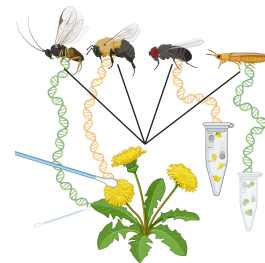


Apr 30, 2025

🌐 Detecting arthropod leaf and flower visitation using DNA metabarcoding

🔗 Forked from [High-throughput individual insect metabarcoding for identification and interaction data](#)



DOI

<https://dx.doi.org/10.17504/protocols.io.yxmvmvoj5v3p/v1>

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Foraging Ecology Resea...



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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 plant material or swabs for metabarcoding to detect and identify ecosystem service and disservice providers and their interactions with plants. The reagents and methods proposed offer a cost effective and high-throughput method for molecular analysis using standard lab equipment. Where specialist equipment is used, attempts are made to suggest low-cost alternatives.

Image Attribution

Main image was created in BioRender (Cuff, J. (2025) <https://BioRender.com/ih4gopk>). Other images were retained from the forked protocol (created in BioRender; Cuff, J. (2025) <https://BioRender.com/559f6hh>).

Materials

For field collection and initial storage:

- Small tubes for storage of samples
- Chemgene/diluted bleach for sterilisation of equipment
- TNES buffer
- Cotton swabs

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
- Liquid nitrogen
- 5 mL centrifuge tubes

For DNA amplification and subsequent steps:

- Tagged PCR primers
- 2X hot-start Taq polymerase master mix
- Molecular grade water
- 96-well PCR plates
- Mineral oil
- 1X SPRI beads

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)
- Kingfisher Apex or similar
- Qiagen Qiaxcel or similar

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.

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). These methods should not involve killing any invertebrates (and are an excellent alternative to such methods), but if that is incorporated for any reason, only kill as many invertebrates as is necessary, and always do so as humanely as possible.

Collecting plant material

15m

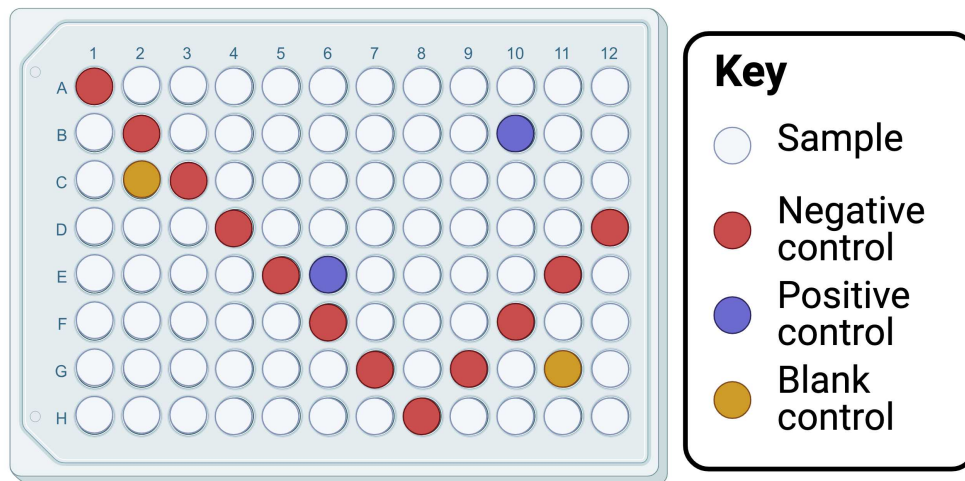
- 1 Select suitable sites and locations for plant surveying. This protocol is based on hand-collected leaves and flowers, or swabs of leaves and flowers, but other collection methods may be viable; be wary of potential sources of contamination though. Consider how systematic the study needs to be and the various constraints imposed on the data by the study design.
- 2 For plant material (e.g., leaves, flowers), tissue can be taken and placed in a labeled tube (5 mL centrifuge tubes are recommended downstream) until it can be frozen in a laboratory. One flower head and 1-2 small leaves should suffice, depending on size. For swabs, a cotton swab can be dipped into TNES buffer and then each side of the swab brushed against the plant tissue in a rolling motion 4-8 times, turning against the brushing motion to maximise contact.

Note

For the TNES buffer, follow the recipe provided by BOMB-Bio (1M 100 millimolar (mM) Tris-HCl, 1M 52 millimolar (mM) NaCl, 1M 10 millimolar (mM) EDTA, 1M 10 Mass / % volume SDS).

- 3 Following collection, store samples in sterile tubes (e.g., 5 mL centrifuge tubes). For flowers and leaves, store samples dry; for swabs, storage should similarly be dry except for the TNES used for swabbing. Only take as much material as is necessary to minimise any impact.
- 4 Transfer individual swabs into deep-well (e.g., 2.2 mL) 96-well plates. Consider the distribution of experimental controls ahead of subsequent steps to streamline downstream liquid handling. Plant material will transfer to a plate format later, but this is still important to consider early on; instead, plant material can be placed into separate 5 mL centrifuge tubes.

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 26m

- 5 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.

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

LINK

- 6 Two protocols are presented below, which simply differ in whether samples are ground or not. For plant material, we recommend grinding to facilitate analysis of internal DNA (e.g., internal herbivores like leaf miners). For swabs, we recommend simply soaking to extract DNA (otherwise exactly the same protocol, but different initial treatment). These protocols could both be used for a wider range of potential applications.



STEP CASE

Plant material 1 step

- 7 Add two 3 mm hardened carbon steel beads to each 5 mL sample 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.

- 8 Place each sample tube into an aluminium tube rack in a robust water-right container (e.g., polystyrene delivery box). Pour liquid nitrogen over the sample and the block, allowing it to stand in liquid nitrogen for ~ 00:10:00 to cool sufficiently. 10m

Note

This step is much quicker if the aluminium block is already pre-cooled to -20 °C prior to the extractions.

- 9 Grind the samples in a tissue grinder/homogeniser/lyser at 1750 rpm, Room temperature , 00:00:30 . 1m

Note

You can repeat this step until the sample is adequately homogenised, but so long as most of the tissue is homogenised, that should suffice.

- 10 Add 100 µL of TNES to each sample. For larger samples, consider adding more (up to 500 µL to ensure coverage and mitigate subsequent collection of solid material). 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).

- 11 Add  10 μ L [M] 20 mg/mL papain to each well.

5m

Note

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

If using larger volumes of TNES, adjust papain volumes accordingly.

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

16h

DNA extraction

1h 6m 40s

- 13 Centrifuge the tubes at  2000 x g, Room temperature, 00:02:00 to separate solid material from the supernatant.

2m

- 14 Transfer  100 μ L to a 96-well filter plate with a 96-well plate positioned below to catch and retain the flow-through. Centrifuge at  2000 x g, 00:02:00 to separate the supernatant from the physical material and discard the filter plate.

2m

**Note**

This is **not** referring to silica membrane spin columns, which are commonly used in spin column-based DNA extractions. Instead, these plates filter out physical material and let liquid through.

If more than $100\ \mu\text{L}$ TNES was added in STEP 10, you can transfer more to the filter plate, but take note of the filter and capture plate volumes and do not exceed them.

If less than $100\ \mu\text{L}$ can be collected, so long as $60\ \mu\text{L}$ is available for STEP 15, that's okay. If that is not possible (e.g., due to physical plant material), add more TNES to dilute the lysate and take that forward.

15 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.

- 15.1** For the sample plate, to each well of a 96-well deep-well plate, add $60\ \mu\text{L}$ of the filtered lysate from STEP 14, $120\ \mu\text{L}$ of 1.5X GITC buffer, $120\ \mu\text{L}$ of $1\ \text{mg/mL}$ SeraMag Speed Beads in TE and $240\ \mu\text{L}$ isopropanol.

5m

Note

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

- 15.2** For the two ethanol plates, to each well of two 96-well deep-well plates, add $400\ \mu\text{L}$ $80\ \%$ ethanol.

2m



- 15.3 For the isopropanol plate, to each well of a 96-well deep-well plate, add  400 μL isopropanol. 1m
- 15.4 For the elution plate, to each well of a 96-well standard plate, add  100 μL molecular biology grade water. 1m
- 16 Insert plates into the Kingfisher Apex and run a preset programme with the below steps. 30m
- 16.1 Pick up the 96 deep-well tip comb from a 96-well standard plate. 30s
- 16.2 Bind DNA to the beads by mixing at medium speed for  00:05:00 with a slow post-mix for  00:05:00 . 10m
- 16.3 Collect the beads in three  00:00:01 collections. 10s
- 16.4 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
- 16.5 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
- 16.6 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
- 16.7 Dry the beads above the well for  00:02:00 . 2m
- 16.8 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
- 16.9 Leave the tip comb in an empty 96-well standard plate. 30s

- 17 Store the eluted DNA at -20 °C until ready for subsequent steps.

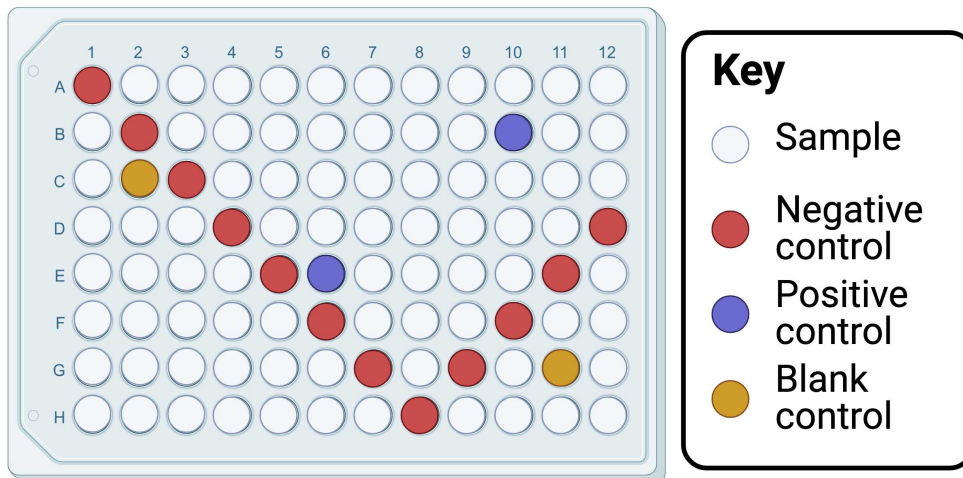
PCR

3h 22m

- 18 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).



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

- 19 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)	1131.6
2X hot-start PCR mastermix	1380

Note

These values are for  25 μL reaction volumes with  1 μL of template given the potentially high concentration of inhibitors in plant tissue samples, although smaller reaction volumes should be accurate too. Consider running them in triplicate for more accurate results.

- 20 Using tagged primers, unique identifiers can be established for a full plate with 8 uniquely tagged forward primers and 12 uniquely tagged reverse primers. 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. For  25 μL reaction volumes, this will subsequently involve adding  22.75 μL hot-start Taq polymerase and water master mix (described in the step above) to each well, followed by  1.25 μL of each primer mix to its corresponding well. It is possible to do this with a multichannel pipette.

15m



Distribution of tagged PCR primers across the 96-well plate. Created in BioRender. Cuff, J. (2025) <https://BioRender.com/559f6hh>

21 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

22 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.

23 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



- 24 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.

- 25 Run your PCR programme.

2h

- 26 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

Equalisation

2h 29m

- 27

5m

Note

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. This protocol presents equalisation, but see the protocol from which this is forked for a step-by-step description of normalisation.

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 that may arise from contamination or error. To merge triplicates, assuming use of  25 μL reaction volumes, pipette  22 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.

- 28 Analyse the PCR products via digital electrophoresis (e.g., Qiagen Qiaxcel). This provides data on the concentration of specific amplicons (i.e., the target amplicon of the PCR primers used), which will be used in the next two steps.

30m

Note

If digital electrophoresis (or an equivalent platform that facilitates quantification of concentration by band size) isn't available, you could proceed through the steps from STEP 31 in this section to remove primers and other non-target DNA, and quantify concentration using a fluorescence-based assay (e.g., Qubit dsDNA assay) to pool (as in STEPS 29-30) based on those values. It is important that primer dimers and other small fragments are removed (e.g., via magnetic bead-based purification) prior to these fluorescence-based assays as they can otherwise inflate and distort concentration values.

- 29 Based on the concentration of the target amplicon in each sample, calculate how much to pool from each sample in order to achieve equimolarity (i.e., equal representation of samples). This can be achieved by dividing the maximum concentration within the plate by the concentration of each individual sample.

5m

- 30 Pool the value calculated for each sample in the previous step as a volume in microlitres. Positive controls can be treated as samples. For negative controls, pool the average volume pooled across the samples. For blanks, do not pool anything. If the volume to be pooled exceeds the total volume available, pool the total volume available. For samples with low or no concentration, the PCRs for these samples could be repeated, the samples could be excluded, or these can be pooled in the same manner as negative controls (although be wary in any interpretation of the data given increased potential for inaccuracy).

30m

Note

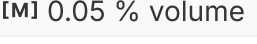
If most of the volumes are similar and small, you can double (or otherwise multiply) the values.

If there is a large variation in the volumes to be pooled, the values can be halved to facilitate pooling more dilute samples proportionally.



31 To purify these pools ahead of library preparation, prepare a 1X solid phase reversible immobilisation (SPRI) bead solution and bring it to room temperature. The below steps detail how to make this solution, but it is also commercially available.

20m

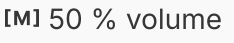
31.1 If using beads such as Sera-Mag Magnetic SpeedBeads (carboxylated, 1 μ m, 3 EDAC/PA5), take  1 mL of well-mixed bead solution and wash the beads twice with TE+Tween buffer ( 10 millimolar (mM) Tris base,  1 millimolar (mM) EDTA,  0.05 % volume Tween 20, pH 8.0) by magnetising the beads, removing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

5m

31.2 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

31.3 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

31.4 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

- 32 If $\geq$  20 μL of PCR product is available for each pool, pipette  20 μL of 1X SPRI bead solution into each corresponding well of a 96-well plate or individual tubes. If less DNA is available, add a volume of SPRI bead solution equivalent to the full available volume of PCR product to the each corresponding well of the new 96-well plate or individual tubes.

2m

Note

When working with magnetic bead solutions, ensure they are at room temperature and fully mixed, with no residue at the bottom of the container.

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. Refer to manufacturer details for more information on size selection.

Larger volumes can be used, so long as the corresponding volume of beads is used. For volumes greater than  100 μL , consider doubling the ethanol and water volumes in the remaining steps of this section.

- 33 Add  20 μL of PCR product (or whatever volume of beads was used in the last step) to each corresponding well of 1X bead solution, avoiding oil, and mix by vortexing ( 1500 rpm, Room temperature , 00:01:00).

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.

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

5m

35 Place on a magnetic stand for 00:05:00 .

5m

36 Remove all but 5 μL of the mixture from each well via pipette without disturbing the beads, which should be settled on the magnet.

5m

Note

We recommend leaving 5 μL behind simply to avoid pipetting the beads themselves, but this can be avoided with care and experience.

37 Add 200 μL 80 % volume ethanol to each well.

2m

38 Remove the ethanol and add a further 200 μL 80 % volume ethanol to each well.

4m

39 Remove the ethanol as completely as possible by pipetting and allow the beads 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).

4m

40 Add 11 μL molecular grade water to each well, shake at 1500 rpm, Room temperature , 00:01:00 and incubate at Room temperature for 00:05:00 .

7m



41 Place on a magnetic stand for  00:05:00 .

5m

42 Remove  10 μL of the supernatant and place it into a new tube. This will be the library used in any subsequent library preparation.

1m

Library preparation and sequencing

5m

43 Quantify the concentration of the library from the previous step (e.g., using Qubit dsDNA assay) and take this forward for library preparation. For Oxford Nanopore Technologies sequencing, follow the manufacturer protocols (or adaptations of them on here). For Illumina sequencing, consider protocols such as the one this is forked from if your primers include a Nextera overhang; otherwise, consider ligation-based library preparation methods. 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.

5m

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.

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

LINK

Citations

Step 5

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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