# Introduction to library preparation chemistry

**Introduction to library preparation chemistry**

Library preparation is the conversion of a DNA or RNA sample to a suitable format for sequencing on Oxford Nanopore Technologies devices. The flow cells used for sequencing the samples contain ion-permeable nanopores embedded in an electrically-resistant membrane enabling an ionic current to pass through the nanopore when a voltage is applied across the membrane. This creates a measurable current that is disrupted when a strand of DNA or RNA passes through the nanopore. The disruption of current is measured and is used to identify the bases passing through the nanopore. Modified bases can also be identified as the nucleic acids are directly sequenced. This means PCR is not required, preventing PCR bias or polymerase error. However, PCR is available to use with our kits to generate more input or repair template damage.

DNA and RNA libraries are prepared by attaching sequencing adapters to strand ends, using either ligation-based or rapid chemistry methods. The sequencing adapters are oligonucleotides that are loaded with a motor protein. The motor protein associates with the nanopore in the flow cell and controls the DNA or RNA strand movement through the nanopores at a defined speed. A hydrophobic tether is also used to localise the template to the membrane into closer contact with the nanopores, improving sensitivity by approximately 10,000 fold.

There are two types of chemistry used in our sequencing kits:

- ligation-based chemistry: The sequencing adapter is ligated onto the DNA ends during library preparation.
- rapid-based chemistry: The sequencing adapter is attached onto the DNA when the transposase cuts the DNA without ligation enzymes. In some kits, adaptation is also be done by PCR.

Find out more information about our sequencing kits in this document.

# Store terminology

**Store terminology**

Throughout the Community and Store, we use the following terminology to explain the capabilities of our kits:

**Sample:**

The starting material from which DNA or RNA is extracted from and used as input in a library preparation protocol.

**DNA/RNA sample:**

A collection of DNA or RNA fragments to be used as input in a library preparation protocol.

**Library:**

A collection of DNA or RNA fragments that have sequencing adapters attached in preparation for sequencing on a flow cell. This can

be a collection of fragments from one sample or multiple DNA/RNA samples that have been barcoded and pooled together.

**Barcodes:**

A known sequence attached to a DNA fragment of a sample during library preparation, enabling users to combine multiple samples into a singular library pool. Before the samples are pooled together, each sample must be uniquely barcoded individually. The multiplexed library is sequenced and demultiplexed using MinKNOW for identification of the individual samples based on the known barcode sequences.

For example, six samples can be individually barcoded and pooled together using the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) before sequencing on a single flow cell. The samples are then demultiplexed by MinKNOW, allowing for analysis of individual samples based on their barcodes.

**Reactions:**

The number of times a kit can be used for either a library preparation, flow cell priming or as extra reagents to make the full use of a barcoding kit.

For example, the Native Barcoding Kit 96 V14 (SQK-NBD114.96) can be used to sequence six libraries of 48 barcoded DNA samples. To make use of the remaining barcodes, a Native Barcoding Auxiliary Kit V14 (EXP-NBA114) is required to provide more library preparation reagents, along with a Sequencing Auxiliary Vials V14 (EXP-AUX003) expansion to provide the flow cell priming reagents.

**Pack size:**

The number of times a kit can be used to prepare a reaction. For example, the Flow Cell Priming Kit V14 (EXP-FLP004) has a pack size of six reactions, meaning enough reagents are supplied to prime six flow cells.

For the sequencing kits, pack size refers to the number libraries and number of flow cells that can be primed for sequencing using the reagents supplied in the kit. For example, the Rapid Sequencing Kit V14 (SQK-RAD114) has a pack size of six reactions, that means the kit can prepare six libraries and contains enough reagents to prime six flow cells for sequencing.

For the barcoding kits, pack size also refers to the number of barcoded libraries and number of flow cells that can be primed for sequencing using the reagents supplied in the kit. However, for the kits containing 96 barcodes, up to 12 barcoded libraries can be prepared and sequenced, depending on how many DNA samples are barcoded. The below options outline how to maximise the kit for either barcodes or flow cell priming reagents:

- 3 libraries of 96 barcodes
- 6 libraries of 48 barcodes
- 12 libraries of 24 barcodes

To maximise the use of unused barcodes, the relevant expansion packs are available on the store.

### Product phases

All our products fit into one of the product phases below that are displayed on the store to make it clear where the product is in their lifecycle and their degree of availability, warranty, and change notification. Product warranties and change notification periods increase through each phase. All products receive regular software and other platform updates that are rigourously checked and

tested before release. These tests become more formalised as the products progress.

Product phases have no impact on ordering processes, they are simply there to provide users with guidance on how rapidly we expect to be applying changes to the product and accuracy of shipping dates in store.

# Ligation-based sequencing kits

**What is ligation-based sequencing?**

Ligation-based chemistry is the method that the sequencing adapter is attached (by ligation) to the DNA ends during library preparation using ligation enzymes.

Our ligation sequencing kits are optimised for output and accuracy. These kits are the most popular kits and require double-stranded DNA as input, including gDNA, amplicons, or cDNA.

Our current ligation sequencing kits available are:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- Ligation Sequencing Kit XL V14 (SQK-LSK114-XL)
- Native Barcoding Kit 24 V14 (SQK-NBD114.24)
- Native Barcoding Kit 96 V14 (SQK-NBD114.96)
- PCR Barcoding Expansion 1-12 (EXP-PBC001)
- PCR Barcoding Expansion 1-96 (EXP-PBC096)
- Multiplex Ligation Sequencing Kit V14 XL (SQK-MLK114.96-XL)

Below, we outline the sample input requirements and library preparation workflows for our simplex kits. For information on the barcoding kits, please see the Barcoding kits section.

**Sample input recommendations**

Before starting the Ligation Sequencing Kit library preparation, it is important to ensure that you are using the correct amount of starting material to ensure a successful sequencing experiment. After DNA extraction, we recommend quantifying your DNA samples:

| Quantification | Method |
|---|---|
| Mass | Qubit Fluorometer and Qubit dsDNA BR Assay Kit |
| Size | • Agilent 2100 Bioanalyser for samples of <10 kb<br>• Agilent FemtoPulse for samples of >10 kb<br>• Oxford Nanopore Flongle |
| Purity | Nanodrop 2000 Spectrophotometer |

For information regarding how to quantify the mass of DNA samples for library preparation input, please refer to the Sample input and recommendations section.

Depending on fragment lengths, we recommend different starting inputs for the Ligation Sequencing Kit V14 (SQK-LSK114) with R10.4.1 flow cells.

**Note:** These input recommendations are for our standard protocols and may vary depending if a protocol is written for a specific application.

| Samples | Input |
| --- | --- |
| Short fragment samples (<10 kb) | • **100-200 fmol** for MinION and PromethION Flow Cells<br>• **50-100 fmol** for Flongle Flow Cells |
| Long fragment samples (>10 kb) | • **1 µg** for MinION and PromethION Flow Cells<br>• **500 ng** for Flongle Flow Cells |

Molar quantification is difficult and unreliable for samples consisting of long fragments and we have found these starting inputs are almost always enough for optimal pore occupancy. The graph below illustrates pore occupancy of long fragment libraries using different mass input.

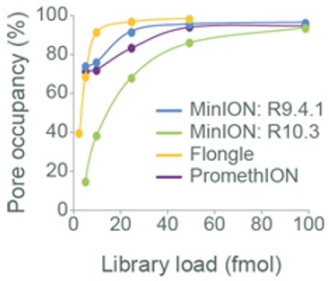



**Figure 1.** Sequencing libraries were prepared using various starting inputs of a gDNA sample consisting of long molecules, and then run on the various flow cell types. The resulting pore occupancy is shown, and it was found that an input of ~1 µg is sufficient for optimal pore occupancy on R9.4.1 flow cells and an input of ~2 µg was sufficient for optimal pore occupancy on R10.3 flow cells.

If you use less than 1 µg in your library preparation, you may have a reduced yield, meaning you will have fewer DNA strands with sequencing adapters (adapted DNA) on each end which may negatively affect sequencing output. This is because optimal pore occupancy may not be reached and it may deteriorate faster as the pores will not always be sequencing, compromising output. For more information on pore occupancy, please see the Sample input and recommendations section.

However, even when starting with as little as 100 ng of high molecular weight (HMW) DNA, we have observed outputs of approximately 10 Gb from R9.4.1 MinION/GridION Flow Cells and approximately 30 Gb from PromethION Flow Cells (Figure 2.). As you decrease input below 100 ng, pore occupancy significantly deteriorates, and we recommend considering amplification (by PCR or multiple displacement amplification) to generate more template. HMW DNA can also be sheared using Covaris g-TUBE or MegaRuptor® to increase strands of DNA for low input samples. For further information, see the optional fragmentation of gDNA in the Sample input and recommendations section.

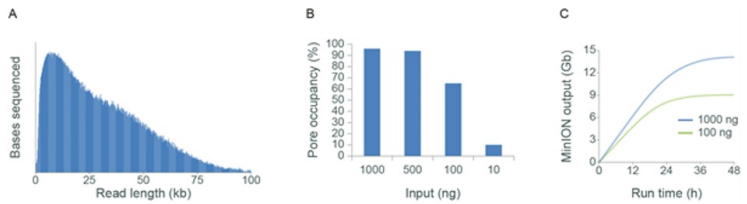



**Figure 2.** Flow cell output may be decreased when starting with less than 1 µg of HMW gDNA. In the experiment above, gDNA extracted from human cells (GM24385) was titrated as input into the Ligation Sequencing Kit. The resulting libraries were sequenced on MinION (R9.4.1 flow cells). **Panel A:** The typical read length distribution of a library. **Panel B:** Starting with 1 µg of HMW DNA results in efficient pore occupancy. As input mass decreases, the number of adapted DNA strands decreases and this leads to reduced pore occupancy. **Panel C:** The reduction in pore occupancy at a lower input mass means that the pores spend less time sequencing, which results in a reduced sequencing output.

### Ligation Sequencing Kit V14

The Ligation Sequencing Kit V14 (SQK-LSK114) is our newest ligation-based sequencing kit optimised to achieve sequencing accuracies of over 99% (Q20+), with high output on our latest nanopore: R10.4.1. Further improvements include duplex sequencing which allows users to sequence both the template DNA strand and the complement strand. For more information, see the Kit 14 sequencing and duplex basecalling document.

This Kit 14 upgrade includes previous updates such as higher capture rate of DNA to enable lower flow cell loading amounts, and fuel fix technology. Note, due to the higher capture of the adapter, it is important to follow the flow cell loading recommendations in the protocols.

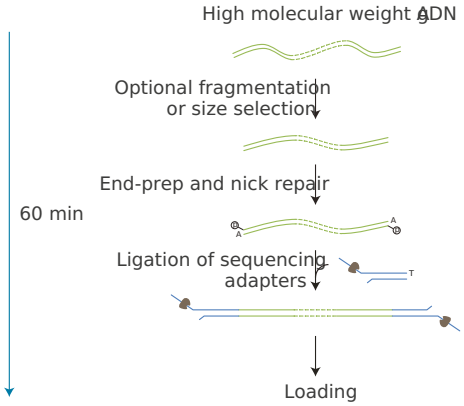
The library preparation is simple and highly versatile, accommodating any double-stranded DNA sample input of 100–200 fmol of short fragment libraries (<10 kb) or 1 µg of long fragment libraries (>10 kb). Fragment length can be controlled by optional fragmentation or size selection methods which can be found in our DNA/RNA handling page.

This kit is also compatible with upstream processes such as targeted enrichment by sequence capture and whole-genome amplification.

Details of our previous Ligation Sequencing kits are available in the Legacy kit page of this document.

## Workflow:

The library preparation involves two enzymatic steps to prepare the DNA ends for sequencing. The first enzymatic step repairs any damage in the DNA molecules, such as nicks and generates uniform ends with 5’ phosphates and 3’ adenine overhangs. The second enzymatic step ligates the sequencing adapters that have complementary thymine tails with the dA-tailed template.




This kit has a standard protocol and multiple associated protocols due to the multiple applications this kit can be used for:

- Ligation Sequencing gDNA V14 (SQK-LSK114)
- Ligation Sequencing Amplicons V14 (SQK-LSK114)
- Ligation Sequencing gDNA - Lambda Control V14 (SQK-LSK114)
- Ligation sequencing gDNA V14 - low input by PCR (SQK-LSK114 with EXP-PCA001)
- Ligation sequencing V14 - PCR Barcoding (SQK-LSK114 with EXP-PBC001 or EXP-PBC096)
- Ligation sequencing V14 - Direct cDNA sequencing (SQK-LSK114)
- Ligation sequencing gDNA V14 - reduced representation methylation sequencing (RRMS) of human samples (SQK-LSK114)
- Ligation Sequencing gDNA V14 - human sample (N50 30 kb) on PromethION (SQK-LSK114)
- Ligation sequencing gDNA V14 - human sample (N50 10 kb) on PromethION (SQK-LSK114)
- Ligation sequencing gDNA V14 - whole genome amplification (SQK-LSK114)
- High duplex ligation sequencing DNA V14 (SQK-LSK114)
- Ligation sequencing V14 - Single-cell transcriptomics with cDNA prepared using 10X Genomics on PromethION (SQK-LSK114)

**Ligation Sequencing Kit XL**

The Ligation Sequencing Kit V14 XL (SQK-LSK114-XL) is a scaled-up version of the Ligation Sequencing Kit V14 and contains larger quantities of the same components.

In this kit, there are sufficient reagents to generate 48 sequencing libraries and is recommended for users who would like to process multiple samples simultaneously, either with a multichannel pipette or a liquid handling robot.

# Rapid-based sequencing kits

**What is rapid-based sequencing?**

Rapid-based chemistry is the method that the sequencing adapter is attached (rapid attachment) to the DNA ends. Typically, in the transposase step, the DNA is cut and the adapter is attached at the same time without any ligation enzymes. However, in the barcoding kits, the barcodes are attached during the transposase step and the sequencing adapters are attached in a later step using rapid attachment. It is also worth noting that in the PCR-based kits, adaptation is completed by PCR.

These kits are optimised for speed and simplicity or for applications where turnaround times are more critical or laboratory equipment is limited.

In comparison to the Ligation Sequencing Kit V14, the Rapid Sequencing Kit V14 sequencing output is slightly reduced due to the lack of purification steps and the presence of transposases; both of which may cause pore blockages, preventing the pore from sequencing adapted DNA (Figure 3). Read length is also slightly reduced due to the requisite transposase fragmentation.

As the Rapid Sequencing Kit focuses on a rapid library preparation, certain steps, such as bead purification, are omitted and may reduce sequencing yield in some cases. For further information on improving flow cell performance, please refer to the Flow cell performance section in the Sample input and recommendations section.

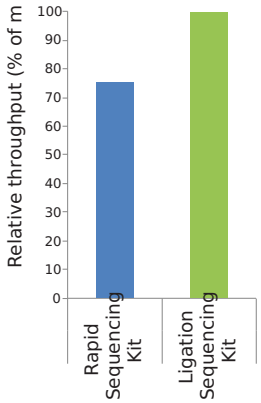



**Figure 3.** The graph illustrates typical output achieved with the Rapid Sequencing Kit compared to the Ligation Sequencing Kit.

Our current rapid sequencing kits available are:

- Rapid Sequencing Kit V14 (SQK-RAD114)
- Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114)
- Rapid Barcoding Kit 96 (SQK-RBK110.96)
- Rapid Barcoding Kit V14 24 (SQK-RBK114.24)
- Rapid Barcoding Kit V14 96 (SQK-RBK114.96)
- Rapid PCR Barcoding Kit (SQK-RPB114.24)
- 16S Barcoding Kit 24 V14 (SQK-16S114.24)
- Midnight RT PCR Expansion (EXP-MRT001)

Below, we outline the sample input requirements and library preparation workflows for our simplex kits. For information on the

barcoding kits, please see the Barcoding kits section.

### Sample input recommendations

Before starting the Rapid Sequencing Kit library preparation, it is important to ensure that you are using the correct amount of starting material to ensure a successful sequencing experiment. After DNA extraction, we recommend quantifying your DNA samples:

| Quantification | Method |
|---|---|
| Mass | Qubit Fluorometer and Qubit dsDNA BR Assay Kit |
| Size | • Agilent 2100 Bioanalyser for samples of <10 kb<br>• Agilent FemtoPulse for samples of >10 kb<br>• Oxford Nanopore Flongle |
| Purity | Nanodrop 2000 Spectrophotometer |

For information regarding how to quantify the mass of DNA samples for library preparation input, please refer to the Sample input and recommendations section.

For our latest Kit 14 upgrade of the kit (SQK-RAD114), a reduced starting input of 100 ng of HMW gDNA containing long fragments is recommended. Using less than 100 ng or samples with shorter fragments can compromise sequencing output because yield from the library preparation will be reduced. Where only lower inputs are available, we recommend using the Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24) to increase the number of template molecules.

Please refer to the Sample input and recommendations section of this document for further information about sample input recommendations and how to improve library quality.

Data output from the flow cell is influenced by the amount and quality of the input DNA sample fragmented by the fixed amount of transposase in the sequencing kit to generate tagged fragments.

- To generate long fragments:
  - Add more than the recommended starting input as there will be fewer cuts per molecule
  - Long fragments of DNA must be present initially in the sample input
- To generate short fragments:
  - Add less than the recommended starting input as there will be more cuts per molecule

Due to the simple nature of the workflow and the fact that little sample manipulation is required (e.g. minimal pipetting steps and no clean-up steps) some very long reads can be achieved with this kit, despite the required transposase fragmentation. However, in order for long reads to be observed in sequencing, long fragments need to be present in the sample in the first place. However, to generate ultra-long reads, we recommend using the Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114), which uses rapid-based chemistry. Further information for this sequencing kit is available below.

### Rapid Sequencing Kit V14

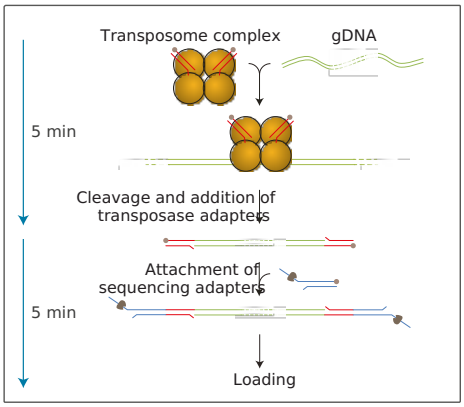
The Rapid Sequencing Kit V14 (SQK-RAD114) is our newest rapid-based sequencing kit optimised for speed and simplicity, using limited laboratory equipment, and a lower starting input of 100 ng of HMW gDNA. This kit has been upgraded to use our newest Kit 14 chemistry, which includes improved modal raw read sequencing accuracies with higher output on our latest nanopore: R10.4.1. This Kit 14 upgrade also includes updates such as the higher capture rate of DNA to enable lower flow cell loading amounts, and fuel fix

technology. Note, due to the higher capture of the adapter, it is important to follow the flow cell loading recommendations in the protocols.

This sequencing kit and protocol is optimised for speed and simplicity, requiring 100 ng of HMW gDNA input. Due to the minimal sample manipulation, required, very long reads can be generated despite the requisite transposase fragmentation as long as there are very long DNA fragments present in the input sample. However, for ultra-long reads, we recommend using the Ultra-Long DNA Sequencing Kit V14.

**Workflow:**

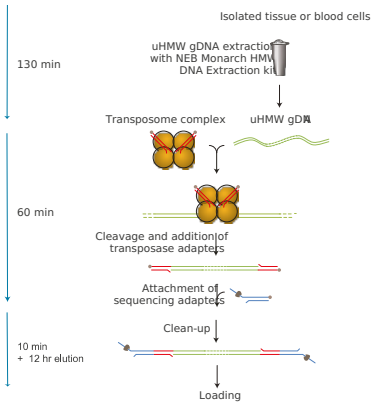
The library preparation uses transposase-based fragmentation of the gDNA input whilst simultaneously tagging the fragmented template with transposase adapter sequences. Post-transposition, sequencing adapters are then attached to the transposase adapters in an enzyme-free reaction.




### Ultra-Long DNA Sequencing Kit V14

This is our updated Ultra-Long DNA Sequencing kit using rapid-based Kit 14 chemistry to sequence ultra-long reads. The kit includes new reagents to simplify the library preparation and to improve DNA precipitation and recovery. This kit uses rapid-based chemistry due to the simple workflow, which enables very long reads to be achieved due to the minimal pipetting required during library preparation.

This kit requires ultra-high molecular weight (uHMW) gDNA to be extracted. In our protocol, we recommend using the NEB Monarch® HMW DNA Extraction Kit for Tissue (T3060) to extract the uHMW gDNA from either frozen cells or whole blood. After gDNA extraction, a diluted fragmentation mix containing transposases are added to the extracted gDNA to fragment and simultaneously tag the fragmented template with transposase adapter sequences. Post-transposition, sequencing adapters are then attached to the transposase adapters in an enzyme-free reaction. After an overnight elution with the Precipitation Star (PS), the library is ready for sequencing.




# RNA and cDNA sequencing kits

### Introduction to RNA and cDNA sequencing

Using nanopore technology, RNA can be directly sequenced without sequencing complementary DNA (cDNA) intermediates, enabling the exploration of the attributes of native RNA, such as base modifications. The recently released Direct RNA Sequencing Kit (SQK-RNA004) has improved sequencing outputs and accuracy on our newly released RNA flow cells.

We have also developed kits to sequence cDNA, which is a reverse-transcribed copy of native mRNA. Therefore, characteristics of cDNA can be exploited to select for fully reverse-transcribed cDNA molecules, using the cDNA-PCR Sequencing Kit V14 (SQK-PCS114) and the cDNA-PCR Barcoding Kit V14 (SQK-PCB114.24).

Our current RNA and cDNA kits available are:

- Direct RNA Sequencing Kit (SQK-RNA004)
- cDNA-PCR Sequencing Kit V14 (SQK-PCS114)
- cDNA-PCR Barcoding Kit V14 (SQK-PCB114.24)

Below, we outline the sample input requirements and library preparation workflows for our simplex kits. For information on the barcoding kits, please see the Barcoding kits section.

### Sample input recommendations

For our RNA and cDNA sequencing kits, poly(A)-tailed mRNA is required as input as the tails are used for adapter attachment and primer annealing. However, total RNA can be used as input to the library preparation as the primers and adapters used can select for the poly(A) tails. However, using total RNA requires larger amount of input, as mRNA typically does not make up a large proportion of total RNA.

To overcome the input issue with total RNA input, poly(A) polymerase can be used to polyadenylate the RNA sample, as described in the Polyadenylation of non-poly(A) transcripts using *E. coli* poly(A) polymerase document. Poly(A)-tailed mRNA can also be enriched using different methods, however, regardless of methods used, there may be a slight bias towards molecules with longer poly(A) tails during library preparation as longer tails may be better targets for selection protocols. Therefore, users should be mindful of which enrichment methods are most appropriate for their needs.

To ensure high-quality sample preparation, RNA samples should be free from contaminants. If total RNA is used, an RNA Integrity Number >7 is recommended before enriching the sample using poly(A) selection. A clean RNase-free environment is crucial with good RNase-cleaning practices to prevent RNA degradation. Further information is available in the RNA contaminants and RNA stability documents.

### Direct RNA Sequencing Kit

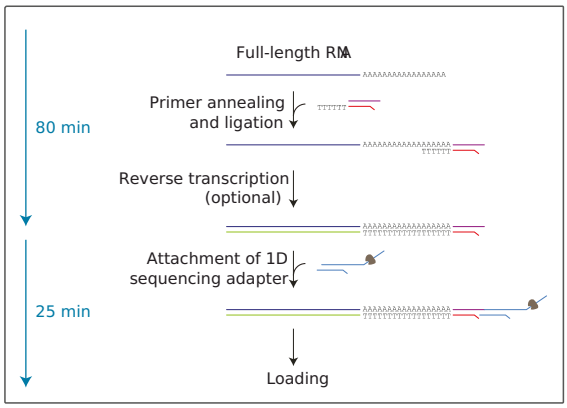
The Direct RNA Sequencing Kit (SQK-RNA004) is our recently updated RNA kit with improved outputs and accuracy compared to the previous version of this kit. Improvements of this kit include a faster motor protein, a new RNA-specific reader pore and an optimised library preparation kit.

An input of 300 ng of poly(A)-tailed RNA or 1 µg of total RNA is required. Other possible RNA inputs includes eukaryotic mRNA, viral RNA with a poly(A) tail, or any RNA prepared with a poly(A)-tailing kit.

#### Workflow

**Note:** Only the RNA strand, not the reverse transcribed strand, is sequenced.

The library preparation prepares the RNA for sequencing by ligating a reverse transcription adapter to the RNA ends before performing reverse transcription to synthesise a complementary strand. This is to stabilise the RNA against secondary structure formation. However, only the RNA strand is sequenced. If reverse transcription is bypassed, the workflow is shortened to 30 minutes but sequencing output is reduced; this is likely due to an RNA tertiary structure blocking the pores. The sequencing adapters supplied in the kit are attached to the ends of the RNA-cDNA hybrid for sequencing.




### cDNA-PCR Sequencing Kit V14

The cDNA-PCR Sequencing Kit (SQK-PCS114) is our recently updated cDNA sequencing kit and is recommended for users starting with a low input of 10 ng of poly(A)-tailed RNA or 500 ng of total RNA, avoiding the need to poly(A)-select the template molecules.

This kit can be used for the identification and quantification of full-length transcripts. Isoforms, splice variants, and fusion transcripts can also be sequenced for analysis.

The Kit 14 chemistry upgrade in this kit reduces the level of free sequencing adapter and improves sequencing accuracies and output. Other updates include the addition of a cDNA RT adapter and RT primer to reduce transcript overlaps during the reverse transcription step to enable the measurement of the poly(A) tail length and a unique molecular identifier (UMI) for the identification of splice variants.

#### Workflow:

The protocol starts with a reverse transcription step to prepare full-length cDNA from the input RNA and incorporates the UMI. During reverse transcription, the poly(dT) reverse transcription adapter is ligated to the 3’ terminal poly(A) tail of the template molecule. The bottom strand of the adapter is removed and a reverse transcription primer is annealed, anchoring the start of transcription to include the entire 3’ terminal poly(A) tail. Then a strand-switching primer, containing a UMI, is added during reverse transcription, allowing strand switching to occur and generate a full-length cDNA strand. This is tagged with universal sequences on both ends.

**Note:** The reverse transcriptase inhibits downstream PCR and the enzymes must be heat-inactivated and the reverse transcribed sample to be split across four PCR reactions to dilute the inhibitors. This is to allow the amplification of cDNA with maximum efficiency, without losing sensitivity in the next step.

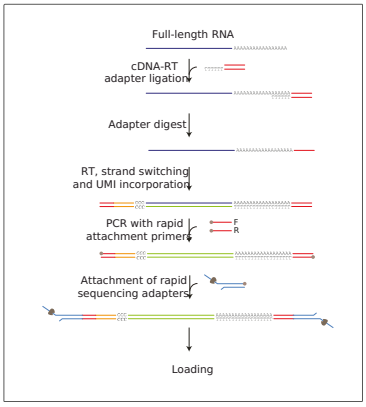
Once the full-length cDNA is prepared, PCR amplification is performed and rapid attachment primers are added to the cDNA simultaneously. Finally, rapid sequencing adapters are attached to the primers for sequencing.

Using this kit, specific transcripts can be selected if one or both ends of the target cDNA are known:

- **Both target ends are known:** Reverse-transcribe the entire template molecule and use selective primers to anneal to both ends of the cDNA before carrying out PCR with the sequence-specific primers.
- **Only one end of the target is known:** Reverse-transcribe the template molecule and use selective primers to anneal to the known end of the cDNA with universal primers on the unknown end. Then carry out PCR with the sequence-specific primers for the known end of the target molecule and a universal primer for the unknown end.

A specific transcript can also be selected by altering the reverse transcription primer to replace the poly(dT) sequence with a sequence-specific primer so only the transcript of interest will be reverse-transcribed. This reverse-transcribed and strand-switched product is then amplified with the universal primers in the PCR-cDNA Sequencing Kit.

PCR-cDNA Barcoding Kit (SQK-PCB114.24) is the barcoding version of this kit and can be found in the Barcoding kits section.




# Barcoding kits

### Introduction to barcoding kits

The barcoding kits are designed to allow the pooling and running of multiple libraries on our flow cell by attaching a known DNA sequence (barcode) to your samples within a pooled library. The pooled library is then sequenced in a single run and MinKNOW uses the barcodes to demultiplex the library during a sequencing run. The barcodes have been carefully designed and extensively purified to minimise cross-talk.

We recommend using our barcoding kits:

- To reduce cost per sample.
- For more efficient use of flow cells when less data per sample is required than the total amount of data that can be generated from a single flow cell.
- For optimal re-use of a flow cell when used with the Flow Cell Wash kit as any barcoded residual reads on a flow cell will be identified between barcoded samples.

**Note:** As more samples are multiplexed, less data per sample is generated.

Multiplexing samples onto one flow cell can reduce the cost per sample for a user. In the table below, we illustrate how barcoding 1 to 96 samples onto a single flow cell can alter the cost per sample.
**Note:** These costs are an example and do not reflect our current store prices.

| Barcodes | 0 | 6 | 12 | 24 | 48 | 96 |
|---|---|---|---|---|---|---|
| Flow cell price | $500 | $500 | $500 | $500 | $500 | $500 |
| Library price | $99 | $99 | $99 | $99 | $99 | $99 |
| Barcode price | - | $18.75 | $37.50 | $75 | $150 | $300 |
| Price per sample | $599 | $102.95 | $53.04 | $28.08 | $15.60 | $9.36 |

There are four types of barcoding kits:

- Ligation-based barcoding kits
- Ligation-based PCR barcoding kits
- Rapid chemistry-based barcoding kits
- Rapid chemistry-based PCR barcoding kits

The recently released barcoding kits have been updated to combine all the reagents required for library preparation, barcoding and flow cell priming into a kit.

All our kits with 96 barcodes have been developed to allow flexibility in the number of samples that can be multiplexed. Users can prepare multiplexed libraries using various options without extra expansion packs:

- 12 libraries containing 24 barcodes
- 6 libraries containing 48 barcodes
- 3 libraries containing 96 barcodes

Expansion packs for library preparation and flow cell priming reagents are available to make use of any remaining barcodes in a kit.

The barcode sequences are available at the back of the document in theappendix.

**Ligation-based barcoding kits**

**Native Barcoding Kit 24 and 96 V14**

There are two standalone native barcoding kits and one expansion:

- Native Barcoding Kit 24 V14 (SQK-NBD114.24)
- Native Barcoding Kit 96 V14 (SQK-NBD114.96)
- Native Barcoding Auxiliary Kit V14 (EXP-NBA114)

The Native Barcoding Kits 24 and 96 V14 are the newest native barcoding sequencing kit and contain all required reagents for barcoding 24 or 96 samples, preparing libraries for sequencing and flow cell priming. The kits are optimised to achieve sequencing accuracies of over 99% (Q20+), with high output. The native barcodes in these kits are shipped at 640 nM.

The Native Barcoding Kits are recommended for users who want to multiplex their samples with a PCR-free method to preserve base modifications. There are up to 24 or 96 unique barcodes which can be used for gDNA or amplicons. The barcoding kits are optimised to generate maximum output without the need for PCR.

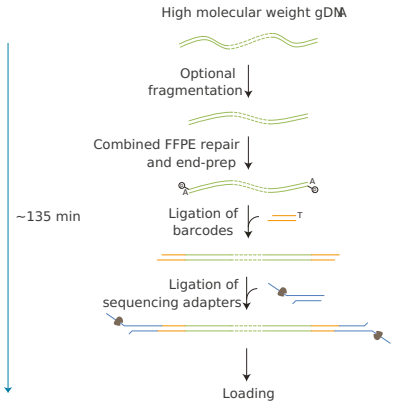
These kits require double-stranded DNA as input with the following recommendations below. Due to the higher capture of the adapter, it is important to follow the flow cell loading recommendations in the protocols.

| Kit | Input requirements |
|---|---|
| **Native Barcoding Kit 24 V14** (SQK-NBD114.24) for barcoding up to 24 samples | • <4 barcodes, 1000 ng of gDNA per sample<br>• >4 barcodes, 400 ng of gDNA per sample<br>• 200 fmol (130 ng for 1 kb amplicons) DNA per sample |
| **Native Barcoding Kit 96 V14** (SQK-NBD114.96) for barcoding 96 samples | • 400 ng of gDNA per sample<br>• 200 fmol (130 ng for 1 kb amplicons) DNA per sample |

The Native Barcoding Auxiliary Kit V14 (EXP-NBA114) contains additional library preparation reagents for use with the Native Barcoding Kits for another 12 reactions. For further information on our available expansions, please see the Expansion packs section.

**Workflow**

The DNA is repaired and the ends dA-tailed in preparation for the dT-tailed native barcode ligation. After barcoding, the samples are pooled together and sequencing adapters are ligated to the barcode ends for sequencing.

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**Multiplex Ligation Barcoding Kit V14 96 XL**

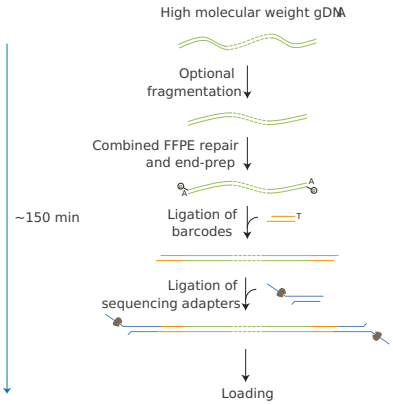
This kit is our recently updated kit for the high output and PCR-free, low-plex sequencing of up to three samples of double-stranded DNA. This kit is recommended to perform low-plex whole genome sequencing. All required reagents for barcoding up to 96 samples, preparing libraries for sequencing and flow cell loading are included, using our Kit 14 chemistry.

We recommend using 1 µg gDNA per sample to sequence two samples per flow cell. This results in the sequencing of up to 96 samples across 48 flow cells. Three samples across two flow cells can also be used to sequence up to 96 samples across 64 flow cells to maximise sequencing output.

This kit uses our native barcodes and is compatible with the Native Barcoding Auxiliary Kit V14 (EXP-NBA114) for additional library preparation reagents.

**Workflow**

The library preparation method is similar to the Ligation Sequencing Kit protocol; DNA ends are repaired and dA-tailed in preparation for the dT-tailed native barcode ligation. After barcoding, the samples are pooled together and the sequencing adapters are ligated to the barcode ends for sequencing.




**Ligation-based PCR barcoding kits**

**PCR Barcoding Expansion 1-12 and 1-96**

For low starting inputs, there are two PCR barcoding expansion packs with 12 or 96 barcodes; PCR Barcoding Expansion 1-12 (EXP-PBC001) and PCR Barcoding Expansion 1-96 (EXP-PBC096). These barcoding expansions are available to use in combination with our Ligation Sequencing Kit, to enable the pooling and running of multiple PCR amplified sequencing libraries for low inputs of gDNA, amplicon, and cDNA. The kits include adapters and up to 96 forward and reverse PCR primers with the same barcode in both forward and reverse primers.

An input of <100-200 fmol per DNA sample is required or <100-200 fmol of first-round PCR product with tailed primers per sample.

**Note:** cDNA and amplicons have to be prepared in advance for barcoding, as reverse transcription or the first round of amplification are not included in the library preparation protocol. Details of recommendations are outlined in the protocol.

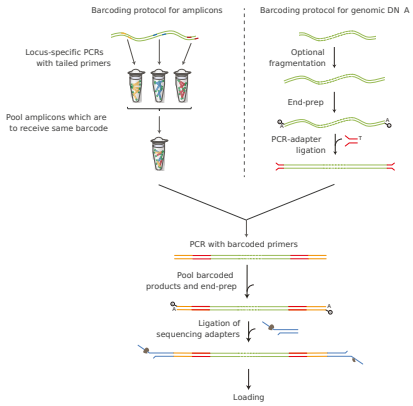
**Workflow**

Depending on your input, the sample preparation step will very before the library preparation step.

For low gDNA inputs, you will need to prepare your DNA ends for PCR adapter ligation and perform PCR amplification with barcoded primers before pooling your samples prior to library preparation.

For low cDNA and amplicon inputs, you will need to perform a round of PCR to incorporate tailed primers. A second round of PCR amplification step with barcoded primers will be performed and the samples are pooled prior to library preparation.

The library preparation step is the same across all sample inputs and the pooled barcoded samples are end-prepped for the sequencing adapters to ligate to the barcodes before sequencing.

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### Dual barcoding protocol

To perform massively parallel sequencing of up to 2,304 samples of gDNA or amplicons on a single flow cell, the PCR Barcoding Expansion 1-96 (EXP-PBC096) can be combined with the Native Barcoding Kit V14 24 (SQK-NBD114.24). The dual barcoding protocol combines both kits to dual barcode the libraries for pooling and sequencing on a single flow cell.

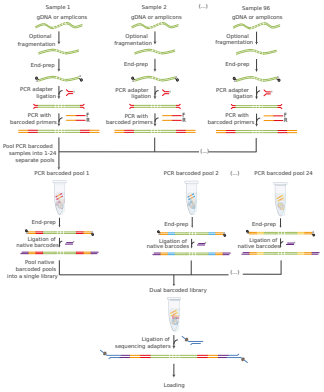
We recommend dual barcoding for users wishing to sequence more than 96 samples or want to sort libraries into multiple categories as each barcode can be used to denote a different sample feature. The inner barcode is a PCR barcode and the outer barcode is a native barcode.

An input of <100-200 fmol per DNA sample is required or <100-200 fmol of first-round PCR product with tailed primers per sample.

**Note:** cDNA and amplicons have to be prepared in advance for barcoding, as reverse transcription or the first round of amplification are not included in the library preparation protocol. Details of recommendations are outlined in the protocol.

### Workflow

The ends of the DNA input are first prepared for PCR adapter ligation and PCR is performed with barcoded primers to barcode up to 96 samples. The 96 samples are pooled into a single library and this is repeated to create 24 pools of 96 barcoded samples. Next, prepare the DNA ends for native barcode ligation for each pool. After barcoding, pool the dual barcoded libraries into a single pool and ligate the sequencing adapters to the native barcodes before sequencing on a single flow cell.




### Rapid chemistry-based barcoding kits

### Rapid Barcoding Kit 24 and 96

There are two standalone rapid barcoding kits and one expansion using Kit 14 chemistry:

- Rapid Barcoding Kit 24 V14 (SQK-RBK114.24)
- Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)
- Rapid Adapter Auxiliary V14 (EXP-RAA114)

The Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) and Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) are both standalone kits for barcoding and sequencing up to 24 or 96 samples, respectively, using a library preparation optimised for speed and simplicity, requiring minimal laboratory equipment. For users requiring high accuracy and output, we recommend the Native Barcoding Kit 24 or 96 V14 kits (SQK-NBD114.24 and SQK-NBD114.96).

This kit has been upgraded to use Kit 14 chemistry with our updated sequencing adapter to improve sequencing accuracies and higher outputs. The barcodes in our rapid-based kits are shipped at 10 µM.

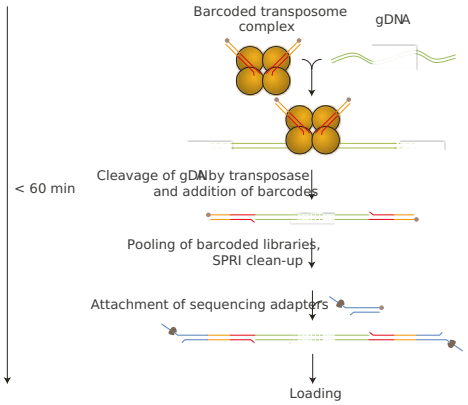
Depending on the number of samples, a different input is required:

| Number of samples | Input required |
|---|---|
| >4 samples | 50 ng of gDNA |
| <4 samples | 200 ng of gDNA |

**Note:** We also have our Rapid Barcoding Kit 96 (SQK-RBK110.96) that uses our previous Kit 10 chemistry. The library preparation workflow and input are the same but uses our previous sequencing adapter and flow cell type.

**Workflow**

The DNA input is undergoes a transposase step to fragment the samples whilst attaching rapid barcodes to the ends simultaneously. The samples can be pooled and a bead clean-up performed to remove any excess transposases in the reaction. The sequencing adapters are then attached to the barcodes and the library can be sequenced.

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**Midnight RT PCR Expansion**

The Midnight RT PCR Expansion (EXP-MRT001) has been developed to work in conjunction with the Rapid Barcoding Kit (SQK-RBK110.96) or the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) to enable PCR tiling of overlapping 1.2 kb amplicons across the SARS-CoV-2 genome.

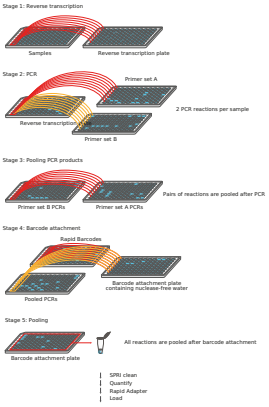
The primers provided in this kits were initially developed by Freed *et al.,* 2020, using the Primal Scheme. These primers are updated as new mutations are identified in the SARS-CoV-2 lineages and we will work closely with the community (including the entire ARTIC

Nextwork, Nikki Freed, Olin Silander, Josh Quick, John Tyson, Nick Loman and many more) to rapidly respond and ensure performance of Oxford Nanopore Technologies' protocols are always kept up-to-date, providing the best performance for robust SARS-CoV-2 whole genome sequencing.

For this kit, an input of extracted RNA in 10 mM of Tris-HCl, pH 8.0 is required.

**Workflow**

The extracted RNA is reverse transcribed with random hexamers and the samples are split into pools A and B for tiled PCR. The primer pools are combined with their associated sample, making sure to not combine different samples together. The DNA samples are then fragmented using a transposase and the rapid barcodes are attached to the fragment ends simultaneously. After barcoding, the samples can be combined into a single pool and a bead clean-up performed to remove any excess barcodes and transposases before sequencing on a flow cell.

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**16S Barcoding Kit 24 V14**

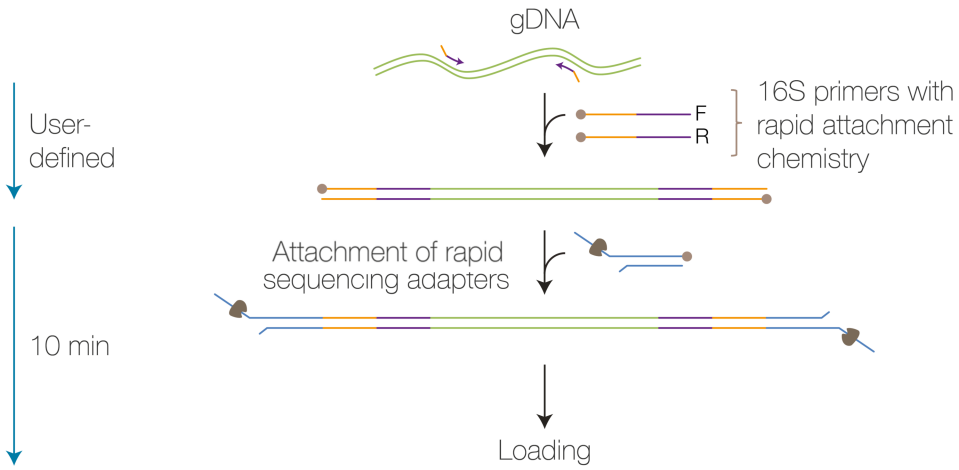
This is our updated 16S kit using our latest Kit 14 chemistry to amplify the entire ~1,500 bp 16S rRNA gene from extracted gDNA. With our chemistry upgrade, sequencing accuracies and output are improved and a lower flow cell loading amount is required. The number of barcodes have also been increased to enable barcoding of up to 24 samples into a single library. The barcodes in our rapid-based kits are shipped at 10 µM.

We recommend this kit for rapid 16S sequencing for bacterial identification as the highly conserved and variant regions across different bacterial species are sequenced. A specific region of interest can also be selected for before preparing the library with this kit to enable the sequencing of the organisms in a sample without unnecessary regions of the genome to improve the efficiency of identification by reducing speed and costs.

An input of 10 ng of gDNA per sample is required.

**Workflow**

The gDNA undergoes PCR amplification with 16S primers (27F and 1492R) to amplify the 16S gene present in the sample whilst simultaneously attaching rapid adapters to the DNA ends. The rapid barcodes are then attached to the rapid adapters and the samples pooled before a bead clean-up. The rapid sequencing adapters are then attached to the rapid barcodes before sequencing.




**Rapid chemistry-based PCR barcoding kits**

**Rapid PCR Barcoding Kit 24 V14**

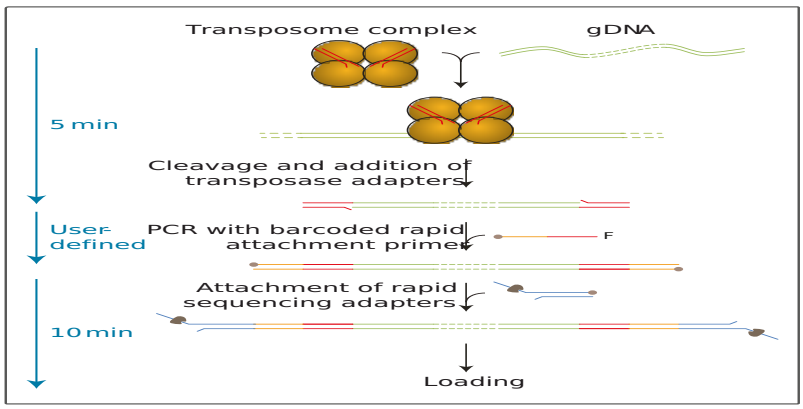
The Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24) is the fastest and simplest method of preparing barcoded libraries starting with low inputs of gDNA (1–5 ng). PCR amplification is performed to increase the number of templates for sequencing. This kit has been upgraded to use Kit 14 chemistry and the number of barcodes available in the kit have been increased to 24. The Kit 14 upgrade includes improved raw read sequencing accuracies and higher output. The barcodes in our rapid-based kits are shipped at 10 µM.

A low starting input of 1-5 ng of gDNA per sample is required.

**Note:** The DNA must be at least 4 kb in length to ensure correct tagmentation and PCR amplification.

**Workflow**

The gDNA is prepared for sequencing by undergoing tagmentation to fragment the DNA and to simultaneously attach PCR primers to the fragment ends. PCR is performed and rapid barcode primers are attached to the DNA ends in the same step. After barcoding, the samples are pooled and the rapid sequencing adapter is attached to the ends of the DNA samples for sequencing.




**cDNA barcoding kit**

**cDNA-PCR Barcoding Kit V14**

This our recently updated cDNA barcoding kit to barcode up to 24 RNA samples for sequencing of the cDNA. Identification and qualtification of full-length transcripts can be performed with this kit and isoforms, splice variants, and fusion transcripts can be sequenced. The barcodes in our rapid-based kits are shipped at 10 µM.

The Kit 14 chemistry upgrade in this kit reduces the level of free sequencing adapter and improves sequencing accuracies and output. Other updates include the addition of a cDNA RT adapter and RT primer to reduce transcript overlaps during the reverse transcription step to enable the measurement of the poly(A) tail length and a unique molecular identifier (UMI) for the identification of splice variants.

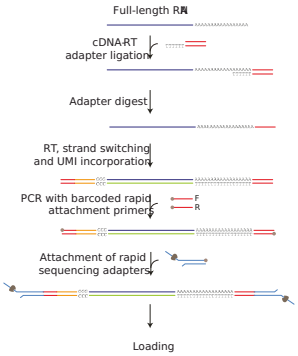
A low input of 10 ng of enriched RNA per sample is required which can be poly(A)-tailed or ribodepleted RNA. Total RNA can also be used but requires 500 ng per sample.

**Workflow**

The RNA undergoes a reverse transcription step to prepare full-length cDNA from the input RNA and incorporates the UMI. During reverse transcription, the poly(dT) reverse transcription adapter is ligated to the 3’ terminal poly(A) tail of the template molecule. The bottom strand of the adapter is removed and a reverse transcription primer is annealed, anchoring the start of transcription to include the entire 3’ terminal poly(A) tail. Then a strand-switching primer, containing a UMI, is added during reverse transcription, allowing strand switching to occur and generate a full-length cDNA strand. This is tagged with universal sequences on both ends.

**Note:** The reverse transcriptase inhibits downstream PCR and the enzymes must be heat-inactivated and the reverse transcribed sample to be split across four PCR reactions to dilute the inhibitors. This is to allow the amplification of cDNA with maximum efficiency, without losing sensitivity in the next step.

Once the full-length cDNA is prepared, PCR amplification is performed and rapid barcode primers are attached to the cDNA samples simultaneously. After barcoding, the samples are pooled into a single library and the rapid sequencing adapters attached are to the rapid barcode primers for sequencing.




# Expansion packs

**Introduction to expansion packs**

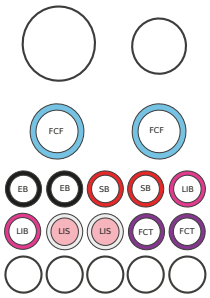
Expansion packs are available to be used alongside the sequencing kits and include extra reagents or barcodes for more efficient use of our sequencing kits.

Below is an overview of our current expansion kits, however, the store expansion pack page also includes additional simple

expansions containing additional reagents.

### Sequencing Auxiliary Vial

This expansion pack (EXP-AUX003) is recommended for users who want to split a library across multiple flow cells, need to add more library to an existing run, or wish to top up a flow cell with an additional library. This kit contains sufficient reagents for six reactions with our V14 kits, including additional flow cell priming reagents.

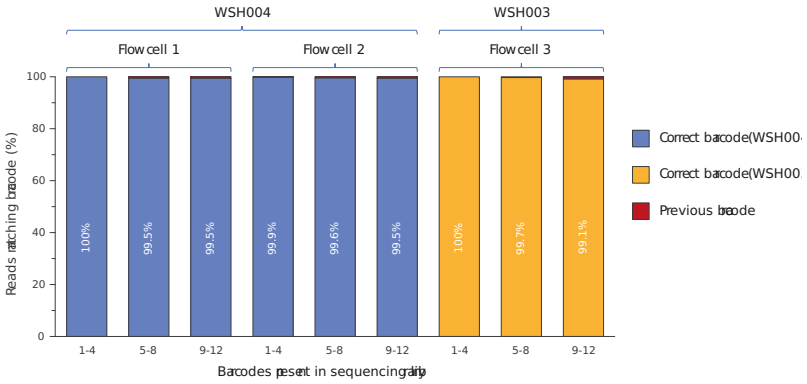



### Flow Cell Wash Kit and XL

This kit (EXP-WSH004) is recommended for users who want to run multiple sequencing libraries on the same flow cell and is compatible with R9.4.1 and R10.4.1 flow cells. It can also be used for when a flow cell has accumulated a high number of ‘unavailable’ pores during a run and users want to revert these pores to the available state.

This kit provides a highly effective means of removing a library that has been loaded onto a flow cell. Once the flow cell is washed, it is available immediately for use again or to store. There are sufficient reagents for six flow cell washes. The Flow Cell Wash Kit XL (EXP-WSH004-XL) is a scaled up version of the kit, providing enough reagents for 48 reactions.

We recommend barcoding samples when running multiple samples sequentially to allow for filtering of sequences remaining from previous runs as there may be some residual DNA on the flow cell.

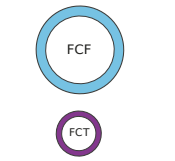



A sample with four barcodes was sequencing before the flow cell was washed using EXP-WSH004 before a samples with four different barcodes was loaded. This was repeated for a third sequencing run. We repeated this using EXP-WSH003 to illustrate the comparable washing between the wash kits.

### Flow Cell Priming Kit

We have released a separate expansion containing just V14 flow cell priming reagents. These are to be used alongside our sequencing kits to prime a MinION/GridION and PromethION Flow Cells, or to add more library to a flow cell during a sequencing experiment for another six reactions.

**Note:** The Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114) requires the specific flow cell priming reagents included in the kit and is not compabitible with the Flow Cell Priming Kit (EXP-FLP004).

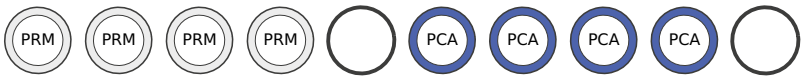



FCF : Flow Cell Flush
FCT: Flow Cell Tether

### PCR Expansion

This expansion pack (EXP-PCA001) contains PCR Primers (PRM) and PCR Adapters (PCA) and is recommended for users needing reagents for a PCR-based sequencing experiment with the Ligation Sequencing Kit V14 (SQK-LSK114). This kit is recommended when users have a low starting input that requires PCR for preparing enough DNA for sequencing. For more information, please see the relevant protocol:

- Ligation Sequencing Kit V14 - low input by PCR (SQK-LSK114)

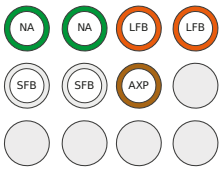



PRM : PCR primer PCA : PCR adapter

### Native Barcoding Auxiliary Kit V14

This expansion (EXP-NBA114) contains additional library preparation reagents for use with unused barcodes in the Native Barcoding Kits 24 and 96 V14 (SQK-NBD114.24 and SQK-NBD114.96). There are sufficient reagents for another 12 reactions supplied with this kit.

**Note:** This kit must by used in conjunction with the Sequencing Auxiliary Vials V14 expansion (EXP-AUX003) to provide the flow cell priming reagents.




NA : NativeAdapter
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads

**Note:** This Product Contains AMPure XP Reagent Manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

### Rapid Adapter Auxiliary V14

This expansion (EXP-RAA114) provides additional Rapid Adapter (RA) and Adapter Buffer (ADB) to maximise any unused barcodes in the Rapid Barcoding Kit 24 and 96 V14 (SQK-RAD114.24 and SQK-RAD114.96).

**Note:** This kit must by used in conjunction with the Sequencing Auxiliary Vials V14 expansion (EXP-AUX003) to provide the flow cell priming reagents.


RA : Rapid Adapter
ADB : Adapter Buffer

# Sample input and recommendations

### The importance of good quality library

To make the most of a flow cell, load the amount of recommended good quality library recommended in the appropriate protocol for your sequencing kit. A good quality library is made up of sample molecules that have sequencing adapters ligated at both ends. To achieve high sequencing output, the flow cell membrane must not be damaged from bubbles and pore blocking should be at a minimum. There must also be enough library input to ensure the pores are always sequencing with minimal idle time between sequencing strands. In this section, we discuss how to achieve this.

How much to load on a flow cell is dependent on library preparation protocols, starting input and fragment length. For example, for the standard library preparation using the Ligation Sequencing Kit, we recommend a starting input of ~100-200 fmols for short fragments of <10 kb, or 1 µg of longer fragments of >10 kb. Starting input will also differ depending on user needs and starting input quality. For example, to generate ultra-long reads, ultra-long fragments must be present in the starting input sample.

It is important to load the recommended amount of library because this will affect data output. For example, not adding enough library onto the flow cell will impact pore occupancy and the optimal number of pores will not be sequencing DNA/RNA strands, reducing sequencing output.

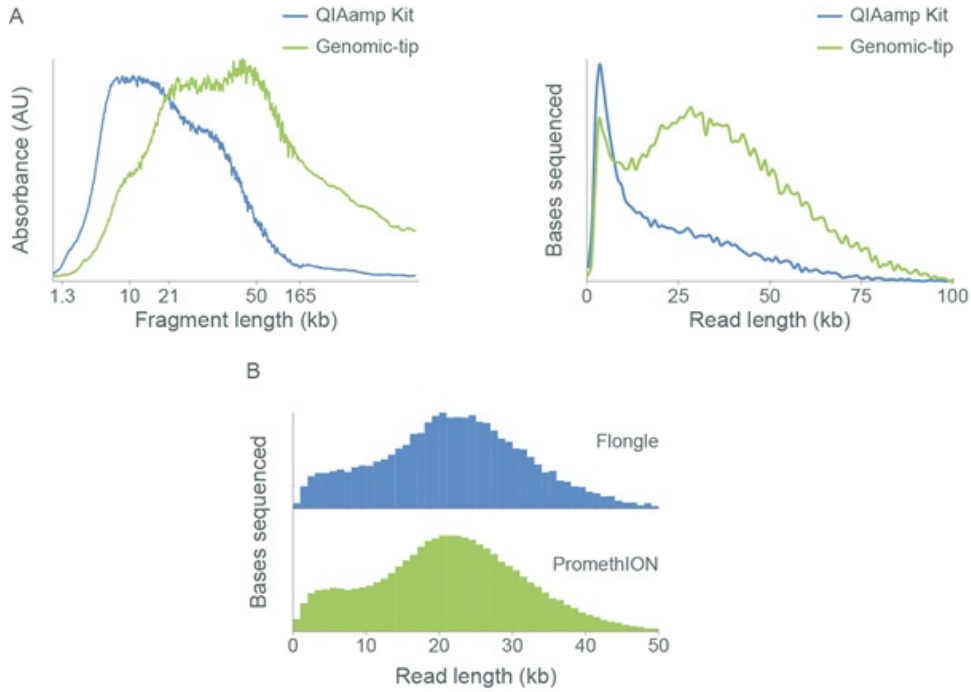
Please refer to the Making the most of your flow cellfor further information on how to interpret and use real-time data to improve sequencing output.

**Quantifying DNA sample mass input for library preparation**

To quantify the mass of DNA samples, we recommend the Qubit dsDNA BR Assay Kit. Both mass and length measurements are needed for a molar quantification of short-fragment samples.

To assess length, we recommend gel-based analysis or the Agilent 2100 Bioanalyzer. While molar quantification isn't required for samples that comprise of long fragments, we still recommend measuring the length of your sample. This is to ensure your sample contains long fragments if you are interested in generating long reads. For this, we recommend using the pulsed-field gel electrophoresis or the Agilent Femto Pulse (the Agilent 2100 Bioanalyzer is not suitable for measuring the length of molecules >10 kb).

However, these are expensive pieces of equipment and are not accessible to every laboratory. Instead, it is possible to effectively QC the fragment length by running a small fraction of the library on a Flongle Flow Cell and evaluating the performance before deciding whether or not to commit to running the remainder of the library on a more expensive flow cell.




Assessing fragment length of DNA samples using the Agilent Femto Pulse and the Oxford Nanopore Technologies Flongle Flow Cell. **Panel A:** Genomic DNA was extracted from rabbit blood (collected in K2-EDTA) using either the QIAamp DNA Blood Midi Kit or a QIAGEN Genomic-tip. The extracted gDNA was analysed using the Agilent Femto Pulse (left). Libraries were prepared using the

Ligation Sequencing Kit and sequenced on a MinION Flow Cell, and the read length distributions are shown (right). The Femto Pulse trace shows that the QIAamp extracted sample consists of mainly short fragments (<10 kb) and this is reflected in the sequencing. Whereas the Genomic-tip extracted sample is shown to contain a higher proportion of longer fragments and therefore, produces a higher proportion of longer reads. **Panel B:** Genomic DNA was extracted from GM12878 cells using the QIAGEN Gentra Puregene Cell Kit and a sequencing library was prepared using the Ligation Sequencing Kit. A portion of the sequencing library was run on a Flongle Flow Cell and the remainder on PromethION Flow Cell. The observed read length distributions are reproducible between the platforms.

### Purity of DNA samples

It is also recommended to measure the purity of extracted DNA. DNA extraction methods often involve chemicals that can inhibit enzymatic activity, including those enzymes in library preparation.

To measure the presence of certain contaminants, the Nanodrop 2000 Spectrophotometer can be used. Pure DNA gives an A260/A280 ratio of ~1.8. A ratio lower than this can be indicative of a contaminant such as phenol. A A260/A230 ratio lower than 2.0–2.2 can also be indicative of phenol contamination but can also indicate carryover of guanidine or carbohydrate. However, while Nanodrop readings are a good place to start, it is not the only authority on purity and subsequent performance. Details of the effects of certain contaminants on the efficiency of the Ligation Sequencing Kit can be found in the DNA contaminants and RNA contaminants documents.

If you have low starting amounts of DNA (<100 ng), PCR is recommended to generate more template. There are library preparation kits and protocols available specifically for PCR-based preparation:

1. Ligation sequencing V14 - low input by PCR (SQK-LSK114 with EXP-PCA001)
2. Rapid sequencing DNA - PCR Barcoding Kit 24 V14 (SQK-RPB114.24)

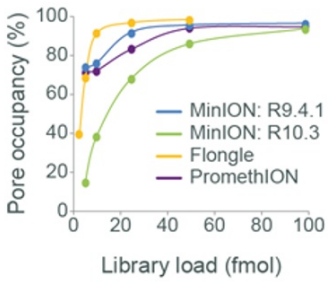
The choice of kit is dependent on the priorities of the user.

If inputs below the protocol recommendations are used, the sequencing output may be negatively affected due to the low pore occupancy.

To maximise sequencing output, it is important that the pores are kept filled with DNA to minimise the time that they are “idle” in-between strands. This metric can be monitored by viewing the pore occupancy graph on the MinKNOW UI. For more information, see the MinKNOW protocol.

### Pore occupancy and library quality

A good quality library is also required and refers to the extent of how well the DNA has been adapted with sequencing adapters. Molecules without sequencing adapters cannot be sequenced and diminish library quality. Libraries that consist of molecules with adapters and tethers on both ends are more potent than those with only one end modified. **Note:** Equivalent library loads may carry slightly different potency (in terms of pore occupancy) between Flongle, MinION and PromethION Flow Cells which is reflective of the differences in flow cell volume (37.5 µl for Flonge, 75 µl from MinION and 200 µl for PromethION).

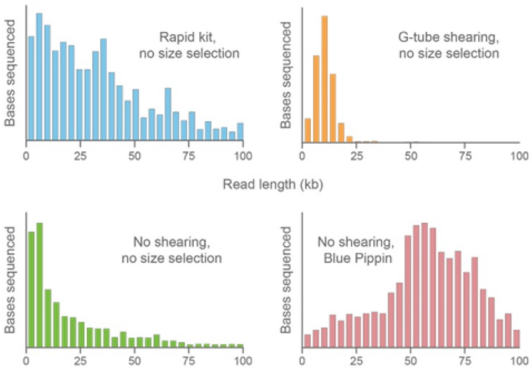



The relationship between the amount of library loaded onto an Oxford Nanopore Technologies flow cell and the resulting pore occupancy that was obtained. We found that pore occupancy is maximised when ~5–50 fmol of good quality library is loaded onto R9.4.1 flow cells and ~25–75 fmol is loaded onto R10.3 flow cells.

The less library you load, 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, then output could be compromised. It is important to note that we do not observe a linear relationship between input onto the flow cell and sequencing yield, but loading less could give reduced output. Conversely, loading more library does not guarantee good performance.

**Determinants of read length**

We have found that long reads can be achieved with both ligation- and rapid-based library preparation methods, as read length reflects input fragment length and is not platform-limited. For example, fragmenting input DNA results in a narrower distribution of read lengths. In some kits, we recommend fragmenting input DNA to ease handling and quantification or to make multiple samples a uniform size. We have also validated several methods of enrichment for long reads, including using SPRI beads and the Short Fragment Eliminator Kit (EXP-SFE001) that are located in the linked Size Selection folder.




A DNA library was prepared using the Rapid Sequencing Kit. **Top left:** Read length from the sequencing kit. **Top right:** Read length with only g-TUBE shearing. **Bottom left:** No fragmentation or size selection. **Bottom right:** Read length using BluePippin for size selection.

**Size selection**

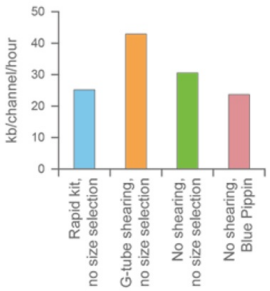
Size selection is an optional step to enrich longer fragments in library input. SPRI-bead purification is used in many Oxford Nanopore Technologies library preparation protocols as this technique is effective at removing fragments of 1.5 kb and can slightly shift read length towards the longer end. The Short Fragment Eliminator Kit (EXP-SFE001) may be used to remove fragments of <25 kb and almost completely removing fragments under 10 kb. However, both methods effectively remove shorter fragments and boost read N50 without compromising data output. To view our protocols for size selection, please refer to the linked Size selection page.

**Fragment length**

The pores in a flow cell can sequence all sizes of fragments and read lengths are only limited by template molecule starting length. Nanopores capture and process both long and short DNA and RNA strands. However, the length of fragments can affect output as the concentration of free DNA ends will be different. For example, short fragments have a higher concentration of DNA ends that will increase pore occupancy and output. This is because there are more threadable ends for the pores to capture.

Libraries composed of long fragments typically have lower data outputs than short fragments as there are fewer molecules available for sequencing and they take longer to be captured by the pore. This also results in more pores not sequencing due to the lack of pore occupancy. If size selection is performed as part of the library preparation, this can lower output further due to fragments outside a particular range being excluded.

Fragmentation in a Covaris g-TUBE has been found to yield the highest output with no size selection. Therefore, we recommend using this fragmentation method in many protocols to optimise for highest output yield.




G-tube fragmentation yields the highest output.

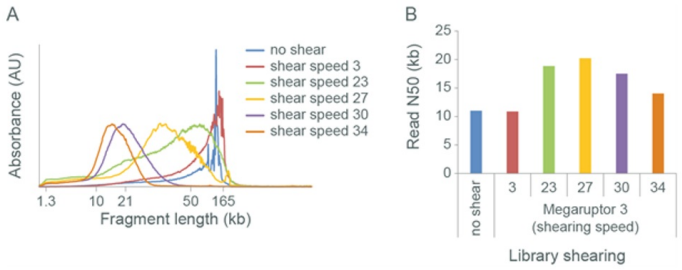
**Flow cell performance**

Flow cell output is governed by various factors, including DNA/RNA library input, loading amounts, and pore blocking. Below, we review how controlling the input material size distribution by fragmentation impacts these factors and what Oxford Nanopore Technologies' recommendations are to generate the best data, based on experimental aims.

**Increasing read N50**

It has been observed that some shearing of gDNA samples can lead to an increase in observed read length: this seems counterintuitive – how can breaking up the DNA fragments give longer reads? It has been suggested that certain fragments may be so long that they become "lost" during the library preparation and therefore are not observed, leaving only the short fragments (for example, the very longest fragments may not efficiently bound to, or elute from the AMPure beads used after end-prep or ligation). Light shearing, for example using the Megaruptor, can break up the very longest molecules into chunks that the library preparation can more readily process, leading to increased read N50s.

This approach is suggested for users where samples appear to be very high molecular weight in gel or Femto Pulse analysis but the observed read length N50 is <15 kb. Other users within the Nanopore Community have also attempted other shearing methods to increase read lengths. However, the more aggressive the fragmentation, the higher the risk of over-fragmenting, leading to a reduction in observed read lengths.

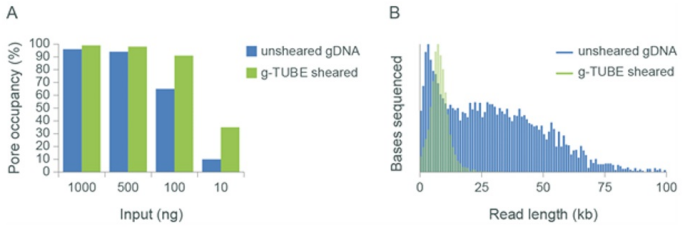



The effect of shearing very high molecular weight gDNA with Megaruptor 3. Human gDNA was extracted from cell culture, with the aim to recover the longest possible fragments. The resulting gDNA was sheared with Megaruptor 3 using a selection of shearing speeds. **Panel A:** The sheared DNA was analysed by Femto Pulse. The sequencing libraries were prepared using the Ligation Sequencing Kit and run on a MinION Flow Cell. **Panel B:** The read N50 values were recorded. The read length distribution of the input (no shear) shows that most of the DNA is above 100 kb, with a spike at 165 kb (area where fragments become compressed). However, this does not correspond to a high read N50 in sequencing. The lowest shearing speed had little-to-no effect on the fragment length distribution or the observed read N50, suggesting unsuccessful fragmentation. However, increasing the shearing speed to 20–30 did show successful fragmentation and led to an increase in observed read N50. Increasing the shearing speed still further led to over-fragmentation and a drop in observed read N50.

**Input amount and pore occupancy**

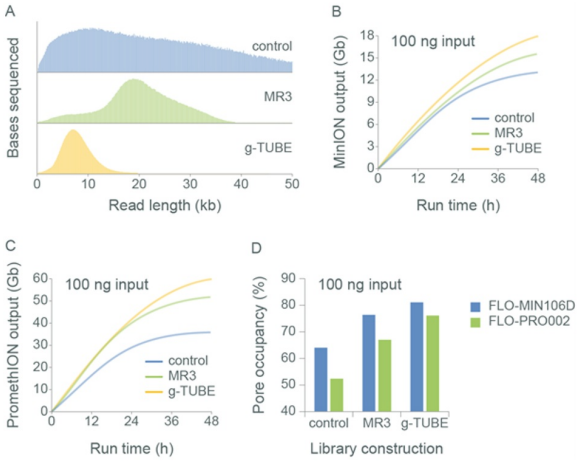
Loading too much or too little library can compromise flow cell performance. For example, 5–50 fmol is optimal for our previously available R9.4.1 flow cells. If sufficient starting material is unavailable, users can start with lower inputs, however we have found that data output can drop at lower inputs as there are insufficient molecules available to maximise pore occupancy. Fragmenting the sample (for example using a Covaris g-TUBE or Megaruptor®) can be used to increase the number of molecules/ends to thread into the nanopores. This increases pore occupancy and recovers the output: an input of 100 ng unsheared Lambda DNA results in a flow cell load of ~1 fmol, which can be increased to ~6 fmol by shearing with a g-TUBE.

It is worth noting that fragmenting DNA to boost the output can mean it may not be possible to achieve ultra-long reads. If you have <100 ng of DNA, we advise performing PCR to increase the amount of DNA available for sequencing.




The relationship between input and output (R9.4.1 flow cells) for sheared and unsheared libraries using our previous sequencing chemistry. **Panel A:** As the input of unsheared gDNA into the library preparation drops below ~500 ng, the pore occupancy decreases, leading to a decrease in flow cell output (Gbases). Shearing the sample using a Covaris g-TUBE increases the molar concentration of the sample leading to more efficient use of the pores and an increase in flow cell output. **Panel B:** Shearing samples with a g-TUBE impacts the read length distribution.

We investigated the performance that can be obtained from human gDNA when starting with 100 ng, with and without shearing, using our previously available R9.4.1 flow cells using MinION and PromethION. It was observed that even without shearing, ~10 Gbases and ~30 Gbases could be obtained from as little as 100 ng of HMW gDNA. Pore occupancy and flow cell output was increased when the sample was sheared with both Megaruptor® 3 and Covaris g-TUBE.




Output on MinION and PromethION Flow Cells with 100 ng of HWM human gDNA. Human gDNA was extracted from GM24385 cells using the QIAGEN Gentra Puregene Cell Kit. The HMW gDNA was sheared using either Megaruptor® 3 (speed setting 30) or Covaris g-TUBE. Libraries were prepared for sequencing with the Ligation Sequencing Kit using 100 ng of sheared and unsheared template DNA. The libraries were run on R9.4.1 flow cells using MinION and PromethION. **Panel A:** Shearing the input gDNA decreases the read lengths that are observed in the subsequent sequencing. **Panel B and C:** The flow cell output (Gbases) obtained from 100 ng of input is increased by shearing on both MinION and PromethION, respectively. **Panel D:** The increased shearing of the sample increases the pore occupancy efficiency, leading to the higher outputs from the sheared samples. At low inputs (100 ng of gDNA) the pore occupancy is observed to be higher on MinION compared to PromethION.

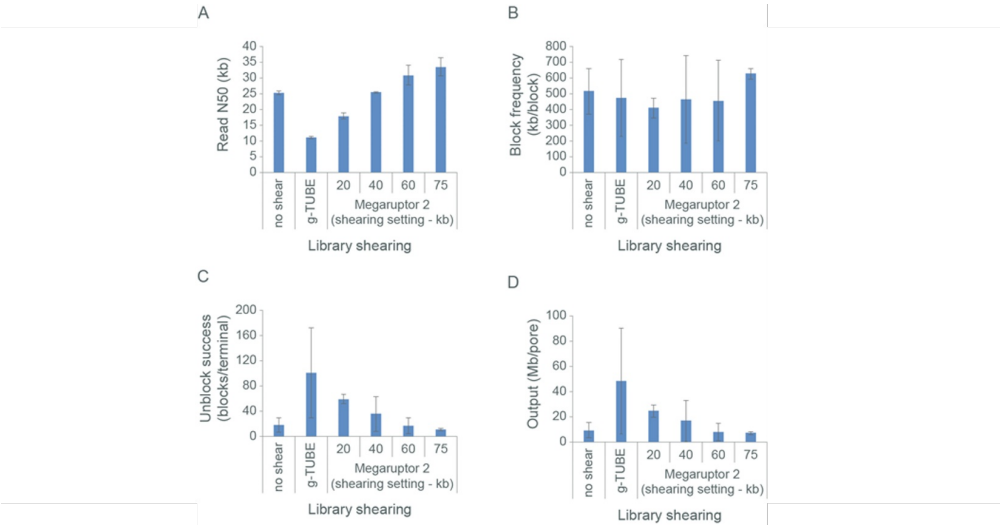
**Blocking**

Pore blocking is another factor that can affect flow cell output. During a sequencing run, pores can become “blocked”, preventing the pore from accepting a new strand for sequencing or continuing to sequence the occupying strand. Such blocks are detected by the MinKNOW software, which changes the channel state from “single pore” to “unavailable”. For the duration of a blockage, the pore acquires no sequencing data. MinKNOW attempts to drive out whatever has blocked the pore by reversing the voltage. The unblocking scheme is progressive, increasing the duration of the voltage reversal until the blockage is cleared. Most of the time (~98%), attempts to unblock a pore are successful and it reverts to the single pore state, where it is available to accept new strands and continue sequencing. However, in a minority of cases, the progressive unblocking scheme will not be able to recover a blocked pore. If a pore becomes terminally blocked and cannot be recovered, a new pore is swapped in from a different well in the channel, if available. Typically, a blockade occurs every 250–500 kb and is successfully removed ~98% of the time (in other words, around 1 in 50 attempts will be unsuccessful). This gives an average output of ~10–20 Mbases per pore, which for a flow cell containing ~1500 pores could lead to a total output of ~15–30 Gbases (note, other factors may limit the actual output obtained). If there is an increase in the rate of blocking, then pores spend less time sequencing and more unblocks are triggered. If there are more unblocks, or if the success of unblocking decreases, then the rate at which pores are lost increases and total flow cell output is reduced.

To determine if fragment length played a role in the rate of blocking, we took DNA extracted from human cells grown in culture (GM12878) and sheared it with Megaruptor 2. We were not able to establish a relationship between read length and blocking rate, although we observed a decrease in the success rate of the unblock for the longer libraries, indicating that our unblocking scheme is less capable of removing blocks from longer fragments. Given this observation, if users are obtaining a low output, then some shearing of the sample could be performed to see if unblocking success can be improved to help boost output.

Recommendations for libraries with high levels of blocking:

- Fragment the starting DNA to increase pore occupancy
- Flow cell washing may be used to unblock pores and revert ‘unavailable’ state to ‘single pore’ state. The nuclease in the Flow Cell Wash Kit is able to digest all of the sample remaining on the flow cell and contaminants blocking pores to increase output for more library to be loaded and sequenced.



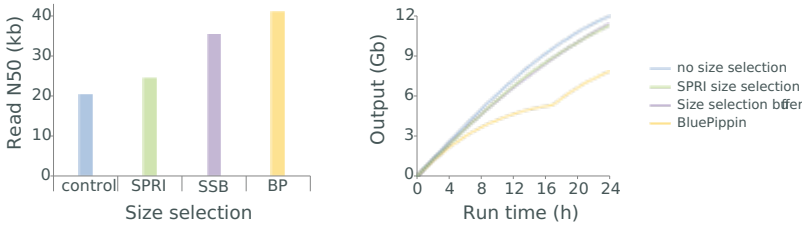

The effect of read length on blocking, unblocking and flow cell output. Extracted gDNA was sheared with Megaruptor 2 using different shearing settings, or with a Covaris g-TUBE. Libraries were then prepared using the Ligation Sequencing Kit and sequenced on the MinION. **Panel A:** The read N50 values for the differently sheared libraries. Note, some Megaruptor shearing produces a slightly elevated read N50. **Panel B:** The block frequency (kb/block) for the differently sheared libraries suggests that there is little relationship between the frequency at which blockades occur and the length of the fragments that are being sequenced, at least for this sample. **Panel C:** As the fragment length of the library increased, a decrease in rate of the success of the unblock (number of blocks before a terminal block was encountered) was observed. **Panel D:** As a result of the decrease in unblock success with

increased read lengths, it follows that the output from any pore may also decrease with increased read length.

**Optional size selection**

Users of Oxford Nanopore Technologies platforms have observed reads of >1 Mbases and the longest read observed approaches 2.3 Mbases. To obtain long reads, long fragments must be present in the extracted DNA sample. All extraction techniques that we recommend yield some short fragments (<10 kb) and even if they do not appear prevalent during quantification, it is likely that you will observe some short reads. Size selection can be used to enrich for long fragments or deplete short fragments. Our size selection protocols are available on the size selection page.

The SPRI bead method enriches for molecules above ~1.5 – 2 kb and the size selection buffer (SSB) promotes semi-selective precipitation and enrichment of molecules above ~10 kb. The BluePippin has a tuneable cut-off limit up to 40 kb. To demonstrate the performance, gDNA was size selected using each method; the BluePippin instrument settings were set to enrich for molecules >40 kb. Using the Ligation Sequencing Kit, 1 µg of each size-selected DNA was prepared for sequencing and the libraries were run on the MinION. A library where no size selection had been performed was also sequenced as control. The data below shows the read length distributions observed and the output generated for each of the libraries. Very little difference in output was observed over the course of the 24 hour sequencing run for SPRI and SSB size-selected libraries, compared with the control. However, an accumulation of pores in the unavailable state were observed throughout the course of the run for the BluePippin size selected library, so a flow cell wash was performed after ~16 hours and more library loaded before recommencing the run.




Size selection of gDNA samples. Genomic DNA was size selected and then prepared for sequencing using the Ligation Sequencing Kit. The read N50 values of the control, SPRI bead size-selected, our Short Fragment Eliminator (EXP-SFE001) size-selected, and BluePippin size-selected libraries were ~20 kb, ~25 kb, ~35kb and ~41 kb, respectively. Very little difference in output was observed over the course of the 24 hour sequencing run for SPRI and EXP-SFE001 size-selected libraries, compared with the control. However, an accumulation of pores in the unavailable state was observed for the BluePippin size selected library, so a flow cell wash were performed after ~16 hours and more library loaded before continuing the run.

# Making the most of your flow cell

**Overview of flow cells**

The flow cell is the platform an DNA/RNA library is loaded onto and sequenced in a device. Sequencing devices can only use compatible flow cells but they all contain the proprietary sensor array, Application-Specific Integrated Circuit (ASIC) and nanopores for

sequencing DNA/RNA libraries. The flow cell contains sufficient buffer to run for up to or more than 72 hours under optimal conditions depending on the flow cell. A user can choose to run continuously or run, stop, wash and load a new library until the buffer and nanopores are exhausted. This enables a single flow cell to be used multiple times before the nanopores are exhausted. An individual flow cell may also be used for different experiments.

| Flow cell | Flongle | MinION/GridION | PromethION |
|---|---|---|---|
| Device | MinION Mk1B, Mk1C and GridION | MinION Mk1B, Mk1C and GridION | PromethION |
| Storage and stability (unopened) | 2-8°C: 4 weeks | Room temp: 1 month<br>2-8°C: 12 weeks | Room temp: 1 month<br>2-8°C: long term |
| Pore count warranty | ≥ 50 | ≥ 800 | ≥ 5000 |

Nanopore technology allows for real-time assessment of sequencing experiments with the visualisation of run statistics on the MinKNOW UI. This makes it easier for the user to adjust sequencing parameters during run set up and to make the most of a flow cell and experiment.

For libraries of high purity and quality, MinKNOW will be able to generate high data output. We recommend monitoring the pore activity plot to ensure pore occupancy remains high, as well as the pore scan plot to check whether there is a build up of unavailable channels. The translocation speed plot should also be checked to ensure sequencing speed remains between the median target. This is discussed in more depth further in the document.

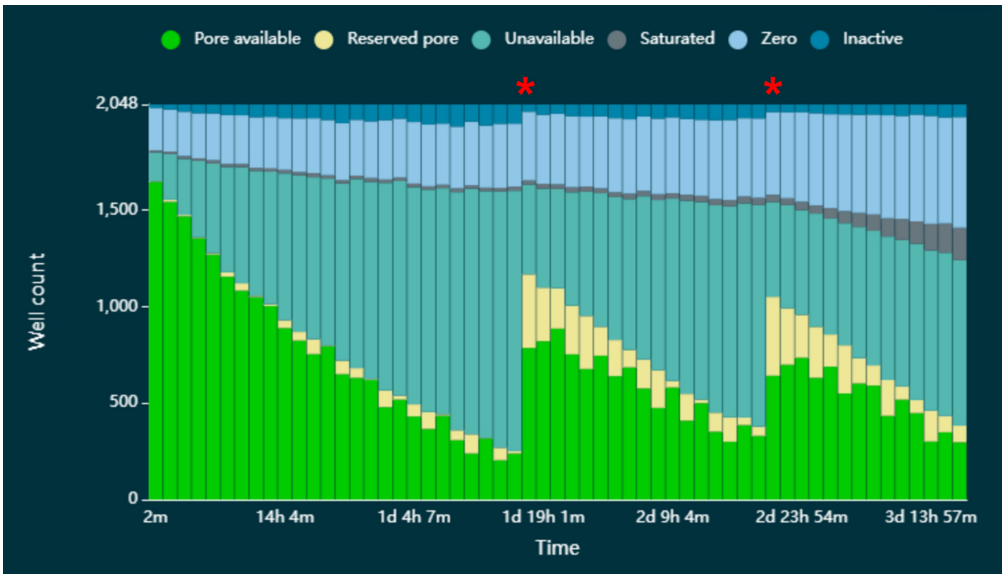
We recommend live basecalling to allow the user to assess in real-time how the experiment is being conducted on the flow cell. However, fast basecalling also allows real-time assessment on computers that struggle to keep up with basecalling and the raw data can be saved and basecalled at a later date.

For further information on how to monitor your sequencing experiment, we go into further detail in the MinKNOW protocol.

**Flow cell wash**

The Flow Cell Wash Kit (EXP-WSH004) contains a nuclease, DNase I, which is used to digest any remaining library on a flow cell for immediate reuse. The wash step is recommended when there is an accumulation of pores in the ‘recovering’/’unavailable’ state. In the figure below, we have demonstrated pores can be reverted to the 'active'/'single pore' state by pausing sequencing and washing the flow cell with DNase I in the Flow Cell Wash Kit (EXP-WSH004). Below, in figure a), the astrisks indicate where sequencing has been paused and the flow cell washed. **Note:** If the sequencing run is paused in MinKNOW for the flow cell wash, you will only see the restoration of sequencing pores after a new pore scan has been performed.

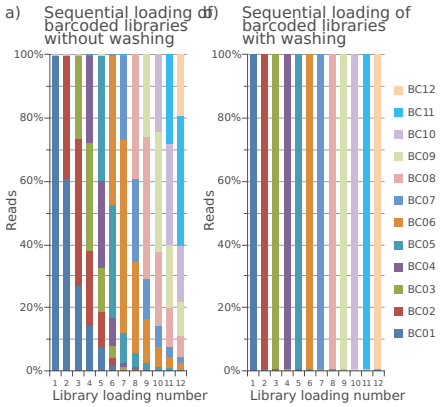
The wash step is only recommended where sequencing channels are lost to the 'recovering/unavailable' state. Where pores are in the ‘saturated’ state, the wash step will not be able to revert to the ‘active’ pore state.




Pore states observed on a flow cell before and after wash steps are performed. A flow cell has been loaded with a sequencing library that has resulted in an accumulation of pores in the “unavailable” state, leading to a decrease in the rate of data acquisition. The red asterisks indicate when a wash step has been performed. A significant number of the pores that had been lost to the “unavailable” state have reverted to the “Pore available” state and are available for sequencing once again.

The flow cell wash kit allows multiple uses of a flow cell with different samples and the recovery of unavailable channels during an experiment. Samples can be multiplexed to reduce costs and sequence many samples simultaneously on a single flow cell, for example using one of the Native Barcoding kits or the PCR Barcoding Expansions.

**Note:** The wash kit should remove 99% of the library. However, some residual DNA may remain on the flow cell. Therefore, we recommend users barcode their libraries when used in conjunction with the Flow Cell Wash Kit, to ensure reads from different libraries can be separated from each other. RNA is also efficiently flushed out of the flow cell but RNA is not digested. Successful deconvolution of DNA reads has been demonstrated in Oxford Nanopore’s internal development.



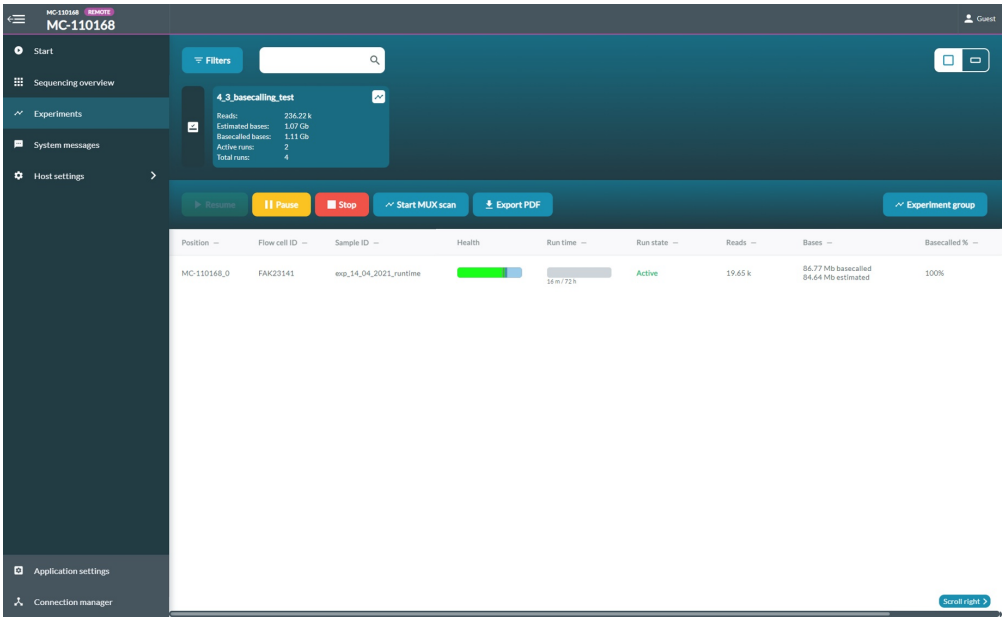

**a)** Sequential loading of barcoded libraries without washing. **b)** Sequential loading of barcoded libraries with washing. Only residual library remained on the flow cell with washing and barcoding samples that are to be sequentially run can help avoid cross-contaimination.

The Flow Cell Wash Kit (EXP-WSH004) presents two options:

1. Wash and store the flow cell

- Allows for flow cells to be used for multiple experiments but not to be reused immediately after one experiment is complete

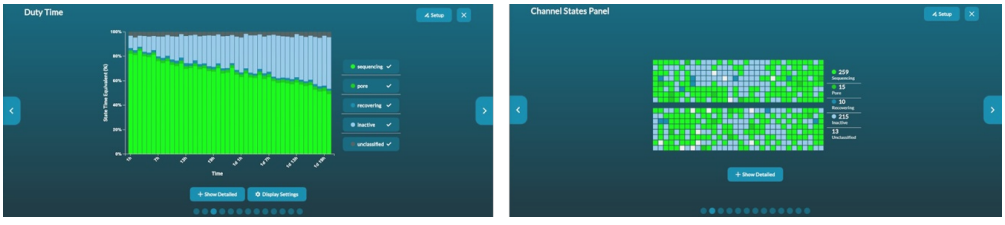
1. Wash and reuse the flow cell straight away
    - We recommend using the pause option in the MinKNOW UI to pause the experiment and to restart as soon as the wash is complete and library loaded.
    - This is recommended when there is a build-up of ‘unavailable’ pores.
    - **Note:** When the **Pause** option is used, all data produced pre- and post-wash is stored in the same data file. Therefore, if changing the sample, be sure to use the **Stop** function rather than pausing to ensure data is stored in a new file when sequencing is restarted.




Navigate to the experiments page and select a flow cell to either **Pause** or **Stop** a sequencing run to wash a flow cell.

### Pore occupancy

The pore occupancy plot is a summary of time spent in each of the channel states during the run. The channel panel illustrates the real-time representation of each of the channels. Pore occupancy is driven by the number of sequenceable ends (sample molecules with sequencing adapters) loaded onto the flow cell. We recommend monitoring ‘inactive’ and ‘recovering’ channels to keep track of saturated and blocked pores which may reduce output of a run if not dealt with appropriately.

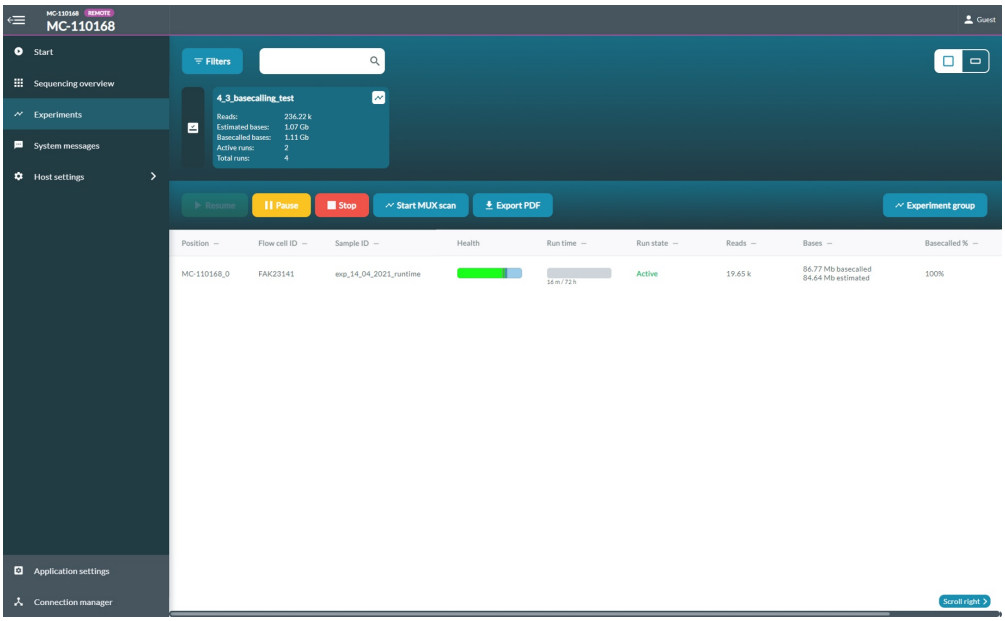



**Left:** Pore occupancy plot illustrates all the channel states during the entire sequencing run.**Right:** Channel states panel illustrates the current channel states.

### Pore scan

The pore scan analyses pore status and selects the best performing pores for sequencing. We recommend triggering a pore scan after pausing or stopping an experiment, whether it is for refueling, reloading or washing a flow cell. This is to ensure all the nanopore channels are assessed most recently with the best selection of pores for sequencing.

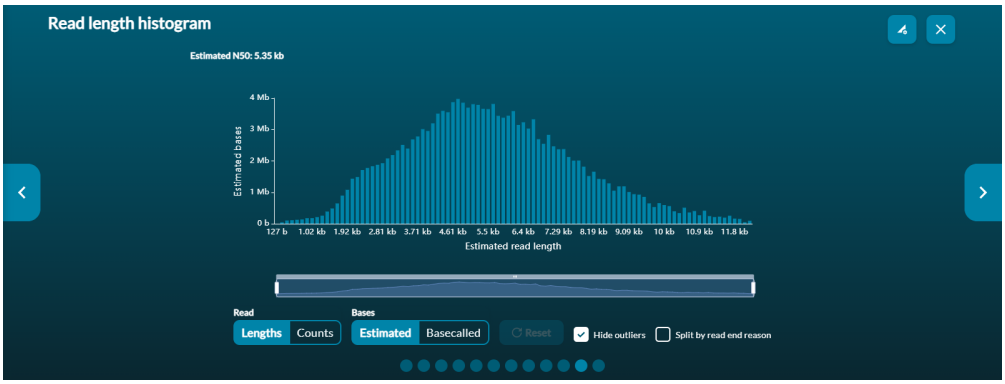
The pore scan results can be used to assess sequencing efficiency with the duty time plot. If there is a high level of ‘unavailable’ pores, we recommend washing the flow cell as there is blocking occuring which will reduce sequencing and data output. The rate of blocking is mainly due to the purity of the sample; low quality DNA/RNA or contamination from either extraction, sample prep or secondary structure can be some of the causes of an increase of pores in the unavailable state.




To start a pore scan, navigate to the experiments page and click **Start pore scan** and select the flow cell to scan in the dialogue box that has opened. This will start a pore scan on the selected flow cell(s) and select a new set of channels for sequencing.

### Read length histogram

This visualises the read lengths of the sequencing libraries in real-time and changes throughout the experiment. This can be used to assess if the sequencing library contains an expected sample if the read length distribution roughly matches the input library fragment sizes. If read length does not reflect the input library fragment sizes, the experiment can be stopped and the flow cell washed and stored whilst the library preparation can be optimised, preventing the waste of a flow cell. **Note:** This graph will take slightly longer to populate for longer reads.




### Disk space plot

This allows the user to monitor the amount of data to be written on the device memory during sequencing. If all the disk space will be used up, the user can pause the experiment whilst making more disk space and preserve the flow cell.

# Ligation-based sequencing kits

### Ligation-based sequencing kits

These are the previously available Ligation Sequencing Kits available using our Kit 9, 10 and 12 chemistry:

- Ligation Sequencing Kit (SQK-LSK112)
- Ligation Sequencing Kit XL (SQK-LSK112-XL)
- Ligation Sequencing Kit (SQK-LSK110)
- Ligation Sequencing Kit (SQK-LSK109)
- Ligation Sequencing Kit XL (SQK-LSK109-XL)
- Cas9 Sequencing Kit (SQK-CS9109)
- Native Barcoding Kit 1-12 (EXP-NBD104)
- Native Barcoding Kit 13-24 (EXP-NBD114)
- Native Barcoding Kit 96 (EXP-NBD196)
- Multiplex Ligation Sequencing Kit XL (SQK-MLK111.96-XL)

### Ligation Sequencing Kits

### SQK-LSK112

The Ligation Sequencing Kit (SQK-LSK112) was our previous upgrade from the Ligation Sequencing Kit (SQK-LSK110) and is optimised for high accuracy. The upgrade included a new sequencing adapter (Adapter Mix H, AMX H) which improved accuracies to over 99% (Q20+), required lower flow cell loading amounts and contained the fuel fix technology to run long experiments without the need for fuel addition during a run.

**Note:** This kit (SQK-LSK112) is a legacy product and will soon be discontinued. We recommend all customers to upgrade to the latest

chemistry for their relevant kit which is available on the Store. If customers require further support for any ongoing critical experiments using a Legacy product, please contact Technical Support via email: support@nanoporetech.com.

**SQK-LSK110**

The Ligation Sequencing Kit (SQK-LSK110) is an upgrade from the Ligation Sequencing Kit (SQK-LSK109) and is optimised for high output. The upgrade contains new fuel fix adapter (Adapter Mix F, or AMX-F) to use significantly less fuel during a sequencing run to improve sequencing output. We have also upgraded the loading beads to Loading Beads II (LBII) and Loading Solution (LS) for users who do not use loading beads for their experiments.

**SQK-LSK109**

This is a ligation-based sequencing kit to offer a flexible method of preparing sequencing libraries from dsDNA (e.g. gDNA, cDNA or amplicons) and is optimised to generate maximum output. Since release, this kit has undergone multiple upgrades and we recommend customers to upgrade to our latest chemistry to achieve the best results.

These are the previously available expansion packs available to work alongside the Ligation Sequencing Kits to enable users to barcode their samples for more efficient use of the kits.

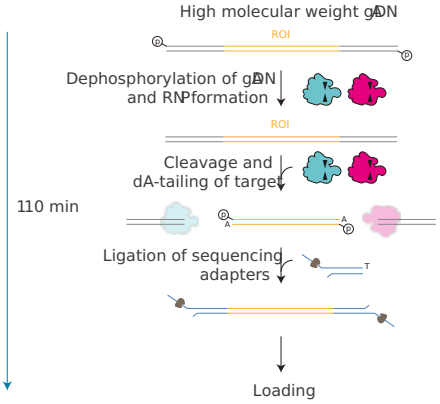
Barcoding expansion packs available for the Ligation Sequencing Kits:

- Compatible with Ligation Sequencing Kit (SQK-LSK109):
  - Native Barcoding Expansion 1-12 (EXP-NBD104)
  - Native Barcoding Expansion 13-24 (EXP-NBD114)
  - Native Barcoding Expansion 96 (EXP-NBD196)

**Cas9 Sequencing Kit (SQK-CS0109)**

The Cas9 Sequencing Kit (SQK-CS9109) uses ligation-based chemistry and is a fast and flexible way to target specific regions of interest within a genome without amplification, increasing the depth of coverage. This is particularly useful for areas not amenable to PCR, “dark” areas of the genome, or for users wishing to target specific regions but maintain DNA modifications. The Targeted, amplification-free DNA sequencing using CRISPR/Cas document explains how to design and order crRNA probes and shares best practices for performing targeted sequencing.

Using the recommended 5 µg high molecular weight gDNA, the DNA is dephosphorylated before adding Cas9 ribonucleoprotein particles (RNPs) with bound crRNA and tracrRNA to the gDNA for binding and cleavage of the Region of Interest (ROI). The cleavage by Cas9 reveals blunt ends with 5’ phosphates which are dA-tailed to prepare the blunt ends for barcode ligation. Sequencing adapters are then ligated to the Cas9-cut sides. Finally, a clean-up step is performed to remove excess adapters and the library is sequenced.



# Rapid-based sequencing kits

### Rapid-based kits

Oxford Nanopore has previously offered the following rapid-based sequencing kits:

- Rapid Sequencing Kit (SQK-RAD004)
- Rapid Barcoding Kit (SQK-RBK004)
- Ultra-Long DNA Sequencing Kit (SQK-ULK001)
- Field Sequencing Kit (SQK-LRK001)
- PCR Sequencing Kit (SQK-PSK004)
- PCR Barcoding Kit (SQK-PBK004):
- Rapid PCR Barcoding Kit (SQK-RPB004)
- 16S Barcoding Kit (SQK-RAB204)
- 16S Barcoding Kit 1-24 (SQK-16S024)

### Rapid Sequencing Kit

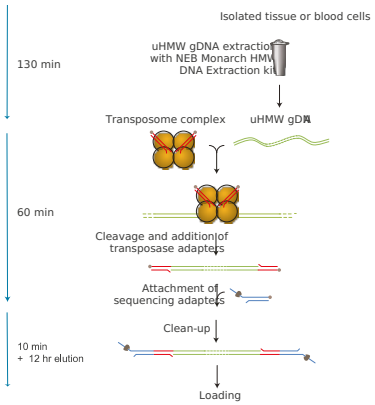
#### SQK-RAD004

The Rapid Sequencing Kit (SQK-RAD004) is optimised for speed and simplicity, using limited laboratory equipment. The transposase-based workflow takes approximately 10 minutes to generate sequencing libraries from extracted gDNA in two steps. We recommend an input of 400 ng of HMW gDNA (>30 kb).

### Ultra-Long DNA Sequencing Kit

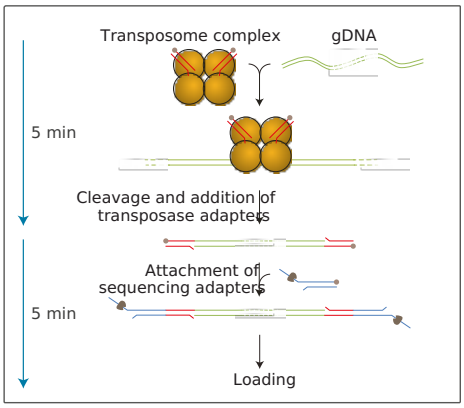
As our transposase-based kits are relatively simple, very long reads can be achieved due to the minimal pipetting required during library preparation. The Ultra-Long DNA Sequencing Kit (SQK-ULK001) was developed using our rapid-based kit.

This kit requires ultra-high molecular weight (uHMW) gDNA to be extracted. In our protocol, we recommend using the NEB Monarch® HMW DNA Extraction Kit for Tissue (T3060) to extract the uHMW gDNA from either frozen cells or whole blood. After gDNA extraction, a diluted fragmentation mix containing transposases are added to the extracted gDNA to fragment and simultaneously tag the fragmented template with transposase adapter sequences. Post-transposition, sequencing adapters are then attached to the transposase adapters in an enzyme-free reaction. After an overnight elution with Elution Buffer, the library is ready for sequencing.




### Field Sequencing Kit

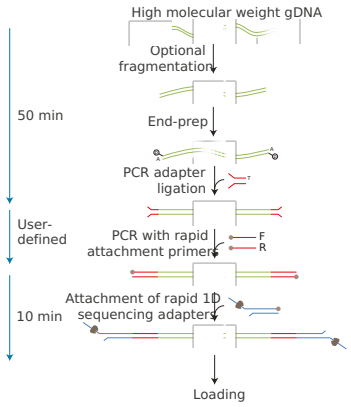
The Field Sequencing Kit (SQK-LRK001) is recommended for those working in the field with limited access to cold storage and laboratory equipment. It is recommended to use ~400 ng high molecular weight gDNA as input for the library preparation. This kit uses a simple two-step protocol, using a transposase to simultaneously cleave the template molecules and attach tags to the cleaved ends. The Rapid Sequencing Adapters are then added to the tagged ends in approximately 10 minutes. This kit is stable at ambient temperatures for extended periods of time and many of the reagents are lyophilised.




### PCR Sequencing Kit

The PCR Sequencing Kit (SQK-PSK004) is designed to prepare sequencing libraries when the available gDNA input is limited (<100 ng). We recommend starting with ~100 ng gDNA but less can be used. The gDNA is fragmented in a Covaris g-TUBE and the sheared ends are repaired and dA-tailed before adapters containing primer binding sites are ligated onto the prepared ends. An amplification step uses primers containing 5’ tags for the ligase-free attachment of Rapid Sequencing Adapters. Prior to PCR, this workflow takes approximately 50 minutes and post-PCR, the attachment of sequencing adapters takes about 10 minutes.

The barcoding version is the PCR Barcoding Kit (SQK-PBK004), enabling the multiplexing of up to 12 samples.




# RNA and cDNA sequencing kits

### RNA and cDNA sequencing kits

These are the previously available RNA and cDNA kits.

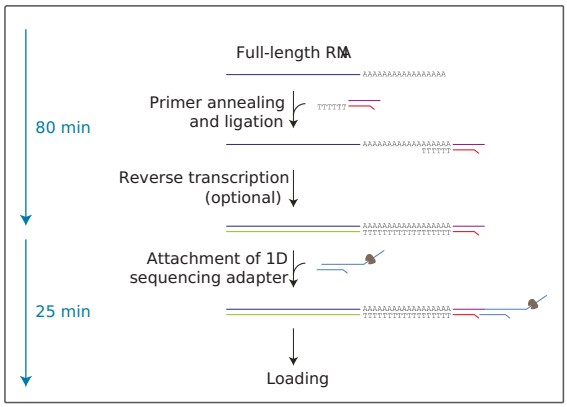
- cDNA-PCR Sequencing Kit (SQK-PCS109)
- cDNA-PCR Barcoding Kit (SQK-PCS111)
- Direct cDNA Sequencing Kit (SQK-DCS109)
- PCR-cDNA Barcoding Kit (SQK-PCB109)
- PCR-cDNA Barcoding Kit (SQK-PCB111.24)
- Direct RNA Sequencing Kit (SQK-RNA002)

### Direct RNA Sequencing Kit

The Direct RNA Sequencing Kit (SQK-RNA002) is used to prepare any RNA with a 3’ poly(A) tail for sequencing. Other possible RNA input includes eukaryotic mRNA, viral RNA with a poly(A) tail, or any RNA prepared with a poly(A)-tailing kit. For RNA without a poly(A) tail, users can follow simple instructions to design their own custom adapter to ligate a specific terminal 3’ sequence like the 3’ of tRNA or 16S rRNA. We recommend an input of ~500 ng of poly(A) RNA template of >200 nucleotides in length, with no upper limit.

**Note:** Only the RNA strand, not the RT strand, is sequenced.

Only the native RNA passes through the nanopore, meaning the read length reflects the length of the RNA molecules in the sample. The first step of the workflow requires the ligation of a reverse transcription adapter which ligates to the 3’ poly(A) tail on the RNA template molecule. Next, the optional reverse transcription step can be carried out to generate cDNA for stabilisation against RNA secondary structure formation. However, only the RNA strand is sequenced. If reverse transcription is bypassed, the workflow is shortened to 30 minutes but there is a reduction in sequencing output which is likely due to an RNA tertiary structure blocking the pores. The sequencing adapters supplied in the kit are attached to the ends of the RNA-cDNA hybrid and the library is ready for sequencing.

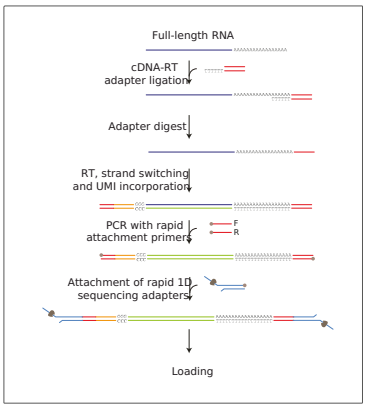



### cDNA-PCR Sequencing Kit

The cDNA-PCR Sequencing Kit (SQK-PCS111) has been to Kit 11 chemistry, which requires starting inputs of 4 ng poly(A) RNA or 200 ng of total RNA. We have included a new cDNA RT adapter (CRTA), Annealing Buffer (AB) and RT primer (RTP) to prime cDNA synthesis from the end of a transcript to reduce overlaps during the reverse transcription step and to allow users to measure poly(A) tail lengths. The Kit 11 upgrade includes a new Rapid Adapter T (RAP T), with two key features which includes higher capture rate and fuel fix technology for longer experiments without the need for fuel addition during the run.

**Note:** Reverse transcriptase inhibits downstream PCR. Therefore, the enzymes must be heat-inactivated and the reverse transcribed sample to be split across four PCR reactions to dilute the inhibitors. This is to allow the amplification of cDNA with maximum efficiency, without losing sensitivity.

The poly(dT) reverse transcription adapter is ligated to the 3’ terminal poly(A) tail of the template molecule. The bottom strand of the adapter is removed and a reverse transcription primer is annealed, anchoring the start of transcription to include the entire 3’ terminal poly(A) tail. Then a strand-switching primer containing a unique sequence (UMI) is added during reverse transcription, allowing strand switching to occur and generates a full-length cDNA strand with universal sequences on both ends. Next, PCR amplification is performed using primers with 5' tags and rapid attachment groups on the ends for adding the Rapid Sequencing Adapter (RAP T).




This kit can be used to select specific transcripts if one or both ends of the target are known:

- **Both target ends are known:** Reverse-transcribe the entire template molecule and use selective primers to anneal to both ends of the cDNA before carrying out PCR with the sequence-specific primers.
- **Only one end of the target end is known:** Reverse-transcribe the template molecule and use selective primers to anneal to the known end of the cDNA with universal primers on the unknown end. Then perform PCR with the sequence-specific primers for the known end of the target molecule and a universal primer for the unknown end.

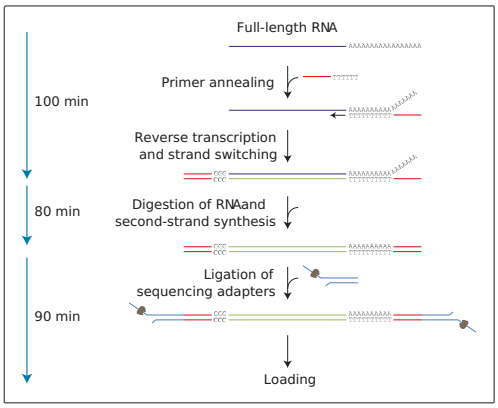
A specific transcript can be selected by altering the reverse transcription primer to replace the poly(dT) sequence with a sequence-specific primer so only the transcript of interest will be reverse-transcribed. This reverse-transcribed and strand-switched product is then amplified with the universal primers in the PCR-cDNA Sequencing Kit.

PCR-cDNA Barcoding Kit (SQK-PCB111.24) is the barcoding version of this kit which can be found in the Barcoding kits section.

### Direct cDNA Sequencing Kit

This kit is used to prepare cDNA for nanopore sequencing without using PCR. Preparing a library using the Direct cDNA Sequencing Kit (SQK-DCS109) takes approximately two hours longer than the cDNA-PCR Sequencing Kit (SQK-PCS109), and requires more third-party reagents. This is a PCR-free protocol and requires a higher input of 100 ng of poly(A)-tailed RNA but prevents PCR bias or polymerase error. However, data output is lower compared to the PCR version (PCR-cDNA Sequencing Kit). We recommend starting with 100 ng poly(A)-tailed RNA, or 70-200 ng of already prepared cDNA.

The first step of the workflow starts with annealing the reverse transcription primer and adding the strand-switching primer before performing reverse transcription to allow strand-switching to occur. An RNase cocktail is used to remove the RNA template strands before performing a second strand synthesis. This generates double-stranded cDNA which is treated similarly to gDNA. The fragment ends are dA-tailed before the dT-tailed sequencing adapter is ligated onto the fragments for sequencing on a flow cell.




# Barcoding kits

### Ligation-based barcoding kits

### Native Barcoding Expansions 1-12, 13-24 and 96

For the Kit 9 Ligation Sequencing Kit (SQK-LSK109), there are three native barcoding expansions to enable barcoding:

- Native Barcoding Expansion 1-12 (EXP-NBD104)
- Native Barcoding Expansion 13-24 (EXP-NBD114)
- Native Barcoding Expansion 96 (EXP-NBD196)

These native barcoding expansions are recommended for users who want to multiplex their samples with a PCR-free method to preserve base modifications. There are up to 96 unique barcodes which can be used with the Ligation Sequencing Kits (SQK-LSK109) to sequence gDNA or cDNA with the Direct cDNA Sequencing Kit (SQK-DCS109). For more information about sequencing cDNA, please see the RNA and cDNA sequencing and kits section.

The barcode expansion packs are optimised to generate maximum output without the need for PCR. For highest data yields, we recommend the following inputs:

Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) for barcoding up to 24 samples:

- 1 µg or 1.5-3 µg pure input DNA for R9.4.1 or R10.3 flow cells.

Native Barcoding Expansion 96 (EXP-NBD196) for barcoding up to 96 samples:

- ~400 ng DNA per barcode for R9.4.1 or R10.3 flow cells.

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**Multiplex Ligation Sequencing Kit XL**

The Multiplex Ligation Sequencing Kit XL is a standalone kit providing 96 unique barcodes to enable PCR-free multiplexing of dsDNA samples such as gDNA and amplicons. This kit provides an easier workflow to enable Whole Genome Sequencing (WGS) by enabling low-plex sequencing of 2-3 samples, allowing users to identify regions more common and others more prone to mutations.

The library preparation is similar to the Ligation Sequencing Kit protocol; DNA ends are repaired and dA-tailed using the NEBNext End Repair/dA-tailing module, and then a unique dT-tailed barcode adapter is ligated on the dA-tailed template. Barcoded samples are then pooled together. Each barcode adapter also has a cohesive end and this is used as a hook to ligate to the supplied sequencing adapters.

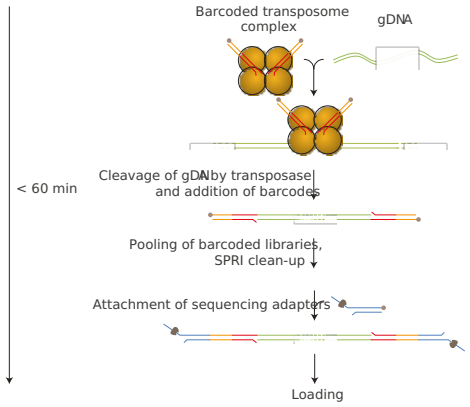
We recommend starting with 1 µg gDNA per sample and our manual protocol recommends sequencing two samples per flow cell enabling the sequencing of 96 samples across 48 flow cells. There is also an automated protocol on the Hamilton NGS STAR 96 (NGS STAR with Multi-Probe Head 96) to sequence either two samples per flow cell or three samples across two flow cells which is fully supported by Oxford Nanopore Technologies.

**Rapid-based barcoding kits**

**Rapid Barcoding Kit**

The Rapid Barcoding Kit (SQK-RBK004) can be used to barcode up to 12 samples quickly with limited laboratory equipment. It is recommended to use ~400 ng high molecular weight gDNA to generate barcoded libraries in 15 minutes using the simple two-step protocol.

This kit uses the transposase simultaneously cleaves template molecules and attaches barcoded tags to the cleaved ends. Barcoded samples are pooled and Rapid Sequencing Adapters are then added to the tagged ends.

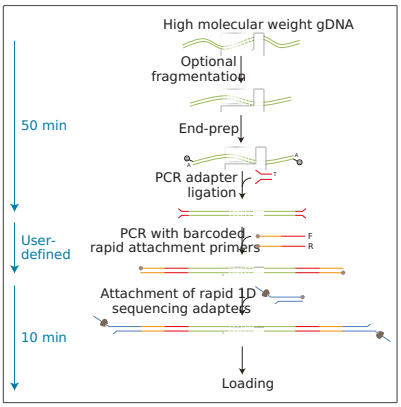


**Rapid-based PCR Barcoding Kits**

**PCR Barcoding Kit (SQK-PBK004):**

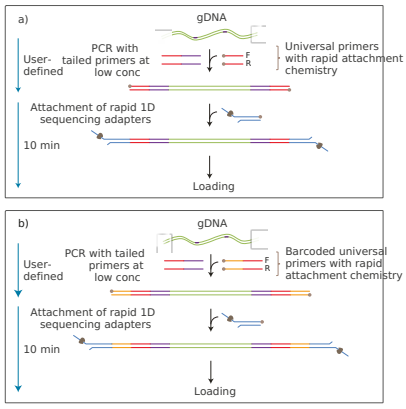
The PCR Barcoding Kit (SQK-PBK004) is the multiplexing version of the PCR Sequencing Kit (SQK-PSK004), allowing up to 12 samples to be multiplexed on one flow cell when starting with low input. When the quantity of input DNA is low or users want to maximise output, we recommend using PCR to increase the amount of template molecules. This kit implements PCR and it is recommended to start with ~100 ng gDNA. However, less input can be used but it may be necessary to increase the number of PCR cycles.

The gDNA samples are fragmented in a Covaris g-TUBE and the sheared ends are repaired and dA-tailed before the adapters containing primer binding sites are ligated onto the prepared ends. This kit contains 12 primer pairs which are used to amplify each sample during PCR. After amplification, the Rapid Sequencing Adapters are attached to the pooled barcoded samples. Each primer pair contains a barcode and 5’ tags which facilitates the ligase-free attachment of the Rapid Sequencing Adapter. Prior to PCR, this workflow takes approximately 50 minutes and post-PCR, the attachment of sequencing adapters takes about 10 minutes.




**Locus-specific protocols:**

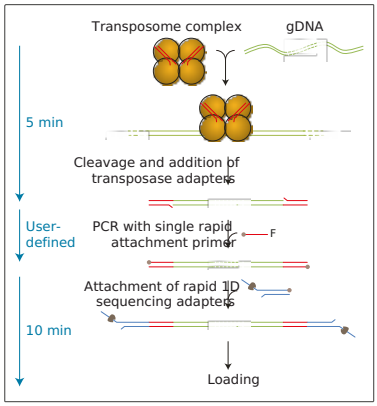
We also offer methods to enrich for loci of interest using the PCR Barcoding Kit (SQK-PBK004) and PCR Sequencing Kit (SQK-PSK004) where users tag their own specific amplicons with the same 5’ group before barcoding to simplify post-PCR processing. This is done via a four-primer PCR outlined in the Four-Primer PCR (SQK-PSK004 or SQK-PBK004) protocol with a recommended input of 30 ng DNA. Users add a 5’ tail to their own primer sequences and this acts as a priming site for a set of barcoded “outer” primers which are supplied in the PCR Barcoding Kit. These primers contain the 5’ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters.




**Rapid PCR Barcoding Kit (SQK-RPB004):**

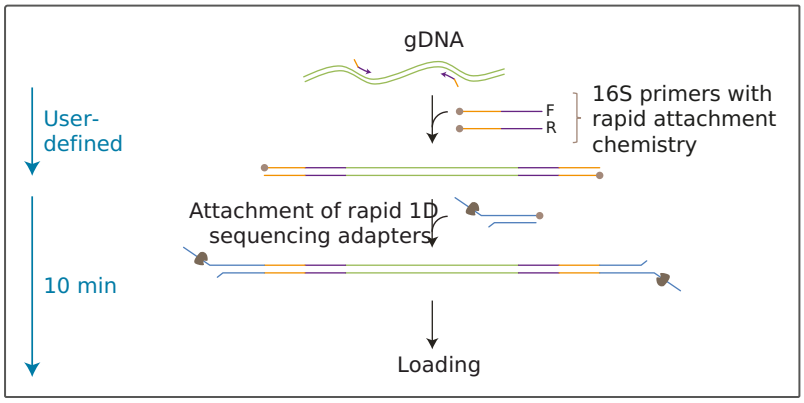
The Rapid PCR Barcoding Kit (SQK-RPB004) is a fast and simple method of preparing barcoded libraries for low quantities of gDNA (1–5

ng), with only ~15 minutes of hands-on preparation time. The transposase simultaneously cleaves the template molecules in each sample and attaches tags, which contain primer binding sites to the cleaved ends. The kit contains 12 primers which are then used to amplify each sample. Each primer contains a barcode and 5’ tag which facilitates the ligase-free attachment of Rapid Sequencing Adapters. Amplified and barcoded samples are then pooled together and Rapid Sequencing Adapters are added to the pooled mix.



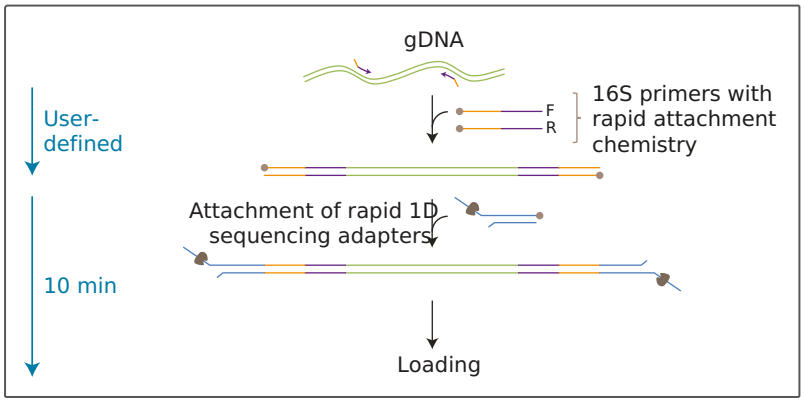

**16S Barcoding Kit (SQK-RAB204)**

The 16S Barcoding Kit (SQK-RAB204) offers a method of amplifying and barcoding the ~1500 bp 16S rRNA gene from multiple samples and sequencing them together. By narrowing down to a specific region of interest, a user can see all the organisms in the sample without sequencing unnecessary regions of the genome, making the identification quicker and more economical. There are up to 12 unique barcodes, allowing the user to pool up to 12 different samples in one sequencing experiment using 10 ng of high molecular weight gDNA per sample as input. The DNA is amplified by PCR using the specific 16S barcode primers (27F and 1492R) and 5’ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters.




**16S Barcoding Kit 1-24 (SQK-16S024):**

The 16S Barcoding Kit 1-24 (SQK-16S024) offers a method of amplifying and barcoding the ~1500 bp 16S rRNA gene from multiple samples and sequencing them together. By narrowing down to a specific region of interest, a user can see all the organisms in the sample without sequencing unnecessary regions of the genome, making the identification quicker and more economical. There are up to 24 unique barcodes, allowing the user to pool up to 24 different samples in one sequencing experiment with 10 ng of high molecular weight gDNA per sample as input. The DNA is amplified by PCR using the specific 16S barcode primers (27F and 1492R) and 5’ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters.

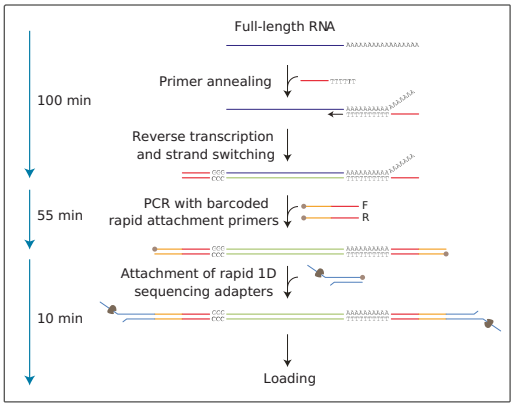



**cDNA barcoding kits**

**PCR-cDNA Barcoding Kit (SQK-PCB109)**

This kit is the barcoding version of the cDNA-PCR Sequencing Kit (SQK-PCS109) and is recommended for users who want to multiplex cDNA samples with limited input material, whilst optimising for output. This kit may also be used to identify and quantify full-length transcripts or characterise and quantify isoforms, splice variants and fusion transcripts. This is also appropriate for users who are interested in differential gene expression or differential transcript usage. Up to 12 samples of cDNA can be prepared from an input as low as 1 ng of poly(A) RNA. Users who do not have poly(A)+ enriched RNA can use 50 ng of total RNA but additional optimisation may be required, as further explained below.

Complementary strand synthesis and strand switching are performed on full-length poly(A) RNA using the kit supplied oligonucleotides. There are 12 primer pairs that are used to generate and then amplify double-stranded cDNA by PCR amplification. Each primer pair contains a barcode and a 5’ tag which facilitates the ligase-free attachment of Rapid Sequencing Adapters. Amplified and barcoded samples are then pooled together and sequencing adapters are added to the pooled mix.




**PCR-cDNA Barcoding Kit (SQK-PCB111.24):**

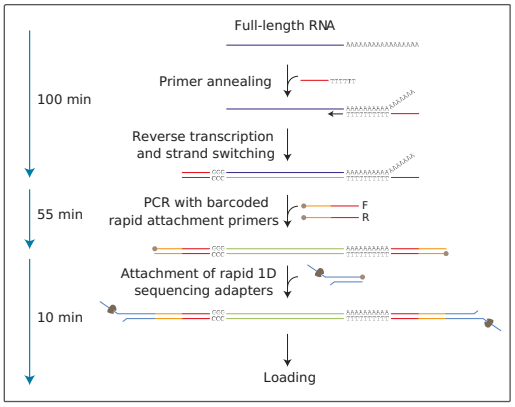
This kit is the barcoding version of the cDNA-PCR Sequencing Kit (SQK-PCS111) and is recommended for users who want to multiplex cDNA samples with limited input material, whilst optimising for output. This kit may also be used to identify and quantify full-length transcripts or characterise and quantify isoforms, splice variants and fusion transcripts. This is also appropriate for users who are interested in differential gene expression or differential transcript usage. Up to 12 samples of cDNA can be prepared from an input as low as 1 ng of poly(A) RNA. Users who do not have poly(A)+ enriched RNA can use 50 ng of total RNA but additional optimisation may be required, as further explained below.

We have recently upgraded this kit to Kit 11 chemistry (SQK-PCB111.24) and increased the number of barcodes available to 24. This

upgrade requires starting inputs of 4 ng poly(A) RNA or 200 ng of total RNA. Further the Kit 11 upgrades includes a new Rapid Adapter T (RAP T), which has a higher capture rate and fuel fix technology for longer experiments without the need for fuel addition during the run.

We have also included a new cDNA RT adapter (CRTA), Annealing Buffer (AB) and RT primer (RTP) to prime cDNA synthesis from the end of a transcript to reduce overlaps during the reverse transcription step. This also enables users to measure poly(A) tail lengths.

The kit workflow includes complementary strand synthesis and strand switching of full-length poly(A) RNA using the kit supplied oligonucleotides. There are 24 primer pairs that are used to generate and then amplify double-stranded cDNA by PCR amplification. Each primer pair contains a barcode and a 5’ tag which facilitates the ligase-free attachment of Rapid Sequencing Adapters. Amplified and barcoded samples are then pooled together and sequencing adapters are added to the pooled mix.




# Barcode sequences

### Native barcode sequences

Below is the full list of our native barcode (NB01-96) sequences. The first 24 unique barcodes are available in the Native Barcoding Kit 24 V14 (SQK-NBD114.24). The Native Barcoding Kit 96 V14 (SQK-NBD114.96) include the first 24 native barcodes, with the additional 72 unique barcodes. The native barcodes are shipped at 640 nM.

In addition to the barcodes, there are also flanking sequences which add an extra level of context during analysis.

**Barcode flanking sequences:**

Forward sequence: 5' - AAGGTTAA - barcode - CAGCACCT - 3'
Reverse sequence: 5' - GGTGCTG - barcode - TTAACCTTAGCAAT - 3'

### Native barcode sequences

| Component | Forward sequence | Reverse sequence |
|---|---|---|
| NB01 | CACAAAGACACCGACAACTTTCTT | AAGAAAGTTGTCGGTGTCTTTGTG |
| NB02 | ACAGACGACTACAAACGGAATCGA | TCGATTCCGTTTGTAGTCGTCTGT |

| Component | Forward sequence | Reverse sequence |
|---|---|---|
| NB03 | CCTGGTAACTGGGACACAAGACTC | GAGTCTTGTGTCCCAGTTACCAGG |
| NB04 | TAGGGAAACACGATAGAATCCGAA | TTCGGATTCTATCGTGTTTCCCTA |
| NB05 | AAGGTTACACAAACCCTGGACAAG | CTTGTCCAGGGTTTGTGTAACCTT |
| NB06 | GACTACTTTCTGCCTTTGCGAGAA | TTCTCGCAAAGGCAGAAAGTAGTC |
| NB07 | AAGGATTCATTCCCACGGTAACAC | GTGTTACCGTGGGAATGAATCCTT |
| NB08 | ACGTAACTTGGTTTGTTCCCTGAA | TTCAGGGAACAAACCAAGTTACGT |
| NB09 | AACCAAGACTCGCTGTGCCTAGTT | AACTAGGCACAGCGAGTCTTGGTT |
| NB10 | GAGAGGACAAAGGTTTCAACGCTT | AAGCGTTGAAACCTTTGTCCTCTC |
| NB11 | TCCATTCCCTCCGATAGATGAAAC | GTTTCATCTATCGGAGGGAATGGA |
| NB12 | TCCGATTCTGCTTCTTTCTACCTG | CAGGTAGAAAGAAGCAGAATCGGA |
| NB13 | AGAACGACTTCCATACTCGTGTGA | TCACACGAGTATGGAAGTCGTTCT |
| NB14 | AACGAGTCTCTTGGGACCCATAGA | TCTATGGGTCCCAAGAGACTCGTT |
| NB15 | AGGTCTACCTCGCTAACACCACTG | CAGTGGTGTTAGCGAGGTAGACCT |
| NB16 | CGTCAACTGACAGTGGTTCGTACT | AGTACGAACCACTGTCAGTTGACG |
| NB17 | ACCCTCCAGGAAAGTACCTCTGAT | ATCAGAGGTACTTTCCTGGAGGGT |
| NB18 | CCAAACCCAACAACCTAGATAGGC | GCCTATCTAGGTTGTTGGGTTTGG |
| NB19 | GTTCCTCGTGCAGTGTCAAGAGAT | ATCTCTTGACACTGCACGAGGAAC |
| NB20 | TTGCGTCCTGTTACGAGAACTCAT | ATGAGTTCTCGTAACAGGACGCAA |
| NB21 | GAGCCTCTCATTGTCCGTTCTCTA | TAGAGAACGGACAATGAGAGGCTC |
| NB22 | ACCACTGCCATGTATCAAAGTACG | CGTACTTTGATACATGGCAGTGGT |
| NB23 | CTTACTACCCAGTGAACCTCCTCG | CGAGGAGGTTCACTGGGTAGTAAG |
| NB24 | GCATAGTTCTGCATGATGGGTTAG | CTAACCCATCATGCAGAACTATGC |
| NB25 | GTAAGTTGGGTATGCAACGCAATG | CATTGCGTTGCATACCCAACTTAC |
| NB26 | CATACAGCGACTACGCATTCTCAT | ATGAGAATGCGTAGTCGCTGTATG |
| NB27 | CGACGGTTAGATTCACCTCTTACA | TGTAAGAGGTGAATCTAACCGTCG |
| NB28 | TGAAACCTAAGAAGGCACCGTATC | GATACGGTGCCTTCTTAGGTTTCA |
| NB29 | CTAGACACCTTGGGTTGACAGACC | GGTCTGTCAACCCAAGGTGTCTAG |
| NB30 | TCAGTGAGGATCTACTTCGACCCA | TGGGTCGAAGTAGATCCTCACTGA |
| NB31 | TGCGTACAGCAATCAGTTACATTG | CAATGTAACTGATTGCTGTACGCA |
| NB32 | CCAGTAGAAGTCCGACAACGTCAT | ATGACGTTGTCGGACTTCTACTGG |
| NB33 | CAGACTTGGTACGGTTGGGTAACT | AGTTACCCAACCGTACCAAGTCTG |
| NB34 | GGACGAAGAACTCAAGTCAAAGGC | GCCTTTGACTTGAGTTCTTCGTCC |
| NB35 | CTACTTACGAAGCTGAGGGACTGC | GCAGTCCCTCAGCTTCGTAAGTAG |
| NB36 | ATGTCCCAGTTAGAGGAGGAAACA | TGTTTCCTCCTCTAACTGGGACAT |

| Component | Forward sequence | Reverse sequence |
|---|---|---|
| NB37 | GCTTGCGATTGATGCTTAGTATCA | TGATACTAAGCATCAATCGCAAGC |
| NB38 | ACCACAGGAGGACGATACAGAGAA | TTCTCTGTATCGTCCTCCTGTGGT |
| NB39 | CCACAGTGTCAACTAGAGCCTCTC | GAGAGGCTCTAGTTGACACTGTGG |
| NB40 | TAGTTTGGATGACCAAGGATAGCC | GGCTATCCTTGGTCATCCAAACTA |
| NB41 | GGAGTTCGTCCAGAGAAGTACACG | CGTGTACTTCTCTGGACGAACTCC |
| NB42 | CTACGTGTAAGGCATACCTGCCAG | CTGGCAGGTATGCCTTACACGTAG |
| NB43 | CTTTCGTTGTTGACTCGACGGTAG | CTACCGTCGAGTCAACAACGAAAG |
| NB44 | AGTAGAAAGGGTTCCTTCCCACTC | GAGTGGGAAGGAACCCTTTCTACT |
| NB45 | GATCCAACAGAGATGCCTTCAGTG | CACTGAAGGCATCTCTGTTGGATC |
| NB46 | GCTGTGTTCCACTTCATTCTCCTG | CAGGAGAATGAAGTGGAACACAGC |
| NB47 | GTGCAACTTTCCCACAGGTAGTTC | GAACTACCTGTGGGAAAGTTGCAC |
| NB48 | CATCTGGAACGTGGTACACCTGTA | TACAGGTGTACCACGTTCCAGATG |
| NB49 | ACTGGTGCAGCTTTGAACATCTAG | CTAGATGTTCAAAGCTGCACCAGT |
| NB50 | ATGGACTTTGGTAACTTCCTGCGT | ACGCAGGAAGTTACCAAAGTCCAT |
| NB51 | GTTGAATGAGCCTACTGGGTCCTC | GAGGACCCAGTAGGCTCATTCAAC |
| NB52 | TGAGAGACAAGATTGTTCGTGGAC | GTCCACGAACAATCTTGTCTCTCA |
| NB53 | AGATTCAGACCGTCTCATGCAAAG | CTTTGCATGAGACGGTCTGAATCT |
| NB54 | CAAGAGCTTTGACTAAGGAGCATG | CATGCTCCTTAGTCAAAGCTCTTG |
| NB55 | TGGAAGATGAGACCCTGATCTACG | CGTAGATCAGGGTCTCATCTTCCA |
| NB56 | TCACTACTCAACAGGTGGCATGAA | TTCATGCCACCTGTTGAGTAGTGA |
| NB57 | GCTAGGTCAATCTCCTTCGGAAGT | ACTTCCGAAGGAGATTGACCTAGC |
| NB58 | CAGGTTACTCCTCCGTGAGTCTGA | TCAGACTCACGGAGGAGTAACCTG |
| NB59 | TCAATCAAGAAGGGAAAGCAAGGT | ACCTTGCTTTCCCTTCTTGATTGA |
| NB60 | CATGTTCAACCAAGGCTTCTATGG | CCATAGAAGCCTTGGTTGAACATG |
| NB61 | AGAGGGTACTATGTGCCTCAGCAC | GTGCTGAGGCACATAGTACCCTCT |
| NB62 | CACCCACACTTACTTCAGGACGTA | TACGTCCTGAAGTAAGTGTGGGTG |
| NB63 | TTCTGAAGTTCCTGGGTCTTGAAC | GTTCAAGACCCAGGAACTTCAGAA |
| NB64 | GACAGACACCGTTCATCGACTTTC | GAAAGTCGATGAACGGTGTCTGTC |
| NB65 | TTCTCAGTCTTCCTCCAGACAAGG | CCTTGTCTGGAGGAAGACTGAGAA |
| NB66 | CCGATCCTTGTGGCTTCTAACTTC | GAAGTTAGAAGCCACAAGGATCGG |
| NB67 | GTTTGTCATACTCGTGTGCTCACC | GGTGAGCACACGAGTATGACAAAC |
| NB68 | GAATCTAAGCAAACACGAAGGTGG | CCACCTTCGTGTTTGCTTAGATTC |
| NB69 | TACAGTCCGAGCCTCATGTGATCT | AGATCACATGAGGCTCGGACTGTA |
| NB70 | ACCGAGATCCTACGAATGGAGTGT | ACACTCCATTCGTAGGATCTCGGT |

| Component | Forward sequence | Reverse sequence |
|---|---|---|
| NB71 | CCTGGGAGCATCAGGTAGTAACAG | CTGTTACTACCTGATGCTCCCAGG |
| NB72 | TAGCTGACTGTCTTCCATACCGAC | GTCGGTATGGAAGACAGTCAGCTA |
| NB73 | AAGAAACAGGATGACAGAACCCTC | GAGGGTTCTGTCATCCTGTTTCTT |
| NB74 | TACAAGCATCCCAACACTTCCACT | AGTGGAAGTGTTGGGATGCTTGTA |
| NB75 | GACCATTGTGATGAACCCTGTTGT | ACAACAGGGTTCATCACAATGGTC |
| NB76 | ATGCTTGTTACATCAACCCTGGAC | GTCCAGGGTTGATGTAACAAGCAT |
| NB77 | CGACCTGTTTCTCAGGGATACAAC | GTTGTATCCCTGAGAAACAGGTCG |
| NB78 | AACAACCGAACCTTTGAATCAGAA | TTCTGATTCAAAGGTTCGGTTGTT |
| NB79 | TCTCGGAGATAGTTCTCACTGCTG | CAGCAGTGAGAACTATCTCCGAGA |
| NB80 | CGGATGAACATAGGATAGCGATTC | GAATCGCTATCCTATGTTCATCCG |
| NB81 | CCTCATCTTGTGAAGTTGTTTCGG | CCGAAACAACTTCACAAGATGAGG |
| NB82 | ACGGTATGTCGAGTTCCAGGACTA | TAGTCCTGGAACTCGACATACCGT |
| NB83 | TGGCTTGATCTAGGTAAGGTCGAA | TTCGACCTTACCTAGATCAAGCCA |
| NB84 | GTAGTGGACCTAGAACCTGTGCCA | TGGCACAGGTTCTAGGTCCACTAC |
| NB85 | AACGGAGGAGTTAGTTGGATGATC | GATCATCCAACTAACTCCTCCGTT |
| NB86 | AGGTGATCCCAACAAGCGTAAGTA | TACTTACGCTTGTTGGGATCACCT |
| NB87 | TACATGCTCCTGTTGTTAGGGAGG | CCTCCCTAACAACAGGAGCATGTA |
| NB88 | TCTTCTACTACCGATCCGAAGCAG | CTGCTTCGGATCGGTAGTAGAAGA |
| NB89 | ACAGCATCAATGTTTGGCTAGTTG | CAACTAGCCAAACATTGATGCTGT |
| NB90 | GATGTAGAGGGTACGGTTTGAGGC | GCCTCAAACCGTACCCTCTACATC |
| NB91 | GGCTCCATAGGAACTCACGCTACT | AGTAGCGTGAGTTCCTATGGAGCC |
| NB92 | TTGTGAGTGGAAAGATACAGGACC | GGTCCTGTATCTTTCCACTCACAA |
| NB93 | AGTTTCCATCACTTCAGACTTGGG | CCCAAGTCTGAAGTGATGGAAACT |
| NB94 | GATTGTCCTCAAACTGCCACCTAC | GTAGGTGGCAGTTTGAGGACAATC |
| NB95 | CCTGTCTGGAAGAAGAATGGACTT | AAGTCCATTCTTCTTCCAGACAGG |
| NB96 | CTGAACGGTCATAGAGTCCACCAT | ATGGTGGACTCTATGACCGTTCAG |

**Rapid barcode sequences**

Below is a full list of the rapid barcode sequences used across the rapid-based barcoding kits, with the component acronym for the specific sequencing kit. The barcodes in our rapid-based kits are shipped at 10 µM.

**Note:** These are not the full sequences due to proprietory information.

In addition to the barcodes, there are also flanking sequences which add an extra level of context during analysis.

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**Rapid barcoding kits**

Rapid Barcoding Kit 24 V14 (SQK-RBK114.24): **RB01-24**
Rapid Barcoding Kit 96 V14 (SQK-RBK114.96): **RB01-96**
*Legacy:* Rapid Barcoding Kit (SQK-RBK004): **RB01-12**
Rapid Barcoding Kit 96 (SQK-RBK110.96): **RB01-96**

Barcode flanking sequence:
5' - GCTTGGGTGTTTAACC - barcode - GTTTTCGCATTTATCGTGAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCA - 3'

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**PCR barcoding kits**

PCR-cDNA Barcoding Kit 24 V14 (SQK-PCB114.24): **BP01-24**
*Legacy* PCR-cDNA Barcoding Kit 24 (SQK-PCB111.24): **BP01-24**
*Legacy:* PCR-cDNA Barcoding Kit (SQK-PCB109): **BP01-12**
*Legacy:* PCR Barcoding Kit (SQK-PBK004): **BP01-12**

Barcode flanking sequence:
Top strand: 5' - ATCGCCTACCGTGA - barcode - TTGCCTGTCGCTCTATCTTC - 3'
Bottom strand: 5' - ATCGCCTACCGTGA - barcode - TCTGTTGGTGCTGATATTGC - 3'

The top and bottom barcode flanking sequences are different to avoid 5' and 3' end sequences annealing to each other and forming a loop.

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**16S barcoding kits**
16S Barcoding Kit 24 V14 (SQK-16S114.24): **16S01-24**
*Legacy* 16S Barcoding Kit 1-24 (SQK-16S024): **16S01-24**
*Legacy:* 16S Barcoding Kit (SQK-RAB204): **16S01-12**

Barcode flanking sequence:
Forward 16S primer: 5' - ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG - 3'
Reverse 16S primer: 5' - ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT - 3'

The 3' flanking sequence of the forward primer contains a wobble base (denoted by M; in the primer the base is either an A or a C) in a variable region of the 16S gene.

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**Rapid PCR barcoding kits**
Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24): **RLB01-24**
*Legacy:* Rapid PCR Barcoding Kit (SQK-RPB004): **RLB01-12A**

Barcode flanking sequence:
5' - ATCGCCTACCGTGAC - barcode - CGTTTTTCGTGCGCCGCTTC - 3'

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**PCR barcoding expansions**
PCR Barcoding Expansion 1-12 (EXP-PBC001): **BC01-12**
PCR Barcoding Expansion 1-96 (EXP-PBC096): **BC01-96**

Barcode flanking sequence:
5' - GGTGCTG - barcode - TTAACCT - 3'

| Component | Sequence |
|---|---|
| BP01 / BC01 / RB01 / 16S01 / RLB01 | AAGAAAGTTGTCGGTGTCTTTGTG |

| Component | Sequence |
| --- | --- |
| BP02 / BC02 / RB02 / 16S02 / RLB02 | TCGATTCCGTTTGTAGTCGTCTGT |
| BP03 / BC03 / RB03 / 16S03 / RLB03 | GAGTCTTGTGTCCCAGTTACCAGG |
| BP04 / BC04 / RB04 / 16S04 / RLB04 | TTCGGATTCTATCGTGTTTCCCTA |
| BP05 / BC05 / RB05 / 16S05 / RLB05 | CTTGTCCAGGGTTTGTGTAACCTT |
| BP06 / BC06 / RB06 / 16S06 / RLB06 | TTCTCGCAAAGGCAGAAAGTAGTC |
| BP07 / BC07 / RB07 / 16S07 / RLB07 | GTGTTACCGTGGGAATGAATCCTT |
| BP08 / BC08 / RB08 / 16S08 / RLB08 | TTCAGGGAACAAACCAAGTTACGT |
| BP09 / BC09 / RB09 / 16S09 / RLB09 | AACTAGGCACAGCGAGTCTTGGTT |
| BP10 / BC10 / RB10 / 16S10 / RLB10 | AAGCGTTGAAACCTTTGTCCTCTC |
| BP11 / BC11 / RB11 / 16S11 / RLB11 | GTTTCATCTATCGGAGGGAATGGA |
| BP12 / BC12 / RB12 / 16S12 / RLB12 | CAGGTAGAAAGAAGCAGAATCGGA |
| RLB012A | GTTGAGTTACAAAGCACCGATCAG |
| BP13 / BC13 / RB13 / 16S13 / RLB13 | AGAACGACTTCCATACTCGTGTGA |
| BP14 / BC14 / RB14 / 16S14 / RLB14 | AACGAGTCTCTTGGGACCCATAGA |
| BP15 / BC15 / RB15 / 16S15 / RLB15 | AGGTCTACCTCGCTAACACCACTG |
| BP16 / BC16 / RB16 / 16S16 / RLB16 | CGTCAACTGACAGTGGTTCGTACT |
| BP17 / BC17 / RB17 / 16S17 / RLB17 | ACCCTCCAGGAAAGTACCTCTGAT |
| BP18 / BC18 / RB18 / 16S18 / RLB18 | CCAAACCCAACAACCTAGATAGGC |
| BP19 / BC19 / RB19 / 16S19 / RLB19 | GTTCCTCGTGCAGTGTCAAGAGAT |
| BP20 / BC20 / RB20 / 16S20 / RLB20 | TTGCGTCCTGTTACGAGAACTCAT |
| BP21 / BC21 / RB21 / 16S21 / RLB21 | GAGCCTCTCATTGTCCGTTCTCTA |

| Component | Sequence |
| --- | --- |
| BP22 / BC22 / RB22 / 16S22 / RLB22 | ACCACTGCCATGTATCAAAGTACG |
| BP23 / BC23 / RB23 / 16S23 / RLB23 | CTTACTACCCAGTGAACCTCCTCG |
| BP24 / BC24 / RB24 / 16S24 / RLB24 | GCATAGTTCTGCATGATGGGTTAG |
| BC25 / RB25 | GTAAGTTGGGTATGCAACGCAATG |
| BC26 / RB26 | CATACAGCGACTACGCATTCTCAT |
| BC27 / RB27 | CGACGGTTAGATTCACCTCTTACA |
| BC28 / RB28 | TGAAACCTAAGAAGGCACCGTATC |
| BC29 / RB29 | CTAGACACCTTGGGTTGACAGACC |
| BC30 / RB30 | TCAGTGAGGATCTACTTCGACCCA |
| BC31 / RB31 | TGCGTACAGCAATCAGTTACATTG |
| BC32 / RB32 | CCAGTAGAAGTCCGACAACGTCAT |
| BC33 / RB33 | CAGACTTGGTACGGTTGGGTAACT |
| BC34 / RB34 | GGACGAAGAACTCAAGTCAAAGGC |
| BC35 / RB35 | CTACTTACGAAGCTGAGGGACTGC |
| BC36 / RB36 | ATGTCCCAGTTAGAGGAGGAAACA |
| BC37 / RB37 | GCTTGCGATTGATGCTTAGTATCA |
| BC38 / RB38 | ACCACAGGAGGACGATACAGAGAA |
| BC39 / RB39 | CCACAGTGTCAACTAGAGCCTCTC |
| BC40 / RB40 | TAGTTTGGATGACCAAGGATAGCC |
| BC41 / RB41 | GGAGTTCGTCCAGAGAAGTACACG |
| BC42 / RB42 | CTACGTGTAAGGCATACCTGCCAG |
| BC43 / RB43 | CTTTCGTTGTTGACTCGACGGTAG |
| BC44 / RB44 | AGTAGAAAGGGTTCCTTCCCACTC |
| BC45 / RB45 | GATCCAACAGAGATGCCTTCAGTG |
| BC46 / RB46 | GCTGTGTTCCACTTCATTCTCCTG |
| BC47 / RB47 | GTGCAACTTTCCCACAGGTAGTTC |
| BC48 / RB48 | CATCTGGAACGTGGTACACCTGTA |
| BC49 / RB49 | ACTGGTGCAGCTTTGAACATCTAG |
| BC50 / RB50 | ATGGACTTTGGTAACTTCCTGCGT |
| BC51 / RB51 | GTTGAATGAGCCTACTGGGTCCTC |
| BC52 / RB52 | TGAGAGACAAGATTGTTCGTGGAC |
| BC53 / RB53 | AGATTCAGACCGTCTCATGCAAAG |

| Component | Sequence |
|---|---|
| BC54 / RB54 | CAAGAGCTTTGACTAAGGAGCATG |
| BC55 / RB55 | TGGAAGATGAGACCCTGATCTACG |
| BC56 / RB56 | TCACTACTCAACAGGTGGCATGAA |
| BC57 / RB57 | GCTAGGTCAATCTCCTTCGGAAGT |
| BC58 / RB58 | CAGGTTACTCCTCCGTGAGTCTGA |
| BC59 / RB59 | TCAATCAAGAAGGGAAAGCAAGGT |
| BC60 / RB60 | CATGTTCAACCAAGGCTTCTATGG |
| BC61 / RB61 | AGAGGGTACTATGTGCCTCAGCAC |
| BC62 / RB62 | CACCCACACTTACTTCAGGACGTA |
| BC63 / RB63 | TTCTGAAGTTCCTGGGTCTTGAAC |
| BC64 / RB64 | GACAGACACCGTTCATCGACTTTC |
| BC65 / RB65 | TTCTCAGTCTTCCTCCAGACAAGG |
| BC66 / RB66 | CCGATCCTTGTGGCTTCTAACTTC |
| BC67 / RB67 | GTTTGTCATACTCGTGTGCTCACC |
| BC68 / RB68 | GAATCTAAGCAAACACGAAGGTGG |
| BC69 / RB69 | TACAGTCCGAGCCTCATGTGATCT |
| BC70 / RB70 | ACCGAGATCCTACGAATGGAGTGT |
| BC71 / RB71 | CCTGGGAGCATCAGGTAGTAACAG |
| BC72 / RB72 | TAGCTGACTGTCTTCCATACCGAC |
| BC73 / RB73 | AAGAAACAGGATGACAGAACCCTC |
| BC74 / RB74 | TACAAGCATCCCAACACTTCCACT |
| BC75 / RB75 | GACCATTGTGATGAACCCTGTTGT |
| BC76 / RB76 | ATGCTTGTTACATCAACCCTGGAC |
| BC77 / RB77 | CGACCTGTTTCTCAGGGATACAAC |
| BC78 / RB78 | AACAACCGAACCTTTGAATCAGAA |
| BC79 / RB79 | TCTCGGAGATAGTTCTCACTGCTG |
| BC80 / RB80 | CGGATGAACATAGGATAGCGATTC |
| BC81 / RB81 | CCTCATCTTGTGAAGTTGTTTCGG |
| BC82 / RB82 | ACGGTATGTCGAGTTCCAGGACTA |
| BC83 / RB83 | TGGCTTGATCTAGGTAAGGTCGAA |
| BC84 / RB84 | GTAGTGGACCTAGAACCTGTGCCA |
| BC85 / RB85 | AACGGAGGAGTTAGTTGGATGATC |
| BC86 / RB86 | AGGTGATCCCAACAAGCGTAAGTA |
| BC87 / RB87 | TACATGCTCCTGTTGTTAGGGAGG |

| Component | Sequence |
|---|---|
| BC88 / RB88 | TCTTCTACTACCGATCCGAAGCAG |
| BC89 / RB89 | ACAGCATCAATGTTTGGCTAGTTG |
| BC90 / RB90 | GATGTAGAGGGTACGGTTTGAGGC |
| BC91 / RB91 | GGCTCCATAGGAACTCACGCTACT |
| BC92 / RB92 | TTGTGAGTGGAAAGATACAGGACC |
| BC93 / RB93 | AGTTTCCATCACTTCAGACTTGGG |
| BC94 / RB94 | GATTGTCCTCAAACTGCCACCTAC |
| BC95 / RB95 | CCTGTCTGGAAGAAGAATGGACTT |
| BC96 / RB96 | CTGAACGGTCATAGAGTCCACCAT |

# Adapter sequences

**Sequencing adapter sequences**

Below are the adapter sequences for our current kits.

**Ligation Adapter (LA)**

Top strand:
5'-TTTTTTTTCCTGTACTTCGTTCAGTTACGTATTGCT-3’

Bottom strand:
5’-GCAATACGTAACTGAACGAAGTACAGG-3’

**Rapid Adapter (RA)**

Top strand:
5’-TTTTTTTTCCTGTACTTCGTTCAGTTACGTATTGCT-3’

**RT Primer (RTP)**
5’-CTTGCCTGTCGCTCTATCTTCAGAGGAG-3’

**Strand Switching Primer II (SSPII)**
5’-TTTCTGTTGGTGCTGATATTGCTTTVVVVTTVVVVTTVVVVTTVVVVTTTmGmGmG-3’

**cDNA RT Adapter (CRTA)**
5'-CTTGCGGGCGGCGGACTCTCCTCTGAAGATAGAGCGACAGGCAAG-3'

**Rapid Adapter T (RAP T)**
5'-TTTTTTTTCCTGTACTTCGTTCAGTTACGTATTGCT-3’

**cDNA Primer (cPRM)**
Forward sequence:
5'-ATCGCCTACCGTGACAAGAAAGTTGTCGGTGTCTTTGTGACTTGCCTGTCGCTCTATCTTC-3’

Reverse sequence:
5’-ATCGCCTACCGTGACAAGAAAGTTGTCGGTGTCTTTGTGTTTCTGTTGGTGCTGATATTGC-3’

**Native Adapter (NA)**
Top strand:
5'-TTTTTTTT CCTGTACTTCGTTCAGTACGTATTGCT-3'

Bottom strand:
5'-ACGTAACTGAACGAAGTACAGG-3'

# Kit reagents

**Sequencing kit reagents**

Below is a list of our common reagents included in our sequencing kits.

**Ligation Adapter (LA)**
The sequencing adapter is ligated onto the dA-tailed DNA fragments during library preparation. A motor protein is loaded onto the sequencing adapter and regulates the DNA passage through the nanopore.

**Native Adapter (NA)**
The sequencing adapter ligates to the native barcodes ligated onto the dA-tailed DNA fragments during the library preparation of the native barcoding kits. A motor protein is loaded onto the sequencing adapter and regulates the DNA passage through the nanopore.

**Fragmentation Mix (FRA)**
This reagent contains a transposase to fragment DNA strands during library preparation and simultaneously attaches transposase adapters to the DNA ends.

**Rapid Adapter (RA)**
The sequencing adapter is attached to the transposase adapters in the Fragmentation Mix (FRA). A motor protein is loaded onto the sequencing adapter and regulates the DNA passage through the nanopore.

**Adapter Buffer (ADB)**
This buffer is used in conjunction with the Rapid Adapter (RA) during the adapter attachment step to reach the correct concentration for optimal adaptation.

**Native Barcodes (NB01-96)**

Barcodes are a known sequence used to identify samples in a pooled library. The Native Barcodes are specific to this kit and are ligated onto the DNA ends after end-prep. Native barcodes are shipped at 640 nM.

**EDTA (EDTA)**
This reagent is used during the barcoding step of the native barcoding kits to stop the barcode ligation reaction after incubation.

**AMPure XP Beads (AXP)**
These beads are manufactured by Beckman Coulter, Inc. and are used in the clean up steps of library preparations to purify a reaction e.g. excess barcodes or transposases present in a reaction.

**Sequencing Buffer (SB)**
The sequencing buffer provides fuel and the optimal chemical conditions for powering DNA translocation through the nanopore. In SQK-LSK114, this component has been upgraded to improve sequencing by reducing variability in current levels during experiment runs.

**Short and Long Fragment Buffers**
Short Fragment Buffer (SFB) is used to enrich fragments of all sizes after adapter ligation.
Long Fragment Buffer (LFB) is used to enrich fragments of ≥3 kb after adapter ligation.

**Ligation Buffer (LNB)**
This buffer is used to aid ligation of the sequencing adapters.

**Elution Buffer (EB)**
This buffer is optimised to keep the integrity of the motor protein on the Y-shaped adapter.

**Library Beads (LIB)**
These beads help reduce frequency of blocking.

**Library Solution (LIS)**
This reagent is the same solution that the Library Beads are stored in and are used to ease the loading of viscous libraries.

**Flow Cell Flush (FCF)** and **Flow Cell Tether (FCT)**
These reagents are combined to make up the flow cell priming mix. The tether is used to bring the DNA strand to the membrane and to improve DNA capture by approximately 10,000-fold compared with capture without tether.

**DNA Control Sample (DCS)**
The DNA Control Sample (DCS) is a 3.6 kb amplicon of the Lambda phage genome. The control sample is included in the library and added to DNA samples during DNA repair and end-prep for troubleshooting purposes.

**Lambda DNA (LMD)**
The Lambda DNA (LMD) is a pure, high-quality DNA sample that can be used to prepare a library and run a control experiment. The lambda control protocols allows a new user to practise the process of library preparation, and can also be used to troubleshoot a poorly performing experiments.