Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

U of S-ETL-EDNA-14

Isolating eRNA 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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Supervisor Review By: _____ Date: 04/30/2018___

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 extract, purify and quantify environmental RNA (eRNA) from environmental water samples.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for eRNA 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 15 ml tubes (≤ 4000 x g)

- Microcentrifuge (20,000 x g)
- 15 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 RNeasy® Mini Kit
- ZYMO RESEARCH OneStep™ PCR Inhibitor Removal Kit
- 70% ethanol (Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- Vortex Adapter for 6 (5 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 water samples using Qiagen RNeasy Mini kit

Notes before starting

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Add either 10 µl β-mercaptoethanol (β-ME) to 1 ml Buffer RLT. Buffer RLT with β-ME or DTT can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.
  1) Centrifuge membrane filter in 5-mL tube at 4000 x g for 5 min to collect the particles. Remove the LifeGuard Solution from the tube.
  2) Add 1,000 µl RLT buffer into sample tube with pellet or membrane filter. Add 2 2.8-mm and 5 1.4-mm Zirconium Ceramic Oxide Beads in to the 5-mL tube.
  3) Disruption and homogenization using the 5-ml Vortex adaptor at maximum speed for 5 min.
  4) Centrifuge the tubes ≤ 4000 x g for 1 min.

5) Transfer all the supernatant to a clean 2 ml Collection Tube (provided). Draw up the supernatant using a 1 ml pipette tip by placing it down into the beads.
6) Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.
7) Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.
8) Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 13,000 x g. Discard the flow-through. Reuse the collection tube in step 7). If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
9) Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 13,000 x g to wash the spin column membrane. Discard the flow-through.
10) Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 13,000 x g to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.
11) Repeat step 10) once. Reuse the collection tube in step 10.
12) Centrifuge for 3 min at 16,000 x g to dry the spin column membrane.
13) 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. Close the lid gently, and centrifuge for 1 min at 16,000 x g to elute the RNA.

5.4 Optional: remove contamination of eRNA

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

14) 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.
15) Inhibitor Removal:
    Add 50 µl eRNA from step 13) to the prepared Zymo-Spin ™ III-HRC Column and centrifuge at 16,000 x g for 3 minutes. The filtered RNA is suitable for PCR, (RT), and other downstream applications.

5.5 Quality control of isolated eRNA

1) 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.
2) Quantify the eRNA with low concentration (< 10 ng/µl) 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.
3) 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 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

Qiagen. RNeasy® Mini Handbook. June 2012.
Qiagen LifeQuard® Soil Preservation Handbook. May 2017.
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® 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.