

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

Ohio EPA DES 705.0

Version 1.0

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## 1. SCOPE AND APPLICATION

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

Reporting Limit (RL) for 16S, mcyE, cyrA, and sxtA genes:  $\leq 0.18$  Gene Copies/ $\mu$ L

This method assumes the use of an Applied Biosystems (AB) Sequence Detector as the default platform. The Cepheid Smart Cycler® may also be used.

## 2. SUMMARY OF METHOD

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. Fluorescently labeled primers in the qPCR Master Mix then anneal to the single stranded DNA, allowing the binding of DNA Polymerase. 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.

## 3. DEFINITIONS

- 3.1. **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. The DNA copy number can be established after each cycle of amplification.
- 3.2. **C<sub>t</sub>** – The cycle at which amplification exceeds the background threshold.
- 3.3. **Anneal** – The process by which primers attach to single-stranded DNA, allowing action of DNA Polymerase.
- 3.4. **Amplification** – The production of replicate DNA by PCR.
- 3.5. **NTC** – No Template Control; used to detect contamination or excess non-specific amplification in a reaction. It is prepared by pipetting 5  $\mu$ L of PCR-grade water and 20  $\mu$ L of respective MasterMix into a well.
- 3.6. **IAC** – Internal Amplification Control; used to verify negative amplification results.

## 4. SAMPLE COLLECTION AND PRESERVATION

- 4.1. A minimum of 200 mL raw sample should be collected in a glass or polyethylene terephthalate glycol (PETG) container.

**NOTE (1):** Cleaning of approved sample collection containers is acceptable as long as

the laboratory can demonstrate effectiveness of the cleaning procedure by collecting and analyzing reagent water in 5% of the cleaned containers. The reagent water results must be less than the reporting limit. The laboratory must maintain these records.

- 4.2. All samples must be protected from sunlight and cooled on ice at 0-4°C immediately after collection and maintained at 0-4°C until analysis. Samples must be extracted within 48 hours from the time of collection. Once extracted, holding time can be extended by freezing the extract.

## 5. 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.

## 6. SAFETY

- 6.1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.
- 6.2. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Safety Data Sheets (SDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

## 7. APPARATUS

- 7.1. Log Book
- 7.2. Micropipettors capable of 1 to 100 µL
- 7.3. Micropipettor tips with aerosol barrier, low retention and PCR-grade disposable for 1 to 100 µL capacity micropipettors.
- 7.4. Freezer capable of -20°C
- 7.5. Refrigerator capable of 4°C
- 7.6. Filter manifold, with in-line vacuum or electrical vacuum pump, to hold several filter bases
- 7.7. Polycarbonate track etch membrane filters: sterile, white, 47 mm diameter, with 0.8 µm pore size (e.g., Whatman Nuclepore 111109 or equivalent)
- 7.8. Disposable Sterile Filter Cups
- 7.9. Forceps: straight or curved, with smooth tips, non-serrated metal or sterile disposable
- 7.10. Small, wide-mouth container, for flame-sterilizing forceps and alcohol burning lamp.
- 7.11. Bead Lysis Tubes with Lysis Buffer, BioGX # 800-1000
- 7.12. Microcentrifuge

- 7.13. Microcentrifuge tubes, low retention, clear, 1.5 mL
- 7.14. Bead Beater-Type Homogenizer
- 7.15. Vortex mixer (ideally heavy-duty)
- 7.16. Applied Biosystems 7500 Fast Real-Time PCR System Capable of 495 to 610 nm wavelengths.
- 7.17. Optical 96 well PCR reaction tray (e.g., Applied Biosystems N801-0560 or equivalent)
- 7.18. Optical adhesive PCR reaction tray tape (e.g., Applied Biosystems 4311971 or equivalent) or MicroAmp caps (e.g., Applied Biosystems N8010534 or equivalent)
- 7.19. Mini Plate Spinner

## 8. REAGENTS

- 8.1. Liquid disinfectant
- 8.2. Reagent-grade water: water free of contaminants
- 8.3. PCR-Grade Water. Water must be DNA/DNase free.
- 8.4. PCR Master Mix kit with Primers, BioGX #205-0050, #205-0051
- 8.5. Alcohol, 95%, for flame-sterilization
- 8.6. 7500 Fast Real-Time PCR Systems Spectral Calibration Kit I; ThermoFisher #4360788
- 8.7. 7500 Fast Real-Time PCR Systems Spectral Calibration Kit II; ThermoFisher # 4362201
- 8.8. TaqMan® RNase P Instrument Verification Plate, Fast 96-well; ThermoFisher #4351979
- 8.9. PhytoXigene™ CyanoNAS Standards, 200,000 copies/μL – 20 copies/μL

## 9. SAMPLE PREPARATION and EXTRACTION

**NOTE (2):** The extraction and assay procedure must be performed away from direct sunlight.

- 9.1. Disinfect the work area
- 9.2. 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.
- 9.3. Vigorously shake each sample for 10 seconds to homogenize immediately prior to measuring volume.
- 9.4. Measure 25 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 9.4 – 9.10). A method blank must be analyzed once per analytical batch.
- 9.5. Measure 25 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 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/ $\mu$ L and the concentration of the analyte is below the modified reporting limit, the sample must be appropriately qualified (UJ). See Section 14 for qualifier definitions.

- 9.6. During filtration, rinse the sides of the filter cup with additional reagent-grade water to ensure complete filtration of sample.
- 9.7. 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.
- 9.8. Beat the bead lysis tube using a dedicated mini bead beater for 2 minutes at its highest speed setting. Conversely, use a heavy-duty vortexer with microcentrifuge tube adapter for 60 minutes at its highest setting.
- 9.9. Centrifuge the tubes using a microcentrifuge for 3 minutes at its highest setting to pellet the beads and debris.
- 9.10. Excluding both lysis beads and filter, transfer supernatant (top layer) to another microcentrifuge tube. This extract will be used as the PCR template. Extract can be frozen to increase holding time indefinitely.

## 10. CALIBRATION

- 10.1. Instrument Calibration: Follow manufacturer's instruction for instrument calibration.
- 10.2. Analyst Calibration: Using PhytoXigene™ CyanoNAS Standards, generate a calibration curve and set thresholds for each parameter including the IAC according to the manufacturer.
  - 10.2.1. Pipette 5  $\mu$ L of the most concentrated standard and 20  $\mu$ L of respective MasterMix into a well. Repeat for all standards.
  - 10.2.2. Analyze the plate using the run method (Section 11.8).
  - 10.2.3. Adjust the  $C_t$  values of each standard to the  $C_t$  suggested by the manufacturer by manually adjusting the threshold of the parameter. These thresholds should be used for every subsequent sample run until a new calibration curve is generated.
  - 10.2.4. Plot the  $C_t$  value of each standard against the Log Value of the gene copies per well.

## 11. SAMPLE ANALYSIS

- 11.1. Verify kit standards and reagents are used prior to the expiration date.
- 11.2. Bring all standards and reagents to room temperature and vortex for a minimum of 10 seconds.
- 11.3. Re-constitute each tube of Master Mix with 80  $\mu$ L PCR-grade water and vortex thoroughly.
- 11.4. Using a capable micropipette and tip, dispense 20 $\mu$ L of the rehydrated Master Mix into

each well of the 96-well plate which will contain sample.

- 11.5 Pipette 5µL sample/control/standard into each reaction well.
- 11.6 Cover the plate with adhesive film and briefly agitate to ensure mixture of reagents. Do not allow any reagent to splash onto the film.
- 11.7 Centrifuge the plate in a plate-spinner for 20 seconds.
- 11.8 Select the following cycling conditions on PCR thermocycler: 2 minutes initial denaturation at 95°C, 15 second denaturation at 95°C, 30 second annealing/extension phase at 60°C. Set number of cycles to 40. Data collection will take place during the annealing/extension phase.
- 11.9 Analyze the plate on a PCR plate-reader according to software and manufacturer instructions.
- 11.10 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).
- 11.11 Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol.

## 12 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:

$$((10^{\text{LOG}})(100))/((V)(1000))$$

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

## 13 QUALITY CONTROL (QC) AND DATA REPORTING

The Ohio EPA requires at a minimum the following program specific analytical QC requirements be met.

- 13.1 PCR thermocycler must be calibrated according to manufacturer's instructions.
- 13.2 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.
- 13.3 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 (3):** Efficiency can be calculated as follows:  $E = 10^{(-1/\text{slope})}$ , where E is efficiency and slope is the slope of the standard curve.

- 13.4 Method Blank (BL): A BL must be analyzed with each batch of samples to verify the reagent-grade water is free of contaminants.
- 13.5 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.
- 13.6 Internal Amplification Control (IAC): An IAC must be analyzed with each sample. The  $C_t$  value acceptance limits should be within  $\pm 1.5$  cycles 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  $\pm 2.0 C_t$  from the NTC IAC, the sample must be diluted and re-analyzed and applicable qualifiers must be used.
- 13.7 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  $\pm 2 C_t$  from the manufacturer's asserted  $C_t$  value.
- 13.8 Samples not extracted within the required holding time must be appropriately qualified (PT) and noted in the final report.

#### 14 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 RL is estimated.

#### 15 REFERENCES:

- 15.1 PhytoXigene™ CyanoDTec a quantitative Real Time PCR assay for the detection and quantitation for the presence of Cyanobacteria and their toxin producing genes from aquatic environment samples.
- 15.2 USEPA Method 1609.1: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay, April 2015.
- 15.3 ABI 7500 user's manual.

#### 16 REVISIONS:

- 16.1 Added References Section; Revision section; added UJ qualifier (4/2018)
- 16.2 Modified Sample Preparation and Extraction section, changed vortex duration from 15 minutes to 60 minutes (9/2018).

