

Environmental DNA Sampling Protocol

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Collection and Laboratory Protocols

1. Sample Collection and Filtration

Avoiding contamination of samples is extremely important for obtaining reliable eDNA results. Samples should always be handled while wearing nitrile gloves, and gloves should be changed between each sample when filtering multiple samples. Nalgene containers should be washed with a 10% bleach solution after filtering and rinsed thoroughly with water before reusing.

1. Collect samples by submerging sterilized, labeled Nalgene containers in water or placing them so that water flows or drips into container (depending on water source). Once containers are filled, store in cool, dark place until filtering. Samples should be filtered as soon as possible and within 24 hours of collection.
2. Connect peristaltic pump to power source (plug into outlet or use charged battery pack if pumping in the field). Set Speed Control to maximum speed.
3. Run section of tubing through peristaltic pump head and place outflow end of tubing in sink (if filtering in lab), bucket or other container, or directly into water source (if filtering in the field). Ensure that the Reverse-Forward switch is turned to the direction of the outflow.
4. With gloved hands, open a filter pack and attach other end of tubing to filter housing. Attach straw to other end of filter housing, open Nalgene container, and rest filter pack on opened Nalgene with straw inserted into the water sample.
5. Turn on pump to begin filtering water. When water level in Nalgene becomes low enough that pumping with straw becomes difficult, remove straw and invert filter pack. Pour remaining water from Nalgene directly into filter housing to complete filtering.
6. If you are filtering more than one Nalgene container's volume of water through one filter (we recommend using at least three containers to maximize DNA yield), repeat process with remaining containers.
7. When water filtering is complete, crack the seal of the filter housing (do not open) and allow pump to run for 20 seconds to air dry filter membrane.
8. Reseal filter housing and remove tubing. Place filter housing back into wrapper, seal, label the wrapper with sample name and date, and store at room temperature until ready for DNA extraction.

2. DNA Extraction

This section of the protocol uses reagents and spin columns that can be found in a Qiagen DNEasy Blood and Tissue Kit.

1. Fill 5 ml centrifuge tube with 720 μ l Buffer ATL and 80 μ l proteinase K. Open filter housing and, using gloves and sterilized forceps, carefully roll filter and fold in half. Add filter to tube, centrifuge, and incubate in water bath overnight at 56 C.

2. Carefully transfer filter and lysate to QIAshredder (with collection tube) and centrifuge at 16,000 RCF for 1 minute. Transfer flowthrough to original 5 ml tube and discard QIAshredder and collection tube.
3. Add 800 μ l Buffer AL to the 5 ml tube. Vortex and incubate in water bath at 70 C for 10 minutes.
4. Add 800 μ l 100% ethanol to 5 ml tube and vortex. Add mixture to spin column over a collection tube from Qiagen kit in batches (~600 μ l at a time for a total of 4 batches). For each batch, centrifuge for 1 minute at 16,000 RCF, and discard the flowthrough. Add next batch to the spin column and repeat. Discard collection tube after last batch.
5. Using a new collection tube, add 500 μ l Buffer AW1 to the spin column and centrifuge for 1 minute at 16,000 RCF. Discard flow through and collection tube.
6. Using a new collection tube, add 500 μ l Buffer AW2 and centrifuge for 3 minutes at 16,000 RCF. Discard flow through and collection tube.
7. Place spin column over an open, labeled 1.5 ml centrifuge tube and elute DNA using 80 μ l of nuclease free water. Incubate at room temperature for 10 minutes.
8. Centrifuge 1.5 ml tube with spin column for 1 minute at 16,000 RCF. Remove spin column and store DNA sample in 1.5 ml tube at -20 C.

3. Quantitative Polymerase Chain Reaction (qPCR)

Before beginning qPCR, choose the samples that you will be testing. This will determine the qPCR assay(s) (primer-probe sets) and the serial dilutions needed for the reactions. Assays are designed for particular target species (Table 1) and should be chosen to test for any possible species of interest present at the site of sample collection. Information about designing primers and probes can be found at <https://www.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes>. Separate master mixes are needed for each assay used. Serial dilutions contain standardized quantities of target species DNA and provide a means to estimate DNA quantity in test samples. A five-fold serial dilution should be used with each assay in a qPCR run. Each assay should also have one negative control (master mix with no DNA sample added).

Name	Forward Primer	Reverse Primer	Probe
<i>sosorum-nana</i>	AGGTGGAGTAATAGCCTTATTA GCCTCTAT	GAAATGACATGCTTCGGTG CT	CTATTCCAGCTATTCATAC
<i>tonkawae-chisholmensis</i>	TCTTCACGAAACCGGATCAAG	TGGGTGAAACGGGATTTT GT	AACCCAACAGGACTAAA
<i>rathbuni-waterlooensis</i>	CACATTTGCCGCGATGTAAA	TGACGCTCCGTTAGTGTGA ATATT	TACGGCTGACTTATGC
<i>naufragia</i>	CACGAAACCGGATCAAGCA	AACGGGATTTGTCTGGGT TAG	CCCAACAGGACTAAAC
sp. 2	CACTAATTACACCCACACATT C	CGAAGGATTGCGTAGGCA AA	ACCAGAGTGATACTTCT

sp. 3	GCAACTCACCCTGGTTTT	TGCTGACCCCTGCAATTAT AAA	CTTCCACTTTATTTTGCC
sp. 1	TCCATGAAACCGGATCAAACA	GAATGGGATTTGTCTGG GTTAGA	CCCAACAGGAATTAA
<i>trogodytes</i>	ACTAACTACACCCACACATTC A	CGAAGGATTGCGTAGGCA AA	CCAGAGTGATACTTCTT
<i>latitans-neotenes- pterophila</i>	CACGAAACCGGGTCAAACA	GAATGGGATTTGTCTGG GTTAGA	CCCAACAGGAATTAA
<i>E. sp. Georgetown</i>	TTCACGAAACCGGGTCAA	GAATAATATGGGTGGAATG GGATT	TAGGTTTCGAGTTAATCCTGTT GGTTGC

Table 1. Primer and probe sequences for all Central Texas *Eurycea* species and clades.

1. Draw a plate map layout with each well identified by assay and sample, serial dilution, or negative control.
2. Fill a bucket with ice. Remove samples, serial dilutions, and primer and probe working solutions from freezer. After thawing, place on ice until ready to return to freezer.
3. Use the master mix formula worksheet (Table 2). Calculate the volumes needed for each component of the master mix. This will be determined by counting the number of samples to be tested and adding seven (five stages of the serial dilution, one negative control, plus one extra to account for pipetting error). Multiply this number by each volume in the master mix formula. Combine master mix ingredients in a 1.5 ml centrifuge tube and mix by pipetting. Spin down briefly in a mini centrifuge.
4. Pipette 18 μ l of the appropriate master mix into each well as designated in the plate map layout. Add 2 μ l sample DNA or serial dilution DNA into the appropriate wells. Do not add DNA to the negative control wells.
5. Place adhesive seal over qPCR plate and ensure that each well is well is securely sealed. Tap plate against counter to remove any bubbles and spin down in a centrifuge with a plate spinner.

Master Mix Formula Worksheet		
Single Sample Volume (SSV)	Component	SSV x (No. Samples + 7)
10 μ l	TaqMan Fast Advanced Master Mix	
1.6 μ l	forward primer (10 μ M)	
1.6 μ l	reverse primer (10 μ M)	
2 μ l	probe (2 μ M)	
2.8 μ l	Nuclease free H ₂ O	

Table 2. Worksheet for calculating the Master Mix volumes for a qPCR run. For each assay, single sample volumes should be multiplied by seven plus the number of test samples. This allows for sufficient master mix for samples, serial dilution, and negative control.

4. Reaction Setup Using StepOne Software

This manual provides instructions for running qPCRs with a StepOne Real-Time PCR System using a PC-controlled configuration.

1. Place PCR plate in the sample block with well A1 at the back left corner.
2. Open StepOne Software v2.3 on your connected PC. After logging in with your username, select File > New Experiment > Advanced Setup.
3. In the Experiment Properties screen, name your experiment in the “Experiment Name” field. In response to the next four questions on this screen, select “StepOne Instrument (48 Wells)”, “Quantitation – Relative Standard Curve”, “TaqMan Reagents”, and “Fast (~40 minutes to complete a run)”.
4. Navigate to the Plate Setup screen. Under “Define Targets”, enter the name of the target species for the first assay in your run in the “Target Name” field. If you are using more than one assay, select “Add New Target” and continue until you have named all target species (i.e., assays) in your run. Under “Define Samples”, enter your first sample name, then select “Add New Sample” and continue until you have entered all test samples in your run. Then select “Assign Targets and Samples”. Referring to your plate map layout, assign wells to the appropriate target species (assays) and sample names. For wells with test samples, select “U”. For wells with serial dilutions, select “S”. For wells with negative controls, select “N”.
5. Navigate to the Run Method screen. Enter the “Reaction Volume Per Well”, which will be 20 μ L for this protocol. Set “Number of Cycles” to 50 and keep all other cycling parameters at the default setting.
6. Navigate to the Run screen and select “Start Run”. After run is completed, remove PCR plate from sample block, discard plate, and check results on Analysis screen.

Equipment

1. We used a **Geopump II Peristaltic Pump** to pump sampled water through filters. This pump is available through Geotech Environmental Equipment (https://www.geotechenv.com/peristaltic_geopump.html) and can be ordered with pump head, modular battery, 5 ft. tubing, carrying case, and power cord.
2. We used **Smith-Root Self-Preserving eDNA Filter Packs** (<https://store.smith-root.com/collections/edna-filter-packs/products/edna-self-preserving-filter-pack>) to filter water samples and preserve DNA until ready for extraction. These packs absorb moisture from the filter membrane and prevent the need for cold storage, or storage in ethanol or buffers, making them ideal for projects that rely on citizen science sampling. For our study, we ordered packs containing 1.2- μ M polyethersulfone filter papers.
3. The eDNA extraction protocol uses reagents and spin columns from the Qiagen **DNeasy Blood and Tissue Kit** (<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit>).

Because the eDNA protocol uses more Buffer ATL, proteinase K, and Buffer AL than the kit's protocol requires, you will also need to order these reagents individually. Buffer ATL and Buffer AL can be ordered individually from Qiagen, and proteinase K can be ordered from Thermo Fisher Scientific.

4. The **QIAshredder** from Qiagen (<https://www.qiagen.com/us/products/instruments-and-automation/accessories/qiashredder>) uses a biopolymer-shredding system in a microcentrifuge spin-column format to homogenize samples and reduce loss of sample material. We used this product in our DNA extractions to maximize yields from the filter before discarding it.
5. We used the **TaqMan Fast Advanced Master Mix** (<https://www.thermofisher.com/order/catalog/product/4444556?SID=srch-srp-4444556>) in qPCR reactions. This reagent includes buffers, dNTPs, and DNA polymerase necessary for amplification of DNA and facilitates faster reaction times.
6. We used the **StepOne Real-Time PCR System** (<https://www.thermofisher.com/order/catalog/product/4376357>) with a PC-controlled configuration to run qPCRs. **MicroAmp Fast Optical 48-Well Reaction Plates** (<https://www.thermofisher.com/order/catalog/product/4375816>) and **MicroAmp 48-Well Optical Adhesive Film** (<https://www.thermofisher.com/order/catalog/product/4375928>) are recommended for this system.

Storage, Handling, and Shipping

eDNA Filter Packs

The Smith-Root Self Preserving Filter Packs can be stored at room temperature for up to six months before DNA extraction. Before shipping, ensure that filter pack wrappers are labeled and completely sealed. If shipping in warm conditions, we recommend packing with cold packs.

Extracted DNA

Extracted DNA should be stored at -20 C before running qPCRs and at -80 C for longer term storage. When shipping DNA samples in 1.5 ml tubes, ensure that all samples are clearly labeled and that caps are shut tightly. Samples should be frozen, packed in cryo/freezer boxes, and shipped with dry ice.

eDNA Laboratories

The following is a list of laboratories that can provide eDNA consultation and/or services.

eDNA and Genomics Core Facility at Cornell University (<https://genomics-edna.cornell.edu/>)

Pacific Northwest eDNA Laboratory (<https://www.usgs.gov/labs/pacific-northwest-environmental-dna-laboratory>)

U.C. Santa Cruz Genomics Institute (<https://genomics.ucsc.edu/conservation-genomics/>)

University of Maine eDNA Laboratory (<https://umaine.edu/ednalab/services/>)

U.S. Fish and Wildlife Service Regional Geneticists and Conservation Genetics Labs
(<https://www.fws.gov/node/378526>)