Quantifying ecosystem service provider interactions via bulk sample DNA metabarcoding

Forked from High-throughput and cost effective pan trap DNA extraction

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Protocol status: Working
We use this protocol and it's working

Created: Sep 05, 2023

ABSTRACT

This protocol is designed for extracting DNA from pan trap-collected invertebrates for biomonitoring and community metabarcoding to identify and quantify ecosystem service and disservice providers. The reagents and methods proposed offer a cost effective and high-throughput method for molecular diversity analyses of bulk samples using standard lab equipment.

IMAGE ATTRIBUTION

Biorender.com

MATERIALS

For field collection and initial storage:

- Pan traps
- Detergent diluted in water for collection of invertebrates
- Small collection pots for storage of samples
- Sieves for isolating invertebrates
- 100 % ethanol
- Chemgene/diluted bleach for sterilisation of sieves
- 50 mL falcon tubes
- 125 mL sample pots (for full sample lysis)

For DNA extraction:

- Hardened carbon steel ball bearings
- 2.2 mL deep well plates (or up to 1.5 mL) for initial protein denaturation
- 2.2 mL deep well plates for archiving
- 2.2 mL deep well plates for spin-column flow-through (these can be bleached and reused across sessions)
- Silica membrane 96-well spin-column plates
- 0.5 mL deep-well 96-well plates to collect eluted DNA
- Breathable plate seals

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Keywords: metabarcoding, biomonitoring, entomology, high-throughput sequencing, community ecology, field techniques

- Plate seals for long-term storage

For DNA amplification and subsequent steps:

- Tagged PCR primers with bridge primer sequence for Nextera library preparation by PCR
- 2X hot-start Taq polymerase mastermix
- Molecular grade water
- 96-well PCR plates
- Mineral oil
- 0.1X and 1X SPRI beads
- Nextera Illumina adapter index primers

Buffers and reagents:

- Sodium chloride
- 1 M Tris-HCl
- 0.5 M EDTA
- Nuclease-free water
- SDS
- PEG
- HCl
- Tris
- Guanidine HCl
- 100 % ethanol
- Proteinase K (10 mg/mL) OR papain (10 mg/mL)

Equipment:

- -20 °C freezer
- -80 °C freezer
- Geno/Grinder 2010 or similar bead beater for homogenisation
- Thermocycler
- Magnetic stand (for plates and tubes)
- Centrifuge
- Microcentrifuge
- Vortex
- Pipettes (preferably including multichannel, ideally including 96-well)
- Illumina sequencer

SAFETY WARNINGS

Check safety guidelines for individual reagents before commencing work. Some reagents will be toxic, corrosive or otherwise present health and safety risks. Appropriate personal protective equipment should be used at all times, not only for personal safety but also reduction of contamination risk.

Trapping of invertebrates

3d 0h 30m

1 Select suitable sites and locations for bulk sample traps (e.g., pan traps). Consider how systematic the study needs to be and the various constraints imposed on the data by the study design. The following steps will focus on the example of water pan traps.

2 Set a pan trap at approximately the same height as the surrounding vegetation. 15m

Note

Ideally, use a pan trap design that prevents overflow if the site is likely to experience heavy rainfall.

Ensure the trap is clean and free of DNA contaminants by cleaning it with Chemgene or 10 % bleach prior to use.

3 Fill the traps with water containing detergent to reduce surface tension. Leave the traps out for 72 hours. 3d

Note

Standardise the volume of soapy water across traps but ensure it is sufficient to avoid evaporation yet not so much as to make overflow likely.

Ensure appropriate permissions are in place for the site. If your site is publicly accessible or likely to be visited, consider using signage to reduce the risk of tampering (e.g., vandalism or benevolent liberation of the dead invertebrates).

Consider as well the ethical implications of your traps. Limit unnecessary collection and implement measures to limit bycatch of non-target organisms where applicable.

4 Pour the trap contents through a sterilised funnel into a suitably sized pot labelled with all necessary sample information for transport to the laboratory. Store samples at -20 °C until ready to process. 15m

Preparation and homogenisation of samples 1d 9h

5 Wash samples with water in a fine mesh sieve to remove external contaminants, and transfer to a 50 mL falcon tube. 5m

Note

Throughout the below processes, include at least one negative control for each 95 samples which is treated identically to the samples but without any sample material added.

6 Add three 3 mm hardened carbon steel beads to the tube. 5m

Note

Beads are usually shipped coated in manufacturing oil (especially the carbon steel beads). To remove this, place beads in a borosilicate glass beaker or Duran bottle with plastic pouring lip and lid removed then bake for at least 12 hours at 250 °C.

7 Freeze the samples overnight at -80 °C. 16h

8 Directly from the freezer, grind the bulk samples in a tissue grinder/homogeniser/lyser at 1750 RPM for 4 minutes. 4m

Note

If the sample is not fully homogenised, repeat this step.

9 Two lysis options are presented below. One standard protocol using Proteinase K to lyse a subsample of each trap (this may reduce detectable diversity but increases efficiency), and the other uses papain (a cheap alternative to Proteinase K) to lyse whole samples.

Step 9 includes a Step case.
Subsample Proteinase K lysis
Bulk papain lysis

step case 1m

Subsample Proteinase K lysis

10 Add an appropriate volume of fresh **Lysis Buffer 1** (detailed in the sub-step below) to sufficiently cover the specimens.

Note

To sterilise borosilicate bottles prior to making up the buffers, acid washing with ~100 mL 0.4 M hydrochloric acid followed by neutralisation with ~100 mL 0.4 M sodium bicarbonate is ideal for sterilisation without introducing bleach or other chemicals that might destroy or contaminate the DNA. Following neutralisation, wash twice with ~100 mL water to remove the reagents. Residual amounts of NaCl may remain, but this is inert and will be present in many of the buffers anyway.

For 0.4 M sodium bicarbonate, into 1000 mL water, add 33.604 g NaHCO3.

10.1 **Lysis Buffer 1** should be pH 9 and comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	NaCl	120 mM	58.44	0.701 g
	1 M Tris-Hcl	50 mM	-	5 mL
	0.5 M EDTA	20 mM	-	4 mL
	Water	-	-	91 mL

11 Grind the samples in a Geno/Grinder at full speed (1750 RPM) for 2 minutes. 2m

12 Centrifuge at 4,000 x *g* for 2 minutes. 2m

13 Take a subsample of 1 mL lysate from the supernatant and transfer it to a 2.2 mL 96-well plate. Store the remaining lysate and sample from the falcon tube at -20 °C. 1m

Note

It is possible to freeze the plate at this stage or proceed with overnight digestion depending on the desired completion time.

14 To the 1 mL lysate, add 500 µL of freshly-prepared **Proteinase Buffer**, a master mix of **Lysis Buffer 2** and proteinase K (detailed in the sub-steps below) and vortex to mix. 1m

14.1 **Lysis Buffer 2** should be pH 9 and comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	Sodium chloride	120 mM	58.44	0.701 g
	1 M Tris-Hcl	50 mM	-	5 mL
	0.5 M EDTA	20 mM	-	4 mL
	SDS	3 %	-	3 g
	H2O	-	-	91 mL

14.2 Per plate, the **Proteinase Buffer** master mix should comprise: 1m

	A	B
	Reagent	Amount per 96-well plate
	Lysis solution 2	51.2 mL
	Proteinase K (10 mg/mL)	1584 µL

15 Vortex each sample to mix and incubate at 37 °C overnight (12-16 hours). 16h

16 Centrifuge at 4,000 x *g* for 4 minutes. 1m

17 Transfer 1 mL of the supernatant to a clean 2.2 mL deep-well 96-well plate for archiving/backup. 1m

18 Transfer 200 µL of the supernatant to a clean 2.2 mL deep-well 96-well plate. 1m

Note

The remaining lysate can now be stored at -20 °C for backup/future work.

DNA extraction and purification 22m

19 Add 400 µL of master mix of **Protein Denaturation Buffer and ethanol** (detailed in the sub-step below) to each well of the 2.2 mL 96-well plate. 1m

19.1 **Protein Denaturation Buffer** should be comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	Guanidine HCl	5 M	95.53	47.7 g
	H2O	-	-	100 mL

19.2 Per 96-well plate, the **Protein Denaturation Buffer and ethanol** master mix should comprise: 1m

	A	B	C
	Reagent	Amount per sample	Amount per 96-well plate
	Protein Denaturation Buffer	220 µL	21.1 mL
	Ethanol (100 %)	220 µL	21.1 µL

20 Add all of the sample solution (~ 600 µL) to a well in a 96-well silica membrane spin-column plate and cover with a breathable seal. 1m

Note

Ensure there is a suitable reservoir beneath into which the flow-through will go (e.g., 2.2 mL deep-well plate).

21 Centrifuge at ≥ 6,000 x *g* for 10 minutes and discard the flow-through. 1m

Note

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation will work.

22 Add 500 µL **Wash Buffer 1** to each spin column and cover with a breathable seal. 1m

22.1 **Wash Buffer 1** should be comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	Guanidine HCl	7 M	95.53	29.4 g
	Ethanol	56 %	-	56 mL
	H2O	-	-	44 mL

23 Centrifuge at ≥ 6,000 x *g* for 5 minutes and discard the flow-through. 1m

Note

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation will work.

24 Add 500 µL **Wash Buffer 2** to each spin column and cover with a breathable seal. 1m

24.1 **Wash Buffer 2** should be pH ~7 and comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	Ethanol (100 %)	70 %	58.44	70 mL
	1 M Tris-Hcl	10 mM	-	1 mL
	H2O	-	-	29 mL

25 Centrifuge at ≥ 6,000 x *g* for 15 minutes and discard the flow-through. 3m

Note

If the centrifuge cannot reach 20,000 x *g*, a longer centrifugation will work.

26 Carefully move the spin column plate to a new 0.5 mL DNA collection plate. 1m

Note

The liquid level following the final wash will be close to the base of the spin column, so take care not to let it touch to prevent ethanol transfer to the soon-to-be eluted DNA.

27 Add 200 µL **Elution Buffer** directly to the silica membrane and leave it at room temperature for 5 minutes, covering with a breathable seal. 1m

27.1 **Elution Buffer** should be pH ~7 and comprised of the following reagents: 2m

	A	B	C	D
	Reagent	Required concentration in buffer	Chemical molarity	Amount per 100 mL
	1 M Tris-Hcl	10 mM	-	1 mL
	H2O	-	-	99 mL

28 Centrifuge at ≥ 6000 x *g* for 2 minutes. The DNA is now in the collection plate and can be taken forward to molecular analysis. 1m

Note

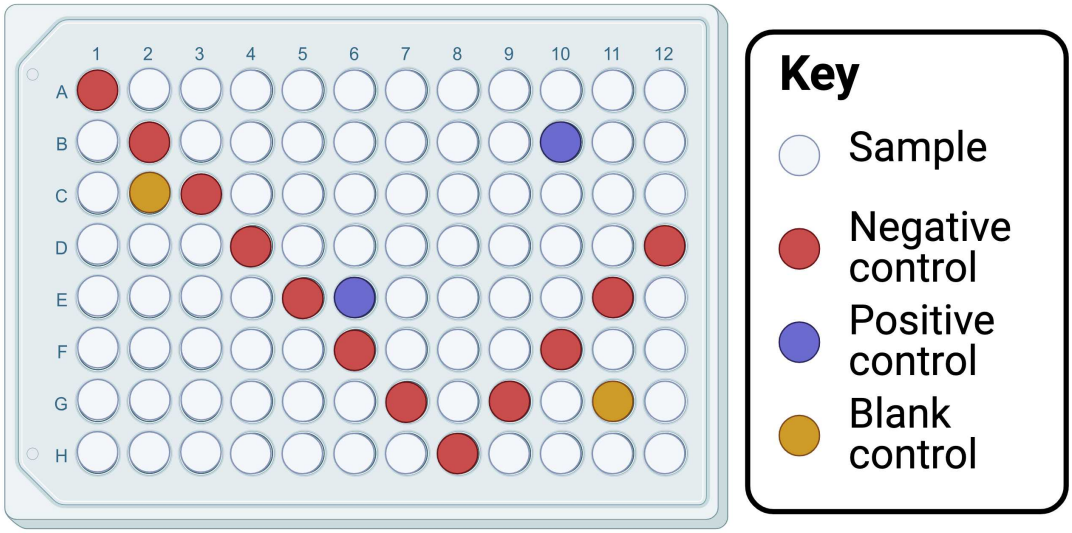
Steps 20-21 can be repeated for increased DNA yield but a lower overall concentration.

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation (e.g., 5 minutes) will work, although should not be necessary for this step.

DNA amplification 1h 22m

29 Decide how samples will be distributed across plates (but don't distribute DNA yet). Consider including a negative control in each row and column to detect any contaminants in each tagged forward and reverse primer. Among these wells, include any DNA extraction negative controls. Include positive controls (ideally mixed samples of species not found in the same study system), perhaps one adjacent to negative controls and the other adjacent only to samples (but both on separate rows and columns). Include blank controls (ideally wells into which no reagents or at least no primers are added), perhaps one adjacent to negative controls and the other adjacent only to samples (but both on separate rows and columns). 10m

If using multiple PCR primer pairs, familiarise yourself with the annealing temperatures for each and prepare separate PCR plates for each. For optimal accuracy, consider running replicates of each reaction (e.g., triplicates).



Example layout of samples and controls in a 96-well plate.

30 Distribute one drop of mineral oil into each well of the PCR plate (~20 µL). 2m

Note

This can be achieved by taking a large volume of mineral oil into the pipette tip and then gently plunging so that a drop forms and falls from the tip into each well.

Mineral oil improves sealing of reactions by preventing evaporation and condensation. This reduces evaporation and thus loss of product, but also reduces potential contamination.

31 Prepare enough PCR mastermix for each sample. 2m

For a full plate, these values will usually suffice (with some overage to account for pipetting error), but check your specific Taq polymerase mix for any differences:

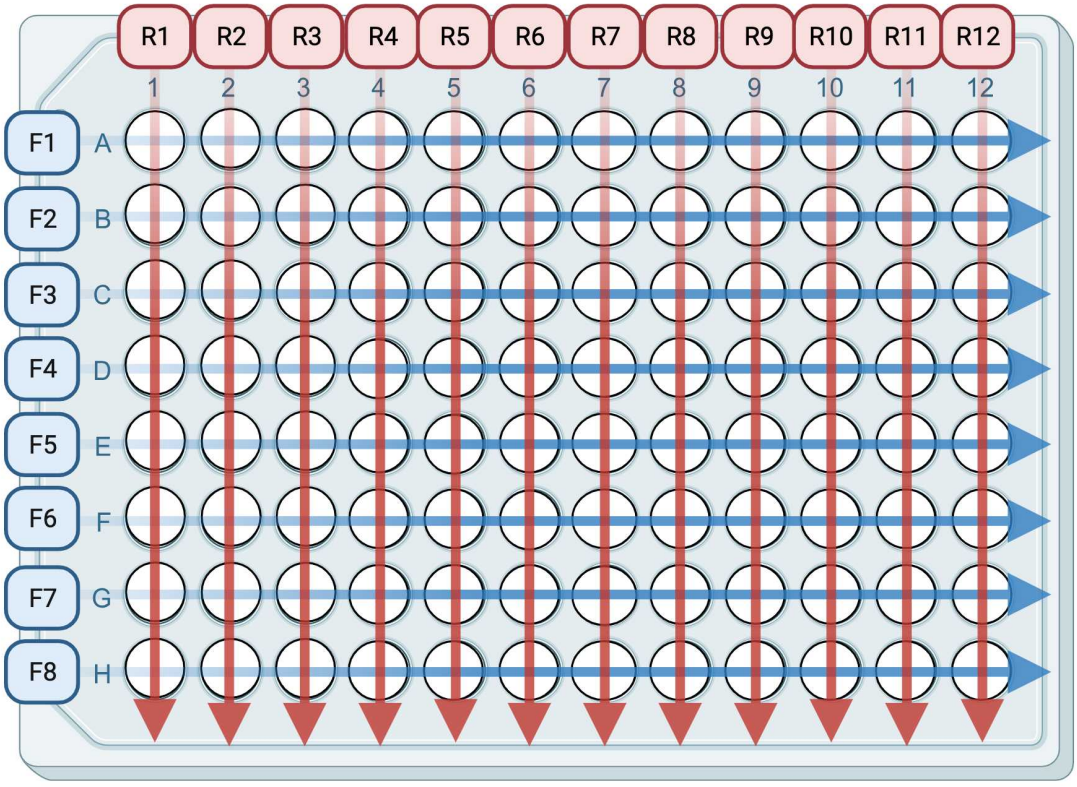
	A	B
	Reagent	Volume (µL)
	Molecular grade water (DNase free)	422.4
	2X hot-start PCR mastermix	528

Note

These values are for 10 µL reaction volumes, which have been demonstrated to be effective for bulk samples. Consider running them in triplicate for more accurate results.

32 For ease, if able to use a 96-well pipette, consider creating a "primer plate" containing both PCR primers for each well at 5 µM concentration; this is especially effective when using multiple plates. Add 7.75 µL mastermix to each well, followed by 1.25 µL of each primer mix to its corresponding well. It is possible to use this strategy with a multi-channel or even single-channel pipette, but the suggestion immediately below may be easier. 15m

If not using a 96-well pipette, consider making eight mastermixes each with sufficient reagents for 12 samples (7.75 µL mastermix per well, so 97.65 µL for 12 with some overage for pipetting error), each containing a different forward primer (with 0.625 µL of 5 µM forward primer per well, so 7.875 µL per mastermix for 12 with some overage for pipetting error). Distribute 8.375 µL of forward primer + mastermix to each well across the rows corresponding to each forward primer. Then distribute 0.625 µL of reverse primer to each well in the corresponding columns **making sure to change pipette tip for each sample to avoid cross-contaminating the forward primers**.



Distribution of tagged PCR primers across the 96-well plate

33 Add 1 µL DNA to each corresponding sample and positive control well, and 1 µL molecular grade water to each negative control other than extraction negative controls (which will be added as samples). 10m

34 Briefly centrifuge the plate to ensure that the oil is above the PCR mix and everything is at the bottom of each well without air bubbles. 1m

35 Load the PCR plate into a thermocycler. Ensure that the temperature regime matches the enzyme used (including any heat activation for hot-start Taq) and that the annealing temperature matches the PCR primers used. 2m

> Note
>
> Given differences between labs and sample material, and inaccuracies in temperature calibration, considering running a temperature gradient PCR with known samples to check optimal temperatures first if unsure.

36 The samples should now be checked for successful amplification, contamination in negatives and any secondary banding. Gel electrophoresis will achieve this, but digital systems like the Qiagen Qiaxcel will do this and facilitate equalisation by generating amplicon-specific DNA concentations. 40m

DNA normalisation 1h 35m

37 If the PCRs were replicated (i.e., each sample run multiple times for each PCR primer pair used), these can be merged together into one plate at this point, or carried forward separately. Keeping the replicates separate increases the number of libraries to prepare and sequence later, but better facilitates identification of inconsistencies between samples. To merge triplicates, assuming use of 10 µL reaction volumes, pipette 8 µL from each well of two of the three plates into the corresponding well of the third. Briefly centrifuge the merged plate to move the oil to the top of the product again. 5m

Note

To avoid pipetting oil from the oil-sealed PCR products, plunge the pipette to the first stop and fully insert the pipette tip into the bottom of the well, then release sharply. The PCR product will be taken up quickly, whereas the relatively viscous oil will be taken up slowly, thus being outcompeted by the PCR product.

38 Prepare 0.1X SPRI bead solution and bring to room temperature. The below steps detail how to make this solution, but it is also commercially available.

38.1 If using beads such as Sera-Mag Magnetic SpeedBeads (carboxylated, 1 µm, 3 EDAC/PA5), take 1 mL of well-mixed bead solution and wash the beads twice with TE+Tween buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 8.0) by magnetising the beads, removing the supernatant, adding the TE+Tween, remagnetising the beads and removing the supernatant, and repeating the addition and removal of TE+Tween once more. 5m

38.2 To the beads, add the following mix: 5m

	A	B
	Reagent	Volume
	5 M NaCl	25 mL
	Molecular grade water	3.582 mL
	1 N HCl	0.168 mL
	1 M Tris base	0.5 mL
	0.1 M disodium EDTA	0.5 mL

38.3 Add 20 mL of 50 % PEG to the tube to reach a 1X bead solution (alongside making the 0.1X solution, this will be useful later). 2m

38.4 Add 5 mL of 1X bead solution to 45 mL of the following mix to make a 0.1X solution: 5m

	A	B
	Reagent	Volume

	A	B
	5 M NaCl	25 mL
	Molecular grade water	3.582 mL
	1 N HCl	0.168 mL
	1 M Tris base	0.5 mL
	0.1 M disodium EDTA	0.5 mL
	50 % PEG	20 mL

39 If ≥20 µL of PCR product is available in each well, pipette 20 µL of 0.1X SPRI bead solution into each well of a new 96-well plate. If less DNA is available, add the available volume of 0.1X SPRI beads and add the same volume of PCR product to these beads in the next step. 2m

> Note
>
> When working with magnetic beads, ensure they are fully mixed with no residue at the bottom of the container.

40 Add 20 µL of PCR product (or whatever volume of beads was used in the last step) to each corresponding well of 0.1X beads, avoiding oil, and mix by vortexing (1500 RPM for 1 min). 2m

> Note
>
> To avoid pipetting oil from the oil-sealed PCR products, plunge the pipette to the first stop and fully insert the pipette tip into the bottom of the well, then release sharply. The PCR product will be taken up quickly, whereas the relatively viscous oil will be taken up slowly, thus being outcompeted by the PCR product.

41 Incubate at RT for 5 mins. 5m

42 Place on a magnetic stand for 5 mins. 5m

43 Remove all but 5 µL of the mixture from each well via pipette without disturbing the beads, which should be settled on the magnet (although can be hard to see with 0.1X bead solutions). 5m

44 Add 200 µL 80 % ethanol to each well. 2m

45 Remove the ethanol and add a further 200 µL 80 % ethanol to each well. 4m

46 Remove the ethanol as completely as possible by pipetting. 4m

> Note
>
> A small amount of remaining ethanol isn't debilitating in this case, unlike usual magnetic bead cleaning protocols, because we will complete a subsequent purification immediately after.

47 Add 10 µL molecular grade water to each well, shake at 1500 RPM for 1 min and incubate for 5 mins. 7m

48 Place on a magnetic stand for 5 mins. 5m

49 Pool 8 µL from each well into a single tube, leaving magnetic beads behind in the 96-well plate. 4m

Note

To pool samples using a multi-channel pipette, pipette each column of samples into a new strip of 200 µL tubes, then merge all of these tubes into a single 1.5 mL tube at the end.

50 Mix the pooled samples by flicking and inverting, and transfer 700 µL into a new 2.5 mL microcentrifuge tube. 1m

51 Add 700 µL 1X magnetic beads for DNA purification (see note below for size selection) and mix by vortexing. Incubate for 5 mins. 6m

Note

This is a good opportunity for size selection as well, especially if the PCR product contains any secondary bands. If using amplicons around 350 bp, consider a 0.8X bead ratio (i.e., add 560 µL beads instead).

52 Place on a magnetic stand for 5 mins. 5m

53 Add 400 µL 80 % ethanol. 1m

54 Remove the ethanol and add a further 400 µL 80 % ethanol. 1m

55 Remove the ethanol completely. Briefly centrifuge the sample to collect and remove any residual ethanol. Allow to air-dry until the aggregation of magnetic beads transitions from 'glossy' (shiny reflection of light) to 'matte' (dull dark brown mass), but not so long that it dries completely (i.e., begins to turn a rusty red and shows cracks). 2m

56 Add 100 µL molecular grade water and mix by vortexing. Incubate for 5 mins. 6m

57 Place the sample on a magnetic stand for 5 mins. 5m

58 Remove 95 µL of the water and place it in a new tube. This will be the library used in the subsequent section. 1m

DNA library preparation and sequencing 1h 9m

59 Quantify the concentration of the library from the previous step (e.g., using Qubit dsDNA assay). 5m

60 Dilute library to ≤5 ng/µL in molecular grade water. 2m

> **Note**
>
> Without diluting the library, there is a risk that the adapters will not be added to all DNA (i.e., there is too much DNA) which will compromise the sequencing yield.

61 Add a drop of mineral oil to one PCR tube for each library, alongside an additional tube for a negative control. 1m

62 Per library, assemble the following reaction: 5m

	A	B
	Reagent	Volume

	A	B
	2X hot-start PCR mastermix	7.5
	Molecular grade water	1
	Nextera sequencing adapter primer mix	1.5
	DNA library	5

63 Briefly centrifuge the tubes to ensure that the oil is above the PCR mix. 1m

64 Load the PCR reactions into a thermocycler. Ensure that the temperature regime matches the enzyme used (including any heat activation for hot-start Taq) and that the annealing temperature matches the PCR primers used. Run for 12 cycles. 5m

65 Quantify the concentration of the library from the previous step (e.g., using Qubit dsDNA assay). 5m

66 Check that the adapters have been added to the DNA by determining and comparing the amplicon sizes for libraries before and after this second PCR. Amplicons should be longer after the second PCR. This is ideally assessed using a digital system like TapeStation, but even gel electrophoresis will work. 40m

67 Once successful adapter addition is confirmed, libraries can be pooled so that each is equimolar in the final mixture based on the concentrations determined above (e.g., by Qubit). 5m

Note

This can be achieved by dividing the maximum concentration across the libraries by each library concentration and pooling that many µL from each. These values may need to be multiplied up to ensure adequate yield for sequencing.

Be careful if using multiple PCR primer pairs! Different amplicons have different molecular weights, so pool based on fmol rather than ng/µL.

68 Check with your sequencing provider how many fmol they will need in how many µL. This will be based on the sequencer, sequencing cartridge and any QC processes they follow. The libraries should be ready for sequencing.

Bioinformatics

69 Optimal bioinformatics workflows depend on a variety of factors including target taxa, study system, the genetic marker used, computational resources available and various other factors. Other resources with code integration will be much better suited to detailing the steps involved. The process will likely follow a similar progression of steps though:

1. Read processing: demultiplex, trim primers, merge pairs.
2. Filtering and error correction: quality filtering, dereplication, denoising, chimera filtering.
3. Taxonomic unit aggregation: OTU/ASV delimitation.
4. Taxon assignment: reference database curation, taxon assignment.

Ecosystem service provider quantification

70 Following taxon assignment to ASVs, taxa must be assigned as ecosystem service providers, ecosystem disservice providers, or neutral taxa. For an example, see the supplementary information in Cuff *et al.* (2022).

CITATION

Cuff, J. P., Tercel, M. P. T. G., Drake, L. E., Vaughan, I. P., Bell, J. R., Orozco-terWengel, P., Müller, C. T., & Symondson, W. O. C. (2022). Density-independent prey choice, taxonomy, life history, and web characteristics determine the diet and biocontrol potential of spiders (Linyphiidae and Lycosidae) in cereal crops. Environmental DNA.

LINK

https://doi.org/10.1002/edn3.272

71 Ecosystem service and disservice providers can then be summed for each sample, giving quantities of each which can be analysed against sample metadata.