TOS STANDARD OPERATING PROCEDURE: Sampling for Soil Microbial Reference Standard

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Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A	09/25/2020	ECO-06423	Initial release
B	05/05/2021	ECO-06608	• Clarified steps for field collection (spatial and temporal aspects) and centralized processing. • Added more shipping details.

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1 DESCRIPTION

1.1 Overview

The ability to quantify and taxonomically identify microbial taxa using amplicon-based sequencing methods (e.g. marker gene sequencing) has revolutionized our understanding of microbial diversity and ecology. With the advent of high-throughput sequencing methods, it is now possible to more completely characterize the high diversity of microbes in soils. This, combined with decreasing per sample costs and improving data quality, help to improve understanding of the ecology of microbes through time and space.

However, the high-throughput sequencing methods present unique and complex QAQC challenges that can make it difficult to determine whether variation in microbial communities through space and time is real, or is an artifact of the sequencing methodology. The goal of developing a soil microbial reference standard is to help data users identify potential sources of methodological variation. This reference standard could be used to:

- Evaluate data consistency – how variable are our results over time?
- Evaluate data precision – how closely do our data reflect 'true' microbial diversity and taxonomic composition?

This document describes how to develop a soil microbial sequencing standard in a cost-effective manner that enables NEON to:

1. Control for variability resulting from changing sequencing laboratories and methods over time.
2. Provide a metric by which data consistency can be evaluated.
3. Provide a control that mimics the normal, environmentally hardened microbial populations present in soil environments.
4. Develop a soil resource for re-analysis as new technologies and methods come on-line. New methods could be applied to the soil DNA standard to develop a reference by which existing NEON data can be compared to new data.
5. Provide a resource to the user community. External researchers may be interested in using the NEON reference standard to enable cross-comparisons with NEON data streams.

The NEON soil microbial reference standard is created and used as part of the soil microbial marker gene sequencing workflow. From the subset of selected NEON sites listed in **Table 3**, a common soil reference standard is generated from field-collected samples originating from the same locations as standard NEON soil microbe samples. As determined by the NEON Microbial Technical Working Group, it is not critical that samples be collected at any specific time of year, or that they be spread across the year, as the goal is simply to capture a portion of the genetic diversity of native microbes living in different kinds of soil. After standard field sampling at each of the sites listed in **Table 3**, individual field samples from that site are sub-sampled and pooled to make the site-level soil reference sample. This sample is

shipped to NEON Science Staff for additional processing, sub-sampling, and compositing with the soil samples from the remaining sites to create an Observatory-wide soil reference standard.

1.2 Purpose

This document outlines the NEON Standard Operating Procedure (SOP) for collecting, compositing, sub-sampling, packaging and storing soil samples that will be used to generate a soil microbial DNA reference standard. This SOP complements the existing SOPs for soil microbial sub-sampling described in the Biogeochemical and Microbial Sampling Protocol (RD[04]). This SOP is only carried out when explicitly requested and only for requested sites, and should be implemented concurrently with 'standard' soil collection bouts.

1.3 Scope

This document provides a change-controlled version of an Observatory procedure. Documentation of content changes (i.e. changes in particular tasks or safety practices) will occur via this change-controlled document, not through field manuals or training materials.

1.4 Applies To

The procedure described in this document is used with the following protocols:

Doc #	Title
NEON.DOC.014048	TOS Protocol and Procedure: Soil Physical, Chemical, and Microbial Measurements

1.5 Acknowledgments

The procedure was developed with extensive contributions from the members of the NEON Microbial Technical Working Group.

2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain higher-level information that is implemented in the current document. Examples include designs, plans, or standards.

AD[01]	NEON.DOC.004300	NEON Environmental, Health, Safety, and Security (EHSS) Policy, Program, and Management Plan
AD[02]	NEON.DOC.004316	Operations Field Safety and Security Manual
AD[03]	NEON.DOC.001155	NEON Training Plan
AD[04]	NEON.DOC.050005	Field Operations Job Instruction Training Plan

2.2 Reference Documents

Reference documents contain information that supports or complements the current document. Examples include related protocols, datasheets, or general-information references.

RD[01]	NEON.DOC.000008	NEON Acronym List
RD[02]	NEON.DOC.000243	NEON Glossary of Terms
RD[03]	NEON.DOC.005003	NEON Scientific Data Products Catalog
RD[04]	NEON.DOC.010408	TOS Protocol and Procedure: Soil Biogeochemical and Microbial Sampling
RD[05]	NEON.DOC. 001577	Datasheets for TOS Protocol and Procedure: Physical, Chemical, and Microbial Measurements
RD[06]	NEON.DOC. 005244	Datasheets for TOS Protocol and Procedure: Sampling for Soil Microbial Reference Standard
RD[07]	NEON.DOC.001271	AOS/TOS Protocol and Procedure: Data Management
RD[08]	NEON.DOC.001716	TOS Standard Operating Procedure (SOP): Toxicodendron Biomass and Handling
RD[09]	NEON.DOC.0055224	Protocol and Procedure: Shipping Ecological Samples and Equipment

2.3 Acronyms

All acronyms used in this document are defined in RD[01] or RD[04].

2.4 Definitions

DNA: Self-replicating material present in nearly all living organisms and the carrier of genetic information.

3 SAFETY

This document identifies procedure-specific safety hazards and associated safety requirements. It does not describe general safety practices or site-specific safety practices.

Personnel working at a NEON site must be compliant with safe field work practices as outlined in the Operations Field Safety and Security Manual (AD[02]) and EHSS Policy, Program, and Management Plan (AD[01]). Additional safety issues associated with this field procedure are outlined below. The Field Operations Manager and the Lead Field Ecologist have primary authority to stop work activities based on unsafe field conditions; however, all employees have the responsibility and right to stop their work in unsafe conditions.

3.1 Soil Safety

Work that involves disturbance of soils or plant litter may increase the concentration of fungal spores and bacterial pathogens in the air. Take precautions to prevent inhalation of dust from soils and plant litter. Review zoonotic diseases in AD[02] for information on areas of high risk and symptoms of fungal infection. If *Toxicodendron spp* are present at a given site, Field Operations should utilize the procedures outlined in TOS Standard Operating Procedure: *Toxicodendron* Biomass and Handling (RD[08]) in order to minimize exposure while sampling and to properly clean equipment that came in contact with toxic soils.

Soil sampling equipment can be sharp and/or heavy (i.e., hori hori knife, coring device). Staff must take precautions to handle these tools with appropriate care. Dry ice used for preserving microbial samples must be handled with appropriate safety protection and must never be stored in airtight containers. Shipment of samples to external laboratory facilities on dry ice requires additional safe handling techniques, the availability of a Safety Data Sheet, and additional safety labels.

3.2 Soil Shipment and Quarantine

Shipment of soils is regulated by the USDA Animal and Plant Health Inspection Services (APHIS) and all requirements must be followed when handling and shipping soils. Refer to the guidelines listed in Section 5.1 of the TOS Protocol and Procedure: Soil Physical, Chemical, and Microbial Measurements (RD[04]) for more details.

4 PERSONNEL

4.1 Training Requirements

All technicians must complete required safety training as defined in the Field Operations Job Instruction Training Plan (AD[04]). Additionally, technicians must complete procedure-specific training for safety and implementation of this procedure as required in AD[04].

Technicians must be trained in the proper implementation of clean and sterile methods as defined by NEON.

4.2 Specialized Skills

All technicians should have the skills and training described in TOS Protocol and Procedure: Soil Biogeochemical and Microbial Sampling (RD[04]).

5 CONTINGENCIES AND NOTES

NA

6 STANDARD OPERATING PROCEDURES

SOP A Preparing for the Field

A.1 Additional Equipment

Carrying out this SOP will require collecting a larger mass of soil than is required for a typical soils bout. Be prepared to estimate the mass of soil in the field to ensure sufficient material will be available for sub-sampling. It is recommended that an appropriately sized spring scale be brought to the field (100-1000 g ranges, **Table 4**) to double check sample masses before leaving the field. This is recommended in particular for sites where O horizons are being sampled or at sites where soil quantity is often limited.

A.2 Ensure Sufficient Soil Has Been Collected

In general, each discrete X, Y location sampled during standard soil sampling (n = 30) will contribute to the site-level soil reference sample. The estimates listed in **Table 1** for minimum total mass of each homogenized sample assume that nearly all bags will be used to obtain the target mass for the reference standard. Note that these masses are slightly higher than the ones listed in SOPs B and C of RD[04], to account for the extra mass needed for site-level reference soil sample creation. For sites that regularly collect 30 cm deep mineral soil cores, there will likely be no change from standard soil sampling. However, for sites with thin horizons, ensure sufficient mass is collected.

Table 1. Estimated quantity of homogenized soil remaining for lab subsampling and analyses when sub-sampling for Soil Reference Standard. Estimate based on minimal presence of rocks, roots and debris in homogenized soil sample. For Core sites conducting a peak green bout: add 5 g additional mass if the metagenomics ('-COMP') sub-sample is not generated in the field.

nTransBoutType	BoutType	SampleTiming	Horizon	Total Target Mass (g)
No	Microbes, microbesBiomass	T1, T2, peak green	O	35
Tinitial	microbesBiomass	T1, T2	O	55
Tinitial	microbesBiomassBGC	Peak green	O	80
No	Microbes, microbesBiomass	T1, T2, peak green	M	85
Tinitial	microbesBiomass	T1, T2	M	125
Tinitial	microbesBiomassBGC	Peak green	M	185

SOP B Laboratory Processing of Soil Reference Standard

One site-level soil reference sample will be generated from this SOP to contribute to the Observatory-wide DNA soil reference standard. This site-level sample will be a composite of all homogenized bags of soil collected at each X, Y location (unless sufficient soil is not available) from a single soils bout. Any bout (T1, PG, T2) is theoretically acceptable, but Science will instruct which bout to choose per site. Only soil from one horizon type should be selected for creating this site-level reference standard: do not mix O and M horizons. Refer to **Table 2** for the horizon and target mass of soil for each selected site.

Sub-sampling for the site-level soil reference standard should be completed within 1 day of field collection on the field-moist soils (e.g., samples collected on a Monday must be sub-sampled by end of the day Tuesday). Sub-sampling is to take place *after* sub-sampling for soil moisture on *unsieved* soil that has been picked clean of non-soil debris such as rocks, roots, insects, etc. Ensure there is sufficient soil for all other sub-samples and analyses as described in the Soil Protocol (RD[04]). If the remaining homogenized soil for downstream measurements is limited, do not sub-sample for the reference standard and exclude this sample.

To avoid sample degradation, keep homogenized field sample bags at 4 ˚C (e.g., in a refrigerator or on ice packs), and move to room temperature for processing for no more than 10 minutes. Best practice is to only leave one sample out of the refrigerator at any given time, and to replace the sample in the refrigerator once the sample is no longer needed. For the site-level reference sample, prepare a cooler with ice packs in which to place the sample bag, as this will need to be kept nearby for most of the processing.

Table 2. Sites and target horizons of soils collected for the site-level soil reference sample generation. Total target mass represents the site-level mass of soil. Total target masses provided here are minimum values; it is OK if more material than the target is collected.

Site ID	Expected horizon	Total Target mass (g)
HARV	O	100
JORN	M	500
CPER	M	500
DELA	M	500
KONZ	M	500
WOOD	M	500

Site ID	Expected horizon	Total Target mass (g)
OAES	M	500
TREE	O	100
UKFS	M	500
UNDE	O	100

B.1 Laboratory Sub-sampling

Prepare to sub-sample

- Either print out a copy of the Soil Reference Standard Datasheet (RD[06]), or create an equivalent electronic version to automatically calculate the total mass of soil added to the reference standard sample.
- Prepare the homogenized bags of soil from each X, Y coordinate that will be sub-sampled for the site-level soil reference standard that day. Ensure that any samples used will have sufficient soil to complete all sub-sampling and analyses after sub-sampling for the reference standard is completed (refer to **Table 2**). Set aside any homogenized bags of soil that will not be used in the refrigerator, and keep separate from the bags that will be used.

1. Clean work area with 70% ethanol (squirt bottle or sterile ethanol wipe OK). Wipe down work area as needed to keep area clean.
2. Put on a new, clean pair of gloves and wipe gloves with sterile ethanol wipe. Re-wipe gloves between samples; replace gloves when visibly dirty or damaged.
3. Label a new, clean 1-quart (or larger if needed) freezer-safe resealable bag with the **siteID**, **horizon**, **date**, and **processedBy**.
4. Place the bag onto the balance and tare the balance.
5. Wipe down a spoon/scoopula with a sterile 70% ethanol wipe. Measure out 4-6 g (O horizon) or 15-20 g (M horizon) field-moist soil into the bag, excluding rocks, roots and debris. More or less soil per individual sample is OK as long as the target total mass is achieved. Record **soilFreshMass** in the datasheet.
    a. The level of effort for removal of non-soil material should match what is done in RD[04] when preparing the various microbial field subsamples.
6. Close bag and store in a cooler on ice packs so that the sample stays chilled (not frozen).

7. Record required metadata in the datasheet.
8. If possible, try to use only soils of the same horizon type. It is okay if an O horizon sample incorporates a small amount of mineral soil.
9. For the next homogenized soil sample, replace the bag containing the composite reference sample onto the balance and zero the balance.
10. Measure out 4-6 g or 15-20 g into the tared bag and record **soilFreshMass** added from this sample on the datasheet.
11. Repeat these steps for all remaining soil samples that will be subsampled that day.
12. If needed, repeat this entire section for each day that additional soil samples will be composited, using a new freezer-safe 1-quart bag. This ensures that samples collected and processed in previous days do not undergo multiple freeze/thaw cycles.
    a. Summing all soil together, ensure the minimum target masses are met, namely 100 g (O horizons) or 500 g (M horizons) as outlined in **Table 1**.
    b. If you are significantly (>20%) below the target mass after sub-sampling for all plots of a particular horizon, submit an issue ticket.
13. Double-bag the final sample in a 1-gallon, freeze-safe bag (may be in multiple smaller bags, following step 12 above). Place the soil sample in the -80 ˚C freezer and store until shipment.

SOP C Sample shipment

- Ship site-level soil reference samples on dry ice to NEON headquarters according to the Shipping Protocol (RD[09]).
- For domains that are creating more than one site-level reference sample, hold until all sites are complete and then ship them together.
- Ensure that all required federal, state and local permits are included and that the receiver (e.g., the NEON Science staff member overseeing reference standard creation) has received an email notification of the sample shipment.
- Include a printed hard-copy of the datasheet in the shipment.

SOP D Preparing site-level samples for Observatory-wide reference standard generation

This SOP is carried out at a centralized facility where site-level soil reference samples are shipped. Due to the potential difficulty in extracting DNA from soils collected from particular sites, it is possible that soils from certain sites may need to be excluded from the final reference standard. Therefore, maintaining the original site-level soil stocks is critical. Site-level issues may be addressed during initial standards testing.

D.1 Sample receipt and storage

1. Upon receipt, check samples to ensure they remain frozen and un-damaged.
2. Records for sample contents and condition are retained locally and electronically for reference.
3. Samples are stored at -80 ˚C until ready to process.

D.2 Prepare to process samples

NOTE: Processing the samples should happen in a way that is as sterile as possible to maintain sample integrity and minimize contamination because the introduction of foreign DNA during processing may degrade the integrity and repeatability of the reference material over time.

1. All equipment and materials (**Table 5**) used for sample handling and processing must be either certified sterile or cleaned prior to use (e.g. glass beakers, soil sieving trays, etc). Cleaning for this procedure is performed as follows:
    a. Wash items in soap and water and rinse with DI water 3 times.
    b. Perform a final rinse with sterilize DI water and allow items to air dry inverted.
    c. Once dry, wrap/cover items with new aluminum foil.
    d. Working surfaces are rinsed clean and dried, then sanitized with 70% ethanol prior to use.

D.3 Processing samples for standards generation

1. If possible, the day before processing, move the samples to be processed from the freezer to a refrigerator to allow them to thaw overnight. However, do not allow samples to remain thawed in a refrigerator for more than one day. If this is not possible, then on the day of processing, remove samples from freezer and allow to just thaw, then move samples onto ice.
2. Wipe down a workspace with 70% ethanol. Process each site-level soil reference sample separately. Don new nitrile gloves and sterilize them with 70% ethanol.
    a. Remove any coarse debris and roots, homogenize remaining soil, and pass through a sterilized, 2 mm sieve. A 4 mm sieve may be used to pre-sieve before moving onto a 2 mm sieve.

3. Sieve all of the soil for the same site-horizon and combine.
4. For each site-horizon combination, place a pre-cleaned (as in D.1) 500-mL beaker on a balance and tare. Weigh out the soil for each site-horizon combination into the corresponding tared beaker in roughly equal quantities of approximately 50.0 g for organic soils and 250.0 g for mineral soils. Repeat for each site-horizon until all the soil has been weighed.
5. Place weighed samples in a dehumidifier environmental chamber until the relative humidity is as low as possible (maximum 2% RH).
6. While samples are drying, prepare labels for individual containers. Print the following text on cryo-safe labels, with up to 20 labels per site/horizon combination.
    a. 'Site-level soil microbe reference standard. HARV-O-__. Prepared by ____ on _____'
7. Shake dried soil samples vigorously to homogenize. If aggregates formed during drying, pass through a sterilized 2 mm sieve a second time. If aggregates do not pass through a sieve, place soil in a new ziplock bag and using a rubber mallet, break up aggregates as much as possible, then sieve.
    a. The soil must be thoroughly homogenized in order to generate consistent sub-samples that can be used across the life of the Observatory. Any soils that are unable to be sufficiently homogenized (aggregates > 2 mm) should be excluded from the final pooled reference sample. If the majority of the soils are not sufficiently homogenized, other options may be required, including freeze-drying.
8. Weigh out the homogenized soil into pre-labelled 15-mL sterile conical tubes. The same mass of soil should be weighed into each aliquot: **3 g organic or 15 g mineral ± 0.01 g**. When these masses can no longer be met, discard remaining soil. For each aliquot from a site-horizon, append a replicate number to the existing label on the vial as '-1', '-2', etc and add initial(s) for who prepared the aliquots and when (YYYYMMDD).
9. Repeat aliquoting for the material from all sites.
    a. Accounting for loss during processing, approximately 200-2000 g of material should be produced, depending on the horizon type and soil moisture content.
10. The site-level aliquots are stored frozen (-80 ˚C) at NEON HQ to preserve the microbial taxonomic signatures. Retaining separate vials for individual sites provides the flexibility to add or remove soils from the reference standard if technical issues for soil from a particular site are encountered.
    a. Storage requirement: 120-200 aliquots of site-level soil is expected to require less than 1 shelf of ultra-low freezer space.
11. When preparing the Observatory-wide soil reference standard, one soil aliquot from each site is combined in a clean 1-L HDPE bottle to create a pooled, multi-site soil sample representative of all selected sites. The sample is homogenized thoroughly by closing the bottle and shaking for at least 30 seconds. Using a soil splitter, the homogenized soil is split into 15-mL sterile conical tubes as 10-mL aliquots (± 1 mL, a representative subsample size).

a. Each conical tube should be labeled as followed using a cryo-safe label:

    i. 'Observatory-wide soil microbe reference standard. Includes sites _____. Prepared by____ on _____'

b. One aliquot of the standard is shipped to the analytical laboratory for active use.

c. Remaining aliquots of the reference standard are stored at -80 ˚C at NEON HQ or at the ASU biorepository, where they are available on an as-needed basis either by the analytical laboratory or by external researchers via the assignable assets program.

7 REFERENCES

Yeh, Yi-Chun, David M. Needham, Ella T. Sieradzki, and Jed A. Fuhrman. 2018. "Taxon Disappearance from Microbiome Analysis Reinforces the Value of Mock Communities as a Standard in Every Sequencing Run." *MSystems* 3 (3).

APPENDIX A QUICK REFERENCES

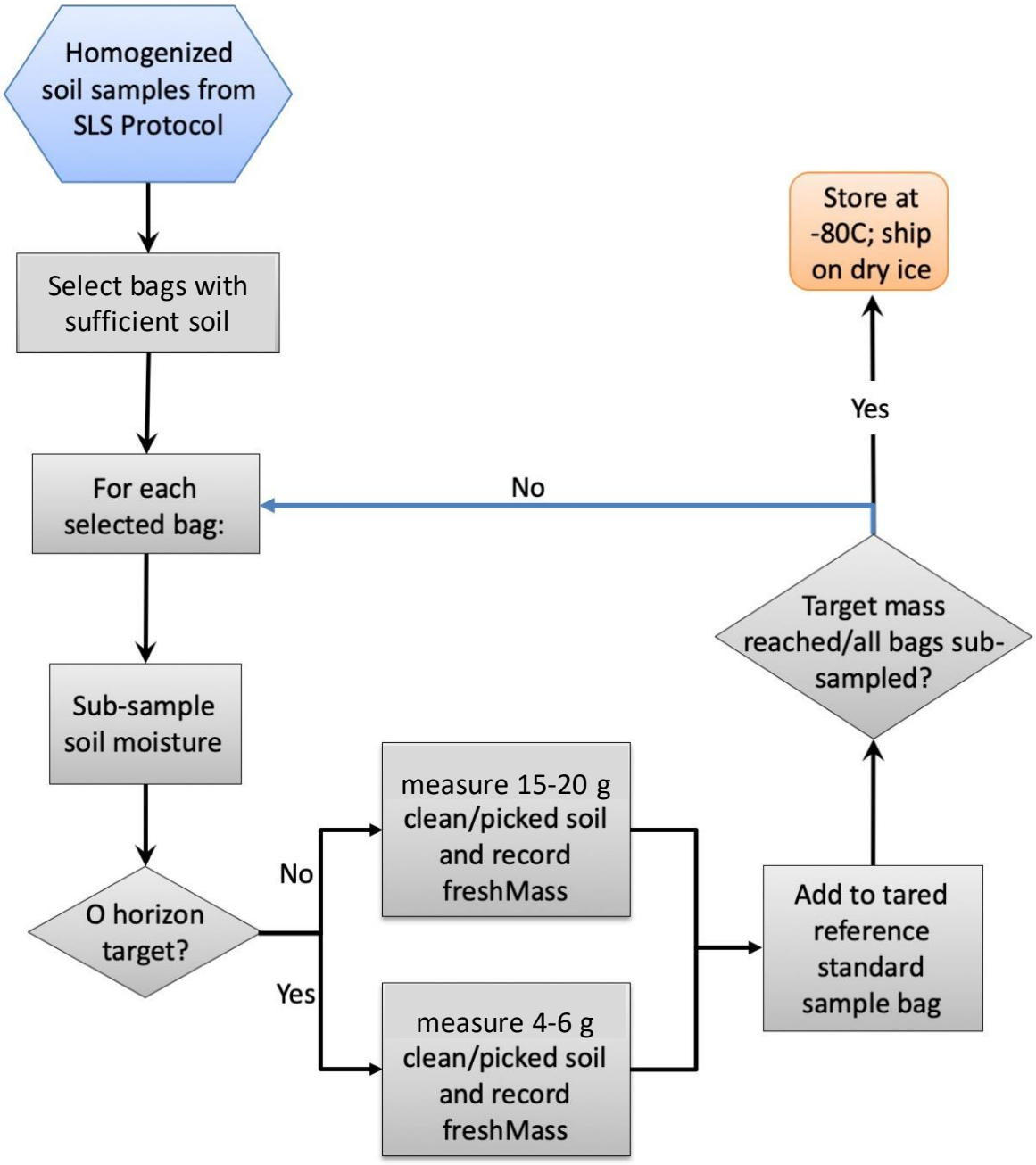

Figure 1. Overview of workflow for creating the site-level soil reference sample.

APPENDIX B SITE SPECIFIC INFORMATION

Table 3. Sites where sub-sampling for the soil reference standard will initially occur.

Domain	Site
D01	HARV
D05	UNDE
D05	TREE
D06	KONZ
D06	UKFS
D08	DELA
D09	WOOD
D10	CPER
D11	OAES
D14	JORN

APPENDIX C EQUIPMENT

The following equipment is needed in addition to the field collection supplies listed in RD[04] in order to implement the procedures in this document. Equipment lists are organized by task. They do not include standard field and laboratory supplies such as charging stations, first aid kits, drying ovens, ultra-low temperature freezers, etc.

Table 4. Equipment list – Creating a site-level soil reference sample.

Supplier/Item No.	Exact Brand	Description	Purpose	Quantity
Forestry Suppliers 93012; 93013	N	Spring scale, 300g	Weigh soil samples in field	1
	N	Resealable, freeze-safe, plastic bag, 1-quart	Storing site-level reference soil sample	1-4 per site
	N	Resealable, freeze-safe, plastic bag, 1-gallon	Double containment to prevent sample loss/contamination	1 per site
Fisher 0190200; Mettler Toledo 11144914	N	Balance, 0.01 g accuracy	Weigh soil in lab	1
	N	Metal spoon or scoopula	Transferring soil	1-2
VWR TWTX3044P	N	Sterile, 70% ethanol pre-wetted wipers	Sterilizing work surfaces, gloves and equipment	10-15
	N	Squirt bottle containing sterilized, 70% ethanol	Alternative for sterilizing work surfaces, gloves and equipment	1
FisherSci SVGPL10RC; Y0974103	N	0.2 micron filtration unit, sterile, polyethersulfone	To create sterile ethanol	1
	N	Nitrile gloves	Handling samples	1 box
	N	Lab marker, alcohol safe	Labeling sample	1-2

Table 5. Equipment for laboratory processing of site-level and Observatory-level reference sample.

Supplier/Item No.	Exact Brand	Description	Purpose	Quantity
	N	Resealable, freeze-safe, plastic bag, 1-gallon	Double containment to prevent sample loss/contamination	1 per site
Fisher 0190200; Mettler Toledo 11144914	N	Balance, 0.01 g accuracy	Weighing soil	1
	N	Metal spoon or scoopula	Transferring soil	1-2
VWR TWTX3044P	N	Sterile, 70% ethanol pre-wetted wipers	Sterilizing work surfaces, gloves and equipment	10-15
	N	Squirt bottle containing sterilized, 70% ethanol	Alternative for sterilizing work surfaces, gloves and equipment	1
	N	Sterile water	Final rinse after cleaning durable equipment	5 L
	N	Beaker, 500 mL	Measuring site-level samples	20
Cincinnati Sub Zero Model Z-Plus (32)	N	Temperature and humidity chamber	Dry site-level reference samples	1
	N	15 mL sterile conical tubes	Hold the reference standard aliquots	200
Fisher 15-930-E	Y	Cryogenic, adhesive labels	Label conical tubes	200
	N	Nitrile gloves	Handling samples	1 box
	N	Lab marker, alcohol safe	Labeling as needed	1-2
	N	HDPE bottle with lid, 1 L	Homogenizing reference material	1
Gilson SP300 precision splitter	N	Soil splitter	Generating homogeneous aliquots of reference material	1