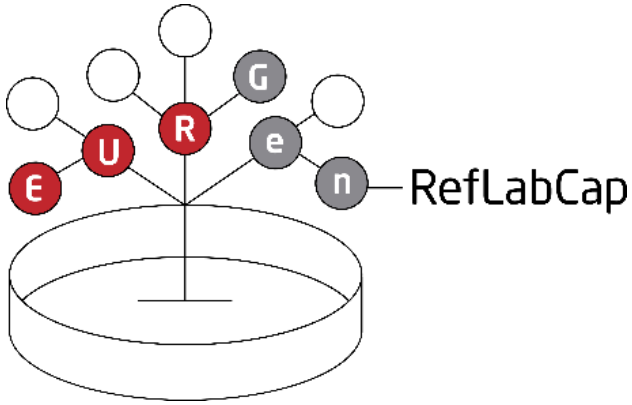
EURGen-RefLabCap

Best practice workshop – Nanopore sequencing

First day (virtual)

Thursday, 30 November 2023

9:00 - 12:30 CET



Funded by the European Union

Introduction

Four-day best practice workshop for Nanopore training

First day: Thursday 30 November (virtual) - Introduction to Nanopore sequencing

Second day: Wednesday 6 December at DTU - DNA extraction for Nanopore sequencing

Third day: Thursday 7 December at DTU - Library preparation and loading ONT flowcells

Fourth day: Friday 15 December (virtual) - Analysis and interpretation of ONT data

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Agenda

First day (virtual) – Thursday 30 November 2023, 9:00 - 12:30 CET

9:00 – 9:05: Introduction and agenda of the workshop (Ana Rita Rebelo, DTU)

9:05 – 9:20: Biochemical principles of Oxford Nanopore Technologies (Natasia Rebekka Thornval, DTU)

9:20 – 9:50: Comparison between ONT and Illumina sequencing (Ana Rita Rebelo, DTU)

9:50 – 10:00: Coffee break

10:00 – 10:30: State-of-the-art – literature on ONT sequencing applied to microbiology research and public health surveillance (Jette Sejer Kjeldgaard, DTU)

10:30 – 11:00: DNA extraction and library preparation for ONT sequencing (Ana Rita Rebelo, DTU)

11:00 – 11:20: Troubleshooting ONT runs (Niamh Lacy-Roberts, DTU)

11:20 – 11:30: Coffee break

11:30 – 12:00: Introduction of bioinformatics analyses of ONT data (Niamh Lacy-Roberts, DTU)

12:00 – 12:20: Explanation regarding purchase of Nanopore sequencer and reagents (Ana Rita Rebelo, DTU)

12:20 – 12:30: Q&A, wrap-up (Ana Rita Rebelo, DTU)

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Natasia Rebekka Thornval
nareth@food.dtu.dk

Biochemical principles of Oxford Nanopore Technologies

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What is DNA sequencing?

DNA sequencing is the determination of the exact **order** of **nucleotides** – adenine, guanine, cytosine and thymine - in a DNA fragment.

Nucleobases

Purines:

A Adenine

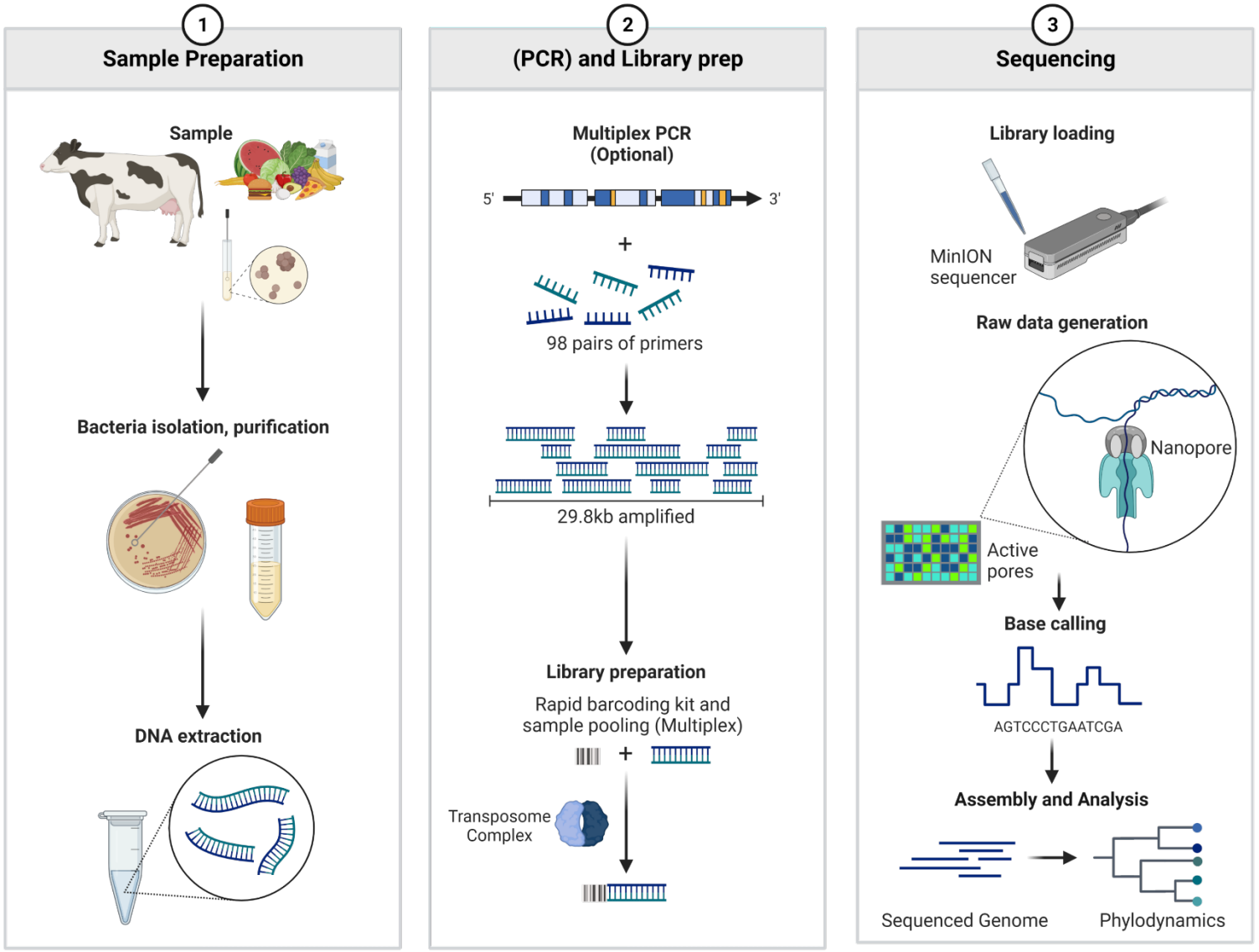
G Guanine

Pyrimidines:

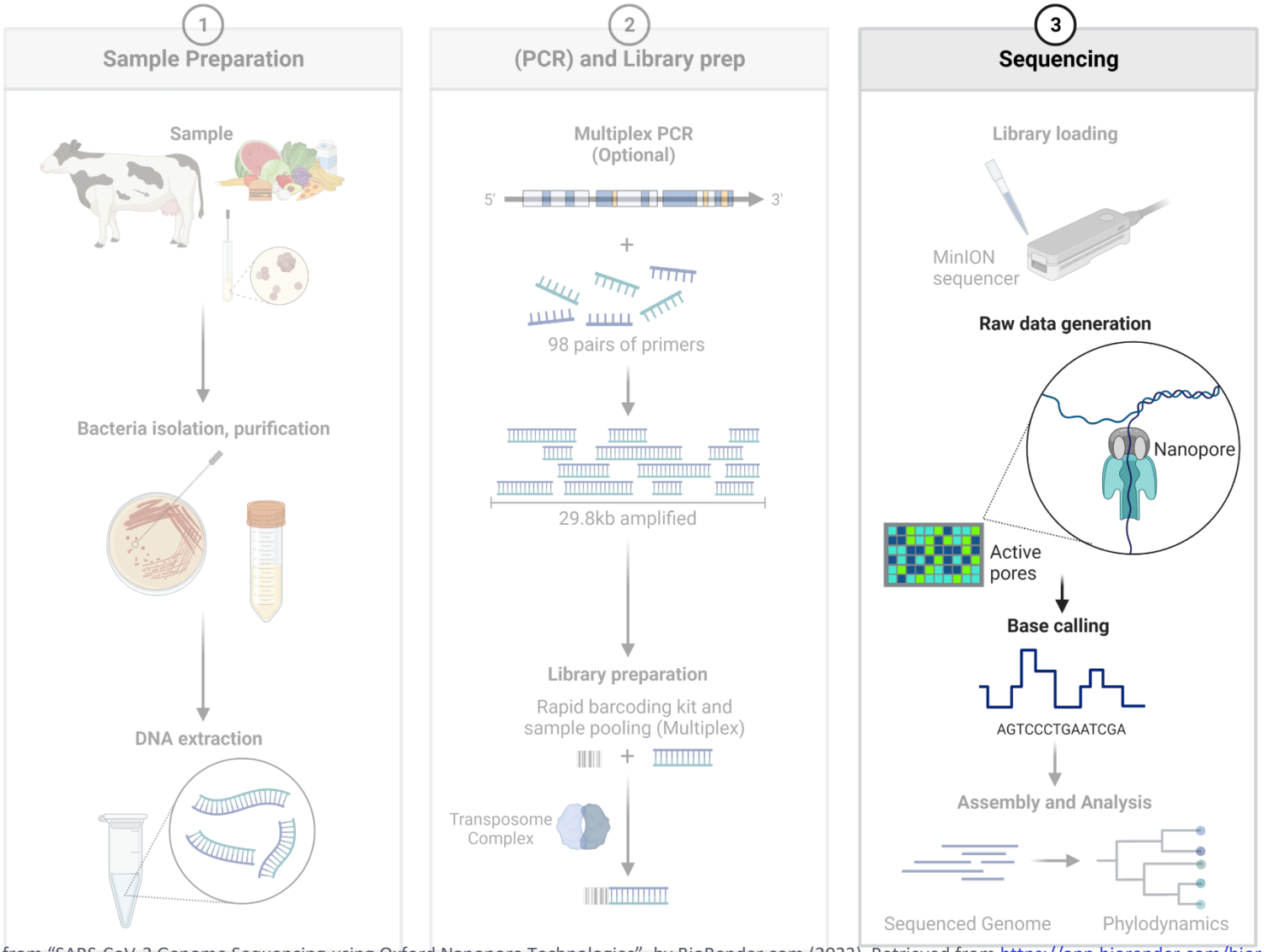
T Thymine

C Cytosine

Nanopore workflow

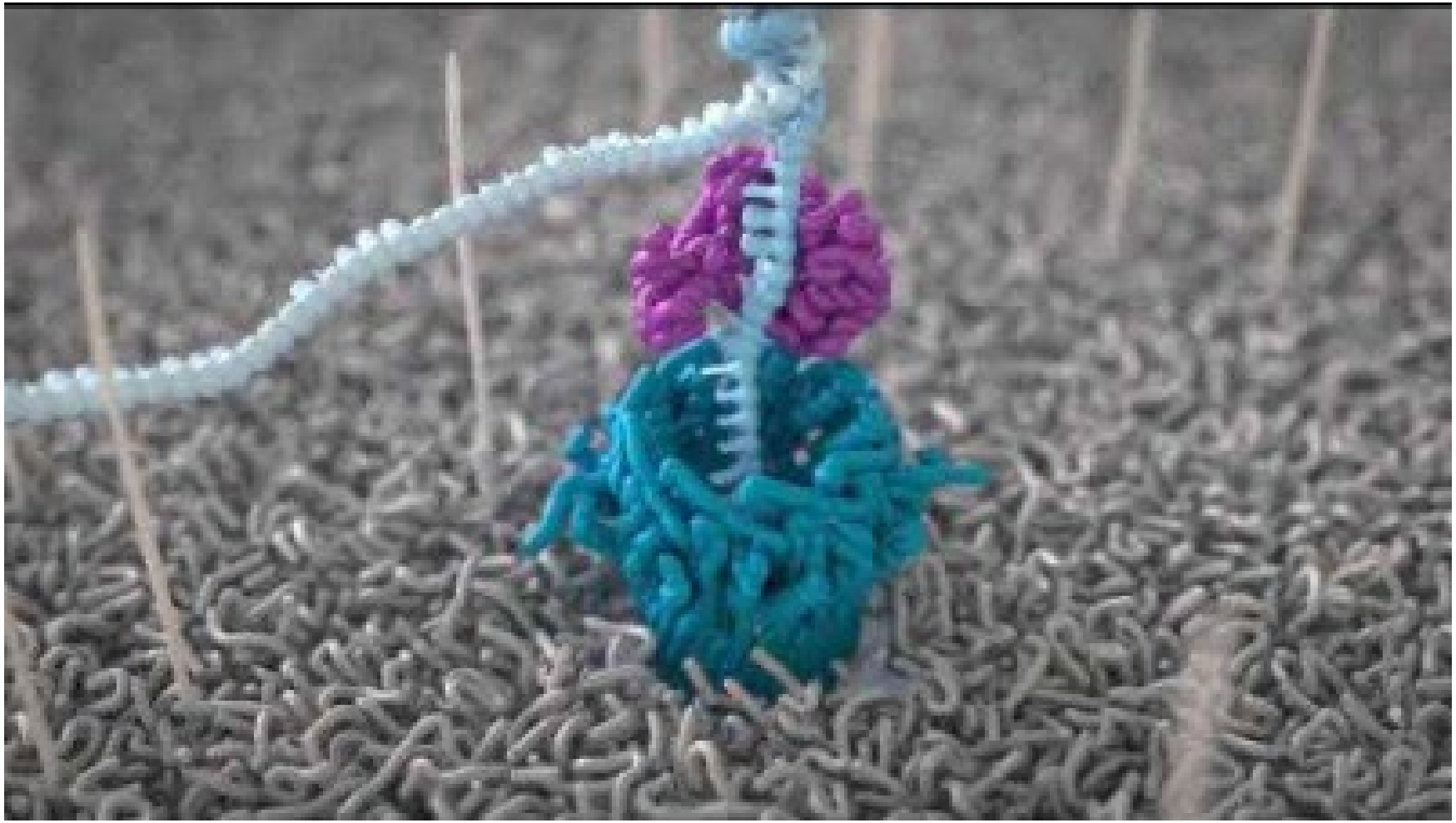


Raw data, base calling and analysis



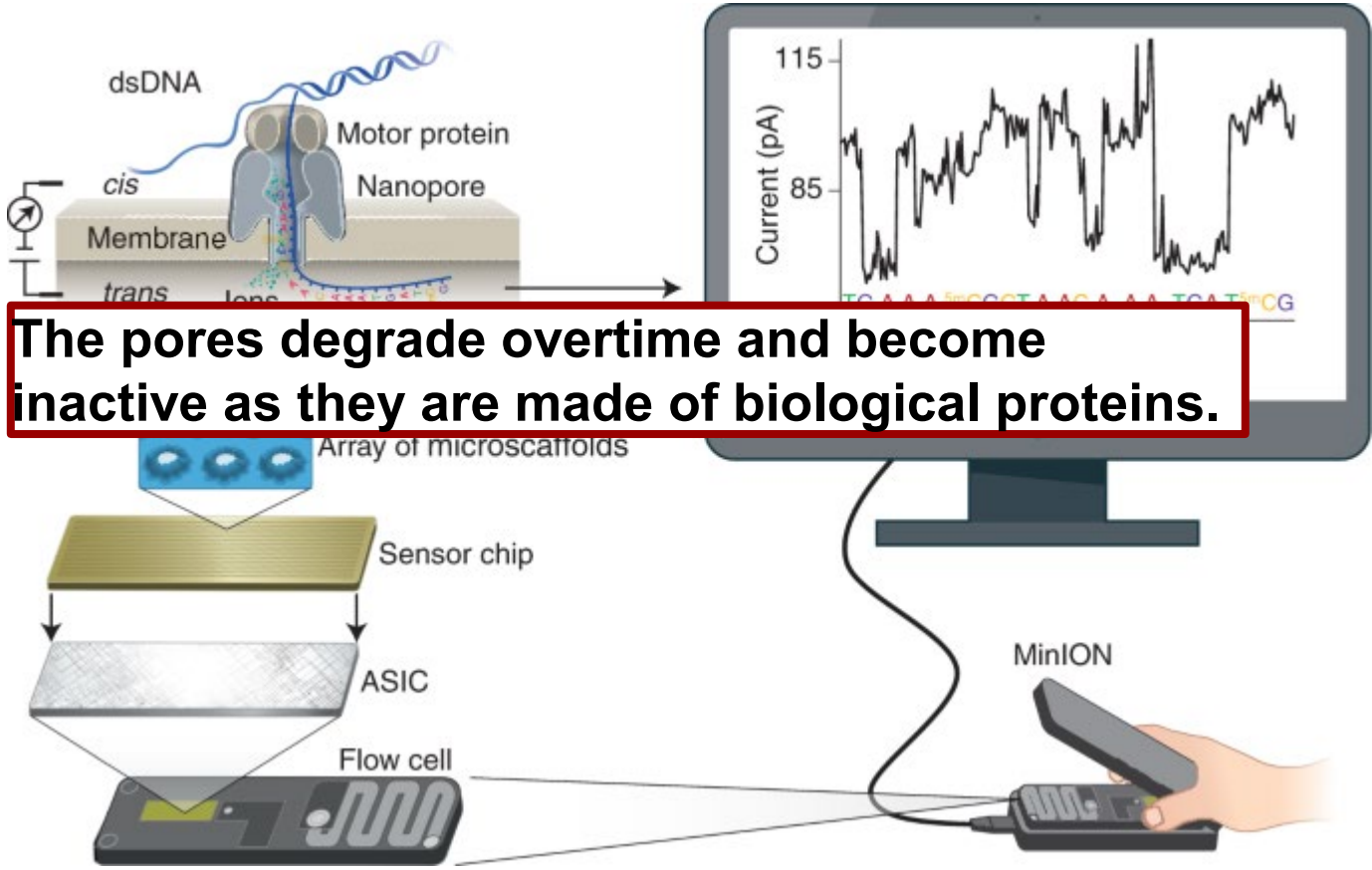
Adapted from "SARS-CoV-2 Genome Sequencing using Oxford Nanopore Technologies", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

How does it work?



What does it mean?

- **Application-Specific Integrated Circuit** (ASIC)
  - Controls and measures each experiment.
- **Sensor chip**
  - An array of channels with connected electrodes, that each corresponds to its own scaffold.
  - On a MinION flow cell there are 512 channels, each connected to 4 nanopores (2048 pores total).
- **Microscaffolds**
  - Each microscaffold contains a synthetic membrane with nanopores.
- **Nanopores**
  - Each nanopore is a protein-channel imbedded in a synthetic membrane connected to individual electrode.

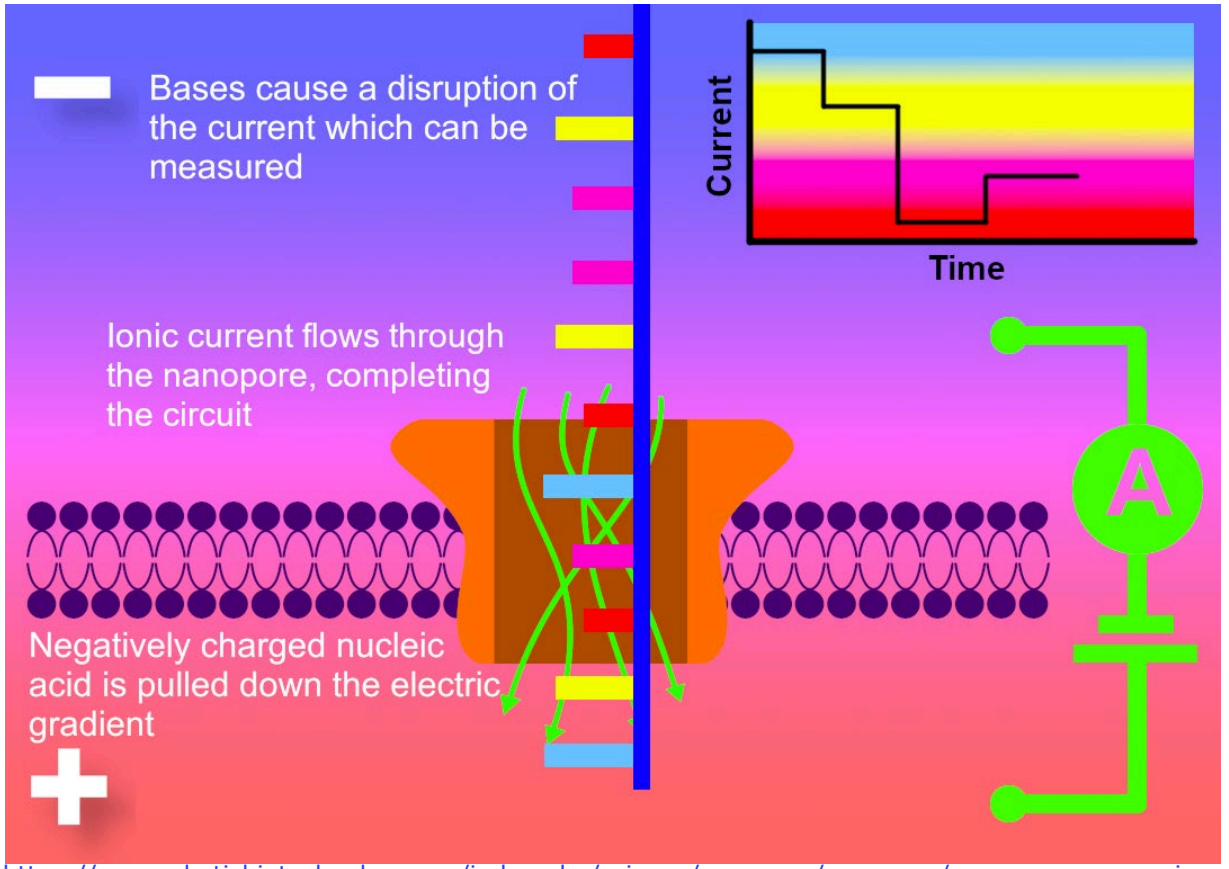


The pores degrade overtime and become inactive as they are made of biological proteins.

Wang, Y., Zhao, Y., Bollas, A. *et al.* Nanopore sequencing technology, bioinformatics and applications. *Nat Biotechnol* **39**, 1348–1365 (2021). https://doi.org/10.1038/s41587-021-01108-x

Mechanism of the pores

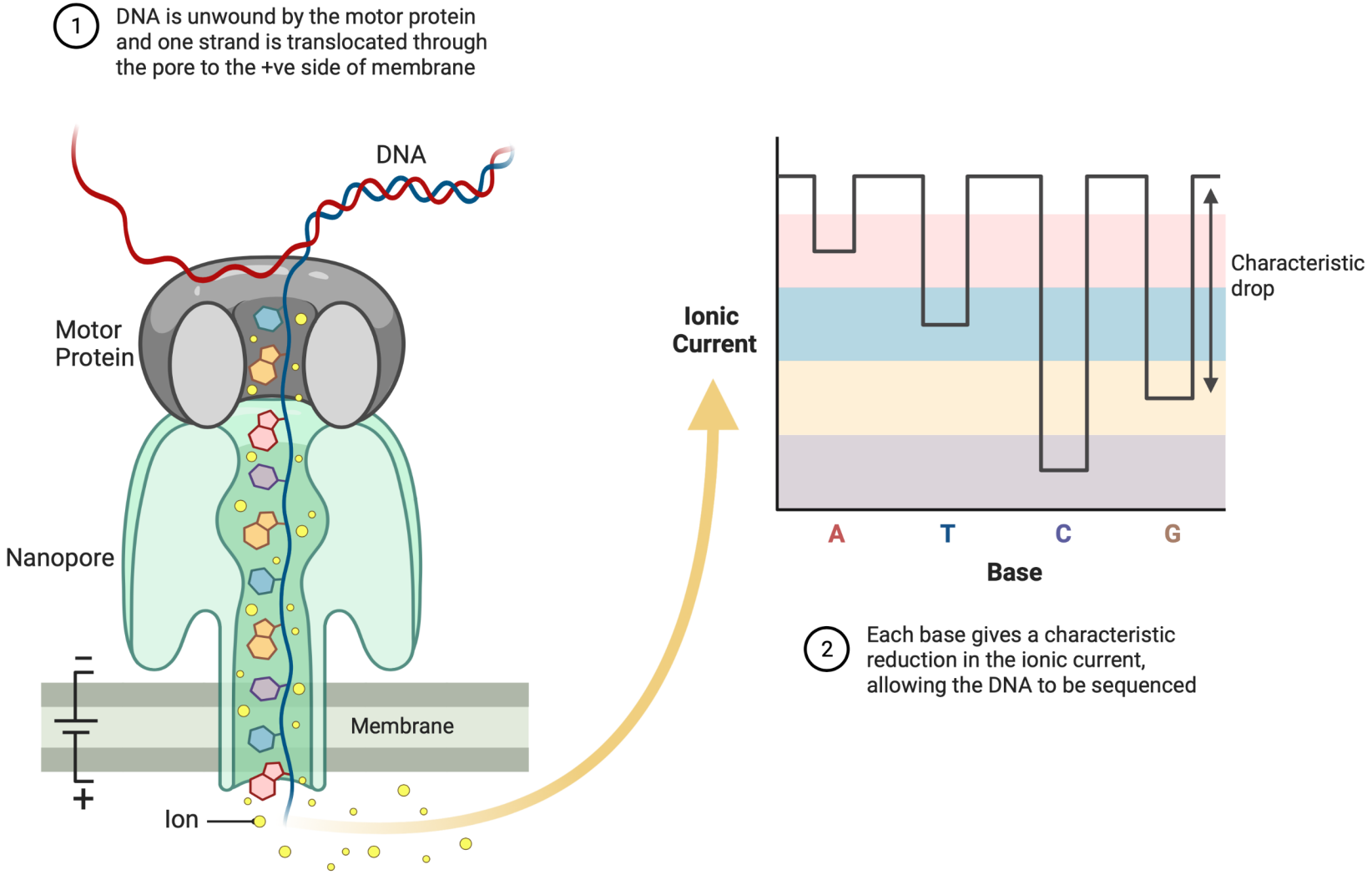
- An electrically resistant polymer membrane separates 2 chambers with electrolyte solutions.
- When a small voltage is added, the electrical circuit is completed by ions flowing through the pores.
- Negatively charged ssDNA is pulled through the pore by the electrical current to the positively charged side.



https://www.whatisbiotechnology.org/index.php/science/summary/nanopore/nanopore-sequencing-makes-it-possible-to-decode-the

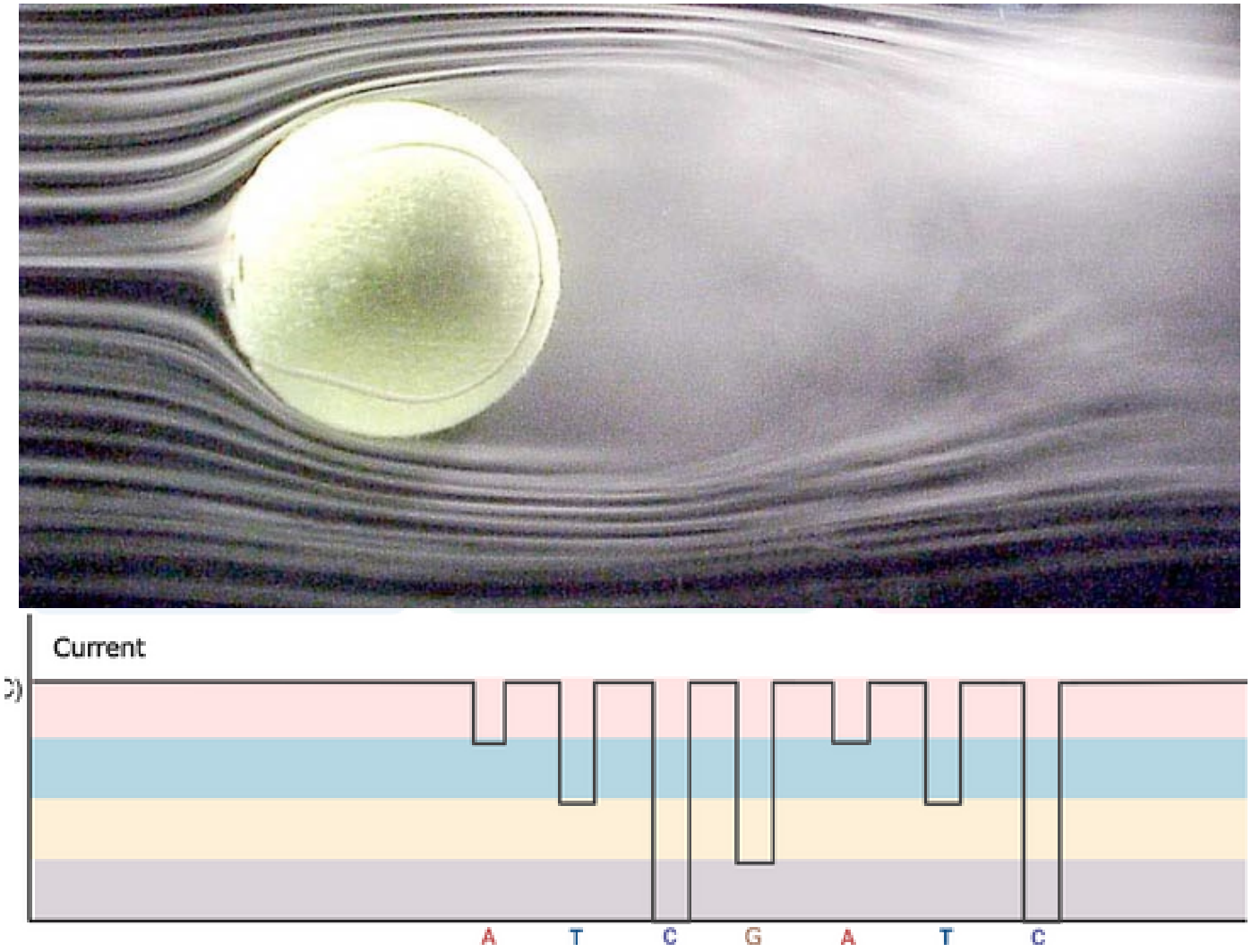
What does it mean?

- A motor protein controls the speed on the DNA strain through the pore that “unzips” the ds-DNA to ss-DNA.
- The motor protein ensures only a single strain is let through the pore in a “step-wise” manner.
- Each DNA base blocks the pore in a distinctive pattern in the electrical current.



How does it translate?

- Each DNA base blocks the pore in a distinctive pattern in the electrical current.
- When ssDNA passes through the Nanopore a distinct disruption in the electrical current can be measured.
- Specialized software translate the patterns by basecalling.
- **The disruption can be measured real-time** and allow real-time analysis!



Ana Rita Rebelo
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Comparison between ONT and Illumina sequencing

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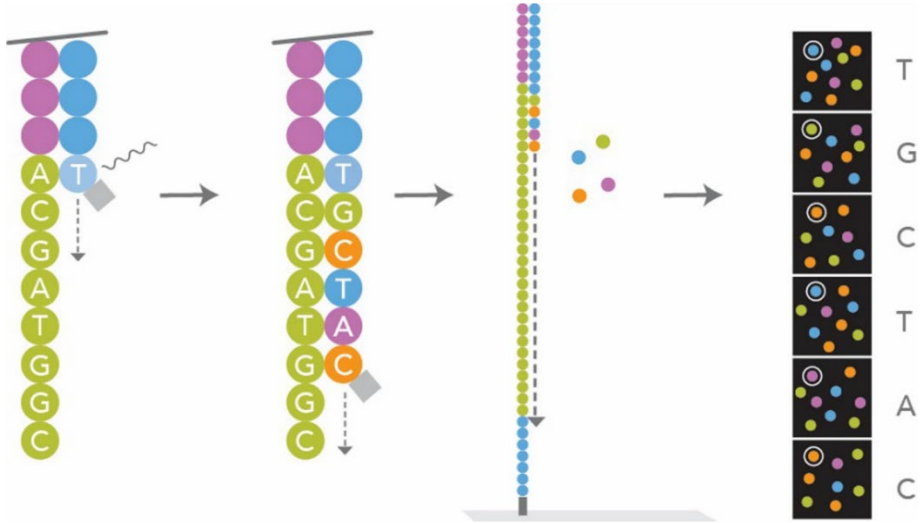
Biochemical principles

The biochemical principles behind Illumina and Nanopore sequencing are different

Illumina

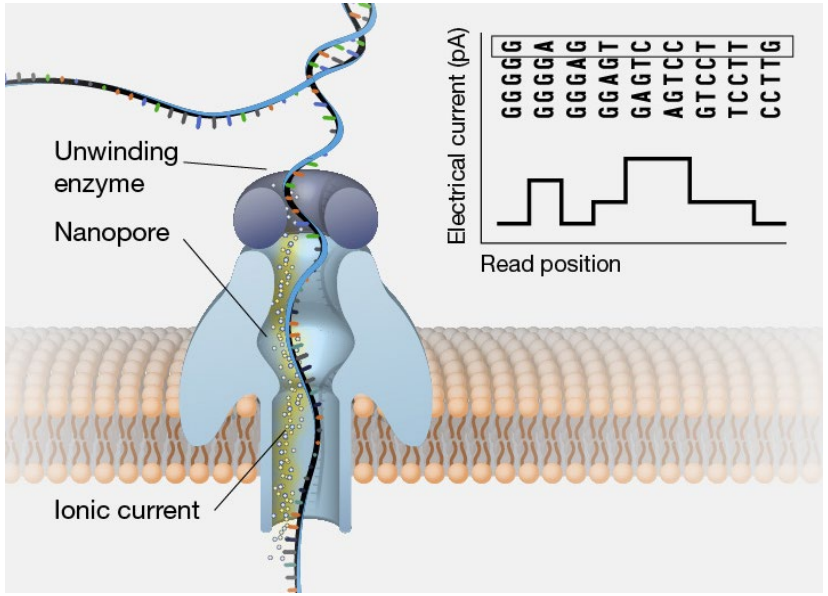
Sequencing by synthesis



EFSA journal 2018;16(S1):e16086

Nanopore

Sequencing through the nanopores



Wetterstrand 2023; https://www.genome.gov/genetics-glossary/Nanopore-DNA-Sequencing

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Biochemical principles

The raw data produced by the technologies are therefore also different

Illumina	**Nanopore**
Short paired end reads	Long single end reads
Not real time	Real time
24 – 48h to complete run	User decides when to stop the sequencing

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Sequencing yield

The amount of data produced in each sequencing run is different

Illumina

MiSeq: 15 Gb

NextSeq: 120 - 360 Gb

NovaSeq: 6 Tb (=6.000 Gb)

Nanopore

MinIon: 50 Gb (72h)

GridIon (5 flowcells): 250 Gb

PromethIon: 7 Tb (= 7.000 Gb)

Numbers are maximum estimates as described by the companies

Not always the case in real life – often lower

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Sequencing yield

The number of bacterial isolates that can be sequenced with proper accuracy and coverage are different

Illumina

MiSeq: 48

NextSeq: 96 - 192

Nanopore

MinIon: 12 - 24

Theoretically up to 96

Theoretically the flowcells can be re-used

Numbers are from in-house experience

Not always the case in real life – can be lower or higher depending on setting

Very dependent on the desired application: how accurate do we need to be and what coverage do we need?

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Sequencing quality

Difficult to estimate → lack of comparative studies

Quainoo et al. 2017, https://doi.org/10.1128/CMR.00016-17 :

Illumina: Error rate < 0.1% (1 incorrect bp / 1.000)

Nanopore: Error rate 12-15% (120-150 incorrect bp / 1.000)

Latest updates to nanopore sequencing achieve: November 2023

Flow cell	Kit	Sequencing & basecalling parameters	Sample	Raw read accuracy	Output
R10.4.1	Ligation Sequencing Kit V14	400 bps, 5 kHz, HAC basecalling	Human HG002	99.0% (Q20)	●●●
R10.4.1	Ligation Sequencing Kit V14	400 bps, 5 kHz, SUP basecalling	Human HG002	99.5% (Q23)	●●●
R10.4.1	Ligation Sequencing Kit V14	400 bps, 5 kHz, Duplex basecalling	Human HG002	>99.9% (Q30)	●

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Sequencing quality

Linde et al. 2023. **Comparison of Illumina and Oxford Nanopore Technology for genome analysis of Francisella tularensis, Bacillus anthracis, and Brucella suis.** *https://doi.org/10.1186/s12864-023-09343-z*

Abstract - Results

(…) Flow cell version 10.4 improved sequencing accuracy over version 9.4.1.

(…) The correct (sub-)species were inferred from all tested technologies, individually.

(…) The long reads of ONT allowed to assemble not only chromosomes of all species to near closure, but also virulence plasmids of Ba. anthracis.

(…) For F. tularensis, high-resolution genotyping using cgMLST and core-genome Single-Nucleotide-Polymorphism (cgSNP) typing produced highly comparable results between data from Illumina and both ONT flow cell versions.

(…) For Ba. anthracis, only data from flow cell version 10.4 produced similar results to Illumina for both high-resolution typing methods.

(…) However, for Br. suis, high-resolution genotyping yielded larger differences comparing Illumina data to data from both ONT flow cell versions.

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Sequencing quality

Ranasinghe et al. 2022.

Comparison of different sequencing techniques for identification of SARS-CoV-2 variants of concern with multiplex real-time PCR.

https://doi.org/10.1371%2Fjournal.pone.0265220

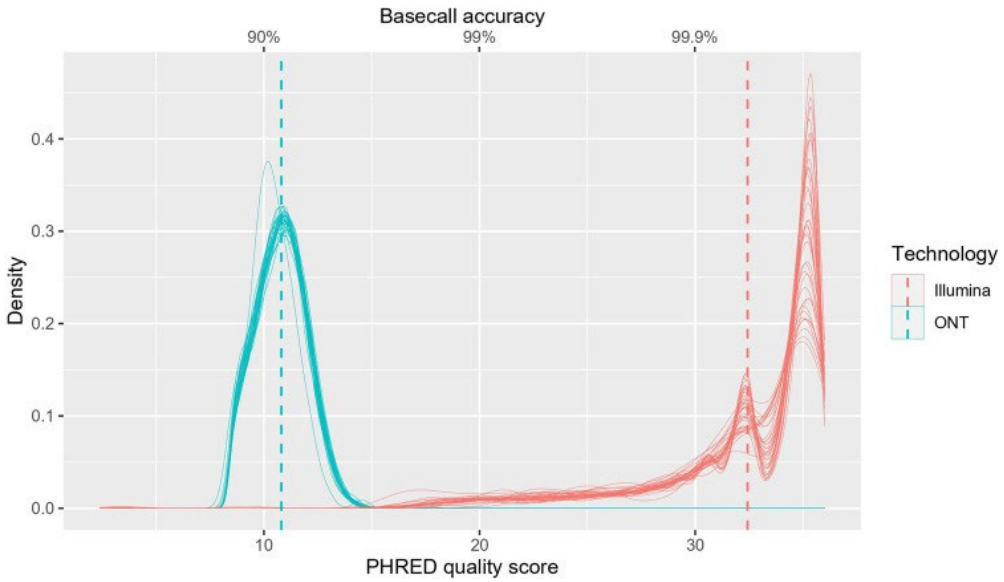

Fig. 1 - PHRED base call quality score distribution of samples sequenced by Illumina and ONT.

Table 1

Basic sequencing matrices for Illumina and Oxford Nanopore (ONT) outputs of 37.

Sequencing metrics	Illumina	ONT
Number of samples	37	37
Alignment start and end positions	30–29842	54–29903
Mean Coverage depth	109	266
Total Number of reads	372,654	9741
Yielded bases	50,576,802	9,307,884
Fraction of bases aligned	0.928	0.897
Mean Read length	140	945
Average per read identity	99.6	91.4
Average PHRED score	32.35	10.78
No of SNPs	31	36
No of amino acid substitutions	22	25
No of deletions	14	15
No of Amino acid substitutions	22	25
No of frameshift mutations	0	2
% of ambiguous bases	9%	6%
No of samples with < 10% ambiguous bases	25	29
Successful Pangolin calls	31	34
Successful Scorpio calls	25	31
Run time (h) for 96 samples	26	14
Cost per sample (USD)	~150–250	~10–40

99.9 - 99.99% accuracy

90% - 99% accuracy

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Price

Starting costs

Illumina

MiSeq: 96.000 EUR

NextSeq: 260.000 EUR

NovaSeq: ? 1M ?

Nanopore

MinIon: 950 EUR

GridIon: 47.500 EUR

PromethIon: 215.000 EUR

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Price

Price per isolate after implementation → very difficult to calculate

Depends on desired application (i.e. necessary quality and coverage) which influences how many isolates are multiplexed

To be considered:

- Price per library preparation kit / number of libraries that can be prepared by kit
- Price per flowcell
- Person-hours
- Longevity of reagents after first use

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Price

Example:

	NextSeq 500	MinIon
Price per library preparation kit	2900	945
Nr. of libraries that can be prepared by kit	96	96
Price per isolate for library preparation	= 30,2 EUR	= 9,8 EUR

	NextSeq 500	MinIon
Price per flowcell	1340	855
Price per isolate for sequencing	Depends on Nr. of isolates that are multiplexed	

	NextSeq 500	NextSeq 500 (after optimization)	MinIon	MinIon (after optimization)	MinIon (in theory)
Nr. of isolates multiplexed	96	192	12	24	96
Price per isolate for sequencing	= 14 EUR	= 7 EUR	= 71,3 EUR	= 35,6 EUR	= 8,9 EUR
Total price per isolate	**44 EUR**	**37 EUR**	**81 EUR**	**45 EUR**	**20 EUR**

Plus other reagents/instruments, etc.

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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DNA extraction

The library preparation protocols are different and therefore influence the choice for DNA extraction

Illumina	**Nanopore**
DNA can be more fragmented	DNA should not be fragmented
Less amount of DNA needed	More amount of DNA needed
“General” extraction kits	Specific extraction kits – examples in upcoming presentations Generally more expensive

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Library preparation

The library preparation protocols are different

	Illumina	**Nanopore**
DNA input	Nextera XT: **1 ng** DNA prep: **100-500 ng**	PCR Barcoding Kit 24 V14: **1-5 ng** Rapid Barcoding Kit 96 V14: **50 ng**
Nr. of samples	**96** / microtiter plate	PCR Barcoding Kit 24 V14: **24** Rapid Barcoding Kit 96 V14: **96**
Time	Nextera XT: **177 min** DNA prep: **185 min**	PCR Barcoding Kit 24 V14: **180 min** Rapid Barcoding Kit 96 V14: **60 min**
Special equipment	Thermal cycler Magnetic rack for microtiter plates Plate shaker	Thermal cycler or heating blocks Magnetic racks for eppendorfs Hula mixer (rotator) (opt.) Plate shaker
Safe stopping points	Up to one week	Not described
Library QC	Qubit Bioanalyzer (opt.)	Qubit Bioanalyzer (opt.) Nanodrop (opt.)

Plus loading, starting the run

Illumina, Inc., San Diego, CA, USA (https://www.illumina.com)

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Applications

Due to the sequencing quality, read length and biochemical principles of the technologies

Illumina

For applications that require high accuracy:

- Cluster analyses
- Detection of chromosomal point mutations (e.g. associated with AMR)

Nanopore

For applications that require long sequences:

- Plasmid analyses
- Circularizing genomes

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Comparable results, literature, guidance, etc.

Illumina was available years earlier

Illumina

Publications describing:

- Optimization of sequencing
- Bioinformatics tools
- Comparison of tools
- QC control and benchmarking
- Thresholds for clustering
- Performance and expected results per species

Benchmarking datasets

Harmonised protocols

Etc..

Nanopore

Not a lot yet - examples in upcoming presentations

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Jette Sejer Kjeldgaard
jetk@food.dtu.dk

State-of-the-art – literature on ONT sequencing applied to microbiology research and public health surveillance

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EURGenRefLabCap Best Practice Workshop

State-of-the-art – literature on ONT sequencing applied to microbiology research and public health surveillance

Jette S. Kjeldgaard (jetk@food.dtu.dk)
Global Capacity Building

Current applications of ONT sequences

- Broad range of applications
  - DNA/RNA
  - Transcriptomics
  - Metagenomics
  - ….

WGS of a cluster of MDR Shigella sonnei utilising Oxford Nanopore R10.4.1 long-read sequencing

MICROBIOLOGY | INFECTIOUS DISEASES | WHOLE GENOME

Publication | 15 November 2023

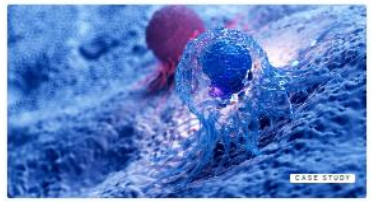
From Journal of Antimicrobial Chemotherapy

Routine metagenomics service for intensive care unit patients with respiratory infection

MINION | GRIDION | METAGENOMICS

Publication | 7 November 2023

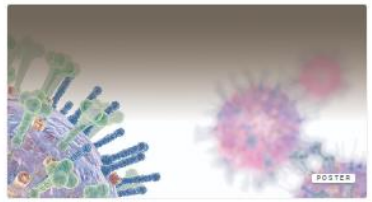
From American Journal of Respiratory and Critical Care Medicine



Characterising somatic structural variation in colorectal cancer with long nanopore reads

STRUCTURAL VARIATION | WHOLE GENOME | PROMETHION

Case study | 25 May 2023



Rapid decentralized nanopore sequencing of full-length influenza A genomes PDF

INFECTIOUS DISEASES

Poster | 15 May 2023

Comprehensive characterisation of repeat expansions in neurodegenerative diseases with long nanopore reads

HUMAN GENOMICS

Case study | 2 February 2022

Targeting sex determination to suppress mosquito populations

ANIMAL | MINION | RNA/CDNA

Publication | 15 November 2023

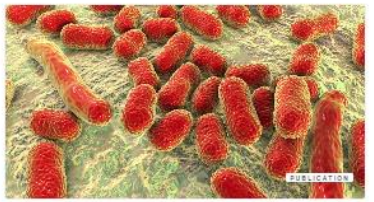
From bioRxiv

Creation of de novo cryptic splicing for ALS/FTD precision medicine

HUMAN GENOMICS | RNA/CDNA

Publication | 15 November 2023

From bioRxiv

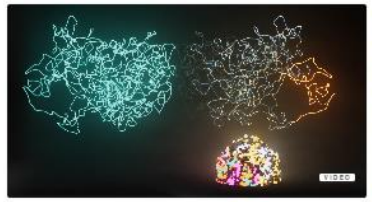


WGS of a cluster of MDR Shigella sonnei utilising Oxford Nanopore R10.4.1 long-read sequencing

MICROBIOLOGY | INFECTIOUS DISEASES | WHOLE GENOME

Publication | 15 November 2023

From Journal of Antimicrobial Chemotherapy



Nanopore sequencing: insights from neonatal intensive care to cancer

PROMETHION | CANCER RESEARCH | CLINICAL RESEARCH

Video | 15 November 2023

Insights into spinach domestication from genome sequences of two wild spinach progenitors, Spinacia turkestanica and S. ...

PLANT | DE NOVO ASSEMBLY

Publication | 13 November 2023

From bioRxiv

Targeted haplotyping in pharmacogenomics using Oxford Nanopore Technologies' adaptive sampling

TARGETED | HUMAN GENOMICS | PROMETHION

Publication | 13 November 2023

From Frontiers in Pharmacology

Applications

Get started | Talk to us | Subscribe

Nanopore sequencing offers advantages in all areas of research. Our offering includes DNA sequencing, as well as RNA and gene expression analysis and future technology for analysing proteins.

Research areas

- Microbiology
- Microbiome
- Cancer
- Transcriptome
- Population genomics
- Environmental research & conservation
- Human genetics
- Clinical research
- Plant
- Animal
- COVID 19
- Infectious disease

Investigations

- Structural variation
- Gene expression
- Splice variation
- Fusion transcripts
- Single cell
- SNVs and phasing
- Identification
- Assembly
- Epigenetics
- Chromatin conformation

Techniques

- Whole genome
- RNA & cDNA sequencing
- Short fragment mode
- Targeted
- Metagenomics

Current applications of ONT sequences

- Broad range of applications
  - Diagnostics
  - AMR analysis
  - Plasmid analysis
  - Outbreak detection

WGS of a cluster of MDR Shigella sonnei utilising Oxford Nanopore R10.4.1 long-read sequencing

MICROBIOLOGY | INFECTIOUS DISEASES | WHOLE GENOME

Publication | 15 November 2023

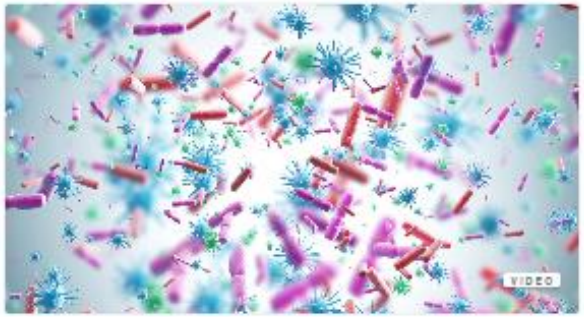
From Journal of Antimicrobial Chemotherapy

Routine metagenomics service for intensive care unit patients with respiratory infection

MINION | GRIDION | METAGENOMICS

Publication | 7 November 2023

From American Journal of Respiratory and Critical Care Medicine



Targeted nanopore sequencing for bacterial and viral classification

VIDEO | INFECTIOUS DISEASES | MICROBIOLOGY

Video | 12 October 2023

Application of nanopore sequencing to identify antimicrobial resistance genes, mobile genetic elements and virulence fac...

CLINICAL RESEARCH | MICROBIOLOGY | INFECTIOUS DISEASES

Publication | 26 October 2023

From bioRxiv

Diagnostics

- Single isolate sequencing
- Community sequencing
  - Metagenomics
    - Direct specimen testing
    - Wastewater surveillance
- Nanopore White Papers:

Portable sequencing

Malaria genomic surveillance using nanopore sequencing: Shifting the focus into endemic countries

https://communities.springernature.com/amp/posts/malaria-genomic-surveillance-using-nanopore-sequencing-shifting-the-focus-into-endemic-countries

In case of allegations of an intentional cause for a biological threat or disease outbreak, an investigation for verification purposes needs to be able to discriminate between a deliberate release of an agent and the natural background.

doi: 10.1093/gigascience/giy033

Diagnostics using portable sequencing - example

2. Case presentation

On February 15, 2022 a male toddler 3 years of age, was brought to Machame Lutheran Hospital located in Hai district in Kilimanjaro region with a history of diarrhea and vomiting for the past week. The patient has been vomiting three times a day and passing out watery stool occasionally with blood stains. This was accompanied by on-and-off fever, especially during the night. The patient had been sent to a nearby health center before coming to Machame Lutheran Hospital. At the previous health center, the patient was treated (medication could not be tracked) with no improvement and hence referred to Machame Lutheran Hospital for further management. The patient had no history of admission due to the current illness.

On examination, the toddler was looking ill, clinically pale, with no jaundice observed, wasted, unhappy, weak with a swollen face, and not dehydrated with a body temperature of 38.5 °C. The provisional diagnosis declared acute watery diarrhea with no dehydration, amoebic dysentery, acute malnutrition, and severe anemia. The management plan was as follows admission at the children's

Check for updates

Nanopore sequencing technology for clinical diagnosis of infectious diseases where laboratory capacity is meager: A case report

Happiness H. Kumburu [a,b,c,*], Mariana Shayo [c,**], Marco van Zwetslaar [b], Judith Njau [b], Davis J. Kuchaka [b,f], Ignas P. Ignas [b], Boaz Wadugu [b], Robert Kasworm [e], Lazaro J. Masaki [e], Malte B. Hallgren [d], Philip T.L.C. Clausen [d], Blandina Theophil Mmbaga [a,b,c], Frank M. Aarestrup [d], Tolbert B. Sonda [a,b,c]

https://doi.org/10.1016/j.heliyon.2023.e17439

Diagnostics using portable sequencing – example
Faecal microbiota

Table 1
Relative abundance of bacterial species identified.

Bacterial species	Number of bases	Relative abundance
*Escherichia coli**	1103065	22.66%
Comamonas kerstersii	904446	18.58%
BIfidobacterium kashiwanohense	499159	10.25%
Desulfovibrio vulgaris	341562	7.02%
Parabacteroides distasonis	311669	6.40%
Collinsella aerofaciens	274892	5.65%
Lactobacillus ruminis	189420	3.89%
Sutterella wadsworthensis	156074	3.21%
Roseburia hominis	130953	2.69%
Olsenella sp.	127369	2.62%
Bifidobacterium longuM	94299	1.94%
Prevotella melaninogenica	93160	1.91%
Dysosmobacter welbionis	89796	1.84%
Prevotella intermedia	89723	1.84%
Faecalibacterium prausnitzii	74165	1.52%
Bacteroides uniformis	68538	1.41%
Intestinimonas Butyriciproducens	68394	1.40%
Bifidobacterium bifidum	62769	1.29%
Veillonella parvula	45998	0.94%
Bifidobacterium pseudocatenulatum	38444	0.79%
*Campylobacter jejuni**	29508	0.61%
Bifidobacterium catenulatum	27401	0.56%
Prevotella multiformis	24272	0.50%
Prevotella denticola	23245	0.48%

*Fast response
-Species ID
-Virulence factors
-AMR genes
*High resolution!
-Non-culturable
-Low abundance

Table 1
Relative abundance of bacterial species identified.

Bacterial species	Number of bases	Relative abundance
*Escherichia coli**	1103065	22.66%
Comamonas kerstersii	904446	18.58%
Bifidobacterium kashiwanohense	499159	10.25%
Desulfovibrio vulgaris	341562	7.02%
Parabacteroides distasonis	311669	6.40%
Collinsella aerofaciens	274892	5.65%
Lactobacillus ruminis	189420	3.89%
*Campylobacter jejuni**	29508	0.61%
Bifidobacterium catenulatum	27401	0.56%
Prevotella multiformis	24272	0.50%
Prevotella denticola	23245	0.48%

Pathogens. 2022 Sep; 11(9): 1032.
Published online 2022 Sep 12. doi: 10.3390/pathogens11091032
PMCID: PMC9504711
PMID: 36145464

The Emergence of the Genus *Comamonas* as Important Opportunistic Pathogens

Metagenomics for diagnostics

- Respiratory metagenomics (RMg) holds promise as a first-line diagnostic test for lower respiratory tract infections.
- In principle, it rapidly detects all potential pathogens along with antimicrobial resistance determinants and provides sequence typing for infection control or public health actions.


American Journal of Respiratory and Critical Care Medicine

Home > American Journal of Respiratory and Critical Care Medicine > List of Issues > Just Accepted

Routine Metagenomics Service for Intensive Care Unit Patients with Respiratory Infection

Themoula Charalampous , Adela Aloclea-Medina , Luke B. Snell , Christopher Alder , Mark Tan , Tom G. S. Williams , Noor Al-Yaakoubi , Gul Humayun , Christopher I S Meadows , Duncan L.A. Wyncoll , Richard Paul , Show All...

Fastidious or unexpected organisms were reported in 21 samples including anaerobes (n=12)

Mycobacterium tuberculosis, Tropheryma whipplei Cytomegalovirus

Legionella pneumophila ST1326

Staphylococcus aureus, Streptococcus pyogenes,

S. dysgalactiae subspecies equisimilis

Aspergillus fumigatus

https://doi.org/10.1164/rccm.202305-0901OC

Metagenomics pilot study

- See workflow in the paper linked below

- During the first 15-weeks RMg provided same-day results for 110 samples (86%) with median turnaround time of **6.7hrs**
- RMg was **93% sensitive** and 81% specific for clinically-relevant pathogens compared with routine testing.
- **48%** of RMg results informed **antimicrobial prescribing changes** (22% escalation; 26% de-escalation) with escalation based on speciation in 20/24 cases and detection of acquired-resistance genes in 4/24 cases

https://doi.org/10.1164/rccm.202305-0901OC

AMR analysis - plasmids

- Advantages
  - Fast answer
    - Real-time sequencing
  - Full genome assembly
  - Full plasmid assembly

Application of nanopore sequencing to identify antimicrobial resistance genes, mobile genetic elements and virulence factors in clinical isolates

Rachel Kimani, Sebastian Musundi, Patrick Wakaba, David Mbogo, Suliman Essuman, Bernard N. Kanoi, Jesse Gitaka

Efficient generation of complete sequences of MDR-encoding plasmids by rapid assembly of MinION barcoding sequencing data

Ruichao Li,[1,2] Miaomiao Xie,[1] Ning Dong,[1] Dachuan Lin,[1,] Edward Wai-Chi Chan,[2] and Sheng Chen[1,2]

In an experiment by Li *et al.*, a single nanopore read from a carbapenem-resistant *Escherichia coli* strain was shown to span an entire plasmid of >90 kb in length[30].

https://doi.org/10.1101/2023.10.25.563907 doi: 10.1093/gigascience/gix132

Plasmid hybrid assembly

Highly accurate-single chromosomal complete genomes using IonTorrent and MinION sequencing of clinical pathogens

Karthick Vasudevan [1], Naveen Kumar Devanga Ragupathi [1], Jobin John Jacob, Balaji Veeraraghavan

Requires both short-read and long-read sequences

 https://doi.org/10.1016/j.ygeno.2019.04.006

Plasmid hybrid assembly approaches

- We **benchmarked** the hybrid assembly approaches of
- **MaSuRCA, SPAdes, and Unicycler**
- for bacterial pathogens using Illumina and Oxford Nanopore sequencing by determining genome completeness and accuracy, antimicrobial resistance (AMR), virulence potential, multilocus sequence typing (MLST), phylogeny, and pan genome

Research article | Open access | Published: 14 September 2020

Benchmarking hybrid assembly approaches for genomic analyses of bacterial pathogens using Illumina and Oxford Nanopore sequencing

Zhao Chen, David L. Erickson & Jianghong Meng

https://doi.org/10.1186/s12864-020-07041-8

Plasmid analysis – ONT only

- Before – Illumina/Sanger sequencing
  - high-quality plasmid sequence is achieved through capillary-based sequencing, requiring customized sets of primers for each plasmid

Complete sequence verification of plasmid DNA using the Oxford Nanopore Technologies' MinION device

Scott D. Brown, Lisa Dreolini, Jessica F. Wilson, Miruna Balasundaram & Robert A. Holt

BMC Bioinformatics **24**, Article number: 116 (2023) | Cite this article

https://doi.org/10.1186/s12859-023-05226-y

Comparison of methods for plasmid assembly

- See table in paper listed below

https://doi.org/10.1186/s12859-023-05226-y

Comparison of ONT library preps

- Oxford Nanopore Technologies (ONT) sequencing platforms currently offer two approaches to whole-genome native-DNA library preparation: **ligation and rapid**.
- Comparison of these two approaches for assessing their ability to recover small plasmid sequences.
- DNA sequenced from seven plasmid-rich bacterial isolates in three different ways:
  - ONT ligation, ONT rapid and Illumina.
- Using the Illumina read depths to approximate true plasmid abundance
  - small plasmids (<20 kbp) were underrepresented in ONT ligation read sets but were not underrepresented in ONT rapid read sets.

› Microb Genom. 2021 Aug;7(8):000631. doi: 10.1099/mgen.0.000631.

Recovery of small plasmid sequences via Oxford Nanopore sequencing

Ryan R Wick [1], Louise M Judd [1], Kelly L Wyres [1], Kathryn E Holt [1 2]

- Smallest plasmids being the most underrepresented in ONT ligation read sets.

- Lower rates of chimaeric reads in the rapid read sets relative to ligation read sets.

- When small plasmid recovery is important, ONT rapid library preparations are preferable to ligation-based protocols

DOI: 10.1099/mgen.0.000631

Outbreak investigation with ONT

- ONT applied to
  - produce complete reference sequences
  - cluster detection

To utilize long-read nanopore sequencing (R10.4.1 flowcells) for WGS of a cluster of MDR *Shigella sonnei*, specifically characterizing genetic predictors of antimicrobial resistance

WGS of a cluster of MDR *Shigella sonnei* utilizing Oxford Nanopore R10.4.1 long-read sequencing

Gordon Ritchie ✉, Samuel D Chorlton, Nancy Matic, Jennifer Bilawka, Leah Gowland, Victor Leung, Aleksandra Stefanovic, Marc G Romney, Christopher F Lowe

https://doi.org/10.1093/jac/dkad346

Outbreak investigation with ONT

Detection of AMR genes AND chromosomal mutations

Cluster isolates were resistant to:

ampicillin (*bla*$_{\text{TEM-1}}$)

trimethoprim/sulfamethoxazole (*dfA1*, *dfrA17*; *sul*1, *sul*2),

azithromycin (*ermB*, *mphA*)

ciprofloxacin (*gyrA* S83L, *gyrA* D87G, *parC* S80I)

See figure in paper linked below

Figure 1. Phylogenetic tree of Shigella sonnei isolated in our laboratory during the study period, including genomic ...

J Antimicrob Chemother, dkad346, https://doi.org/10.1093/jac/dkad346

Beyond DNA sequencing

- applications of nanopores in
  - molecular sensing and sequencing, chemical catalysis and biophysical characterization.
  - single-protein analysis and sequencing, single-molecule covalent chemistry,
  - clinical sensing applications for single-molecule liquid biopsy,
  - synthetic biomimetic nanopores as experimental models for natural systems.

Nanopore-based technologies beyond DNA sequencing

Yi-Lun Ying, Zheng-Li Hu, Shengli Zhang, Yujia Qing, Alessio Fragasso, Giovanni Maglia, Amit Meller, Hagan Bayley, Cees Dekker & Yi-Tao Long

Nature Nanotechnology **17**, 1136–1146 (2022) | Cite this article

https://doi.org/10.1038/s41565-022-01193-2

References:

Field sequencing:

Pomerantz et al 2018. Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building; doi: 10.1093/gigascience/giy033

Hamilton 2023; https://communities.springernature.com/amp/posts/malaria-genomic-surveillance-using-nanopore-sequencing-shifting-the-focus-into-endemic-countries

Kumburu et al 2023; Nanopore sequencing technology for clinical diagnosis of infectious diseases where laboratory capacity is meager: A case report https://doi.org/10.1016/j.heliyon.2023.e17439

Diagnostics:

Charalampous et al 2023 Routine Metagenomics Service for Intensive Care Unit Patients with Respiratory Infection https://doi.org/10.1164/rccm.202305-0901OC

https://nanoporetech.com/resource-centre

Nanopore is linking to new studies including ONT technology

-NB: some are not yet peer reviewed

The also made ‘White papers’:

-Large insights into microorganisms

-New approaches for clinical research

Ana Rita Rebelo
anrire@food.dtu.dk

DNA extraction and library preparation for ONT sequencing

Funded by the European Union

DNA extraction

The first step in any sequencing process

The design of the protocol depends on the sequencing technology to be used → different specifications for different technologies

In general DNA quality needs to be much higher than for other molecular methods (e.g. PCR)

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DNA extraction

In house protocols or commercial kits

Cell lysis

-- Cell burst with release of intracellular components

-- Enzymes, temperature, mechanical lysis, detergents, etc.

Precipitation

-- Separation of the DNA and debris

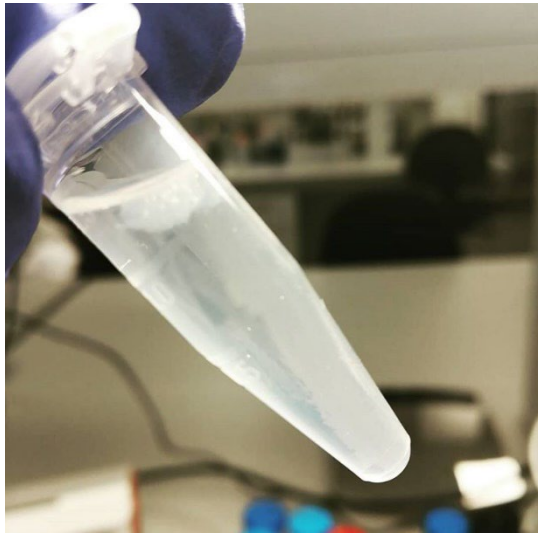
-- Organic solvents/alcohols and salts

Clean up

-- Recovery of DNA and removal of remaining salts and reagents

-- Organic solvents/alcohols

Resuspension



Funded by the European Union

DNA extraction kits

Many different kits in the market

To consider:

Price
Length/duration of protocol
Material that is needed
Extra reagents

Number of DNA samples in parallel
Scalability
Potential for automatization

Storage conditions of materials
Longevity of materials

Safety

Characteristics of the extracted DNA
(yield (ng), fragment length, purity, etc.)

Applicability to your purpose
Existing literature

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DNA extraction kits

Recommended by ONT

Examples of kits

Kit	**Beckman GenFind V3**	**ThermoFisher MagMax Viral/Pathogen Ultra Nucleic Acid Isolation**	**Monarch HMW DNA Extraction Kit for Tissue**	**QIAGEN Genomic-tips 20/G**
Principle	Magnetic beads	Magnetic beads	Precipitation of the DNA onto the surface of large glass beads	Gravity-flow, anion-exchange tips (DNA binds to the Resin in the column)
DNA yield	Not described	Not described	> 8 ug	20 µg (500 ug in 500/G kit)
Fragment size	Not described	Not described	> 500 kb	50 – 100 kb
Price approx.	250	600	500	350 (450 for 500/G)
Nr. samples	50	100	50	25
Price per sample	5 EUR	6 EUR	10 EUR	14 EUR

Previously tested at DTU, good performance for ONT

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DNA extraction kits

We will use protocol and in-house prepared checklist:

INSTRUCTION MANUAL

NEW ENGLAND BioLabs Inc.

be INSPIRED drive DISCOVERY stay GENUINE

Monarch® HMW DNA Extraction Kit for Tissue

NEB #T3060S/L

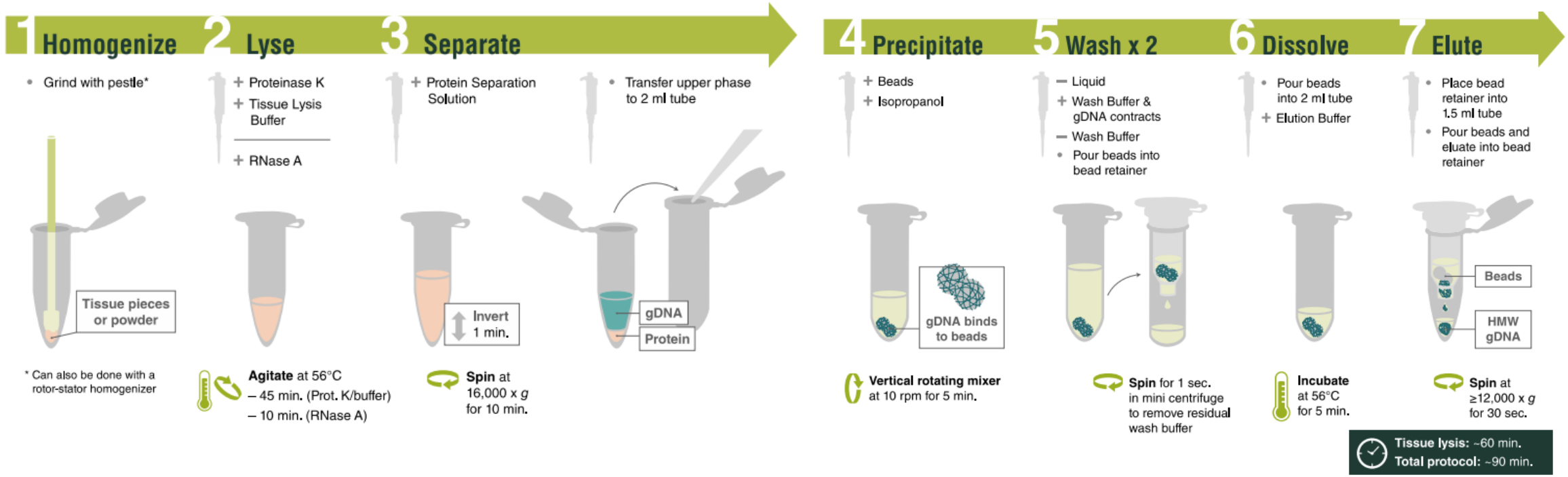
5/50 preps

Version 2.1_4/21

Funded by the European Union

DNA extraction kits

Figure 2: Workflow for Tissue Samples



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DNA extraction kits

Bacterial Samples (Gram-negative and Gram-positive): In addition to tissue, the Monarch HMW DNA Extraction Kit for Tissue provides a rapid and reliable process for extracting HMW genomic DNA from bacteria. Separate protocols for processing Gram-negative and Gram-positive bacteria are provided and differ slightly in the initial lysis step. Lysozyme is required to efficiently lyse the bacterial cell wall in these tough-to-lyse samples, and for processing Gram-positive bacteria, a STET buffer is also required. Alternative lysis enzymes (e.g., lysostaphin) may be required for certain Gram-positive bacteria. Ensure that the recommended input amounts of cells are used by measuring the density of bacteria cells in liquid culture (see "Choosing Input Amounts", page 9-10). Cells should be pelleted, and all culture medium removed prior to sample processing. Standard and low input protocols are provided to ensure the buffer volumes are appropriate for the sample input amount used.

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DNA dilution and quality control

From the DNA extraction protocol:

- UHMW DNA needs to be measured several times (~5 times with different aliquots) then average the results for a to estimate the sample concentration
- Qubit
- Nanodrop
- Spectrophotometric analysis of gDNA eluates (absorbance at 260 nm)
- *“Analysis by standard gel electrophoresis or other electrophoretic methods (e.g. Bioanalyzer) may not provide suitable resolution. Typically, more than 80% of the material is ≥ 50 kb in length.”*

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DNA dilution and quality control

From ONT:

"We have had good results with DNA which meets the following criteria:"

- Purity as measured using Nanodrop – OD 260/280 of 1.8 and OD 260/230 of 2.0–2.2
- Input mass, as measured by Qubit – 1 µg
- Average fragment size, as measured by pulsed-field gel analysis (>30 kb)

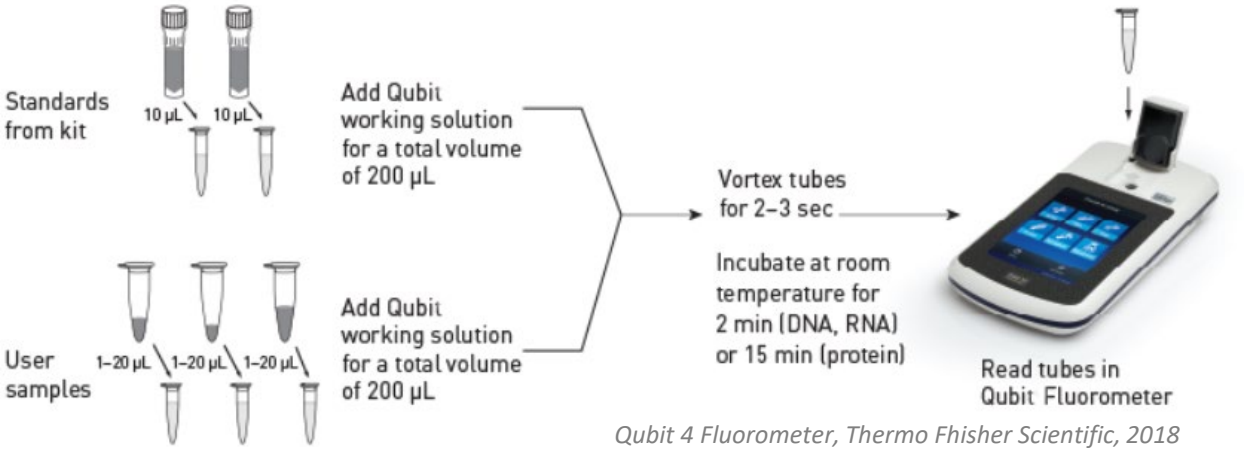
Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

Funded by the European Union

DNA dilution and quality control

Procedure during the workshop:

Qubit fluorometer (Invitrogen, Carlsbad, CA, USA)



Qubit 4 Fluorometer, Thermo Fhisher Scientific, 2018

Aim: > 100 ng DNA in 1-10 uL

Approx. Min: 10 ng/uL and Max: 100 ng/uL

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Library preparation

Parameter	Library prep kits
DNA input	PCR Barcoding Kit 24 V14: **1-5 ng** Rapid Barcoding Kit 96 V14: **50 ng**
Nr. of samples	PCR Barcoding Kit 24 V14: **24** Rapid Barcoding Kit 96 V14: **96**
Time	PCR Barcoding Kit 24 V14: **180 min** Rapid Barcoding Kit 96 V14: **60 min**
Special equipment	~~Thermal cycler~~ or heating blocks Magnetic racks for eppendorfs ~~Hula mixer (rotator) (opt.)~~ ~~Plate shaker~~
Safe stopping points	Not described
Library QC	Qubit Bioanalyzer (opt.) Nanodrop (opt.)

We will use 100 ng

We will use 24 kit

We will have 2 h

We will use Qubit

…and other adjustments

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

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Library preparation

Rapid sequencing DNA V14 - barcoding (SQK-RBK114.24 or SQK-RBK114.96)

- This protocol uses genomic DNA
- For multiplexing 1-96 samples
- Library preparation time ~60 minutes
- High yield
- Fragmentation
- Compatible with R10.4.1 flow cells

This kit is recommended for users who:

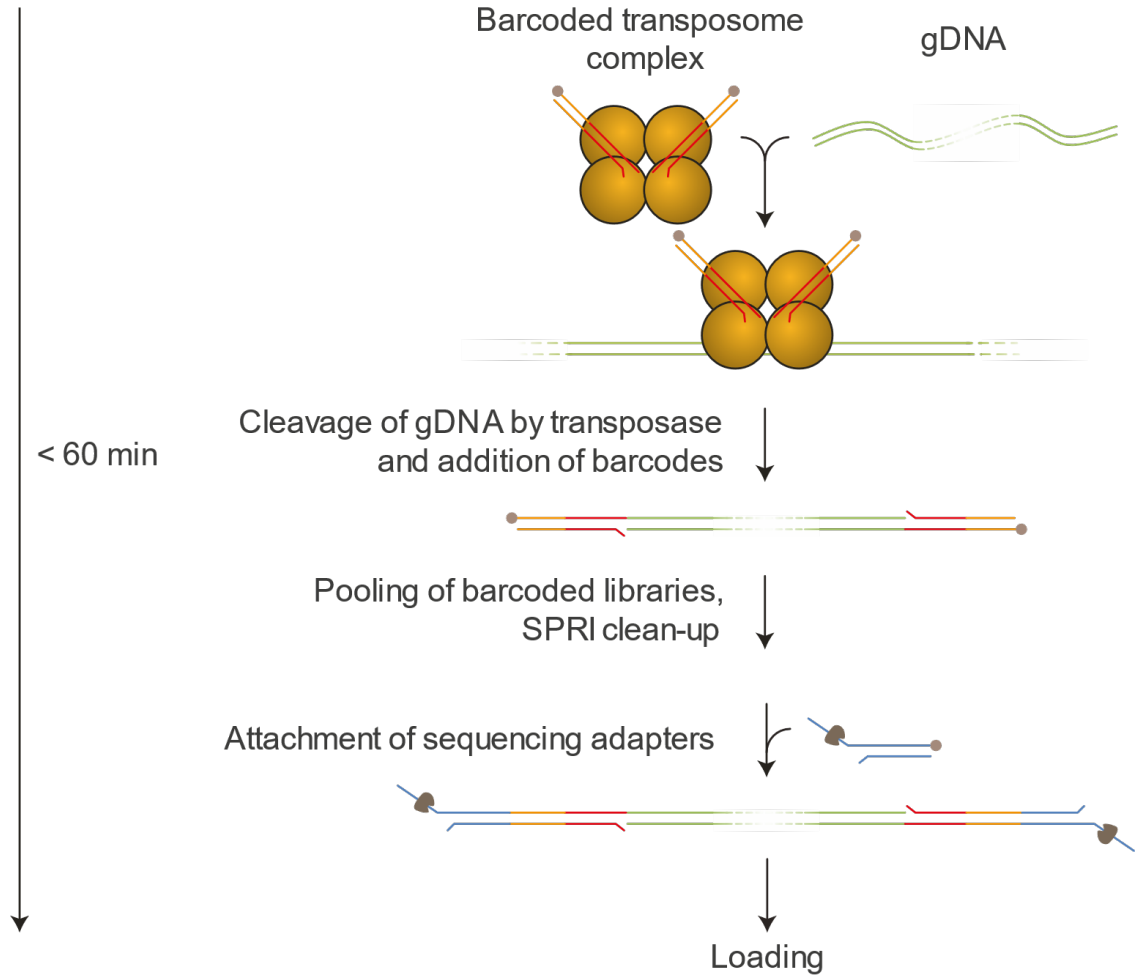
- Wish to multiplex samples to reduce price per sample
- Need a PCR-free method of multiplexing to preserve additional information such as base modifications
- Require a short preparation time
- Have limited access to laboratory equipment

Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

Funded by the European Union

Library preparation

- Tagment your DNA using the Rapid Barcodes in the kit; this simultaneously attaches a pair of barcodes to the fragments
- Pool and clean-up the barcoded samples
- Attach sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell



Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

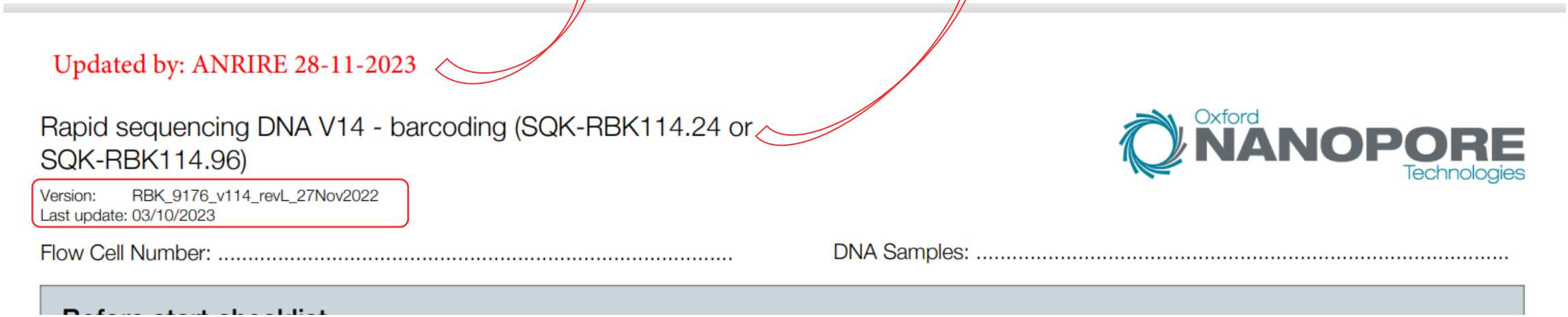
Funded by the European Union

Library preparation

We will use the protocol and checklist:

Optimized

New chemistry



Oxford Nanopore Technologies, Inc., Oxford, UK (https://nanoporetech.com)

Funded by the European Union

Library preparation

Quality control:

Qubit measurement of the pooled libraries

Aim: 20 ng/uL → because for the following step we want > 200 ng of DNA in 11 uL

Funded by the European Union

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Troubleshooting ONT runs

Funded by the European Union

Contaminants

- Chemical components used in DNA extraction methods can be carried over from the extraction procedure and contaminate the extracted DNA sample.
- These contaminants can have a significant effect on downstream library preparation efficiency, and therefore **sequencing throughput**.

Ethanol and Isopropanol

- The presence of residual ethanol and isopropanol in extracted DNA can lead an overestimation of the concentration of DNA that is present in your sample.
- They can also lead to a reduction in the measured A260/280 and A260/230 ratios.

- ONT found that the presence of some ethanol in the input sample can be tolerated before performance might start to be adversely affected:
  - the Ligation Sequencing Kit can tolerate up to 20% ethanol contamination
  - and the Rapid Sequencing Kit can tolerate up to ~7.5% ethanol contamination.

- ONT found that the presence of up to ~7.5% isopropanol in the input sample can be tolerated by the Rapid Sequencing Kit, but the presence of any isopropanol might adversely affect the performance of the Ligation Sequencing Kit.

EDTA

- The presence of EDTA in extracted DNA can lead large perturbations in the nanodrop spectrum and A260/280 and A260/230 ratios.
- It can also lead to an overestimation of the concentration of DNA that is present in your sample.
- ONT found that the presence of some EDTA in the input sample can be tolerated by both the Ligation Sequencing Kit and the Rapid Sequencing Kit, before performance might start to be significantly affected:
  - the Ligation Sequencing Kit can tolerate up to 10 mM EDTA contamination
  - and the Rapid Sequencing Kit can tolerate up to 5 mM EDTA contamination

NaCl

- The presence of NaCl in extracted DNA does not appear to perturbation nanodrop spectra or A260/280 and A260/230 ratios.
- ONT found that the presence of up to 100 mM NaCl in the input sample can be tolerated by both the Ligation Sequencing Kit and the Rapid Sequencing Kit.

Guanidinium chloride and Guanidinium isothiocyanate

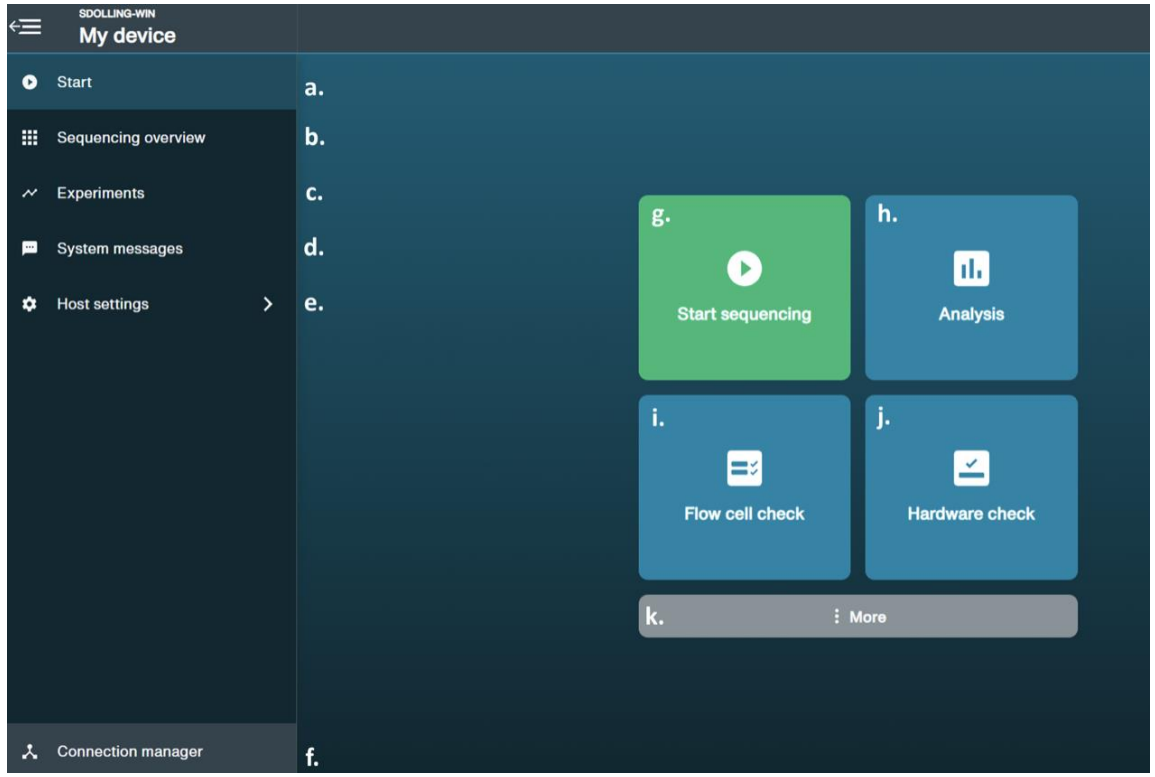
- The presence of guanidinium chloride in extracted DNA can significantly perturb nanodrop spectra, particularly with respect to the A260/230 ratio.
- ONT found that the presence of up to 100 mM guanidinium chloride in the input sample can be tolerated by both the Ligation Sequencing Kit and the Rapid Sequencing Kit.

- The presence of guanidinium isothiocyanate in extracted DNA can significantly perturb nanodrop spectra, giving atypical A260/280 and A260/230 ratios, and leading to mis-quantification of the DNA concentration.
- ONT found that the presence of up to 50 mM guanidinium isothiocyanate in the input sample can be tolerated by both the Ligation Sequencing Kit and the Rapid Sequencing Kit.

Phenol

- The presence of residual phenol in extracted DNA can significantly perturb nanodrop spectra, giving atypical A260/280 and A260/230 ratios, and lead to overestimation of the DNA concentration.
- ONT found that the presence of up to 1% phenol in the input sample can be tolerated by both the Ligation Sequencing Kit and the Rapid Sequencing Kit, before performance might start to be significantly affected.

MinKNOW Interface



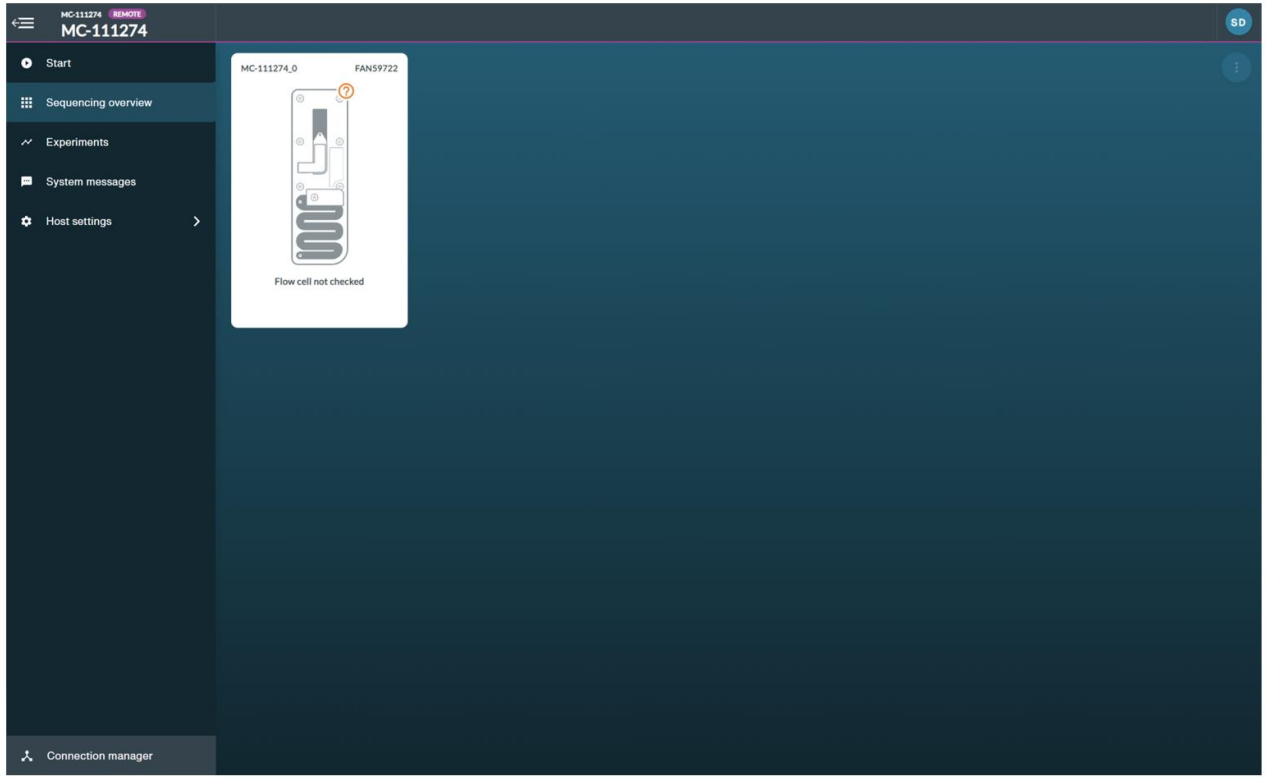
Homepage overview

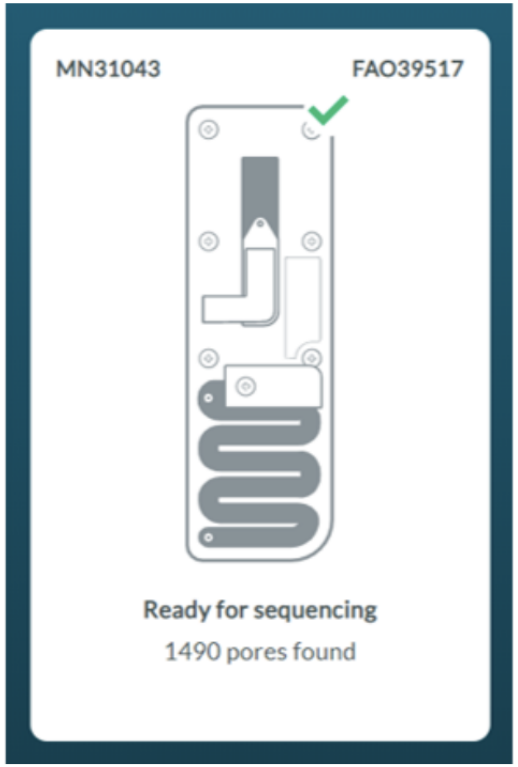
The MinKNOW Homepage enables the user to navigate to:

a. **Start** homepage
b. **Sequencing Overview** of connected flow cells
c. Recent and current **Experiments**
d. **System Messages**
e. **Host Settings**
f. **Connection Manager** to connect with other available devices
g. **Start Sequencing** experiment
h. Post-run **Analysis**
i. **Flow Cell Check**
j. **Hardware Check**
k. **More** includes option to generate .mmi from .fasta file or to import a sample sheet
l. **Guest/initials** to logout

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

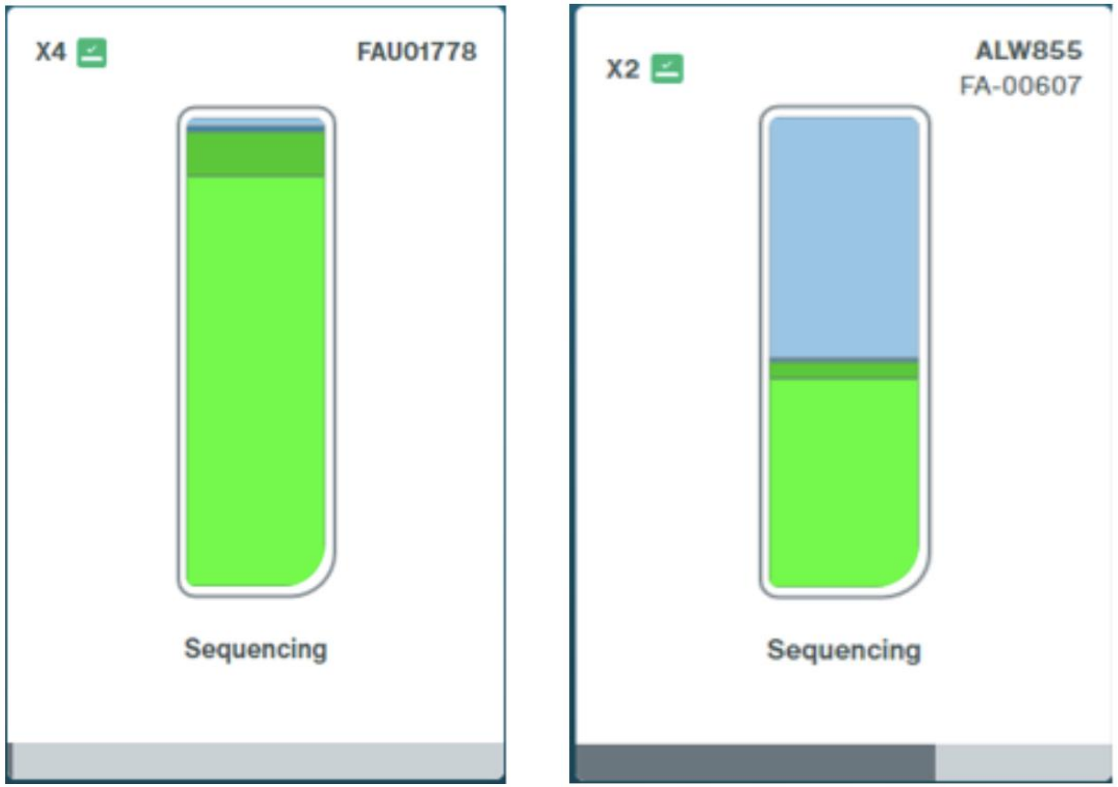
Flow Cell Check





Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

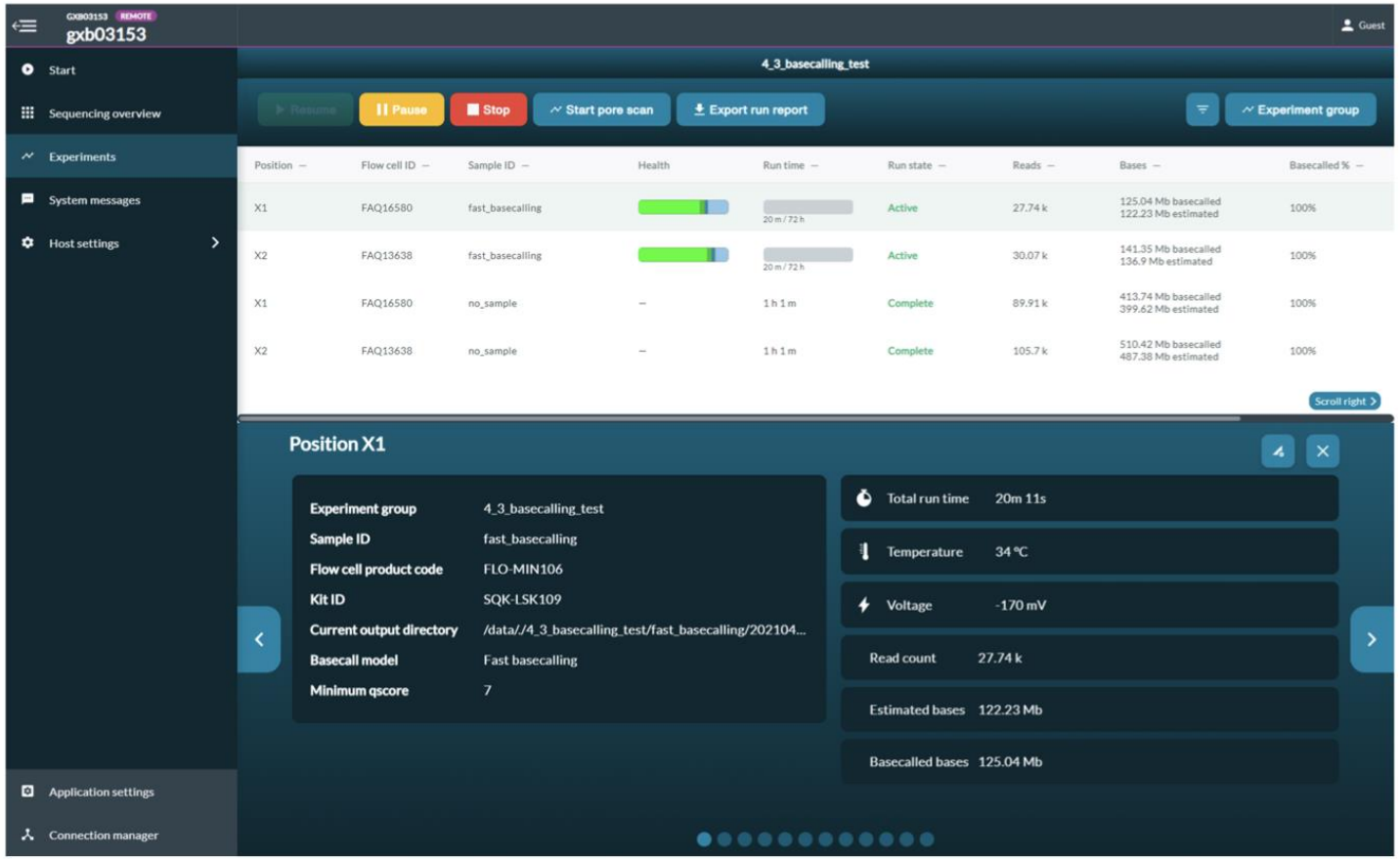
Flow cell health



- During a sequencing experiment, the Sequencing Overview page shows a flow cell icon with coloured bars.
- The bars represent the combined health of all pores in a flow cell

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

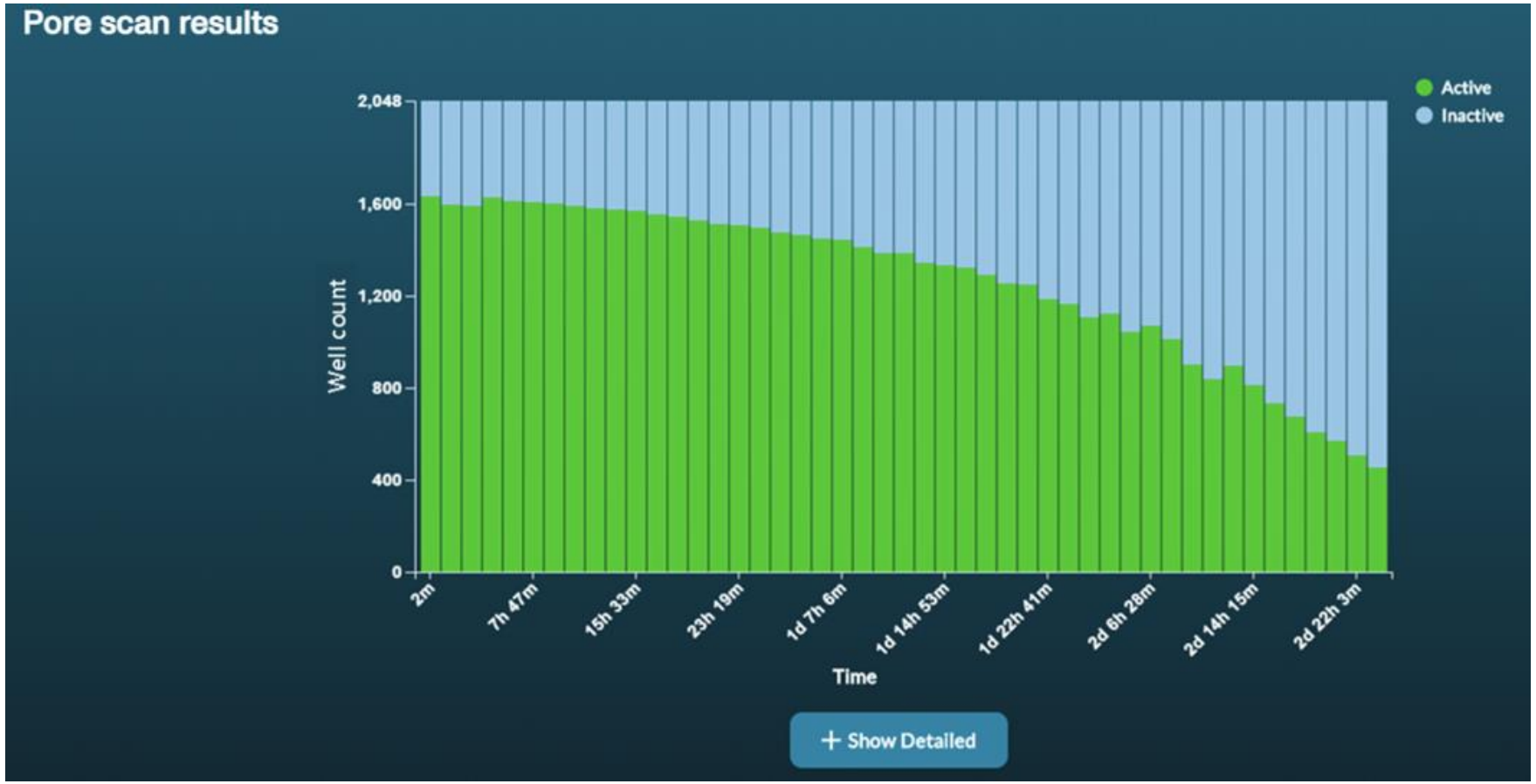
Experiment Summary Information



Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

- Minknow will basecall and demultiplex live
- Real time information on flow cell health and sequencing

Pore Scan

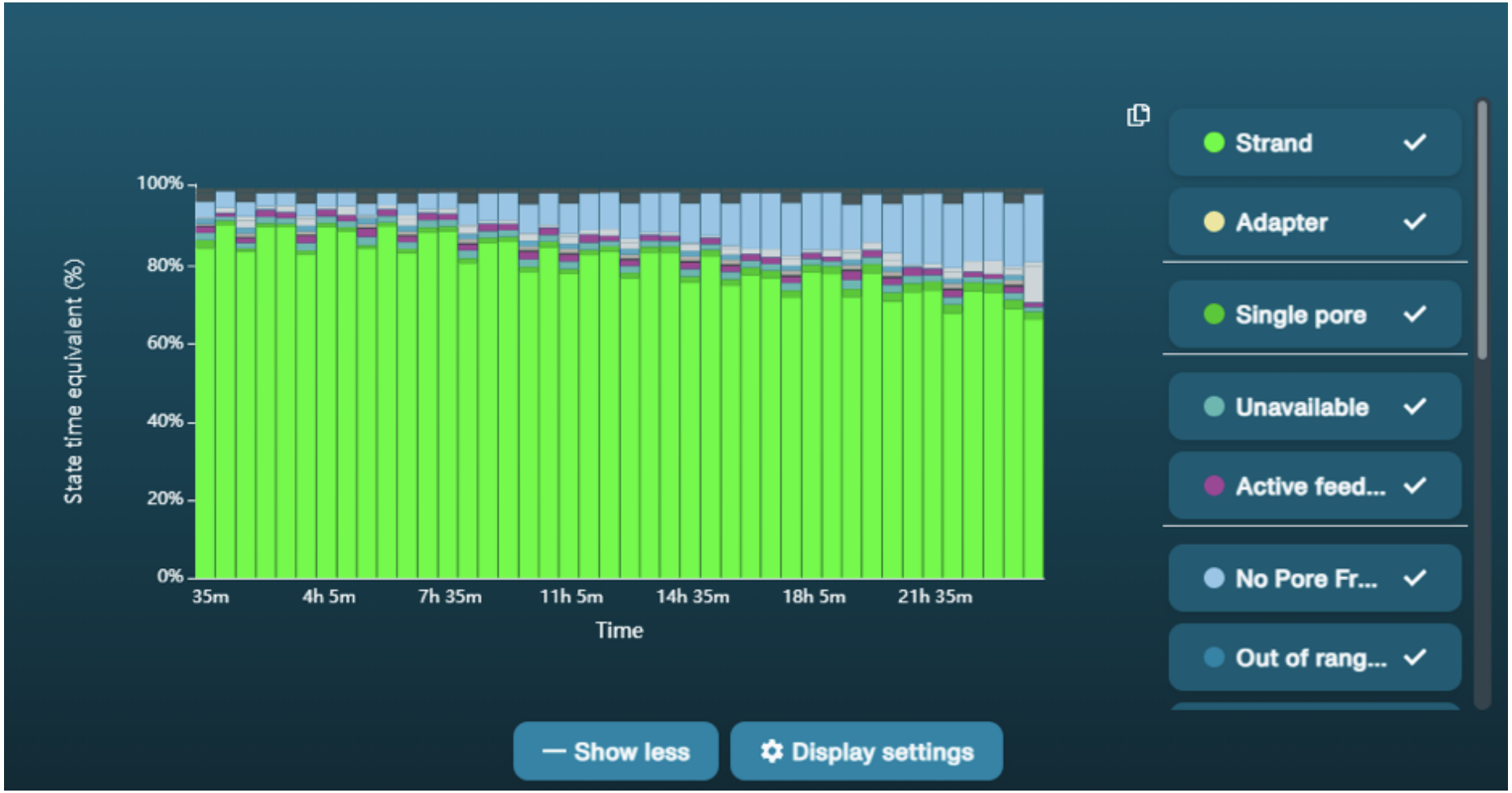


Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Pore Occupancy

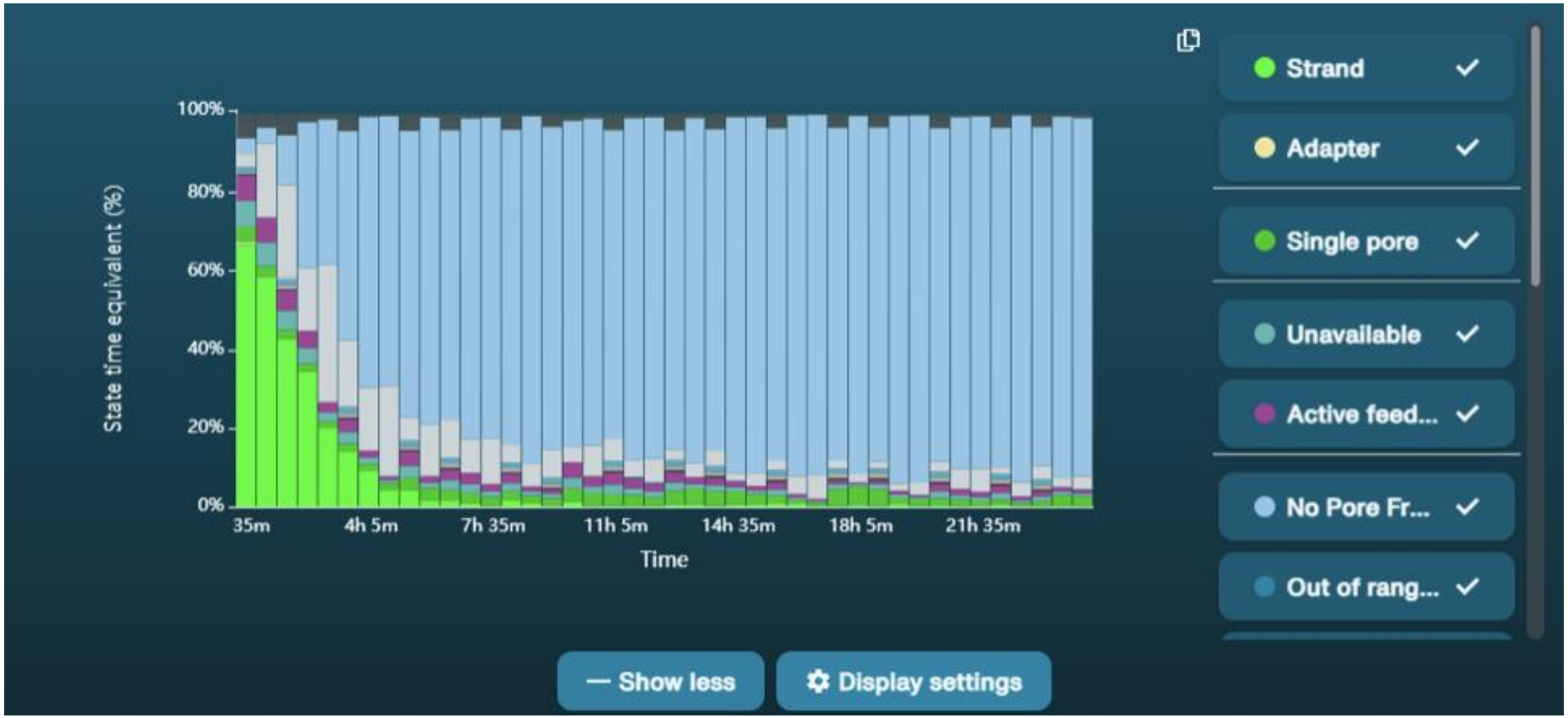
Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Good library



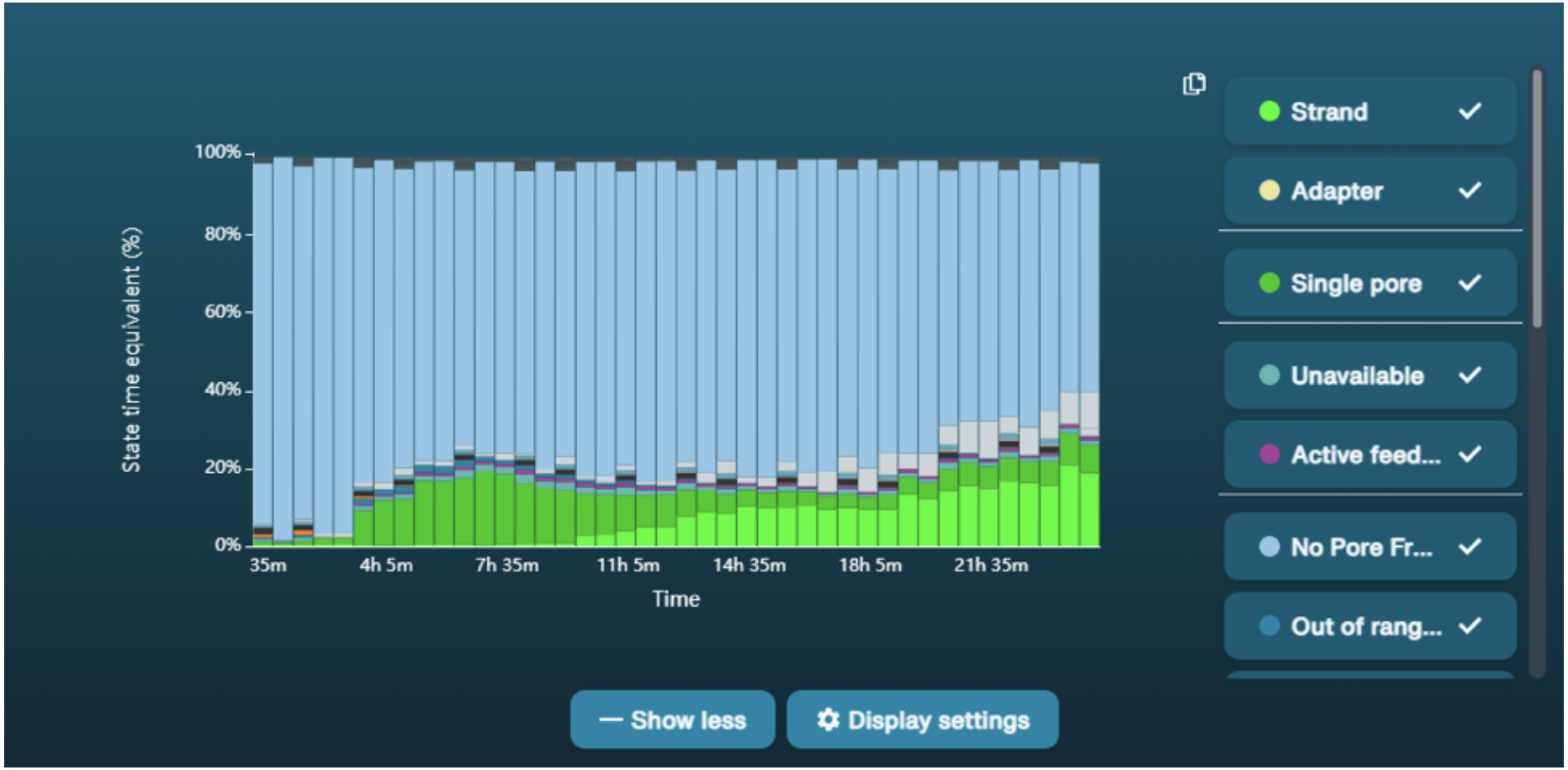
Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Channel Blocking



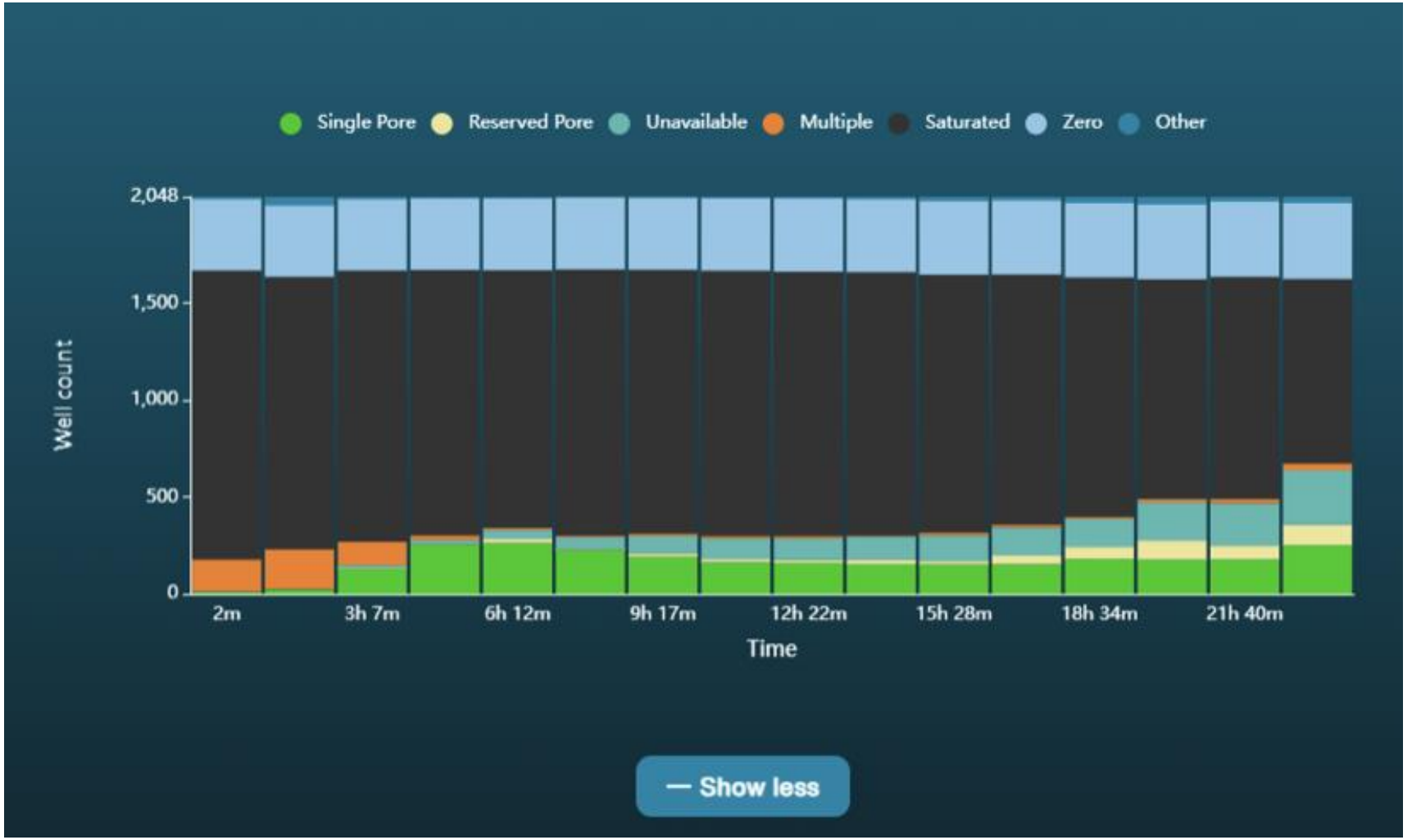
Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Osmotic Imbalance



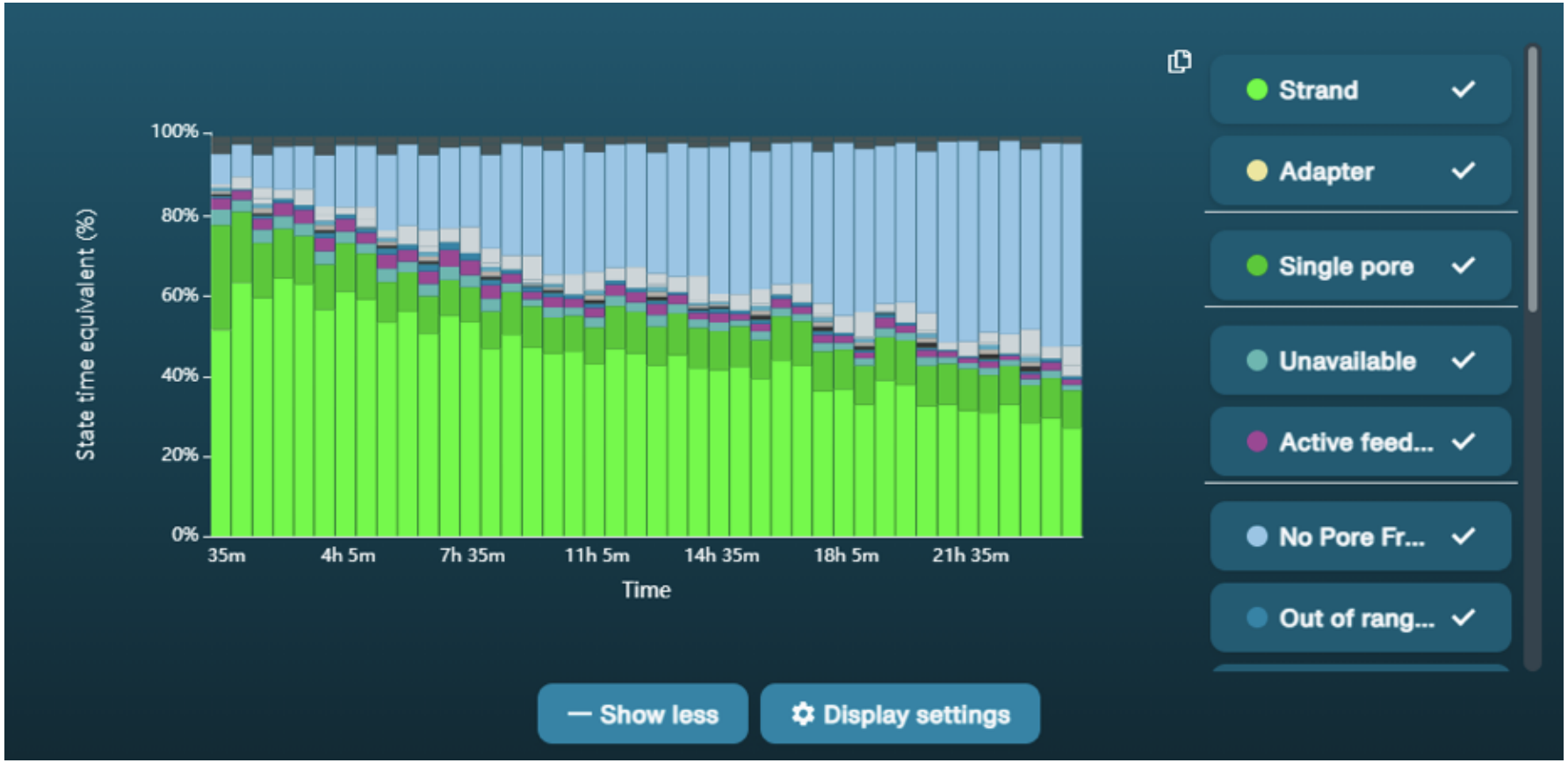
Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Osmotic Imbalance – channel scan



Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Low Pore Occupancy



Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

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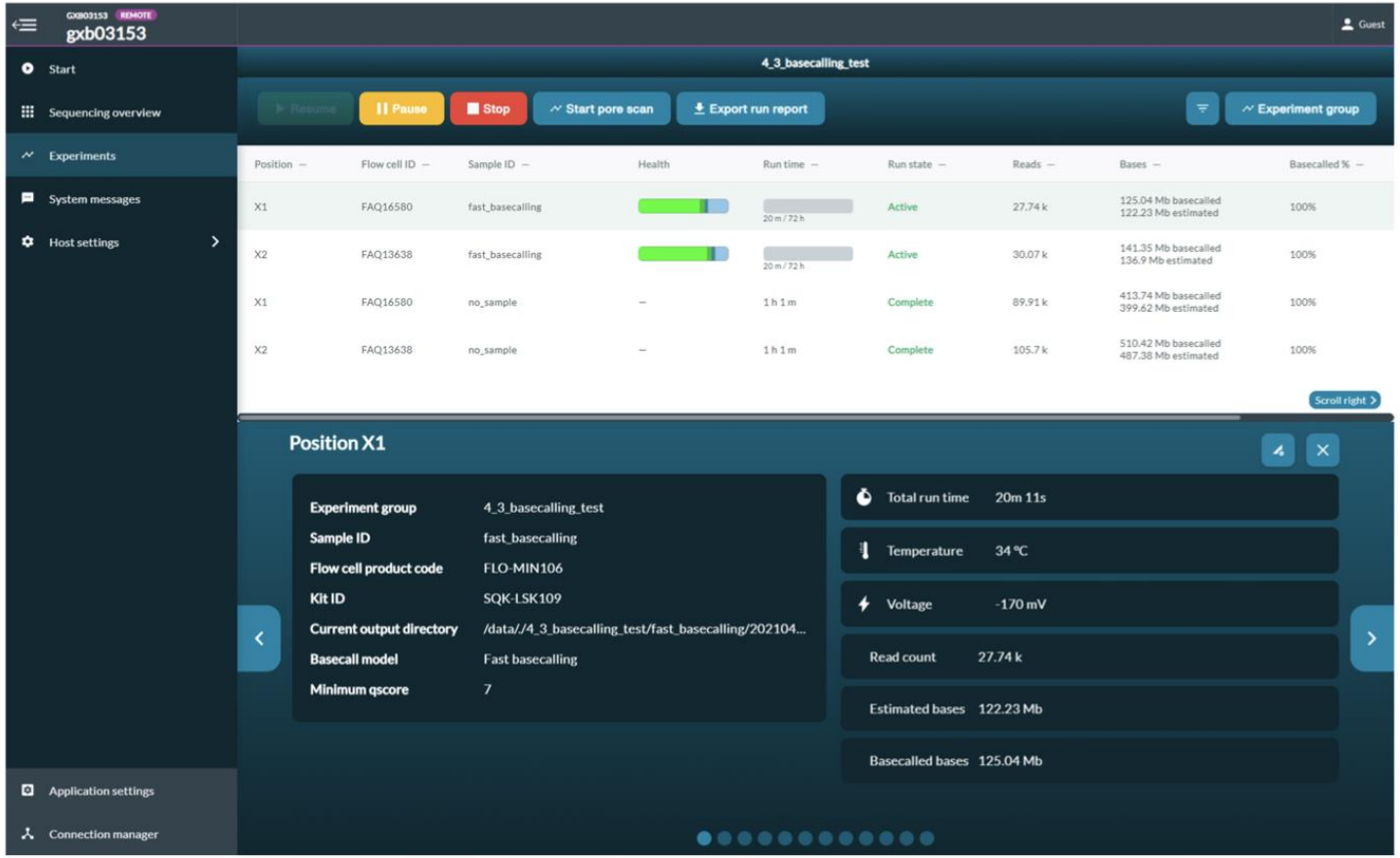
Introduction of bioinformatics analyses of ONT data

Funded by the European Union

Experiment Summary Information



- Minknow will basecall and demultiplex live
- Real time information on flow cell health and sequencing

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

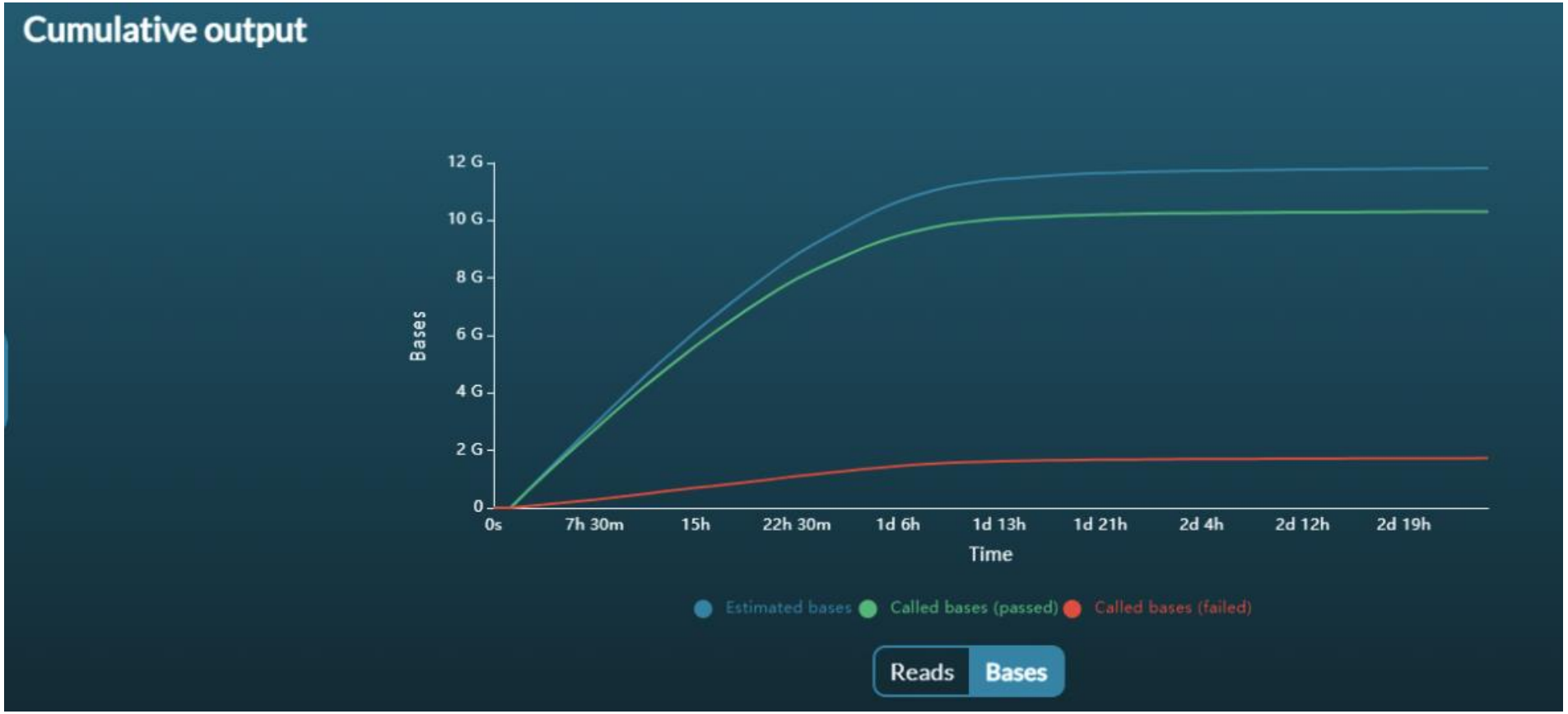
Pore Occupancy

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Read Length Histogram

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Cumulative Output

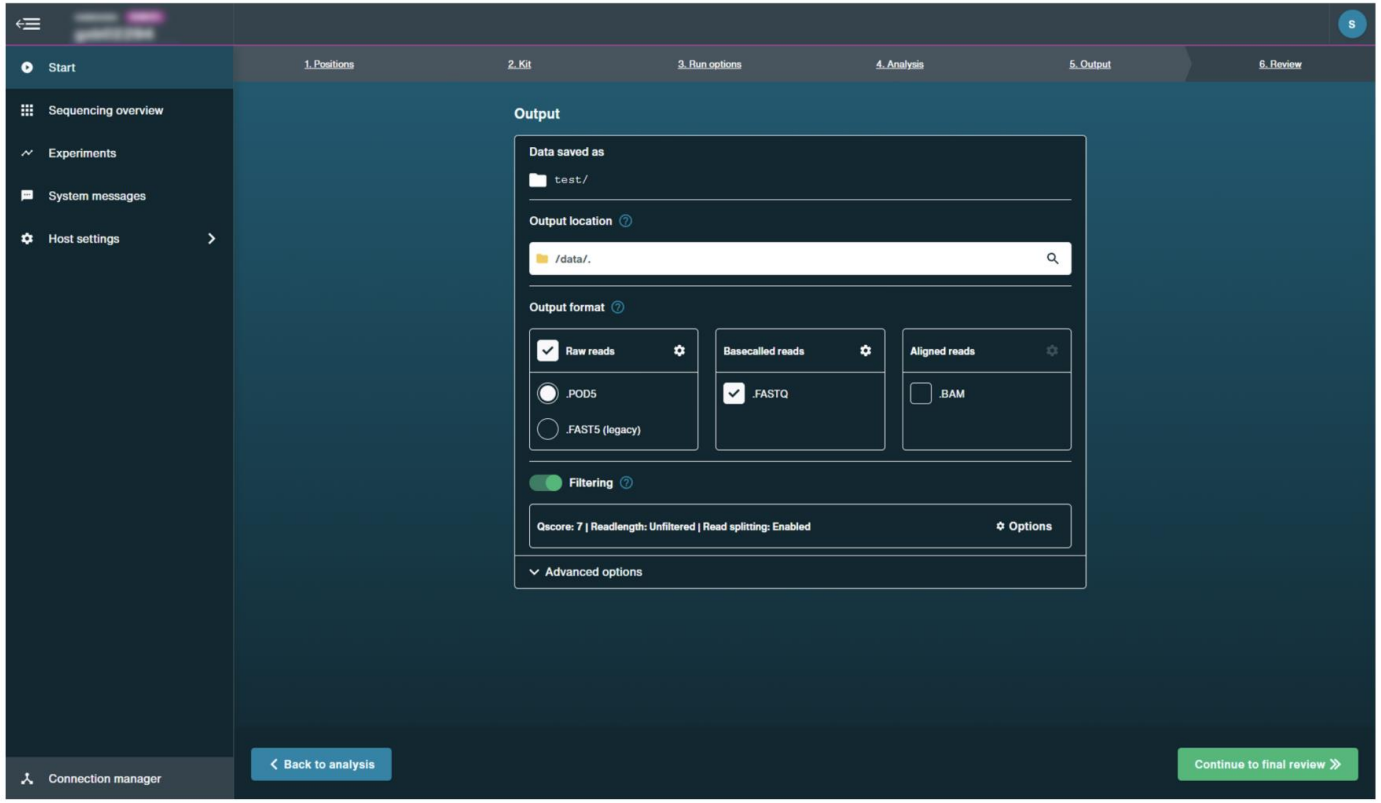


Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Barcode read counts

Picture belongs to oxford nanopore (https://store.nanoporetech.com/eu/rapid-barcoding-kit-1.html)

Output files



Picture belongs to oxford nanopore (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/experiment-companion-minknow/v/mke_1013_v1_revcy_11apr2016/starting-a-sequencing-run-on-minion)

- Raw reads output can be saved as .POD5 or .FAST5.
- Basecalled reads are saved as .FASTQ
- Run report contains real-time feedback, such as read quality and length.
- sequencing_summary.txt also contains read quality and length infotmation.

Run Report

- Run reports contain information about the sequencing run and include performance graphs.
- These graphs can be manually generated during a run as well as being automatically generated once sequencing is complete for MinION Mk1C, GridION and PromethION and MinION Mk1B running on Linux.
- However, for MinION Mk1B running on Windows or Mac, the run reports need to be manually generated by clicking Export run report and selecting which experiment to export to html.

- The run report includes the run summary and configuration, sequence output, run health and run log.
- Troubleshooting suggestions are also available throughout the report, with links to further information available on the Community.

Next steps

- Additional QC metrics can be generated post-sequencing using one of several tools.
- Third party tools exist such as PycoQC which can also be used to generate QC metrics from the sequencing_summary.txt file.
- ONT has a cloud-based tool called EPI2ME, with different workflows that take fastq or fastq.gz files as the input. ONT also has a Desktop application called EPI2ME labs that allows users to run many different workflows.
- Alternatively, you can use third-party tools like NanoPlot and NanoStat.
- For third-party tools, please see their GitHub pages for installation and usage.
- After QC if the FASTQ files, next step is assembling the reads to generate FASTA files…

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Thank you on behalf of the EURGen-RefLabCap team

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