Oregon Department of Environmental Quality

Standard Operating Procedure Amendment

SOP Title	Toxigenic Cyanobacteria by Quantitative Polymerase Chain Reaction (qPCR and RT-qPCR)
Doc Control Number	DEQ23-LAB-0005-SOP
Revision Number	1.0
Revision Date	April 26, 2023
Amendment Date	August 29, 2024
Amended Section	**Reason for Amendment**
14.0 Records Management	To detail new electronic records process that is replacing the historic paper process.

Approvals

Kale Clauson, Chemist — Date

Signed copy on file

Erica Figliulo, Interim Laboratory Quality Assurance Officer — Date

Amendments

Section 14. Records Management (Replace original with the following)

For detailed instructions on how to generate and review data electronically, please refer to DEQ24-LAB-0007-SOP: “Generating Instrument Data Packages Electronically”.

Instrument sequence documents are generated in an individual subfolder labeled by Sequence ID in the **!In_Process** folder under the instrument folder on the instrument network drive: e.g. **\\deqlead-lims\Organic_InstData\qPCR\Data \!In_Process\S24A123**.

The sequence folder should include the following documentation, saved as PDF:

1. Completed Analytical Review Checklist
   e.g. 01_SequenceID_CHK.pdf
2. Element® Field Blank and Field Duplicate Crystal reports e.g. 02_SequenceID_FBK.pdf
   e.g. 02_SequenceID_FDU.pdf
3. Element® Sequence Bench Sheet with list of reagents, pipettes, and standards used for amplification.

e.g. 03_SequenceID_SEQ.pdf

4. Element® Preparation Bench Sheet with list of reagents, pipettes, and standards used during preparation of sample batch.

e.g. 04_BatchID.pdf

5. Element® Cleanup Bench Sheet if purification was performed on samples.

e.g. 05_CleanupID.pdf

6. Calculation Reports if Phytoxigene assay performed and/or results calculated in accordance with a continuing calibration in Excel template.

e.g. 06_SequenceID_CALC.pdf

7. Experiment Results Report

e.g. 07_SequenceID_RUN.pdf

8. Melt Curve Report for SYBR assays (not required for detections <MRL)

e.g. 08_SequenceID_MLT.pdf

The analyst generates the preceding documentation and passes it on to a second chemist for peer review by cutting the sequence folder from the **!In_Process** folder and pasting it to the **!Needs_Review** folder. There should be no copy of the folder remaining in the **!In_Process** folder after this step.

In other instances, the analyst may combine the multiple pdfs into one master report pdf, named as SequenceID.pdf, e.g. S24A123.pdf. The individual files listed above will be ordered in the master report as designated; the contents of report 01 will be the first pages of the master report (which should be the checklist), followed by the contents of report 02, then 03, etc. In this instance, the pdf, not a folder containing multiple pdfs, is removed from the **!In_Process** folder and transferred to the **!Needs_Review** folder for peer review.

Standard Operating Procedure

Toxigenic Cyanobacteria by Quantitative Polymerase Chain Reaction (qPCR and RT-qPCR)

April 2023

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DEQ is a leader in restoring, maintaining and enhancing the quality of Oregon's air, land and water.

State of Oregon
Department of Environmental Quality

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Approvals

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Signed Copy on File

Documents can be provided upon request in an alternate format for individuals with disabilities or in a language other than English for people with limited English skills. To request a document in another format or language, call DEQ in Portland at 503-229-5696, or toll-free in Oregon at 1-800-452-4011, ext. 5696; or email deqinfo@deq.state.or.us.

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1. Scope and Application

This method uses the technique known as Quantitative Polymerase Chain Reaction (qPCR) for the detection of toxin-producing cyanobacteria in water samples. By quantifying toxin-related genes present in a water sample, the occurrence and quantity of toxigenic cyanobacteria can be estimated. In addition to assessing the toxigenic potential, the application of Reverse Transcription qPCR (RT-qPCR) is used to quantify the active expression of toxin genes, which is related to measured toxin concentrations. (1)

1.1. Applicable Matrices

This procedure was developed using water samples collected from Detroit Reservoir (Marion and Linn County, OR) and the Willamette River (Multnomah County, OR). This method is applicable for the following matrices:

- Surface water from lakes, rivers, and streams
- Untreated source water
- Cyanobacteria 'scum', periphyton

1.2. Detection Limits

The Limit of Quantitation (LOQ) for the listed qPCR assays are established experimentally and are based on a default sample volume filtered. In addition, the assay techniques described in this method have a characteristic detection limit – refer to Table 1 for this information.

Table 1: Target Assays and Limits of Quantitation (LOQ)

Assay	Instrument Calibration Range	Instrument	Element LOQ[1]	
		LOQ	Screening	Purified
Cyanobacteria (16Scya)	20 – 2.0 × 10^5 copies/µL	22 copies/µL	132 copies/mL	30 copies/mL
Cylindrospermopsin Producers (cyrA)	20 – 2.0 × 10^5 copies/µL	22 copies/µL	132 copies/mL	30 copies/mL
Microcystin Producers (mcyE-ndaF)	20 – 2.0 × 10^5 copies/µL	22 copies/µL	132 copies/mL	30 copies/mL
Saxitoxin Producers (stxA)	20 – 2.0 × 10^5 copies/µL	22 copies/µL	132 copies/mL	30 copies/mL
AnaC SYBR®-based qPCR[2]	50 – 5.0 × 10^6 copies/µL	50 copies/µL	300 copies/mL	60 copies/mL

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[1] The Limit of Quantitation (LOQ) is also commonly known as the method reporting limit (MRL). In general, the DEQ LEAD policy is to prepare the lowest calibration standard at concentrations less than or equal to the LOQ.

[2] Successful amplification of 5 copies per well of the target sequence, as described in Method 1609.1 (11), demonstrates that the sensitivity of this method is acceptable.

Because the fluorometric measurement used for qPCR analysis and quantitation is not normally distributed, and is heteroscedastic, the use of parametric statistics is not valid. As a result, LOD procedures required by EPA 40CFR part 136, Appendix B, Revision 2, and the Limits of Detection (LOD) and Quantitation (LOQ) SOP (DEQ18-LAB-0053-SOP), are not applicable to qPCR. The on-instrument LOD and LOQ are treated as equivalent and correspond to the lowest on-instrument concentration at which the mean recovery of seven replicate LCS samples is 50-200%, with a ≥95% degree of confidence. Results below the LOD/LOQ are reported as non-detects.

2. Summary

This method is based on the procedures described in “Molecular Marker Analysis for HAB” (2). Modifications have been made to the procedure to fit our monitoring goals and to demonstrate method performance under our stated conditions.

Untreated water will be collected in a sterile PETG bottle and transported to the DEQ laboratory. Alternatively, the cyanobacteria may be collected on site by using a sterile membrane filter cartridge and filtering 100 mL of sample and sending the filter back to DEQ. In the laboratory, water samples are then filtered aseptically through membrane filters. Lab or field prepared filter membranes are folded and inserted into lysis tubes and processed by bead beating. After centrifuging, the supernatant containing lysed cellular material is removed and subsequently processed immediately or stored at ≤-70°C.

An important factor which affects the outcome of qPCR is loss of genetic material during sample preparation. Cell lysis alone was shown to be a reliable method compared to conventional DNA extraction methods for use with cyanobacteria (3). Through minimizing the number of steps and reducing the potential for incomplete recovery, the method becomes more efficient to perform while improving yields. An aliquot of the lysate is used for rapid analysis of nucleic acid concentration by Qubit fluorometric assay and qPCR. A spin-column based extraction protocol is used to separate, purify, and concentrate DNA and RNA from the remaining lysate which will be used for qPCR (for DNA) to confirm detections of genes or RT-qPCR (for RNA) to quantify active gene expression. The sets of primers and probes described in this method are considered suitable for qPCR quantification of the genera specified in Appendix B.

3. Personnel/Qualifications

The analyst should meet the minimum qualifications for a Chemist II position. When possible, a chemist who has previously demonstrated their proficiency at performing the method should train the analyst. An Initial Demonstration of Capability (IDOC) is to be conducted and passed prior to reporting data.

4. Interferences

4.1. General Considerations

The performance of this procedure is dependent on the analyst technique, consistent lysis conditions, and the accuracy of pipetted volumes and dilutions. Potential areas where error can be introduced are addressed in the procedure and are mitigated as much as practically possible. Differences in lysis and extraction efficiency in field samples is to be expected due to strain-specific variation in polysaccharide composition among cyanobacteria (4). During development, DEQ observed significant loss of genetic material during purification and significant bias due to lysis duration and bead material. Specific references to equipment and supplies do not preclude the use of other manufacturers and does not imply endorsement of these products. The materials listed in Section 6 and Section 7 have been evaluated and selected based on method performance. The method is intended to represent suitable analytical conditions and specifications - changes may not be made to sample collection, preservation, storage procedures, or target primers.

4.2. Cross Contamination

Workspaces should be designed to prevent cross-contamination between samples, contamination of current work from previous products and contamination of assays by reagents/primers/standards. Physical separation of steps into workstations will be used to minimize potential sources of contamination.

Separate any potential qPCR products from sample preparation by locating the qPCR instrument in another room and never bringing analyzed plates (especially open ones) into the space used for preparation.

Preparation and storage of the qPCR Master Mix components should occur in a PCR workstation laminar hood to prevent introduction of contaminants present in the room. Gloves, supplies, coats, and other consumables should be designated for this workstation. The working surface should be stainless steel or similar nonporous surface that can be sterilized, and the hood should have UV integrated as well.

4.3. Sample Matrix Effects

During method development, DEQ observed bias in ambient water from a single source and identified several factors that contributed to variability, including suspended materials, chemical inhibition, and non-target genetic material.

Suspended material can overload the filter, therefore limiting the volume of water that can be processed. This can include inorganic particulates, like clay, as well as phytoplankton and cyanobacteria themselves. The use of IAC assays, matrix spikes, and sample dilutions should be considered as an important component when assessing this method's performance with field samples. Studies of EPA qPCR enterococci analysis method performance support a recovery range of 50–200% to err on the side of being more protective of public health (5). They found a high likelihood (94.5-96%) that conclusions drawn from the QA/QC will be supported by analyses of undiluted sample extracts.

A broad range of PCR inhibitors occur in environmental samples. The sources typically encountered in water samples are organic substances like tannins, fulvic and humic acids, and dissolved calcium. Serial dilutions during analysis in conjunction with purification are used to remove and reduce these effects.

Non target genetic material – bioburden – can overload the purification procedure. The binding capacity of the columns used in this procedure have an upper limit of 100 micrograms of nucleic acid. If a large amount of nucleic acid is present in the sample, it will saturate the column, resulting in low bias.

4.4. Amplification Inhibitors

When a new matrix is analyzed, or interference is suspected, verify that interfering properties or substances from the isolation of the nucleic acids do not inhibit the PCR. To determine whether inhibition is present, perform the Internal Amplification Control (IAC) assay in the Phytoxigene kit or by diluting the sample to mitigate the issue.

5. Safety

Analysts working in the LEAD facility must review the laboratory’s Chemical Hygiene Plan / Laboratory Safety Plan (DEQ04-LAB-0006-SFTY) and the Emergency Operation Plan (EOP/DEQ04-LAB-0050-SFTY).

Refer to the Job Safety Assessment (JSA) for this procedure and conduct analysis in accordance with the safety precautions specified (Appendix A).

Safety Data Sheets are now stored online. Please visit the Health & Safety page on QNet for the database.

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6. Equipment and Supplies

6.1. General Supplies

- Centrifuge tubes, Sterile, 5, 15, 25 and 50 mL
- Chilling container or rack for microtubes
- Clean powderless nitrile gloves for each work station
- Dedicated lab coats for each work station, if available
- ELIMINase™ Decontaminant (Fisher cat. 04-355-31)
- Alcohol decontamination solution (Ethanol or isopropanol solution, 70%, Fisher cat. 04-355-122)
- Freezer, low temperature (-20°C)
- Freezer, ultra-low temperature(≤-70°C)
- Household bleach diluted to 30% with water (ca. 2% sodium hypochlorite)
- Microcentrifuge tubes, 0.5, 1.5 and 2.0 mL, low binding
- Permanent marking pens (fine point) for labeling tubes
- Pipette tips, sterile with aerosol barrier, various sizes
- Racks for microtubes (polypropylene or acrylic)
- Repeating pipette, with 5- and 10-mL PCR-clean tips (Eppendorf 4982000322)
- Safety glasses or goggles for each workstation
- Wypalls™ lab towels (VWR cat. 37002-024)

6.2. Sampling Supplies

- Caps and plugs for Luer fittings
- Container for Swin-Lok™ filter holders (Simport Scientific cat. C577-90W)
- Glass fiber filters, 25 mm Whatman Grade 934-AH (Cytiva cat. 1827025)
- Sample collection containers: 250 mL or 500 mL polyethylene terephthalate (PETG), sterile media bottles. (Wheaton cat. WPBGC0250S; Thermo Nalgene cat. 3420200250)
- Swin-Lok™ Filter Holder, 25 mm (Whatman cat. 420200)
  - Recommended cleaning instructions:
    1. Disassemble completely and put parts into a 1 liter wide mouth bottle.
    2. Fill the bottle with a mild detergent and warm water. Shake the bottle for ~30 seconds to mix. Let soak for 5 minutes then shake and soak again.
    3. Drain detergent, leaving the parts in the bottle.
    4. Fill the bottle with clean water and shake and rinse three times.
    5. Fill the bottle with 30% bleach solution, cap, mix, and let soak >1 hr.
    6. Drain and rinse in clean water. Air dry on a clean surface.
    7. Store disassembled holders in a clean container.
  - Assembly instructions:
    1. Press O-ring securely into the molded groove on the underside of the cap.
    2. Using filter forceps, gently place the filter onto holder base.
    3. Place the cap over the filter so that the anti-twist tabs fall into the open slots on the base.
    4. Replace the assembly ring over the cap and screw finger tight onto the base.
  - Sterilization:
    1. Holders and filters can be autoclaved at 250°F for 15 minutes at 15 PSI.
    2. Loosen the assembly ring one-eighth of a turn before processing; wrap in foil.
    3. Re-tighten assembly after autoclaving and cooling.
- Syringes, 50-60 mL polypropylene

6.3. Lab Filtering Supplies

- Alcohol burner or equivalent flame source
- Filter holders, 47 mm (Millipore cat. XX1014720)
- Filter manifold (3-6 filter bases) and tubing, accessories and vacuum source
- Forceps, with filter-specific tips
- Forceps, with smooth tips
- Graduated Cylinders, 100 mL, TC
- Membrane filters, 47 mm Durapore® (PVDF) 0.45µm (Millipore cat. HVLP04700)

6.4. Lysis and Sample Storage

- Bead-Beater, MP Biomedicals™ FastPrep -24™ Classic (PN 116004500)
  - 4-6.5 m/s in 0.5 inch
  - 24 x 2 mL tube holder
- Centrifuge, with compatible rotor

    - 1.5-2 mL tubes capable of ≥16,000 rcf
- Centrifugal filters, Ultrafree®-MC (0.5 mL, PVDF) 0.65 µm (Millipore cat. UFC30DV00)
- Centrifugal filters, Zymo-Spin III-F (Zymo Research cat. C1057-50)
- Cryo storage boxes for microtubes
- Micropipettors, 20-200 µL and 100-1000 µL
- Pipet Tips
    - 200 µL Sterile Filtered Extended Length, Wide Orifice (Thermo cat. 2160G)
    - 1000 µL Sterile Aerosol Filter Wide Orifice (VWR cat. 89049-168)
- Sample lysing tubes, 2 mL polypropylene tubes with screw cap and o-ring.
    - Lysis Tubes, ZR BashingBead, 0.1 & 0.5 mm (Zymo Research cat. S6012-50)
- Mini Vortex Mixer

6.5. Nucleic Acid Purification

- Centrifuge, with rotor for 1.5-2 mL tubes capable of 16,000 RCF
- Mini Vortex Mixer
- Micropipettors, 20-200 µL and 100-1000 µL

6.6. Total Nucleic Acid Analysis

- Micropipettor, 20-200 µL
- Mini Vortex Mixer
- Qubit™ assay tubes (cat. Q32856)
- Qubit™ 4 Fluorometer (Thermo Fisher Scientific)
- Rack for assay tubes

6.7. Reagent Preparation

- Dedicated PCR Workstation or biological safety cabinet - laminar recirculating airflow with ultraviolet light source. Stainless work surface preferred.
- Micropipettors, 20-200 µL and 100-1000 µL
- Micropipettor, electronic repeating (VWR 10827-920) or multichannel (8 Channel)
- MicroAmp Fast 96-well reaction plate

6.8. Amplification and qPCR Analysis

- Dedicated PCR Workstation - laminar recirculating airflow with ultraviolet light source. Stainless work surface preferred.
- Micropipettors, 0.2-2.0 µL, 2.0-20 µL, 20-200 µL
- Optical adhesive qPCR plate seal
- Plate centrifuge
- Plate sealing tool or roller
- StepOnePlus™ Real-Time PCR System (Applied Biosystems)
- Sterile, aerosol barrier tips, 0.2-2.0 µL, 2.0-20 µL, 20-200 µL

7. Reagents

7.1. General

- Ethanol, 96-100%, molecular biology grade
- Isopropanol, 100%, molecular biology grade
- PCR grade water –Sterile, nuclease-free water (e.g., EMD 9610 or equivalent) for dilutions, etc.
- TE Buffer, 1X Solution, pH 8.0, Low EDTA, Molecular Biology Grade. Also used diluted 1:10 with PCR-grade water

7.2. Lab Filtering

- PBS (Phosphate-Buffered Saline) Tablets, (Invitrogen, cat. 00-300-2)
    - Add 500 mL reagent water to an autoclave-safe 1000 mL polypropylene bottle
    - Add 5 PBS tablets and dissolve
    - Leave cap loose on bottle and autoclave at 121-123 °C (250 - 253 °F) at 1.0 bar for 30 minutes (Cycle 11 -Liquids 250)
    - Cool to room temperature before tightening cap

7.3. Lysis and Sample Storage

- DNA/RNA Shield, 1X (Zymo cat. R1100)
- DNA/RNA Shield, 2X (Zymo cat. R1200)
- Lysis tubes, ‘BioGx’ (Phytoxigene cat. 800-1000)
    - prepared - contain 500 µL of lysis buffer and 300 mg of 0.1 µm glass beads

7.4. Nucleic Acid Purification

- Purification Kit(s):
    - ZymoBIOMICS™ DNA Miniprep Kit (D4300, 50pk)
    - ZymoBIOMICS™ DNA/RNA Miniprep Kit (R2002, 50pk)

7.5. Total Nucleic Acid Analysis

- High Sensitivity dsDNA Quantitation Kit
    - Qubit™ 1X dsDNA HS Assay Kit (Invitrogen #Q33230)
    - or Qubit™ DNA HS Assay Kit (Invitrogen #Q32854)
    - or AccuGreen™ HS dsDNA Quantitation Kit (Biotium #31066)

7.6. Reagents for Phytoxigene™ qPCR Analysis

- CyanoDTec Complete Kit (cat. 205-0100)
    - Total Cyanobacteria Kit, includes IAC (24)

- Cyano Toxin Gene Kit (24) (Cylindrospermopsin, Microcystin/Nodularin, Saxitoxin)

7.7. Reagents for SYBR® qPCR Analysis and RT-qPCR Analysis

- KAPA FAST qPCR MasterMix, 2X (Roche Cat. 07959435001)
- Blocking agent, Bovine Serum Albumen (BSA), Ultrapure (Invitrogen Cat. AM2616)
    - Working stock concentration of 5 mg/mL
- Luna® Universal One-Step RT-qPCR Kit (New England Biolabs Cat. E3005)
- Antarctic Thermolabile UDG (New England Biolabs Cat. M0372S)
    - 1,000 units/mL
    - For carryover prevention, add to MasterMix at concentration 0.025 units/µL
    - Include a 10 minute incubation at 25°C (i.e. RT<40°C) before initial denaturation step.

8. Standards

8.1. Primers

Dilute forward and reverse primers to a working concentration of 10 µM with TE buffer, divide into several 200 µL aliquots and store at ≤-70°C until needed. Before use, thaw the complimentary forward and reverse primers and combine at 1:1 for a mixed primer working solution.

See Appendix B for qPCR primer sequences.

8.2. Calibration Standards

Follow manufacturer recommended procedure to store and reconstitute reference material.

- Cyanobacteria Standards, certified (Phytoxigene™ cat. CyanoNAS)
    - 20 copies/µL - 200,000 copies/µL
- Plasmid DNA Template Suspensions
    - Working stock concentration of 3 to 5 × 10^7 copies/µL
- Synthetic DNA Templates, GeneBlock (Integrated DNA Technologies)
    - See Appendix B for template sequences
    - Suggested yield of 1000 ng dried DNA (1000 ng = 1.086 x 10^{12} copies)
    - Primary stock of 2.50 x 10^9 copies/mL prepared by adding 435 µL TE buffered water to the dried template.

8.3. Check Standards

- ICV/CCV for Phytoxigene assays is prepared at a concentration obtained close to the midrange of the calibration curve response cycles. This is approximately 24 cycles or 5000 copies/mL.

    - Cyanobacteria Standard, 200,000 copies/µL, certified (Phytoxigene™ cat. NA026)
        - Dilute 5 µL with 195 µL PCR-grade water
- LowCV for Phytoxigene assays is a calibration standard with a concentration at the LOQ and is used to confirm amplification and quantitation.

9. Sample Collection, Preservation, Shipment, and Storage

9.1. Sample Bottle Preparation

The suggested sample bottles are unused, pre-sterilized 250 mL polyethylene terephthalate (PETG) media bottles. To reduce potential contamination and degradation of nucleic acids, disposable materials are recommended. However, if an effective cleaning and sterilization procedure can be demonstrated, samples may be collected in reusable polypropylene or glass containers. Reagent water collected in reused containers must be less than the reporting limit.

For analyses performed at the DEQ LEAD lab, the use of laboratory-provided sample containers to collect water samples is encouraged. The laboratory may not accept containers from other sources, or results may be invalidated.

9.2. Sample Collection

Sampling procedures outlined in the SAP/QAPP should be followed, otherwise follow the procedures cited in Microbiological Methods for Monitoring the Environment: Water and Wastes (6), described below. Sampling methods may vary slightly between standard operating procedures used by samplers and different conditions encountered in the field.

Composite samples should not be collected as they will not display the range of values found in individual samples.

Drinking Water Intake

1. Open the tap and allow the water to flush for approximately 5 minutes.
2. Rinse hands with distilled or deionized water and dry. Put on a new pair of powder-free nitrile/latex/rubber gloves.
3. Do not rinse the new, sterile, sample bottle.
4. Hold the capped sample bottle near base with gloved hand.
5. Continuing to hold only the outside of the bottle, remove the cap while pointing the open bottle away from your body.
6. Tip the open bottle back toward your body and into the flowing water, taking care not to contact the bottle with the tap.
7. Fill the bottle completely, then cap the bottle tightly. If the cap is tamper-evident, check that it has seated properly.
8. Immediately put the labeled bottle on ice, away from light.

Recreational Areas

Samples should be collected with the same PETG bottle used for microcystins and cylindrospermopsin toxin analysis to control for variability associated with multiple sample grabs. If the purpose of the sampling event is to assess the maximum risk and a representative location cannot be found, use professional judgment to determine whether the site should be sampled. Observations of non-representativeness and explanation of rationale for choosing to collect or not collect a sample must be noted. For some locations, it will be necessary to collect a second sample (field duplicate). Subsequent samples should be collected at a different, but representative location from the same sample site.

The recommended long-term monitoring site sample would be taken at a single representative site at 1 meter depth, in open, deep water using a Van Dorn sampler (7). If the use of a Kemmerer or Van Dorn sampler is unsafe or unpractical, samples should be collected by hand as follows:

1. Rinse hands with distilled or deionized water and dry. Put on a new pair of powder-free nitrile/latex/rubber gloves.
2. Do not rinse the sample bottle.
3. Hold the capped sample bottle near base with gloved hand.
4. Continuing to hold only the outside of the bottle, remove the cap while pointing the open bottle away from your body.
5. Turn bottle upside down, submerge completely.
6. Tip the bottle away from your body to fill (pointing upstream or toward the center of the lake).
7. Fill the bottle completely (250 or 500 mL), then cap the bottle tightly. If the cap is tamper-evident, check that it has seated properly.
8. Put the labeled bottle on ice away from light immediately.

Filtering Samples in the Field

For projects where samples may not meet the holding times required for filtering in the lab, it will be necessary for the sampling team to filter and preserve the sample. Follow the grab sampling procedure for Recreational or Drinking Water intake, then use the procedure outlined in Section 13.2 to process a Swin-Lok filter. Collecting and preserving with DNA/RNA shield in a lysis tube is recommended, as the sensitivity to temperature and time is reduced.

Training for proper cleanliness and technique in the laboratory is recommended before performing in the field.

Each water sample will need:

- (1) Lysis tube with 750 microliters DNA/RNA Shield (1X)
- (1) Swin-Lok filter holder[3]
- (1) Clean/sterile 50 mL syringe
- (2) Clean/sterile forceps (reusable if flame-sterilized)
- (1) Small bag to keep each finished tube clean

General equipment and supplies:

- 100 mL graduated cylinder – Glass or Plastic
- Clean gloves

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[3] Section 6.2

- Microcentrifuge tube rack
- Labels, if tubes are not pre-labeled

If a thick bloom is expected, it is recommended to also bring a kit to collect scum and periphyton. The procedure is described in section 12.3.

9.3. Sample Shipment

Water samples must be transported on ice, at temperatures between 0°C and 10°C and must not be frozen.

Samples preserved in DNA/RNA Shield are stable for at least 30 days at temperatures between 0°C and 25°C, or for 7 days at up to 35°C[4].

Sample temperature must be verified at the time of receipt by the laboratory to determine if the temperature limit has been exceeded or if the sample has been frozen. This limit may be exceeded only if the transport time was too short to allow the samples to chill below 10°C, AND accompanying ice packs remain frozen. If the 10°C temperature limit has been exceeded and ice packs have melted, samples must be rejected and resampled if possible.

If resampling is not possible, the sample results must be qualified as failing to meet preservation requirements and the data downgraded. Synthetic ice (i.e., blue ice) is not recommended.

9.4. Sample Holding Time

Water samples and field-filters must be stored at 0-4°C, away from direct light. Water must be filtered, and the filters preserved within 48 hours from the time of collection. Preserved filters are frozen for up to six months at -20°C, or up to twelve months at ≤-70°C to prevent degradation. Lysate and purified DNA/RNA must be stored at ≤-70°C and analyzed within 1 year of collection.

If water samples do not meet the above conditions or cannot be processed immediately, see section 11.5 for contingencies.

10. Calibration and Standardization

10.1. Calibration Procedure

For the Phytoxigene kit assays, a calibration curve must be generated at least every 6 months, or whenever a new kit lot is used. For SYBR®-based assays, a calibration is generated for every primer and target run.

- The calculated LOQ may not be below the lowest non-zero calibration standard.
- The calibration should cover a standard concentration range of 5 to 6 logs.

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[4] Zymo Research, DNA/RNA Shield Instructions ver 2.0.0

10.2. Calibration Acceptance Criteria

The calibration curve is validated by evaluating the CV of the C_T values for the well replicates representing each calibration level, the correlation coefficient of the calibration curve, and the amplification efficiency of the calibration curve.

Calculate the CV (see Section 14.2) for each of the paired C_T values. The CV for each pair must be less than or equal to 1.5 cycles. The square of the correlation coefficient (R^2) of the four-parameter curve must be greater than or equal to 0.980. Efficiency of the calibration curve must be 100 ± 20% (see Section 14.2).

If the calibration fails the above limits, then the entire analysis batch is invalid. Assay the samples in a subsequent analysis batch.

Table 2: Calibration Requirements for Phytoxigene Assays

No. of calibration standards	5 (5 minimum)
Low calibration standard	20 copies/µL
High calibration standard	200,000 copies/µL
Signal Response	Fluorescent Probes: Total Cyanobacteria (16S rRNA) FAM (495/516nm) IAC - CalFluor Orange (538/559nm) mcyE/ndaF FAM (495/516nm) CyrA CalFluor Orange (538/559nm) SxtA CalFluor Red (590/610nm)
Calibration Method	Linear regression
Acceptance criteria	$R^2 \geq 0.980$; Eff% 100±20%
Calibration frequency	At least every 6 months or with new MasterMix lot
No Template Control (NTC)	Per Analysis Batch Acceptance Criteria: < LOQ (on-instrument)
CV between replicate pairs	$\leq 1.5\ C_T$

Table 3: Calibration Requirements for SYBR® AnaC Assay

No. of calibration standards	7 (5 minimum)
Low calibration standard	50 copies/µL,
High calibration standard	5×10^6 copies/µL
Signal Response	Fluorescent intercalating dye: SYBR® Green (495/520nm) With passive reference (ROX)
Calibration Method	Linear regression
Acceptance criteria	$R^2 \geq 0.980$; Eff% 100±20%
Calibration frequency	Per Analysis Batch
No Template Control (NTC)	Per Analysis Batch Acceptance Criteria: < LOQ (on-instrument)
CV between replicate pairs	$\leq 1.5\ C_T$

All volume measurements for standard additions and dilutions will be performed using calibrated micropipettors. See SOP (DEQ07-LAB-0011-SOP) for the method on calibration of pipettes and syringes.

11. Quality Control

11.1. Data Assessment and QC Acceptance Criteria

Data assessment and QC acceptance criteria are of the primary importance in assessing the quality of data resulting from an analytical batch. Be especially attentive to analytical bias; negative bias is of equal concern to positive bias. Any results above the upper end of the calibration range or with amplification inhibition must be diluted and re-analyzed so that all detections quantitate within the calibration range and minimize matrix effects. The following data assessment and QC data are required for all analyses (Table 1). These criteria use applicable elements outlined in *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples* (9).

Table 4 Quality Control Elements and Acceptance Criteria

QC Element	Frequency	Acceptance Criteria	Comments
Initial Demonstration of Capability (IDOC)	At end of method development and prior to reporting data. Each new analyst prior to reporting data.	Successful completion of three QC components.	Three components: demonstration of calibration, demonstration of acceptable system background, confirmation of the MRL.
Continuing Demonstration of Capability	Annually	Successful completion of two QC components.	Two components: demonstration of acceptable system background, confirmation of the MRL.
Standard Curve	See Table 4 and 5 for assay specific requirements.	Eff% between ≥80% and ≤120%; R^2 value ≥ 0.980	See Table 2 and Table 3 for assay specific requirements.

QC Element	Frequency	Acceptance Criteria	Comments
Laboratory Control Sample (LCS/BS)	N/A	Under development. Percent recovery between ≥50% and ≤200%, if known.	A source and technique are under investigation to verify the performance of the entire method so that target genes from a positive control culture can be quantified.
Method Blank (MB/BLK)	With every preparatory batch.	<LOQ (on-instrument)	To identify unacceptable levels of contamination during preparation. If no change has been made to the consumable sources, a field blank may be used to evaluate contamination.
Continuous Calibration Verification standard (CCV)	At least once per analytical sequence, unless a calibration is included	Percent recovery between ≥50% and ≤150%	To verify the performance of the PCR reagent master mix and cycling conditions at the midrange of the calibration
Low-Level Calibration Verification standard (LowCV)	At least once per analytical sequence, unless a calibration is included	Percent recovery between ≥50% and ≤200% (i.e. ±1 cycle of ICAL)	To verify the performance of the PCR reagent master mix and cycling conditions at the LOQ
No Template Control (NTC/CCB)	At least once per analytical sequence	<LOQ (on-instrument)	To identify unacceptable levels of contamination of master mix or PCR setup
Sample Duplicate (Field and Lab)	Lab Duplicate (DUP), with every preparatory batch. Field Duplicate according to the DQO of the QAPP	RPD between pair ≤80%, for concentrations >5x LOQ	Duplicate filter is prepared to evaluate the homogeneity of the sample as collected.

QC Element	Frequency	Acceptance Criteria	Comments
Matrix Spike (MS)	N/A	Under development. Percent recovery ≥50% and ≤200% of LCS after correction for native sample result.	Sample spiked with same positive control culture as the LCS and carried through entire method.
IAC Assay	All samples assayed by Phytoxigene kit	Sample C_T ±1.5 cycles of analytical batch CCB CCB C_T ±2.25 cycles of ICAL NTC	Demonstrate that matrix components are not inhibiting the PCR
Serial Dilutions of Samples	All non-zero samples assayed by SYBR®	RPD between pair ≤50%	1:10 dilution to demonstrate that matrix components are not inhibiting the PCR
Melting Curve Analysis	At the end of each SYBR®-dye analytical sequence	Single peak for detections at similar Tm of template	See Appendix C
Passive Reference Signal	All samples assayed by SYBR®	Consistent signal across all wells (≤0.6)	Not flagged by instrument QC “BADROX”

11.2. Initial Demonstration of Capability (IDOC)

A successful IDOC must be completed before the analysis of field samples. This includes three determinations: demonstration of accuracy and precision during calibration, demonstration of acceptable system background (MB), and confirmation of the MRL (LowCV). These components may be completed in the same analysis batch. The IDOC is performed for the Phytoxigene™ assay and one target for the SYBR® assay – anaC by SYBR® is preferred.

11.2.1 Demonstration of Acceptable Calibration

In the same analysis batch constructed for the IDOC the target must be successfully calibrated and meet all requirements of a passing calibration.

11.2.2 Demonstration of Acceptable Background

In the same analysis batch constructed for the precision and accuracy component, filter, and assay five Method Blanks (MB), also known as LRBs. These must be distributed throughout the plate and must each recover less than the on-instrument LOQ.

11.2.3 Determination of Minimum Reporting Level

Establish a target LOQ based on the intended use of the method. Confirm that LOQ using the following procedure. Prepare seven replicate MRL samples by diluting a plasmid or synthetic DNA standard. Calculate the mean percent recovery and standard deviation of the seven replicates. Verify the LOQ as shown in Section 13.3.

11.3. Ongoing QC Requirements

11.3.1 Preparatory Batch Quality Control Requirements

Oregon DEQ defines a preparatory batch performed for qPCR as 20 or fewer samples and the quality control elements using identical lots of reagents and consumables within a single 24 hour period. Each preparatory batch includes the minimum following elements:

- 1 Method Blanks (Filter Blanks, LRBs)
- 1 Sample Duplicate (DUP)

11.3.2 Phytoxigene Analysis Sequence Quality Control Requirements

Oregon DEQ defines an analytical sequence performed for qPCR as; standards, samples and the quality control elements assayed on a 96-well plate using identical production lots of reagents and consumables within a single 24 hour period. Each analytical sequence includes the minimum following elements:

- 1 Continuing calibration verification standard (CCV – Positive Control)
- 1 Low-Level Calibration Verification standard (LowCV – MRL Control)
- 1 Instrument Blank (CCB/NTC - No Template Control)
- 1 IAC Assay per standard and sample

11.3.3 SYBR® Analysis Sequence Quality Control Requirements

Oregon DEQ defines an analytical sequence performed for qPCR as; standards, samples and the quality control elements assayed on a 96-well plate using identical lots of reagents and consumables within a single 24 hour period. Each analytical sequence includes the minimum following elements:

- Calibration Curve
- 3 No Template Control wells (NTC/IBL)
- 1 Serial Dilution per non-zero sample
- Melting Curve Analysis

11.3.4 Melting Curve Analysis

Every SYBR® qPCR assay must have a melting curve analysis program added after the amplification cycles. The program should start 3-5°C above the annealing temperature (ex. 65°C) and ramp to 97°C, measuring the fluorescence at a rate of 1% (at least one read every 0.3°C).

Method-acceptable real-time PCR thermocyclers, like the Applied Biosystems StepOnePlus, are equipped with programs that determine melting point curves. The melting temperature, Tm, of nucleic acids is affected by properties like amplicon length and GC content, as well as master mix composition. Non-specific targets may have a different Tm versus the specific target, and melting point curves should be run at the end of SYBR® assays to confirm amplicon specificity.

See Appendix C for melting curve data analysis procedure and examples.

Probe-based qPCR (TaqMan®) methods cannot be used to determine melt curves.

11.3.5 Internal Amplification Control (IAC) Analysis

For assays utilizing the IAC template, the analytical sequence C_T value is obtained from the C_T of the first NTC (CCB). The analytical NTC must be within 2.25 cycles of the ICAL NTC. Sample C_T values cannot be offset by more than 1.5 units when compared to the analytical batch NTC.

11.4. Corrective Actions for QC Failures

Table 5: List of corrective actions for QC failures.

QC Element	Corrective Action
Replicate Wells	For each set of well replicates, the SD is calculated from the C_T values. The SD of the C_T values for calibration standards must meet criteria stated in Section 10.2. The SD of the C_T values for all field and QC samples must meet a SD of ≤1.5. If SD fails, reanalyze QC or field sample; for concentrations less than the confirmed LOQ in a field sample, reanalysis is not necessary. If the failure is in the calibration curve, the entire analysis batch must be reanalyzed.
No Template Control (NTC/CCB)	If NTC recovery is ≥ the LOQ, this indicates master mix or contamination during plate preparation. The source of contamination should be identified and eliminated. Once determined, the source should be documented, and the samples in the batch reanalyzed, if the contamination can be mitigated.
Method Blank (Filter Blank)	If MB recovery is ≥ the LOQ and NTC is < the LOQ, this confirms MB contamination. Flag the sample result as potentially false-positive if target detected in associated method blank and the samples (BK1::B or BK2::C). The source of contamination should be identified, documented, and eliminated.
LCS (Laboratory Control Sample/BS)	Under development. A failed recovery <50% of LCS renders the preparatory batch **invalid** and negative field samples should be listed as potentially false-negative samples and flagged as estimated (LC2::B). If the recovery is >200%, qualify sample results >RL as suspected high bias (LC1::B) and results <RL considered valid.
Matrix Spike (MS)	Under development. The matrix should be evaluated to determine the cause of the interference. If confirmed, flag parent sample as having matrix effects. If the IAC or serial dilution shows evidence of inhibition, it may be possible to report a diluted result if matrix effects are removed. A failed recovery <50% in the MS indicates the parent sample as having matrix effects and the parent sample should be listed as potentially false-negative and samples flagged as estimated. If the recovery is >200%, qualify sample results >RL as suspected high bias and results <RL considered valid.
Serial Dilution	A recovery >50% RPD of a serial dilution indicates the parent sample as potentially having matrix effects and the undiluted parent sample should be flagged as estimated. Report the result obtained from the serial dilution if: • >10 times the LOQ (otherwise DU6::A) • And no evidence of inhibition or nonspecific amplification present • Or the IAC indicates matrix effects are removed
Sample Duplicate	A recovery ≥80% RPD of a preparatory batch (filter) duplicate indicates the parent sample as potentially having matrix effects – if parent sample and dup are from the same container, flag all samples in the

QC Element	Corrective Action
	batch (DU1::B). If sample was noted as exceptionally heterogeneous during sample preparation the qualifier may be applied to only the source sample work order.
Melting Curve	Samples with a suspected non-specific target may have one or more different Tm peaks versus the specific target. If multiple peaks are present, or if the Tm is substantially different from that of the calibration standards, the sample should be flagged as estimated with high-bias (SA20::B). Examples are provided in Appendix C.
Continuous Calibration Verification standard (CCV)	A failed recovery <50% or >150% renders the analytical batch **invalid.** The cause of the failure should be identified and eliminated. Verify the PCR reagent Master Mix and cycling conditions (e.g. plate sealed, correct primers added). Assay the samples in a subsequent analysis batch.
Low-Level Calibration Verification standard (LowCV)	A failed recovery <50% or >200% renders the analytical batch invalid. The cause of the failure should be identified and eliminated. Verify the PCR plate setup equipment and process (e.g. plate sealed evenly, pipet performance, contamination, etc.). Assay the samples in a subsequent analysis batch.
IAC Assay	The matrix should be evaluated to determine the cause of the interference. If offset by 1.5-2 cycles of the sequence CCB, flag parent sample as having minor inhibitory effects (SA19::B). If the IAC or serial dilution shows evidence of major inhibition (offset by more than 2 cycles from CCB) dilute the sample until matrix effects are mitigated. If reported from dilution apply RL1::A, and set the undiluted sample to not-reportable (NR11::D).
Passive Reference Signal (BADROX)	If a well is flagged by instrument QC, confirm the results. View the multicomponent plot and review the passive reference signal for abnormalities. View the amplification plot (Rn vs. Cycle) and review the data in the C_T region for abnormalities. Examine the reaction plate, and check for condensation and/or inconsistent reaction volumes.

11.5. Contingencies for Out-of-Control Data

If the samples cannot be reanalyzed or the analysis otherwise brought under-control:

a. Identify if there is any guidance in the relevant QAPP or SAP and follow that guidance.
b. Contact the end user and verify the importance of the data and to see if the analyte can be reported as estimated.
    - If so, report the affected analyte(s) with a data qualifier and set the DQL to B.
    - If not, void the analysis and set the DQL to D.
c. Determine with the end user if an alternate sample can be used for his/her evaluation purposes.
d. If it seems appropriate in your best professional judgment, speak with the lab sample tracker or sample collector about obtaining an alternate sample that may provide the required information.
e. If the end user cannot be reached, consult with the section manager or QA officer to determine if the data should be reported as an estimate (DQL B), rejected (DQL C) or voided (DQL D).

f. In every case, the problem(s) and attempted corrections should be noted in the laboratory notebook (when instrumental problems have occurred), with the analytical batch data, and in the LIMS system for final reporting.

For water samples that cannot be processed immediately (8):

- Filter the water and then store the frozen filter (≤20°C) in lysis tubes with preservative for up to six months before DNA extraction. This results in no appreciable loss of detectable PCR signal. DQL of A.
- Water samples stored at 4°C for longer than one week is not recommended. DQL of C.
- DNA extracted and preserved in Shield (or equivalent preservative) can be stored at 4°C-25°C for up to two years without considerable loss of PCR signal. DQL of A

12. Procedure

12.1. General Considerations

When performing these procedures, properly cleaned and disinfected work areas are essential. The specifications are provided in this section for workstation separation to minimize potential sources of sample and reagent contamination.

- If the bench material is compatible, a 70% alcohol solution is used to quickly decontaminate a work surface as needed.
- Household bleach diluted to 30% with water (ca. 2% sodium hypochlorite) can damage some surfaces and equipment but is appropriate for cleaning after work is performed.
- Specialty decontaminants, like ELIMINase™ or RNAseAWAY™, are used in critical portions of the procedure and where alcohol or bleach could damage equipment or surfaces.

12.2. Water sample filtration using Swin-Lok™

1. Prepare one lysis tube for each water sample.
    a. Samples requiring unpurified lysate – use BioGX tubes as prepared by manufacturer
    b. Samples requiring purified DNA/RNA - Add 750 microliters DNA/RNA Shield (1X) to a ZR BashingBead tube and screw the cap back on.
2. Assemble the filter holder[5]
3. Remove the plunger from a clean/sterile syringe.
4. Screw the male end of the Luer Lock of the syringe body to the female Luer Lock inlet of the filter holder.
5. Shake the sample bottle vigorously for 30 seconds to distribute the cyanobacteria uniformly. Keep samples cool and away from light when not in use.
6. Pour 50 mL sample into the syringe body and reinsert the plunger.
7. While holding the device vertically, pass the sample through the filter while capturing the filtrate in a 100 mL graduated cylinder.
    - If the filter clogs due to very high cell counts, reduce the volume used.

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[5] Section 6.2

- If the filter clogs with less than 10 mL, proceed to section 12.3 and process as periphyton.
- If cell densities are very low, the volume filtered may be increased.

8. Unscrew the syringe from the filter holder, remove the plunger from the syringe and repeat steps 3-6 to obtain a recommended volume of 100 mL.
9. Fill the syringe with air, reattach to the holder and ‘dry’ the filter. Excessive liquid retained in the device and filter should be avoided.
10. Record the volume of filtrate in the graduated cylinder to the nearest 1 mL.
11. Cap the inlet and outlet and store the filter holders in a labeled container. If more samples are being filtered, keep the prepared filters cool and away from light.
12. Repeat steps until filters have been prepared for each water sample.
13. Unscrew the holder ring and carefully open the holder – the filter tends to stick to the upper cap.
14. Using sterile blunt or curved tweezers, pick up the filter and fold in half into a semicircle with the sample side facing inward, being careful to handle the filter only on the edges where the filter has not been exposed to the sample. This step usually requires a tweezer in each hand.
15. Insert the folded filter into the lysis tube and carefully push down so the cap can be screwed on.
16. Quickly vortex the sample.
17. If more samples are being preserved, keep the prepared tubes cool until storage.
18. Repeat steps until lysis tubes have been prepared for each water sample.
19. Store tubes at -20°C or ≤-70°C or continue with lysis in section 12.5.

12.3. Periphyton and ‘scum’ sample preparation

Note: Samples processed using this procedure should be qualified to account for increased LOQ and potential method bias. Samples must be collected and preserved with DNA/RNA Shield and therefore must be purified accordingly. For larger amounts of sample scale ratios proportionately.

1. Add 2 mL DNA/RNA Shield (2X) to one 5 mL PCR-Clean centrifuge tube for each sample, and any sample duplicates and blanks.
2. Shake the sample bottle vigorously for 30 seconds to distribute the cyanobacteria uniformly.
3. Use a wide-bore pipet tip to transfer 2 mL sample to the centrifuge tube containing DNA/RNA Shield (2X). Mix the tube by inverting or vortexing for 15 seconds. The sample is stable in this state for at least 30 days at temperatures between 0°C and 25°C.

   Note: If sample cannot be transferred with a pipet, the analyst may select an appropriate method to quantitatively transfer the sample to a tube. This must be recorded on the bench sheet.

4. Prepare one ZR BashingBead lysis tube for each sample.
   a. Add 750-1000 microliters preserved sample to the lysis tube and screw the cap back on.
5. Store tubes at -20°C or ≤-70°C or continue with lysis in section 12.5.

12.4. Water sample filtration using manifold

Note: This procedure is only performed when collecting sample splits to send to EPA for analysis.

1. Assemble the filtration apparatus and connect it to a vacuum line. Use a different sterilized filtration funnel and base for each sample.
2. Center a fresh filter membrane on top of the filtration support screen and attach the filtration funnel to the base.
3. Shake the sample bottle vigorously 25 times (30 seconds) to distribute the cyanobacteria uniformly. Keep samples cool and away from light when not in use.
4. Measure 100 mL[6] of water sample in a sterile graduated cylinder, transfer the sample into the funnel, and apply the vacuum. Method blank filters are prepared by substituting the 100 mL of water sample with 100 mL of sterile PBS (sample diluent).
5. Rinse the sides of the graduated cylinder and funnel with approximately 20 mL of sterile 1X PBS, transfer to the funnel, and continue filtration until filtrate has been pulled through the filter.
6. Turn off the vacuum and remove the funnel from the filter base.
7. Carefully remove the large ceramic bead from a lysis tube, keeping it in the cap.
8. Using sterile forceps, fold filter in half into a semicircle with the sample side facing inward, being careful to handle the filter only on the edges where the filter has not been exposed to the sample.
9. Insert the folded filter into the lysis tube. Tap the ceramic bead back into the tube and screw the top back on.
10. Add 600 microliters of prepared lysis buffer to the tube. Quickly centrifuge at low RCF (<6000 rpm) to settle beads and filters.
11. Store tubes at -20°C or ≤-70°C or continue with lysis in section 12.5.

12.5. Crude Nucleic Acid Extractions

Bead Beating with the FastPrep-24

1. Insert the sample lysis tubes into the bead beater tube holder. **(20 tubes max)**[7]
2. Firmly secure the holder on the spindle and close the top.
3. Bead beat a batch of tubes at low (4.0 m/s) setting for 60 seconds (thaw samples if frozen).
    a. cool tubes with icepack for 3-5 minutes
    b. repeat bead beating at medium (5.0 m/s) setting and cool again
    c. repeat bead beating at high (6.5 m/s) setting and cool before proceeding

Clarifying BioGX Lysate

1. Centrifuge tubes at 12,000 RCF for 4 minutes.
2. Transfer supernatant (~500 µL) to a 1.5 mL microcentrifuge tube with a wide-bore pipet tip.
3. Centrifuge tubes with supernatant at 12,000 RCF for 15 sec.
4. Prepare one 2 mL centrifuge tube with an Ultrafree®-MC filter for each sample.
5. Transfer supernatant to the filter – avoiding solids from the lysis beads and filter material (400 µL)
6. Centrifuge the filters at 12,000 RCF for 1 min.

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[6] Adjust accordingly: If the filter clogs due to very high cell counts or other matrix issues, reduce the volume used. If cell densities are very low, the volume filtered can be increased.

[7] The weight of more than 20 tubes may cause a system error when using the standard 24-place holder.

7. Dispose of the filters, cap the tubes and vortex briefly to mix lysate. Use a micropipettor to split the clarified lysate into two 1.5 mL low retention microcentrifuge tubes:
    a. 90 µL for initial screening analysis.
    b. 10 µL diluted with 90 µL PCR-Grade water as a 10x dilution for screening analysis.
    c. The remaining 300 µL is archived for purification - leave in the 2mL tube. Add 300 µL DNA/RNA Shield and freeze.

Clarifying DNA/RNA Shield Lysate

1. Centrifuge tubes at 12,000 RCF for 4 minutes.
2. Transfer supernatant (~700 µL) to a 1.5 mL microcentrifuge tube with a wide-bore pipet tip.
3. Centrifuge tubes with supernatant at 12,000 RCF for 15 sec.
4. Prepare one 2 mL centrifuge tube with a Zymo-Spin III-F filter for each sample.
5. Quantitatively transfer supernatant to the filter – avoiding solids from the lysis beads and filter material (400-600 µL)
    a. record volume on worksheet ‘SUBSAMPLE’ for each sample.
6. Centrifuge the filters at 8,000 RCF for 1 min.
    a. Proceed with nucleic acid purification.

12.6. Nucleic Acid Purification

Nucleic acids are extracted from sample lysate using a commercially available kit. PCR inhibitors are removed after separating and concentrating high quality DNA and RNA. The purified products are used to verify the results obtained from crude lysate, for RT-qPCR assays, or other downstream applications such as sequencing. The use of the ZymoBIOMICS™ DNA/RNA Miniprep Kit when performing this step is recommended because it has been evaluated to provide high yields with low bias in microbial communities in environmental samples. Actual abundance may not be representative if changes are made to chemical, enzymatic or physical elements of this procedure. Method performance is being evaluated based on this kit. The kit or purification procedure used must be stated along with the results.

Prepare Reagents (as needed)

- Before starting, add 96 mL 100% ethanol or isopropanol to the 24 mL DNA/RNA Wash Buffer Concentrate.
- Reconstitute the lyophilized DNase I by adding 275 µL water per vial. Mix by gentle inversion. Split into five 55 µL aliquots and store frozen at -20ºC.
- Gather all necessary reagents.

Parallel Purification of DNA and RNA

- Centrifuge steps should be performed at 12,000 RCF for 30 seconds, unless specified.

1. Add an equal amount[8] of Lysis Buffer to the samples preserved in 1X DNA/RNA Shield, cap, and vortex to mix.
2. Transfer each sample into a **yellow** Spin-Away filter in a collection tube and centrifuge.
3. Transfer the **yellow** Spin-Away Filters into a new collection tube. DNA is bound to the column. Store at room temperature, or at 4°C if not performing DNA purification immediately. (Do not freeze)

Save the flow-through – RNA is in the flow-through

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[8] The default ‘equal amount’ is assumed to be 600 µL in Step 1 and 1200 µL in Step 4.

4. Add an equal amount[7] of ethanol or isopropanol (95-100%) to the flow-through and mix well using the pipette.
5. Transfer 600 µL of flow-through into a **green** Zymo-Spin IIICG column in a collection tube and centrifuge. Discard this flow-through and reload the column with the remaining flow-through from Step 4.
6. Continue until all flow-through has been passed through the column. RNA is bound to the column.
7. Wash the columns with 400 µL Wash Buffer and centrifuge. Discard the flow-through.
8. Add 80 µL DNase I Reaction Mix (below) directly to each column. (Make as needed)
    a. 5 µL DNase I (1 U/µL)
    b. 75 µL DNA Digestion Buffer
9. Incubate the columns at room temperature (20-30ºC) for 15 minutes. While RNA columns are incubating, DNA Purification may be continued, if desired.

RNA Purification (Green Columns)

1. Add 400 µL Prep Buffer to the columns and centrifuge. Discard the flow-through.
2. Add 700 µL Wash Buffer to the columns and centrifuge. Discard the flow-through.
3. Add 400 µL Wash Buffer and centrifuge the columns for 2 minutes to remove wash buffer. Carefully transfer the columns into new 1.5 mL microcentrifuge tubes.
4. Add 100 µL DNase/RNase-Free water directly to each column, let stand for 2 minutes, and then centrifuge to elute RNA from the column.
5. Split the eluted RNA into 2 or more tubes.
6. The filtered RNA can be stored at ≤-70ºC or used immediately for RT-qPCR.

DNA Purification (Yellow Columns)

1. Add 400 µL Prep Buffer to the columns and centrifuge. Discard the flow-through.
2. Add 700 µL Wash Buffer to the columns and centrifuge. Discard the flow-through.
3. Add 400 µL Wash Buffer and centrifuge the columns for 2 minutes to remove wash buffer. Carefully transfer the columns into new 1.5 mL microcentrifuge tubes.
4. Add 100 µL DNase/RNase-Free water directly to each column, let stand for 2 minutes, and then centrifuge to elute DNA from the column.
5. Split the eluted DNA into 2 tubes.
    a. 90 µL for undiluted concentration.
    b. 10 µL diluted with 90 µL PCR-Grade water as a 10x dilution for analysis.
6. The purified DNA can be stored at ≤-70ºC or used immediately for qPCR.

12.7. Phytoxigene Assay with the StepOnePlus™

Experiment Design

Parameters have been modified to accommodate equipment cycling conditions when using the StepOnePlus. The change is meant to improve efficiency and well variation by reducing the reaction volume from the assay manufacturer’s protocol document (10).

- Experiment name: SEQUENCE-ptx
- Instrument: StepOnePlus Instrument (96 Wells)
- Experiment Type: Quantitation - Standard Curve
- Reagents: Other (Uncheck “Include Melt Curve”)
- Ramp Speed: Standard
- Passive Reference Dye: None
- Reaction Volume: 20 µL

Table 6: Instrument Parameters – Phytoxigene Assay

Target Name	Reporter	Quencher
Px_16S	FAM	None
Px_IAC	VIC	None
Px_STX	ROX	None
Px_MCY	FAM	None
Px_CYN	VIC	None

Holding Stage	Cycling Stage (Number of cycles: 40)	
Initial Denaturation	Denaturation	Annealing-Extension
95.0°C, 3 min	95.0°C, 20 sec	60.0°C, 60 sec Data Collection On

1. Turn on the StepOnePlus and begin the block/cover preheating routine.
2. Use the manufacturer's instructions[9] and the following outline to start a new experiment. A template should be saved once the test conditions are defined.
3. Open the template file defined for a Phytoxigene run (e.g. Phytoxigene_template.edt)

Prepare Master Mix (in PCR Workstation designated for Reagent Prep)

1. Allow reagents reach room temperature (about 15 minutes).
2. In the cleaned PCR Workstation, reconstitute enough tubes to cover the requirements of the number of tests being performed in the analysis batch. Rehydrate sufficient tubes in each of the respective test pouch by adding 90 µL PCR grade water.
   <u>Note: Each tube is enough for five tests.</u>
3. Quickly vortex each tube to mix, then briefly spin down to settle liquid.
4. Combine the volumes into a single tube.
5. Vortex briefly and spin down.
6. Keep the master mix cool and use immediately.

Component	Final Concentration	20 µL Reaction
Master Mix (dry) Contains enzymes, dNTP, probes and primers	1X	18 µL
Nuclease-free Water	-	-
Template DNA	-	2 µL

Prepare plate for analysis (in PCR Workstation designated for Reaction Setup)

1. Allow sample extracts and standards to reach room temperature.

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[9] *StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*

2. In the cleaned workstation, pipette 18 µL rehydrated Master Mix into each respective plate well.
3. Mix sample or standard by pulse-vortexing or flicking the microtube prior to loading.
4. Add 2 µL of respective sample or standard into each respective well. Use a plate loading worksheet to ensure correct sample placement.

   Note: Samples are analyzed by default at 10x dilution in the 16Scya and IAC wells because concentrations of gene copies typically exceed the calibration range.
5. Seal the plate with optical film and spin for 1-2 minutes in a plate centrifuge.

Start the run

1. Open the instrument drawer.
2. Place the reaction plate in the sample block. Orient the well A1 in the back left corner.
3. Close the instrument drawer carefully.
4. In the instrument software, open the previously defined experiment and click the “Run” pane.
5. Click “Start Run”

Finishing the run

1. After the instrument completes a run, open the instrument drawer and remove the plate.
2. Carefully close the instrument drawer.
3. Save the instrument experiment file to the local computer as SEQUENCE-ptx.eds then transfer the saved file to the deqlead-lims directory for qPCR data.
4. Follow the data processing instructions in the Phytoxigene calculation template excel file to export data and format it for Element data upload.

12.8. SYBR® qPCR Assay with the StepOnePlus™

Experiment Design

- Experiment name: SEQUENCE-ASSAY
- Instrument: StepOnePlus Instrument (96 Wells)
- Experiment Type: Quantitation - Standard Curve
- Reagents: SYBR® Green Reagents (Check “Include Melt Curve”)
- Ramp Speed: Standard
- Passive Reference Dye: ROX
- Reaction Volume: 20 µL

Table 7: Instrument Parameters – SYBR qPCR anaC

Holding Stage	Cycling Stage (Number of cycles: 33)		Melt Curve
Initial Denaturation	Denaturation	Annealing-Extension	Continuous
95.0°C, 5 min	95.0°C, 20 sec	62.0°C, 60 sec Data Collection On	62.0°C, 1 min Ramp @ 1% to 95°C

Table 8: Instrument Parameters – SYBR qPCR 16Scya (*Under Development)

Holding Stage	Cycling Stage (Number of cycles: 40)			Melt Curve
Initial Denaturation	Denaturation	Annealing	Extension	Continuous
95.0°C, 2 min	95.0°C, 20 sec	60°C*, 30 sec	72.0°C, 20 sec Data Collection On	55.0°C*, 1 min Ramp @ 1% to 97°C

Table 9: Instrument Parameters – SYBR qPCR cyr7

Holding Stage	Cycling Stage (Number of cycles: 40)			Melt Curve
Initial Denaturation	Denaturation	Annealing	Extension	Continuous
95.0°C, 2 min	95.0°C, 20 sec	62.8°C, 30 sec	72.0°C, 20 sec Data Collection On	67.0°C, 1 min Ramp @ 1% to 97°C

Table 10: Instrument Parameters – SYBR qPCR mcyA

Holding Stage	Cycling Stage (Number of cycles: 40)			Melt Curve
Initial Denaturation	Denaturation	Annealing	Extension	Continuous
95.0°C, 3 min	95.0°C, 20 sec	60.0°C, 30 sec	72.0°C, 20 sec Data Collection On	65.0°C, 1 min Ramp @ 1% to 97°C

Table 11: Instrument Parameters – SYBR qPCR stxA

Holding Stage	Cycling Stage (Number of cycles: 40)			Melt Curve
Initial Denaturation	Denaturation	Annealing	Extension	Continuous
95.0°C, 3 min	95.0°C, 20 sec	60.3°C, 30 sec	72.0°C, 20 sec Data Collection On	62.0°C, 1 min Ramp @ 1% to 97°C

1. Turn on the StepOnePlus and verify the block is preheating.
2. Use the manufacturer's instructions[10] and the following outline to start a new experiment. A template should be saved once the test conditions are defined.
3. Open the template file defined for a SYBR run (e.g. anaC_template.edt)
4. Verify the assay target matches the "Target Name" with the reporter as "SYBR"

Prepare Master Mix (in PCR Workstation designated for Reagent Prep)

1. Allow reagents reach room temperature (about 15 minutes).

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[10] *StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*

2. In the cleaned PCR Workstation, reconstitute enough master mix to cover the requirements of the number of tests being performed in the analysis batch.

Table 12: SYBR Master Mix – anaC

Component	Final Concentration	20 µL Reaction	For a 96 well Plate
Master Mix (2X)	1X	10 µL	1000 µL
Forward Primer (10 µM)	0.15 µM	0.3 µL	30 µL
Reverse Primer (10 µM)	0.15 µM	0.3 µL	30 µL
Nuclease-free Water	-	7.4 µL	740 µL
Template DNA	< 100 ng	2 µL	-

Table 13: SYBR Master Mix – 16Scya, cyr7, mcyA, stxA

Component	Final Concentration	20 µL Reaction	For a 96 well Plate
Master Mix (2X)	1X	10 µL	1000 µL
Primer Pool (10 µM)	0.2 µM	0.4 µL	40 µL
BSA (5 mg/mL)	0.2 mg/mL	0.8 µL	80 µL
Nuclease-free Water	-	6.8 µL	680 µL
Template DNA	< 100 ng	2 µL	-

3. Vortex briefly and spin down.
4. Keep the master mix cool and use immediately.

Prepare plate for analysis (in PCR Workstation designated for Reaction Setup)

1. Allow sample extracts and standards to reach room temperature.
2. In the cleaned workstation, pipette 18 µL rehydrated master mix into each respective plate well.
3. Mix sample or standard by pulse-vortexing or flicking the microtube prior to loading.
4. Add 2 µL of respective sample or standard into each respective well. Use a plate loading worksheet to ensure correct sample placement.
5. Seal the plate with optical film and spin for 1-2 minutes in a plate centrifuge.

Start the run

1. Open the instrument drawer.
2. Place the reaction plate in the sample block. Orient the well A1 in the back left corner.
3. Close the instrument drawer carefully.
4. In the instrument software, open the previously defined experiment and click the “Run” pane.
5. Click “Start Run”

Finishing the run

1. After the instrument completes a run, open the instrument drawer and remove the plate.
2. Carefully close the instrument drawer.
3. Save the instrument experiment file to the local computer as SEQUENCE-ASSAY.eds then transfer the saved file to the deqlead-lims directory for qPCR data.
4. Export run data by selecting the Export Properties tab and select ‘Results’ only.
5. Change the Export File Name to the following format: SEQUENCEID-ASSAY_data
6. Change the Export File Location to the desired directory.
7. Export the data in both .XLS and .TXT format by clicking the Start Export button
8. Close the Export dialog
9. In the File menu select Print Report to open the run report dialog
10. Select all content checkboxes except ‘Amplification Plot (CT vs Well)’
11. Print the report as a PDF and save in the desired directory.

12.9. SYBR® RT-qPCR Assay with the StepOnePlus™

Experiment Design

- Experiment name: YYYYMMDD_ASSAY_RT
- Instrument: StepOnePlus Instrument (96 Wells)
- Experiment Type: Quantitation – Standard Curve
- Reagents: SYBR® Green Reagents (Check “Include Melt Curve”)
- Ramp Speed: Standard
- Passive Reference Dye: ROX
- Reaction Volume: 20 µL

Table 14: Instrument Parameters – SYBR RT-qPCR

Holding Stages			**Cycling Stage (Number of cycles: 45)**		**Melt Curve**
UNG Incubation	Reverse Transcription	Initial Denaturation	Denaturation	Annealing-Extension	Step and Hold
25.0°C, 10 min	55.0°C, 10 min	95.0°C, 60 sec	95.0°C, 10 sec	60.0°C, 60 sec Data Collection On	60.0°C, 1 min Ramp @ 0.3°C to 95°C

1. Turn on the StepOnePlus and verify the block is preheating.
2. Use the manufacturer’s instructions[11] and the following outline to start a new experiment. A template should be saved once the test conditions are defined.
3. Open the template file defined for a SYBR-RT run (e.g. mcyA_RT_template.edt)
4. Save as a new experiment, with the unique current analytical batch name.

Prepare Master Mix (in PCR Workstation designated for Reagent Prep)

1. Allow reagents reach room temperature (about 15 minutes).

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[11] *StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*

2. In the cleaned PCR Workstation, reconstitute enough master mix to cover the requirements of the number of tests being performed in the analysis batch.

Component	Final Concentration	20 µL Reaction	For 36 wells
One-Step Reaction Mix (2X)	1X	10 µL	400 µL
WarmStart RT Enzyme Mix (20X)	1X	1 µL	40 µL
Thermolabile UDG (1 unit/µL)	0.025 units/µL	0.5 µL	20 µL
Forward Primer (10 µM)	0.4 µM	0.8 µL	32 µL
Reverse Primer (10 µM)	0.4 µM	0.8 µL	32 µL
Nuclease-free Water	-	4.9 µL	196 µL
Template RNA	< 1 µg (Total)	2 µL	-

3. Vortex briefly and spin down.
4. Keep the master mix cool and use immediately.

Prepare plate for analysis (in PCR Workstation designated for Reaction Setup)

1. Allow sample extracts and standards to reach room temperature.
2. In the cleaned workstation, pipette 18 µL rehydrated master mix into each respective plate well.
3. Mix sample or standard by pulse-vortexing or flicking the microtube prior to loading. Purified samples generally do not need dilution.
4. Add 2 µL of respective sample or standard into each respective well. Use a plate loading worksheet to ensure correct sample placement. Each sample and QC undergoing RT should be assayed in triplicate to account for variation.
5. Seal the plate with optical film and spin for 1-2 minutes in a plate centrifuge.

Start the run

1. Open the instrument drawer.
2. Place the reaction plate in the sample block. Orient the well A1 in the back left corner.
3. Close the instrument drawer carefully.
4. In the instrument software, open the previously defined experiment and click the “Run” pane.
5. Click “Start Run”

Finishing the run

1. After the instrument completes a run, open the instrument drawer and remove the plate.
2. Carefully close the instrument drawer.
3. Save the instrument experiment file to the local computer as SEQUENCE-ASSAY.eds then transfer the saved file to the deqlead-lims directory for qPCR data.
4. Export run data by selecting the Export Properties tab and select ‘Results’ only.
5. Change the Export File Name to the following format: SEQUENCEID-ASSAY_data
6. Change the Export File Location to the desired directory.
7. Export the data in both .XLS and .TXT format by clicking the Start Export button
8. Close the Export dialog
9. In the File menu select Print Report to open the run report dialog

10. Select all content checkboxes except ‘Amplification Plot (CT vs Well)’
11. Print the report as a PDF and save in the desired directory.

13. Calculations

13.1. Quantitation

The data system will calculate the concentrations of all detected targets as well as the range of the IAC using the linear calibration curves generated during initial calibration. Other data of interest: cycle threshold (C_T) in all analytical runs and melt temperature for all target “Hits” will be calculated by the data system.

When well replicates are used, the mean concentration value and cycle threshold is calculated by the data system. The mean concentration must be used for reporting sample results and in all method calculations and for evaluating results against QC limits. The standard deviation of C_T in the well replicates must meet the requirements in Section 11.1, Table 4 to be considered acceptable.

13.2. Exceeding the Calibration Range

If a sample result exceeds the range of the calibration curve or exhibits amplification inhibition, dilute an aliquot of the lysate or purified sample with PCR-grade water and reanalyze in a subsequent analysis batch.

Incorporate the dilution factor into the final concentration calculations in LIMS. For example, a 10x dilution would be entered by adding ‘@10’ to the end of the sample ID in the instrument software and is interpreted by Element. Report the dilution factor with the sample result.

Report over-range samples as estimated using the J1::B qualifier if the IAC also passes recovery.

13.3. Equations

Equation 1: Sample Concentration

$$Sample\ Result\ (copies/mL) = \frac{R_I \times V_L \times C \times D}{V_I}$$

Where:
R_I = Initial Result (copies/µL)
V_I = Initial Volume Filtered (mL)
V_L = Volume of Lysate (µL)
C = Cleanup Factor, if used
D = Dilution Factor, if used

Equation 2: Cleanup Factor

$$Cleanup\ Factor = \frac{Final\ Vol.Eluted\ (\mu L)}{Initial\ Vol.Extracted\ (\mu L)}$$

Note: Element cleanup batches must be entered in reverse to correctly calculate as a concentration factor instead of as a dilution factor. In batch entry, the 'Initial (mL)' column is the final eluted volume and the 'Final (mL)' column is the initial volume purified. Verify the factor during data review in the analytical sequence.

Equation 3: Copy Number

$$copy\ number\ (as\ dsDNA) = (M \times N)/(L \times D + E)$$

Where:
M = Concentration of nucleic acid detected (g/mL)
N = Avogadro's number (6.022×10^{23} molecules/mol)
L = Length of nucleic acid, in base pairs (bp; total length of oligo or plasmid and insert)
D = Conversion from base pairs to molecular weight (avg. dsDNA per bp = 617.96 g/mol)
E = Molar mass of the 2 hydroxy and 2 –H at the ends (dsDNA = 36.04 g/mol)

Equation 4: Amplification Standard Curve

$$C_T = m \log Q + b$$

Where:
C_T = Cycle threshold
m = Slope of regression line
Q = Quantity of standard
b = y-intercept of regression line

Equation 5: Amplification Efficiency

$$Amplification\ Efficiency\ (Eff\%) = \left(10^{-1/m} - 1\right) * 100\%$$

Where:
m = Slope of regression line from standard curve

Equation 6: Precision and Accuracy

$$\mathrm{RPD\%} = \frac{|X_s - X_r|}{0.5 \times (X_s + X_r)} \times 100\%$$

Where:
X_s = Result for the sample
X_r = Result for the replicate/duplicate sample.

$$\mathrm{R\%} = \frac{AV}{TV} \times 100\%$$

Where:
AV = Analyzed value
TV= True value

Equation 7: Limit of Quantitation[12]

Calculate the mean and standard deviation of the seven LCS replicates spiked at the estimated LOQ of the method. The LOQ is verified if the mean percent recovery of the replicates is 50-200% of the true value, with a ≥95% degree of confidence (%CI), based on the following equation:

$$\%\mathrm{CI} = \left(\bar{x} \pm 1.96\frac{s}{\sqrt{n}}\right) \times 100\%$$

Where:
$\bar{x}$ = Mean of the replicates
s = Standard deviation of replicates
n = Number of replicates

Note: 1.96 is the Z-score corresponding to a confidence level of 95%.

Equation 8: Matrix Spike Recovery

$$R\% = \frac{(A - B)}{C}\ \mathrm{X}\ 100$$

Where:
A = Measured concentration of the MS
B = Measured concentration in the unspiked sample
C = Spiked concentration

14. Records Management

Laboratories shall maintain SOPs that accurately reflect all phases of current laboratory activities, such as assessing data integrity, corrective actions, and all methods. Each SOP shall clearly indicate the effective date of the document, the revision number, and the signature(s) of the approving authority. Documents that contain sufficient information to perform the tests do not need to be supplemented or rewritten as internal procedures if the documents are written in a way that they can be used as written. Any changes, including the use of a selected option, shall be documented, and included in the laboratory's records. The laboratory shall retain all records necessary to facilitate reconstruction of the preparation, processing, and reporting of analytical results for samples for a minimum of ten (10) years.

The following data files generated by the instrument must be saved onto the instrument computer and data server.

1. Instrument Report, .pdf format (Name convention YYYYMMDD_ASSAY)
   a. Experiment Summary
   b. Results Summary
   c. Amplification Plot (ΔRn vs. Cycle)
   d. Standard Curves
   e. Results Table (By Well)
   f. QC Summary
   g. Melt Curve (Derivative Reporter)

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[12] Clinical and Laboratory Standards Institute. Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline. Wayne, PA USA: CLSI; CLSI document EP17. 2004.(15)

2. Export Data, .xls format (Name convention YYYYMMDD_ASSAY)
    a. Results

Data files must be backed up to the instrument server on a regular basis and should be done at least monthly. Data are to be collated based on run order and combined with the following documentation:

1. **Analytical Data Review Checklist**
2. **Preparation Benchsheet(s)** with reagent and consumable lot information.
3. **Cleanup Batch Benchsheet(s)** with reagent and consumable lot information.
4. **Plate Template** showing assay plate layout, with reagent and consumable lot information.
5. **Instrument Report** listing the run date and time, well position, sample ID, target(s) assayed, C_T mean and std dev, concentration with calculated mean and std dev. and Tm peaks.
6. **Amplification Plot** (ΔRn vs. Cycle) showing the cycle threshold.
7. **Standard Curve**, showing run date and time, assay target, slope, Y-intercept, R^2, and Eff%
8. **Melt Curve** by derivative reporter.

The preceding documentation is put into the order listed above and is provided to a second chemist who performs a review. After the initial review, the data packet is stored with previously generated instrument data and sorted chronologically. Secondary data review will be performed by a chemist meeting the minimal requirements of a Chemist 2. Ideally, QA and Manager Review will be performed on all data. Access to the data is limited to DEQ employees since the facility is secured. Data packets should be maintained in accordance with the State records retention schedule.

15. Method Performance

The latest method validation data may be found in the Organics Section of the Shared directory:

\\deqlab1\sp-organics\method validation data

16. Maintenance

Daily inspection of the qPCR thermal cycler is a good practice prior to operating the instrument. Any instrument maintenance and inspections should be recorded in the maintenance logbook.

Use the instrument software to view a summary of maintenance data for the instrument. To view the maintenance information, select Instrument → Instrument Maintenance Manager.

The following maintenance should be performed weekly, as needed:

- Check the computer disk space. If necessary, archive or back up experiment files to the server.
- Power cycle the computer controlling the instrument.
- Clean the instrument surface with a lint-free cloth.

The following maintenance should be performed monthly:

- Perform a background calibration to check for contamination.

- If a high fluorescent background is observed and is suspected to be contamination, see maintenance manual[13] pp. 145-147 and 164 for the decontamination procedure.

The following maintenance should be performed every 18 months:

- Perform a spatial calibration and a dye calibration to verify optical performance.

17. Pollution Prevention

Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

No solvents are utilized in this method except the very small volumes of ethanol and isopropanol required to purify nucleic acids. The only other chemicals used in this method are the materials used in preparing standards, sample preservatives, and PCR reagents. All are used in very small amounts and pose little or no danger to the environment after being autoclaved.

For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 115[5] 16th Street N.W., Washington D.C. 20036, 202.872.4477.

18. Waste Management

The Oregon Department of Environmental Quality requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions.

Waste Disposal: All samples, standards and sample extracts that are no longer of use and that meet the criteria to be defined as hazardous waste must be treated and/or disposed of as such following all appropriate procedures/protocols in the Lab. Concentrated nucleic acids, used assay plates, pipet tips and tubes can be collected and autoclaved at 250°F for 15 minutes at 15 PSI to deactivate any potentially hazardous enzymes or organisms.

The aqueous liquid (water sample) that remains in the sample bottles following analysis should be removed from the bottle and the bottle can then be recycled. The liquid should be disposed of in the sink.

For further information on waste management consult The Waste Management Manual for Laboratory Personnel, available from the American Chemical Society.

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[13] *StepOne™ and StepOnePlus™ Real-Time PCR Systems: Installation, Networking, and Maintenance* (14)

19. Definitions

Standard Definitions applicable to laboratory quality systems can be found in Appendix A of the LEAD Quality Manual DEQ91-LAB-0006-LQM

Specific definitions are explained below.

Amplicon: A segment of DNA amplified during PCR.

Amplification: In a PCR instrument run, the step in which extra copies of a gene or a DNA sequence are formed. Fluorescence data are collected during amplification and displayed in the amplification plot.

Amplification Efficiency (EFF%): In PCR, an efficiency of 100% is obtained when each target sequence present in the PCR reaction doubles during each round of amplification. A slope close to -3.32 indicates optimal efficiency.

Cycle Threshold (C_T): The PCR cycle at which the fluorescence meets the threshold in the amplification plot.

Complementary: A strand of DNA or RNA that is capable of forming specific pairing of the purine and pyrimidine base sequences with those of a second strand.

Expressed: A gene able to function (active) within a cell in such a way that the gene product is formed.

GC content: In DNA (or RNA), the molar percentage of bases in a segment that are guanosine-cytosine.

Gene: A unit of heredity; a segment of DNA specifying a particular protein, polypeptide, tRNA, or rRNA.

Master Mix: Solution containing all reagents (except for the test or sample nucleic acid) that is required to perform PCR.

Melting Temperature (Tm): The temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA.

No Template Control (NTC): Well assayed with water or buffer instead of sample. No amplification should occur in negative control wells.

Passive Reference: A dye that produces fluorescence signal used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations and minor well-to-well differences. ROX dye is used as the passive reference in the StepOnePlus™.

Primer: A segment of DNA that is complementary to a given DNA sequence and that is needed to initiate replication by DNA polymerase.

Probe: A fluorescently labeled substance, such as TaqMan®, that is designed to detect or identify amplification of a target sequence in a sample.

Ramp Speed: The rate at which the temperature changes during the instrument run.

Reporter: A fluorescent dye used to detect amplification.

RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction. In 1-Step RT-qPCR, an enzyme that coverts RNA to cDNA is added to the qPCR reaction mix.

Target: See Amplicon.

Total: A measurement of gene copies amplified in the PCR by a primer and used for broad coverage of potential targets, either due to the result of a common evolutionary origin or toxin synthetase gene cluster.

20. Deviations from Referenced Methods

NA.

21. References

1. *Use of qPCR and RT-qPCR for monitoring variations of microcystin producers and as an early warning system to predict toxin production in an Ohio inland lake.* **Lu, Jingrang, et al.** March 1, 2020, Water Research, Vol. 170, p. 115262.

2. **U.S. EPA.** *Molecular Marker Analysis for HAB (D-EMMD-MEB-SOP-2372-0).* May 2017.

3. *Quantitative PCR detection and improved sample preparation of microcystin-producing Anabaena, Microcystis and Planktothrix.* **Hautala, Henna, et al.** 2013, Ecotoxicology and Environmental Safety, Vol. 87, pp. 49-56.

4. *Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water.* **Schober, E and Kurmayer, R.** 4, 2006, Letters in applied microbiology, Vol. 42, pp. 412-417.

5. *Multi-laboratory survey of qPCR enterococci analysis method performance in US coastal and inland surface waters.* **Haugland, R A, et al.** 2016, Journal of microbiological methods, Vol. 123, pp. 114-125.

6. **Bordner, Robert, Winter, John A and Scarpino, Pasquale.** *Microbiological Methods for Monitoring the Environment: Water and Wastes.* Environmental Monitoring and Support Laboratory, U.S. EPA Office of Research and Development. Washington, D.C., EPA-600/8-78-017 : s.n., 1978.

7. **USGS.** *Guidelines for Design and Sampling for Cyanobacterial Toxin and Taste-and-Odor Studies in Lakes and Reservoirs.* 2008. Technical Report 2008-5083.

8. *Recommendations for the processing and storage of water samples before polymerase chain reaction (PCR) analysis.* **Gilpin, BJ, et al.** 4, 2013, New Zealand Journal of Marine and Freshwater Research, Vol. 47, pp. 582-586.

9. **U.S. EPA.** *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples (EPA 815-B-04-001).* October 2004.

10. **Phytoxigene, Inc.** 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 environmental samples. Version 9 August 2019.

11. **U.S. EPA.** *EPA Method 1609.1: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay (EPA-820-R-15-099).* April 2015.

12. **Applied Biosystems, Inc.** StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments (PN 4376784 Rev. F). August 2010.

13. **Thermo Fisher Scientific.** Qubit™ 4 Fluorometer User Guide, Pub. No. MAN0017209. April 9, 2018.

14. **Applied Biosystems, Inc.** StepOne™ and StepOnePlus™ Real-Time PCR Systems: Installation, Networking, and Maintenance (PN 4376782 Rev. H). February 2016.

15. **Clinical Laboratory Standards Institute.** Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline. *CLSI document EP17.* Wayne, PA : CLSI, 2004.

22. Revision History

Revision	Date	Changes	Editor
1.0	2023	New Method	KMC

Appendix A: Job Safety Assessment

Hazard Analysis	**Activity:**	Organics HAB Analysis
	Program/Location	DEQ Laboratory
	Position # (s):	3012, 3437
	Analysis by:	Health & Safety
	Date:	04/26/2023

Required PPE:

Gloves - Nitrile	Gloves - Thermal	Gloves – Cut Resistant	Safety Glasses	Lab Coat
				

Required/Recommended Trainings:

1. Review of Chemical Hygiene Plan
2. SIM-plicity Training
3. Review of relevant lab SOPs
4. Compressed Gas Safety Training
5. PPE Policy Review

1. TASK	HAZARDS	SEVERITY	CONTROLS
2. Computer Use/Data Entry	Repetitive motion injuries	Low	Follow ergonomic recommendations
3. Sample removal from refrigerators	(E) – Wet and cold temperatures (CW) – Glass shards from broken sample vials; unknown samples and sample preservatives	Medium Medium	Gloves; Use thermal gloves as necessary Inspect extracts for hazards prior to removing contents
4. Sample Preparation and Analysis	(E) – Microcystin exposure from preparing standards from dry, neat sources:	Very High	Always work in approved hood wearing appropriate PPE: lab coat, safety glasses/goggles, gloves suitable for chemicals in use. Among cyanotoxins are some

	unknown sample contaminant exposure;		of the most powerful natural poisons known, including poisons which can cause rapid death by respiratory failure. The toxins include potent neurotoxins, hepatotoxins, cytotoxins, and endotoxins. All samples should be handled with extreme care utilizing all PPE listed above.
	(CW) – Glass shards from broken sample vials, glass pipettes or needles on microsyringes.	Low	Inspect glassware and extract vials prior to handling. Store syringes in a safe manner to prevent accidental punctures.
	(CW) – shock hazard from electrical components	Low	Turn off and unplug all instruments prior to performing any work that may result in a shock hazard, such as board replacement or repair.

* Codes for Potential Hazards

(BIO) Biological		(CO) Caught On		(FS) Fall – Same Level
(CB) Contacted By		(CW) Contact With		(OE) Overexertion
(CBT) Caught Between		(E) Exposure		(SA) Struck Against
(CI) Caught In		(FB) Fall To Below		(SB) Struck By

Risk Severity Level Key	Low	Medium	High	Very High

Appendix B: List of Primers and Intended Target Sequences

Table 15: Phytoxigene Assay Targets

Target	Specificity[14]	Target Sequence
Px_16S	Cyanobacteria	16S rRNA gene
Px_IAC	Quality Control	Internal Amplification Control (IAC)
Px_CYN	Cyanobacteria	Cylindrospermopsin synthetase gene (cyrA)
Px_MCY	Cyanobacteria	Microcystin or nodularin synthetase gene (mcyE/ndaF)
Px_STX	Cyanobacteria	Saxitoxin synthetase gene (stxA)

Table 16: SYBR® Assay Targets

Target	Specificity	Target Sequence	Reference
16Scya	Cyanobacteria	Ribosomal 16S subunit RNA gene (16Scya)	Shaw et al. (1999)
AnaC	Cyanobacteria	Anatoxin synthetase gene (anaC)	
Cyl7	Cyanobacteria	Cylindrospermopsin synthetase gene (cyrA)	
mcyAcya	Cyanobacteria	Microcystin synthetase gene (mcyAcya)	Hisbergues et al., 2003
mcyA-Cd	Cyanobacteria	Microcystin or nodularin synthetase gene (mcyA/ndaA) *Anabaena, Microcystis, Nostoc, Nodularia, Planktothrix*	Hisbergues et al., 2003 Rantala et al., 2004
StxA	Cyanobacteria	Saxitoxin synthetase gene (stxA) *Aphanizomenon, Anabaenopsis, Chrysosporum, Cuspidothrix, Cylindrospermum Dolichospermum, Geitlerinema, Phormidium, Scytonema*	Ballot et al., 2010
stxB	Cyanobacteria	Saxitoxin synthetase gene (stxB)	Savela et al., 2015

Primers have been selected from referenced methods and should amplify only the target of interest. According to the EPA guidelines for PCR, the primers should meet the following:

- Length of 18 to 27 base pairs
- Melting tempe™res (Tm) less than 3°C different

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[14] Specificity shown in Tables 1-3 of "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 environmental samples. (Ver. 9)"

- GC content between 40% and 70%
- No guanine-cytosine (GC) stretches greater than four base pairs
- Avoid primer-dimer formation
    - No homology within or between primers, especia'ly at the 3' end.
- No hairpin loops with an ΔG energy of -0.5 kcal/mol or less

Primers and standards may be purchased from commercial sources. Primers should be desalted. Additional purification by HPLC or PAGE may not be necessary.

Table 17: SYBR® Primers

Target Name	bp	Tm (°C)	(Oligo Name) Primer'Se'uence, 5'-3'	Optimal Anneal [15](°C)
16Scya	512	59*	(16ScyaF) TGTAAAACGACGGCCAGTCCAGACTCCTACG	48.0*
			(16ScyaR) CGCGTTAGCTACGGCACGGCTC	
AnaC	190	81.4	(AnaCF1) GGTCCTGGGTTGATGACAGG	60.7
			(AnaCR1) CGGTAGCCCCGACTCTTAATC	
Cyl7	112	81.6	(cyrAf7) AACACGGCTTTGAGGTCTATC	60.6
			(cyrAr7) GAAGTAATCCTCACAGGTTCCC	
mcyAcya	99	75.2	(mcyAf1) AAAAGTGTTTTATTAGCGGCTCATT	56.4
			(mcyAr1) TCTAACCGTCCATTAGAGACTAAACC	
mcyA-Cd	*	*	(mcyA-Cd1F) AAAATTAAAAGCCGTATCAAA	*
			(mcyA-Cd1R) AAAAGTGTTTTATTAGCGGCTCAT	
SxtA	110	81.0	(SxtAF1) GCGGGACTTTATGCTCTACTAC	60.3
			(SxtAR1) TACTCCGTCATCGGCATTTG	
stxB	*	*	(sxtB_F2) TGTTGTGCTTGCTGCTCTATCAG	*
			(sxtB_R2) CAGCGTTTTCAGCGTAYCGAC	

Table 18: Synthetic DNA Standard Sequences

Standard Name	Sequence Properties	Reference Target(s)
AnaC-sp54	897 bp 44.37%GC 554081.2 AMU	Anabaena sp. 54 AnaC (anaC) gene GenBank: JF803646.1
5'- TGAGCGAACAAGTGAGTTGTTCGGAAAAGTCTTCCCATGGTCAGAGGTTTTACAAGCTCCTTGCA GCCCG CCACTAGACCCACATCTGATTGAAAACGACTTAGCTTATATTCTTTATACATCTGGCTCAACAGGAA CCC CCAAAGGGGTGATGATTAGCCATCGTGCCTCTCTTACTTTTGTAAATTGGGCCTATGATACGTTTC AGGT GACTGCGGAAGACCGTGTCTCCAGTCATGCACCGTTCCATTTTGATCTTTCCATTTTTGATATCTTC ACA		

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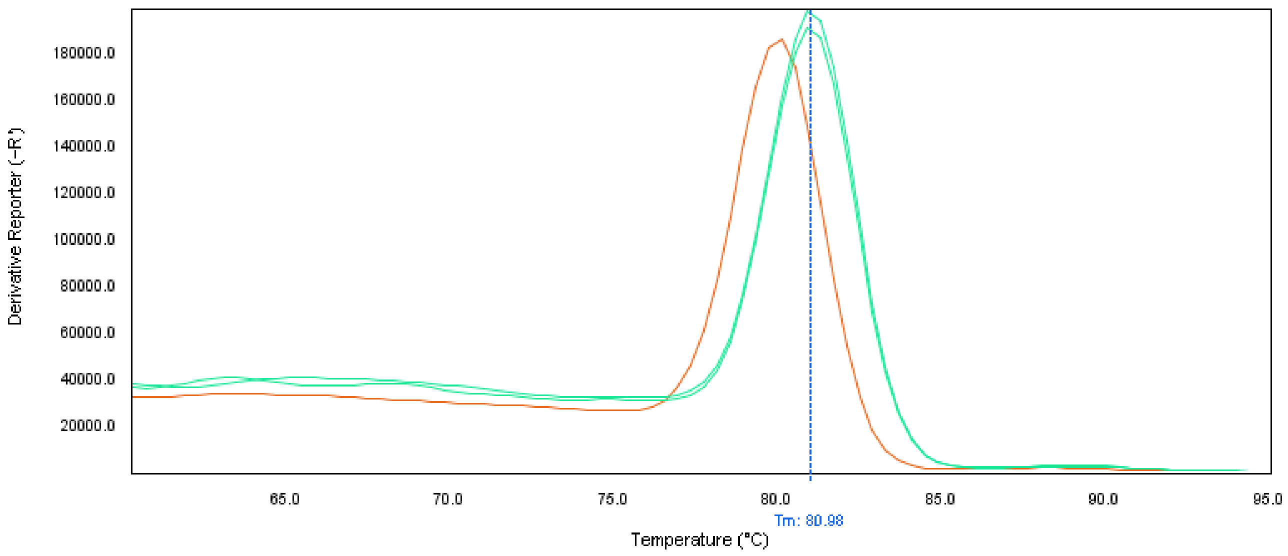
[15] Rychlik W, Spencer WJ, Rhoads RE (1990) Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res 18(21):6409–6412.

Standard Name	Sequence Properties	Reference Target(s)
ACTATCAAAGCAGGGGGTACGGTCATTTTGCTCTCGGCGGCTTTATCAGTCTTTCCAATTAATCTGGCCA AATTTATTGCCGCAGAAAGGATTTCAATCTGGTATTCAGTCCCCTCTATTCTGACAAATTTAGTCCTATA TGGTCGGTTAGAGCAACATACCTACCCCGATCTTAGAAGCATTCTGTTTGCAGGTGAAGTTTTTCCTAAC AAATATTTGCATCAGTTGATGGTGCATATTCCAGATGCTGGCTATTACAACCTCTATGGTCCGACGGAGA CAAATGTCTGCACATATTACCGAGTCTCACCGCCCGATATTGAAACAAGTGAAGCAGTTCCTATTGGACA AGCCTGTGCCAACACTGAAGTTTTTGTCTTGGGTACAAGTAATGAATTGGTAGCTAGAGGTGAAGTGGGT GAGCTTTGCGTGCGCGGTCCTGGGTTGATGACAGGCTATTGGGATTTGCCTGAAAAGACTGCTCAAGTCC TTGTTCCTTTTACATTGCATCATGGATTGGGATCGGAGATGATTTACCGCACGGGGGATCTTGTTAAACA ATCTCCAGAGGGTGATTATATCTTTTTAGGTCGTAGAGATCGCATGATTAAGAGTCG -3’		

Appendix C: Melting Curve Analysis Procedure

Melt curve analysis will be performed on samples with detectable amplification in the SYBR® assay to assess whether any nonspecific amplification is affecting results.

Figure 1: Melt curve with minor Tm difference in sample



The example shown in Figure 1 shows an acceptable melt profile comparing a known amplicon, in red, with the melt profile from the amplified material in a sample, in aqua. The single peak for the standard material (80°C) demonstrates the amplification of a single product from the primers used in the reaction. Likewise, the single peak of the unknown sample (81°C) is indicative of a valid amplification with a minor difference in the melting temperature. This change in melting temperature may be indicative of population-specific gene variants present in the sample.

Figure 3: Melt curve with valid target peak and non-target amplification at higher temperature

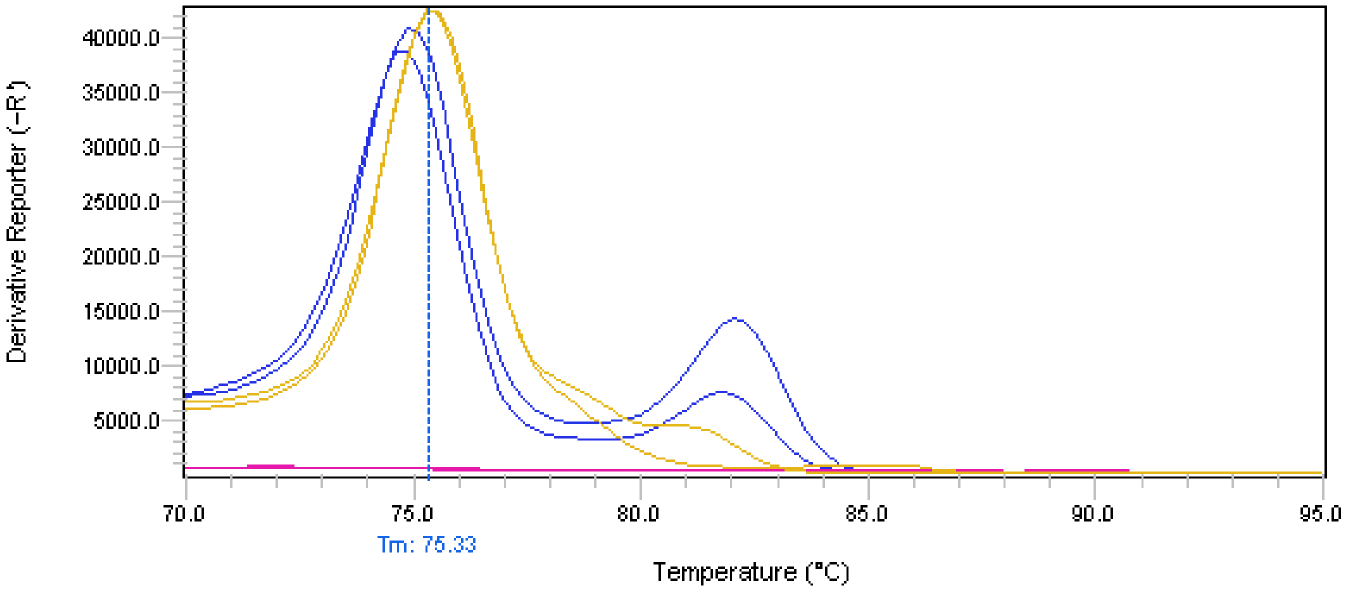
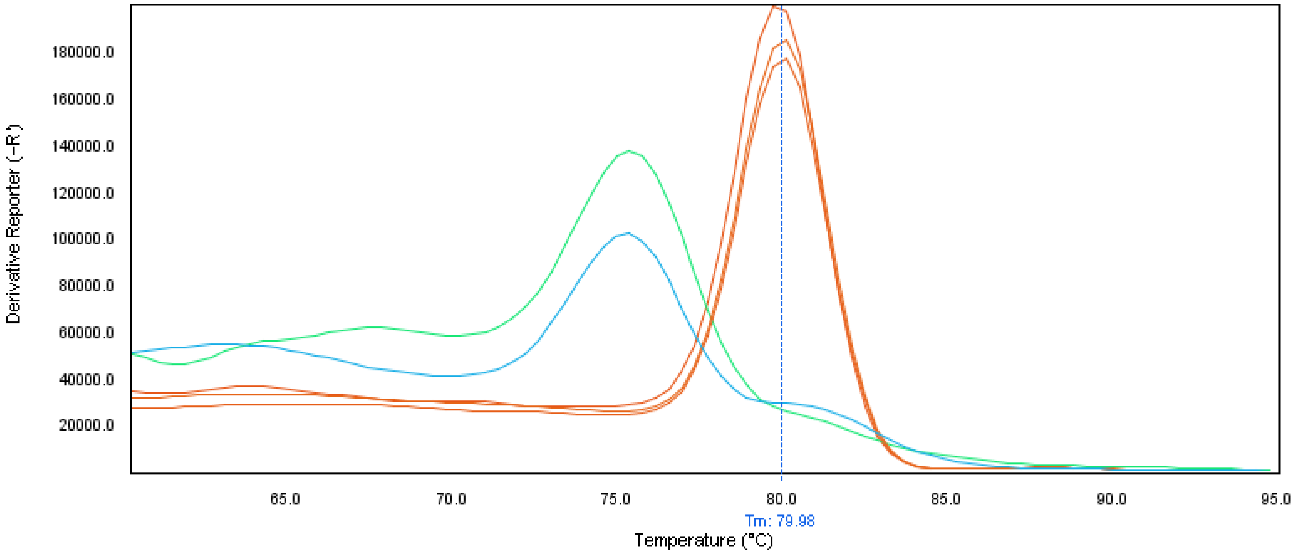


Figure 2: Melt curve with non-specific amplification



It is difficult to provide an exhaustive list of diagnostic examples for non-target amplification in environmental samples. However, a typical example is shown in Figure 2. By comparing the melt profile of a known amplicon (80°C) the difference in melt profiles from samples can be visualized. The broad peak at 75-76°C and 'noisy' profile in Figure 4, indicates a problematic result that should be evaluated.

Figure 4: Melt curve with broad non-target amplification peaks

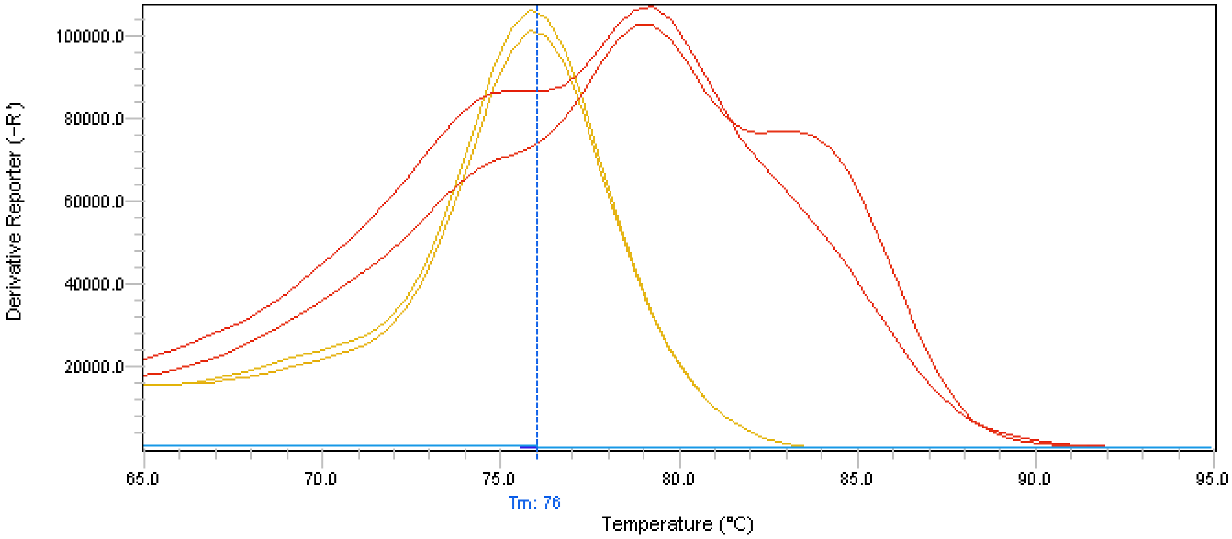
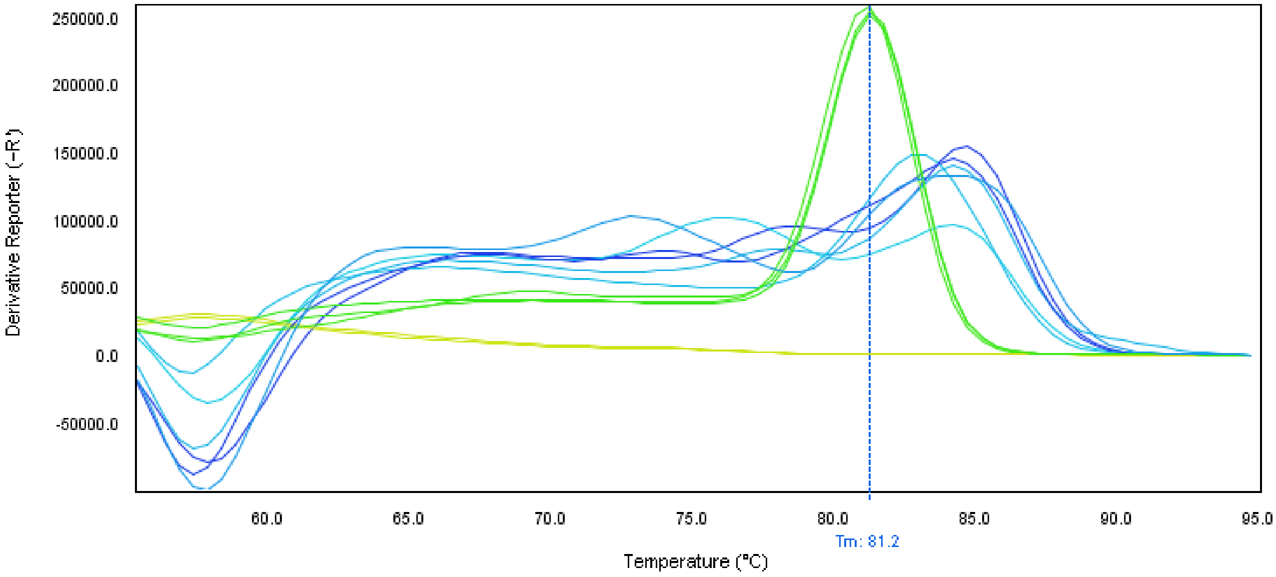


Figure 5: Melt curves with background in method blank and samples



If amplification occurs in NTC or method blanks.

View the melt curves of NTC and/or method blanks. If a similar profile is present in samples or standards, they should be removed from quantitation and standard curves. If the chemist performing the analysis suspects this as affecting the sample result, the comments and melt curve must be documented and sample flagged. An example of matrix interfering with results is shown below in Figure 5. The method blank is shown in yellow and has no peaks in the melting profile. The undiluted samples are shown in blue and have broad, raised melt profiles relative to the method blank which indicate that interfering compounds are likely present. The melt profiles of samples diluted 1:10 are shown in green. The single peak at 81°C demonstrates that interfering compounds have been mitigated by dilution.