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Foot-and-Mouth disease virus whole genome sequencing using a SISPA approach and nanopore technology

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Mathilde Gondard¹, Cindy Bernelin-Cottet¹, Aurore Romey¹, Anne-Laure Salomez¹, Anthony Relmy¹, Sandra Blaise-Boisseau¹, Guillaume Girault¹, Labilb Bakkali-Kassimi¹

¹Virologie Unit, Laboratory of animal Health, ANSES, Maisons-Alfort, France



mathilde Gondard

ANSES, Laboratory of Animal Health, Virology Unit

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We use this protocol and it's working

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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 Foot-and-Mouth Disease Virus (FMDV) from clinical samples or isolates. We hope this will be useful for other teams working on FMDV. Feel free to reuse, adapt, or cite this protocol using the DOI provided.

Materials

Checklist

1. Materials for sample preparation

- RNA eluted in Nuclease free water (be careful if using commercial kit or automate, avoid EDTA)
- Specific Pan-FMDV real-time RT-PCR (rtRT-PCR) assay targeting a conserved region of the FMDV genome (the 3D polymerase coding region) (Gorna et al., 2016 (1)).
- DNase I Amp grade Invitrogen™ (18068015, Life Technologies, USA)
- SuperScript™ IV Reverse Transcriptase, Invitrogen™ (18090010, Life Technologies, USA)
- 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-) (M0212L, New England Biolabs, USA)
- Q5® Hot Start High-Fidelity DNA Polymerase (M0493S, New England Biolabs, USA)
- HighPrep™ PCR Clean-up System (AC-60050, MagBio Genomics Inc, USA)
- Qubit™ ds DNA high sensitivity (HS), Invitrogen™ (Q32851, Life Technologies, USA)
- Genomic DNA ScreenTape kit (Agilent Technologies, USA)
- Magnetic rack (for bead pelleting)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease free water
- SISPA primers (both random and FMDV specific primers)

	A	B	C
	Primers name	Sequences 5'-3'	Reference
	FR26RV-N	GCCGGAGCTCTGCAGATATC NNNNNNNN	Djikeng <i>et al.</i> , 2008 (2)
	SISPA_rev6	GCCGGAGCTCTGCAGATATC GGCGGCCGCTTTTTT TTTTTTTTT	ANSES, adapted from Logan <i>et al.</i> , 2014 (3)
	SISPA_FMDV_N K72	GCCGGAGCTCTGCAGATATC GAAGGGCCCAGGGT TGGA CTC	ANSES, adapted from Logan <i>et al.</i> , 2014 (3)
	SISPA_FMDV_80 9F	GCCGGAGCTCTGCAGATATC AYGGGACGTCWGCG CACG	ANSES, adapted from Dill <i>et al.</i> , 2017 (4)
	FR20 Rv	GCCGGAGCTCTGCAGATATC	Djikeng <i>et al.</i> , 2008 (2)



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), include barcodes
- 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 (M0367, 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™ ds DNA high sensitivity (HS), Invitrogen™ (Q32851, Life Technologies, USA)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease free water
- AMPure XP Beads (AXP) (used at 1.8X for amplicon clean-up and 1.5X for end-prep clean-up)

3. Equipments

- Pipettes and pipette tips with filters P2, P3, P10, P20, P100, P200, P1000
- Plastic consumables (Eppendorf DNA LoBind 1.5 mL 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).
- Thin-walled 0.2 mL PCR tubes or clean 96-well plate
- 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 some modifications: "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)

Before start

Checklist - See '1. Materials for sample preparation' and '2. Materials for nanopore library preparation' for items to prepare 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 the specific pan-FMDV real-time RT-PCR assay targeting the conserved 3D polymerase coding region as previously described (Gorna *et al.*, 2016).

DNase treatment

27m

- 3 Place RNA samples  On ice .
- 4 Prepare DNase reaction mix for the number of samples to be processed, allowing for at least two extra samples.

	A	B	C
	Reagent	Quantity	Final concentration
	DNase I Reaction buffer 10X	1 μ L	1X
	DNase I Amp Grade (1U/ μ L)	1 μ L	1U

- 5 Add 2 μ L of DNase reaction mix to 8 μ L of RNA ($\leq$ 1 μ g of RNA).
- 6 Mix by pipetting, close the tube and briefly spin down.
- 7 Incubate for  00:15:00 at  Room temperature .
- 8 Add 1 μ L of EDTA (25 mM) to inactivate the reaction.

15m



9 Using a thermal cycler, incubate:

- 65 °C for 00:10:00
- 4 °C for 00:02:00

12m

10 Place RNA samples On ice and proceed quickly to the next step.

SISPA preparation, Reverse Transcription

1h 17m

11 Prepare the RT1 reaction mix for the number of samples to be processed, allowing for at least two extra samples.

A	B	C
Reagent for RT1 reaction mix	Quantity	Final concentration
dNTP mix (10 mM each)	1 µL	0.25 mM each
FR26RV-N (50 µM)	1 µL	2.5 µM
SISPA_FMDV_NK72 (10 µM)	1 µL	0,5 µM
SISPA_rev6 (10 µM)	1 µL	0,5 µM
SISPA_FMDV_809F (10 µM)	1 µL	0,5 µM

12 Add 5 µL of the RT1 reaction mix to the RNA sample, mix and briefly centrifuge to collect components.

13 Using a thermal cycler, incubate:

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

7m

14 Place the RNA sample On ice and proceed quickly to the next step.

15 Prepare the RT2 reaction mix for the number of samples to be processed, 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

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

17 Mix, and then briefly centrifuge the contents.

18 Using a thermal cycler, incubate:

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

1h 10m

19 Move on quickly to the next step.

SISPA preparation, cDNA Second Strand Synthesis

1h 10m

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

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



22 Using a thermal cycler, incubate:

1h 10m

- 37 °C – 01:00:00 (polymerisation)
- 75 °C – 00:10:00 (inactivation)
- 16 °C – Hold

23 Tenfold dilution of the ds cDNA sample:

- 3 µL ds cDNA
- 27 µL Nuclease free water

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

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

SISPA preparation, labeled DNA amplification

9m 40s

25 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

26 Distribute 45 µL of PCR reaction mix per sample.

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



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

29 Using a thermal cycler, incubate:

9m 40s

- 98 °C – 00:01:00

- 40 cycles of:

- 98 °C – 00:00:10

- 65 °C – 00:00:30

- 72 °C – 00:03:00

- 72 °C – 00:05:00

- 16 °C – Hold

30 Move on to the next step.

31 Optionnal: 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

32 Resuspend the AMPure (or equivalent) beads by vortexing.

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

34 Incubate 00:05:00 at Room temperature .

5m

35 Spin down the sample and pellet the beads on a magnetic rack until the supernatant is clear and colourless (at least 00:05:00).

5m

36 Pipette off and discard the supernatant.

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



- 38 Incubate  00:00:30 . 30s
- 39 Remove the ethanol and discard.
- 40 Repeat the washing step by adding again 200 μ L of freshly prepared 80% ethanol without disturbing the pellet, incubate  00:00:30 and remove the ethanol. 30s
- 41 Spin down and place the tube back on the magnet. Pipette off any residual ethanol using a P10.
- 42 Allow to dry, but do not dry the pellet to the point of cracking.
- 43 Remove the tube from the magnetic rack and resuspend the pellet in 27 μ L of nuclease-free water.
- 44 Incubate for  00:02:00 at  Room temperature . 2m
- 45 Pellet the beads on a magnet until the eluate is clear and colorless (at least  00:05:00). 5m
- 46 Transfer 25 μ L of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.
- 47 Quantify your sample using a Qubit fluorometer and the Qubit™ dsDNA high sensitivity (HS) kit (Invitrogen™, ThermoFisher, Q32851).
- 48 Check the amplicon size distribution of your sample and determine the average size (value used during library preparation input quantity calculation).
- 49 Optional: you can check the amplification by testing your DNA with the specific pan-FMDV real-time RT-PCR assay targeting the conserved 3D polymerase coding region (Gorna *et al.*, 2016 (1)).
- 50 *Comment: Volume of elution can be adapted according to the input required for library preparation.*



- 51 *Comment: Keep the bead ratio during the clean-up at 1.8X or 1.5X to avoid losing small DNA fragments.*
- 52 Safe stop – short term conservation at 4 °C long term conservation at -80 °C or -20 °C .

Library prep – input normalization

- 53 *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”.*
- 54 *Comment: Approximately 200 nanopores should be active per FMDV sample following this protocol to reach a good sequencing depth and coverage.*
- 55 Determine the molarity of your sample using concentration (ng/μL) and the DNA amplicon average size (bp).
- 56 Use the formula: Molar concentration (fmol/μL) = [(concentration (ng/μL) / (Size * 660)) * 1.10⁶] or the online tool: <https://nebiocalculator.neb.com/#/dsdnaamt>.
- 57 In clean 0.2 mL thin-walled PCR tubes (or a clean 96-well plate), aliquot 300 fmol of DNA per sample.
- 58 Make up each sample to 12.5 μL using nuclease-free water. Mix gently by pipetting and spin down.

Library prep – End-prep

1h 40m

- 59 Thaw the reagents at Room temperature : 1h
- AMPure XP Beads (AXP): mix by vortexing (00:30:00 before use).
 - Ultra II End-prep reaction buffer: mix by vortexing (to dissolve any precipitate).
- 60 Remove the purification beads from the fridge at least 00:30:00 before use. 30m
- 61 Set the heating block to 25 °C .



- 62 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

- 63 Add 2.5 µL of the NEBNext Ultra II End-prep mix to the sample (300 fmoles DNA in 12.5 µL).
- 64 Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.
- 65 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 .

10m

Library prep – Clean-up & Quantification

18m

- 66 Resuspend the AMPure XP beads (AXP) by vortexing.
- 67 Add 22.5 µL (1.5X) of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.
- 68 Incubate 00:05:00 at Room temperature .
- 69 Prepare fresh 80% ethanol for all purification steps to be performed during the day.
- 70 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

5m



- 71 Wash the beads with 200 μ L of freshly prepared 80% ethanol without disturbing the pellet (incubate at least  00:00:30). 30s
- 72 Pipette off the ethanol using a pipette and discard.
- 73 Repeat the washing step: add 200 μ L 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
- 74 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.
- 75 Remove the tubes from the magnetic rack and resuspend the pellet in 12 μ L nuclease-free water.
- 76 Spin down and incubate for  00:02:00 at  Room temperature . 2m
- 77 Pellet the beads on a magnet until the eluate is clear and colorless ( 00:05:00). 5m
- 78 Transfer 10 μ L of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.
- 79 Quantify 1 μ L of each eluted sample using a Qubit fluorometer.
- 80 Safe stop – short term conservation at  4 °C long term conservation at  -80 °C or  -20 °C .
- 81 *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

- 82 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 .

83 Set the heating block to  25 °C .

84 Prepare and 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

85 Thoroughly mix the reaction by gently pipetting and briefly spin down.

86 Incubate for  00:20:00 at  25 °C .

20m

87 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

88 Set the heating block to  37 °C .

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



- 90 Resuspend the AMPure XP beads (AXP) by vortexing.
- 91 Add 1.5X of resuspended AMPure XP Beads (AXP) to the pool.
- 92 Incubate  00:10:00 at  Room temperature . 10m
- 93 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
- 94 Wash the beads with 700 μ L of freshly prepared 80% ethanol without disturbing the pellet (incubate at least  00:00:30). 30s
- 95 Pipette off the ethanol using a pipette and discard.
- 96 Repeat the washing step: add 700 μ L 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
- 97 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.
- 98 Remove the tubes from the magnetic rack and resuspend the pellet in 37 μ L nuclease-free water.
- 99 Incubate for  00:10:00 at  37 $^{\circ}$ C . Every  00:02:00 , agitate the sample by gently flicking for  00:00:10 to encourage DNA elution. 12m 10s
- 100 Pellet the beads on a magnet until the eluate is clear and colorless ( 00:05:00). 5m
- 101 Transfer 35 μ L of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.
- 102 Quantify 1 μ L of the library using a Qubit fluorometer.



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

Library prep – Adapter ligation

20m

- 104 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 to ensure the reagent is thoroughly mixed.

- 105 Add the reagents in the following order per sample.

A	B
Reagent	Quantity
Pooled barcoded sample	30 µL
Native adapter (NA)	5 µL
NEBNext Quick Ligation Reaction Buffer 5X	10 µL
Quick T4 DNA Ligase	5 µL

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

- 107 Incubate for 00:20:00 at Room temperature .

20m

Library prep – Clean-up & Quantification

45m 10s

- 108 *Comment: To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB) rather than 80% ethanol for post-adapter-ligation clean-up; the use of ethanol will be*



detrimental to the sequencing reaction.

- 109 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.
- 110 Resuspend the AMPure XP beads (AXP) by vortexing.
- 111 Add 75 μ L (1.5X) of resuspended AMPure XP Beads (AXP) to the pool.
- 112 Incubate 00:10:00 at Room temperature . 10m
- 113 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
- 114 Wash the beads by adding 125 μ L 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
- 115 Repeat the previous step: add 125 μ L 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
- 116 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).
- 117 Remove the tubes from the magnetic rack and resuspend the pellet in 17 μ L nuclease-free water.
- 118 Incubate for 00:10:00 at 37 $^{\circ}$ C . Every 00:02:00 , agitate the sample by gently flicking for 00:00:10 to encourage DNA elution. 12m 10s
- 119 Pellet the beads on a magnet until the eluate is clear and colorless (00:05:00). 5m
- 120 Transfer 15 μ L of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.



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

122 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.

3m

Library prep – Library dilution

123 Determine the molarity of your sample using concentration (ng/µL) and the DNA average size (bp).

124 Use the formula: Molar concentration (fmol/µL) = [(concentration (ng/µL) / (Size * 660)) * 1.10⁶] or the online tool: <https://nebiocalculator.neb.com/#/dsdnaamt>.

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

126 Make up to 12.5 µL using Elution buffer (EB). Mix gently by pipetting and spin down.

Library prep – Priming and loading the SpotON flow cell

5m

127 If not already done, check your flowcell before loading:

- Open the MinkNOW software
- From the menu, select the option allowing to check your flowcell.

128 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)

129 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

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

131 Prepare the following library mix as below. 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
DNA library	12 µL

132 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.

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

134 After opening the priming port, check for a small air bubble under the cover. 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 you can see a small volume of buffer entering the pipette tip
- Visually check that there is continuous buffer from the priming port across the sensor array.



- 135 Load 800 μ L of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for  00:05:00 .
- 136 Complete the flow cell priming:
- Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
 - Load 200 μ L of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
- 137 Mix the prepared library gently by pipetting up and down just prior to loading.
- 138 Add 75 μ L 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.
- 139 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.
- 140 Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.
- 141 Close the device lid and set up a sequencing run on MinKNOW.

Sequencing - MINKNOW software

- 142 Open the MinKnow software
- 143 From the menu, select the option allowing to start the sequencing.
- 144 Fulfill the required information (flowcell Id, run name, kit name, barcoding, etc.).
- 145 Do not enable real-time basecalling (unless your computer's performance is outstanding).
- 146 Start sequencing.



Protocol references

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Additional resources cited in the Library prep section:

- NEB online dsDNA calculator: <https://nebiocalculator.neb.com/#/dsdnaamt>
- MinION kit manual reference used: "ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24-NBA9168v114revL15Sep2022-minion"