Overview of ONT Sequencing

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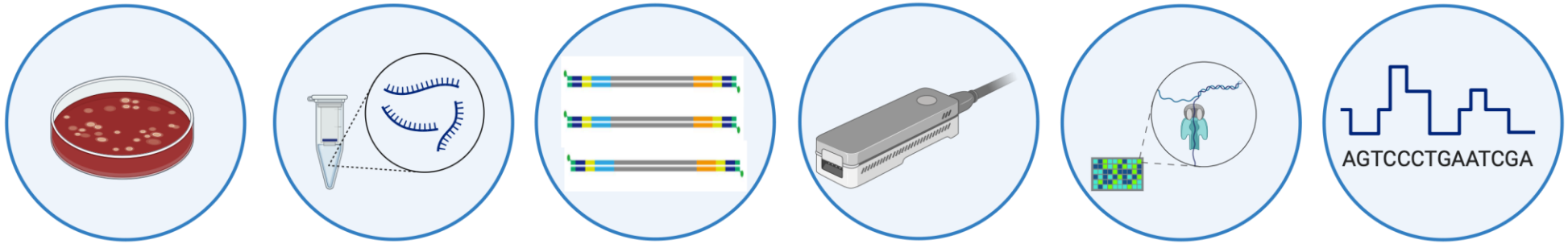
Lead Laboratory Scientist and Genomics Trainer

CENTRE FOR PATHOGEN GENOMICS

World Health Organization
Western Pacific Region

IPSN International Pathogen Surveillance Network
WHO hosted network

ONT Sequencing Workflow



Generate Pure Bacterial Isolates

Extract Pure HMW DNA

Or generate tiled amplicons

Make ONT Sequencing Library

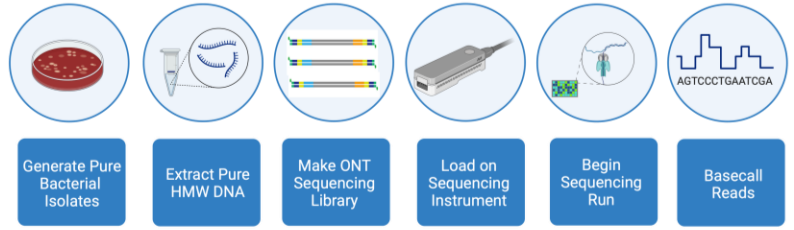
Load on Sequencing Instrument

Begin Sequencing Run

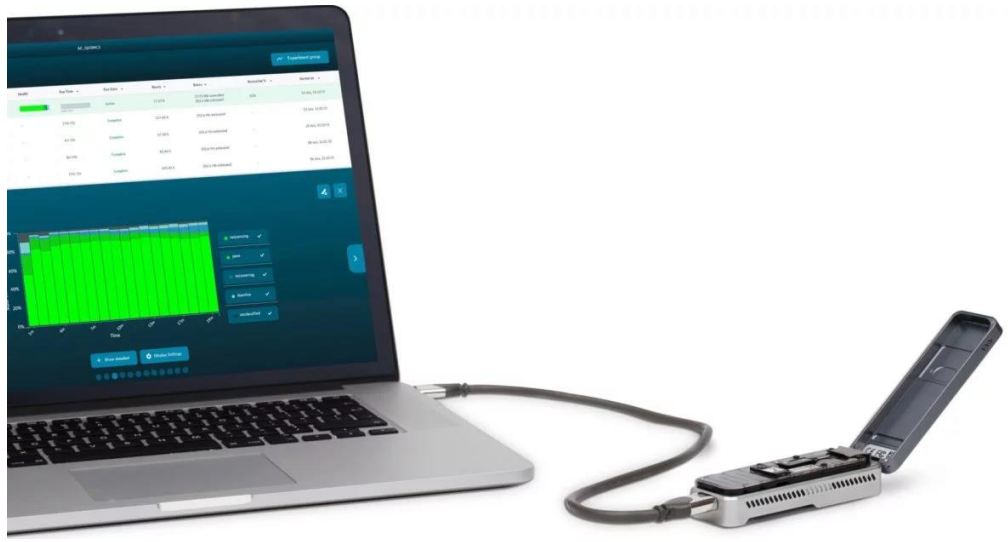
Basecall Reads

Created with BioRender.com

Load on Sequencing instrument
MinION Mk1b



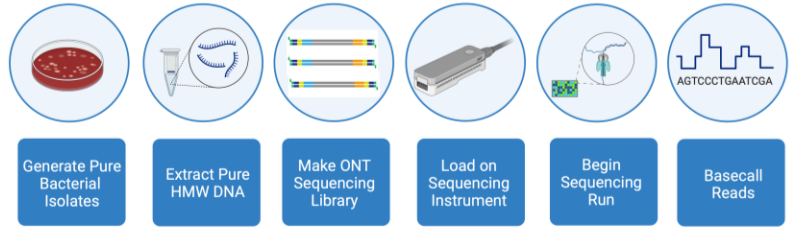
Load on Sequencing Instrument



MinION
- 2048 pores
- 512 channels
- 15-25 Gb

- Plug into USB-B of computer
- Any computer can be used for sequencing
- Efficient basecalling will need GPU
- No cost- provided with purchase of flow cells
- Can plug multiple MinIONs in to HPC

Load on Sequencing instrument
Pores and Channels- Mux Scans



Load on Sequencing Instrument

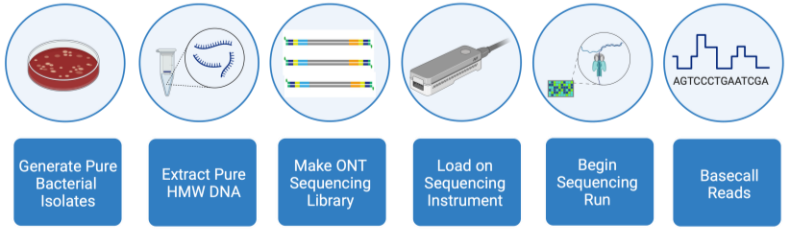
MinION Flow cell

- 2048 pores
- 512 channels
  - Pores grouped in batches of four
  - Only one of four pores connects to single channel

Pore scan (Mux scan)

- Start of run AND every 90 minutes during run
- All DNA ejected from pores
- For each channel “best” of the four pores is selected and used for sequencing for the next 90 minutes
- Can increase frequency of Pore scans for “blocky” libraries

Load on Sequencing instrument
MinION Mk1c

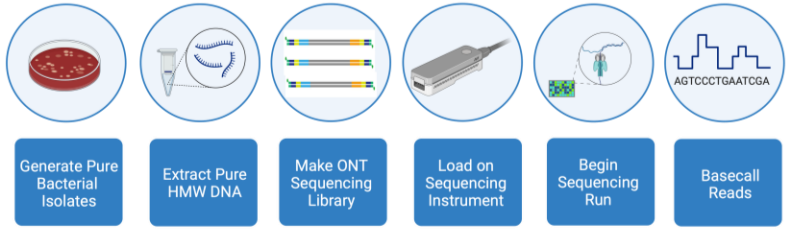


Load on Sequencing Instrument

MinION
- 2048 pores
- 512 channels
- 15-25 Gb

- Built in screen and compute
- Saves raw sequence data
- Will only basecall in “fast” mode
- Set up and connections can be challenging
- ~$USD5,000

Load on Sequencing instrument GridION



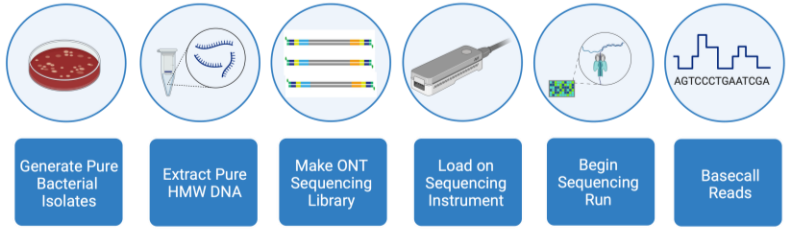
Load on Sequencing Instrument

MinION

- 2048 pores
- 512 channels
- 15-25 Gb
- 5 flow cell positions

- Built in compute
- Saves raw sequence data
- Will basecall in ALL modes
- ~$USD10,000 plus ~$USD12,000 annual service fee

Load on Sequencing instrument OnION



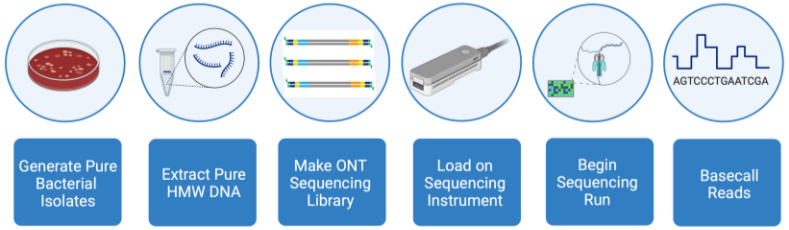
Load on Sequencing Instrument

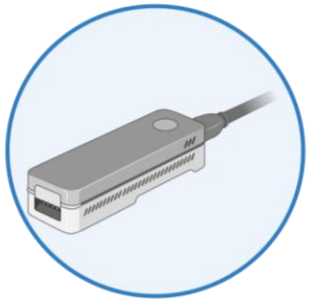
MinION Mk1b connected to Gaming Desktop computer

- Can run 4 Mk1b simultaneously
- O/S Ubuntu
- Intel i9-13900K CPU (32 threads)
- 128 GB of RAM
- NVIDIA RTX 4090 GPU
- 10 TB of SSD storage (2 TB Samsung 990 Pro and 8 TB PNY CS3140)

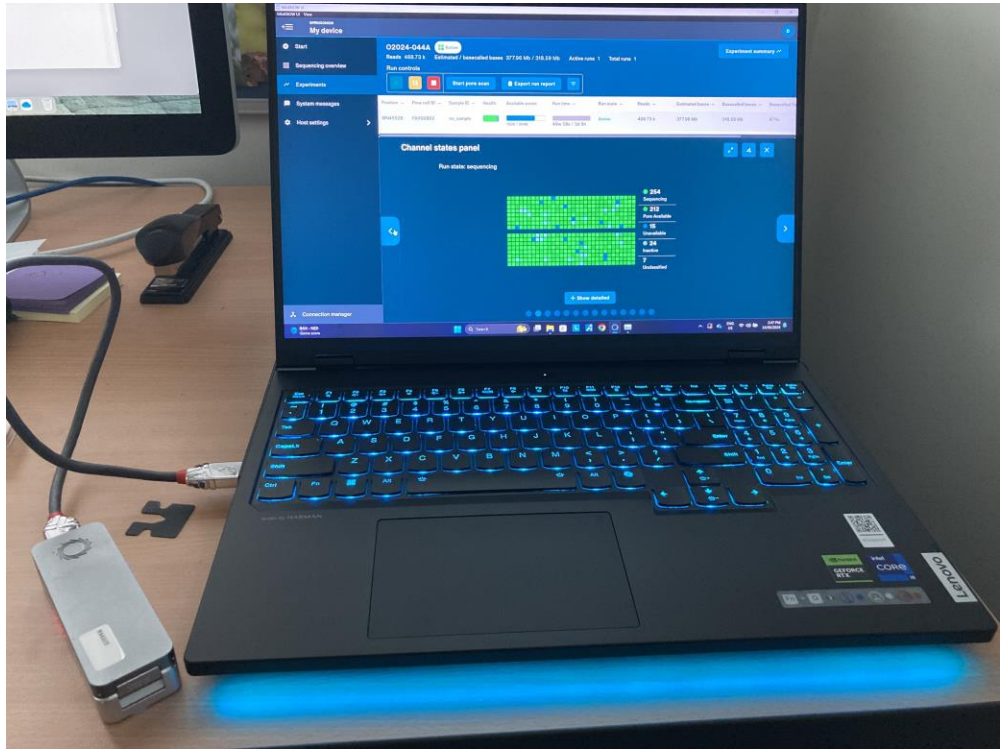
- Saves raw sequence data
- Have had up to 4 Mk1
- Will basecall in ALL modes
- ~$USD5,000

Load on Sequencing instrument SpringOnION





Load on Sequencing Instrument

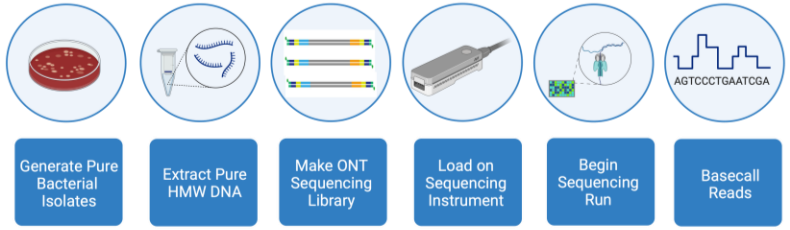


MinION Mk1b connected to Gaming Laptop computer

- Only tested with 1 Mk1b
- O/S Windows
- Intel i9-14900HX Processor
- 32 GB of RAM
- NVIDIA geForce RTX 4090 Laptop GPU
- 2 TB of SSD storage

- Saves raw sequence data
- Will basecall in ALL modes
- ~$USD3,500

Load on Sequencing instrument
PromethION- P2 Solo



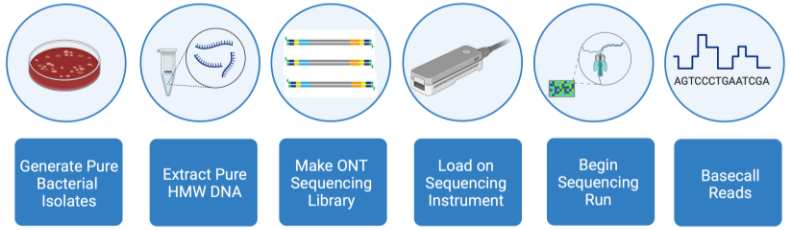
Load on Sequencing Instrument

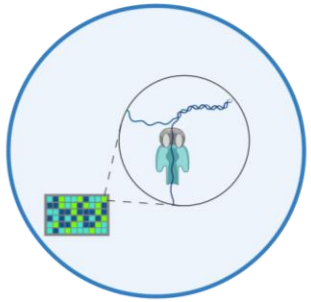
PromethION

- 10,700 pores
- 2675 channels
- 150-200 Gb
- 2 flow cell positions

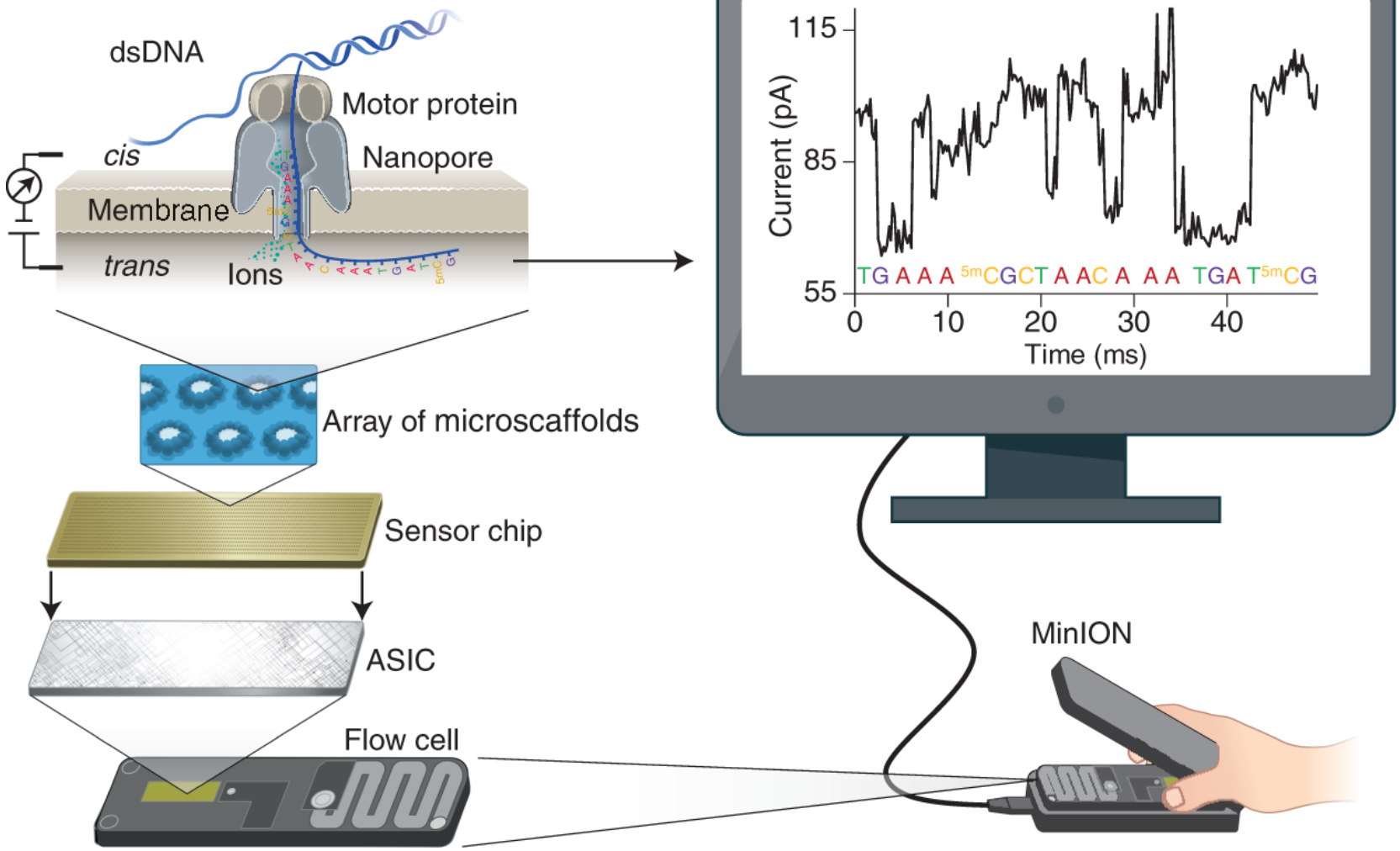
- Plug in to HPC or GridION
- Saves raw sequence data
- Will basecall in ALL modes
- ~$USD10,000 plus ~$USD21,000 annual service fee

Begin Sequencing Run
Overview





Begin Sequencing Run



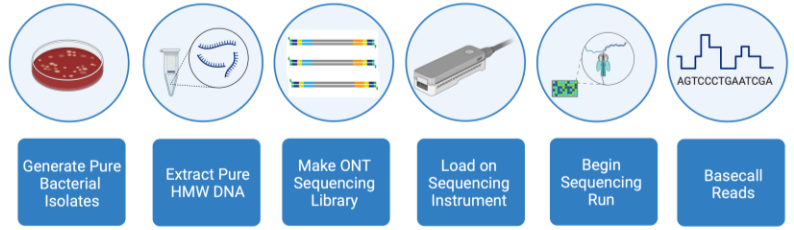
How does ONT sequencing work?

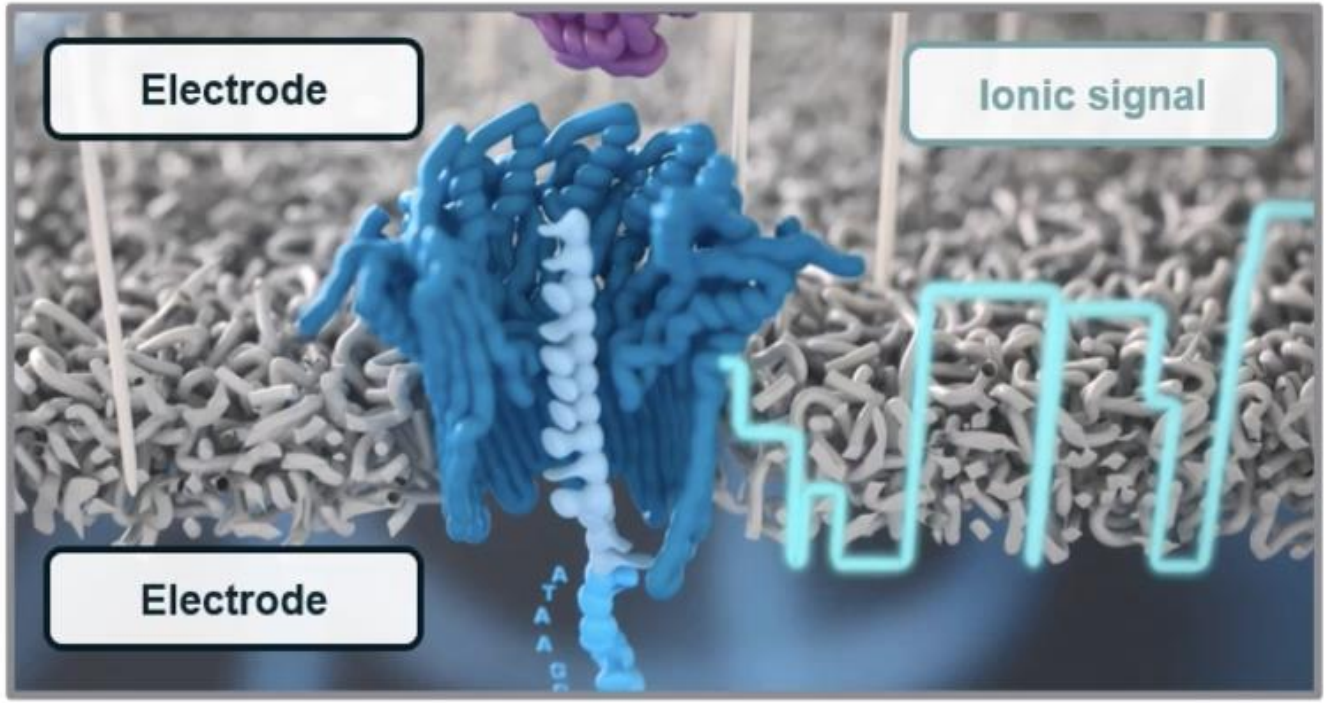
CENTRE FOR PATHOGEN GENOMICS
Doherty Institute
THE UNIVERSITY OF MELBOURNE
The Royal Melbourne Hospital
A joint venture between The University of Melbourne and The Royal Melbourne Hospital

Oxford NANOPORE Technologies

Begin Sequencing Run

How a flow cell works



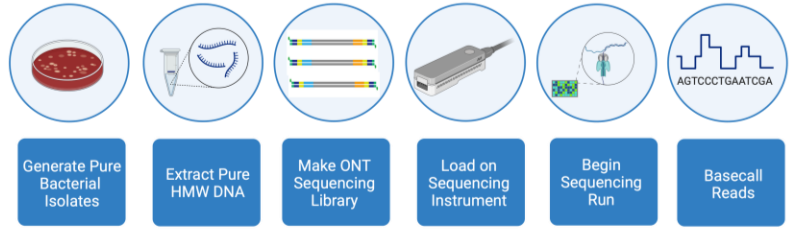


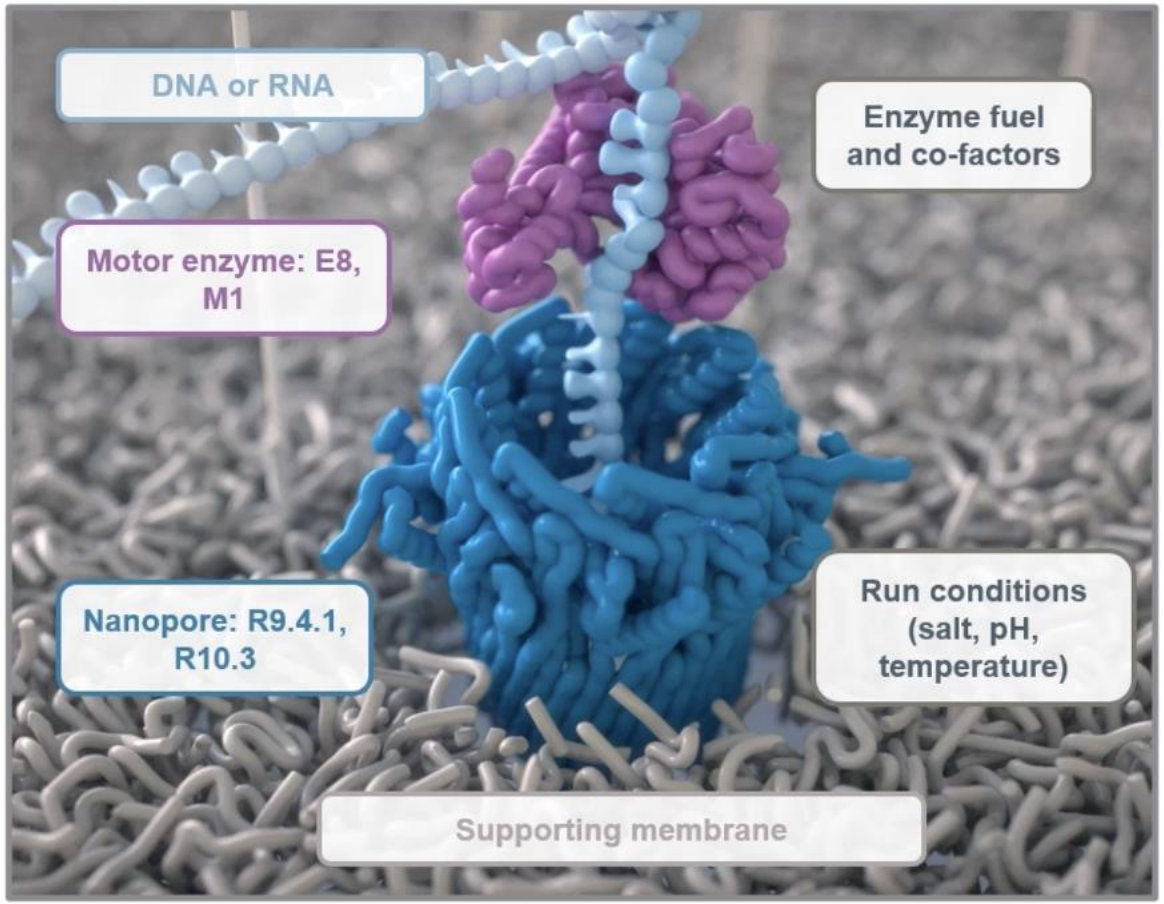
ONT flow cell

1. Two electrodes insulated by a membrane
2. Single nanopore in membrane allow ion flow
3. Any blocking of ion flow results in a drop in current
4. Sequences of DNA block the ion flow to give different currents
5. DNA moves through the pore to give the signal of the DNA strand
6. Signal can be computationally converted (basecalled) into a DNA sequence

Begin Sequencing Run

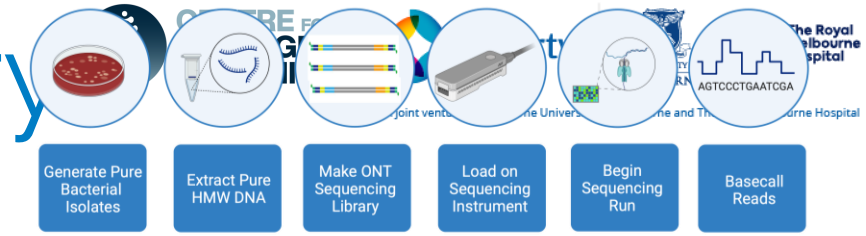
What is required for ONT sequencing?

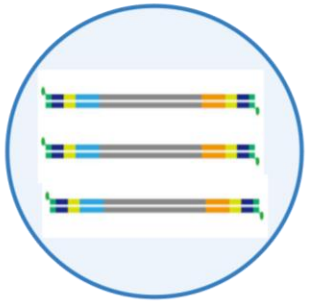




1. Molecules for sequencing DNA or RNA
   - pure sample with no contaminants that will damage a biological system
2. Nanopore
   - modified bacterial protein pore
3. Adapter protein
   - Topoisomerase unzips dsDNA molecule
   - Motor protein regulates the rate at which molecules migrate through pore
4. Enzyme fuel and co-factors
   - May need to be replenished during a run
5. Run conditions
   - Biological system (salt, pH and temperature)

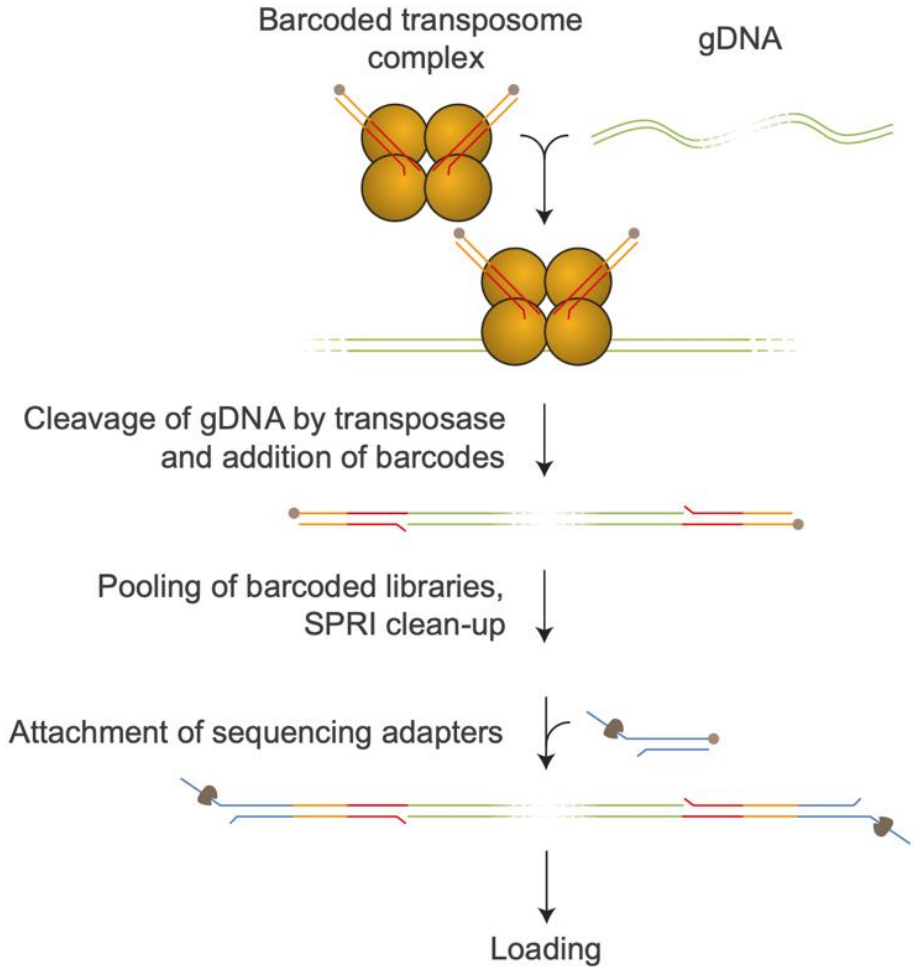
Make ONT sequencing library
Rapid Libraries RBK





Make ONT Sequencing Library

< 60 min



- 96 samples can be barcoded
- MinION yield 7-15 Gb
- ~15 bacterial isolates (100 x depth) on each flow cell
- Will sequence ALL DNA in sample
- Very fast library prep (<60 minutes)

Rapid Library Preparation

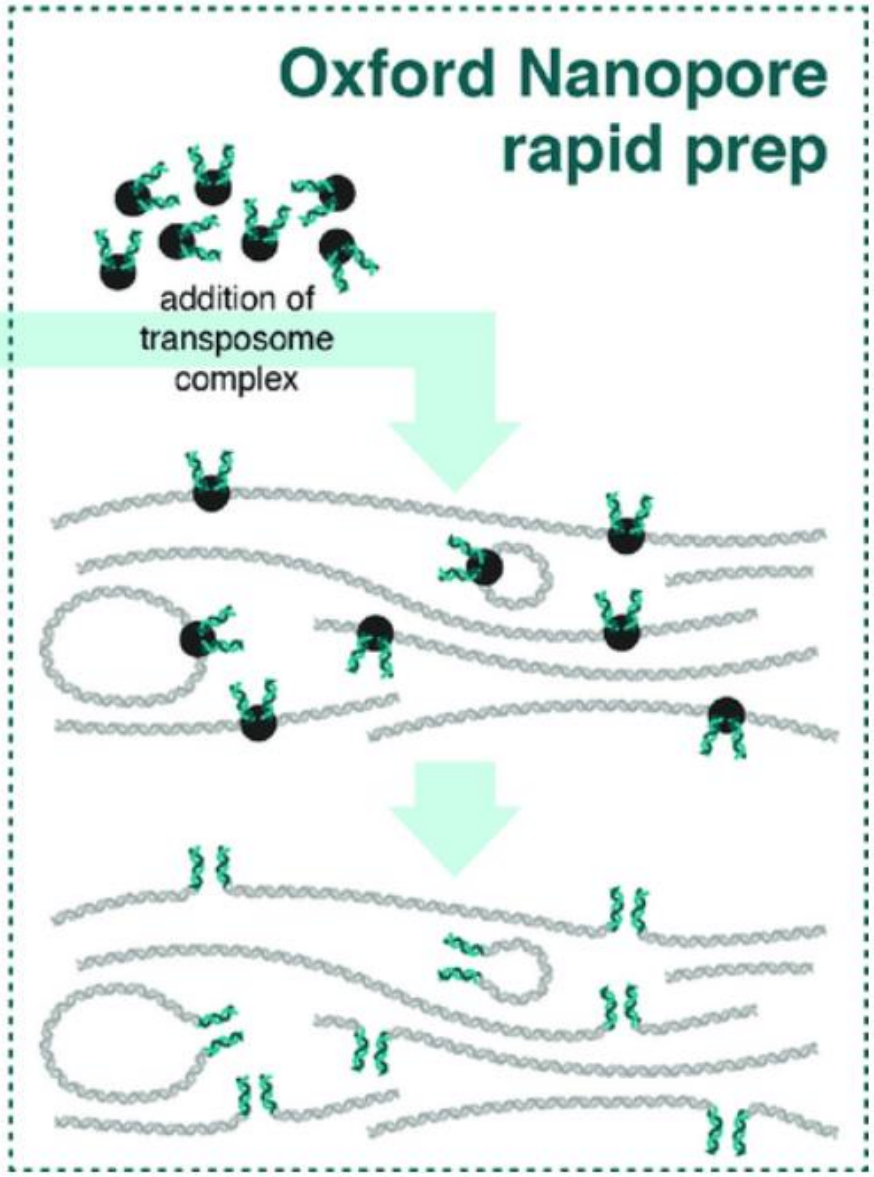
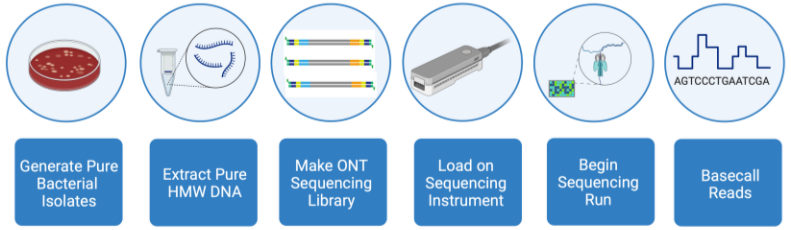


Image Source: Recovery of Small Plasmid Sequences via Oxford Nanopore Sequencing (Wick *et al.*, 2021)

Multiplexing samples on the ONT platform

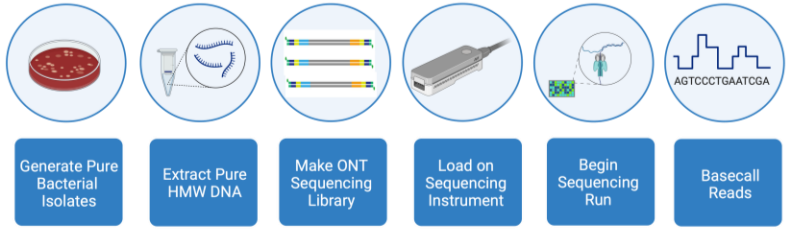
- Multiplexing works by addition of barcodes to the DNA fragments
- Barcode sequence becomes part of read sequence
  - Barcode should be removed by basecaller but does not always work efficiently
- Available with both ligation and rapid library preps
  - Ligation should see barcode on both ends of read
  - Rapid should only see barcode at start of read
- Up to 96 samples can be plexed into one library
- Normal to see >20% reads unclassified

Begin Sequencing Run
Tips for loading a flow cell

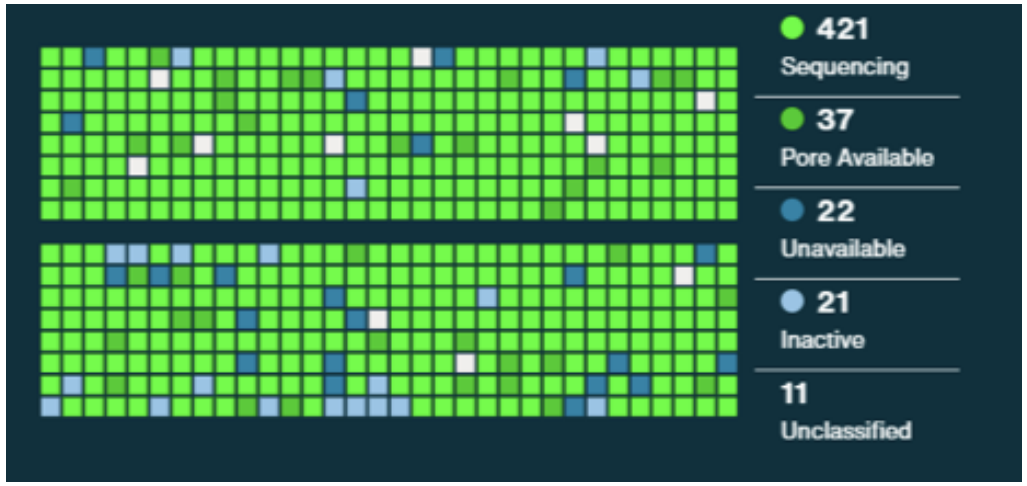


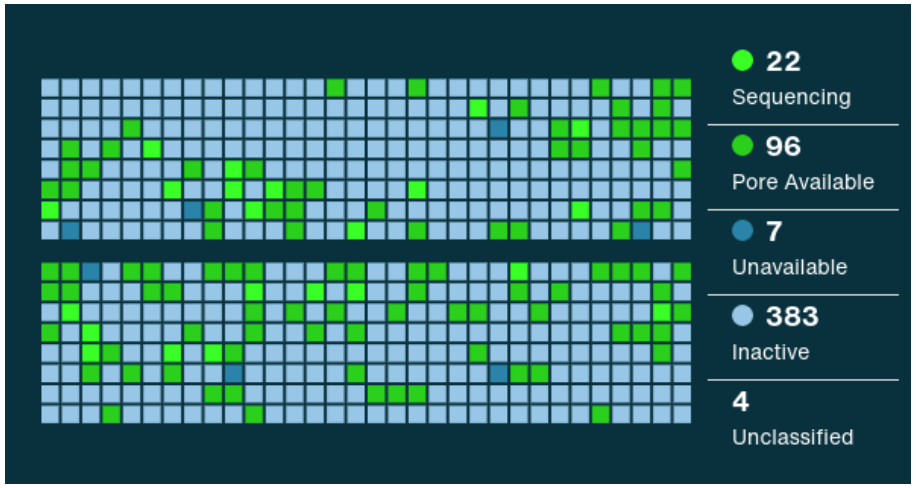
1. Never introduce air into the Priming or Spot-On ports – check for air in the port before dispensing
2. Find tips that fit snugly into the Priming port
3. Hold pipette vertically as you dispense liquid
4. Pipette into ports slowly and smoothly
5. Never pipette to second stop as this will expel air into the flow cell
6. Inspect tip after aspirating and before dispensing liquid to ensure there are no air bubbles in the tip
7. Leave a small amount of liquid in the tip to avoid dispensing air

Begin Sequencing Run
Monitoring ONT runs



Channels state- Snapshot in time



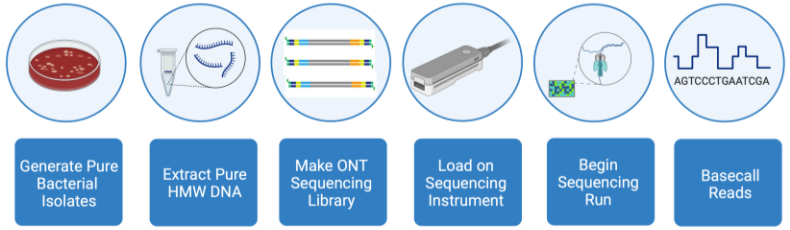


Good library
- Most pores Sequencing
- % viable pores = 89.4% (green squares)
- % currently sequencing = Sequencing/viable = 91.9%

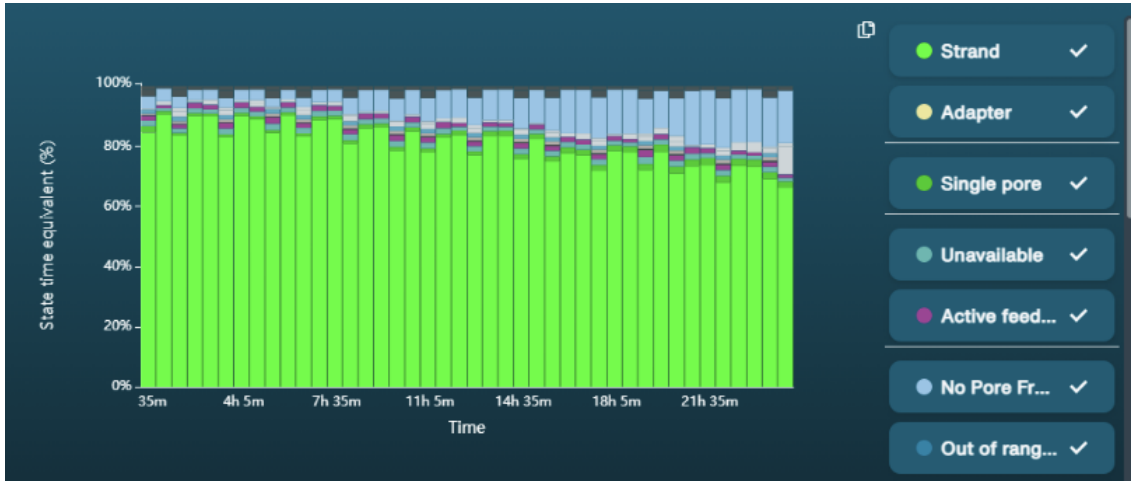
Poor library
- Most pores Inactive
- % viable pores = 23.0% (green squares)
- % currently sequencing = Sequencing/viable = 18.6%

Aim: Start of run % viable pores and % sequencing both >

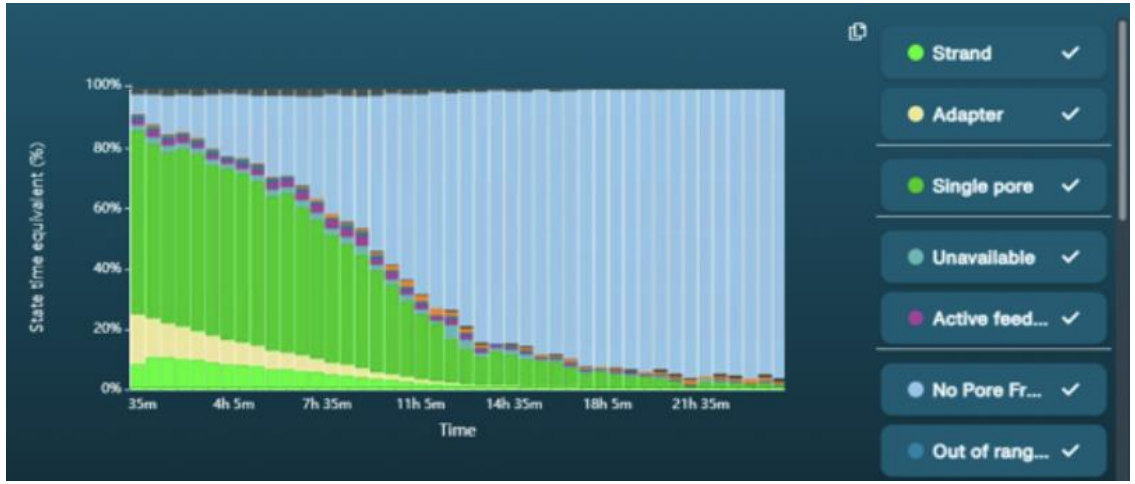
Begin Sequencing Run
Monitoring ONT runs



Pore Activity

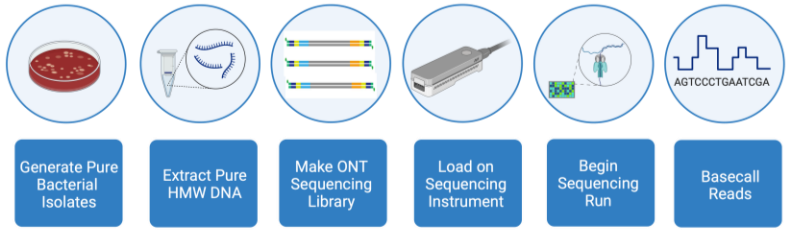


Good library

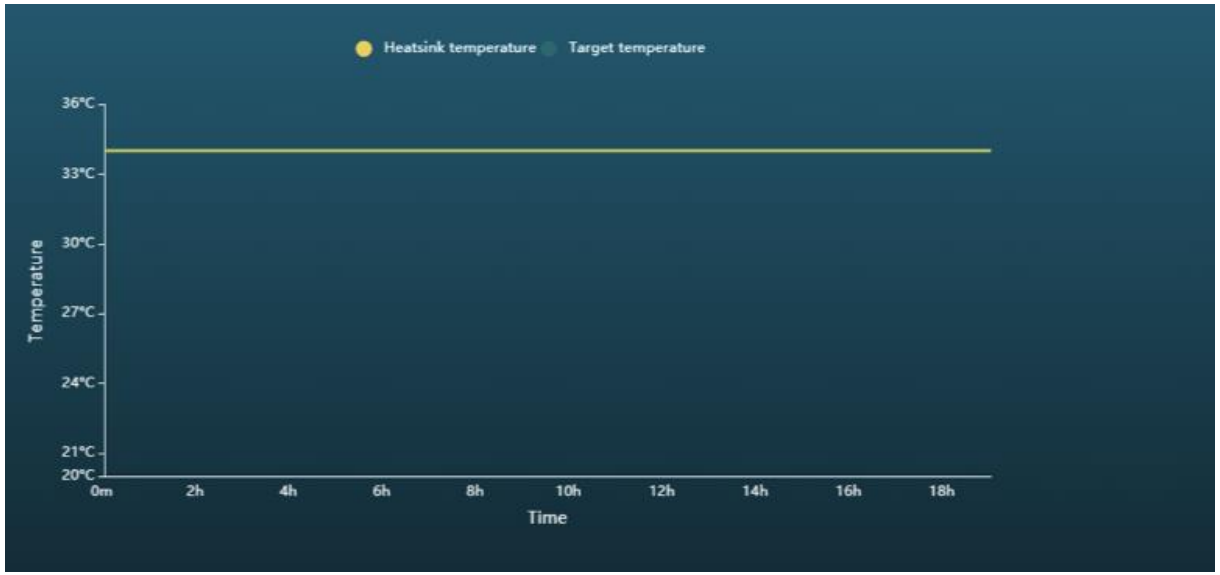


Poor library

Begin Sequencing Run
Monitoring ONT runs

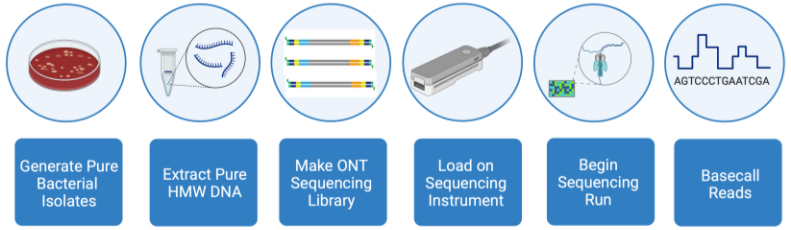


Temperature History

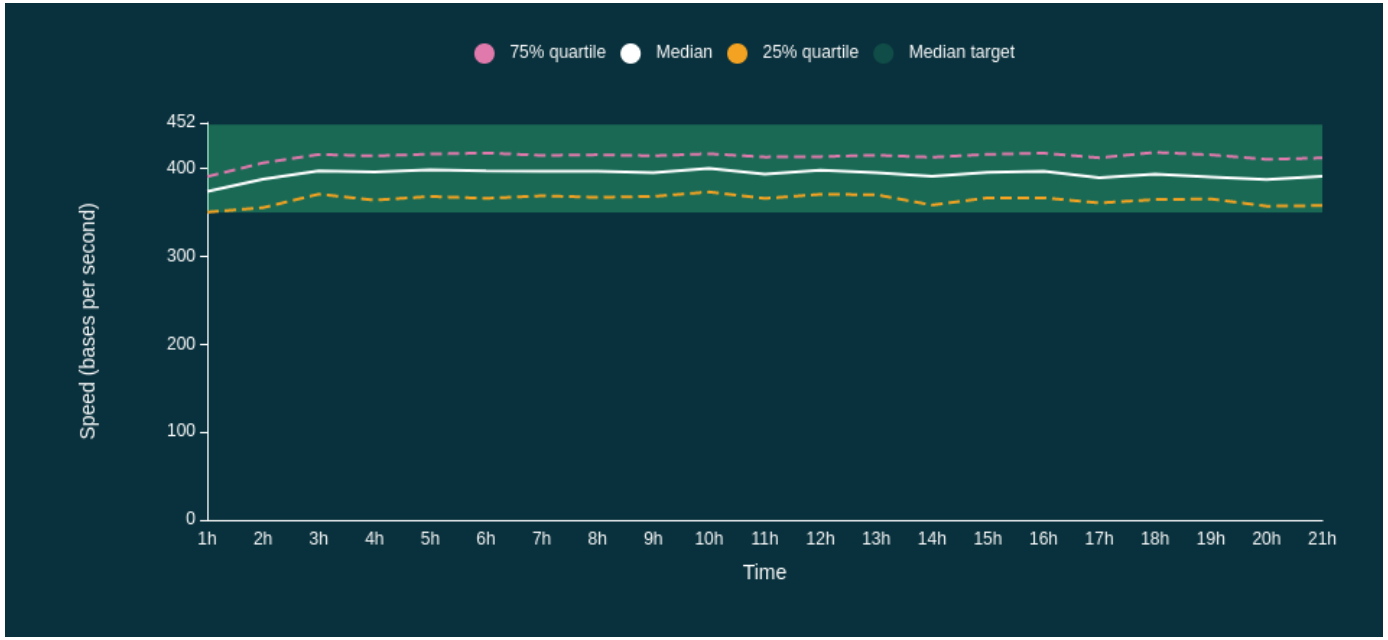


Should be stable at 34°C
If it varies >1°C monitor room temperature
Implement room engineering to cool or heat as required

Begin Sequencing Run
Monitoring ONT runs

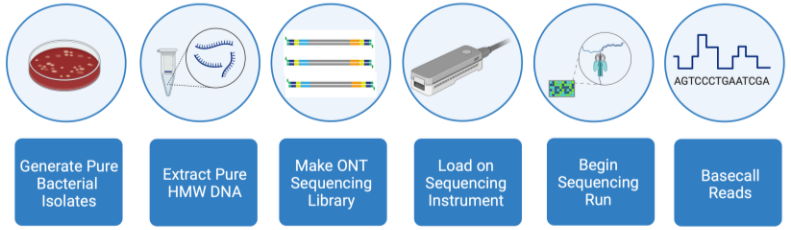


Translocation Speed

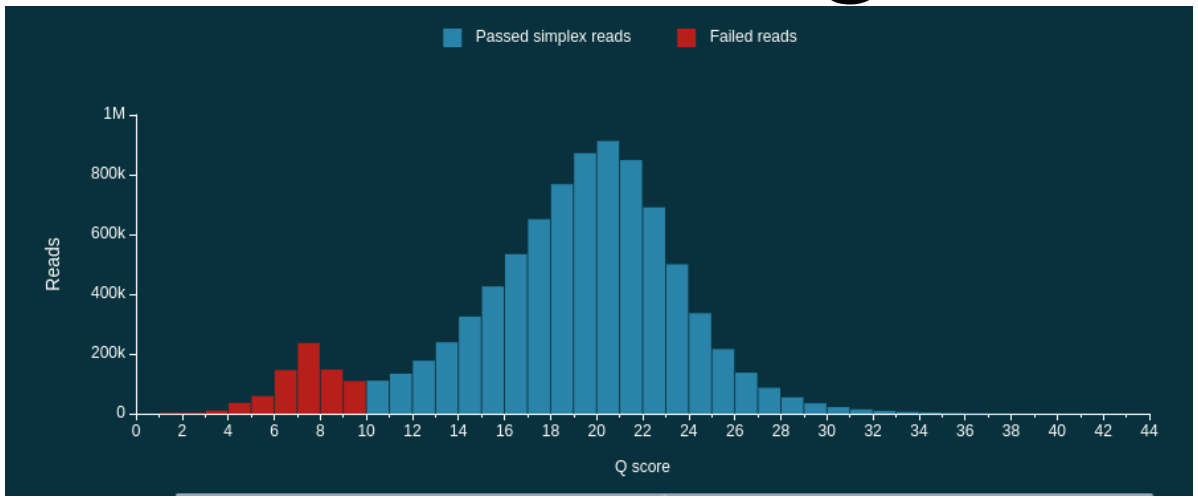


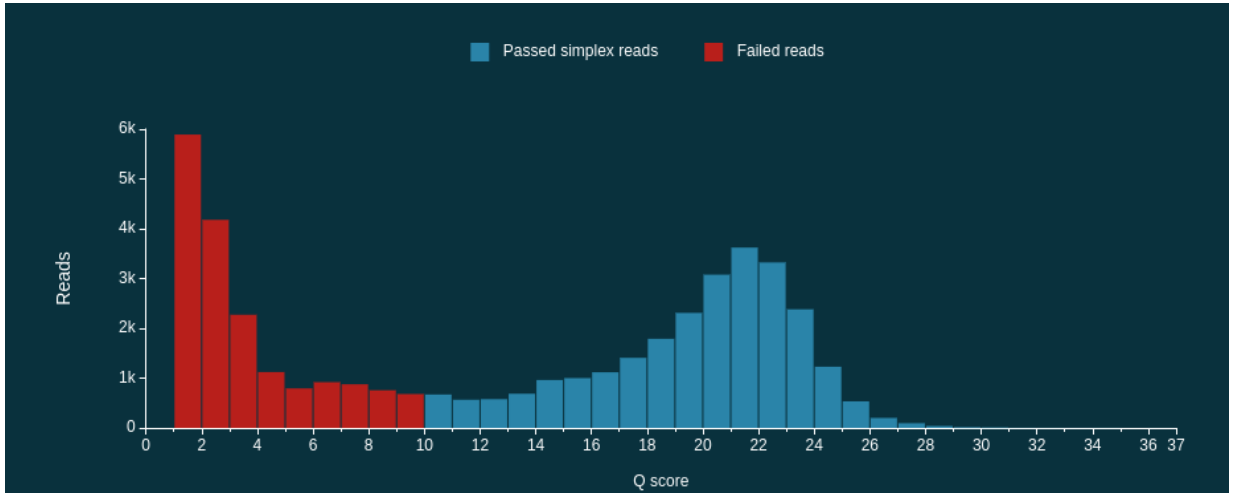
Should be stable at 400 bps
If it varies considerably then run may be running out of fuel (ATP)
Mostly happened with very strong runs with older sequencing kits

Begin Sequencing Run
Monitoring ONT runs



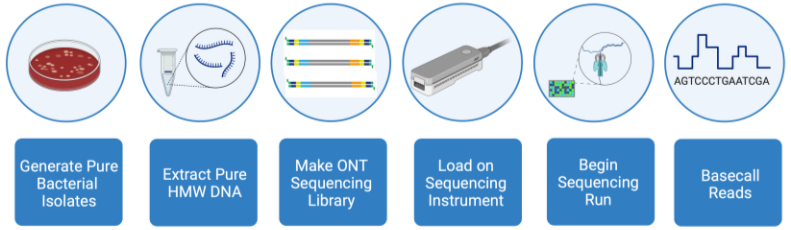
Q Score Histogram



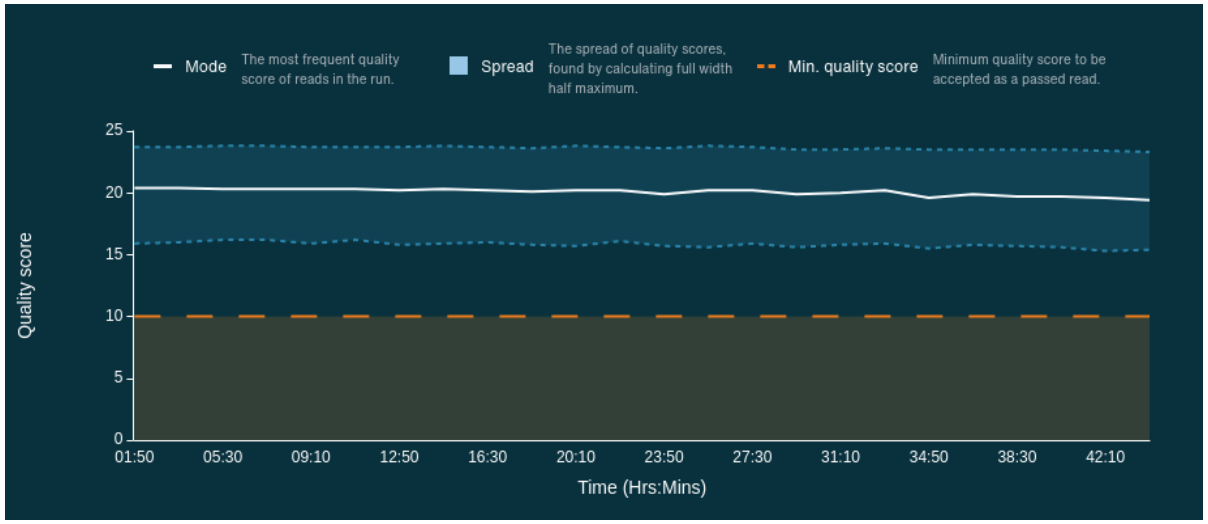


- Q score 20 – 99% accuracy – 1:100 base incorrect
- Q score < 10 – failed reads – not analysed

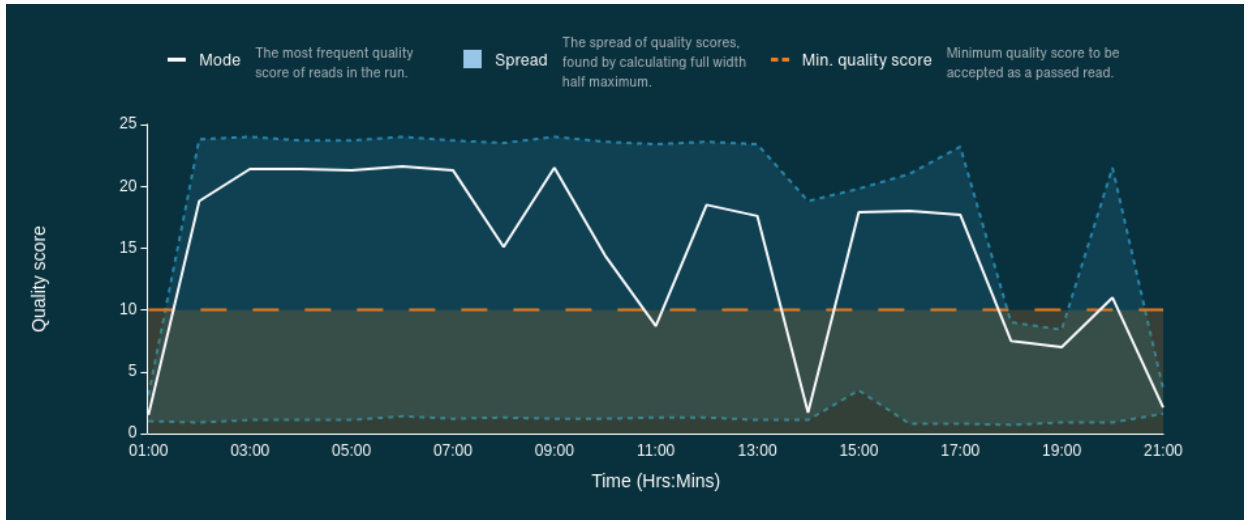
Begin Sequencing Run
Monitoring ONT runs



Q Score Over time

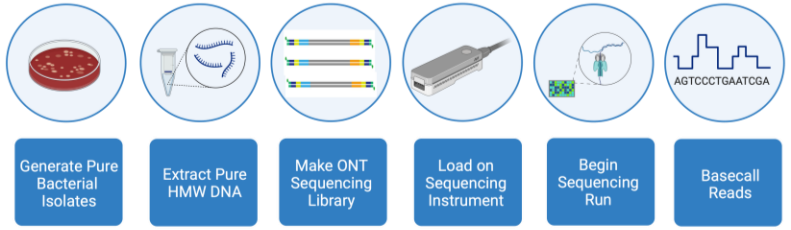


Good library



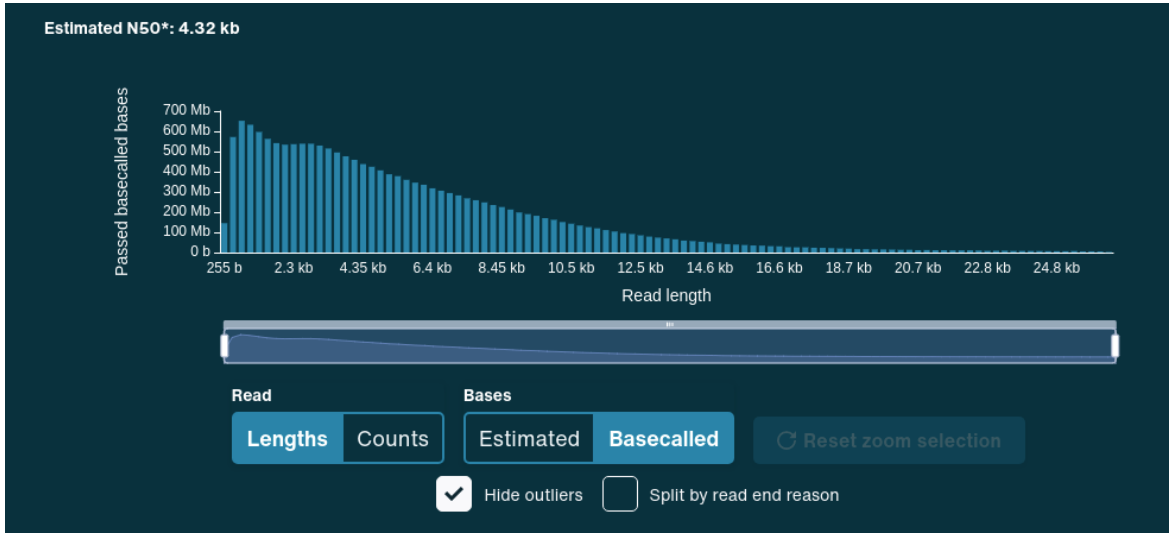
Poor library

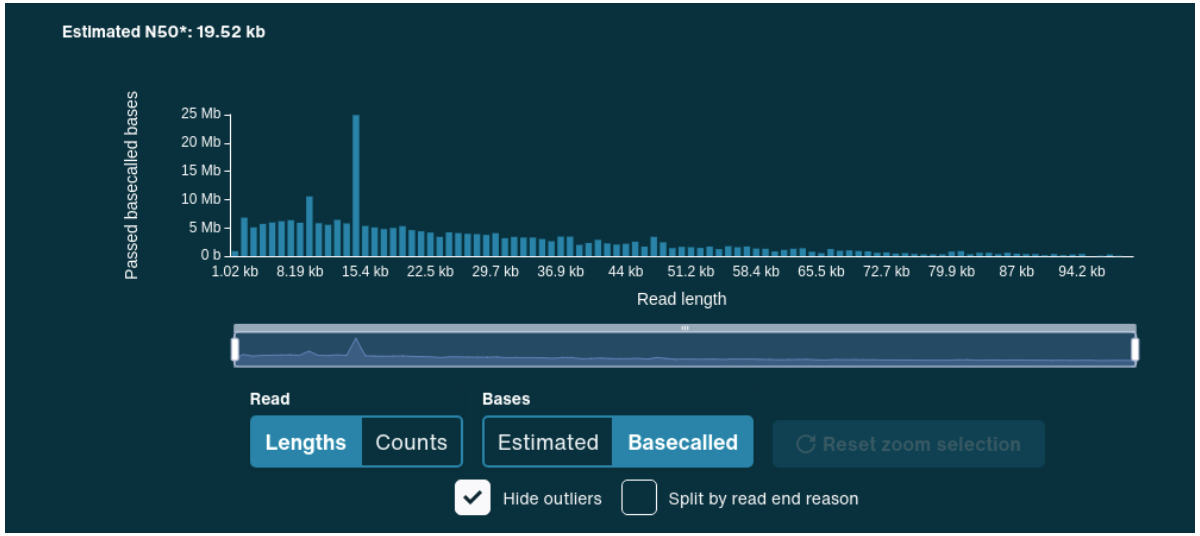
Begin Sequencing Run
Monitoring ONT runs



Read Length Histogram

1. Lengths toggle
2. Basecalled toggle

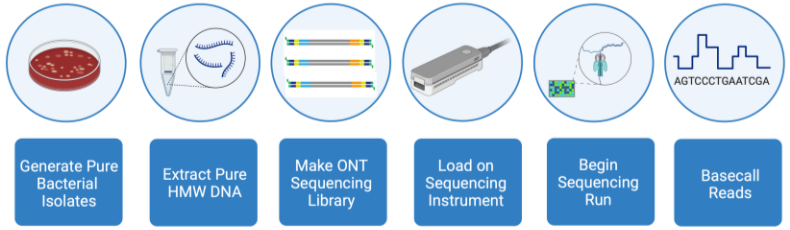




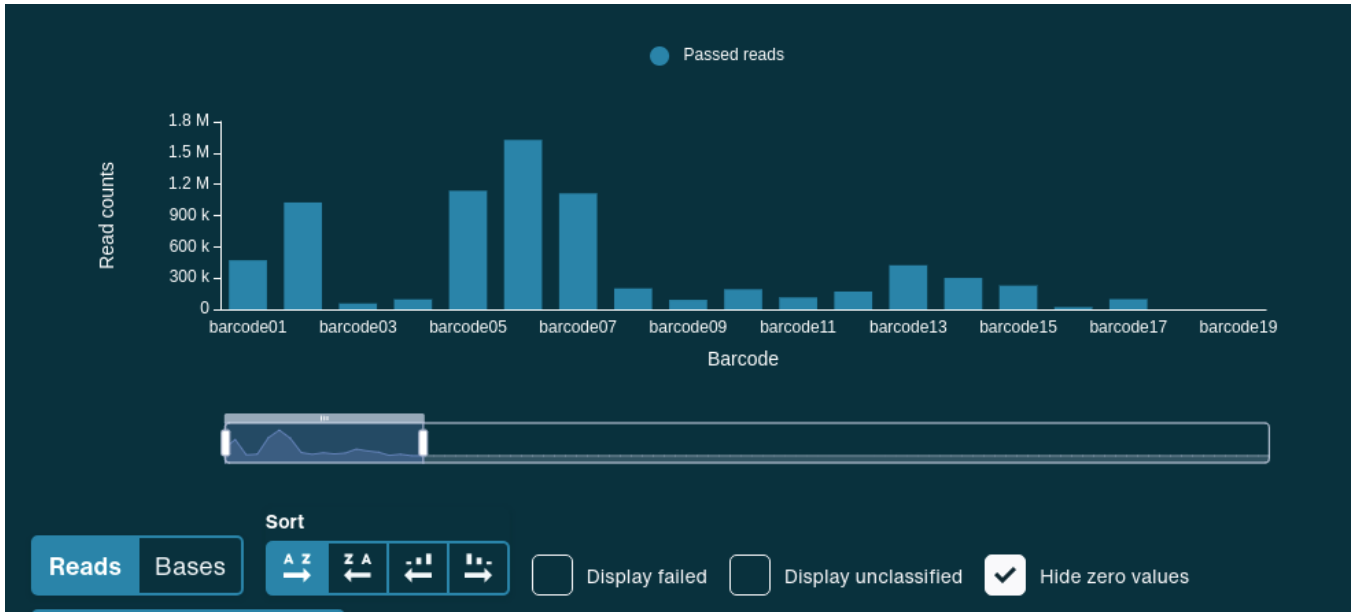
- Estimated N50 4.32 kb
- N.B. Samples were bead beaten for DNA extraction

- Estimated N50 19.52
- Note peak at ~15.5 kb - most likely a 15.5 kb plasmid in samples

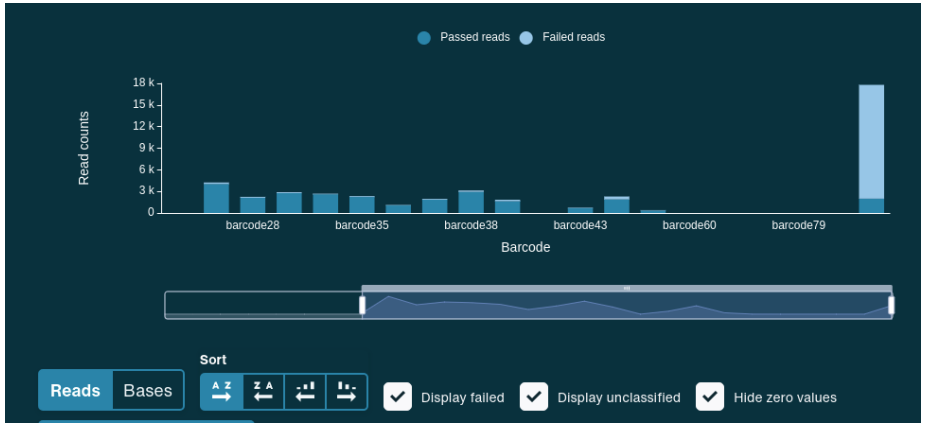
Begin Sequencing Run
Monitoring ONT runs



Barcode hits

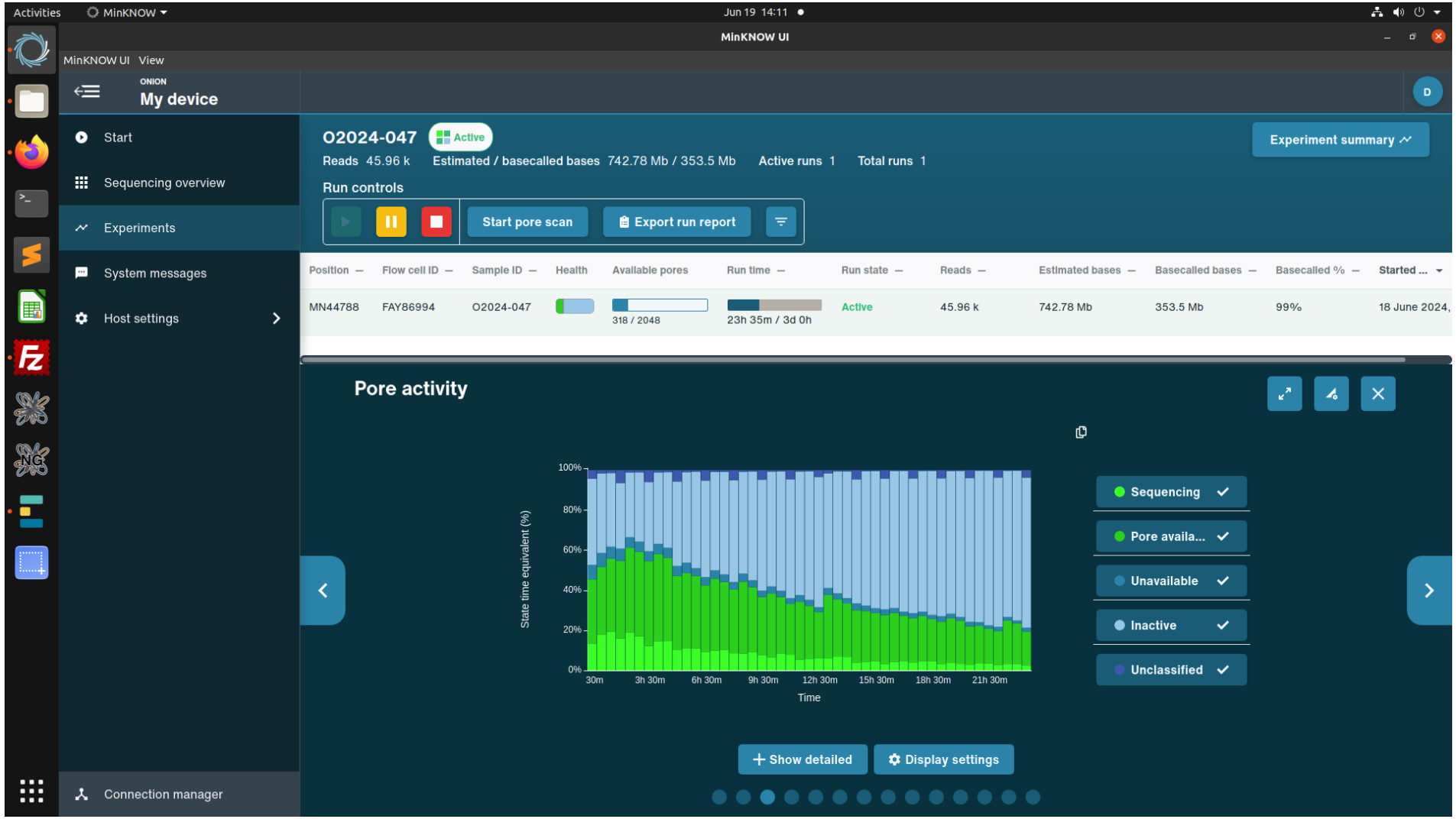


- Very difficult to get even barcode distribution
- Different samples – different molarities
- Toggle between reads and bases to get indication of per sample read length distribution

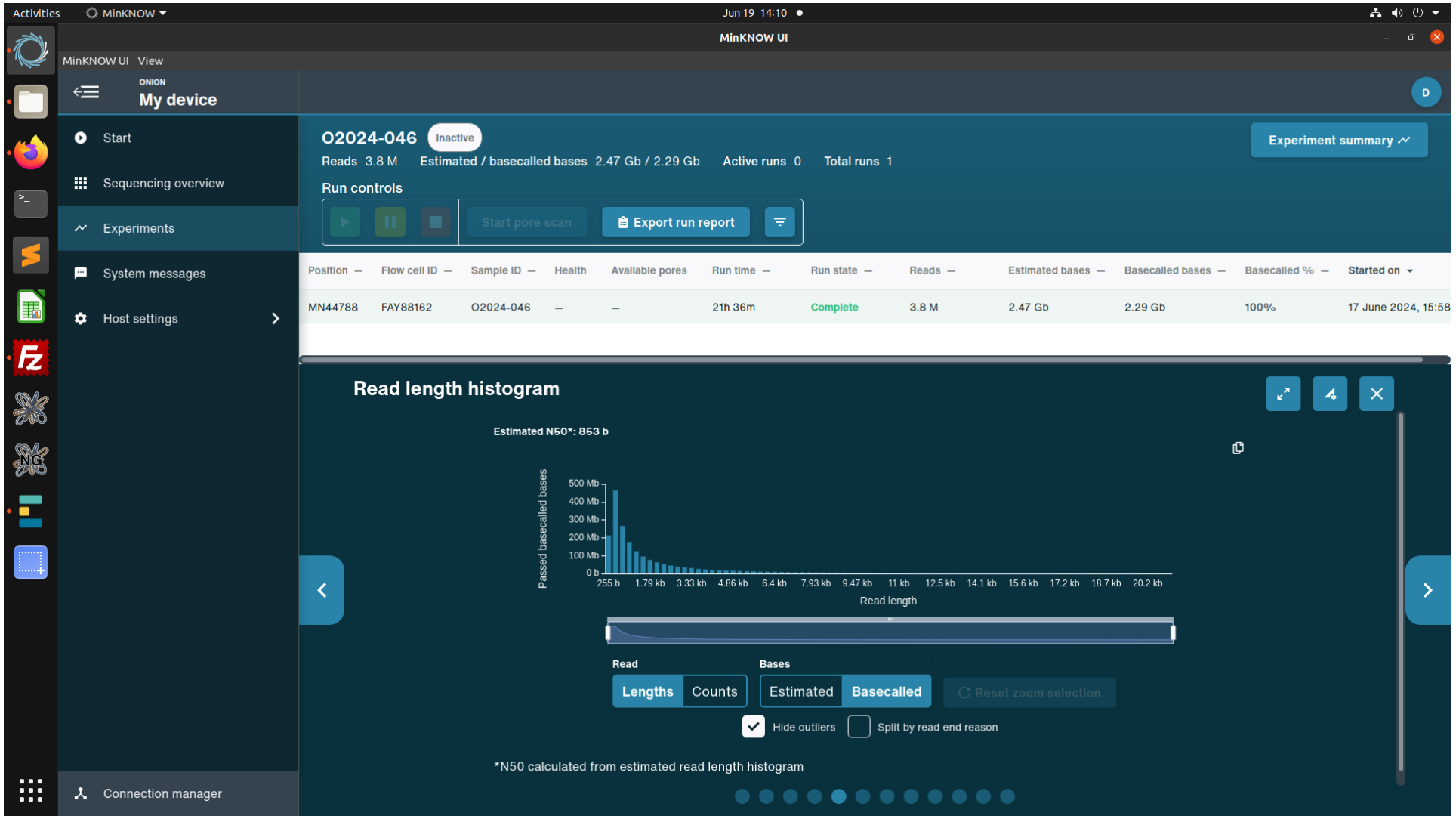


- Display unclassified reads
- Display failed reads

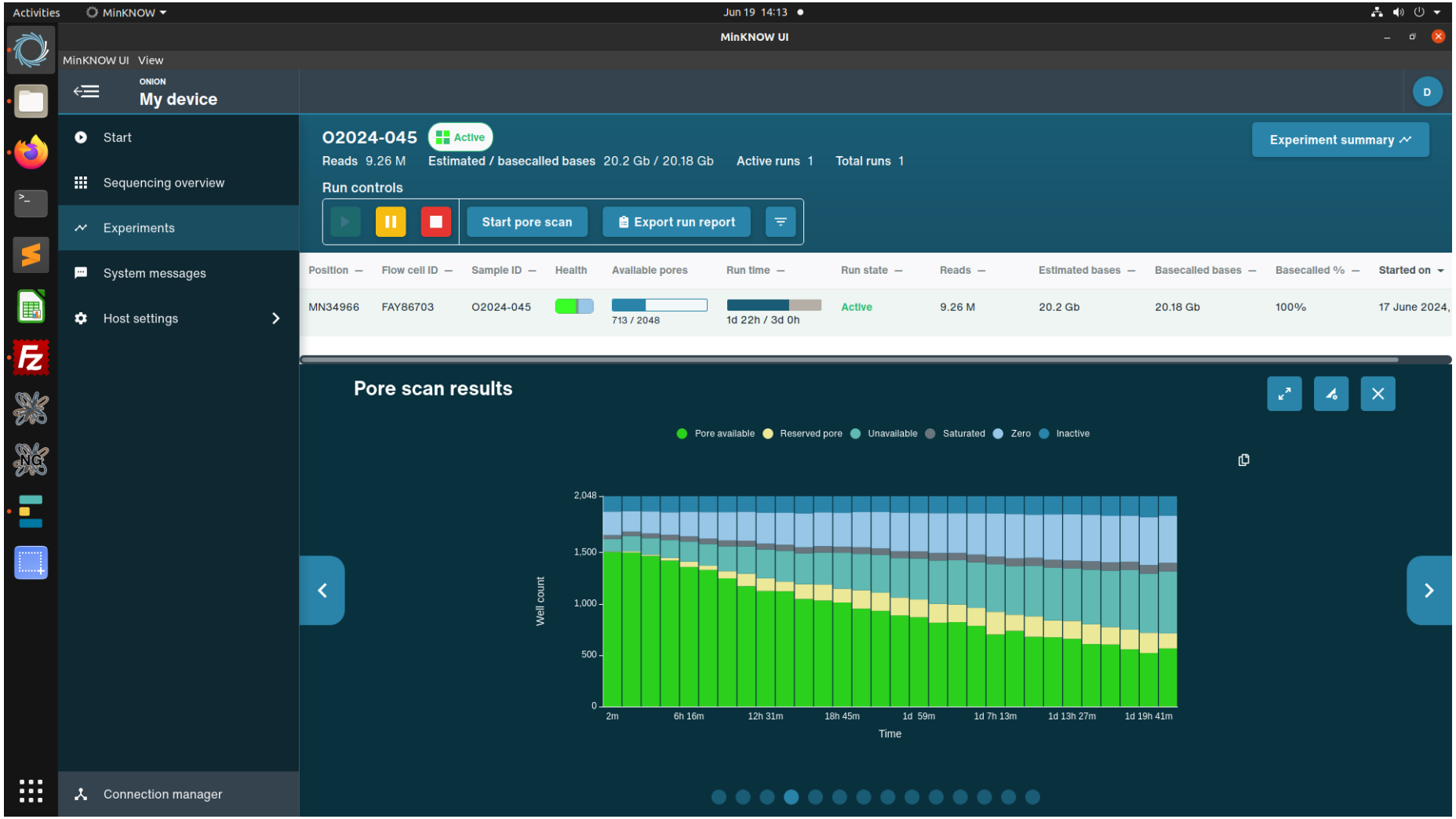
What is happening here?



What is happening here?

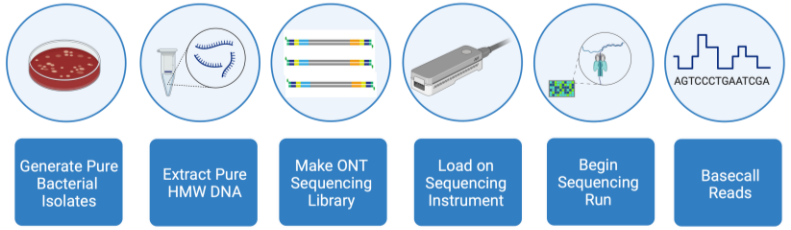


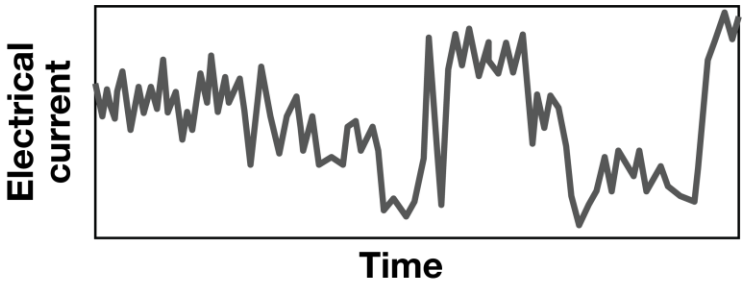
What is happening here?

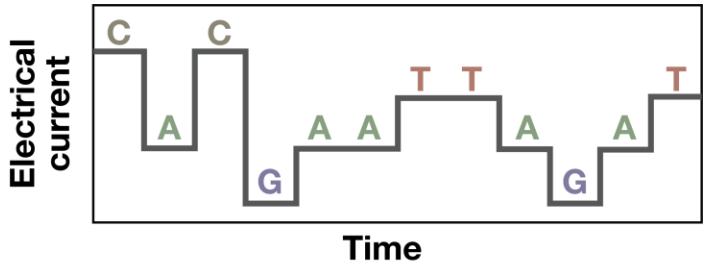


Basecall Reads

Basecall Reads



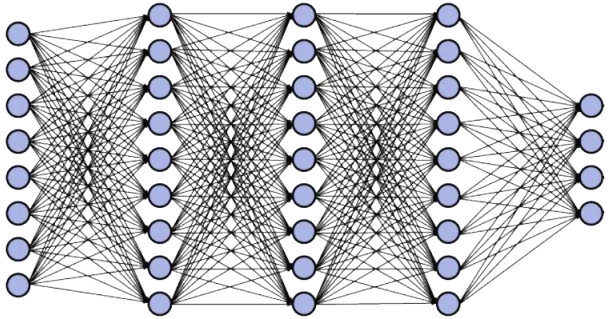




CACGAATTAGAT

pod5 files

Neural network

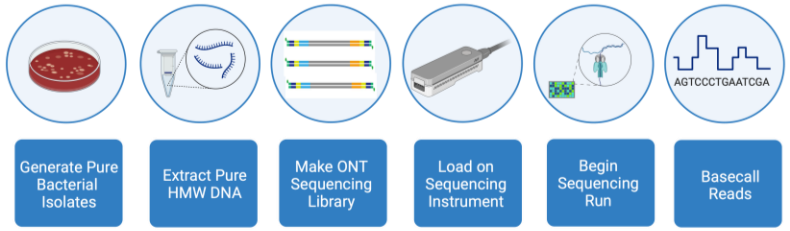


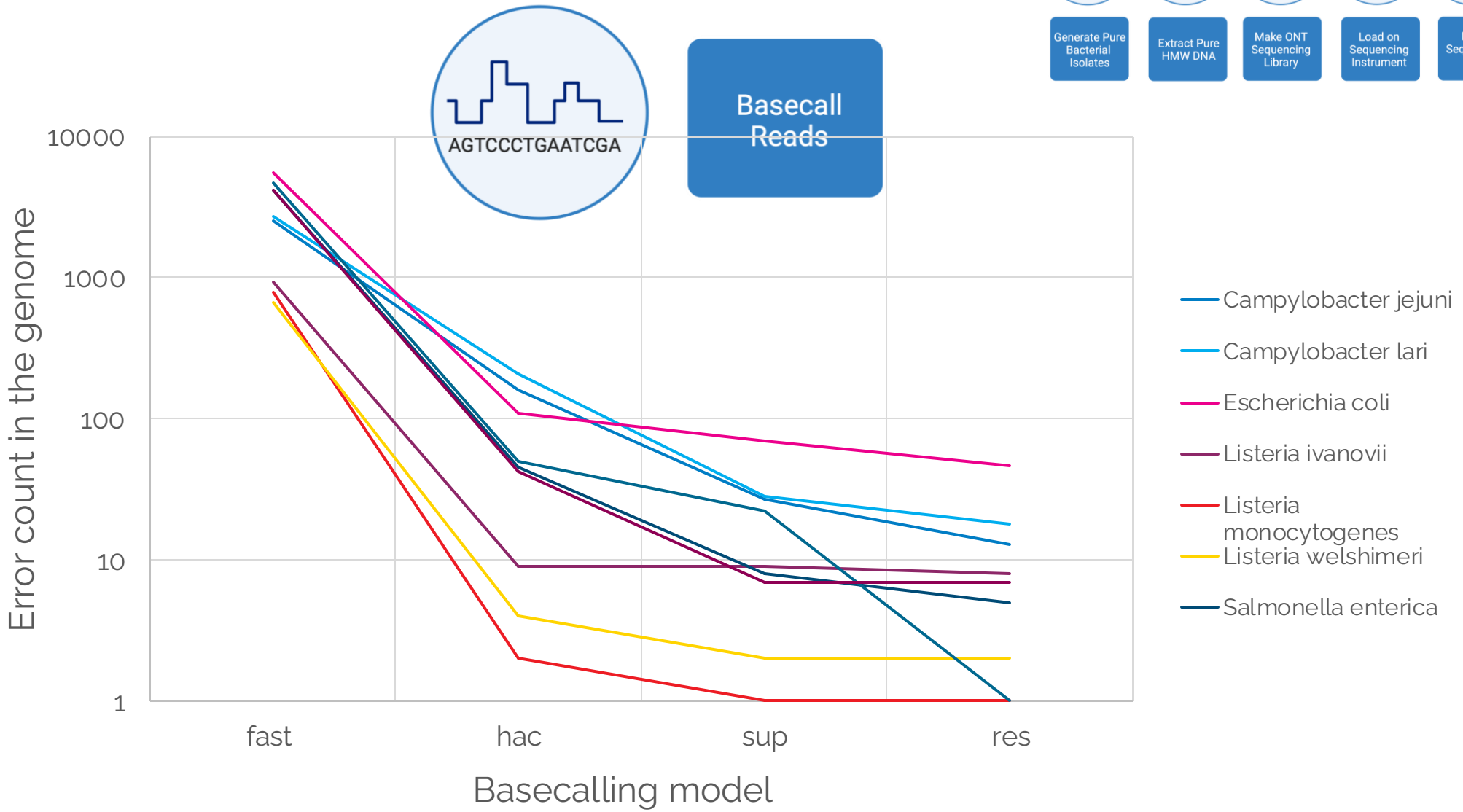
fastq files

Dorado/Guppy

- current ONT basecaller
- Used by MinKNOW
- Available in FAST, HAC and Sup mode

Basecall Reads





https://rrwick.github.io/2023/10/24/ont-only-accuracy-update.html Note: Analysis is from October 2023 and there have been improvements in ALL basecalling models since then

Wash and Store Flow Cells for Re-use

Aims:

- Remove old library from the flow cell
- Unblock any pores that have become “Unavailable”

Note: If unable to wash flow cell after run is finished store flow cell at 4°C and come back at a later date to perform wash

1. Remove all solution from the waste channel
2. Degrade all library on the flow cell by adding DNAse I wash solution
3. Incubate for 1 hour
4. Add storage buffer to flow cells
5. Store flow cell at 4°C
6. Perform flow cell check before loading new library

Pores available on flow cell check always lower than following first pore scan after loading new library
Eg. 200 pores on flow cell check becomes 400-500 pores on first pore scan after loading new library

Wash and Store Flow Cells for Re-use

What to do with old flow cells

Flongle has maximum of 126 pores (guarantee of 60)
A MinION flow cells with >120 pores is at least as good as a Flongle

What to do with a flongle

- Amplicon sequencing
- Plasmid sequencing
- Piloting sequencing of new libraries or samples
- Sequencing single bacterial isolates

THANK YOU

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