Version 2

Aug 30, 2022

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

Padmini Ramachandran[1], Tamara Walsky[1], Amanda Windsor[1], Chris Grim[1], Maria Hoffmann[1]

[1]FDA CFSAN

In Development Share

dx.doi.org/10.17504/protocols.io.3byl4bwervo5/v2

GenomeTrakr Coronavirus Method Development Community

Padmini Ramachandran

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.

DOI

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EXTERNAL LINK

https://www.neb.com/-/media/nebus/files/manuals/manuale7660.pdf?rev=48c42313dcb64b0dbb16c4bfd1563a27

PROTOCOL CITATION

Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Chris Grim, Maria Hoffmann 2022. Modified NEBNext® VarSkip Short SARS-CoV-2 Enrichment and library prep for Oxford Nanopore Technologies- adapted for wastewater samples. **protocols.io** https://protocols.io/view/modified-nebnext-varskip-short-sars-cov-2-enrichme-cfyitpue
Version created by Padmini Ramachandran

FORK NOTE

FORK FROM

Forked from NEBNext® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies®) E7660, Isabel Gautreau

KEYWORDS

NEBNext, NEB, ARTIC, SARS-CoV-2, Oxford, Nanopore, wastewater, SARS-CoV-2, Oxford, Nanopore, NEBNext

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GUIDELINES

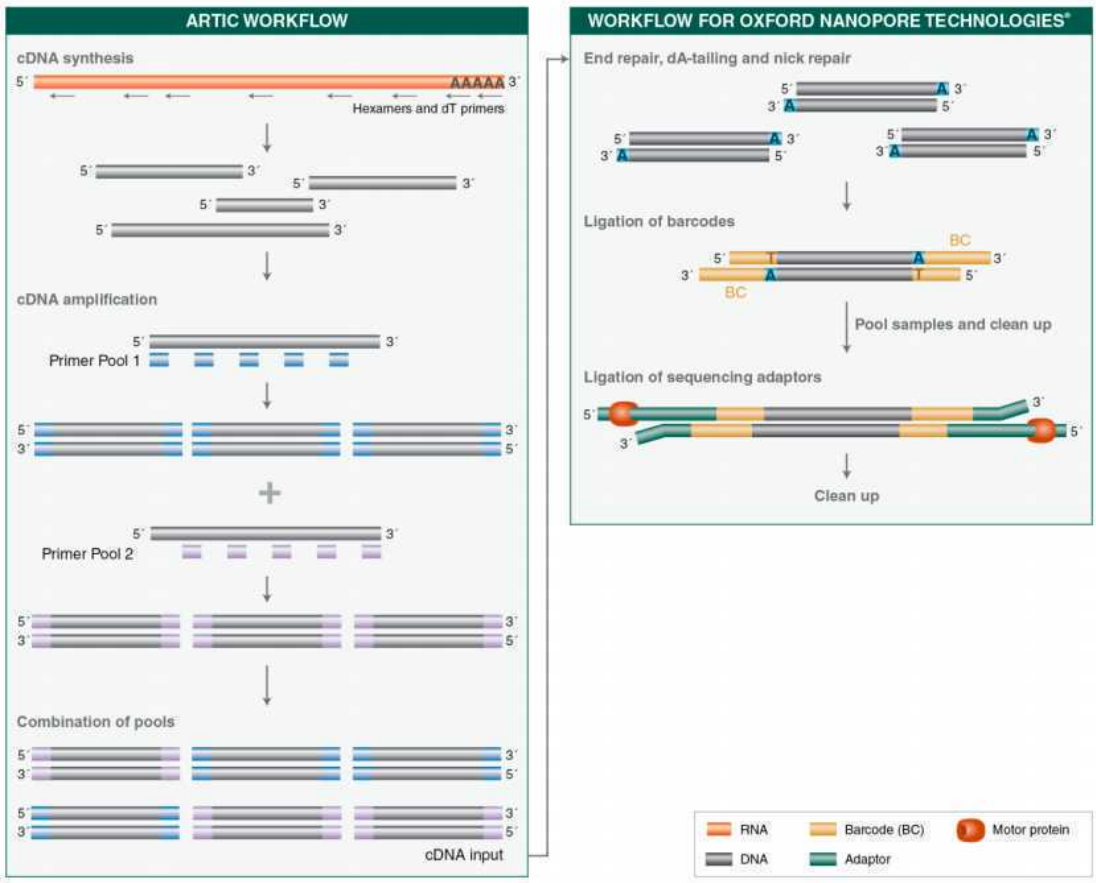
Overview

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 TEXT

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

This kit includes two sets of for primers:

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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)

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- 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

SAFETY WARNINGS

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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 STARTING

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

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
Total Volume	10 µl

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

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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
(lilac) LunaScript RT SuperMix	2 µl
Total Volume	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	∞	

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

Targeted cDNA Amplification

7

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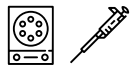
5 µl cDNA input is recommended. If using less than 5 µl of cDNA, add nuclease-free water to a final volume of 4.5 µl. We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of subsequent reactions.

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
Total Volume	13 µl

For Pool Set B:

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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
Total Volume	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.

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.

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.

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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 µL (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  10m

Incubate samples at **Room temperature** for **00:10:00** .

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.

Caution: do not discard the beads.

18  30s

Add **200 µL 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.

19  31s

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Repeat previous step once for a total of two washes:
Add **200 µL 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.

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 3m

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

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 µL Nuclease-free water**.

22 2m

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 µL** to clean PCR tubes.

24

Assess the concentration of the DNA targets. We recommend using a Qubit fluorometer for

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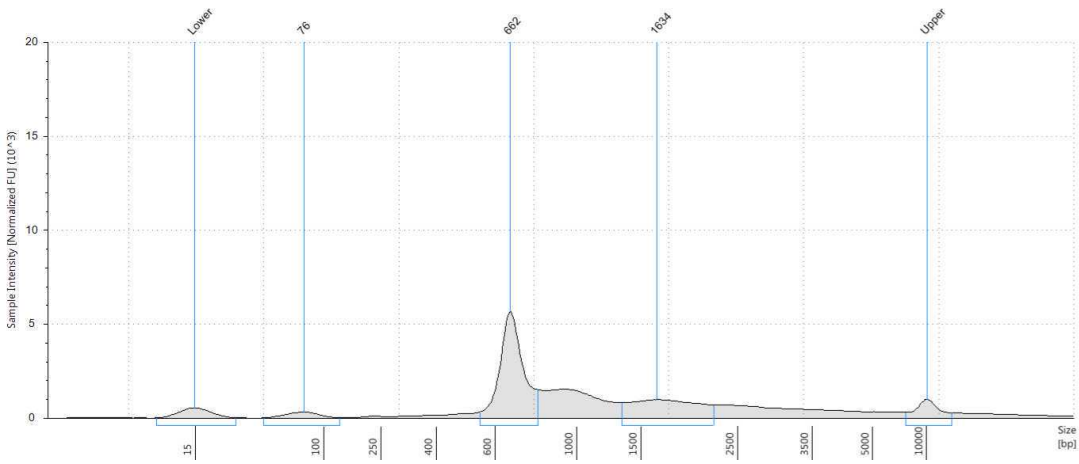
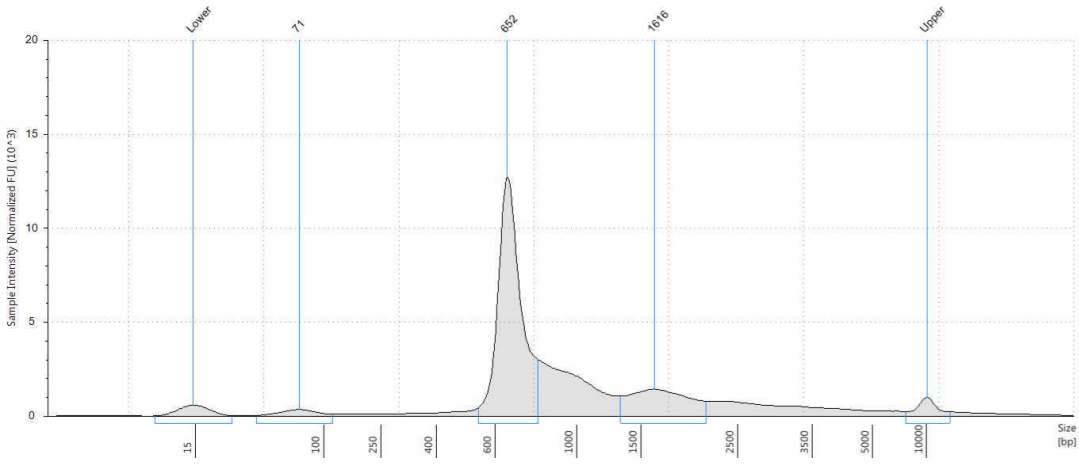
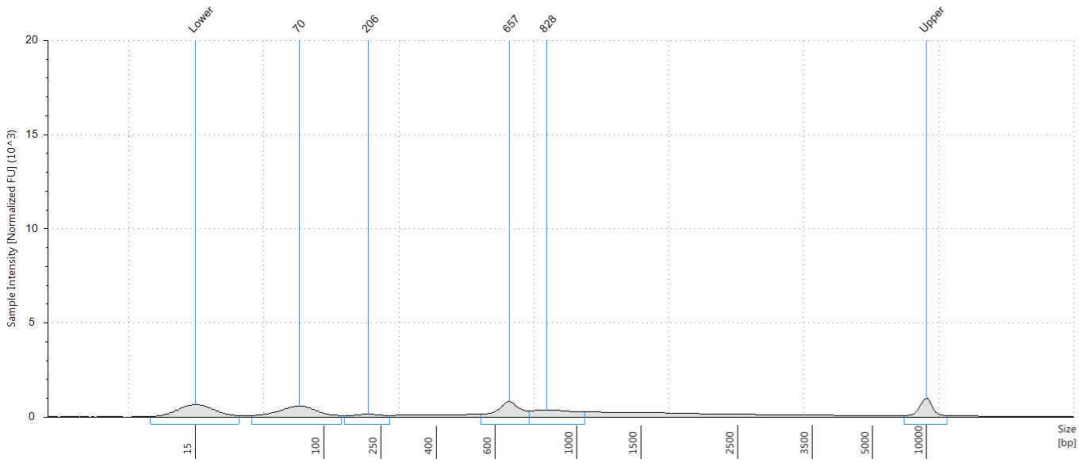
concentration assessment. Use 1 µl of sample for the Qubit fluorometer. Amplicons may also be run on a Bioanalyzer® or Tape Station using High Sensitivity (HS) 5000 tape or HS 1000 tape to confirm ~560-650 bp size of amplicons.



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

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

25

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
Total Volume	15 µl

27

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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.

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

28 20m

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**

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

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:

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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

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.

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

31m

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

25 °C for **00:20:00**

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65 °C for 00:10:00 .

Place On ice for 00:01:00 .

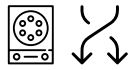
34 **Pool all barcoded samples into one 1.5 ml DNA LoBind Tube.**

Cleanup of Barcoded DNA

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

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  1s

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 µl total), add **192 µL of resuspended Sample Purification beads** to the 480 µl 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  10m

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.

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Be careful not to disturb the beads that contain DNA targets.

Caution: do not discard the beads.

41  1s

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 **00:00:01** 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.

43  3s

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. 30s
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.

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46 Air dry the beads for up to **00:03:00** while the tube is on the magnetic stand with the lid open. 3m

> **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  1s

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.

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.

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

Adapter Ligation

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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
Total Volume	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 1s

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

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 20m

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

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

Cleanup of Adapter Ligated DNA 20m 3s

55

The volumes of SPRIselect or NEBNext Sample Purification Beads provided

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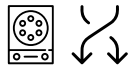
here are for use with the sample contained in the exact buffer at this step. AMPure XP beads can be used as well. If using AMPure XP beads, allow the beads to warm to Room temperature for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow.

56 Vortex NEBNext Sample Purification Beads to resuspend.

57  1s

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** .

58  15m

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

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.

Caution: do not discard the beads.

61 

Wash the beads by adding **250 µL 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.

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63

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 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 µL Elution Buffer (EB)** provided in SQK-LSK109 kit from Oxford Nanopore.

65

15m

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 µL** to a new DNA LoBind tube.

67

Use Qubit to quantify **1 µL 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.

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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.

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