

RECLAMATION

Managing Water in the West

Technical Memorandum No.

Polymerase Chain Reaction: Preparation and Analysis of Veliger Water Samples

PCR Laboratory Standard Operating Procedure (PCR SOP)

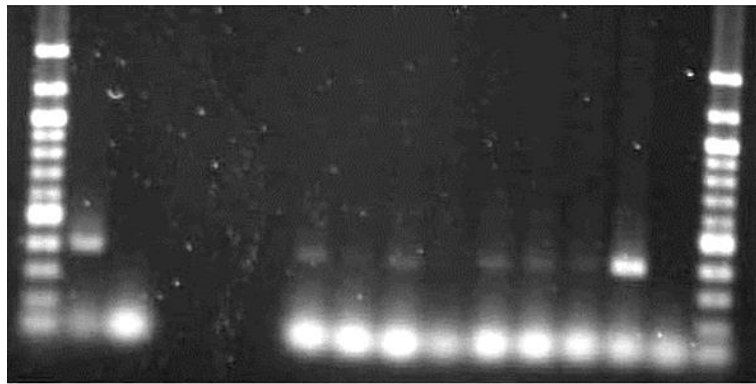
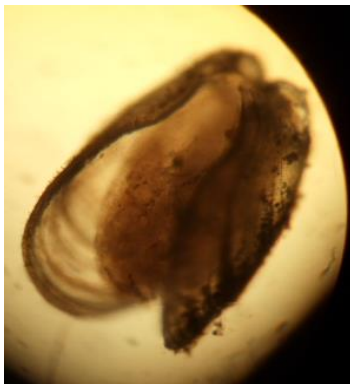
PCR SOP Version 5

Date Revised: September 2015

Bureau of Reclamation

Technical Service Center

Reclamation Detection Laboratory for Exotic Species



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

September 2015

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.

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 5

Date Revised: September 2015

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**Bureau of Reclamation
Technical Service Center
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Previous Versions:

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**U.S. Department of the Interior
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PEER REVIEW DOCUMENTATION

Project and Document Information

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1. Review Certification

Peer Reviewer: I have reviewed the assigned items/sections(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  (Signature)

Date reviewed 9/23/2015



U.S. Department of the Interior
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Denver, Colorado

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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 Exotic 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 raw water samples for the presence of mussel DNA.

4. INTERFERENCES

Keep the DNA extraction and PCR work stations separate to ensure that there is no cross contamination. To test for cross contamination wipe tests are performed (See Appendix 2). Always use sterile pipette tips and tubes so that there is no cross contamination of reagents or DNA. It is possible that the PCR reaction can be inhibited by humic acid and other inhibitory substances. To overcome this issue, a soil DNA extraction kit that is designed to remove these substances is used.

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: 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. Ingestion: 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. DNA Away (Alkali hydroxide solution)

5.4.1. Health Hazard: The product is mildly to moderately irritating to skin, eyes, mucous membranes and other tissues which may be contaminated (depending on duration and concentration of exposure).

5.4.2. Flammability Hazards: this product is not flammable. If this product is involved in a fire, the decomposition produces generated will include irritating vapors and toxic gases (including sodium oxides).

5.4.3. Reactivity Hazards: This products is not reactive.

5.4.4. Environmental Hazards: Large quantities released to the environment may have an adverse effect.

5.4.5. Emergency Considerations: Emergency responders should wear appropriate protection for situation to which they respond.

5.5. Gel Star (contains dimethyl sulfoxide (DMSO))

5.5.1. Health Hazard: Inhalation- no special precautions required; Skin contact- wash with water and soap as a precaution, if skin irritation persists, call a physician; Eye contact- immediately flush eye(s) with plenty of water, if eye irritation persists, consult a specialist; Ingestion-immediately give large quantities of water to drink, do not induce vomiting without medical advice, never give anything by mouth to an unconscious person.

5.5.2. Environmental Hazards: Prevent product from entering drains.

5.5.3. Emergency Considerations: Contain spillage, and then collect with non-combustible absorbent material and place in container for disposal according to local/national regulations.

5.5.4. Personal precautions: Use personal protective equipment.

5.6. Glassware

5.6.1. 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.

5.7. Material Safety Data Sheets (MSDS)

5.7.1. MSDS are located in the microscopy lab documentation binder.

6. MATERIALS

- 6.1. Squirt bottle for DI water
- 6.2. Squirt bottle for ethanol
- 6.3. Squirt bottle and/or spray bottle for 20% 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. Enumeration File
- 6.6. Gel Logic 200 Imaging System (connected to computer in gel running area)
 - 6.6.1. Kodak MI program for taking gel images
- 6.7. Falcon tubes (50 mL)
- 6.8. Centrifuge and rotors (2 mL microcentrifuge and 50 mL)
- 6.9. Vortex
- 6.10. Heat block
- 6.11. Microcentrifuge tubes (2 mL, 0.5 mL)
- 6.12. Pipettes (1000 μ L, 20-200 μ L, 1-10 μ L)
 - 6.12.1. Dedicated pipettes for the DNA, PCR, and gel running areas of the laboratory
- 6.13. Pipette tips and pipette filter tips to fit all pipettes
- 6.14. PCR strip tubes with attached caps
- 6.15. PCR hood with UV light
- 6.16. Gel electrophoresis units and power supply
- 6.17. Gel casting trays and combs
- 6.18. Thermal cycler
- 6.19. Microwave
- 6.20. Plastic containers with lids
- 6.21. Refrigerator and freezer (-80°C)
- 6.22. Latex or nitrile gloves
- 6.23. PCR racks

7. REAGENTS AND CHEMICALS

- 7.1. Bleach
- 7.2. Concentrated ethanol
- 7.3. Lake water preserved with 20% ethanol and buffered with baking soda (added in the field).
- 7.4. PowerSoil[®] DNA Isolation Kit (Mo Bio, 12888-100), store at room temperature in the DNA prep room.
- 7.5. Qiagen DNeasy Blood and Tissue Kit (Qiagen, 69506), store at room temperature in the DNA prep room.
- 7.6. Positive DNA controls (adult QM and ZM) stored at -20°C.
 - 7.6.1. Make a 1:50 dilution of mussel DNA to DI water for both ZM and QM to use as positive controls for the PCR reaction.
 - 7.6.2. Label the top of each microcentrifuge tube of each diluted positive control with either QM or ZM.
 - 7.6.3. Write the date that the dilution was made on the side of the microcentrifuge tube.
 - 7.6.4. Store both diluted and non-diluted positive control DNA in the freezer in the PCR prep room.

- 7.7. Taq polymerase (AmpliTaq with GeneAmp, Applied BioSystems, N808-0242), store at -20°C.
 - 7.7.1. The Taq does not have to be diluted.
 - 7.7.2. Taq that is being used for the PCR reactions is stored in the blue ice block in the PCR prep room freezer.
- 7.8. dNTPs (Promega, U1515)
 - 7.8.1. 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.
 - 7.8.2. It is recommended to mix several tubes of working stocks at one time and store the stocks in the freezer.
 - 7.8.3. On the top of each microcentrifuge tube write dNTP.
 - 7.8.4. Store the aliquots in the PCR working stock box in the freezer in the PCR prep room.
 - 7.8.5. Once thawed, a tube of working stock should either be used or discarded within one week.
 - 7.8.6. Store at 4°C once thawed
- 7.9. MgCl₂ (Thermo Scientific, AB-0359)
 - 7.9.1. Aliquot the MgCl₂ into sterile 0.5 mL microcentrifuge tubes.
 - 7.9.2. On top of the microcentrifuge tube write the label Mg.
 - 7.9.3. Store the aliquots in the PCR working stock box in the freezer in the PCR prep room.
 - 7.9.4. Once thawed, an aliquot of MgCl₂ should either be used or discarded within one week.
- 7.10. GeneAmp 10X PCR Buffer (AmpliTaq with GeneAmp, Applied BioSystems, N808-0242).
 - 7.10.1. Store at 4°C .
 - 7.10.2. 10X buffer comes in 2 mL aliquots and should be aliquoted into sterile 0.5 mL microcentrifuge tubes.
 - 7.10.3. Label the top of the microcentrifuge tube with 10X.
 - 7.10.4. Store the aliquots in the PCR working stock box in the PCR prep room freezer.
 - 7.10.5. Once thawed, a tube of 10X buffer should either be used or discarded within one week.
- 7.11. Primers
 - 7.11.1. Order all primers from Integrated DNA Technologies (IDT), www.idtdna.com.
 - 7.11.2. When the primers arrive they need to be rehydrated.
 - 7.11.2.1. To create the master stock for a primer, look on the primer specification sheet that came with the primers to find the Oligo information.
 - 7.11.2.2. Under the heading Oligo there are three numbers listed: OD₂₆₀, nMoles, and mg.
 - 7.11.2.3. Take the number of nMoles of Oligo and multiply by 10 to determine the amount of molecular grade water to add to the tube to make a 100 µM master stock.
 - 7.11.3. Primers 332/333 for Zebra COX1
 - 7.11.3.1. Sequence 332: 5' TGT CAC CAC TCA TGG GCT TGT T 3'
 - 7.11.3.2. Sequence 333: 5' TGC AGA ACA AAG GGA CCC GGT AAA 3'
 - 7.11.4. Primers 334/335 for Quagga COX1
 - 7.11.4.1. Sequence 334: 5' GAA ACT GGT TGG TCC CGA TA 3'
 - 7.11.4.2. Sequence 335: 5' TAA GGC ACC GGC TAA AAC AG 3'
 - 7.11.5. Working stocks of each primer are prepared by combining 198 µL of molecular grade water with 2 µL of the designated master stock primer in a sterile 0.5 mL microcentrifuge tube.
 - 7.11.6. Label the top of each microcentrifuge tube with the number of the primer (e.g. 332).
 - 7.11.7. Store the primer stocks in the PCR primer QM/ZM box in the PCR prep room freezer.

- 7.11.8. Once thawed, working stocks of primers should be either used or discarded within one week.
- 7.11.9. Store at 4°C once thawed
- 7.12. Molecular grade water (Research Products International Corp, 248700)
 - 7.12.1. Pipette 0.5 mL of water into 1.5 mL Eppendorf tubes.
 - 7.12.2. Label the cap of each tube with a W.
 - 7.12.3. Store the aliquots in the PCR working box in the PCR rooms freezer.
 - 7.12.4. Once thawed, the working stock of water should be either used or discarded within one week.
 - 7.12.5. Store at 4°C once thawed.
- 7.13. Agarose (Agarose Unlimited, PS 1200)
 - 7.13.1. Agarose is stored in the gel running area of the laboratory.
- 7.14. EDTA (Fisher, S312-500)
 - 7.14.1. Use 1000 mL autoclavable bottle.
 - 7.14.2. Add a stir bar.
 - 7.14.3. Add 148.9 grams of EDTA disodium salt dehydrate into 800 mL DI water to make 0.5 M EDTA.
 - 7.14.4. Stir using the magnetic stir plate until dissolved.
 - 7.14.5. Adjust pH to 8.0 by adding NaOH pellets (approximately 20 grams will be needed). Additional, NaOH (~5 grams) may help with the dissolution of EDTA.
 - 7.14.6. Store at room temperature.
- 7.15. TAE buffer (50X)
 - 7.15.1. Use 1000 mL autoclavable bottle.
 - 7.15.2. Add stir bar.
 - 7.15.3. Add 242.0 grams Tris base, 57.1 mL glacial acetic acid, and 100 mL of 0.5M EDTA.
 - 7.15.4. Add DI water to adjust total volume to 1000 mL and turn on stir plate. Everything should dissolve.
 - 7.15.5. Store at room temperature.
- 7.16. TAE (1X)
 - 7.16.1. Add 400 mL of 50X TAE to a 20 L carboy. Fill to the 20 L mark with DI water.
 - 7.16.2. Store in carboy at room temperature.
- 7.17. Molecular weight ladder (New England BioLabs, N3231L)
 - 7.17.1. Molecular weight ladder (MWL) should be diluted in molecular grade water at a 1:10 ratio before using.
 - 7.17.2. The 1:10 MWL is then combined with loading dye.
 - 7.17.2.1. Use 30% diluted MWL and 70% loading dye.
 - 7.17.3. This is labeled as pre-mix ladder and stored in the -20°C freezer when not in use.
- 7.18. Loading dye (New England BioLabs, B7021S)
 - 7.18.1. In a 15 mL Falcon tube, dissolve 3 g of reagent grade sucrose in 7.5 mL of molecular grade water.
 - 7.18.2. Let the sucrose dissolve completely.
 - 7.18.3. Add 2 mL of 10X TAE to the sucrose solution.
 - 7.18.4. Add 0.83 mL of 6X Blue/Orange loading dye to the sucrose solution.
 - 7.18.5. Mix well.
 - 7.18.6. Aliquot into 2 mL microcentrifuge tubes.
 - 7.18.7. Label tubes as “load dye”.

- 7.18.8. Aliquots of loading dye should be stored in the freezer and thawed just before each use.
- 7.18.9. Aliquots of load dye can undergo multiple freezing and thawing without degrading. Therefore, it is possible to use the whole aliquoted tube up.
- 7.19. GelStar (Lonza, 50535)
 - 7.19.1. GelStar contains DMSO, gloves are required.
 - 7.19.2. GelStar stain is stored in the -20°C freezer when not in use 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.
 - 9.1.2.2.1 DNA from These suspects will be extracted using the Qiagen DNeasy Blood and Tissue kit.
 - 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. All raw water samples are analyzed using the PowerSoil[®]DNA Isolation Kit (Mo Bio Cat #12888-100).
- 8.3. Store samples for PCR in refrigerator on designated shelf until ready for prep.
- 8.4. Open PCR Login File located.
 - 8.4.1. H:\EnvResShare\MUSSEL SAMPLES\DATA\PCR LOG
 - 8.4.2. Password
- 8.5. Log-in samples to the PCR login sheet by copying and pasting the information for the samples from the Master Login File.

9. RAW WATER 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. While the samples are centrifuging the following tasks need to be completed.
 - 9.3.1. In the sample notebook, start a new page with Prep, Extraction, PCR, and Gel written across the top.
 - 9.3.2. Fill out the date as each of these steps take place for the samples being analyzed.
 - 9.3.3. Under these columns make a list of the samples being analyzed.
 - 9.3.3.1. Write out the sample ID, location, and collection date in the notebook.
 - 9.3.4. Fill out and complete the PCR calculations sheet for both QM and ZM.
 - 9.3.5. Fill out and complete the gel running sheet for both QM and ZM.
 - 9.3.6. Get all of the PowerBead, Spin Column, and Collection tubes labeled for the DNA extraction procedure.

10. SAMPLE LYSIS

This procedure is modified from the PowerSoil[®] DNA Isolation Kit and from the Metropolitan Water District (2010). All chemicals listed are part of the PowerSoil[®] DNA Isolation Kit (Mo Bio 12888-50). The kit used for the extraction process is subject to change as companies selling these kits continue to change, update, or discontinue kits.

- 10.1. Lysis with the PowerSoil[®] DNA Isolation Kit (Mo Bio, #12888-100)
 - 10.1.1. Before beginning the DNA isolation have all of the necessary plastic tubes labeled.
 - 10.1.1.1. Tubes that are used for the different steps in the isolation process should be numbered to match the list in the sample notebook (one PowerBead Tube, one Spin Filter Tube, and three Collection Tubes per DNA extraction).
 - 10.1.1.2. An additional Collection Tube is used as the DNA Collection Tube and needs to be labeled in two ways.
 - 10.1.1.3. A Tough Spot microcentrifuge sticker is labeled with the sample ID and attached to the top of the Collection Tube.
 - 10.1.1.4. On the side the tube the sample ID and extraction data are written in black sharpie.
 - 10.1.2. As the tubes and pipette tips are used in the DNA extraction protocols throw they need to be disposed of in the trash.
 - 10.1.2.1. All Collection Tubes should be closed and then placed in the trash.
 - 10.1.2.2. None of the plastics used in this protocol are reusable.
 - 10.1.2.3. New tips are used for all of the steps and samples
 - 10.1.3. The following steps begin after the completion of section 10.3.
 - 10.1.4. To the PowerBead Tubes, add 0.25 mL (approximately 0.25 grams) of the pellet.
 - 10.1.4.1. Vortex to mix.
 - 10.1.5. Add 60 μ L of Solution C1 and invert several times.
 - 10.1.6. Secure the PowerBead Tubes horizontally to the tube holders on the vortex.
 - 10.1.6.1. Vortex at max speed for 20 minutes.
 - 10.1.7. Centrifuge the PowerBead Tubes at 10,000 x g for 30 seconds at room temperature.
 - 10.1.8. Place 250 μ L of Solution C2 into Collection Tube (#1).
 - 10.1.9. Transfer the sample supernatant to the Collection Tube containing C2 Solution and vortex for 5 seconds.
 - 10.1.9.1. Expect between 400-500 μ L of supernatant.
 - 10.1.9.2. Incubate at 4°C for 5 minutes.
 - 10.1.10. Centrifuge the Collection Tubes at room temperature for 1 minute at 10,000 x g.
 - 10.1.11. Place 200 μ L of Solution C3 into Collection Tube (#2).
 - 10.1.12. Avoiding the pellet, transfer 600 μ L of supernatant into the Collection Tube with Solution C3, vortex for 5 seconds.
 - 10.1.12.1. Incubate at 4°C for 5 minutes.
 - 10.1.13. Centrifuge the Collection Tubes at room temperature for 1 minute at 10,000 x g.
 - 10.1.14. Shake to mix Solution C4 before use.
 - 10.1.15. Place 1200 μ L of Solution C4 into a Collection Tube (#3).
 - 10.1.16. Avoiding the pellet, transfer up to 750 μ L of supernatant into the Collection Tube containing Solution C4 and vortex for 5 seconds.
- 10.2. Two alternative methods may be used to bind the DNA to the spin filter: centrifugation or vacuum manifold

- 10.3. Centrifugation to bind the DNA to the Spin Filter.
 - 10.3.1. 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.
 - 10.3.1.1. Repeat 11.2.1 until all of the supernatant has passed through the Spin Filter.
 - 10.3.2. Add 500 μL of solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.
 - 10.3.3. Discard the flow through.
 - 10.3.4. Centrifuge again at room temperature for 1 minute at 10,000 x g.
 - 10.3.5. Carefully place the Spin Filter in a new Collection Tube (#4).
 - 10.3.6. Add 100 μL of Solution C6 to the center of the white filter membrane.
 - 10.3.6.1. Be careful not to touch the filter with the pipette tip, this can damage the filter and reduce DNA yield.
 - 10.3.7. Centrifuge at room temperature for 30 seconds at 10,000 x g to elute the DNA sample.
 - 10.3.8. Discard the Spin Filter.
 - 10.3.9. Close the Collection Tube.
 - 10.3.10. Store the Collection Tubes with the DNA in the PCR room refrigerator until the PCR assay is set up.
 - 10.3.11. Once PCR assay has been performed store the DNA in the -80°C freezer.
- 10.4. PowerVac™ Manifold to bind the DNA to the Spin Filter.
 - 10.4.1. This is the preferred method for binding DNA to the Spin Filter.
 - 10.4.2. The Spin Filter is inside its own Collection Tube.
 - 10.4.3. 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.
 - 10.4.4. Transfer 650 μL of prepared sample lysate (from step 11.1.16) to the Spin Filter.
 - 10.4.5. Turn on the vacuum source and open the stopcock on the port. Allow the lysate to pass through the Spin Filter.
 - 10.4.5.1. Continue to add lysate until all of it has gone through the Spin Filter column.
 - 10.4.5.2. Close the vacuum stopcock.
 - 10.4.6. 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.
 - 10.4.7. Add 500 μL of Solution C5 to the Spin Filter. Open the stopcock and allow the solution to pass through the filter.
 - 10.4.8. Turn off the vacuum source and open an unused port to vent the manifold.
 - 10.4.9. Remove the Spin Filter and place in back into its Collection Tube.
 - 10.4.10. Place into the centrifuge and spin at 13,000 x g for 1 minute to completely dry the membrane.
 - 10.4.11. To elute the DNA, transfer the Spin Filter the final Collection Tube (#4) that is labeled with the sample ID and date. Add 100 μL of Solution C6 to the center of the white filter membrane.
 - 10.4.12. Allow the Spin Filter to sit for 5 minutes at room temperature so that the elution buffer can soak into the membrane.
 - 10.4.13. Centrifuge at room temperature for 30 seconds at 10,000 x g.
 - 10.4.14. Discard the Spin Filter. The DNA is now ready for PCR.
 - 10.4.15. Store the DNA in the refrigerator in the PCR prep room until the PCR assay is ran.

- 10.4.16. Once the PCR has been performed store the DNA in the -80°C freezer.
- 10.5. Analysis of Suspect Individuals: Lysis with the Qiagen Blood and Tissue Kit (From the FDA SOP for Fish Barcoding).
- 10.5.1. For the DNA extraction one Spin Column and one Eppendorf tube are needed.
- 10.5.1.1. Number the Spin column.
- 10.5.1.2. Label the Eppendorf tube with the sample ID on top and date on the side.
- 10.5.2. Place the suspect(s) into an 1.5 mL Eppendorf tube.
- 10.5.3. Add 50 µL of buffer ATL.
- 10.5.4. Add 5.56 µL of proteinase K.
- 10.5.5. Incubate 3-4 hours at 56°C with vortexing every hour or incubate overnight.
- 10.5.6. Centrifuge the sample for 1 minute at 6000 x g (8000 rpm).
- 10.5.7. Add 55.6 µL Buffer AL, mix by vortexing.
- 10.5.8. Add 55.6 µL of ethanol (96-100%), mix by vortexing.
- 10.5.9. Pipette the mixture of DNA and buffers into the DNeasy Mini Spin Column.
- 10.5.10. Centrifuge at 6000 x g (8000 rpm) for 1 minute.
- 10.5.11. Place column in a fresh collection tube.
- 10.5.12. Add 140 µL Buffer AW1.
- 10.5.13. Centrifuge for 1 minute at 6000 x g (8000 rpm).
- 10.5.14. Place DNeasy Mini spin column in a fresh collection tube.
- 10.5.15. Add 140 µL Buffer AW2.
- 10.5.16. Centrifuge for 3 min at 20,000 x g (14,000 rpm).
- 10.5.17. Place DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube.
- 10.5.18. Pipette 50 µL Buffer AE (warmed to 37°C) directly onto the DNeasy membrane.
- 10.5.19. Incubate at room temperature for 1 min.
- 10.5.20. Centrifuge for 1 minute at 6000 x g (8000 rpm) to elute.
- 10.5.21. Store the DNA in the refrigerator in the PCR prep room until the PCR assay is set up.
- 10.5.22. Once the PCR assay has been completed transfer the DNA to long term storage in the -80°C freezer.

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. Two different PCR primers sets will be used to analyze each sample
 - 11.3.2.1. Both zebra mussel (ZM) and quagga mussel (QM)
 - 11.3.3. Multiply the quantity needed for one reaction tube by the number of samples to be run.
 - 11.3.3.1. Add two extra samples for each gel (the negative and positive controls).
 - 11.3.3.2. 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 out two gel template sheets for the samples that will be run.
 - 11.4.1.1. One template sheet will be for ZM and the other will be for QM gel results.
 - 11.4.1.2. The gel should include ladder in lanes 1 and 16.
 - 11.4.1.3. Each sample will be run using both the ZM and QM primers.
 - 11.4.1.4. Each sample will be analyzed in two separate PCR reactions for ZM and QM.
 - 11.4.1.5. Lanes 14 and 15 will always be the negative and positive controls.
- 11.5. Sterilize the PCR hood
 - 11.5.1. Place the PCR reagents into the PCR hood.
 - 11.5.2. Turn on the UV light for at least 15 minutes.
 - 11.5.2.1. Make sure that the PCR hood doors are closed.
 - 11.5.3. 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.
 - 11.6.1. On one of the tubes place the label Z, while on the other place the label Q.
- 11.7. Set up the PCR tubes needed to analyze the samples.
 - 11.7.1. On top of the first of the PCR tubes put the label Z or Q to indicate which organism is being analyzed.
 - 11.7.2. On top of the second of the PCR tubes write the date.
 - 11.7.3. On the sides of the PCR tubes write the sample ID number.
 - 11.7.4. 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 (either ZM or QM).
- 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. This program is on all three of the PCR instruments.
 - 12.4.2. The PCR program is as follows: Pre-heat 95°C for 9 minutes, 40 cycles of (95°C for 20 seconds, 59°C for 90 seconds, 72°C for 90 seconds), then 72°C for 10 minutes, and hold 4°C for ∞

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 a 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 1 and 16 on each gel.

- 13.2.9. Transfer 7 μ L 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.
- 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 μ L 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.
 - 13.3.6. Place to discarded GelStar stain liquid in the waste container.

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. Gel images are saved as JPEG FILES.
- 14.10. To save the gel image, go to FILE and select EXPORT DATA and then IMAGE.
 - 14.10.1. A new window will pop up.
 - 14.10.2. Click SAVE AS and select type of file as JPEG FILE.
- 14.11. Name the file with the date, gel number, and organism analyzed.
 - 14.11.1. Example: 8.12.2015 GEL1 ZM F0001-F0012 and 8.12.2015 GEL 2 QM F0001-F0012.
 - 14.11.2. Click SAVE.
- 14.12. These images are saved to the desktop in the folder YEAR SAMPLES and EXPERIMENTS.
- 14.13. Print a copy of the gel image and tape it into the PCR notebook on the appropriate page.
- 14.14. Record the PCR results in the PCR notebook and also in the PCR Login.
- 14.15. Record the PCR results in the Enumeration Log.
- 14.16. See the Table 1 below for the possible outcomes.

Table 1: Possible outcome for gels run at RDLES. The positive and negative control outcome determines if the gel passes QA/QC.

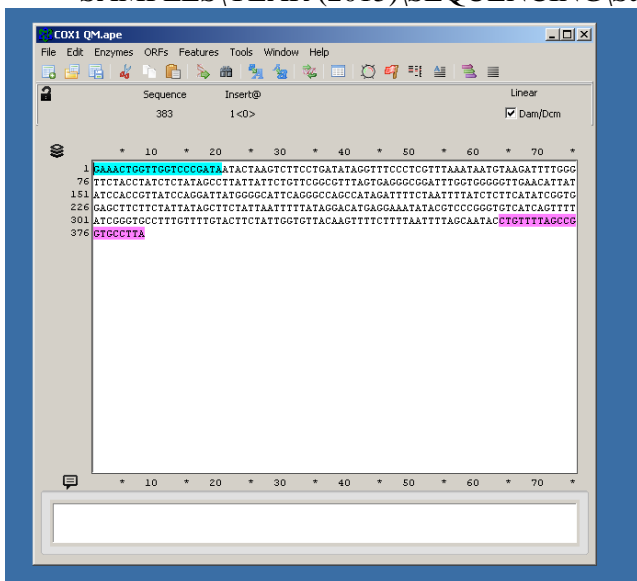
Positive Control	Positive	Negative	Positive	Negative
Negative Control	Negative	Negative	Positive	Positive
Outcome	Passes	Fails	Fails	Fails

- 14.17. Samples need to come back positive twice to be considered a reproducible positive.
- 14.18. Negative Control
- 14.18.1. A negative control is run for each PCR sample set.
 - 14.18.2. The negative control is the PCR reagents without any DNA present.
 - 14.18.3. 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.
- 14.19. Positive Control
- 14.19.1. A positive control is also run for each PCR sample set.
 - 14.19.2. The positive control DNA is obtained from an adult mussel of the appropriate species.
 - 14.19.3. DNA from the whole organism is extracted using the Qiagen Blood and Tissue Kit as described in Section 11.4.
 - 14.19.4. Control DNA should be diluted to provide a clear, bright band. This may require up to a 500X dilution.
- 14.20. Sequencing
- 14.20.1. Sequencing allows us to validate if the band that is on the gel is actually QM or ZM COX1, or some other organism.
 - 14.20.2. Once samples are analyzed on an agarose gel, positive samples are reanalyzed by PCR to confirm the result. If the sample is positive for a second time, the sample will be sent for sequencing. If a sample is not positive for the second time, it is considered a non-reproducible result and reported as a negative finding
 - 14.20.2.1. 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).
 - 14.20.3. Fill out the on-line order form for Sanger-based sequencing located at:
<http://seqwright.com/placeorder/orderforms.html>.
 - 14.20.4. Check the box to tell SeqWright that the samples have not been cleaned up and that SeqWright needs to perform this task prior to sequencing.
 - 14.20.5. Copy and paste the forward and reverse primers and size of the DNA product from the Primer Library Excel spreadsheet in the Sequencing Folder on the H drive into the on-line order form (H:\EnvResShare\MUSSEL SAMPLES\YEAR (2015)\SEQUENCING).
 - 14.20.6. Use the sample ID to identify each sample, and if it was from ZM or QM.
 - 14.20.7. Save the order form with the days date and sample ID number as the file name.
 - 14.20.8. Create a folder on the H drive (H:\EnvResShare\MUSSEL SAMPLES\YEAR (2015)\SEQUENCING) and name it with the sample ID and date.
 - 14.20.9. Save the order form in the Sequencing Folder on the H drive.
(H:\EnvResShare\MUSSEL SAMPLES\YEAR (2015)\SEQUENCING\Sample Name.
 - 14.20.10. E-mail the order form to SeqWright.
 - 14.20.11. 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.
 - 14.20.12. Transfer all of the PCR product to a pre-labeled 0.5 mL microcentrifuge tube.
 - 14.20.13. Aliquot ~30 µL of each of the necessary PCR primers into eppendorf tubes to also send to the sequencing company.
 - 14.20.14. Samples are placed in a plastic bag with the order form and sent FedEx overnight to SeqWright.
 - 14.20.14.1. SeqWright, Inc., 2575 West Bellfort, Suite 2001, Houston, TX 77054.

- 14.20.14.2. Phone number: 800-720-GENE (4363).
- 14.20.15. If other labs are used for DNA sequencing, follow the company's 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.
 - 15.2.1.1. These files on the H drive in the sequencing folder.
- 15.3. Open the notepad file.
 - 15.3.1. Paste the sample sequence into A Plasmid Editor (see example below).
 - 15.3.2. Save the file under the sample name.
 - 15.3.2.1. Save the file onto the H drive under (H:\EnvResShare\MUSSEL SAMPLES\YEAR (2015)\SEQUENCING\Sample Name).

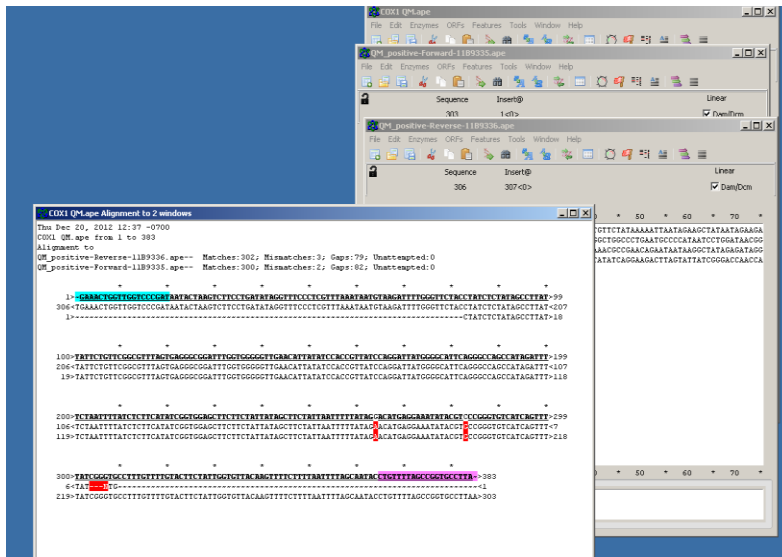


- 15.4. Align the sample sequence in the following order
 - 15.4.1. To the published COX1 ZM or QM sequence, forward and reverse sequences
 - 15.4.2. To both the forward and reverse sequences of previously sequenced positive control samples
 - 15.4.3. Open all four files in A Plasmid Editor, then go 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 (see example below)

PCR: Preparation and Analysis of Veliger Water Samples
Laboratory Standard Operating Procedures (Lab SOP)

SOP NO. PCR-5

Date Revised: 9/2015

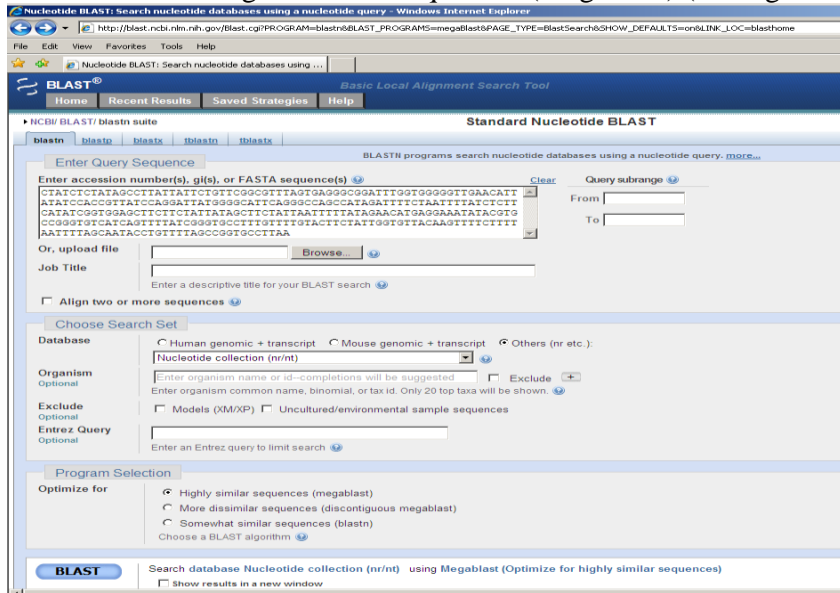


15.5. A second DNA analysis program is used to confirm the finding: NCBI BLAST

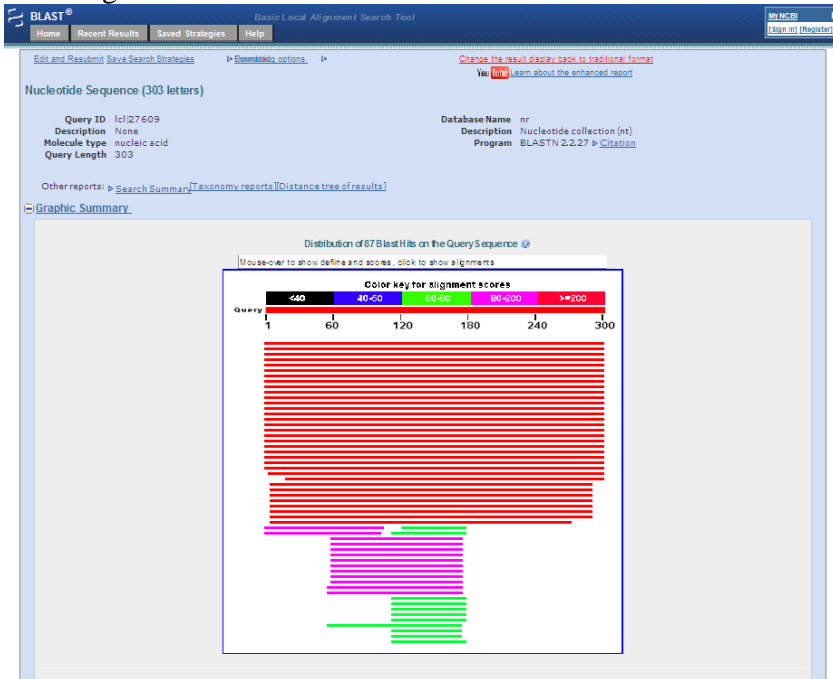
15.5.1. Copy the sample sequence and paste it into nucleotide NCBI BLAST
(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>)

15.5.2. Select the database

15.5.3. Select the higher similar sequences (Megablast) (See figure below)



15.6. BLAST generates a readout that looks like this:



15.7. A list of organisms is generated that have similar sequences is below the first readout:

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
<input type="checkbox"/> Dreissena rostriformis bugensis isolate DbBalaton1 cytochrome oxidase subunit I gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	JQ771943.1
<input type="checkbox"/> Dreissena rostriformis bugensis isolate DbBalaton2 cytochrome oxidase subunit I gene, partial cds, mitochondrial bobUQ771945.1 Dreissena rostriformis bugensis isolate DbBalaton2 cytochrome oxidase subunit I gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	EU484438.1
<input type="checkbox"/> Dreissena bugensis voucher DU33.1 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	EF303081.1
<input type="checkbox"/> Dreissena rostriformis bugensis isolate 5174 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	EF303082.1
<input type="checkbox"/> Dreissena rostriformis bugensis isolate 5178 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	DQ849132.1
<input type="checkbox"/> Dreissena bugensis cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	500	500	100%	2e-155	100%	AF510504.1
<input type="checkbox"/> Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial gene for mitochondrial product	500	500	100%	2e-155	100%	AF496877.1
<input type="checkbox"/> Dreissena bugensis cytochrome oxidase subunit I gene, partial cds, mitochondrial gene for mitochondrial product	500	500	100%	2e-155	100%	AF478937.1
<input type="checkbox"/> Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	500	500	100%	2e-155	100%	AF398765.1
<input type="checkbox"/> Dreissena bugensis cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	500	500	100%	2e-155	100%	U47950.1
<input type="checkbox"/> Dreissena bugensis var. profunda cytochrome c oxidase subunit I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	555	555	100%	9e-155	99%	U47950.1
<input type="checkbox"/> Dreissena rostriformis bugensis cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	549	549	100%	4e-153	99%	JQ435818.1
<input type="checkbox"/> Dreissena rostriformis cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	549	549	100%	4e-153	99%	DQ849133.1
<input type="checkbox"/> 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
<input type="checkbox"/> 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
<input type="checkbox"/> Dreissena rostriformis type 2 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial gene for mitochondrial product	544	544	100%	2e-151	99%	AF510508.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD7 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	394	394	100%	2e-108	90%	DQ849106.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD4 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	394	394	100%	2e-108	90%	DQ849102.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD6 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	388	388	100%	1e-104	90%	DQ849104.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD5 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	388	388	100%	1e-104	90%	DQ849103.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD3 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	388	388	100%	1e-104	90%	DQ849101.1
<input type="checkbox"/> Dreissena caputlacus haplotype SD2 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	388	388	100%	1e-104	90%	DQ849100.1

15.8. This list will give both query coverage and the maximum identity of the DNA sequence that was analyzed by BLAST.

15.8.1. Record the query coverage for the top hit and max identity in the PCR spreadsheet.
 Note if it does not come back as Dreissena.

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

15.8.3. 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. Under the list of organisms it is possible to see how the sequence aligned with each of the organisms on the list.

15.10. Below is an alignment of the sample sequence query to sequences in the database.

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	CTATCTCTATAGCCTTATTATTCTGTTGCGCGTTTAGTGAGGGCGGATTGGTGGGGGTT	60		
Sbjct 212	CTATCTCTATAGCCTTATTATTCTGTTGCGCGTTTAGTGAGGGCGGATTGGTGGGGGTT	271		
Query 61	GAACATTATATCCACCGTTATCCAGGATTATGGGGCATTGAGGGCCAGCCATAGATTTTC	120		
Sbjct 272	GAACATTATATCCACCGTTATCCAGGATTATGGGGCATTGAGGGCCAGCCATAGATTTTC	331		
Query 121	TAATTTTATCTCTTCATATCGGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTATA	180		
Sbjct 332	TAATTTTATCTCTTCATATCGGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTATA	391		
Query 181	GAACATGAGGAAATATACGTGCCGGGTGTCATCAGTTTTATCGGGTGCCCTTTGTTTTGTA	240		
Sbjct 392	GAACATGAGGAAATATACGTGCCGGGTGTCATCAGTTTTATCGGGTGCCCTTTGTTTTGTA	451		
Query 241	CTTCTATTGGTGTACAAAGTTTTCTTTTAAATTTAGCAATACCTGTTTTAGCCGGTGCCT	300		
Sbjct 452	CTTCTATTGGTGTACAAAGTTTTCTTTTAAATTTAGCAATACCTGTTTTAGCCGGTGCCT	511		
Query 301	TAA 303			
Sbjct 512	TAA 514			

15.11. Determination if the sequencing results pass QA/QC.

15.11.1. 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.

15.11.2. 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. DATA LOGGING PROCEDURES

16.1. Input results into the PCR login file.

16.1.1. Located in H:\EnvRes Share\MUSSEL SAMPLES\DATA and Enumeration.

16.1.2. Password

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

16.1.3.1. H:\EnvRes Share\MUSSEL SAMPLES\DATA\PCR Log.

16.1.4. Move old spreadsheet into "Old Data Files" folder.

16.1.5. In the PCR log enter the sample information, the date that the gel was ran, and if the sample is positive or negative.

- 16.1.5.1. If the sample was positive and there are sequencing results, enter the results in this spreadsheet. In this spreadsheet it is possible to enter details about the PCR assay (such as if one of the controls failed).
- 16.1.6. In the Enumeration file enter the outcome of the PCR testing. Samples can be positive or negative.

17. DISPOSAL OF PCR PRODUCTS AND DNA SAMPLE STORAGE

17.1. DNA storage

- 17.1.1. DNA extracts are stored in the -80°C freezer in a box or plastic bag labeled with the DNA extraction date. These should be kept indefinitely.

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

18. ONLINE DATABASE

The online Share Point is currently being used to report results.

19. SPILLS

19.1. When spills occur.

- 19.1.1. Clean spill with paper towels.
- 19.1.2. Decontaminate counter with bleach.

20. REFERENCES

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

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

ZM COX1

AGCGTCCTTGGTGATTTTCAATTATATAATTTAAT**TGTCACCACTCATGGGCTT**
GTTAATAATTTTTTTTCTAGTAATACCTATAATAATGGGGGGATTTCGGAAATTGAT
TGGTACCAATAATACTGAGTCTTCTGATATAGGTTTCCCTCGTCTTAATAATGTT
AGTTTTTGGGTTTTACCTGTCTCTATAGGACTTCTGTTTTGTTTCAGCTTTTAGGGA
AGGAGGATTCGGGGGTGGTTGAACCTTATATCCTCCTTATCTAGAGTTATAGGA
CATTTCAGGCCCTGCGATAGATTTTTTATTCTCTTCATATTGGGGGAGCTTC
TTCGATTATGGCTTCTATTAATTTTTATAGGACATGAGGTAATATACGTGCTGGAT
GTCATCAAT**TTTACCGGGTCCCTTTGTTCTGCA**CATCTATTGGTGTGACCAGAT
TCCTTTTAATCTTAGCAATGCCTGTATTAGCTGGGGCTTTAACAATATTATTA
ACTGATCGAAATTTTAACA

Primer-332 (forward): 5'-TGTCACCACTCATGGGCTTGT-3'

Primer-333 (reverse): 5'-TGCAGAACAAAGGGACCCGGTAAA-3'

QM COX1

GAAACTGGTTGGTCCCGATAAATACTAAGTCTTCTGATATAGGTTTCCCTCGTT
TAAATAATGTAAGATTTTGGGTCTACCTATCTCTATAGCCTTATTATTCTGTTCG
GCGTTTAGTGAGGGCGGATTTGGTGGGGTTGAACATTATATCCACCGTTATCCA
GGATTATGGGGCATTTCAGGGCCAGCCATAGATTTTCTAATTTTATCTCTTCATATC
GGTGGAGCTTCTTCTATTATAGCTTCTATTAATTTTTATAGGACATGAGGAAATAT
ACGTCCCGGTGTCATCAGTTTTATCGGGTGCCTTTGTTTTGTACTTCTATTGGTG
TTACAAGTTTTCTTTAATTTAGCAATAC**CTGTTTTAGCCGGTGCCTT**AACAAT
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

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GAGCTTAGTGCTCCTGGAAGAGTAGTAGGAGATTATCAATTATATAATTTAATTG
TTACTACTCACGGGCTTATCATAATTTTTTTTCTTGTAATACCTATAATGATAGGT
GGATTTGGAAA CTGGTTGGTCCCGATAATACTAAG TCTTCCTGATATAGGTTTCC
CTCGTTTAAATAATGTAAGATTTTGG GGTTCTACCTATCTCTATAGCCTTATT AT
TCTGTTTCGGCGTTTAGTGAGGGCGGATTTGGTGGGGGTTGAACATTATATCCACC
GTTATCCAGGATTATGGGGCATTACAGGGCCAGCCATAGATTTTCTAATTTTATCT
CTTCATATCGGTGGAGCTTCTTC TATTATAGCTTCTATTAATTTTATAGGACATG
AGGAAATATACGTCCCGGGTGT CATCAGTTTTATCGGGTGCCTTTGTTTTGTAC
TTCTATTGGTGTTACAAGTTTTCTTTAATTTTAGCAATACCTGTTTTAGCCGGTG
CCTTAACAATGCTCTTAACTGATCGTAATTTTAAACAC
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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'-CGGGACGTATATTTCCCTCATGTC-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 section 11.2), 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: TESTING FOR CONTAMINATION USING A WIPE TEST

- 23.1. Each month a wipe test of key areas of the laboratory is performed to test for contamination.
 - 23.1.1. The areas of the laboratory that are tested include:
 - 23.1.2. The microscopes, DNA hood, centrifuge, PCR hood, PCR instrument thermal block.
 - 23.1.2.1. In addition, there are two positive controls.
 - 23.1.2.2. Veliger's pipette directly from a veliger stock.
 - 23.2. Two samples are taken from each area for a total of 12 samples.
 - 23.2.1. Each location is sampled twice (Sample A and Sample B).
 - 23.2.1.1. Sample A does not have the DNA extracted from it. This is done to test for any ambient DNA in the environment.
 - 23.2.1.2. Sample B has the DNA extracted using the Qiagen Blood and Tissue Kit.
 - 23.3. To collect samples, twelve Eppendorf tubes (1-12) are labeled with the site that is being tested.
 - 23.3.1. Aliquot into each tube 400 μ L of Qiagen elution buffer.
 - 23.3.2. Wet a sterile Q-tip with the elution buffer.
 - 23.3.3. Using the Q-tip, wipe the area being tested.
 - 23.3.4. Place the Q-tip back into the Eppendorf tube and stir it in the remaining elution buffer.
 - 23.3.5. Discard the Q-tip.
 - 23.3.6. Close the Eppendorf tubes.
 - 23.3.6.1. The sample A set, no DNA extraction, are transferred to a new rack and placed in the refrigerator.
 - 23.3.7. For the sample B set, DNA extraction is carried out using the Qiagen method (See Section 10.4).
 - 23.4. Once the DNA extraction is completed, both sample sets A and B are analyzed using the COI PCR method for QM (See Sections 11-14).
 - 23.4.1. Following the PCR reaction, the PCR products are analyzed on an agarose gel.
 - 23.4.1.1. The date and test results are recorded in an Excel Spreadsheet: Wipe Test Log that is on the desktop of the PCR computer.
 - 23.4.1.2. The wipe test log book is also updated with a picture of the gel and any comments about the monthly wipe test.
 - 23.4.2. The results are discussed with the laboratory manager if there is a positive finding.
 - 23.5. Maintaining Laboratory Hygiene
 - 23.5.1. Bleach
 - 23.5.1.1. 20% working solution- fill the spray bottle up to the 200 mL mark with bleach, then add DI water to the top of the fill mark.
 - 23.5.1.2. Make fresh every two weeks.
 - 23.5.1.3. Bleach is used to decontaminate the DNA extraction hood and surfaces in the DNA extraction room, the PCR set up hood and surfaces in the PCR room, and the gel running area.
 - 23.5.1.4. Spray the bleach on the area that is being decontaminated.
 - 23.5.1.5. Let the bleach sit for a few minutes.
 - 23.5.1.6. Wipe the bleach up with a paper towel.
 - 23.5.2. DNA Away (Catalog #7010)
 - 23.5.2.1. This reagent is an alkali hydroxide (strong base).
 - 23.5.2.2. This cleaner is used in all areas of the laboratory where DNA is being extracted, PCR is set up, and the gels analyzed.

- 23.5.2.3. It can be used without mixing or adding any additional reagents.
 - 23.5.2.4. Soak the areas that are being decontaminated with DNA Away.
 - 23.5.2.5. Use paper towels to wipe up the DNA Away.
- 23.6. Unusual sources of contamination
- 23.6.1. The PCR instruments
 - 23.6.1.1. If the reagents and plastics used to make the PCR reactions were all fresh) then an additional source of contamination could be the PCR instruments themselves
 - 23.6.1.2. To overcome this issue it is important to decontaminate the PCR instruments.
 - 23.6.1.3. Equipment needed: DNA Away, Q-tips, paper towels, short wave UV light, 50 mL conical tube, ring stand.
 - 23.6.1.4. Place approximately 20 mL of DNA Away in the 50 mL conical tube.
 - 23.6.1.5. The PCR instrument should be shut off.
 - 23.6.1.6. Open the PCR instrument so that the PCR wells are all visible.
 - 23.6.1.7. Soak a Q-tip in DNA Away.
 - 23.6.1.8. Gently swish the Q-tip into the PCR thermal block wells.
 - 23.6.1.9. Soak the Q-tip in the DNA away after cleaning a row of wells.
 - 23.6.1.10. Change the Q-tip as its cotton become soaked.
 - 23.6.1.11. After cleaning the wells, then take the Q-tip to the top of the heated lid.
 - 23.6.1.12. Take a Kimwipe and squirt DNA Away on it.
 - 23.6.1.13. Gently wipe the PCR block down with the Kimwipe.
 - 23.6.1.14. Set up the ring stand so that it is over the PCR block.
 - 23.6.1.15. Set the UV lamp onto the ring stand so that the light is going to be able to hit the PCR block (the lamp will not be able to cover the whole thermal block).
 - 23.6.1.16. Turn the lamp on.
 - 23.6.1.17. Leave the lamp on for at least 10 minutes.
 - 23.6.1.18. Move the lamp to a different region of the PCR block.
 - 23.6.1.19. Leave the lamp on for at least 10 minutes.
 - 23.6.1.20. Rotate the UV lamp so that it is hitting the top of the heated lid.
 - 23.6.1.21. Leave the lamp on for at least 10 minutes.
 - 23.6.1.22. Once the PCR thermal blocks and heated lid have been decontaminated set up a test PCR to check that if the decontamination has worked.
 - 23.6.1.23. Make up a master mix for 15 samples for QM and ZM COXI gene.
 - 23.6.1.24. Set up a strip of 12 PCR tubes and load the master mix into each of the tubes
 - 23.6.1.25. Do not include an positive control (this test is to determine if there is any DNA or PCR product contamination in the PCR machine).
 - 23.6.1.26. If the resulting gel comes back negative, then the decontamination worked, and it is now possible to analyze real samples.
 - 23.6.1.27. If the resulting gel comes back with a positive band, then repeat the decontamination, use fresh PCR reagents and plastics, wipe the PCR hood down with DNA away, repeat the UV light treatment of the PCR hood, try to eliminate any potential sources of contamination.
 - 23.6.1.28. If all else fails, stop all PCR work for the rest of the day. Come back the next day and try again.
 - 23.6.2. Additional things to remember.
 - 23.6.2.1. Aliquot all PCR reagents do decrease the risk of cross contamination.
 - 23.6.2.2. Make fresh QM primers from the stocks.

- 23.6.2.3. Avoid a lot of freezing and thawing the PCR reagents, at the end of each week throw out the PCR stocks that had been used.
- 23.6.2.4. Do not throw out the Taq!! It is temperature stable and can last a while.
- 23.6.2.5. Change pipette tips after every single use.
- 23.6.2.6. Avoid if possible performing DNA extractions after loading and running PCR products on gels.
- 23.6.2.7. Make sure to clean the Eppendorf tube racks on a regular basis.
- 23.6.2.8. Wear and change gloves often.
- 23.6.2.9. Use the PCR tubes from USA Scientific that are colored.
- 23.6.2.10. Clean the lab on a weekly basis.
- 23.6.2.11. All areas are wiped down every Friday with bleach and DNA Away.
- 23.6.2.12. The centrifuge rotor is taken out of the centrifuge in the DNA extraction room and washed with soap and running water.
- 23.6.2.13. The inside of the centrifuge is wiped down with bleach and DNA away.
- 23.6.2.14. After gels are run, put away all of the gel equipment and wipe the area down with bleach and DNA Away.
- 23.6.2.15. The gel area is a hot spot in the lab for potential contamination because the PCR tubes are opened in this area so that the gels can be loaded.
- 23.6.2.16. Avoid having additional people in the lab when DNA extractions, PCR, and gels are being loaded.