4- Oxford Nanopore Technologies (ONT) Library Prep & Sequencing

Special focus on SARS-CoV-2 genome sequencing protocols

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ONT Sequencing

Oxford NANOPORE Technologies

PromethION, GridION, MinION Mk 1C, MinION Mk 1B, Flongle

ONT Sequencing Overview

1 minute, 41 secs

https://www.youtube.com/watch?v=RcP85JHLmnI

ONT Sequencing Overview- Long Version

6 minutes, 33 secs

https://www.youtube.com/watch?v=sv9fFeSd3kE

Oxford Nanopore Library Prep

	Ligation Sequencing Kit	Rapid /Field Sequencing Kit	Ultra-Long DNA Sequencing Kit	PCR Sequencing Kit	Rapid PCR Sequencing Kit
Preparation time	60 min	10 min	610 min	60 min + PCR	15 min + PCR
Input requirement	1,000 ng HMW gDNA	400 ng HMW gDNA	Tissue culture cells: 6 million Gram-positive and -negative bacteria: ~2 ml of OD1 culture Blood: 1–2 ml	100 ng	10 ng
Fragmentation	Optional	Transposase based	Transposase based	N/A	Transposase based
Read length	Equal to fragment length	Random distribution, dependent on input fragment length	0–100+ kb N50 (R9.4.1 flow cells)	Equal to fragment length post-PCR	~2-5 kb
Multiplexing options	Yes	Yes	In development	Yes	This kit offers barcoding for up to twelve samples

https://nanoporetech.com/products/kits

SARS-CoV-2 Genome Sequencing
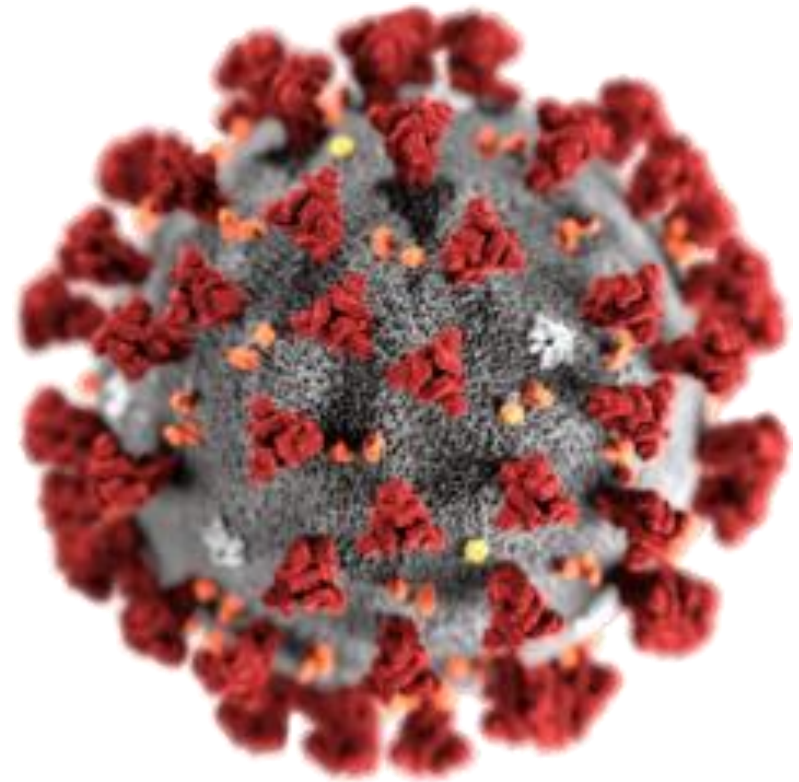
ONT SARS-CoV-2 Sequencing Options

▶ **Sequencing of SARS-CoV-2: how does it work?**

▶ There are two methods available for whole-genome nanopore sequencing of SARS-CoV-2: Midnight and ARTIC Classic. Both methods employ a PCR tiling approach in which the viral genome is amplified in overlapping sections, maximising coverage across the full genome.

▶ **ARTIC Classic** was the first SARS-CoV-2 nanopore sequencing protocol to be utilised, and has been used by scientists around the world. In this method, the SARS-CoV-2 genome is amplified in ~400 bp fragments. This shorter length may help improve coverage for RNA samples that are likely to be degraded - for example, due to freeze-thaw cycles or storage at temperatures above -80°C.

▶ **Midnight** is a simple, rapid method of sequencing SARS-CoV-2 genomes at low cost per sample. The approach is highly flexible, allowing the on-demand sequencing of small numbers of samples or scaling up to high-throughput sequencing needs. Hands-on time is also minimal, facilitating automation. In the Midnight protocol, the SARS-CoV-2 genome is amplified in ~1,200 bp overlapping segments, making it more resilient to drop-out caused by mutations in the viral genome.
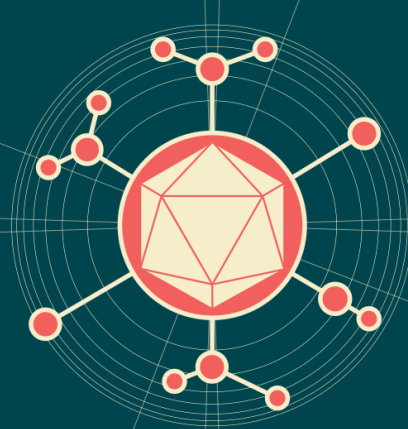
https://nanoporetech.com/covid-19

SARS-CoV-2 Primer Sets

Primer Set	Amplicon Length	Protocol
ARTIC v3	~400	Ligation
ARTIC v4	~400	Ligation
ARTIC v4.1	~400	Ligation
Midnight	~1200	Ligation/ Rapid Barcoding

ARTIC Network

https://artic.network/
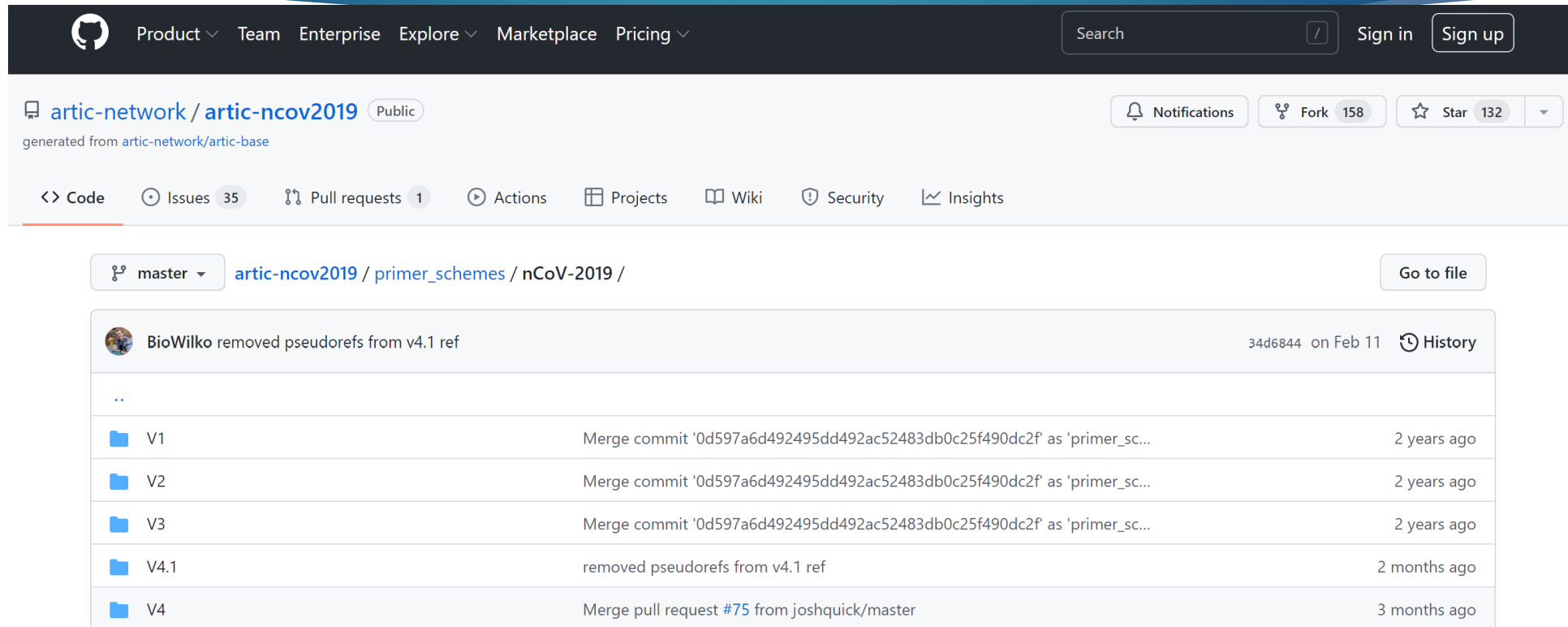
https://artic.network/ncov-2019

ARTICnetwork

REAL-TIME MOLECULAR EPIDEMIOLOGY FOR OUTBREAK RESPONSE

ARTIC Primer Sets

https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019

ARTIC vs Midnight

	Midnight	ARTIC Classic
Experience level required	●●○○	●●●○
Third-party reagent usage	○○○○	●●●○
Amplicon length generated	1,200 bp	400 bp
Normalisation step included	No	Yes
Library prep method	Rapid	Ligation
Turnaround time of workflow	●●○○	●●●●
Cost per sample (including third-party reagents)	●○○○	●●○○

https://nanoporetech.com/covid-19

ARTIC v4.1

209 lines (209 sloc)		14.9 KB				
1	MN908947.3	25	50	SARS-CoV-2_1_LEFT	1	+	AACAAACCAACCAACTTTCGATCTC
2	MN908947.3	324	344	SARS-CoV-2_2_LEFT	2	+	TTTACAGGTTCGCGACGTGC
3	MN908947.3	408	431	SARS-CoV-2_1_RIGHT	1	-	CTTCTACTAAGCCACAAGTGCCA
4	MN908947.3	644	666	SARS-CoV-2_3_LEFT	1	+	GTAATAAAGGAGCTGGTGGCCA
5	MN908947.3	705	727	SARS-CoV-2_2_RIGHT	2	-	ATAAGGATCAGTGCCAAGCTCG
6	MN908947.3	944	966	SARS-CoV-2_4_LEFT	2	+	GTGTATACTGCTGCCGTGAACA
7	MN908947.3	1017	1044	SARS-CoV-2_3_RIGHT	1	-	GCCAATTTAATTTCAAAAGGTGTCTGC
8	MN908947.3	1245	1266	SARS-CoV-2_5_LEFT	1	+	TGAAACTTCATGGCAGACGGG
9	MN908947.3	1337	1362	SARS-CoV-2_4_RIGHT	2	-	ACAACAGCATTTTGGGGTAAGTAAC
10	MN908947.3	1540	1562	SARS-CoV-2_6_LEFT	2	+	CGTGCTAGCGCTAACATAGGTT

https://github.com/artic-network/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V4.1/SARS-CoV-2.primer.bed

Updates v4.1

- Added to pool 1:
- SARS-CoV-2_23_RIGHT_alt1
- SARS-CoV-2_27_RIGHT_alt1
- SARS-CoV-2_79_RIGHT_alt1
- SARS-CoV-2_89_LEFT_alt1
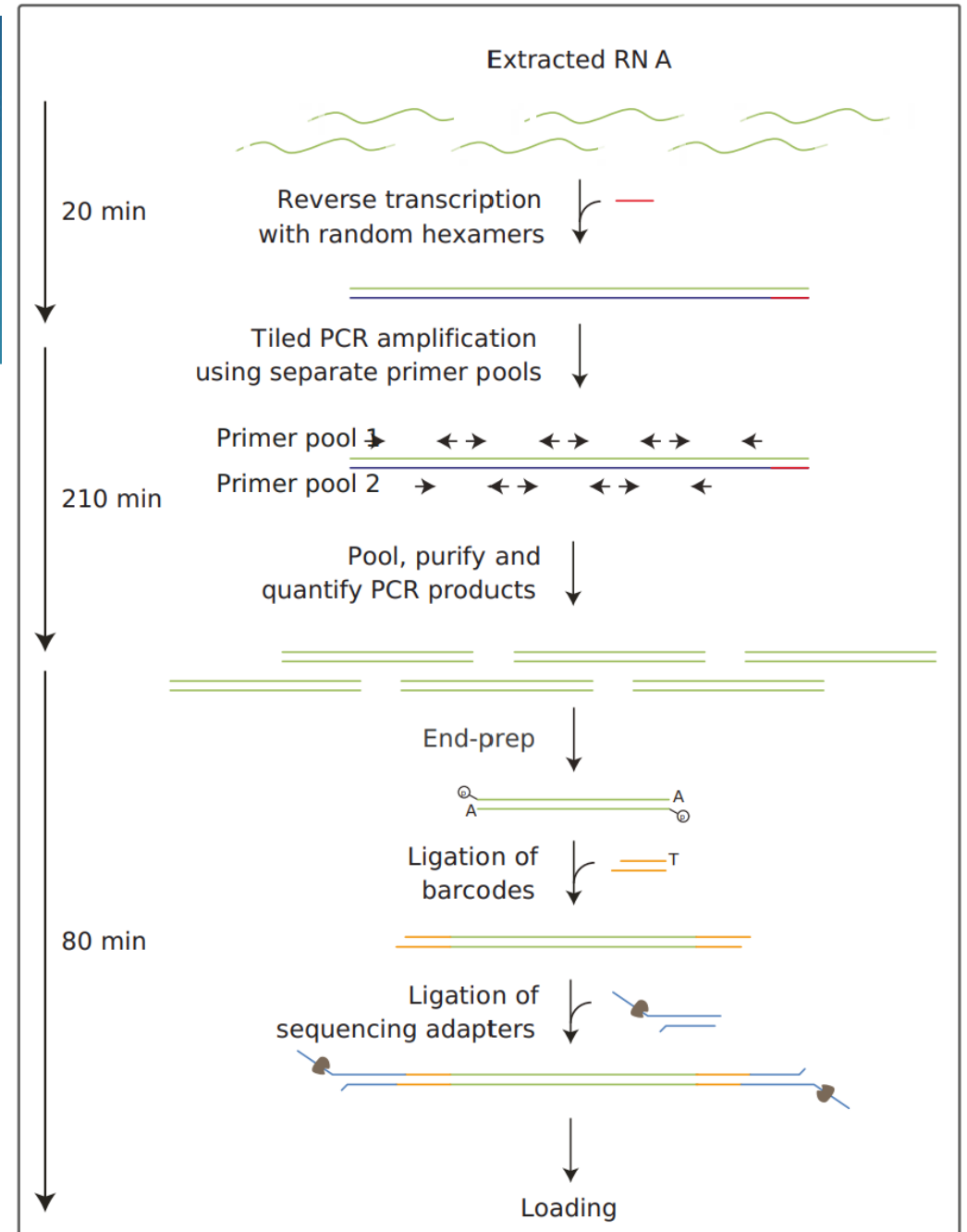- SARS-CoV-2_89_RIGHT_alt1

- Added to pool 2:
- SARS-CoV-2_10_LEFT_alt1
- SARS-CoV-2_10_RIGHT_alt1
- SARS-CoV-2_76_LEFT_alt1
- SARS-CoV-2_76_RIGHT_alt1
- SARS-CoV-2_88_LEFT_alt1
- SARS-CoV-2_90_RIGHT_alt1

Updates v4.1
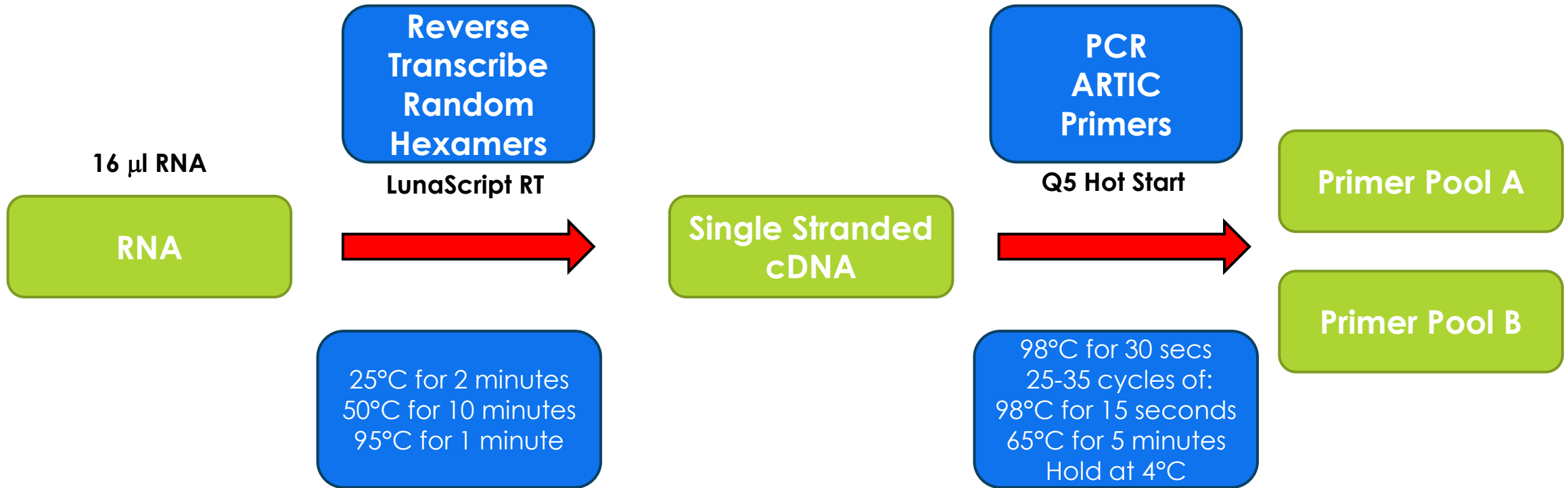
- 2x volume:
- SARS-CoV-2_1_LEFT & SARS-CoV-2_1_RIGHT
- SARS-CoV-2_7_LEFT & SARS-CoV-2_7_RIGHT
- SARS-CoV-2_13_LEFT & SARS-CoV-2_13_RIGHT
- SARS-CoV-2_17_LEFT & SARS-CoV-2_17_RIGHT
- SARS-CoV-2_27_LEFT & SARS-CoV-2_27_RIGHT
- SARS-CoV-2_45_LEFT & SARS-CoV-2_45_RIGHT

- SARS-CoV-2_59_LEFT & SARS-CoV-2_59_RIGHT
- SARS-CoV-2_60_LEFT & SARS-CoV-2_60_RIGHT
- SARS-CoV-2_61_LEFT & SARS-CoV-2_61_RIGHT
- SARS-CoV-2_64_LEFT & SARS-CoV-2_64_RIGHT
- SARS-CoV-2_79_LEFT & SARS-CoV-2_79_RIGHT
- SARS-CoV-2_90_LEFT & SARS-CoV-2_90_RIGHT
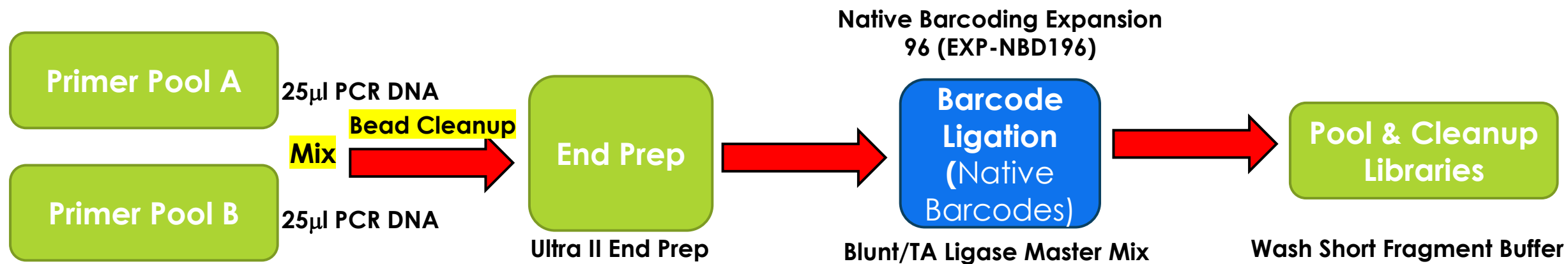- SARS-CoV-2_91_LEFT & SARS-CoV-2_91_RIGHT

ARTIC Ligation

- Reverse Transcription
- SARS-CoV-2 PCR, 2 Pools
- Pool PCR Amplicons
- End Prep
- Ligation of Barcodes
- Ligation of Sequencing Adaptors
- Sequencing

ARTIC Ligation

16 µl RNA

RNA

Reverse Transcribe Random Hexamers

LunaScript RT

25°C for 2 minutes
50°C for 10 minutes
95°C for 1 minute

Single Stranded cDNA

PCR ARTIC Primers

Q5 Hot Start

98°C for 30 secs
25-35 cycles of:
98°C for 15 seconds
65°C for 5 minutes
Hold at 4°C

Primer Pool A

Primer Pool B

ARTIC Ligation

Primer Pool A

25μl PCR DNA

Mix

Bead Cleanup

Primer Pool B

25μl PCR DNA

End Prep

Ultra II End Prep

Native Barcoding Expansion 96 (EXP-NBD196)

Barcode Ligation (Native Barcodes**)**

Blunt/TA Ligase Master Mix

Pool & Cleanup Libraries

Wash Short Fragment Buffer

ARTIC Ligation

Eluted DNA → Adapter Ligation (AMII) → Bead Clean Up → Elute ONT Library for Sequencing
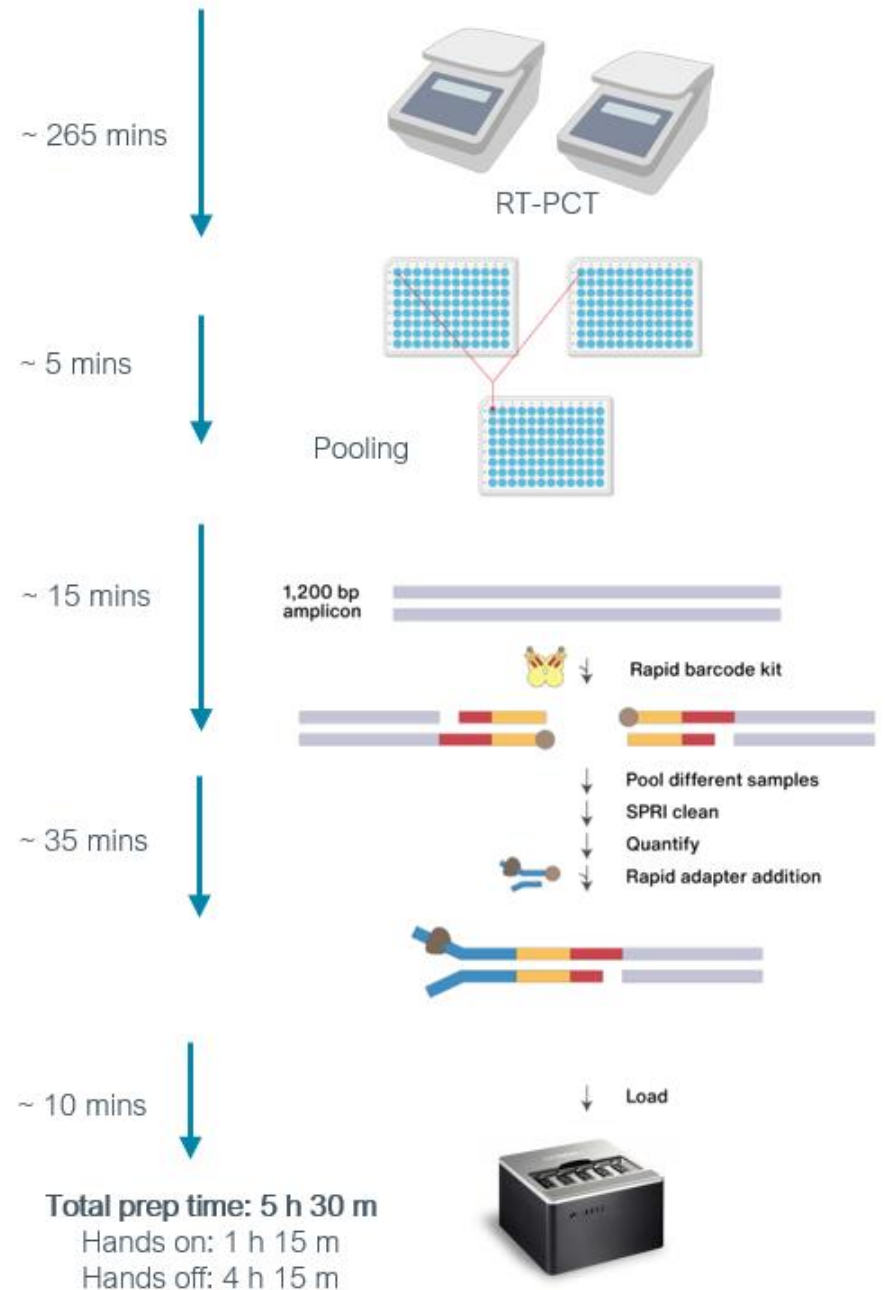
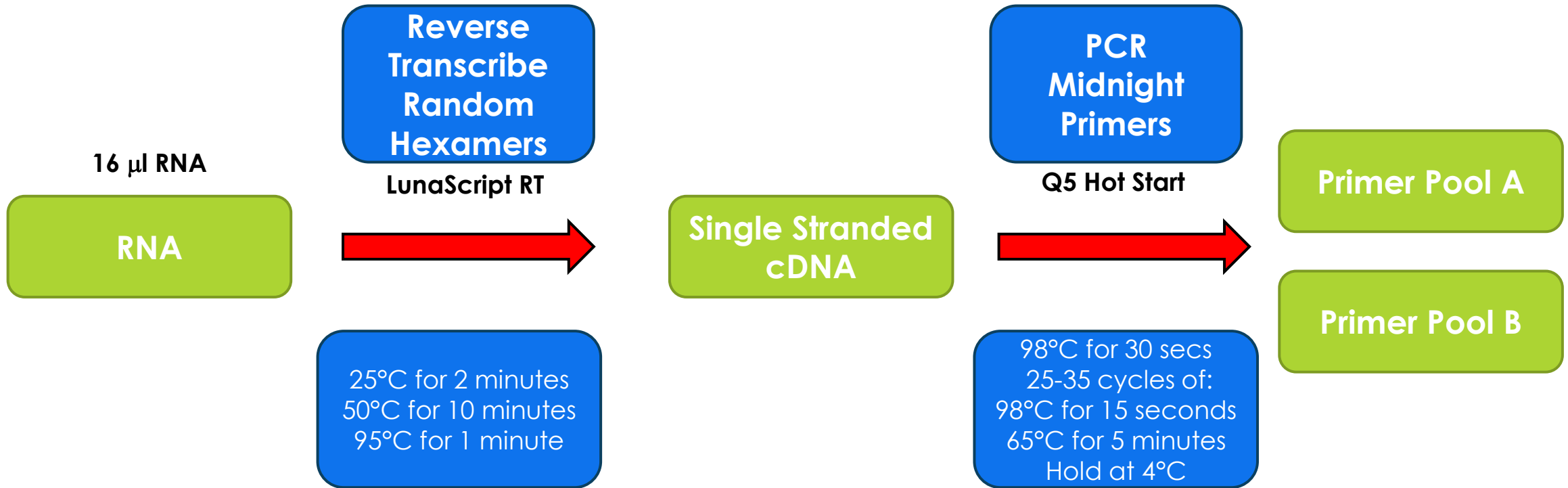**NEBNext Quick T4 Ligase Master Mix**

Wash Ethanol
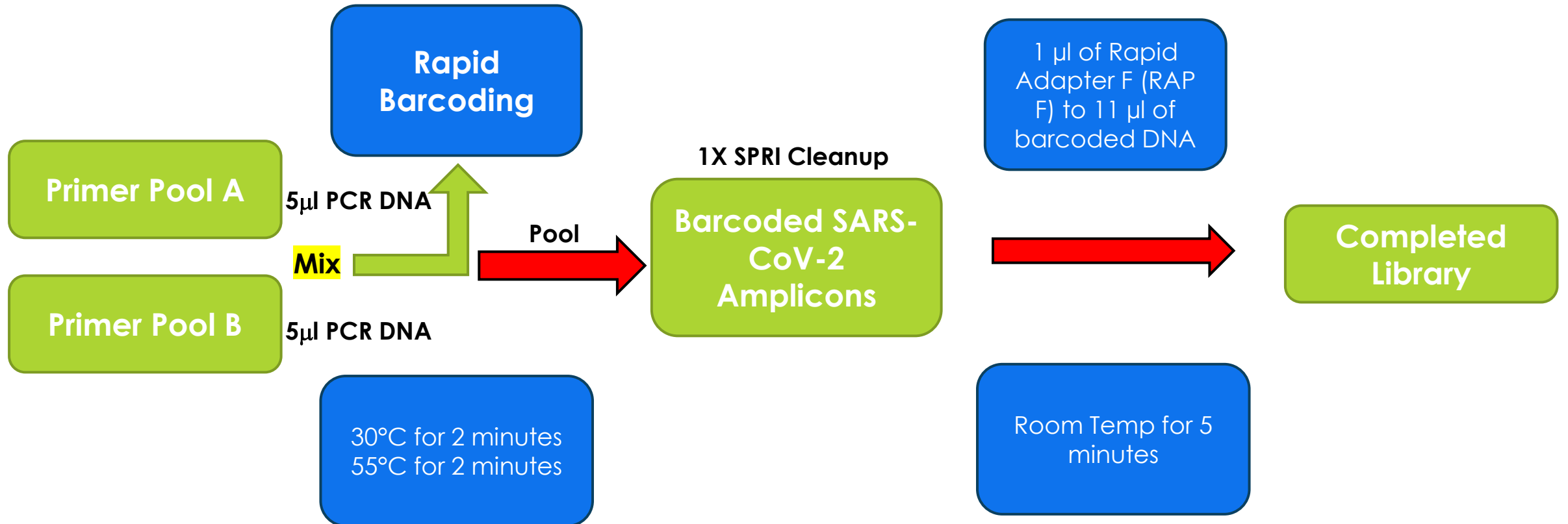
Midnight Rapid Barcoding

- **Reverse Transcription**
- **SARS-CoV-2 PCR, 2 Pools**
- **Pool PCR Amplicons**
- **Transposase Fragmentation**
- **Rapid Adapter Ligation**
- **Sequencing**

~ 265 mins

RT-PCT

~ 5 mins

Pooling

~ 15 mins

1,200 bp amplicon

Rapid barcode kit

Pool different samples
SPRI clean
Quantify
Rapid adapter addition

~ 35 mins

~ 10 mins

Load

Total prep time: 5 h 30 m
Hands on: 1 h 15 m
Hands off: 4 h 15 m

Midnight Rapid Barcoding

16 µl RNA

RNA

Reverse Transcribe Random Hexamers

LunaScript RT

25°C for 2 minutes
50°C for 10 minutes
95°C for 1 minute

Single Stranded cDNA

PCR Midnight Primers

Q5 Hot Start

98°C for 30 secs
25-35 cycles of:
98°C for 15 seconds
65°C for 5 minutes
Hold at 4°C

Primer Pool A

Primer Pool B

Midnight Rapid Barcoding

Primer Pool A

Primer Pool B

5µl PCR DNA

5µl PCR DNA

Mix

Rapid Barcoding

30°C for 2 minutes
55°C for 2 minutes

Pool

1X SPRI Cleanup

Barcoded SARS-CoV-2 Amplicons

1 µl of Rapid Adapter F (RAP F) to 11 µl of barcoded DNA

Room Temp for 5 minutes

Completed Library

Prepackaged SARS-CoV-2 Kits

Name	Product #	Samples	Flowcells
COVID Mini	C19MINI	576	6
COVID Midi	C19MIDI	2,304	24
COVID Maxi	C19MAXI	9,216	96

ONT Flowcells

Waste port 2

Waste port 1

Priming port cover

SpotON activator

SpotON sample port cover

Waste channel

Close both i. the Priming port and ii. the SpotON sample port*

Sensor array

FAB20748
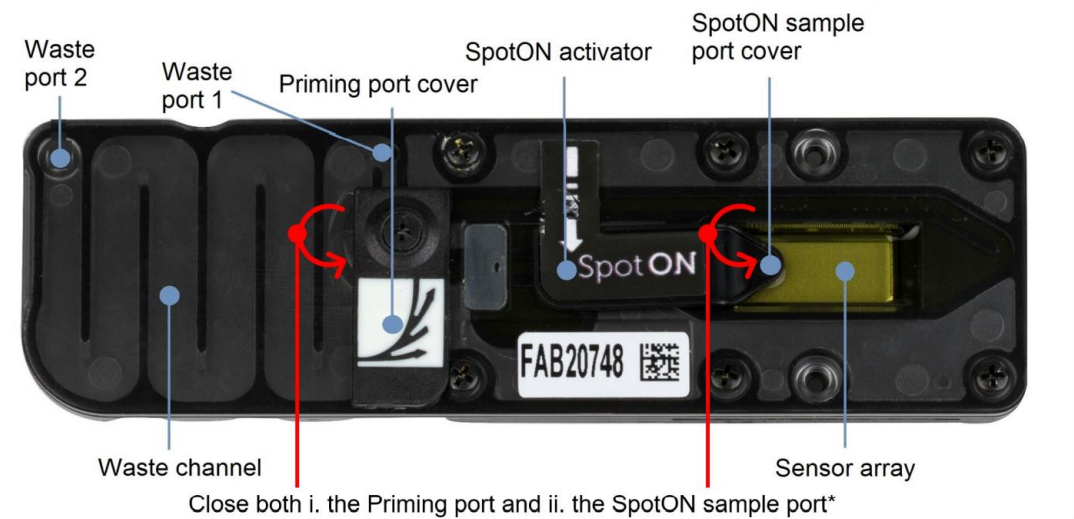
*Both ports are shown in a closed position

Flow Cell Pores

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

Waste port 2 · Waste port 1 · Priming port cover · SpotON activator · SpotON sample port cover

Waste channel · Close both i. the Priming port and ii. the SpotON sample port* · Sensor array
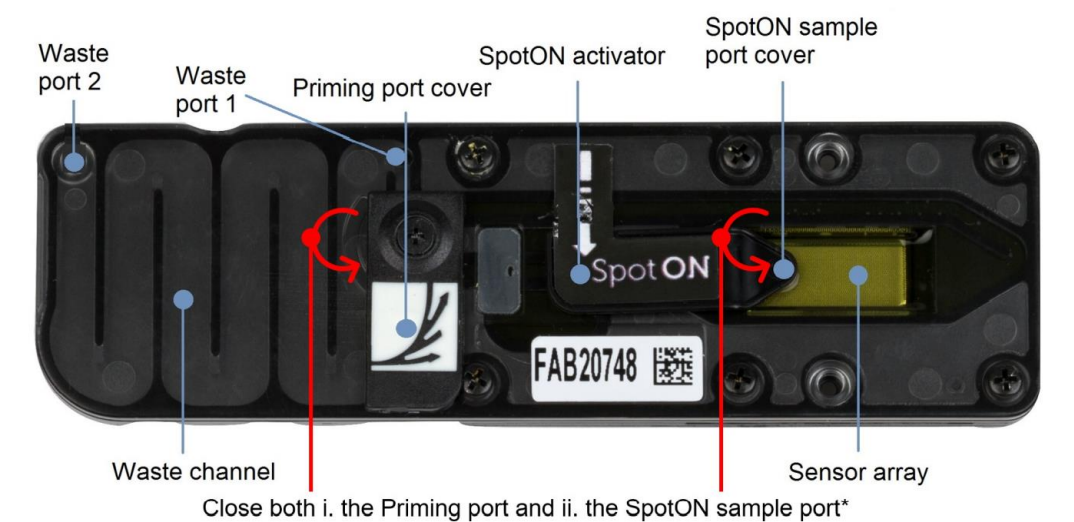
*Both ports are shown in a closed position

Flowcell Loading

Flowcell Priming

Waste port 2 · Waste port 1 · Priming port cover · SpotON activator · SpotON sample port cover

Waste channel · Close both i. the Priming port and ii. the SpotON sample port* · Sensor array

FAB20748
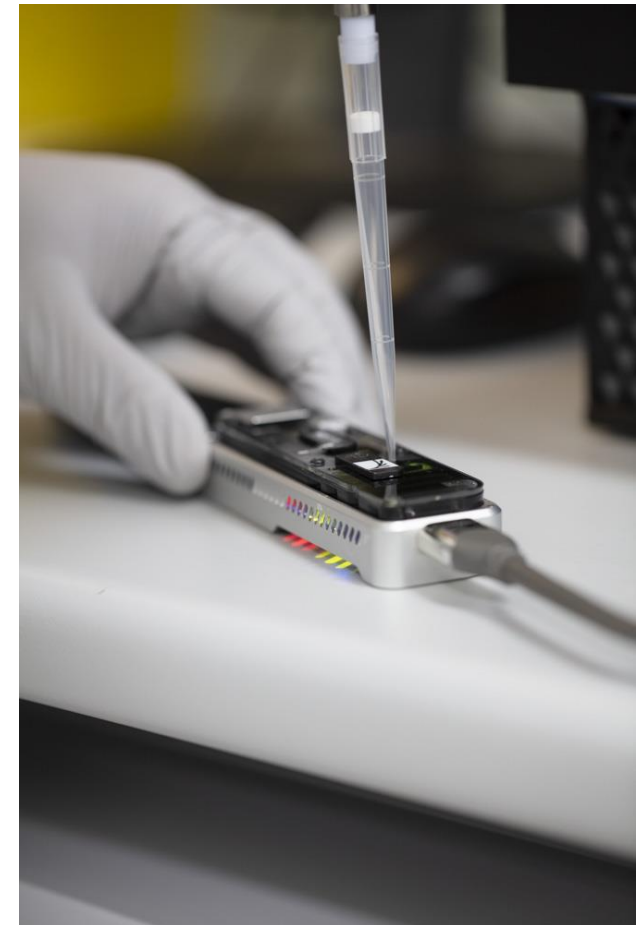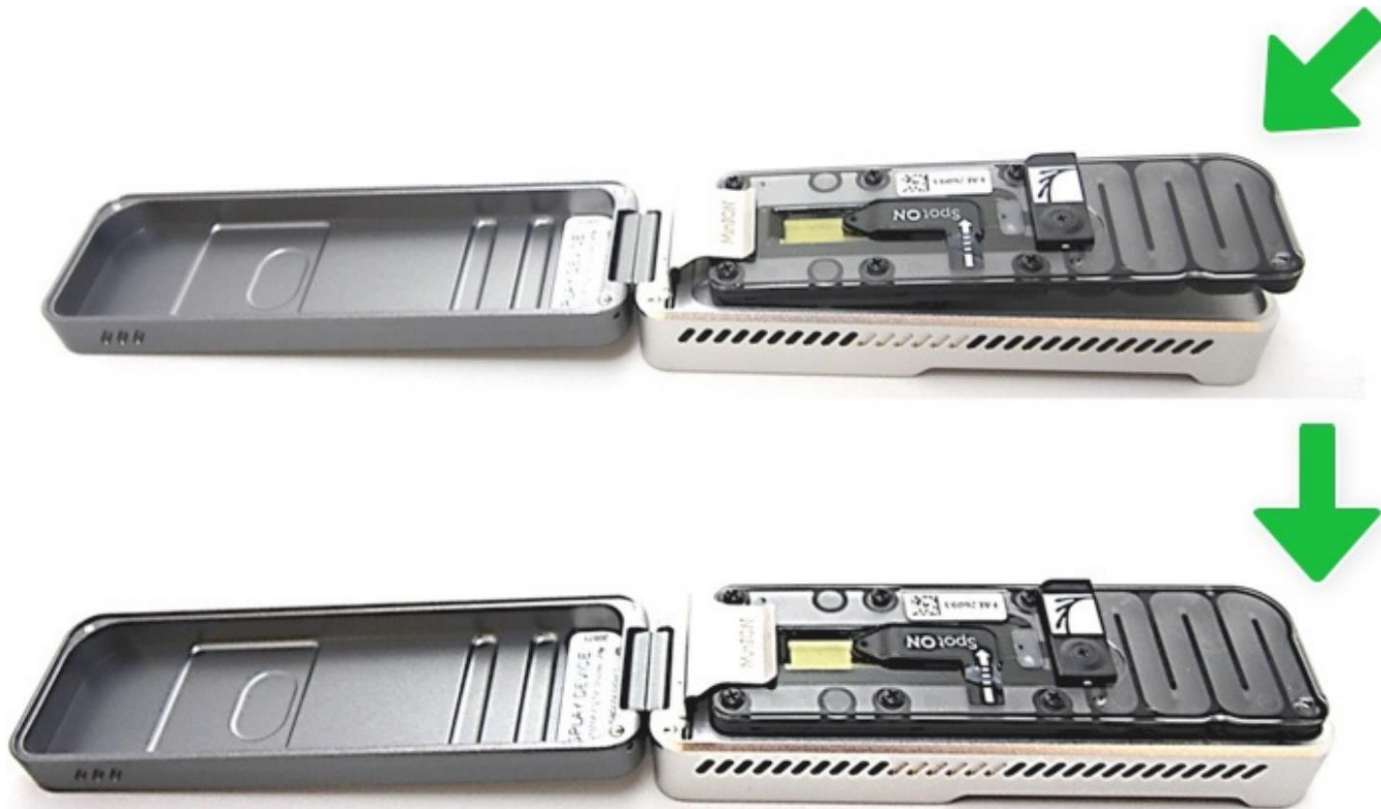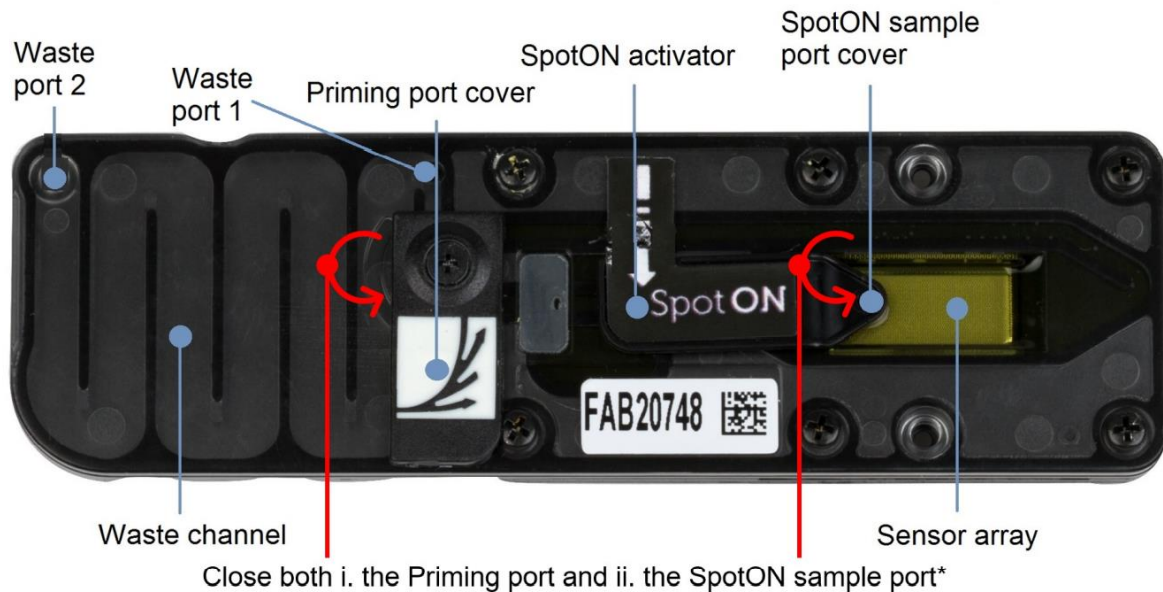
*Both ports are shown in a closed position

After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl): 1. Set a P1000 pipette to 200 µl 2. Insert the tip into the priming port 3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip

Flowcell Priming
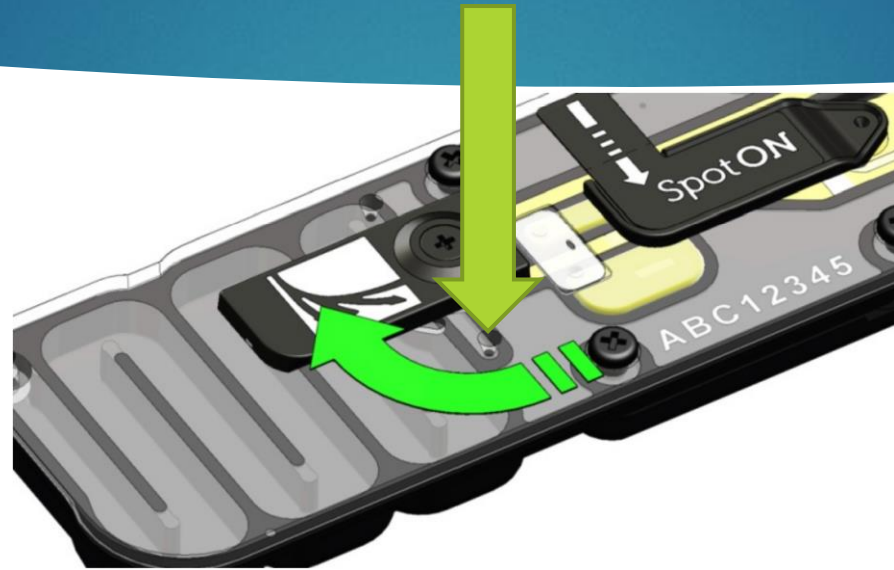
To prepare the flow cell priming mix, add 30 μl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.

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

During this time, prepare the library for loading by following the steps below.

Prepare Library for Sequencing

Reagent	Volume
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
Total	**75 µl**

Flowcell Priming
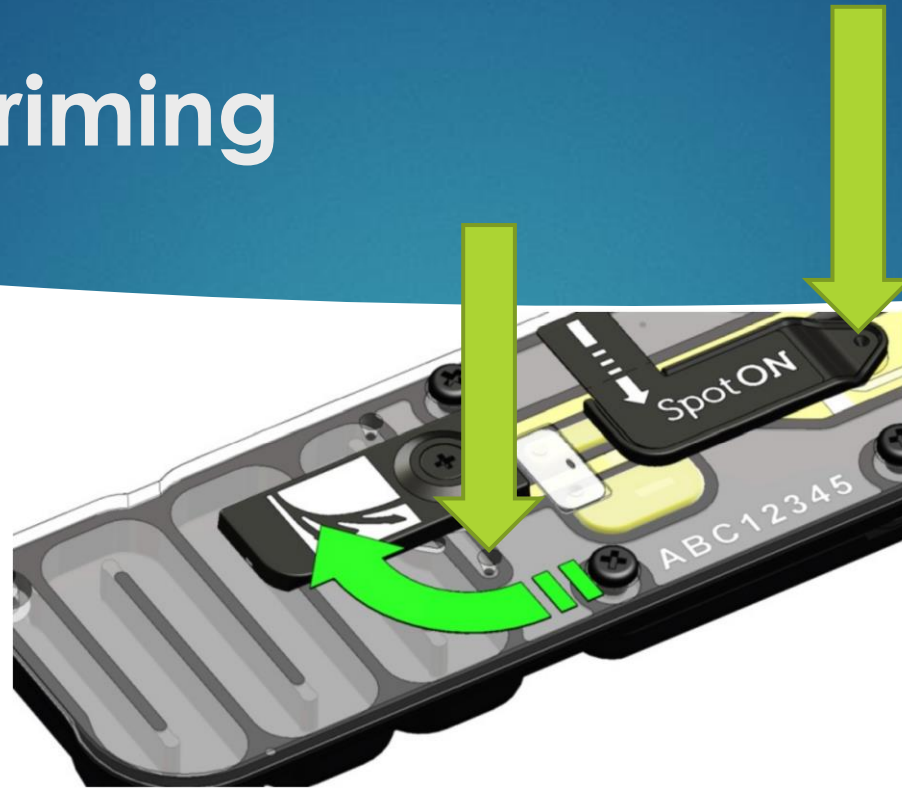
Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

Loading Library
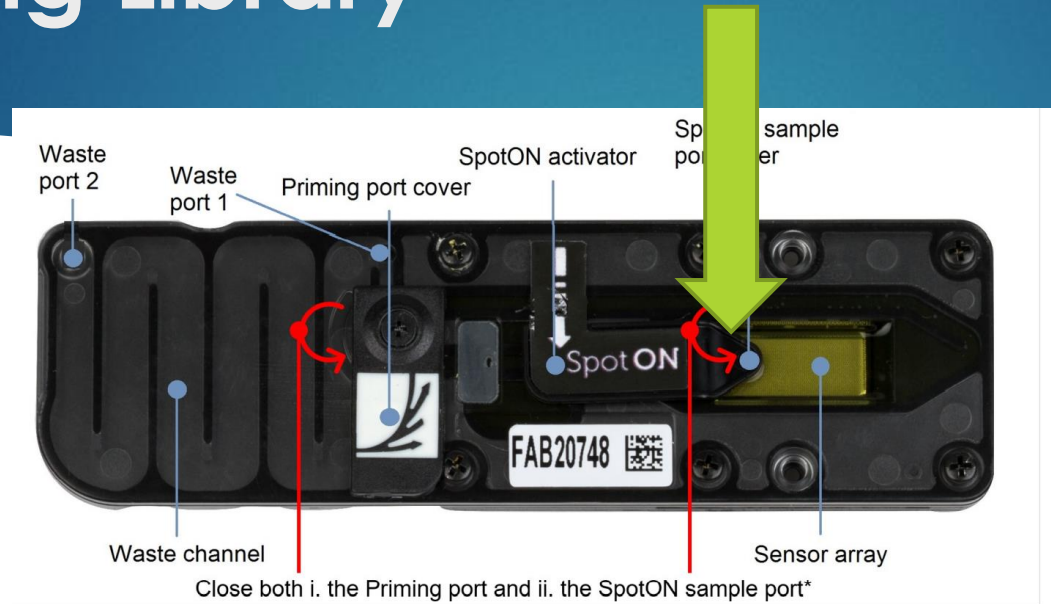
Mix the prepared library gently by pipetting up and down just prior to loading.

Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

ONT Sequencing & Metrics
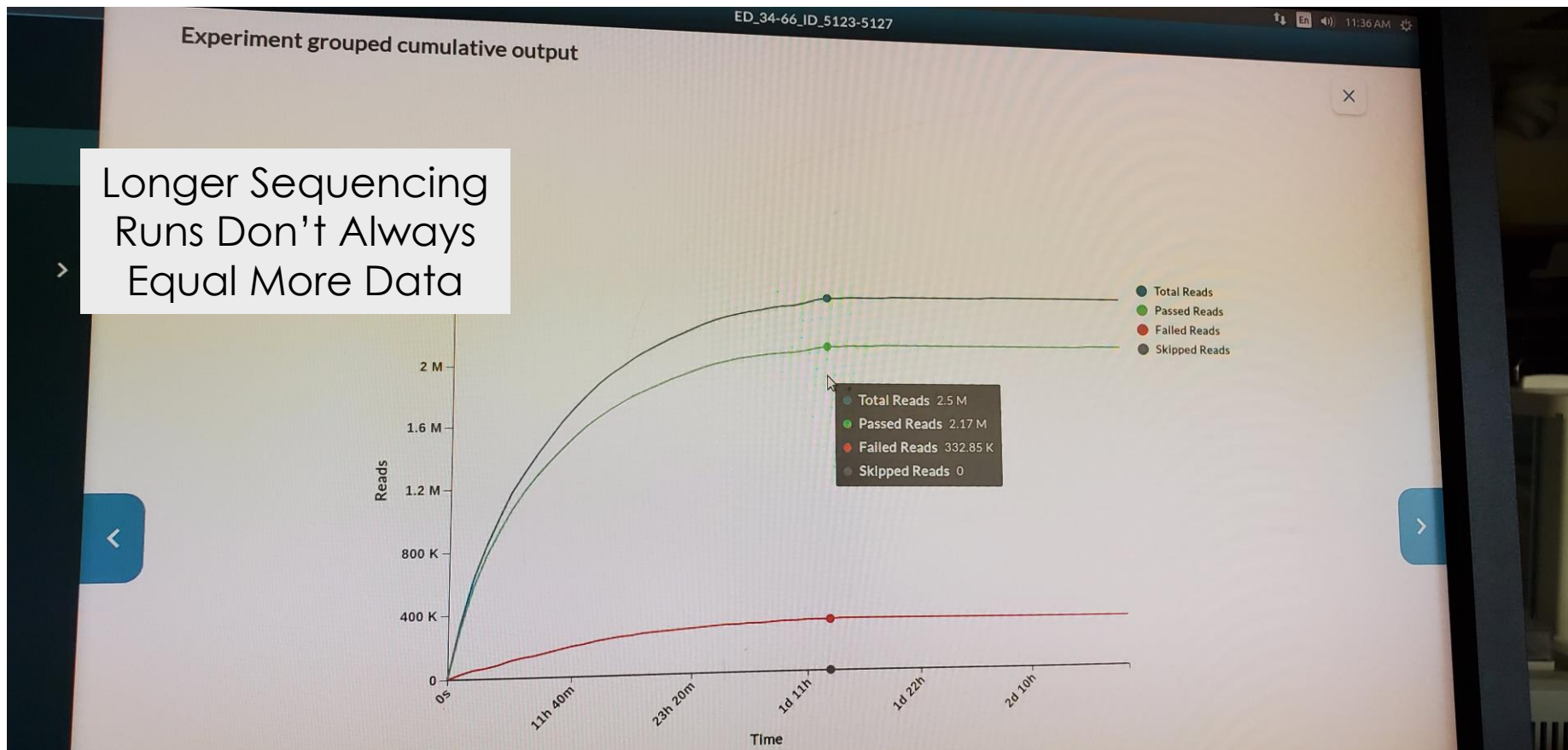
Pore Channels Panel
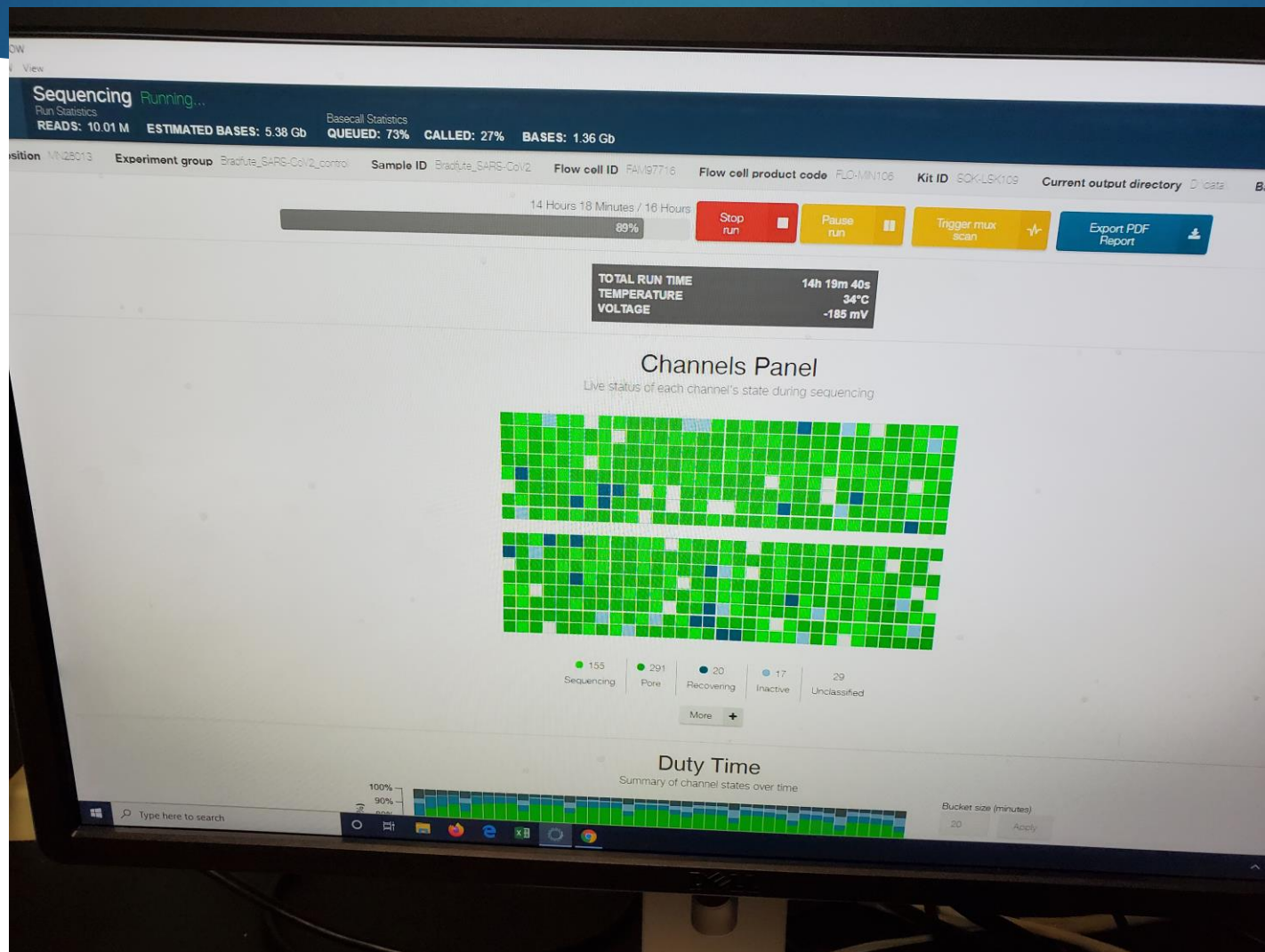
Pore Channels Panel
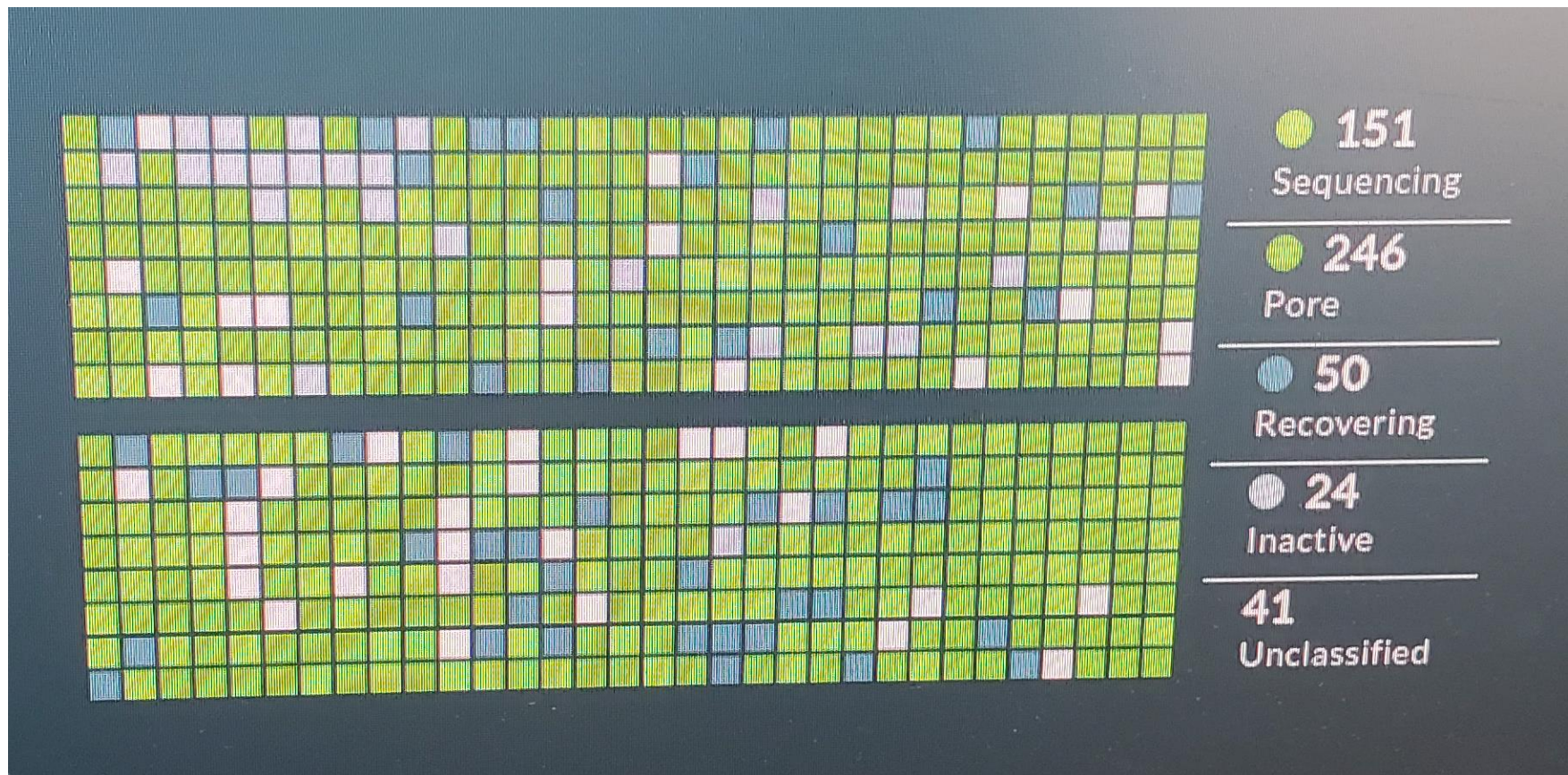
Read Output

Cumulative Output Reads
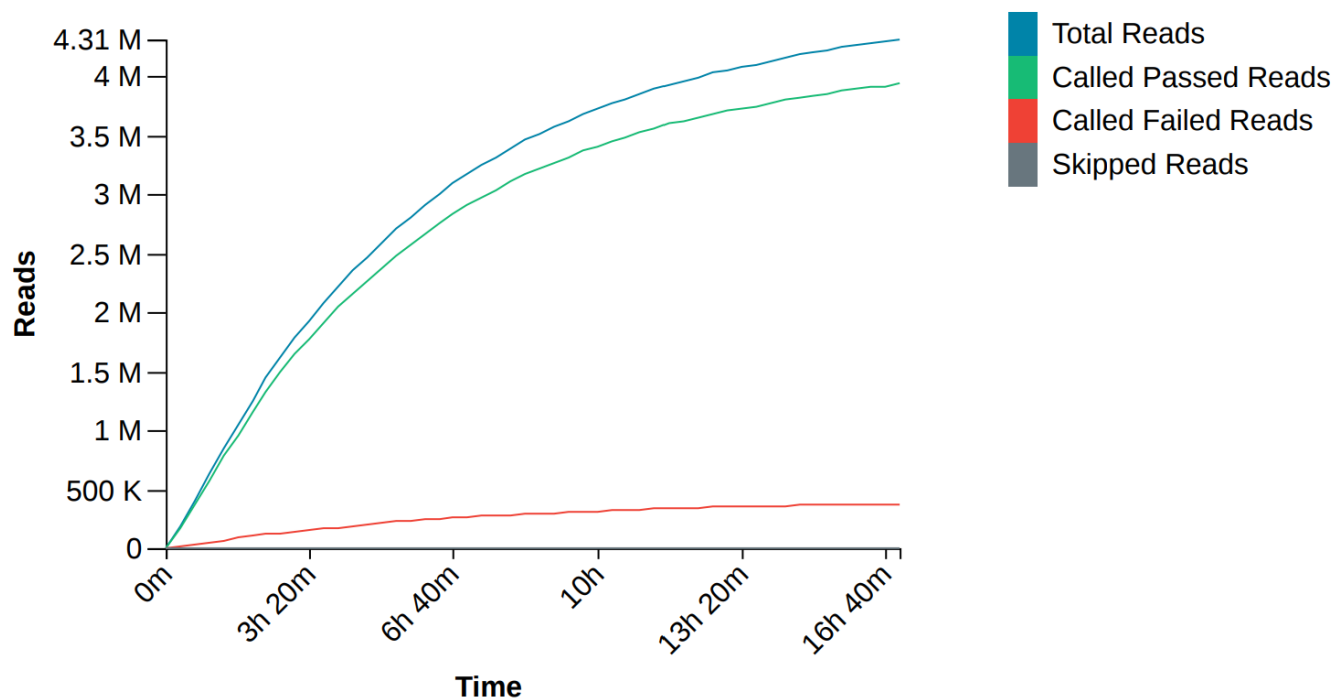
Total Reads
Called Passed Reads
Called Failed Reads
Skipped Reads

17 hour run time
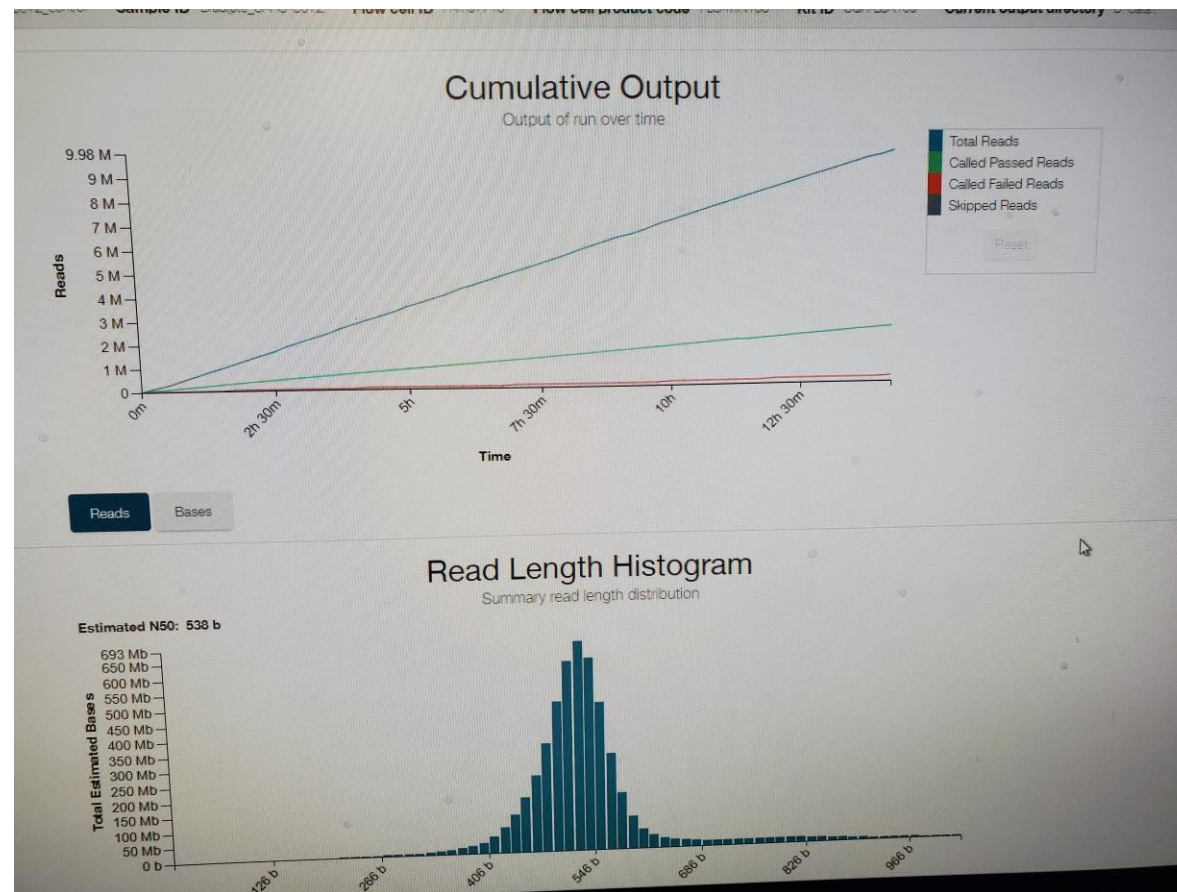
Base Output

Cumulative Output Bases
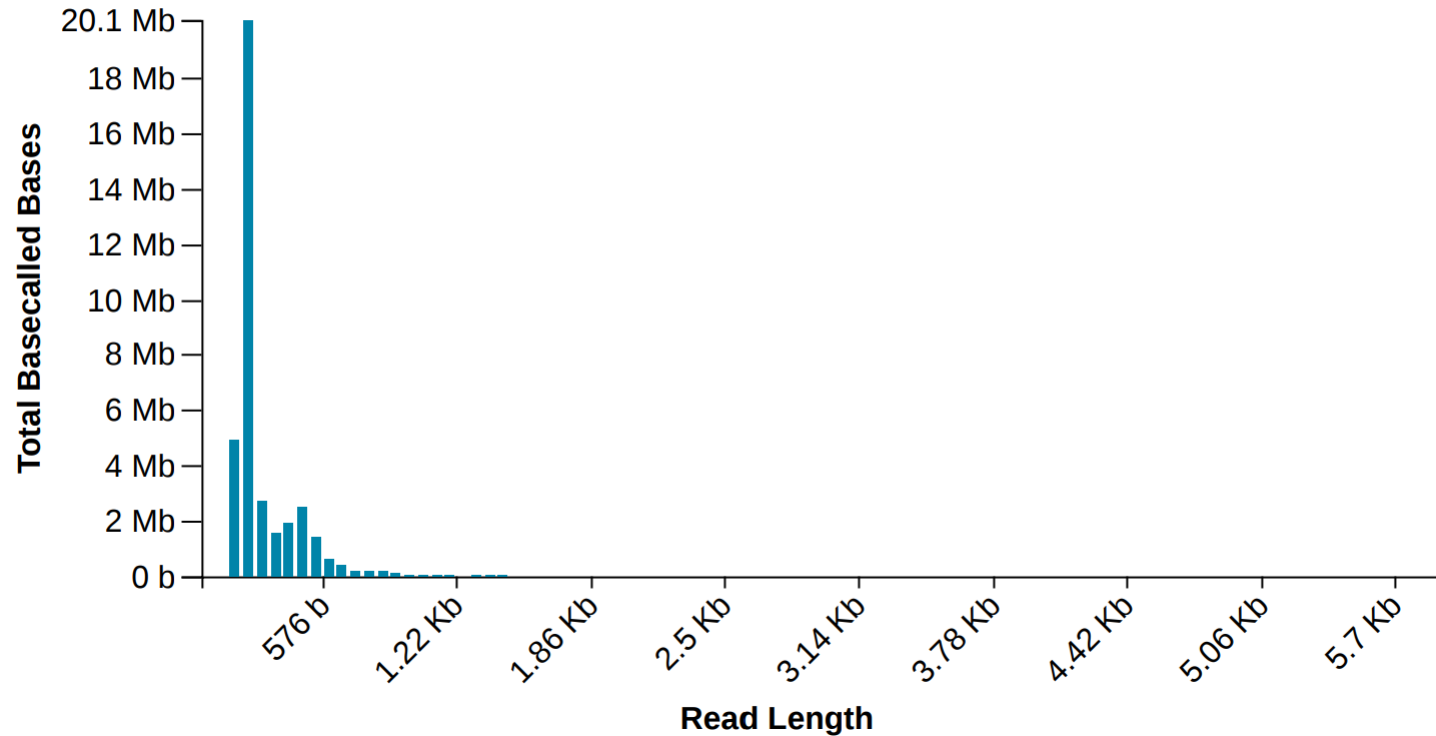

Estimated Bases
Called Bases
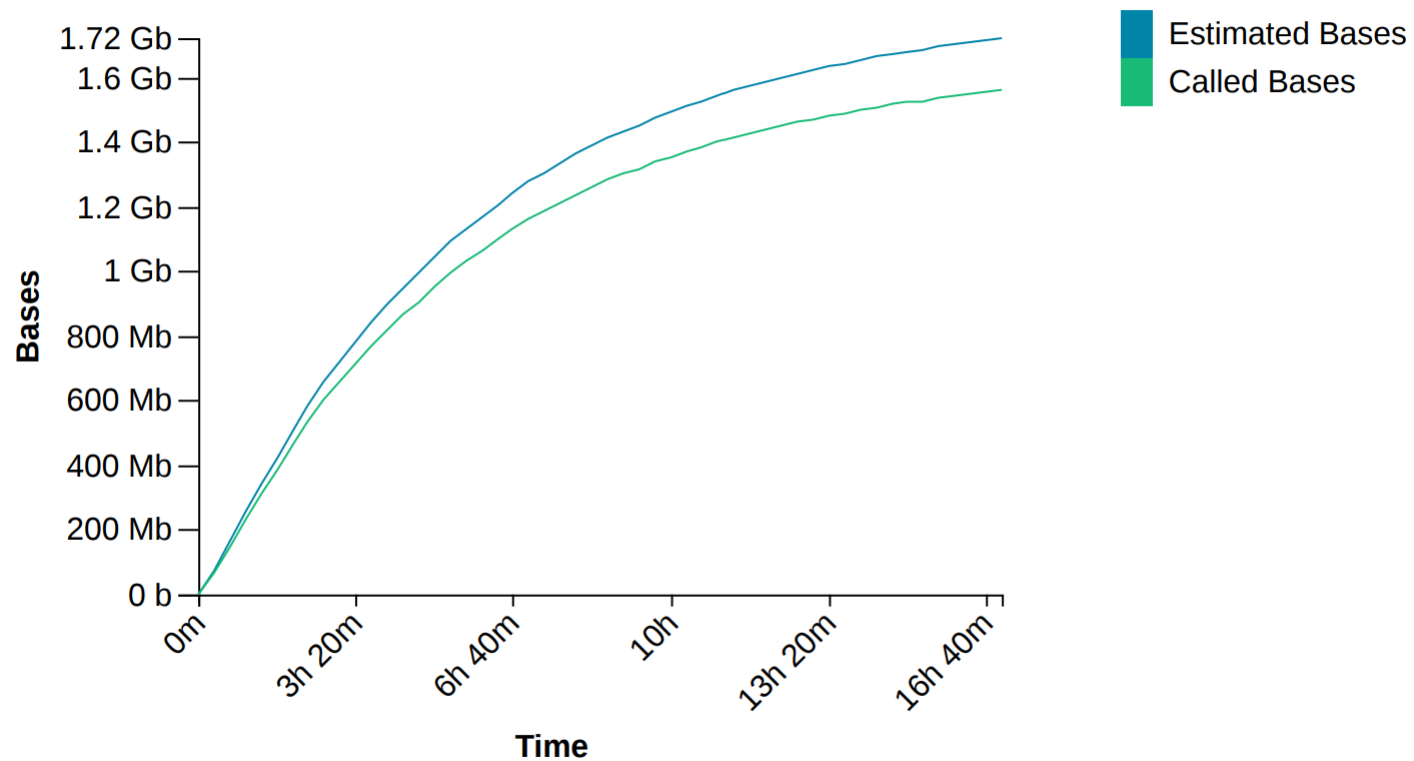
17 hour run time

Read Length Histogram

Read Length- ARTIC v2
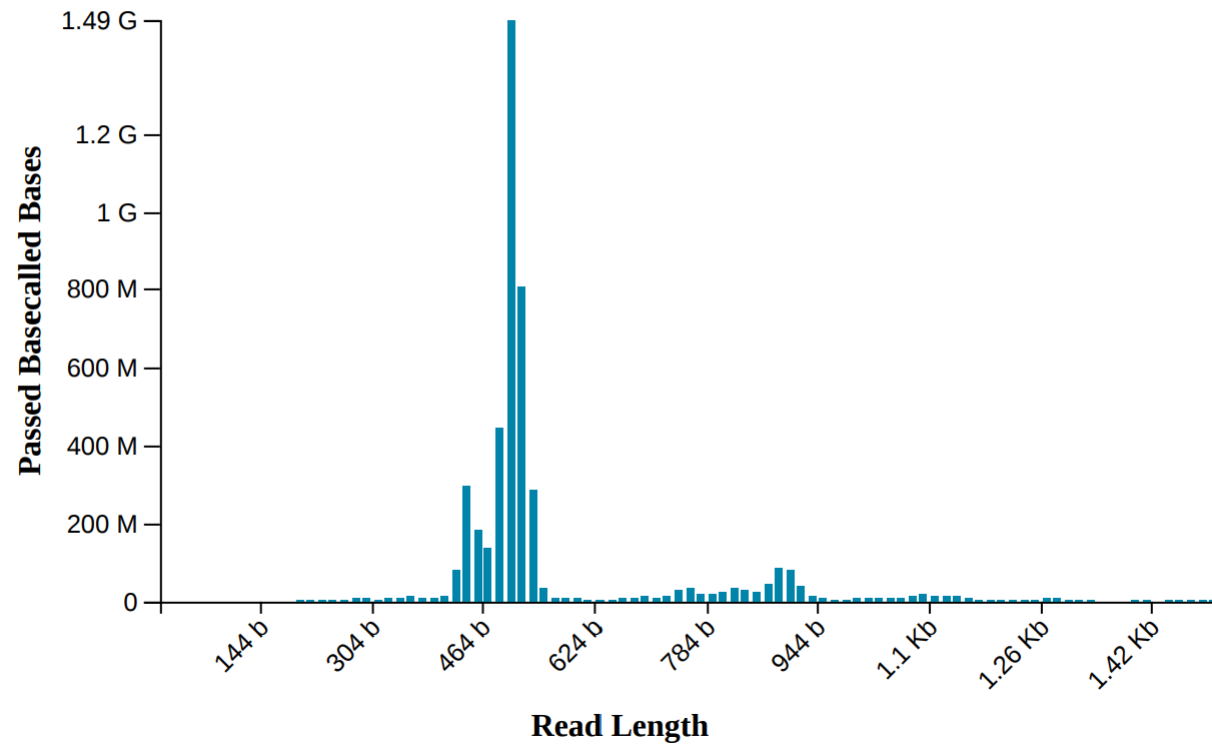
Read Length Histogram Basecalled Bases
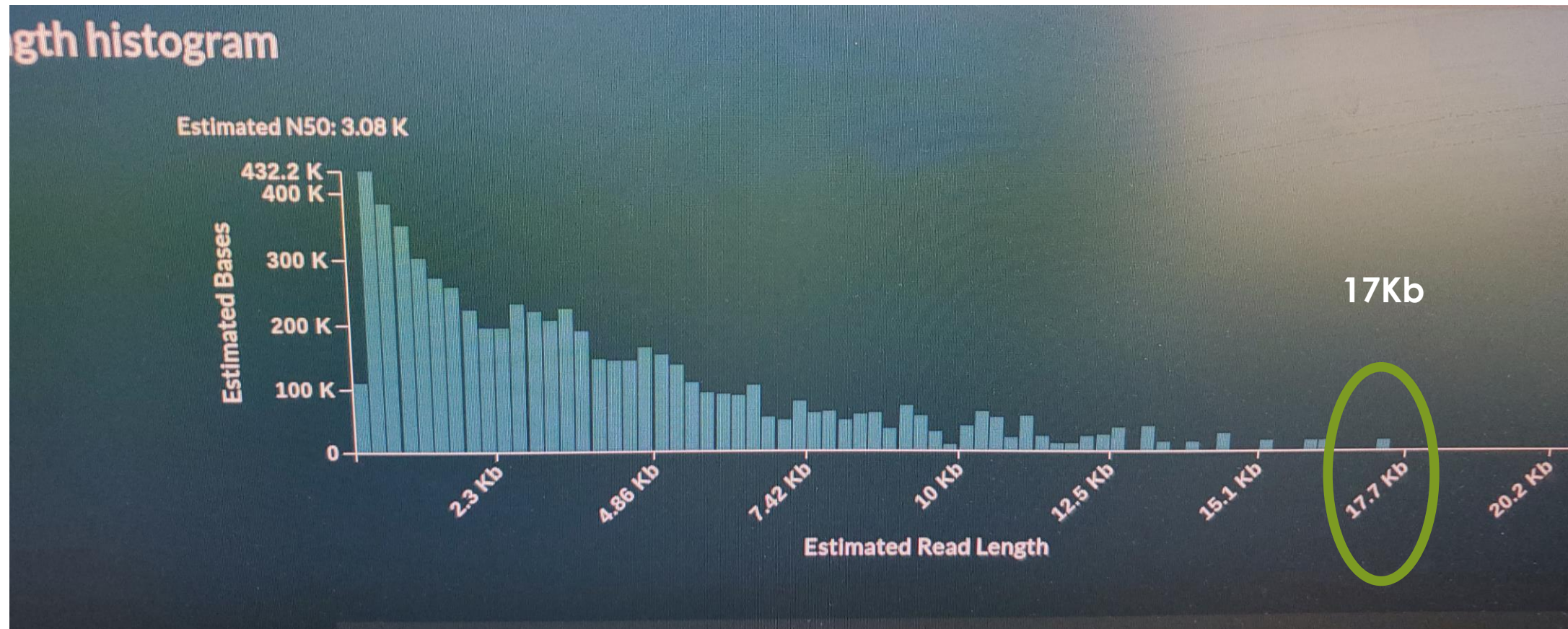
Estimated N50: 223 b

Read Length- ARTIC v4

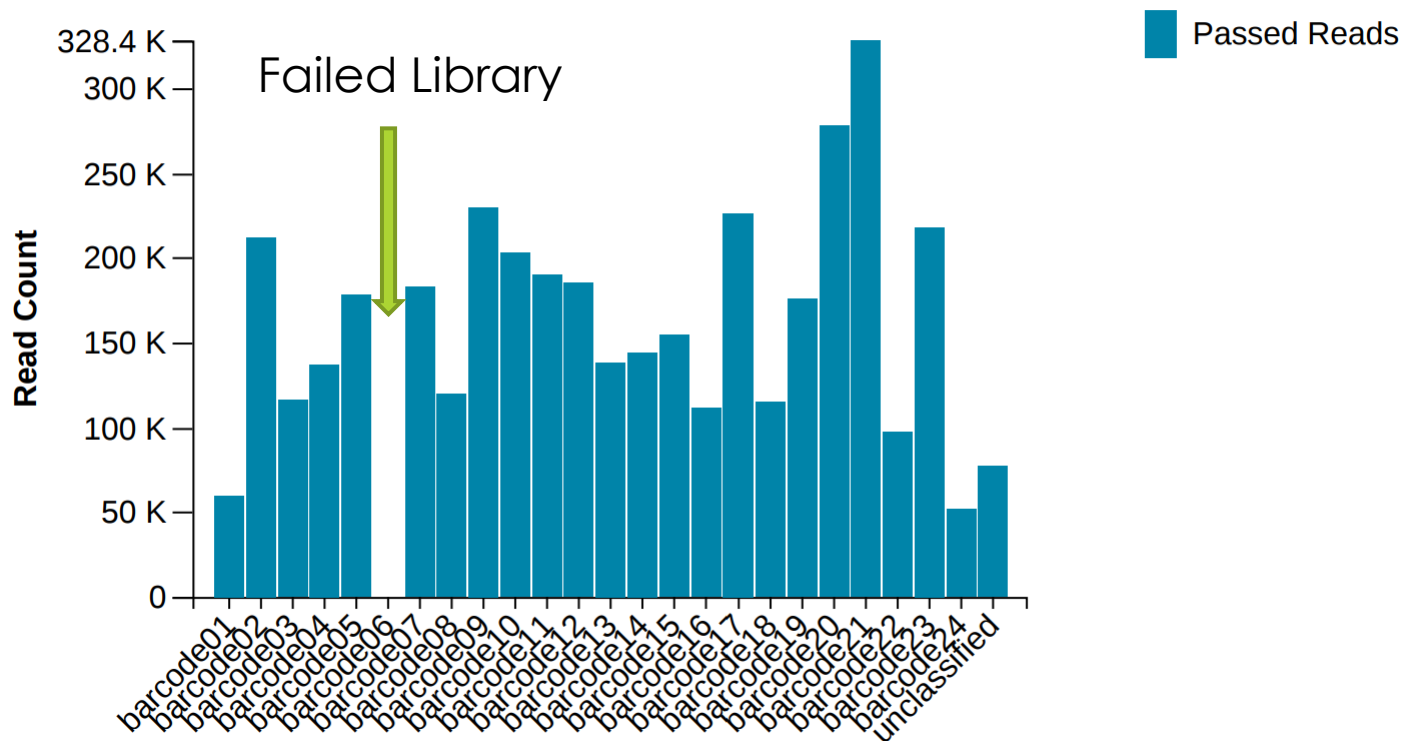**Read Length Histogram Basecalled Bases**
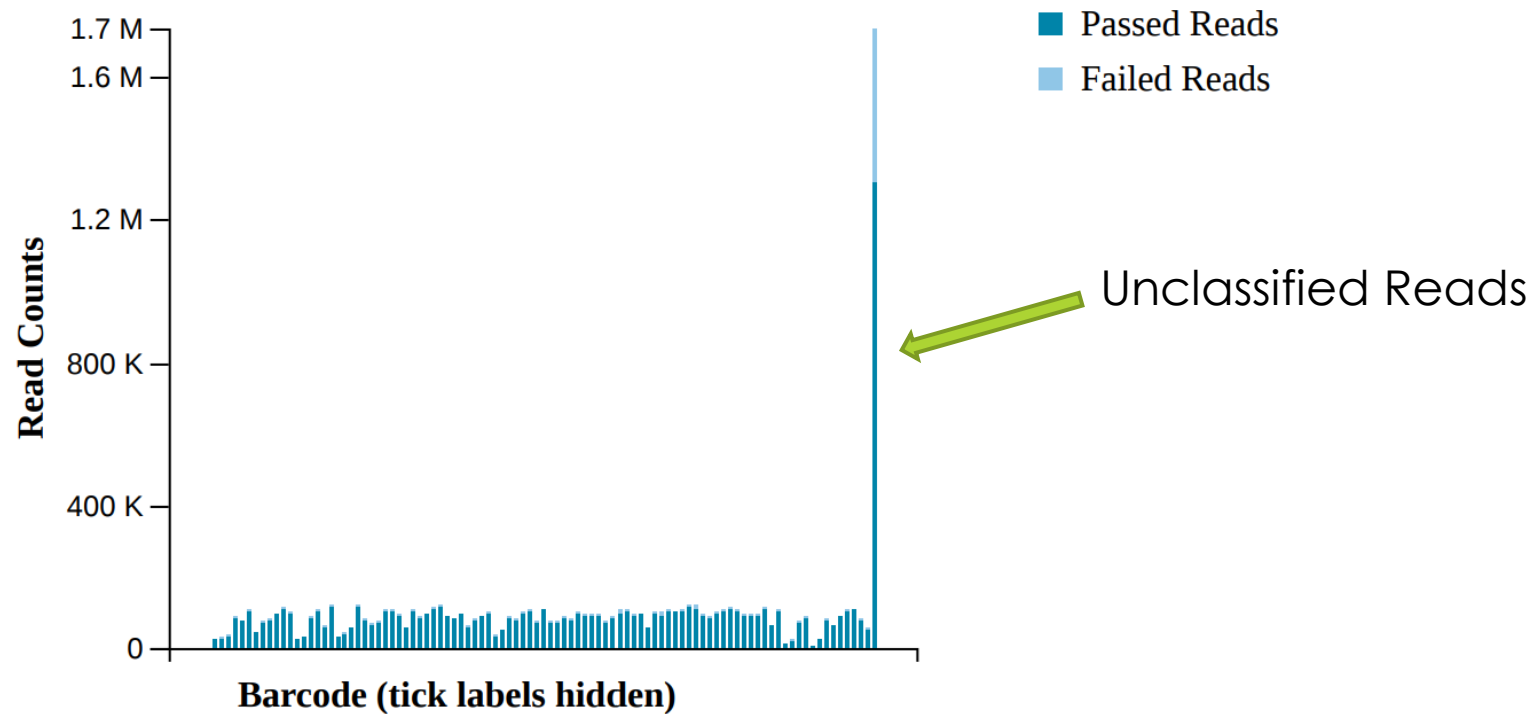
Estimated N50: 507

Read Length- Metagenomic
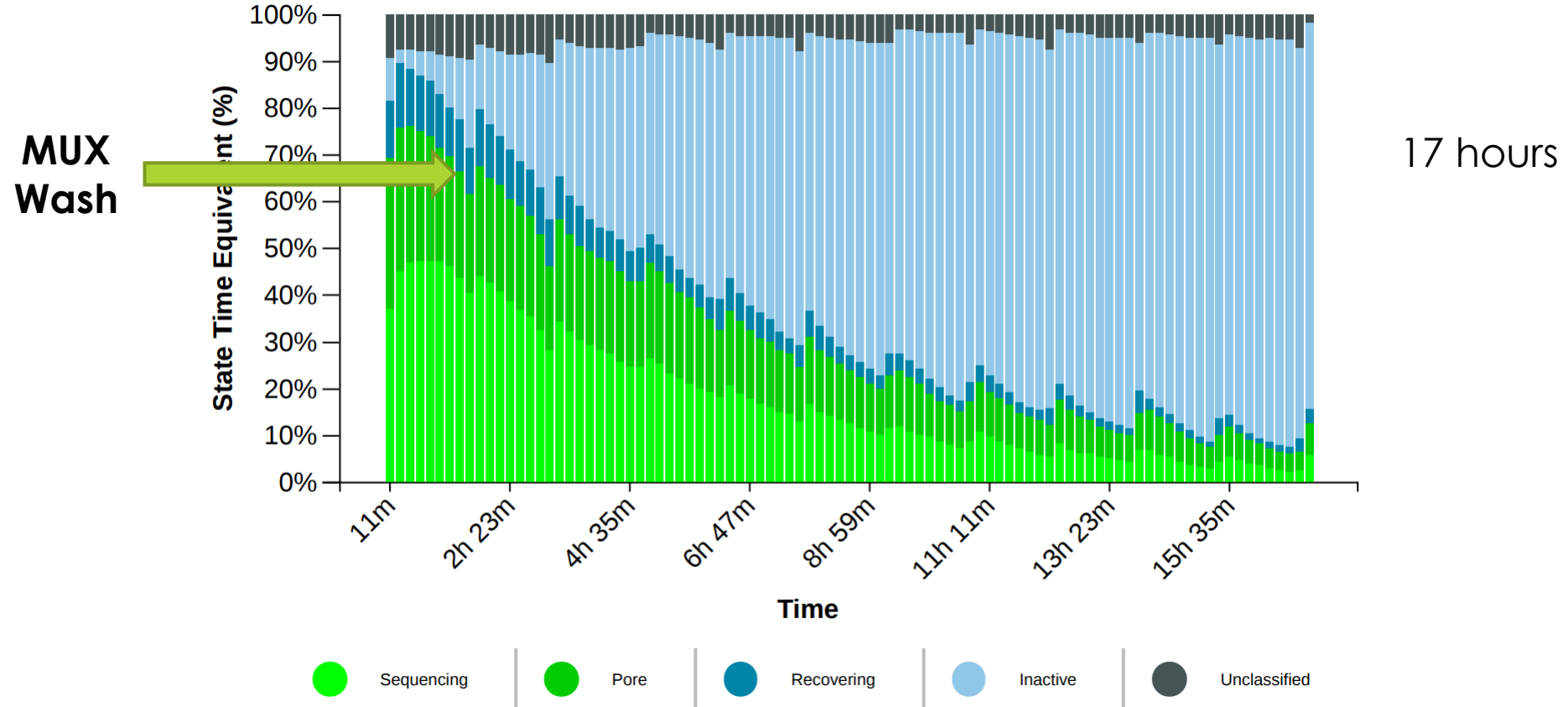
Barcode Read Counts- 24 plex

Barcode Read Counts
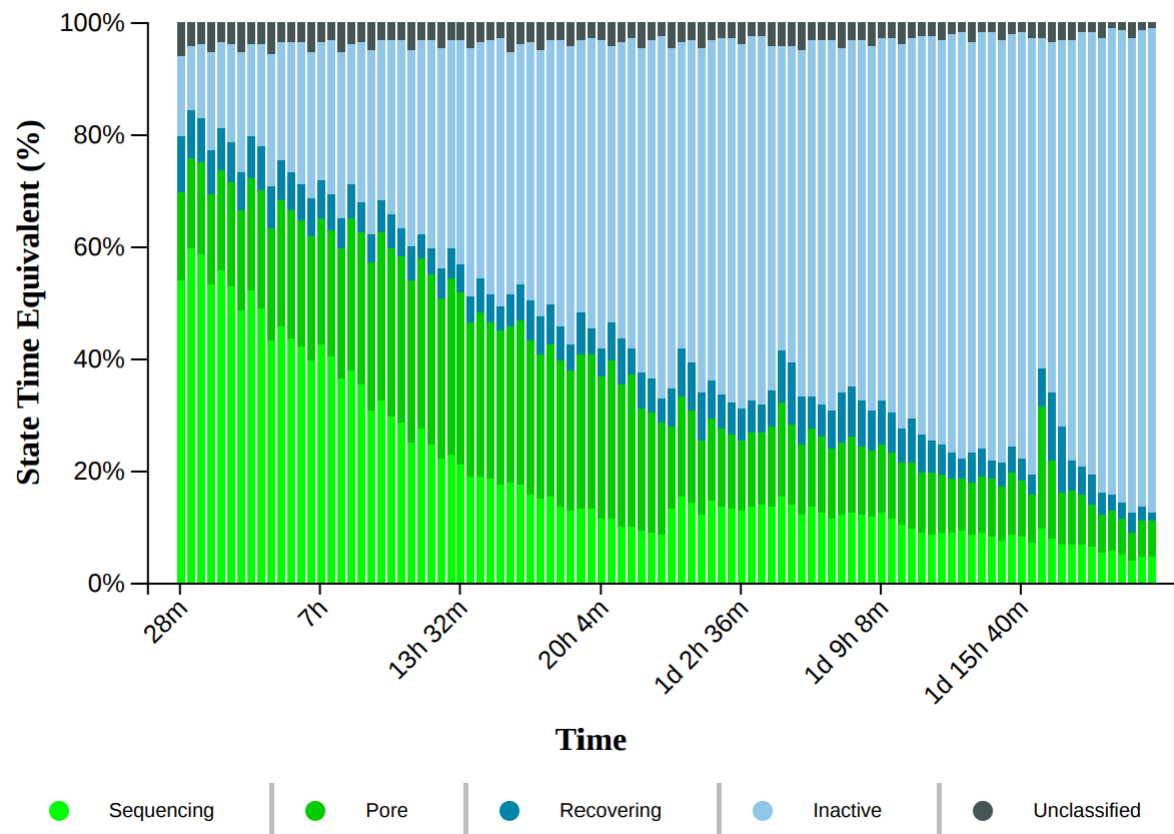
Barcode Read Counts- 96 plex

Barcode Read Counts

Legend:
- Passed Reads
- Failed Reads

Y-axis (Read Counts): 1.7 M, 1.6 M, 1.2 M, 800 K, 400 K, 0

X-axis: Barcode (tick labels hidden)

Unclassified Reads

ONT Pore Status- 2020

Duty Time Grouped

MUX Wash

17 hours

Legend: Sequencing, Pore, Recovering, Inactive, Unclassified

ONT Pore Status- 2021

Duty Time Grouped

1 day 21 hours

Flow Cells Can Be Reused

- **Flow Cell Wash Kit (EXP-WSH004)**
  - **~30-45 minutes, 5 minutes hands on time**

- **Active # of pores will be decreased**

- **Longer original sequencing run, more decreased output of second use**

- **Possible sample & read carry over**
  - **Sequence bacterial samples using washed SARS-CoV-2 flow cells**

Questions?