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High-throughput individual insect metabarcoding for identification and interaction data

Forked from [Quantifying ecosystem service provider interactions via bulk sample DNA metabarcoding](#)

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Jordan P Cuff¹, Thomas Howells¹, James JN Kitson¹, Ben SJ Hawthorne¹, darren.evans¹

¹Newcastle University

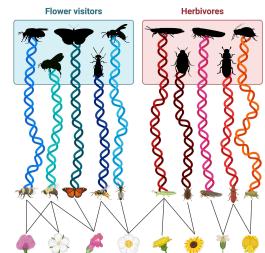
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Network Ecology Group



Jordan P Cuff

Newcastle University



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We use this protocol and it's working

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Abstract

This protocol is designed for extracting DNA from individual invertebrates for metabarcoding to detect and identify ecosystem service and disservice providers and their interactions. The reagents and methods proposed offer a cost effective and high-throughput method for molecular analyses of individual invertebrates using standard lab equipment. Where specialist equipment is used, attempts are made to suggest low-cost alternatives.

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Materials

For field collection and initial storage:

- Small collection pots or tubes for storage of samples
- 100 % ethanol for preservation
- Chemgene/diluted bleach for sterilisation of equipment

For DNA extraction:

- Hardened carbon steel ball bearings
- 2.2 mL deep well plates for initial lysis
- Deep-well and standard Kingfisher 96-well plates
- 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
- Tris-HCl
- EDTA
- GITC
- Nuclease-free water
- SDS
- PEG
- Tris-HCl
- 100 % ethanol
- Papain

Equipment:

- -20 °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)

- Ideally, Kingfisher Apex or similar
- Illumina sequencer

Safety warnings

- 1 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.

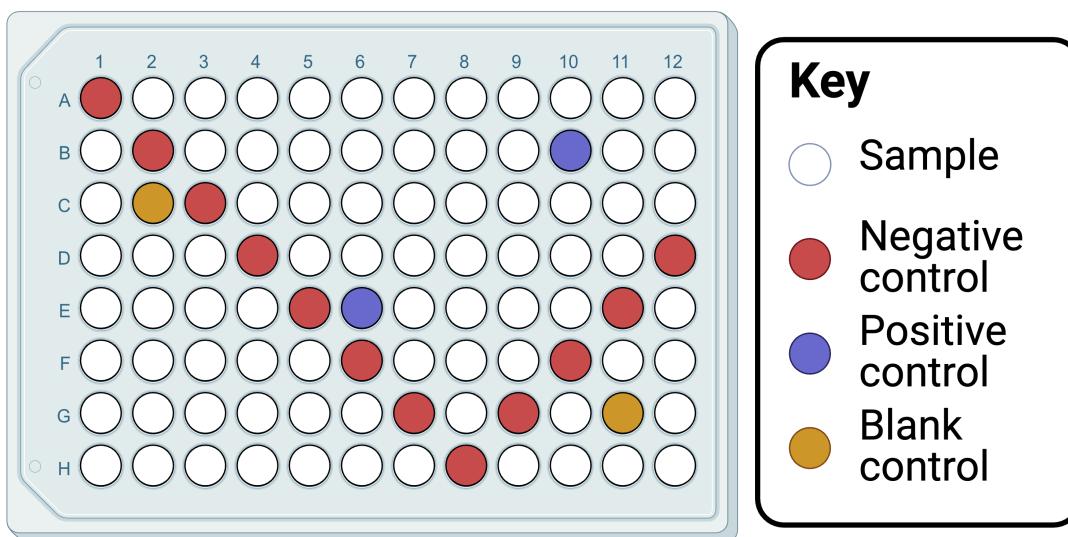
Ethics statement

Check national and institutional policies for insect research. Follow best practice guidelines and stay up to date with the latest developments in insect welfare (e.g., through the Insect Welfare Research Society). Only kill as many invertebrates as is necessary, and always do so as humanely as possible.

Sampling of invertebrates

3d 0h 30m

- 1 Select suitable sites and locations for insect sampling. This protocol is based on hand-collected foliar and flower visitor invertebrates, but other collection methods are viable; be wary of cross-contamination and surface DNA though, and consider surface sterilisation accordingly. Consider how systematic the study needs to be and the various constraints imposed on the data by the study design.
- 2 Following collection, store insects in sterile tubes. For flower visitors, store insects dry and consider killing them with ethyl acetate on a cotton ball; for other insects, storage in 100 % ethanol may be ideal. Kill invertebrates as humanely as possible. Freezing is widely considered to be among the optimal methods. 3d
- 3 Transfer individual invertebrates into 96-well plates (ideally deep-well, e.g., 2.2 mL, with fixable lids for grinding/washing). Consider the distribution of experimental controls ahead of subsequent steps to streamline downstream liquid handling. 15m



Our recommended PCR plate layout, which could be adopted here for streamlining downstream. Created in BioRender. Cuff, J. (2025) <https://BioRender.com/559f6hh>

Store samples at -20 °C until ready to process.

Preparation and homogenisation of samples

16h 8m

- 4 The DNA extraction protocol is largely adapted from the BOMB-Bio tissue nucleic acid extraction protocol. See their documentation for additional detail.

CITATION

Oberacker P, Stepper P, Bond DM, Höhn S, Focken J, Meyer V, Schelle L, Sugrue VJ, Jeunen GJ, Moser T, Hore SR, von Meyenn F, Hipp K, Hore TA, Jurkowski TP (2019). Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation..

LINK

<https://doi.org/10.1371/journal.pbio.3000107>

- 5 Add  100 µL TNES lysis buffer to each well of a 96-well plate.

5m

Note

For the TNES buffer, follow the recipe provided by BOMB-Bio (
[M] 100 millimolar (mM) Tris-HCl, [M] 52 millimolar (mM) NaCl, [M] 10 millimolar (mM) EDTA, [M] 10 Mass / % volume SDS).

- 6 Two protocols are presented below, which simply differ in whether samples are ground or surface-washed. For herbivorous insects, we recommend grinding to facilitate analysis of internal DNA (either consumed plants or parasitoids). For flower visitors, we recommend surface washing (exactly the same protocol, but without ball bearings during the grinding step). This still facilitates analysis of DNA from the insect itself, but also pollen on the surface of the insect. These protocols could both be used for a wider range of potential applications.

STEP CASE

Herbivorous insects 1 step

- 7 Add one 3 mm hardened carbon steel bead to each well.

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.

8 Grind the samples in a tissue grinder/homogeniser/lyser at 1750 RPM for 1 minute.

1m

9 Add  10 µL  20 mg/mL papain to each well.

2m

Note

For streamlining, you could add the papain prior to grinding, but be aware that this may reduce its efficacy.

10 Incubate overnight (~  16:00:00) at  37 °C.

16h

DNA extraction

1h 5m

11 Centrifuge the plate at  2000 x g, Room temperature, 00:02:00.

2m

12 Prepare Kingfisher Apex reagent plates as below.

Note

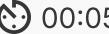
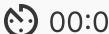
The Kingfisher Apex is ideal for automated high-throughput extraction of DNA, but will not always be available. Alternative equipment can achieve similar results, including just using magnetic racks. In that case, rather than transferring beads between plates, remove the supernatant and replace it with the next reagent, as described for normalisation below.

12.1 For the sample plate, to each well of a 96-well deep-well plate, add  60 µL of the lysate from STEP 11,  120 µL of 1.5X GITC buffer,  120 µL of  1 mg/mL SeraMag Speed Beads in TE ( 10 millimolar (mM) Tris and  1 millimolar (mM) EDTA) and  240 µL isopropanol.

5m

Note

For the 1.5X GITC buffer, follow the recipe provided by BOMB-Bio ([M] 6 Molarity (M) GITC, [M] 75 millimolar (mM) Tris-HCl, [M] 3 % volume sarkosyl, [M] 30 millimolar (mM) EDTA, [M] 0.15 % volume antifoam).

- 12.2 For the two ethanol plates, to each well of two 96-well deep-well plates, add  400 μ L 80 % ethanol. 2m
- 12.3 For the isopropanol plate, to each well of a 96-well deep-well plate, add  400 μ L isopropanol. 1m
- 12.4 For the elution plate, to each well of a 96-well standard plate, add  100 μ L molecular biology grade water. 1m
- 13 Insert plates into the Kingfisher Apex and run a preset programme with the below steps. 30m
 - 13.1 Pick up the 96 deep-well tip comb from a 96-well standard plate. 30s
 - 13.2 Bind DNA to the beads by mixing at medium speed for  00:05:00 with a slow post-mix for  00:05:00. 10m
 - 13.3 Collect the beads in three  00:00:01 collections. 10s
 - 13.4 Release the beads into the isopropanol plate and mix for  00:01:00 at medium speed, and collect the beads in three  00:00:01 collections. 1m 30s
 - 13.5 Release the beads into one of the 80 % ethanol plates and mix for  00:01:00 at medium speed, and collect the beads in three  00:00:01 collections. 1m 30s

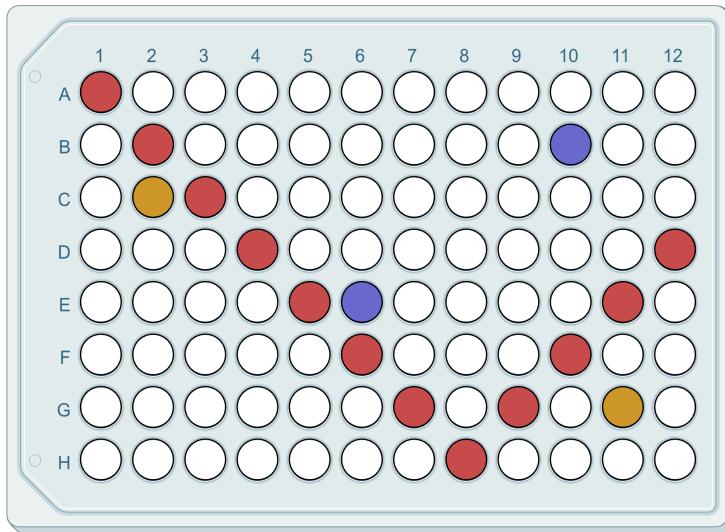
- 13.6 Release the beads into one of the 80 % ethanol plates and mix for  00:01:00 at medium speed, and collect the beads in three  00:00:01 collections. 1m 30s
- 13.7 Dry the beads above the well for  00:02:00. 2m
- 13.8 Release the beads into the elution plate and mix for  00:02:00 at fast speed with a slow post-mix for  00:03:00, and collect the beads in four  00:00:20 collections. 6m
- 13.9 Leave the tip comb in an empty 96-well standard plate. 30s
- 14 Store the eluted DNA at  -20 °C until ready for subsequent steps.

PCR

3h 22m

- 15 Decide how samples will be distributed across plates (but don't distribute the 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).



Key

- Sample
- Negative control
- Positive control
- Blank control

Our recommended PCR plate layout, which could be adopted here for streamlining downstream. Created in BioRender. Cuff, J. (2025) <https://BioRender.com/559f6hh>

- 16 Prepare enough PCR mastermix for each sample.

2m

For a full plate, the below 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

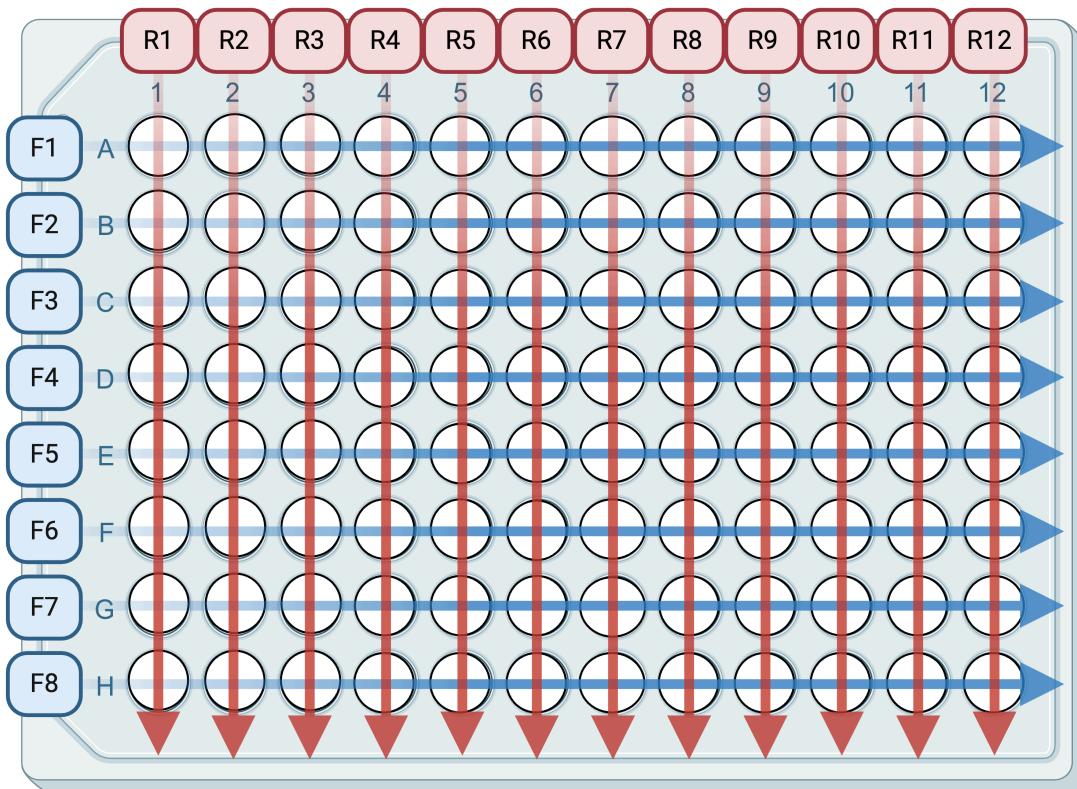
These values are for $\text{10 } \mu\text{L}$ reaction volumes, which have been demonstrated to be effective for community metabarcoding. Consider running them in triplicate for more accurate results.

- 17 For ease, if able to use a 96-well pipette, consider creating a "primer plate" containing both PCR primers for each well at 5 μM concentration; this is especially effective when

15m

using multiple plates. For $\text{10 } \mu\text{L}$ reaction volumes, this will subsequently involve adding $\text{7.75 } \mu\text{L}$ hot-start Taq polymerase and water mastermix (described in the step above) to each well, followed by $\text{1.25 } \mu\text{L}$ of each primer mix to its corresponding well. It is possible to use this strategy with a multichannel pipette, but the next suggestion may be easier.

If not using a 96-well pipette, consider making eight mastermixes each with sufficient reagents for 12 samples ($\text{7.75 } \mu\text{L}$ mastermix per well, so $\text{97.65 } \mu\text{L}$ for 12 with some overage for pipetting error), each containing a different forward primer (with $\text{0.625 } \mu\text{L}$ of $5 \text{ micromolar } (\mu\text{M})$ forward primer per well, so $\text{7.875 } \mu\text{L}$ per mastermix for 12 with some overage for pipetting error). Distribute $\text{8.375 } \mu\text{L}$ of forward primer + mastermix to each well across the rows corresponding to each forward primer. Then distribute $\text{0.625 } \mu\text{L}$ 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. Created in BioRender. Cuff, J. (2025) <https://BioRender.com/559f6hh>

18 Add  1 μL DNA to each corresponding sample or positive control well, and  1 μL molecular grade water to each negative control other than extraction negative control(s). 10m

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

Note

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

Mineral oil improves sealing of reactions by preventing evaporation and condensation. By reducing evaporation and thus loss of product, this also reduces potential cross-contamination.

20 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

21 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 samples, and inaccuracies in temperature calibration, considering running a temperature gradient PCR with known samples to check the specificity of your PCR primers.

22 Run your PCR programme. 2h

23 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. 40m

Normalisation

24

1h 55m

Note

5m

Equalisation is more effective than normalisation, but requires amplicon concentration data. Both can be time and labour intensive though, so can be skipped if time restricted for large-scale projects, although at the expense of data recovery and evenness.

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 inconsistencies between samples. To merge triplicates, assuming use of $\text{PCR} 10 \mu\text{L}$ reaction volumes, pipette $\text{PCR} 8 \mu\text{L}$ 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

5m

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.

25 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.

20m

25.1 If using beads such as Sera-Mag Magnetic SpeedBeads (carboxylated, 1 μm , 3 EDAC/PA5), take $\text{PCR} 1 \text{ mL}$ of well-mixed bead solution and wash the beads twice with TE+Tween buffer ($[M] 10 \text{ millimolar (mM)}$ Tris base, $[M] 1 \text{ millimolar (mM)}$ EDTA, $[M] 0.05 \% \text{ volume}$ 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.

5m

25.2 To the beads, add the following mix:

5m

A	B
Reagent	Volume
5 M NaCl	25 mL
Molecular grade water	3.582 mL

A	B
1 N HCl	0.168 mL
1 M Tris base	0.5 mL
0.1 M disodium EDTA	0.5 mL

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

2m

- 25.4 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
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

- 26 If \geq  20 μ L of PCR product is available in each well, pipette  20 μ L 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.

- 27 Add  20 μ L 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 (

2m

⌚ 1500 rpm, Room temperature , 00:01:00).

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.

28 Incubate at 🌡 Room temperature for ⏱ 00:05:00 .

5m

29 Place on a magnetic stand for ⏱ 00:05:00 .

5m

30 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 solutions).

5m

31 Add 🧨 200 µL [M] 80 % volume ethanol to each well.

2m

32 Remove the ethanol and add a further 🧨 200 µL [M] 80 % volume ethanol to each well.

4m

33 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.

34 Add 🧨 10 µL molecular grade water to each well, shake at

7m

⌚ 1500 rpm, Room temperature , 00:01:00 and incubate at 🌡 Room temperature for ⏱ 00:05:00 .

- 35 Place on a magnetic stand for  00:05:00 . 5m
- 36 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.
- 37 Mix the pooled samples by flicking and inverting, and transfer  700 µL into a new 2.5 mL microcentrifuge tube. 1m
- 38 Add  700 µL 1X magnetic beads for DNA purification (see note below for size selection) and mix by vortexing. Incubate for  00:05:00 . 6m
- Note**

This is a good opportunity for size selection as well, especially if the PCR product contains any secondary bands. If using SPRI beads, adjust the volume added to select different fragment sizes.
- 39 Place on a magnetic stand for  00:05:00 . 5m
- 40 Add  400 µL  80 % volume ethanol. 1m
- 41 Remove the ethanol and add a further  400 µL  80 % volume ethanol. 1m
- 42 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 2m

'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).

43 Add 100 µL molecular grade water and mix by vortexing. Incubate for

00:05:00 at Room temperature .

6m

44 Place the sample on a magnetic stand for 00:05:00 .

5m

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

1m

Library preparation and sequencing

3h 9m

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

5m

47 Dilute library to \leq 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.

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

1m

49 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

- 50 Briefly centrifuge the tubes to ensure that the oil is above the PCR mix. 1m
- 51 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. 5m
- 52 Run your PCR programme for 12-20 cycles, with 12 for ~5 ng/ μ L libraries and more for lower concentrations. 2h
- 53 Quantify the concentration of the library from the previous step (e.g., using Qubit dsDNA assay). 5m
- 54 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
- 55 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 μ L 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/ μ L.

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

Protocol references

CITATION

Oberacker P, Stepper P, Bond DM, Höhn S, Focken J, Meyer V, Schelle L, Sugrue VJ, Jeunen GJ, Moser T, Hore SR, von Meyenn F, Hipp K, Hore TA, Jurkowski TP (2019). Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation..

LINK

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Citations

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Step 4

Oberacker P, Stepper P, Bond DM, Höhn S, Focken J, Meyer V, Schelle L, Sugrue VJ, Jeunen GJ, Moser T, Hore SR, von Meyenn F, Hipp K, Hore TA, Jurkowski TP. Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation.

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