



Jul 25, 2025

Multi-tool Monitoring: eDNA Sample Collection and Processing Protocol: Water, Tree roller, Soil and Scat sampling

DOI

<https://dx.doi.org/10.17504/protocols.io.eq2lyqm8mvx9/v1>

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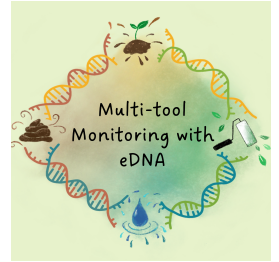
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eDNA, acoustics, cameras



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Protocol Citation: Clare Cowgill 2025. Multi-tool Monitoring: eDNA Sample Collection and Processing Protocol: Water, Tree roller, Soil and Scat sampling. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.eq2lyqm8mvx9/v1>



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Protocol status: Working

We use this protocol and it's working

Created: June 24, 2025

Last Modified: July 25, 2025

Protocol Integer ID: 220871

Keywords: eDNA, environmental DNA, rewilding, vertebrates, tree rolling, multi-method, assessing vertebrate taxonomic, dna protocols preparation of sample, vertebrate dna bioinformatics, vertebrate taxonomic, initial processing of environmental dna, environmental dna, filtering dna extraction, dna extraction, sample collection from water, dna protocols preparation, taxonomic assignment, scat contamination control practice, other terrestrial field study, trophic diversity, dna, integrated monitoring approach, sample collection, mitochondrial gene region, extraction

Abstract

This protocol describes the collection, extraction, and initial processing of environmental DNA (eDNA) samples used in a multi-tool vertebrate monitoring study in the Scottish Highlands, UK. Sampling was conducted in June 2023 with the aim to understand the comparative effectiveness of different sample-types.

The methods outlined here cover:

- **Sample collection** from water, soil, tree surfaces, and scat
- **Contamination control** practices throughout sampling and filtering
- **DNA extraction** using modified Mu-DNA protocols
- **Preparation of samples for sequencing**, amplifying the 12S mitochondrial gene region to detect vertebrate DNA
- **Bioinformatics** and **taxonomic assignment**, including quality control

This protocol is part of an integrated monitoring approach that also includes camera traps and passive acoustic recorders, with the goal of assessing vertebrate taxonomic, functional, and trophic diversity.

The procedures are modular and may be adapted for other terrestrial field studies involving eDNA.

Section 1: General Sample Collection Procedures

- 1 All sampling was conducted in June 2023 at the Natural Capital Laboratory rewilding site in the Scottish Highlands. The following general procedures were applied across all sample types (water, soil, tree rollers, scat):

1.1 Equipment and Materials

- Sterile nitrile gloves (changed between samples)
- 10% bleach solution (for surface and tool sterilisation)
- 70% ethanol (for surface and tool sterilisation)
- 5% lipsol detergent (for tool sterilisation)
- Distilled water (for field blanks)
- Bleach-sterilised cool bag for transport in the field

1.2 Preventing Contamination

- Wear sterile gloves for each sample and change them between samples.
- Sterilise all collection equipment and work surfaces between samples by wiping with 10% bleach solution, followed by 70% ethanol.
- For additional sterilisation, either of lab-based equipment or at the end of a day of sampling, a soaking protocol of 20 minutes in 10% bleach, 5 minutes in 5% lipsol and rinsed with distilled water was used
- For each day of sampling, take a field blank (sterile water) into the field and process alongside samples.
- If transporting all samples in the same sterilised cool bag, double-bag each sample and separate samples by their type (i.e. water, tree roller etc.) and also by the habitat taken from to prevent cross-contamination.

Section 2: Water Sample Collection and Filtration

- 2 The protocol for collecting water samples from relatively small water bodies (e.g. small rivers and streams, woodland drainage channels, puddles).

2.1 Equipment

For sample collection:

- Sterile nitrile gloves
- 10% bleach solution
- 70% ethanol solution
- Blue roll
- Sterile Whirl-Pak bags (Sigma-Aldrich, DE): 1 for each sample, plus extras to transport field blanks
- 100 mL sterile plastic beakers

For filtration:

- Manifold vacuum filtration units
- 0.45 µm cellulose ester membrane filters with pads (47 mm diameter, Whatman, GE Healthcare, UK)
- Sterile metal tweezers
- 5 mL sterile screw-cap tubes (Axygen, Fisher Scientific, UK) containing garnet beads: 1 g each of 0.15 mm and 1–1.4 mm size.
- -20°C freezer

3 **Sample Collection**

- Collect water (~1 L where possible) from any available site sources (e.g. puddles, drainage channels, streams or rivers)
- Use Whirl-Pak® bags to collect water directly from the source
- Where the water is too shallow to fill the bag, scoop with a sterile beaker
- Ensure a field blank is carried into the field in the same cool bag as samples
- Wipe the Whirl-Pak bag with bleach before placing in the cool bag and bring samples back for filtration and filtering within 24 hours of collection

4 **Sample Filtration:**

1. Set up manifold vacuum filtration equipment (ensuring sides have been wiped down with bleach and ethanol)
2. Place a Whatmann filter on the pad

4.1 **Sample Filtration:**

1. Pour 500ml of sample into the cup and run for 20 minutes
2. Add additional sample if all the initial water has been filtered within this time

4.2 **Sample Filtration:**

1. Following 20 minutes, tip away sample not filtered
2. Leave the vacuum pump running for an additional 10 seconds to dry the filter
3. Remove the cup and roll up the filter paper with sterile tweezers
4. Place the filter in a prepared 5ml sterile screw-cap tube, containing 1 g each of two garnet bead sizes

4.3 **Sample Filtration:**

1. Repeat steps 4.1 and 4.2 again and run the sample for a second time for 20 minutes
2. Insert the second filter within the first (rolled tighter) inside the screw-cap tube

4.4 **Store samples at -20°C for transport and future processing**

5 **Notes**

- Make a note of the total volume of water passed through both filters
- If less than 1L of water was collected, consider using less than 500ml for each of the filtration steps (i.e. using half the available sample each time)
- Ensure that the field blanks are filtered after filtering all other samples

Section 3: Soil Sample Collection

6 Equipment

- Metal teaspoons (sterilised by soaking in 10% bleach for 20 mins, then 5% lipsol detergent for 5 mins, then thoroughly rinsed with distilled water)
- Sterile nitrile gloves
- 50ml Falcon tubes (sterile and placed under a UV lamp for 20 minutes prior to sampling)

7 Plan Sample Points

Use QGIS tools to generate random sampling points, stratified across habitat types according to area

- A sample at each passive monitoring point (camera/ acoustic recorder setup) was also included

8 Sample Collection

At the sampling point, set up a 1m² quadrat using a tape measure

- Ensure that the quadrat is representative of the point

8.1 Sample Collection

Ensure a sterile collection spoon is used and sterile gloves are worn

1. Using a spoon, collect four subsamples (12.5 ml each) from the top 5–10 cm of soil at the four corners of a 1 m² quadrat.
2. Combine subsamples into a single 50 ml sterile Falcon tube.

8.2 Sample Collection

- Ensure used spoons are kept separate from sterile spoons (e.g. using a different compartment/ pocket of the cool bag)
- Within a few hours of sample collection, freeze samples at –20°C for storage and transport

9 Notes

- Where there is no immediate soil substrate at the surface (e.g. conifer pine needles, moss, leaf litter etc.), use the spoon to dig down to the closest soil substrate or remove loose substrate covering soil prior to sampling

Section 4: Tree Roller Sample Collection and Filtration (*Adapted from Valentin et al. 2020*)

10 Equipment

- Thick pile, 10.16 cm, Harris Trade paint rollers (sterilised by soaking in 10% bleach for 20 minutes, 5 % lipsol detergent for 5 minutes, double rinsing with distilled water, stored in sterile ziploc bags and placed under a UV lamp for 20 minutes)
- Paint roller frame (sterilised by soaking in bleach and lipsol, then rinsing)
- Paint roller pole (end wiped with bleach)

- Sterile nitrile gloves
- Tight-fitting pipe: Modified Push Fit Reducer 40 × 32mm White (Toolstation): modified to remove the rubber ring and filed down so that the inner plastic lip is removed - sterilised with soaking protocol
- Filtering equipment (same as water sampling)

11 Sample Collection

1. Attach roller to sterilised frame and extendable pole.
2. Spray roller with distilled water to help DNA transfer (not necessary if stored wet following sterilisation)
3. Roll up and down tree trunks and large branches from ground level up to 3 m, for 30 seconds with moderate force.
4. Place used roller into sterile ziploc bag (ideally the same as it was stored in to reduce plastic waste)
5. Wipe the roller frame and pole with 10% bleach and 70% ethanol between samples

11.1 Sample Collection: Field Blank

- For every day of sampling, create a field blank
- Attach a roller to the frame, then place directly into a ziploc bag without touching any trees

12 Sample Filtration

1. Once samples have been brought back to the filtration location, add 300ml of distilled water to each of the ziploc bags
2. Massage the roller in the bag to release surface DNA and debris
3. Let the rollers soak for 10 minutes
4. Still within the ziploc bag, squeeze the rollers through a modified, tight-fitting pipe to remove as much water as possible from the roller
5. Remove the roller from the bag
6. Vacuum-filter the recovered water as per the water sample protocol (Section 2, Step 4), however only use one filter.

13 Store samples at -20°C for transport and future processing

14 Notes

- If conditions are warm, during the 10 minute soaking step, ideally rest the bags on ice blocks
- Bowls can be used to store the roller-bags to ensure stability
- Rollers should ideally be kept and sterilised again for reuse and to prevent single usage. If rollers are to be reused, reapply the full sterilisation protocol (bleach, lipsol, rinse, UV) before next use.

Section 5: Scat Sample Collection

15 Equipment

- Sterile nitrile gloves
- Sterile spoon
- Sterile 50ml falcon tube

16 Sample Collection

- Opportunistically collect any scat samples found during fieldwork
- Use a sterile spoon to collect either the whole scat or part of the scat, such that it fits within a 50ml falcon tube
- Within a few hours of sample collection, freeze samples at -20°C for storage and transport

Section 6: DNA Extraction (*Mu-DNA Protocol with Modifications*)

- 17 This extraction protocol follows the Mu-DNA workflow (Sellers et al. 2018), using the *water protocol* for water and tree roller samples, and the *soil protocol* for soil and scat samples. Minor modifications are detailed below.

17.1 Equipment and Materials

- TissueLyser II (Qiagen, Germany)
- 10 mL Teflon grinding jars (Qiagen)
- Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, US)
- Extraction reagents: Mu-DNA-compatible reagents (see to Sellers et al. 2018)
- 10% bleach solution
- 5% Lipsol detergent
- Distilled water
- -20°C freezer for storage
- Sterile nitrile gloves
- Pipettes and sterile filtered tips
- Sterile microcentrifuge tubes (e.g., 1.5 mL or 2 mL)
- Elution buffer (manufacturer and concentration as per Mu-DNA protocol)

18 Soil and Scat Sample Extraction

1. Weigh out 2.5 g of sample a 10 mL Teflon grinding jars
2. Add 5.5 mL lysis solution
3. Add one 20 mm steel ball
4. Homogenise using the TissueLyser II (Qiagen) according to Mu-DNA protocol settings (30 hz for 10 mins)
5. Pour the homogenised sample lysate into a 5ml screw-cap Axygen tube (without garnet beads). At this point, samples can be frozen for later extraction if necessary
6. Following all samples being homogenised, create a blank by adding just 5.5ml of lysis solution to the grinding jars
7. Proceed using the Mu-DNA soil protocol after lysis. Before the inhibitor removal step, to ensure sufficient supernatant is collected, centrifuge the 5ml tubes at 4,000 xg for

- 1 min at room temperature, then collect the supernatant
8. Elute final DNA in 100 μL of elution buffer
9. Include at least one extraction blank per batch of extractions

18.1 Notes

- For some soil and scat substrates (e.g. soil with a lot of moss collection, pellets) the mass of sample included in the homogenisation step may need to be reduced. Where this is the case, also reduce the amount of lysis solution added such that it remains in the same ratio of sample : lysis buffer
- Creating separate blanks for the lysis step, then extraction step allows downstream identification of any contamination origins

19 Water and Tree Roller Sample Extraction

1. For water samples, extraction is carried out on both filters in one extraction (resulting in one sample)
2. Follow the Mu-DNA water protocol steps
3. At the elution stage, for water samples: elute in 100 μL of elution buffer. For tree roller samples: elute into 50 μL of elution buffer (adapted for the lower concentration of DNA, as found in test samples)
4. Include one extraction blank per batch, using the corresponding amount of elution buffer as the samples being extracted

20 Post-Extraction Processing

1. Quality check: Using a Nanodrop 1000 Spectrophotometer, assess the DNA concentration (ng/ μL) and purity ratios (260/280, 260/230) for each sample
2. Storage: Store DNA extracts at -20°C until PCR amplification

Section 7: Library Preparation and Sequencing (*Griffiths et al., 2023*)

- 21 This protocol uses a nested metabarcoding workflow with a two-step PCR approach. It targets the 12S mitochondrial gene region to detect vertebrate DNA, applying dual MID tags for sample identification.

21.1 Equipment and Reagents

- UV- and bleach-sterilised laminar flow hood (for pre-PCR setup)
- Applied BiosystemsTM VeritiTM Thermal Cycler (Fisher Scientific, UK)
- Q5[®] High-Fidelity 2X Master Mix (New England Biolabs, US)
- MyTaq HS Red Mix (Meridian Bioscience, US)
- Mag-BIND RxnPure Plus magnetic beads (Omega Bio-tek, US)
- NEBNext Library Quant Kit for Illumina (New England Biolabs, US)
- Qubit 3.0 Fluorometer (Invitrogen)
- dsDNA HS Assay Kit (Invitrogen)
- 2% agarose gel electrophoresis setup
- Illumina MiSeq platform with V3 2 × 300 bp chemistry

22 Step 1: Primer Selection and first PCR (PCR1)

1. Use vertebrate-specific primers 12S-V5-F (5'-ACTGGGATTAGATACCCC-3') and 12S-V5-R (5'-TAGAACAGGCTCCTCTAG-3') ([Kelly et al. 2014](#); [Riaz et al. 2011](#)), modified to include:
 - Illumina sequencing adapters
 - MID tags (8-nucleotide multiplex identifiers) - [Kitson et al. 2019](#)
 - Heterogeneity spacers
2. Prepare PCR1 reactions in triplicate for each sample:
 - 12.5 µL Q5® Master Mix (for water/tree roller samples) or MyTaq™ Red Mix (for soil/scat)
 - 0.5 µL BSA (Thermo Scientific)
 - 7 µL molecular-grade water
 - 1.5 µL of each 10 µM tagged primer
 - 2 µL template DNA
3. Run PCR1 on the thermal cycler with the following conditions:
 - 98°C for 5 mins
 - 35 cycles of 98°C for 10s, 58°C for 20s and 72°C for 30s
 - 72°C for 7 minutes
 - Held at 4°C
4. Include the following controls in each 24-sample sub-library
 - At least 1 extraction blank
 - Associated field blanks
 - 1 negative PCR control
 - 1 positive control: *Astatotilapia calliptera* DNA (0.05 ng/µL) — a tropical species not present in Scotland

23 **Step 2: Sub-library Construction**

Each group of samples (up to 24) and associated controls were treated as a sub-library, with each sample tagged with one of 24 uniquely identifiable MID tags.

1. Pool PCR1 triplicates by band intensity visualised on a 2% agarose gel:
 - No/very faint = 20 µL
 - Faint = 15 µL
 - Bright = 10 µL
 - Very bright = 5 µL

2 µL of the PCR positive control in each library was added, and 10 µL of any blanks or controls

2. Purify each sub-library using double-size magnetic bead selection:

Following pooling, libraries were 'cleaned' with Mag-BIND RxnPure Plus beads to remove non-target DNA.

100 µL pooled PCR product yields 25 µL cleaned DNA.

Beads were added at the following ratios:

- 0.9× ratio (keep supernatant)
- 0.15× ratio (discard supernatant)

Store purified sub-library DNA at 4°C

24



NCL_sample_sheet.csv 1.4KB

Step 3: PCR2 - Indexing and Adapter Addition

The second PCR adds Illumina-compatible adapters and a second round of MID tags for dual-indexing.

1. Prepare duplicate 50 µL PCR2 reactions for each sub-library:

- 25 µL Q5® Master Mix
- 13 µL molecular-grade water
- 3 µL of each 10 µM Illumina-indexed primer
- 4 µL cleaned template DNA

2. Run PCR2 with the following cycling conditions:

- 95°C for 3 minutes
- 10 cycles of 98°C (20s), 72°C (1 min)
- Final extension: 72°C for 5 minutes
- Hold at 4°C

3. Pool duplicates and purify using double-size selection:

- 50 µL of PCR product used in the clean up

Beads added at the following ratios:

- 0.7× ratio (keep supernatant)
- 0.15× ratio (discard supernatant)

Elute and store at 4°C

Sample sheet shows the primer and tag combinations used for each sub-library.

25

Step 4: Library Quantification and Pooling

1. Quantify each sub-library using a Qubit™ 3.0 Fluorometer with the dsDNA HS Assay Kit.

2. Pool sub-libraries proportionally based on:

- Number of samples
- DNA concentration

3. Create two pooled libraries at 4 nM and 6 nM final concentrations.

4. Quantify the final pool using the NEBNext® Library Quant Kit via qPCR.

5. Adjust final library concentration to 4 nM.

26

Step 5: Illumina MiSeq Sequencing

1. Denature and dilute the final library to 13 pM.
2. Spike in 10% PhiX Control v3.
3. Load the library for sequencing on the Illumina MiSeq using V3 2 × 300 bp chemistry.

27 Notes

- Perform all pre-PCR steps in UV-sterilised rooms.
- Run inhibited samples at 1:10 dilution and process alongside originals in separate sub-libraries.
- Include original and diluted versions in sequencing.
- Follow strict contamination controls throughout all steps.

Section 8: Bioinformatics

28 Software and environment

- Use the Tapirs metabarcoding workflow (<https://github.com/EvoHull/Tapirs>), which runs via Snakemake within a conda virtual environment to ensure reproducibility and software compatibility.
- Perform all steps on a high-performance computing cluster or equivalent local system with sufficient memory and processing power to handle demultiplexed FASTQ files.

29 Demultiplexing

- Allow Illumina MiSeq Reporter software to automatically demultiplex raw sequence data into forward and reverse FASTQ files for each library
- Further demultiplex reads to sample level using a custom Python script that matches unique MID tag combinations from the first and second PCR rounds.

30 Quality filtering and sequence clustering

1. Trim adapters and low-quality regions using fastp with the following settings: 5 bp sliding window, minimum Phred score of Q30 across ≥60% of each read
2. Remove the first 18 bp of both forward and reverse reads to eliminate primer sequences.
3. Apply length filtering to retain reads between 90–110 bp.
4. Merge paired-end reads using fastp with the following criteria: minimum 20 bp overlap, ≤5% mismatches, ≤5 total mismatched bases
5. Retain only forward reads from unmerged pairs.
6. Remove exact duplicate sequences using VSEARCH with --derep_fulllength
7. Discard clusters represented by fewer than three reads.
8. Perform denoising using VSEARCH with --cluster_unoise, followed by chimera detection using --uchime3_denovo

31 Taxonomic assignment

1. Compare the final filtered query sequences against the reference database using BLAST+ (Zhang et al. 2000).
2. Assign taxonomy using a Majority Lowest Common Ancestor (MLCA) approach:
 - Retain only hits with ≥90% query coverage and ≥98% identity.

- Select the top 2% of hits by bit-score.
- Require $\geq 80\%$ of unique taxonomic lineages to agree at a given taxonomic level for assignment.

3. If only one BLAST hit meets the above criteria, assign taxonomy directly to that hit.

4. Record read counts based on cluster sizes for each taxonomic assignment.

5. Classify sequences that cannot be assigned below order as unassigned.

32 **Contamination control and thresholds**

- Use species read counts detected in controls (field, extraction, PCR negatives and positives) to calculate a limit of detection (LOD) per species and sample type.
- Calculate LOD as:

$$\text{LOD} = \text{mean}(\text{blank reads}) + (3 \times \text{standard deviation of blank reads})$$

- Set to zero any read count below the species-specific LOD for the corresponding sample type.
- For additional confidence in results, apply a 0.1% read threshold, removing any taxonomic assignment that comprises less than 0.1% of the total reads in a given sample.

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