Direct RNA sequencing (SQK-RNA002)

Overview

Version: DRS_9080_v2_revB_22Nov2018

Protocols are updated regularly, please check this is the latest version before proceeding. This protocol is for research only.

Overview

Overview of the Direct RNA Sequencing protocol

Direct RNA Sequencing Kit features

This kit is highly recommended for users who:

- are exploring attributes of native RNA such as modified bases
- would like to remove RT or PCR bias
- have transcripts that are difficult to reverse transcribe

Introduction to the Direct RNA Sequencing protocol

This protocol describes how to carry out sequencing of native RNA using the Direct RNA Sequencing Kit (SQK-RNA002).

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:
- Extract your RNA, and check its length, quantity and purity.

The quality checks performed during the protocol are essential in ensuring experimental success.

- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell(s) to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:
- Synthesise the complementary strand of the RNA
- Attach sequencing adapters supplied in the kit to the ends of the RNA-cDNA hybrid
- Prime the flow cell, and load your RNA library into the flow cell

Direct RNA sequencing (SQK-RNA002)

Prepare for your experiment

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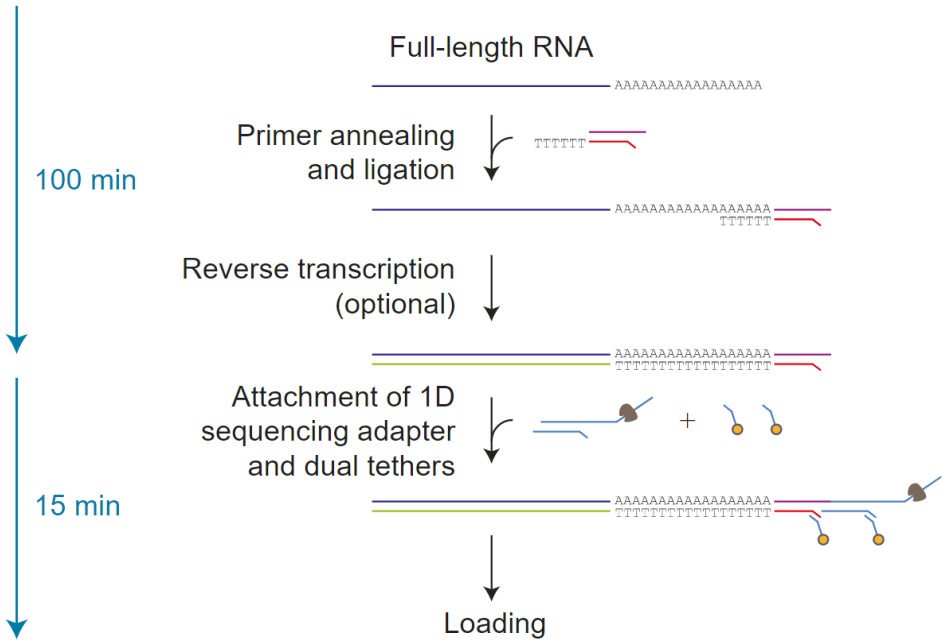


Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads

IMPORTANT

Please note that, unlike DNA, RNA is translocated through the nanopore in the 3'-5' direction. However, the basecalling algorithms automatically flip the data, and the reads are displayed 5'-3'.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Direct RNA Sequencing Kit (SQK-RNA002)
- FLO-MIN106 flow cells (FLO-MIN107 not advised)
- Albacore software for basecalling
- Wash Kit EXP-WSH002

Direct RNA sequencing (SQK-RNA002)

Preparing input RNA

Check the quality, quantity and formulation of the input polyA RNA

Materials	• 500 ng input polyA-tailed RNA
Consumables	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Optional Equipment	• Agilent Bioanalyzer (or equivalent) • Qubit fluorometer (or equivalent for QC check)

IMPORTANT

Importance of RNA QC

It is important that you check your input RNA for quality before beginning library preparation. Incorrectly quantified and/or contaminated RNA (e.g. salt, EDTA, protein, organic solvents) can have a significant impact on downstream processes and ultimately, your sequencing runs.
Below are some guidelines for how to check the RNA quality to ensure the highest possible throughput.

Access to laboratory equipment is not always possible in field conditions, however the recommendations should be used to optimise extraction and purification in the laboratory before doing fieldwork.

IMPORTANT

Input RNA mass and molarity

Oxford Nanopore's protocols recommend an input quantity in mass (e.g. 50 ng), as it is relatively easy to measure. The library preparation kit components are prepared with these input amounts in mind, but are robust to deviations from the input amount. However, if you are unble to quantify your input RNA mass, please use to the table below as a guide. Then, take forward the appropriate amount of RNA based on the average fragment length and known concentration.

Mass	Molarity if fragment length = 0.5 kb	Molarity if fragment length = 1.5 kb	Molarity if fragment length = 3 kb
50 ng	294 fmol	98 fmol	49 fmol
250 ng	1.5 pmol	490 fmol	245 fmol
500 ng	2.9 pmol	979 fmol	490 fmol

Prepare the RNA in nuclease-free water.

- Transfer 500 ng RNA into a DNA LoBind tube
- Adjust the volume to 9 µl with nuclease-free water
- Mix thoroughly by inversion
- Spin down briefly in a microfuge

Record the quality, quantity and size of the input RNA.

IMPORTANT

Criteria for input RNA

Before beginning library preparation, please ensure that the RNA sample meets the following criteria:

- Average fragment size: >500 bp
- Input mass, as measured by Qubit RNA HS assay: 500 ng
- No detergents or surfactants in the buffer

Assessment of RNA quality

- Chemical impurities such as detergents, denaturants, chelating agents and high concentrations of salts should be avoided as these may affect the efficiency of enzymatic steps.
- Other contaminants such as DNA, proteins and dyes may also reduce the efficiency of steps in the library preparation.
- The quality of RNA may be assessed by Nanodrop (for samples with concentration >20 ng/µl).
- We recommend that sample RNA has a 260/280 ~ 2.0 and a 260/230 ~2.0-2.2.
- A 260/280 which is lower than ~2.0 indicates the presence of DNA.
- A 260/280 which is lower than ~1.8 can indicate the presence of protein or phenol
- If the 260/230 is significantly lower than 2.0-2.2 indicates the presence of contaminants, and the RNA may need additional purification.
- Use the Agilent Bioanalyzer together with RNA Analysis Kits to assess whether the RNA has degraded.

Direct RNA sequencing (SQK-RNA002)

Equipment and consumables

Check the quality, quantity and formulation of the input polyA RNA

Materials	• Direct RNA Sequencing Kit (SQK-RNA002)	• Flow Cell Priming Kit (EXP-FLP001)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044) • Concentrated T4 DNA Ligase 2M U/ml (NEB M0202) • Agencourt RNAClean XP beads • Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)	• 0.2 ml thin-walled PCR tubes • Freshly prepared 70% ethanol in nuclease-free water • 10 mM dNTP solution (e.g. NEB N0447) • NEBNext® Quick Ligation Reaction Buffer (NEB B6058) • Qubit RNA HS Assay Kit (ThermoFisher Q32852)
Equipment	• Hula mixer (gentle rotator mixer) • Microfuge • Ice bucket with ice • Thermal cycler • P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips	• Magnetic separator, suitable for 1.5 ml Eppendorf tubes • Vortex mixer • Timer • Qubit fluorometer (or equivalent for QC check) • P200 pipette and tips • P20 pipette and tips • P2 pipette and tips
Optional Equipment	• Agilent Bioanalyzer (or equivalent)	• Eppendorf 5424 centrifuge (or equivalent)

Direct RNA Sequencing Kit contents

Contents	Colour	No. of tubes
RT Adapter (RTA)	blue	1
RNA Adapter Mix (RMX)	green	1
RNA CS (RCS)	yellow stripe	1
Wash Buffer (WSB)	orange stripe	2
Elution Buffer (ELB)	black	1
RNA Running Buffer (RRB)	red	1

Flow Cell Priming Kit contents

Contents	Description	No. of tubes
FLB (blue cap)	Flush Buffer	6
FLT (purple stripe cap)	Flush Tether	1

Direct RNA sequencing (SQK-RNA002)

Computer requirements and software downloads

Check the quality, quantity and formulation of the input polyA RNA

Direct RNA sequencing (SQK-RNA002)

Computer requirements and software downloads

Version: DRS_9080_v2_revB_22Nov2018

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the MinION device, collects sequencing data in real-time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment.

Albacore

The Albacore command-line software can be used for basecalling instead of MinKNOW. Albacore is the only software that can currently basecall reads from a Direct RNA sequencing experiment.

EPI2ME (optional)

The EPI2ME cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You will use the EPI2ME platform *only* if you would like further analysis of your data post-basecalling.

IMPORTANT

Software installation and updates

This section assumes that you have already installed the necessary software for your data analysis.
If not, please proceed to the Downloads page in the Community and complete the installation.

Otherwise, please check that you are using the latest version of our software, as described below.

Check for software updates

1. Open MinKNOW from the desktop icon, and follow the on-screen instructions to complete the automatic updates
2. If using an EPI2ME workflow, open the EPI2ME Desktop Agent and follow the on-screen instructions to complete the automatic updates

Required disk space for data

A minimum of 1 TB storage space is recommended. To avoid the risk of losing experimental data due to running out of disk space, it is recommended that the SSD is always cleared of old read data before a run.

IMPORTANT

Disabling sleep modes

To ensure that the experiment runs to completion, all sleep modes (including screensavers and log-offs) should be disabled on the host computer.

Direct RNA sequencing (SQK-RNA002)

Check your flow cell

Check the quality, quantity and formulation of the input polyA RNA

Consumables	• SpotCN Flow Cell	
Equipment	• MinICN	• Host computer connected to the Internet with MinKNCW and the Desktop Agent installed

In this step, you will use the MinKNOW software to check the number of pores in your flow cell. This has to be done within 10 days of receiving your flow cell. Flow cells can then be stored until required. If there is a delay between receiving and using the flow cells, another check should be performed before library preparation begins.

How it works

Flow cells are shipped with a QC DNA molecule present in the buffer. This molecule produces a distinctive nanopore signal. The MinKNOW™ software uses this signal to validate the integrity of the nanopore array before use and provides the user with an estimate of the number of simultaneously available channels for the experiment. Active pores are reported in four groups, each of which may be used in turn when running long experiments e.g. a 48 hour sequencing run. Shorter experiments will use fewer than four groups.

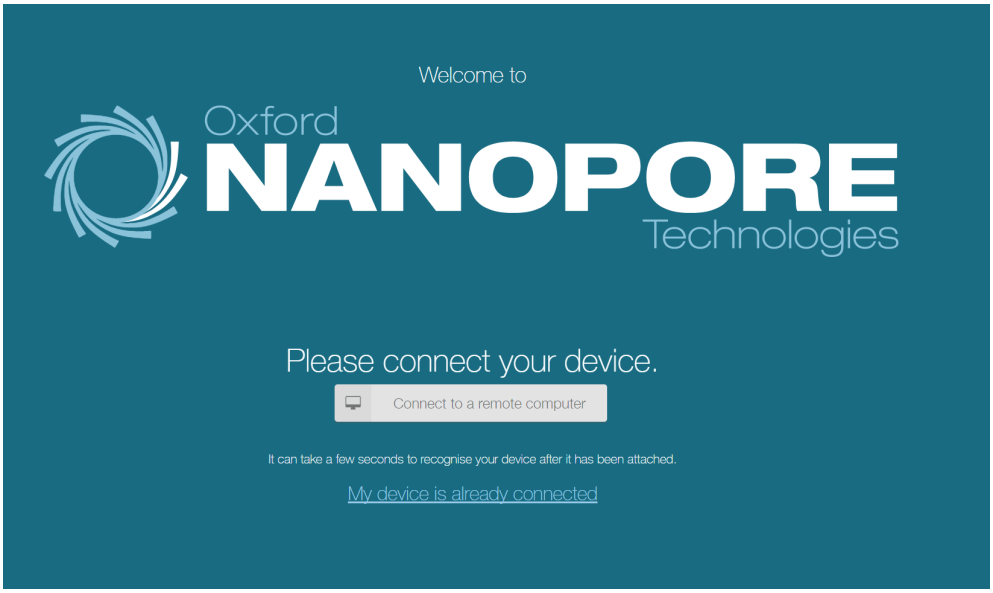
Set up the MinION, flow cell and host computer

Figure SSFC: Assembled MinION and MinION SpotON Flow Cell connected to through the USB 3.0 port to the host computer

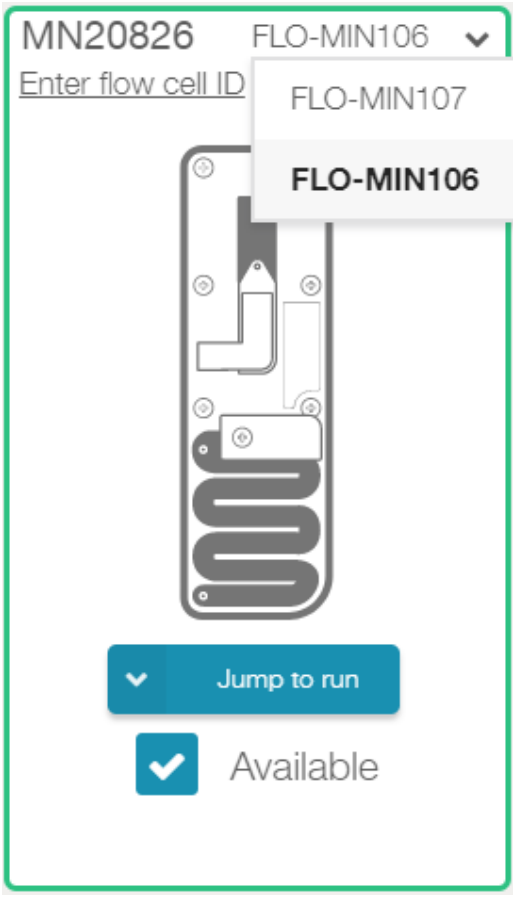
Once successfully plugged in, you will see a light and hear the fan.

Open the MinKNOW GUI from the desktop icon and establish a local or remote connection.

- If running a MinION on the same host computer, plug the MinION into the computer.
- If running a MinION on a remote computer, first enter the name or IP address of the remote host under **Connect to a remote computer** (if running from the Connection page), or **Connections** (if running from the homepage) and click **Connect**.



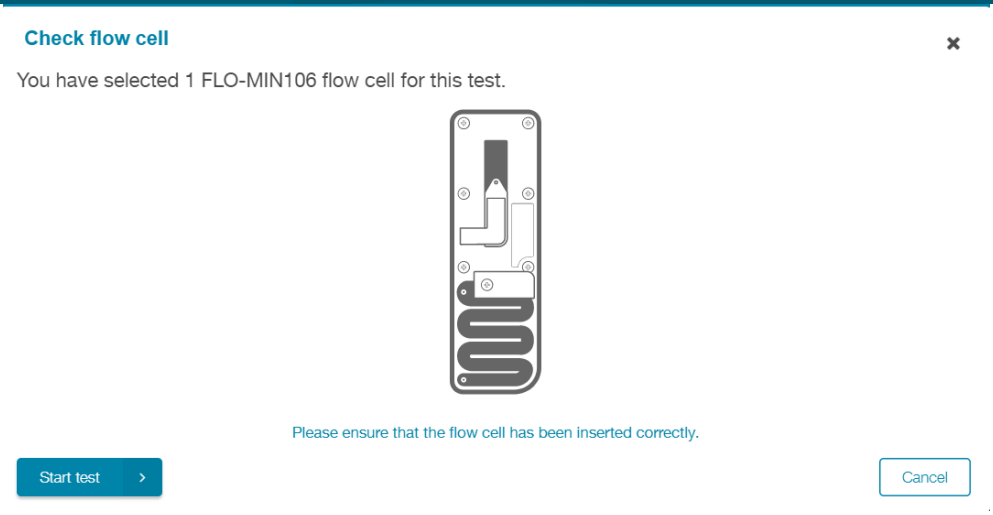
- Choose the flow cell type from the selector box. Then mark the flow cell as "Selected":



Click "Check flow cells" at the bottom of the screen.

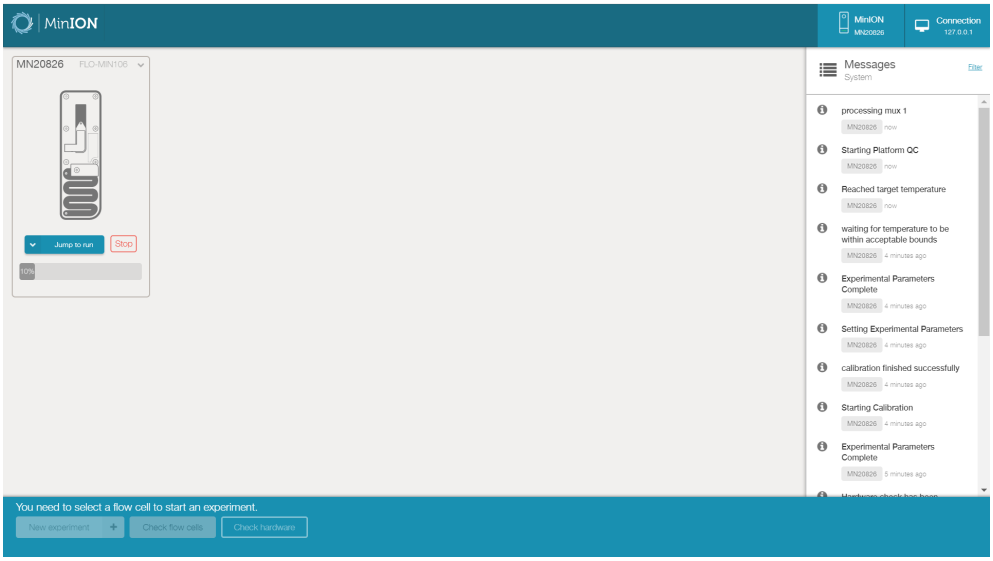
A screen will load displaying the number of flow cells selected for your test, and offering a drop down for flow cell type. It is important the correct flow cell type is chosen to obtain an accurate result:

Flow cell code	Pore type
FLO-MIN106	R9.4.1
FLO-MIN107	R9.5.1

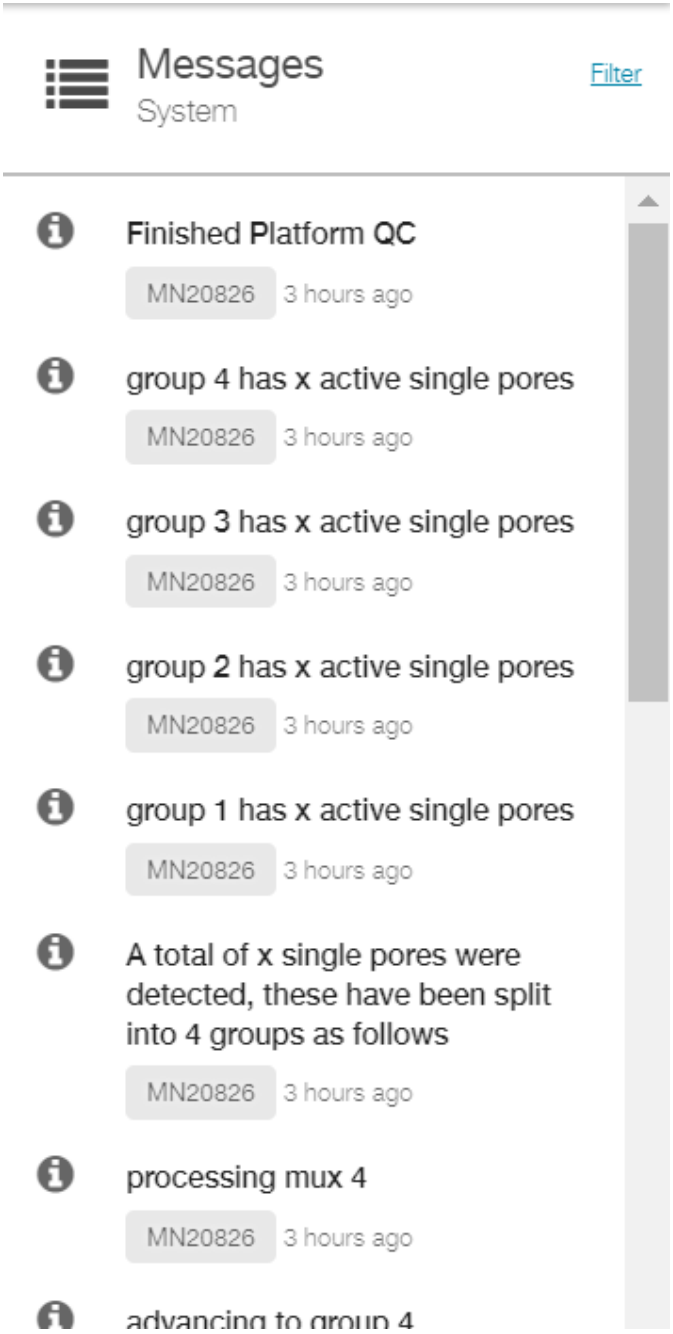


Click "Start test".

The flow cell will become greyed out, and will show the run progress:



Check the number of active pores available for the experiment, reported in the System History panel when the check is complete.



END OF STEP

Flow cell check complete.

The total number of pores available will be reported in the **notification panel.**

If the flow cell check is completed within 5 days of receipt, for the flow cell warranty will be activated.

Warranty for flow cells: 800 nanopores or above

Direct RNA sequencing (SQK-RNA002)

Library preparation

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes

Materials

- 500 ng input polyA-tailed RNA in 9 µl
- RNA CS (RCS)
- Wash Buffer (WSB)
- RT Adapter (RTA)
- RNA Adapter (RMX)
- Elution Buffer (ELB)

Consumables

- Concentrated T4 DNA Ligase 2M U/ml (NEB M0202)
- 0.2 ml thin-walled PCR tubes
- Agencourt RNAClean XP beads
- 1.5 ml Eppendorf DNA LoBind tubes
- 10 mM dNTP solution (e.g. NEB N0447)
- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Thermal cycler
- Hula mixer (gentle rotator mixer)

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

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

Reagent	Volume
NEBNext Quick Ligation Reaction Buffer	3.0 µl
RNA	9.0 µl
RNA CS	0.5 µl
RT Adapter (RTA)	1.0 µl
T4 DNA Ligase	1.5 µl
Total	**15 µl**

Mix by pipetting and spin down.

Incubate the reaction for 10 minutes at room temperature.

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

Reagent	Volume
Nuclease-free water	9.0 µl
10 mM dNTPs	2.0 µl
5x first-strand buffer	8.0 µl
0.1 M DTT	4.0 µl
Total	**23.0 µl**

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 1.5 ml DNA LoBind Eppendorf tube.

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 room temperature.

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.

1. 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.
2. 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 room temperature.

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.

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

Reagent	Volume
Reverse-transcribed RNA from the "Reverse Transcription" step	20.0 µl
NEBNext Quick Ligation Reaction Buffer	8.0 µl
RNA Adapter (RMX)	6.0 µl
Nuclease-free water	3.0 µl
T4 DNA Ligase	3.0 µl
Total	**40 µl**

Mix by pipetting.

Incubate the reaction for 10 minutes at room temperature.

Resuspend the stock of Agencourt RNAClean XP beads by vortexing.

Add 40 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 room temperature.

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.

> **IMPORTANT**
>
> **Agitating the beads by flicking results in a more efficient removal of free adapter, compared to adding the wash buffer and immediately aspirating.**

Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer. Incubate for 10 minutes at room temperature.

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 ~200 ng.

> **END OF STEP**
>
> **The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.**

Direct RNA sequencing (SQK-RNA002)

Priming and loading the SpotON flow cell

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes

Materials

- Prepared RNA library
- Flush Tether (FLT)
- RNA Running Buffer (RRB)
- Flush Buffer (FLB)

Consumables

- SpotCN Flow Cell
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Thaw the RNA Running Buffer (RRB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at room temperature before placing the tubes on ice as soon as thawing is complete.

Mix the RNA Running Buffer (RRB) and Flush Buffer (FLB) tubes thoroughly by vortexing, spin down and return to ice.

Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

Flip back the MinION lid and slide the sample port cover clockwise to that the sample port is visible.

Priming and loading the SpotON Flow Cell

Priming and loading: The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.

Take care to avoid introducing any air during pipetting.

IMPORTANT

Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks damaging the pores in the array.

After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 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.

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 (FLB), and mix by pipetting up and down.

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.

> **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:

Reagent	Volume
RRB	37.5 µl
RNA library in nuclease-free water	37.5 µl
Total	**75 µl**

Complete the flow cell priming:

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the 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.

Add 75 µl of sample 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 lid.

Direct RNA sequencing (SQK-RNA002)

Starting a sequencing run

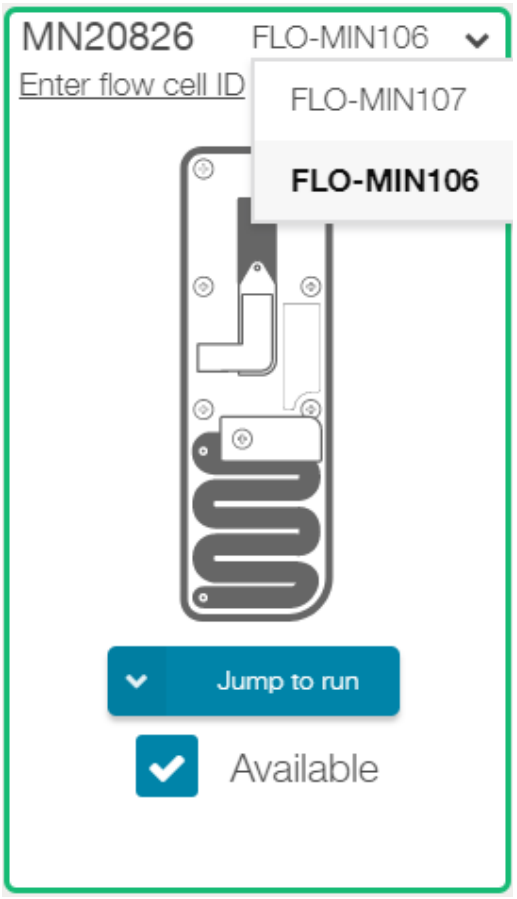
Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes ~5 minutes

Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI.

If your MinION was disconnected from the computer, plug it back in.

Alternatively, if running a MinION on a remote computer, first enter the name or IP address of the remote host under **Connect to a remote computer** (if running from the Connection page), or **Connections** (if running from the homepage) and click **Connect**.

Choose the flow cell type from the selector box. Then mark the flow cell as "Selected".

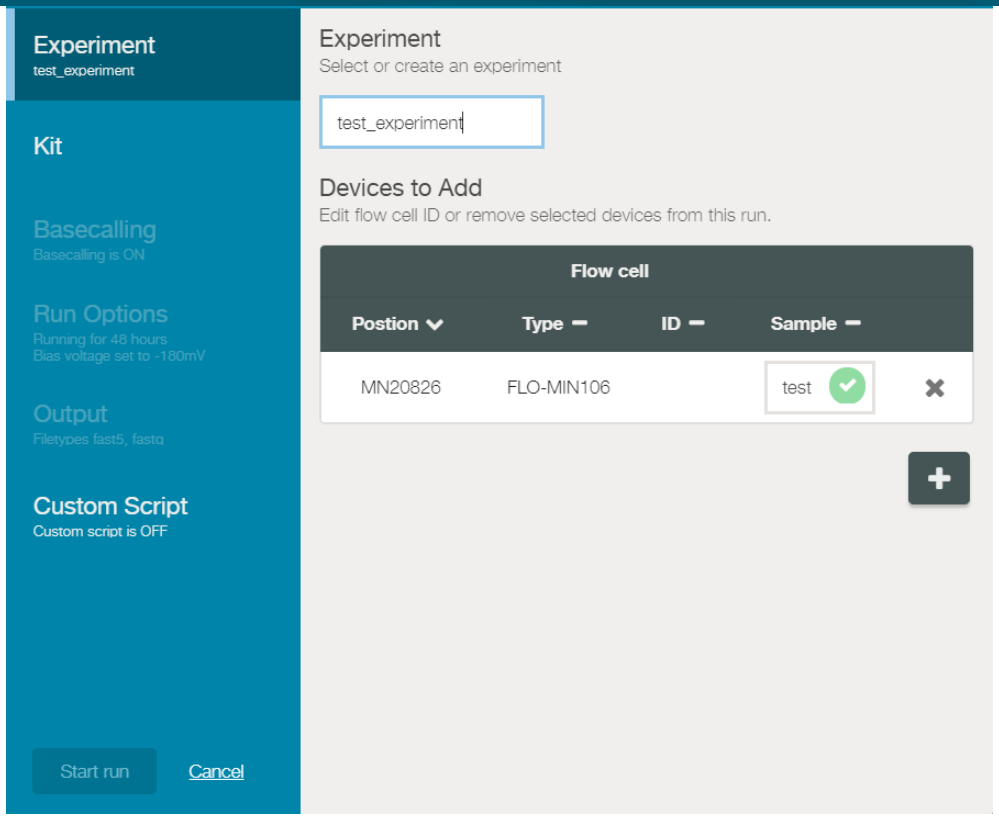


Click the "New Experiment" button at the bottom left of the GUI.

On the New experiment popup screen, select the running parameters for your experiment from the individual tabs.

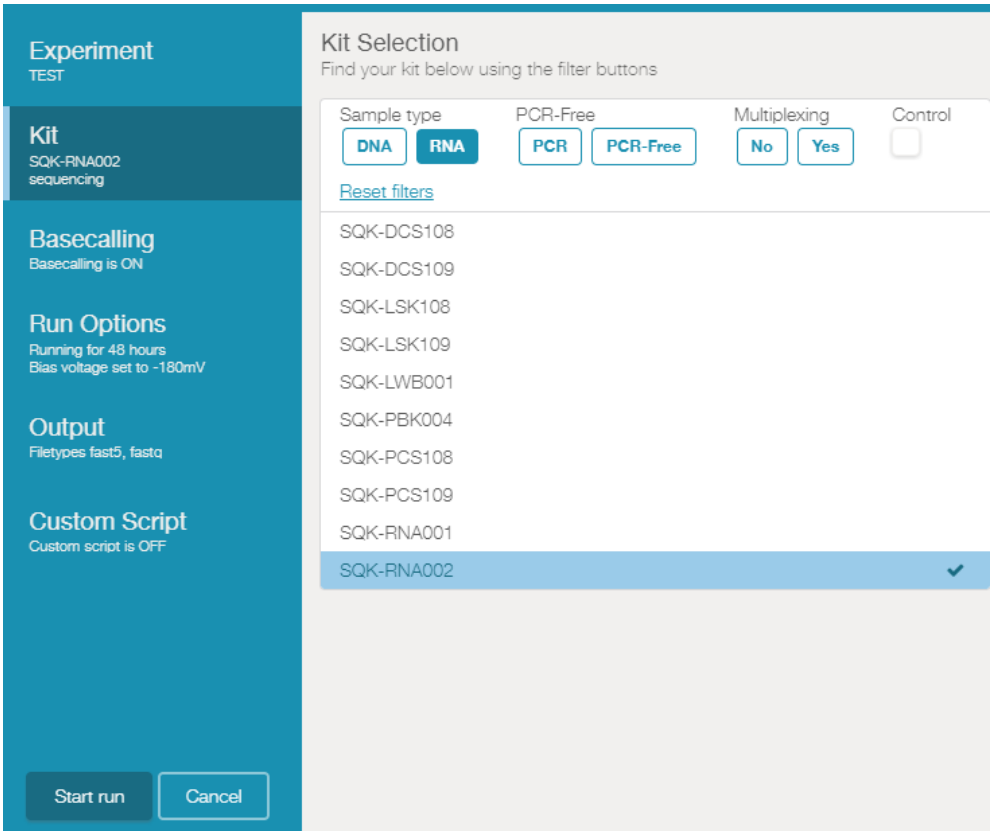
Experiment name

The experiment name tab will show the chosen flow cell. An experiment name can then be assigned.
The other tabs will not become available until an experiment name has been provided.



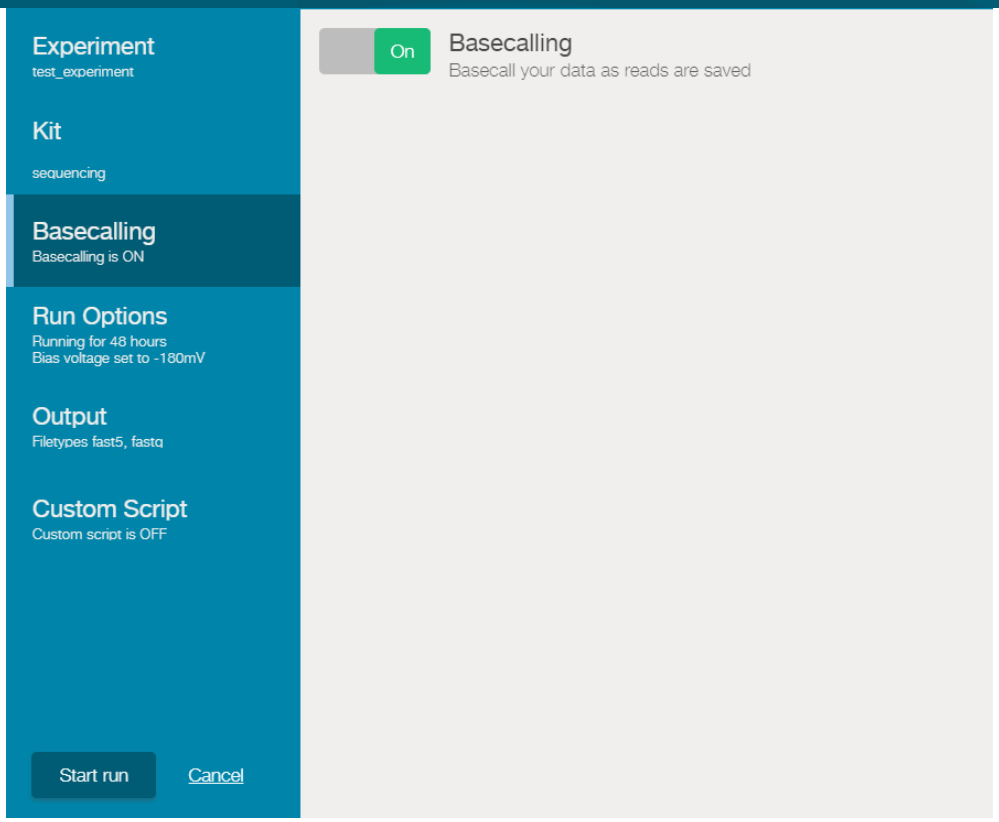
Kit selection

The kit selection tab will provide a dropdown of available kits. Select **SQK-RNA002**.



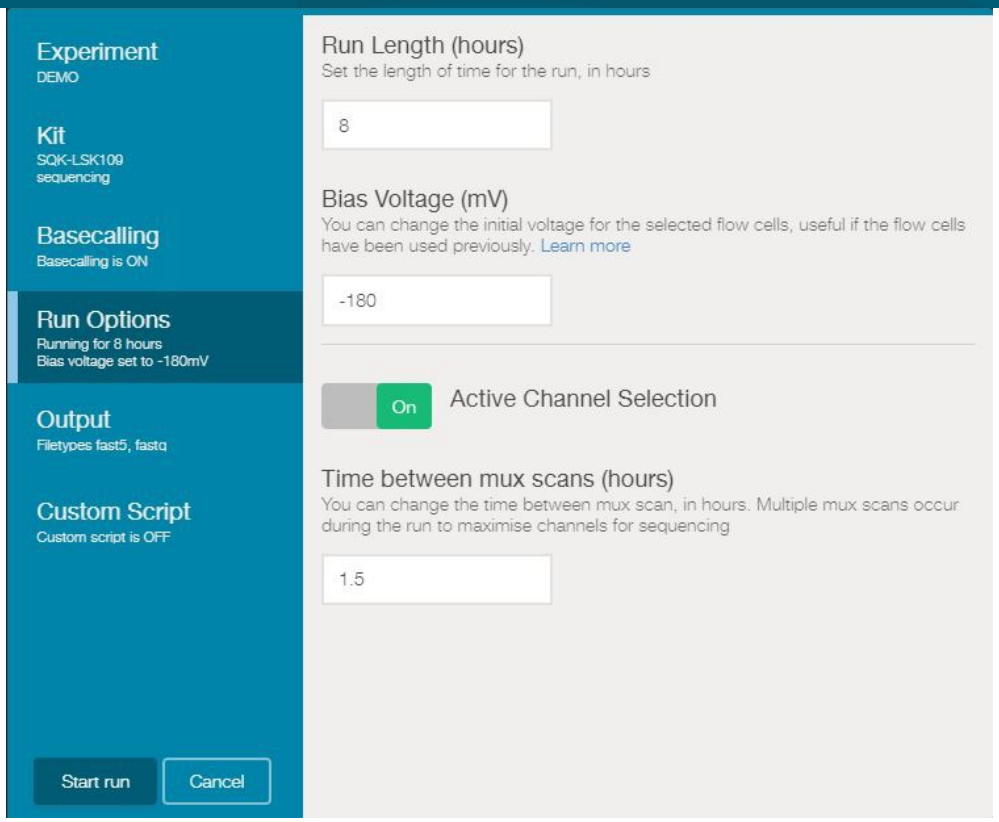
Basecalling

Select whether or not you want your data basecalled live on the instrument.



Run Options

The run options tab provides variables for run time and starting voltage. By default these will be 48 hours and -180 mV. Active Channel Selection refers to a feature introduced in MinKNOW v2.2. If a channel is in the "Saturated" or "Multiple" state, the software instantly switches to a new channel in the group. If a channel is "Recovering", MinKNOW will attempt to revert the channel back to "Pore" or "Sequencing" for ~5 minutes, after which it will select a new channel in the group. This maximises the number of channels sequencing at the start of the experiment.



Output

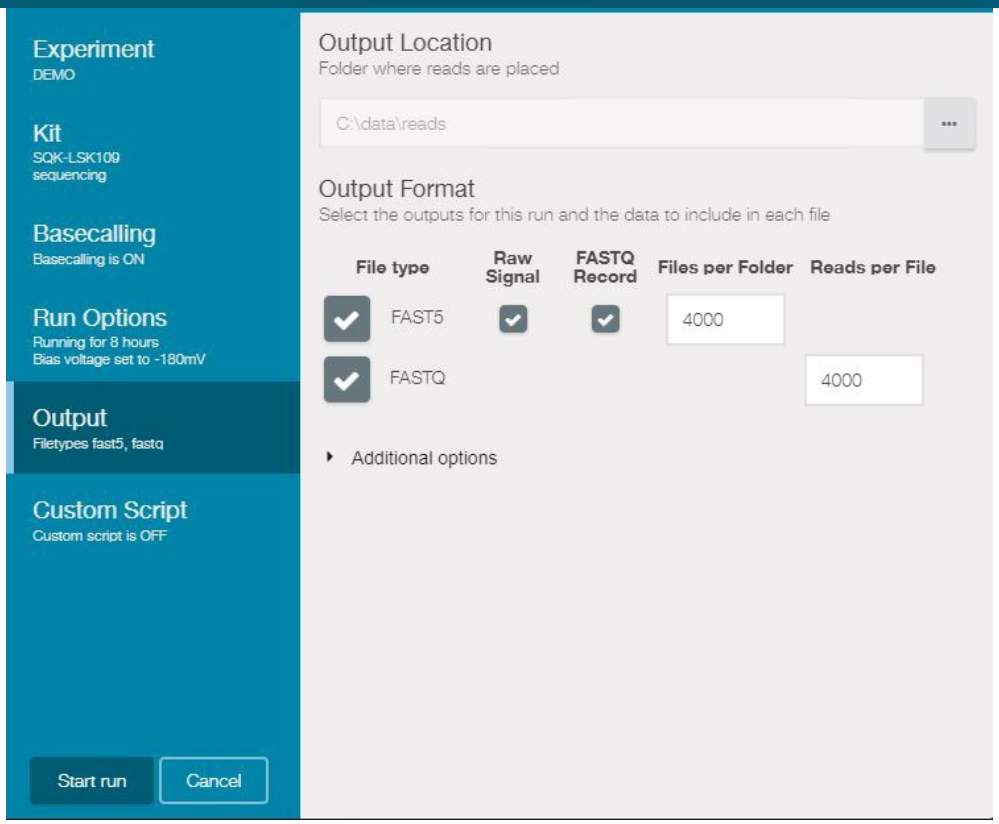
You can specify to produce:

1) Just .fast5 files (with basecall information in them)
2) Just .fastq files
3) Both .fast5 and .fastq files

The options available for each file type are:

- **Output settings - FASTQ:** The number of basecalls that MinKNOW will write in a single file. By default this is set to 4000
- **Output settings - FAST5:** The number of files that MinKNOW will write to a single folder. By default this is set to 4000

There are additional options for the information contained in .fast5 files. By default these are all stored within the .fast5 file:
- raw data
- event data
- .fastq basecalls



IMPORTANT

Please note that, unlike DNA, RNA is translocated through the nanopore in the 3'-5' direction. However, the basecalling algorithms automatically flip the data, and the reads are displayed 5'-3'.

Click "Start run".

The pop up box will disappear, and the flow cell will become greyed out.

Allow the script to run to completion.

- The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen
- Monitor messages in the Message panel in the MinKNOW GUI

Basecalled read files

The basecalled read files are stored in the Output Location specified during MinKNOW installation, or experiment set-up. By default, this is:

:\data\ for MinION
/data/ for GridION

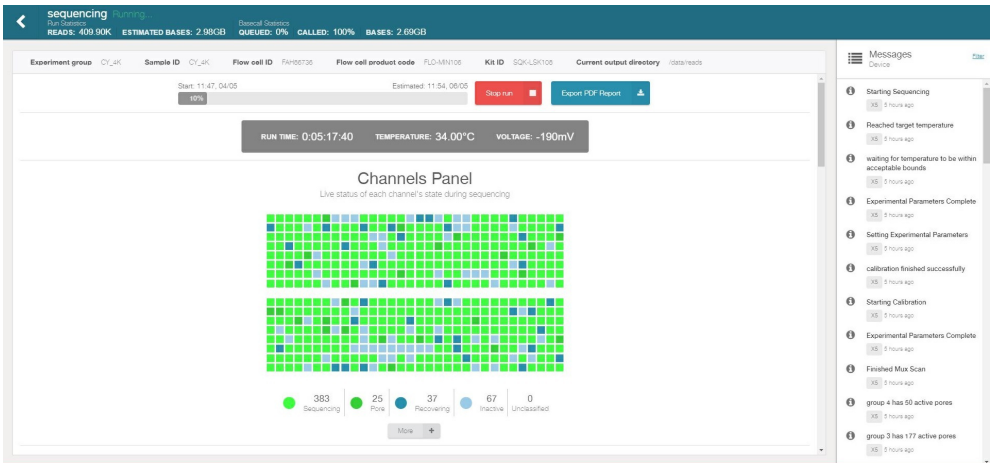
Direct RNA sequencing (SQK-RNA002)

Progression of MinKNOW protocol script

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes ~5 minutes

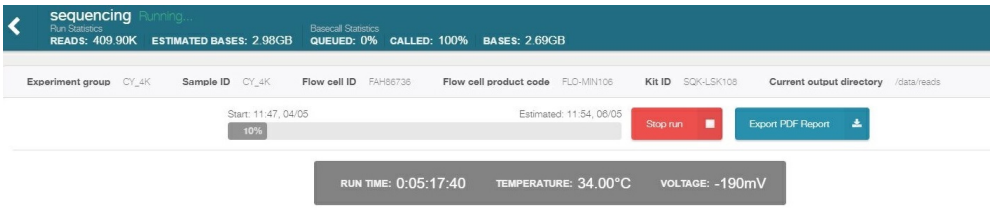
The running experiment screen

As your experiment progresses, you can obtain real-time feedback for your flow cell from the running experiment screen. For additional information regarding the plots on this screen, see below.



Experiment summary information

In addition to the flow cell information, the status bar at the top of the screen will display information relating to the expeirment as a whole.



- **Run statistics:** The total number of reads and bases produced across the experiment
- **Basecall statistics:** There are two values for basecalled reads:

1. Basecalled reads as a percentage of the total reads produced across the experiment. This gives an indication as to the size of the queue for reads to be basecalled
2. Total number of reads basecalled across the experiment

- **Run time:** The duration of the experiment
- **Temperature:** The heatsink temperature of the selected position
- **Voltage:** The applied potential of the position at that point in time

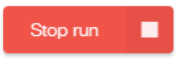
TIP

As the MinKNOW script progresses, you can check the following:

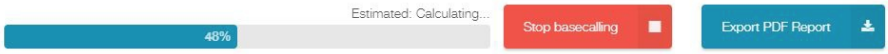
- Number of active pores
- Heatsink temperature
- Development of the read histogram
- Pore occupancy
- Local basecalling report

Check the number of active pores reported in the MUX scan are similar (within 10-15%) to those reported at the end of the Flow Cell Check

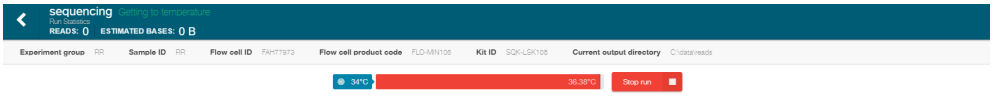
- If there is a significant reduction in the numbers, restart MinKNOW.
- If the numbers are still significantly different, close down the host computer and reboot.
- When the numbers are similar to those reported at the end of the Flow Cell Check, restart the experiment on the Connection page. There is no need to load any additional library after restart.
- Stopping the experiment is achieved by clicking "Stop run" button at the top of the screen.



Data acquisition will stop, but the software will continue basecalling unless the user clicks the "Stop basecalling" button.



Check that the temperature has reached 34° C.



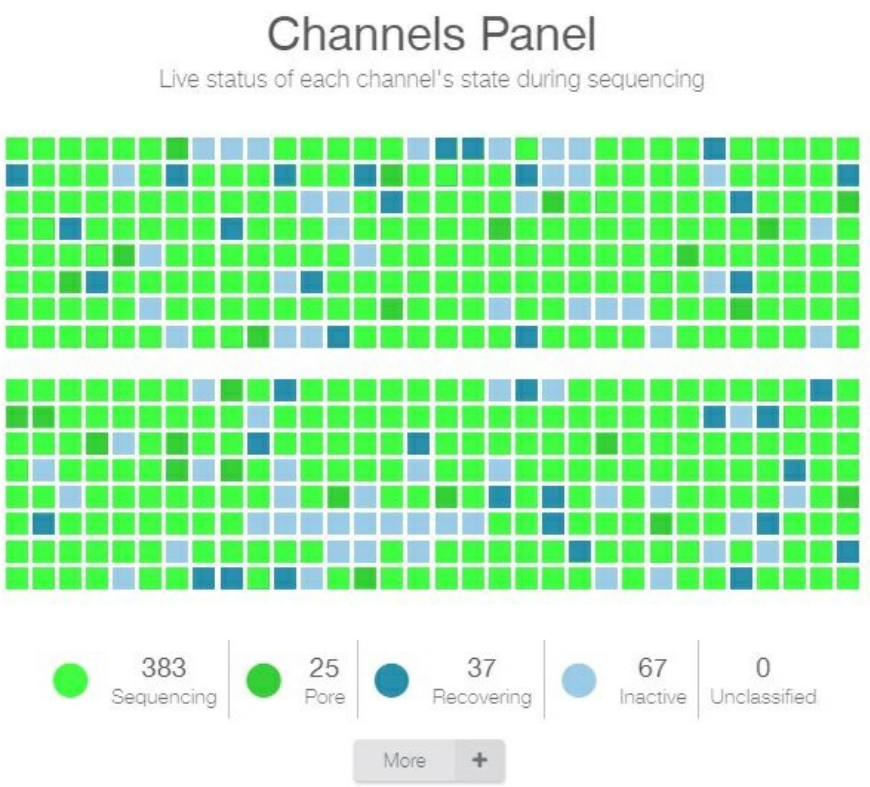
MinION temperature control

The MinION is able to maintain a temperature of 34° C on a typical lab bench when the local ambient conditions are between 19.5° C and 24.5° C. However, there are a number of external factors which can disrupt the local conditions and which need to be taken into account, for example warm air expelled from laptops, or cool air from a fan or air conditioning system increasing airflow around the MinION.

The MinION takes approximately 10 minutes to get to temperature.

Check pore occupancy in the channel panel at the top of the experimental view.

- A good library will be indicated by a higher proportion of light green channels in **Sequencing** than are in **Pore**. The combination of Sequencing and Pore indicates the number of active pores at any point in time. A low proportion of Sequencing channels will reduce the throughput of the run.
- **Recovering** indicates channels that may become available for sequencing again. A high proportion of this may indicate additional clean up steps are required during your library preparation.
- **Inactive** indicates channels that are no longer available for sequencing. A high proportion of these as soon as the run begins may indicate an osmotic imbalance.
- **Unclassified** are channels that have not yet been assigned one of the above classifications



Clicking on the "More" button reveals a more detailed array of channel states:

- **Strand:** the channel is in strand
- **Adapter:** the channel has just captured a new strand
- **Single pore:** the channel appears to show a single pore. Available for sequencing
- **Unavailable:** the channel appears to show a pore that is currently unavailable for sequencing
- **Active feedback:** the channel is reversing the current flow to eject the analyte
- **Out of range 2:** current level is between 10 and 9999 pA. Currently unavailable for sequencing
- **Possible multiple:** the channel appears to show more than one pore. Unavailable for sequencing
- **Saturated:** the channel has switched off due to current levels exceeding hardware limitations
- **Out of range 1:** current level is between -5 and -9999 pA. Currently unavailable for sequencing
- **Zero:** Current level is between -5 and 10 pA. Currently unavailable for sequencing.

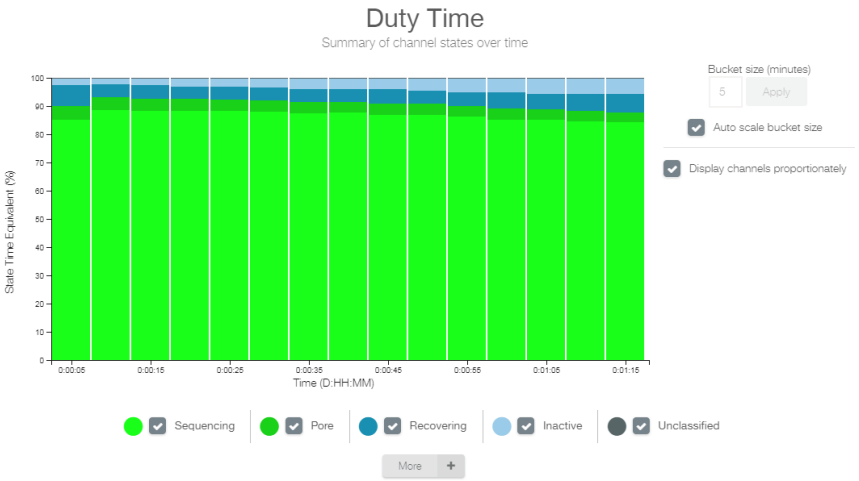
Monitor the pore occupancy

It is recommended that you monitor the pore occupancy for the first 30 minutes of your sequencing experiment. If fewer than 70% of all active pores are in strand, please follow the instructions in this FAQ.

Duty time plots

The duty time plot summarises the channel states over time.

Each bar shows the sum of all channel activity in a particular amount of time. This time bucket defaults to 1 minute, and scales to 5 minutes automatically after reaching 48 buckets. However, bucket size can be adjusted in the "Bucket size" box to the right of the graph.

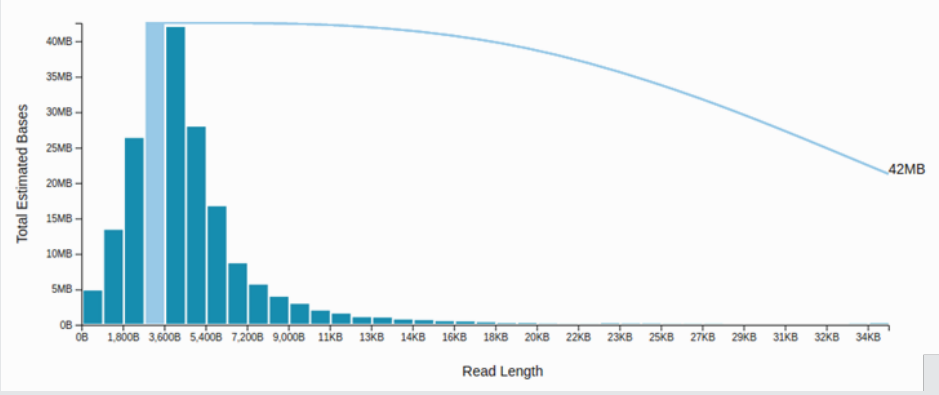


The graph populates over time, and can be used as a way to assess the quality of your sequencing experiment, and make an early decision whether to continue with the experiment or to stop the run.

Monitor the development of the read length histogram.

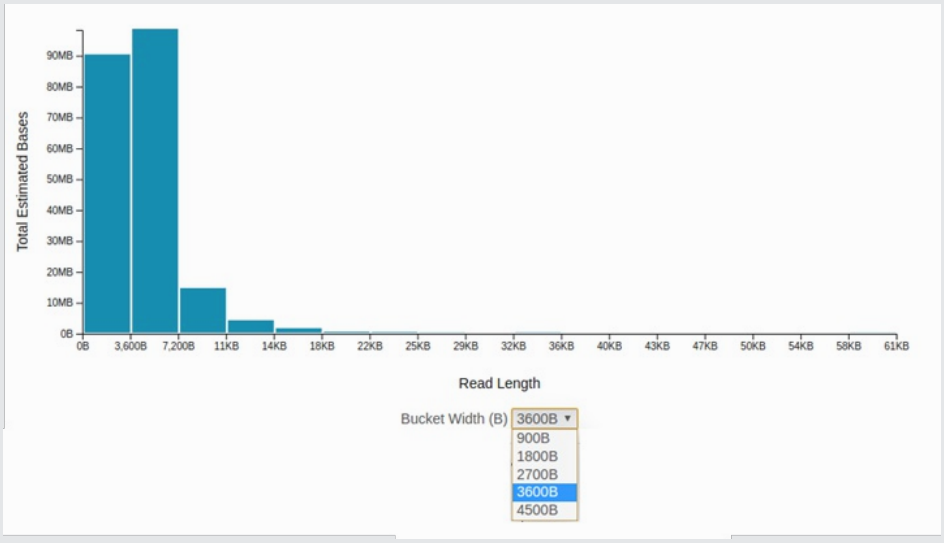
EXAMPLE

Read length histogram



The histogram will reflect expected read lengths for the experimental design being used. It plots **read length** (x axis) vs the **total number of estimated bases in that read length** bin (y axis). You can see the number of bases in a bin by hovering over the bar in question; a tool tip then appears as shown above.

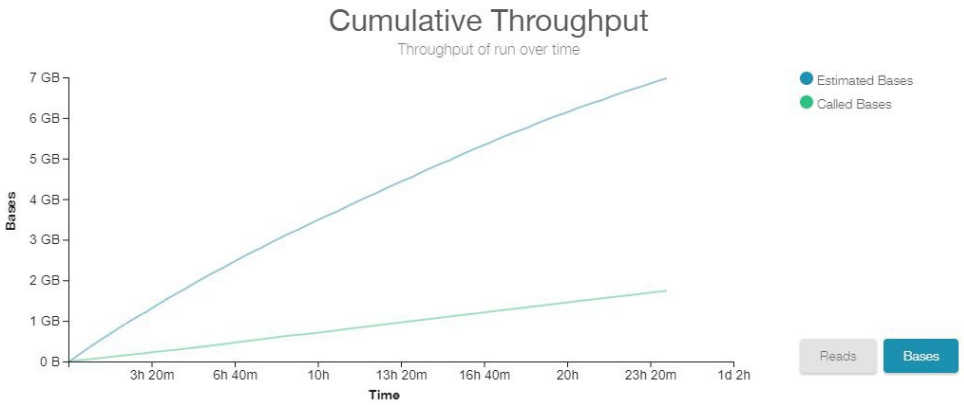
Additional control is available over the bin size in the histogram; the drop down below the x axis allows selection of 5 different bin sizes, with the size of the bin shown in bases i.e. 3600B is a bin size of 3600 bases.



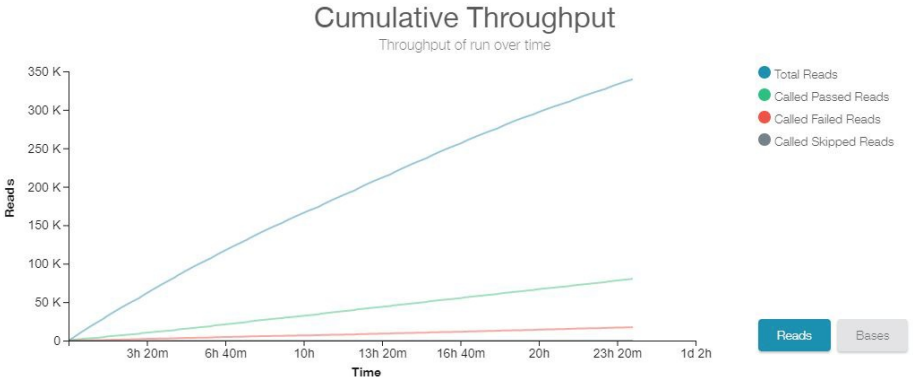
Cumulative throughput

The cumulative throughput graph shows:

- the number of bases that have been sequenced and basecalled



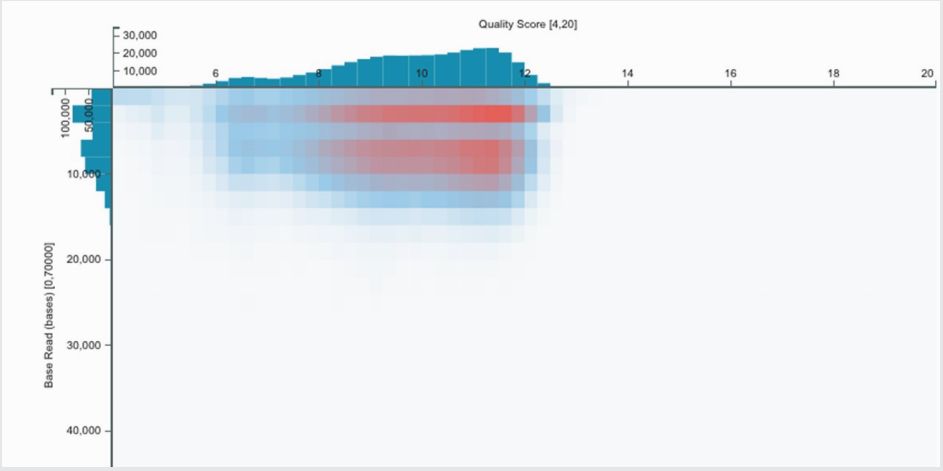
- the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters



EXAMPLE

Local basecalling report in the MinKNOW GUI

The progress of the local basecalling can be monitored via the **Basecalling** panel on the experiment page of the GUI. The histogram shows the number of reads against q-score, with red boxes representing a higher number of reads.



Trace viewer

The trace viewer displays the current levels from individual channels. By default it is set to show 10 channels, this number can be changed through the selection boxes beneath the viewer. Additional parameters that can be altered:

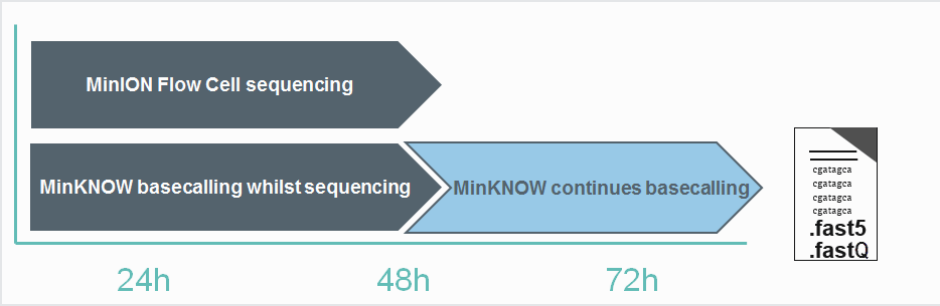
- **Time:** The length of time plotted on one screen
- **Maximum:** The highest current level to be shown on the y axis

Please note that viewing a high number of channels in the trace viewer may impact the speed at which the GUI is able to function

END OF STEP

End of sequencing protocol script.

The length of the sequencing scripts are indicated during selection, e.g. 48 Hr. However, if live basecalling is selected, a minimum-spec laptop may not keep up with the speed of data acquisition, and not all reads will be basecalled by the end of the experiment. The reads that had not finished being basecalled during the experiment will continue to be basecalled in Catch-Up mode.



The reads from the experiment will be found in the location set during experiment set-up in MinKNOW. The read file structure is described here.

If the Desktop Agent is running simultaneously to MinKNOW, the full report will also be available. If it is being run at a different times, the reads will be present in the data/reads folder ready to be processed.

Direct RNA sequencing (SQK-RNA002)

Assessing the quality of your run

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes ~5 minutes

Assessing the quality of your run - introduction

The Duty Time feature in the MinKNOW software can be used to judge the quality of your experiment. The duty time plot shows the distribution of channel states over time, grouped by time chunks, or 'buckets'. The basic view shows the five main channel states: Sequencing, Pore, Recovering, Inactive, and Unclassified. Clicking the "More" button shows a more detailed breakdown of channel states.

It is recommended to observe the duty time plot populating over the first 30 min-1 hr of the sequencing run. By this time, the channel state distribution will give an indication whether the DNA/RNA library is of a good quality, and whether the flow cell is performing well.

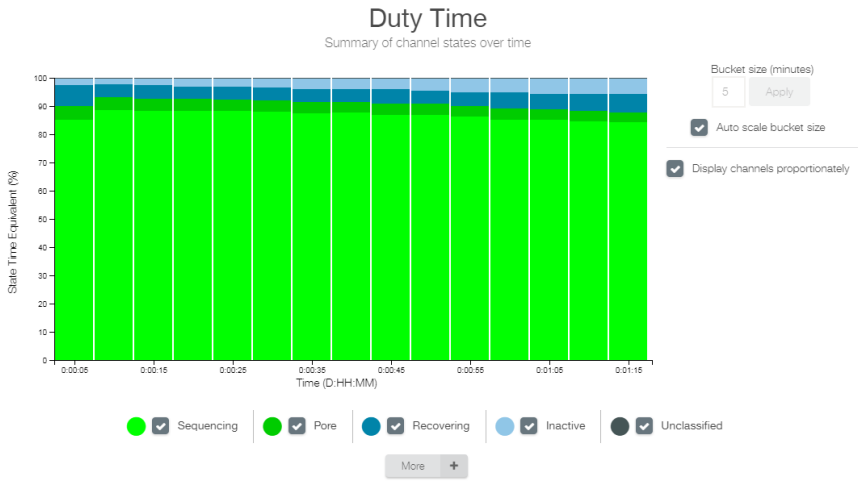
If Active Channel Selection is enabled during the run, the software instantly switches to a new channel in the group if a channel is in the “Saturated” or “Multiple” state, or after ~5 minutes if a channel is “Recovering”. This feature maximises the number of channels sequencing at the start of the experiment, however this may also result in an artificially high number of "Sequencing" or "Pore" channels in the duty time plot. For this reason, we recommend referring to the Mux Scan Results plot, which shows the true distribution of channel states at the point of the most recent mux scan.

Below are some examples of good and bad sequencing runs. For more detailed examples and troubleshooting steps, please refer to the MinKNOW protocol.

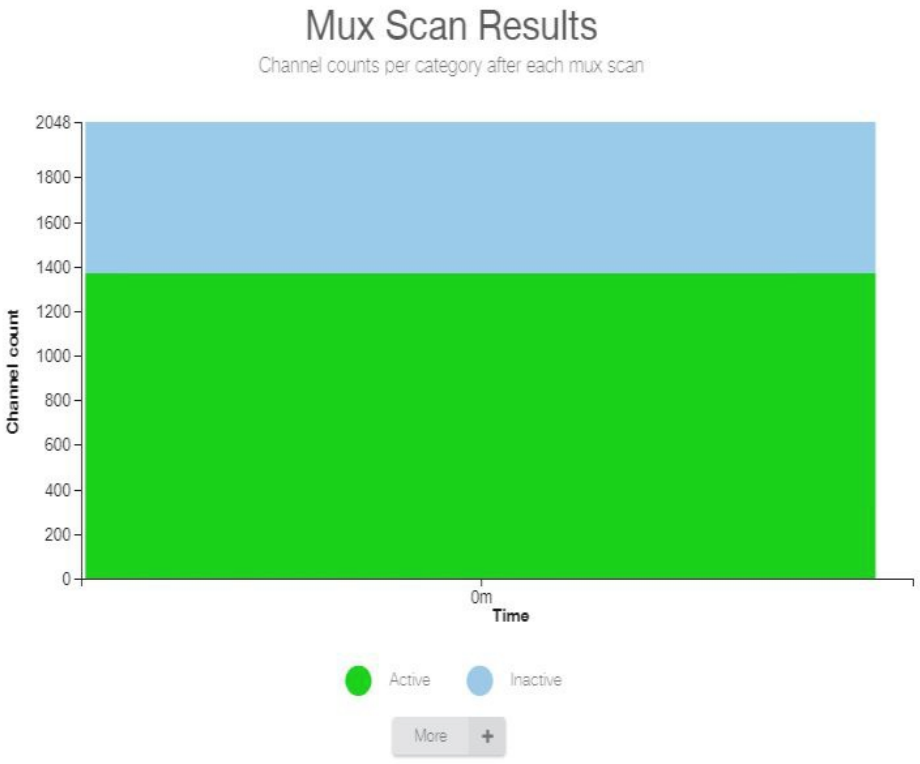
Good quality library

A good quality library will result in most of the pores being in the "Sequencing" state, and very few in "Pore", "Recovering" or "Inactive". A library that looks like this is likely to give a good sequencing throughput.

Duty time



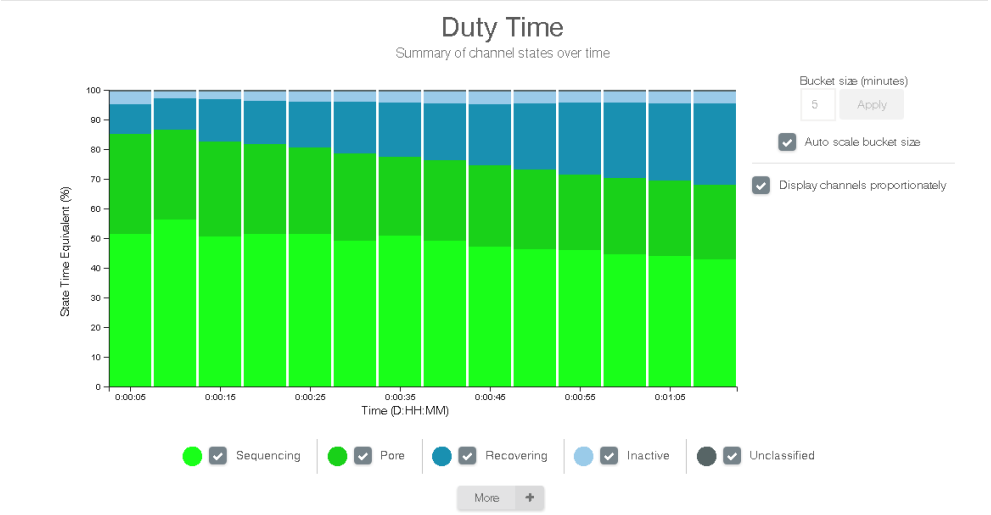
Mux scan results



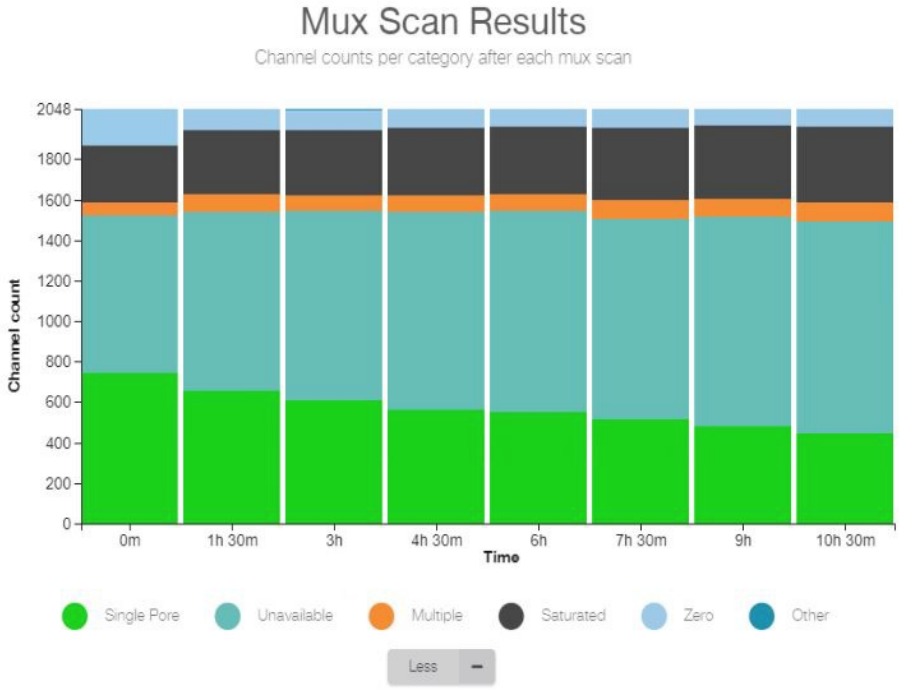
Channel blocking

Under certain conditions (usually the presence of contaminants in the library), pores may become blocked and therefore unable to sequence. This manifests itself as a build-up of "Recovering" pores over time.

Duty time



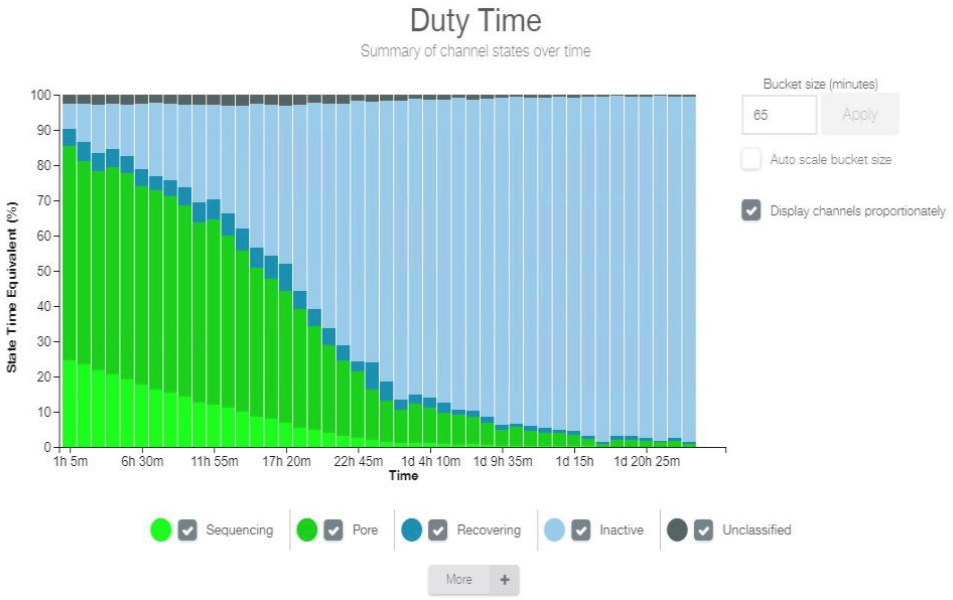
Mux scan results



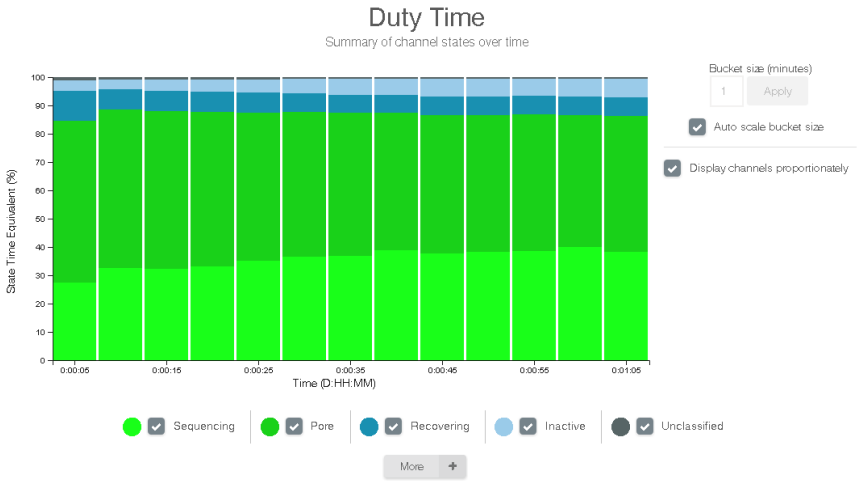
Low pore occupancy

If there was insufficient starting material, or some sample has been lost during library prep, or the sequencing adapters did not ligate well to the strand ends, the duty time plot will show a high ratio of "Pore" to "Sequencing" states, meaning that only a limited number of pores are sequencing at any one time.

Duty time: with Active Channel Selection switched on



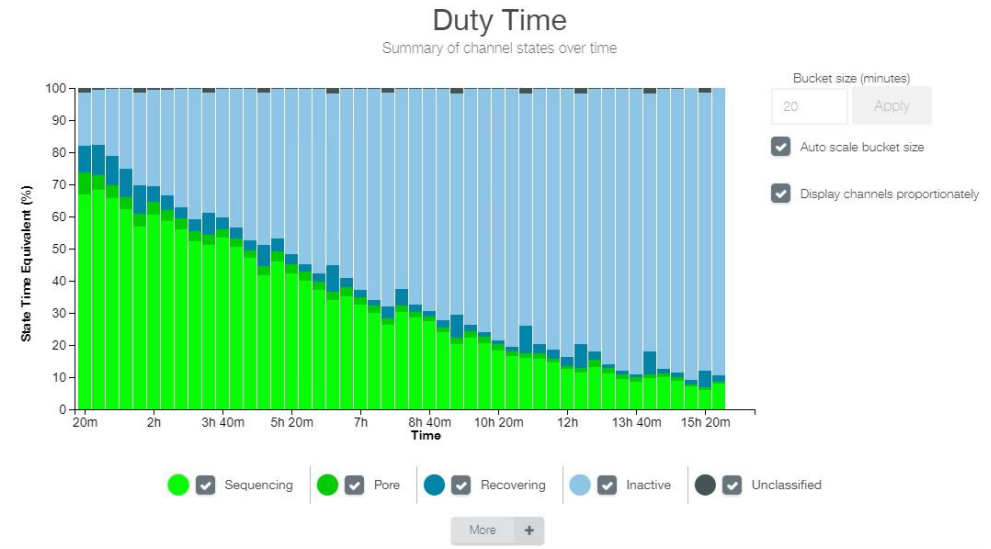
Duty time: with Active Channel Selection switched off



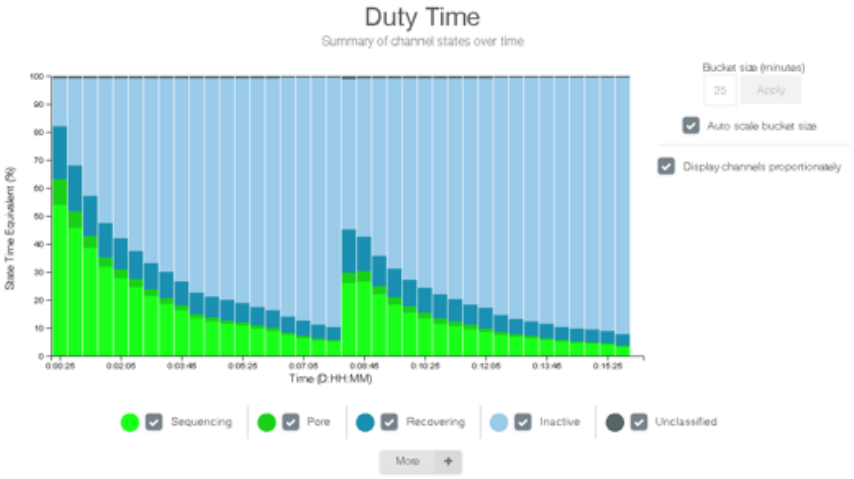
Flow cell failure due to osmotics or surfactants

If the duty time plot shows a high number of 'Inactive' channels building up over time, this could indicate that the channels or membranes have been damaged by e.g. air bubbles, osmotic imbalance, or the presence of detergents or surfactants in the library.

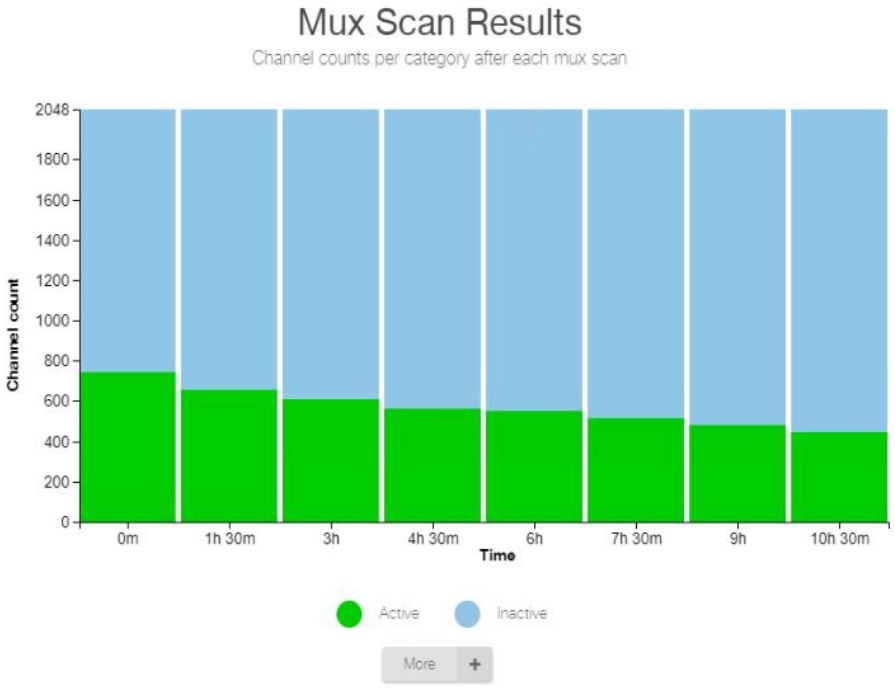
Duty time: with Active Channel Selection switched on



Duty time: with Active Channel Selection switched off

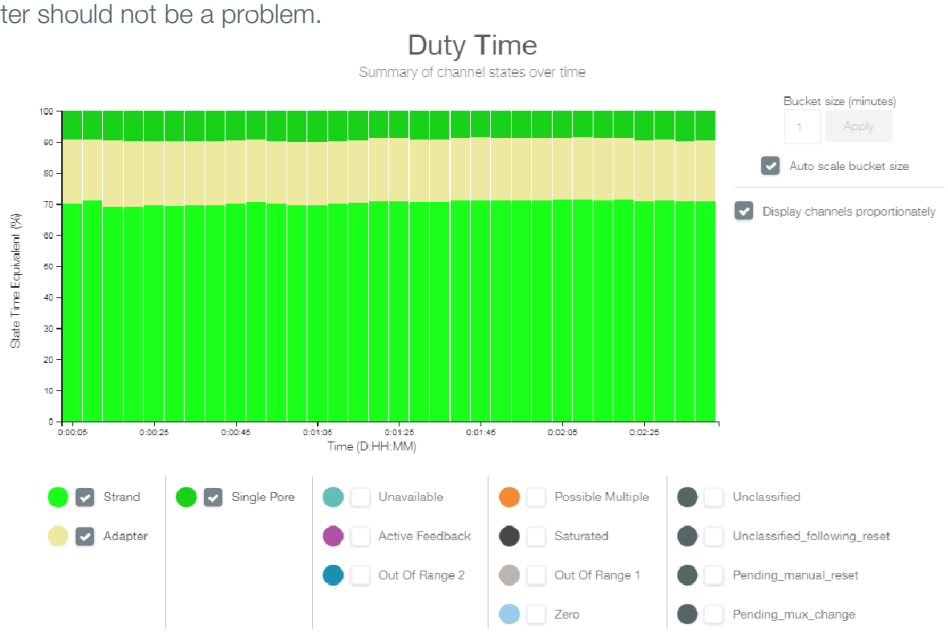


Mux scan results



RNA adapter

Opening up the "More" view gives more granular detail about channel states. For RNA in particular, this view may show a large proportion of pores sequencing Adapter. This happens because RNA strands are usually shorter than DNA, and the adapter takes up a larger proportion of the strand. Additionally, the RNA sequencing chemistry is optimised for sequencing RNA, whereas the adapter is DNA, and is processed slower. As long as the 'basic' duty time plot view shows the majority of pores in "Sequencing", a high proportion of Adapter should not be a problem.



Direct RNA sequencing (SQK-RNA002)

Close down MinKNOW and the Desktop Agent

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes ~5 minutes ~2 minutes

Quit Desktop Agent using the close x.

Quit MinKNOW by closing down the web GUI.

Disconnect the MinION.

Direct RNA sequencing (SQK-RNA002)

Prepare the flow cell for re-use or return to Oxford Nanopore.

Check the quality, quantity and formulation of the input polyA RNA
~115 minutes ~15 minutes ~5 minutes ~2 minutes

Materials

- Wash Kit (EXP-WSH002)

If you would like to reuse the flow cell, follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR

The Wash Kit protocol is available here.

Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.

Instructions for returning flow cells can be found here.