

Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-15

Isolating eRNA from Sediments

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

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1.0 PURPOSE

The primary purpose of this SOP is to extract, purify and quantify environmental RNA (eRNA) from environmental sediment samples.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for eRNA isolation from sediment 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 °C, -80 °C)
- Permanent markers
- Chain of custody forms
- Kim wipes
- Nitrile gloves
- Absolute ethanol
- Centrifuge for 50 ml tubes ($\leq 4000 \times g$)

- Centrifuge for 15 ml tubes ($\leq 4000 \times g$)
- Microcentrifuge (20,000 $\times g$)
- 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)
- Phenol/chloroform/isoamyl alcohol solution
- RNeasy® PowerSoil® Total RNA Kit
- RNeasy PowerSoil DNA Elution Kit
- Vortex Adapter for four 15-ml tubes
- Heater
- Biosafety cabinet
- Nanodrop
- Qubit V4
- Qubit® RNA HS Assay Kits
- Qubit™ RNA IQ 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 eRNA 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 eRNA from sediment using the RNeasy PowerSoil Total RNA Kit

Notes before starting

- The sediment samples should be preserved in LifeGuard Solution.
- Equilibrate frozen sample to room temperature.
- Perform all centrifuge steps at room temperature (15-25 °C).
- If Solution IRS has precipitated, heat at 60°C until precipitate dissolves
- Wear RNase-free gloves at all times and remove RNase from the work area.
- Preheat an incubator to 45 °C.
 - 1) Add up to 2 g of sediment sample to the 15 ml PowerBead Tube (provided).
 - 2) Add 2.5 ml of PowerBead Solution, 0.25 ml of Solution SR1 and 0.8 ml of Solution IRS.
 - 3) Add 3.5 ml of phenol/chloroform/isoamyl alcohol (pH 6.5–8.0, [User supplied]). Cap and vortex the PowerBead Tube to mix until the biphasic layer disappears.
 - 4) Place the PowerBead Tube on a 15 mL-Vortex Adapter and vortex at maximum speed for 15 min.
 - 5) Remove the PowerBead Tube and centrifuge at 2500 x g for 10 min.
 - 6) Transfer the upper aqueous phase (avoid the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided). Discard the phenol/chloroform/isoamyl alcohol. Note: The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.
 - 7) Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 2–8 °C for 10 min and then centrifuge at 2,500 x g for 10 min at room temperature.

- 8) Transfer the supernatant, without disturbing the pellet (if there is one), to a new 15 ml Collection Tube (provided).
- 9) Add 5 ml of Solution SR4 to the supernatant in the Collection Tube and invert or vortex to mix. Incubate at -20°C for 30 min.
- 10) Centrifuge at $2500 \times g$ for 30 min.
- 11) Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 min.
- 12) Shake Solution SR5 to mix and add 1 ml to the 15 ml Collection Tube. Resuspend the pellet completely by repeatedly pipetting or vortexing. Note: If the pellet is difficult to resuspend, place the tube in a heat block or water bath at 45°C for 10 min, followed by vortexing. Repeat until the pellet is resuspended.
- 13) Prepare one JetStar Mini Column (provided) for each RNA isolation sample:
 - 13)-1 Remove the cap of a 15 ml Collection Tube (provided) and place the JetStar Mini Column inside it. The column will hang in the Collection Tube.
 - 13)-2 Add 2 ml of Solution SR5 to the JetStar Mini Column. Allow it to completely gravity flow through the column and collect in the 15 ml Collection Tube.Note: Do not allow the column to dry out before loading the RNA isolation sample.
- 14) Add the RNA isolation sample from Step 12 onto the JetStar Mini Column and allow it to gravity flow through the column into the 15 ml Collection Tube.
- 15) Add 1 ml of Solution SR5 to the JetStar Mini Column and allow it to completely gravity flow into the 15 ml Collection Tube.
- 16) Transfer the JetStar Mini Column to a new 15 ml Collection Tube (provided). Shake Solution SR6 to mix and then add 1 ml to the JetStar Mini Column to elute the bound RNA. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube.
- 17) Transfer the eluted RNA to a 2.2 ml Collection Tube (provided). Add 1 ml of Solution SR4. Invert at least once to mix and incubate at -15°C to -30°C for a minimum of 10 min.
- 18) Centrifuge the 2.2 ml Collection Tube at $13,000 \times g$ for 15 min to pellet the RNA.
- 19) Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the pellet.
- 20) Resuspend the RNA pellet in $100 \mu\text{l}$ of Solution SR7.

5.4 Optional: Co-isolating eDNA from the JetStar Mini Columnst

Note: After RNA is eluted from the JetStar Mini Columns, DNA may also be eluted from the same columns using the RNeasy PowerSoil DNA Elution Kit.

- 21) Transfer the JetStar Mini Column from step 16 of the RNeasy PowerSoil Total RNA Kit to a 15 ml Collection Tube (provided).
- 22) Add 1 ml of Solution SR8 to the JetStar Mini Column to elute the bound DNA into the 15 ml Collection Tube.

- 23) Transfer the eluted DNA to a 2.2 ml Collection Tube (provided) and add 1 ml of Solution SR4. Invert at least once to mix and incubate at -15°C to -30°C for 10 min.
- 24) Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 min at room temperature to pellet the DNA.
- 25) Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the DNA pellet.
- 26) Resuspend the DNA pellet in 100 μl of Solution SR7.
Note: When the absence of RNA contamination is critical, an RNase treatment of the isolated DNA is recommended. Follow UosS-ETL-EDNA-13 to proceed
Quality control of isolated eDNA from sediments.

5.5 Quality control of isolated eRNA

- 27) Purity check and quantify the isolated eRNA 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, RNA-40 mode.
 - 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 eRNA 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.
- 28) Quantify the eRNA with low concentration ($< 10 \text{ ng}/\mu\text{l}$) with Qubit[®] RNA HS Assay Kits.
 - a. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit[®] RNA HS Assay requires 2 standards.
 - b. Label the tube lids.
 - c. Prepare the Qubit[®] working solution by diluting the Qubit[®] RNA HS Reagent 1:200 in Qubit[®] RNA 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 eRNA 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 eRNA samples using Qubit[®] 4.0 Fluorometer.
 - j. Transfer data file to an appropriate folder on the computer and print report.
- 29) Check the degradation of eRNA sample using the Qubit[™] RNA IQ Assay Kit
- a. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit[®] RNA IQ Assay requires 3 standards.
 - b. Label the tube lids.
 - c. Prepare the Qubit[®] working solution by diluting the Qubit[®] RNA IQ Reagent 1:200 in Qubit[®] RNA IQ 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 eRNA 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 eRNA samples using Qubit[®] 4.0 Fluorometer.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve isolated eRNA sample under -80 °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. RNeasy® PowerSoil® Total RNA Kit Handbook. June 2017.
- Qiagen. RNeasy PowerSoil DNA Elution Kit Handbook. May 2017.
- Qiagen LifeGuard® Soil Preservation Handbook. May 2017.
- 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® RNA HS Assay Kits manual. Pub. No. MAN0002327. Rev. A.0.
- Thermo Scientific. Qubit® RNA IQ Assay Kits manual. Pub. No. MAN0017405. Rev. B.0.