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Standard Operating Procedure: Quantitative Polymerase Chain Reaction (qPCR) for the Early Detection of Invasive Mussel eDNA

Quantitative Polymerase Chain Reaction (qPCR) Standard Operating Procedure (SOP)
Version 5 (Date Revised: 2022)
Document No. EcoLab-F436A-2022-04

Bureau of Reclamation
Ecological Research Laboratory



Mission Statements

The Department of the Interior (DOI) conserves and manages the Nation's natural resources and cultural heritage for the benefit and enjoyment of the American people, provides scientific and other information about natural resources and natural hazards to address societal challenges and create opportunities for the American people, and honors the Nation's trust responsibilities or special commitments to American Indians, Alaska Natives, and affiliated island communities to help them prosper.

The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

Standard Operating Procedure: Quantitative Polymerase Chain Reaction (qPCR) for the Early Detection of Invasive Mussel eDNA

**Quantitative Polymerase Chain Reaction (qPCR) Standard Operating Procedure (SOP)
Version 5 (Date Revised: 2022)**

Document No. EcoLabF436A-2022-04

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PCR SOP Version 2	7/2012
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1. ACRONYMS / DEFINITIONS

Cq: Quantification cycle value. The PCR cycle number at which the sample's reaction curve intersects with the threshold line (i.e., the cycle number at which sample fluorescence is detected above background).

DNA: Deoxyribonucleic acid

DNA probe: Oligonucleotides that are labeled with a fluorescent reporter which is only detected after the probe is hybridized with its complementary sequence

DI water: Deionized water

Dreissenid: Members of the group of freshwater mussels which includes quagga mussel (*Dreissena rostriformis bugensis*) and zebra mussel (*Dreissena polymorpha*)

eDNA: environmental DNA - DNA collected from sampling of the environment, rather than from direct sampling of cells or tissue from the target organism(s)

gBlock: Synthetic DNA fragments designed to match the gene region targeted by primers and probe in an eDNA assay. gBlocks are used to create standard curves and serve as a positive control for qPCR reactions.

L: Liter

Melt curve: Assessment of the dissociation characteristics of double stranded DNA during heating.

mL: Milliliter

qPCR: Quantitative Polymerase Chain Reaction. A PCR technique

Quagga mussel: (*Dreissena rostriformis bugensis*) Invasive species of freshwater mussel

RFU: Relative Fluorescence Units

Veliger: The larval life stage for both zebra and quagga mussels

µL: Microliter

Zebra mussel: (*Dreissena polymorpha*) Invasive species of freshwater mussel

2. SCOPE AND APPLICABILITY

This Standard Operating Procedure (SOP) is used to establish a uniform format for the use of qPCR for the analysis of *Dreissena polymorpha* (zebra mussel) and *Dreissena rostriformis bugensis* (quagga mussel) environmental DNA (eDNA) in bulk water samples. This method is applicable to raw water samples that are prepared and analyzed by the Ecological Research Laboratory at the Bureau of Reclamation, Technical Service Center, Denver, CO. The goal of this SOP is to standardize how each task in the laboratory is performed by every lab technician. This SOP is not a replacement for training. This document is focused on the setting up, running, and analysis of qPCR data for the detection of quagga and zebra mussel eDNA isolated from bulk water samples. The DNA extraction kit currently used is *Quick-DNA*TM Fecal/Soil Microbe Microprep Kit (Zymo Research). The extraction kit used is subject to change based on availability and advances to DNA extraction methods. DNA from individual suspects is isolated using a Proteinase K digestion method. Other extraction methods (i.e., filters) are not detailed in this document and will be addressed in future SOP's. The method that will be used for extracting eDNA from filters is currently under review by researchers in the Ecological Research Laboratory. Past extraction and conventional PCR methods are available upon request.

eDNA analysis of bulk samples will be performed to detect invasive mussels. The use of eDNA methods to detect species based upon DNA in tissue, cells, and any biological material that an organism may shed into the environment is a growing field of study for many researchers. eDNA is also increasingly being used to inform management decisions. Many different assays have been developed for the detection of invasive, endangered, and threatened species with eDNA techniques.

While there is still research that must be done to understand both the limitations and full potential of this technique, it should be used as a method (in addition to microscopy) to expand our detection methods for invasive mussels.

The scope of this SOP is focused on the use of qPCR for the detection of eDNA from invasive dreissenid mussels. This SOP details the extraction of eDNA from bulk water samples, setting up qPCR for the detection of dreissenid mussels, and the operation and daily use of the CFX Maestro™ qPCR instrument and software for users in the Ecological Research Laboratory. Methods and reagents for DNA extraction directly from dreissenid veliger tissue samples are also included.

3. SUMMARY OF METHOD

DNA extraction of both bulk water samples and individual suspects requires using either the *Quick-DNA*™ Fecal/Soil Microbe Microprep Kit, or the Proteinase K method. The CFX Maestro™ software is used to perform quagga and zebra mussel DNA analysis for both bulk water samples and individual suspects. The amplification protocol and PCR plate protocol have been programmed into the instrument and will be consistent for each analysis. Data produced by the qPCR instrument for the detection of invasive dreissenid mussels will be reported as either positive or negative in the Reclamation Mussel SharePoint Site.

4. INTERFERENCES

eDNA extraction, qPCR set up, and qPCR instrument workstations are in separate places to prevent contamination. Once the qPCR plate is used it is never opened in the main laboratory. Plates can only be opened in the gel electrophoresis area located in a room separate from the main lab. Always use sterile filter pipette tips and Eppendorf tubes. Dedicated pipets are used for the eDNA extraction, qPCR set up, and gel electrophoresis workstations. qPCR is a more sensitive method than conventional PCR. It can also be inhibited by environmental substances, such as humic acid and other substances. Soil extraction kits contain reagents that can help remove the PCR inhibitors. Using an internal qPCR control will help to check for inhibition in the sample. The gBlocks used to create the standard curve are only used with a dedicated pipette, tips, and waste container. Maintaining good quality assurance and quality control is vital to ensure that the results can be trusted. Use 20% bleach to clean down the countertops and work areas daily. Perform monthly wipe tests to assess for laboratory contamination.

5. CHEMICAL HAZARDS

Reagents that are used for the eDNA extraction and in the qPCR, assay are not hazardous. See MSDS binders for information on specific reagents. Also, MSDS information is available in the online file: <https://access.cheminventory.net/filestore.php>

6. MATERIALS

Quick-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research D6012)

Proteinase K (Qiagen #69504)

Dedicated Pipettes for both eDNA extractions and qPCR set up (P1000, P200, P20, and P10)

Multichannel pipettes (P300, P200, P10)

Filter pipette tips (P1000, P300, P200, P20, and P10) (USA Scientific, TipOne Graduated Filter Tips or equivalent)

Eppendorf tubes (1.5 mL and 0.5 mL volumes, USA Scientific)

PCR strip tubes (USA Scientific 1402-4708)
Hard-shell PCR plates (Thermo Scientific AB-2800/W)
Sealing film for plates (Thermo Scientific AB-1170)
Nuclease-free water (IDT)
IDTE pH 8.0 (1X TE solution) (IDT)

7. SAMPLE PREPARATION

Two different DNA extractions are performed: for bulk water samples (eDNA) the *Quick-DNA*TM Fecal/Soil Microbe Microprep Kit (Zymo Research) is used; for individual veliger suspects direct digestion in a buffered solution containing Proteinase K is used. The DNA extraction kits used for the extraction process are subject to change as companies selling these kits continue to change and update their kits. As the DNA extraction kits continue to evolve, the kits will be evaluated for their use in the detection of quagga and zebra mussel DNA. The SOP's sections on eDNA extractions will be updated to reflect any major changes in the kits and methods used. DNA extractions from filters is still under review, and several different methods are being investigated.

1. See Appendix 1 for the *Quick-DNA*TM Fecal/Soil Microbe Microprep Kit for bulk water samples
2. See Appendix 2 for the Proteinase K Digestion Solution Method for individual suspects

8. FIELD AND LABORATORY CONTROLS

1. Both field and laboratory controls must be used to ensure QA/QC is maintained and that samples pass current USGS reporting guidelines
2. Field blanks
 - a. Field blanks that are from priority water bodies (where QM/ZM suspects have been found in the past)
 - i. Onsite in the field purified water is poured through the decontaminated net and codend. Water remaining in the codend is collected, and preserved prior to using the net to collect samples
 - ii. Log the sample in as a PCR only sample
 - iii. Print off labels for both a 50 mL conical tube and the field blank bottle
 - iv. Shake field blank bottle
 - v. Gently pour 40 mL of the field blank into the conical tube
 - vi. Place the 50 mL conical tube onto the PCR shelf with the rest of the samples from the site
 - vii. Place the field blank bottle into the designated refrigerator space for field blanks
 1. It will be available if additional analysis is needed
 - viii. Following the qPCR analysis of the field blank, store the field blank bottle in the refrigerator until it is time to purge the sample
 - ix. Purge the field blank by dumping it down the sink, and update the database to show that the field blank has been purged
 - b. Field blanks from non-priority sites

- i. Onsite in the field purified water is poured through the decontaminated net and codend. Water remaining in the codend is collected, and preserved prior to using the net to collect samples
- ii. Log the sample in as a PCR only sample
- iii. Print off a label for the field blank bottle
- iv. Store the field blank bottle in the refrigerator space designated for field blanks.
- v. Store the field blank bottle in the refrigerator until the site's microscopy samples have been analyzed and it is time to purge the samples
- vi. Purge the field blank by dumping in down the sink, and update the database to show that the field blank has been purged
- vii. If a suspect(s) is found in the sample, then pull the field blank, and use in qPCR analysis

3. Lab blanks

- a. Label 50 ml conical tube "lab blank"
- b. Pour 40 mL of molecular grade water (Fisher Scientific) in the tube
- c. For every eleven samples that are extracted there should be one lab blank included

9. eDNA SAMPLE EXTRACTION METHODS

Appendix 1 contains the SOP for the current DNA extraction method. As the availability and changes to the manufacturing of Soil DNA kits continue, the extraction kit that is used is subject to change. New DNA extraction methods will be added as additional Appendices as needed. In the PCR laboratory notebook and PCR log the name of the DNA extraction kit that is used will be noted. Appendix 2 contains the protocol for the extraction of DNA from single veligers.

10. eDNA SAMPLE PREPARATION OF BULK WATER SAMPLES

1. Once samples are logged in:
 - a. Print off label for a 50-mL conical tube
 - b. Shake bulk water sample
 - c. Gently pour 40 mL of the bulk water sample into the conical tube
 - d. Place bulk eDNA sample onto the PCR shelf for analysis
 - e. Put bulk water sample onto the microscopy shelf
2. Eleven samples can be extracted at once plus one lab blank for twelve samples at a time
3. eDNA samples will be centrifuged
 - a. 4,500 x g for 30 minutes
 - b. Pour off the supernatant
 - c. Use the pellet in the eDNA extraction
4. Pipette up to 250 μ L of the eDNA pellet into the DNA extraction kit
5. Follow the manufactures instructions to perform the extraction (See Appendix 1 for SOP of the current extraction method)
6. Once the eDNA is extracted, store in the PCR room in the -20°C freezer
7. Following the qPCR analysis, eDNA samples are then moved into the -80°C for long term storage

11. REAGENTS FOR qPCR MASTER MIX

1. The reagents listed below are for the qPCR reaction
 - a. Master Mix for qPCR: Quantbio PerfeCTa qPCR ToughMix (VWR catalog # 97065-956)
 - b. qPCR primers- each set consists of a forward, reverse, and internal probe that are ordered from Integrated DNA Technologies (IDT)
 - i. Quagga mussel (QM) COI Primers and probe (Sepulveda et al. 2019):

QMCOI-Forward: 5'-CTCTTCATATCGGTGGAGCTTC-3'
QMCOI-Reverse: 5'-CAAAGGCACCCGATAAAACTG-3'
QMCOI-Probe: 5'-6FAM-CCCGGCACGTATATTTCTCATGTT-MGBNFQ-3'
 - ii. Zebra mussel (ZEB) COI Primers and probe (Gingera et al. 2017):

ZEBCOI-Forward: 5'- SCCTGCGATAGATTTTTTGATTTTA-3'
ZEBCOI-Reverse: 5'- GCAGAACAAAGGGACCCG-3'
ZEBCOI-Probe: 5'-6FAM- CGTGCTGGATGTCAT-MGBNFQ-3'
 - c. Make aliquots of each primer and probe
 - i. Working primer stock concentration- 10 µM
 - ii. Working probe stock concentration- 2.5 µM
 - iii. Use IDTE pH 8.0 (1X TE solution) to make working stocks
 - iv. Place the working primer and probe stocks in the freezer until used

11. CONTROLS FOR qPCR

1. Several controls are used for qPCR
 - a. Positive control- known adult DNA from quagga and zebra mussels, or a gBlock
 - i. Dilution of positive control: genomic DNA (gDNA) extracted from quagga or zebra mussel tissue can be used as a positive control. Dilutions of the stock will need to be tested by qPCR to identify a concentration that provides a reliable positive reaction. Cq values of 35 to 36 cycles should be targeted. gDNA cannot be used for absolute quantification of qPCR results.
 - ii. gBlocks: gBlocks are synthetic double-stranded oligonucleotides that can be purchased from Integrated DNA Technologies (IDT). They provide a known amount of template, allowing for absolute quantification of qPCR results.

gBlock sequences:

QMCOI-gBlock-scramble:

CTAATTTTATCTCTTCATATCGGTGGAGCTTCTTATTTTCCAT
TATATATTCGTTATAAGTTAACATGAGGAAATATACGTGCCG
GGTTTACGCAGTTTTATCGGGTGCCTTTGTTTTGTA CTTC

ZEBCOI-gBlock scramble:

GACATTCAGGSCCTGCGATAGATTTTTTTGATTTTATATGTTTC
TTCAGGCTAGGAATAAGTGGTTTTATTGAACCGTCAAGTTATAT
CTTTTGGATATTCGCATCGTGCTGGATGTCATCCATTAATCGG
GTCCCTTTGTTCTGCACATCTATTG

gBlock notes:

Primer and probe recognition sequences are highlighted in **bold** type

Regions between the primer and probe recognition sequences retain the same nucleotide frequency at the reference sequence, with the nucleotide order scrambled randomly. This allows for identification of any potential gBlock contamination in qPCR reactions through DNA sequencing.

- iii. gBlock dilutions: gBlocks are supplied lyophilized at a standardized mass of 250 ng. gBlocks should be resuspended to generate a full-strength stock of 1×10^{10} copies/ μ l (IDTE volume added is based upon the molecular weight of the gBlock; see calculations below). The 1×10^{10} copies/ μ l full strength stock should be diluted to generate a 1×10^7 copies/ μ l diluted stock. Serial dilutions can then be used to generate working stocks with concentrations ranging from 1×10^5 copies/ μ l down to 1×10^1 copies/ μ l for generation of standard curves. All dilutions should be done with IDTE buffer to maximize gBlock stability. Aliquot the gBlocks into smaller volumes ($\sim 50 \mu$ L) for use.

Note: gBlock stocks are highly concentrated. They should be handled with extreme care to minimize potential contamination. Dedicated pipets should be used only for gBlocks solutions. Workspace surfaces should be decontaminated with a dilute bleach solution following handling of gBlocks.

Resuspension of gBlocks: The calculation for resuspending gBlocks to a stock concentration of 1×10^{10} copies/ μl is:

$$\mu\text{l of IDTE} = \frac{250 \text{ ng (gBlock mass)} * \text{fmol/ng (from spec sheet)} * 1 \times 10^{-15} \text{ fmol/mol} * 6.022 \times 10^{23}}{1 \times 10^{10} \text{ copies}/\mu\text{l}}$$

<u>Dilutions:</u>	<u>Input dilution</u>	<u>Input vol. (μl)</u>	<u>IDTE vol (μl)</u>
1×10^7 copies/ μl	1×10^{10} copies/ μl	5	4995
1×10^5 copies/ μl	1×10^7 copies/ μl	10	1000
1×10^4 copies/ μl	1×10^5 copies/ μl	100	900
1×10^3 copies/ μl	1×10^4 copies/ μl	100	900
1×10^2 copies/ μl	1×10^3 copies/ μl	100	900
1×10^1 copies/ μl	1×10^2 copies/ μl	100	900

b. Negative controls

- i. No template control - no DNA present, just the master mix and DI water in the place of sample
- ii. Laboratory blank - DI water that has been extracted along with the real samples
- iii. Field blank - DI water that has been used to rinse the net and cod end.
- iv. Negative control - IPC block – Blocking reagent as negative control for Internal positive control (see below)

c. Internal control

- i. Reagent: TaqMan Exogenous Internal Positive Control (IPC) Reagents (ThermoFisher catalog number 430821)
- ii. Amount added to each reaction: 2.0 μl of 10x Exo IPC Mix, 0.4 μl of 50x Exo IPC DNA

2. All controls must pass QA/QC standards for the results to be considered valid

12. qPCR MASTERMIX SET UP

1. Master mix components and volumes (total volume for each individual reaction 20 μl)
 - a. Master mix reagent, 10 μl per reaction
 - b. Primers, 1 μl each of 10 μM forward and reverse primer working stocks per reaction (0.5 μM final concentration)
 - c. Probe, 2 μl of 2.5 μM working stock per reaction (0.25 μM final concentration)
 - d. Internal control, 0.6 μl per reaction (0.5 μl of 10x Exo IPC Mix, 0.1 μl of 50x Exo IPC DNA)
 - e. Molecular grade water: 3.4 μl per reaction

2. Calculating the master mix for a 96 well plate (Appendix 3 for calculation sheet)
 - a. 104 reactions worth of master mix is prepared for a 96 well plate to ensure enough reagents for dispensing with an 8-channel automatic pipet. This equals 13 reactions worth of Mastermix per row of 12 wells. A full 96 well can be used to analyze 21 samples (including field blanks), along with negative and positive controls. 3 samples with 4 replicates can be loaded per row, with 1 row reserved for positive and negative controls. The amount of Mastermix can be decreased proportionally for fewer samples (e.g., 65 reactions worth of master mix would be prepared for 12 samples: 4 rows of 3 samples each and 1 row of controls)
3. Amount of DNA per reaction
 - a. Experimental DNA: 2 μ l per reaction – 4 reactions per sample
 - b. Negative control: 2 μ l per reaction – 6 reactions per plate
 - c. Laboratory blank: 2 μ l per reaction – 4 reaction per sample
 - d. Field blank: 2 μ l per reaction – 4 reactions per sample
 - e. IPC block: 2 μ l per reaction – 1 reaction per plate
 - f. Positive controls (gBlock): 2 μ l per standard – 5 standards: 1 x 10⁵ copies/ μ l; 1 x 10⁴ copies/ μ l; 1 x 10³ copies/ μ l; 1 x 10² copies/ μ l; 1 x 10¹ copies/ μ l

13. SETTING UP qPCR REACTION

1. Turn the UV lamp on in the eDNA PCR hood
2. Pull out the reagents and samples from the freezer and allow them to thaw at room temperature
 - a. Use separate racks for the DNA samples, Mastermix reagents, and gBlocks
 - b. Keep the gBlocks out of the eDNA PCR hood until needed
3. Aliquot ~13 μ l of each DNA sample into strip tubes (see Appendix 3)
4. Create master mix according to the master mix calculations (See Appendix 3)
5. See Appendix 4 for an example of how the plate is to be set up
 - a. Location of samples, blanks, and controls on the plate
6. Pull out the 96 well hard plate and label the side
7. Aliquot master mix into the plate wells, adding 18 μ l/well – Mastermix will nearly fill the 0.2 ml strip tube wells. Set the aspiration speed on the 300 μ L Xplorer electronic pipette to the minimum speed of “1”. Place the pipet tips below the surface of the Mastermix, taking care not to cause the Mastermix to overflow. Begin aspirating the Mastermix and slowly lower the pipet tips as the Mastermix is drawn up and the level in the tubes decreases
8. Add 2 μ l of DNA to the appropriate wells
 - a. Use the 20- μ L multichannel pipettes to add the DNA samples
9. Add 2 μ l of molecular grade water to each of the no template control (NTC) wells
 - a. Use the 10- μ L pipette
10. Add 2 μ l of IPC Block Reagent to the negative control (NC) well
 - a. Use the 10- μ L pipette
 - b. Place all reagents and samples back in the freezer

11. Add 2 μ l of each gBlock dilution to the appropriate Standard wells. Use a dedicated 10- μ L pipet and tips do dispense gBlock solutions
 - a. Remove gBlocks from the hood and place back in the freezer
12. Seal the plate with clear sealing film
13. Place all tips and plastic waste into the trash
14. Turn the UV lamp on in the eDNA PCR hood
15. Centrifuge the plate to ensure that all of the reagents are in the bottom of the wells
16. Place plate into the qPCR instrument

14. qPCR INSTRUMENT SET UP

1. Turn the instrument on before starting to set up the qPCR plate
2. Log on to the qPCR PC and launch the CFX Maestro software.
3. qPCR protocol files and plate templates are store on the t: drive in the directory:
T:\Programs\Ecological Research Laboratory\DATA\MUSSEL SAMPLES\DATA & LOGIN\PCR & Sequencing\qPCR templates
4. Use the appropriate qPCR protocol for analysis (QMCOI_protocol_2step.prc1 or ZEBCOI_protocol_2step.prc1). See protocol profiles in Appendix 5.
5. Open the appropriate plate template file (QMCOI-plate-12samples.pltd, QMCOI-plate-21samples.pltd, ZEBCOI-plate-12samples.pltd, or ZEBCOI-plate-21samples.pltd). See plate setup templates in Appendix 4.
6. Fill in the sample names and other plate information
7. Set up the qPCR reaction in the PCR room
8. Once the plate is ready, place in the qPCR instrument, and hit start
9. Save the plate with the first sample ID, operators initials, and date and time (date, time, and instrument name are auto-populated when the run is started)

15. DATA ANALYSIS

1. Copy the qPCR data file from the qPCR PC to the mussel detection qPCR folder on the T: drive (T:\ Programs\Ecological Research Laboratory\DATA\MUSSEL SAMPLES\DATA & LOGIN\PCR & Sequencing). Place the file in the folder for the appropriate year and month the experiment was run.
2. Once the amplification is completed the results can be accessed under the Start Up Wizard under Analyze
3. This window shows the amplification, standard curve, plate layout, and the Cq values
4. From this window it is possible to export the Cq and other values as an Excel Spreadsheet
 - a. Export the spreadsheet and save in the PCR results folder located here: pathway to where the data should go
5. It is also possible to go back into the plate set up and add information (such as sample names) if anything was left out prior to the amplification

16. REPORTING RESULTS

All results are recorded as either positive or negative in the PCR results spreadsheet. Results are downloaded from the CFX Maestro™ program as an Excel spreadsheet that can then be saved to the PCR result folder. Results are reported as either positive or negative in the Mussel Database and the Mussel SharePoint site where the results are given to clients.

1. Positive qPCR reactions can be sent for DNA sequencing for secondary conformation if the detection is from a negative location or DNA sequencing is requested by the client
 - a. qPCR products will be sent to certified laboratory
 - b. Primers for sequencing will also be sent
 - c. Sequencing results will be analyzed using NCBI BLAST to confirm either ZM or QM origin
 - d. Sequencing results will be uploaded into the PCR spreadsheet
2. Positive results will be discussed with the client as needed to explain the qPCR process and QA/QC that the lab performs

17. POSITIVE RESULTS

1. If a qPCR result has two Cq values below of 38 it is considered a positive finding
 - a. All QA/AC must be passing
 - b. The amplification curve should also be examined for any unusual features (e.g. a Cq value of less than that of the 1×10^5 copies/ μ l gBlock reaction)
2. To confirm the finding, a second set of qPCR reactions will be performed
3. The qPCR product will be sent for sequencing to confirm the result
4. If a qPCR result has one Cq value below 38 and no additional findings in the second round of qPCR it will be considered suspect. These finding will be reports following our reporting protocols.
5. The results will be discussed internally so that there is agreement that the sample passes all QA/QC
6. Results will be reported to the clients via the Reclamation Invasive Mussel Detection Notification Protocol
7. Results will be uploaded to the Mussel Database

18. NEGATIVE RESULTS

1. If a qPCR result is above the Cq value of 38 or negative, that is a negative finding
2. The results will be reported in the PCR log
3. Results will be uploaded to the Mussel Database

19. TROUBLESHOOTING ISSUES

1. If QA/QC fails, the samples will be reanalyzed
2. If positive controls fail, then fresh controls will be used, and the samples reanalyzed
3. If gBlocks result in a poor standard curve, then a fresh aliquot of the gBlocks will be used and the older aliquot discarded
4. If the IPC Block Reagent internal control come back positive, then a fresh aliquot of the control will be used, and the older aliquot discarded
5. Field blank comes back positive

- a. If both field blanks fail and samples from the site come back positive, then the samples will be reanalyzed
 - b. If both the field blanks fail and samples come back positive after being reanalyzed, then fresh DNA extractions will be done on the samples for confirmation of findings
 - c. If field blanks and samples come back positive after reanalysis, then the results will be discussed with the client to determine possible sources of contamination in the field blank and best ways to move forward
6. Any unusual results or issues will be discussed internally to resolve and if necessary outside expertise will be sought

20. DISPOSAL OF PCR PRODUCTS AND DNA SAMPLE STORAGE

1. All DNA samples are stored at -80°C following analysis. They are kept indefinitely
2. Aliquots of eDNA samples can be sent to secondary testing laboratories when requested
3. At the end of each month, qPCR products are disposed of by placing the hard PCR plates into the trash

21. SPILLS

1. All spills should immediately be cleaned up to avoid contamination.
2. Use 20% bleach solution to gently spray down the area
3. Let the bleach soak on the surface for a few minutes then wipe up with a paper towel

22. HELP MANUALS FOR THE SOFTWARE

1. The CFX Maestro™ software contains a Software Help Guide
2. The software help guide contains a table of contents, index, and search functions
 - a. If there is an issue that needs to be investigated this guide is a good resource
 - b. The guide is also a good resource for learning about the instrument and the software

23. REFERENCES

CFX Maestro™ Software User Guide, Version 1.0, 2017 Bio-Rad Laboratories
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24. CONTACT INFORMATION

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25. APPENDIX 1: QUICK-DNA™ FECAL/SOIL MICROBE MICROPREP KIT

This protocol has been adapted for the *Quick-DNA™* Fecal/Soil Kit, Zymo Research, Catalog # D6012, January 2022

1. Assemble and label all the necessary tubes
2. Pour 40 mL of the bulk water sample into a 50-mL conical tube
3. Centrifuge for at 4,500 x g for 30 minutes
4. Pour off the supernatant leaving the pellet
5. Add up to 250 mg (250 µL) of pellet to a ZR BashingBead™ Lysis tube
6. Add 750 µL of BashingBead™ Buffer to the tube and cap tightly
7. Place tube in bead beater at max speed for 5 minutes
8. Centrifuge the ZR BashingBead™ Lysis tube at 10,000 x g for 1 minute
9. Transfer 400 µL of supernatant to a Zymo-Spin™ III-F Filter in a collection tube
10. Centrifuge at 8,000 x g for 1 minute
11. Discard the Zymo-Spin™ III-F filter
12. Add 800 µL of Genomic Lysis Buffer and 400 µL of 95% ethanol to the filtrate in the collection tube. Mix well
13. Transfer 800 µL of the mixture to a Zymo-Spin™ IC Column in a collection tube
14. Centrifuge for 10,000 x g for 1 minute
15. Discard the flow through and repeat step 13 with the rest of the mixture
16. Discard the flow through
17. Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin™ IC column
18. Centrifuge for 10,000 x g for 1 minute
19. Discard the flow through
20. Add 500 µL g-DNA Wash Buffer to the Zymo-Spin™ Column IC
21. Centrifuge at 10,000 x g for 1 minute
22. Transfer the Zymo-Spin™ Column IC to a new collection tube
23. Add 100 µL of DNA Elution Buffer to the column
24. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.
25. Place a Zymo-Spin™ II- µHRC Filter into a clean collection tube and add 600 µL of Prep Solution
26. Centrifuge at 8,000 x g for 3 minutes
27. Transfer the Zymo-Spin™ II- µHRC filter to pre-labeled 1.5 mL microcentrifuge tube and pipette the eluted DNA onto the column
28. Centrifuge the sample at 16,000 x g for 3 minutes
29. Remove the filter and place the DNA extract in the PCR rooms freezer until the sample is analyzed

26. APPENDIX 2: PROTEINASE K DIGESTION OF INDIVIDUAL VELIGER SUSPECTS

Suspect Sample Handling

1. When single or multiple suspects are found pictures need to be taken of the suspect(s) to document its size, shape, and condition. These images are part of the reporting process
2. Suspects are then placed into a 0.2 mL PCR tube and sent of the PCR shelf for analysis

Digestion procedure

1. Transfer veliger to a 0.2 mL PCR tube. Remove as much liquid as possible. Dry in hood for ~5 minute.
2. Add 19 μ L working solution
3. Add 1 μ L Qiagen Proteinase K
4. Incubate in thermocycler @ 55°C for 1hr
5. Inactivate @ 95°C for 10 min

Sample Analysis

1. Suspects are analyzed by qPCR for both QM and ZM DNA
2. Just like bulk water samples, the qPCR results need to pass QA/QC for the suspect to be called positive or negative for either QM or ZM DNA presence

DNA Digestion Solution

<u>Stock solutions</u>	<u>Mass</u>	<u>Water Volume</u>	<u>1N HCl</u>
500 mM KCl (MW 74.55)	0.932 g	25 mL	
200 mM Tris Base pH 8.4 (MW 121.14)	0.606 g	23.28 mL	1.72 mL
20 mM EDTA (MW 372.24)	0.186 g	25 mL	
5% Triton-X 100	500 μ L	9.5 mL	
5% Tween 20	500 μ L	9.5 mL	

Digestion solution mix

<u>Component</u>	<u>Volume</u>
500 nM KCl	0.5 mL
200 mM Tris pH 8.4	0.5 mL
20 mM EDTA	0.25 mL
5% Triton-X 100	0.5 mL
5% Tween 20	0.5 mL
Molecular grade water	2.75 mL

27. APPENDIX 3: CALCULATING qPCR MASTERMIX FOR 96 WELL PLATE

Mastermix calculations:

Reagent	Volume per well (μl)	Volume for 12 samples (μl)*	Volume for 21 samples (μl)*
Forward primer (10uM)	1	65	104
Reverse primer (10uM)	1	65	104
Probe (2.5uM)	2	130	208
Quantbio 2x PerfeCTa Mastermix	10	650	1040
10X Exo IPC Mix	0.5	32.5	52
50X Exo IPC DNA	0.1	6.5	10.4
Molecular grade water	3.4	221	
Total	18	1170	1872

The Quantbio 2X PerfeCTa Mastermix has been pre-aliquoted into 650 μL volumes. This was done to avoid freezing and thawing out larger volumes of the Mastermix. When setting up the qPCR reaction, add all reagents to the tube with the Mastermix.

Add 234 μL of Mastermix to each well of an 8-well strip tube. This will facilitate dispensing to the plate with a 300 μL 8-channel Xplorer electronic pipette.

*Note: Calculations for 12 samples are for 4 technical replicates each, and a row of positive and negative controls (5 rows total). Calculations for 21 samples are for 4 technical replicates each, and a row of positive and negative controls (8 rows total). See plate setup diagrams in Appendix 4.

SOP: qPCR for the Early Detection of Invasive Mussel eDNA
qPCR SOP (Updated:2022)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date
	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date
	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date
Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date
NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	Unk ZEBCOI IPC Extraction_blnk DNAext.date	Neg ZEBCOI IPC IBP_Block	Neg ZEBCOI IPC IBP_Block	Std ZEBCOI gBlock_2e1	Std ZEBCOI gBlock_2e2	Std ZEBCOI gBlock_2e3	Std ZEBCOI gBlock_2e4	Std ZEBCOI gBlock_2e5
F												
G												
H												

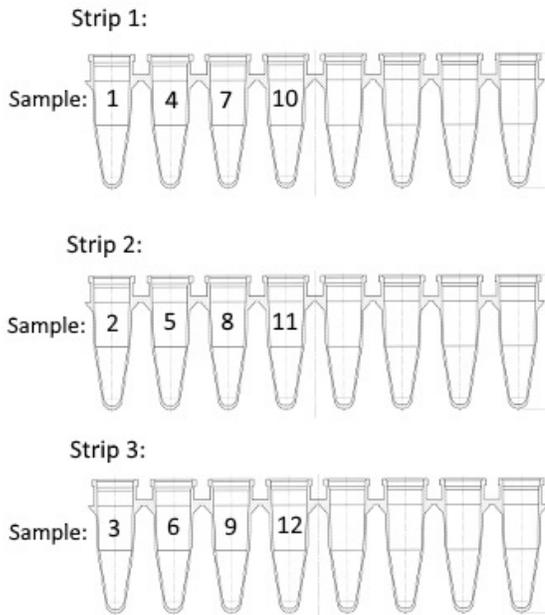
Plate setup for 21 samples with ZEBCOI primer/probe set:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-1 ZEBCOI IPC Sample01 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-2 ZEBCOI IPC Sample02 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date	Unk-3 ZEBCOI IPC Sample03 DNAext.date
	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-4 ZEBCOI IPC Sample04 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-5 ZEBCOI IPC Sample05 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date	Unk-6 ZEBCOI IPC Sample06 DNAext.date
	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-7 ZEBCOI IPC Sample07 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-8 ZEBCOI IPC Sample08 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date	Unk-9 ZEBCOI IPC Sample09 DNAext.date
Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-10 ZEBCOI IPC Sample10 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-11 ZEBCOI IPC Sample11 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date	Unk-12 ZEBCOI IPC Sample12 DNAext.date
Unk-13 ZEBCOI IPC Sample13 DNAext.date	Unk-13 ZEBCOI IPC Sample13 DNAext.date	Unk-13 ZEBCOI IPC Sample13 DNAext.date	Unk-13 ZEBCOI IPC Sample13 DNAext.date	Unk-14 ZEBCOI IPC Sample14 DNAext.date	Unk-14 ZEBCOI IPC Sample14 DNAext.date	Unk-14 ZEBCOI IPC Sample14 DNAext.date	Unk-14 ZEBCOI IPC Sample14 DNAext.date	Unk-15 ZEBCOI IPC Sample15 DNAext.date	Unk-15 ZEBCOI IPC Sample15 DNAext.date	Unk-15 ZEBCOI IPC Sample15 DNAext.date	Unk-15 ZEBCOI IPC Sample15 DNAext.date	Unk-15 ZEBCOI IPC Sample15 DNAext.date
Unk-16 ZEBCOI IPC Sample16 DNAext.date	Unk-16 ZEBCOI IPC Sample16 DNAext.date	Unk-16 ZEBCOI IPC Sample16 DNAext.date	Unk-16 ZEBCOI IPC Sample16 DNAext.date	Unk-17 ZEBCOI IPC Sample17 DNAext.date	Unk-17 ZEBCOI IPC Sample17 DNAext.date	Unk-17 ZEBCOI IPC Sample17 DNAext.date	Unk-17 ZEBCOI IPC Sample17 DNAext.date	Unk-18 ZEBCOI IPC Sample18 DNAext.date	Unk-18 ZEBCOI IPC Sample18 DNAext.date	Unk-18 ZEBCOI IPC Sample18 DNAext.date	Unk-18 ZEBCOI IPC Sample18 DNAext.date	Unk-18 ZEBCOI IPC Sample18 DNAext.date
Unk-19 ZEBCOI IPC Sample19 DNAext.date	Unk-19 ZEBCOI IPC Sample19 DNAext.date	Unk-19 ZEBCOI IPC Sample19 DNAext.date	Unk-19 ZEBCOI IPC Sample19 DNAext.date	Unk-20 ZEBCOI IPC Sample20 DNAext.date	Unk-20 ZEBCOI IPC Sample20 DNAext.date	Unk-20 ZEBCOI IPC Sample20 DNAext.date	Unk-20 ZEBCOI IPC Sample20 DNAext.date	Unk-21 ZEBCOI IPC Sample21 DNAext.date	Unk-21 ZEBCOI IPC Sample21 DNAext.date	Unk-21 ZEBCOI IPC Sample21 DNAext.date	Unk-21 ZEBCOI IPC Sample21 DNAext.date	Unk-21 ZEBCOI IPC Sample21 DNAext.date
NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	NTC ZEBCOI IPC NC_water	Unk ZEBCOI IPC Extraction_blnk DNAext.date	Neg ZEBCOI IPC IBP_Block	Neg ZEBCOI IPC IBP_Block	Std ZEBCOI gBlock_2e1	Std ZEBCOI gBlock_2e2	Std ZEBCOI gBlock_2e3	Std ZEBCOI gBlock_2e4	Std ZEBCOI gBlock_2e5

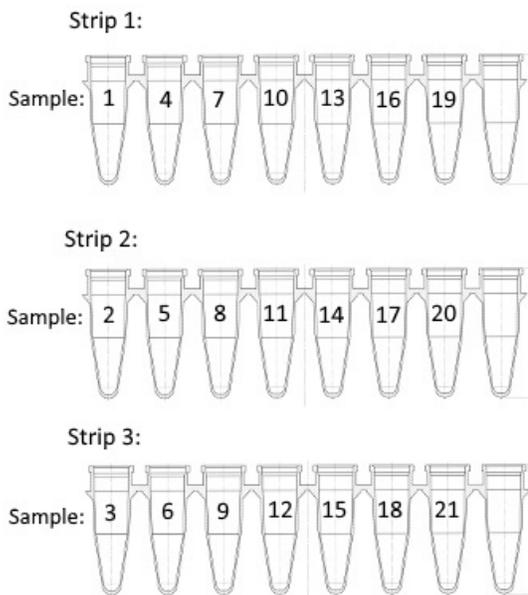
Note that field blanks are treated as regular unknown samples are run in quadruplicate. Lab extraction blanks are run single in the bottom controls and standards row. One lab extraction blank is shown in the templates. If samples from more than one set of extractions are run on the same plate, an additional lab extraction blank can be substituted for one of the NTC samples. A minimum of 4 NTC wells should be included on the plate for proper QA/QC.

29. APPENDIX 5: LOADING SAMPLES IN 8-WELL STRIP TUBES

8-well tubes for 12 samples:

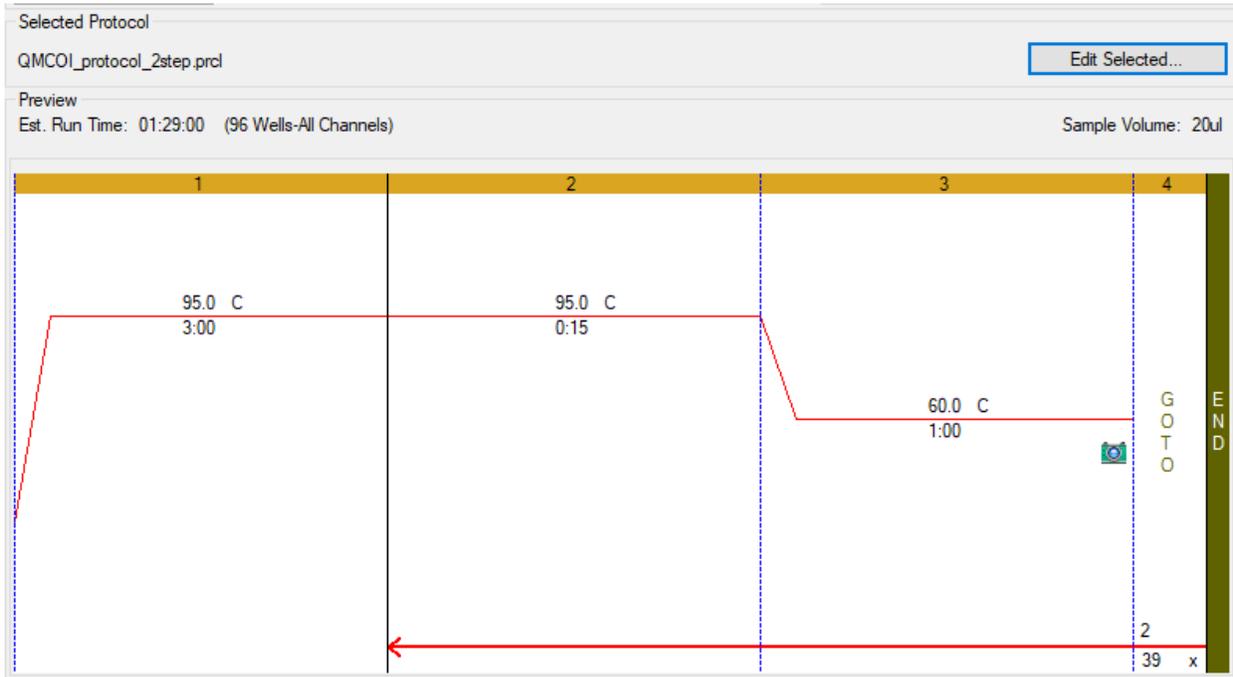


8-well tubes for 21 samples:



30. APPENDIX 5: qPCR PROTOCOL PROFILES

QMCOI protocol profile:



ZEBCOI protocol profile:

