RNA Harvest

Add 200 uL of input sample to MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (Thermofisher, A48383)

Run on Thermofisher Kingfisher extraction machine

Elute in 50uL Elution Buffer

RT

Lunascript Supermix (NEB, E3010L)

2 µL of MM with 8 µL of RNA

Machine:

25C-2 min

55C-10 min

95C-1 min

Multiplex PCR

Q5 Hot Start Master Mix (NEB, M0494s)

6.25 µL Master Mix 660µL MM

1.75 µL (P1/P2 primer pools). 185µL Primer Pool

4.5 µL cDNA from RT

add 8 µL of MM into each well, add 4.5 µL of cDNA

Machine:

98°C-30 sec

35 cycles

95°C-15 sec

63°C-5 min

4°C hold

Run a 1% agarose gel to check negative controls of the post-RTPCR RSV DNA plate.

Combine M-PCR pools together (12.5uL pool 2 into 12.5 uL pool 1)

Pre-library prep bead cleanup (1.0X)

Vortex NEB Next Sample Purification Beads.

Add 25 uL beads to each sample, mix thoroughly. Incubate for 5 minutes.

Transfer liquid to a round bottom plate, place on magnet for 3 minutes.

Remove supernatant. Add 150 uL 80% EtOH.

Remove supernatant. Add 150 uL 80% EtOH.

Remove supernatant. Remove remaining liquid with a P10 pipette.

Dry on magnet for 3 minutes.

Elute beads in 15 uL H2O off magnet. Mix thoroughly. Incubate for 3 minutes.

Incubate on magnet for 3 minutes.

Transfer 12.5 uL to new plate.

End prep

Prepare end prep master mix. Add the following in a 1.5 mL tube and mix thoroughly.

202 uL Ultra II End Prep buffer

80 uL Ultra II End Prep enzyme

Transfer 22 uL end prep master mix to 12 (8-strip) tubes.

Add 2.5 uL of end prep master mix to each well of a new 96-well plate (end prep plate).

Add 12.5 uL of eluted DNA from the bead cleanup to the end prep plate. Mix thoroughly.

End prep incubation

20 C for 5 minutes

65 C for 5 minutes

4 C hold

*Place AXP (kit) at room temperature.*

Barcoding

Prepare blunt TA master mix. Add the following in a 1.5 mL tube and mix thoroughly.

576 uL NEB Blunt/TA Ligase Master Mix

288 uL H2O

Transfer 68 uL blunt TA master mix to 12 (8-strip) tubes.

Add 7.75 uL of blunt TA master mix to each well of a new 96-well plate (barcoding plate)

Add 1.25 uL of each V14 barcode from SQK-NBD114.96 kit to the respective well of the

barcoding plate. Mix thoroughly.

Barcoding incubation

Room temperature for 20 minutes.

While incubating, add 10 uL EDTA (kit) to 12 (8-strip) tubes.

After 20 minute incubation, add 1 uL EDTA to each well of the barcoding plate. Mix

thoroughly.

Pooling and cleanup (0.4X)

Pool the entire 11 uL volume of each sample from the barcoding plate together into a 1.5 mL tube for a total pool volume of 1056 uL.

Vortex Ampure XP (AXP) beads (kit).

Add 422 uL AXP to pool. Mix thoroughly.

Incubate at room temperature for 10 minutes.

Place on magnet for 5 minutes.

Remove supernatant. Add 1 mL 80% EtOH.

Remove supernatant. Add 1 mL 80% EtOH.

Remove supernatant. Spin down tube. Remove remaining liquid with P20/P200 pipette.

Dry on magnet for 1 minute.

Resuspend beads in 35 uL H2O. Mix thoroughly.

Incubate at room temperature for 10 minutes, flick tube every 2 minutes.

Place on magnet for 2 minutes, move 30 uL supernatant to a new 1.5 mL tube.

*Place Qubit High Sensitivity kit at room temperature.*

*Place ONT R10 flow cell at room temperature.*

*Place FCF (kit) and FCT (kit) at room temperature.*

Native Adaptor ligation and cleanup (0.4X)

Add the following to the 30 uL cleaned library pool for a total volume of 50 uL.

5 uL NA (kit)

10 uL NEB Quick Ligation buffer 5X

5 uL NEB Quick T4 ligase

Mix thoroughly. Incubate at room temperature for 20 minutes.

Vortex AXP beads. Add 20 uL AXP beads to 50 uL adaptor-ligated pool.

Incubate at room temperature for 10 minutes.

Place on magnet for 3 minutes.

Remove supernatant. Resuspend beads off magnet with 125 uL SFB (kit).

Place back on magnet for 2 minutes.

Remove supernatant. Resuspend beads off magnet with 125 uL SFB.

Place back on magnet. Remove supernatant.

Spin down tube. Place back on magnet. Remove remaining liquid with P20/P200 pipette.

Resuspend beads with 15 uL EB (kit) off magnet.

Incubate at room temperature for 10 minutes, flick tube every 2 minutes.

Place on magnet, incubate for 2 minutes.

Library quantification and flow cell loading

Place R10 flow cell on GridION, carry out flow cell check.

Prepare 1:20 dilution of the cleaned library using 1 uL of the 15 uL elution.

Quantify library concentration using Qubit High Sensitivity kit.

Using the quantification value, prepare a diluted pool with EB in a new 1.5 mL tube that has a final mass of 20 ng DNA and a final volume of 12 uL.

Add 30 uL FCT to 1170 uL FCF, mix thoroughly.

Add 5 uL of BSA (50 mg/mL) to FCF+FCT

Load 800 uL FCF+FCT+BSA mixture into the priming port of the flow cell, leave the priming port open.

Wait 5 minutes.

With the priming port open, load 200 uL FCF+FCT mixture onto the spot-on port. Ensure

vacuum pulls the mixture into the flow cell. Leave the priming port open.

Prepare the final loading pool. To the 12 uL 20 ng pool, add 37.6 uL SB (kit) and 25.4 uL

LIB (kit). Mix thoroughly.

Load the entire 75 uL final loading pool into the spot-on port. Close spot-on port and

priming port.

Starting the run

Select “Start Sequencing”

Select flow cell being used.

Enter run name.

Continue to kit selection.

Select multiplex kits.

Select SQKNBD114.96

24 hours runtime.

All else default settings.

Start run.