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Protocol status: Working
We use this protocol and it's working

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B-4 BLOOD TESTING

REDI-NET Consortium[1]

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DISCLAIMER

This work is supported by the US Army Medical Research and Development Command under Contract No.W81XWH-21-C-0001, W81XWH-22-C-0093 and HT9425-23-C-0059. The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army or Navy position, policy or decision unless so designated by other documentation.

ABSTRACT

This protocol details standard operating procedure for blood testing.

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PROTOCOL integer ID: 81626

Keywords: SEQUENCING LIBRARY PREPARATION, ONT Native barcoding kit v14, cDNA sequencing, TNA sequencing, Total nucleic acid, gDNA sequencing, Blood samples, Whole genome shotgun sequencing

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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 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

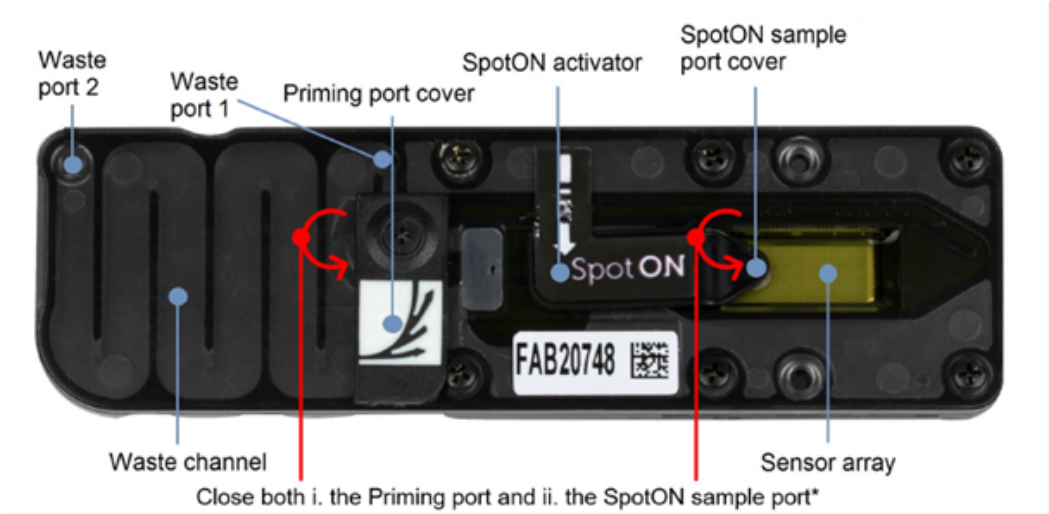
- Manual: riboPOOL Kit

Host DNA depletion

- Instruction manual: NEBNext® Microbiome DNA Enrichment Kit

APPENDICES

APPENDIX 2. FLOW CELL



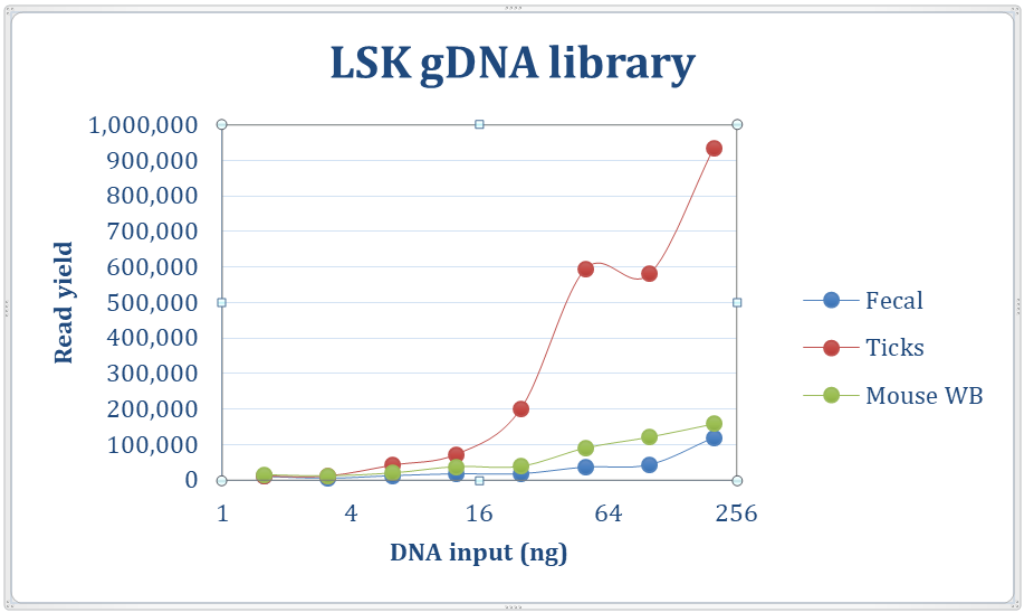
APPENDIX 3. cDNA END-PREP MASTER MIX PREPARATION

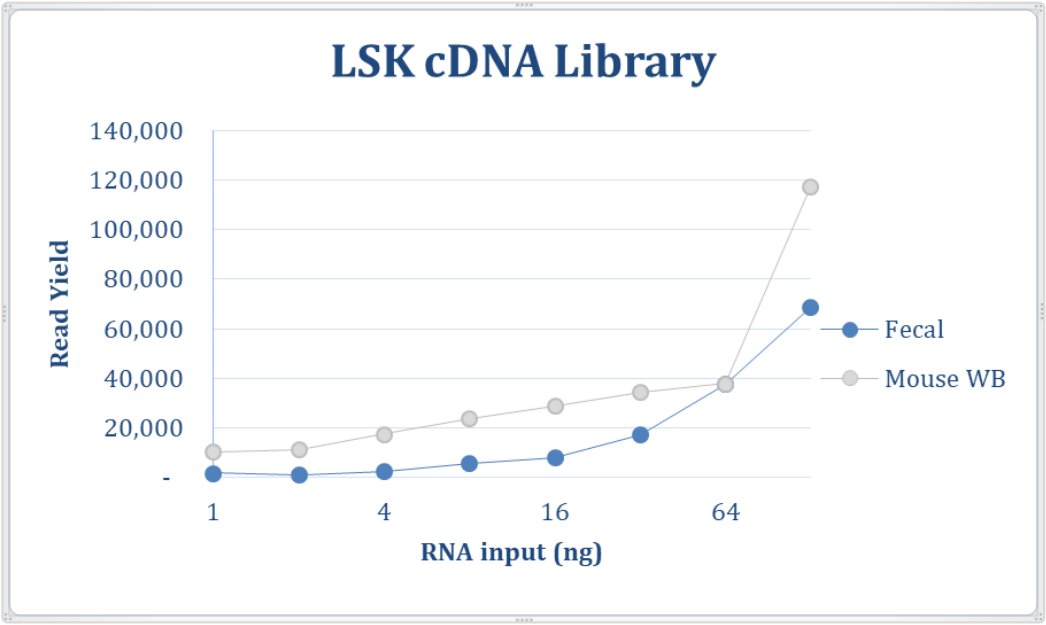
	A	B	C
	Component	Volume for 1 reaction	Volume for n+1 reactions

	A	B	C
	cDNA sample	20 µl	20 µl
	Nuclease-free water	30 µl	… µl
	Ultra II End-prep reaction buffer	7 µl	… µl
	Ultra II End-prep enzyme mix	3 µl	… µl
	Final total volume	60 µl	… µl

APPENDIX 4. EXPECTED OUTCOMES

The DNA or RNA inputs vs the sequencing read yields.




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

MATERIALS

EQUIPMENT AND MATERIALS

Note

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

	A	B
	Equipment	**Mfg / Product #**
	Oxford Nanopore GridION or MinION Mk1C device	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	REDI-NET DNA sample

	A	B	C
		Preparation)	
	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
	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

	A	B	C
	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
	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
	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**

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

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

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

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

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

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

SAFETY WARNINGS

RISKS AND PERSONAL PROTECTION

Gloves should be worn all the time when handling samples.

BEFORE START INSTRUCTIONS

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/µl, 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 from section cDNA SYNTHESIS step 40 to a new 200 µl PCR tube or a well of 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* **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 ic 7m 5s 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.

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 100mM 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 60 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 Enzyme Mix are not frozen, should be briefly centrifuged and placed On ice before use. 5s

15 Add the following reagents into the ezDNase-treated RNA from step 13.6. For processing multiple samples, make a master mix for the 60 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
	Final 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. Using a pipette, remove any residual ethanol. Allow to d 30s for ~ 00:00:30 , but do not dry the pellet to the point of cracking.

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. 10m 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.

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 4 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 d 30s for ~ 00:00:30, but do not dry the pellet to the point of cracking.

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

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 seq…

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 5m pipette, discard the supernatant.

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 d 30s for ~ 00:00:30, but do not dry the pellet to the point of cracking.

67 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water. 10m

Incubate for 00:10:00 at 37 °C temperature.

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, 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

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 10m min.

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.

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 2*** *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

	A	B
	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.

97.4 Apply the light shield to cover the pore window.

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

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

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

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

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

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

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

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

104.3 Do not turn on the Alignment option.

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

105.1 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.

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

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

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

109 48 hrs later, check the sequencing data. Use 1 mL pipette to remove 1 mL waste solution in the waste channel via waste port 1 (see Appendix 2 under Guidelines & Warning 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 BLOO...

1h 29m 5s

110

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.

110.1 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 1.5mL tubes, 1 mL for each tube, then aliquot the rest of the 42 mL buffer into fourteen 15mL tubes, 3 mL for each tube.

Note

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

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

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

111.2 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.

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

Note

Do not vortex.

111.4 In one 1.5mL tube, add 8 µL of MBD2-Fc protein and 80 µL of Protein A Magnetic Beads. Mix by pipetting up and down (avoid bobbling) 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.

111.5 Mix the beads in a Hula mixer for 00:03:00 at Room temperature. 3m

111.6 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 µL mix to 1.5mL tubes. 5s

111.7 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.

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

111.9 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.

111.10 Mix the beads in a Hula mixer for 00:03:00 at Room temperature. 3m

111.11 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.

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

111.13 Repeat steps 113.9 to 113.12 one time.

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

111.15 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.

112 Prepare riboPOOL Pan-Mammal Kit.

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

112.2  When using the kit for the first time, centrifuge the riboPOOL probe tube (RP) at 30s 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 1.5ml tubes after resuspension. Store RP aliquots at -20 °C. Avoid freeze-thaw cycles.

113 Host rRNA depletion

113.1 **Before starting**, set up 2 programs on a thermocycler. 30m

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

113.2 Transfer TNA containing around 500 ng RNA to a 200µL 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/ul, transfer 5 µL of the sample to a 200µL 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.

113.3 **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	
	TNA	13.7
	RP	0.3
	4X Hybridization Buffer (HB)	5
	RNaseOUT (40U/ µL)	1
	Total	20

113.4 Mix by pipetting, then spin down briefly.

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

113.6 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.

113.7 Briefly spin down, place the tube on a magnetic rack and wait for 00:01:00. 1m

113.8 Aspirate with a pipette and discard all supernatant.

113.9 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.

113.10 Repeat steps 115.7 to 115.9. Beads are now resuspended in Depletion Buffer.

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

113.12 Add 40 µL beads from step 115.10 to the PCR tube and mix by pipetting.

113.13 Put the tube back into the thermocycler for incubation at 37 °C for 00:15:00, then 55 °C for 00:05:00. 20m

113.14 After incubation, briefly spin down droplets.

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

113.16 Place the 1.5mL tube on the magnet rack for 00:02:00 then carefully transfer the 60 µL supernatant to a new 1.5mL tube. The supernatant now contains host rRNA-depleted TNA. Keep the supernatant On ice for the next section of Host DNA depletion. 2m

114 Host DNA depletion.

114.1 Add 60 µL host rRNA depleted sample from the previous step (115.16) to a tube containing 80 µL MBD2-Fc-bound magnetic beads prepared at step 113.14.

Note

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

114.2 Add 35 µL 5X Bind/wash buffer from the 1 mL aliquot prepared at step 112.1.

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

114.4 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

114.5 Carefully transfer 175 µL supernatant with a pipette, without disturbing the beads to a clean 1.5mL microcentrifuge tube. The sample at this stage is host rRNA and DNA depleted in buffer. Store this sample at -20 °C or proceed directly to the sample cleanup described in steps 117 - 117.15

115 AMPure XP Bead Cleanup.

115.1 Vortex AMPure XP Beads to resuspend.

115.2 Add 175 µL resuspended AMPure XP beads to the sample at step 116.5.

115.3 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.

115.4 Incubate samples for at least 00:05:00 at Room temperature. 5m

115.5 Quickly spin the sample, then place the tube on the magnet rack to separate the beads from the supernatant.

115.6 After 2-5 minutes (or when the solution is clear), using a pipette, remove and discard the supernatant without disturbing the beads .

115.7 Add 400 µL of freshly prepared 80% ethanol to the tube on a magnetic rack. Incubate at 30s

Room temperature for 00:00:30, then with a pipette, carefully remove and discard the 80% ethanol without disturbing the beads.

115.8 Repeat the previous step X1.

115.9 Briefly spin the tube, place it back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.

115.10 Air dry the beads for 00:02:00 while the tube is on the magnetic rack with the lid open. 2m

115.11 Add 30 µL of nuclease-free water to the beads.

115.12 Briefly spin the sample and mix well by pipetting up and down 10 times.

115.13 Incubate for 00:02:00 at Room temperature . 2m

115.14 Briefly spin the sample, then place the tube on the magnetic rack for 2-5 minutes (or when the solution is clear).

115.15 Transfer the supernatant to a new 1.5mL tube.

115.16 Use 1 µL for DNA/ RNA concentration measurement. Refer to REDI-NET SOP B-2 for the method of DNA/RNA quantification and storage. Recode the concentration on REDI-NET DCS B-4 Blood Testing.