Microbial ID, Inc.

Soil Microbial Biomass by High Throughput PLFA Analysis

Extraction and Analysis Protocol

NSF NEON Project

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Reagents

Bligh-Dyer Extractant

PO_4 buffer: 10.66 grams of Dipotassium Phosphate (K_2HPO_4) with 5.28 grams of Monopotassium Phosphate (KH_2PO_4) added to 2 liters of DI water

A. 80 mL 50 mM PO_4 buffer
B. 200 mL methanol
C. 100 mL chloroform
D. This reagent can be mixed in a ratio of 4:10:5 PO_4 buffer:methanol:chloroform depending on how many samples are being extracted.
E. Mix fresh daily and dispose of any extra.

Transesterification Reagent

A. 0.561 grams KOH
B. 75 mL methanol
C. 25 mL toluene
D. Dissolve KOH in methanol and then add toluene.
E. Expiration date: 6 months from manufacturing date.

Chromatography Wash Solution

A. 50mL methanol
B. 50mL chloroform
C. 10mL DI H_2O
D. This reagent can be mixed in a ratio of 5:5:1 methanol:chloroform:DI H_2O.
E. Expiration date: 6 months from manufacturing date.

Internal Standard

1,2-dinonadecanoyl-sn-glycero-3-phosphocholine
(Avanti Polar Lipids Catalog # 850367P – white powder)

A. Dissolve 12.28 mg in 10 mL of chloroform (1.5 mM solution).
B. Store powder at -20°C.
C. Just before extracting, add to an appropriate volume of extractant at a rate of 0.5 µL internal standard per mL of extractant and mix.
D. This is equivalent to adding 6.1 nanomoles of 19:0.

Soil Drying

1. Weigh each soil scintillation vial before drying and record.
2. Store at least overnight in -80°C freezer.
3. Freeze-dry using Lyophilizer* for at least 24 hours.
4. Weigh each vial after freeze-drying and record.

5. Unless a tare weight is provided, while weighing for extraction select ~5 vials from each batch. Empty, weigh, and record the average tare weight.
6. Any vials that are different than the rest of the batch must be weighed individually, and their tare weight recorded.

Extraction

1. Use one additional test tube as a reagent blank per 20 samples.
2. Depending on the density of the soil, homogenize the soil in one of three ways:
    a. For samples which are compacted, grind the sample using a sterilized scoopula/spatula then shake the vial to further homogenize.
    b. For samples with many plant parts, sift and mix while sifting.
    c. For loose samples, shake the vial to homogenize.
3. Remove between 0.05 – 1.02 grams of soil, record the weight, and place in a 16x100 mm test tube **(Fisher Part # 1495935AA)**.
4. Add 4 mL of Bligh-Dyer extractant containing internal standard and cap tubes with 16x100 mm PTFE-lined screw caps **(Kimble Chase Part # 45066C-15)**.
5. Vortex* for 5 seconds on high, then sonicate* for 10 minutes at room temperature.
6. Repeat step 4 two more times, drying the outside of the tubes in between rounds of sonication, for a total of 30 minutes of sonication.
7. Centrifuge* 15 minutes.
8. Transfer liquid phase to clean 16x100 mm test tubes.

Separation

1. Add 1 mL each of chloroform and deionized water.
2. Cap and vortex* 5 seconds on high, then centrifuge* 15 minutes.
3. Transfer bottom phase to 13x100 mm test tubes **(Fisher Part # 1495935C)**.
4. SpeedVac* to dryness at ~30°C (for ~1 hour).
5. Samples may be stored, capped or covered, overnight in a -20°C freezer.

Chromatography

1. Dissolve samples in 1 mL chloroform and vortex* for 5 seconds on low.
2. 50 mg silica gel SPE 96-well plate **(Phenomenex Part # 8E-S012-DGB – <u>No Substitutes</u>)**.
3. Wash each well 3 times with 1 mL methanol then 3 times with 1 mL chloroform.
4. Add extract to wells.
5. Let sample drain into column.
6. Repeat transfer with another 1 mL chloroform and drain into column.
7. Wash with 1 mL chloroform.
8. Wash with 1 mL acetone.

9. Place clean 1.5 mL glass vials into Multi-Tier microplate **(Thomas Scientific Part # 1149B46, E&K Scientific Part # 99236)** in bottom of 96-Well Plate Manifold **(Supelco Part # 57192-U)**.
10. Elute phospholipids with 0.5 mL of Chromatography Wash Solution (5:5:1 methanol:chloroform:DI H_2O).
11. Transfer to clean 13x100 mm test tubes.
12. SpeedVac* to dryness (~1.5 hours).

Transesterification and Transfer to GC Vials

1. Add 0.2 mL transesterification reagent, cap with 13x100 mm PTFE-lined caps **(Kimble Chase Part # 45066C-13)**, and vortex* 5 seconds on high.
2. Incubate at 37°C for 15 minutes.
3. Add 0.4 mL of 0.075 M acetic acid and 0.5 mL chloroform.
4. Vortex* for 10 seconds on low and allow phases to separate.
5. Transfer bottom phase to 2 mL screw cap GC vials.
6. Place the GC vials into the SpeedVac* and dry for 15 minutes. If samples are not dry, continue additional 5-minute increments until dry. Do not over dry.
7. Add 100 µL hexane, cap with PTFE-lined GC screw caps **(Restek Part # 24498)**, and vortex* for 5 seconds on low.
8. Transfer extract to a 100 uL limited volume glass vial insert **(LabSphere Catalog # 05090129)** and place the insert into the original sample vial.

Glassware and Cap Cleaning

1. All glassware is to be scrubbed carefully with detergent and thoroughly rinsed with DI water while wearing gloves (to avoid any lipids from forming a monolayer and spreading over the entire surface of the wet glass).
2. Where necessary, bake glassware in a dedicated oven* at 220°C for at least 2 hours.
3. Use an Ultrasonic Cleaning Bath* to clean PTFE-lined caps:
    a. Soak caps in 10% bleach for 30 minutes
    b. Add a few drops of detergent and sonicate in DI water for 1 hour.
    c. Rinse and sonicate in DI water for 1 hour 3 additional times.
    d. Make sure PTFE-lining is in place before using.

Notes

1. All organic solvents should be HPLC grade or better.
2. Contamination is a potential problem.
    a. Run at least 1 reagent blank per 20 samples.
    b. Wear nitrile gloves during the extraction process.
    c. GC caps must have PTFE/Silicone/PTFE Septa.
    d. Test tube caps must be PTFE-lined.
    e. Limited volume inserts must <u>not</u> have polyspring feet.

*** Major Equipment**

1. Lyophilizer (Labconco or functionally equivalent).
    a. Labconco FreeZone Benchtop Freeze Dryer
        i. Part # 700401000
    b. Freeze Dry Vacuum Pump
        i. Fisher # 01-096-21
    c. Clear Drying Chamber
        i. Part # 7318700
    d. Shelves for FreeZone Freeze Dryer
        i. Part # 7317200
2. SpeedVac & Accessories (Savant or functionally equivalent).
    a. Savant Automatic Environmental SpeedVac Concentrator System
        i. Part # 7810014.
    b. Savant 12-13mm Rotor – Model RH200-12.
    c. Savant Glass Condensation Flask (GCF400).
3. Ultrasonic Cleaning Bath (Branson or functionally equivalent).
    a. Branson Ultrasonic Cleaner w. Manual Timer – Model M3800 5.7L. For larger batch sizes, up to 96 test tubes.
        i. Part # CPX-952-316R.
4. Vortex Mixer (Baxter Scientific or functionally equivalent).
    a. Baxter Scientific SP Vortex Mixer
        i. Part # S8223-1.
5. Centrifuge (Eppendorf or functionally equivalent)
    a. Eppendorf Centrifuge 5804
        i. Catalog # 022622501.
6. Single Channel Pipettor (Eppendorf or functionally equivalent).
    a. Elemental Scientific Pre-Cleaned PFA PureTip pipette tips (Part # ES70001011).
    b. Eppendorf Reference 2 100 µL Automatic Pipettor
        i. Part # 4924000053.
    c. Eppendorf Reference 2 1000 µL Automatic Pipettor
        i. Part # 4924000088.
7. Multichannel Pipettor (Thermo Scientific or functionally equivalent).
    a. Thermo Scientific E-1 ClipTip Electronic Pipettor 8 channel 10-300 µL – Part # 4672080BT.
        i. Thermo Scientific ClipTip 300 µL Pipet Tips – Part # 94410510.
    b. Thermo Scientific E-1 ClipTip Electronic Pipettor 8 channel 15-1250 µL – Part # 4672100BT.
        i. Thermo Scientific ClipTip 1250 µL Pipet Tips – Part # 94410810.
8. Oven (Fisher Scientific or functionally equivalent).
    a. Fisher Scientific Isotemp Oven
        i. Catalog # 13247625G

Soil Microbial Biomass by High Throughput PLFA Analysis
Part II. Data Analysis Protocol

Overview

The ***Microbial Zoe**** LIMS system is used to track samples through the analysis process. This specialized system was designed specifically for NEON.
The ***MIDI Sherlock**** software is used to process the samples. It consists of three applications: *Sample Processor* used for analyzing samples on the Gas Chromatograph; *CommandCenter* used for inspecting processed samples and exporting data; and *PLFA Tools* used for transforming data into absolute moles based on an internal standard.
A specialized program, ***CSVExport****, creates the files in the format needed to upload into the NEON portal by collecting information from *Zoe* and from the exported data.

Sample Reception

Samples are received in batches from a specific site, described by a four-letter code. Each sample has a bar-code containing a sample identifier.

7. Create a new batch in *Zoe* for the samples using the four-letter code as the *Customer.*
8. Enter each sample into the batch, including the bar-code as the *Customer Sample Code.*
9. *Zoe* will assign a unique *Lab Number* for each sample.
10. Have a second technician verify the sample information in *Zoe***.**

Extraction

Extraction of PLFA from soil follows the *Microbial ID, Inc. Soil Microbial Biomass by High Throughput PLFA Analysis for NSF NEON Project* protocol.

9. Enter the recorded wet and dry weight for each full sample (including the vial) into *Zoe*.
10. Enter the weight of soil used for extraction into *Zoe*.
11. If a unique tare weight was calculated for this sample, enter that into the *Comments* field, in the form “VTW=<Weight>”.
12. Have a second technician verify the weight information in Zoe.

Running on the Gas Chromatograph

Samples are run using the *Sherlock* system.

1. Enter each sample into the *Sherlock Sample Processor* using this specific sample ID structure: “C-<Cust>-<LabNum>(G=<Weight>” where *Cust* is the four-letter code for that site, *LabNum* is the *Zoe* assigned lab number, and *weight* is the weight of soil used in the extraction. Example: “C-UNDE-MI190620-0424(G=0.49”
   Other information (such as the sample’s bar code) may follow the weight.

2. Include in the batch the reagent blanks processed with the batch (at least 1 per 20 samples). Use a sample ID beginning with “QC-BLANK” for each reagent blank.
3. Include in the batch positive controls to be processed with the batch (at least 1 per 20 samples). The positive control to be used is the **MIDI 1208*** standard.
   Use “QC-CALMIX-<LotNumber>” as the beginning of the sample ID.
4. All samples are to be processed with the **MIDI PLFAD2*** method.
5. When the runs are complete, inspect the sample reports for any flagged samples and rerun as necessary (concentrating if *Low Total Response*, diluting if *Column Overload*).

Transforming to Absolute Moles

Transform to absolute moles by using the *PLFA Tools TransformSamps* application.

9. Open the *TransformSamps* tool and select the data files containing the batch of interest.
10. Set the transform using method *PLFAD2* and the D2_MOLE text file.
11. Verify that the internal standard is 19:0 and amount is set to 6100.
12. Set the suffix of the transform file to *.MOL* to indicate moles.
13. Transform all selected files.
14. Check the log for any issues.
15. Open the Sherlock *CommandCenter Samples* mode and select the first sample in the batch from the original file and the transformed file.
16. Verify that the first compound has increased in percent, and the last compound has decreased in percent, indicating correct molarity transformation.

Exporting to Access Database

1. Open Sherlock *CommandCenter Data Export* mode.
2. Select the .MOL files for this batch.
3. Select the *Include Flagged Samples* checkbox (as some samples may be above or below the expected threshold once the molarity transform has been completed).
4. Include the prefixes “C-<Cust>”.
5. Include the prefixes “QC-BLANK”
6. Include the prefixes “QC-CALMIX”
7. Update the profile list and verify that the expected samples have been included.
8. DB Export these into an Access database whose name includes the customer code and batch number (“NEON_<Cust>_<BatchNum>”).

Generate Neon Upload files

1. Open *CSVExport*.
2. Select the Access database for this batch.
3. Enter the calculated or provided Tare weight for this batch, calculated during the extraction procedure.
4. Press the *Make Reports* button to generate the NEON microbialBiomass and the batchResults files.
5. Inspect the files, **but do not save**, as that will impact the format. CSVExport should have flagged samples under the following conditions:
    a. Extraction efficiency above 111%.
    b. A single compound has response above 30,000 nanomoles.
    c. A QC value is outside a +/- 25% range from the expected value.
6. Upload the files to the NEON Data Portal.

*** Major Components**

1. **Microbial Zoe.**
   A specialized LIMS system developed to store NEON information in a SQL database. It maintains sample information, including wet and dry weights, and assigns a unique lab number to each sample. It also includes information for harmonizing MIDI and NEON peak names.

2. **MIDI Sherlock.**
   Software system for analyzing GC samples. Includes:

a. Sherlock Method *PLFAD2* containing the instrument method as well as the peak naming table for PLFA compounds.
b. *Sample Processor* for automatically processing samples through the Gas Chromatograph using method *PLFAD2*.
c. *CommandCenter* for manually inspecting sample results and generating Access databases containing necessary data.
d. *PLFA Tools TransformSamps* for automatically converting raw data into absolute moles based on an internal standard. The D2_MOLE file is provided with the tools.
e. MIDI 1208 Calibration mixture. Used both to calibrate the Gas Chromatograph and used as the positive control for NEON.

3. **CSVExport.**
   A specialized program developed to extract information from Zoe and from the Sherlock-generated Access database to produce the microbialBiomass and batchResult files for each batch in the format needed by NEON.

Introduction

The MIDI, Inc. Sherlock PLFA Analysis System is designed to automate the process of analyzing soil sample extracted through the PLFA procedure.
Samples for the Neon project are analyzed using a combination of the standard Sherlock PLFA analysis methods and an additional step specific to Neon data formation.

As an aid to quantitation, an internal standard (19:0 PLFA) is added at a specific concentration in the first step of the extraction procedure; this standard both determines the scaling for the data and is used to evaluate the extraction efficiency.
Once the extract is placed on the Gas Chromatograph (GC), the main Sherlock software manages the characterization of the compounds and their relative amounts.
After Sherlock creates sample information in relative amounts, the PLFA Tools add-on uses the internal standard to scale to absolute amounts, and adjusts for the amount of soil actually used, giving values in picomoles/gram.

The specialized program *CSVExport* handles the final calculations necessary for Neon, and generates the .csv files in the appropriate format.
This document will describe the calculations for each phase of processing of a sample for the Neon project.

Part I: Sherlock GC Analysis

The Sherlock PLFA Analysis System automatically names fatty acids by calculating an Equivalent Carbon Length (ECL) for each peak using a calibration mix processed with the batch, and comparing peaks to the peak naming table. (Sasser, 1990; Ford, 2019)
The ECL method begins by assigning integral ECLs to the straight-chain fatty acids with C10 being 10.000, C11 being 11.000 and so on. A peak's ECL is determined by a simple linear interpolation, comparing the peak's Retention Time (RT) with that of the previous and following calibration compound. The formula is a standard interpolation:

$$ECLs = ECLp + (ECLf - ECLp) \times (RTs - RTp) \div (RTf - RTp)$$

where

ECLs is the calculated ECL for the sample peak
ECLp is the ECL for the previous calibration compound
ECLf is the ECL for the following calibration compound
RTs is the RT for the sample peak
RTp is the RT for the previous calibration compound
RTf is the RT for the following calibration compound

Once the ECL is calculated for a peak, the compound name is determined by a lookup in the peak naming table. For example, a compound such as 15:0 iso is described in the peak naming table as ECL 14.6170 ± 0.0200, so any peak whose ECL is calculated between 14.5970 and 14.6370 is assigned the name "15:0 iso".

While the Flame Ion Detector (FID) for a GC is a nearly universal detector, it operates by burning carbon and as such is selective: responding higher to compounds with more carbons. The Sherlock software automatically adjusts for this effect by calculating a response factor based on a quantitative calibration mix run with every batch.

For each quantitative compound in the calibration mix, a response factor is determined using the following formula:

$$RFi = (AMTi \div RSPi) \times (SumRSP \div SumAMT)$$

where

RFi is the calculated response factor for compound i
AMTi is the actual amount of compound i in the calibration mix
RSPi is the GC-FID response (area) for the peak associated with compound i.
SumRSP is the sum of the responses of all calibration compounds in the calibration mix
SumAMT is the sum of the actual amounts of all of the compounds in the calibration mix

The response factor for each compound is listed on the calibration report.
When a sample is processed, the responses for **all** compounds, those in the calibration mix and those not in the calibration mix, are multiplied by the response factor to yield an "amount" for that compound. Note that this is a relative weight amount, that is, it gives an amount for that compound which corresponds to its relative weight compared to the other compounds in the sample.

For compounds that were part of the calibration mix, the multiplying response factor is obvious. For a sample compound that is not part of the calibration mix, the response factor for the compound is calculated by interpolating the immediately previous and the immediately following calibration compounds' response factors, using the relative distance of the sample compound from the two calibration compounds.

As described above, the Sherlock software converts Retention Times (RT) to Equivalent Carbon Lengths (ECL) for peak identification; it also uses those units for interpolation calculation of response factor (though RT could be used with insignificant differences):

$$RFs = RFp + (RFf - RFp) \times (ECLs - ECLp) \div (ECLf - ECLp)$$

where

RFs is the calculated response factor for the sample compound
RFp is the response factor for the previous calibration compound
RFf is the response factor for the following calibration compound
ECLs is the ECL for the sample compound
ECLp is the ECL for the previous compound
ECLf is the ECL for the following compound

To discern that this formula is correct, note that if ECLs = ECLp then RFs = RFp; and if ECLs = ECLf then RFs = RFp + (RFf – RFp) thus RFp.
The relative amount of each compound is then computed by simply multiplying its response by its response factor.

Part II: Sherlock PLFA Tools Scaling

Part I yields for each sample a list of named compounds with relative weight amounts. The PLFA Tools application converts those into absolute moles using the information available from the internal standard.

While the Buyer/Sasser PLFA method (Buyer, 2012) suggests adding 10,000 picomoles to each sample, for historical reasons the Microbial ID method adds 6,100 picomoles. The PLFA Tools software has a parameter that is set appropriately for this use.
There are two aspects to scaling a sample to absolute moles; first, the relative weights for each compound needs to be converted to relative moles; then the mole values need to be scaled to take into account the known amount of the internal standard and the weight of soil used in the original sample.

Because the internal standard used is 19:0, relative scaling is achieved using 1.000 as the scale for 19:0. Other compounds are scaled based on the ratio of the molecular weight of 19:0 to their molecular weight. For example, the 15:0 iso (FAME) has molecular weight 256.4 while 19:0 (FAME) has weight 312.5; thus the 15:0 iso is scaled by a factor of 312.5/256.4 or 1.219.

As stated above 6,100 picomoles of internal standard is added to each sample. Having scaled to relative moles, the sample is scaled so that the value for 19:0 will be 6,100. Now all values are in absolute picomoles. However, if an amount of soil other than 1.00 grams is used, that amount is recorded with the sample and induces a final scaling by 1/weight. In this manner the final result is in absolute picomoles per gram.

All of the above calculations are handled automatically by the PLFA Tools application, yielding a new data file with values in absolute picomoles per gram.

Part III: Specialized Analysis for Neon

The typical procedure would be to report the values determined by PLFA Tools, but for the Neon project some specialized analysis has been added. A small program called *CSVExport* was written for the express purpose of generating the final export files in the form necessary for Neon.

Extraction efficiency is determined by comparing that original GC-FID response of the internal standard compound to the historical average maximum for that compound. Based on several years of data, it was determined that the 19:0 response of 30,000 units indicated full extraction. (The units that the Sherlock system uses for response is as a scaled peak area for the peak: 2000 × picoAmps × seconds.) A sample that has a 19:0 response of 20,000 units will thus be reported as 66.67% extraction efficiency.

Because the values generated by PLFA tools have already been scaled to the internal standard, the final values reported are multiplied by extraction efficiency, so that when the efficiency is applied the total value will be restored. Further, the values are divided by 1,000 to report nanomoles per gram rather than picomoles per gram.

Part IV: Batch Results and Lab Summary

The batch results are calculated for each batch by analyzing the positive controls run during the batch. The positive control, MIDI 1208, is a mixture of fatty acids made with precise relative amounts. The C20:0 saturated fatty acid in the mixture is at twice the amount as the C19:0 saturated fatty acid. The ratio of amounts is 2:1, but values are reported in moles, not amounts. Thus, the expected ratio of C20:0 to C19:0 is:

$$C20{:}0\ /\ C19{:}0\ ratio = 1.913\ =\ 2\ \times \frac{312.5}{326.6}$$

where 312.5 and 326.6 are the molecular weights of the C19:0 and C20:0 FAMEs, respectively.

For each positive control processed with a batch, the actual ratio is reported in the Batch Results File. (As a ratio, these values are unitless.)

The Lab Summary report uses this same ratio. The absolute difference of each positive control from the expected value is calculated for each positive control processed during the reporting period. The mean and standard deviation of these differences are reported.

Summary

Using the MIDI, Inc. Sherlock Chemical Analysis Software with PLFA Tools add-on allows the bulk of the analysis required for Neon to be completed automatically using off-the shelf software. The addition of the *CSVExport* tool automates the final analysis and reporting steps required for Neon.

In this manner, the full set of calculations for Neon are handled without human intervention.

References

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