Ligation sequencing influenza whole genome (SQK-LSK109 with EXP-NBD196)

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Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials

- [ ] Input influenza RNA
- [ ] Influenza A primers
- [ ] Influenza B primers
- [ ] Ligation Sequencing Kit (SQK-LSK109)
- [ ] Native Barcoding Expansion 96 (EXP-NBD196)
- [ ] Or Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing 1-24 samples.
- [ ] Flow Cell Priming Kit (EXP-FLP002)
- [ ] Adapter Mix II Expansion (EXP-AMII001)
- [ ] SFB Expansion (EXP-SFB001)
- [ ] Sequencing Auxiliary Vials (EXP-AUX001)

Consumables

- [ ] SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (ThermoFisher, cat # 12574018 or 12574026)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 5 ml Eppendorf DNA LoBind tubes
- [ ] 15 ml Eppendorf DNA LoBind tubes
- [ ] Reagent reservoirs for multichannel pipetting
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals

Equipment

- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Magnetic rack suitable for 96 well plates, e.g. DynaMag™-96 Side Skirted Magnet (Thermo Fisher CAT#12027)
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Reverse transcription, PCR and clean-up	
IMPORTANT ☐ Keep the RNA sample on ice as much as possible to prevent nucleolytic degradation, which may affect sensitivity.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS

To reduce risk of contamination, we recommend the use of PCR hoods with a UV steriliser when setting up the PCR plates.

In a clean template-free pre-PCR hood, prepare the primer mixes for influenza A and influenza B as follows in 1.5 ml Eppendorf DNA LoBind tubes:

- ☐ Nuclease-free water
  - Concentration: -
  - Volume: 378 µl
- ☐ Tuni 12
  - Concentration: 100 µM
  - Volume: 16.8 µl
- ☐ Tuni 12.4
  - Concentration: 100 µM
  - Volume: 4.2 µl
- ☐ Tuni 13
  - Concentration: 100 µM
  - Volume: 21 µl
- ☐ Nuclease-free water
  - Concentration: -
  - Volume: 378 µl
- ☐ B-PBs-UniF
  - Concentration: 100 µM
  - Volume: 5 µl
- ☐ B-PBs-UniR
  - Concentration: 100 µM
  - Volume: 5 µl
- ☐ B-PA-UniF
  - Concentration: 100 µM
  - Volume: 2.5 µl
- ☐ B-PA-UniR
  - Concentration: 100 µM
  - Volume: 2.5 µl
- ☐ B-HANA-UniF
  - Concentration: 100 µM
  - Volume: 5 µl
- ☐ B-HANA-UniR
  - Concentration: 100 µM
  - Volume: 5 µl
- ☐ B-NP-UniF
  - Concentration: 100 µM
  - Volume: 3 µl
- ☐ B-NP-UniR
  - Concentration: 100 µM
  - Volume: 3 µl
- ☐ B-M-Uni3F
  - Concentration: 100 µM
  - Volume: 1.5 µl
- ☐ B-Mg-Uni3F
  - Concentration: 100 µM
  - Volume: 1.5 µl
- ☐ B-M-Uni3R
  - Concentration: 100 µM
  - Volume: 3 µl
- ☐ B-NS-Uni3F
  - Concentration: 100 µM
  - Volume: 2.5 µl
- ☐ B-NS-Uni3R

- Concentration: 100 µM

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS

In the template-free pre-PCR hood, prepare the following master mixes in Eppendorf DNA LoBind tubes and mix thoroughly as follows:

- ☐ Nuclease free water
  - Influenza A RT-PCR Master Mix: 280 µl
  - Influenza B RT-PCR Master Mix: 280 µl
- ☐ Influenza A primer mix
  - Influenza A RT-PCR Master Mix: 28 µl
  - Influenza B RT-PCR Master Mix: -
- ☐ Influenza B primer mix
  - Influenza A RT-PCR Master Mix: -
  - Influenza B RT-PCR Master Mix: 28 µl
- ☐ 2X Reaction Mix
  - Influenza A RT-PCR Master Mix: 350 µl
  - Influenza B RT-PCR Master Mix: 350 µl
- ☐ SuperScript™ III RT/Platinum™ Taq Mix
  - Influenza A RT-PCR Master Mix: 28 µl
  - Influenza B RT-PCR Master Mix: 28 µl
- ☐ Nuclease free water
  - Influenza A RT-PCR Master Mix: 560 µl
  - Influenza B RT-PCR Master Mix: 560 µl
- ☐ Influenza A primer mix
  - Influenza A RT-PCR Master Mix: 56 µl
  - Influenza B RT-PCR Master Mix: -
- ☐ Influenza B primer mix
  - Influenza A RT-PCR Master Mix: -
  - Influenza B RT-PCR Master Mix: 56 µl
- ☐ 2X Reaction Mix
  - Influenza A RT-PCR Master Mix: 700 µl
  - Influenza B RT-PCR Master Mix: 700 µl
- ☐ SuperScript™ III RT/Platinum™ Taq Mix
  - Influenza A RT-PCR Master Mix: 56 µl
  - Influenza B RT-PCR Master Mix: 56 µl
- ☐ Nuclease free water
  - Influenza A RT-PCR Master Mix: 1120 µl
  - Influenza B RT-PCR Master Mix: 1120 µl
- ☐ Influenza A primer mix
  - Influenza A RT-PCR Master Mix: 112 µl
  - Influenza B RT-PCR Master Mix: -
- ☐ Influenza B primer mix
  - Influenza A RT-PCR Master Mix: -
  - Influenza B RT-PCR Master Mix: 112 µl
- ☐ 2X Reaction Mix
  - Influenza A RT-PCR Master Mix: 1400 µl
  - Influenza B RT-PCR Master Mix: 1400 µl
- ☐ SuperScript™ III RT/Platinum™ Taq Mix
  - Influenza A RT-PCR Master Mix: 112 µl
  - Influenza B RT-PCR Master Mix: 112 µl
- ☐ Nuclease free water
  - Influenza A RT-PCR Master Mix: 2240 µl
  - Influenza B RT-PCR Master Mix: 2240 µl
- ☐ Influenza A primer mix
  - Influenza A RT-PCR Master Mix: 224 µl
  - Influenza B RT-PCR Master Mix: -
- ☐ Influenza B primer mix
  - Influenza A RT-PCR Master Mix: -
  - Influenza B RT-PCR Master Mix: 224 µl
- ☐ 2X Reaction Mix

- Influenza A RT-PCR Master Mix: 2800 µl

- Influenza B RT-PCR Master Mix: 2800 µl

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS

- [ ] For each influenza type, place one clean 96-well RT-PCR plate into a PCR-cooler (if using).
- [ ] Using a stepper pipette or a multichannel pipette, aliquot 49 µl of influenza A RT-PCR Master Mix into the influenza A RT-PCR plate.
- [ ] Using a stepper pipette or a multichannel pipette, aliquot 49 µl of influenza B RT-PCR Master Mix into the influenza B RT-PCR plate.
- [ ] Seal the RT-PCR plate(s) and transfer to a template-addition pre-PCR hood.

IMPORTANT

- [ ] We recommend having a negative control for every plate of samples.

- [ ] Transfer 1 µl of influenza A samples to the wells containing influenza A RT-PCR Master Mix in the influenza A RT-PCR plate and mix thoroughly by pipetting the contents of each well up and down.
- [ ] Transfer 1 µl of influenza B samples to the wells containing influenza B RT-PCR Master Mix in the influenza B RT-PCR plate and mix thoroughly by pipetting the contents of each well up and down.
- [ ] Seal the RT-PCR plate(s) and spin down in a centrifuge.

Incubate the influenza A RT-PCR plate using the following program, with the heated lid set to 105°C:

- [ ] cDNA synthesis 60 min @ 42°C (1 cycle)
- [ ] Initial denaturation 2 min @ 94°C (1 cycle)
- [ ] Denaturation Annealing and extension 30 sec 30 sec 3 min @ 94°C 45°C 68°C (5 cycles)
- [ ] Denaturation Annealing and extension 30 sec 30 sec 3 min @ 94°C 57°C 68°C (31 cycles)
- [ ] Hold @ 4°C

Incubate the influenza B RT-PCR plate using the following program, with the heated lid set to 105°C:

- [ ] cDNA synthesis 60 min @ 45°C (1 cycle)
- [ ] cDNA synthesis 30 min @ 55°C (1 cycle)
- [ ] Initial denaturation 2 min @ 94°C (1 cycle)
- [ ] Denaturation Annealing and extension 20 sec 30 sec 3 min 30 sec @ 94°C 40°C 68°C (5 cycles)
- [ ] Denaturation Annealing and extension 20 sec 30 sec 3 min 30 sec @ 94°C 58°C 68°C (30 cycles)
- [ ] Final extension 10 min @ 68°C (1 cycle)
- [ ] Hold @ 4°C

IMPORTANT

- [ ] If available, a clean post-PCR hood should be used for all steps that involve handling amplified material. Decontamination with UV and or DNAzap between sample batches is recommended.

- [ ] Resuspend the AMPure XP beads by vortexing.
- [ ] Add 50 µl of resuspended AMPure XP beads to each well of the RT-PCR plate(s) and mix by gently pipetting.
- [ ] Incubate the PT-PCR plate(s) at RT for 10 minutes.
- [ ] Prepare at least 500 µl 80% ethanol in Nuclease-free water per sample.

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Spin down the RT-PCR plate(s) and pellet the beads on a magnet for 5 minutes. Keep the plate on the magnet until the eluate is clear and colourless, and pipette off the supernatant. ☐ Keep the plate on the magnet and wash the beads in each well with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the plate back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the plate from the magnetic rack and resuspend each pellet in 15 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 15 µl of eluate containing the DNA per well, into a clean 96-well plate(s).	
Quantify 1 µl of each eluted sample using a Qubit fluorometer.	
Take forward your quantified sample to the end-prep step.	
End-prep	
IMPORTANT ☐ We recommended carrying the negative control through this step until sequencing.	
☐ Determine the volume of the cleaned-up PCR reaction that yields 200 fmol of DNA per sample and aliquot into a clean 96-well plate (End-prep plate). Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice: ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. Note: Do not vortex the Ultra II End Prep Enzyme Mix. ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep Buffer may have a little precipitate. Allow the mixture to come to RT and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate. ☐ Make up each sample per well to 12.5 µl using Nuclease-free water.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the following end-prep master mix in 1.5 ml Eppendorf DNA LoBind tube and mix thoroughly by pipetting: ☐ Ultra II End-prep reaction buffer - Volume per reaction: 1.75 µl - For X24 samples: 52.5 µl - For X48 samples: 105 µl - For X96 samples: 210 µl ☐ Ultra II End-prep enzyme mix - Volume per reaction: 0.75 µl - For X24 samples: 22.5 µl - For X48 samples: 45 µl - For X96 samples: 90 µl ☐ Using a stepper pipette or multi-channel pipette, add 2.5 µl of the end-prep master mix to each well containing 12.5 µl sample. ☐ Ensure the reactions are thoroughly mixed by pipetting. Seal the End-prep plate and spin down briefly. ☐ Using a thermal cycler, incubate the plate at 20°C for 5 minutes and 65°C for 5 minutes.	
Take forward the end-prepped DNA into the native barcode ligation step.	
Native barcode ligation	
IMPORTANT ☐ To monitor cross-contamination events, we recommend that the negative control is carried through this process and a barcode is used to sequence this control.	
Thaw Native Barcodes at RT. Use one barcode per sample. ☐ Spin down barcodes before opening tubes/piercing plates. ☐ Pipette mix the entire content of each barcode 10 times before use. ☐ Once thawed, keep the barcodes on ice. Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. ☐ Thaw the Short Fragment Buffer (SFB) at RT and mix by vortexing. Then spin down and place on ice. In a clean 96-well plate (Native barcode ligation plate), add the following reagents in the following order per well: ☐ 3 µl Nuclease-free water ☐ 0.75 µl End-prepped DNA ☐ 1.25 µl Native Barcode ☐ 5 µl Blunt/TA Ligase Master Mix ☐ Mix contents thoroughly by pipetting, seal the Native barcode ligation plate and spin down briefly. ☐ Using a thermal cycler, incubate the Native barcode ligation plate at 20°C for 20 mins and at 65°C for 10 mins.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Pool all the barcoded samples in a clean 1.5 ml Eppendorf DNA LoBind tube, checking the base of the plate to ensure all the liquid has been pooled. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add a 0.4X volume of AMPure XP beads to the pooled reaction and mix thoroughly by pipetting: ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet the beads on a magnet for 5 minutes. Keep the tube on the magnet until the eluate is clear, and pipette off the supernatant. ☐ Wash the beads by adding 700 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Keep the tube on the magnetic rack and wash the beads with 100 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ 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. ☐ Remove the tube from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnetic rack until the eluate is clear and colourless. ☐ Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
Take forward the barcoded DNA library to the adapter ligation and clean-up step. However, at this point it is also possible to store the sample at 4°C overnight.	
Adapter ligation and clean-up	
Adapter Mix II Expansion use Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice: ☐ Thaw the reagents at RT. ☐ Spin down the reagent tubes for 5 seconds. ☐ Ensure the reagents are fully mixed by performing 10 full volume pipette mixes. Note: Do NOT vortex the Quick T4 DNA Ligase.	
IMPORTANT ☐ Do not vortex the Quick T4 DNA Ligase.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Thaw the Elution Buffer (EB) and the Short Fragment Buffer (SFB) at RT, before mixing by vortexing. Then spin down and place on ice. ☐ Spin down the Adapter Mix II (AMII), pipette mix and place on ice. Perform the adapter ligation of the pooled and barcoded DNA. In a clean 1.5 ml Eppendorf LoBind tube, add the reagents in the following order: ☐ 30 µl Pooled barcoded sample ☐ 5 µl Adapter Mix II (AMII) ☐ 10 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 5 µl Quick T4 DNA Ligase ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	
IMPORTANT ☐ The next clean-up step uses Short Fragment Buffer (SFB) and not 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.	
☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Spin down the sample and pellet the beads on a magnet for 5 minutes. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant. ☐ Wash the beads by adding 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.	
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
IMPORTANT ☐ We recommend loading 50 fmol of final prepared library onto a flow cell.	
The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
Priming and loading the SpotON flow cell	

☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing, and spin down at RT.

☐ To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at RT.

☐ Open the MinION device lid and slide the flow cell under the clip.

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

IMPORTANT

☐ Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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

  Note: Visually check that there is continuous buffer from the priming port across the sensor array.

☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.

☐ Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

IMPORTANT

☐ The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

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

- ☐ 37.5 µl Sequencing Buffer (SQB)
- ☐ 25.5 µl Loading Beads (LB), mixed immediately before use
- ☐ 12 µl DNA library

Complete the flow cell priming:

- ☐ 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 sample port), avoiding the introduction of air bubbles.

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

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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. ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.	
IMPORTANT ☐ Required settings in MinKNOW	
Flow cell reuse and returns	
☐ After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C. ☐ Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT ☐ If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.	