WP6 Barcode Sequencing

T6.2 Metabarcoding for Biomonitoring

Invertebrate characterization from bulk arthropod samples

Lab Standard Operating Procedure (SOP)

Co-funded by the European Union

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
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UK Research and Innovation

BIODIVERSITY GENOMICS EUROPE

receives funding from the European Union's Horizon Europe Research and Innovation Action.

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Background

The Biodiversity Genomics Europe (BGE) Consortium has the overriding aim of accelerating the use of genomic science to enhance understanding of biodiversity, monitor biodiversity change, and guide interventions to address its decline. The objective is to establish functioning biodiversity genomics networks, data generation and pipelines to characterize biodiversity, and to improve management intervention and biomonitoring programs by practical application of genomic tools.

Within the BGE project, in terrestrial field sampling, arthropods were sampled across Europe to discover biodiversity and to assess pan-European patterns of species diversity and community composition in key systems, using DNA barcoding (Hebert et al., 2003) and metabarcoding (Taberlet et al., 2012) techniques. More specifically, the case study High Mountain Systems (HMS) aimed establishing a baseline sampling in mountain ranges across Europe to track biodiversity shifts associated with climate change; and the Pollinator Communities (PC) case study aimed to set a baseline for future monitoring efforts on pollinator temporal trends in European urban and agricultural habitats, to quantify differences in pollinator community attributes between urban and agricultural habitats, and to identify pollinator species traits associated with urban living.

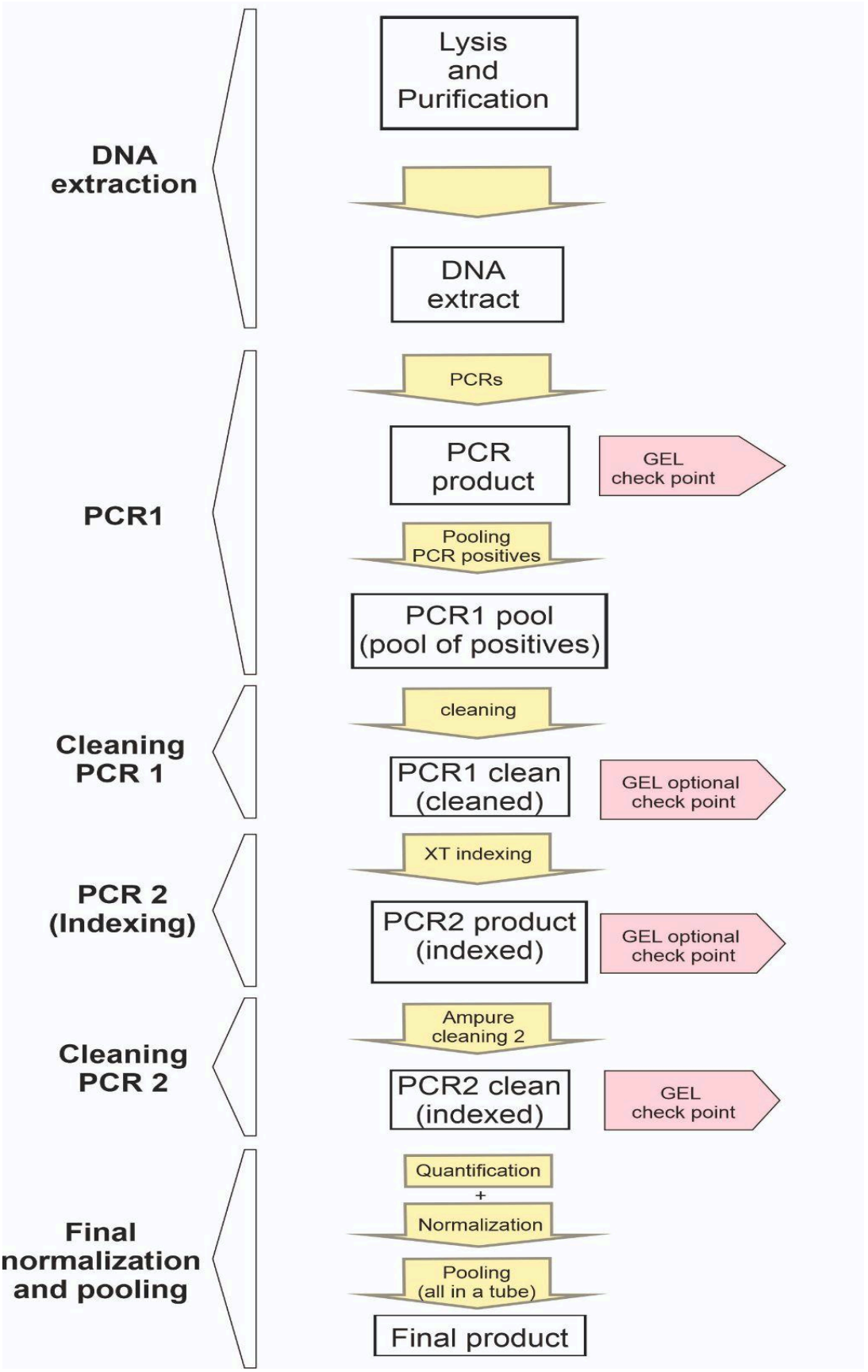
For fieldwork of these studies, Malaise traps were used to sample arthropods bulk samples, contained in ethanol. For the HMS case study, two Malaise traps (one for sampling and one as back up) were placed in five altitudinal stages, to be collected each week, during 20 weeks. For the PC case study, a simultaneous paired sampling, one trap in an urban garden and one in an agricultural field, was carried out on each selected site, during five weeks. For more information about the arthropod bulk sampling with Malaise traps please consult the BGE | High Mountain Systems - Arthropod sampling with Malaise traps Standard Operating Procedure (SOP) (Najera-Cortazar et al., 2024a), and the BGE | Pollinator Communities - Malaise trap sampling SOP (Najera-Cortazar et al., 2024b) in WorkflowHub (Gustafsson et al., 2025).

For both BGE case studies laboratory work, the FAVIS: Fast and Versatile protocol for metabarcoding of bulk Insect Samples (Iwaszkiewicz-Eggebrecht et al., 2023a; 2023b), a non-destructive metabarcoding protocol optimized for high-throughput processing of bulk arthropod samples, was used. We describe the modifications performed within the BGE project, according to each step of the FAVIS protocol, and we include the detailed instructions for using a modular robotic workstation for automated liquid handling and sample processing (TECAN Freedom Evo 150) during library preparation. The protocol is divided in four sections, and each section has different subsections and steps, format that we will preserve within this document, in order to facilitate following the modifications performed within the BGE lab work. Please consult alongside the full FAVIS protocol (Iwaszkiewicz-Eggebrecht et al., 2023a; 2023b) following the link provided, which includes multiple resources to correctly follow the protocol.

Specifications

- Collecting bottle size was specified at the beginning of both case studies, in order to have the adequate size and width of mouth to be compatible with the lab bottle heated shaker (VWR Incu-Line ILS6). Partners were provided with bottles, and were advised to buy the following model or similar: 500 mL Thermo Scientific™ Nalgene™ Wide-Mouth LDPE Bottles with Closure
- The sampling SOPs (Najera-Cortazar et al., 2024a, 2024b) provided to each partner specified to use at least 96% ethanol for filling the bottles while sampling
- We did not include an identification label for the samples inside the bottles. The information for each sample is contained in a QR code affixed to the outside of each collection bottle. This way, we avoided handling the samples before the alcohol had settled.
- For international shipping of Malaise trap samples, ethanol was decanted from each bottle, leaving less than 10 mL per bottle. For more information about the shipping process, please consult Malaise traps - Bulk sample shipping instructions. Sufficient ethanol was added upon arrival so that the sample is submerged. Bottles were stored at -20°C before processing
- We did not use a pre-heating bath to preheat the insect bottles. We pre-heated buffers and bottles with specimens independently in the dry oven. Once they reached a working temperature of around 56º (after about an hour), we pooled the appropriate volume of buffer and proteinase *K* to the samples..
- Sample bottles were sent with fresh ethanol, therefore, the FAVIS steps involving the reuse of the original ethanol,were not performed.
- Each sample requires a bench working station approximately 30 cm wide. We processed samples in batches of 22 + one lysis blank (negative control) for digestion.
- The lysate generated was transferred to a 50 ml Falcon tube and then transferred to a 2 ml tube. The 2 ml tubes from each batch were kept for long-term storage at -20°C.
- After four lysis batches, enough lysate-samples (92) were available for DNA extraction using a Kingfisher Flex in 96 well plates format. Extractions were performed with the commercial kit Omega BIO-TEK, using 200 ul of lysate.
- Library preparation will follow a two-step PCR, consistent with the procedure described in FAVIS (Iwaszkiewicz-Eggebrecht et al., 2023a; 2023b), with some modifications. We made two replicates of the first PCR (PCR1) per sample using Qiagen Multiplex PCR Kit (100).
- Cycling conditions and primers remained the same as in FAVIS (primers BF3-BR2). For the second PCR (PCR2), we used TaKaRa Ex Taq® DNA Polymerase Hot-Start, which is the polymerase that we routinely use for bulk sample metabarcoding. We carried out a second cleaning of PCR2 products. Finally, we measured and pooled the samples.
- Up to 384 unique dual indexes (Nextera XT Index Kit; Illumina, San Diego, CA, USA) were used to multiplex samples, allowing simultaneous sequencing in a single run.
- Samples were sequenced in Illumina NovaSeq 6000 platform (paired-end; 2x250 bp), sequencing four plates simultaneously within a full run.
- Collections tubes with 50 ml of lysate per sample will be temporarily stored until finalisation of the project, and 2 ml tubes per sample were kept for long-term storage.

BGE Metabarcoding Workflow



FAVIS | Sample preparation and DNA extraction

We followed the FAVIS protocol, but with some modifications, thus the implementation of the BGE protocol should side the FAVIS protocol as the fundamental protocol. Below we list the steps of the FAVIS protocol, indicating where relevant the changes made for the BGE project workflow. Therefore, changes are described within each modified step only.

FAVIS: Fast and Versatile protocol for metabarcoding of bulk Insect Samples
(Iwaszkiewicz-Eggebrecht et al., 2023a; 2023b)

SECTION 1: Preparation

This step sets up five working (washing, working, spike-in, lysis and weighting) stations and the use of a heating station; bulk samples are processed within their original bottle during the complete preparation workflow. Please keep in mind that each sample (or batch of samples) may come from different origins and they have been transported under different conditions. Therefore, it is important to inspect each sample to assess if the bottle is intact and arthropods are in good condition; and externally clean the bottles to remove additional contaminants for the working stations.

1. Set up washing stations

2. Set up working stations

2.2 Each sample requires a bench working tray, approximately 30 cm wide. Samples will be processed in batches of 22 + lysis blank (negative control) per day for digestion (if one person is working alone). It is estimated that after four days, there will be enough lysate-samples ready for DNA extraction using 96 well plates in the kingfisher robot.

3. Set up spike-in stations

Proceed to step 4 - As suggested by the protocol, this step was skipped, as there was no need to add biological spike-ins to the samples.

4. Set up lysis buffer and proteinase K station

5. Set up weighing station

6. Preheat lysis buffer

SECTION 2: Sample Processing

7. Decant ethanol

   For this study, there was no archive paper label placed inside any bottle, thus there will not be any label to be removed. All metadata was taken during sampling and recorded with PlutoF and the PlutoF Go app (Abarenkov et al., 2010) under each unique sample ID code

8. Wet weigh samples

   Before closing the bottles, a termite was added as control, before weighting.
   8.5 Not adding spike-ins, but adding a termite for control

9. Add biological spike-ins (OPTIONAL)

Not done for the BGE project

10. Adding lysis buffer and proteinase K

Inverted steps 10-11

11. Pre-heated buffers and bottles with specimens independently in a dry oven

Inverted steps 11-10

12. Incubate samples at 56°C in a shaking dry incubator

13. Measure EtOH concentration (OPTIONAL)

Not done for the BGE project

14. Decant lysate solution and preserve insect sample for long term storage

In the next steps we will decant the lysate solution to the lysate collecting bottle which will be stored at -20°C for long term storage.

15. Take aliquot of the lysate for DNA purification (optional)

In the next steps we will transfer a 1.8 mL aliquot of the lysate to the 2 mL lysate microtube which will be used as a working stock for DNA purification. If you will proceed with DNA purification straight away you can save time by transferring the precise aliquot of lysate (225 µL if you use step 16 below or 390 µL if you use the alternative option 17) directly from the lysate bottle into a deep well plate and proceed with DNA purification from step 16.2 or step 17 onwards. Dilute samples 1:8 using ultrapure water.

SECTION 3: DNA Purification

This step uses Omega Mag-Bind Blood and Tissue DNA HDQ 96 Kit (M6399) to isolate DNA from lysed bulk samples using the KingFisher Flex.

16. For our project we purify a 200 µL aliquot of lysate from each sample using Omega Mag-Bind Blood and Tissue DNA HDQ 96 Kit (M6399) on a King Fisher Flex 96 robot (modifying the manufacturer's instructions). We have reduced the volumes by half compared to the original protocol. The following table shows the volumes used in each purification step.

Plate	Content	Volumen (µl) per sample
Plate 1	Lysate	200
	Al Buffer	230
	Binding Bead Mix	340 (320 HDQ Bind+20 MBP)
Plate 2	VHB Buffer	300
Plate 3	VHB Buffer	300
Plate 4	SPM Buffer	300
Plate 5	Elution Buffer	100

17. DNA purification using homemade magnetic beads - (ALTERNATIVE OPTION)
Before proceeding with this step, prepare Magnetic Bead Solution as described in the "Materials" section.

SECTION 4: Library preparation and Sequencing

18. Library preparation using a two step PCR approach.

For amplicon library preparation we use a two-step PCR approach. In the first step (PCR 1) we amplify the target gene: 418 base pairs of the mitochondrial cytochrome c oxidase subunit I (COI) gene region. In the second step (PCR 2) we add the Illumina indexes to the amplified sequences and fill up Illumina adapters. 18.1

Target Group	Marker	Primer Sequences	*Ta*	*TDa*	Expected Amplicon size*
Metazoa	BF3_P5	FWD: TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCHGAYATRGCHTTYCCHCG	**50º**	**90s**	**418bp**
Metazoa	BR2_P7	REV: GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGCDGGRTGNCCRAARAAYCA			

** Expected amplicon size = insert length + primer lengths + 3bp NNs (avg.).*

19. Pooling strategy and sequencing

Because our inserts are ca. 462bp long (418bp region of interest, plus primers, plus variable-length inserts), we require a sequencing platform providing reads with total length >500 bp. NovaSeq SPrime 2x250bp flow cell is the most cost-effective solution at the time of publishing this protocol. We sequence 768 libraries (8x96 well plates) per NovaSeq SPrime 2x250bp flow cell: 384 samples (4x96-well plates) pooled per lane. Before submitting libraries for sequencing we create two Master Pools (A and B) composed of 384 libraries each. Each Master pool is sequenced in 1 lane of a NovaSeq SPrime 2x250bp flow cell. According to Illumina specifications, the NovaSeq SPrime 2x250bp should provide up to 800M read pairs per flow cell, but in our experience, 900M or more reads were often obtained. This results in an average of >1M read pairs for each of 768 libraries sequenced per flow cell.

20. Spotlight video with authors Elzbieta Iwaszkiewicz-Eggebrecht and Andreia Miraldo

Amplicon library prep workflow | Custom protocol using the Freedom TECAN EVO 150 workstation

The Tecan Freedom EVO® 150 (Tecan Austria GmbH, 2024) platform provides a robust and automated solution for high-throughput library preparation in metabarcoding workflows. Designed to streamline complex multi-step processes such as PCR amplification, indexing, and clean-up, this system minimizes manual intervention while ensuring reproducibility and accuracy.

1 PCR1 amplification of mitochondrial cytochrome c oxidase subunit I (COI) gene region (418bp)

- Two PCR1 replicates, total volume 40 µL (26.25 mix, 8.75 extract)
- PCR1 master mix is performed manually as described below, but the master mix and template DNA are dispensed robotically.

1.1 PCR1 Preparation

To prepare the master mix for a 96-well plate, use a 5 mL microcentrifuge tube compatible with the robotic unit holder. Multiply the volumes below by 95 (as one well of the plate is left empty for PCR2 negative control).

Consumables and reagents

	Component	Volume (µL)
1	2 x Qiagen Multiplex	16.1
2	10µM CO1_BF3_P5 primer mix	3.5
3	10µM CO1_BR2_P5 primer mix	3.5
4	ddH2O	3.15
5	Master mix	26.25
	+ DNA template	8.75

1.2 PCR1 Procedure

1. Dispense the PCR master mix and DNA extract into a plate compatible with the thermal cycler. This procedure is replicated, such that two amplification plates are produced, labelled as “MBC_BGE_PCR1.1” and “MBC_BGE_PCR1.2”.

Items	Quantity
96-well PCR plate, non-skirted	1
Box of 50 µL tips	1
Microcentrifuge tube 5mL	1
Adhesive object cover for PCR plate	1

The consumables and reagents used in this step are placed on work table 1 (Figure 1).

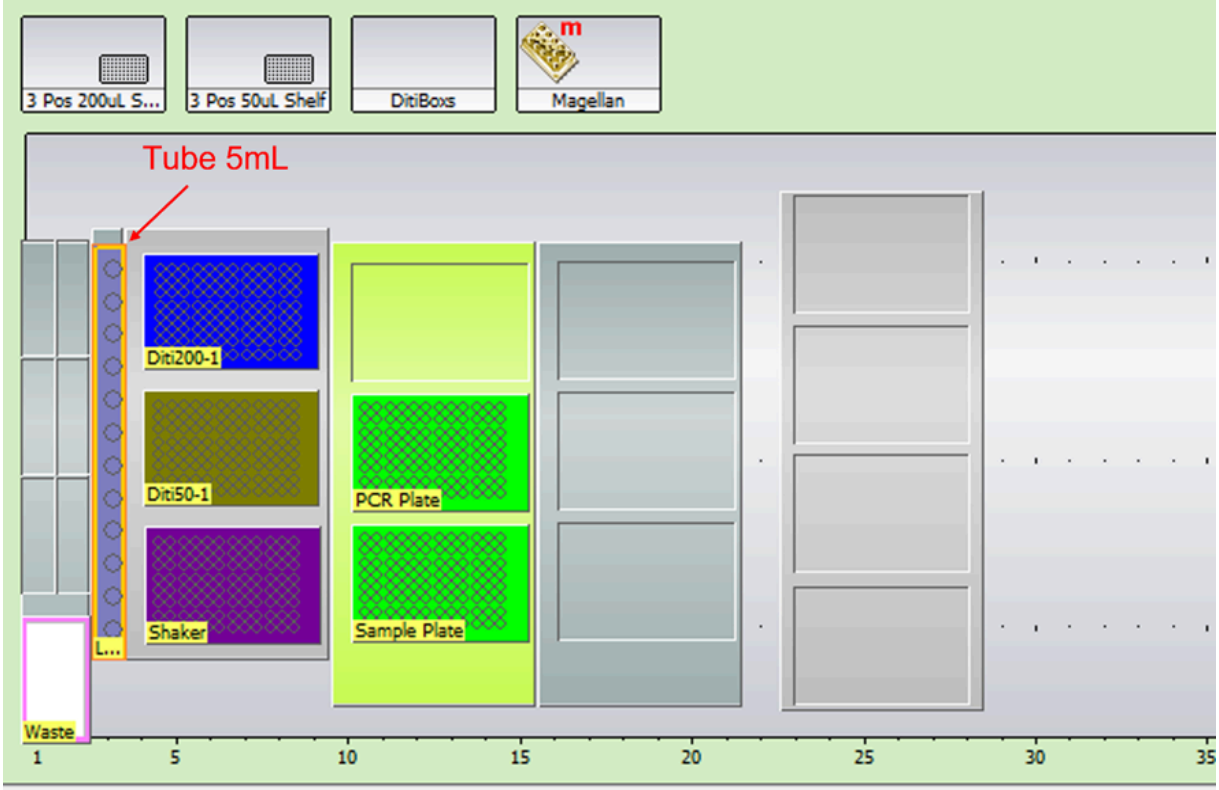


Figure 1. Work table 1. The position of each consumable is shown. Abbreviations: Waste, container where used tips are discarded; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Shaker, shaker position; Sample plate, 96-well PCR with DNA extract; PCR plate, plate with the final result.

2. Run PCR1 using the following conditions:

	Step	Temp. (ºC)	Time (s)	Cycles
1	Initial denaturation	95	900	1
2	Denaturation	94	30	30
3	Annealing	50	90	30
4	Extension	72	90	30
5	Final extension	72	600	1
6	Store	4	∞	

Note: Performed outside the robotic station.

3. Check point

Run PCR1 products on a 2.5% agarose gel (2 µL PCR product + 2 µL loading dye).

Note: Performed outside the robotic station.

1.3 PCR1 products mixture

Combine the two PCR1 replicates in a rigid 96-well PCR plate with skirt. The resulting plate will contain 30 µL of PCR product (15 µL from each PCR1) labelled “MBC_BGE_PCR1_pool”.

Consumables and reagents

Items	Quantity
96-well PCR plate, non-skirted	1
Box of 50 µL tips	1
Rigid 96-well PCR plate with skirt	1
Adhesive object cover for PCR plate	1

The consumables and reagents used in this step are placed on the work table 2 (Figure 2).

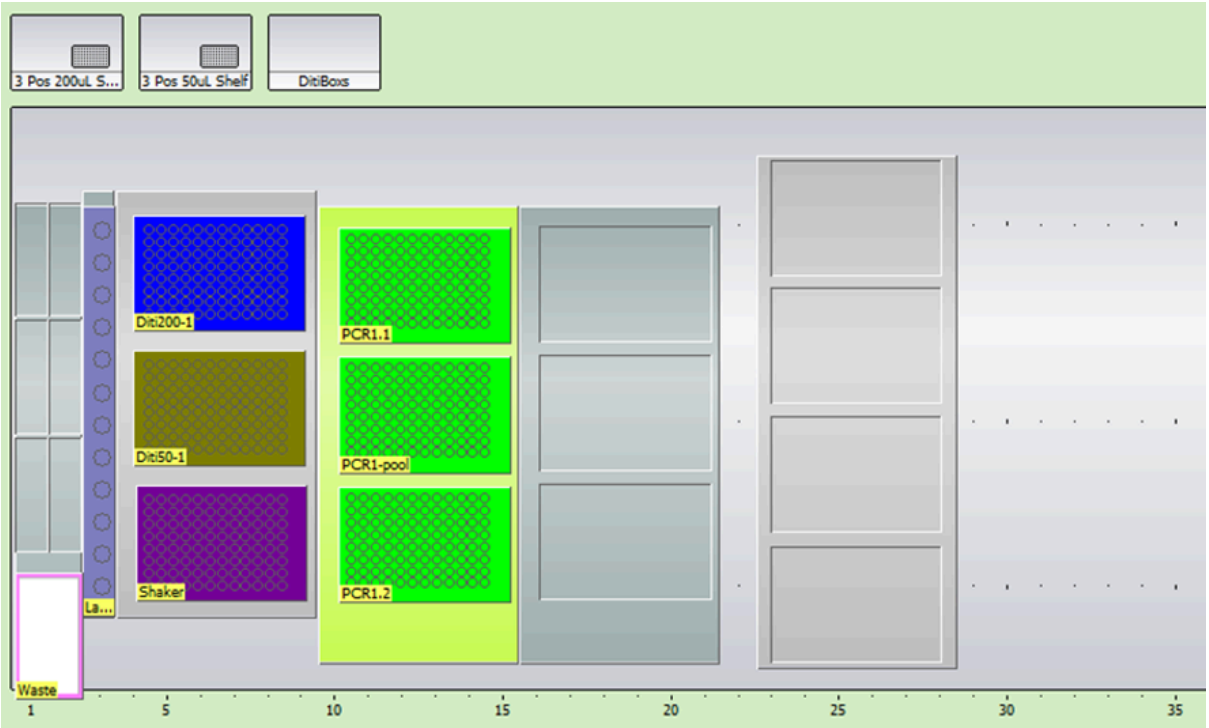


Figure 2. Work table 2. The position of each consumable is shown. Abbreviations: Waste, container where used tips are discarded; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Shaker, shaker position; PCR1.1 and PCR1.2, PCR plates; PCR1-pool, plate with the final result.

2 Cleaning PCR1 products using magnetic beads (Clean1)

This step will remove primer dimer and enzymes that may affect PCR2 (indexing). The final result will be a 96-well PCR plate, without skirt, with the clean PCR1 product labelled as “MBC_BGE_Clean1.”

2.1 Clean1 preparation

- Prepare fresh aliquots of 80% EtOH.

- Keep magnetic beads at room temperature.

- Spin the plate with the samples to remove any air bubbles.

Consumables and reagents

Items	Quantity
96-well PCR plate, non-skirted	1
Rigid 96-well PCR plate with skirt	1
Box of 50 µL tips	1
Box of 200 µL tips	9
Elution buffer	35 µL/sample
80% EtOH	180 µL /sample x 2
Magnetic beads	22.5 µl/sample
Adhesive object cover for PCR plate	1

The consumables and reagents used in this step are placed on the work table 3 (Figure 3).

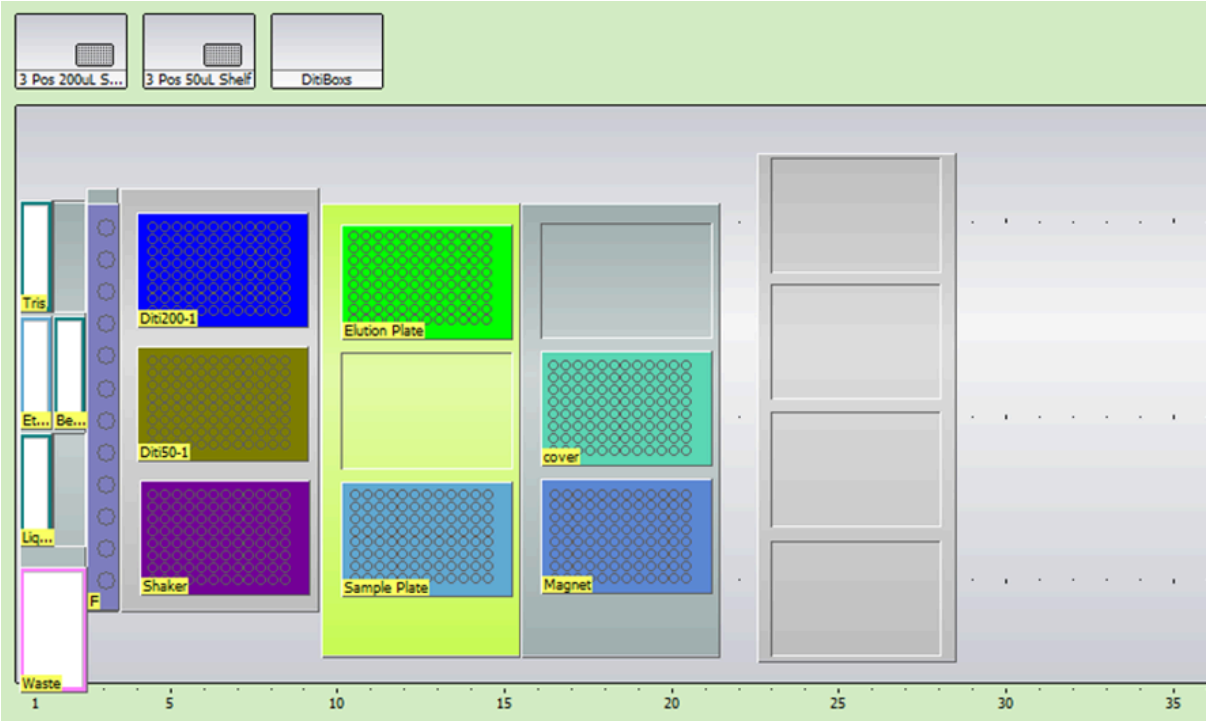


Figure 3. Work table 3. The position of each consumable is shown. Abbreviations:Tris, 25 mL reagent reservoir with tris buffer; EtOH, 100 mL reagent reservoir with 80% ethanol; Beads, 25 mL reagent reservoir with magnet beads; Liqu…, container where waste liquids are dispensed; Waste, container where used tips are discarded; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Shaker, shaker position; cover plate cover position; Magnet, magnet position; Sample plate, rigid 96-well PCR plate with sample; Elution plate, plate with the final result.

2.2 Clean1 procedure

1. Transfer 30 µL of PCR1 product to a rigid 96-well PCR plate with skirt and add 22.5 µL (x 0.75) of Magnetic Bead Solution, mix by pipetting and vortex the entire plate using the plate shaker (1,800 rpm/1 min).
2. Incubate mixture for 300 s at room temperature.
3. Place the plate on a magnetic stand for 4 min.
4. Remove supernatant.
5. From a 100 mL reagent reservoir, transfer 180 µL of 80% EtOH to each well.
6. Incubate on a magnetic stand for 30 s.
7. Remove supernatant.
8. Repeat steps 5 – 7 above.
9. Wait 6 min. until the magnetic bead pellet is dry (on magnetic stand).
10. Remove samples from the magnetic stand, add 35 µL of Tris buffer, vortex the entire plate using the plate shaker (1,800 rpm/1 min.), mix by pipetting up and down and vortex again (1,800 rpm/1 min.). It is good to centrifuge the plate before the next step.
11. Place the plate back on the magnetic stand for 5 min.
12. Transfer supernatant (30 µL) to a new 96-well plate, non-skirted.
13. Mark the plate with a unique identifier and store at 4ºC (for a few days) or at -20ºC (for a longer period).

Note: rigid 96-well PCR plate, with skirt must be compatible with the robotic manipulator arm so that they can be placed in different positions on the robotic unit

14. Check point (Optional)

Run PCR1 products on a 2.5% agarose gel (2 µL PCR product + 2 µL loading dye).

Note: Performed outside the robotic station.

3 PCR2 indexing

3.1 PCR2 Preparation

- PCR 2 master mix is performed manually as described below.

Consumables and reagents.

	PCR2 Master Mix	Volume (µL) /sample
1	ddH2O	29.92
2	Buffer (NH4)	3.5
3	MgCl2	0.7
4	dNTPs	0.7
5	DNA polymerase	0.18
	Total	35

3.2 PCR 2 procedure

1. Mix 35 µL of PCR2 master mix, 5 µL of clean PCR1 product, 5 µL of index i5, and index i7 per sample. The final result will be a plate with the indexing PCR product labelled “MBC_BGE_PCR_index”

Items	Quantity
96-well PCR plate, non-skirted	1
Box of 50 µL tips	3
Adhesive object cover for PCR plate	1

The consumables and reagents used in this step are placed on the work table 4 (Figure 4).

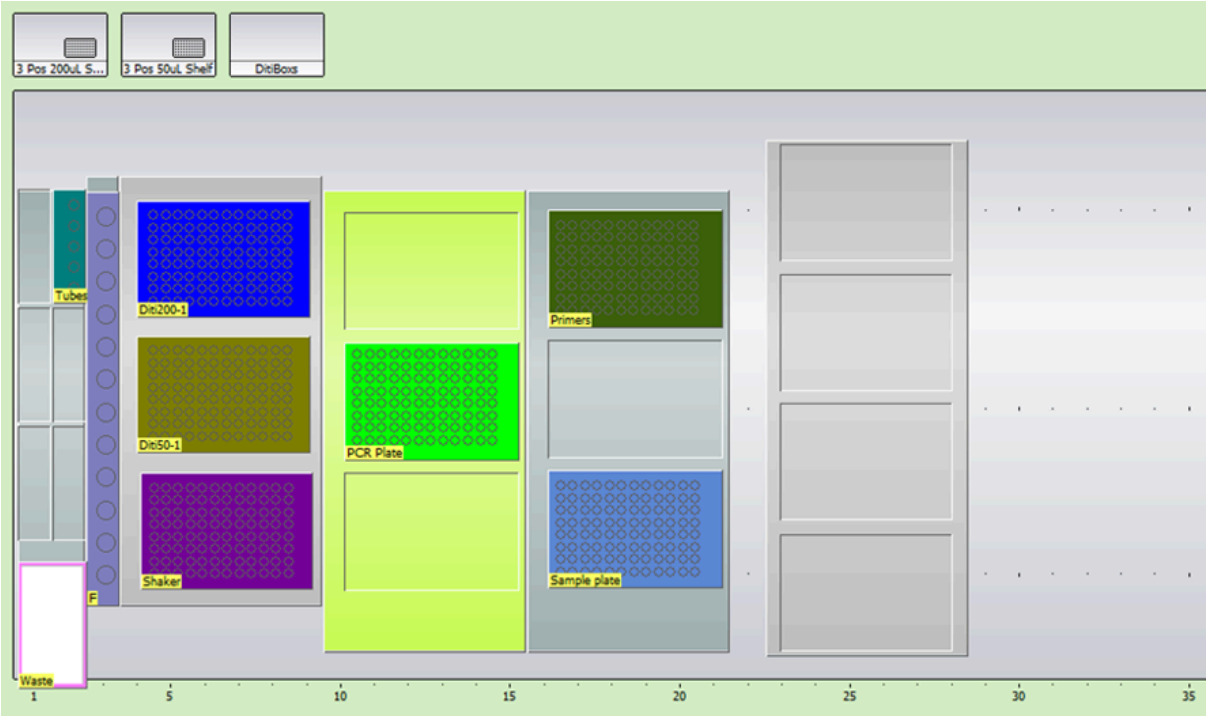


Figure 4. Work table 4. The position of each consumable is shown. Abbreviations: Waste, container where used tips are discarded; Tube, Eppendorf tube with PCR master mix; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Shaker, shaker position; cover, plate cover position; Magnet, magnet position; Primers, i5 and i7 index support; Sample plate, rigid 96-well PCR plate with sample; PCR plate, plate with PCR products.

2. Run PCR 2 using the following conditions.

	Step	Temp. (ºC)	Time (s)	Cycles
1	Initial denaturation	95	900	1
2	Denaturation	94	30	8
3	Annealing	50	90	8
4	Extension	72	90	8
5	Final extension	72	600	1
6	Store	4	∞	

Note: Performed outside the robotic station.

3. Check point (Optional)

   Run clean PCR index products on a 2.5% agarose gel (2 µL PCR product + 2 µL loading dye).

Note: Performed outside the robotic station.

4 Cleaning PCR indexing using magnetic beads (Clean2)

This step removes primer dimer and enzymes.

4.1 Clean 2 preparation

- Prepare fresh aliquots of 80% EtOH.

- Keep magnetic beads at room temperature.

- Spin the plate with the samples to remove any air bubbles.

Consumables and reagents

Items	Quantity
96-well PCR plate, non-skirted	1
Box of 50 µL tips	4
Box of 200 µL tips	7
Elution buffer	50 µL /sample
80% EtOH	180 µL /sample x 2
Magnetic beads	31.5 µL /sample
Adhesive object cover for PCR plate	1

The consumables and reagents used in this step are placed on the work table 5 (Figure 5).

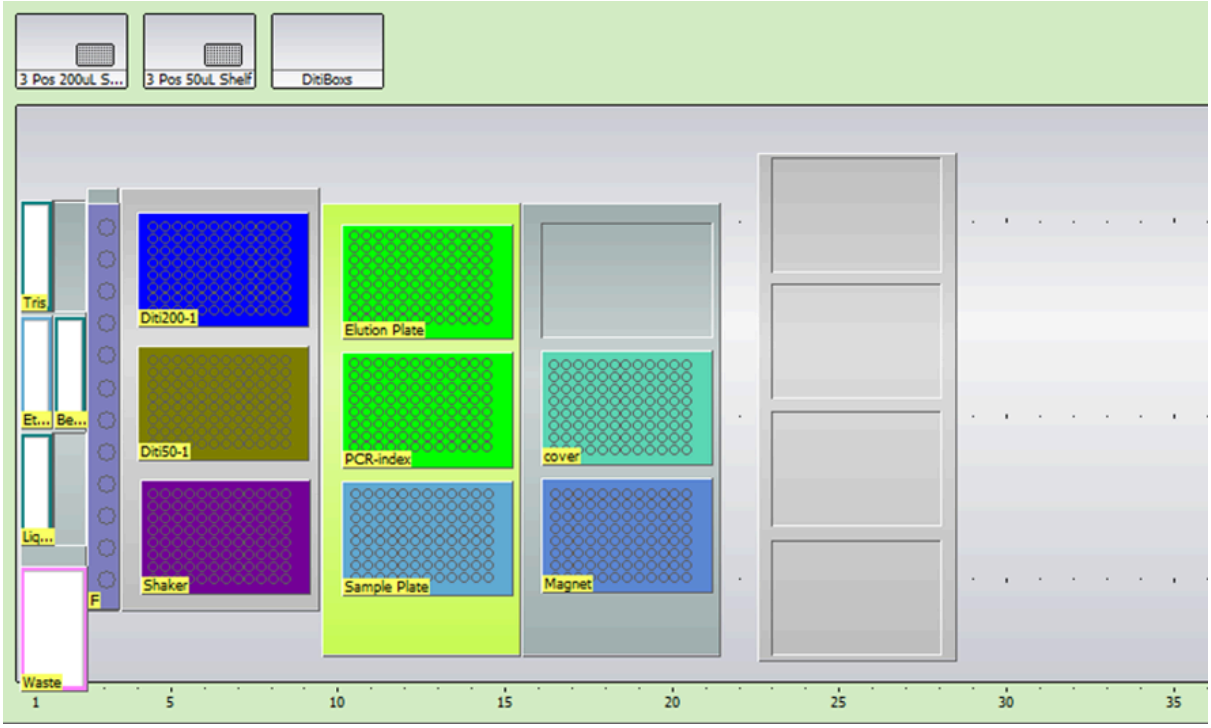


Figure 5. Work table 5. The position of each consumable is shown. Abbreviations:Tris, 25 mL reagent reservoir with tris buffer; EtOH, 100 mL reagent reservoir with 80% ethanol; Beads, 25 mL reagent reservoir with magnet beads; Liqu…, container where waste liquids are dispensed; Waste, container where used tips are discarded; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Shaker , shaker position; cover, plate cover position; Magnet, magnet position; Sample plate, rigid 96-well PCR plate; PCR-index, plate with sample; Elution plate, plate with the final result.

4.2 Clean 2 procedure

1. Transfer 45 µL of PCR index product to a 96 deep-well plate and add 31.5 µL (x 0.7) of Magnetic Bead Solution, and mix by pipetting and vortex the entire plate using the plate shaker (1,800 rpm/2 min.).
2. Incubate mixture for 5 min. at room temperature.
3. Place the plate on a magnetic stand for 4 min.
4. Remove supernatant.
5. From a 100 mL reagent reservoir, transfer 180 µL of 80% EtOH to each well.
6. Incubate on a magnetic stand for 30 s.
7. Remove supernatant.
8. Repeat steps 6 – 8 above.

9. Wait 6 min. until the magnetic bead pellet gets dry (on magnetic stand).

10. Remove samples from the magnetic stand, add 50 µL of Tris buffer, vortex the entire plate using the plate shaker (1,800 rpm/1 min.), mix by pipetting up and down and vortex again (1,800 rpm/1 min.). It is good to centrifuge the plate before the next step.

11. Place back the plate on the magnetic stand for 5 min.

12. Transfer supernatant (45 µL) to a new 96-well plate.

13. Mark the plate with a unique identifier and store at 4ºC (for a few days) or at -20ºC (for a longer period).

Note: 96-well PCR plate, non-skirted with clean PCR2 product labelled as "MBC_XXX_Clean2".

14. Check point

Run clean PCR index products on a 2.5% agarose gel (2 µL PCR product + 2 µL loading dye).

Note: Performed outside the robotic station.

5 Library preparation for sequencing

Create a pool of samples by mixing PCR2 products in an 1.5 mL microtube, based on DNA concentration of each library.

Consumables and reagents.

Items	**Quantity**
96-well PCR plate, non-skirted	1
Adhesive object cover for PCR plate	1
fluorescence buffer	200 µL/ sample
Box of 50 µL tips	3
1.5 mL Eppendorf tube	1

The consumables and reagents used in this step are placed on the work table 6 (Figure 6).

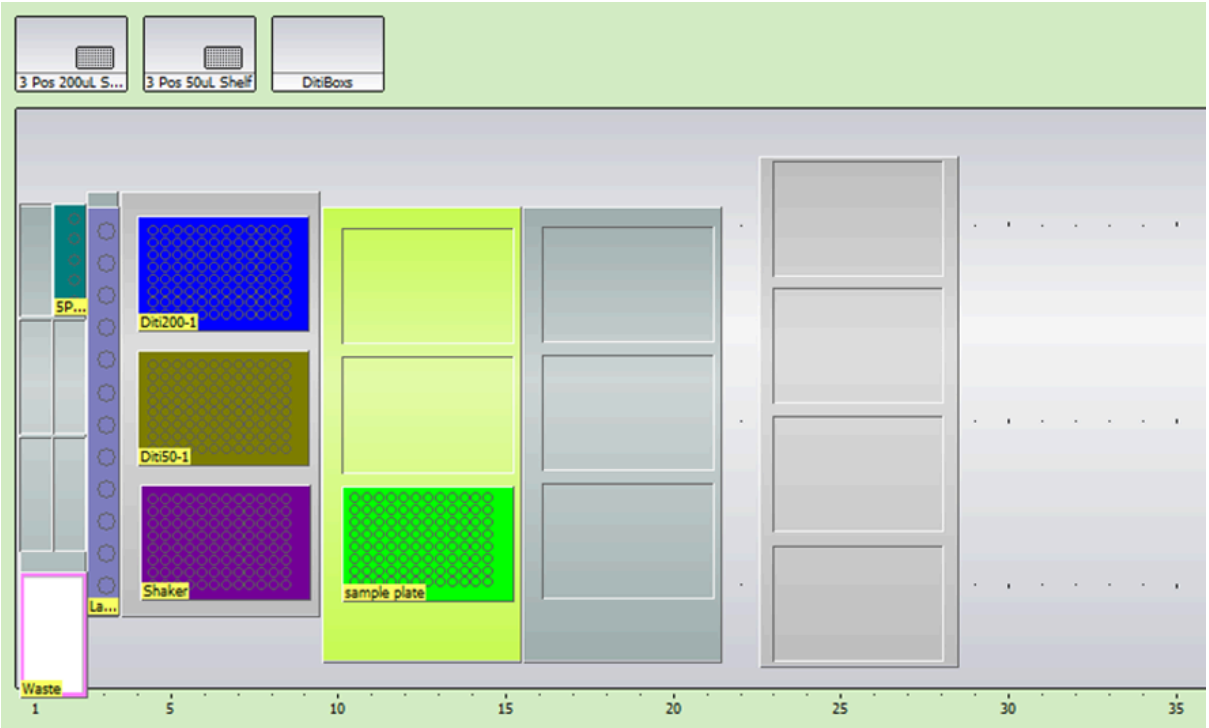


Figure 6. Work table 6. The position of each consumable is shown. Abbreviations: Waste, container where used tips are discarded; SP…, Eppendorf tube with final pool; Diti200-1, position of new 200 µL tips; Diti50-1, position of new 50 µL tips; Sample plate, clean PCR2 product.

5.1 Fluorescence quantification of the PCR plate

Operation performed manually

5.2 Normalization and pooling

Dispense the determined volume into the Eppendorf tube so that each sample has the same concentration, resulting in a 1.5 mL tube with equivalent molarities for each library labelled “MBC_XXX.”

Acknowledgments

Biodiversity Genomics Europe (Grant no.101059492) is funded by Horizon Europe under the Biodiversity, Circular Economy and Environment call (REA.B.3); co-funded by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract numbers 22.00173 and 24.00054; and by the UK Research and Innovation (UKRI) under the Department for Business, Energy and Industrial Strategy's Horizon Europe Guarantee Scheme.

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