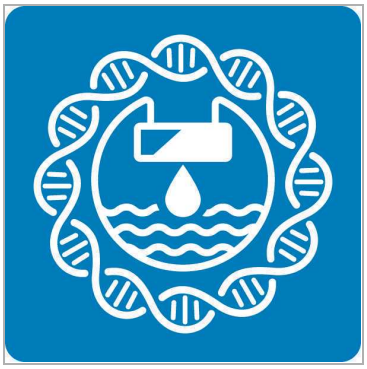

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External link:
https://www.neb.com/-/media/nebus/files/manuals/manuale7660.pdf?rev=48c42313dcb64b0dbb16c4bfd1563a27

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Protocol status: In development
We are still developing and optimizing this protocol

Modified NEBNext® VarSkip Long SARS-CoV-2 Enrichment and library prep (Native Barcoding Kit V14 Oxford Nanopore Technologies)- adapted for wastewater samples

Forked from Modified NEBNext® VarSkip Long SARS-CoV-2 Enrichment and library prep (SMRTbell prep kit 3.0 Pacific Biosciences)- adapted for wastewater samples

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Kathryn Judy

ABSTRACT

This protocol details methods for the preparation of SARS-CoV-2 sequencing library using VSL primers from NEB, adapted for wastewater samples. This protocol produces multiplexed amplicon libraries suitable for sequencing on Oxford Nanopore Technologies® (ONT) MinION systems using ONT V14 chemistry (SQK-NBD114).

GUIDELINES

Overview

Sequences and information on the NEBNext VarSkip Long primers can be found at https://github.com/nebiolabs/VarSkip. All other enzymes, buffers, beads and oligos required to convert cDNA into targeted, high quality libraries for next-generation sequencing on the Oxford Nanopore platform are available.

MATERIALS

We recommend multiplexing samples to lower sequencing cost. This protocol lists reagents for the Native Barcoding Kit 96 V14 (SQK-NBD114.96) but is compatible with the Native Barcoding Kit 24 V14 (SQK-NBD114.24). Reagents for VSL amplification must be purchased individually. Information on NEBNext® VarSkip Long primers is available at https://github.com/nebiolabs/VarSkip.

Kit Components

Native Barcoding Kit 96 V14 (SQK-NBD114.96) **Table of Components**

	A	B	C	D	E

Created: Apr 11, 2023

Last Modified: Apr 19, 2023

PROTOCOL integer ID: 80353

Keywords: NEBNext, NEB, SARS-CoV-2, wastewater, VarSkip Long, Oxford Nanopore

	A	B	C	D	E
	Component	Acronym	Quantity	Color	Volume per vial
	Native barcode plate	NB01-96	3	-	8 µl per well
	DNA control sample	DCS	3	Yellow	35 µl
	Native adapter	NA	2	Green	40 µl
	Sequencing buffer	SB	2	Red	700 µl
	Library beads	LIB	2	Pink	600 µl
	Library solution	LIS	2	White	600 µl
	Elution buffer	EB	1	Black	1500 µl
	AMPure XP beads	AXP	1	Amber	6000 µl
	Long fragment buffer	LFB	1	Orange	7500 µl
	Short fragment buffer	SFB	1	Clear	7500 µl
	EDTA	EDTA	1	Clear	700 µl
	Flow cell flush	FCF	1	Blue	15500 µl
	Flow cell tether	FCT	2	Purple	200 µl

SQK-NBD114.96 is an Early Access product. Reagent packaging (color, # vials) may vary

Required Materials Not Included

- **Q5® Reaction buffer** (NEB #B9027S)
- **Q5® Hot Start High-Fidelity DNA Polymerase** (NEB #M0493L)
- **NEBNext® VarSkip Long primer mixes 1** and **2** (NEB, https://github.com/nebiolabs/VarSkip)
- **50mM $MgCl_2$** (Thermo Fisher Scientific, Inc.® V0216 or equivalent)
- **Deoxynucleotide (dNTPs) Solution** (NEB #N0447L)
- Nuclease-free water, molecular biology grade
- AMPure® XP beads (Beckman Coulter A63880) or equivalent
- 80% Ethanol (freshly prepared, molecular biology grade)
- DNA LoBind Tubes (Eppendorf® #022431021)
- 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)
- **NEB Blunt/TA Ligase Master Mix** (NEB #M0367)
- **NEBNext Ultrall End repair/dA-tailing Module** (NEB #E7546)
- **NEBNext Quick Ligation Module** (NEB #E6056)

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 INSTRUCTIONS

Note: We recommend setting up a no template control reaction and **all reactions are set-up in a biological safety cabinet**.

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

To use this protocol, we recommend wastewater extraction using either of the protocols linked below. Extraction using the Promega Enviro Total Nucleic Acid Kit may be more robust to PCR inhibitors in wastewater. Other wastewater extraction methods have not been tested.

Protocol

NAME

Extraction of Total Nucleic Acid from Wastewater Using the Promega Wizard Enviro Total Nucleic Acid Kit

CREATED BY

Chris Grim

PREVIEW

Protocol

NAME

SARS-CoV-2 RNA extraction with Ceres Nanotrap and Zymo Environ Water

CREATED BY

Amanda Windsor

PREVIEW

Note

This protocol requires cDNA as input.

We recommend 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.). Before cDNA synthesis, samples must be DNase-treated (with Invitrogen™ ezDNase™ (Catalog number:11766051) or equivalent).

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.

Targeted cDNA Amplification

2

Note

4.5 µl cDNA input is recommended. If using less than 4.5 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.

3 Prepare master mixes fresh immediately before performing cDNA amplification.

- **Q5 Hot Start High-Fidelity Polymerase should stay on ice at all times. Do not vortex.**
- Thaw Q5 Reaction Buffer, $MgCl_2$, dNTPs, and water.
- Mix thawed tubes, spin down, and place on ice.
- Thaw VarSkip Long Primer Mix 1 and VarSkip Long Primer Mix 2.
- Mix by flicking and spin down both the tubes.
- Keep on ice.

Prepare the split pool amplification reactions as described below:

For Pool set A:
Prepare the master mix below in sufficient volume for your samples.

	A	B
	COMPONENT	VOLUME
	Q5 Reaction Buffer	2.5 µl
	50mM Magnesium Chloride	0.5 µl
	Deoxynucleotide (dNTP) Solution	0.75 µl
	Nuclease-free water	1.75 µl
	NEBNext VarSkip Long Primer Mix 1	2.25 µl
	Total Volume	7.5 µl

For Pool Set B:
Prepare the master mix below in sufficient volume for your samples.

	A	B
	COMPONENT	VOLUME
	Q5 Reaction Buffer	2.5 µl
	50mM Magnesium Chloride	0.5 µl
	Deoxynucleotide (dNTP) Solution	0.75 µl
	Nuclease-free water	1.75 µl
	NEBNext VarSkip Long Primer Mix 2	2.25 µl
	Total Volume	7.5 µl

Mix the two master mix tubes by flicking and spin down. Dispense 7.5 µl master mix from each tube into separate PCR tube strips (**A** and **B**), two PCR tubes (one for each master mix) per sample to amplify.

4 Add 4.5 µl cDNA into each pre-filled PCR tube, ensuring each sample to be amplified is added into exactly 1 tube in strip A and 1 tube in strip B.

5 While keeping the polymerase on ice, add 0.5 µL Q5 Hot Start High-Fidelity Polymerase to each tube.

6 Gently flick the tube strips to mix and spin down briefly.

7 Incubate **Pool A** 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	38
	Annealing	59°C	1 minute	
	Extension	72°C	2 minutes	
	Hold	4°C	∞	1

* Set heated lid to 105°C.

Incubate **Pool B** 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	38
	Annealing	61°C	45 seconds	
	Extension	72°C	2 minutes	
	Hold	4°C	∞	1

* Set heated lid to 105°C.

Note

Samples can be stored at 4 °C **if they are not used immediately.**

Cleanup of cDNA Amplicons

21m 1s

8 We highly recommend this clean up step using AMPure® XP beads, though NEBNext sample purification beads can be used as well.

Note

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.

9 For each sample, combine pool A and pool B PCR products (amplicons), measuring the pooled volume.

10 Vortex AMPure® XP beads for 00:00:30 to resuspend. 30s

11 Add 0.6 X resuspended AMPure® XP beads to the combined PCR product. Mix well by flicking the tube and a very short 2-3 seconds quick centrifugation. Be sure to stop the centrifugation before the beads start to settle out.

12 Incubate samples at Room temperature for 00:05:00. 5m

13 Quickly spin samples to collect the liquid from the sides of the tube before placing on the magnetic stand for 00:05:00 to separate the beads from the supernatant. 5m

14 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.

15 Add 200 µL freshly prepared 80% 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

16 Repeat previous step once for a total of two washes: 31s
Add 200 µL freshly prepared 80% 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.

17 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.

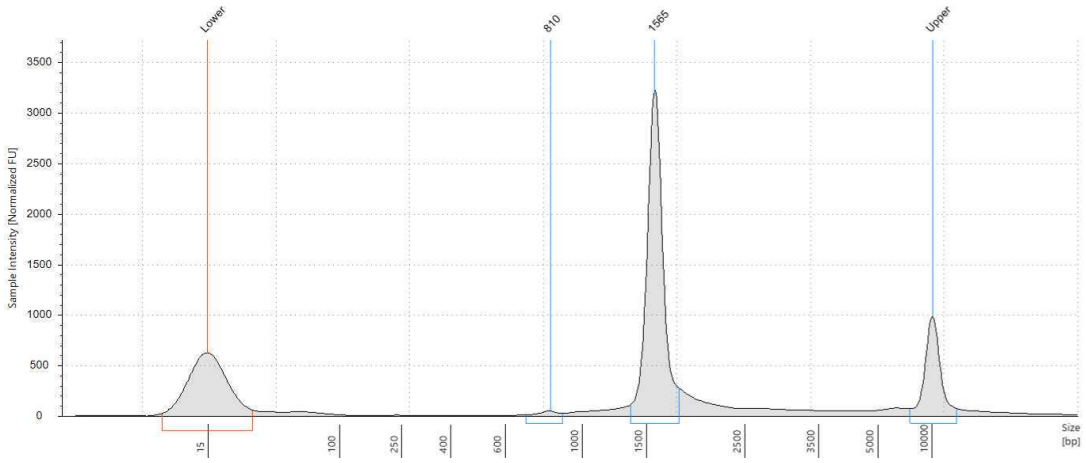
18 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 18 µL 0.1x TE buffer.

19 Mix well by flicking the tube followed by a very short centrifugation. Incubate for 00:05:00 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. 5m

20 Place the tube on the magnetic stand. After 00:02:00 (or when the solution is clear), transfer 17 µL to clean PCR tubes. 2m

21 Assess the concentration of the DNA targets. We recommend using a Qubit fluorometer for concentration assessment. Use 1 µl of sample for the Qubit fluorometer. Amplicons should also be run on Femto or Bioanalyzer® or Tape Station using High Sensitivity (HS) 5000 tape to confirm ~1500-1600 bp size of amplicons.

Note



A high positive wastewater sample.

Note

Samples can be stored at 4 °C if they are not used immediately.

End-Prep

22

Calculate the volume of each sample needed to bring forward at least 200 fmol DNA per sample using the amplicon size determined after cleanup. We recommend bringing forward approximately the same moles of DNA for each sample.

Aliquot the volume of each sample calculated into fresh PCR tubes and make up each sample to 11.5 µL using nuclease-free. Excess amplicons should be returned to 4 °C

23 Make the end repair and dA-tailing master mix by combining the reagents below in the order and amounts listed in the table. Adjust component volumes for your number of samples plus 20% overage.

- Thaw DNA Control Sample (DCS) at room temperature, vortex briefly, and place on ice. If this is the first use of the DCS tube, dilute by adding 105 µL elution buffer, mix gently by pipetting, and spin down. Diluted DCS can be stored at -20 °C after use in Step 24.
- Thaw Ultra II End-Prep Buffer at room temperature, then vortex and spin down briefly.
- Thaw Ultra II End Prep Enzyme mix on ice, spin down briefly, and return to ice. Do not vortex.

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

Mix the master mix components by pipetting or gentle flicking and quickly centrifuge. Master mix can remain stable On ice for 4 hours.

24 Add 1 µL diluted DCS to each sample, mix by gentle flicking, and spin down briefly.

25 Add 2.5 µL master mix to each sample, mix by gently flicking the tubes, and spin down briefly.

Incubate samples in a thermocycler* with the following settings:

26

	A	B	C	D
	TEMP	TIME	CYCLES	
	20°C	5 minutes	1	
	65°C	5 minutes	1	
	4°C	∞	1	

* Set heated lid to 105°C

Native Barcode Ligation and Cleanup

24m

27

Note

This cleanup requires AMPure XP beads at room temperature. Allow beads to come to room temperature for 30 minutes before use.

In PCR tubes or a 96-well plate, add reagents below **in the order listed** in the table.

- Thaw Blunt/TA Ligase master mix at room temperature, spin down 5 seconds, then mix with 10 full volume pipette mixes and place on ice. Do not vortex.
- Thaw EDTA at room temperature, mix by vortexing, spin down, and place on ice.
- Thaw Native Barcodes (ex: NB01-96) required for your number of samples at room temperature, individually mix by pipetting, spin down, and place on ice.
- **Add a unique barcode to each sample to be run together on a single flowcell (to be pooled).**
- 2-3 barcoding reactions per sample may be desired to increase input DNA. If multiple reactions per sample are performed, be sure to use the same barcode for the same sample in each reaction.

	A	B
	Component	Volume per Sample
	End-prepped DNA	3 µl
	Native Barcode	5 µl
	Blunt/TA Ligase master mix	5 µl

	A	B
	Total	13 µl

Mix components by gently flicking the tubes, then centrifuge briefly.

28 Incubate samples at Room temperature for 00:20:00 20m

29 Add 1 µL EDTA to each tube to stop the reaction. Mix well by flicking and spin down briefly.

30 Pool all barcoded samples to be run on a single flowcell in a 1.5 µl LoBind tube, measuring the volume each sample as it is added. Calculate the final volume of the pooled samples.

31 Add 0.4 X room temperature AMPure XP beads to each pool and mix by gently flicking followed by a short spin to collect the liquid. Stop the centrifugation before the beads begin to settle.

32 Incubate at room temperature for 00:10:00 to bind DNA to the beads, agitating the pool(s) every two minutes. If available, pools can be incubated on a Hula mixer (rotator mixer) to agitate the beads instead. 10m

33 Spin down the pool(s) and place tube(s) in an appropriate magnetic separation rack until the beads have separated, 00:05:00 5m

34 Carefully pipette off the supernatant without disturbing the beads, discarding the supernatant. **Do not discard the beads, which contain your DNA target**.

35 Wash the beads with 700 µL freshly prepared 80% ethanol. Pipette off the ethanol and discard. If the pellet was disturbed, wait for the beads to pellet again before pipetting off the

ethanol. **Do not discard the beads.**

36 Repeat the previous step once for a total of two washes:
Wash the beads with 700 µL freshly prepared 80% ethanol. Pipette off the ethanol and discard. If the pellet was disturbed, wait for the beads to pellet again before pipetting off the ethanol. **Do not discard the beads.**

37 To remove residual ethanol, quickly spin pool(s) and return the tube(s) to the magnetic rack, allowing beads to separate fully. Pipette off residual ethanol with a P20 pipette and discard. **Do not discard the beads.**

38 Remove samples from the magnetic rack and immediately add 35 µL nuclease-free water. Resuspend beads by flicking, then quickly spin to collect liquid.

39 Incubate pool(s) at 37 °C for 00:10:00 to elute DNA. Every two minutes, agitate the sample by gentle flicking for 10 seconds to encourage elution. 10m

40 Place samples on the magnetic rack until beads separate fully from the solution, 00:05:00 5m

41 Slowly pipette 35 µL of clear eluate without disturbing the beads and transfer to new 1.5 µl LoBind tube(s). Discard the old sample tube(s) with beads. **Do not discard the supernatant.**

Note

Cleaned barcoded samples can be safely stored at 4°C overnight.

Adapter Ligation and Cleanup

42

Note

This cleanup requires AMPure XP beads at room temperature. Ensure beads have warmed at room temperature for 30 minutes before use.

Add the following components to a 1.5 µl LoBind tube.

- Thaw NEBNext Quick Ligation Reaction Buffer at room temperature, pipette up and down several times to break up precipitate, vortex for several seconds, then spin down briefly.
- Spin down Quick T4 DNA Ligase, pipette mix, and place on ice. Do not vortex.
- Spin down Native Adapter, pipette mix, and place on ice.
- Thaw Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down, and place on ice.

	A	B
	Component	Volume per Sample
	Pooled barcoded sample	30 µl
	Native Adapter	5 µl
	NEBNext Quick Ligation Reaction Buffer	10 µl
	Quick T4 DNA Ligase	5 µl
	Total volume	50 µl

Thoroughly mix components by pipetting or gently flicking the tube, then quickly centrifuge to mix.

43 Incubate the reaction for 00:20:00 at room temperature. 20m

44
- Thaw elution buffer at room temperature.

Add 20 µL room temperature AMPure XP beads (0.4X) to each nuclease-treated sample and mix by gently flicking followed by a short spin to collect the liquid. Stop the centrifugation before the beads begin to settle.

45 Incubate at room temperature for 00:10:00 to bind DNA to the beads, agitating every two minutes. If available, the library can be incubated on a Hula mixer (rotator mixer) to agitate the beads instead. 10m

46 Spin down and place tube in an appropriate magnetic separation rack until the beads have separated, 00:05:00 5m

47 Carefully pipette off the supernatant without disturbing the beads, discarding the supernatant. **Do not discard the beads, which contain your DNA target**.

48 Add 125 µL Short Fragment Buffer to each sample tube. Flick the tube to resuspend beads, collect liquid with a quick spin, and return the tube to the magnetic rack.

When the beads have pelleted, remove the supernatant and discard without disturbing the beads. **Do not discard the beads.**

49 Repeat the previous step once for a total of two washes:
Add 125 µL Short Fragment Buffer to each sample tube. Flick the tube to resuspend beads, collect liquid with a quick spin, and return the tube to the magnetic rack.

When the beads have pelleted, remove the supernatant and discard without disturbing the beads. **Do not discard the beads.**

50 To remove residual Short Fragment Buffer, quickly spin the tube and return to the magnetic rack, allowing beads to separate fully. Pipette off residual supernatant with a P20 pipette and discard. **Do not discard the beads.**

51 Remove the tube from the magnetic rack and immediately add 15 µL elution buffer. Resuspend beads by flicking, then quickly spin to collect liquid.

52 Incubate at 37 °C for 00:10:00 to elute DNA. Every two minutes, agitate the sample by gentle flicking for 10 seconds to encourage elution. 10m

53 Place tube on the magnetic rack until beads separate fully from the eluate, usually less than 00:05:00

54 Slowly pipette 15 µL of clear supernatant without disturbing the beads and transfer to a new LoBind tube. Discard the old tube with beads. **Do not discard the supernatant.**

55 Measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit. The final library should also be run on Femto or Bioanalyzer® or Tape Station using High Sensitivity (HS) 5000 tape to confirm the size of the final library.

Note

The final library can be safely stored at 4°C overnight. For long-term storage of more than 3 months, store libraries at -80°C.

End Protocol

56 Based on the concentration and library size determined in step 55, aliquot 10-20 fmol final library and make up to 12 µL using nuclease-free water in a new 0.5 µl LoBind tube. This will be used to load the flowcell.

Prime and load the R10.4.1 (FLO-MIN114) SpotON flowcell following the Oxford Nanopore SQK-LSK114 protocol. We recommend using High Accuracy Basecalling (HAC) at 260bps ("Accurate") speed.