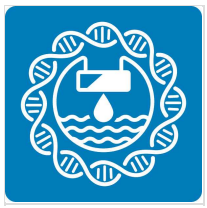

3

Mar 31, 2022

Modified NEBNext® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples V.3

Standard Protocol NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®) (NEB #E7650S/L)

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

[1]FDA CFSAN

dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

GenomeTrakr Coronavirus Method Development Community

Chris Grim

PURPOSE:
This method was developed at the FDA's Center for Food Safety and Applied Nutrition for GenomeTrakr's pandemic response project, monitoring SARS-CoV-2 variants in wastewater. Protocols developed for this project cover wastewater collection, concentration, RNA extraction, RT-qPCR, library prep, genome sequencing, quality control checks, and data submission to NCBI.

This modified protocol details methods for cDNA synthesis and library preparation for sequencing of wastewater samples containing SARS-CoV-2. The protocol is based primarily on the NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®), NEB #E7650S/L 24/96 reactions, with a few modifications. Primarily, VarSkip Short primers are used in place of the ARTIC V3 primers. These primers are available in the NEBNext®ARTIC SARS-CoV-2 FS Library Prep Kit (Illumina®); however, for optimal variant detection from wastewater, sequenced fragments should be as large as possible, so we discourage fragmentation prior to end prep.

There are a couple of decision points in this protocol. Examining cDNA amplicon samples on an Agilent TapeStation system or similar fragment analyzer is extremely helpful in making these decisions.

Version Updates: V3- Controlled vocabulary for positiveControl and negativeControl has been added to the Sample Sheet.

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÃÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

DOI

dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim 2022. Modified NEBNext® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3
Padmini Ramachandran

Standard Protocol NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®) (NEB #E7650S/L), Isabel Gautreau

SARS-CoV-2, Library Prep, NEB, COVID, VarSkip Short, wastewater

protocol ,

Mar 31, 2022

Mar 31, 2022

Mar 31, 2022 Chris Grim

60136

Overview

The NEBNext SARS-CoV-2 Library Prep Kit (Illumina) contains the enzymes, buffers and oligos required to convert a broad range of total RNA input amounts into targeted, high quality libraries for next-generation sequencing on the Illumina platform. Primers targeting the human EDF1 (NEBNext ARTIC Human Primer Mix 1) and NEDD8 (NEBNext ARTIC Human Primer Mix 2) genes are supplied as optional internal controls. The fast, user-friendly workflow also has minimal hands-on time.

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 Illumina 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.

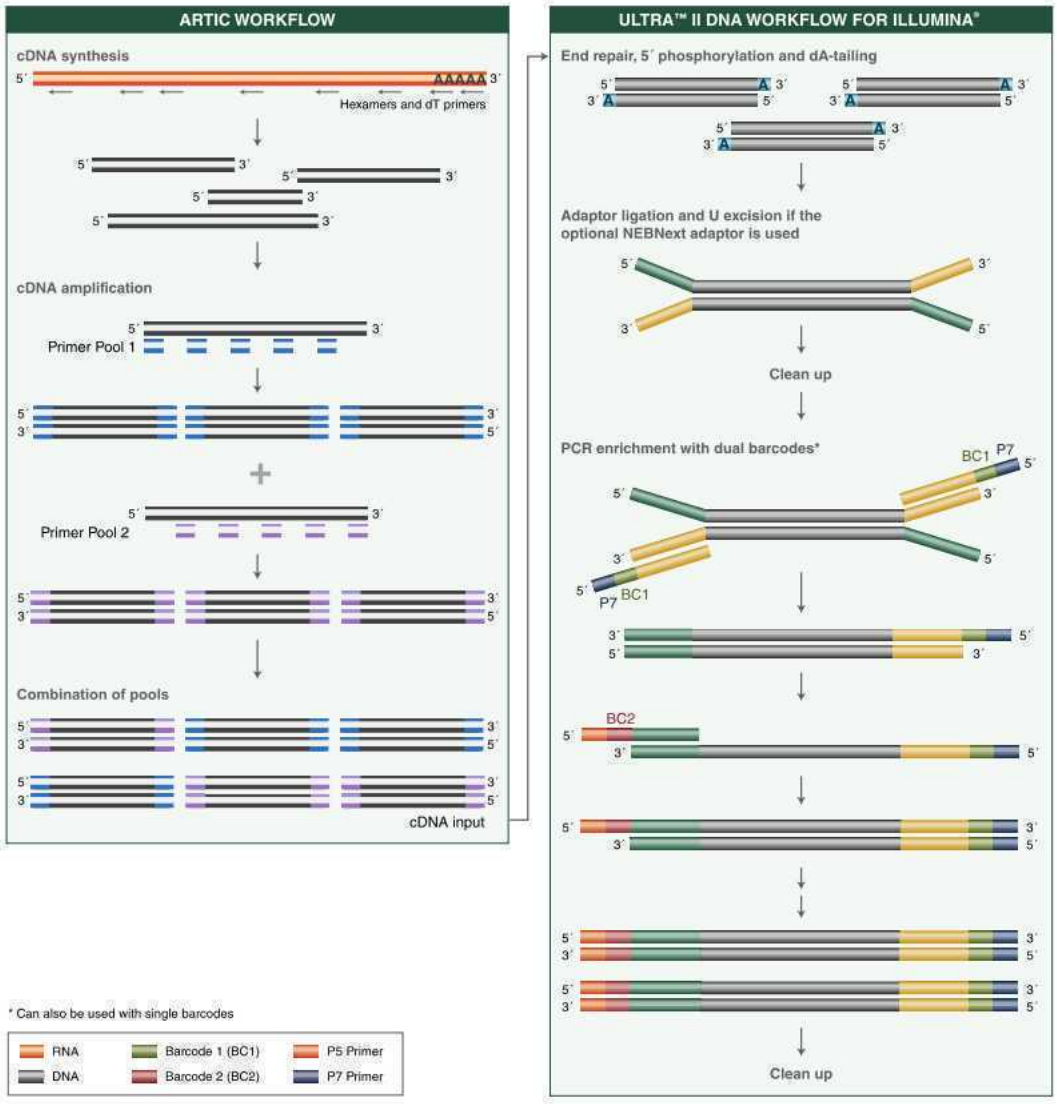

Figure 1. Workflow demonstrating the use of NEBNext SARS-CoV-2 Library Prep Kit for Illumina

Kit:

NEBNext®ARTIC SARS-CoV-2 RT-PCR Module- E7626S/L-

This kit includes two sets of for 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 in this kit.

2. The V3 ARTIC primers have been balanced, using methodology developed at NEB based on

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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)**

Once the amplicon is generated, the library prep of the amplicons can be done with either/or

Option 1) NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®)- E7650S/L
Option 2) Illumina® DNA Prep, (M) Tagmentation (24/96 Samples) 20018704/20018705

The Library Kit Includes:
The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7650S) and 96 reactions (NEB #E7650L).

Package 1: Store at -20 °C

- LunaScript™ RT SuperMix (E7651) — Store at -20°C Q5®
- Hot Start High-Fidelity 2X Master Mix (E7652) — Store at -20°C
- NEBNext® ARTIC SARS-CoV-2 Primer Mix 1 (E7725) — Store at -20°C **(not used)**
- NEBNext® ARTIC SARS-CoV-2 Primer Mix 2 (E7726) — Store at -20°C **(not used)**
- NEBNext® ARTIC Human Control Primer Pairs 1 (E7727) — Store at -20°C **(not used)**
- NEBNext® ARTIC Human Control Primer Pairs 2 (E7728) — Store at -20°C **(not used)**
- NEBNext® Ultra II™ End Prep Enzyme Mix (E7653) — Store at -20°C
- NEBNext® Ultra II™ End Prep Reaction Buffer (E7654) — Store at -20°C
- NEBNext® Ultra II™ Ligation Master Mix (E7655) — Store at -20°C
- NEBNext® Library PCR Master Mix (E7656) — Store at -20°C
- 0.1X TE (E7657) — Store at -20°C
- Nuclease-free Water (E7667) — Store at -20°C

Package 2: Store at Room temperature . Do not freeze.
NEBNext Sample Purification Beads

Required Materials Not Included:
- NEBNext Multiplex Oligos for Illumina – www.neb.com/oligos- E6440S- Dual index primer pairs
- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf #022431021)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermocycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)

Either one of the index kit for Illumina DNA prep
- IDT® for Illumina® DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) 20027213
- IDT® for Illumina® DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

20027214
- IDT® for Illumina Nextera DNA Unique Dual Indexes Set C (96 Indexes, 96 Samples) 20027215
- IDT® for Illumina Nextera DNA Unique Dual Indexes Set D (96 Indexes, 96 Samples) 20027216

Please refer to the 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.

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, however, results may vary depending on the quality of the input. In addition, we recommend setting up a no template control reaction and **that 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.

cDNA Synthesis

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.**

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.).

2

Gently mix and spin down the LunaScript RT SuperMix reagent. Prepare the cDNA synthesis reaction as described below:

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

A	B
COMPONENT	VOLUME
RNA Sample	8 µl
(lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

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

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

4

Incubate reactions in a thermocycler* with the following steps:

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

*Set heated lid to 105°C

Samples can be stored at -20 °C for up to a week.

Targeted cDNA Amplification

5

4.5 µl cDNA input is recommended. If using less than 4.5 µl of cDNA, add

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÃÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
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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 future reactions. The cDNA reaction can be set up in hood to minimize cross-contamination.

6 

Gently mix and spin down reagents. Prepare the split pool cDNA amplification reactions as described below:

For Pool Set A:

A	B
COMPONENT	VOLUME
cDNA (Step 2)	4.5 µl
(lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	1.75 µl
Total Volume	12.5 µl

For Pool Set B:

A	B
COMPONENT	VOLUME
cDNA (Step 2)	4.5 µl
(lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	1.75 µl
Total Volume	12.5 µl

7 Flick the tubes or pipet up and down 10 times to mix followed by a quick spin.

8 

Incubate reactions in a thermocycler* with the following steps:

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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

9

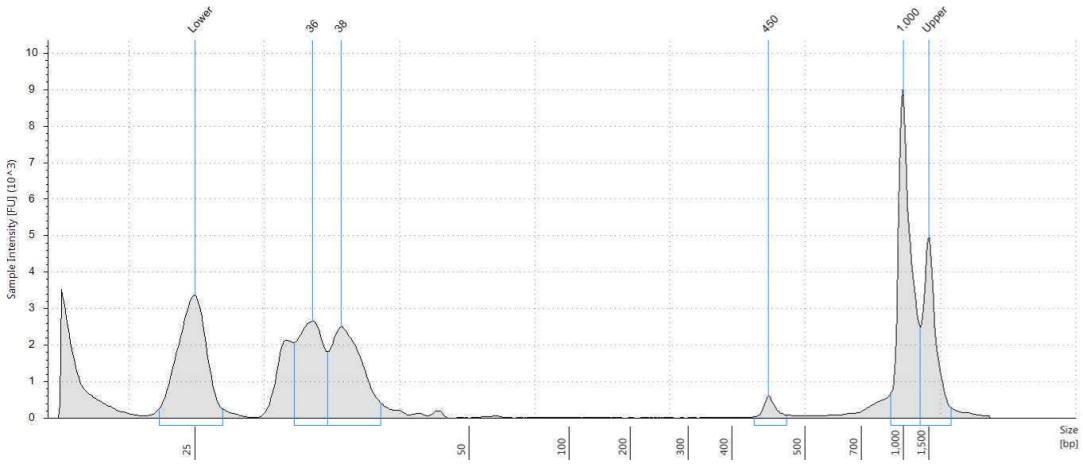
Combine the Pool A and Pool B PCR reactions for each sample.

Samples can be stored at -20 °C for up to a week.

10 Assess the quantity of the cDNA amplicon using the Qubit HS kit. (

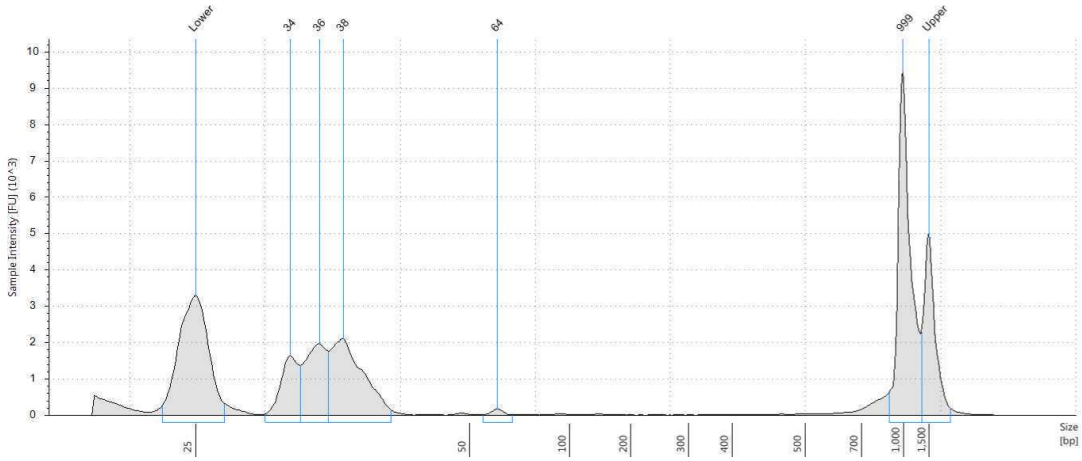
1 µL each combined pool

11 Assess the size of the cDNA amplicon using Agilent TapeStation System, Bioanalyzer, or similar size fragment analyzer.

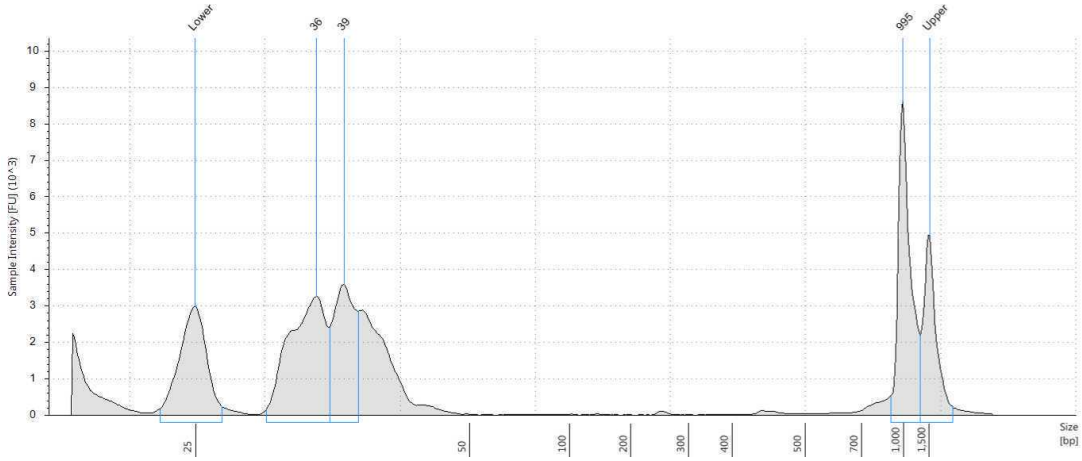


TapeStation profile of cDNA amplicons of positive wastewater sample

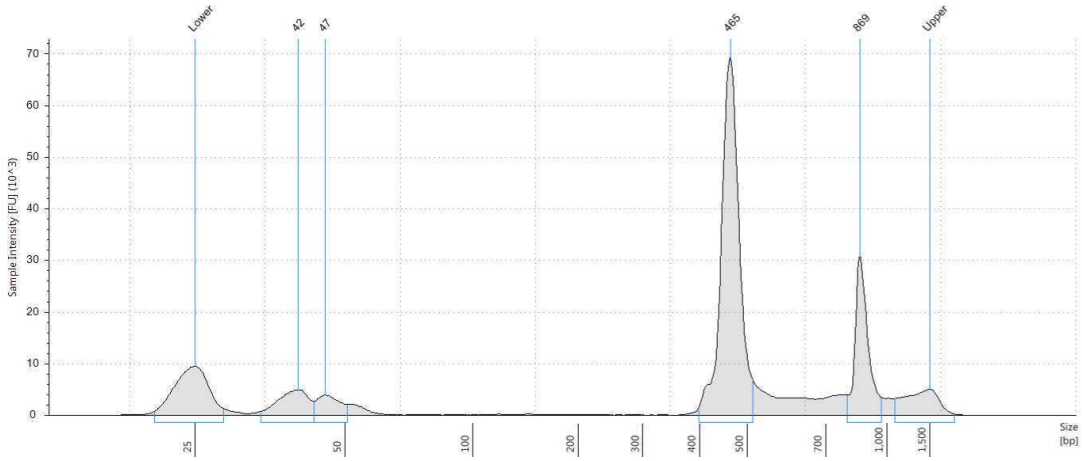
Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÃÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3



TapeStation profile of cDNA amplicons of high Ct value or degraded wastewater sample. Notice measurable peak at 64 bp and unmeasurable peaks to the right.



TapeStation profile of cDNA amplicons of high Ct value or degraded wastewater sample. Notice unmeasurable peaks at approximately 250 and 450 bp.



TapeStation profile of cDNA amplicons of Twist SARS-CoV-2 control

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÃÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

12

Based on the viral load in the sample, you will see a spectrum of fragment profiles. If there is a single peak around 400-600 bp, the clean-up step can be omitted and you can proceed directly to library preparation using Illumina DNA Prep
https://www.protocols.io/edit/modified-illumina-dna-prep-m-tagmentation-library-b2yzqfx6

If there is not a single peak, check the amplicon size distributions. If there are lots of smaller peaks, for example, we typically see one at 44, 75, 128, and 213, these PCR reaction pools need to be cleaned up. Follow the cleanup protocol and prepare libraries using the Ultra II DNA workflow by NEB Next ARTIC SARS-CoV-2 Library Prep Kit (Illumina). If you are not able to perform the PCR pool assessment above, proceed to PCR cleanup and prepare libraries using the Ultra II DNA workflow by NEB Next ARTIC SARS-CoV-2 Library Prep Kit (Illumina).

Cleanup of cDNA Amplicons.

13

The volume of NEBNext Sample Purification Beads provided here are for use with the sample composition at this step (25 µl; Step 6). These bead volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions the volumes may need to be experimentally determined.

14 Vortex the NEBNext Sample Purification Beads to resuspend.

15

Add **20 µL (0.8X) resuspended beads** to the combined PCR reaction. Mix well by

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pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

16 

5m

Incubate samples at **Room temperature** for at least **00:05:00** .

17 

Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant.

18 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.

19 

30s

Add **200 µL 80% freshly prepared ethanol** to the tube/plate 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.

20 

30s

Perform second Ethanol wash:

Add **200 µL 80% freshly prepared ethanol** to the tube/plate 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/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

21 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid

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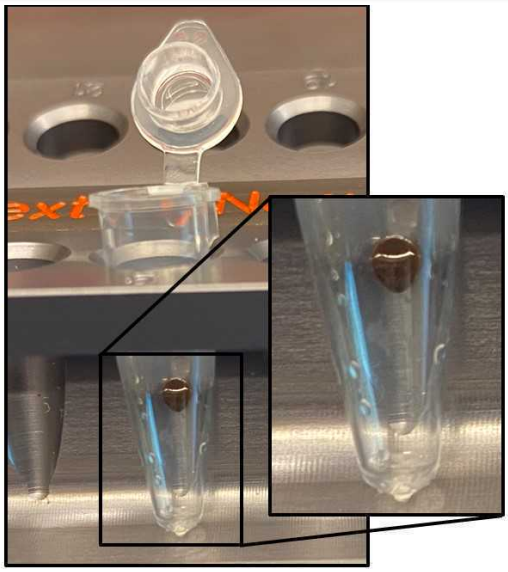
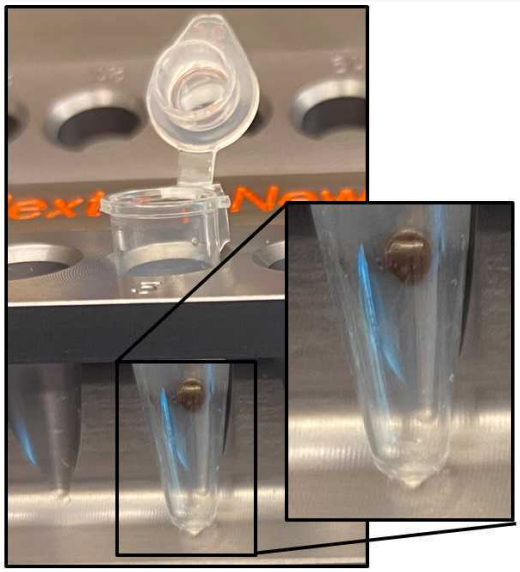
open.

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, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

22

Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **28 µL 0.1X TE** . If not assessing cDNA (Step 19) elute DNA in **27 µL 0.1X TE** .

23

Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes 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.

24

Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer **26 µL** to a new PCR tube. If not assessing cDNA (Step 19) transfer **25 µL** to a new PCR tube.

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

25

We recommend assessing cDNA amplicon (from Step24) concentrations with a Qubit fluorometer.

Amplicons may also be run on a Bioanalyzer or TapeStation® to confirm 400 bp size of amplicons. To run on a Bioanalyzer, dilute amplicon 10-fold with 0.1X TE Buffer and run 2 µL on a DNA High Sensitivity ScreenTape. (See Figure 19 below for example of amplicon size profile on a Tapestation).

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

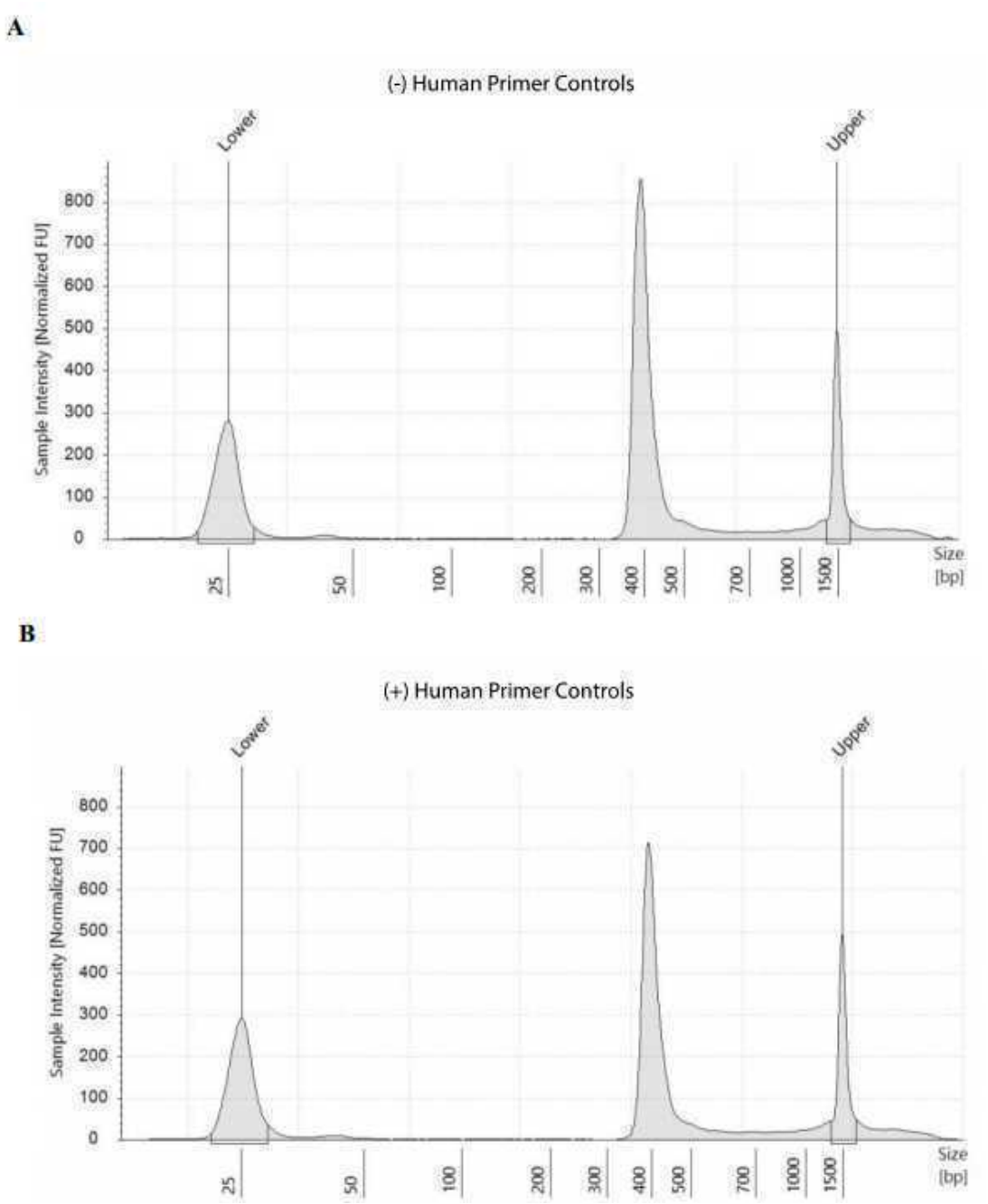

Figure 19: Example of cDNA amplicons generated from 1000 genome copies of SARS CoV-2 in the absence (A) and presence (B) of human primer controls.

Samples can be stored at -20 °C for up to a week.

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Quality checks on cleaned amplicons before proceeding to library prep

26 **We have observed the following output for VSS amplicons based on viral titer in the wastewater samples:**

1)Moderate to high viral titer samples: The cleaned amplicon quantity is expected to be **0.5 ng/µl to 8 ng/µl**

2)Low viral titer samples: The cleaned amplicon quantity is expected to be **0 - 0.2 ng/µl**

We recommend to proceed to library preparation for samples at 0.2 ng/µl or higher. Cleaned VSS amplicons below this value have not yielded sequencing results.

27 We recommend assessing cDNA amplicon (from Step 23) on Tape Station or Bioanalyzer using High Sensitivity kit.

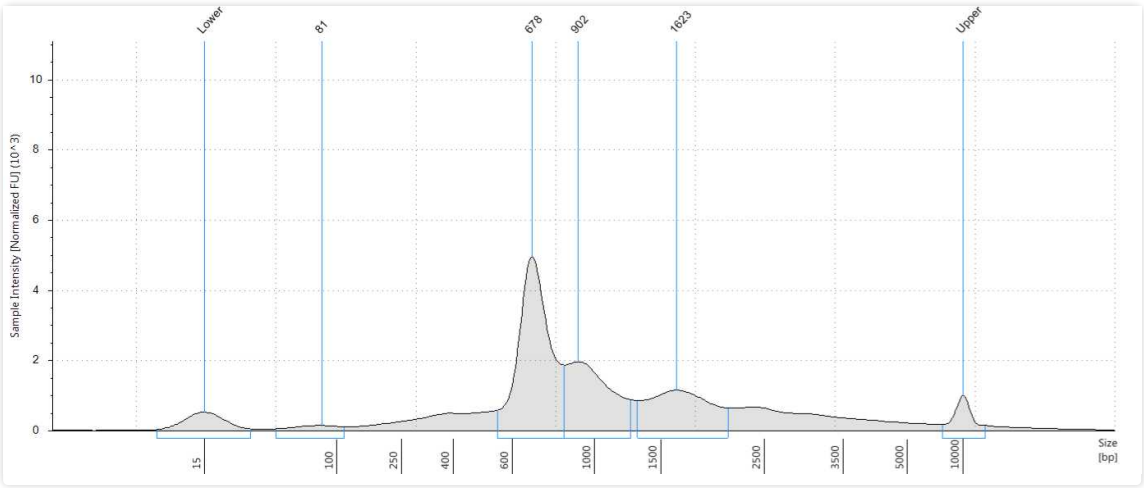

Figure 20: Tape station profile of cleaned amplicon from a moderate to high positive sample.

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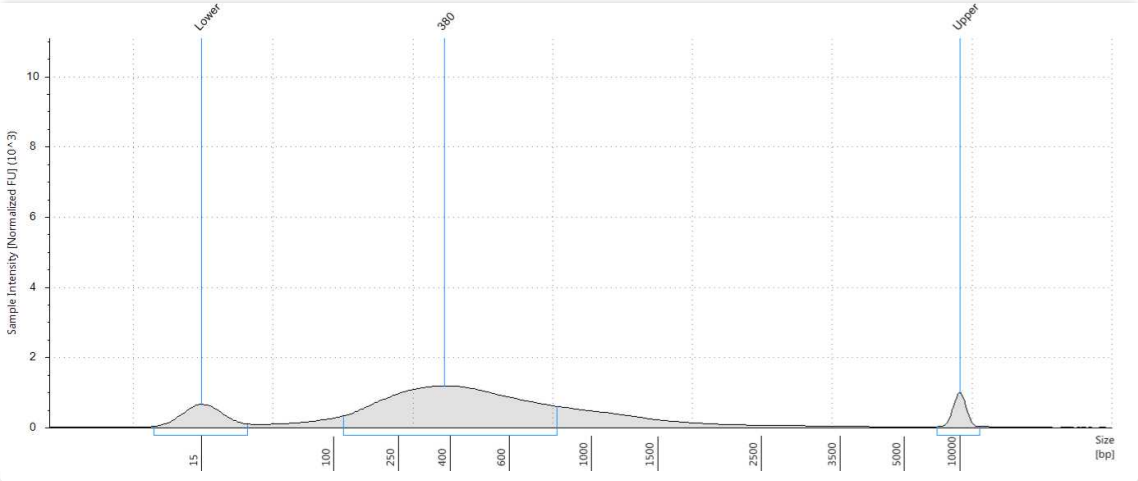

Figure 21: Tape station profile of cleaned amplicon from a low positive sample.

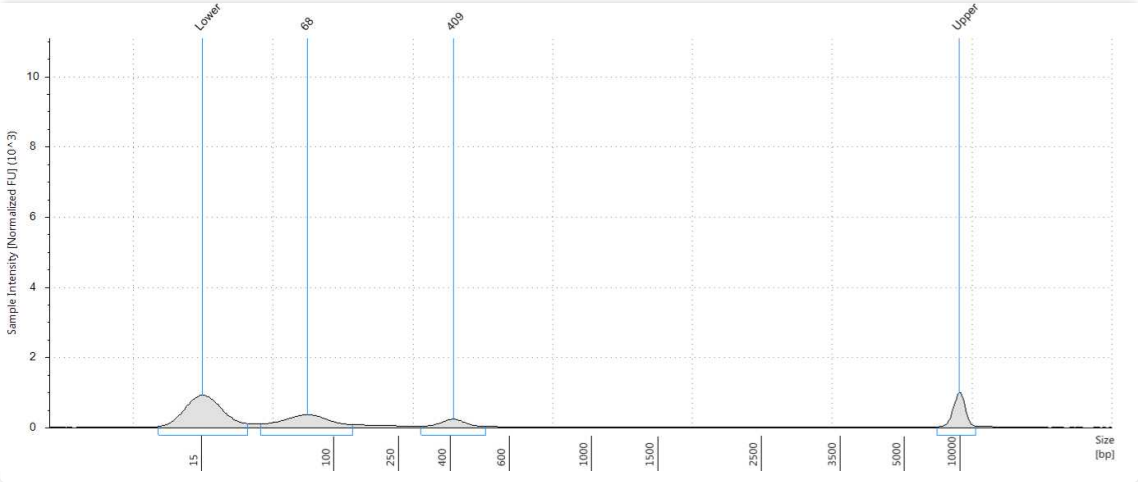

Figure 22: Tape station profile of cleaned amplicon from a low positive sample measured at ~0 ng/ul.

NEBNext End Prep

28 ⚠

This is library preparation Option 1, and the most common that will be used. You will use the Ultra II DNA workflow as described in the NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®) (cat# E7650S/L), with NEBNext®Multiplex Oligos for Illumina®(96 Unique Dual Index Primer Pairs) (cat# E6440S/L).

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29

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

A	B
COMPONENT	VOLUME
(green) NEBNext Ultra II End Prep Enzyme Mix	1.5 µl
(green) NEBNext Ultra II End Prep Reaction Buffer	3.5 µl
Targeted cDNA Amplicons*	25 µl
Total Volume	30 µl

*When cleanup of the pooled cDNA amplicons is skipped, the amplicons must be diluted prior to library prep, please dilute the cDNA as mentioned here. Transfer 2.5 µl of the pooled cDNA amplicons to a fresh tube. Add 22.5 µl of 0.1X TE for a final volume of 25 µl. If cleanup is performed, use the cleaned targeted cDNA amplicons as the input.

30

Set a 100 µl or 200 µl pipette to 25 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. 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

31

Place in a thermocycler* and run the following program:

A	B
TEMP	TIME
20°C	30 minutes
65°C	30 minutes
4°C	∞

*Set heated lid to 75°C

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

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

Adaptor Ligation

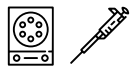
32 

Add the following components directly to the End Prep Reaction Mixture:

A	B
COMPONENT	VOLUME
End Prep Reaction Mixture (previous step)	30 µl
(red) NEBNext Adaptor for Illumina**	1.25 µl
(red) NEBNext Ultra II Ligation Master Mix*	15 µl
Total Volume	46.25 µl

* Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.
** The NEBNext adaptor is provided in NEBNext Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Do not premix adaptor with the Ligation Master Mix.

33 

Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext Ultra II Ligation 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.

34 

15m

Incubate at **20 °C** for **00:15:00** in a thermocycler with the heated lid off.

35 

Add **1.5 µL (red or blue) USER® Enzyme** to the ligation mixture from the previous step.

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÃÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

Steps 26 and 27 are only required for use with NEBNext non-indexed Adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

36 15m

Mix well and incubate at **37 °C** for **00:15:00** with the heated lid set to ≥ **47 °C** .

Samples can be stored overnight at -20 °C .

Cleanup of Adaptor-ligated DNA

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

The volume of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step (47.75 µl; Step 27). These bead volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions the volumes may need to be experimentally determined.

38 Vortex the NEBNext Sample Purification Beads to resuspend.

39

Add **43 µL (0.9X) resuspended beads** to the Adaptor Ligation reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

40 5m

Incubate samples on bench top for at least **00:05:00** at **Room temperature** .

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https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

41 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant.

42 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 beads.

43  30s

Add **200 µL 80% freshly prepared ethanol** to the tube/ plate 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.

44  30s

Repeat the previous step once for a total of two washes:

Add **200 µL 80% freshly prepared ethanol** to the tube/ plate 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/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

45 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

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.

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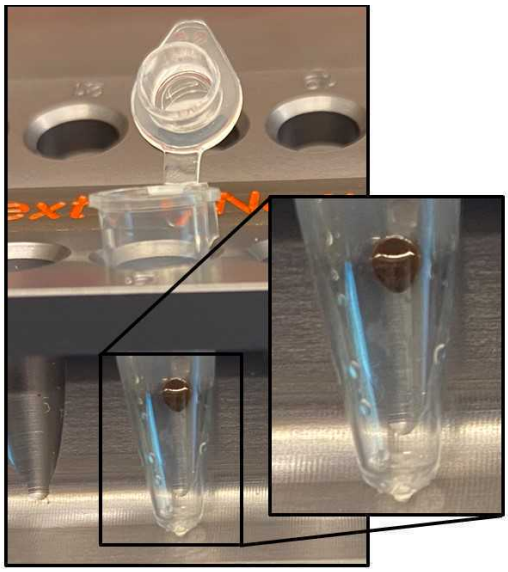
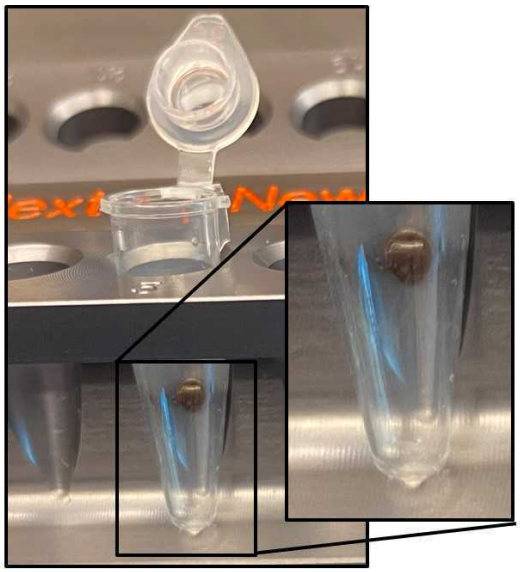


After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

46

Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **10 µL 0.1X TE** .

47

2m

Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least **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.

48

5m

Place the tube/plate on the magnetic stand. After **00:05:00** (or when the solution is clear), transfer **7.5 µL** to a new PCR tube.

Samples can be stored at -20 °C .

PCR Enrichment of Adaptor-ligated DNA

49

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Modified NEBNextÂÂ® VarSkip Short SARS-CoV-2 Library Prep Kit for Illumina Platforms - adapted for wastewater samples
https://dx.doi.org/10.17504/protocols.io.5jyl89n26v2w/v3

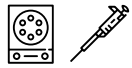
We only recommend using NEBNext Oligo kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied ay 10 µM (combined). We observed addtion of spurious nucleotides when using primers supplied in separate tubes

50 

Add the following components to a sterile strip tube:

A	B
COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 45)	7.5 µl
(blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer Mix (E6440S)*	5.0 µl
Total Volume	25 µl

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

51 

Set a 100 µl pipette to 20 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

52 

Place the tube on a thermocycler** and perform PCR amplification using the following PCR cycling conditions:

A	B	C	D
CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	6-8*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles recommended should be viewed as a starting point and may need to be optimized for particular sample types. If you have cleaned up cDNA as input, use 8 cycles. If you have diluted cDNA as input use 6/7 cycles.
**Set heated lid to 105°C.

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Cleanup of PCR Reaction 8m

53

The NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. These 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.

54 Vortex NEBNext Sample Purification Beads to resuspend.

55 

Add **22.5 µL (0.9X) resuspended beads** to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

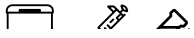
56  5m

Incubate samples on bench top for at least **00:05:00** at **Room temperature** .

57 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant.

58 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.

 30s

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59

Add **200 µL 80% freshly prepared ethanol** to the tube/plate 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.

60 30s

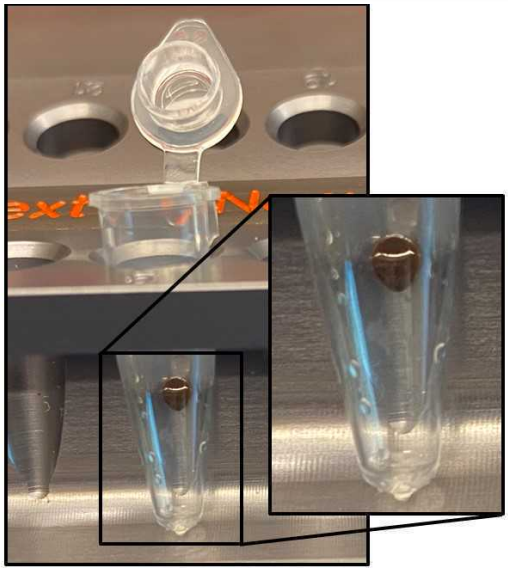
Repeat the previous step once for a total of two washes:

Add **200 µL 80% freshly prepared ethanol** to the tube/plate 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.

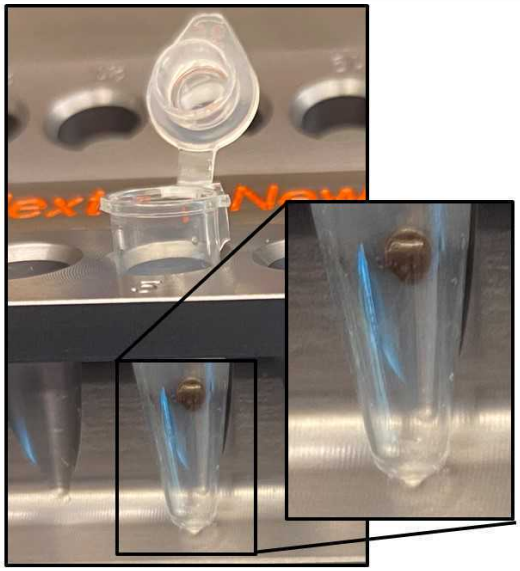
Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

61 Air dry the beads for up to 5 minutes while the tube/plate 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, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

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62 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **17 µL** **0.1X TE** .

63 2m

Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least **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.

64

Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer **15 µL** to a new PCR tube and store at **-20 °C** .

65

Check the size distribution on an Agilent Bioanalyzer or TapeStation. The sample may need to be diluted before loading. A peak size of ~520 bp is expected (Figure 56).

Samples can be stored at -20 °C or proceed to MiSeq denature and dilution guideline to load it on to the MiSeq.

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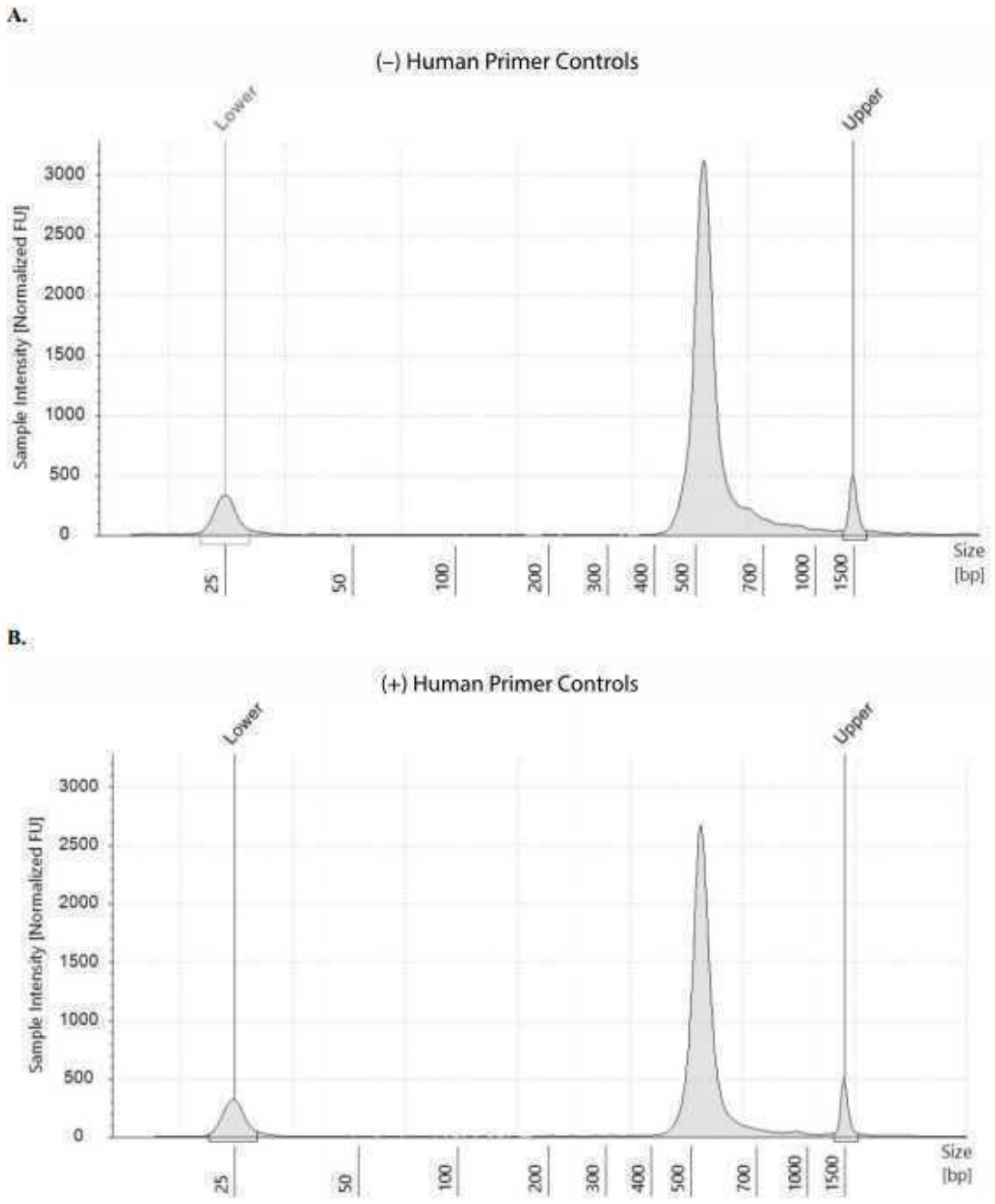

Figure 56: Example of final library size distributions on a TapeStation. ARTIC SARS-CoV-2 libraries were generated from 1000 viral copies in the absence (A) or presence (B) of the human primer controls.

66 Sample Sheet generation for NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®) with E6440 Index plate. Controlled vocabulary for labelling/naming positiveControl and negativeControl has been added to the sample sheet.

E6440_ Forward Strand Workflow Sample Sheet (1).csv

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