Northeast Hellbender RCN – eDNA Protocol

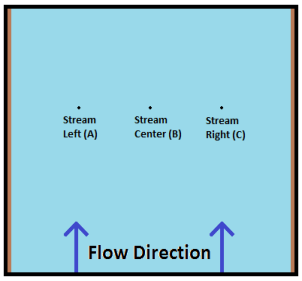
Updated 12 March 2015

Disinfection

- Prior to sample collection at each site, all equipment (collection bottles, coolers, flasks, stoppers, etc) must be washed and soaked in **10% bleach for 30 minutes**. This equipment can be rinsed with tap water.
- Tweezers should be disinfected with **50% bleach for 30 min** and rinsed with distilled/deionized water. Alternately, single-use tweezers may be used (see equipment list).
- Collection bottles should be rinsed an additional **3 times in stream water** immediately before samples are collected.
- Gloves should be changed between sites. Additionally, change gloves immediately before handling filter papers.

Water Collection

- Samples should be collected **50 m downstream of the location where hellbenders are suspected to occur.** If there are multiple sites within 200 m of one another, collect a sample at the downstream site only.
- Ideally, samples should be collected from pools or areas of low stream flow, as DNA tends to accumulate in these places.
- Enter downstream from where samples are going to be taken. When collecting samples, face upstream with the bottle in front of your body to avoid contamination. Rinse each collection bottle 3 times with stream water.
- Collect 3 samples (**2 liters each**) at the site, for a total of 6 liters. Collect one sample in the center of the stream (sample B) and the other 2 samples equal distance from the center and shore (samples A and C). If river volume restricts sampling to one bank, collect all 3 samples from this bank. Make sure collection bottles are labeled so you can keep track of the samples.



- Cap bottles immediately. Filter on site OR dry the outside of each bottle, place it in a cooler on ice, and filter within 48 hours.
- Be sure you have a negative control to filter at the same time as the sample (see below).

Stream Data Collection

Record the following information at each collection site using the standardized datasheet in Dropbox:

- Date, time, crew names, institution, state, site name, GPS coordinates, current weather, and any rain events in the last 7 days
- Habitat quality (i.e., river substrate)
- Average stream width, depth, turbidity, and flow rate
- Water temperature, pH, TDS, and conductivity (measured near stream center)
- Stream depth and flow at each of the 3 spots where samples were collected
- Labels for each sample
- Label and source of negative control

Filtration

- Assemble the filtering apparatus;
    - Attach the hose to the side arm of the filtering flask. Make sure it is a tight fit. Attach the other end of the hose to the 'vacuum' valve of the vacuum pump. Make sure that this is also a tight fit.
    - Place the small plastic filter cup adaptor (comes with the MoBio water filters) in to the ¼" hole of the rubber stopper (Fisher Scientific Rubber Stopper No. 9) and place rubber stopper on top of the filtering flask.
    - Attach a new sterile filtering cup with 0.45µm pore membrane to the filter cup adaptor.

- Once assembled, slowly fill the filtering cup with collected water and turn on the vacuum pump. Continue pouring the water sample into the cup until the entire 2-liters are filtered.
- Remove the filtering cup from the flask and carefully separate top of the cup from the bottom. Using sterile tweezers, carefully remove the filter paper from the filter cup.
- Using the tweezers, gently fold the filter paper in half repeatedly until it fits in the 1.5mL tube. Fill tube with 95% ethanol and store in freezer.

Extraction

Day 1

1. Wipe the bench you'll be working at with 50% bleach.
2. Put on a new pair of latex or nitrile gloves.
3. Set a paper towel on the dry bench and place two pairs of sterile tweezers on the towel.
4. Place the 3 replicate samples from one site in a rack on the paper towel, along with the corresponding negative control.
5. Using sterile tweezers, remove the NEGATIVE CONTROL filter paper from the tube. Tear the folded filter paper in half. Place one half back in the original tube. Place the second half in a pre-labeled tube with 800 µl of 95% ethanol and close the cap.
6. Place the original tube in a drying rack (with the cap open).

7. Repeat steps 5-6 for the three replicate samples (you can use the same pairs of tweezers, as long as you start with the negative control).
8. Place your used tweezers in a beaker containing 50% bleach, or in a trash/recycling bin (if single use).
9. Repeat steps 1-8 for each site.
10. Place the extra filter paper halves back in the freezer.
11. Rinse the used metal tweezers, wipe off any rust with a dry paper towel, and autoclave.

Day 2

- Turn heat block to 55C.
- Add 180µl ATL to each tube.
- Add 20 µl ProK to each tube individually with a **filter tip**. Use the tip to push all the filter material down into the liquid at the bottom. It should be fully immersed but seem to take up the whole amount of liquid. Squish it around a little. **Vortex each one as you finish with that sample.** If there really doesn't seem to be enough liquid to cover the filter material, set it aside and top it off with ATL at the end.
- Incubate at 55 degrees and vortex a 1-2 more times that day. Incubate overnight.

Day 3

- Remove samples from incubator and turn up to 70C.
- Vortex samples 15s each.
- Set out Qiashredder spin columns and label the sides of the tubes.
- Move each sample to a Qiashredder spin column by moving the filter with sterile tweezers and pipetting the rest of the liquid. Try to get all of it.
- Spin Qiashredder columns 5 minutes at 8000rpm.
- Dispose of the shredder column containing the dry filter paper (KEEP the collection tube containing your liquid sample).
- Add 200ul Buffer AL to each sample. Cap each tube and vortex.
- Incubate at 70C for 10 minutes.
- Centrifuge tubes briefly to remove condensation from the caps.
- Remove caps and add 200µl 100% ethanol to each tube, vortexing **immediately** each time (without caps on the tubes).
- Transfer the mixture to a Qiamp Spin column using a **filter** pipette tip. Label the top of the tube and centrifuge at 8000rpm for 1 minute.
- Place the filter in a new collection tube and discard the old collection tube (including the liquid).
- Add 500 µl AW1 to the Qiamp Spin column of each sample. Centrifuge at 8000 rpm for 1 minute.
- Place the filter in a new collection tube and discard the old collection tube (including the liquid).
- Add 500 µl of AW2 to the Qiamp Spin column of each sample and spin at 11,000 rpm for 3 minutes.
- Carefully remove each filter and place in a sterile, pre-labeled microcentrifuge tube.

- Elute the DNA with 100 µl Buffer AE (preheated to 70C) added directly to the Qiamp Spin column of each sample. Incubate at room temperature for 5 minutes then spin at 8000 rpm for 1 minute. Allow 2 empty spaces between samples in the centrifuge to accommodate the open caps of the microcentrifuge tubes.
- Store at -80°C until ready for PCR.

PCR

- Reconstituting desalted primers and probe to a 100 uM stock solution.
    - To obtain a 100 uM stock solution take the number of nanomoles (nm) provided (information found on the tube label and/or the technical datasheet) and multiply the number of nanomoles by ten. The result provides the number of microliters of liquid to add to the tube for reaching a final concentration of 100 µMolar. Note that this is equivalent to a stock solution of 100 pmol/µL.
        1. Forward Primer (CRALQF): Add 496 µl of nuclease free water to forward primer CRALQF to obtain 100 uM stock solution
        2. Reverse Primer (CRALQR): Add 305 µl nuclease free water to reverse primer CRALQR to obtain 100uM stock solution
        3. Probe: Add 64 µl nuclease free water to the probe to obtain 100 uM stock solution

    - Mix primers and probes together so that the concentration of each primer is 8 uM and the probe is 4 uM.
        1. Take 5.12 µl of the 100 uM probe stock solution to make 4 uM in final solution
        2. Take 39.68 µl of the forward primer CRALQF 100 uM stock solution to make 8 uM in final solution
        3. Take 24.4 µl of reverse primer CRALQR 100 uM stock solution to make 8 uM in final solution
        4. Mix all 3 amounts together and vortex. This primer/ probe mix will be used for PCR.
- Standards, Negatives & Samples: A total of 8 standards of different concentrations as well as 2 negatives will be run in each PCR. A total of 31 samples including standards will be run in each PCR, unless otherwise stated. Standards are made using extracted blood in the following concentrations; 100%, 10%, 1%, 0.1 and 0.01%. The following table details the amount of each reagent and DNA for each of the standards, negatives, and samples per each capillary in every PCR run. (All volumes added in µl)

Standards	100%	10	1%	0.1%	0.01%	0 (Neg)	Sample
dH2O	2.85	2.85	2.85	2.85	2.85	2.85	2.85
Master Mix	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Primer/Probe	0.75	0.75	0.75	0.75	0.75	0.75	0.75

- "Pre-PCR Master Mix": Before each PCR run a 'Master Mix" of all of the reagents should be mixed together in volumes based on the amount of samples running, except for the DNA. 12 µl of this 'Master Mix' will then be pipetted into each capillary tube. DNA will be added individually to each capillary tube right before PCR is run. The following table explains how much of each reagent should be added to the 'pre-PCR Master Mix'(All volumes added are in µl)

Reagent	X1	X17	X18	X31	X32	X33
dH2O	2.85	48.45	51.3	88.35	91.2	94.05
Quantitect Master Mix	7.5	127.5	135	232.5	240	247.5
Primer/Probe	0.75	12.75	13.5	23.25	24	24.75

*3.0 µl DNA added to each capillary individually for a final volume of 15 µl.

- PCR Cycle: The PCR cycle should start with an initial denaturing step of 95 °C for 15 minutes. This 15 minute hot start will also help activate the enzyme in the reaction. Following this there will be 50 cycles of a 60 second denaturing step at 94°C and a 60 second annealing step at 60°C.