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F-4 FECES TESTING V.1

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REDI-NET Consortium[1]
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Remote Emerging Disea...

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We use this protocol and it's working

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Disclaimer

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Abstract

This protocol details standard operating procedure for feces testing.

Guidelines

OBJECTIVE

To outline the procedures for properly using the Oxford Nanopore Sequencing platforms (GridION or MinION Mk1C) to sequence gDNA and TNA extracted from collected feces 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

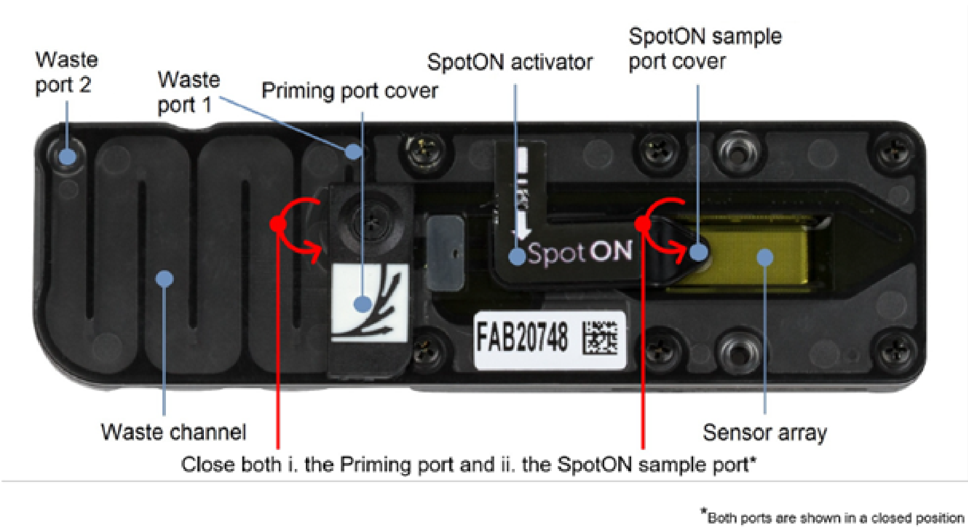
- ezdnase_PI

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

APPENDICES

APPENDIX 1. FLOW CELL

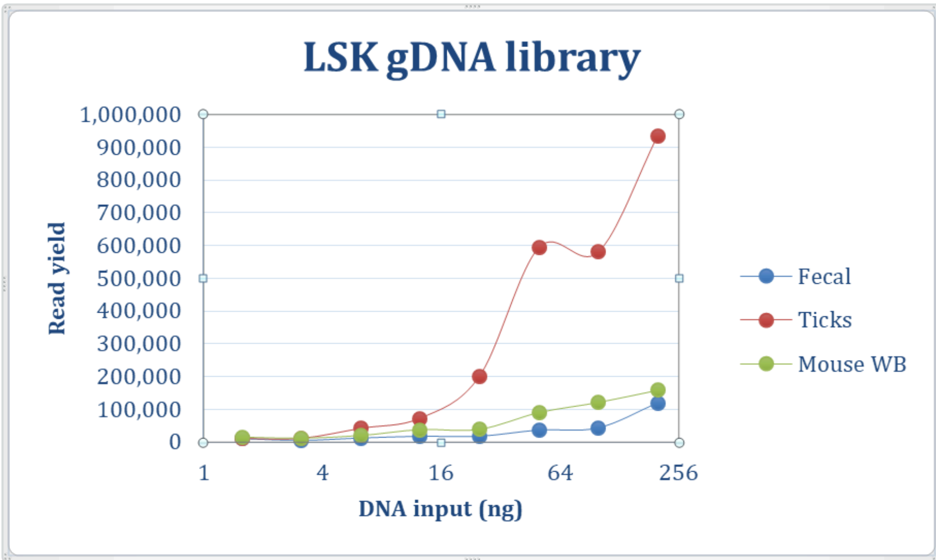


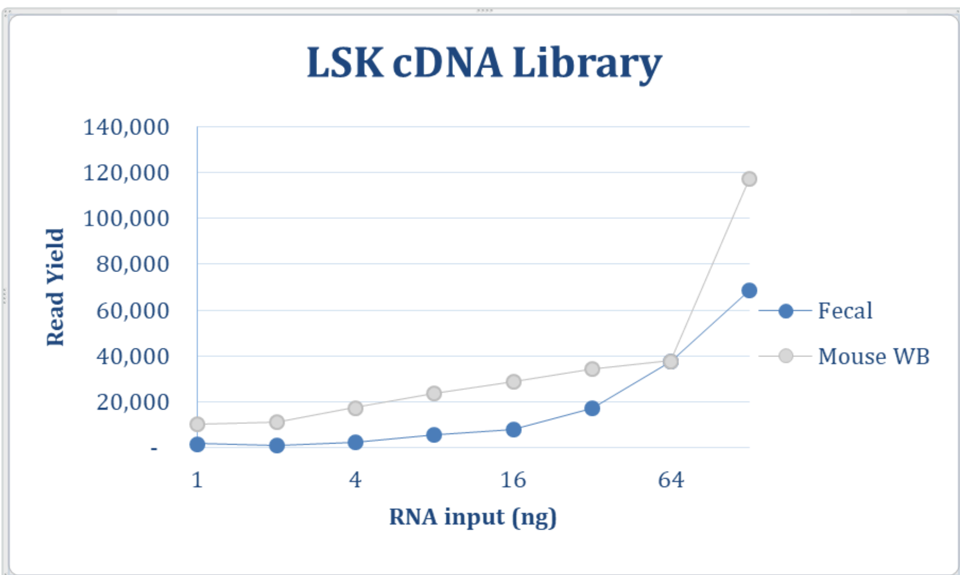
APPENDIX 2. cDNA END-PREP MASTER MIX PREPARATION

	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 3. 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	Oxford Nanopore Technologies, GRD-CapEx or Oxford Nanopore Technologies, M1CCapEx
	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
	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 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
	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

	A	B	C
	Agencourt AMPure XP beads	(Sequencing Library Preparation)	Beckman Coulter, A63881
	NEBNext End repair / dA-tailing Module	(Sequencing Library Preparation)	New England Biolabs, E7546L
	NEBNext FFPE Repair Mix	(Sequencing Library Preparation)	New England Biolabs, M6630L
	NEB Blunt/TA Ligase Master Mix	(Sequencing Library Preparation)	New England Biolabs, M0367L
	NEBNext Quick Ligation Module	(Sequencing Library Preparation)	New England Biolabs, E6056L
	R10.4.1 flow cells	Flow cells for sequencing experiment (*consumable*)	Oxford Nanopore, FLO-MIN114
	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
	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
	Freshly prepared 80% ethanol in nuclease-free water	Prepared from 100% molecular biology grade ethanol (*consumable*)	Locally sourced
	Freshly prepared 70% ethanol in nuclease free water	Prepared from 100% molecular biology grade ethanol (*consumable*)	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

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 the

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

Troubleshooting

Safety warnings

RISKS AND PERSONAL PROTECTION

Gloves should be worn all the time when handling samples.

Before start

BEFORE START

1. Check the DNA and RNA concentrations in each sample of total nucleic acid (TNA) extraction.
2. If the concentrations are detectable, choose the sequencing approach following the table below.
3. If DNA or RNA concentration is not detectable, prepare the library for the detectable one.
4. Use sections **gDNA PREPARATION** and **TNA PREPARATION** for gDNA and TNA preparation, respectively, then subject the prepared gDNA and TNA to Section SEQUENCING LIBRARY PREPARATION.

A	B	C	D	E
		DNA concentration (ng/ul)		
		< 1 ng/ul	**1-10 ng/ul**	**> 10 ng/ul**
RNA concentration (ng/ul)	**< 4 ng/ul**	TNA	DNA	DNA
	4-20 ng/ul	TNA	TNA	TNA
	>20 ng/ul	TNA	TNA	TNA

gDNA PREPARATION

1 When the RNA concentration of the sample is lower than the detectable range of the Qubit High Sensitivity Assay (< 0.01 ng/µl), the sample is subjected to gDNA sequencing. The cDNA synthesis can be skipped.

2 When the DNA concentration > 10 ng/ul, calculate the required volume of 200 ng DNA, then transfer the volume to a new 200µl 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.

3 Prepare 100 ng gDNA from positive control extraction in 20 µL nuclease-free water in a new 200µl PCR tube or a well of a 96-well PCR plate.

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

5 All samples are subjected to section SEQUENCING LIBRARY PREPARATION.

TNA PREPARATION

6 To prepare TNA for sequencing both cDNA and gDNA, cDNA needs to be prepared separately and then mixed with TNA from the original sample.

7 Prepare cDNA following section cDNA SYNTHESIS (positive control and negative control included) until step 40.

8 Transfer 10 µL double-stranded cDNA of section cDNA SYNTHESIS step 40 to a new 200 µl PCR tube or a well of a 96-well PCR plate. Add 10 µL of TNA from the original sample to make the final volume 20 µL.

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

Note

***NOTE**: Twenty-four samples must be pooled in one sequencing run to make the most out of a sequencing flow cell. For collecting 24 samples, the samples from gDNA and TNA preparations can be placed in the same 96-well plate for* End-prep *and* Barcode Ligation *before pooling, then pooled for the subsequent steps of library preparation.*

cDNA SYNTHESIS: DNase treatment

10 If RNA concentration > 20 ng/µl, calculate the required volume of 160 ng RNA,then transfer the volume to a new 200µl PCR tube or a well of a 96-well PCR plate. Adjust the volume with nuclease-free water to a final volume of 8 µL. If the concentration of the RNA < 20 ng/µl, directly transfer 8 µL of RNA to a new 200µl PCR tube or a well of a 96-well PCR plate. Keep the tube or plate On ice.

11 Prepare 40 ng RNA from positive control extraction and adjust the volume to final 8 µL with nuclease-free water in a new 200µl PCR tube or a well of a 96-well PCR plate.

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

13 **Remove contaminated DNA (~ 15 mins)**: Thaw total nucleic acid, 10x ezDNase Buffer, and DTT on the ice at Room temperature. Vortex 10x ezDNase Buffer and DTT briefly, spin down by centrifugation for 00:00:05, and place On ice. 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. 7m 5s

13.1 Mix the following components in an RNase-free tube or plate. For processing multiple samples, make a master mix for 10× 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**
	10× ezDNase Buffer	1 µl
	ezDNase	1 µl
	RNA from step 6	8 µl
	Total volume	10 µl

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

13.3 Incubate the sample for 00:02:00 at 37 °C . 2m

13.4 Add 1 µL of [M] 100 millimolar (mM) DTT into the reaction tube.

13.5 Incubate the sample at 55 °C for 00:05:00 to inactivate the enzyme. 5m

13.6 Chill the tube On ice to bring the sample to Room temperature , then spin down and place the tube On ice .

cDNA SYNTHESIS: First strand cDNA Synthesis (~ 1hr)

14 **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 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 5s

Enzyme Mix are not frozen, should be briefly centrifuged and placed On ice before use.

15 Add the following reagents into the ezDNase-treated RNA from step 13.6. For processing multiple samples, make a master mix for the [M] 60 micromolar (µM) Random Primer Mix and nuclease-free water with 10% overage.

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

16 Mix gently, spin down and incubate at 65 °C for 00:05:00 . Chill On ice , spin down again and place On ice . 5m

17 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

18 Mix gently and spin down.

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

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

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

22 Continue immediately with the second strand synthesis reaction as described below.

cDNA SYNTHESIS: Second strand cDNA Synthesis (~ 1hr)

23 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

24 Mix gently and centrifuge briefly.

25 Incubate at 16 °C for 01:00:00 (heated lid set at ≤ 40 °C). 1h

26 Proceed with cDNA purification or store the reaction mixture at -20 °C before the subsequent cDNA purification (the double-stranded cDNA is ready to be shipped to Gold Labs if necessary).

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

27

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.

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

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

30 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at Room temperature . 5m

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

32 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.

33 Repeat the previous step X1.

34 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

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

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

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

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

39 **Optional**: Analyze 1 µL of the purified double-stranded cDNA for quantity using Qubit fluorometer and Qubit 1X dsDNA HS Assay Kit.

40 Subject 10 µL purified double-stranded cDNA for section SEQUENCING LIBRARY PREPARATION.

Note

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

SEQUENCING LIBRARY PREPARATION

41 **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

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

42 Mix the following reagents in a 0.2ml PCR tube. To process 24 samples, prepare a master mix by multiplying gradients except for cDNA by 24 with a 10% overage. Aliquot the master mix into a 96-well plate, then add cDNA or TNA (see Appendix 2 for master mix preparation):

	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

43 Mix gently by pipetting and spin down.

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

10m

45 Resuspend the AMPure XP beads by vortexing.

46 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).

47 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at Room temperature .

5m

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

49 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.

50 Repeat the previous step X1.

51 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

52 Remove the tube from the magnetic rack and resuspend the pellet in 12 µL nuclease-free water. Incubate for 00:02:00 at Room temperature . 2m

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

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

SEQUENCING LIBRARY PREPARATION: Barcode ligation (~ 25 minutes)

55 Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:

Note

NOTE: *When working on 24 End-prepped gDNA/TNA, set up the reactions in a low DNA binding 96-well plate. The Native barcodes can be transferred by an 8-channel pipette directly punching through the sealing foil with tips of the barcode plate. Please reseal the used wells with trimmed adhesive foil. Each well provides sufficient volume for two barcoding ligations.*

	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

56 Mix gently by flicking the tube and spin down.

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

58 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

59 Pool every 24 barcoded samples 12 µL from each sample, (total 288 µL /pool) in a new 1.5ml low DNA binding tube.

60 Resuspend the AMPure XP beads by vortexing.

61 Add 518 µL (1.8x volume of the pooled library) of resuspended AMPure XP beads to the pooled library and mix by pipetting.

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

63 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

64 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.

65 Repeat the previous step X1.

66 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

67 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water. Incubate for 00:10:00 at 37 °C temperature. 10m

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

69 Remove and retain 35 µL of eluate into a clean 1.5ml low DNA binding tube.

SEQUENCING LIBRARY PREPARATION: Adapter ligation (~ 45 minutes)

70 **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 .

71 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

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

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

74 Resuspend the AMPure XP beads by vortexing.

75 Add 90 µL of resuspended AMPure XP beads to the reaction and mix by pipetting.

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

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

78 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.

79 Repeat the previous step X1.

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

81 Remove the tube from the magnetic rack and resuspend the pellet in 13 µL of Elution Buffer (EB).

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

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

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

85 Quantify 1 µL of eluted sample using a Qubit fluorometer and Qubit 1X dsDNA HS Assay Kit (recovery aim ~ 430 ng in total).

86 Make up the library to 12 µL at 10-20 fmol.

87 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

88 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.*

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

88.2 Depending on the number of pooled samples, get one to four new flow cells from the fridge and check the expiration date.

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

88.4 Use Oxford Nanopore Community username and password to login.

88.5 Select the device shown on the screen.

88.6 Open the lid of GridION (or MinION Mk1C) and insert the flow cells under the clips, press down the flow cell to ensure good thermal and electrical contact.

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

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

88.9 Select the flow cells to assign the flow cell type FLO-MIN114 from the dropdown menu.

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

88.11 Record the port number and date of checking on the original package of the flow cell. The flow cell with less than 800 pores should not be used for the sequencing. If the flow cell is not expired, contact Oxford Nanopore Company for customer service.

88.12 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

89

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 .

90 Check the air bubble of priming pore.

91 Slide open the GridION lid (or MinION Mk1C) and insert flow cell with minimum 800 pores.

92 Slide the priming port cover clockwise to open the priming port.

Note

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

93 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume (20-30 µl) to remove any bubbles:

93.1 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.

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

94.1 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
Component	**Volume**
Bovine Serum Albumin (BSA) (50 mg/ml)	5 µl
Flow Cell Tether (FCT)	30 µl
Flow Cell Flush (FCF)	1170 µl
Final total volume	1205 µl

94.2 Load 800 µL of the priming mix into each flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 00:05:00 . 5m

95 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.

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

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

	A	B
	Component	**Volume**
	Sequencing Buffer (SB)	37.5 µl
	Library Beads (LIB)	25.5 µl
	DNA library	12 µl
	Final total volume	75 µl

96 Complete the flow cell priming.

96.1 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

96.2 Load 200 µL of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

97 Loading samples.

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

97.2 Add 75 µL of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next drop.

97.3 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the GridION lid.

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

2d

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

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

100 Select the device shown on the screen.

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

101.1 Type in the **Experiment Name** using the scheme, **[YYYY_MM_DD_Approach(gDNA or TNA)_Sample type (soil, water,… etc.)]**

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

101.3 Choose flow cell FLO-MIN114 from the drop-down menu

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

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

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

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

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

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

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

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

105.3 Do not turn on the Alignment option.

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

106.1 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.

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

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

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

110 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). Remove the flow cells on the device, put it back in the original package, and turn off the device.

Protocol references

REDI-NET Overview Summary