Oxford Nanopore (ONT) sequencing using Rapid Barcoding Kit

This protocol is to determine the viral genome sequence of FluA/FluB/RSV. The relevant genomes were amplified by various RT-PCR methods using virus specific primers. Amplicons will be fragmented and simultaneously attached with a unique barcoded tag and a sequencing adapter by a transposase to make a pooled library. The library will be loaded onto an Oxford Nanopore Flow Cell attached to a MinION device for sequencing.

RT-PCR product quantification

The TapeStation system is used as both a quality control (QC) check and to quantify the concentration of PCR product in each reaction. An alternative QC check can be a conventional agarose gel to visualise the products in each sample. The DNA concentration can be measured using alternative means such as Qubit or Nanodrop.

1. Bring all reagents to room temperature for at least 30 mins prior to use.
2. Connect the unit to a laptop or PC with the Agilent TapeStation software installed. Switch the unit ON and open the software.
3. In the software, select which samples are to be tested in either a strip tube or plate. Ensure the A1 position of the **strip tube** is left empty as the ladder will go here.
4. Place a screen tape into the device. The software will tell you how many samples the tape can do, what volume of ladder to add (11 µL for less than 15 samples or 22 µL for more than 15) and how many additional screen tapes are required.
5. Make up the ladder in a strip tube according to the instructions in the software. This will either be:
    a. For up to 16 samples: 10 µL of Dye (Green tube) plus 1 µL of ladder (yellow tube) or,
    b. For 17-96 samples: 20 µL of Dye (Green tube) plus 2 µL of ladder (yellow tube).
6. Make up each sample according to the layout selected in the software, adding 10 µL of dye to 1 µL of sample. If in a plate, seal with a foil seal.
7. Vortex both the sample and ladder at 2000 rpm for 1 min. Spin down to collect contents.
8. Uncap any strip tubes and place the samples and ladder into the device in the positions shown in the software. Plates can be placed with the foil seal still on.
9. Add a box of tips to the device, removing the lid.
10. Press "Start". A series of checks will be displayed on the screen. Ensure each one is met, and press "Start".
11. Once finished, remove and store any partially used tapes – any fully used ones can be discarded. Remove and discard any tubes containing samples. Remove the tips and place the lid back on for storage.
12. Results will open in another window. Write down sample concentrations of each sample in worksheet.

Amplicon normalization

13. Calculate the volume of the PCR products (200 ng for Flu, 100 ng for both RSV1 and RSV2) and record in worksheet.
14. Calculate the volume of water, so that the total volume add up to 10 µL.
15. Label a new strip of PCR tubes, add the required volume water first, then PCR products of each individual samples.

Library preparation

16. To each sample add 1 µL of Fragmentation Mix RB01-96 (a different barcode for each sample). Mark down on the worksheet the unique barcode for each sample.
17. Mix gently by flicking the tube and briefly spinning down.
18. Place on a thermocycler with the following conditions:

Temperature	Time
30°C	2 mins
80°C	2 mins
4°C	30 secs
4°C	∞

19. Pool all samples into a singular 1.5mL Eppendorf DNA LoBind tube. Check the final pooled volume.
20. Vortex well the AMPure XP beads to resuspend and add **equal volume** of beads to the pooled sample tube. (For example, if your total pooled volume is 44 µL, then add 44 µL of resuspended AMPure XP beads).
21. Mix by flicking the tube – avoid vortexing as this could shear the DNA.
22. Incubate the tube at room temperature for 5 mins, gently flicking occasionally.
23. During incubation period, prepare 1000 µL of freshly prepared 80% ethanol in nuclease-free water (800 µL ETOH+ 200 µL H_2O).
24. After the 5 min incubation, pulse spin the tube to collect the contents and place on a magnet. Allow the beads to pellet (around 2 mins).
25. With the tube on the magnet, carefully pipette off the supernatant without disturbing the bead pellet.
26. Wash the beads with 500 µL freshly prepared 80% ethanol to cover the pellet without disturbing it. Remove the ethanol with a pipette and discard.
27. Repeat the wash step above for a total of 2 rinses.
28. Spin down the tube and place back on the magnet. Remove any residual ethanol and allow the bead to dry briefly (30 seconds). Take care not to over-dry the pellet until it cracks. Just until the pellet appears matte and has lost its shine.
29. Remove the tube from the magnet and resuspend the pellet in 15 µL of Elution Buffer (EB). Incubate at room temperature for 10 mins flicking occasionally.
30. Pellet the beads once again on the magnet. Leave until the solution goes clear (around 2 mins).

31. Remove and retain 15 µL of the eluate in a clean and labelled 1.5mL Eppendorf DNA LoBind tube.
32. Measure the concentration of 1 µL of the eluate using BR Qubit.
33. Use the Qubit value to dilute the library down to a final concentration of 100 ng/µL in 11 µL. It is ok to load less than 100 ng/µL, but less than 30 ng/µL may affect the quality score of the run.
34. **For this workshop only,** we are combining the Flu and RSV libraries together for loading. Combine 5.5 µL of each library for the final combined library for each group.
35. Make up a Rapid Adapter Mix by combining 1.5 µL of Rapid Adapter (RA) to 3.5 µL of Adapter Buffer (ADB). **Note: For this workshop, the mix will be premade for you.**
36. To 11 µL of library, add 1µL of the freshly prepared Rapid Adapter Mix and incubate for 5 minutes at room temperature.
37. After the incubation, keep this mix on ice until ready to load.

Priming and loading the flow cell

Thaw the sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

38. Make up a working flow cell priming mix by combining the following:

Reagent	Volume (µL)
Flow Cell Flush (FCF)	1,170
Bovine Serum Albumin (BSA) at 50mg/ml	5
Flow Cell Tether (FCT)	30
Total	**1,205**

39. Open the priming port of the flow cell by sliding it to the left.
40. Perform a bubble check:
    a. Set a P1000 to 200 µL.
    b. Insert the tip into the priming port.
    c. Turn the dial on the P1000 up to draw back 20-30 µL liquid and discard.
41. Load 800 µL of flow cell priming mix into the priming port. Avoid introducing air bubbles. Set a timer for 5 mins.
42. During this 5 min wait prepare the final library mix by combining:

Reagent	Volume (µL)
Sequencing buffer (SB)	37.5
Resuspended Library Beads (LIB)	25.5
DNA in EB with RAP added: 100 ng/µL	12

Total	**75**

Note: Ensure the LIB beads are WELL mixed to ensure homogeneity as they settle quickly.

43. After 5 mins, gently lift the SpotON sample port.
44. Gently load 200 µL of the flow cell priming mix into the **priming port** (Not the SpotON port). The SpotON port will pulse as the priming mix is added to the priming port. Ensure liquid doesn't leave the SpotON port by **dispensing slowly**.
45. Ensure the loading solution is homogenous by pipetting it up and down a few times just prior to loading. Immediately load 75 µL of the library mix in a dropwise fashion onto the SpotON port. Each drop will be drawn into the flow cell by the vacuum created.
46. Close the SpotON port by replacing the bung. Press gently to ensure it's closed properly.
47. Close the priming port by sliding the cover back to the right.
48. If working on the bench (not recommended), return the flow cell to the sequencing unit (Mk1B or Mk1C).
49. Navigate to start run on MinKNOW. Follow the set-up wizard as follows:
    a. Positions – Type the experiment name and select the flow cell type which will be on the packaging.
    b. Kit – Select the kit type used. For the rapid barcoding it will be: SQK-RBK114. If unsure, check the box of the kit used for the product code.
    c. Run options – Set the duration of the run. 4 hours is recommended. Minimum read length collected is 20 bp. Leave all else default.
    d. Analysis – Basecalling should be set to Super High Accuracy if using a PC. **Only select High Accuracy if using the Mk1C**, leave all other settings as default.
    e. Output – select a different output folder if not using the default. Leave all other settings as default.
    f. Final review – Review your final settings and select Start to begin the run.

Data retrieval

49. At the end of the run, transfer data to USB.

Washing of Flow Cell (not in use for this Workshop)

50. Put the Wash Mix (WMX) on ice, do not vortex. Flick the tube a few times to mix the WMX.

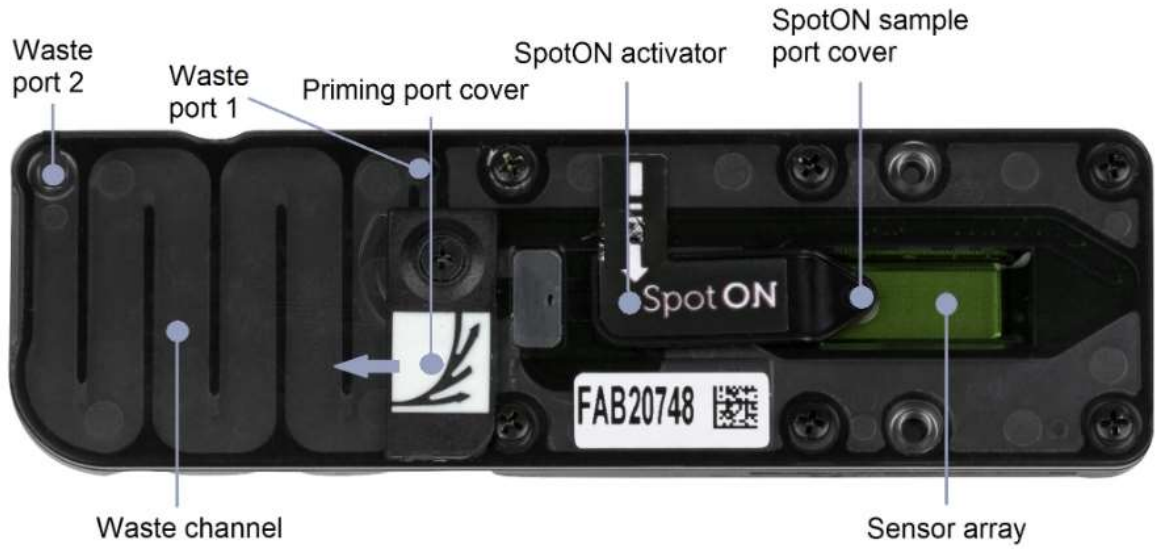
51. Thaw one tube of Wash Diluent (DIL) at room temperature. Mix thoroughly by vortexing, then spin down briefly and place on ice.

52. In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix:

Reagent	**Volume (µL)**
Wash Mix (WMX)	2
Wash Diluent (DIL)	398
Total	**400**

53. Mix well by pipetting and place on ice, do not vortex.

54. Remove the waste buffer from the flow cell which has finished the sequence run: with the priming port and SpotON sample port closed, insert a P1000 pipette into waste port 1 and remove the waste buffer.



55. Slide the flow cell priming port cover clockwise to open, insert a P1000 pipette into the flow cell priming port, withdraw 20-30 µL, visually check that there is continuous buffer from the flow cell priming port across the sensor array.

56. Use a P1000 pipette, add 200 µL of the flow cell wash mix into the priming port slowly, ensuing no bubbles are introduced, take out the pipette when there is a small volume of buffer in the pipette tip.

57. Incubate for 5 minutes.

58. Once the incubation is complete, carefully load the remaining 200 µL of the prepared flow cell wash mix into the priming port, as previous step.

59. Close the priming port and wait for 1 hour.

60. **To use the flow cell immediately for a second library loading**, close the priming port and SpotON sample port cover before removing the waste buffer, insert a P1000 pipette into waste port 1 and remove the waste buffer. Now this flow cell is ready to be primed for a second library loading.

62. **Or to store the flow cell** follow the steps below to add 500 µL sample buffer.

A. Thaw one tube of Storage Buffer (S) at room temperature.

B. Mix contents thoroughly by pipetting and spin down briefly.

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

D. Use a P1000 pipette setting to 200 µL, insert the tip into the flow cell priming port, withdraw 20-30 µL liquid slowly. Visually check that there is continuous buffer from the flow cell priming port across the sensor array.

E. Slowly add 500 µL of Storage Buffer (S) through the flow cell priming port.

F. Close the priming port.

G. Remove all fluid from the waste channel through waste port 1 using a P1000 pipette.

H. Store the flow cell at 4^{O}C.