Rapid Sequencing (SQK-RAD004)

Overview

Version: RSE_9046_v1_revB_17Nov2017

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

Overview

Overview 1

Overview 2

- Overview of the Rapid Sequencing protocol 2

Prepare for your experiment 3

- Preparing input DNA 4
- Equipment and consumables 9
- Computer requirements and software downloads 11
- Check your flow cell 12

Prepare your Rapid library 17

- Library preparation 18
- Priming and loading the SpotON Flow Cell 20

Start sequencing and data analysis 22

- Starting a sequencing run 22
- Progression of MinKNOW protocol script 27
- Assessing the quality of your run 32
- Further analysis with EPI2ME (optional) 35

Complete the experiment 38

- Close down MinKNOW and the Desktop Agent 38
- Prepare the flow cell for re-use or return to Oxford Nanopore. 38

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

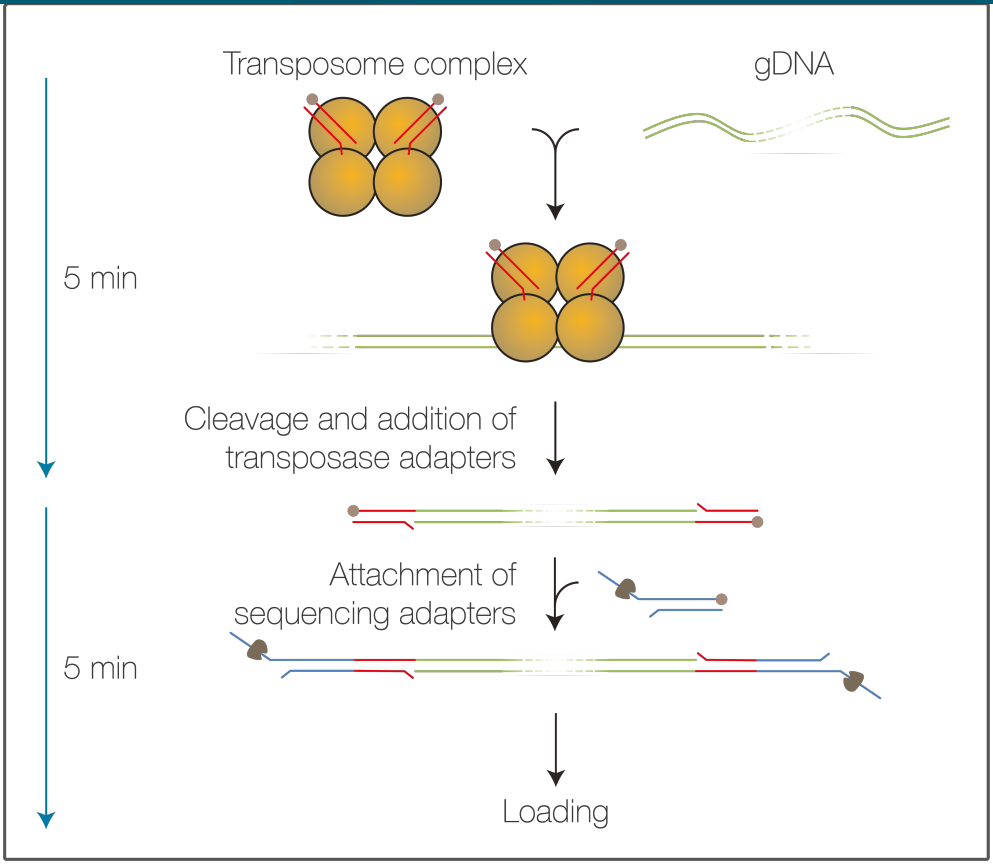
Library preparation

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

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revB_17Nov2017



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)
- MinKNOW script for local basecalling ending in *_plus_1D_Basecaller.py*

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

Oxford Nanopore's protocols recommend an input quantity in mass (e.g. 1 µg), 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.

For example, if you start library prep with 50-100 fmoles of DNA, you can expect to load 5-50 fmoles onto 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

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
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
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.

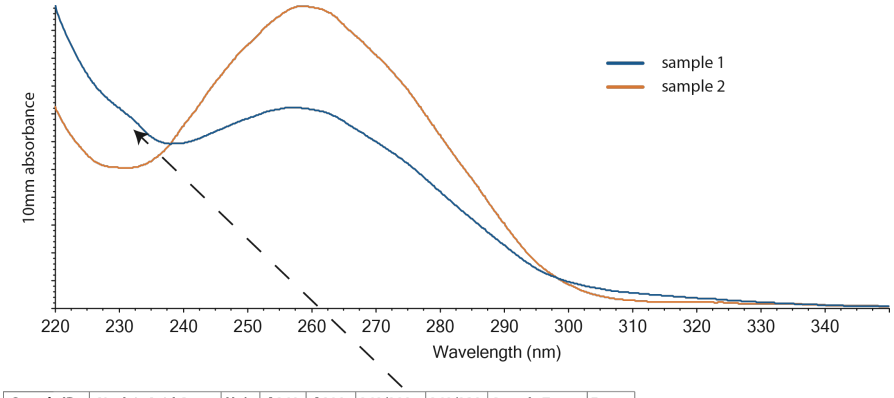
We recommend that the DNA stock is quantified using Qubit analysis. A Qubit measures DNA specifically. Even after RNAse digestion, residual RNA is a common contaminant in gDNA preparations and is not well identified by Nanodrop measurements. Incorrect quantification could mean that you will proceed with 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.
- Prior to fragmentation, 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 (figure DQND), 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

Figure DQND. NanoDrop trace of absorbance.

Assessing molecular weight

Nanopore sequencing devices 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 (figure AGLD).

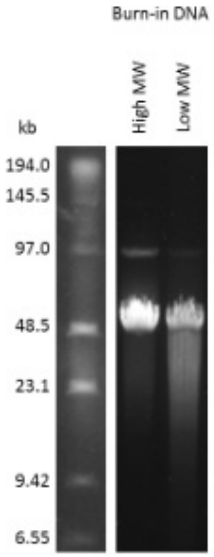


Figure AGLD: Two samples of Lambda DNA: one of intact high molecular weight fragments and one containing a significant proportion of low molecular weight fragments

Low % agarose gel analysis can be used to detect substantial degradation / shearing (figure AGDS):

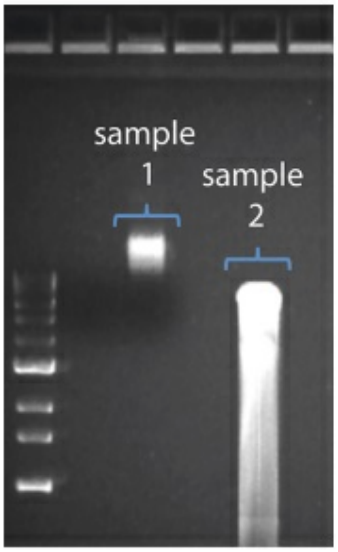


Figure AGDS: The figure shows two samples of input DNA: Sample #1 is of high molecular weight and sample #2 is of lower

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

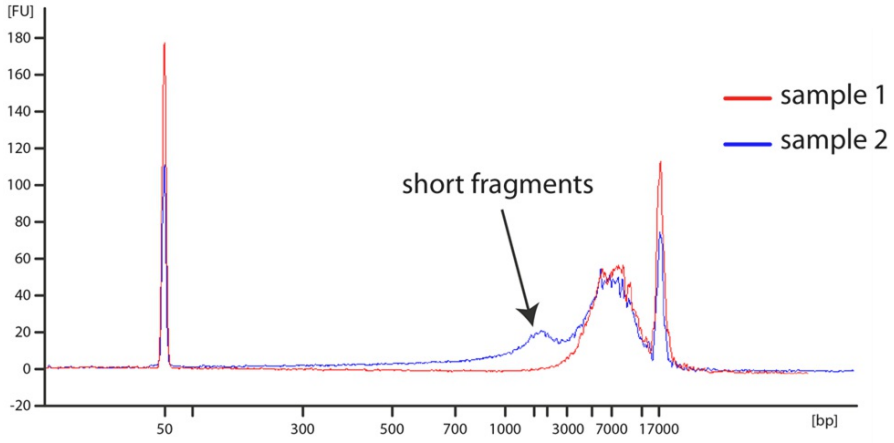


Figure DQFA: Agilent Bioanalyzer trace of two DNA samples after fragmentation

The figure above (figure DQFA) shows successful (sample 1) and unsuccessful (sample 2) fragmentation. Sample #2 contains a substantial proportion of low molecular weight fragments. This is possibly as a result of improper fragmentation, or these low MW weight 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-FLP001)	• 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 (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revB_17Nov2017

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.

Flow Cell Priming Kit contents

FLB : Flush buffer
FLT : Flush tether

Contents	Description	No. of tubes
FLB (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.

Albacore (optional)

The Albacore 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	• MinION 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 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

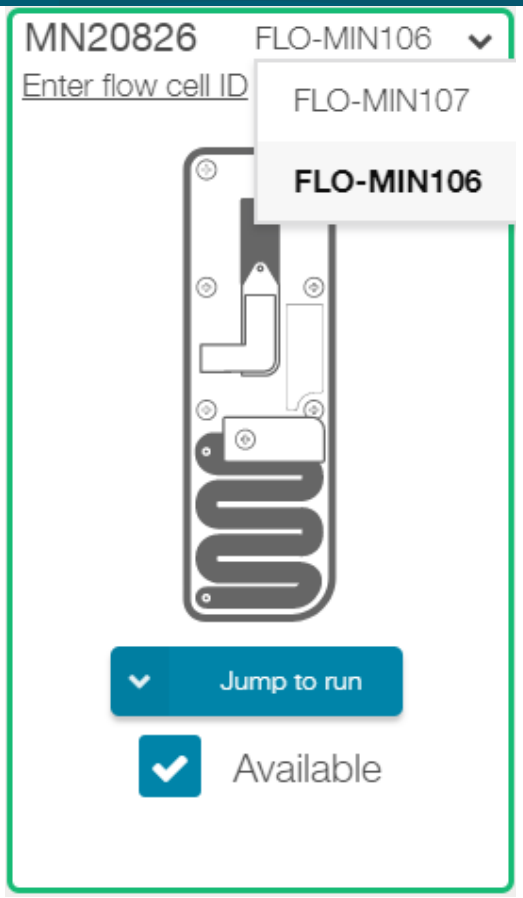
Figure SSFC: Assembled MinION and MinION SpotON Flow Cell connected to through the USB 3.0 port to the host computer

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**.

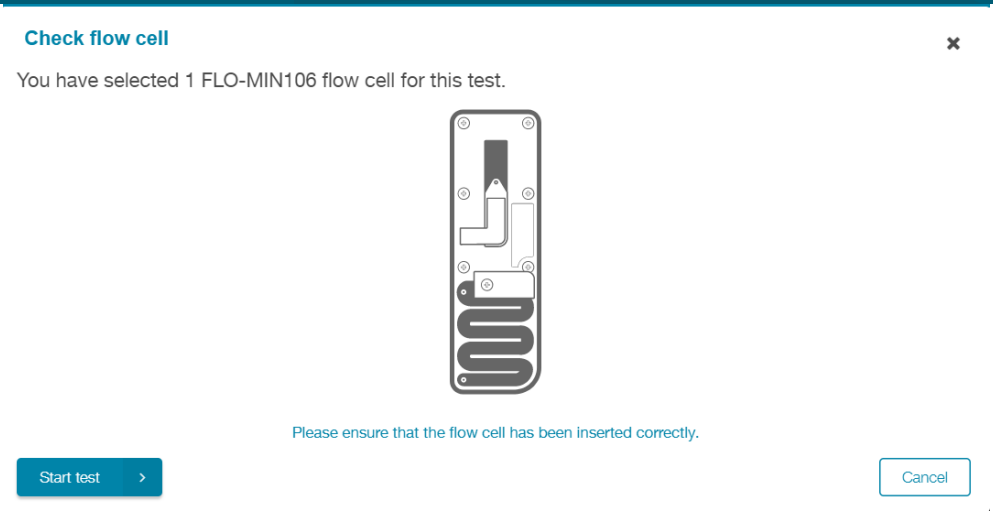
- Choose the flow cell type from the selector box. Then mark the flow cell as "Selected":



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

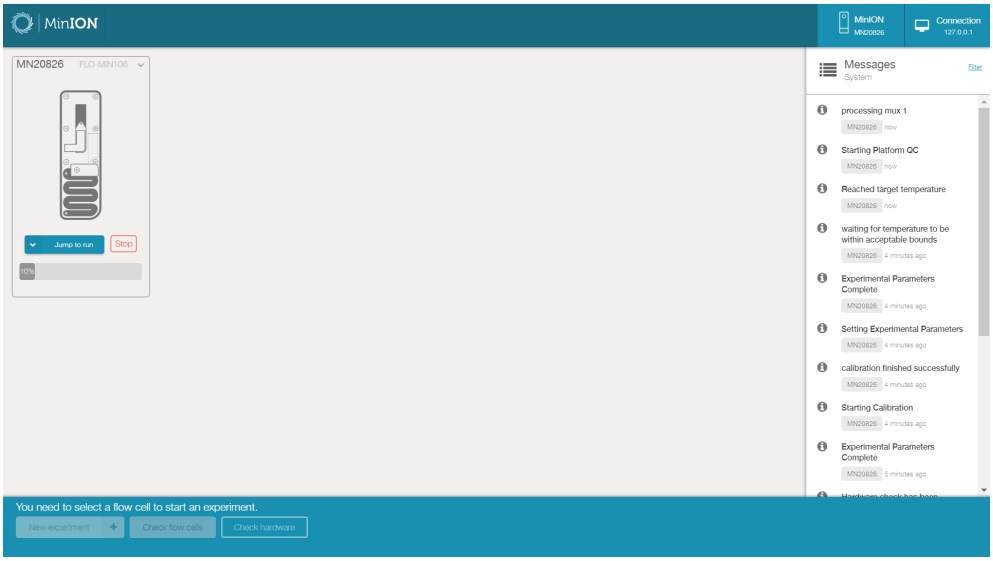
A screen will load displaying the number of flow cells selected for your test, and offering a drop down for flow cell type. It is important the correct flow cell type is chosen to obtain an accurate result at Platform QC:

Flow cell code	Pore type
FLO-MIN106	R9.4
FLO-MIN107	R9.5

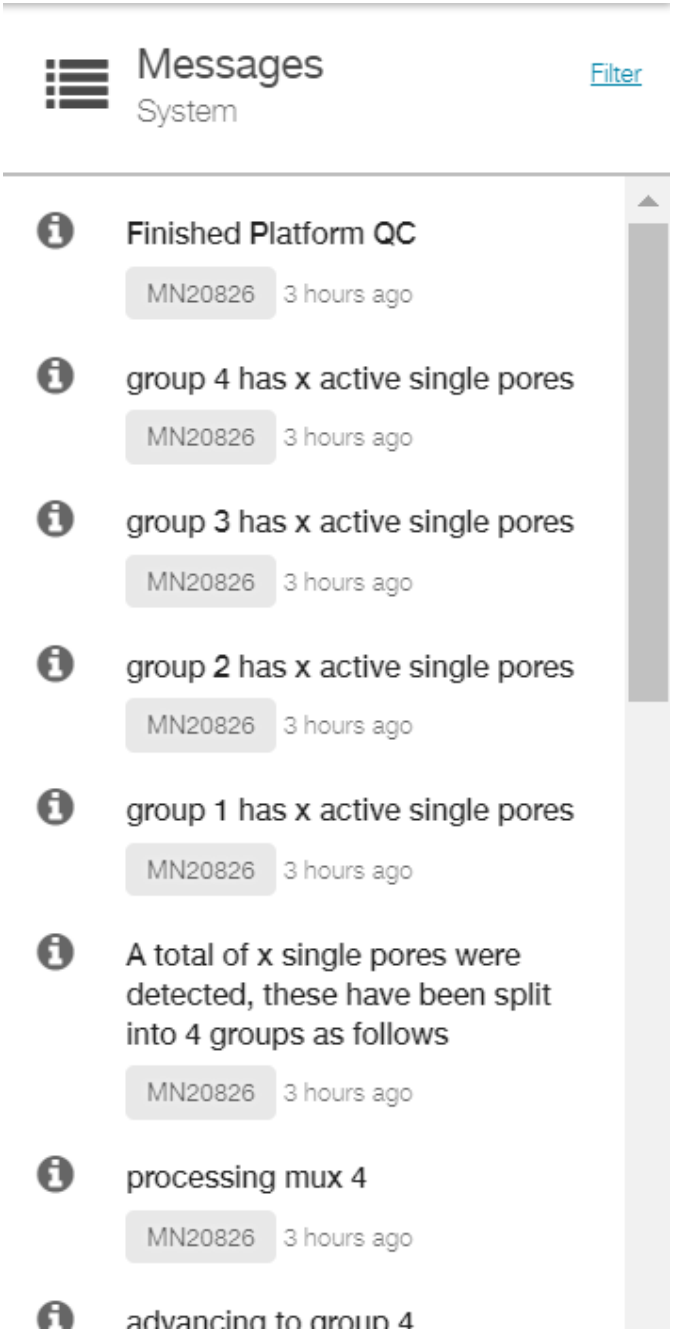


Click "Start test".

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

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

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 (FLB) • Loading Beads (LB)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • SpotCN flow cell	• 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

Thaw the Sequencing Buffer (SQB), Loading Beads (LB), 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 Sequencing Buffer (SQB) and Flush Buffer (FLB) 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 (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.

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.

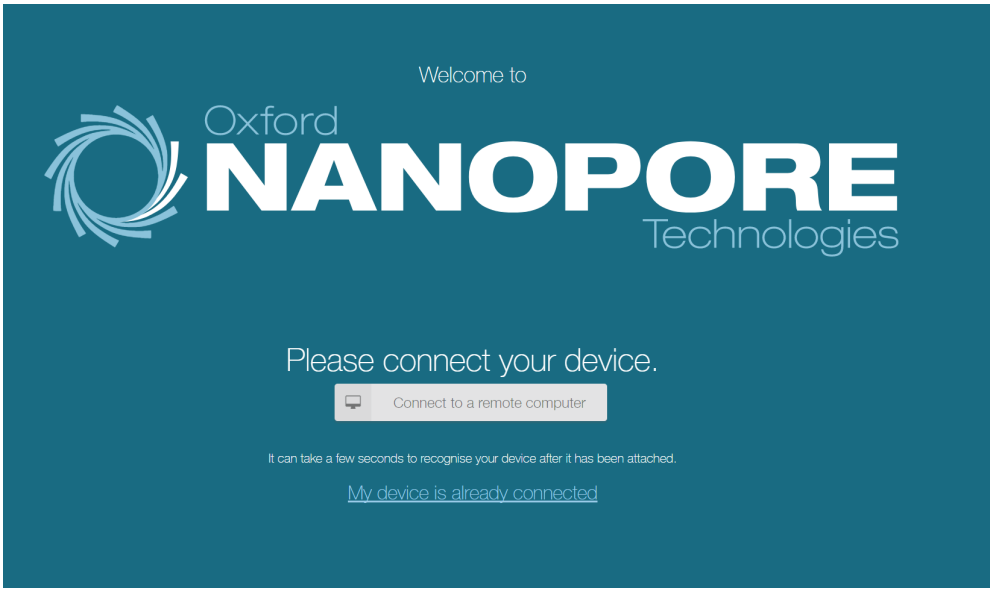
Starting a sequencing run

~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.

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**.

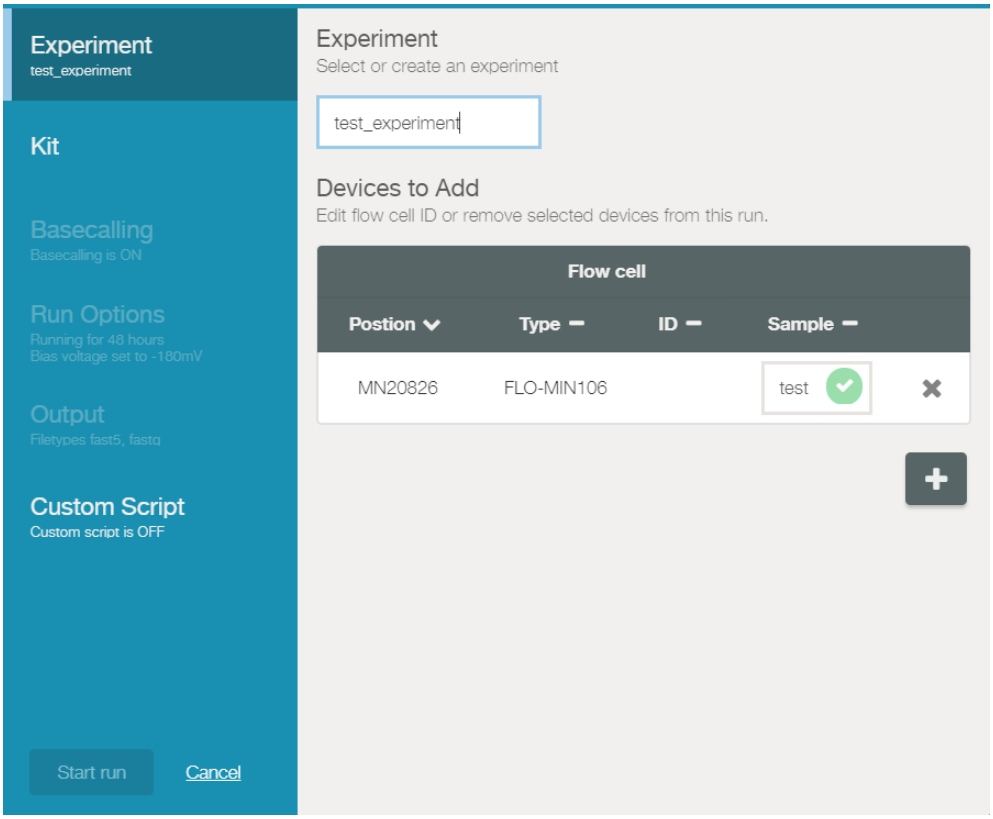


Click the "New Experiment" button at the bottom left of the GUI.

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

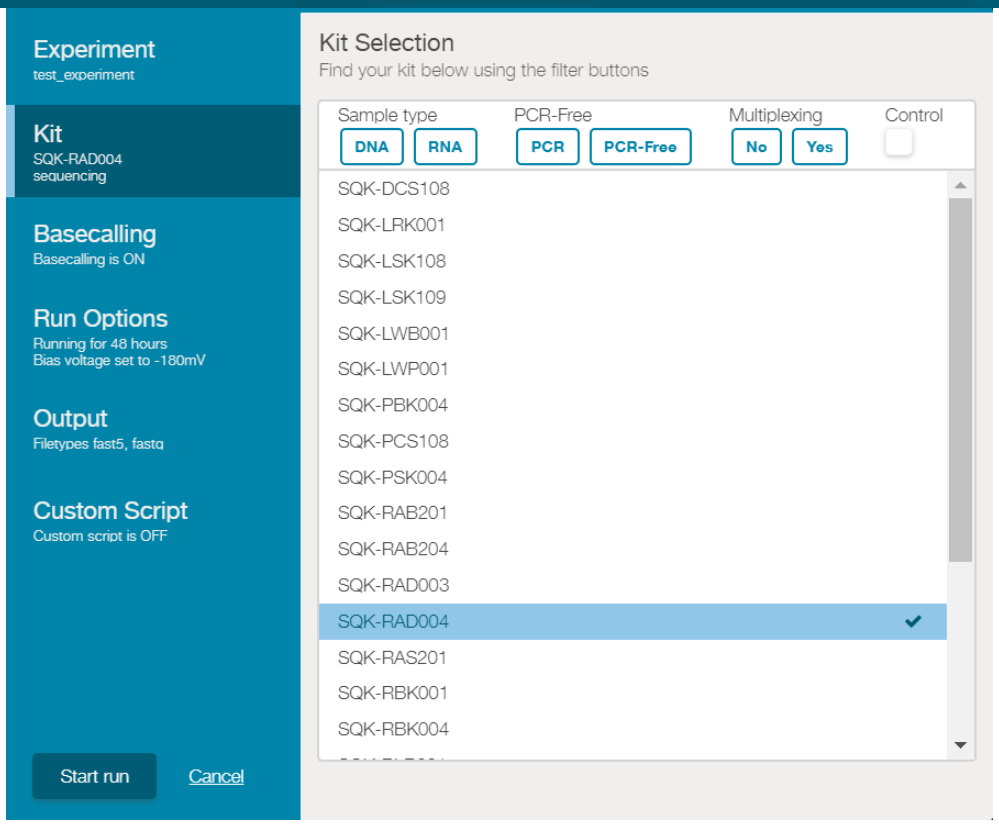
Experiment name

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.



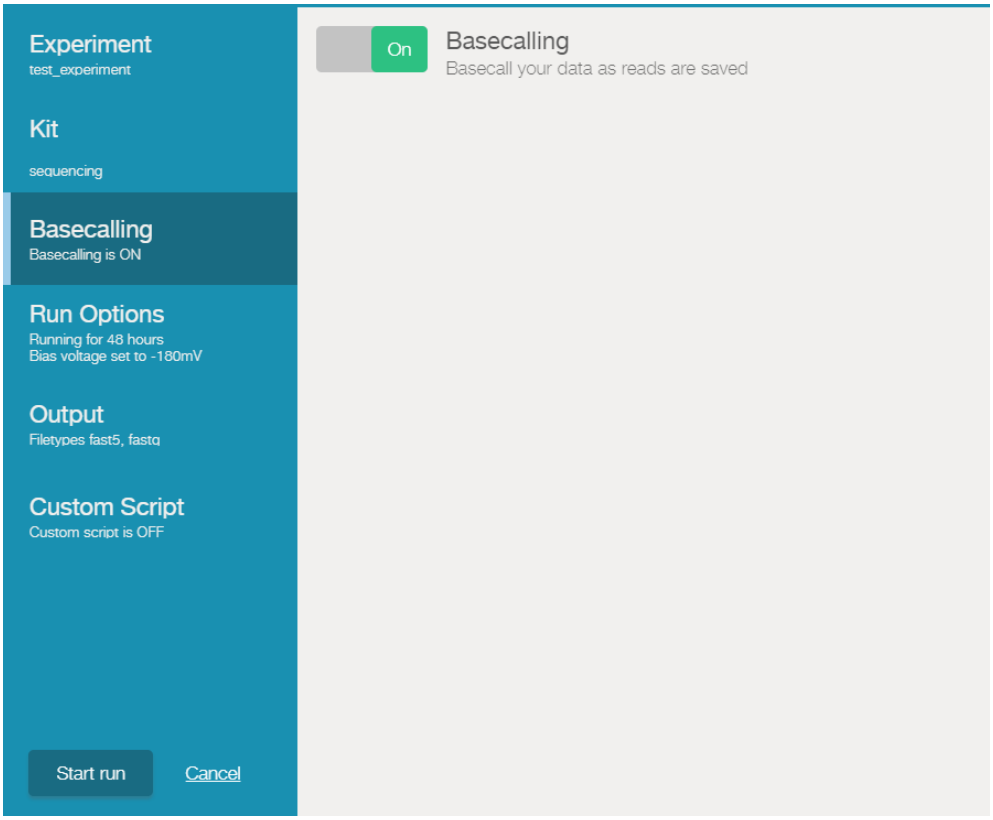
Kit selection

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



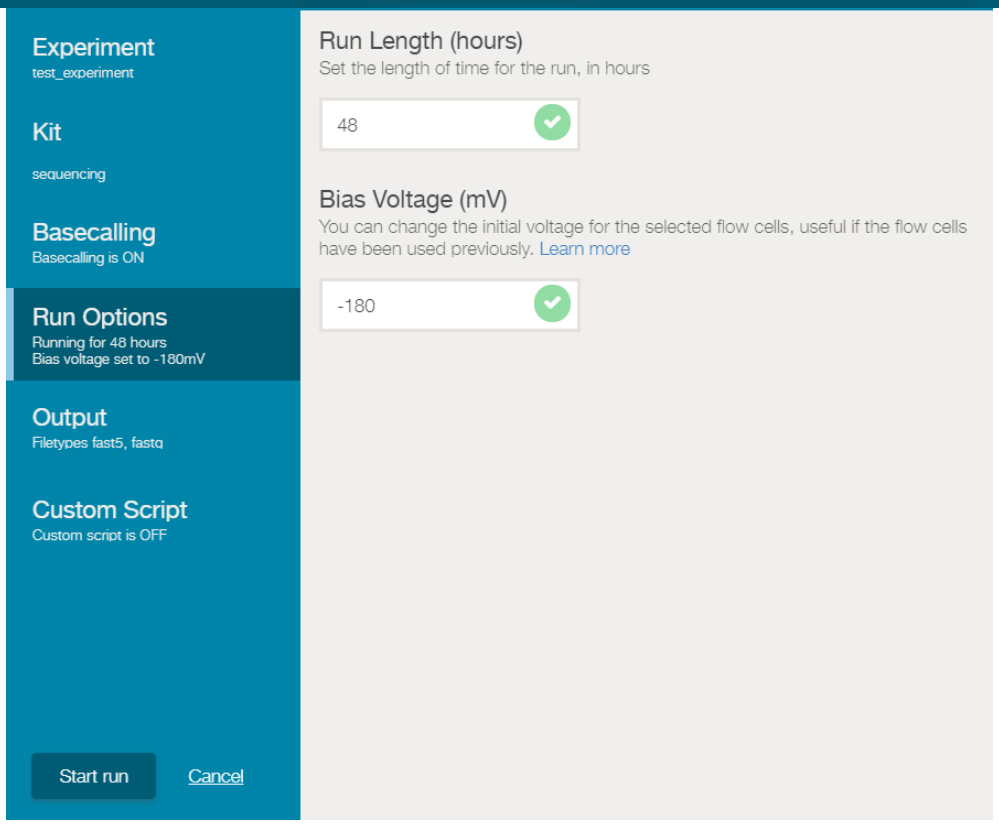
Basecalling

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.



Output

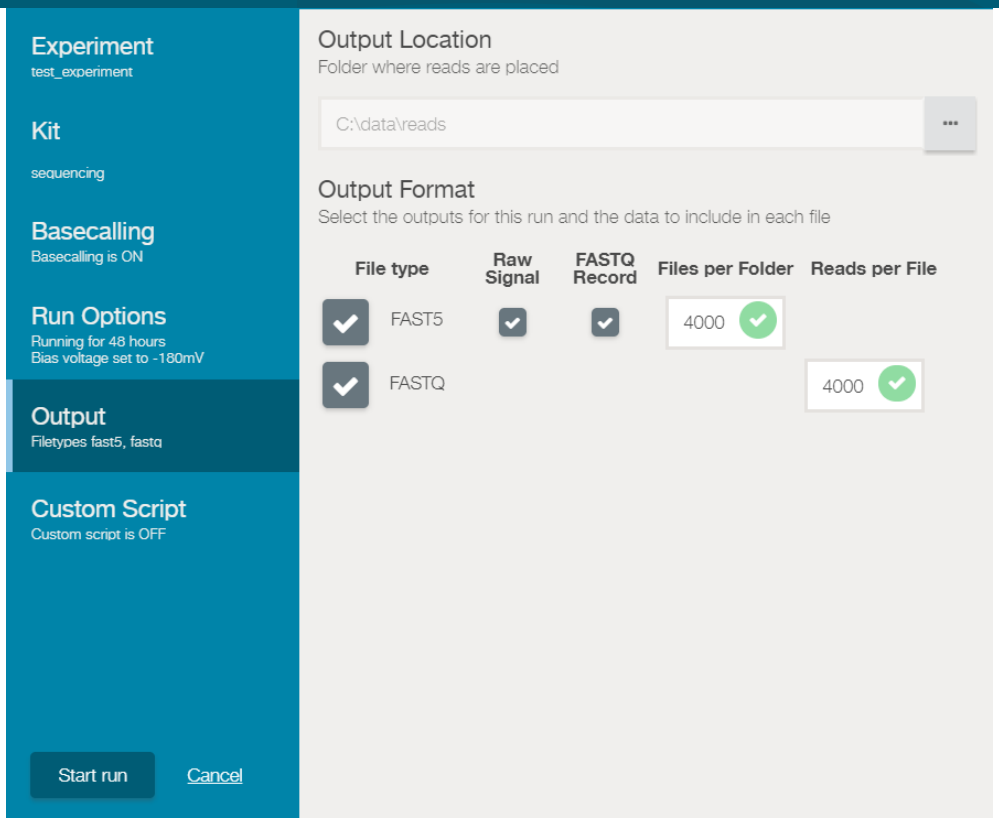
You can specify to produce:

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 "Begin Experiment".

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

Allow the script to run to completion.

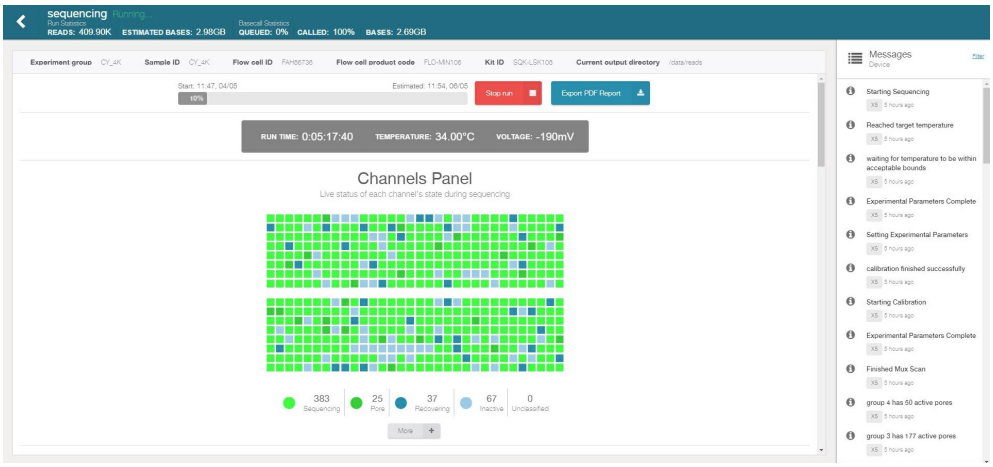
- The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen
- Monitor messages in the Message panel in the MinKNOW GUI

The basecalled read files are stored in :\data\reads

Progression of MinKNOW protocol script

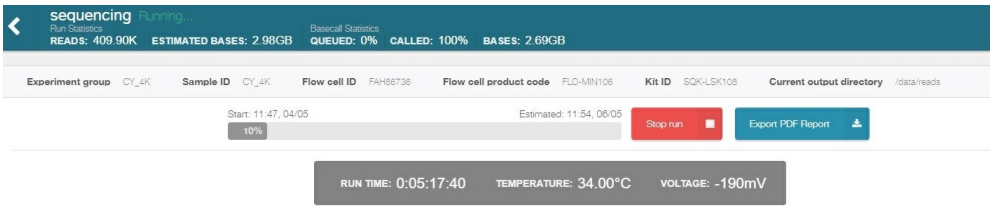
The running experiment screen

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 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

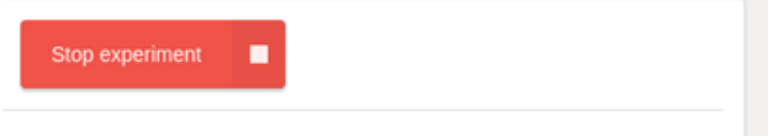
TIP

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

- Number of active pores
- Heatsink termperature
- 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 Platform QC

- 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 Platform QC, 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 experiment" button at the top of the screen.



Check the heatsink temperature is approximately 34° C.

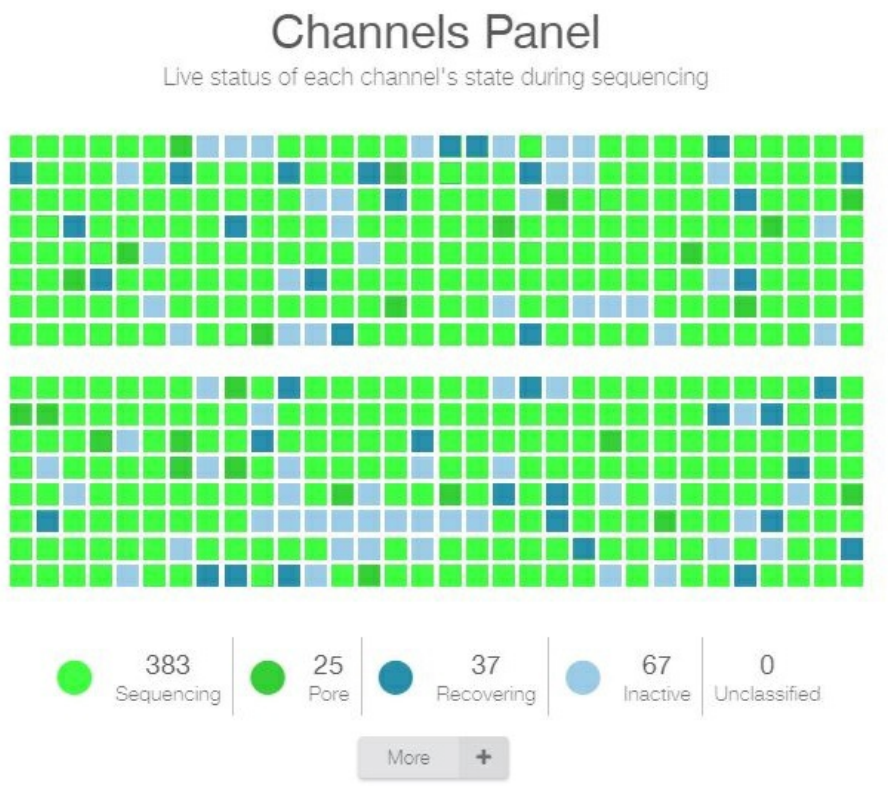
MinION temperature control

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



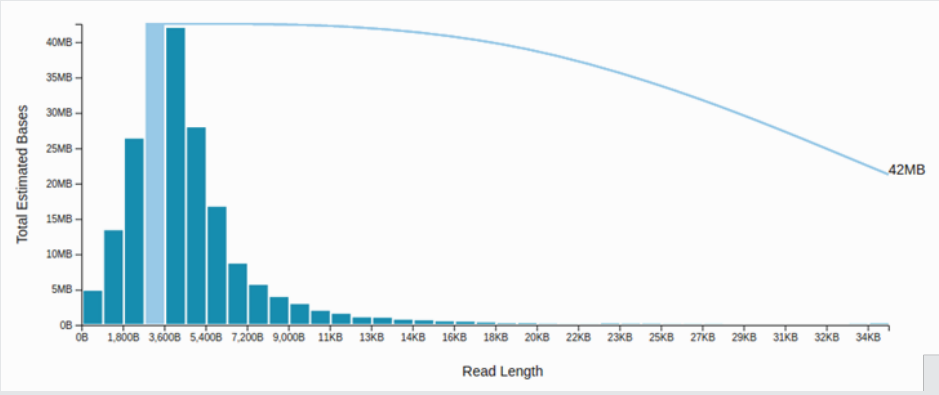
Monitor the pore occupancy

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 development of the read length histogram.

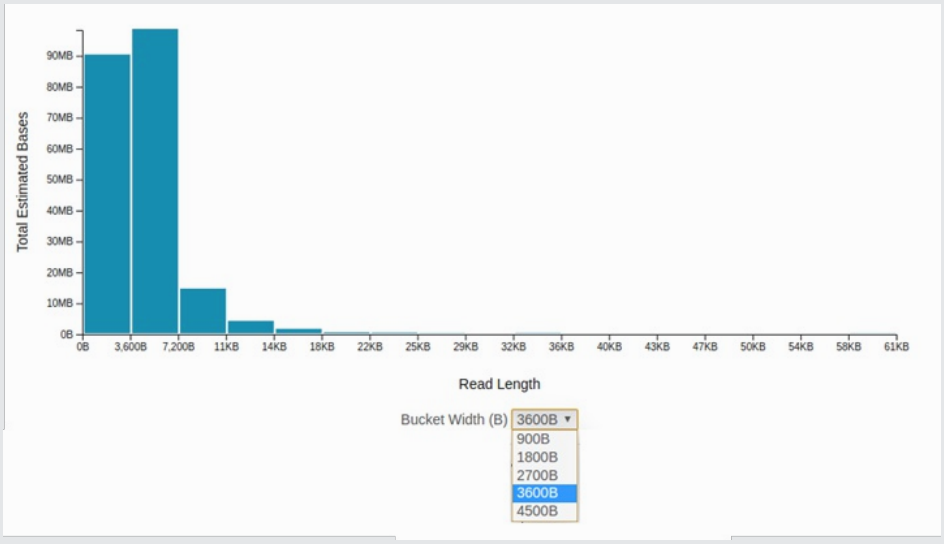
EXAMPLE

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.

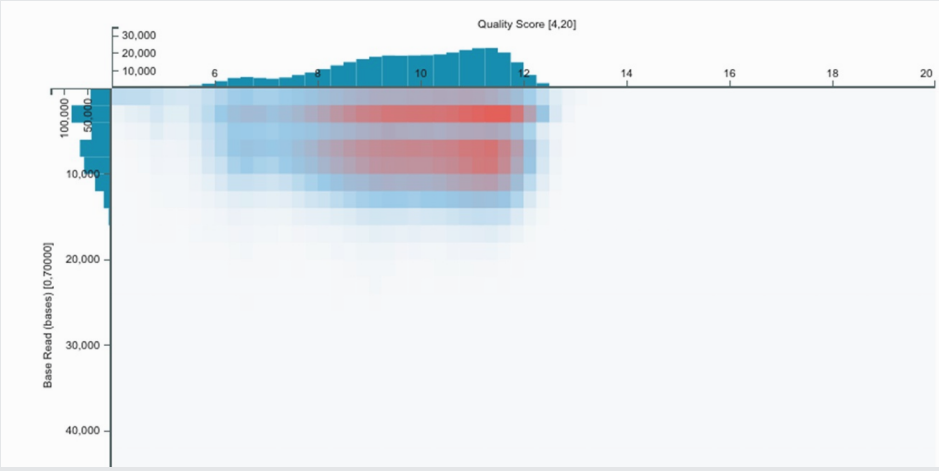
Additional control is available over the bin size in the histogram; the drop down below the x axis allows selection of 5 different bin sizes, with the size of the bin shown in bases i.e. 3600B is a bin size of 3600 bases.



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:

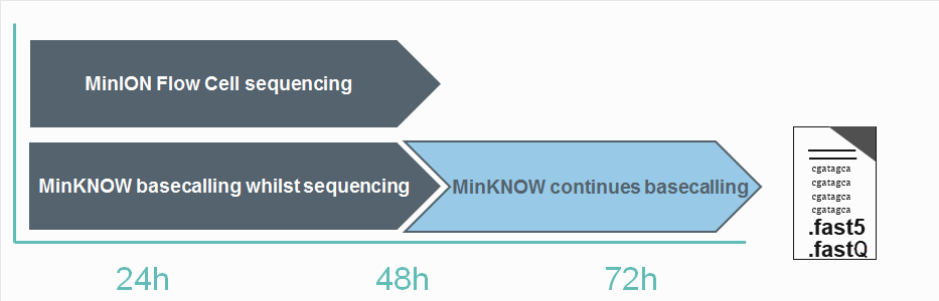
- **Time:** The length of time plotted on one screen
- **Maximum:** The highest current level to be shown on the y axis

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 installation of the software. 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

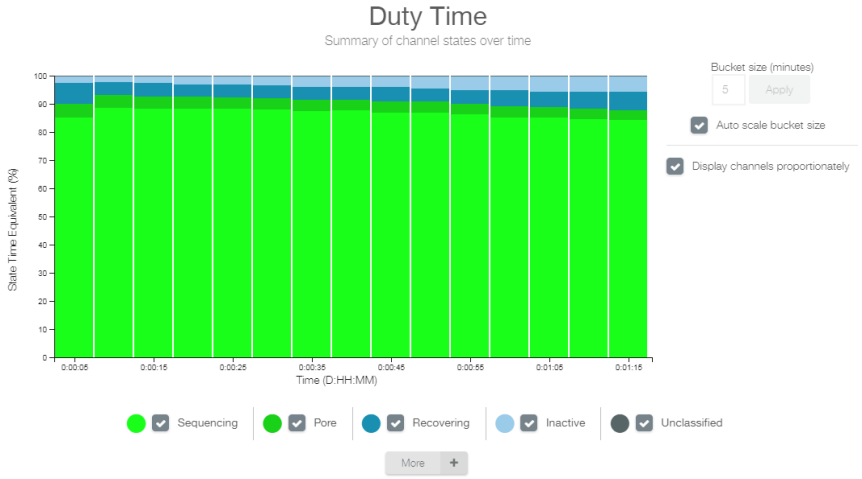
The Duty Time feature in the MinKNOW software can be used to judge the quality of your experiment.

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.

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

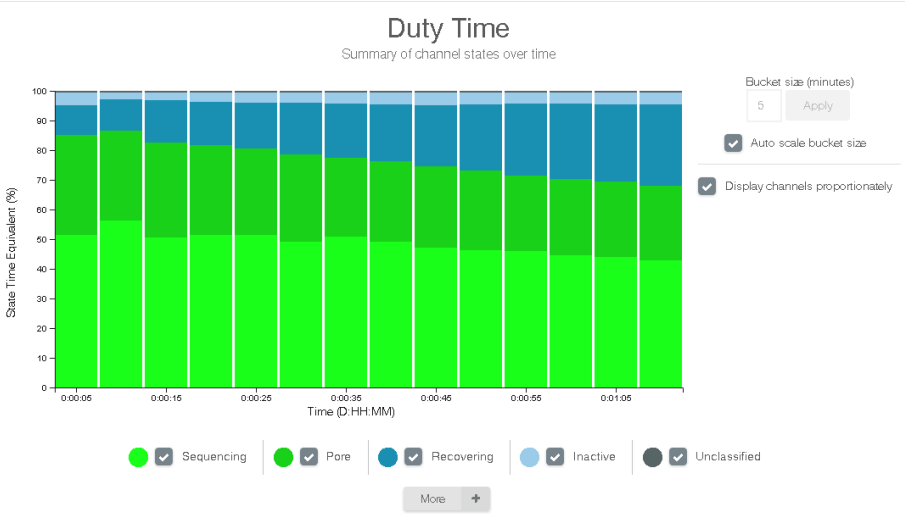
Good quality library

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.



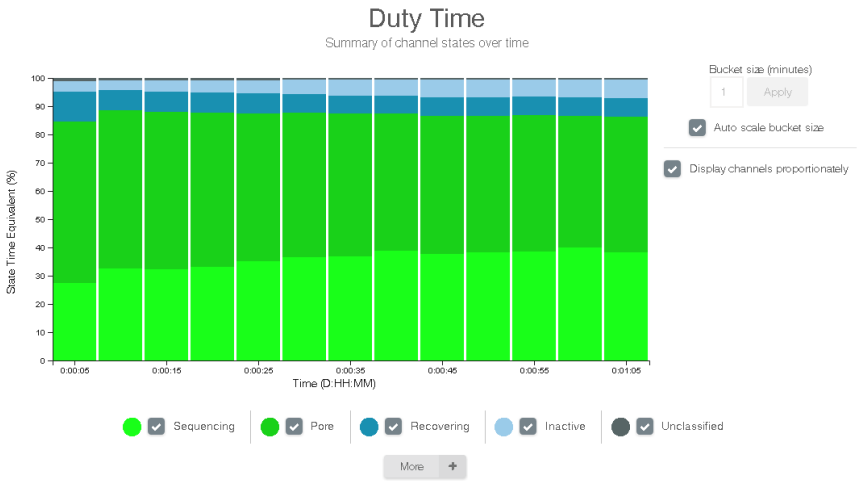
Channel blocking

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.



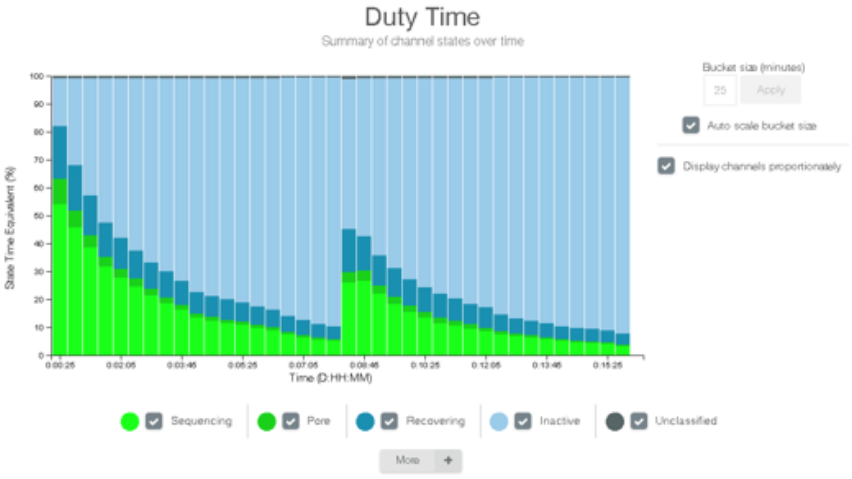
Low pore occupancy

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.



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.



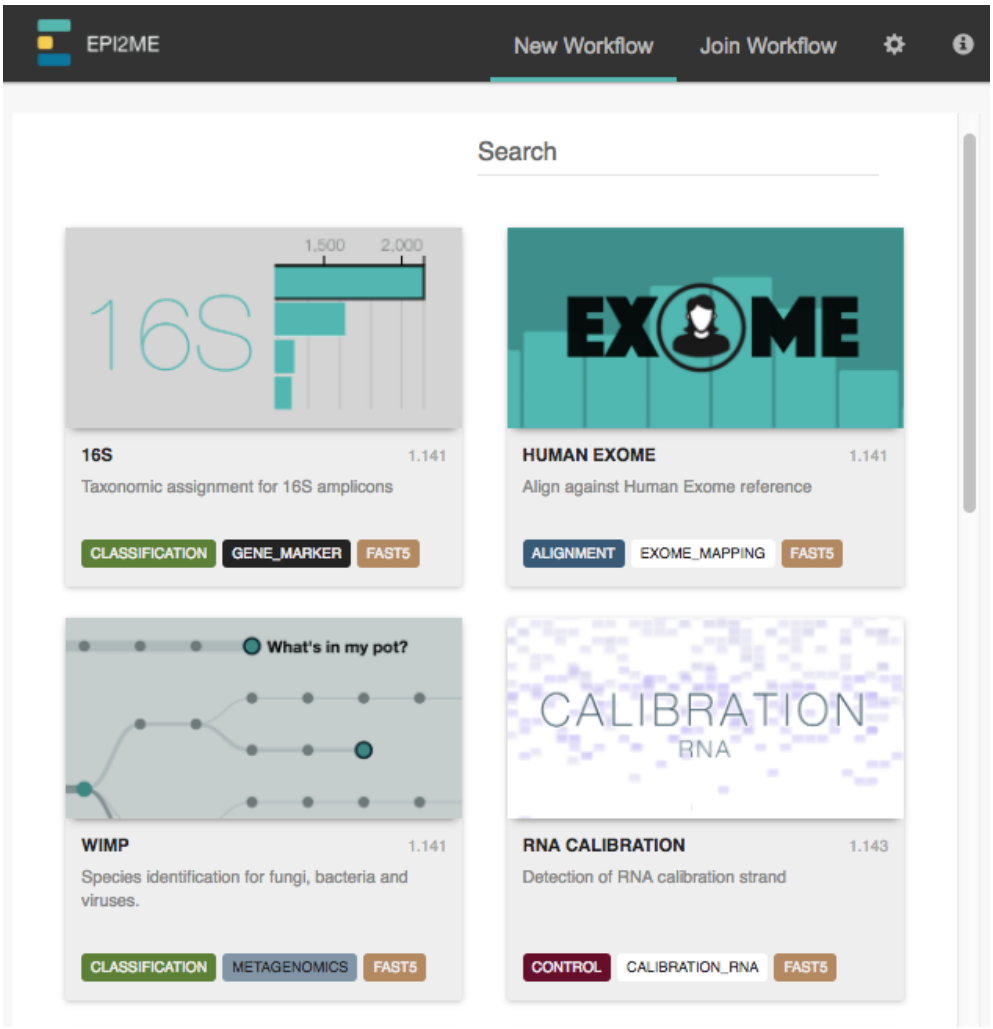
Further analysis with EPI2ME (optional)

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

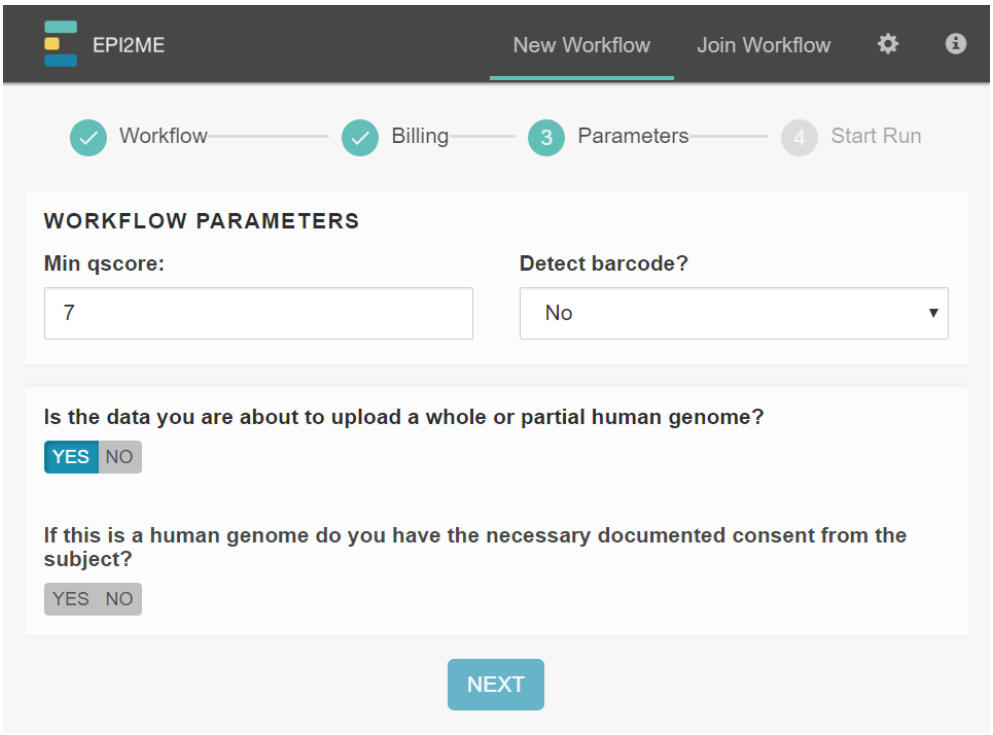
Open the Desktop Agent using the desktop shortcut.

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



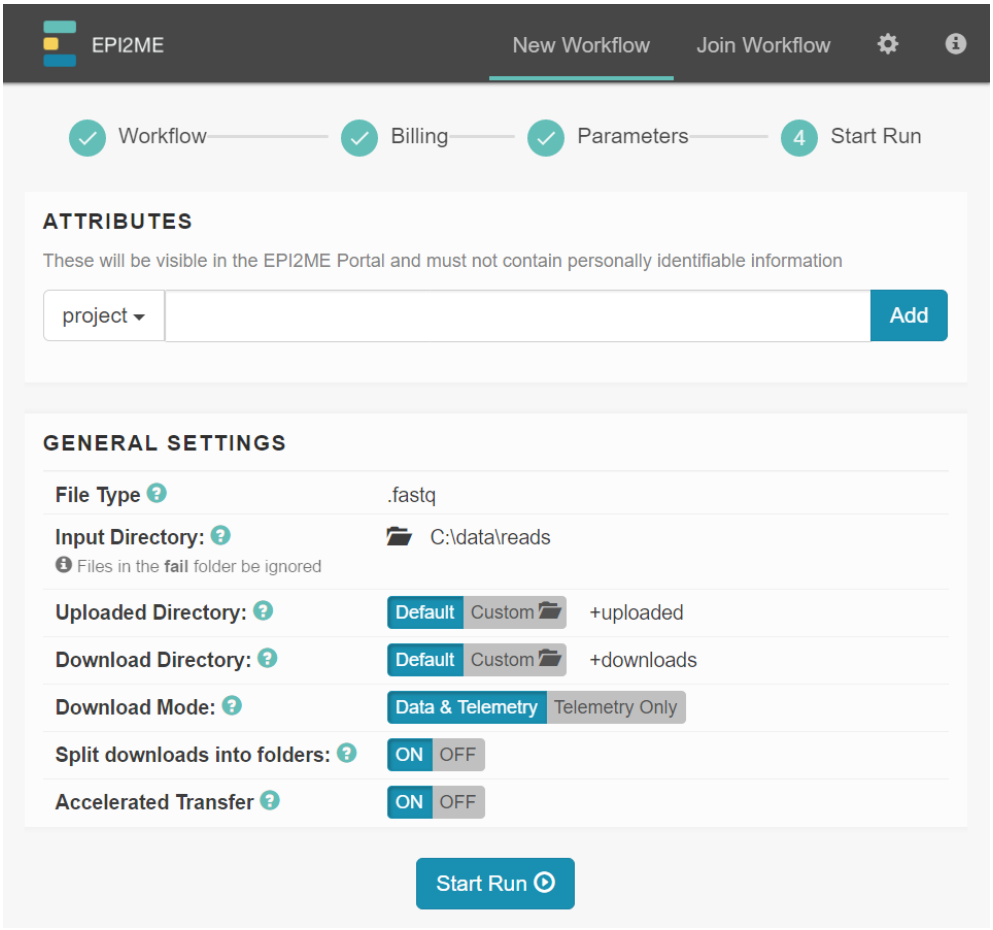
Select the workflow parameters.

- Select the quality score cut-off (this defaults to 7 unless changed)
- Select whether the reads should be demultiplexed by barcode
- 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, Split downloads into folders, and Download Mode.



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.

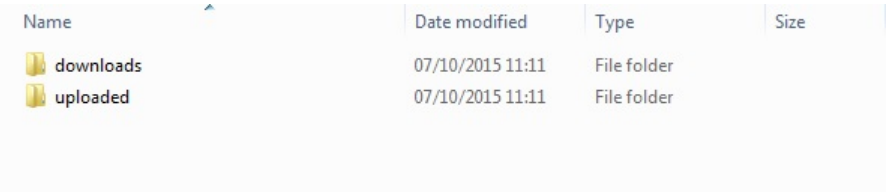


Figure MADF: Folder structure on the host computer. As the reads are uploaded to the cloud, they are transferred into the uploaded folder. Once they have been processed by the workflow, the reads are returned to the downloads folder.

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.

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.