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REDI-NET FF-4 FILTH FLY TESTING V.1

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Remote Emerging Disea...

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We use this protocol and it's working

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Disclaimer

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Abstract

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

Guidelines

OBJECTIVE

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

SUMMARY/SCOPE

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

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

Note

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

MAINTENANCE OF EQUIPMENT

CAUTION ON RNA HANDLING:

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

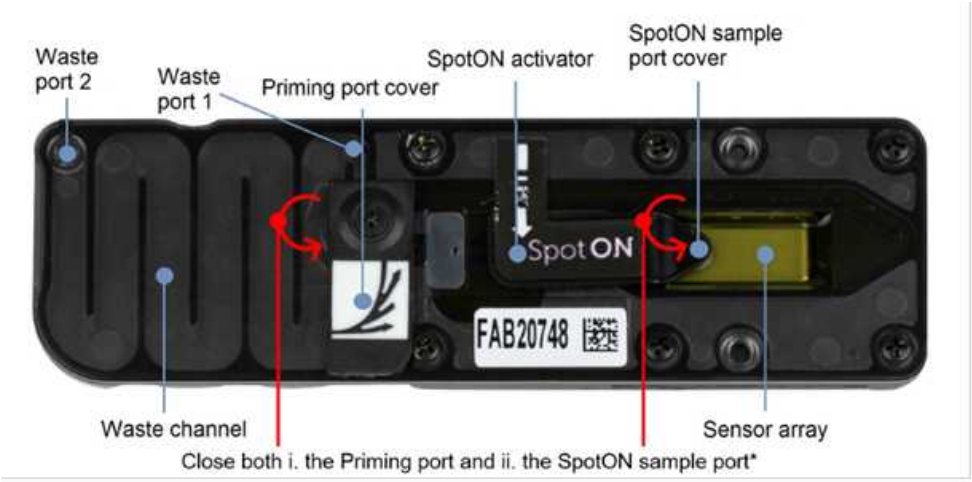
HANDLING ENZYMATIC REACTIONS

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

APPENDICES

APPENDIX 1. FLOW CELL

MinION flow cell:



PromethION flow cell:

APPENDIX 2. MinKNOW SOFTWARE INSTRUCTIONS

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

Select device shown on the screen.

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

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

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

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

Turn on Basecalling and Barcoding.

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

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

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

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

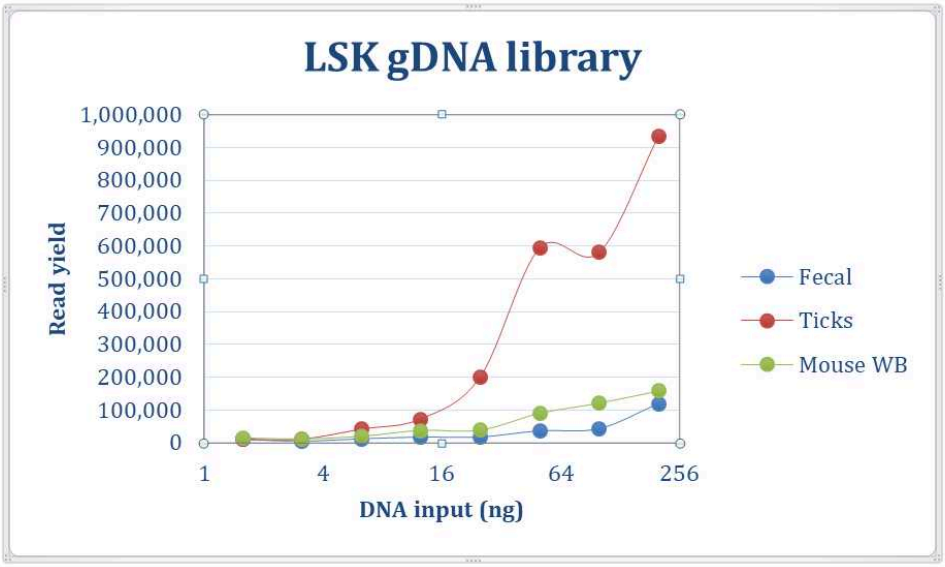
The system will automatically navigate the Sequencing Overview when sequencing starts.

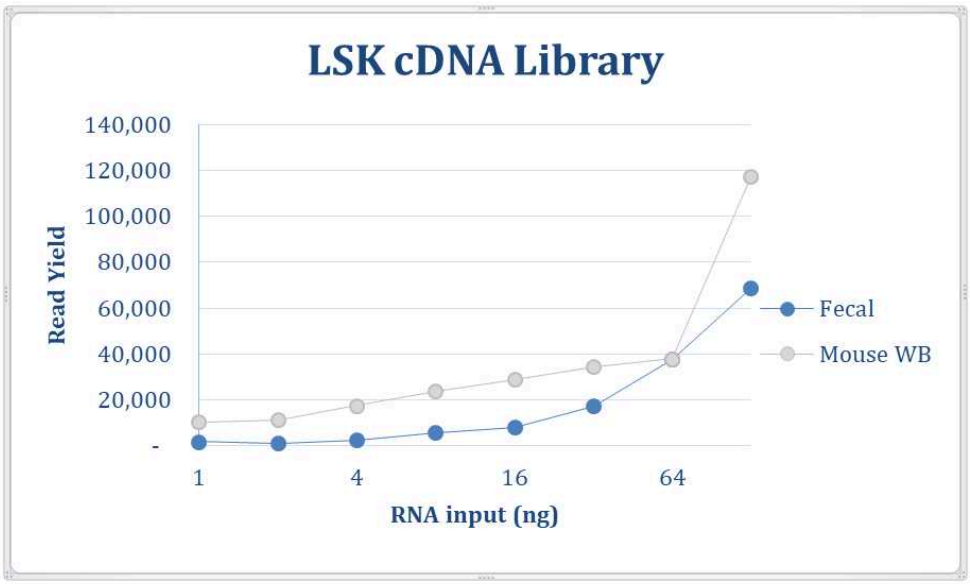
APPENDIX 3. cDNA END-PREP MASTER MIX PREPARATION

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

APPENDIX 4. EXPECTED OUTCOMES

The DNA or RNA inputs vs the sequencing read yields.




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

Materials

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
	P300 8-channel pipette	Locally sourced

	A	B	C
	Material	Description	Mfg / Product #
	200 ng DNA from a sample	Per sample from SOP S-2 (gDNA Preparation)	REDI-NET DNA sample
	20 ul eluents from negative control extraction	From SOP S-2 (gDNA Preparation)	REDI-NET negative control
	100 ng DNA from positive control extraction	From SOP S-2 (gDNA Preparation)	REDI-NET positive control
	160 ng RNA from a sample	Per sample from SOP S-2 (TNA preparation)	REDI-NET RNA sample
	40 ng RNA from positive control extraction	from SOP S-2 (TNA preparation)	REDI-NET negative control
	8 µl total nucleic acid negative control extraction	From SOP S-2 (TNA preparation)	REDI-NET positive control
	10 µl total nucleic acid	Per sample from SOP S-2 (TNA Preparation)	REDI-NET TNA sample
	10 µl total nucleic acid from negative control extraction	From SOP S-2 (TNA Preparation)	REDI-NET negative control
	10 µl total nucleic acid from positive control extraction	from SOP S-2 (TNA Preparation)	REDI-NET positive control
	Native Barcoding Kit 96 V14	(Sequencing Library Preparation)	Oxford Nanopore, SQK-NBD114.96
	ezDNase	(cDNA synthesis)	ThermoFisher, Invitrogen 11766051
	NEBNext Ultra II RNA First Strand Synthesis Module	(cDNA synthesis)	New England Biolabs, E7771L
	NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module	(cDNA synthesis)	New England Biolabs, E6111L
	Random primer mix (Random hexamer and poly-T mixture)	(cDNA synthesis)	New England Biolabs, S1330
	USB Dithiothreitol (DTT), 0.1M Solution	(cDNA synthesis)	ThermoFisher,707265ML
	Agencourt AMPure XP beads	(Sequencing Library Preparation)	Beckman Coulter, A63881

	A	B	C
	NEBNext End repair / dA-tailing Module	(Sequencing Library Preparation)	New England Biolabs, E7546L
	NEBNext FFPE Repair Mix	(Sequencing Library Preparation)	New England Biolabs, M6630L
	NEB Blunt/TA Ligase Master Mix	(Sequencing Library Preparation)	New England Biolabs, M0367L
	NEBNext Quick Ligation Module	(Sequencing Library Preparation)	New England Biolabs, E6056L
	R10.4.1 flow cells	Flow cells for sequencing experiment (consumable)	Oxford Nanopore, FLO-MIN114, FLO-PRO114M
	Ultra pure Bovine Serum Albumine (50mg/ml)	(Sequencing Library Preparation)	ThermoFisher, AM2616
	low DNA binding tubes	1.5 mL (consumable)	Eppendorf, 022131021 or equivalent
	low DNA binding tubes	2.0 mL (consumable)	Eppendorf, 022431048 or equivalent
	PCR tubes	0.2 mL thin-walled (consumable)	Eppendorf, 951010006 or equivalent
	PCR plate	96 well, low DNA binding, semi-skirted with heat seals (consumable)	Eppendorf, 0030129504 or equivalent
	riboPool pan-mammal Kit	For Host rRNA depletion (for TNA form whole blood and buffy coat samples only)	SiTools Biotech, 24 reactions
	NEBNext Microbiome DNA enrichment Kit	For Host DNA depletion (for TNA form whole blood and buffy coat samples only)	New England Biolabs, E2612
	RNaseOUT Recombinant Ribonuclease Inhibitor	For Host DNA/rRNA depletion (for TNA form whole blood and buffy coat samples only)	ThermoFisher, 10777019
	BRAND Self-adhesive Plate Sealing Film	Aluminum (consumable)	Fisher Scientific, 13-882-329
	Clear Adhesive Film	For PCR plate sealing	ThermoFisher, 4306311
	Qubit Assay Tubes	For Qubit DNA/RNA measurement (consumable)	Thermo Fisher, Q32856
	Qubit 1X dsDNA HS Assay Kit	(consumable)	ThermoFisher, Q33230
	Qubit RNA HS Assay Kit	(consumable)	ThermoFisher, Q32852
	Nuclease-free water	To prepare ethanol dilutions (consumable)	Locally sourced

Safety warnings

RISKS AND PERSONAL PROTECTION

Gloves should be worn all the time when handling samples.

Before start

BEFORE START

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

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

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

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

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

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

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

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

gDNA APPROACH

1 If the DNA concentration > 10 ng/µl, calculate the required volume of 200 ng DNA, then transfer the volume to a new 200 µL PCR tube or a well of a 96-well PCR plate. Adjust the volume with nuclease-free water to a final volume of 20 µL.
Directly use a 20 µL sample for the downstream preparation if the DNA concentration is below 10 ng/µL.

2 Prepare 50 ng gDNA from positive control extraction in 20 µL nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below 2.5 ng/µl, directly transfer 20 µL positive control eluent to a tube or a well.

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

4 All samples are subjected to section SEQUENCING LIBRARY PREPARATION.

TNA APPROACH

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

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

7 Prepare 50 ng of gDNA from a positive control extraction in 10 µL of nuclease-free water in a new 200 µL PCR tube or a well of a 96-well PCR plate. If the DNA concentration is below 5 ng/µl, directly transfer 10 µL of the positive control eluent to the tube or well.

8 Transfer 10 µL 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 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

5m

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 done 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 Add 1 µL [M] 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 [M] 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) 5m

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. Pipette off 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 (rotator mixer) 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

10m

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 .

10m

Note

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

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

10m

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 . 5m

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 µL 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. 30s

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

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

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

SEQUENCING LIBRARY PREPARATION: Barcode ligation (~ 25 minutes) 20m

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 form 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 10m

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)**
	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 . 10m

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. 5m

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. 30s

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. 10m

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 1.5ml low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Adapter ligation (~ 45 minutes) 20m

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 or 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, mixing by flicking the tube between each sequential addition.

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

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 (R10.4.1)**
	EB (µL)	13	33

90 Incubate at 37 °C for 00:10:00 , 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

5m

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

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 1*** *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 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 poach 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 2d

2d

106

Note

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

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

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. 2d

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.

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.

Protocol references

REFERENCES

REDI-NET Overview Summary

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

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

Oxford Nanopore Manufacturer's protocols:

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