eDNA PROTOCOL

SAMPLE COLLECTION FROM MEADOWS

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INTRODUCTION

This protocol was developed during a project that sought to test the effectiveness of the recently developed Smith-Root™ eDNA Sampler backpack (Thomas et al. 2018) and self-desiccating filter packs (Thomas et al. 2019) in montane meadows (Pope et al. 2020). The backpack Sampler was designed to overcome limitations of traditional peristaltic pump methods (e.g. Laramie et al. 2015) by reducing filtering time, contamination risk, and variability in sample quality (Thomas et al. 2018). The backpack is portable, battery-powered, and contains a negative pressure inline filtration system that allows for efficient collection of eDNA samples from a variety of aquatic habitats. Flow rate, sample volume, and filtration pressure can be programmed and recorded for each sample collected (Thomas et al. 2018). In addition, Thomas et al. (2019) developed self-desiccating filter packs that contain and preserve eDNA samples without the need for removing, folding, and placing filters in ethanol-filled vials in the field, potentially increasing collection efficiency and decreasing contamination risk for eDNA samples.

While promising, the effectiveness of the Smith-Root™ eDNA Sampler backpack and self-desiccating filter packs had yet to be demonstrated for many sampling applications. eDNA sampling is also highly sensitive to differences in sampling and analysis protocols as well as site characteristics that may influence detection (Goldberg et al. 2016), so pilot studies are recommended for each new application to assess detection probabilities for target species. During the Pope et al. (2020) study, a protocol was developed to maximize the site-level detection of rare amphibians in complex Sierra Nevada meadows, with a standardized sampling design, sample volume, and collection and preservation methods. This protocol has been adapted for general use with a variety of aquatic habitats and target organisms.

Pope et al. (2020) found that rare target species could be efficiently and accurately detected in complex mountain meadow habitats using the eDNA Sampler backpack. The Sampler backpack allowed for highly efficient sampling by filtering larger volumes of water more rapidly than traditional hand-pumping methods. eDNA detection did not differ between self-desiccating filters and the traditional method of sample preservation in ethanol. The authors noted, however, that large, complex habitats require thorough visual and eDNA surveys to yield accurate assessments of occupancy for rare species. Knowledge of the life history and habitat relationships of target taxa is also essential to ensure that samples can be collected from the most suitable localities.

MATERIALS

1. Smith-Root™ eDNA Sampler backpack lithium combo (includes pack frame, tubing, lithium battery, and charger; Figure 1)
2. Smith-Root™ eDNA Sampler pole (Figure 1)
3. Optional: dual filter mount and tube splitter (for taking duplicate samples; Figure 1)
4. Smith-Root™ eDNA Sampler remote control (can be attached to the mount on the Sampler pole or a wristband; Figure 2)
5. Smith-Root™ eDNA 1.2 µm filter pack (contains filter housing loaded with filter, forceps, and tube extension) **or** Smith-Root™ eDNA 1.2 µm self-preserving filter pack (contains filter housing loaded with filter and tube extension; Figure 3)
6. Latex or nitrile gloves (non-powdered; Figure 4)
7. Ethanol-proof laboratory pen (do not use a regular Sharpie marker; Figure 4)
8. High quality, o-ring screw cap 2mL tubes (e.g., Sarstedt brand) with 1mL 100% molecular-grade ethanol (not denatured; Figure 4)
9. 1 gallon distilled water (for negative control sample) and clean plastic container(s) (at least 1 gallon capacity; **do not bleach**; Figure 5)
10. Water, bleach, scrub brush, rubber gloves, and plastic tubs (for decontaminating between sites; Figure 5)

Figure 1. eDNA Sampler backpack (1), eDNA Sampler pole (2), and dual filter mount with tube splitter (3).

Figure 2. eDNA Sampler remote control (4).

Figure 3. eDNA 1.2 µm filter pack (5a) and eDNA 1.2 µm self-preserving filter pack (5b).

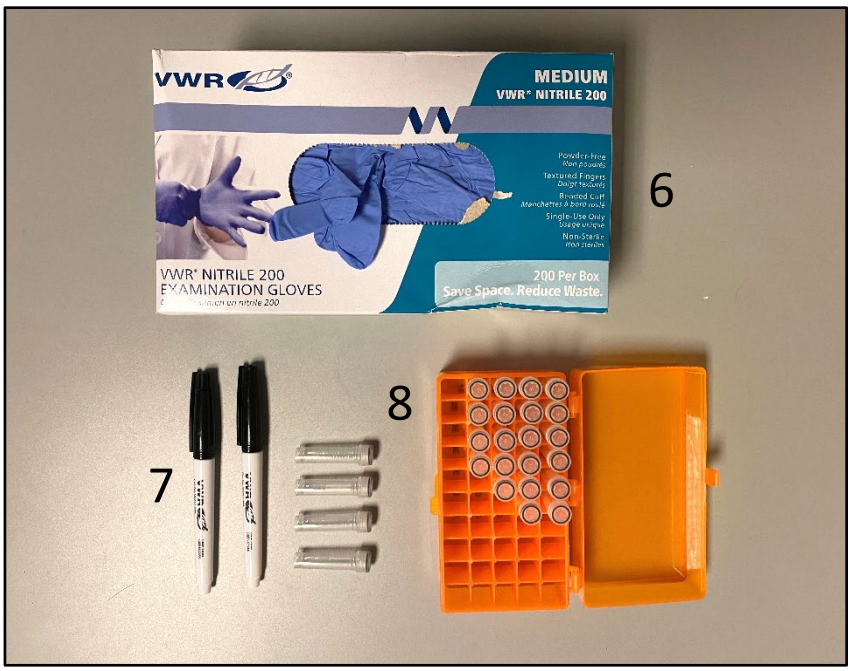

Figure 4. Sample collection materials: latex or nitrile gloves (6), ethanol-proof laboratory pens (7), and o-ring screw cap 2mL tubes containing 1mL 100% molecular-grade ethanol (8).

Figure 5. Negative control materials: 1 gallon distilled water (9a) and clean plastic container (9b). Decontamination materials: bleach (10a), scrub brush (10b), rubber gloves (10c), and plastic tubs (10d).

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.
2. Guidelines for wearing gloves:
    - Keep gloves in zipped 2.5 gallon plastic bags and work gloves out of the box while holding the outside of the bag, ensuring that only the inside of the bag touches the gloves. If gloves touch items that are not “clean”, they are no longer “clean” and should be disposed of.
    - Wear clean gloves when attaching tubing extensions to filter housing units.
    - Wear clean gloves when removing filters and placing in ethanol storage tubes. Do not touch anything other than the filter or disposable forceps before you handle the filter. If your gloves touch anything that you’re not certain is clean, replace them with clean gloves. Change gloves between handling different filters.
    - You do not need to wear gloves when handling the outside of the filter housing unit, tube splitter, and eDNA Sampler tubing, 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. Ensure that eDNA filter packs are not punctured.
4. If using eDNA 1.2 µm filter packs (not self-preserving) that come with disposable forceps: do not re-use forceps, discard after use.
5. Use caution to avoid cross-contamination between different sampling locations at the same field site.
    - Avoid getting in the water if possible.
    - Try to keep all parts of the sampling apparatus other than the tubing extensions attached to the filter housing units out of the water.
6. Avoid handling target species while engaged in eDNA sample collection.
7. Avoid entering water while engaged in eDNA sample collection.
8. Clean and decontaminate boots and equipment between sites using a 10% bleach solution and a tap water rinse (Figure 6). All items should be in contact with bleach solution for at least one minute before rinsing.

- NOTE: remove all dirt, mud, pebbles, etc. prior to decontamination
- Pump and tubing:
    1. Pump bleach solution through instrument, then rinse.
    2. Remove tubing and decontaminate, along with the end of the sampling apparatus that holds the filter housing units.

9. To test for field contamination, collect 1 field negative before entering each site (Figure 7). 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 (clean bottle or plastic tub that has not been bleached) with distilled water. Using methods for filtering samples as described in Step 4 below, filter the same volume of distilled water as the volume of samples. Remove and preserve filter as described in Step 5 below.

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

Figure 7. Collect 1 field negative before entering each site by filtering and preserving distilled water using the same equipment and procedures as the water samples.

SAMPLE COLLECTION

Step 1. Sample Site Selection

1. Knowledge of the target species' ecology should always be used to select sampling locations in the habitats likely to be used by the species.
2. The Smith-Root™ eDNA Sampler backpack allows for the collection of eDNA samples at single points or along transects in a variety of habitats.
    - 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 boat[1] will increase detection.
    - Distribution of eDNA in streams is also likely to be heterogeneous. Samples can be collected from the stream margin, thalweg, or, in larger streams, from a 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.
    - When sampling in meadows, it is important to sample all the habitat types present within the meadow (stream channels, backwater, off-channel pools). At least one sample should also be taken at the bottom of the meadow, where the primary stream leaves the meadow habitat, to capture eDNA that has collected there from upstream (Pope et al. 2020).

[1] Boats that are used at multiple sites should be decontaminated between sites, as with other field gear.

Step 2. eDNA Sampler Backpack Setup[2]

1. Charge the backpack unit battery. Mount charged 12v battery in the battery tray (behind lower dry bag), connecting to power with the slide connectors.
2. Make sure that fresh AA batteries are in the remote control unit.
3. Connect long tubing to the "IN" port on the right-hand side of the Sampler backpack (if facing display) and run through the guides on the pole. Connect short length of tubing to the "OUT" port on the left-hand side of unit.
4. If collecting duplicate samples, attach the double mount to allow use of two filter housings simultaneously. Securely attach the tube splitter to the intake tubing.
5. Open the water-tight box on the backpack Sampler and power it on (you will hear 1 beep on startup), waiting 15 seconds for the system to boot up.
6. Set volume limit, flow limit, and pressure limit using the blue buttons next to the display panel (Figure 1). When finished, return to home screen using the back arrow.
    - The pressure and flow limits are thresholds. The motor controller will modulate pump speed to stay below these values.
    - The volume limit is used when the system is in Auto Mode to alert you when to remove the filter from water in order to attain your desired sample volume. In manual mode there is no volume-based alert.
7. Choose Auto or Manual Mode.
    - The sole difference between modes is that in Auto Mode you will be alerted by 2 beeps when to remove the filter from the water to in order to achieve your volume limit. If using Manual mode, you can zero out the Volume Filtered by holding down the start/stop button for 3 seconds. If using Auto mode, the Volume Filtered will zero out each time the start/stop button is depressed.
8. If needed, adjust the Volume Offset.
    - This value accounts for the fact that water volume is measured in the pump apparatus, not at the point of filtration. When the system is in Auto Mode, the volume offset is used to indicate when to stop collecting water in order to achieve the desired sample volume. The appropriate Volume Offset differs between the 2 filter configuration and the one filter configuration.

[2] Details regarding operation of Smith-Root eDNA samplers vary between models; make sure you consult the appropriate documentation for the model you are using. This protocol is based on Beta release eDNA sampler.

Step 3: Filter Assembly

1. Remove the filter housing and the tube extension from the filter pack. Attach the tubing extension to the filter housing. If collecting duplicate samples, repeat with the second filter housing.
    - To avoid contamination, this step is best achieved with two people. One person opens the filter pack and holds it open while the other person, wearing clean gloves, removes the filter pack contents and assembles them.
2. Put one filter housing into one of the mount's clamps and tighten the clamp to secure it. Attach the intake tubing tightly to the filter housing. If collecting duplicate samples, repeat for the second filter.
    - Make sure the tube extension is facing away from the remote control mount on the sample pole.

Step 4. Water Collection and Filtration[2]

1. Place the end of the tubing extension(s) (attached to the filter housing(s)) in the water and begin sampling by pressing the Start/Stop button on the eDNA Sampler remote control (Figure 8).
    - Start walking a transect when water starts entering the tubing extensions and adjust the rate of movement to maintain consistent sampling.
2. Continue filtering until a stopping point is indicated by: a) the system beeps 2 times indicating you need to remove the tubing extensions from the water to achieve target volume (Auto Mode), b) you have achieved your desired sampling volume (Manual Mode), or c) the system beeps 5 times indicating that flow has dropped below a registerable level.
    - Raise the elevation of the filter(s) to help to clear the line of any remaining water and dry the filter membrane(s) (Figure 9a). Under most circumstances you will hear a low flow alarm (5 rapid beeps) after 10 seconds when the tubing line is sufficiently cleared. If collecting duplicate samples and one of the eDNA filters is clogged (water is not passing through it), clear the line by pinching the tubing connected to the other filter which will increase pressure on the clogged filter (Figure 9b).
3. Turn off the pump using the Sampler remote control Start/Stop button.

Figure 8. Collect eDNA samples by placing the end of the tubing extensions into the water and pressing the Start/Stop button on the Sampler remote control.

Figure 9. After sampling, raise the elevation of the filters to help to clear the line of any remaining water (a). If one of the eDNA filters is clogged, pinch the tubing connected to the other filter to increase pressure to help clear the line (b).

Step 5. Filter Membrane Preservation

1. Lean the pole up against a sturdy object with the mount inverted so that the tubing extensions are pointing up.
2. If using self-preserving filters:
    a. Loosen the clamp to release the filter housing, and carefully detach the filter housing from the intake tubing and extension tube.
    b. Put the entire intact filter housing back in the original filter pack and ensure that the pack is sealed. Label the sample pack with sample ID and date.
    c. Discard the extension tube.
3. If using non-self-preserving filters:
    a. Grasp the bottom of the filter housing in one hand and top of the filter housing in the other. Gently lift the top of the filter housing by the tab on the side to disconnect it from the bottom, exposing the 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. Repeat with the second housing if collecting duplicate samples.
    b. Open 2 mL o-ring tube to prepare for filter.
    c. Wearing clean gloves, use the forceps from the filter pack to fold the filter membrane in quarters by folding it in half (with the filtrate on the inside of the fold) and then in half again (Figure 10a). Still using the clean glove, roll the folded filter membrane into a cylinder (Figure 10b).
        - To avoid contamination, this step is best achieved with two people. One person opens the filter pack and holds it open while the other person, wearing clean gloves, removes the forceps and folds the filter membrane.
        - Do not touch anything other than this filter membrane with these gloves and the tips of these forceps.
    d. Slide the folded filter membrane into a 2 mL vial filled with 1 mL ethanol (Figure 11), then push it down to the bottom of the vial using one arm of the forceps.
    e. Cap vial firmly and label with sample ID and date, using an ethanol-proof marker. Label cap with sample ID. Remove glove.
    f. Discard the extension tube, filter housing, forceps, and gloves.
    g. If collecting duplicate samples, repeat with the other filter.
    h. 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.

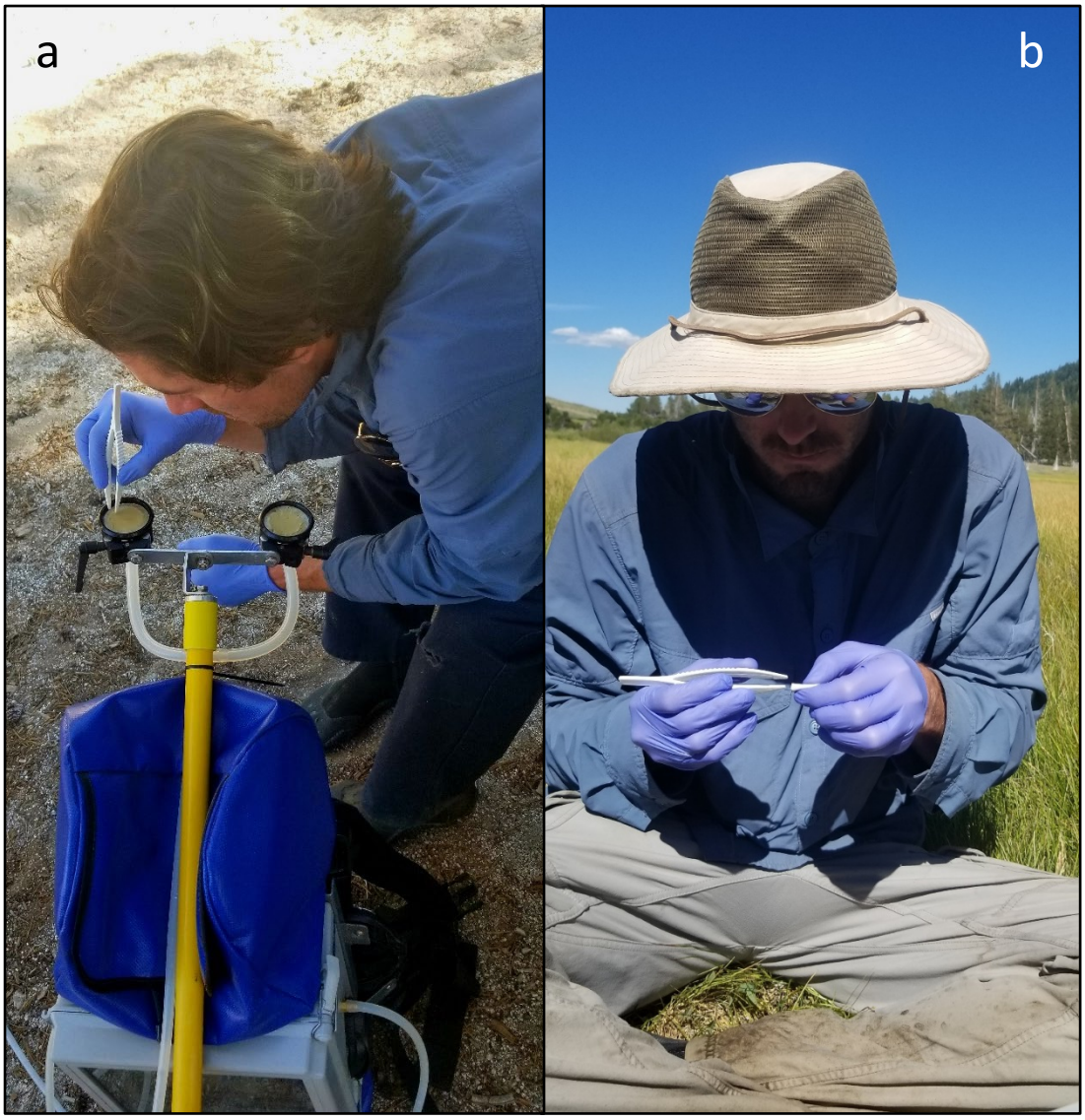

Figure 10. If using regular (non-self-preserving) filters, carefully remove the top of the filter housing and then use clean gloves and forceps to fold the filter membrane in quarters, with the filtrate on the inside of the folds (a). Roll the folded membrane into a cylinder (b).

Figure 11. If using regular (non-self-preserving) filters, place the folded filter membrane into a 2 mL vial filled with 1 mL ethanol.

eDNA SAMPLER TROUBLESHOOTING

System cannot achieve prime (water does not reach pump)
The particulate load in the water may be too high for the given filter pore size, the max pressure is set too low, or head pressure is too great. First, try lowing the elevation of the pump. Next, try increasing the pump pressure. If you still cannot achieve prime, try a larger pore size filter.

Filtering below Flow Limit, and Pressure is not increasing up to Pressure Limit
Check the tubing is well connected to “IN” port. If the max achievable pressure seems low, there is likely entrained gas in the pump. Try increasing the initial flow rate, and ensure that the system is sealed (no air bubbles in the line).

Remote control display light is flickering or remote not communicating with pump
Change remote control batteries. It’s advised to carry spare AA batteries and small screwdriver when sampling.

Pump shuts down in the middle of a run
This can occur if the backpack loses communication with the remote device on the pole. Try changing the batteries in the remote device, as low battery levels can be the cause of the lost connection.

CITATIONS

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