NEBNext® ARTIC SARS-CoV-2 FS Library Prep Kit (Illumina®)

NEB #E7658S/L 24/96 reactions

Version 4.1_9/21

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7658S) and 96 reactions (NEB #E7658L). Colored bullets represent the color of the cap of the tube containing the reagent.

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
- (yellow) NEBNext Ultra II FS Enzyme Mix
- (yellow) NEBNext Ultra II FS Reaction Buffer
- (red) NEBNext Ultra II Ligation Master Mix
- (blue) NEBNext Library PCR Master Mix
- (white) 0.1X TE
- (white) Nuclease-free Water

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

NEBNext Sample Purification Beads

Required Materials Not Included

- NEBNext Singleplex or Multiplex Oligos for Illumina
  - www.neb.com/oligos
- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf® #022431021)
- Magnetic rack/stand (NEB #S1515S, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- DNase RNase free PCR strip tubes (USA Scientific® 1402-1708)

Protocol Descriptions

Chapter 1: Express Protocol without PCR Bead Cleanup for NEBNext ARTIC SARS-CoV-2 Primers: 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 step for each sample after cDNA synthesis and after adaptor ligation. Performing RNA input normalization prior to cDNA synthesis and targeted amplification and/or normalizing final libraries prior to sequencing promotes more even distribution of reads across libraries. Skipping RNA input normalization, final library normalization, and cleanups reduces hands on time but may require deeper sequencing depth to reach sufficient coverage of each sample.

Chapter 2: Standard Protocol with PCR Bead Cleanup for NEBNext ARTIC SARS-CoV-2 Primers: This protocol utilizes NEBNext ARTIC SARS-CoV-2 Primer Mixes, which are balanced ARTICv3 primers. This protocol includes a cleanup step for each sample after cDNA synthesis. Normalizing final libraries prior to sequencing promotes more even distribution of reads across libraries. These pools will likely achieve sufficient and equal coverage with less sequencing depth, but they take more hands on time.

Chapter 3: Express Protocol without PCR Bead Cleanup for NEBNext VarSkip Short SARS-CoV-2 Primers: 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 step for each sample after cDNA synthesis and after adaptor ligation. Performing RNA input normalization prior to cDNA synthesis and targeted amplification and/or normalizing final libraries prior to sequencing promotes more even distribution of reads across libraries. Skipping RNA input normalization, final library normalization, and cleanups reduces hands on time but may require deeper sequencing depth to reach sufficient coverage of each sample.

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community. Please look for E7658 on https://www.protocols.io/

Overview

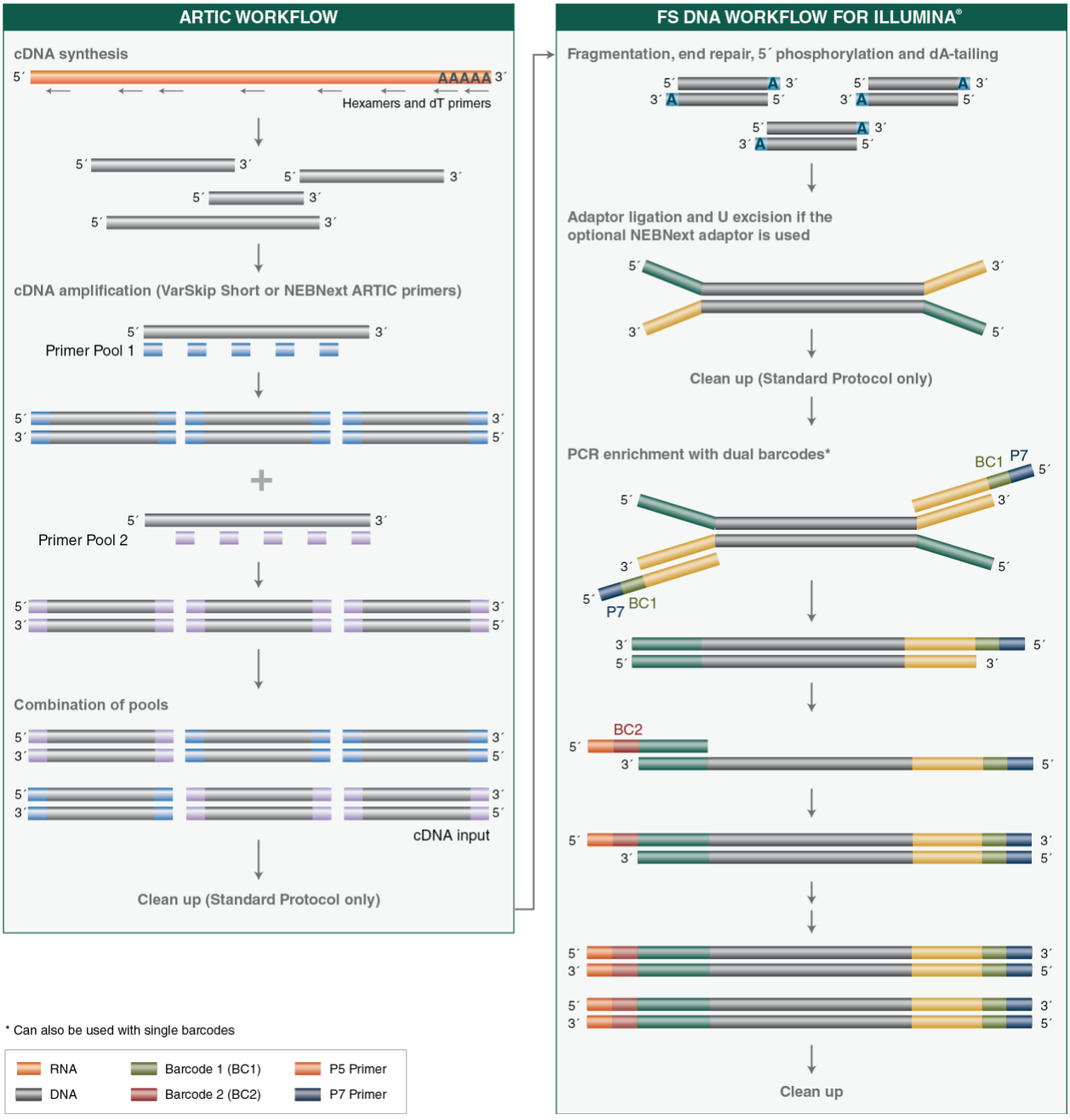
The NEBNext ARTIC SARS-CoV-2 FS Library Prep Kit for Illumina contains the enzymes, buffers and oligos required to convert a broad range of total RNA into high quality, targeted, libraries for next-generation sequencing on the Illumina platform. Primers targeting the human EDF1 (NEBNext ARTIC Human Control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

The NEBNext VarSkip Short SARS-CoV-2 Primer mixes included provide an alternate variant-tolerant approach for targeting SARS-CoV-2. 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. The NEBNext ARTIC Human Control Primer Pairs can be used as internal controls with the NEBNext VarSkip Short SARS-CoV-2 Primer mixes.

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 indexed libraries on an 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 ARTIC SARS-CoV-2 FS Library Prep Kit.



Chapter 1
NEBNext ARTIC Express Protocol without cDNA Amplicon and Ligation Bead Cleanups (One clean-up step)

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: We recommend using the express protocol for inputs of ≥ 100 copies of the (SARS-CoV-2) viral genome. The use of lower input amounts may result in significant levels of adaptor dimer in the sequencing data. In addition, we recommend setting up a no template control reaction. It is advisable to set up your reactions in the hood.

1.1. 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.

1.1.1. 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

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

1.1.2. 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.

1.2. 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.

1.2.1. 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 ARTIC 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 ARTIC 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 1.1.2)	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 ARTIC 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 ARTIC 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 ARTIC 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 1.1.2)	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 ARTIC SARS-CoV-2 Primer Mix 2 and NEBNext ARTIC Human Control Primer Pairs 2.

1.2.2. 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

*Set heated lid to 105°C

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

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

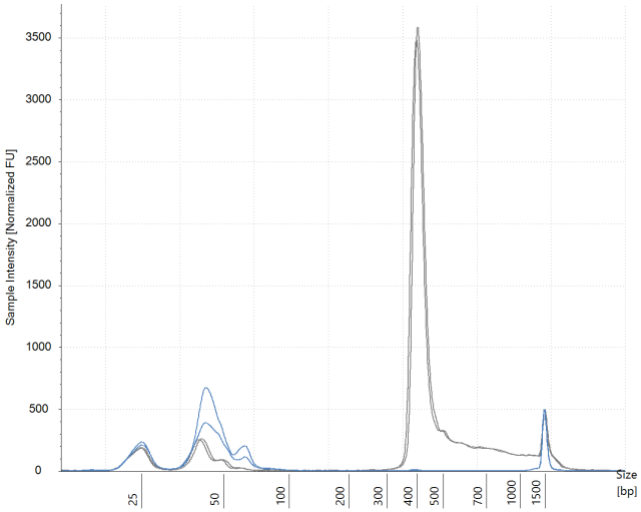
Note: When cDNA amplification cleanup steps are skipped, the cDNA amplicon pool must be diluted before to proceeding to library preparation. Description of sample dilution is detailed below in Steps 1.2.5-1.2.7.

1.2.5. Transfer 1.3 µl of the pooled cDNA amplicons to a fresh tube.

1.2.6. Add 11.7 µl of 0.1X TE for a final volume of 13 µl.

Note: The pooled cDNA amplicons may be run on a TapeStation® to confirm 400 bp size of amplicons without cleaning up. Cleanup of cDNA amplicon pool is not needed for assessing on a TapeStation. To run on a TapeStation, dilute an aliquot of the pooled amplicons 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape. (See Figure 1.2.7. below for example of amplicon size profile on a Bioanalyzer)

Figure 1.2.7: ARTIC SARS-CoV-2 cDNA amplicons generated from 1000 total viral copies.



1.3. Fragmentation/End Prep

FRAGMENTATION SIZE RANGE	INCUBATION @ 37°C	OPTIMIZATION
80 bp–300 bp	20 min	20–30 min

Note: Fragmentation occurs during a 37°C incubation step. A fragmentation time of 20 minutes should be sufficient for most samples to generate mainly ~120 bp fragments, however, the time may need to be optimized.

1.3.1. Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

1.3.2. Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.

Note: It is important to vortex the enzyme mix prior to use for optimal performance.

1.3.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME PER ONE LIBRARY
ARTIC SARS-CoV-2 cDNA (**Step 1.2.7**)	13 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	3.5 µl
○ (yellow) NEBNext Ultra II FS Enzyme Mix	1 µl
Total Volume	17.5 µl

1.3.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

1.3.5. In a thermal cycler*, run the following program:

TEMP	TIME
37°C	20 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.

1.4. Adaptor Ligation

1.4.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 1.3.5)	17.5 µl
• (red) NEBNext Adaptor for Illumina**	1.25 µl
• (red) NEBNext Ultra II Ligation Master Mix*	15 µl
Total Volume	33.75 µl

* Mix the NEBNext 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.

Note: Do not premix adaptor with the Ligation Master Mix.

1.4.2. 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. **(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).**

1.4.3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

1.4.4. Add 1.5 µl of (red or blue) USER® Enzyme to the ligation mixture from Step 1.4.3.

Note: Steps 1.4.4. and 1.4.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

1.4.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

Samples can be stored overnight at –20°C.

Note: Only a portion of the ligation reaction (7.5 µl) will move forward to PCR enrichment.

1.5. PCR Enrichment of Adaptor-ligated DNA

Follow Section 1.5.1A. if you are using the following oligos:
Use Option A for any NEBNext Oligo kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 µM each.

Follow Section 1.5.1B. if you are using the following oligos:
Use Option B for any NEBNext Oligo kit w here 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 at 10 µM combined.

1.5.1. Add the following components to a sterile strip tube:

1.5.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 1.4.3 or 1.4.5)	7.5 µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer/i7 Primer*,**	2.5 µl
Universal PCR Primer/i5 Primer*,**	2.5 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.

** Use only one i7/primer/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

1.5.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 1.4.3 or 1.4.5)	7.5 µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer Mix*	5 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

1.5.2. 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.

1.5.3. Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

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

Set heated lid to 105°C.

* The number of PCR cycles recommended should be viewed as a starting point and may need to be optimized for particular sample types.

1.6. Cleanup of PCR Reaction

Follow Section 1.6.1.A. if you are normalizing individual final libraries prior to pooling for sequencing. Option A is recommended for samples with a large range of SARS-CoV-2 viral genome copies input into the cDNA synthesis reaction (Section 1.1), such as a set of samples with inputs outside of the 100–100,000 genome copies range.

Follow Section 1.6.1B. if you are not normalizing individual final libraries prior to pooling for sequencing. Option B is recommended for samples with a small range of SARS-CoV-2 viral genome copies input into the cDNA synthesis reaction (Section 1.1), such as a set of samples with inputs within the 100–100,000 genome copies range. Otherwise, a sequencing depth of at least 1 million reads per library may be required.

1.6.1A. Cleanup of Individual PCR Reactions

Note: The amount of NEBNext Sample Purification Beads added in Step 1.6.1A.2. is specific for samples suspended in the buffer described in Section 5.1. Using the amount of beads at Step 1.6.1A.2 may not work properly for sample cleanup at other steps in the workflow. If your samples are suspended in a different buffer at this point, the amount of beads required may need to be experimentally determined.

1.6.1A.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.6.1A.2. Add 17.5 µl (0.7X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Take care 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.

1.6.1A.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.6.1A.4. Place the tube/plate 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.

1.6.1A.5. 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)**.

1.6.1A.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

1.6.1A.7. Repeat Step 1.6.1A.6 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 ethanol with a p10 pipette tip.

1.6.1A.8. 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.

1.6.1A.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE.

1.6.1A.10. 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.

1.6.1A.11. 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.

1.6.1A.12. Assess the library size distribution with Agilent Bioanalyzer or TapeStation high sensitivity DNA reagents. The sample may need to be diluted before loading. A peak size of 200–400 bp is expected, based on a 20-minute fragmentation time (Figure 1.6.12).

Note: If excess adaptor dimer peak is observed at 150–180 bp, a second 0.7X bead cleanup can be performed. The second 0.7X cleanup will result in slightly larger libraries which will not affect sequencing results.

1.6.1B. Cleanup of Pooled PCR Reactions

Note: For cleanup of pooled PCR reactions, we recommend cleanup of a 50 µl pool volume. For non-normalized pools using a 24-reaction kit, add 3 µl of each library to the pool, then mix, spin down, and take 50 µl of the pool to cleanup. For non-normalized pools using a 96-reaction kit, add 2 µl of each library to the pool, then mix, spin down, and take 50 µl of the pool on to cleanup. The amount of NEBNext Sample Purification Beads added in Step 1.6.1B.2. is specific for pooled samples suspended in the buffer described in Section 5.1. Using the exact amount of beads as described in Step 1.6.1B.2 may not work properly for sample cleanup at other steps in the workflow. If your samples are suspended in a different buffer at this point, the amount of beads needed may need to be experimentally determined.

1.6.1B.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.6.1B.2. Add 35 µl (0.7X) resuspended beads to the 50 µl pool. Mix well by pipetting up and down at least 10 times. Take care 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 pool after mixing, be sure to stop the centrifugation before the beads start to settle out.

1.6.1B.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.6.1B.4. Place the tube/plate 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 well before placing on the magnetic stand.

1.6.1B.5. 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)**.

1.6.1B.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

1.6.1B.7. Repeat Step 1.6.1B.6 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 ethanol with a p10 pipette tip.

1.6.1B.8. 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 pool 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.

1.6.1B.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 25 µl of 0.1X TE.

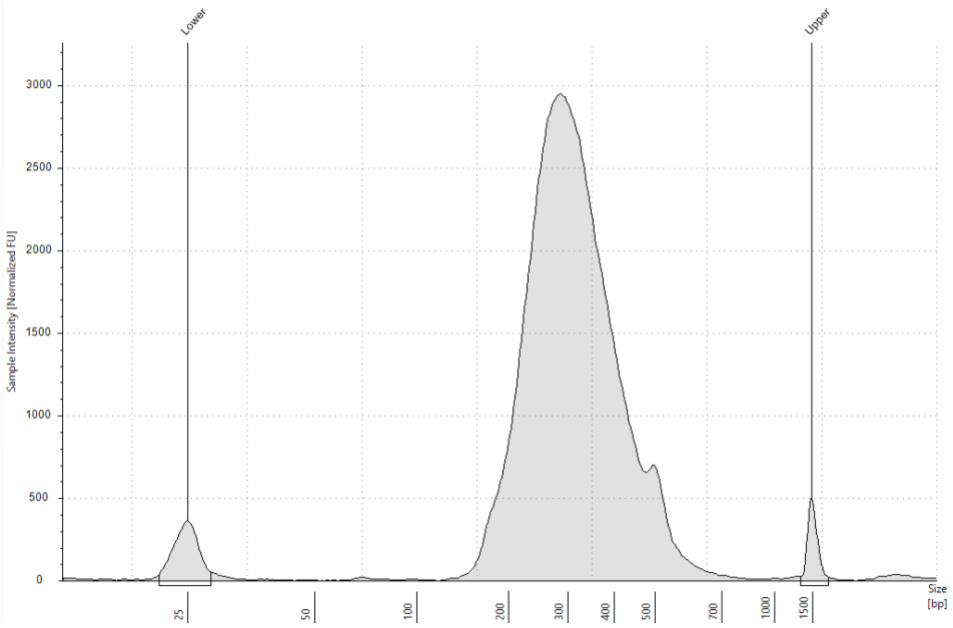
1.6.1B.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the pool to collect the liquid from the sides of the tube or plate well before placing back on the magnetic stand.

1.6.1B.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 23 µl to a new PCR tube and store at –20°C.

1.6.1B.12. Assess the library size distribution with Agilent Bioanalyzer or TapeStation high sensitivity DNA reagents. The pool may need to be diluted before loading. A peak size of 200–400 bp is expected, based on a 25-minute fragmentation time (Figure 1.6.12).

Note: If excess adaptor dimer peak is observed at 150–180 bp, a second 0.7X bead cleanup can be performed. The second 0.7X cleanup will result in slightly larger libraries which will not affect sequencing results.

Figure 1.6.12: Example of final library pool size distributions on a TapeStation. ARTIC SARS-CoV-2 libraries were generated from 10,000 viral copies. Library pool was cleaned up twice with a 0.7X bead ratio.



Chapter 2
NEBNext ARTIC Standard Protocol with cDNA Amplicon and Ligation Bead Cleanups (Three 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, however, results may vary depending on the quality of the input. In addition, we recommend setting up a no template control reaction. It is advisable to set up your reactions in the hood.

2.1. 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.

2.1.1. 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

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

2.1.2. 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.

2.2. 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 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 ARTIC 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 ARTIC 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 2.1.2)	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 ARTIC 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 ARTIC 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 ARTIC 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 2.1.2)	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 ARTIC SARS-CoV-2 Primer Mix 2 and NEBNext ARTIC Human Control Primer Pairs 2.

2.2.2. 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

*Set heated lid to 105°C

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

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

2.3. Cleanup of cDNA Amplicons

Note: The volume of NEBNext Sample Purification Beads provided here are for use with the sample composition at this step (25 µl; Step 2.2.3). 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.

2.3.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.3.2. Add 20 µl (0.8X) resuspended beads to the combined PCR reaction. Mix well by pipetting up and down at least 10 times. Take care 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.

2.3.3. Incubate samples at room temperature for at least 5 minutes.

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

2.3.5. 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)**.

2.3.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

2.3.7. Repeat Step 2.3.6. 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 ethanol with a p10 pipette tip.

2.3.8. 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.

2.3.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 16 µl 0.1X TE. If not assessing amplicons (Step 2.3.12.) elute DNA in 15 µl of 0.1X TE.

2.3.10. 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.

2.3.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 14 µl to a new PCR tube. If not assessing amplicons (Step 2.3.12) transfer 13 µl to a new PCR tube.

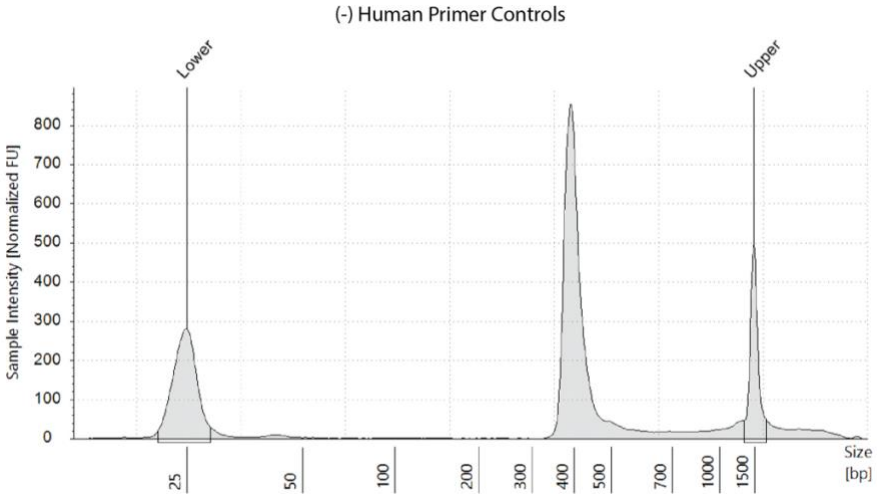
2.3.12. We recommend assessing cDNA amplicon (from Step 2.3.11) concentrations with a Qubit fluorometer.

Note: Amplicons may also be run on a Bioanalyzer or TapeStation® to confirm 400 bp size of amplicons. To run on a TapeStation, dilute amplicon 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape. (See Figure 2.3.13. below for example of amplicon size profile on a Bioanalyzer).

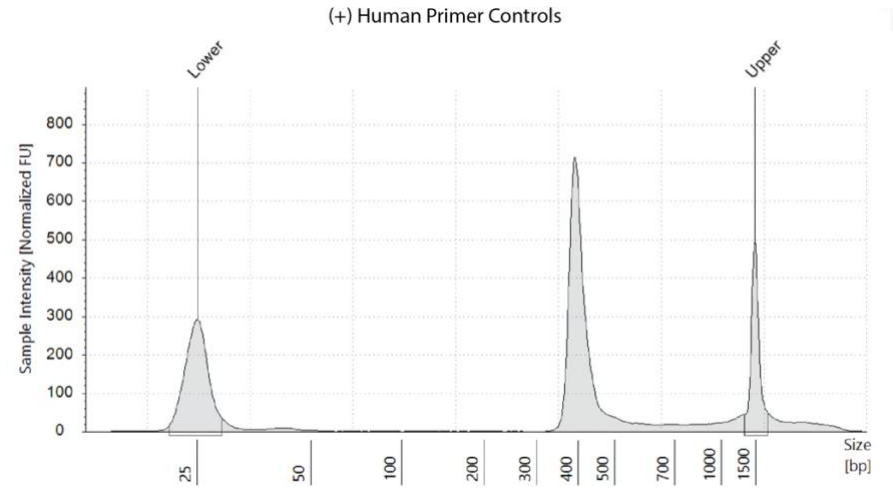
Samples can be stored at –20°C for up to a week.

Figure 2.3.13: ARTIC SARS-CoV-2 cDNA amplicons generated from 1000 total viral copies in the absence (A) or presence (B) of the human primer controls.

A.



B.



2.4. Fragmentation/End Prep

FRAGMENTATION SIZE RANGE	INCUBATION @ 37°C	OPTIMIZATION
80 bp–250 bp	30 min	30–40 min

Note: Fragmentation occurs during a 37°C incubation step. A fragmentation time of 30 minutes should be sufficient for most samples to generate mainly ~120 bp fragments, however, the time may need to be optimized. For high input samples (1 µg), fragmentation time may need to be increased.

2.4.1. Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

2.4.2. Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.

Note: It is important to vortex the enzyme mix prior to use for optimal performance.

2.4.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME PER ONE LIBRARY
ARTIC SARS-CoV-2 cDNA (Step 2.3.11)	13 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	3.5 µl
○ (yellow) NEBNext Ultra II FS Enzyme Mix	1 µl
Total Volume	17.5 µl

2.4.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

2.4.5. In a thermal cycler*, run the following program:

TEMP	TIME
37°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.

2.5. Adaptor Ligation

2.5.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 2.4.5)	17.5 µl
● (red) NEBNext Adaptor for Illumina**	1.25 µl
● (red) NEBNext Ultra II Ligation Master Mix*	15 µl
Total Volume	33.75 µl

* Mix the NEBNext 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.

Note: Do not premix adaptor with the Ligation Master Mix.

2.5.2. 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. **(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).**

2.5.3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

2.5.4. Add 1.5 µl of (red or blue) USER® Enzyme to the ligation mixture from Step 2.5.3.

Note: Steps 2.5.4. and 2.5.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

2.5.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

Samples can be stored overnight at –20°C.

2.6. Cleanup of Adaptor-ligated cDNA

The following section is for cleanup of the ligation reaction.

Note: The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step (35.25 µl; Step 2.5.5). 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.

2.6.1. Vortex the NEBNext Sample Purification Beads to resuspend.

2.6.2. Add 28 µl (0.8X) 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.

2.6.3. Incubate samples at room temperature for at least 5 minutes.

2.6.4. Place the tube/plate 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.

2.6.5. 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).**

2.6.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

2.6.7. Repeat Step 2.6.6 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 ethanol with a p10 pipette tip.

2.6.8. 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.

2.6.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 10 µl of 0.1X TE.

2.6.10. 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.

2.6.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 7.5 µl to a new PCR tube.

Samples can be stored at –20°C.

2.7. PCR Enrichment of Adaptor-ligated DNA

Follow Section 2.7.1A. if you are using the following oligos (10 µM primer):
Use Option A for any NEBNext Oligo kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Follow Section 2.7.1B. if you are using the following oligos (10 µM primers):
Use Option B for any 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.

2.7.1. Add the following components to a sterile strip tube:

2.7.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.6.11)	7.5 µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer/i7 Primer*,**	2.5 µl
Universal PCR Primer/i5 Primer*,**	2.5 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.

** Use only one i7/primer/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

2.7.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.6.11)	7.5µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer Mix*	5 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

2.7.2. 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.

2.7.3. Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

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

Set heated lid to 105°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 cleanup of the cDNA amplification step was skipped (Step 2.6) increase PCR cycles to 6.

2.8. Cleanup of PCR Reaction

Note: 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.

2.8.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.8.2. 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.

2.8.3. Incubate samples on bench top for at least 5 minutes at room temperature.

2.8.4. Place the tube/plate 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 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)**.

2.8.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

2.8.7. Repeat Step 2.8.6. 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 ethanol with a p10 pipette tip.

2.8.8. 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.

2.8.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE.

2.8.10. 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.

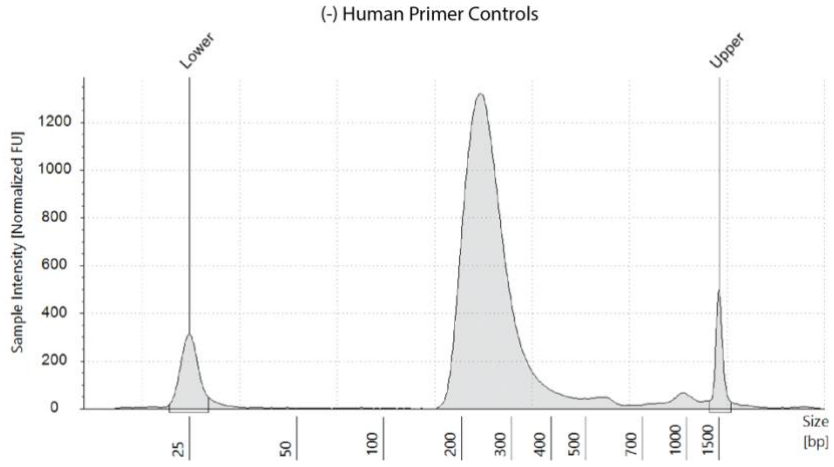
2.8.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube and store at –20°C.

2.8.12. Assess the library size distribution with Agilent Bioanalyzer or TapeStation high sensitivity DNA reagents. The sample may need to be diluted before loading. A peak size of 200–250 bp is expected, based on a 30-minute fragmentation time (Figure 2.8.12).

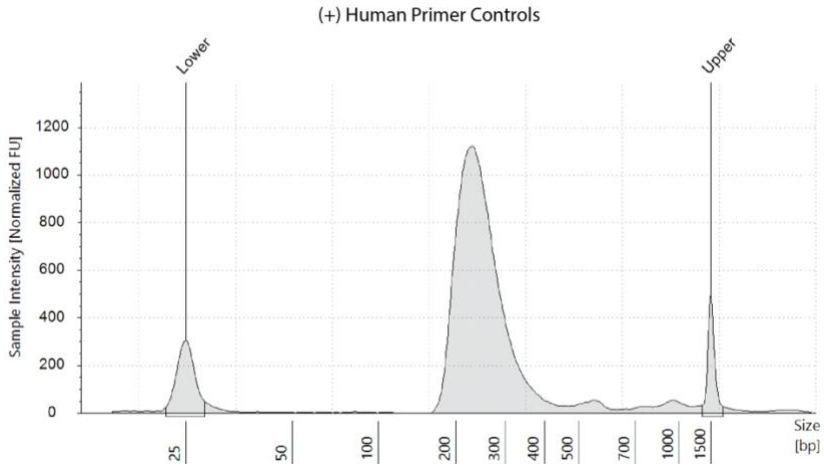
Note: If the cDNA amplification cleanup step was skipped, an additional peak of 150-180 bp may be observed in the final library. If this peak is observed, libraries should be pooled for sequencing and a 0.8X bead cleanup performed prior to sequencing. The cleanup will reduce the concentration of the pooled samples.

Figure 2.8.12: 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.

A.



B.



Chapter 3
NEBNext VarSkip Short Express Protocol without cDNA Amplicon and Ligation Bead Cleanups (One clean-up step)

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: We recommend using the express protocol for inputs of ≥ 100 copies of the (SARS-CoV-2) viral genome. The use of lower input amounts may result in significant levels of adaptor dimer in the sequencing data. In addition, we recommend setting up a no template control reaction. It is advisable to set up your reactions in the hood.

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

For no template controls, mix the following components:

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

3.1.2. 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. 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 3.1.2)	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

* 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 3.1.2)	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

* 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

*Set heated lid to 105°C

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.**

Note: When cDNA amplification cleanup steps are skipped, the cDNA amplicon pool must be diluted before to proceeding to library preparation. Description of sample dilution is detailed below in Steps 3.2.5-3.2.7.

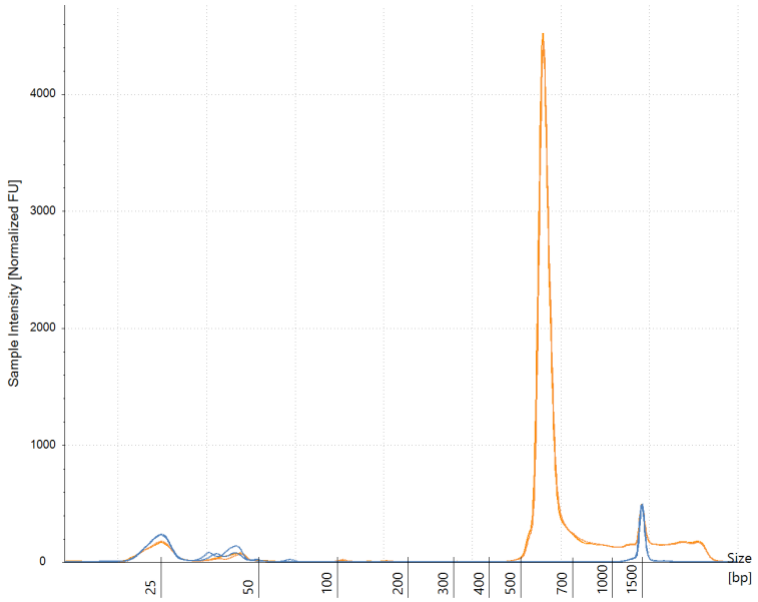
3.2.5. Transfer 1.3 µl of the pooled cDNA amplicons to a fresh tube.

3.2.6. Add 11.7 µl of 0.1X TE for a final volume of 13 µl.

3.2.7. Proceed to Fragmentation/End Prep (Step 3.3.1).

Note: The pooled cDNA amplicons may be run on a TapeStation® to confirm 560 bp size of amplicons without cleaning up. Cleanup of cDNA amplicon pool is not needed for assessing on a TapeStation. To run on a TapeStation, dilute an aliquot of the pooled amplicons 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape. (See Figure 3.2.7. below for example of amplicon size profile on a TapeStation)

Figure 3.2.7: 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.



3.3. Fragmentation/End Prep

FRAGMENTATION SIZE RANGE	INCUBATION @ 37°C	OPTIMIZATION
80 bp–300 bp	25 min	20–30 min

Note: Fragmentation occurs during a 37°C incubation step. A fragmentation time of 25 minutes should be sufficient for most samples to generate mainly ~120 bp fragments, however, the time may need to be optimized.

3.3.1. Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

3.3.2. Vortex the Ultra II FS Enzyme Mix 5–8 seconds prior to use and place on ice.

Note: It is important to vortex the enzyme mix prior to use for optimal performance.

3.3.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME PER ONE LIBRARY
ARTIC SARS-CoV-2 cDNA (**Step 3.2.7**)	13 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	3.5 µl
○ (yellow) NEBNext Ultra II FS Enzyme Mix	1 µl
Total Volume	17.5 µl

3.3.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

3.3.5. In a thermal cycler*, run the following program:

TEMP	TIME
37°C	25 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.

3.4. Adaptor Ligation

3.4.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 3.3.5)	17.5 µl
• (red) NEBNext Adaptor for Illumina**	1.25 µl
• (red) NEBNext Ultra II Ligation Master Mix*	15 µl
Total Volume	33.75 µl

* Mix the NEBNext 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.

Note: Do not premix adaptor with the Ligation Master Mix.

3.4.2. 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. **(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).**

3.4.3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

3.4.4. Add 1.5 µl of (red or blue) USER® Enzyme to the ligation mixture from Step 3.4.3.

Note: Steps 3.4.4. and 3.4.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

3.4.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

Samples can be stored overnight at –20°C.

Note: Only a portion of the ligation reaction (7.5 µl) will move forward to PCR enrichment.

3.5. PCR Enrichment of Adaptor-ligated DNA

Follow Section 3.5.1A. if you are using the following oligos:
Use Option A for any NEBNext Oligo kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 µM each.

Follow Section 3.5.1B. if you are using the following oligos:
Use Option B for any NEBNext Oligo kit w here 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 at 10 µM combined.

3.5.1. Add the following components to a sterile strip tube:

3.5.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 3.4.3 or 3.4.5)	7.5 µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer/i7 Primer*,**	2.5 µl
Universal PCR Primer/i5 Primer*,**	2.5 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.

** Use only one i7/primer/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

3.5.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 3.4.3 or 3.4.5)	7.5 µl
• (blue) NEBNext Library PCR Master Mix	12.5 µl
Index Primer Mix*	5 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

3.5.2. 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.

3.5.3. Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

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

Set heated lid to 105°C.

* The number of PCR cycles recommended should be viewed as a starting point and may need to be optimized for particular sample types.

3.6. Cleanup of PCR Reaction

Follow Section 3.6.1A if you will normalize individual final libraries prior to pooling for sequencing. Option A is recommended for samples with a large range of SARS-CoV-2 viral genome copies input into the cDNA synthesis reaction (Section 3.1), such as a set of samples with inputs outside of the 100–100,000 genome copies range.

Follow Section 3.6.1B if you are not normalizing individual final libraries prior to pooling for sequencing. Option B is recommended for samples with a small range of SARS-CoV-2 viral genome copies input into the cDNA synthesis reaction (Section 3.1), such as a set of samples with inputs within the 100–100,000 genome copies range. Otherwise, a sequencing depth of at least 1 million reads per library may be required.

3.6.1A. Cleanup of Individual PCR Reactions

Note: The amount of NEBNext Sample Purification Beads added in Step 3.6.1A.2 is specific for samples suspended in the buffer described in Section 3.5.1. Using the amount of beads at Step 3.6.1A.2 may not work properly for sample cleanup at other steps in the workflow. If your samples are suspended in a different buffer at this point, the amount of beads required may need to be experimentally determined.

3.6.1A.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.6.1A.2. Add 17.5 µl (0.7X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Take care 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.

3.6.1A.3. Incubate samples on bench top for at least 5 minutes at room temperature.

3.6.1A.4. Place the tube/plate 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.

3.6.1A.5. 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)**.

3.6.1A.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

3.6.1A.7. Repeat Step 3.6.1A.6 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 ethanol with a p10 pipette tip.

3.6.1A.8. 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.

3.6.1A.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 0.1X TE.

3.6.1A.10. 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.

3.6.1A.11. 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.

3.6.1A.12. Assess the library size distribution with Agilent Bioanalyzer or TapeStation high sensitivity DNA reagents. The sample may need to be diluted before loading. A peak size of 200–400 bp is expected, based on a 25-minute fragmentation time (Figure 3.6.12).

Note: If excess adaptor dimer peak is observed at 150–180 bp, a second 0.7X bead cleanup can be performed. The second 0.7X cleanup will result in slightly larger libraries which will not affect sequencing results.

3.6.1B. Cleanup of Pooled PCR Reactions

Note: For cleanup of pooled PCR reactions, we recommend cleanup of a 50 µl pool volume. For non-normalized pools using a 24-reaction kit, add 3 µl of each library to the pool, then mix, spin down, and take 50 µl of the pool to cleanup. For non-normalized pools using a 96-reaction kit, add 2 µl of each library to the pool, then mix, spin down, and take 50 µl of the pool on to cleanup. The amount of NEBNext Sample Purification Beads added in Step 3.6.1B.2. is specific for pooled samples suspended in the buffer described in Section 3.5.1. Using the exact amount of beads as described in Step 3.6.1B.2 may not work properly for sample cleanup at other steps in the workflow. If your samples are suspended in a different buffer at this point, the amount of beads needed may need to be experimentally determined.

3.6.1B.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.6.1B.2. Add 35 µl (0.7X) resuspended beads to the 50 µl pool. Mix well by pipetting up and down at least 10 times. Take care 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 pool after mixing, be sure to stop the centrifugation before the beads start to settle out.

3.6.1B.3. Incubate samples on bench top for at least 5 minutes at room temperature.

3.6.1B.4. Place the tube/plate 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 well before placing on the magnetic stand.

3.6.1B.5. 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)**.

3.6.1B.6. Add 200 µl of 80% freshly prepared ethanol to the tube/plate 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.

3.6.1B.7. Repeat Step 3.6.1B.6 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 ethanol with a p10 pipette tip.

3.6.1B.8. 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 pool 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.

3.6.1B.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 25 µl of 0.1X TE.

3.6.1B.10. 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 pool to collect the liquid from the sides of the tube or plate well before placing back on the magnetic stand.

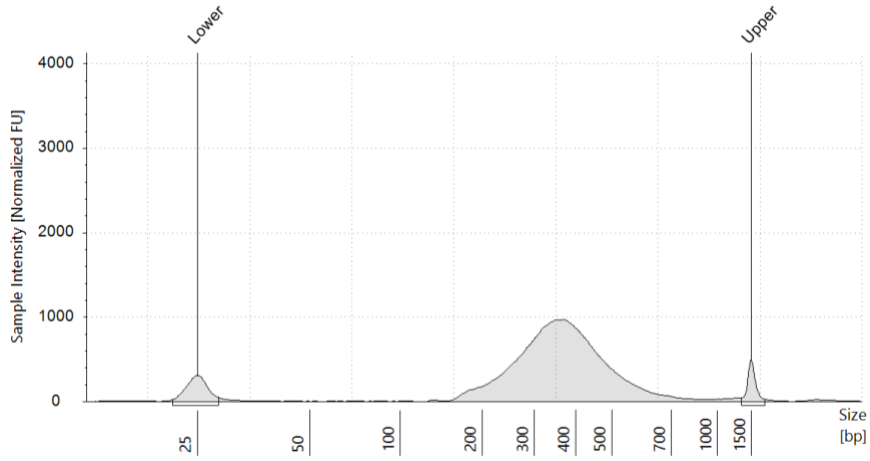
3.6.1B.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 23 µl to a new PCR tube and store at –20°C.

3.6.1B.12. Assess the library size distribution with Agilent Bioanalyzer or TapeStation high sensitivity DNA reagents. The pool may need to be diluted before loading. A peak size of 200–400 bp is expected, based on a 25-minute fragmentation time (Figure 3.6.12).

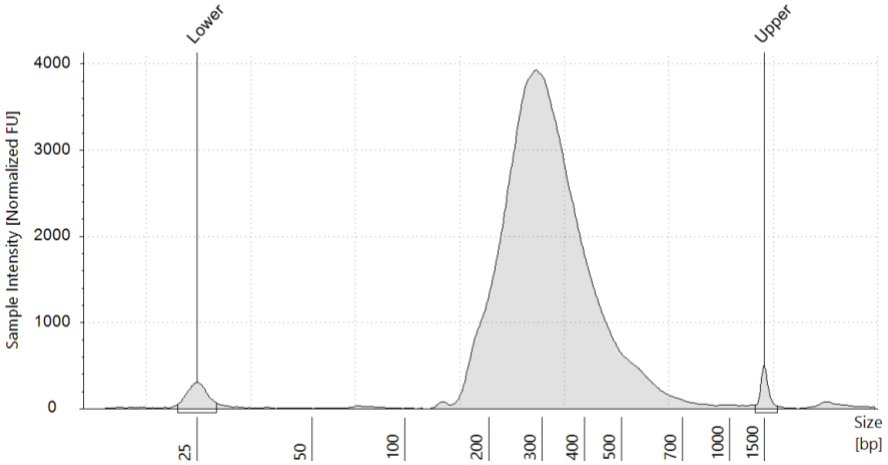
Note: If excess adaptor dimer peak is observed at 150–180 bp, a second 0.7X bead cleanup can be performed. The second 0.7X cleanup will result in slightly larger libraries which will not affect sequencing results.

Figure 3.6.12: Example of final library pool size distributions on a TapeStation. VarSkip Short SARS-CoV-2 libraries were generated from 1,000 viral copies. Normalized library pool after 0.7X bead cleanup of individual libraries, pooling, and 0.7X bead ration cleanup of normalized pool (A). Non-normalized library pool cleaned up once with a 0.7X bead ratio (B).

A.



B.



Kit Components

NEB #E7658S Table of Components

NEB #	PRODUCT	VOLUME
E7651A	LunaScript RT SuperMix	0.048 ml
E7652A	Q5 Hot Start High-Fidelity 2X Master Mix	0.3 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	7 µl
E7728A	NEBNext ARTIC Human Control Primer Pairs 2	7 µl
E7668A	NEBNext Ultra II FS Enzyme Mix	0.024 ml
E7669A	NEBNext Ultra II FS Reaction Buffer	0.084 ml
E7655A	NEBNext Ultra II Ligation Master Mix	0.36 ml
E7656A	NEBNext Library PCR Master Mix	0.3 ml
E7657A	0.1X TE	1.3 ml
E7667A	Nuclease-free Water	1.5 ml
E7659S	NEBNext Sample Purification Beads	2.1 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 #E7658L 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
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	7 µl
E7728A	NEBNext ARTIC Human Control Primer Pairs 2	7 µl
E7668AA	NEBNext Ultra II FS Enzyme Mix	0.096 ml
E7669AA	NEBNext Ultra II FS Reaction Buffer	0.336 ml
E7655AA	NEBNext Ultra II Ligation Master Mix	2 x 0.72 ml
E7656AA	NEBNext Library PCR Master Mix	1.2 ml
E7657AA	0.1X TE	5.2 ml
E7667A	Nuclease-free Water	1.5 ml
E7659L	NEBNext Sample Purification Beads	4 x 2.1 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

Companion Products

NEB #	PRODUCT	VOLUME
T2010S	Monarch® Total RNA Miniprep Kit	50 preps

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 Mix 1 and 2 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 protocols, add 2 Chapters	6/21
4.0	Update protocols, add Chapter 3	9/21
4.1	Update protocols	9/21