Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-30

Metabarcoding of Cyanobacteria Assembly

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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

TABLE OF CONTENTS

1.0 PURPOSE

This SOP is developed for environmental DNA (eDNA) metabarcoding of cyanobacteria assembly.

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 spills occur, 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 ($\leq$ 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
- Thermo Scientific Phusion Green High-Fidelity DNA Polymerase

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

The following procedures describe methods for metabarcoding of cyanobacteria assemblies in a stepwise fashion and explain the reasoning behind the techniques selected. Detailed below are the preparatory procedures, molecular techniques, and procedures for documentation and preservation of samples.

5.1 **Mobilization and Training**

This section describes requirements for mobilization for the necessary lab-work, as well as necessary safety training. The objective of this section is to ensure that all of the necessary preparatory work has been conducted to enable 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).

Note: The following precautions must be observed to minimize risks of cross contamination when setting up a PCR reaction.

- PCR should ideally be performed in a dedicated ‘clean’ area which is free from other work involving DNA.
- All stocks of pipette tips, microcentrifuge tubes, etc. to be used should be DNase, RNase and nuclease-free, and stored in a dust free environment. The use of filter tips is advisable.
- Pipettes designated solely for PCR should be used to set up the reaction if available.
- Wear gloves at all times.
- Prepare the PCR master mix in a “Master Mix laboratory” which is DNA/RNA isolation and gel gel-electrophoresis free lab.

- Template DNA/cDNA should be added in the "DNA extraction laboratory".

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 surfaces.**
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 **Prepare primer stock solution and work solution**

A. Preparing primer stock solution for long-term storage
Reconstitute dual-tagged forward and reverse primers to 100 μM using 1 x TE Buffer (DNase and DNA free). Carefully transfer the primer stock solutions into the 96-well plate (primer stock solution plate).

B. Preparing primer working solution for PCR setup
Reconstitute dual-tagged primers to 10 μM using DNase and RNase free sterile deionized water (diH_2O). Add 90 μL diH_2O into each well of a new 200 μL 96-well plate (primer working solution plate), then transfer 10 μL primer stock solution into the primer working solution (10 μM) plate. Seal the working solution plate with a film, mix the solution and centrifuge at 1500 rpm for 2 min. Transfer 20 μL working solution from each well of both the forward and reverse primer working solution plate into a new 96-well plate (primer mixture working solution plate, 40 μL, totally 10 μM for each dual-tagged primer pair, 5 μM for each primer) to prepare a primer mixture working solution. Dual-tagged primer pairs are listed in Appendix I at the end of this document.

5.4 **Preparing the 96-well plates of eDNA/cDNA samples for PCR setup**

Aliquot 20 μL template into a 96-well plate. A NON-Template Control Sample (NTC) should be prepared in each sample plate by use of diH_2O instead of DNA template. The location (well) of this NTC should be randomly assigned. Make electric records for the sample list, location and ID of sample plates.

5.5 **PCR amplification for metabarcoding of cyanobacteria**

Avoid carry over between tubes or contamination of stock.

PCR amplification is conducted in triplicate to reduce the bias introduced by the PCR reaction.

Note: Include the following control reactions for each DNA template.

- Positive DNA control if appropriate
- Negative DNA control if appropriate
- No template control using sterile water instead of DNA

To profile the composition of cyanobacteria, the V6 fragment of the 16s rRNA gene is amplified by use of Phusion Green Hot Start II High-Fidelity DNA Polymerase (CyanoB1328F: GCTAACGCGTTAAGTATCCCGCCTGG; CyanoB1664R: GTCTCTCTAGAGTGCCCAACTTAATG). Primer set (dual-tagged CyanoB1328F and CyanoB1664R) is used to profile the bacterial communities. The length of this PCR products is about 400 bp.

A. Set-up master mix of PCR reaction

Component	*20-µL rxn*
diH_2O	12.4 µL
5X Phusion Green HF Buffer	4.0 µL
10 mM dNTP mix	0.4 µL
10 µM primer mix	2.0 µL
Phusion Hot Start II DNA Polymerase	0.2 µL
Template DNA or controls	1.0 µL
Total	20 µL

B. PCR amplification
Set the PCR tubes or 96-well plate on a thermocycler, run the amplification program below:

	Step	*Temperature*	*Time*
A	Initial Denaturation	98 °C	30 s
B	Denaturation	98 °C	30 s
C	Annealing	58 °C	30 s
D	Extension	72 °C	30 s
E	Cycling	From B to D	40 cycles
F	Final extension	72 °C	10 min
G	Hold	4 °C	∞

C. Quality check products of PCR by use of agarose gel-electrophoresis;
D. Check and products of PCR or store it at –20 °C.

6.0 RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

Preserve products of PCR at -20 ^{o}C. 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

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.

APPENDIX I PRIMER SET

CyanoV6 Primer set PLAT-I

	1	2	3	4	5	6	7	8	9	10	11	12
A	F01 R.A	F02 R.A	F03 R.A	F04 R.A	F05 R.A	F06 R.A	F07 R.A	F08 R.A	F09 R.A	F10 R.A	F11 R.A	F12 R.A
B	F13 R.A	F14 R.A	F15 R.A	F16 R.A	F17 R.A	F18 R.A	F19 R.A	F20 R.A	F21 R.A	F22 R.A	F23 R.A	F24 R.A
C	F25 R.B	F26 R.B	F27 R.B	F28 R.B	F29 R.B	F30 R.B	F31 R.B	F32 R.B	F33 R.B	F34 R.B	F35 R.B	F36 R.B
D	F37 R.B	F38 R.B	F39 R.B	F40 R.B	F41 R.B	F42 R.B	F43 R.B	F44 R.B	F45 R.B	F46 R.B	F47 R.B	F48 R.B
E	F49 R.C	F50 R.C	F51 R.C	F52 R.C	F53 R.C	F54 R.C	F55 R.C	F56 R.C	F57 R.C	F58 R.C	F59 R.C	F60 R.C
F	F61 R.C	F62 R.C	F63 R.C	F64 R.C	F65 R.C	F66 R.C	F67 R.C	F68 R.C	F69 R.C	F70 R.C	F71 R.C	F72 R.C
G	F73 R.D	F74 R.D	F75 R.D	F76 R.D	F77 R.D	F78 R.D	F79 R.D	F80 R.D	F81 R.D	F82 R.D	F83 R.D	F84 R.D
H	F85 R.D	F86 R.D	F87 R.D	F88 R.D	F89 R.D	F90 R.D	F91 R.D	F92 R.D	F93 R.D	F94 R.D	F95 R.D	F96 R.D

B CyanoV6 Primer set PLAT-II

	1	2	3	4	5	6	7	8	9	10	11	12
A	F01 R.E	F02 R.E	F03 R.E	F04 R.E	F05 R.E	F06 R.E	F07 R.E	F08 R.E	F09 R.E	F10 R.E	F11 R.E	F12 R.E
B	F13 R.E	F14 R.E	F15 R.E	F16 R.E	F17 R.E	F18 R.E	F19 R.E	F20 R.E	F21 R.E	F22 R.E	F23 R.E	F24 R.E
C	F25 R.F	F26 R.F	F27 R.F	F28 R.F	F29 R.F	F30 R.F	F31 R.F	F32 R.F	F33 R.F	F34 R.F	F35 R.F	F36 R.F
D	F37 R.F	F38 R.F	F39 R.F	F40 R.F	F41 R.F	F42 R.F	F43 R.F	F44 R.F	F45 R.F	F46 R.F	F47 R.F	F48 R.F
E	F49 R.G	F50 R.G	F51 R.G	F52 R.G	F53 R.G	F54 R.G	F55 R.G	F56 R.G	F57 R.G	F58 R.G	F59 R.G	F60 R.G
F	F61 R.G	F62 R.G	F63 R.G	F64 R.G	F65 R.G	F66 R.G	F67 R.G	F68 R.G	F69 R.G	F70 R.G	F71 R.G	F72 R.G
G	F73 R.H	F74 R.H	F75 R.H	F76 R.H	F77 R.H	F78 R.H	F79 R.H	F80 R.H	F81 R.H	F82 R.H	F83 R.H	F84 R.H
H	F85 R.H	F86 R.H	F87 R.H	F88 R.H	F89 R.H	F90 R.H	F91 R.H	F92 R.H	F93 R.H	F94 R.H	F95 R.H	F96 R.H