Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-16

First strand cDNA synthesis of eRNA

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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)

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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

PCR Polymerase chain reaction

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1.0 PURPOSE

This SOP is developed for first strand cDNA synthesis and quantification of environmental RNA (eRNA).

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for eDNA metabarcoding 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
- Kim wipes
- Nitrile gloves
- Absolute ethanol
- Centrifuge for 0.2-mL 96-well PCR plate (≤ 4000 x g)
- Microcentrifuge (20,000 x g)
- PCR thermal cycler

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- 15-mL sterile Graduated Conical-Bottom Tubes with rack
- 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)
- PCR PRE-station
- Qubit V4
- Qubit® ssDNA 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
- Invitrogen ezDNase (Catalog Number 11766051)
- Invitrogen SuperScript™ IV First-Strand Synthesis System

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

The following procedures describe methods for synthesis and quantification of cDNA of 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.

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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 **First strand cDNA synthesis for eRNA samples**

This section is developed for cDNA synthesis for eRNA samples, while for eDNA samples, directly proceeded to 5.4.

A. Removal of contaminating double-stranded DNA from RNA preparations
  a. Mix the following components in a 0.2-mL RNase-free tube.

Component	*Volume*
10X ezDNase Buffer	1 µL
ezDNase	1 µL
Template RNA	up to 8 µL
Nuclease-free Water	to 10 µL
Total volume	10 µL

  b. Gently mix the samples then centrifuge the tube.
  c. Incubate the sample for 5 minutes at 37°C on a thermocycler.
  d. Chill the tube on ice to bring the sample to room temperature, then briefly centrifuge and place the tube on ice.

B. First-strand cDNA synthesis
  a. Mix the following components in a 0.2-mL RNase-free tube for primer annealing

Component	*20-µL rxn*	*Final Conc.*
DEPC-treated water	1.0 µL	N/A
10 mM dNTP mix (10 mM each)	1.0 µL	0.5 mM each
50 ng/µL random hexamers	1.0 µL	2.5 ng/µL
Template RNA decontaminated from 5.3 A	10 µL	< 5 µg total RNA

  Mix and briefly centrifuge the components.
  Heat the RNA-primer mix at 65°C for 5 minutes, and then incubate on ice for at least 1 minute.

  b. Vortex and briefly centrifuge the 5× SSIV Buffer.
  c. Prepare a RT reaction mix following the receipt below in a 1.5 mL-tube.

Component	*20-µL rxn*	*Final Conc.*
5× SSIV Buffer	4.0 µL	1 ×
100 mM DTT	1.0 µL	5 mM

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Ribonuclease Inhibitor (40 U/µL)	1.0 µL	2.0 U/µL
SuperScript™ IV Reverse Transcriptase (200 U/µL)	1.0 µL	10 U/µL

d. Cap the tube, mix, and then briefly centrifuge the contents.
e. Add 7.0 µL RT reaction mix to each annealed RNA.
f. Run RT program on a thermocycler.
23°C for 10 minutes; 55°C for 10 minutes; 80°C for 10 minutes.
g. Optional: Remove RNA
Note: Amplification of some PCR targets (>1 kb) may require removal of RNA. To remove RNA, add 1 µL E. coli RNase H, and incubate 37°C for 20 minutes.
h. Use your RT reaction immediately for PCR amplification or store it at –20 °C.
i. Quality check of the cDNA using Nanodrop 1000 or Qubit ssDNA Assay kit (Appendix II).

5.4 Quantify the cDNA

5.5.1 Quality check of the nucleic acids 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. Check the appropriate setting for the sample type.
   a) Note: The default setting is DNA-50 for dsDNA, ssDNA-33 for cDNA and RNA-40 for RNA.
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 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.

5.5.2 Quantify the ssDNA with low concentration (< 10 ng/µl) with Qubit® ssDNA Assay Kit

A. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® ssDNA Assay requires 2 standards.
B. Label the tube lids.
C. Prepare the Qubit® working solution by diluting the Qubit® ssDNA 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.

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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 cDNA 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 cDNA 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

The product of the first strand cDNA synthesis can be used directly in PCR and qPCR or stored at −20 °C for up to one week. For longer storage, −70 °C is recommended. Avoid freeze/thaw cycles of cDNA. 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.

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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

Invitrogen ezDNase Product Information Sheet. Pub. No. MAN0015899. Rev. A.0.
Invitrogen SuperScript™ IV First-Strand Synthesis System User Guide. Pub. no. MAN0013442 Rev. B.0.
Thermo Scientific. Qubit™ 4 Fluorometer manual. Pub. No. MAN0017209. Rev. B.0.
Thermo Scientific. Qubit® ssDNA Assay Kit. MAN0001988. Rev. A.0.

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