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Orbivirus (BTV, EHDV, AHSV) whole genome sequencing using a SISPA approach and nanopore technology

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We use this protocol and it's working, and we are currently working on optimizations.

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Abstract

This protocol describes a SISPA (Sequence-Independent Single Primer Amplification) approach combined with Oxford Nanopore sequencing for the detection and characterization of three economically most important orbiviruses from clinical samples or isolates.
This protocol has been validated on field samples and isolates of Bluetongue Virus (BTV) and Epizootic hemorrhagic virus (EHDV), and on isolates of African Horse Sickness virus (AHSV).
We hope this will be useful for other teams working on orbiviruses. Feel free to reuse, adapt, or cite this protocol using the DOI provided.

Materials

1. Materials for sample preparation

- RNA eluted in Nuclease free water (be careful if using commercial kit or automate)
- Group specific real-time RT-PCR (rtRT-PCR) assay

Example:
-- Pan-BTV (Segment 10): commercial real-time RT-PCR (rtRT-PCR) kit mix (ADI-352, Bio-X Diagnostics S.A., Ploufragan, France).
-- Pan-EHDV (segment 9): Viarouge et al., 2015 (1)
-- Pan-AHSV (segment 7): Quan et al., 2010 (2)

- NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads (E6350L, New England Biolabs, USA) (note: kit may include Agencourt RNAClean XP or NEBNext RNA Sample Purification Beads; if not, order separately)
- SuperScript™ IV Reverse Transcriptase, Invitrogen™ (18090010, Life Technologies, USA) (200 U/µL)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor 40 U/µL, Invitrogen™ (10777019, Life Technologies, USA)
- dNTP mix, 10 mM each (N0447S, New England Biolabs, USA)
- Klenow Fragment (3'→5' exo-) / 3'-5' Klenow Polymerase (2.5 U)
- Q5® Hot Start High-Fidelity DNA Polymerase (M0493S, New England Biolabs, USA)
- HighPrep™ PCR Clean-up System (AC-60050, MagBio Genomics Inc, USA)
- Qubit™ dsDNA high sensitivity (HS), Invitrogen™ (Q32851, Life Technologies, USA)
- Genomic DNA ScreenTape kit (Agilent Technologies, USA)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease free water
- SISPA primers (both random and orbivirus-specific primers)

	A	B	C
	Primers name	**Sequences 5′-3′**	**Reference**
	FR26RV-N (50 µM)	GCCGGAGCTCTGCAGATATC**NNNNNNN**	Djikeng *et al.*, 2008 (3)
	FR-BT_F (10 µM)	GCCGGAGCTCTGCAGATATC**GTTAAAN**	Marcacci et al., 2016 (4), Sghaier et al., 2022 (5)
	FR-BT_R (10 µM)	GCCGGAGCTCTGCAGATATC**GTAAGTN**	Marcacci et al., 2016 (4), Sghaier et al., 2022 (5)
	FR20 Rv (40µM)	GCCGGAGCTCTGCAGATATC	Djikeng *et al.*, 2008 (3)

	A	B	C

2. Materials for nanopore library preparation

- 200-300 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded
- Native Barcoding Kit 24 V14 (SQK-NBD114.24, Oxford Nanopore)
- Sequencing Auxiliary Vials V14 (EXP-AUX003, Oxford Nanopore)
- Native Barcoding Expansion V14 (EXP-NBA114, Oxford Nanopore)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- NEB Blunt/TA Ligase Master Mix (M0367T, New England Biolabs, USA)
- NEB Next Ultra II End repair/dA-tailing Module (E7546, New England Biolabs, USA)
- NEB Next Quick Ligation Module (E6056, New England Biolabs, USA)
- Qubit™ dsDNA high sensitivity (HS), Invitrogen™ (Q32851, Life Technologies, USA)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease free water

3. Equipment (listed in document)

- Pipettes and pipette tips with filters P2, P3, P10, P20, P100, P200, P1000
- Plastic consumables (Eppendorf DNA LoBind tubes, plates, microtubes...)
- Conventional and real-time Thermal cycler
- Microfuge, vortex mixer
- Ice bucket with ice
- Dry bath / Heating block capable of 25°C, 37°C, 65°C
- Magnetic separator, suitable for 1.5 mL Eppendorf tubes (Magnarack, CS15000, Life Technologies, USA)
- Qubit® 2.0 Fluorometer (or later)
- Qubit™ Assay Tubes Invitrogen™ (Q32856, Life Technologies, USA)
- 2200 TapeStation instrument (Agilent Technologies, USA)
- Note: MinION libraries are prepared using NBD114.24 kit and following the manual version with somemodifications: "ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24-NBA9168v114revL15Sep2022-minion"
- MinION or GridION device (with lid and clip to seat flow cell)
- Light shield for flow cell (installed after loading for optimal sequencing output)

Troubleshooting

Before start

Checklist - See '1. Materials for sample preparation' and '2. Materials for nanopore library preparation' for items toprepare before starting.

RNA input

1 RNA should be eluted in nuclease-free water instead of other commercial elution buffer(with content that might interfere with NGS preparation protocols, such as EDTA).

2 Optional: Validate RNA samples using group specific real-time RT-PCR assay.

Ribosomal RNA depletion – Probe Hybridization 20m

3 Comment: rRNA depletion is advised when working with host matrices rich in ribosomal RNA.

4 Comment: This step uses the NEBNext® rRNA Depletion Kit (Human/Mouse/Rat). For updated versions, follow the manufacturer's instructions.

5 Prepare Probe Hybridization (PH) reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B
	Reagent	**Quantity**
	NEBNext rRNA Depletion Solution	1 µl
	Probe Hybridization Buffer	2 µl

6 Add 3 µL of PH reaction mix to 12 µL of RNA (5 ng – 1 µg of RNA).

7 Mix by pipetting, close the tube and spin down quickly.

8 Incubate 00:15:00 at Room temperature . 15m

9 Add 1 µL of EDTA 25 mM to inactivate the reaction.

10 Using a thermal cycler, incubate:
- Heated lid to 105 °C
- 95 °C to 22 °C – temperature decrease of 0.1°C/sec
- 22 °C for 00:05:00
- 4 °C - hold.

11 Spin down, place RNA samples on ice and move on quickly to the next step.

Ribosomal RNA depletion – RNase H Digestion

30m

12 Prepare RNase H (RH) reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B
	Reagent	**Quantity**
	NEBNext RNase H	1 µl
	RNase H Reaction Buffer	2 µl
	Nuclease-free Water	1 µl

13 Add 5 µL of RH reaction mix to the RNA from the previous step.

14 Mix by pipetting, close the tube and spin down quickly.

15 Using a thermal cycler, incubate:
- Heated lid off (or 40 °C)
- 37 °C for 00:30:00
- 4 °C - hold

16 Spin down, place RNA samples on ice and move on quickly to the next step.

Ribosomal RNA depletion – DNase I Digestion

30m

17 Prepare DNase I (DI) reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B
	Reagent	**Quantity**
	DNase I Reaction Buffer	5 µl
	DNase I (RNase-free)	2.5 µl
	Nuclease-free Water	22.5 µl

18 Add 30 µL of DI reaction mix to the RNA from the previous step.

19 Mix by pipetting, close the tube and spin down quickly.

20 Using a thermal cycler, incubate: 30m

- Heated lid off (or 40 °C)
- 37 °C for 00:30:00
- 4 °C - hold

21 Spin down, place RNA samples on ice and move on quickly to the next step.

RNA purification

28m

22 Comment: The NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) includes either Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads; if not, they should be ordered separately.

23 Vortex RNA sample purification beads to resuspend.

24 Add 110 µl (2.2X) of purification beads to the RNA sample. Mix well by pipetting up and down at least 10 times, close the tube and spin down quickly.

25 Incubate samples On ice for 00:15:00 . 15m

26 Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

27 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA. 5m

28 Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA. 30s

29 Repeat the washing step: add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant. 30s

30 Air dry the beads for up to 00:05:00 while the tube/plate is on the magnetic stand with the lid open; do not over-dry the beads. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. 5m

31 Remove the tube/plate from the magnetic stand. Elute the RNA from the beads by adding 12 µl of nuclease-free water. Mix well by pipetting up and down 10 times.

32 Incubate for at least 00:02:00 . If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

33 Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 10 µl to a new PCR tube.

34 Place the tube On ice and proceed with downstream application. Alternatively, the sample can be stored at -80 °C .

RNA DENATURATION 8m

35 Thermal denaturation of the double stranded RNA genome using a thermal cycler, incubate: 8m

- 95 °C for 00:05:00
- 4 °C for 00:03:00 (or place On ice).

36 Place RNA samples On ice and move on quickly to the next step.

SISPA preparation, Reverse Transcription 1h 17m

37 Prepare RT1 reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B	C
	Reagent	**Quantity**	**Final concentration**
	10 mM dNTP mix (10 mM each)	1 µl	0.25 mM
	FR26RV-N (50 µM)	1 µl	2.5 µM
	FR-BT_F (10 µM)	1 µl	0,5 µM
	FR-BT_R (10 µM)	1 µl	0,5 µM

38 Add 4 µL of RT1 reaction mix to the RNA sample, mix and briefly centrifuge the components.

39 Using a thermal cycler, incubate: 7m

- 65 °C for 00:05:00
- 4 °C for 00:02:00

40 Place RNA sample On ice and move on quickly to the next step.

41 Prepare RT2 reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B	C
	Reagent for RT2 reaction mix	**Quantity**	**Final concentration**
	SSIV Buffer 5X (heated at +37°C)	4 µL	1X
	DTT (100 mM)	1 µL	5 mM
	RNaseOUT‰ Recombinant RNase Inhibitor (40 U/µL)	1 µL	40 U
	SuperScript‰ IV Reverse Transcriptase (200 U/µL)	1 µL	200 U

42 Add 7 µL of RT2 reaction mix to the annealed RNA.

43 Mix, and then briefly centrifuge the contents.

44 Using a thermal cycler, incubate: 1h 10m

- 23 °C for 00:10:00
- 53 °C for 00:50:00
- 80 °C for 00:10:00
- 4 °C - Hold.

45 Move on quickly to the next step.

SISPA preparation, cDNA Second Strand Synthesis 1h 10m

46 Add 1 µL of 3'-5' Klenow Polymerase (2.5 U) directly to the sample.

47 Mix by pipetting, close the tube and spin down.

48 Using a thermal cycler, incubate: 1h 10m

- 37 °C for 01:00:00
- 75 °C for 00:10:00
- 16 °C - Hold

49 Tenfold dilution of the ds cDNA sample:

- 3 µL ds cDNA
- 27 µL Nuclease free water

50 Comment: The tenfold dilution is essential to avoid PCR inhibition. Alternatively, you can purify your sample.

51 Safe stop – short term conservation at 4 °C, long term conservation at -80 °C or -20 °C.

SISPA preparation, labeled DNA amplification 9m 40s

52 Prepare PCR reaction mix as described below, for the number of samples to be prepared allowing for at least two extra samples.

	A	B	C
	Reagent	**Quantity**	**Final concentration**
	Nuclease-free water	32.5 µL	/
	Q5 Reaction Buffer 5X	10 µL	1X
	dNTP mix (10 mM each)	1 µL	0.25 mM each
	Primer FR20-Rv (40 µM)	1 µL	0.8 mM
	Q5 Hot Start High-Fidelity DNA Polymerase	0.5 µL	0.02 U

53 Distribute 45 µL of PCR reaction mix per sample.

54 Add 5 µL of tenfold diluted ds cDNA (or nuclease-free water for negative PCR control).

55 Mix by pipetting, close the tube and spin down.

56 Using a thermal cycler, incubate: 9m 40s

- 98 °C for 00:01:00
- 40 cycles:
  - 98 °C for 00:00:10
  - 65 °C for 00:00:30
  - 72 °C for 00:03:00
- 72 °C for 00:05:00
- 16 °C – Hold

57 Move on quickly to the next step.

58 Comment: Before the purification step, which is expensive, you can control the concentration of the diluted ds cDNA and your fresh amplicon using Qubit and Qubit™ ds DNA high sensitivity (HS), Invitrogen™ (ThermoFisher, Q32851) kit.

Amplicon Clean-up & quantification 18m

59 Resuspend the beads by vortexing.

60 Add 90 µL of beads (ratio 1.8X) to the sample and mix by pipetting (ten times).

61 Incubate 00:05:00 at Room temperature . 5m

62 Spin down the sample and pellet on a magnet rack until supernatant is clear and colorless (at least 00:05:00). 5m

63 Pipette off the supernatant.

64 Add 200 µL of freshly prepared 80% ethanol without disturbing the pellet.

65 Incubate 00:00:30 . 30s

66 Remove the ethanol and discard.

67 Repeat the washing step by adding again 200 µL of freshly prepared 80% ethanol without disturbing the pellet; incubate 00:00:30 and remove the ethanol. 30s

68 Spin down and place the tube back on the magnet, pipette off any residual ethanol using P10.

69 Allow to dry, but do not dry the pellet to the point of cracking.

70 Remove the tube from the magnetic rack and resuspend pellet in 27 µL of nuclease-free water.

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

72 Pellet the beads on a magnet until the eluate is clear and colorless (at least 00:05:00). 5m

73 Transfer 25 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

74 Quantify your sample using a Qubit fluorometer and the Qubit™ ds DNA high sensitivity (HS) kit.

75 Check the amplicon size distribution of your sample and determine the average size (value used during library preparation input quantity calculation).

76 Optional: Quantify your sample using specific real time PCR to check viral enrichment.

77 Comment: Volume of elution can be adapted according to the input required for library preparation.

78 Comment: Keep the bead ratio during the clean-up at 1.8X or 1.5X to avoid losing small DNA fragments.

79 Safe stop – short term conservation at 4 °C, long term conservation at -80 °C or -20 °C.

Library prep – input normalization

80 Comment: MinION libraries are prepared using the NBD114.24 kit and following the manual version with some modifications: "ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24-NBA*9168*v114*revL*15Sep2022-minion".

For the most up-to-date information on library preparation, please consult the official Oxford Nanopore Technologies documentation on their website, which includes protocols for preparing sequencing libraries with their kits. Oxford Nanopore provides a comprehensive set of library preparation protocols and updates as part of their Prepare documentation.

For detailed instructions on how to install and use the MinKNOW software and start a sequencing run, please refer to the MinKNOW documentation and user guides provided by Oxford Nanopore Technologies. These resources explain how to set up a sequencing experiment, select kits and parameters, and begin data acquisition with MinKNOW.

81 Comment: Approximately 120 nanopores should be active per FMDV sample following this protocol to reach a good sequencing depth and coverage.

82 Determine the molarity of your sample using concentration (ng/µL) and the DNA amplicon average size (bp).
- Use the formula: Molar concentration (fmol/µL) = [(concentration (ng/µL) / (Size * 660)) * 1.10^6]
- or use the online tool: https://nebiocalculator.neb.com/#!/dsdnaamt

83 In clean 0.2 mL thin-walled PCR tubes (or a clean 96-well plate), aliquot 300 fmol of DNA per sample.

84 Make up each sample to 12.5 µL using nuclease-free water. Mix gently by pipetting and spin down.

Library prep – End-pre

10m

85 Thaw the reagents at Room temperature :

- AMPure XP Beads (AXP): mix by vortexing (beads should be at room temperature 30 minutes before use).
- Ultra II End-pre reaction buffer: mix by vortexing (to dissolve any precipitate).

86 Remove the purification beads from the fridge at least 30 minutes before use.

87 Set the heating block to 25 °C .

88 Prepare the NEBNext Ultra II End prep mix as described below, for the number of samples to be prepared allowing for at least three extra samples.

	A	B
	Reagent	**Quantity**
	Ultra II End-prep reaction buffer	1.75 µL
	Ultra II End-prep enzyme mix	0.75 µL

89 Add 2.5 µL of the NEBNext Ultra II End prep mix to the sample (300 fmoles DNA in 12.5 µL).

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

91 Using a thermal cycler, incubate: 10m

- 20 °C for 00:05:00
- 65 °C for 00:05:00

Library prep – Clean-up & Quantification

18m

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

93 Add 22.5 µL (1.5X) of resuspended AMPure XP Beads (AXP) to each end-pre reaction and mix by flicking the tube.

94 Incubate 00:05:00 at Room temperature . 5m

95 Prepare fresh 80% ethanol for all the purifications to be done during the day.

96 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). Keep the tubes on the magnet and pipette off the supernatant. 5m

97 Wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet (incubate at least 00:00:30). 30s

98 Pipette off the ethanol using a pipette and discard.

99 Repeat the washing step: add 200 µL of freshly prepared 80% ethanol without disturbing the pellet (incubate at least 00:00:30), pipette off the ethanol using a pipette and discard. 30s

100 Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol using P10. Allow to dry but do not dry the pellets to the point of cracking.

101 Remove the tubes from the magnetic rack and resuspend the pellet in 12 µL nuclease-free water.

102 Spin down and incubate for 00:02:00 at Room temperature . 2m

103 Pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). 5m

104 Transfer 10 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

105 Quantify 1 µL of each eluted sample using a Qubit fluorometer.

106 Store amplicons at 4 °C if used within 24 hours, or at -20 °C for long-term storage.

107 Comment: Keep the bead ratio during the clean-up at 1.8X or 1.5X to avoid losing small DNA fragments.

Library prep – Native barcode ligation

20m

108 Thaw the reagents at Room temperature :

- Blunt/TA Ligase Master Mix: ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
- EDTA: mix by vortexing.
- Native Barcodes (NB01-24): thaw the number of NB required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place them On ice .

109 Heating block set to 25 °C .

110 Add the reagents in the following order per well, between each addition, pipette mix 10 - 20 times

	A	B
	Reagent	**Quantity**
	End-prepped DNA	7.5 µL
	Native Barcode (NB01-24)	2.5 µL
	Blunt/TA Ligase Master Mix	10 µL

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

112 Incubate for 00:20:00 at 25 °C . 20m

113 Add 2 µL of EDTA (clear cap) or 4 µL of EDTA (blue cap) to each well and mix thoroughly by pipetting and spin down briefly.

Library prep – Pool, clean-up & quantification 33m 10s

114 Heating block set to 37 °C .

115 Pool all the barcoded samples in a 1.5 mL Eppendorf DNA LoBind tube.

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

117 Add 1.5X of resuspended AMPure XP Beads (AXP) to the pool.

118 Incubate 00:10:00 at Room temperature . 10m

119 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). Keep the tubes on the magnet and pipette off the supernatant. 5m

120 Wash the beads with 700 µL of freshly prepared 80% ethanol without disturbing the pellet (incubate at least 00:00:30). 30s

121 Pipette off the ethanol using a pipette and discard.

122 Repeat the washing step: add 700 µL of freshly prepared 80% ethanol without disturbing the pellet (incubate at least 00:00:30), pipette off the ethanol using a pipette and discard. 30s

123 Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol using P10. Allow to dry but do not dry the pellets to the point of cracking.

124 Remove the tubes from the magnetic rack and resuspend the pellet in 37 µL nuclease-free water.

125 Incubate for 00:10:00 at 37 °C. Every 00:02:00, agitate the sample by gently flicking the sample for 00:00:10 to encourage elution. 12m 10s

126 DNA elution.

127 Pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). 5m

128 Transfer 35 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

129 Quantify 1 µL of the library using a Qubit fluorometer.

130 Store amplicons at 4 °C if used within 24 hours, or at -20 °C for long-term storage.

Library prep – Adapter ligation 20m

131 Thaw the reagents at Room temperature :

- Native Adapter (NA): Spin down and place On ice .
- 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.

132 Add the reagents in the following order per sample

	A	B
	Reagent	**Quantity**
	Pooled barcoded sample	30 µL
	Native adapter (NA)	5 µL

	A	B
	NEBNext Quick Ligation Reaction Buffer 5X	10 µL
	Quick T4 DNA Ligase	5 µL

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

134 Incubate for 00:20:00 at Room temperature . 20m

Library prep – Clean-up & Quantification 40m

135 Thaw the reagents at Room temperature :

- Elution Buffer (EB): Mix by vortexing, then spin down.
- Short Fragment Buffer (SFB): Mix by vortexing, then spin down.

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

137 Add 75 µL (1.5X) of resuspended AMPure XP Beads (AXP) to the pool.

138 Incubate 00:10:00 at Room temperature . 10m

139 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). Keep the tubes on the magnet and pipette off the supernatant. 5m

140 Wash the beads by adding 125 µL 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 (00:05:00). Remove the supernatant using a pipette and discard. 5m

141 Repeat the previous step: add 125 µL 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 (00:05:00). Remove the supernatant using a pipette and discard. 5m

142 Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual supernatant using P10 (no need to wait here).

143 Remove the tubes from the magnetic rack and resuspend the pellet in 17 µL nuclease-free water.

144 Incubate for 00:10:00 at 37 °C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage. 10m

145 DNA elution.

146 Pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). 5m

147 Transfer 15 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

148 Quantify 1 µL of the library using a Qubit fluorometer.

149 Safe point: libraries in Eppendorf DNA LoBind tubes can be stored at 4 °C for short-term storage or at -80 °C for single use and long-term storage of more than 3 months.

Library prep – Library dilution

150 Determine the molarity of the library using concentration (ng/µL) and the DNA amplicon average size (bp).
- Use the formula: Molar concentration (fmol/µL) = [(concentration (ng/µL) / (Size * 660)) * 1.10^6]
- or use the online tool: https://nebiocalculator.neb.com/#!/dsdnaamt.

151 In clean 0.2 mL thin-walled PCR tubes (or a clean 96-well plate), aliquot 45 fmol of library.

152 Make up to 12 µL using Elution Buffer (EB).

Library prep – Priming and loading the SpotON flow cell

5m

153 If not already done, check your flowcell before loading:
- Open the MinKnow software.
- From the menu, select the option allowing to check your flowcell.

154 Thaw the reagents at Room temperature : mix by vortexing, then spin down and store On ice
- Sequencing Buffer (SB)
- Library Beads (LIB)
- Flow Cell Tether (FCT)
- Flow Cell Flush (FCF)

155 Prepare the following flowcell priming mix as below, mix by vortexing, then spin down and store On ice :

	A	B
	Reagent	**Quantity**
	Flow Cell Flush (FCF)	1170 µL
	Flow Cell Tether (FCT)	30 µL

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

157 Prepare the following library mix and mix well by pipetting before use (beads must be resuspended):

	A	B
	Reagent	**Quantity**
	Sequencing Buffer (SB)	37.5 µL
	Library Beads (LIB) mixed immediately before use	25.5 µL

	A	B
	DNA library	12 µL

158 Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

159 Slide the flow cell priming port cover clockwise to open the priming port.

160 After opening the priming port, check for a small air bubble under the cover and, if present, draw back a small volume to remove any bubbles:
- Set a P1000 pipette to 200 µL
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 µL, to draw back 20-30 µL, or until youcan see a small volume of buffer entering the pipette tip
- Visually check that there is continuous buffer from the priming port across the sensorarray.

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

162 Complete the flow cell priming by gently lifting the SpotON sample port cover to make the SpotON sample port accessible.
- Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
- Load 200 µL of the priming mix into the flow cell priming port (not the SpotON sampleport), avoiding the introduction of air bubbles.

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

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

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

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

166 Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

167 Close the device lid and set up a sequencing run on MinKNOW.

Sequencing - MINKNOW software

168 Open the MinKNOW software.

169 From the menu, select the option allowing to start the sequencing.

170 Fulfill the required information (flowcell Id, run name, kit name, barcoding, etc.).

171 Avoid enabling real-time basecalling, except when using a computer with sufficient performance to handle the process.

172 Start sequencing.

Protocol references

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