



# 🔒 Semi-automated nCoV-2019 LoCost protocol using the OT-2 (Opentrons) and its python API

 nCoV-2019 sequencing protocol v3 (LoCost)

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Automation of standard lab workflows allows for greater ease in upscaling processes while reducing the chances of errors and contamination. Using a liquid handling robot coupled with a python api, it has been possible to adapt a previously established protocol for Covid-19 sequencing (Quick, 2019 Amplicon sequencing protocol for SARS-CoV-2 v3 LoCost) in order to automate the majority of steps post-amplicon sequencing. This is performed using an Opentrons OT-2 robot and its API written using python to design scripts for each step. The automated process involves four stages of dilution, end preparation, barcode ligation, and library pooling. Following on from pooling it is easy perform the remaining steps of the protocol manually. Samples can be prepared in batches of 48 or 96 (including positive and negative controls) and there are variations of the script to facilitate different initial conditions and total samples. Additionally, the OT-2 has been designed with an open-source approach meaning that a variety of different types of labware and reagent reservoirs can be utilised. In this protocol specific pre-designated types of labware already recognised by the API have been used as these allow for a wide variety of labware to be used depending on what is available in the lab. This is possible as calibration is performed to the base of each type of labware being used. The labware defined in the protocol is similar to a deep well plate that requires a high clearance which allows for a range of different 96 well plates to be used without a risk of collision. The adaptability of this protocol provides a possible easy upscaling method for a wide variety of labs performing genome sequencing not just with Covid-19 but also for other high throughput processes.

1\_dilution\_run\_per\_plate.p  
y  
1\_dilution\_pool\_per\_plate.py  
2\_endrepair.py  
3\_barcoding.py  
4\_pooling\_48\_samples.py  
4\_pooling\_96\_samples.py

Jeremy D Mirza . Semi-automated nCoV-2019 LoCost protocol using the OT-2 (Opentrons) and its python API . [protocols.io](#)  
<https://protocols.io/view/semi-automated-ncov-2019-locost-protocol-using-the-bx6tpren>

 nCoV-2019 sequencing protocol v3 (LoCost), Josh Quick

Covid-19, Automation, Whole genome sequencing, Opentrons, OT-2

Sep 13, 2021

Jan 11, 2022

53171

Set the robot to calibrate from the base of the labware to ensure more accurate liquid handling

Perform the first stages of the protocol prior to PCR in an amplicon free environment to avoid contamination

Clean the OT-2 robot before and after each run using 10% bleach, distilled water and 70-80% ethanol in that order

A	B	C
Component	Supplier	Part number
ARTIC nCoV-2019 Amplicon Panel v4	IDT	See links below
LunaScript RT SuperMix Kit	NEB	E3010
Q5 Hot Start HF Polymerase or	NEB	M0493
Q5 Hot Start High-Fidelity 2X Master Mix	NEB	M0494
dNTP Solution Mix (10 mM ea.)	NEB	N0447
Nuclease-free water (100 mL)	NEB	B1500
NEBNext Ultra II End Repair/dA-tailing module	NEB	E7546
Blunt/TA Ligase Master Mix	NEB	M0367
Native Barcoding Expansion Kit 96	ONT	EXP-NBD196
AMPure XP beads	Beckman	A63881
NEBNext Quick Ligation Module	NEB	E6056S
Sequencing Auxiliary Vials	ONT	EXP-AUX001
Short Fragment Buffer Expansion Kit	ONT	EXP-SFB001
Qubit dsDNA HS Assay Kit	Thermo	Q32854
Flow Cell Priming Kit	ONT	EXP-FLP002
R9.4.1 flow cells	ONT	FLO-MIN106
Opentrons OT-2 liquid handling robot	Opentrons	999-00047
P300 8-channel pipette (GEN 2)	Opentrons	999-00006
P20 8-channel pipette (GEN 2)	Opentrons	999-00005

IDT premixed ARTIC nCoV-2019 [V4](#) panel along with the oligos required for the [4.1 spike-in](#) for omicron. Alternatively it is possible to order the oligos [individually](#).

Prepare either 48 or 96 RNA samples (including 1 positive and 1 negative control per 48 samples)

Download the required scripts attached here or on other parts of the protocol:

[!\[\]\(cead67df4d82d6c83effe4f8699a7d8f\_img.jpg\) 1\\_dilution\\_run\\_per\\_plate.py](#)

[!\[\]\(1d3a1175dd4902218e694b9c098adb83\_img.jpg\) 1\\_dilution\\_pool\\_per\\_plate.py](#)

[!\[\]\(c507f772dba2b921f86777f01218e570\_img.jpg\) 2\\_endrepair.py](#)

[!\[\]\(4729e517bc6a7cd81c8025b9646574fb\_img.jpg\) 3\\_barcoding.py](#)

[!\[\]\(cbe80b694ebd74fcfe136a095b608235\_img.jpg\) 4\\_pooling\\_48\\_samples.py](#)

[!\[\]\(a03a7eb2f4046e1d3c76772003e549ea\_img.jpg\) 4\\_pooling\\_96\\_samples.py](#)

## cDNA preparation

30m

- 1 Prepare 92 RNA samples plus 1 negative control of nuclease-free water per library. Ideally also use a positive control of a known sequence during cDNA preparation. Whether you will load 96 total samples on 1 flow cell or split this into 2 separate runs, it is better to have a positive and negative control for every 48. If previously frozen, mix by briefly vortexing and pulse spin to collect liquid. Keep samples on ice at all times.

Ideally, it is recommended to run 48 total samples per flow cell including one negative and one positive control. However it is possible with the native barcoding kit to run up to 96 samples on a single flow cell.

cDNA and PCR preparation can also be automated however this will require a separate liquid handling robot maintained in an amplicon free environment to avoid the chance of contamination.

For this protocol it is important to use the same type of 96-well plate for all automated processes as the OT-2 robot will calibrate from one of them and use these settings for every plate.

- 2 Mix the following components in a PCR strip-tubes/plate. Gently mix by pipetting and pulse spin the tube to collect liquid.

A	B
Component	Volume
LunaScript RT SuperMix (5X)	2 µL
Template RNA	8 µL
<b>Total</b>	<b>10 µL</b>

To prevent pre-PCR contamination the mastermix should be added to the PCR strip-tubes/plate in the **mastermix** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

RNA samples should be added in the **extraction/sample addition** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

### 3 Incubate the reaction as follows:

↓ 25 °C for ↗ 00:02:00

↓ 55 °C for ↗ 00:10:00

↓ 95 °C for ↗ 00:01:00

Hold at ↓ 4 °C

Multiplex PCR

4h

### 4 Set up the two PCR reactions per sample as follows in plates with one run per plate (i.e. 48 samples Reaction 1 and Reaction 2 on the same plate). Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

A	B	C
Component	Reaction 1	Reaction 2
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
V4 Pool 1 (10µM)	4 µL	0 µL
V4 Pool 2 (10µM)	0 µL	4 µL
Nuclease-free water	12.75 µL	12.75 µL
<b>Total</b>	<b>22.5 µL</b>	<b>22.5 µL</b>

For M0493

or

A	B	C
Component	Reaction 1	Reaction 2
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µL	12.5 µL
V4 Pool 1 (10µM)	4 µL	0 µL
V4 Pool 2 (10µM)	0 µL	4 µL
Nuclease-free water	6 µL	6 µL
<b>Total</b>	<b>22.5 µL</b>	<b>22.5 µL</b>

For M0494

Q5 Hot Start High-Fidelity 2X Master Mix can also be used instead of the component kit. Half-scale PCR reactions can also be used to save costs as you will only require **2.5 µL** for downstream steps.

To prevent pre-PCR contamination the mastermix for each pool should be made up in the **mastermix** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use and aliquoted into PCR strip-tubes/plate

- Add **2.5 µL** cDNA to each of the PCR reactions, gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

cDNA should be added in the **extraction and sample addition** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

- Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	<b>98 °C</b>	<b>00:00:30</b>	1
Denaturation	<b>98 °C</b>	<b>00:00:15</b>	35
Annealing	<b>65 °C</b>	<b>00:05:00</b>	35
Hold	<b>4 °C</b>	Indefinite	1

Thermocycler calibration can vary instrument to instrument. If you see amplicon 64 dropout then decrease the annealing/extension temperature to **63 °C**. Denaturation temperature of **95 °C** can also be used and may slightly increase PCR yields.

### Python Script Downloads

- 7 Download the following 4 python scripts for the subsequent stages of the protocol:

1. PCR Dilution [1\\_dilution\\_run\\_per\\_plate.py](#) [1\\_dilution\\_pool\\_per\\_plate.py](#)
2. End Preparation [2\\_endrepair.py](#)
3. Native Barcoding [3\\_barcoding.py](#)
4. Library Pooling [4\\_pooling\\_48\\_samples.py](#) [4\\_pooling\\_96\\_samples.py](#)

Alternate versions from part 1 and part 4 are available above. If PCR plates are organised by Primer Pool rather than run use the alternate script for this step. Additionally choose the appropriate version of part 4 for pooling if running 48 or 96 samples per flow cell.

Prior to using the OT-2 robot for each library preparation, it is recommended to clean the OT-2 robot before and after each run using 10% bleach, distilled water and 70-80% ethanol in that order. UV

This script is set up to use the **P300 8-channel pipette** on the **left position** and the **P20 8-channel** on the **right position**. Opentrons tips are recommended however it is possible to use other brands provided that they are accurately calibrated before the run.

### OT-2 Part 1: PCR dilution

20m

- 8 Load protocol named “**1\_dilution\_run\_per\_plate**” and set up the robot with the following layout: [!\[\]\(c33cb967c8fc4f5e27188a389b621c8e\_img.jpg\)](#)

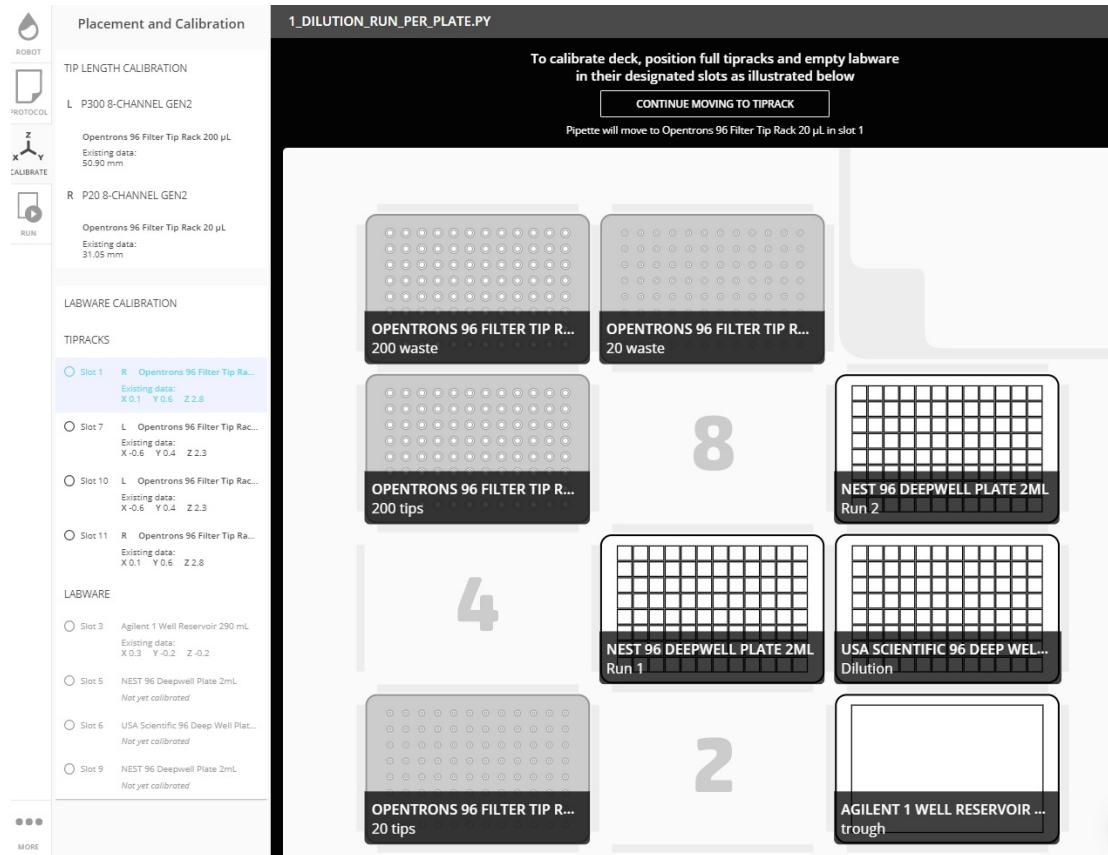


Figure demonstrating the layout of the OT-2 for the dilution step of the protocol. Tips required are labelled 20 tips and 200 tips respectively. 20 waste and 200 waste are empty tip racks where used tips are discarded to reduce the risk of dispersing amplicon. For the Dilution, Run 1 and Run 2 plates any 96 well plate can be used provided it fits within the grid position (127x86 mm). Run 1 and Run 2 must be the same type of plate to allow for the robot to calibrate properly whilst the dilution plate can be different or the same. Finally for the reagent trough any type can be used provided it can be used with an 8 channel pipette. This is placed at position 3 on the robot so that it doesn't need to fit within the dimensions of the position if this is required.

- 9 Add sufficient nuclease free water to the reagent trough for the required number of samples (e.g., 4800 uL per 96 samples)
- 10 Calibrate and run the protocol "1\_dilution\_run\_per\_plate" [1\\_dilution\\_run\\_per\\_plate.py](#)
- 11 Seal the plates labelled Run 1 and Run 2 and store at **-20 °C**, and seal the Dilution plate until it is required for the next stage of the protocol.

## OT-2 Part 2: End Preparation

45m

- 12 Barcode the amplicon pools with the one-pot native barcoding approach using the OT-2 robot to perform the steps of end preparation, barcoding and amplicon pooling. After pooling libraries, SPRI bead clean up can be performed manually as per the one-pot protocol shown on step 24.

- 13 Load protocol named “2\_endrepair” and set up the robot with the following layout:

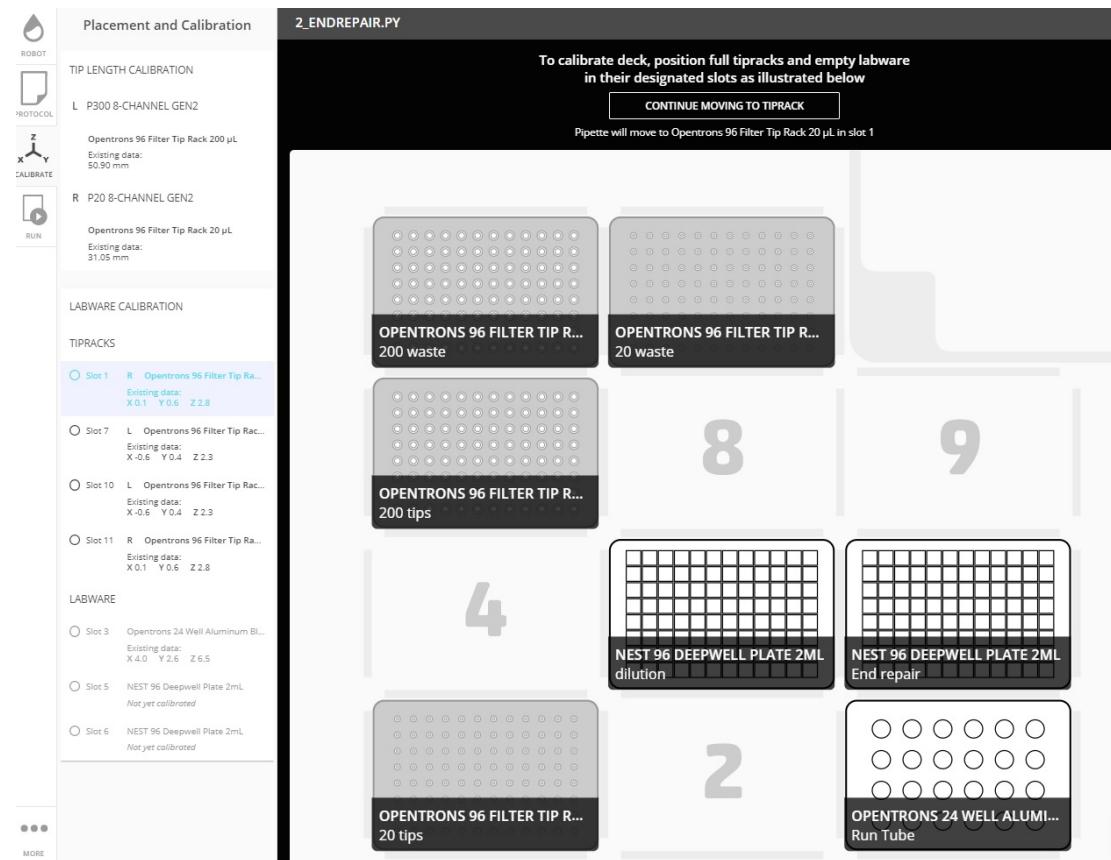


Figure demonstrating the layout of the OT-2 for the end preparation step of the protocol. Tips required are labelled 20 tips and 200 tips respectively. 20 waste and 200 waste are empty tip racks where used tips are discarded to reduce the risk of dispersing amplicon. For the Dilution, and End Repair plates any 96 well plate can be used provided it fits within the grid position (127x86 mm), and that they are the same type of plate. For the run tube as only one eppendorf tube is required it is possible to use any tube holder that can fit into this position as this is calibrated during the run.

- 14 Prepare the following mastermix for each set of 96 samples in an Eppendorf tube and place in position A1 of the labware being utilised:

A	B
Nuclease free water	588 µL
NEBNext Ultra II End Prep Reaction Buffer	141 µL
NEBNext Ultra II End Prep Enzyme Mix	59 µL
<b>Total</b>	<b>788 µL</b>

The volumes used for this master mix and the subsequent one for the barcoding step have been optimised with the robot in mind which is why they are slightly higher than if

these steps were performed with manual pipetting.

15 Calibrate and run the protocol "**2\_endrepair**" on the OT-2 and once the run is complete, seal the End repair plate

16 Incubate the End repair plate at **Room temperature** for 15 minutes

Incubate at **65 °C** for 15 minutes

Incubate on ice for at least 1 minute

This process can be performed on a bench top with a heat block or by using a thermocycler. Plate can be left on ice or even frozen if a pause is required at this stage.

#### OT-2 Part 3: Native Barcoding

45m

17 Take the Native Barcoding Expansion Kit from ONT, and transfer the contents of each tube to a 96-well plate (ideally the same type of plate as used for the different stages of library preparation), seal and then freeze at **-20 °C**.

This only needs to be done once per kit and will allow for much easier reuse for these reagents as there is a higher chance of contamination when reopening the tubes they are in for this kit. To ensure it is defrosted when it is ready to use this should be removed from the freezer when starting **Step 8** (PCR dilution) of the protocol.

18 Load protocol named "**3\_barcoding**" and set up the robot with the following layout:



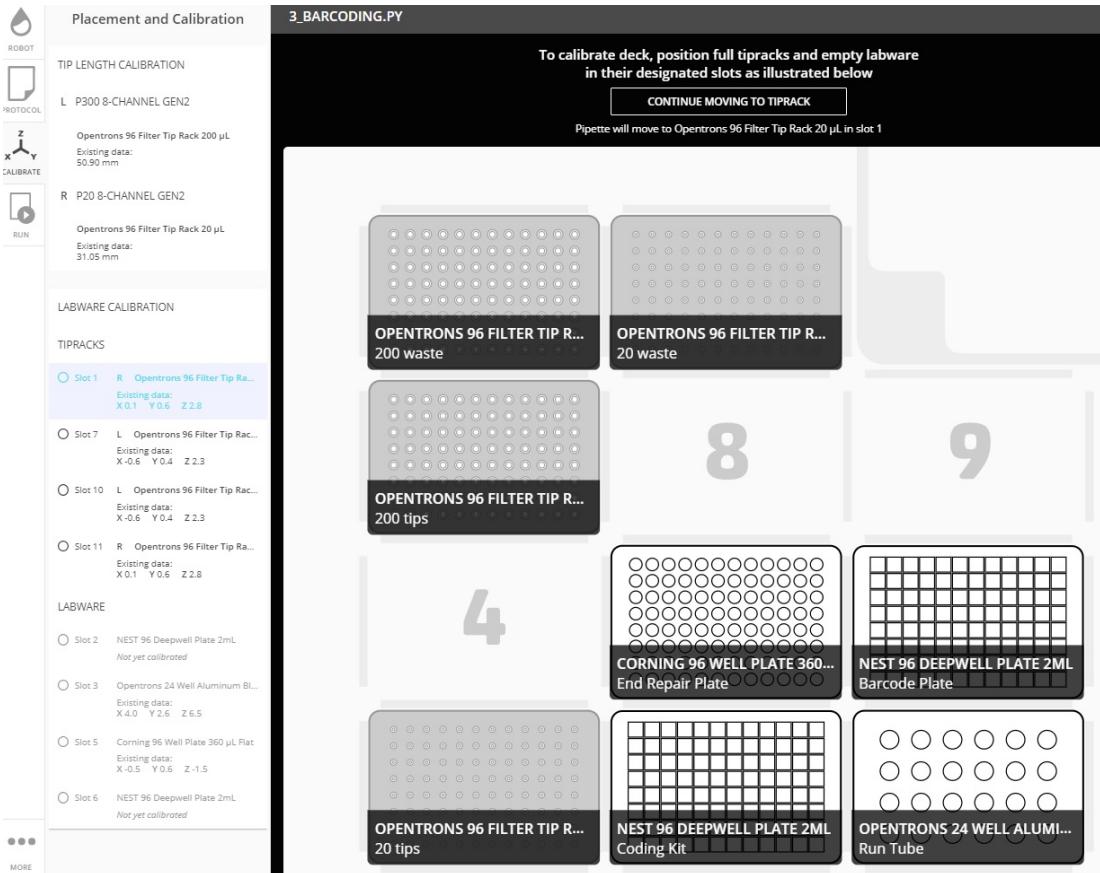


Figure demonstrating the layout of the OT-2 for the barcoding step of the protocol. Tips required are labelled 20 tips and 200 tips respectively. 20 waste and 200 waste are empty tip racks where used tips are discarded to reduce the risk of dispersing amplicon. The End Repair Plate refers to the plate prepared in the previous section of the protocol. The Coding kit refers to the aliquoted Native Barcoding Expansion Kit that was aliquoted in step 17. For the Coding Kit and Barcode plate any 96 well plate can be used provided it fits within the grid position (127x86 mm), and that they are the same type of plate. For the run tube as only one eppendorf tube is required it is possible to use any tube holder that can fit into this position as this is calibrated during the run.

The End Repair Plate is calibrated separately from the other plates used in this protocol as it requires a volume of **1  $\mu$ L** which is the lower limit of the pipette and thus requires more accuracy when calibrating.

- 19** Prepare the following mastermix for each set of 96 samples in an Eppendorf tube and place in position A1 of the labware being utilised:

A	B
Nuclease free water	323 $\mu$ L
Blunt TA / Ligase Master Mix	539 $\mu$ L
<b>Total Volume</b>	<b>862 <math>\mu</math>L</b>

20 Calibrate and run the protocol "**3\_barcoding**" refilling the tip boxes when prompted by the OT-2.

21 Seal the barcode plate and incubate at **Room temperature** for 20 minutes

Then, incubate this plate at **65 °C** for 10 minutes

Hold at **4 °C** until ready for the next stage of the process

This process can be performed on a bench top with a heat block or by using a thermocycler

OT-2 Part 4: Library pooling

5m

22 Load protocol named "**4\_pooling\_48\_samples**" or "**4\_pooling\_96\_samples**" and set up the robot with the following layout: 

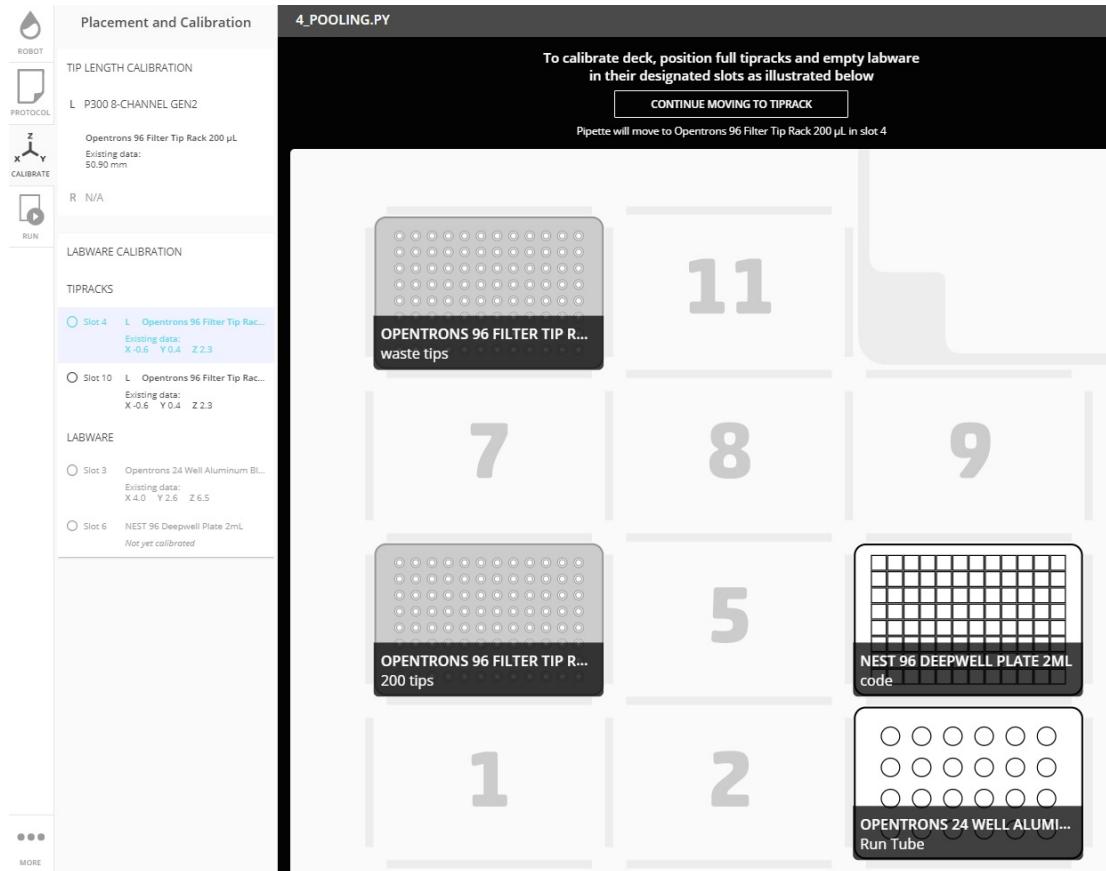


Figure demonstrating the layout of the OT-2 for the pooling step of the protocol whether processing 48 or 96 samples for a run. Tips required are labelled 200 tips and 200 waste is an empty tip racks where used tips are discarded. The code plate is the barcode plate from the previous step once ligation has been completed, and any 96 well plate can be used provided it fits within the grid position (127x86 mm). For the run tube as only one eppendorf tube is required it is possible to use any tube holder that can fit into this position as this is calibrated during the run.

- 23 Calibrate and run the protocol “[4\\_pooling\\_48\\_samples](#)” or “[4\\_pooling\\_96\\_samples](#)” depending on how many samples will be loaded onto each flow cell

When running the “[4\\_pooling\\_48\\_samples](#)” script for the second set of samples, simply rotate the “code” plate 180 degrees and add a new empty Eppendorf tube at position A1 on the “Run Tube” labware

- 24 Carry out the SPRI bead clean-ups and washes with SFB and ethanol as per the original one pot protocol from step 24.6 onwards



One-pot native barcoding of amplicons v3 (LoCost)  
by **Josh Quick**

[PREVIEW](#)

[RUN](#)



- 24.1 In a new PCR strip-tube/plate set up the following reaction for each sample:

Component	Volume
PCR dilution from previous step	3.3 µL
Ultra II End Prep Reaction Buffer	1.2 µL
Ultra II End Prep Enzyme Mix	0.5 µL
Nuclease-free water	5 µL
<b>Total</b>	<b>10 µL</b>

Make a master mix of end-preparation reagents and nuclease-free water and aliquot into strip-tube/plate to improve reproducability.

#### 24.2 Incubate at room temperature for 00:15:00

Incubate at 65 °C for 00:15:00

Incubate on ice for 00:01:00

#### 24.3 In a new PCR strip-tube/plate set up the following reaction for each sample:

Component	Volume
End-preparation reaction mixture	0.75 µL
NBXX barcode	1.25 µL
Blunt/TA Ligase Master Mix	5 µL
Nuclease-free water	3 µL
<b>Total</b>	<b>10 µL</b>

Use one native barcode from the EXP-NBD104 (1-12), EXP-NBD114 (13-24) or EXP-NBD196 per sample. Use 12 or more barcodes per library or there will be insufficient total material to achieve good yields.

#### 24.4 Incubate at room temperature for 00:20:00

Incubate at 65 °C for 00:10:00

Incubate on ice for 00:01:00

The 65°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

**24.5** In a new **1.5 mL** Eppendorf tube pool all one-pot barcoding reactions together.

If processing 12-24 samples pool all **10 µl** from each native barcoding reaction.

if processing 48 samples pool **5 µl** from each native barcoding reaction.

If processing 96 samples pool **2.5 µl** from each native barcoding reaction so as not to exceed a pool volume of **240 µl** which would make the clean-up volume too large.

**24.6** Add 0.4x volume of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **96 µl** SPRI beads to **240 µl** pooled one-pot barcoding reactions.

0.4x volume of SPRI is sufficient to bind 400 bp amplicons in the presence of ligation buffer, do not use 1x as this will result in an excessive large bead pellet.

**24.7** Mix by vortexing and pulse centrifuge to collect all liquid at the bottom of the tube. Incubate for **00:05:00** at room temperature.

**24.8** Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

**24.9** Add **250 µl** SFB and resuspend beads completely by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube and place on the magnet. Remove supernatant and discard.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

- 4.10 Repeat steps 11.9 to perform a second SFB wash. Pulse centrifuge and remove any residual SFB.

You do not need to allow to air dry with SFB washes.

- 4.11 Add **200 µl** of room-temperature **[M]70 % volume** ethanol to bathe the pellet. Carefully remove and discard ethanol, being careful not to touch the bead pellet.

Only perform 1x 70% ethanol wash

- 4.12 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

- 4.13 With the tube lid open incubate for **00:01:00** or until the pellet loses its shine (if the pellet dries completely it will crack and become difficult to resuspend).

- 4.14 Resuspend pellet in **30 µl [M]10 Milimolar (mM)** Tris pH 8.0, mix gently by either flicking or pipetting and incubate for **00:02:00**.

- 4.15 Place on magnet and transfer sample to a clean **1.5 mL** Eppendorf tube ensuring no beads are transferred into this tube.

Adapter Ligation

1h

- 25 Quantify **1 µl** of the barcoded amplicons using the Quantus Fluorometer using the ONE dsDNA assay. Concentration will vary depending on number and Ct of samples and but you need about **30 ng** total at this stage to achieve maximum run yield.



DNA quantification using the Quantus fluorometer  
by Josh Quick

PREVIEW

RUN

25.1 Remove Lambda DNA 400 ng/ $\mu$ L standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

 QuantiFluor(R) ONE dsDNA System,

500rxn Promega Catalog #E4870

25.2 Set up two **0.5 mL** tubes for the calibration and label them 'Blank' and 'Standard'

25.3 Add **200  $\mu$ L** ONE dsDNA Dye solution to each tube.

25.4 Mix the Lambda DNA standard 400 ng/ $\mu$ L standard by pipetting then add **1  $\mu$ L** to one of the standard tube.

25.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

25.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

25.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

25.8 Set up the required number of **0.5 mL** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

25.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

5.10 Add **199 µl** ONE dsDNA dye solution to each tube.

5.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

5.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

5.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

5.14 On the Home screen of the Quantus Fluorometer, select 'Protocol', then select 'ONE DNA' as the assay type.

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

5.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

5.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

5.17 Repeat step 16 until all samples have been read.

5.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

26 Set up the following AMII adapter ligation and clean-up with SFB.

Adapter ligation with AMII v2  
by Josh Quick

PREVIEW    RUN

26.1 In a new **1.5 µl** Eppendorf tube set up the following AMII adapter ligation reaction.

Component	Volume
Barcoded amplicon pool	30 µL
NEBNext Quick Ligation Reaction Buffer (5X)	10 µL
Adapter Mix (AMII)	5 µL
Quick T4 DNA Ligase	5 µL
<b>Total</b>	<b>50 µL</b>

26.2 Incubate at room temperature for **00:20:00**

26.3 Add **50 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. Pulse centrifuge to collect all liquid at the bottom of the tube.

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

There will be some variation in clean-up efficiencies but expect to carry around 50% through this clean-up

26.4 Incubate for **00:05:00** at room temperature.

26.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

26.6 Add **250 µl** SFB and resuspend beads completely by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube. Remove supernatant and discard.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

26.7 Repeat steps 13.6 to perform a second SFB wash.

26.8 Pulse centrifuge and remove any residual SFB. Add **15 µl** EB (ONT) and resuspend beads by pipette mixing.

You do not need to allow to air dry with SFB washes.

26.9 Incubate at room temperature for **00:02:00**.

6.10 Place on magnetic rack until clear. Transfer final library to a new 1.5mL Eppendorf tube.

27 Quantify **1 µl** of the final library using the Quantus Fluorometer using the ONE dsDNA assay. Concentration will vary depending on number and Ct of samples but **15 ng** final library is usually required to achieve maximum run yield.



DNA quantification using the Quantus fluorometer  
by Josh Quick

PREVIEW

RUN



Final library can be now be stored in **10 Milimolar (mM)** Tris **pH 8** at **4 °C** for up to a week if needed otherwise proceed directly to MinION sequencing.

- 27.1 Remove Lambda DNA 400 ng/ $\mu$ L standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

 **QuantiFluor(R) ONE dsDNA System,**  
**500rxn Promega Catalog #E4870**

- 27.2 Set up two **0.5 mL** tubes for the calibration and label them 'Blank' and 'Standard'

- 27.3 Add **200  $\mu$ L** ONE dsDNA Dye solution to each tube.

- 27.4 Mix the Lambda DNA standard 400 ng/ $\mu$ L standard by pipetting then add **1  $\mu$ L** to one of the standard tube.

- 27.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

- 27.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

- 27.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

- 27.8 Set up the required number of **0.5 mL** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

27.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

7.10 Add **199 µl** ONE dsDNA dye solution to each tube.

7.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

7.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

7.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

7.14 On the Home screen of the Quantus Fluorometer, select 'Protocol', then select 'ONE DNA' as the assay type.

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

7.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

7.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

Repeat step 16 until all samples have been read.

7.17

- 7.18 The value displayed on the screen is the dsDNA concentration in ng/ $\mu$ L, carefully record all results in a spreadsheet or laboratory notebook.

MinION sequencing

1d

- 28 Prime the flowcell and load **15 ng** sequencing library onto the flowcell.



Priming and loading a MinION flowcell v2  
by Josh Quick

PREVIEW

RUN



From experience we know **15 ng** is optimum loading input for short amplicons. Speed drop during the run indicates excessive library was loaded. Low run yield <20M reads indicates insufficient library.

- 28.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)  
Loading beads (LB)  
Flush buffer (FLB)  
Flush tether (FLT)

- 28.2 Add **30  $\mu$ l** FLT to the FLB tube and mix well by vortexing.

- 28.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

- 28.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

- 28.5 Take a P1000 pipette and tip and set the volume to **800  $\mu$ l**. Place the tip in the inlet port

and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

28.6 Load **800 µL** of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

28.7 Wait for **00:05:00**.

28.8 Gently lift the SpotON cover to open the SpotON port.

28.9 Load another **200 µL** of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

8.10 In a new tube prepare the library dilution for sequencing:

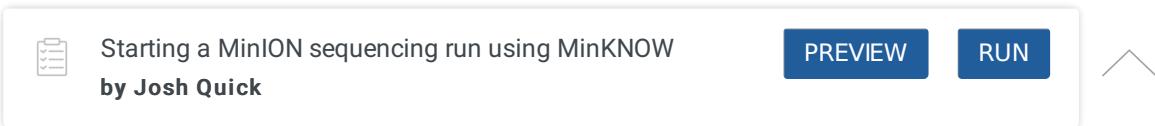
Component	Volume
SQB	37.5 µL
LB	25.5 µL
Library	12 µL
<b>Total</b>	<b>75 µL</b>

Mix LB immediately before use as they settle quickly.

Make up with EB if less than 12 µL library is required.

8.11 Mix the prepared library gently by pipetting up and down just prior to loading.

- 8.12 Add the **75 µl** library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.
- 8.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.
- 29 Start the sequencing run using MinNOW.



- 29.1 If required plug the MinION into the computer and wait for the MinION and flowcell to be detected.
- 29.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.
- 29.3 Then select the flowcell so a tick appears.
- 29.4 Click the 'New Experiment' button in the bottom left of the screen.
- 29.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:
- Experiment:** Name the run in the experiment field, leave the sample field blank.

**Kit: Selection:** Select LSK109 as there is no option for native barcoding (NBD104).

**Run Options:** Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

**Basecalling:** Leave basecalling turned but select 'fast basecalling'.

**Output:** The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

## 29.6 Monitor the progress of the run using the MinKNOW interface.