Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-31

Preparation of Sequencing Library for Illumina Platform

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Yuwei Xie, Ph.D. and John P. Giesy, Ph.D., FRSC, FSETAC

Supported through:
Toxicology Centre and
Department of Veterinary Biomedical Sciences

Correspondence to:
Environmental Toxicology Laboratory
Toxicology Centre
44 Campus Drive,
Saskatoon, Saskatchewan, S7N 5B3
Canada

Phone: (306) 966-5062; 966-2096
Facsimile: (306) 966-4796

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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)

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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

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1.0 PURPOSE

This SOP is developed for sequencing library construction of metabarcoding. This SOP is only compatible with the Illunima high throughput sequencing platform.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for eDNA metabarcoding under 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® dsDNA HS Assay kit
- Qubit® assay tubes (500 tubes, Life Technologies, Cat. #: Q32856)
- NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB, UK, Cat. #: E7645S)
- NEBNext® Library Quant Kit for Illumina® (NEB, UK, Cat. #: E7630S)
- 80% Ethanol (freshly prepared)
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- NEBNext® Singleplex or Multiplex Oligos for Illumina (NEB #E7350, #E7335, #E7500, #E6609, #E7710, #E7730 or #E7600)
- Magnetic rack/stand
- ABI qPCR plate
- ABI optical clear film for qPCR plate
- QuantStudio Flex 6 real-time qPCR thermocycler (ThermoFisher)
- Spray bottle with Invitrogen™ RNase Away™ Decontamination Reagent
- 0.2 mL PCR tube
- 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 sequencing and construction of a library in a stepwise fashion and explain the reasoning behind the techniques selected. 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).

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The NEBNext Ultra II DNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of DNA into high quality libraries for next-generation sequencing on the Illumina platform.

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.

5.3 **Quantify the concentration of pooled PCR products from SOP# ETL-EDNA-24**

A. Thaw pooled PCR products on ice.
B. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA HS Assay requires 2 standards.
C. Label the tube lids.
D. 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.
E. Add 190 µl of Qubit® working solution to each of the tubes used for standards.
F. Add 10 µl of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
G. Add 198 µl Qubit® working solution to individual assay tubes.
H. 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.
I. Allow all tubes to incubate at room temperature for 2 minutes.
J. Measure the concentration of DNA samples using Qubit® 4.0 Fluorometer.
K. Transfer data file to an appropriate folder on the computer and print report.

5.4 Purify the pooled PCR products with AMPure XP beads

A. Sit the AMPure XP Beads (hereafter beads) at room temperature for at least 30 minutes before use
B. Aliquot 500 ng pool PCR products into a new 2.0 microcentrifuge tube.

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C. Vortex AMPure XP Beads. Add 1.6 X volume resuspended beads to the PCR products. Vortexing for 3-5 seconds on high speed can also be used.
D. Incubate samples on bench top for at least 5 minutes at room temperature.
E. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
F. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).
G. Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
H. Repeat last step once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.
I. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
Caution: Do not over-dry the beads. This might result in lesseer recovery of the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.
J. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 54 µl of 0.1X TE.
K. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
L. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 50 µl purified PCR products to a new PCR tube.

5.5 DNA End repair

A. Add the following components to the PCR tube with 50 µl:
3 µl NEBNext Ultra II End Prep Enzyme Mix (green cap)
7 µl NEBNext Ultra II End Prep Reaction Buffer (green cap)
B. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. **Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**
C. Place in a thermocycler, with the **heated lid set to 75°C**, and run the following program: {30 minutes @ 20°C; 30 minutes @ 65°C; Hold at 4°C}

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5.6 **Adaptor ligation**

A. Add the following components directly to the End Prep Reaction Mixture:

30 µl	NEBNext Ultra II Ligation Master Mix (Red cap; **Note: Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction**)
1 µl	NEBNext Ligation Enhancer (Red cap)
2.5 µl	Adaptor for Illumina (NEBNext Singleplex or Multiplex kit)

B. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. (**Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance**).

C. Incubate at 20°C for 15 minutes in a thermocycler **with the heated lid off**.

D. Add 3 µl of USER™ Enzyme (Red Cap) to the ligation mixture from Step 5.6-C.

E. Mix well and incubate at 37°C for 15 minutes with the heated lid set to 47°C.

5.7 **Cleanup of Adaptor-ligated DNA**

A. Sit the AMPure XP Beads (hereafter beads) at room temperature for at least 30 minutes before use.

B. Vortex AMPure XP Beads. Add 87 µl resuspended beads (0.9 X) to the PCR products. Vortexing for 3-5 seconds on high speed can also be used.

D. Incubate samples on bench top for at least 5 minutes at room temperature.

E. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

F. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

G. Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

H. Repeat last step once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

I. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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J. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE.
K. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
L. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl purified ligation products to a new PCR tube.

5.8 **PCR Enrichment of Adaptor-ligated DNA**

A. Prepare PCR mixtures (50 µl)
15 µl Adaptor Ligated DNA (Step 5.7-L)
25 µl NEBNext Ultra II Q5 Master Mix (Blue cap)
5 µl Index Primer/i7 Primer (NEBNext Singleplex or Multiplex Oligos Kit)
5 µl Universal PCR Primer/i5 Primer

Caution: one index for one library, more than six different indexes are preferred for one sequencing run.

B. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

C. Place the tube on a thermocycler and perform PCR amplification using the following PCR cycling conditions: 30 s @ 98 °C; {10 s @ 98 °C; 75 s @ 65 °C;}6 cycles; 5 minutes @ 65 °C

5.9 **Cleanup of PCR Reaction**

A. Set the AMPure XP Beads (hereafter beads) at room temperature for at least 30 minutes before use.
B. Vortex AMPure XP Beads. Add 45 µl resuspended beads (0.9 X) to the PCR products. Vortexing for 3-5 seconds on high speed can also be used.
D. Incubate samples on bench top for at least 5 minutes at room temperature.
E. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
F. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).
G. Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
H. Repeat last step once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

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I. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.
Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

J. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE.

K. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

L. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl purified ligation products to a new 1.5 mL tube.

M. Quantify the concentration of sequencing library with Qubit (follow steps of Section 5.3). **Samples can be stored at –20°C.**

5.10 Quantify the sequencing library with qPCR

Use NEBNext® Library Quant Kit for Illumina®

A. Thaw Kit Reagents at room temperature. Mix well and centrifuge briefly to collect material from the sides of the tubes. Place reagents on ice.

B. Prepare Master Mix + Primer Mix
Add 100 µl NEBNext Library Quant Primer Mix and 20 µl ROX (low) to the tube of NEBNext Library Quant Master Mix (1.5 ml). Mix by vortexing for 10 seconds. Write the date on the master mix tube to indicate that primer mix has been added.

C. Prepare NEBNext Library Quant Dilution Buffer (1X)
Prepare the NEBNext Library Quant Dilution Buffer (1X) by making a 1:10 dilution of the 10X buffer in nuclease-free water. Prepare sufficient buffer for the desired number of libraries to be quantitated, allowing 1.2 ml for each library.

D. Prepare Library Dilutions
Prepare an initial 1:1,000 dilution of each library sample in NEBNext Library Quant Dilution Buffer (1X).
Add 1 µl library sample to 999 µl NEBNext Library Quant Dilution Buffer (1X) to create a 1:1,000 dilution.
Add 10 µl of the 1:1,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) (creates 1:10,000 dilution).
Add 10 µl of the 1:10,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) (creates 1:100,000 dilution).

E. Prepare qPCR Assays
Note: run each DNA standard and library sample in triplicate.
Prepare DNA standards and diluted library samples per reaction:
16 µl NEBNext Library Quant Master Mix (with primers)

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4 µl DNA standard or library dilution

Prepare a no-template control:

16 µl NEBNext Library Quant Master Mix (with primers)

4 µl Library Dilution Buffer (1X)

Note: Mix reactions by pipetting sample or buffer at least 5X. Try to minimize bubbles in plate wells, but 1–2 bubbles per well will be removed by heating and not affect results. If replicates are prepared outside of the qPCR plate, load 19 µl per well to minimize bubble formation.

F. Real-time qPCR program a Real-time Thermal Cycler Using FAM/SYBR (Fast mode): 60 s @ 95 °C; {15 s @ 95 °C; 45 s @ 63 °C (signal capture)}35 cycles; melting curve.

G. Analyze Data and Calculate Library Concentrations

SAMPLE NAME	CONCENTRATION (pM)
DNA Standard 1	10
DNA Standard 2	1
DNA Standard 3	0.1
DNA Standard 4	0.01

Note: Confirm PCR efficiency for the DNA standards is 90–110%.
Adjusted Conc. (pM) = Calculated Conc. × 399 / library size (bp)
Samples can be stored at –20°C.

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

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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

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.

NEB biotech. NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®. Instruction Manual, 10/16.

NEB biotech. NEBNext® NEBNext® Library Quant Kit for Illumina®. Instruction Manual.

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