Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-12

Isolating eDNA from Water

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APPROVAL PAGE

Revisions to an existing SOP, addition of a SOP change form, or preparation of a new SOP must be reviewed, approved, and signed by the following:

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DEFINITIONS AND ACRONYMS

ETL	Environmental Toxicology Laboratory (University of Saskatchewan)
DHSE	Department of Health Safety and Environment (University of Saskatchewan)
DQO	Data Quality Objective
QA	Quality Assurance
QC	Quality Control
QAPP	Quality Assurance Project Plan
SOP	Standard Operating Procedure
GWF	Global Water Futures
eDNA	Environmental DNA

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1.0 PURPOSE

The primary purpose of this SOP is to extract, purify and quantify environmental DNA (eDNA) from environmental water samples.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for eDNA isolation from water samples from the Global Water Futures (GWF) program titled "Next generation solutions to ensure healthy water resources for future generations" (eDNA project).

3.0 SAFETY CONSIDERATIONS

Biosafety training, laboratory training and medical monitoring requirements are described in the Health and Safety Plan for eDNA project of the GWF program. In addition, there are various safety concerns regarding the preparing, storage, and disposal of sample containers at the ETL. If biological spill happens, the appropriate personnel will be immediately notified and the Department of Health, Safety and Environment (DHSE) will be called in order to assess the hazard. DHSE will also be contacted in the case of chemical spills and will be responsible for the disposal of hazardous wastes.

3.1 PERSONAL PROTECTIVE EQUIPMENT

Personal protective equipment (PPE), consisting of nitrile gloves will be worn at all times when handling samples.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

- Freezer (-20 ^{o}C, -80 ^{o}C)
- Permanent markers
- Chain of custody forms
- Kim wipes
- Nitrile gloves
- Absolute ethanol
- Centrifuge for 50-ml tubes (≤ 4000 x g)

- Centrifuge for 15-ml tubes (≤ 4000 x g)
- Microcentrifuge (20,000 x g)
- Electronic scales (maximum measure: 1000 g) for balance of centrifuge
- 50-mL sterile Graduated Conical-Bottom Tubes with rack
- 2-mL sterile microcentrifuge tubes
- 1.5-mL sterile microcentrifuge tubes
- Adjustable Pipettes (1000-µl, 200-µl and 10-µl)
- Disposable aerosol filter pipette tips (1000-µl, 200-µl and 10-µl)
- Qiagen DNeasy® Blood & Tissue Kit
- ZYMO RESEARCH OneStep™ PCR Inhibitor Removal Kit
- Qiagen Proteinase K
- Roller
- Heater
- Biosafety cabinet
- Nanodrop
- Qubit V4
- Qubit® dsDNA HS Assay kit
- Qubit® assay tubes (500 tubes, Life Technologies, Cat. no. Q32856)
- Spray bottle with Invitrogen™ RNase Away™ Decontamination Reagent
- Spray bottle with 70% ethanol
- Spray bottle with 10% bleach
- Spray bottle with di Water

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

The following procedures describe methods for isolating eDNA in a stepwise fashion and explain the reasoning behind the techniques. Detailed below are the preparatory procedures, molecular techniques, and procedures for sample documentation and preservation.

5.1 Mobilization and Training

This section describes requirements for mobilization for the necessary lab-work, as well as the necessary safety training. The objective of this section is to ensure that all of the necessary preparatory work has been conducted to enable the successful completion of the overall project. Mobilization for the necessary lab-work entails maintenance and decontamination of equipment and biosafety training in accordance with the site Health and Safety Plan (HASP).

5.2 **Decontamination of instruments and laboratory areas before and after isolation**

A. Wipe down surfaces of all points of contact, including bench tops, pipettes, and fridge/freezer handles, using 10% bleach. Let bleach remain on surfaces for 15 minutes.
B. After 15 minutes, use a DI water-dampened paper towel to remove bleach residue.
C. Use a 70% alcohol dampened paper towel to help quickly dry the surfaces. **Note**: **For PCR PRE-station, DO NOT use buffers containing ethanol to clean the clear acrylic surface.**
D. If necessary, repeat the cleaning procedure from step B.
E. For biosafety cabinet and PCR PRE-station, turn on the UV-light for at least 15 min.
F. Decontaminate laboratory equipment during work using RNase AWAY™ Surface Decontaminant (Thermo Scientific™)
G. Wipe down surfaces of bench tops, pipettes, and racks using RNase AWAY™ Surface Decontaminant, then wipe dry with dust-free paper or rinse clean.

5.3 Isolating eDNA from water samples using Qiagen DNeasy Blood & Tissue kit

Notes before starting

- Redissolve any precipitates in Buffer AL and Buffer ATL.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen sample to room temperature.
- Preheat an incubator to 56°C.

1) Sample preparing

1a. Frozen membrane filters

Filter water samples using a filter funnel attached to a vacuum source following SOP: UofS-ETL-EDNA-01. Thaw the frozen membrane filter on ice. Go to step 2 of Section 5.2.

1b. Ethanol preserved membrane filters

Filter water samples using a filter funnel attached to a vacuum source following SOP: UofS-ETL-EDNA-01.

Centrifuge the 5 mL tube with ethanol preserved membrane filters at 4150 rpm for 15 min. Avoiding the pellet, carefully remove the supernatant. Go to step 2 of Section 5.2.

1c. Preparing water samples preserved with ethanol

a) Chill the 1-L PP bottle with preserved water in a -20 ^{o}C freezer;
b) Pre-chill the centrifuge for 50 mL tubes to -8 ^{o}C;
c) Invert 10 times to mix the chilled preserved water, aliquot 50 mL preserved water into a labeled 50 mL sterile tubes;

d) Centrifuge 4150 rpm for 60 min at -8 ^{o}C;
e) Avoiding the pellet, carefully discard the supernatant;
f) Repeat step 3) – 4) until all the preserved water has been processed;
NOTE: totally need 24 hours per batch; STOP POINT: keep samples in a freezer;
g) Add 50 mL absolute ethanol into each 1-L PP bottle, re-suspended and transfer to the correspondent 50 mL tube, centrifuge 4150 rpm for 60 min at -8 ^{o}C;
h) STOP POINT: After bulk sample participation, store the bulk sample in -80 ^{o}C before eDNA isolation;
Note: If the turbidity of water is high and the weight of pellet is more 0.5 g, use Qiagen DNeasy PowerSoil kit or PowerMax Soil kit to isolate the water eDNA depend on the weight of pellet, following the SOP: UofS-ETL-EDNA-13.

2) Add 900 µl ATL buffer into sample tube with pellet or membrane filter. Add 100 µl proteinase K. Incubate at 56°C on a roller (Speed: 10 rpm) in the incubator for 1 hour or until completely lysed.
3) Vortex 15 s. Add 1 mL Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56 °C for 10 min.
4) Add 1 mL ethanol (96–100%). Mix thoroughly by vortexing.
5) Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 13,000 x g for 1 min. Discard the flow-through and collection tube.
6) Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at 13,000 x g. Discard the flow-through and collection tube.
7) Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 1 min at 13,000 x g. Discard the flow-through.
8) Dry column. Centrifuge for 2 min at 16,000 x g.
9) Transfer the spin column to a new 1.5 mL microcentrifuge tube.
10) Elute the DNA by adding 50 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at 13,000 x g.
11) Repeat step 10 with another 50 µl Buffer AE for increased DNA yield.

5.4 Optional: remove contamination of eDNA

Note: If the eluted DNA solution is brown and contaminated with polyphenolic compounds, humic/fulvic acids, use the OneStep™ PCR Inhibitor Removal Kit to remove contaminants from eDNA.

12) Zymo-Spin™ III-HRC Columns need to be prepared prior to use:
    1) Insert column into a Collection Tube.

2) Open the cap, add 600 µl of Prep-Solution and centrifuge at 8,000 x g for 3 minutes.
3) Transfer the column to a clean1.5 ml microcentrifuge tube.
Note: Resin may appear dispersed or dislodged prior to column preparation. This is normal.

13) Inhibitor Removal:
Add 100 µl eDNA from step 11 to the prepared Zymo-Spin ™ III-HRC Column and centrifuge at 16,000 x g for 3 minutes. The filtered DNA is suitable for PCR, (RT), and other downstream applications.

5.5 Quality control of isolated eDNA

14) Purity check and quantify the isolated eDNA using NanoDrop 1000.
a. Turn on NanoDrop computer and open ND-1000 software.
b. Wipe NanoDrop pedestal with a clean Kimwipe.
c. In NanoDrop software, select Nucleic Acid.
d. Pipette 1.5 µl of sterile DNase-free water onto the pedestal of NanoDrop. Press OK. After calibrating, wipe NanoDrop pedestal with a clean Kimwipe.
e. Load 1.5 µl of elution buffer onto the pedestal of NanoDrop. Select "Blank". Waiting for Blank to be read (about 10 s).
f. Wipe NanoDrop pedestal with a clean Kimwipe.
g. Enter first sample and name. Apply 1.5 µl of eDNA sample onto the pedestal and select "Measure".
h. Repeat f-g for each sample.
i. Save file to an appropriate folder on the computer and print report.
j. Reapply 1.5 µl of sterile DNase-free water onto the pedestal and wipe pedestal with a clean Kimwipe.
k. Close NanoDrop.

15) Quantify the eDNA with low concentration (< 10 ng/µl) with Qubit® dsDNA HS Assay Kits.
a. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA HS Assay requires 2 standards.
b. Label the tube lids.
c. Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Note: Do not mix the working solution in a glass container.
d. Add 190 µl of Qubit® working solution to each of the tubes used for standards.
e. Add 10 µl of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
f. Add 198 µl Qubit® working solution to individual assay tubes.

g. Add 2 µl of each eDNA sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µl.
h. Allow all tubes to incubate at room temperature for 2 minutes.
i. Measure the concentration of eDNA samples using Qubit® 4.0 Fluorometer.
j. Transfer data file to an appropriate folder on the computer and print report.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve isolated eDNA sample under -80 ^{o}C for long-term storage. Sample management will also follow a scripted procedure (ETL-SOP-EDNA-09: Management of eDNA sample: Receiving, Preservation, Storage, Documentation, Decontamination, and Disposal) to ensure samples will not be lost or destroyed. The results of quality control of eDNA samples should be archived properly (hard-copy and electric copy).

7.0 RESPONSIBILITIES

Project Director — Will oversee and approve all project activities.

Project Manager — Will oversee and approve all project activities; review QA reports; approve final project QA needs; authorize necessary actions and adjustments to accomplish program QA objectives; and act as liaison between agencies, field staff.

Quality Assurance (QA) Manager — Will oversee all QA activities to ensure compliance with contract specifications; initiate audits on work completed by project personnel and subcontractors, including analytical laboratories and independent data validation contractors; review program QA activities, quality problems, and quality-related requests. In response to field and analytical findings, this person will approve the corrective actions. This person will report quality non-conformances to the Project Manager and review all pertinent portions of the deliverables before they are transmitted to ensure conformance with QA/QC procedures and quality work product.

Data Manager — Will oversee data management for this project. This person is responsible for the structure, organization, format, implementation, and operation of the project database.

Field Team Leader — Will oversee field activities and supervise the field crews. This person will ensure that proper sample collection, preservation, storage, transport, and COC QC procedures are followed. This person will inform the Project QA Manager

when field problems occur, and will communicate and document corrective actions taken. The Field Team Leader will discuss field activities with the Project Manager.

Laboratory Project Manager — The laboratory project manager for this project is the person responsible for assuring that the analysis of all samples is performed in accordance with the QAPP and the laboratory's quality assurance manual. In addition, the Laboratory Project Manager performs the final laboratory review of project data packages for completeness and compliance with project requirements.

8.0 REFERENCES

Qiagen. DNeasy® Blood & Tissue Handbook. 07/2006.
ZYMO RESEARCH CORP. OneStep™ PCR Inhibitor Removal Kit manual. Version 2.0.1.
Thermo Scientific. NanoDrop 1000 Spectrophotometer User's Manual. Rev. V3.8
Thermo Scientific. Qubit™ 4 Fluorometer manual. Pub. No. MAN0017209. Rev. B.0.
Thermo Scientific. Qubit® dsDNA HS Assay Kits manual. Pub. No. MAN0002326. Rev. B.0.