Centre for Biodiversity Genomics
INNOVATION
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DNA barcoding on Oxford Nanopore: multiplexing up to 24 x 96-well plates

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ABSTRACT

This protocol describes laboratory methods for sequencing a standard COI marker (i.e. DNA barcoding), multiplexing up to 2,280 specimens (24 x 96 well plates, with one negative control well per plate), to run on an Oxford Nanopore Technologies Flongle 10.4.1 Flow Cell on a MinION sequencer. All indexing is accomplished by PCR using tagged primers, meaning that library prep is only carried out in a single tube with all 2,280 PCRs pooled. This is accomplished by asymmetrical indexing, where forward primers carrying 96 Unique Molecular Identifiers (UMIs) provide mapping to a well of a 96-well plate, and reverse primers with 24 UMIs provide mapping to the plate.

The protocol details all primer sequences (attached as a text table) and gives instructions for manufacturing batches of pre-made indexed plates, carrying out PCR, pooling and library prep for the ONT platform, and loading onto the sequencing device.

ATTACHMENTS

COI_UMI_primers.txt

IMAGE ATTRIBUTION

Oxford Nanopore Technologies plc.

OPEN ACCESS

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GUIDELINES

This protocol describes laboratory protocols for sequencing a standard COI marker (i.e. DNA barcoding), multiplexing up to 2,280 specimens (24 x 96 well plates, with one negative control well per plate), to run on an Oxford Nanopore Technologies Flongle 10.4.1 Flow Cell on a MinION sequencer. All indexing is accomplished by PCR using tagged primers, meaning that library prep is only carried out in a single tube with all 2,280 PCRs pooled. This is accomplished by asymmetrical indexing, where forward primers carrying 96 Unique Molecular Identifiers (UMIs) provide mapping to a well of a 96-well plate, and reverse primers with 24 UMIs provide mapping to the plate.

The protocol details all primer sequences (attached as a text table) and gives instructions for manufacturing batches of pre-made indexed plates, carrying out PCR, pooling and library prep for the ONT platform, and loading onto the sequencing device.

It is recommended that:
-reagent prep
-DNA extraction
-all post-PCR steps including Library prep
should be carried out in three different rooms or otherwise physically separated spaces to avoid cross-contamination.

MATERIALS

Consumables:

Ligation Sequencing Kit V14 (SQK-LSK114)
Flongle Sequencing Expansion (EXP-FSE002)
R10.4.1 Flongle flow cells (FLO-FLG114)
Platinum Taq DNA Polymerase (Invitrogen 15966025)
Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Trehalose
10 mM dNTP mix
E-Gels (optional)
AMPure XP Beads (or other brand SPRI beads)
NEBNext Ultra II End repair/dA-tailing Module (E7546)
NEBNext Quick Ligation Module (E6056)
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes
Freshly prepared 70-80% ethanol in nuclease-free water
Qubit Assay Tubes (ThermoFisher, Q32856)
Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment:
MinION Mk1B Sequencer with Flongle Adapter
E-Gel bases and Gel imaging system (optional)
Magnetic separator, suitable for 1.5 ml Eppendorf tubes
Microfuge
Vortex mixer
Thermal cycler
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
Timer
Qubit Fluorometer

SAFETY WARNINGS

Appropriate PPE should be worn during this procedure (lab coat, gloves).
Ethanol is highly flammable and must be kept away from heat sources and flames. Waste ethanol should be disposed of by appropriate chemical waste route in accordance with local regulations.

BEFORE START INSTRUCTIONS

DNA extracts or lysates from specimens of interest should be arrayed in 96-well plates.
Forward indexing primers should be ordered in 96-well plate format.

1. Prepare Indexed PCR Plates

1 To prepare the partial mastermix (no primers), combine the following components in a sterile bottle of at least 250 mL volume:

Component	Volume (uL)
Trehalose (10%)	145,200
Molecular Grade Water	37,636
10x PCR Buffer (-MgCl2)	29,040
MgCl2 (50mM)	14,520
dNTPs (10mM)	1,452
Platinum Taq (5U/ul)	1,394
Total volume	229,242

This volume is calculated to make a slight excess of partial PCR mix, more than sufficient for 8 reactions x 96 wells x 24 plates.

2 Dilute forward UMI primer plate to 2 uM (e.g. 20 uL of stock primer at 10 uM, added to 80 uL molecular-grade water or Tris buffer pH 8), e.g. using Liquidator™ to transfer the contents from all wells to a new 96-well plate in the same orientation.

[M] 2 micromolar (µM)

3 Dispense 9,552 uL of partial mastermix (from Step 1) into each of 24 sterile falcon tubes.

9552 µL

4 Add 12 uL of each 100 uM reverse primer to a tube of mastermix, carefully labelling which tube contains which reverse primer. Mix well.

12 µL

Tubes can be stored in fridge for use later the same day, or at -20°C for later use.

5 Transfer mastermix (containing reverse primer) to a reservoir and dispense 87 uL to each well of a 96-well plate using a multichannel pipette.

87 µL

6 Using a Liquidator™ or multichannel pipette, add 5.5 uL of primer from the **diluted (2uM)** forward primer plate to each well of the 96-well plate, in the same orientation.

5.5 µL

7 Using a Liquidator™ or multichannel pipette, mix all wells by pipetting up and down several times, then dispense 10.5 uL of complete mastermix (containing forward and reverse primers) into eight (8) 96-well PCR plates. Seal each plate and store at -20C for up to 6 months.

10.5 µL

Ensure all plates are clearly labelled to indicate primers and plate UMI used.

2. COI PCR

8 Thaw the number of PCR plates required. Add 2 uL of extracted DNA or specimen lysate to each 10.5 uL PCR reaction. Mix by pipetting up and down several times and spin down plate in centrifuge.

2 µL

Label all plates carefully, indicating both plate ID/name and UMI index (i.e. reverse primer).

9 Place in thermocycler and carry out the following program:

	Temperature	Duration	No. cycles
	94°C	2 min	1
	94°C	40 seconds	5
	45°C	40 seconds	
	72°C	1 minute	
	94°C	40 seconds	

	Temperature	Duration	No. cycles
	51°C	40 seconds	33
	72°C	1 minute	
	72°C	2 minutes	1
	4°C	HOLD	1

10 Optional: run 4 uL of each PCR product on an E-gel™ or other gel electrophoresis device to confirm amplification.

4 µL

3. Pool and purify PCRs

11 Using a multichannel pipette or liquid handling robot, pool each PCR plate into a single 1.5ml Eppendorf tube. Mix well by pipetting and label all tubes carefully with both plate ID/name and UMI index (i.e. reverse primer).

12 Combine equal volumes of each plate pool (maximum 24), such that the final volume will be at least 200 uL (e.g. 50 uL from each tube). Mix well and take 200 uL of the single pool into a new 1.5ml eppendorf tube (exact amount is not critical, 200 uL provides an excess of final DNA in most cases). Store excess pools at -20°C in case repeat runs are needed.

13 Carry out magnetic bead cleanup:

13.1 Add 0.8x volume of Ampure XP beads (e.g. for 200 uL pooled PCRs, add 160 uL beads). Mix well by pipetting.

160 µL

13.2 Incubate ~10 minutes at room temperature, mixing occasionally by gently flicking the tube.
During this incubation, prepare 70% ethanol (e.g. mix 1.4 mL 100% ethanol + 0.6 mL molecular-grade water for 2 ml, sufficient for this wash and the later washes in Step 16).

13.3 Place bead-pool tube on magnetic rack. Wait 1-2 minutes until supernatant is clear, then remove and

discard supernatant.

13.4 Leaving tube on magnetic rack, add 500 ul of freshly-prepared 70% ethanol.
500 µL

13.5 Remove and discard ethanol.

13.6 Repeat steps 13.4 and 13.5 for a total of two ethanol washes.

13.7 After the second ethanol removal, place tube in mini centrifuge and spin for a few seconds to collect all remaining ethanol at the bottom of the tube. Return tube to magnetic rack and use a P20 pipette to remove the last ethanol.

13.8 Leave tube open at room temperature for 1-2 minutes to allow residual ethanol to dry (beads are sufficiently dry when they take on a matte appearance; do not over-dry).

13.9 To elute DNA, remove tube from magnetic rack and add 40 ul of molecular-grade water. Resuspend beads by pipetting up and down or by flicking tube.
40 µL

13.10 Incubate 10 minutes at room temperature, mixing occasionally by gently flicking the tube.

13.11 Return tube to magnetic rack. Wait 1 minute until supernatant is clear, then transfer 38 uL supernatant to fresh 1.5 ml eppendorf tube.

38 µL

4. Quantification and End Prep

14 Quantify 1 uL of the eluted DNA using Qubit HS kit (following manufacturer's instructions).

15 Set up End Prep reaction:

15.1 From the Qubit result in step 14, calculate the volume of DNA needed for ~100ng (this is a guide, not a critical amount). Transfer this volume to a fresh 0.2ml PCR tube. Unused purified pool may be stored at -20°C for future use.
100 ng

15.2 Add molecular grade water to make up a total volume of DNA+water to 12.5 uL.

15.3 Combine the following components in the tube:

	Component	Volume (uL)
	DNA amplicons (~100ng)	12.5
	NEBNext Ultra II End Prep Buffer	1.75
	NEBNext Ultra II End Prep Enzyme	0.75
	Total volume	15

15.4 Carry out the following program in thermocycler 20°C for 5 minutes, 65°C for 5 minutes, hold at 4 10m
20 °C 00:05:00
65 °C 00:05:00

16 End Prep cleanup:

16.1 Transfer all 15 uL End Prep reaction to a new 1.5 mL LoBind tube and mix with 15 uL Ampure XP beads. Mix well by pipetting.

16.2 Incubate 5 minutes at room temperature, mixing occasionally by gently flicking the tube.

16.3 Place tube on magnetic rack. Wait 1-2 minutes until supernatant is clear, then remove and discard supernatant.

16.4 Leaving tube on magnetic rack, wash with 200 uL of 70% ethanol (prepared on same day).
200 µL

16.5 Remove and discard ethanol.

16.6 Repeat steps 16.4 and 16.5 for a total of two washes.

16.7 After the second ethanol removal, place tube in mini centrifuge and spin for a few seconds to collect all remaining ethanol at the bottom of the tube. Return tube to magnetic rack and use a P20 pipette to remove the last ethanol.

16.8 Leave tube open at room temperature for 1-2 minutes to allow residual ethanol to dry (beads are sufficiently dry when they take on a dull appearance; do not over-dry).

16.9 To elute DNA, Remove tube from magnetic rack and add 16 uL of molecular-grade water. Resuspend beads by pipetting up and down or by flicking tube.

16 µL

16.10 Incubate 5 minutes at room temperature, mixing occasionally by gently flicking the tube.

16.11 Return tube to magnetic rack. Wait ~1 minute until supernatant is clear, then transfer 15 uL supernatant to fresh 1.5 ml LoBind tube.

15 µL

5. Adapter Ligation and Final Cleanup

17 Combine the following components in the 1.5ml tube:

	Component	Volume (uL)
	End-prepped DNA (step 16)	15
	Ligation Buffer (LNB)*	6.25
	NEBNext Quick T4 Ligase	2.5
	Ligation Adapter (LA)*	1.25
	Total volume	25

** From ONT Native Barcoding Kit (SQK-LSK114).*

Note

This recipe uses half-volumes of all components compared to the protocol published on Oxford Nanopore Technologies' own website; we have found this smaller-volume ligation reaction to perform equally well while providing cost savings on reagents.

Mix by pipetting and incubate at room temperature (~25°C) for 10 minutes.

18 Cleanup final library using Ampure XP beads:

18.1 Add 12.5 uL (0.5x) volume of Ampure XP beads and mix well by pipetting.
12.5 µL

18.2 Incubate at room temperature for 10 minutes.

18.3 Place tube on magnetic rack. Wait 1-2 minutes until supernatant is clear, then remove and discard supernatant.

18.4 Remove tube from rack and add 100 uL Short Fragment Buffer (SFB - from Ligation Sequencing Kit SQK-LSK114). Pipette up and down or flick tube to resuspend beads.
100 µL

18.5 Return tube to magnetic rack. Wait 1-2 minutes until supernatant is clear, then remove and discard supernatant.

18.6 Repeat steps 18.4 and 18.5 for a total of two washes in SFB.

18.7 After the second wash, place tube in mini centrifuge and spin for a few seconds to collect all remaining SFB at the bottom of the tube. Return tube to magnetic rack and use a P20 pipette to remove the last SFB.

18.8 Air dry pellet for no more than 30 seconds, then add 11 uL Elution Buffer (EB - from Ligation Sequencing Kit SQK-LSK114) and resuspend pellet by pipetting up and down.

11 µL

18.9 Incubate for 10 minutes at room temperature or optionally, for increased recovery, place on thermocycler block set to 37C.

18.10 Return tube to magnetic rack. Wait ~ 1 minute until supernatant is clear then collect 10 uL supernatant to fresh 1.5ml tube.

10 µL

19 Quantify 1ul of library using Qubit HS kit (following manufacturer's instructions).

6. Flow Cell Loading

20 Prepare Flongle and carry out Flow Cell Check:

20.1 Remove one Flongle Flow Cell from the fridge and insert into MinION, ensuring the Flongle Adapter is in place.

Image credit: Oxford Nanopore Technologies plc.

20.2 In MinKNOW software, navigate to “Flow Cell Check”. Enter the Flongle ID (3 letter+3 number code written on the Flongle) and select “FLO-FLG-114” if using 10.4.1 cell.

The check takes ~5 minutes. When complete, record the pore count. If below 50, it is considered to fail warranty and is eligible for a free replacement if within 28 days of receipt; record details and report to ONT.

21 While Flow Cell Check is running, prepare flow cell priming mix and sequencing mix:

21.1 In 1.5ml Eppendorf tube, mix 117 uL Flow Cell Flush (FCF) with 3 uL Flush Tether (FT) – both from Flongle Expansion Kit.

117 µL FCF

3 µL FCT

21.2 Pull back the plastic film covering the Flongle loading port, holding in place by pressing the dots (adhesive) against the lid of the MinION device.

Image credit: Oxford Nanopore Technologies plc.

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21.3 Pipette the priming mix (FCF/FT) into the loading port using a P200 pipette. It is important to dispense slowly and not to introduce air bubbles; draw all 120 uL into the pipette, ensure there is no air gap at the tip and dispense by turning the pipette plunger rather than pushing down. Leave the last 1-2 uL in the tip and discard to ensure no air is introduced.

120 µL

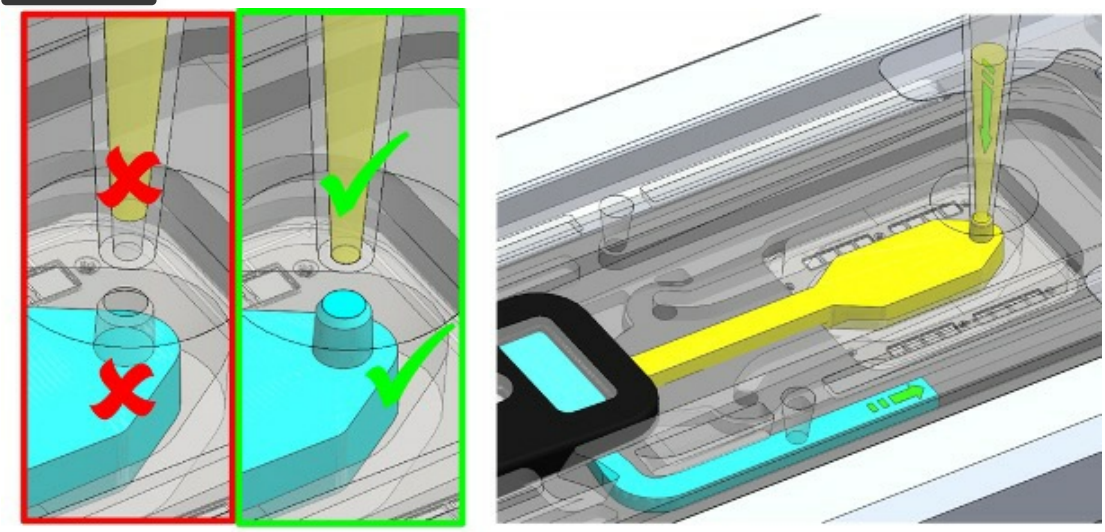

Image credit: Oxford Nanopore Technologies plc.

21.4 From Qubit reading in Step 19, calculate volume of library needed for 5ng DNA in a final volume of 5 uL. If dilution is necessary, dilute using EB.

5 ng

Note

Optimal loading amount for Flongle flow cell is 5-10 fmol. For the standard COI barcode fragment (~750bp with primers, UMIs and adapters), 10 fmol equals ~5ng of DNA. If sequencing a different fragment size, calculate and adjust as appropriate.

When ready to load, combine the following components in a 1.5ml Eppendorf tube:

	Component	Volume (uL)
	Sequencing Buffer (SB)	15
	Library Beads (LIB)*	10
	DNA library (10 fmol) in EB	5
	Total volume	30

** Library beads must be thoroughly mixed by vortexing immediately before use and pipetting up and down several times before taking from vial.*

21.5 Pipette library mix up and down several times in the tube to resuspend Library Beads, then pipette into the loading port. NOTE: as in Step 22.4, dispense slowly to avoid introducing air bubbles and leave the last 1-2 uL in the tip.

30 µL

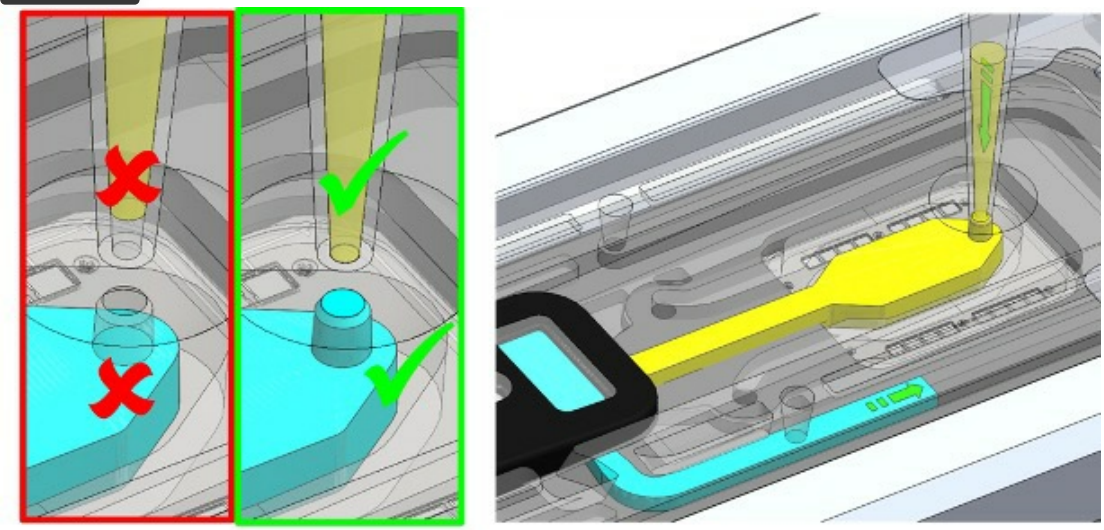

Image credit: Oxford Nanopore Technologies plc.

21.6 Close the seal tab over the loading port, pressing down on the adhesive spots to stick down. Close the lid of the MinION.

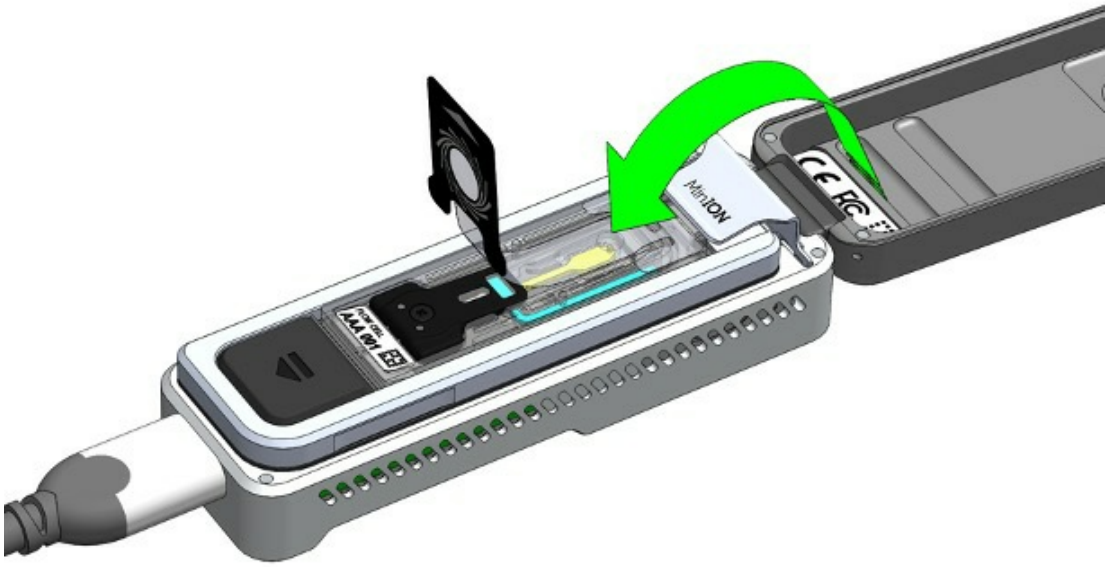

Image credit: Oxford Nanopore Technologies plc.

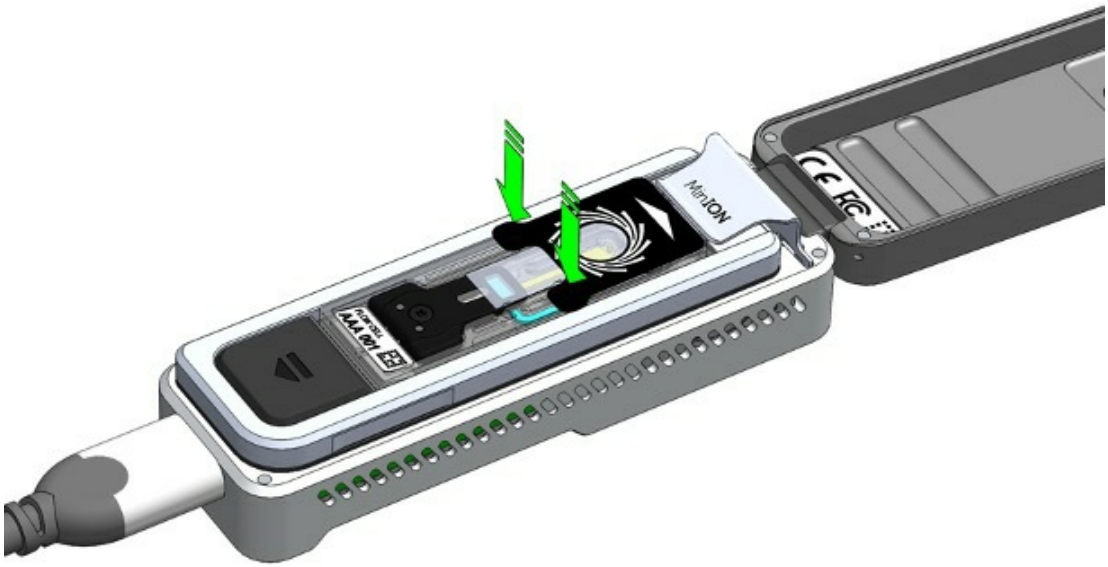

Image credit: Oxford Nanopore Technologies plc.

21.7 Navigate to “Start Sequencing” in MinKNOW software and begin run.