

# Rapid Sequencing (SQK-RAD004)

## Introduction to the protocol

Version: RSE\_9046\_v1\_revK\_14Aug2019

# Overview of the protocol

### 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

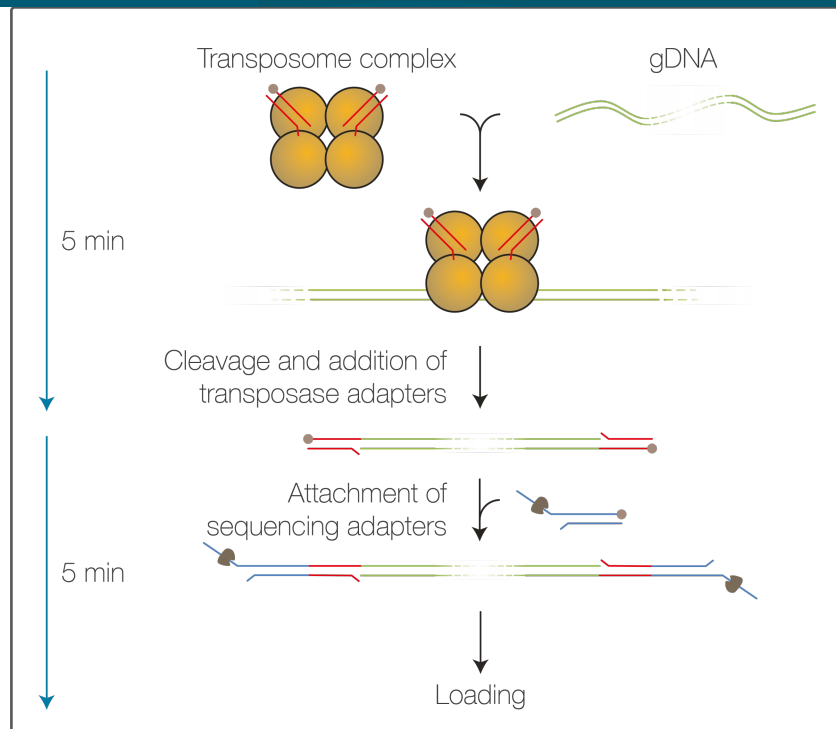
You will need to:

- 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)

### Equipment and consumables

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### Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads
- **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-WSH003)

## Equipment and consumables

# Rapid Sequencing (SQK-RAD004)

## Equipment and consumables

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### Materials

- ~400 ng high molecular weight genomic DNA
- Rapid Sequencing Kit (SQK-RAD004)
- Flow Cell Priming Kit (EXP-FLP002)

### 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
- 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

**For this protocol, you will need ~400 ng high molecular weight genomic DNA**

### Input DNA

#### How to QC your input DNA

It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

For instructions on how to perform quality control of your DNA sample, please read the [Input DNA/RNA QC protocol](#).

### Chemical contaminants

Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the [Contaminants page](#) of the Community.

## Rapid Sequencing (SQK-RAD004)

### Computer requirements and software

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#### Rapid Sequencing Kit contents



LMD : Lambda DNA

FRA : Fragmentation mix

RAP : Rapid adapter

SQT : Sequencing tether

LB : Loading beads

SQB : Sequencing buffer

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

#### IMPORTANT

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

#### Flow Cell Priming Kit contents (EXP-FLP002)



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

## Computer requirements and software

# Rapid Sequencing (SQK-RAD004)

## Computer requirements and software

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### MinION IT requirements

Unless you are using a MinIT device, sequencing on a MinION requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the [MinION IT Requirements document](#).

### Software for nanopore sequencing

#### MinKNOW

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

#### EPI2ME (optional)

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

#### Guppy (optional)

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

### MinKNOW installation and use

For instructions on how to install the MinKNOW software, please refer to the relevant "Download and install MinKNOW" section in the [MinKNOW protocol](#).

### EPI2ME installation and use

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the [EPI2ME Platform protocol](#).

### Guppy installation and use

If you would like to use the Guppy basecaller instead of basecalling live in MinKNOW, please refer to the [Guppy protocol](#).

### Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment, and within three months of purchasing the flow cell. Oxford Nanopore Technologies will replace any flow cell with fewer than:

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	60
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the [Flow Cell Check document](#).

# Rapid Sequencing (SQK-RAD004)

## Library preparation

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# Library preparation

~10 minutes

### Materials

- ~400 ng high molecular weight genomic DNA
- Fragmentation Mix (FRA)
- Rapid Adapter (RAP)

### 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
- 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)

### Priming and loading the SpotON Flow Cell

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#### 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

#### 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
<b>Total</b>	<b>10 µl</b>

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

~10 minutes

# Rapid Sequencing (SQK-RAD004)

## Priming and loading the SpotON Flow Cell

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<b>Materials</b>	<ul style="list-style-type: none"> <li>• Flush Tether (FLT)</li> <li>• Sequencing Buffer (SQB)</li> </ul>	<ul style="list-style-type: none"> <li>• Flush Buffer (FB)</li> <li>• Loading Beads (LB)</li> </ul>
<b>Consumables</b>	<ul style="list-style-type: none"> <li>• 1.5 ml Eppendorf DNA LoBind tubes</li> <li>• Flongle device - flow cell and adapter</li> </ul>	<ul style="list-style-type: none"> <li>• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)</li> </ul>
<b>Equipment</b>	<ul style="list-style-type: none"> <li>• MinION</li> <li>• P100 pipette and tips</li> <li>• P10 pipette and tips</li> </ul>	<ul style="list-style-type: none"> <li>• P1000 pipette and tips</li> <li>• P20 pipette and tips</li> </ul>

### IMPORTANT

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

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

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

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

Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.

Priming and loading the SpotON Flow Cell

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

Take care to avoid introducing any air during pipetting.

### IMPORTANT

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

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

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

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



## Rapid Sequencing (SQK-RAD004)

### Sequencing and data analysis

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Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by pipetting up and down.

Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

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

#### TIP

##### Using the Loading Beads

Demo of how to use the Loading Beads.

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

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

#### 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.

## Rapid Sequencing (SQK-RAD004)

### Data acquisition and basecalling

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# Data acquisition and basecalling

#### Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the [Data Analysis](#) document.

#### How to start sequencing

MinION device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing on the MinION:

#### 1. Data acquisition and basecalling in real-time using MinKNOW on a computer/GridION/PromethION

Please follow the instructions in the [MinKNOW protocol](#) beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

#### 2. Data acquisition and basecalling in real-time using the MinIT device

Please follow the instructions in the [MinIT protocol](#).

#### 3. Data acquisition using MinKNOW on a computer/GridION/PromethION and basecalling at a later time using Guppy

Please follow the instructions in the [MinKNOW protocol](#) beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the *Basecalling* tab to OFF.** After the sequencing experiment has completed, please follow the instructions in the [Guppy protocol](#) starting from the "Quick Start Guide for Guppy" section.

## Downstream analysis

# Rapid Sequencing (SQK-RAD004)

## Ending the experiment

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### Post-basecalling analysis

There are several options for further analysing your basecalled data:

#### 1. EPI2ME platform

The EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of Oxford Nanopore Technologies. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment, and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results. For instructions on how to run an analysis workflow in EPI2ME, please follow the instructions in the [EPI2ME protocol](#), beginning at the "Starting an EPI2ME workflow" step.

#### 2. Bioinformatics tutorials

For more in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials, which are available in the [Bioinformatics resource](#) section of the Community. The tutorials take the user through installing and running pre-built analysis pipelines, which generate a report with the results. The tutorials are aimed at biologists who would like to analyse data without the help of a dedicated bioinformatician, and who are comfortable using the command line.

#### 3. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, which are available in the Oxford Nanopore [GitHub repository](#). The tools are aimed at advanced users, and contain instructions for how to install and run the software. They are provided as-is, with minimal support.

#### 4. Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, we recommend the [Community-developed data analysis tool library](#). Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

## Ending the experiment

### Materials

- Flow Cell Wash Kit (EXP-WSH003)

# Rapid Sequencing (SQK-RAD004)

## Troubleshooting

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**After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR**

The [Flow Cell Wash Kit protocol](#) is available on the Nanopore Community.

**Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be found [here](#).

### IMPORTANT

**If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.**

## Issues during DNA/RNA extraction and library preparation

**Below is a list of the most commonly-encountered issues, with some suggested causes and solutions.**

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email ([support@nanoporetech.com](mailto:support@nanoporetech.com)) or via [LiveChat](#) in the [Nanopore Community](#).

### Low sample quality

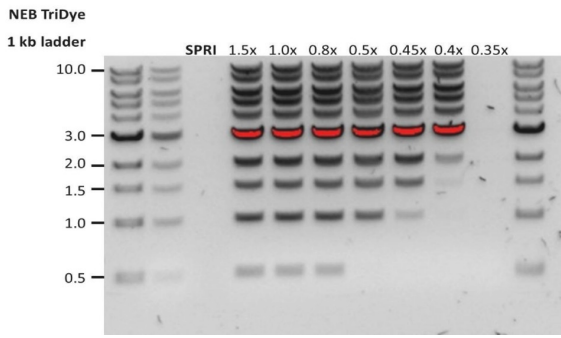
Observation	Possible cause	Comments and actions
<b>Low DNA purity (Nanodrop reading for DNA OD 260/280 is &lt;1.8 and OD 260/230 is &lt;2.0–2.2)</b>	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the <a href="#">Contaminants Know-how</a> piece. Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.  Consider performing an additional SPRI clean-up step.
<b>Low RNA integrity (RNA integrity number &lt;9.5 RIN, or the rRNA band is shown as a smear on the gel)</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how</a> piece.
<b>RNA has a shorter than expected fragment length</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how</a> piece.  We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

# Rapid Sequencing (SQK-RAD004)

## Troubleshooting

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### Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
<b>Low recovery</b>	DNA loss due to a lower than intended AMPure beads-to-sample ratio	<ol style="list-style-type: none"> <li>1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.</li> <li>2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.</li> </ol>
<b>Low recovery</b>	DNA fragments are shorter than expected	<p>The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use.</p> 
<b>Low recovery after end-prep</b>	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

### The VolTRAX run terminated in the middle of the library prep

Observation	Possible cause	Comments and actions
<p><b>The green light was switched off</b></p> <p><b>or</b></p> <p><b>An adapter was used to connect the VolTRAX USB-C cable to the computer</b></p>	Insufficient power supply to the VolTRAX	The green LED signals that 3 A are being supplied to the device. This is the requirement for the full capabilities of the VolTRAX V2 device. Please use computers that meet the requirements listed on the <a href="#">VolTRAX V2 protocol</a> .

## Rapid Sequencing (SQK-RAD004)

### Issues during the sequencing run

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#### The VolTRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
<b>The VolTRAX software shows an inaccurate amount of reagents loaded</b>	Pipette tips do not fit the VolTRAX cartridge ports	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VolTRAX cartridge. Rainin 20 µl is the most suitable.
<b>The VolTRAX software shows an inaccurate amount of reagents loaded</b>	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VolTRAX software before loading.

## Issues during the sequencing run

**Below is a list of the most commonly-encountered issues, with some suggested causes and solutions.**

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email ([support@nanoporetech.com](mailto:support@nanoporetech.com)) or via [LiveChat](#) in the Nanopore Community.

## Rapid Sequencing (SQK-RAD004)

### Issues during the sequencing run

Version: RSE\_9046\_v1\_revK\_14Aug2019

#### Fewer pores at the start of sequencing than after Flow Cell Check

Observation	Possible cause	Comments and actions
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in <a href="#">this video</a> .
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the <a href="#">Contaminants Know-how piece</a> . Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.

#### MinKNOW script failed

Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Script failed"</b>		Restart the computer and then restart MinKNOW. If the issue persists, please collect the <a href="#">MinKNOW log files</a> and contact Technical Support.

# Rapid Sequencing (SQK-RAD004)

## Issues during the sequencing run

Version: RSE\_9046\_v1\_revK\_14Aug2019

### Pore occupancy below 40%

Observation	Possible cause	Comments and actions
<b>Pore occupancy &lt;40%</b>	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION/GridION flow cell. Please quantify the library before loading and calculate mols using tools like the <a href="#">Promega Biomath Calculator</a> , choosing "dsDNA: µg to pmol"
<b>Pore occupancy close to 0</b>	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK109 kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
<b>Pore occupancy close to 0</b>	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
<b>Pore occupancy close to 0</b>	No tether on the flow cell	Tethers are adding during flow cell priming (FLT tube). Make sure FLT was added to FB before priming.

### Shorter than expected read length

Observation	Possible cause	Comments and actions
<b>Shorter than expected read length</b>	Unwanted fragmentation of DNA sample	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> <li>Please review the <a href="#">Extraction Methods</a> in the Nanopore Community for best practice for extraction.</li> <li>Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.</li> </ol>  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> <li>During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.</li> </ol>



# Rapid Sequencing (SQK-RAD004)

## Issues during the sequencing run

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### Large proportion of recovering pores

Observation	Possible cause	Comments and actions
<b>Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)</b>	Contaminants are present in the sample	<p>Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases:</p> <ol style="list-style-type: none"> <li>1. A <a href="#">nuclease flush</a> can be performed, or</li> <li>2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.</li> </ol> <div data-bbox="798 734 1289 1008"> <p><b>Duty Time</b> Summary of channel states over time</p> </div> <p>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment</p>

### Large proportion of inactive pores

Observation	Possible cause	Comments and actions
<b>Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)</b>	Air bubbles have been introduced into the flow cell	Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the <a href="#">Priming and loading your flow cell</a> video for best practice
<b>Large proportion of inactive pores</b>	Certain compounds co-purified with DNA	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none"> <li>1. Please refer to the <a href="#">Plant leaf DNA extraction method</a>.</li> <li>2. Clean-up using the QIAGEN PowerClean Pro kit.</li> <li>3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.</li> </ol>
<b>Large proportion of inactive pores</b>	Contaminants are present in the sample	The effects of contaminants are shown in the <a href="#">Contaminants</a> Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

## Rapid Sequencing (SQK-RAD004)

### Issues during the sequencing run

Version: RSE\_9046\_v1\_revK\_14Aug2019

#### Reduction in sequencing speed and q-score later into the run

Observation	Possible cause	Comments and actions
<b>Reduction in sequencing speed and q-score later into the run</b>	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–50 fmol of library is recommended).	Add more fuel to the flow cell by following the instructions in the <a href="#">MinKNOW protocol</a> . In future experiments, load lower amounts of library to the flow cell.

#### Temperature fluctuation

Observation	Possible cause	Comments and actions
<b>Temperature fluctuation</b>	The flow cell has lost contact with the device	Check that there is a heat pad covering the metal plate on the back of the flow cell. Re-insert the flow cell and press it down to make sure the connector pins are firmly in contact with the device. If the problem persists, please contact Technical Services.

#### Failed to reach target temperature

Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Failed to reach target temperature"</b> (37°C for Flow Cell Check, 34°C for sequencing on MinION/PromethION flow cells, and 35°C for sequencing on Flongle)	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to <a href="#">this FAQ</a> for more information on MinION temperature control.

#### Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
<b>No input .fast5 was found or basecalled</b>	<i>input_path</i> did not point to the .fast5 file location	The <i>--input_path</i> has to be followed by the full file path to the .fast5 files to be basecalled, and the location has to be accessible either locally or remotely through SSH.
<b>No input .fast5 was found or basecalled</b>	The .fast5 files were in a subfolder at the <i>input_path</i> location	To allow Guppy to look into subfolders, add the <i>--recursive</i> flag to the command

# Rapid Sequencing (SQK-RAD004)

## Issues during the sequencing run

Version: RSE\_9046\_v1\_revK\_14Aug2019


### Guppy – no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
<b>No Pass or Fail folders were generated after basecalling</b>	The <code>--qscore_filtering</code> flag was not included in the command	The <code>--qscore_filtering</code> flag enables filtering of reads into Pass and Fail folders inside the output folder, based on their strand q-score. When performing live basecalling in MinKNOW, a q-score of 7 (corresponding to a basecall accuracy of ~80%) is used to separate reads into Pass and Fail folders.

### Guppy – unusually slow processing on a GPU computer

Observation	Possible cause	Comments and actions
<b>Unusually slow processing on a GPU computer</b>	The <code>--device</code> flag wasn't included in the command	The <code>--device</code> flag specifies a GPU device to use for accelerate basecalling. If not included in the command, GPU will not be used. GPUs are counted from zero. An example is <code>--device cuda:0 cuda:1</code> , when 2 GPUs are specified to use by the Guppy command.

### MinIT – the MinKNOW interface is not shown in the web browser

Observation	Possible cause	Comments and actions
<b>The MinKNOW interface is not shown in the web browser</b>	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing <code>//mt-xxxxxx</code> (x is a number) in the address bar, type in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
<b>The MinKNOW interface is not shown in the web browser</b>	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	<p>Make sure the computer or mobile device is using the MinIT Wi-Fi. It should be shown as MT-xxxxxx (x is a number) on the underside label on the MinIT:</p>  <p>Disable the Ethernet connection from the computer or mobile device as needed. If necessary, contact your IT department to determine if the MinIT Wi-Fi is blocked (MinIT generic IP: 10.42.0.1). Please white-list MinIT as needed.</p>
<b>The MinKNOW interface is not shown in the web browser</b>	The MinIT was not on the same network that the computer was connected to.	Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network.

## Rapid Sequencing (SQK-RAD004)

### Issues during the sequencing run

Version: RSE\_9046\_v1\_revK\_14Aug2019

#### MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
<b>The MinIT software cannot be updated</b>	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the <a href="#">following AWS IP ranges</a> . Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
<b>The MinIT software cannot be updated</b>	The device already has the latest version of the software	Occasionally, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the <a href="#">Software Downloads page</a> . Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitwise or Putty, as described in the <a href="#">MinIT protocol</a> ) on a Windows computer or the terminal window on a Mac, run the command, <code>dpkg -l   grep minit</code> , to find out the version of the MinIT software and <code>sudo apt update</code> if an update is needed. If the issue still persists, please contact Technical Services with details of the error.