

Rapid Sequencing (SQK-RAD004)

Overview

Version: RSE_9046_v1_revC_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 | 10 |
| Check your flow cell | 12 |
| Prepare your Rapid library | 17 |
| Library preparation | 17 |
| Priming and loading the SpotON Flow Cell | 19 |
| Start sequencing and data analysis | 21 |
| Starting a sequencing run | 21 |
| Progression of MinKNOW protocol script | 27 |
| Assessing the quality of your run | 34 |
| Further analysis with EPI2ME (optional) | 40 |
| Complete the experiment | 43 |
| Close down MinKNOW and the Desktop Agent | 44 |
| Prepare the flow cell for re-use or return to Oxford Nanopore. | 44 |

Rapid Sequencing (SQK-RAD004)

Overview

Version: RSE_9046_v1_revC_17Nov2017

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

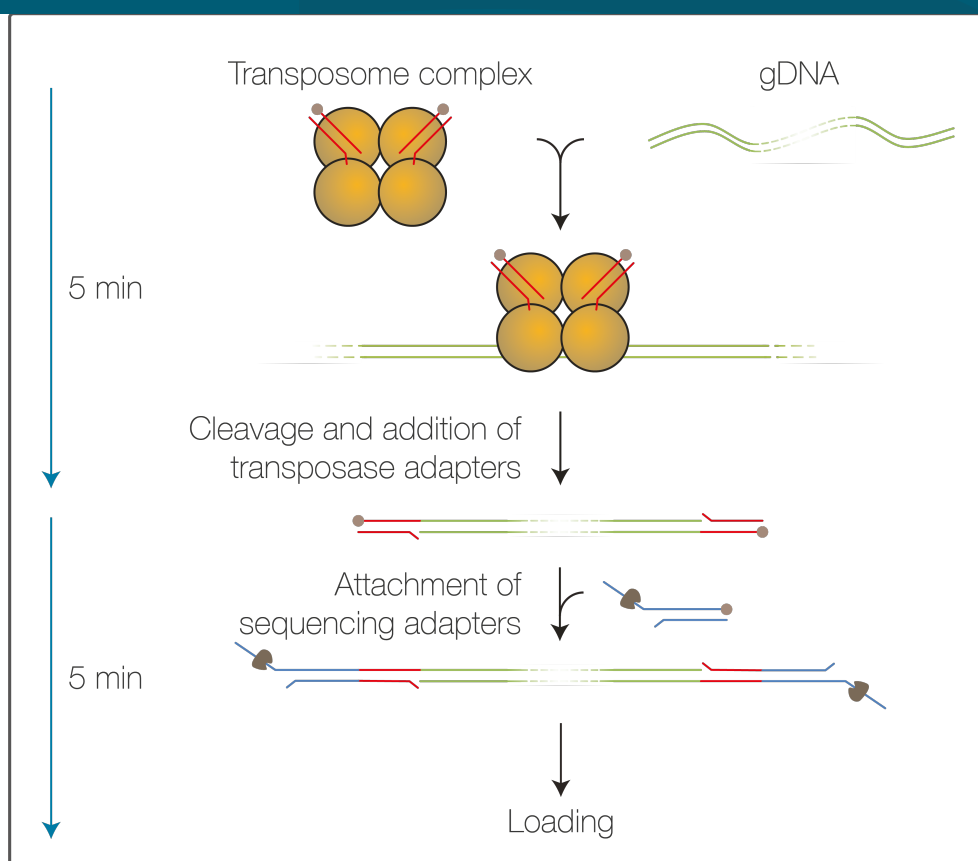
You will need to:

- 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_revC_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`

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

| 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 |
| 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.

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

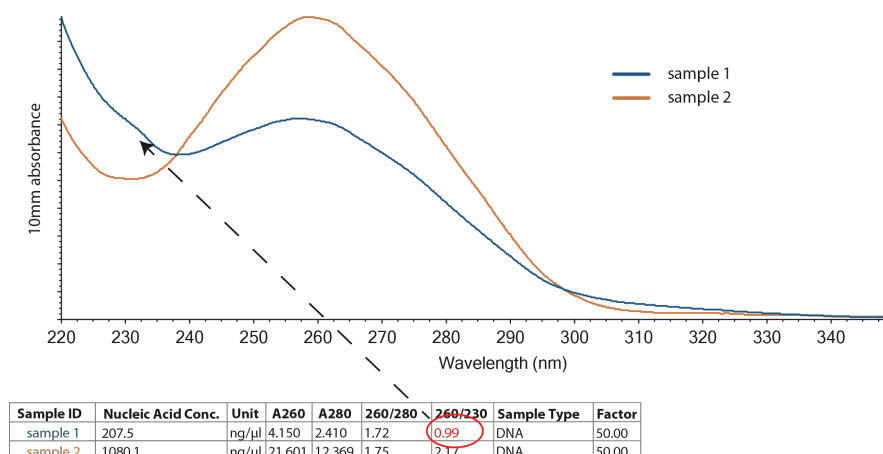
Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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/ μ l).
- 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.



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:

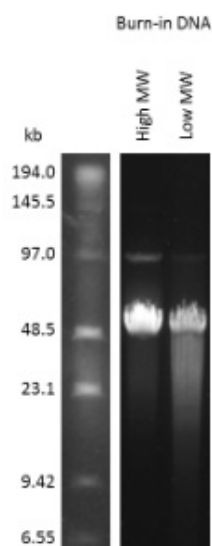
- Using wide-bore pipette tips to handle the gDNA
- Mixing gently but thoroughly by inversion, as opposed to vortexing or pipetting
- Avoiding unnecessary freeze-thaw cycles
- Avoiding pH <6 and >9
- Avoiding high temperatures, which can lead to degradation

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.

Rapid Sequencing (SQK-RAD004)

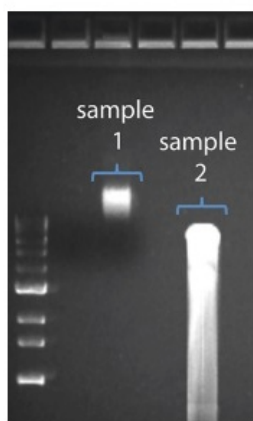
Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017



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

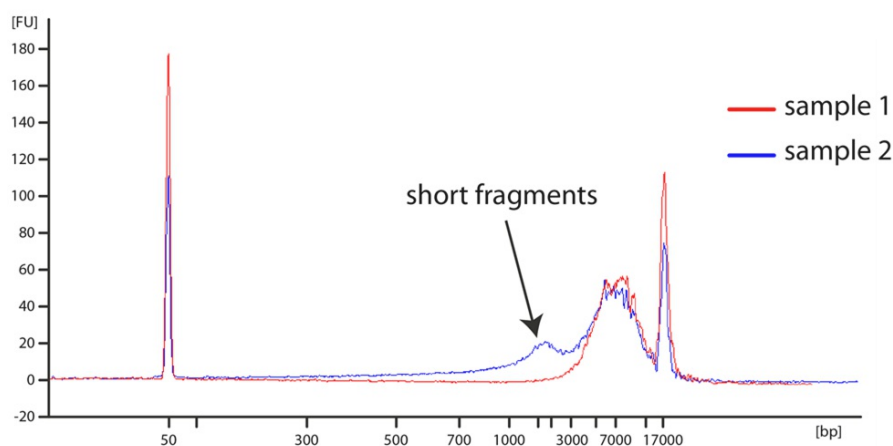
Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

Assessing fragmentation

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



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
- Rapid Sequencing Kit (SQK-RAD004)
- Flow Cell Priming Kit (EXP-FLP001)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

- Microfuge
- P1000 pipette and tips
- P200 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Timer
- 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_revC_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. It is provided in the kit for potential future product compatibility.

Flow Cell Priming Kit contents



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

Computer requirements and software downloads

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

Check your flow cell

Consumables

- SpotCN 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 through the USB 3.0 port to the host computer

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

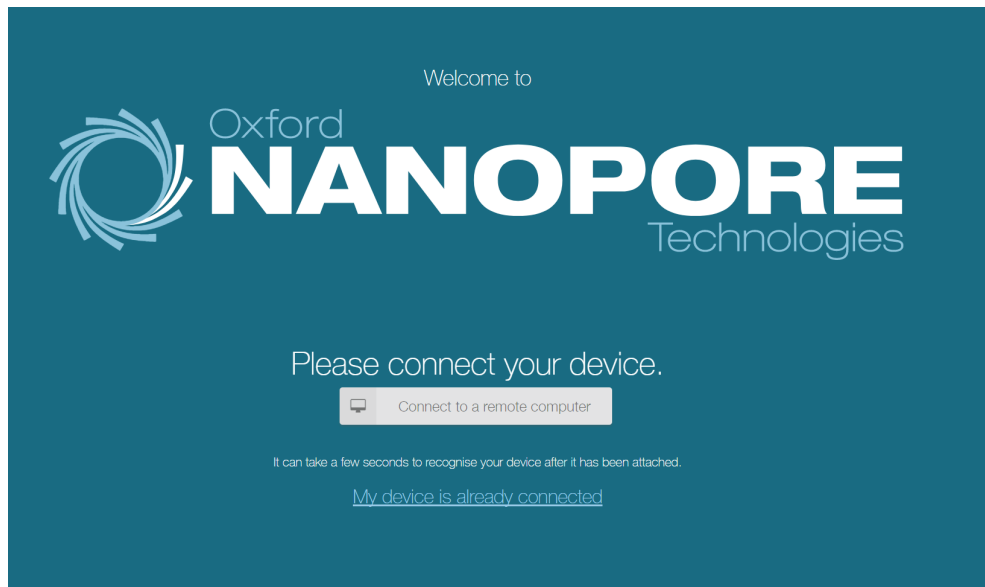
Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

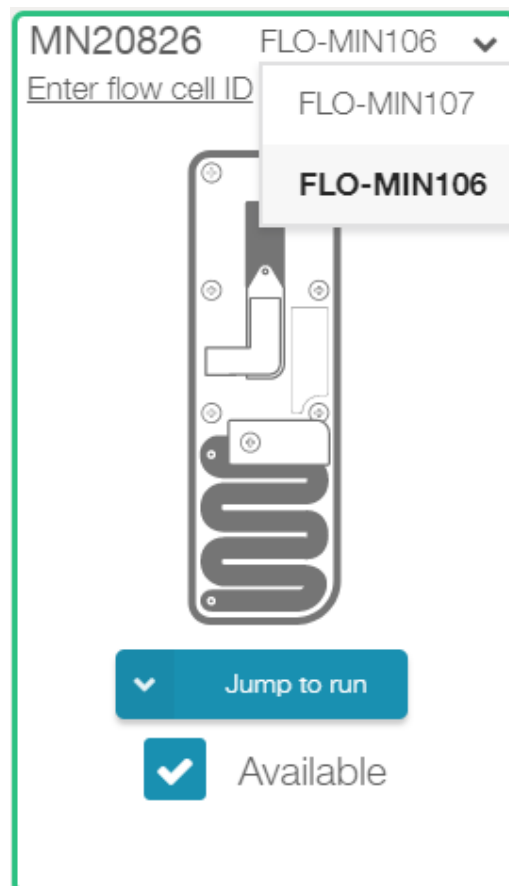
Version: RSE_9046_v1_revC_17Nov2017

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":

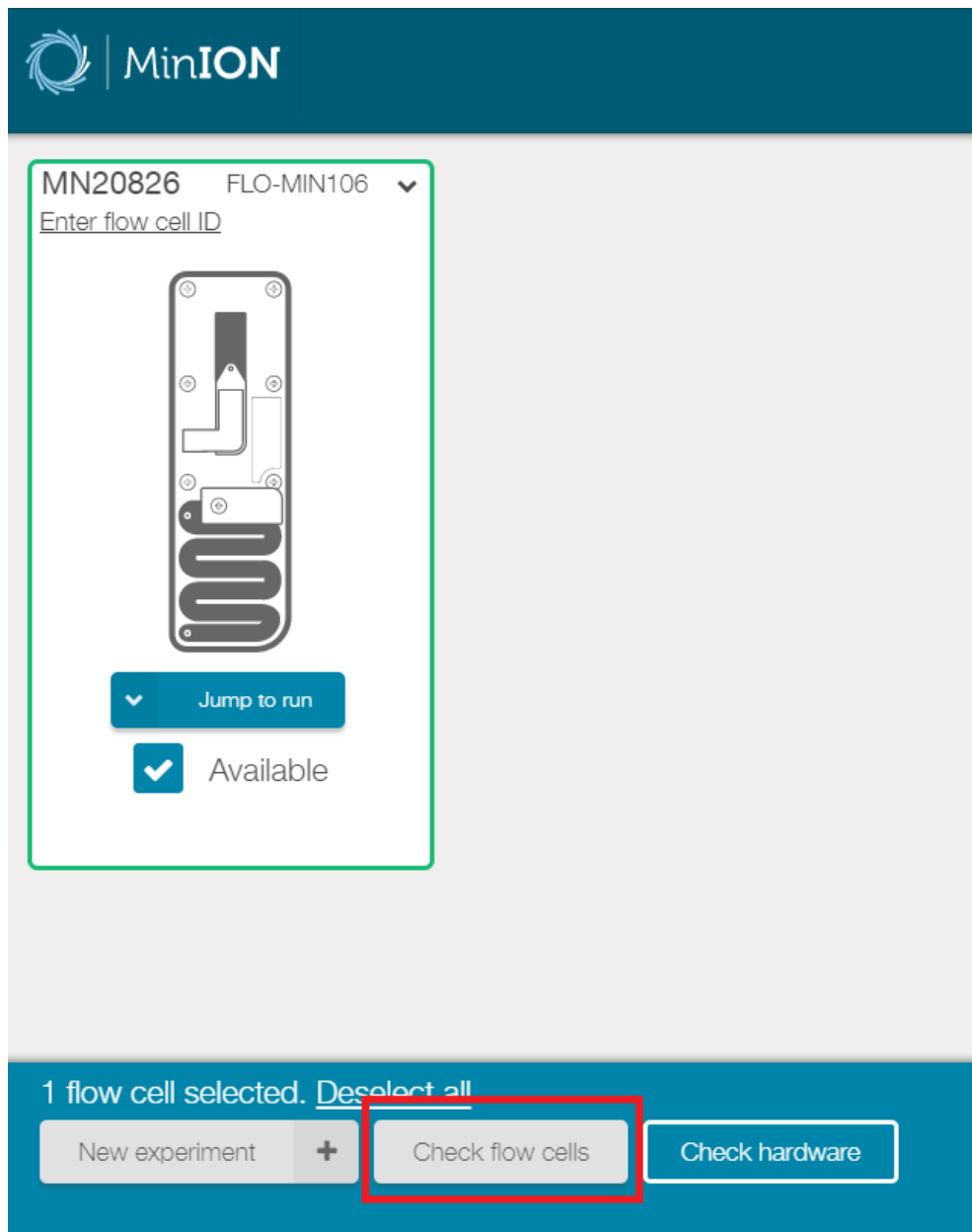


Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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 |

Rapid Sequencing (SQK-RAD004)

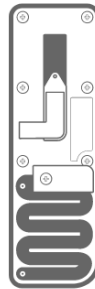
Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

Check flow cell



You have selected 1 FLO-MIN106 flow cell for this test.



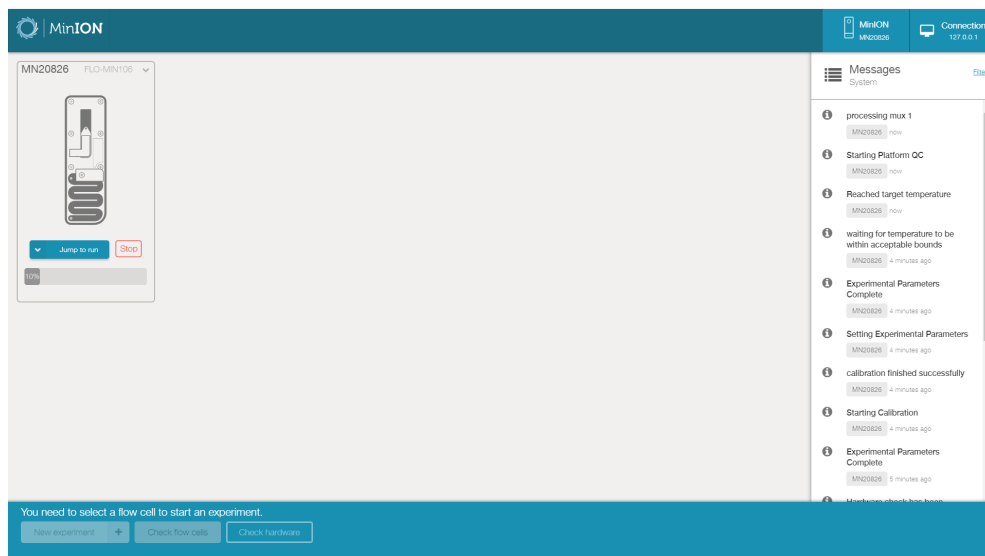
Please ensure that the flow cell has been inserted correctly.

Start test >

Cancel

Click "Start test".

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

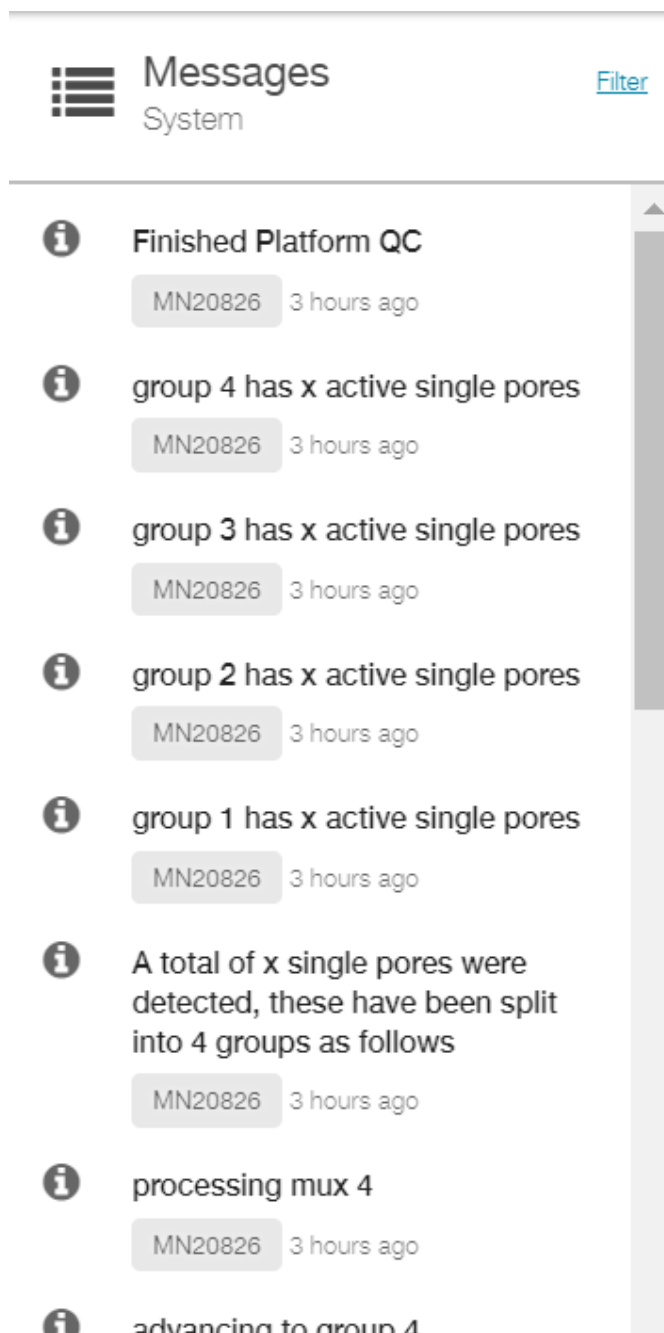


Rapid Sequencing (SQK-RAD004)

Prepare for your experiment

Version: RSE_9046_v1_revC_17Nov2017

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

Prepare your Rapid library

Version: RSE_9046_v1_revC_17Nov2017

Library preparation

~10 minutes

| | |
|-------------|---|
| Materials | <ul style="list-style-type: none">• ~400 ng high molecular weight genomic DNA• Fragmentation Mix (FRA)• Rapid Adapter (RAP) |
| Consumables | <ul style="list-style-type: none">• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)• 0.2 ml thin-walled PCR tubes |
| Equipment | <ul style="list-style-type: none">• Thermal cycler at 30° C and 80° C• P2 pipette and tips• P10 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.

Rapid Sequencing (SQK-RAD004)

Prepare your Rapid library

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

Prepare your Rapid library

Version: RSE_9046_v1_revC_17Nov2017

Priming and loading the SpotON Flow Cell

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

| | | |
|-------------|---|---|
| Materials | <ul style="list-style-type: none">• Flush Tether (FLT)• Sequencing Buffer (SQB) | <ul style="list-style-type: none">• Flush Buffer (FLB)• Loading Beads (LB) |
| Consumables | <ul style="list-style-type: none">• 1.5 ml Eppendorf DNA LoBind tubes• SpotCN flow cell | <ul style="list-style-type: none">• Nuclease-free water (e.g. ThermoFisher, cat # AM9937) |
| Equipment | <ul style="list-style-type: none">• MinION• P100 pipette and tips• P10 pipette and tips | <ul style="list-style-type: none">• 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 (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.

Rapid Sequencing (SQK-RAD004)

Prepare your Rapid library

Version: RSE_9046_v1_revC_17Nov2017

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 |

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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.

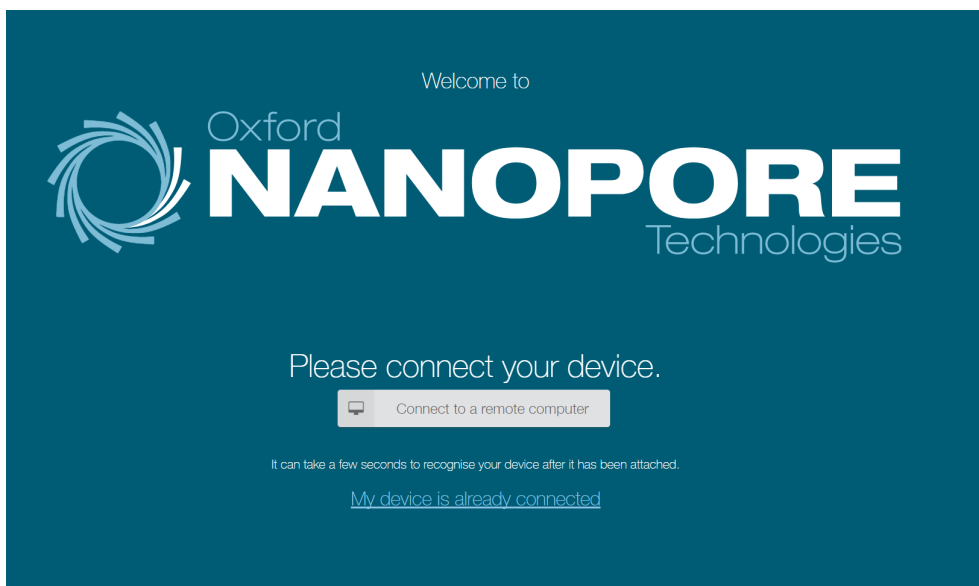
Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

Experiment
test_experiment

Kit

Basecalling
Basecalling is ON

Run Options
Running for 48 hours
Bias voltage set to -180mV

Output
Filetypes fast5, fastq

Custom Script
Custom script is OFF

Start run

Cancel

Experiment
Select or create an experiment

test_experiment

Devices to Add
Edit flow cell ID or remove selected devices from this run.

| Flow cell | | | |
|------------|------------|------|-------------------------------------|
| Position ▾ | Type ▾ | ID ▾ | Sample ▾ |
| MN20826 | FLO-MIN106 | test | <input checked="" type="checkbox"/> |

+

Kit selection

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

Experiment
test_experiment

Kit
SQK-RAD004 sequencing

Basecalling
Basecalling is ON

Run Options
Running for 48 hours
Bias voltage set to -180mV

Output
Filetypes fast5, fastq

Custom Script
Custom script is OFF

Start run

Cancel

Kit Selection
Find your kit below using the filter buttons

Sample type

DNA

RNA

PCR-Free

PCR

PCR-Free

Multiplexing

No

Yes

Control

☐

SQK-DCS108
SQK-LRK001
SQK-LSK108
SQK-LSK109
SQK-LWB001
SQK-LWP001
SQK-PBK004
SQK-PCS108
SQK-PSK004
SQK-RAB201
SQK-RAB204
SQK-RAD003
SQK-RAD004 ✓
SQK-RAS201
SQK-RBK001
SQK-RBK004

Basecalling

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

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

Experiment
test_experiment

Kit
sequencing

Basecalling
Basecalling is ON

Run Options
Running for 48 hours
Bias voltage set to -180mV

Output
Filetypes fast5, fastq

Custom Script
Custom script is OFF

Start run

Cancel

On

Basecalling
Basecall your data as reads are saved

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.

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

Experiment
DEMO

Kit
SQK-LSK109
sequencing

Basecalling
Basecalling is ON

Run Options
Running for 8 hours
Bias voltage set to -180mV

Output
Filetypes fast5, fastq

Custom Script
Custom script is OFF

Run Length (hours)
Set the length of time for the run, in hours
8

Bias Voltage (mV)
You can change the initial voltage for the selected flow cells, useful if the flow cells have been used previously. [Learn more](#)
-180

☒ **On** Active Channel Selection

Time between mux scans (hours)
You can change the time between mux scan, in hours. Multiple mux scans occur during the run to maximise channels for sequencing
1.5

Start run **Cancel**

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

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

Experiment
DEMO

Kit
SQK-LSK109
sequencing

Basecalling
Basecalling is ON

Run Options
Running for 8 hours
Bias voltage set to -180mV

Output
Filetypes fast5, fastq

Custom Script
Custom script is OFF

Output Location
Folder where reads are placed

Output Format
Select the outputs for this run and the data to include in each file

| File type | Raw Signal | FASTQ Record | Files per Folder | Reads per File |
|---|-------------------------------------|-------------------------------------|-----------------------------------|-----------------------------------|
| <input checked="" type="checkbox"/> FAST5 | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | <input type="text" value="4000"/> | |
| <input checked="" type="checkbox"/> FASTQ | | | | <input type="text" value="4000"/> |

► Additional options

Start run

Cancel

Click "Start run".

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



Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

Allow the script to run to completion.

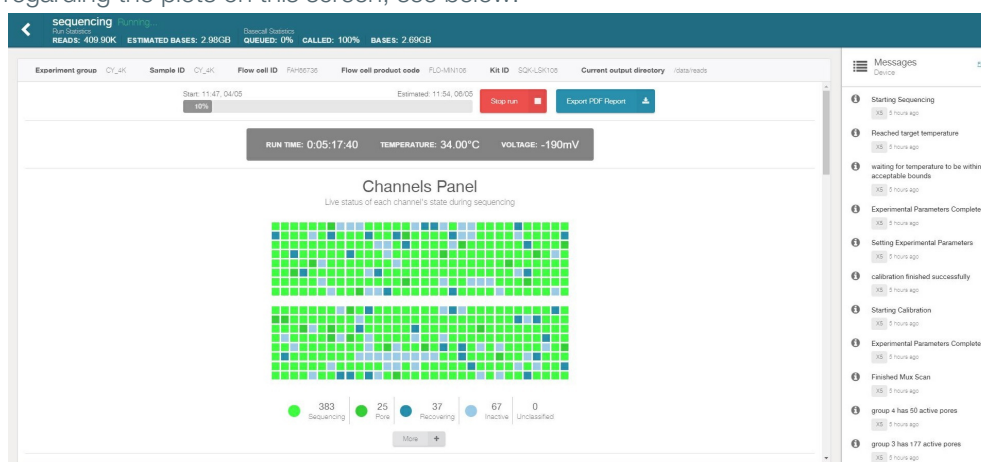
- The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen
- Monitor messages in the Message panel in the MinKNOW GUI

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.



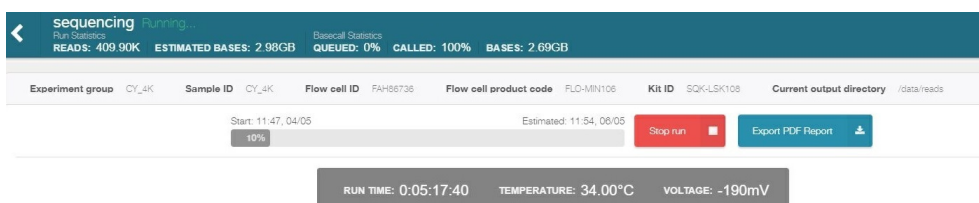
Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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

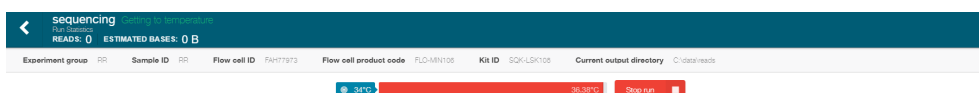
- **Voltage:** The applied potential of the position at that point in time

TIP

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

- Number of active pores
- Heatsink temperature
- 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.

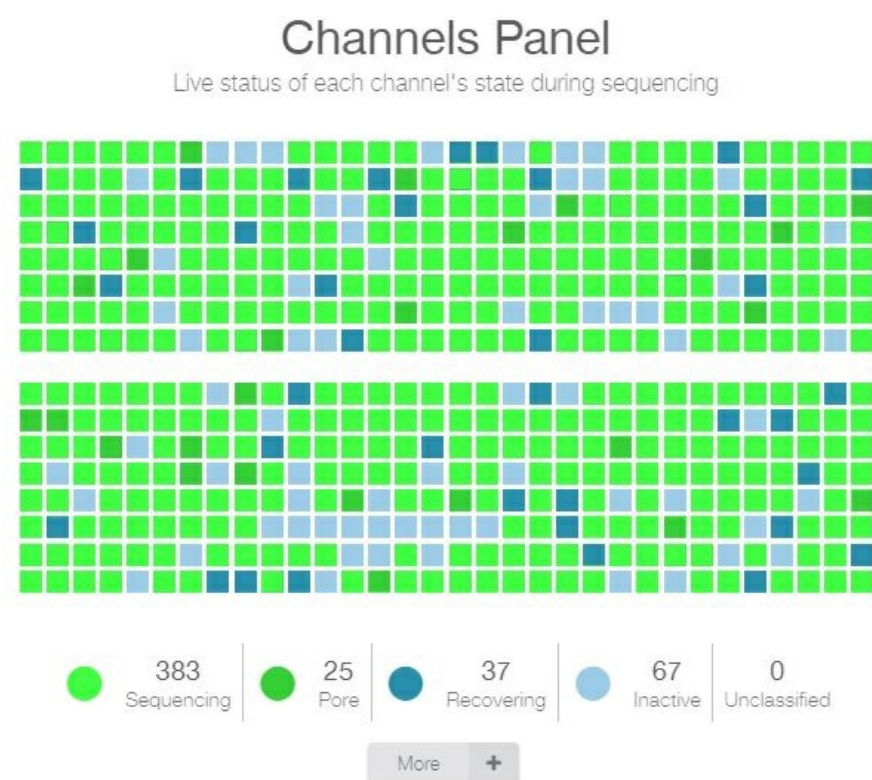
Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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

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



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

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

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

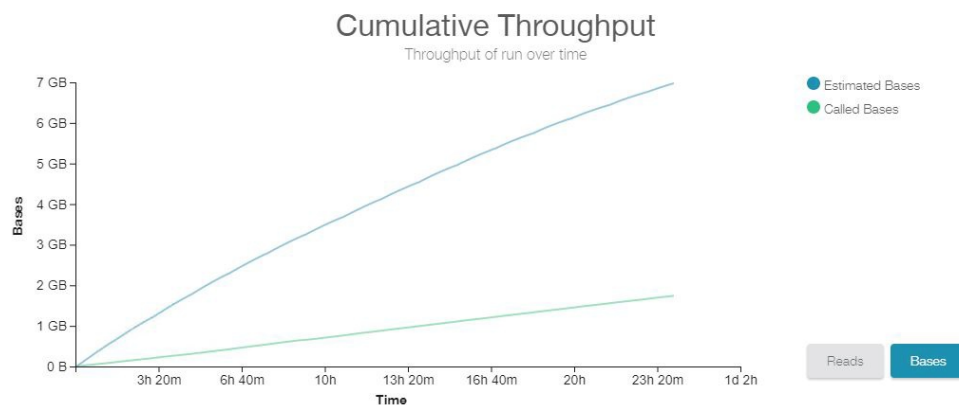
Monitor the pore occupancy

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](#).

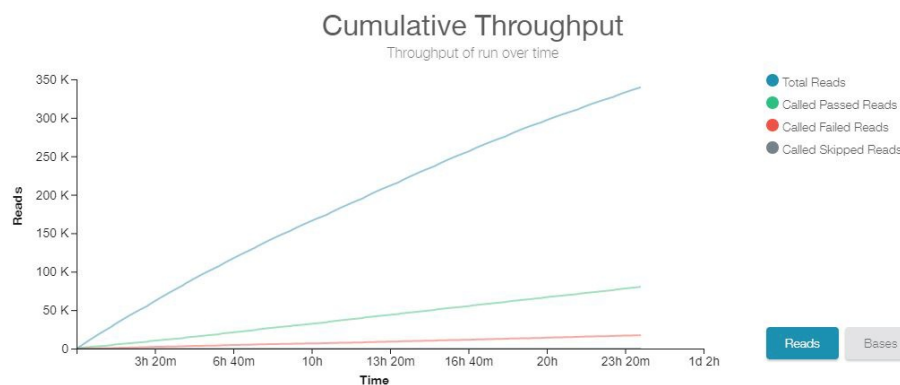
Cumulative throughput

The cumulative throughput graph shows:

- the number of bases that have been sequenced and basecalled



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



Rapid Sequencing (SQK-RAD004)

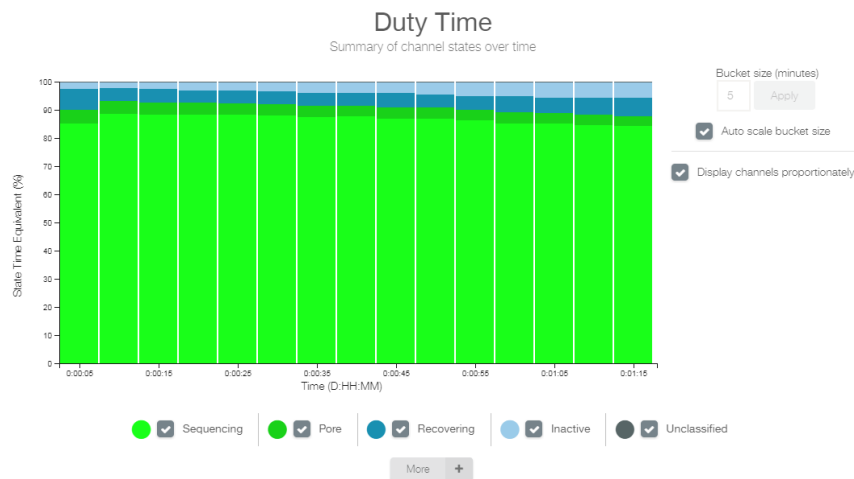
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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.

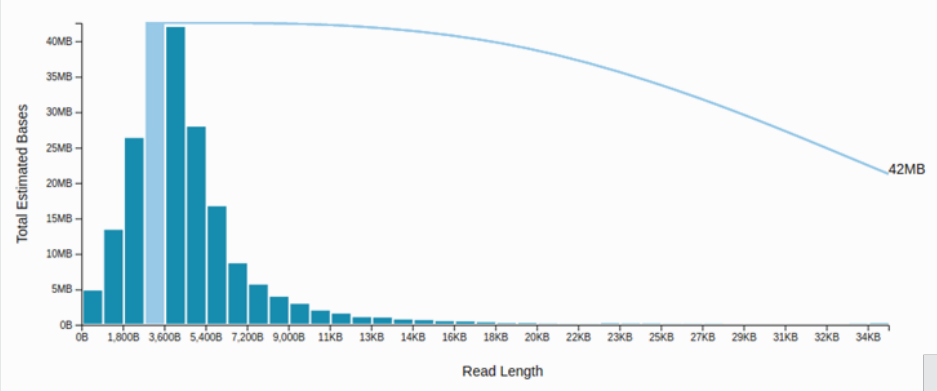
Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

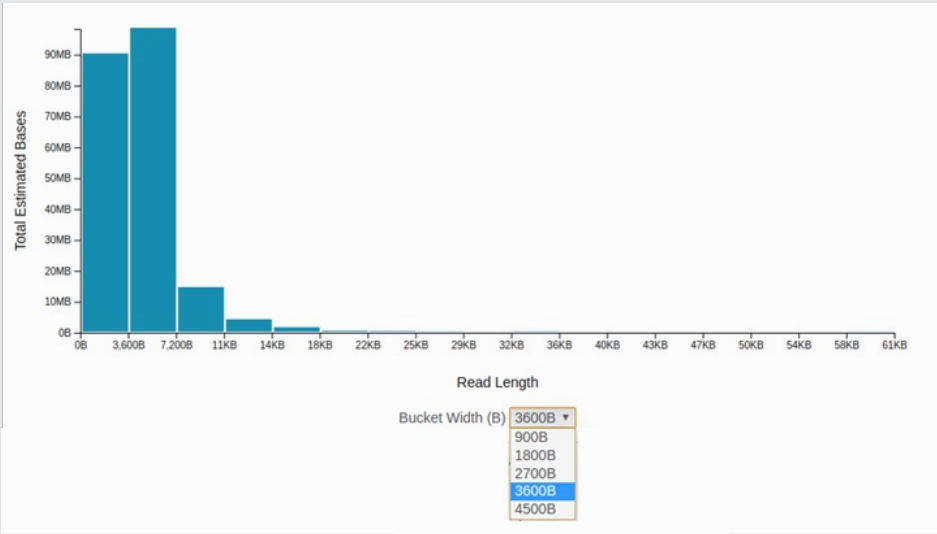
EXAMPLE

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.



Rapid Sequencing (SQK-RAD004)

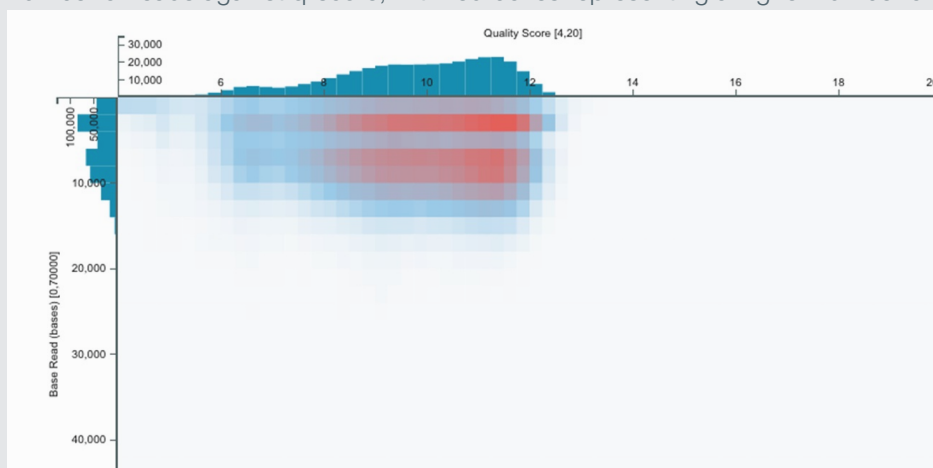
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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

Rapid Sequencing (SQK-RAD004)

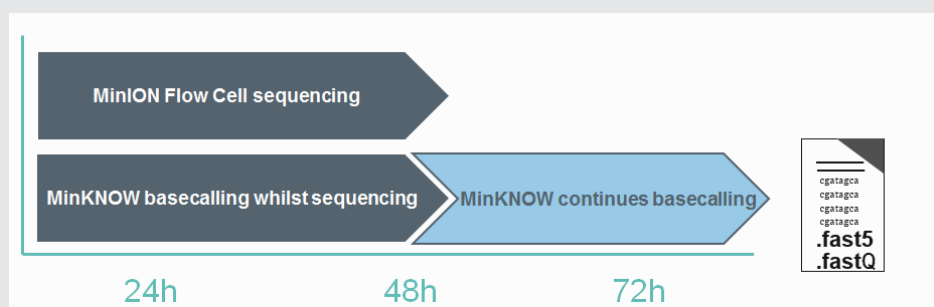
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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.

Rapid Sequencing (SQK-RAD004)

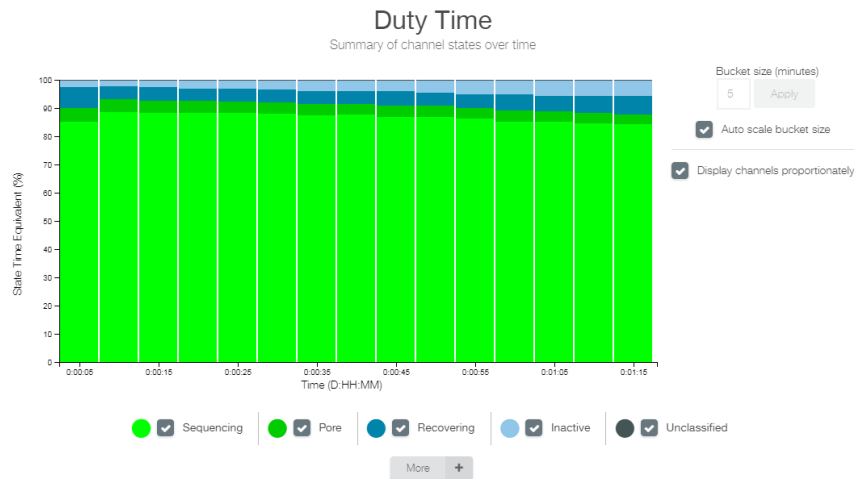
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

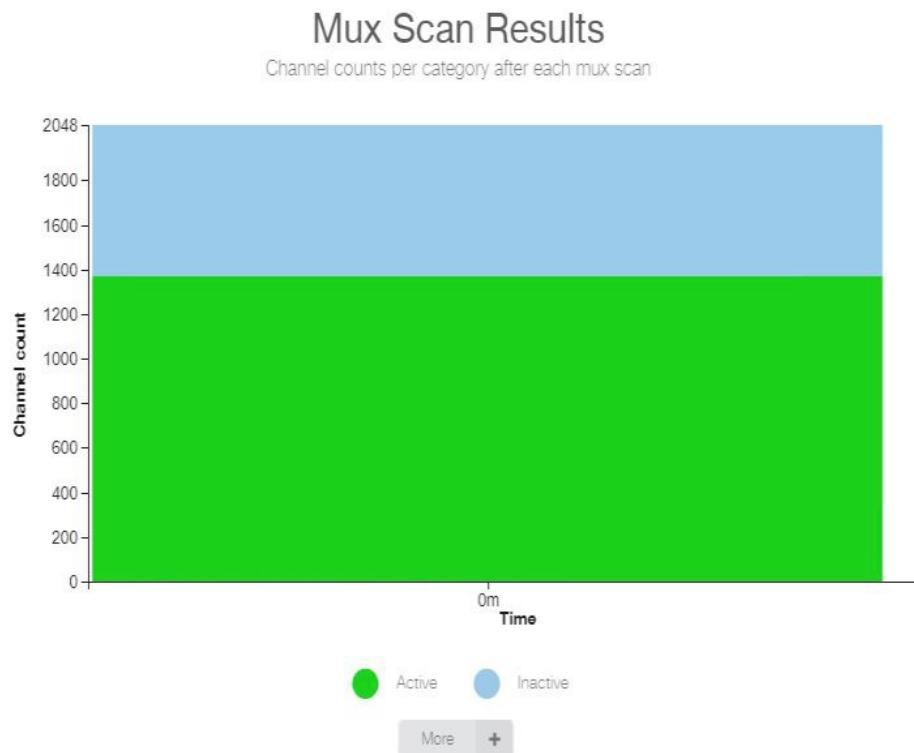
Good quality library

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



Rapid Sequencing (SQK-RAD004)

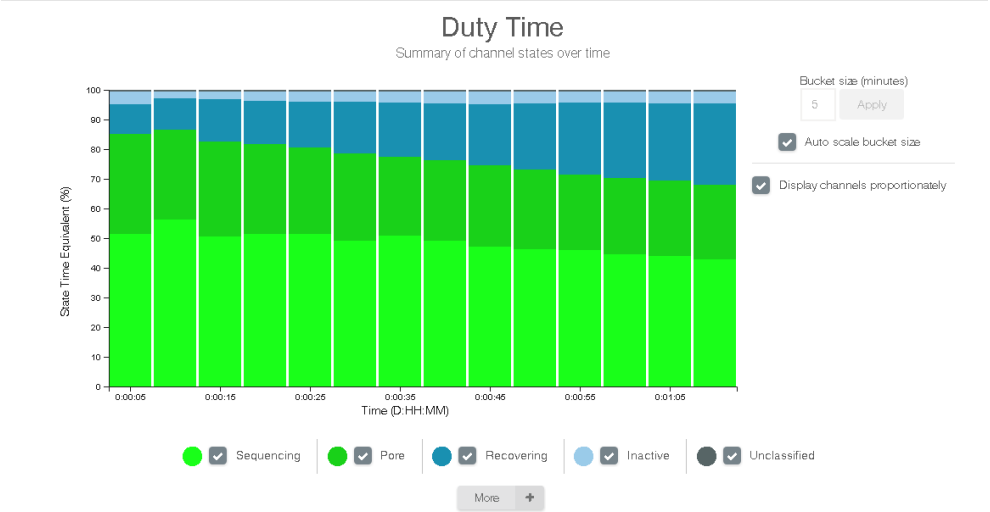
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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



Rapid Sequencing (SQK-RAD004)

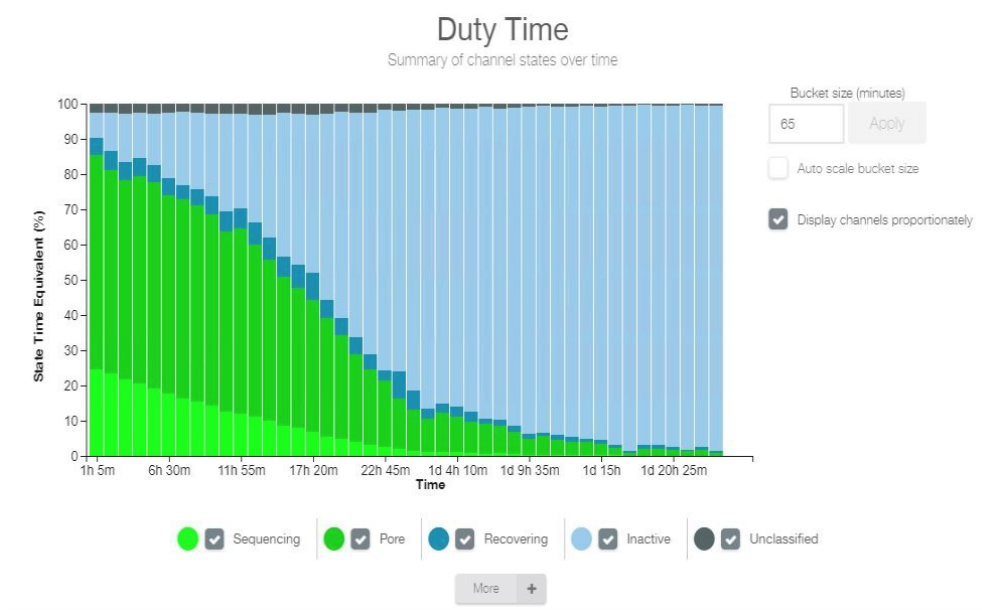
Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

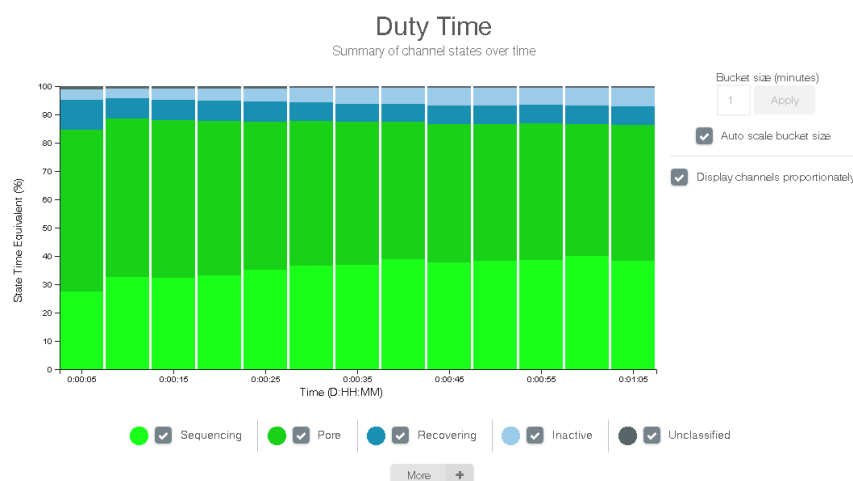
Low pore occupancy

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

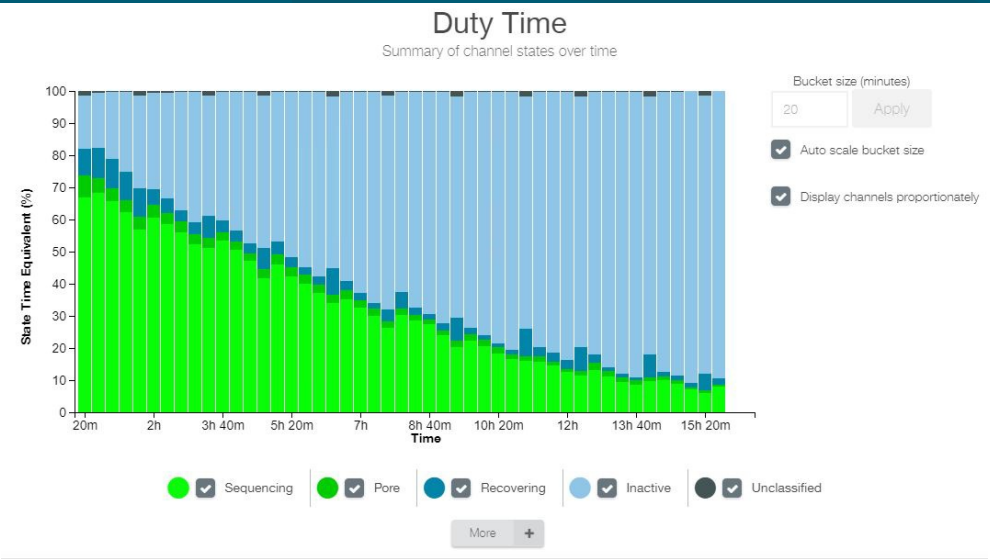
If the duty time plot shows a high number of 'Inactive' channels building up over time, this could indicate that the channels or membranes have been damaged by e.g. air bubbles, osmotic imbalance, or the presence of detergents or surfactants in the library.

Duty time: with Active Channel Selection switched on

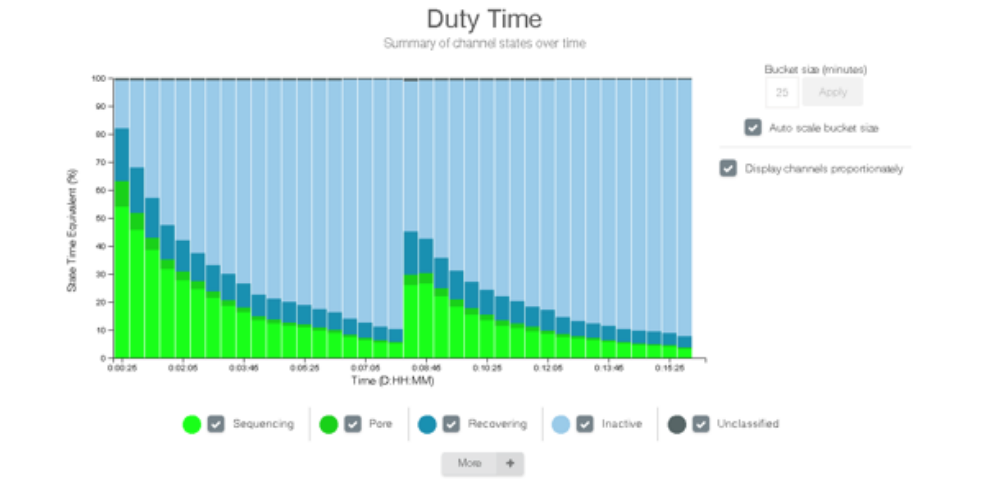
Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017



Duty time: with Active Channel Selection switched off

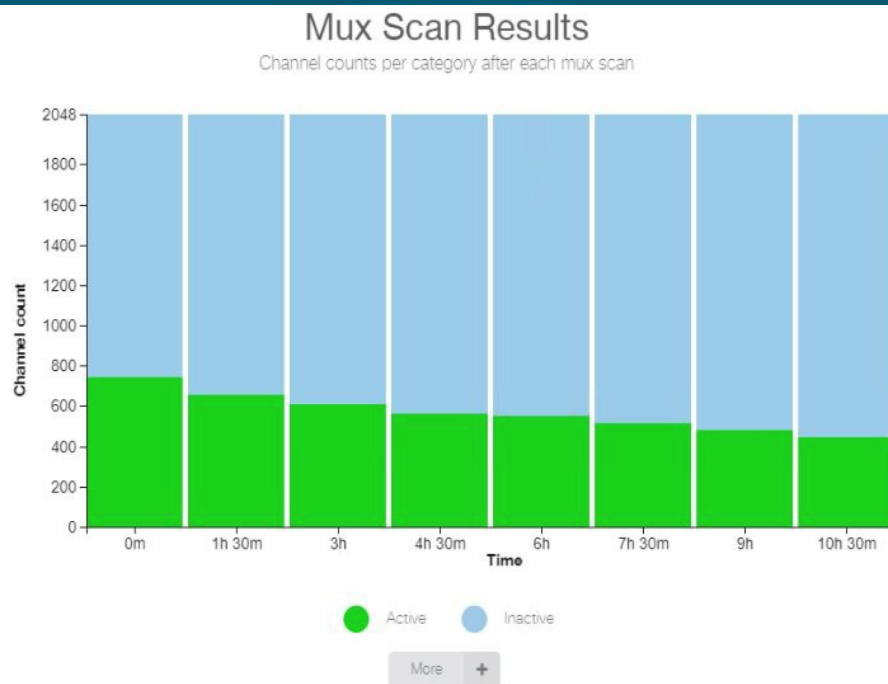


Mux scan results

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017



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 MinKNOW and the Desktop Agent installed

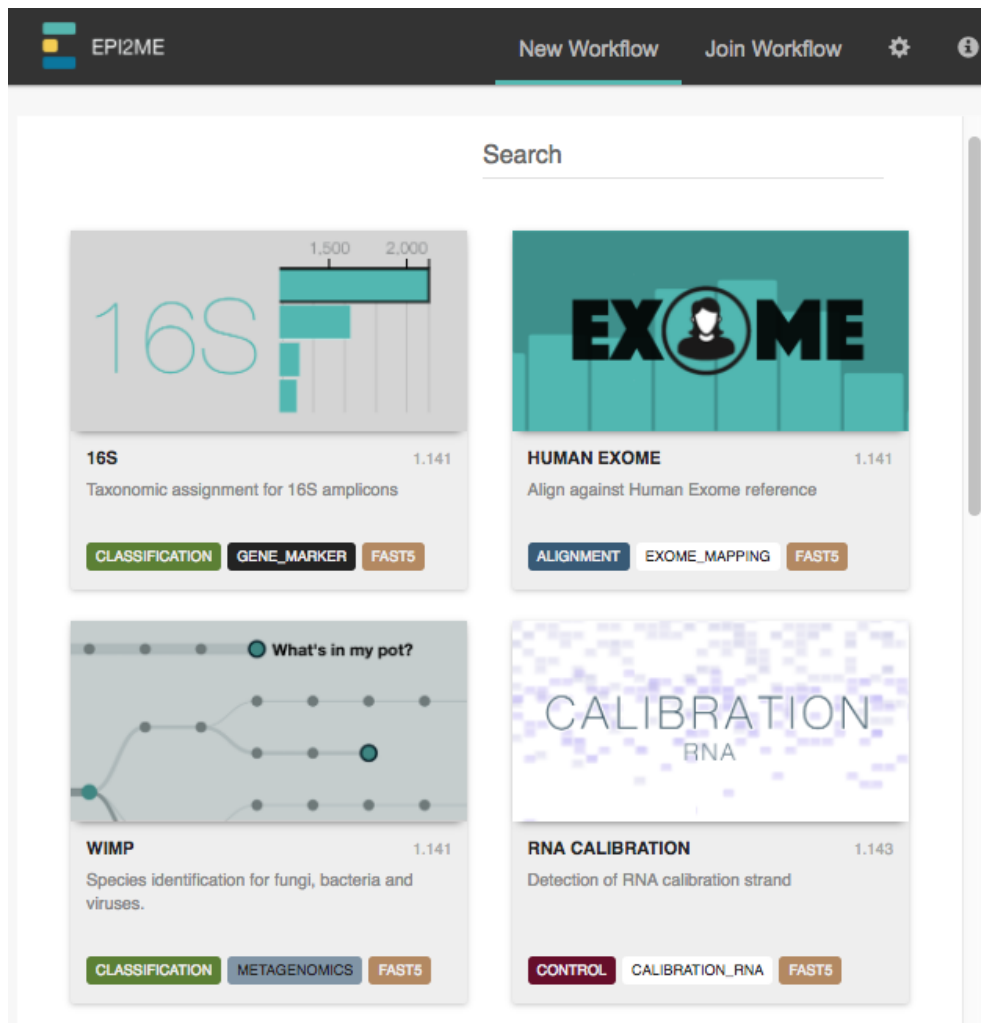
Open the Desktop Agent using the desktop shortcut.

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

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



Select the workflow parameters.

Rapid Sequencing (SQK-RAD004)

Start sequencing and data analysis

Version: RSE_9046_v1_revC_17Nov2017

EPI2ME New Workflow Join Workflow ⚙️ ⓘ

✓ Workflow — ✓ Billing — 3 Parameters — 4 Start Run

WORKFLOW PARAMETERS

Min qscore:

Detect barcode?

EPI2ME STORAGE
If you enable storage the dataset may be downloaded at any time and can be used to start new workflows.
Charges may apply against your billing account.

Is the data you are about to upload a whole or partial human genome?

☒ YES ☐ NO

- Select the quality score cut-off

Min qscore:

- Select "No" under "Detect barcode?"

Detect barcode?

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

EPI2ME STORAGE
If you enable storage the dataset may be downloaded at any time and can be used to start new workflows.
Charges may apply against your billing account.

- 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.

Is the data you are about to upload a whole or partial human genome?

☒ YES ☐ NO

If this is a human genome do you have the necessary documented consent from the subject?

☒ YES ☐ NO

Rapid Sequencing (SQK-RAD004)

Complete the experiment

Version: RSE_9046_v1_revC_17Nov2017

Check the correct settings are selected in the Desktop Agent.

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

Workflow — Billing — Parameters — 4 Start Run

ATTRIBUTES

These will be visible in the EPI2ME Portal and must not contain personally identifiable information

project ▾ Add

GENERAL SETTINGS

| | |
|---|---------------------------------|
| File Type ? | .fastq |
| Input Directory: ? Files in the fail folder be ignored | /data |
| Uploaded Directory: ? | Default Custom +uploaded |
| Download Directory: ? | Default Custom +downloads |
| Download Mode: ? | Data & Telemetry Telemetry Only |
| Accelerated Transfer ? | ON OFF |

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.

| Name | Date modified | Type | Size |
|-----------|------------------|-------------|------|
| downloads | 07/10/2015 11:11 | File folder | |
| uploaded | 07/10/2015 11:11 | File folder | |

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.

Rapid Sequencing (SQK-RAD004)

Complete the experiment

Version: RSE_9046_v1_revC_17Nov2017

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](#).