STANDARD OPERATING PROTOCOL · EnGeL-MOLB-MB

DNA metabarcoding of soil fauna: two-step PCR amplicon library preparation (COI for microarthropods, 18S rRNA for nematodes)

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Main hazards	 DNA intercalating dye (GreenSafe or other): handle with gloves; UV/blue-light transilluminator: eye and skin protection; Ethanol (80% washes): flammable

1 Purpose and scope

This protocol prepares Illumina amplicon libraries from bulk soil fauna DNA extracts for DNA metabarcoding: a fragment of the mitochondrial COI gene for microarthropods, and a fragment of the 18S rRNA gene for nematodes. It starts from DNA extracts (EnGeL-MOLB-DNA-MAG or -DNA-COL) and ends with a pooled, quantified library diluted to 4 nM for short-read sequencing. Bioinformatic processing is described in EnGeL-BIOINF-OBITOOLS4. Other markers can be added to the Primer table once validated.

2 Principle

A first PCR amplifies the target region with fusion primers that carry a TruSeq-type (iTru) Illumina adapter overhang and 0–3 extra bases (“phased” primers) to increase sequence diversity at the start of the reads. A second, short PCR adds unique dual indexes and the P5/P7 adapters, so every sample can be identified after sequencing. Each indexed PCR product is cleaned with magnetic beads, quantified and normalised; the products are pooled,

the pool is cleaned again and checked on the TapeStation, and its molar concentration is measured by qPCR before dilution for sequencing (Elbrecht et al., 2019; Glenn et al., 2019a, b; Porazinska et al., 2009).

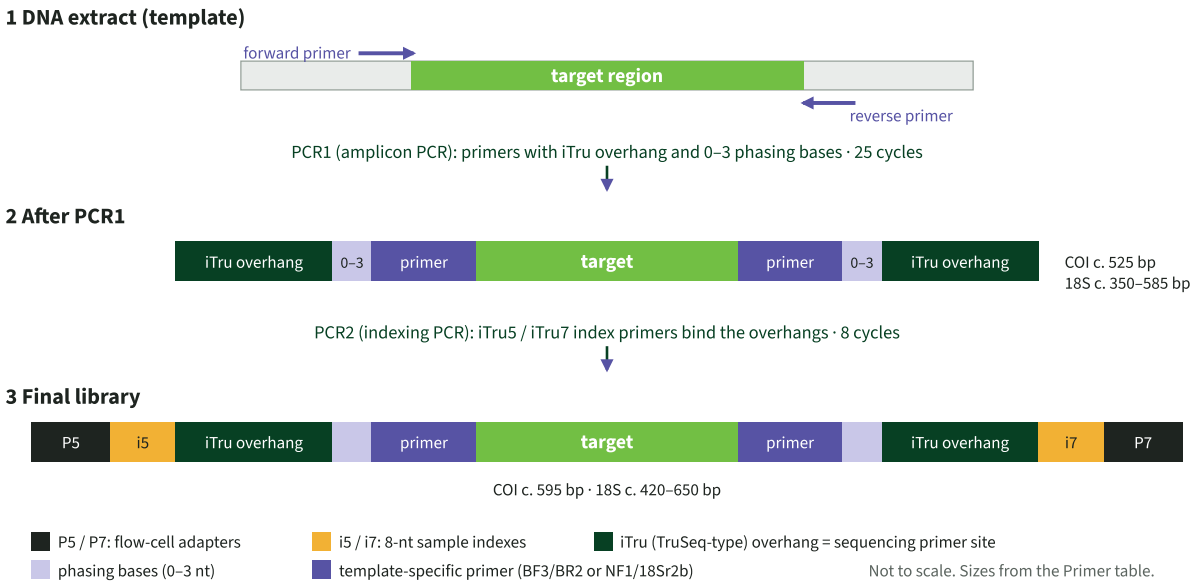

Figure 1: Two-step PCR and final library structure (iTru system). PCR1 adds the iTru overhang and 0–3 phasing bases to the template-specific primers; PCR2 adds the i5/i7 indexes and the P5/P7 adapters. Not to scale; sizes from the Primer table. Figure: EnGeL-UC, CC BY 4.0.

3 Before you start

Item	Details
Hands-on time	[TO CONFIRM]
Total time	[TO CONFIRM] PCR1 c. [TO CONFIRM], PCR2 c. [TO CONFIRM]
Input	DNA extracts with their plate map; concentrations known (EnGeL-MOLB-QUBIT or EnGeL-MOLB-PICOGREEN)
Prepare in advance	Plate map with index assignment (Annex 1); Lab Pipeline workbook for the project; primer and index plates thawed; KAPA Pure Beads at room temperature
Key safety points	See Safety and waste

3.1 Primer table

Markers and PCR settings.

	COI (microarthropods)	18S rRNA (nematodes)
Primers (target-specific, 5′–3′)	BF3 `CCHGAYATRGCHTTYCCHCG` (Elbrecht et al., 2019); BR2 `TCDGGRTGNCCRAARAAYCA` (Elbrecht & Leese, 2017)	NF1 `GGTGGTGCATGGCCGTTCTTAGTT`; 18Sr2b `TACAAAGGGCAGGGACGTAAT` (Porazinska et al., 2009)
Target length (excluding primers)	c. 418 bp	237–471 bp, mean c. 320 bp (Ficetola et al., 2024)
DNA input for PCR1	Undiluted; dilute with nuclease-free water only if PCR inhibition is suspected	Diluted to 25–35 ng/µL (suggested 35 ng/µL)
PCR1 annealing	50 °C, 90 s	60 °C, 90 s
Expected size after PCR1 / PCR2	c. 525 bp / c. 595 bp	c. 350–585 bp / c. 420–650 bp

	COI (microarthropods)	18S rRNA (nematodes)
Pool clean-up bead ratio (pool : beads)	1:0.65	1:0.75 (0.65× may remove short amplicons and bias the community)
Read overlap with 2 × 250 bp	c. 40 bp, enough to merge	Most amplicons merge; the longest (up to 471 bp + c. 45 bp primers) do not; 2 × 300 bp would cover the full range

3.2 First-PCR primers with overhang (5′–3′)

Overhang (P5 side): `ACACTCTTTCCCTACACGACGCTCTTCCGATCT`; overhang (P7 side): `GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT`. Phased versions add 0–3 bases between overhang and primer (ins0–ins3) to increase sequence diversity at the start of the reads. These TruSeq-type (iTru) overhangs are the binding sites of the iTru index primers used in the second PCR.

Name	Sequence
iTru_COI_BF3_P5_ins0	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCHGAYATRGCHTTYCCHCG`
iTru_COI_BF3_P5_ins1	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCHGAYATRGCHTTYCCHCG`
iTru_COI_BF3_P5_ins2	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACCHGAYATRGCHTTYCCHCG`
iTru_COI_BF3_P5_ins3	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCHGAYATRGCHTTYCCHCG`
iTru_COI_BR2_P7_ins0	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCDGGRTGNCCRAARAAYCA`
iTru_COI_BR2_P7_ins1	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCDGGRTGNCCRAARAAYCA`
iTru_COI_BR2_P7_ins2	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCDGGRTGNCCRAARAAYCA`
iTru_COI_BR2_P7_ins3	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGATCDGGRTGNCCRAARAAYCA`
iTru_NF1_P5_ins0	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGGTGCATGGCCGTTCTTAGTT`
iTru_NF1_P5_ins1	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGTGGTGCATGGCCGTTCTTAGTT`
iTru_NF1_P5_ins2	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGGTGGTGCATGGCCGTTCTTAGTT`
iTru_NF1_P5_ins3	`ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAGGTGGTGCATGGCCGTTCTTAGTT`
iTru_18Sr2b_P7_ins0	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTACAAAGGGCAGGGACGTAAT`
iTru_18Sr2b_P7_ins1	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTACAAAGGGCAGGGACGTAAT`
iTru_18Sr2b_P7_ins2	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATTACAAAGGGCAGGGACGTAAT`
iTru_18Sr2b_P7_ins3	`GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGATTACAAAGGGCAGGGACGTAAT`

Second-PCR index primers: iTru_i5_P5 `AATGATACGGCGACCACCGAGATCTACAC[i5 index]ACACTCTTTCCCTAC`; iTru_i7_P7 `CAAGCAGAAGACGGCATACGAGAT[i7 index]GTGACTGGAGTTCAG`. Adapterama iTru5/iTru7 primers (Glenn et al., 2019a): 384 unique dual-index pairs on four 96-well plates (1–384); index names and sequences are in the primer workbooks and the index sheets of the Lab Pipeline workbook.

4 Materials

4.1 Equipment

- Thermocyclers (three new units, one older unit; NextGenPCR ultra-fast thermocycler available) and PCR plates/tubes
- Gel electrophoresis apparatus, power supply and gel imaging system (UV/blue-light transilluminator)
- 96-well magnetic extractor for U-bottom plates (clean-up of individual PCR products)
- Magnetic stand for tubes (clean-up of the pooled library)
- Fluorometer (Qubit) or fluorescence plate reader (PicoGreen) — see EnGeL-MOLB-QUBIT and EnGeL-MOLB-PICOGREEN
- Agilent TapeStation (library size check)
- qPCR instrument: QuantStudio 3 (library quantification)

- Multichannel pipettes, pipettes; vortex mixer; plate centrifuge

4.2 Consumables

- U-bottom 96-well plates
- PCR plates and seals; PCR strip tubes
- Filter tips, including 20 µL filter tips for bead dispensing (10 µL tips may clog)
- Low-binding tubes
- Agilent D1000 ScreenTape

4.3 Reagents and kits

- Qiagen Multiplex PCR Master Mix [TO CONFIRM: catalogue number]
- First-PCR primers with iTru overhang, phased (BF3/BR2 or NF1/18Sr2b; Primer table), 10 µM
- KAPA HiFi HotStart ReadyMix (Roche)
- Adapterama iTru5/iTru7 dual-index primers (four plates, 1–384), 5 µM
- KAPA Pure Beads (Roche)
- Ethanol 80%, freshly prepared
- 10 mM Tris-HCl (pH 8.0–8.5)
- Elution buffer for bead clean-ups (sterile water or low-TE)
- Nuclease-free water
- Agarose, DNA loading dye, DNA size marker, electrophoresis buffer (TAE or TBE), GreenSafe or other DNA intercalating dye
- Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA assay
- Agilent D1000 ScreenTape reagents (D1000 Sample Buffer, D1000 Ladder)
- KAPA Library Quantification Kit for Illumina (Roche) — see EnGeL-MOLB-KAPA

5 Reagent preparation

- **First-PCR primers (with overhang):** 10 µM working solutions [TO CONFIRM: how the phased versions ins0–ins3 are combined, e.g. equimolar mix per direction; storage].
- **iTru index primers:** synthesised at 25 nmol scale, standard desalting, with one phosphorothioate bond; diluted to 5 µM and aliquoted in strip tubes (primer workbook) [TO CONFIRM: storage].
- **Ethanol 80%:** prepare fresh on the day.
- **KAPA Pure Beads:** equilibrate to room temperature and vortex c. 30 s before use.

6 Procedure

6.1 Before starting

- Clean the working space and material with bleach and water and leave the UV light on for at least 15 min.
- Use filter tips at all steps.
- Clean forceps and spatulas with 3% sodium hypochlorite and 96% ethanol between samples.
- Prepare or update the plate map and index assignment (Annex 1) and the project's Lab Pipeline workbook.
- Include a PCR negative control (water) on each plate and, whenever possible, a positive control per run (a mock community of known composition). Extraction blanks from the DNA extraction are carried through. Every control gets its own index combination and is sequenced.

6.2 First PCR (amplicon PCR)

1. Prepare the DNA input for the marker (Primer table): COI — undiluted; 18S — diluted to 25–35 ng/µL with nuclease-free water.
2. Set up one 10 µL reaction per sample: 5 µL Qiagen Multiplex PCR Master Mix, 1 µL of each primer with overhang (10 µM), 2 µL nuclease-free water and 1 µL of DNA. Up to 3 µL of DNA may be used for low-concentration extracts, reducing the water so that the volume stays at 10 µL.

3. Cycling: 95 °C for 15 min; 25 cycles of 94 °C for 30 s, annealing for 90 s (COI 50 °C; 18S 60 °C), 72 °C for 90 s; final extension 72 °C for 10 min.
4. Check amplification on a 2% agarose gel. All field samples should show bands of the expected size; extraction and PCR negative controls should show no amplification. Record the outcome per well (Gel_Success, Faint, Failure, Lost, Pending) in the Lab Pipeline workbook.

6.3 Second PCR (indexing PCR)

5. Set up one 14 µL reaction per sample: 7 µL KAPA HiFi HotStart ReadyMix, 0.7 µL iTru5 index primer (5 µM), 0.7 µL iTru7 index primer (5 µM), 2.8 µL nuclease-free water and 2.8 µL of first-PCR product. Record the index plate and well of every sample in the plate map.

CRITICAL Each sample in a pool must carry a unique iTru5/iTru7 index combination. Combinations must never be repeated within the same pool: duplicated combinations cannot be demultiplexed. Check the plate map against the indexes actually used.

6. Cycling: 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; final elongation 72 °C for 5 min.

6.4 Clean-up of individual PCR products (KAPA Pure Beads, 0.7×)

7. Transfer 10 µL of each indexing PCR product to a new U-bottom 96-well plate.
8. Vortex the KAPA Pure Beads (room temperature) for c. 30 s. Add 7 µL of beads per well (1:0.7, product : beads), pipetting into the bottom of the well. For a full plate, dispense from a PCR strip (e.g. 91 µL per strip tube for a row of 12 wells plus excess) and re-vortex the beads every 3 columns. Use 20 µL filter tips.
9. Mix by pipetting up and down 10–12 times without bubbles; incubate for 5 min at room temperature.
10. Meanwhile, prepare three U-bottom plates: two wash plates with 150 µL of freshly prepared 80% ethanol per well, and one elution plate with 30 µL of elution buffer per well.
11. Place the magnetic extractor on the bead plate, let the beads collect (c. 2–3 min), then lift the beads.
12. Wash: place the extractor in the first wash plate for 30 s, then in the second for 30 s.
13. Air-dry the beads on the extractor for 30–60 s (no longer than 2–3 min). Do not over-dry: this makes elution harder.
14. Elute: place the extractor in the elution plate, release the beads and leave them to resuspend for 5 min (optional: 5 min at 55 °C to improve recovery). Re-attach the magnet to remove the beads; the cleaned products stay in the elution plate.
15. Optional (recommended): check c. 1 µL on an agarose gel or the TapeStation for a single band of the expected size (Primer table) and no primer dimers (< 150 bp).

NOTE At the end of a bead tube, spin it down and transfer the remainder to a new tube rather than using it directly, as it may no longer be homogeneous; vortex the new tube well.

6.5 Quantification, normalisation and pooling

16. Measure the concentration of each cleaned product by fluorometry (EnGeL-MOLB-QUBIT or EnGeL-MOLB-PICOGREEN) and record the values in the plate map.
17. Normalise each product to 20 nM with nuclease-free water or 10 mM Tris-HCl, using the measured concentration and the expected fragment size (Calculations; *PCR2_Plate* sheet of the Lab Pipeline workbook). Mix gently.
18. Combine the normalised products into one tube to make the pooled library (*Pooling* sheet). Check the plate map before pooling and label and document the samples and indexes in each pool.

6.6 Clean-up of the pooled library

19. Add KAPA Pure Beads to the pool at the ratio for the marker (Primer table: COI 1:0.65; 18S 1:0.75).
20. Mix by vortexing and incubate for 10 min at room temperature.
21. Place the tube on a magnetic stand for 5 min; remove and discard the supernatant without disturbing the beads.
22. Wash the beads twice with 200 µL of freshly prepared 80% ethanol for 60 s. Remove the ethanol completely and air-dry for 3–5 min (do not over-dry).

23. Remove the tube from the magnet and resuspend the beads in 200 µL of elution buffer (10 mM Tris-HCl, pH 8.0–8.5, or low-TE). Incubate for 5–10 min at room temperature. The elution volume sets the final concentration: use less to concentrate low-yield pools, more to dilute, keeping enough for TapeStation, qPCR and sequencing.
24. Place the tube on the magnetic stand for 5 min and transfer the eluate (cleaned pool) to a clean tube.

6.7 Pooled library check on the TapeStation (D1000)

25. Take the D1000 ScreenTape and reagents from 4 °C and equilibrate to room temperature for 30 min; keep the ScreenTape in its pouch, protected from light.
26. Ladder: mix 3 µL D1000 Sample Buffer + 1 µL D1000 Ladder in the first tube or well. Samples: mix 3 µL D1000 Sample Buffer + 1 µL library.
27. Seal with caps or foil, vortex and centrifuge briefly.
28. Insert the ScreenTape and the tubes into the TapeStation; remove caps or foil and check that the tip box is full. Run.
29. Confirm a single peak at the expected size and no primer/adapter dimers. If dimers persist, repeat the pool clean-up. Record the average fragment size.

6.8 Library quantification by qPCR (KAPA)

Quantify the cleaned pool with the KAPA Library Quantification Kit, as in EnGeL-MOLB-KAPA:

Kit preparation (first use)

30. Add the 1 mL Primer Mix (10X) to the 5 mL bottle of SYBR FAST Master Mix (2X); vortex briefly.
31. Store the combined mix at −20 °C (or 4 °C for short-term use, per kit insert); keep away from light.
32. Thaw the standards on ice, vortex and spin down. Do not dilute the standards further.

Library dilutions (serial, in library dilution buffer)

Dilution	Make from	Volume taken (µL)	Dilution buffer (µL)	Use
1:1 000	Pool	1	999	Intermediate only
1:10 000	1:1 000	100	900	Run in qPCR
1:20 000	1:1 000	50	950	Run in qPCR
1:100 000 (optional)	1:10 000	10	90	If PicoGreen > c. 50 nM

33. Prepare the dilutions above. Vortex and spin each dilution before taking the next step. Check against the PicoGreen value: the diluted library should be about 0.2–20 pM, inside the standard range.

Positive control: treat the positive control like a library. It starts at 200 nM and is diluted in the same series: 1:1 000 (intermediate), then 1:10 000 and 1:20 000, which should give about 20 pM and 10 pM in the reaction.

Reaction set-up (20 µL per well)

Component	Per well (µL)
Master Mix + Primer Mix (combined)	12
PCR-grade water	4
Diluted library, standard or NTC buffer	4

34. Prepare a master mix of 12 µL combined mix + 4 µL water per well, plus 10% excess.
35. Dispense 16 µL per well, then add 4 µL of diluted library, standard or dilution buffer (for the NTC).
36. Seal, spin briefly, and keep the plate in the dark until loading.

Plate layout (96-well): Standards 1–6 in triplicate (18 wells); NTC (dilution buffer as template) in duplicate (2 wells); positive control and each library at 1:10 000 and 1:20 000 in triplicate (6 wells each). With the positive control, up to 11 libraries fit on one plate (18 + 2 + 6 + 6 × 11 = 92 wells). In the software, enter the standards as *Standard* with quantities 20, 2, 0.2, 0.02, 0.002 and 0.0002 pM (S1–S6).

Cycling

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	35
Annealing/extension + read	60 °C	45 s	
Melt curve	65 °C, then to 95 °C at 0.15 °C/s	15 s at each temperature	1

Ramp rate 1.6 °C/s for all other steps (instrument settings in use).

37. Run the plate on the qPCR instrument (QuantStudio 3) with the cycling above.
38. Export the results as .csv (tab *Export*) and check the run against the run QC criteria before calculating.

6.9 Dilution to 4 nM and submission

39. Calculate the library concentration (Calculations) and dilute the library to 4 nM with an appropriate buffer [TO CONFIRM: buffer]. Mix gently.
40. Prepare the sequencing submission from the *Novogene_Submission* sheet of the Lab Pipeline workbook (sequencer choice sets the i5 orientation) and the provider's sample information form [TO CONFIRM: provider and form for each project].

> **PAUSE POINT** Cleaned PCR products and the pooled library can be stored at [TO CONFIRM: −20 °C?] between steps.

6.10 After finishing

- Clean the bench with 70% ethanol.
- Empty the small bench bin into the right waste bin.
- Wash your hands.
- Turn off equipment and lights.
- Lock doors, fridge and freezer.

7 Calculations

Normalisation to 20 nM (as in the Lab Pipeline workbook):

$$c_{\mathrm{nM}} = \frac{c_{\mathrm{ng}}}{0.00066 \times L}$$

$$V_{\mathrm{water}} = V_{\mathrm{p}} \times \left(\frac{c_{\mathrm{nM}}}{20} - 1 \right)$$

- c_{nM}: molar concentration of the PCR product (nM)
- c_{ng}: mass concentration measured by fluorometry (ng/µL)
- L: fragment size (bp); 0.00066: mass of 1 nmol of 1 bp of dsDNA (µg), as used in the workbook
- V_{p}: volume of PCR product (µL); V_{water}: volume of water or 10 mM Tris-HCl to add (µL)

Worked example (illustrative values): 15 ng/µL, 620 bp → 15 / (0.00066 × 620) = 36.7 nM; for 10 µL of product add 10 × (36.7 / 20 − 1) = 8.3 µL to reach 20 nM.

Library concentration by qPCR

On the *Results* sheet of the exported file, use the **Quantity Mean** (pM) reported for each dilution:

$$c_{\mathrm{lib}} = \frac{Q \times \frac{452}{L} \times D}{1000}$$

- c_{lib}: library concentration (nM)
- Q: Quantity Mean reported by the software for that dilution (pM)
- 452: size of the kit DNA standard (bp)
- L: average fragment size of the library (bp), from the TapeStation

- D: dilution factor (e.g. 20 000; dimensionless)
- 1000: conversion from pM to nM

Calculate c_{lib} for each valid dilution, then average them.

Worked example (illustrative values): Quantity Mean 1.5 pM at 1:20 000, library size 595 bp: 1.5 × (452 / 595) × 20 000 / 1000 = 22.8 nM.

Dilution of the pool for loading: dilution factor = pool concentration (nM) / target concentration (nM); volume of pool = required volume / dilution factor; buffer = required volume − volume of pool (*Pooling* sheet).

8 Quality control

Control	Where	Purpose	Acceptance criterion
Extraction blanks	Carried from the DNA extraction	Contamination during extraction	No band on the PCR1 gel; contaminant reads removed in bioinformatics
PCR negative (water)	Each PCR plate	Contamination during PCR	No band on the PCR1 gel
Positive control / mock community	Each run, whenever possible	PCR and sequencing performance	[TO CONFIRM]
PCR1 gel	Every sample	Amplification	Band of the expected size (Primer table)
Cleaned product check (optional)	Subset or all	Size and dimers	Single band of the expected size; no dimers < 150 bp
Pooled library (TapeStation)	Each pool	Size and dimers	Single peak at the expected size; no primer/adapter dimers
qPCR run	Each quantification	Reliable concentration	See table below

qPCR run acceptance

Check	Accept
Standard curve efficiency	90–110% (slope −3.6 to −3.1)
Standard curve R^2	≥ 0.99
ΔCt between consecutive standards	c. 3.3 (3.1–3.6)
Replicate Ct SD	≤ 0.2–0.3; omit a clear outlier
NTC	Undetermined
ΔCt between 1:10 000 and 1:20 000	c. 1.0 (0.8–1.2)
Sample Ct	Within the Ct range of Standards 1–6
Melt curve	Single peak, no primer-dimer peak
Positive control (200 nM)	Measured within ±20% of 200 nM (160–240 nM)

9 Data and metadata

- **Outputs:** gel images; concentrations; pooled library with TapeStation trace and qPCR concentration; after sequencing, paired fastq files (R1, R2) per sample.
- **Sequencing:** Illumina NovaSeq, 2 × 250 bp paired-end, at least 30 000 reads per sample.
- **Where:** the project's Metabarcoding Lab Pipeline workbook (*Sample_Tracking*, *PCR1_Plate*, *PCR2_Plate*, *Pooling*, *Library_QC*, *Novogene_Submission*), the plate map and the lab book. Sequence data are deposited in [TO CONFIRM: public archive, e.g. ENA].

Minimum to record

- EnGeL label of each DNA extract; plate ID and well; sample type (sample, extraction blank, PCR negative, mock)
- Marker and primer pair; PCR1 and PCR2 plate IDs, dates, operators and thermocycler; PCR outcomes (Gel_-Success, Estimated_Success, Faint, Failure, Lost, Pending)
- Index plate and well, iTru5 and iTru7 index names and sequences; pool ID
- Lot numbers: Qiagen Multiplex PCR Master Mix, KAPA HiFi HotStart ReadyMix, KAPA Pure Beads, KAPA Library Quantification Kit; primer and index plate IDs
- Concentrations after clean-up; normalisation volumes; TapeStation size; qPCR concentration; final dilution
- MIxS fields for the sequence submission [TO CONFIRM: checklist and package, e.g. MIMARKS-survey]

10 Safety and waste

Follow the manufacturer's instructions and safety data sheets for each reagent. Biological samples are potentially hazardous and are handled following the facility's procedures. EnGeL lab rules:

- Before you enter: wear your lab coat, use appropriate gloves, closed shoes only, tie back long hair.
- Not allowed: eating or drinking; touching phones or laptops with contaminated gloves; bags or coats on benches.
- Label all reagents (name, concentration, date) and treat all chemicals as hazardous.
- DNA intercalating dyes: handle with gloves [TO CONFIRM against the SDS].
- Gel imaging: protect eyes and skin from UV light.
- Ethanol and isopropanol are flammable (to be confirmed against the SDS). Keep away from flames and heat sources.

Waste

EnGeL waste streams (choose the right bin):

- **Biological solid waste** (black bin, white bag): contaminated gloves, contaminated disposable materials, solid biological laboratory waste, contaminated PPE. No liquids or sharps.
- **Specific hazardous waste** (red bin, red bag): chemicals and pharmaceuticals designated for this waste stream, specific hazardous laboratory waste, anatomical material; sharps only in a rigid sharps container. No loose sharps.
- **Biological liquid waste** (jerrican, UN 3291): liquid biological waste and contaminated biological liquids only. Use a funnel; rinse the original container and the funnel with dH_2O and discard the rinse water in the same jerrican.

For this protocol: gels and gel-contaminated material [TO CONFIRM: route] PCR plates, bead plates, tips and tubes to biological solid waste (black bin); bead supernatants and ethanol washes [TO CONFIRM: route].

11 Troubleshooting

Problem	Likely cause	Solution
No band after PCR1 (COI)	PCR inhibition	Dilute the DNA extract with nuclease-free water and repeat.
Band in a negative control	Contamination	[TO CONFIRM: repeat the plate? sequence and filter?]
Primer/adapter dimers in the pool	Incomplete clean-up	Repeat the pool clean-up.
Low recovery after bead clean-up	Beads over-dried, or not homogeneous	Dry for no longer than 2–3 min; vortex beads; transfer the end of a bead tube to a new tube.

Problem	Likely cause	Solution
Tips clog when dispensing beads	10 µL tips	Use 20 µL filter tips.
Samples cannot be demultiplexed	Index combination repeated in the pool	Check the plate map before pooling; each combination once per pool.

12 Remarks

The Metabarcoding Lab Pipeline workbook is the working record for this protocol: one copy per project. Its built-in PCR calculator (25 µL, 2× master mix) is for standard PCRs, not for these metabarcoding reactions. The adapter and index system is the one used in the lab (FAVIS primer workbooks; BENCHMARKS mesofauna 2024 runs): TruSeq-type (iTru) phased primers and Adapterama iTru5/iTru7 indexes. The BENCHMARKS handbook (D4.3) describes Nextera-type overhangs with iNext indexes instead; the two systems are not interchangeable (iTru indexes need the iTru overhang, iNext indexes the Nextera-type overhang).

13 References

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14 Version history and citation

Version	Date	Change	By
1.0.0	2026-11-16	First EnGeL-UC version, merging the UC BENCHMARKS protocols BM-BIO-MAR-MB and BM-BIO-NEM-MB v2.0 (2026-09-28; Luísa Fraga, Luís Cunha) into one protocol with a primer table; primer sequences and index design from the lab's primer workbooks and the Metabarcoding Lab Pipeline workbook. Re-	Luísa Fraga, Luís Cunha

Version	Date	Change	By
		places the template test EnGeL-MOLB-NEM.	
1.1.0	2026-11-16	Adapter and index system follows the BENCHMARKS handbook (D4.3): Nextera-type overhangs and Adapterama iNext5/iNext7 indexing primers (10 µM, 0.7 µL each); phased iTru primer table removed.	Luís Cunha
1.2.0	2026-11-16	Back to the iTru system as used in the lab (FAVIS primer workbooks; BENCHMARKS mesofauna 2024 runs): TruSeq-type phased primers and iTru5/iTru7 indexes at 5 µM, 0.7 µL each; PCR1 DNA 1–3 µL.	Luís Cunha
1.2.1	2026-11-16	Schematic of the two-step PCR and library structure (figure).	Luís Cunha
1.2.2	2026-11-17	Contributor: Andreia Quaresma.	Luís Cunha
1.3.0	2026-11-17	Shared KAPA qPCR text updated: diluted library range 0.2–20 pM, positive control treated like a library, melt-curve and ramp settings.	Luís Cunha
1.3.1	2026-11-17	Authorship: all who contributed are authors, with their roles; lead author first where identified, reviewer (Luís Cunha) last.	Luís Cunha
1.4.0	2026-11-20	Library quantification check (shared with EnGeL-MOLB-KAPA): positive control within ±20% of 200 nM (160–240 nM).	Luís Cunha
1.4.1	2026-11-20	GHS pictograms for ethanol (harmonised EU classification).	Luís Cunha
1.4.2	2026-11-20	Cross-references to the nucleic acid QC protocols, now split into EnGeL-MOLB-NANODROP, EnGeL-MOLB-QUBIT and EnGeL-MOLB-PICOGREEN.	Luís Cunha

How to cite

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15 Open questions

1. How the phased first-PCR primers (ins0–ins3) are combined (e.g. equimolar mix per direction).
2. Storage of the primer and index working solutions.
3. Positive control / mock community: composition and acceptance criterion; action when a negative shows a band.
4. Storage of PCR products and pools between steps; buffer for the 4 nM dilution.
5. Sequencing provider and form per project; public archive for the data; MIxS checklist.
6. Waste routes for gels, bead supernatants and ethanol washes; intercalating dye hazards.
7. Hands-on and total time.
8. DOI.

Annex 1 – Plate map guidance

Each laboratory should keep its own plate map template, but every plate map must contain at least the elements below. The plate map is the key that links each sample to its well, its DNA extract, its index combination and its sequencing reads. It must therefore be completed before extraction starts and kept up to date through every step of the workflow.

1. Plate layout and identification

- Use one plate map per 96-well plate (rows A–H, columns 1–12). Give each plate a unique plate ID, e.g. Project_-Marker_Plate##_YYYYMMDD.
- Keep the same layout from the Bind plate through the elution plate, the PCR plates, indexing and pooling. The well position is the sample's tracking key throughout the workflow.
- Always orient plates with A1 at the top left, and mark the A1 corner on every plate.

2. Controls

- Extraction blanks: 4 per plate, at fixed positions H3, H6, H9 and H12. They are spread across the plate so that cross-contamination can be traced back to a position.
- Extraction positive control (when establishing a new starting material or community): record its well and composition.
- All controls are recorded in the plate map, carried through library preparation and sequenced. PCR controls and index assignment: see EnGeL-MOLB-MB.

3. Sample placement

- Fill the plate column by column (A1→H1, A2→H2, …), skipping the blank positions. This matches multichannel pipetting and the robot's orientation.
- Where possible, randomise or interleave samples from different sites or treatments rather than grouping them in one area of the plate. This avoids confounding position or batch effects with treatment effects.
- If a sample is split into two or more extractions, give each extraction its own well and its own suffixed ID (e.g. S045a, S045b).

4. Information recorded for each well (one row per well)

- Plate ID and well position
- EnGeL sample label from the sample master data file (e.g. EnGeL_24_AAA_SYBER_Raw_Soil), matching the label on any archived tube; DNA extracts get their own label (sample nature DNA), with the source sample code kept in a separate column
- Sample type: sample, extraction blank, or positive control / mock community
- Taxonomic group and pre-treatment used, and a link to site/treatment metadata
- Extraction date and operator
- DNA concentration (Qubit), A_{260}/A_{280} and A_{260}/A_{230} ratios, and the dilution used for PCR
- Index IDs and sequences, and pool ID (filled in during library preparation, EnGeL-MOLB-MB)
- Notes (e.g. low biomass, split extraction, failed PCR, re-run)

5. Format and storage

- Keep the plate map as a spreadsheet (.xlsx/.csv) with one row per well, plus a visual 8 × 12 grid. Store it in the lab database and keep a printed copy in the lab book.
- Use the same file to generate the sequencing sample sheet, so that sample and index information is never transcribed twice.
- Version the file whenever it changes (e.g. after a re-run), and record the date and the person who made the change.

Example layout (92 samples + 4 extraction blanks, filled column by column)

	1	2	3	4	5	6	7	8	9	10	11	12
A	S01	S09	S17	S24	S32	S40	S47	S55	S63	S70	S78	S86
B	S02	S10	S18	S25	S33	S41	S48	S56	S64	S71	S79	S87
C	S03	S11	S19	S26	S34	S42	S49	S57	S65	S72	S80	S88
D	S04	S12	S20	S27	S35	S43	S50	S58	S66	S73	S81	S89
E	S05	S13	S21	S28	S36	S44	S51	S59	S67	S74	S82	S90
F	S06	S14	S22	S29	S37	S45	S52	S60	S68	S75	S83	S91
G	S07	S15	S23	S30	S38	S46	S53	S61	S69	S76	S84	S92
H	S08	S16	**EB1**	S31	S39	**EB2**	S54	S62	**EB3**	S77	S85	**EB4**

S = sample; EB = extraction blank.

Index assignment

- Each well must have a unique iTru5/iTru7 combination within a pool. A simple scheme is one iTru5 index per row and one iTru7 index per column, giving 96 unique combinations per plate.
- When several plates are pooled together, each plate must use a different index set, so that no combination is repeated within the pool.
- Check the plate map against the indexes actually used before pooling.