NEON DNA Extraction Standard Operating Procedure v.6

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

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I. Version History

Version 6	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	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	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	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	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	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 PowerSoil Pro Kit in either 96-well plate or tube format, and from Sterivex water filters using the 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 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
X-Pierce Film	Sigma Aldrich	Z722502-100EA
ZymoBIOMICS Spike-in Control I (High Microbial Load)	Zymo Research	D6320

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 -60°C to -85°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. Positive Extraction Control (PEC)

The ZymoBIOMICS Spike-in Control I (High Microbial Load, Catalog # D6320) stored at -80°C when not in use. **This control is used for the soil and biofilm samples only; a PEC for filter samples has not yet been developed.** In preparation for extraction, thaw the standard completely. Mix thoroughly by vortexing to ensure there is no cellular aggregation. Once the PEC is aliquoted for extraction, return the standard to -80°C.

The Spike-in Control I is stored in DNA/RNA Shield, which is incompatible with the initial steps of the extraction kits. The DNA/RNA Shield is removed by centrifuging a 20 µL aliquot of the standard to produce a cell pellet and transferring the supernatant (containing the DNA/RNA Shield) to a new, clean tube. The pellet is used as the soil and biofilm PEC and is resuspended as described in their respective sections. The supernatant containing the DNA/RNA Shield may contain free DNA and will be added back to the PEC later in the DNA extraction workflow, as described in Section C.

2. 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.2.1. 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.2.2.

2.1. **96-Well Plate Extraction Format.** Disposable weighing funnels and scoopulas are autoclaved for 80 minutes to ensure sterility. In addition, the sealing film that covers the 96-well plate is UV-crosslinked for 5 minutes. A full extraction batch consists of 94 soil samples, one negative extraction control (NEC), and one PEC. For each extraction plate, well A1 is left empty as the NEC. Prior to loading soil into plate wells, the ZymoBIOMICS Spike-in Control I (High Microbial Load) standard with the DNA/RNA Shield removed is resuspended in 20 µL 1X Phosphate Buffered Saline (PBS) and added to well H12 as the PEC. A pierce-able sealing film is then applied to plates to prevent cross contamination. Next, 0.25 ± 0.03 g of thawed soil sample is weighed and placed into the appropriate well by using a sterilized disposable weighing funnel to pierce the seal. Each well is then individually sealed with a square of adhesive. After all the wells have been filled, the plate may be stored at 2-8°C for up to 48 hours if not immediately proceeding to Section IV.C.1.1.

2.2. **Individual Tube Extraction Format**. Disposable weighing funnels and scoopulas are autoclaved for 80 minutes to ensure sterility. Up to 24 soil samples may be included in one tube extraction batch. One NEC and one PEC will be prepared with each PowerSoil Pro Kit (each kit can extract approximately 50 samples). This results in one NEC and one PEC with approximately every two extraction batches using the same PowerSoil Pro Kit. Each extraction batch will contain up to 22 soil samples when both a PEC and NEC accompany 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 PEC will consist of the ZymoBIOMICS Spike-in Control I (High Microbial Load) standard with the DNA/RNA Shield removed and resuspended in 20 µL 1X PBS before being added to a 2 mL collection 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 proceeding to Section IV.C.1.2.

3. Soil Samples

3.1. **Biofilm**. Biofilm samples from sediment and from plant tissues (epiphyton, epipsammon, and epipelon sample collections) are extracted in tubes. Up to 24 biofilm samples are included in the same extraction batch. One NEC and one PEC will be prepared with each PowerBiofilm Kit (each kit can extract approximately 50 samples). This results in one NEC and one PEC with approximately every two extraction batches using the same PowerBiofilm Kit. Each extraction batch will contain up to 22 biofilm samples when both a PEC and NEC accompany the batch.

For sediment biofilm material, 0.20 ±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 0.10-0.25 g of the material into a 2 mL collection tube with an appropriate label. The biofilm PEC will consist of the ZymoBIOMICS Spike-in Control I (High Microbial Load) standard with the DNA/RNA Shield removed and resuspended in 350 µL MBL solution before being added to a 2 mL collection tube. 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.

3.2. **Filters**. **The filter PEC is in development and not currently implemented in this SOP version.** 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.

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 in 96-well plates using the Qiagen DNeasy 96 PowerSoil Pro Kit (cat #47017) according to the manufacturer's instructions (Manual: Qiagen_HB-2675-002_HB_DNeasy_PowerSoil_ProKit96_0721_WW) with the following change:

> At Step 12 (addition of CD3), add in the entire PEC supernatant containing the DNA/RNA Shield to the sample and continue with the subsequent steps.

Solution CD3 adjusts the salt concentration of the DNA solution to allow binding of DNA, but not non-DNA material that may still be present at low levels.

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 96-well plates using the Qiagen DNeasy 96 PowerSoil Pro Kit (cat #47017) according to the manufacturer's instructions (Manual: Qiagen_HB-2495-005_HB_DNY_PowerSoil_Pro_0321_WW) with the following change:

> At Step 7 (addition of CD3), add in the entire PEC supernatant containing the DNA/RNA Shield to the sample and continue with the subsequent steps.

Once extraction is complete, proceed to Section D.

1.3. Biofilm Samples

Genomic DNA (gDNA) from biofilm samples (epiphyton, epipsammon, epipelon) is extracted using the Qiagen DNeasy PowerBiofilm Kit (cat #24000-50) according to the manufacturer's instructions (Qiagen_HB-2274-002_HB_DNY PowerBiofilm_0120_WW) with the following change:

> At Step 9 (addition of Solution MR), add in the entire PEC supernatant containing the DNA/RNA Shield to the sample and continue with the subsequent steps.

Solution MR adjusts the salt concentration of the DNA solution to allow binding of DNA, but not non-DNA material that may still be present at low levels.

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.

D. Quantus dsDNA Assay

After extraction and isolation, the gDNA of each sample, including the PECs and NECs, is quantified using a Promega Quantus Fluorometer with a QuantiFluor ONE dsDNA Kit (#E4870) according to the manufacturer's instructions (Manual: Quantus_FluorometerManual_TM396_ rev 01/2020). DNA concentrations must meet the acceptance criteria of both Tables 1 and 2 to proceed to downstream analyses. Soil and biofilm samples that fail the sample-level QAQC criteria will be re-extracted one time starting in Section IV.B. Sterivex filter samples that fail the sample-level QAQC criteria will not be re-extracted as the entire sample is consumed during the initial extraction. See the corrective action in Table 2 for guidance on how to proceed with failed samples.

VI. 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).

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) – one per DNA extraction kit lot # Biofilm – one per DNA extraction kit lot # 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.
DNA concentration in Positive Extraction Control (PEC)	Soil (96-Well Plate Format)– one per 96-well extraction plate Soil (Individual Tube Format) – one per DNA extraction kit lot # Biofilm – one per DNA extraction kit lot # Sterivex Filters – not applicable	DNA concentration that represents at least 20% of manufacturer's expected yield given the elution volume used *Example: DNA concentration of at least 0.25 ng/µL if the expected yield is 1.25 ng/µL (125 ng in 100 µL elution volume)*	Suspected DNA extraction method failure. Halt processing the batch and contact NEON immediately to evaluate batch-level resolution. NEON must approve the resolution. Follow up with manufacturer if commercial standard fails to generate high-quality DNA of sufficient concentration to meet the NEON requirements.

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 PECs or NECs, which are evaluated using Table 1)	Per sample	≥1 ng/µL	If <1 ng/µL on initial extraction, 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.