Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-27

Co-isolation of eDNA/eRNA from Sediment

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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:

Authored By: Yuwei Xie and John P. Giesy Date: 09/12/2018

Supervisor Review By: John P. Giesy Date: 09/19/2018

Reviewed By: _____ Date: _____
(QA Coordinator)

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 co-extract, purify and quantify environmental DNA and RNA from sediments. This protocol also works for co-isolation eDNA/eRNA from soil.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for co-isolation of eDNA/eRNA from sediment 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 ºC, -80 ºC)
- Permanent markers
- Chain of custody forms
- Kim wipes
- Absolute ethanol
- Microcentrifuge (16,000 x g)

- Vortex-Genie 2 Vortex
- Vortex Adapter for 15-mL tubes
- 2 mL sterile microcentrifuge tubes (DNase/RNase free)
- Serological pipettes (1 ml and 10 ml)
- 1.5 mL sterile microcentrifuge tubes (DNase/RNase free)
- 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
- 70% ethanol
- Dry heat block
- Biosafety cabinet
- Nanodrop 1000
- Qiagen® RNeasy PowerSoil Total RNA kit
- Qiagen® RNeasy PowerSoil DNA Elution kit
- DNase I stock solution (Dissolve 1500 Kunitz units solid DNase I in 550 µl of the RNase-free water)
- 14.3 M β-mercaptoethanol (β-ME)
- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- Centrifuge (with Swing Rotor for 15 ml tubes, 4000 g RCF available)
- Vortex with 5-mL tube adaptor
- RNase free 1.4-mm Ceramic beads
- RNase free 2.8-mm Ceramic beads
- Qiagen Tissue Lyser II
- 96–100% ethanol
- 70% ethanol in water
- Disposable gloves
- 5% (w/v) sodium dodecyl sulfate (SDS)
- Qubit V4
- Qubit® DNA HS Assay Kits
- 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 co-isolating eDNA/eRNA from sediment in a stepwise fashion and explain the reasoning behind the techniques. Qiagen RNeasy PowerSoil Total RNA Kit and RNeasy PowerSoil DNA Elution Kit are used for the co-isolation. 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 clear, acrylic surface.**
D. If necessary, repeat the cleaning procedure from step B.
E. For the 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 Co-isolation of eDNA/eRNA/eProtein from membrane filter Using Qiagen RNeasy PowerSoil Total RNA Kit and DNA Elution Kit.

Notes before starting

- If Solution IRS has precipitated, heat at 60 °C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15 – 25 °C).
- Wear RNase-free gloves at all times and remove RNase from the work area.
- Preparing Phenol/Chloroform/Isoamyl Alcohol Solution

Mix 25 parts phenol, 24 parts chloroform, and one part isoamyl alcohol. This solution can be stored under TE buffer (10mM Tris, 1mM EDTA, pH 8.0) or 0.1M Tris, pH 8.0, for periods up to 3 months at 2 – 8°C. Store in an amber bottle to protect from light. If storing under TE Buffer, we recommend you add a small volume of Tris buffer.

Note: At least one negative control should be conducted during isolation.

Extraction of total nucleic acid

1) Thaw the sediment sample preserved in LifeGuard Soil Preservation Solution on ice, centrifuge sample at 4000 x g for 5 min to collect the sediment. Remove the LifeGuard Solution from the tube.
2) Aliquot up to 1 ~ 2 g of Sediment to the 15-mL PowerBead Tube (provided).
3) Add 2.5 ml of PowerBead Solution, 0.25 ml of Solution SR1 and 0.8 ml of Solution IRS.
4) Add 3.5 ml of the prepared phenol/chloroform/isoamyl alcohol (pH 6.5–8.0). Cap and vortex the PowerBead Tube to mix until the biphasic layer disappears.
5) Place the PowerBead Tube on a Vortex Adapter and vortex at full speed for 15 min.
6) Remove the PowerBead Tube and centrifuge at 4000 x g for 10 min.
7) 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.
8) Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4 °C for 10 min and then centrifuge at 2,500 x g for 10 min at room temperature.
9) Transfer the supernatant, without disturbing the pellet (if there is one), to a new 15-mL Collection Tube (provided).
10) Add 5 ml of Solution SR4 to the supernatant in the Collection Tube and invert or vortex to mix. Incubate at room temperature for 30 min.
11) Centrifuge at 2500 x g for 30 min.
12) Decant the supernatant and invert the 15-mL Collection Tube on a paper towel for 5 min.
13) 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.

Purification of total eRNA

14) Prepare one JetStar Mini Column (provided) for each RNA isolation sample:
    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.

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.

15) 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.
16) Add 1 ml of Solution SR5 to the JetStar Mini Column and allow it to completely gravity flow into the 15 ml Collection Tube.
17) 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.
18) Transfer the JetStar Mini Column from step 16) of the RNeasy PowerSoil Total RNA Kit to a 15 ml Collection Tube (provided) for eDNA co-isolation from step 22) to 26).
19) 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.
20) Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 min to pellet the RNA.
21) Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the pellet.
22) Resuspend the RNA pellet in 100 µl of Solution SR7.

Total eRNA Cleanup using Qiagen RNeasy Mini Kit

23) Add 350 µl Buffer RLT, and mix well.
24) Add 250 µl ethanol (96–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed next step immediately.
25) Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 16,000 x g. Discard the flow-through.
26) Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g to wash the spin column membrane. Discard the flow-through.
27) Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
28) Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30 °C) for 15 min.
29) Add 500 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g. Discard the flow-through.
30) Repeat step 29) once.
31) Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 16,000 x g to wash the spin column membrane. Discard the flow-through.

32) Repeat step 31) once.
33) Place the RNeasy spin column back to the used 2 ml collection tube. Centrifuge at full speed for 3 min to dry the column.
34) Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 50 µl RNase-free water directly to the spin column membrane. Centrifuge for 1 min at 16,000 x g to elute the RNA.
35) Store purified eRNA at –80 °C after quality check.

Purification of total eDNA

36) Add 1 ml of Solution SR8 to the JetStar Mini Column (from step 17) to elute the bound DNA into the 15 ml Collection Tube. Allow Solution SR8 to gravity flow into the Collection Tube.
37) 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.
38) Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 min at room temperature to pellet the DNA.
39) Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 min to air dry the DNA pellet.
40) Resuspend the DNA pellet in 100 µl of Solution SR7. Store purified eDNA at – 20°C after QC.

5.4 Quality control of isolated eRNA

5.4.1 Purity check and quantify the isolated eRNA using NanoDrop 1000

41) Turn on NanoDrop computer and open ND-1000 software.
42) Wipe NanoDrop pedestal with a clean Kimwipe.
43) In NanoDrop software, select Nucleic Acid, RNA-40 mode.
44) 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.
45) Load 1.5 µl of elution buffer onto the pedestal of NanoDrop. Select “Blank”. Waiting for Blank to be read (about 10 s).
46) Wipe NanoDrop pedestal with a clean Kimwipe.
47) Enter first sample and name. Apply 1.5 µl of eRNA sample onto the pedestal and select “Measure”.
48) Save file to an appropriate folder on the computer and print report.
49) Reapply 1.5 µl of sterile DNase-free water onto the pedestal and wipe pedestal with a clean Kimwipe.
50) Close NanoDrop.

5.4.2 Quantify the eRNA with low concentration (< 10 ng/µl) with Qubit® RNA HS Assay Kits

51) Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® RNA HS Assay requires 2 standards.
52) Label the tube lids.
53) 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.
54) Add 190 µl of Qubit® working solution to each of the tubes used for standards.
55) Add 10 µl of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
56) Add 198 µl Qubit® working solution to individual assay tubes.
57) 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.
58) Allow all tubes to incubate at room temperature for 2 minutes.
59) Measure the concentration of eRNA samples using Qubit® 4.0 Fluorometer.
60) Transfer data file to an appropriate folder on the computer and print report.

5.4.3 Check the degradation of eRNA sample using the Qubit™ RNA IQ Assay Kit

61) Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® RNA IQ Assay requires 3 standards.
62) Label the tube lids.
63) Prepare the Qubit® working solution by diluting the Qubit® eRNA 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.
64) Add 190 µl of Qubit® working solution to each of the tubes used for standards.
65) Add 10 µl of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
66) Add 198 µl Qubit® working solution to individual assay tubes.
67) 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.
68) Allow all tubes to incubate at room temperature for 2 minutes.
69) Measure the concentration of eRNA samples using Qubit® 4.0 Fluorometer.

5.5 Quality control of isolated DNA

5.5.1 Purity check and quantify the isolated DNA using NanoDrop 1000

70) Turn on NanoDrop computer and open ND-1000 software.
71) Wipe NanoDrop pedestal with a clean Kimwipe.

72) In NanoDrop software, select Nucleic Acid.
73) 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.
74) Load 1.5 µl of elution buffer onto the pedestal of NanoDrop. Select "Blank". Waiting for Blank to be read (about 10 s).
75) Wipe NanoDrop pedestal with a clean Kimwipe.
76) Enter first sample and name. Apply 1.5 µl of DNA sample onto the pedestal and select "Measure".
77) Save file to an appropriate folder on the computer and print report.
78) Reapply 1.5 µl of sterile DNase-free water onto the pedestal and wipe pedestal with a clean Kimwipe.
79) Close NanoDrop.

5.5.2 Quantify the DNA with low concentration (< 10 ng/µl) with Qubit® dsDNA HS Assay Kits

80) Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA HS Assay requires 2 standards.
81) Label the tube lids.
82) 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.
83) Add 190 µl of Qubit® working solution to each of the tubes used for standards.
84) Add 10 µl of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
85) Add 198 µl Qubit® working solution to individual assay tubes.
86) 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.
87) Allow all tubes to incubate at room temperature for 2 minutes.
88) Measure the concentration of eDNA samples using Qubit® 4.0 Fluorometer.
89) Transfer data file to an appropriate folder on the computer and print report.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve isolated DNA/RNA/protein sample under -80 ºC 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

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MO BIO. PowerWater® RNA Isolation Kit. Version: 05302013.
Qiagen. LifeGuard® Soil Preservation Handbook. 25/2017.
Qiagen. TissueLyser Handbook. Ocectmober 2010, 2nd edition.
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.
Thermo Scientific. Qubit® Protein Assay Kits manual. Pub. No. MAN0002349. Rev. A.0.