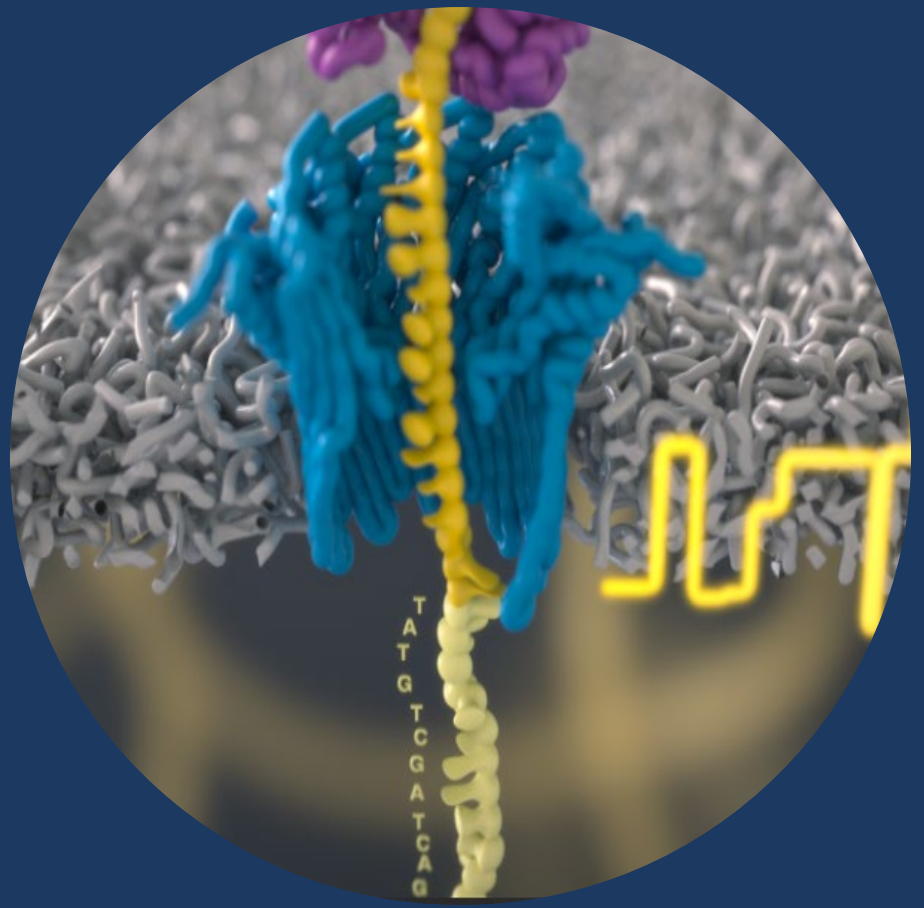
Nanopore sequencing

Workshop session 3
MB&C Course 2024

by Jozef Dingemans, Ph.D.
(Molecular Biologist, Jessa Hospital)



Introduction: Evolution of sequencing methods

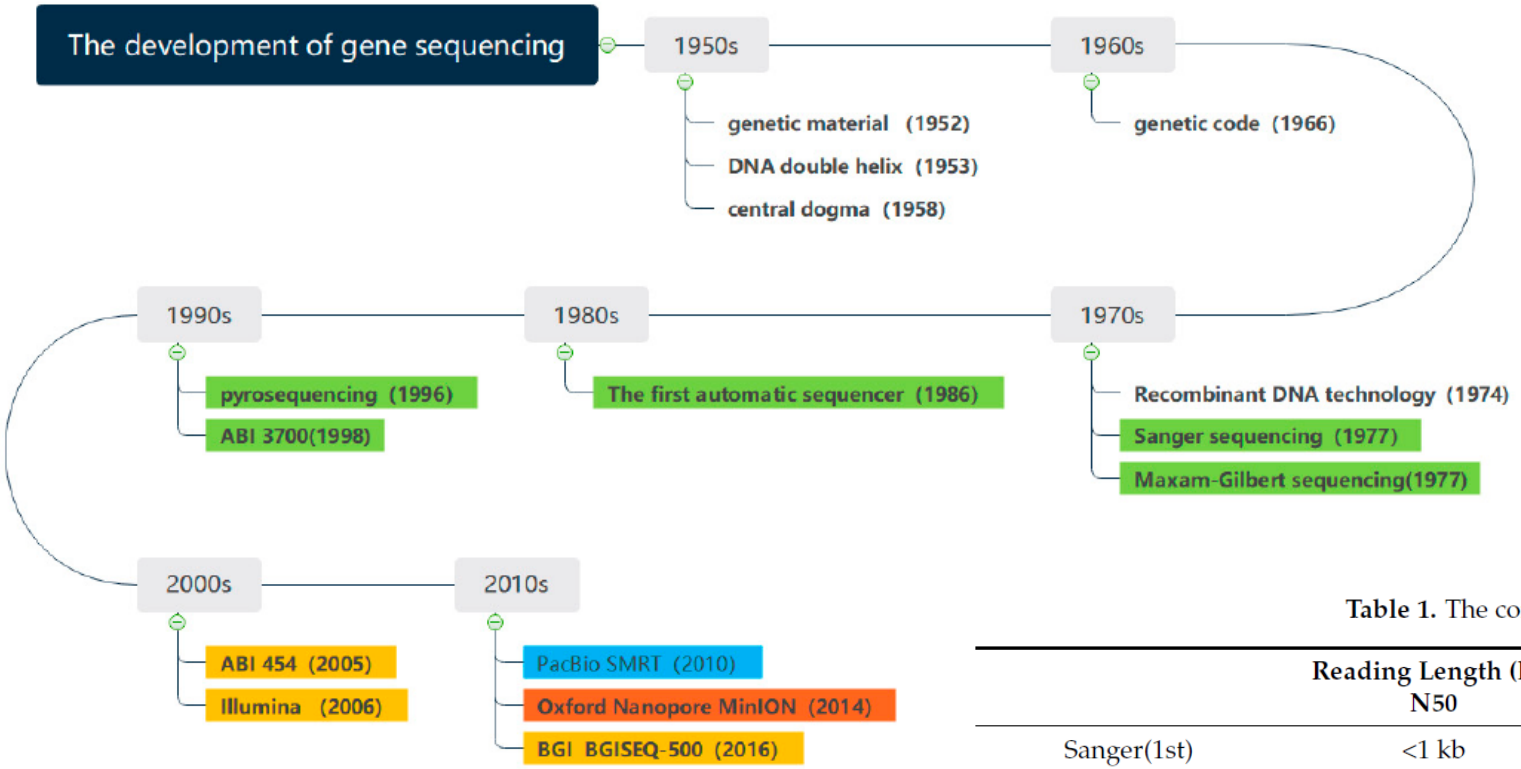
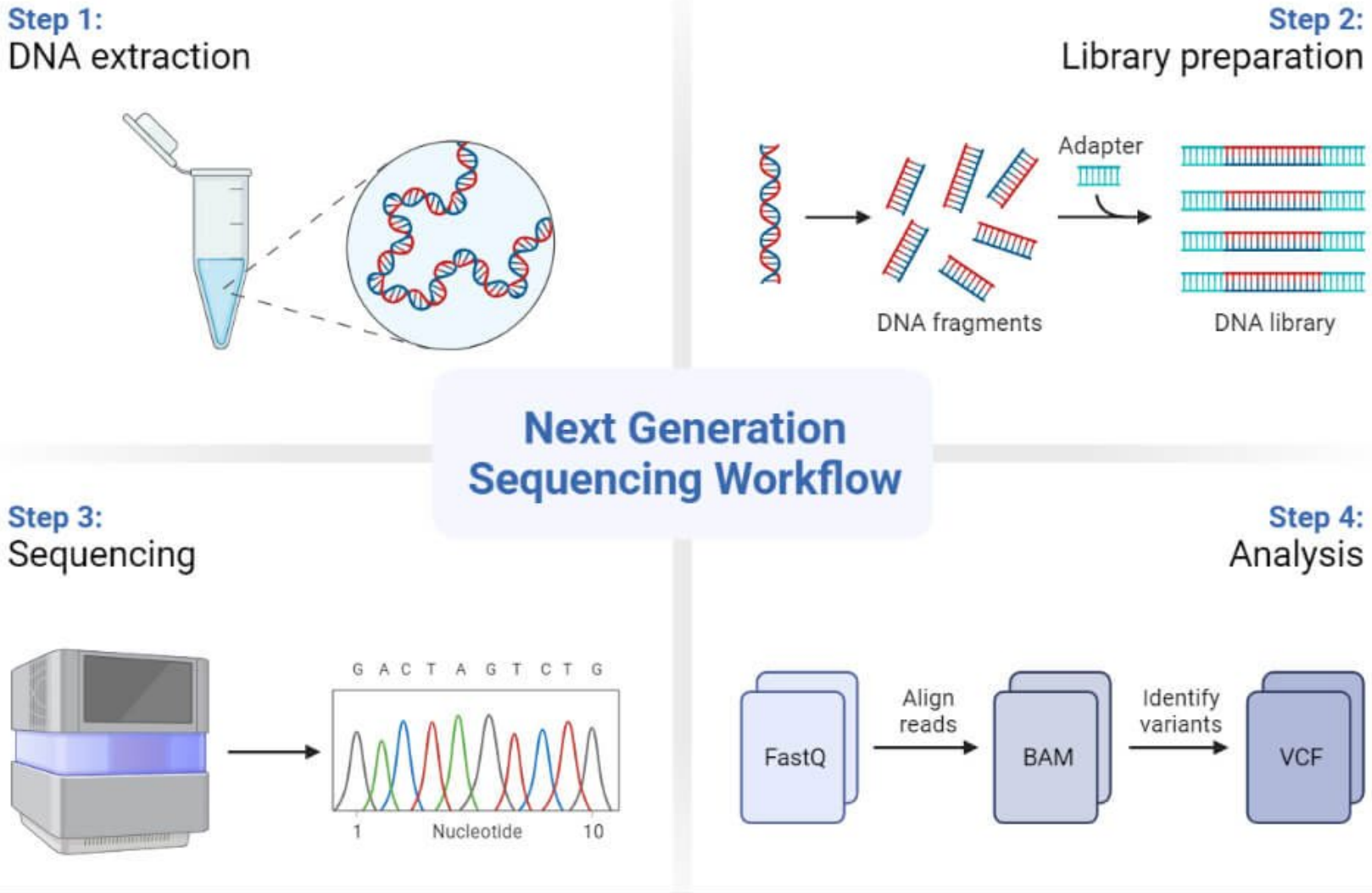


Table 1. The comparison of different generation of gene sequencing.

	Reading Length (kb) N50	Estimated Cost per Gb (US $)	Throughput per Flow Cell (Gb)	Read Accuracy (%)
Sanger(1st)	<1 kb	13,000 [d]	/	>99.9
Illumina(2nd)	0.075–0.15 [a]	50–63	16–30	>99.9
PacBio(3rd)	10–20 [b]	43–86	15–30	>99
ONT(4th)	10–60 [c]	21–42	50–100	87–98

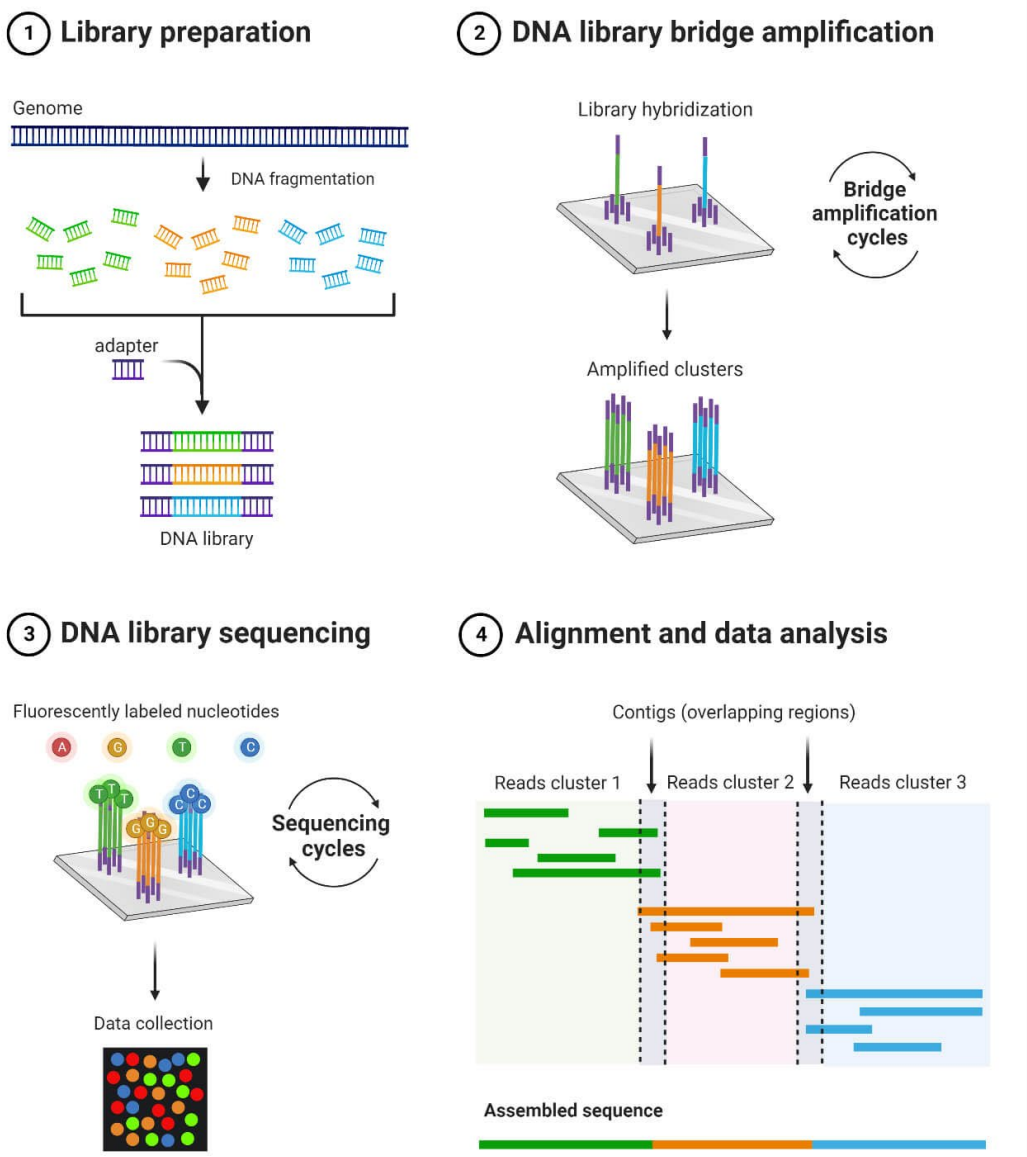
Ref: Lin, B.; Hui, J.; Mao, H. Nanopore Technology and Its Applications in Gene Sequencing. Biosensors 2021, 11, 214. https://doi.org/10.3390/bios11070214.

Introduction: Next-Generation sequencing



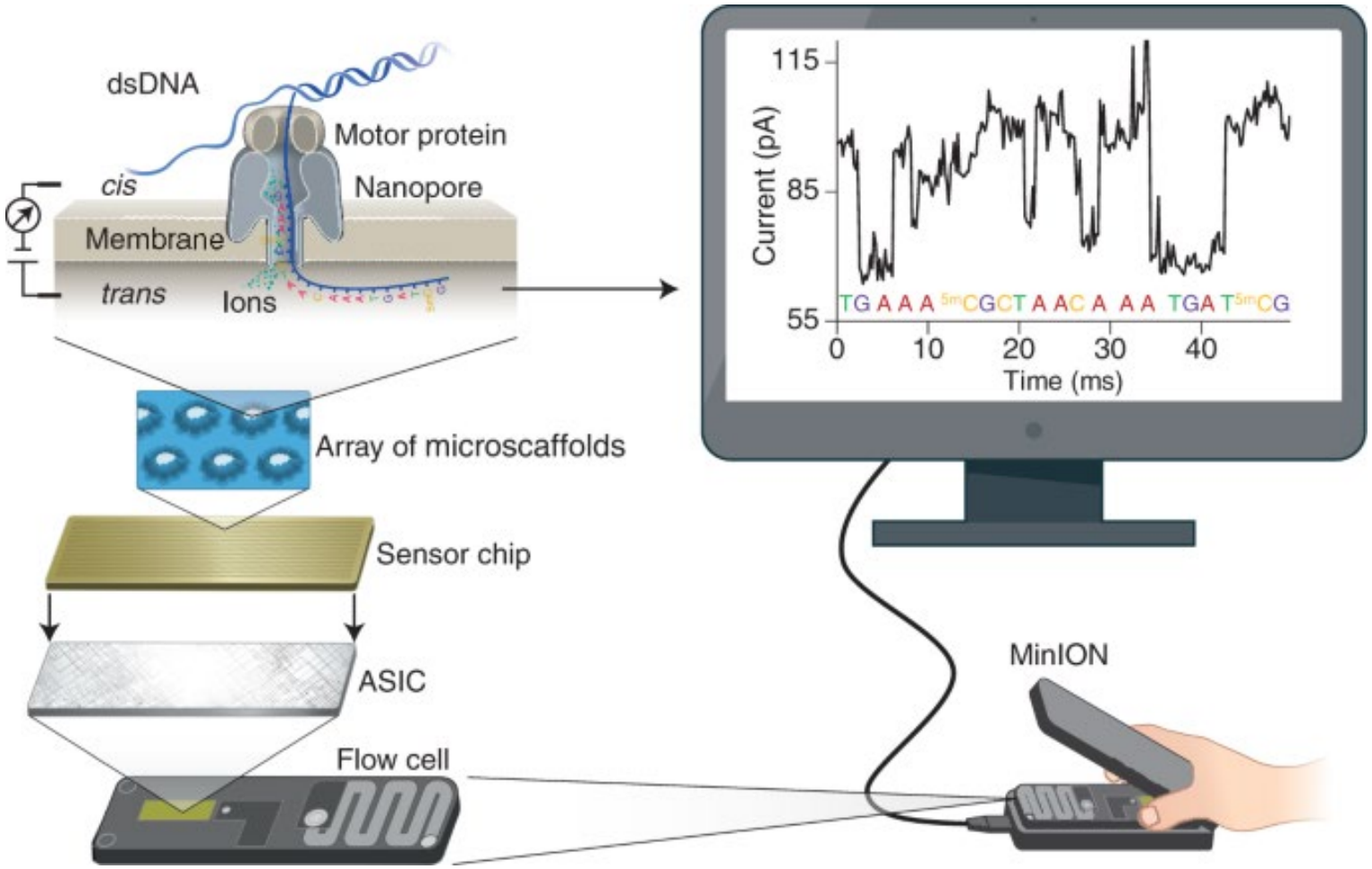
Ref: https://microbenotes.com/next-generation-sequencing-ngs/

Introduction: Illumina sequencing



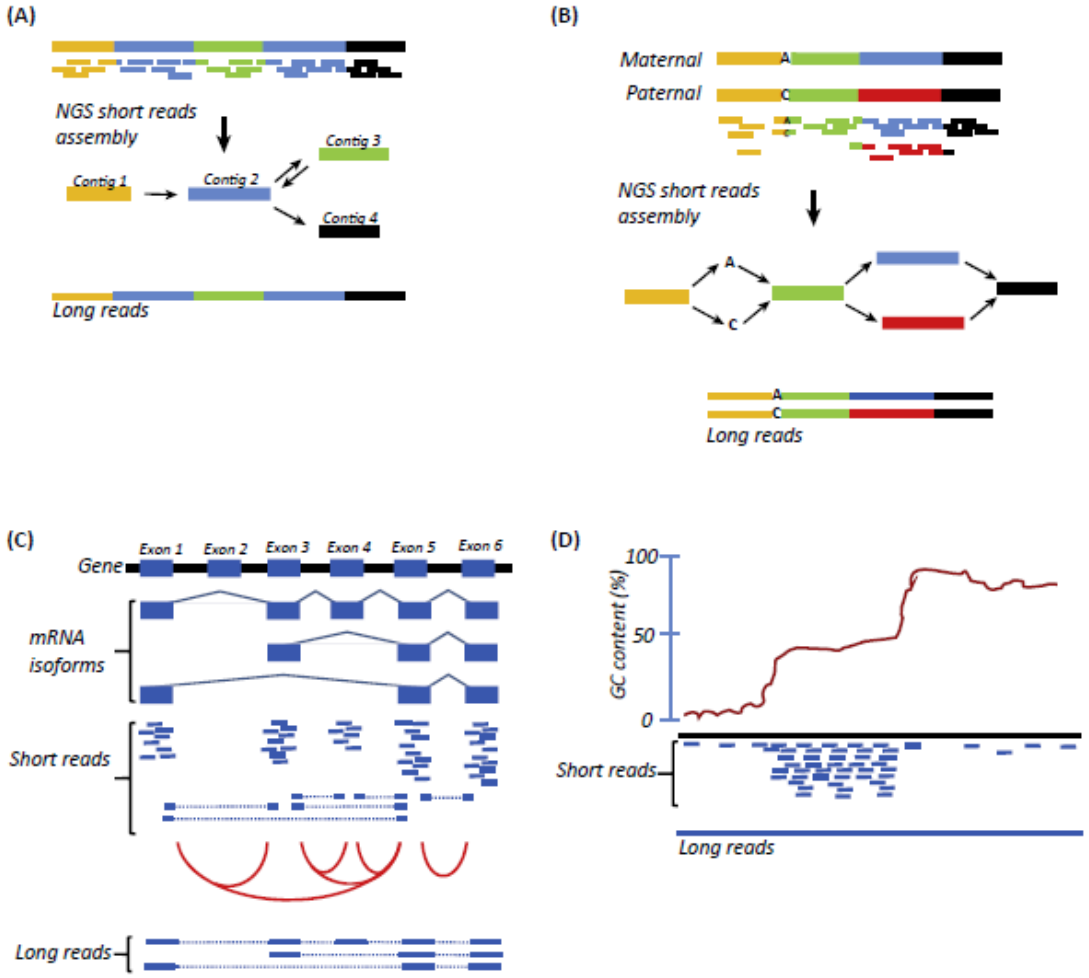
Ref: https://microbenotes.com/next-generation-sequencing-ngs/

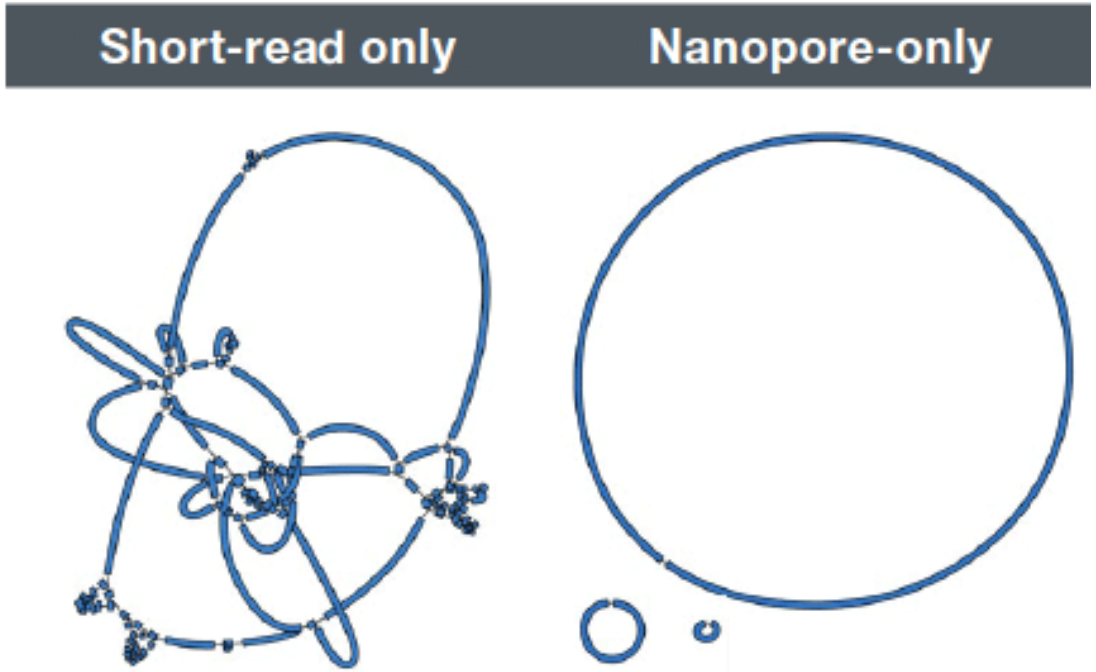
Introduction: Nanopore sequencing



Ref: 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

Short read vs long read sequencing





Ref: van Dijk EL, Jaszczyszyn Y, Naquin D, Thermes C. The Third Revolution in Sequencing Technology. Trends Genet. 2018 Sep;34(9):666-681. doi: 10.1016/j.tig.2018.05.008. Epub 2018 Jun 22. PMID: 29941292.

Pros and cons of Nanopore sequencing

Pros

- Long reads => complete genome coverage & assembly possible
- Fast => Data-analysis possible in real-time
- Relatively low cost
- Scalable
- No amplification or labeling

Cons

- Lower accuracy than Illumina or Sanger sequencing

Cost of Nanopore vs Illumina sequencing

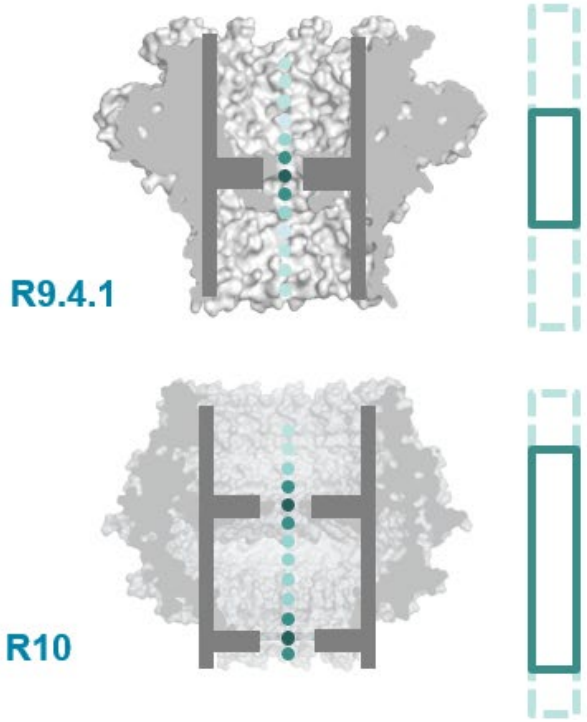
Sequencing System	iSeq™	MiniSeq™	MiSeq®	NextSeq®	HiSeq® 4000	HiSeq® X Five/Ten	NovaSeq® 6000
Output per run	1.2 Gb	7.5 Gb	15 Gb	120 Gb	1.5 Tb	1.8 Tb	1 Tb - 6 Tb[1]
Instrument price	$19.9K	$49.5K	$99K	$275K	$900K	$6M[2]/$10M[2]	$985K
Installed base[3]	NA	~600	~6,000	~2,400	~2,300[4]		~285

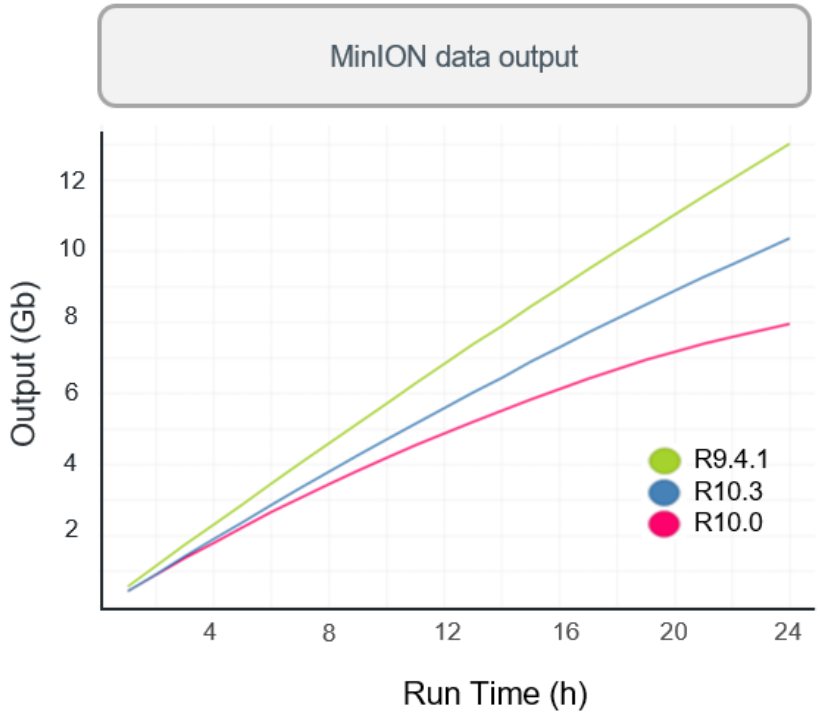
Illumina

Platform	Instrument	Average read length (kb)	Maximum read length (kb)	Instrument cost (US$)	Cost per run (US$)	Cost per Gb (US$)	Input requirement	Throughput per run	Run time	Refs
ONT	MinION	Variable[a]	Variable[a]	1000	475–900[e]	24[g]	~1 µg DNA	Up to 20 Gb	Up to 48 h	[25] https://nanoporetech.com/products#modal=comparison
	GridION	Variable[a]	Variable[a]	49 955[b] 125 000[c] Free (U$142 500 for reagents)[d]	475–900[e] per flow cell	24[g]	~1 µg DNA	Up to 100 Gb (five flow cells)	Up to 48 h	
	PromethION	Variable[a]	Variable[a]	135 000	625–2000[e] per flow cell	5[g]	~1 µg DNA	Up to 125 Gb (one flow cell) Up to 6 Tb (48 flow cells)	Up to 64 h	

Nanopore

Nanopore: tackling the accuracy problem



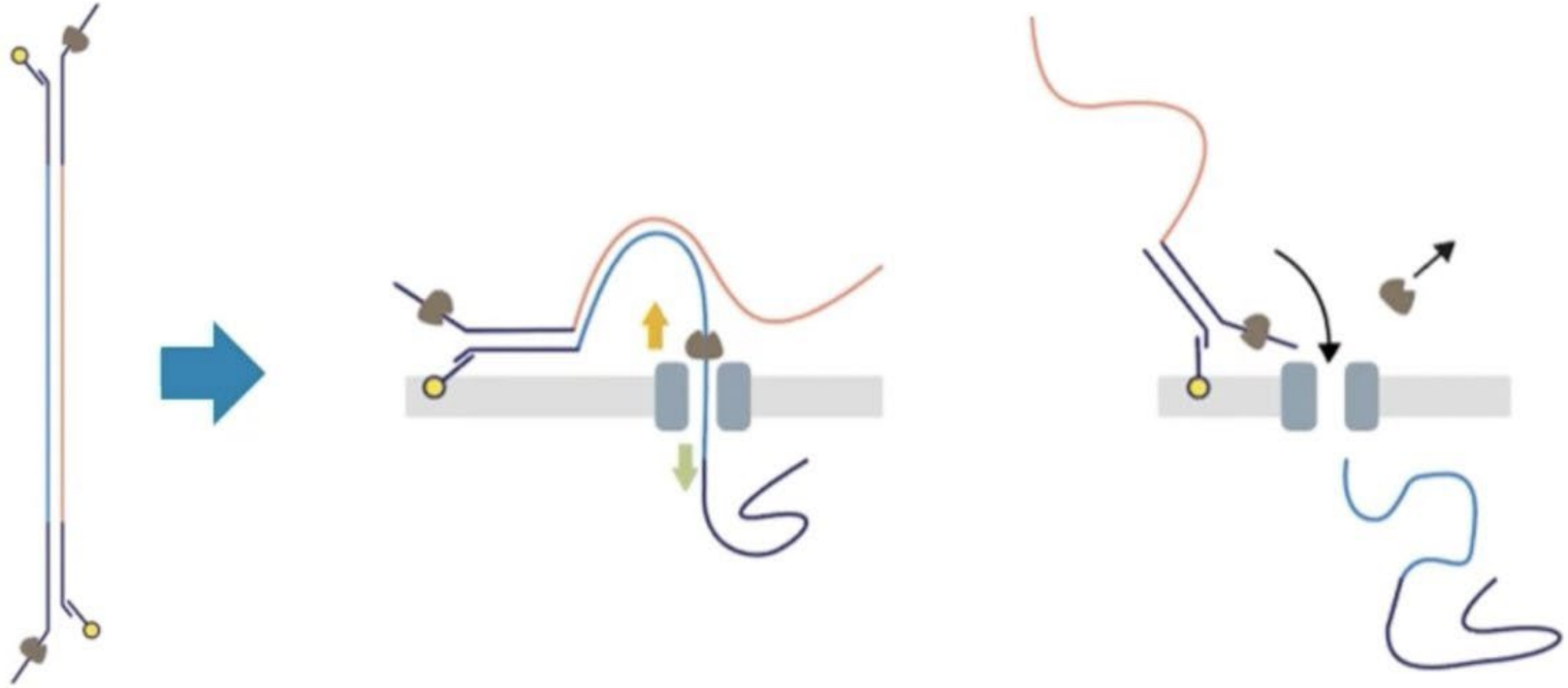


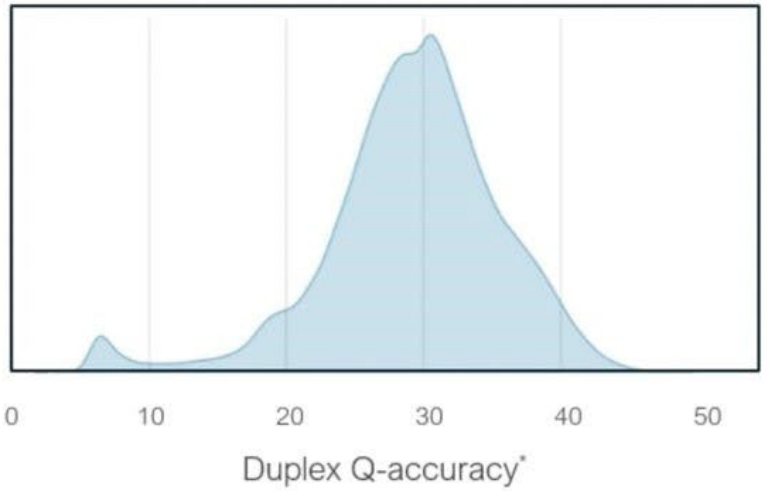
Input Requirements:	R9.4.1	R10.3	R10.0
	5 – 50 fmol	25 – 75 fmol	50 – 100 fmol

Ref: Lin, B.; Hui, J.; Mao, H. Nanopore Technology and Its Applications in Gene Sequencing. Biosensors 2021, 11, 214. https://doi.org/10.3390/bios11070214.

Nanopore: tackling the accuracy problem

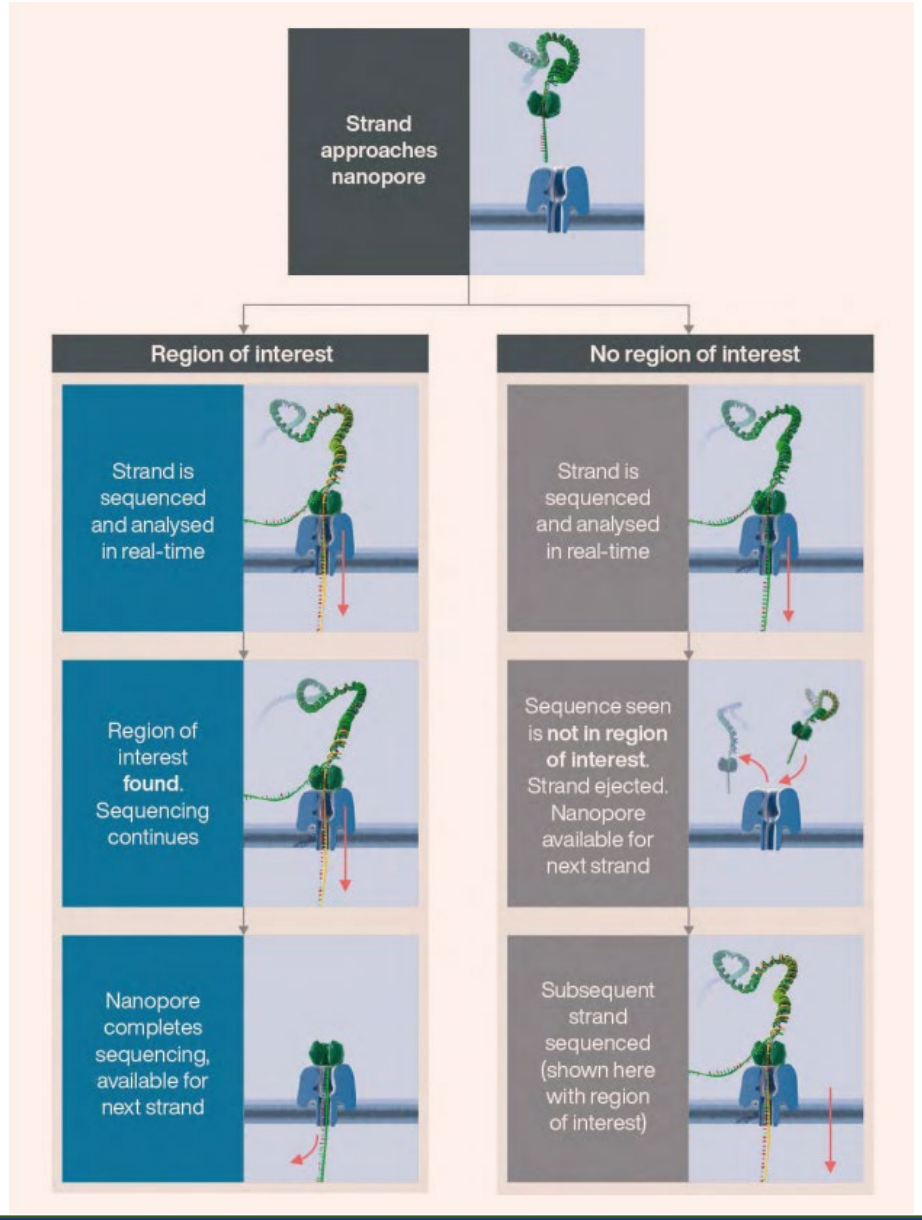
Duplex sequencing => Q30 (99,9% accuracy), but 5x slower





https://nanoporetech.com/resource-centre/clive-brown-ncm-update-2021

Adaptive sampling: finding the needle in the haystack



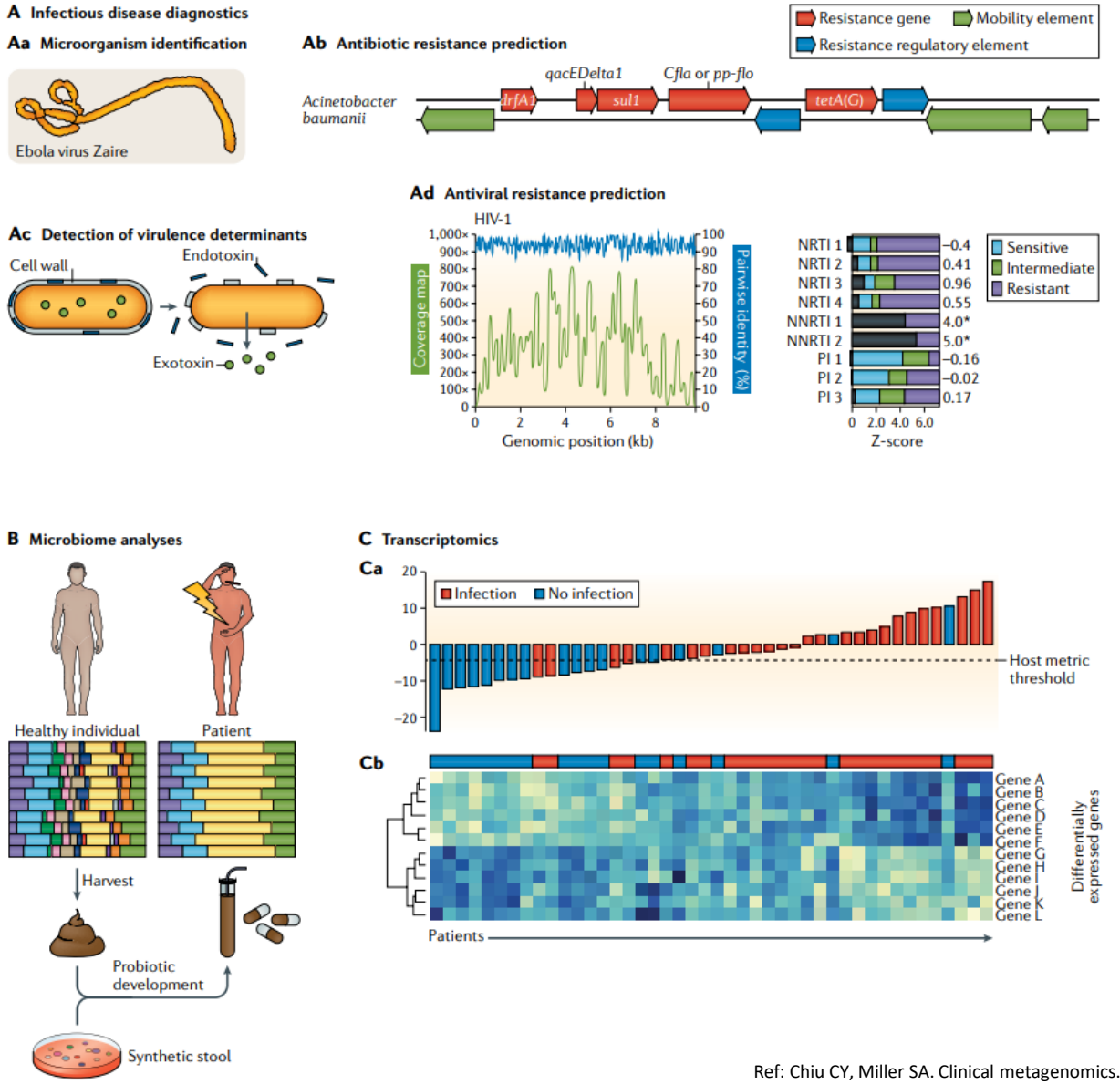
Ref: https://nanoporetech.com/sites/default/files/s3/white-papers/Cancer-research-white-paper.pdf

Overview of Nanopore devices

	Flongle	MinION & MinION Mk1C	GridION Mk1	PromethION 2/2 Solo	PromethION 24/48
Read length	Fragment length = read length. Longest read now >4 Mb[9]				
Run time	1 min – 16 hrs	1 min – 72 hrs	1 min – 72 hrs	1 min – 72 hrs	1 min – 72 hrs
Number of flow cells per device	1	1	5	2	24/48
DNA sequencing yield per flow cell*	Up to 2.8 Gb	Up to 50 Gb	Up to 50 Gb	Up to 290 Gb	Up to 290 Gb
DNA sequencing yield per device*	Up to 2.8 Gb	Up to 50 Gb	Up to 250 Gb	Up to 580 Gb	Up to 7Tb /14 Tb
Multiplexing	1 – 96 samples	1 – >2,000 samples	1 – >2,000 samples	1 – >2,000 samples	1 – >2,000 samples

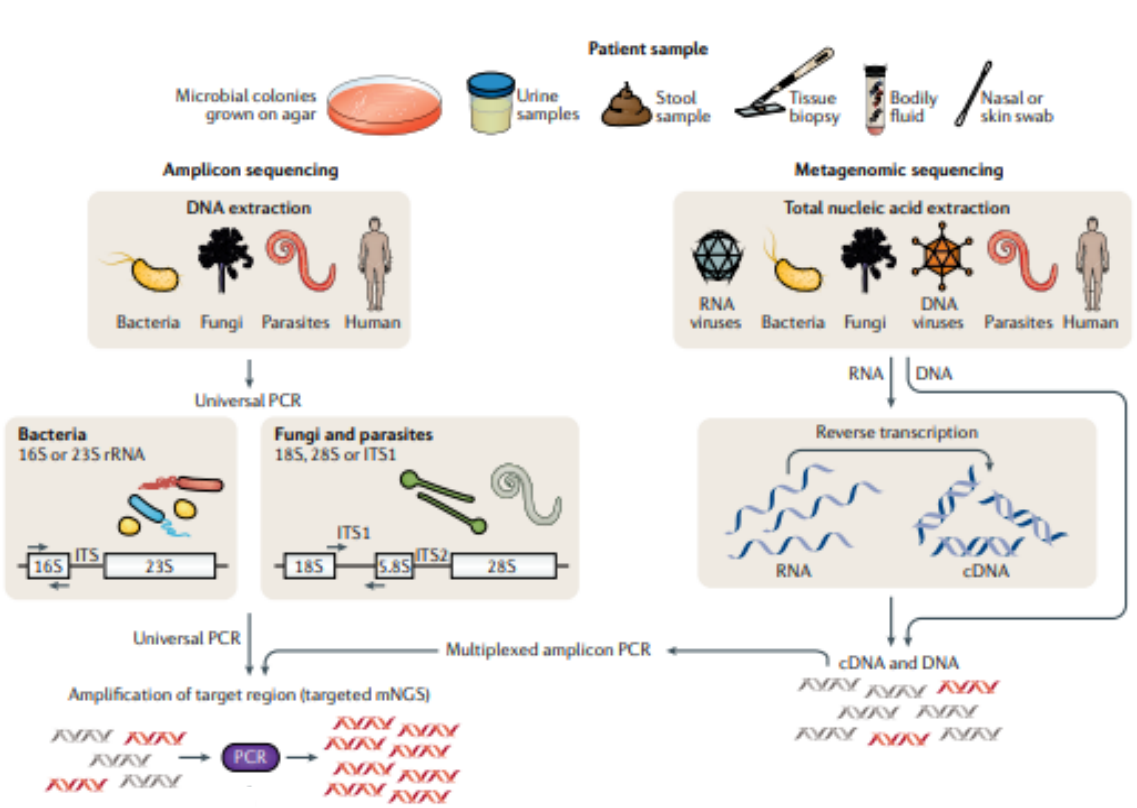
Ref: https://nanoporetech.com/sites/default/files/s3/white-papers/Cancer-research-white-paper.pdf

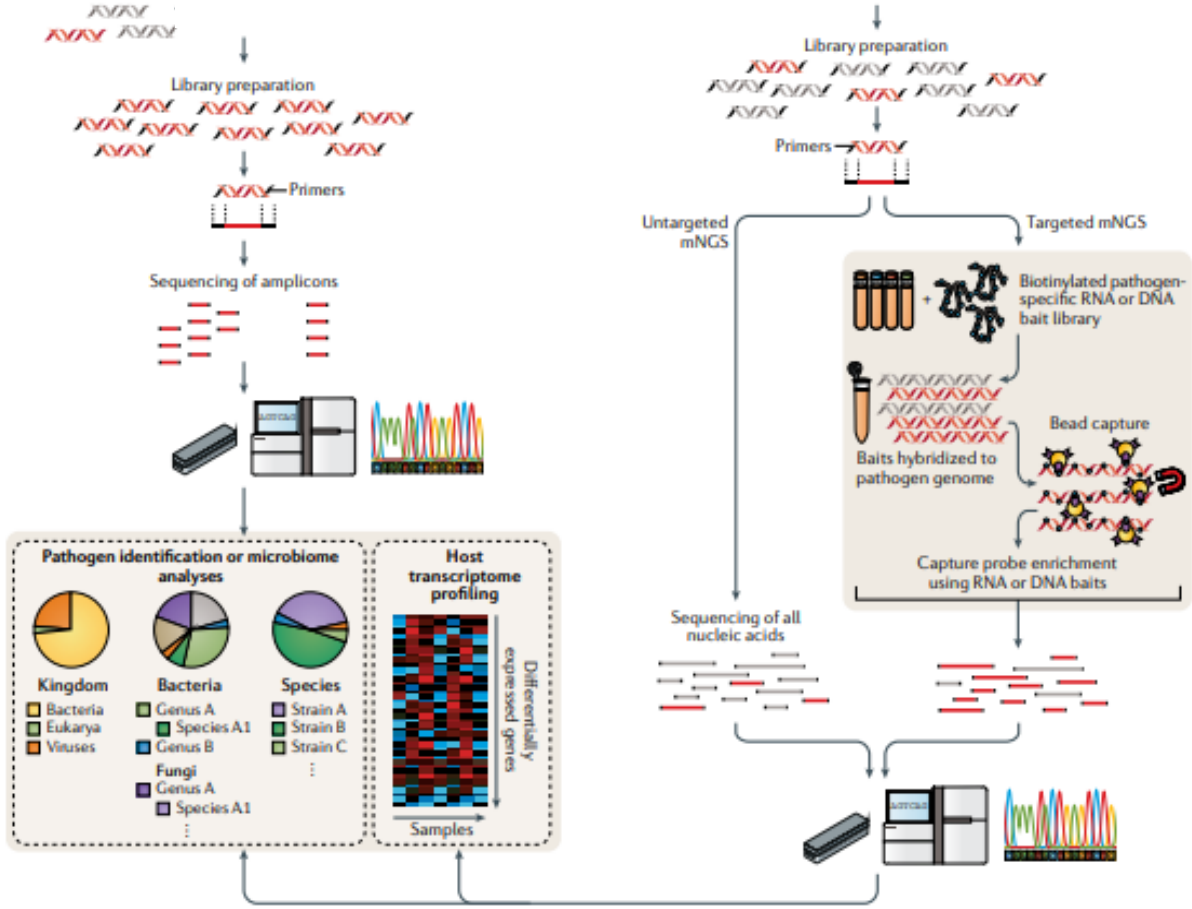
Applications of Nanopore sequencing in Microbiology



Ref: Chiu CY, Miller SA. Clinical metagenomics. Nat Rev Genet. 2019 Jun;20(6):341-355. doi: 10.1038/s41576-019-0113-7.

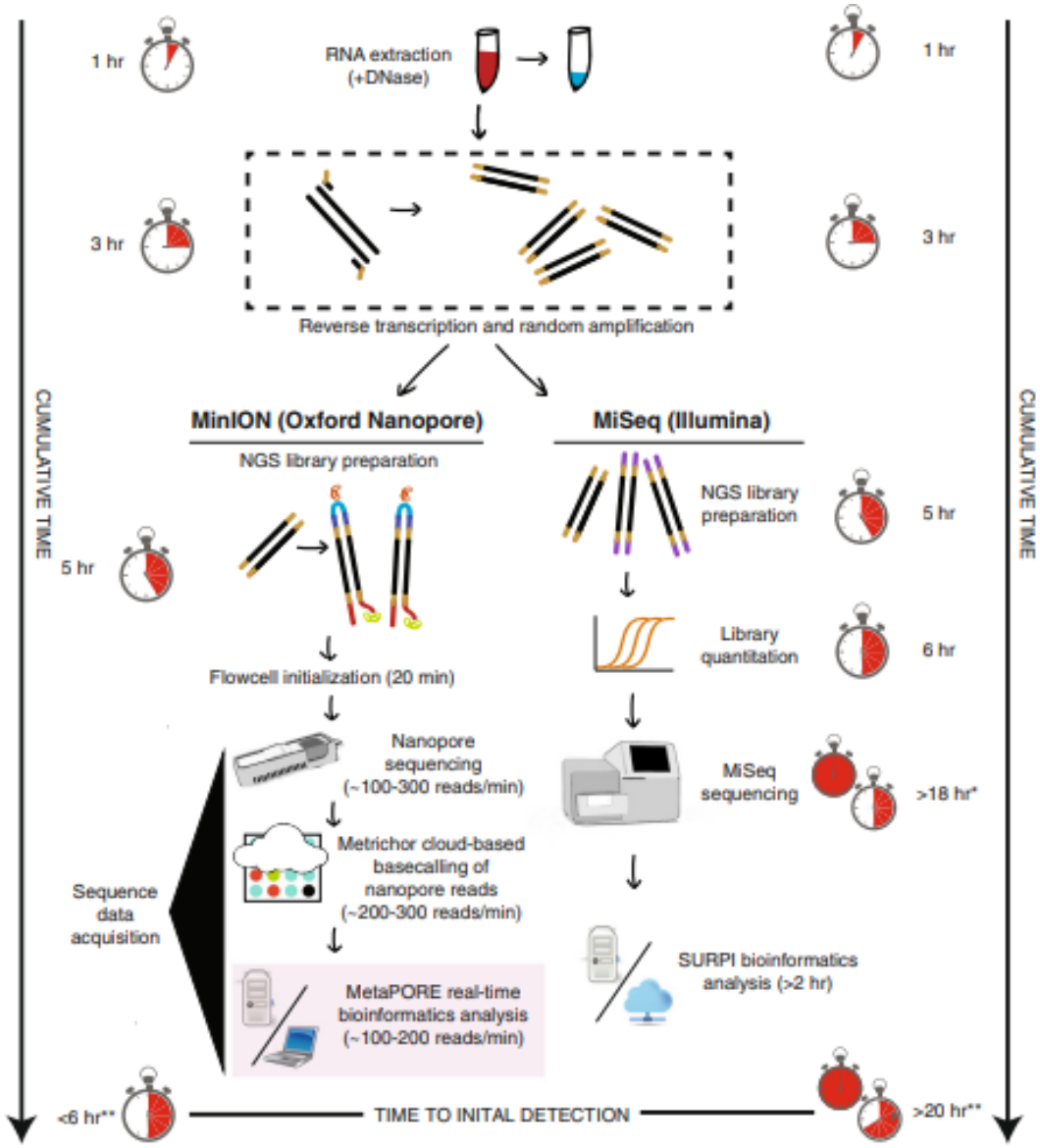
Applications of Nanopore sequencing in Microbiology





Ref: Chiu CY, Miller SA. Clinical metagenomics. Nat Rev Genet. 2019 Jun;20(6):341-355. doi: 10.1038/s41576-019-0113-7.

Applications of Nanopore sequencing in Microbiology



Ref: Greninger et al. Genome Medicine (2015) 7:99 DOI 10.1186/s13073-015-0220-9.

Applications of Nanopore sequencing in Oncology

Key challenges of traditional sequencing technologies

Structural variants

The short reads generated by traditional sequencing technologies cannot span large or complex structural variants (or repeat regions), requiring the use of computational analyses to infer results. As a result, many important variants may be missed or misinterpreted.

Methylation calling

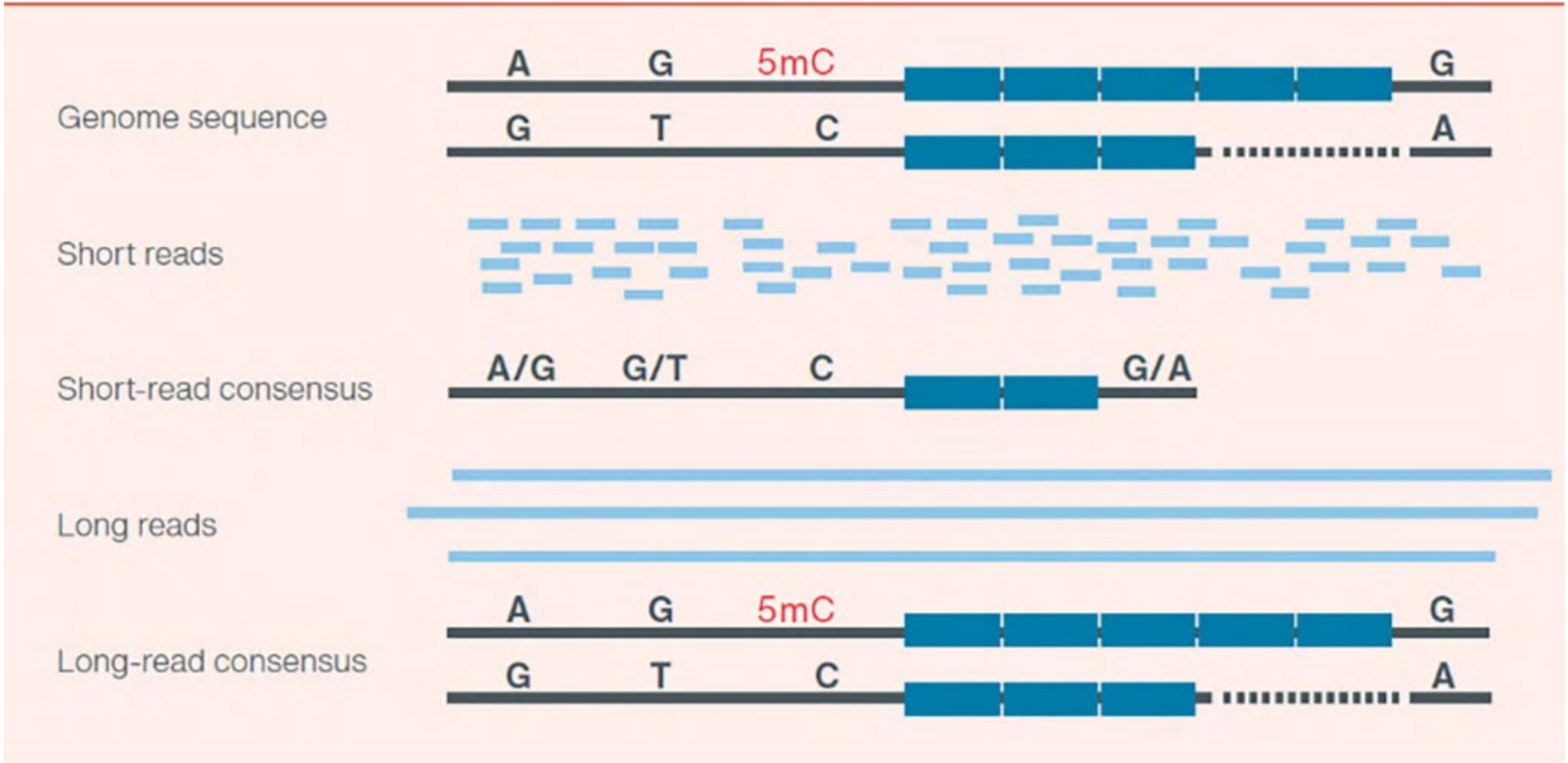
Traditional sequencing technologies require bisulphite conversion (a harsh and laborious sample prep technique) to indirectly infer methylation. Where a suitable reference sequence is not available, additional sequencing runs may also be required. Together these factors can increase experimental variability, time, and costs.

Transcript isoforms

Different transcript isoforms can be associated with different disease stages, but the short reads generated by traditional RNA-Seq techniques only partially cover a transcript's length, making it challenging to accurately assemble and quantify transcript isoforms — providing an incomplete picture of gene expression.

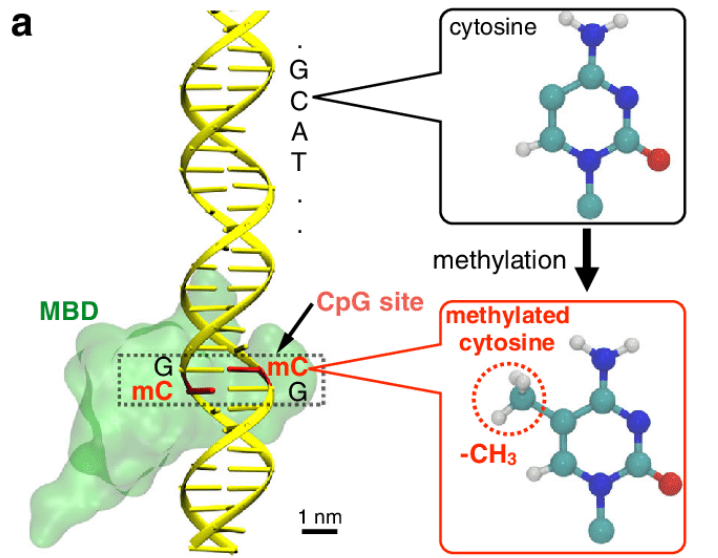
Ref: https://nanoporetech.com/sites/default/files/s3/white-papers/Cancer-research-white-paper.pdf

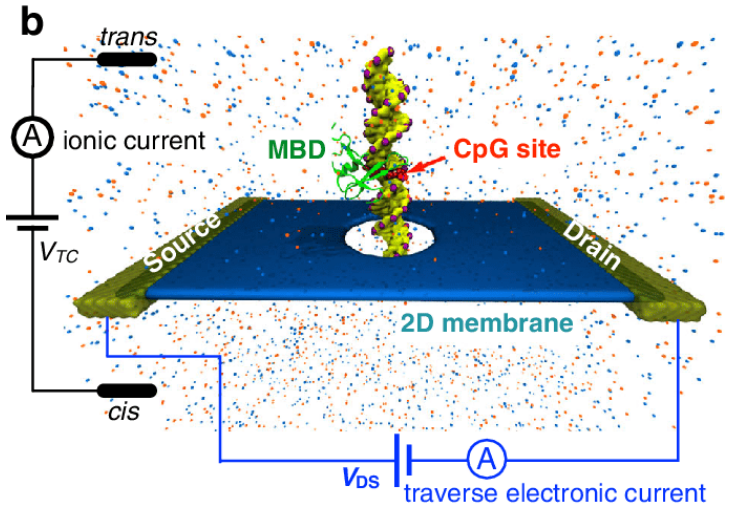
Applications of Nanopore sequencing in Oncology

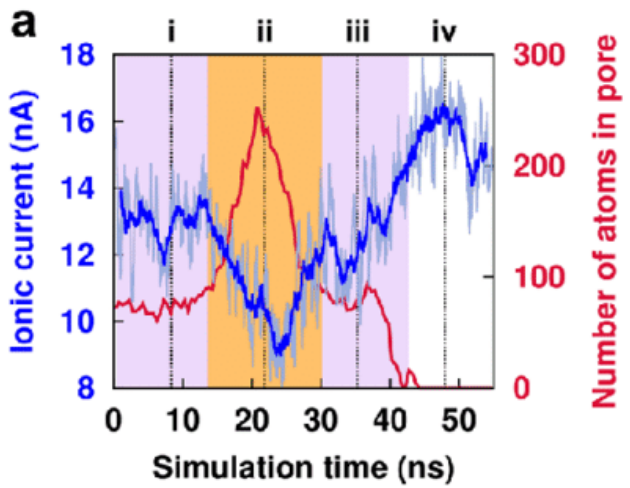


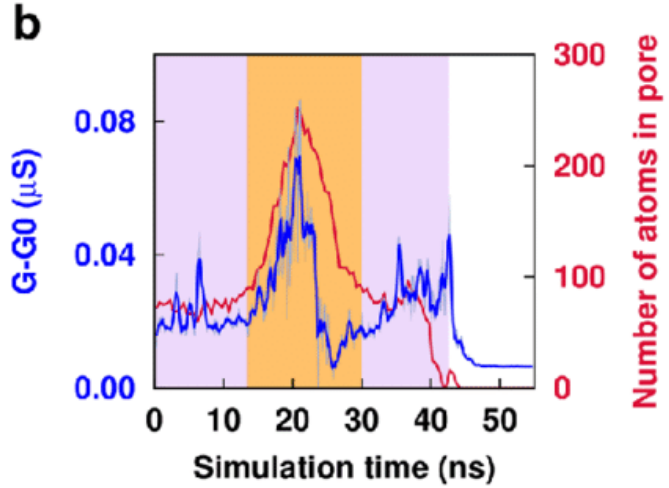
Ref: https://nanoporetech.com/sites/default/files/s3/white-papers/Cancer-research-white-paper.pdf

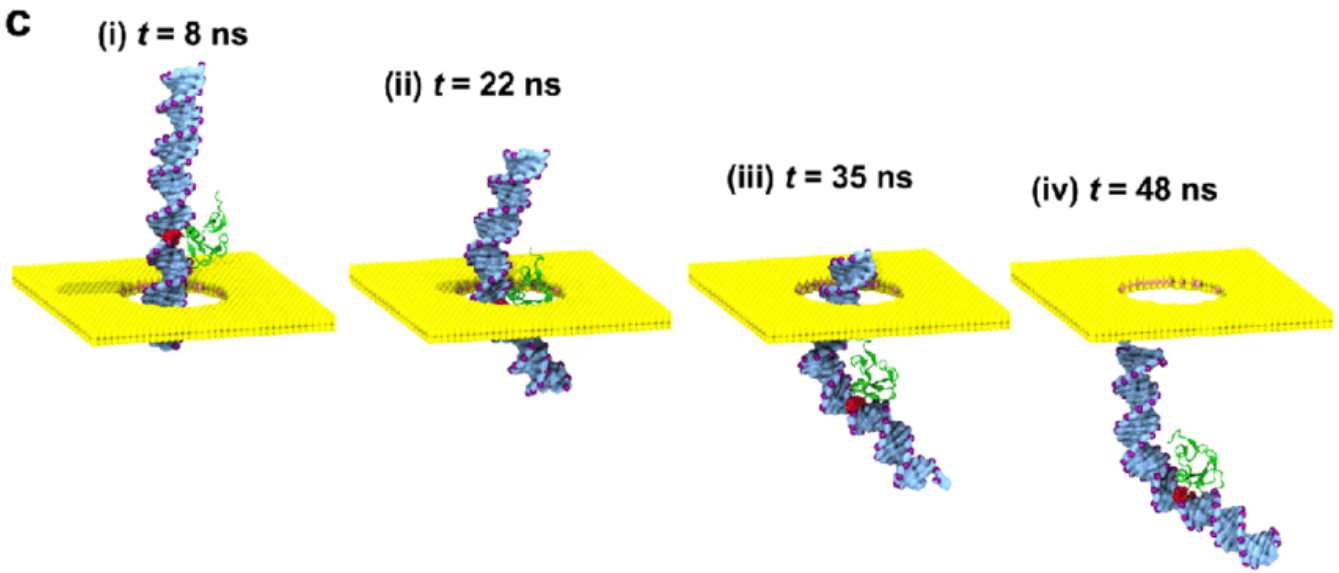
Applications of Nanopore sequencing in Oncology





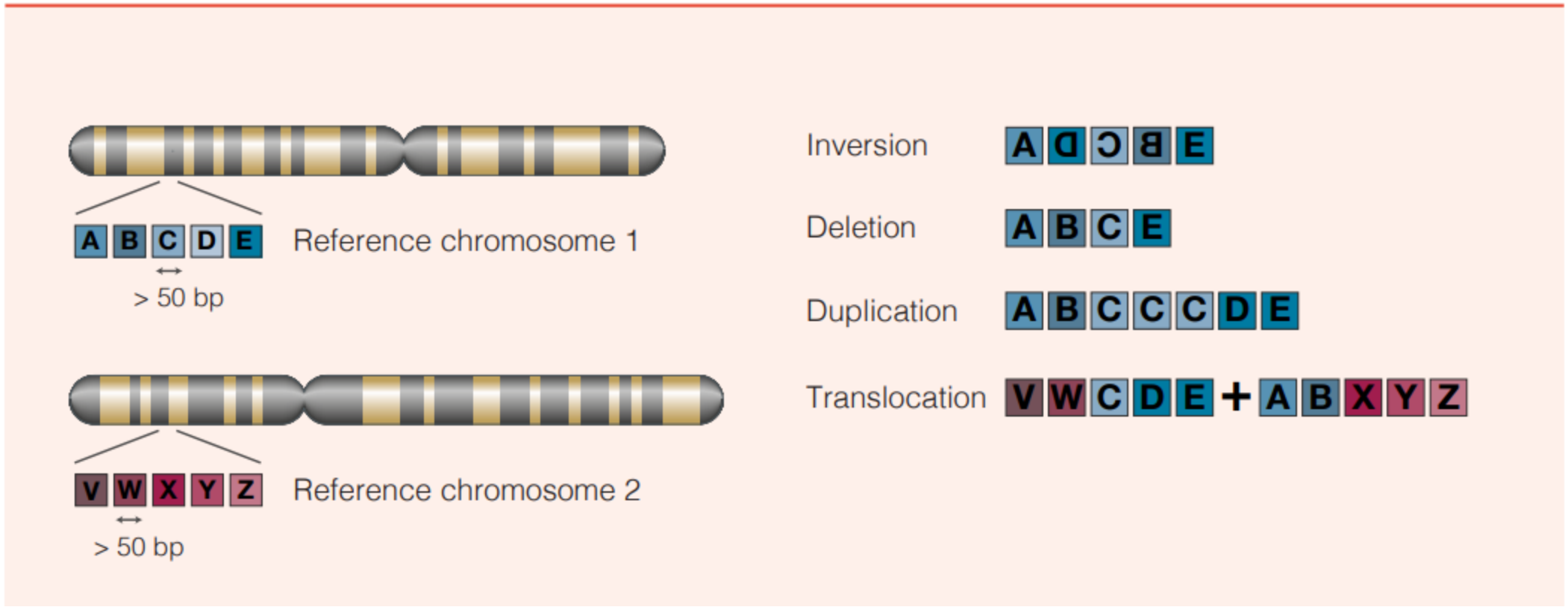






Ref:Qiu, H., Sarathy, A., Schulten, K. et al. Detection and mapping of DNA methylation with 2D material nanopores. npj 2D Mater Appl 1, 3 (2017). https://doi.org/10.1038/s41699-017-0005-7.

Applications of Nanopore sequencing in Oncology



Ref: https://nanoporetech.com/sites/default/files/s3/white-papers/Cancer-research-white-paper.pdf

Applications of Nanopore sequencing in Oncology

nature

Explore content ⌄ About the journal ⌄ Publish with us ⌄

nature › articles › article

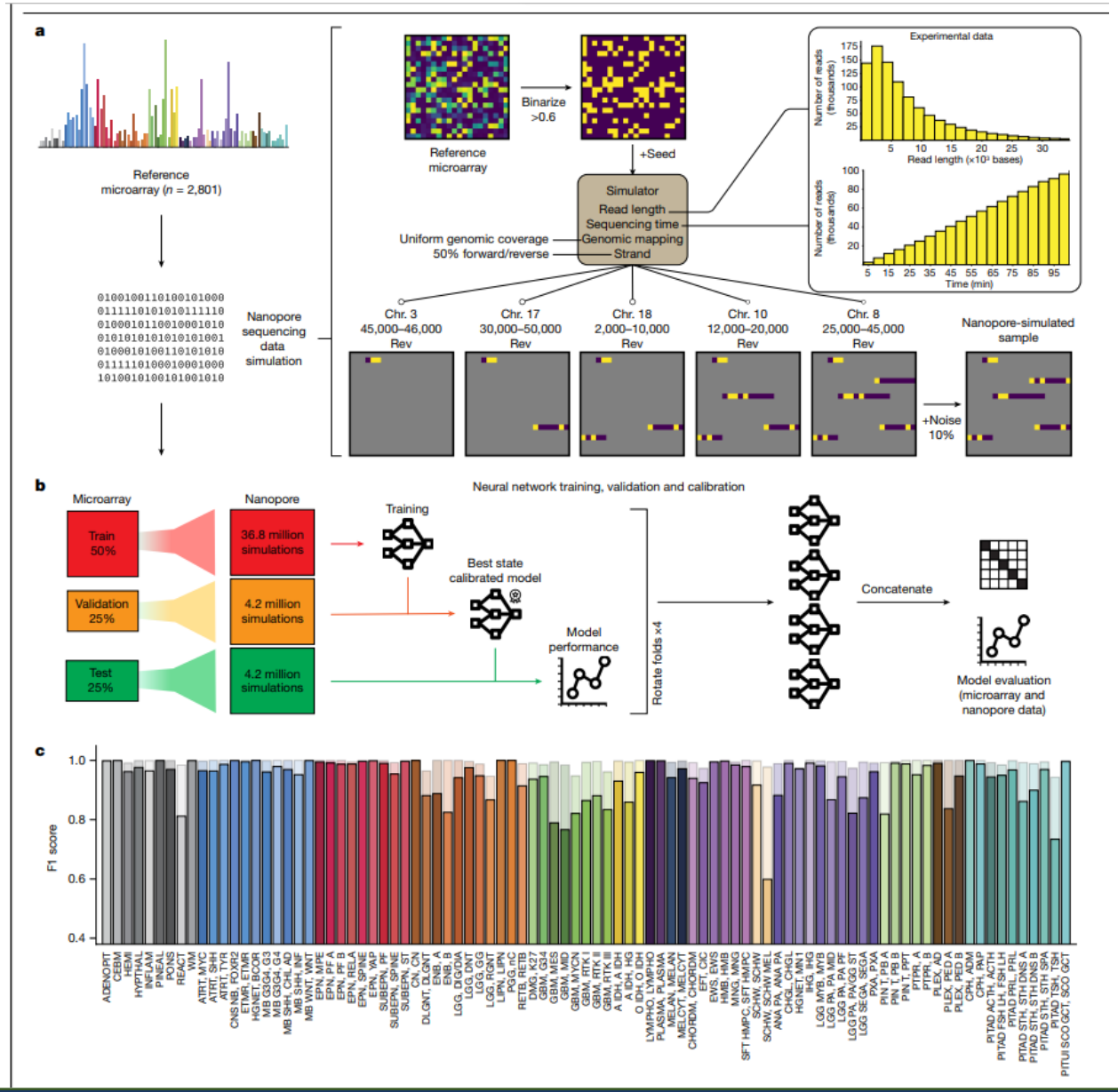
Article | Open access | Published: 11 October 2023

Ultra-fast deep-learned CNS tumour classification during surgery

C. Vermeulen, M. Pagès-Gallego, L. Kester, M. E. G. Kranendonk, P. Wesseling, N. Verburg, P. de Witt Hamer, E. J. Kooi, L. Dankmeijer, J. van der Lugt, K. van Baarsen, E. W. Hoving, B. B. J. Tops ✉ & J. de Ridder ✉

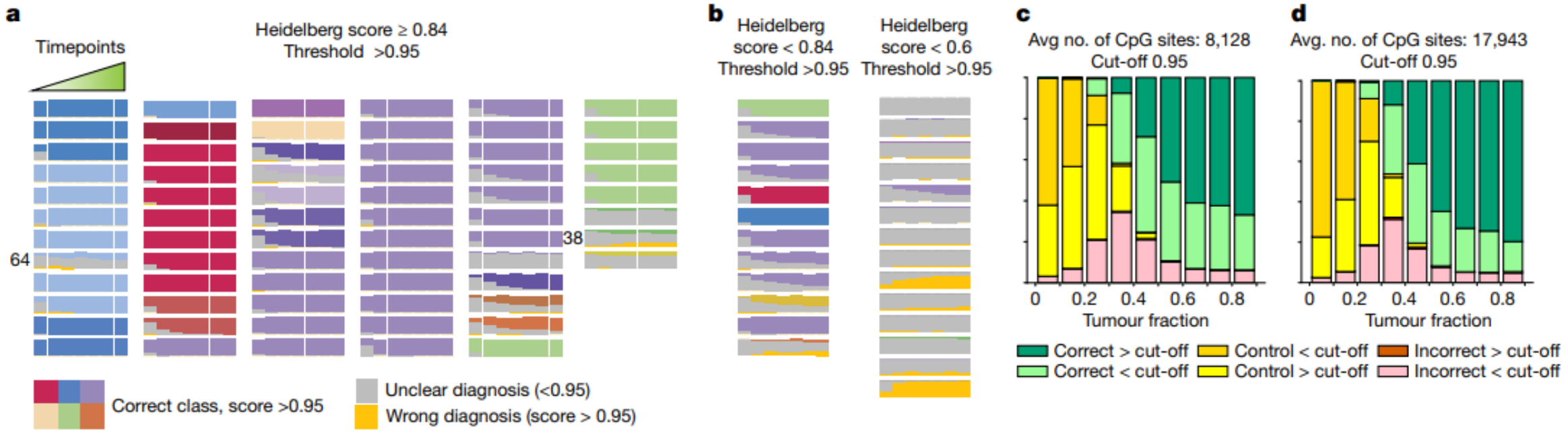
Nature **622**, 842–849 (2023) | Cite this article

Applications of Nanopore sequencing in Oncology



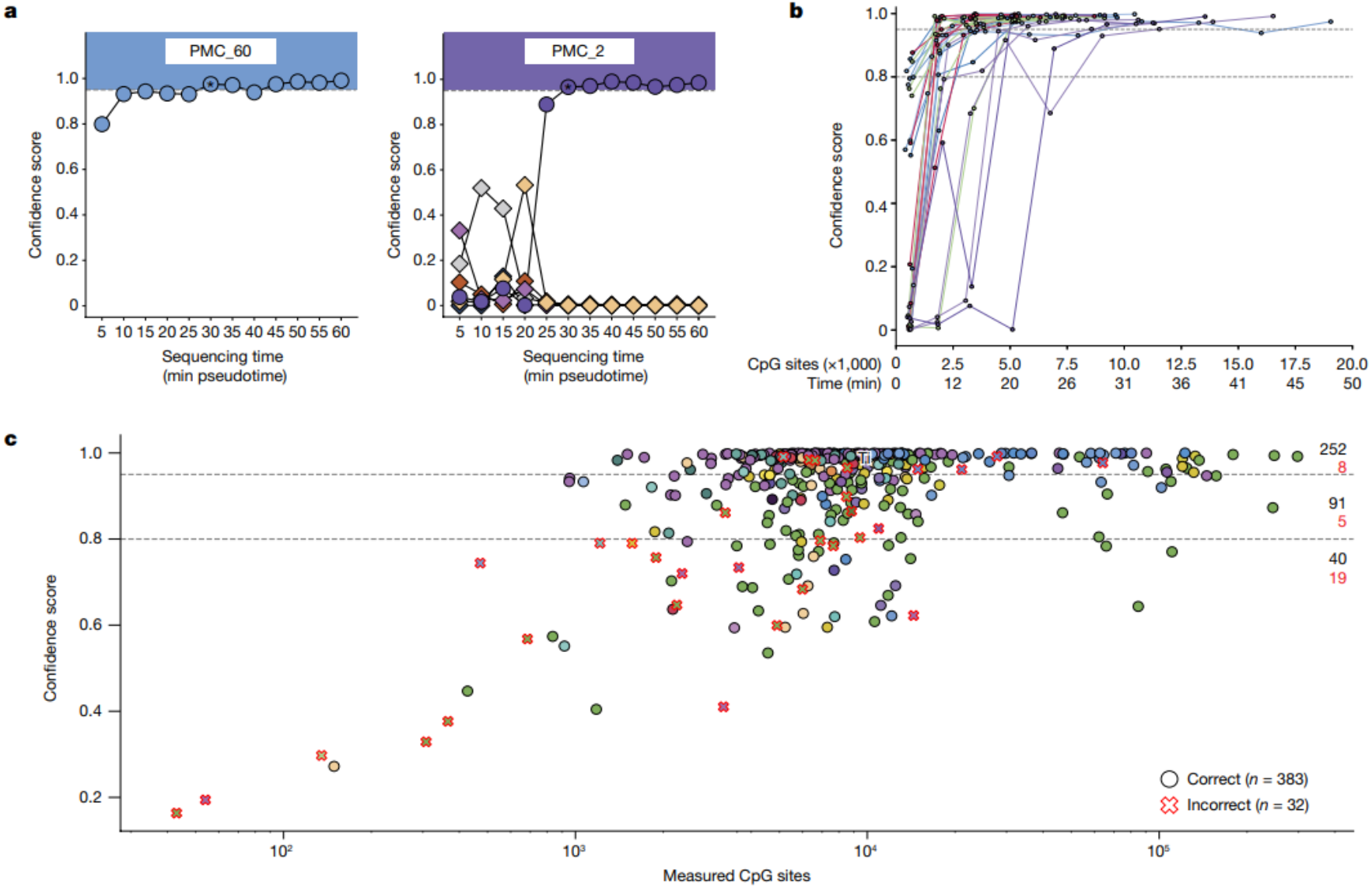
Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

Applications of Nanopore sequencing in Oncology



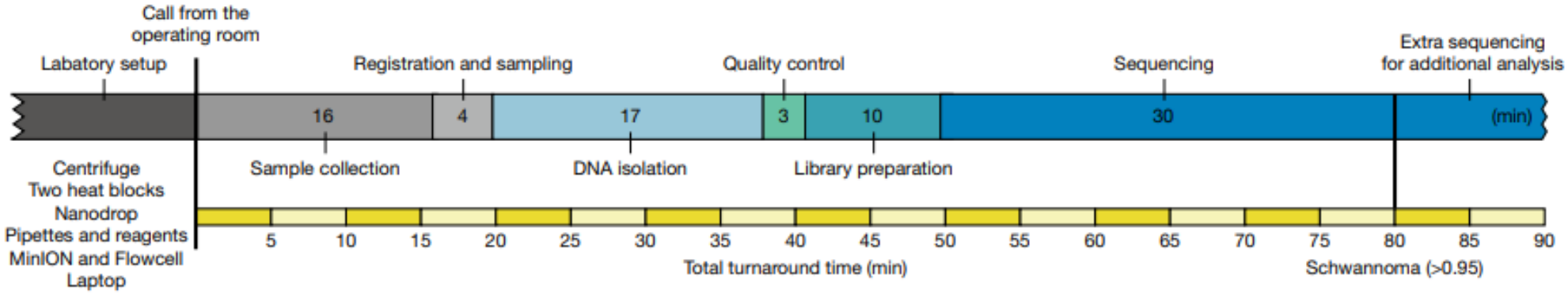
Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

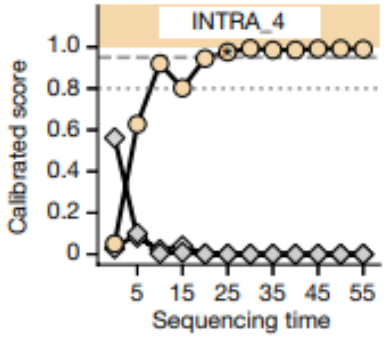
Applications of Nanopore sequencing in Oncology



Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

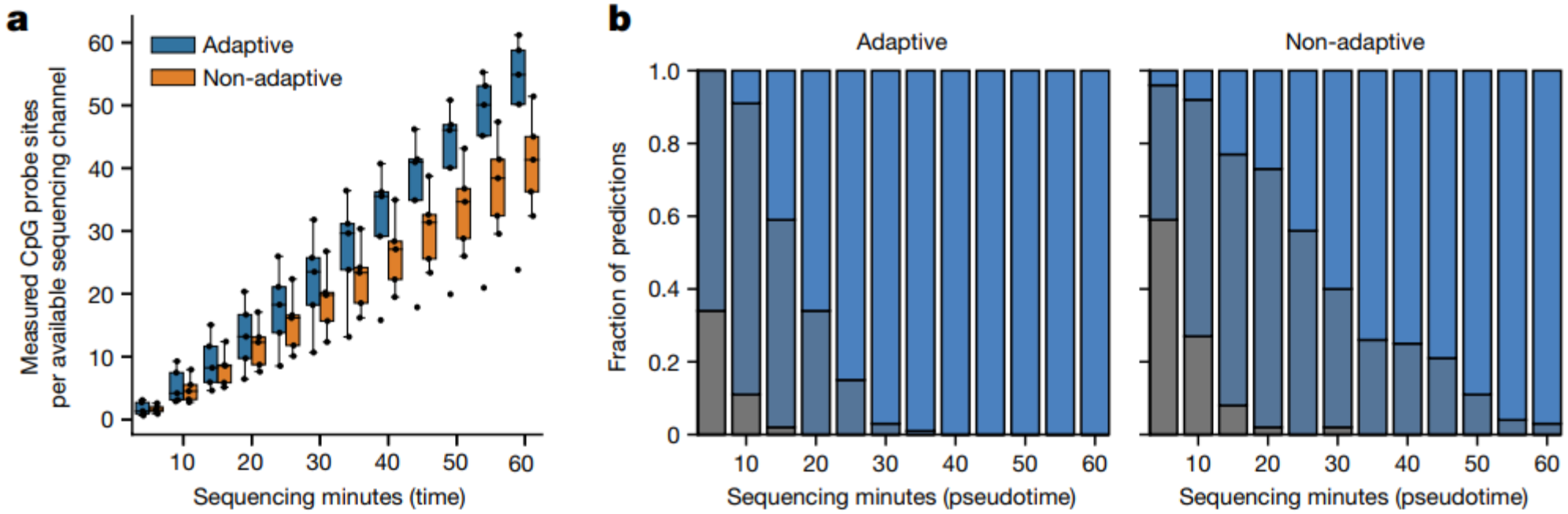
Applications of Nanopore sequencing in Oncology





Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

Applications of Nanopore sequencing in Oncology



Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

Applications of Nanopore sequencing in Oncology

Extended Data Table 1 | Overview of the intraoperative sequencing cases

	Sturgeon	CV	ST	Heidelberg	Diagnosis	*Notes*
1.	Inconclusive	R9	-	Not performed	Adamantinomatous Craniopharyngioma	*Sample used for intraoperative sequencing not representative. Diagnosis based on histology*
2.	Medulloblastoma, non-*WNT* / non-*SHH* subtype: group 3	R9	20	Medulloblastoma, non-*WNT* / non-*SHH* subtype: group 3 (0.99)	Medulloblastoma, non-*WNT*/non-*SHH*	
3.	Inconclusive	R9	-	Pilocytic Astrocytoma, subtype: low grade glioma (0.59)	Pilocytic Astrocytoma	*Sample used for intraoperative sequencing not representative (blood clot). A later sample was used for array and integrated diagnosis*
4.	Schwannoma	R9	30	Schwannoma (0.99)	Schwannoma	*Process captured on film. (https://zenodo.org/record/8261128)*
5.	Pilocytic Astrocytoma	R10	20	Midline Pilocytic Astrocytoma (0.99)	Pilocytic Astrocytoma	*3 samples collected, two with low tumor percentage. Highest tumor percentage sample used in intraoperative sequencing*
6.	IDH-wildtype Glioblastoma RTKII	R9	35	IDH-wildtype Glioblastoma RTKII (0.87)	Glioblastoma, IDH-wildtype	*Adult Glioma case*
7.	Choroid Plexus Papiloma type A	R10	15	Plexus tumor, subtype pediatric A (0.90)	Atypical Chorioid Plexus Papiloma	
8.	Inconclusive	R10	-	Reactive tissue (0.47), Pilocytic Astrocytoma (0.43)	Pilocytic Astrocytoma	*Unusual histological features for pilocytic astrocytoma, histomolecular diagnosis partly based on KIAA1549-BRAF fusion*
9.	Pilocytic Astrocytoma	R10	15	Posterior fossa Pilocytic Astrocytoma (0.99)	Pilocytic Astrocytoma	
10.	IDH-mutant Astrocytoma	R9	10	IDH-mutant, Glioma, High- or Low-Grade Astrocytoma (0.99)	IDH-mutant, Astrocytoma, CNS WHO grade 4	*Adult Glioma case*
11.	Inconclusive	R10	-	Not performed	Germinoma	*Germinoma; tumor subtype not in reference Capper et al., 2018 datset. Diagnosis based only on intraoperative frozen section histology*
12.	Inconclusive	R9	-	White matter (0.99)	IDH-mutant, Astrocytoma, CNS WHO grade 4	*Adult Glioma case. Sample used for intraoperativesequencing was not representative, Integrated molecular diagnosis based on an other tissue sample.*
13.	Inconclusive	R10	-	Low-Grade Glioma, *MYB/MYBL1* (0.33)	Diffuse Glioma, Not Elsewhere Classified	*Case considered as 'gliomatosis cerebri' in context of complex genetic background*
14.	Inconclusive	R10	-	Not performed	Pilocytic Astrocytoma	*Biopsy sample, not tumor representative*
15.	Pilocytic Astrocytoma	R10	15	Pilocytic Astrocytoma subtype: posterior fossa	Pilocytic Astrocytoma	*Resection sample from patient 14*
16.	Adamantinomatous Craniopharyngioma	R10	10	Not performed	Adamantinomatous Craniopharyngioma	*Diagnosis based on histology*
17.	IDH-mutant Astrocytoma	R9	45	Not performed	IDH-mutant, Astrocyto - ma, CNS WHO Grade 2	*Adult Glioma case*
18.	Pilocytic Astrocytoma	R10	40	Pilocytic Astrocytoma, subtype: posterior fossa (0.95)	Pilocytic Astrocytoma	
19.	Medulloblastoma Group 4	R10	10	Medulloblastoma, Group 3 and 4, subtype: Group 4 (0.99)	Medulloblastoma, non-*WNT*/non-*SHH*	
20.	Medulloblastoma Group 3	R10	15	Medulloblastoma, non-*WNT* / non-*SHH*, subtype: Group 3 (0.99)	Medulloblastoma, non-*WNT*/non-*SHH*	
21.	Pilocytic Astrocytoma	R10	30	Pilocytic Astrocytoma (0.97)	Pilocytic Astrocytoma	
22.	Pilocytic Astrocytoma *	R10	-	Not performed	Pilocytic Astrocytoma	*Recurrent tumor, diagnosis the same as in initial tumor sample*
23.	Ependymoma, subtype *RELA* fusion	R10	10	Ependymoma, subtype *RELA* fusion (0.99)	Supratentorial Ependymoma, *ZFTA* fusion-positive **	*Classification used to support frozen section diagnosis during surgery*
24.	IDH-wildtype Glioblastoma, subtype Mesenchymal *	R9	-	IDH-wildtype Glioblastoma, subtype Mesenchymal (0.4)	Diffuse High-Grade Glioma, results further analysis pending	*Adult Glioma case; Imaging consistent with Low-Grade Diffuse Glioma*
25.	Ependymoma, posterior fossa type A	R10	25	Ependymoma, posterior fossa type A (0.99)	Ependymoma, posterior fossa type A	*Classification used to support frozen section diagnosis during surgery*

Ref: Vermeulen, C., Pagès-Gallego, M., Kester, L. et al. Ultra-fast deep-learned CNS tumour classification during surgery. Nature 622, 842–849 (2023). https://doi.org/10.1038/s41586-023-06615-2

Getting started with Nanopore sequencing

- Invest in the right type of sequencer and PC for your needs!

MinION Mk1B IT requirements

Component	Required specification: GPU high-accuracy basecalling	Required specification: data acquisition/CPU basecalling (note: CPU basecalling performance is limited - a GPU is recommended)
Operating system	Windows – 10 Linux – Ubuntu 20.04 and 18.04	Windows – 10 OSX – Mojave, Catalina Linux – Ubuntu 20.04 and 18.04
Memory/RAM	16 GB RAM or higher	16 GB RAM or higher
CPU	Intel i7, i9, Xeon, or better, with at least 4 cores/8 threads Ryzen 5, 7, or better, with at least 4 cores/8 threads	Intel i7, i9, Xeon, or better, with at least 4 cores/8 threads Ryzen 5, 7, or better, with at least 4 cores/8 threads
GPU	NVIDIA GPU RTX 2060 SUPER or better, with at least 8 GB of GPU memory. Theoretical performance is only a guide, but more is better. Technical information can be found on various websites, for example https://www.techpowerup.com/gpu-specs/. Widely-available examples include the RTX 2060 SUPER, RTX 2070, RTX 3060, RTX 3070. Ampere-based GPUs (the 3000 series, A series etc.) are particularly recommended for optimal performance. If you are working with a different type of GPU than the models listed above, please ensure that it has a CUDA Compute Capability >6.1 (for more information about CUDA-enabled GPUs, see the NVIDIA website).	-
Storage	1 TB internal SSD or higher	1 TB internal SSD or higher
Ports	USB3.0‡	USB3.0‡

Set-up your own experiment

- Amplicon-based? => Native barcoding kit or Ligation sequencing kit (barcoded primers)
- Untargeted approach => Rapid barcoding kit
- Ready to use (including primers, barcodes & adapters) => 16S Barcoding kit

Set-up your own experiment

Native Barcoding Kit 96 V14 SQK-NBD114.96

A versatile method of preparing barcoded sequencing libraries optimised for modal raw read accuracy of Q20+ (99%+) and long read multiplexed samples.

This is an Early Access product

This uses our latest Kit 14 chemistry

- Preparation time: 140 minutes
- Read length: = to fragment length
- Input amount: 400 ng per sample of gDNA, 200 fmol per sample (130 ng for 1 kb amplicons)

€760.00

Buy >

2 Early Access

Ligation Sequencing Kit V14 SQK-LSK114

A versatile sequencing kit optimised for modal raw read accuracy of Q20+ (99%+) and long read singleplex samples.

This uses our latest Kit 14 chemistry

- Preparation time: 60 minutes
- Read length: = fragment length
- Input amount: 1000 ng dsDNA, 100+ ng DNA if performing fragmentation or PCR,100-200 fmol of amplicons or cDNA

€570.00

Buy >

3 Released

Rapid Barcoding Kit 96 V14 SQK-RBK114.96

Simple and rapid library preparation, with barcoding for up to 96 gDNA samples.

This is an Early Access product

This uses our latest Kit 14 chemistry

- Preparation time: 60 minutes
- Read length: Random distribution, dependent on input fragment length
- Input amount: 50 ng dsDNA per sample

€945.00

Buy >

2 Early Access

16S Barcoding Kit 24 V14 SQK-16S114.24

Genus-level bacterial identification with barcoding for up to 24 samples.

This is an Early Access product

Product lead time: 1 week

This uses our latest Kit 14 chemistry.

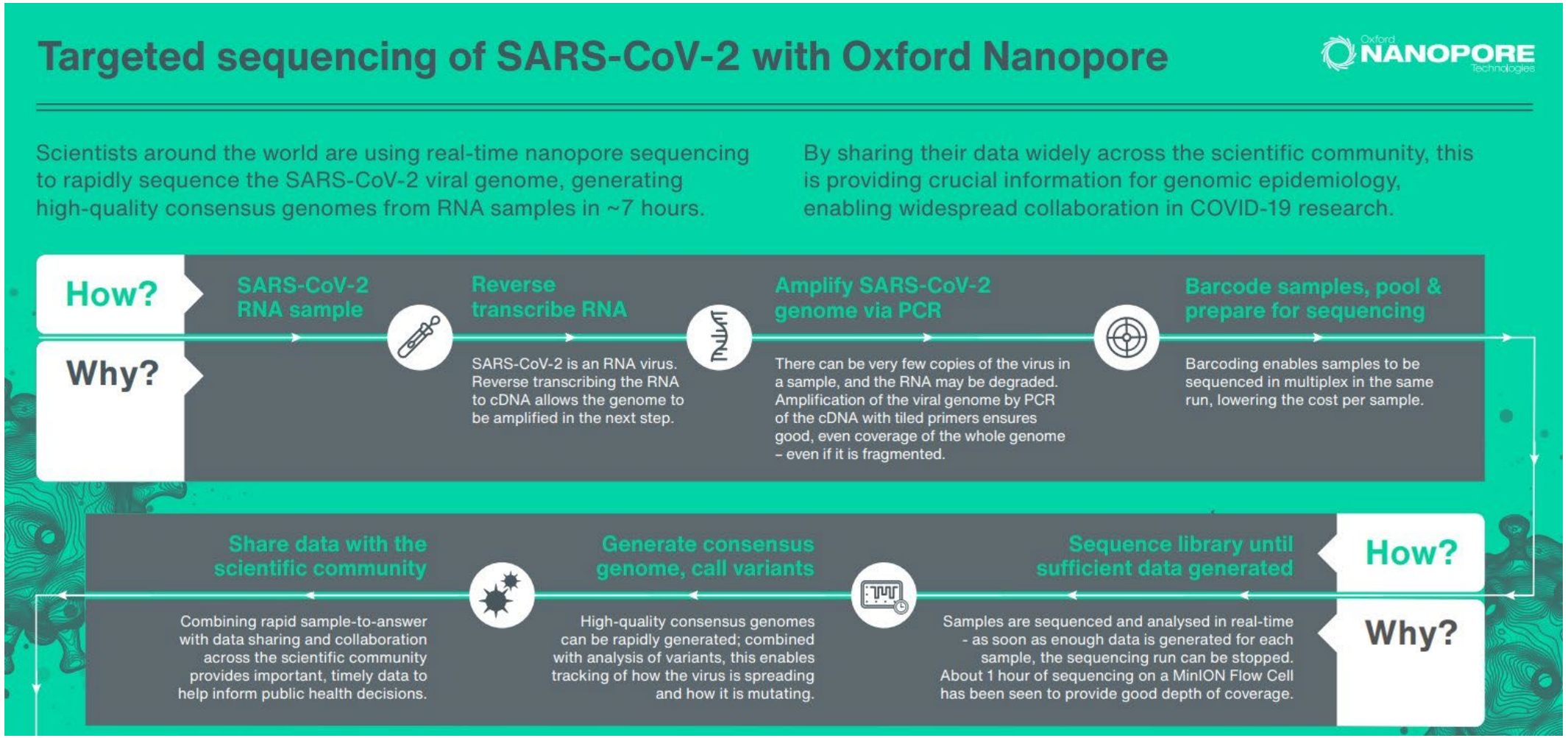
- Preparation time: 25 minutes + PCR
- Read length: Full-length 16S gene (~1.5 kb)
- Input amount: 10 ng gDNA per sample

€855.00

Buy >

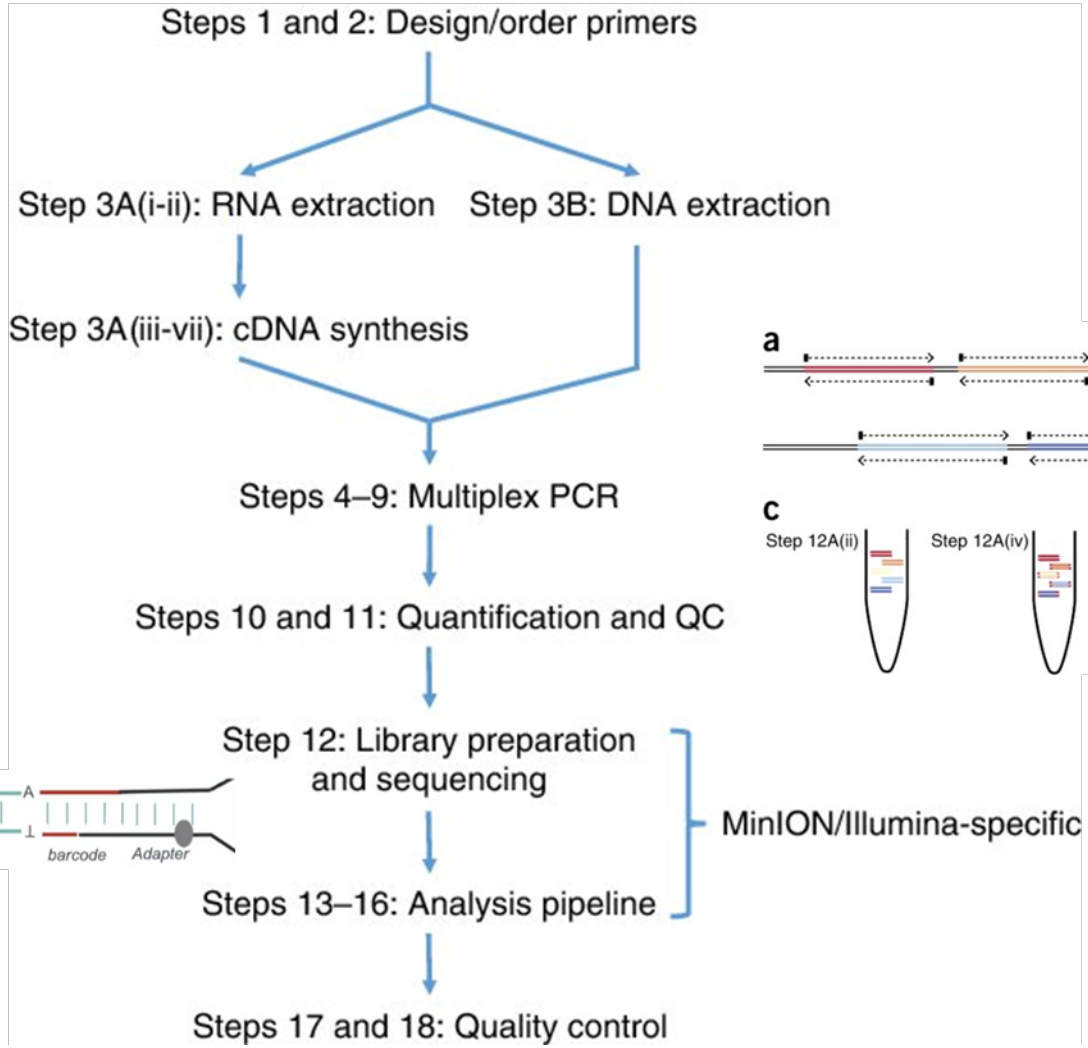
2 Early Access

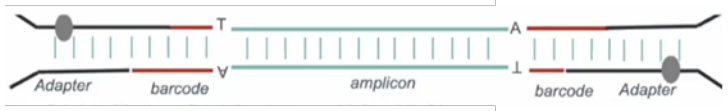
Set-up your own experiment: SARS-CoV-2 WGS

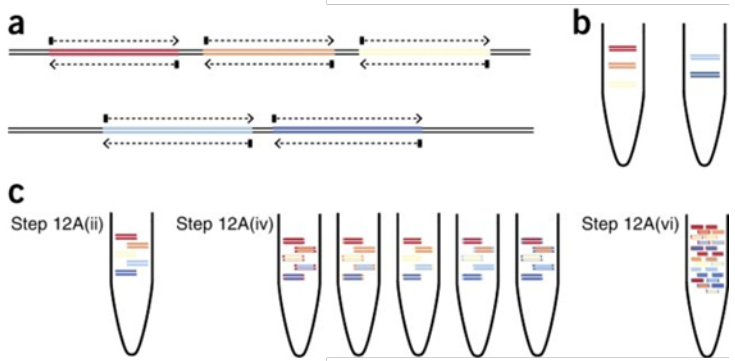


Set-up your own experiment: SARS-CoV-2 WGS

Workflow







Set-up your own experiment: SARS-CoV-2 WGS

Reverse transcription

~15 minutes

1 **In a clean pre-PCR hood, mix together the following components in each well of a 96-well plate on ice or in a PCR cool rack, such as the Eppendorf PCR-Cooler:**

Reagent	Volume
RNA sample	16 µl
LunaScript RT SuperMix (5x)	4 µl
Total	**20 µl**

Note: We recommend using up to 16 µl of RNA sample. Use nuclease-free water to make up the final volume to 16 µl if required.

2 **Mix gently by pipetting, and spin down. Return the plate to ice.**

3 **Preheat the thermal cycler to 25°C.**

4 **Incubate the samples in the thermal cycler using the following program:**

Temperature	Time
25°C	2 minutes
55°C	10 minutes
95°C	1 minute
4°C	hold

Set-up your own experiment: SARS-CoV-2 WGS

PCR and clean-up

~210 minutes

1 **Add 5 µl of each primer from pool A per sample to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool.**

2 **Add 5 µl of each primer from pool B per sample to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool.**

3 **Dilute each 100 µM stock 1 in 10 with nuclease-free water to form a working stock of each pool at 10 µM.**

Note: To achieve the desired final concentration of each primer in the pool at 0.015 µM in the PCR reaction, 3.7 µl of the 10 µM working stock is needed for each PCR reaction. Two separate PCR reactions will be performed per sample, one for pool A primers and one for pool B. This results in tiled amplicons that have approximately 20 bp overlap.

4 **In a clean pre-PCR hood, set up two individual reactions using primer pool A and primer pool B in a clean 5 ml centrifuge tubes:**

Reagent	Volume (pool A)	Volume (pool B)
Q5® Hot Start High-Fidelity 2X Master Mix	1250 µl	1250 µl
Primer pool at 10 µM (A or B)	370 µl	370 µl
Nuclease-free water	380 µl	380 µl
Total	**2 ml**	**2 ml**

Note: the above have been calculated to allow for excess.

5 **In a clean 96-well plate, aliquot 20 µl of pool A reaction to each well. Repeat in a new 96-well plate with pool B reaction.**

Add 5 µl of the reverse-transcribed samples per well.

Set-up your own experiment: SARS-CoV-2 WGS

PCR and clean-up

~210 minutes

6 **Mix gently by pipetting and spin down.**

7 **Incubate using the following program, with the heated lid set to 105°C:**

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	15 sec	25–35
Annealing and extension	65°C	5 min	25–35
Hold	4°C	∞	

Note: Cycle number should be varied for low or high viral load samples. Guidelines provided by Josh Quick suggest that 25 cycles should be used for Ct 18–21 up to a maximum of 35 cycles for Ct 35, however this has not been tested here.

8 **Combine the 25 µl reaction from pool A and the 25 µl reaction from pool B per sample, into a new deep well plate; one well per sample.**

Note: ensure the sample from pool A corresponds to the sample from pool B.

9 **Resuspend the AMPure XP beads by vortexing.**

10 **Add 50 µl of resuspended AMPure XP beads to each well and mix by gently pipetting.**

11 **Allow DNA to bind to the beads for 5 minutes at room temperature.**

12 **Prepare 50 ml of fresh 80% ethanol in nuclease-free water.**

Set-up your own experiment: SARS-CoV-2 WGS

PCR and clean-up

~210 minutes

13 **Spin down the 96-well plate and pellet the beads on a magnet for 5 minutes. Keep the plate on the magnet until the eluate is clear and colourless, and pipette off the supernatant.**

14 **Keep the plate on the magnet and wash the beads in each well with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Keeping the magnetic rack on the benchtop, rotate the plate by 180°. Wait for the beads to migrate towards the magnet and form a pellet. Remove the ethanol using a pipette and discard.**

15 **Repeat the previous step.**

16 **Spin down and place the plate back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 30 seconds, but do not dry the pellet to the point of cracking.**

17 **Remove the plate from the magnetic rack and resuspend each pellet in 15 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

18 **Pellet the beads on a magnet until the eluate is clear and colourless.**

19 **Remove and retain 15 µl of eluate containing the DNA library per well, into a clean 96-well plate.**

Dispose of the pelleted beads.

20 **Quantify 1 µl of each eluted sample using a Qubit fluorometer.**

21 **Store any unused amplified material at -20°C for use in later experiments.**

Set-up your own experiment: SARS-CoV-2 WGS

End-prep

~20 minutes

1 **Determine the volume of the cleaned-up PCR reaction that yields 200 fmol (50 ng) of DNA per sample and aliquot in a clean 96-well plate.**

2 **Prepare the NEBNext Ultra II End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.**

3 **Make up each sample per well to 12.5 µl using nuclease-free water.**

4 **In a 1.5 ml Eppendorf DNA LoBind tube, combine the following:**

Reagent	Volume
Ultra II End-prep reaction buffer	175 µl
Ultra II End-prep enzyme mix	75 µl
Total	**250 µl**

5 **Mix gently by pipetting and spin down.**

6 **Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins.**

END OF STEP

Take forward the end-prepped DNA into the native barcode ligation step.

Set-up your own experiment: SARS-CoV-2 WGS

Native barcode ligation

~60 minutes

1 **Thaw the native barcodes at room temperature, enough for one barcode per sample. Individually mix the barcodes by pipetting, and place them on ice.**

2 **Thaw the tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.**

3 **Select a unique barcode for every sample to be run.**

4 **In a new 96-well plate, add the reagents in the order given below per well:**

Reagent	Volume
Nuclease-free water	3 µl
End-prepped DNA	0.75 µl
Native Barcode	1.25 µl
Blunt/TA Ligase Master Mix	5 µl
Total	**10 µl**

5 **Mix contents thoroughly by pipetting and spin down briefly.**

6 **Using a thermal cycler, incubate at 20°C for 20 mins and at 65°C for 10 mins.**

7 **Pool the barcoded library together and carry forward 480 µl of the library.**

Set-up your own experiment: SARS-CoV-2 WGS

Native barcode ligation

~60 minutes

8 Resuspend the AMPure XP beads by vortexing.

9 Add 192 µl of resuspended AMPure XP beads to the 480 µl of pooled reaction and mix by pipetting.

10 Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

11 Prepare 500 µl of fresh 80% ethanol in nuclease-free water.

12 Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant.

13 Wash the beads by adding 700 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard.

14 Repeat the previous step.

15 Keep the tube on the magnet and wash the beads with 100 µl of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

16 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

Set-up your own experiment: SARS-CoV-2 WGS

Native barcode ligation

~60 minutes

17 **Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

18 **Pellet the beads on a magnet until the eluate is clear and colourless.**

19 **Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

20 **Quantify 1 µl of eluted sample using a Qubit fluorometer - recovery aim 2 ng/µl.**

Set-up your own experiment: SARS-CoV-2 WGS

Adapter ligation and clean-up

~70 minutes

1 **Thaw the Elution Buffer (EB), Short Fragment Buffer (SFB), and NEBNext Quick Ligation Reaction Buffer (5x) at room temperature, mix by vortexing, spin down and place on ice. Check the contents of each tube are clear of any precipitate.**

2 **Spin down the T4 Ligase and the Adapter Mix II (AMII), and place on ice.**

3 **Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.**

Reagent	Volume
Pooled barcoded sample	30 µl
Adapter Mix II (AMII)	5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
Quick T4 DNA Ligase	5 µl
Total	**50 µl**

4 **Mix gently by flicking the tube, and spin down.**

5 **Incubate the reaction for 20 minutes at room temperature.**

6 **Resuspend the AMPure XP beads by vortexing.**

7 **Add 20 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.**

Set-up your own experiment: SARS-CoV-2 WGS

Adapter ligation and clean-up

~70 minutes

8 **Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.**

9 **Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant.**

10 **Wash the beads by adding 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard.**

11 **Repeat the previous step.**

12 **Spin down and place the tube back on the magnet. Pipette off any residual supernatant.**

13 **Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at room temperature.**

14 **Pellet the beads on a magnet until the eluate is clear and colourless.**

15 **Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

- Dispose of the pelleted beads

16 **Quantify 1 µl of eluted sample using a Qubit fluorometer.**

Set-up your own experiment: SARS-CoV-2 WGS

Priming and loading the SpotON flow cell for GridION

~10 minutes

1 **Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice as soon as thawing is complete.**

2 **Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down and return to ice.**

3 **Slide open the GridION lid and insert the flow cell.**

Press down firmly on the flow cell to ensure correct thermal and electrical contact.

4 **Slide the priming port cover clockwise to open the priming port.**

Set-up your own experiment: SARS-CoV-2 WGS

Priming and loading the SpotON flow cell for GridION

~10 minutes

5 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

Visually check that there is continuous buffer from the priming port across the sensor array.

6 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.

7 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.

8 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

9 In a new tube, prepare the library for loading as follows:

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**

Set-up your own experiment: SARS-CoV-2 WGS

Priming and loading the SpotON flow cell for GridION

~10 minutes

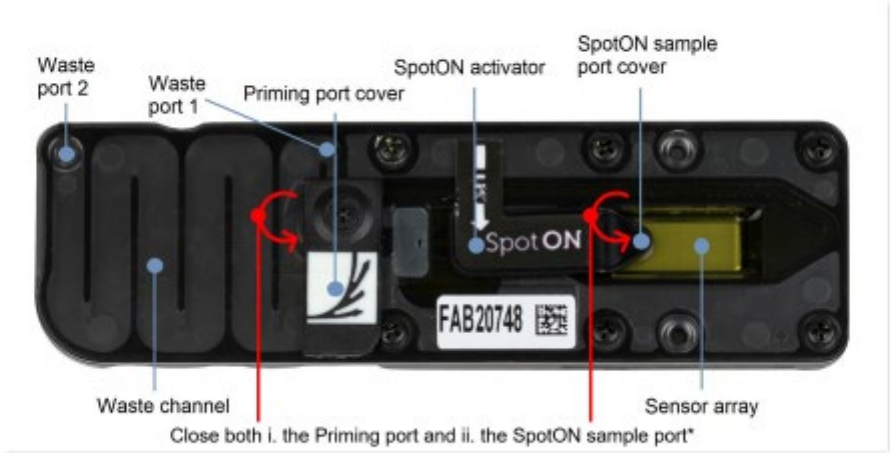
10 Complete the flow cell priming:

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. 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.

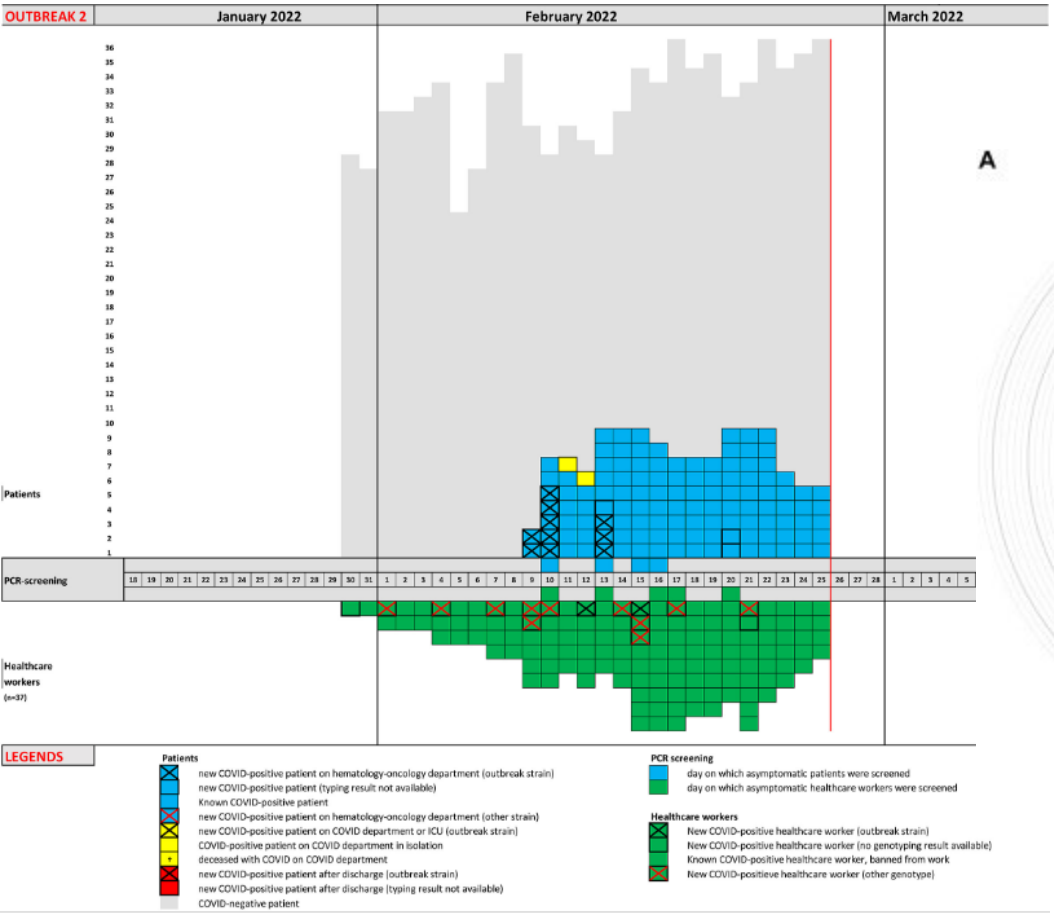
11 Mix the prepared library gently by pipetting up and down just prior to loading.

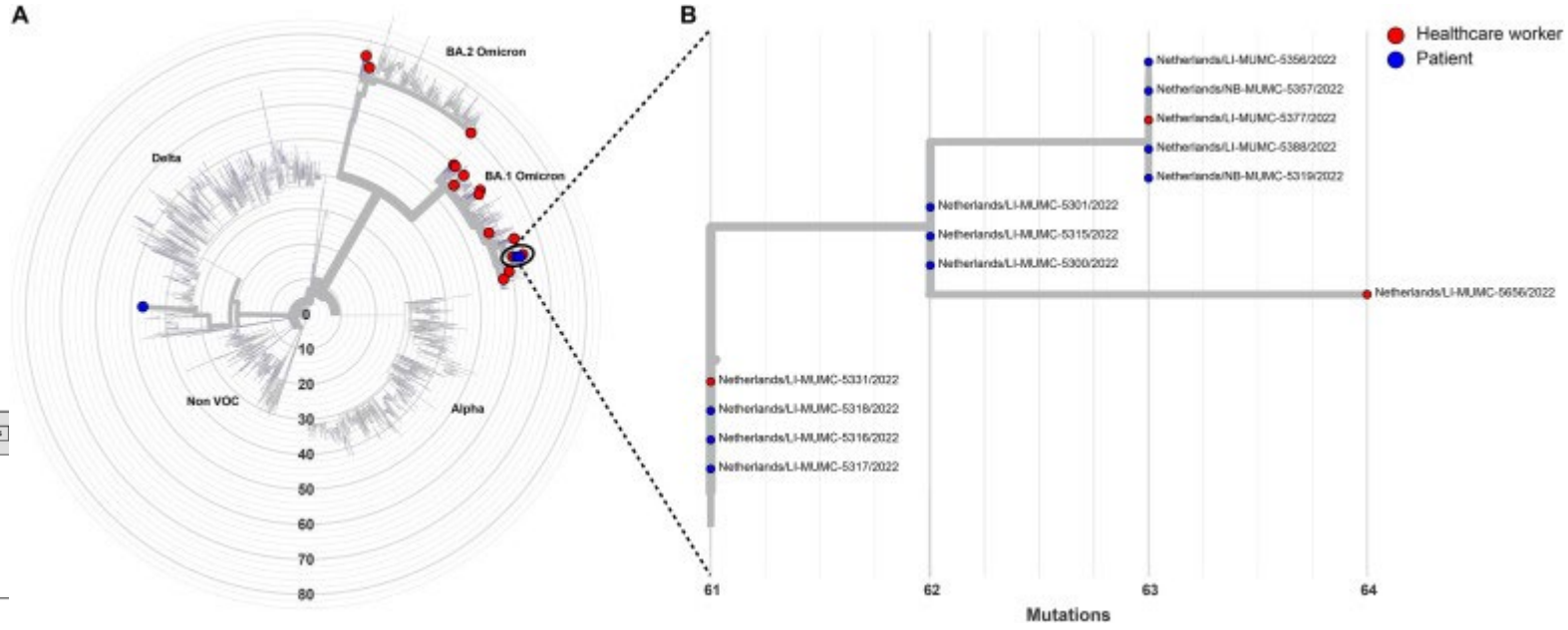
12 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

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



Set-up your own experiment: SARS-CoV-2 WGS





Ref: W.C. van der Zwet, E.A. Klomp-Berens, A.M.P. Demandt, J. Dingemans, B.M.J.W. van der Veer, L.B. van Alphen, J.A.M.C. Dirks, P.H.M. Savelkoul, Analysis of two sequential SARS-CoV-2 outbreaks on a haematology-oncology ward and the role of infection prevention, Infection Prevention in Practice, Volume 6, Issue 1, 2024, 100335,

Set-up your own experiment: rapid barcoding

Library preparation

~50 minutes

1 Program the thermal cycler: 30°C for 2 minutes, then 80°C for 2 minutes.

2 Thaw kit components at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Rapid Barcodes (RB01-24) or Rapid Barcode Plate (RB01-96)	Not frozen	✓	✓
Rapid Adapter (RA)	Not frozen	✓	✓
AMPure XP Beads (AXP)	✓	✓	Mix by pipetting or vortexing immediately before use
Elution Buffer (EB)	✓	✓	✓
Adapter Buffer (ADB)	✓	✓	Mix by vortexing

3 Prepare the DNA in nuclease-free water.

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with nuclease-free water.
3. Pipette mix the tubes for 10-15 times to avoid unwanted shearing
4. Spin down briefly in a microfuge

Set-up your own experiment: rapid barcoding

Library preparation

~50 minutes

3 Prepare the DNA in nuclease-free water.

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with nuclease-free water.
3. Pipette mix the tubes for 10-15 times to avoid unwanted shearing
4. Spin down briefly in a microfuge

4 In the 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind, mix the following:

Reagent	Volume per sample
Template DNA (200 ng from previous step)	10 µl
Rapid Barcodes (RB01-24 or RB01-96, one for each sample)	1.5 µl
Total	**11.5 µl**

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

6 Incubate the tubes or plate at 30°C for 2 minutes and then at 80°C for 2 minutes. Briefly put the tubes or plate on ice to cool.

7 Spin down the tubes or plate to collect the liquid at the bottom.

Set-up your own experiment: rapid barcoding

Library preparation

~50 minutes

8 **Pool all barcoded samples in a clean 2 ml Eppendorf DNA LoBind tube, noting the total volume.**

	Volume per sample	For 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Total volume	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,104 µl

9 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

10 **To the entire pooled barcoded sample, add an equal volume of resuspended AMPure XP Beads (AXP) and mix by flicking the tube.**

	Volume per sample	For 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Volume of AMPure XP Beads (AXP) added	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,000 µl

11 **Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.**

12 **Prepare at least 2 ml of fresh 80% ethanol in nuclease-free water.**

13 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**

14 **Keep the tube on the magnet and wash the beads with 1 ml of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

15 **Repeat the previous step.**

Set-up your own experiment: rapid barcoding

Library preparation

~50 minutes

16 Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

17 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB) per 24 barcodes used.

	For 24 barcodes	For 48 barcodes	For 96 barcodes
Volume of Elution Buffer (EB)	15 µl	30 µl	60 µl

18 Incubate for 10 minutes at room temperature.

19 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

20 Remove and retain the full volume of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

- Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
- Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

21 Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

Set-up your own experiment: rapid barcoding

Library preparation

~50 minutes

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

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

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

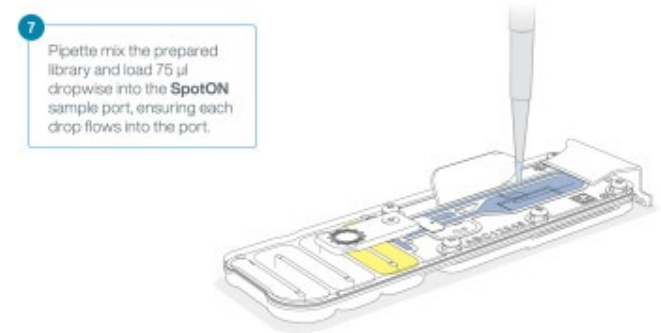
24 Mix gently by flicking the tube, and spin down.

25 Incubate the reaction for 5 minutes at room temperature.

Tip: While this incubation step is taking place you can proceed to the Flow Cell priming and loading section of the protocol.

Priming and loading the MinION and GridION Flow Cell

~10 minutes

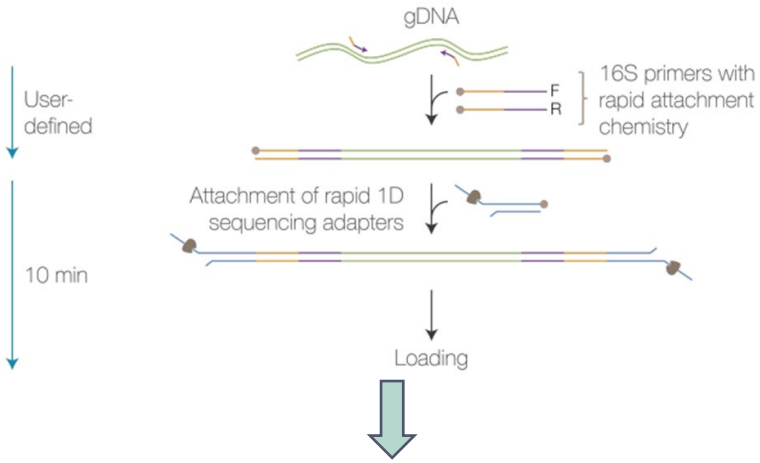


Set-up your own experiment: 16S sequencing

16S NGS on clinical samples

Sample spiking (ZymoBIOMICS Spike-in Control II (Low Microbial Load) & ZymoBIOMICS DNA Miniprep Kit)

16S library prep & sequencing (Oxford Nanopore)



<8 hours

Data analysis (EPI2ME 16S workflow (ONT) vs Species ID 16S tool 1928 Diagnostics)

Set-up your own experiment: 16S sequencing

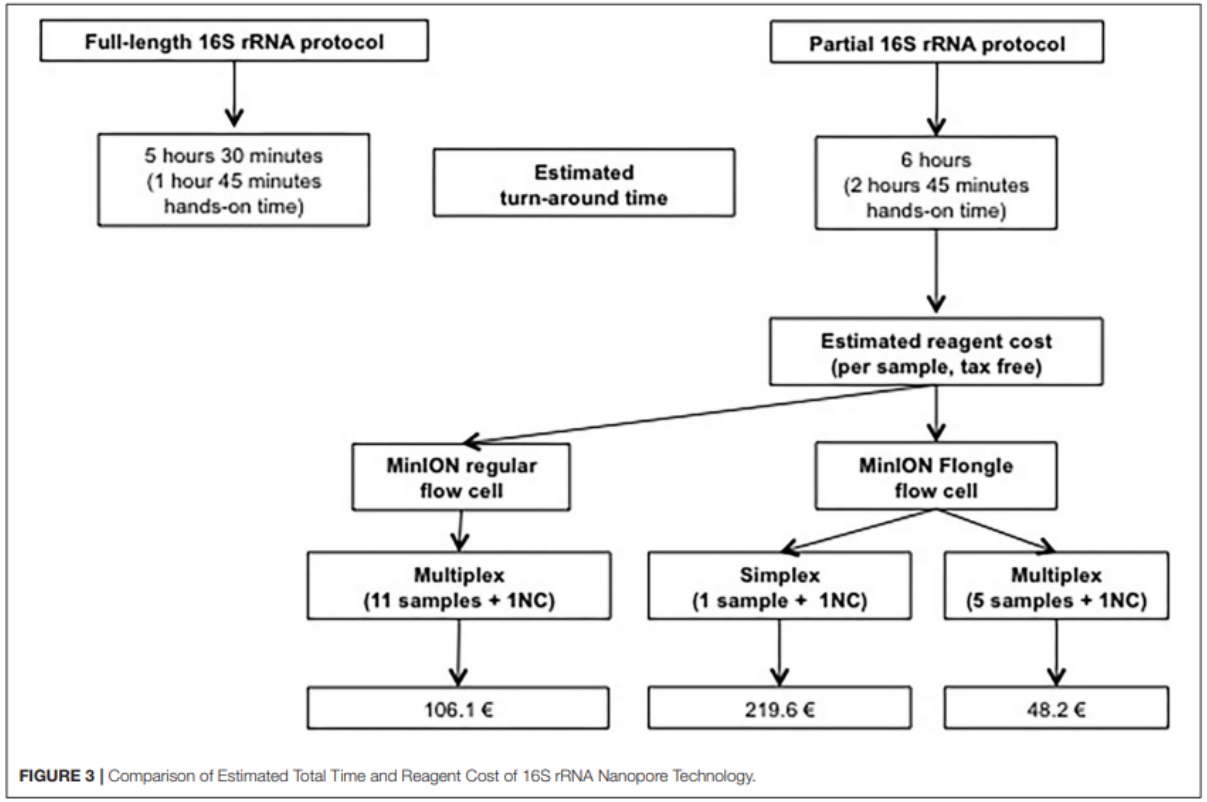


FIGURE 3 | Comparison of Estimated Total Time and Reagent Cost of 16S rRNA Nanopore Technology.

Ref: Bouchiat *et al*. Improving the Diagnosis of Bacterial Infections: Evaluation of 16S rRNA Nanopore Metagenomics in Culture-Negative Samples. Front Microbiol. 2022 Jul 14;13:943441. doi: 10.3389/fmicb.2022.943441.

	No barcodes	12 barcodes	24 barcodes	24 barcodes (Flongle)
Flow cell price	$500	$500	$500	$90 (Flongle flow cell)
Library price	$90	$120	$150	$150
Price per sample	**$590**	**$51.67**	**$27.08**	**$10**

Acknowledgements

- Thank you for your attention!
- Thanks to all laboratory staff and collaborators at MUMC+ & Jessa!
- Thanks to the organization for the invitation!

Questions?