Overview of the Rapid Sequencing protocol

Introduction of the Rapid Sequencing Kit

Rapid Sequencing kit features

This kit is recommended for users who:

- require a short preparation time
- have limited access to laboratory equipment

Introduction to Rapid Sequencing protocol (SQK-RAD004)

This protocol describes the step-by-step instructions to complete a rapid sequencing of genomic DNA using the Rapid Sequencing Kit (SQK-RAD004). It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

Steps in the sequencing workflow:

Prepare for your experiment

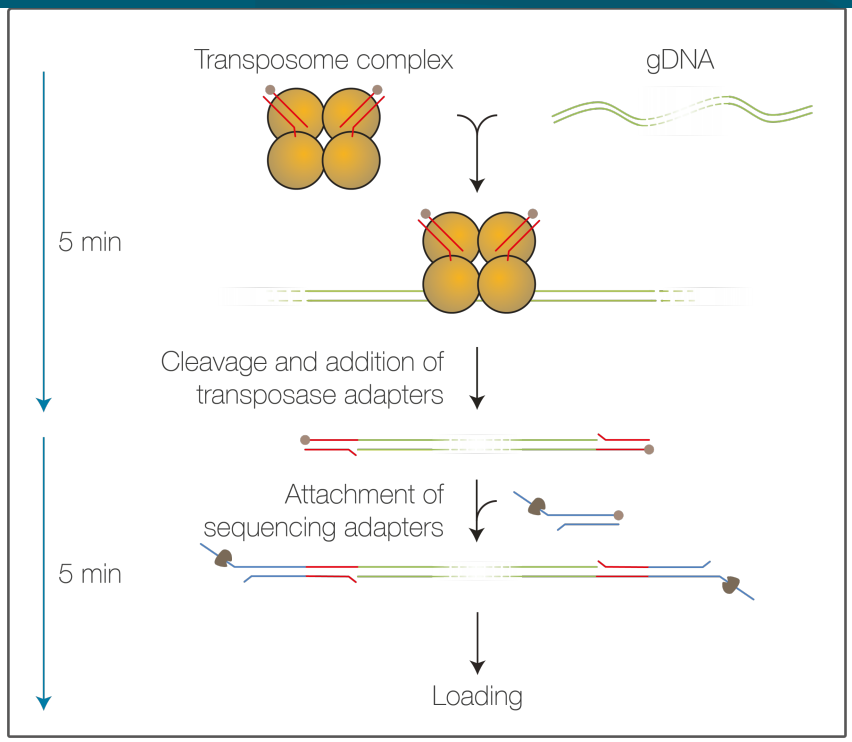
You will need to:
- Extract your DNA, and check its length, quantity and purity.

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
- If not already installed, 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:
- Tagment your DNA using the Fragmentation Mix in the kit
- Attach sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell



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
- ***optional*** Start the EPI2ME software and select a workflow for further analysis, e.g. metagenomic analysis or drug resistance mapping

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Sequencing Kit (SQK-RAD004)
- FLO-MIN106 flow cells (FLO-MIN107 not advised)
- Wash Kit (EXP-WSH002)

Preparing input DNA

Check the quality, quantity and formulation of the input DNA

Materials	• ~400 ng high molecular weight genomic DNA
Consumables	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • 1.5 ml Eppendorf DNA LoBind tubes
Equipment	• Microfuge
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

IMPORTANT

Importance of DNA QC

It is important that you check your input DNA for quality before beginning library preparation. Low molecular weight, incorrectly quantified and/or contaminated DNA (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 DNA 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 DNA mass and molarity

In order to maximise sequencing yield, it is important that the nanopores are kept filled with DNA to minimize the time they are idle between strands. The less material goes into the flow cell, the fewer “threadable ends” will be present to be captured by the pores. Therefore, the pores will be searching for molecules for longer, and if the pores are not always sequencing, throughput could be compromised. We have found that in order to keep the pores full, the current R9.4.1 and R9.5.1 pores require about **5-50 fmol of good quality library put into the flow cell**.

We expect many users to try and optimise protocols for maximum performance, and input amounts can mean different things in moles depending on the average length of the DNA fragments.

If you are unable to quantify your input DNA mass, please use the table below as a guide. Then, take forward the appropriate amount of DNA based on the average fragment length and known concentration.

Mass	No. of moles if fragment length = 2 kb	No. of moles if fragment length = 8 kb	No. of moles if fragment length = 50 kb
10 µg	7.7 pmol	1.9 pmol	308 fmol
5 µg	3.9 pmol	963 fmol	154 fmol
3.5 µg	2.7 pmol	674 fmol	108 fmol
2 µg	1.5 pmol	385 fmol	62 fmol
1.5 µg	1.2 pmol	289 fmol	46 fmol
1 µg	770 fmol	193 fmol	31 fmol

Mass	No. of moles if fragment length = 2 kb	No. of moles if fragment length = 8 kb	No. of moles if fragment length = 50 kb
500 ng	385 fmol	96 fmol	15 fmol
400 ng	308 fmol	77 fmol	12 fmol
200 ng	154 fmol	39 fmol	6.2 fmol
100 ng	77 fmol	19 fmol	3.1 fmol
30 ng	23 fmol	5.8 fmol	0.9 fmol
10 ng	7.7 fmol	1.9 fmol	0.3 fmol
10 pg	0.0077 fmol	0.009 fmol	0.0003 fmol

Prepare the DNA in nuclease-free water.

- Transfer ~400 ng genomic DNA into a DNA LoBind tube
- Adjust the volume to 7.5 µl with nuclease-free water
- Mix by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

Record the quality, quantity and size of the DNA.

IMPORTANT

Criteria for input DNA

Ensure that your DNA meets the following criteria:

- Purity as measured using Nanodrop - OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2
- Average fragment size, as measured by pulse-field, or low percentage agarose gel analysis >30 kb
- Input mass, as measured by Qubit - ~400 ng
- No detergents or surfactants in the buffer

The presence of a transposase in the Fragmentation Mix means that DNA fragmentation is unavoidable. To avoid a library entirely composed of short fragments, it is recommended to start with DNA >30 kb.

For long-term storage of high molecular weight gDNA, we recommend the use of TE buffer.

Correct quantification

The majority of RNA should be removed by RNase digestion. We have found RiboShredder (Epicentre RS 12500) to be particularly effective. However, since Riboshredder is being discontinued, you can use the RNase Cocktail Enzyme Mix (ThermoFisher, AM2286) instead.
Please be aware that certain RNase treatments can lead to digestion of DNA, as well as RNA.

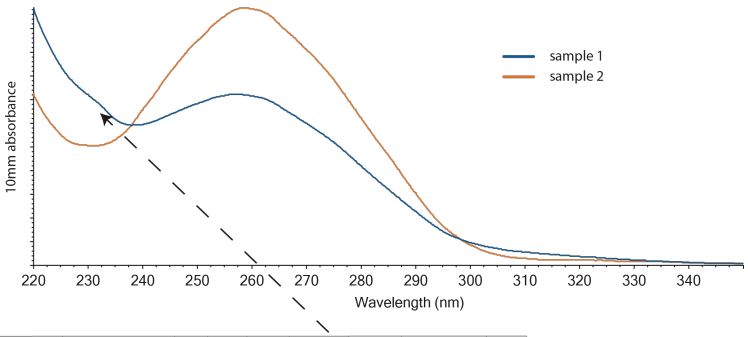
We recommend that the DNA stock is quantified using Qubit analysis. A Qubit measures DNA specifically. Even after RNase digestion, residual RNA is a common contaminant in gDNA preparations and is not well identified by Nanodrop measurements. Incorrect quantification could mean that you will proceed with less DNA than intended, resulting in poor performance. Also, contamination from bases (dNTPs and NTPs) will interfere with Nanodrop measurements. Therefore, we recommend that Qubit is used for all quantification measurements (i.e. after all clean-up steps).

Additionally, high concentration, high molecular weight DNA preparations (and those with heavy RNA contamination) can lack homogeneity, which will give rise to inaccurate quantification. If you encounter this with your RNase-treated DNA sample, we recommend that you dilute the DNA further with TE, and that you rotate the tube gently until the suspension is homogeneous. Vortexing the DNA or pipetting up and down will cause shearing, which will limit the fragment sizes available to the nanopore.

Assessment of DNA 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 single stranded DNA, RNA, proteins and dyes may also reduce the efficiency of steps in the library preparation.
- The quality of DNA may be assessed by Nanodrop (for samples with concentration >20 ng/µl).
- We recommend that sample DNA has a 260/280 ~ 1.80 and a 260/230 ~2.0-2.2.
- A 260/280 which is higher than ~1.8 indicates the presence of RNA.
- 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 DNA may need additional purification.

In the Nanodrop trace shown below, Sample 1 had a 260/230 of ~1.0 and the resulting library performed badly in a sequencing run. If additional purification is not possible, amplification of the library by PCR (following the low input protocol) can be performed to improve library cleanliness.



Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
sample 1	207.5	ng/µl	4.150	2.410	1.72	0.99	DNA	50.00
sample 2	1080.1	ng/µl	21.601	12.369	1.75	2.17	DNA	50.00

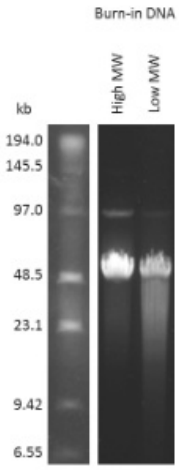
Assessing molecular weight

Nanopore sequencing devices generate reads that reflect the lengths of the fragments loaded into the flow cell. To have control over the size of the fragments generated in the library prep it is important to begin with high molecular weight DNA.

The shearing of HMW DNA can be minimised by:

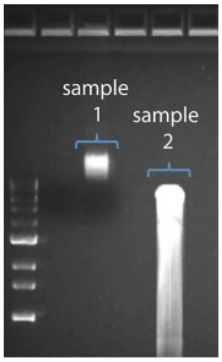
- Using wide-bore pipette tips to handle the gDNA
- Mixing gently but thoroughly by inversion, as opposed to vortexing or pipetting
- Avoiding unnecessary freeze-thaw cycles
- Avoiding pH <6 and >9
- Avoiding high temperatures, which can lead to degradation

Conventional agarose gels cannot resolve DNA fragments greater than 15–20 kb, but the molecular weight of starting material can be measured by pulsed-field gel analysis.



The figure shows two samples of Lambda DNA: one of intact high molecular weight fragments and one containing a significant proportion of low molecular weight fragments

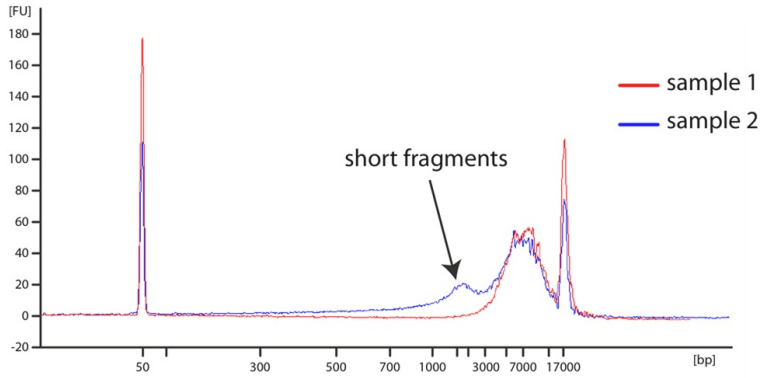
Low % agarose gel analysis can be used to detect substantial degradation / shearing:



The figure shows two samples of input DNA: Sample #1 is of high molecular weight and sample #2 is of lower molecular weight and has sheared

Assessing fragmentation

Post-fragmentation, the quality of the fragmented material may be assessed by different methods e.g. Agilent Bioanalzyer.



The figure above shows successful (sample 1) and unsuccessful (sample 2) fragmentation, demonstrated by an Agilent Bioanalyzer trace of two DNA samples. Sample #2 contains a substantial proportion of low molecular weight fragments. This is possibly as a result of improper fragmentation, or these low MW fragments may have been present in the input sample.

Equipment and consumables

Materials	• ~400 ng high molecular weight genomic DNA • Flow Cell Priming Kit (EXP-FLP002)	• Rapid Sequencing Kit (SQK-RAD004)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937)	• 0.2 ml thin-walled PCR tubes
Equipment	• Microfuge • P100 pipette and tips • P10 pipette and tips • Timer	• P1000 pipette and tips • P20 pipette and tips • P2 pipette and tips • Thermal cycler at 30° C and 80° C
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)	

Rapid Sequencing Kit contents

LMD : Lambda DNA
FRA : Fragmentation mix
RAP : Rapid adapter
SQT : Sequencing tether
LB : Loading beads
SQB : Sequencing buffer

Contents	Description	No. of tubes
LMD (yellow cap)	Lambda DNA Identical to that found in the SQK-RAD001 kit	1
FRA (amber cap)	Fragmentation Mix Contains the transposase with transposase adapters	1
RAP (green cap)	Rapid Adapter Contains leader adapters with loaded motor protein; this is a direct replacement of the AMX tube in the Ligation Sequencing kits	1
SQT (violet cap)	Sequencing Tether	1
LB (pink cap)	Loading beads	1
SQB (red cap)	Sequencing buffer	1

IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

Flow Cell Priming Kit contents (EXP-FLP002)

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

Computer requirements and software downloads

Software for nanopore sequencing

MinKNOW

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

Guppy (optional)

The Guppy command-line software can be used for basecalling instead of MinKNOW. You can use it if you would like to re-basecall old data, or integrate basecalling into your analysis pipeline.

> **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.

Check your flow cell

Consumables	• SpotON Flow Cell	
Equipment	• MinION	• Host computer connected to the Internet with MinKNOW 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 prior to loading your DNA or RNA library onto the flow cell.

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.

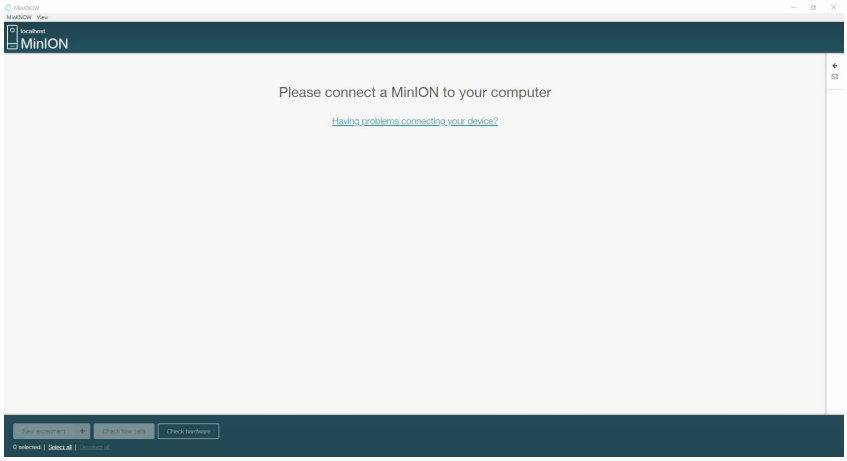
Set up the MinION, flow cell and host computer

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

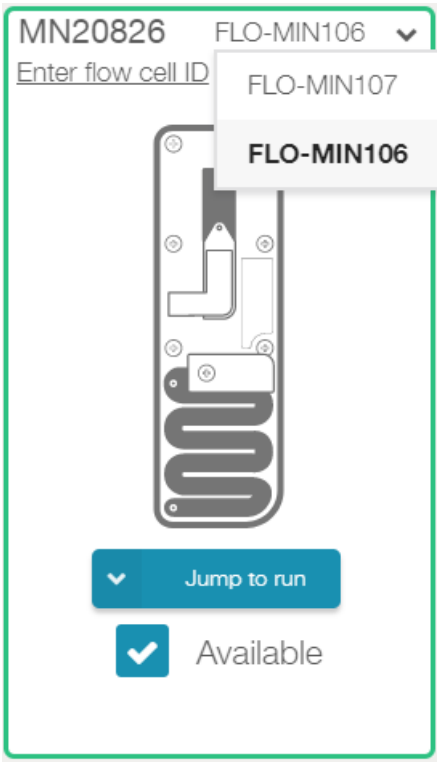
Once successfully plugged in, you will see a light and hear the fan.

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

- Plug the MinION into the computer.



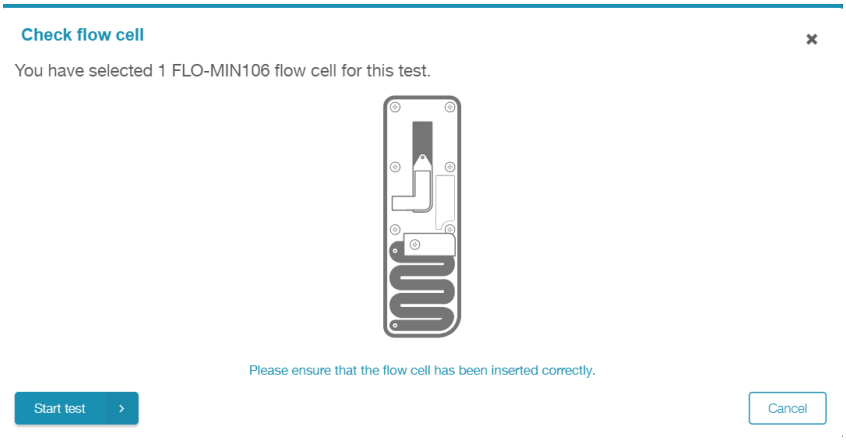
- Choose the flow cell type from the selector box. Then check the "Available" box.
  Note: if you are using flow cells from your Starter Pack, please select FLO-MIN106.



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

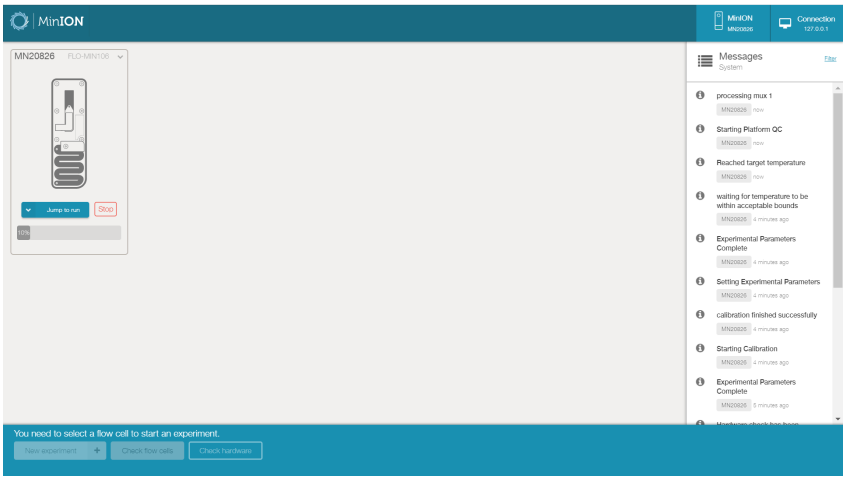
A screen will load displaying the number of flow cells selected for your test, and offering a drop down for flow cell type. It is important the correct flow cell type is chosen to obtain an accurate result:

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

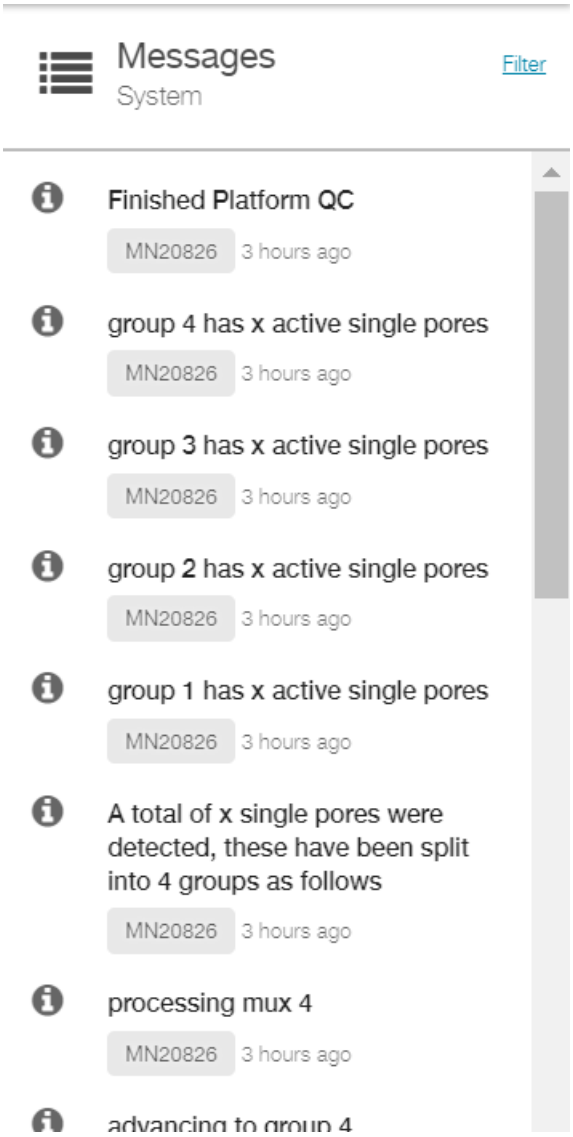


Click "Start test".

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.**

Warranty for flow cells: 800 nanopores or above

Library preparation

~10 minutes

Materials	• ~400 ng high molecular weight genomic DNA • Rapid Adapter (RAP)	• Fragmentation Mix (FRA)
Consumables	• 0.2 ml thin-walled PCR tubes	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Equipment	• Thermal cycler at 30° C and 80° C • P10 pipette and tips	• P2 pipette and tips

DNA tagmentation

Thaw kit components at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix (FRA)	Not frozen	✓	✓
Rapid Adapter (RAP)	Not frozen	✓	✓
Sequencing Buffer (SQB)	✓	✓	✓*
Loading Beads (LB)	✓	✓	Mix by pipetting or vortexing immediately before use
Flush Buffer (FLB) - 1 tube	✓	✓	✓*
Flush Tether (FLT)	✓	✓	✓

*Vortexing, followed by a brief spin in a microfuge, is recommended for Sequencing Buffer (SQB) and Flush Buffer (FLB).

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

Once thawed, keep all the kit components on ice.

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

Reagent	Volume
400 ng template DNA	7.5 µl
FRA	2.5 µl
Total	**10 µl**

Mix gently by flicking the tube, and spin down.

Incubate the tube at 30° C for 1 minute and then at 80° C for 1 minute. Briefly put the tube on ice to cool it down.

> **TIP**
>
> **If heat blocks are used instead of a thermal cycler, incubation at both temperatures should be extended to 2 minutes.**

400 ng tagmented DNA in 10 µl is taken into the next step.

Adapter attachment

Add 1 µl of RAP to the tube.

Mix gently by flicking the tube, and spin down.

Incubate the reaction for 5 minutes at room temperature.

> **END OF STEP**
>
> **The prepared DNA library is used for loading into the flow cell. Store the library on ice until ready to load.**

Priming and loading the SpotON Flow Cell

Preparing and loading the library into the flow cell
~10 minutes

Materials	• Flush Tether (FLT) • Sequencing Buffer (SQB)	• Flush Buffer (FB) • Loading Beads (LB)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Flongle device - flow cell and adapter	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Equipment	• MinICN • P100 pipette and tips • P10 pipette and tips	• P1000 pipette and tips • P20 pipette and tips

> **IMPORTANT**
>
> **Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.**

Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice as soon as thawing is complete.

Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.

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

Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming 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 (FB), 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.

Thoroughly mix the contents of the SQB and LB tubes by pipetting.

> **TIP**
>
> **Using the Loading Beads**
>
> Demo of how to use the Loading Beads.

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

Reagent	Volume
Sequencing Buffer (SQB)	34 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
Nuclease-free water	4.5 µl
DNA library	11 µl
Total	**75 µl**

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.

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.

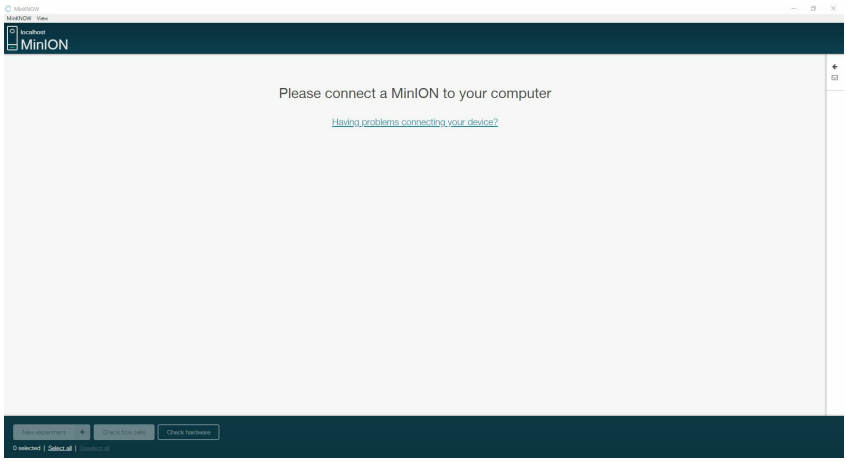
Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

Starting a sequencing run

~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.



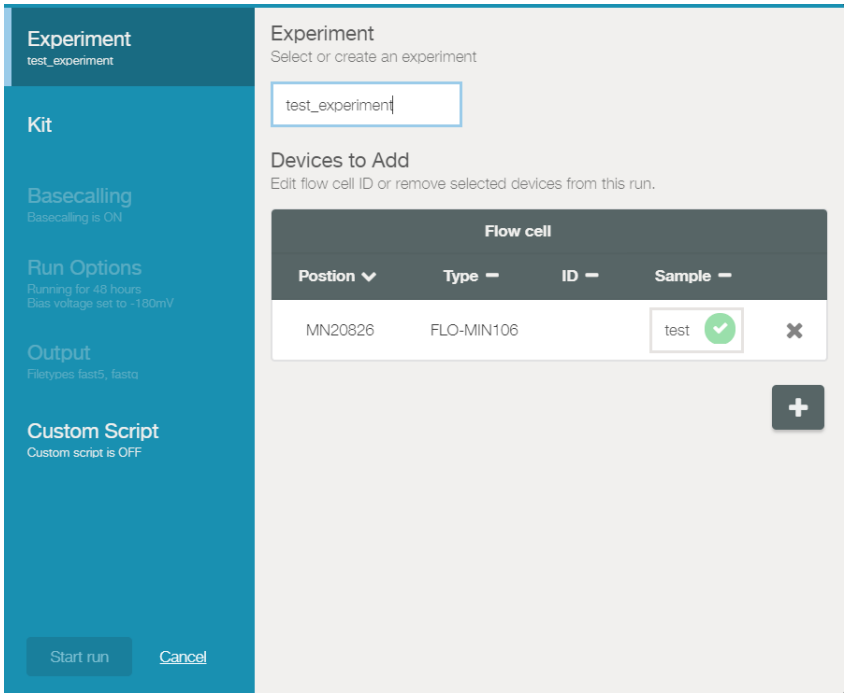
Click the "New Experiment" button at the bottom left of the GUI.

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

Experiment name

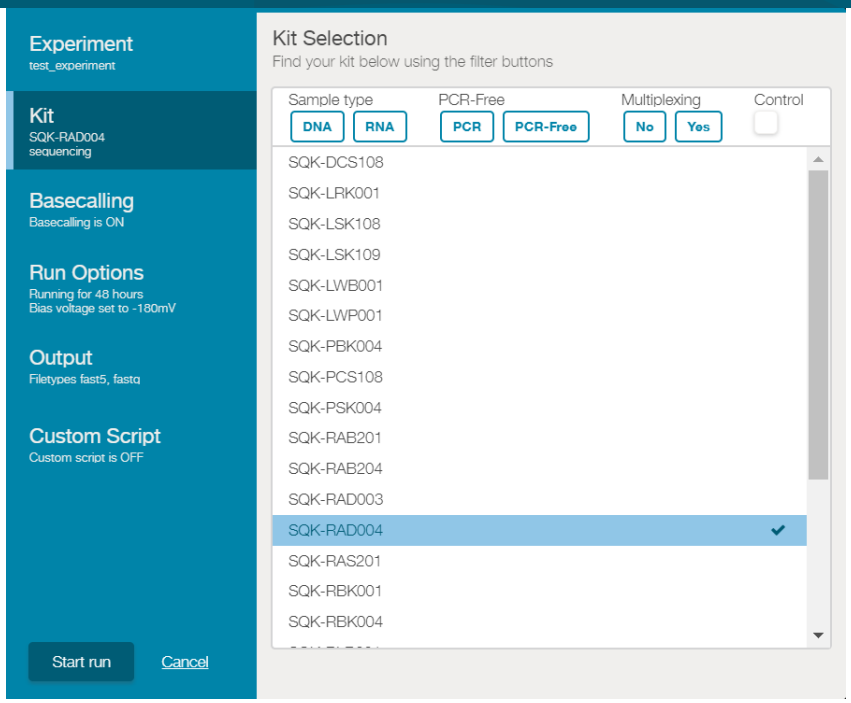
The experiment name tab will show the chosen flow cell. An experiment name can then be assigned.

The other tabs will not become available until an experiment name has been provided.



Kit selection

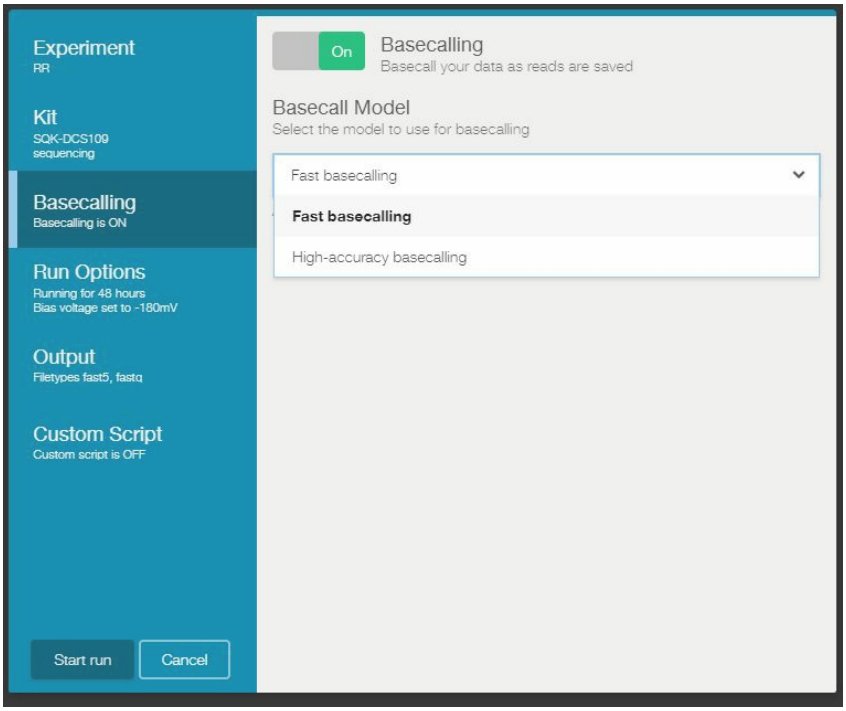
The kit selection tab will provide a dropdown of available kits. Select **SQK-RAD004**.



Basecalling

At this stage you select whether or not you want your data basecalled live on the instrument and choose the basecalling model you wish to basecall your nanopore data.

- **Basecalling live**

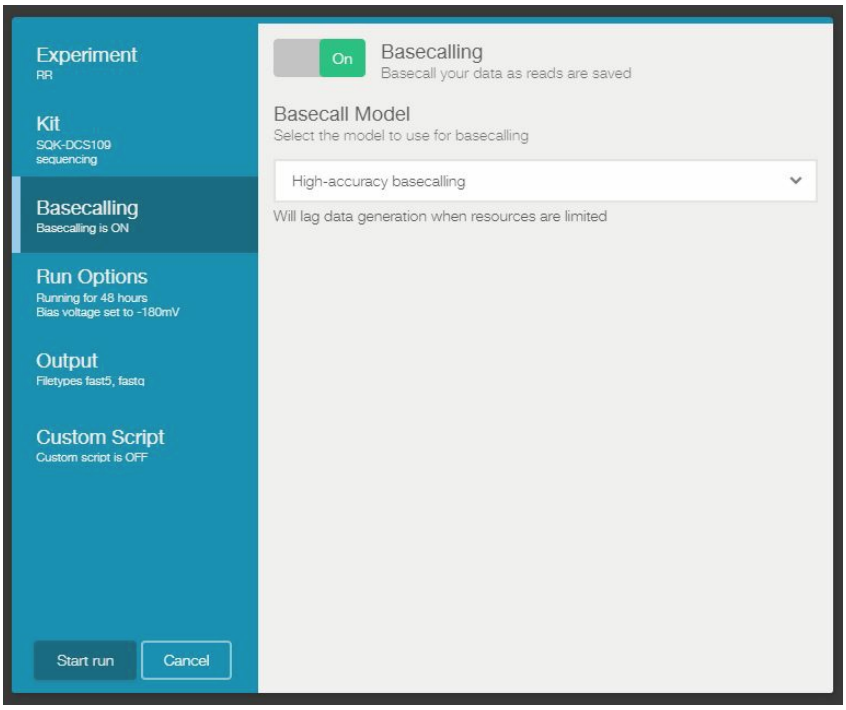


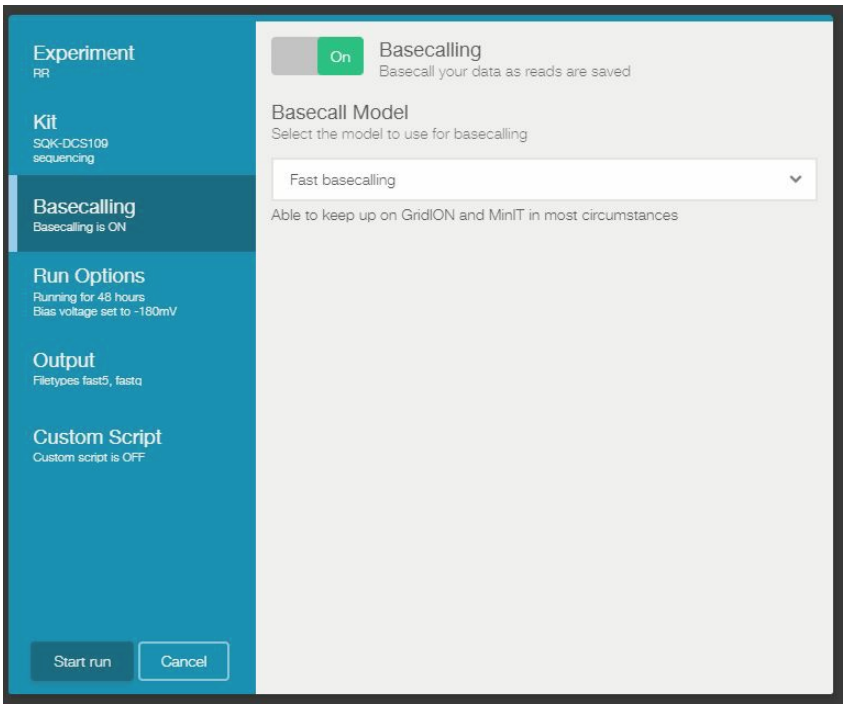
- **Model selection** The MinKNOW's basecaller - Guppy - now provides two basecalling models for nanopore data. These are:

1. **Fast** - This model is able to keep up with a full experiment on a GridION or MinIT. It implements a lite version of the Flip-flop algorithm. This delivers the same level of accuracy as the previous default transducer models, which have been deployed prior to the introduction of Flip-flop.

2. **HAC** - The High accuracy (HAC) Flip-flop model provides a higher consensus/raw read accuracy than the 'Fast' model. It contains a more computationally intense Flip-flop architecture that can deliver higher accuracy using the same data produced by nanopore sequencing. It is currently 5-8 times slower than the fast model so users should ensure their data transfer, disk-space and device utilisation is scaled appropriately for this.

In order to select your model, use the drop down menu as appropriate:

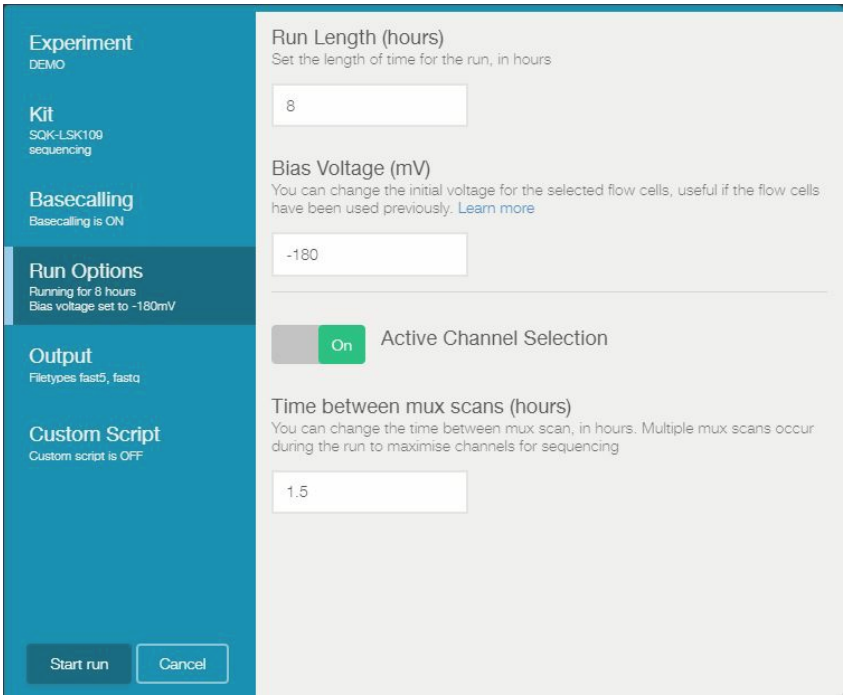




Run Options

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

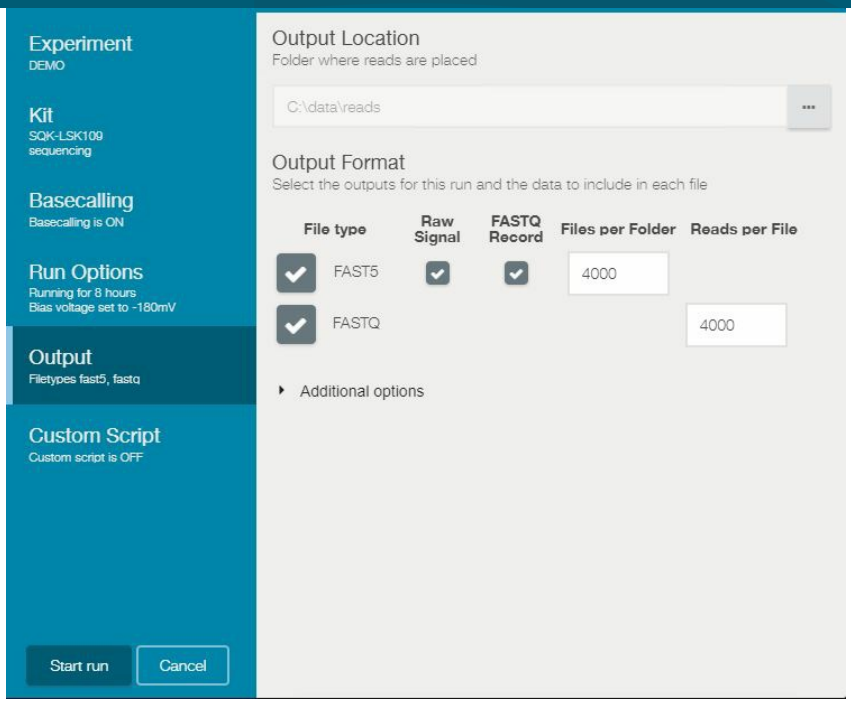
You can specify to produce:

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



Click "Start run".

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

Optional Action
Refuelling a MinION™/GridION™ flow cell

This is for a user that:
- is sequencing DNA
- notices a reduction in speed and Qscore over the lifetime of the run
- has the EXP-FLP002 Kit

If the translocation speed drops below 300 bases per second for DNA, you may start to see a reduction in quality of data, reflected in the Qscore. We therefore recommend topping up the flow cell with fuel, using the Flush Buffer (FB) from EXP-FLP002 - the new Flow Cell Priming Kit. Please use the following instructions if you wish to top up the fuel during an experiment:

Note: Please consult the 'Priming and loading the SpotON flow cell' step earlier in this protocol for advice on the adding solutions to the flow cell.

Instructions for refuelling a MinION/GridION flow cell:

1. Remove one tube of Flush Buffer (FB) from the freezer and thaw by bringing to room temperature
2. After opening the priming port, check for a small bubble under the cover. Draw back a small volume to remove the bubble (a few µls):

a. Set a P1000 pipette to 200 µl
b. Insert the tip into the priming port
c. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip

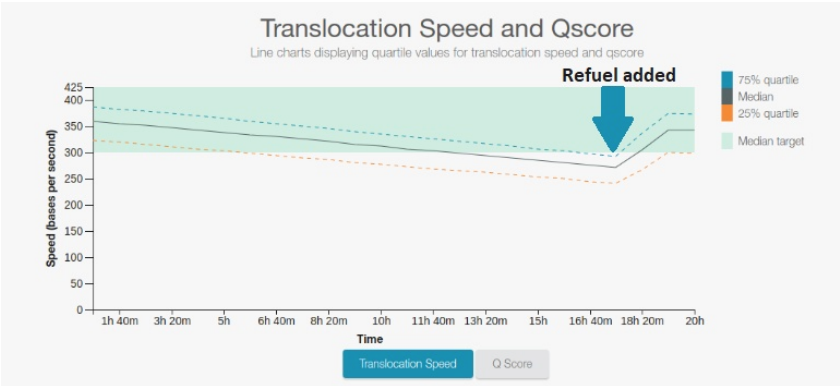
Visually check that there is continuous buffer from the priming port across the sensor array.

Complete the flow cell refuelling:

1. Load 250 µl of the FB into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
2. Close the priming port and replace the MinION lid.

Translocation speed and Qscore over time

Below is a graph that shows what is expected for translocation speed after the addition of FB to the flow cell.



Speed of bases through nanopores before and after refuelling. As the speed drops below 300 bases per second, the Qscore will begin to decline for the reads processed through the nanopores at this speed. After refuelling at the 17.5 hour mark, the speed begins to increase and returns to an improved rate (~400 bases per second), which is similar to the speed at the start of the experiment. After the addition of fuel using FB, the quality of the data may increase and return to Qscores equivalent to those seen at the start of your run.

Refuelling multiple times in a run

You can refuel a sequencing run multiple times over an experiment. When you should refuel will depend on when the translocation speed drops below 300 bases per second on the speed graph in the MinKNOW GUI.

Warning - overloading the flow cell when refuelling

Please be aware that the addition of FB will require you to monitor the buffer fill volume in the waste reservoir and waste port, if you refuel multiple times. For information on the location of the waste port on a Flongle™, MinION or PromethION™ flow cell, see the

technical documents, here.

The array and waste reservoir of the below platforms (Flongle, MinION/GridION, PromethION) will take the following dosings:

- a flush
- a prime
- a library
- **one** refuel - for Flongle
- **three** refuels - for MinION/GridION
- **four** refuels - for PromethION

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

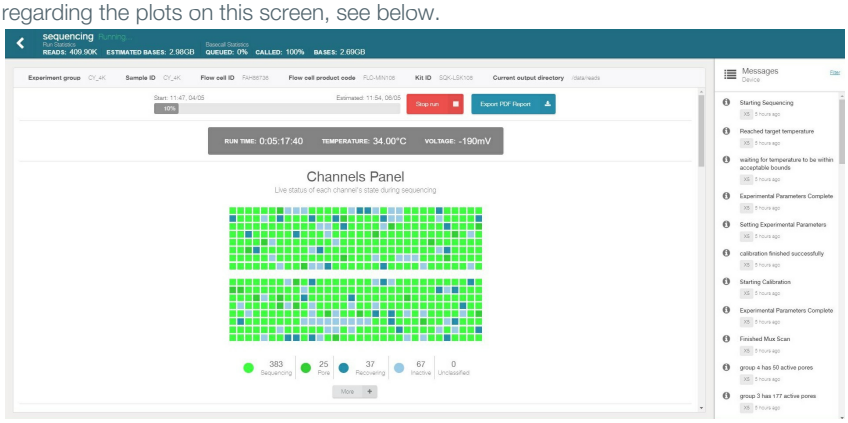
The basecalled read files are stored in the Output Location specified during MinKNOW installation, or experiment set-up. By default, this is:

:\data\ for MinION
/data/ for GridION

Progression of MinKNOW protocol script

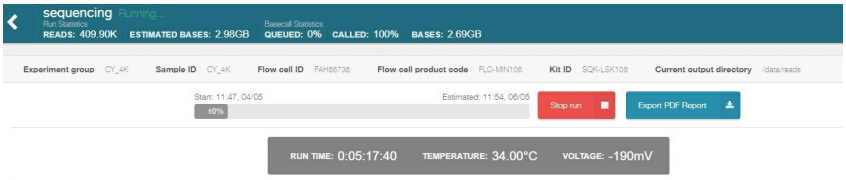
The running experiment screen

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 experiment as a whole.



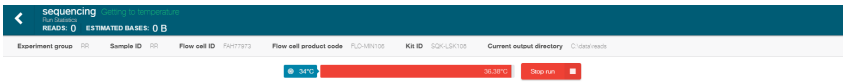
- **Run statistics:** The total number of reads and bases produced across the experiment
- **Basecall statistics:** There are two values for basecalled reads:

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 termperature
> - Development of the read histogram
> - Pore occupancy
> - Local basecalling report

Check that the temperature has reached 34° C.



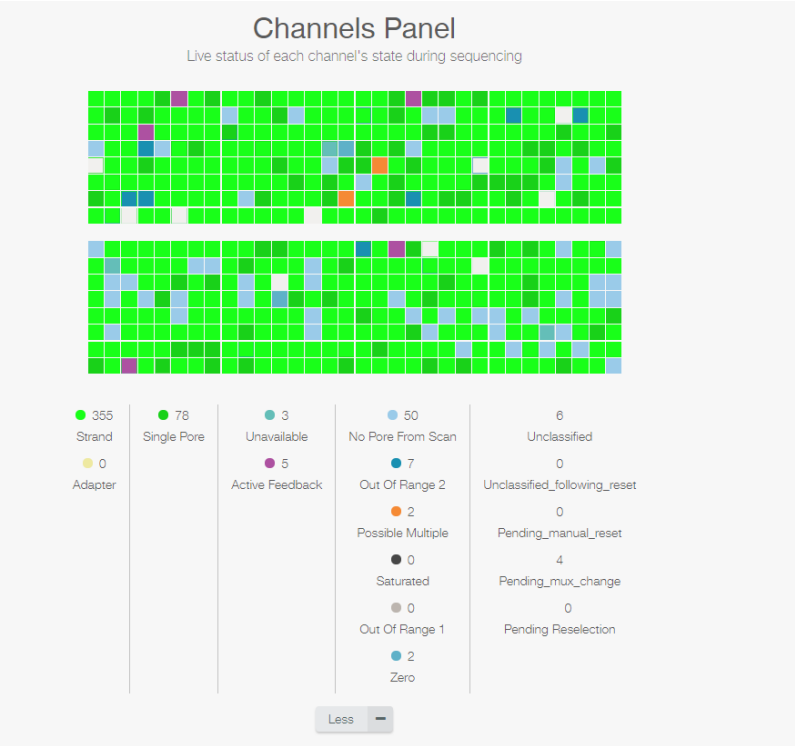
MinION temperature control

The MinION is able to maintain a heatsink 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



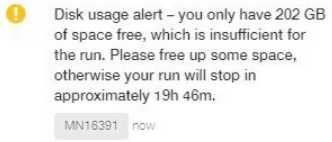
Clicking on the "More" button reveals a more detailed array of channel states:
- **Strand:** the channel is in strand
- **Adapter:** the channel has just captured a new strand
- **Single pore:** the channel appears to show a single pore. Available for sequencing
- **Unavailable:** the channel appears to show a pore that is currently unavailable for sequencing
- **Active feedback:** the channel is reversing the current flow to eject the analyte
- **No pore from scan:** the Mux scan has not detected a pore in the well
- **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.

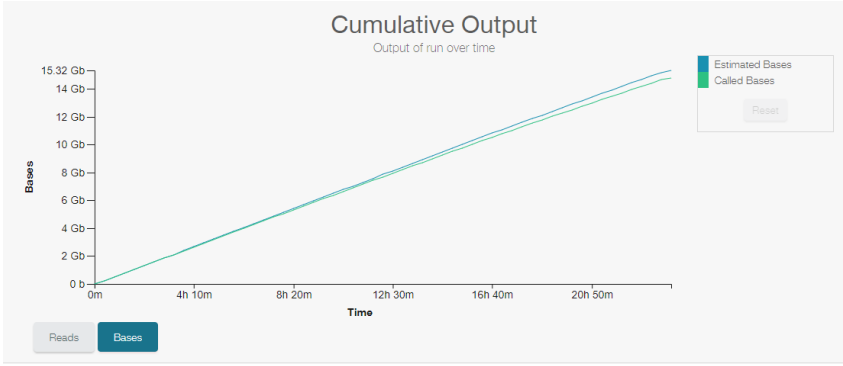
Monitor the disk space

MinKNOW will display a warning message when the disk space is too low to finish the sequencing run. It is advised to periodically check the messages, and free up disk space if needed.

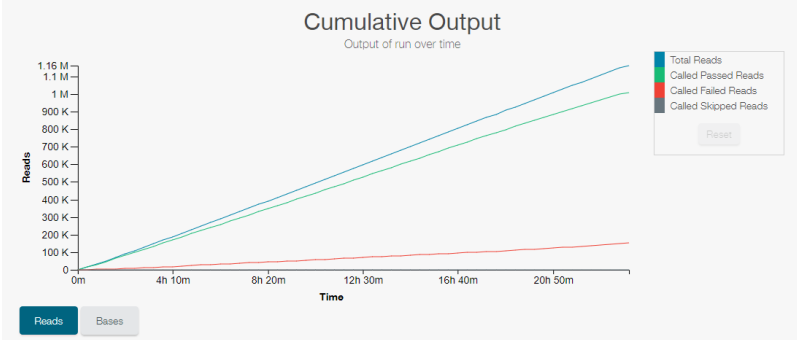


Cumulative output

The cumulative output graph shows:
- the number of bases that have been sequenced and basecalled



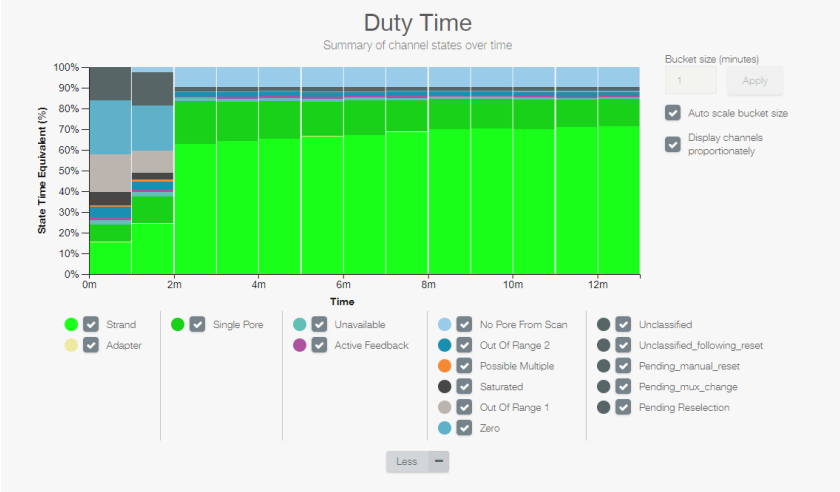
- the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters
-



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.



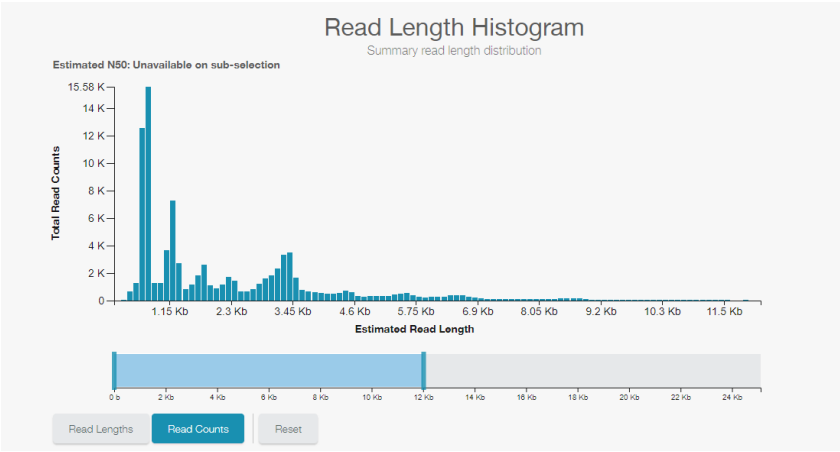
The graph populates over time, and can be used as a way to assess the quality of your sequencing experiment, and make an early decision whether to continue with the experiment or to stop the run.

Monitor the development of the read length histogram.

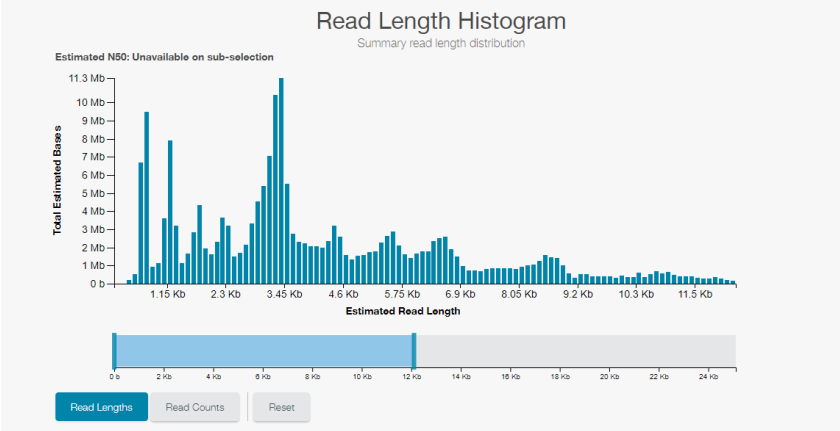
Read length histogram

There are two cumulative histograms that can be toggled between in MinKNOW. These are:

1. Read count - this shows the number of reads per bin and the distribution of these bins' aggregate values. This enables the user to understand how the read lengths vary in number and size; this helps the user visualise the N50 value.

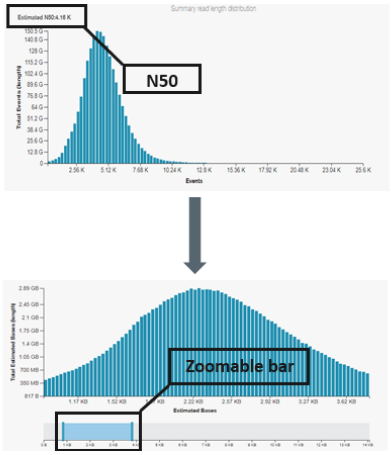


1. Read length - this shows the cumulative number of bases vs the read length. This enables the user to compare the skew from more numerous reads or longer reads towards the N50 i.e. if more bases are implicated in a particular bin (through numerosity or length) then this will pull the averages in *this* direction.



The N50 value is presented (only for the whole set of passed reads) in the top left corner of the histogram.

Each histograms X-axis (read length) can be zoomed into using the scaled bar under the histogram (shown with the blue box as labelled below. This facilitates the user to visualise distribution of 1. read counts or 2. number of bases with read length, more accurately over a size of interest. The reset button refocuses the zoom bar and histogram for the entire 'passed read' data set.



The histograms will reflect expected read lengths or cumulative number of bases for the experimental design being used.

The histogram for read count plots:

x-axis - **read length** vs y-axis the **total number of estimated *reads* in that read length** bin

The histogram for read length plots:

x-axis - **read length** vs y-axis the **total number of estimated *bases* in that read length** bin

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.

MinKNOW 19.05 has several new interpretations and graphics of the data obtained from a sequencing run.

The new graphs are displayed with 10-minute bin intervals over the course of the experiment and include:

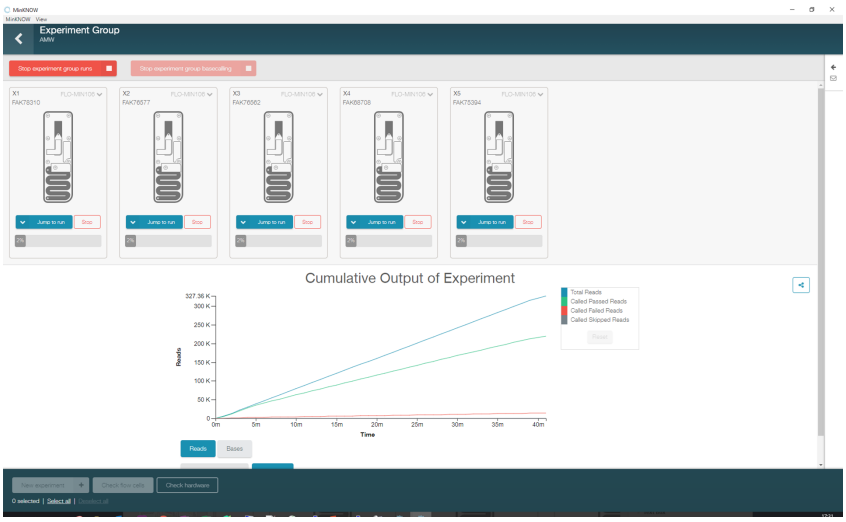
- Temperature
- Bias voltage
- Translocation speed
- Qscore
- Cumulative and individual output

Cumulative output of multiple flow cells - experiment view

If running multiple flow cells under the same experiment name, you will see the **Experiment view**. This gives outhput information on all assigned flow cells, plus a running cumulative total of *bases* or *reads* sequenced.

Total (cumulative) output v time

The cumulative output graph shows the running total number of Gbases sequenced by the multi-flow cell platforms, like GridION™ and PromethION™.



Multi-flow cell output v time

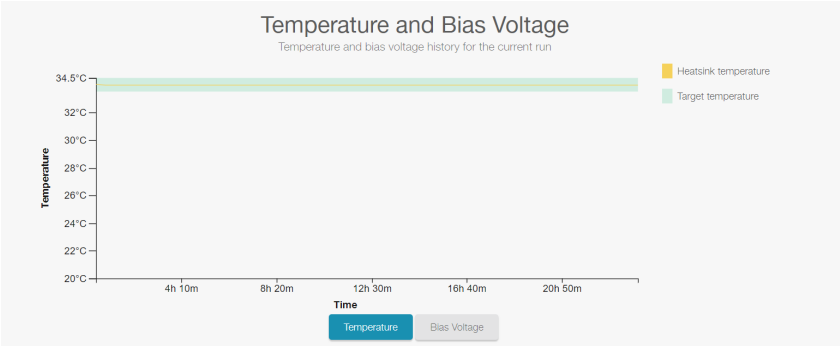
The output generated by each flow cell to make the total cumulative output can be represented by individual output plots (different coloured lines on the graph below).

Both graphs can be toggled between in the MinKNOW GUI. Passed, failed and skipped reads can be selected and deselected to tailor the output graph, as required.

Temperature and Bias voltage graph

Temperature vs time graph

The temperature graph gives a real-time representation of the temperature below the flow cell. If the temperature reading drifts out of the target zone, please consult Technical Services, otherwise the quality of your data may be compromised.

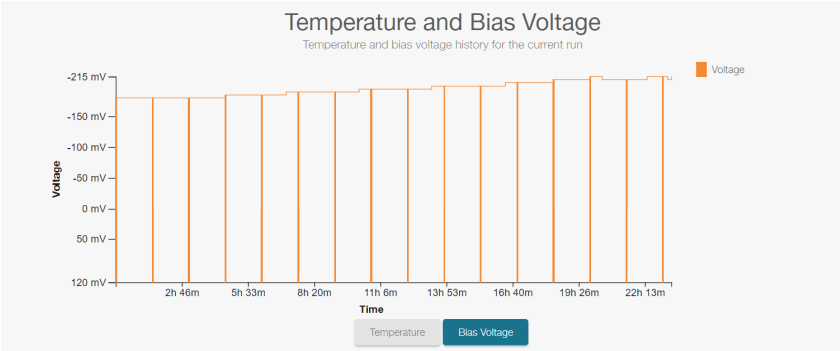


Bias voltage v time graph

The bias voltage graph provides the running voltage in real-time. MinKNOW will automatically adjust the applied voltage based on the strand range and will naturally drift to higher voltages as the electrochemistry in the well is depleted. This graph is useful for running a flow cell multiple times.

If you set the voltage for a subsequent run as the final running voltage of the previous run, then MinKNOW will find it easier to identify the appropriate running voltage.

You will notice drops in the voltage at regular intervals and these will correspond to the MUX scans that are defaulted to occur every one and a half hours. Here, each MUX - the options available to each channel - will be scanned to look for its availability for sequencing. The common voltage is reversed before and after each MUX assessment for clearer results.



The user can toggle between the graphs above in the MinKNOW GUI.

Translocation speed and Qscore graphs

Note: *These graphs are only present if Basecalling is turned on.*

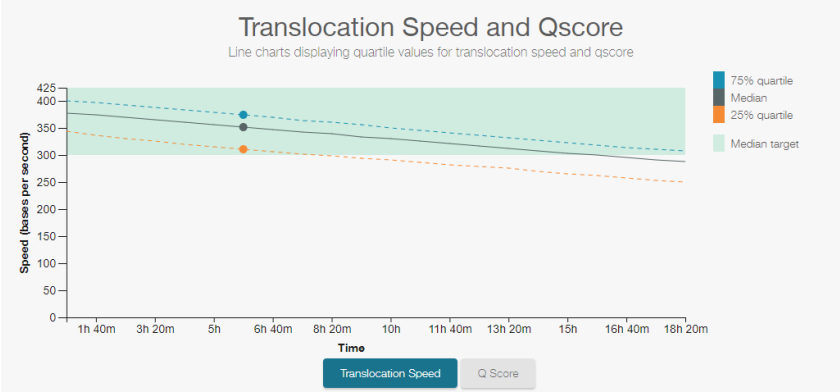
Translocation speed vs time

The translocation speed graph gives a new insight into the workings of the nanopore sequencing.

The **rate at which the DNA/RNA is passed through a nanopore** by the motor protein is proportional to the concentration of the **ATP** in the flow cell and **ATP turnover** will be proportional to the concentration of **motor protein** present in solution and runtime. As a result, we therefore recommend not to overload a flow cell with library, otherwise more motor proteins will turn over ATP in the bulk solution, limiting the ATP available for the motors above the pore.

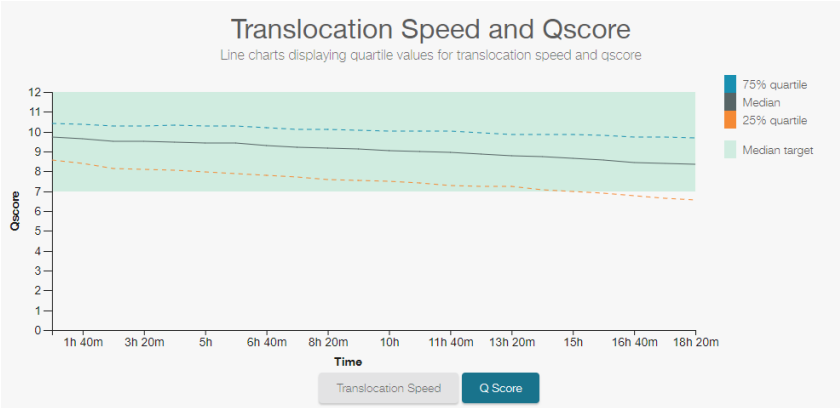
The translocation graph gives a real time representation of the speed at which DNA/RNA strands are passed through the pore. If the translocation speed drops below this window, then quality may be compromised, as well as potential output, as strands take longer to move through the pore.

Specific details on how to increase the speed will be given elsewhere in the near future



Qscore vs time

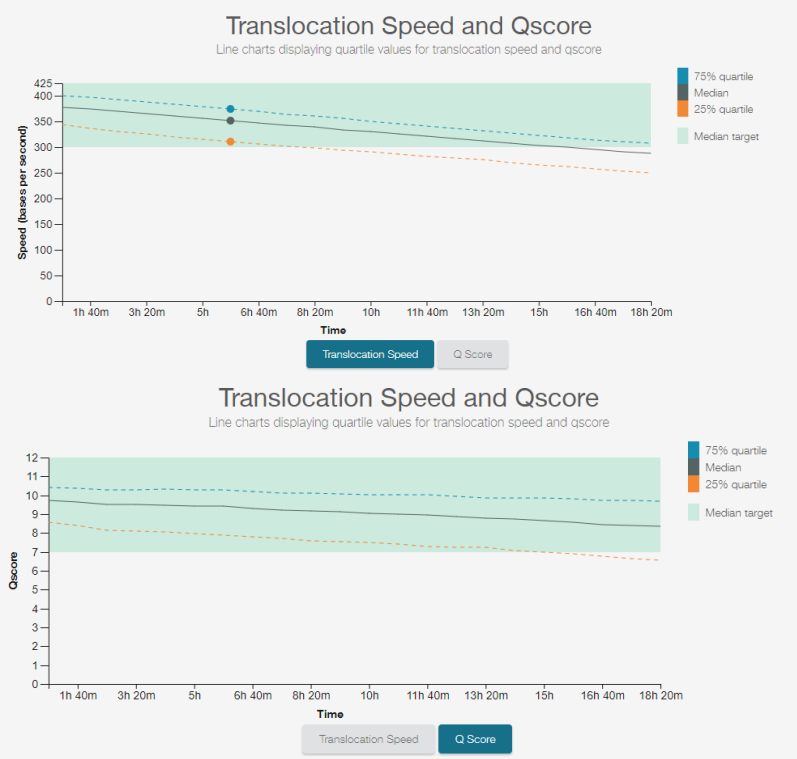
The Qscore graph gives a live representation of the median strand Qscore over time, plus a window that gives the pass/fail folder divide.



EXAMPLE

Local basecalling report in the MinKNOW GUI

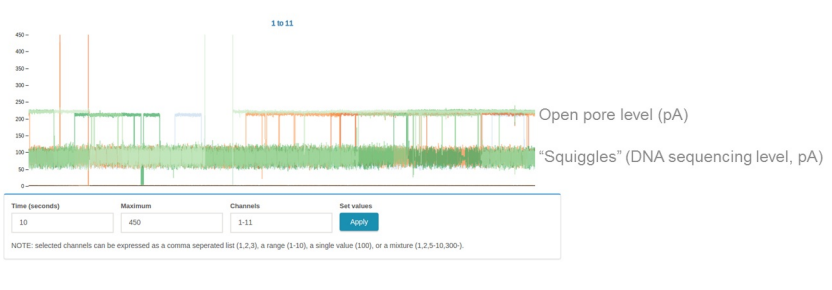
The progress of the local basecalling can be monitored via the **Basecalling** panel on the experiment page of the GUI. The graph shows the translocation speed or q-score against time.



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

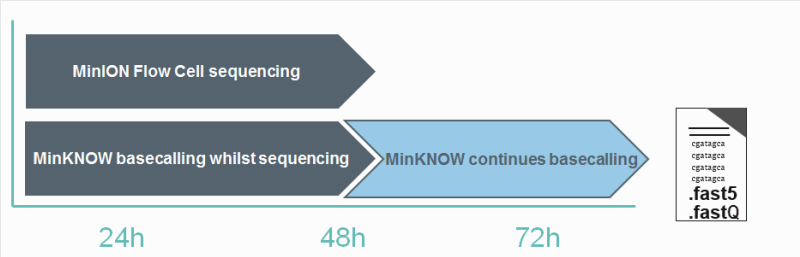


Please note that viewing a high number of channels in the trace viewer may impact the speed at which the GUI is able to function

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.

Assessing the quality of your run

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.

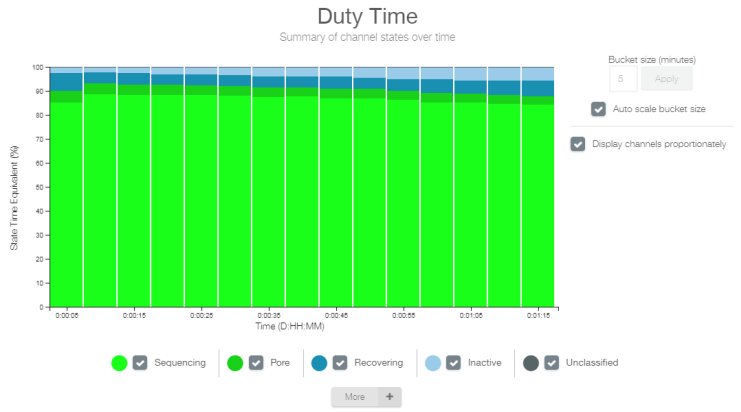
If Active Channel Selection is enabled during the run, the software instantly switches to a new channel in the group if a channel is in the “Saturated” or “Multiple” state, or after ~5 minutes if a channel is “Recovering”. This feature maximises the number of channels sequencing at the start of the experiment, 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.

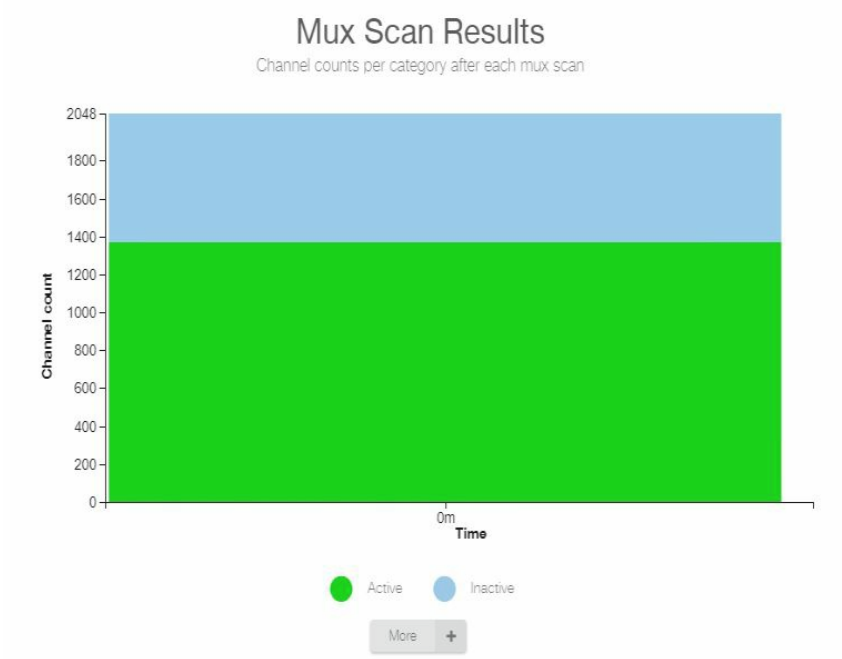
Good quality library

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



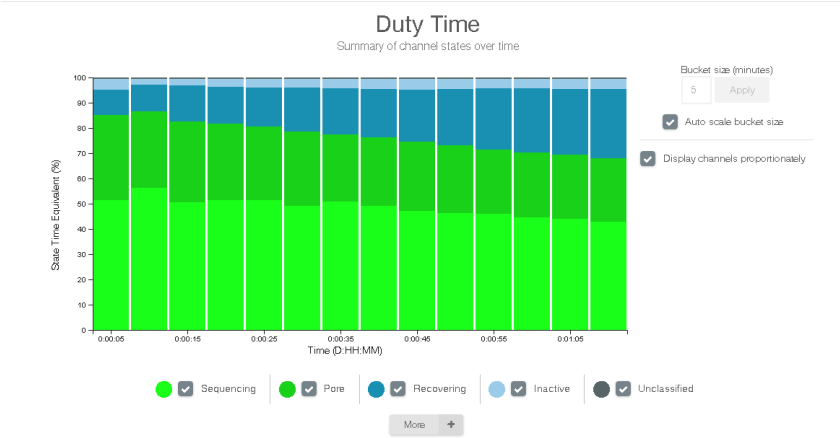
Mux scan results



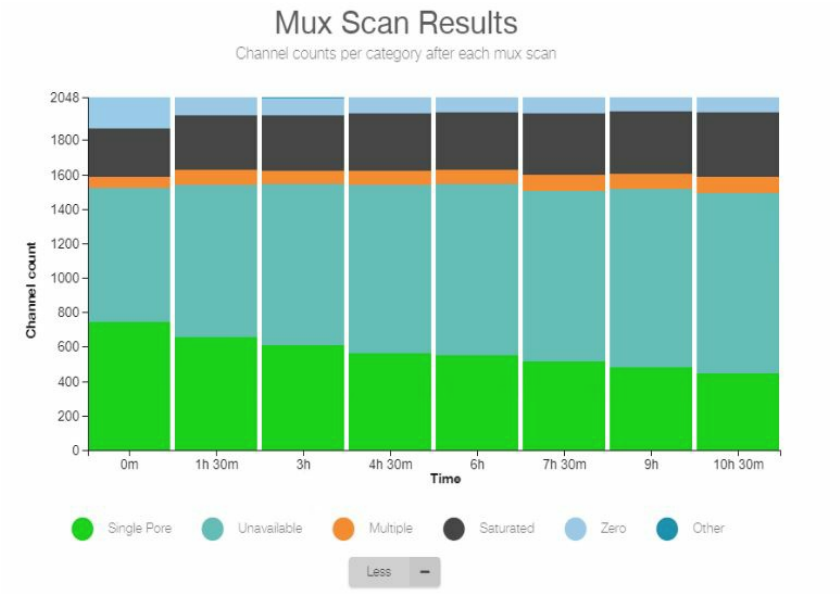
Channel blocking

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



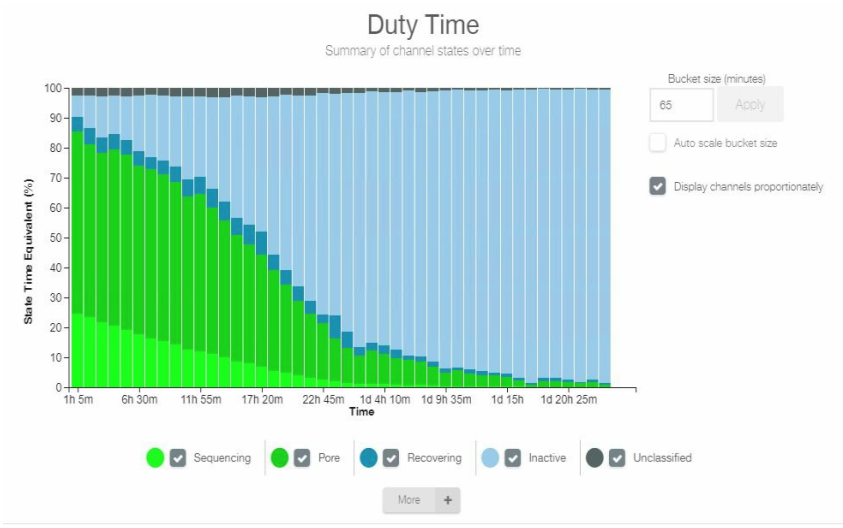
Mux scan results



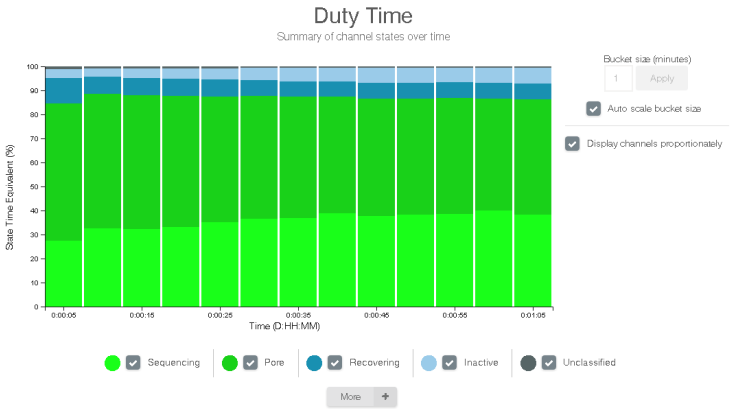
Low pore occupancy

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



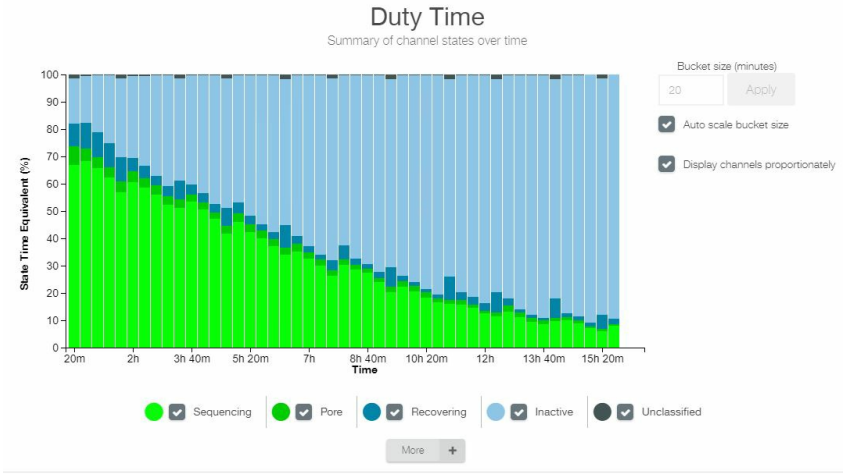
Duty time: with Active Channel Selection switched off



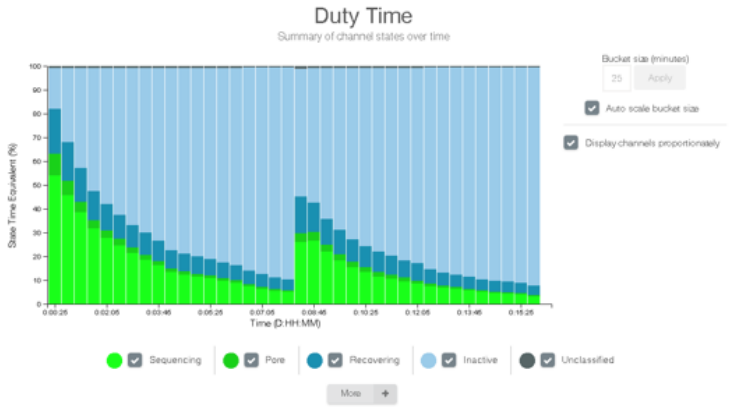
Flow cell failure due to osmotics or surfactants

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.

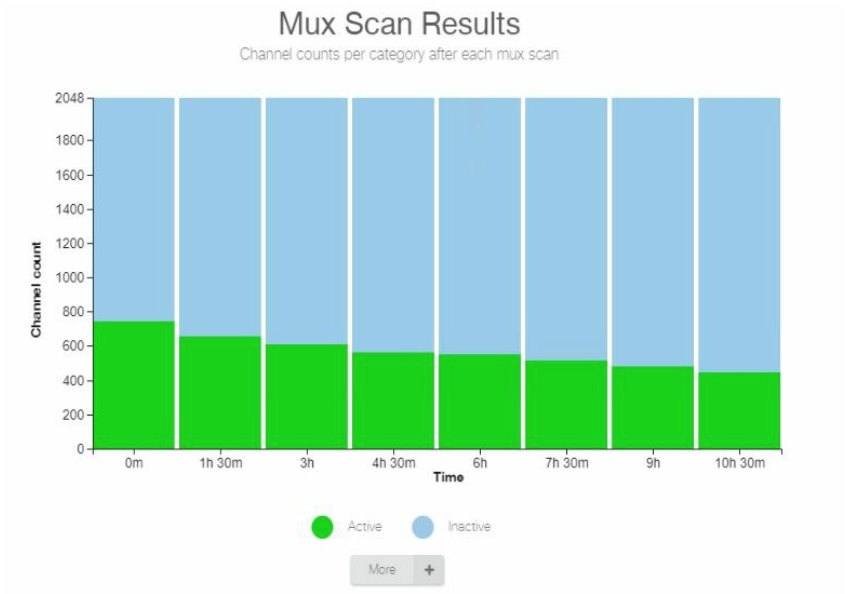
Duty time: with Active Channel Selection switched on



Duty time: with Active Channel Selection switched off



Mux scan results



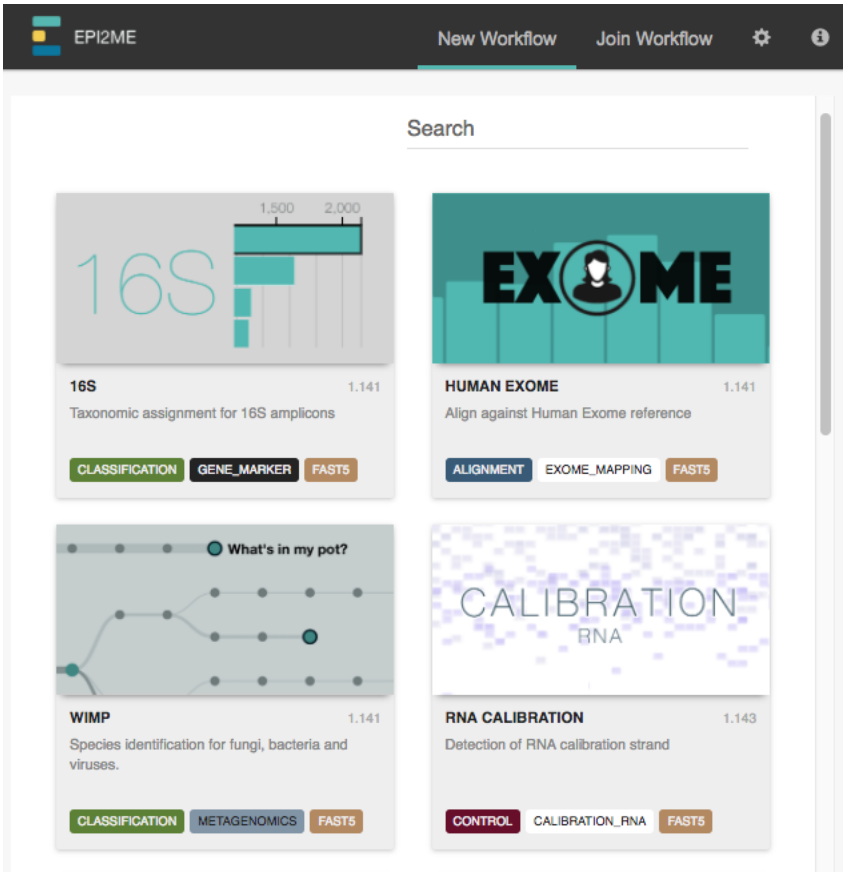
Further analysis with EPI2ME (optional)

Setting up and starting the upload and download of reads
~5 minutes

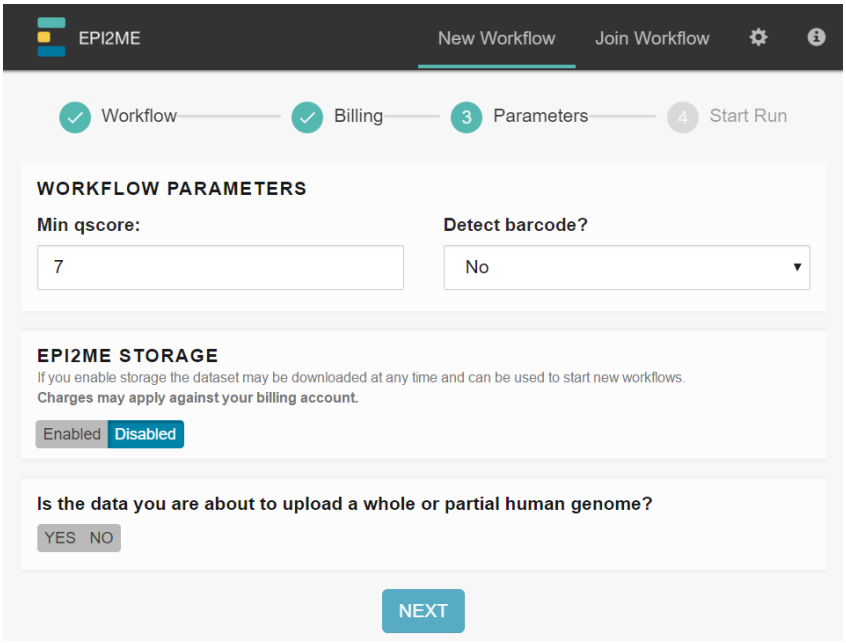
Consumables	• EPI2ME account
Equipment	• Host computer connected to the Internet with MinKNCW and the Desktop Agent installed

Open the Desktop Agent using the desktop shortcut.

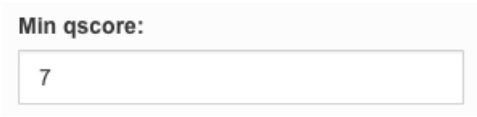
Click on the New Workflow tab in the Desktop Agent and select the workflow to be used in the analysis.



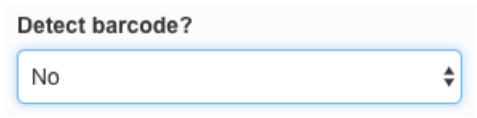
Select the workflow parameters.



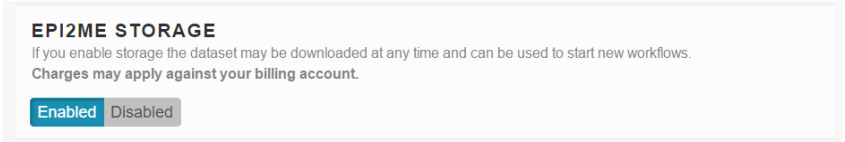
- Select the quality score cut-off



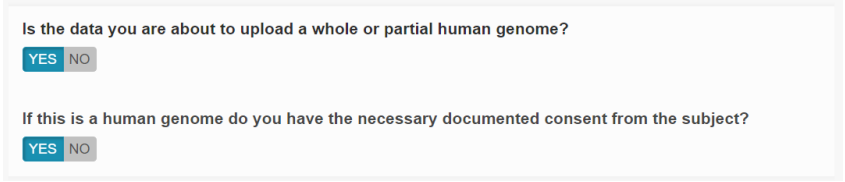
- Select "No" under "Detect barcode?"



- Select whether you would like to store the data in the Data Manager

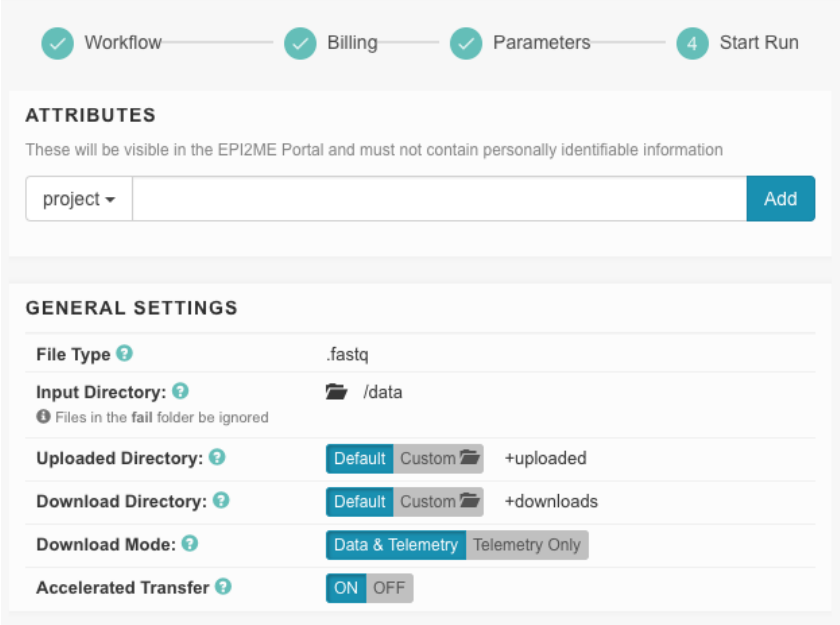


- If you are working with human data, please tick "Yes" in answer to "Is the data you are about to upload a whole or partial human genome?", and confirm that you have consent from the subject to upload the data.



Check the correct settings are selected in the Desktop Agent.

Check Input Directory, Uploaded Directory, Download Directory, Download Mode, and Accelerated transfer.



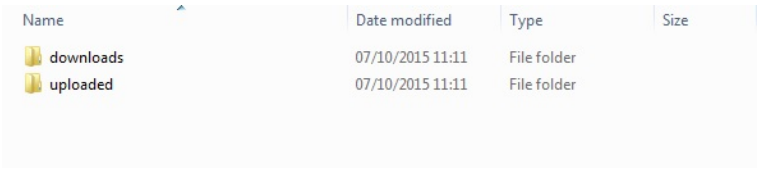
Click "Start Run" to start data analysis.

Follow the progression of upload and download of read files in the Desktop Agent.

Click on VIEW REPORT.

- Click on VIEW REPORT to navigate to the EPI2ME website, this can be done at any point during data exchange
- Return to the Desktop Agent to see progression of the exchange

When the upload and download numbers are the same, the data exchange is complete. The processed reads will be in downloads folder in the selected location on the host computer.



Close down MinKNOW and the Desktop Agent

~2 minutes

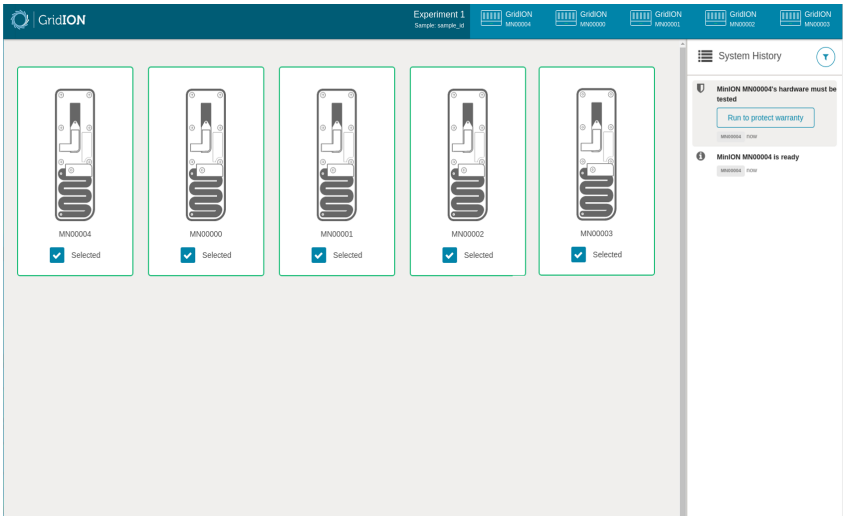
Quit Desktop Agent using the close x.

Quit MinKNOW by closing down the web GUI.

Disconnect the MinION.

Powering off the device

Ensure that no experiments are running.



Close the GUI using the "x" in the top left corner.

Select "Shut down" from the power options at the top right of the desktop.

DO NOT select "Restart". This will lead to errors in recognising the flow cells.

Wait for the screen to turn black.

If using mains power, turn off the device at the mains supply.

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

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.