OCT 05, 2023

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External link:
https://www.neb.com/-/media/nebus/files/manuals/manuale7658.pdf?rev=7253d569aa3040069cb09a9aa3724f2d&hash=53C7EDF0D2FDEDAE75308379A5EB2D89

Protocol Citation: Parker Montfort 2023. RSV Nested Amplicon Prep (from VarSkip Workflow). **protocols.io** https://protocols.io/view/rsv-nested-amplicon-prep-from-varskip-workflow-c2y5yfy6

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

Created: Oct 04, 2023

RSV Nested Amplicon Prep (from VarSkip Workflow)

Forked from RSV Tiled Amplicon Prep (from VarSkip Workflow)

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New England Biolabs (NEB) NEBNext 2 more workspaces

aglazer

ABSTRACT

Use this modified protocol to prepare RSV Tiled amplicons for sequencing using the VarSkip Reagents and Workflow, with cleanup steps.This original protocol details methods for the NEBNext® ARTIC SARS-CoV-2 FS Library Prep Kit (Illumina®), NEB #E7658S/L 24/96 reactions.

GUIDELINES

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 Primer Mix 1) and NEDD8 (NEBNext ARTIC Human Primer Mix 2) genes are supplied as optional internal controls. The fast, user-friendly workflow also has minimal hands-on time.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of indexed libraries on an Illumina sequencing platform.

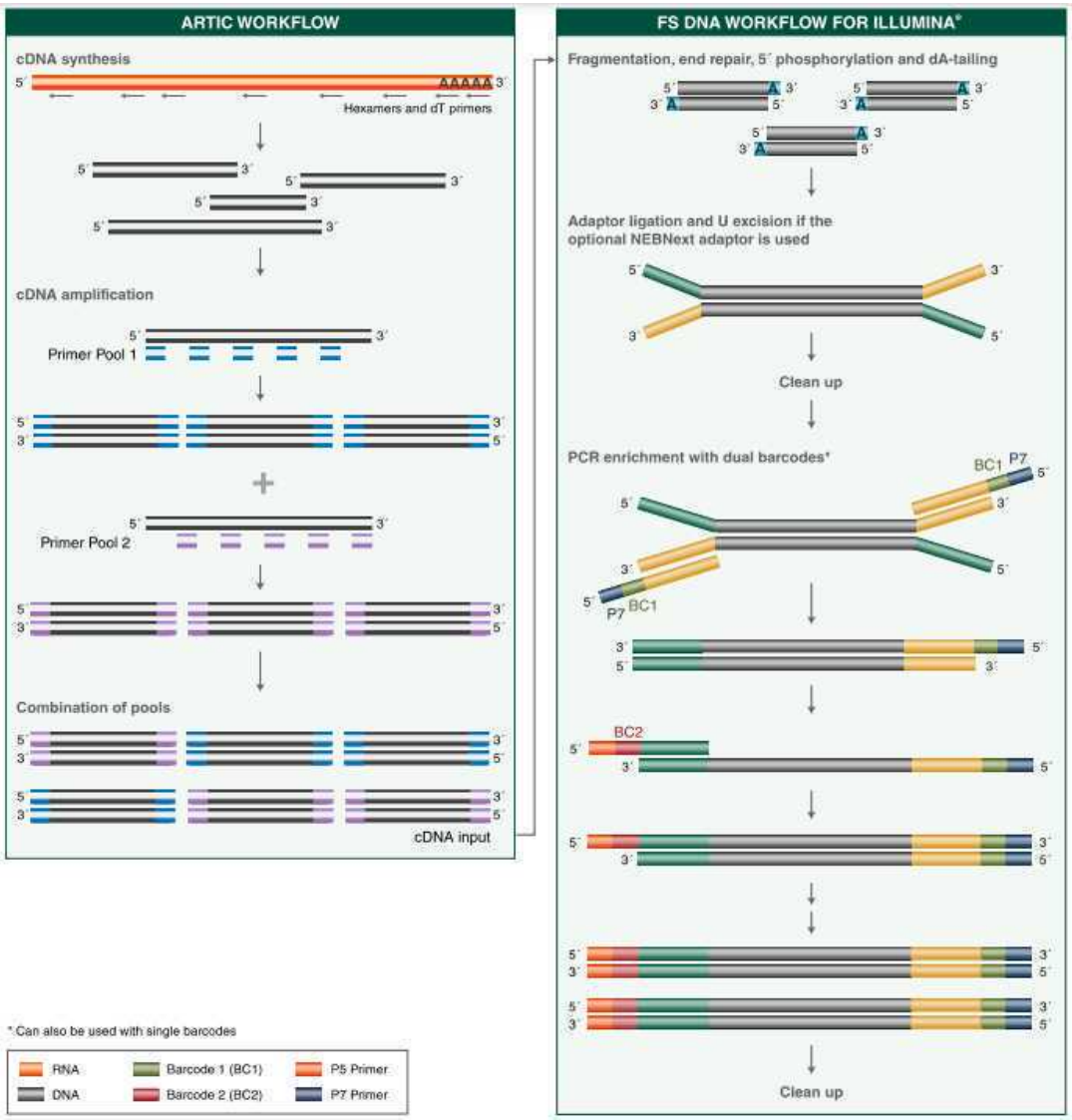
For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

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

Last Modified: Oct 05, 2023

PROTOCOL integer ID: 88829

Keywords: NEBNext, NEB, ARTIC, SARS-CoV-2, FS, Library Prep, Illumina



MATERIALS

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7658S) and 96 reactions (NEB #E7658L).

Package 1: Store at –20°C.

(lilac) LunaScript® RT SuperMix
(lilac) Q5® Hot Start High-Fidelity 2X Master Mix
(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 Buffer
(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 #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermocycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- DNase RNase free PCR strip tubes (USA Scientific® 1402-1708)

Kit Components

NEB #E7658S Table of Components

	A	B	C
	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 Primer Mix 1	0.010 ml
	E7728A	NEBNext ARTIC Human Primer Mix 2	0.010 ml
	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

NEB #E7658L Table of Components

	A	B	C
	NEB #	PRODUCT	VOLUME

	A	B	C
	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
	E7727AA	NEBNext ARTIC Human Primer Mix 1	0.034 ml
	E7728AA	NEBNext ARTIC Human Primer Mix 2	0.034 ml
	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
	E7667AA	Nuclease free-Water	1.5 ml
	E7659L	NEBNext Sample Purification Beads	4 x 2.1 ml

Primer Sequences for HRSV A and B genome sequencing using 10-amplicon one-step PCR

	A	B	C	D
	Primer	**Sequence (5'-3')**	Location	**Amplicon size (bp)**
	RSVA-F1	ACGSGAAAAAATGCGTACAAC	1-21	1835
	RSVA-R1	GAAGATTGTGCTATACCAAAATGAACA	1809-1835	
	RSVA-F2	ACAGGCATGACTCTCCTGAT	1586-1605	1873
	RSVA-R2	TTGGGTGTGGATATTTGTTTCAC	3436-3458	
	RSVA-F3	GGGCAAATATGGAAACATACGTG	3254-3276	1633
	RSVA-R3	GTTTGCYGAGGCTATGAATATGAT	4863-4886	
	RSVA-F4	ACCTGGGACACTCTCAATCA	4734-4753	1797
	RSVA-R4	GACATGATAGAGTAACTTTGCTGTCT	6505-6530	

	A	B	C	D
	RSVA-F5	GAACAACAGACTACTAGAGATTACCAG	6339-6365	1576
	RSVA-R5	AGGAGTTTGCTCATGGCAA	7896-7914	
	RSVA-F6	GTCACGAAGGAATCCTTGCA	7609-7628	1900
	RSVA-R6	CCCTCTACCTCTTTTATTATGTAGAACC	9481-9508	
	RSVA-F7	AGCTTAGGCTTAAGATGYGGA	9390-9410	1595
	RSVA-R7	TGAGTTTGACCTTCCATGAGT	10964-10984	
	RSVA-F8	GGTGTACAATCTCTATTTTCCTGGT	10671-10695	1937
	RSVA-R8	CGATTAATAGGGCTAGTATCAAAGTG	12582-12607	
	RSVA-F9	GGGTTGGTTCATCTACACAAGAG	12283-12305	1801
	RSVA-R9	CGCAAYAATAAATTCCCTGCTCC	14061-14083	
	RSVA-F10	CGTCTACAATGATTAGAACCAATTAC	13759-13784	1465
	RSVA-R10	ACGAGAAAAAAAGTGTCAAAAACTAA	15198-15223	
	RSVB-F1	ACGCGAAAAAATGCGTACTACA	1-22	1704
	RSVB-R1	CATTGTTTGCCCTCCTAATTACTG	1681-1704	
	RSVB-F2	CAGRTTAGGAAGGGAAGACACTA	1355-1377	1671
	RSVB-R2	CAAGTCACTCAATTTTTTGGAGGTTGG	2999-3025	
	RSVB-F3	GATCAAACAAATGACAACATTACAGCA	2720-2746	1634
	RSVB-R3	ATAGGGCCAAAATTTGCTTGTG	4332-4353	
	RSVB-F4	TGGAAGCAYACAGCTACACG	3986-4005	1793
	RSVB-R4	CTACATGTYGATTGGTAAAACTCC	5755-5778	

	A	B	C	D
	RSVB-F5	AGTGCAATCTTCCTAACTCTTGC	5690-5712	1678
	RSVB-R5	TGATTCCACTTAGTTGGTCTTTGC	7344-7367	
	RSVB-F6	CCTCTAGTGTTTCCTTCTGATGAG	7103-7126	1723
	RSVB-R6	GTTGTAGCAATTTGTTCAGACGAG	8802-8825	
	RSVB-F7	GGTGAACTGAAATTAGAAGAACCAAC	8737-8762	1762
	RSVB-R7	CACCATATCTTGTCAAACTCTCAGG	10474-10498	
	RSVB-F8	AAGTTCTCTGAAAGCGACAGATC	10222-10244	1960
	RSVB-R8	TAATACTTGGYGATGTTACTCCTAC	12157-12181	
	RSVB-F9	GAACCAACTTACCCTCATGGATT	11851-11873	1858
	RSVB-R9	TTCTGGGGTTGGGTGATATAG	13621-13641	
	RSVB-F10	TAGTCAATCAAGACACAAGTTTGC	13280-13303	1946
	RSVB-R10	ACGAGAAAAAAAGTGTCAAAAACTAATG	15198-15225	

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

BEFORE START INSTRUCTIONS

Note: The amount of RNA required for detection depends on the abundance of the RNA of interest. In general, we recommend using > 10 copies of the (SARS-CoV-2) viral genome as input, however, results may vary depending on the quality of the input. In addition, we recommend setting up a no template control reaction and that reactions are set-up in a hood.

The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful. It is advisable to set up your reactions in the hood.

cDNA Synthesis

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

	A	B
	COMPONENT	VOLUME
	RNA Sample	12 µl
	(lilac) LunaScript RT SuperMix	3 µl
	Total Volume	15 µl

1.1 Add 3 µL LunaScript cDNA to each reaction well of a midskirt plate in the clean room. Be sure to add cDNA NTC.

1.2 Bring cDNA plate into Template Addition (TA/ Low Titer). Add 12 µL RSV RNA to each reaction well and seal with PCR seal.

> **Note**
>
> RNA samples are stored long-term in -80 °C Garage Freezer

1.3 Centrifuge plate and load on thermocycler. It is ok to use the BioRad cycler in TA for cDNA

synthesis.

2 Incubate reactions in a thermocycler* with the following steps:

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

*Set heated lid to 105°C

Note

cDNA samples can be stored at -20 °C for up to a week in TA freezer with foil seal

cDNA Amplification

3

Note

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

Gently mix and spin down reagents. Prepare the split pool cDNA amplification reactions as described below: (Follow this protocol for Primer Pooling)

Tiled RSV Amplicon reaction setup

	A	B
	COMPONENT	VOLUME
	cDNA (Step 2)	6.25 µl
	(lilac) Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl
	15x (5 uM each) RSV A/B 20 primer pair pool (evens or odds)	1.67 µl
	Molecular Grade water	4.58 µl

	A	B
	Total Volume	25 µl

This chart outlines the reaction setup for any RSV primer pool; use the following substeps to adjust the mastermix necessary for the number of RSV A and B positives, respectively.

3.1 **RSV A Mastermix:** scale as necessary

Combine 12.5 µL Q5 Hot Start 2x SMX , 1.67 µL 15x (5uM each) RSV A 20 primer pair pool (evens or odds) , and 4.58 µL molecular-grade water . Plate into alignment with each RSV A sample on the plate map.

3.2 **RSV B Mastermix:** scale as necessary

Combine 12.5 µL Q5 Hot Start 2x SMX , 1.67 µL 15x (5uM each) RSV B 20 primer pair pool (evens or odds) , and 4.58 µL molecular-grade water . Plate into alignment with each RSV B sample on the plate map.

3.3 Add 6.25 µL cDNA to both Pool 1 and Pool 2 reactions. Mix, seal, and centrifuge.

4 Incubate reactions in a thermocycler with the following steps:

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	95°C	30 seconds	1
	Denature	94°C	30 seconds	45
	Annealing	*55°C*	30 seconds	
	Extension	72°C	1:15 minutes	
	Final Extension	72°C	7 minutes	1
	Hold	4°C	∞	1

*Touchdown PCR, temperature change -0.2C / cycle

5

Note

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

Cleanup of First PCR Product

6

Note

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

Vortex NEBNext Sample Purification Beads to resuspend.

7

Add 25 µL (1X) resuspended beads to each PCR Plate (evens or odds). 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. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

8

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

9

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

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.

Note

Caution: do not discard the beads.

11 Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. 30s
Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

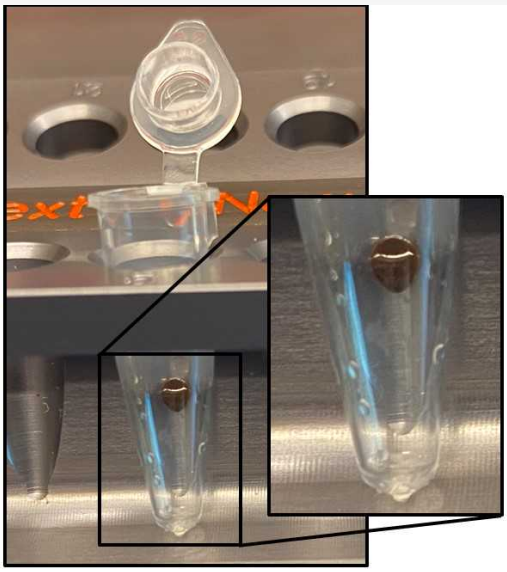
12 Repeat previous Step once for a total of two washes: 30s
Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand.
Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

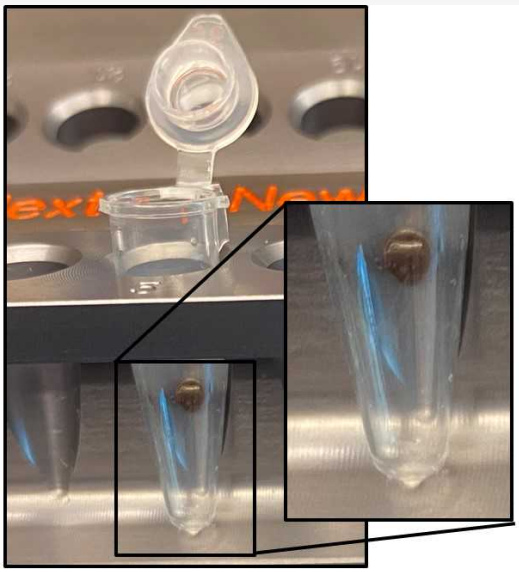
13 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



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



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

14 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding

25 µL 0.1X TE .

15 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at Room temperature . If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

16 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.

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

Nested PCR

17

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

Gently mix and spin down reagents. Prepare the split pool cDNA amplification reactions as described below: (Follow this protocol for Primer Pooling)

Tiled RSV Amplicon reaction setup

	A	B
	COMPONENT	VOLUME
	First PCR Product (Step 16)	1 µl
	(lilac) Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl
	15x (5 uM each) RSV A/B nested primer pool (evens or odds)	1.67 µl
	Molecular Grade water	9.83 µl
	Total Volume	25 µl

This chart outlines the reaction setup for any RSV primer pool; use the following substeps to adjust the mastermix necessary for the number of RSV A and B positives, respectively.

17.1 **RSV A Mastermix:** scale as necessary
Combine 12.5 µL Q5 Hot Start 2x SMX ,
1.67 µL 15x (5uM each) RSV A nested primer pool (evens or odds) , and
9.83 µL molecular-grade water . Plate into alignment with each RSV A sample on the plate map.

17.2 **RSV B Mastermix:** scale as necessary
Combine 12.5 µL Q5 Hot Start 2x SMX ,
1.67 µL 15x (5uM each) RSV B nested primer pool (evens or odds) , and
9.83 µL molecular-grade water . Plate into alignment with each RSV B sample on the plate map.

17.3 Add 1 µL of the First PCR Product to both even and odd reactions. Mix, seal, and centrifuge.

18 Incubate reactions in a thermocycler with the following steps:

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	94°C	2 min	1
	Denature	94°C	30 seconds	35
	Annealing	56°C	30 seconds	
	Extension	72°C	1 min	
	Final Extension	72°C	7 minutes	1
	Hold	4°C	∞	1

Note

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

Cleanup of Nested PCR Product

19

Note

The volume of NEBNext Sample Purification Beads provided here are for use with the sample composition at this step (25 µl; Step 18). 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.

Vortex NEBNext Sample Purification Beads to resuspend.

20 Add 25 µL (1X) resuspended beads to each PCR Plate (evens or odds). 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. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

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

23 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note

Caution: do not discard the beads.

24 Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 30s

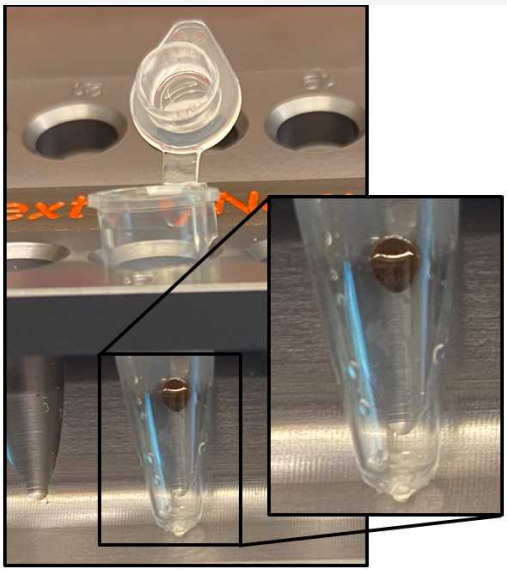
25 Repeat previous Step once for a total of two washes: 30s
Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

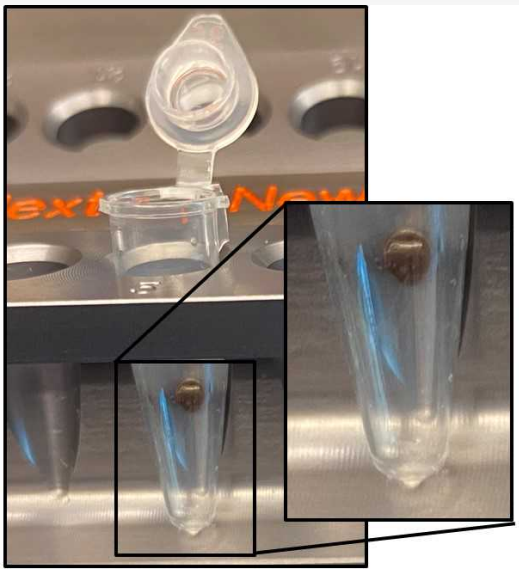
26 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



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



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

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

28 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

29 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.

30 Combine the paired Pool 1 and Pool 2 PCR reactions for each sample.

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

Fragmentation/End Prep

31 Historic RSV FS NEB Preps utilized a 5 min incubation period.

TEST A SUBSET ON 5 MIN, AND RUN ON TAPESTATION TO CONFIRM ~300bp

	A	B	C
	FRAGMENTATION SIZE	INCUBATION @ 37°C	OPTIMIZATION
	100 bp–250 bp	30 min	30–40 min
	150 bp–350 bp	20 min	20–30 min
	200 bp–450 bp	15 min	15–20 min
	300 bp–700 bp	10 min	5–15 min
	500 bp–1 kb	5 min	5–10 min

Fragmentation occurs during the 37°C incubation step. Use the chart below to determine the incubation time required to generate the desired fragment sizes. Incubation time may need to be optimized for individual samples.

32 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.

There are short videos on NEB.com about handling the FS reagents:
Preparing FS Reagents: https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-the-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix
Making FS master mix: https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix-master-mix

33 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

34 Add the following components to a 0.2 ml thin wall PCR tube On ice :

	A	B
	COMPONENT	VOLUME PER ONE LIBRARY
	Tiled RSV Amplicons (Step 30)	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

*This is a half reaction of what has been historically run

35 Vortex the reaction for 00:00:05 and briefly spin in a microcentrifuge. 5s

36 In a thermocycler* run the following program:

	A	B
	TEMP	TIME
	37°C	5 minutes
	65°C	30 minutes
	4°C	∞

*Set heated lid to 75°C

Note

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.

Adaptor Ligation

37 Determine whether adaptor dilution is necessary, by following the table below;

	A	B	C
	INPUT	ADAPTOR DILUTION (volume of adaptor: total volume)	WORKING ADAPTOR CONCENTRATION
	100 ng–500 ng	No Dilution	15 µM
	5 ng–99 ng	10-Fold (1:10)	1.5 µM
	less than 5 ng	25-Fold (1:25)	0.6 µM

*Historically, adaptor was diluted 10-fold (10uL adaptor -> 90uL adaptor dilution buffer)

38 Add the following components directly to the FS Reaction Mixture:

	A	B
	COMPONENT	VOLUME
	FS Reaction Mixture (Step 36)	17.5 µl
	(red, or diluted) NEBNext Adaptor for Illumina**	1.25 µl
	(red) NEBNext Ultra II Ligation Master Mix*	15 µl
	Total Volume	33.75 µl

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

-> Ligation enhancer is included in the base FS prep mix, but is not absolutely necessary (we usually run RSV samples without it)

Note

Do not premix adaptor with the Ligation Master Mix

39 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.

Note

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.

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

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

Note

Steps 29 and 30 are only required for use with NEBNext Adaptors.
USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

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

Note

Samples can be stored Overnight at -20 °C.

Cleanup of Adaptor-ligated cDNA

43 **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 28). 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

44 Vortex the NEBNext Sample Purification Beads to resuspend.

45 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.

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

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

48 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

> Note
>
> **Caution: do not discard the beads.**

49 Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

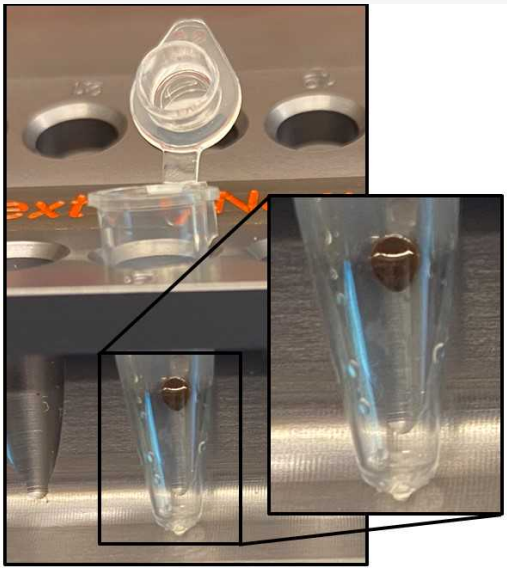
50 Repeat the previous step once for a total of two washes:
Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

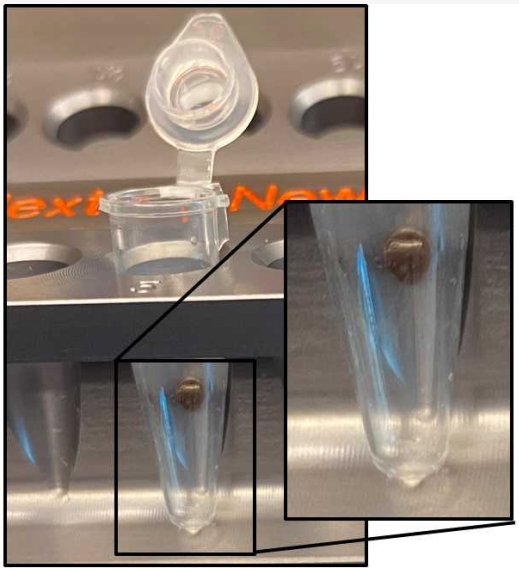
51 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



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



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

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

53 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

54 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.

Note

Samples can be stored at -20 °C.

PCR Enrichment of Adaptor-ligated DNA

55 Add the following components to a sterile strip tube:

	A	B
	COMPONENT	VOLUME
	Adaptor Ligated DNA Fragments	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

56 

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.

57 

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

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	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. For past NEB RSV preps, only 5 PCR cycles have been run.

Cleanup of PCR Reaction

58 **The following section is for cleanup of the 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.**

59 Vortex NEBNext Sample Purification Beads to resuspend.

60 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.

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

62 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.

63 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

> Note
>
> **Caution: do not discard the beads.**

64 Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

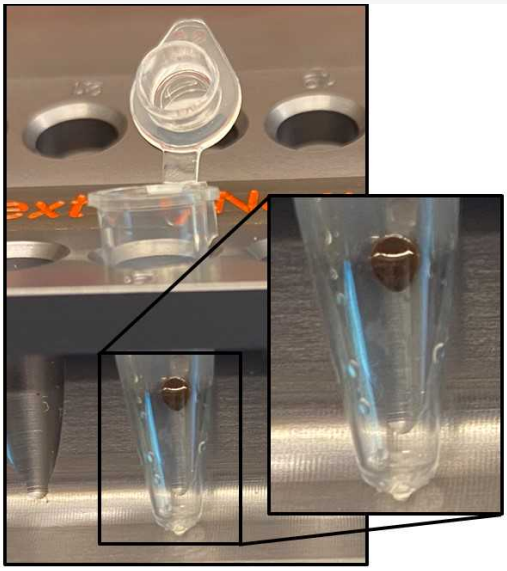
Add 200 µL 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

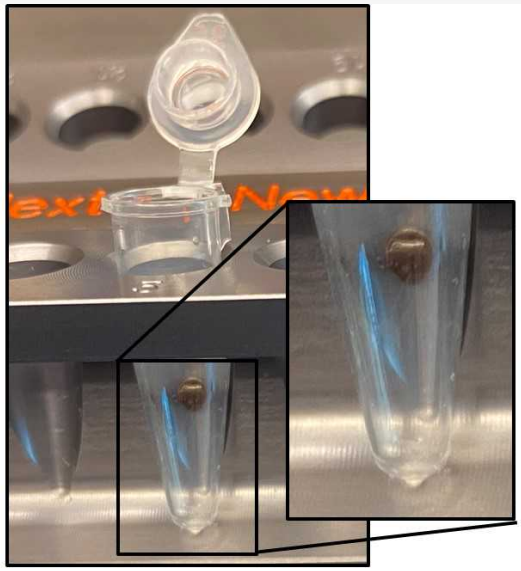
66 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



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



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

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

68 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

69 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.

70 Assess the library size distribution with TapeStation high sensitivity DNA 1000 reagents. The sample may need to be diluted before loading. A peak size of 200–250 bp is expected.

Note

Note: If a peak ~80 bp (primers) or ~128-140 bp (adaptor-dimer) is visible in the TapeStation analysis, bring up the sample volume (from Step 55) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction (Steps 44-55). You may see adaptor-dimer when starting with inputs ≤ 1 ng.