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A Protocol for Collecting Environmental DNA Samples From Streams

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Abstract

Environmental DNA (eDNA) is DNA that has been released by an organism into its environment, such that the DNA can be found in air, water, or soil. In aquatic systems, eDNA has been shown to provide a sampling approach that is more sensitive for detecting target organisms faster, and less expensively than previous approaches. However, eDNA needs to be sampled in a manner that has been tested and found effective and, because single copies of target DNA are detected reliably, rigorous procedures must be designed to avoid sample contamination. Here we provide the details of a sampling protocol designed for detecting fish. This protocol, or very similar prototypes, has been used to collect data reported in multiple peer-reviewed journal articles and from more than 5,000 additional samples at the time of publication. This process has been shown to be exceedingly sensitive and no case of field contamination has been detected. Over time, we have refined the process to make it more convenient. Our policy at the National Genomics Center for Wildlife and Fish Conservation is to provide collaborators with kits that contain all of the materials necessary to properly collect and store eDNA samples. Although the instructions in this protocol assume that the collaborator will have this same equipment, we also describe how users can create their own kit, and where we think there is flexibility in the equipment used.

Keywords: sampling protocol, fish, environmental DNA, eDNA, stream, survey

Cover photo— A bull trout (*Salvelinus confluentus*) swimming in the West Fork Clearwater River in western Montana. (Photo Credit: Aubree Benson, USDA Forest Service.)

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Introduction

Environmental DNA (eDNA) is DNA that has been released by an organism into its environment and that can be found in air, water, or soil. Collection of eDNA was first used to detect and describe microbial communities (Barnes and Turner 2016), but there has been an explosion of research implementing eDNA sampling to detect macrobial species—fishes, amphibians, mollusks, crustaceans, and insects—in aquatic environments over the last decade (Thomsen and Willerslev 2015). Of particular interest is the use of eDNA sampling to detect organisms that are rare or difficult to sample, whether they are invasive nonnative species (e.g., Dejean et al. 2012; Goldberg et al. 2013; Moyer et al. 2014) or native species of conservation concern (Spear et al. 2014; Thomsen et al. 2012; Wilcox et al. 2013).

In many instances, eDNA sampling has proven to be at least as effective as conventional sampling for determining species' presence, and it can often be performed more rapidly and efficiently (Dejean et al. 2012; Wilcox et al. 2016). We have found that it typically takes less than 15 minutes (from arrival to departure) to collect and catalog a sample. This efficiency enabled us to use eDNA sampling to describe the distribution of bull trout (*Salvelinus confluentus*) across 98 km (60 miles) of first- to third-order streams in 8 days (McKelvey et al. 2016). This survey detected bull trout presence in all locations where they were expected to be, based on electrofishing, and discovered habitats that were not previously known to be occupied. Furthermore, eDNA-based methods have detected species at very low densities in streams. Jane et al. (2015) achieved 100-percent detection of caged brook trout (*S. fontinalis*) across 162 samples at distances of up to 240 m (800 feet) downstream, despite order-of-magnitude changes in stream discharge. Wilcox et al. (2013) found that samples containing an average of 2 DNA copies led to positive detections of target species in 72 to 86 percent of trials, whereas DNA release rates averaged 495 copies per fish per second (Wilcox et al. 2016). Wilcox et al. (2016) used these data to estimate that the detection probability of a single subadult fish in 100 m (300 feet) of stream was 84 percent, double or triple the capture probabilities associated with electrofishing for many stream salmonids. This combination of sensitivity, reliability, and efficiency suggests that eDNA sampling has the potential to transform species assessment and monitoring in streams.

Moreover, each eDNA sample represents a snapshot in time of aquatic biodiversity, and can be tested for the presence of many species if eDNA assays are available. In that regard, the National Genomics Center for Wildlife and Fish Conservation (NGC) has developed taxon-specific eDNA assays for a number of salmonid fishes including brook trout and bull trout (Wilcox et al. 2013); westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), Yellowstone cutthroat trout (*O. c. bouvieri*), and rainbow trout (*O. mykiss*; Wilcox et al. 2015); brown trout (*Salmo trutta*; Carim et al. 2016a); and Arctic grayling (*Thymallus arcticus*; Carim et al. 2016b). There are eDNA assays for many other species such as North American river otters (*Lontra canadensis*; Padgett-Stewart et al. 2015), Rocky Mountain tailed frogs (*Ascaphus montanus*), and Idaho giant salamanders (*Dicamptodon aterrimus*; Goldberg et al. 2011). Developing eDNA assays for additional species is relatively straightforward, as long as care is taken in their design and testing (Wilcox et al. 2013, 2015). Eventually, there will be genomic-based tools permitting the simultaneous assessment of many species, which the NGC and other facilities are actively exploring (e.g., Valentini et al. 2016). Among the many challenges to implementation of multispecies eDNA sampling is the possibility that it may fail to attain levels of sensitivity comparable to the

single-species method (Kelly et al. 2014). Regardless, the eDNA samples being collected now will be suitable for later analyses with these more advanced approaches.

Although we have extolled the advantages of eDNA sampling for species detection, it is vulnerable to two problems: concluding that a species is absent when it is present (false negative), and concluding that a species is present when it is absent (false positive; also called false detection). Most research has focused on reducing the frequency of false negatives by improving laboratory techniques or sampling methods to increase sensitivity. Similarly, avoiding false positives involves strict adherence to laboratory standards. Another major concern is the avoidance of sample contamination in the field. The ability to detect single copies of eDNA allows robust detection of species at low densities, but also makes samples highly sensitive to contamination. As a result, it is imperative that eDNA-based methods follow protocols that allow for efficient sampling while taking precautions to prevent contamination.

Here we present protocol that has been rigorously tested on salmonid fishes in small- to medium-sized streams. It minimizes contamination issues while maximizing species detection and sampling efficiency of stream fishes, based on our experiences and those of our partners throughout the western United States. We have analyzed more than 5,000 eDNA samples collected using this protocol, and it has been outlined in several peer-reviewed publications (e.g., McKelvey et al. 2016; Wilcox et al. 2016). Many cooperators have collected samples using this protocol, and we have had no instances of field contamination. Our step-by-step guide describes how to collect reliable eDNA samples from streams. Please note that this protocol has not been extensively tested in ponds, lakes, or large rivers; it will require modification when used in these environments.

Throughout this document we refer to items such as bags and desiccant as being either "clean" or "dirty." "Clean" in this context means that the item is unlikely to be contaminated by the target organism's DNA. "Dirty" means that it could be contaminated. We use these terms to differentiate our needs from those associated with sterile equipment, which is guaranteed to be free of all living organisms. Bags contained in a box bought at a retail store are therefore considered initially clean, though they are not formally sterile. Similarly, the sampling site, such as the grass next to the stream, is considered clean; it is far from sterile, but contains DNA only from organisms found at that site. This is an important distinction, and what is considered "clean" will vary somewhat depending on which organism is targeted. For example, if bacterial eDNA were the target, different protocols would need to be developed.

Before Heading to the Field

Three points:

1. The kit described in the next section contains all of the materials needed to collect eDNA. You will need to provide a global positioning system (GPS) unit or GPS-equipped device to determine the sampling location.
2. Start each day with fully charged batteries and check the electrical connection and switches on the pump.
3. Once collected, eDNA samples should be kept cool, dry, and dark. When you are camping overnight with access to a vehicle, a cooler makes a convenient and secure storage box for multiple samples.

The Environmental DNA Sampling Kit

The NGC provides collaborators with eDNA kits (Box 1) that contain all the equipment and supplies required for field sampling. Although we do not endorse any specific brand, the instructions assume the use of a battery-powered peristaltic pump. Many of these supplies are "disposables" that are retained and returned to the NGC to be cleaned and reused. You may also assemble your own kit as described in Appendix A. But be sure that the filters, filter assemblies, tools used to handle samples, and interiors of bags used to store samples are clean. Precisely adhere to these specifications to ensure that your results are reliable and consistent with other eDNA samples. This document assumes that you have a kit supplied by NGC, or one containing identical elements. Also, note that although the pump comes in a hard-sided case, our field teams often transfer the unit to a well-protected portion of a backpack for transit into the field.

Box 1: The Environmental DNA Sampling Kit

The displayed kit contains the materials supplied by the National Genomics Center for Wildlife and Fish Conservation. If you are building your own kit, see Appendix A for additional information.

1. Duffelbag containing eDNA sampling equipment
2. Pump
3. Tubing with adapter
4. Filter holder with filter (in clean bag)
5. eDNA sampling protocol
6. Forceps (in clean bag)
7. Clean gloves
8. Outflow bucket
9. Clean sample bags with desiccant (for storing filters after sample is taken)
10. Sample box with letter-sized envelopes, pencils, and indelible ink markers
11. Black bag for used equipment **(please return used supplies for cleaning and reuse**)
12. White bag with unused site kits
13. Battery charger
14. Pump battery (with backup battery)
15. Alligator clip adapter for battery
16. GPS unit (**not shown and not provided; note that most cellphones have apps for determining geolocation)**

Note: Items 4, 6, 7, and 9 constitute a site kit, and come in a 1-gallon resealable bag. A site kit has all the supplies needed for collecting one sample.

Procedures for Avoiding Contamination

The most important thing you can do to ensure the accuracy of your eDNA results is to avoid contamination of the field sample. The primary sources of contamination are anything that has previously come in contact with the target species or its DNA: hands, clothes, waders, the pump, used supplies, even the field vehicle. If you suspect that any forceps, filter holders, samples, or anything else has become contaminated, stop sampling and start over with a new site kit. Carry spare site kits for these circumstances (see note in Box 1).

The pump, tubing, outflow bucket, and used equipment are sources of contamination because they are exposed to the surrounding environment at every sampling site. Avoid handling these items once you have donned the clean gloves. We have adopted the convention of storing clean items in a white bag, and used or contaminated items in a black bag. Generally, remove the clean site kits from the white plastic bag only as needed. If you are backpacking to a sampling location, it is fine to remove enough field kits—plus spares—to complete the day's work; it is a good idea to transfer them in a new clean bag prior to use. However, a kit that leaves the white bag should never be returned to the white bag. Used equipment goes into the black "used equipment" bag and is never touched again in the field.

Choosing a Sampling Location at a Site

Choose a bank or island location—the flatter, the better—that allows you to lay out all of the sampling equipment without risk of it being blown away or falling into the stream. During sampling, always stay downstream of the sampling location. This includes you and all the sampling equipment. During sampling, the filter cup should be placed in a well-mixed portion of the flow (the center of the main current if possible). Avoid eddies or splash pools where DNA could wash off contaminated materials (like waders or tubing), flow upstream, and contaminate the sample.

Field Collection Protocol

Step 1: Connect the Pump

- Remove the pump and battery and set in a stable area. Connect the pump to the battery using the power cord or alligator clips.

Step 2: Install the Tubing

- Ensure the tubing has been loaded into the pump following the orientation indicated by the sticker placed on the pump. Lift the quick-release lever to load the tubing into the pump head. Center the tubing in the track, and lower the quick-release lever. Once the tubing has been loaded, thread the outflow end of the tubing from the pump through the hose clamp on the bucket to keep it in place for an accurate measure of how much water has been pumped (fig. 1). The hose clamp will keep the tubing in the bucket during pumping.

Figure 1—Outflow end of tubing threaded through hose clamp and placed into outflow bucket.

Step 3: Start the Pump

- Turn the pump on. Ensure that the pump switch is in the FORWARD direction, and test that air is being sucked through the filter adapter on the palm of your hand. If the tubing is oriented in the wrong direction through the pump head, or the pump switch is in the reverse direction, air will be blowing out of the adapter end of the tube, and may contaminate the sample. If the pump will not turn on or pull water, see Troubleshooting.

Step 4: Remove the Site Kit

- Remove a site kit from the white bag and immediately close the white bag. Put on a pair of gloves from the site kit. Once you have gloves on, be careful not to touch anything that may be contaminated with DNA (such as yourself and the pump).

Step 5: Forceps and Sample Bag

- Remove the bag containing clean forceps and the sample bag containing the silica desiccant. Unseal these bags without removing the contents and set them in an area where they are easily accessible, and away from contaminated materials (such as the bucket and pump).

Step 6: Install the Filter Assembly

- Remove the packaged filter assembly from the site kit. Open the bag containing the filter assembly, but do not remove it from the bag. Instead, orient the filter holder in the bag so that it can be attached to the tubing without removing it from the bag. In

Figure 2—Attaching the filter assembly to the adapter while using the packaging bag.

essence, you are treating the bag as a secondary "glove" to prevent contamination before collecting the sample. Press the filter holder on to the filter holder adapter on the tubing (fig. 2) so the connection is snug (enough to prevent the filter assembly from falling off). Here you are touching the tubing, which is considered dirty, with a gloved hand. If you are right-handed, use the left hand to hold the tubing (vice versa if left-handed). This is the natural thing to do, and this way the hand that is closer to the filter when you are removing it from the cup (fig. 2) will be clean.

Step 7: Stream Placement of Filter Assembly

- Place the filter assembly into the stream (fig. 3). Be careful not to sample in any area with backflow that could result in contamination of the sample. You may prefer to hold the filter assembly in place, but we often place a rock on the tubing just behind the assembly and pin it facing upstream on or near the stream bottom. Take care to avoid stirring up sediment that may clog the filter, or using a rock so large that it compresses the tubing. Sometimes even this approach will not work, so be creative. Whichever method you use to hold the cup under water during sample collection, remember to keep the cup upstream and in the water column where it will not pick up sediment from the bottom. Note that placing the dominant (clean) hand in the water and grabbing a rock does not render the hand unclean; at that point, the stream and everything in it is part of the sample.
- Assuming that you are not holding the filter assembly to collect the sample, then the main job is to monitor things: Make sure that the current does not dislodge the cup and make sure that the bucket is filling as you watch for the water to reach the 5-L mark. Things will happen. The tube may fall out of the bucket and need to be reinserted, the bucket may tilt and almost spill, or the filter may clog. While you are monitoring, if you ever need to touch the bucket, tube, pump, or anything else considered to be dirty, try to remember to use your nondominant (=dirty) hand.
- If the bucket appears to be filling very slowly, look for signs of filter clogging (or check to see whether the pump speed was set at less than maximum). It generally

Figure 3—Collecting a field sample. Filter cup is submerged in the main current upstream from the pump and bucket. In this case, the cup is held in place in the current with a rock.

takes 8 to 9 minutes to filter 5 L of water from clear streams, so if you have filtered that long but have collected only 2 to 3 L, it is likely that the filter is clogged. Other signs are an excessive amount of bubbles in the tubing and outflow, or the discharge of very little water from the tubing. See Troubleshooting for instructions on how to proceed.

Step 8: Drain and Dry the Filter

- When 5 L of water have been pumped, lift the cup up and away from the stream and allow the pump to run for approximately 30 seconds to dry the filter.

Step 9: Remove Cup From Filter Assembly

- Remove the cup from the filter assembly (fig. 4); it does not unscrew, but pops off. At this point you are finished with this cup and you can set it aside.

Step 10: Fold Filter and Secure in Sample Bag

- Remove the forceps from their protective bag, being careful to not touch the tips to anything except the filter. Use forceps to fold the filter paper in half and then in quarters with the filtrate side facing in (fig. 5). Place the filter into the sample bag containing the desiccant beads, ensuring that it is at the bottom of the bag and in contact with the beads. Allow nothing to come in contact with the inside of the silica bag except for the filter. Push out any excess air from the bag and seal completely.

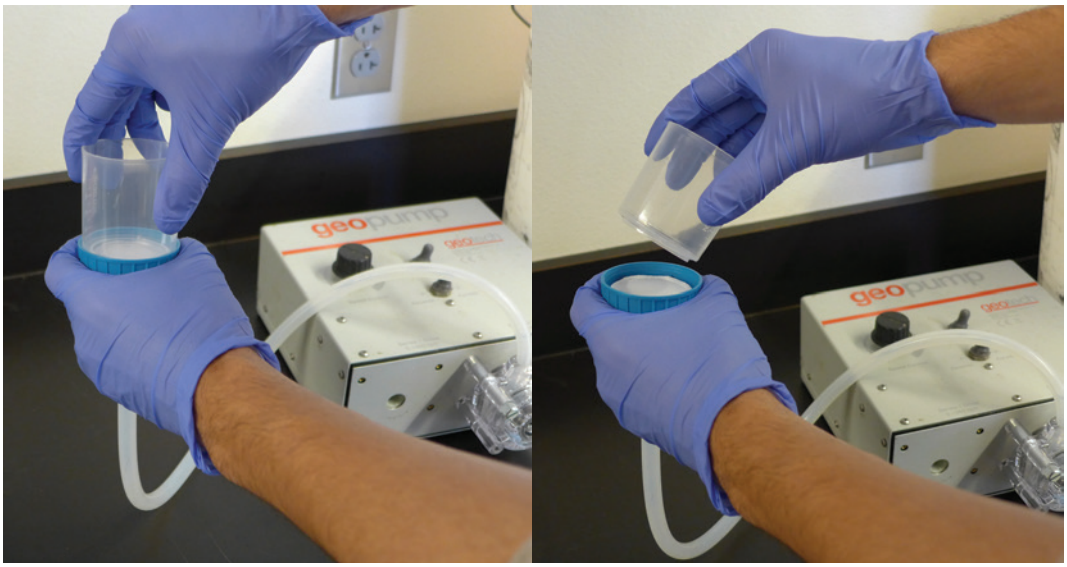

Figure 4—Removing the cup from the filter assembly.

Figure 5—Folding the filter paper, filtrate (usually brown) side in.

Step 11: Label Sample Bag

- At this point, you have secured an eDNA sample in a clean environment. Turn off the pump, take off your gloves, and label the bag with the date, sample identification (e.g., stream name and sample number), GPS coordinates, number of filters sampled, and your initials (fig. 6).

Step 12: Place Sample Bag in Envelope

- Label a letter-sized envelope with the same information on the sample bag, place the sample bag in the envelope, and seal it (see Troubleshooting if you used multiple filters to collect the sample at a site because of clogging). This envelope provides extra protection for the sample and provides redundant labeling.

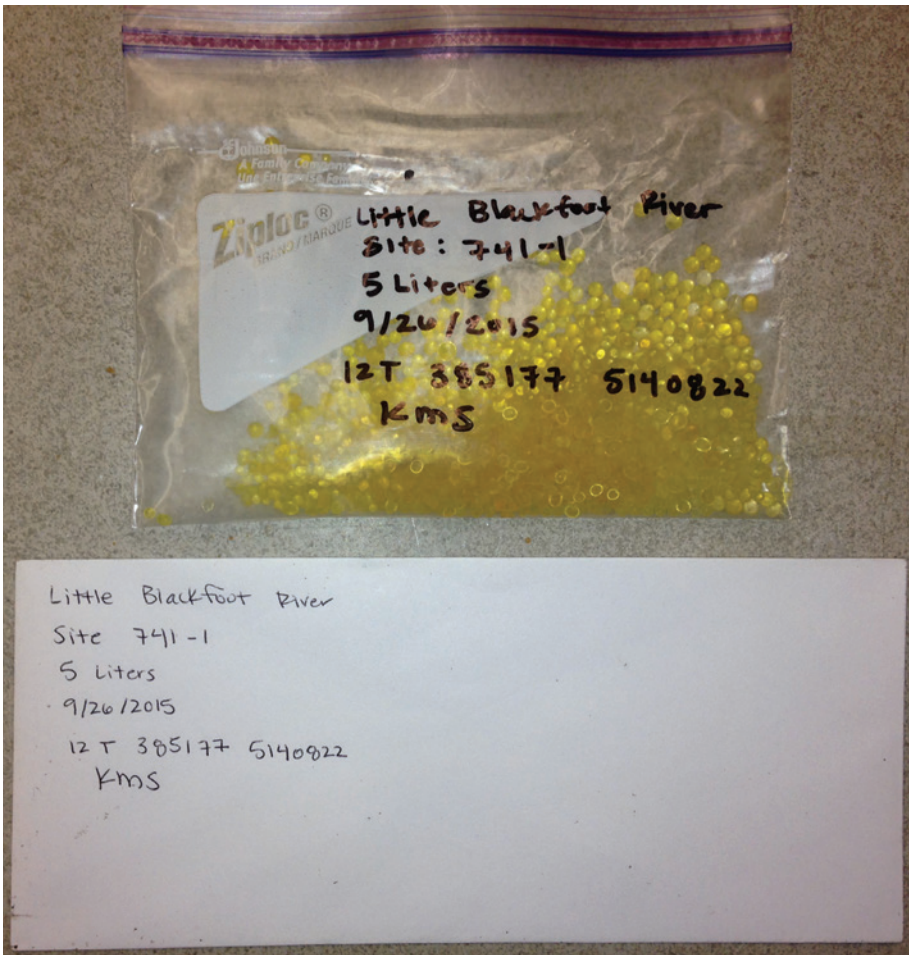

Figure 6—An example of a properly labeled sample.

Step 13: Clean Up

- Clean up the used supplies. Put all of the used plastic and forceps back into the 1-gallon (4-L) site kit bag. Make sure that you have removed the blue base of the filter cup from the tubing. Double-check to ensure you have not forgotten to pack up anything—the forceps are easy to overlook. Place the used bag in the black "used equipment bag," discard the water from the outflow bucket, and pack up—you are ready to move to the next site. If you are backpacking to remote sites, it is good practice to store clean and dirty kits separately, preferably in white and black garbage sacks.

Step 14: Storing Samples

- Environmental DNA samples are stable in silica desiccant beads for several weeks. Nevertheless, keep them away from water, heat, and sunlight. We recommend that samples be stabilized no more than 2 weeks after collection either by storing the bagged filters in a freezer or, preferably, by sending them to us at NGC so we can extract the DNA. Desiccant beads contain a colored indicator, generally blue, orange, or yellow, indicating that the desiccant is dry and can absorb water. Desiccant that has lost its color cannot absorb water and therefore cannot dry out a

filter or keep it dry. If a sample bag contains desiccant that has lost its color, don't use it. If desiccant in a bag containing a sample loses its color, get out a new site kit and, using the sterile forceps, transfer the sample to a new bag. Should a dry sterile bag not be available, place the bag containing the sample in a freezer.

Step 15: What to Return to NGC

- Return the samples, pump, and used and unused supplies to the NGC. There is no need to separate or dispose of used kit materials. Upon receipt, we recycle all plastic bags and sterilize the filter assembly and forceps for use in future sample collection.

Troubleshooting

If the pump will not turn on or provide suction, or initially seems slow:

- Check that the battery is properly connected. Adjusting the alligator clips or detaching and reinserting the circular adapter can help. Also make sure that the alligator clips are clipped to the proper terminal (e.g., red to red).
- Make sure the speed control knob is at the maximum setting.
- Try using a backup battery if available, or charge the original battery. After extended use and recharging, batteries will sometimes fail to hold a charge. Emergency replacements are often available. Standard 12-volt lantern batteries provide an adequate (though disposable) alternative power source.
- Check that the quick-release tubing lever is lowered.
- Loosen the pump head and move the tubing a few inches forward or backward; once the tubing gets worn, it will not pump as efficiently. Also, lift the quick-release lever and move the tubing laterally. At times it gets pinched and is not in the designated groove.
- Double-check that the tubing direction is correct (adapter end is loaded in the way indicated on pump) and that the switch is in the FORWARD position. NOTE: If the tubing is threaded backwards or the switch is not in the FORWARD position, the pump will be pushing air toward the filter rather than pulling air through it. Replace the filter assembly if this happens.

If pumping slows because the filter clogs:

- If a 5-L sample cannot be collected with a single filter because of clogging, follow the instructions for filter removal and labeling (steps 8–11), then use materials from a fresh kit to resume sampling until a total of 5 L of water have been filtered. In this case, label the plastic sample bags of each filter to indicate both the order of use and the approximate number of liters that were pumped through each filter. For example, you may have one sample bag labeled "SAMPLE #1- 4L" and the other "SAMPLE #2-1L." Label the sample bags with the same date and identification number as in Step 12. Store the filters in individual silica bags, but together in the same letter-sized envelope.

If pumping seems exceptionally fast:

- Ensure that the filter was properly seated (i.e., water was being drawn only through the filter, not to one side). If so, simply recollect the sample, using a new filter assembly.
- Ensure the filter assembly is secured to the tubing adapter.

Control Samples

In many studies, field "control" samples are collected by filtering a quantity of distilled water. There is, however, reason to question the need for these samples and the value of the information obtained. Filtering a sample of distilled water from a bottle verifies the general cleanliness of the field equipment (e.g., the filter assembly). However, it cannot measure any contamination that may occur in the process of collecting a sample from a stream or lake. For example, standing upstream of the sampling area in waders that have brook trout DNA on them while a sample is being collected may result in a false positive detection of brook trout in that sample. Similarly, sampling for a target species from a boat that was previously in a waterbody containing that species may also contaminate a sample. After collecting hundreds of field controls with the protocol outlined in this document and failing to observe any positive detections, we deemed these samples expensive and uninformative about the risks and causes of false-positive detections in an eDNA sample.

The protocol is designed to make field contamination extremely unlikely, and the NGC has designed cleaning protocols that effectively remove DNA from all critical components. Note that eDNA, being more sensitive than other sampling methods, may produce results that cannot be verified by other methods. Also note that errors such as sample misidentification, mislabeling, and laboratory-based contamination may occur at low levels. Because results cannot always be verified by independent methods, the best approach to evaluating unusual or suspect results is simply to resample at that location. The ease and sensitivity of eDNA sampling tends to produce results that are highly replicable (e.g., McKelvey et al. 2016).

At the NGC, we include positive and negative control samples in every laboratory analysis to ensure that contamination does not influence our results. If groups are creating their own kits and cleaning components, they may wish, as we did, to run field control samples until certain that cleaning methods are effective. If you are using NGC kits and wish to collect field control samples, please notify us to that we may provide the necessary extra supplies.

Sampling Objectives

Although stated last here, the objectives of any eDNA sampling project should be identified well before any sampling is done. The first decision is whether eDNA sampling is appropriate (see Appendix B). Although there are many reasons for using eDNA sampling, they can usually be divided into two categories: those involving populations and those involving one or few individuals. We address each of these in turn, but also note that the sampling design will vary depending on the species to be investigated. As of this

writing, our research has focused on detection of stream-dwelling salmonids. We assume that this protocol will be applicable for other stream fishes that occupy the water column (as opposed to benthic fish, amphibians, or macroinvertebrates). Extensive evaluations of the efficacy of eDNA sampling for other taxa may not have been conducted and detection efficiency appears variable (Pilliod et al. 2013; Thomsen et al. 2012), so we urge caution in comparing detection rates among taxa. We also note that timing of eDNA sampling is critical, because many aquatic species are mobile, transiently occupy certain habitats, and exhibit seasonal variation in eDNA detection rates (Laramie et al. 2015). Simply put, ecological understanding of a species is essential to designing an appropriate eDNA sampling scheme.

Aquatic populations contain multiple individuals that are usually distributed across many stream habitats. Both factors play to the strengths of eDNA sampling. In small streams, we expect high detection probabilities (84 percent) from even a single individual if it is located within 100 m (300 feet) of the sample location, and at least some level of detectability even when 1 km upstream (Wilcox et al. 2016). Because salmonid population densities, especially in natal habitat, are generally much higher (Copeland and Meyer 2011), eDNA-based surveys for populations are effective. By sampling nearly 1,000 sites at 1-km (0.6-mile) intervals across the northern Rocky Mountains, we have also found that salmonid occupancy of natal habitats is typically characterized by contiguous strings of positive eDNA samples (McKelvey et al. 2016). The high rates of site-level detection and feasibility of rapid, intensive, broad-scale surveys imply that eDNA sampling is a superior tool for assessing the presence and distribution of salmonid populations. It should be noted that, when populations are being evaluated, quantities of eDNA collected can be meaningful. Wilcox et al. (2016) found a reasonably high correlation ($r^2 = 0.592$) between fish abundance in the reach immediately upstream from an eDNA sample and quantity of DNA in the sample. Formal quantification of eDNA requires a "standard curve" for calibration, and this requires additional laboratory work.

Alternatively, there are instances in which a species of interest is represented by very few individuals that may be erratically distributed. This may be the case after efforts to eradicate nonnative species, during early stages of a nonnative species invasion, or when searching for a few migratory adults of a native species. In these circumstances, shorter spatial intervals between sampling sites, filtering more water, or both, are logical approaches to increasing the probability of detection. Mean estimates of the variables influencing detection probability of stream salmonids (and the R code to simulate detection probabilities assuming different covariate values) are available in Wilcox et al. (2016). Additionally, there is no reason that samples collected throughout a given area need to be analyzed all at once (analysis is generally the most expensive part of eDNA surveys). Instead, incremental analyses of samples from areas with the highest pretreatment densities, highest likelihood of incomplete treatment, or most suitable habitat for the species of interest are the most likely to reveal whether the target species is truly present.

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Appendix A—Making Your Own Kit

Sampling eDNA from water, like sampling many aquatic species, is intrinsically simple. And like sampling most aquatic species, there are many different tools and techniques for collecting eDNA. As eDNA sampling becomes more widely adopted to assess the presence of aquatic species, the need for standardization—or at least a set of best practices—that permits some level of comparison among different eDNA collections will grow. Recognizing that eDNA sampling to support natural resources management is in its infancy, we have focused on identifying and implementing practical and dependable methods that promote this standardization.

Developing a reliable field kit was among those efforts. We were guided by the notion that biologists require a field kit that is portable, permits rapid sampling, allows for immediate preservation of eDNA to minimize degradation (Takahara et al. 2014; Turner et al. 2014), and can be used to collect many samples in a single field day. From this, we concluded that collecting and transporting water samples from the field to the laboratory (for either filtration or precipitation) was too cumbersome and that field filtration was a more reliable method for eDNA preservation. Additionally, a number of drying agents—such as alcohol—were considered for sample preservation and may be preferable for some applications (Renshaw et al. 2015). We opted for storage in plastic bags with desiccant, in part because this had the advantage of providing a large surface to record all metadata associated with a sample (as opposed to a vial with alcohol, which might be prone to leakage).

Even within the constraints of field filtration, there are many alternative methods and details, a number of which we have tested. For example, a variety of filter materials and pore sizes are available; some produce higher eDNA yields than the 1.5-µm-pore, fiberglass filters we use. In field trials, however, higher-yield filters proved to be fragile, clogged more easily, and were sometimes subject to higher filter-to-filter variation in eDNA yield (Minamoto et al. 2016). Similarly, in-line filter holders are available, but we found them to be prone to air leakage, difficult to clean, bulkier, and more expensive than the cup-style holders we selected. Thus, while acknowledging alternative choices are available, we recommend adopting our equipment choices and copying as much as possible the kit described in Box 1.

Finally, a note concerning pumps: The type of pump need not be peristaltic or driven by a motor. To follow this protocol, however, the pump must be able to move 5 L of water through a fine and often fairly clogged filter many times over the course of a day's sampling. As time spent pumping will be a significant portion of the total sampling time, a pump must be reasonably powerful. If you choose a peristaltic pump, we have found that size 24 (interior diameter = 0.25 inch [6.35 mm]) silicone tubing works well. Smaller tubing slows down water flow whereas larger tubing can collapse due to high vacuum pressure created when filters clog.

If you build your own kits, we suggest using rechargeable 12-volt lithium batteries. We recommend 20+ ampere-hour batteries. These batteries are lightweight and reliable even in low temperatures and will hold enough charge to sample over 30 sites.

Specifications for Sampling Equipment and Materials

We do not endorse any specific brands, but we do list the materials in the kits that we supply**.** We have included the precise filter holder and filter types that we use. Using other filters or filters of different diameters will lead to different eDNA capture efficiencies.

Equipment and Materials

Pump: Portable electric peristaltic pump with a pump head that allows easy removal/adjustment of the plastic tubing. Pump specifications for the pumps we provide are: DC external power source, 12 to18 volts @70 watts, maximum 600 rpm, liquid delivery rate of 1.67 ml per revolution.

Battery: 12-volt lithium ion battery and charger. We recommend a 20+ ampere-hour battery.

Tubing: Size 24 silicon tubing, cut to desired length (minimum 4 m [12 feet] recommended).

Filter holder: Nalgene™ Analytical (Thermo Fisher Scientific Inc., Waltham, MA) test filter funnels (holds a 47-mm-diameter filter; pack of 50 comes with adapters for tubing connection).

Filters: Whatman® 1827-047 Glass Microfiber Binder-Free Filter (Thermo Fisher Scientific Inc.), 1.5 µm, 3.7 s/100 ml flow rate, Grade 934-AH, 47 mm diameter.

Forceps: We recommend plastic forceps because they tend to be cheaper than metal forceps and are more robust against corrosion from repeated cleaning and field use. We have also found that flat or blunt-edge forceps allow for the best dexterity when handling filters.

Silica desiccant: We use roughly 30 g (1 ounce) of silica gel beads to store filters between field collection and laboratory analysis. We recommend using beads with a humidity indicator to assess the viability of the silica gel for keeping samples dry.

Plastic bags: Any household brand of sealable plastic bags will work for packaging materials for field use. We use a 1-gallon (4-L) bag to hold the contents of a site kit, and smaller sizes to package the filter assembly and the forceps. For storing the eDNA filters in silica desiccant, we recommend using heavy-gauge, 1-pint (0.5-L) or 1-quart (1-L) freezer bags that are durable and seal more tightly than regular bags, preventing moisture entry and DNA degradation.

Kit Assembly and Cleaning

Assembly of all sampling materials should occur in a room that is clean and free of any extracted DNA or PCR products. Ideally, the target species of your eDNA sampling will have never been present inside this room. We assume that plastic bags and factory packaged instruments are free from target DNA when new. Always wear clean gloves when assembling kit materials. A clean lab coat is also a good idea, particularly if you have recently been in a laboratory with PCR products or other high concentrations of DNA. When reusing equipment and tools, clean all forceps and filter cups in a manner that will destroy all DNA before each field use. Our protocol is to soak them in a 50 percent solution of household chlorine bleach for 20 minutes, rinse with deionized water, and allow to air-dry on a clean surface or drying rack.

Appendix B—Cautions About the Use of eDNA Sampling

For assessment and monitoring of wild populations, eDNA sampling has been seen as a breakthrough because of its efficiency, specificity (and therefore extremely low rates of misidentification), and extreme sensitivity (e.g., Wilcox et al. 2016). Fundamentally, however, eDNA sampling should be regarded as a sampling tool for detecting species, and like all tools is more useful for particular applications. Often, addressing questions about wild populations requires that individuals be observed or handled or that tissues be collected; such questions cannot be answered by using eDNA sampling. Also, if a target organism is extremely easy to find and identify, other methods may be more efficient. Moreover, even the strength of eDNA sampling—the indirect assessment of species presence—has limitations.

An eDNA sample contains no information about which members of a population (e.g., adults, juveniles, or individuals with particular life histories) produced the DNA. This means that eDNA sampling can be uninformative about the age or size structure of a population. In some instances, however, this limitation can be overcome if the timing or location of suitable habitats for a species is unique to a subset of a population. For example, because juvenile bull trout are thermally constrained to very cold water temperatures, and adult bull trout typically occupy such habitats only during the spawning season, sampling within such habitats can lead to strong inference about the likely presence of juveniles of this species (Isaak et al. 2015).

As currently practiced, eDNA detection of an organism relies on capturing and amplifying short fragments of that organism's mitochondrial DNA. Thus, it shares many of the advantages and limitations of DNA barcoding for species identification (Hebert et al. 2003). For example, only groups with substantial genetic differences—such as species and perhaps subspecies—can be identified, and recently evolved species that differ little from their sister taxa may be indistinguishable based on eDNA sampling. By contrast, haplotype variation among populations within a single species may render an eDNA assay less efficient or completely unable to identify all populations of its target species (Wilcox et al. 2015). This situation may require the development of additional eDNA assays for sampling where distinct populations may be encountered.

Furthermore, because mitochondrial DNA is maternally inherited and does not recombine during mating, eDNA sampling may be of little use in evaluating hybridization. However, hybridization among some pairs or groups of species may be commonplace, particularly among fishes (Scribner et al. 2011). Thus, it may be reasonable to infer that if the eDNA of two potentially hybridizing species is present, their hybrids may also be present.

The quantity of eDNA can, to a certain extent, indicate the relative abundance of a single species through space and time. Wilcox et al. (2016) found, for example, that the number of eDNA copies in a sample was reasonably correlated with fish numbers immediately upstream from the eDNA sampling location, and a number of other studies have linked eDNA quantity to the local abundance or biomass of individuals (see Barnes and Turner 2016). This level of correlation may be sufficient in many biological contexts, but

we caution that eDNA quantity is an index of animal abundance that is uniquely sensitive to the proximity of individuals of the target species. There is also substantial uncertainty about how environmental variables, such as discharge, water chemistry, water velocity, channel roughness, temperature, or productivity, influence eDNA abundance and persistence. Much more work is needed to assess these variables.

The bulk of our research has been directed at assessing drift-feeding salmonid fishes in relatively small mountain streams. Our inferences about detection rates are restricted to those environments and to our field and laboratory protocols. Not all species share similar rates of detection; different environments will dictate different eDNA sampling designs, and field and laboratory practices have a dramatic influence on eDNA amplification. As a result, we believe that it is unwise to generalize about species detection rates using eDNA sampling and that further studies are needed to understand detection rates across taxa and ecosystems.

In conclusion, we believe eDNA sampling is most advantageous when the target species is scarce or cryptic, or alternative sampling methods problematic (that is, they are extremely laborious, have low detection rates, or are excessively stressful or disruptive to the target species). Some of the most powerful applications of eDNA sampling may occur when it is coupled with traditional methods for biological assessment. We can envision using eDNA sampling to locate habitats occupied by a particular species, followed by targeted electrofishing to evaluate the size, age structure, and condition of a population and its community. In turn, genetic analyses of tissues from the captured individuals could yield information on isolation, effective population size, and hybridization.

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