NEON DNA Extraction Standard Operating Procedure v.9

Prepared for:
Battelle/National Ecological Observatory Network (NEON) Program

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I. Version History

Version 9	Updated sample storage temperature to match the statement of work (Section IV.A). Added details about using a plate reader for the quantification of DNA extracts (Section IV.D). Updated repeat quantification guidelines to only include aquatic filter samples (Section IV.D, Table 3).
Version 8 11/26/2024	Added details about duplicate aquatic filter samples (Section IV.B.2.2). Added guidelines for performing repeat quantification of DNA extracts (Section IV.D). Added section to cover pooling and concentration of duplicate aquatic filter sample extracts (Section IV. E). Updated table numbers. Added storage temperatures for DNA extracts (Section IV.C.2). Added change from performing the bead beating step in the 96 well plate to doing it in tubes (Section IV.C.1.1).
Version 7 06/13/2022	Removed use of Positive Extraction Control (PEC), with approval of NEON, due to incompatibility of the ZymoBIOMICS Spike-in Control I (High Microbial Load) with Qiagen DNA extraction kits
Version 6 02/10/2022	Soil extraction kit changed from DNeasy PowerSoil HTP 96 Kit to DNeasy PowerSoil Pro kit and includes instructions for use of this extraction kit in both 96-well plate format and individual tube format. Added clarification on repeating extractions based on DNA concentration of the initial extraction. This SOP includes the soil, biofilm, and Sterivex water filter extractions for samples destined for all analyses. Minor editorial updates were also made throughout the SOP.
Version 5 08/12/2021	Added steps for removal of DNA/RNA Shield from the ZymoBIOMICS Spike-in Control I (High Microbial Load) prior to extraction and later adding back in to the samples to capture any free DNA left in solution. This is necessary in order to obtain enough DNA from the Spike-in Control I when using DNeasy PowerSoil kits. Clarified the impact of batch-level and sample-level QAQC failures. This SOP is for the use of the DNeasy PowerSoil HTP 96 kit to extract composite soil samples destined for metagenomics sequencing only.
Version 4 02/04/2021	Added a microbial community standard as a positive extraction control. Clarified negative extraction controls used with each sample type. Clarified the frequency of both positive and negative extraction controls. Updated acceptance criteria in Section V to include results of the positive and negative extraction controls.
Version 3 07/27/2020	Removed the manual references in Section II; updated materials information in Section III; clarified the use of sterile consumables throughout Section IV.B; revised the concentration level below which the extraction is considered a fail to 1 ng/µL in Section V; updated user manual references.

Version 2 09/30/2019	Disposable polypropylene weighing funnels (that are autoclavable) are now used to weigh soil samples and insert samples into plate wells; revised acceptance criteria.
Version 1 06/01/2017	Initial release.

II. Objective and Overview

Samples from the National Ecological Observatory Network (NEON) program are extracted to isolate DNA for use in subsequent qPCR, marker gene, and/or metagenomic sequencing analyses. DNA is extracted from soil samples using the DNeasy PowerSoil Pro Kit in either 96-well plate with PowerBead Pro tubes to replace the PowerBead Pro plate or the full tube format (DNeasy PowerSoil Pro kit), and from Sterivex water filters using the DNeasy PowerWater Sterivex Kit. DNA is extracted from the benthic biofilms of sediment samples (sand and silt) and adherent biofilms that are scraped from plant grab samples with sterile spatulas using the DNeasy PowerBiofilm Kit. Extraction batches are accompanied by quality control samples as described in Section IV. Kits are detailed in Section III.

III. Recommended Materials

Material	Manufacturer	Catalog #
DNeasy 96 PowerSoil Pro Kit	Qiagen	47017
DNeasy PowerSoil Pro Kit	Qiagen	47016
DNeasy PowerBiofilm Kit	Qiagen	24000-50
DNeasy PowerWater Sterivex Kit	Qiagen	14600-50-NF
QuantiFluor ONE dsDNA Kit	Promega	E4870
Disposable Weighing Funnels	TWD Scientific	DDWF-PP-XS
PowerBead Pro Tubes	Qiagen	19301
NucleoSpin® gDNA Clean-up Kit	Macherey-Nagel	740230

IV. Procedure

A. Sample Receipt and Storage

Soil and aquatic samples are held in cryostorage before extraction. Upon receipt Battelle will ensure the samples are in good condition and sort them according to post-extraction analysis type. Samples are stored at -65°C to -80°C until extraction.

B. Sample Preparation

Note: All extraction work takes place in a biosafety cabinet (BSC). Prior to beginning work the BSC is wiped down with 10% bleach solution followed by 70% isopropyl alcohol. The germicidal light is turned on for a minimum of 10 minutes. All consumables used during sample processing either come in sterile, unopened bags that are only opened in a BSC (sealing film, collection tubes) or are autoclaved prior to use (disposable weighing funnels).

1. Soil Samples

If there are enough soil sample extractions to fill a 96-well plate, use the 96-well plate extraction process as described in Section IV.B.1.1. If there are not enough samples to extract using the 96-well plate protocol, use the individual tube extraction process for batches of up to 24 samples at a time, as described in Section IV.B.1.2.

1.1. **96-Well Plate Extraction Format.** Disposable weighing funnels and scoopulas are autoclaved for 80 minutes to ensure sterility. A full extraction batch consists of 95 soil samples and one negative extraction control (NEC).

Prior to DNA extraction, label PowerBead Pro tubes with appropriate well number and briefly spin tubes so that beads are pulled to the bottom of the tube. Next, 0.25 ± 0.03 g of thawed soil sample is weighed and placed into the PowerBead Pro tube by using a sterilized disposable weighing funnel or directly into the tube. For each extraction plate, PowerBead Pro tube A1 is left empty as the NEC. After all the tubes have been filled and caps secured, the tubes may be stored at 2-8°C for up to 48 hours if not immediately continuing to Section IV.C.1.1.

1.2. **Individual Tube Extraction Format**. Disposable weighing funnels and scoopulas are autoclaved for 80 minutes to ensure sterility. Up to 24 samples may be included in one tube extraction batch. One NEC will be prepared with each DNeasy PowerSoil Pro Kit (each kit can extract approximately 50 samples). This results in one NEC with approximately every two extraction batches using the same DNeasy PowerSoil Pro Kit. Each extraction batch will contain up to 23 soil samples when a NEC accompanies the batch.

Prior to DNA extraction, 0.25 ± 0.03 g of thawed soil sample is weighed and placed into the appropriate 2 mL microcentrifuge tube. The soil tube extraction NEC will consist of an empty 2 mL collection tube. After all the tubes have been filled, they may be stored at 2-8°C for up to 48 hours if not immediately continuing to Section IV.C.1.2.

2. Aquatic Samples

2.1. **Biofilm**. Biofilm samples from sediment and from plant tissues (epiphyton, epipsammon, and epipelon sample collections) are extracted in tubes. Up to 24 samples are included

in the same extraction batch. One NEC will be prepared with each DNeasy PowerBiofilm Kit (each kit can extract approximately 50 samples). This results in one NEC with approximately every two extraction batches using the same DNeasy PowerBiofilm Kit. Each extraction batch will contain up to 23 biofilm samples when a NEC accompanies the batch.

For sediment biofilm material, 0.25 ± 0.03 g of the material is weighed in a 2 mL collection tube with an appropriate label. For plant biofilm material, a sterile spatula is used to scrape 0.25 ± 0.03 g of the material into a 2 mL collection tube with an appropriate label. The biofilm NEC will consist of an empty 2 mL collection tube. Biofilm samples are extracted the day they are prepared following Section IV.C.1.3.

2.2. **Filters**. For surface water and benthic filter samples (originating from epixylon and epilithon biofilm sample collections), samples will be extracted as-is with no additional preparation steps. Up to 24 filter samples are included in the same extraction batch. One NEC will be prepared with each PowerWater Sterivex Kit (each kit can extract approximately 50 samples). This results in one NEC with approximately every two extraction batches using the same PowerWater Sterivex Kit. Each extraction batch will contain up to 23 filter samples when a NEC accompanies the batch. The filter NEC will consist of an empty Sterivex filter unit. Filter samples are extracted the day they are prepared following Section IV.C.1.4.

Aquatic biofilm and filter samples collected during mid-summer may be collected in duplicate. The duplicate sample will have ".MET" at the end of the sample ID. For duplicate samples, each sample will be individually extracted following Section IV.C.1.3 or IV.C.1.4, as appropriate.

C. DNA Extraction and Isolation

1. DNA Extraction by Sample Type

1.1. Soil Samples in 96-Well Plate Extraction Format

Genomic DNA (gDNA) is extracted from soil samples using the Qiagen DNeasy 96 PowerSoil Pro Kit (cat #47017) according to the manufacturer's instructions, with the exception of the bead beating step, which will be done with DNeasy PowerBead Pro tubes (cat# 19301) following the individual tube extraction method described in Section 1.2 (Manual: Qiagen_HB-2675-002_HB_DNeasy_PowerSoil_ProKit96_0721_WW). In short, samples are weighed into DNeasy PowerBead Pro tubes, CD1 solution is added, tubes are shaken via TissueLyser II and then spun down, supernatant is transferred to the collection microtubes provided in the Qiagen DNeasy 96 PowerSoil Pro kit and the kit instructions followed.

Once extraction is complete, proceed to Section D.

1.2. Soil Samples in Individual Tube Extraction Format

Genomic DNA (gDNA) is extracted from soil samples in individual tubes using the Qiagen DNeasy 96 PowerSoil Pro Kit (cat #47016) according to the manufacturer's instructions (Manual: Qiagen_HB-2495-005_HB_DNY_PowerSoil_Pro_0321_WW).

Once extraction is complete, proceed to Section D.

1.3. Biofilm Samples

Genomic DNA (gDNA) is extracted from biofilm samples (epiphyton, epipsammon, epipelon) in individual tubes using the Qiagen DNeasy PowerBiofilm Kit (cat #24000-50) according to the manufacturer's instructions (Qiagen_HB-2274-002_HB_ DNY_PowerBiofilm_0120 _ WW). Once extraction is complete, proceed to Section D.

1.4. Sterivex Samples

Genomic DNA (gDNA) from aquatic samples in Sterivex filter units (including epilithon and epixylon biofilm samples) is extracted using the Qiagen DNeasy PowerWater Sterivex™ Kit (cat #14600-50-NF) according to the manufacturer's instructions (Manual: Qiagen_HB-2266-002_HB_DNY PowerWater_Sterivex_0519_WW).

Once extraction is complete, proceed to Section D.

2. DNA Extract Storage

Following quantification and pooling/concentration (as applicable), DNA extracts will be stored at -20°C while downstream analysis is ongoing (to prevent freeze thaw damage from ultra-low conditions), samples will then be transferred to long-term storage in an ultra-low freezer (-65 to -80°C) until ready for shipment to archive at the NEON Biorepository.

D. Quantus dsDNA Assay

After extraction and isolation, the gDNA of each sample, including the NECs, is quantified using either a Promega Quantus Fluorometer or a fluorescent plate reader (such as the SpectroMax i3 Multi-Mode Detection Platform) with a QuantiFluor ONE dsDNA Kit (#E4870) according to the manufacturer's instructions (Manuals: Quantus_Fluorometer-Operating-Manual_TM396_ rev 11/2024 and QuantiFluor ONE dsDNA System_Manual_TM405_rev 10/2022). DNA concentrations must meet the acceptance criteria of both Tables 1 and 2 to proceed to downstream analyses. Because aquatic filter samples are completely consumed in the extraction process and cannot be re-extracted, extracts that meet the criteria of Table 1, but not Table 2 will be re-quantified, as described in Table 3 below.

Table 1. Batch- level Quality Control and Acceptance Criteria

QA/QC measurement	Frequency	Requirement	Corrective Action
DNA concentration in Negative Extraction Control (NEC) (no DNA control)	Soil (96-Well Plate Format)– one per 96-well extraction plate Soil (Individual Tube Format) / Biofilm / Sterivex Filters – one per DNA extraction kit lot #	< 1 ng/µL	Suspected batch-level contamination. Halt processing the batch and contact NEON immediately to evaluate batch-level resolution. NEON must approve the resolution. Potential outcomes of evaluation: continue with analysis as normal; continue with analysis with additional quality flags; re-extract batch of samples; replacement of reagents/kits before proceeding with more DNA extractions.

Table 2. Sample-level Quality Control and Acceptance Criteria

QA/QC measurement	Frequency	Requirement	Corrective Action
DNA concentration in each NEON field sample extract (does not include NECs, which are evaluated using Table 1)	Per sample	≥1 ng/µL	If <1 ng/µL on initial extraction, follow guidance in Table 3 for re-quantification. If re-extraction is necessary, then re-extract one time for soil or biofilm samples. After 2 unsuccessful attempts (initial and one repeat) where all batch-level QAQC criteria passed, notify NEON of sample-level failure. Because there is only one Sterivex filter sample, failed samples cannot be repeated and NEON should be notified of the initial Sterivex filter failure. NEON may instruct laboratory to continue downstream analyses (e.g. marker gene sequencing) with a failed sample DNA extract or to report the extract as a “fail” in the extraction ingest.

Table 3. Repeat DNA Quantification Guidelines

Re-Quantified Extract DNA Concentration	Action
<1 ng/ µL	**Aquatic Filters**: Re-prepare extract for Quantus ONE dsDNA System analysis. Report repeat DNA concentration to NEON to discuss whether extract should be concentrated or reported as a failure **Soil/Biofilm**: Add to repeat extraction list (maximum one repeat)
1.0 - 5.0 ng/ µL	Report repeat DNA concentration, report extract as pass and continue to downstream analysis
> 5 ng/ µL	Quantify a 3rd aliquot and report concentration from 3rd re-quantification as pass if > 1.0 ng/µL and continue to downstream analysis; if <1.0 ng/uL on 3rd aliquot, add sample to repeat extraction list (maximum one repeat)

E. Pooling and Concentration of Aquatic DNA Extracts for Metagenomic Analysis

Duplicate aquatic samples collected in mid-summer may have their DNA extracts combined post-quantification. The principal investigator will contact NEON to discuss the DNA concentrations of duplicate sample pairs so that NEON can provide guidance on whether the duplicates should be pooled or if pools should also be concentrated. For those samples that NEON approves for pooling, the two extracts should be combined in a single well of a 96-well plate prior to DNA quantification of the pool following Section IV.D.

Individual or pooled extracts that NEON approves for concentrating prior to downstream analyses, will be concentrated using the Macherey-Nagel NucleoSpin® genomic DNA clean-up and concentration Kit (cat #740230) or equivalent NEON approved kit according to the manufacturer's instructions (Manual: User Manual NucleoSpin® gDNA Clean-up September 2023/ Rev.05). Following concentration, the extract will be quantified following Section IV.D.

V. Technical and QA Review

The internal technical review is conducted by qualified personnel not involved in conducting the work under review.

The internal quality assurance review will be conducted after the technical review.

Batch-level quality control criteria are outlined in Table 1 and sample-level quality control criteria are outlined in Table 2. Batch-level QAQC failures will not impact repeats performed for sample-level failures (e.g. if an entire extraction plate fails batch-level criteria, samples on the repeat extraction plate may still be re-extracted if they fail sample-level criteria).