

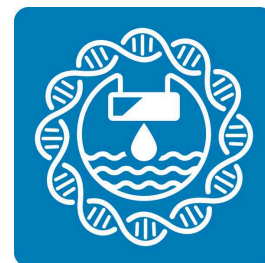
Aug 30, 2022

Version 2

Modified NEBNext® VarSkip Short SARS-CoV-2 Enrichment and library prep for Oxford Nanopore Technologies-adapted for wastewater samples V.2



Version 1 is forked from [NEBNext® ARTIC SARS-CoV-2 Companion Kit \(Oxford Nanopore Technologies®\) E7660](#)



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GenomeTrakr

Coronavirus Method De...



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Protocol status: In development

We are still developing and optimizing this protocol

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Keywords: NEBNext, NEB, ARTIC, SARS-CoV-2, Oxford, Nanopore, wastewater, SARS-CoV-2, Oxford, Nanopore, NEBNext, cov2 genome, sequencing library, wastewater samples this protocol details method, wastewater sample, standard protocol with pcr bead cleanup, nanopore, pcr bead cleanup, vss primers from library preparation, sequencing, library pool, wastewater, reads for each library, cDNA synthesis

Abstract

This protocol details methods for the preparation of SARS-CoV-2 sequencing library using VSS primers from library preparation from NEBNext® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies®), NEB #E7660S/L 24/96 reactions adapted for wastewater samples.

Standard Protocol with PCR Bead Cleanup: This protocol includes a cleanup and normalization step for each sample after cDNA synthesis. Performing the cleanup and normalization step creates library pools where the reads for each library are more evenly distributed. These pools will likely achieve sufficient and equal coverage in less run time, but they take more hands-on time

This protocol also includes options for barcoding with dual indexing or single indexing of the samples.

Version updates V2: Recommending the addition of VSS v2b (spike-in primers) to the primer pool1 to increase the coverage across multiple regions of SARS-CoV2 genome. Optimized the input of cDNA. Optimized wash steps to get rid of shorter fragments of library.

Guidelines

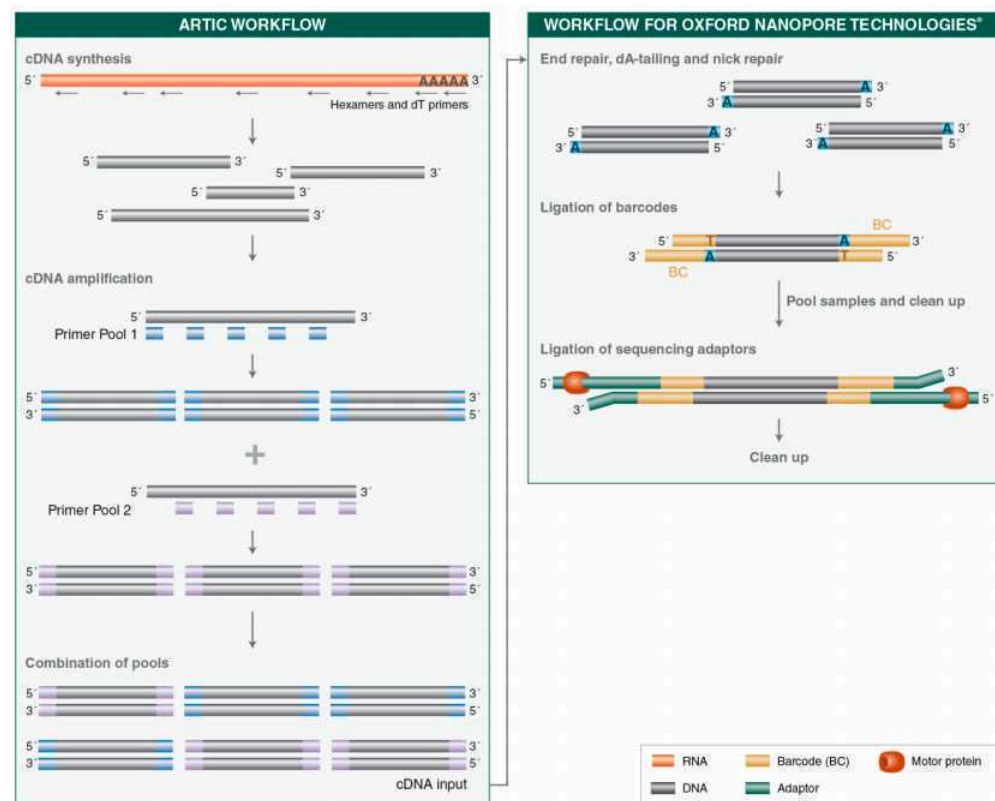
Overview

The NEBNext ARTIC SARS-CoV-2 Companion Library Prep Kit (Oxford Nanopore Technologies) contains the enzymes, buffers, beads and oligos required to convert a broad range of total RNA input amounts into targeted, high quality libraries for next-generation sequencing on the Oxford Nanopore platform.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of an indexed library on the Oxford Nanopore sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Figure 1. Workflow demonstrating the use of NEBNext ARTIC SARS-CoV-2 Companion Library Prep Kit for Oxford Nanopore.



Materials

The Library Kit Includes NEB #E7660L/S and Oxford Nanopore Technologies Native Barcoding Expansion kits.

This kit includes two sets of primers:

1. The VarSkip (for Variant Skip) Short primers have been designed at NEB to provide improved performance with SARS-CoV-2 variants, including the Omicron and Delta variants. The Omicron variant can be called confidently using NEBNext VarSkip Short (VSS) primers. Note that there are two dropouts (amplicons 56 and 67), and two amplicons (20 and 64) have lower coverage. Starting Feb 14th, 2022 NEB has V2 VSS primers in the kit, for improved Omicron coverage.
2. The V3 ARTIC primers have been balanced, using methodology developed at NEB based on empirical data from sequencing. In combination with optimized reagents for RT-PCR, the kits deliver improved uniformity of amplicon yields from gRNA across a wide copy number range. **(not used)**

Package 1: Store at -20°C.

(lilac) LunaScript® RT SuperMix
(lilac) Q5® Hot Start High-Fidelity 2X Master Mix
(green) NEBNext Ultra II End Prep Enzyme Mix
(green) NEBNext Ultra II End Prep Reaction Buffer
(red/white) Blunt/TA Ligase Master Mix
(red) NEBNext Quick T4 Ligase
(red) NEBNext Quick Ligation Reaction Buffer
(lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 1 (not used)
(lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 2 (not used)
(lilac) NEBNext ARTIC Human Control Primer Pairs 1 (not used)
(lilac) NEBNext ARTIC Human Control Primer Pairs 2 (not used)
(white) Nuclease-free water

Package 2: Store at room temperature. Do not freeze.

NEBNext Sample Purification Beads or Ampure beads

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- DNA LoBind Tubes (Eppendorf® #022431021)
- Oxford Nanopore Technologies Native Barcoding Expansion kits 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) or
- **Oxford Nanopore Technologies Native Barcoding Expansion kits- Dual barcoding 1-96 (EXP-NBD196)**
- **Oxford Nanopore Technologies Ligation Sequencing Kit (SQK-LSK109)**



- Qubit[®] dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.[®] Q32851)
- Magnetic rack/stand (NEB #S1515, Alpaqua[®], cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent[®] Bioanalyzer[®] or similar fragment analyzer and associated consumables (#4150 or #4200 TapeStation System)
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)
- 1.5 ml tube magnet stand (NEB #S1506)

Kit Components

NEB #E7660S Table of Components

	A	B	C
	NEB #	PRODUCT	VOLUME
	E7651A	LunaScript RT SuperMix (5X)	0.048 ml
	E7652A	Q5 Hot Start High-Fidelity 2X Master Mix	0.30 ml
	E7661A	NEBNext Ultra II End Prep Enzyme Mix	0.018 ml
	E7662A	NEBNext Ultra II End Prep Reaction Buffer	0.042 ml
	E7663A	Blunt/TA Ligase Master Mix	0.24 ml
	E7664A	NEBNext Quick T4 DNA Ligase	0.020 ml
	E7665A	NEBNext Quick Ligation Reaction Buffer	0.040 ml
	E7725A	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.042 ml
	E7726A	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.042 ml
	E7727A	NEBNext ARTIC Human Control Primer Pairs 1	0.007 ml
	E7728A	NEBNext ARTIC Human Control Primer Pairs 2	0.007 ml
	E7667A	Nuclease free-Water	1.50 ml
	E7666S	NEBNext Sample Purification Beads	0.872 ml

NEB #E7660L Table of Components



	A	B	C
	NEB #	PRODUCT	VOLUME
	E7651AA	LunaScript RT SuperMix (5X)	0.192 ml
	E7652AA	Q5 Hot Start High-Fidelity 2X Master Mix	1.2 ml
	E7661AA	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
	E7662AA	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
	E7663AA	Blunt/TA Ligase Master Mix	0.96 ml
	E7664A	NEBNext Quick T4 DNA Ligase	0.020 ml
	E7665A	NEBNext Quick Ligation Reaction Buffer	0.040 ml
	E7725AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.168 ml
	E7726AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.168 ml
	E7727A	NEBNext ARTIC Human Control Primer Pairs 1	0.007 ml
	E7728A	NEBNext ARTIC Human Control Primer Pairs 2	0.007 ml
	E7667AA	Nuclease free-Water	4.7 ml
	E7666L	NEBNext Sample Purification Beads	2.90 ml

Troubleshooting

Safety warnings

⚠ Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.



Before start

Note: The amount of RNA required for detection depends on the abundance of the RNA of interest. In general, we recommend, using > 10 copies of the (SARS-CoV-2) viral genome as input. In addition, we recommend setting up a no template control reaction and **all reactions are set-up in a hood.**

The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.



Before you start

1

Note

The presence of genomic DNA or carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

Absolutely no vortexing of cDNA, amplicons, or libraries at any point.

We have also verified cDNA synthesis using the Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Catalog number:18091200), as described in the SNAP protocol with modifications (random hexamers, RT incubation of 30 min.).

cDNA Synthesis

- 2 Gently mix 10 times by pipetting and spin down the LunaScript RT SuperMix reagents (contains primers). Prepare the cDNA synthesis reaction as described below:



A	B
COMPONENT	VOLUME
RNA Sample*	8 µl
(lilac) LunaScript RT SuperMix	2 µl
<i>Total Volume</i>	10 µl

*Up to 0.5 µg total RNA can be used in a 10 µl reaction.

- 3 Flick the tube or pipet up and down 10 times to mix followed by a quick spin.



- 4 For no template controls, mix the following components:



A	B
COMPONENT	VOLUME
(white) Nuclease-free Water	8 µl



A	B
(lilac) LunaScript RT SuperMix	2 µl
<i>Total Volume</i>	10 µl

- 5 Flick the tube or pipet up and down 10 times to mix followed by a quick spin.



- 6 Incubate reactions in a thermocycler with lid temperature at 105°C with the following steps:



A	B	C	D
CYCLE STEP	TEMP	TIME	CYCLE
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	20 minutes	
Heat Inactivation	95°C	1 minute	
Hold	4°C	∞	

Note

Samples can be stored at  -20 °C if they are not used immediately.

Targeted cDNA Amplification

7

Note

5 µl cDNA input is recommended. If using less than 5 µl of cDNA, add nuclease-free water to a final volume of 4.5 µl. We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of subsequent reactions.

- 8 Addition of spike-in to improve coverage across certain regions of SARS-COV2 genome.



**For 96 reaction kits:**

- Thaw BA2 Spike-in Mix and VarSkip Short v2 Primer Mix 1.
- Spin down both the tubes.
- Add 1 ul of the BA2 Spike-in Mix to the VarSkip Short v2 Primer Mix 1.
- Mix and quick spin updated VarSkip Short v2 Primer Mix 1.

For 24 reaction kits:

- Thaw BA2 Spike-in Mix and VarSkip Short v2 Primer Mix 1.
- Spin down both the tubes.
- Add 1 ul of BA2 Spike-in Mix to 3ul 0.1x TE to make a ¼ dilution of the BA2 Spike-in Mix.
- Add 1 ul of the diluted BA2 Spike-in Mix to the VarSkip Short v2 Primer Mix 1.
- Mix and quick spin updated VarSkip Short v2 Primer Mix 1.

Gently mix Q5 Hot Start High Fidelity 2X master mix 10 times by pipetting and spin down reagents. Prepare the split pool amplification reactions as described below:

For Pool set A:

A	B
COMPONENT	VOLUME
cDNA (Step 6)	5 µl
(lilac) Q5 Hot Start High-Fidelity 2X MM	6.25 µl
NEBNext VSS SARS-CoV-2 Primer Mix 1 with spike in	1.75 µl
<i>Total Volume</i>	13 µl

For Pool Set B:

A	B
COMPONENT	VOLUME
cDNA (Step 6)	5 µl
(lilac) Q5 Hot Start High-Fidelity 2X MM	6.25 µl
NEBNext VSS SARS-CoV-2 Primer Mix 2	1.75 µl
<i>Total Volume</i>	13 µl



9 Flick the tube or gently pipet up and down 10 times to mix followed by a quick spin.

10 Incubate reactions in a thermocycler* with the following steps:



	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denature	95°C	15 seconds	35
	Annealing/Extension	63°C	5 minutes	
	Hold	4°C	∞	1

* Set heated lid to 105°C.

Note

Samples can be stored at -20 °C if they are not used immediately.

Cleanup of cDNA Amplicons


11 We highly recommend the clean up step using either NEBNext sample purification beads or Ampure beads.

Note



SPRIselect or AMPure[®] XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to Room temperature for at least 30 minutes before use. These bead volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

12 For each sample, combine pool A and pool B PCR Reactions.




- 13 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.
- 14 Add  20 μ L (0.76X) resuspended beads to the combined PCR reaction. Mix well by flicking the tube or pipetting up and down 10 times to mix and a very short 2-3 seconds quick centrifugation. Be sure to stop the centrifugation before the beads start to settle out.



- 15 Incubate samples at  Room temperature for  00:10:00 .

10m






- 16 Place the tubes on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample  00:00:01 to collect the liquid from the sides of the tube before placing on the magnetic stand.

1s




- 17 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note**Caution: do not discard the beads.**

- 18 Add  200 μ L 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at  Room temperature for  00:00:30 , and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.


30s




- 19 Repeat previous step once for a total of two washes:
Add  200 μ L 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at  Room temperature for  00:00:30 , and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

31s



Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube for  00:00:01 , place back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.











- 20 Air dry the beads for up to  00:03:00 while the tube is on the magnetic stand with the lid open.

3m

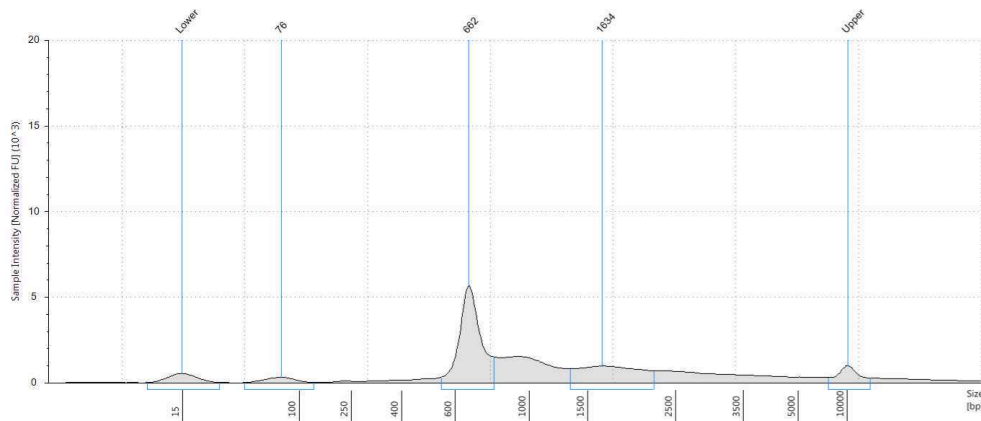


Note

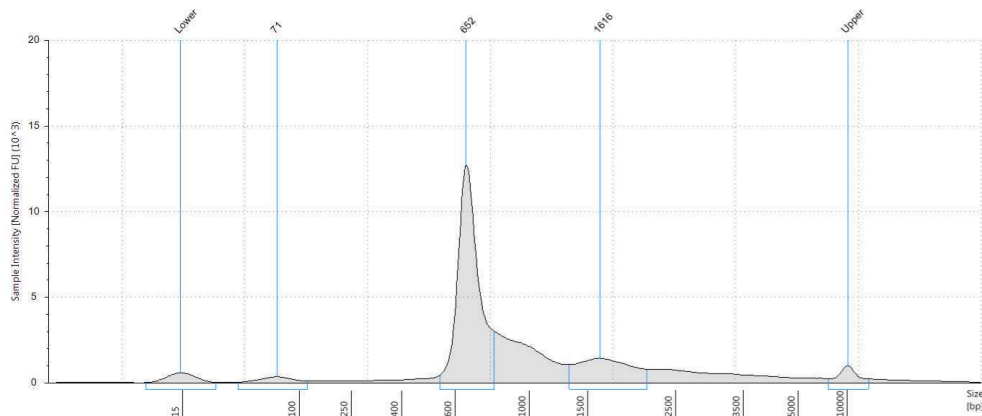
Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking. When the beads turn lighter brown and start to crack, they are too dry.

- 21 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding  18 μ L Nuclease-free water . 
- 22 Mix well by flicking the tube or pipetting up and down 10 times to mix and followed by a very short centrifugation. Incubate for  00:02:00 at  Room temperature . If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 

- 23 Place the tube on the magnetic stand. After 5 minutes (or when the solution is clear), transfer  17 μ L to clean PCR tubes. 
- 24 Assess the concentration of the DNA targets. We recommend using a Qubit fluorometer for concentration assessment. Use 1 μ l of sample for the Qubit fluorometer. Amplicons may also be run on a Bioanalyzer[®] or Tape Station using High Sensitivity (HS) 5000 tape or HS 1000 tape to confirm ~560-650 bp size of amplicons.  

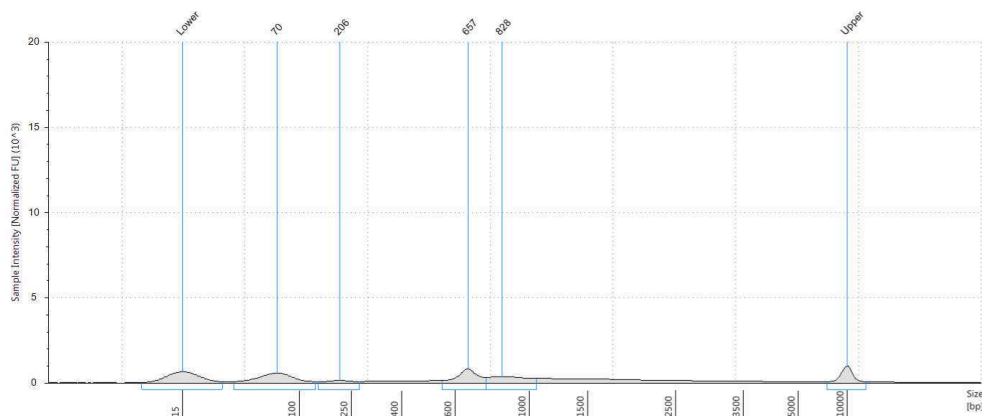
Note



Tape station profile of moderately positive wastewater sample.



Tape station profile of a high positive wastewater sample.



Tape station profile of a low positive wastewater sample

Note


Samples can be stored at  -20 °C if they are not used immediately.

25

Note

Based on the qubit readings of the cleaned VSS amplicons(step 24), we recommend to adjust the concentration of the amplicons going into end prep and barcoding. In step 26, the amount recommended is 4ng/ul. In low titer wastewater samples, it is very hard to achieve this desired concentration. We do not recommend proceeding to end prep for VSS amplicons with a concentration less than 1.5 ng/ul.

NEBNext End Prep

- 26 Use the Qubit readings from Step 24 to determine the amount of the VSS Amplicons. Dilute each amplicon sample into 50 ng/12.5 µl (4ng/ul) concentration using Nuclease-free water. Add the following components to a PCR tube (End Prep Reaction and Buffer can be pre-mixed and stable  On ice for 4 hours):



A	B
COMPONENT	VOLUME
Targeted cDNA Amplicons (Step 24)	12.5 µl
(green) NEBNext Ultra II End Prep Reaction Buffer	1.75 µl
(green) NEBNext Ultra II End Prep Enzyme Mix	0.75 µl
<i>Total Volume</i>	15 µl

- 27 Flick the tube or gently pipet up and down 10 times to mix the solution. Perform a quick spin to collect all liquid from the sides of the tube.



Note

It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

28 Place in a thermocycler, with the heated lid set to = 75°C, and run the following program:

⌚ 00:10:00 @ 🌡️ 20 °C

⌚ 00:10:00 @ 🌡️ 65 °C

Hold at 🌡️ 4 °C

20m



Note

If necessary, samples can be stored at 🌡️ -20 °C for a few days; however, a slight loss in yield (~20%) may be observed. We recommend continuing with barcode ligation before stopping.

29

Note

If the concentration of cDNA amplicons going to end prep is ~3-4 ng/ul, we recommend using 3ul of End-prepped DNA for barcoding (as mentioned in Step 30). If the concentration of cDNA amplicons is less than 3ng/ul (1.5ng/ul - 3ng/ul) going into end prep, we recommend using 5ul of End-prepped DNA into barcoding.

Barcode Ligation

30 Add the following components directly to a sterile nuclease-free PCR tube:



A	B
COMPONENT	VOLUME
End-prepped DNA (Previous Step)	3 ul
Dual Barcode*	8 ul
(red) Blunt/TA Ligase Master Mix**	10 µl
Total Volume	21 µl



* Barcodes are provided in Oxford Nanopore Technologies Dual Barcoding Expansion kit EXP- NBD 196. Adding 8ul of barcode to 3-5ul of End prepped DNA helps with better barcoding efficiency for wastewater samples.
** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction.

31

Note

Since these are wastewater samples, low viral load samples are routinely expected. It is highly recommended that you set up 2-3 barcode reactions per sample. You will be setting up 2-3 sets following Step 30. Since, only 3ul - 5ul of End-prepped DNA (from step 28) is used per barcode ligation reaction, there is sufficient material to set up 3-5 barcode reactions per sample.

This is done to improve the volume of the total pooled barcoded sample, which will help in increasing the yield of cleaned barcoded DNA and ultimately more sequencing pores.

- 32 Flick the tube or gently pipet up and down 10 times to mix solution. Perform a quick spin to collect all liquid from the sides of the tube.

**Note**

Caution: The Blunt/TA Ligase Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

- 33 Place in a thermocycler, with the heated lid set to = 75°C, and run the following program:

🌡 25 °C for ⌚ 00:20:00

🌡 65 °C for ⌚ 00:10:00 .

Place 🌡 On ice for ⌚ 00:01:00 .

31m



- 34 **Pool all barcoded samples into one 1.5 ml DNA LoBind Tube.**

Cleanup of Barcoded DNA

- 35 **The following section is for cleanup of the ligation reaction.**



Note

The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to

Room temperature **for at least 30 minutes before use.**

- 36 Vortex NEBNext Sample Purification Beads to resuspend.
- 37 Add 0.4X resuspended beads to pooled, barcoded samples (Step 30), for example, if you are pooling 12 samples with 2 barcode set up, which will be 24 libraries (which amounts to 480 μ L total), add 192 μ L of resuspended Sample Purification beads to the 480 μ L of pooled sample. Flick the tube or pipet up and down 10 times to mix to resuspend pellet. Perform a quick spin for 00:00:01 to collect all liquid from the sides of the tube.
- 38 Incubate samples on bench top for 00:10:00 at Room temperature .
- 39 Place the tube on a 1.5 ml magnetic stand (such as NEB S1506) to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 40 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note

Caution: do not discard the beads.

- 41 Wash the beads by adding 250 μ L Short Fragment buffer (SFB) . Flick the tube or pipet up and down to mix to resuspend pellet. If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 42 Place the tube on an appropriate magnetic stand for 4 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

1s



10m



1s





43 Repeat previous 2 steps once for a total of two washes:

Wash the beads by adding 250 µL Short Fragment buffer (SFB) . Flick the tube or pipet up and down to mix to resuspend pellet. If necessary, quickly spin the sample for 00:00:03 to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

Place the tube on an appropriate magnetic stand for 4 minutes (or when the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube, place back on the magnetic stand and remove traces of SFB with a p10 pipette tip

44 Add 500 µL 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at Room temperature for 00:00:30 , and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. **Repeat this wash step once for a total of two washes.**

45 Perform a quick spin and place the sample tube on the magnetic stand, to remove any residual ethanol.

46 Air dry the beads for up to 00:03:00 while the tube is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

47 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µL Nuclease-free water .

48 Resuspend the pellet by flicking the tube or pipetting up 10 times and down to mix. Incubate for at least 2 minutes at Room temperature . If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube before placing back on the magnetic stand.

3s




30s

3m



1s



49 Place the tube on the magnetic stand. After 2 minutes (or when the solution is clear), transfer  32 µL to a new 1.5 ml microcentrifuge DNA LoBind Tube or PCR tube.



50 **We recommend assessing cDNA concentrations with a Qubit fluorometer. Use 1 µl for the Qubit fluorometer.**



Note

Samples can be stored at  -20 °C if they are not used immediately.

Adapter Ligation


51 Add the following components into a 1.5 ml microcentrifuge DNA LoBind Tube or nuclease-free PCR tube:



A	B
COMPONENT	VOLUME
Dual barcoded and purified DNA (Step 45)	30 µl
Adapter Mix II (AMII)**	5 µl
(red) NEBNext Quick Ligation Reaction Buffer *	10 µl
(red) NEBNext Quick T4 Ligase	5 µl
<i>Total Volume</i>	50 µl

* Mix the NEBNext Quick Ligation Reaction Buffer by pipetting up and down several times prior to adding to the reaction.

** Adapter Mix II is provided by Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104), 13-24 (EXP-NBD114) and 1-96 (EXP-NBD-196) kits.

52 **Flick the tube** to mix solution. Perform a quick spin for  00:00:01 to collect all liquid from the sides of the tube.

1s



Note

Caution: The NEBNext Quick Ligation Buffer is viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.



53 Incubate at 25 °C or at Room temperature for 00:20:00 .

20m



54 Proceed to Cleanup of Adapter-ligated DNA in the next section.

Cleanup of Adapter Ligated DNA

20m 3s

55

Note

The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. AMPure XP beads can be used as well. If using AMPure XP beads, allow the beads to warm to

Room temperature **for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow.**

56 Vortex NEBNext Sample Purification Beads to resuspend.

57 Add 40 µL (0.8X) resuspended beads to the ligation mix. Mix well by flicking the tube to mix followed by a quick spin for 00:00:01 .

1s



58 Incubate samples for 00:15:00 at Room temperature .

15m


















59 Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

60 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA libraries.

Note

Caution: do not discard the beads.



- 61 Wash the beads by adding  250 μ L Short Fragment Buffer (SFB) . Flick the tube to mix to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. Place the tube on an appropriate magnetic stand. 
- 62 Wait for 5 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.
- 63 Repeat previous 2 steps once for a total of two washes:
Wash the beads by adding  250 μ L Short Fragment Buffer (SFB) . Flick the tube to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. Place the tube on an appropriate magnetic stand.
Wait for 5 minutes (or when the solution is clear) to separate the beads from the supernatant. Remove the supernatant. 
- Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of SFB with a p10 pipette tip.**
- 64 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding  15 μ L Elution Buffer (EB) provided in SQK-LSK109 kit from Oxford Nanopore. 
- 65 Resuspend the pellet well in EB buffer by flicking the tube. Incubate for  00:15:00 at  Room temperature . If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.   
- 66 Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer  15 μ L to a new DNA LoBind tube. 
- 67 Use Qubit to quantify  1 μ L DNA sample . Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION[®] flow cell and DNA library sequencing mix and load the flow cell. **We recommend not multiplexing more than 10 samples(9 samples + NTC) in one R9.4.1 flow cell.** We highly recommend using a Negative template control and label them as 'water', 'negative', 'blank', 'ntc' if using our custom analysis pipeline for the data analysis. 

The base calling options can be one among the three: Fast base calling, High accuracy base calling, or Super accurate base calling. We have observed improved average read quality with High accuracy and Super accurate base calling, but little difference in read



numbers, or variant calling compared to Fast base calling, using our custom analysis pipeline.