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Metabarcoding using MinION: PCR, Multiplexing and Library Preparation V.2

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A protocol for the metabarcoding of DNA samples using nanopore technology, for the purposes of biomonitoring, biodiversity assessment, DNA-based diet analysis, or other related applications.

The protocol is being used in our laboratory for detecting aquatic invasive bivalve species and for measuring biodiversity of fish and other vertebrates from water eDNA samples, as well as for species identification of scats.

The key steps are a first PCR to amplify metabarcoding fragments, a 2nd PCR to add indexes (barcodes) to allow pooling of multiple samples, and the preparation of the pool for sequencing on a MinION sequencer.

The protocol is adapted from PCR barcoding (96) amplicons (SQK-LSK109) by Nanopore.

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Note: Steps for DNA extraction and associated DNA quality checks are outside the scope of this protocol. It is assumed that suitable DNA samples are being used as input for the initial PCR.

Also, note that this protocol is optimised for fragments <500bp (using Qiagen Master Mix for the barcoding PCR reaction). If targeting longer amplicons (particularly over 1kb), modification of steps 3-5 should be considered, e.g. using LongAmp Taq 2x Master Mix (New England Biolabs [NEB] USA).

Preparing first PCR

1 Prepare first PCR with a **25 µL** total PCR volume using optimised conditions for the primers / enzyme in use. Primers should include MinION adapters (5´-TTTCTGTTGGTGCTGATATTGC-forward primer-3´, 5´-ACTTGCCTGTCGCTCTATCTTC-reverse primer-3´).

2 Test PCR product to assess amplification success, e.g. via gel electrophoresis with 2 % agarose gels stained with GelRed (Biotium, USA).

Suitable stopping point. Can store sample at -20° C long-term.

Barcoding PCR: 96-BARCODING KIT (EXP-PBC096)

3 In a 0.2 mL 96-well plate, set up a barcoding PCR reaction for each library, as follows:

1 µL PCR Barcode: one of the BC1-BC96 per well (PCR Barcoding Expansion 1-96; EXP-PBC096; ONT, UK)

24 µL of amplified first PCR product (if <24µL available, adjust up with ddH2O)

25 µL QIAGEN Multiplex PCR Master Mix (Qiagen, Germany)

4 Mix by pipetting.

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5 Amplify, in a thermal cycler (e.g. T100 Thermal Cycler, BioRad, USA), using the following cycling conditions:

- Initial denaturation **00:15:00** @ **95 °C** (1 cycle),
- Denaturation **00:00:15** @ **95 °C** (12 cycles),
- Annealing **00:00:15** @ **62 °C** (12 cycles),
- Extension **00:00:30** @ **72 °C** (12 cycles),
- Final extension **00:10:00** @ **72 °C** (1 cycle),
- Hold @ **4 °C** .

Suitable stopping point. Can store sample at -20° C long-term.

6 Pool **5 µL** of each sample into a single 1.5 mL tube. If multiple primers were used in the first PCRs, and libraries from the different primer sets will be combined in the same sequencing run, we suggest preparing a separate pool for each primer set.

- Note that this may result in substantial variation in the number of reads/sample obtained, particularly if amplicon concentrations are highly variable. If aiming to reduce such variation, it is recommended to purify each PCR product using bead purification, calculate the concentration of each PCR product (e.g. using spectrophotometry or fluorometry), and normalise all samples to be equimolar prior to pooling. Note that amplicon length increases c. 94bp during the previous step. 48bp are the barcodes themselves (24 X 2bp), we assume the other 46bp are flanking adapter sequences.

7 Use automated electrophoresis (e.g. 2200 Tapestation System using a D1000 assay; Agilent Technologies, USA) to perform fragment analysis of each pooled library.

8 Bead Purification Protocol for each pooled library (adjust ratio of beads according to fragment lengths observed in previous steps, use a ratio of 0.6 X to clean fragments <300 bp):

- Prepare the Agencourt AMPure XP beads (Beckman Coulter, USA) for use (30 minutes at RT - room temperature) and freshly prepared 70% ethanol.

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- Resuspend AMPure XP beads by vortexing.
- Transfer **100 µL** of the pooled library to a clean 2 mL Eppendorf tube (adjust this volume if low number of samples used).
- Add **60 µL** of resuspended AMPure XP beads and mix by flicking the tube or pipetting up and down 10 times.
- Incubate for 7-10 minutes at RT and invert occasionally for mixing.
- Spin down the tube and place it on a magnetic rack (e.g. DynaMag-2 Magnet; Thermo Fisher Scientific, USA) for 5 mins (or until supernatant is clear).
- Keeping the tube on the magnet, pipette off the supernatant and discard.
- Taking care not to disturb the beads, add **200 µL** of 70% ethanol, then pipette off the ethanol and discard.
- Repeat the previous step.
- Spin down the tube and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow to air dry at RT for ~30 seconds.
- Remove the tube from the magnetic rack and resuspend the pellet in 80 µL nuclease-free water. Incubate for 2 minutes at RT.
- Place the tube back on the magnetic rack for 5 min (or until the eluate is clear and colourless).
- Transfer **70 µL** of eluate into a clean 1.5 mL Eppendorf tube.

9 Quantify each cleaned pooled library using fluorometer assay (e.g. Qubit dsDNA HS or BR assay kit; Thermo Fisher Scientific, USA).

10 If multiple pooled libraries were made, combine the pools in the ratios desired for sequencing, e.g. if twice as many reads are desired for Pool1 compared to Pool2, combine the pools so that Pool1 has twice the DNA mass of Pool2.

11 Prepare 1 µg of final pooled library in **47 µL** Nuclease-free water:

- The volume of the final pooled library to take equals 1000 divided by the pooled library concentration (ng/µL)
- Use nuclease-free water to bring final volume to **47 µL**

Suitable stopping point. Can store sample at -20° C long-term.

DNA repair and end-prep (SQK-LSK109)

12 Prepare the AMPure XP beads for use (30 minutes at RT) and prepare fresh 70% ethanol.

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13 Thaw DNA CS (DCS, Ligation Sequencing Kit SQK-LSK109,ONT, UK) at RT, spin down, mix by pipetting, and place on ice.

14 Place NEBNext FFPE Repair Mix (NEB, USA) and NEBNext Ultra II End Repair/dA-Tailing Module (NEB, USA) on ice.

15 In a 0.2 mL thin-walled PCR tube, mix the following:

- **1 µL DNA CS**
- **47 µL final pooled library**
- **3.5 µL NEBNext FFPE DNA Repair Buffer**
- **2 µL NEBNext FFPE DNA Repair Mix**
- **3.5 µL Ultra II End-prep reaction buffer**
- **3 µL Ultra II End-prep enzyme mix**

16 Mix gently by flicking the tube, and spin down.

17 Using a thermal cycler, incubate at **20 °C** for **00:07:00** and **65 °C** for **00:07:00**, with lid heating turned off.

18 Repeat Bead Purification Protocol (see **go to step #8 from Barcoding PCR section**), starting with the full **60 µL** from the previous step and using **60 µL** beads (here this is a 1 X ratio). Use **61 µL** for final resuspension.

Suitable stopping point. Can store sample at 4° C for 24h or -20° C long-term.

Adapter ligation and clean-up

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19 Spin down Adapter Mix (AMX, Ligation Sequencing Kit SQK-LSK109, ONT, UK) and T4 Ligase (Quick Ligation Module NEB, USA), and place on ice.

20 Thaw Ligation Buffer (LNB, Ligation Sequencing Kit, SQK-LSK109,ONT, UK) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.

21 Thaw Elution Buffer (EB, Ligation Sequencing Kit, ONT, UK) at RT, mix by vortexing, spin down and place on ice.

22 To retain DNA fragments shorter than 3 kb (by purifying fragments of all sizes), thaw one tube of S Fragment Buffer (SFB, Ligation Sequencing Kit, SQK-LSK109, ONT, UK) at RT, mix by vortexing, spin down and place on ice. If targeting longer fragments, use Long Fragment Buffer (LFB).

23 In a 2 mL Eppendorf tube, mix in the following order:

- **60 µL pooled library from the previous step**
- **25 µL Ligation Buffer (LNB)**
- **10 µL NEBNext Quick T4 DNA Ligase**
- **5 µL Adapter Mix (AMX)**

24 Mix gently by flicking the tube, and spin down.

25 Incubate tube for 10 minutes at RT.

26 Bead Purification with a ratio of 0.4 X ratio after adapter ligation:

- Prepare the AMPure XP beads for use; resuspend by vortexing.
- Add **40 µL** of resuspended AMPure XP beads, to tubes from previous step, and mix by flicking the tube.
- Incubate for 7-10 minutes at RT and invert occasionally for mixing.
- Spin down the tube, place it on a magnetic rack for 5 mins (or until supernatant is clear).
- Keeping the tube on the magnet, pipette off the supernatant and discard.
- Add **250 µL** S Fragment Buffer (SFB) and flick the tube to resuspend, spin down the tube, place it on a magnetic rack for 5 mins (or until supernatant is clear). Keeping the tube on the magnet, pipette off the supernatant and discard.

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- Repeat previous step.
- Spin down the tube and place the tube back on the magnetic rack. Pipette off any residual S Buffer. Allow to air dry at RT for ~30 seconds.
- Remove the tube from the magnetic rack and resuspend the pellet in **15 µL** Elution Buffer (EB). Incubate for 10 minutes at RT. For high molecular weight DNA, incubate at 37° C to improve the recovery of long fragments.
- Spin down the tube, place it on a magnetic rack for 5 mins (or until supernatant is clear).
- Transfer **15 µL** of eluate to a clean 1.5 mL Eppendorf tube.

27 Quantify **1 µL** of eluate using a fluorometer assay (e.g. Qubit e.g. Qubit dsDNA HS or BR assay kit; Thermo Fisher Scientific, USA).

28 Using the concentration assessed in the previous step, calculate the molarity of the library (in fmol), using the formula below:

$$PoolConc\ (fmol/\mu L) = \frac{Concentration\ of\ pool\ (ng/\mu L) \times 10^6}{length\ of\ fragment\ (bp) \times 617.96 + 36.04}$$

29 Dilute the library to 35 fmol (the range can vary from 5 - 50 fmol) in a final volume of **12 µL** , using the concentration assessed previously. Use the formula below to assess how many µL from the pool you need.

$$Pool\ volume\ (\mu L) = \frac{35 \times fragment\ length\ (bp) \times 617.96\ + 36.04}{Pool\ concentration\ \ (ng/\mu L)\ \times 10^6}$$

The prepared library is ready for loading into the flow cell. Store the library at 4° C until ready to load (up to 4 days).

Priming and loading the SpotON flow cell

30 Thaw the Sequencing Buffer (SQB, Ligation Sequencing Kit, ONT UK), Loading Beads (LB, Ligation Sequencing Kit, ONT UK), Flush Tether (FLT, Flow Cell Priming Kit, EXP-FLP001, ONT,

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UK) and one tube of Flush Buffer (FLB, Flow Cell Priming Kit, EXP-FLP001, ONT, UK) to RT before placing the tubes on ice as soon as thawing is complete.

31 Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, spin down and return to ice.

32 Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

33 Place the R9.4 flow cell (FLO-MIN106D, ONT, UK) in the MinION sequencer (Mk1B, ONT, UK).

34 Open the lid of the sequencer and slide the flow cell's priming port cover clockwise so that the priming port is visible.

> Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks damaging the pores in the array, by exposing them to air.

35 After opening the priming port, check for small bubbles under the cover. Draw back a small volume to remove any air bubbles:
- Set a P1000 pipette to 200 µL,
- Insert the tip into the priming port,
- Turn the wheel until the dial shows 220-230 µL, or until you can see a small volume of buffer entering the pipette tip.

36 Prepare the flow cell priming mix: add **30 µL** of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB), and mix by pipetting.

37 Load **800 µL** of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

38 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

> The Loading Beads (LB) tube contains a suspension of beads. These beads settle very

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quickly. It is vital that they are mixed immediately before use.

39 In a new 1.5 mL Eppendorf tube, prepare the library for loading as follows:

37.5 µL Sequencing Buffer (SQB)

25.5 µL Loading Beads (LB), mixed immediately before use

12 µL pooled library

40 Complete the flow cell priming:

- Gently lift the SpotON sample port cover to make the SpotON sample port accessible,
- Load **200 µL** of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

41 Mix the sequencing pool gently by pipetting up and down just prior to loading.

42 Add **75 µL** of the sequencing pool to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

43 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and close the MinION lid.

44 The MinION is now ready to commence sequencing according to user specifications.

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