Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-24

Quality-check, cleaning, quantification and pooling of PCR products

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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: 04/12/2018

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
PCR	Polymerase chain reaction

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1.0 PURPOSE

This SOP is developed for quality-checking, cleaning, quantification and pooling for environmental DNA (eDNA) metabarcoding of multiple communities from microbiota to macrobiota.

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 ^{o}C, -80 ^{o}C)
- 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
- 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® 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 validating, cleaning and pooling PCR products 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**

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 **Quality check of PCR products using agarose gel electrophoresis**

A. Remove 96 well plate from thermocycler and centrifuge to collect liquid at the bottom of the wells.
B. Combine the triplicate PCR products into a new 96-well plate.
C. Prepare 1.2% (wt./vol.) agarose gel for electrophoresis (1.2 g agarose in 100 mL 1X TAE), after boiling, add 4 ul SYBR safe per 100 mL gel.
D. Load 5 µl PCR product onto a 1.2% gel, at one 5 µl 100-bp DNA ladder for each row.
E. Run electrophoresis for approximately 40 minutes at 120 V or until the PCR products run far enough into the gel to confirm that the PCR was successful.
F. Validate the NTC results to confirm that there was no contamination.

5.4 **PCR cleaning**

A. Determine the volume of each PCR reaction.
B. Clean the PCR products using the Omega bio-tech@ E-Z96 Cycle-Pure Kit and QIAvac 96 system.
C. Prepare the QIAvac 96 with an E-Z 96-well plate, placing the waste tray inside the QIAvac base and the QIAvac top plate squarely over the base. Attach the QIAvac to a vacuum source. Seal unused wells of the E-Z 96-well plate with sealing film, and place the EZ 96-well plate securely in the QIAvac top plate.
D. Add 3 volumes CP Buffer to 1 volume of the PCR sample and mix thoroughly. Apply the samples to the wells of the E-Z 96-well plate. Switch on vacuum.
E. After all liquid has passed through the membrane, switch off vacuum. Wash the E-Z 96-well plate by adding 800 µl DNA Wash Buffer to each well and switch on vacuum to draw the buffer through the plate. Note: Add ethanol (96–100%) to DNA wash Buffer concentrate before first use (see bottle label for volume).
F. Repeat step 5.8-C.
G. Switch off vacuum and ventilate the QIAvac 96 slowly. Carefully transfer the EZ 96-well plate onto a new 96-well Square-well well plate. Seal the wells with a

piece of AirPore Tape Sheet. Centrifuge the EZ 96-well plate/deep well plate assembly at 4,000 x g for 10 min to dry the columns.

H. Place the EZ 96-well plate on top of a 96-well Microplate.
I. To elute, add 60 µl Elution Buffer directly onto the center of each well of the EZ 96-well plate, incubate for 2 min at room temperature. Centrifuge the EZ 96-well plate/96-well plate assembly at 4,000 x g for 5 min to elute the cleaned products.
J. Quantify the PCR products or store it at -20 °C.

5.5 **Quantify the cleaned PCR products using 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 DNA 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 DNA samples using Qubit® 4.0 Fluorometer.
J. Transfer data file to an appropriate folder on the computer and print report.

5.6 **Polling the PCR products for library construction of next-generation sequencing**

A. Calculate the aliquot volume for each PCR product in excel using the formula below:
$$\text{Volume}_{i\text{th sample}} = \text{Min(Concentration)} * (\text{Volume}_{\text{Elution}} - 3) / \text{Concentration}_{i\text{th sample}}$$
B. Aliquot the calculated volume of each samples into one 2 mL tube. Mix thoroughly.
C. Quantify the pooled sample using Qubit HS dsDNA kit following Section 5.5.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve cleaned PCR products and pooled products under -70 ^{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

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 Phusion Green High-Fidelity DNA Polymerase User Guide. Pub. No. MAN0012395. Rev. Date 23 September 2016 (Rev. B.00).
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.