

Direct RNA Sequencing Experiment (Sunday 25th June)

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Direct RNA Experiment

- Aim to sequence mRNA transcripts without PCR directly sensing RNA nucleotides as they pass through pores
 - No PCR bias (1 molecule, 1 sequence read = more quantitative)
 - Possibility to detect RNA methylation (dubious currently)
 - 3' end focused
 - Long reads possible, aim to maximise length (gentle handling to reduce shearing)
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- **6 Samples**
 - 3 Wildtype
 - 3 Scrambled controls

Direct RNA Sample Requirements

- **Total RNA (e.g. Trizol extracted, or RNeasy (Qiagen))**
 - 500ng starting requirement (in 9ul)
- **polyA enriched RNA**
 - 50ng starting requirement (in 9ul)
 - NEBnext (polyA+) - large loss of material (96-98%)
 - Ribo minus ?

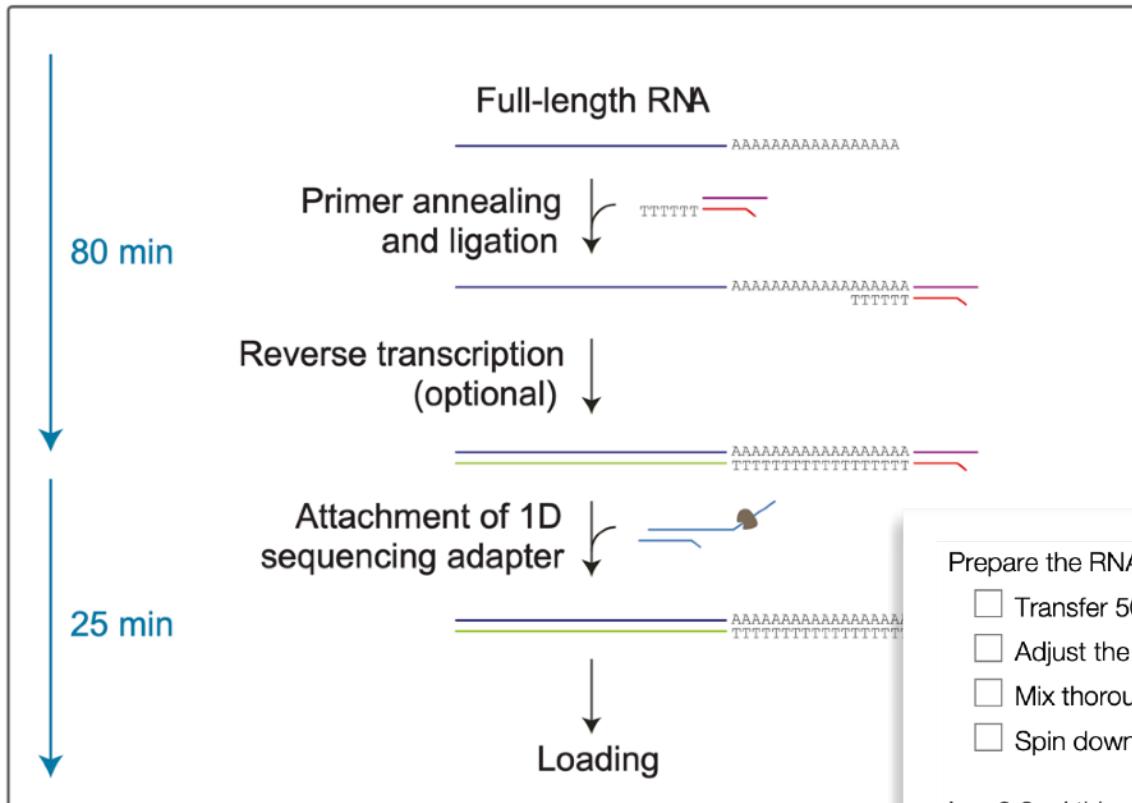
Samples

- **Tapestation analysis of samples yesterday showed issues**
 - Ribosomal peaks diminished, evidence of RNA degradation
 - microRNA fraction was probably ok
- **Vedrana brought total RNA and pellets**
 - Last night Vedrana and Carme used the pellets to harvest another batch of total RNA
 - Hoping for 400-600ng of RNA
 - Hoping for good tape station results
 - Awaiting news this morning on the samples

Direct RNA Setup

- 3 Computers available (2 Macintosh, 1 Linux)
 - USB 3.0 (4.8 Gbit/s, 600Mb per sec, Blue or Red USB port)
 - Fast Solid State Drives (SSDs) internal
 - Approx 100Gb-150Gb needed per flow cell
- We will run 2 minIONs per computer
- We have laptops in case of problems and can run 1 flow cell on each

The Protocol - Step I RT Primer Annealing



Prepare the RNA in Nuclease-free water.

- Transfer 50 ng of poly(A)-tailed RNA or 500 ng of total RNA into a 1.5 ml Eppendorf tube.
- Adjust the volume to 9 µl with Nuclease-free water.
- Mix thoroughly by flicking the tube to avoid unwanted shearing.
- Spin down briefly in a microfuge.

In a 0.2 ml thin-walled PCR tube, mix the reagents in the following order:

- 3.0 µl NEBNext Quick Ligation Reaction Buffer
 - 9.0 µl RNA
 - 0.5 µl RNA CS (RCS), 110 nM
 - 1.0 µl RT Adapter (RTA)
 - 1.5 µl T4 DNA Ligase
- Mix by pipetting and spin down.



RTA : RT adapter

RMX : RNA adapter mix

RCS : RNA CS

WSB : Wash buffer

ELB : Elution buffer

RRB : RNA running buffer

The Protocol - Step 2 Reverse Transcription

Mix the following reagents together to make the reverse transcription master mix:

- 9.0 µl Nuclease-free water
 - 2.0 µl 10 mM dNTPs
 - 8.0 µl 5x first-strand buffer
 - 4.0 µl 0.1 M DTT
-
- Add the master mix to the 0.2 ml PCR tube containing the RT adapter-ligated RNA from the "RT Adapter ligation" step. Mix by pipetting.
 - Add 2 µl of SuperScript III reverse transcriptase to the reaction and mix by pipetting.
 - Place the tube in a thermal cycler and incubate at 50°C for 50 min, then 70°C for 10 min, and bring the sample to 4°C before proceeding to the next step.
 - Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.



RTA : RT adapter

RMX : RNA adapter mix

RCS : RNA CS

WSB : Wash buffer

ELB : Elution buffer

RRB : RNA running buffer

The Protocol - First Clean (Ampure Beads)

- Resuspend the stock of Agencourt RNAClean XP beads by vortexing.
- Add 72 µl of resuspended RNAClean XP beads to the reverse transcription reaction and mix by pipetting.
- Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
- Prepare 200 µl of fresh 70% ethanol in Nuclease-free water.
- Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

Keep the tube on magnet, and wash the beads with 150 µl of freshly prepared 70% ethanol without disturbing the pellet as described below.

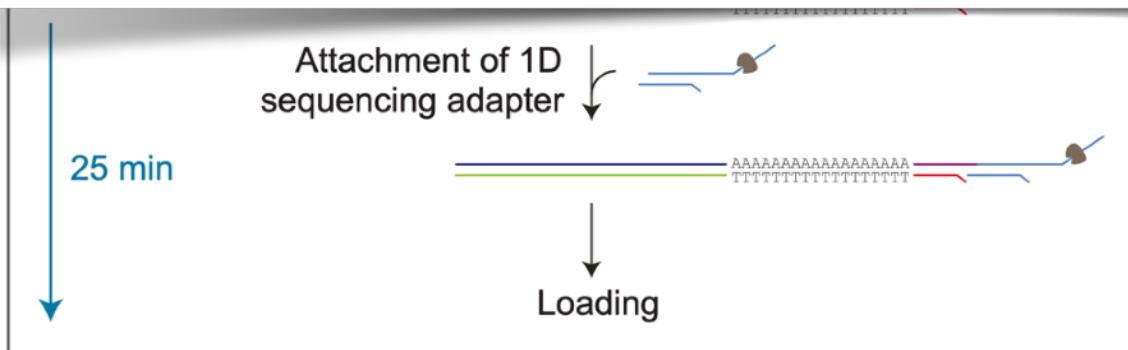
- Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet.
- Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet.
- Remove the 70% ethanol using a pipette, and discard.
- Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol.
- Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water. Incubate for 5 minutes at RT.
- Pellet the beads on a magnet until the eluate is clear and colourless.
- Pipette 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

The Protocol - Step 3 - Adapter Ligation (DNA+RNA+Protein)

In the same 1.5 ml Eppendorf DNA LoBind tube, mix the reagents in the following order:

- 8.0 µl NEBNext Quick Ligation Reaction Buffer
- 6.0 µl RNA Adapter (RMX)
- 3.0 µl Nuclease-free water
- 3.0 µl T4 DNA Ligase

- Mix by pipetting.



RTA : RT adapter

RMX : RNA adapter mix

RCS : RNA CS

WSB : Wash buffer

ELB : Elution buffer

RRB : RNA running buffer

The Protocol - Final Clean (Ampure Beads)

- Resuspend the stock of Agencourt RNAClean XP beads by vortexing.
- Add 16 µl of resuspended RNAClean XP beads to the adapter ligation reaction and mix by pipetting.
- Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
- Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
- Add 150 µl of the Wash Buffer (WSB) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.
- Repeat the previous step.
- Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer by gently flicking the tube. Incubate for 10 minutes at RT.
- Pellet the beads on a magnet until the eluate is clear and colourless.
- Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay - recovery aim ~20 ng.

The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.

Flow Cell Anatomy 2048 wells, 512 channels (4 muxes)

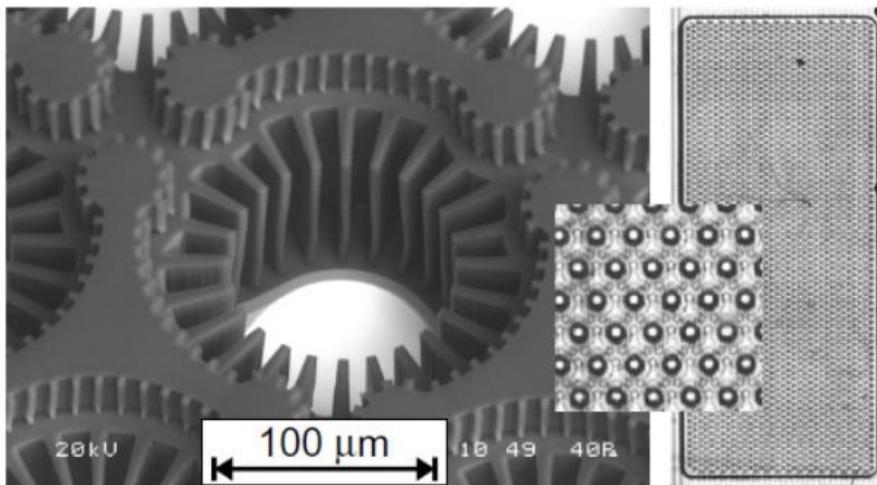


Figure 6.4. Scanning electron microscope image of a single sensor well (left) and the hexagonal pattern of wells in the array (right).

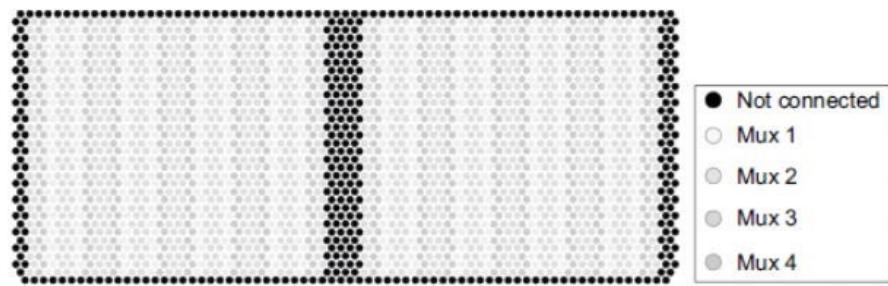
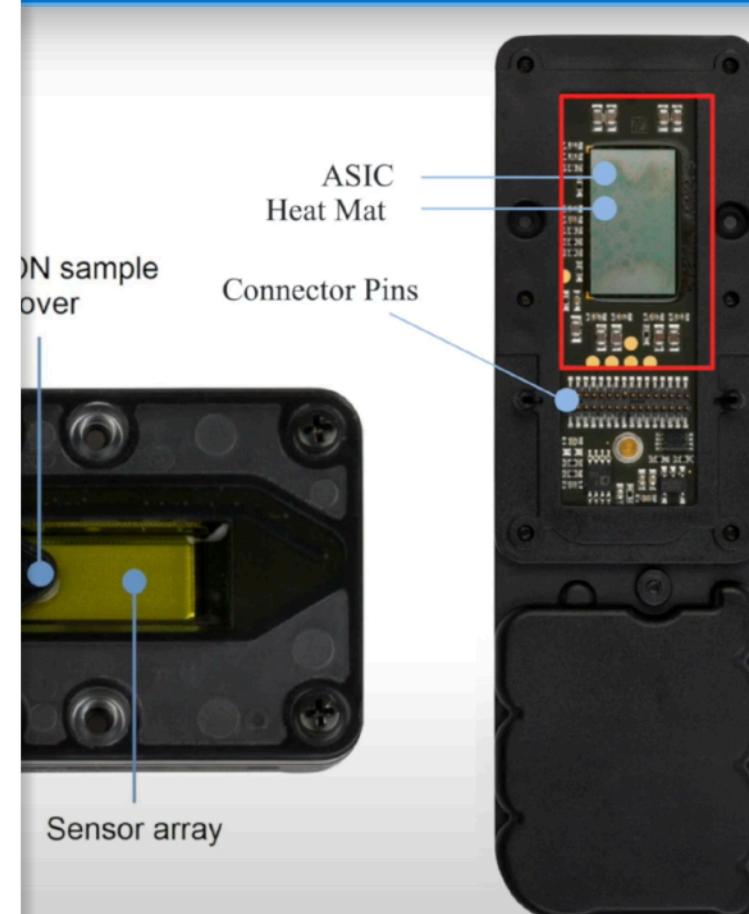


Figure 6.5. Multiplex layout of the nanopore sensor array for the MinION flow cell.

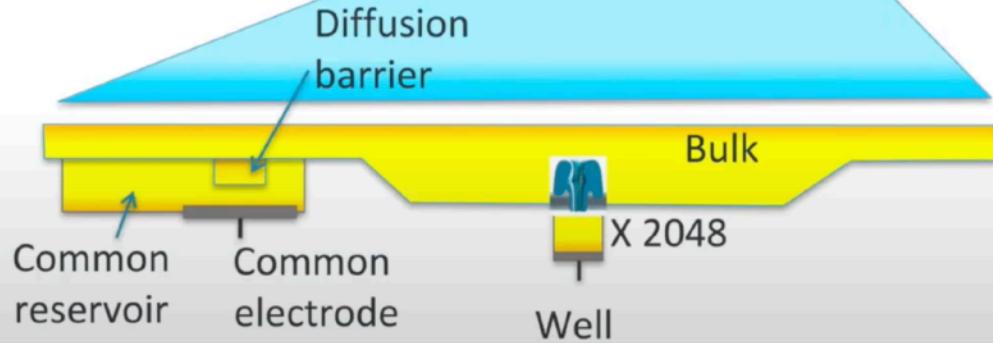
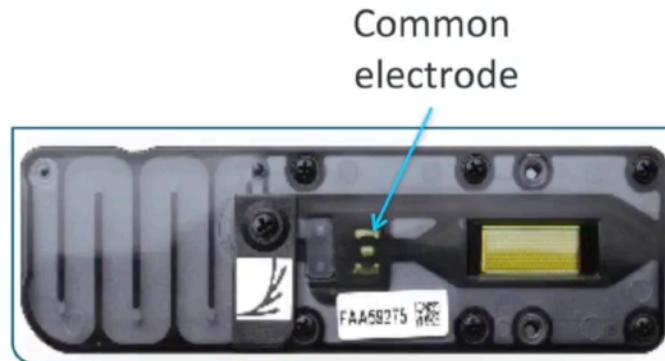


Flow Cell Anatomy

WHAT MAKES UP A FLOW CELL?

FLOW CELL

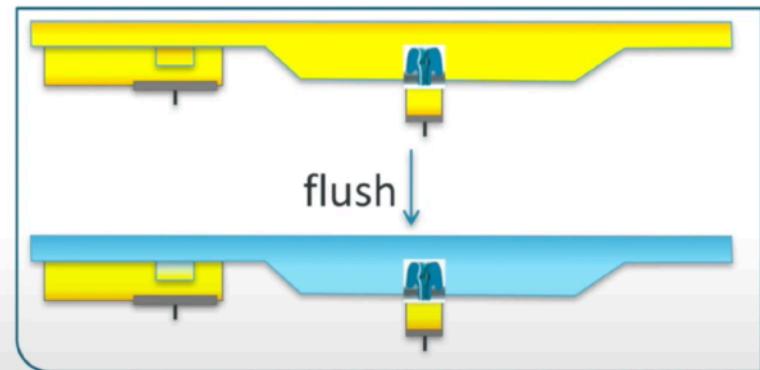
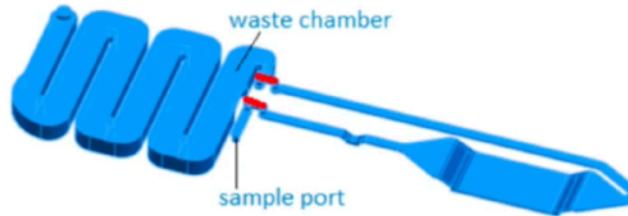
- Three main compartments
 - Common Reservoir
 - Bulk Buffer
 - Buffer in Wells
- Shipped with storage buffer in all 3 compartments.
- Separation allows change in running buffer without changing electrochemistry



Flow Cell Anatomy

FLOW CELL PRIMING

- Flush from the priming port.
- Priming mix made up of running buffer and nuclease free water.
- Aim to displace the storage buffer from the bulk compartment.
- Storage buffer remains in the reservoir and wells of the sensor array
- Important to avoid introducing air bubbles into the flow cell.



The Protocol - Priming the Flow Cell - Part I (Prep)

Priming and loading the SpotON flow cell

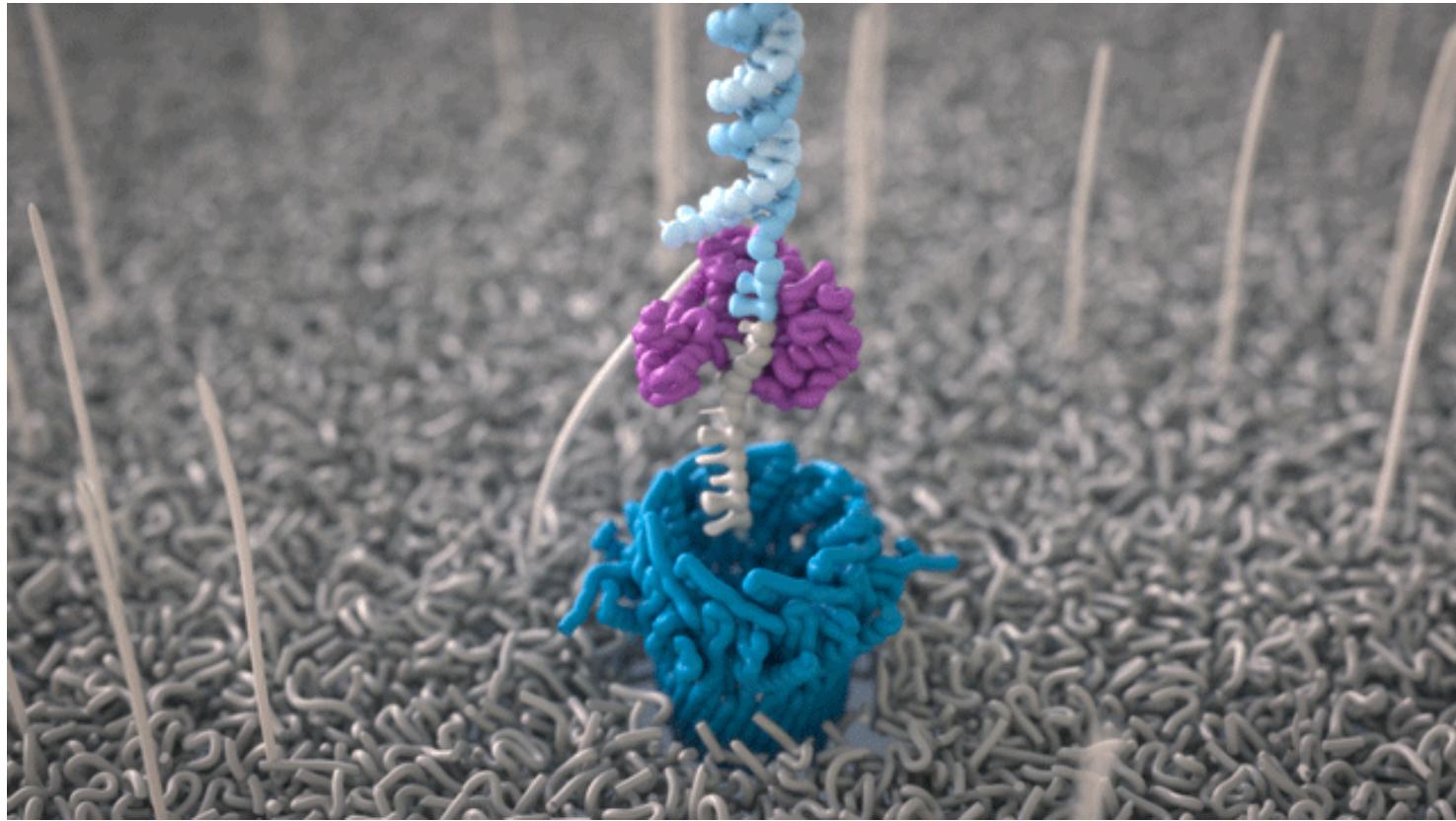
- Thaw the RNA Running Buffer (RRB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT.
- Mix the RNA Running Buffer (RRB), Flush Buffer (FB) and Flush Tether (FLT) tubes thoroughly by vortexing and spin down at RT.
- To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush 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 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.

FLT - Flush tether



Priming Part I - Priming Port

- **P1000 pipette**

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 μ l
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 μ l, to draw back 20-30 μ l, 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.

Priming the Flowcell

pulling a small volume of storage buffer back through priming port



The Protocol - Priming the Flow Cell - Part 2

- Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.

IMPORTANT

- Thoroughly mix the contents of the RRB tube by vortexing or pipetting, and spin down briefly.

- Take 20 µl of the prepared RNA library and mix it with 17.5 µl of Nuclease-free water.

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

- 37.5 µl RNA Running Buffer (RRB)
- 37.5 µl RNA library in Nuclease-free water

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.

Priming the Flowcell 2

Push Running Buffer through the sensor array - displacing storage buffer (waste channel)



Priming the Flowcell 2

Push Running Buffer through the sensor array - displacing storage buffer (waste channel)

Do not Pipette entire volume!!



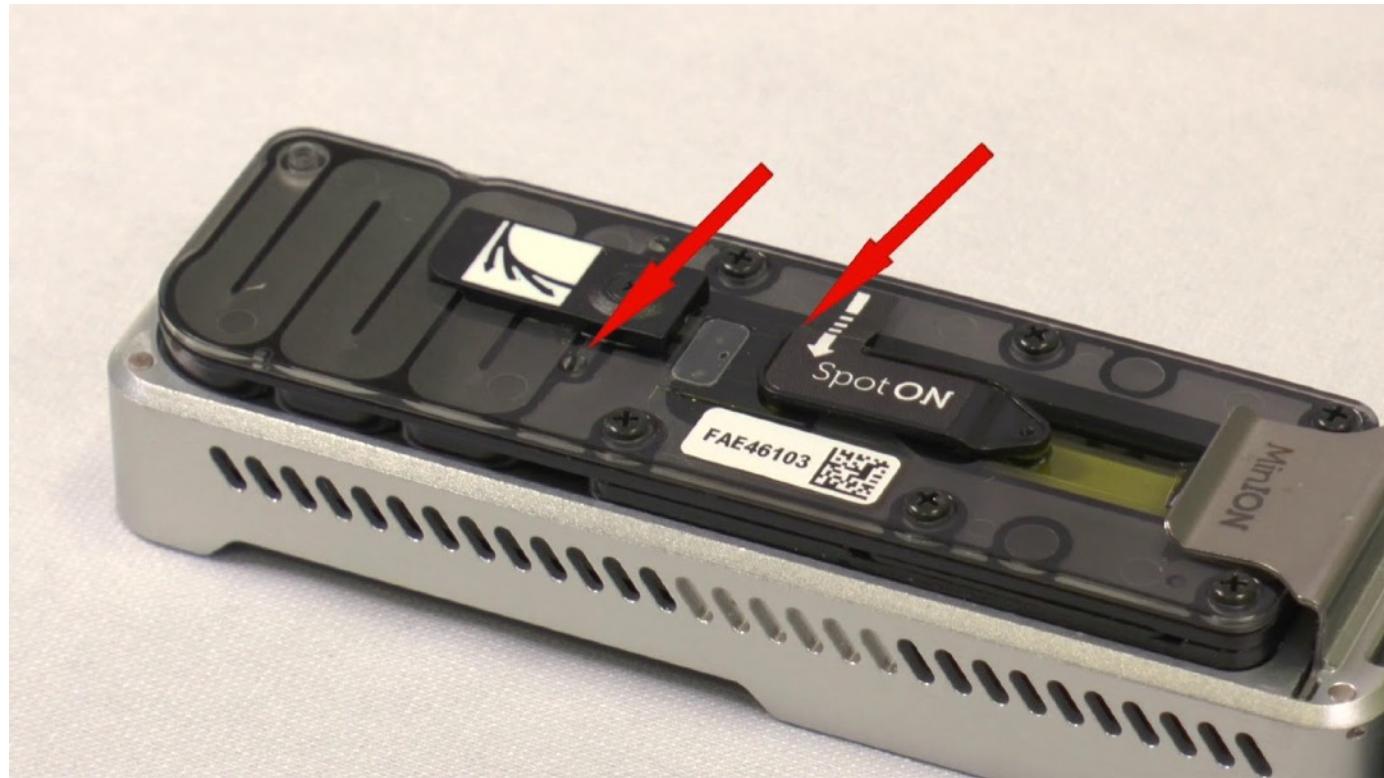
Priming the Flowcell 3

Push more priming mix up towards the sample port (SpotON port) **Both ports open**



Sample Loading

- SpotON Port -
NOT priming port
for sample
- Both ports open -
or sample will just
sit on top
- Drop Wise
- Do NOT touch
port with tip
- **P200 pipette**



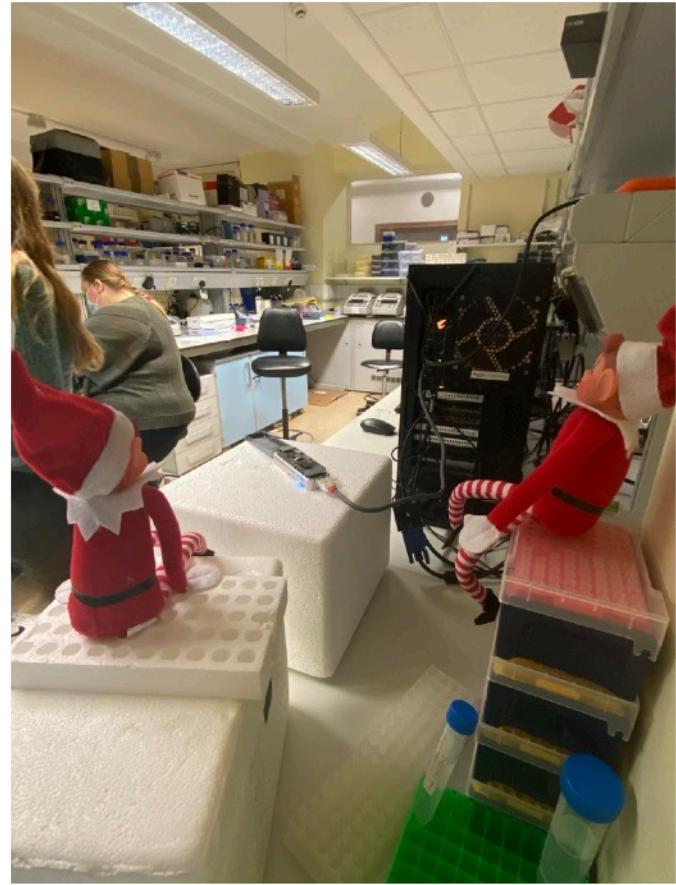
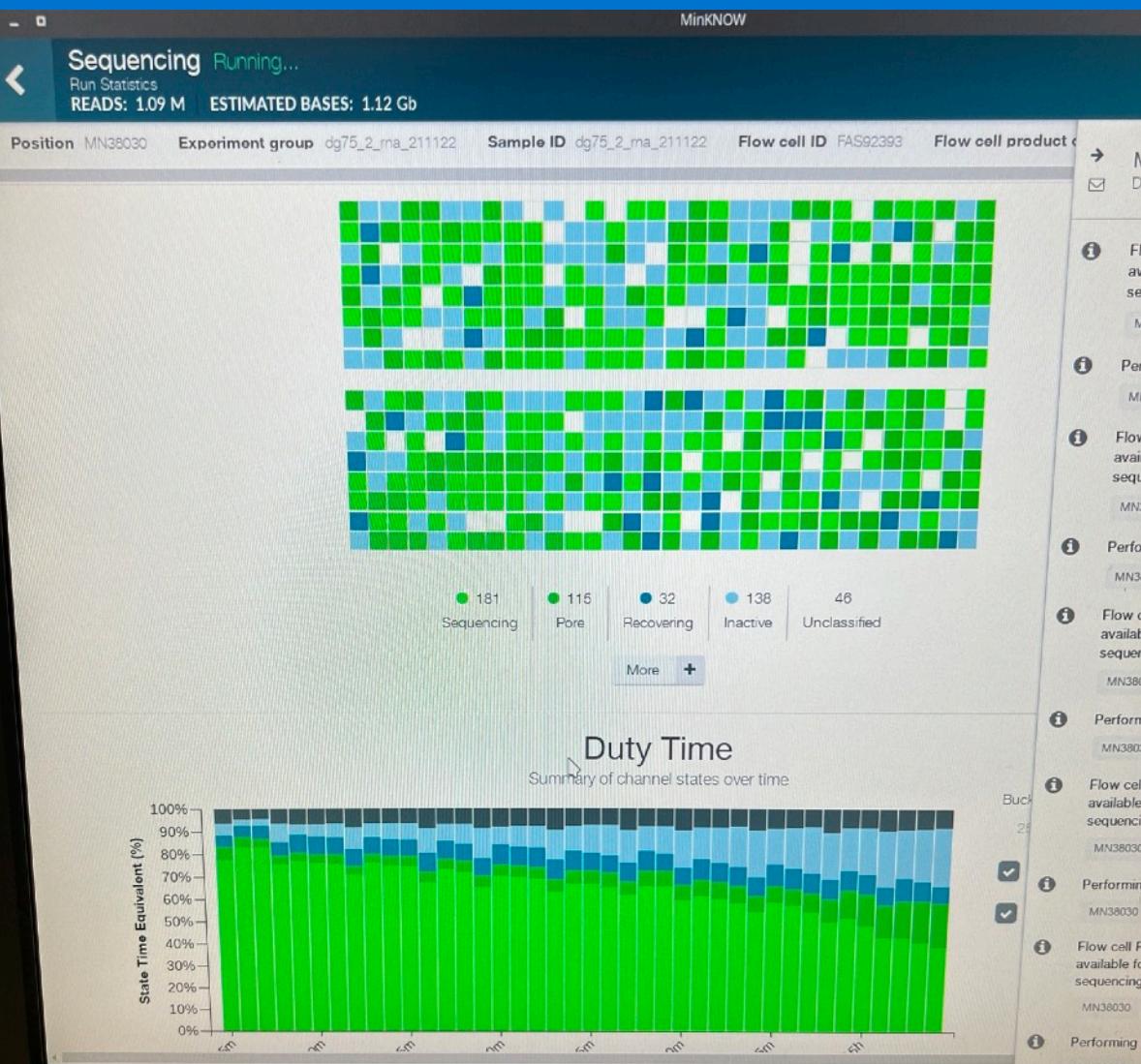
Flow Cell Practicalities

- **3 month shelf life (refrigerated)**
 - Reality is longer
- **On average 1200-1600 pores**
 - Largest we saw yesterday was 1700
 - Average 1500-1600
 - Can return for refund if less than a certain amount (900?)
 - Number of pores will influence number of reads (half the pores, half the reads)
 - Check pores when flow-cells arrive (write it on a label and then store again for next use)
- **3 Backup flow cells (pre-covid origin ?)**
 - 700, 800 and 1300 pores

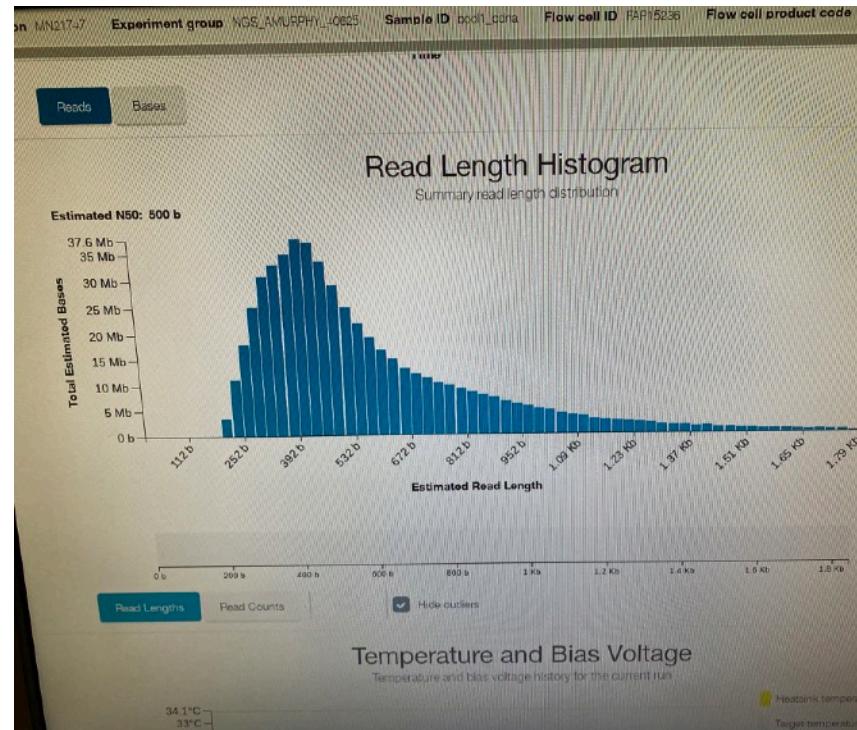
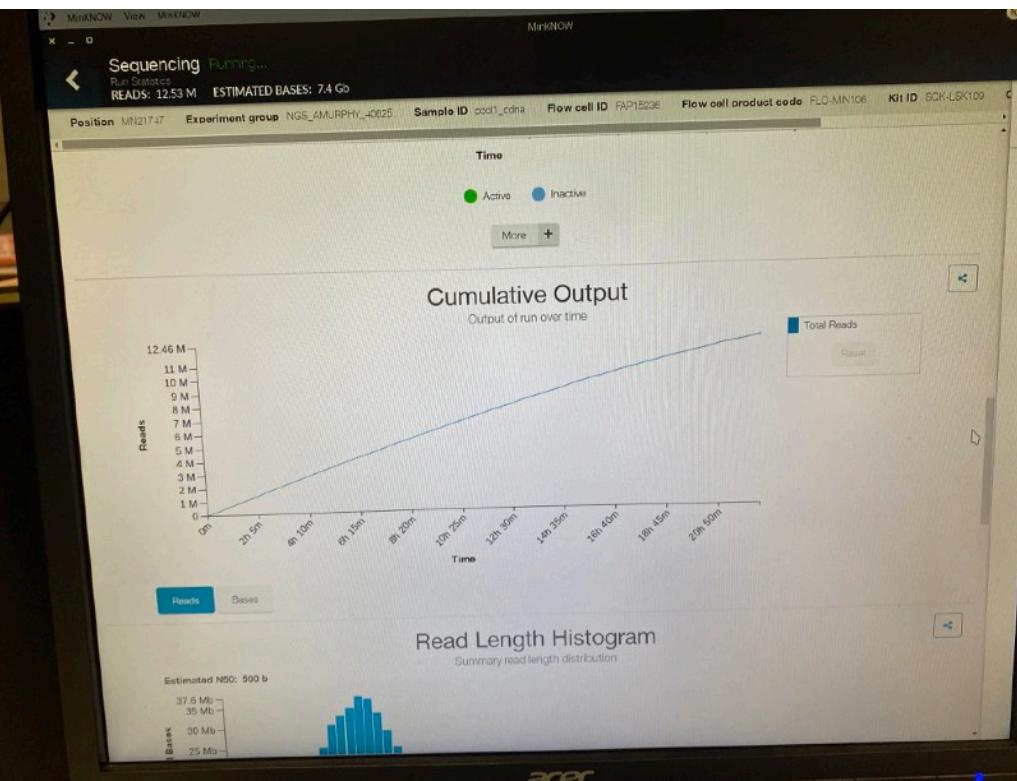
MinNOW Software

- MinION hardware check
- Flow-cell check
- **Starting and monitoring a sequencing run**
 - Choose experiment name, Flow cell version and Kit used (SQKRNA-002)
 - Run time (24, 48 or 96 hours common)
 - FAST5 output (compressed)
 - Make sure output folder is onto a fast solid state drive (SSD)

Graphical interface - Pore Health



Graphical Interface - Output and Lengths



Graphical Interface - Traceviewer

