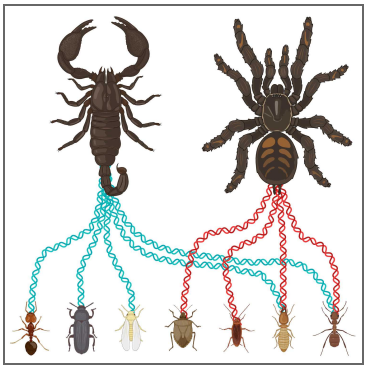

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We use this protocol and it's working

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Metabarcoding based gut content analysis of arachnids

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ABSTRACT

This protocol describes the workflow used for the gut content analysis via DNA metabarcoding of scorpions and spiders for some work conducted in the Hebrew University of Jerusalem, Israel. The protocol describes sample processing, DNA extraction, PCR amplification, gel electrophoresis, DNA normalisation, PCR-based library preparation, DNA purification and (briefly) sequencing and bioinformatics.

IMAGE ATTRIBUTION

Created with Biorender.

MATERIALS

For field collection and initial storage:

- Arachnid survey equipment (study specific)
- Small collection tubes for storage of samples (e.g., 1.5 mL microcentrifuge tubes)
- Sample labels (consider alcohol and freezer resistant labels, e.g., laser printed)
- 100 % ethanol
- Chemgene/diluted bleach for sterilisation of equipment

For DNA extraction:

- Tissue homogeniser with sufficient number of 'heads' for running multiple samples in parallel
- Qiagen DNeasy Blood and Tissue kit reagents and plasticware
- 1.5 mL microcentrifuge tubes for final elution

For DNA amplification and subsequent steps:

- Tagged PCR primers (with bridge primer sequence for Nextera library preparation by PCR if following that process)
- 2X hot-start Taq polymerase mastermix (e.g., Qiagen Multiplex PCR kit or MyFi)

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Keywords: ecology, diet analysis, molecular dietary analysis, DNA metabarcoding, gut content analysis, spiders, scorpions, trophic interactions

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- Molecular grade water
- 96-well PCR plates
- Mineral oil
- 0.1X and 1X SPRI beads
- Nextera Illumina adapter index primers (unless library prep to be completed by an external sequencing facility)

Buffers and reagents:

- Sodium chloride
- 1 M Tris-HCl
- 0.5 M EDTA
- Nuclease-free water
- PEG
- HCl
- Tris
- Guanidine HCl
- 100 % ethanol
- 1X TBE
- Agarose
- Gel stain (e.g., SYBRSafe)
- DNA dye for gel electrophoresis
- Gel ladder (50 bp - 500 bp)

Equipment:

- -20 °C freezer
- Tissue homogeniser or similar for homogenisation
- Thermocycler
- Magnetic stand (for plates and tubes)
- Centrifuge
- Microcentrifuge
- Vortex
- Pipettes (preferably including multichannel, ideally including 96-well)
- Gel imaging dock
- Gel tank and power pack
- 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 sample cross-contamination risk.

ETHICS STATEMENT

The welfare of invertebrates, although poorly regulated, is of crucial importance when collecting and analysing them. Limiting suffering, but also taking only what is necessary is key. See the below citations for some considerations for this.

Crump A, Gibbons M, Barrett M, Birch J, Chittka L (2023) Is it time for insect researchers to consider their subjects' welfare? *PLoS Biol* 21(6): e3002138. https://doi.org/10.1371/journal.pbio.3002138

Cuff, J. P., Barrett, M., Gray, H., Fox, C., Watt, A., & Aimé, E. (2023). The case for open research in entomology: reducing harm, refining reproducibility and advancing insect science. *OSF Preprints* https://doi.org/10.31219/osf.io/cufpq

BEFORE START INSTRUCTIONS

Plan your experiment carefully. Ensure appropriate safety of all researchers throughout the work. Ensure welfare of the invertebrates used is appropriately considered. For the reduction of sample cross-contamination, consider using separate pre- and post-PCR work spaces and use bleach/Chemgene and/or UV decontamination of surfaces and equipment throughout.

Sample preparation

2m

1 Collect arachnids from study sites, place them into [M] 100 % volume ethanol and store them at -20 °C until ready to proceed with DNA extraction.

Note

Consider the experimental design carefully to ensure that there is sufficient replication of the taxa sampled to answer the research questions. Consider the welfare and conservation of the invertebrate individuals and populations throughout this process too.

2 Depending on the arachnid, the optimal manner of extracting gut contents can differ. Here, we present two subtly different methods for scorpion and spider gut content analysis. These will be more broadly applicable to other arachnids (e.g., pseudoscorpions, mites, solifugids) but also many other invertebrates.

STEP CASE

Isolation of gut contents from scorpions

68 steps

3 Using bleach-sterilised tools, cut the scorpion's abdomen open and scrape the innards of the abdomen into a 1.5 mL microcentrifuge tube.

Note

Given the size of scorpions used for this analysis, specific organs were not extracted, but see the citation below to guide selection of organs for optimal prey DNA prevalence.

CITATION

Simone, Y., Chaves, C., van der Meijden, A. & Egeter, B. (2022). Metabarcoding analysis of different portions of the digestive tract of scorpions (Scorpiones, Arachnida) following a controlled diet regime shows long prey DNA half-life. Environmental DNA.
LINK
https://doi.org/10.1002/edn3.311

DNA extraction

2h 15m 3s

4 DNA extraction follows the Qiagen DNeasy Blood & Tissue Kit protocol. 1m

Add 180 µL Buffer ATL to the isolated tissue in the 1.5 mL microcentrifuge tubes.

Note

As per the Qiagen protocol, sub-sample tissue exceeding approximately 25 mg.

5 Homogenise abdomens with a tissue homogeniser for approximately 00:00:03 or until fully homogenised. 3s

> **Note**
>
> You can reuse the 'head' of the tissue homogeniser if appropriately sterilised between samples. For this, wash thoroughly in bleach, ensuring any remaining tissue is removed, then rinse in water and ethanol, and air-dry.

6 Add 20 µL Proteinase K to tubes. 1m

7 Incubate on a thermoshaker at 150 rpm, 56°C, 02:00:00. 2h

8 Add 200 µL Buffer AL and vortex/shake to mix. 1m

9 Add 200 µL [M] 100 % (v/v) ethanol and vortex/shake to mix 1m

10 Pipette the mixture (~ 600 µL) into a spin column held within a 2 mL collection tube and centrifug 2m for 6000 x g, 00:01:00. Discard the flow-through and collection tube.

> **Note**
>
> Collection tubes can be cleaned and re-used if washed thoroughly with bleach. This reduces costs for additional extractions and reduces plastic waste.

11 Place the spin column into a new collection tube, add 500 µL Buffer AW1 and centrifuge for 6000 x g, 00:01:00. Discard the flow-through and collection tube. 2m

12 Place the spin column into a new collection tube, add 500 µL Buffer AW2 and centrifuge for 20000 x g, 00:03:00. Discard the flow-through and collection tube. 4m

13 Place the spin column into a new 1.5 mL microcentrifuge tube, add 200 µL Buffer AE, incubate at Room temperature for 00:01:00 and centrifuge for 6000 x g, 00:01:00. 3m

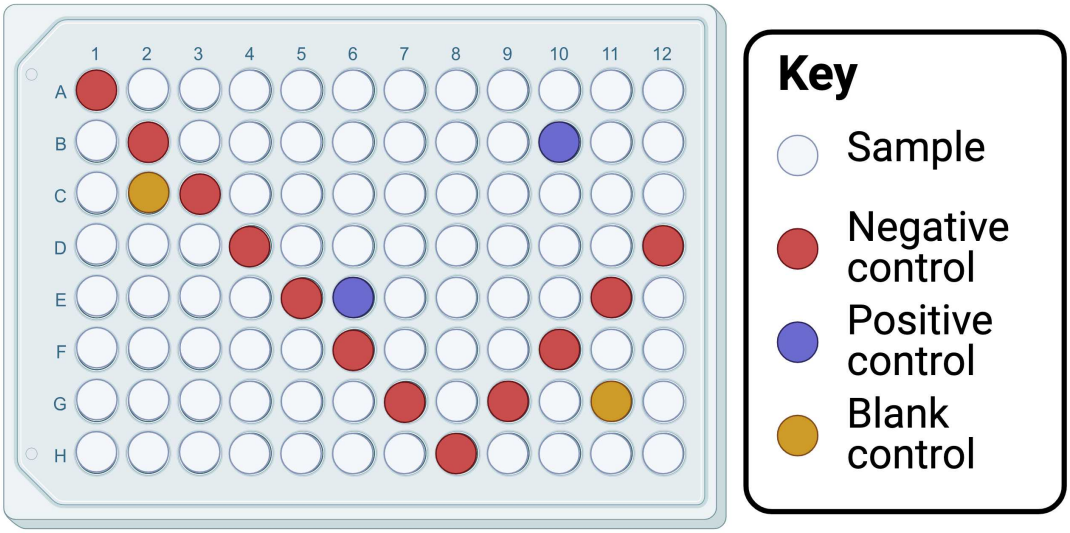
> **Note**
>
> You can repeat this step, eluting into the same tube, to increase mass yield, but the concentration of the DNA may be lower as a result.

PCR amplification

2h 47m

14 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).

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.

Note

Consider your PCR primers carefully, ensuring your recovery of data is sufficient to answer your questions, particularly considering amplification of predator DNA (see the citation below).

CITATION

Cuff, J. P., Kitson, J. J. N., Hemprich-Bennett, D., Tercel, M. P. T., Browett, S. S., & Evans, D. M. (2023). The predator problem and PCR primers in molecular dietary analysis: Swamped or silenced; depth or breadth?. Molecular Ecology Resources.

LINK

https://doi.org/10.1111/1755-0998.13705

15 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.

16 Prepare enough PCR mastermix for each sample. 2m

For a full plate, these values should 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)	1056
	2X hot-start PCR mastermix	1320

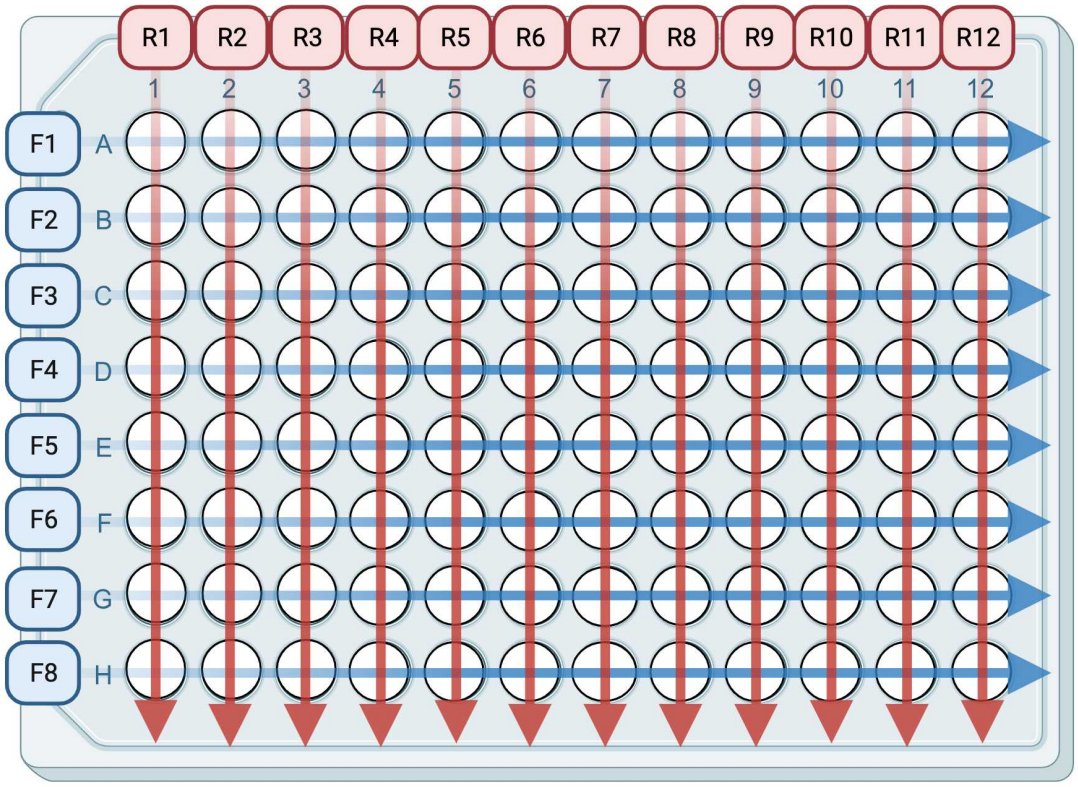
Note

These values are for 25 µL reaction volumes, which have been demonstrated to be effective for bulk samples. Consider running them in triplicate for more accurate results.

17 For ease, if able to use a 96-well pipette, consider creating a "primer plate" containing both PCR prime 5m for each well at [M] 2.5 micromolar (µM) concentration; this is especially effective when using multiple plates. Add 20 µL mastermix to each well of the PCR plate, followed by 2 µL of each primer mix from the primer plate to its corresponding well in the PCR plate. It is possible to use this strategy with a multi-channel or even single-channel pipette, but the alternative described immediately below may be easier.

If not using a 96-well pipette, consider making eight mastermixes each with sufficient reagents for 12 samples (20 µL µL mastermix per well, so 264 µL µL for 12 with some overage for pipetting

error), each containing a different forward primer (with 2 µL µL of [M] 2.5 micromolar (µM) forward primer per well, so 26.4 µL µL per mastermix for 12 with some overage for pipetting error). Distribute 22 µL µL of forward primer + mastermix to each well across the rows corresponding to each forward primer. Then distribute 2 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

18 Add 1 µL DNA to each corresponding sample and positive control well, and 1 µL molecular 5m grade water to each negative control other than extraction negative controls (which should be added as samples).

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

20 Load the PCR plate into a thermocycler. Ensure that the temperature regime matches the enzyme (including any heat activation for hot-start Taq) and that the annealing temperature matches the PCR primers used. 2h 30m

> **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.

21 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 concentrations. Gel electrophoresis is nevertheless described below.

Gel electrophoresis 1h 15m

22 Prepare a gel tank containing sufficient 1X TBE buffer to submerge the gel. 1m

> **Note**
>
> TBE buffer can be purchased pre-made or made from its constituent reagents. Alternative buffers like borax can, however, be cheaper, easier and quicker to use.

23 Prepare a [M] 2 Mass / % volume agarose gel for the samples to go into (detailed in the below substeps).

23.1 Prepare a gel mould with sufficient comb tips for the number of samples to be run. Ensure these a 1m spaced out enough to allow the gel to run and separate without running into another well.

23.2 Determine a suitable size (and therefore volume) of gel based on the size of the tank available. For 2m 150 mL gel, weigh 3 g of agarose and add it to 150 mL of 1X TBE buffer.

23.3 Heat this mixture in a microwave in a conical flask until it begins bubbling. 2m

23.4 Mix the solution by gently swirling it and heat again until bubbling. Repeat this as necessary until there are no large lumps of agarose remaining. 1m

> **Note**
>
> It can be beneficial to let the mixture cool for 00:00:30 before proceeding.

23.5 Add 3 µL of gel dye (e.g., SYBRSafe) and mix by swirling. 1m

23.6 Pour the mixture into the gel mould and allow to set for 00:25:00. 25m

24 Add 2 µL of each sample to a new plate and add 2 µL of 1X DNA dye for visualisation of th products in the gel. 10m

25 Once set, place the gel into the tank and add 4 µL of DNA gel ladder to each side of the gel in eac row, and then 4 µL of dyed PCR product to each other well. 5m

> **Note**
>
> Ensure that the DNA will run in the correct direction when placing the gel in the tank. TIP: it will 'run to the red'.

26 Once filled, run the gel power pack at 120 V and 120 A for 00:25:00 . 25m

27 Visualise the gel under a UV or blue light in a gel dock to confirm amplification and a lack of contamination in negative controls. 2m

> **Note**
>
> Ensure you are not exposed to UV by only using the UV light in a contained dock and/or with appropriate protective equipment.

PCR product normalisation 1h 21m

28 If the PCRs were replicated (i.e., each sample run multiple times for each PCR primer pair used), these 5m can be merged together into one plate per PCR primer pair 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 25 µL reaction volumes, pipette 20 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.

> **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 and mostly left behind.

29 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.

29.1 If using beads such as Sera-Mag Magnetic SpeedBeads (carboxylated, 1 µm , 3 EDAC/PA5), t 5m 1 mL of well-mixed bead solution and wash the beads twice with TE+Tween buffer (

[M] 10 millimolar (mM) Tris base, [M] 1 millimolar (mM) EDTA, [M] 0.05 % volume 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.

29.2 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

29.3 Add 20 mL of [M] 50 % volume PEG to the tube to reach a 1X bead solution (alongside maki the 0.1X solution, this will be useful later). 1m

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

	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
	50 % PEG	20 mL

30 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 PCR product is available, add a volume equivalent to the available PCR product volume of 0.1X SPRI beads to a new 96-well plate. 2m

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

31 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, 00:01:00). 3m

> **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.

32 Incubate at Room temperature for 00:05:00 . 5m

33 Place on a magnetic stand for 00:05:00 . 5m

34 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). 2m

35 Add 200 µL [M] 80 % volume ethanol to each well. 1m

36 Remove the ethanol and add a further 200 µL [M] 80 % volume ethanol to each well. 2m

37 Remove the ethanol as completely as possible by pipetting. 1m

> **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.

38 Add 10 µL molecular grade water to each well, shake at 1500 rpm, 00:01:00 and incubate at 7m Room temperature for 00:05:00.

39 Place on a magnetic stand for 00:05:00. 5m

40 Pool 8 µL from each well into a single tube, leaving the magnetic beads behind in the 96-well pla 2m

> **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.

41 Mix the pooled samples by flicking and inverting, and transfer 700 µL into a new 1.5 mL microcentrifuge tube. 1m

42 Add 700 µL 1X magnetic beads for DNA purification (see note below for size selection) and mix 6m

vortexing. Incubate at Room temperature for 00:05:00.

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).

43 Place on a magnetic stand for 00:05:00. 5m

44 Add 400 µL [M] 80 % volume ethanol. 1m

45 Remove the ethanol and add a further 400 µL [M] 80 % volume ethanol. 1m

46 Remove the ethanol completely. Briefly centrifuge the sample to collect and remove any residual etha 3m
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).

47 Add 100 µL molecular grade water and mix by vortexing. Incubate at Room temperature for 6m
00:05:00.

48 Place the sample on a magnetic stand for 00:05:00. 5m

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

PCR2 and library preparation 2h 21m

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

> **Note**
>
> At this point, many sequencing facilities will accept the samples and can carry out the subsequent steps themselves. This may be preferable if the appropriate library preparation/indexing primers are not available. Below, the process for PCR-based library preparation will be detailed as an example.

51 Dilute library to ≤ [M] 5000 µg/µL (≤5 ng/µL) in molecular grade water. 1m

> **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.

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

53 Per library, assemble the following reaction: 5m

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

	A	B
	DNA library	5

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

55 Load the PCR reactions into a thermocycler. Ensure that the temperature regime matches the enzy 1h 30m
used (including any heat activation for hot-start Taq) and that the annealing temperature matches the PCR primers used. Run for 12 cycles.

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

57 Check that the adapters have been added to the DNA by determining and comparing the amplicon si 30m
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.

58 Once successful adapter addition is confirmed, libraries can be pooled so that each is equimolar in th 3m
final mixture based on the concentrations determined above (e.g., by Qubit).

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.

Sequencing

59 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. Sequencing times will depend on the machines, protocols and any queues involved at the sequencing facility.

Bioinformatics

60 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.