Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-33

Amplicon Next-generation sequencing by use of Illumina NextSeq 500 platform

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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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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

PCR Polymerase chain reaction

NGS Next-generation sequencing

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1.0 PURPOSE

This SOP is developed for next-generation sequencing of eDNA metabarcoding libraries. This SOP is developed for NextSeq 500 sequencer with high output sequencing chemistry kit. Length of sequencing library should be shorter than 200 base pairs.

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
- 50 mL sterile Graduated Conical-Bottom Tubes with rack

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- 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)
- Spray bottle with 70% ethanol
- Spray bottle with 10% bleach
- Spray bottle with di-Water
- Illumina NextSeq 500
- NextSeq 500/550 High Output Kit v2.5 (300 Cycles)

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

The following procedures describe methods for NGS of eDNA metabarcoding library 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.

5.3 **Perform a Manual Quick Wash before sequencing (20 minutes)**

Flushes the system with a user-supplied wash solution of laboratory-grade water and Tween 20 (buffer wash cartridge).

A. Prepare fresh wash solution (0.05% Tween 20): Combine 100% Tween 20 (20 µl) and Laboratory-grade water (40 ml) in a new 50-mL falcon tube; Invert five times to mix.

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B. Add 40 ml wash solution to the center reservoir of the buffer wash cartridge.
C. select **Start Wash** of Sequencing Control Software.
D. Select **Perform Wash**, and then select **Quick Wash**.
E. If a used flow cell is not present, load a used flow cell. Select Load, and then select **Next**.
F. Remove the spent reagents container and discard the contents in accordance with applicable standards.
G. Slide the empty spent reagents container into the buffer compartment until it stops.
H. Remove the used buffer cartridge from the previous run, if present.
I. Load the buffer wash cartridge containing wash solution.
J. Remove the used reagent cartridge from the previous run, if present.
K. Load the reagent wash cartridge.
L. Select **Next**. The prewash check begins automatically
M. Select **Start**.
N. When the wash is complete, select **Home**.
O. After the wash, the sippers remain in the down position to prevent air from entering the system. Leave the cartridges in place until the next run.

5.4 **Create a run in the Local Run Manager software module**

A. From the home screen, select **Edit Runs**
B. Select **Create Run** from the Local Run Manager dashboard
C. Enter a run name, enter samples for the run, and if applicable, import manifests
D. Save the run and close the Local Run Manager dashboard window

5.5 **Prepare the Reagent Cartridge (60 minutes)**

A. Remove the reagent cartridge from -25°C to -15°C storage.
B. Thaw in a room temperature water bath until thawed (~60 minutes). Do not submerge the cartridge.
C. Gently tap on the bench to dislodge water from the base, and then dry the base.
D. Invert the cartridge five times to mix reagents.
E. Inspect positions 29, 30, 31, and 32 to make sure that reagents are thawed.
F. Gently tap on the bench to reduce air bubbles.

5.6 **Prepare the Flow Cell**

A. Remove a new flow cell package from 2°C to 8°C storage.
B. Set the unwrapped flow cell package aside at room temperature for 30 minutes.
Note: If the foil package is intact, the flow cell can remain at room temperature up to 12 hours. Avoid repeated cooling and warming of the flow cell.
C. Remove the flow cell from the foil package.
D. Open the clear plastic clamshell package and remove the flow cell.
E. Clean the glass surface of the flow cell with a lint-free alcohol wipe. Dry the glass with a low-lint lab tissue.

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5.7 **Prepare Sequencing Libraries (Denature and diluter protocol)**

A. Prepare a Fresh Dilution of NaOH:
   Prepare **1N NaOH**: combine 900 µl diH$_2$O and 100 µl **10N-NaOH** and mix
   Prepare **0.2N NaOH**: combine 800 µl diH$_2$O and 200 µl **1N-NaOH** and mix
   Note: Use the fresh dilution within 12 hours
B. Thaw HT1 and sit HT1 on ice.
C. Thaw RSB solution and sit RSB on ice.
   Note: RSB can be replaced by 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20.
D. Denature a 4-nM Library
   a. Dilute sequencing library to 4 nM
   b. Combine 4 nM library (5 µl) and 0.2N NaOH (5 µl) into a new microcentrifuge tube.
   c. Vortex briefly and then centrifuge at 280 × g for 1 minute.
   d. Incubate at room temperature for 5 minutes.
   e. Add 5 µl 200 mM Tris-HCl solution, pH 7
   f. Vortex briefly and then centrifuge at 280 × g for 1 minute.
   g. Add 985 µl prechilled HT1 to the microcentrifuge tube containing denatured library. The result is 1 ml of a 20 pM denatured library.
   h. Vortex briefly and then centrifuge at 280 × g for 1 minute.
   i. Place the 20 pM libraries on ice until you are ready to proceed to final dilution.
D. Dilute Denatured 20 pM Library (1.3 mL) to 1.8 pM for high throughout kit
   Combine 117 µl 20 pM denatured library and 1183 µl prechilled HT1.
   Invert to mix and then pulse centrifuge.
E. Denature PhiX Control
   a. Determine the concentration of PhiX Control with Qubit
   b. Diluter PhiX to 4 nM with RSB buffer. 25 µl diluted PhiX is needed for library loading.
   c. Vortex briefly and then pulse centrifuge.
   d. Combine 4 nM PhiX (5 µl) and fresh 0.2N NaOH (5 µl) into a new microcentrifuge tube.
   e. Vortex briefly and then pulse centrifuge.
   f. Incubate at room temperature for 5 minutes.
   g. Add 5 µl 200 mM Tris-HCl, pH 7.0.
   h. Vortex briefly, and then centrifuge at 280 × g for 1 minute.
F. Dilute Denatured PhiX to 20 pM. Add 985 µl prechilled HT1 to the denatured PhiX library. The result is 1 ml of a 20 pM denatured PhiX library. Invert to mix.
G. Dilute the denatured 20 pM PhiX to 1.8 pM: combine Denatured PhiX (117 µl) and Prechilled HT1 (1183 µl) into a new microcentrifuge tube. The result is 1.3 ml of a 1.8 pM denatured PhiX library. Invert to mix and then centrifuge at 280 × g for 1 minute.

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G. Combine Library and PhiX Control (~ 10% Spike-In). Combine 130 µl 1.8-pM PhiX library and 1170 µl 1.8-pM denatured sequencing library.
H. Store library on ice for loading.

5.8 Load Sample Libraries onto the Reagent Cartridge

A. Clean the foil seal covering reservoir #10 labeled Load Library Here using a low-lint tissue.
B. Pierce the foil seal with a clean 1 ml pipette.
A. Load 1.3 ml of prepared 1.8 pM libraries into reservoir #10 labeled Load Library Here. Avoid touching the foil seal as you dispense the libraries.
D. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface.

5.9 Set Up a Sequencing Run

A. From the Home screen, select **Experiment,** On the Select Assay screen, select **Sequence**.
B. Select Run (Local Run Manager Run Mode): Select a run name from the list of available runs.
C. Confirm run parameters: **Run Name**, **Library ID**, **Recipe**, **Read Type**, **Read Length** and/or **Custom Primers**, if applicable
D. [Optional] Select the Edit icon to change run parameters. When finished, select Save
E. Select **Next.**

5.10 Clean and load the Flow Cell

A. Remove the used flow cell from a previous run
B. Align the flow cell over the alignment pins and place the flow cell on the stage.
C. Select **Load**.
D. Select **Next**.

5.11 Empty the Spent Reagents Container and Load the Buffer Cartridge

A. Empty the Spent Reagents Container. Remove the spent reagents container and discard the contents in accordance with applicable standards (chemical hazard water bottles). Slide the empty spent reagents container into the buffer compartment until it stops. An audible click indicates that the container is in position.
B. Remove the used buffer cartridge from the upper compartment.
C. Slide a new buffer cartridge into the buffer compartment until it stops.
D. Close the buffer compartment door, and select **Next**.
E. Remove the used reagent cartridge from the reagent compartment. Dispose of unused contents in accordance with applicable standards.

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F. Slide the reagent cartridge into the reagent compartment until the cartridge stops, and then close the reagent compartment door.
G. Select **Load** and then select **Next**.
H. After you have removed the used reagent cartridge from the instrument, remove the protective rubber cover over the slot next to position #6. Press down on the clear plastic tab and push towards the left to eject the reservoir. Dispose of the reservoir in accordance with chemical hazard containers.
I. Review Automated Check.
J. Start the Run and monitor run progress.

5.12 Automatic Post-Run Wash

When the sequencing run is complete, the software initiates an automatic post-run wash. The post-run wash uses wash solution provided in the buffer cartridge and NaOCl provided in the reagent cartridge.

5.13 Download data from sequencer after the Run

Download and backup all the result of sequencing run following the Data Management Plan of eDNA project.
Copy the fast.gz files to the work station for bioinformatics analyses.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve PCR products under -20 ºC. 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

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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

Illumina. NextSeq system guide. December 2018. Document # 15046563 v05.
Illumina. NextSeq System: Denature and Dilute Libraries Guide. April 2019. Document # 15048776 v11.

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