

**Whitney Genetics Lab Invasive Carp eDNA Sample Processing
Standard Operating Procedure**
IC eDNA Monitoring Program



U.S. Fish and Wildlife Service, Region 3

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IC eDNA Monitoring Program
WGL eDNA Sample Processing
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Application/Purpose:

Standardized lab procedures for all sample processing (post shipment) including decanting, extraction and PCR amplification of samples collected for the Region 3 Invasive Carp eDNA Monitoring Program.

Roles and Responsibilities:

All staff in Whitney Genetics Lab (WGL) processing any Invasive Carp eDNA samples should be trained in each part of the process that WGL staff will be expected to perform. For example, if staff are expected to decant and dry samples, staff should be trained on that procedure and read the following SOP before the task is performed. Training documents should be placed here (eDNA Program SharePoint site/WGL).

A. Sample Handling and Custody

Samples should be shipped as soon as logistics in the field allow to prevent samples from sitting in preservative longer than needed.

A1. Receipt of Tubes

Once the samples have been shipped to WGL, designated personnel will receive them. NOTE: alcohol-preserved centrifuged samples are shipped at ambient temperature, there is no need to send refrigerated.

- Upon receipt of samples from the field, the shipped box(es) should be opened. The general condition of the box(es) should also be assessed.
- Centrifuged samples can be stored with preservative at ambient temperatures for up to three weeks, post receipt at the lab. In effort to minimize DNA degradation, after three weeks samples must be decanted, dried, and frozen. Emergency storage of samples in $-20\text{ }^{\circ}\text{C}$ freezers is allowable until room in the $-80\text{ }^{\circ}\text{C}$ can be made.
- If samples cannot be spun and dried within 3 weeks, samples still in ethanol can be stored at $-80\text{ }^{\circ}\text{C}$ until they are processed.
- Review the Survey123 Chain of Custody (COC) forms that correspond to the samples. Note any condition issues (broken tape or seals or damaged containers) with the samples on the COC forms. Note any samples that must be discarded due to condition issues and the reason for discard. Any corrected error on form should be communicated to the person who made the error, and the correction must be noted, dated, and initialed in the comments section of the electronic form.
- Enter sample data into internal WGL Case Log including noting any samples that are being discarded and that should not be analyzed, and complete WGL check in form. Note any observations about samples such as condition issues. Log samples into the Bench Top Shelving Log.
- Alert shipping office that samples have been received through Microsoft Teams.

A2. Preservative Decanting and Alcohol Evaporation Procedure

1. Centrifuged samples need to be re-spun to re-concentrate sample at the bottom of the tube after shipping. Swirl liquid to re-capture any observable debris from the side of the tubewall.

2. Centrifuge at max speed for 5 minutes. Effort should be taken to minimize the time between centrifuging and decanting ethanol.
3. Centrifuged samples preserved with alcohol must have the alcohol evaporated away before extracting the samples.
4. Remove cap from tube and gently pour the preservative from the tube into an appropriate collection vessel with funnel. The tube can be placed back into the original rack or into a new rack depending upon personal preference. Caps should be placed on a clean tray in numerical order of removal. Tubes will be open in the hood at this point, the drying process in the hood aids in the evaporation of the alcohol preservative from the tubes.
5. Move samples in tube racks to the laminar flow hood. Turn on the air flow and leave the samples to dry until all traces of ethanol or isopropanol smell are gone, because these are both PCR inhibitors. Do not let samples sit at room temperature longer than it takes to evaporate the preservative, and never over a weekend. If some samples in a case are taking longer than the rest to dry, put dry samples in the freezer, and allow the remaining samples to evaporate until they are dried. If working in advance, evaporated samples should be stored at -80 °C until extraction.
6. There should be at least one hood control before and after 8-10 sample racks in a hood. A maximum of 3 hood controls for a full hood. A minimum of 2 hood controls should be in place for sample that do not fill a full hood. Hood controls are prepared by placing a clean 50 mL centrifuge tube in a clean rack in the hood along with the range of samples that were placed in the hood to evaporate. The hood control should contain the case number and sample range included with the control. The lid should be labeled as well since the tube and lid are separated during the evaporation process.

B. General Sample Processing Considerations

- Non-sterile microcentrifuge tubes (MCT), swabs, and glassware used in the lab must be autoclaved at 121°C for 30 min before being used. Any re-used items must be soaked in a freshly made 10% bleach solution for 10 minutes followed by a thorough rinse. (Sterile, certified DNA- free MCT do not need to be autoclaved prior to use).
- Good laboratory practices should be always considered, including but not limited to: Reagents that have passed expiration dates should not be used, unless they have been tested in the lab and demonstrate that they are still effective at the same level as non-expired reagents. Reagents that have been stored at insufficient temperatures should not be used. All reagents must be clearly labeled with lot numbers and expiration dates and recorded on the appropriate data sheet.
- The temperatures of cold storage units must be monitored at a minimum of at least once per week and recorded in the Survey123 application. For cold storage units that are not on an alarm system or generator back-up temperature should be monitored more frequently. Temperature files will be downloaded at the end of each season and kept on an internal server.
- Sterile techniques and good laboratory practices should be used in the DNA laboratory to prevent contamination through the transfer of DNA between surfaces,

and to prevent cross-contamination between samples. Contamination can adversely affect the outcome of a case; therefore, it is essential to follow procedures listed in this section to limit the chance of contamination.

- Gloves (e.g., powder-free nitrile or latex) must be worn throughout sample processing. At a minimum, gloves should be changed at the completion of each step of the process. If gloves become contaminated or if contamination is suspected, discard them, and replace them with new ones.
- Centrifuge tubes before opening the reagents. Uncap and close tubes carefully to prevent contamination through the spread of aerosolized DNA.
- To prevent damaging equipment, ensure that centrifuges are always balanced, pipettes are regularly calibrated, and freezers are defrosted yearly or as needed.
- All freezers should have the ability to be locked (with non-universal locks or marine brackets attached that can be used with keyed locks) or be housed in a secure facility.
- Ensure that all equipment, including paper, pens, and lab coats, are dedicated for use only in that particular laboratory (e.g., laboratory coat for each stage of procedure rooms). Each room dedicated to eDNA sample processing should have a lab notebook to contain data sheets related to that specific area. Lab notebooks shall remain in each dedicated space until the end of each season when they are removed to be archived. Each room should have note-taking materials (e.g., loose-leaf paper, networked PCs) that can be used for consolidation in the Case Log. Other solutions for preventing contamination of sensitive areas may be used following approval by the eDNA Processing Lead and eDNA Processing QA/QC Specialist. Any changes should be incorporated into a revised QAPP. Laboratory notes/notebooks should be written or printed with tamper-proof procedures:
 - Lab notebooks should be unique and have unique identifiers, with consecutive page numbering, note/data entry dates, and initials (of the note-taker) on each page. A user identification page should be included with the printed, signed, and initialed name of each person entering notes/data into the lab book.
 - Only permanent blue ink should be used. Special pens may be required for certain paper types.
 - Any changes to the notebooks must be dated and initialed by the person who made the change. Any incorrect information should have a single line drawn through it and not be completely obscured.
 - Lab notebooks should contain all data images (when appropriate, e.g., gel photographs and denaturing curves). Images should be permanently affixed to the notebook and signed across both the edge of the insert and the page.
 - Any large data images or images that are not suitable for permanently affixing into a notebook should be kept on permanent record and its location, document, name, and relation to the assay referenced in the lab notebook.
 - Notebooks must be kept in a locked drawer or cabinet with restricted access when not in use.

C. Invasive Carp eDNA Sample Extraction Procedures

To prepare centrifuged samples for extraction, 50 mL centrifuge tubes will be swabbed and extracted according to the below procedures.

C1. Extraction Considerations:

- Extraction of DNA must be performed where PCR products and stocks of cloned material are not handled.
- A PCR hood with a built-in ultraviolet (UV) light and HEPA filter may be used to further isolate DNA extraction kit solutions and elutes from ambient DNA when a dedicated DNA extraction space is not available.
- A separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for use in DNA extraction.
- Positive and negative extraction controls should be added to each eDNA extraction procedure batch.
- Before proceeding with extraction, a positive control swab is prepared by pipetting mixture which contains a lower dilution ratio of Bluegill (BLG) or Brook Trout (BKT) cell lines or tissue slurry directly onto a sterile cotton swab in 1.5-ml MCT with sterile lab DI water. The dilutions should be below the highest standard range (6250). A batch of extraction positives can be prepared ahead of time and frozen at -20 °C.
- Additionally, an extraction negative control sample should be prepared by pipetting 100 µl of sterile lab DI water onto a sterile cotton swab in a 1.5-ml MCT. A batch of extraction negative controls can be prepared in advance and kept frozen at -20 °C.

C2. IBI Scientific gMAX Kit Extraction Procedure

1. Move dried samples in 50 mL centrifuge tubes to the extraction room making sure all tubes are dry.
2. Obtain one Qiagen lyse and spin column per sample to be extracted, open and organize tubes in rack. Label both caps of each sample. You will have time to label the rest (1 lab-supplied 1.5-mL MCT, 1 GD spin column, and 3 IBI collection tubes) during the 30-minute incubation. Be sure to add one positive and negative extraction control to each eDNA extraction procedure batch.
3. Add 350 µL **GSB** to each lyse and spin basket tube. If precipitate has formed in GSB buffer, dissolve by incubating at 60 °C for 10 minutes, before dispensing into tubes.
4. Add 35 µL **proteinase K** to each lyse and spin basket tube.
5. Remove a sterile swab from pack and dip it into a numbered tube of GSB/proteinase K mix. Use the moistened swab to swab the bottom of each tube included in the field sample (e.g. there is 5 tubes per sample, swab the bottom of all 5 tubes with the moistened swab). If the swab becomes covered with debris, rinse swab in tube of GSB/proteinase K solution for that sample and proceed swabbing the rest of the replicates. Be careful to avoid cross-contamination at this step.

NOTE: If a situation arises where a swab falls during the swabbing process (lab bench, floor, garbage, or MCT rack) the swab should be discarded. A note should be made in the logbook and in the appropriate case notes on the eDNA Case Log spreadsheet. Also indicate what tube number the swab was discarded at (ex: swab

fell on floor during tube 2 of 5, new swab for tubes 3 through 5). If a sample has a lot of organic material, there may be a need to make an additional tube of the GSB/Proteinase K mix. The tube would get the same label as the original tube (ex: 22001001a) and both tubes will be combined when the sample is loaded onto the GS spin column in steps 10-12. If multiple tubes of GSB/ProK are used, that should also be noted in the logbook and in the appropriate case notes on the eDNA Case Log spreadsheet.

6. Place the swab back into the GSB mixture, break the wooden stick as close to the swab as possible, and close the tube. Change gloves between samples. For particularly dirty samples, add mixed GSB and ProK to any samples that appear dry or low so that the level appears to be about 350 μ L. A sufficient combination of GSB and ProK should be added to maintain enough liquid for incubation. Ensure maximum extraction of the sample.
7. Incubate at 60°C for 30 minutes. Label the rest of the tubes, place GD spin columns in tube rack and print final archive labels for extracts. Archive labels are the case number and three-digit sample number (e.g., 22001001). Also, during this time place the Elution Buffer into the 60°C bead bath.
8. Remove lyse and spin tube from incubator and centrifuge at max speed for at least 1 minute, up to 3 minutes depending on level of sediment in tubes. IF samples are particularly dirty, spin at least 3 minutes. If the basket is clogged, use a pipette to remove the supernatant from the sample, recovering as much as you can. Make notes in the Case Log.
9. Add 500 μ L **ethanol** (100%, molecular grade) to the extract. Mix thoroughly by vortexing. If liquid collects around the cap, spin briefly before opening to reduce contamination risk.
10. Transfer up to 780 μ L of the mixture by pipette into a GD spin column placed in a 2 mL collection
11. tube. Centrifuge at 18,000 rpms for 1 minute. Discard flow-through and collection tube.
12. Transfer any remaining mixture by pipette onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at 18,000 rpms for 1 minute. Discard flow-through and collection tube.
13. Place GD spin column in a new 2ml collection tube. Add 400 μ L Buffer **W1**. Centrifuge at 18,000 rpms for 30 seconds. Discard flow-through and collection tube.
14. Place spin column in a new 2 mL collection tube. Add 600 μ L **Wash Buffer**. Centrifuge at 18,000rpms for 3 minutes. Discard flow-through and collection tube.
15. Transfer the spin column to a new 1.5 mL MCT.
16. Add 200 μ L of the pre-heated **Elution buffer** to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at \geq 18000 rpms for 30 seconds.
17. Discard the spin column and store the eluted DNA samples at -20°C. If DNA is to be immediately used for PCR, keep in 4 °C refrigerator.

D. Invasive Carp eDNA PCR Amplification Procedures

To determine if the DNA of a species is present in the concentrated water samples taken in the field, the total DNA extracted from the centrifuged samples must be amplified using species-specific primers. All samples will be screened with an assay that consists of two general Invasive carp markers that detect two loci within the cytochrome oxidase I gene of both species of carp mitochondria (ACTM 1/3). The assay also consists of two markers specific to silver carp that detects two loci within the ND6 and ND2 regions of the mitochondria (SCTM 4/5), and finally two markers specific to bighead carp that detects two loci on the ND4 and ND6 regions of the mitochondria (BHTM 1/2). Any sample that has at least one detection for any loci of eight replicates will be considered a positive detection.

D1: Reagent Room Considerations

To prevent carry-over of amplified DNA sequences, when available, PCR reactions should be set up in a separate room from that used for post-PCR manipulations. If a separate and designated space is unavailable, PCR reactions should be set up in a dedicated clean fume hood with UV lights and a HEPA filter. A set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for pre-PCR manipulations. Reagents and supplies should be taken directly from clean storage into the PCR area and should never be taken to or shared between areas where post-PCR analyses are being performed. Equipment such as pipettes should never be taken to a pre-PCR area after use with amplified material.

D2. Standard Curve Material

Standard curve materials will be synthetic strands of DNA purchased from a reliable production company. Standard curves are only used to assess assay performance and address Acceptance Criteria and are not used to for quantification purposes. WGL will purchase gBlock product from Integrated DNA Technologies, but other suitable vendors are allowed. To get reproducible and high-quality standard curve data, it is important to store materials properly, and make serial dilutions fresh each week. Storing for longer periods than one year can produce inaccurate results. The gBlock standard is purchased so that it contains all 6 qPCR marker targets (ACTM1/3, SCTM4/5, BHTM1/2).

1. gBlock is hydrated with TE buffer to make an initial dilution that is 1.00×10^{10} copies per microliter (an excel stock dilution calculator spreadsheet is on the shared drive at WGL).
2. Volume TE to add = $fmoles * 0.0000000000000001 * 6.022E+23 / 1.00E+10$. Mix well.
3. Immediately use 1.0 μ l of the hydrated gBlock into 999.0 μ l of TE buffer to make storage stock with 1.0×10^7 copies/ μ l.
4. Make several aliquots of this stock and store in screw-cap tubes with o-rings to prevent evaporation in the freezer. Freeze remainder of stock from step 1 above.
5. Add 1.0 μ l of 1.0×10^7 copies/ μ l stock to 31.0 μ l 100 ng/ μ l yeast tRNA in water. This working stock has 312,500 copies/ μ l.
6. Serial dilutions for use in assays should be made fresh weekly with the stock and 100 ng/ μ l tRNA as a diluent. They may be stored frozen and thawed as needed throughout the week.

7. A **new** pipette tip **is required for each** dilution for accurate results.
8. After each addition, use the pipette to mix a **minimum** of 17 times.
9. Currently, a 5X curve is used at WGL.
 - 30 μ l working stock into 270 μ l tRNA. Mix 17 times = 31,250 copies
 - 60 μ l of 31,250 mix into 240 μ l tRNA, mix 17 times = 6250 copies.
 - 60 μ l of 6250 mix into 240 μ l tRNA, mix 17 times = 1250 copies
 - 60 μ l of 1250 mix into 240 μ l tRNA, mix 17 times = 250 copies
 - 60 μ l of 250 mix into 240 μ l tRNA, mix 17 times = 50 copies
 - 60 μ l of 50 mix into 240 μ l tRNA, mix 17 times = 10 copies

D2. PCR Amplification Considerations

This stage of DNA processing is particularly susceptible to contamination and, subsequently, inaccurate results. Carefully follow the bullets listed below:

- This room is a contaminated area; therefore, no reagents, equipment, laboratory coats, etc. from this room should be used in any of the other lab areas.
- A biological or PCR-type hood may be used for setting up cloning or sequencing reactions.
- A set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for PCR assays.
- Every effort should be made to ensure that equipment, work areas, and solutions are free from DNA contamination. All surfaces should be wiped clean with 10% bleach solution (or commercial DNA eliminating solution such as DNAway) before and after use. If equipped with UV lamps, clean lab rooms or PCR hoods should be irradiated with UV light 30 minutes at the end of the workday.
- DNA elutes from samples should either be located in designated -20°C freezer or carried from the DNA extraction room to PCR room. Ensure thermalcyclers are available before preparing the master mix.
- If using automated system for plating it should be UV sterilized after each use and the sample deck and racks wiped down with DNAway or 10% bleach if necessary.
- Centrifuges, tube racks, pipettes, and any other equipment used for PCR amplification should be cleaned before and after each use.
- Use autoclaved, filtered, or commercially sterile molecular grade water for setting up PCR reactions.
- Aerosol-resistant pipette tips should be used. Place the sterile tip on the pipette immediately prior to use. If the pipette is set down with the tip on, discard the tip. A new pipette tip must be used for the addition of each reagent to a sample tube.
- Use PCR clean or autoclaved sample tubes for PCR master mix.
- Include a positive and negative PCR control in the plate set-up.
- A negative PCR control is prepared by pipetting 200 μ l of autoclaved sterile lab water into a clean 1.5-ml Eppendorf MCT.
- A positive PCR control is prepared by previously extracting Invasive carp cell lines or tissue slurry (both Bighead and Silver Carp) and diluting to an appropriate volume to not overwhelm the reaction. Positive PCR controls

should be tested ahead of using for official eDNA monitoring cases.

- Close each tube immediately after labeling and after the addition of sample or reagents to prevent cross-contamination.
- Be sure to only touch the tip of the MCT cap or use a tube opener, clean Kimwipe®, or other suitable barrier to open MCT.
- Record all solution batch numbers used for reactions in lab notes.
- PCR reagents should be aliquoted (a portion of the original stock) to avoid excessive repetitive freeze-thaw cycles and to protect stock reagents if contamination occurs.
- Lightly vortex and quick-spin tubes before opening the reagents to avoid splashes or drips from cap when opening. Uncap and close tubes carefully to prevent aerosol contamination.
- A log should be used to track which PCR reactions happened on each thermal cycler. Log information should include the assay name, sample case, plate number, and the thermalcycler head ID.
- Note for Exhibit 9-10: Plate maps have been included for scenarios most processed at WGL.

D3. PCR Amplification Procedure

1. If DNA samples (extraction elutes) are removed from long term storage freezer, note this on the appropriate logbook.
2. Use pre-printed plate map (Section E) or build plate map to determine which samples will be pipetted into which wells. Clearly mark plate identification on bottom edge skirt of plate. Write plate identification information (ddMONyy_initials_ASSAY_case(sample#-sample#)_Plate#) in lab notes.
3. Make sure sample map for each plate is entered into the pipetting robot or attached to lab notes and a signature is written across the map and lab book page.
4. Follow DNA amplification protocol detailed below.
5. Eight reactions are set up for each sample, in addition to negative and positive controls for each master mix.
6. In the clean reagent room, obtain all PCR master mix reagents (using only those that have not expired or that have been tested and found viable).
 - TaqMan Environmental Master Mix 2.0.
 - One tube with combined ACTM1/3 forward and reverse primers at 10 μ M working dilution.
 - One tube with combined SCTM4/5 forward and reverse primers at 10 μ M working dilution.
 - One tube with combined BHTM1/2 forward and reverse primers at 10 μ M working dilution.
 - One tube with a combined FAM-labeled probe for ACTM1/3 and HEX-labeled (or another appropriate fluorophore for your instrument) probe for BHTM1/2 and CY5-labeled probe for SCTM4/5, all diluted to 2.5 μ M. Double-quenched probes are preferred that include a ZEN quencher located about 9 base pairs from the fluorophore as well as an Iowa Blackhole Quencher on the 3' end of the probe,

but regular TAMRA-quenched probes may also be used.

7. Sterile molecular grade water (commercially sterile or Millipore filtered, autoclaved).
8. Allow reagents to thaw. Do not vortex primers or Taq too violently. Briefly spin down tubes to minimize aerosolization.
9. Record on data sheets the lot number and expiration date of all reagents used.
10. The master mix volume can be adjusted according to the number of samples to be processed. In order to make sure that master mix does not run out prior to supplying all the desired reactions (this may occur as a result of minor errors or variations in pipetting volumes), it is generally helpful to make more than enough master mix than is needed for the desired number of reactions. For example, if using the 8-channel pipette for the robot, calculate 14 extra reactions for a 384 well plate. If using a 96 well plate, calculate 4 extra reactions. NOTE: If positive extraction controls consist of a different species of DNA, be sure to make a small separate master mix for those samples and use primers specific to the content of the control sample. Negative extraction controls should be amplified with the Bighead and Silver Carp master mix and a second time with the extraction positive control assay.
11. Each IC Combo Assay PCR 1X reaction should contain:

Table 1. 1X PCR reaction for IC Combo Assay, total volume of 20 μ L per reaction

Volume μL	Master Mix Reagent Component
10.0	TaqMan Environmental Master Mix 2.0
1.0	each primer (10 μ M each, working dilution)
1.0	probe mix (2.5 μ M each, working dilution)
3.0	sterile water (*5.0 μ L in standard curve mix)
3.0	sample extract as template (*1.0 μ L gBlock template in standards)

12. TaqMan Environmental Master Mix is very viscous, and requires careful pipetting to ensure accuracy, and a new tip for each aliquot removed. The tip must be left in the mix for a few seconds after aspiration to ensure complete aspiration of the desired volume, and upon dispensing, a pause is required to allow the mix to pool in the tip before the final plunge to dispense the last drop of mix. If this mix is pipetted too quickly, there will be too little volume to fill the required wells. The electronic pipetting epMotion 5075s were specifically calibrated for this viscous mix. If other mixes are validated for use, new programs for the epMotion 5075s must also be developed.
13. Mix the master mix well by slowly inverting the tube several times. Avoid shaking or inverting too quickly to prevent creating bubbles. Move prepared mix from reagent room into PCR room.
14. Remove DNA extracts from freezer or fridge (fill out sample logs as needed), vortex (quick touch) or finger flick and quick spin down the extract tubes. Take them into the PCR room.
15. WGL has epMotion 5075s (automated liquid handling robots) that will fill plates for cycling. If these are unavailable, detailed directions for loading plates follows.

Fill all plate wells with 17.0 μL PCR mix. FOR STANDARD WELLS only, add 2.0 μL water. To avoid creating bubbles, which interferes with data collection, place the pipette tip gently in the very bottom of the wells. Fill wells from the bottom up. Do not dispense air into the mix.

16. Carefully pipette 3.0 μL of each extract (or 1.0 μL gblock + 2.0 μL water in standards) to be delivered onto the side of each well of a column, changing the pipette tip between each sample. Again, placement of the tip is important to avoid creating bubbles, so set the tip against the side of the well, above the level of master mix, and then deposit the aliquot of template.
17. Each column of eight wells should be filled with the same sample (i.e., eight replicates per sample to be tested). Twenty-three columns of the PCR plate can test 46 different samples, the last remaining column is for PCR negatives and positive controls and standard curve dilutions. Pipette 3.0 μL of water into wells G, H, O, and P to serve as PCR negative samples. Five-point, five-fold standard curves with 10, 50, 250, 1250, and 6250 copies/ μL will be used. The highest standard should be placed in well B followed by the next lowest standards through well F, and again in wells J-N. Place a mixed sample of both Silver and Bighead carp cell extract into wells A and I to serve as PCR positive samples.
18. Extraction negative controls and hood controls will be run as a sample. Extraction positive controls will be run separately on a Bluegill assay (Takahara et al. 2013) or Brook Trout (Wilcox et al. 2013) placed consecutively as they were extracted in the case.
19. Each Bluegill PCR 1X reaction should contain:

Table 2. 1X PCR reaction for Bluegill Assay, total volume of 20 μL per reaction

Volume μL	Master Mix Reagent Component
13.4	TaqMan Environmental Master Mix 2.0
1.8	forward primer (10 μM each, working dilution)
1.8	reverse primer (10 μM each, working dilution)
1.0	probe (2.5 μM , working dilution)
2.0	template

Table 3. 1X PCR reaction for Brook Trout Assay, total volume of 20 μL per reaction

Volume μL	Master Mix Reagent Component
13.4	TaqMan Environmental Master Mix 2.0
1.8	forward primer (10 μM each, working dilution)
1.8	reverse primer (10 μM each, working dilution)
1.0	probe (2.5 μM , working dilution)
2.0	template

20. If filling plates by hand in 96-well formats, before the PCR plate is sealed, check every well for bubbles.
21. If filling plates on the epMotion robots, place PCR film over the PCR plate and use an automatic plate sealer to ensure the edges of all wells are sealed. Lightly vortex the sealed plate to mix contents, spin down the plate in the plate spinner to ensure

- mixture is in the bottom of wells and there are no bubbles.
22. WGL has a BioRad brand plate sealer. Use the seal guard to keep the seal from shifting. Turn on the sealer and ensure it is up to temperature. Seal 96 well plates at 180°C for 3 seconds and 384 well plates at 167°C for 3 seconds. Inspect the seal to ensure it is not loose, and all wells are sealed. To obtain reliable seals, temperature and time may be adjusted.
 23. If you do not have a plate sealer, use adhesive seals and a plate roller or paddle to ensure a good seal. It is imperative to have a good seal to prevent evaporation which will prevent data collection, and potentially contaminate the lab with amplified DNA. Place the PCR plate in the thermal cycler, close and secure lid, and select the appropriate PCR thermal program. The thermal programs for the combo assay reaction is:
 - Initial denaturation at 95°C for 10 min
 - 95°C for 15 sec
 - 60°C 1 min with picture
 - Repeat 40 times
 24. Record the plate ID, thermal cycler unit or head, and time of run start for the PCR plate in the PCR log.
 25. Place cycled PCR plates and product in trash without opening the seal. Remove PCR plates promptly. Under no circumstances should a cycled PCR plate be opened or uncovered in the PCR room.

D4. PCR Data Analysis

1. Export data file from Bio-Rad and open using Bio-Rad CFX Manager software.
2. File names must be carefully entered without any mistakes in spacing or coding. File names will follow this format: ddMONyy_initials_ASSAY_case#(sample#-sample#)_Plate#_cyclerSN. There should not be any spaces. Sample range should be integers separated with a dash. Plate numbers are designated as P1, P2, P3.....and no plate numbers should be repeated within any single case. Add EN range to plate name.
3. Positive control plates (BRK or BLG) will be designated as BRK or BLG.
4. Dates must be as depicted with a two-digit day, three-letter CAP month, and two-digit year. Initials are not case sensitive, but assay **MUST BE CAPS**.
5. Immediately upon opening a BioRad result file, click on PLATE SETUP, then REPLACE PLATE FILE and replace with the appropriate template. Under EDITING_TOOLS, click SETUP WIZARD and enter the sample names.
6. Then use the baseline correction function (Apply Fluorescence Drift Collection found under SETTINGS menu in CFX software). This will adjust for wells that have abnormal drifting RFU values.
during the initial few cycles of a run and derives an estimated baseline.
7. Assay quality must be assessed before data is accepted. With a 3 assay multiplex it may still be necessary to manually adjust the baseline threshold. To do this click and hold on the assay's baseline and adjust the threshold to where a maximum R² coefficient is reached or adjust the baseline to the linear region of exponential growth on the amplification plot. The threshold line should not be placed in the plateau phase nor in the initial linear phase of amplification, which may be too low

- and in the background fluorescence.
8. Next, be sure that all assays have efficiencies between 80-120% and R^2 equal to or greater than 0.95. Then manually check results of PCR positives, PCR negatives and any Extraction Negatives that may be within the samples so that all are acceptable.
 9. Once quality measures are deemed acceptable, export the data as a *.txt file and save into a folder named with the case number.
 10. Once text files are in the folder, the program R should be used to analyze the data. Output will include the following:
 - a. Summary statistics on assay quality including the efficiency (E) and R^2 of the duplicate standard curves on each plate.
 - b. Summary data for the total number of negative controls that were clean and the total number of positive controls that were positive.
 - c. A list of samples that had cycle thresholds less than or equal to 15 cycles that were changed to negative.
 - d. A list of positive detection samples will be provided.
 11. Rerun data should be exported as a *.txt file with the plate name noting rerun after the sample range in parenthesis but before any underscore and saved within a folder nested within the folder with case results. The folder with re-run data must be named "reruns", all lowercase, one word.
 - a. Original BioRad file and rerun BioRad file are both saved into the BIORAD folder. The original (file that rerun would replace) .txt file is saved in case folder with other .txt files.
 12. Enter results and QC summary data into the Case Log file. Biologists who are entering results should review entries for accuracy and completion. The eDNA Processing Lead will then review all associated detection data (Case Log entries and BioRad files).
 13. Notify the eDNA Processing Lead immediately of any cases with positive detection results.
 14. All results will be reviewed by the eDNA Processing QA/QC Specialist then reported by the eDNA Processing Lead.
 15. See 2023 QAPP for reporting procedures (Section D. Data Review, Verification and Usability).

E. Example Data Sheets

Combined ERDC ACTM1/3, BHTM1/2, SCTM 4/5 qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ Date _____ Sample batch _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

SAMPLE PLATE MM

Reagent name	Volume per rxn	Volume for rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10.0 µl	µl		
AC TM1/3 Primer Mix(10 µM)	1.0 µl	µl		
BH TM1/2 Primers(10 µM)	1.0 µl	µl		
SC TM4/5 Primers(10 µM)	1.0 µl	µl		
AC TM1/3 Probe(2.5 µM)	1.0 µl	µl		
BH TM1/2 Probe(2.5 µM)				
SC TM4/5 Probes(2.5 µM)				
H2O	3.0 µl	µl	NA	NA

Samples: 3.0ul template + 17.0ul master mix

Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONvy Initials_ASSAY_case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A																								POS	
B																									6250
C																									1250
D																									250
E																									50
F																									10
G																									NTC
H																									NEG
I																									POS
J																									6250
K																									1250
L																									250
M																									50
N																									10
O																									NTC
P																									NEG

Cycler ID _____

"COPY" watermark is grey on original.

Extraction data sheet: to be filled out and taped into Extraction room data book

Analyst _____ Room# _____ Date _____ Sample batch _____

Reagents used: note lot ID and expiration date for kit components

Reagent/tubes	Lot #	Expiration date
GSB Buffer		
Proteinase K		
Ethanol		
W1 Buffer		
Wash Buffer		
Elution Buffer		

Tube ID	Sample ID	Tube ID	Sample ID
1		25	
2		26	
3		27	
4		28	
5		29	
6		30	
7		31	
8		32	
9		33	
10		34	
11		35	
12		36	
13		37	
14		38	
15		39	
16		40	
17		41	
18		42	
19		43	
20		44	
21		45	
22		46	
23		47	
24		48	

Write notes for any deviations from QAPP or lab blunders on facing page of notebook. Be sure to add the same notes to the case log.

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BRK qPCR data sheet: to be filled out and taped into PCR room data book

Analyst _____ Date _____ Samples _____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

Reagent name	Volume per rxn	Volume for rxns	Lot or ID #	Expiration date
TM Env. Master Mix	10 µl	µl		
BRK F Primer(10 µM)	1 µl	µl		
BRK R Primer(10 µM)				
BRK Probe (2.5 µM)	1 µl	µl		
H2O	5 µl	µl	NA	NA

Samples: 3.0ul template + 17.0ul master mix

Standards: 1µl standard + 2µl H₂O + 17.0 µl master mix

Plate Name _____

ddMONvy_Initials_ASSAY_case(sample#-sample#)_P#

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								POS
B																								
C																								
D																								
E																								
F																								
G																								NTC
H																								NEG
I																								POS
J																								
K																								
L																								
M																								
N																								
O																								NTC
P																								NEG

Notes for any lab blunders or deviations from protocol:

Cycler ID _____