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REDI-NET B-4 BLOOD TESTING V.3

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Protocol status: Working

We use this protocol and it's working

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Disclaimer

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Abstract

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

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 blood 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.

REFERENCES

REDI-NET Overview Summary

cDNA synthesis and amplification:

- Claro IM, Ramundo MS, Coletti TM et al. Rapid viral metagenomics using

SMART-9N amplification and nanopore sequencing. Wellcome Open Research 2023, 6:241. <https://doi.org/10.12688/wellcomeopenres.17170.2>

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

Host rRNA depletion

- Manual: riboPOOL Kit

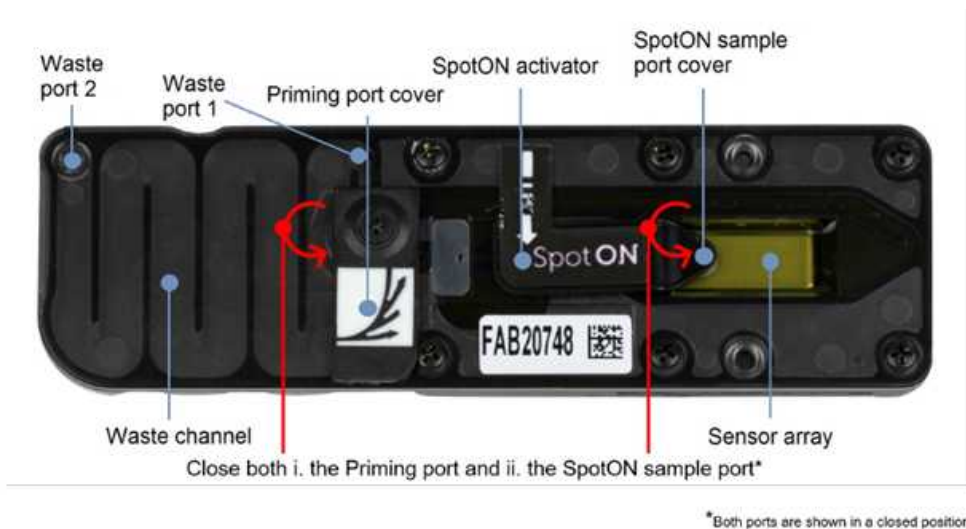
Host DNA depletion

- Instruction manual: NEBNext® Microbiome DNA Enrichment Kit

APPENDICES

APPENDIX 2. FLOW CELL

MinION flow cell:



PromethION flow cell:



APPENDIX 3. 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.

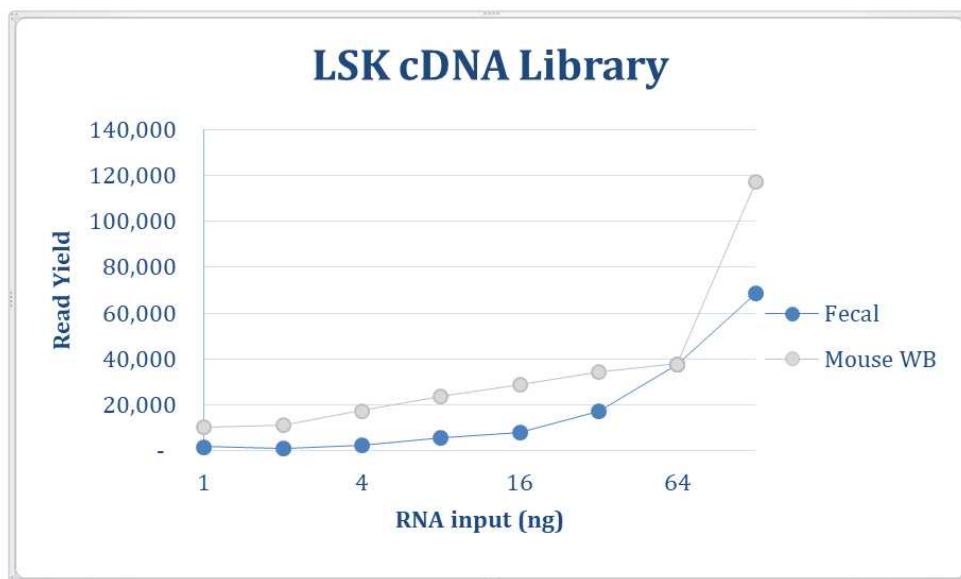
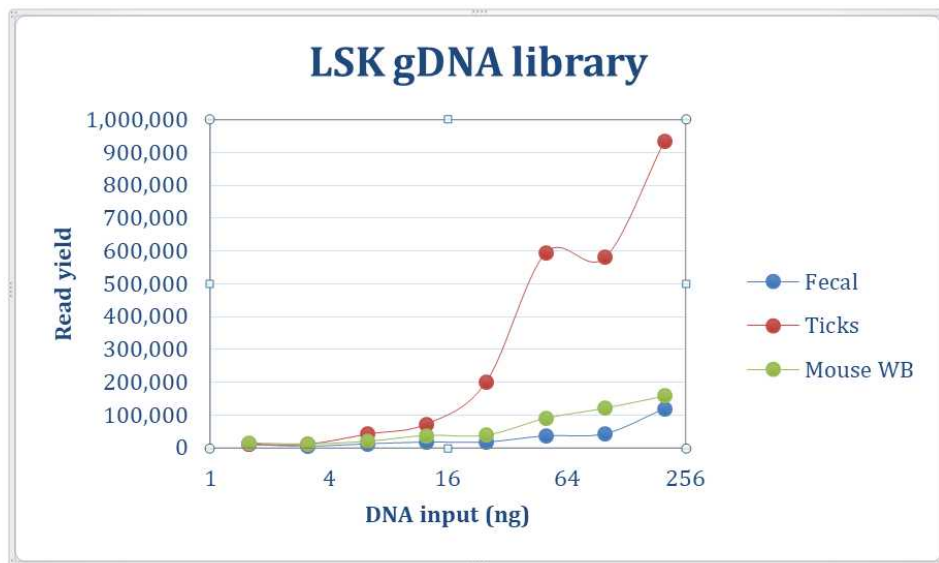


APPENDIX 4. cDNA END-PREP MASTER MIX PREPARATION

	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 5. EXPECTED OUTCOMES

The DNA or RNA inputs vs the sequencing read yields.



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
P10 8-channel pipette	Locally sourced



A	B
P300 8-channel pipette	Locally sourced

A	B	C
Material	Description	Mfg / Product #
DNA from a sample	Per sample from SOP B-2 (gDNA Preparation)	REDI-NET DNA sample
Eluents from negative control extraction	From SOP B-2 (gDNA Preparation)	REDI-NET negative control
ONT DNA Control Standard (ONT DCS)	10 ng/ μ L	Oxford Nanopore, SQK-NBD114.96
RNA from a sample	Per sample from SOP B-2 (TNA preparation)	REDI-NET RNA sample
MS2 bacteriophage RNA	500 ng/ μ L	Roche, 10165948001
RNA Storage Solution	Nuclease-free, 1 mM sodium citrate, pH 6.5 buffer	ThermoFisher, AM7000
Native Barcoding Kit 96 V14	(Sequencing Library Preparation)	Oxford Nanopore, SQK-NBD114.96
Turbo DNase and reaction buffer	(cDNA synthesis and amplification)	ThermoFisher, AM2238
SuperScript IV Reverse Transcriptase	(cDNA synthesis and amplification)	ThermoFisher, 18090010
RNase inhibitor	For cDNA synthesis and amplification and Host DNA/rRNA depletion	New England Biolabs, M0314L
RLB RT-9N (DNA oligo)	DNA Oligonucleotide, 5'-TTTTTCGTGCGCCGCTTCAA CNNNNNNNNN-3'	Integrated DNA Technologies, Custom DNA oligo, 100 nmole Standard desalting
RLB TSO (chimeric oligo)	Chimeric oligonucleotide, 5'-GCTAATCATTTGCTTTTTTCGTG CGCCGCTTCAACATrGrGrG-3'	Integrated DNA Technologies, Custom RNA oligo, 100nmole Standard desalting
RLB PCR primer (DNA oligo)	DNA Oligonucleotide, 5'-TTTTTCGTGCGCCGCTTCA-3'	Integrated DNA Technologies, Custom DNA oligo, 100nmole Standard desalting
10 mM dNTP	(cDNA synthesis and amplification)	New England Biolabs, N0447S

A	B	C
Q5 High-Fidelity 2X Master Mix	(cDNA synthesis and amplification)	New England Biolabs, M0492S
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 (<i>consumable</i>)	Oxford Nanopore, FLO-MIN114, FLO-PRO114M
UltraPure Bovine Serum Albumin (50mg/ml)	(Sequencing Library Preparation)	ThermoFisher, AM2616
low DNA binding tubes	1.5 mL (<i>consumable</i>)	Eppendorf, 022131021 or equivalent
low DNA binding tubes	2.0 mL (<i>consumable</i>)	Eppendorf, 022431048 or equivalent
PCR tubes	0.2 mL thin-walled (<i>consumable</i>)	Eppendorf, 951010006 or equivalent
PCR plate	96 well, low DNA binding, semi-skirted with heat seals (<i>consumable</i>)	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 (<i>consumable</i>)	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 (<i>consumable</i>)	Thermo Fisher, Q32856
	Qubit 1X dsDNA HS Assay Kit	(<i>consumable</i>)	ThermoFisher, Q33230
	Qubit RNA HS Assay Kit	(<i>consumable</i>)	ThermoFisher, Q32852
	Nuclease-free water	To prepare ethanol dilutions (<i>consumable</i>)	Locally sourced
	Freshly prepared 80% ethanol in nuclease-free water	Prepared from 100% molecular biology grade ethanol (<i>consumable</i>)	Locally sourced
	Freshly prepared 70% ethanol in nuclease free water	Prepared from 100% molecular biology grade ethanol (<i>consumable</i>)	Locally sourced
	Data sheets	REDI-NET DCS B-4 Testing	REDI-NET Data Portal

Equipment

Qubit Fluorometer

Fluorometer

Invitrogen

Q33238

<https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238>^{LINK}

NAME

TYPE

BRAND

SKU

Equipment

DynaMag™-2 Magnet	NAME
Magnet	TYPE
DynaMag™	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/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 bacteriophage RNA **Roche Catalog #10165948001**

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

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

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



⊗ RNase Inhibitor, Murine - 15,000 units **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**

⊗ Agencourt AMPure XP beads **Beckman Coulter 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**

⊗ Nanopore 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.

Ethics statement

The protocols.io team notes that research involving animals and humans, including their tissues, must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

Before start

BEFORE START

1. Must check the DNA and RNA concentrations of your samples of total nucleic acid (TNA).
2. If TNAs were prepared from whole blood or buffy coat, follow **Appendix 1** for host DNA and rRNA depletion (process either one or both depending on downstream applications), measure the concentration again, and then choose the appropriate approach for sequencing library preparation.
3. Select the sequencing approach based on the target of interest: a) For viral targets only, use cDNA to prepare the sequencing library. b) For bacterial targets only, use gDNA. c) If both viral and bacterial targets are of interest, use TNA to prepare the sequencing library.
4. gDNA sequencing does not involve any PCR steps and should be handled exclusively in the designated pre-PCR area of the lab.
5. The gDNA preparation for TNA sequencing can be handled in the designated pre-PCR area of the lab before being mixed with amplified cDNA.
6. **Any step involving PCR-amplified cDNA must be handled in the designated post-PCR area of the lab to avoid library contamination.**
7. The designated post-PCR area of the lab should be cleaned with 1% bleach followed by 70% ethanol before use.
8. Use sections **gDNA APPROACH**, **TNA APPROACH**, and **cDNA APPROACH** for gDNA, TNA, and cDNA preparation, respectively, then proceed with the prepared gDNA, TNA or cDNA to Section **SEQUENCING LIBRARY PREPARATION**.
9. The capacity of the sequencing flow cells is limited. The table below shows the maximum allowable DNA and RNA amount and volumes of one 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)
	40 ng, RNA	x	20-x	20



	A	B	C	D
	200 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	20-x	20

gDNA APPROACH

- 1 If the DNA concentration >  10 ng/ μ L , calculate the required volume of  200 ng DNA, then transfer the volume to a new  200 μ L PCR tube or a well of a 96-well PCR plate. Adjust the volume with nuclease-free water to a final volume of  20 μ L .
Directly use a 20 μ L sample for the downstream preparation if the DNA concentration is below 10 ng/ μ L (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 μ L nuclease-free water in a new  200 μ L PCR tube or a well of a 96-well PCR plate. Add  1 μ L of ONT DNA Control Standard (DCS) from ONT SQK-NBD114.96 kit to the well containing  19 μ L nuclease free water. Mix well by pipetting.
- 3 Transfer  20 μ L 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 μ L of nuclease-free water in a new  200 μ L PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below  20 ng/ μ L , directly transfer  10 μ L of the sample to the tube or well.
- 7 Prepare the gDNA from a positive control using  9 μ L of nuclease-free water in a new  200 μ L PCR tube or a well of a 96-well PCR plate. Add  1 μ L of ONT DCS from ONT SQK-NBD114.96 kit to the well containing  9 μ L 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

 50 ng/ μL to  300 ng/ μL , typically between  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  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 $-80\text{ }^{\circ}\text{C}$. When using the first-time, take $1\text{ }\mu\text{L}$ of RNA stock and mix with $9\text{ }\mu\text{L}$ of nuclease-free water in a 1.5 mL tube to make a 10-fold dilution. Use $1\text{ }\mu\text{L}$ of the 10-fold diluted RNA to measure the concentration using Qubit RNA HS Assay Kit. After the concentration traction measurement, aliquote $50\text{ }\mu\text{L}$ of the stock RNA to 1.5 mL nuclease-free, low-binding tubes and label the concentration on the aliquots for $-80\text{ }^{\circ}\text{C}$ storage and future use. Use one $50\text{ }\mu\text{L}$ aliquot to prepare a diluted stock in RNA storage solution at a final concentration of $250\text{ ng}/\mu\text{L}$. Aliquot the diluted stock into 1.5 mL nuclease-free, low-binding tubes for $2\text{ }\mu\text{L}$ per tube. Store the aliquot diluted stocks at $-80\text{ }^{\circ}\text{C}$ 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 $2\text{ }\mu\text{L}$ MS2 RNA aliquot ($250\text{ ng}/\mu\text{L}$) from the $-80\text{ }^{\circ}\text{C}$ freezer.
- 13.2 Add $98\text{ }\mu\text{L}$ of RNA storage solution directly to the aliquot, which contains $2\text{ }\mu\text{L}$ of concentrated bacteriophage MS2 RNA. This will prepare an RNA working solution with a final concentration of $5\text{ ng}/\mu\text{L}$ in $100\text{ }\mu\text{L}$.
- 13.3 Keep the tube on ice at all times to maintain RNA stability.
- 13.4 In the 96-well sample plate, add $16\text{ }\mu\text{L}$ of nuclease-free water to a designated well.
- 13.5 Add $4\text{ }\mu\text{L}$ of the RNA working solution to the same well, pipetting mix with the $16\text{ }\mu\text{L}$ of nuclease-free water.
- 13.6 The remaining $96\text{ }\mu\text{L}$ of the RNA working solution can be stored at $-80\text{ }^{\circ}\text{C}$ 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  50 ng/µL to

 300 ng/µL , typically between  80-150 ng/µL .

cDNA SYNTHESIS AND AMPLIFICATION

3h 30m

17 Remove contaminated DNA (~ 30 mins):

25m

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)

	A	B	C
	10× Turbo DNase Buffer	3 µL	$(3 \times N) \times 1.1 =$
	Turbo DNase (2U/µL)	1 µL	$(1 \times N) \times 1.1 =$
	RNase Inhibitor (40U/µL)	0.75 µL	$(0.75 \times N) \times 1.1 =$
	Nuclease-free water	5.25 µL	$(5.25 \times N) \times 1.1 =$
	Total volume	10 µL	$(10 \times N) \times 1.1 =$

- 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)
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). 3m
- 24 Resuspend the AMPure XP beads by vortexing for  00:00:20 . 
- 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). 5m
- 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. 5m
- 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 μ L 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 μ L 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

 00:10:00

44 Smart-9N First-Strand cDNA synthesis (~ 2.5 hr)

5m

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  $-20\text{ }^{\circ}\text{C}$. 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*
 $-80\text{ }^{\circ}\text{C}$.

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  $-20\text{ }^{\circ}\text{C}$ for repeated use.

- Making **1M 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 **1M 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 **1M 10 millimolar (mM)** dNTP, along with the 5× SuperScript IV RT buffer and **1M 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 **1M 10 millimolar (mM)** dNTP and **1M 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	$(1 \times N) \times 1.2 =$
2 μM RLB RT 9N	1 μL	$(1 \times N) \times 1.2 =$
Total volume	2 μL	$(2 \times N) \times 1.2 =$

- 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	$(4 \times N) \times 1.1 =$
RNase inhibitor	1 μL	$(1 \times N) \times 1.1 =$
2 μM RLB TSO oligo	1 μL	$(1 \times N) \times 1.1 =$
0.1M DTT	1 μL	$(1 \times N) \times 1.1 =$
SuperScript IV reverse transcriptase (SS4RT)	1 μL	$(1 \times N) \times 1.1 =$
Total volume	8 μL	$(8 \times N) \times 1.1 =$

- 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 .

15m

- 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 $-20\text{ }^{\circ}\text{C}$.

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 $-20\text{ }^{\circ}\text{C}$ On ice. In a new RNase-free low-binding 1.5 mL tube, combine $10\text{ }\mu\text{L}$ of oligo stock with $90\text{ }\mu\text{L}$ 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 $-20\text{ }^{\circ}\text{C}$ for repeated use.
- Set up thermal cycler: Set lid temperature at $100\text{ }^{\circ}\text{C}$, and set up the cDNA amplification program following the table below.

Step	Temp ($^{\circ}\text{C}$)	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	$(2 \times N) \times 1.1 =$
Q5 High-Fidelity 2X Master Mix	15 μL	$(15 \times N) \times 1.1 =$
Nuclease-free water	8 μL	$(8 \times N) \times 1.1 =$
Total volume	25 μL	$(25 \times N) \times 1.1 =$

- 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 (~ 15 mins)

16m 30s

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

Note

NOTE: Always keep PCR tubes and plates sealed when not in use. Briefly spin down tubes before opening to prevent aerosols. Open tubes and plates gently and use slow pipetting techniques to avoid cross-contamination.

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 $\times$ g, depending on the model. If the speed $<1000 \times$ g, centrifuge the plate for  00:03:00).

2m

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 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.
- 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 Centrifuge the plate for  00:01:00 and place the plate back on the magnet until the eluate is clear and colorless. 1m
- 80 Remove and retain  11 μL of eluate into a new 96-well PCR plate.
- 81 Analyze  1 μL of the purified double-stranded cDNA for quantity using Qubit fluorometer and Qubit 1X dsDNA HS Assay Kit.
- 82 For the downstream TNA or cDNA sequencing approach, refer to **TNA APPROACH** or **cDNA APPROACH**. II

**Note**

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

SEQUENCING LIBRARY PREPARATION

10m

83 BEFORE START

10m

- When preparing a TNA or cDNA sequencing library with PCR-amplified cDNA, the reactions must be handled in the designated **post-PCR** area of the lab.
- Briefly spin down the sample plate before opening to prevent aerosols.
- Spin down Ultra II End-prep Enzyme Mix, FFPE DNA Repair Mix and Blunt/TA Ligase Master Mix, then place On ice .
- Thaw the Ultra II End-prep reaction buffer, NEBNext FFPE DNA Repair Buffer, and items from the ONT SQK-NBD 114.96 Kit (Barcode Plate, EDTA, AMPure XP bead) On ice .
- After thawing, Ultra II End-prep reaction buffer and NEBNext FFPE DNA Repair Buffer may form precipitates. If this occurs, equilibrate the reagents to room temperature and vortex before use.
- Prepare fresh 70% ethanol in nuclease-free water (1 mL per sample).
- Prepare fresh 80% ethanol in nuclease-free water (1.5 mL per sample).
- Program a thermal cycler: 20 °C for 00:05:00 and 65 °C for 00:05:00 .
- Spin down all reagents briefly before use.

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)

19m 30s

- 84 Mix the following reagents to make a master mix for End-prep. When working with multiple samples, prepare a master mix by multiplying the ingredients (except gDNA/cDNA/TNA) with a 20% overage account for pipetting variability (N: number of samples plus controls).



Component	Volume per reaction	Master mix volume (μL)
Nuclease-free water	4 μL	$(4 \times N) \times 1.2 =$
Ultra II End-prep reaction buffer	1.75 μL	$(1.75 \times N) \times 1.2 =$
Ultra II End-prep enzyme mix	1.5 μL	$(1.5 \times N) \times 1.2 =$
NEBNext FFPE DNA Repair Buffer	1.75 μL	$(1.75 \times N) \times 1.2 =$
NEBNext FFPE DNA Repair Mix	1 μL	$(1 \times N) \times 1.2 =$
Final total volume	10 μL	$(10 \times N) \times 1.2 =$

- 85 Spin down the plate, and remove the plate seal carefully. Aliquot  10 μL of the master mix to each well of a 96-well PCR plate containing  20 μL of samples or controls. Mix  30 μL End-prep reactions gently by pipetting and spin down.



- 86 Using a thermal cycler, incubate at  20 °C for  00:05:00 and  65 °C for  00:05:00 .

10m



- 87 Resuspend the AMPure XP beads by vortexing.

- 88 Add  50 μL of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting (use an 8-channel pipette for reagent transfer of multiple samples).



- 89 Incubate on a Hula mixer (a rotator mixer >1600 rpm) for  00:05:00 at  Room temperature . (If a mixer is unavailable, vortex the plate every 2 minutes)

5m

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

19m 30s

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

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

19m 30s

- 91 Centrifuge the plate for 00:02:00 and pellet the beads on the magnet until the supernatant looks clear. (DynaMag-2 for 1.5mL tubes and DynaMag-96 for a PCR plate) and pipette off the supernatant without disturbing the beads 2m
- 92 Remove the seal. Keep the sample on the magnet and wash the beads with 200 μ L of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 93 Repeat the previous step X1.

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

19m 30s

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

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

19m 30s

- 95 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
- 96 Remove the tube from the magnetic rack and resuspend the pellet in 12 μ L nuclease-free water by pipetting. 2m

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

19m 30s

- 97 Seal the plate with a new self-adhesive plate-sealing film.
- 98 Incubate for 00:02:00 at Room temperature .

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

19m 30s

- 99 Spin down and place the tube back on the magnet until the eluate is clear and colorless.
- 100 Remove the seal and transfer  11 µL of eluate into a cleanwells on the same plate, maintaining the original column/row pattern while skipping previously used columns and rows. (For example, if columns 1-6 were used, transfer the elutes in parallel to columns 7-12).

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

19m 30s

- 101 Directly use the product to barcode ligation or seal the plate with a new self-adhesive plate-sealing film.
- STOP POINT: The purified End-prep reactions can be stored at  -20 °C before barcode ligation.

SEQUENCING LIBRARY PREPARATION: Barcode ligation (~ 25 minutes)

- 102 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

- 103 Mix gently by pipetting 10-15 times, seal, and spin down the plate.



104 Incubate the reaction for  00:20:00 at  Room temperature .

20m

105 Spin down the plate and remove the plate seal. 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.

106 Seal the plate and spin down.

SEQUENCING LIBRARY PREPARATION: Library pooling for multiplex sequencing

107 Resuspend the AMPure XP beads by vortexing.

108 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



- 109 Incubate on a Hula mixer (or a rotator mixer) for 00:10:00 at Room temperature . 10m
- 110 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
- 111 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.
- 112 Repeat the previous step X1.
- 113 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
- 114 Remove the tube from the magnetic rack and resuspend the pellet in 32 μL nuclease-free water. Incubate for 00:10:00 at 37 °C temperature, agitating the sample for 10 seconds every 2 minutes. Alternatively, if available, mix the sample on a thermomixer at 37°C at ≥ 1600 rpm. 10m
- 115 Spin down and pellet the beads on a magnet until the eluate is clear and colorless.
- 116 Remove and retain 31 μL of eluate into a clean 1.5 mL low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Adapter ligation (~ 45 minutes)

30m

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



118

Taking the pooled and barcoded DNA, perform adapter ligation as follows, mix 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

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

120 Incubate the reaction for  00:10:00 at  Room temperature .

10m

121 Resuspend the AMPure XP beads by vortexing.

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



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

10m

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

125 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.



126 Repeat the previous step X1.

- 127 Spin down and place the tube back on the magnet. Using a pipette, remove any residual supernatant.
- 128 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 N (R10.4.1)
	EB (μL)	13	33

- 129 Incubate on at  37 °C for  00:10:00 at, agitate the sample for 10s every 2 min. Alternatively, if available, mix the sample on a thermomixer at 37°C at ≥1600 rpm.

10m



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

- 131 Remove and retain  13 μL (MinION) or  33 μL (PromethION) of eluate into a clean  1.5 mL low DNA binding tube.

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

- 133 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 N (R10.4.1)
	Library volume	12	32
	Expected recovery quantity (ng)	100-400	200-1000



	A	B	C
	Expected library molar conc. (fmol)	30	100
	Recommended library loading upper limit	1000 ng total (83 ng/μL)	4000 ng total (125 ng/μL)

- 134 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

- 135 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.

- 135.1 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.
- 135.2 Turn on GridION (or MinION Mk1C) device. Make sure all the connections for the display, mouse, keyboard, and internet are ready.
- 135.3 Get a MinION or a PromethION flow cell from the refrigerator.
- 135.4 Double-click the MinKNOW icon shown on the desktop to initiate the program.
- 135.5 Use Oxford Nanopore Community username and password to login.
- 135.6 Select the device shown on the screen.

- 135.7 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.
- 135.8 The Sequencing Overview tab should show the **flow cell not checked** in each position in use.
- 135.9 Navigate to the Start tab and select **Flow Cell Check**.
- 135.10 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.
- 135.11 Click **Start** to begin the flow cell check.
- 135.12 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.
- 135.13 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

- 136 **BEFORE STARTING:**
Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at TemperatureRoom temperature . Mix SB by tapping or pipetting (DO NOT Vortex) and vortex the other tubes. Spin down tubes at TemperatureRoom temperature .
- 137 Check the air bubble of priming pore.
- 138 Open the sequencer lid and insert the pore checked flow cell.
- 139 Slide the priming port cover (MinION) or inlet port cover (PromethION) clockwise to open the port.

Note

NOTE: Please see **Appendix 2** for the positions of the flow cell ports.

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

140.1 Set a P1000 pipette to  200 μL . Insert the tip into the priming 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.

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

141.1 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
Flow Cell Flush (FCF)	1170 μl	1170 μl
Final total volume	1205 μl	1200 μl

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

5m

142 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.

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

142.2 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

143 Complete the flow cell priming.

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

143.2 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.

144 Loading samples.

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



- 144.2 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.
- 144.3 (MinION) Gently replace the SpotON/inlet port cover, making sure the bung enters the SpotON port, close the priming port.
- 144.4 Press the light shield included in the flow cell pouch around the SpotON/inlet port cover firmly to block the light from the pore window.
- 144.5 Close the lid of the sequencer.



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

145

Note

NOTE: A series of snapshots showing the way to MinKNOW is listed in **Appendix 3**.

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

- 146 Use Oxford Nanopore Community username and password to login or continue as Guest.
- 147 Select the device shown on the screen.
- 148 Go to the Start tab, and click the Start Sequencing option to choose the running parameters.
- 149 Type in the **Experiment Name** use scheme as shown below,
[YYYY_MM_DD_Approach(gDNA or TNA)_Sample type]
Example: 2024_11_25_cDNA_Water
- 149.1 **Approaches: gDNA, cDNA, TNA**



- 149.2 **Sample type names: Soil, Water, Tick, Leech, Feces, Blood, Swab, Filth Fly**
- 150 Type in **Sample ID** (same as experiment name)
- 151 Choose flow cell FLO-MIN114 for MinION and FLO-PRO114M for PromethION from the drop-down menu.
- 152 Use Select all available to select all the connected flow cells or use the diagram above to select specific flow cells to run.
- 153 Click **Continue to Kit Selection** to move to the next page.
- 154 Click the kit **SQK-NBD114-96** from the Kit Selection menu.
- 155 Click **Continue to Run Options** to choose run parameters.
- 156 Set run length to  48:00:00 and minimum read length 200 bp. Leave adaptive sampling unchecked.
- 157 Click **Continue to Analysis** to choose basecalling and Barcoding parameters.
- 157.1 In the Basecalling options, checkup the basecalling with configuration: **High accuracy basecalling**.
- 157.2 In the Barcoding options, turn on the **Trim barcodes** and **Mid-read barcoding** filtering.
- 157.3 Do **not** turn on the Alignment option.
- 158 Click **Continue to output** to the next page.

2d



- 158.1 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.
- 159 Click **Continue to final review** to proceed.
- 160 Review the settings listed in the Run Setup page. Correct any errors. Select **Start** to run the experiment.
- 161 The system will automatically navigate the Sequencing Overview when sequencing starts.
- 162 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 2 under Guidelines & Warnings tab). Remove the flow cells on the device, put it back in the original package, and turn off the device.

APPENDIX 1. HOST DNA & rRNA DEPLETION for TNA from WHOLE BLOOD & BUFFY COAT

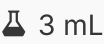
1h 29m 5s

163

Note

IMPORTANT: Host-depletion is required for TNA from whole blood and buffy coat only, but NOT for other blood sample types including plasma, serum and dried blood spot on FTA cards.

Prepare NEBNext Microbiome DNA Enrichment Kit.

- 163.1 When using the kit for the first time, thaw 5X NEBNext Bind/wash Buffer (48ml bottle)  On ice . After defrosting, aliquot the buffer into six  1.5 mL tubes,  1 mL for each tube, then aliquot the rest of the  42 mL buffer into fourteen  15 mL tubes,  3 mL for each tube.

Note

All tubes should be stored at  -20 °C before use.



164 Prepare MBD2-Fc Protein and Magnetic Beads in Microbiome DNA Enrichment Kit for host DNA depletion.

164.1 Place one tube of  3 mL 5X Bind/wash buffer, NEBNext Protein A Magnetic Beads, and NEBNext MBD2-Fc Protein  On ice .

164.2 Add  12 mL Nuclease-free water into the  3 mL 5X Bind/wash buffer in 15mL tube to make 1X Bind/wash buffer and relabel the tube as 1X buffer.

164.3 Resuspend NEBNext Protein A Magnetic Beads by gently pipetting the slurry up and down until the suspension is homogeneous; avoid bubbling.

Note

Do not vortex.

164.4 In one  1.5 mL tube, add  8 μ L of MBD2-Fc protein and  80 μ L of Protein A Magnetic Beads. Mix by pipetting up and down (avoid bubbling) until the beads are completely homogeneous, at least 5-10 times.

Note

One mix is used for one sample. If preparing the MBD2-Fc protein and Protein A Magnetic Beads mix for multiple samples, scale up by the number of samples.

164.5 Mix the beads in a Hula mixer for  00:03:00 at  Room temperature .

3m



164.6 Spin the tube for  00:00:05 and use pipetting to resuspend the bottom beads if any. If the mix is prepared for multiple samples, aliquot  80 μ L mix to  1.5 mL tubes.

5s

164.7 Briefly spin the tube and place in the magnetic rack for 2–5 minutes or until the beads have collected to the wall of the tube and the solution is clear.



164.8 Carefully remove the supernatant with a pipette without disturbing the beads.

164.9 Add  500 μL of 1X Bind/wash Buffer (kept  On ice) to the tube to wash the beads. Pipette up and down until the beads are completely homogeneous, at least 5-10 times.



164.10 Mix the beads in a Hula mixer for  00:03:00 at  Room temperature .

3m



164.11 Briefly spin the tube and place on the magnetic rack for 2–5 minutes or until the beads have collected to the wall of the tube and the solution is clear.

164.12 Carefully remove the supernatant with a pipette without disturbing the beads.

164.13 Repeat steps 119.9 to 119.12 one time.

164.14 Remove the tube from the rack and add  80 μL of 1X Bind/wash Buffer (kept  On ice) to resuspend the beads.



164.15 The MBD2-Fc-bound magnetic beads now are ready to use for host DNA depletion and stable for up to **7 days at**  4 °C .

165 Prepare riboPOOL Pan-Mammal Kit.

165.1 Upon arrival, store the reagents in the kit as instructed on the bottles.

165.2 When using the kit for the first time, centrifuge the riboPOOL probe tube (RP) at  11000 x g, 00:00:30 before opening. Add  30 μL kit provided nuclease-free water and vortex well to resuspend the riboPOOL probe. Aliquot the  5 μL RP in  1.5 mL tubes after resuspension. Store RP aliquots at  -20 °C . Avoid freeze-thaw cycles.

30s



166 Host rRNA depletion

166.1 **Before starting**, set up 2 programs on a thermocycler.

30m

1. 68 °C , 00:10:00 , then slowly cool down to 37 °C at a speed 3 °C /min (0.05°C/sec); when reached, keep the temperature at 37 °C .
2. 37 °C , 00:15:00 , then 50 °C , 00:05:00 .

166.2 Transfer TNA containing around 500 ng RNA to a 200 µL PCR tube, adjust volume with nuclease-free water to 13.7 µL . For example, if the RNA concentration of the TNA sample is 100 ng/µl , transfer 5 µL of the sample to a 200 µL PCR tube and add 8.7 µL nuclease-free water. When the RNA concentration is lower than 36.5 µL use 13.7 µL sample without volume adjustment.

166.3 **Volume (Probe Hybridization):** add reagents as the following table for one sample. For processing multiple samples, make a master mix with a 10% overage.

A	B
Component	Volume (µL)
TNA	13.7
RP	0.3
4X Hybridization Buffer (HB)	5
RNaseOUT (40U/ µL)	1
Total	20

166.4 Mix by pipetting, then spin down briefly.

166.5 Place the tube in the thermocycler and run the first program 68 °C cool down to 37 °C set at step 115.1.



166.6 Prepare streptavidin-coated magnetic beads (SMB): Vortex SMB beads at medium speed. Transfer  45 μL bead suspension per sample to a new 1.5mL tube. For multiple sample processing, calculate the amount of SMB needed by multiply 45 with the sample number.

166.7 Briefly spin down, place the tube on a magnetic rack and wait for  00:01:00 .

1m

166.8 Aspirate with a pipette and discard all supernatant.

166.9 Add  40 μL Depletion Buffer per sample (i.e.  240 μL for 6 samples,  480 μL for 12 samples) and tap the tube to resuspend beads.



166.10 Repeat steps 121.7 to 121.9. Beads are now resuspended in Depletion Buffer.

166.11 Move the tube in the thermocycler at step 115.5 to a PCR tube rack.

166.12 Add  40 μL beads from step 121.5 to the PCR tube and mix by pipetting.

166.13 Put the tube back into the thermocycler for incubation at  37 $^{\circ}\text{C}$ for  00:15:00 , then  55 $^{\circ}\text{C}$ for  00:05:00 .

20m



166.14 After incubation, briefly spin down droplets.

166.15 Transfer  60 μL sample-bead mix in the PCR tube to a new  1.5 mL tube.

166.16 Place the  1.5 mL tube on the magnet rack for  00:02:00 then carefully transfer the  60 μL supernatant to a new  1.5 mL tube. The supernatant now contains host rRNA-depleted TNA. Keep the supernatant  On ice for the next section of Host DNA depletion.

2m



167 Host DNA depletion.

- 167.1 Add  60 μL host rRNA depleted sample from the previous step (121.16) to a tube containing  80 μL MBD2-Fc-bound magnetic beads prepared at step 119.14.

Note

NOTE: make sure the MBD2-Fc-bound magnetic beads were prepared within 7 days.

- 167.2 Add  35 μL 5X Bind/wash buffer from the  1 mL aliquot prepared at step 118.1.

- 167.3 Mix the sample in the Hula mixer for  00:15:00 at  Room temperature .

15m



- 167.4 Briefly spin the tube and place it on the magnetic rack for  00:05:00 until the beads have collected to the wall of the tube and the solution is clear.

5m

- 167.5 Carefully transfer  175 μL supernatant with a pipette, without disturbing the beads to a clean  1.5 mL microcentrifuge tube. The sample at this stage is host rRNA and DNA depleted in buffer. Store this sample at  -20 $^{\circ}\text{C}$ or proceed directly to the sample cleanup described in steps 123 - 123.16

168 AMPure XP Bead Cleanup.

- 168.1 Vortex AMPure XP Beads to resuspend.

- 168.2 Add  175 μL resuspended AMPure XP beads to the sample at step 122.5.

- 168.3 Mix well by pipetting up and down at least 10 times.





Note

Be careful to expel all the liquid from the tip during the last mix.

- 168.4 Incubate samples for at least 00:05:00 at Room temperature . 5m
- 168.5 Quickly spin the sample, then place the tube on the magnet rack to separate the beads from the supernatant.
- 168.6 After 2-5 minutes (or when the solution is clear), using a pipette, remove and discard the supernatant without disturbing the beads .
- 168.7 Add 400 μL of freshly prepared 80% ethanol to the tube on a magnetic rack. Incubate at Room temperature for 00:00:30 , then with a pipette, carefully remove and discard the 80% ethanol without disturbing the beads. 30s
- 168.8 Repeat the previous step X1.
- 168.9 Briefly spin the tube, place it back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.
- 168.10 Air dry the beads for 00:02:00 while the tube is on the magnetic rack with the lid open. 2m
- 168.11 Add 30 μL of nuclease-free water to the beads.
- 168.12 Briefly spin the sample and mix well by pipetting up and down 10 times.
- 168.13 Incubate for 00:02:00 at Room temperature . 2m
- 168.14 Briefly spin the sample, then place the tube on the magnetic rack for 2-5 minutes (or when the solution is clear).



168.15 Transfer the supernatant to a new  1.5 mL tube.

168.16 Use  1 µL for DNA/ RNA concentration measurement. Refer to REDI-NET SOP B-2 for the method of DNA/RNA quantification and storage.

Protocol references

[REDI-NET Overview Summary](#)