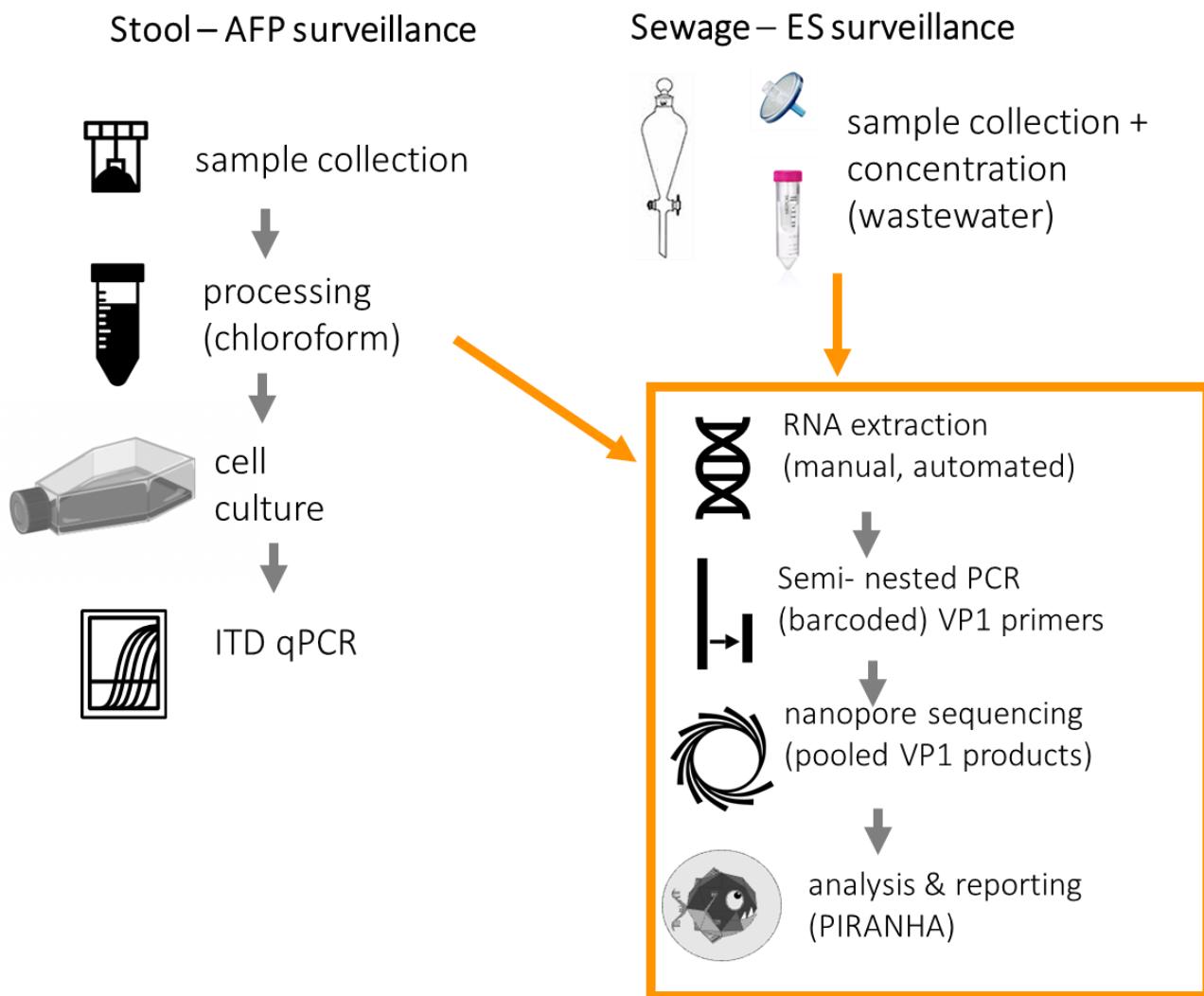


1. Overview of Direct molecular Detection and Nanopore Sequencing (DDNS) for Poliovirus

Presentation and discussion

Poliovirus Direct Detection and Nanopore Sequencing (DDNS)



PoSeCo | Poliovirus Sequencing Consortium

piranha | Poliovirus Investigation Res

sample08 report 2022-09-12

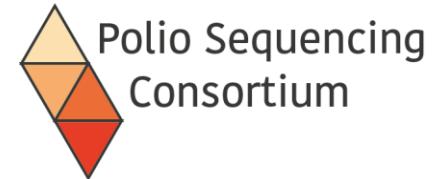
Table 1 | Summary of sample content

Sample	Barcode	Reference Group
sample08	barcode33	Sabin1-related
sample08	barcode33	Sabin3-related

VP1 sequences

<http://polionanopore.org>

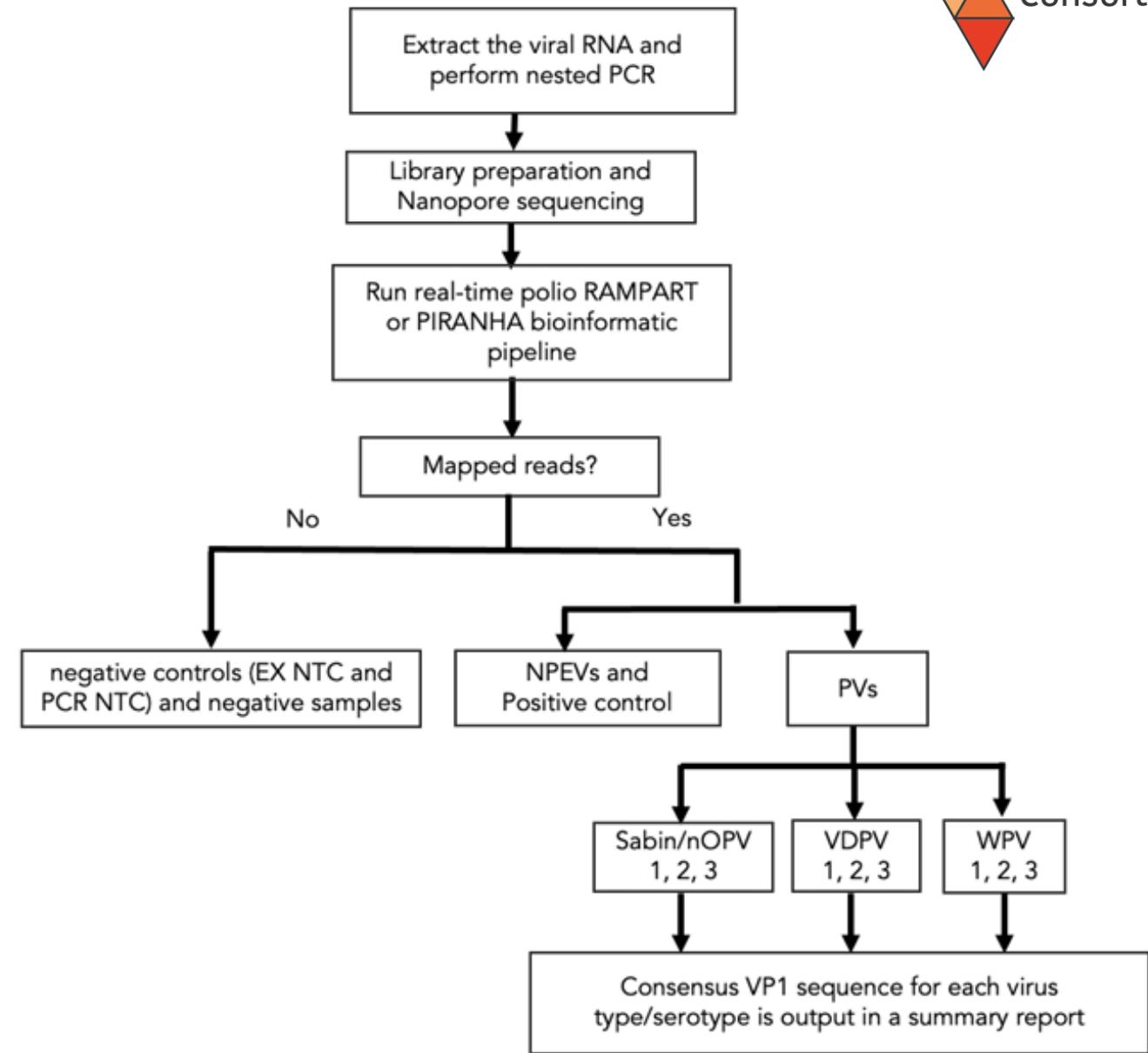
Advantages of poliovirus direct detection by nanopore sequencing



- Protocol detects and generates a VP1 sequence for poliovirus in stool samples 2-3 days after arrival in the laboratory
- Non-inferior sensitivity and specificity compared with cell-culture
- Accurate consensus sequences for every poliovirus detected
- Faster detection of outbreaks
- Cost-effective at ~\$15 per sample assuming >90 samples/week
- Ongoing training in GPLN labs to implement method and generate further data on performance and costs -> accepted/recommended by GPLN SWG



Diagnostic algorithm



<http://polionanopore.org>

- Updated protocols maintained on protocols.io – can be reached through
<http://polionanopore.org>

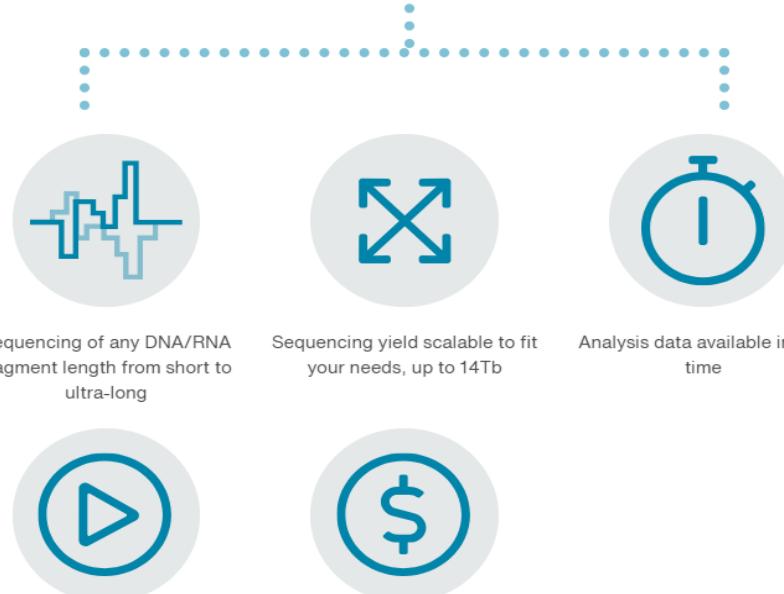


Free, open source protocols and software for poliovirus detection and sequencing using nanopore.

You can find all you need here for direct detection and nanopore sequencing (DDNS) of poliovirus from stool and environmental samples. Helping you detect and respond to poliovirus outbreaks faster. Funded by the Bill and Melinda Gates Foundation.

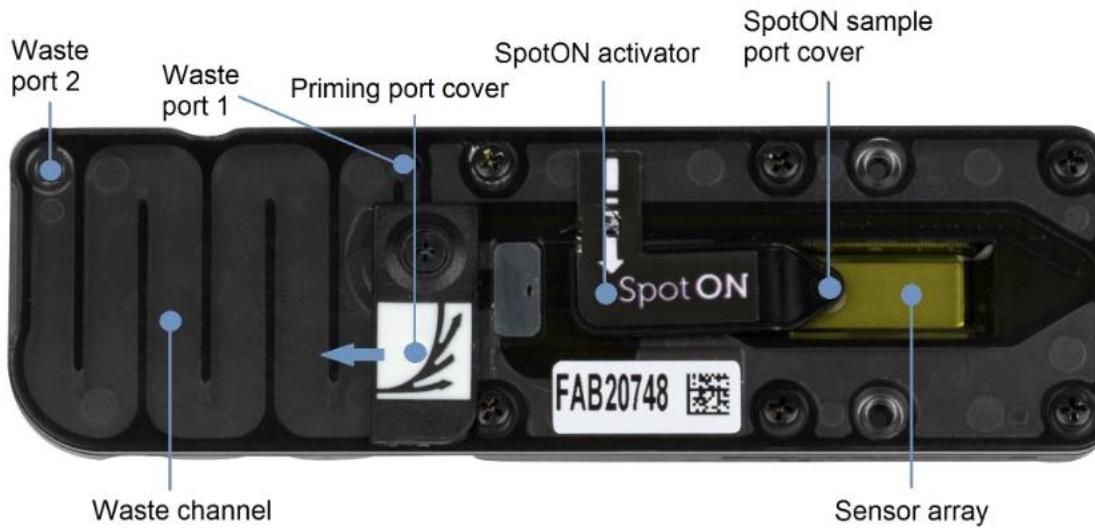


Nanopore (MinION) Sequencing



- It provides a direct electrical analysis of the target molecule
- Unrestricted read length: from short (20b) to ultra long reads (>4Mb reads)
- Fast- 1,000,000 reads per hour and can be processed in real time
- Raw read error rate per base was ~5% - new chemistry offers accuracy >99.0%
- Variant calling and consensus accuracy dependent on sample quality and analysis tools
- “Cheap” (depending on multiplexing)

MinION Flow cell



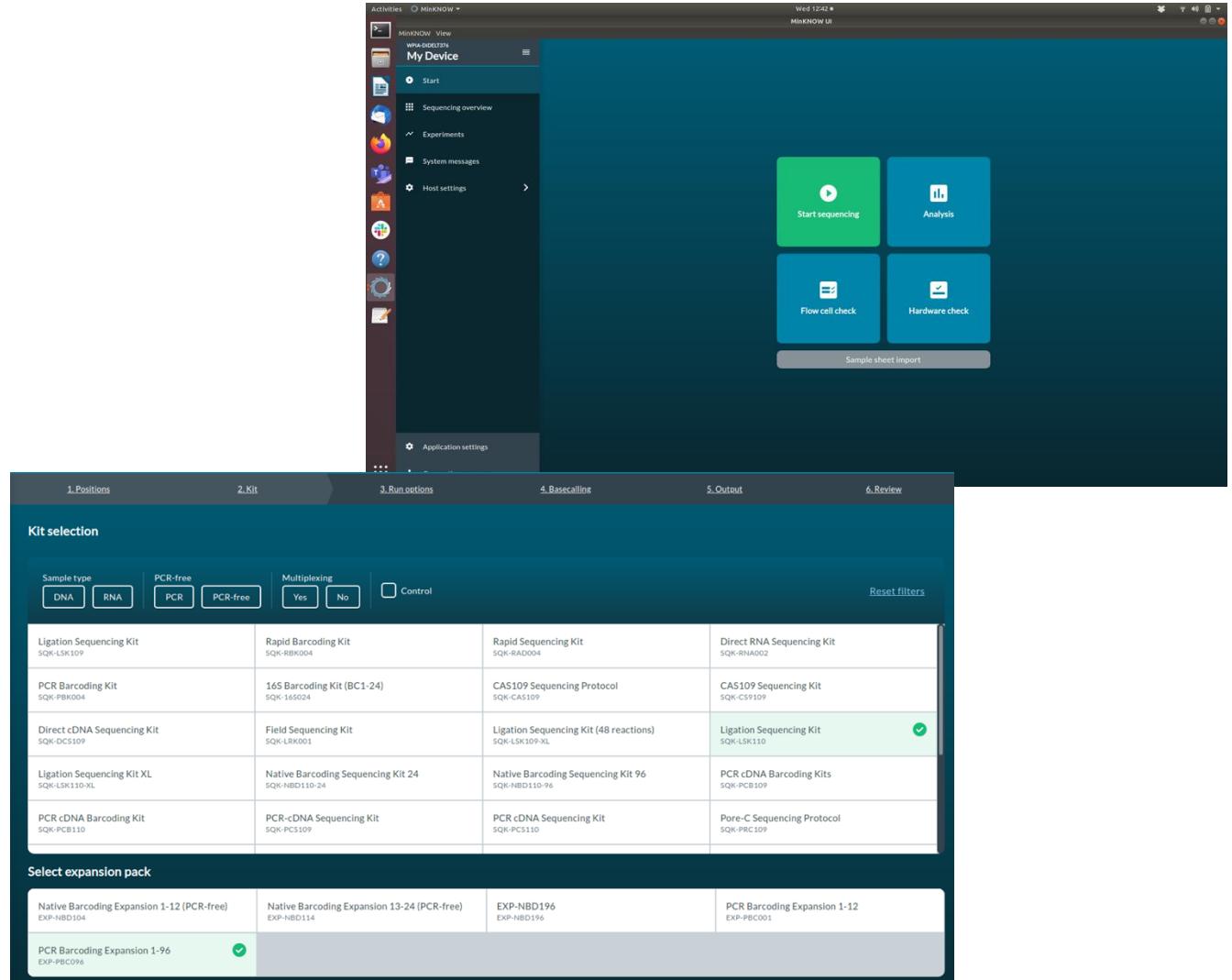
In this picture both the priming and sample loading ports are closed.



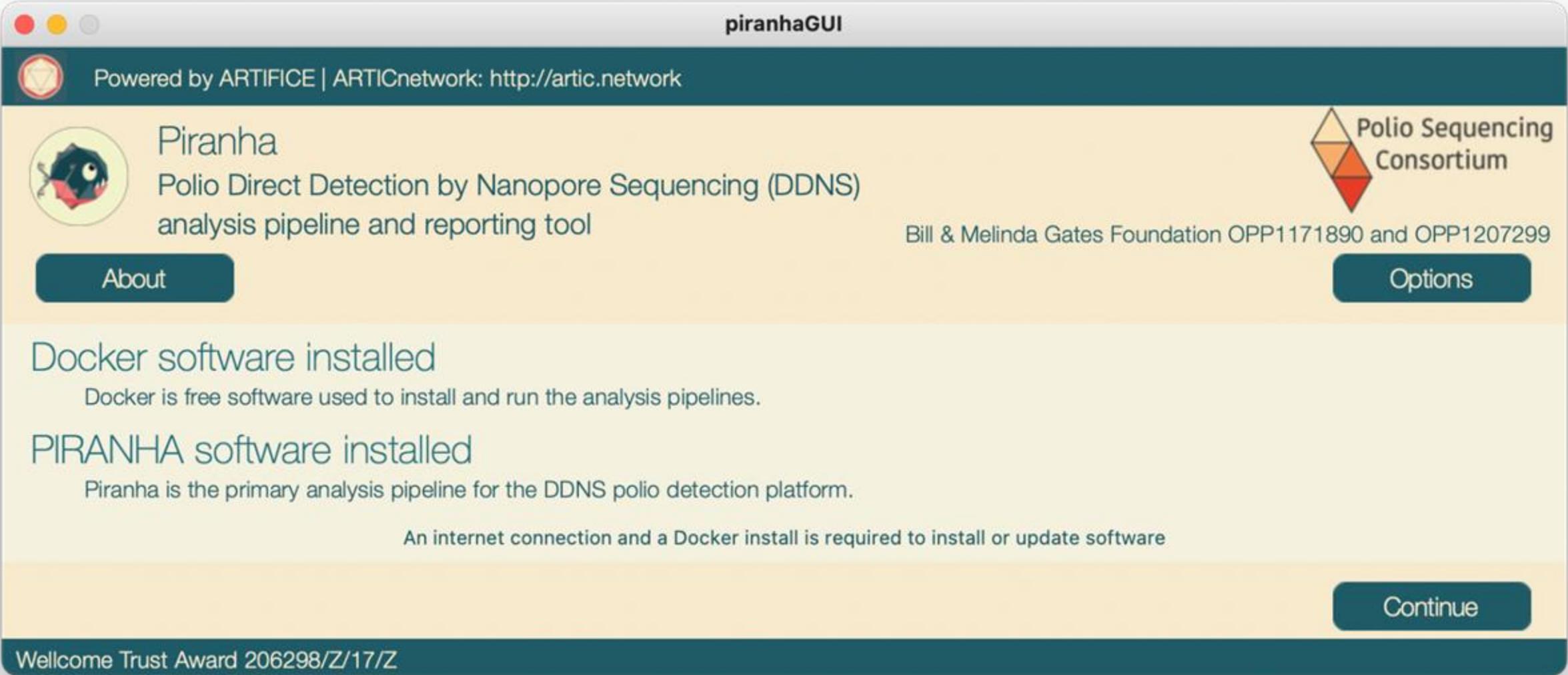
- Has sufficient buffer to run for up to 72 hours
- Can generate up to 50Gb data
- Can be stopped, restarted, reloaded
- Can be washed and reused until nanopores are exhausted
- Can be stored at RT or refrigerated
- Costs £400-650
- Should eventually be returned to ONT for partial recycling

MinKNOW – nanopore software

- Easy to use GUI for setting up and running your sequencing
- Install following the instructions on the ONT website
- Live basecalling and demultiplexing of reads



Data analysis - PIRANHA



piranhaGUI

Powered by ARTIFICE | ARTICnetwork: <http://artic.network>

 Piranha
Polio Direct Detection by Nanopore Sequencing (DDNS)
analysis pipeline and reporting tool

Bill & Melinda Gates Foundation OPP1171890 and OPP1207299

About Options

Docker software installed
Docker is free software used to install and run the analysis pipelines.

PIRANHA software installed
Piranha is the primary analysis pipeline for the DDNS polio detection platform.

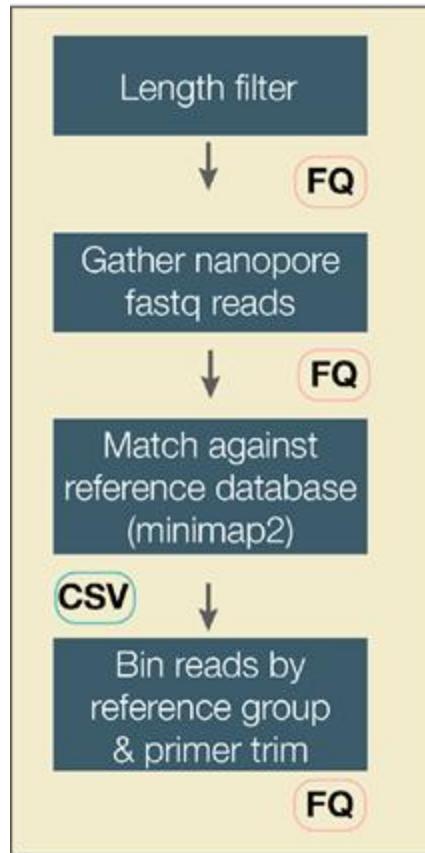
An internet connection and a Docker install is required to install or update software

Continue

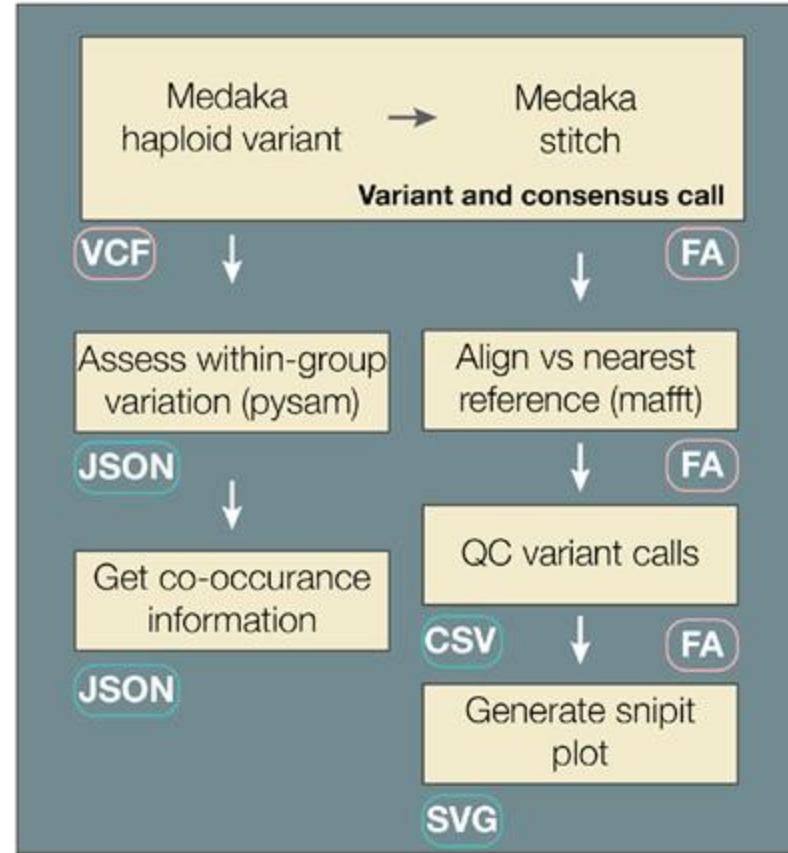
Wellcome Trust Award 206298/Z/17/Z

Workflow schema

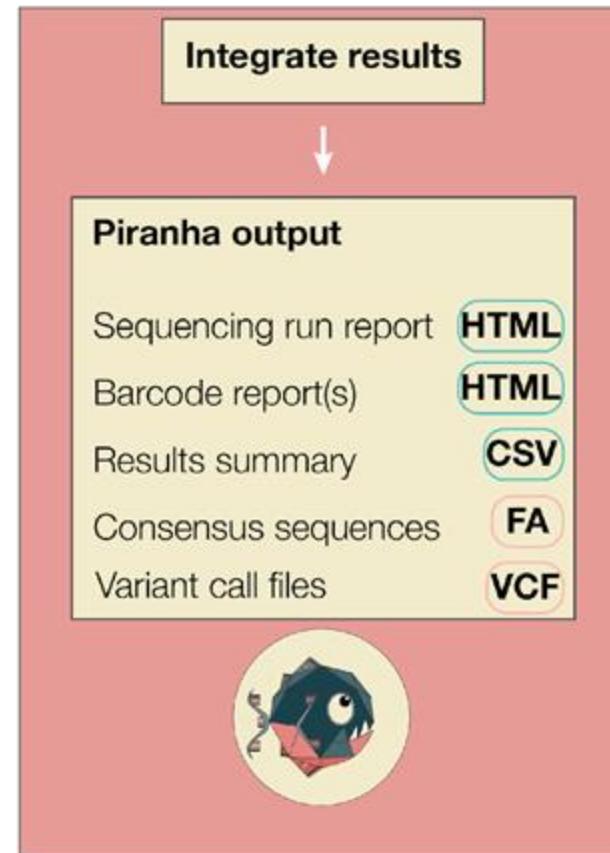
A. Barcode analysis



B. Reference group analysis



C. Results reporting





PIRANHA Outputs- Report



Sample	Barcode	Sample Call	Reference Group	Number Of Mutations	VP1 sequence
sample01	barcode25	Sabin-like	Sabin3-related	1	Download FASTA
sample02	barcode26	Sabin-like	Sabin1-related	0	Download FASTA
sample02	barcode26	Sabin-like	Sabin3-related	1	Download FASTA
sample02	barcode26	Sabin-like	Sabin2-related	4	Download FASTA
sample03	barcode27	VDPV	Sabin2-related	106	Download FASTA
sample07	barcode32	Sabin-like	Sabin3-related	2	Download FASTA
sample07	barcode32	Sabin-like	Sabin2-related	0	Download FASTA
sample08	barcode33	Sabin-like	Sabin1-related	1	Download FASTA
sample08	barcode33	Sabin-like	Sabin3-related	1	Download FASTA
sample09	barcode34	Sabin-like	Sabin1-related	0	Download FASTA
sample09	barcode34	Sabin-like	Sabin3-related	1	Download FASTA
sample09	barcode34	Sabin-like	Sabin2-related	2	Download FASTA
sample11	barcode36	VDPV	Sabin2-related	126	Download FASTA
sample12	barcode37	WPV2	WPV2	NA	Download FASTA
sample12	barcode37	NonPolioEV	NonPolioEV	NA	Download FASTA
sample12	barcode37	VDPV	Sabin2-related	135	Download FASTA
sample13	barcode38	WPV2	WPV2	NA	Download FASTA
sample13	barcode38	VDPV	Sabin2-related	142	Download FASTA

Table 2 | Composition of samples

Sample	Barcode	Sabin1-Related	Sabin2-Related	Sabin3-Related	Wpv1	Wpv2	Wpv3	Nonpolioev	Unmapped
neg1	barcode31	0	0	0	0	0	0	0	0
neg2	barcode39	0	0	0	0	0	0	0	0
sample01	barcode25	144	0	2408	3	0	0	1	0
sample02	barcode26	200	499	1003	0	0	0	0	0
sample03	barcode27	0	2803	1	1	221	0	0	1
sample04	barcode28	0	0	0	0	0	0	0	0
sample05	barcode29	0	0	0	0	0	0	0	0
sample06	barcode30	0	0	0	0	0	0	0	0
sample07	barcode32	0	1133	1367	71	0	0	200	1
sample08	barcode33	445	1	2800	0	0	0	7	1
sample09	barcode34	558	436	1696	2	0	0	54	1
sample10	barcode35	0	0	0	0	0	0	0	0
sample11	barcode36	0	68	3	0	0	0	0	1
sample12	barcode37	n	4 13.4	4	0	14 00	n	2777	4

sample08 report 2022-09-12

Table 1 | Summary of sample content

Sample	Barcode	Reference Group
sample08	barcode33	Sabin1-related
sample08	barcode33	Sabin3-related

VP1 sequences

```
>sample08|barcode33|Sabin1-related|Poliovirus1-Sabin_AY184219|1|268:AT
GGGTTAGGTCAAGATGCTTGAAGCATGATTGACAACACAGTCGGTAAACGGTGGGGCGGCAACGTCAGAGCCTCTCCAAACACTGAAGCCAGTGACAGCACACTCCAAGGAAATCCGCAC
AAATCCACTAGTCCTCTGTACAGTGCAAACACAGACATGGTCAACATAGGTCAAGGTCAGAGGCTAGCATAGAGTCTTCTCGGGGGGGTGCATGGTGGGATAACTCAGCTCCACCAAGAA
TATTACAGTGTGGAAAGATCACTTAAAGATACTGTCCAGTTACGGAGGAATTGGAGTTCTCACCTATTCTAGATTGGATATGGAAATTACCTTGTGGTTACTGCAAATTTC
ACTGAGACTAACATGGCATGCCCTAAATCAAGTGTACAAATTGCTTACCTACGGAAACGCTCCAGGCCGATCTCGGTACCGTATGGTGGTATTTCGAACGCTTATTCA
CAAATTATGTACGTACCACCAAGGCGCTCAGTGCCCAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCTTACCTACGGAAACGCTCCAGGCCGATCTCGGTACCGTATGGTGGT
ATTTCGAACGCTTATTCACTGAGACTAACATGGCATGCCCTAAATCAAGTGTACAAATTGCTTACCTACGGAAACGCTCCAGGCCGATCTCGGTACCGTATGGTGGTATTTCGAACGCTTATTCA
CTTACGACGGTTTCCAAAGTACCACTGAAGGACCAAGTCGGCAGCACTAGGTGACTCCCTCATGGTGGCATCTAAATGACTTCGTTAGAGTAGTCAATGATCACAAACCCGACCAAGGTCA
CTCCAAATCAGAG
```

[Download](#)

QA/QC

- Careful QA/QC essential (clean PCR, data management)
- QC guidelines available and QA programme under development
- SOPs include recommended best practices for preventing and detecting cross contamination, minimising sample handling
- Positive control (lyophilized CV-A20 standard prepared by NIBSC) and negative (water) control included in each sample batch from RNA extraction step
- Sequencing accuracy can be confirmed by testing reference panel and repeats/comparison with alternative sequencing platforms in a subset
- Long term monitoring of lab performance (including NPEV detection) and proficiency testing
- Accreditation of GPLN laboratories for DDNS will follow a similar process as for virus isolation, ITD, sequencing and environmental surveillance based on minimum requirements checklist

Expected Outcomes

- Be able to perform the DDNS protocol including RNA extraction, nested PCR, barcode file preparation, nanopore sequencing, report generation by PIRANHA
- Understand the QC/QA process
- Be ready to implement in your own laboratories with GPLN and PSC support
- Appreciate the other potential applications of poliovirus nanopore sequencing including generation of whole genomes

Post training expectations

- Trainees should identify any additional equipment their lab would require to perform DDNS
- Trainees should identify the areas in their lab where the steps of the DDNS protocol could be performed
- Trainers should explain the DDNS method to their other lab members
- Trainees should liaise with the WHO for procurement of required equipment and reagents for DDNS implementation in their lab
- Trainees should provide a point of contact for the PSC to provide additional reagents (e.g. the positive control virus)
- Further training should be arranged to support implementation of DDNS in their own laboratory

Acknowledgments

Study team

Imperial College London: Alex Shaw, Catherine Troman, Joyce Akello, Shannon Fitz, Ben Bellekom, Nicholas Grassly

NIBSC/MHRA: Erika Bujaki, Kafayat Arowolo, Victory Poloamina, Manasi Majumdar, Javier Martin

NIH Islamabad: Adnan Khurshid, Yasir Arshad, Masroor Alam

University of Edinburgh: Áine O'Toole, Rachel Colquhoun, Corey Ansley, Zoe Vallance, Andrew Rambaut

INRB DRC: Tresor Kabeya, Emmanuel Lokilo, Yogolelo Riziki, Amuri Aziza, Eddy Lusamaki, Jean-Claude Makangara, Marceline Alonga, Yvonne Lay, Bibiche Nsunda, Elisabeth Pukuta, Steve Ahuka, Jean-Jacques Muyembe, Placide Mbala

Biosurv International: Catherine Pratt, Kirsten Williamson, Shean Mobed

Collaboration and funding

BMGF: Ananda Bandyopadhyay, Kathleen Rankin, Corey Peak and colleagues

WHO HQ: GPLN SWG, Surveillance Group

WHO regional offices: Anfumbom Kitu Womeyi Kfutwah (Jude) (AFRO), Salmaan Sharif (EMRO), Eugene Saxentoff (EURO), Lucky Sangal (SEARO)

2. Sequencing Run preparation, RNA extraction and RT-PCR, semi-nested PCR, Library preparation and flow cell loading

Presentation and discussion

Planning a run

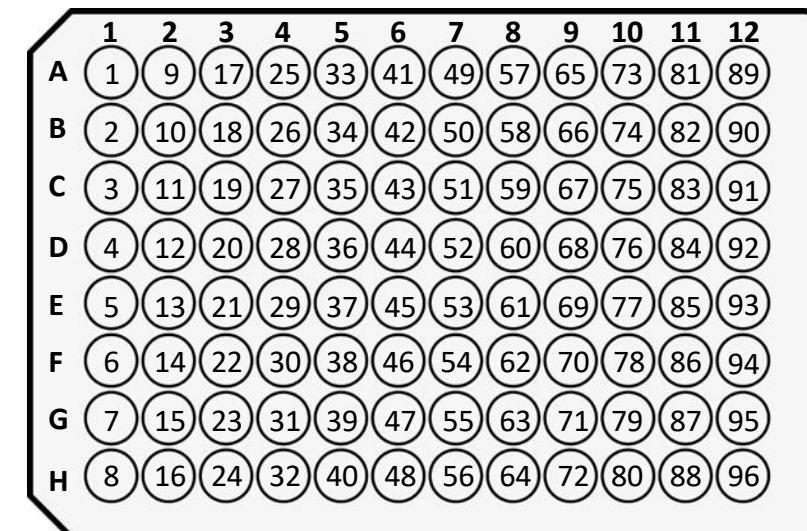
- The protocol requires 2 days once RNA is extracted
- Can process up to 96 samples (including negatives) by barcoding samples
- Negative and positive run controls should be included in every run
- Samples are pooled to allow simultaneous sequencing
- Samples only tested once, contamination likely to be identified through identical sequences for non-vaccine strains.

Run preparation

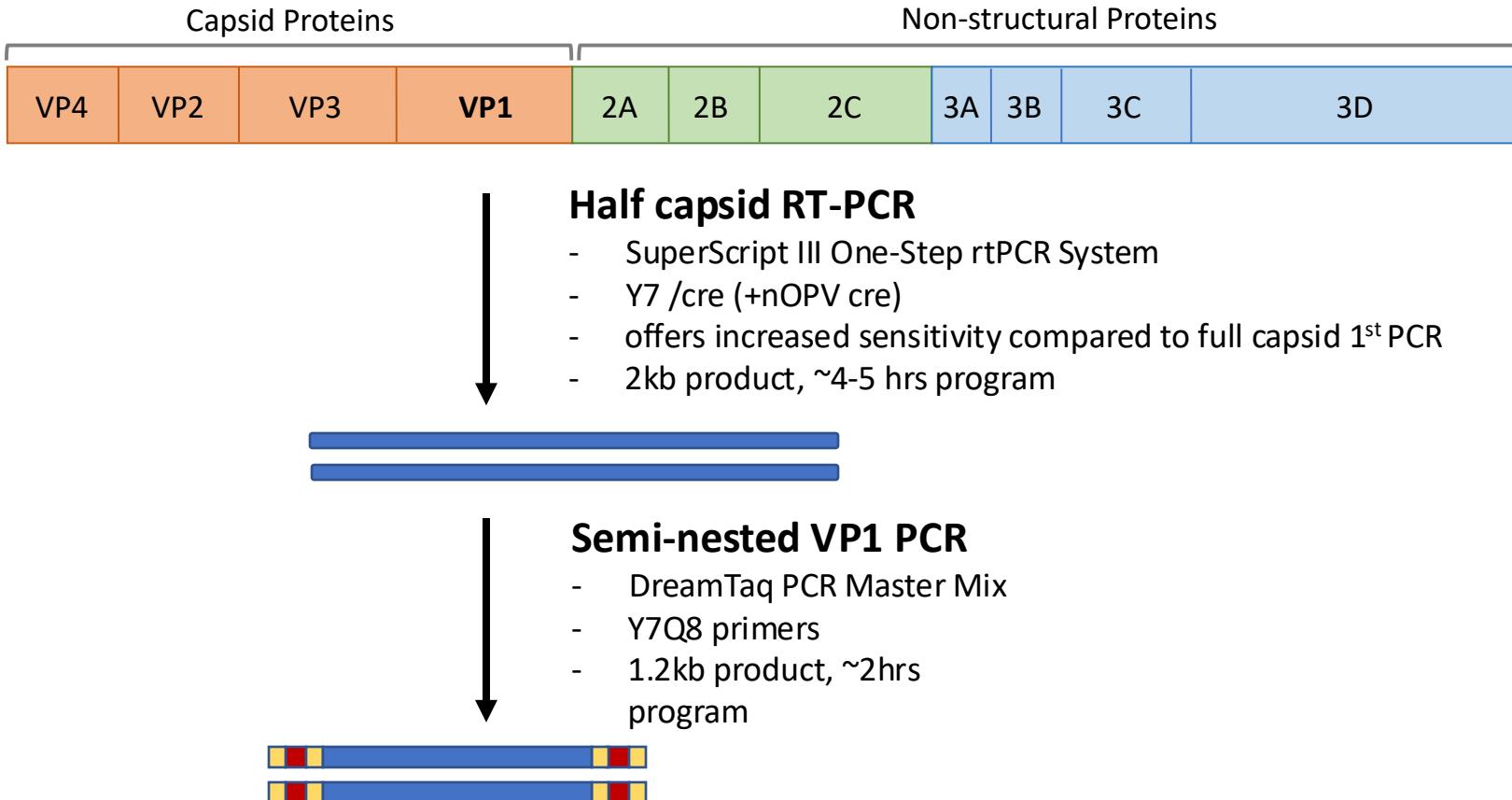
- All sample metadata can be entered into a csv file and each sample assigned a barcode.
- The analysis software (PIRANHA) will append the sequencing results to each sample.

	A	B	C	D	E
1	barcode	sample	EPID	institute	...
2	barcode01	sample01	ARA-HIG-TOR-22-01		
3	barcode02	sample02	ARA-HIG-TOR-22-01		
4	barcode03	sample03	ARA-KAN-ERF-22-01		
5	barcode04	sample04	ARA-KAN-ERF-22-01		
6	barcode05	sample05	ARA-HIG-TOR-22-02		

The barcoded primers are stored in the format shown below, each number being a unique barcoded sequence attached to both Q8 and Y7

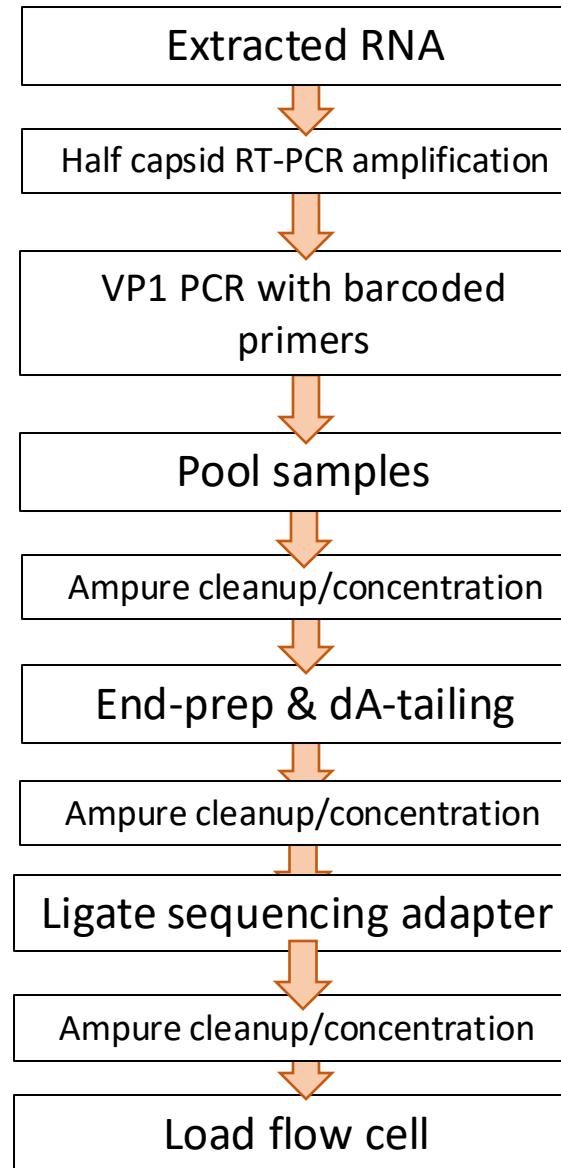


Our PCR strategy in direct detection



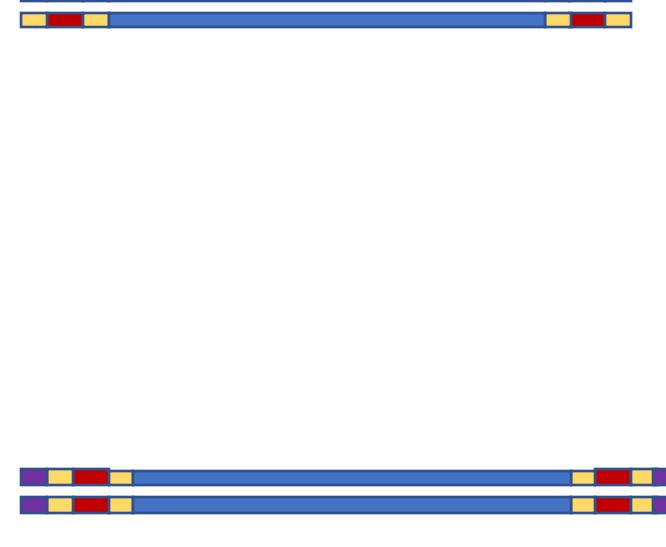
Library preparation overview – stool DDNS

Part 1:
RNA
extraction,
RT-PCR
amplification



Part 2:
Pooling,
cleaning and
end-prep

Part 3:
Preparation
for
sequencing

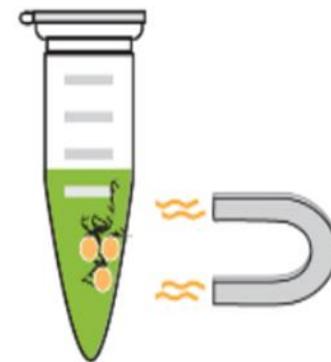


Run controls

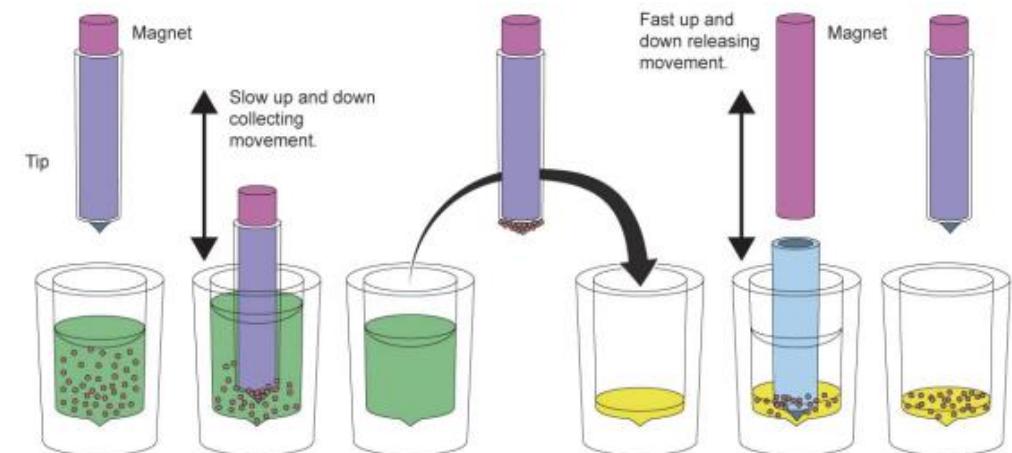
- Both negative and positive controls go through the whole process from RNA extraction to sequencing
- Negative run control: Nuclease free water used for RNA extraction
- Positive run control: CVA20 formulated by NIBSC, distributed freeze-dried and known to amplify in both PCR steps
- Instructions for use of the positive control are provided with the vials
- The controls demonstrate if the required sensitivity and specificity of the method is achieved
- They confirm negative or positive signals and can indicate if any issues arise during processing steps

RNA extraction

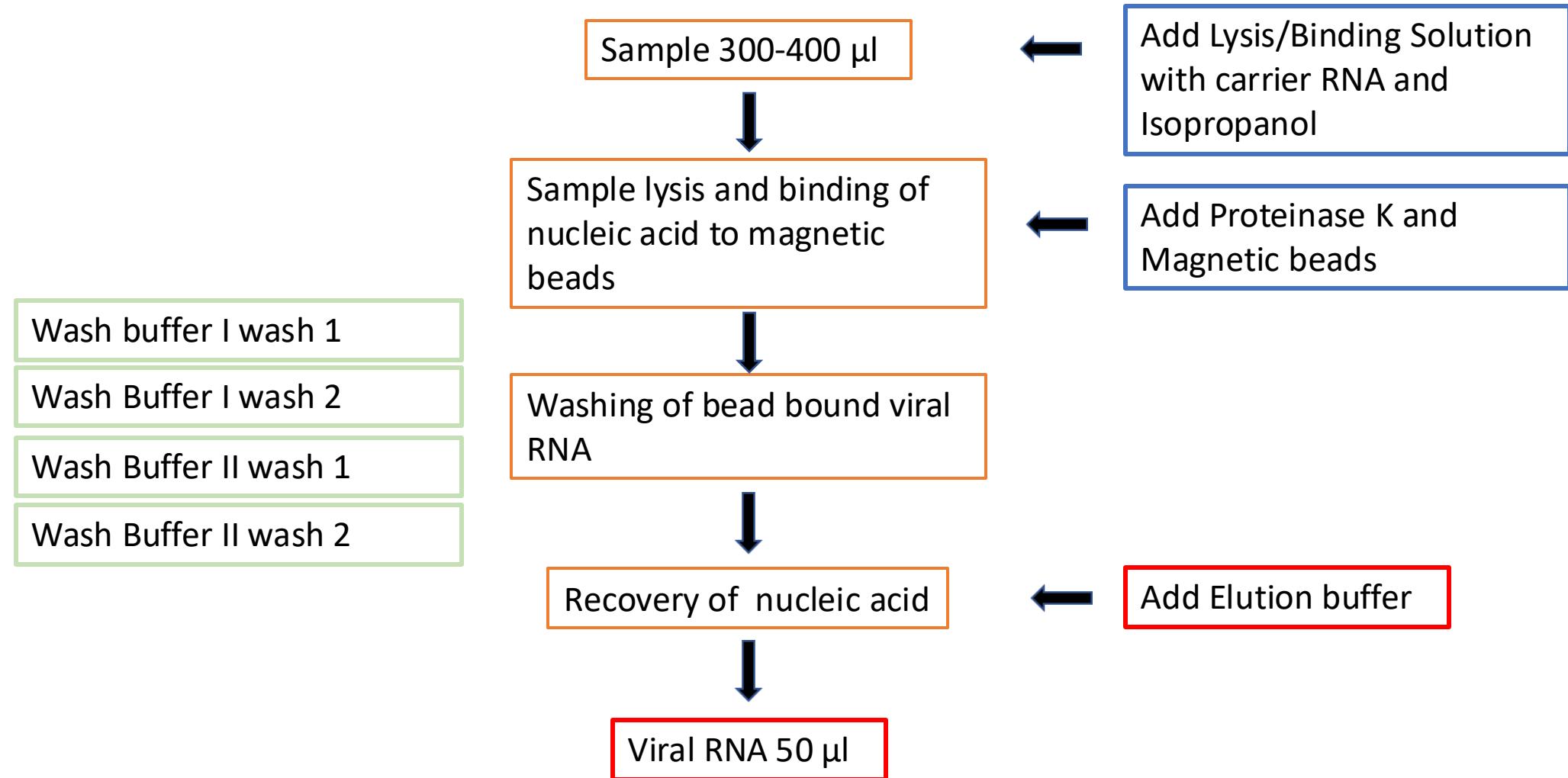
- Recommended kit: MagMAX Viral RNA Isolation kit cat no: AM1939
- Magnetic bead based method
- Can be performed manually in microcentrifuge tubes or in deep-well plates using KingFisher equipment



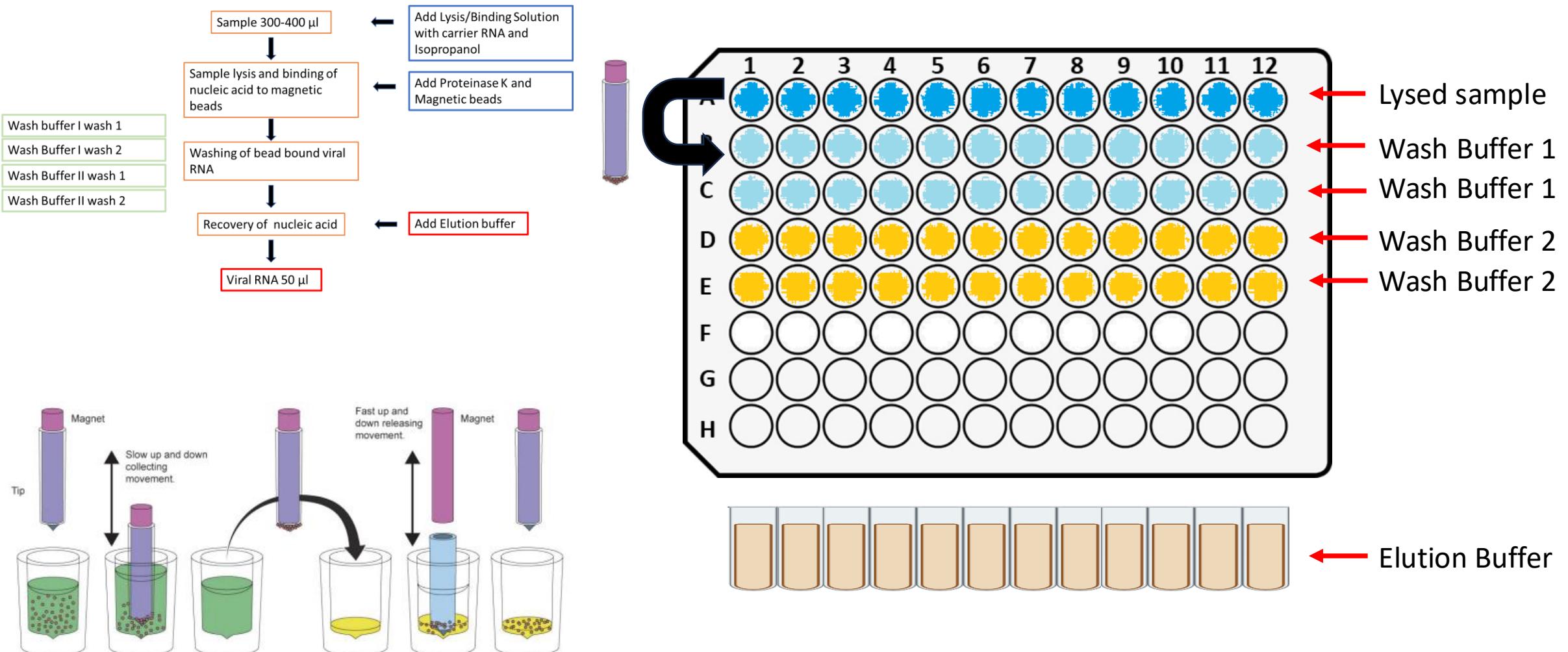
or



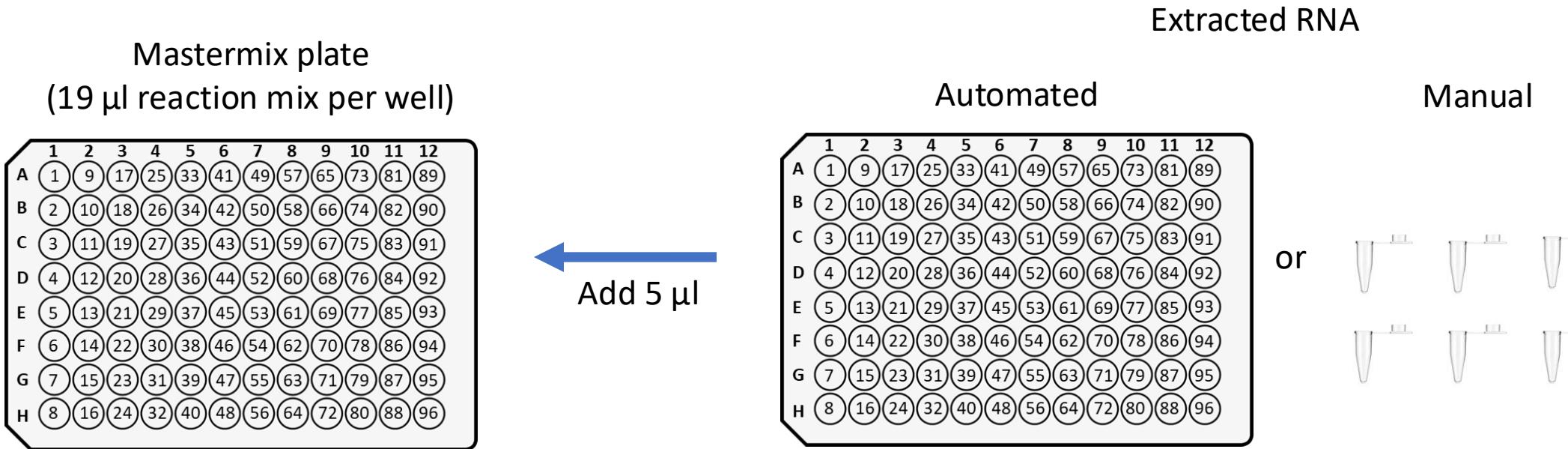
RNA extraction - MagMAX Viral RNA Isolation Kit



RNA extraction - Automated extraction (Kingfisher Duo)



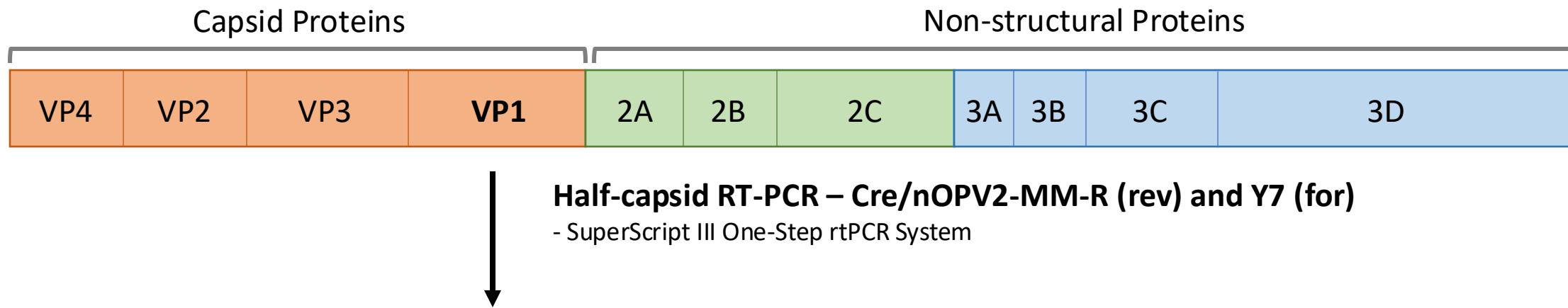
Setting up the half-capsid RT-PCR



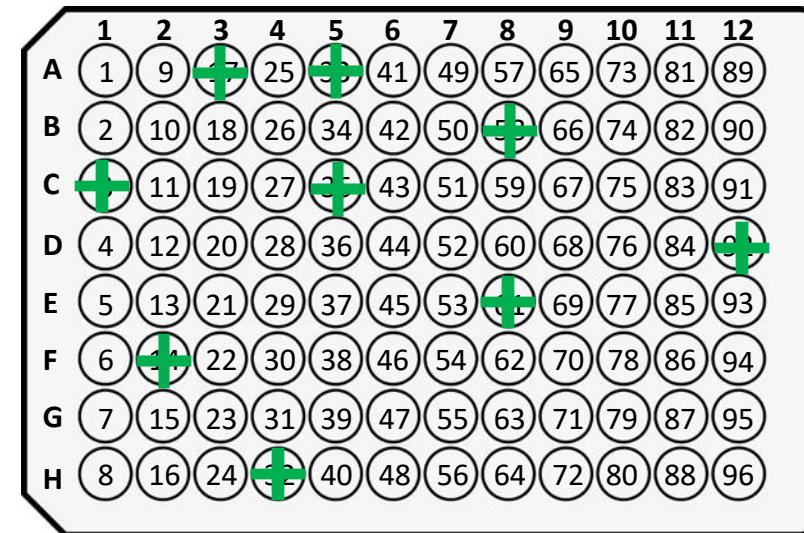
	1 Reaction (µL)
2x Master Mix	12.5
SSIII Platinum Taq mix	1
Reverse Primer (10 µM, Cre/nOPV2-MM-R mix)	1
Nuclease free Water	4.5
Total	19

- 30 minutes of incubation at 50 °C (reverse transcription)
- Add 1 µL of 10µM forward primer (Y7) for the PCR

Half-capsid RT-PCR



- Can run a gel to confirm, expect a band around 2kb.
 - Only indicates the presence of an enterovirus.
 - We do not run this gel routinely
 - We do not exclude samples at this stage



Semi-nested VP1 PCR

- Performed with Dreamtaq
- Uses 2 µl of each of the 1st PCR reaction (no cleaning required)
- Primers Q8 and Y7 for poliovirus specific amplification of VP1
- Primers have a barcode to allow multiplexing of samples; each sample is assigned a barcode, which is the same in each of the primers.
- Barcoded Q8 and Y7 primers can be organised in a 96 well plate for ease of addition.
- Duration: 2 hrs for PCR

A	1	2	3	4	5	6	7	8	9	10	11	12
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

VP1 PCR primers

Flanking sequence

Y7 GGTGCTGACCGAGATCCTACGAATGGAGTGT^{TTAACCTGGGTTGTGTCAGCCTGTAATGA}

Barcode sequence

Primer sequence

TACACCTRTCTGGAGAA^{TCCAATTACCGAGATCCTACGAATGGAGTGTGTCGTGG} Q8

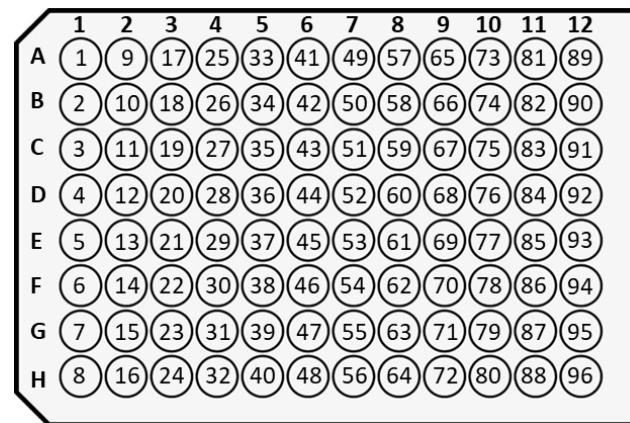
Primer sequence

Barcode sequence

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

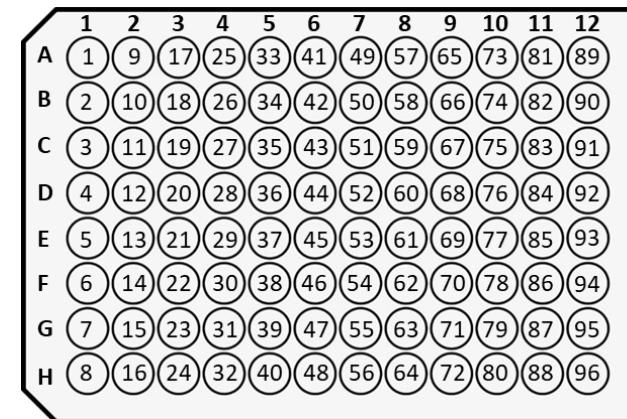
Setting up the semi-nested VP1 PCR

Mastermix plate
(21 µl reaction mix per well)



	1 Reaction (µL)
Water	8.5
DreamTaq 2x master mix	12.5
Total volume	21

Q8/Y7 barcoded primer plate (10µM)



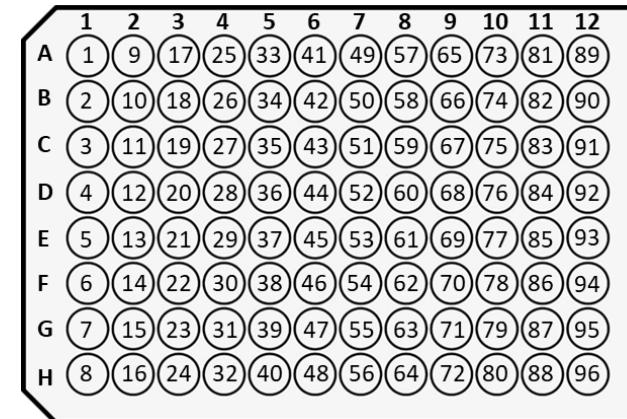
Add 2 µl



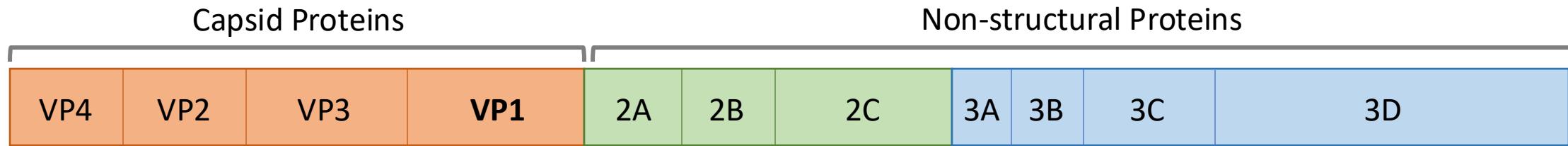
Add 2 µl



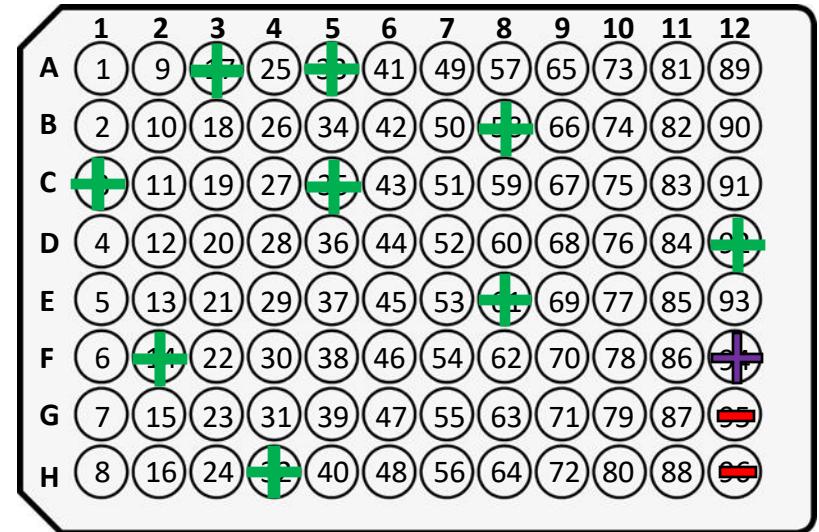
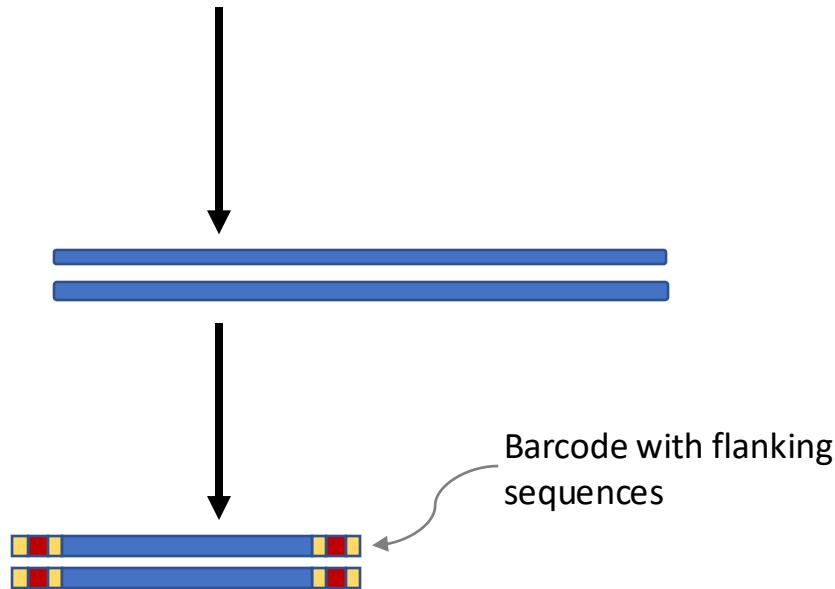
1st reaction plate



Post PCR

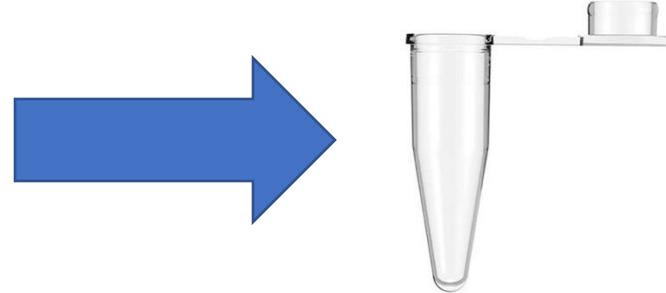
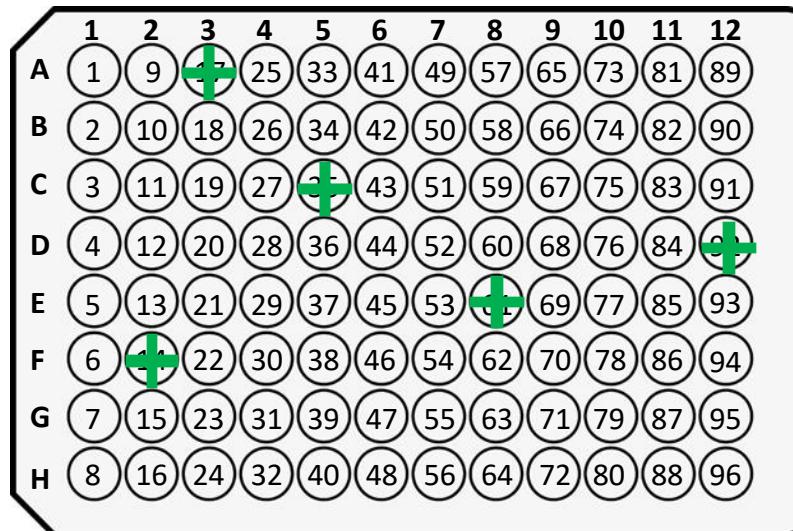


- Can run a gel to confirm samples, expect a band around 1.2kb.
- Only indicates the presence of poliovirus or enterovirus with similar primer binding sites
- DO run the controls on a gel for QC



Library Pooling

- Barcoded samples equally pooled by volume (2 μ l each)
- Previously pooling equal amounts of product, we have found for routine stool testing that it is easier to pool by volume without losing sensitivity



Why pool everything?

- Equal volume pooling removes quantification and dilution steps
 - Reduces cost
 - Reduces scope for error
 - Quicker
- Samples tend to have a fairly narrow range of concentrations after the nested PCR- generally 10-100 ng/ μ l
- Do not need great sequencing depth with stool samples- likely low complexity
- Negative samples do not detract from the success of sequencing

Library cleaning and concentration

- Clean and concentrate the pool using 1:1 ratio of AmpureXP beads.
- In detail:
 - Add ampure beads to the pool which will bind the DNA and allow it to be separated by a magnet
 - Wash the pool with 80 % ethanol twice
 - Allow to briefly dry (bead pellet will become matt brown)
 - Resuspend pellet in 51 µl of water
 - Use magnet to pull the beads out of solution
 - Retain 50 µl of the eluate (the concentrated pool).

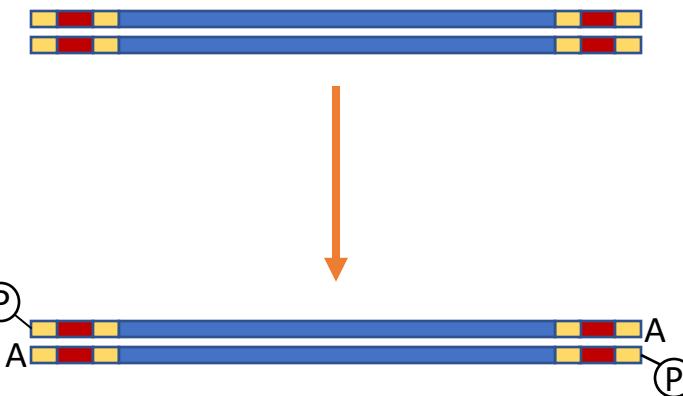


Ampure beads,
Beckman Coulter

Pause point – store library at 4°C

End preparation

- Treatment of DNA with Ultra II End-prep
- Ensures DNA has 3' dA tailed and 5' phosphorylated ends making them sticky for ligation of the sequencing adapter



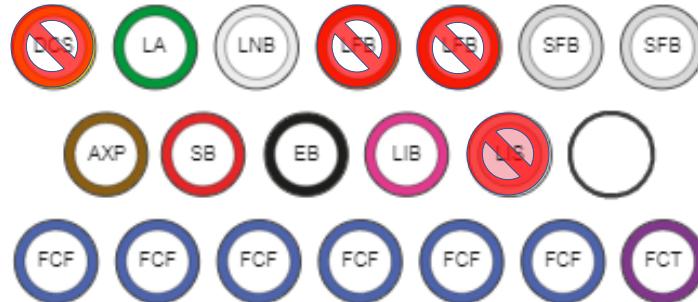
- Clean sample with ampure beads to remove enzyme

Library preparation

- Performed using the Ligation Sequencing Kit from Oxford Nanopore
- Current version is LSK-114
- Contents:



There vials in the kit that we will not use in this protocol (these are crossed out to the right)

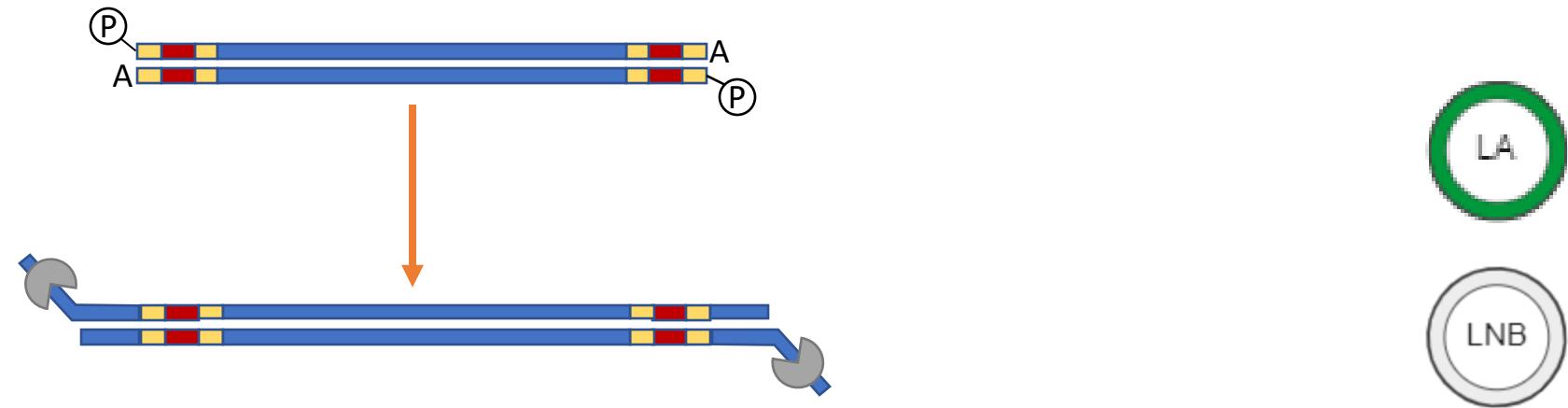


DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads

SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

Adaptation for Sequencing

- Ligate the Ligation Adapter (LA) which includes the motor protein



- The motor protein controls the speed at which the DNA passes through the pore
- Ligation performed with Quick T4 Ligase (supplied by NEB) and the LNB ligation buffer

Cleaning of Library

- Clean using Ampure beads

BUT

washing is performed with Short Fragment Buffer (SFB) and elution in elution buffer (EB)

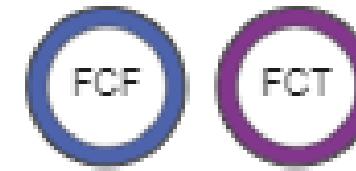


- SFB used for washing where no size selection is required.
- Long fragment buffer (LFB) can be used to select for DNA >3kb



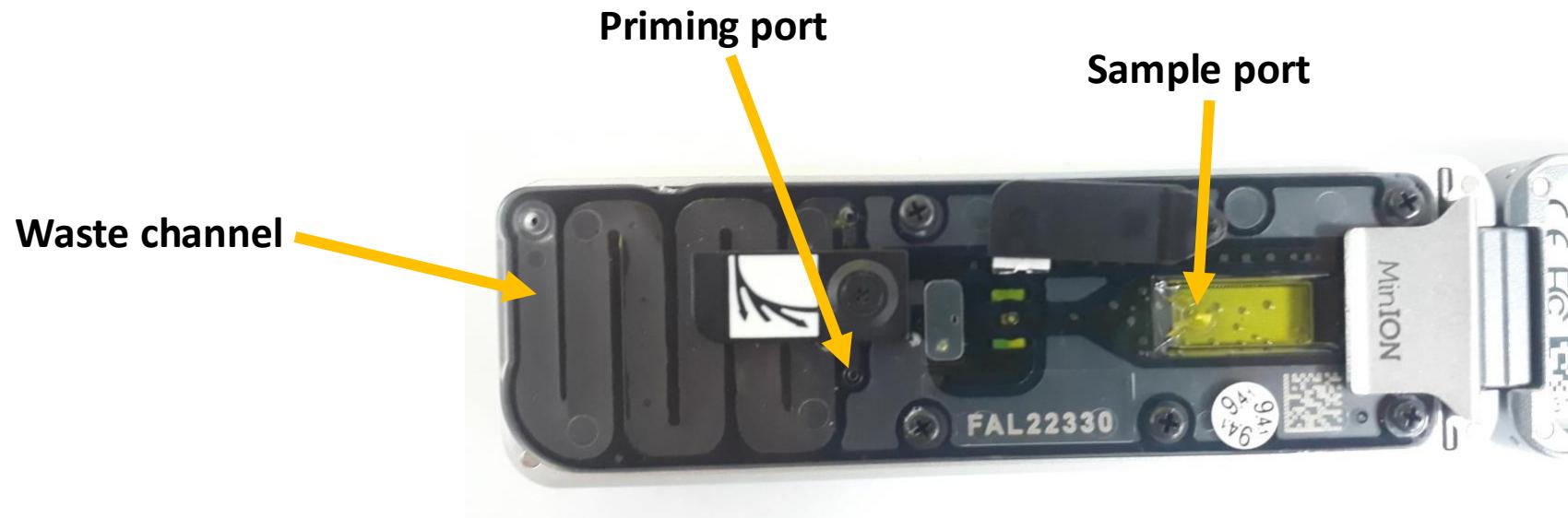
Priming of the flow cell

- Flowcell priming – Flow Cell Flush (FCF), Flow Cell Tether (FCT), and BSA



- The function of the tethers is to help pull DNA down towards the pores for sequencing
- The BSA (not included in the kit) is said to improve stability and throughput
- Added via the priming port in two steps
 - First time with sample port closed
 - Second time with sample port open

Loading the MinION

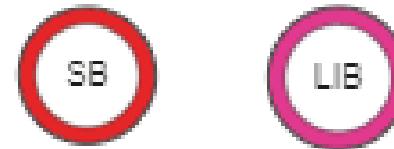


In this picture both the priming and sample loading ports are open.

Final Library

- The mix loaded onto the MinION contains:

- Your adapted DNA library
- Sequencing buffer (SB)
- Library beads (LB)



- Loaded via the spot-on port, one drop at a time.

Loading the MinION



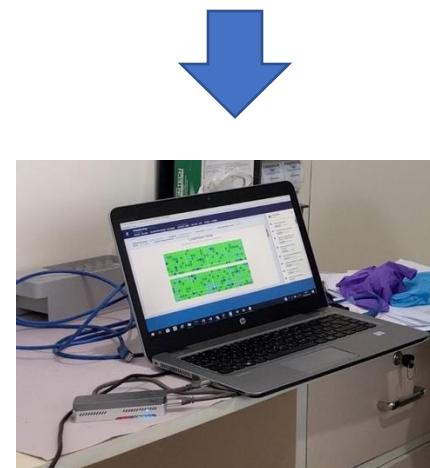
- Before flowcell priming/loading any small air bubbles are removed from beneath the priming port ()
- Waste reagents are removed from the waste port () with **both** priming port and sample port covers **closed**
- *Video guide: See “[Loading the MinION](#)”*

3. Nanopore sequencing

Presentation and discussion

Library Preparation and Nanopore Sequencing

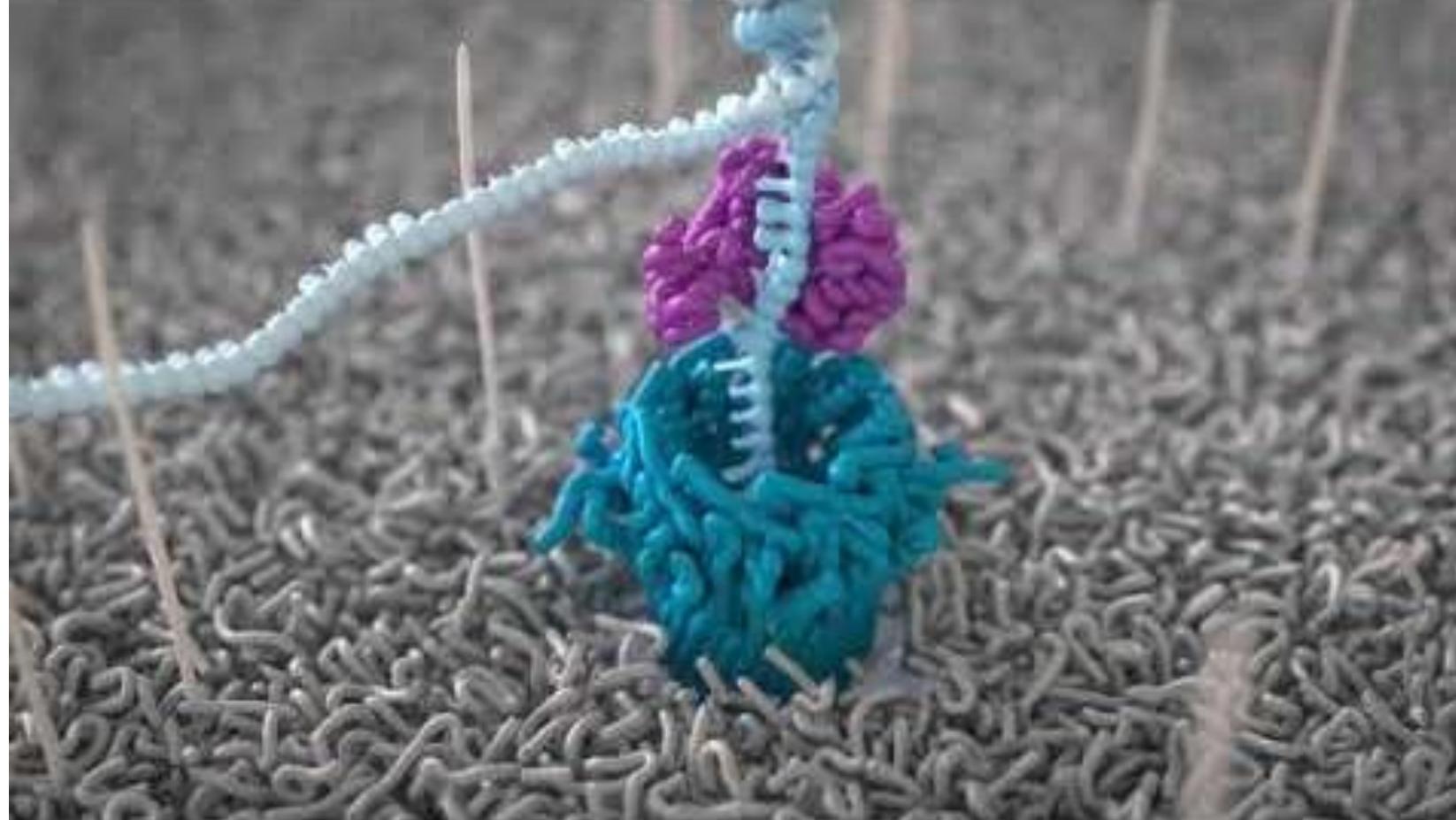
Extracted RNA → RT-PCR with panEV primers → Nested PCR with barcoded VP1 primers → Sequencing library preparation



PIRANHA (command line) or
piranhaGUI (point and click)



Nanopore Sequencing



<https://youtu.be/RcP85JHLmnI>

MinION Sequencing

- Long Reads- potentially 100,000s of bp
- Fast- 1,000,000 reads per hour and can process in real time
- “Cheap” (depending on multiplexing)

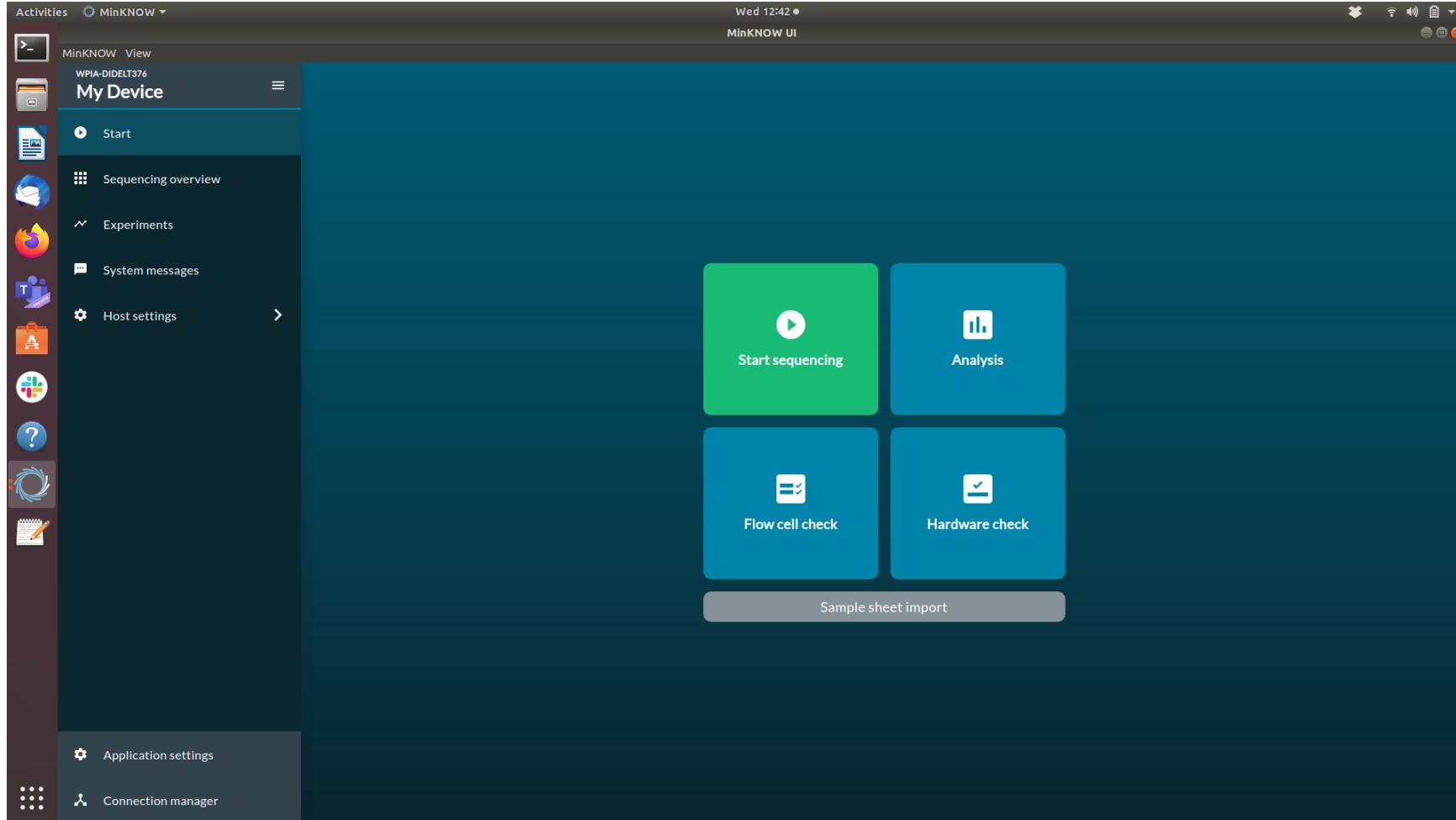
but

- Error rate per base (~0.5% with current highest accuracy settings)
 - can still give accurate consensus sequences.

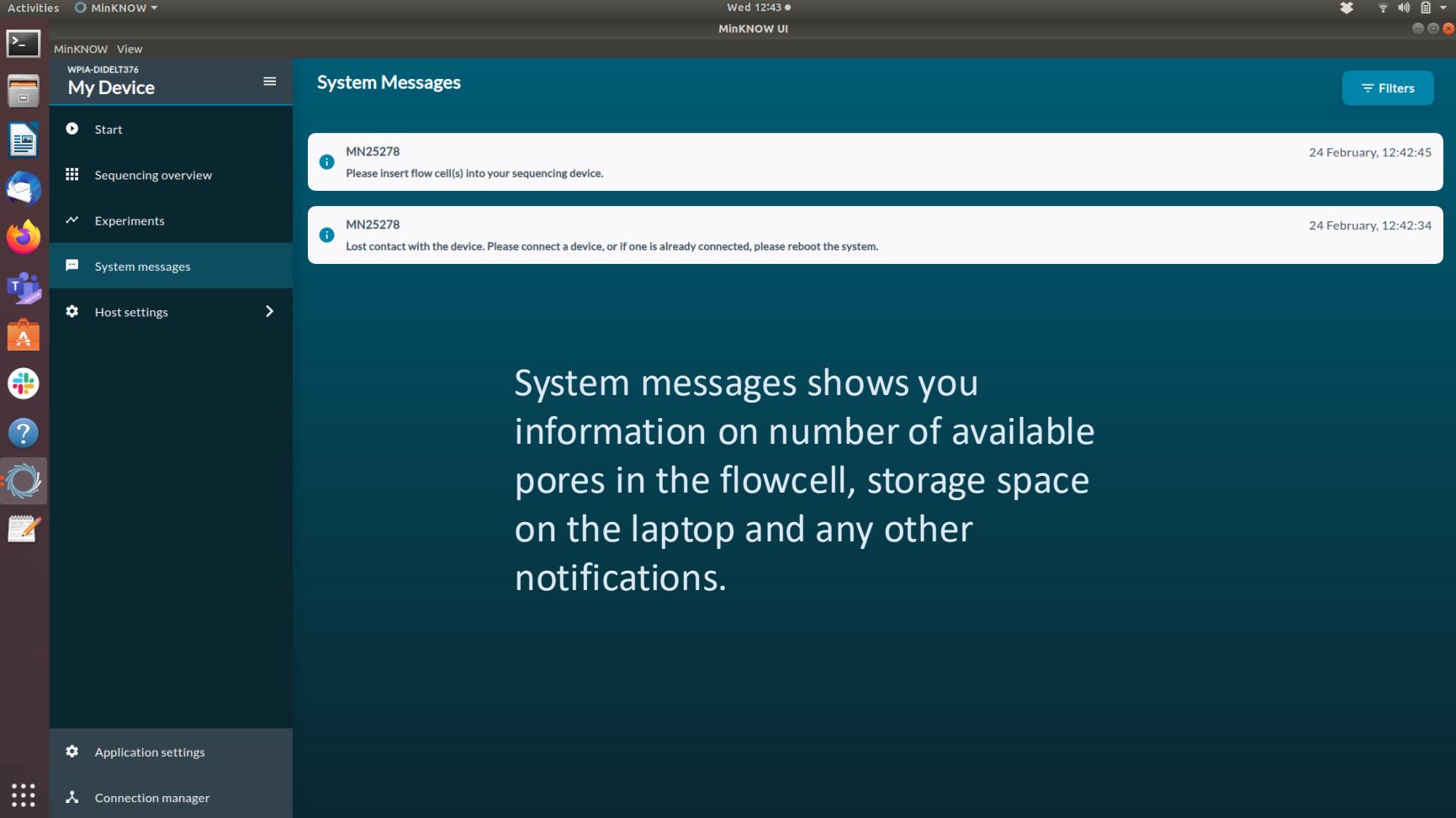
MinKNOW

- Easy to use for setting up and running your sequencing run
- Install following the instructions on the Nanopore website
- Basecalling and demultiplexing performed within MinKNOW

MinKNOW GUI



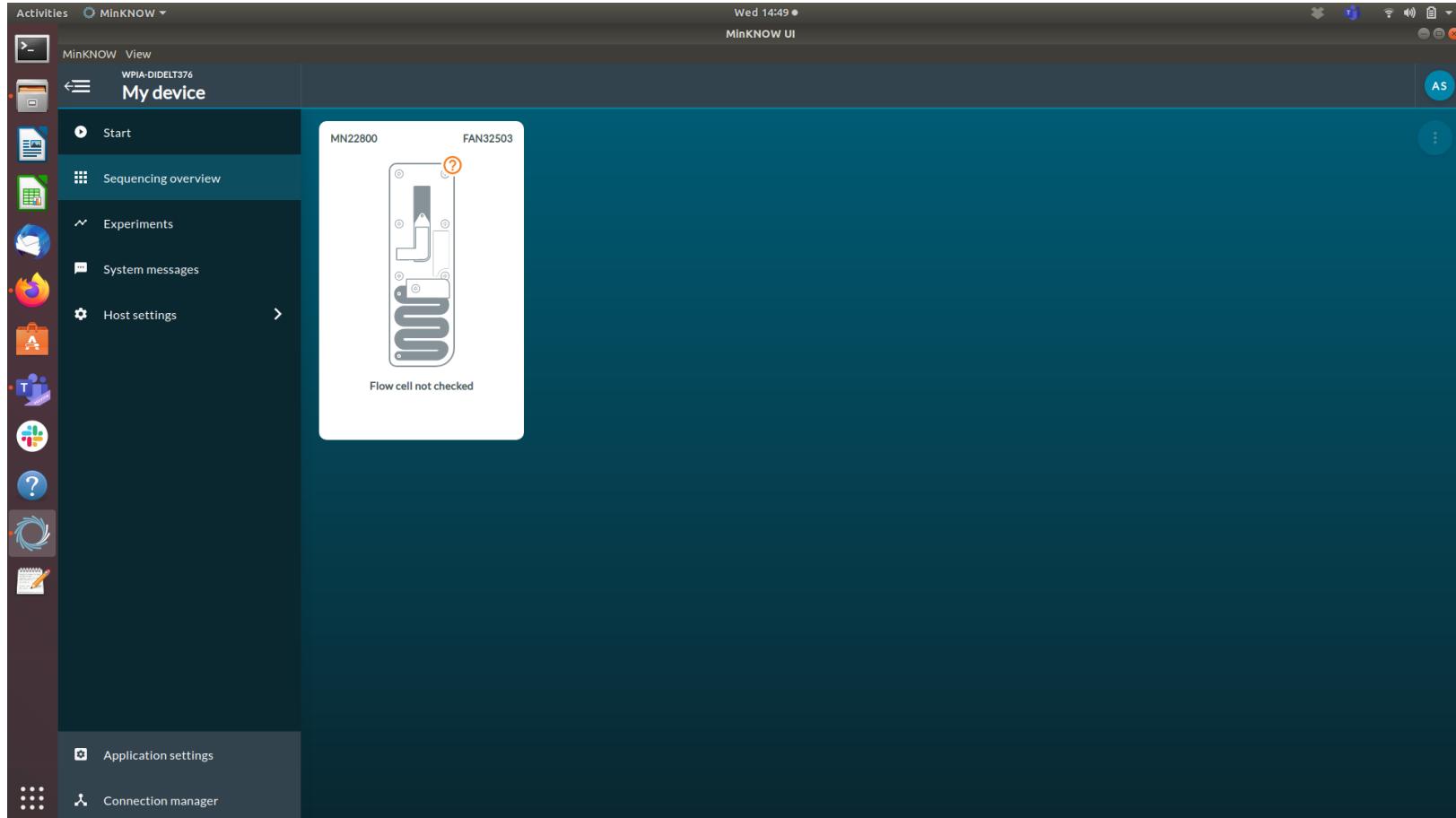
System messages



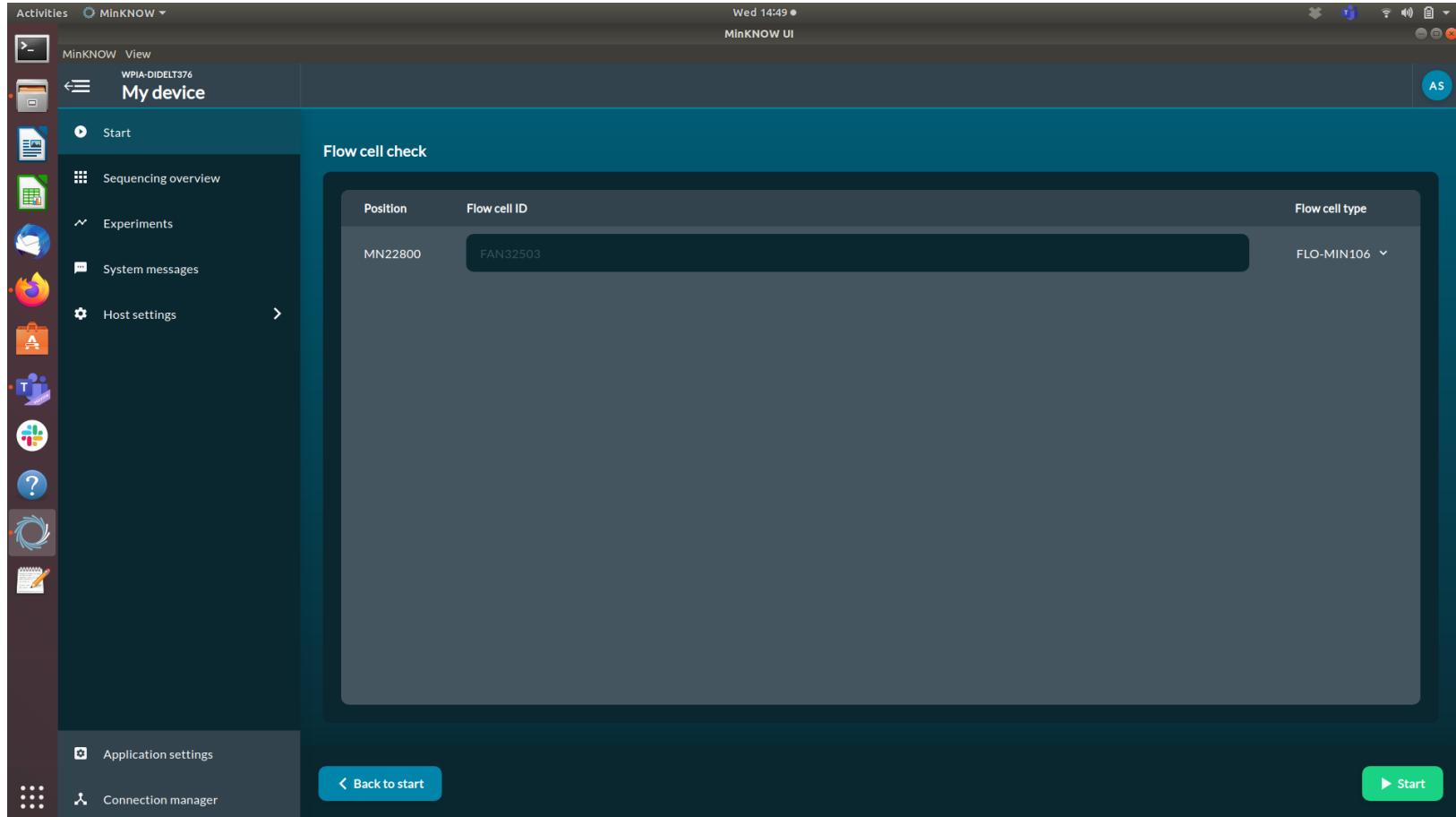
The screenshot shows the MinNOW UI interface. On the left is a vertical sidebar with icons for various applications like Activities, MinNOW View, My Device, Sequencing overview, Experiments, System messages (which is selected), Host settings, Application settings, and Connection manager. The main window title is "System Messages". At the top right of the main window are "Filters" and a date/time stamp "Wed 12:43 • MinNOW UI". Below the title, there are two system message notifications. The first message, timestamped "24 February, 12:42:45", says "MN25278 Please insert flow cell(s) into your sequencing device." The second message, timestamped "24 February, 12:42:34", says "MN25278 Lost contact with the device. Please connect a device, or if one is already connected, please reboot the system."

System messages shows you information on number of available pores in the flowcell, storage space on the laptop and any other notifications.

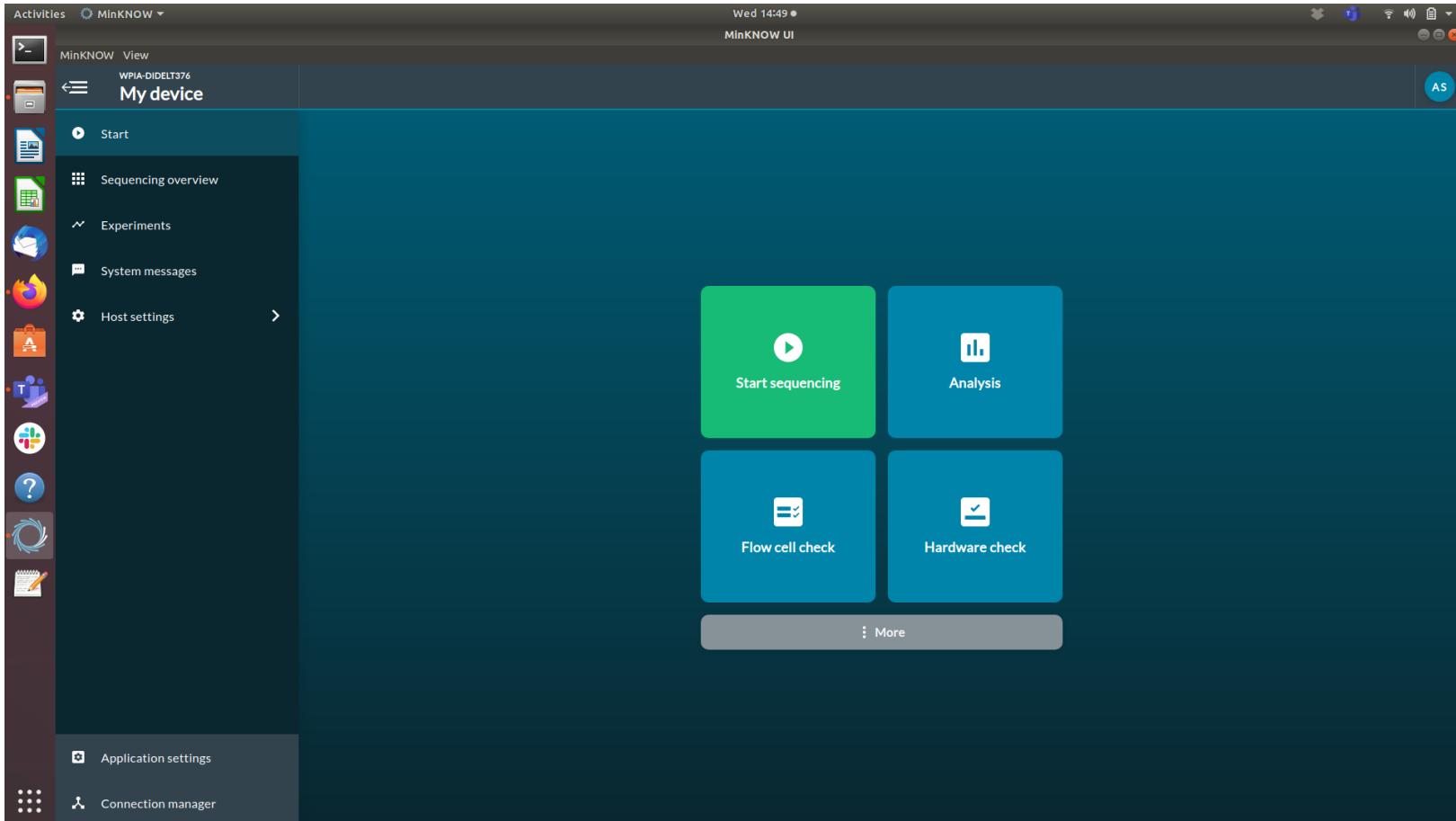
Sequencing overview



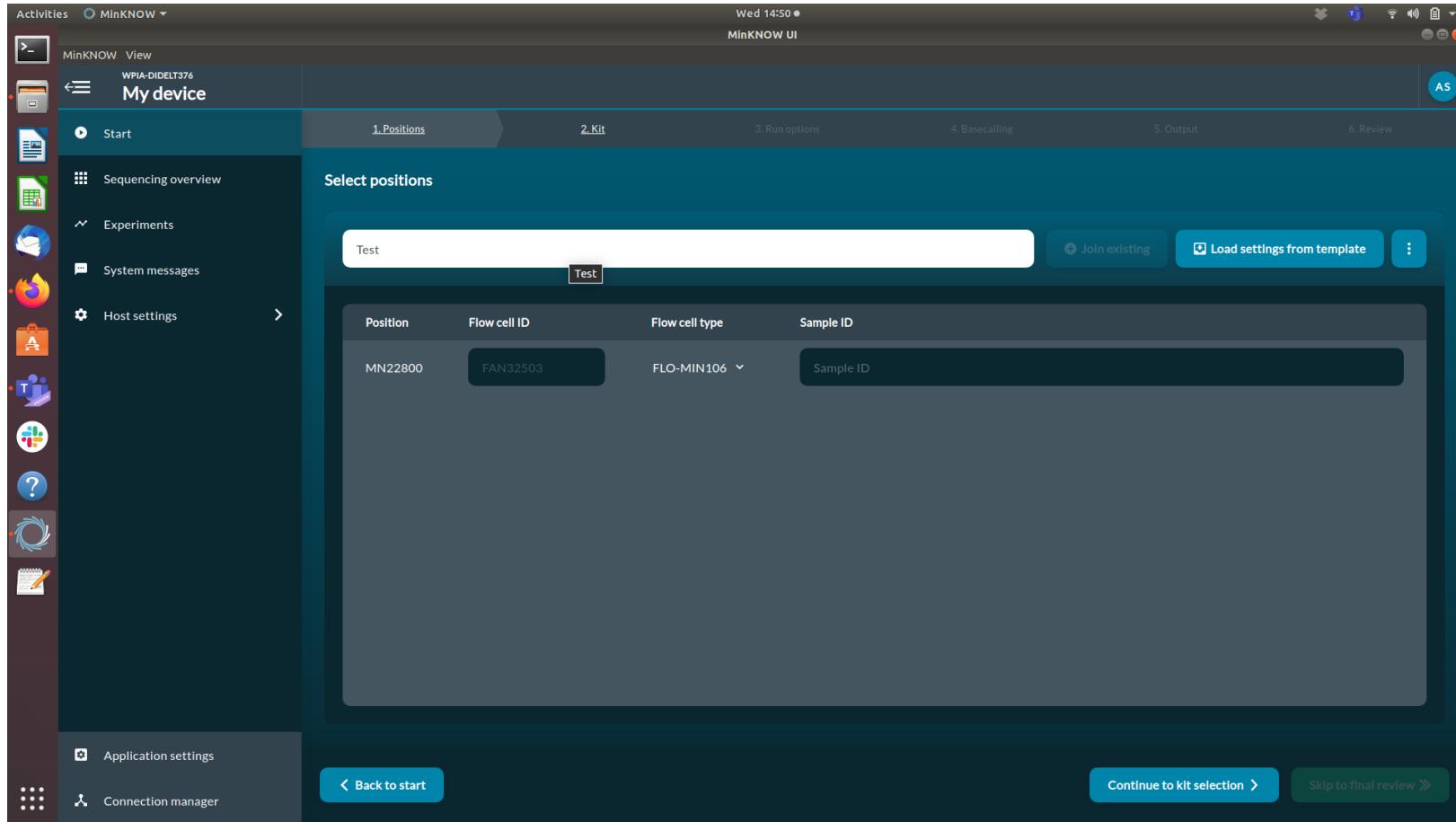
Running a flow cell check



Starting a sequencing run

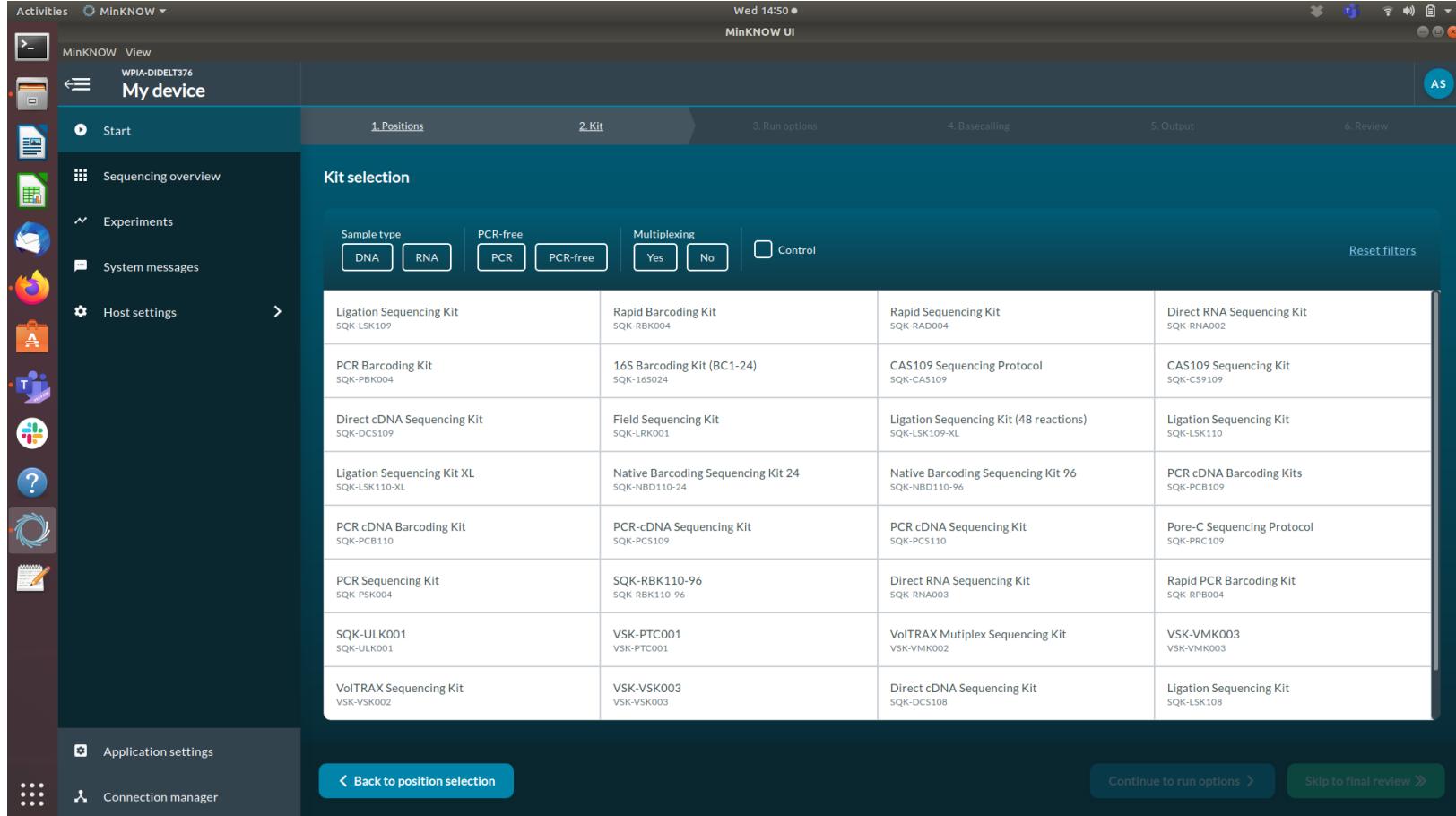


Starting a sequencing run



Follow an agreed naming pattern and folder structure for good traceability in your group.

Selecting the sequencing kit



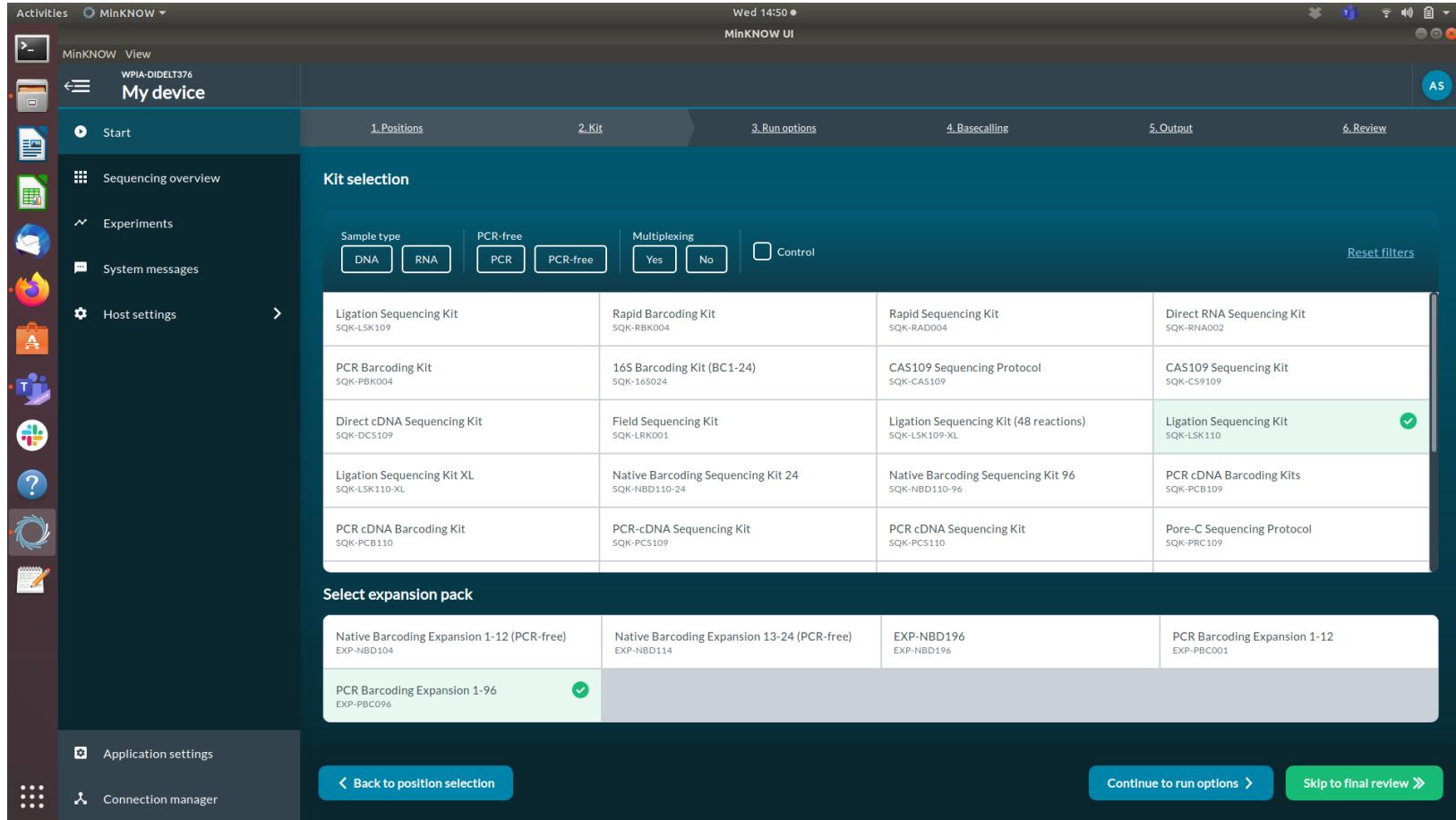
The screenshot shows the MinKNOW User Interface (UI) for selecting a sequencing kit. The main window title is "MinKNOW UI" and the date and time are "Wed 14:50". The left sidebar has a "My device" section with "WPIA-DIDELT376" and a "Start" button. Other sections include "Sequencing overview", "Experiments", "System messages", and "Host settings". The main content area is titled "Kit selection" and shows a grid of sequencing kit options:

Sample type	PCR-free	Multiplexing	
DNA	PCR	Yes	<input type="checkbox"/> Control
RNA	PCR	No	
Ligation Sequencing Kit SQK-LSK109	Rapid Barcoding Kit SQK-RBK004	Rapid Sequencing Kit SQK-RAD004	Direct RNA Sequencing Kit SQK-RNA002
PCR Barcoding Kit SQK-PBK004	16S Barcoding Kit (BC1-24) SQK-16S024	CAS109 Sequencing Protocol SQK-CAS109	CAS109 Sequencing Kit SQK-CS9109
Direct cDNA Sequencing Kit SQK-DCS109	Field Sequencing Kit SQK-LRK001	Ligation Sequencing Kit (48 reactions) SQK-LSK109-XL	Ligation Sequencing Kit SQK-LSK110
Ligation Sequencing Kit XL SQK-LSK110-XL	Native Barcoding Sequencing Kit 24 SQK-NBD110-24	Native Barcoding Sequencing Kit 96 SQK-NBD110-96	PCR cDNA Barcoding Kits SQK-PCB109
PCR cDNA Barcoding Kit SQK-PCB110	PCR-cDNA Sequencing Kit SQK-PCS109	PCR cDNA Sequencing Kit SQK-PCS110	Pore-C Sequencing Protocol SQK-PRC109
PCR Sequencing Kit SQK-PSK004	SQK-RBK110-96 SQK-RBK110-96	Direct RNA Sequencing Kit SQK-RNA003	Rapid PCR Barcoding Kit SQK-RPB004
SQK-ULK001 SQK-ULK001	VSK-PTC001 VSK-PTC001	VolTRAX Multiplex Sequencing Kit VSK-VMK002	VSK-VMK003 VSK-VMK003
VolTRAX Sequencing Kit VSK-VSK002	VSK-VSK003 VSK-VSK003	Direct cDNA Sequencing Kit SQK-DCS108	Ligation Sequencing Kit SQK-LSK108

At the bottom of the main panel are buttons for "Back to position selection", "Continue to run options", and "Skip to final review".

For v14 chemistry
and sequencing
with the
barcoded VP1
primers select
SQK-LSK114

Selecting barcodes



Activities MinNOW View WPIA-DIDELT376 My device Wed 14:50 • MinNOW UI AS

Start Sequencing overview Experiments System messages Host settings >

1. Positions 2. Kit 3. Run options 4. Basecalling 5. Output 6. Review

Kit selection

Sample type: DNA RNA PCR PCR-free Multiplexing: Yes No Control Reset filters

Ligation Sequencing Kit SQK-LSK109	Rapid Barcoding Kit SQK-RBK004	Rapid Sequencing Kit SQK-RAD004	Direct RNA Sequencing Kit SQK-RNA002
PCR Barcoding Kit SQK-PBK004	16S Barcoding Kit (BC1-24) SQK-16S024	CAS109 Sequencing Protocol SQK-CAS109	CAS109 Sequencing Kit SQK-CS9109
Direct cDNA Sequencing Kit SQK-DCS109	Field Sequencing Kit SQK-LRK001	Ligation Sequencing Kit (48 reactions) SQK-LSK109-XL	Ligation Sequencing Kit SQK-LSK110
Ligation Sequencing Kit XL SQK-LSK110-XL	Native Barcoding Sequencing Kit 24 SQK-NBD110-24	Native Barcoding Sequencing Kit 96 SQK-NBD110-96	PCR cDNA Barcoding Kits SQK-PCB109
PCR cDNA Barcoding Kit SQK-PCB110	PCR-cDNA Sequencing Kit SQK-PC5109	PCR cDNA Sequencing Kit SQK-PC5110	Pore-C Sequencing Protocol SQK-PRC109

Select expansion pack

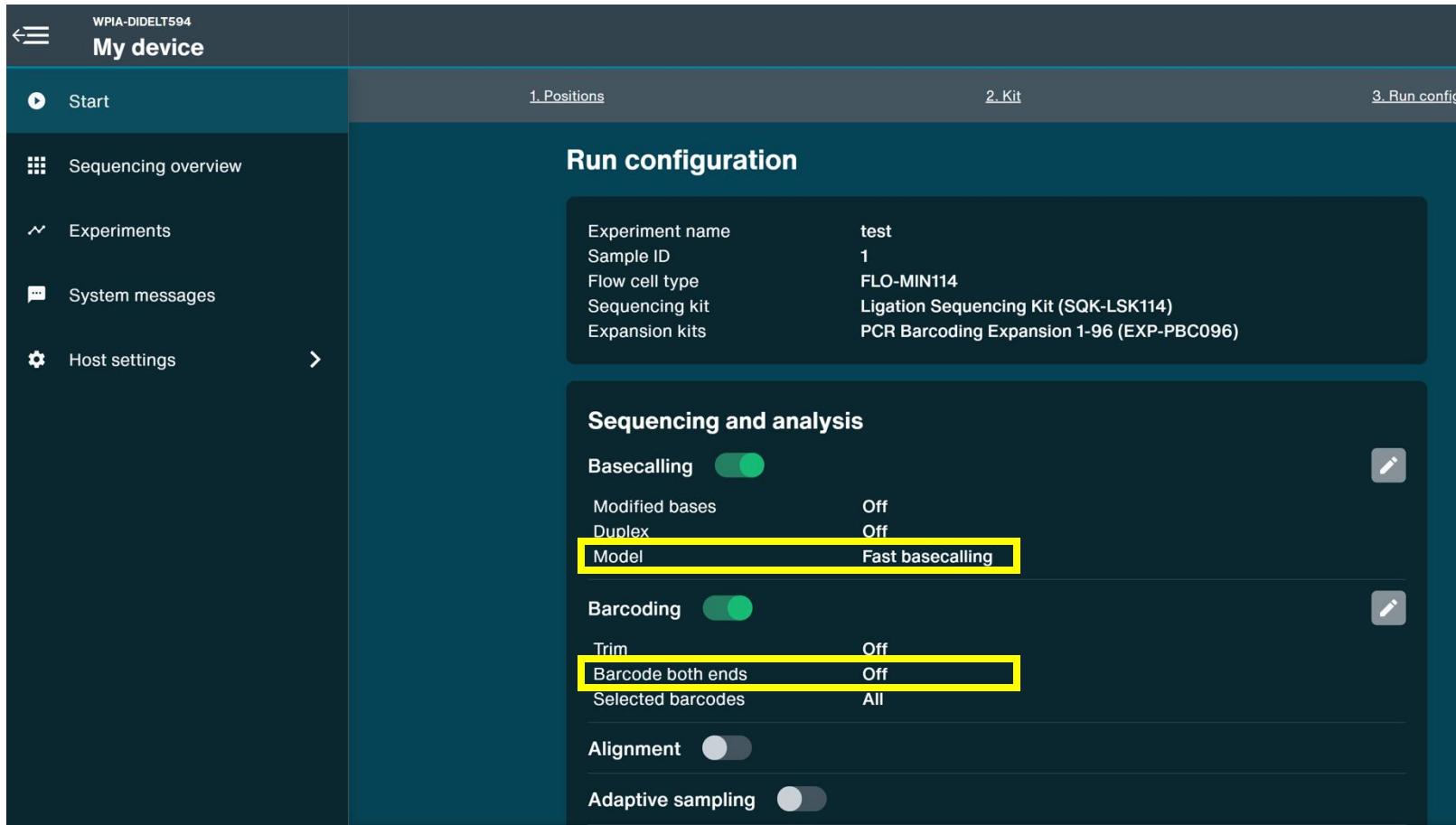
Native Barcoding Expansion 1-12 (PCR-free) EXP-NBD104	Native Barcoding Expansion 13-24 (PCR-free) EXP-NBD114	EXP-NBD196 EXP-NBD196	PCR Barcoding Expansion 1-12 EXP-PBC001
PCR Barcoding Expansion 1-96 EXP-PBC096			

< Back to position selection Continue to run options > Skip to final review >

For the barcoded VP1 primers select EXP-PBC096

MinNOW will look for the barcode set selected to de-multiplex reads.

Run Configuration



The screenshot shows the 'Run configuration' screen in a software interface. The left sidebar lists 'My device' with options like Start, Sequencing overview, Experiments, System messages, and Host settings. The main area has tabs for Positions, Kit, and Run configuration. The 'Run configuration' tab is active, displaying the following details:

Experiment name	test
Sample ID	1
Flow cell type	FLO-MIN114
Sequencing kit	Ligation Sequencing Kit (SQK-LSK114)
Expansion kits	PCR Barcoding Expansion 1-96 (EXP-PBC096)

Sequencing and analysis

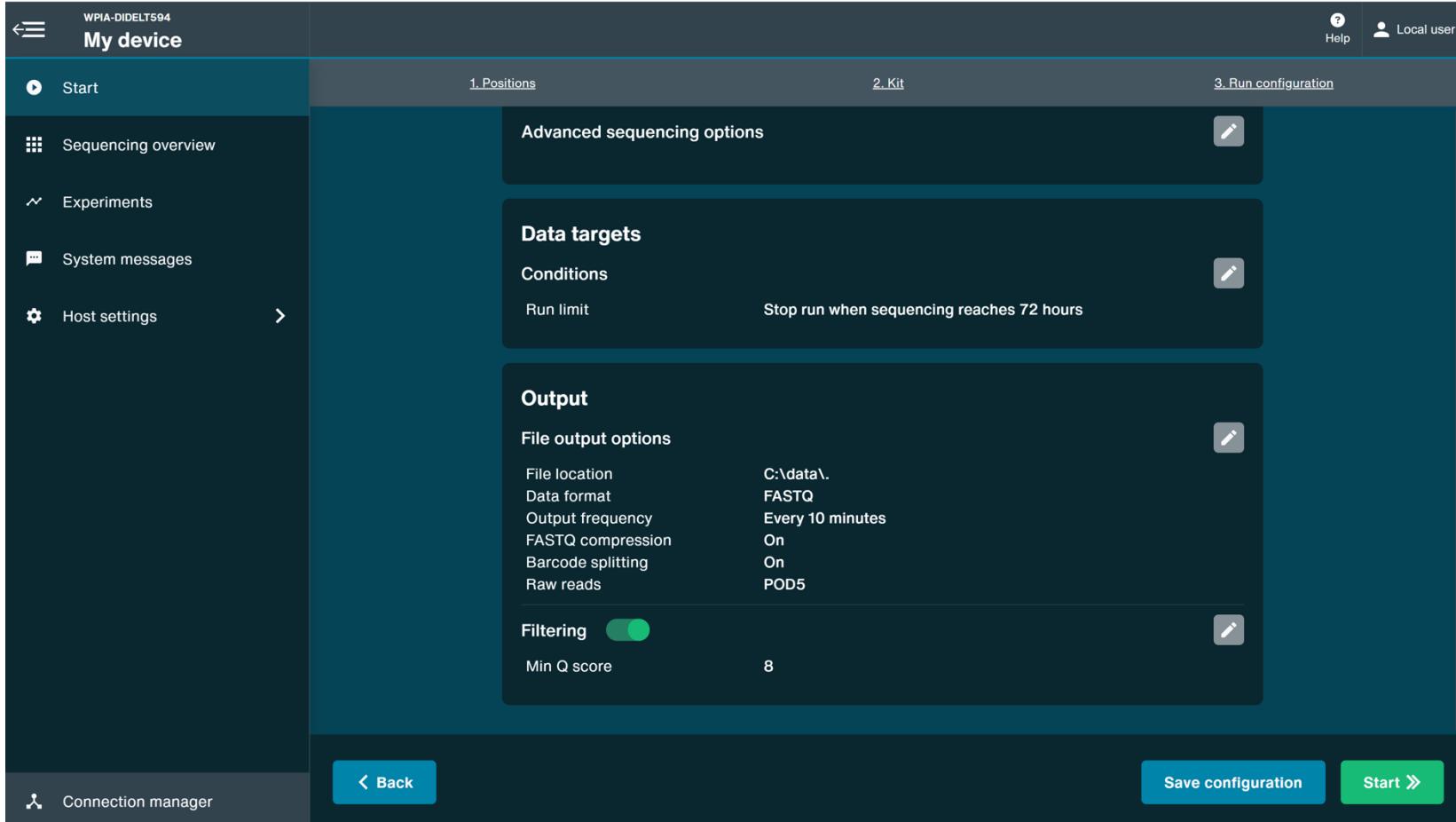
- Basecalling: On
- Modified bases: Off
- Duplex: Off
- Model:** Fast basecalling (highlighted with a yellow box)
- Barcode both ends: Off (highlighted with a yellow box)
- Trim: Off
- Selected barcodes: All
- Alignment: Off
- Adaptive sampling: Off

Basecalling speed will depend on computer processing power.

We expect to have barcodes on both ends of our reads as we used barcoded forward and reverse primers for the VP1 amplicon.

Basecalling/Barcoding can also be performed post-run from the raw data when accessed from the START menu.

Setting run length in run options



The screenshot shows the 'Run options' configuration screen for a sequencing run. The left sidebar includes 'Start', 'Sequencing overview', 'Experiments', 'System messages', and 'Host settings'. The main area has tabs for '1. Positions', '2. Kit', and '3. Run configuration'. The '3. Run configuration' tab is active, displaying 'Advanced sequencing options' and 'Data targets' sections. In the 'Data targets' section, there is a 'Conditions' row with 'Run limit' set to 'Stop run when sequencing reaches 72 hours'. The 'Output' section contains 'File output options' with settings: File location (C:\data\), Data format (FASTQ), Output frequency (Every 10 minutes), FASTQ compression (On), Barcode splitting (On), and Raw reads (POD5). A 'Filtering' toggle switch is turned on, and the 'Min Q score' is set to 8. At the bottom are 'Save configuration' and 'Start >' buttons.

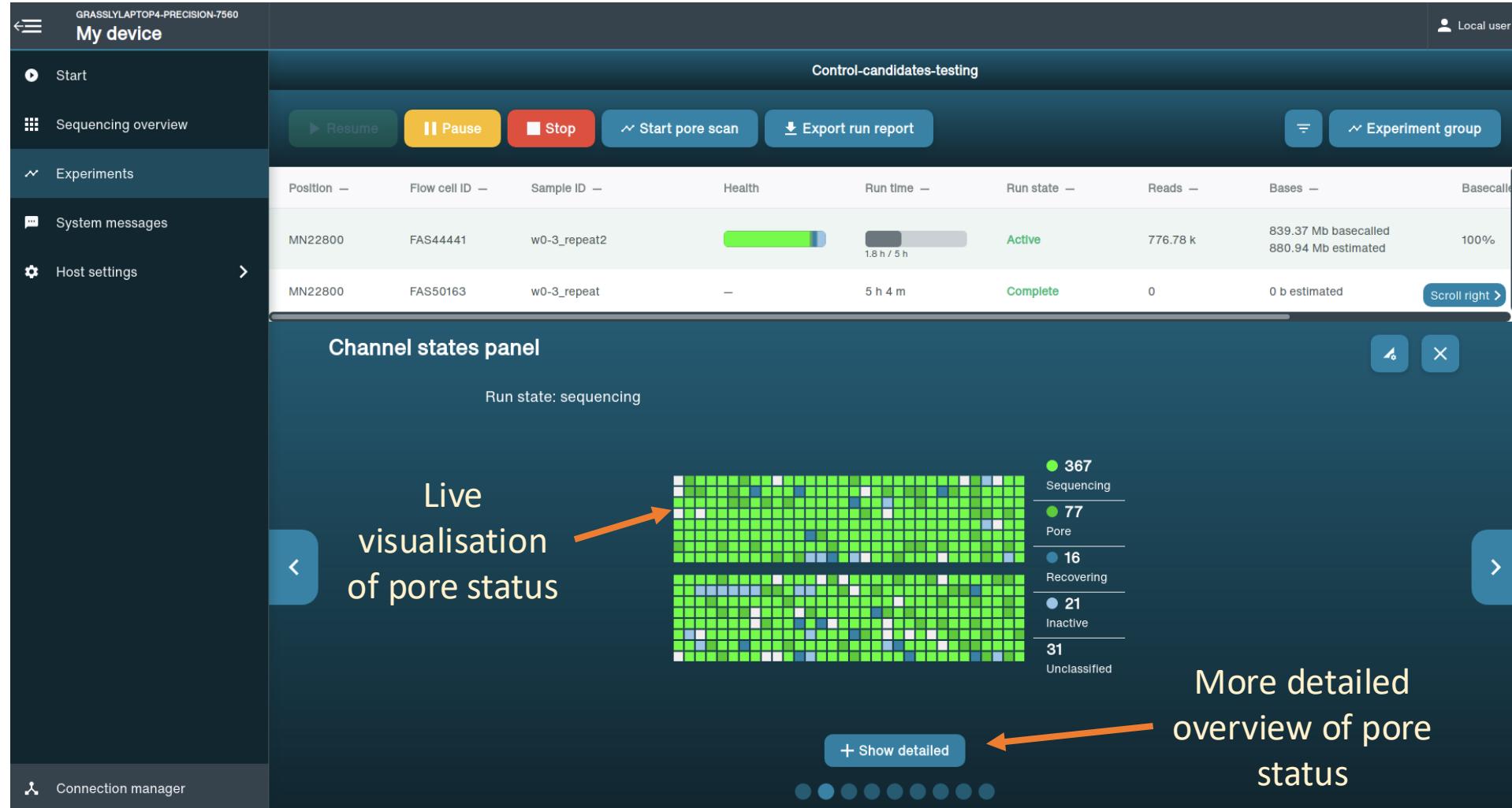
Run can be extended or stopped earlier as required.

You can select an analysis folder on your desktop for easy access

On a Windows computer the default is C:\data\

Raw data file format is POD5

Sequencing Run



GRASSLYLAPTOP4-PRECISION-7560

My device

- Start
- Sequencing overview
- Experiments
- System messages
- Host settings

Local user

Control-candidates-testing

Position — Flow cell ID — Sample ID — Health — Run time — Run state — Reads — Bases — Basecalling

MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%;"> </div>	1.8 h / 5 h	Active	776.78 k	839.37 Mb basecalled 880.94 Mb estimated	100%
MN22800	FAS50163	w0-3_repeat	—	5 h 4 m	Complete	0	0 b estimated	

Channel states panel

Run state: sequencing

Live visualisation of pore status

More detailed overview of pore status

+ Show detailed

Legend:

- 367 Sequencing
- 77 Pore
- 16 Recovering
- 21 Inactive
- 31 Unclassified

Sequencing Run

GRASSYLAPTOP4-PRECISION-7560

My device

Local user

Start

Sequencing overview

Experiments

System messages

Host settings

Control-candidates-testing

Resume | Pause | Stop | Start pore scan | Export run report | Experiment group

Position	Flow cell ID	Sample ID	Health	Run time	Run state	Reads	Bases	Basecalling
MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%;"> </div>	1.8 h / 5 h	Active	777.98 k	840.66 Mb basecalled 882.41 Mb estimated	100%
MN22800	FAS50163	w0-3_repeat	—	5 h 4 m	Complete	0	0 b estimated	Scroll right >

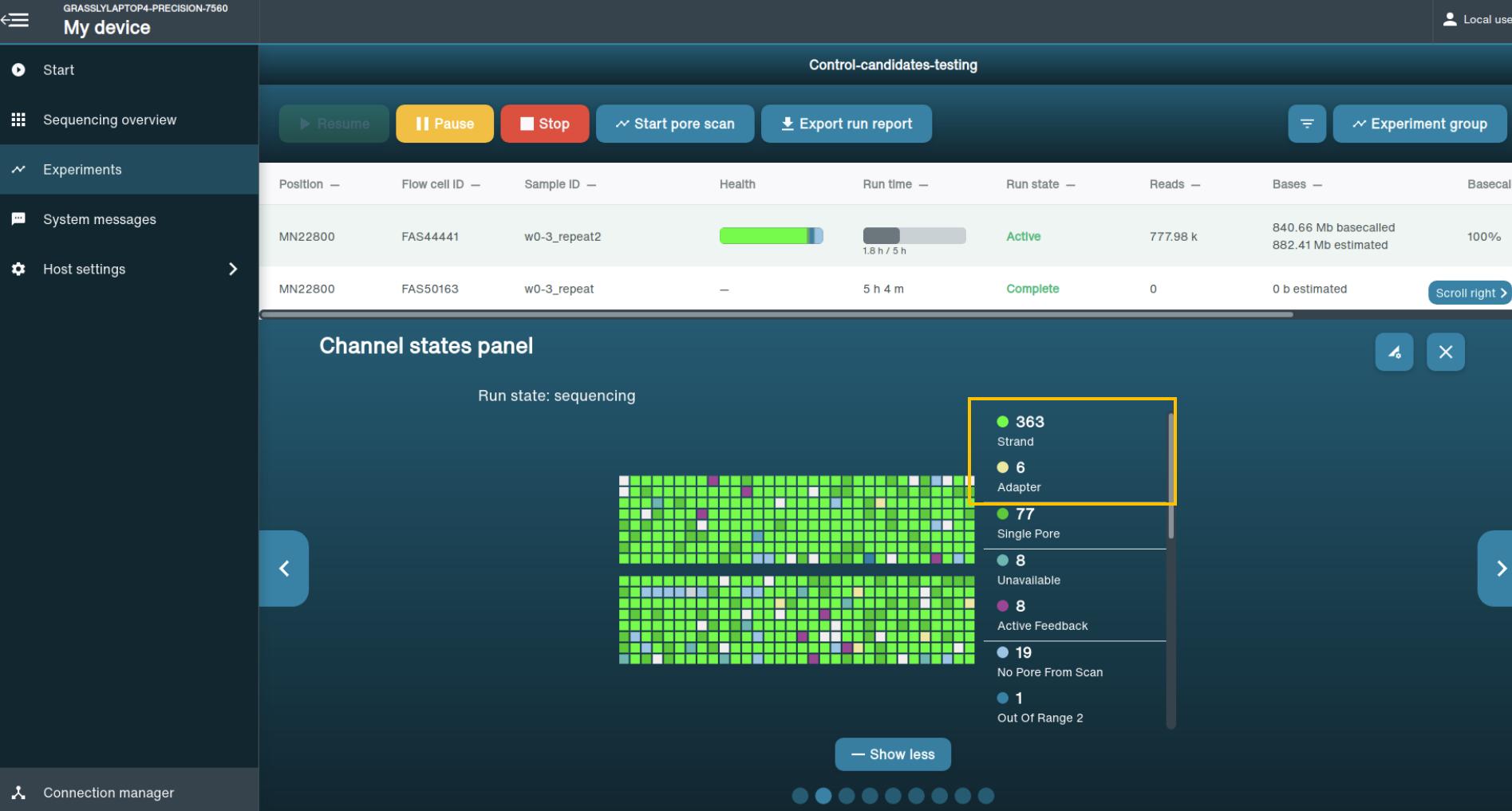
Channel states panel

Run state: sequencing

363 Strand
6 Adapter
77 Single Pore
8 Unavailable
8 Active Feedback
19 No Pore From Scan
1 Out Of Range 2

Show less

Connection manager



Sequencing Run

GRASSYLAPTOP4-PRECISION-7560 Local user

Control-candidates-testing

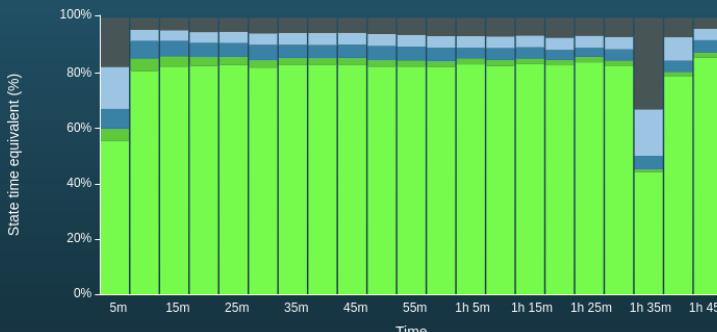
Position — Flow cell ID — Sample ID — Health — Run time — Run state — Reads — Bases — Basecaller —

MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%;">80%</div>	1.8 h / 5 h	Active	780.32 k	843.26 Mb basecalled 885.35 Mb estimated	100%
MN22800	FAS50163	w0-3_repeat	—	5 h 4 m	Complete	0	0 b estimated	—

Pore activity

State time equivalent (%)

Time: 5m, 15m, 25m, 35m, 45m, 55m, 1h 5m, 1h 15m, 1h 25m, 1h 35m, 1h 45m



sequencing ✓
pore ✓
recovering ✓
inactive ✓
unclassified ✓

+ Show detailed Display settings

Connection manager

Sequencing Run

GRASSLYLAPTOP4-PRECISION-7560
My device

- Start**
- Sequencing overview**
- Experiments**
- System messages**
- Host settings**

Control-candidates-testing

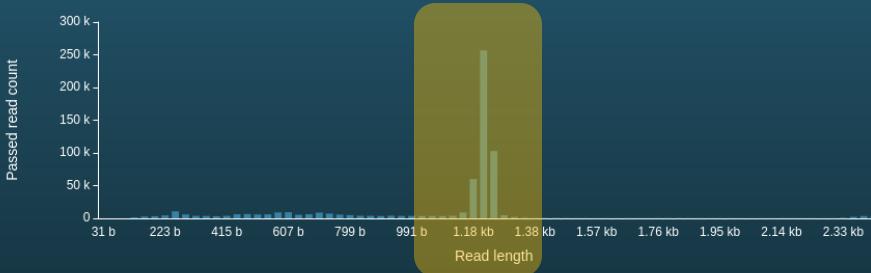
Position — Flow cell ID — Sample ID — Health — Run time — Run state — Reads — Bases — Basecaller —

MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%; background-color: green;"></div>	1.8 h / 5 h	Active	781.45 k	844.48 Mb basecalled 886.77 Mb estimated	100%
MN22800	FAS50163	w0-3_repeat	—	5 h 4 m	Complete	0	0 b estimated	Scroll right >

Read length histogram
Estimated N50: 1.2 kb

Assess size of reads being sequenced

Passed read count



Read length

Read lengths: 31 b, 223 b, 415 b, 607 b, 799 b, 991 b, 1.18 kb, 1.39 kb, 1.57 kb, 1.76 kb, 1.95 kb, 2.14 kb, 2.33 kb

Read bases: Estimated, Basecalled

Counts (selected)

Reset Hide outliers Split by read end reason

Adjust here to focus on specific read lengths

Sequencing Run

GRASSYLAPTOP4-PRECISION-7560

My device

- Start
- Sequencing overview
- Experiments
- System messages
- Host settings

Control-candidates-testing

Position — Flow cell ID — Sample ID — Health — Run time — Run state — Reads — Bases — Basecalling —

Position	Flow cell ID	Sample ID	Health	Run time	Run state	Reads	Bases	Basecalling
MN22800	FAS44441	w0-3_repeat2	<div style="width: 90%;"> </div>	1.8 h / 5 h	Active	782.6 k	845.78 Mb basecalled 888.33 Mb estimated	100%
MN22800	FAS50163	w0-3_repeat	—	5 h 4 m	Complete	0	0 b estimated	

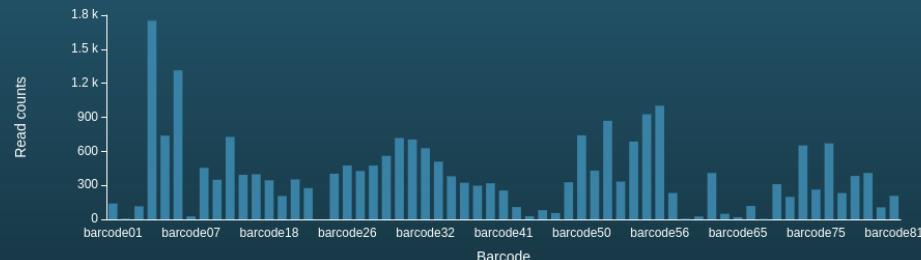
Barcode hits

See which barcodes have been detected and how many reads each

Read counts

Barcode

● Passed reads



Sort

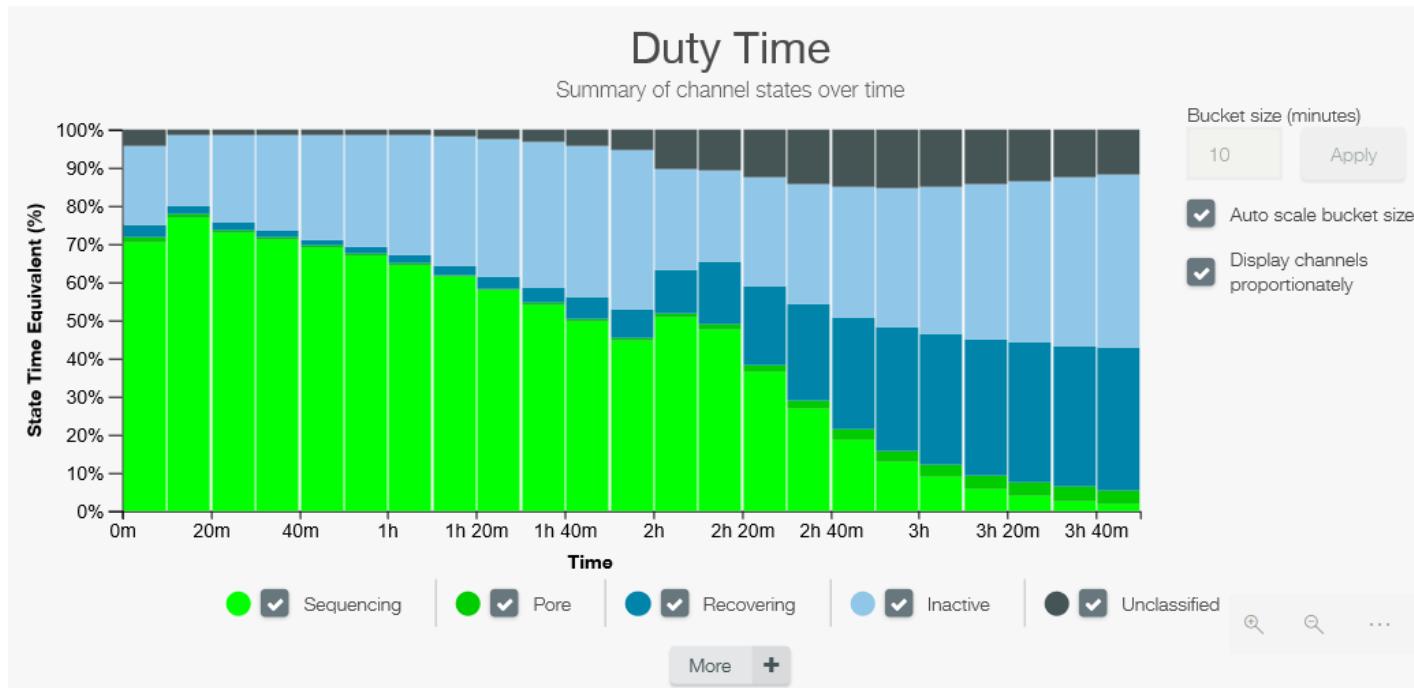
Reads Bases

Display failed Display unclassified Hide zero values

Reset selection

Connection manager

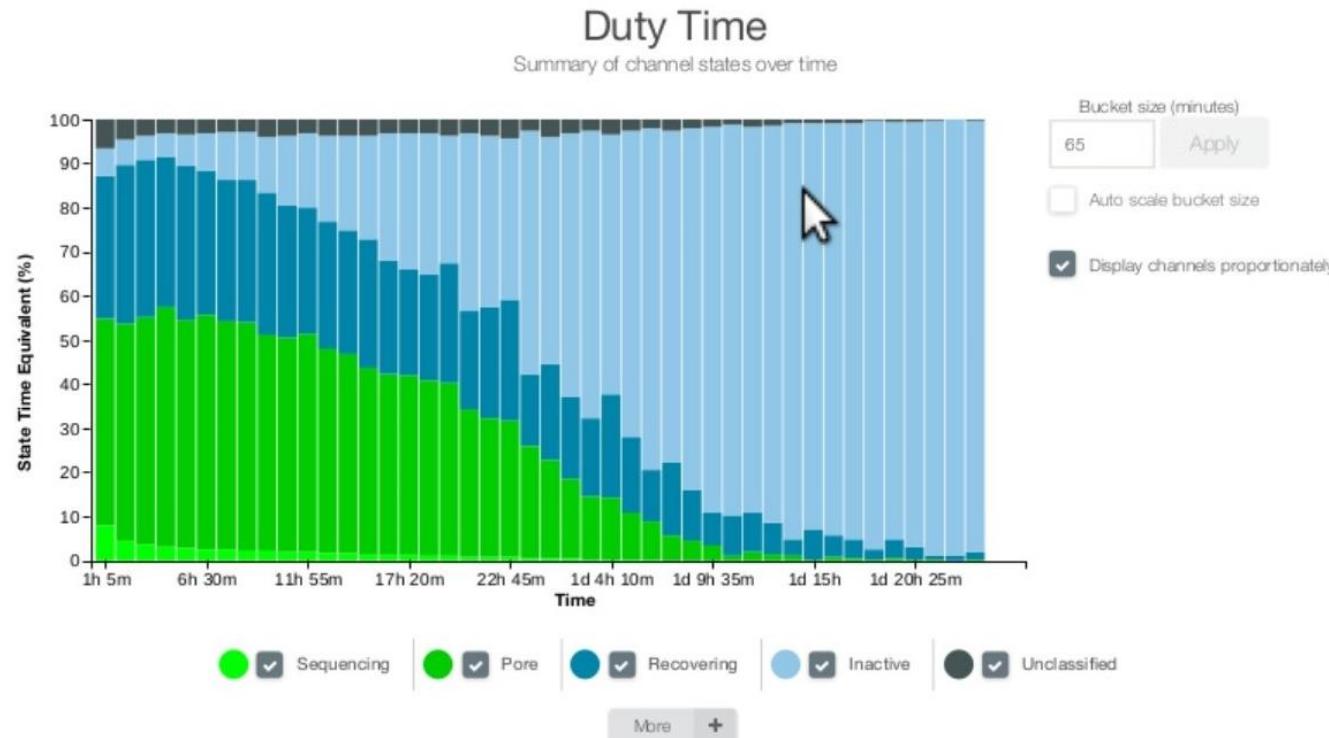
Examples of a bad start



Rapid pore death may be caused by:

- Contamination
- Using an old flowcell
- Software fault

Examples of a bad start



Low pore occupancy may be caused by:

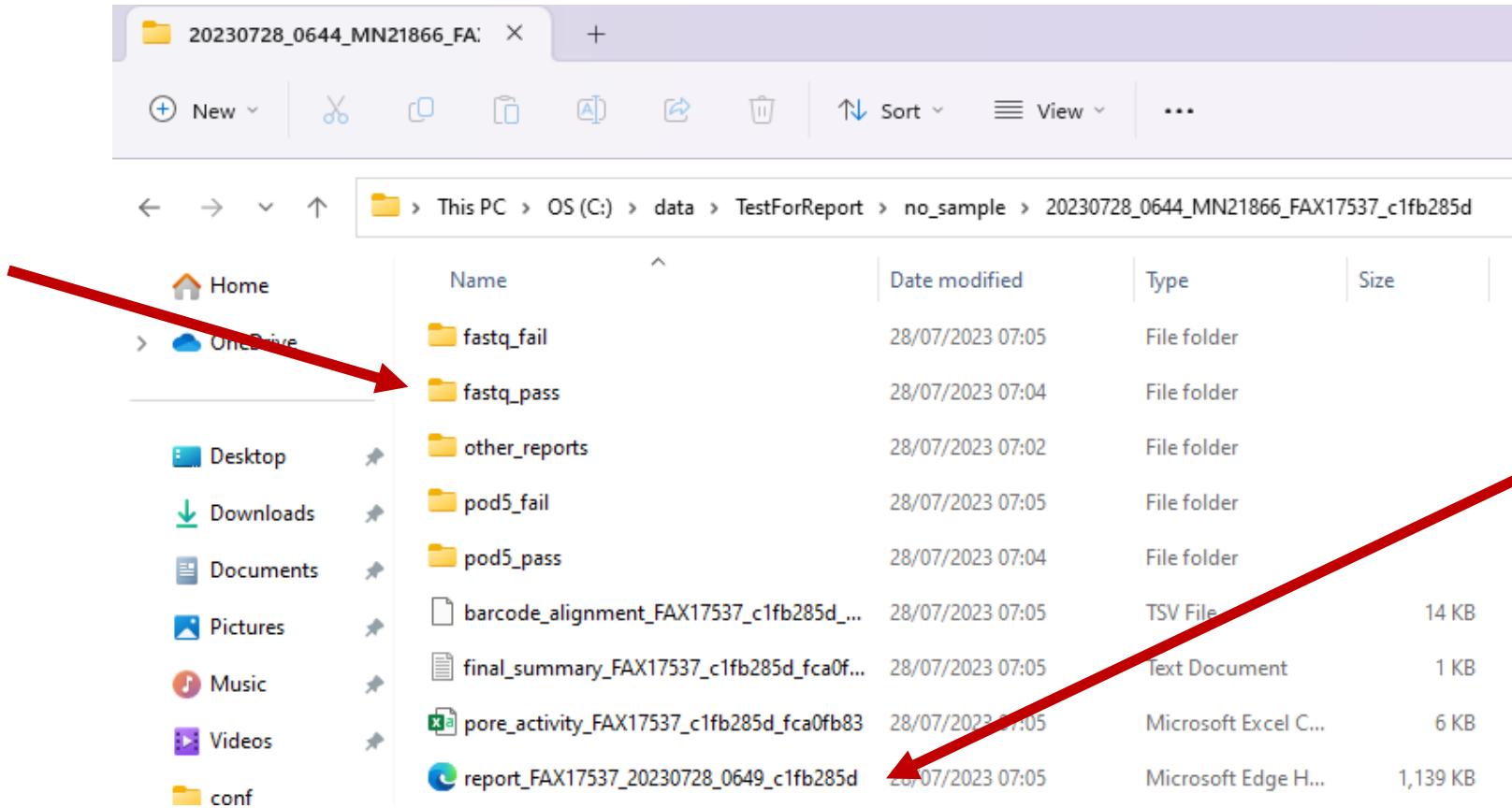
- Not loading enough sample onto the flowcell
- Failed adaptation for sequencing

4. Post-sequencing run checks

Presentation and discussion

1. Confirm that your run created data

Confirm that there is data in the fastq_pass folder



Name	Date modified	Type	Size
fastq_fail	28/07/2023 07:05	File folder	
fastq_pass	28/07/2023 07:04	File folder	
other_reports	28/07/2023 07:02	File folder	
pod5_fail	28/07/2023 07:05	File folder	
pod5_pass	28/07/2023 07:04	File folder	
barcode_alignment_FAX17537_c1fb285d...	28/07/2023 07:05	TSV File	14 KB
final_summary_FAX17537_c1fb285d_fca0f...	28/07/2023 07:05	Text Document	1 KB
pore_activity_FAX17537_c1fb285d_fca0fb83	28/07/2023 07:05	Microsoft Excel C...	6 KB
report_FAX17537_20230728_0649_c1fb285d	28/07/2023 07:05	Microsoft Edge H...	1,139 KB

Confirm that your run report has been created (this contains a summary of the run performance)

If there is no data, you can restart your run in MinKNOW.

If there is not enough data, you can click “join existing” when you start a run to ADD the data to your prior run

2. Check your run is complete in MinKHOW

MinKHOW UI

MinKHOW UI View

WPIA-DIDELT547 My device

Start

Sequencing overview

Experiments

System messages

Host settings

Experiments (13)

Experiments active in the last 7 days.

Search experiments

TestForReport groupe_1_ddns2 groupe_2_ddns2 Test4_FAX17330

TestForReport Inactive

Reads 82.93 k Estimated / basecalled bases 97.8 Mb / 101.18 Mb Active runs 0 Total runs 1

Experiment summary ↗

Run controls

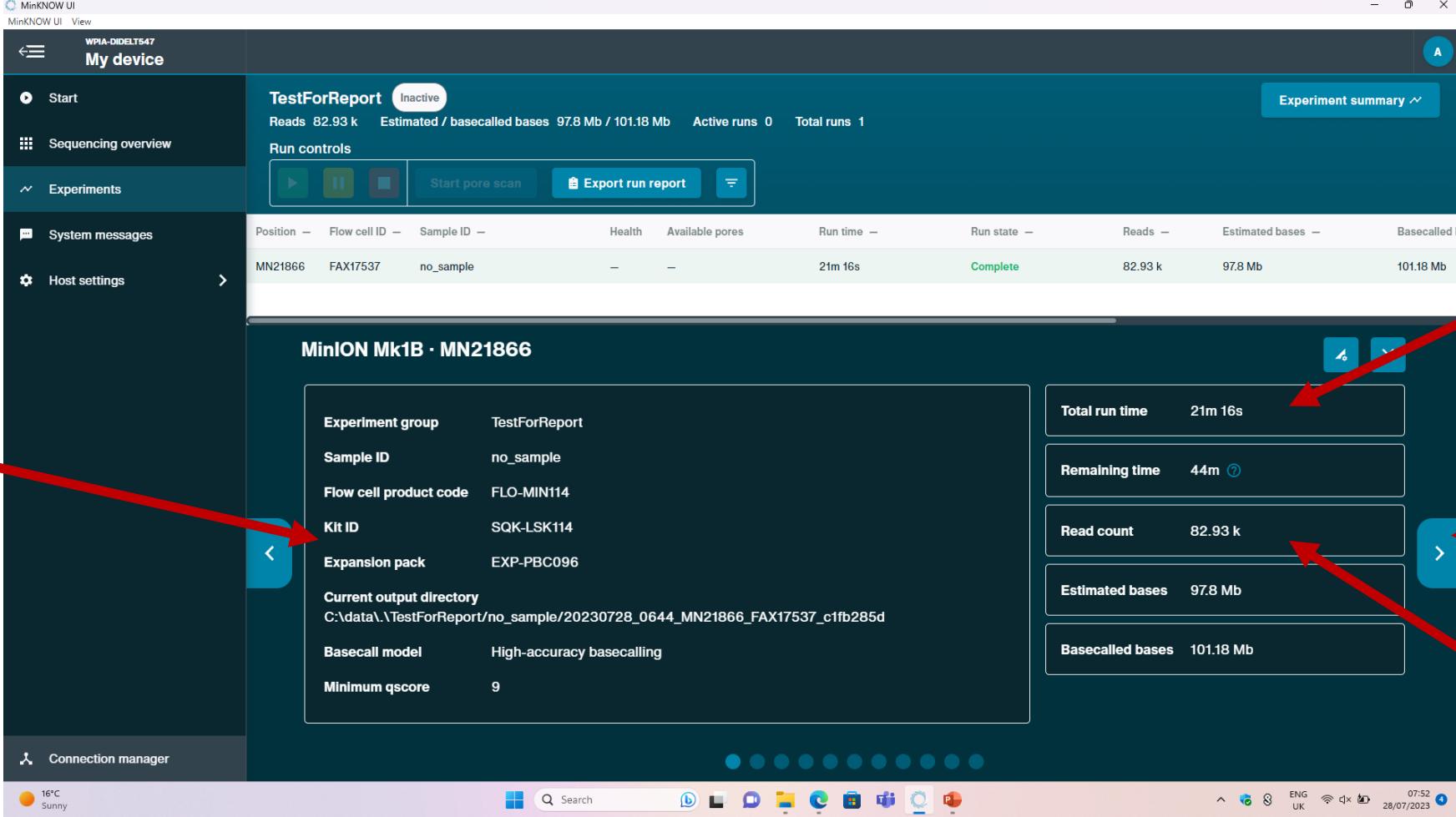
Position — Flow cell ID — Sample ID — Health Available pores Run time — Run state — Reads — Estimated bases — Basecalled |

Position	Flow cell ID	Sample ID	Health	Available pores	Run time	Run state	Reads	Estimated bases	Basecalled
MN21866	FAX17537	no_sample	—	—	21m 16s	Complete	82.93 k	97.8 Mb	101.18 Mb

Click on your run to see more details (will take you to the screen on the next slide)

3. Check your run performance

Your run settings



MinKNOW UI
WPIA-DIDELT547
My device

TestForReport Inactive

Reads 82.93 k Estimated / basecalled bases 97.8 Mb / 101.18 Mb Active runs 0 Total runs 1

Run controls

Position — Flow cell ID — Sample ID — Health Available pores Run time — Run state — Reads — Estimated bases — Basecalled bases —

MN21866	FAX17537	no_sample	—	—	21m 16s	Complete	82.93 k	97.8 Mb	101.18 Mb
---------	----------	-----------	---	---	---------	----------	---------	---------	-----------

MinION Mk1B · MN21866

Experiment group	TestForReport	Total run time	21m 16s
Sample ID	no_sample	Remaining time	44m ⓘ
Flow cell product code	FLO-MIN114	Read count	82.93 k
Kit ID	SQK-LSK114	Estimated bases	97.8 Mb
Expansion pack	EXP-PBC096	Basecalled bases	101.18 Mb
Current output directory	C:\data\.\TestForReport\no_sample\20230728_0644_MN21866_FAX17537_c1fb285d		
Basecall model	High-accuracy basecalling		
Minimum qscore	9		

Connection manager

16°C Sunny

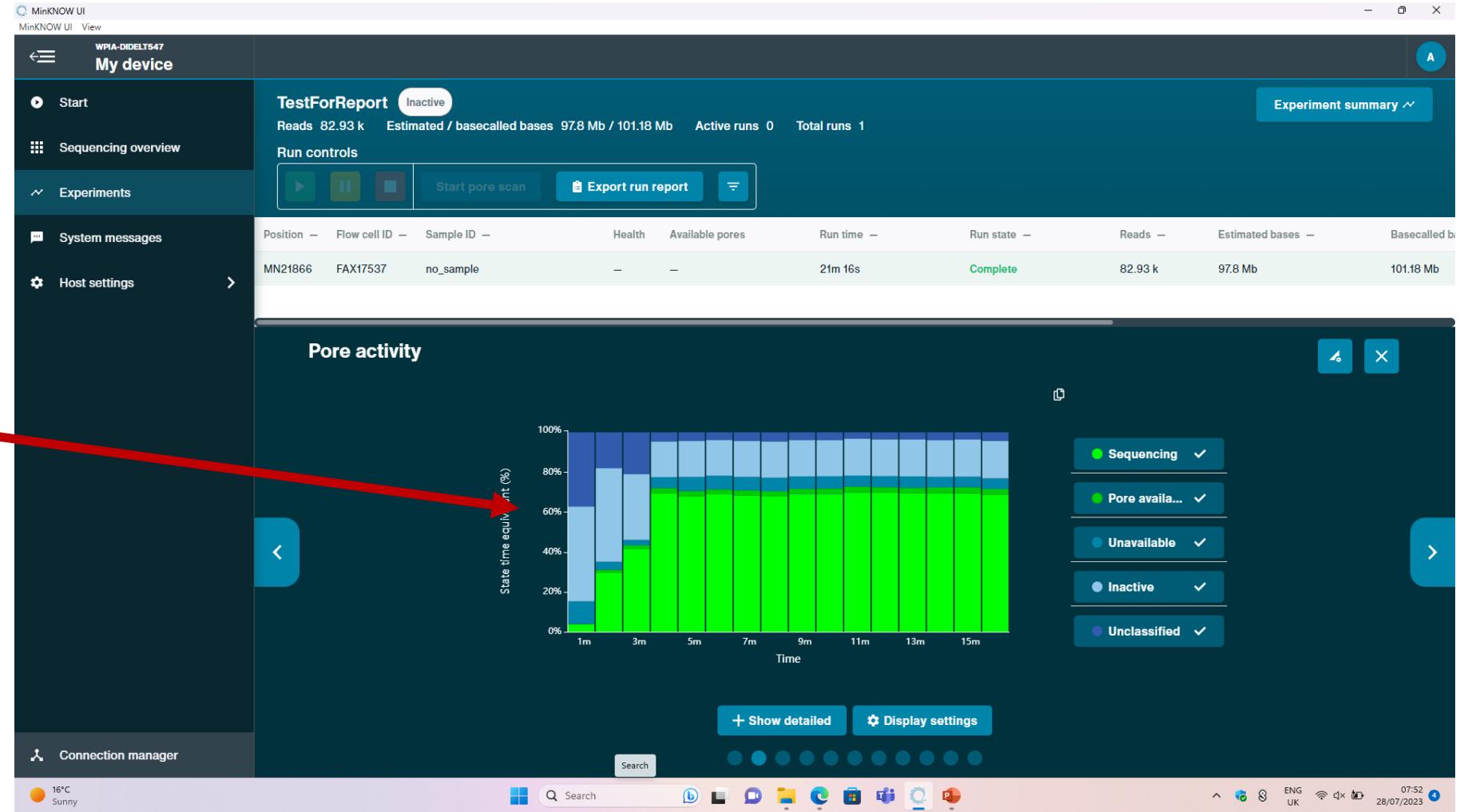
Confirm your run length (4 hours for routine stool testing by DDNS)

Click for more figures

The total number of sequencing reads generated

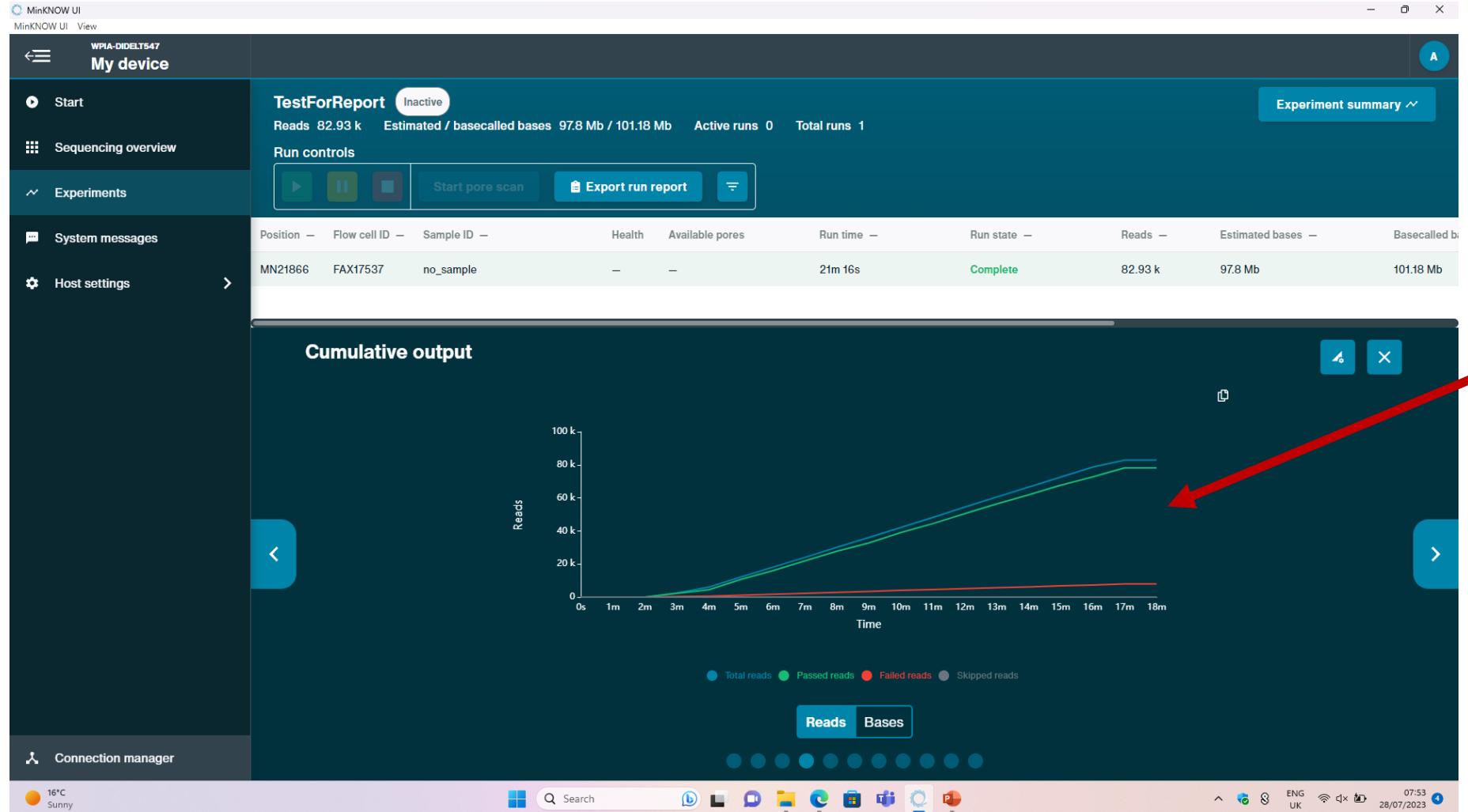
4. Check the number of pores remaining

Check how many pores are remaining at the end of your run (and again after washing). Green pores are available to sequence.



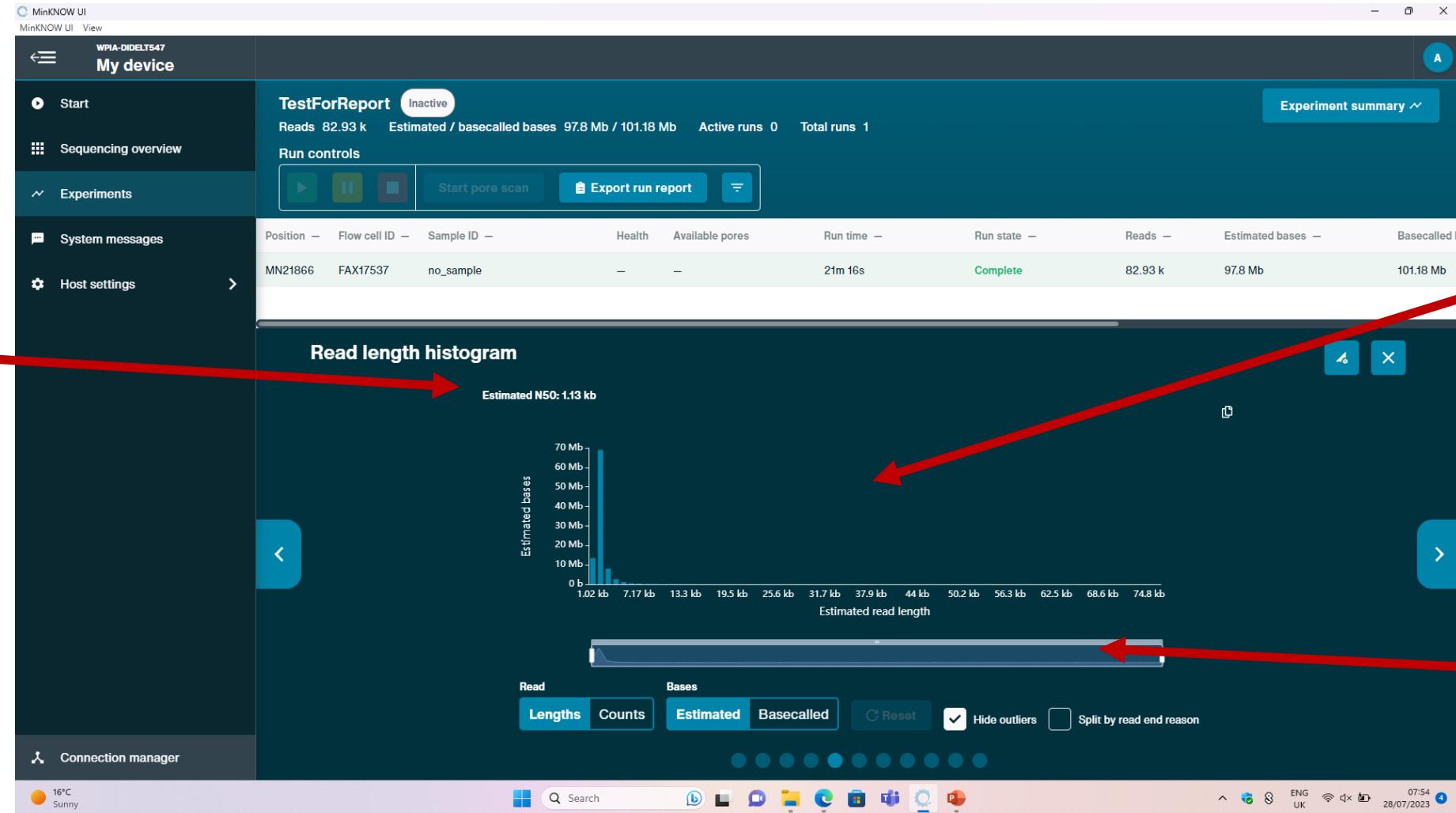
The screenshot shows the MinKNOW UI interface for a sequencing run labeled "TestForReport". The main panel displays a "Pore activity" chart showing the percentage of available pores over time (1m to 15m). A red arrow points to the y-axis label "State time equiv int (%)" on the chart. The chart area includes a legend for sequencing status: Sequencing (green), Pore available (green), Unavailable (light blue), Inactive (dark blue), and Unclassified (purple). Below the chart are buttons for "Show detailed" and "Display settings". The top right corner of the main panel shows an "Experiment summary" button. The left sidebar contains links for "Start", "Sequencing overview", "Experiments", "System messages", and "Host settings". The "Experiments" section is expanded, showing a table with columns: Position, Flow cell ID, Sample ID, Health, Available pores, Run time, Run state, Reads, Estimated bases, and Basecalled bases. The table row for the run shows: MN21866, FAX17537, no_sample, -, -, 21m 16s, Complete, 82.93 k, 97.8 Mb, 101.18 Mb. The bottom of the screen shows a taskbar with various icons and system status information.

5. Check the quality of your data



Check the proportion of reads that are passing quality checks (green line). The vast majority of reads should pass.

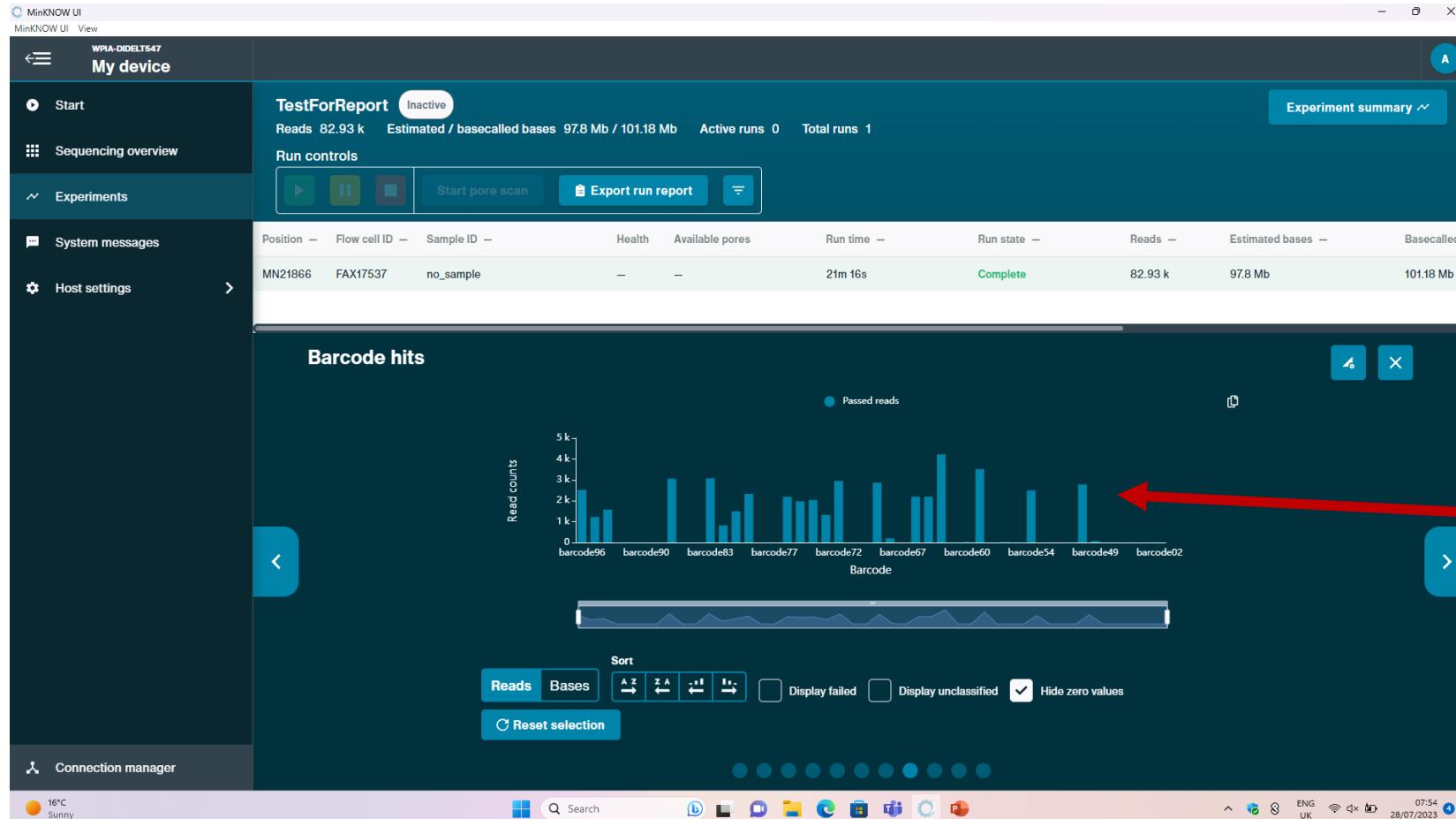
6. Check the length of your sequences



This chart shows the distribution of read lengths

Use this slider to change the width of the chart

7. Check your data has been demultiplexed



If barcoding was turned on, this chart will show the data assigned to each barcode

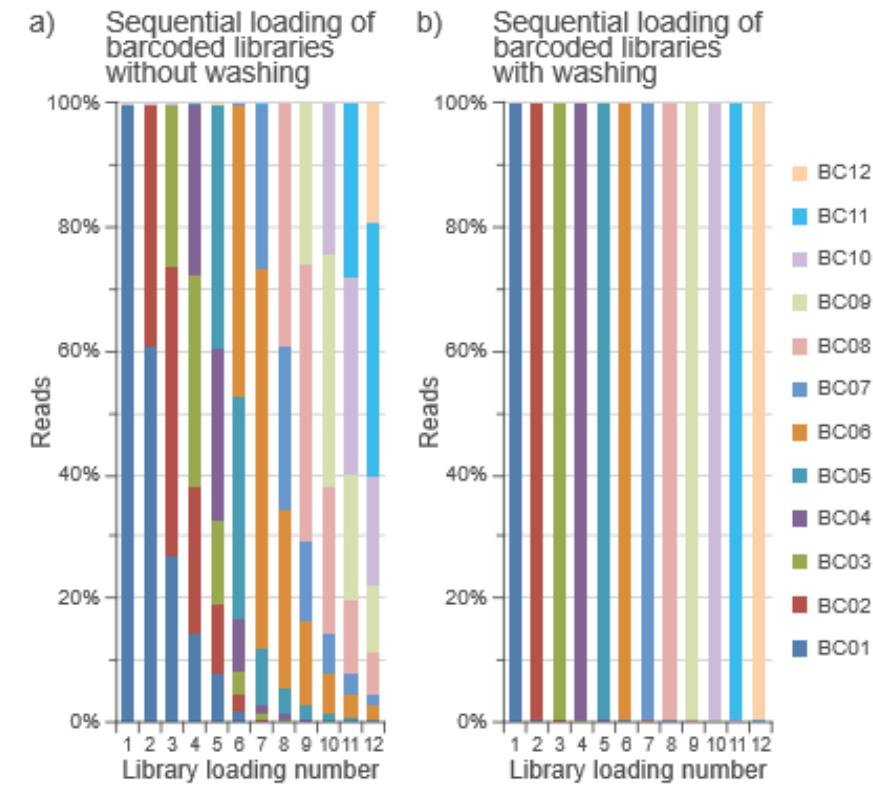
If your data has not been demultiplexed you can go to “Start”, “Analysis” and “Barcoding” in MinKNOW to perform the demultiplexing.

5. Washing a flow cell after a sequencing run

Presentation and discussion

What does washing do?

- Wash kit (WSH004) contains
 - Wash mix (DNase I, WMX)
 - Wash diluent (enzyme buffer, DIL)
 - Storage buffer (S)
- DNase I removes DNA that remains in the pores after the run ends
 - Avoids contaminating the next run
 - Improves pore availability for the next run



Protocol

- Combine 2ul of the DNase enzyme (WMX) with 398ul of the wash buffer (DIL)
- Remove any air bubbles under the priming port
- Put 200ul wash solution onto the flow cell through the priming port with the SpotOn port closed
- Incubate at room temperature for 5 minutes
- Put 200ul wash solution onto the flow cell through the priming port with the SpotOn port closed
- Incubate at room temperature for 60 minutes

Protocol

- For immediate use:
 - Flush the flow cell with Flush Buffer as described for flow cell priming
- For storage:
 - Add 500ul of the storage buffer (S) through the priming port, put the flow cell back into its packaging
 - Store in the fridge until the next use



6. Installation and use of piranha

Poliovirus investigation resource automating nanopore
haplotype analysis

Presentation and discussion



piranha



Poliovirus investigation resource automating nanopore haplotype analysis

Áine O'Toole^{1,*}, Rachel Colquhoun¹, Corey Ansley¹, Catherine Troman², Daniel Maloney¹,
Zoe Vance¹, Joyce Akello², Erika Bujaki³, Manasi Majumdar³, Adnan Khurshid⁴, Yasir
Arshad⁴, Muhammad Masroor Alam⁴, Javier Martin³, Alexander G Shaw², Nicholas C
Grassly^{2,\$}, Andrew Rambaut^{1,\$}

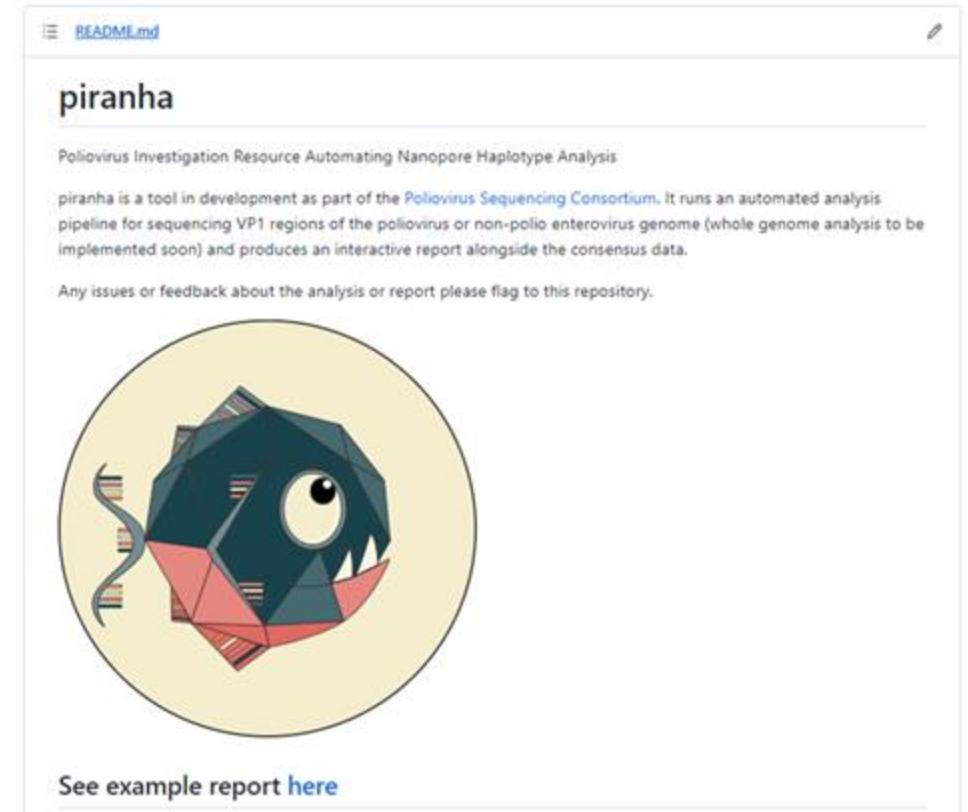
Affiliations

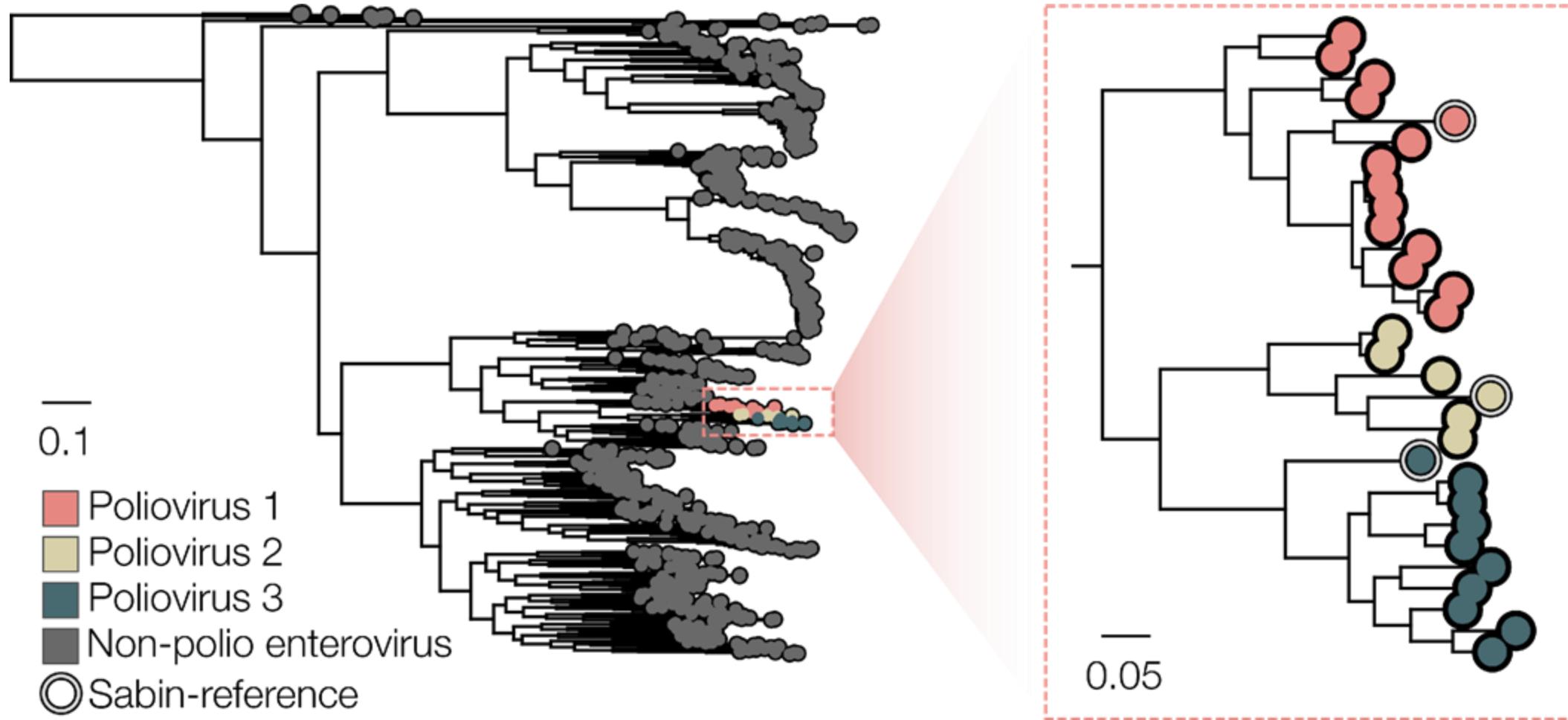
1. Institute of Ecology & Evolution, University of Edinburgh, United Kingdom
2. Department of Infectious Disease Epidemiology, Imperial College London, United Kingdom
3. Division of Virology, National Institute for Biological Standards and Control (NIBSC), Hertfordshire, United Kingdom
4. Department of Virology, National Institute for Health, Islamabad, Pakistan

PIRANHA - overview

- Takes demultiplexed fastq files, maps to reference sequences, filters by length, and produces consensus sequences and reports as output
- Requires command line
- Installation instructions and basic usage available on github:

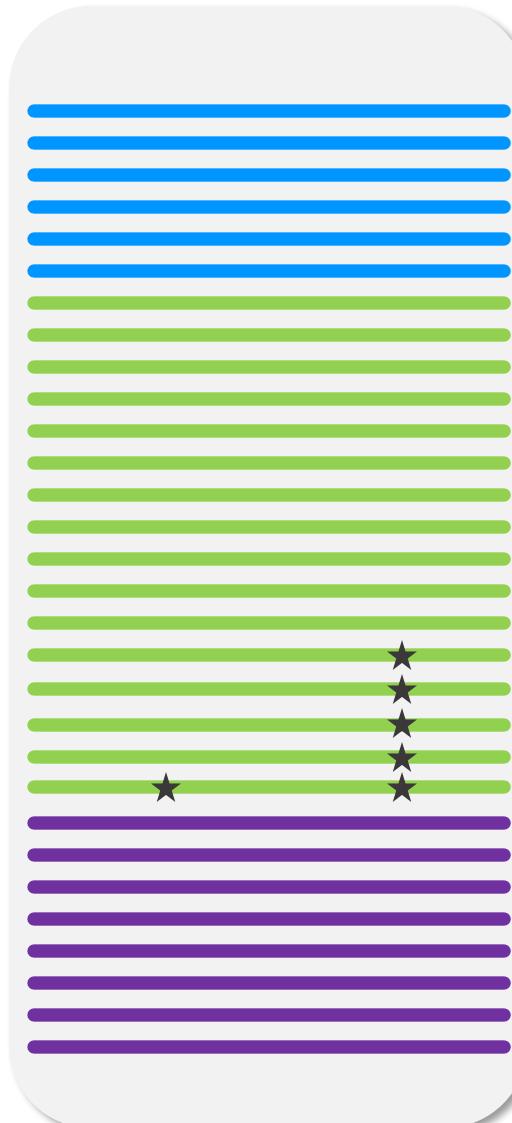
<https://github.com/polio-nanopore/piranha>



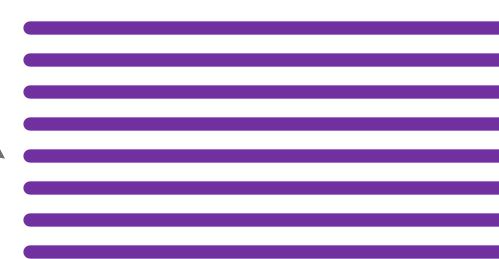
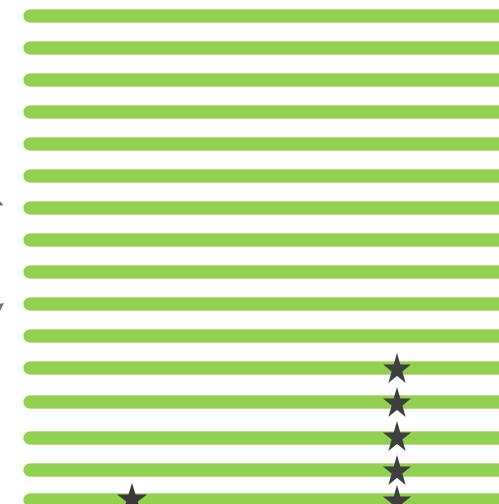


Background database: 959 VP1 sequences

Mixed Sample



Reference groups



Consensus/ haplotype





piranhaGUI



Powered by ARTIFICE | ARTICnetwork: <http://artic.network>



Piranha
Polio Direct Detection by Nanopore Sequencing (DDNS)
analysis pipeline and reporting tool

[About](#)

Polio Sequencing
Consortium

Bill & Melinda Gates Foundation OPP1171890 and OPP1207299

[Options](#)

Docker software installed

Docker is free software used to install and run the analysis pipelines.

PIRANHA software installed

Piranha is the primary analysis pipeline for the DDNS polio detection platform.

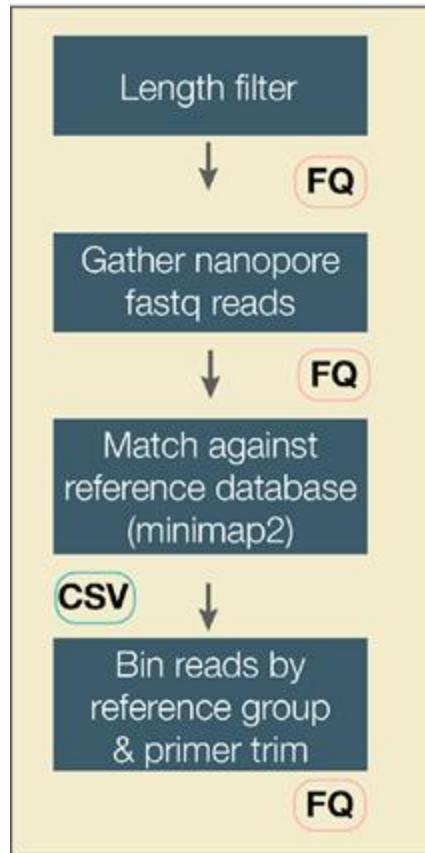
An internet connection and a Docker install is required to install or update software

[Continue](#)

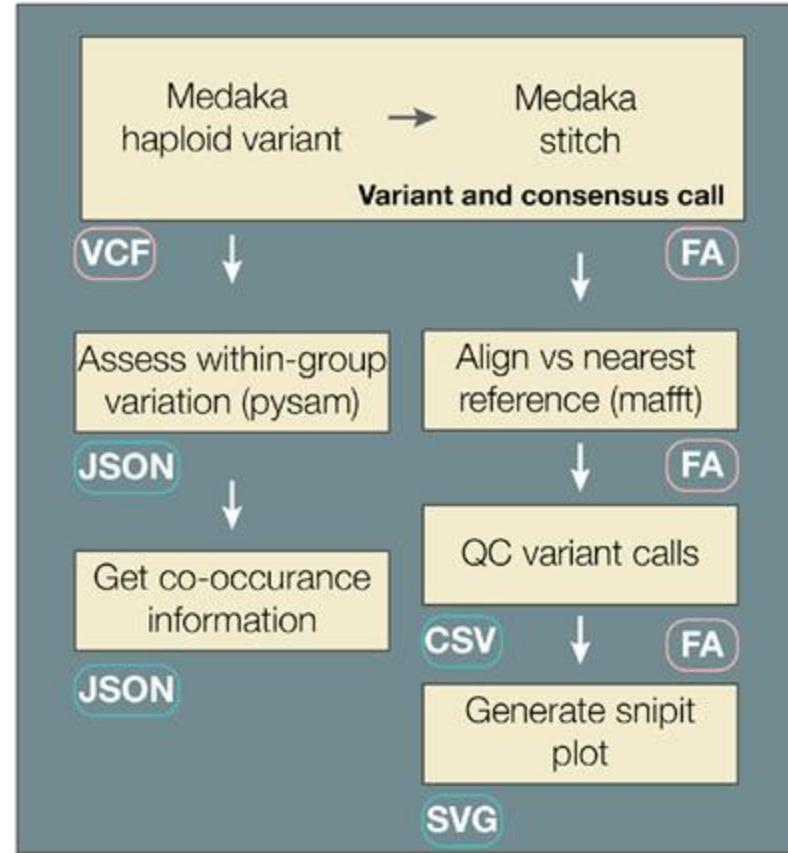
Wellcome Trust Award 206298/Z/17/Z

Workflow schema

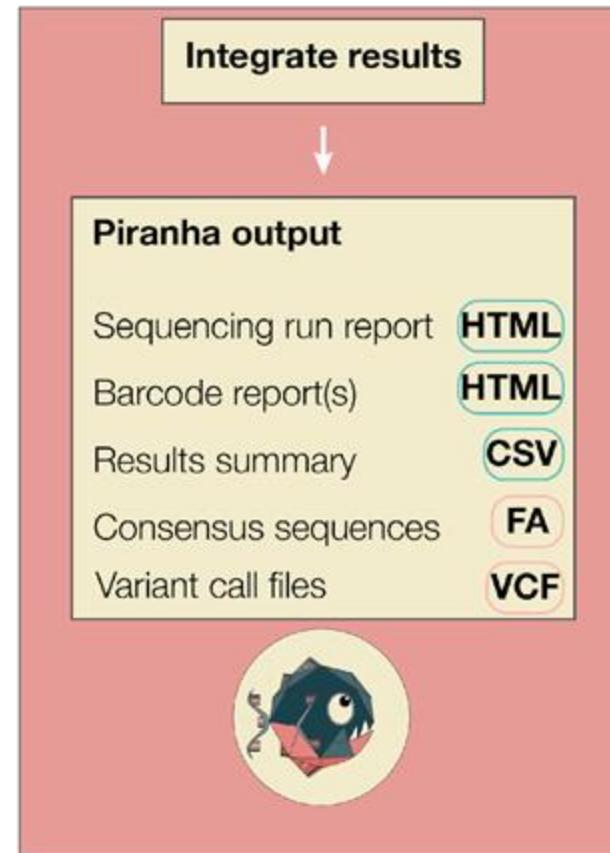
A. Barcode analysis



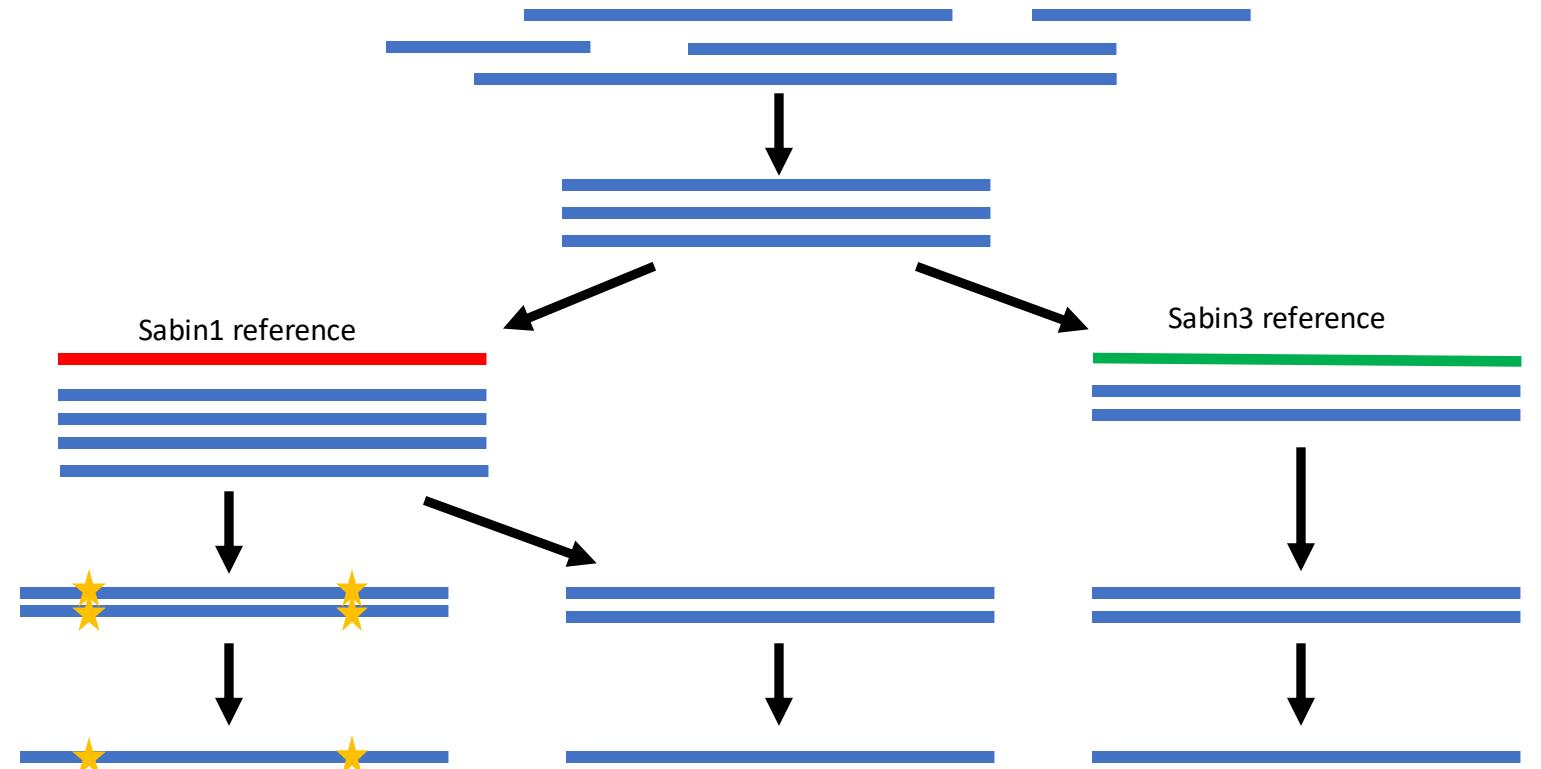
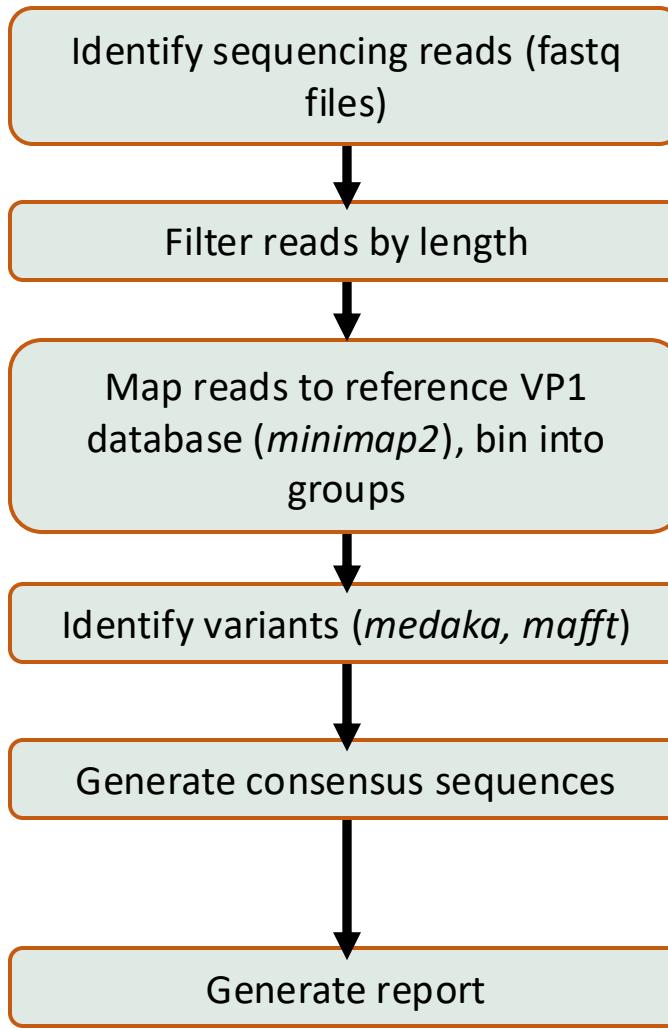
B. Reference group analysis



C. Results reporting

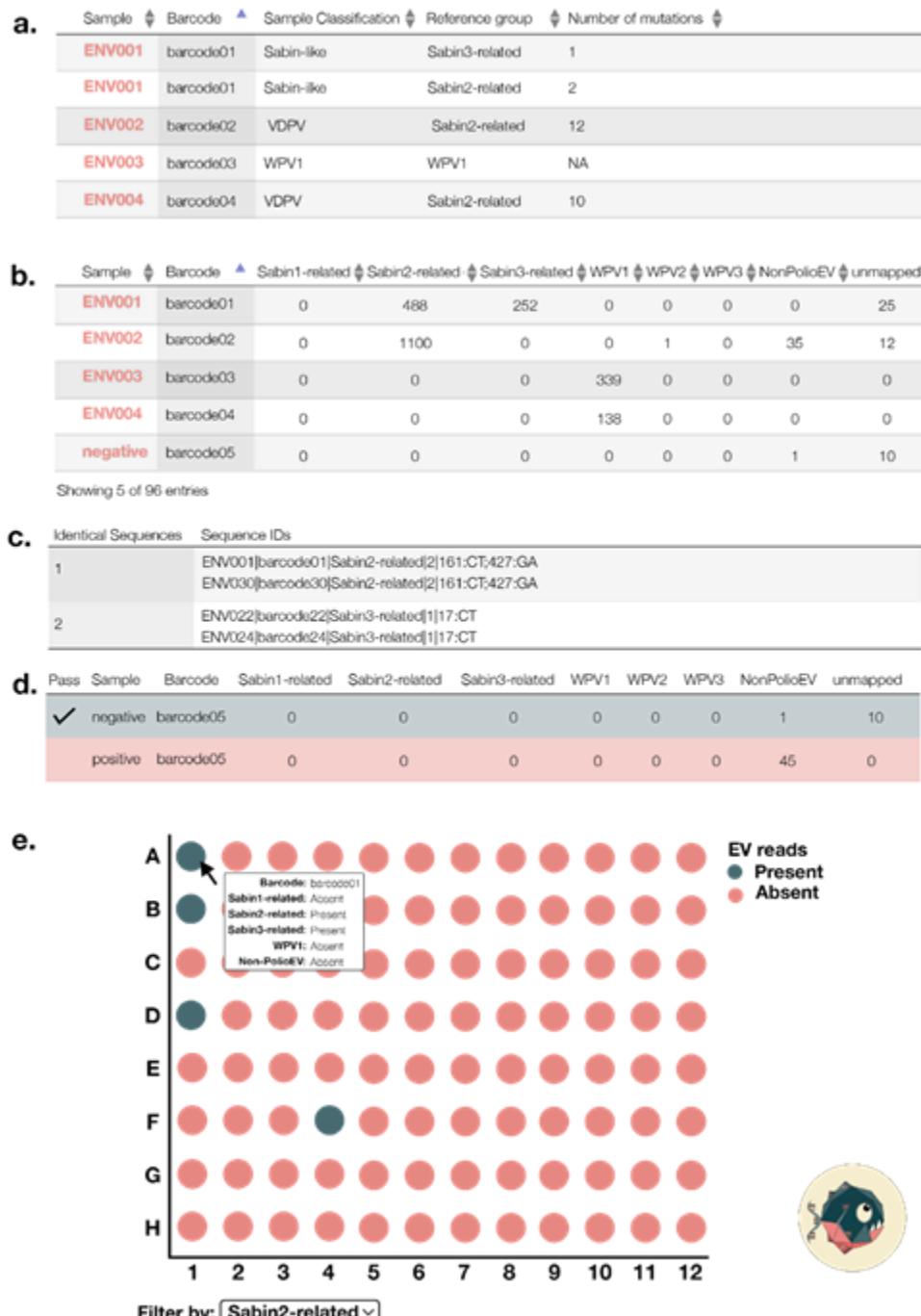


Graphic workflow



Sample	Barcode	Sample classification	Reference Group	Number of Mutations	Sequence (VP1)
Sample01	barcode01	Sabin-like	Sabin1-related	2	Download FASTA
Sample01	barcode01	Sabin-like	Sabin1-related	0	Download FASTA
Sample02	barcode02	Sabin-like	Sabin3-related	0	Download FASTA

Example report



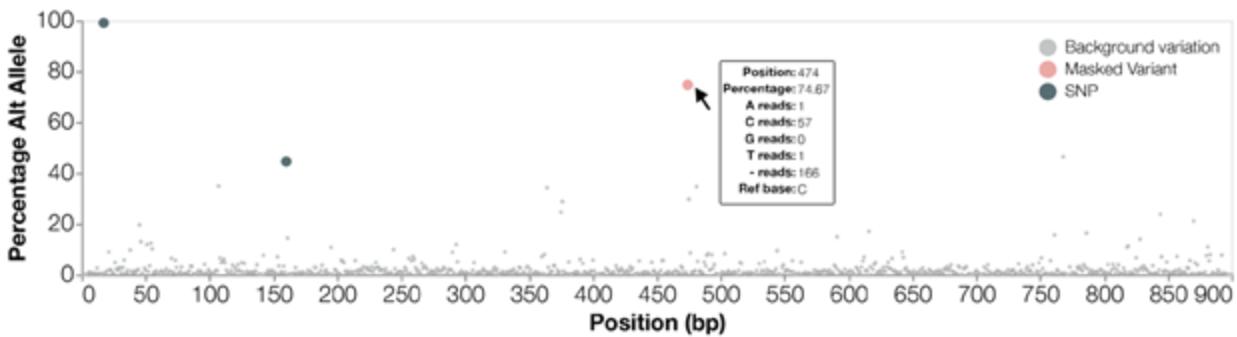
a.

Sample	Barcode	Reference group
ENV001	barcode01	Sabin3-related
ENV001	barcode01	Sabin2-related

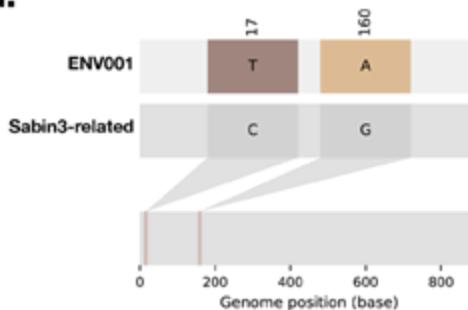
b. VP1 sequences

```
>ENV001|barcode01|Sabin3-related|Poliovirus3-Sabin_AY184221|2|17:CT;160:GA[date=2022-03-01
GGTATTGAAGATTGATTCTGAAGTTGCACAGGGCGCCCTAAGTCACCTGGCAACTCCCAGAACAGGATAGCTTACCTGATACTAAGGCCAG
TGGCCCGGGCGCATTCAGGAGGTACCTGCACACTGCAGTCGAGACTGGAGGCCAACATCCTCTGACACCACCGACACAGTTCAA
CGCGGCCACGTAGTCCAACCGACGCCAGCAGGTAGAGTCCACAATAGAAATCATTCTTGCACGCGGGCGTGCCTCGTATTATTGAGGTGG
ACAATGAACAAACCAACCCACGGGACAGAAACTATTTGCATGTGGCGCATTACATAACAAAGATAACAGTGCAGTTGCAGCGTAAGTTGA
```

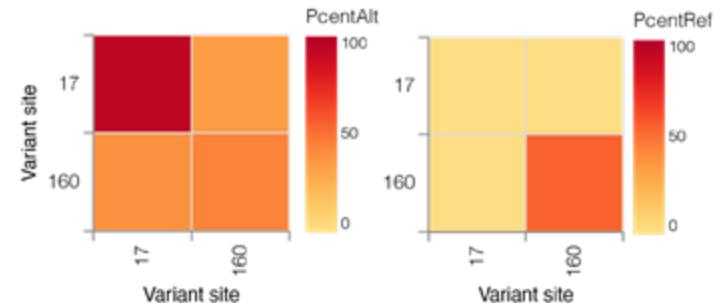
c.



d.



e.



PiranhaGUI

- Uses *Docker* to allow running of PIRANHA on different operating system (e.g. Windows, Linux) and to avoid using the command line!
- Available on github here:
<https://github.com/polio-nanopore/piranha>
- Requires same input as piranha and produces the same output

PiranhaGUI Installation

- Download from <https://github.com/polio-nanopore/piranha/releases>
- To install for windows, download the [PiranhaGUIvX.Y.Z_installer_windows.exe](#) (where X.Y.Z is the version)
- Double click the downloaded file to install
- Open the PiranhaGUI when installed
- Click on the “Install Docker” button to go to the Docker website to download Docker
- Click on the “Install piranha” button to install piranha inside piranhaGUI.

Barcodes file

	A	B	C	D	E	F
1	sample	barcode	EPID	Well	IsQCTest	IfRetestOriginalRun
2	sample01	barcode01	HQU-JAD-JAS-04	A01	No	
3	sample02	barcode02	HQU-JAD-JAS-04	B01	No	
4	sample03	barcode03	ANB-NSD-AJD-03	C01	No	
5	sample04	barcode04	ANB-NSD-AJD-03	D01	No	
6	positive_control	barcode05		E01		
7	negative_ext	barcode06		F01		
8	negative_RTPCR	barcode07		G01		

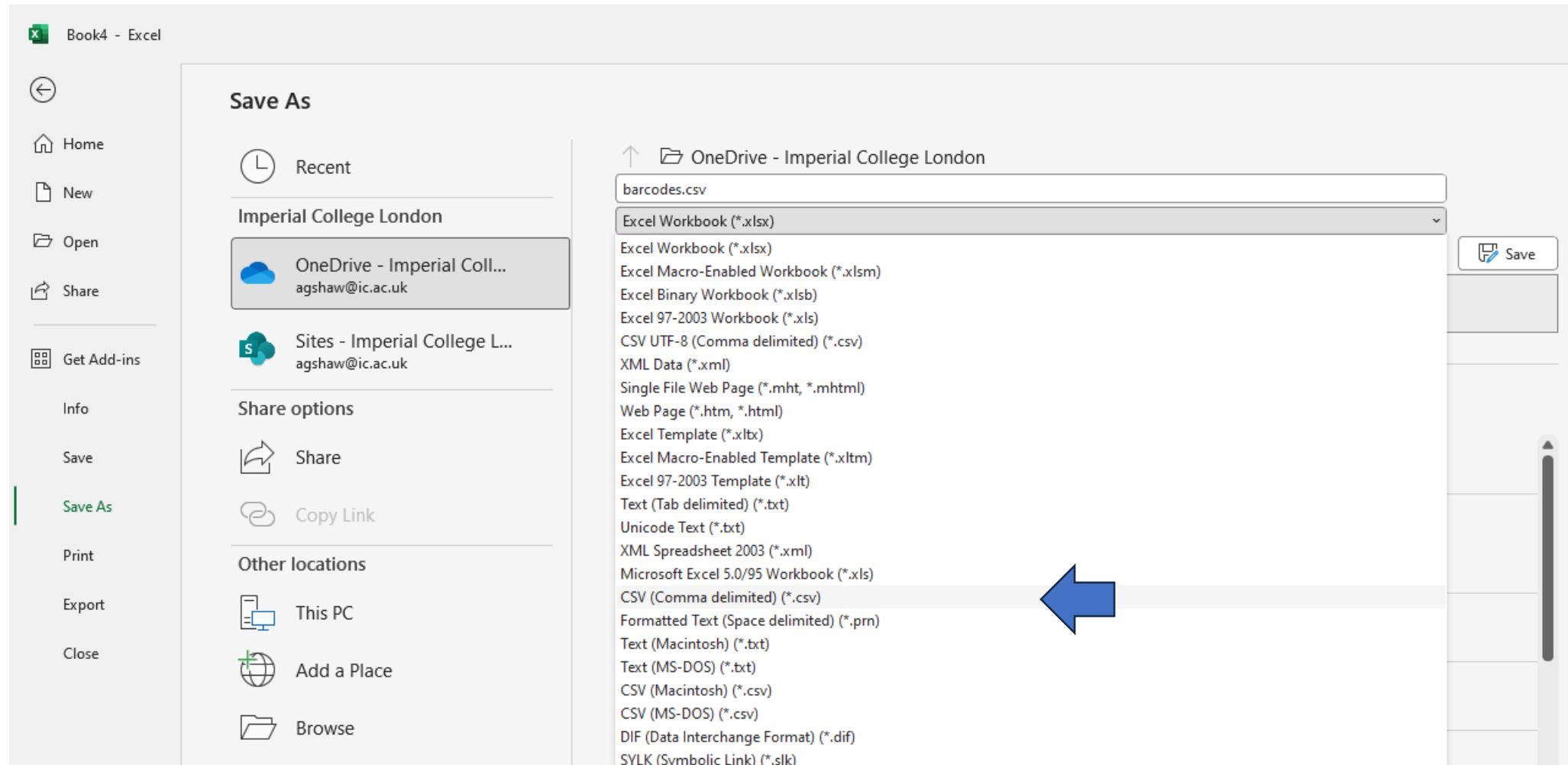
Links your barcodes and your samples.

Use the headers “sample” and “barcode”

Barcodes must be entered as “barcode##”

More metadata columns can be added

Saving as a .csv from excel

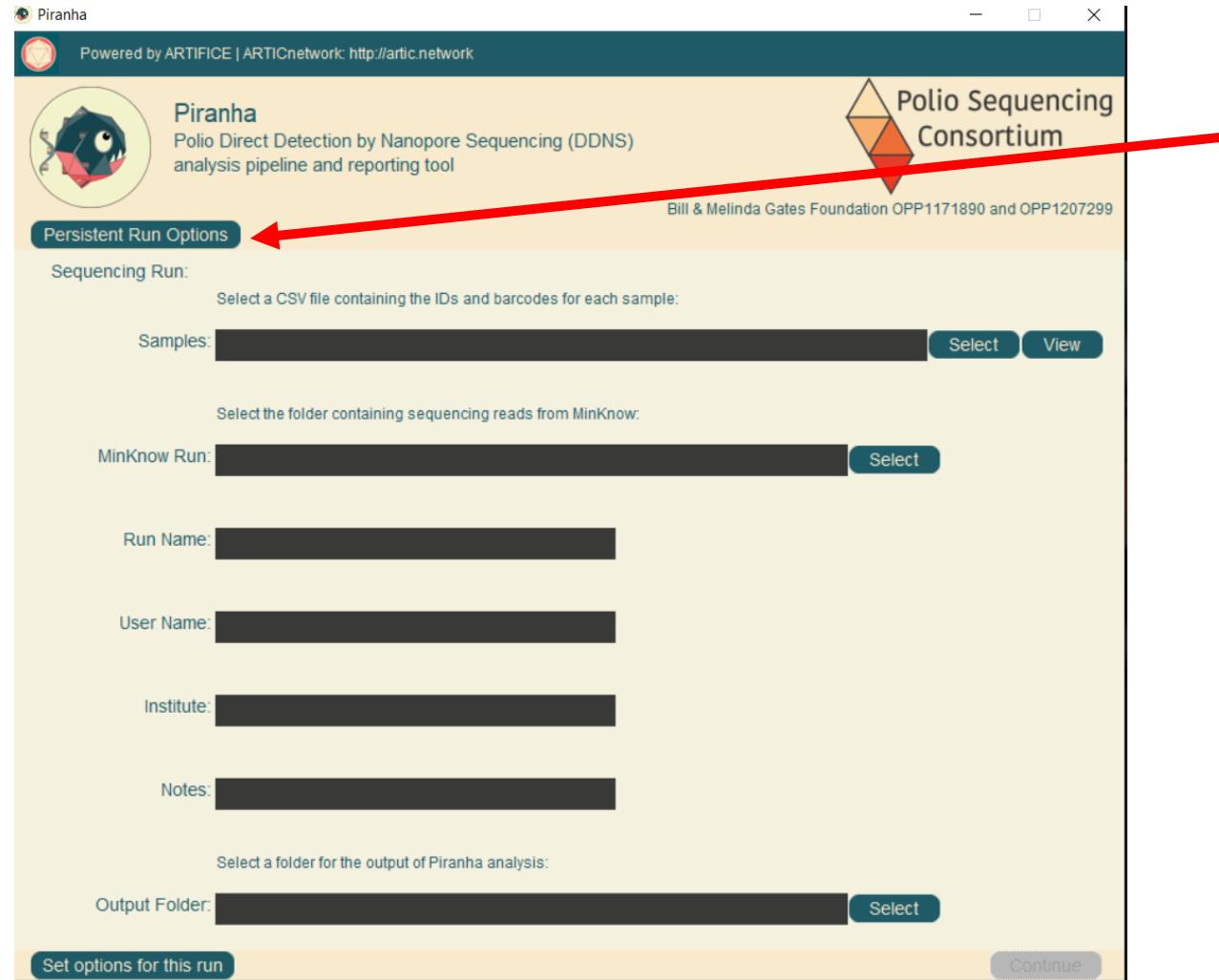


Appearance of a .csv file

File Edit View

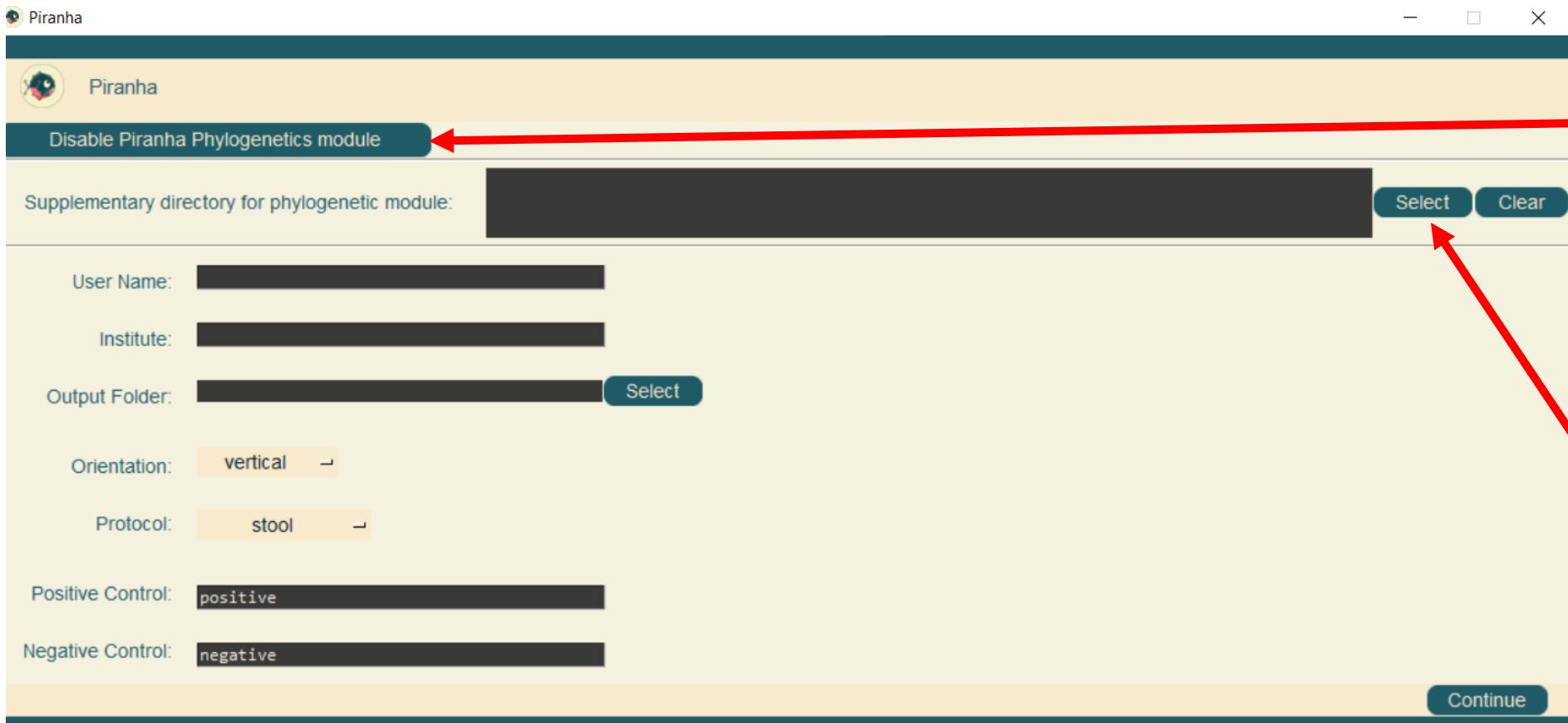
```
sample,barcode,EPID,Well,IsQCTest>IfRetestOriginalRun
sample01,barcode01,HQU-JAD-JAS-04,A01,No,
sample02,barcode02,HQU-JAD-JAS-04,B01,No,
sample03,barcode03,ANB-NSD-AJD-03,C01,No,
sample04,barcode04,ANB-NSD-AJD-03,D01,No,
positive_control,barcode05,,E01,,
negative_ext,barcode06,,F01,,
negative_RTPCR,barcode07,,G01,,
```

Running PiranhaGUI



- Click here to set up the phylogenetic module

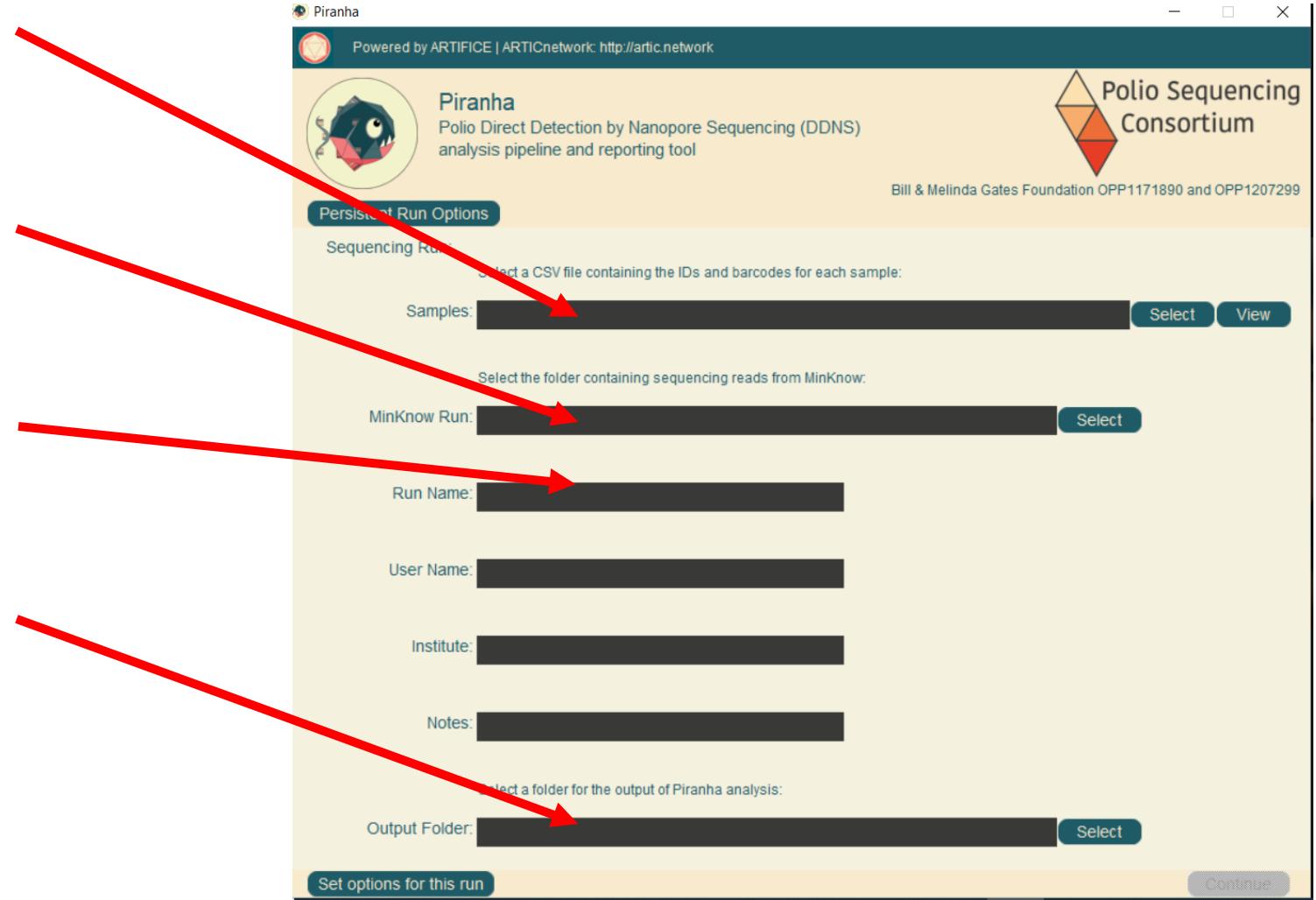
Running PiranhaGUI



- Turn on/off phylogenetics module
- Select a folder containing sequences to add to the tree (e.g. your laboratory sequence QC database)

Running PiranhaGUI

- Select your barcode.csv file
- and your demultiplexed sequencing data
- Set your run name
- Select an output folder for your results
- Click “Continue”



- Set run options. For DDNS stool testing:

Minimum length - 1000
bp

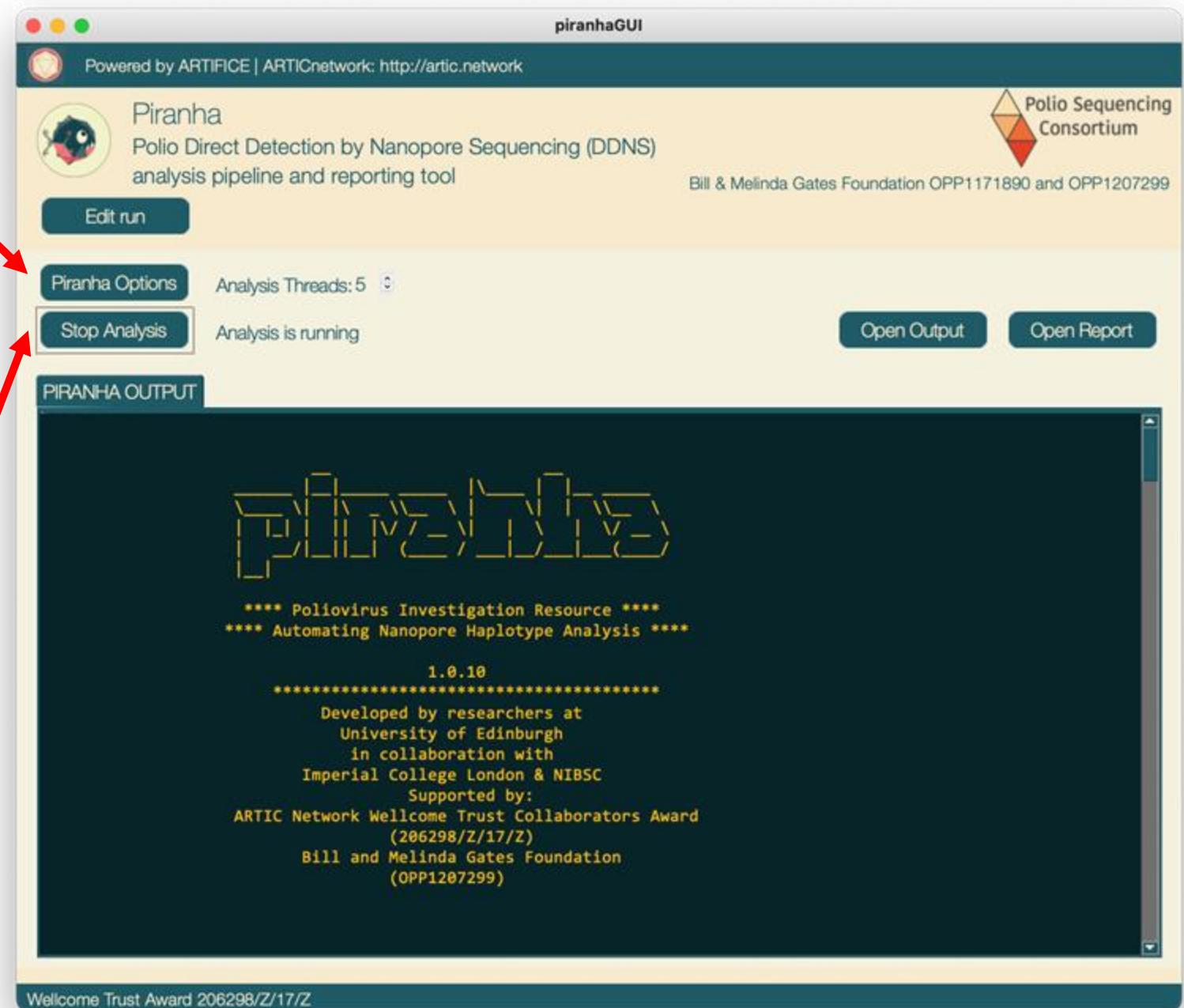
Maximum length - 1300
bp

Minimum read depth –
50

Minimum read
percentage – 0

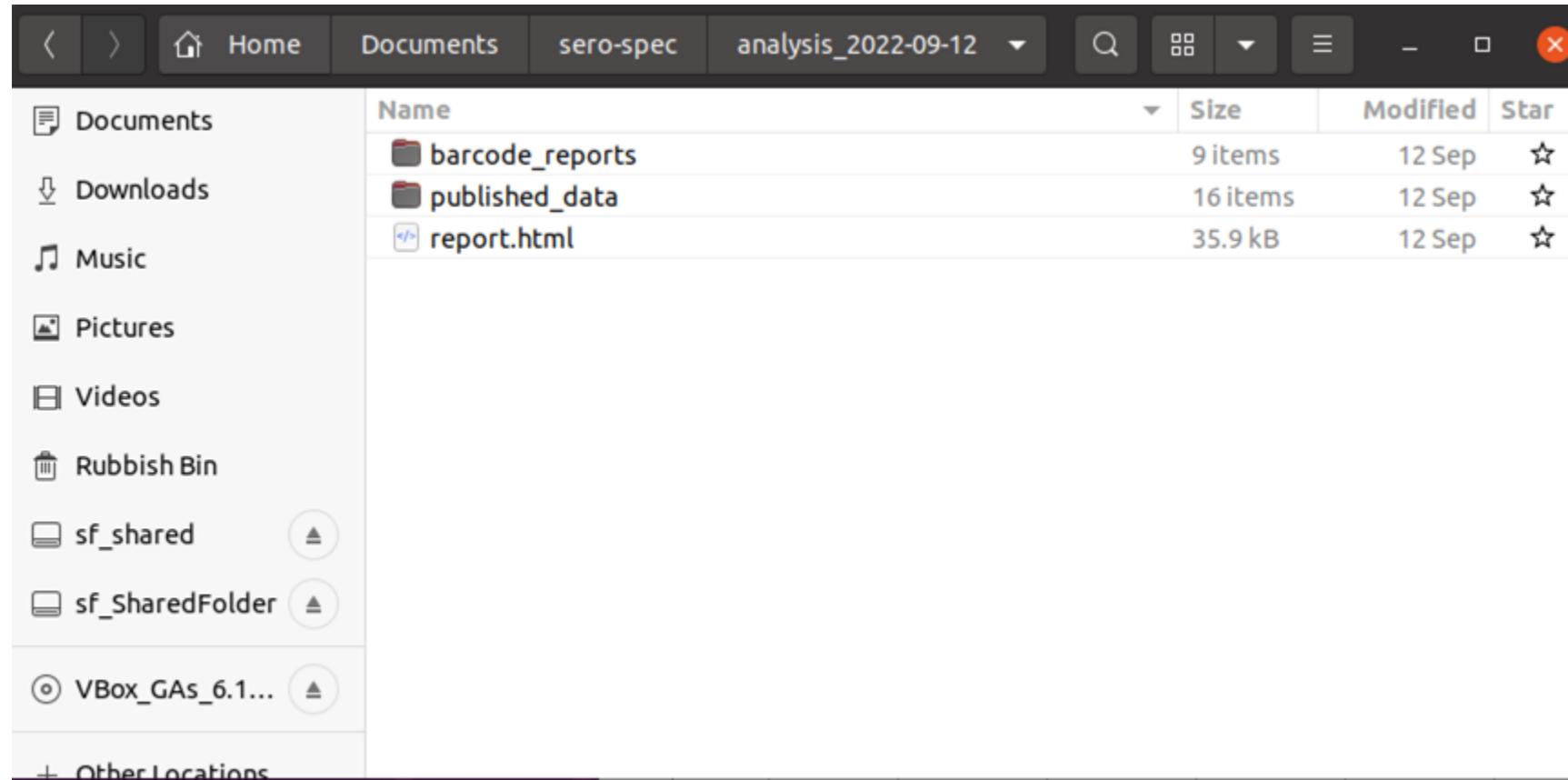
(see QC document)

- Start analysis



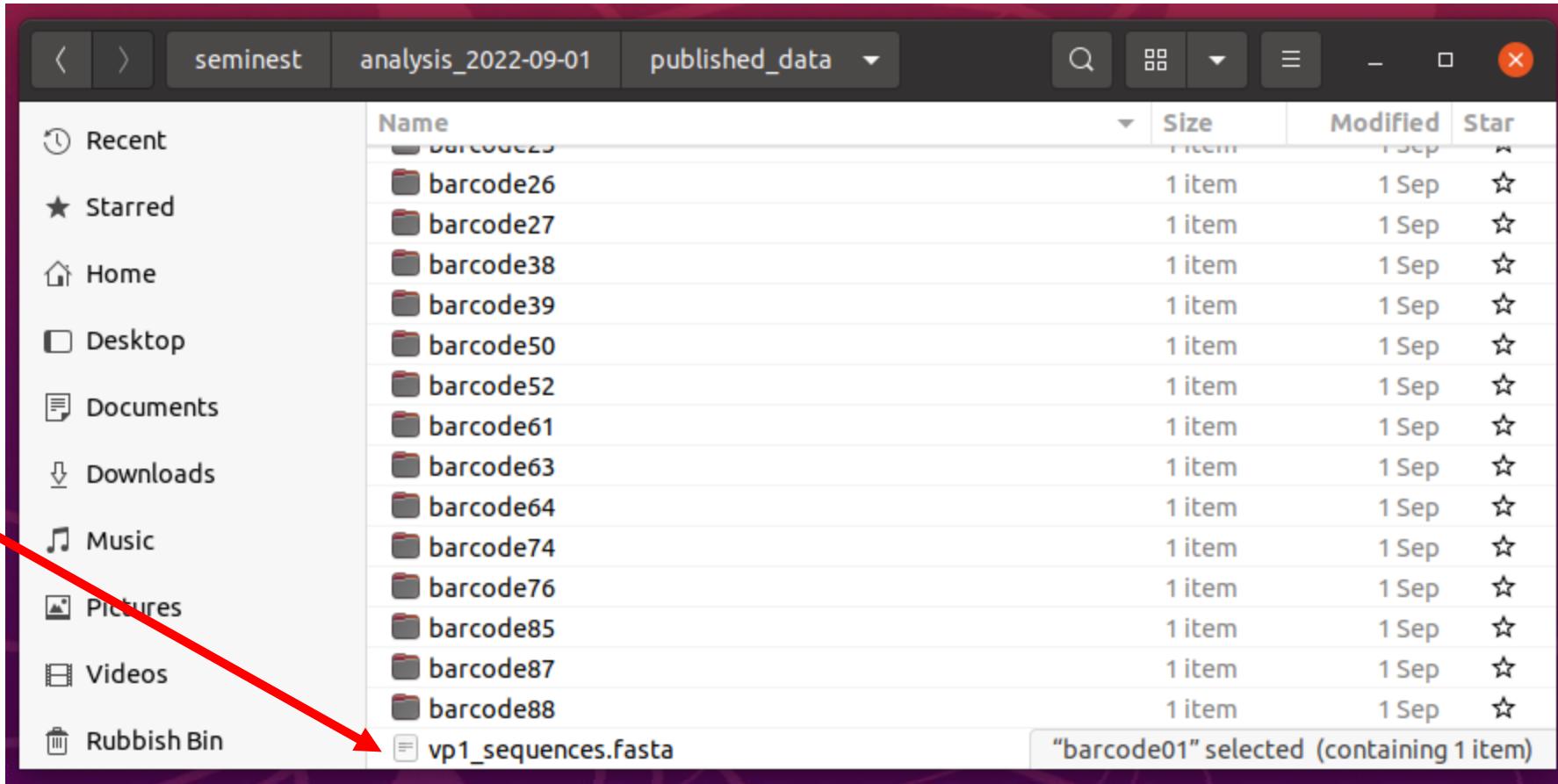
PIRANHA - output

- Outputs a summary report html, individual barcode reports, and consensus sequences as a fasta (both individual and in one file)



PIRANHA fasta output

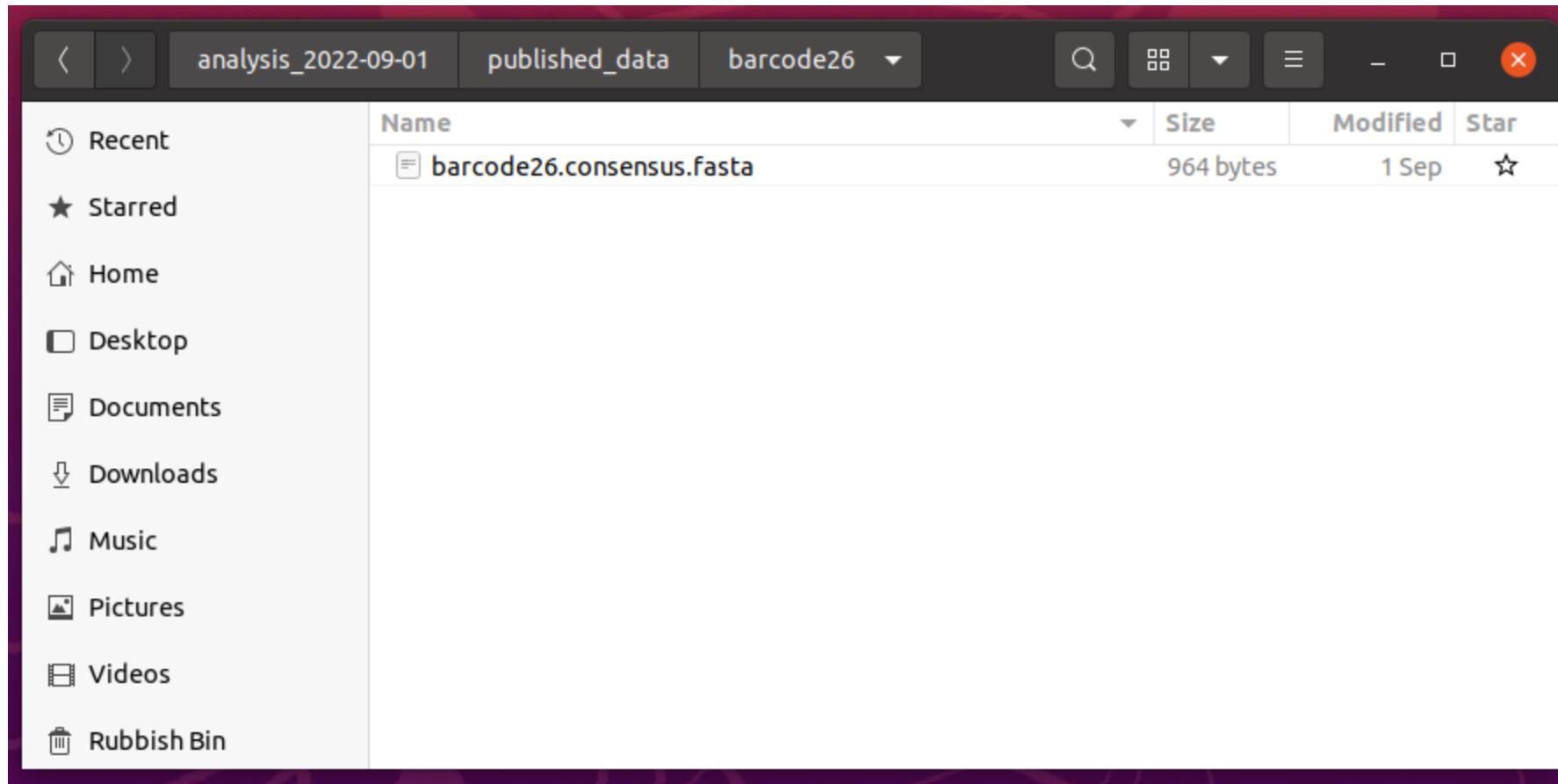
All VP1
sequences
for the
sequencing
run



	Name	Size	Modified	Star
Recent	barcode23	1 item	1 Sep	☆
Starred	barcode26	1 item	1 Sep	☆
Home	barcode27	1 item	1 Sep	☆
Desktop	barcode38	1 item	1 Sep	☆
Documents	barcode39	1 item	1 Sep	☆
Downloads	barcode50	1 item	1 Sep	☆
Music	barcode52	1 item	1 Sep	☆
Pictures	barcode61	1 item	1 Sep	☆
Videos	barcode63	1 item	1 Sep	☆
Rubbish Bin	barcode64	1 item	1 Sep	☆
	barcode74	1 item	1 Sep	☆
	barcode76	1 item	1 Sep	☆
	barcode85	1 item	1 Sep	☆
	barcode87	1 item	1 Sep	☆
	barcode88	1 item	1 Sep	☆
	vp1_sequences.fasta	1 item	1 Sep	☆

"barcode01" selected (containing 1 item)

PIRANHA fasta output



Sample contents overview

Navigate to individual sample reports

Sample	Barcode	Sample Call	Reference Group	Number Of Mutations	Vp1 sequence	Search: <input type="text"/>
sample01	barcode25	Sabin-like	Sabin3-related	1	Download FASTA	
sample02	barcode26	Sabin-like	Sabin1-related	0	Download FASTA	
sample02	barcode26	Sabin-like	Sabin3-related	1	Download FASTA	
sample02	barcode26	Sabin-like	Sabin2-related	4	Download FASTA	
sample03	barcode27	VDPV	Sabin2-related	106	Download FASTA	
sample07	barcode32	Sabin-like	Sabin3-related	2	Download FASTA	
sample07	barcode32	Sabin-like	Sabin2-related	0	Download FASTA	
sample08	barcode33	Sabin-like	Sabin1-related	1	Download FASTA	
sample08	barcode33	Sabin-like	Sabin3-related	1	Download FASTA	
sample09	barcode34	Sabin-like	Sabin1-related	0	Download FASTA	
sample09	barcode34	Sabin-like	Sabin3-related	1	Download FASTA	
sample09	barcode34	Sabin-like	Sabin2-related	2	Download FASTA	
sample11	barcode36	VDPV	Sabin2-related	126	Download FASTA	
sample12	barcode37	WPV2	WPV2	NA	Download FASTA	
sample12	barcode37	NonPolioEV	NonPolioEV	NA	Download FASTA	
sample12	barcode37	VDPV	Sabin2-related	135	Download FASTA	
sample13	barcode38	WPV2	WPV2	NA	Download FASTA	
sample13	barcode38	VDPV	Sabin2-related	142	Download FASTA	

Table 2 | Composition of samples

Export table

Sample	Barcode	Sabin1-Related	Sabin2-Related	Sabin3-Related	Wpv1	Wpv2	Wpv3	Nonpolioev	Unmapped	Search: <input type="text"/>
neg1	barcode31	0	0	0	0	0	0	0	0	
neg2	barcode39	0	0	0	0	0	0	0	0	
sample01	barcode25	144	0	2408	3	0	0	1	0	
sample02	barcode26	200	499	1003	0	0	0	0	0	
sample03	barcode27	0	2803	1	1	221	0	0	1	
sample04	barcode28	0	0	0	0	0	0	0	0	
sample05	barcode29	0	0	0	0	0	0	0	0	
sample06	barcode30	0	0	0	0	0	0	0	0	
sample07	barcode32	0	1133	1367	71	0	0	200	1	
sample08	barcode33	445	1	2800	0	0	0	7	1	
sample09	barcode34	558	436	1696	2	0	0	54	1	
sample10	barcode35	0	0	0	0	0	0	0	0	
sample11	barcode36	0	68	3	0	0	0	0	1	
sample12	barcode37	0	1134	4	9	1198	0	370	1	

Download fasta files of consensus sequences

Read numbers per sample

sample08 report 2022-09-12

Sample reports give details of determined contents and fasta consensus sequences

Table 1 | Summary of sample content

Search:

Sample	Barcode	Reference Group
sample08	barcode33	Sabin1-related
sample08	barcode33	Sabin3-related

VP1 sequences

```
>sample08|barcode33|Sabin1-related|Poliovirus1-Sabin_AY184219|1|268:AT  
GGGTTAGGTAGATGCTTGAAGCATGATTGACAACACAGTCGGTAAACCGTGCGCAACGTCTAGAGACGCTCTCCAAACACTGAAGCCAGTGGACCAGCACACTCCAAGGAAATTCCGGCACTCACCAGTGGAAACTGGGGCAC  
AAATCCACTAGTCCCTTCTGATACAGTGCAAACCAAGACATGTTGTACAACATAGGTCAAGGTCAAGAGTCTAGCATAGAGTCTTCTTCGCGCGGGGTGCATCGTGGCCATTAAACCGTGGATAACTCAGCTTCCACCAAGAATAAGGATAAGC  
TATTACAGTGTGGAAGATCACTTATAAGATACTGTCCAGTTACGGAGGAAATTGGAGTTCTTCACCTATTCTAGATTGATATGGAATTACCTTGTGGTTACTGCAAATTCACTGAGACTAACAAATGGGCATGCCTTAATCAAGTGTAC  
CAAATTATGTACGTACCAACCAAGCGCTCAGTGCCCCAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTACACCTACGGAACAGCTCAGGCCGATCTCGGTACCGTATTTGGTATTTGAACGCCATTCA  
CTTTACGACGGTTTTCAAGTACCACTGAAGGGACCAAGTCGGCAGCACTAGGTGACTCCCTCTATGGTGAGCATCTCTAAATGACTTCGGTATTGGCTGTTAGAGTAGTCAATGATCACAAACCCGACCAAGGTCAACCTCAGAG
```

[Download](#)

Sabin1-Related variant report

Table 2 | Sabin1-related

Information	
Reference group	Sabin1-related
Number of mutations	1
Mutations	268:AT

For each consensus it shows mutation details including location on the VP1 region

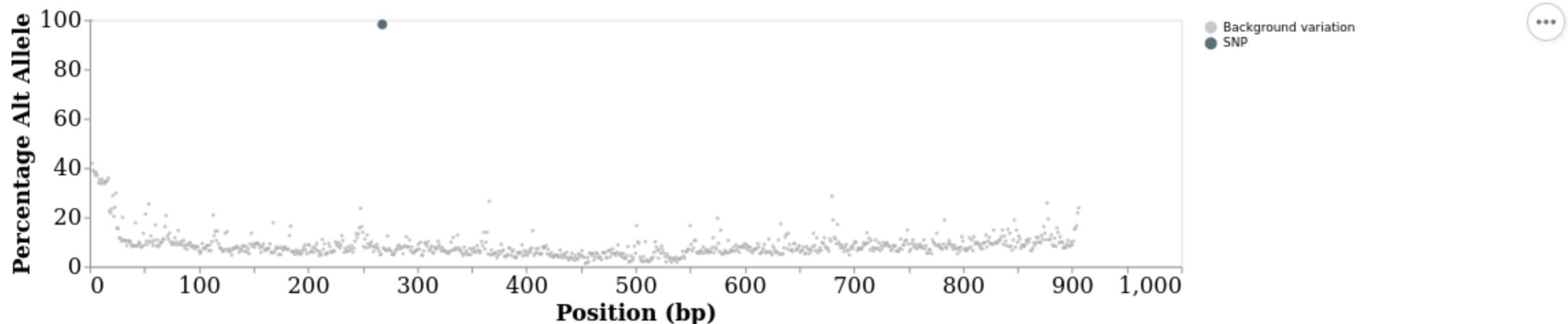


Figure 1 | Variation (errors + mutations) across Sabin1-Related reference in sample08

~1SNP

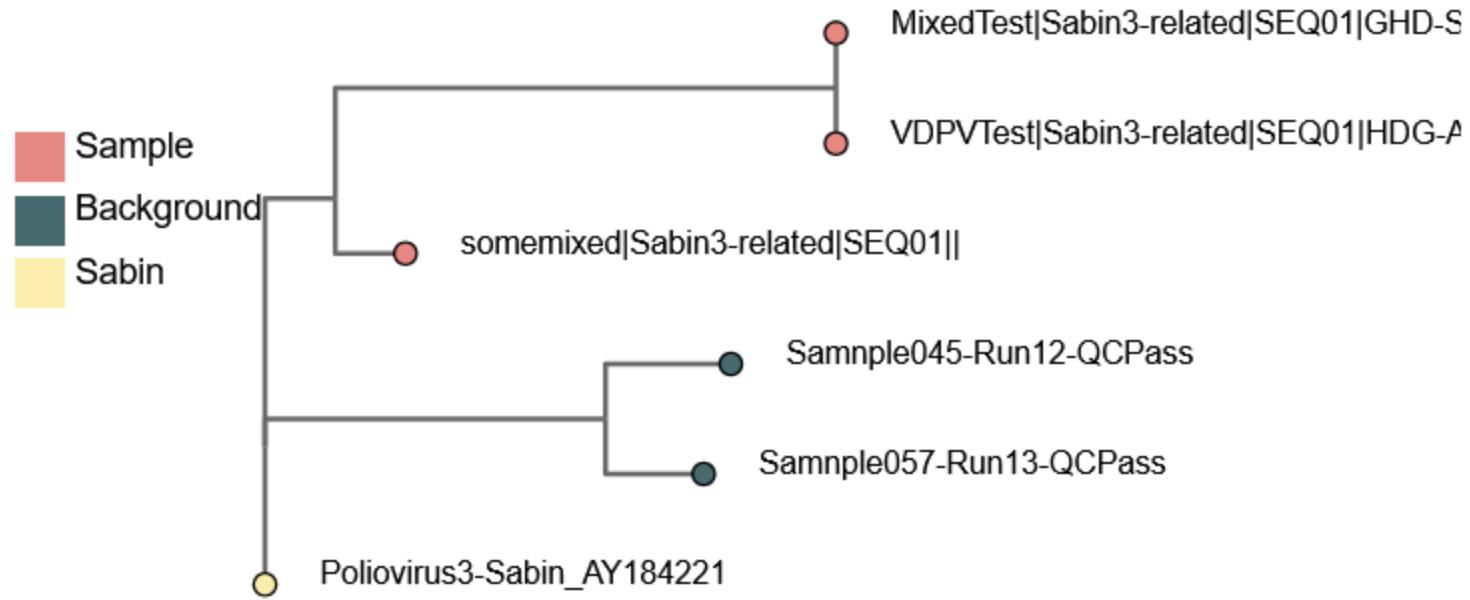


Figure 3 | Sabin3-related phylogeny

- Trees can be used to identify contamination within the run.
- Historical sequences from the lab (stored in the laboratory sequence QC database) can help identify contamination from experiments.

Detailed run report

	A	B	C	D	E	F	G
1	sample	barcode	EPID	institute	well	IsQCRetes	IfRetestOri
2	MixedTest	barcode01	GHD-SHD-AKD-02		A01	No	
3	PureTest	barcode02	HDG-AKS-UHE-03		B01	No	
4	WTTest	barcode03	GHD-SHD-AKD-10		C01	No	
5	VDPVTest	barcode04	HDG-AKS-UHE-12		D05	No	
6	negative	barcode05			D06		
7	positively	barcode06			D07		
8	somemixed	barcode07			H12		
9							

...

AI	AJ	AK	AL	AM	AN
Sabin1-rel	Sabin2-rel	Sabin2-rel	Sabin2-rel	Sabin2-rel	Sabin2-rel
	Poliovirus2	246	1	99.89	Sabin-like F
	Poliovirus2	708	10	98.89	VDPV
		1			
		0			
		0			
		0			
	Poliovirus2	137	0	100	Sabin-like F

- Includes data from barcodes.csv with the sequencing results now appended
- Can be annotated during QC process, marking samples to “pass” and report, or those that are “pending” and need further investigation.
- Additional metadata can be added as it becomes available (e.g. ITD results, Sanger results)

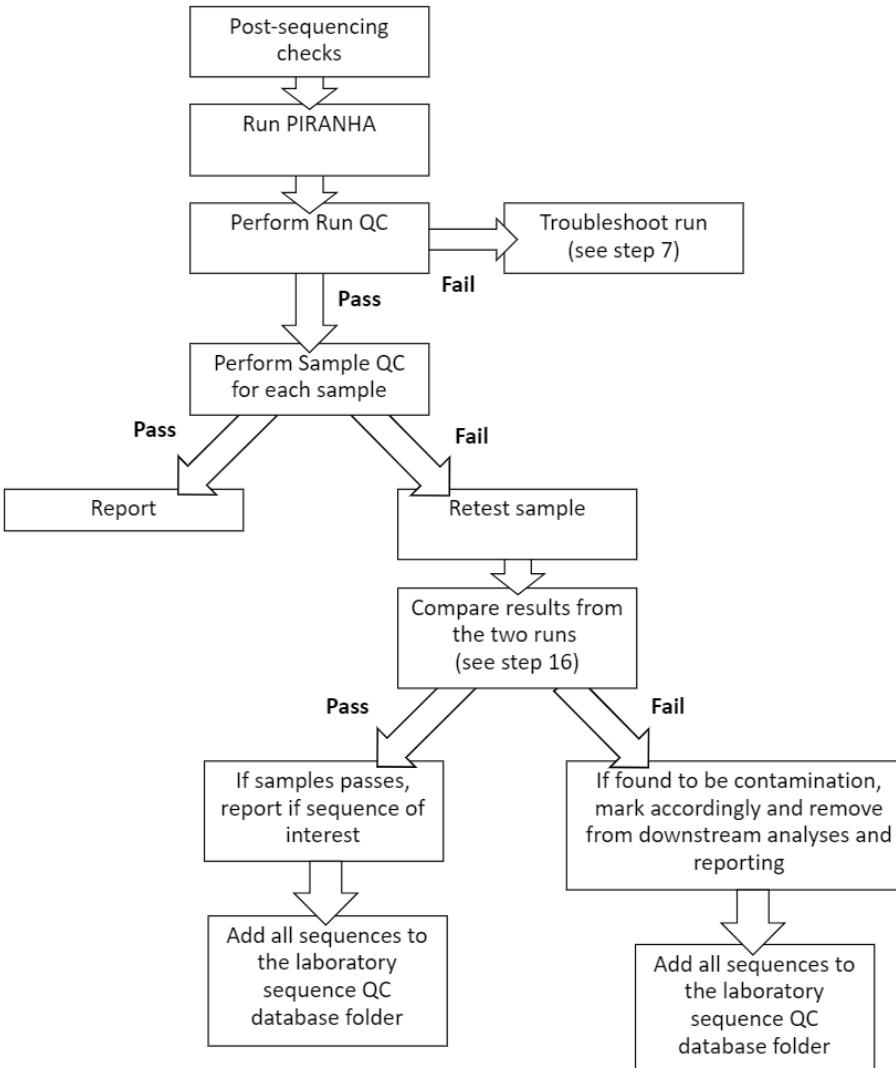
Things to look out for

- Spaces
 - Don't put spaces in folder names
- Special characters
 - Try to stick to using only ‘–’ and ‘_’ when naming samples, files, and directories: e.g., run_2023-07-26_barcodes.csv
- Capital letters
 - Some bioinformatics software is case sensitive so be careful when writing commands and file paths
- Dates: always use the ISO 8601 date format: 2023-07-26

7. Quality Control and Reporting

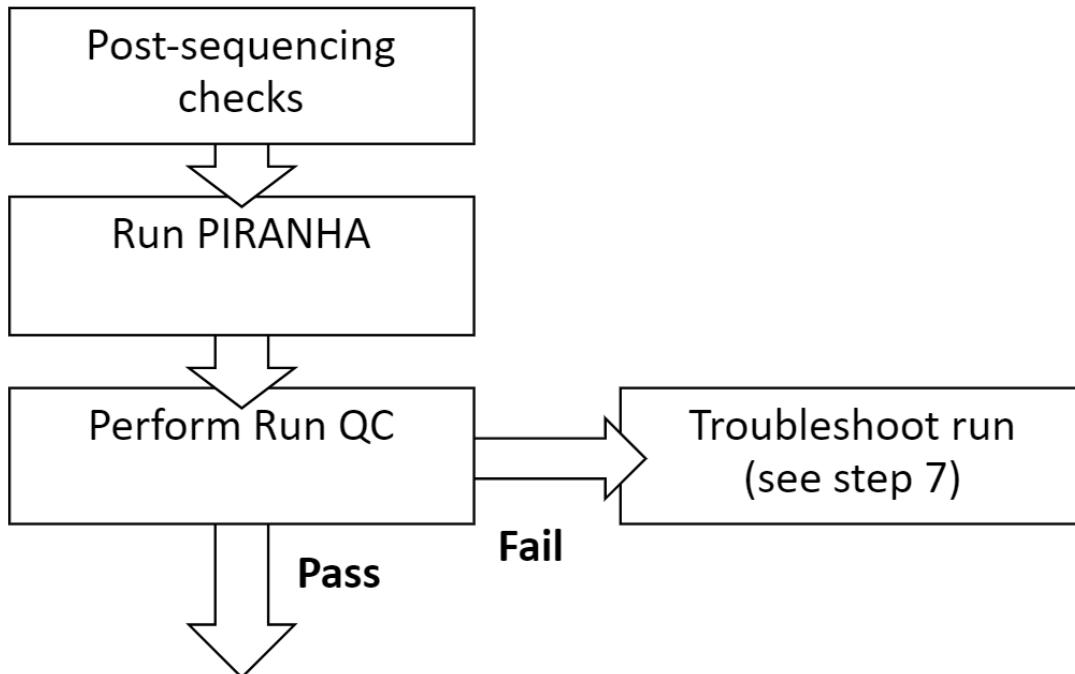
Presentation and discussion

Post-run QC for DDNS stool testing



- QC process documented in:
SOP_Metadata and QC.docx – available via
<https://polionanopore.org/>
- This QC routine is designed for routine direct testing of poliovirus from stool samples where the majority of samples will be negative. Other applications may require other QC thresholds (e.g. minimum pore numbers, run duration)

Sequencing Run QC



- PIRANHA will add sequencing and QC data to the barcodes.csv and save the file as a report.
- Check run QC
 - Did Positive control have > 500 reads mapping to A20?
 - Does the Negative control have <50 reads mapping to PV or NPEVs?

Sequencing Run QC

Check your paperwork and the barcodes.csv!

Too few positive control reads:

Confirm that your earlier positive control check has passed QC checks. Repeat the library pooling and confirm the presence of your library after the cleanup steps using a Tapestation or a Qubit fluorometer.

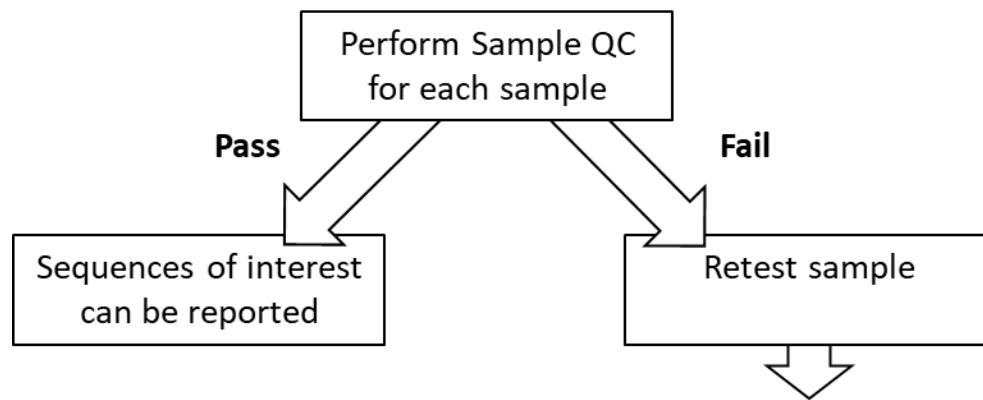
Check that you are ligating the correct adaptor (LA) and are using the short fragment buffer (SFB) during library preparation.

Too many negative control reads:

Confirm that your earlier negative control check has passed QC checks.

Rewash the flow cell with a DNase wash and repeat the library pooling and sequencing run.

Sample QC

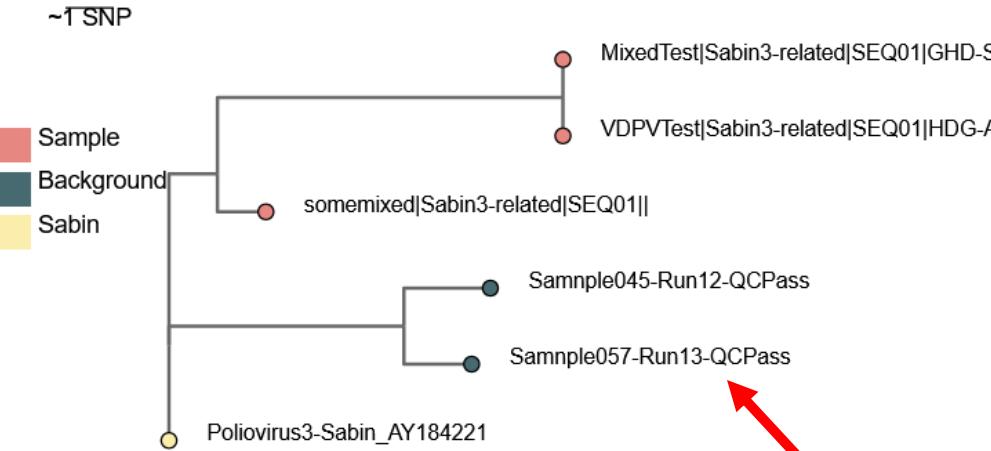


- If run passes, check samples where PV was detected. Samples may be classified as “Fail” if:

A pair of samples with the same EPID (i.e. from the same case) are 3 or more nucleotides different from each other over VP1.

A sample is identical to any other sample with a different EPID (i.e. they are from different cases), unless the sequences are both the same Sabin serotype with no mutations from the original vaccine.

Sample QC

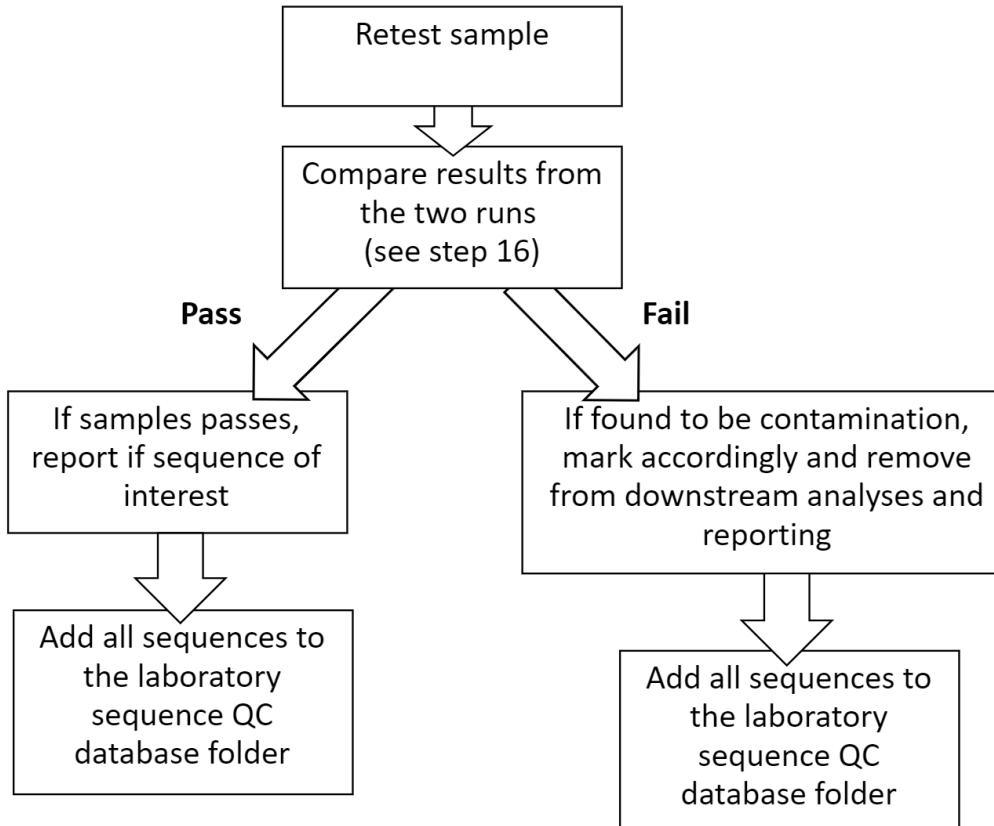


- Wild type and VDPVs should be compared to the laboratory sequence QC database (e.g. via the tree generated in the Piranha report)
- Identical sequences may indicate amplicon contamination and should be marked as “Fail” for retesting.

Sequences from previous
DDNS runs

Figure 3 | Sabin3-related phylogeny

Sample retesting



- Try to avoid using the same layout if checking a group of samples.
- Flag as a QC-check on the new sequencing run.
- Annotate the original run depending on the result. Report the sample if it passes the repeat.
- Talk to the appropriate person to arrange the retesting

Laboratory Sequence QC Database

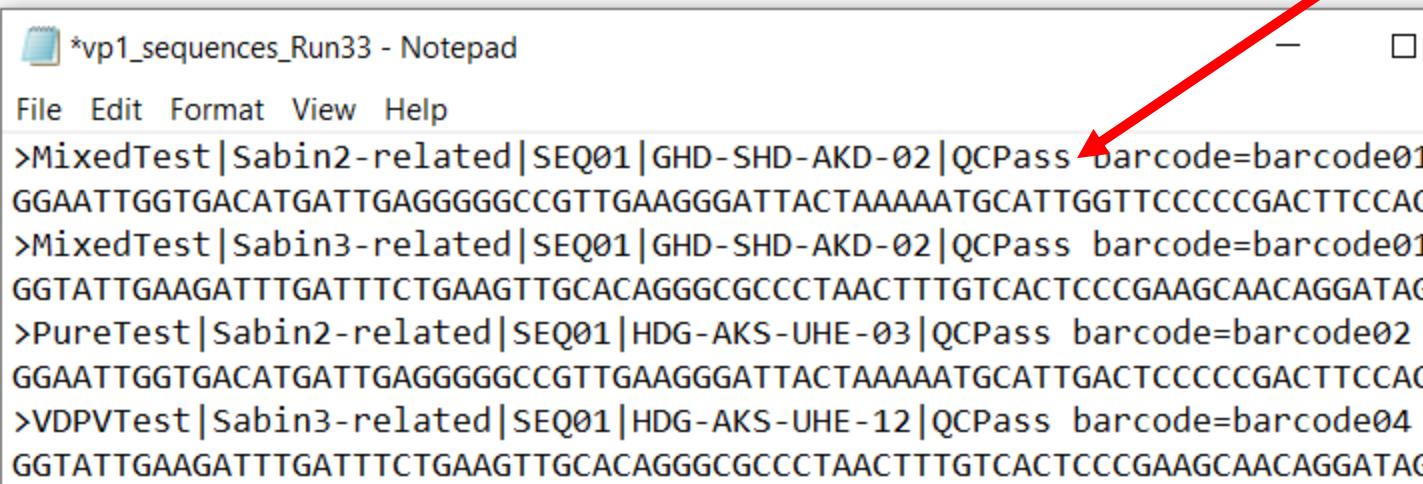
- Copy the vp1_sequences.fasta from the piranha output folder to your Laboratory Sequence QC Database folder.
- Edit the filename to include the run number e.g. vp1_sequences.fasta becomes vp1_sequences_Run33.fasta

Run33 > piranha_output > published_data		
Name	Date modified	Type
barcode01	25/09/2024 11:47	File folder
barcode02	25/09/2024 11:47	File folder
barcode03	25/09/2024 11:47	File folder
barcode04	25/09/2024 11:47	File folder
barcode05	25/09/2024 11:47	File folder
barcode06	25/09/2024 11:47	File folder
barcode07	25/09/2024 11:47	File folder
vp1_sequences_Run33	25/09/2024 11:47	FASTA File

Laboratory Sequence QC Database

📁 barcode01	25/09/2024 11:47	File folder
📁 barcode02	25/09/2024 11:47	File folder
📁 barcode03	25/09/2024 11:47	File folder
📁 barcode04	25/09/2024 11:47	File folder
📁 barcode05	25/09/2024 11:47	File folder
📁 barcode06	25/09/2024 11:47	File folder
📁 barcode07	25/09/2024 11:47	File folder
📄 vp1_sequences_Run33	25/09/2024 11:47	FASTA File

Sequences passing QC can
be annotated



*vp1_sequences_Run33 - Notepad

File Edit Format View Help

```
>MixedTest|Sabin2-related|SEQ01|GHD-SHD-AKD-02|QCPass barcode=barcode01
GGAATTGGTGACATGATTGAGGGGGCCGTGAAGGGATTACTAAAAATGCATTGGTCCCCGACTTCCAC
>MixedTest|Sabin3-related|SEQ01|GHD-SHD-AKD-02|QCPass barcode=barcode01
GGTATTGAAGATTGATTCTGAAGTTGCACAGGGGCCCTAACCTTGTCACTCCCGAACGAAACAGGATAG
>PureTest|Sabin2-related|SEQ01|HDG-AKS-UHE-03|QCPass barcode=barcode02 |
GGAATTGGTGACATGATTGAGGGGGCCGTGAAGGGATTACTAAAAATGCATTGACTCCCCGACTTCCAC
>VDPVTest|Sabin3-related|SEQ01|HDG-AKS-UHE-12|QCPass barcode=barcode04 |
GGTATTGAAGATTGATTCTGAAGTTGCACAGGGGCCCTAACCTTGTCACTCCCGAACGAAACAGGATAG
```

8. Preparation of the positive control for DDNS

Presentation and discussion

The positive control

- Positive control: Coxsackie virus A20 (CVA20) Contact Dr. Erika Bujaki to order
- Supplied as pre-measured, lyophilised virus
- Must be reconstituted before use in extraction step



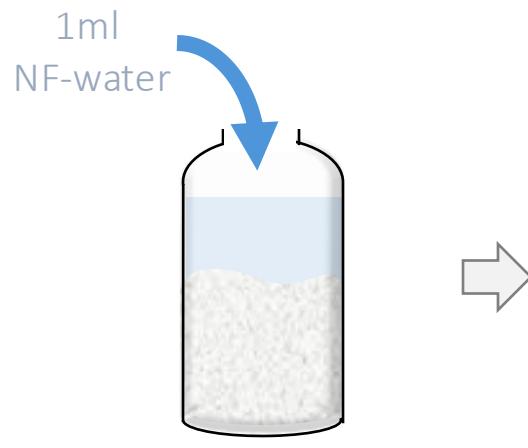
Lyophilised CVA20
positive control

Preparation of positive control - stocks

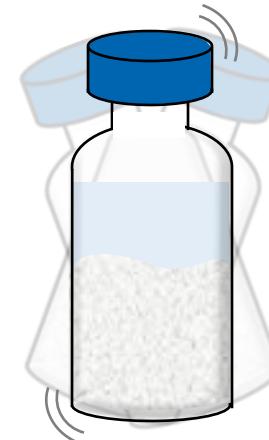


BIOLOGICAL HAZARD
Must be handled in
Class 2 safety cabinet

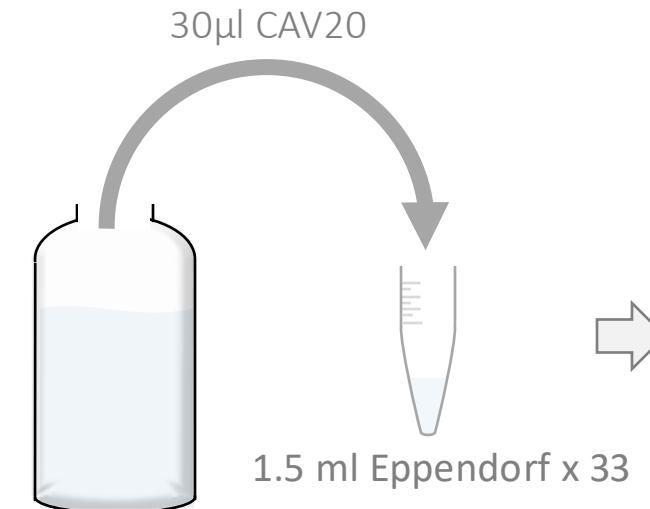
Step One : Add 1ml of
nuclease-free water



Step Two:
Vortex briefly



Step Three: Make 33
single-use aliquots



Step Four: Store
at -20°C



-20°C
Do not freeze thaw
Discard aliquots after
5 weeks

Preparation of positive control for extraction

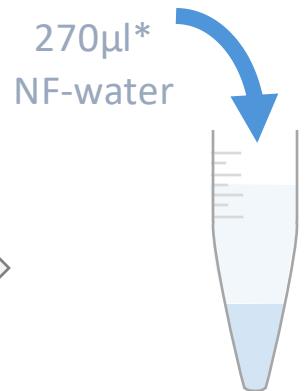


BIOLOGICAL HAZARD
Must be handled in
Class 2 safety cabinet

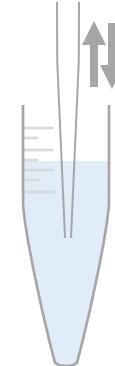
Step One: Defrost one
30ul aliquot



Step Two: Add nuclease-
free water



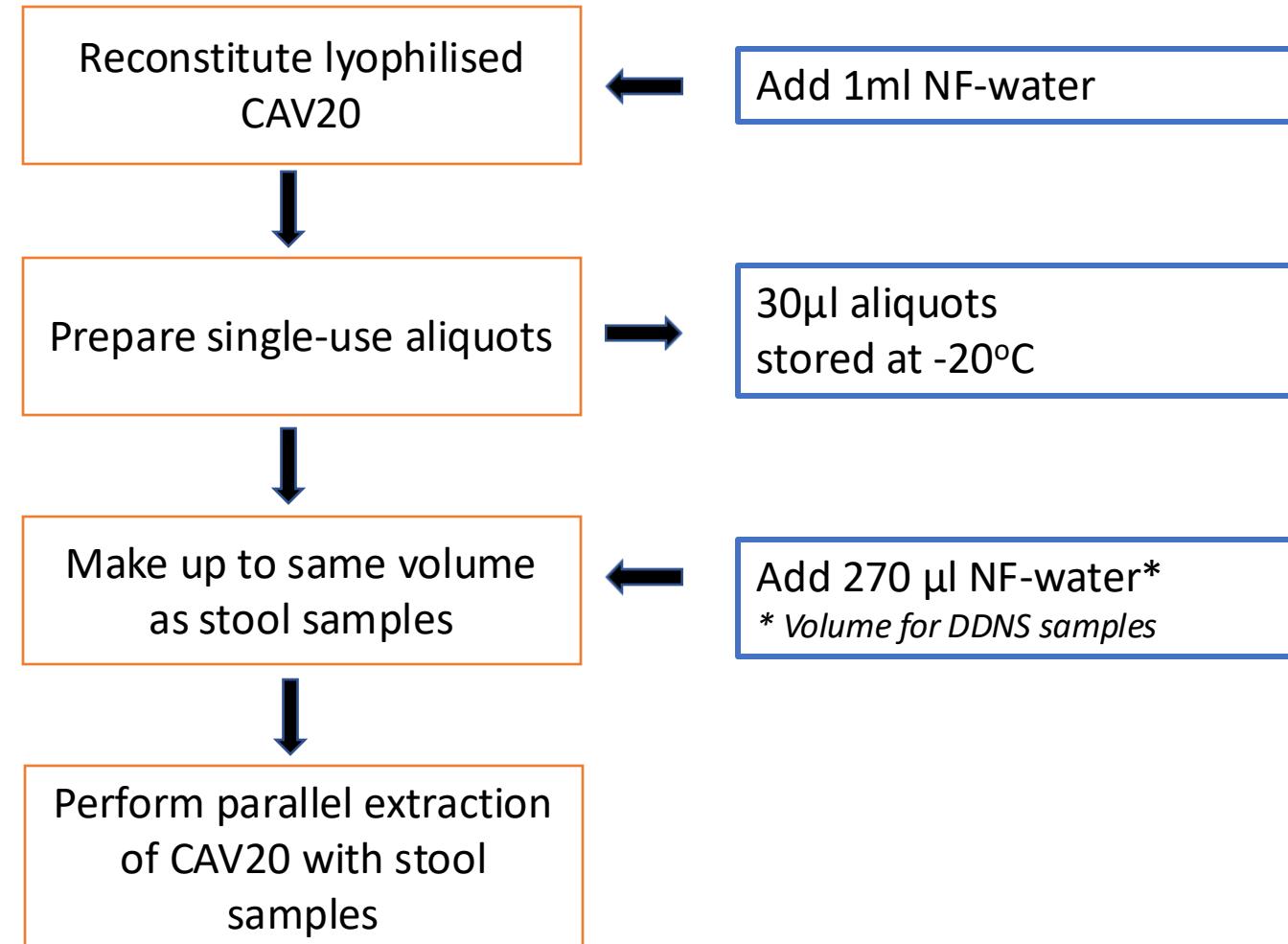
Step Three: Pipette to
mix



Proceed to RNA
extraction

* Volume for preparation
of DDNS controls

Preparation of positive control - overview



9. In-house Verification

Presentation and discussion

In-house verification

❖ In-house verification process— available via <https://polionanopore.org/>

❖ In-house verifications aims to:

- ✓ confirm that the required performance characteristics can be met within the settings
- ✓ Prove that the lab can adequately perform the method as intended achieving the DDNS performance requirements for the sample matrix (stool) to which the DDNS method is applied to.

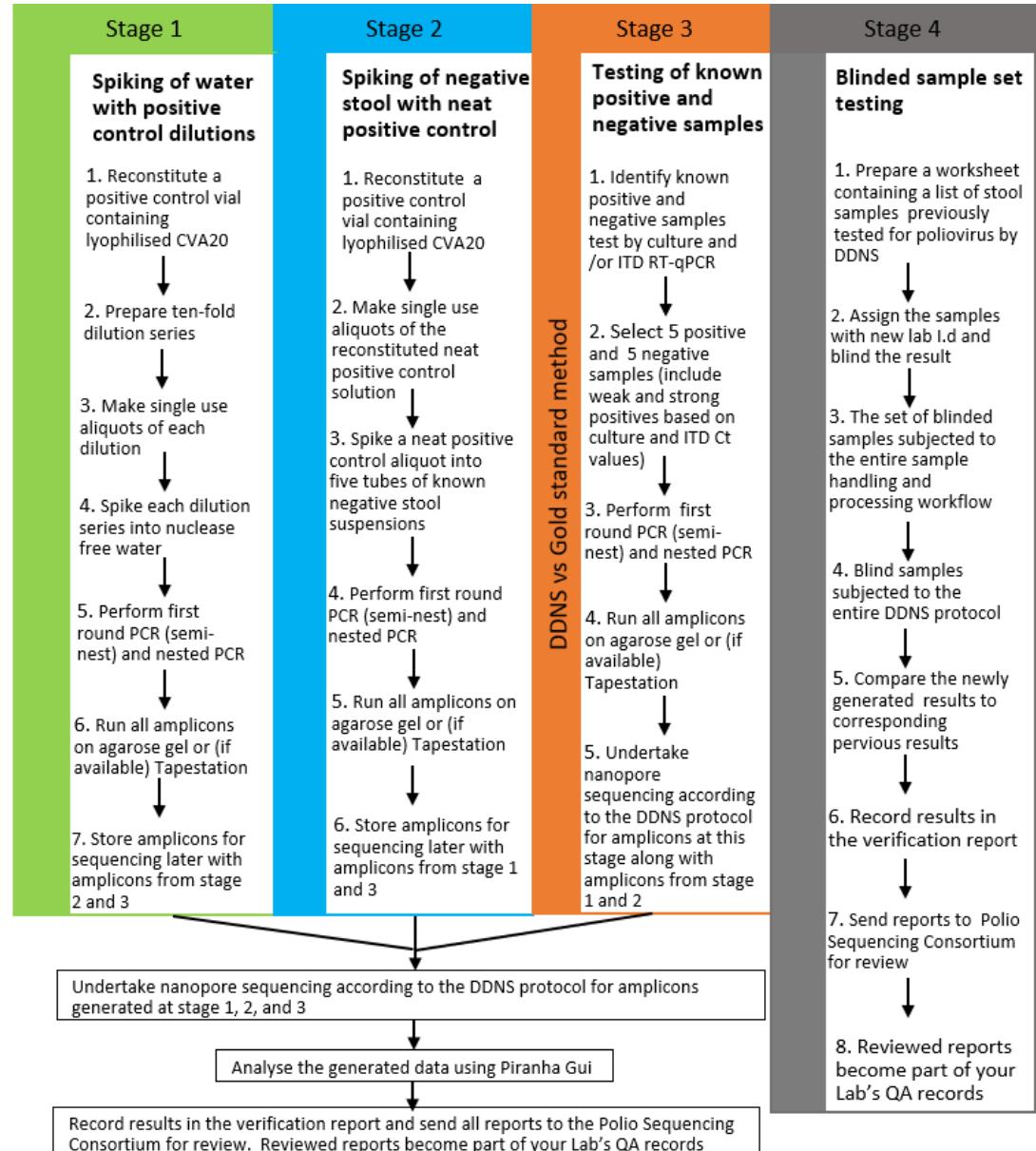
❖ Positive controls – supplied by MHRA

- ✓ Candidate 1 (CVA20-IH35)
- ✓ Candidate 2 (CVA20-Cecil)

In-house verification

❖ Four stages

- ✓ Stage 1: To show if RNA extraction & PCR amplification worked efficiently resulting in PCR amplicons and consensus sequences.
- ✓ Stage 2: To show if the assay worked efficiently with the stool matrix.
- ✓ Stage 3. DDNS vs gold standard method result comparison.
- ✓ Stage 4: Lab to test its entire quality system and provide a real time assessment of the lab's proficiency



In-house verification Report



DDNS In-House Verification Report

This form should be completed to record the laboratory's DDNS in-house verification results. It aims to demonstrate that your lab results are in line with how the DDNS method has been designed to perform. The completed report becomes part of your laboratory's QA records.

Name of Institute/Organisation.....

Date of in-house verification: Start/...../..... Completion/...../.....

Positive control candidate used: Lot/batch number.....

Date of control material vial resuspension:/...../..... Aliquot storage temperature.....°C

Please summarise results in the tables below, ensuring to attach RT-PCR and VP1 PCR electrophoresis images/reports, MinKNOW sequencing run reports and PIRANHA analysis report files for each stage to enable review.

Stage 1

Spiking of water with positive control dilutions

Aim: To show if RNA extraction & PCR amplification worked efficiently resulting in PCR amplicons and consensus sequences.

Samples	Results				For Official Use Only Outcome	
	PCR amplicon		Sequence generated			
	Yes	No	Yes	No		
Neat						
10-1						
10-2						
10-3						
10-4						
Positive control						
Negative control(s)						

Comments (if applicable):

Stage 2

Spiking of negative stool with positive control neat stock

Aim: To show if the assay worked efficiently with the stool matrix.

Samples	Results				For Official Use Only Outcome	
	PCR amplicon		Sequence generated			
	Yes	No	Yes	No		
Spiked stool 1						
Spiked stool 2						
Spiked stool 3						
Spiked stool 4						
Spiked stool 5						
Positive control						
Negative control(s)						
Unspiked stool						

Comments (if applicable):



DDNS In-House Verification Report

Stage 3
Testing of known positives and negative samples

Aim: DDNS vs gold standard method result comparison.

Samples	Expected	Observed	For Official Use Only Outcome	
			Pass	Fail
Positives				
Negatives	No PCR amplicons or consensus sequence			
Positive control				
Negative control(s)	No PCR amplicons or consensus sequence			

Comments (if applicable):

Stage 4
Blinded sample set testing

Aim: Lab to test its entire quality system and provide a real time assessment of the lab's proficiency

Samples	Expected	Observed	For Official Use Only Outcome	
			Pass	Fail
Positives				
Negatives	No PCR amplicons or consensus sequence			
Positive control				
Negative control(s)	No PCR amplicons or consensus sequence			

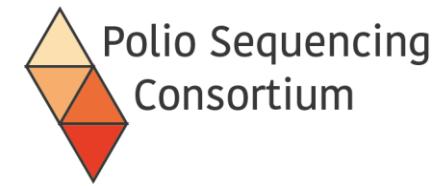
Comments (if applicable):

Approval

Laboratory's Lead Signature & Date

QC&QA Lead Signature & Date

GSL Signature & Date



End