USERS GUIDE

Environmental DNA as a Tool for Inventory and Monitoring of Aquatic Vertebrates

Environmental DNA Sampling for Species Detection

ESTCP Project RC-201204

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This guide is intended for managers and technicians that are first-time users of environmental DNA techniques. The focus is on species-specific detection; projects focused on whole ecosystem surveys (metabarcoding) may need to modify these guidelines. The field protocol details the approach we developed for remote wetlands and small to mid-order streams and is designed for portability and ease of use. In this approach, water samples are concentrated onto filter membranes using simple hand vacuum pumps, eliminating the need for heavy or expensive filtration equipment. Filters are preserved in ethanol in the field, with no need for cold storage during transport to the laboratory.

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User guide introduction. This guide is intended for managers and technicians that are first-time users of environmental DNA techniques. The focus is on species-specific detection; projects focused on whole ecosystem surveys (metabarcoding) may need to modify these guidelines. The field protocol details the approach we developed for remote wetlands and small to mid-order streams and is designed for portability and ease of use. In this approach, water samples are concentrated onto filter membranes using simple hand vacuum pumps, eliminating the need for heavy or expensive filtration equipment. Filters are preserved in ethanol in the field, with no need for cold storage during transport to the laboratory. In larger rivers or lakes, or in areas in which portability is not a concern, automated pump systems may be more efficient for filtering samples. Additional adjustments will likely be required for different systems and should be developed in collaboration with a partner laboratory. There are also some developing technologies for field-based analysis of eDNA that are beginning to be commercially available and may be useful for practitioners.

When using eDNA to detect species, sampling design considerations include when to sample, how much volume to sample, what type of filter to use, and where to collect samples within the aquatic system. Generally, samples should be collected when the biomass of target species in the system is highest, but when to sample will also be informed by the objectives of the survey (e.g., breeding individuals vs. successful reproduction). Some general guidance is incorporated into this user guide, but consultation with the literature and an experienced practitioner is recommended before conducting pilot sampling. This guidance was developed as a companion to the report *Environmental DNA as a Tool for Inventory and Monitoring of Aquatic Vertebrates*. CS Goldberg, KS Strickler, A Fremier. 2017. ESTCP Project RC-201204. This report contains extensive additional information about eDNA technology and implementation.

Sample filtering when the filter is well-suited to the site should take 10-15 minutes per sample. If pore size is too small relative to the amount of organic debris or other agents that may clog filters, or if the filter cup is not well-sealed, filtration time could be much longer. In that case, it is up to the user to decide on when to stop filtering if the goal volume cannot be reached in a reasonable amount of time. Note that having multiple pumps and flasks at a site can be useful for decreasing overall filtering time because replicates can be filtered simultaneously.

This guide includes materials and equipment that we have found are optimal for collecting, filtering, and preserving eDNA water samples using this sampling protocol. Similar materials may be substituted, but all sampling materials and equipment should be either single-use or able to be thoroughly decontaminated to prevent cross-contamination of samples.

The second part of this user guide details laboratory practices that should be followed by any laboratory that processes eDNA samples. Practitioners seeking to partner with a commercial, academic, or governmental lab should verify that potential lab partners adhere to these practices.

ENVIRONMENTAL DNA PROTOCOL FOR SAMPLE COLLECTION

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MATERIALS

1. Cellulose nitrate disposable filter funnels or other field-tested, disposable filter funnels
2. Vacuum flask (1L)
3. Silicone tubing
4. Vacuum hand pump (from auto parts store)
5. Rubber stopper with hole for funnel stem
6. Latex or nitrile gloves (non-powdered)
7. Forceps, either stainless steel or disposable plastic (flat-ended filter forceps if possible)
8. *If using steel forceps:* 50 mL tubes with 30 mL of 50% bleach solution (15 mL household bleach and 15 mL distilled water) in a holder to stabilize tubes (a foam drink holder such as a koozie works well)
9. High quality, o-ring screw cap 2mL tubes (e.g., Sarstedt brand) with 1mL 95% molecular-grade ethanol (not denatured)
10. Ethanol-proof laboratory pen (do not use a regular Sharpie marker)
11. Polypropylene grab bottles and cooler with ice (for off-site filtering) or Whirl-Pak® bags (for on-site filtering)
12. Water, bleach, scrub brush, and tubs (for decontaminating between sites)

This protocol is adapted from
Protocol Version 04/12/2012 (D.S. Pilliod, R.S. Arkle, and M.B. Laramie)
USGS Snake River Field Station

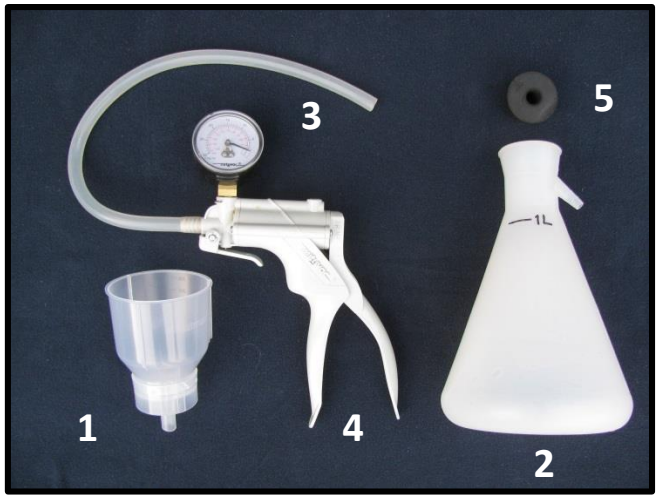

Figure 1. Filter funnel (1), vacuum flask (2), silicone tubing (3), vacuum pump (4), and rubber stopper (5).

Figure 2. Latex or nitrile gloves (6), forceps (7), 2 mL o-ring tubes with 1 mL ethanol (8), ethanol-proof lab marker (9), and 2 50 mL tubes with 50% household bleach/distilled water solution (10).

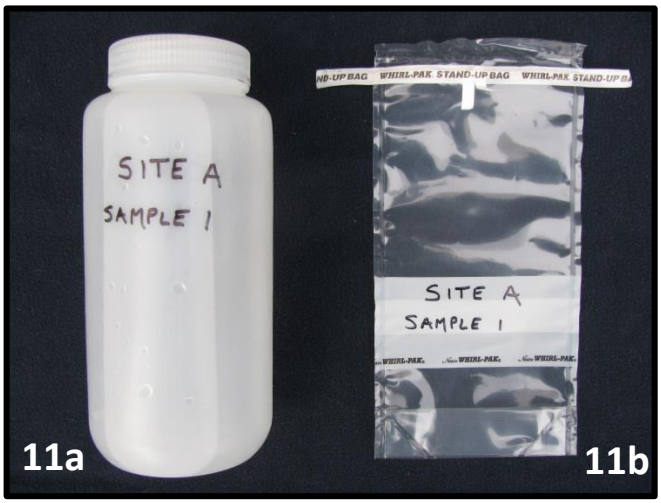

Figure 3. Polypropylene grab bottles (11a) and Whirl-Pak® bag (11b).

Figure 4. Water (12a), bleach (12b), scrub brushes (12c), and tubs (12d) for decontaminating boots and equipment between sites.

CONTAMINATION PREVENTION

Avoid cross-contamination between samples! Contamination can result from a variety of factors at every step in the sample collection process. Be vigilant.

1. Be careful with gloves and other supplies. Do not leave them unprotected and do not toss them in a backpack. Keep everything clean and in plastic bags. Keep grab bottles and Whirl-Paks in clean bags.
2. Guidelines for wearing gloves:
    - Wear new gloves when pulling Whirl-Paks or grab bottles from bags and collecting water for sampling unless hands have been decontaminated with bleach while decontaminating boots and other gear between consecutive sites.
    - Wear clean gloves when removing filter and placing in ethanol storage tubes. Do not touch anything other than the filter or decontaminated tips of the forceps before you handle the filter. If your gloves touch anything that you're not certain is clean, replace them with clean gloves.
    - You do not need to wear gloves when handling the outside of the filter funnel, vacuum flask, and rubber stopper, as these are downstream from the filter (that is, they are below the filter and do not come into contact with sample water before it is filtered).
    - Use non-powdered gloves only.
3. Open filter funnel package from bottom (stem end) and keep closed between sites.
4. When filtering samples, be careful not to touch the top or inside of the filter cup.
5. *If reusing forceps:* Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled or deionized water (Figure 5). *Other methods do not remove DNA and will cross-contaminate your samples!*
6. *If using disposable forceps*: use new forceps for each sample, discarding after use. Remove disposable forceps from plastic wrapper by the hinged end, careful not to touch the tips.
7. Clean boots thoroughly between sites. Remove all dirt, pebbles, etc. from soles and sides of boots. Decontaminate in 10% bleach if they came in contact with water or mud during sampling. Rinse well in tap water (not water from the site) (Figure 6).
8. Bleach vacuum flask and stopper in 10% bleach between sites to prevent disease transport. If vacuum pump and tubing got wet during sampling or filtering, bleach them as well. Submerge equipment in 10% bleach for at least 1 minute, then rinse thoroughly with tap water.
9. To re-use Nalgene grab bottles, bottles must be decontaminated prior to collecting new samples. Submerge bottles in 50% bleach/50% tap water solution for at least 1 minute. Rinse thoroughly with clean tap water (fill, cap, shake, and rinse; repeat at least 3

times). At the sampling site, rinse again with water from the water body 3 times (shaking with cap on each time) before collecting sample to make sure there is no bleach residue in the bottle. Discard rinse water on the shore where it won't run back into the water body. If the water body you're sampling isn't deep enough for the second round of rinsing, consider using single-use bottles.

10. To test for field contamination, collect 1 field negative per site. The field negative is distilled water that is filtered and preserved using the same equipment and procedures as the water samples. Fill a collection receptacle (Whirl-Pak or bottle, whichever is being used for the samples) with distilled water. Using methods for filtering samples as described in Step 3 below, filter the same volume of distilled water as the volume of samples. Remove and preserve filter as described in Step 4 below.

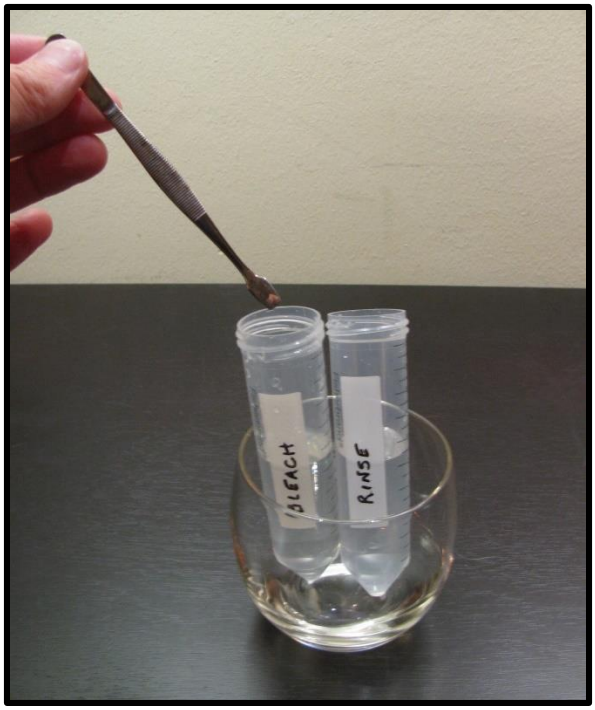

Figure 5. Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled water.

Figure 6. Clean boots thoroughly between sites. Decontaminate with 10% bleach and rinse well with tap water.

SAMPLE COLLECTION

Step 1. Sample Site Selection

1. Environmental DNA is not homogenously distributed within lentic aquatic systems, so within-site sample location can be important for detection. Samples can be collected in association with particular habitat characteristics or evenly spaced. It is easiest to sample from the edge of aquatic sites, but space use by species may indicate that sampling from a (decontaminated) boat will increase detection.

   Distribution of eDNA in streams is also likely to be heterogeneous. Knowledge of the target species' ecology can be used to select sampling locations in the stream habitats likely to be used by the species. Samples can be collected from the stream margin, thalweg, or, in larger streams, from a decontaminated boat. In all cases, collect samples upstream of your position and equipment. When sampling multiple sites on the same stream, always begin sampling at the site that is furthest downstream and sample other sites sequentially as you move upstream.

Step 2: Filter Assembly (Figure 7)

1. Attach rubber stopper to top of the vacuum flask.
2. Attach disposable filter funnel to rubber stopper by inserting stem of funnel into hole in stopper, creating airtight seal.
3. Attach vacuum pump to tube on vacuum flask using silicone tubing.

Figure 7. Filter assembly. Note pressure release lever on underside of vacuum pump near the nozzle.

Step 3. Water Collection and Filtration

If filtering on-site:

1. Collect water in new Whirl-Pak for filtering (Figure 8a). Wear new gloves when touching Whirl-Pak and collecting sample.
2. Pour sample slowly into filter funnel, tracking water volume with gradations on filter funnel (Figure 9). Pause several times to swirl water in Whirl-Pak or bottle before pouring remaining water into funnel.
3. Engage vacuum pump to begin filtration (Figure 10). During filtering, make sure vacuum pressure is sustained (monitor pump gauge if available, or watch water level to make sure water is flowing between the funnel and vacuum flask).
4. If > one filter funnel of volume is being collected, disengage vacuum pump when adding more volume if you are using the funnel to measure volume. Otherwise, use mark on flask to determine when target volume has been reached. Do not use the pressure release lever on the vacuum pump or water from hose may contaminate the filter sample. (The pressure release is the small plastic lever located on underside of the pump, below the pressure gauge.)
5. In some aquatic systems, the filter may clog before the target water volume has been filtered. The filtering rate may slow to individual drips separated by several seconds. Consider setting a cutoff time or drip rate for ending filtering. For example, you might end filtering when the drip rate slows to 3 drips every 10 seconds.
6. Make note of the volume of water filtered, whether samples were collected using Whirl-Paks or grab bottles, and any unusual events, conditions, or problems. Be sure to make a note if you suspect there might have been any sort of contamination of the sample.

If taking grab samples for later filtering off-site:

1. Collect water in decontaminated Nalgene bottle (Figure 8b). Wear new gloves for removing bottle from bag and collecting sample.
2. Rinse grab bottle 3 times with water from sample site. Cap and shake water during each rinse. Dispose of rinse water away from spot where you'll collect water sample.
3. Fill grab bottle with water away from where rinsing occurred, while standing in one place to the extent possible. Avoid stirring up sediment while collecting sample.
4. Cap firmly, label with site name and sample number, and place in a cooler with ice.
5. Filter as soon as possible (within 12 hours) using steps 2-6 described above for filtering on-site. Keep grab samples refrigerated or in a cooler filled with ice until they can be filtered.

Figure 8. Collect water in (a) disposable Whirl-Pak® bag or (b) decontaminated Nalgene bottle.

Figure 9. Pour sample slowly into filter funnel.

Figure 10. Engage hand pump to begin filtration.

Step 4. Filter Membrane Removal

1. Decontaminate forceps by soaking in 50% bleach solution for at least 1 minute and then in deionized or distilled water, each stored in a 50 mL tube. Replace water frequently to ensure that forceps are free of bleach before touching filter. After decontamination, the tips of the forceps should not come into contact with anything other than the filter or clean gloves.
2. Remove silicone tubing from the vacuum flask to release vacuum pressure on the filter.
3. Remove funnel cup. Grasp funnel cup in one hand and the funnel base in the other. Gently squeeze and lift funnel cup to disconnect the funnel cup from the base, exposing filter membrane (Figure 11). Remember that the outside of the funnel cup and flask may be contaminated. Gloves are not needed for this step, and if worn, gloves must be replaced for the following step.
4. Open 2 mL o-ring tube to prepare for filter.
5. Put new glove on one hand. Do not touch anything other than the filter membrane with gloved hand.
6. Using decontaminated (or disposable) forceps and gloved fingers, fold filter membrane as described below. In Nalgene cellulose nitrate and some other filter funnels, the filter membrane sits on top of a paper disc. Discard this thicker paper disc and preserve the thinner, uppermost filter membrane. Fold the filter membrane in quarters by folding it in half and then in half again.
7. Roll the folded filter membrane into a cylinder that fits easily into the ethanol tube (Figure 12). Keep filter stable and prevent it from unrolling by using gloved finger. Place filter in 2 mL vial filled with 1 mL ethanol (Figure 13).
8. Cap vial firmly and label with sample ID and date, using an ethanol-proof marker. Label cap with sample ID. Remove glove.
9. Remove filter funnel from rubber stopper and discard funnel.
10. Repeat filtration and filter preservation for each sample and field negative, making sure to empty vacuum flask between samples to prevent it from overfilling. For each sample, wear clean gloves whenever touching filter or forceps tips.
11. Store sample vials at room temperature or colder, and away from light.

Note for shipping samples: Ethanol is prohibited in some methods of shipping. Check with your carrier.

Figure 11. Remove funnel cup.

Figure 12. Fold filter.

Figure 13. Place filter in 2 mL o-ring tube of ethanol.

Suggested Materials

Item[1]	Product number[2]	Estimated cost[3]
Disposable filter funnel, sterile, with 0.45 micron mixed cellulose ester filter membrane[4]	Sterlitech product #AF045W50	$139.74 per pack of 50 filter funnels
1 L (1000 mL) Nalgene polypropylene vacuum flask with tubulation	Fisher Scientific Cat. #: 10 182 50B, Vendor Cat. #: DS41011000	$28.79 each
Masterflex platinum-cured silicon tubing (size L/S 15)	Fisher Scientific Cat. #: 13 310 110, Vendor Cat. #: 9641015	$119.44 per pack of 25’
Hand-operated Nalgene PVC vacuum pump	O’Reilly Auto Parts Part #MV8010	$57.99 each
#8 Fisherbrand rubber stopper (must be drilled to accommodate stem of filter funnel)	Fisher Scientific Cat. #:14 130M	$16.76 per pack of 12
Powder-free latex gloves (powder-free nitrile gloves may be substituted)	Fisher Scientific Cat. #: 11 4692 67A (X-Small) 11 462 67B (Small) 11 462 67C (Medium) 11 462 67D (Large) 11 462 67E (X-Large)	$34.87 per box of 100 gloves
Fisherbrand filter forceps	Fisher Scientific Cat. #: 09 753 50	$8.18 each
Single-use sterile forceps	Fisher Scientific Cat. #:NC0266178, Vendor Cat. #: DF8988PS	$114.43 per pack of 100
Falcon 50mL centrifuge tube	Fisher Scientific Cat. #: 14 432 22, Vendor Cat. #: 352070	$134.45 per case of 500
2mL screw cap micro tubes for sample collection	Sarstedt 2 mL micro tubes: Fisher Scientific Cat. # NC9739217, Vendor Cat. #72.609.001 o-ring screw caps: Fisher Scientific Cat. #NC9649319, Vendor Cat. #65.716.002	$41.20 per pack of 250 $70.49 per pack of 500
Chemical-resistant laboratory marker	VWR Cat. #95042-566	$37.29 per pack of 12

Item[1]	Product number[2]	Estimated cost[3]
Nalgene wide-mouth polypropylene sample bottles	250 mL: Fisher Scientific Cat.#: 02-893B, Vendor Cat. #: 21050008 500 mL: Fisher Scientific Cat.#: 02-893C, Vendor Cat. #: 21050016 1 L: Fisher Scientific Cat.#: 02-893D, Vendor Cat. #: 21050032	$67.42 per pack of 12 $90.25 per pack of 12 $83.96 per pack of 6
Whirl-Pak polyethylene easy-to-close sample bags	710 mL (good for 250 mL samples): Fisher Scientific Cat. #: 01 812 78C; Vendor Cat. #: B01196WA 1.24 L (good for 1 L samples): Fisher Scientific Cat. #: 01-812-5Q; Vendor Cat. #: B01027WA	$168.68 per pack of 500 $174.61 per pack of 500

[1] This materials list represents the protocol authors' suggestions for materials currently available and in use. Similar products from other manufacturers may be substituted.

[2] Product numbers are valid for the manufacturers noted.

[3] Prices are valid for the manufacturers noted as of 1 March 2017. Estimated costs for Fisher Scientific and VWR products include discounts for account holders.

[4] The protocol authors have found that the Sterlitech filter funnels listed here hold vacuum pressure better than other filter funnels, resulting in more efficient filtering. Alternative single-use filter funnels with filter membranes may be used. Recommended filter membrane materials are mixed cellulose ester, cellulose nitrate, or polyethersulfone. Filters with 0.45 micron pore size work well for most eDNA applications, but larger pore sizes (e.g., 1.2 micron or 5 micron) may be needed in aquatic systems with abundant organic material or suspended sediment that can clog filters and reduce filtration rates.

GUIDELINES FOR SELECTING A LAB FOR PROCESSING ENVIRONMENTAL DNA SAMPLES

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When laboratory analysis of eDNA samples can't take place in-house, samples will need to be sent to a commercial, academic, or government laboratory for analysis. Environmental DNA laboratories should follow a set of practices and procedures designed specifically for handling eDNA samples. When selecting a lab for processing samples from eDNA monitoring, make sure the following practices are part of the lab's operating procedures.

1. Environmental DNA samples are very low quality and quantity compared with DNA samples collected directly from organisms. They therefore need to be handled in a designated room separate from high-quality samples and from the products of polymerase chain reactions (PCR). This designated room needs to have dedicated equipment, including pipettors, centrifuges, and any other item that is needed for sample processing. Technicians should be required to shower and change clothes or go through equivalent decontamination procedures before entering this room after having been in a lab containing PCR products.

2. For assays to be specific enough to detect only target species in eDNA samples, quantitative PCR or next-generation sequencing is required. Conventional PCR tests are not adequate unless every positive result is sequenced.

3. Filter tips should be used at all times when handling samples or reagents pre-PCR to prevent cross-contamination.

4. Fifty percent household bleach should be used to clean all items that come into contact with samples (e.g., forceps) between uses and at least 10% household bleach for regular decontamination by wiping, or UV directly in contact with surfaces. Autoclaving at typical settings, ethanol, and products such as DNA-away are not sufficient to destroy DNA.

5. A negative control should be extracted with each batch of extractions and tested in all downstream processes.

6. A test for inhibition should be incorporated with each sample analysis. This consists of an assay that should always amplify at a known concentration, such as an added internal control (sold as IC or IPC by several companies). Environmental DNA samples are often inhibited and false negatives reported if this control is not included.

7. The laboratory should be able to archive samples after processing (preferably at -80°C) for future analysis, if that is requested by the agency (it is reasonable to expect an additional fee for this service).

8. The agency should collect a series of samples from known positive and negative sites and send them for a blind test to the laboratory (it is reasonable to expect to pay for this service, although some laboratories may waive this cost). All sites with the species should test positive and without should test negative. However, detection probabilities may not be perfect at positive sites and sometimes field crews can introduce small amounts of DNA into samples when first learning techniques (or if clean field practices are not applied absolutely). This testing should be an iterative process that involves working with a lab to understand where errors are occurring and fix problems during a pilot phase. Laboratories should be willing to work collaboratively with the agency during this phase and produce accurate data from blind samples to the satisfaction of the agency before embarking on extensive sample processing.

At this time, there are not many laboratories that analyze eDNA samples, but the number is likely to increase as eDNA sampling becomes more widely used. A list labs advertising eDNA processing services is available at https://labs.wsu.edu/edna/edna-labs/ and will be updated periodically.