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# Assessing multiplex tiling PCR sequencing approaches for detecting genomic variants of SARS-CoV-2 in municipal wastewater V.1

 In 1 collection

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**We use this protocol and it's working**

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## Abstract

In this work, we aim to assess the performance of three different multiplex primer schemes, i.e. Swift amplicon SARS-CoV-2 panel (150bp amplicons), ARTIC V3 panel (400bp amplicons), and SARS-CoV-2 midnight panel (1200bp amplicons), for metatranscriptomic sequencing of SARS-CoV-2 for influent wastewater and primary sludge.

This protocol is adapted from the Swift amplicon™ SARS-COV-2 protocol (150bp amplicon), ARTIC V3 protocol (400bp amplicon), and "midnight" protocol (1200bp amplicon).

Sequencing libraries are prepared with 1) Oxford Nanopore Ligation Sequencing Kit (SQK-LSK109) with Native Barcoding kit (EXP-NEB104 and EXP-NEB114), or 2) NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® with NEBNext® Multiplex Oligos for Illumina®.

Links to the protocols are:

Swift amplicon protocol (150bp): <https://swiftbiosci.com/swift-amplicon-sars-cov-2-panel/>

ARTIC V3 protocol (400bp): [https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye?version\\_warning=no](https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye?version_warning=no)

"midnight" protocol V4 (1200bp): [dx.doi.org/10.17504/protocols.io.bh7hj9j6](https://dx.doi.org/10.17504/protocols.io.bh7hj9j6)



## Materials

### Primers

Swift primers were purchased as two pools from IDT

☒ SARS-CoV2-Midnight-1200 500rxn **IDT Catalog #10007184**

☒ ARTIC nCoV-2019 V3 Panel **IDT**

### Reverse Transcription

☒ SuperScript™ IV First-Strand Synthesis System **Invitrogen - Thermo Fisher Catalog #18091200**

### cDNA cleanup

☒ DNA Clean & Concentrator™-5 **Zymo Research Catalog #D4003**

### Multiplex PCR

☒ Q5® Hot Start High-Fidelity 2X Master Mix **New England Biolabs Catalog #M0494L**

☒ Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01**

### Oxford Nanopore Sequencing

☒ NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

☒ Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs Catalog #M0367L**

☒ Native Barcoding Expansion 1-12 (PCR-free) **Catalog #EXP-NBD104**

☒ Native Barcoding Expansion 13-24 (PCR-free) **Oxford Nanopore Technologies Catalog #EXP-NBD114**

☒ NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

☒ SFB expansion **Oxford Nanopore Technologies Catalog #EXP-SFB001**

☒ Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK109**

☒ ONT MinION Flow Cell R9.4.1 **Oxford Nanopore Technologies Catalog #FLO-MIN106D**

### Illumina Sequencing

☒ NEBNext Ultra II DNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #E7645S**

☒ NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #E7335S**

## Protocol materials

- ✕ SuperScript™ IV First-Strand Synthesis System **Invitrogen - Thermo Fisher Catalog #18091200**
- ✕ NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**
- ✕ SARS-CoV2-Midnight-1200 500rxn **IDT Catalog #10007184**
- ✕ ARTIC nCoV-2019 V3 Panel **IDT**
- ✕ Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01**
- ✕ NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**
- ✕ SFB expansion **Oxford Nanopore Technologies Catalog #EXP-SFB001**
- ✕ Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK109**
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- ✕ Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs Catalog #M0367L**
- ✕ ONT MinION Flow Cell R9.4.1 **Oxford Nanopore Technologies Catalog #FLO-MIN106D**
- ✕ NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #E7335S**
- ✕ DNA Clean & Concentrator™-5 **Zymo Research Catalog #D4003**
- ✕ Native Barcoding Expansion 1-12 (PCR-free) **Catalog #EXP-NBD104**
- ✕ Native Barcoding Expansion 13-24 (PCR-free) **Oxford Nanopore Technologies Catalog #EXP-NBD114**
- ✕ Zymo DNA Binding Buffer **Zymo Research Catalog #D4003-1-25**
- ✕ Zymo DNA Wash Buffer **Zymo Research Catalog #D4003-2-6**
- ✕ Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-1**

## Before start

Time can be saved by preparing master mixes first, before PCR steps. The master mix for cDNA and PCR steps should be prepared in Master Mix (PCR) Hood. To avoid cross-contamination make sure that your original stock reagents have no contact with RNA or any amplified DNA material.

A Negative Control (nuclease-free H<sub>2</sub>O ) should be included from cDNA synthesis step until the end. Keep the enzymes on ice and thaw the other reagents at room temperature before placing on ice.

Sequencing library preparation should be performed in post-PCR area.



## First strand cDNA synthesis

1h 30m

### 1 Preparation of master mix

- 1.1 Prepare the following components in a 1.5 mL Eppendorf DNA LoBind tube for the number of samples that will be tested and dispense  4  $\mu\text{L}$  Master Mix RT\_1 to 0.2mL each PCR tubes. Briefly spin down the 0.2 mL tubes containing **Master Mix RT\_1** and keep  On ice

| A                                | B               |
|----------------------------------|-----------------|
| Component                        | Volume          |
| 50 $\mu\text{M}$ random hexamers | 2 $\mu\text{L}$ |
| 10mM dNTPs mix (10mM each)       | 2 $\mu\text{L}$ |

- 1.2 Prepare the following components in a 1.5mL tube and keep the **Master Mix RT\_2**  On ice .

| A                                             | B                        |
|-----------------------------------------------|--------------------------|
| Component                                     | Volume ( $\mu\text{L}$ ) |
| 100 mM DTT                                    | 2                        |
| Ribonuclease Inhibitor (40 U/ $\mu\text{L}$ ) | 2                        |
| 5x SSIV Buffer                                | 8                        |
| SSIV Reverse transcriptase                    | 2                        |
| Nuclease free H <sub>2</sub> O                | 10                       |

- 2 This step should be conducted in the **pre-PCR area (e.g. cleaned DNA hood)**. Keep all the Master Mix  On ice while doing this step.

- 2.1 Add  12 µL RNA extract to each 0.2 mL tube containing the **Master Mix RT\_1**
- 2.2 Mix by pipetting or flicking the tube, spin down briefly.
- 2.3 Incubate the reaction mix in a thermocycler at  65 °C for  00:05:00 , then spin down briefly, and incubate immediately  On ice for at least  00:01:00 .
- 2.4 While on ice, add to each tube  24 µL Master Mix RT\_2 ,
- 2.5 Gently mix by pipetting or flicking the tubes and briefly spin down.
- 2.6 Incubate the reaction mix in the thermocycler for:

6m

|  | A    | B     | C        |
|--|------|-------|----------|
|  | Step | Temp  | Time     |
|  | 1    | 42 °C | 00:50:00 |
|  | 2    | 70 °C | 00:10:00 |
|  | 3    | 4°C   | hold     |

#### Note

The cDNA can be stored at  -20 °C . If needed, it is a safe stop point. The remaining RNA should be stored at  -80 °C .

## cDNA cleanup and concentration

1h

- 3 cDNA cleanup with  DNA Clean & Concentrator™-5 **Zymo Research Catalog #D4003** (all centrifuge steps at 13,000xg)
- 3.1 Add  8 µL 0.5M EDTA and  8 µL 1N NaOH to the  40 µL RT reaction .
- 3.2 Incubate the reaction mix in a thermocycler at  65 °C for  00:15:00 to hydrolyze RNA. 15m
- 3.3 Transfer the hydrolysis reaction mix from the last step to a new 1.5 mL Eppendorf DNA LoBind tube and add  392 µL (7x volume)  Zymo DNA Binding Buffer **Zymo Research Catalog #D4003-1-25** to  56 µL hydrolysis reaction mix . Briefly vortex to mix, and pulse spin to collect the sample.
- 3.4 Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.
- 3.5 Centrifuge for  00:00:30 and discard the flow-through. 30s
- 3.6 Add  200 µL  Zymo DNA Wash Buffer **Zymo Research Catalog #D4003-2-6** to the column. Centrifuge for  00:00:30 . 30s
- 3.7 Repeat step 3.6.
- 3.8 Add  Zymo DNA Elution Buffer **Zymo Research Catalog #D3004-4-1** to the column matrix and incubate at  Room temperature for  00:05:00 . 5m

|  | A             | B                 |
|--|---------------|-------------------|
|  | Primer Scheme | Elute volume (µL) |
|  | Swift_150bp   | 14                |



|  | A               | B    |
|--|-----------------|------|
|  | ARTIC V3_400bp  | 18.5 |
|  | Midnight_1200bp | 25   |

- 3.9 Transfer the column to a 1.5 ml microcentrifuge tube, then centrifuge for  00:00:30 to elute the DNA and keep on ice.

30s

## Multiplex PCR

4h

- 4 Prepare the following PCR mastermixs and keep  On ice .

- 4.1 Prepare PCR mastermix for Swift\_150bp primer scheme.

|  | A                                        | B                 | C                 |
|--|------------------------------------------|-------------------|-------------------|
|  | <b>Component</b>                         | <b>Reaction 1</b> | <b>Reaction 2</b> |
|  | Q5 Hot Start High-Fidelity 2X Master Mix | 12.5 µL           | 12.5 µL           |
|  | Swift Pool 1 (15nM each primer)          | 6.25 µL           | 0                 |
|  | Swift Pool 2 (15nM each primer)          | 0                 | 6.25 µL           |
|  | Total                                    | 18.75 µL          | 18.75 µL          |

- 4.2 Prepare PCR mastermix for ARTIC V3\_400bp primer scheme.

|  | A                                        | B                 | C                 |
|--|------------------------------------------|-------------------|-------------------|
|  | <b>Component</b>                         | <b>Reaction 1</b> | <b>Reaction 2</b> |
|  | Q5 Hot Start High-Fidelity 2X Master Mix | 12.5 µL           | 12.5 µL           |
|  | V3 Pool 1 (15nM each primer)             | 4 µL              | 0                 |
|  | V3 Pool 2 (15nM each primer)             | 0                 | 4 µL              |
|  | Total                                    | 16.5 µL           | 16.5 µL           |





#### 4.3 Prepare PCR mastermix for midnight\_1200bp primer scheme.

| A                                        | B            | C            |
|------------------------------------------|--------------|--------------|
| Component                                | Reaction 1   | Reaction 2   |
| Q5 Hot Start High-Fidelity 2X Master Mix | 12.5 $\mu$ L | 12.5 $\mu$ L |
| midnight Pool 1 (15nM each primer)       | 1.1 $\mu$ L  | 0            |
| midnight Pool 2 (15nM each primer)       | 0            | 1.1 $\mu$ L  |
| Total                                    | 13.6 $\mu$ L | 13.6 $\mu$ L |

- 5 Add the corresponding amount of cDNA to each of the PCR reactions, mix by pipetting or flicking the tube and spin down.

| A               | B                              |
|-----------------|--------------------------------|
| Primer Scheme   | Volume per reaction ( $\mu$ L) |
| Swift_150bp     | 6.25                           |
| ARTIC V3_400bp  | 8.5                            |
| Midnight_1200bp | 11.4                           |

- 6 Run in a thermal cycler using the following program:

##### Swift\_150bp primer scheme

| A                                   | B                                  | C             | D      |
|-------------------------------------|------------------------------------|---------------|--------|
| Step                                | Temperature                        | Time          | Cycles |
| Initial denaturation                | 98 $^{\circ}$ C                    | 30 s          | 1      |
| Denaturation<br>Annealing/Extension | 98 $^{\circ}$ C<br>65 $^{\circ}$ C | 15 s<br>2 min | 35     |



| A    | B    | C | D |
|------|------|---|---|
| Hold | 4 °C | ∞ | 1 |

OR

**ARTIC V3 and midnight primer scheme**

| A                                   | B            | C             | D      |
|-------------------------------------|--------------|---------------|--------|
| Step                                | Temperature  | Time          | Cycles |
| Initial denaturation                | 98 °C        | 30 s          | 1      |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 15 s<br>5 min | 35     |
| Hold                                | 4 °C         | ∞             | 1      |

**Note**

For ARTIC V3 400bp primer scheme and midnight 1200bp primer scheme (sequencing with nanopore), continue with steps 7-16.  
For Swift 150bp primer scheme (sequencing with Illumina), continue with steps 17-26.

**PCR amplicon cleanup and concentrate (400bp & 1200bp)**

30m

- 7 Combine the  25 µL PCR reaction mixtures for the two pools per sample into new 1.5 mL Eppendorf tubes, one per sample.
- 7.1 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.
- 7.2 Add  50 µL of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down.  
Incubate at  Room temperature for  00:05:00 .
- 7.3 Prepare fresh 80% ethanol in nuclease-free water that enough for  400 µL per sample.

5m



- 7.4 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, and pipette off the supernatant. 5m
- 7.5 Keep the tubes on the magnet and wash the beads with  200  $\mu\text{L}$  of freshly prepared 80% ethanol without disturbing the pellet. Incubate for  00:00:30 and pipette off the ethanol. 30s
- 7.6 Repeat the previous step (step 7.5).
- 7.7 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about  00:00:30 , but do not dry the pellet to the point of cracking. 30s
- 7.8 Remove the tubes from the magnetic rack and resuspended each pellet in  15  $\mu\text{L}$  nuclease-free water. Incubate for  00:05:00 at  Room temperature . 5m
- 7.9 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 7.10 Remove and retain  15  $\mu\text{L}$  of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.
- 8 Quantify  1  $\mu\text{L}$  of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

#### Note

This is a safe stop point.

For short-term storage, samples can be stored at  4 °C overnight;

For long-term storage, samples should be stored at  -20 °C .



## DNA End-Prep (nanopore)

20m

- 9 Thaw the NEBNext Ultra II End repair / dA-tailing Module reagents on ice.
- 9.1 Determine the volume of the cleaned-up PCR reaction that yields  $200 \text{ fmol}$  (  $50 \text{ ng}$  ) of DNA per sample and aliquot in new 0.5 mL PCR tubes.
- 9.2 Make up each sample per tube to  $12.5 \text{ }\mu\text{L}$  using nuclease-free water.
- 9.3 Prepare end-prep mastermix, mix by pipetting at least 10 times or flicking the tube, spin down and place  $\text{On ice}$  .

| A                                 | B                                           |
|-----------------------------------|---------------------------------------------|
| Component                         | Volume                                      |
| Ultra II End-prep reaction buffer | $1.75 \text{ }\mu\text{l}$                  |
| Ultra II End-prep enzyme mix      | $0.75 \text{ }\mu\text{l}$                  |
| <b>Total</b>                      | <b><math>2.5 \text{ }\mu\text{l}</math></b> |

- 9.4 Add  $2.5 \text{ }\mu\text{L}$  end-prep mastermix to each tube, mix by pipetting or flicking the tube.
- 9.5 Using a thermal cycler, incubate at  $20 \text{ }^{\circ}\text{C}$  for  $00:05:00$  and  $65 \text{ }^{\circ}\text{C}$  for  $00:05:00$

10m

## Native barcode ligation and cleanup (nanopore)

1h 30m

- 10 Thaw the native barcodes at room temperature, enough for one barcode per sample. Individually mix the barcodes by pipetting, and place them  $\text{On ice}$  .

Thaw the tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place  On ice .

- 10.1 Add the reagents in the following order per tube, mixing by flicking the tube between each sequential addition (one barcode per sample):

| A                          | B            |
|----------------------------|--------------|
| Component                  | Volume       |
| Nuclease-free water        | 6 µl         |
| End-prepped DNA            | 1.5 µl       |
| Native Barcode             | 2.5 µl       |
| Blunt/TA Ligase Master Mix | 10 µl        |
| <b>Total</b>               | <b>20 µl</b> |

- 10.2 Mix contents thoroughly by pipetting or flicking the tube and spin down briefly.

- 10.3 Using a thermal cycler, incubate at  20 °C for  00:20:00 and at  65 °C for  00:10:00 .

30m

- 11 Pool each barcoded library into a new 1.5 Eppendorf DNA LoBind tube.

- 11.1 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.

- 11.2 Add **0.4x volume** of pooled barcoded reaction of resuspended Mag-Bind® TotalPure NGS beads to the pooled barcoded reaction. Briefly vortex to mix and spin down.

- 11.3 Incubate on a Hula mixer (rotator mixer) at  Room temperature for  00:10:00 .

10m

- 11.4 Prepare  500 µL fresh 80% ethanol in nuclease-free water.



- 11.5 Spin down and pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, and pipette off the supernatant.
- 11.6 Wash the beads by adding  700  $\mu\text{L}$  Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.
- 11.7 Repeat the previous step (step 11.6).
- 11.8 Keep the tubes on the magnet and wash the beads with  500  $\mu\text{L}$  of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 11.9 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about  00:00:30 , but do not dry the pellet to the point of cracking.
- 11.10 Remove the tubes from the magnetic rack and resuspended each pellet in  35  $\mu\text{L}$  nuclease-free water. Incubate for  00:02:00 at  Room temperature . 2m
- 11.11 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 11.12 Remove and retain  35  $\mu\text{L}$  of eluate into a new 1.5 mL Eppendorf tubes.
- 12 Quantify  1  $\mu\text{L}$  of eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

## Adapter ligation and cleanup (nanopore)

1h 30m

- 13 Thaw the Elution Buffer (EB), Short Fragment Buffer (SFB), and NEBNext Quick Ligation Reaction Buffer (5x) at  Room temperature , mix by vortexing, spin down and place  On ice . Check the contents of each tube are clear of any precipitate.

Spin down the T4 Ligase and the Adapter Mix II (AMII), and place  On ice .

- 13.1 Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

| A                                           | B            |
|---------------------------------------------|--------------|
| Component                                   | Volume       |
| Pooled barcoded sample                      | 30 µl        |
| Adapter Mix II (AMII)                       | 5 µl         |
| NEBNext Quick Ligation Reaction Buffer (5X) | 10 µl        |
| Quick T4 DNA Ligase                         | 5 µl         |
| <b>Total</b>                                | <b>50 µl</b> |

- 13.2 Mix gently by flicking the tube, and spin down.

- 13.3 Incubate the reaction for  00:20:00 at room temperature.

20m

- 14 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.

- 14.1 Add  20 µL (0.4x volume) of resuspended Mag-Bind® TotalPure NGS beads to the reaction. Briefly vortex to mix and spin down.

- 14.2 Incubate on a Hula mixer (rotator mixer) at  Room temperature for  00:10:00 .

- 14.3 Spin down and pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, and pipette off the supernatant.

- 14.4 Wash the beads by adding  125 µL Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.



14.5 Repeat the previous step (step 14.4).

14.6 Spin down and place the tubes on the magnet. Pipette off any residual supernatant.

14.7 Remove the tubes from the magnetic rack and resuspended each pellet in  15  $\mu\text{L}$  Elution Buffer (EB). Incubate for  00:05:00 at  Room temperature .

5m

14.8 Pellet the beads on a magnetic rack until the eluate is clear and colourless.

14.9 Remove and retain  15  $\mu\text{L}$  of eluate into a new 1.5 mL Eppendorf tubes.

15 Quantify  1  $\mu\text{L}$  of eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

## Priming and loading the SpotON flowcell (nanopore)

30m

16 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at  Room temperature .

Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by vortexing and spin down at  Room temperature .

16.1 Open the MinION Mk1B lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

16.2 Slide the priming port cover clockwise to open the priming port.

16.3 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few  $\mu\text{L}$ ).



16.4 To 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 (FB), and mix by vortexing at  Room temperature .

16.5 Load  800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for  00:05:00 . During this time, prepare the library for loading by following the steps below.

5m

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

16.7 In a new tube, prepare the library for loading as follows:

| A                                                | B       |
|--------------------------------------------------|---------|
| Reagent                                          | Volume  |
| Sequencing Buffer (SQB)                          | 37.5 µl |
| Loading Beads (LB), mixed immediately before use | 25.5 µl |
| DNA library (50 fmol)                            | 12 µl   |
| Total                                            | 75 µl   |

16.8 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

16.9 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.

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

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



- 16.12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

## PCR amplicon cleanup and concentrate (150bp)

45m

- 17 Combine the  25  $\mu\text{L}$  PCR reaction mixtures for the two pools per sample into new 1.5 mL Eppendorf tubes, one per sample.
- 17.1 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.
- 17.2 Add  42.5  $\mu\text{L}$  (0.85x beads/sample ratio) of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down. Incubate at  Room temperature for  00:05:00 .
- 17.3 Prepare fresh 80% ethanol in nuclease-free water that enough for  400  $\mu\text{L}$  per sample.
- 17.4 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, pipette  92.5  $\mu\text{L}$  supernatant to new 1.5 mL Eppendorf tubes (keep supernatant).
- 17.5 Add  47.5  $\mu\text{L}$  (1.8x total beads/sample ratio) of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down. Incubate at  Room temperature for  00:15:00 .
- 17.6 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, pipette off the supernatant (keep beads).
- 17.7 Keep the tubes on the magnet and wash the beads with  200  $\mu\text{L}$  of freshly prepared 80% ethanol without disturbing the pellet. Incubate for  00:00:30 and pipette off the ethanol.
- 17.8 Repeat the previous step (step 17.7).



- 17.9 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about 00:00:30 , but do not dry the pellet to the point of cracking.
- 17.10 Remove the tubes from the magnetic rack and resuspended each pellet in 15  $\mu\text{L}$  nuclease-free water. Incubate for 00:05:00 at Room temperature .
- 17.11 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 17.12 Remove and retain 15  $\mu\text{L}$  of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.
- 18 Quantify 1  $\mu\text{L}$  of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

#### Note

This is a safe stop point.

For short-term storage, samples can be stored at 4 °C overnight;

For long-term storage, samples should be stored at -20 °C .

## DNA End-Prep (Illumina)

1h

- 19 Thaw the NEBNext Ultra II End repair / dA-tailing Module reagents on ice.
- 19.1 Determine the volume of the cleaned-up PCR reaction that yields 100 ng of DNA per sample and aliquot in new 0.5 mL PCR tubes.
- 19.2 Make up each sample per tube to 20  $\mu\text{L}$  using nuclease-free water.

- 19.3 Prepare end-prep mastermix, mix by pipetting at least 10 times or flicking the tube, spin down and place  On ice .

| A                                 | B           |
|-----------------------------------|-------------|
| Component                         | Volume      |
| Ultra II End-prep reaction buffer | 2.8 µl      |
| Ultra II End-prep enzyme mix      | 1.2 µl      |
| <b>Total</b>                      | <b>4 µl</b> |

- 19.4 Add  4 µL end-prep mastermix to each tube, mix by pipetting or flicking the tube.

- 19.5 Using a thermal cycler, incubate at  20 °C for  00:30:00 and  65 °C for  00:30:00 , then hold at  4 °C .

1h

## Adapter ligation and cleanup (Illumina)

1h

20

Dilute the NEBNext Adaptor for Illumina 10x in Tris/NaCl, pH 7.5-8.0.

- 20.1 Prepare adapter ligation mastermix, mix by pipetting at least 10 times or flicking the tube, spin down and place  On ice

| A                                    | B           |
|--------------------------------------|-------------|
| Component                            | Volume (µl) |
| NEBNext Adaptor for Illumina         | 1           |
| NEBNext Ultra II Ligation Master Mix | 12          |
| NEBNext Ligation Enhancer            | 0.4         |



|  |                     |             |
|--|---------------------|-------------|
|  | A                   | B           |
|  | <b>Total Volume</b> | <b>13.4</b> |

**Note**

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C.

20.2 Add  13.4 µL 1adapter ligation mastermix directly to the  24 µL End Prep Reaction Mixture, mix by pipetting or flicking the tubes and spin down.

20.3 Incubate at  20 °C for  00:15:00 in a thermocycler with the heated lid off (or open lid).

15m

20.4 Add  1.2 µL 11.2µl of USER<sup>®</sup> Enzyme to the ligation mixture.

20.5 Mix well and incubate in a thermocycler at  37 °C for  00:15:00 .

15m

21 Transfer the  36 µL ligation reaction mix into new 1.5 mL Eppendorf tubes, one per sample.

21.1 Resuspend the Mag-Bind<sup>®</sup> TotalPure NGS beads by vortexing.

21.2 Add  43.2 µL (1.2x beads/sample ratio) of resuspended Mag-Bind<sup>®</sup> TotalPure NGS beads to each tube. Briefly vortex to mix and spin down. Incubate at  Room temperature for  00:05:00 .

5m

21.3 Prepare fresh 80% ethanol in nuclease-free water that enough for  400 µL per sample.

- 21.4 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, and pipette off the supernatant.
- 21.5 Keep the tubes on the magnet and wash the beads with  200  $\mu\text{L}$  of freshly prepared 80% ethanol without disturbing the pellet. Incubate for  00:00:30 and pipette off the ethanol.
- 21.6 Repeat the previous step (step 21.5).
- 21.7 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about  00:00:30 , but do not dry the pellet to the point of cracking.
- 21.8 Remove the tubes from the magnetic rack and resuspended each pellet in  15  $\mu\text{L}$  nuclease-free water. Incubate for  00:05:00 at  Room temperature .
- 21.9 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 21.10 Remove and retain  15  $\mu\text{L}$  of eluate containing the DNA library per tube into new 0.5 mL PCR tubes.

## PCR Barcoding and cleanup(Illumina)

2h 30m

- 22 Prepare barcoding mastermix, mix by pipetting or flicking the tube, spin down and place

 On ice

| A                              | B                        |
|--------------------------------|--------------------------|
| Component                      | Volume ( $\mu\text{L}$ ) |
| NEBNext Ultra II Q5 Master Mix | 25                       |
| Index Primer (one per sample)  | 5                        |
| Universal PCR Primer           | 5                        |



|                     |           |
|---------------------|-----------|
| A                   | B         |
| <b>Total Volume</b> | <b>35</b> |

22.1 Add  35 µL barcoding mastermix to  15 µL cleaned adapter ligation mixture, mix well by pipetting or flicking the tubes.

22.2 Run in a thermal cycler using the following program:

|                                     |                    |              |               |
|-------------------------------------|--------------------|--------------|---------------|
| A                                   | B                  | C            | D             |
| <b>Step</b>                         | <b>Temperature</b> | <b>Time</b>  | <b>Cycles</b> |
| Initial denaturation                | 98 °C              | 30 s         | 1             |
| Denaturation<br>Annealing/Extension | 98 °C<br>65 °C     | 10 s<br>75 s | 4             |
| Final extension                     | 65 °C              | 5 min        | 1             |
| Hold                                | 4 °C               | ∞            | 1             |

23 Transfer  20 µL barcoded library into new 1.5 mL Eppendorf tubes, one per sample.  
Add  30 µL nuclease-free water to each tube.

23.1 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.

23.2 Add  35 µL (0.7x beads/sample ratio) of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down. Incubate at  Room temperature for  00:05:00 .

23.3 Prepare fresh 80% ethanol in nuclease-free water that enough for  400 µL per sample.

23.4 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, pipette  85 µL supernatant to new 1.5 mL



Eppendorf tubes (keep supernatant).

- 23.5 Add  15  $\mu\text{L}$  (1.0x total beads/sample ratio) of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down. Incubate at  Room temperature for  00:15:00 .
- 23.6 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, pipette off the supernatant (keep beads).
- 23.7 Keep the tubes on the magnet and wash the beads with  200  $\mu\text{L}$  of freshly prepared 80% ethanol without disturbing the pellet. Incubate for  00:00:30 and pipette off the ethanol.
- 23.8 Repeat the previous step (step 23.7).
- 23.9 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about  00:00:30 , but do not dry the pellet to the point of cracking.
- 23.10 Remove the tubes from the magnetic rack and resuspended each pellet in  20  $\mu\text{L}$  nuclease-free water. Incubate for  00:05:00 at  Room temperature .
- 23.11 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 23.12 Remove and retain  20  $\mu\text{L}$  of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.
- 24 Quantify  1  $\mu\text{L}$  of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.



**Note**

This is a safe stop point.

For short-term storage, samples can be stored at  4 °C overnight;

For long-term storage, samples should be stored at  -20 °C .

- 25 Pool equal mass of each barcoded library to form a pooled barcoded library.
- 25.1 Resuspend the Mag-Bind® TotalPure NGS beads by vortexing.
- 25.2 Add **1.0x volume of pooled barcoded reaction** of resuspended Mag-Bind® TotalPure NGS beads to each tube. Briefly vortex to mix and spin down.
- 25.3 Incubate at  Room temperature for  00:05:00 .
- 25.4 Prepare fresh 80% ethanol in nuclease-free water that enough for  400 µL per sample.
- 25.5 Pellet the beads on a magnet rack for  00:05:00 . Keep the tubes on the magnet until the eluate is clear and colourless, and pipette off the supernatant.
- 25.6 Keep the tubes on the magnet and wash the beads with  200 µL of freshly prepared 80% ethanol without disturbing the pellet. Incubate for  00:00:30 and pipette off the ethanol.
- 25.7 Repeat the previous step (step 25.6).
- 25.8 Spin down and place the tubes on the magnet. Pipette off any residual ethanol. Allow to air dry for about  00:00:30 , but do not dry the pellet to the point of cracking.



- 25.9 Remove the tubes from the magnetic rack and resuspended each pellet in  $20\ \mu\text{L}$  nuclease-free water. Incubate for 00:05:00 at Room temperature .
- 25.10 Pellet the beads on a magnetic rack until the eluate is clear and colourless.
- 25.11 Remove and retain  $20\ \mu\text{L}$  of eluate containing the DNA library per tube into new 0.5 mL PCR tubes.
- 26 Quantify  $1\ \mu\text{L}$  of pooled barcoded library using a Qubit fluorometer with Qubit dsDNA HS Assay Kit. This sequencing library is ready for submission.

#### Note

This is a safe stop point.  
Store this sequencing library at  $-20\ ^\circ\text{C}$  .