eDNA PROTOCOL
SAMPLE COLLECTION

Caren Goldberg and Katherine Strickler, Washington State University
Revised January 2017

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 100% 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

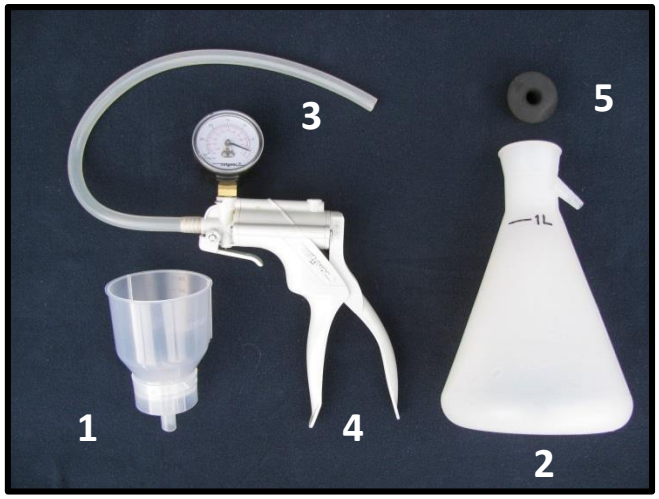

Figure 1. Filter funnel (1), vacuum flask (2), silicone tubing (3), vacuum pump (4), and rubber stopper (5).

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).

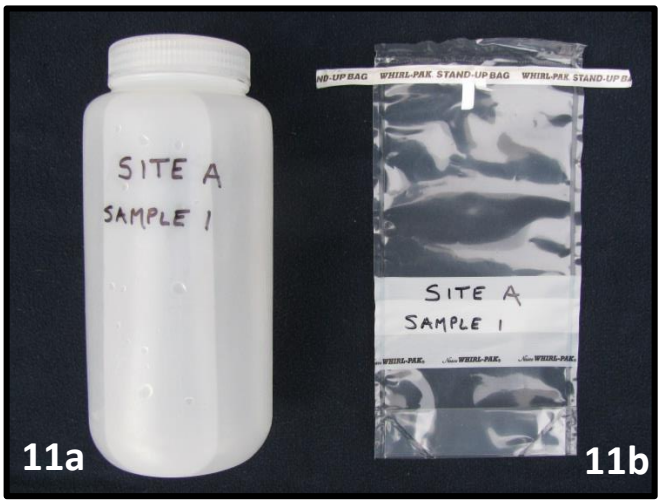

Figure 3. Polypropylene grab bottles (11a) and Whirl-Pak® bag (11b).

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.

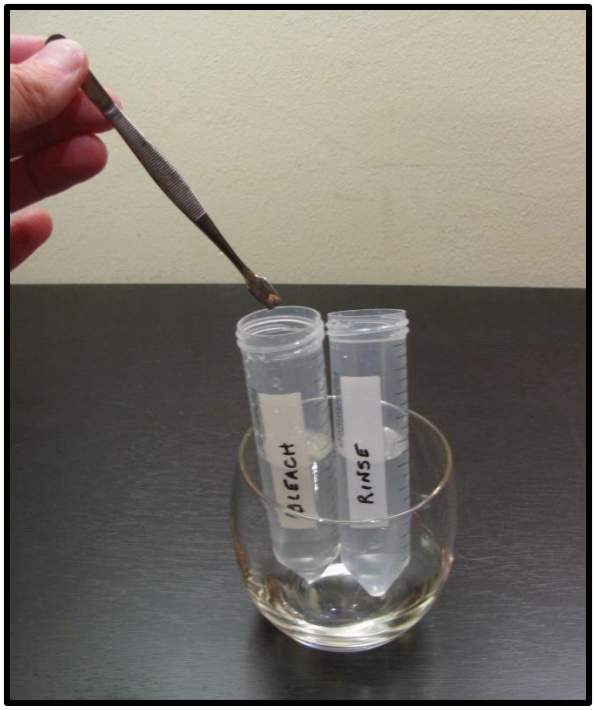

Figure 5. Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled water.

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.