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We use this protocol and it's working

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Sequencing SARS-CoV-2 from Animal Samples with NEBNext® ARTIC SARS-CoV-2 Companion Kit

Forked from NEBNext® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies®) E7660 -- Express Protocol without PCR Bead Cleanup

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ABSTRACT

This protocol is adapted for animal samples and is based on the methods for the NEBNext® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies®), NEB #E7660S/L 24/96 reactions.

This protocol does not include a cleanup and normalization step for each sample after cDNA synthesis. Performing the cleanup and normalization step creates library pools where the reads for each library are more evenly distributed. Skipping these steps reduces hands on time but may require a longer sequencing run to obtain sufficient coverage for each sample. To obtain more even sample to sample coverage, we recommend normalizing the RNA samples prior to starting the protocol.

Changes in this version:

- Removal of the human primers
- Combining multiple barcoding reactions (step 15 and 21)
- Addition of Loading Flongle Flow Cell section
- Addition of Analysis section

GUIDELINES

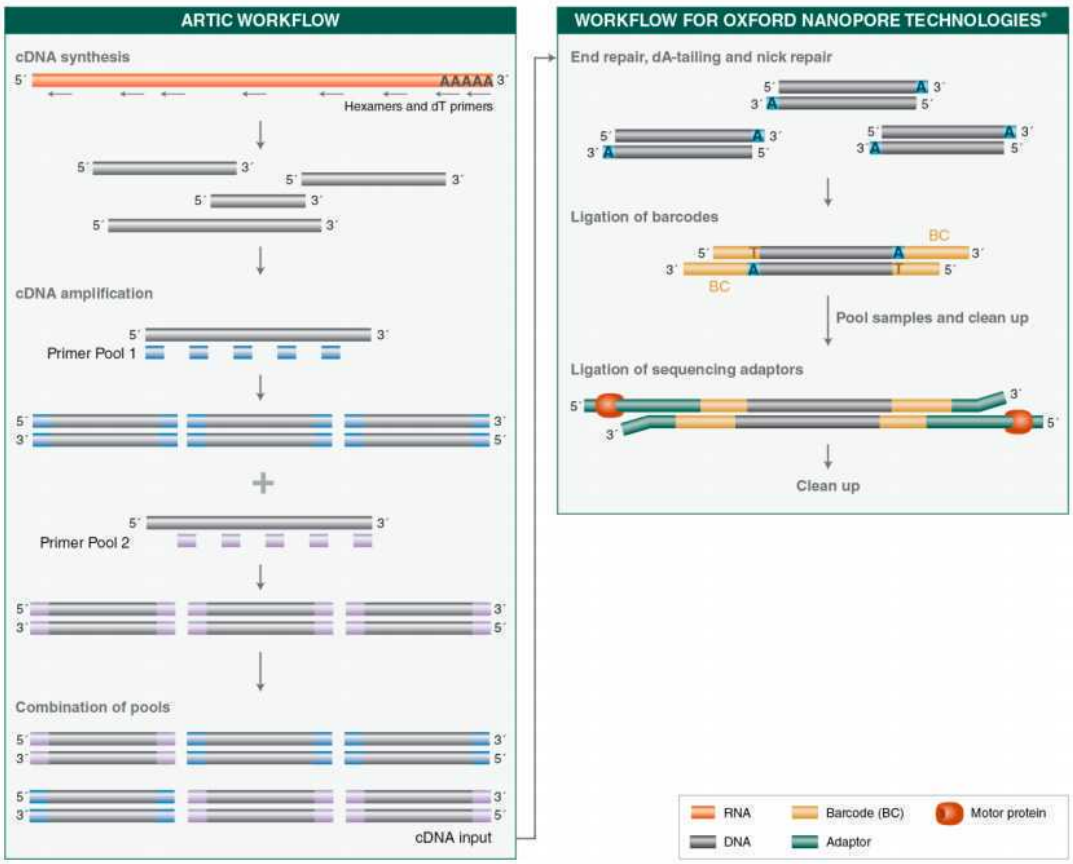
Overview

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

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

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

Figure 1. Workflow demonstrating the use of NEBNext ARTIC SARS-CoV-2 Companion Library Prep Kit for Oxford Nanopore.



MATERIALS

The Library Kit Includes

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

Package 1: Store at –20°C.

(lilac) LunaScript® RT SuperMix
(lilac) Q5® Hot Start High-Fidelity 2X Master Mix
(green) NEBNext Ultra II End Prep Enzyme Mix
(green) NEBNext Ultra II End Prep Reaction Buffer
(red) Blunt/TA Ligase Master Mix
(red) NEBNext Quick T4 Ligase
(red) NEBNext Quick Ligation Reaction Buffer
(lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 1
(lilac) NEBNext ARTIC SARS-CoV-2 Primer Mix 2

(white) Nuclease-free water

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

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- DNA LoBind Tubes (Eppendorf® #022431021)
- Oxford Nanopore Technologies Native Barcoding Expansion kits 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114)
- Oxford Nanopore Technologies Ligation Sequencing Kit (SQK-LSK109)
- Oxford Nanopore Technologies SFB Expansion Kit (EXP-SFB001)
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.® Q32851)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables (#4150 or #4200 TapeStation System)
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)
- 1.5 ml tube magnet stand (NEB #S1506)

Kit Components

NEB #E7660S 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	0.30 ml
	E7661A	NEBNext Ultra II End Prep Enzyme Mix	0.018 ml
	E7662A	NEBNext Ultra II End Prep Reaction Buffer	0.042 ml
	E7663A	Blunt/TA Ligase Master Mix	0.24 ml
	E7664A	NEBNext Quick T4 DNA Ligase	0.020 ml
	E7665A	NEBNext Quick Ligation Reaction Buffer	0.040 ml
	E7725A	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.042 ml
	E7726A	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.042 ml
	E7727A	NEBNext ARTIC Human Control Primer Pairs 1	0.007 ml

	A	B	C
	E7728A	NEBNext ARTIC Human Control Primer Pairs 2	0.007 ml
	E7667A	Nuclease free-Water	1.50 ml
	E7666S	NEBNext Sample Purification Beads	0.872 ml

NEB #E7660L Table of Components

	A	B	C
	NEB #	PRODUCT	VOLUME
	E7651AA	LunaScript RT SuperMix	0.192 ml
	E7652AA	Q5 Hot Start High-Fidelity 2X Master Mix	1.2 ml
	E7661AA	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
	E7662AA	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
	E7663AA	Blunt/TA Ligase Master Mix	0.96 ml
	E7664A	NEBNext Quick T4 DNA Ligase	0.020 ml
	E7665A	NEBNext Quick Ligation Reaction Buffer	0.040 ml
	E7725AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 1	0.168 ml
	E7726AA	NEBNext ARTIC SARS-CoV-2 Primer Mix 2	0.168 ml
	E7727A	NEBNext ARTIC Human Control Primer Pairs 1	0.007 ml
	E7728A	NEBNext ARTIC Human Control Primer Pairs 2	0.007 ml
	E7667AA	Nuclease free-Water	4.7 ml
	E7666L	NEBNext Sample Purification Beads	2.90 ml

NEBNext ARTIC SARS-CoV-2 Primers

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

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. In addition, we recommend setting up a no template control reaction and **all reactions are set-up in a hood**.

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

cDNA Synthesis

1 The presence of genomic DNA or carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

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

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

*Up to 0.5 µg total RNA can be used in a 10 µl reaction.

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

4 For no template controls, mix the following components:

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

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

6 Incubate reactions in a thermocycler with lid temperature at 105°C with the following steps:

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

Note

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

Targeted cDNA Amplification

7

Note

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

Gently mix Q5 Hot Start High Fidelity 2X Master Mix 10 times by pipetting and spin down reagents. Prepare the split pool amplification reactions as described below:

For Pool Set A: .

	A	B
	COMPONENT	VOLUME
	cDNA (Step 6)	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

For Pool Set B:

	A	B
	COMPONENT	VOLUME
	cDNA (Step 6)	4.5 µl
	(lilac) Q5 Hot Start High-Fidelity 2X MM	6.25 µl
	NEBNext ARTIC SARS-CoV-2 Primer Mix 2*	1.75 µl
	Total Volume	12.5 µl

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

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

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES

	A	B	C	D
	Initial Denaturation	98°C	30 seconds	1
	Denature	95°C	15 seconds	35
	Annealing/Extension	63°C*	5 minutes	
	Hold	4°C	∞	1

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

Note

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

PCR Reaction Pooling

10

Note

Please note, there is also a protocol that includes a cleanup and normalization step at this point . Performing the cleanup and normalization step creates library pools where the reads for each library are more evenly distributed. These pools will likely achieve sufficient coverage in less run time.

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

Note

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

NEBNext End Prep

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

	A	B

	A	B
	COMPONENT	VOLUME
	Targeted cDNA Amplicons (Step 11)	1 µl
	(white) Nuclease-free water	11.5 µl
	(green) NEBNext Ultra II End Prep Reaction Buffer	1.75 µl
	(green) NEBNext Ultra II End Prep Enzyme Mix	0.75 µl
	Total Volume	15 µl

13 Flick the tube or pipet up and down 10 times to mix the solution. Perform a quick spin to collect all liquid from the sides of the tube.

Note

It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

14 Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program: 20m

00:10:00 @ 20 °C
00:10:00 @ 65 °C
Hold at 4 °C

Note

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

Barcode Ligation

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

	A	B
	COMPONENT	VOLUME
		single

	A	B
	(white) Nuclease-free water	6 µl
	End-prepped DNA (Previous Step)	1.5 µl
	Native Barcode*	2.5 µl
	(red) Blunt/TA Ligase Master Mix**	10 µl
	Total	20 µl***

* Native Barcodes are provided in Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) or 1-96 (EXP-NBD196)
** Mix the Blunt/TA Ligase Master Mix by pipetting up and down several times prior to adding to the reaction.
*** Multiple ligation reactions for a sample with the same barcode can be pooled (see #21) to increase the amount of product for cleanup. Recommend separate tubes for individual 20uL reactions and then combine in step 21. Unsure how a 40uL reaction will respond to the cycling conditions.

16 Flick the tube or pipet up and down 10 times to mix solution. Perform a quick spin to collect all liquid from the sides of the tube.

Note

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.

17 31m

Incubate at 25 °C for 00:20:00.
Incubate at 65 °C for 00:10:00.
Place On ice for 00:01:00.

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

Cleanup of Barcoded DNA

19

Note

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

20 Vortex NEBNext Sample Purification Beads to resuspend.

21 Add 0.4X resuspended beads to pooled, barcoded samples (Step 20) 1s

There is a maximum of 480uL of pooled samples. So multiple barcoded samples can be pooled. As an example, for seven samples, including four duplicated samples:
40+40+40+40+20+20+20uL = 220 µL with 88 µL of resuspended Sample Purification beads

Mix well by flicking the tube or pipetting up and down 10 times. Perform a quick spin for 00:00:01 to collect all liquid from the sides of the tube.

22 Incubate samples on bench top for 00:10:00 at Room temperature . 10m

23 Place the tube on a 1.5 ml magnetic stand (such as NEB S1506) to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

24 After 00:03:00 (or when the solution is clear), carefully remove and discard the supernatant. 3m
Be careful not to disturb the beads that contain DNA targets.

Note

Caution: do not discard the beads.

25 Wash the beads by adding 250 µL Short Fragment buffer (SFB). Flick the tube or pipet up and down 10 times to mix to resuspend pellet. If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 1s

26 Place the tube on an appropriate magnetic stand for 00:03:00 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant. 3m

27 Repeat Step 26 and 27 once for a total of two washes: 3m 3s
Wash the beads by adding 250 µL Short Fragment buffer (SFB). Flick the tube or pipet up and down to mix to resuspend pellet. If necessary, quickly spin the sample for 00:00:03 to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
Place the tube on an appropriate magnetic stand for 00:03:00 (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

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.

28 Add 500 µL 80% freshly prepared ethanol to the tube while on 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

29 Repeat the previous step once to make it a total of 2 washes.

30 Perform a quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

31 Air dry the beads for 00:00:30 while the tube is on the magnetic stand with the lid open. 30s

Note

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.

32 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µL Nuclease-free water.

33 Resuspend the pellet by flicking the tube or pipetting up and down 10 times to mix. Incubate for at least 2 minutes at Room temperature. If necessary, quickly spin the sample for 00:00:01 to collect the liquid from the sides of the tube before placing back on the magnetic stand. 1s

34 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.

35 Assess the concentration of DNA targets using 1 µL of sample with a DeNovix with FX fluorometer module. Use

It is also recommend using a Qubit fluorometer for concentration assessment. (Nanodrop is **NOT** recommended since it may overestimate the DNA concentration).

Note

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

Adapter Ligation

36 Use the Qubit readings from the previous Step to dilute purified Native barcoded DNA pool in nuclease-free water. Dilute the sample to a concentration of 60 ng total in a final volume of 30 µl (or 2 ng/µl in 30 µl). Add the following components into a 1.5 ml Eppendorf DNA LoBind Tube or

nuclease-free PCR tube:

	A	B
	COMPONENT	VOLUME
	Native barcoded and purified DNA (Step 35, up to 60 ng)	30 µl
	(red) NEBNext Quick Ligation Reaction Buffer *	10 µl
	Adapter Mix II (AMII)**	5 µl
	(red) NEBNext Quick T4 Ligase	5 µl
	Total Volume	50 µl

* Mix the NEBNext Quick Ligation Reaction Buffer by pipetting up and down several times prior to adding to the reaction.
** Adapter Mix II is provided by Oxford Nanopore Technologies Native Barcoding Expansion 1-12 (EXP-NBD104), 13-24 (EXP-NBD114) and 1-96 (EXP-NBD-196) kits.

37 **Flick the tube** to mix solution. Perform a quick spin for 00:00:01 to collect all liquid from the sides of the tube. 1s

Note

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.

38 Incubate at 25 °C for 00:20:00. 20m

39 Proceed to Cleanup of Adapter-ligated DNA in the next section.

Cleanup of Adapter Ligated DNA

20m 3s

40 Vortex NEBNext Sample Purification Beads to resuspend.

41 Add 50 µL (1X) resuspended beads to the ligation mix. Mix well by flicking the tube followed by a quick spin for 00:00:03. 3s

42 Incubate samples for 00:10:00 at Room temperature. 10m

43 Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

44 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.

Note

Caution: do not discard the beads.

45 Wash the beads by adding 250 µL Short Fragment Buffer (SFB). **Flick the tube** to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. Place the tube on an appropriate magnetic stand.

46 Wait for 00:03:00 (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant. 3m

47 Repeat Step 46 and 47 once for a total of two washes:
Wash the beads by adding 250 µL Short Fragment Buffer (SFB). Flick the tube or pipet up and down to mix to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. Place the tube on

an appropriate magnetic stand.
Wait for 3 minutes (or when the solution is clear) to separate the beads from the supernatant.
Remove the supernatant.

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.

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

49 Resuspend the pellet well in EB buffer **by flicking**. Incubate for 00:10:00 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. 10m

50 Place the tube/plate on the magnetic stand. After 00:03:00 (or when the solution is clear), transfer 15 µL to a new DNA LoBind tube. 3m

51 Determine DNA concentration by quantifying 1 µL DNA sample.

Loading Flongle Flow Cell

52 Follow Oxford Nanopore Protocol SQK-LSK109 to prepare MinION® flow cell and DNA library sequencing mix using up to 20 ng adapter-ligated DNA sample (previous step).

52.1 Combine 117 µL of Flush Buffer + 3 µL Flush Tether in a LoBind tube and mix by pipetting

52.2 Prime the flongle flow cell by slowly pipetting the majority of the previous mix, avoid pushing too fast and bubbles

52.3 In another LoBind tube, combine 15 µL of Sequencing Buffer II + 10 µL of freshly vortexed Loading Beads II + 5 µL of ~20fmol DNA library (if volume was less than 5uL, bring up to 5uL with Elution Buffer.

> Note
>
> Based on what ONT recommends load ~20fmol of the adapter-ligated final pooled library on a flongle flow cell

52.4 Load ~30uL on the flongle flow cell, avoid bubbles.

Analysis

53

Used the EPI2ME Fastq + ARTIC + NextClade workflow for analyses.

> Note
>
> Raw files can be retrieved from MinKNOW and put through other pipelines.