RECLAMATION

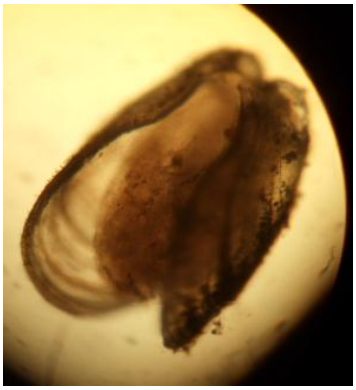
Managing Water in the West

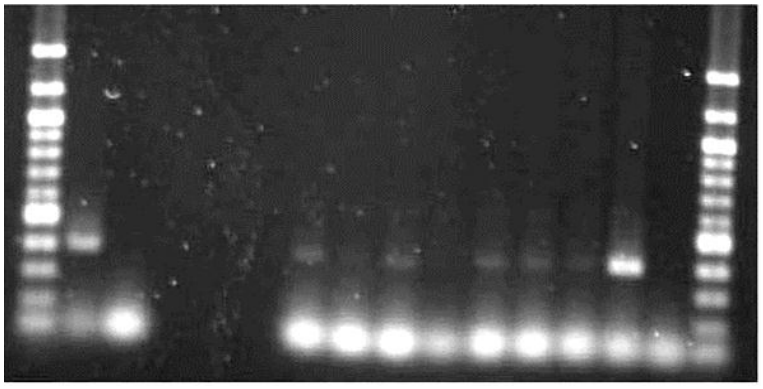
Technical Memorandum No. 86-68220-13-13

Polymerase Chain Reaction: Preparation and Analysis of Veliger Water Samples

PCR Laboratory Standard Operating Procedure (PCR SOP)
PCR SOP Version 4
Date Revised: September 2013

Bureau of Reclamation
Technical Service Center
Reclamation Detection Laboratory for Invasive and Native Species





U.S. Department of the Interior
Bureau of Reclamation
Technical Service Center
Denver, Colorado

September 2013

Mission Statements

The mission of the Department of the Interior is to protect and provide access to our Nation's natural and cultural heritage and honor our trust responsibilities to Indian Tribes and our commitments to island communities.

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.

Technical Memorandum No. 86-68220-13-13

Polymerase Chain Reaction: Preparation and Analysis of Raw Water Samples for the Detection of Dreissenid Mussels

PCR Standard Operating Procedure (PCR SOP)
PCR SOP Version 4
Date Revised: September 2013

Prepared for:

Bureau of Reclamation
Technical Service Center
Research and Development Office

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SOP PCR Version 1	**Susan McGrath**	**8/04/2011**
SOP PCR Version 2	**Jamie Carmon**	**7/11/2012**
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PEER REVIEW DOCUMENTATION
PROJECT AND DOCUMENT INFORMATION

Project Name: Polymerase Chain Reaction: Preparation and Analysis of Raw Water Samples for the Detection of Dreissenid Mussels(PCR Standard Operating Procedure Version 4)

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REVIEW REQUIREMENT

Part A: Document Does Not Require Review

Explain_____

Part B: Document Requires Peer Review: SCOPE OF PEER REVIEW
Peer review restricted to the following items/section(s):
Reviewer:

REVIEW CERTIFICATION

Peer Reviewer- I have reviewed the assigned items/section(s) noted for the above document and believe them to be in accordance with the project requirements, standards of the profession, and Reclamation policy.

Reviewer: Jamie Carmon Signature Review Date: 9/23/2013

Reviewer: _____ Signature Review Date: 9/5/13

Reviewer: _____ Signature Review Date: 9/10/13

Preparer- I have discussed the above document and review requirements with the Peer Reviewer(s) and believe that this review is completed, and that the document will meet the requirements of the project.

Team Member: Denise M. Hosler Signature Date: Sept 23, 2013

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1. ACRONYMS / DEFINITIONS

Dreissenid: Genus of freshwater mussel
Zebra Mussel: (*Dreissena polymorpha*) Invasive species of mussel
Quagga Mussel: (*Dreissena rostriformis bugensis*) Invasive species of mussel
Veliger: Larval mussel
DI water: Deionized water
mL: Milliliter
µL: Microliter
L: Liter
PCR: Polymerase Chain Reaction. The technique used to amplify DNA

2. SCOPE AND APPLICABILITY

This Standard Operating Procedure (SOP) is used to establish a uniform format for duties performed in the dreissenid mussel lab. This method is applicable to raw water samples that are prepped and analyzed by The Reclamation Detection Laboratory for Invasive and Native Species, 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

3. SUMMARY OF METHOD

Preparation, analysis, and disposal of raw water samples specifically analyzed for PCR. As DNA extraction and PCR methods continue to improve and evolve the methods in this SOP will be updated to reflect new knowledge and understanding of the best ways to process and analyze for the presence of mussel DNA in raw water samples

4. INTERFERENCES

Keep the DNA extraction and PCR work stations separate. Always use sterile pipette tips and tubes. A common interference is the inhibition of the PCR reaction by humic acid and other inhibitory substances.

5. CHEMICAL HAZARDS

5.1. Ethanol, EtOH, denatured with up to 5% V.V Ether: CAS-No-60-29-7 UN-No1170 Hazard

5.1.1. Handling: Wash hands thoroughly after handling. Use only in a well-ventilated area. Take precaution to avoid static discharges. Avoid contact with eyes, skin, and clothing. Do not breathe fumes. Empty containers retain product residue (liquid and/or vapor), and can be dangerous. Keep container tightly closed. Avoid contact with heat, sparks and flame. Avoid ingestion and inhalation. Do not pressurize, cut, weld, braze, solder, drill, grind, or expose empty containers to heat, sparks or open flames

5.1.2. Storage: Keep away from heat, sparks, and flame. Keep away from sources of ignition. Store in a tightly closed container. Keep from contact with oxidizing materials. Store in a cool, dry, well-ventilated area away from incompatible substances. Store in a designated area marked flammables. Do not store near perchlorates, peroxides, chromic acid or nitric acid

5.1.3. Engineering Controls: Use explosion-proof ventilation equipment. Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower

close to workstation location. Use adequate general or local exhaust ventilation to keep airborne concentrations below the permissible exposure limits

5.1.4. Personal Protective Equipment: Eyes: Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166. Skin: Wear appropriate protective gloves to prevent skin exposure. Clothing: Wear appropriate protective clothing to prevent skin exposure. Respirators: A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant a respirator's use (MSDS, No. 270660000, 2010)

5.2. Clorox Regular Bleach: EPA Regulation No. 5813-50

5.2.1. Health Hazard Data: Corrosive: May cause irritation or damage to eyes and skin. Vapor or mist may irritate. Harmful if swallowed. No special protection or precautions have been identified with using this product under directed consumer use conditions

5.2.2. First Aid: Eyes: Hold eye open and rinse with water for 15 to 20 minutes. Remove contact lenses, after first five minutes. Continue rinsing eye. Call physician. Skin: Wash skin with water for 15 to 20 minutes. If irritation develops, call a physician. Ingestion: Do not induce vomiting. Drink a glassful of water. If irritation develops, call a physician. Do not give anything by mouth to an unconscious person. Inhalation: Remove to fresh air. If breathing is affected, call a physician

5.2.3. Spill Procedures: Control spill; use absorbents to clean spill. Wash area and let dry. Dispose of in accordance with all applicable federal, state and local regulations (MSDS EPA Regulation No. 5813-50, 2009)

5.3. Lemon Fresh Clorox Disinfecting Wipes: EPA Regulation No. 5813-5

5.3.1. Health Hazard: Eyes: Can cause moderate eye irritation. Ingestion: Liquid should be non-toxic if ingested. Skin: Prolonged skin contact may produce minor irritation. No special protection or precautions have been identified for using this product under directed consumer use conditions

5.3.2. First Aid: Eyes: Flush with water. Call a physician if irritation. Ingested: Drink a glassful of water. Call a physician or poison control center. Skin: Wash thoroughly with soap and water after handling. Inhalation: If breathing is affected, move to fresh air. Under normal consumer use conditions the likelihood of any adverse health effects are low

5.3.3. Spill Procedures: Absorb and containerize. Wash residual down to sanitary sewer. Contact the sanitary treatment facility in advance to assure ability to process washed down material. Waste disposal: Disposal must be made in accordance with applicable federal, state and local regulations

5.4. Glassware

5.4.1. In the case of broken glassware, obtain a dustpan and broom and sweep up the pieces. Discard broken glassware in the container marked ‘Recycled Glass’ in the lab. Do not handle broken glass by hand, if it can be avoided. Broken glassware with chemical residue should either be cleaned (if there is a way to do so safely), or placed into the trash container. Only place clean broken glass in the ‘Recycled Glass’ container. Glass pipettes are rinsed and disposed of in the ‘Recycled Glass’ container after each sample

5.5. Material Safety Data Sheets (MSDS)

5.5.1. MSDS are located in the microscopy lab documentation binder

6. MATERIALS

6.1. Squirt bottles for DI water
6.2. Squirt bottles for ethanol
6.3. Squirt bottles for bleach
6.4. Parafilm
6.5. Computer. Access to H:/ drive
  6.5.1. Master Login File
  6.5.2. PCR Login File
  6.5.3. Gel Logic 200 Imaging System (hooked up to computer)
6.6. Falcon tubes (50 mL)
6.7. Centrifuge and rotors (2 mL microcentrifuge and 50 mL)
6.8. Vortex
6.9. Heat block
6.10. Microcentrifuge tubes (2 mL, 0.5 mL)
6.11. Pipettes (1000 µL, 20-200 µL, 1-10 µL)
6.12. Pipette tips and pipette filter tips to fit all pipettes
6.13. PCR hood with UV light
6.14. Gel electrophoresis units and power supply
6.15. Gel casting trays and combs
6.16. Thermal Cycler
6.17. Microwave
6.18. Plastic containers with lids
6.19. Refrigerator and freezer (-80^{o}C)
6.20. Latex or nitrile gloves
6.21. PCR Racks

7. REAGENTS AND CHEMICALS

7.1. Bleach
7.2. Concentrated ethanol
7.3. Lake water preserved with ethanol
  7.3.1. 20% ethanol added in field
7.4. PowerSoil®DNA Isolation Kit (Mo Bio, 12888-100)
  7.4.1. Stored at room temperature
7.5. Positive DNA controls (adult QM and ZM)
  7.5.1. Stored at 4˚C
7.6. Taq polymerase (AmpliTaq with GeneAmp, Applied BioSystems, N808-0242)
  7.6.1. Stored at -20^{o}C
7.7. dNTPs (Promega, U1515)
  7.7.1. Working stock of the dNTPs is made by combining 150 µL of molecular grade water with 50 µL of dNTPs in a sterile 0.5 mL microcentrifuge tube
  7.7.2. It is recommended to mix several tubes of working stocks at one time and store the stocks in the freezer
  7.7.3. Once thawed, a tube of working stock should either be used or discarded within one week
7.8. MgCl (Thermo Scientific, AB-0359)
  7.8.1. It is recommended to aliquot the MgCl into sterile 0.5 mL microcentrifuge tubes

7.8.2. Aliquots (200 µL) should then be stored in the freezer
7.8.3. Once thawed, an aliquot of MgCl should either be used or discarded within one week
7.9. GeneAmp 10X PCR Buffer (AmpliTaq with GeneAmp, Applied BioSystems, N808-0242)
7.9.1. Stored at -20^{o}C
7.9.2. 10X buffer comes in 2 mL aliquots and should be aliquoted into sterile 0.5 mL microcentrifuge tubes
7.9.3. Aliquots (250 µL) of the 10X buffer should be made and stored in the freezer
7.9.4. Once thawed, a tube of working stock should either be used or discarded within one week
7.10. Primers
7.10.1. Primers 332/333 for Zebra COX1
7.10.1.1. Sequence 332: 5' TGT CAC CAC TCA TGG GCT TGT T 3'
7.10.1.2. Sequence 333: 5' TGC AGA ACA AAG GGA CCC GGT AAA 3'
7.10.2. Primers 334/335 for Quagga COX1
7.10.2.1. Sequence 334: 5' GAA ACT GGT TGG TCC CGA TA 3'
7.10.2.2. Sequence 335: 5' TAA GGC ACC GGC TAA AAC AG 3'
7.10.3. Working stocks of each primer are prepared by combining 198 µL of molecular grade water with 2 µL of the designated primer in a sterile 0.5 mL microcentrifuge tube
7.10.4. Working stocks can be made ahead and stored in the freezer
7.10.5. Once thawed, working stocks of primers should be either used or discarded within one week
7.11. Molecular grade water (Research Products International Corp, 248700)
7.12. Agarose (Agarose Unlimited, PS 1200)
7.13. EDTA (Fisher, S312-500)
7.13.1. Use 1000 mL autoclavable bottle.
7.13.2. Add a stir bar
7.13.3. Add 148.9 grams of EDTA disodium salt dehydrate into 800 mL DI water to make 0.5 M EDTA
7.13.4. Stir using the magnetic stir plate until dissolved
7.13.5. Adjust pH to 8.0 by adding NaOH pellets (approximately 20 grams will be needed), may need to add NaOH initially to help with the dissolution of EDTA
7.13.6. Store at room temperature
7.14. TAE buffer (50X)
7.14.1. Use 1000 mL autoclavable bottle. Add stir bar
7.14.2. Add 242.0 grams Tris base, 57.1 mL glacial acetic acid, and 100 mL of 0.5M EDTA
7.14.3. Add DI water to adjust total volume to 1000 mL and turn on stir plate. Everything should dissolve
7.14.4. Store at room temperature
7.15. TAE (1X)
7.15.1. Add 400 mL of 50X TAE to a 20 L carboy. Fill to the 20 Lmark with DI water
7.15.2. Store in carboy at room temperature
7.16. Molecular weight ladder (New England BioLabs, N3231L)
7.16.1. Molecular weight ladder (MWL) should be diluted in molecular grade water at a 1:10 ratio before using
7.16.2. The 1:10 MWL is then combined with loading dye
7.16.2.1. Use 30% diluted MWL and 70% loading dye
7.16.3. This is labeled as pre-mix ladder and stored in the -20^{o}C freezer when not in use

7.17. Loading dye (New England BioLabs, B7021S)
- 7.17.1. In a 15 mL Falcon tube, dissolve 3 g of reagent grade sucrose in 7.5 mL of molecular grade water
- 7.17.2. Let the sucrose dissolve completely
- 7.17.3. Add 2 mL of 10X TAE to the sucrose solution
- 7.17.4. Add 0.83 mL of 6X Blue/Orange loading dye to the sucrose solution
- 7.17.5. Mix well
- 7.17.6. Aliquot into 2 mL microcentrifuge tubes
- 7.17.7. Label tubes as "load dye"
- 7.17.8. Test newly made dye by mixing with positive controls that have been amplified by PCR and running gel electrophoresis as described in Section 15
- 7.17.9. Aliquots of loading dye should be stored in the freezer and thawed just before each use

7.18. GelStar (Lonza, 50535)
- 7.18.1. GelStar contains ethidium bromide, gloves are required
- 7.18.2. GelStar stain is stored in the -20^{o}C freezer and is light sensitive

8. SAMPLE RECEIVING

8.1. Samples that require PCR testing fall into three categories
- 8.1.1. Samples that are submitted for PCR only
  - 8.1.1.1. May be a duplicate of a sample sent for microscopy
- 8.1.2. Suspect organisms are found by microscopy
  - 8.1.2.1. Raw water sample will be analyzed by PCR
  - 8.1.2.2. If multiple suspects are found, some suspects may be analyzed by PCR
- 8.1.3. Samples that had a prior positive and have been put on a priority list
  - 8.1.3.1. When microscopy is completed the raw water sample is tested by PCR

8.2. Store sample for PCR in refrigerator on designated shelf, until ready for prep

8.3. Open PCR Login File located
- 8.3.1. H:\EnvResShare\MUSSEL SAMPLES\DATA and LOGIN
- 8.3.2. Password

8.4. Log-in samples to the PCR login sheet by copying and pasting the information for the samples from the Master Login File

9. SAMPLE PREPARATION PROCEDURE

9.1. Label a 50 mL Falcon tube with the sample ID number
- 9.1.1. Gently mix the contents of the sample bottle by inverting several times
  - 9.1.1.1. Pour 40 mL of sample into the pre-labeled Falcon tube

9.2. Centrifuge the sample at 4500 rpm for 30 minutes
- 9.2.1. Pour off the supernatant while taking care to not disrupt the pellet

9.3. In sample notebook, start new page with Prep, Extraction, PCR, and Gel written across the top. Fill in the date as each of these steps take place. Under this header write Samples and record the sample ID number and location of the sample being prepared

10. SAMPLE LYSIS

This procedure is modified from the PowerSoil®DNA Isolation Kit and from the Metropolitan Water District. All chemicals listed are part of the PowerSoil®DNA Isolation Kit (Mo Bio 12888-50). Currently there are two different methods for samples lysis used in the Reclamation Detection Laboratory. PCR on suspect organisms and PCR on the raw water sample. Suspect organisms will be pipetted into a sterile microcentrifuge tube and the DNA extracted using the Qiagen Blood and Tissue Kit (FDA Fish Barcoding SOP). For sample analysis of the raw water sample the PowerSoil®DNA Isolation Kit is used. The kits used for the extraction process are subject to change as companies selling these kits continue to change and update their kits

10.1. Lysis with the PowerSoil® DNA Isolation Kit (Mo Bio)

- 10.1.1. To the PowerBead Tubes, add 0.25 mL (approximately 0.25 grams) of the pellet
  - 10.1.1.1. Vortex to mix
- 10.1.2. Add 60 µL of Solution C1 and invert several times
- 10.1.3. Secure the PowerBead Tubes horizontally to the tube holders on the vortex.
  - 10.1.3.1. Vortex at max speed for 20 minutes
- 10.1.4. Centrifuge the tubes at 10,000 x g for 30 seconds at room temperature
- 10.1.5. Transfer the supernatant to a clean 2 mL collection tube
  - 10.1.5.1. Expect between 400-500 µL of supernatant
- 10.1.6. Add 250 µL of Solution C2 and vortex for 5 seconds
  - 10.1.6.1. Incubate at 4°C for 5 minutes
- 10.1.7. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
- 10.1.8. Avoiding the pellet, transfer 600 µL of supernatant to a clean 2 mL collection tube
- 10.1.9. Add 200 µL of Solution C3 and vortex briefly
  - 10.1.9.1. Incubate at 4°C for 5 minutes
- 10.1.10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
- 10.1.11. Avoiding the pellet, transfer up to 750 µL of supernatant into a clean 2 mL collection tube
- 10.1.12. Shake to mix Solution C4 before use. Add 1200 µL of Solution C4 to the supernatant and vortex for 5 seconds

10.2. Use the centrifuge to bind the DNA to the Spin Filter

- 10.2.1. Load approximately 675 µL onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add additional supernatant to the Spin filter and centrifuge at 10,000 x g for 1 minute at room temperature
  - 10.2.1.1. Repeat 10.13.13-10.13.14 until all of the supernatant has passed through the Spin Filter
- 10.2.2. Add 500 µL of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g
- 10.2.3. Discard the flow through
- 10.2.4. Centrifuge again at room temperature for 1 minute at 10,000 x g
- 10.2.5. Carefully place the spin filter in a clean 2 mL collection tube.
- 10.2.6. Add 100 µl of Solution C6 to the center of the white filter membrane
- 10.2.7. Centrifuge at room temperature for 30 seconds at 10,000 x g
- 10.2.8. Discard the Spin Filter
- 10.2.9. The DNA is now ready for PCR. Once PCR has been performed store the DNA in the -80°C freezer

10.3. Using the PowerVac™ Manifold to Bind the DNA to the Spin Filter

10.3.1. For each sample, gently press a Spin Filter into the aluminum PowerVac™ Mini Spin Filter Adapter into the Luer-Lok® fitting on one port in the manifold. Make sure that all of the unused ports of the vacuum manifold are closed

10.3.2. Transfer 650 µL of prepared sample lysate (from step 10.1.12) to the Spin Filter

10.3.3. Turn on the vacuum source and open the stopcock on the port. Allow the lysate to pass through the Spin Filter

10.3.3.1. Continue to add lysate until all of it has gone through the Spin Filter column

10.3.3.2. Close the stopcock

10.3.4. Load 800 µL of 100% ethanol into the Spin Filter so that it completely fills the column. Open the stopcock and allow the ethanol to pass through the column completely

10.3.5. Add 500 µL of Solution C5 to the Spin Filter. Open the stopcock and allow the solution to pass through the filter

10.3.6. Turn off the vacuum source and open an unused port to vent the manifold

10.3.7. Remove the Spin Filter and place in the original labeled 2 mL collection tube. Place into the centrifuge and spin at 13,000 x g for 1 minute to completely dry the membrane

10.3.8. To elute the DNA, transfer the Spin Filter to a labeled sterile 2 mL collection tube and add 100 µL of Solution C6 to the center of the white filter membrane

10.3.9. Centrifuge at room temperature for 30 seconds at 10,000 x g

10.3.10. Discard the Spin Filter. The DNA is now ready for PCR. Once PCR has been performed store the DNA in the -80^{o}C freezer

10.4. Lysis with the Qiagen Blood and Tissue Kit (From the FDA SOP for Fish Barcoding)

10.4.1. Place the suspect(s) into an 1.5 mL eppendorf tube

10.4.2. Add 50 µL of buffer ATL

10.4.3. Add 5.56 µL of proteinase K

10.4.4. Incubate 3-4 hours at 56^{o}C with vortexing every hour or incubate overnight

10.4.5. Vortex for 15 seconds

10.4.6. Add 55.6 µL Buffer AL, mix by vortexing

10.4.7. Add 55.6 µL of ethanol (96-100%)

10.4.8. Vortex

10.4.9. Pipette the mixture into the DNeasy Mini spin column. Centrifuge at 6000 x g (8000 rpm) for 1 minute

10.4.10. Place column in a fresh collection tube

10.4.11. Add 140 µL Buffer AW1

10.4.12. Centrifuge for 1 minute at 6000 x g (8000 rpm)

10.4.13. Place DNeasy Mini spin column in a fresh collection tube

10.4.14. Add 140 µL Buffer AW2

10.4.15. Centrifuge for 3 min at 20,000 x g (14,000 rpm)

10.4.16. Place DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube

10.4.17. Pipette 50 µL Buffer AE (warmed to 37^{o}C) directly onto the DNeasy membrane.

10.4.18. Incubate at room temperature for 1 min

10.4.19. Centrifuge for 1 min at 6000 x g (8000 rpm) to elute

11. PCR REACTION

11.1. Cytochrome oxidase 1 (COX1) is analyzed to test for the presence of QM/ZM DNA in samples

11.2. Master Mix recipe for analysis of QM/ZM by COX1

11.2.1. Quantities given are for one reaction tube

11.2.1.1. Molecular grade water - 9.8 µL

11.2.1.2. 10X buffer - 2.0 µL

11.2.1.3. dNTP's - 1.6 µL

11.2.1.4. Primer up - 1.0 µL

11.2.1.5. Primer down - 1.0 µL

11.2.1.6. MgCl - 2.4 µL

11.2.1.7. Taq polymerase - 0.2 µL

11.2.1.8. Template (extracted DNA from sample) - 2.0 µL

11.3. PCR run sheets

11.3.1. Use the PCR run sheet to calculate the quantities needed for the master mix cocktail

11.3.2. Multiply the quantity needed for one reaction tube by the number of samples to be run

11.3.2.1. Add two extra samples for each gel (the negative and positive controls)

11.3.2.2. It is recommended to include in the calculations 1-2 extra reactions so the master mix does not run out when it is being aliquoted

11.4. Gel template sheet

11.4.1. Fill in a gel template for the samples that will be run

11.4.1.1. The gel should include ladder in lanes 1 and 16

11.4.1.2. Each sample should be run using both the ZM and QM primers

11.4.1.3. Lanes 14 and 15 will always be the negative and positive controls

11.4.1.4. Each sample will be ran against both QM and ZM primers

11.5. Sterilize the PCR hood

11.5.1. Turn on the UV light for at least 15 minutes. Make sure that the PCR hood doors are closed

11.5.2. Remember to turn off the UV light before opening the doors

11.6. Set up closed 2 mL sterile microcentrifuge tubes for the master mix cocktail to be mixed in

11.7. Set up the PCR tubes needed and label on of the tops with the date, QM or ZM, and on the side place the sample ID number

11.7.1. Close the lids to all tubes

11.8. Make the master cocktail

11.8.1. Use the quantities calculated on the PCR run sheet, mix the master cocktail

11.9. Aliquot 18 µL of master cocktail mix into each of the 0.2 mL PCR reaction tubes

11.10. Using the gel template as a guide, transfer 2.0 µL of each sample into the appropriate tube

11.10.1. In the negative control tube, do not add any DNA

11.10.2. In the positive control tube, add 2.0 µL of the appropriate adult mussel DNA

11.11. Once the template DNA has been added to each tube, spin the PCR reaction tubes on the mini centrifuge to make sure that all liquid is at the bottom of the tube

11.12. Sterilize the PCR hood by turning on the UV light for 15 minutes

12. THERMAL CYCLER

12.1. Turn on the thermal cycler before setting up the PCR reaction
12.2. Place the PCR reaction tubes in the wells of the thermal cycler
12.3. Close the lid to the thermal cycler
12.4. Start the COX1 program (the program is the same for both QM/ZM)
- 12.4.1. Pre 95˚C for 9 minutes
- 12.4.2. Step 1 95˚C for 20 seconds
- 12.4.3. Step 2 59˚C for 90 seconds
- 12.4.4. Step 3 72˚C for 90 seconds
  - 12.4.4.1. Repeat for 40 cycles
- 12.4.5. Post 72˚C for 10 minutes
- 12.4.6. Hold 4˚C for infinity

13. AGAROSE GELS

13.1. Gel Casting
- 13.1.1. Using a digital scale and weigh paper, weigh out 0.6 g of agarose and place in a clean 125 mL flask. This will make an 1.5% agarose gel
- 13.1.2. Add 40 mL of 1X TAE buffer
- 13.1.3. Swirl to combine
- 13.1.4. Microwave the flask for approximately 2 minutes, swirling every 30 seconds or until the agarose is completely dissolved
  - 13.1.4.1. Four flasks can be in the microwave at the same time
  - 13.1.4.2. Dissolved agarose looks completely clear, not milky or jelly like
- 13.1.5. Assemble the gel casting tray with a comb and use a bubble level to level the tray
- 13.1.6. Gently pour the molten agarose into the prepared gel casting tray
  - 13.1.6.1. Pour slowly to avoid making bubbles
  - 13.1.6.2. If there are bubbles, pop the bubbles with a sterile pin
  - 13.1.6.3. Be careful not to disrupt the comb
- 13.1.7. Allow the gel to cool and solidify
  - 13.1.7.1. Transfer the gel casting tray to a plastic container with a lid and cover the gel completely with 1X TAE buffer for storage in the refrigerator

13.2. Gel electrophoresis
- 13.2.1. Carefully remove combs from the casting tray
- 13.2.2. Remove the gel from the casting tray
- 13.2.3. Transfer the gel to the gel electrophoresis chamber, using the handled portion of the gel casting tray
  - 13.2.3.1. Add 1X TAE to completely cover the gel
- 13.2.4. Label PCR tubes with sample ID number
- 13.2.5. Place 7 µL of loading dye into each PCR tube
- 13.2.6. Add 3 µL of amplified DNA to the load dye
- 13.2.7. Close the PCR tube lid and gently tap the tubes so that the DNA and loading dye are mixed and at the bottom of the tube
- 13.2.8. Load 7 µL of pre-mixed ladder into wells one and sixteen on each gel
- 13.2.9. Transfer 7 µL of sample mixture to the correct well of the gel
- 13.2.10. Place the lid on the electrophoresis chamber, with the electrodes in the proper orientation (black goes to the top and red to the bottom)
- 13.2.11. Connect the electrophoresis chamber to the power supply

13.2.12. Turn on the power supply
13.2.13. Run the gel for one hour at 100V, 500mA
13.3. Staining the gel
13.3.1. When the gel is finished running, transfer the gel (with transfer tray) to a gel casting tray dedicated to staining
13.3.2. Allow GelStar to thaw in its closed container
13.3.3. Mix 40 mL 1X TAE buffer and 5 µL GelStar in a 125 mL flask (dedicated to staining)
13.3.4. Pour mixture over the gel
13.3.5. Stain gel for 30 minutes in the dark

14. GEL IMAGING

14.1. Open the Kodak program on the computer
14.2. Using the gel carrying tray, transfer the gel to the photo chamber
14.3. Turn the knob on the front of the photo system from OFF to TRANS UV
14.4. In the Kodak program, click on CAPTURE GL100
14.5. Click PREVIEW to see the gel image
14.5.1. If the gel needs to be adjusted, turn off the UV before opening the door and moving the gel
14.6. Once the image is in focus, click on the CAPTURE button to take a picture of the gel.
14.6.1. This will open the IMAGE DISPLAY
14.7. Check SATURATION OFF to remove the red lines from the gel image.
14.8. Also, click INVERT so that the bands are dark and gel background is light
14.9. To save the gel, go to FILE and select EXPORT DATA and then IMAGE. A new window will pop up. Click SAVE AS and select type of file as JPEG FILE
14.10. Name the file with the date, gel number, and organism analyzed
14.10.1. Click SAVE
14.11. These images are saved to the desktop in the folder YEAR SAMPLES and EXPERIMENTS
14.12. Print a copy of the gel image and tape it into the PCR notebook on the appropriate page
14.13. Record the PCR results in the PCR notebook and also in the PCR spreadsheet
14.14. See the Table 1 below for the possible outcomes

Table 1: Possible outcome for gels ran in Reclamations Detection Laboratory for Invasive and Native Species. The positive and negative control outcome determines if the gel passes QA/QC.

Positive Control	Positive	Negative	Positive	Negative
Negative Control	Negative	Negative	Positive	Positive
Outcome	**Passes**	**Fails**	**Fails**	**Fails**

14.15. Negative Control
- 14.15.1. A negative control is ran for each PCR sample set
- 14.15.2. The negative control is the PCR reagents without any DNA present
- 14.15.3. This control should come back blank, if there is a band present then the gel fails QA/QC standards and all of the samples that used that master mix need to be redone

14.16. Positive Control
- 14.16.1. A positive control is also ran for each PCR sample set
- 14.16.2. The positive control DNA is obtained from an adult mussel of the appropriate species
- 14.16.3. DNA from the whole organism is extracted using the PowerSoil® DNA Isolation Kit as described in Section 10
- 14.16.4. Control DNA should be diluted to provide a clear, bright band. This may require up to a 500X dilution

14.17. Sequencing
- 14.17.1. Sequencing allows us to validate if the band that is on the gel is actually QM or ZM COX1, or some other organism
- 14.17.2. Once samples are analyzed on an agarose gel, positive samples are reanalyzed by COX1 to confirm the positive result. If the sample is positive for a second time, the sample will be sent for sequencing
  - 14.17.2.1. There must be enough DNA for the sequencing reaction; if the original COX1 reaction did not yield a sufficient amount of DNA then it is necessary to use the nested COX1 primers to obtain the needed DNA (See Appendix 1)
- 14.17.3. Fill out the on-line order form for Sanger-based sequencing located at: http://seqwright.com/placeorder/orderforms.html
- 14.17.4. Check the box to tell SeqWright that the samples have not been cleaned up and that they need to perform this task prior to sequencing
- 14.17.5. Copy and paste the primers and size of the DNA product from the excel spreadsheet that serves as the primer library
- 14.17.6. Give each order a number (initials/number) so that the order can be kept track of
- 14.17.7. E-mail the order form to SeqWright
- 14.17.8. Print two copies of the order form. One will be mailed with the samples to SeqWright and the other will be kept in the sequencing notebook
- 14.17.9. Add the sequencing order to the PCR lab supply spread sheet
- 14.17.10. Transfer all of the PCR product to a pre-labeled 0.5 mL microcentrifuge tube
- 14.17.11. Samples are placed in a plastic bag with the order form and mail to SeqWright
  - 14.17.11.1. SeqWright, Inc., 2575 West Bellfort, Suite 2001, Houston, TX 77054
  - 14.17.11.2. Phone number: 800-720-GENE (4363)
- 14.17.12. If other labs are used for DNA sequencing, follow the companies specific instructions for sample shipment

15. SEQUENCING ANALYSIS

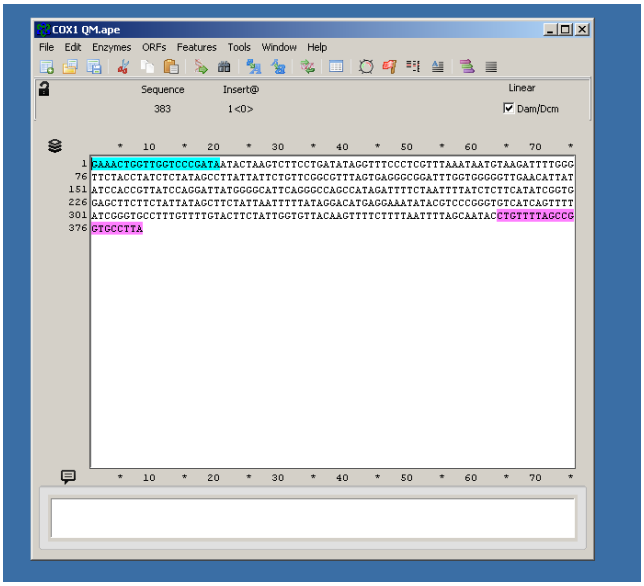
15.1. SeqWright and other sequencing companies will send back both the sequence and the chromatograph
- 15.1.1. Convert the sequence data file into a notepad file

15.2. Load A Plasmid Editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/)
- 15.2.1. Open the sequence files for the published positive control and the positive control sequences that our lab has generated

15.3. Open the notepad file

15.3.1. Paste the sample sequence into A Plasmid Editor

15.3.2. Save the file under the sample name



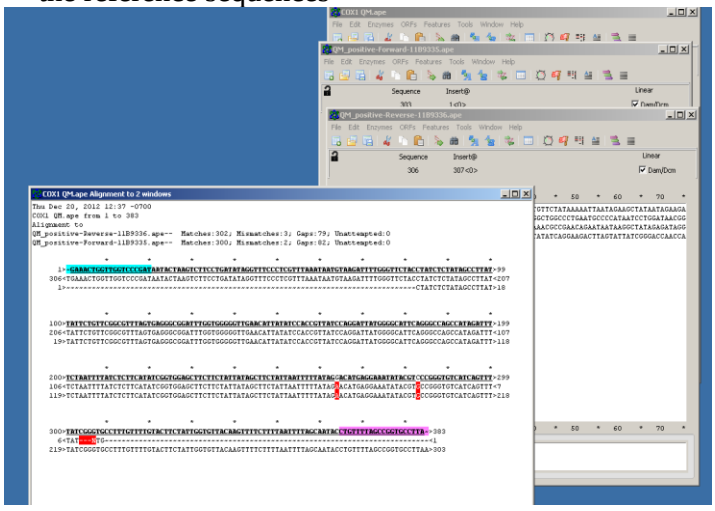
15.4. Align the sample sequence in the following order

15.4.1. To a published COX1 ZM, or QM sequence, forward and reverse sequences

15.4.2. To both the forward and reverse sequences of previously sequenced positive control samples

15.4.3. Open all four files in A Plasmid Editor, then go to Tools, click align sequences, and select the sequences that will be aligned from SeqWright

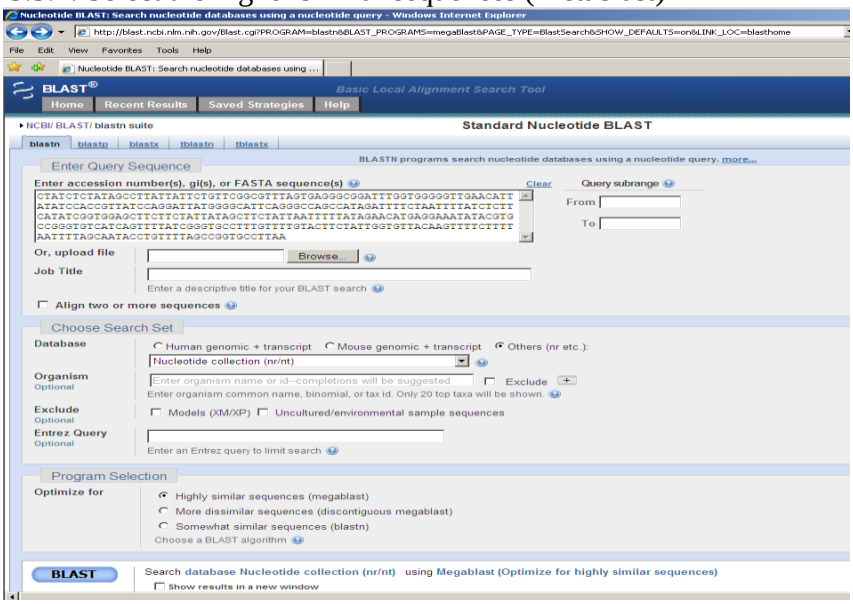
15.4.4. Once the sequences are aligned record how many matches the sample sequence has to the reference sequences



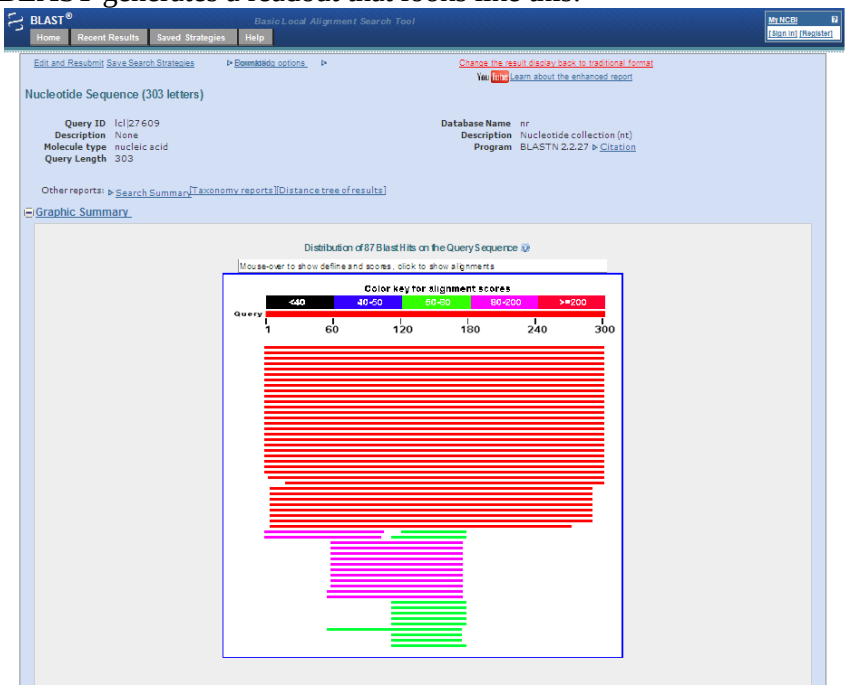
15.5. Following the alignment, copy the sample sequence and paste it into nucleotide NCBI BLAST

15.5.1. Select the others database

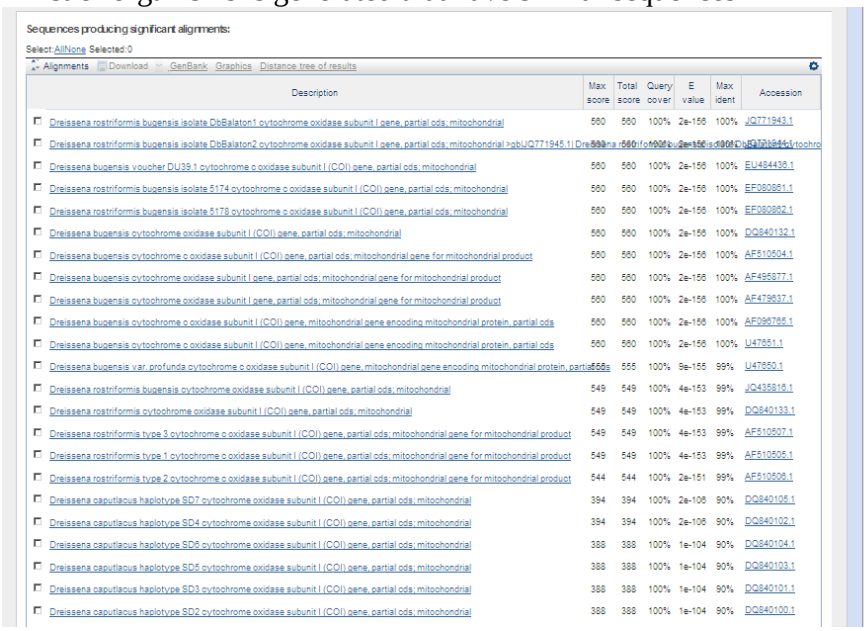
15.5.2. Select the higher similar sequences (Metablast)



15.6. BLAST generates a readout that looks like this:



15.7. A list of organisms is generated that have similar sequences



Sequences producing significant alignments:

Select: All None Selected: 0

Alignments | Download | GenBank | Graphics | Distance tree of results

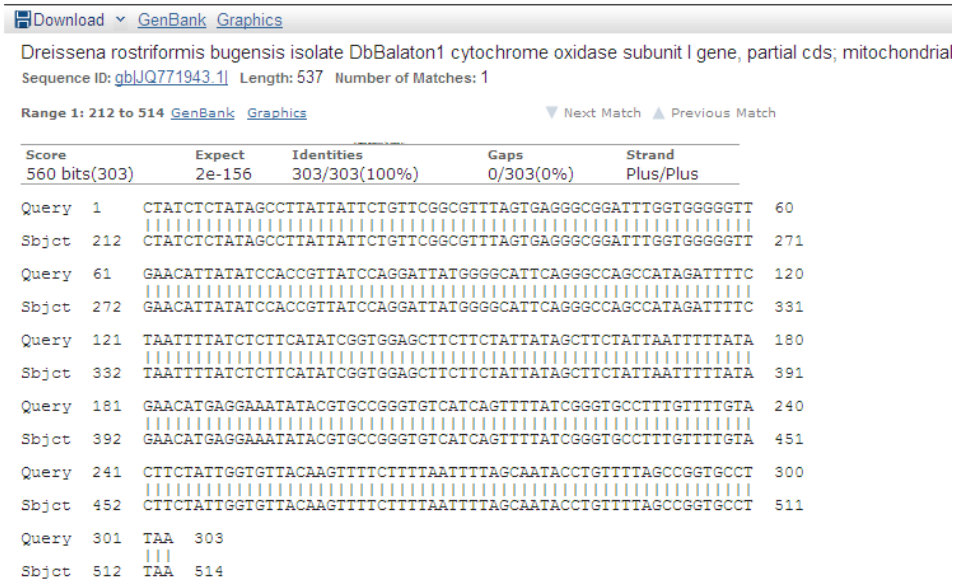
Description	Max score	Total score	Query cover	E value	Max ident	Accession
Dreissena rostriformis bugensis isolate DbBalaton1 cytochrome oxidase subunit I gene, partial cds; mitochondrial	560	560	100%	2e-156	100%	JQ771943.1
Dreissena rostriformis bugensis isolate DbBalaton2 cytochrome oxidase subunit I gene, partial cds; mitochondrial >gb|JQ771945.1|Dre	560	560	100%	2e-156	100%	JQ771944.1
Dreissena bugensis voucher DU39.1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	560	560	100%	2e-156	100%	EU484436.1
Dreissena rostriformis bugensis isolate 5174 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	560	560	100%	2e-156	100%	EF080861.1
Dreissena rostriformis bugensis isolate 5178 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	560	560	100%	2e-156	100%	EF080862.1
Dreissena bugensis cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	560	560	100%	2e-156	100%	DQ840132.1
Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	560	560	100%	2e-156	100%	AF510504.1
Dreissena bugensis cytochrome oxidase subunit I gene, partial cds; mitochondrial gene for mitochondrial product	560	560	100%	2e-156	100%	AF495877.1
Dreissena bugensis cytochrome oxidase subunit I gene, partial cds; mitochondrial gene for mitochondrial product	560	560	100%	2e-156	100%	AF479837.1
Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	560	560	100%	2e-156	100%	AF096766.1
Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	560	560	100%	2e-156	100%	U47651.1
Dreissena bugensis var. profunda cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	555	555	100%	9e-155	99%	U47650.1
Dreissena rostriformis bugensis cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	549	549	100%	4e-153	99%	JQ435816.1
Dreissena rostriformis cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	549	549	100%	4e-153	99%	DQ840133.1
Dreissena rostriformis type 3 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	549	549	100%	4e-153	99%	AF510507.1
Dreissena rostriformis type 1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	549	549	100%	4e-153	99%	AF510505.1
Dreissena rostriformis type 2 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	544	544	100%	2e-151	99%	AF510506.1
Dreissena caputlacus haplotype SD7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	394	394	100%	2e-106	90%	DQ840105.1
Dreissena caputlacus haplotype SD4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	394	394	100%	2e-106	90%	DQ840102.1
Dreissena caputlacus haplotype SD6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	388	388	100%	1e-104	90%	DQ840104.1
Dreissena caputlacus haplotype SD5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	388	388	100%	1e-104	90%	DQ840103.1
Dreissena caputlacus haplotype SD3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	388	388	100%	1e-104	90%	DQ840101.1
Dreissena caputlacus haplotype SD2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	388	388	100%	1e-104	90%	DQ840100.1

15.8. This will give both query coverage and the maximum identity of the DNA sequence that was analyzed by BLAST. Record both of these numbers in the PCR spreadsheet.

15.8.1. The query coverage tells how much of the sequence that BLAST analyzed is the same as sequences in the database.

15.8.2. The maximum identity is a score that BLAST gives for how certain the program is of the identity of the sequence that was analyzed

15.9. Below is an alignment of the sample sequence query to sequences in the databas



Download GenBank Graphics

Dreissena rostriformis bugensis isolate DbBalaton1 cytochrome oxidase subunit I gene, partial cds; mitochondrial

Sequence ID: gb|JQ771943.1| Length: 537 Number of Matches: 1

Range 1: 212 to 514 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
560 bits(303)	2e-156	303/303(100%)	0/303(0%)	Plus/Plus

```
Query  1    CTATCTCTATAGCCTTATTATTCTGTTCGGCGTTTAGTGAGGGCGGATTTGGTGGGGGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  CTATCTCTATAGCCTTATTATTCTGTTCGGCGTTTAGTGAGGGCGGATTTGGTGGGGGTT  271

Query  61   GAACATTATATCCACCGTTATCCAGGATTATGGGGCATTCAGGGCCAGCCATAGATTTTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  GAACATTATATCCACCGTTATCCAGGATTATGGGGCATTCAGGGCCAGCCATAGATTTTC  331

Query  121  TAATTTTATCTCTTCATATCGGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTTATA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  TAATTTTATCTCTTCATATCGGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTTATA  391

Query  181  GAACATGAGGAAATATACGTGCCGGGTGTCATCAGTTTTATCGGGTGCCTTTGTTTTGTA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  392  GAACATGAGGAAATATACGTGCCGGGTGTCATCAGTTTTATCGGGTGCCTTTGTTTTGTA  451

Query  241  CTTCTATTGGTGTTACAAGTTTTCTTTTAATTTTAGCAATACCTGTTTTAGCCGGTGCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  452  CTTCTATTGGTGTTACAAGTTTTCTTTTAATTTTAGCAATACCTGTTTTAGCCGGTGCCT  511

Query  301  TAA  303
            |||
Sbjct  512  TAA  514
```

15.10. Determination if the sequencing results pass QA/QC

15.10.1. For a sequence result to pass QA/QC there should be at least a 150 bp match with the alignment of the sample to the positive control and published sequences in A Plasmid Editor

15.10.2. In addition, the samples should have BLAST query coverage and maximum ID scores of at least 95% certainty that it is QM/ZM. The higher these two numbers the more certainty there is in the identification of the sample as a QM/ZM

16. SPILLS

16.1. When spills occur

16.1.1. Clean spill with paper towels

16.1.2. Decontaminate counter with bleach

17. DATA LOGGING PROCEDURES

17.1. Input results into the PCR login file

17.1.1. Located in H:\EnvRes Share\MUSSEL SAMPLES\DATA and LOGIN

17.1.2. Password

17.1.3. Save PCR login file as a new spreadsheet with current date

17.1.4. Move old spreadsheet into “Old Data Files” folder

17.1.5. Enter the date on which the 40 mL subsample was pulled as the “Set aside for PCR”

17.1.6. Enter the last date the sample was tested as the “Date Analyzed”

18. DISPOSAL OF PCR PRODUCTS AND DNA SAMPLE STORAGE

18.1. DNA storage

18.1.1. DNA extracts are stored in the -80^{o}C freezer in a box or plastic bag labeled with the DNA extraction date. These should be kept indefinitely

18.2. PCR products are stored in the refrigerator for at least one month, after which they are disposed

19. ONLINE DATABASE

The online share point in currently under construction

20. REFERENCES

Acetic Acid. MSDS (2010). No. A/0407/PB08; Fisher Scientific: Loughborough, Leicestershire, Great Britain, 11/9/2010, www.Acros.com (accessed 12/20/2010)

Ethanol, denat. with up to 5% v/v ether (2010). No. 270660000; Acros Organics: Geel, Belgium, 11/16/2010, www.Acros.com (accessed 12/20/2010)

Single Laboratory Validated Method for DNA-Barcoding for the Species Identification of Fish for FDA Regulatory Compliance (September 2011)

Metropolitan Water District of Southern California. Paul Rochelle. SOP rough draft (2010).

PowerSoil®DNA Isolation Kit, Instruction Manual, Mo Bio Laboratories, Inc., ver: 01102013

SOP Z/Q Mussel Lab-3 (2012) Developed for the Denver Environmental Laboratory, Technical Services Center, Denver CO, Technical Memorandum No. 86-68220-12-08

SOP Z/Q Mussel Lab-2 (2010) Developed for the Denver Environmental Laboratory, Technical Services Center, Denver CO, Technical Memorandum No. 86-68220-12-08

SOP PCR-1 (2010) Developed from the Denver Environmental Laboratory, Technical Services Center, Denver CO, Technical Memorandum No. 86-68220-12-08

Standard Methods (2001) For the Examination of Water and Wastewater, Counting Techniques for Phytoplankton (10200F) and zooplankton (10200G)

http://www.standardmethods.org/store/ProductView.cfm?ProductID=85 (accessed 2/6/2009)

US Army Corps of Engineers, *Zebra Mussel Information Systems* (ZMIS 2002), 3/14/2002, http://el.erdc.usace.army.mil/zebra/zmis/zmishelp.htm, (accessed 12/22/10)

U.S. EPA (2003) Standard Operating Procedure for Zooplankton Analysis. http://epa.gov/grtlakes/monitoring/sop/chapter_4/LG403.pdf (accessed 2/6/2009)

21. CONTACT INFORMATION

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22. APPENDIX 1: DNA SEQUENCES OF ZM AND QM COX1

ZM COX1

AGCGTCCTTGGTGATTTTCAATTATATAATTTAAT**TGTCACCACTCATGGGCTT**
GTTATAATTTTTTTTCTAGTAATACCTATAATAATGGGGGGATTCGGAAATTGAT
TGGTACCAATAATACTGAGTCTTCCTGATATAGGTTTCCCTCGTCTTAATAATGTT
AGTTTTTGGGTTTTACCTGTCTCTATAGGACTTCTGTTTTGTTCAGCTTTTAGGGA
AGGAGGATTCGGGGGTGGTTGAACCTTATATCCTCCTTTATCTAGAGTTATAGGA
CATTCAGGCCCTGCGATAGATTTTTTGATTTTATCTCTTCATATTGGGGGAGCTTC
TTCGATTATGGCTTCTATTAATTTTTATAGGACATGAGGTAATATACGTGCTGGAT
GTCATCAAT**TTTACCGGGTCCCTTTGTT**CTGCACATCTATTGGTGTGACCAGAT
TCCTTTTAATCTTAGCAATGCCTGTATTAGCTGGGGCTTTAACAATATTATTAACT
GATCGAAATTTTAACA

Primer-332 (forward):5’-TGTCACCACTCATGGGCTTGTT-3’
Primer-333 (reverse): 5’-TGCAGAACAAAGGGACCCGGTAAA-3’

QM COX1

GAAACTGGTTGGTCCCGATAATACTAAGTCTTCCTGATATAGGTTTCCCTCGTT
TAAATAATGTAAGATTTTGGGTTCTACCTATCTCTATAGCCTTATTATTCTGTTCG
GCGTTTAGTGAGGGCGGATTTGGTGGGGGTTGAACATTATATCCACCGTTATCCA
GGATTATGGGGCATTCAGGGCCAGCCATAGATTTTCTAATTTTATCTCTTCATATC
GGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTTATAGGACATGAGGAAATAT
ACGTCCCGGGTGTCATCAGTTTTATCGGGTGCCTTTGTTTTGTACTTCTATTGGTG
TTACAAGTTTTCTTTTAATTTTAGCAATAC**CTGTTTTAGCCGGTGCCTTA**ACAAT
GCTCTTAACTGATCGTAATTTTAACAC

Primer-334 (forward): 5’-GAAACTGGTTGGTCCCGATA-3’
Primer-335 (reverse): 5’-TAAGGCACCGGCTAAAACAG-3’

Nested Primers (NP) on QM sequence

Each primer pair is highlighted in the COX1 sequence. Publication pending

GAGCTTAGTGCTCCTGGAAGAGTAGTAGGAGATTATCAATTATATAATTTAATTG
TTACTACTCACGGGCTTATCATAATTTTTTTTCTTGTAATACCTATAATGATAGGT
GGATTTGGAAACTGGTTGGTCCCGATAATACTAAGTCTTCCTGATATAGGTTTCC
CTCGTTTAAATAATGTAAGATTTTGGGTTCTACCTATCTCTATAGCCTTATTAT
TCTGTTCGGCGTTTAGTGAGGGCGGATTTGGTGGGGGTTGAACATTATATCCACC
GTTATCCAGGATTATGGGGCATTCAGGGCCAGCCATAGATTTTCTAATTTTATCT
CTTCATATCGGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTTATAGGACATG
AGGAAATATACGTCCCGGGTGTCATCAGTTTTATCGGGTGCCTTTGTTTTGTAC
TTCTATTGGTGTTACAAGTTTTCTTTTAATTTTAGCAATACCTGTTTTAGCCGGTG
CCTTAACAATGCTCTTAACTGATCGTAATTTTAACAC

NP1 Forward: 5'-CTGGTTGGTCCCGATAATACTAAG-3'
NP1 Reverse: 5'-GAAGAAGCTCCACCGATATGAA-3'
Expect: 231 bp band (yellow)

NP2 Forward**:** 5'-GGTTCTACCTATCTCTATAGCCTTATT-3'
NP2 Reverse: 5'-GACACCCGGGACGTATATTT-3'
Expect: 218 bp band (red)

NP3 Forward: 5'-TCCTGATATAGGTTTCCCTCG-3'
NP3 Reverse: 5'-CGGGACGTATATTTCCTCATGTC-3;
Expect: 254 bp band (grey)

To amplify the original COX1 PCR product with the nest primers

22.1. Decide which nested primer (NP) set that will be used.
  22.1.1. The NP3 primer set is the preferred set to use because it produces a band that is over 250 bp in size
22.2. Set up the PCR master mix as if for the whole length COX1 (See section11.2), except with the NP
22.3. Aliquot 18 µL of the master mix into each tube
22.4. Take 2 µL of the original PCR product and add it to the master mix
  22.4.1. For a positive control use 2 µL of positive control
  22.4.2. Also include a negative control without any DNA added
22.5. Run the COX1 PCR program
22.6. After the PCR has been run, analyze the sample on an agarose gel to confirm the presence of a band
22.7. Send the PCR product for sequencing (Sections 14 & 15)

23. APPENDIX 2: ADDITIONAL DNA EXTRACTION METHOD

Reclamations Detection Laboratory for Invasive and Native Species previously used the Mo Bio Ultra Clean Soil Kit to perform DNA extractions from water samples. Below is the method that had previously been used by.

23.1. Lysis with the Mo Bio Ultra Clean Soil Kit (12800-100)

- 23.1.1. Add up to 0.5 grams of the concentrated pellet to each Bead Solution tube
- 23.1.2. Gently vortex to mix
- 23.1.3. Add 60 µL of Solution S1, and invert once to mix
- 23.1.4. Add 200 µL of Solution IRS
- 23.1.5. Using a Vortex adaptor pad, attach the samples horizontally to pad
  - 23.1.5.1. Vortex at maximum speed for 20 minutes
- 23.1.6. Centrifuge tubes at 7800 rpm for 1 minute
- 23.1.7. Transfer 725 µL of supernatant to a clean microcentrifuge tube (provided in kit)
- 23.1.8. Add 250 µL of Solution S2, vortex and incubate at 4^{o}C for 5 minutes
  - 23.1.8.1. Samples can be stored at 4^{o}C until ready to complete lysis
- 23.1.9. Centrifuge tubes at 7800 rpm for 1 minute
- 23.1.10. Avoiding pellet, transfer all supernatant by dividing into 2 clean microcentrifuge tubes (provided in kit)
- 23.1.11. Add 650 µL of Solution S3 to the supernatant in each tube (total of 1300 uL)
  - 23.1.11.1. Vortex for 5 seconds
- 23.1.12. Load approximately 600 µL onto the Spin Filter (provided in kit)
  - 23.1.12.1. Centrifuge at 7800 rpm for 1 minute
- 23.1.13. Discard the flow-through, add remaining supernatant to the Spin Filter
  - 23.1.13.1. Centrifuge at 7800 rpm for 1 minute
- 23.1.14. Repeat step 10.12 until all supernatant has been processed
- 23.1.15. Add 300 µL of Solution S4
  - 23.1.15.1. Centrifuge at 7800 rpm for 30 seconds
  - 23.1.15.2. Discard flow-through, and re-centrifuge for 1 minute
- 23.1.16. Carefully place Spin Filter into a new clean labeled microcentrifuge tube (provided in kit)
- 23.1.17. Avoid splashing any Solution S4 onto the Spin Filter
- 23.1.18. Add 50 µL of Solution S5 to the center of the white filter membrane
- 23.1.19. Centrifuge at 7800 rpm for 30 seconds
- 23.1.20. Discard the Spin Filter
- 23.1.21. Store samples at 4^{o}C for up to one month, archive samples at -80^{o}C