Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-32

Amplicon Next-generation sequencing by use of Illumina Miseq

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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: 07/10/2019

Supervisor Review By: John P. Giesy Date: 07/20/2019

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
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 only compatible with the Illunima MiSeq sequencer. Length of sequencing library should be shorter than 500 base pairs and longer than 200 base pairs.

2.0 SCOPE AND APPLICATION

This SOP applies to research done by the ETL for eDNA metabarcoding during 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
- 50 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)
- Spray bottle with 70% ethanol
- Spray bottle with 10% bleach
- Spray bottle with di-Water
- Illumina Miseq
- MiSeq Reagetn kit V3 (600 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 Post-Run Wash before sequencing**

A. Prepare fresh wash solution with Tween 20 and laboratory-grade water
10% Tween 20: Add 5 ml 100% Tween 20 to 45 ml laboratory-grade water

0.5% Tween 20 wash solution: Add 25 ml 10% Tween 20 to 475 ml laboratory-grade water.
Invert five times to mix.

B. Prepare the wash components with fresh wash solution:
Add 6 ml wash solution to each reservoir of the wash tray;
Add 350 ml wash solution to the 500 ml wash bottle
C. select **Start Wash** of Sequencing Control Software.
D. Open the reagent compartment door and reagent chiller door, and slide the used reagent cartridge from the chiller.
E. Slide the wash tray into the reagent chiller until it stops, and then close the reagent chiller door.
F. Raise the sipper handle in front of the PR2 bottle and waste bottle until it locks into place.
G. Remove the PR2 bottle and replace it with the wash bottle.
H. Remove the waste bottle and discard the contents appropriately. Return the waste bottle to the reagent compartment.
I. Slowly lower the sipper handle, making sure that the sippers lower into the wash bottle and waste bottle.
J. Close the reagent compartment door.
K. Select **Next**. When the wash is complete, leave the used flow cell, wash tray, and wash bottle containing the remaining.

5.4 **Power flushing and perform another Post-Run Wash**

A. Power off the sequencer, and wait for 5 minutes, power on the sequencer.
B. Run another Post-Run Wash following the Section 5.3 Step A-K.

5.5 **Thaw Reagent Cartridge**

Thaw the reagent cartridge using a room temperature water bath for about 60 to 90 minutes. **Caution: Do not allow the water to exceed the maximum water line printed on the reagent cartridge.**

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

A. Prepare a Fresh Dilution of NaOH:
Prepare **1N NaOH**: combine 900 µl diH_2O and 100 µl **10N-NaOH** and mix
Prepare **0.2N NaOH**: combine 800 µl diH_2O and 200 µl **1N-NaOH** and mix
Note: Use the fresh dilution within 12 hours
B. Thaw HT1 and store HT1 on ice.
C. Denature a 4-nM Library
a. Dilute sequencing library to 4 nM (SOP: ETL-EDNA-31)
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 990 µl prechilled HT1 to the tube containing denatured library. The result is 1 ml of a 20 pM denatured library.

D. Dilute Denatured 20 pM Library to 10 pM
Combine 300 µl 20 pM denatured library and 300 µl prechilled HT1.
Invert to mix and then pulse centrifuge.

E. Denature and Dilute PhiX Control
a. Determine the concentration of PhiX Control with Qubit
b. Diluter PhiX to 4 nM with EBT buffer (10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20). Five µl diluted PhiX is needed for library loading.
c. Combine 4 nM PhiX (5 µl) and fresh 0.2N NaOH (5 µl) into a new microcentrifuge tube.
d. Vortex briefly and then centrifuge at 280 × g for 1 minute.
e. Incubate at room temperature for 5 minutes.

F. Dilute Denatured PhiX to 20 pM. Add 990 µl prechilled HT1 to the denatured PhiX library. The result is 1 ml of a 20 pM PhiX library. Invert to mix.

G. Combine Library and PhiX Control (~ 10% Spike-In). Combine 30 µl 20-pM PhiX library and 570 µl 10-pM denatured sequencing library.

H. Store library on ice for loading.

5.7 **Inspect the Reagent Cartridge**

A. Invert the reagent cartridge ten times to mix the thawed reagents, and then inspect that all positions are thawed.

B. Inspect the reagents in positions 1, 2, and 4 to make sure that they are fully mixed and free of precipitates.

C. Gently tap the cartridge on the bench to reduce air bubbles in the reagents.

D. Place the reagent cartridge on ice for up to six hours, or set aside at 2°C to 8°C until ready to set up the run. For best results, proceed directly to loading the sample and setting up the run.

5.8 **Load Sample Libraries**

A. Clean the foil seal covering the reservoir labeled Load Samples with a low-lint lab tissue.

B. Pierce the foil seal with a clean 1 ml pipette.

C. Pipette 600 µl of prepared libraries into the reservoir Load Samples. Avoid touching the foil seal.

D. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface.

5.9 **Set Up a Run Using MCS**

From the Home screen, select Sequence to begin the run setup steps.

5.10 Clean and load the Flow Cell

A. Put on a new pair of powder-free gloves.
B. Using plastic forceps, grip the flow cell by the base of the plastic cartridge and remove it from the flow cell container.
C. Lightly rinse the flow cell with laboratory-grade water until both the glass and plastic cartridge are thoroughly rinsed of excess salts.
D. Using care around the black flow cell port gasket, thoroughly dry the flow cell and cartridge with a lint free lens cleaning tissue. Gently pat dry in the area of the gasket and adjacent glass.
E. Dry excess alcohol with a lint-free lens cleaning tissue.
F. Make sure that the flow cell ports are free of obstructions and that the gasket is well-seated around the flow cell ports.
G. Raise the flow cell compartment door, and then press the release button to the right of the flow cell clamp. The flow cell clamp opens.
H. Make sure that the flow cell stage is free of lint. If lint or other debris is present, clean the flow cell stage using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol. Carefully wipe the surface of the flow cell stage until it is clean and dry.
I. Holding the flow cell by the edges, place it on the flow cell stage.
J. Gently press down on the flow cell clamp to close it over the flow cell.
K. Close the flow cell compartment door.
L. Select **Next**.

5.11 Load PR2 and Check the Waste Bottle

A. Remove the bottle of PR2 from 2° to 8°C storage. Invert to mix, and then remove the lid.
B. Open the reagent compartment door.
C. Raise the sipper handle until it locks into place.
D. Remove the wash bottle and load the PR2 bottle.
E. Empty the contents of the waste bottle into the appropriate waste container.
F. Slowly lower the sipper handle. Make sure that the sippers lower into the PR2 and waste bottles.
G. Select **Next**.

5.12 Load the Reagent Cartridge

A. Open the reagent chiller door.
B. Hold the reagent cartridge on the end with the Illumina label, and slide the reagent cartridge into the reagent chiller until the cartridge stops.
C. Close the reagent chiller door.
D. Close the reagent compartment door.
E. Select **Next**.

5.13 Change Sample Sheet

A. Prepare a new sample sheet.
B. Select Change Sample Sheet on the Load Reagents screen.
D. Select Browse to navigate to the sample sheet.
E. Select Open.
F. Select Save and Continue.
G. Select **Next**.

5.14 Start the Run

A. Review Run Parameters.
B. Select Change Folder to review the folder locations.
C. Modify as needed and then select Save.
D. Select **Next**.
E. Review Pre-Run Check.
F. select **Start Run.**

5.15 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 workstation for bioinformatics analyses.

5.16 Run the Post-Run Wash

Run another Post-Run Wash following the **Section 5.3 Step A-K.**

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 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. MiSeq system guide. July 2018. Document # 1000000061014 v00.
Illumina. MiSeq System: Denature and Dilute Libraries Guide. Document # 15039740 v09.