A1. Title Page

QUALITY ASSURANCE PROJECT PLAN eDNA MONITORING OF BIGHEAD AND SILVER CARPS

Prepared for:

U.S. Fish and Wildlife Service
Region 3

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Modified for:

U.S. Fish and Wildlife Service
Region 3
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REVIEW CERTIFICATION SHEETS

FOR

Final Quality Assurance Project Plan for the eDNA Monitoring of Bighead and Silver Carp

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A3. ACRONYMS/ABBREVIATIONS

Table 1. Common accronyms/abbreviations commonly referenced in this document

Acronym/Abbreviation	Definition
CAWS	Chicago Area Waterway System
COC	Chain-of-custody
CSO	combined sewer overflow
CSSC	Chicago Sanitary and Ship Canal
DI	deionized (water)
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates, also known simply as nucleotides
eDNA	environmental Deoxyribonucleic acid
FWCO	Fish and Wildlife Conservation Office
GPS	Global Positioning System
LDB	left descending bank
LFHC	La Crosse Fish Health Center
LRC	Chicago District, USACE
MRWG	Monitoring and Response Work Group
MSDS	Material Safety Data Sheets
PCR	polymerase chain reaction
QAPP	Quality Assurance Project Plan
RDB	right descending bank
Taq	*Thermus aquaticus*
USACE	United States Army Corps of Engineers
USFWS	United States Fish and Wildlife Service
UV	ultraviolet
WGL	Whitney Genetics Lab

SECTION 1. PROJECT DESCRIPTION AND PERSONNEL REQUIREMENTS

1.1 Background

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Within Illinois, the manmade Chicago Sanitary & Ship Canal (CSSC), constructed in the early 1900s, provided an unnatural portal for invasive species dispersal between the geologically separated Mississippi River and Great Lakes drainage basins. In 2002, in an effort to curtail the spread of invasive species between the two basins, the U.S. Army Corps of Engineers (USACE), constructed a dispersal barrier system within the CSSC. The primary objective of the barrier system when initiated was to stop the dispersal of the invasive Round Goby into the Mississippi River basin; however, once the project was completed, it was found that the Round Goby had already surpassed the barrier. Since then, a new threat to the Great Lakes from the Mississippi River basin has become the primary objective of the dispersal barrier system. Invasive Asian carps, including Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp (*H. molitrix*) have been steadily dispersing upstream through the Mississippi, Illinois, and Des Plaines rivers. Their potential dispersal through the dispersal barrier system within the CSSC poses a potential threat to the Great Lakes ecosystem. In the past, traditional fishery techniques were used to detect the leading edge of the Asian carp population; however, this method was somewhat ineffective at targeting these species at low densities. The University of Notre Dame, with funding from the USACE, developed a method that detected "environmental" DNA (eDNA) left behind in the aquatic system by the targeted species. Environmental DNA enters the system through a variety of mechanisms, some of which include sloughing of external epidermal cells into the water, sloughing of internal epidermal cells into feces and into the water, and as tissue residues following injury or predation. The detection of eDNA in water samples is based on whole DNA extraction from particulate organic and inorganic matter found in the water and polymerase chain reaction (PCR) assays for species-specific mitochondrial DNA markers. Use of this method is to provide detection of Asian carp at low densities and to serve as an early detection system of the spread of Asian carp into previously uninhabited areas. The original Quality Assurance Project Plan (QAPP) detailing the eDNA monitoring process, including methodologies and quality controls, was requested from the U.S. Army Engineer Research and Development Center (ERDC) as the USACE assumed eDNA monitoring responsibility from the University of Notre Dame. This modified QAPP will be followed by the U.S. Fish and Wildlife Service Fish (USFWS) offices responsible for field sampling and lab processing of eDNA samples. Fish and Wildlife Conservation offices (FWCO) will handle field sampling and the Whitney Genetics Lab (WGL) at the La Crosse Fish Health Center will process eDNA samples once the USFWS assumes responsibility for monitoring Asian carp in the Chicago Area Waterway System (CAWS) and other water bodies in 2013. This version of the QAPP has been modified to be a general guide to field and laboratory methods that should be employed during eDNA monitoring programs. Specific sampling plans and schedules for particular water bodies will be developed by regional sampling agencies and their partners, as well as overseeing governing bodies (such as the Asian Carp Regional Coordinating Committee and Great Lakes Commission). These plans are available online on various agency websites and will not be included in this document.

1.2 General Requirement

Region 3 USFWS requires a Quality Assurance Project Plan (QAPP) for eDNA monitoring. Full-scale eDNA monitoring commenced in September 2010 by the USACE, and based on consultation with experts in processing of litigable DNA evidence (which applies to eDNA monitoring), a final, comprehensive QAPP was not in place within that time frame. Instead a provisional QAPP was used during the first year of eDNA monitoring, to be followed later by a finalized version under a different scope of work. The USFWS will continue to maintain and update a QAPP for use in eDNA monitoring programs that allows for inclusion of any beneficial technical or strategic modifications that become apparent from past monitoring events, research conducted by the eDNA calibration research team, or research published in the literature and vetted by the eDNA calibration team or another USFWS genetics laboratory.

1.3 Project History

The University of Notre Dame, Department of Biological Sciences, Center for Aquatic Conservation, prepared a Standard Operating Procedure (SOP) in 2010. The SOP provided details regarding eDNA monitoring protocol and was given to USACE in May 2010 in compliance with Cooperative Ecosystem Study Unit agreement #W912HZ-08-2-0014, modification P00007. On 15 and 16 December 2009, a technical and quality systems audit of the Center for Aquatic Conservation Lodge Laboratory at the University of Notre Dame was conducted by the US Environmental Protection Agency. The laboratory audit report dated 5 February 2010 was provided to USACE in addition to the eDNA monitoring protocol. These documents served as the basis for the QAPP followed by ERDC. Additionally, USACE submitted the eDNA methodology for an Independent External Peer Review (IEPR), which is a requirement to examine decision documents and supporting work products where there are public safety concerns, significant controversy, a high level of complexity, or significant economic, environmental and social effects to the nation. Released fall of 2011, the eDNA IEPR report, conducted by objective panelists with technical expertise in genetics and population ecology, confirmed eDNA sampling and testing methodology is sound for detecting Silver and Bighead carp DNA but cannot indicate the source of Asian carp DNA (information on the size, gender, age and number of individuals present and cannot distinguish between pure Silver or Bighead carp and their hybrids). In 2013, the WGL received the QAPP and made changes so that it would be applicable to the new field sample processing and eDNA processing personnel and their specific locations. It was also modified according to results of the eDNA Calibration Studies (ECALS) and lab validations carried out by the ECALS team. Modified versions of the QAPP will be approved by the ECALS research team and leadership of Region 3 USFWS.

1.4 Objective

The objective of this QAPP is to provide detailed procedures for Asian carp eDNA sample collection, sample processing (including filtering, centrifuging, DNA extraction, PCR, biomarker analysis, DNA sequencing), data reporting, and quality control/quality assurance procedures to ensure that data are as technically defensible, consistent, and usable as possible. The specific goals and objectives of sampling plans for particular bodies

of water are currently directed by the appropriate governing body. For example, the Chicago Area Waterway sampling plan has been issued by the Asian Carp Regional Coordinating Committee's Monitoring and Response Workgroup in the Monitoring and Response Plan. The Great Lakes, Ohio River, and Upper Mississippi River plans will be or have been issued by Region 3 USFWS in conjuction with state and other partners.

1.5 Project Personnel

The eDNA monitoring project must have personnel appointed to the following positions:

- Field sampling point of contact for each field station responsible for sampling and field processing. For each sampling trip, specific personnel will differ for each role as previously listed in the QAPP, but each role will be filled on each sampling trip
    - Sampling Leader
    - Sampling Quality Assurance Specialist
    - Filtering/Centrifuging Leader
    - Filtering/Centrifuging Quality Assurance Specialist
- eDNA Project Coordinator
- DNA Processing Leader
- DNA Processing Quality Assurance Specialist
- Data Documentation & Reporting Specialist
- Supporting Agency contacts

The minimal responsibilities of the above positions are detailed below. Specific personnel assigned to the project are listed in Appendix A.

The first four roles may be filled by the same person or more than one person on each sampling trip.

Prior to each trip, the eDNA Program Coordinator and the eDNA processing leader need the name and mobile phone number for the field sampling and filter/centrifuge leaders. After each trip, a brief summary report (Exhibit 16) covering quality assurance issues should be provided to the eDNA Program Coordinator.

Sampling Leader: Responsible for obtaining water samples for eDNA monitoring and providing those to filtering/centrifgue team. Responsible for reporting results to Filtering/Centrifuging and DNA Processing Team Leaders, the eDNA Program Coordinator, as well as other designated USFWS personnel.

Sampling Quality Assurance Specialist: Responsible for knowing all quality assurance/quality control (QA/QC) measures for eDNA sampling efforts. Advises Sampling Leader on any potential QA/QC problems. Reviews procedures, field logs, data collection methodology; ensures that all agencies participating in sampling are conforming to procedures, and documents this after each sampling trip (Exhibit 16). Recommends corrective actions for non-conformities.

Filtering/Centrifuge Leader: Responsible for filtering/centrifuging water samples for eDNA monitoring and providing those processed samples to eDNA Processing team.

Responsible for reporting results to Sampling and DNA Processing Team Leaders, the eDNA Program Coordinator, as well as other designated USFWS personnel.

Filtering/Centrifuge Quality Assurance Specialist: Responsible for knowing all QA/QC measures for filtering/centrifuging efforts. Advises Filtering/Centrifuge Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for filtering; ensures all personnel are conforming to procedures, and documents this after each sampling trip (Exhibit 16). Recommends corrective actions for non-conformities.

DNA Processing Leader: Responsible for processing eDNA samples through DNA extraction, PCR, and sequencing. Responsible for reporting results to eDNA Program Coordinator, as well as other designated USFWS personnel.

DNA Processing Quality Assurance Specialist: Responsible for knowing all QA/QC measures for eDNA processing efforts. Advises eDNA Processing Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for DNA processing; ensures all personnel are conforming to procedures. Recommends corrective actions for non-conformities.

Data Documentation & Reporting Specialist: Assists DNA Processing Leader in maintaining laboratory database for eDNA sample processing. Performs data completeness and data verification checks, and ensures that all data are documented completely.

Assigned Project Leaders and Specialists: Others serving on the project may include researchers, technicians, and budgetary personnel. Sampling may employ personnel from other agencies in coordinated efforts. All personnel must meet a minimum standard for training and/or experience before independently conducting any portion of the eDNA monitoring protocol. The supporting agency contacts are given in Appendix A. Minimum personnel training requirements are given below.

Personnel Training Requirements

Minimum training and/or experience requirements for the different major components of the eDNA monitoring protocol are detailed below.

Boat Operator:

- Must meet USFWS boat operator requirements as a minimum.

Sampling:

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid and/or boating safety course.
- Minimum 1 year experience in collecting field samples for biological analyses.

Filtering/Centrifuging:

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid training.
- Facility-specific safety training.
- Minimum one semester college level laboratory experience, plus eDNA-specific training.

DNA Processing:

- A minimum BA/BS degree or its equivalent in biology or related area and successful completion of college course work (graduate or undergraduate level) covering the subject areas of biochemistry, genetics, and molecular biology (molecular genetics, recombinant DNA technology) or other subjects that provide a basic understanding of the foundation of DNA analysis, as well as course work and/or training in PCR amplification as it applies to eDNA analysis.
- A minimum of 6 months of general DNA laboratory experience, including experience with DNA extraction and PCR. Additionally, 2 weeks of training on Asian carp eDNA protocols.
- Successful completion of a qualifying test demonstrating effective execution of eDNA-type assays before beginning independent work on the project.

1.6 Reporting

All agencies, need to submit resumes (Curriculum Vitaes) for proposed staff, to be reviewed and approved by USFWS. Those documents will be kept with the project file. For each sampling event, specific staff members conducting the various activities will be documented (e.g., on the field sampling log). That information will also become part of the project file.

SECTION 2. SAMPLE COLLECTION

Prior to any field sampling work, all field employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Field employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Field employees will also review the sampling safety plan (separate document) and participate in a safety briefing.

Prior to any sample processing or analysis work, all laboratory employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Laboratory employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Laboratory employees will also review the laboratory safety plan (separate document) and participate in a safety briefing.

2.1 Pre-Trip Planning and Site Selection

Refer the internet for annual monitoring plans issued by the agency responsible for each particular body of water. For example, the CAWS monitoring plan can be found at www.asiancarp.us.

2.1.1 Purpose

Accurate planning of a general collection site is necessary to effectively manage the time of crews collecting samples, as well as to ensure complete and correct sampling procedures are used.

2.1.2 Pre-trip Planning Procedure

Refer to the annual Plans to properly plan for sampling and provide this information to the sampling agency.

The sampling agency should use interactive aerial imagery software (i.e., Google Earth) to scope out reaches to be sampled, placement of samples, and unique features that should be targeted during sampling.

(1) Aerial maps should be detailed enough to show unique features (e.g., barge slips, factory, etc.) that can be identified in the field and used as markers for location when sampling. The recommended minimum scale is 1':500'.
(2) Aerial maps should be marked with sample locations and should ensure spatial coverage and overall representativeness of the sample area.
(3) Target specific areas (backwaters, island side channels, pooled areas, below and around structures, confluence of tributaries) as well as integrating transect plots in the sampling plan.
(4) Print map(s) with detailed sample plan.

- Locate access points for boat launch and acquire permission to use if necessary.
- If sampling around locks, or if sampling will require lockage, notify the Lockmaster at least 1 day before sampling.

- Coordinate sample plan with sample crew, which should comprise four people at a minimum: one boat operator, one lead sampler, and two sampling assistants. All participants involved in the sampling must have their resume (CV) on file with USFWS, prior to the sampling event. All participants involved in the sampling must have read this QAPP and must have a signed certification statement (Exhibit 15) on file with USFWS, prior to the sampling event. All participants involved in the sampling must meet the minimum qualifications given for their role in Section 1.3 of this document.
- A field equipment checklist (Exhibit 1) and datasheets (Exhibit 2) should be printed prior to each sampling trip on Rite-in-the-Rain® paper or other waterproof paper. Datasheets are printed on front and back.
- Check river stage and weather forecast.

(5) No eDNA sampling should occur within 5 days after a significant rainfall event (more than 1.5 inches in a 24-hr period), on the rising limb of a hydrograph of the river as it exceeds flood stage, and/or within 2 days of a combined sewer overflow (CSO) event. Weather data and river stage for the area to be sampled can be checked at: http://waterdata.usgs.gov/nwis. The occurrence of a CSO event can be verified by contacting the FWCO contact for that particular sampling event listed in Appendix A.

2.1.3 Field equipment list (procedures are in section 2.2)

(1) Minimum 18-ft boat with specified motor, including trailer and vehicle to pull, provided by the sampling agency.

(2) Personal flotation devices for each crew member, using the type of devices listed in the safety plan. Minimum PFD requirements are Type I within the Safety Zone near the existing electric barrier (on the Chicago Sanitary and Ship Canal) or Type II for the remainder of the CAWS.

(3) If filtering: 100-qt coolers (each cooler capable of holding 20 2L samples); sterilized and filled with 2-L bottles will need to be provided initially by the sampling agency. Once used, WGL will clean and return kits to the sampling agency.

If centrifuging: enough 100-qt coolers capable of holding 5 50-ml centrifuge tubes per planned field sample and ice will need to be provided by the sampling agency.

50-ml centrifuge tubes, polypropylene rated to withstand 6000 x g. Tubes will not be re-used, but WGL will return shipping containers to be sterilized and re-used by sampling agencies if requested.

(4) If filtering: 2L sample bottles, labeled by sampling agency.

If centrifuging: 50ml sterile polypropylene centrifuge tubes with maximum RCF of at least 6,000 x g, labeled by sampling agency.

(5) 3-gallon sprayer (yard sprayer type for spraying down boats)

(6) Habitat measurement equipment (Global Positioning System [GPS], Digital Depth Sounder); provided by the sampling agency.

(7) Field Collection Summary sheets (Exhibit 1) and Field datasheets (Exhibit 2) provided by USFWS or by sampling agency.

(8) Chain-of-custody form (Exhibit 3) provided by USFWS or by the sampling agency.

(9) Sharpie® permanent marker in black provided by the sampling agency.

(10) Powderless nitrile or latex gloves provided by the sampling agency.

(11) Ice provided by the sampling agency.

(12) Drinking water for crew provided by the sampling agency.

(13) Safety plan – USFWS plan represents minimum requirements; agency-specific alternative plans are allowable as long as all hazards are addressed and minimum requirements are met.

2.1.4 Site Selection Procedure (in the field)

Using the sampling plan as guidance, refinement of exact sampling should depend on the following factors:

(1) In lotic systems, sampling should occur in a downstream to upstream direction to minimize the potential for surface water disturbance caused by the vessels wake within the sample reach. The only exception where sampling may occur in an upstream to downstream direction would be if the nearest boat launch is upstream of the reach to be sampled. Sample direction should be noted on the Field Collection Summary datasheet (Exhibit 1).

(2) Samples could be collected in two ways – transect and targeted sampling.

(a) Transect: Location of transects will be determined by USFWS prior to the start of a sampling event. The first transect will be set across the downstream end of the reach to be sampled with subsequent transects set 500 m apart heading upstream (see exception to protocol above (1)). Transects will run perpendicular to flow, and three samples will be collected along each transect using the following scheme: one collected near the left descending bank (LDB), one in mid-channel (MC), and one near the right descending bank (RDB). Where samples are collected should be recorded in the "Habitat" column of the Sampling datasheet (Exhibit 2). Samples should be collected on the upstream side of the boat or off the bow.

- When collecting samples near the bank, be observant of wake-disturbed surfaces. To compensate for the wake created by a passing boat, samples may need to be collected 2–3 ft off the bank to obtain displaced surface film.

(b) Targeted sampling is collecting samples in the most probable places of eDNA accumulation, such as (but not limited to):

- Backwater areas
- Island side channels
- Confluences of tributary waters
- Effluent areas
- Eddies or pooled areas
- Near structures that create slack-water (e.g., sunken barges)
- Bays
- Below Lock and Dam structures
- Other areas where organic material has accumulated on the water surface

Avoid sampling under or downstream of bird rookeries or storm sewer outflows since these have been shown to be vectors of eDNA from sources other than a live fish.

2.2 Boat and Equipment Preparation

2.2.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, vessels and equipment must be sanitized in accordance with the following protocols to eliminate introduction of outside DNA sources in the sampling regime.

Cautions: Precautions should be made to avoid direct skin contact with bleach; bleach solution may also stain clothing or other materials. Be aware of pollutants in the aquatic environment and related health hazards.

2.2.2 Equipment Procedure

(1) A 10% bleach (hypochlorous acid) solution with deionized (DI) water will be prepared in a 3-gallon hand-held sprayer that is dedicated to the project. The bleach solution must be prepared immediately prior to use, and each time disinfection activities will be occurring.

(2) Sample transport coolers will be sterilized with freshly made 10% bleach solution. Using the hand-held sprayer, the inside surfaces of the cooler will be thoroughly covered with the bleach solution. At least 10 min of contact between the solution and the inside surfaces of the cooler will be allowed before rinsing with water. Coolers will then be left to air dry.

(3) If filtering: Sterilized disposable 2L bottles may be used for sample collection or 2L autoclavable reusable bottles sterilized in the following way may be used:

(a) Reusable 2L bottles will first be thoroughly rinsed with water to remove any material that may be on the outside or inside of the bottle and cap.

(b) Prepare a 10% bleach solution and soak bottles and caps for a minimum of 10 minutes. Rinse each bottle by partially filling and shaking water so that all areas of the bottle are rinsed, repeat three times per bottle. Air dry, cap, and place in sanitized coolers.

(c) If you have access to an autoclave, you may autoclave bottles, but it is optional. Place the threaded caps on the bottles and lightly screw on cap (cap will not be able to come off, but will still be able to move).

(d) Place capped 2L bottles into autoclave and set cycle for 1 hour at 121°C (15 psig or 1 bar).

(e) Once the autoclave cycle is complete, carefully remove bottles and allow them to cool to room temperature before fully screwing on the caps.

If centrifuging: purchase sterile disposable 50-ml polypropylene tubes with maximum RCF of at least 6,000 x g.

(4) Once all 2L bottles have been sanitized, sample labels can be printed by the sampling agency, or WGL can send pre-printed labels to affix to the outside of the bottles/tubes prior to going into the field. Sample containers will be labeled with an unique barcode ID or agency generated code that does not indicate location (to allow blind processing). Labels will be printed on Rite-N-Rain® or some type of waterproof labels and affixed to the outside of the sample bottles/tubes using clear duct tape prior to the initiation of field activities.

(5) Once bottles/tubes have been labeled, they will be placed in the sterilized sample coolers in numerical order. Sample containers will be stored in the sterilized coolers until use, and will be transported only in the coolers. Although the number of the samples is not relevant except for identification purposes, collecting in consecutive order will aid in determining where samples were taken in case of a recording error.

(6) A minimum of 10% of the total number of samples collected should be cooler blanks. If filtering, a minimum of one per cooler is required. If centrifuging, collect blanks to meet 10% of total number of samples collected. Note, if the sampling design for your particular body of water requires a specific sample size in order to meet a precise detection probability, then containers for control samples will have to be added to the total number of sample containers dictated by your sampling plan.

2.2.3 Boat Preparation Procedure

(1) Vessels to be used for eDNA sample collection must be washed with a commercial power washer and detergent prior to the disinfection process. All detergent must be thoroughly rinsed and the boat must be allowed to air dry 1 day prior to disinfection. A 10% bleach solution with DI water will be prepared in a 3-gal hand-held sprayer that is dedicated to the project. The bleach solution must be prepared immediately prior to use, and each time disinfection activities will be occurring.

(2) The outer surfaces of the sampling vessel (i.e., hull, motor, etc.) as well as the transport trailer will be thoroughly rinsed with the prepared bleach solution using a hand-held sprayer. Outer surfaces should be thoroughly covered with the bleach solution and left in contact with no rinsing. Three gallons of solution may not be sufficient to rinse the entire boat and trailer, and additional batches of bleach solution must be mixed when needed to ensure thorough rinsing of the hull, trailer, motor, etc. A minimum of 10 minutes contact time must be used for the boat to allow sufficient time for the bleach solution to oxidize organic matter that is present on the boat.

2.3 Sample Collection Procedure

2.3.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, samples must first be collected from the appropriate aquatic environment in accordance with the following protocols.

Cautions: Lifejackets must be worn at all times in transport vessels (boats). Additionally, disposable poweder free latex or nitrile gloves must be worn when collecting water samples and measuring water depth and temperature. Be aware of pollutants in the aquatic environment and related health hazards.

2.3.2 Water Collection Procedure

(1) Prior to launch, crew members will have reviewed this QAPP, will have signed the QAPP certification form, and will understand their assigned roles in the sample collection procedure. All sampler identification information and other field data will be recorded on the Field Collection Summary (Exhibit 1).

(2) The transport vessel will be launched from an appropriate area that allows access to the reaches to be sampled.

(3) Sampling will commence at the first transect located at the downstream end of the reach to be sampled and will proceed in an upstream direction. The only exception to this protocol is when the boat launch is located upstream of the sampling reach. Then sampling will commence at the first transect located at the upstream end of the reach to be sampled and will proceed in an downstream direction. The direction traveled for sampling should be recorded on the Field Collection Summary (Exhibit 1).

(4) When arriving at a sample location (within either a transect or targeted area), the lead sampler and sampling assistant 1 will put on sterile exam gloves (powderless latex or nitrile). **REMINDER – Gloves must be changed before each new transect is taken to prevent cross contamination. The same gloves may be worn when collecting duplicate or blank samples in tandem with a regular sample in a transect.**

(5) Going in consecutive numerical order based on the bottle labels, the lead sampler will remove a labeled sample bottle from the sample cooler.

(6) Just prior to collecting the sample, the lead sampler will unscrew and remove the lid from the sample bottle.

(7) The lead sampler will then reach over the upstream side or the bow of the transport vessel with the sample bottle and fill the bottle by skimming the surface of the field water. The sample bottle must not be submerged or dipped beyond the upper 2 inches of the surface water for sample collection, since the intent of the sampling is to collect floatable materials that are on the water surface.

(8) Once the sample bottle is filled (approximately 1 in. of space should be left within the sample bottle), the lead sampler will screw the lid back on to the bottle until it is tight. The closed bottle will then be returned to the sample cooler from which it was removed.

(9) While the lead sampler is collecting the water sample, sampling assistant 1 will take habitat measurements: water temperature, depth, GPS coordinates in Decimal Degrees, military time of sample, location (e.g., left-bank decending (LBD), center, right-bank decending (RBD), and relate the information to sampling assistant 2, who will record the information on the datasheet next to the appropriate sample ID.

(10) If the lead sampler pulls a transport (cooler)blank (DI water filled prior to trip) from the cooler, the sampler will unscrew the lid and remove to expose the bottle contents to the atmosphere for 5 sec, reseal the bottle, fully submerge the bottle in the field water, and return the bottle to the sample cooler from which it was removed. The lead sampler should relay to sampling assistant 2 that the sample was a blank, so that it can be recorded on the data sheet next to the appropriate ID. BLANKS ARE TAKEN IN TANDEM WITH THE NEXT ACTUAL SAMPLE AND DO NOT REPLACE A SAMPLE IN THAT LOCATION. If a blank has been pulled, the boat will remain at the same location and an actual sample will be taken.

(11) Steps 1 through 10 will be repeated at each sampling location until sampling has been completed for the targeted reach.

(12) Once sampling is complete, ice will be added to the sample coolers as soon as possible. Enough ice should be added to each cooler to completely surround each sample bottle and maintain an inside temperature of 4.4°C. If at any time during transport the inside temperature of the cooler(s) rises above 4.4°C, additional ice should be added.

(13) Chain-of-custody (COC) forms (Exhibit 3) will be completed for every sample and every cooler. All samples, including blanks, will be logged onto COC forms. The forms will be collected and signed whenever the coolers are transferred between parties. If you must split samples into different configurations than listed on the original COC, make an entry for those samples on the original COC and create a new COC for any new shipping containers. Be sure that each container has its own COC.

SECTION 3. SAMPLE FILTERING/CENTRIFUGING

3.1 Purpose

In order to isolate eDNA from water samples collected in the field, particulate matter must be concentrated. It can be filtered from the sample using a vacuum filtration system, or concentrated by centrifugation.

Passing each water sample through the appropriate sterile filter (1.5 micron, 5.5 cm diameter glass fiber filter) will collect particulate matter from the water sample, including sloughed cellular materials containing eDNA, on the filter paper. DNA will later be extracted from the filtered particulates and utilized in subsequent analyses.

If samples are centrifuged, matter collected at the bottom of multiple centrifuge tubes can be collected on sterile cotton swabs and eDNA can be extracted from the swab.

Cautions: Wear powder-free latex or nitrile gloves when handling samples (a glove change is required for each sample). Be careful to avoid unintentional punctures of gloves when using forceps. Punctured gloves must be changed immediately. Be careful not to touch commonly used items in the laboratory when wearing sample gloves (i.e., writing utensils, stationary lab equipment). If in doubt, change your gloves!

3.2 Filtering Procedure

Water samples collected in the field need to be filtered within 12–16 hours after the last field sample is collected.

Equipment needed:

- Manifolds (Millipore 3-place stainless steel Hyrrosol Manifold, item #XX2504735)
- Magnetic 500-ml filter funnels (Pall item #4238; VWR item #28150-496)
- Glass fiber filters, 1.5 micron, 5.5 cm diameter glass fiber filter paper (Type 934-AH; Fisher item #1827-055)
- Forceps (microforceps) – at least two pair, labeled with different colors or other identifier
- Carboy (3.5 gal or larger), for wastewater generated during filtering
- Rubber vacuum tubing (14-176-24, 1.25 in. inner diameter), double hole stopper (Fisher item #14-140P) that fits carboy opening, and glass connectors or plastic connectors (Fisher item #509557177) for connecting manifold to carboy and manifold to vacuum line
- Sterile conical tubes (50mL plastic, Fisher item #14-959-49A) with caps and labels
- Sterile conical tubes (15mL plastic, Fisher item #14-959-49D) with caps and labels
- Paper towels
- Black permanent markers (e.g., Sharpie®)
- Sterile powderless latex or nitrile gloves
- Vacuum system capable of −75 kPa vacuum

- Bleach
- Molecular-grade water (deionized, distilled, DNase and RNase-free)
- Dedicated lab equipment cleaning sink
- Waste water disposal location such as nonspecified-use sink
- Sterile bench paper
- Dedicated water bottles: one for DI water; one for bleach solution
- Washbin for manifolds, such as a dedicated 10 qt plastic tub

3.2.1 Laboratory Preparation

(1) Wash hands thoroughly prior to starting. Prepare a dedicated plastic wash bottle with 10% bleach solution for wiping down lab tables and manifold surfaces prior to processing samples. Sanitize all equipment, in freshly made 10% bleach solution, prior to starting. Collect all supplies.

(2) Rinse down each workstation with bleach solution prior to beginning the filtration process. Cover each workstation surface with sterile bench paper or clean paper towels. Bench paper must be changed between samples.

(3) Put on new sterile powder-free latex or nitrile gloves. Prior to filtering a sample, each work station should have one black waterproof permanent marker for labeling sample tubes (or pre-printed labels), one sterile 50-mL and one sterile 15-mL plastic conical tube, sterile filter paper, one set of sterile forceps for placing filter paper on filter apparatus, one set of forceps for handling used filter paper, dedicated wash bottle with DI water, and dedicated wash bottle with freshly made 10% bleach solution.

(4) At each workstation, connect a 3-place sterile stainless steel filter funnel manifold to a large carboy bottle using rubber vacuum tubing, two-hole stopper, and plastic tubing connector. Connect carboy bottle to vacuum line with second piece of rubber tubing and plastic connector. Glass connectors may be used instead if available.

3.2.2 Sample Preparation

(1) Put on new sterile powder-free latex or nitrile gloves prior to handling each sample.

(2) Remove first sample from transport cooler and rinse bottle thoroughly under running water to remove residual biological materials. Place prepared bottle at workstation.

(3) Label one sterile 15-mL conical tube and one sterile 50-mL conical tube with sample number; indicate that the filter to be stored in the 15-mL tube is the equipment control by labeling this tube with a “C”. Ensure gloves that come into contact with labeling marker are not used again for handling other samples.

3.2.3 Filtering the Equipment Control

(1) Put on clean powder-free latex or nitrile exam gloves prior to processing each new sample.

(2) Place bottom portion of sterile (section 3.2.6) magnetic filter funnel equipped with rubber stopper on manifold and open vacuum line.

(3) Take designated forceps for handling clean filter paper, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion.

(4) A sterile and clean magnetic filter funnel must be used for each sample. The cleaning process is described under the Equipment and Work Area Cleaning section.

(5) Once the magnetic filter funnel top is secured to the bottom portion, pour the 1L of DI water from the graduated cylinder into the magnetic filter funnel top as a control. In order to capture any potential contaminant DNA in the funnel, be sure to pour the DI water quickly so as to immerse all the internal surfaces of the magnetic filter funnel top with DI water. Once the DI water has been poured into the filter funnel top, turn the vacuum on to draw the water down quickly and filter the material as quickly as possible.

(7) Once the 1L of DI water has been filtered through the funnel, remove the filter funnel top. Take the sterile forceps designated for used filter paper and grasp the edge of the filter paper. Roll or fold the filter paper until it is of a size to fit into the 15mL conical tube labeled as a control for the appropriate sample ID.

(8) Place the control filter paper into the 15mL tube, screw on top, and place tube with control sample filter paper into cold storage. If work is at a laboratory, use a −20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.

3.2.4 Filtering the Sample

(1) Take designated forceps for handling clean filter, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion. Avoid touching the funnel base with clean forceps. If forceps accidentally touch the base, get a new pair of forceps for handling clean filter paper.

(2) Take the sample bottle and gently shake to distribute the contents within the sample evenly.

(3) Once the magnetic filter funnel top is secured to the bottom portion, turn on the vacuum and then pour approximately one-third of the sample into the magnetic filter funnel top.

(4) DI water from a wash bottle may be used to rinse any particulates attached to the sides of the magnetic filter funnel onto the filter paper. Make sure that DI in wash bottle is fresh and was replaced prior to the filtering process for the entire collection.

(5) Once the one-third portion of the sample has been filtered through the funnel, remove the filter funnel top. Take the forceps designated for used filter paper and grasp the edge of the filter paper. Neatly roll or fold the filter paper until it is of a size to fit into the 50mL conical tube labeled for the appropriate sample ID.

(6) Repeat steps 1–4 at least two more times or until the entire sample has been filtered. All filter paper used to process the sample is placed in a single 50mL conical tube labeled for the appropriate sample ID, which should then be placed into cold storage. If work is at a laboratory, use a −20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.

(7) The number of filters used to process the sample is up to the discretion of personnel processing the sample, however in the DNA lab, filters must be extracted one at a time. Therefore, if the water sample exhibits an excessively slow filtration rate, multiple filters should be used. Also up to the discretion of the personnel processing the sample is the amount of sample water to run through a single filter. A general rule is to run one-third of a 2L sample through a single filter; however, if the sample water is extremely turbid, for example, less water should be put through a filter. At the other end of the spectrum, if the sample water is extremely clear, more than one-third of a 2L sample may be run through a single filter. Do not place more than 10 filters into a single 50-mL tube. If more than 10 filters are generated, use additional 50-mL tubes, making sure they are clearly labeled with the sample ID.

(8) On the field datasheet (Exhibit 2) next to the appropriate sample ID, mark the military time of filter completion and the initials of the person that processed the sample.

(9) Change gloves and sterilize the workstation between samples. Repeat steps 1–8 until all samples have been processed.

(10) When filtering, if the water collection carboy becomes full, disconnect the carboy from the vacuum and manifold and dispense water in a sink separate from the one used to clean equipment. Once emptied, reconnect the carboy to the vacuum and manifold and proceed with the filtering process. **CAUTION: Be sure to open manifold valve and turn off the vacuum air supply when disconnecting and connecting the carboy so as to prevent explosion of the glass.**

(11) If sample is accidentally spoiled during the filtering process (e.g., bleach was used to rinse filter funnel instead of DI water, forceps from previous sample used, etc.), immediately throw away ruined samples. If portions of the sample are still viable, place in 50mL conical tube. On the outside of the sample tube, label with the amount of the viable sample (e.g., 2/3 sample). On datasheet, label with the same information

(e.g., 2/3 sample) next to appropriate sample ID. Note the reason for the ruined or diminished sample (i.e., spilled bottle, bleach solution used for rinsing instead of DI water, suspected cross contamination due to dirty gloves, etc.).

3.2.5 Equipment and Work Area Cleaning After Filtering Each Sample

(1) Fill a 500mL glass beaker with 10% bleach solution. Forceps designated for used filter paper must be switched out for each sample. Used forceps will be placed in beaker with 10% bleach solution for a minimum of 10 min for sterilization. Once sterilized, remove forceps from bleach solution and rinse thoroughly with DI water before use.

(2) Fill at least a 10-qt plastic tub (e.g., Rubbermaid® plastic storage bin) with 10% bleach solution. Once a sample has been processed, the filtering apparatus must be dismantled (i.e., the magnetic filter funnel should be separated into the upper and lower parts), rinsed with DI water to remove any particles and/or film, and placed in the plastic tub with the 10% bleach solution for a minimum of 10 min for sterilization. Once sterilized, remove the two parts of the magnetic filter funnel and thoroughly rinse with DI water before use. Rinsing should continue until all residues and scent from the hypochlorous acid can no longer be detected.

(3) In between each sample, dispose of bench paper. Wipe down surface with 10% bleach solution using wash bottle and paper towels. CHANGE GLOVES! Cover work station with new bench paper.

3.3 Centrifuging Procedure

Water samples collected in the field need to be centrifuged within 12–16 hours after the last field sample is collected.

Equipment needed:

- Sterile centrifuge tubes made of polypropylene that can withstand 6000 xg (50mL plastic, Fisher item #14-959-49A) with caps and labels.
- Paper towels
- 95% ethanol or 70% isopropyl alcohol
- Black permanent markers
- Sterile powderless latex or nitrile gloves
- Refrigerated centrifuge(s) with rotors and adaptors for 50-ml tubes (Fisher item # listed for: Sorvall Legend XTR centrifuge; rotor GS25F7087G item #75-033-607; 750 ml round bucket GS25F7087G item #75-003-608; 50-ml tube adaptors item #75-003-638)
- Bleach
- Molecular-grade water (deionized, distilled, DNase and RNase-free)
- Dedicated lab equipment cleaning sink
- Waste water disposal location such as nonspecified-use sink
- Sterile bench paper
- Dedicated water bottles: one for DI water; one for bleach solution
- Washbin for centrifuge adaptors and buckets, such as a dedicated 10 qt plastic tub

3.3.1 Laboratory Preparation

Hands must be washed thoroughly prior to starting. A dedicated plastic wash bottle with 10% bleach solution should be prepared for wiping down lab tables and other surfaces prior to processing samples. All equipment must be sterilized and all supplies collected prior to starting.

Each workstation must be rinsed with bleach solution and the surface covered with one or more clean paper towels or bench paper prior to beginning the centrifuging process. Paper must be switched between each batch of sampling sites. New sterile powder-free latex or nitrile gloves must be worn for processing each batch. Prior to centrifuging samples, each work station should have pre-printed labels or one black, waterproof permanent marker for labeling sample tubes, a dedicated wash bottle with DI water, a wastewater container (with lid), and a dedicated wash bottle with 10% bleach solution, and a dedicated alcohol bottle with 95% ethanol or 70% isopropanol. At least a 10-qt plastic tub (e.g., Rubbermaid® plastic storage bin) should be filled with 10% bleach solution for the sterilization bath.

At each workstation a refrigerated centrifuge set at 4^0 C. If in mobile trailer, check horizontal alignment, trailer should be as level as possible.

3.3.2 Sample Preparation

New sterile powder-free latex or nitrile gloves must be worn prior to handling each sample set.

The first sample set of replicate 50-ml tubes should be removed from the transport cooler and examined to ensure the meniscus of the water is at the 50 ml line, caps checked/tightened, and dipped in a 10% bleach solution to clean the outside of tube prior to centrifuging. Tubes will then be rinsed with DI water, dried with a clean paper towel, and placed in a clean sterilized 50ml holding rack on a prepared lab bench.

Process tubes in sets of replicates, until a full centrifuge batch is prepared.

A centrifuge equipment control comprised of 50 ml of distilled water should be included with each batch of tubes in each centrifuge. It should be labled with the range of sample numbers included in the batch.

3.3.3 Centrifuging the Samples

(1) Place the 50ml tubes that have been cleaned and dried in the refrigerated centrifuge set at 4 ^{0}C. Tubes must be evenly distributed within the centrifuge to maintain the rotor balance. NOTE: Always follow the manufacturer's guidelines for centrifuge operation.

(2) Once 50 ml tubes are in position, close and secure centrifuge lid. Set centrifuge to spin the samples for 30 min at max speed (~4500-5000 xg) and begin centrifuging the samples. During this period the other tubes may be decontaminated and placed on a sterilized surface or rack. DO NOT PLACE DECONTAMINATED TUBES ON ANY

SURFACE OR RACK THAT HAS NOT BEEN STERILIZED. Once centrifuge cycle begins, sterilize the empty Styrofoam rack the 50ml tubes came in by placing in bleach solution for 10 minutes.

(3) Once samples have been centrifuged the eDNA will be on the bottom of the tube. Wearing a new, sterile pair of gloves for each set tubes comprising one sample, carefully remove cap and GENTLY pour off water into a wastewater container. Change gloves after each sample set. Add ~5 ml of 95% ethanol or 70% isopropanol to the tube to stabilize the eDNA. Replace cap and swirl alcholol around tube covering the entire internal wall. Centrifuge the samples with ethanol for 10 minutes (or more as needed if pellet is too loose) at max speed (~4500-5000 xg). Decant excess alcohol until the alcohol is less than half-way up the conical end of the tube. Place tubes back into the sterilized styrofoam container. Change gloves between sample sets. When styrofoam racks are full, put them back into the plastic shipping bags so that the tubes are secured in the rack. Tape bags closed and sign across the tape where it overlaps. Place in box to be shipped. NOTE: If a stabilizing solution (i.e., ethanol) is not used eDNA samples must be frozen (traditional commercial freezer or −20 °C freezer) or placed on ice immediately. To conserve dry ice, samples may be placed in a cooler filled with ice during the working day. At the end of the day, quickly transfer all samples into a dry ice cooler for long-term storage. Be sure to monitor temperature twice a day and replenish dry ice as needed.

(4) Prior to centrifuging the next batch of 50 ml samples remove the 50 ml bucket inserts and examine for any water or debris. If anything is found, place in 10% bleach bath for 10 minutes, rinse with DI water, dry with new paper towels, and replace. If bucket inserts are clean and dry only perform this step during daily lab cleanup.

Steps 1–4 should be repeated until all samples have been processed.

(5) On the field datasheet next to the appropriate sample ID, record the time of centrifuge completion and the initials of the person that processed the sample.

As always, gloves must be changed and the workstation sterilized between batches.

When the wastewater collection carboy is full, it should be disposed of in a sink separate from the one used to bleach equipment. If a second sink is not available, dispose of the wastewater in any drain that is connected to a sewage treatment facility or system.

If a sample is accidentally spoiled during the centrifuging process (e.g., the pellet was lost during decanting, bleach was squirted into a tube, or cross-contamination is suspected), it should immediately be thrown away. Record on the corresponding datasheet the appropriate sample ID as well as the reason for the ruined sample (i.e., spilled bottle, acid solution used for rinsing instead of DI water, suspected cross contamination due to dirty gloves).

SECTION 4. SAMPLE SHIPMENT

4.1 Purpose

Samples must be shipped to the DNA processing lab (WGL lab in Onalaska, WI) within 24 hours if samples were filtered. If samples were centrifuged, samples may be shipped at the end of the sampling event). The Filtering/Centrifuge Leader is responsible for ensuring that samples are properly packed and shipped according to the procedure below.

Please note: the COC forms are as important as the samples themselves. If COC forms are not filled out properly, then sample intergrity is lost and the samples cannot be processed because their custody cannot be accounted for. Therefore, please be sure to accurately and completely fill out the COC forms. If you have questions, do not hesitate to call the eDNA Program Coordinator or the eDNA laboratory.

Cautions: Wear gloves and use caution when working with dry ice.

4.2 Shipping Procedure

(1) If filters: Corrugated boxes (minimum outer dimensions 12" X 12" X 12") with styrofoam cooler inserts will be prepared for shipment. The number of boxes to prepare depends upon the number of samples collected (e.g., a 120-sample collection will require more boxes for shipping than a 50-sample collection); however, at least two boxes will be prepared for every collection: one for the controls and one for the samples.

If centrifuge tubes: Any shipping containers that will hold the tubes and prevent damage to the tubes will work.

(2) For filters or non-preserved centrifuge samples: The bottom of the coolers will be lined with solid blocks of dry ice (approximately 1–2 inch thickness). Oven-mitt type gloves must be worn by personnel that are handling dry ice to protect hands.

(3) For filtered samples: Remove the 15mL conical tubes with control filter paper from secure (i.e., locked) −20°C freezer and place in clean 1-gal resealable bag (e.g., Ziploc®). Multiple bags may be used if the entire sample does not fit in one bag. Seal over the opening of all bags used with tamper-evident tape (e.g., Evidence Tape – NC9709516). Be sure to remove as much air as possible from all resealable bags used before sealing.

Place a ~10-lb block of dry ice on the bottom of cooler. Place a bag of samples on the block, layer approximately 1 in. of dry ice pellets on top of bag before placing another bag in the cooler. Repeat until only 2 inches of space is left at the top of cooler and fill the space with dry ice pellets. If pellets are not available, use a minimum of 20 pounds of block dry ice. Leave the bottom block whole, but break up the other block and distribute among the bags of samples. Before closing the styrofoam cooler, record the inside temperature on the datasheet (Exhibit 2). Place the styrofoam lid on top of the sample contents and seal with tamper-evident tape, ensuring the tape crosses the lid and body of the cooler. If tamper-evident tape is not available, use packing tape and sign across where the tape overlaps as well as across the lid meets the body of the cooler.

For centrifuge samples: Be sure tubes are securely closed to prevent leaking. If tubes have been chilled, lids may become loose, so double-and triple-check before packing. Place tubes back into the

foam rack in numerical order. Place the rack of tubes back into the plastic bag the tubes came in, wrap it tight to keep tubes from falling out, tape it closed, signing across the tape. Pack racks of tubes into boxes, using packing material to keep them from shifting. These can be shipped at ambient temperature since they are preserved in alcohol.

(4) Fill out a COC form for each shipping container that lists exactly and only the samples shipped in that particular container. The individual employee packing and sealing the containers should list their name in the "from" line, be sure to include agency and print clearly. The container should be packed and released on the same date. Sign and place COC forms (Exhibit 3) in a clean 1-gal resealable bag, place evidence tape across the seal, and place the bag on top of the cooler before closing the corrugated shipping box and sealing with packing tape.

(5) Repeat steps 1–4 for 50mL conical tubes and additional boxes. **Each box MUST** have a separate signed COC form included to document the specific samples included therein. If you must split samples into different configurations than listed on the original COC, make a new entry for those samples going into one container on the original COC and create a new COC for any new shipping containers.

(6) Fill out a Federal Express (FedEx) air bill shipping label with appropriate information. Be sure to affix a dry ice warning label with the amount of dry ice in each cooler on the cardboard shipping container. On the label be sure to designate FedEx Overnight Express (delivery is usually the following day between 8 and 10 AM) as well as to identify the weight of the dry ice in the package. When ready, drop off at FedEx or call FedEx (1-800-463-3339) for pickup. Be sure to tell the operator that the package contains dry ice and ask for an approximate pickup time. Be sure to record tracking numbers for all boxes being shipped.

(7) Items will be shipped to the following address:

Whitney Genetics Lab
555 Lester Avenue
Onalaska, WI 54650
608-783-8444

See item 8 for WGL contacts

(8) Once items have been picked up for shipment, at least one person at WGL must be contacted and notified of approximate delivery date and time. At least one of the following personnel should be contacted via telephone and email regarding shipment, continue calling down the list until you speak with a human on the phone:

Emy Monroe
Phone: 608-783-8402
Email: emy_monroe@fws.gov

Maren Tuttle-Lau
Phone: 608-783-8403
Email: maren_tuttle-lau@fws.gov

Jennifer Bailey
Phone: 608-783-8451
Email: jennifer_bailey@fws.gov

Nick Grueneis
Phone: 608-783-8404
Email: Nikolas_Grueneis@fws.gov

Nick Berndt
Phone: 608-783-8419
Email: Nicholas_Berndt@fws.gov

Kyle Von Ruden
Phone: 608-783-8411
Email: Kyle_VonRuden@fws.gov

(9) Upon receipt of the samples at WGL, if the samples were shipped with dry ice, the inside temperature of all the coolers must be taken and recorded. Samples shipped with dry ice that have remained at room temperature (approximately 20°C) for more than 24 hours will be discarded, and the sample names and reason(s) for discarding will be noted in the laboratory log. Samples that are shipped at ambient temperature do not need a temperature recorded upon receipt.

(10) Personnel receiving the shipment must immediately sign the COC form, scan it and email it back to the sender of the COC. Receiving personnel should also call the sender if they do not have email access in the field.

SECTION 5. DNA ASSAYS

5.1 General Quality Assurance and Chain-of-Custody Considerations

(1) Any change to described DNA handling, storage, or processing procedures must not result in reduction of eDNA sensitivity relative to current values and must be cleared with the eDNA Processing Leader. Table 2 represents the results produced with the current protocol at different concentrations of purified DNA amplicons in sterile water (Concentations are as reported in Jerde et al. (2012) in response to Casey et al.'s sensitivity of detecting environmental DNA comment. Conservation Letters 0:1-2.)

Table 2. Purified DNA amplicon, in copies and nanograms (ng) per microliter (µl) of water, for Bighead and Silver Carp detection sensitivity

Species	**Purified DNA amplicon (copies/µl water)**	**Purified DNA amplicon (ng/µl water)**
Bighead Carp	207	3.30×10^{-8}
Silver Carp	7	7.25×10^{-10}

(2) Each stage of eDNA genetic processing procedures (eDNA sample extraction, PCR setup, and post-PCR processes) should be performed in a separate room in order to minimize the risk of sample cross-contamination. If separate rooms are not available, extractions and PCR set up can be combined in one room, ONLY if PCRs are set up in a PCR hood with a hepa filter and UV light. All post-PCR processing *must* be in a separate room.

(3) 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 DNA Away) 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 beginning and end of the work day.

(4) All new microcentrifuge tubes (non-sterile) and glassware used in the lab must be autoclaved at 121˚C for 20 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 and autoclave.

(5) Good housekeeping policy should be practiced at all times. Reagents that have passed expiration dates should not be used, nor should any reagents that have been kept at incorrect storage temperatures for a significant length of time. All reagents, reaction tubes, etc., must be clearly labeled. Records of batch numbers of all reagents used in individual assays should be made whenever reagents are signed out from the designated freezer. The temperatures of cold storage units must be monitored once a day, using the forms given in Exhibits 7-8.

(6) Positive and/or negative reactions should be used to test all new batches of critical components prior to or concurrent with their application to eDNA samples.

(7) Standard sterile techniques should be used in the DNA laboratory to prevent the unintended 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 that the laboratory have procedures in place to limit, recognize, and address contamination.

(8) Gloves (e.g., sterile, 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. For example, gloves should not be worn when using or handling keyboards, notebooks, pens, telephones, etc. and must be replaced immediately before recommencing bench work.

(9) Centrifuge tubes before opening the reagents. Uncap and close tubes carefully to prevent aerosol contamination.

(10) Ensure that centrifuges are always balanced when centrifuging samples.

(11) 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. Workbooks that have been in contaminated areas shall not be taken into clean PCR areas. A Project Lab Book should be kept in a room separate from the DNA Extraction Room and DNA PCR Room. Each room (Extraction, PCR, Post-PCR) should have note-taking materials (e.g., loose-leaf paper, networked tablet PCs) that can be transported or viewed for consolidation in the Project Lab Book. Other solutions for preventing contamination of sensitive areas via lab notes may be used following approval by the eDNA Processing Leader. Any changes should be incorporated into a revised QAPP. Laboratory notes/notebooks should:

- Be written or printed on tamper-proof paper (e.g., does not exactly photocopy).
- Have lab book identification, with consecutive numbering, dates, and signatures (of the note-taker) on each page.
- Be made using permanent ink. Special pens may be required for certain paper types.
- Have any changes to notebooks 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.
- Contain all data images (e.g., gel photographs, denaturing curves, DNA sequence electropherograms). Images should be permanently affixed to the notebook and signed across both the edge of the insert and the page.
- Be kept in a locked drawer or cabinet with restricted access when not in use.

(12) A log of all batches of critical components should be kept. This log should include material safety data sheets (MSDS) and product information sheets. Dates of receipt, opening, testing, and disposal for each component should be recorded in the log.

5.2 Quality Control for Sample Custodian Procedure and Storage

(1) An internal log book should be kept for all samples. Tamper-proof paper should be used. The log book should be kept in a locked drawer or cabinet when not in use.

(2) Separate freezers should be designated for storage of (a) concentrated water samples, (b) DNA extracts, (c) PCR and sequencing product, and (d) PCR, cloning, and sequencing kit components. A dedicated refrigerator should also be maintained for any PCR, gel electrophoresis, cloning, and sequencing kit components that require 4˚C storage.

(3) Maps or other designations of the location of samples within freezers should be maintained.

(4) All items in freezers should have indelible ink identifications.

(5) All freezers should have non-universal locks or marine brackets attached that can be used with keyed locks, or be housed in a secure facility.

(6) All samples placed into or removed from freezers should be signed for on freezer log or ambient storage log (see Exhibits 7, 8, and 9).

5.3 Physical Separation of Pre-PCR and Post-PCR Assay Stages

5.3.1 The eDNA Extraction Room

- 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.
- A complete separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for use in DNA extraction.

5.3.2 Pre-PCR Room

- To prevent carry-over of amplified DNA sequences, PCR reactions should be set up in a separate room from that used for post-PCR manipulations.
- A completely separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for the specific use of pre-PCR manipulations.
- Reagents and supplies should be taken directly from clean storage into the PCR area and should never be taken or shared with areas in which post-PCR analyses are being performed. Equipment such as pipettes should never be taken to the post-PCR area after use with amplified material.
- A sign-in log system should be implemented for use of the thermal cyclers (PCR machines), including the run name, plate orientation, and the number of thermal cycler heads.

5.3.3 PCR Product Analysis Room

This is the post-PCR Room where post-PCR manipulations are performed, including agarose gel electrophoresis of products and sequencing of presumptive positives.

- 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.

5.4 Receipt of Filters/Tubes

5.4.1 Source

Water samples have been taken in the field according to Section 2 and filtered or centrifuged according to Section 3. Samples have been shipped to WGL and personnel assigned to the WGL eDNA Team have received packages from the overnight service. NOTE: filters are shipped cold, but alcohol-preserved centrifuged samples are shipped at ambient temperature.

(1) Upon receipt of samples from the eDNA sample filtering team, the shipped box(es) should be opened and if shipment was on dry ice, the temperature inside each box recorded. The general condition of the box(es) should also be recorded.

(2) To measure temperature upon opening the box, either (a) place a glass thermometer inside the Styrofoam container, replace lid, and leave the thermometer in place for at least 2 min before removing it and immediately recording temperature or (b) immediately aim an infrared laser thermometer at the samples and press the MEASURE button to record the temperature inside the cooler.

(3) Place filtered samples in filter sample storage freezer (–80°C) and log samples on freezer sheet (Exhibit 8). Place centrifuged sample tubes in ambient sample storage (shelves) and log samples on bench-top log sheet (Exhibit 9).

(4) Sign and date the COC forms that accompanied the samples. Place them in a designated file; a copy should also be provided to sampling agency. If the forms were inside sealed bags, slit the bag to remove the COC forms. Note any condition issues (broken tape or seals, damaged containers or bottles, etc.) with the samples on the COC forms. Note any samples that must be discarded due to condition issues and the reason for discard.

(5) Enter sample data into internal sample log book (or LIMS), including noting any samples that are being discarded and that should not be analyzed, and create new WGL COC form for samples. Note any observations about samples such as condition issues. Store COC forms in a secure area.

(6) Alert Sampling and Filtering/Centrifuging Leader that samples have been received. Use e-mail addresses with return/receipt requested and directly contact via telephone; team contacts are listed in Appendix A. This reporting must be done within 1 hour of receipt.

5.5 DNA Extraction from Filters or Tubes

5.5.1 Source

Filters or tubes from eDNA sampling have been received by WGL eDNA Team and logged. Filter samples should be in designated −80°C freezer, centrifuged sample tubes may be in ambient storage or if they are not preserved with alcohol in designated -80°C freezer.

5.5.2 DNA Extraction Quality Assurance and Chain-of-custody

At this stage, a critical component of quality control should be to correctly label all sample extraction processing tubes so that there is no question about the origin of samples.

(1) Bench areas in DNA extraction laboratory and PCR-type hood (if used) should be wiped before and after use with 10% bleach. Validated, commercially available sterilization reagents, such as LookOut® DNA Erase®, may be preferred. Extraction rooms should be irradiated with UV lights for 30 minutes prior to use.

(2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach must be rinsed to avoid corrosion.

(3) It is common practice for moisture barrier paper towels to be placed on the bench top while processing samples to act as a barrier. The paper barriers must be changed and the bench top cleaned between sample batches.

(4) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for the extraction process should be cleaned before and after each use.

(5) Instruments such as forceps and scissors should be cleaned just prior to use. Sterile disposable equipment should be opened just prior to sample processing and discarded after one use.

(6) When using pipettors, use filtered tips and never allow the liquid in a pipette tip to rise up to the barrier.
- Do not rest the pipette on a dirty surface.
- Avoid cross-contamination by changing pipette tips after each use.
- Watch that the tip -- and only the tip -- is allowed to go into a bottle of reagent, never the pipette itself.

(7) Record all solution batch/lot numbers used for reactions in lab notes.

(8) Always mix tubes by vortex or finger flicking, and then briefly spin down tubes before opening.

(9) No deviations to the DNA extraction protocol are allowed without written approval by the eDNA Processing Leader. Any errors in processing should be noted in the laboratory log. Samples affected by errors in the extraction protocol should be clearly identified.

5.5.3 Alcohol evaporation from centrifuged samples procedure

(1) Centrifuged samples preserved with alcohol must have the alcohol evaporated away before extracting the samples. Prepare laminar flow hood by wiping down the work surface with 10% bleach or DNA away and/or use the UV lamp for 15 mintues.

(2) Remove samples from freezer or ambient storage; note on freezer/storage and sample log (see Exhibits 7-9).

(3) Move samples in tube racks to the laminar flow hood. Carefully remove tube lids and place in the same order as the samples next to each rack. 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.

(4) Include a hood negative control sample for each extraction batch set out to dry. For our extraction batches of 30, this equals 27 field samples, an extraction positive and negative, and one hood negative control. Make a negative hood control by placing an empty, sterile centrifuge tube in the racks with the lid removed alongside the field samples.

(4) 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 100 µl of Silver and Bighead carp cell lines or tissue slurry directly onto a sterile swabs in 1.5-ml flip-top tubes. Alternative species (e.g. – sturgeon) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA and (2) can be run on the same thermocycler settings as carp samples. 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 ul of sterile lab DI water onto a sterile swab in a 1.5-ml flip-top tube. A batch of extraction blanks can be prepared in advance and kept frozen at −20°C

- For every 28 filter samples processed or 27 centrifuged samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control and one prepared positive control.

(5) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below.

Cautions: As with all components of eDNA processing, quality control and sterilization procedures must be carefully followed in order to avoid contamination of downstream procedures.

5.5.4 PowerWater Procedure for *filters*

(1) Remove samples from freezer if filters, note on freezer and sample logs (see Exhibits 7-9).

(2) Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.

(1) Before proceeding with extraction, a positive control filter is prepared by pipetting 100 µl of a mixed slurry of homogenized Silver and Bighead carp tissue or cells directly onto a sterile filter paper. Alternative species (e.g. – sturgeon) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA and (2) can be run on the same thermocycler settings as carp samples .
(2) Additionally, an extraction negative control sample should be prepared by placing a new filter paper in a new sterile 1.5mL microcentrifuge tube (MCT). A batch of extraction blanks can be prepared in advance and kept frozen at −20°C.
(3) For every 28 filter samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control filter and one prepared positive control filter.

(3) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below. For each filter, you will need one spin column and 4 1.5/1.7 ml lab supplied MCT and 5 Powerwater collection tubes.

This DNA extraction utilizes the PowerWater DNA Isolation Kit (MoBio Laboratories, MoBio Inc.) and the protocol is adapted from the manufacturer's protocol (http://www.mobio.com/images/custom/file/14900.pdf).

(a) Place Solution PW1 in a 55°C water bath for 5–10 min to dissolve any precipitates that have formed at room temperature. Remove Solution PW1 from the water bath immediately prior to use.

(b) Remove the appropriate filter sample from −20°C freezer and transfer the filter(s) to a labeled 5mL PowerWater Bead Tube. If DNA will be extracted from multiple filter samples, continue to remove each filter sample from −20°C freezer immediately prior to filter transfer. Each PowerWater Bead Tube will hold up to two filters per sample. If any filtered water samples required more than two pieces of filter paper, split the filters into two per PowerWater Bead Tube.

Note: Change gloves between the transfers of each sample to avoid cross-contamination of samples.

(c) Add 1mL of Solution PW1 to the PowerWater Bead Tube and secure the cap tightly. Mount the tube on a vortex adaptor (MoBio Inc.) and vortex on high for 5–10 min, or until the contents of the bead tube appear liquefied. Times can vary depending on the number of filter papers being extracted. A bead beater can be used on a large batch of bead tubes if available.

(d) Centrifuge the tubes at 4,000 x *g* for 8 min at room temperature. Ensure centrifuge is balanced before centrifuging. Transfer 650-800 µl supernatant using a 1mL pipette to a labeled 1.7mL lab supplied MCT.

(e) Centrifuge tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled 1.7 mL lab supplied MCT. Be sure to avoid any beads or filter debris.

(f) Add 200µL of Solution PW2, vortex briefly, and incubate at 4°C for 5 min. It should appear cloudy.

(g) Centrifuge the tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled lab-supplied 1.7 mL MCT.

(h) Add 650µL of Solution PW3 and vortex briefly. Load 650µL of supernatant onto a spin filter, place spin filter into a MoBio 2mL tube and centrifuge at 13,000 x *g* for 1 min. Discard the flow through and collection tube. Place spin filter into a new MoBio 2mL tube and load another 650 µl , centrifuge and repeat until all the supernatant has been pass through the spin filter.

(i) Place the spin filter basket into a new labeled MoBio 2mL collection tube and add 600µL of Solution PW4.

(j) Centrifuge the tubes at 13,000 x *g* for 1 min and discard flow through. Place spin filter in a new, labeled MoBio 2mL tube.

(k) Add 550µL of Solution PW5 and centrifuge at 13,000 x *g* for 1 min. Discard flow through, place spin filter into a new MoBio 2mL labeled tube and centrifuge again at 13,000 for **2 min**. Be sure all traces of EtOH are gone.

(l) Place the spin filter into a new labeled 1.7mL lab supplied MCT labeled with the sample identification number.

(m) Add 100µL of sterile water (autoclaved, de-ionized) to the center of the white filter membrane, let it sit for 1-2 minutes, then centrifuge at 13,000 x *g* for 1 min.

(n) Discard the spin filter and store the eluted DNA samples at −20°C. If more than one extraction tube was required for a single sample, combine all replicates into one final extraction tube.

(4) DNA extraction elutes should be placed into a designated freezer for overnight or longer storage, or, if used for PCR within 1-4 hours, stored in the refrigerator. Make note of sample addition to freezer log if necessary (see Exhibit 7). Note completion of extraction on sample log.

5.5.5 Qiagen DNeasy Kit (or equivalent kit) Procedure for *filters*

(1) Label one set of 1.5ml MCT for the extraction (one tube per filter). You will have time to label the rest (3 more 1.5mL lab supplied MCT, one spin filter tube, 3 Qiagen collection tubes) during the 1-hour incubation.

(2) Add 370 µL ATL to each tube.

(3) Add 30 µL protease K to each tube. Vortex for 5 - 10 seconds.

(4) Remove samples that have been designated to be extracted using the DNEasy Blood and Tissue DNA Isolation Kit from freezer.

(5) Remove the appropriate filter sample and transfer to the labeled tube. Only one filter can be extracted per MCT. If more than two extractions are required for one sample, combine products into a single tube once extractions have been completed (during step 17). Be sure to push the filter down into the ATL/proteinase K mixture (use a clean pipet tip).

(6) Incubate at 55°C for 1 hour.

(7) Remove from incubator and centrifuge at ≥ 16,000 x *g* for 5 minutes.

(8) Transfer about 200-300 µl of supernatant, or whatever amount is easily pipetted off of the filter or swab, to a new 2 mL MCT. If the filters absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

(9) Archive tube and filter at -80°C. Archive these until the samples have been completely processed. Once results are finalized, these can be disposed of because extracts will be permanently archived.

(10) Add 400 µL Buffer AL.

(11) Add 400 µL ethanol (96 – 100% molecular grade). Mix thoroughly by vortexing.

(12) Transfer about half of the mixture by pipet into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(13) Transfer the remaining mixture by pipet onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(14) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(15) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge at 18,000 x *g* for 3 minutes. Discard flow-through and collection tube.

(16) Transfer the spin column to a new 1.5 mL or 2 mL MCT.

(17) If there are 8 or fewer filters per sample, elute the DNA by adding 200 µL Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at ≥ 6000 x *g* for 1 minute. If there are more than 8 filters, elute with only 100 µL Buffer AE so that all of the replicates can be pooled into one extraction tube.

(18) Discard the spin column, combine multiple extractions for a single sample if necessary. Store the eluted DNA samples at −20°C. If DNA is to be immediately used for PCR, keep on ice.

5.5.6 Qiagen DNeasy Kit (or equivalent kit) Procedure for *centrifuged* samples

(1) Label one set of 1.5-ml MCT for the extraction. You will have time to label the rest (3 lab-supplied 1.5-mL MCT, 1 Qiagen spin tube, and 3 Qiagen collection tubes) during the 1-hour incubation.

(2) Add 370 µL ATL to each tube.

(3) Add 30 µL proteinase K to each tube. Vortex for 5 - 10 seconds. Place tubes in rack and open all tubes.

(4) Remove sterile swabs from pack and place one swab into each tube of ATL/proteinase K mix.

(5) Move dried samples in centrifuge tubes to the extraction room. Use the moistened swab to swab the bottom of each tube included in the field sample (e.g. if there are 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 ATL solution in the extraction tube and proceed swabbing the rest of the replicates.

(6) Place the swab back into the ATL mixture, break the wooden stick and close the tube.

(7) Incubate at 55°C for 1 hour.

(8) Remove from incubator and centrifuge at ≥ 16,000 x *g* for 5 minutes.

(9) Transfer about 250-300 µl of supernatant to a new 2 mL centrifuge tube. If the swabs absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

(10) Archive tube with swab at -80°C. Archive these until the samples have been completely processed. Once results are finalized, these can be disposed of because extracts will be permanently archived.

(11) Add 400 µL Buffer AL.

(12) Add 400 µL ethanol (96 – 100%, molecular grade). Mix thoroughly by vortexing.

(12) Transfer about half of the mixture by pipet into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(13) Transfer the remaining mixture by pipet onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(14) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge at 18,000 x *g* for 3 minutes. Discard flow-through and collection tube.

(15) Transfer the spin column to a new 1.5 mL or 2 mL MCT.

(16) If there are 8 or fewer filters per sample, elute the DNA by adding 200 µL Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at ≥ 6000 x *g* for 1 minute. If there are more than 8 filters per sample, elute with 100 µL Buffer AE so that all of the replicates can be pooled at the end.

(17) Discard the spin column, combine replicates if needed, and store the eluted DNA samples at −20°C. If DNA is to be immediately used for PCR, keep on ice.

5.6 PCR Amplification of eDNA Samples

5.6.1 Purpose

In order to determine if the DNA of a specific species is present in the concentrated water samples taken in the field, the total DNA extracted from the filtered samples must be amplified using species-specific primers.

5.6.2 Source

Filters or centrifuge tubes from eDNA sampling have been received by WGL eDNA Team and DNA has been extracted. DNA elutes from samples should either be located in designated −20°C freezer or carried from the DNA extraction room to PCR room. Be sure 96-well PCR plates to be used have been set out on clean bench paper on the bench-top and the room and plates are irradiated with UV light for 30 minutes prior to use. Ensure thermalcyclers are available before mixing the master mix. Master mix with or without template DNA can not sit longer than it takes to prepare the samples for cycling.

5.6.3 PCR Quality Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to contamination and, subsequently, inaccurate results. Carefully follow quality control and COC steps listed below:

(1) PCR-type hood bench should be wiped before and after use with 10% bleach. Validated commercially available sterilization reagent such as LookOut® DNA Erase® may be preferred. PCR room should be sterilized using a built-in UV lights if available.

(2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach should be rinsed to avoid corrosion.

(3) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for PCR amplification should be cleaned before and after each use.

(4) Use autoclaved, filtered, or commercially sterile water prior to use for setting up PC reactions.

(5) 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.

(6) Use autoclaved sample tubes for PCR master mix.

(7) Only one MCT should be open at a time. Close each tube immediately after labeling and after the addition of sample or reagents to prevent cross-contamination.

(8) Use a tube opener, clean Kimwipe®, or other suitable barrier, rather than gloved fingers, to open MCT.

(9) Record all solution batch numbers used for reactions in lab notes.

(10) PCR reagents should be aliquoted (a portion of the original stock) to avoid excessive freeze-thawing and to protect stock reagents if contamination occurs.

(11) Lightly vortex (quick touch, because vigorous vortexing can damage *Taq*) to mix sample and quick-spin/centrifuge tubes before opening the reagents to avoid splashes or drips from cap when opening. Uncap and close tubes carefully to prevent aerosol contamination.

(12) Any revisions to the DNA amplification protocol must be approved by the e DNA Project Leader and documented in writing.

Cautions: Wear powder-free latex or nitrile gloves throughout the DNA amplification and gel electrophoresis procedures. Ethidium bromide, used in DNA gel electrophoresis to visualize DNA, is a known mutagen that affects biological processes.

5.6.4 Procedure

(1) If DNA samples (extraction elutes) are removed from freezer, note on freezer/ambient log (see Exhibits 7-9). Also note on sample log (Exhibit 6).

(2) Use preprinted 96-well plate map (Exhibits 12-13) or build plate map in LIMS to determine which samples will be pipetted into which wells. Clearly mark plate identification on bottom edge skirt of plate. Write plate identification information (e.g. FY_case#_sample numbers_species_initials_date) in lab notes. Mark the skirts on left side (technician's left) of the plate with L, right side (technician's right) with R, top (T; 90° clockwise from L side) with T, and bottom (B; 270° clockwise from L side) with B using indelible marker.

(3) Official plate identification will be the barcode, but legible labels for use while working must be complete to allow for easy down-stream processing in the sequencing lab.

(4) Make sure sample map for each plate is entered into the LIMS or attached to lab notes and a signature is written across the map and lab book page.

(6) Follow DNA amplification protocol detailed below.

Primers specific to either *Hypophthalmichthys molitrix* (Silver Carp) or *H. nobilis* (Bighead Carp) are used to screen eDNA samples and amplify unique DNA sequences in each species potentially present in the eDNA samples by PCR. The PCR programs used to amplify the extracted DNA are specific to the

primer set used. The PCR protocol has been optimized to utilize Platinum® *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA) in the eDNA screening. If other brands of *Taq* are used, optimization of the recipe and thermal profiles must be executed. Eight reactions are set up for each sample, in addition to negative (DNA blank) and positive controls for each master mix (DNA extracted from tissue using commercial DNA extraction kit (e.g., Qiagen DNeasy Blood & Tissue Kit), and manufacturer protocol. Run test PCR before relying on any DNA extract for eDNA assay positive controls). The PCR reactions are prepared as follows:

(1) Wipe lab bench area with 10% bleach, 75% Ethanol, or commercial DNA sterilization wipes. Also wipe down work area with PCR hood. Use built-in UV lamps to radiate clean room for 30 min prior to PCR set-up.

(2) Electronic pipettors should be wiped down with one of the solutions or wipes listed in Step 1.

- In the clean reagent room, obtain all PCR master mix reagents (using only those that have not expired and that have been tested and found viable).
- 10X PCR buffer (comes in *Taq* kit)
- 10mM equally mixed dNTP solution (2.5 mM per nucleotide)
- 50 mM Mg2+ solution (concentration in Platinum *Taq* kit; use what comes with your brand)
- Species-appropriate forward primer
- Species-appropriate reverse primer
- *Taq* DNA polymerase (we use Platinum, but any hot-start *Taq* can be used)
- Sterile DI water (commercially sterile or Millipore filtered, autoclaved, and UV cross-linked)

Allow reagents to thaw. Do not vortex primers or *Taq* too violently.

(3) Record in lab notebook the lot number of all reagents used.

(4) Prepare PCR master mixes in clean reagent room. 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, make enough master mix for 100 reactions when actually preparing for 96 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 Silver or Bighead Carp master mix.

(a) Each Initial PCR 1X reaction should contain:

- 2.5 µL 10X PCR buffer
- 0.5 µL dNTP (10 mM mixed dNTP)
- 0.75 µL Mg2+ solution (50mM)
- 0.5 µL forward primer (10µM working dilution)
- 0.5 µL reverse primer (10µM working dilution)
- 0.25 µL Platinum® *Taq* polymerase (= 1.25 U)
- 19.0 µL sterile water.

Move prepared mix from reagent room into PCR room.

(5) Remove DNA extracts from freezer or fridge (fill out logs as needed), vortex (quick touch) and quick-spin down the extract tubes. Take them into the PCR room. Place the 96-well PCR plate onto a clean surface, positioned from left to right.

(6) Fill the plate wells with 24 µl PCR mix. Carefully pipette 1 µL of each sample to be screened into each well of a column, changing the pipette tip between each sample. Each column of eight wells should be filled with the same sample (i.e., eight replicates per sample to be tested). The first 11 columns of the PCR plate can test 11 different samples for one target species. Into the 12th column, pipette 1 µL of sterile water into the bottom three wells (F, G, H) and pipette 1µL of each of the target species positive control DNA into each of the top four wells (A, B, C, D). Leave the intervening well (E) empty.

(7) Place the positive control DNA back in to the appropriate −20°C freezer and change gloves immediately in order to reduce risk of contamination.

(8) Place PCR film over the PCR plate and press firmly (or use an automatic plate sealer) to ensure the edges of all wells are sealed. Gently tap a few times on the lab bench to ensure thorough mixing of each reaction. Spin down the plate in the plate spinner to ensure all DNA is down in the master mix.

(9) Place the 96-well PCR plate in the thermal cycler, close and secure lid, and select the appropriate PCR thermal program (thermal cycle programs for Silver Carp and Bighead Carp utilize different annealing temperatures). The thermal programs for the current eDNA markers (Jerde et al. 2011) both consist of:

- Initial denaturation at 94°C for 10 min

Followed by 45 cycles of:
- 94°C for 1 min,
- 50°C for Silver Carp program or 52°C for Bighead Carp for 1 min
- 72°C for 1.5 min.

Followed by:
- final extension at 72°C for 7 min
- 4°C hold temperature until plate removed from thermal cycler.

(10) Record the plate ID, thermal cycler unit or head, plate orientation, and run times for the PCR plate in the PCR log (Exhibit 10).

(11) Place cycled PCR plates and product in designated −20°C freezer for long-term (more than overnight) storage, in the designated 4°C refrigerator for short- or mid-term storage (1–12 hours). If you leave for the night, the thermal cyclers are set to hold at 4^{0}C forever. Remove plates promptly in the morning.

(12) Under no circumstances should you open or uncover PCR plates that have been cycled in the PCR room. Only open cycled plates in the post-PCR rooms.

5.7 Gel Electrophoresis of eDNA PCR Assays

5.7.1 Purpose

Once amplified, the DNA samples should then be subjected to gel electrophoresis in order to visualize the amplified DNA. This method is useful in determining the presence of DNA from the target species in different aquatic environments.

5.7.2 Source

PCR product following amplification can be taken either from cold storage (see #11 above) or directly from the thermal cycler.

5.7.3 Gel Electrophoresis Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to pipetting error. It is also highly susceptible to mislabeling and, consequently, confounding of sample results.

(1) Draw or otherwise produce a map of which sample will be electrophoresed on which gel and in which lane of the select gel.

(2) Carefully pipette samples so as to avoid:
- Injecting samples to incorrect wells.
- Piercing the bottom of sample wells and losing PCR product.
- Spill over from adjacent wells.

(3) Record all solution batch numbers or name/date identification for stock solutions. Record precast gel batch identification number if appropriate.

(4) Centrifuge plates, strip tubes, etc. before removing film or caps in order to prevent aerosol cross-contamination.

(5) Any revisions to the DNA amplification protocol must be approved by the e DNA Project Leader (and documented) and incorporated into a revised QAPP.

5.7.4 Option A Procedure

(1) Prepare 2% agarose gels with SB (sodium hydroxide and boric acid) buffer and allow the gel to polymerize for a minimum of 25–30 min prior to loading samples. Gels can be prepared at any time prior to PCR or immediately after PCR. However, once you begin loading a gel finish loading and run the gel immediately.

(2) To prepare PCR samples for gel electrophoresis, transfer 10µl from each well of the 96-well plate to new wells in an identically labeled 96-well plate. Add 2µl of loading dye (see recipe below) to each well with PCR product (loading dye = 500µl 6X loading dye, 500 µl DMSO, 1µl SYBR Green I)

(3) Place the 2% agarose gel in the electrophoresis chamber that contains SB Buffer and remove the gel combs. In the first well of each row on the agarose gel, load 100bp DNA ladder/loading dye mix. Next, load 10µL of each sample mixture (i.e., each PCR reaction and loading dye), and negative controls into the remaining wells. Be sure 10 µl of a positive control PCR product is in the last lane of each row. Run electrophoresis at ~250V for ~45 min depending on migration times through the

gel. Times and voltages required to run each gel are approximate (different buffers and higher content agarose gels will require different run times and voltages, use what works in your lab).

(4) Be sure to annotate the gel loading map in the notebook as you observe the gel in the geldoc. Interpret positive samples and note successful positive and negative controls on each gel. After documentation (5.8), dispose of gel in the garbage.

5.7.5 Option B Procedure

Pre-cast 2% E-Gel® 48 gels (Invitrogen) are used with the E-Base™ system (Invitrogen).

(1) Open E-gels and load into the bases.
- Remove gel from the pouch. Remove comb from the gel.
- Slide gel into the two electrode connections on the Mother E-Base™ or Daughter E-Base™. If gel is properly inserted, a fan in the base begins to run, a red light illuminates, and digital display shows 20 min.
- Load each gel within 30 min of removing gel from the pouch and run within 15 min of loading.

(2) Selecting Program on E-Base™
- Connect the Daughter E-Base™ to a Mother E-Base™ or another Daughter E-Base™ if running multiple gels.
- Select program EG by pressing and releasing the pwr/prg (power/program) button on the base.

(3) Loading E-Gel® 48 Gels
- Load 15µL PCR product into each well of an E-Gel® 48 gel. Keep all sample volumes uniform. Load with a multichannel pipettor.
- Load appropriate DNA ladder (distinct bands between 0-500 bp) into far left well (labeled M) and positive control PCR product into the far right marker well. Ensure the marker salt concentration is similar to that of adjacent samples (2% gel uses 100 bp DNA Ladder).
- Load 15µL of sample buffer containing the same salt concentration as the sample into any empty wells.

(4) Run Conditions
- To begin electrophoresis, press and release the pwr/prg button on the Mother E-Base™ and Daughter E-Base™. The red light changes to green.
- At the end of the run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button on the base to stop the beeping and flashing red light.
- Remove gel cassette from the base and analyze results.

Note: if a gel is removed before a run is complete, a gel must be replaced and the unit allowed to run out until the timer counts down to zero. There is no option to reset the base.

5.8 eDNA Gel Documentation and Storage

5.8.1 Purpose

Once eDNA gels have been visualized, the results must be documented, interpreted (i.e., scored), and recorded. In some cases, very light bands may be visible, making scoring difficult. Documentation, labeling and saving for sequence confirmation, and storage are critical for later quality control review.

5.8.2 Source

Following electrophoresis, agarose gels should be immediately documented. Following documentation, PCR products requiring sequencing must be labeled and organized for sequencing.

5.8.3 Gel Documentation and Storage Assurance and Chain-of-custody

Because of the difficult nature of scoring some results, careful records must be kept of all gels and results. These results must be maintained so as to minimize the risk of tampering or data loss.

(1) Gel image quality must be assessed at the time images are obtained. Images should exhibit all bands on gels as clearly as possible, if this is not possible, it must be noted on the gel data sheet (Exhibit 14). All gel digital image files should be saved and should be archived at the end of each working day. All gel image data are referenced to the samples case number to make sure the consistency of the sample custody.

(2) Gel score data should be entered and stored in the appropriate database in an Excel file and on the data sheet in the laboratory notebook, or in LIMS.

(3) All reports should reviewed by the eDNA Processing Leader before being reported.

(4) A paper copy of the report should be held in the files for 5 years.

(5) Electronic copies of all reports should be held for 5 years or longer, as space permits.

(6) Any substantive revisions to the DNA amplification protocol must be approved by the eDNA Processing Leader and approved by the eDNA Coordinator. Any such changes must be incorporated into a revised QAPP.

5.8.4 Procedure

(1) After electrophoresis is complete, remove casting tray with gel from the electrophoresis chamber and place the gel onto the gel scanner (BioRad Molecular imager FX), select DNA ethidium bromide stain gel, set up scanning area, and then select 100 micrometer to start scanning the gel.

(2) Alternatively, place the gel on a UV transilluminator equipped with a digital camera, such as the Alpha red Imager (Cell Biosciences, Inc.), and capture a digital photograph of the gel.

(3) After the gel scanning is done, properly label file name and save the file on the hard drive immediately.

(4) If not using LIMS, print out a picture of the gel image and insert into lab book. A copy should be kept with the Project Lab Book. Be sure to sign across the print out and the lab book page.

5.9 Gel Interpretation

5.9.1 Purpose

Once a gel is visualized, the quality of the results and presence of potential positive bands must be assessed in order to determine which samples need to be further assayed.

5.9.2 Source

Immediately following the cessation of electrophoresis, the agarose gel containing the eDNA PCR products should have been visualized on either the UV-based imager or the laser-based imager. In both cases, the gel image should be captured (saved to hard disk) immediately and then printed.

5.9.3 Gel Interpretation Quality Assurance and Chain-of-custody

(1) The positive controls should have bright bands at the appropriate migration distance (number of base pairs), indicating a positive reaction.

(2) No bands at the targeted sizes (~200bp silver, ~300bp bighead) should be observed in the negative controls.

(3) If any of the initial PCR reactions are positive (i.e., a visible band at the appropriate migration distance), the initial sample is designated a "presumptive positive".

(4) Record the number of presumptive positive reactions for each sample both in the gel electrophoresis lab notebook and in the excel file on the lab computer.

(5) Presumptive positive results will initiate a series of results confirmation mechanisms (see below). These mechanisms include screening the transport and equipment controls, and DNA sequencing.

(6) Once presumptive positives are documented:
   a. Fill in the quality control results in the eDNA sample log (Exhibit 6).
   b. Notifiy the eDNA Processing leader.
   c. Color in the plate seal above the wells containing presumptive positive samples on the PCR product plate. Move all plates requiring sequence confirmation into the freezer in the sequencing room. Plates without positives can be discarded.

5.10 Confirmation of Positive Results

5.10.1 Purpose

These confirmation mechanisms are initiated if a sample returns as a positive for the PCR test (any number of the eight reactions, e.g., one of eight up to eight of the eight PCR reactions).

5.10.2 Source

Positive results for Asian carp eDNA require that those positive samples be further assayed. The original DNA elutes from samples should be located in designated −20°C freezer.

5.10.3 Gel Interpretation Assurance and Chain-of-custody

Presumptive positive assays (PCR reactions) are validated through DNA sequencing and testing of additional control samples.

Any revisions to the DNA QA/QC amplification protocol must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.10.4 Procedure

(1) Conduct PCR assays of the paired equipment control for each presumptive positive. DNA extraction, amplification, documentation, and interpretation following protocols detailed above (Sections 5.6–5.9).

(2) Ensure that the transport blanks (see 2.2.2 (6) and 2.3.2 (10)) have been tested for that sample group (i.e., from the same cooler in which the presumptive positive sample was transported).

(3) For all presumptive positive samples, bidirectional sequencing confirmation is performed. This is done by using a commercially available gel extraction kit (e.g., Qiagen Qiaquick Gel Extraction kit) per the manufacturer's recommendations on the positive PCR reactions, or E-Gel® CloneWell Agarose Gels (Invitrogen) per manufacturer's recommendation.

(4) If the equipment control and transport blanks test negative, the sample is designated a "**confirmed positive**."

(5) The following sequencing reaction can be done either by cloning (TA TOPO cloning kit used for sequencing per manufacturer's recommendation) then sequencing, or by a direct Sanger sequencing method (ABI BigDye® Terminator v3.1 or v1.1 Cycle Sequencing Kit) modified by WGL. BigDye Terminator Reaction Master Mix for 1X reaction:

- 1 µL BigDye terminator mix,
- 4 µL 5X reaction buffer,
- 0.8 µL either SC/BH forward primer, and
- 10.2 µL of water.

Add 16 µL of master mix to the 4 µL of purified DNA. Total reaction size is 20 µL.
The positive control reaction of sequencing was done per manufacturer's recommendation.

For each pGEM control PCR master mix:

- 1 µL BigDye terminator mix,
- 4 µL 5X reaction buffer,
- 2 µL m13 primer,
- 8 µL water, and
- 2 µL pGEM.

Add 20 µL to each control well.

Place the PCR plate on the thermal cycler and begin temperature cycling protocol. Program the thermal cycler as follows: 25 cycles of [96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min], then ramp to 4°C.

(6) Option 1: To clean the BigDye reaction with EDTA precipitation:

- Spin plate after removing from thermal cycler (just to make sure that everything is at the bottom of the well).
- Add 5 µL of 125mM EDTA to each well.
- Add 60 µL of 100% EtOH.
- Seal the plate and mix by inverting four times.
- Incubate at room temperature (RT) for 15 min.
- Spin plate at 3000xg for 30 min (at 4°C) or at 2000 x g for 45 min. Proceed to next step **immediately**.
- Invert the plate and spin at 185 x g for 1 min (time from when rotor starts).

- Add 60 µL of 60% EtOH.
- Spin at 1650 x g for 15 min at 4°C.
- Invert plate and spin at 185 x g for 1 min.
- Resuspend samples in 20 µL Hi-Dye.

Option 2: To clean the BigDye reaction with Sephadex in Centri-SEP spin columns or Millipore plates, use columns for 1-16 samples and plates for 17 or more.

Centri-SEP column procedure

Materials list:

Spin columns (preloaded with dry sephadex, caps, and bottom caps), one per sample
Pipets and tips
Centrifuge with MCT rotor
Reagent grade water
Labeled 1.5-mL MCT and tube rack
Vortexer

(1) Column Hydration

a. Tap column on counter to settle sephadex gel into the bottom.
b. Remove cap and add 800 µl sterile ddH_2O. Replace cap, invert, and mix well on vortexer, ensuring all gel is hydrated. Invert again, vortex and place in rack.
c. Let sit 30 minutes at room temp in a tube rack.
d. While columns sit, label 1.5ml MCT with sample names for collecting cleaned product.

(2) Remove excess water

a. Check each tube for bubbles, if there are bubbles in the gel, invert the tubes and vortex so all the slurry moves toward the cap.
b. Invert and gently place in tube rack, allowing gel to settle.
c. Remove caps, then bottom, and place column in collection tube to drain.
d. Allow to drain until about 200-250 µl water has drained. If needed, gently apply pressure with a gloved finger to get the water draining.
e. Discard fluid, replace column in collection tube.
f. Place columns into centrifuge, making sure the indicator tabs are up.
g. Spin at 750xg(rcf) for 2 min
h. Discard the collection tube, blot any water off the bottom of columns and place column into labeled sample collection tube.

(3) Sample processing

a. Hold the column up to eye level, and carefully place all of the cycle sequencing product onto the center of the packed column, being careful to not disturb the column. Don't touch the side of the column with the product or the pipet tip.
b. Place collection tube into centrifuge with caps open and facing inward and the indicator tab of the column facing up.
c. Spin at 750xg(rcf) for 2 min. Discard spin column.
d. If you have more than 32 samples, you must dry filtrates in the spin vac at ~60* for ~45minutes. Once dry, you may freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.

Millipore plate sephadex procedure
Materials list:

- Millipore filter plate with centrifuge alignment frame
- Plain 96-well plate to catch waste water (can reuse the same one)
- Sequencing plate (with bar code)
- Sephadex G50-fine
- Millipore column loader and tray
- Centrifuge with plate rotor
- Reagent grade water

(1) Prepare sephadex plate:

a. Pour dry sephadex into the black metal loading plate which is in the plastic catchment tray.
b. Use the clear squeegee to level each well you need for cleanup (tape off unused wells).
c. Scrape excess powder into the catchment tray so you don't waste too much sephadex.
d. Invert a multiscreen plate over the metal tray and hold them together while you invert the tray to fill the wells with sephadex. Make sure you are filling the wells you wish to use! Tap gently to make sure all of the sephadex moves into the filter plate.
e. Pour excess sephadex from the tray back into the sephadex container and cap tightly.
f. Add 300 µl of water to each well of the plate and let sit *3 hours at room temp*. (these plates can be prepared ahead of time, wrapped tightly in plastic wrap, placed in a Tupperware with a moist towel and refrigerated for two weeks.)

(2) Remove excess water and pack columns prior to use:

a. Place the filter plate over a plain 96-well plastic plate, and load into the plate rotor. Don't use the interior lid because it does not fit over stacked plates.
b. Centrifuge at 910xg(rcf) for 5 min.
c. Dump the waste water into the sink and rinse catchment plate (set aside for another use).

(3) Clean sequencing reactions with the columns.

a. Use the multichannel pipet to carefully add reactions to the *center* of each column.
b. Place the filter plate over a 96-well sequencer plate making sure wells A1 are aligned.
c. Centrifuge at 910xg(rcf) for 5 minutes.
d. Dry filtrates in the spin vac at ~60^0C for ~20 minutes. You may seal plate and freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.
e. Once samples are dry, add 15 µl HiDi to each sample. Samples in Hi-Dye can be stored at 4°C overnight, but may not be left any longer than 12 hours.

(4) To sequence:

- Denature samples with HiDi for 5 min at 95°C in thermocycler (do not denature samples run wet)
- Place immediately on ice
- Load into sequencer plate and onto sequencer.

Resulting sequencing reactions that are successful are screened in GenBank (http://www.ncbi.nlm.nih.gov/blast) using the BLAST (Basic Local Alignment Search Tool) algorithm. If the resulting sequence is a positive match to the targeted species of Asian carp, the sample is designated a "**confirmed positive – sequenced**".

References: User Manual, "BigDye® Terminator v3.1 Cycle Sequencing Kit"
http://www.ibt.lt/sc/files/BDTv3.1_Protocol_04337035.pdf
"TOPO TA Cloning® Kit for Sequencing" http://tools.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf
"QIAquick Gel Purification Kit"
http://molecool.wustl.edu/krolllab/Kroll_Lab_Protocols/Molecular%20Biology%20protocols/Cloning%20protocols%20folder/Gel%20extraction-Qiagen.pdf

5.11 Communication of eDNA Assay Results from WGL to Region 3 Leadership

5.11.1 Purpose

To convey to USFWS designated personnel the progress and results of eDNA assays.

5.11.2 Source

WGL keeps a record of each samples progress through the eDNA assay procedure. These records are summarized for each batch for reporting to the eDNA Coordinator in Region 3 of USFWS.

Each field sampling agency keeps a record of field data. These records are summarized and reported to the eDNA Coordinator in Region 3 of USFWS.

The eDNA Coordinator in Region 3, USFWS collates field and lab data for reporting results to partners and the public.

5.11.3 Quality Control

The WGL eDNA Processing Leader will provide updates and reports to the Region 3 eDNA Coordinator. At this time, Emy Monroe is the WGL eDNA Processing Leader and Kelly Baerwaldt is the Region 3 eDNA Coordinator. Any permanent or temporary changes to either position should be communicated immediately to the assigned USFWS senior executive. Any permanent changes should be incorporated into a revised QAPP.

Any revisions to the reporting procedures must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.11.4 General Procedure (for details see Appendix E for SOP)

The USFWS eDNA Processing Leader should, on every Friday during the period over which the WGL eDNA Team is processing samples, provide updates on all sample batches to the USFWS eDNA Coordinator no later than 1200 CST/CDT. Reports should be organized by batch and should consist of spreadsheets showing the stage of processing for each sample. Additionally, following approval by the eDNA Processing Leader, the WGL PRR should convey final results for each batch (all samples confirmed as positive or negative for AC eDNA) within 18 hours of completion of processing for the last sample within a batch.

The USFWS eDNA Coordinator may request updates from the eDNA processing leader at any point. The WGL eDNA Processing Leader is expected to respond as soon as possible during normal working hours.

The following is a list of critical components for eDNA Processing, and component information for ERDC. No vendor is listed for generally available supplies:

- Enough sets of manual pipettes ranging from 0.1µl to 1000µl so that each designated area has its own set
- Electronic 8 or 12 channel multi-dispense pipettes ranging from 0.5µl to 125µl
- ERDC: Finpipettes models # (0.1-25 µl)
- Programmable thermal cyclers equipped with four 96-well plate heads. Thermal cycler should be capable of self-test upon instrument startup. All thermal cyclers and heads should be equipped with the heated lid function.
- Centrifuge equipped with programmable speeds and time.
- Interchangeable Rotors capable of holding 96-well plates and tubes of various sizes (5mL, 1.5mL, and 2mL).
- Electrophoresis Chambers including gel casts and gel combs
- Sterile Hoods equipped with UV light and Hepa filters
- Autoclave
- Locking Refrigerator monitored at 4˚C equipped with a temperature sensitive alarm
- Locking Freezer monitored at −20˚C equipped with a temperature sensitive alarm
- Locking Freezer monitored at −80˚C equipped with a temperature sensitive alarm
- Sequencer
- Gel Image Scanner
- UV Stratalinker
- LookOut DNA Erase (Sigma Aldrich Cat# L8917 refill cat # L9042)
- Power Water DNA Isolation Kit (Mo Bio Laboratories Cat # 14900-100-NF)
- QIAquick Gel Extraction Kit (Qiagen Cat# 28706)
- Taq DNA Polymerase (Platinum taq Life Technologies Cat# 10966083)
- Big Dye Terminator Sequencing Kit v3.1 (Applied Biosystem Cat # 4337455)
- Nuclease Free Water (Ambion Cat # AM9932) (Autoclaved before use.)
- pGEM-3Z(+) Vector 20 g (Promega Cat # P2271)
- Ethanol (Sigma Aldrich Cat # E7023)
- dNTP
- MgCl2
- 10X buffer
- Primers (forward and reverse)
- 20X SB buffer
- Cloning Reagents
- Sequencing Reagents
- Agarose
- Bleach Solution (10%)
- Ethidium Bromide Gel Stain
- 100 bp DNA ladder
- Gel Loading Dye
- Sterile Pipette Tips: various sizes and types to fit manual and electronic pipettes.
- Sterile Nuclease Free conical tubes: 1.5 mL, 2.0 mL, 5.0 mL
- Sterile 96-well PCR Plates – UV cross-linked prior to use.
- Gloves: Nitrile or Latex, various sizes

- Forceps: cleaned by soaking a minimum of 10 min in a 10% bleach solution or by LookOut DNA Erase following manufacturer protocol.
- Sterile seals for 96 well plates.
- Sterile Optical Sealing Tape for 96 well plates.
- Kimwipes®
- Paper Towels
- Countertop moisture barrier papers
- Black Permanent Markers

SECTION 6. INTERNAL QUALITY CONTROL CHECKS

Details on quality control are found within each of the various protocol sections. In summary, however, quality control relative to sample contamination is covered by the transport (or cooler), equipment, DNA extraction, PCR, and sequencing blanks. Quality control for efficacy of methodology, solutions, etc. is covered by positive DNA controls for each sample handling step (sampling, filtering, extraction, PCR, and sequencing) of the eDNA protocol. Furthermore, each new solution or kit to be used in eDNA processing will be tested with positive and negative controls before use.

The WGL eDNA processing facility and protocols was reviewed by an ERDC audit shortly after completion of the transition plan and full deployment of Asian carp eDNA dedicated equipment.

6.1 Laboratory Quality Control Evaluation Criteria

Quality control is measured in two ways:

- If transport, filtering, centrifuge, hood, extraction, PCR, and DNA sequencing negative controls show product (e.g., bands in PCR or DNA sequence), the associated data are negated and, when possible, samples are reprocessed. Contamination of DNA extract will require that samples be removed from consideration.
- Positive controls are currently employed for extraction, PCR, and sequencing. If the positive controls fail to behave as expected, any sample showing an apparent lack of results will be rerun at the same time or following rerunning of the positive controls. This will be done until all positive controls produce the expected results.
- We incorporate two types of positive controls during sequencing. One positive control PCR products from positive control reactions and one sequencing reaction per plate with a standard DNA sequencing template (pGem) provided by the manufacturer. If less than a full plate are sequenced, one pGEM per set of 16 eDNA samples that are sequenced. In the case that any of these fail, any samples that fail to produce sequence data that were run at the same time will be rerun at the same time as positive controls are rerun.
- In cases where fewer than 16 eDNA samples are sequenced, both types of positive sequence controls are still run.

SECTION 7. SPECIFIC ROUTINE PROCEDURES TO ASSESS DATA PRECISION, ACCURACY, AND COMPLETENESS

7.1 Field Methods and Laboratory Data

Every 3 months, a dilution series of Asian carp water will be processed starting with the filtering and centrifuging, and then extraction through sequencing to ensure that current practice, instruments, and personnel are maintaining the same level of sensitivity and accuracy. A brief report will be provided by the DNA Processing Lead to the eDNA Coordinator. Asian carp water will be either collected from the Asian carp holding tanks at the USGS Upper Midwest Environmental Sciences Center (UMESC), or made by collecting slime and feces from Asian carp at UMESC, or made with Asian Carp cell lines from the USFWS Fish Health Center Virology lab.

SECTION 8. CORRECTIVE ACTIONS

Corrective actions may be required for two classes of problems: analytical/equipment problems and noncompliance problems. Analytical and equipment-related problems may develop during sampling and sample handling, sample preparation, laboratory instrumental analysis, and data review. Noncompliance issues arise when eDNA sampling, filtering, or processing execution deviates from procedures described in the QAPP.

In the case of analytical/equipment problems or deviations from set procedures (as outlined in QAPP), the responsible lead will determine if the problem or deviation will impact the accuracy of the resulting data. If it is determined that the problem or deviation does impact data accuracy, two courses of action may be followed:

(1) If possible, the procedure is repeated until it is performed without any problem or deviation, or

(2) The sample or samples are removed and not processed any further.

In either case, a corrective action report must be completed. Careful notes of any corrective actions and what incident led to them, as well as the resolution or preventative measure(s) identified will be carefully noted in the corrective action report, which must be provided electronically to all Leaders (Project Leader, Sampling Leader, etc) as an after action report. The paper copy of the corrective action report will be maintained in the project file as a long-term record.

In the case that the responsible lead determines that data accuracy is not affected by the analytical/equipment problem or deviation from procedure, the sample or samples may continue to be processed. The responsible lead will make careful note of the incident in project records and include the rationale for continuing processing.

SECTION 9. PREVENTATIVE MAINTENANCE PROCEDURES

9.1 Field Equipment/Instruments

Hand-held sonar: Batteries will be changed at least once a month (if not required sooner) to ensure accurate readings of the instrument. In addition, reading accuracy should be checked once a month. Depth readings may be checked by filling a container of a known depth with water and submerging the instrumentation. Temperature readings of the sonar may be checked against a thermometer.

GPS equipment: Batteries will be changed at least once a month (if not required sooner) to ensure accurate readings of the instrument. In addition, coordinate accuracy will be checked against known benchmarks.

Plastic 2L sample bottles: After bleaching and autoclaving, bottles will be inspected for dents and/or warping of the material. Any bottle failing inspection will be disposed of and replaced.

Forceps: Forceps will be inspected monthly, and those exhibiting large amounts of rust will be disposed of and replaced.

Carboys: Carboys will be inspected monthly for cracks in the glass that could pose a safety hazard to filtering personnel. Any carboy failing inspection will be disposed of and replaced.

Plastic tubing: Plastic tubing used to connect the carboy to the manifold will be inspected monthly for cracks in the plastic. Any plastic tubing failing inspection will be disposed of and replaced.

All other laboratory equipment will be inspected monthly and undergo proper maintenance to maintain their ideal working condition. Any equipment not performing accurately or to established standards will be disposed of and replaced.

9.2 Laboratory Instruments

Pipettes: Annually all pipettes will be inspected, calibrated, and certified. Any pipette failing inspection and certification will be disposed and replaced.

Any thermal-cycler head that fails the manufacturers self-test upon instrument startup will be removed and replaced with the manufacturer's certified replacement part.

Equipment maintenance contracts, with annual maintenance check-ups, will be used for any appropriate equipment (i.e., DNA sequencer).

SECTION 10. PERFORMANCE AND SYSTEM AUDITS

10.1 Field Audits

Internal audits of field crew performance and quality controls for sampling and filtering will be made semi-annually by the FWCO field staff to make sure that all procedures in the sample collection portions of the QAPP are being followed. On sampling trips where more than two FWCO offices are on the team, the visiting FWCO eDNA leader will serve as the sampling auditor. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures (Exhibit 16).

10.2 Laboratory Audits

Internal audits of WGL laboratory performance and quality controls will be made semi-annually by the DNA Processing Lead to make sure that all procedures in the DNA processing portions of the QAPP are being followed. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures. Every 2 years an external review of WGL eDNA processing will be undertaken. The review panel or consultant(s) will be selected by the Project Lead. The DNA Processing Lead may assist the Program Coordinator in identifying one or more potential reviewers

EXHIBITS

Exhibit 1. Field Collection Summary

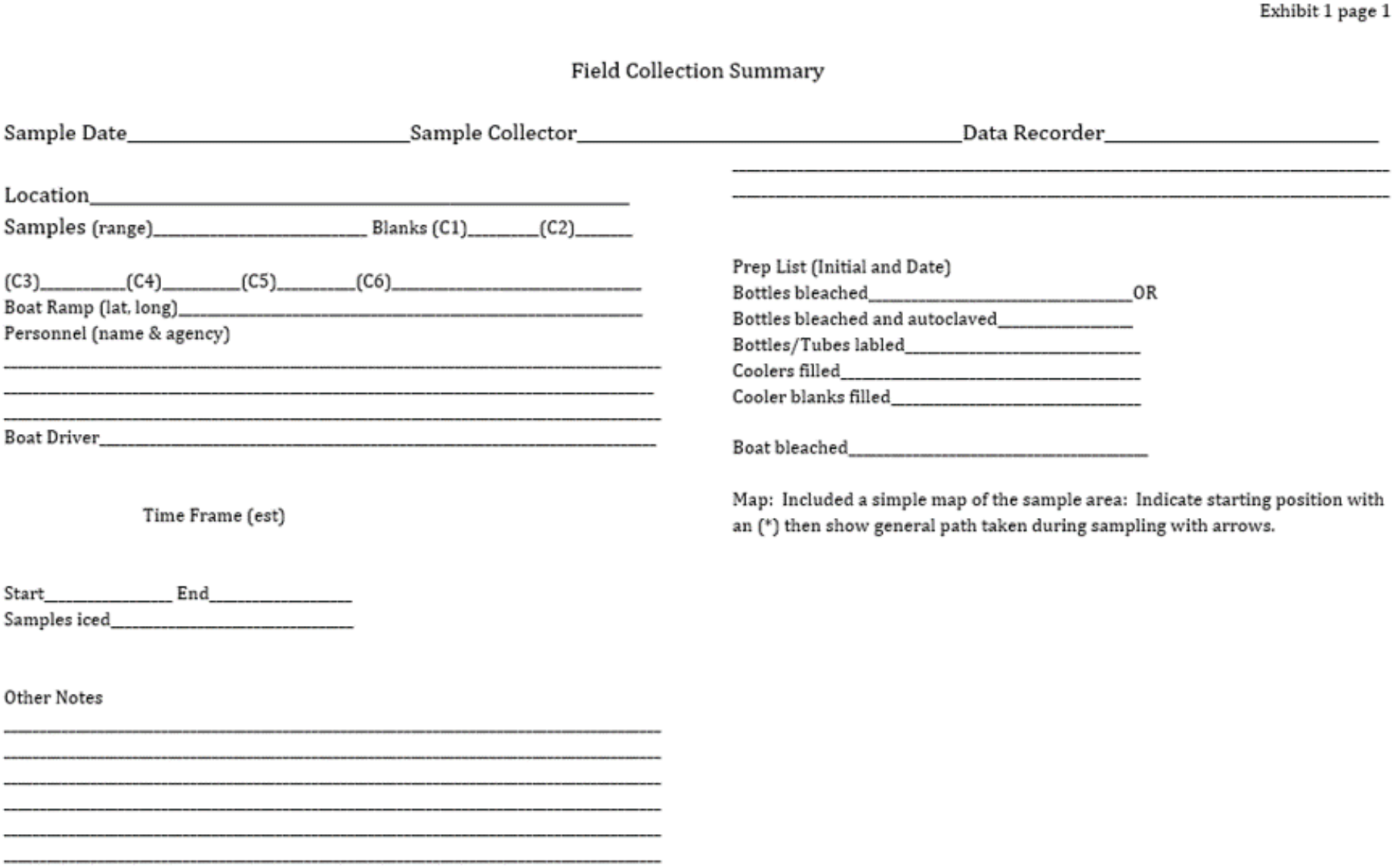


Exhibit 1 page 1

Field Collection Summary

Sample Date_____Sample Collector_____Data Recorder_____

Location_____

Samples (range)_____ Blanks (C1)_____(C2)_____

(C3)_____(C4)_____(C5)_____(C6)_____

Boat Ramp (lat, long)_____

Personnel (name & agency)

_____

_____

_____

Boat Driver_____

Time Frame (est)

Start_____ End_____

Samples iced_____

Other Notes

_____

_____

_____

_____

_____

_____

_____

_____

Prep List (Initial and Date)

Bottles bleached_____OR

Bottles bleached and autoclaved_____

Bottles/Tubes labled_____

Coolers filled_____

Cooler blanks filled_____

Boat bleached_____

Map: Included a simple map of the sample area: Indicate starting position with an (*) then show general path taken during sampling with arrows.

Figure 1. Exhibit 1 page 1 of 2 of a template for the Field Collection Summary. Its main purpose is to collect metadata and other important information for sampling event.

Field Collection Supply List

Coolers
___Labeled bottles

Clipboard
___pencils/pens
___Data sheets
___GPS
___Extra batteries for GPS
___Maps

Drybag
___Gloves
___Depth/temp device
___Wet Wipes
___Sunblock

Drinking water

Others
___bleach
___mop
___bucket
___camera
___Ipass
___Life jackets
___Filed float plan
___sunglasses

Figure 2. Exhibit 1 page 2 of 2 of a template for the Field Collection Supply List.

Exhibit 2. Field Collection Data Sheet

DATE_____ NAME_____ START TIME_____ SHEET ___of___ Temp at shipping:_____

ID	Volume (2L or 5x50ml)	Latitude (dec.degrees)	Longitude (dec.degrees)	Temp (F/C)	Depth (ft/m)	Double (D) Blank (B)	Habitat (LDB/MC/RDB)	Collect time	Filter/fuge time

Figure 3. A template for the Field Collect Data Sheet. Each sample should have all the corresponding metadata fields filled out at the time and location in which the sample is taken.

Exhibit 3. Chain of Custody Form

US Fish and Wildlife Service	CHAIN-OF-CUSTODY RECORD		File no. Inv.
Date and time of collection:	Water body:	Collected by (first and last names):	
Case Number:			

Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	
Collection No.	From: (Print name, Agency)	Release signature:	Release Date:	Delivered via: ☐ FedEx ☐ In Person ☐ Other:
	To: (Print name, Agency)	Receipt signature:	Receipt Date:	

Figure 4. A template of the Chain of Custody Form. This form should be updated throughout the progression of the case and completed for each case of samples. The main purpose of this form is to document the exchange of samples via shipping or drop off.

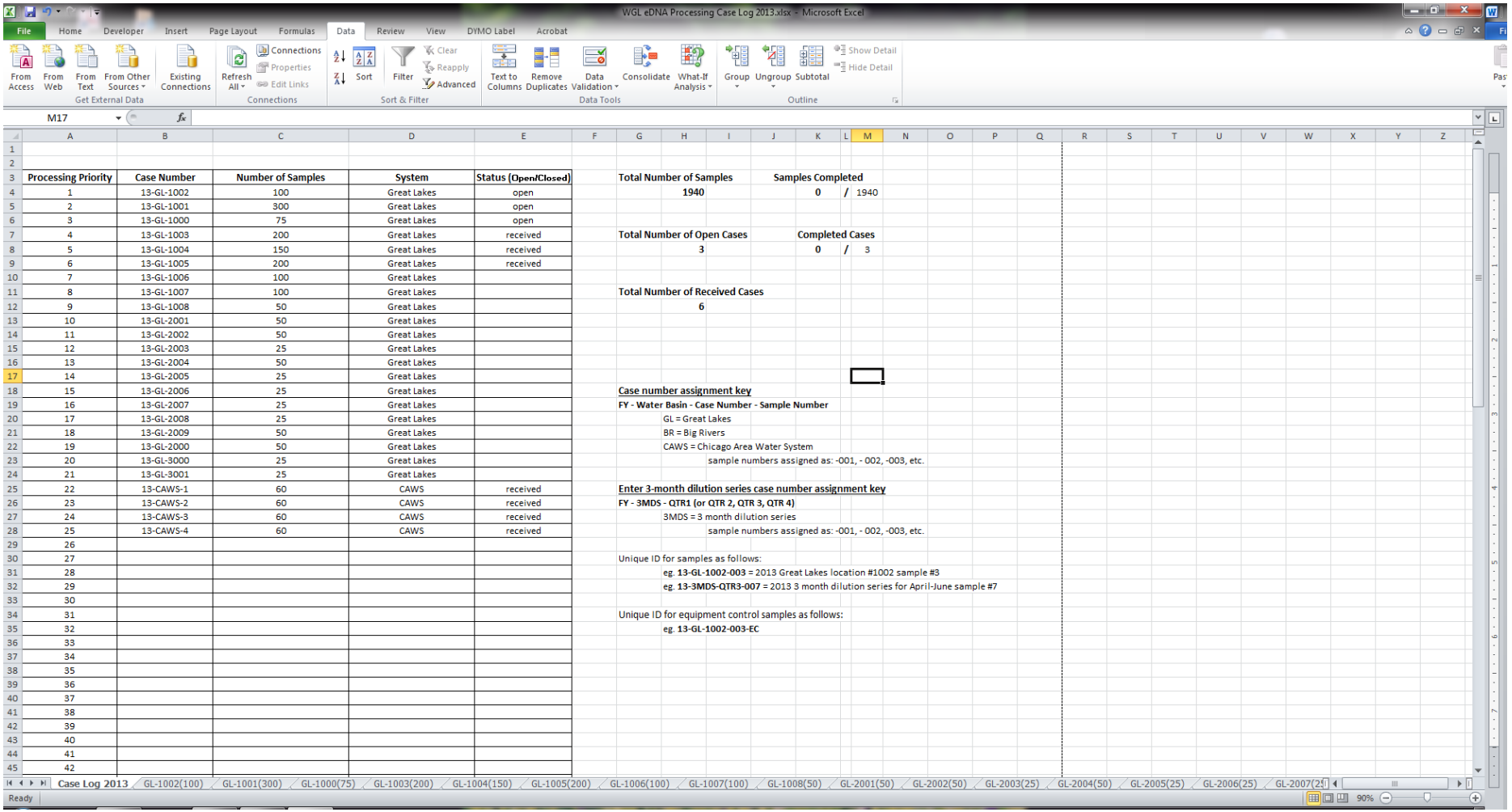
Exhibit 4. USFWS WGL Sample Receipt Checklist

Project/case:	Receipt Date:
Other:	Rec'd by:

Were samples shipped? Yes, FEDEX/UPS/Other No, Courier pickup/hand delivered	Comments:
Cooler temp upon arrival _____ ^{0}C/NA	
Chain-of-custody (COC) present? Yes/no Complete? Yes/no	
Custody seals present on cooler? Yes/no Samples? Yes/no	
Were sample containers intact? Yes/no	
Samples and COC match? Yes/no	
If any problems, was project manager notified? Yes/no By whom?_____	
Appropriate sample containers? Yes/no	
Date/time of collection on COC Yes/no	
Location and ID of sample storage: Freezer_____ Refrigerator_____	
Temperature log updated for storage? Yes/no	

Figure 5. A template of the USFWS WGL Sample Receipt Checklist. This document serves as a record of the condition of samples when they are received into the lab and as a record of receipt into WGL custody.

Exhibit 5. Screen Capture of Excel Log File, First Worksheet



WGL eDNA Processing Case Log 2013.xlsx - Microsoft Excel

Processing Priority	Case Number	Number of Samples	System	Status (Open/Closed)
1	13-GL-1002	100	Great Lakes	open
2	13-GL-1001	300	Great Lakes	open
3	13-GL-1000	75	Great Lakes	open
4	13-GL-1003	200	Great Lakes	received
5	13-GL-1004	150	Great Lakes	received
6	13-GL-1005	200	Great Lakes	received
7	13-GL-1006	100	Great Lakes	
8	13-GL-1007	100	Great Lakes	
9	13-GL-1008	50	Great Lakes	
10	13-GL-2001	50	Great Lakes	
11	13-GL-2002	50	Great Lakes	
12	13-GL-2003	25	Great Lakes	
13	13-GL-2004	50	Great Lakes	
14	13-GL-2005	25	Great Lakes	
15	13-GL-2006	25	Great Lakes	
16	13-GL-2007	25	Great Lakes	
17	13-GL-2008	25	Great Lakes	
18	13-GL-2009	50	Great Lakes	
19	13-GL-2000	50	Great Lakes	
20	13-GL-3000	25	Great Lakes	
21	13-GL-3001	25	Great Lakes	
22	13-CAWS-1	60	CAWS	received
23	13-CAWS-2	60	CAWS	received
24	13-CAWS-3	60	CAWS	received
25	13-CAWS-4	60	CAWS	received

Total Number of Samples: 1940 — Samples Completed: 0 / 1940

Total Number of Open Cases: 3 — Completed Cases: 0 / 3

Total Number of Received Cases: 6

Case number assignment key

FY - Water Basin - Case Number - Sample Number

GL = Great Lakes

BR = Big Rivers

CAWS = Chicago Area Water System

sample numbers assigned as: -001, - 002, -003, etc.

Enter 3-month dilution series case number assignment key

FY - 3MDS - QTR1 (or QTR 2, QTR 3, QTR 4)

3MDS = 3 month dilution series

sample numbers assigned as: -001, - 002, -003, etc.

Unique ID for samples as follows:

eg. 13-GL-1002-003 = 2013 Great Lakes location #1002 sample #3

eg. 13-3MDS-QTR3-007 = 2013 3 month dilution series for April-June sample #7

Unique ID for equipment control samples as follows:

eg. 13-GL-1002-003-EC

Figure 6. Screen capture of Excel log file. First worksheet in workbook outlines log for all sampling events or cases which correspond to samples from a particular system. Subsequent worksheets are created for each case and allow for sample tracking through the eDNA lab.

Exhibit 6. Screen Capture of Excel Log File, Sample Log Open

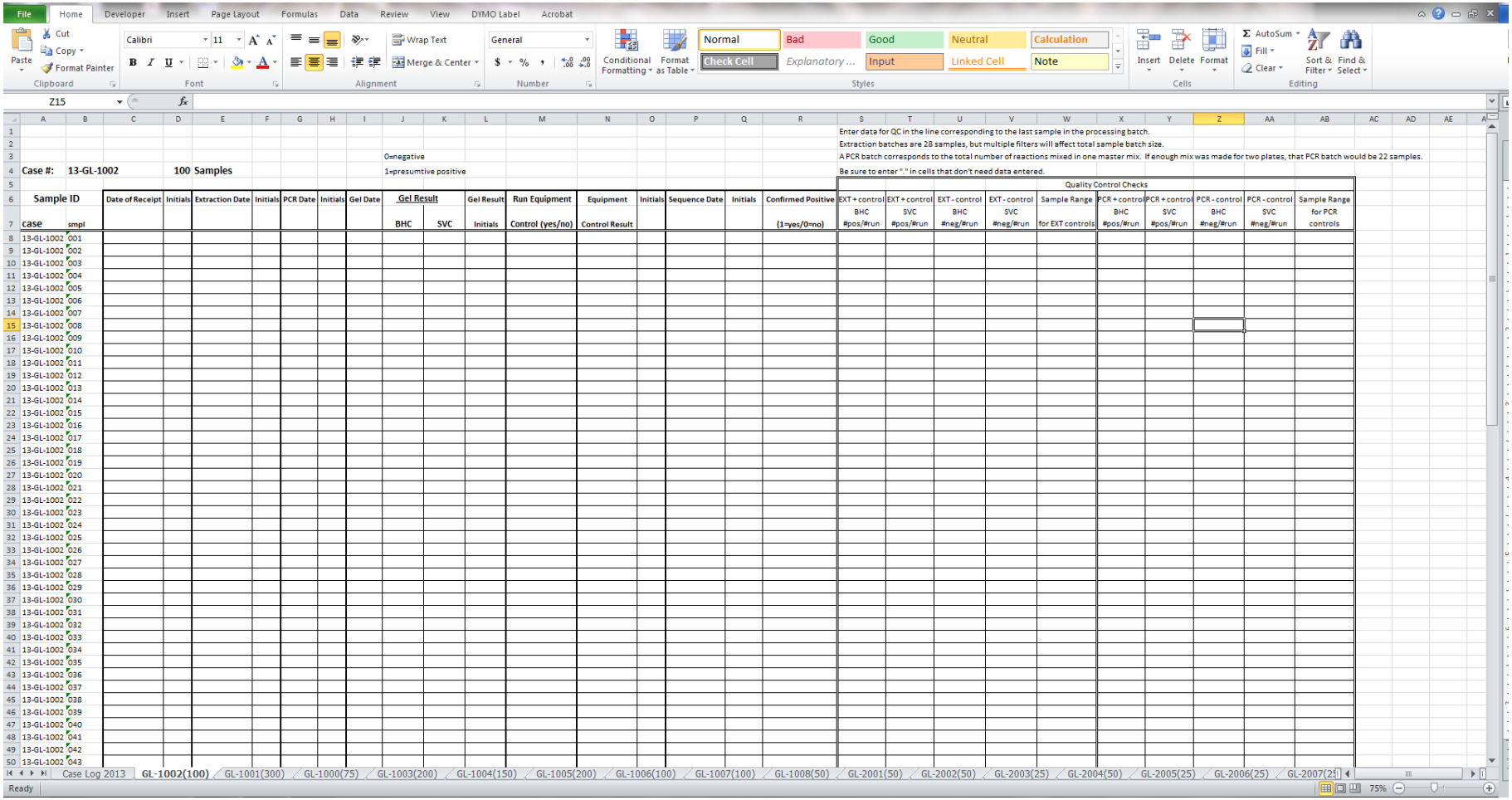


Figure 7. Screen capture of Excel log file with the first case sample log open. Samples are tracked by date and process step through the lab. Corresponding quality control results by extraction batch and PCR batch are reported with sample results.

Exhibit 7. Example of -20 Freezer Temperature Log

Freezer Temperature Log

Month: _____ Year: _____

Freezer ID: _____ Freezer Location: _____

Day	Displayed Temp	Date	Initials	Maintenance (Y/N)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				
31				

Temperature should be between -23 and -17 °C for recording.

Figure 8. A template of -20 freezer temperature log. All cold storage appliances have a designated log which is filled out daily.

Exhibit 8. Example of -80 Feezer Temperature and Sample Storage Log

Example of -80 freezer temperature and sample storage log. All cold storage appliances that store samples after receipt but before extraction, or store archived extracts have a temperature log and a sample log. The temperature log is filled out daily and the sample log each time samples are removed or stored.

Ultra Low Freezer Temperature Log

Month: _____ Year: _____

Freezer ID: _____ Freezer Location: _____

Day	Temp in range (Y/N)	Date	Initials	Maintenance (Y/N)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Figure 9. A template of the Ultra Low Freezer Temperature Log

-80 °C Sample Log

Freezer ID and Location: _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.			

Figure 10. A template of the -80°C Sample log

Exhibit 9. Example of Ambient Temperature Sample Storage Log

Bench-top Shelving Log

Room _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.							

Figure 11. A template of ambient temperature (alcohol-preserved centrifuge samples only) sample storage log. The sample log is filled out each time samples are removed or stored

Exhibit 10. Thermocycle log

PCR Log

Month: _____ Year: _____

Machine ID: _____ Machine Location: _____

Plate ID	PCR Machine ID	Plate Orientation	PCR Program	Run Start Time	Run Finish Time	Date/Initials

Figure 12. A template of a PCR Thermocycle log. It should be filled out at the start of a PCR run and completed at the conclusion of a PCR run.

Exhibit 11. Data sheets for extraction, amplification, gel loading, and sequencing

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

Analyst_____ Date_____ Sample batch_____

Extraction room # 124 or 125 (circle)

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

Reagent/tubes	Lot #	Expiration date
ATL		
Proteinase K		
AL		
Ethanol		
AW1		
AW2		
Molecular grade water		
2-mL collection tube		
Spin filter		

Start time_____

Tube ID	Sample ID	Number Filters		Tube ID	Sample ID	Number Filters
1				16		
2				17		
3				18		
4				19		
5				20		
6				21		
7				22		
8				23		
9				24		
10				25		
11				26		
12				27		
13				28		
14				29	Extraction Negative	1
15				30	Extraction Positive	1

Notes for any deviations from QAPP or lab blunders:

_____

_____

_____

_____

Finish time:_____

Figure 13. A template data sheet for extraction. This should be completed for each extraction event.

Exhibit 12. PCR Data Sheet

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

Analyst_____ Date_____ Sample batch_____ Start time_____

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
10X PCR buffer	2.5 µl	µl		
dNTP (10 mM mixed)	0.5 µl	µl		
MgCl (50 mM)	0.75 µl	µl		
Forward primer (10µM)	0.5 µl	µl		
Reverse primer (10µM)	0.5 µl	µl		
Platinum *taq* (5U/ µl)	0.25 µl	µl		
Sterile ddH_2O	19 µl	µl		

Positive control ID_____ Negative control _____

Silver (SV) or Bighead (BH)(circle) use SV or BH in plate name

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Notes for any lab blunders or deviations from QAPP:

_____

_____

_____

_____

_____

Cycler ID _____ Did cycle complete Y N

Figure 14. Exhibit 12 page 1 of 2 of a template for a data sheet used during PCR for Asian carp.

Time started_____ Time removed_____
Plate Name_____ Date_____
Cycler ID _____ Did cycle complete Y N

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Cycler ID _____ Did cycle complete Y N

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Cycler ID _____ Did cycle complete Y N

Figure 15. Exhibit 12 page 2 of 2 of a template data sheet used during PCR for Asian carp.

Exhibit 13. Gel Data Sheet

Gel data sheet: thermal print of gel should be taped on the facing page. Sign across the photo and this page.

Analyst_____ Date_____ Plate name: _____

Lane #	Gel #_____ Sample loaded, loading notes	+	Lane #	Gel #_____ Sample loaded, loading notes	+
M	ladder		M	ladder	
1			1		
2			2		
3			3		
4			4		
5			5		
6			6		
7			7		
8			8		
9			9		
10			10		
11			11		
12			12		
13			13		
14			14		
15			15		
16			16		
17			17		
18			18		
19			19		
20			20		
21			21		
22			22		
23			23		
24			24		
M	PCR +		M	PCR +	
M	ladder		M	ladder	
25			25		
26			26		
27			27		
28			28		
29			29		
30			30		
31			31		
32			32		
33			33		
34			34		
35			35		
36			36		
37			37		
38			38		
39			39		
40			40		
41			41		
42			42		
43			43		
44			44		
45			45		
46			46		
47			47		
48			48		
M	PCR +		M	PCR +	

Figure 16. A template data sheet using during gel reading.

Exhibit 14. Sequencing Data Sheet

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

Analyst_____ date_____ Kit or sample batch_____

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

Reagent name	Volume per rxn	Volume for ___ rxns (f & r mixes)	Lot or ID #	Expiration date
5X sequencing buffer	4.0 µl	µl		
Forward OR reverse primer (10µM)	0.8 µl	µl		
BigDye V3.1	1.0 µl	µl		
ddH2O	10.2 µl	µl		

Indicate which species in sample ID

Plate Name_____ Date_____

Case#_ddMONyy_Initials

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Notes for any lab blunders or deviations from QAPP:

_____

_____

_____

_____

Cycler ID _____ Did cycle complete Y N

Time started_____ Time removed_____

Figure 17. A template data sheet for sequencing confirmation of presumptive positive samples.

Exhibit 15. Quality Assurance Project Plan Certification Statement

I, the undersigned, certify that I have read and that I understand the Quality Assurance Project Plan (QAPP) for the eDNA monitoring of Invasive Asian Carp. I further certify that I will follow the procedures listed in this QAPP.

Signed:

_____

Name

_____

Agency

_____

Date

Exhibit 16. Quality Control Audit Checklist for Field Sampling and Water Processing

I, _____, observed sampling and sample processing for samples

_____Collected on _____, from the following

locations:_____.

During this time, I compared the actions of the crew to the QAPP and witnessed adherence to the QAPP in the following: A check indicates compliance with the QAPP. An X indicates the QAPP was not followed and a short explanation follows. If more space is needed, attach a written report.

Contamination Prevention:

Boat and equipment preparation _____

Sample collection and navigation_____

Sample processing equipment_____

Sample processing procedures_____

Explanation for any X in the categories above:

_____

_____

_____

_____

_____

_____

_____

Figure 18. A template of a quality control audit checklist for field sampling and water processing.

APPENDIX

Appendix A: Staff Assignments

As of 31 May 2013, the following staff are assigned to the eDNA monitoring project.

Project Leader:

eDNA Program Coordinator: Kelly Baerwaldt, USACE, 309-794-5285 // 309-230-3804

eDNA Processing Leader: Emy Monroe, WGL, 608-783-8402

DNA Processing Quality Assurance Specialist: Maren Tuttle-Lau, WGL, 608-783-8403

Data Documentation & Reporting Specialist: Jennifer Bailey, WGL, 608-783-8451

Fish and Wildlife Conservation Office Points of Contact:

La Crosse: Nick Bloomfield, 608-783-8441

Columbia: Patty Herman, 573-234-2132 x 170

Carterville: Sam Finney, 618-997-6869 x 17

Green Bay: Mark Holey, 920-866-1760

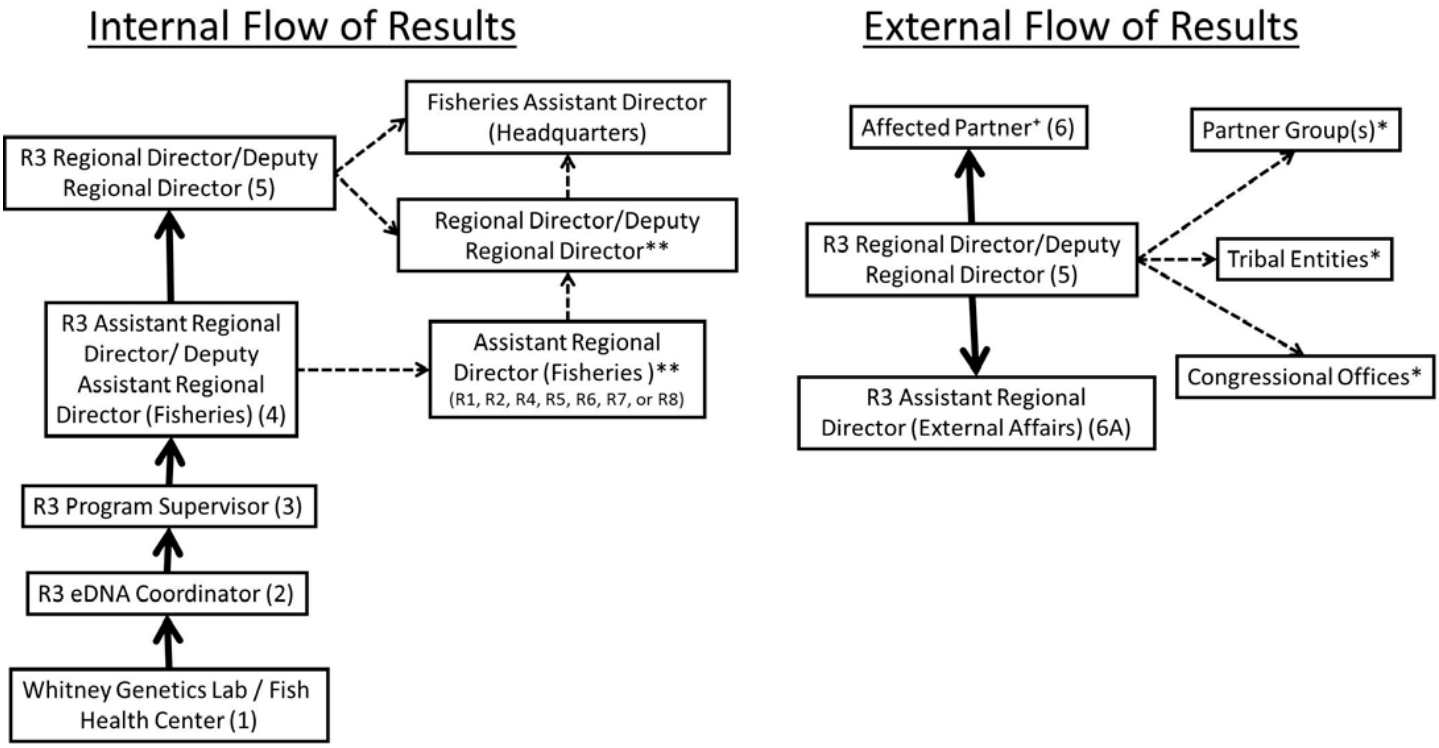
Ashland: Mark Brouder, 715-682-6185 x11

Alpena: Scott Koproski, 989-356-3052 x 1023

Appendix B: Communication of Results SOP

B.1 Standard Operating Procedure: Notification of U.S. Fish and Wildlife Service eDNA Results

This Standard Operating Procedure (SOP) is intended to guide U.S. Fish and Wildlife Service (Service) employees involved in the sampling and analysis of environmental DNA (eDNA) as they disseminate the results of early detection and monitoring for Bighead and Silver carp genetic surveillance. Included is a flowchart (Figure 19) depicting how information will be transmitted through the Midwest Region, as well as specific guidance relating to the format and types of information that will be crucial to include as eDNA results are communicated. Further guidance regarding Service eDNA sampling can be found in the Asian Carp Regional Coordinating Committee's Monitoring and Response Plan and other regionally specific plans. All eDNA sampling should be completed in accordance with the Quality Assurance Project Plan; eDNA Monitoring of Bighead and Silver Carp.



⁺Affected Partner refers to an entity, such as a state, province, or tribe, who has management authority of the body of water being sampled.

*Partners Group(s), Tribal Entities, and Congressional Offices communication will be carried out as determined appropriate by the Region 3 (R3) Regional Director/Deputy Regional Director. See "Post Analysis Coordination" section for further detail.

**Information sharing cross regionally within the Service, communication will be carried out as determined appropriate by the R3 Assistant Regional Director and R3 Regional Director/Deputy Regional Director.

- - - Dashed lines indicate situation dependent lines of communication based on case specific information.

Figure 19. The flow of information from the Whitney Genetics Lab (WGL) / Fish Health Center (FHC) within the Service to the Regional Director (RD), and then from the RD externally to our affected partner. Numeric order of the primary flow of results information noted at each step

B.2 Data sharing from FWCO's and Service Whitney Genetics Laboratory (WGL)

- Once sample processing is complete and positives identified, results will be communicated through the flowchart (Figure 19) within Region 3 (R3) Fisheries to the Assistant Regional Director (ARD) level. Reporting internally to the eDNA Coordinator level as the results are determined by the Whitney Genetics Lab (WGL) will help streamline the process and allow time for the eDNA

Coordinator to prepare for disseminating the results up and out to the affected partner

- All eDNA results information is to be kept confidential, being transmitted as shown in the flow chart (Figure 19). Any potential communication with partner groups or other entities outside the Service will not occur prior to informing the affected partner. For further information on the dissemination of results to others, see the "Post Analysis Coordination" Section below.
- eDNA results information will be formatted as described in the Results Procedure Section (below) by the eDNA Coordinator and prepared as a briefing for the Fisheries Deputy Assistant Regional Director (DARD), and ARD.
- Once briefed, the R3 Fisheries ARD will brief the R3 Regional Director (RD), or Deputy Regional Director (DRD), which will allow the RD or DRD to provide input and disseminate the results as appropriate to affected partner and partner group(s) as outlined in the Post Analysis Coordination Section (below).
- The communication and briefing process from eDNA Coordinator to RD and/or DRD should occur quickly. This will allow R3 to disseminate results promptly and maintain our responsiveness to affected partner and partner group(s).
- When communications are made which transmit results or data from one step to the next in the flowchart (Figure 19), the person sending it must confirm receipt of the information via email response to ensure the message was received and maintain a record of the exchange.
- Communication of results beyond the eDNA Coordinator will only be made once all samples from the sampling event are fully analyzed.

Results Procedure:

- The sampling event data will be packaged together by the eDNA Coordinator in a uniform template to be used to inform the affected partner. Sampling events are defined by the geographic boundaries sampled or by the number of samples taken.
- Data and maps related to the sampling event will be provided to the eDNA Coordinator from each respective Fish and Wildlife Conservation Office (FWCO) upon their completion.
- The analysis results associated with each sampling event will be provided to the eDNA Coordinator by the WGL once processing at the lab is completed.
- The eDNA Coordinator, as part of gathering this information, will compare the datasets provided by the WGL and the FWCO to ensure the data matches each other, i.e. labeling and unique identifiers match, and combine them to create the final data package to be sent to the affected partner.

- The data package to be sent to the affected partner will include:
    - Geo-referenced map indicating all sites eDNA samples were taken, highlighting those sights that were determined to be positive for eDNA (Example included as Figure 20). This map will be formatted as a .jpeg file indicating positive results as red triangles (silver carp), blue squares (bighead carp) and negative results at yellow circles.
    - Digital Excel file with each sample uniquely identified. Data columns will include: Sample ID, Latitude and Longitude in decimal degrees, Date of Collection, Water Body Name, Silver Carp Results, and Bighead Carp Results.
    - A transmission memo from R3 Fisheries ARD to the affected partner relating a summary of the information that has been collected and our proposed next steps (Memo template attached in section B.3). This memo will be sent via email for timeliness as well as in hard copy.
    - A press release template with eDNA specific language will also be included in the transmission to assist the affected partner as needed (Section B.4).

Post Analysis Coordination

- The data package of results will be emailed from the Deputy Regional Director (delegated to Assistant Regional Director or Deputy Assistant Regional Director as appropriate) directly to the affected partner point of contact (as determined by the affected partner). The Deputy Assistant Regional Director will follow up with secondary communication via telephone and/or email to the affected partner point of contact to ensure the data was received.
- Following transmission of the data package to the affected partner we will be prepared to work with them, to assist in interpreting the results or supporting further sampling if possible, understanding that in some cases the affected partner may not decide to carry out follow up investigation. We will also assist as requested in a partner led press release sharing the results with the general public.
- When notifying the affected partner, the data package will also be transmitted via email to our website manager and External Affairs Office. The Service External Affairs team will be prepared to assist the affected partner with outreach or press release materials upon request. This transmission will start a 3 business day waiting period at the end of which the results will be posted to a Service website. However, upon written request from an affected partner, the Service will extend the timeframe for up to an additional 5 business days. The request will specify the reason(s) for the extension (e.g. new geographic area of eDNA detection, high number of positive results, etc.), the name and position of the requesting official, and the number of additional days requested. See appendix D for further information regarding Service External Affairs role in eDNA results notification process.
- The website manager will review the materials and format them as needed to prepare them for posting on the FWS public website: www.fws.gov/midwest/fisheries/eDNA.html
- The results will be posted concurrent with any press releases issued by affected partner, **OR** by 3

PM CST on the third business day after delivery of the data package to the affected partner, whichever comes first. This is to ensure transparency in the system and to not restrict information sharing.

- Immediately upon posting the results online, the website manager will inform the ARD/DARD, who will then inform the affected partner via confirmed email.
- In addition, Partner Groups, Tribal Entities and Congressional Member Offices will be notified on a case dependent basis.
    - Partner Groups – Groups, such as the Asian Carp Regional Coordinating Committee, the Council of Great Lakes Fisheries Agencies, the Affected Great Lakes Fishery Commission Lake Committee, the Mississippi River Interstate Cooperative Resource Association, etc. will be notified via email and/or phone call from the DRD (delegated to ARD as appropriate) to their chairperson upon issuance of a press release by the affected partner.
    - Tribal Entities – Notifications will be made to each Tribe's leadership respectively as an affected partner when sampling has taken place within or in direct proximity to tribal lands. In this case, they will receive the same data package via email as any other affected partner (See Results Procedure section). If the samples were taken in treaty waters, but not in proximity to tribal lands, their leadership will be notified via email and/or phone call from the DRD (delegated to ARD as appropriate) either upon issuance of a press release by the affected partner or when the results are posted on the FWS website.
    - Congressional Member Offices – Congressional Office contacts will be notified upon issuance of a press release by the affected partner on their behalf. Notification will be made via email and/or phone call from DRD (delegated to ARD as appropriate).

Figure 20. Example of geo-referenced map of negative results, although none occur on this example, positive Silver carp results should be highlighted as red triangles and positive Bighead carp results should be highlighted as blue squares.

B.3 Template Letter for Partner Sample Results

Dear **(Insert name of Partner),**

Enclosed with this letter you will find the U.S. Fish and Wildlife Service (Service) Whitney Genetics Lab results relating to the recent Environmental DNA (eDNA) sampling which occurred in **(Enter Name of Sampling Location or Water Body).** These results are presented geographically to provide an overview of the locations where each individual sample was collected, **as well as indicating those that were found to be positive for Asian Carp eDNA (include if positives are found).** Additionally, there is a table provided Which identifies each individual sample collected by unique identifier and its corresponding data.

If one or more samples were positive for Asian carp eDNA, use this paragraph:

Of the **X** samples taken, positive results were found in **X** samples. Of those, **X** were positive for silver carp **and/or X** were positive for bighead carp. Working with you as our partner, we would recommend that…

If no samples were positive for Asian carp eDNA, use this paragraph:

After reviewing the data, you will see that none of the water samples collected in this effort were found to be positive for Asian carp eDNA. We will keep you appraised as further information is collected through future sampling efforts.

Please be aware that these results will be posted on our public website no later than (Enter approximate time (noon) and date of posting if no press release if issued), 3 business days after the transmission of this message. Upon your request, we will work with you to interpret these results and issue a press release.

Include if more than one affected partner:

This information has also been sent to **(Enter names of other affected partner)** as affected partners. Please work together with the Service and the other affected partners to coordinate next steps in responding to these results and providing information to the public to ensure a clear and consistent message.

Please contact eDNA Coordinator **XXXX** at **(Phone and email)** if you have any questions.

B.4 Template Memo for Partner Sample Results

FOR IMMEDIATE RELEASE

Month **XX, XXXX** Contact(s):

Name of Agency, Contact Name, Phone Number

Name of Partner Agency, Contact Name, Phone Number

MAIN TITLE

Subtitle

Analysis of water samples taken from **[insert water body]** on **[insert date]** have tested positive for the presence of Asian carp environmental DNA, also known as eDNA. Out of **[insert total number of water samples]** water samples collected by the **[insert agency]**, **[insert number]** have traces of genetic material from **[insert Asian carp species, e.g. bighead and/or silver]**.

The eDNA samples were collected as part of an extensive monitoring effort in **[insert all water bodies sampled]**. The samples were processed by the U.S. Fish and Wildlife Service's Whitney Genetics Lab in La Crosse, Wisconsin.

[Insert agency VIP quote explaining what the positive eDNA findings mean]

In response to the positive findings **[insert next steps].**

eDNA can be left in the environment in the form of scales, cells, feces or mucus. At present, eDNA evidence cannot verify whether live Asian carp are present, whether the DNA may have come from a dead fish, or whether water containing Asian carp DNA may have been transported from other sources such as bilge water, storm sewers or fish-eating birds. The U.S. Fish and Wildlife Service, U.S. Army Corps of Engineers and the U.S. Geological Survey are leading a multi year Asian Carp Environmental DNA Calibration Study (ECALS), funded through the Great Lakes Restoration Initiative, to improve the understanding and interpretation of Asian carp eDNA results. For more information on ECALS, please visit www.asiancarp.us/ecals.

For more information on the science of eDNA in the fight against Asian carp, watch the video at: http://youtu.be/xXwply6ahQ8 .

[Insert agency boiler plate]

B.5 U.S. Fish and Wildlife Service External Affairs Flow Chart

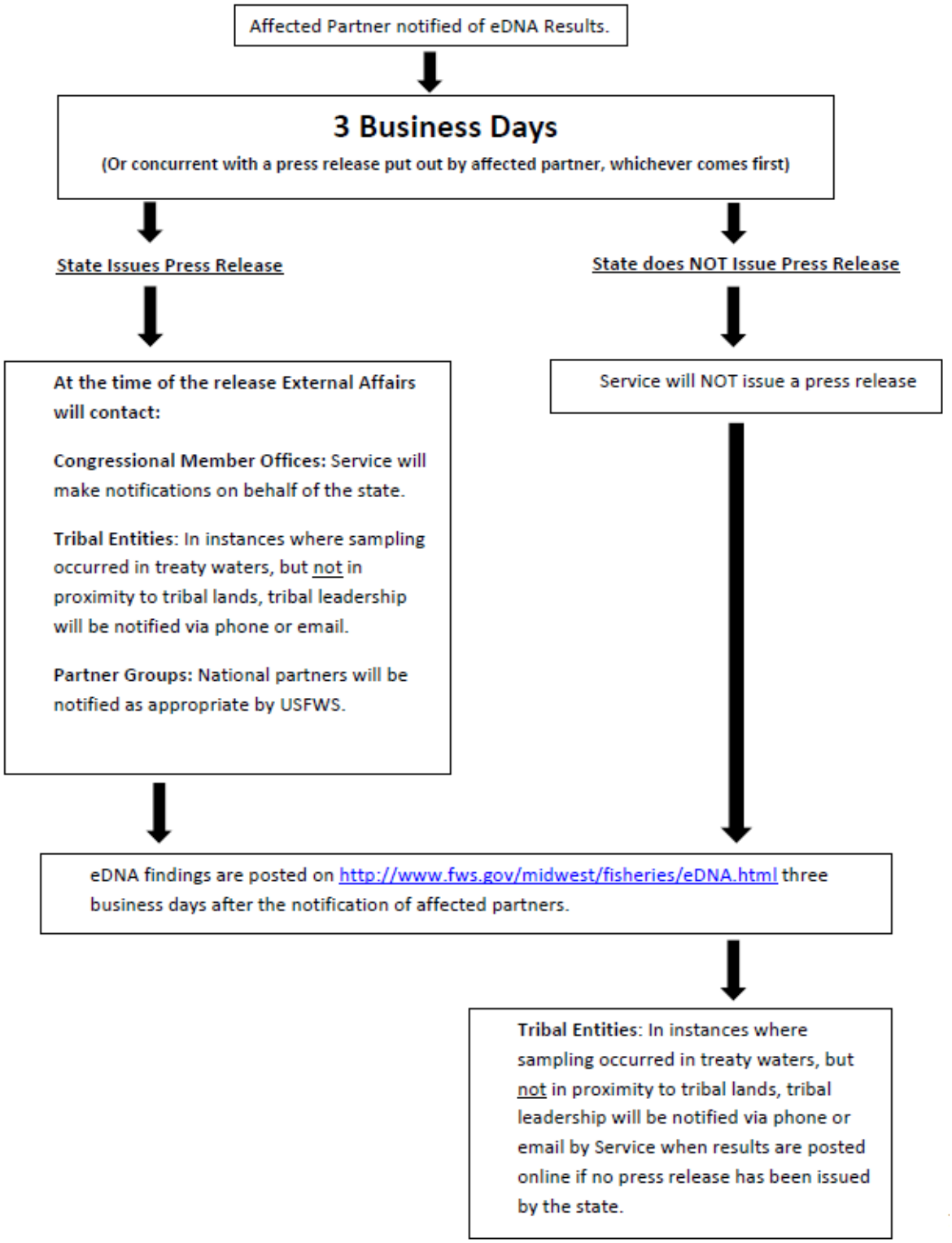


Figure 21. A flow chart describing the flow of eDNA results through U.S Fish and Wildlife External Affairs.

This plan is available on: http://www.fws.gov/midwest/fisheries/eDNA.html

Appendix C: Data Management

Purpose

In order to keep accurate records of eDNA sample collections, personnel associated with sampling and processing of collections, and data associated with a specific collection sample, datasheets associated with sample collections must be kept in accordance with the following protocols for quick reference and to prevent loss. This appendix describes procedures for data reporting and data management specific to USFWS.

Monthly reports will be produced by the eDNA Processing Leader, the eDNA Program Coordinator, and each of the FWCOs responsible for sampling during the month. Reports should include:

(a) Summary of results from the month and context of results to previous sampling events.
(b) Maps and tables generated during the reporting period
(c) News regarding staff, equipment, incident reports, etc.
(d) Results for the previous month in a tabular format:
    a. Field data from FWCOs
    b. Lab data from WGL
(e) Forthcoming sampling efforts (location, dates, and crews)

The eDNA Program Coordinator will collate information from the field crews and the lab.

Data management

(1) Field data and data sheets are the responsibility of the FWCO.
    a. FWCO staff are responsible for entering field data, proofing data and maintaining data at their location. Data should be backed up regularly.
    b. Field data sheets should be scanned and saved in a PDF with all of the data sheets for a particular location on one document. The filename should include sampling dates and location information.
    c. The COC forms are sent to WGL with the samples. FWCOs will receive a scanned COC that should be printed and filed in a project binder and the electronic copy saved with electronic field data and scanned data sheets.
        i. The original COC forms are kept in a project binder at WGL.
(2) Lab data and data sheets are the responsibility of WGL.
    a. WGL will maintain hard copies of laboratory notebooks and the project data file cataloging each case in an excel file. The gel photos are printed and placed into lab notebooks, and electronic files are also saved on the lab network, which is backed up.
    b. WGL will scan data notebooks into PDFs by cases and these will be saved on the lab networked computer with a back-up.
    c. Eventually an electronic Laboratory Information Management System will be utilized to track cases and samples through the lab workflow.
        i. Final reports will be generated as each case is completed, and hard-copies will be printed and filed at WGL as well as saved electronically in the lab network.
        ii. The LIMS will include PDFs of the scanned field COC as well as the gel images and sequencing results.
(3) Collated field and lab data
    a. Region 3 will maintain a database complete with field and lab data as well as electronic copies of reports provided to partner agencies and the public.