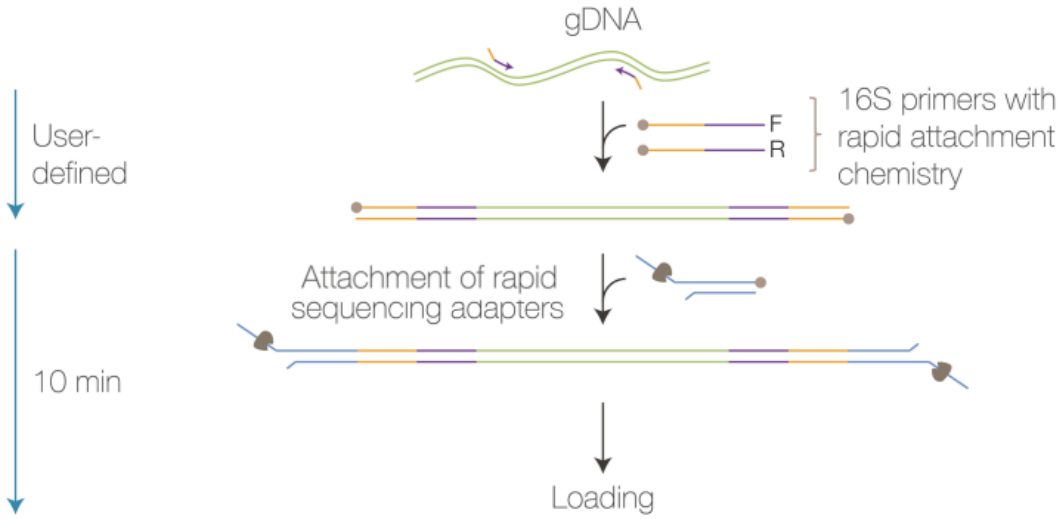
Nanopore 16S rRNA amplicon sequencing

Rapid sequencing DNA 16s barcoding kit-v14

(SQK-16S114.24)



Prepare your library

Library preparation

The table below is an overview of the steps required in the library preparation, including timings and stopping points.

Library preparation step	Process	Time	Stop option
16S barcoded PCR amplification	Amplify the 16S gene using barcodes supplied in the kit	10 minutes + PCR	4°C overnight

Library preparation step	Process	Time	Stop option
Barcoded sample pooling and bead clean-up	Quantify and pool the barcoded samples and perform a library clean-up using beads	15 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage.
Adapter ligation	Attach the rapid sequencing adapters to the to the DNA ends.	5 minutes	We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared DNA library for sequencing	5 minutes	

Materials :

- 10 ng high molecular weight genomic DNA
- 16S Barcoding Kit 24 V14 (SQK-16S114.24))

Consumables :

- LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- Freshly prepared 80% ethanol in nuclease-free water
- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- Nuclease-free water
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) / Qubit™ Assay Tubes (Invitrogen, Q32856)

Sample :

N° sample	Sample name	Concentration (ng/µl)	Barcode number
1	Leila	0.122	1
2	Adrien	1.1	2
3	ME_M	142.0	3
4	ME_S	6.7	4
5	Camille_Sophie forest sediment	90.6	5
6	Camille_Sophie canal water	169.0	6
7	Beatrice_Manon_SED	5.8	7
8	Beatrice_Manon_Forest	40.6	8
9	Lucille	39.8	9
10	Mathilde	82.8	10
11	Clara_Laura_C1	1.0	11
12	Clara_Laura_C2	1.3	12
13	Clara_Laura_B	0.7	13

Protocol :

1. Take one 96-well plate containing 16S barcodes. Break one set of barcodes (1-24, or as desired) away from the plate and return the rest to storage. Thaw the desired barcodes at room temperature.

2. Briefly centrifuge barcodes in a microfuge to make sure the liquid is at the bottom of the tubes and place on ice.

3. Thaw the LongAmp Hot Start Taq 2X Master Mix, spin down briefly, mix well by pipetting and place on ice.

4. Prepare the DNA in nuclease-free water .
   - Transfer 10 ng of each genomic DNA sample into a 0.2 ml thin-walled PCR tube.
   - Adjust the volume to 15 µl with nuclease-free water.
   - Mix thoroughly by flicking avoiding unwanted shearing Spin down briefly in a microfuge

5. In each 0.2 ml thin-walled PCR tube containing a sample to be tested, prepare the following mixture:

Reagent	Volume
10 ng input DNA (from previous step)	15 µl
LongAmp Hot Start Taq 2X Master Mix	25 µl
Total	**40 µl**

6. Ensure the components are thoroughly mixed by pipetting and spin down briefly.

7. Using clean pipette tips, carefully pierce the foil surface of the required barcodes. Use a new tip for each barcode to avoid cross-contamination. Make a note of which barcode numbers will be run for each sample.

8. Using a multichannel pipette, mix the 16S barcodes by pipetting up and down 10 times. Transfer 10 µl of each 16S Barcode into respective sample-containing tubes.

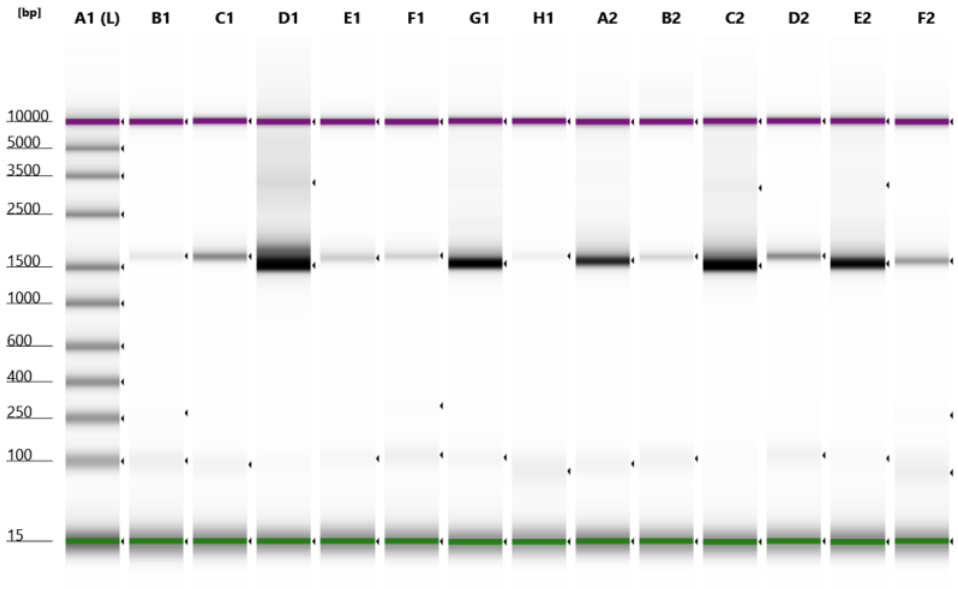
10. Ensure the components are thoroughly mixed by pipetting the contents of the tubes 10 times and spin down.

11. Amplify using the following cycling conditions:

STEP	TEMP	TIME
Initial Denaturation	95°C	1 minutes
30 Cycles	95°C 55°C 65°C	20 seconds 30 seconds 120 seconds
Final Extension	65°C	10 minutes
Hold	4-10°C	

TapeStation result : D5000 screenTape and reagents

Sample: 1 2 3 4 5 6 7 8 9 10 11 12 13



Note : Only samples showing good amplification were used for sequencing.
sample used : N°2, N°3, N°6, N°8, N°10, N°12

12. Add 4 µl of EDTA to each barcoded sample, mix thorougly by pipetting and spin down briefly.

13. Incubate for 5 minutes at room temperature.

14. Quantify 1 µl of each barcoded sample using a Qubit fluorometer for QC check.

N° sample	Sample name	Concentration (ng/µl)	180ng per sample (µl)
1	Leila	1.3	-
2	Adrien	6.6	27.0
3	ME_M	42.4	4.3
4	ME_S	2.6	-
5	Camille_Sophie forest sediment	2.9	-
6	Camille_Sophie canal water	26.4	6.8
7	Beatrice_Manon_SED	1.3	-
8	Beatrice_Manon_Forest	16.9	10.6
9	Lucille	3.8	-
10	Mathilde	45.0	4.0
11	Clara_Laura_C1	7.2	-
12	Clara_Laura_C2	22.2	8.1
13	Clara_Laura_B	4.84	-

15. Pool all barcoded samples in equimolar ratios in a 1.5 ml Eppendorf DNA LoBind tube.
16. Resuspend the AMPure XP Beads (AXP) by vortexing.
17. To the pool of barcoded samples, add a 0.6X volume ratio of resuspended AMPure XP Beads (AXP) and mix by pipetting.

    Total volume = 60.8 µl volume beads = 37 µl
18. Incubate at room temperature for 5 minutes.
19. Prepare 2 ml fresh 80% ethanol in nuclease-free water.
20. Place on a magnetic rack, allow beads to pellet and pipette off supernatant.
21. Keep the samples on the magnet and wash the beads with 1 ml of freshly prepared 80% ethanol without disturbing the pellets. Remove the ethanol using a pipette and discard.
22. Repeat the previous step.
23. Spin down and place the samples back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking.
24. Remove the samples from the magnetic rack and resuspend each pellet in EB buffer.
25. Incubate for 5 minutes at room temperature.
26. Pellet the beads on a magnetic rack until the eluate is clear and colourless.
27. Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify using a Qubit fluorometer.

- Qubit 1x dsDNA HS Assay kit
- 1 µl DNA is used for quantification
- ➢ Result = 44.2 ng/µl

28. Transfer 50 fmol (=50 ng) of your eluted sample into a clean 1.5 ml Eppendorf DNA LoBind tube. Make up the volume to 11 µl with Elution Buffer (EB).
    - 1.2 µl of eluate + 9.8 µl EB buffer

29. In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

Reagent	Volume
Rapid Adapter (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5 µl**

30. Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.

31. Mix gently by flicking the tube, and spin down.

32. Incubate the reaction for 5 minutes at room temperature.

Priming and loading the MinION and GridION Flow Cell

1. Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

2. To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at room temperature:

Reagents	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Final total volume in tube	**1,205 µl**

3. Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

4. Slide the flow cell priming port cover clockwise to open the priming port.

5. After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles.

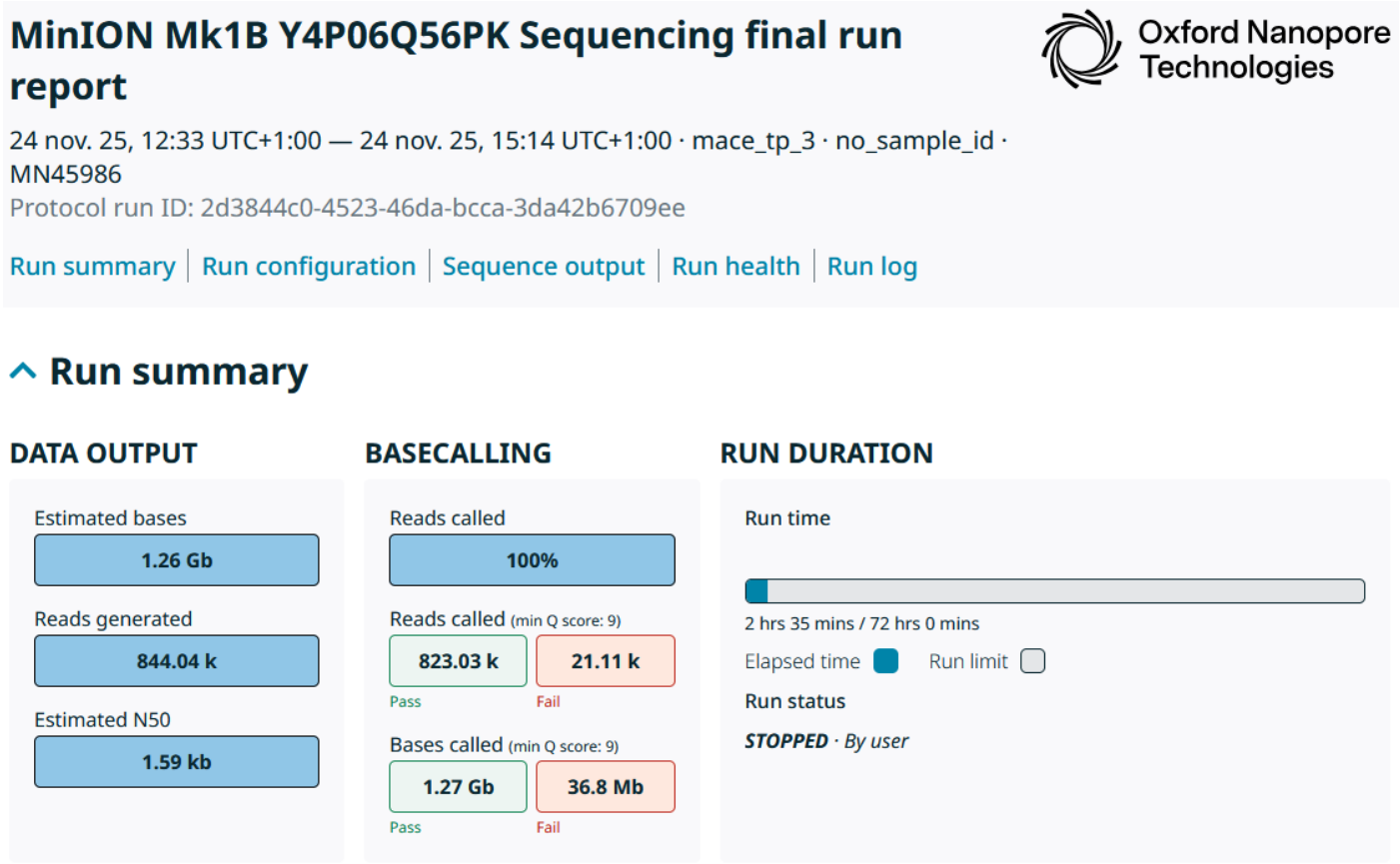
6. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.

7. Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

8. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

9. Gently lift the SpotON sample port cover to make the SpotON sample port accessible. Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
10. Mix the prepared library gently by pipetting up and down just prior to loading. Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
11. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.
12. Close the device lid and set up a sequencing run on MinKNOW.



Run configuration

RUN SETUP

Flow cell type	FLO-MIN114
Flow cell type alias	FLO-MIN114
Flow cell ID	FBE31539
Kit type	SQK-16S114-24

RUN SETTINGS

Run limit	72 hrs
Pore scan freq.	1.5 hrs
Reserved pores	On
Basecalling	High-accuracy model 400bps
Modified basecalling	Off
Trim barcodes	Off
Min Q score	9
Pooled data	Off

DATA OUTPUT SETTINGS

FAST5 output	4000 reads per file
FASTQ data output	One file per hour
POD5 data output	One file per hour, or 500000000 bases per batch
BAM file output	Off
Bulk file output	Off
Data location	/Library/MinKNOW/data/mace_tp_3/no_sample_id/20251124_1233_MN45986_FBE31539_2d3844c0

SOFTWARE VERSIONS

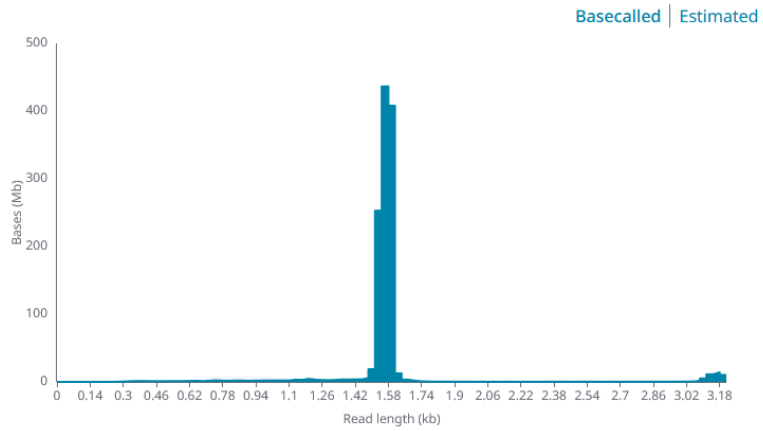
MinKNOW	25.09.16
Bream	8.8.3
Configuration	6.8.9
Dorado	7.11.2
MinKNOW Core	6.8.11

Sequence output

READ LENGTHS · OUTLIERS REMOVED

The read length graph shows the total number of bases vs the read length. The longest 1% of strands are classified as outliers, and excluded to allow focus on the main body of data.

N50*	% Basecalled
1.59 kb	100 %



*N50 calculated from basecalled read length histogram.

OUTLIERS

The longest 1% of strands are classified as outliers, and aggregated into groups to show their relative amounts.

Read length (kb)	Bases (Mb)
2.048 - 67.584	5.08
67.584 - 133.12	None
133.12 - 198.656	None
198.656 - 227.328	0.23

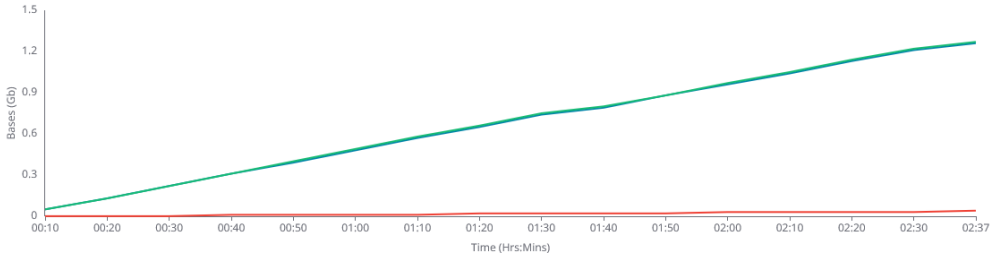
CUMULATIVE OUTPUT

The cumulative output shows the total amount of bases or reads sequenced over time by your device.

Bases

Legend

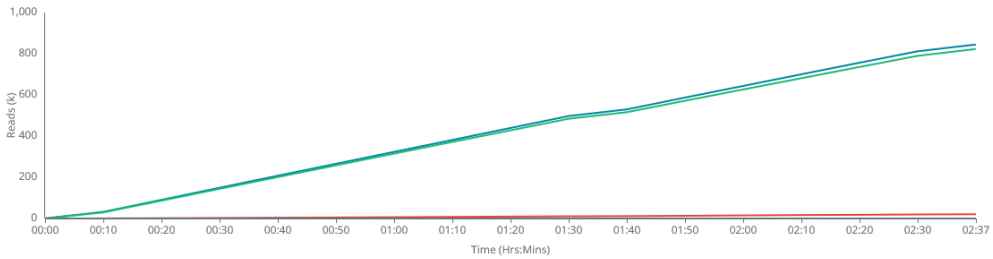
- Estimated — Predicted total number of bases, prior to basecalling
- Passed — Bases equal to or above the quality score threshold.
- Failed — Bases below the quality score threshold.



Reads

Legend

- Total — Total number of reads, including passed, failed and skipped.
- Passed — Reads equal to or above the quality score threshold.
- Failed — Reads below the quality score threshold.
- Skipped — Reads that will not be basecalled. Post run basecalling is possible.



BARCODES

Detected barcodes Bases graph Reads graph

Detected barcodes

The total number of bases and reads for each barcode detected are displayed in table below. Reads/bases must have a quality score above 9 to pass.

Unclassified data

35.68 k (4.3%)	67.71 Mb (5.3%)
Passed Reads	Passed Bases

Search barcodes

Export CSV

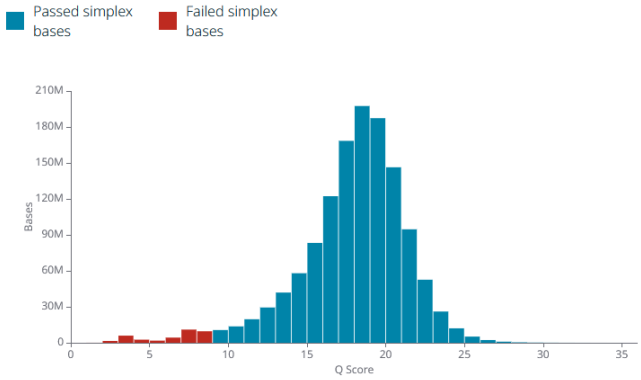
Barcode	Total bases (Mb)	Passed bases (%)	Total reads (k)	Passed reads (%)
barcode01	0.	0.00	0.	0.00
barcode02	134.25	98.70	85.30	98.80
barcode03	213.13	98.60	141.02	98.60
barcode04	0.01	100.00	0.01	100.00
barcode05	0.	100.00	0.	100.00
barcode06	204.59	98.60	132.53	98.60
barcode07	0.	0.00	0.	0.00
barcode08	206.75	98.50	134.59	98.60
barcode09	0.	0.00	0.	0.00
barcode10	208.37	98.40	137.79	98.40
barcode11	0.	34.60	0.	33.30
barcode12	255.51	98.60	167.16	98.70

First Prev 1 2 Next Last

QUALITY SCORE

The quality score is calculated as basecalling is performed on your device. Reads that fall below the minimum value of 9 will be classified as failed reads. You can alter the accepted minimum quality score in MinKNOW.

Q score histogram



PORE SCAN

A Pore scan is performed at configurable time intervals to determine the current status of pores within channels on a Flow Cell. For this run a Pore scan is performed every 1.5 hrs.

Legend

Pore available	Reserved pore	Unavailable	Saturated	Zero	Inactive
Pore in channel available for sequencing	Pore in reserve, will return to available when required	Pore inhibited from sequencing	Possible contamination in the sample	No current is passing through this pore, possibly due to bubbles on the membrane	Pore no longer suitable for further sequencing