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

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ABSTRACT

In this work, we aim to access 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
"midnight" protocol V4 (1200bp): dx.doi.org/10.17504/protocols.io.bh7hj9j6

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MATERIALS TEXT

Primers

Swift primers were purchased as two pools form 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** Step 3

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) **Contributed by users 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**

BEFORE STARTING

Time can be saved by preparing master mixes first, before PCR steps. The master mix for cDNA and PCR steps

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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_2O) 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 µl 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**
50µM random hexamers	2 µL
10mM dNTPs mix (10mM each)	2 µL

1.2 Prepare the following components in a 1.5mL tube and keep the **Master Mix RT_2** **On ice** .

A	B
Component	**Volume (µL)**
100 mM DTT	2
Ribonuclease Inhibitor (40 U/µL)	2
5x SSIV Buffer	8
SSIV Reverse transcriptase	2
Nuclease free H2O	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 6m

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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:

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

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 DNA Clean & Concentrator™-5 **Zymo**
cDNA cleanup with **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 thesample.

Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.

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3.4

3.5 Centrifuge for **00:00:30** and discard the flow-through. 30s

3.6 Add **200 µl** Zymo DNA Wash Buffer **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 **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
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
Component	**Reaction 1**	**Reaction 2**
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

Prepare PCR mastermix for ARTIC V3_400bp primer scheme.

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4.2

A	B	C
Component	**Reaction 1**	**Reaction 2**
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 µL	12.5 µL
midnight Pool 1 (15nM each primer)	1.1 µL	0
midnight Pool 2 (15nM each primer)	0	1.1 µL
Total	13.6 µL	13.6 µL

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 (µL)**
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 °C	30 s	1
Denaturation Annealing/Extension	98°C 65°C	15 s 2 min	35
Hold	4 °C	∞	1

OR

ARTIC V3 and midnight primer scheme

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

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 mixtrues 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. 5m
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.

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 µl** 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 µl** nuclease-free water. Incubate for **00:05:00** at **Room temperature** . 5m

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

7.10 Remove and retain **15 µl** of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.

8 Quantify **1 µl** of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

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 fmol** (**50 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 µl** 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 **On ice** .

A	B
Component	**Volume**
Ultra II End-prep reaction buffer	1.75 µl
Ultra II End-prep enzyme mix	0.75 µl
Total	**2.5 µl**

9.4 Add **2.5 µl** end-prep mastermix to each tube, mix by pipetting or flicking the tube.

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9.5 Using a thermal cycler, incubate at **20 °C** for **00:05:00** and **65 °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 **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
Total	**20 µl**

10.2 Mix contents thoroughly by pipetting or flcking 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.

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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 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.

11.7 Repeat the previous step (step 11.6).

11.8 Keep the tubes on the magnet and wash the beads with **500 µl** 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 µl** 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 µl** of eluate into a new 1.5 mL Eppendorf tubes.

12 Quantify **1 µl** 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.

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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
Total	**50 µl**

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 µl** Elution Buffer (EB). 5m
Incubate for **00:05:00** at **Room temperature** .

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

14.9 Remove and retain **15 µl** of eluate into a new 1.5 mL Eppendorf tubes.

15 Quantify **1 µl** 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 µl).

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:

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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 µl** 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 µl (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** 5m .

17.3 Prepare fresh 80% ethanol in nuclease-free water that enough for **400 µl** 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 µl** supernatant to new 1.5 mL Eppendorf tubes (keep supernatant).

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17.5 Add **47.5 µl (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** . 15m

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). 5m

17.7 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.

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 µl** 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 µl** of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.

18 Quantify **1 µl** of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

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

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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 µl** 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
Total	**4 µl**

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
Total Volume	**13.4**

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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® 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® TotalPure NGS beads by vortexing.

21.2 Add **43.2 µl (1.2x 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** . 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 µl** 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).

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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 µl** 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 µl** 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 (µl)**
NEBNext Ultra II Q5 Master Mix	25
Index Primer (one per sample)	5
Universal PCR Primer	5
Total Volume	**35**

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
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	30 s	1
Denaturation Annealing/Extension	98 °C 65 °C	10 s 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.

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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 µl (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 µl** 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 µl** 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.

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23.12 Remove and retain **20 µl** of eluate containing the DNA library per tube into new 1.5 mL Eppendorf tubes.

24 Quantify **1 µl** of each eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

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.

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25.9 Remove the tubes from the magnetic rack and resuspended each pellet in **20 µl** 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 µl** of eluate containing the DNA library per tube into new 0.5 mL PCR tubes.

26 Quantify **1 µl** of pooled barcoded library using a Qubit fluorometer with Qubit dsDNA HS Assay Kit. This sequencing library is ready for submission.

This is a safe stop point.
Store this sequencing library at **-20 °C** .

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