NEBNext® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies®)

NEB #E7660S/L

24/96 reactions

Version 5.0_9/21

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The Kit Includes

The volumes provided are sufficient for preparation of up to 24 barcoding reactions (NEB #E7660S, minimum 6 barcoding samples per run for total 4 runs) and 96 barcoding reactions (NEB #E7660L, minimum 24 barcoding samples per run for total 4 runs). Colored bullets represent the color of the cap of the tube containing the reagent. If one plans to follow a different protocol, additional reagents can be purchased separately)

Package 1: Store at –20°C.

- (lilac) LunaScript® RT SuperMix
- (lilac) Q5® Hot Start High-Fidelity 2X Master Mix
- (lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 1
- (lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 2
- (lilac) NEBNext ARTIC Human Control Primer Pairs 1
- (lilac) NEBNext ARTIC Human Control Primer Pairs 2
- (orange) NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1
- (orange) NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (green) NEBNext Ultra II End Prep Reaction Buffer
- (red) Blunt/TA Ligase Master Mix
- (red) NEBNext Quick T4 DNA Ligase
- (red) NEBNext Quick Ligation Reaction Buffer
- (white) Nuclease-free water

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

NEBNext Sample Purification 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), 13-24 (EXP-NBD114) or 1-96 (EXP-NBD196)
- Oxford Nanopore Technologies Ligation Sequencing Kit (SQK-LSK109) or Oxford Nanopore Technologies Adapter Mix II Expansion (EXP-AMII001), Flow Cell Priming kit (EXP-FLP002) and Sequencing Auxiliary Vials (EXP-AUX001)
- Oxford Nanopore Technologies SFB Expansion Kit (EXP-SFB001)
- 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)

Protocol Descriptions

Chapter 1: NEBNext ARTIC Express Protocol without PCR Bead Cleanup (Two clean-up steps): This protocol utilizes NEBNext ARTIC SARS-CoV-2 Primer Mixes for targeting SARS-CoV-2, these are balanced ARTICv3 primers, This protocol does not include 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. Skipping these steps reduces hands on time but may require a longer sequencing run to obtain sufficient coverage for each sample.

Chapter 2: NEBNext ARTIC Standard Protocol with PCR Bead Cleanup (Three clean-up steps): 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.

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community. Please look for E7660 on https://www.protocols.io/

Chapter 3: NEBNext VarSkip Short Express Protocol without PCR Bead Cleanup (Two clean-up steps): This protocol follows an alternate variant-tolerant approach for targeting SARS-CoV-2 by utilizing NEBNext VarSkip Short SARS-CoV-2 Primer Mixes. The NEBNext VarSkip Short SARS-CoV-2 Primer mixes cannot be added to the same cDNA amplification reaction as the NEBNext ARTIC SARS-CoV-2 Primer Mixes.This protocol does not include 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. Skipping these steps reduces hands on time but may require a longer sequencing run to obtain sufficient coverage for each sample.

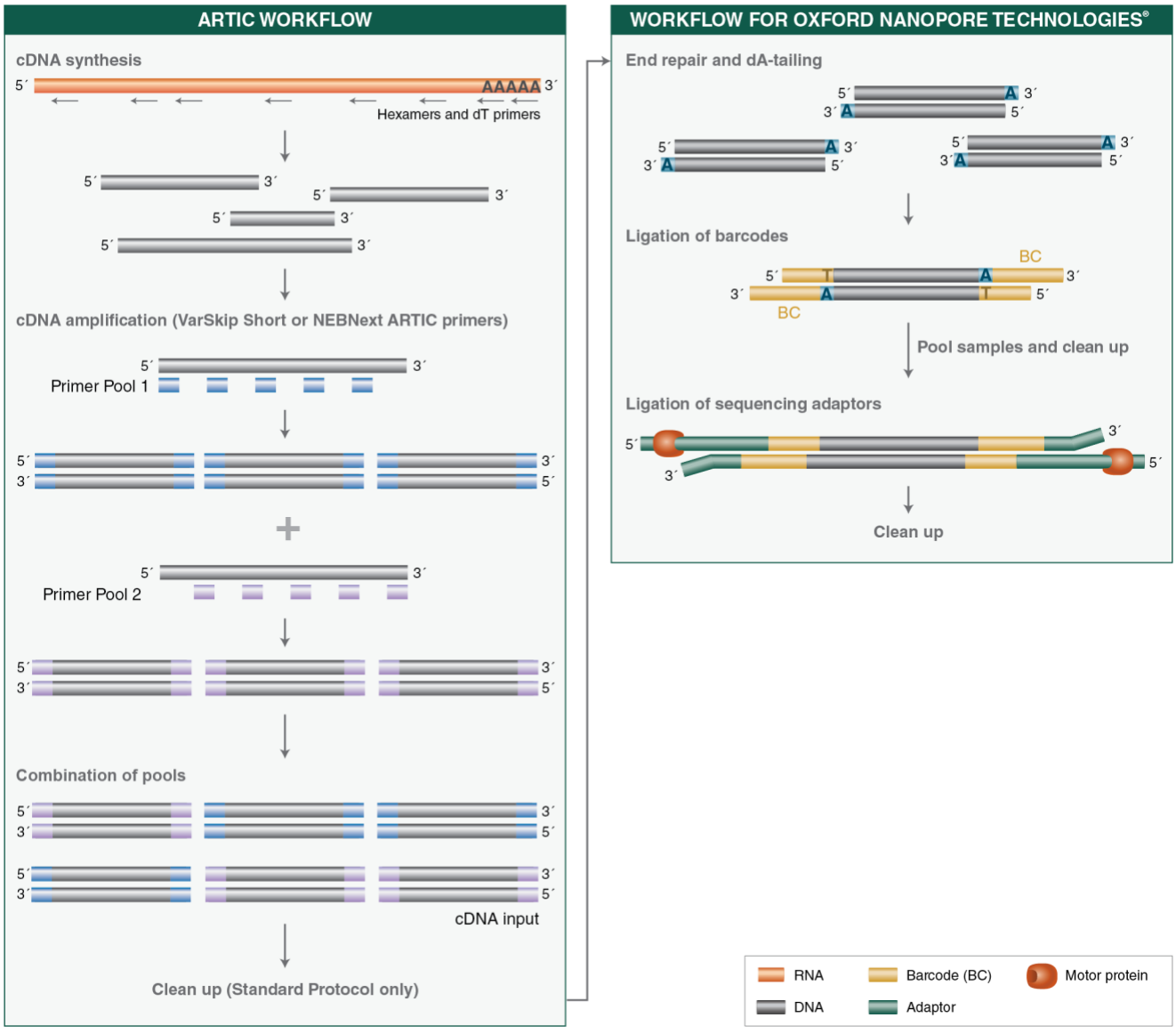
Overview

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. Primers targeting the human EDF1 and NEDD8 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 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.



Chapter 1
NEBNext ARTIC Express Protocol without PCR Bead Cleanup (Two clean-up steps)

This protocol does not include 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. Skipping these steps reduces hands on time, but may require a longer sequencing run to obtain sufficient coverage for each sample. To obtain more even sample to sample coverage, we recommend normalizing the RNA samples prior to starting Express Protocol without PCR Bead Cleanup (this Chapter).

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

- *Colored bullets indicate the cap color of the reagent to be added to a reaction.*

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.**

Note 2: If sample Ct is between 12-15, then it is recommended per the ARTIC network nCoV 2019 sequencing protocol v3 LoCost to dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

1.1. cDNA Synthesis

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.

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

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.

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

1.1.3. For no template controls, mix the following components

COMPONENT	VOLUME
○ (white) Nuclease-free Water	8 µl
• (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

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

1.1.5. Incubate reactions in a thermal cycler with lid temperature at 105°C with the following steps:

CYCLE STEP	TEMP	TIME	CYCLE
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	20 minutes	
Heat Inactivation	95°C	1 minute	

*Set heated lid to 105°C

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

1.2. Targeted cDNA Amplification

Note: We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of future reactions.

Use of the NEBNext ARTIC Human Control Primer Pairs 1 and 2 are optional. If used, the appropriate NEBNext ARTIC Human Control Primer Pairs and NEBNext ARTIC SARS-CoV-2 Primer Mix should be combined prior to use. More specifically, NEBNext ARTIC Human Control Primer Pairs 1 should be combined with NEBNext ARTIC SARS-CoV-2 Primer Mix 1 and NEBNext ARTIC Human Control Primer Pairs 2 with NEBNext ARTIC SARS-CoV-2 Primer Mix 2. Mixing directions are listed below.

1.2.1. 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:

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 1 with 42 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 1, vortex and spin down reagents. If using a 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Mix 1 with 168 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 1, vortex and spin down reagents. Use 1.75 µl of the combined mix for each Pool Set A reaction.

COMPONENT	VOLUME
cDNA (Step 1.1.5)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext ARTIC SARS-CoV-2 Primer Mix 1*	1.75 µl
Total Volume	12.5 µl

* If using NEBNext ARTIC Human Control Primer Pairs 1, add 1.75 µl of the combined NEBNext ARTIC Human Control Primer Pairs 1 and NEBNext ARTIC SARS-CoV-2 Primer Mix 1.

1.2.2. Flick the tube or pipet up and down to mix followed by a quick spin.

1.2.3. **For Pool Set B:**

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 2 with 42 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 2, vortex and spin down reagents. If using 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Pairs 2 with 168 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 2. Use 1.75 µl of the combined mix for each Pool Set B reaction.

COMPONENT	VOLUME
cDNA (Step 1.1.5)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext ARTIC SARS-CoV-2 Primer Mix 2*	1.75 µl
Total Volume	12.5 µl

* If using NEBNext ARTIC Human Control Primer Pairs 2, add 1.75 µl of the combined NEBNext ARTIC Human Control Primer Pairs 2 and NEBNext ARTIC SARS-CoV2 Primer Mix 2.

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

1.2.5. Incubate reactions in a thermal cycler** with the following steps:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 second	1
Denature	95°C	15 seconds	35
Annealing and Extension	63°C*	5 minutes	
Hold	4°C	∞	1

*** It is very important to set up the annealing and extension temperature to 63°C**
** Set heated lid to 105°C.

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

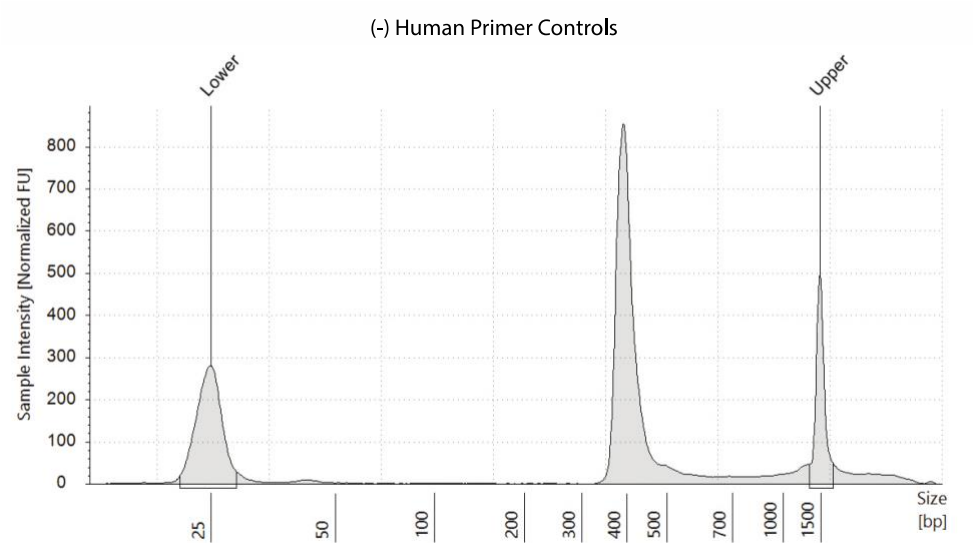
1.3. PCR Reaction Pooling

Please note, there is also a protocol that includes a cleanup and normalization step at this point (chapter 2 in this manual). 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 coverage in less run time

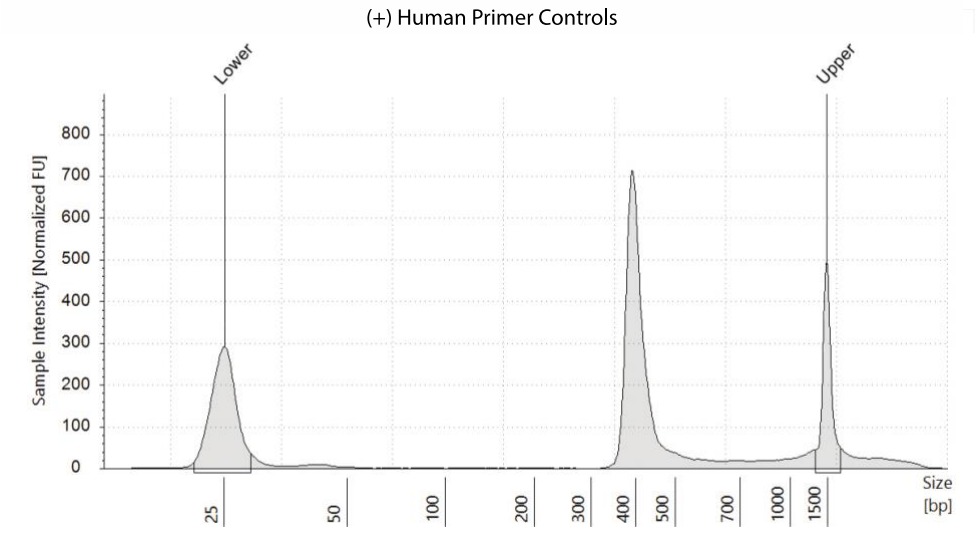
1.3.1. For each sample, combine pool A and pool B PCR Reactions.

Figure 1.3.1: Example of cDNA amplicons generated from 1000 genome copies of SARS CoV-2 in the absence (A) and presence (B) of human primer controls.

A



B



1.4. NEBNext End Prep

1.4.1. Add the following components to a PCR tube (End Prep Reaction and Buffer can be pre-mixed and master mix is stable on ice for 4 hours):

COMPONENT	VOLUME
Targeted cDNA Amplicons (1.3.1)	1 µl
○ (white) Nuclease-free water	11.5 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

1.4.2 Flick the tube or 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.

1.4.3 Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:

10 minutes @ 20°C

10 minutes @ 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.

1.5. Barcode Ligation

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

COMPONENT	VOLUME
○ (white) Nuclease-free water	6 µl
End-prepped DNA (1.4.3.)	1.5 µl
Native Barcode*	2.5 µl
● (red) Blunt/TA Ligase Master Mix**	10 µl
Total Volume	20 µl

* Native Barcodes are provided in Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) or 1-96 (EXP-NBD196)

** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction

1.5.2. Flick the tube or 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).**

1.5.3. Incubate at 25°C for 20 minutes.
Incubate at 65°C for 10 minutes.
Place on ice for 1 min.

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

1.6. Cleanup of Barcoded DNA

Note: Use the pooled barcoded DNA samples (from Step 1.5.4.), up to 480 µl for bead cleanup. Remaining pooled DNA can be stored at -20°C

1.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.6.2. Add 0.4X resuspended beads to pooled barcoded samples (1.5.4.) (for example, if you are pooling 24 barcoded DNA samples [which amounts to 480 µl total], add 192 µl of resuspended sample purification beads to the 480 µl of pooled sample). Mix well by flicking the tube or pipetting up and down 10 times. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.

1.6.3. Incubate samples on bench top for 10 minutes at room temperature.

1.6.4. 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.

1.6.5. After 3 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).**

1.6.6. Wash the beads by adding 250 µl of Short Fragment buffer (SFB). Flick the tube or pipet up and down 10 times to mix to resuspend pellet. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

1.6.7. Place the tube on an appropriate magnetic stand for 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

1.6.8. Repeat step 1.6.6. and 1.6.7. once for a total of two washes. 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

1.6.9. Add 500 µl of 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. Repeat the wash once to make it a total of two washes.

1.6.10. A quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

1.6.11. Air dry the beads for 30 seconds 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 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.

1.6.12. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of Nuclease-free water.

1.6.13. Resuspend the pellet by flicking the tube or pipetting up and down 10 times to mix. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.6.14. 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 Eppendorf DNA LoBind Tube or PCR tube.

1.6.15. Assess the concentration of the purified barcoded DNA sample. We recommend using a Qubit fluorometer for concentration assessment. Nanodrop is NOT recommended since it may overestimate the DNA concentration. Use 1µl of sample for the Qubit fluorometer.

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

1.7. Adapter Ligation

1.7.1. Use the Qubit readings from Step 1.6.15. to dilute 60 ng of the Native barcoded DNA pool with nuclease-free water to a final volume of 30 µl (2 ng/µl). Add the following components into a 1.5 ml Eppendorf DNA LoBind Tube or nuclease-free PCR tube:

COMPONENT	VOLUME
Native barcoded and purified DNA (1.6.14.; up to 60 ng)	30 µl
● (red) NEBNext Quick Ligation Reaction Buffer*	10 µl
Adapter Mix II (AMII)**	5 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.

1.7.2. Flick the tube to mix solution. Perform a quick spin for 1 second 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).**

1.7.3. Incubate at 25°C or room temperature for 20 minutes.

1.7.4. Proceed to Cleanup of Adapter-ligated DNA in Section 1.8.

1.8. Cleanup of Adapter Ligated DNA

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.

1.8.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.8.2. Add 50 µl (1X) resuspended beads to the ligation mix. Mix well by flicking the tube followed by a quick spin for 1 second.

1.8.3. Incubate samples for 10 minutes at room temperature.

1.8.4. 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.

1.8.5. After 3 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)**.

1.8.6. Wash the beads by adding 250 µl of 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.

1.8.7. Wait for 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

1.8.8. Repeat steps 1.8.6. and 1.8.7. once for a total of two washes. 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.

1.8.9. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of Elution Buffer (EB) provided in SQK-LSK109 kit from Oxford Nanopore.

1.8.10. Resuspend the pellet well in EB buffer by flicking. Incubate for 10 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.

1.8.11. Place the tube/plate on the magnetic stand. After 3 minutes (or when the solution is clear), transfer 15 µl to a new DNA LoBind tube.

1.8.12. Use Qubit to quantify 1 µl DNA sample. Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION® flow cell and DNA library sequencing mix using up to 20 ng adapter-ligated DNA sample (Step 1.8.11).

Note: After normalizing the DNA to 20 ng, if the volume is less than 12 µl, then top off the sample volume to 12 µl with EB.

Chapter 2
NEBNext ARTIC Standard Protocol with PCR Bead Cleanup (Three clean-up steps)

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.

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

●

Colored bullets indicate the cap color of the reagent to be added to a reaction.

Note 1: 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.

Note 2: If sample Ct is between 12-15, then it is recommended per the ARTIC network nCoV 2019 sequencing protocol v3 LoCost to dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

2.1. cDNA Synthesis

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.

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

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.

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

2.1.3. For no template controls, mix the following components

COMPONENT	VOLUME
○ (white) Nuclease-free Water	8 µl
● (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

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

2.1.5. Incubate reactions in a thermal cycler with lid temperature at 105°C with the following steps:

CYCLE STEP	TEMP	TIME	CYCLE
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	20 minutes	
Heat Inactivation	95°C	1 minute	

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

2.2. Targeted cDNA Amplification

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 future reactions.

Use of the NEBNext ARTIC Human Control Primer Pairs 1 and 2 are optional. If used, the appropriate NEBNext ARTIC Human Control Primer Pairs and NEBNext ARTIC SARS-CoV-2 Primer Mix should be combined prior to use. More specifically, NEBNext ARTIC Human Control Primer Pairs 1 should be combined with NEBNext ARTIC SARS-CoV-2 Primer Mix 1 and NEBNext ARTIC Human Control Primer Pairs 2 with NEBNext ARTIC SARS-CoV-2 Primer Mix 2. Mixing directions are listed below.

2.2.1. 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:

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 1 with 42 µl of ARTIC SARS-CoV-2 Primer Mix 1, vortex and spin down reagents. If using a 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Mix 1 with 168 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 1, vortex and spin down reagents. Use 1.75 µl of the combined mix for each Pool Set A reaction.

COMPONENT	VOLUME
cDNA (Step 2.1.5)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext ARTIC SARS-CoV-2 Primer Mix 1*	1.75 µl
Total Volume	12.5 µl

* If using NEBNext ARTIC Human Control Primer Pairs 1, add 1.75 µl of the combined NEBNext ARTIC Human Control Primer Pairs 1 and NEBNext ARTIC SARS-CoV-2 Primer Mix 1.

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

2.2.3. **For Pool Set B:**

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 2 with 42 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 2, vortex and spin down reagents. If using 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Pairs 2 with 168 µl of NEBNext ARTIC SARS-CoV-2 Primer Mix 2. Use 1.75 µl of the combined mix for each Pool Set B reaction.

COMPONENT	VOLUME
cDNA (Step 2.1.5)	4.5 µl
• (lilac) Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µl
NEBNext ARTIC SARS-CoV-2 Primer Mix 2*	1.75 µl
Total Volume	12.5 µl

* If using NEBNext ARTIC Human Control Primer Pairs 2, add 1.75 µl of the combined NEBNext ARTIC Human Control Primer Pairs 2 and NEBNext ARTIC SARS-CoV-2 Primer Mix 2.

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

2.2.5. Incubate reactions in a thermal cycler* with the following steps:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 second	1
Denature	95°C	15 seconds	35
Annealing and Extension	63°C**	5 minutes	
Hold	4°C	∞	1

* Set heated lid to 105°C.
** It is very important to set up the annealing and extension temperature to 63°C

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

2.3. Cleanup of cDNA Amplicons

If you prefer to omit the cleanup step follow chapter 1 in this manual. **Otherwise continue to Cleanup of cDNA Amplicons (Step 2.3.1).**

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.

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

2.3.2. Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

2.3.3. Add 20 µl (0.8X) 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.

2.3.4. Incubate samples at room temperature for 10 minutes.

2.3.5. Place the tubes on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample 1 second to collect the liquid from the sides of the tube before placing on the magnetic stand.

2.3.6. After 2 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)**.

2.3.7. Add 500 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.3.8. Repeat step 2.3.7. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube for 1 second, place back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.

2.3.9. Air dry the beads for 30 seconds 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.

2.3.10. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 21 µl Nuclease-free water.

2.3.11. Mix well by flicking the tube or pipetting up and down 10 times to mix and followed by a very short centrifugation. Incubate for 10 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.

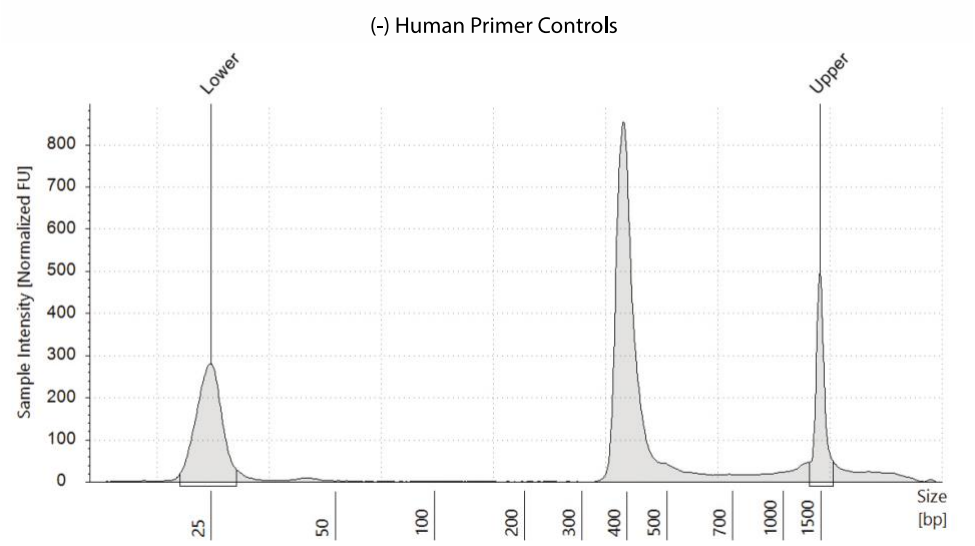
2.3.12. Place the tube on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 20 µl to clean PCR tubes.

2.3.13. Assess the concentration of the purified samples. We recommend using a Qubit fluorometer for concentration assessment. Use 1 µl of sample for the Qubit fluorometer.

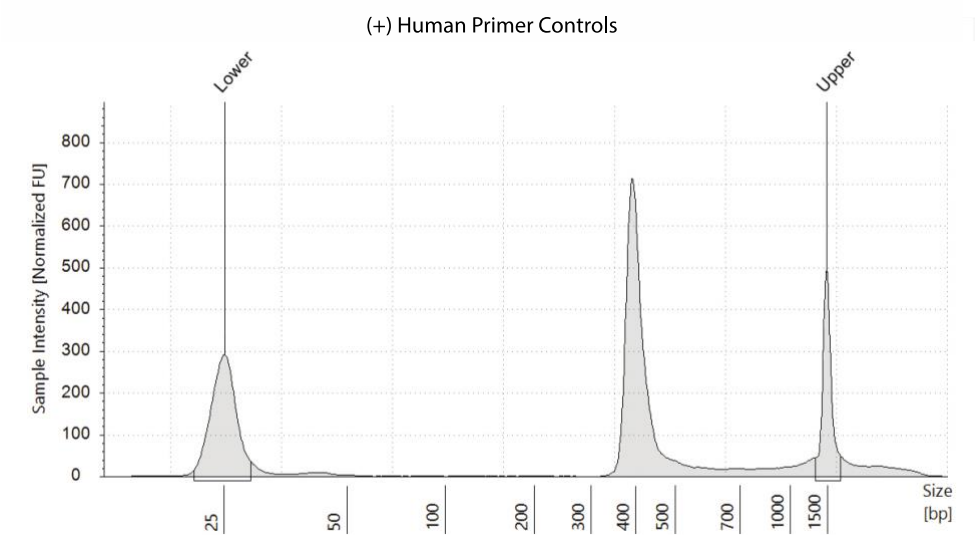
Samples can be stored at –20°C if they are not used immediately.

Figure 2.3.13: Example of cDNA amplicons generated from 1000 genome copies of SARS CoV-2 in the absence (A) and presence (B) of human primer controls.

A



B



2.4. NEBNext End Prep

2.4.1. Use the Qubit readings from Step 2.3.13. to dilute 50 ng of the Targeted cDNA Amplicons sample with nuclease-free water to a final volume of 12.5 µl (4 ng/µl). Add the following components to a PCR tube (NEBNext Ultra II End Prep Reaction Buffer and NEBNext Ultra II End Prep Enzyme Mix can be pre-mixed and this master mix is stable on ice for 4 hours):

COMPONENT	VOLUME
Targeted cDNA Amplicons (2.3.12)	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

2.4.2 Flick the tube or 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.

2.4.3 Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:

10 minutes @ 20°C

10 minutes @ 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.

2.5. Barcode Ligation

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

COMPONENT	VOLUME
○ (white) Nuclease-free water	6 µl
End-prepped DNA (2.4.3.)	1.5 µl
Native Barcode*	2.5 µl
● (red) Blunt/TA Ligase Master Mix**	10 µl
Total Volume	20 µl

* Native Barcodes are provided in Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) or 1-96 (EXP-NBD196)

** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction

2.5.2. Flick the tube or 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).**

2.5.3. Incubate at room temperature for 20 minutes.
Incubate at 65°C for 10 minutes.
Place on ice for 1 min.

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

2.6. Cleanup of Barcoded DNA

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.

2.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.6.2. Add 0.4X resuspended beads to pooled barcoded samples (2.5.4.) (for example, if you are pooling 24 barcoded DNA samples [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. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.

2.6.3. Incubate samples on bench top for 10 minutes at room temperature.

2.6.4. 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.

2.6.5. After 2 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).**

2.6.6. Wash the beads by adding 250 µl of Short Fragment buffer (SFB). Flick the tube or pipet up and down 10 times to mix to resuspend pellet. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

2.6.7. Place the tube on an appropriate magnetic stand for 2 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

2.6.8. Repeat Step 2.6.6. and 2.6.7. once for a total of two washes. 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

2.6.9. Add 200 µl of 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.6.10. A quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

2.6.11. Air dry the beads for 30 seconds 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 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.

2.6.12. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of Nuclease-free water.

2.6.13. Resuspend the pellet by flicking the tube or pipet up and down 10 times to mix. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube before placing back on the magnetic stand.

2.6.14. 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 Eppendorf DNA LoBind Tube or PCR tube.

2.6.15. Assess the concentration of the purified barcoded DNA sample. We recommend using a Qubit fluorometer for concentration assessment. Nanodrop is NOT recommended since it may overestimate the DNA concentration. Use 1µl of sample for the Qubit fluorometer.

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

2.7. Adapter Ligation

2.7.1. Use the Qubit readings from Step 2.6.15. to dilute 60 ng of the Native barcoded DNA pool with nuclease-free water to a final volume of 30 µl (2 ng/µl). Add the following components into a 1.5 ml Eppendorf DNA LoBind Tube or nuclease-free PCR tube:

COMPONENT	VOLUME
Native barcoded and purified DNA (2.6.14.)	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.

2.7.2. Flick the tube to mix solution. Perform a quick spin for 1 second 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).**

2.7.3. Incubate at 25°C or room temperature for 20 minutes.

2.7.4. Proceed to Cleanup of Adapter-ligated DNA in Section 2.8.

2.8. Cleanup of Adapter Ligated DNA

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.

2.8.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.8.2. Add 50 µl (1X) resuspended beads to the ligation mix. Mix well by flicking the tube to mix followed by a quick spin for 1 second.

2.8.3. Incubate samples for 10 minutes at room temperature.

2.8.4. 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.

2.8.5. After 2 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)**.

2.8.6. Wash the beads by adding 250 µl of 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.

2.8.7. Wait for 2 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

2.8.8. Repeat steps 2.8.6. and 2.8.7. once for a total of two washes. 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.

2.8.9. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of Elution Buffer (EB) provided in SQK-LSK109 kit from Oxford Nanopore.

2.8.10. Resuspend the pellet well in EB buffer by flicking the tube to mix. Incubate for 10 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.

2.8.11. Place the tube/plate on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 15 µl to a new DNA LoBind tube.

2.8.12. Use Qubit to quantify 1 µl DNA sample. Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION® flow cell and DNA library sequencing mix using up to 20 ng adapter-ligated cDNA sample (2.8.11).

Note: After normalizing the DNA to 20 ng, if the volume is less than 12 µl, then top off the sample volume to 12 µl with EB.

Chapter 3
NEBNext VarSkip Short Express Protocol without PCR Bead Cleanup (Two clean-up steps)

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

Colored bullets indicate the cap color of the reagent to be added to a reaction.

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.

Note 2: If sample Ct is between 12–15, then it is recommended per the ARTIC network *nCoV 2019 sequencing protocol v3 LoCost* to dilute the sample 100-fold in water, if between 15–18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

3.1. cDNA Synthesis

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.

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

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

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

3.1.3. For no template controls, mix the following components:

COMPONENT	VOLUME
○ (white) Nuclease-free Water	8 µl
● (lilac) LunaScript RT SuperMix	2 µl
Total Volume	10 µl

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

3.1.5. Incubate reactions in a thermal cycler* with the following steps:

CYCLE STEP	TEMP	TIME
Primer Annealing	25°C	2 minutes
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.

3.2. Targeted cDNA Amplification

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 future reactions. NEBNext VarSkip Short Primer Mixes cannot be combined with NEBNext ARTIC Primer Mixes in the same targeted amplification reaction.

Use of the NEBNext ARTIC Human Control Primer Pairs 1 and 2 are optional. If used, the appropriate NEBNext ARTIC Human Control Primer Pairs and NEBNext VarSkip Short SARS-CoV-2 Primer Mix should be combined prior to use. More specifically, NEBNext ARTIC Human Control Primer Pairs 1 should be combined with NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1 and NEBNext ARTIC Human Control Primer Pairs 2 with NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2. Mixing directions are listed below.

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

For Pool Set A:

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 1 with 42 µl of NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1 in a new tube, vortex and spin down reagents. If using a 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Mix 1 with 168 µl of NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1 in a new tube, vortex and spin down reagents. Use 1.75 µl of the combined mix for each Pool Set A reaction.

COMPONENT	VOLUME
cDNA (Step 3.1.5)	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

* If using NEBNext ARTIC Human Control Primer Pairs 1, add 1.75 µl of the combined VarSkip Short SARS-CoV-2 Primer Mix 1 and NEBNext ARTIC Human Control Primer Pairs 1.

For Pool Set B:

If using the NEBNext ARTIC Human Control Primer Pairs and a 24 reaction kit, combine 0.7 µl of NEBNext ARTIC Human Control Primer Pairs 2 with 42 µl of NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2 in a new tube, vortex and spin down reagents. If using a 96 reaction kit, combine 2.8 µl of the NEBNext ARTIC Human Control Primer Pairs 2 with 168 µl of VarSkip Short SARS-CoV-2 Primer Mix 2 in a new tube, vortex and spin down reagents. Use 1.75 µl of the combined mix for each Pool Set B reaction.

COMPONENT	VOLUME
cDNA (Step 3.1.5)	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

* If using NEBNext ARTIC Human Control Primer Pairs 2, add 1.75 µl of the combined VarSkip Short SARS-CoV-2 Primer Mix 2 and NEBNext ARTIC Human Control Primer Pairs 2.

3.2.2. Incubate reactions in a thermal cycler* with the following steps:

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

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

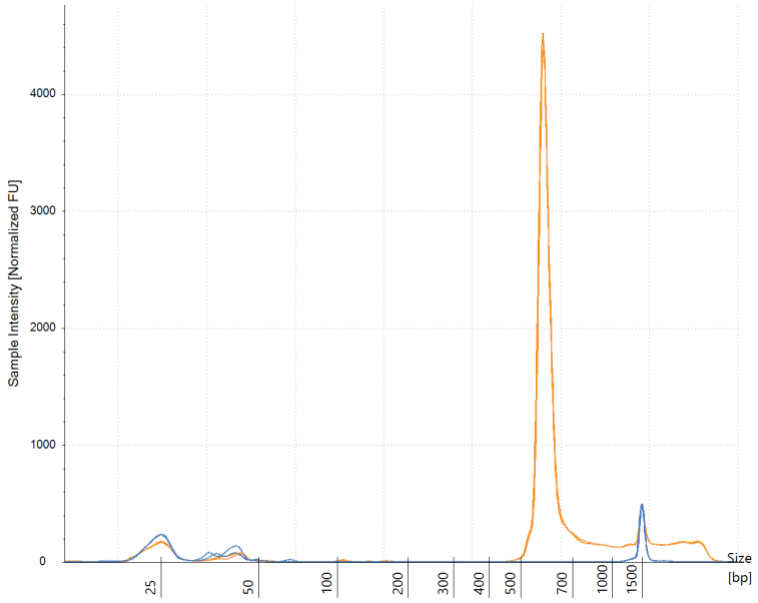
3.2.4. **Samples can be stored at -20°C for up to a week.**

3.3. PCR Reaction Pooling

Please note, 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 coverage in less run time

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

Figure 3.3.1: VarSkip Short SARS-CoV-2 cDNA amplicons generated from 1000 total viral copies. 1/10 diluted cDNA amplicons without bead cleanup run on a TapeStation.



3.4. NEBNext End Prep

3.4.1. Add the following components to a PCR tube (End Prep Reaction and Buffer can be pre-mixed and master mix is stable on ice for 4 hours):

COMPONENT	VOLUME
Targeted cDNA Amplicons (3.3.1)	1 µl
○ (white) Nuclease-free water	11.5 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

3.4.2 Flick the tube or 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.

3.4.3 Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:

10 minutes @ 20°C

10 minutes @ 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.

3.5. Barcode Ligation

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

COMPONENT	VOLUME
○ (white) Nuclease-free water	6 µl
End-prepped DNA (3.4.3.)	1.5 µl
Native Barcode*	2.5 µl
● (red) Blunt/TA Ligase Master Mix**	10 µl
Total Volume	20 µl

* Native Barcodes are provided in Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) or 1-96 (EXP-NBD196)

** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction

3.5.2. Flick the tube or 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).**

3.5.3. Incubate at 25°C for 20 minutes.
Incubate at 65°C for 10 minutes.
Place on ice for 1 min.

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

3.6. Cleanup of Barcoded DNA

Note: Use the pooled barcoded DNA samples (from Step 3.5.4.), up to 480 µl for bead cleanup. Remaining pooled DNA can be stored at -20°C

3.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.6.2. Add 0.4X resuspended beads to pooled barcoded samples (3.5.4.) (for example, if you are pooling 24 barcoded DNA samples [which amounts to 480 µl total], add 192 µl of resuspended sample purification beads to the 480 µl of pooled sample). Mix well by flicking the tube or pipetting up and down 10 times. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.

3.6.3. Incubate samples on bench top for 10 minutes at room temperature.

3.6.4. 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.

3.6.5. After 3 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).**

3.6.6. Wash the beads by adding 250 µl of Short Fragment buffer (SFB). Flick the tube or pipet up and down 10 times to mix to resuspend pellet. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

3.6.7. Place the tube on an appropriate magnetic stand for 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

3.6.8. Repeat steps 3.6.6. and 3.6.7. once for a total of two washes. 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

3.6.9. Add 500 µl of 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. Repeat the wash once to make it a total of two washes.

3.6.10. A quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

3.6.11. Air dry the beads for 30 seconds 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 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.

3.6.12. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of Nuclease-free water.

3.6.13. Resuspend the pellet by flicking the tube or pipetting up and down 10 times to mix. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube before placing back on the magnetic stand.

3.6.14. 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 Eppendorf DNA LoBind Tube or PCR tube.

3.6.15. Assess the concentration of the purified barcoded DNA sample. We recommend using a Qubit fluorometer for concentration assessment. Nanodrop is NOT recommended since it may overestimate the DNA concentration. Use 1µl of sample for the Qubit fluorometer.

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

3.7. Adapter Ligation

3.7.1. Use the Qubit readings from Step 3.6.15. to dilute 75 ng of the Native barcoded DNA pool with nuclease-free water to a final volume of 30 µl (~2.5 ng/µl). Add the following components into a 1.5 ml Eppendorf DNA LoBind Tube or nuclease-free PCR tube:

COMPONENT	VOLUME
Native barcoded and purified DNA (3.6.14.; up to 75 ng)	30 µl
● (red) NEBNext Quick Ligation Reaction Buffer*	10 µl
Adapter Mix II (AMII)**	5 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.

3.7.2. Flick the tube to mix solution. Perform a quick spin for 1 second 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).**

3.7.3. Incubate at 25°C or room temperature for 20 minutes.

3.7.4. Proceed to Cleanup of Adapter-ligated DNA in Section 3.8.

3.8. Cleanup of Adapter Ligated DNA

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.

3.8.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.8.2. Add 50 µl (1X) resuspended beads to the ligation mix. Mix well by flicking the tube followed by a quick spin for 1 second.

3.8.3. Incubate samples for 10 minutes at room temperature.

3.8.4. 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.

3.8.5. After 3 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)**.

3.8.6. Wash the beads by adding 250 µl of 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.

3.8.7. Wait for 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

3.8.8. Repeat steps 3.8.6. and 3.8.7. once for a total of two washes. 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.

3.8.9. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of Elution Buffer (EB) provided in SQK-LSK109 kit from Oxford Nanopore.

3.8.10. Resuspend the pellet well in EB buffer by flicking. Incubate for 10 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.

3.8.11. Place the tube/plate on the magnetic stand. After 3 minutes (or when the solution is clear), transfer 15 µl to a new DNA LoBind tube.

3.8.12. Use Qubit to quantify 1 µl DNA sample. Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION® flow cell and DNA library sequencing mix using up to 30 ng adapter-ligated DNA sample (Step 3.8.11).

Note: After normalizing the DNA to 30 ng, if the volume is less than 12 µl, then top off the sample volume to 12 µl with EB.

Kit Components

NEB #E7660S Table of Components

NEB #	PRODUCT	VOLUME
E7651A	LunaScript RT SuperMix	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
E8005A	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	0.042 ml
E8006A	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	0.042 ml

NEB #E7660L Table of Components

NEB #	PRODUCT	VOLUME
E7651AA	LunaScript RT SuperMix	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
E8005AA	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1	0.168 ml
E8006AA	NEBNext VarSkip Short SARS-CoV-2 Primer Mix 2	0.168 ml

NEBNext ARTIC Human Primers

PRIMER MIX	GENE	POSITION	PRIMERS
NEBNext ARTIC Human Control Primer Pairs 1	EDF1	113 bp – 501 bp	GGCCAAATCCAAGCAGGCTA GTGTTCATTTCGCCCTAGGC
NEBNext ARTIC Human Control Primer Pairs 2	NEDD8	110 bp – 489 bp	AAAGTGAAGACGCTGACCGG GGGATCCTCACAGTCTCCCA

Detailed information for the ARTIC Human control primers can be found at: https://doi.org/10.5281/zenodo.4495958

NEBNext ARTIC SARS-CoV-2 Primer Mix 1 and 2

NEBNext ARTIC SARS-CoV-2 Primers for SARS-CoV-2 genome amplification are based on hCoV-2019/nCoV-2019 Version 3 (v3) sequences with balanced primer concentrations. Sequence information can be found at:
https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv

NEBNext VarSkip Short SARS-CoV-2 Primer Mix 1 and 2

NEBNext VarSkip Short SARS-CoV-2 Mix 1 and 2 for SARS-CoV-2 genome amplification were designed to reduce the impact of variants on amplification efficiency. Sequence information can be found at:
https://github.com/nebiolabs/VarSkip

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	2/21
2.0	Update protocol	3/21
3.0	Update protocol and add second protocol	4/21
4.0	Update protocols	5/21
5.0	Add Chapter 3. Update protocols	9/21