place the 96-well plate on top of the collection trough making sure they are properly aligned by matching up A1 cell at top left corner.

8. Wash each well with 1 mL of chloroform using the bottle-top chloroform dispenser. Allow the 96-well plate to completely drain between each wash.
9. Repeat 2 more times for a total of 3 mL of chloroform wash.

During the Chloroform washes:
Gather 1.5 flat-bottom vials, 1.0 flat-bottom vials, 96 vial racks (with A1 marked for positioning), tweezers.
Set up 1.5 flat-bottom vials in rack '1' (can use the plastic containers that the glass vials came in to protect vials from contamination until use).
Remove samples from the freezer to bring to room temperature.

10. After the 3rd chloroform wash, mount the 96-well plate and 96-well collection trough on the vacuum manifold and use a very low vacuum (-1 to -2 psi) to drain the last drops of chloroform.
11. Place the 96-well plate back on top of the collection trough making sure they are properly aligned. Add 1 mL of Chloroform to each tube using the bottle-top chloroform dispenser; Swirl each rack a few times to dissolve dried materials on the sides of the tubes.
12. Add sample extracts to the plate wells by pouring from the culture tubes (with the 1 mL chloroform) directly into the SPE plate wells. Tube 1 goes into well A-1 and tube 2 goes into well A-2 etc. (moving left to right, top to bottom in the 96-well plate); be careful to not cross contaminate – If a drop falls into the wrong cell, make a note on the Run Sheet what happened, the sample will need to be rerun.
    NOTE: The top left corner of the well plate (cell A-1) should be marked with a sharpie as well as the well plate number written on the front of the plate to help ensure correct plate positioning.
13. Let the samples completely drain through the wells and discard.
14. Add another 1 mL of chloroform into each culture tube using the bottle-top chloroform dispenser. Swirl the racks once again to wash the sides of the tube to make sure all lipids are collected.
15. Carefully pour the second 1 mL of chloroform into each well of the SPE plate.
16. After the wells are completely drained, mount the SPE plate and 96-well collection trough on the vacuum manifold. **Apply a low vacuum** to drain the last bit of chloroform and discard.
17. Align the 96-well plate and collection trough and carefully add another 1 mL of chloroform directly into each well using the bottle-top chloroform dispenser; Once completely drained, mount the SPE plate and 96-well collection trough on the vacuum manifold; Apply a low vacuum to drain the last bit of chloroform from the 96-well SPE plate and discard. This separates the neutral lipids.
18. Once again, set the 96-well SPE plate on top of the 96-well collection trough; slowly Add 1 mL of Acetone to each well using the Acetone bottle-top dispenser. This step separates the glycolipids.
19. Allow the SPE plate to completely drain and then mount the SPE plate and 96-well collection trough onto the vacuum manifold. Apply a low vacuum to drain the last of the acetone from the 96-well SPE plate and empty the collection trough into the acetone-waste beaker.

While waiting for acetone to drain:
Place standard 1250 µl tips next to 8-channel pipettor and vacuum manifold.
Set 8-channel dispense volume to 500 µl (551 setting).

20. Replace the 96-well collection trough with the set of 1.5 mL vials in the vacuum manifold .

21. Using the 8-channel pipettor with solvent washed 1250 µl size tips, thoroughly mix 5:5:1 solution with pipette tips. Next, elute the phospholipids from well plate by adding 500 µl (0.5 mL) of the 5:5:1 solution; Allow the SPE plate to drain and then apply a low vacuum.
22. Separate the 1.5 mL vials by using tweezers to remove the even-numbered columns of vials from the original rack (#1) and to place them in the even columns of a second vial rack (#2). The odd rows of vials will remain in the first vial rack. (This will allow the 2-place rotor to be balanced while centrifuging); Place the two vial racks onto the 2-plate rotor in the CentriVap.
23. Run the CentriVap on **Program 1 (70°, 30 minute timer)** using cold trap and vacuum.

While waiting for Program 1 to end:
1. If the SPE 96-well plate is to be re-used: Put the disposable collection trough in vacuum manifold,
and wash used 96-well plate with 1mL Methanol before putting back into drawer. Wrap 96-well plate completely in kim wipes so that it does not touch bag.

24. When 29-minute timer goes off, shut off the vacuum pump valve at 1 minute before Program 1 ends (DO NOT turn off the vacuum pump during this step) and use the red plug on the cold trap to slowly release the vacuum in the system by allowing air to enter.
25. Set the CentriVap to **Program 2 (37°, 121-minute timer)** and re-open pump valve to run with vacuum and using the cold trap until the vacuum reaches **500 microns**.

While waiting for Program 2 to end:
1. Set up Fume hood with: two new and one opened boxes of solvent washed 1250 µl tips,
Chloroform bottle-top dispenser,
500 mL Toluene, 100mL graduated cylinder, 8-channel pipettor, clean sheet of foil,
And 2 clean Teflon cap mats (be careful not to touch cap-side of mats.
place mats on clean sheet of foil (cap-side up) and fold foil in half to cover them until use).
2. Mark Teflon cap mats as “1” and “2”
3. Set 8-channel pipettor to 200 µl (transesterification setting), and set Chloroform dispenser to 0.40 mL.

Transesterification and Transfer to GC Vial

26. Add the Toluene (25 mL) to the Transesterification dish (KOH pellets should be completely dissolved). Using the 8-channel pipettor with 1250 µl tips, mix solution.
27. Add 200 µl (0.2 mL) of Transesterification solution to each column of 1.5 mL vials in racks ‘1’ and ‘2’.
28. Make sure the cap mats are labeled “1” and “2”. Place Teflon cap mats on the respective numbered vial rack, and gently shake to mix; place on two-plate rotor and put in CentriVap.
29. Incubate the vials with the cap mats on in the CentriVap without spinning, vacuum, or cold trap for 15 minutes (set timer) at 37°CWhen finished, remove vial racks out of the CentriVap and place in fume hood.

While samples are incubating:
1. Gather 0.075 M Acetic Acid solution,
Acetic Acid beaker, HandyStep with ‘acetic acid’ tip.
Safety glasses, PLFA wood block, paper towels; 1.0 mL glass bottom vials and racks.
2. If not already, separate 1.0 mL vials into the two racks so

odd numbered columns are in rack "1" and even numbered in rack "2"
3. Set 8-channel pipettor to 300 µl (transfer 1 setting).

30. Starting with Rack 1 - 1.5 mL vials, slowly remove the cap mat (one column at a time) and place it cap-side up on foil (so not to contaminate caps); fill Acetic Acid beaker with 0.075 M Acetic Acid solution; Add 400 µl (0.4 mL) of 0.075 M Acetic Acid to each vial using a HandyStep dispenser with the 'Acetic Acid' tip.
31. Add 0.4 mL of Chloroform to each vial using the chloroform bottle-top dispenser.
32. Making sure the cap mats are in the proper orientation, Recap Rack 1 vials with the Teflon cap mat; Shake vigorously to mix and set aside to let the phases separate.
33. Repeat Steps 30 to 32 on Rack 2 – 1.5 mL vials.
34. While Rack 2 phases are separating, slowly remove Rack 1 cap mat so not to cause any sample drops on the caps to contaminate another sample; align Rack 1 - 1 mL vials and Rack 1 - 1.5 mL.
35. Using the 8-channel pipettor and using a new 1250 µl tip for each sample, Transfer 300 µl (0.3 mL) of the bottom phase from the 1.5 mL vial racks to the corresponding 1 mL vials.
    NOTE: IF DOING 2 RUNS: to prevent contamination, wrap cardboard cover in fresh piece of foil and bend sides up (so that drips cannot roll off edge). When transferring, place cover on sample rack covering samples and move 8-channel tips over foil while transferring to next rack.
    NOTE: DO NOT worry about drops coming out of tips and losing sample (vapor pressure inside tips tends to push out solution, but since there is an Internal Standard in the samples, the software will account for sample loss).
    IMPORTANT: if any cross-contamination occurs, make a note of sample #s and what happened on Run Sheet, so they can be re-run; see 96-Well Number chart to identify sample #.
36. Add another 0.4 mL of chloroform to each Rack 1-1.5 mL vial, replace the Teflon cap mat in the proper orientation, shake again, and set aside to allow the phases to separate; gently place Rack 1-1.0 mL vials to back of hood and cover with empty plastic vial container to protect.
37. While Rack 1-1.5 mL vial phases are separating, Repeat Steps 35 to 36 on Rack 2 – 1.5 mL vials.
38. While Rack 2 – 1.5 mL vial phases are separating, carefully remove the Rack 1 cap mat, set on foil, and align the vial columns.
39. Set 8-channel pipettor to 400 µl (0.4 mL) (transfer 2 setting).
40. Using the 8-channel pipettor with a new 1250 µl tip for each sample, transfer 400 µl (0.4 mL) from the bottom phase of the 1.5 mL vials to the 1.0 mL vials (as did above).
41. Carefully set both vial racks to the side, without cap mat.
42. Repeat Steps 39 and 40 on Rack 2 vials.
43. Wipe around CentriVap Lid; Carefully place both 1.0 mL vial racks on the 2-plate rotor and place in CentriVap for dry-down.
44. Set the **CentriVap** to **Program 4** (room temperature) and use vacuum and cold trap until the vacuum reaches **500 microns.**

Gather in Fume Hood:
Set of GC vials with new glass inserts, small container of new blue caps,
Box of Pasteur pipettes, blue pipettor, white bucket for glass trash,
Hexane bottle-top dispenser, small 'hexane' beaker (set on Kimwipe),

Pipetman with 200 µl solvent-safe tip (be careful not to contaminate tip so, set on Kimwipe near hexane beaker).
Set Pipetman dispense volume to 0.075 mL.

45. Once the CentriVap finishes, pull out rotor, and remove vial racks; move all even-numbered vials back to Rack 1 in appropriate columns; place Rack 1 with all vials in fume hood.
46. In Fume Hood: fill small hexane beaker with hexane, and pull GC vial '1' from GC vial tray. Add .075 mL of Hexane using the Pipetman pipettor with Solvent-Safe tip to 1.0 mL vials (starting in upper left corner = A1 position); then, insert a new Pasteur pipette into manual pipettor and hold in hand, pick up A1 vial and rotate to dissolve extract on side of vial; take up the sample with glass pipette, , grab the GC vial 1, carefully dispense sample into vial insert; cap vial with new PTFE lined screw caps, and put in Position 2 of gray auto-sampler tray or back into blue vial holder.
47. Repeat the above steps with other vials, making sure 1.0 mL vial # and GC vial # are the same.
    NOTE: Hexane vaporizes quickly at room temperature; so, if dissolve too many samples at one time, there is a chance the hexane will evaporate from the 1.0 mL vial before transferring to the GC vial, and there won't be enough liquid volume in the glass insert for the GC to sample from. If hexane volume is 0.5 or below too much is evaporating, and smaller batches should be done.
48. If samples cannot be run immediately on GC, samples can be stored for a small period of time in freezer (-20°C) if suspended in hexane. If samples can be run, place on GC.

Clean Up/Maintenance

Checklist:

- Check inventory—keep enough supplies and reagents for at least 20 PLFA runs. Report supply needs to a purchaser.
- Keep all counters, fume hood, tops of refrigerators, machines, and other equipment wiped down and free of dust; can use Ethanol or disinfectant wipes to sterilize surfaces.
- Keep floors swept.
- To empty glass trash: secure lid and bottom with tape and put in dumpster; order glass boxes and bag liners if needed.
- Wash Teflon cap mats. See "Teflon Cap Mats." Clean Cold Trap cap and tubes with deionized (ultrapure DI) water when dirty with dust/glass shards.
- Clean CentriVap lid with ultrapure DI water when dirty with dust/glass shards by removing from machine and taking apart; let dry completely before reassembling.

Teflon Cap Mats

- Immediately following use, rinse off in lab sink with ultrapure DI water and put into the Pyrex water dish (in fresh ultrapure DI water) to soak until washing.
- When washing cap mats:
  - Gather in Fume Hood: Pyrex dishes with dirty cap mats, Pyrex methanol dish, Pyrex chloroform dish, aluminum foil, and tweezers.
  - Set up in hood from left to right: dish with dirty cap mats, Methanol dish, Chloroform dish, and sheet of aluminum foil (big enough to lay out all cap mats).
  - Fill Methanol and Chloroform dishes with enough chemical for a cap mat to be submerged (roughly halfway).

- Rinsed sequentially in methanol, then chloroform. Make sure to hold cap mat between the nubs to decrease potential contamination.
- Drain off chloroform and lay on clean sheet of aluminum foil to dry, then put back in zip-bags in drawer. Make sure only the back of the cap mat touches the foil. Flip occasionally so that they dry completely on both sides.

Transesterification Dish

- When all solvents are gone, a precipitate of potassium hydroxide will remain in the dish.
  - Rinse remaining precipitate into waste beaker with ultrapure DI water squirt bottle.
  - Rinse thoroughly with ultrapure DI water and place dish back in fume hood to air dry. Once dry, move back into drawer placing upside-down on aluminum foil to reduce contamination.

Black Caps

- After using reusable caps, place in dirty cap container until ready to clean.
- If caps are particularly dirty, fill two wash containers with ultrapure DI water, place roughly half of caps inside each one, push inserts on top of caps to hold under water, and place in sonicator for roughly 10 minutes. If caps are not very dirty, skip this step.
- Once caps are sonicated, shake water out over sink.
- Place two pieces of foil large enough to hold 96 caps in fume hood. Gather chloroform, chloroform dish, cotton swaps, enough foil to cover chloroform dish, and caps.
- Pour chloroform into dish and cover with foil, leaving enough of one side uncovered so that a cotton swab can fit. This is to help hold in the fumes.
- Grab a dirty cap and dip cotton swab in chloroform. Swab out all visible dirt, focusing mostly on threads. Once clean, set top down onto the first piece of foil and move onto the next cap. If a liner falls out, throw the cap away.
- Replace cotton swabs when broken or visible dirty.
- Once all 96 caps are washed, grab a "clean" cap and a new cotton swab. Dip cotton swab into chloroform and clean cap again, focusing on the liner this time and making sure cap is completely clean. If cotton swabs are coming back very dirty, clean a third time. Once cap is clean, place on the second sheet of foil to dry.
- Once all caps are clean and dry, place into clean cap container for storage.

Running Samples on GC

1. When ready to run samples, follow directions for **Turning ON GC** in the **Instruments** section.
2. Place CAL on GC in position "1". Make sure CAL level is at least 1.25 mL. If CAL level is not this high, the GC may use all of the CAL solution before the run is finished and stop.
3. Fill wash bottles with hexane. If forgotten, all samples ran with insufficient wash solvent levels will be contaminated. These bottles are used to rinse out the syringe before samples are injected into the inlet of GC.
4. Add all samples into Sherlock Sample Processor. Once in **Sample Processor**, click **Add Samples**, copy the first sample from the **Sherlock ID** column from the **Sample Names Ready for Sherlock** tab from the run sheet. This should have the sender ID and the sample weight in format (g=0.000. Paste this into Processor and click enter on keyboard. Continue to add samples until all are added.

5. Once all maintenance is done, GC is ready, samples are on the GC, and samples are entered correctly into Sample Processor, press **Start Run.** Watch for error codes and correct when seen as noted in **GC Error Codes in Sample Processor** section.
   NOTE: write down the file name (found in Sample Processor in the top blue section) on the run sheet as well as the run date and time at the top of the sheet. Sherlock generates a file name for all samples run together at the same time.
6. Once the run is finished, ensure there are no error codes. If the run looks good, take off all GC bottles and place back into an empty blue vial holder. Dump vial inserts and blue caps into grey cap waste container before placing into glass disposal. Replace vial inserts and put away. If there are errors where a sample needs remeasured, remeasure at least one blank and standard with it.
7. In **Sample Processor**, go to **Table**, and click **Clear If Done.**
8. If another run is to be done, add hexane to CAL until back at 1.25mL. If another run is not to be immediately run, dump CAL vial insert, replace with fresh insert, solution, and cap and place in fridge.
9. If another run is to be done, repeat steps 2-5. If another run is not to be immediately done, follow directions for **Turning OFF GC** in the **Instruments** section.

Data

NOTE: Once all steps in **Running Samples on GC** section are complete, data can be viewed and exported.

Checking Data

NOTE: sample transformations are automated. During transformation, the WT file will be generated from the raw A file where the samples are transformed into picomoles per gram of soil and the internal standard recovery is accounted for by multiplying up to the known amount of 10,000 picomoles.

1. Go into Sherlock Command Center.
2. Go into “Views” tab (top left side bar).
3. Select all A and .WT files.
4. Select Calibrations, blanks, and standards (MO-000-SHAC-STD_).
5. Click “Apply”, go to “Profile tab and check…
    a. Calibration (A file only): check to see if the RMS is trending higher. Fresh Cal mix should be in the 0.002s and dilute trends higher. Once the RMS value (in the top bar of samples) starts trending in the high 0.003s and 0.004s, the new column needs to be checked to ensure that it works and calibrates so that when the column fails, the backup is ready.
    b. Blanks (A file): always have 16:0, 18:0 20:0, 22:0, and 24:0 (20:0, 22:0, and 24:0 are all from ClipTip pipette tips, the others are likely from the SPE plates).12:0 and 14:0 are also common. Check to see how many peaks are present. If contaminant values are trending higher between runs or new contaminants are becoming frequent, investigation is recommended. WT file shows contaminants that are kept.
    c. Standards (WT file): PLFA values for total response in picomoles (bottom left) should be between 40,000 and 80,000. Typically, it trends in the mid 60,000s to low

70,000s. If both standards are in the low 40,000s, the run is a rerun. If response starts to trend lower, the internal standard needs to be replaced.

6. If run is considered valid, then data can be exported.

Creating a Database

1. Go to "Utilities" tab (left side bar) and select "Data Export", then click through the tabs:
    a. Selection Criteria: calculation method = <sampmethod>
    b. Files and samples: NEON: A, .WT (newest samples are at the bottom)
    c. Methods: select all
2. Prefixes: select samples needed for file. After doing above and selecting all samples, click "Update Profile List" in bottom right corner.
3. In top left, select "DB Export".
4. Name file with the run number.
5. Select "Include Sherlock 3 Queries"
6. Make a folder with run number and put files in
    a. **NEON:** name NEON-neon run number.
7. Update folder into SharePoint DB folder **AND** SharePoint Incomplete folder.

Generating Reports

1. Go into the Incomplete folder in SharePoint to see the files that need reports.
    a. In R, pull up the required RScript found in the R folder **NEON:** NeonData.R This makes the 2C and 2D reports as well as updates the 2E info document and the Conversion Log.
2. Change the required inputs indicated at the top of the script.
3. Run the code. It will generate the file and place it into the Incomplete folder. Quality Flags will populate following the conditions listed in Table 1.
4. Open the report. Check to make sure that the correct samples are there, and that the SHAC ID is generated.
5. For complete Reports
    a. Do this for all sheets.
    b. **NEON:** Look over file. Delete the lipidInternalStandardResponse and analysisResultsQF for samples that were diluted during analysis on the GC and make a remark of "sample diluted".
        i. Record all samples that still have quality flags in the analysisResultsQF column in the current NEON Reruns document.
6. Record samples needing reextracted and delete out of report. Once reports have been reviewed, they can be placed into the Complete folder.
7. Send report link to Data Analyst and/or Lab Supervisor for review, changing to .csv, and sending the reports.
8. Go to Trello.
    a. **NEON:** in the description, add the SMB and NEON Run Number. Comment any samples that need rerun. Mark off required boxes.

Quality Control and Assurance

Several things play an important role in quality assurance of analysis as discussed below and in Table 1.

- **GC Checks**: The GC start up includes several internal self-checks that look for large gas leaks, if the gasses are at correct pressures and flowing at correct flow rates, if the oven sensor is working, if the oven is working, if the detectors are working, etc. There are also ongoing checks that the GC does when it's on. If any detector is triggered at any point when the GC is on, it will trigger an emergency shut down for safety, effectively stopping a run.
- **Calibration Standard**: This is a standard that ensures that the GC is working properly. The Cal standard is made up of straight chains 9:0 to 20:0 as well as five hydroxy acids. It is run twice at the beginning of the run and again every eleven samples. If at any point the Cal does not pass, it will be rerun once. If it does not pass again, the GC shuts down. If it passes, the GC will continue analysis. The Cal will also run again if it detects drift within the samples between calibrations to ensure that it is still working properly. The Cal solution lets the software know several things…
    - Hydroxys: these are the first things to degrade when the column degrades. If the hydroxy acids are not present, this indicates to the technician that the column may need trimmed or replaced.
    - Root Mean Square (RMS): the RMS value is what makes a calibration "good" or "bad". The RMS is calculated by how close each retention time for each PLFA found is to the expected retention time. If this value is over 0.005, the calibration will fail. This indicates to the technician that the column may be degraded or need calibrated.
    - Other reasons that the calibration can fail is due to gas leaks, which can be shown by a noisy baseline or misidentified peaks.
- **Blanks**: at least one blank is run per set of samples. Blanks are prepared the same as samples except no soil is included and they are run alongside samples in the analysis. From blanks the technician can observe contamination levels and monitor new/continuing contamination. For NEON samples, quality assurance checks are done as according to Table 1.
- **Soil Standards**: at least one soil standard is run per set of samples. This is a dried soil that has been run many times to get a baseline for total PLFA response. From this, the technician can see if total response is in the expected range. If it is not, the run is rerun, and the cause is investigated. Total response out of range can indicate an extraction issue where PLFAs were damaged or incorrect amounts of internal standard. For NEON samples, quality assurance checks are done as according to Table 1.
- **Internal Standard (19:0)**: a known amount of internal standard is placed into each sample in the beginning of every run through the Bligh-Dyer extraction solution to account for an imperfect extraction through the process, spills, dilutions, etc. Once the sample is done on the GC, the SHERLOCK software then accounts for the how much 19:0 was recovered by scaling everything to meet 10,000 picomoles of 19:0. For NEON samples, the 19:0 response is reported, and quality assurance checks are done as according to Table 1.
- **Routine Preventative Maintenance**: Agilent performs a preventative maintenance check on the GC annually. During this check, they trim the column, service the jet, inlets, and detectors, check for leaks, and deep clean the instrument. Calibration standards are then used to ensure that the instrument is working properly.
- **Annual Calibrations**: All pipetting systems and scales are professionally calibrated once per year.

- **Chemical Logs**: When chemicals come into the lab, their lot number, CoA, and expiration/retest date are recorded in a log of all of the PLFA chemicals in the lab (OneDrive - University of Missouri/General - SHAC PLFA – Ogrp/PLFA/General/CoA and Expiration Dates.) This list is updated and verified for expiration monthly. Once chemicals are used or expired, their records are removed. If chemicals are expiring within the next month, a note on the chemical bottle is made and it is taken out of use.
- **Chemicals**: Opened dates, make dates, and expiration dates are written or highlighted directly onto chemical labels in permanent marker. A complete inventory is completed every six months to ensure that there are no upcoming expirations. If a chemical is expiring soon, new chemicals are ordered, and the original goes out of use. Expiration and retest dates are also included in the CoA and Expiration Dates doc that is updated weekly.
- **Traceability of Tasks**: Individuals that work on different steps of the PLFA process have to initial whenever a task is completed and record any relating details about that step. This allows the technicians to more easily track down issues when they arise, it allows for sample tracking throughout the process and transfer between locations and allows technicians to ensure tasks are being completed correctly or if more training is needed. The Soil Health Assessment Center also uses the sample tracking app Trello, where all of the steps from sample intake to invoicing are recorded per sample. Whenever a sample is freeze dried, it is recorded on a separate freeze dry log. When a run gets loaded, whoever works on it initials the run sheet in the Loaded By line. Whenever a run is finished being loaded, when it gets dried in tubes, when it gets weighed, and when it is being extracted, whoever is responsible initials on the run sheet in the relating line. Whenever the samples are placed on the GC, the date is written on the top of the run sheet in the Date line, and at the bottom of the run sheet, the file that contains the data is recorded.
- **Trainings**: When the Soil Health Assessment Center began running PLFA samples, they observed and were trained by Jeff Buyer, the scientist behind the method. Before one can get involved in the PLFA process, they must be trained in the process of the task as well as be informed about the risks of contamination and special sample handling. Run preparation can be completed by students, but the extraction is only completed by Technicians that have been well versed in the process. All student training and work at SHAC location is completed and overseen by the Sample Intake Lead and all training and work at the campus location is completed and overseen by the PLFA Lead. Trainings will be logged in a training log and SOPs will be revisited annually by all involved in the analyses and signed off for retraining in the training log. Training log will contain details of the trainer showing traceability to the Jeff Buyer original training, the trainee, date training completed and details of level of training with version of the SOP.

Materials (Inventory list)

Glass box for broken/discarded glass
Yellow Jacket pump oil
9V batteries (for vacuum meters)
Aluminum foil
Kim wipes
Nitrile gloves: small, medium, large
Resealable bags, 3x4", 4 mil, Cat.No. S-494
Aluminum weighing tins, 42 mL, Cat.No. 08-732-101
Weighing paper, 3X3", Fisher No. 09-898-12A
Pasteur pipets, glass, 5 ¾", Cat.No. 13-678-6A
Glass culture tubes, Cat.No. 99447-13
Black caps for culture tubes, Cat.No. 73802-13415
96-well plates, Phenomenex, Cat.No. 8E-S012-DGB
Teflon Cap mats (for 96-well plate)
1.5mL glass flat-bottom vials, Cat.No. 4150FB-944VL
1.0mL glass flat-bottom vials, Cat.No. 4100FB-930VL
Blue caps (for GC vials), Cat.No. C5000-53B
Glass inserts (300 µl), Cat.No. C4010-6271
Solvent safe pipet tips for Pipetman (Cat No. 5469)
8-channel pipet:
1250 µl / 102 mm tall tips, Cat.No. 8242-11
1250 µl pipet tips, Cat.NO. 8042
    Or Refill box of tips only, Cat.No. 8046
Fisher chemicals:
Methanol, 4L, Cat.No. A452-4
Acetone, 4L, Cat.No. A949-4
Toluene, 1L, Cat.No. T290-1
Acetic acid, 500 mL, Cat.No. BP2401-500
Potassium hydroxide, 500 mL, Cat.No. P250-500
Potassium phosphate, 500 mL, Cat.No. P288-500
Hexane, 1L, Cat.No. H303-1
Honeywell chemicals
Chloroform, 4L, Cat.No. 34854-4X4L
Other:
PLFA - Internal Standard – stock and active use
MIDI - Calibration Standard for GC – stock and active use
Hydrogen, Nitrogen, and Air Tanks

A list of current materials can be found in the PLFA Inventory spreadsheet (OneDrive - University of Missouri/General - SHAC PLFA – Ogrp/PLFA/General).
A list of CoAs and expirations dates can be found in the CoA and Expiration Dates log (OneDrive - University of Missouri/General - SHAC PLFA – Ogrp/PLFA/General).
To find a list of quality assurance measures, review the Quality Control and Assurance section.

PLFA Chemical Solutions

NOTE: All glassware used to prepare PLFA reagents is kept separate from any other chemistry glassware and is prohibited from being used for any other reagents. Also, any glassware with a single purpose (i.e., Bligh Dyer bottle top dispenser) is used strictly for that reagent and is labeled as such. All reagent containers are rinsed 6 times with ultrapure DI water and left to dry between uses.

NOTE: All **reagents** used in this procedure are kept at room temperature in tightly sealed bottles; With exception of the **PLFA Internal Standard** that is stored in the freezer (-20°C) and **GC Calibration Standard** that is stored in the refrigerator.

PLFA Internal Standard

- *Should be stable for at least 2 months.*
- Gather: gloves, Avanti standard (powder), scale, new culture tube with disposable black cap, new small GC glass vial with blue cap, glass weigh boat/funnel, Chloroform bottle-top. dispenser, Methanol, Kimwipes, small metal scoop, small chloroform and methanol beakers, large Methanol beaker, HandyStep with Methanol-tip, and Pasteur pipets. Clean all materials with chloroform before use, except methanol beaker which is rinsed with methanol.
- Dispense **2.5 mL of Chloroform** into small clean beaker, and set aside.
- Weigh out **10.2 mg (0.0102 g) of Avanti Standard** in glass weigh boat and add to a clean culture tube (there will be some powder remaining attached to glass).
- Using Pasteur pipet, carefully rinse remaining powder from glass weigh boat using part of the 2.5g-chloroform; then carefully pour or pipet remaining chloroform into culture tube.
- Using HandyStep, add **2.5 mL of Methanol** to culture tube.
- Cap culture tube and Vortex; label tube with "PLFA IS" and date.
- Using clean Pasteur pipet, fill a small GC glass vial/blue cap with the new Internal Standard; label vial with date created.
- Clean glass weigh boat with chloroform and wrap in kim wipe.
- **Store Internal Standard in freezer.**

Calibration Standard for GC

NOTE: Make sure that all condensation is evaporated and ampule is room temperature before opening.

- Stock standard (in a box) and active-use Calibration solution (in labeled GC vial) are kept in refrigerator.
- Gather: gloves, Stock Calibration solution, hexane beaker (rinsed with hexane), hexane bottle-top dispenser, new GC vial and blue cap labelled "GC CAL" with the date, ampule opener (plastic piece in ampule box), ampule neck holder (rubber piece in ampule box), Pasteur pipet with new pipette tip.
- Carefully using the ampule opener, place over top of ampule and press away from body with thumb to break. Place neck into neck holder.
- Dispense 2.0 mL of hexane into beaker.
- Using the Pasteur pipet and working fast to prevent hexane evaporation, transfer some hexane into the neck, rinsing the sides well. Transfer this into the ampule body, rinsing well. Transfer this into the "GC CAL" bottle. Repeat until all hexane is in small GC bottle. Mix well.

- Use Pasteur pipet to transfer approx. 1.0 mL "Cal" solution from the GC vial into the glass insert of a separate labeled GC vial labelled "CAL"; Be sure to replace blue cap after each GC run. Change vial and dump extra CAL solution at the end of each week/whenever the concentration is too strong and replace with fresh CAL solution from working stock.
- Store in refrigerator.

50mM Dipotassium Phosphate

- **50 *mM* Potassium Phosphate buffer solution** (K_2HPO_4) is stored in 500 mL bottle.
- Before Day 1 of PLFA analysis, make sure there is enough K_2HPO_4 solution in the 500mL bottle. If not, dump out remaining solution, rinse out bottle with ultrapure DI water, and lay out on paper towel in fume hood to air dry.
- To make the **50 *mM* K_2HPO_4:**
    - Weigh out **4.35 g of Potassium Phosphate Dibasic** into plastic weigh boat.
    - Measure **500 mL ultrapure DI water** into 500 mL graduated cylinder.
    - Carefully dump the granular Potassium Phosphate into 500 mL bottle.
    - With a Pasteur pipette, use some of the 500 mL of measured ultrapure DI water to rinse. remaining chemical from weigh boat into bottle.
    - Pour remaining ultrapure DI water into glass bottle, cap, and shake to mix. Label bottle with initials and date.

Bligh-Dyer Extractant

Make only amount needed for one day; make just before using.

- Gather: gloves, Bligh-Dyer 1L dispenser, 500 mL Methanol, 500 mL Chloroform, Internal Standard (small GC vial), 50 *mM* Dipotassium Phosphate buffer solution (K_2HPO_4), Pipetman pipettor with a new Solvent Safe tip, 100 mL graduated cylinder, 250 mL graduated cylinder, and a few papers towels.
- After adding Bligh-Dyer solution to samples, dispose of remaining solution in Chloroform-waste beaker; thoroughly rinse (6 times minimum) bottle and dispenser with ultrapure DI water, and let air dry for a few hours; lay out onto Kimwipes/paper towels and protect sensitive surfaces and openings from dust and surface contamination.

For 950 mL (192 Samples-Two Runs)

- Add **200 mL of 50 *mM* K_2HPO_4** to Bligh-Dyer dispenser using the 100 mL graduated cylinder.
- Add **250 mL of Chloroform** to Bligh-Dyer dispenser using 250 mL graduated cylinder.
    - The 250 mL graduated cylinder is not washed because chloroform evaporates quickly so there is no volume displacement and any potential residue left from the chloroform does not impact the reagent since all chemicals are being combined in the Bligh-Dyer reagent bottle.
- Add .476 mL of Internal Standard to Bligh-Dyer dispenser using Pipetman pipet with the solvent safe tip; **To do this**, **dispense .119 mL of Internal Standard, four times.**
- Add **500 mL of Methanol** to Bligh-Dyer dispenser using the 250 mL graduated cylinder.
- Swirl and use the recirculation valve to mix the solution in the bottle; Solution MUST be well mixed to ensure accurate dispensing of 19:0. Mix until the two layers combine.
- Rinse all used glassware 6 times with ultrapure DI water and dry the 100 mL graduated cylinder with acetone for later use.

For 475 mL (96 Samples-1 Run)

- Add **100 mL of 50 *mM* K_2HPO_4** to Bligh-Dyer dispenser using the 100 mL graduated cylinder.
- Add **125 mL of Chloroform** to Bligh-Dyer dispenser using 250 mL graduated cylinder.
  - The 250 mL graduated cylinder is not washed because chloroform evaporates quickly so there is no volume displacement and any potential residue left from the chloroform does not impact the reagent since all chemicals are being combined in the Bligh-Dyer reagent bottle.
- Add .238 mL of Internal Standard to Bligh-Dyer dispenser using Pipetman pipet with the solvent safe tip; **To do this**, **dispense .119 mL of Internal Standard, twice.**
- Add **250 mL of Methanol** to Bligh-Dyer dispenser using the 250 mL graduated cylinder.
- Swirl and use the recirculation valve to mix the solution in the bottle; Solution MUST be well mixed to ensure accurate dispensing of 19:0. Mix until the two layers combine.
- Rinse all used glassware 6 times with ultrapure DI water and dry the 100 mL graduated cylinder with acetone for later use.

ultrapure DI

0.075 *M* Acetic Acid

- 0.075 *M* Acetic Acid is stored in 500 mL bottle.
- Each run requires about 50 mL of 0.075 *M* Acetic Acid solution.
- Before Day 1 of PLFA analysis, make sure there is enough Acetic Acid solution in the 500 mL bottle. If not, dump out remaining solution, rinse out bottle with ultrapure DI water, and lay out on paper towel in fume hood to air dry.
- To make 0.075 *M* Acetic Acid solution:
  - Gather: stock Acetic Acid bottle, small Acetic acid beaker, HandyStep with 5 mL-tip, 500 mL graduated cylinder, 500 mL acetic acid bottle.
  - Pour a small bit of stock Acetic acid into the small beaker (<5 mL).
  - Set HandyStep (with tip) to 2.16 mL (2160 µl); take up acetic acid from small beaker and dispense **2.16 mL of concentrated Acetic acid** into 500 mL bottle.
  - Measure **500 mL of ultrapure DI water** in graduated cylinder and add to bottle.
  - Cap, invert several times to mix.
  - Rinse all used glassware 6 times with ultrapure DI water.
  - Label bottle with initials and date.

5:5:1 Solution

Made fresh each run.

- Gather: gloves, round flat-bottom dish labeled "**5:5:1**", watch glass, 500 mL Methanol, 500 mL Chloroform, ultrapure DI water in H2O beaker, 100 mL graduated cylinder, and HandyStep dispenser with "H2O" tip.
- Add **50 mL of Methanol** to 5:5:1 dish using 100 mL graduated cylinder.
  - The 100 mL graduated cylinder is not washed because methanol evaporates quickly so there is no volume displacement and any potential residue left from the methanol does not impact the reagent since all chemicals are being combined in the 5:5:1 reagent dish.
- Add **50 mL of Chloroform** to 5:5:1 dish using 100 mL graduated cylinder.
- Add **10 mL of ultrapure DI water** to dish using the HandyStep dispenser with "H2O" tip.

- Cap dish with watch glass until use.
- Thoroughly mix solution with the 8-channel pipettor using 1250 µl tips.

After use, dump remaining solution into Chloroform-waste beaker; rinse with ultrapure DI water, place dish upside-down on kim wipes in fume hood until dry; put dish and watch glass back into storage drawer.

Transesterification Reagent

Stable for 1 week but made fresh each run.

- Gather: gloves, round dish labeled "**Transesterification**," watch glass, Kimwipes, 100 mL graduated cylinder, 500 mL Methanol, 500 mL Toluene, Potassium Hydroxide (KOH) pellets, 3x3 weighing paper, and metal scoop.
- Add **75 mL of Methanol** to dish using 100 mL graduated cylinder; Cover dish with watch glass.
    - The 100 mL graduated cylinder is not washed because methanol evaporates quickly so there is no volume displacement and any potential residue left from the methanol does not impact the reagent since all chemicals are being combine in the 5:5:1 reagent dish.
- Weigh out **0.561 g of KOH** pellets and place in dish; to do this, fold one sheet of weighing paper to function as weigh boat and place on scale, TARE scale, and add pellets; to get exactly 0.561 g, place a pellet onto another piece of weighing paper and break into pieces with metal scoop.
- Wait for the KOH pellets to completely dissolve before continuing to next step.
- Add **25 mL of Toluene** to dish using 100 mL graduated cylinder.
- Thoroughly mix solution with 8-channel pipettor with 1250 µl tips.
- Rinse all used glassware 6 times with ultrapure DI water.

After use, put watch glass back into storage cabinet (wipe with ultrapure DI and Kimwipe as needed); leave dish in Fume Hood to allow Toluene in the remaining Transesterification solution to evaporate; after the toluene evaporates, a KOH residue will remain and can be washed out with ultrapure DI water into chloroform waste beaker; place upside down in fume hood on kim wipe until dry; place in storage drawer on foil .

Instruments

Vacuum pumps (in fume hood and for Cube)

- Oil needs to be changed periodically when clear oil takes on a light yellowish color or with slow dry downs.
    - Gather plastic funnel, metal funnel, wrench, and new Yellow Jacket pump oil, waste jug for used oil, paper towels, and gloves.
    - Keep pump in place, don't remove tube on Cube pump but remove for PLFA pump, and move closer to edge.
    - Situate a metal stool to ideal height as surface to set used oil jug with plastic funnel below pump while draining oil; put paper towel under jug to catch dripping oil.
    - Unscrew plug to drain oil; once flow rate decreases, tilt pump to drain remaining oil; screw in oil plug.
    - Insert metal funnel into top of pump (will need to remove screen from Cube pump); slowly fill with new oil up to the indicator-lines in window.

    - Securely reconnect all caps and tubes.
- Log oil change date on Log Sheet located in GC binder (for PLFA pump) or on wall above pump (for Cube-pump).

LyphLock12 Freeze Dryer

- Keep inside and door seals clean, drain and wipe out coil chamber after every run.
- Apply vacuum grease as needed.
- Wipe out with disinfectant wipes after drying APHIS-regulated samples.
- Log oil-change date on Log Sheet.

PLFA room - Cold Trap / Vapor Trap

- Before turning on Cold Trap, empty glass jar of liquid from previous run; dump into Chloroform-waste beaker in Fume Hood, and check that Ethanol level in basin is at line.
- **To turn ON Cold Trap**: put black rubber cap securely on glass jar and make sure red plug is completely inserted.
- Before using, the machine needs **45 minutes to cool down** to correct temperature inside jar; the temperature on the display is the temperature inside the machine and may show that the temperature is cooled between -106 and -110° C in about 30 minutes; but, it takes longer for the ethanol bath to cool down the jar, so 45 minutes allows for complete cooling. If the Cold Trap is not completely cooled when running with vacuum pump, then moisture could get into pump oil and degrade oil quality.
- Ethanol needs to be replaced if becomes dirty or gains water (will notice if it is frozen after a run); tools for scooping or withdrawing out old Ethanol are in drawer below fume hood; write date of Ethanol replacement on GC Log in binder.
- Black rubber cap and plastic tubes need to be periodically washed of glass shards with ultrapure DI water.
  ***HINT**: it may be helpful to first take a picture of how they are assembled so you remember how to reassemble.*
- Check red rubber tubes for cracks.
- After turning off machine, MUST remove rubber cap, set off to side, and put rubber stopper (upside down so stopper is not inside jar) over jar opening.

CentriVap - Labconoco

- Wipe around lid and basin with moist Kimwipe before each dry-down to ensure tight seal around lid.
- Periodically, glass shards will build up between the rubber frame and glass in the lid; to clean, unscrew lid from hinge, and carefully take off rubber frame (can take a picture of how the layers are assembled before completely separating), wipe or wash with ultrapure DI water and lay out on paper towel to dry before reassembling.
- Samples must be evenly spaced on rotor (for even weight distribution) when using centrifuge.

Wall Meters (Vacuum Gauges)

- Turn off when not using to save battery power.

Agilent GC 7890A

- Make sure to put on **new** gloves before doing any maintenance on the GC.
- Replace septum and clean syringe before each run; when replacing syringe, make sure it is securely latched in and connected; test springs for easy, smooth movement by lifting the white 'foot' at bottom – add oil to spring when needed.
- Every now and then, add a Hexane-only sample to end of PLFA run to clean/test for contamination in GC Column.
- Log gas pressure levels (Hydrogen, Nitrogen, and Air) after each run.
- GC liner is replaced every 12 runs.
- Annually (October), someone from Agilent comes to the lab and does a Preventative Maintenance check, an extensive maintenance of parts and calibration check of performance on the GC.
- See white binder for SHAC's maintenance logs and guides.
- See a separate white MIDI's binder (in drawer) for history of service checks by MIDI staff.
- See additional manuals (in drawer) for Microbial Identification System info and Data export using Sherlock® software.

Turning ON GC

GC needs about **4 hours to completely warm up**, after completing the Start-up protocol (below); plan ahead so that it is ready when ready to use.

NOTE: make sure to do all maintenance (replacing septum, changing port liner, etc.) before GC is turned on because it gets HOT. Make sure that all gas levels are above 500 PSI.

- Open valves for all three gas tanks (Air, Hydrogen, and Nitrogen) by turning silver knob on each tank all the way to the left.
    - Gas levels are indicated on gauge on the right.
    - Check tank pressures; outlet psi on each gauge (on the left side) should be about 60.
    - Do not adjust pressure regulator (black knob) unless necessary.
- **Turn on GC** – power button on lower left front.
- Enter GC start-up date and time on the log sheet.
- Press **Config**, scroll down to **Front Detector**, scroll down to **LIT Offset**, change to 1.0.
    - The lit offset essentially is a signal that tells the GC that the flame is lit. Generally, it is set to 2.0 and that is sufficient. Because we use such clean gasses and reagents, ours generally runs lower, telling the GC that the flame is not lit when it is, resulting in a safety shut down. To avoid this, the lit offset needs to be lowered.
- Press **OVEN** button (black button on front) to check oven temperature; **Set oven temperature to 50 °C** using number pad and push **ENTER;** let warm up to 50 °C.
- Press **FRONT INLET** button (next to OVEN button) to check front inlet temperature; Once front inlet reaches **~250 °C**, return to OVEN display.
- Press **OVEN**, and **raise oven temperature to 300 °C** by selecting oven temperature using arrow buttons, type in '300' using number pad, and press **ENTER.**
- After oven temperature reaches 300 °C, **set oven temperature to 170 °C.**
- Press the STATUS button to see if detector, inlet, and oven temperatures are 'Ready' before beginning the run.

Turning OFF GC

- Press **OVEN** button and **set temperature to 300 °C.**
- After oven temp reaches 300 °C, **set temperature to 30 °C.**
- After oven temperature reaches 30 °C, **turn OFF GC** using power button on lower left front.

- **Close all gas valves** by turning silver valves all the way to the right; note remaining gas level on Log Sheet.
- Enter DATE, TIME, and Total Run Time on Log Sheet.
- Dump out old CAL vial, replace vial, add new CAL solution, replace blue cap, and place in fridge.
- Empty the two Hexane rinse vials into waste beaker; wipe out vials with cotton swab.

Pipettes

Before use, ensure that the calibrations are current. If not, send them in for calibration as soon as possible.

8 Channel Pipette

Fisher E1-ClipTip BT
TM8 15-1250 (Model)
RH46202 (Serial #)

Pipetman

Gilson
P 50-200 (Model)
HH21438 (Serial #)

HandyStep

Brand
HandyStep Touch (Model)
20G20167 (Serial #)

Pasteur Pipet

Bel-Art
F37897 (Model)

Bottle Top Dispensers

0,5-5mL Dispensette S Organic – Brand
0,5-5mL Dispensette Organic – Brand
1-10mL Dispensette Organic – Brand
1-10mL Dispensette S Organic – Brand

Scale

Scale does an internal adjustment after it wakes from sleep mode. Weekly, ensure scale calibration is valid within 0.0006 of weight tested and log. If not, schedule calibration or calibrate with weight set. If blanks are not measuring close to the same for pre and post weights, check calibration with weights.

GenPure Ultrapure water purifier

- Turn on DI water.
- Turn on machine and wait for interval screen.
- Press “Nonstop.”
- Do a 30 second rinse and continue until the water is ≥ 18.20 MΩ·cm.Press “Enter” x2 to start and x1 to stop.

Table 1. Quality Assurance and Quality Control Measures for NEON Samples.

QA Check	Material Used	Description	Frequency	Calculation
QA Reference Standard	SHAC STD8	SHAC STD8 is a dried, sieved (to 2 mm), and homogenized Missouri soil used as a quality control STD in the laboratory since the mid 1980s and for a PLFA STD since 2018. It's used because of its relatively high organic matter content and its presumed stability from years of storage.	STD8 will typically be placed in tubes 2 and 95 for each 96-well sample plate.	Average and ST DEV values for STD8 total lipid concentrations (nanomolesPerGram) resulting from runs from 2019 to 2023 were calculated (removing outliers). (Average STD8 = 64.968 nanomolesPerGram soil. ST DEV=13.833 nanomolesPerGram soil)
Internal Standard	Internal Standard (Avanti 19:0 PC 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine 850367P)	Commercially available phospholipid standard containing a known concentration of phospholipid C19:0	10,000 pmols of the 19:0 internal standard is added to each sample through the Bligh-Dyer extractant.	GC-FID response for C19:0 response was estimated from previous years blank and STD8 data and from current NEON sample runs. The 19:0 response of 60,000 units indicates full extraction.
Procedural Blank	Extraction tube processed with reagents used for samples added	Blank tube processed with reagents used for sample batch. Process with a sample weight of 1.000g	For each 96-well plate of samples one blank typically will be placed in tubes 1, 36, 61, and 96.	Calculate lipid concentrations in blanks with units of nanomoles (process with a sample weight of 1) Report blank lipid concentrations (as nanomoles) for any lipids above detection limits in batchQA template
Continuing Calibration Check	A calibration solution containing 17 lipids, MIDI PLFA Cal Mix (Part# 1208) is used. The final ending check may also be the SHAC STD 8 QA reference standard analyzed after all unknown samples.	NA	Two consecutive calibrations must pass to begin a run, or the run will be aborted. A calibration standard will be run for every 11 samples. If a calibration does not pass, a second calibration will be run. If the second calibration fails the run will be aborted. Passing calibration is based upon the root mean square error calculated from the calibration analyses analyte? differences from the expected relative retention times. See "Sherlock software" section of SOP.	The MIDI Sherlock software calculates root mean square error. RMSE = $\sqrt{[\ \Sigma(Pi - Oi)2 / n\ ]}$ where Σ represents the sum and Pi is the measured retention time for the ith lipid in the standard and Oi in the formula refers to the expected relative retention times value for the ith lipid in the calibration standard. Lastly, n is the number of lipids in the calibration standard, in this case n=17.
Long term summary	All SHAC STD 8 and procedural blanks processed with NEON samples during a designated time period.	Summary statistics about recovery of analytes in the QA reference standards processed with NEON samples.	Calculate and report in January for the previous calendar year or any time a major update to analytical procedures is made.	Calculate the mean and standard deviation of all absolute error measurements for total phospholipids in the STD 8. Report procedural blank internal standard 19:0 GC response during the reporting period.

Table 1. (Continued) Quality Assurance and Quality Control Measures for NEON Samples.

QA Check	Acceptance Criteria	Corrective Actions	Insufficient Sample for Corrective Action	NEON Data Ingest for Reporting
QA Reference Standard	STD8 total lipids concentrations (nanomolesPerGram) falling outside 2 ST DEV from the mean of STD8 results from runs from 2019 to 2023 will be rejected. (Acceptable range = 37.302 to 92.634 nanomolesPerGram)	Review possibilities. Vacuum leaks, reagent issues, internal standard values would affect all or most samples. If the average response from both STD8s fall outside of range, all samples will be rerun. If only one standard falls outside the range, assess other factors to determine if only one sample was affected vs the entire set. If other factors indicate other samples affected, all affected samples will be rerun.	Report with Quality Flag of "Total lipids out of range in batch QA materials"	SHAC 8 concentrations are reported in batch QA table Batch-level flags to communicate QA issues are reported in sample table
Internal Standard	The minimal acceptable amount of the internal standard in an undiluted sample is 19,800 units and the maximum is 90,000 units.	Reprocess or rerun individual samples with undiluted internal standard response <19,800 or >90,000 units. For diluted samples, there is no criteria. Do not report internal standard recovery and make a standard remark. If the average internal standard response of all QA materials falls out of range, re-extract the entire batch.	Report results with a quality flag.	Internal standard for each sample reported in sample table and for each quality control in batch QA table
Procedural Blank *	Blanks will be reviewed for any unusual or consistent contamination. Specifically: • values of recurrent lipid contaminants must be within 2 standard deviations of their long-term means for each blank • less than 5 lipids other than known recurrent contaminants should be found in each blank. • less than 3 shared, non-recurrent lipids must be found in all blanks in a run	Blanks with contamination as described in the first two "Acceptance Criteria" bullets will be reported with a "high blank, do not use" flag. Runs where only one blank is flagged are reported as usual. Runs with two or more flagged blanks should be discussed with NEON prior to reporting, Any run where the third bullet acceptance criteria is not met will be rerun.	Discuss with NEON for guidance before reporting results.	Report blank lipid concentrations (as nanomoles) for any lipids above detection limits in batchQA table
Continuing Calibration Check	Root mean square error of ≥ 0.0050 will cause a failed calibration as set by the software and the run will be aborted.	Use fresh calibration standard, review failed calibrations to determine if maintenance is needed. Keep adjusting until it passes calibration. Rerun sample extracts not bracketed by passing calibration standards.	The software will not allow samples to run if the calibration standard does not pass. If unbracketed sample extracts cannot be rerun (because calibration standard will not pass), report result with a quality flag.	Keep in study records for review during audits.
Long term summary	NA	NA	NA	LabSummary table

** Due to consumable product impact on contaminant levels, acceptable limits will be reviewed periodically. Lipids, their acceptance criteria, and relevant dates will be published in an accompanying long term table*

Version History Table		
Version	Date	Changes
1.0	October 2022	Initial Document Version
2.0	January 16, 2024	Ultrapure DI water use (Type I) implemented on January 16, 2024. Changes to ethanol cleaning during sample intake, drying, and loading reflected in data on March 08, 2024.
3.0	April 06, 2024	Cleaning steps implemented for 8-channel pipette tips. Clarified blank acceptance parameters included in Table 1.
3.1	May 15, 2025	New blank acceptance criteria outlined in Table 1.