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REDI-NET F-4 FECES TESTING V.3

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REDI-NET Consortium[1]

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Remote Emerging Disea...

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We use this protocol and it's working

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Disclaimer

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Abstract

The overarching aim of the REDI-NET is to develop a collaborative laboratory network between domestic and international partnering institutions to address disease surveillance needs in order to effectively detect, predict and contain potentially emergent zoonosis. This protocol provides guidance on testing of feces samples and their purified nucleic acid to preserve their integrity for downstream sequencing library preparation.

Guidelines

OBJECTIVE

To outline the procedures for properly using the Oxford Nanopore Sequencing platforms (GridION/MinION: MinION flow cell or P2 Solo: PromethION flow cell) to sequence DNA and cDNA extracted from collected fecal samples.

SUMMARY/SCOPE

This SOP provides guidance on procedures of Oxford Nanopore sequencing to generate sequencing reads for downstream data analysis and pathogen detection.

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

Note

NOTE: *All study procedures must be conducted in compliance with national and local policies for the prevention and control of COVID-19 infection.*

MAINTENANCE OF EQUIPMENT

CAUTION ON RNA HANDLING:

1. RNases are very stable and difficult to inactivate and only minute amounts are sufficient to destroy RNA.
2. Care should be taken to avoid inadvertently introducing RNases into the samples during or after the purification procedure.
3. Clean the work surfaces with RNA Zap to remove nucleases, then wipe the surfaces with 70% to 100% molecular biology grade ethanol to remove additional contaminants.

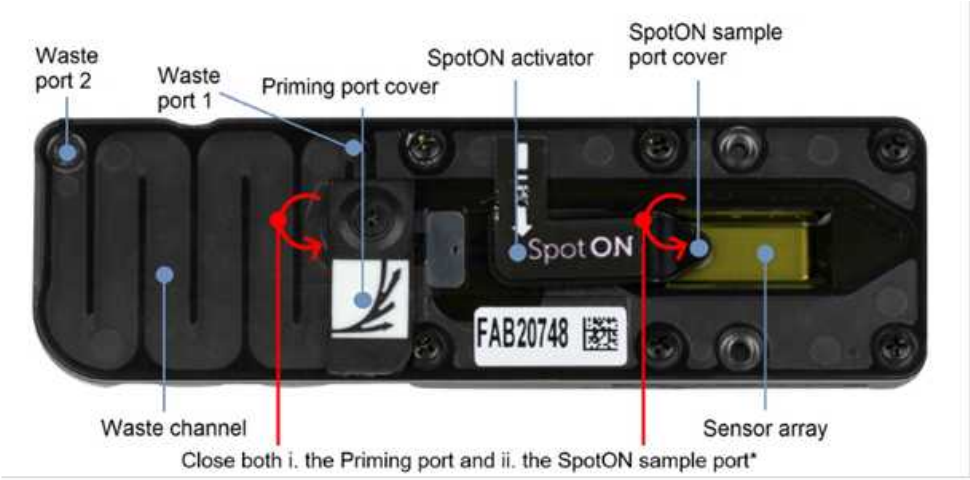
HANDLING ENZYMATIC REACTIONS

Reagents containing enzymes should be handled On ice before mixed and transferred to the assigned activation temperature.

APPENDICES

APPENDIX 1. FLOW CELL

MinION flow cell:



PromethION flow cell:

APPENDIX 2. MinKNOW SOFTWARE INSTRUCTIONS

After Double-clicking the MinKNOW icon, the login page should show up. Use Oxford Nanopore Community username and password to login.

Select device shown on the screen.

Go to Start tab, select the “Start Sequencing” option to choose the running parameters.

Type in the (1) experiment name, (2) “Select all available” to select all the connected flow cells or use the diagram above to select specific flow cells to run, (3) Check the Flow cell ID and Flow cell type are auto-filled correctly, (4) Copy experiment name and past to the space for sample ID, (5) Select Continue to Kit Selection to move to the next page.

Select the kit SQK-NBD114-96 from the Kit Selection menu. Select Continue to Run Options to choose run parameters.

Set run length to 48 hrs and Minimum read length 200 bp. Let adaptive sampling off. Select **Continue to analysis** for output setting.

Turn on Basecalling and Barcoding.

Set up Option of Basecalling model in High-accuracy basecalling. Save the setting.

Set up Barcoding options. Turn on Trim barcodes and Mid-read barcode filtering. Save the settings and **Continue** to output.

Select the output data location, format, and filtering options. Check the box for Raw reads and select POD5. Check the box FASTQ of Basecalled reads. Keep the filter score as the system default. Select "**Continue to final review**" to proceed.

Review the settings listed in the Run Setup page. Correct any errors. Select "**Start**" to run the experiment. The system will automatically navigate the Sequencing Overview when sequencing starts.

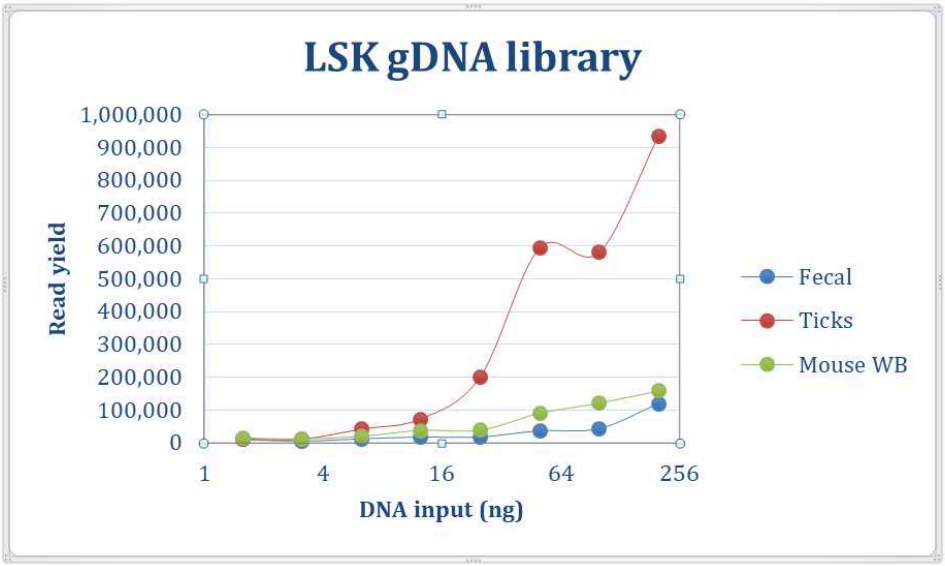
The system will automatically navigate the Sequencing Overview when sequencing starts.

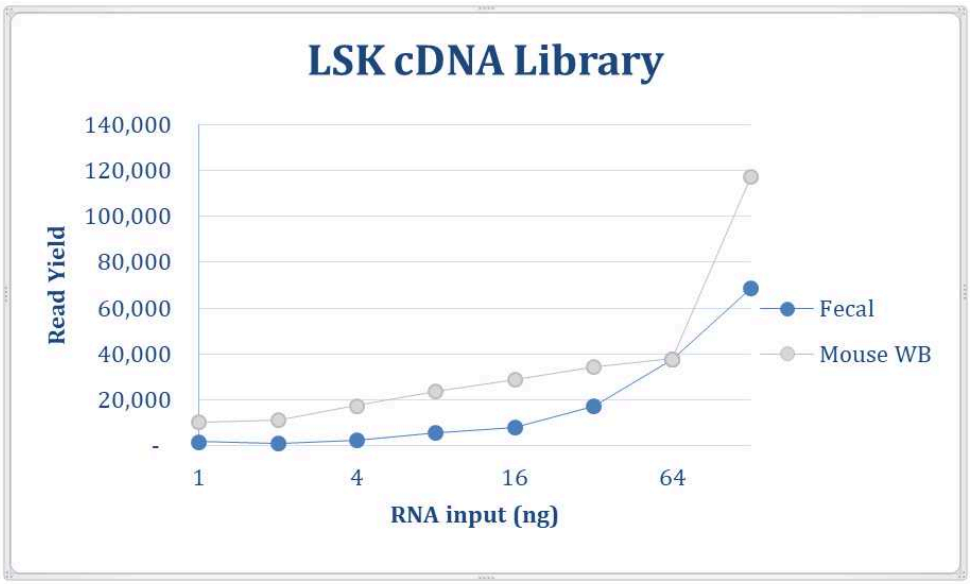
APPENDIX 3. cDNA END-PREP MASTER MIX PREPARATION

	A	B	C
	Component	Volume for 1 reaction	Volume for n+1 reactions
	cDNA sample	20 µl	20 µl
	Nuclease-free water	30 µl	... µl
	Ultra II End-prep reaction buffer	7 µl	... µl
	Ultra II End-prep enzyme mix	3 µl	... µl
	Final total volume	60 µl	... µl

APPENDIX 4. EXPECTED OUTCOMES

The DNA or RNA inputs vs the sequencing read yields.




LSK cDNA Library
140,000
120,000
100,000
80,000
60,000
40,000
20,000
-
Read Yield
1
4
16
64
RNA input (ng)
Fecal
Mouse WB

Materials

EQUIPMENT AND MATERIALS

Note

***NOTE**: If product number is listed, please ensure use of this or equivalent product.*

	A	B
	Equipment	**Mfg / Product #**
	Oxford Nanopore GridION or MinION Mk1C device or P2 Solo device	Oxford Nanopore Technologies, GRD-CapEx or Oxford Nanopore Technologies, M1CCapEx, or PromethION 2 Solo CapEx
	Computer monitor (with HDMI port or Display port), mouse and keyboard	Locally sourced
	MinKNOW - software equipped already in the GridION and MinION Mk1C device	Oxford Nanopore Technologies
	P2 Solo compatible computer with GPU power for real-time basecalling	Locally sourced
	Ice bucket with ice	Locally sourced
	Qubit fluorometer	ThermoFisher, Q33238 or equivalent
	DynaMag-2 magnet	Invitrogen, 12321D or equivalent
	DynaMag-96 Side Magnet	Invitrogen, 12331D or equivalent
	Hula sample mixer	ThermoFisher, 15920D
	Microplate centrifuge	Locally sourced
	Timer	Locally sourced
	Thermal cycler	Locally sourced
	96-well PCR plate holder	Locally sourced
	P1000 pipette and tips	Locally sourced
	P200 pipette and tips	Locally sourced
	P20 pipette and tips	Locally sourced
	P10 pipette and tips	Locally sourced

	A	B
	P10 8-channel pipette	Locally sourced
	P300 8-channel pipette	Locally sourced

	A	B	C
	Material	Description	Mfg / Product #
	200 ng DNA from a sample	Per sample from SOP S-2 (gDNA Preparation)	REDI-NET DNA sample
	20 ul eluents from negative control extraction	From SOP S-2 (gDNA Preparation)	REDI-NET negative control
	100 ng DNA from positive control extraction	From SOP S-2 (gDNA Preparation)	REDI-NET positive control
	160 ng RNA from a sample	Per sample from SOP S-2 (TNA preparation)	REDI-NET RNA sample
	40 ng RNA from positive control extraction	from SOP S-2 (TNA preparation)	REDI-NET negative control
	8 µl total nucleic acid negative control extraction	From SOP S-2 (TNA preparation)	REDI-NET positive control
	10 µl total nucleic acid	Per sample from SOP S-2 (TNA Preparation)	REDI-NET TNA sample
	10 µl total nucleic acid from negative control extraction	From SOP S-2 (TNA Preparation)	REDI-NET negative control
	10 µl total nucleic acid from positive control extraction	from SOP S-2 (TNA Preparation)	REDI-NET positive control
	Native Barcoding Kit 96 V14	(Sequencing Library Preparation)	Oxford Nanopore, SQK-NBD114.96
	ezDNase	(cDNA synthesis)	ThermoFisher, Invitrogen 11766051
	NEBNext Ultra II RNA First Strand Synthesis Module	(cDNA synthesis)	New England Biolabs, E7771L
	NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module	(cDNA synthesis)	New England Biolabs, E6111L
	Random primer mix (Random hexamer and poly-T mixture)	(cDNA synthesis)	New England Biolabs, S1330

	A	B	C
	USB Dithiothreitol (DTT), 0.1M Solution	(cDNA synthesis)	ThermoFisher,707265ML
	Agencourt AMPure XP beads	(Sequencing Library Preparation)	Beckman Coulter, A63881
	NEBNext End repair / dA-tailing Module	(Sequencing Library Preparation)	New England Biolabs, E7546L
	NEBNext FFPE Repair Mix	(Sequencing Library Preparation)	New England Biolabs, M6630L
	NEB Blunt/TA Ligase Master Mix	(Sequencing Library Preparation)	New England Biolabs, M0367L
	NEBNext Quick Ligation Module	(Sequencing Library Preparation)	New England Biolabs, E6056L
	R10.4.1 flow cells	Flow cells for sequencing experiment (consumable)	Oxford Nanopore, FLO-MIN114, FLO-PRO114M
	Ultra pure Bovine Serum Albumine (50mg/ml)	(Sequencing Library Preparation)	ThermoFisher, AM2616
	low DNA binding tubes	1.5 mL (consumable)	Eppendorf, 022131021 or equivalent
	low DNA binding tubes	2.0 mL (consumable)	Eppendorf, 022431048 or equivalent
	PCR tubes	0.2 mL thin-walled (consumable)	Eppendorf, 951010006 or equivalent
	PCR plate	96 well, low DNA binding, semi-skirted with heat seals (consumable)	Eppendorf, 0030129504 or equivalent
	riboPool pan-mammal Kit	For Host rRNA depletion (for TNA form whole blood and buffy coat samples only)	SiTools Biotech, 24 reactions
	NEBNext Microbiome DNA enrichment Kit	For Host DNA depletion (for TNA form whole blood and buffy coat samples only)	New England Biolabs, E2612
	RNaseOUT Recombinant Ribonuclease Inhibitor	For Host DNA/rRNA depletion (for TNA form whole blood and buffy coat samples only)	ThermoFisher, 10777019
	BRAND Self-adhesive Plate Sealing Film	Aluminum (consumable)	Fisher Scientific, 13-882-329
	Clear Adhesive Film	For PCR plate sealing	ThermoFisher, 4306311

	A	B	C
	Qubit Assay Tubes	For Qubit DNA/RNA measurement (consumable)	Thermo Fisher, Q32856
	Qubit 1X dsDNA HS Assay Kit	(consumable)	ThermoFisher, Q33230
	Qubit RNA HS Assay Kit	(consumable)	ThermoFisher, Q32852
	Nuclease-free water	To prepare ethanol dilutions (consumable)	Locally sourced

Equipment

Qubit Fluorometer

NAME

Fluorometer

TYPE

Invitrogen

BRAND

Q33238

SKU

https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238

LINK

Equipment

DynaMag-2

NAME

Magnet

TYPE

Invitrogen

BRAND

12321D

SKU

https://www.thermofisher.com/order/catalog/product/12321D#/12321D

LINK

Equipment

Hula mixer	NAME
Mixer	TYPE
Invitrogen	BRAND
15920D	SKU
Any rotator mixer	SPECIFICATIONS

Native Barcoding Kit 96 V14 **Oxford Nanopore Technologies** **Catalog #**SQK-NBD114.96

MS2 bacteriaphage RNA **Roche** **Catalog #**10165948001

TURBO™ DNase (2 U/µL) **Thermo Fisher Scientific** **Catalog #**AM2238

THE RNA Storage Solution **Thermo Fisher** **Catalog #**AM7000

Superscript IV Reverse Transcriptase **Life Technologies** **Catalog #**18090050

RNase Inhibitor **New England Biolabs** **Catalog #**M0314L

Deoxynucleotide (dNTP) Solution Mix **New England Biolabs** **Catalog #**N0447S

Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs** **Catalog #**M0492S

Ampure XP beads **Beckman** **Catalog #**A63881

NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs** **Catalog #**E7546L

NEBNext FFPE DNA Repair Mix - 96 rxns **New England Biolabs** **Catalog #**M6630L

Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs** **Catalog #**M0367L

NEBNext Quick Ligation Module - 100 rxns **New England Biolabs** **Catalog #**E6056L

Flow Cell (R10.4.1) **Oxford Nanopore Technologies** **Catalog #**FLO-MIN114

DNA LoBind Tubes 2.0 ml **Eppendorf** **Catalog #**022431048

Eppendorf PCR Tubes **Eppendorf** **Catalog #**951010006

PromethION R10.4.1M flow cell **Oxford Nanopore Technologies** **Catalog #**FLO-PRO114M

96 well LoBind PCR plates Semi-skirted **Eppendorf** **Catalog #**0030129504

NEBNext Microbiome DNA Enrichment Kit - 6 rxns **New England Biolabs** **Catalog #**E2612S

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific** **Catalog #**10777019

BRAND™ Self-adhesive Plate Sealing Film **Fisher Scientific** **Catalog #**13-882-329

MicroAmp™ Clear Adhesive Film **Thermo Fisher Scientific** **Catalog #**4306311

Qubit assay tubes **Thermo Fisher Scientific** **Catalog #**Q32856

Qubit 1X dsDNA High Sensitivity Assay Kit **Thermo Fisher Scientific** **Catalog #**Q33230

Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific** **Catalog #**Q32852

Safety warnings

RISKS AND PERSONAL PROTECTION

Gloves should be worn all the time when handling samples.

Before start

BEFORE START

1. Must check the DNA and RNA concentrations of your samples of total nucleic acid (TNA).
2. The sequencing approach should be selected based on the target of interest. For viral targets, use cDNA to prepare the sequencing library. For bacterial targets, use gDNA. If both viral and bacterial targets are of interest, use TNA to prepare the sequencing library.
3. Use sections **gDNA APPROACH**, **TNA APPROACH**, and **cDNA APPROACH** for gDNA, TNA, and cDNA preparation, respectively, then subject the prepared gDNA, TNA and/or cDNA to Section SEQUENCING LIBRARY PREPARATION.
4. The capacity of the sequencing flow cells is limited. Refer to the table below for the maximum allowable DNA and RNA quantities and volumes of a sample for each corresponding sequencing approach.

	A	B	C	D
	DNA amount (gDNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	200 ng, DNA	x	20-x	20

	A	B	C	D
	DNA and RNA amount (TNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	160 ng, RNA	x	8-x	8
	200 ng, DNA	x	10-x	10

	A	B	C	D
	RNA amount (cDNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	160 ng, RNA	x	8-x	8

5. Use the following table to prepare **positive controls** for the corresponding sequencing approach.

	A	B	C	D
	DNA amount (gDNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	50 ng, DNA	x	20-x	20

	A	B	C	D
	DNA and RNA amount (TNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	40 ng, RNA	x	8-x	8
	50 ng, DNA	x	10-x	10

	A	B	C	D
	RNA amount (cDNA approach)	Sample volume	Nuclease-free water (µL)	Total volume (µL)
	40 ng, RNA	x	8-x	8

gDNA APPROACH

1 If the DNA concentration > 10 ng/µl , calculate the required volume of 200 ng DNA, then transfer the volume to a new 200 µL PCR tube or a well of a 96-well PCR plate. Adjust the volume with nuclease-free water to a final volume of 20 µL .
Directly use a 20 µL sample for the downstream preparation if the DNA concentration is below 10 ng/µL (for MinION: 22 samples plus 1 positive and 1 negative control; for PromethION: 46 samples plus 1 positive and 1 negative control).

2 Prepare gDNA from positive control from a ONT DNA Control Standard in 19 µL nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. Add 1 µL of ONT DNA Control Standard (DCS) from ONT SQK-NBD114.96 kit to the well containing 19 µL nuclease free water. Mix well by pipetting.

3 Transfer 20 µL negative control extraction to a new tube or a well of a 96-well PCR plate.

4 All samples are subjected to section SEQUENCING LIBRARY PREPARATION.

TNA APPROACH

5 cDNA and gDNA from the same sample are prepared separately, then combined for a single sequencing library preparation.

6 Use the table in "Before Start" section to prepare 200 ng of DNA in 10 µL of nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below 20 ng/µl , directly transfer 10 µL of the sample to the tube or well.

7 Prepare the gDNA from a positive control using 9 µL of nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. Add 1 µL of ONT DCS from ONT SQK-NBD1114.96 kit to the well containing 9 µL nuclease free water. Mix well by pipetting.

8 Transfer 10 µL of the negative control extraction to a new tube or a well of a 96-well PCR plate.

9 Prepare double stranded cDNA from RNA following the steps described in sections cDNA APPROACH and cDNA SYNTHESIS AND AMPLIFICATION.

10 Add 3 µL of final amplified cDNA products from section cDNA APPROACH and 7 µL of nuclease-free water into each well of the 96-well PCR plate containing the corresponding 10 µL of gDNA prepared in prepared in steps 6-8 (including the samples, positive control, and negative control). **CAUTION**: be careful not to mix materials from different samples or controls.

NOTE: *After cDNA amplification and purification (the final products from section cDNA SYNTHESIS AND AMPLIFICATION, the concentration in each sample may range from* [M] 50 ng/µL *to* [M] 300 ng/µL*, typically between* [M] 80-150 ng/µL*. When preparing cDNA and gDNA in a single library preparation reaction, the ideal cDNA quantity per sample is* 40-300 ng*. For consistency and efficient handling in bulk preparation, a fixed* 3 µL *of cDNA should generally provide the required amount for sequencing. If the concentration falls outside the expected range, adjust the volumes of cDNA and nuclease-free water accordingly. The total volume of cDNA plus water must not exceed 1* 10 µL *.*

11 Subject the 20 µL double-stranded gDNA/cDNA mixture to section SEQUENCING LIBRARY PREPARATION.

cDNA APPROACH

12 Use the tables in the "Before Start" section to prepare 40 ng of RNA in 20 µL of nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. If the RNA concentration is below 2.0 ng/µl, directly transfer 20 µL of the sample to the tube or well.

13 Prepare 20 ng RNA positive control of MS2 Bacteriophage RNA.

NOTE: *MS2 bacteriophage RNA consists of extracted RNA at a concentration of* [M] 500 ng/µL *or higher in a total volume of* 500 µL *(the company labeled*

concentration could be inaccurate). Upon arrival, store the stock below -80 °C *. When using the first-time, take* 1 µL *of RNA stock and mix with* 9 µL *of nuclease-free water in a 1.5 mL tube to make a 10-fold dilution. Use* 1 µL *of the 10-fold diluted RNA to measure the concentration using Qubit RNA HS Assay Kit. After the concentration traction measurement, aliquote* 50 µL *of the stock RNA to 1.5 mL nuclease-free, low-binding tubes and label the concentration on the aliquots for* -80 °C *storage and future use. Use one* 50 µL *aliquot to prepare a diluted stock in RNA storage solution at a final concentration of* [M] 250 ng/µL *. Aliquot the diluted stock into 1.5 mL nuclease-free, low-binding tubes for* 2 µL *per tube. Store the aliquot diluted stocks at* -80 °C *and use one aliquot at a time to prevent repeated freeze/thaw.*

Preparing the MS2 bacteriophage RNA positive control

13.1 Retrieve a tube of the 2 µL MS2 RNA aliquot (250 ng/µL) from the -80 °C freezer.

13.2 Keep the tube on ice at all times to maintain RNA stability.

13.3 Add 98 µL of RNA storage solution directly to the aliquot, which contains 2 µL of concentrated bacteriophage MS2 RNA. This will prepare an RNA working solution with a final concentration of [M] 5 ng/µL in 100 µL .

13.4 In the 96-well sample plate, add 16 µL of nuclease-free water to a designated well.

13.5 Add 4 µL of the RNA working solution to the same well, pipetting mix with the 16 µL of nuclease-free water.

13.6 The remaining 96 µL of the RNA working solution can be stored at -80 °C for two additional uses, after which any leftover solution should be discarded.

cDNA APPROACH

14 Transfer 20 µL negative control extraction to a new tube or a well of a 96-well PCR plate.

15 Use prepared samples, positive control, and negative control for cDNA SYNTHESIS AND AMPLIFICATION (starting at step 17).

cDNA APPROACH

16 Add 10 µL nuclease-free water to the 10 µL purified double-stranded cDNA purified final product for SEQUENCING LIBRARY PREPARATION.

NOTE: After cDNA SYNTHESIS AND AMPLIFICATION, the concentration in each sample may range from [M] 50 ng/µL to [M] 300 ng/µL, typically between [M] 80-150 ng/µL.

cDNA SYNTHESIS AND AMPLIFICATION

5h 5m 20s

17 Remove contaminated DNA (~ 30 mins): 20m

BEFORE START:

- Prepare the reactions in the designated **pre-PCR** area to minimize the risk of cross-contamination.
- Use dedicated pipettes for **pre-PCR** tasks and use disposable, aerosol-resistant filter tips.
- Thaw total nucleic acid and 10x Turbo DNase Buffer on ice
- Vortex 10x Turbo DNase Buffer briefly, spin down by centrifugation for 5 seconds, and place on ice.
- Place Turbo DNase on ice all the time.
- Set up thermal cycler programs: Set lid temperature at 80 °C. Set up thermal cycler programs: 37 °C, 00:20:00 and hold at 4 °C.

18 Prepare a master mix of 10× Turbo DNase buffer and Turbo DNase following the table below with a 10% overage to account for pipetting variability (N: number of samples plus controls).

	A	B	C
	Component	Volume per reaction	Master mix volume (µL)
	10× Turbo DNase Buffer	3 µL	(3 × N)×1.1=
	Turbo DNase (2U/µL)	1 µL	(1 × N)×1.1=

	A	B	C
	RNase Inhibitor (40U/µL)	0.75 µL	(0.75 × N)×1.1=
	Nuclease-free water	5.25 µL	(5.25 × N)×1.1=
	Total volume	10 µL	(10 × N)×1.1=

19 Aliquot 10 µL of the prepared master mix into each well of the sample plate outlined in Section cDNA APPROACH, ensuring it is added to wells containing 20 µL of samples or controls.

20 The final volume per well should be 30 µL. Seal the plate with a self-adhesive plate-sealing film.

21 Gently mix the samples then centrifuge the sample plate for 00:01:00. 1m

22 Incubate the sample for 00:20:00 at 37 °C and hold at 4 °C. 20m

23 DNA-free RNA purification (~ 30 mins) 3m
Centrifuge the sample plate for 00:01:00 1 min (use the highest available speed of a bench-top plate centrifuge, generally between 500-3000 × g, depending on the model. If the speed is <1000 × g, centrifuge the plate for 00:02:00).

24 Resuspend the AMPure XP beads by vortexing for 00:00:20. 20s

25 Carefully remove the plate seal to avoid cross-contamination of the droplets.
Add 30 µL of resuspended AMPure XP beads to the reaction wells in the sample plate and mix by pipetting 5-10 times.

26 Seal the plate with a new self-adhesive plate-sealing film.

27 Incubate the sample plate on a rotator mixer at a speed of >1600 rpm for 00:05:00 at Room temperature (If a mixer is unavailable, vortex the plate every 00:02:00). 7m

28 Centrifuge the sample plate for 00:01:00 . 1m

29 Place the plate on a magnet for a 96-well plate. Keep the plate on the magnet for 00:03:00 to 00:05:00 until the supernatants are clear. 8m

30 While on the magnet, carefully remove the plate seal to avoid cross-contamination of the droplets.

31 Pipette off the supernatants without touching the bead pellet.

32 Keep the plate on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

33 Repeat the previous step.

34 Seal the plate with a new self-adhesive plate-sealing film.

35 Spin down and place the plate back on the magnet. Remove the seal and pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

36 Remove the plate from the magnetic rack and remove the seal. Resuspend the pellet in 13 µL nuclease-free water by pipetting for 00:05:00 to 00:10:00 . 15m

37 Seal the plate with a new self-adhesive plate-sealing film.

38 Incubate the sample plate on a rotator mixer at a speed >1600 rpm for 00:10:00 at Room temperature (If a mixer is unavailable, vortex the plate every 2 minutes). 10m

39 Spin down and pellet beads on the magnet until the elute is clear and colorless.

40 Transfer 11 µL of eluate of each sample to a new 96-well PCR plate. Label the plate as DNA-free RNA with date.

41 **Optional:** Measure the RNA quantity using a Qubit fluorometer and Qubit RNA HS Assay Kit.

42 Seal the plate with a new self-adhesive plate-sealing film.

43 Use the purified 10 µL DNA-free RNA for the cDNA or TNA sequencing approach 10m
00:10:00

44 Smart-9N First-Strand cDNA synthesis (~ 2.5 hr) 1h 45m

NOTE: *Upon the first time use, resuspend the lyophilized RLB RT-9N oligo in nuclease-free water at a molar concentration of 100 µM as stock solution. Store the DNA oligo stock at* -20 °C *. Also, resuspend the lyophilized RLB TSO oligo in RNA storage solution at a molar concentration of* [M] 100 micromolar (µM) *as stock solution. Aliquot the RLB TSO oligo stock into 1.5 mL Nuclease-free, low-binding tubes for* 3 µL *per tube. Store the aliquots at* -80 °C .

BEFORE START:

- Prepare the reactions in the designated **pre-PCR** area to minimize the risk of cross-contamination.
- Use dedicated pipettes for **pre-PCR** tasks and use disposable, aerosol-resistant filter tips.
- Making
- [M] 2 micromolar (µM) RLB RT-9N DNA oligo: Thaw the RLB RT-9N DNA oligo stock on ice. In a new RNase-free low-binding 1.5 mL tube, combine 3 µL of [M] 100 micromolar (µM) RLB RT-9N DNA oligo stock with 147 µL of nuclease-free water to make a [M] 2 micromolar (µM) working solution. Mix the diluted oligo thoroughly by vortexing. The unused [M] 2 micromolar (µM) working solution can be kept at -20 °C for repeated use.
- Making [M] 2 micromolar (µM) RLB TSO oligo: Retrieve a tube of RLB TSO RNA oligo aliquot (3 µL) from the -80 °C freezer. Add 147 µL of nuclease-free water directly to the tube to prepare the [M] 2 micromolar (µM) RLB TSO oligo working solution. Keep it on ice.
- NOTE: The RLB TSO oligo working solution must be freshly prepared each time.

- Thaw a tube of [M] 10 millimolar (mM) dNTP, along with the 5× SuperScript IV RT buffer and [M] 0.1 Molarity (M) DTT provided with the SuperScript IV reverse transcriptase at room temperature. After thawing, keep the tubes on ice.
- Place SuperScript IV reverse transcriptase and RNase inhibitor on ice.
- Set up thermal cycler: Set lid temperature at 100 °C. Set up two programs as follows,
- 1) RNA denature: 65 °C, 00:05:00, then hold at 4 °C.
- 2) Template switch RT: 50 °C for 01:30:00, 70 °C for 00:10:00, then hold at 4 °C

45 Prepare a master mix of [M] 10 millimolar (mM) dNTP and [M] 2 micromolar (µM) RLB RT 9N oligo following the table below with a 20 % overage to account for pipetting variability (N: number of samples plus controls).

	Component	Volume per reaction	Master mix volume (µL)
	10 mM dNTP	1 µL	(1 × N)×1.2=
	2 µM RLB RT 9N	1 µL	(1 × N)×1.2=
	Total volume	2 µL	(2 × N)×1.2=

46 Vortex the master mix gently, then spin down.

47 Aliquot 2 µL master mix into each purified 10 µL DNA-free RNA sample in the plate prepared in Step 40. Mix well the 12 µL reactions by pipetting.

48 Seal the plate with a new self-adhesive plate-sealing film.

49 Heat the plate on the thermocycler to 65 °C for 00:05:00 and hold at 4°C. 5m

50 Prepare a master mix of SuperScript IV reverse transcriptase (SS4RT) following the table below with a 10% overage to account for pipetting variability (N: number of samples plus

controls).

	A	B	C
	Component	Volume per reaction	Master mix volume (µL)
	5× RT buffer	4 µL	(4 × N)×1.1=
	RNase inhibitor	1 µL	(1 × N)×1.1=
	2 µM RLB TSO oligo	1 µL	(1 × N)×1.1=
	0.1M DTT	1 µL	(1 × N)×1.1=
	SuperScript IV reverse transcriptase (SS4RT)	1 µL	(1 × N)×1.1=
	Total volume	8 µL	(8 × N)×1.1=

51 Vortex the master mix gently, then spin down.

52 Make sure the plate in Step 49 has been cooled at 4 °C. Place the plate On ice.

53 Carefully remove the plate seal. Aliquot 8 µL of the SS4RT master mix into each reaction well of the plate. Mix thoroughly by pipetting.

54 Seal the plate with a new self-adhesive plate-sealing film.

55 Mix gently and centrifuge briefly.

56 Incubate at 50 °C for 01:30:00 followed by 70 °C for 00:10:00 and then hold at 4 °C. 1h 40m

57 cDNA amplification (~ 3 hr)

NOTE: *For first time use, resuspend the lyophilized RLB PCR DNA oligo in nuclease-free water at a molar concentration of* [M] 100 micromolar (µM) *as stock solution. Store the DNA oligo stock at* -20 °C .

BEFORE START:

- Prepare the reactions in the designated **Pre-PCR** area to minimize the risk of cross-contamination.
- Use dedicated pipettes for **Pre-PCR** tasks and use disposable, aerosol-resistant filter tips.
- Always work from clean (**pre-PCR**) to contaminated (**post-PCR**) areas, and **never backtrack**.
- Making [M] 10 micromolar (µM) RLB PCR DNA oligo: Thaw the
- [M] 100 micromolar (µM) oligo stock On ice . In a new RNase-free low-binding 1.5 mL tube, combine 10 µL of oligo stock with 90 µL of nuclease-free water to make a [M] 10 micromolar (µM) working solution. Mix the diluted oligo thoroughly by vortexing. The unused [M] 10 micromolar (µM) working solution can be kept at -20 °C for repeated use.
- Set up thermal cycler: Set lid temperature at 100 °C , and set up the cDNA amplification program following the table below.

	Step	Temp (ºC)	Duration	Cycle
	Initial denaturation	95	3 mins	1
	Denaturation	98	15 secs	27
	Annealing	62	15 secs	
	Extension	65	5 mins	
	Final extension	65	10 mins	1
	Storage	4	Hold	1

58 Prepare a master mix for PCR reaction following the table below with a 10 % overage to account for pipetting variability (N: number of samples plus controls).

	Component	Volume per reaction	Master mix volume (µL)
	10 µM RLB PCR oligo	2 µL	(2 × N)×1.1=
	Q5 High-Fidelity 2X Master Mix	15 µL	(15 × N)×1.1=
	Nuclease-free water	8 µL	(8 × N)×1.1=
	Total volume	25 µL	(25 × N)×1.1=

59 Vortex the master mix gently, then spin down.

60 In a new 96-well PCR plate, aliquot 25 µL of the PCR reaction master mix into the wells designated for each sample.

61 Add 5 µL of the first-strand cDNA products prepared in Step 56 into each well containing the PCR master mix. Ensure thorough mixing by pipetting gently.

62 Seal the plate with a new self-adhesive plate-sealing film.

63 Run the 96-well PCR plate in the thermal cycler using the program for cDNA amplification.

64 Remaining cDNA products can be stored at -20 °C in case of test failure. Once sequencing is successfully complete, it can be discarded.

STOP POINT: The amplified double-stranded cDNA can be stored at -20 °C before purification.

cDNA SYNTHESIS: Purification of double-stranded cDNA (~ 30 mins)

18m 30s

65 Purification of amplified double-stranded cDNA (~ 30 mins):

Note

NOTE: *Before starting, prepare fresh 70% ethanol in nuclease-free water sufficient for your samples. (* 500 µL *per sample).*

BEFORE START:

- Always work from clean (**pre-PCR**) to contaminated (**post-PCR**) areas, and never backtrack.
- Wipe the bench top with 1% bleach, then use 70% ethanol to wipe the bench top again.
- Prepare the reactions in the designated **Post-PCR** area to minimize the risk of cross-contamination.
- Use dedicated pipettes for **Post-PCR** tasks and use disposable, aerosol-resistant filter tips.
- Prepare fresh 70% ethanol in nuclease-free water sufficient for your samples. (500 µL per sample).

66 Centrifuge the sample plate for 00:02:00 (use the highest available speed of a bench-top plate centrifuge, generally between 500-3000 × g, depending on the model. If the speed <1000 × g, centrifuge the plate for 00:03:00). 5m

67 Add 30 µL of resuspended AMPure XP beads to the reaction wells in the sample plate and mix by slow pipetting 5-10 times, changing the tip every time to avoid cross-contamination.

68 Seal the plate with a new self-adhesive plate-sealing film.

69 Incubate the sample plate on a rotator mixer at a speed of >1600 rpm for 00:05:00 at Room temperature .(If a mixer is unavailable, vortex the plate every 2 minutes). 5m

70 Centrifuge the plate for 00:02:00 and pellet the beads on the magnet until the supernatant looks clear. 2m

71 Keep the plate on the magnet, and pipette off the supernatant. Be careful not to touch the bead pellet.

72 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and

discard.

73 Repeat the previous step X1.

74 Seal the plate with a new self-adhesive plate-sealing film.

75 Centrifuge the plate for 00:01:00 and place the plate back on the magnet. Using a pipette, remove any residual ethanol. Allow to dry for ~ 00:00:30, but do not dry the pellet to the point of cracking. 1m 30s

76 Remove the tube from the magnetic rack and resuspend the pellet in 13 µL nuclease-free water by slow pipetting to elute the cDNA.

77 Seal the plate with a new self-adhesive plate-sealing film.

78 Incubate on a rotator mixer at speed of>1600 rpm) for 00:05:00 at Room temperature (If a mixer is unavailable, vortex the plate every 2 minutes). 5m

79

80

81

82 Proceed with cDNA purification or store the reaction mixture at -20 °C before the subsequent cDNA purification.

cDNA SYNTHESIS: Purification of double-stranded cDNA (~ 15 mins)

83

Note

NOTE: *Before starting, prepare fresh 70% ethanol in nuclease-free water sufficient for your samples. (* 500 µL *per sample).*

Resuspend the AMPure XP beads by vortexing.

84 Transfer the sample (50 µL) to a clean 1.5 mL low DNA binding tube.

85 Add 40 µL of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

86 Incubate on a Hula mixer (or a rotator mixer >1600 rpm) for 00:05:00 at Room temperature . 5m

87 Spin down the sample and pellet on the magnet. Keep the tube on the magnet, and using a pipette, discard the supernatant.

88 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

89 Repeat the previous step X1.

90 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

91 Remove the tube from the magnetic rack and resuspend the pellet in 13 µL nuclease-free water.

92 Incubate on a Hula mixer (or a rotator mixer >1600 rpm) for 00:10:00 at Room temperature . 10m

93 Spin down and pellet beads on magnet until the eluate is clear and colorless.

94 Remove and retain 11 µL of eluate into a clean 1.5 mL low DNA binding tube.

95 **Optional**: : If using the cDNA sequencing approach for viral target identification, analyze 1 µL of the purified double-stranded cDNA for quantity using Qubit fluorometer and Qubit 1X dsDNA HS Assay Kit.

96 Use the purified 10 µL cDNA for the cDNA or TNA sequencing approach, see **TNA APPROACH** or **cDNA APPROACH**.

Note

STOP POINT: The synthesized double-stranded cDNA can be stored at -20 °C before sequencing.

SEQUENCING LIBRARY PREPARATION

97 **Before starting**, prepare fresh 70% ethanol in nuclease-free water sufficient for your samples (1 mL per sample). Program the thermal cycler or use a heat block for 96 well plate: 20 °C for 00:05:00 and 65 °C for 00:05:00 . Thaw Ultra II End-prep reaction buffer, NEBNext FFPE DNA Repair Buffer, Barcode Plate(from SQK-NBD114.96 Kit),and Blunt/TA Ligase Master Mix On ice . After fully thaw, mix by vortex, spin down briefly, and place On ice . Check that there is no precipitate present (the Blunt/TA Master Mix can sometimes form a precipitate). Spin down Ultra II End-prep enzyme mix and place On ice . 10m

Note

NOTE: Depending on the chosen sequencing approach, the materials subjected to downstream steps at this point should be either gDNA, cDNA, or a mixture of both gDNA and cDNA (called TNA) in 20 µL nuclease-free water.

SEQUENCING LIBRARY PREPARATION: End-prep (~ 50 minutes)

98 Mix the following reagents for one sample. When working with 24 or 48 samples, prepare a master mix by multiplying the ingredients (except gDNA/cDNA/TNA) with a 10% overage. Aliquot 10 µL of the master mix for each sample in a 96 well plate, then add the prepared samples:

	A	B
	Component	**Volume**
	DNA/TNA sample	20 µl
	Nuclease-free water	4 µl
	Ultra II End-prep reaction buffer	1.75 µl
	Ultra II End-prep enzyme mix	1.5 µl
	NEBNext FFPE DNA Repair Buffer	1.75 µl
	NEBNext FFPE DNA Repair Mix	1 µl
	Final total volume	30 µl

99 Mix gently by pipetting and spin down.

100 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

101 Resuspend the AMPure XP beads by vortexing.

102 Add 50 µL of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting (optional: use multi-channel pipette for reagent transfer when working with multiple samples).

103 Incubate on a Hula mixer (a rotator mixer >1600 rpm) for 00:05:00 at Room temperature . 5m

104 Spin down the sample and pellet on a magnet (DynaMag-2 for 1.5 mL tube and DynaMag-96 for PCR plate). Keep the tube on the magnet, and using a pipette, discard the supernatant.

105 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

106 Repeat the previous step X1.

107 Spin down and place the tube back on the magnet. Using a pipette, remove any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

108 Remove the tube from the magnetic rack and resuspend the pellet in 11 µL nuclease-free water. Incubate for 00:02:00 at Room temperature . 2m

109 Pellet the beads on a magnet until the eluate is clear and colorless.

110 Remove and retain 10 µL of eluate into a clean 1.5 mL low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Barcode ligation (~ 25 minutes)

111 The table below lists reagents for one barcoding reaction. Add the reagents to the End-prepped sample following the listed order. Carefully check the plate layout and recode numbers for the barcodes when transferring. (Optional: use multi-channel pipette for barcode transfer when working with 24 or 48 samples). Seal the used barcodes with a cut-off piece of a self-adhesive aluminum foil.

	A	B
	Component	**Volume**
	End-prepped DNA	10 µl
	Native Barcode (pick one form Native Barcoding Expansion 1-96)	2 µl
	Blunt/TA Ligase Master Mix	12 µl
	Final total volume	24 µl

112 Mix gently by flicking the tube and spin down.

113 Incubate the reaction for 00:20:00 at Room temperature . 20m

114 Add 3 µL of EDTA to each well and mix thoroughly by pipetting and spin down briefly.

Note

At this point, samples should be individually barcoded and ready to be subjected to pooling.

SEQUENCING LIBRARY PREPARATION: Library pooling for multiplex sequencing

115 Resuspend the AMPure XP beads by vortexing.

116 Use the table below to pool the barcoded samples in a new 1.5 mL low DNA binding tube depending on the flow cell that is going to be used. Add resuspended AMPure XP beads to the pooled library and mix by pipetting.

	A	B	C
		MinION (R10.4.1)	**PromethION (R10.4.1)**
	Each barcoded sample (µL)	12	12
	Number of samples in a pool	24	48
	Blunt/TA Ligase Master Mix (NEB M0367L, ready-to-use)	288	576
	Total volume of a pool (µL) AMPure XP beads for purification (µL, 0.5x of the pooled library volume)	144	288

117 Incubate on a Hula mixer (or a rotator mixer) for 00:10:00 at Room temperature . 10m

118 Spin down the sample and pellet on a magnet. Keep the tube on the magnet for 00:05:00 , and using a pipette, discard the supernatant. 5m

119 Keep the tube on the magnet and wash the beads with 700 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

120 Repeat the previous step X1.

121 Spin down and place the tube back on the magnet. Using a pipette, remove any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

122 Remove the tube from the magnetic rack and resuspend the pellet in 31 µL nuclease-free water. Incubate for 00:10:00 at 37 °C temperature. 10m

123 Spin down and pellet the beads on a magnet until the eluate is clear and colorless.

124 Remove and retain 30 µL of eluate into a clean 1.5 mL low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Adapter ligation (~ 45 minutes)

125 **BEFORE STARTING:** Thaw Short Fragment Buffer (SFB), Elution Buffer (EB), and NEBNext Quick Ligation Reaction Buffer (5×) at Room temperature, mix by vortexing, spin down, and place On ice. Check that the contents or each tube are clear of any precipitate. Spin down the T4 Ligase and the Native Adapter (NA), and place On ice.

126 Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

	A	B
	Pooled barcoded sample	30 µl
	Native Adapter (NA)	5 µl
	NEBNext Quick Ligation Reaction Buffer (5×)	10 µl
	Quick T4 DNA Ligase	5 µl
	Final total volume	50 µl

127 Mix gently by flicking the tube, and spin down.

128 Incubate the reaction for 00:20:00 at Room temperature. 20m

129 Resuspend the AMPure XP beads by vortexing.

130 Add 50 µL of resuspended AMPure XP beads to the reaction and mix by pipetting.

131 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at Room temperature. 10m

132 Place on the magnetic rack, allow beads to pellet and using a pipette, discard the supernatant.

133 Add 125 µL of the Short Fragment Buffer (SFB) to the beads. Close the tube lid and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and using a pipette, discard the supernatant.

134 Repeat the previous step X1.

135 Spin down and place the tube back on the magnet. Using a pipette, remove any residual supernatant.

136 Remove the tube from the magnetic rack and resuspend the pellet in Elution Buffer (EB), depending on the flow cell to be used.

	A	B	C
		MinION (R10.4.1)	**PromethION (R10.4.1)**
	EB (µL)	13	33

137 Incubate on at 37 °C for 00:10:00 at, agitate the sample for 10s every 2 min. 10m

138 Pellet beads on magnet until the eluate is clear and colorless.

139 Remove and retain 13 µL of eluate into a clean 1.5 mL low DNA binding tube.

140 Quantify 1 µL of eluted sample using a Qubit fluorometer and Qubit 1X dsDNA HS Assay Kit.

141 The recovery of the library and the expected molar concentration for loading are listed in the table below.

	A	B	C
		MinION (R10.4.1)	**PromethION (R10.4.1)**
	Library volume	12	32
	Expected recovery quantity (ng)	100-400	200-1000
	Expected library molar conc. (fmol)	30	100

142 Put the library On ice until ready to load or store the library at -20 °C for future sequencing.

Priming and loading the SpotON Flow Cell

143 Check the number of pores in your flow cell.

Note

NOTE: *before starting the flow cell pore checking, check the hardware following the manufacturer's guidance.*

143.1 If using a P2 Solo device, connect the P2 Solo with the wire included in the P2 Solo package to a GridION or a compatible computer with MinKNOW software via the USB-C port and turn on.

143.2 Turn on GridION (or MinION Mk1C) device. Make sure all the connections for the display, mouse, keyboard, and internet are ready.

143.3 Get a MinION or a PromethION flow cell from the refrigerator.

143.4 Double-click the MinKNOW icon shown on the desktop to initiate the program.

143.5 Use Oxford Nanopore Community username and password to login.

143.6 Select the device shown on the screen.

143.7 Open the lid of the sequencer and insert the flow cells under the clips, press down the flow cell to ensure good thermal and electrical contact.

143.8 The Sequencing Overview tab should show the **flow cell not checked** in each position in use.

143.9 Navigate to the Start tab and select **Flow Cell Check**.

143.10 Check that the sequencer assigns the correct flow cell type: FLO-MIN114 for MinION and FLO-PRO114M for PromethION. If not, select the correct flow cell type from the drop-down list.

143.11 Click **Start** to begin the flow cell check.

143.12 Record the port number and date of checking on the original package of the flow cell. The flow cell with less than 800 (MinION)/5000(PromethION) pores should not be used for the sequencing. If the flow cell is not expired (12 weeks shelf life), contact Oxford Nanopore Technologies to claim a replacement.

143.13 If the flow cell is going to be used immediately, keep it on the GridION or MinION Mk1C sequencer for priming. Otherwise put the flow cell back to the original pouch, store at 4 °C for next day use. The opened flow cell should be used within one week.

Priming and loading the SpotON Flow Cell: Flow cell priming

144 **BEFORE STARTING**:
Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at Room temperature. Mix SB by tapping or pipetting (DO NOT Vortex) and vortex the other tubes. Spin down tubes at Room temperature.

145 Check the air bubble of priming pore.

146 Open the sequencer lid and insert the pore checked flow cell.

147 Slide the priming port cover (MinION) or inlet port cover (PromethION) clockwise to open the port.

Note

NOTE: *Please see* ***Appendix 1*** *for the positions of the flow cell ports.*

148 Check for a small air bubble under the cover. Draw back a small volume (20-30 µL) to remove any bubbles:

148.1 Set a P1000 pipette to 200 µL . Insert the tip into the priming/inlet port. Turn the volume adjustment wheel counter-clockwise until the dial shows 220-230 µL or until you can see a small buffer volume entering the pipette tip.

Note

IMPORTANT: Take care when drawing back the buffer from the flow cell. Do not remove more than 20-30 µL , and make sure that the array of pores is always covered by the buffer. Introducing air bubbles into the array can irreversibly damage pores.

149 Prepare the flow cell priming mix and prime flow cells.

149.1 Using a 2.0 mL low DNA binding tube, prepare flow cell priming mix with components as follows, mix by inverting the tube and pipetting.

	A	B	C
	Component	**MinION**	**PromethION**
	Bovine Serum Albumin (BSA) (50 mg/ml)	5 µl	-
	Flow Cell Tether (FCT)	30 µl	30 µl

	A	B	C
	Flow Cell Flush (FCF)	1170 µl	1170 µl
	Final total volume	1205 µl	1200 µl

149.2 Load 800 µL (MinION) or 500 µL (PromethION) of the priming mix into each flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 00:05:00 . 5m

150 Prepare the library for loading.

Note

IMPORTANT: The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

150.1 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

150.2 In a new tube, prepare each library for loading as follows:

	A	B	C
	Component	**MinION**	**PromethION**
	Sequencing Buffer (SB)	37.5 µl	100 µl
	Library Beads (LIB)	25.5 µl	68 µl
	DNA library	12 µl	32 µl
	Final total volume	75 µl	200 µl

151 Complete the flow cell priming.

151.1 (MinION) Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

151.2 Load 200 µL (MinION) or 500 µL (PromethION) of the priming mix into the flow cell via the priming/inlet port, avoiding the introduction of air bubbles.

152 Loading samples.

152.1 Mix the prepared library gently by pipetting up and down just prior to loading.

152.2 Add the entire volume of the library loading mix via the SpotON sample port (MinION) or inlet port (Note: PromethION which has only one port for both priming and sample loading) in a dropwise fashion. Ensure each drop flows into the port before adding the next drop.

152.3 (MinION)Gently replace the SpotON/inlet port cover, making sure the bung enters the SpotON port, close the priming port.

152.4 Press the light shield included in the flow cell poach around the SpotON/inlet port cover firmly to block the light from the pore window.

152.5 Close the lid of the sequencer.

Priming and loading the SpotON Flow Cell: Data acquisition and basecalling

2d

153

Note

NOTE: A series of snapshots showing the way to MinKNOW is listed in **Appendix 2**.

Double-click the MinKNOW icon displayed on the desktop to initiate the program.

154 Use Oxford Nanopore Community username and password to login or continue as Guest.

155 Select the device shown on the screen.

156 Go to the Start tab, and click the Start Sequencing option to choose the running parameters.

156.1 Type in the **Experiment Name**

156.2 Type in **Sample ID** (same as experiment name)

156.3 Choose flow cell FLO-MIN114 for MinION and FLO-PRO114M for PromethION from the drop-down menu.

156.4 Use Select all available to select all the connected flow cells or use the diagram above to select specific flow cells to run.

157 Click **Continue to Kit Selection** to move to the next page.

157.1 Click the kit **SQK-NBD114-96** from the Kit Selection menu.

158 Click **Continue to Run Options** to choose run parameters.

158.1 Set run length to 48:00:00 and minimum read length 200 bp. Leave adaptive sampling unchecked. 2d

159 Click **Continue to Analysis** to choose basecalling and Barcoding parameters.

159.1 In the Basecalling options, checkup the basecalling with configuration: **High accuracy basecalling.**

159.2 In the Barcoding options, turn on the **Trim barcodes** and **Mid-read barcoding** filtering.

159.3 Do **not** turn on the Alignment option.

160 Click **Continue to output** to the next page.

160.1 Select the output data location, format, and filtering options. Check up the box for Raw reads in POD5 format and Basecalled reads in FASTQ format. Keep the filter score as the system default.

161 Click **Continue to final review** to proceed.

162 Review the settings listed in the Run Setup page. Correct any errors. Select **Start** to run the experiment.

163 The system will automatically navigate the Sequencing Overview when sequencing starts.

164 48 hrs later, check the sequencing data. Use 1 mL pipette to remove 1 mL waste solution in the waste channel via waste port 1 (see Appendix 1 under Guidelines & Warnings tab). Remove the flow cells on the device, put it back in the original package, and turn off the device.

Protocol references

REFERENCES

REDI-NET Overview Summary

Double-stranded cDNA synthesis (NEB first and second strand cDNA synthesis protocols):

- NEBNext Ultra II RNA First Strand synthesis manual E7771
- NEBNext Ultra II Non-directional RNA Second Strand synthesis manual E6111
- ezdnase_PI

Oxford Nanopore Manufacturer's protocols:

- Ligation sequencing gDNA - Native Barcoding Kit 96 V14 (SQK-NBD114.96)-minion.
- ligation-sequencing-gdna-native-barcoding-v14-sqk-nbd114-96-NBE_9171_v114_revG_15Sep2022-minion
- ligation-sequencing-gdna-native-barcoding-v14-sqk-nbd114-96-NBE_9171_v114_revG_15Sep2022-gridion