INSTRUCTION MANUAL

NEW ENGLAND Biolabs®

NEBNext® RSV Primer Module

NEB #E9642S/L

24/96 reactions

Version 1.0_9/24

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The NEBNext RSV Primer Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E9642S) or 96 reactions (NEB #E9642L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

○ (white) NEBNext RSV Primer Mix 1

○ (white) NEBNext RSV Primer Mix 2

Required Materials Not Included for Illumina Sequencing

- NEBNext UltraExpress® FS DNA Library Prep Kit (NEB #E3340S/L)
- NEBNext Multiplex Oligos for Illumina
  - NEBNext Multiplex Oligos options can be found at www.neb.com/oligos.
  - Alternatively, customer-supplied adaptor and primers can be used. Additional information can be found here: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb
- LunaScript® Multiplex One-Step RT-PCR Kit (NEB #E1555S/L)
- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind® Tubes (Eppendorf® #022431021)
- DNase-, RNase-free PCR strip tubes
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® #A001322) or equivalent
- Vortex Mixer
- Thermal Cycler
- Bioanalyzer® or TapeStation® (Agilent Technologies®, Inc.) and associated reagents and consumables
- NanoDrop® or Lunatic® (Unchained Labs®) and associated consumables
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific®, Inc. #Q32851)

Required Materials Not Included for Oxford Nanopore Technologies Sequencing

- NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E7546S/L)
- Blunt/TA Ligase Master Mix (#M0367S)
- NEBNext Quick Ligation Module (NEB #E6056S/L)
- LunaScript Multiplex One-Step RT-PCR Kit (NEB #E1555S/L)
- Oxford Nanopore Technologies SFB Expansion (EXP-SFB001)
- Oxford Nanopore Technologies Native Barcoding Kits; Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96)
- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf #022431021)
- DNase-, RNase-free PCR strip tubes
- SPRIselect Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic racks (e.g. NEB# S1506S and NEB #S1515S), magnetic plate (Alpaqua #A001322), or equivalent
- Vortex Mixer
- Thermal Cycler
- Bioanalyzer or TapeStation (Agilent Technologies, Inc.) and associated reagents and consumables
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc. #Q32851)

Overview

The NEBNext RSV Primer Module is intended for use in cDNA generation and library preparation upstream of Respiratory Syncytial Virus (RSV) sequencing on either Illumina or Oxford Nanopore Technology platforms. It includes two primer mixes (NEBNext RSV Primer Mix 1 and Primer Mix 2); these primer mixes for RSV genome amplification can identify RSV-A and RSV-B and were designed to reduce the impact of variants on amplification efficiency. Sequence information can be found in the Protocols, Manuals & Usage section of the NEBNext RSV Primer Module product page (www.neb.com/e9642). Note: The NEBNext RSV Primer Module contains only sequencing primers; all other required reagents must be purchased separately.

For Illumina sequencing, the NEBNext RSV Primer Module is used in conjunction with the LunaScript Multiplex One-Step RT-PCR Kit and NEBNext UltraExpress FS DNA Library Prep Kit, which contain the enzymes and buffers required for whole-genome targeted amplification of RSV and rapid conversion of amplicons into high-quality libraries for sequencing on Illumina platforms. The fast and simple workflow allows for amplicon library preparation in a single tube, minimizing plastic consumables waste.

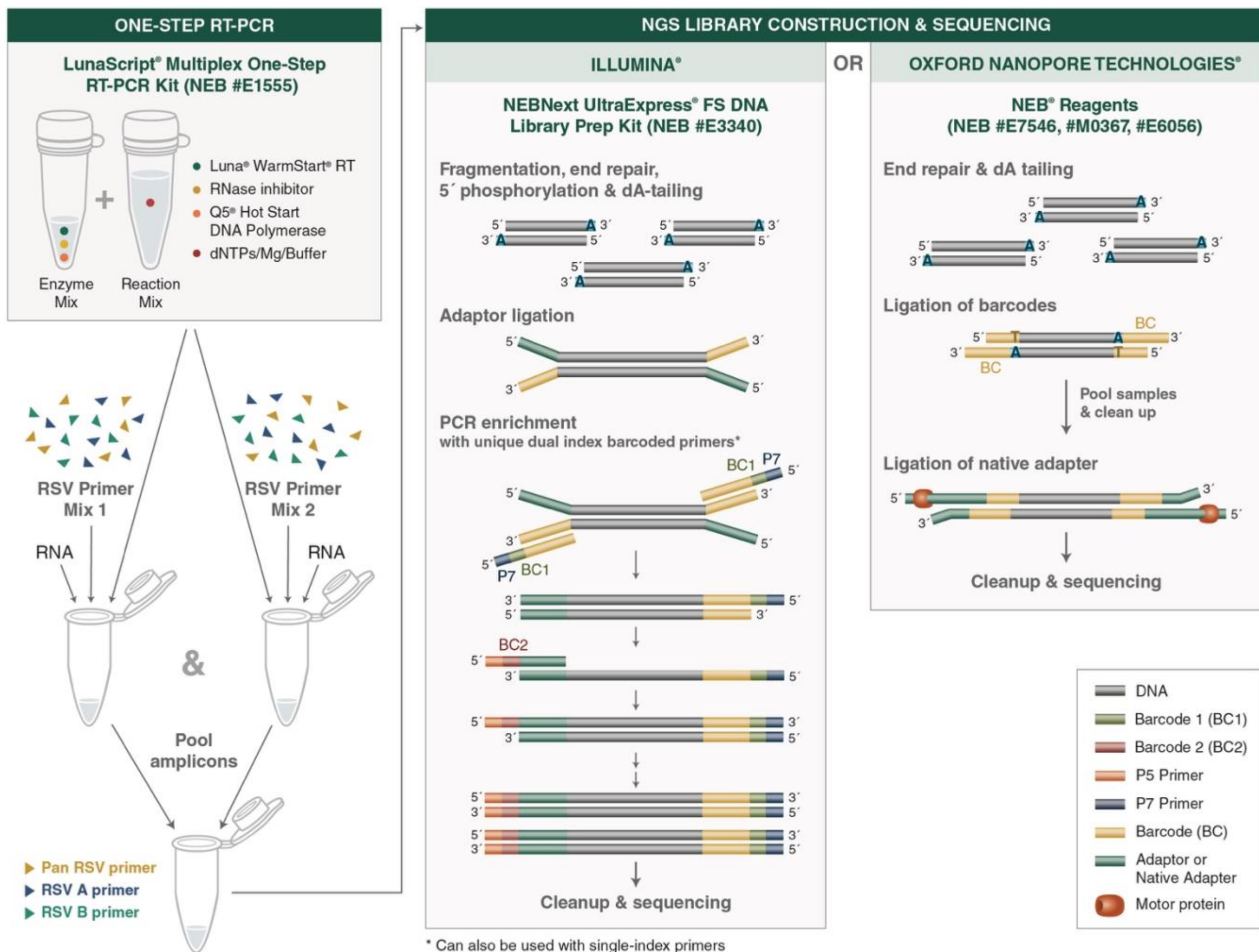
For ONT® sequencing, the NEBNext RSV Primer Module is meant to be used in conjunction with the LunaScript Multiplex One-Step RT-PCR kit and ONT Native Barcoding library prep reagents, allowing for whole-genome targeted amplification of RSV and rapid conversion of amplicons into high-quality libraries for sequencing on Oxford Nanopore Technologies platforms.

All module components must pass rigorous quality control standards. Module components are also functionally validated via amplicon generation, followed by the construction and sequencing of indexed libraries on an Illumina sequencing platform.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

Please refer to the product page on NEB.com for FAQs about this product.

Figure 1. Workflow demonstrating the use of NEBNext RSV Primer Module across two sequencing platforms



Section 1
RSV Amplicon Generation

Symbols

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

This caution sign signifies a step in the protocol that has multiple 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.

1.1. One-Step RT-PCR with NEBNext RSV Primers

Notes:

(1) We recommend setting up the cDNA synthesis and PCR reactions in a room (ideally in a hood) separate from the library construction area to minimize cross-contamination of future reactions.

(2) Inputs of ≥ 1,000 copies of the RSV viral genome are recommended. The use of lower viral gRNA input amounts may result in significant levels of adaptor dimer in the sequencing data. For samples consisting of viral gRNA mixed with host RNA, we recommend not exceeding 100 ng of total RNA input.

(3) 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. NTC reactions should be prepared prior to non-NTC samples following the guidelines outlined below.

(4) Precipitates may appear in the LunaScript Multiplex One-Step RT-PCR Reaction Mix upon thawing, resuspend completely prior to use by vortexing or pipette mixing 10 times.

1.1.1. Briefly centrifuge the LunaScript Multiplex One-Step RT-PCR Enzyme Mix to collect solution to bottom of the tube, then place on ice.

1.1.2. Thaw the LunaScript Multiplex One-Step RT-PCR Reaction Mix, RSV Primer Mix 1, RSV Primer Mix 2, and the nuclease-free water at room temperature, then place on ice. Prior to use, vortex and briefly centrifuge the Reaction Mix and Primer Mixes.

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

For Pool Set 1:

COMPONENT	VOLUME
○ (white) Nuclease-free Water	7.25 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix	2.5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix	1 µl
○ (white) NEBNext RSV Primer Mix 1	1.75 µl
Total Volume	**12.5 µl**

For Pool Set 2:

COMPONENT	VOLUME
○ (white) Nuclease-free Water	7.25 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix	2.5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix	1 µl
○ (white) NEBNext RSV Primer Mix 2	1.75 µl
Total Volume	**12.5 µl**

1.1.4. Prepare the split pool cDNA synthesis and amplification reaction as described below:

For Pool Set 1:

COMPONENT	VOLUME
RNA template*	1 – 7.25 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix	2.5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix	1 µl
○ (white) NEBNext RSV Primer Mix 1	1.75 µl
○ (white) Nuclease-free Water	to 12.5 µl
Total Volume	**12.5 µl**

* For achieving high genome coverage, use an input of ≥ 1,000 copies of RSV viral genomes.

For Pool Set 2:

COMPONENT	VOLUME
RNA template*	1 – 7.25 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix	2.5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix	1 µl
○ (white) NEBNext RSV Primer Mix 2	1.75 µl
○ (white) Nuclease-free Water	to 12.5 µl
Total Volume	**12.5 µl**

* For achieving high genome coverage, use an input of ≥ 1,000 copies of RSV viral genomes.

1.1.5. Mix reactions gently by pipetting up and down or by inverting the tubes, then briefly centrifuge to collect solutions to the bottom of tubes.

1.1.6. Place the tube in a thermal cycler with the heated lid set to 105°C, or on and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C*	25 minutes	1
Reverse Transcriptase Inactivation & Initial Denaturation	98°C	1 minute	1
Denaturation	95°C	15 seconds	35
Annealing	60°C**	3 minutes	
Extension	70°C	2 minutes	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	1

* 55°C is the optimal temperature for reverse transcription using Luna WarmStart Reverse Transcriptase. To ensure best performance and full WarmStart activation, avoid using reverse transcription temperatures below 50°C.

** The annealing temperature is primer-mix dependent, 60°C is optimal for use with NEB #E9642S/L RSV primers.

Samples can be stored at –20°C long-term.

1.1.7. The amplified cDNA product can be stored at 4°C overnight or -20°C for long-term storage prior to proceeding with downstream applications.

Note: We recommend proceeding with the library construction in a different area or room.

1.1.8. Combine amplified cDNA product from Primer Mix 1 and Primer Mix 2 reactions at a 1:1 ratio.

Notes:

(1) The cDNA samples must be diluted before proceeding to library preparation. Description of sample dilution is detailed below in Step 1.1.9.

(2) The cDNA samples may be run on a TapeStation to confirm amplicons in the size range of 400 – 1,400 bp (Refer to Figure 1.1). Cleanup of cDNA amplicons is not needed for assessing on a TapeStation. To run on a TapeStation, dilute an aliquot of the amplicons 10-fold with 0.1X TE Buffer and run 2 µl on a DNA High Sensitivity ScreenTape®. See Figure 1.1 for example of amplicon size profile.

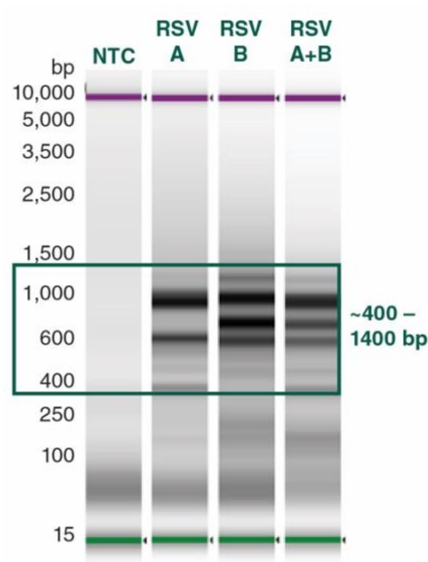
1.1.9. For each cDNA sample, prepare a dilution.

Follow Option A to proceed with Library Prep for Illumina Sequencing in Section 2. Follow Option B to proceed with Library Prep for Oxford Nanopore Sequencing in Section 3.

Option A: Transfer 3 µl of the combined cDNA amplicons to a fresh tube. Add 12 µl of 1X TE for a final volume of 15 µl per sample.

Option B: Transfer 2.5 µl of the combined cDNA amplicons to a fresh tube. Add 10 µl of 0.1X TE or Nuclease-Free Water for a final volume of 12.5 µl per sample.

Figure 1.1: Examples of amplicons prepared from 1,000 genome copies of RSVA, RSV B, or both.



Amplicons made from non-template control (NTC) or 1,000 copies of RSV gRNA were diluted 1:10 and run on a TapeStation HSD5000 ScreenTape. Green box highlights the ~400-1,400 bp range of expected amplicon bands/peaks.

Section 2
RSV Amplicon Library Preparation for Illumina Platform

2.1 Fragmentation/End Prep

Notes:

(1) Ensure that the NEBNext UltraExpress FS Reaction Buffer is completely thawed. If precipitate is seen in the buffer, pipette up and down several times to break it up, vortex to mix, and place on ice prior to use.

(2) It is important to vortex the NEBNext UltraExpress FS enzyme mix prior to use for optimal performance. If making a master mix of NEBNext UltraExpress FS Reaction Buffer and NEBNext UltraExpress FS Enzyme Mix, it is important to mix the individual component before combining into a master mix. It is also important to vortex the master mix before adding to sample.

2.1.1. Vortex the NEBNext UltraExpress FS Enzyme Mix and Reaction Buffer 5–8 seconds prior to use and place on ice. A master mix of the enzyme mix and buffer may be prepared immediately prior to use.

2.1.2. Add the following components to a sterile nuclease-free tube on ice:

COMPONENT	VOLUME
Diluted RSV Amplicons from Step 1.1.9,*,**	15 µl
● (yellow) NEBNext UltraExpress FS Reaction Buffer	4 µl
● (yellow) NEBNext UltraExpress FS Enzyme Mix	1 µl
Total Volume	**20 µl**

* Use 15 µl of diluted amplicons (3 µl of amplicons + 12 µl of 1X TE) as input for NEBNext UltraExpress FS library prep.

** Alternatively, use 10–200 ng of cleaned-up amplicons.

2.1.3. Vortex the reaction for 5 seconds and briefly spin down. A small number of bubbles in the reaction will not inhibit performance.

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

25 minutes at 37°C

15 minutes at 65°C

Hold at 4°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.

2.2. Adaptor Ligation

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

COMPONENT	VOLUME
FS Reaction Mixture (Step 2.1.4.)	20 µl
● (red) NEBNext Adaptor for Illumina*	2 µl
● (red) NEBNext UltraExpress Ligation Master Mix**	20 µl
Total Volume	**42 µl**

* The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit. www.neb.com/oligos

** Mix the NEBNext UltraExpress Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

Note: Do not premix the NEBNext UltraExpress Ligation Master Mix and Adaptor prior to use in the Adaptor Ligation Step.

2.2.2. Set a 100 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. **(Caution: The NEBNext UltraExpress 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 number of bubbles will not interfere with performance).**

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

Note: Steps 2.2.4 and 2.2.5. are only required for use with NEBNext Adaptor (loop adaptor). USER enzyme can be found in NEBNext Multiplex Oligos for Illumina sets.

2.2.4. Add 3 µl of • (red) USER® Enzyme to the ligation mixture from Step 2.2.3.

2.2.5. Mix well and incubate at 37°C for 5 minutes in a thermal cycler with the heated lid set to ≥ 47°C or on.

Samples can be stored at –20°C overnight.

2.3. PCR Amplification of Adaptor-ligated DNA

Use Option A (2.3.1.A.) for any NEBNext Index Primers where the forward and reverse primers are supplied separately in tubes. Primers are supplied at 10 µM.

Use Option B (2.3.1.B.) for any NEBNext Index Primers where index primers are supplied with the forward and reverse primers (i5 and i7) premixed in a 96-well plate format. These primers are supplied at a 10 µM combined concentration (5 µM each).

Notes:

(1) The adaptor amount and PCR cycle numbers were optimized using high-quality amplicon cDNA and NEBNext adaptor for Illumina. For other DNA input types, we recommend optimization of adaptor amount and PCR cycles.

(2) Take out NEBNext Bead Reconstitution Buffer and bring up to room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to room temperature for at least 30 minutes before use.

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

2.3.1.A. Forward and Reverse Primers not already combined (Option A)

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.2.5.)	45 µl
• (blue) NEBNext MSTC High Yield Master Mix	45 µl
• (blue) Index Primer/i7 Primer*,**	5 µl
• (blue) Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	**100 µl**

2.3.1.B. Forward and Reverse Primers already combined (Option B)

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.2.5.)	45 µl
• (blue) NEBNext MSTC High Yield Master Mix	45 µl
Index/Universal Primer*	10 µl
Total Volume	**100 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Multiplex Oligos 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.

2.3.2. Set a 100 µl or 200 µl pipette to 90 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.

2.3.3. Place the tube in a thermal cycler with the heated lid set to 105°C, or on 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	∞	

* For cDNA amplicon inputs greater than 200 ng, 5 cycles of PCR can result in amplification beyond the linear phase during the late cycles. This can cause inaccurate library quantification on migration-based methods like Bioanalyzer and TapeStation. In this case, we recommend using Nanodrop or Lunatic for library quantification.

2.3.4. Proceed to cleanup of PCR amplified libraries in Section 2.4.

2.4. Phased Bead Cleanup of PCR Reaction

Note: The SPRIselect or AMPure Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, keep at room temperature for 30 minutes prior to use.

2.4.1. Vortex SPRIselect or AMPure Beads to resuspend. Thaw and mix NEBNext Bead Reconstitution Buffer at room temperature and afterwards it may be stored in a sealed container at room temperature for future use.

2.4.2. Add 70 µl (0.7X) of resuspended beads to the 100 µl PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all 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.

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

2.4.4. 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.

2.4.5. 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)**.

2.4.6. Remove the tube/plate from the magnetic stand **(Note: No ethanol wash at this step)**. Add 100 µl of 0.X TE (dilute 1X TE Buffer 1:10 in water) to resuspend the beads. Mix well by pipetting up and down at least 10 times.

2.4.7. Add 80 µl (0.8X) of NEBNext Bead Reconstitution Buffer to the 100 µl of resuspended beads. 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.

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

2.4.9. 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.

2.4.10. 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)**.

2.4.11. Add 200 µl of freshly prepared 80% 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.

2.4.12. Repeat Step 2.4.11. once for a total of two washes.

2.4.13. 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.

2.4.14. 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.

2.4.15. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in water).

2.4.16. 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.

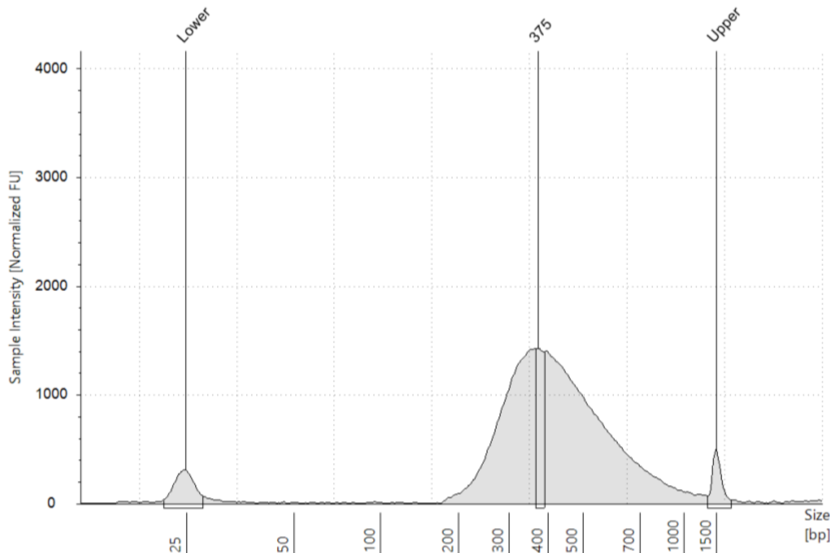
2.4.17. Place the tube/plate on the magnetic stand.

2.4.18. When the solution is clear (about 5 minutes), transfer 30 μl to a new PCR tube.

2.4.19. Check the size distribution on a TapeStation D1000 HS ScreenTape. The sample may need to be diluted before loading.

Samples can be stored at –20°C long term.

Figure 2: Examples of libraries prepared with 1,000 genome copies of RSV



Library made from 1000 copies of RSV B gRNA (ATCC 1580) with 5 PCR cycles, run on a TapeStation HSD1000 ScreenTape.

Note: If excess adaptor dimer peak is observed at 150–180 bp, an additional 0.8X bead cleanup can be performed on individual or pooled libraries. The additional cleanup will result in a reduction in yield and slightly larger libraries, which will not affect sequencing results.

Section 3
RSV Amplicon Library Preparation for Oxford Nanopore Platform

3.1. End Prep

Note: The End Prep Enzyme Mix and Reaction Buffer can be pre-mixed and master mix is stable on ice for 4 hours.

3.1.1. Add the following components to a sterile nuclease-free tube on ice:

COMPONENT	VOLUME
Diluted RSV Amplicons from Step 1.1.9.*	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**

* Use 12.5 µl of diluted amplicons (2.5 µl of amplicons + 10 µl of 0.1X TE or Nuclease-Free Water) as input for ONT Native Barcoding library prep.

3.1.2. Flick the tube or pipette up and down 10 times to mix the solution. Perform a quick spin to collect all liquid from the sides of the tube. A small number of bubbles in the reaction will not inhibit performance.

3.1.3. Place in a thermal cycler, with the heated lid set to ≥ 75°C or on, and run the following program:
10 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

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

3.2. Barcode Ligation

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

COMPONENT	VOLUME
○ (white) Nuclease-free Water	6 µl
End-prepped DNA (Step 3.1.3.)	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 Kit(s); Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96)

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

3.2.2. Flick the tube or pipette 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).**

3.2.3. Place in a thermal cycler, with the heated lid set to off, and run the following program:
20 minutes at 25°C
10 minutes at 65°C
Hold at 4°C

3.2.4. Add 2 µl of EDTA provided in Oxford Nanopore Technologies Native Barcoding V14 Kit(s) to each barcoded sample prior to pooling to hinder non-specific barcode ligation amongst pooled samples.

3.2.5. Pool all barcoded samples by transferring 20 µl of each barcoded sample into a 1.5 ml DNA LoBind Tube.

Samples can be stored at –20°C overnight.

3.3. Cleanup of Pooled Barcoded DNA

Notes:

(1) Use the pooled barcoded DNA samples (from Step 3.2.5.), up to 480 µl, for bead cleanup. Remaining pooled DNA can be stored at -20°C.

(2) The SPRIselect or AMPure Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, keep at room temperature for 30 minutes prior to use.

3.3.1. Vortex SPRIselect or AMPure Beads to resuspend.

3.3.2. Add 0.4X resuspended beads to pooled barcoded samples (Step 3.2.5.) (For example, if the total volume of the pooled barcoded samples is 480 µl, add 192 µl of resuspended sample purification beads to the 480 µl of pooled sample). Flick the tube 15 times to mix, followed by a quick spin for 1 second.

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

3.3.4. Place the tube on a 1.5 ml magnetic stand (e.g., NEB #S1506) to separate the beads from the supernatant.

3.3.5. 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)**.

3.3.6. Wash the beads by adding 125 µl of Short Fragment Buffer (SFB) provided in the Native Barcoding Kit or SFB Expansion from Oxford Nanopore. Flick the tube to resuspend the pellet followed by a quick spin for 1 second before placing back on the magnetic stand.

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

3.3.8. When the solution is clear (about 3 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

3.3.9. Repeat steps 3.3.6. through 3.3.8. 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.

3.3.10. Add 500 µl of freshly prepared 80% 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.

3.3.11. Repeat Step 3.3.10. once for a total of 2 washes.

3.3.12. If necessary, quickly spin the sample for 1 second to collect residual ethanol from the sides of the tube before placing it back on the magnetic stand, then remove any residual ethanol.

3.3.13. 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.

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

3.3.15. Resuspend the pellet by flicking the tube or pipetting up and down gently 10 times.

3.3.16. 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 it back on the magnetic stand.

3.3.17. 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.

3.3.18. 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 overnight if they are not used immediately.

3.4. Native Adapter Ligation

3.4.1. Use the Qubit readings from Step 3.3.18. to dilute up to 130 ng of the Native barcoded DNA pool with nuclease-free water to a final tube:

COMPONENT	VOLUME
Native barcoded and purified DNA (Step 3.3.17.; up to 130 ng)	30 µl
• (red) NEBNext Quick Ligation Reaction Buffer*	10 µl
Native Adapter**	5 µl
(red) Quick T4 DNA 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.

** Native Adapter is provided in Oxford Nanopore Technologies Native Barcoding Kit(s); Native Barcoding Kit 24 V14 (SQK-NBD114.24) or Native Barcoding Kit 96 V14 (SQK-NBD114.96).

3.4.2. 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).**

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

3.4.4. Proceed to Cleanup of Native Adapter-ligated DNA in Section 3.5.

3.5. Cleanup of Native Adapter Ligated DNA

Note: The SPRIselect or AMPure Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

3.5.1. Vortex SPRIselect or AMPure Beads to resuspend.

3.5.2. Add 25 µl (0.5X) resuspended beads to the ligation mix. Flick the tube 15 times to mix, followed by a quick spin for 1 second.

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

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

3.5.5. 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).

3.5.6. Wash the beads by adding 125 µl of Short Fragment Buffer (SFB) provided in the Native Barcoding Kit or SFB Expansion from Oxford Nanopore. Flick the tube to resuspend the pellet followed by a quick spin for 1 second before placing back on the magnetic stand.

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

3.5.8. When the solution is clear (about 3 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads).**

3.5.9. Repeat steps 3.5.6. through 3.5.8. 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.

3.5.10. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of Elution Buffer (EB) provided in the Native Barcoding Kit from Oxford Nanopore.

3.5.11. 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 it back on the magnetic stand.

3.5.12. 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.

3.5.13. Use a Qubit fluorometer to quantify 1 µl of Native Adapter-ligated DNA sample.

3.5.14. Follow Oxford Nanopore Technologies Native Barcoding Kit Protocol for preparing the flow cell and DNA library sequencing mix using 30–50 ng adapter-ligated DNA sample (Step 3.5.13.).

Notes:

(1) After normalizing the DNA to 30 ng - 50 ng, if the volume is less than 12 µl, then top off the sample volume to 12 µl with EB.

(2) Follow ONT SQK-NBD114 protocols and recommendations for MinION® or PromethION® Flow Cell priming and loading.

Kit Components

NEB #E9642S Table of Components

NEB #	COMPONENT	VOLUME
E9643AVIAL	NEBNext RSV Primer Mix 1	0.042 ml
E9644AVIAL	NEBNext RSV Primer Mix 2	0.042 ml

NEB #E9642L Table of Components

NEB #	COMPONENT	VOLUME
E9643AAVIAL	NEBNext RSV Primer Mix 1	0.168 ml
E9644AAVIAL	NEBNext RSV Primer Mix 2	0.168 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	9/24

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