ONTOLOGY™ Laboratory Protocols

DNA barcoding and metabarcoding
on Oxford Nanopore sequencers

Release 1.02.2

Centre for Biodiversity Genomics

Produced 14 September 2026

Contents

REFERENCE

List of Required Materials for ONTOLOGY™

v1.02

Included in the ONTOLOGY™ PCR Kit

- 10× Orange ONTOLOGY™ Freeze-Dried PCR Plates (one each of UMIs 1–10)
- 10× PCR seals (Applied Biosystems 4306311)
- 10× temporary seals (Cytiva 77040001)
- 10× foil seals (Axygen PCRAS600)

Included in the ONTOLOGY™ DNA Extraction Kit

- 10× Clear DNA Extraction Plates
- 1× 1 mL concentrated Lysis Buffer*
- 1× 1 mL concentrated Neutralization Buffer*
- 2× empty 50 mL tubes labeled for Lysis or Neutralization
- 10× PCR seals (Applied Biosystems 4306311)
- 10× temporary seals (Cytiva 77040001)
- 10× foil seals (Axygen PCRAS600)

Equipment

Required

- Refrigerator (4°C)
- Freezer (−20°C)
- Computer with GPU (or Apple ‘M’-series chip) and ≥16 GB RAM
  - For barcoding, typical high-end consumer laptops are fine (∼$3K)
  - For metabarcoding, very high end workstations are recommended
- Camera (smartphone or DSLR)
- Alcohol burner
- Pipettes
  - P1000 pipette (>200 μL up to 1000 μL) and tips
  - P200 pipette (20–200 μL) and tips

  - Either P100 pipette (10–100 μL) or P20 pipette (2–20 μL) and tips
  - P10 pipette (0.5–10 μL) and tips
- 96-well plate thermal cycler equipped to receive semi-skirted PCR plates
  - *Note: Some thermal cyclers require adapters so they do not crush 0.2 mL tubes*
- Magnetic rack, suitable for 1.5 mL Eppendorf tubes[1]
- MinION Sequencer with Flongle Adapter[2,4]
- Gel Imaging System
- Microfuge
- Vortex mixer
- Timer
- Qubit Fluorometer, or other DNA quantification instrument; we do not recommend using a NanoDrop

Recommended

- Multi-channel pipette for P20 and P200 volumes
- Plate centrifuge
- *If using smartphone camera:* Clip-on macro lens
- Plate stand (e.g., https://www.divbio.com/liquid-handling/well-plate-stands/well-plate-stand)

Optional

- Incubator or heated thermal block (can be used for ethanol evaporation, and/or in place of a thermal cycler for lysis incubation)

Consumables

- Ligation Sequencing Kit V14 (SQK-LSK114)[2]
- Flow Cell
  - Flongle Sequencing Expansion (EXP-FSE002)[2]
  - R10.4.1 Flongle flow cell (FLO-FLG114)[1]
    OR
  - MinION Flow Cell (FLO-MIN114)
- Nuclease-free water (e.g., Thermo Fisher, cat # AM9937)
- Prepared agarose gels (or E-Gels)
- AMPure XP Beads (or other brand SPRI beads)[1]
- NEBNext Companion Module v2 for Oxford Nanopore Technologies Ligation Sequencing (E7672)[1]
- (Recommended) 1.5 mL Eppendorf DNA LoBind tubes
- 0.2 mL thin-walled PCR tubes

- 100% EtOH
- Qubit Assay Tubes (ThermoFisher, Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- Reagent reservoirs

Notes

[1]Sergi Lab Supplies (https://sergilabsupplies.com/) offers a relatively inexpensive option for purchasing this product.

[2]Purchase from Oxford Nanopore Technologies (http://store.nanoporetech.com/).

[3]Purchase from New England BioLabs (https://www.neb.com/).

[4]Not needed if using a MinION Flow Cell instead of a Flongle.

[*]One extra tube of each concentrated bufer is included.

PROTOCOL

Sort and Array Specimens

Insecta v1.01

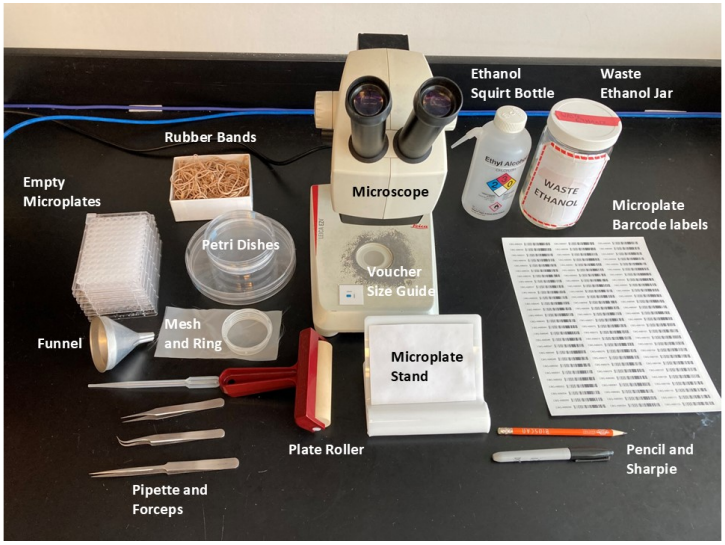
This protocol is designed for bulk samples containing both large (pinned) and small (microplated) arthropods preserved in alcohol (e.g., Malaise traps). If your specimens are either or both, you can just refer to the relevant parts of the protocol.

- *Bullet points show tips for executing the protocol.*

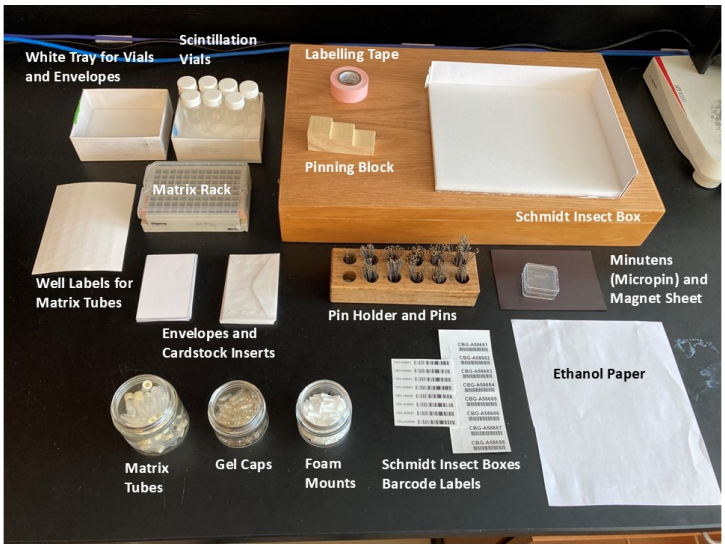
(1) Sort specimens by size

- *Specimen preparation is much more efficient if similarly-sized specimens are processed on the same plates.*
- *If all your specimens have the same preservation method (e.g., all are pinned, or all are plated), you can skip to (2).*
- *Non-arthropods should be removed from the workflow at this stage and preserved separately.*

a. If your work will involve specimens in microplates, assemble a 'plating' workstation containing as many of the items below as you have. (Labels are printed by ONTOLOGY™). ONTOLOGY™ also contains a printout for the Voucher Size Guides.



b. If your work will involve specimens that are too large for microplates, assemble a 'plating' workstation containing as many of the items below as you have. (Labels are printed by ONTOLOGY™).



c. Sort your specimens into two 'piles': (i) small and (ii) large.

i. Prepare a 'destination' Petri dish with Ethanol.

ii. Using forceps, move large specimens—those that do not fully fit inside the box in the voucher size guide (provided by ONTOLOGY™)—into the destination Petri dish. When complete, you should have two size-sorted Petri dishes.

(2) Place specimens into 96-well plate format

- *The later steps of this process will be most efficient if all specimens analyzed on an ONTOLOGY™ plate are arrayed in the same manner—e.g., all pinned or all plated.*
- *If a 'mixed' plate is necessary, process all of one kind then all of the othery.*
- *You can only analyze 95×N specimens, where N is the number of ONTOLOGY™ plates remaining in your batch (e.g., 950 specimens if all 10 plates remain). Therefore, if you have more than this number of specimens, choose carefully which you take into the next steps.*
- *Remember that H12 is always empty.*

a. Process small specimens into microplates

i. Label the plate with the corresponding ONTOLOGY™ plate ID.

ii. Pre-fill all wells with EtOH if you will extract DNA at a later date, or with 50 μL Alkaline Lysis (AL) buffer if you will extract DNA immediately (see *Protocol – DNA Extraction by Alkaline Lysis*).

iii. Using a microscope to aid you, gently pick up individual specimens with forceps and transfer them to your microplate, starting with well A01 and proceeding to A12; then rows B–H, ensuring you leave H12 empty except for buffer or preservative.

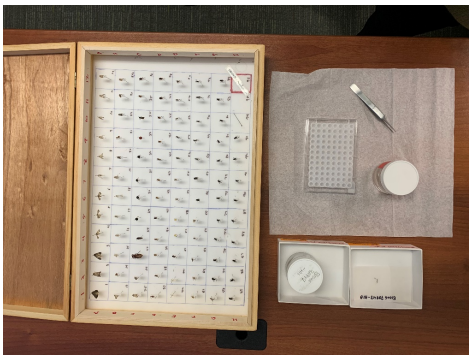
iv. Seal plate with a PCR seal if it contains AL buffer and will be incubated, or seal plate with a

foil seal if it contains EtOH and will be stored.

v. Proceed to the next plate.

b. Pin large specimens

i. For large specimens, pin and place in a labelled pinning box in the positions they will occupy on the plate.



ii. Ensure the box is clearly labelled with the destination ONTOLOGY™ plate.

iii. Proceed to the next plate.

PROTOCOL

Tissue Sampling

v1.01

This protocol applies only to 'large' specimens—those that are pinned or in matrix tubes. Ensure your arrays are complete before proceeding. If your specimens are not labelled, ensure they remain in the same positions in the array.

- *Bullet points show tips for executing the protocol.*

(1) Prepare tissue sampling workstation

- *Working with a 'plate stand', that elevates and angles the DNA extraction plate, can make this workflow more comfortable.*

a. Obtain your DNA extraction plate and, using a multi-channel pipette and reservoir, fill it with *either* 50–100 μL EtOH (if extracting at a later date) *or* 50 μL Alkaline Lysis (AL) buffer (if extracting immediately)—see "DNA Extraction by Alkaline Lysis" below for details.

b. Ensure the correct box of specimens for the plate is at your workstation.

c. Obtain two pairs of forceps.

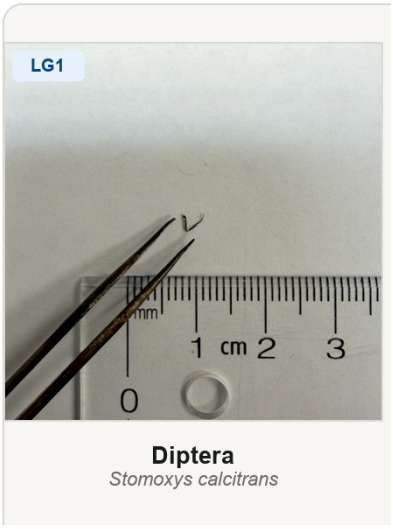
d. Fill a small jar with enough EtOH to fully cover the tips of your forceps; this can be Waste EtOH. A small, stout jar is recommended to reduce the likelihood of spills.

e. Set up an Ethanol burner on the periphery of your workstation, light it, then flame-sterilize your forceps by dipping into the ethanol and then burning it off with the burner.

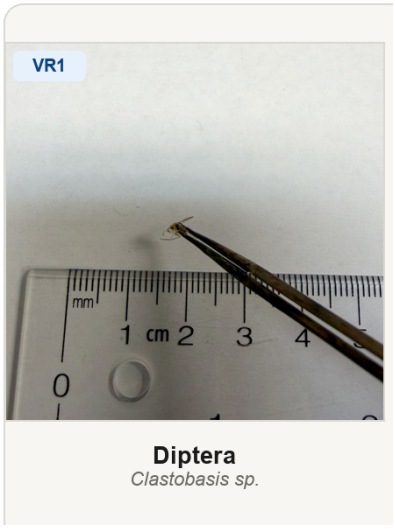
(2) Sample tissue into plates

- *For Alkaline lysis, we find that 'less is best' when it comes to tissue. The amount of tissue should be no larger than one-half of a single grain of rice.*
- *We find it is helpful to 'rotate' the specimens 90° after sampling tissue to help the user follow along.*

Example tissue amounts



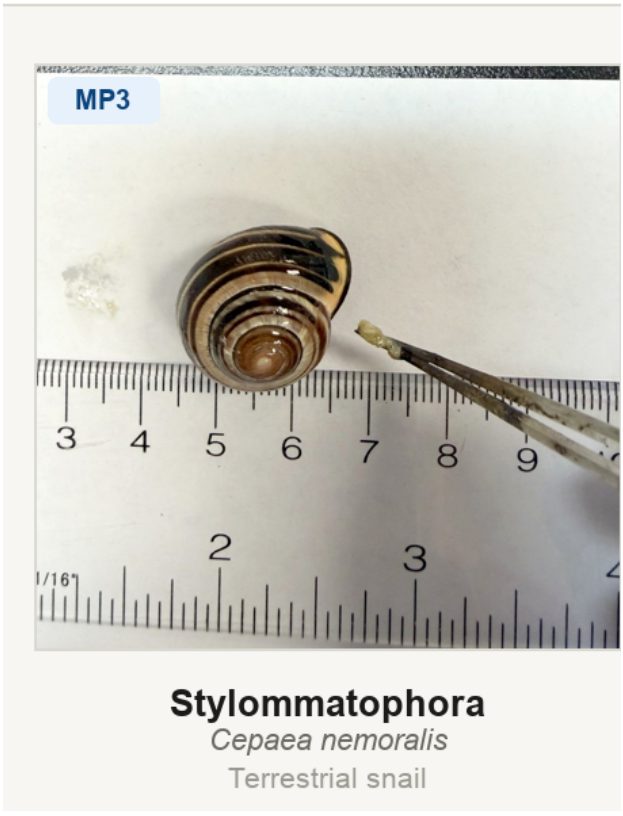
Leg — Stomoxys calcitrans (Diptera)



Voucher — Clastobasis sp. (Diptera)

Larva — Galleria mellonella (Lepidoptera)

Mucopolysaccharide — Lumbricus terrestris (Crassiclitellata)



Mucopolysaccharide — Cepaea nemoralis (Stylommatophora)

Forceps indicate the approximate tissue mass taken for a single extraction, shown against a millimetre ruler.

a. Light your EtOH burner.

b. Flame-sterilize your forceps.

c. Obtain the specimen with tissue bound for well A01 or the first available starting well.

d. Remove the tip of a leg, or—if necessary—a small amount of body tissue, and place into the well, ensuring it is fully covered by the buffer.

e. Repeat b–d until you sample tissue into H11.

f. Seal plate with a PCR seal if it contains AL buffer and will be incubated, or seal plate with a foil seal if it contains EtOH and will be stored.

g. Proceed to DNA extraction.

PROTOCOL

DNA Extraction by Alkaline Lysis

v1.01

*Alkaline lysis is a chemical DNA extraction method that is inexpensive and quick. It involves lysing tissue in an alkaline solution (pH 13) at hot temperatures and then neutralizing the reaction with an acidic solution (pH 5.5). The DNA can be purified after, but this is generally not necessary for PCR-based analyses. A similar protocol using NaOH rather than KOH is also known as 'HotShot' (**Hot** **S**odium **Hydr**o**x**ide & **T**ris).*

- *Bullet points show tips for executing the protocol.*

(1) Prepare buffer solutions from concentrates

a. Prepare Alkaline Lysis (AL) Buffer:

i. Centrifuge one 2 mL tube containing 1 mL AL buffer concentrate.

ii. Pipette entire 1 mL volume into included empty 50 mL tube with AL labels.

iii. Fill 50 mL AL tube to 50 mL mark with molecular grade water.

iv. Invert to mix.

b. Prepare Neutralization Buffer (NB):

i. Centrifuge one 2 mL tube containing 1 mL NB buffer concentrate.

ii. Pipette entire 1 mL volume into included empty 50 mL tube with NB labels.

iii. Fill 50 mL NB tube to 50 mL mark with molecular grade water.

iv. Invert to mix.

Note: Prepared buffer solutions can be stored at room temperature.

(2) Sample tissue into ONTOLOGY™ DNA extraction plates

- *So long as the tips do not contact the wells, the same tips can be reused.*
- *Pour the buffer solutions into a sterile reservoir and use a multi-channel pipette to dispense.*
- *Ensure you label the DNA plates clearly from 1–10, because they come unlabeled.*

a. Fill the wells of the clear (transparent) ONTOLOGY™ DNA extraction plates with 50 μL of **either** prepared AL buffer **or** 95% EtOH (if using ethanol, you can reduce volume to 20 μL).

i. If you will extract DNA today, sample tissue into lysis buffer.

ii. If you will extract DNA later, sample tissue into EtOH.

b. Place a very small piece of tissue—at most 2 mm^2 (half of a grain of rice)—into each well.

c. Seal plate.

i. If you used AL buffer, seal with a **PCR seal**.

ii. If you used ethanol, seal with a **temporary seal**.

d. Briefly centrifuge the plate to ensure that the tissue is submerged.

(3) Alkaline Lysis protocol

*If specimens are already in AL buffer, start from step **b**.*

a. Evaporate EtOH, if used, then add AL buffer (proceed to (b) if AL buffer already used):

i. Place plates containing EtOH uncovered (i.e., remove seal) in an incubator at 56°C.

ii. Keep plates in incubator until all EtOH is evaporated from all wells, typically 30–45 minutes, depending on the volume.

iii. Add 50 μL AL buffer to each well after EtOH is evaporated.

iv. If you will be incubating in a thermocycler with a heated lid, apply a **PCR seal** to plate. If you will be incubating in an incubator, thermomixer, or thermocycler without a heated lid, apply a **foil seal**.

b. Quickly centrifuge the plate to ensure tissue is in contact with the AL buffer.

c. Incubate sealed plate at 95°C for 10 minutes.

d. After incubation, allow sealed plate to cool to room temperature for 3–4 minutes.

e. Centrifuge the sealed plate to remove any condensation that formed on the seal.

f. Add 50 μL of neutralization buffer using a pipette.

g. Mix by gently tapping the plate.

h. Store neutralized lysate at −20°C for long-term storage, 4°C for short-term storage, or use immediately in PCR. If desired, tissue can be recovered at this point.

(4) (Optional) Tissue Recovery

a. If carrying out tissue recovery, e.g., for small organisms where the entire specimen was used for lysis, transfer the full volume (e.g., 100 μL) of neutralized lysate to a new 96-well plate, carefully avoiding the specimen.

b. Add 50–100 μL of 95% ethanol to each original well containing the specimens to be retained. Cover the plate using a **foil seal** (or heat sealer, if available) and store at −20°C, or store the vouchers as desired.

Preparation of stock reagents

1. **1M Tris-HCl, pH 5.15**
   i. Dissolve 78.8 g of Tris-HCl in ~450 mL of deionized water (ideally filtered to 18.2 MΩ·cm).
   ii. Using 5M KOH, adjust pH to 5.15 (10–20 drops should suffice).
   iii. Adjust volume to 500 mL with deionized/filtered water.
   iv. Filter sterilize solution through a 0.22 μm filter into a sterile container.
2. **0.5M EDTA, pH 8.0**
   i. Dissolve 9.31 g of EDTA in ~40 mL of deionized water (ideally filtered to 18.2 MΩ·cm).
   ii. Using NaOH pellets, adjust pH to 8.0. Add one pellet at a time. EDTA will fully dissolve once pH hits 8.0.
   iii. Adjust volume to 50 mL with deionized/filtered water.
   iv. Filter sterilize solution through a 0.22 μm filter into a sterile container.
3. **1M KOH**
   i. Dissolve 28.06 g of KOH in ~450 mL of deionized water (ideally filtered to 18.2 MΩ·cm).
   ii. Adjust volume to 500 mL with deionized/filtered water.
   iii. Filter sterilize solution through a 0.22 μm filter into a sterile container.

Preparation of working solutions

Note: To simplify preparation, the following two solutions are prepared directly in unopened 500 mL bottles of commercial molecular-grade water.

Lysis buffer

Stock	Working conc.	Volume (µL)
1M KOH	25 mM	12,828
0.5M EDTA pH 8.0	0.2 mM	205
100% Triton X-100	0.2%	103
Water	N/A	500,000
TOTAL	**N/A**	**513,136**

Neutralization buffer

Stock	Working conc.	Volume (µL)
1M Tris-HCl pH 5.15	40 mM	20,833
Water	N/A	500,000
TOTAL	**N/A**	**520,833**

Optional pH validation: If desired, measure the pH of lysis buffer (should be ∼13); neutralization buffer should be between 5.5–6.0, and a 1:1 (v:v) mixture of both solutions should be ∼8.5.

Lysate Dilution

Alkaline lysis releases DNA but does not purify it, so the neutralized lysate still carries polymerase inhibitors into PCR alongside the target DNA. Diluting the lysate removes these inhibitors, but diluting too far also removes the target DNA, so the right dilution depends strongly on the type and amount of tissue that was sampled. The table below gives recommended starting dilutions for four commonly encountered tissue types.

These values suit a broad range of taxa, but some taxa may need more or less dilution, and over-sampling tissue can push the required dilution well above what is shown here. Sample the smallest practical amount of tissue—see the examples in the *Protocol – Tissue Sampling* section.

Recommended dilutions

Tissue type	Example specimens	Recommended lysate dilution	Suggested dilution volumes
Leg	Insect legs, all Orders	None	—
Voucher	Whole small-bodied insect specimens	10×	2 µL lysate + 18 µL water
Larva	Whole soft-bodied larvae	50×	2 µL lysate + 98 µL water
Mucopolysaccharide	Annelids & molluscs	100×	1 µL lysate + 99 µL water

PROTOCOL

Polymerase Chain Reaction (PCR)

v1.01

ONTOLOGY™ uses freeze-dried PCR plates that attach unique molecular identifiers (UMIs) to the barcode region during PCR. The software recognizes these UMIs to automate demultiplexing. All components—except for water and DNA—are already in the plates. The user must just rehydrate, add DNA, and then thermocycle.

(1) Rehydrate ONTOLOGY™ PCR plates

a. Prepare one freeze-dried PCR plate (orange) for each DNA plate (clear) to be sequenced.

Critical step: **Ensure the plate numbers match. If you are using DNA plates #1–3 you must use PCR plates #1–3.**

b. For each PCR plate (orange) to be used, use a reservoir and multichannel pipette to dispense 10 μL of molecular-grade water to each well.

c. Cover plate with a **temporary seal** and either quickly centrifuge or gently tap the plate against the bench to ensure all water moves to the bottom of the wells.

d. Leave plate at room temperature for 10 min to allow PCR reagents to dissolve.

e. Quickly centrifuge.

(2) Conduct PCR

- *If diluting lysate to reduce PCR inhibition, see Protocol – DNA Extraction by Alkaline Lysis (Lysate Dilution) for recommended dilutions by tissue type.*

a. **Slowly uncover the seal to avoid cross contamination.**

b. Transfer 2 μL of neutralized lysate from the clear DNA plate into each well of the corresponding orange PCR plate; use a multichannel pipette and transfer row A to row A, row B to row B, etc.

Critical step: **Take extra care to ensure both plates are oriented the same way (A01 in top left corner), and that the number of the DNA plate and UMI plate match. Change tips between samples.**

c. Seal with a **PCR seal**.

d. Pulse down.

e. Thermocycle using the ONTOLOGY™ PCR program:

Stage	Temp (℃)	Duration	No. cycles
Denaturation/activation	94	2 min	1
Amplification 1	94	40 sec	5
	45	40 sec	
	72	1 min	
Amplification 2	94	40 sec	40
	51	40 sec	
	72	1 min	
Final elongation	72	2 min	1
Hold	4	hold	1

PCR plate components

All PCR components except for the DNA are included in the freeze-dried plates; the user need only add water when ready to use. Each PCR contains the following reagents:

Component	1 rxn (µL)
Trehalose (10%)	6.25
Molecular grade water	2
Buffer (10X)	1.25
$MgCl_2$ (50 mM)	0.625
Indexed Forward primer (10 μM)[1]	0.125
Indexed Reverse primer (10 μM)[2]	0.125
dNTPs (10 mM)	0.0625
Platinum Taq (5 U/μL)	0.06
DNA	2
TOTAL	**12.5**

Primer	Sequence (5′ – 3′)
C_LepFolF[1]	RKTCAACMAATCATAAAGATATTGG
C_LepFolR[2]	TAAACTTCWGGRTGWCCAAAAAATC

PROTOCOL

Agarose Gel Check

v1.01

To confirm PCR amplification success from ONTOLOGY™ PCR Plates, completed reactions can be visualized using gel electrophoresis. This protocol uses the Invitrogen E-Gel® 96 with SYBR Safe stain. For other electrophoresis approaches, contact the ONTOLOGY™ development team (dev@ontology.bio) for advice.

- *Bullet points show tips for executing the protocol.*

(1) Loading and running E-Gel® 96 gels

- *The recommended program for 2% Agarose E-Gel® 96 gel is **EG** and the run time is 6–12 min.*

a. Plug the Mother E-Base™ into an electrical outlet. Press and release the pwr/prg (power/program) button on the base to select program EG.

b. Remove E-Gel® from the package and remove plastic comb.

c. Slide gel into the two electrode connections on the Mother or Daughter E-Base™.

d. Load 12 μL of molecular grade H_2O into wells with multichannel pipettor.

e. Optional: Load appropriate DNA markers in the marker wells (“M” column on the right); e.g., 4 μL of Invitrogen E-Gel 1 Kb Plus DNA Ladder, #10488090.

f. Load 4 μL of sample.

g. To begin electrophoresis, press and release the pwr/prg button on the E-Base™. The red light will change to green.

h. At the end of the run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button to stop the beeping.

i. Remove gel cassette from the base and capture a digital image of the gel on a UV transilluminator equipped with digital camera.

j. Analyze the image and align or arrange lanes in the image using the E-Editor™ 2.0 software available at `http://www.invitrogen.com/egels/`.

Example E-Gel® with successful amplification for most samples

PROTOCOL

PCR Pooling

v1.0

This protocol provides technical instructions for combining all PCR reactions into a single tube.

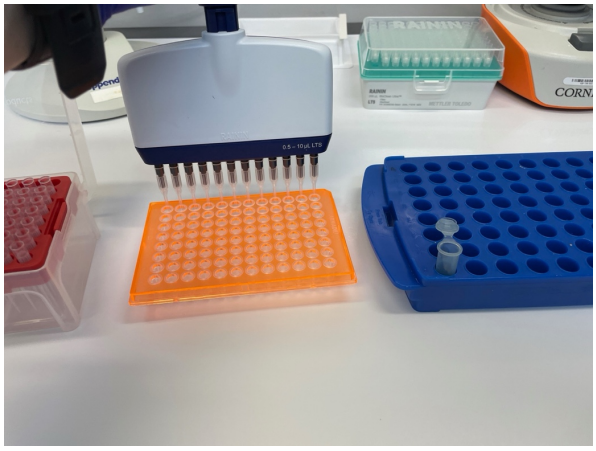
- *Bullet points show tips for executing the protocol.*

(1) Plate pools: pool each PCR plate into its own 1.5 mL tube

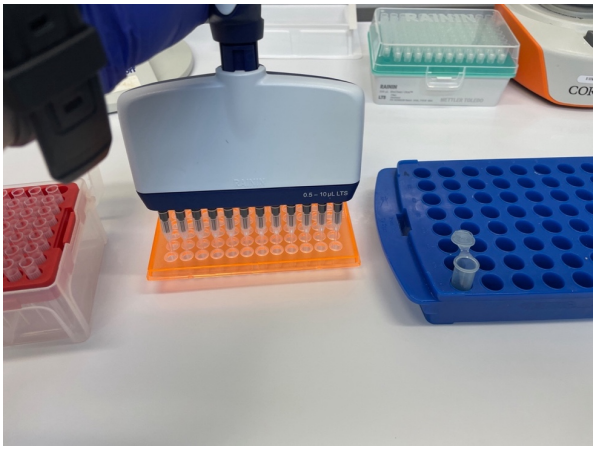
- *Although this protocol can be completed entirely with a single-channel pipette, we recommend using a 12-channel or 8-channel pipette where possible for efficiency.*

a. Obtain all ONTOLOGY™ plates that will be multiplexed into this run. Each plate must have completed PCR and, ideally, had some or all wells checked on a gel.

b. Using a multi-channel pipette, combine all rows into a single row (if 4 μL was taken to run on a gel previously, the remaining volume per well is 8.5 μL; otherwise, the full PCR volume of 12.5 μL remains).

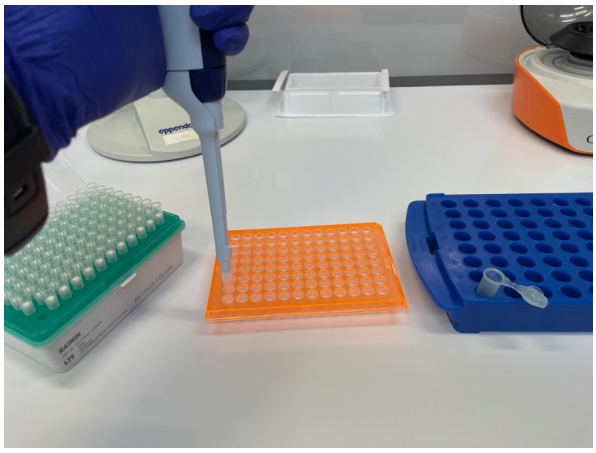


Use a multi-channel pipette to obtain all samples from row A

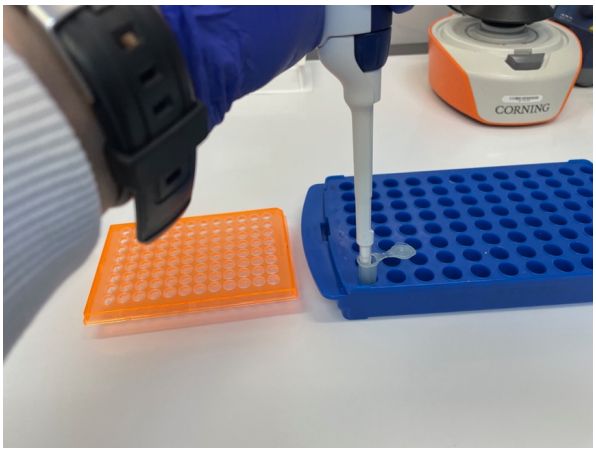


Dispense row A samples into row H; repeat for rows B–G

c. Using a single-channel pipette, and setting the pipette to collect the entire volume in each well, collect the combined row into a single 1.5 mL tube.



Use a single-channel pipette to obtain pool in H1



Transfer H1 pool to destination 1.5 mL tube; repeat for H2–H12

d. Mix well by pipetting after transfers are complete.

e. Label tube carefully with plate ID and other relevant information.

f. Repeat a–e for all plates.

(2) Run-wide pool: pool part of each plate pool into a tube for library preparation

The amount of each plate pool added to the run-wide 'pool of pools' varies depending on the number of plates in the run. Consult the table below to determine the amount of each plate pool to add to the run-wide pool. ONTOLOGY™ will also calculate the volume for you.

a. Gather all plate pools.

b. Using a single-channel pipette, add a portion of each plate pool to a single tube that will become your run-wide pool. Consult the table below and add the volume in (B) that corresponds to the number of plates in your run (A).

(A) # plates in run	(B) Volume per plate pool (µL)	Final volume (µL)
1	200	200
2	100	200
3	66.7	200
4	50	200
5	40	200
6	33.3	200
7	28.6	200
8	25	200
9	22.2	200
10	20	200

c. Carry this run-wide pool forward into library preparation.

PROTOCOL

Library Preparation

v1.03

This protocol is for the Flongle by Oxford Nanopore Technologies. For other sequencers and flow cells, contact the ONTOLOGY™ development team (dev@ontology.bio) for advice.

- *Bullet points show tips for executing the protocol.*

(1) Pool and purify PCRs

- *It is critical to make fresh 70% EtOH every time because evaporation will decrease the concentration of EtOH in a stock solution.*
- *While not necessary, we recommend performing all mag-bead purifications in Eppendorf LoBind 1.5 mL microfuge tubes, as purification will be slightly more efficient compared to regular 1.5 mL microfuge tubes.*

a. Using a multichannel pipette, pool each PCR plate into a single 1.5 mL microcentrifuge tube. Mix well by pipetting or vortexing and label all tubes carefully with both plate number and ONTOLOGY™ batch.

b. Combine equal volumes of each plate pool such that the final volume will be at least 200 μL (e.g., for 10 plates take $\geq$20 μL from each). Mix well and take 200 μL of the single pool into a new 1.5 mL microcentrifuge tube (exact amount is not critical; 200 μL provides an excess of final DNA in most cases). Store excess pools at -20°C in case repeat runs are needed.

c. Perform magnetic bead cleanup:

i. Add 0.8$\times$ volume of AMPure XP beads (e.g., for 200 μL pooled PCR product, add 160 μL beads). Mix well by pipetting or vortexing.

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

iii. During this incubation, prepare 70% EtOH by mixing 1.4 mL 100% EtOH + 0.6 mL molecular-grade water for 2 mL total, sufficient for this wash and subsequent washes.

iv. Place the tube on a magnetic rack and wait 1–2 minutes until supernatant is clear, then remove and discard the supernatant.

v. Leaving tube on magnetic rack, add 500 μL of freshly prepared 70% EtOH.

vi. After a few seconds, remove and discard EtOH.

vii. Repeat v and vi once, for a total of two EtOH washes.

viii. After the second EtOH removal, centrifuge tube for a few seconds to collect all remaining EtOH and beads at the bottom of the tube. Return the tube to the magnetic rack and, after

the beads pellet (∼30 s), use a P20 pipette to remove residual EtOH (if present).

ix. Leave the tube open, on the magnetic rack, at room temperature for 1–2 minutes to allow residual EtOH to dry.
   (i) The pellet will initially appear shiny/glossy when wet.
   (ii) The beads are sufficiently dry when the pellet takes on a matte (non-glossy) appearance but still has a smooth surface.
   (iii) If the pellet starts to show cracking, it is over-dried. Avoid over-drying, but proceed immediately if it happens.

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

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

xii. Return tube to magnetic rack. Wait 2 minutes until supernatant is clear, then transfer 38 μL supernatant to a fresh 1.5 mL microcentrifuge tube. Discard the tube with the pellet.

(2) Quantification and End Prep

a. Quantify 1 μL of the eluted DNA using Qubit HS kit following manufacturer's instructions.

b. Set up End Prep reaction:

i. From the Qubit result in **2a**, calculate the volume of DNA needed for ∼100 ng (this is a guide, not a critical amount). Transfer this volume to a fresh 0.2 mL PCR tube. Unused purified pool may be stored at −20°C for future use.

ii. Add molecular grade water to make up a total volume of DNA + water to 12.5 μL.

iii. Combine the following components in the 0.2 mL tube:

	Component	Volume (µL)
1	DNA amplicons (∼100 ng)	12.5
2	NEBNext® FFPE DNA Repair Buffer	1.75
3	NEBNext Ultra II End Prep Enzyme	0.75
	TOTAL VOLUME	**15**

iv. Mix tube contents by gently pipetting up and down 2–3 times.

v. Carry out the following program in a thermocycler:
20°C for 5 minutes, 65°C for 5 minutes, hold at 4°C.

c. Purify the End-Prep reaction:

i. Transfer all 15 μL End Prep reaction to a new 1.5 mL microcentrifuge tube and mix with 15 μL AMPure XP beads. Mix well by pipetting or vortexing.

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

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

iv. Leaving tube on magnetic rack, wash with 200 μL of prepared-today 70% EtOH.

v. Remove and discard EtOH.

vi. Repeat steps iv and v once, for a total of two washes.

vii. After the second EtOH removal, place tube in centrifuge and spin for a few seconds to collect all remaining EtOH at the bottom of the tube. Return tube to magnetic rack and use a P20 pipette to remove any residual EtOH.

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

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

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

xi. Return tube to magnetic rack. Wait 2 minutes until supernatant is clear, then transfer 15 μL supernatant to fresh 1.5 mL microcentrifuge tube.

(3) Adapter Ligation and Final Cleanup

a. Combine the following reagents in a 1.5 mL microcentrifuge tube.

	Component	Volume (µL)
1	End-prepped DNA amplicons (~100 ng)	15
2	Ligation Buffer (LNB)*	6.25
3	Salt-T4 DNA Ligase	2.5
4	Ligation Adapter (LA)*	1.25
	TOTAL VOLUME	**25**

**From ONT Ligation Sequencing Kit (SQK-LSK114).*

b. Mix by pipetting and incubate at 25°C for 10 minutes.

c. Clean up final library using AMPure XP beads:

i. Add 12.5 μL (0.5×) volume of AMPure XP beads and mix well by pipetting.

ii. Incubate at room temperature for 10 minutes.

iii. Place tube on magnetic rack. Wait ~2 minutes until supernatant is clear, then remove and discard supernatant.

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

v. Return tube to magnetic rack. Wait ~2 minutes until supernatant is clear, then remove and discard supernatant.

vi. Repeat steps iv and v for a total of two washes in SFB.

vii. 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.

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

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

x. Return tube to magnetic rack. Wait 2 minutes until supernatant is clear, then transfer 10 μL supernatant to fresh 1.5 mL microcentrifuge tube.

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

(4) Flow cell loading

a. Prepare Flongle and carry out flow cell check:

i. 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.

ii. 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.

Note: Pore counts in the range of 20–49 will still often generate high quality results and so you may decide to use a warranty-eligible Flongle rather than wait for a replacement. Use your

discretion.

b. While the flow cell check is running, prepare flow cell priming mix, prime the flow cell, and prepare the sequencing library mix:

i. In a 1.5 mL microcentrifuge tube, mix 117 μL Flow Cell Flush (FCF) with 3 μL Flow Cell Tether (FCT). FCF is from the Flongle Expansion Kit; FCT is from the Ligation Sequencing Kit.

ii. 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.

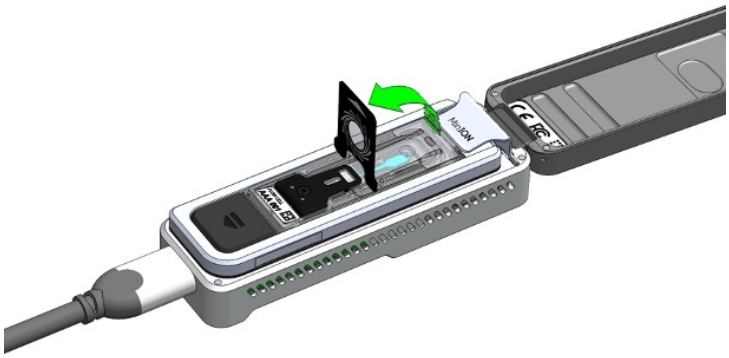


Image credit: Oxford Nanopore Technologies plc.

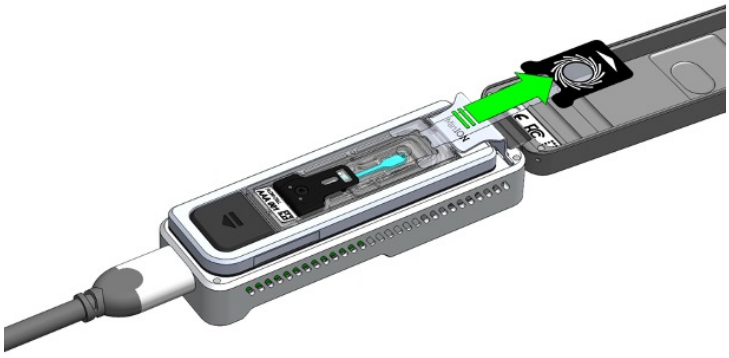


Image credit: Oxford Nanopore Technologies plc.

Image credit: Oxford Nanopore Technologies plc.

iii. Pipette the priming mix (FCF/FCT) into the loading port using a P200 pipette. It is important to dispense slowly and not to introduce air bubbles; draw all 120 μL 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 μL in the tip and discard to ensure no air is introduced.

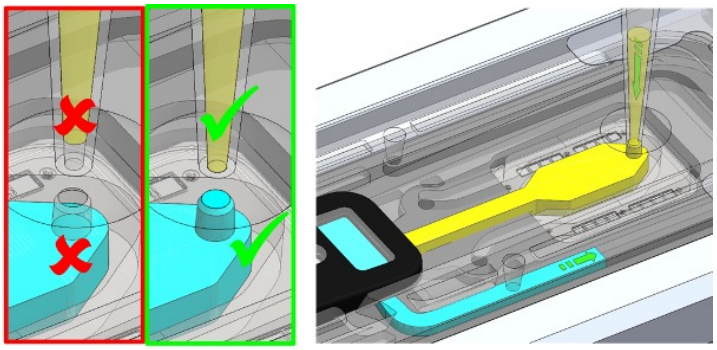


Image credit: Oxford Nanopore Technologies plc.

iv. From the Qubit reading in step **3d**, calculate volume of library needed for 5 ng DNA in a final volume of 5 μL (ONTOLOGY™ contains a calculator for this step). If dilution is necessary, dilute using EB.

v. When ready to load, combine the following components in a 1.5 mL microcentrifuge tube:

	Component	Volume (µL)
1	Sequencing Buffer (SB)	15
2	Library Beads (LIB)*	10
3	DNA library	5
	TOTAL VOLUME	**30**

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

vi. Pipette library mix up and down several times in the tube to resuspend Library Beads, then pipette into the loading port.

Note: As above, dispense slowly to avoid introducing air bubbles and leave the last 1–2 μL in the tip.

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

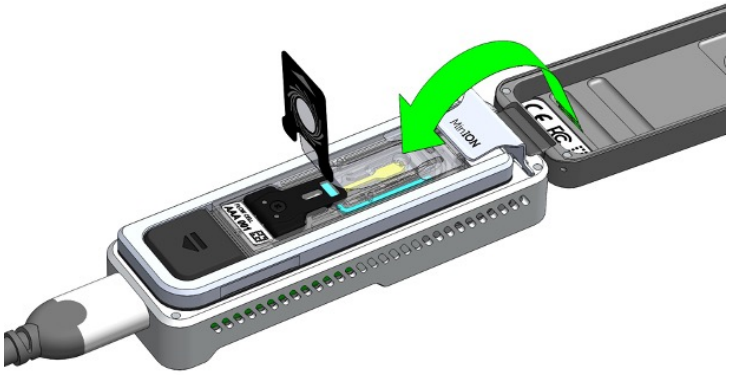


Image credit: Oxford Nanopore Technologies plc.

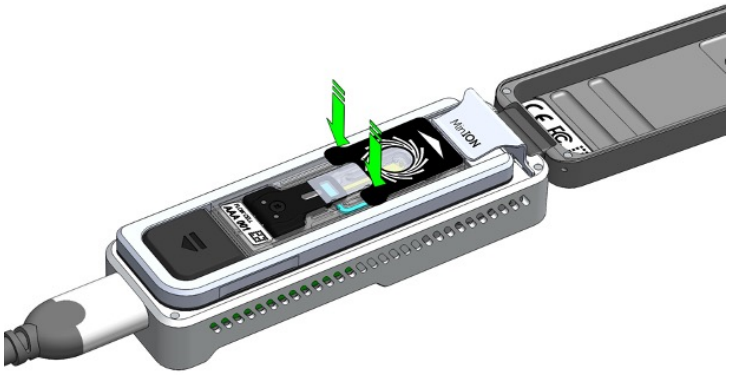


Image credit: Oxford Nanopore Technologies plc.

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

PROTOCOL

MinKNOW Run Setup

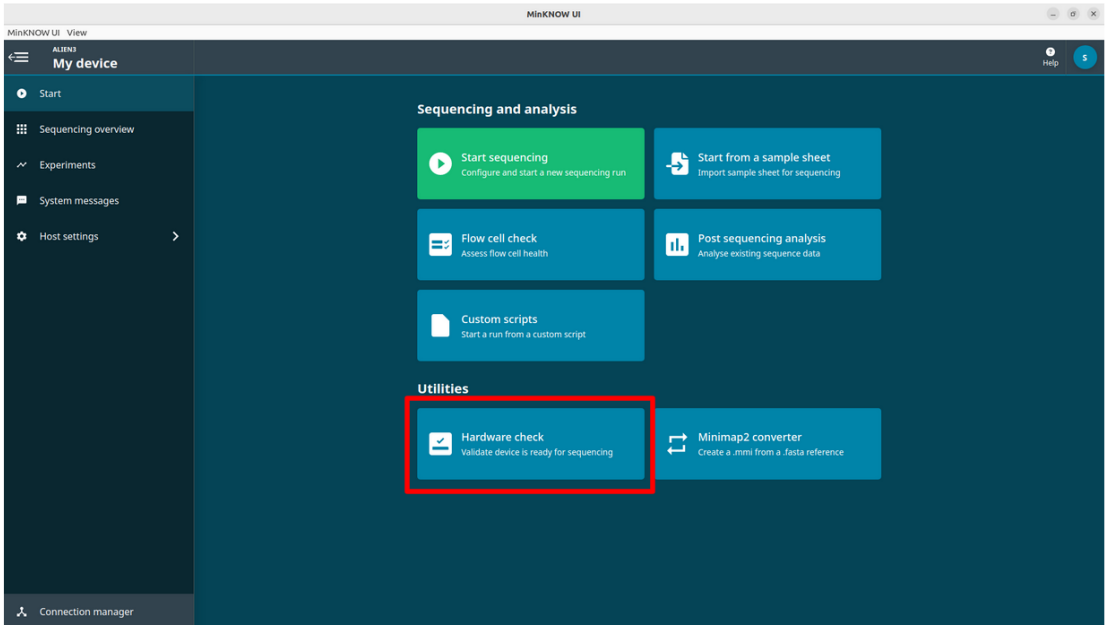
v1.0

This protocol is for the Flongle by Oxford Nanopore Technologies using MinKNOW version 6.8.7 (Nov 2025). For other sequencers, flow cells, or software versions contact the ONTOLOGY™ development team (dev@ontology.bio) for advice.

(1) Start sequencer and MinKNOW

a. Plug in the MinION sequencer and start MinKNOW.

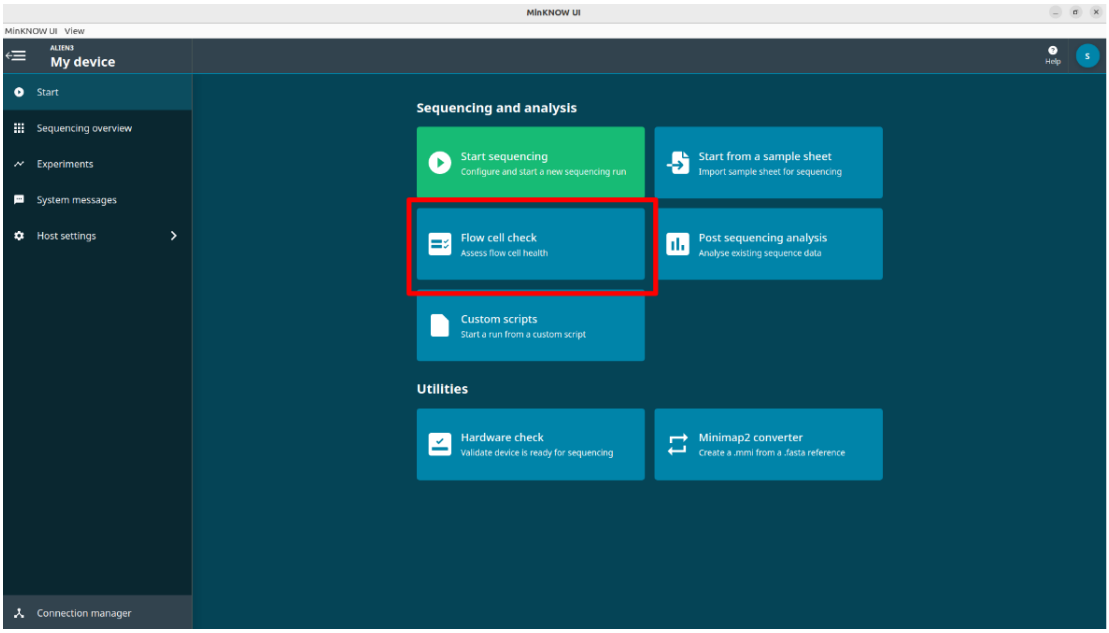
b. If this is the first time using the MinION sequencer, run a hardware check.

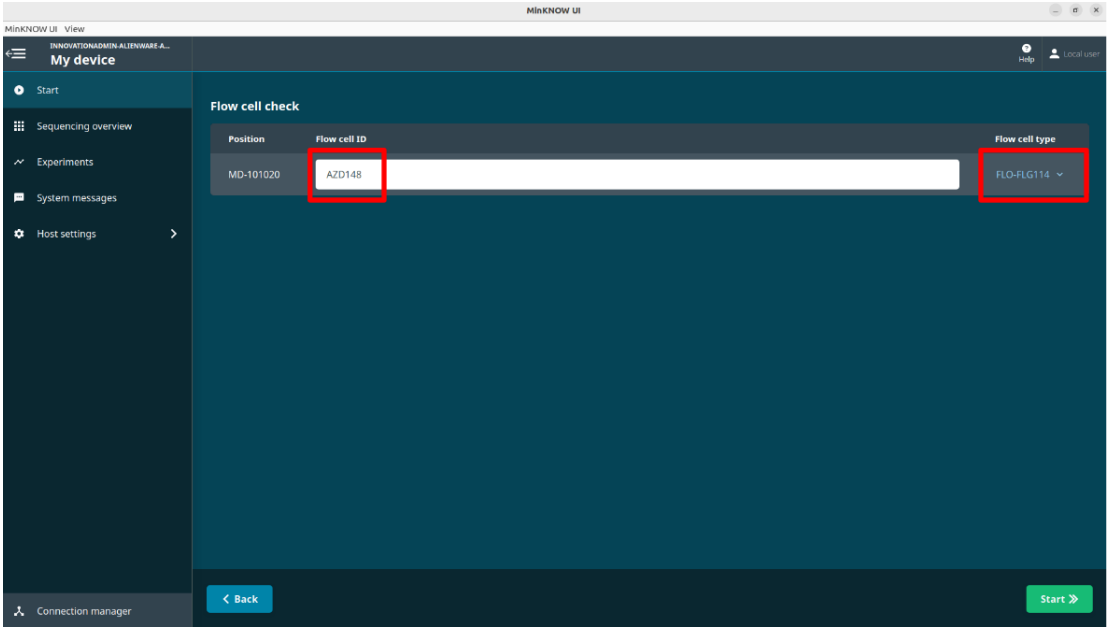


(2) Perform pore scan

a. Insert a new Flongle Flow Cell into the Flongle adapter on the MinION sequencer.

b. Click 'Flow Cell Check'. Enter the Flongle ID found on the Flongle Flow Cell and select "FLO-FLG114" as the flow cell type.

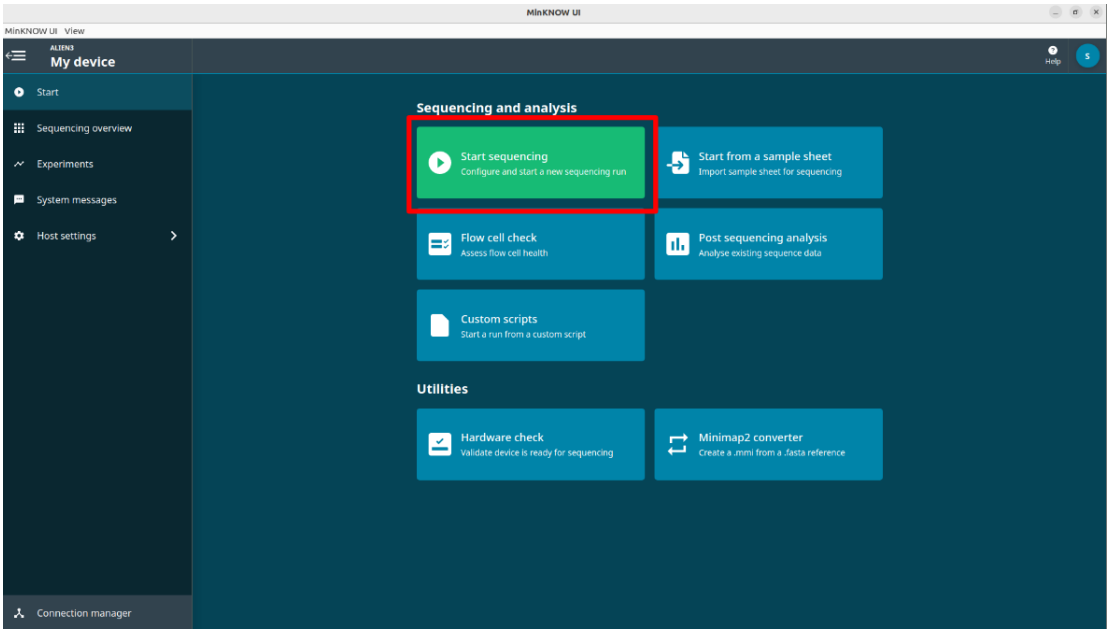




c. Click 'Start' and wait for the flow cell check to complete (~5 min). If the number of pores is below 50 and the flow cell was received less than 30 days prior, take a screenshot; your Flongle is eligible for warranty if you reach out to ONT.

(3) Set up and start sequencing run

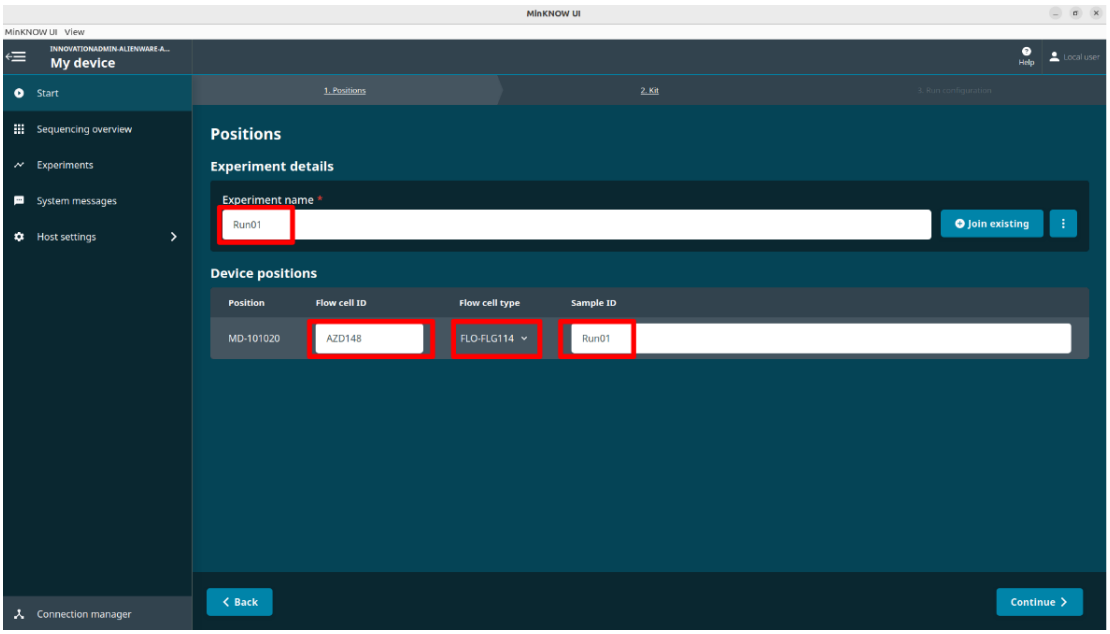
a. Click ‘Start Sequencing’.



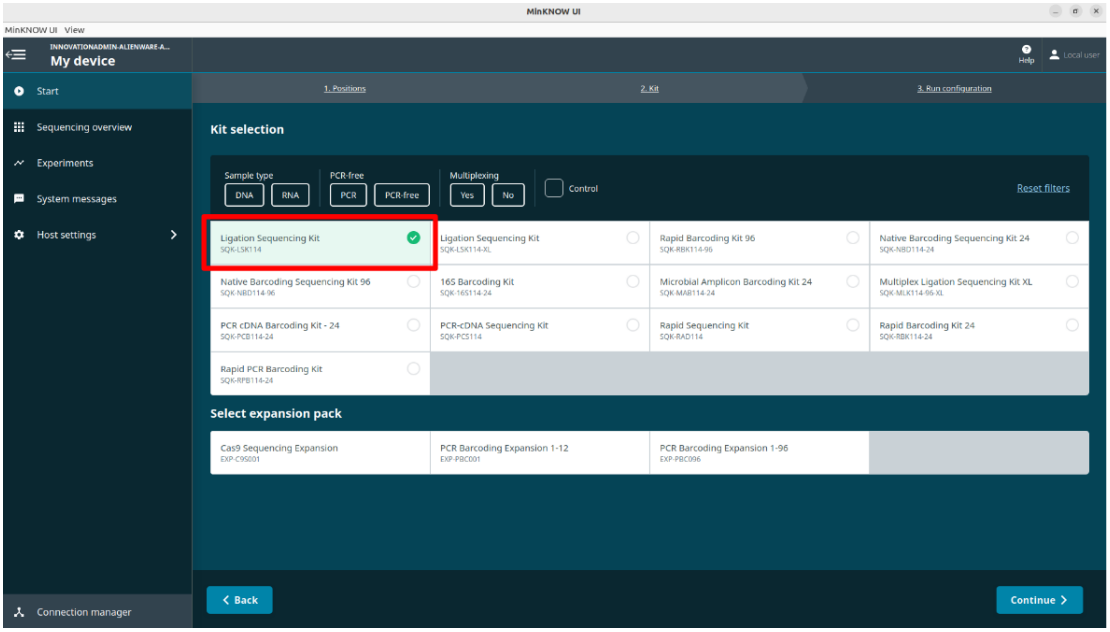
b. Use your ONTOLOGY™ run name as the Experiment Name and Sample ID.

c. Enter the flow cell ID and select “FLO-FLG114” as the flow cell type.

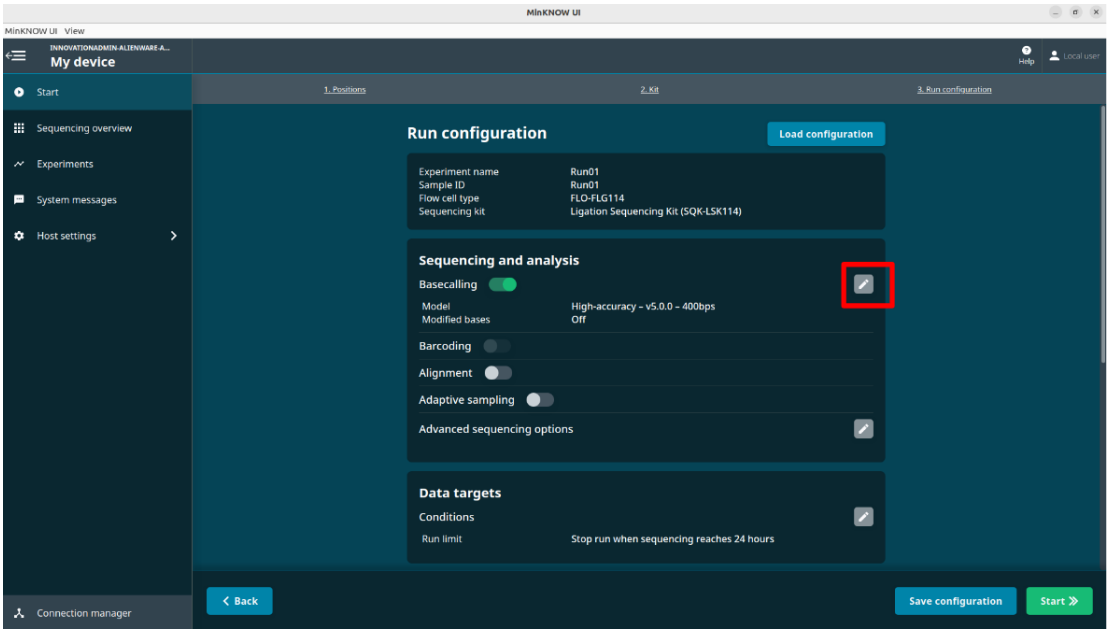
d. Click ‘Continue’.



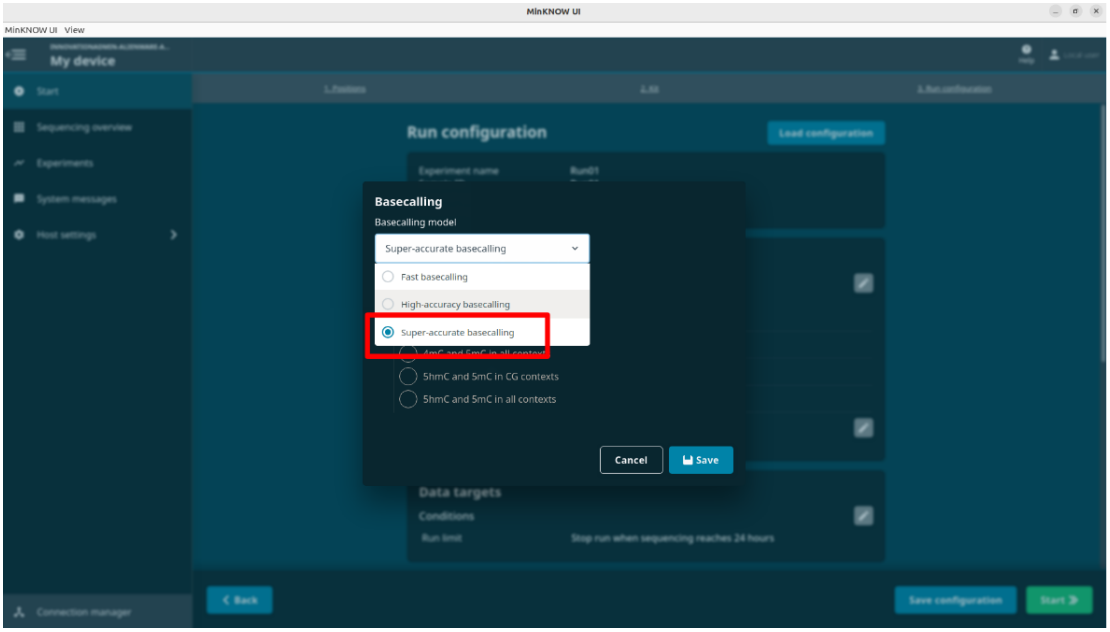
e. Select your library preparation kit (typically “SQK-LSK114”) and click ‘Continue’.



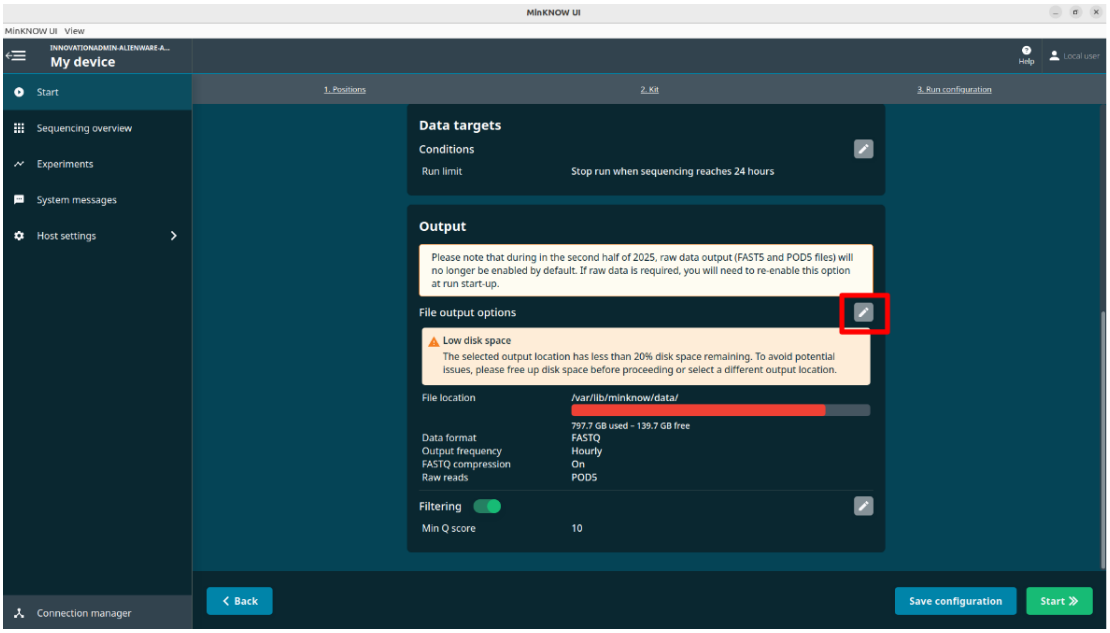
f. Click the 'edit' button under "Sequencing and analysis".



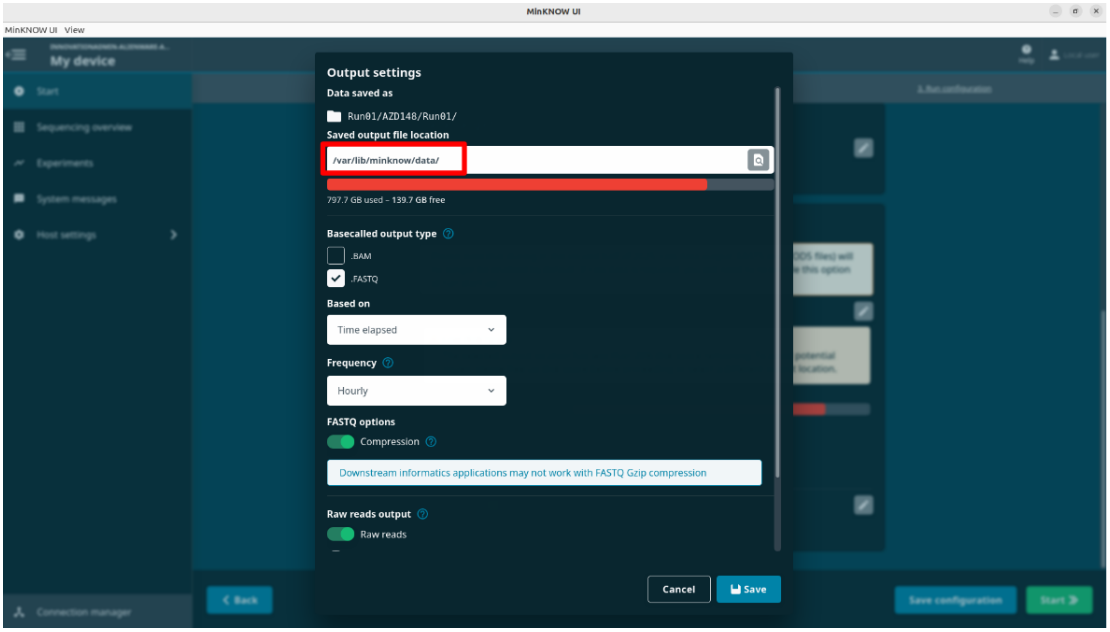
g. Set the basecalling model to "Super-accurate basecalling".



h. Select the 'edit' button under "File output options".



i. Note the location of the output files, as you will need to retrieve them after sequencing is complete.



j. Click ‘Start’ to start sequencing.