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Version 2

REDI-NET B-4 BLOOD TESTING V.2

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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

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

Host rRNA depletion

- Manual: riboPOOL Kit

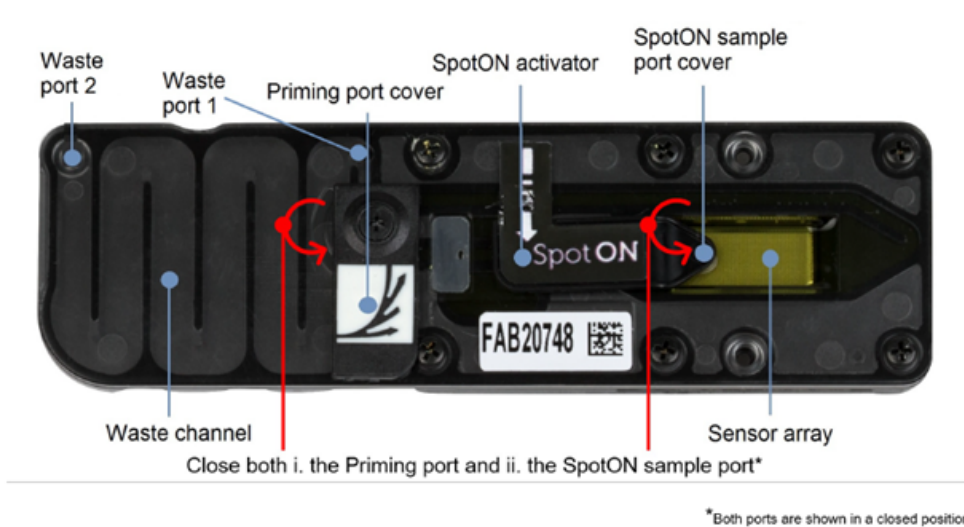
Host DNA depletion

- 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.

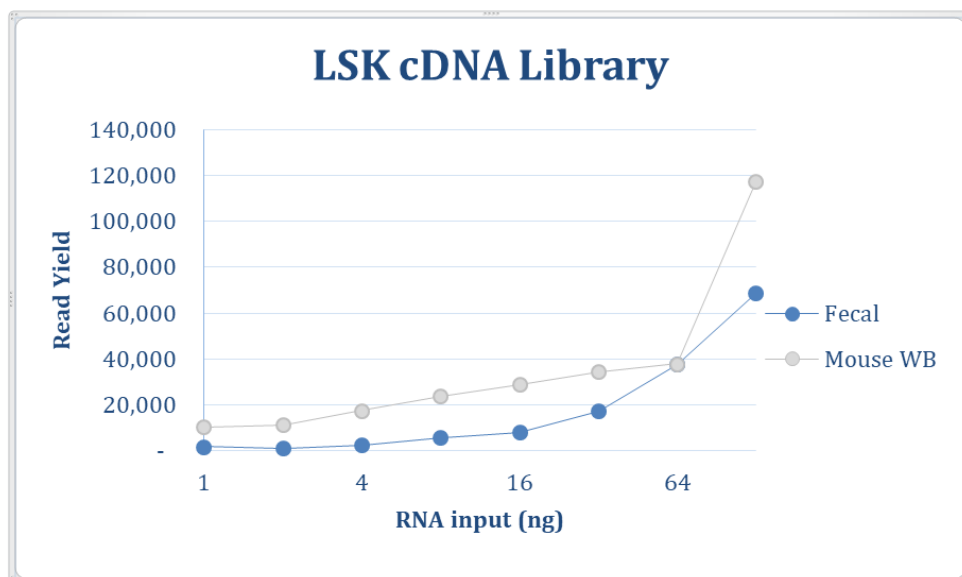
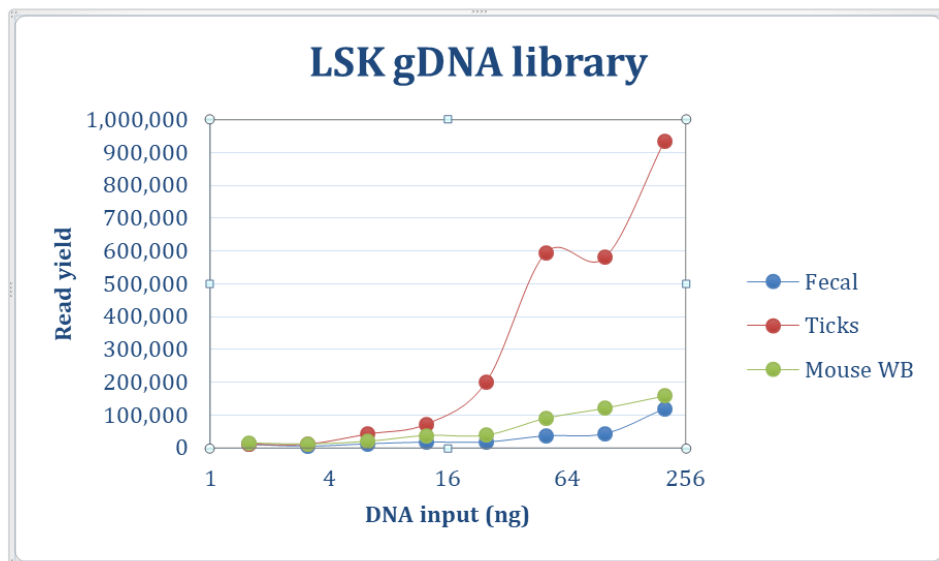


APPENDIX 4. cDNA END-PREP MASTER MIX PREPARATION

	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 #
200 ng DNA from a sample	Per sample from SOP B-2 (gDNA Preparation)	REDI-NET DNA sample
20 ul eluents from negative control extraction	From SOP B-2 (gDNA Preparation)	REDI-NET negative control
100 ng DNA from positive control extraction	From SOP B-2 (gDNA Preparation)	REDI-NET positive control
160 ng RNA from a sample	Per sample from SOP B-2 (TNA preparation)	REDI-NET RNA sample
40 ng RNA from positive control extraction	from SOP B-2 (TNA preparation)	REDI-NET negative control
8 µl total nucleic acid negative control extraction	From SOP B-2 (TNA preparation)	REDI-NET positive control
10 µl total nucleic acid	Per sample from SOP B-2 (TNA Preparation)	REDI-NET TNA sample
10 µl total nucleic acid from negative control extraction	From SOP B-2 (TNA Preparation)	REDI-NET negative control
10 µl total nucleic acid from positive control extraction	from SOP B-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



A	B	C
Random primer mix (Random hexamer and poly-T mixture)	(cDNA synthesis)	New England Biolabs, S1330
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 (<i>consumable</i>)	Oxford Nanopore, FLO-MIN114, FLO-PRO114M
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	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238 ^{LINK}	

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**

⊗ ezDNase™ Enzyme **Thermo Fisher Catalog #11766051**

⊗ NEBNext Ultra II RNA First Strand Synthesis Module - 96 rxns **New England Biolabs Catalog #E7771L**

⊗ NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**

⊗ Random primer mix **New England Biolabs Catalog #S1330S**



⊗ USB Dithiothreitol (DTT) 0.1M Solution **Thermo Fisher Scientific Catalog #707265ML**

⊗ 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**

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

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

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

⊗ 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. 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 > $10 \text{ ng}/\mu\text{L}$, calculate the required volume of 200 ng DNA, then transfer the volume to a new $200 \mu\text{L}$ PCR tube or a well of a 96-well PCR plate. Adjust the volume with nuclease-free water to a final volume of $20 \mu\text{L}$.
Directly use a $20 \mu\text{L}$ sample for the downstream preparation if the DNA concentration is below $10 \text{ ng}/\mu\text{L}$. 
- 2 Prepare 50 ng gDNA from positive control extraction in $20 \mu\text{L}$ nuclease-free water in a new $200 \mu\text{L}$ PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below $2.5 \text{ ng}/\mu\text{L}$, directly transfer $20 \mu\text{L}$ positive control eluent to a tube or a well. 
- 3 Transfer $20 \mu\text{L}$ 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 $10 \mu\text{L}$ of nuclease-free water in a new $200 \mu\text{L}$ PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below $20 \text{ ng}/\mu\text{L}$, directly transfer $10 \mu\text{L}$ of the sample to the tube or well. 
- 7 Prepare 50 ng of gDNA from a positive control extraction in $10 \mu\text{L}$ of nuclease-free water in a new $200 \mu\text{L}$ PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below $5 \text{ ng}/\mu\text{L}$, directly transfer $10 \mu\text{L}$ of the positive control eluent to the tube or well. 
- 8 Transfer $10 \mu\text{L}$ of the negative control extraction to a new tube or a well of a 96-well PCR plate. 



- 9 Prepare cDNA from RNA following the quantities outlined in the tables listed in the "Before Start" section, and the procedure outlined in the cDNA SYNTHESIS section (starting at step 17).
- 10 Add the corresponding  10 μL of purified double-stranded cDNA from the sample, positive control, and negative control to the  10 μL DNA as prepared in steps 6-8.  
- 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  160 ng of DNA in  8 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  20 ng/ μL , directly transfer  8 μL of the sample to the tube or well. 
- 13 Prepare  40 ng RNA from positive control extraction and adjust the volume to final  8 μL with 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  5 ng/ μL , directly transfer  8 μL of the positive control eluent to the tube or well. 
- 14 Transfer  8 μL negative control extraction to a new tube or a well of a 96-well PCR plate. 
- 15 Use prepared  8 μL samples, positive control, and negative control for cDNA SYNTHESIS (starting at step 17).

cDNA APPROACH

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

cDNA SYNTHESIS

- 17 Remove contaminated DNA (~ 15 mins):
Thaw total nucleic acid and 10x ezDNase Buffer on ice at room temperature. Vortex 10x ezDNase Buffer briefly, spin down by centrifugation for 5 seconds, and place on ice.  7m



ezDNase is not frozen and should be placed on ice before use. Set up thermal cycler programs: 37 °C , 00:02:00 and 55 °C , 00:05:00 .

- 18 Mix the following components in an RNase-free tube or plate. For processing multiple samples, make master mix for 10X ezDNase buffer and ezDNase with 10% overage. Aliquot the master mix into the wells of a 96-well plate, then add TNAs.



A	B
Component	Volume
10x ezDNase Buffer	1 µl
ezDNase	1 µl
RNA from step 12	8 µl
Total volume	10 µl

- 19 Gently mix the samples then centrifuge the tube (Include a reaction for extraction positive control and negative control of each batch nucleic acid extraction).

- 20 Incubate the sample for 00:02:00 at 37 °C .

2m



- 21 A 1 µL 100 millimolar (mM) DTT into the reaction tube.

- 22 Incubate the sample at 55 °C for 00:05:00 to inactivate the enzyme.

5m



- 23 Chill the tube on ice to bring the sample to room temperature, then spin down and place the tube on ice.



- 24 **BEFORE START:** Thaw 60 micromolar (µM) stock Random Primer Mix (NEB, S1330S) at Room temperature . DO NOT USE the Random Primer provided by the NEBNext First Strand Synthesis Module. Thaw Random Primer Mix solution, NEBNext First Strand Reaction Buffer, NEBNext Second Strand Reaction Buffer at

5s

🌡️ Room temperature then place 🌡️ On ice . Vortex the vials briefly, spin done by centrifugation for ⌚ 00:00:05 , and place 🌡️ On ice . First and Second Strand Enzyme Mix are not frozen, should be briefly centrifuged and placed 🌡️ On ice before use.

- 25 Add the following reagents into a sterile, RNase-free 🧪 0.2 mL PCR tube on ice. For processing multiple samples, make a master mix for the [M] 60 micromolar (μM) Random Primer Mix and nuclease free water with 10% overage. Aliquot the master mix into the wells of a 96-well plate, then add TNAs.

A	B
Component	Volume
ezDNase treated RNA	10 μl
60 μM Random Primer	1 μl
Nuclease free water	3 μl
Total volume	14 μl

- 26 Mix gently, spin down and incubate at 🌡️ 65 °C for ⌚ 00:05:00 . Chill 🌡️ On ice , spin down again and place 🌡️ On ice .

5m



- 27 Add the following components in the indicated order, if multiple reactions will be processed at the same time, make a master mix with a 10% overage:

A	B
Component	Volume
NEBNext First Strand Synthesis Reaction Buffer	4 μl
NEBNext First Strand Synthesis Enzyme Mix	2 μl
Total volume	20 μl

- 28 Mix gently and spin down.



29 Incubate the tube for 00:10:00 at 25 °C followed by 00:15:00 at 42 °C .

25m



30 Terminate the reaction by heating at 70 °C for 00:15:00 .

15m

31 Place the tube On ice or pre-chilled freezer block.



32 Pipette the following components directly into the first strand reaction tube (with 20 µL mixture) On ice in the indicated order, if multiple reactions will be processed at the same time, make a master mix with a 10% overage:

A	B
Component	Volume
5x NEBNext Second Strand Synthesis Reaction Buffer	5 µl
NEBNext Second Strand Synthesis Enzyme Mix	2.5 µl
Nuclease-free water	22.5 µl
Final total volume	50 µl

33 Mix gently and centrifuge briefly.



34 Incubate at 16 °C for 01:00:00 (heated lid set at ≤ 40 °C).

1h



35 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)

36



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.

- 37 Transfer the sample ( 50 μL) to a clean  1.5 mL low DNA binding tube.
- 38 Add  40 μL of resuspended AMPure XP beads to the reaction and mix by flicking the tube. 
- 39 Incubate on a Hula mixer (or a rotator mixer >1600 rpm) for  00:05:00 at  Room temperature . 5m
- 40 Spin down the sample and pellet on the magnet. Keep the tube on the magnet, and using a pipette, discard the supernatant.
- 41 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.
- 42 Repeat the previous step X1.
- 43 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
- 44 Remove the tube from the magnetic rack and resuspend the pellet in  13 μL nuclease-free water.
- 45 Incubate on a Hula mixer (or a rotator mixer >1600 rpm) for  00:10:00 at  Room temperature . 10m



- 46 Spin down and pellet beads on magnet until the eluate is clear and colorless.
- 47 Remove and retain  11 μL of eluate into a clean  1.5 mL low DNA binding tube.
- 48 **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. 
- 49 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

- 50 **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 . 

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)

- 51 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

- 52 Mix gently by pipetting and spin down.



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

10m



- 54 Resuspend the AMPure XP beads by vortexing.

- 55 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).





- 56 Incubate on a Hula mixer (a rotator mixer >1600 rpm) for  00:05:00 at  Room temperature . 
- 57 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. 
- 58 Keep the tube 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.
- 59 Repeat the previous step X1.
- 60 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. 

- 61 Remove the tube from the magnetic rack and resuspend the pellet in  11 μ L nuclease-free water. Incubate for  00:02:00 at  Room temperature . 
- 62 Pellet the beads on a magnet until the eluate is clear and colorless.
- 63 Remove and retain  10 μ L of eluate into a clean  1.5 mL low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Barcode ligation (~ 25 minutes)

- 64 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 from Native Barcoding Expansion 1-96)	2 µl
Blunt/TA Ligase Master Mix	12 µl
Final total volume	24 µl

65 Mix gently by flicking the tube and spin down.



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

20m

67 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

68 Resuspend the AMPure XP beads by vortexing.

69 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)



	A	B	C
	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

- 70 Incubate on a Hula mixer (or a rotator mixer) for 00:10:00 at Room temperature .
- 71 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.
- 72 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.
- 73 Repeat the previous step X1.
- 74 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.
- 75 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.
- 76 Spin down and pellet the beads on a magnet until the eluate is clear and colorless.
- 77 Remove and retain 30 μL of eluate into a clean 1.5 mL low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Adapter ligation (~ 45 minutes)

78 **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 of each tube are clear of any precipitate. Spin down the T4 Ligase and the Native Adapter (NA), and place  On ice .

79 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

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

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

20m

82 Resuspend the AMPure XP beads by vortexing.

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



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

10m

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



86 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.

87 Repeat the previous step X1.

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

89 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

90 Incubate on at  37 $^{\circ}\text{C}$ for  00:10:00 at, agitate the sample for 10s every 2 min.

10m



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

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

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

94 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

- 95 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

- 96 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.

- 96.1 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.
- 96.2 Turn on GridION (or MinION Mk1C) device. Make sure all the connections for the display, mouse, keyboard, and internet are ready.
- 96.3 Get a MinION or a PromethION flow cell from the refrigerator.
- 96.4 Double-click the MinKNOW icon shown on the desktop to initiate the program.
- 96.5 Use Oxford Nanopore Community username and password to login.



- 96.6 Select the device shown on the screen.
- 96.7 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.
- 96.8 The Sequencing Overview tab should show the **flow cell not checked** in each position in use.
- 96.9 Navigate to the Start tab and select **Flow Cell Check**.
- 96.10 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.
- 96.11 Click **Start** to begin the flow cell check.
- 96.12 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.
- 96.13 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

- 97 **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 .
- 98 Check the air bubble of priming pore.
- 99 Open the sequencer lid and insert the pore checked flow cell.

- 100 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.

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

- 101.1 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.

- 102 Prepare the flow cell priming mix and prime flow cells.

- 102.1 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



102.2 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

103 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.

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

103.2 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

104 Complete the flow cell priming.

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

104.2 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.

105 Loading samples.

- 105.1 Mix the prepared library gently by pipetting up and down just prior to loading. 
- 105.2 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. 
- 105.3 (MinION) Gently replace the SpotON/inlet port cover, making sure the bung enters the SpotON port, close the priming port.
- 105.4 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.
- 105.5 Close the lid of the sequencer.

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

106

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.

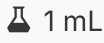
- 107 Use Oxford Nanopore Community username and password to login or continue as Guest.
- 108 Select the device shown on the screen.
- 109 Go to the Start tab, and click the Start Sequencing option to choose the running parameters.
 - 109.1 Type in the **Experiment Name**
 - 109.2 Type in **Sample ID** (same as experiment name)



- 109.3 Choose flow cell FLO-MIN114 for MinION and FLO-PRO114M for PromethION from the drop-down menu.
- 109.4 Use Select all available to select all the connected flow cells or use the diagram above to select specific flow cells to run.
- 110 Click **Continue to Kit Selection** to move to the next page.
- 110.1 Click the kit **SQK-NBD114-96** from the Kit Selection menu.
- 111 Click **Continue to Run Options** to choose run parameters.
- 111.1 Set run length to  48:00:00 and minimum read length 200 bp. Leave adaptive sampling unchecked.
- 112 Click **Continue to Analysis** to choose basecalling and Barcoding parameters.
- 112.1 In the Basecalling options, checkup the basecalling with configuration: **High accuracy basecalling**.
- 112.2 In the Barcoding options, turn on the **Trim barcodes** and **Mid-read barcoding** filtering.
- 112.3 Do **not** turn on the Alignment option.
- 113 Click **Continue to output** to the next page.
- 113.1 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.
- 114 Click **Continue to final review** to proceed.

2d



- 115 Review the settings listed in the Run Setup page. Correct any errors. Select **Start** to run the experiment.
- 116 The system will automatically navigate the Sequencing Overview when sequencing starts.
- 117 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.

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

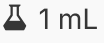
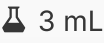
1h 29m 5s

118

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.

- 118.1 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.

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



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

119.2 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.

119.3 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.

119.4 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.

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

3m



119.6 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

119.7 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.

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



119.9 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.



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

3m



119.11 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.

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

119.13 Repeat steps 119.9 to 119.12 one time.

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



119.15 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 .

120 Prepare riboPOOL Pan-Mammal Kit.

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

120.2 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



121 Host rRNA depletion

30m

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

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 .

121.2 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 ng/µl use 13.7 µL sample without volume adjustment.

121.3 **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

121.4 Mix by pipetting, then spin down briefly.

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

121.6 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.



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

1m

121.8 Aspirate with a pipette and discard all supernatant.

121.9 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.



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

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

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

121.13 Put the tube back into the thermocycler for incubation at  37 °C for  00:15:00 , then  55 °C for  00:05:00 .

20m



121.14 After incubation, briefly spin down droplets.

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

121.16 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

122 Host DNA depletion.



122.1 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.

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



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

15m



122.4 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

122.5 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



123 AMPure XP Bead Cleanup.

123.1 Vortex AMPure XP Beads to resuspend.

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



123.3 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.

- 123.4 Incubate samples for at least 00:05:00 at Room temperature . 5m
- 123.5 Quickly spin the sample, then place the tube on the magnet rack to separate the beads from the supernatant.
- 123.6 After 2-5 minutes (or when the solution is clear), using a pipette, remove and discard the supernatant without disturbing the beads .
- 123.7 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
- 123.8 Repeat the previous step X1.
- 123.9 Briefly spin the tube, place it back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.
- 123.10 Air dry the beads for 00:02:00 while the tube is on the magnetic rack with the lid open. 2m
- 123.11 Add 30 μL of nuclease-free water to the beads.
- 123.12 Briefly spin the sample and mix well by pipetting up and down 10 times.
- 123.13 Incubate for 00:02:00 at Room temperature . 2m
- 123.14 Briefly spin the sample, then place the tube on the magnetic rack for 2-5 minutes (or when the solution is clear).



123.15 Transfer the supernatant to a new  1.5 mL tube.

123.16 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](#)