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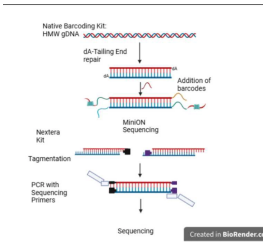
Combining NexteraXT and Native BK to Identify Gene Expression Differences in Staphylococcus aureus Strains



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We are still developing and optimizing this protocol

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Abstract

The purpose of these two protocols combined is to improve the accuracy of genome assembly and gene expression analysis, especially when investigating the mechanisms of antibiotic resistance. Four samples are chosen including MSSA, MRSA, VSSA, and VRSA, with MSSA/VSSA acting as genomic controls and the other two samples acting as the antibiotic resistant genomes.

Long reads provide the ability to span large, complex, and repetitive regions of the genome, which is critical when identifying structural variations and fully assembling genomic regions like those harboring antibiotic resistance genes. Short reads, on the other hand, offer high base-level accuracy and depth, which is important for identifying small mutations or variants, such as SNPs or indels, that may contribute to antibiotic resistance or virulence.

Once both sequencing runs are complete, a workflow aligning both kits results could reveal variations in resistance gene loci and differences in gene expression that influence pathogenicity, with short read sequencing filling in the gaps of information that long read sequencing may have left.

Image Attribution

This image identifies the workflow of first the Native Barcoding Kit, in which the samples are dA-tailed and end repaired prior to ligation, followed by the ligations of barcodes and adapters, then finally the cleanup of the DNA and sequencing. The second portion of the workflow represents the Nextera XT kit in which fresh samples are first tagmented, amplified, cleaned then loaded.
Biorender.com

Guidelines

Epi2me Workflow Guidelines:
Native Barcoding: AMR Gene Analysis

ARIBA Workflow:
Germline Pipeline

Comparing Datae:
Combine files using SamTools
Cross Validate findings from Gene resistance results with ARIBA and AMR Gene

Materials

Nextera
- 10 µl pipette tips
- 10 µl multichannel pipettes
- 10 µl single channel pipettes
- 1000 µl pipette tips
- 1000 µl multichannel pipettes
- 1000 µl single channel pipettes
- 200 µl pipette tips
- 200 µl multichannel pipettes
- 200 µl single channel pipettes
- 96-well storage plates, round well, 0.8 ml (MIDI plate)
- Illumina Purification Beads, 1 x 100 ml
- Illumina Purification Beads, 4 x 100 ml
- Distilled water
- Ethanol 200 proof (absolute) for molecular biology (500 ml)
- Microseal 'A' film
- Microseal 'B' adhesive seals
- Microseal 'F' foil seals
- NaOH 1 N, pH > 12.5, molecular biology grade
- RNase/DNase-free multichannel reagent reservoirs, disposable
- Ultrapure water
- Hard-Shell 96-well PCR plates

Equipment:
Basic lab equipment: safety glasses, lab coats, and powder-free protective gloves
High-Speed microplate shaker VWR, catalog # 13500-890 (110 V/120 V)
VWR, catalog # 14216-214 (230 V)
Magnetic stand-96 Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge General lab supplier
Vortexer
Eppendorf Mastercycler Pro S
Nextera XT DNA Library Prep Kit
(24 Samples)
Nextera XT DNA Library Prep Kit
(96 Samples)
Index adapters IDT for Illumina Nextera UD Indexes Set A
(96 Indexes, 96 Samples)
IDT for Illumina Nextera UD Indexes Set B
(96 Indexes, 96 Samples)
IDT for Illumina Nextera UD Indexes Set C
(96 Indexes, 96 Samples)
IDT for Illumina Nextera UD Indexes Set D
(96 Indexes, 96 Samples)

Nextera XT Index Kit v2 Set A
(96 Indexes, 384 Samples)
Nextera XT Index Kit v2 Set B
(96 Indexes, 384 Samples)
Nextera XT Index Kit v2 Set C
Nextera XT Index Kit v2 Set D
- ATM Amplicon Tagment Mix
- TD Tagment DNA Buffer
- HT1 Hybridization Buffer
- NPM Nextera PCR Master Mix
- RSB Resuspension Buffer
- LNA1 Library Normalization Additives
- LNW1 Library Normalization Wash 1
- Neutralize Tagment Buffer
- LNB1 Library Normalization Beads
- LNS1 Library Normalization Storage Buffer
1- 96 Dual Adapter Index Plate
4 tubes Index Adapters: S502–S504 and S517
6 tubes Index Adapters: N701–N706
i7 Index Tube Caps, Orange
i5 Index Tube Caps, White

NBK
400 ng gDNA per barcode (or 1000 ng gDNA per sample)
AMPure XP Beads (AXP)
DNA Control Sample (DCS)
NEBNext FFPE DNA Repair Mix (NEB, M6630)
NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
Freshly prepared 80% ethanol in nuclease-free water
Nuclease-free water (e.g. ThermoFisher, AM9937)
Qubit™ Assay Tubes (Invitrogen, Q32856)
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
1.5 ml Eppendorf DNA LoBind tubes
Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat #0030129504) with heat seals
0.2 ml thin-walled PCR tubes
Native Barcodes (NB01-24)
NEB Blunt/TA Ligase Master Mix (NEB, M0367)
EDTA
Long Fragment Buffer (LFB)
Short Fragment Buffer (SFB)
Elution Buffer (EB)
Native Adapter (NA)
NEBNext® Quick Ligation Module (NEB, E6056)
Flow Cell Flush (FCF)

Flow Cell Tether (FCT)
Library Solution (LIS)
Library Beads (LIB)
Sequencing Buffer (SB)
MinION Flow Cell
Bovine Serum Albumin (BSA) (50 mg/ml, e.g., Invitrogen™ UltraPure™ BSA, AM2616)
Flow Cell Wash Kit (EXP-WSH004)
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips
Multichannel pipette and tips
Thermal cycler
Microplate centrifuge (e.g., Fisherbrand™ Mini Plate Spinner Centrifuge)
Microfuge
Ice bucket with ice
Magnetic rack
Vortex mixer
Hula mixer (rotator mixer)
Qubit fluorometer (or equivalent)
GridION device
GridION Flow Cell Light Shield

Lysis and Confirmation

1 The purpose of the lysis addition and modification is due to the thickness of Staphylococcus aureus's cell wall. In order to achieve desired results for the following library kits, samples should have high molecular weight DNA, the only way to achieve this is by lysing the cells using lysostaphin. Lysostaphin ensures the cells are lysed efficiently for gDNA isolation (whereas normal gram-negative techniques will not have). Quantification is used to ensure successful lysis and the ability to proceed onto extraction and library prep.

2 Measure Optical Density at 600nm and record the value as a reference.

3 Dilute lysostaphin to a final concentration of [M] 100 µg/µL in nuclease-free water.

4 Store the lysostaphin solution on ice until ready for use.

5 Add 2 µL 2 µL of lysostaphin to 198 µL of the bacterial suspension in nuclease-free water (for a total volume of 200 200 µL µL).

6 Incubate the mixture at 37° 37 °C 00:30:00 to allow efficient lysis of the Staphylococcus aureus cells. 30m

7 Record another set of OD600 values to confirm successful lysis after incubation. Alternatively, take a small aliquot and observe the cell structure under a microscope to confirm lysis.

DNA Repair and End Prep (Native Barcoding Kit)

8 This sections ensures DNA is repaired and prepares the DNA ends by creating blunt ends or A-tails, making the fragments compatible for ligation with sequencing adapters.

9 Thaw the AMPure Beads and mix by vortexing.

10 Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dAtailing Module reagents in accordance with manufacturer's instructions, and place on ice.

11 In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), prepare your DNA samples. For this experiment and 4 barcodes, 1000ng of sample should be aliquoted.

12 Make up each sample to 11 µl using nuclease-free water. Mix gently by pipetting and spin down

13 Combine the following components per tube/well

Between each addition, pipette mix 10 - 20 times.
Reagent Volume DNA sample 12 µl
NEBNext FFPE DNA Repair Buffer 0.875 µl
Ultra II End-prep Reaction Buffer 0.875 µl
Ultra II End-prep Enzyme Mix 0.75 µl
NEBNext FFPE DNA Repair Mix 0.5 µl
Total 15ul

14 Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.

15 Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

16 Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

17 Resuspend the AMPure XP beads (AXP) by vortexing.

18 Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.

19 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

20 Prepare sufficient fresh 80% ethanol in nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess.

21 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.

22 Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

23 Repeat the previous step.

24 Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.

25 Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl nuclease free water. Spin down and incubate for 2 minutes at room temperature.

26 Pellet the beads on a magnet until the eluate is clear and colourless.

27 Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Dispose of the pelleted beads

28 Quantify 1 µl of each eluted sample using a Qubit fluorometer

Barcode Ligation

29 Barcodes are ligated onto the end prepped DNA sequences.

30 Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice:

31 Thaw the reagents at room temperature.

32 Spin down the reagent tubes for 5 seconds.

33 Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.

34 Thaw the EDTA at room temperature and mix by vortexing. Then spin down and place on ice.

35 Thaw the Native Barcodes (NB01-24) required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place them on ice.

36 Select a unique barcode for each sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment. Please note: Only use one barcode per sample.

37 In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well: Between each addition, pipette mix 10 - 20 times.
7.5ul of Reagent Volume
2.5ul of Native Barcode (NB01-24)
10ul of Blunt/TA Ligase Master

38 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

39 Incubate for 20 minutes at room temperature.

40 Add the following volume of EDTA to each PCR tube or well and mix thoroughly
EDTA clear cap: 2ul
EDTA blue cap: 4ul

41 Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube

Total Volume for preps:
EDTA Clear Cap - 22ul, For 6 samples: 132ul
EDTA Blue Cap - 24 ul, For 6 samples: 144 ul

42 Resuspend the AMPure XP Beads (AXP) by vortexing.

43 Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting.

Volume of AXP for preps:
EDTA Clear Cap - 9ul, For 6 samples: 53ul
EDTA Blue Cap - 10ul, For 6 samples: 60ul

44 Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

45 Prepare 2 ml of fresh 80% ethanol in nuclease-free water.

46 Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.

47 Keep the tube on the magnetic rack and wash the beads with 700 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

48 Repeat the previous step.

49 Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

50 Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nucleasefree water by gently flicking.

51 Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.

52 Pellet the beads on a magnetic rack until the eluate is clear and colourless.

53 Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

54 Quantify 1ul of eluted sample using a Qubit fluorometer.

Adapter Ligation and Cleanup

55 This sections is meant to ligate adaptors onto the ends of DNA sequences and enrich for DNA fragments larger than 3kb

56 Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:

57 Thaw the reagents at room temperature.

58 Spin down the reagent tubes for 5 seconds.

59 Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.

Note: Do NOT vortex the Quick T4 DNA Ligase. The NEBNext Quick Ligation Reaction Buffer (5x) may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for several seconds to ensure the reagent is thoroughly mixed.

60 Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice.

61 Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then spin down and place on ice.

62 Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.

63 In a 1.5 ml Eppendorf LoBind tube, mix in the following order

30 ul of Reagent Volume Pooled barcoded sample
5ul of Native Adapter (NA)
10ul of NEBNext Quick Ligation Reaction Buffer (5X)
5ul of Quick T4 DNA Ligase
For a total volume of 50ul

64 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

65 Incubate the reaction for 20 minutes at room temperature.

66 Resuspend the AMPure XP Beads (AXP) by vortexing.

67 Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

68 Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

69 Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

70 Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and

allow the beads to pellet. Remove the supernatant using a pipette and discard.

71 Repeat the previous step.

72 Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

73 Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB).

74 Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.

75 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

76 Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

77 Depending on your DNA library fragment size, prepare your final library in 12 µl of Elution Buffer (EB).
Fragment library length:
Flow cell loading amount: Very short (<1 kb) 100 fmol
Short (1-10 kb) 35–50 fmol
Long (>10 kb) 300 ng

Loading and Sequencing Settings

78 Loading should be done on a MinION flow cell according to these protocols (**Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24) (NBE_9169_v114_revR_15Sep2022) (nanoporetech.com)** and sequencing should be done on a GridION device.

79 Using the MinKNOW software, select Super Accurate Basecalling, the Native Barcoding Kit, 72 hour run time, and enable quality score filtering with a threshold of Phred > 9

Nextera XT: Tagmenting DNA

80 This sections fragments and tags DNA with adaptor sequences.

81 Prepare the following consumables:

	A	B	C
	Item	Storage	Instructions
	ATM	-25C to -15C	Thaw on cie. I nvert the thaw ed tubes 3-5 ti mes and then centrifuge brie fly.
	TD	-25C to -15C	Thaw on ice. I nvert the thaw ed tubes 3-5 ti mes and then centrifuge bie fly.
	NT	15C to 30C	Check for pre cipitates. If pr esent, vortex until all partic ulates are res uspended.

Preparation for Tagmenting consumables

81.1 Save the following tag program on the thermocycler:
- Choose the preheat lid option and set to 100C
- Set the reaction volume to 50ul
- 55C for 5 minutes
- Hold at 10C

82 Add the following volumes in the order listed to each well of a new 96-well PCR plate.
- TD (10 µl)
- 1 ng DNA (5 µl)

83 Pipette to mix.

84 Add 5 µl ATM to each well.

85 Pipette 10 times to mix, and then seal the plate.

86 Centrifuge at 280 × g at 20°C for 1 minute.

87 Place on the preprogrammed thermal cycler and run the TAG program. When the program reaches 10°C, immediately proceed to step 7 because the transposome is still active.

88 Add 5 µl NT to each well.

89 Pipette 10 times to mix, and then seal the plate.

90 Centrifuge at 280 × g at 20°C for 1 minute.

91 Incubate at room temperature for 5 minutes.

Amplify Libraries

92 This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds the i7 adapters, i5 adapters, and sequences required for sequencing cluster generation. This is also a safe stopping point, the plate can be sealed and stored at 2C-8C for up to 2 days.

93 Prepare the following consumables:

	A	B	C
	Item	Storage	Instructions
	Index Adaptors	-25C to -15C	Thaw at room temperature. Vortex to mix tubed and then centrifuge briefly. SPin plates briefly before use.
	NPM	-25C to -15C	Thaw on ice for 20 minutes.

Amplification consumables

93.1 Save the following NXT PCR program on a thermal cycler:

- Choose the preheat lid option and set to 100C
- Set the reaction volume to 50ul
- 72C for 3 minutes
- 95C for 30 minutes
- 12 cycles of:
- (95C for 10 seconds)
- (55C for 30 seconds)
- (72C for 30 seconds)
- 72C for 5 minutes

- Hold at 10C

94 Add the following index adapter volumes per sample according to your index adapter kit type.

	A	B
	Index Adaptor Kit Type	Volume of Index Adapter per Sample
	Index adapter tubes	5ul i7 adapter 5ul i5 adapter
	Index adapter plate	10ul prepared i7 and i5 index adapters

95 Add 15 µl NPM to each well.

96 Pipette 10 times to mix, and then seal the plate.

97 Centrifuge at 280 × g at 20°C for 1 minute.

98 Place on the preprogrammed thermal cycler and run the NXT PCR program.

Clean Up Libraries

99 This section uses single-sided bead purification to purify amplified libraries.

100 Prepare the following consumables:

	A	B	C
	Item	Storage	Instructions
	IPB	15C to 30C	Resuspend IPB beads
	RSB	-25C to -15C	Thaw and bring to room temperature. Vortex to mix. RSB can be stored at 2C to 8C after rthe initial thaw.

100.1 Prepare fresh 80% EtOH from absolute ethanol.

101 Centrifuge at 280 × g at 20°C for 1 minute to collect contents at the bottom of the well.

102 Transfer 50 µl supernatant from each well of the PCR plate to corresponding wells of a new MIDI plate.

Note: The ratio of supernatant to volume of IPB is 3:2. If you transfer less than 50 µl supernatant, adjust the volume of IPB accordingly.

103 If you are using standard DNA input, add 30 µl IPB to each well containing supernatant.

104 Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.

105 Incubate at room temperature for 5 minutes.

106 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

107 Without disturbing the beads, remove and discard all supernatant.

108 Wash two times with 200 µl 80% EtOH as follows.
a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
b. Incubate for 30 seconds.
c. Without disturbing the beads, remove and discard all supernatant.

109 Use a 20 µl pipette to remove and discard residual EtOH.

110 Air-dry on the magnetic stand for 15 minutes.

111 Remove from the magnetic stand.

112 Add 52.5 µl RSB to the beads.

113 Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.

114 Incubate at room temperature for 2 minutes.

115 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

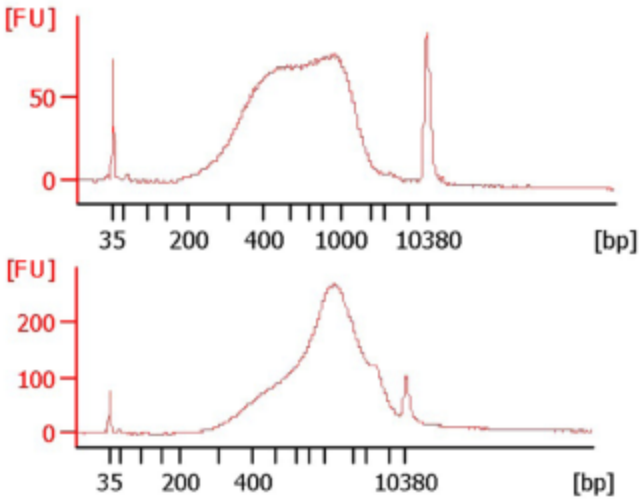
116 Transfer 50 µl supernatant to a new 96-well PCR plate.

Check Library Quality

117 Library quality should be checked to determine if PCR was successful and if there were any contaminnats in the sample.

118 Run 1 µl undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit.
Typical libraries show a broad size distribution of ~250–1000 bp, as shown in the top panel. Various libraries can be sequenced with average fragment sizes as small as 250 bp or as large as 1500 bp.

118.1



Example Bioanalyzer Trace

Quality Metrics

119 This section is to troubleshoot library size and concentration inconsistencies.

119.1 Checking Library Size:
Larger molecules cluster less efficiently than smaller molecules. If the fragment size after tagmentation is larger than expected, low cluster numbers are possible. The inverse is also true. The average expected library size after tagmentation is between 400 bp and 1.2 kb. Check the library size with a high sensitivity Bioanalyzer trace after the PCR cleanup step. Look for a long low plateau. Alternatively, PCR-amplify the library with qPCR primers and run the product on an agarose gel. The sequence for these primers is available in the Sequencing Library qPCR Quantification Guide (document # 11322363).

Short libraries indicate too little input DNA: Requantify the input DNA with a fluorometric method. Start with 10%–25% more input DNA. If the library peak is below 400 bp and you want to continue with this library, dilute the library further.

Long libraries indicate too much input DNA or inhibitor presence: Start with less input DNA, make sure that the input DNA is free from inhibitors, and repeat the quantification step.

119.2 Check library concentration:
Bead-based normalization is most efficient when the library yield after amplification is 10–15 nM, or higher. Measure library concentration using high sensitivity dsDNA Qubit after library cleanup, and measure library size with a Bioanalyzer to calculate molarity.
If you are starting with high-quality DNA and see low yield after library cleanup, there are possible issues with IPB cleanup or the amplification step. If results show either condition, confirm proper storage of the PCR master mix at -25°C to -15°C in a no-frost freezer. Confirm minimal freeze-thaw cycles.

Normalize Libraries

120 This step normalizes the quantity of each library made with Nextera XT Index v2 or Nextera XT Index Kits to ensure more equal library representation in the pooled library.

121 Prepare the following consumables:

	A	B	C
	Item	Storage	Instructions
	LNA1	-25C to -15C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water ba th as needed.
	LNB1	2C to 8C	Prepare under a fume hood.

	A	B	C
			Bring to room temperature. Use a 20°C to 25°C water ba th as needed.
	LNW1	2C to 8C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water ba th as needed.
	LNS1	Room temper ature	Keep at room temperature.

122 Transfer 20 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.

123 Combine the following volumes in a 15 ml conical tube to prepare the LN master mix. Multiply each volume by the number of samples being processed.
- LNA1 (46 µl)
- LNB1 (8 µl)

Reagent coverage is included in the volume to ensure accurate pipetting.

124 Pipette 10 times to mix.

125 Pour the LN master mix into a trough.

126 Use a 200 µl multichannel pipette to transfer 45 µl LN master mix to each well.

127 Seal the plate, and then use a plate shaker at 1800 rpm for 30 minutes.

128 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

129 Without disturbing the beads, remove and discard all supernatant.

130 Wash two times with 45 µl LNW1 as follows.
a. Add 45 µl LNW1 to each well.
b. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.

c. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
d. Without disturbing the beads, remove and discard all supernatant.

131 Add 30 µl 0.1 N NaOH to each well.

132 Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.

133 Add 30 µl LNS1 to each well of a new 96-well PCR plate labeled SGP.

134 After the 5 minute elution completes, make sure that all samples in the MIDI plate are resuspended. If they are not, resuspend as follows.
a. Pipette 10 times to mix or lightly tap the sample plate on the bench.
b. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.

135 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

136 Transfer 30 µl supernatant from each well of the MIDI plate to the corresponding well of the SGP plate.

137 Seal the sample plate, and then centrifuge at 1000 × g for 1 minute. At this point, the libraries are single-stranded DNA, which resolves poorly on an agarose gel or Bioanalyzer chip. For quality control, use the double-stranded DNA saved from step 17 of the cleanup procedure.

Loading and Sequencing

138 Load libraries according to loading protocols for the MiSeq platform. (**https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-guide-15027617-06.pdf**).
Sequencing setting sshould be set to paired end reads and a target of 30x-100x coverage.

Protocol references

OXFORD NANOPORE TECHNOLOGIES | Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24)
(**Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24) (NBE_9169_v114_revR_15Sep2022) (nanoporetech.com)**
(NBE_9169_v114_revR_15Sep2022

Nextera XT DNA Library Prep Illumina
(**Tagment Genomic DNA (illumina.com)**

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