Ohio EPA Quantitative Polymerase Chain Reaction (qPCR) Multi-Plex Molecular Assay for Determination of Cyanobacteria and Cyanotoxin-Producing Genes Analytical Methodology

Quick Reference	**Standard/Reagent**	**Requirements**
Standard/Reagent Storage	Liquid Disinfectant	Manufacturer's Recommendations
	Analysis Kit	Manufacturer's Recommendations
	Calibration Kit	Manufacturer's Recommendations
	Verification Plate	Manufacturer's Recommendations
	Standards	Manufacturer's Recommendations
Standard/Reagent Expiration	**Standard/Reagent**	**Expiration**
	Liquid Disinfectant	Manufacturer's Expiration Date
	Analysis Kit	1 Year After Opening/ Manufacturer's Expiration Date
	Calibration Kit	1 Year After Opening/ Manufacturer's Expiration Date
	Verification Plate	1 Year After Opening/ Manufacturer's Expiration Date
	Standards	1 Year After Opening/ Manufacturer's Expiration Date
Required Quality Control	**QC Procedure**	**Frequency**
	See Chapter 9 of this method	See Chapter 9 of this method
Sample Collection	**Preservation**	**Maximum Hold Time**
	0-6°C	7 days

Method Reference

Ohio EPA Method 705.0, Version 1.0

Survey Requirements

- For Method 705.0, each analyst must submit the assay calibration report, curve and test report to dwlabcert@epa.ohio.gov.
- All reagents, standards and solutions used for this method will be audited for correct labeling and dating.
- All records will be audited.

1.0 General Method Summary

This method is used as a regulatory screening procedure for cyanobacterial genes and genes capable of producing microcystin, saxitoxins, or cylindrospermopsin in surface water.

This method assumes the use of a Real Time PCR System capable of reading the relevant dye sets used in the assay.

The Ohio EPA quantitative Polymerase Chain Reaction multi-plex technique is a method devised for the determination of cyanobacterial genes and genes capable of producing cyanotoxins in water samples. This test is predicated on the action of DNA Polymerase, an enzyme which is capable of synthesizing double-stranded DNA from a single-stranded template. This template DNA is extracted from the sample and denatured by heat. Primers then anneal and DNA polymerase synthesizes the double-stranded DNA. When the DNA polymerase reaches the probe binding site, it degrades the probe releasing the fluorophore. The DNA is subsequently amplified across several orders of magnitude over 40 heating/cooling cycles. During this amplification, the level of fluorescence is proportional to the original amount of target DNA present in the sample and can be detected by a PCR plate reader.

Interferences
Due to the high variability of compounds found in water samples, test interferences caused by matrix effects cannot be completely ruled out. Ohio EPA continues to work with U.S. EPA and other experts to identify and provide more guidance on potential interferences.

2.0 Definitions

a. **qPCR –** A molecular assay used to detect a specific DNA sequence in a sample and determine the actual copy number of this sequence relative to a standard.

b. **C_t** – The cycle at which amplification exceeds the background threshold.

c. **Anneal** – The process by which primers attach to single-stranded DNA, allowing action of DNA Polymerase.

d. **Amplification –** The production of replicate DNA by PCR.

e. **NTC** – No Template Control; used to detect contamination or excess non-specific amplification in a reaction. It is prepared by pipetting 5 µL of PCR-grade water and 20 µL of respective MasterMix into a well.

f. **IAC** – Internal Amplification Control; used to verify negative amplification results.

g. **Calibrator/Positive Control** – A positive QC standard of known target concentration, analyzed with each batch of samples to detect assay drift.

h. **Method Blank** – Control used to verify the absence of measurable levels of contaminating target sequences that may be introduced during filtration. DNA extraction and/or preparation for the reagents of reactions by filtering DNAse/RNAse-free water alongside samples.

3.0 Equipment and Supplies

a. Glass or polyethylene terephthalate glycol (PETG) sampling container: 200.0mL

- Cleaning of approved sample collection containers is acceptable if the laboratory can demonstrate effectiveness of the cleaning procedure by collecting and analyzing reagent water in 5% per batch of the cleaned containers. The reagent water results must be less than the reporting limit. The laboratory must document this procedure and must maintain these records. The **Sample Bottle Cleaning Record** on page 10 may be used to document the required information.

b. Bench sheets or logbooks: See pages 10 – 14.

c. Micropipettors: Capable of 1 - 100 µL

d. Micropipettor tips with aerosol barrier, low retention and PCR-grade disposable, 1-100 µL capacity.

e. Freezer: Must maintain a temperature of ≤ -20.0 ºC

f. Refrigerator: Must maintain a temperature of 4.0 ± 2.0 ºC

g. Filter manifold, with in-line vacuum or electrical vacuum pump, to hold several filter bases

h. Polycarbonate Track Etch Membrane Filters: Sterile, white, 25- or 47-mm diameter, with 0.8 µm pore size (e.g., Whatman Nuclepore 111109 or equivalent)

i. Disposable or Reusable Sterile Filter Cups

j. Forceps: Straight or curved, with smooth tips, non=serrated metal or sterile disposable

k. Small, wide-mouth container, for flame-sterilizing forceps

l. Alcohol burning lamp

m. Bead Lysis Tubes with Lysis Buffer, BioGX #800-1000

n. Microcentrifuge

o. Microcentrifuge Tubes: Low retention, clear, 1.5 mL

p. Bead Beater-Type Homogenizer

q. Vortex Mixer (heavy-duty)

r. Real Time PCR System capable of reading relevant dye sets used in the assay. (DES Lab uses Applied Biosystems 7500 Fast Real-Time PCR System capable of 495 to 610 nm wavelengths.)

s. Optical 96 Well PCR Reaction Tray (e.g., Applied Biosystems N801-0560 or equivalent)

t. Optical Adhesive PCR Reaction Tray Tape (e.g., Applied Biosystems 4311971 or equivalent) or MicroAmp Caps (e.g., Applied Biosystems N8010534 or equivalent)

u. Mini Plate Spinner

4.0 Reagents

a. Liquid Disinfectant: Commercially available, Roccal® or equivalent disinfectant.

b. Reagent-Grade Water: Laboratory-available deionized water meeting *Standard Methods* criteria for bacterial analysis. Quality must meet minimum resistivity of 10.0 MΩ.

c. PCR-Grade Water: Water must be DNA/DNase free

d. PCR Mater Mix kit with Primers, BioGX #205-0050, #205-0051

e. Alcohol, 95%: For flame-sterilization

f. For 7500 Fast Real-Time PCR Systems - Spectral Calibration Kit I; ThermoFisher #4360788 or for non-7500 Thermo instruments - CAL Fluor® Red 610 T10 Calibration Standard ThermoFisher # RD-5082.

g. For 7500 Fast Real-Time PCR Systems Spectral Calibration Kit II; ThermoFisher # 4362201 or for non-7500 Thermo instruments - CAL Fluor® Orange 560 T10 Calibration Standard ThermoFisher #RD-5081-5.

h. TaqMan® RNase P Instrument Verification Plate, Fast 96-well; ThermoFisher # 4351979

i. Phytoxigene™ CyanoNAS Standards, 200,000 copies/µL – 20 copies/µL.

NOTE:

- Reagents, standards and kits must be labeled with the received, opened and expiration dates.
- Prepared reagents must be labeled with content, date made, expiration date, and analyst initials. Prepared reagents must be discarded one year after preparation or the manufacturer's expiration date for items used, whichever comes first.

5.0 Sample Collection/Preservation/Hold Time

a. **Sample Collection:** A minimum of 200 mL should be collected in a glass or polyethylene terephthalate glycol (PETG) container.

b. **Preservation:** All samples must be protected from sunlight, cooled to 0.0 – 6.0°C immediately after collection and maintained at 0.0 – 6.0ºC until analysis.

c. **Holding time:** Drinking Water samples must be extracted within 48 hours from the time of collection and analyzed no later than seven days from the time of collection.

NOTE: If the sample is not to be used for drinking water screening, the hold time **after extraction**, can be extended by freezing the extract. The extract may be kept in the freezer indefinitely to be analyzed later.

6.0 Procedure

6.1 Sample Preparation and Extraction

NOTE: The extraction and assay procedure must be performed away from direct sunlight.

1. Disinfect the work area.

2. Place disposable filter assembly onto filtration manifold. Remove cup from the assembly and replace existing filter with a polycarbonate track etch membrane filter without disturbing the support disc below. Replace cup onto assembly.

3. Vigorously shake each sample for 10 seconds to homogenize immediately prior to measuring volume.

4. Measure 25.0 mL of reagent-grade water and filter through the filtration unit until there is no visible moisture. This will be the method blank, and it must undergo the entire preparation process (Steps 4-11). A method blank must be analyzed once per extraction batch.

5. Measure 25.0 mL of sample and filter through the filtration unit until there is no visible moisture. If, due to high biomass or suspended material, filtering 25.0 mL is not possible, discard the filter and start over with a smaller sample volume. If the filtered sample volume results in a reporting limit higher than 0.18 Gene Copies/µL and the concentration of the analyte is below the modified reporting limit, the sample must be appropriately qualified (UJ). See Section 8.0 for qualifier definitions.

6. During filtration, rinse the sides of the filter cup with additional reagent-grade water to ensure complete filtration of sample.

7. Once sample is completely filtered, remove and dispose of the filter cup.

8. Remove the filter from the filtration unit base with flame-sterilized or sterile disposable forceps, fold into a cylinder with the sample side facing inward. Be careful to handle the filter only on the edges where the filter has not been exposed to the sample. Insert the rolled filter into a labeled bead lysis tube. Forceps must be re-sterilized between samples.

9. Beat the bead lysis tube using a dedicated mini bead beater for 2 minutes at its highest speed setting. Conversely, use a heavy-duty vortex mixer with microcentrifuge tube adapter for a minimum of 15 minutes at its highest setting.

10. Centrifuge the tubes using a microcentrifuge for 3 minutes at its highest setting to pellet the beads and debris.

11. Excluding both lysis beads and filter, transfer supernatant (top layer) to another, properly labeled, microcentrifuge tube. This extract will be used as the PCR template. Extract can be stored frozen to increase holding time indefinitely.

7.0 Calibration

Instrument Calibration: Follow manufacturer's instructions for instrument calibration.

7.1 Analyst Calibration: Using Phytoxigene™ CyanoNAS Standards, generate a calibration curve and for each parameter including the IAC according to the manufacturer.

7.2 Pipette 20.0 µL of the proper Master Mix into a well along with 5.0 µL of the most concentrated standard. Repeat for all standards.

7.3 Analyze the plate using the run method (Section 8.8).

7.4 The thresholds produced by the analyst's calibration must be used for every subsequent sample run until a new calibration curve is generated.

7.5 Plot the C_t value of each standard against the Log Value of the gene copies per well.

8.0 Analysis

Verify kit standards and reagents are used prior to the expiration date.

8.1 Bring all standards and reagents to room temperature and vortex for a minimum of 10 seconds.

8.2 Re-constitute each tube of Master Mix with 80.0 µL PCR-grade water and vortex thoroughly. Micro-centrifuge briefly to bring all Master Mix to bottom of tube.

8.3 Using a capable micropipette and tip, dispense 20.0 µL of the rehydrated Master Mix into each well of the 96-well plate which will contain sample.

8.4 Pipette 5.0 µL sample/control/standard into each reaction well.

NOTE: Pipette tip must be changed after sample has been pipetted into Master Mix to avoid cross-contamination of the Master Mixes

8.5 Cover the plate with adhesive film and briefly agitate to ensure mixture of reagents. Do not allow any reagent to splash onto the film.

8.6 Centrifuge the plate in a plate-spinner for 20 seconds.

8.7 Select the following cycling conditions on PCR thermocycler: 2 minutes initial denaturation at 95.0°C, 15 second denaturation at 95.0°C, 30 second annealing/extension phase at 60.0°C. Set number of cycles to 40. Data collection will take place during the annealing/extension phase.

NOTE: The CyanoDTec Assay has been successfully run on many different qPCR platforms. While the parameters listed above work best for some ABI 7500 instruments, the cycling conditions may require optimization for each individual instrument. These parameters include Annealing Temperature, Time and Ramp Rate. Some instruments may require annealing time to be at 45 seconds.

8.8 Analyze plate on a PCR plate-reader according to software and manufacturer instructions.

8.9 Sample results which are higher than the highest standard in the calibration curve must be diluted within the calibration range and reanalyzed to obtain accurate results. Additionally, if a sample IAC recovery does not meet the QC standard, the sample must be diluted and reanalyzed. If a sample is diluted, the final values must be calculated by multiplying the result by the proper dilution factor. Report calculated values. If after dilution the reporting limit is greater than 0.18 Gene Copies/µL and the analyte is below the modified reporting limit, the sample must be appropriately qualified (UJ).

8.10 Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol. Record the analyst initials, date of analysis, and the kit lot number/expiration date on the results page.

9.0 Data Analysis

C_t values generated by Thermocycler are plotted on the calibration curve. Resulting Log values are then converted into Gene Copies per microliter by the following equation:

((10^LOG)(100))/((V)(1000))

Where LOG is the log value generated by plotting C_t on the calibration curve and V is the filtration volume.

10.0 Analyst QC Requirements

10.1 Calibration Curve Generation

The calibration curve generation procedure must be performed by each analyst prior to initial use for analyzing potable water and at least every six months thereafter, for each new reagent lot number or after the instrument is moved (Refer to Section 7.0). Each calibration curve generation must be dated and recorded.

Note: An Initial Demonstration of Capability (IDC) study must be completed and documented for this method.

10.2 Analyst QC Requirements

Certified Analyst Requirements

All certified analysts are required to perform the calibration curve generation procedure at least once every six months (Refer to Section 6.0). Generations must be dated and initialed by all certified analysts participating in each procedure.

Analysts must only use their own calibration curve to calculated sample results.

Operationally Certified Analyst Requirements

Operational certification is not available for this method.

10.3 QC Requirements with Each Analysis

1. With each sample batch analysis, the following QC samples must be analyzed:

    a. Method Blank (BL): A BL must be analyzed with each batch of samples to verify the reagent-grade water is free of contaminants.

    b. No Template Control (NTC): An NTC must be analyzed with each batch of samples to verify the PCR-grade reagent water is free of contaminants. This value must remain below the analyst threshold for each parameter.

    c. Internal Amplification Control (IAC): An IAC must be analyzed with each sample. The IAC CTs of NTC should be between 29-32 cycles. The C_t value acceptance limits for samples should be within ± 1.5 C_t of the NTC IAC value. C_t values exceeding the acceptance limits require corrective action and reanalysis of sample(s). If reanalysis is not possible, all samples with C_t values outside of the defined acceptance limits must be appropriately

qualified (J) and noted in the final report. If a sample exceeds ± 2.0 C_t from the NTC IAC, the sample must be diluted and re-analyzed, and applicable qualifiers must be used.

d. A positive control sample should be analyzed with each batch of samples to ensure the method is performing properly. Calibrator sample may be used as a Positive control. Ohio EPA recommends that the C_t of this positive control differ no more than ± 2.0 C_t from the manufacturer's asserted C_t value.

2. PCR thermocycler must be calibrated according to manufacturer's instructions.

3. A calibration curve independent of the instrument calibration must be generated for each analyst every 6 months, or new reagent lot number, or after the instrument is moved. Analysts must only use their own calibration curve to calculate sample results.

4. Calibration curves must result in a Correlation Coefficient (R) > 0.995 or a Coefficient of Determination (R^2) > 0.990 to be acceptable. Efficiency of the calibration curve must be 100% ± 10%.

NOTE: Efficiency can be calculated as follows: E = 10^(-1/slope), where E is efficiency and slope, the slope of the standard curve.

5. Samples not extracted within the required holding time must be appropriately qualified (PT) and noted in the final report.

11.0 Qualifiers

B Analytical result is estimated. Analyte was detected in associated reagent blank as well as the samples.

J Analyte was positively identified; the associated numerical value is estimated.

PT The reported result is estimated because the sample was not analyzed within required holding time.

UJ The analyte was not detected above the sample Reporting Limit (RL). However, the reported result is estimated.

12.0 Required Documentation

12.1 The **HAB qPCR Sample Extraction Record** on page 10 of this SOP may be used to track extraction date, time and volume

12.2 The **Sample Bottle Cleaning Record** on page 11 of this SOP may be used to keep these records. The minimum requirements for documenting each procedure are as follows:

a. Analyst
b. Date Bottles Cleaned
c. Number Cleaned
d. Date Tested
e. Number Tested
f. Results: Number < Reporting Limit or Number > Reporting Limit

12.3 The **Reagent/Standard Preparation Record** on page 12 of this SOP may be used to keep records.

13.0 Optional Documentation

Documentation for daily temperature of refrigerator, freezer and water bath are not required for microcystin analysis. Should you wish to keep track of these, they are attached below.

13.1 The **Daily Refrigerator Temperature Record**, page 13, may be used to keep these records.

13.2 The **Daily Freezer Temperature Record**, page 14, may be used to keep these records.

HAB qPCR SAMPLE EXTRACTION RECORD

DATE /		EXTRACTION				
INITIALS	SAMPLE ID	DATE	TIME	VOLUME (ml)	PREP BATCH	COMMENTS

Sample Bottle Cleaning Record

To be recorded for each lot or batch cleaned

Laboratory _____

Analyst	Date Bottles Cleaned	Number Cleaned	Date Tested	Number Tested	Results		Comments[1]
					Number < Reporting Limit	Number > Reporting Limit	

[1]Note action taken if results are unacceptable.

Reagent/Standard Receipt/Preparation Record

Laboratory _____

Supplier/Analyst(s) Initials	Type of Reagent/Standard	Reagent/Standard Lot Number	Date Received/Prepared	Reagent/Standard Expiration Date

Daily Refrigerator Temperature Record for qPCR

To be recorded daily, 4.0 ± 2.0°C

Laboratory _____

Analyst	Date	Temp (°C)	Comments	Analyst	Date	Temp (°C)	Comments

*Note action taken if temperature is out of range.

Daily Freezer Temperature Record for qPCR

To be recorded daily, <0.0°C

Laboratory _____

Analyst	Date	Temp (°C)	Comments	Analyst	Date	Temp (°C)	Comments

*Note action taken if temperature is out of range.