Bench sheet · EnGeL-MOLB-MB

DNA metabarcoding of soil fauna: two-step PCR amplicon library preparation (COI for microarthropods, 18S rRNA for nematodes) · version 1.4.2 (2026-11-20). Full protocol: EnGeL-MOLB-MB. Tick each step when done; write deviations in Notes.

Date	
Operator	
Plate ID / samples	
Qiagen Multiplex PCR Master Mix lot	
KAPA HiFi HotStart ReadyMix lot	
Primer and index plate IDs	
KAPA Pure Beads lot	
KAPA Library Quantification Kit lot	

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
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

Before starting

☐	No.	Step
☐	•	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.

First PCR (amplicon PCR)

☐	No.	Step
☐	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.

Second PCR (indexing PCR)

☐	No.	Step
☐	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.

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

☐	No.	Step
☐	7	Transfer 10 µL of each indexing PCR product to a new U-bottom 96-well plate.

☐	No.	Step
☐	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).

Quantification, normalisation and pooling

☐	No.	Step
☐	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.

Clean-up of the pooled library

☐	No.	Step
☐	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.

Pooled library check on the TapeStation (D1000)

☐	No.	Step
☐	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.

Library quantification by qPCR (KAPA)

☐	No.	Step
☐	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.

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

☐	No.	Step
☐	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.

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

☐	No.	Step
☐	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.

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

☐	No.	Step
☐	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.

Dilution to 4 nM and submission

☐	No.	Step
☐	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].

After finishing

☐	No.	Step
☐	•	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.

Notes / deviations
