Polio Sequencing Consortium

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

Presentation and discussion

Poliovirus Direct Detection and Nanopore Sequencing (DDNS)

Polio Sequencing Consortium

Stool – AFP surveillance

sample collection

processing (chloroform)

cell culture

ITD qPCR

Sewage – ES surveillance

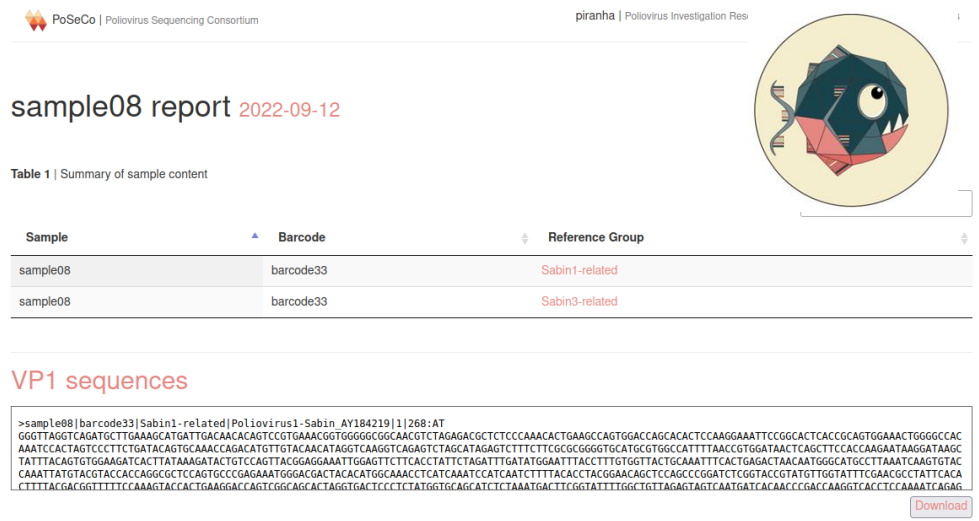
sample collection + concentration (wastewater)

RNA extraction (manual, automated)

Semi- nested PCR (barcoded) VP1 primers

nanopore sequencing (pooled VP1 products)

analysis & reporting (PIRANHA)


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

```
>sample08|barcode33|Sabin1-related|Poliovirus1-Sabin_AY184219|1|268:AT
GGGTTAGGTCAGATGCTTGAAAGCATGATTGACAACACAGTCCGTGAAACGGTGGGGGCGGCAACGTCTAGAGACGCTCTCCCAAACACTGAAGCCAGTGGACCAGCACACTCCAAGGAAATTCCGGCACTCACCGCAGTGGAAACTGGGGCCAC
AAATCCACTAGTCCCTTCTGATACAGTGCAAACCAGACATGTTGTACAACATAGGTCAAGGTCAGAGTCTAGCATAGAGTCTTTCTTCGCGCGGGGTGCATGCGTGGCCATTTTAACCGTGGATAACTCAGCTTCCACCAAGAATAAGGATAAGC
TATTTACAGTGTGGAAGATCACTTATAAAGATACTGTCCAGTTACGGAGGAAATTGGAGTTCTTCACCTATTCTAGATTTGATATGGAATTTACCTTTGTGGTTACTGCAAATTTCACTGAGACTAACAATGGGCATGCCTTAAATCAAGTGTAC
CAAATTATGTACGTACCACCAGGCGCTCCAGTGCCCGAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTTACACCTACGGAACAGCTCCAGCCCGGATCTCGGTACCGATATGTTGGTATTTCGAACGCCTATTCACA
CTTTTACGACGGTTTTTCCAAAGTACCACTGAAGGACCAGTCGGCAGCACTAGGTGACTCCCTCTATGGTGCAGCATCTCTAAATGACTTCGGTATTTTGGCTGTTAGAGTAGTCAATGATCACAACCCGACCAAGGTCACCTCCAAAATCAGAG
```

Download

http://polionanopore.org

Advantages of poliovirus direct detection by nanopore sequencing

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- 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

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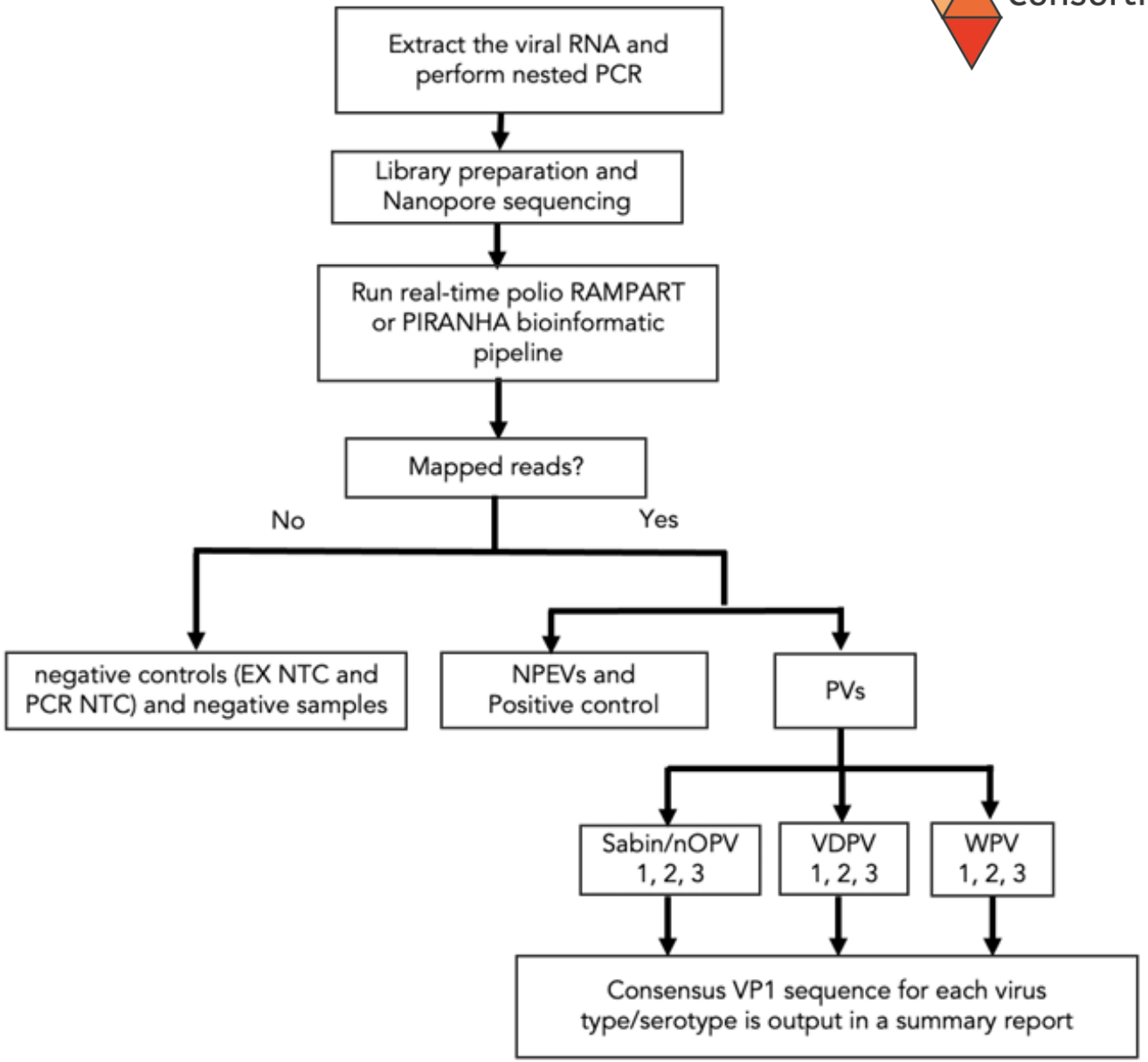
Diagnostic algorithm



http://polionanopore.org

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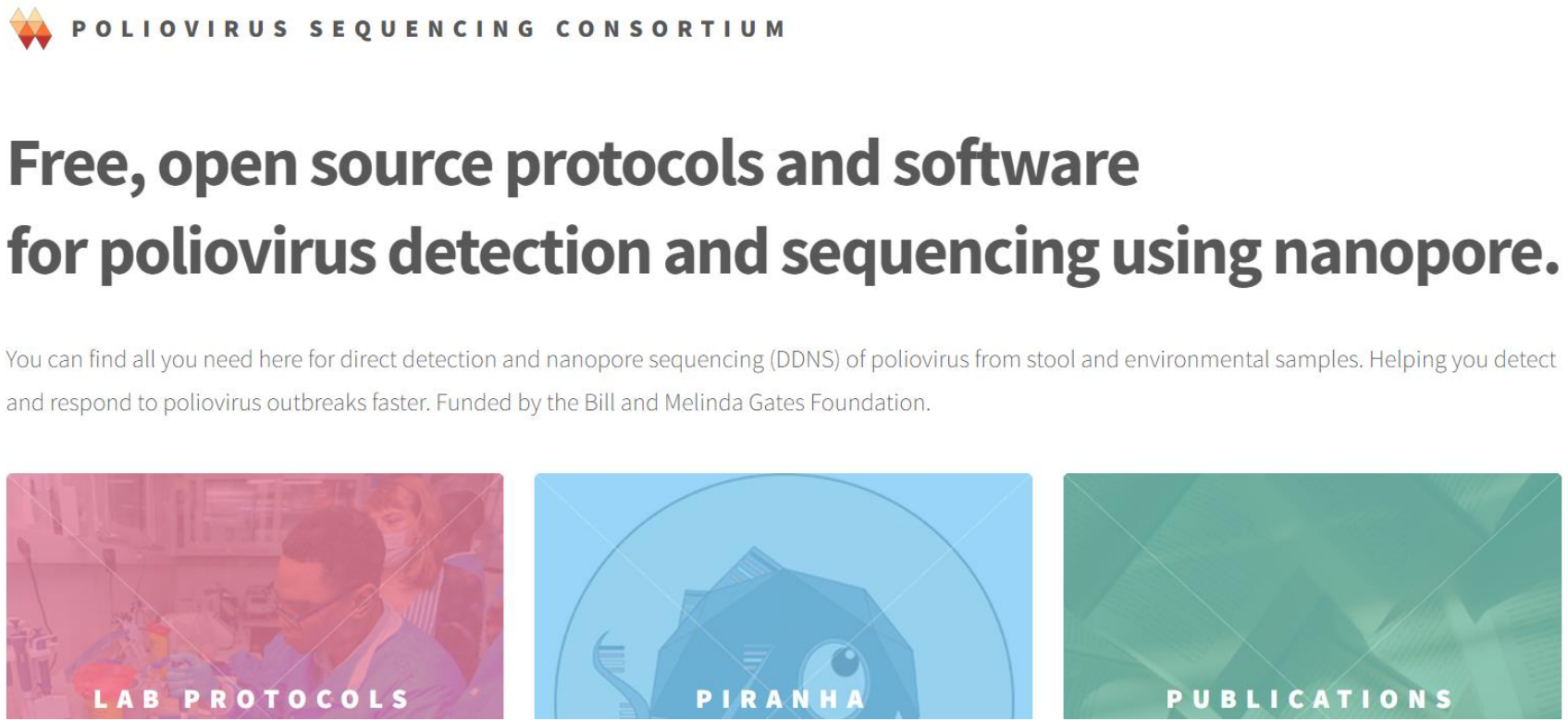
- Updated protocols maintained on protocols.io – can be reached through http://polionanopore.org

- Forums on the protocols.io site to raise any queries you have about the protocol

- New version of the protocols will be published here.



Nanopore (MinION) Sequencing

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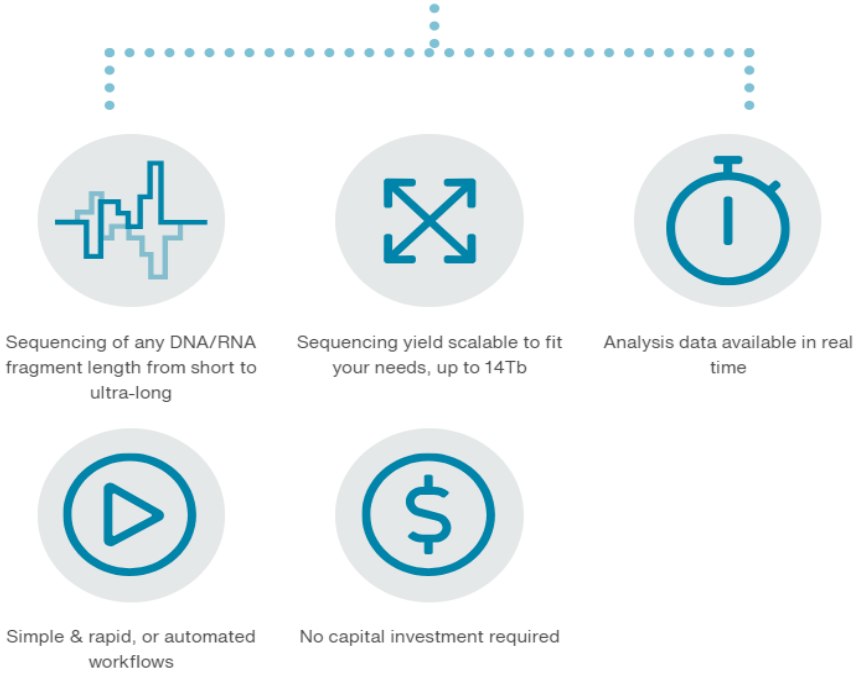
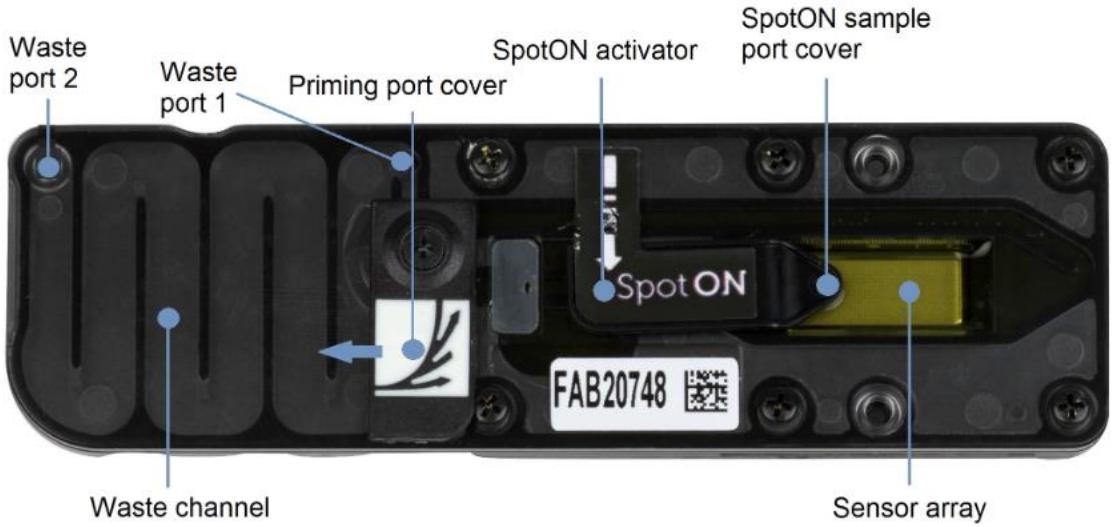


- It provides a direct electrical analysis of the target molecule
- Unrestricted read length: from short (20b) to ultra long reads (>4Mb reads)
- Fast- 1,000,000 reads per hour and can be processed in real time
- Raw read error rate per base was ~5% - new chemistry offers accuracy >99.0%
- Variant calling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

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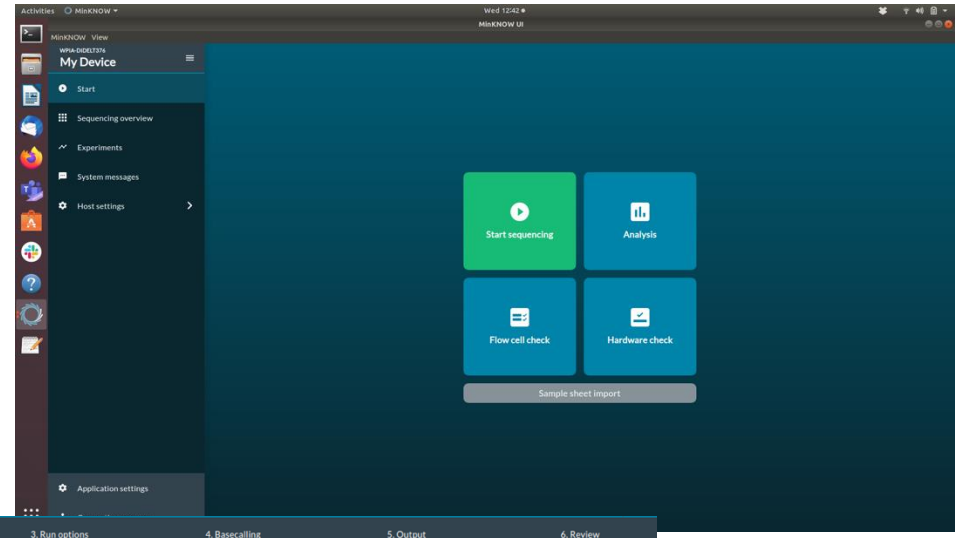
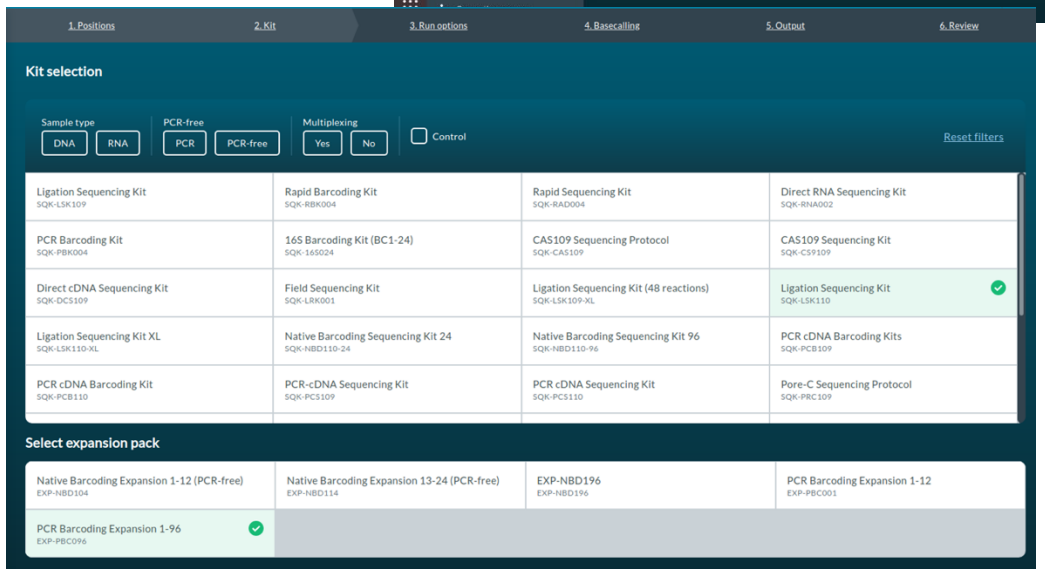
- Easy to use GUI for setting up and running your sequencing
- Install following the instructions on the ONT website
- Live basecalling and demultiplexing of reads





Data analysis - PIRANHA

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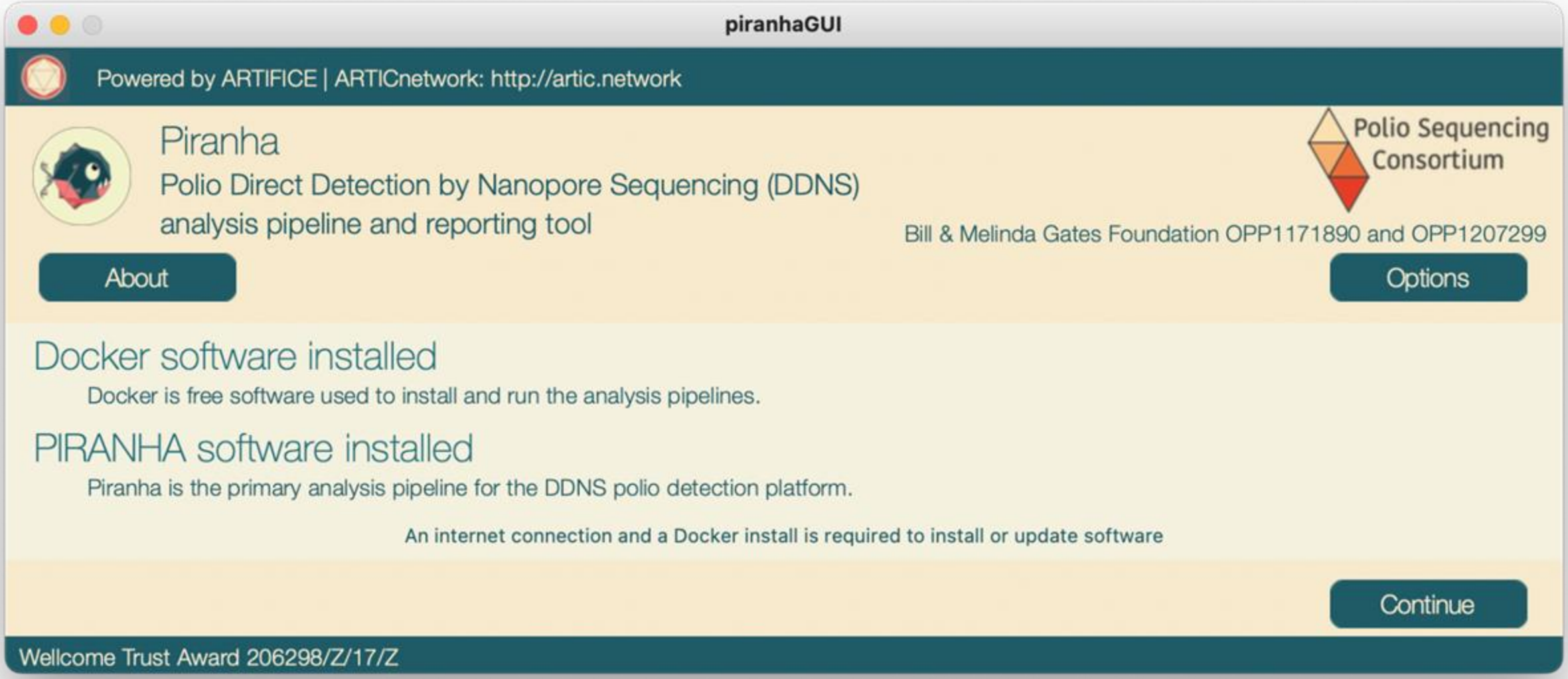

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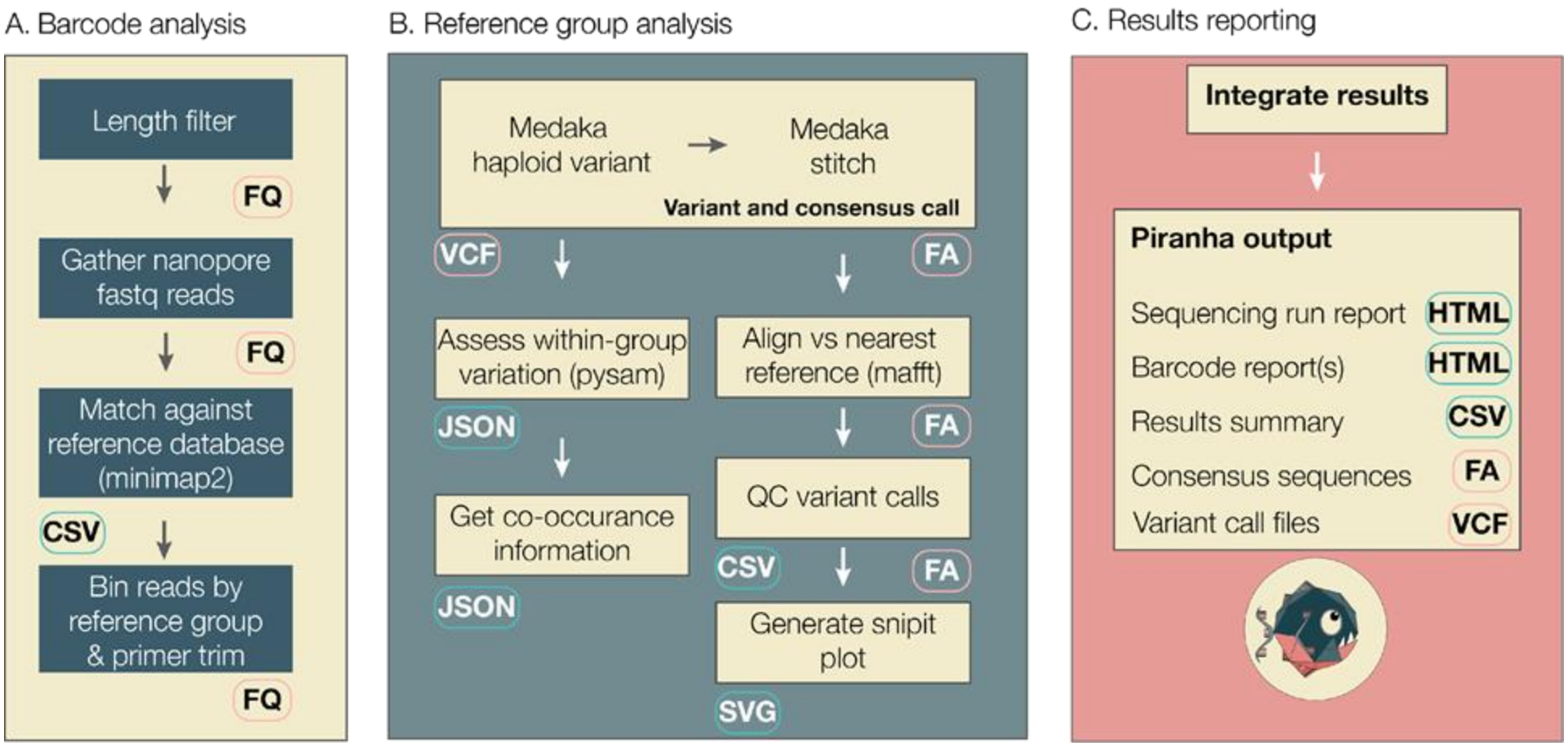
Workflow schema



PIRANHA Outputs- Report

Search:

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

Export table

Search:

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

PoSeCo | Poliovirus Sequencing Consortium

piranha | Poliovirus Investigation Resource Automating Nanopore Haplotype Analysis

sample08 report 2022-09-12

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TATTTACAGTGTGGAAGATCACTTATAAAGATACTGTCCAGTTACGGAGGAAATTGGAGTTCTTCACCTATTCTAGATTTGATATGGAATTTACCTTTGTGGTTACTGCAAATTTCACTGAGACTAACAATGGGCATGCCTTAAATCAAGTGTAC
CAAATTATGTACGTACCACCAGGCGCTCCAGTGCCCGAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTTACACCTACGGAACAGCTCCAGCCCGGATCTCGGTACCGTATGTTGGTATTTCGAACGCCTATTCACA
CTTTTACGACGGTTTTTCCAAAGTACCACTGAAGGACCAGTCGGCAGCACTAGGTGACTCCCTCTATGGTGCAGCATCTCTAAATGACTTCGGTATTTTGGCTGTTAGAGTAGTCAATGATCACAACCCGACCAAGGTCACCTCCAAAATCAGAG
```

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

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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

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- 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

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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)

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

Presentation and discussion

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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.

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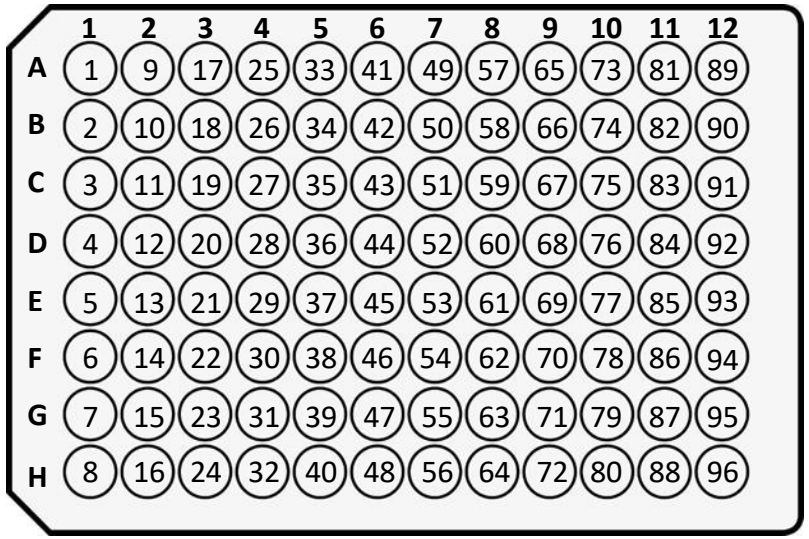
Run preparation

- 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

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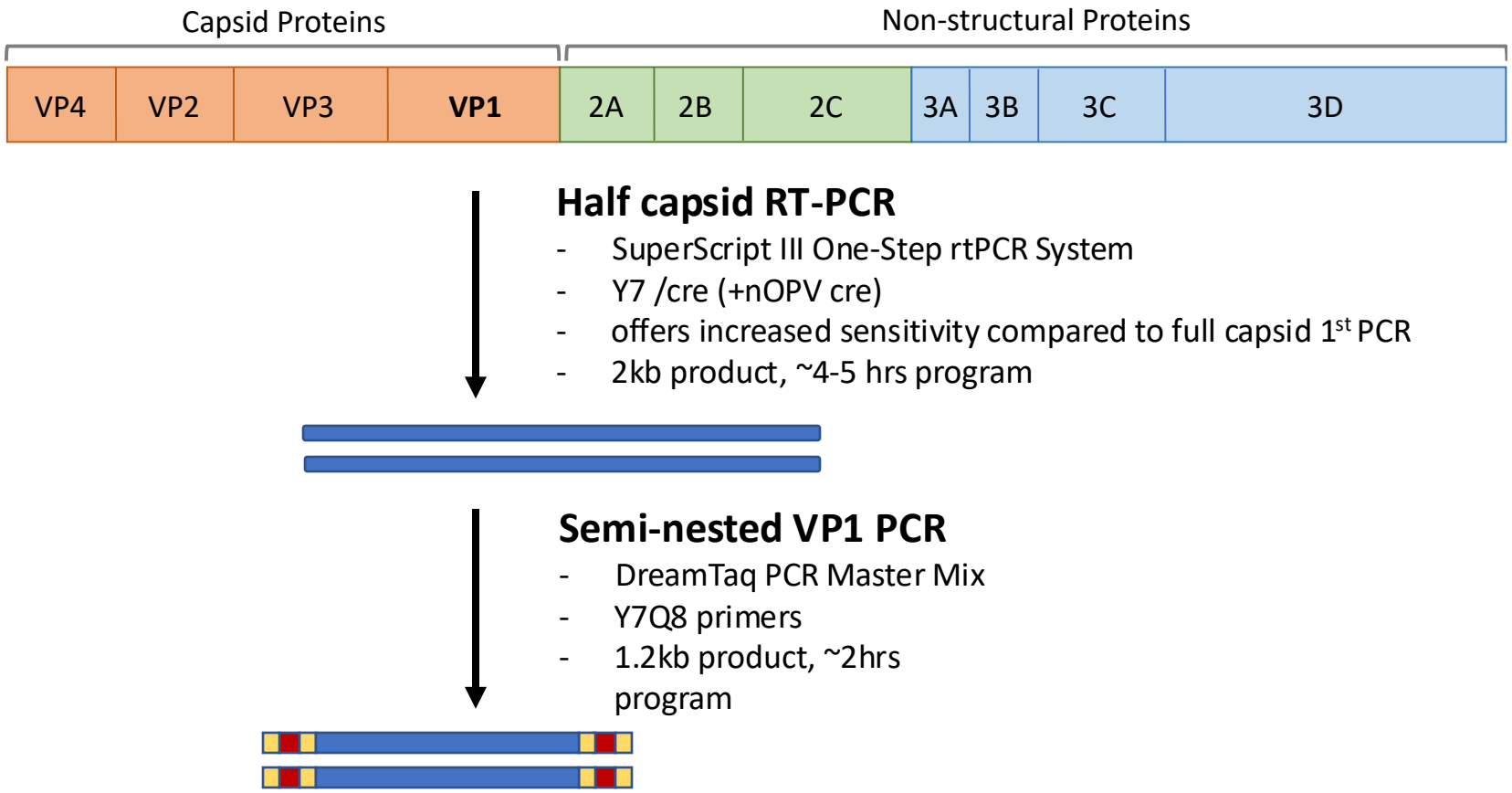

Library preparation overview – stool DDNS

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Part 1: RNA extraction, RT-PCR amplification

Part 2: Pooling, cleaning and end-prep

Part 3: Preparation for sequencing

Extracted RNA

Half capsid RT-PCR amplification

VP1 PCR with barcoded primers

Pool samples

Ampure cleanup/concentration

End-prep & dA-tailing

Ampure cleanup/concentration

Ligate sequencing adapter

Ampure cleanup/concentration

Load flow cell

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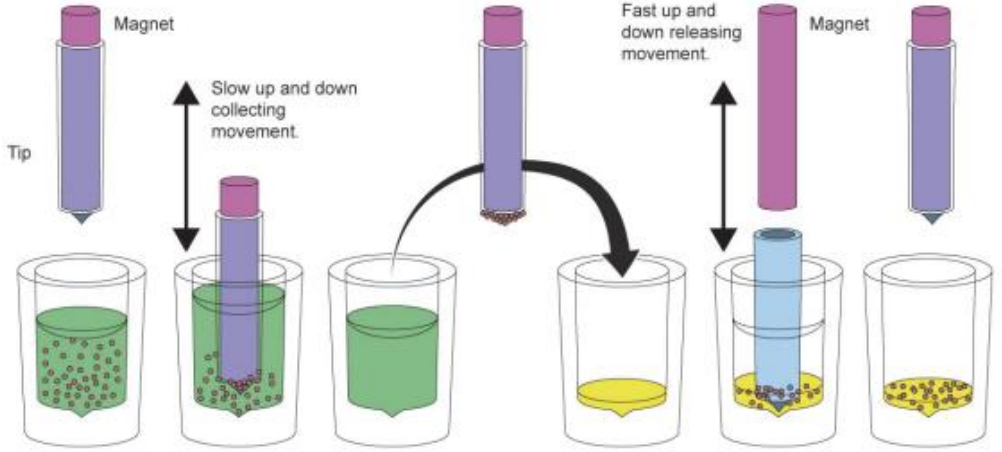
Run controls

- 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

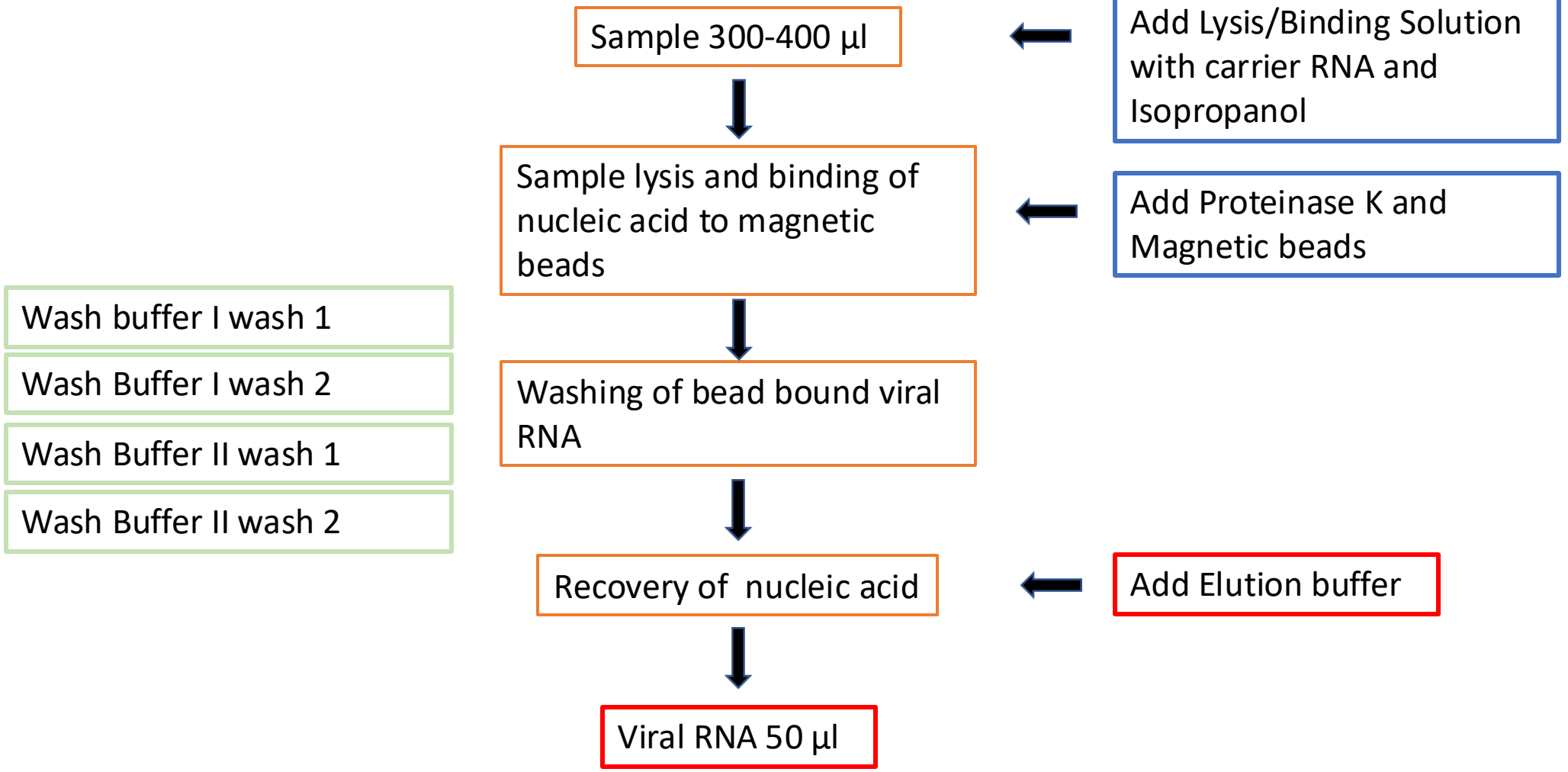
RNA extraction

- 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

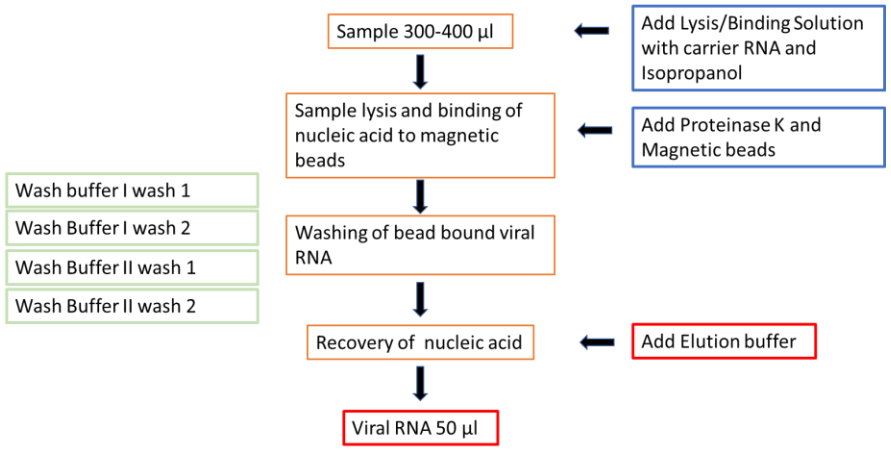
or

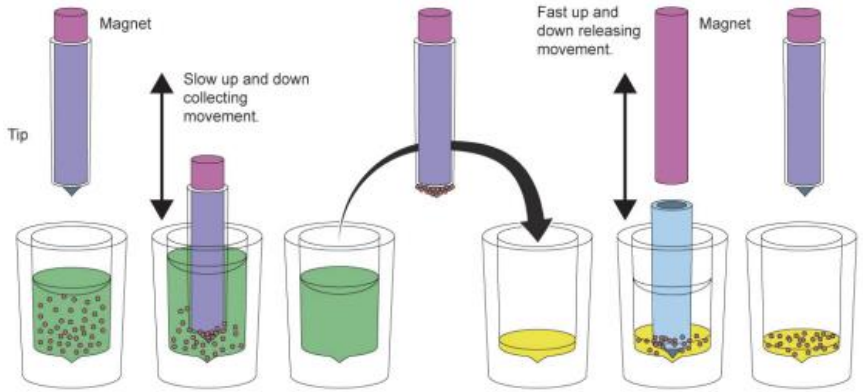


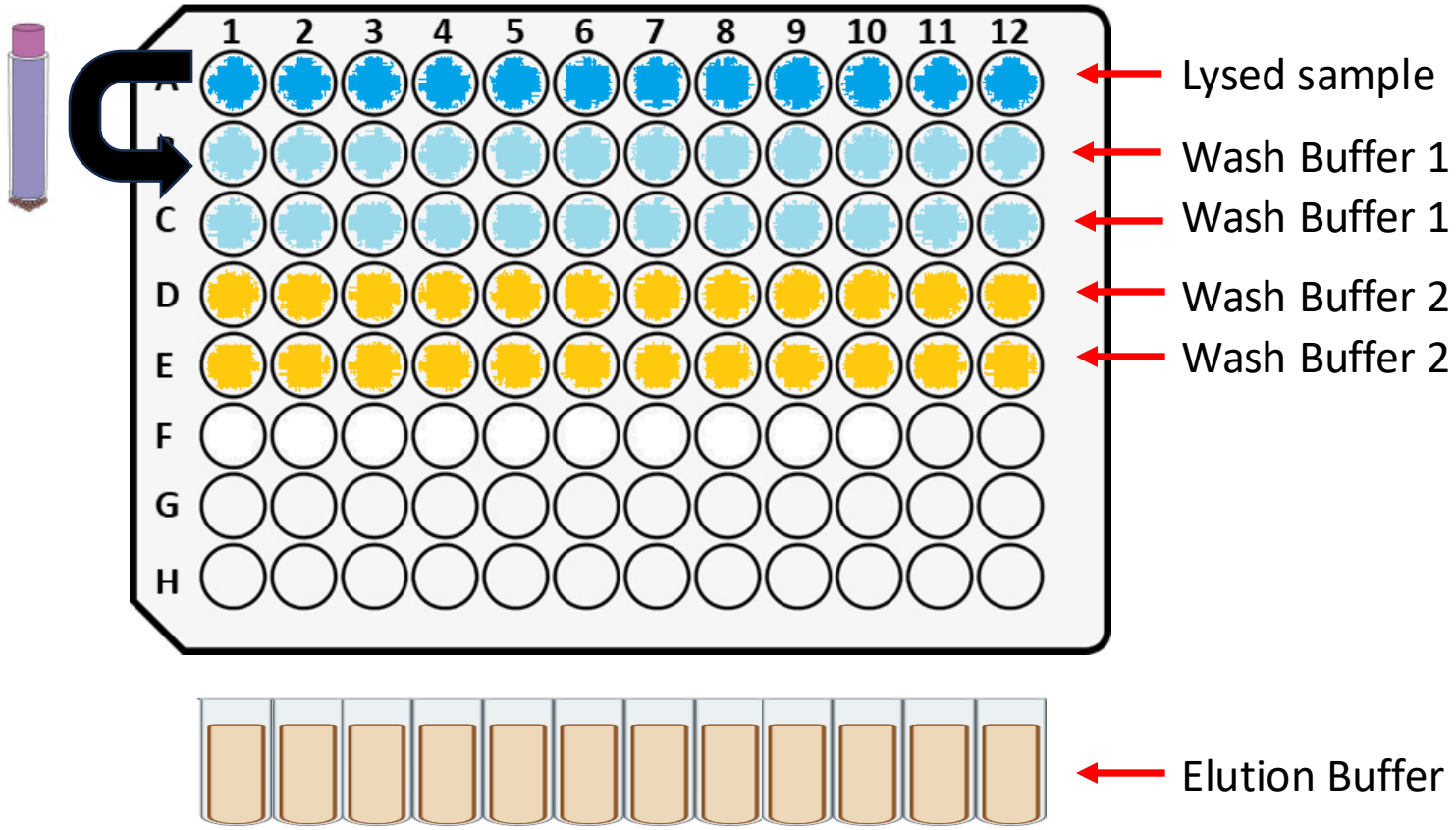
RNA extraction - MagMAX Viral RNA Isolation Kit



RNA extraction - Automated extraction (Kingfisher Duo)

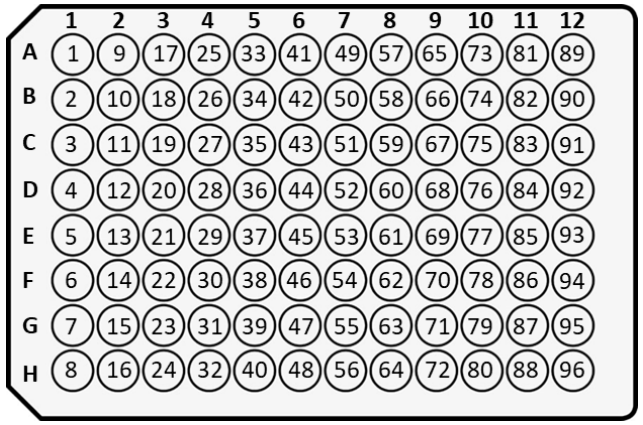






Setting up the half-capsid RT-PCR

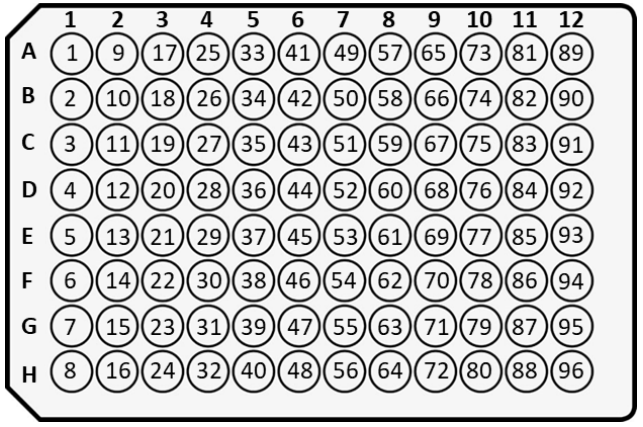
Mastermix plate
(19 µl reaction mix per well)



Add 5 µl

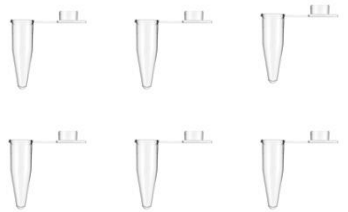
Extracted RNA

Automated



or

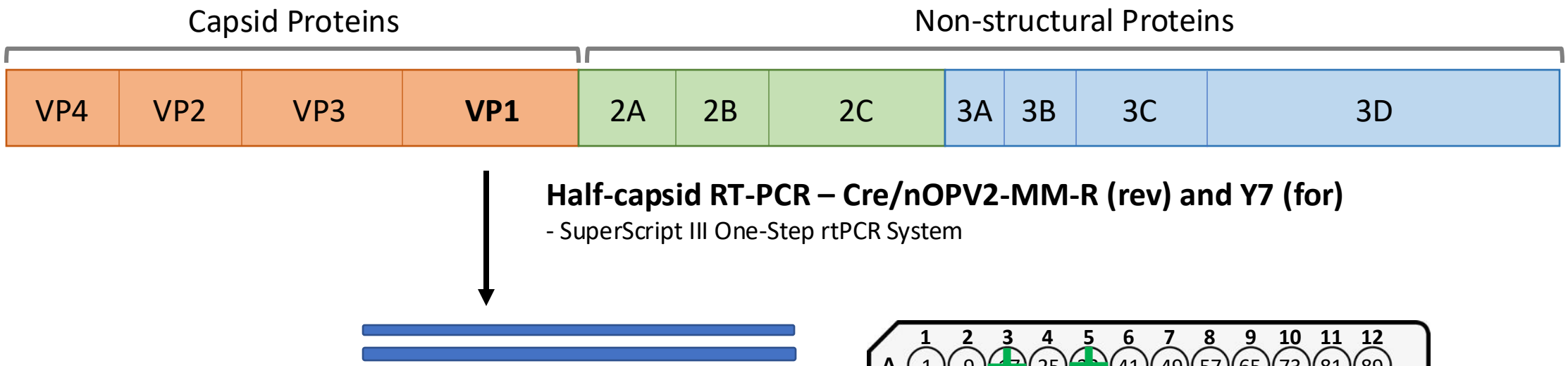
Manual

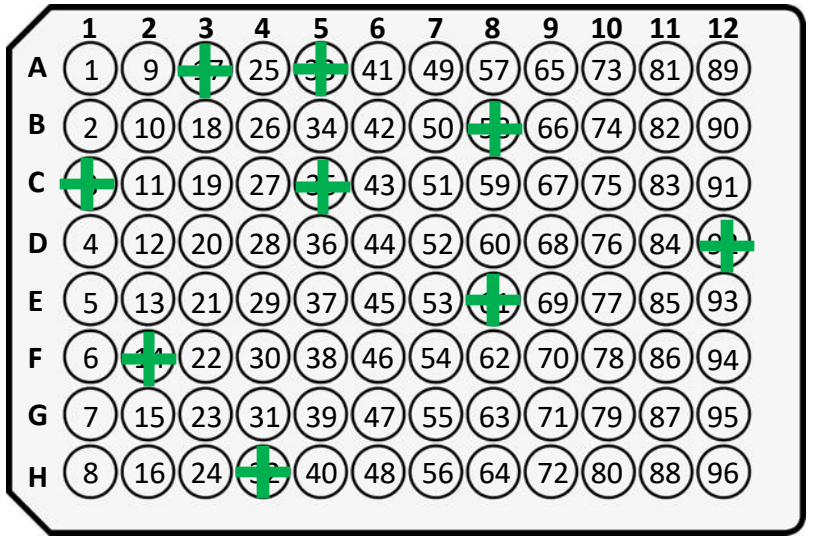


	1 Reaction (µL)
2x Master Mix	12.5
SSIII Platinum Taq mix	1
Reverse Primer (10 µM, Cre/nOPV2-MM-R mix)	1
Nuclease free Water	4.5
Total	**19**

- 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

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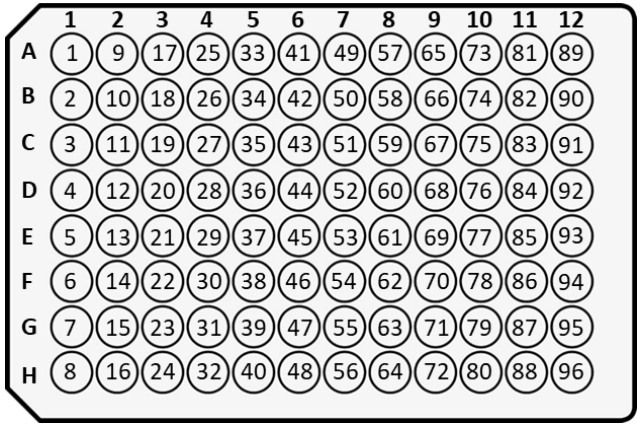
Semi-nested VP1 PCR

- 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



VP1 PCR primers

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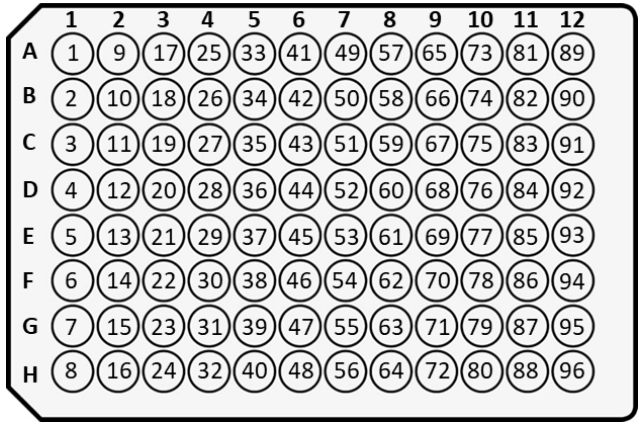
Flanking sequence

Y7 GGTGCTGACCGAGATCCTACGAATGGAGTGTTTAACCTGGGTTTGTGTCAGCCTGTAATGA

Barcode sequence **Primer sequence**

TACACCTTRTCTCTGGAGAATCCAATTACCGAGATCCTACGAATGGAGTGTGTCGTGG Q8

Primer sequence **Barcode sequence**



https://community.nanoporetech.com/technical_documents/chemistry-technical-document/v/chtd_500_v1_revt_07jul2016/barcoding-kits

Setting up the semi-nested VP1 PCR

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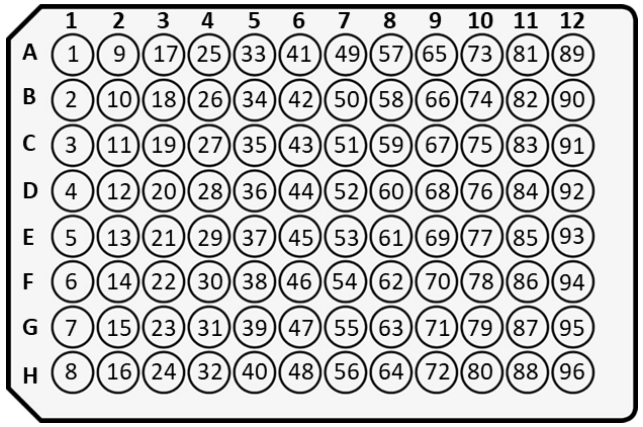
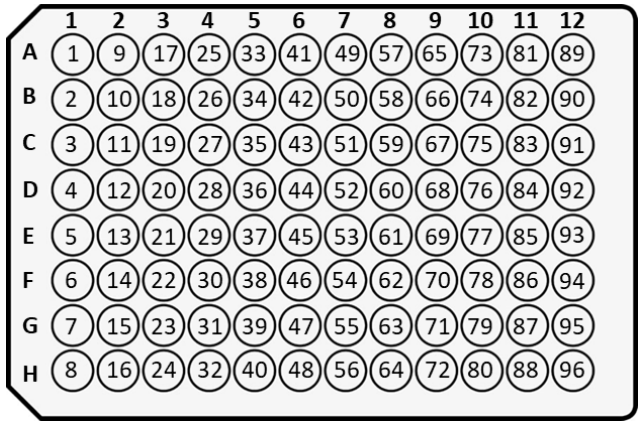
Mastermix plate
(21 µl reaction mix per well)



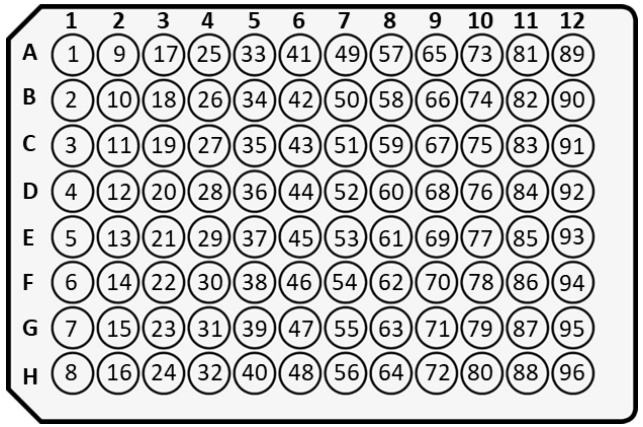
Add 2 µl

Q8/Y7 barcoded primer plate (10µM)



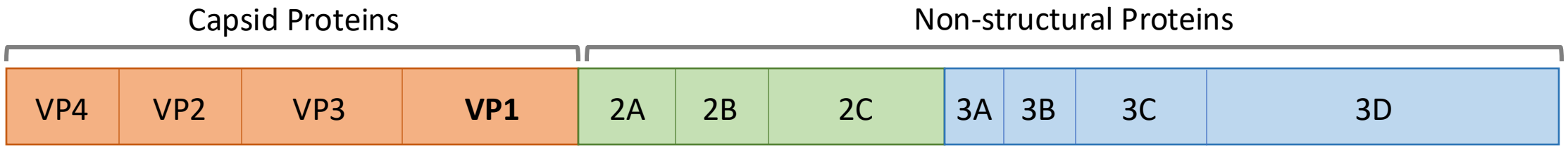
Add 2 µl

1st reaction plate

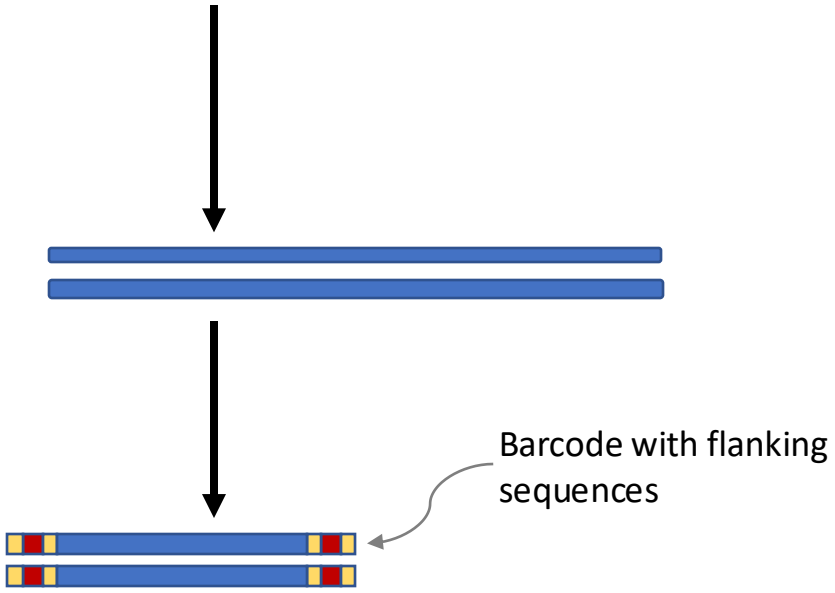


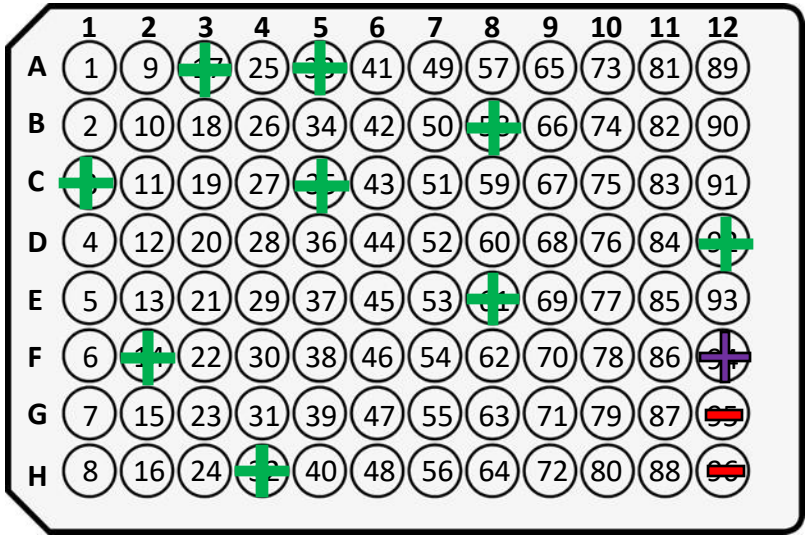
	1 Reaction (µL)
Water	8.5
DreamTaq 2x master mix	12.5
Total volume	**21**

Post PCR



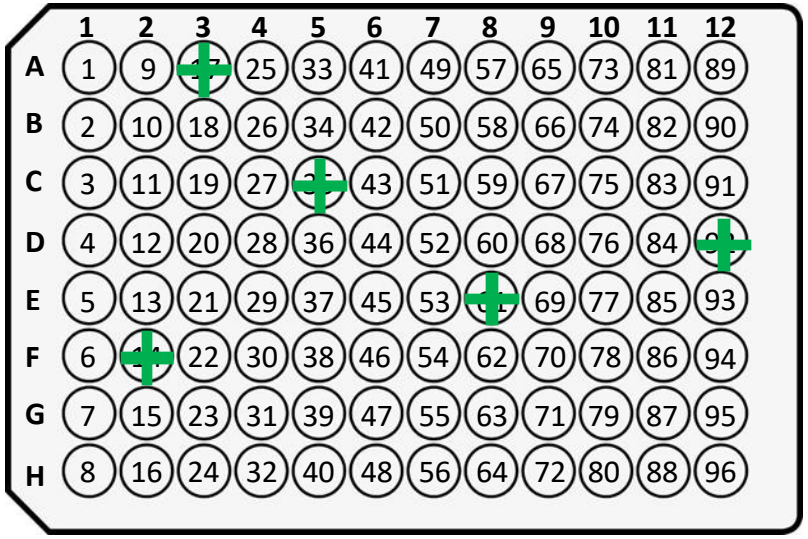
- Can run a gel to confirm samples, expect a band around 1.2kb.
- Only indicates the presence of poliovirus or enterovirus with similar primer binding sites
- DO run the controls on a gel for QC

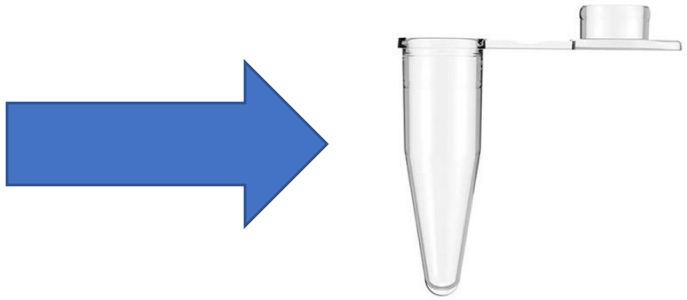




Library Pooling

- Barcoded samples equally pooled by volume (2µl each)
- Previously pooled equal ng 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/µl
- 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.

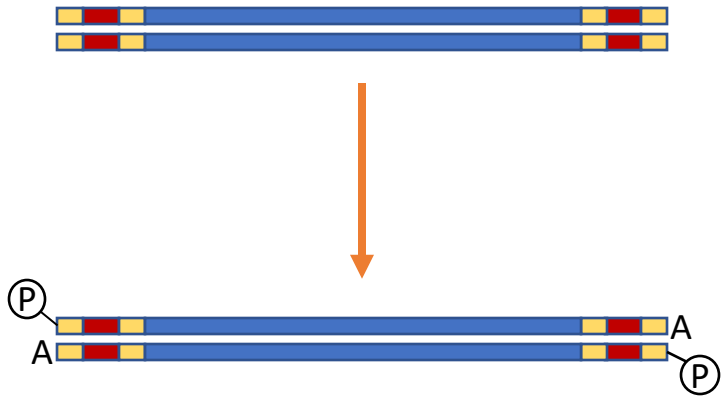
- In detail:
  - Add ampure beads to the pool which will bind the DNA and allow it to be separated by a magnet
  - Wash the pool with 80 % ethanol twice
  - Allow to briefly dry (bead pellet will become matt brown)
  - Resuspend pellet in 51 µl of water
  - Use magnet to pull the beads out of solution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

Ampure beads, Beckman Coulter

Pause point – store library at 4°C

End preparation

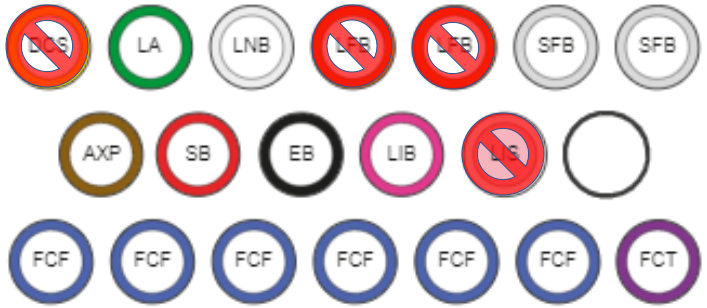
- Treatment of DNA with Ultra II End-prep
- Ensures DNA has 3’ dA tailed and 5’ phosphorylated ends making them sticky for ligation of the sequencing adapter



- 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:

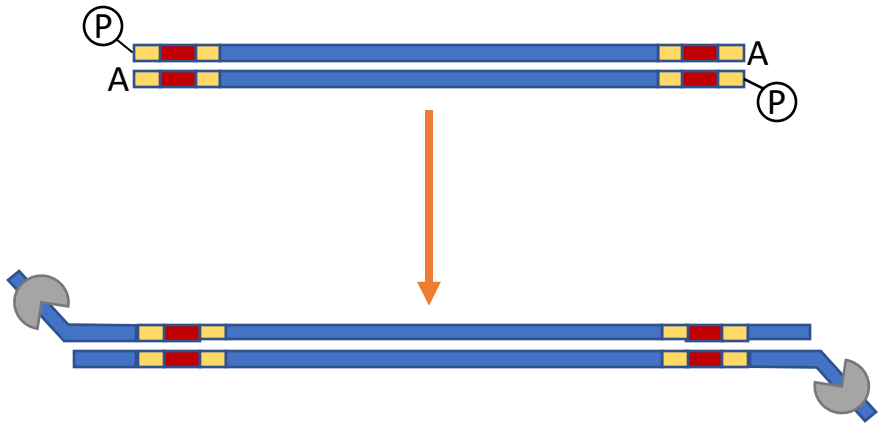


There vials in the kit that we will not use in this protocol (these are crossed out to the right)

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

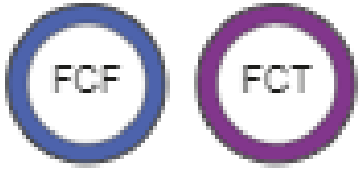
BUT

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

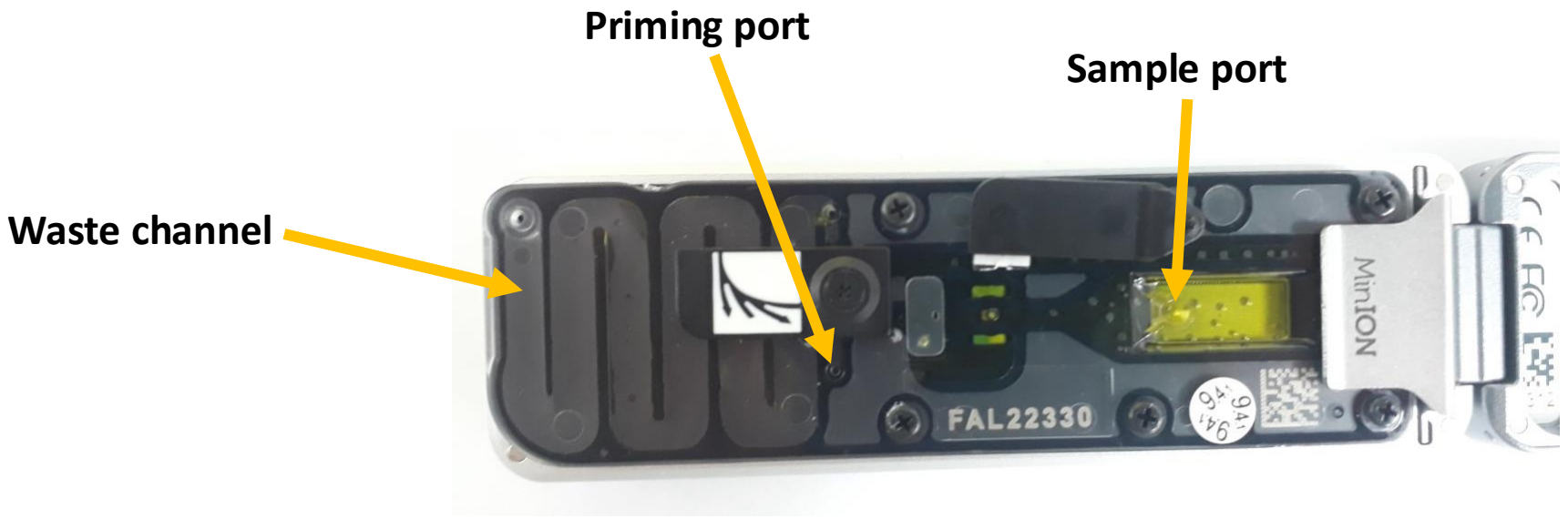
Priming of the flow cell

- 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.

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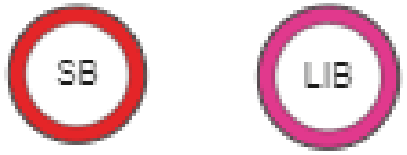
Final Library

- 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.

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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"***

Polio Sequencing Consortium

3. Nanopore sequencing

Presentation and discussion

Library Preparation and Nanopore Sequencing

Polio Sequencing Consortium

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

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

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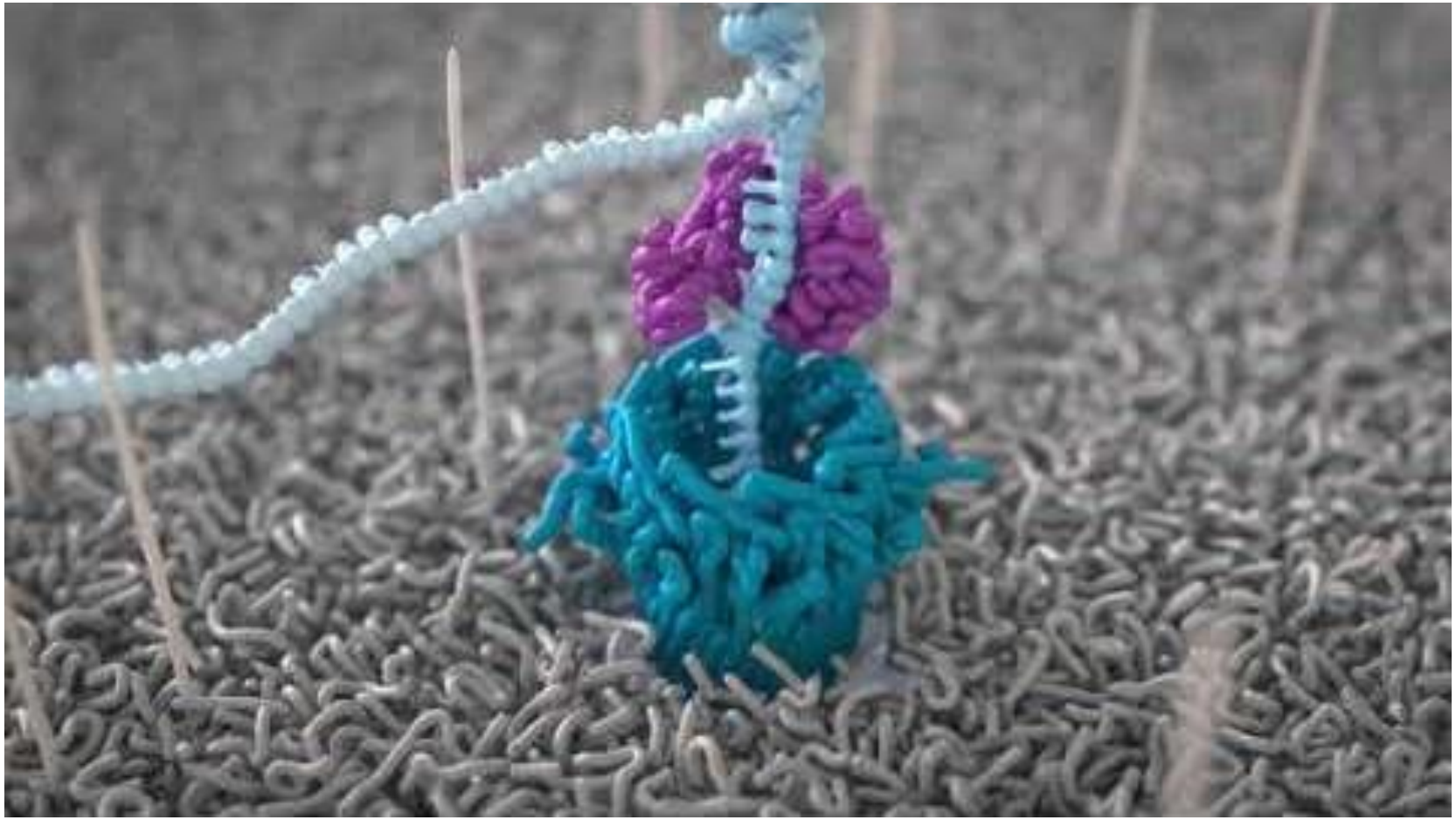
Nanopore Sequencing



https://youtu.be/RcP85JHLmnI

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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.

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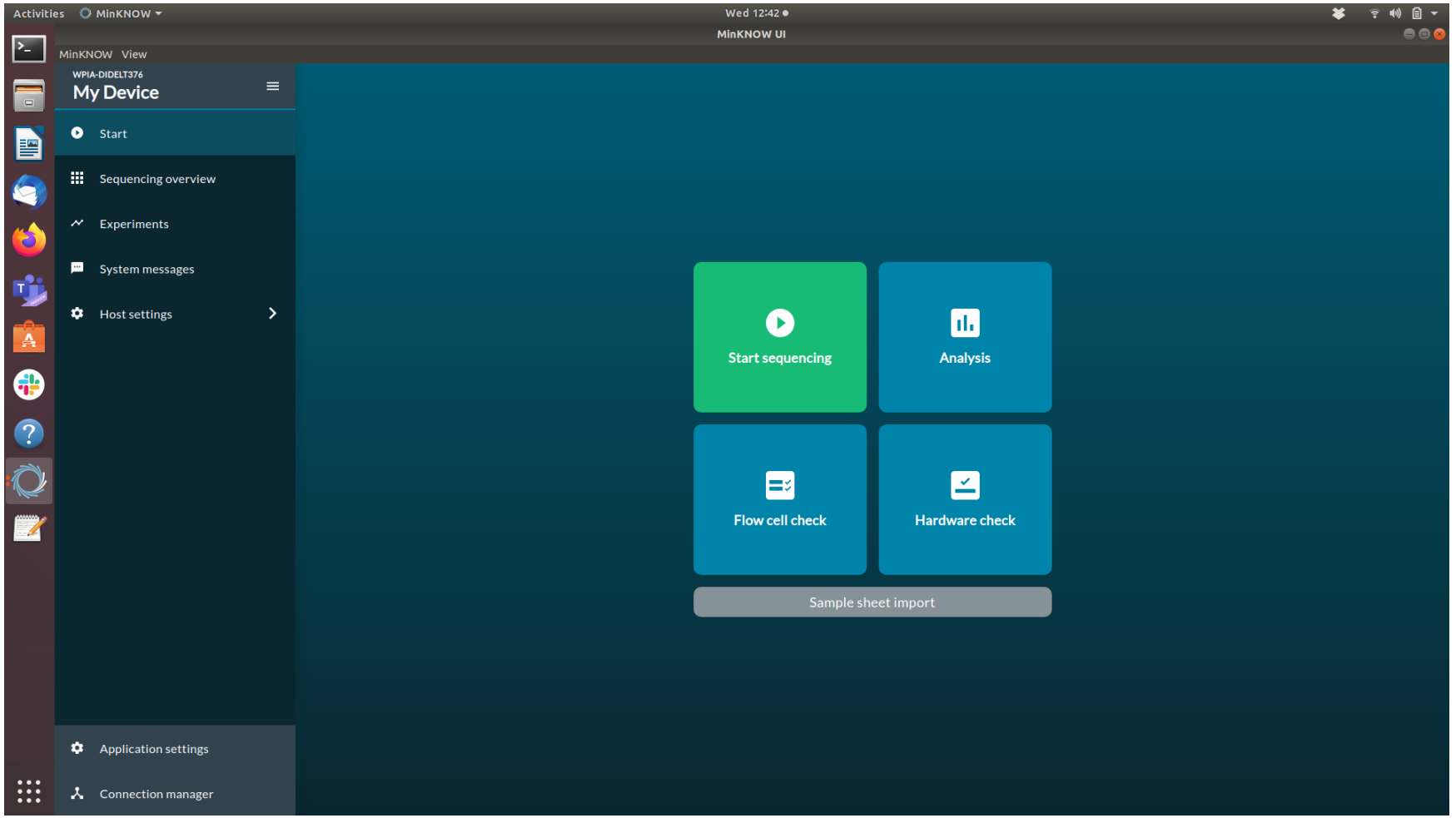
MinKNOW

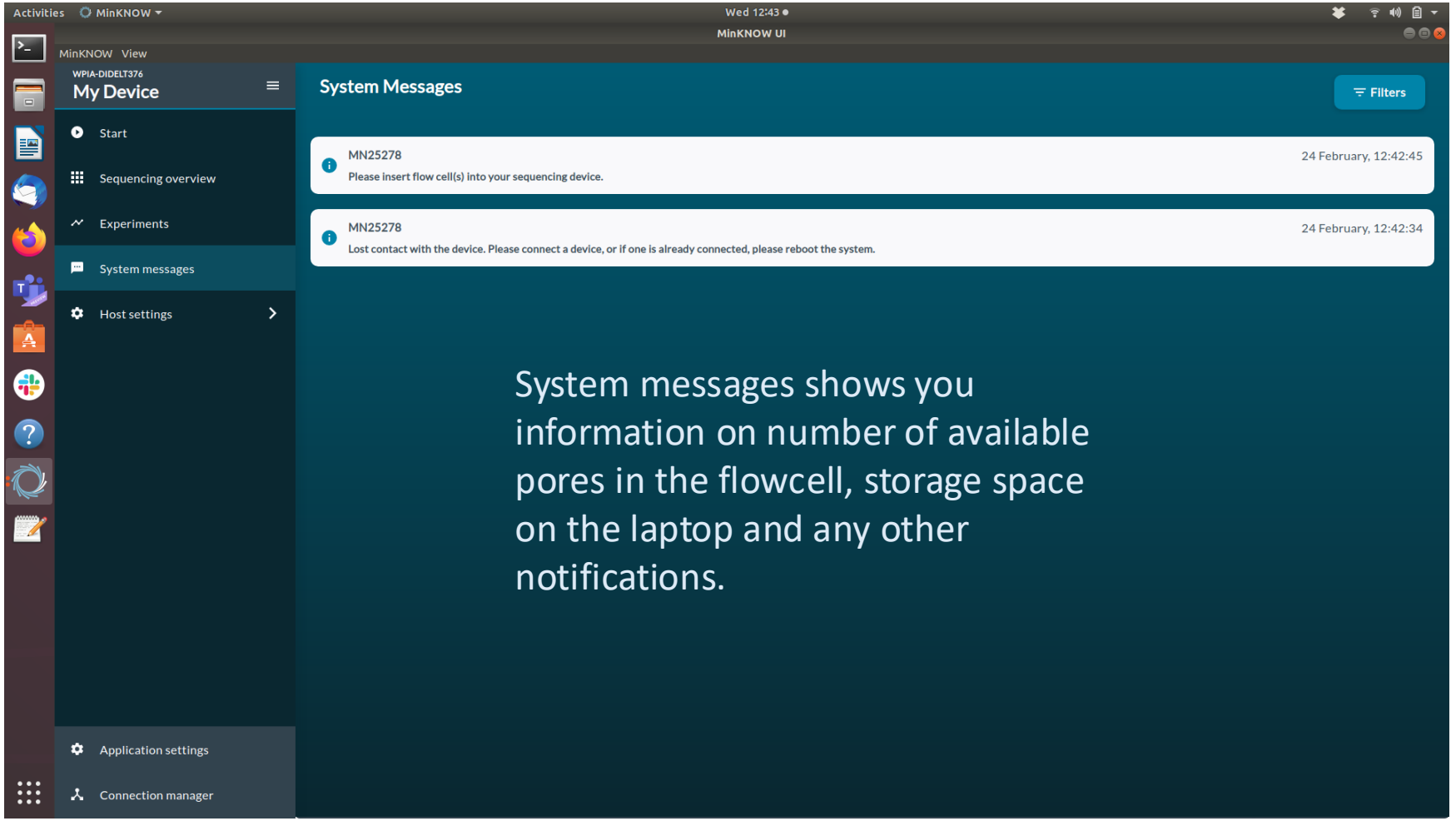
- Easy to use for setting up and running your sequencing run
- Install following the instructions on the Nanopore website
- Basecalling and demultiplexing performed within MinKNOW

MinKNOW GUI


Activities
MinKNOW
Wed 12:42
MinKNOW UI
MinKNOW View
WPIA-DIDELT376
My Device
Start
Sequencing overview
Experiments
System messages
Host settings
Application settings
Connection manager
Start sequencing
Analysis
Flow cell check
Hardware check
Sample sheet import

Polio Sequencing Consortium

System messages



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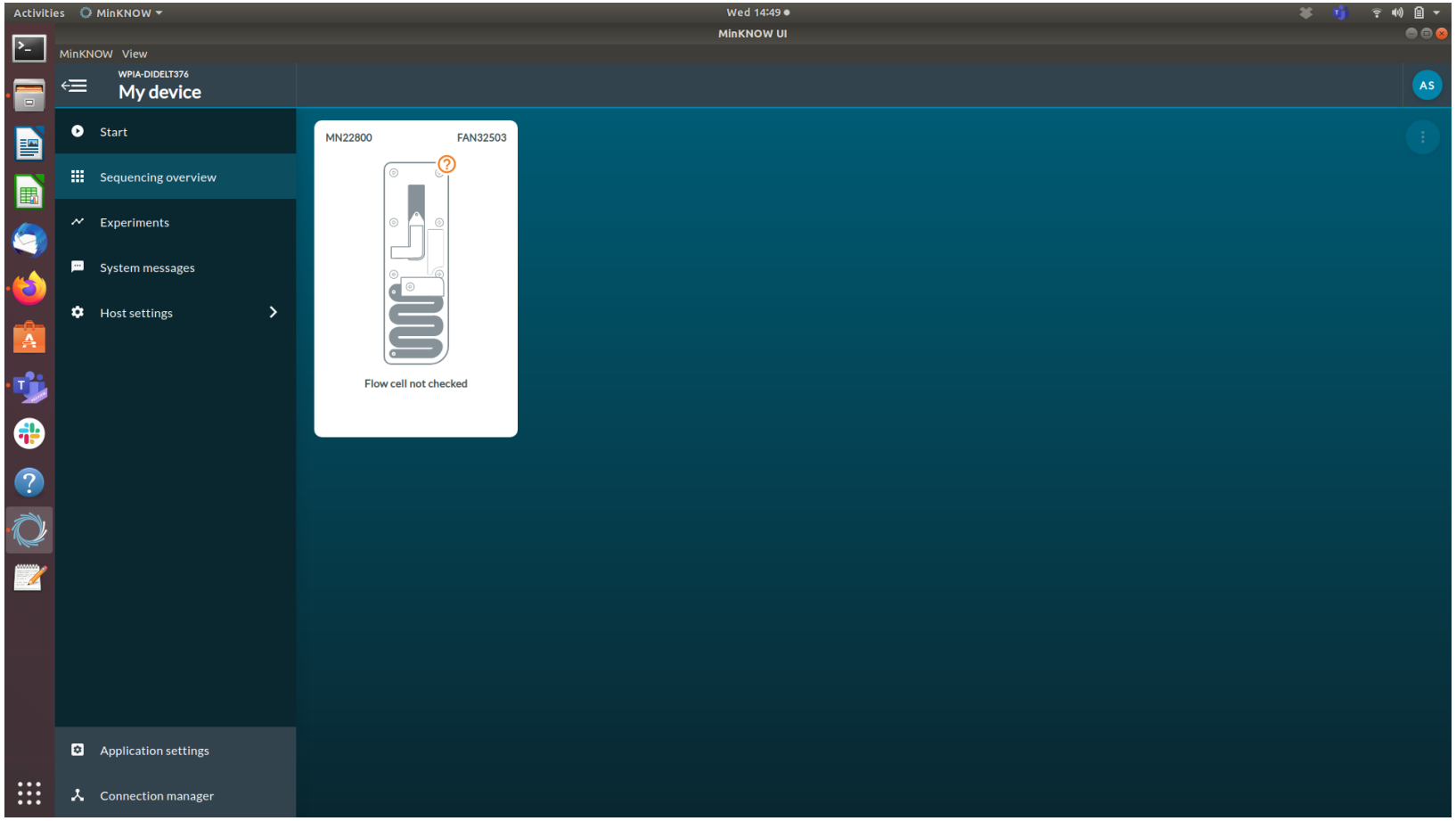
Sequencing overview



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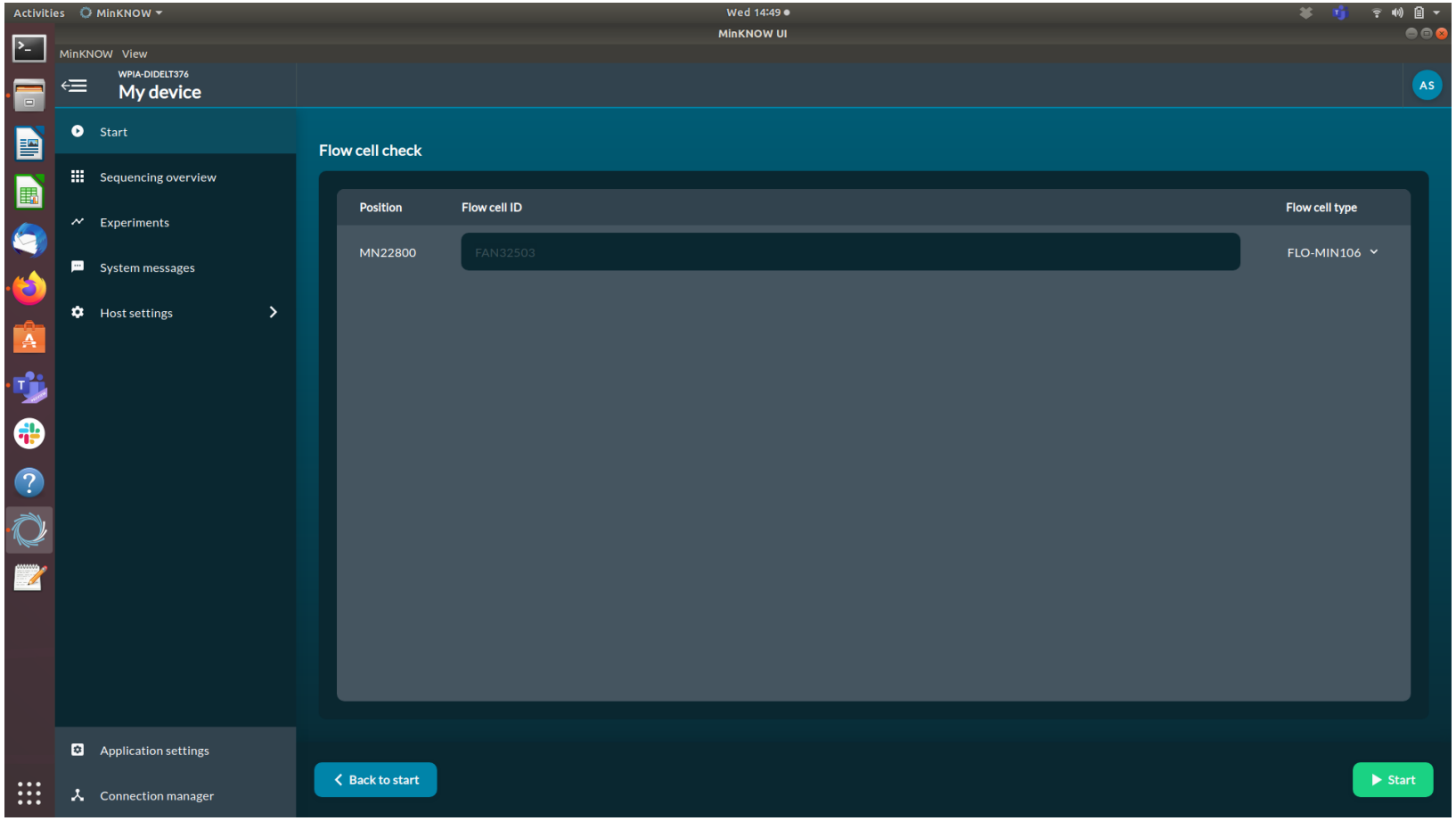
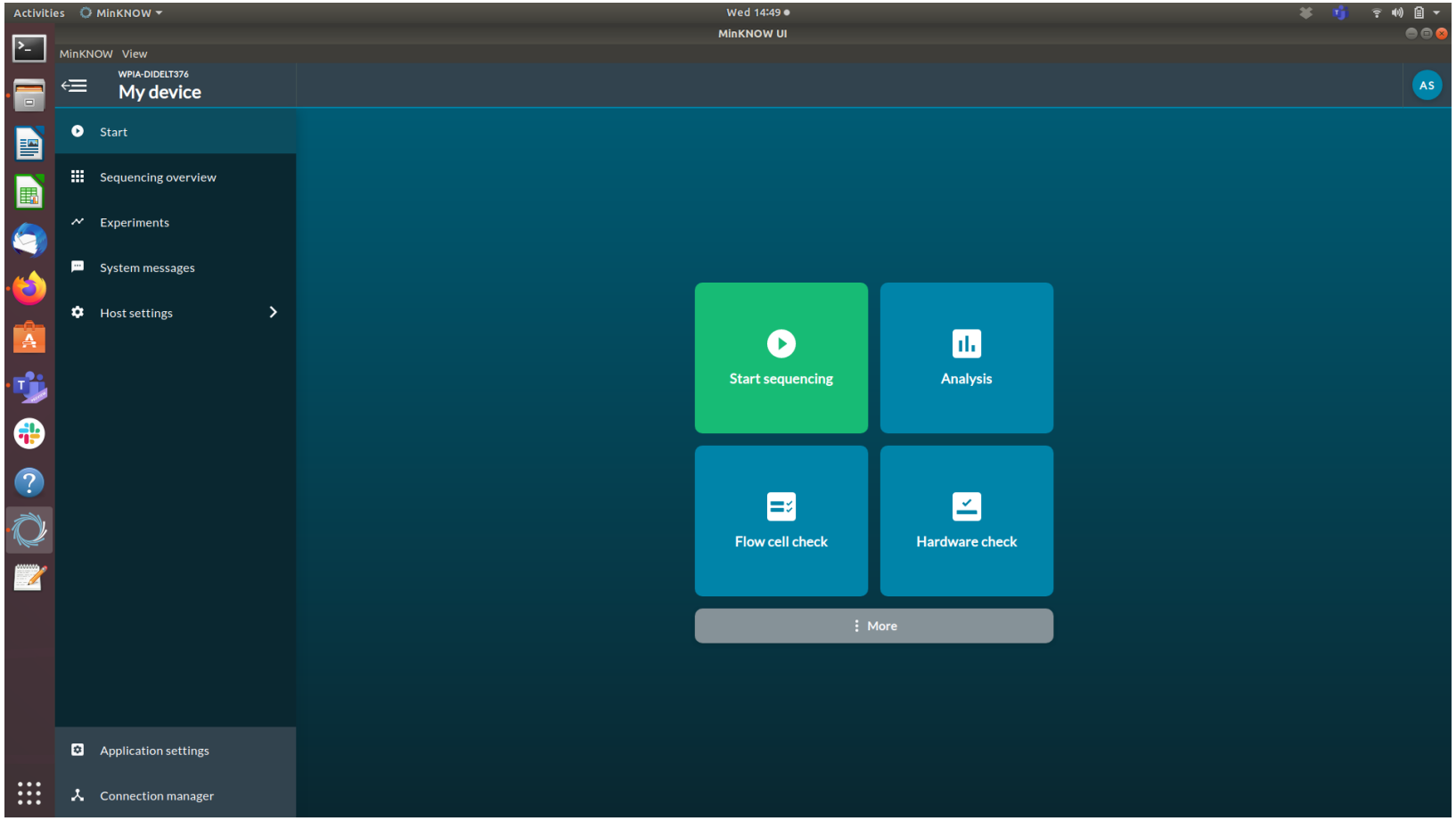
Running a flow cell check

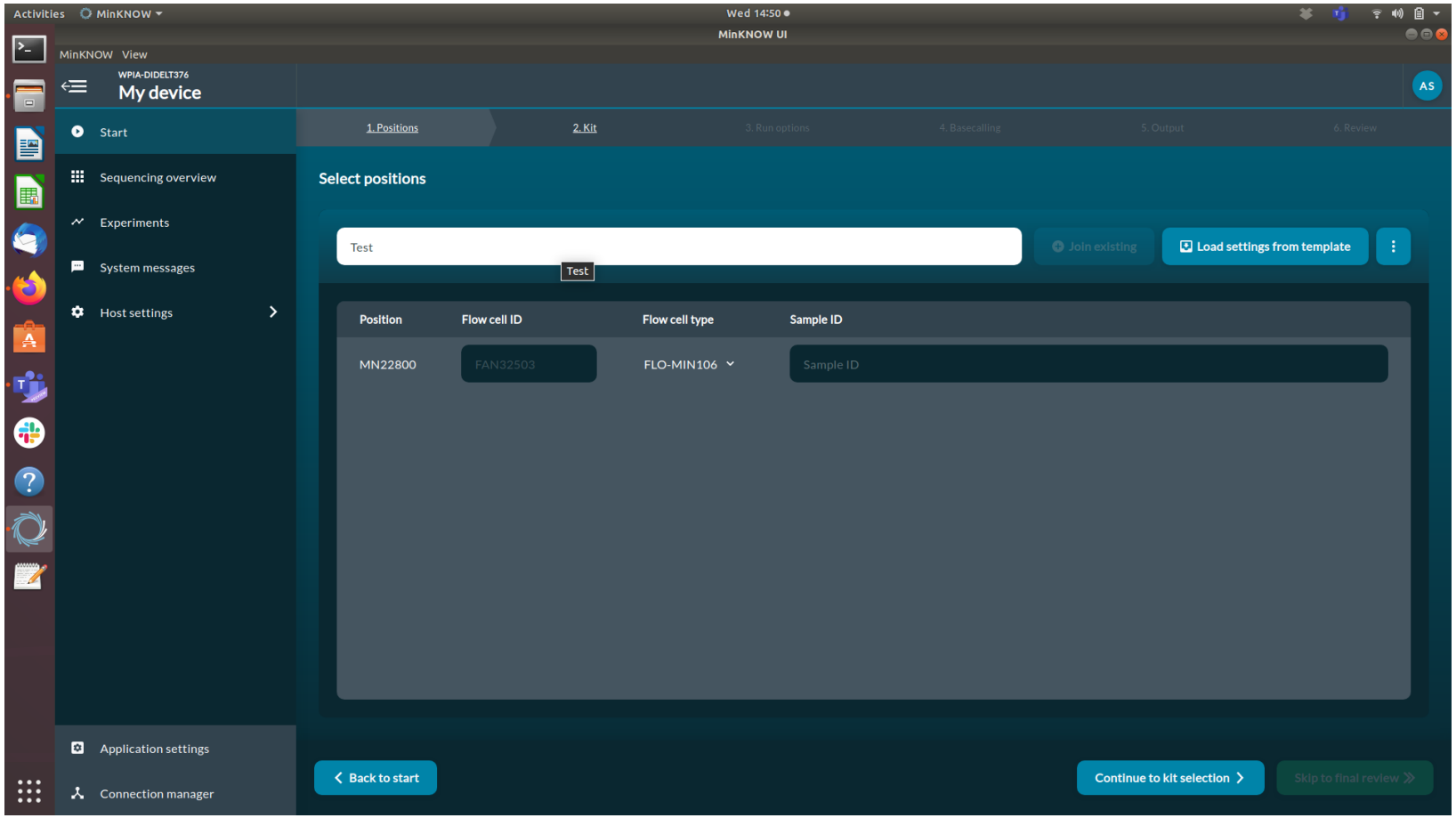


Starting a sequencing run

Polio Sequencing Consortium



Starting a sequencing run



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

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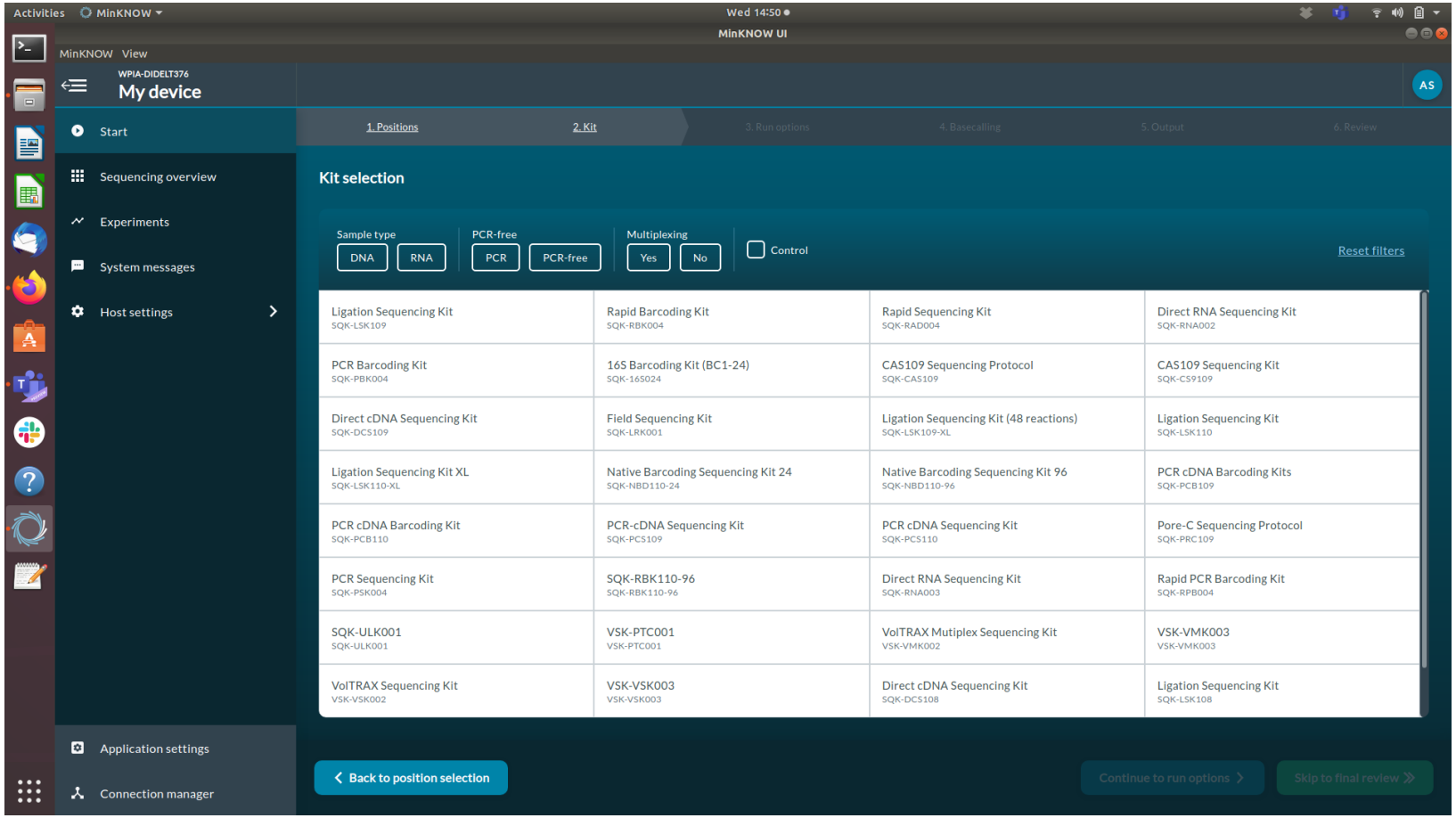
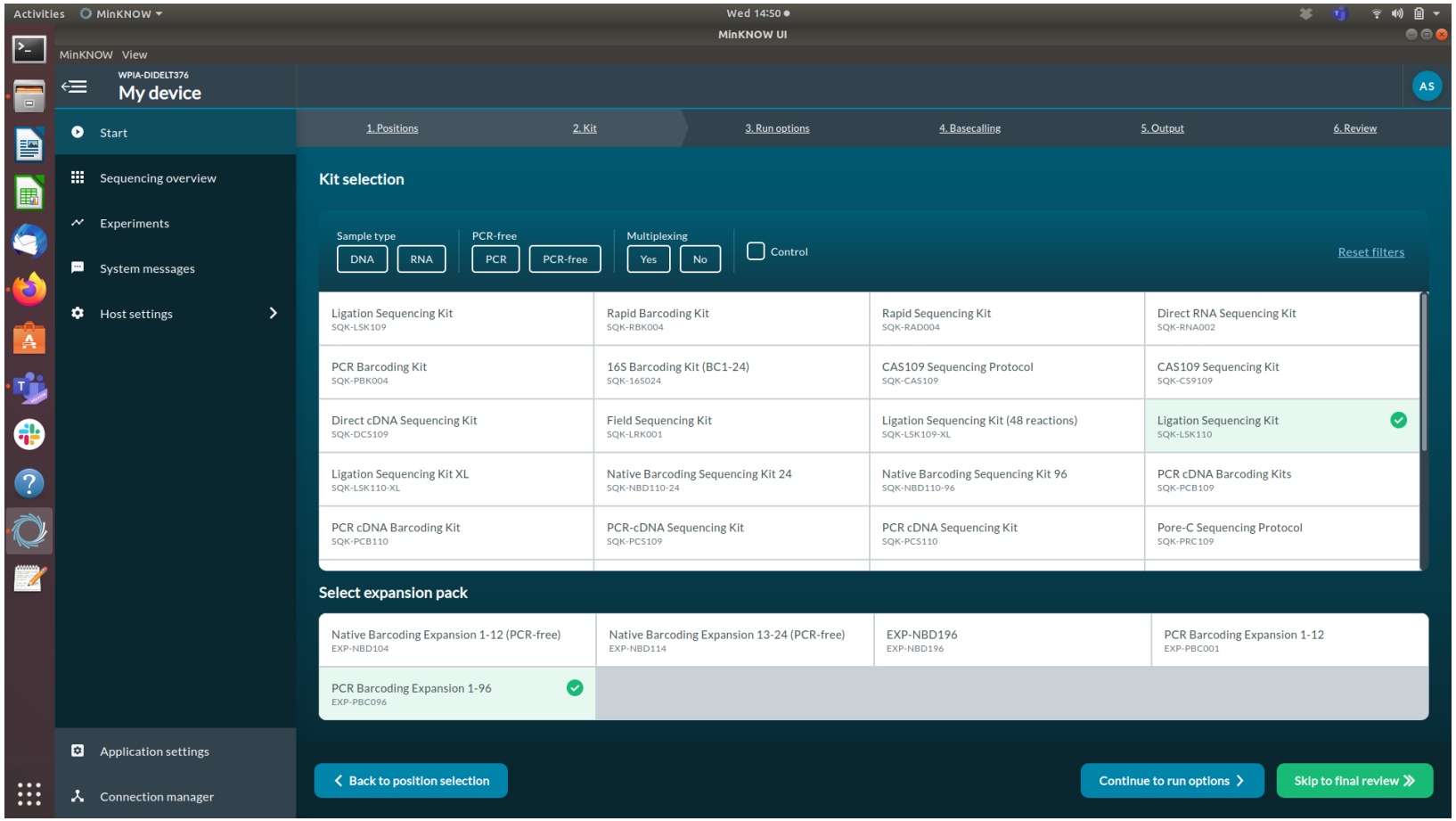
Selecting the sequencing kit



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

Polio Sequencing Consortium

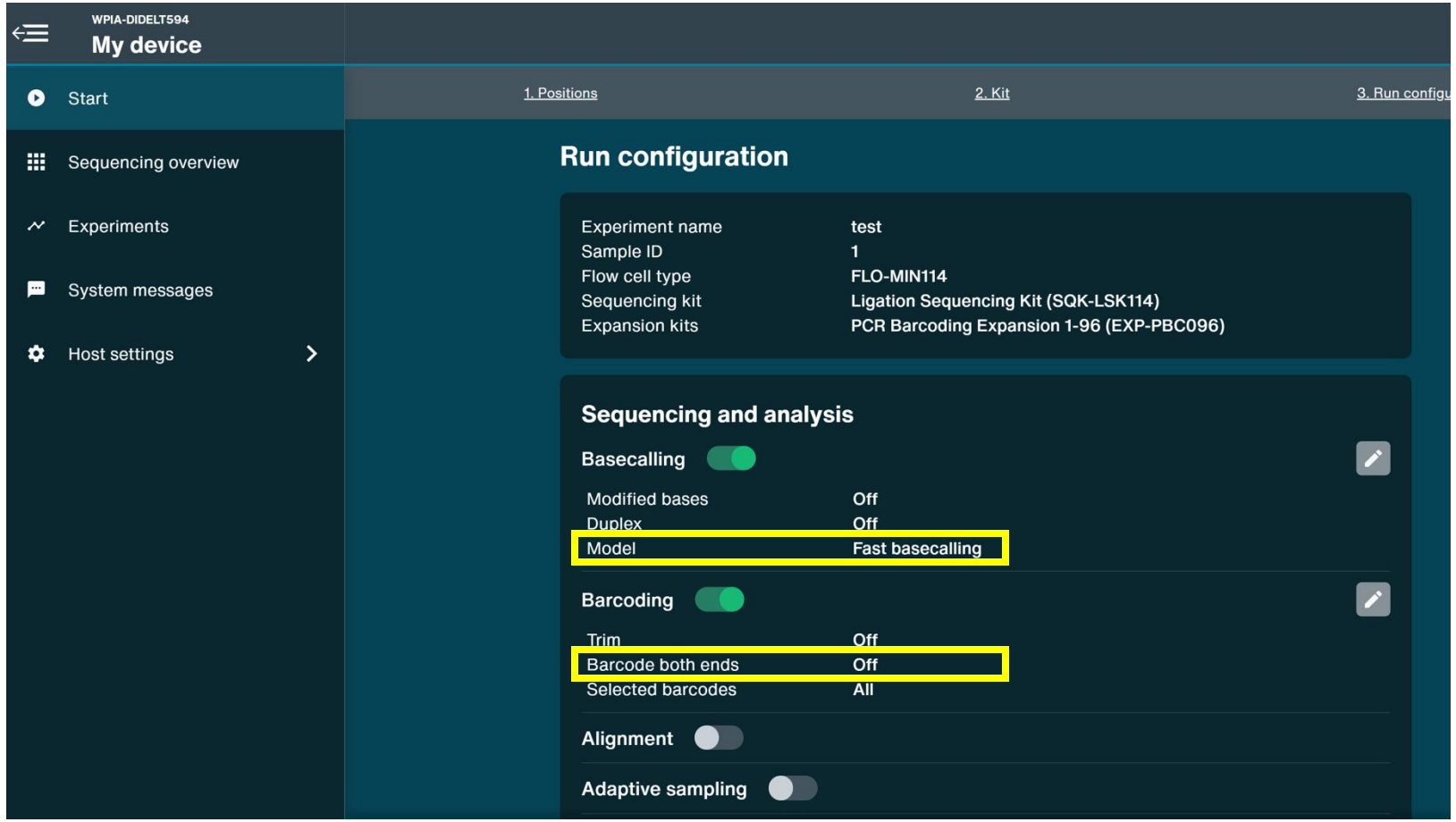
Selecting barcodes



For the barcoded VP1 primers select EXP-PBC096

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

Run Configuration

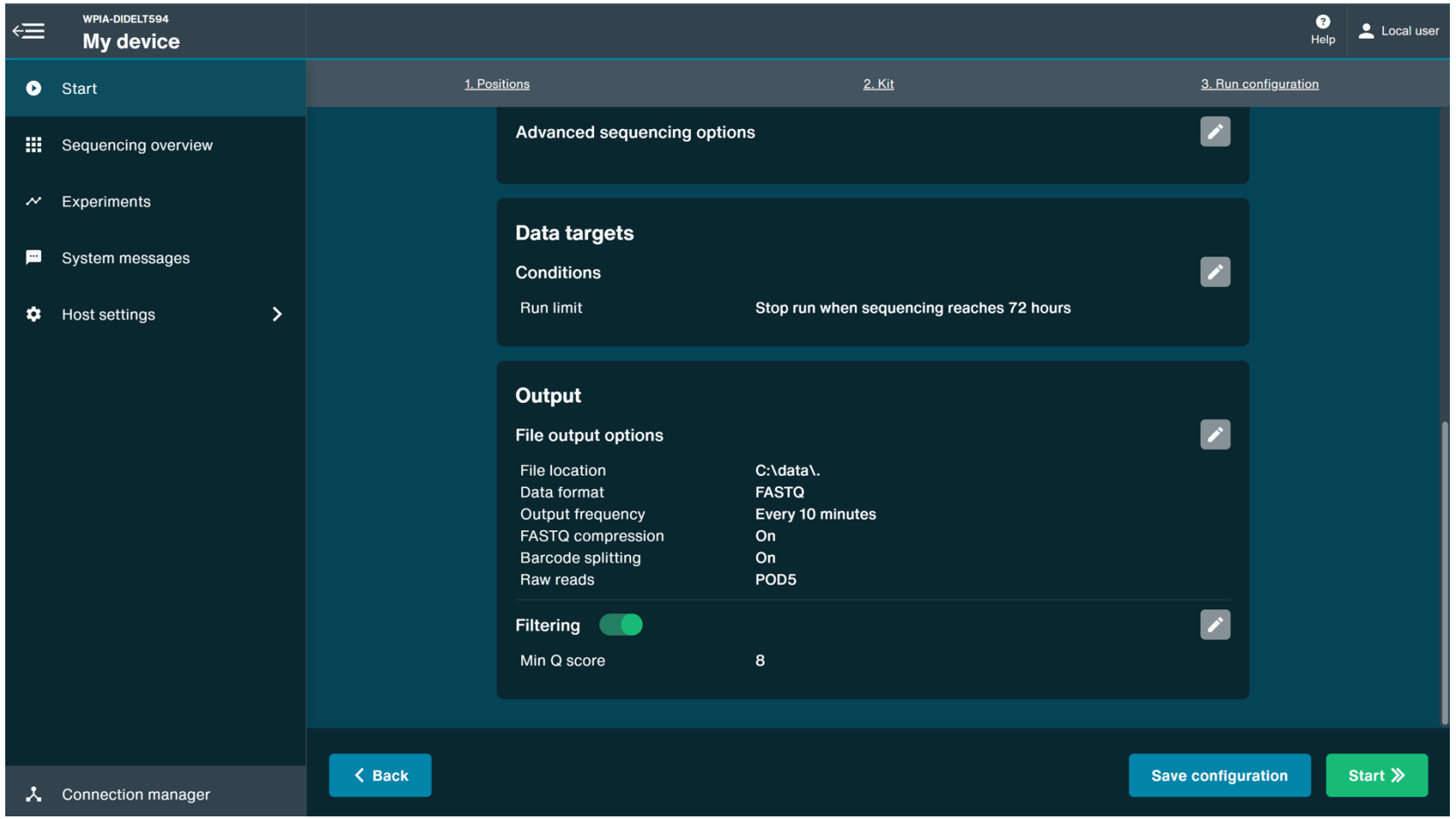


Basecalling speed will depend on computer processing power.

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



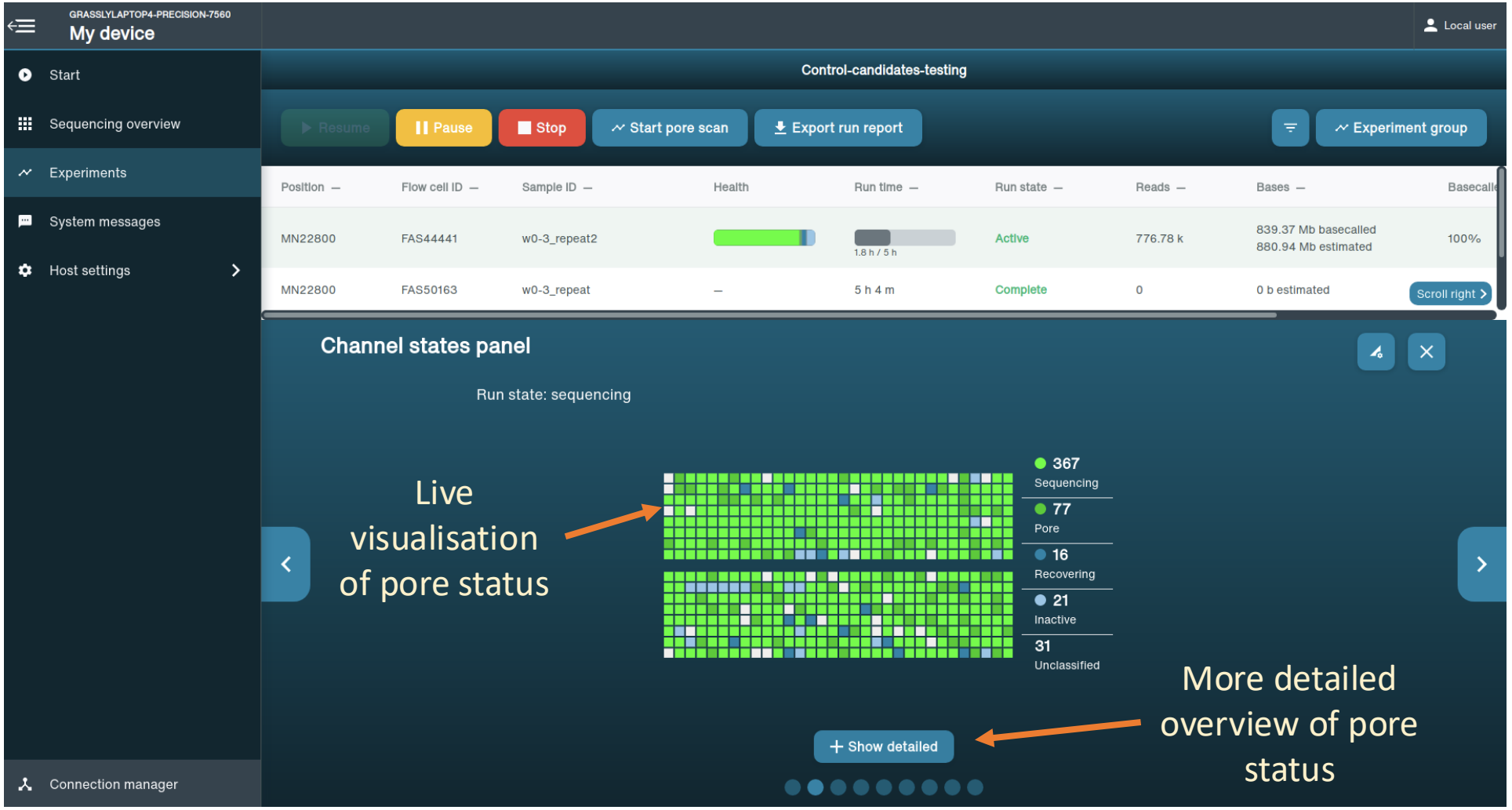
Run can be extended or stopped earlier as required.

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

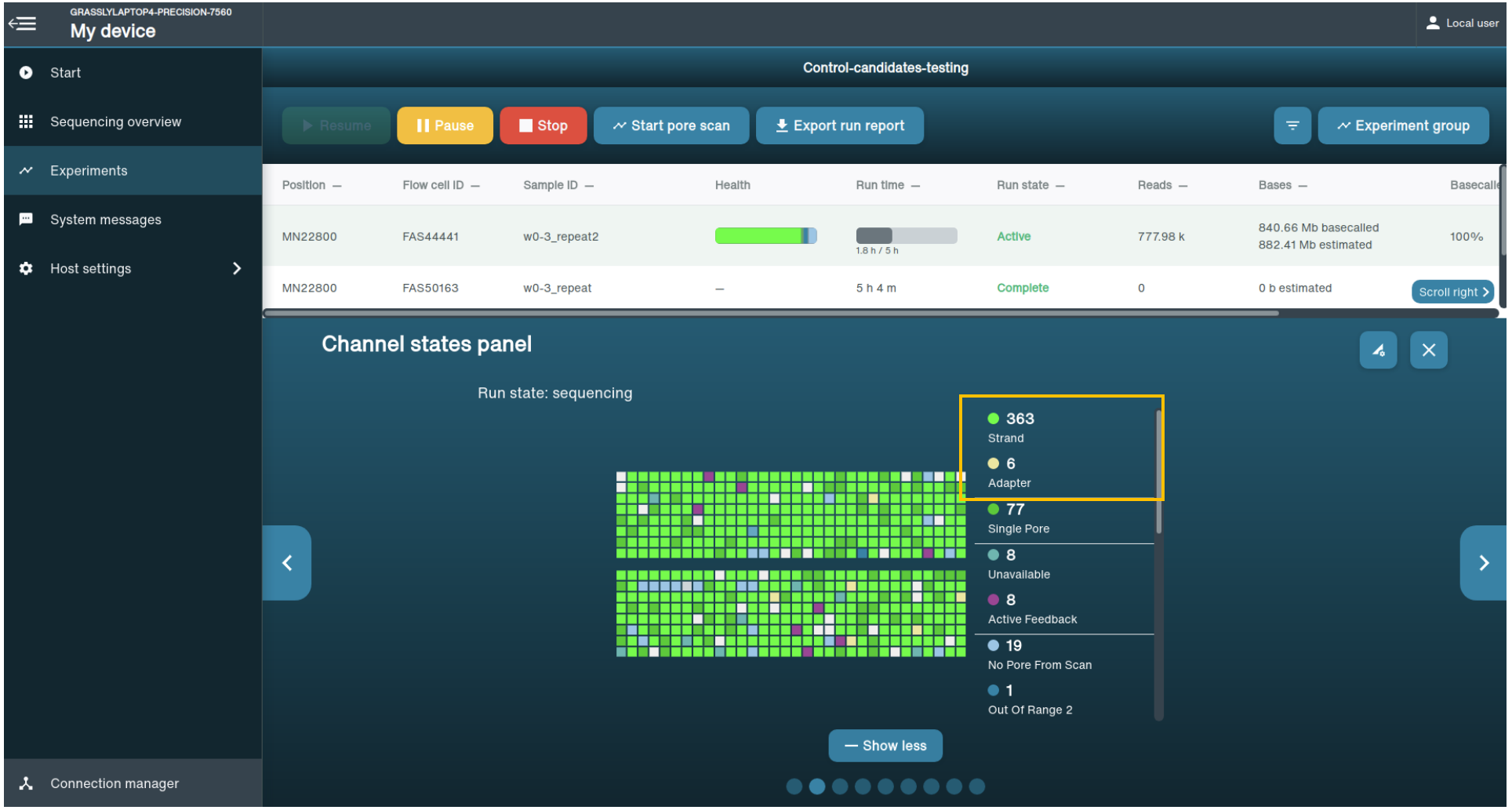
On a Windows computer the default is C:\data\

Raw data file format is POD5

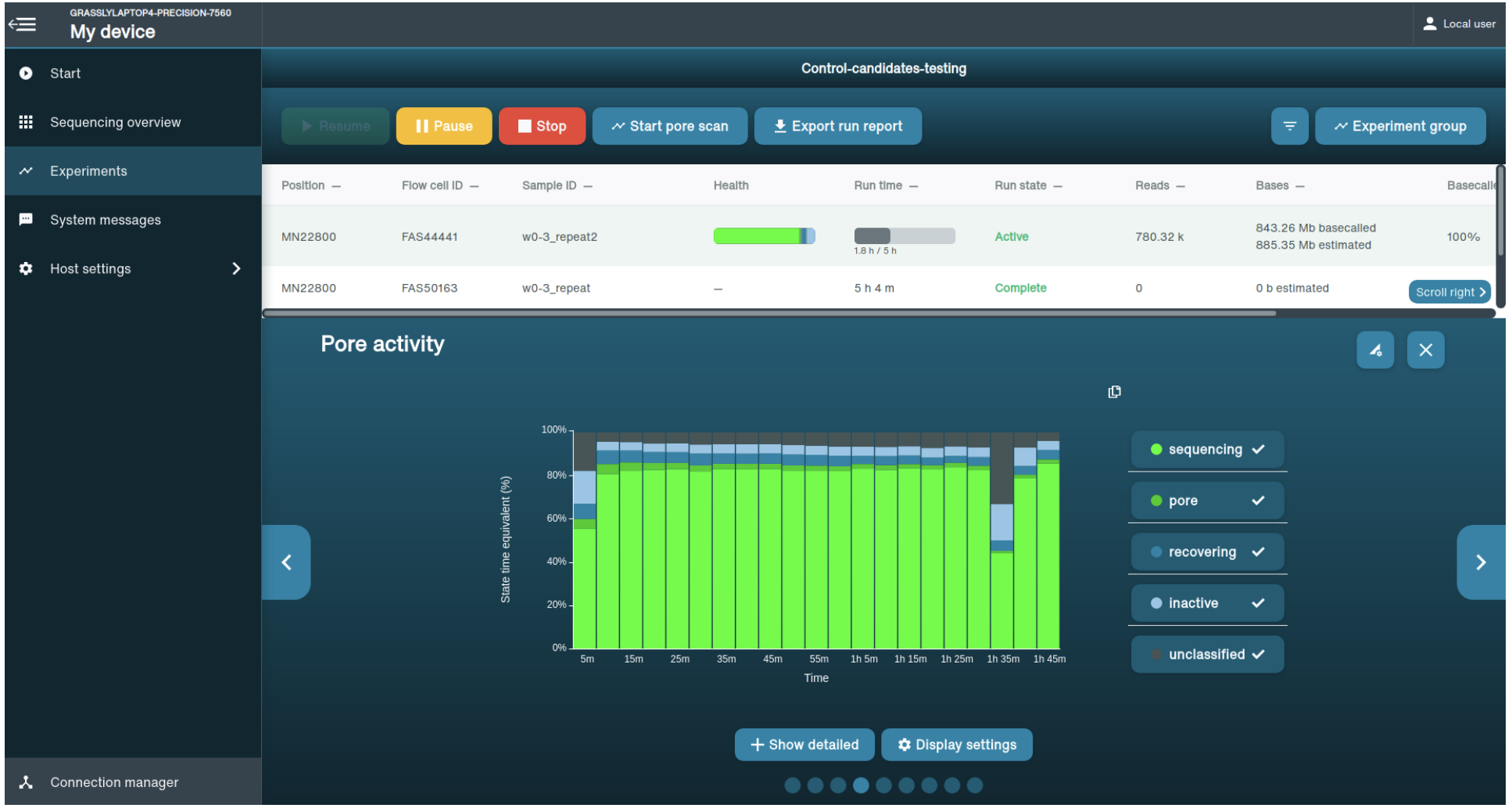
Sequencing Run



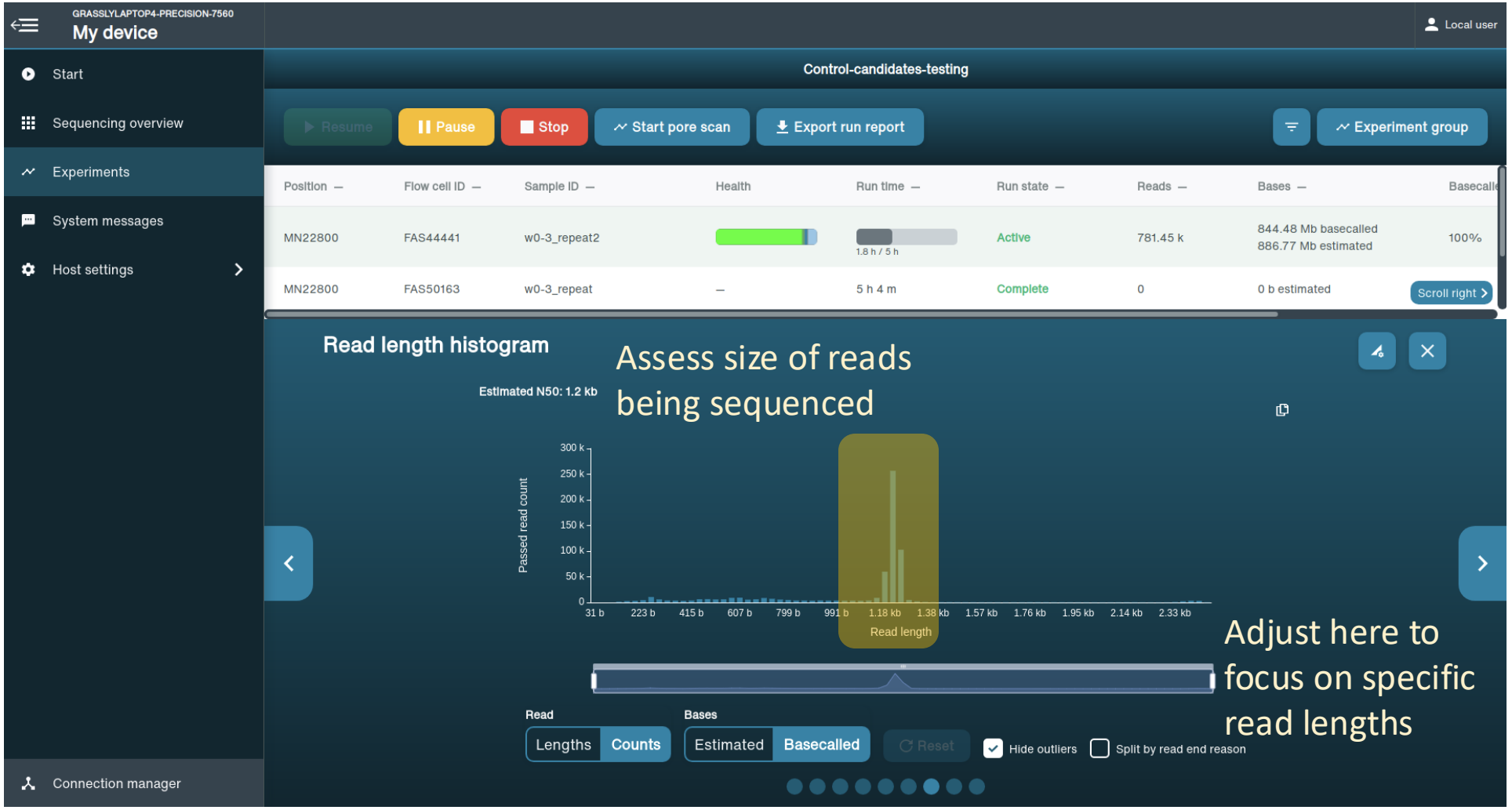
Sequencing Run



Sequencing Run

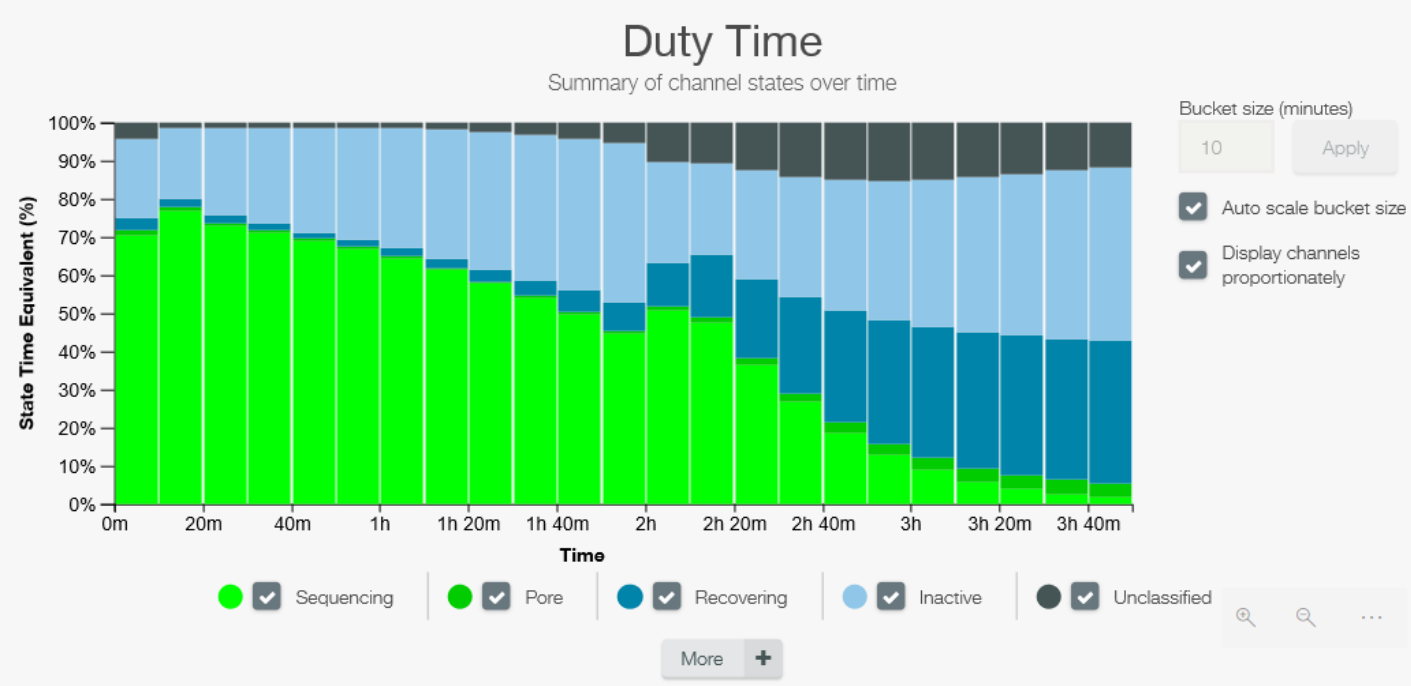


Sequencing Run



Sequencing Run

Examples of a bad start

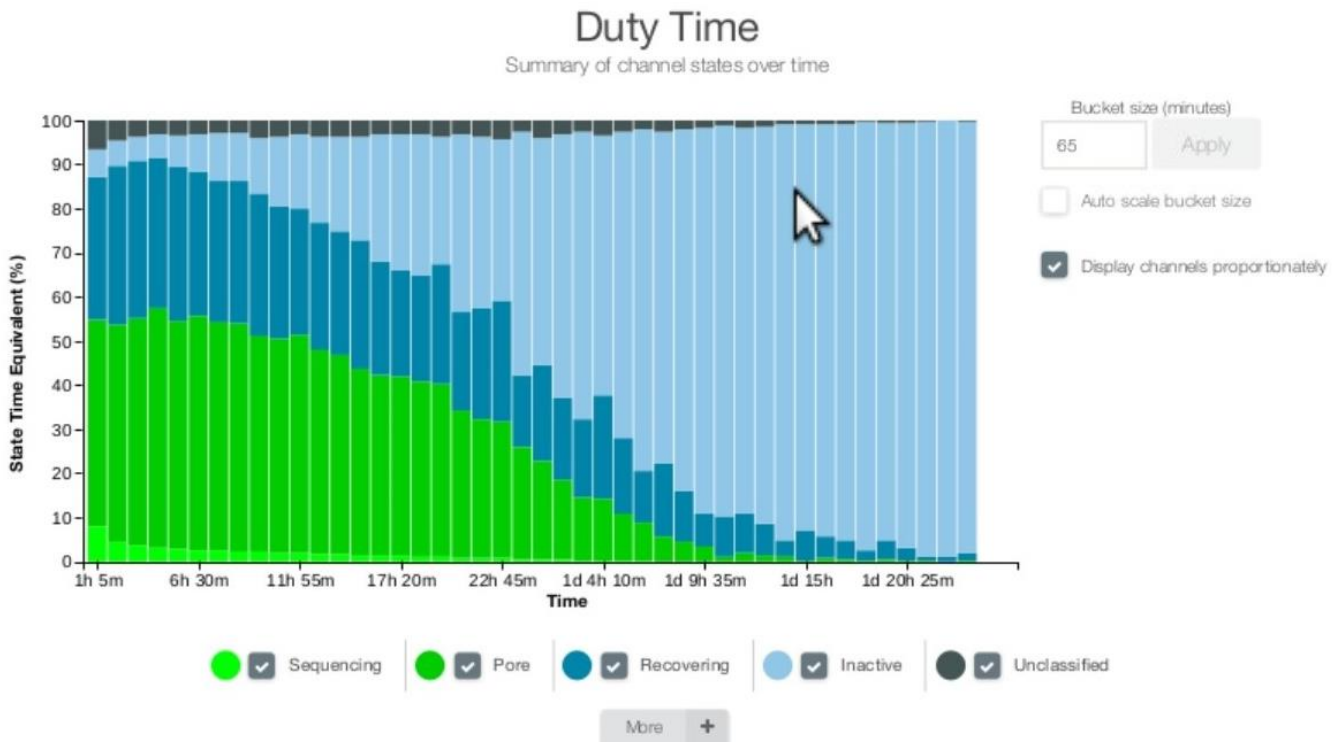


Rapid pore death may be caused by:

- Contamination
- Using an old flowcell
- Software fault

https://community.nanoporetech.com

Examples of a bad start



Low pore occupancy may be caused by:

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

https://community.nanoporetech.com

Polio Sequencing Consortium

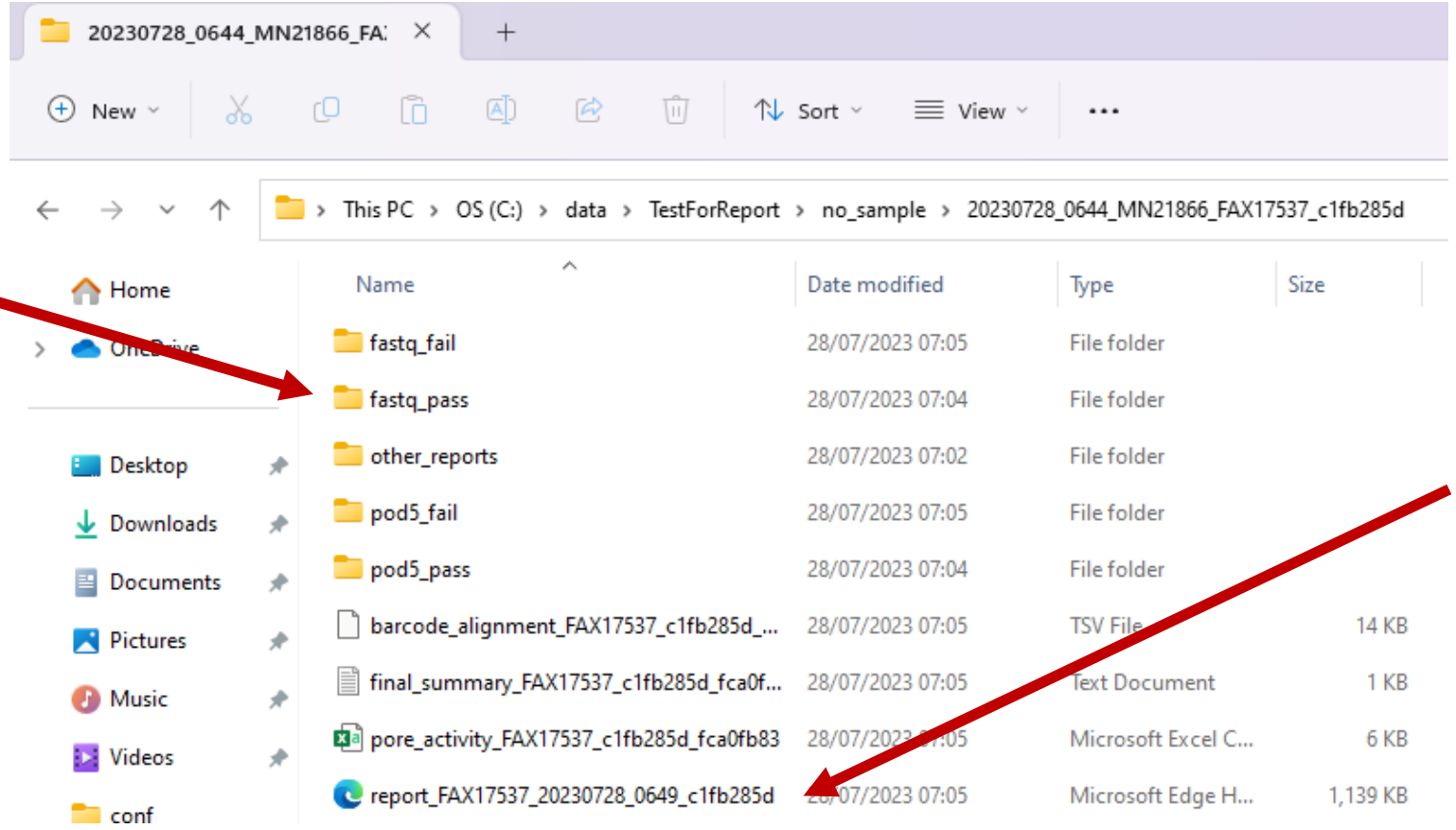
4. Post-sequencing run checks

Presentation and discussion

Polio Sequencing Consortium

1. Confirm that your run created data

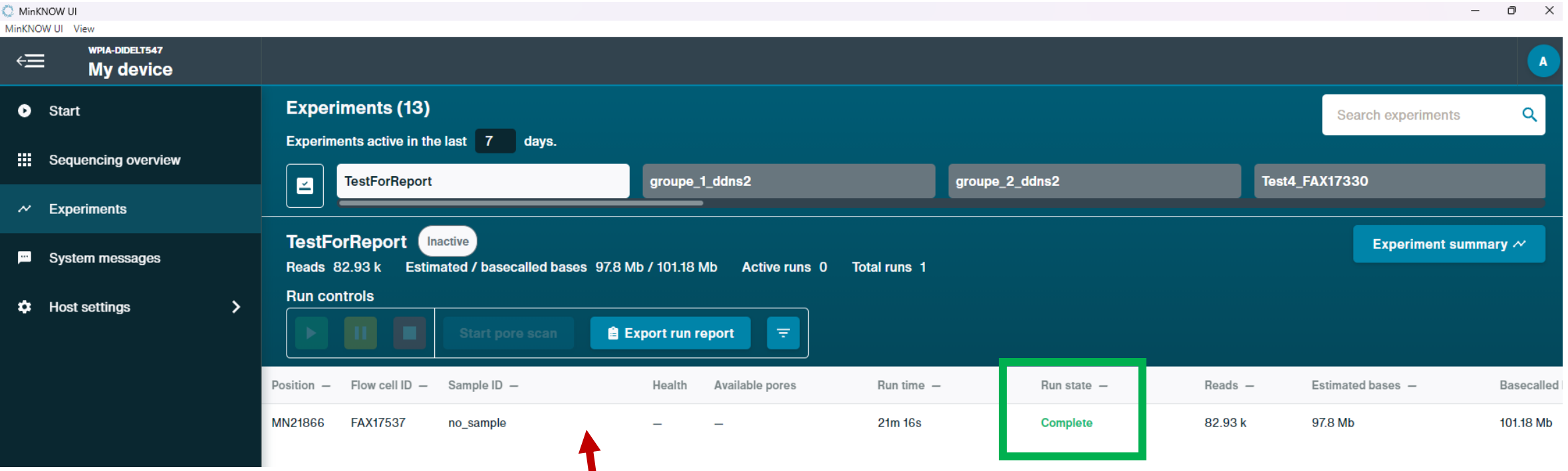
Confirm that there is data in the fastq_pass folder



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

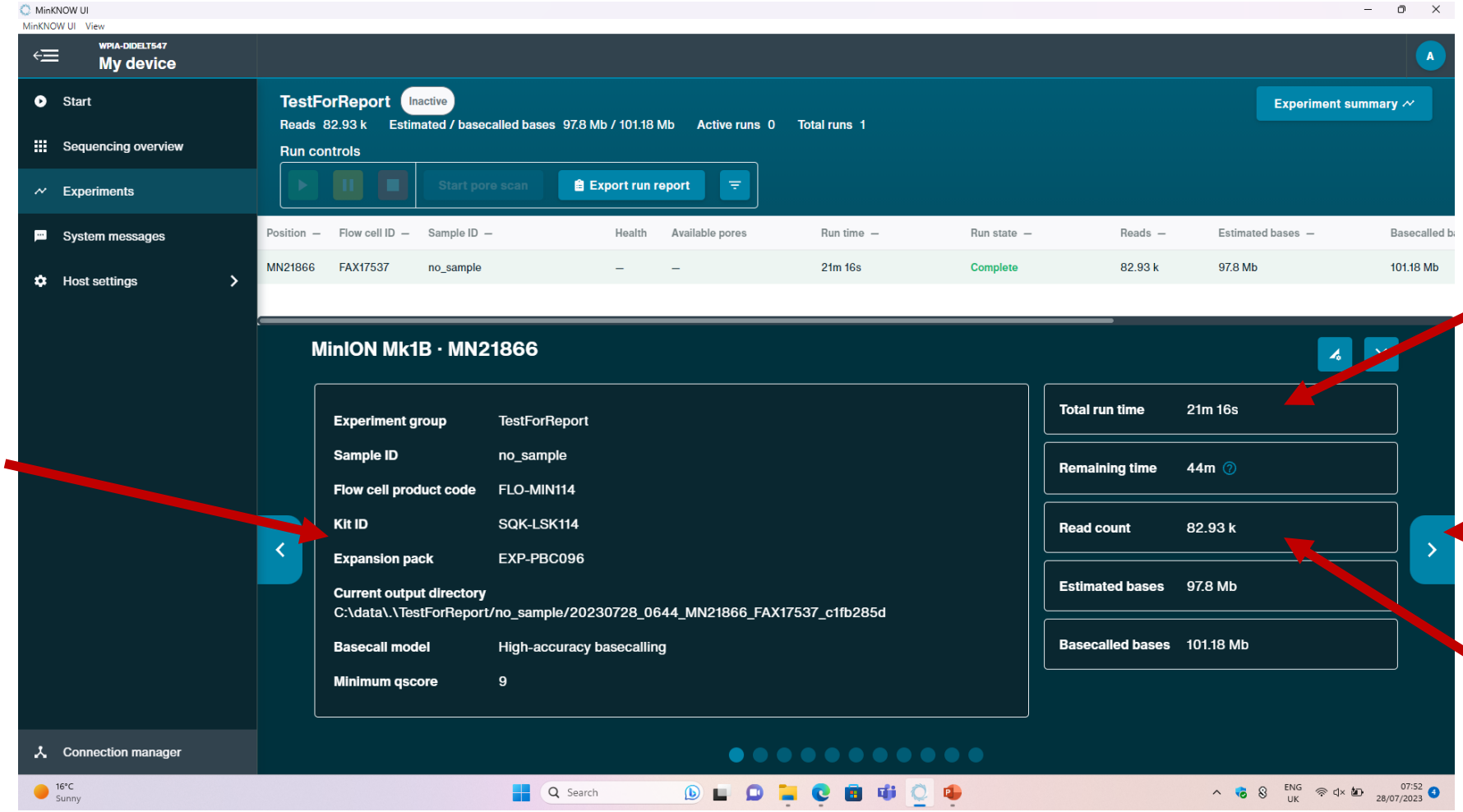
2. Check your run is complete in MinKNOW



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

Polio Sequencing Consortium

3. Check your run performance



Your run settings

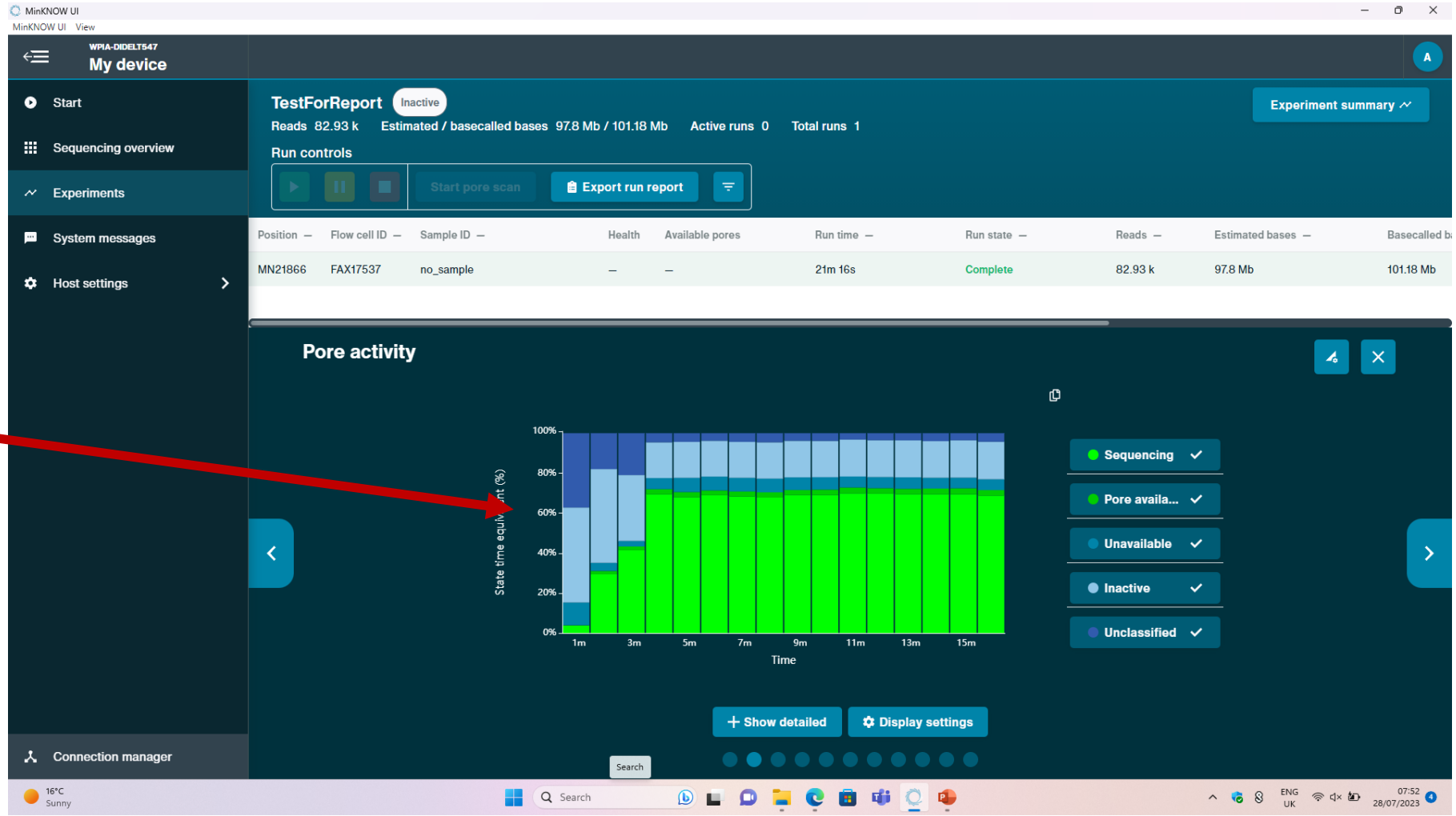
Confirm your run length (4 hours for routine stool testing by DDNS)

Click for more figures

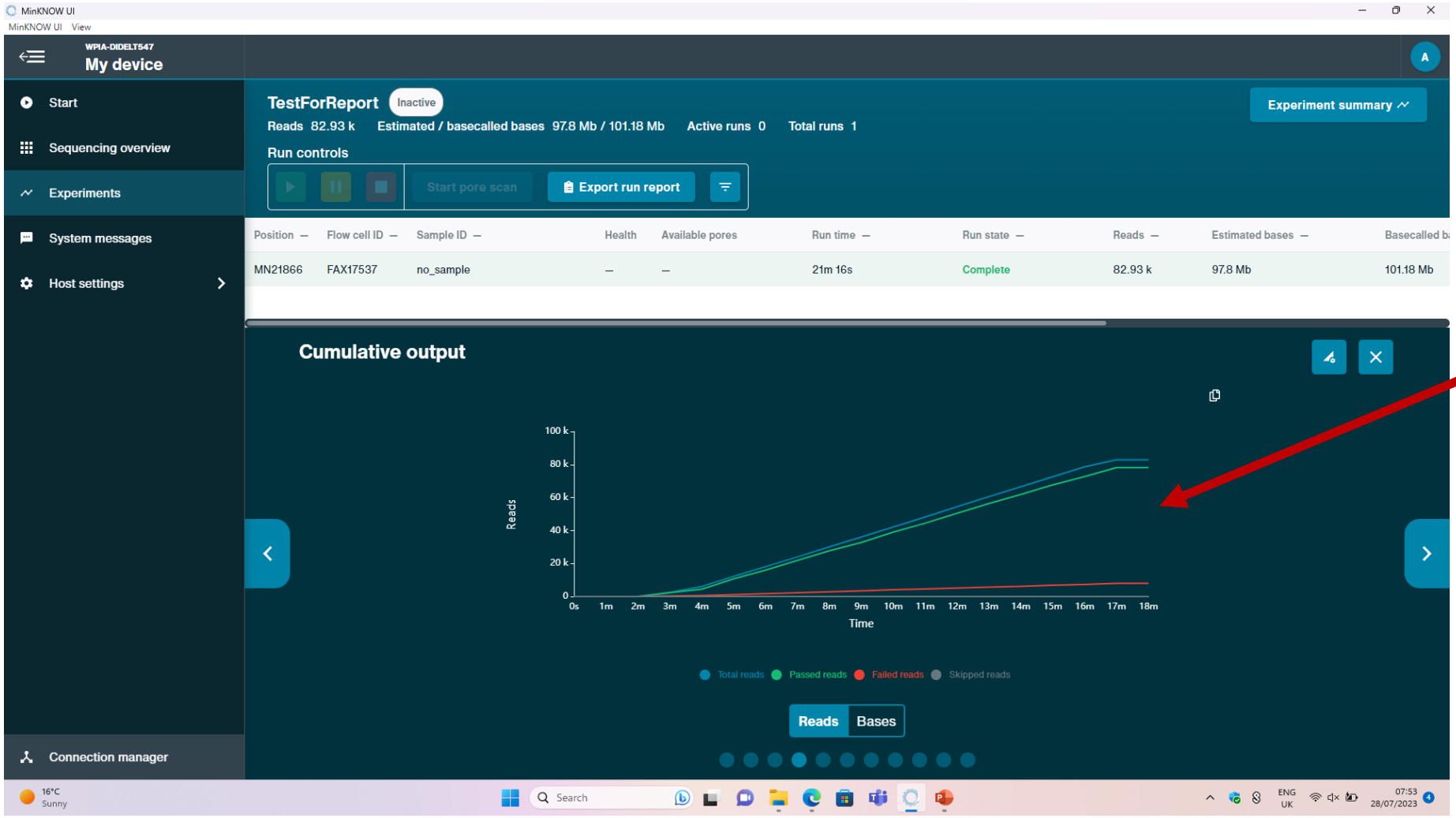
The total number of sequencing reads generated

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.



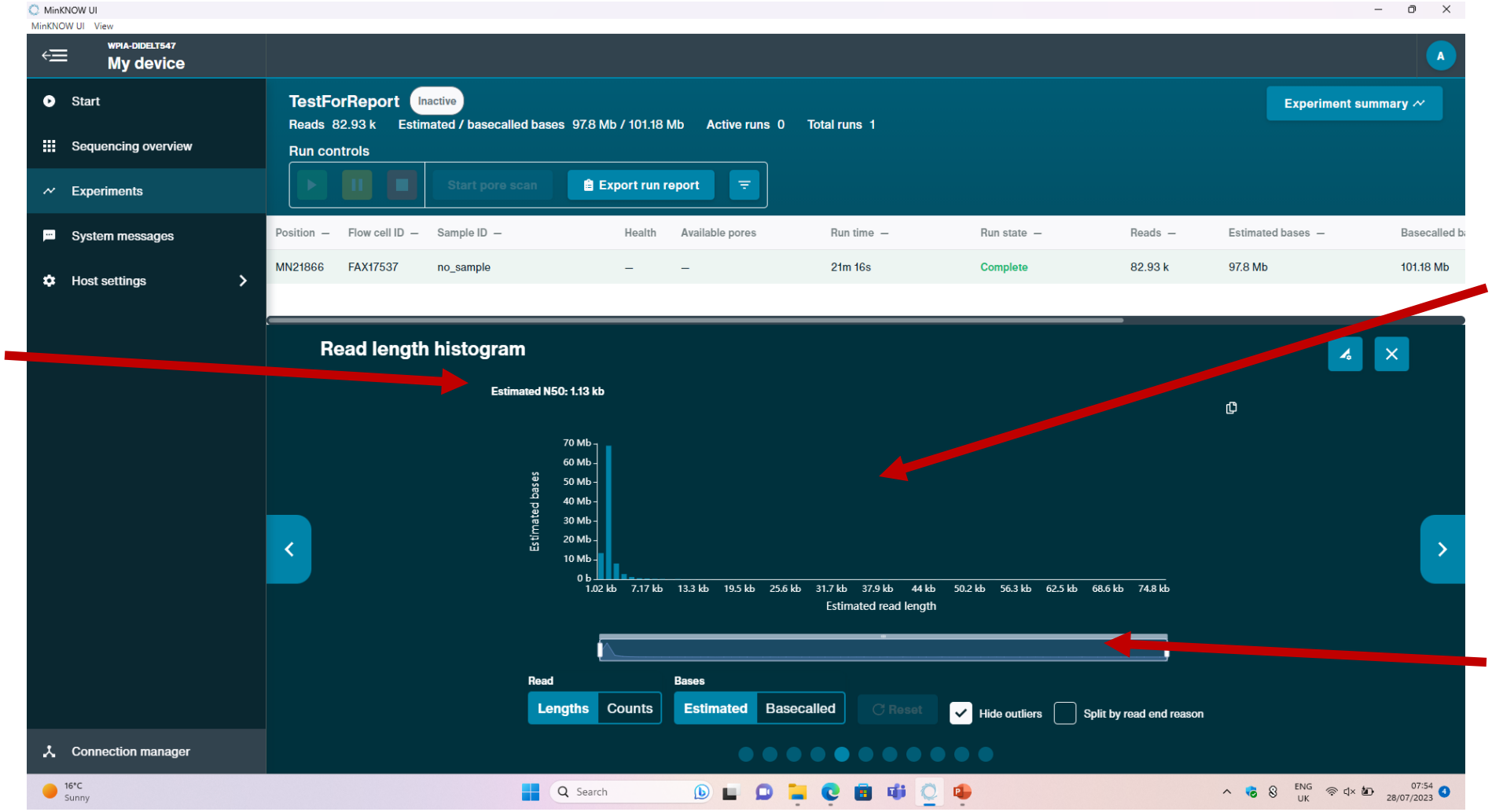
5. Check the quality of your data



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

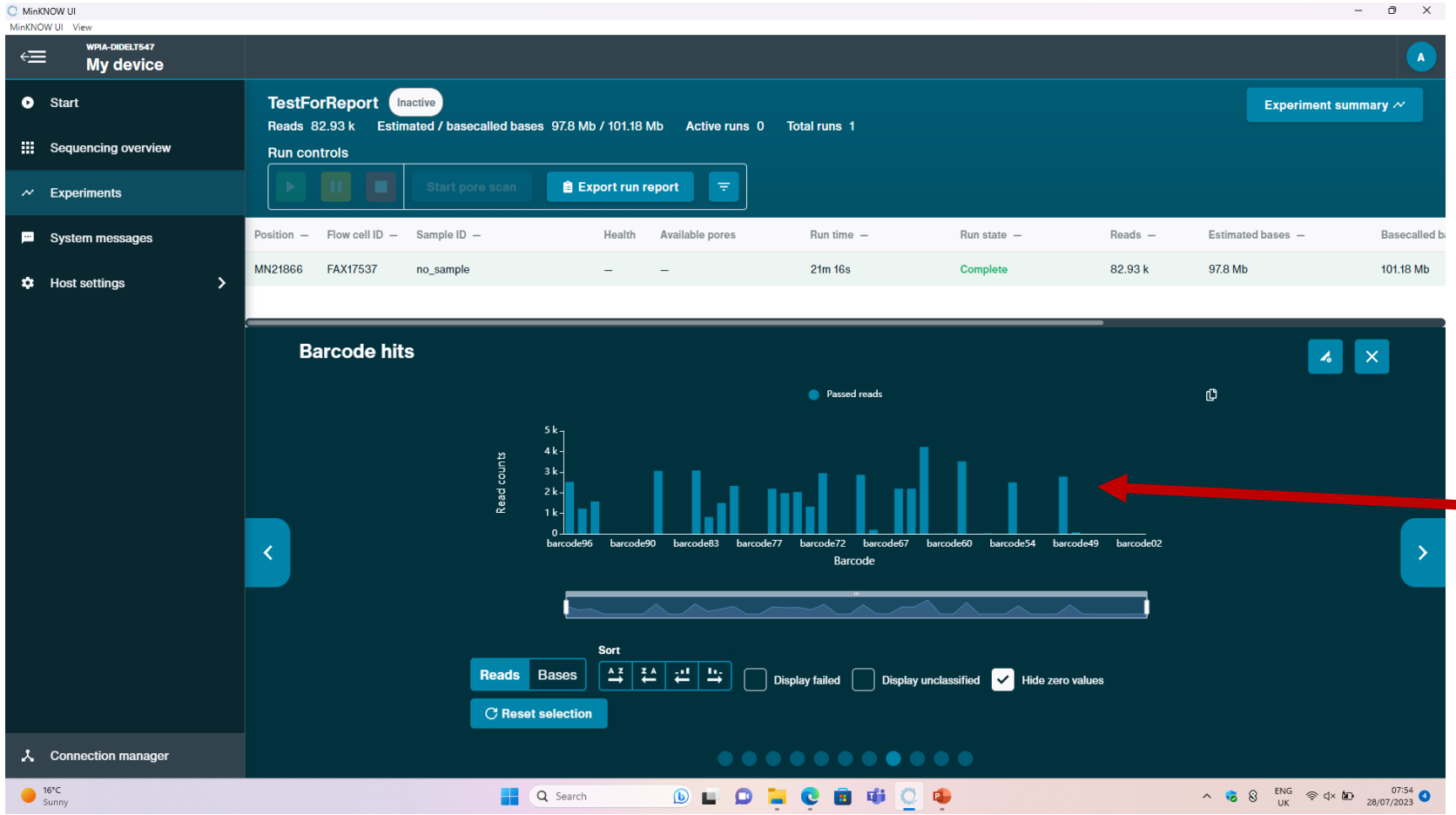
Check the average read length (N50)- for VP1 by DDNS this should be ~ 1.2 kB



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.

Polio Sequencing Consortium

5. Washing a flow cell after a sequencing run

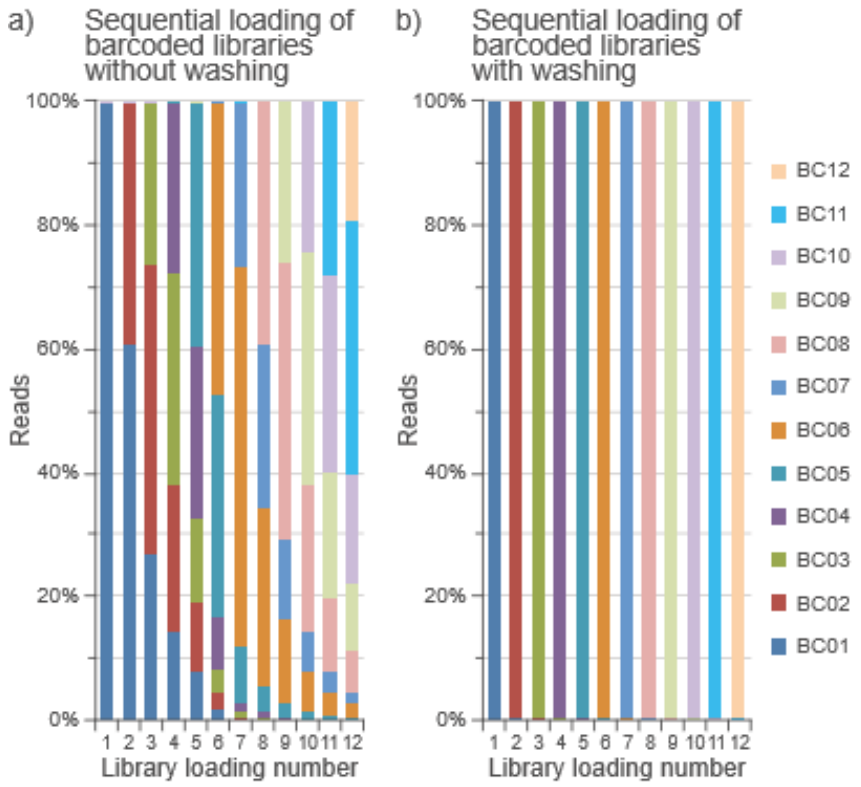
Presentation and discussion

Polio Sequencing Consortium

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



https://store.nanoporetech.com/catalog/product/view/id/122/

Polio Sequencing Consortium

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

Polio Sequencing Consortium

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

Polio Sequencing Consortium

6. Installation and use of piranha

Poliovirus investigation resource automating nanopore haplotype analysis

Presentation and discussion

Polio Sequencing Consortium

piranha

Poliovirus investigation resource automating nanopore haplotype analysis

Áine O'Toole[1,*] Rachel Colquhoun[1], Corey Ansley[1], Catherine Troman[2], Daniel Maloney[1], Zoe Vance[1], Joyce Akello[2], Erika Bujaki[3], Manasi Majumdar[3], Adnan Khurshid[4], Yasir Arshad[4], Muhammad Masroor Alam[4], Javier Martin[3], Alexander G Shaw[2], Nicholas C Grassly[2,$], Andrew Rambaut[1,$]

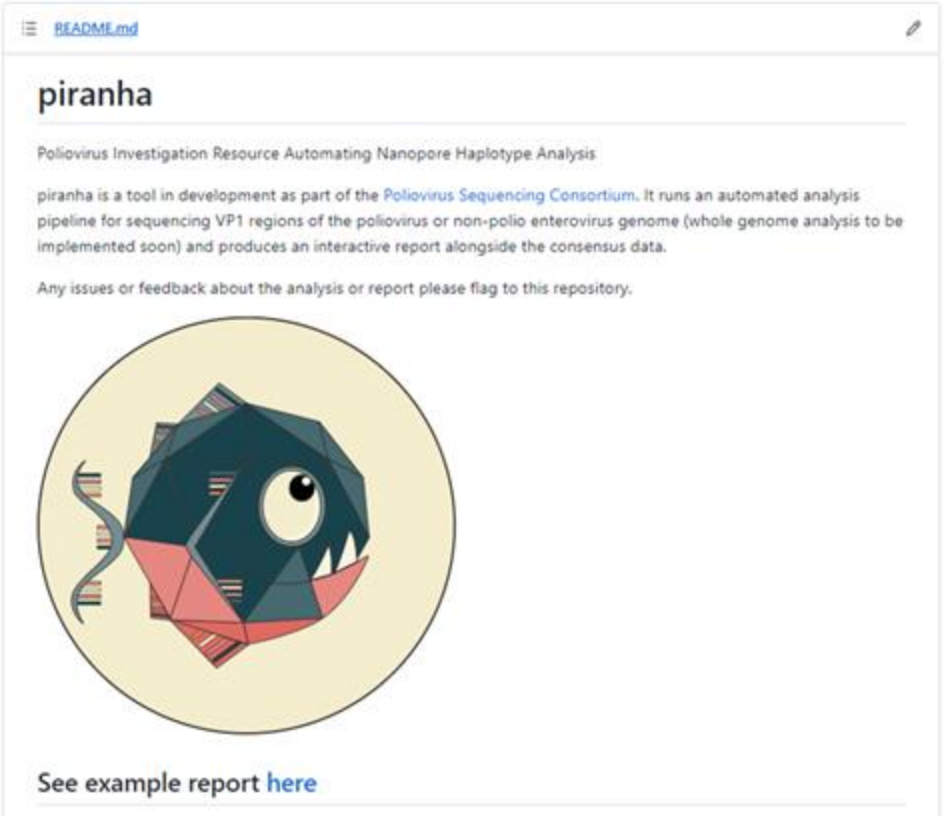
Affiliations

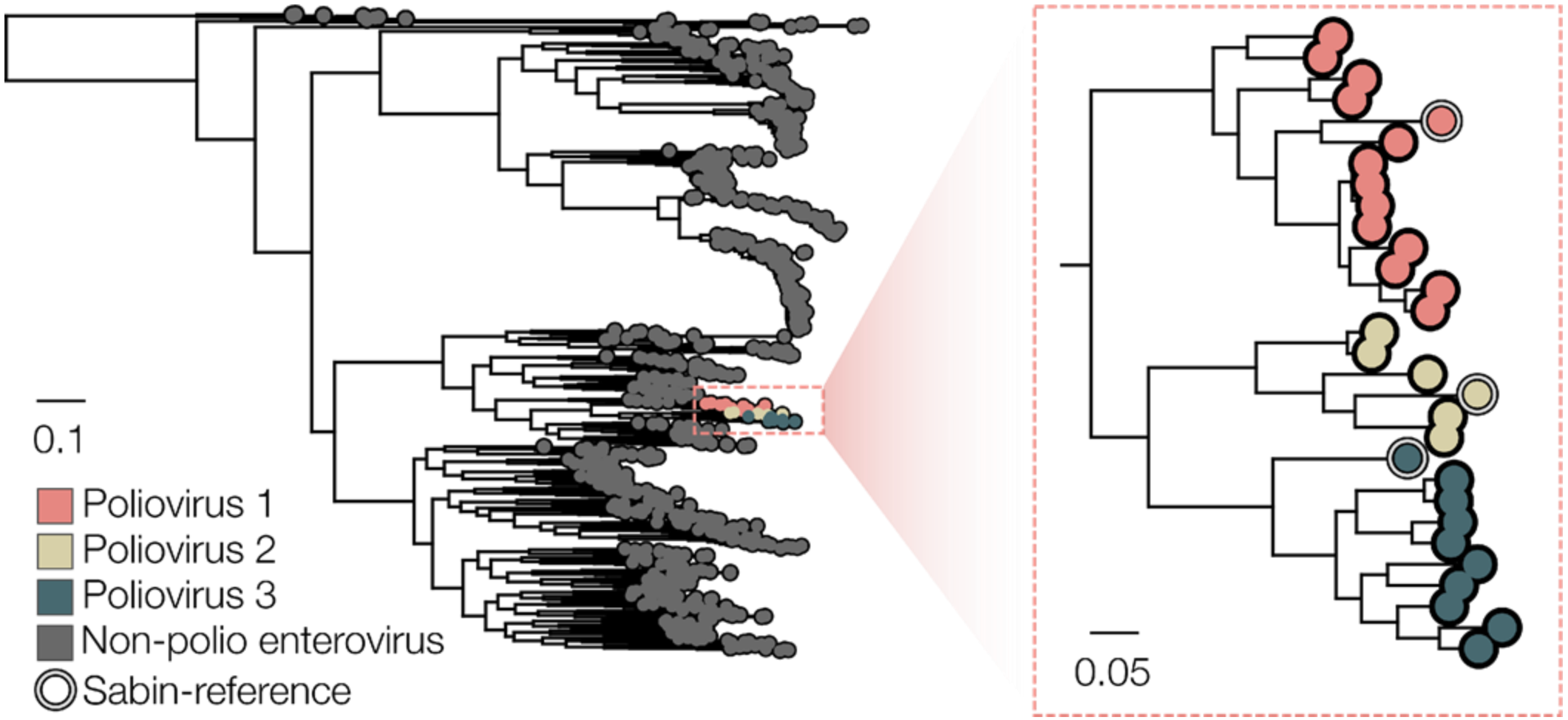
1. Institute of Ecology & Evolution, University of Edinburgh, United Kingdom
2. Department of Infectious Disease Epidemiology, Imperial College London, United Kingdom
3. Division of Virology, National Institute for Biological Standards and Control (NIBSC), Hertz, United Kingdom
4. Department of Virology,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

PIRANHA - overview

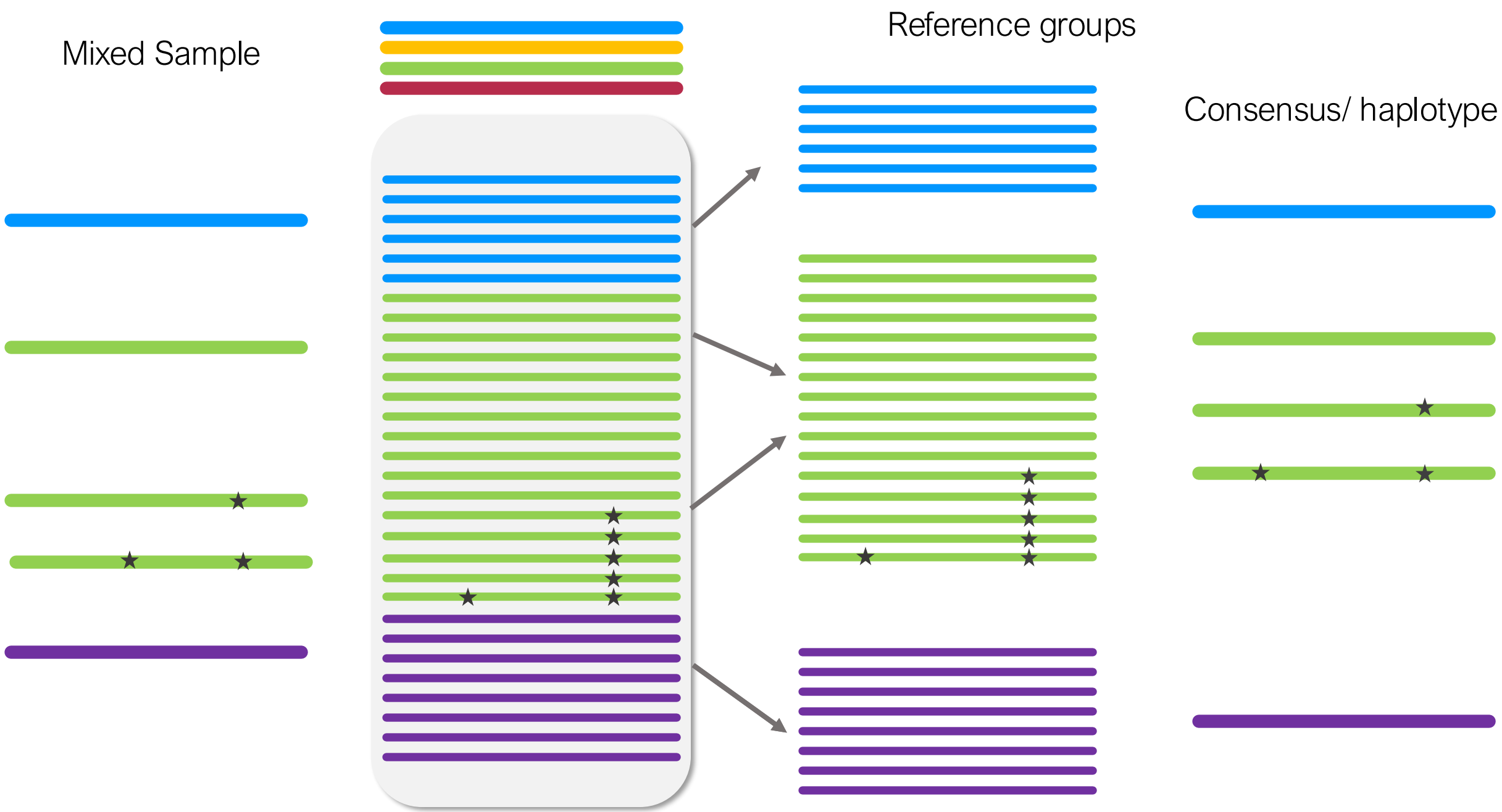
- Takes demultiplexed fastq files, maps to reference sequences, filters by length, and produces consensus sequences and reports as output
- Requires command line
- Installation instructions and basic usage available on github:

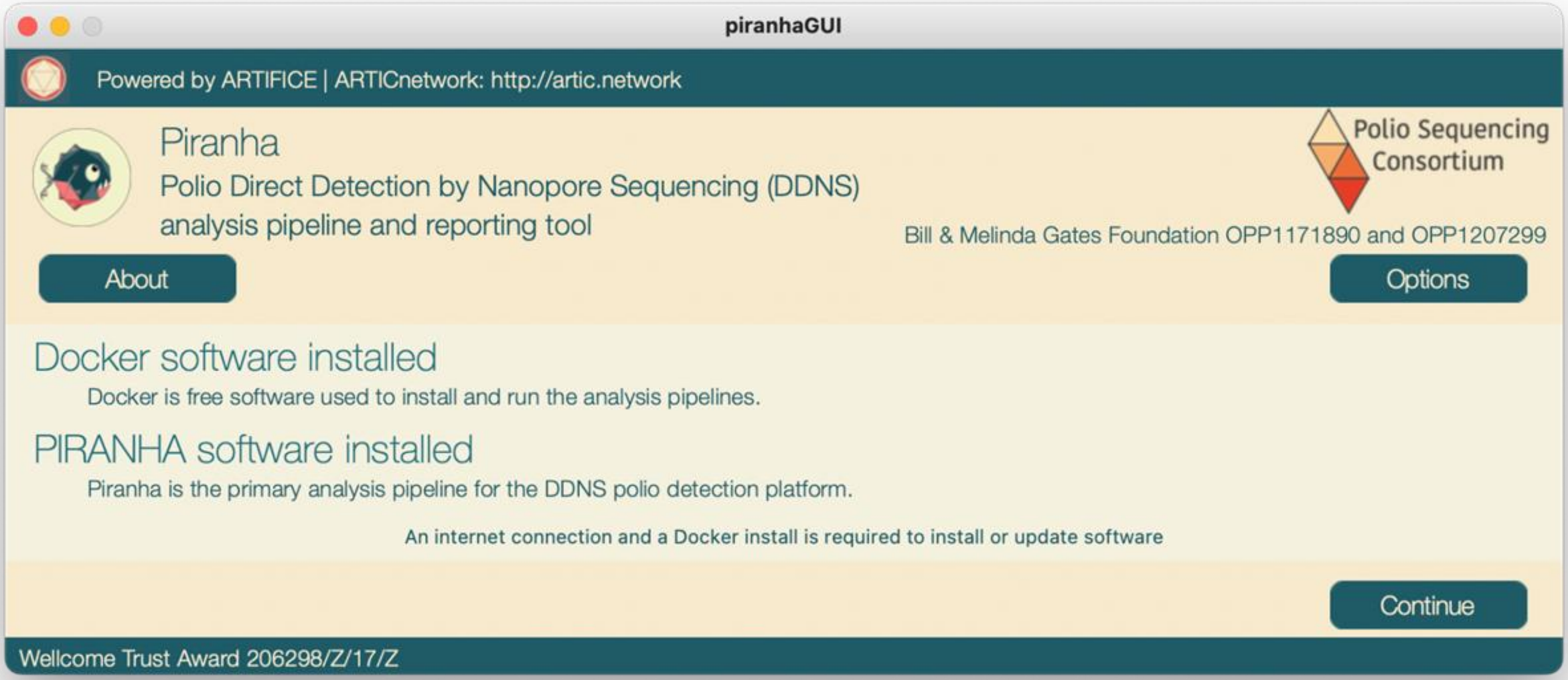
https://github.com/polio-nanopore/piranha





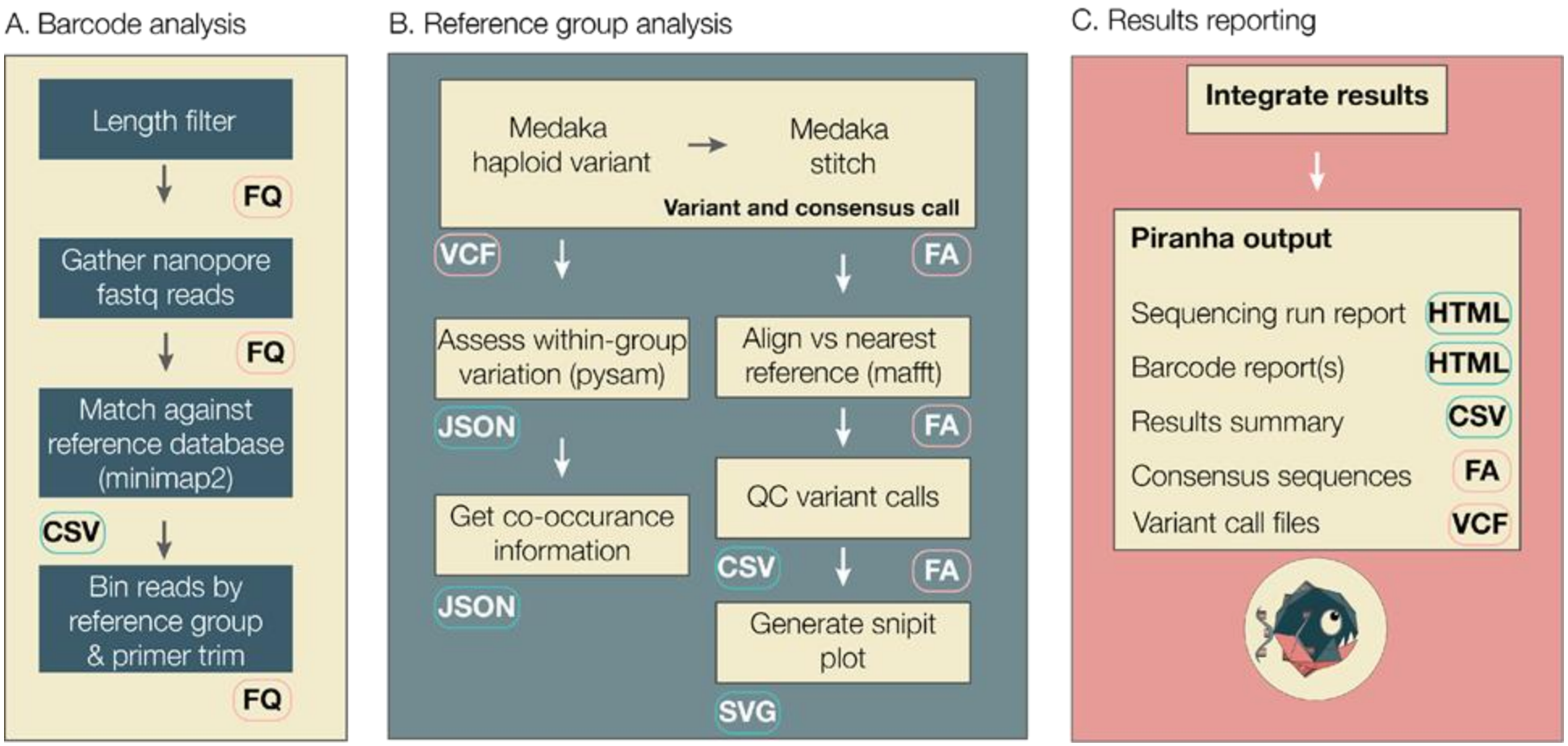
Background database: 959 VP1 sequences


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
Polio Sequencing
Consortium
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

Polio Sequencing Consortium

Workflow schema



Graphic workflow

Identify sequencing reads (fastq files)

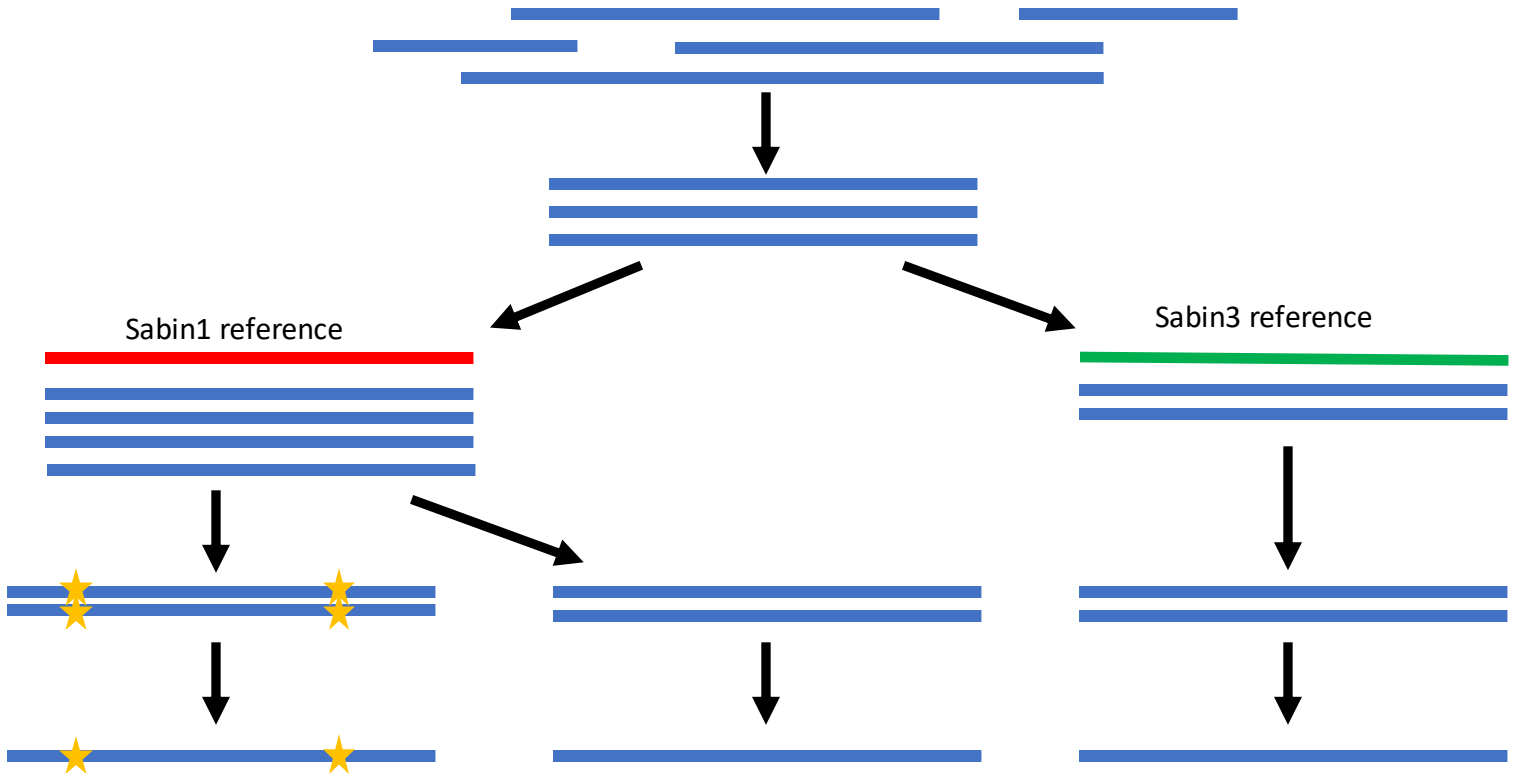
Filter reads by length

Map reads to reference VP1 database (*minimap2*), bin into groups

Identify variants (*medaka, mafft*)

Generate consensus sequences

Generate report



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

Piranha report 2022-12-08

a.

Sample	Barcode	Sample Classification	Reference group	Number of mutations
ENV001	barcode01	Sabin-like	Sabin3-related	1
ENV001	barcode01	Sabin-like	Sabin2-related	2
ENV002	barcode02	VDPV	Sabin2-related	12
ENV003	barcode03	WPV1	WPV1	NA
ENV004	barcode04	VDPV	Sabin2-related	10

b.

Sample	Barcode	Sabin1-related	Sabin2-related	Sabin3-related	WPV1	WPV2	WPV3	NonPolioEV	unmapped
ENV001	barcode01	0	488	252	0	0	0	0	25
ENV002	barcode02	0	1100	0	0	1	0	35	12
ENV003	barcode03	0	0	0	339	0	0	0	0
ENV004	barcode04	0	0	0	138	0	0	0	0
negative	barcode05	0	0	0	0	0	0	1	10

Showing 5 of 96 entries

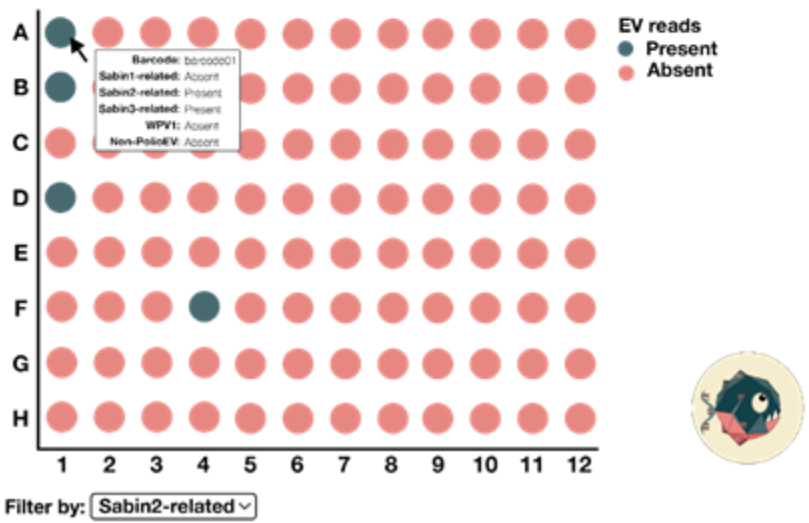
c.

Identical Sequences	Sequence IDs
1	ENV001|barcode01|Sabin2-related|2|161:CT;427:GA ENV030|barcode30|Sabin2-related|2|161:CT;427:GA
2	ENV022|barcode22|Sabin3-related|1|17:CT ENV024|barcode24|Sabin3-related|1|17:CT

d.

Pass	Sample	Barcode	Sabin1-related	Sabin2-related	Sabin3-related	WPV1	WPV2	WPV3	NonPolioEV	unmapped
✓	negative	barcode05	0	0	0	0	0	0	1	10
	positive	barcode05	0	0	0	0	0	0	45	0

e.



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ENV001 report 2022-12-08

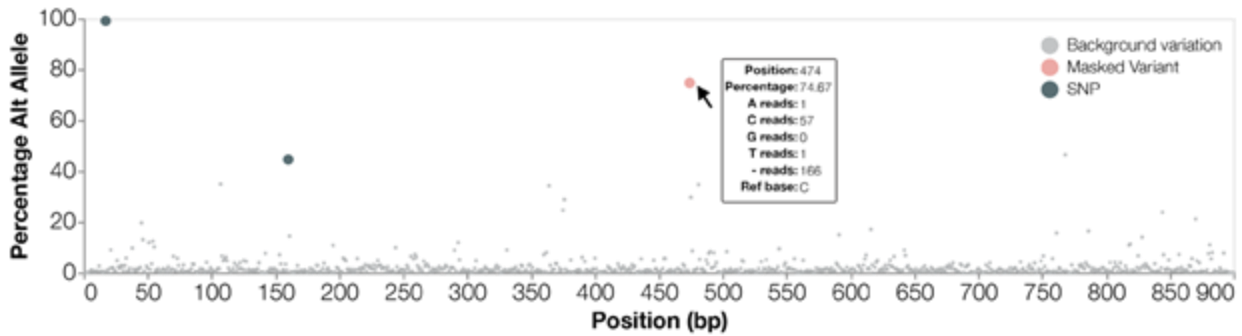
a.

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

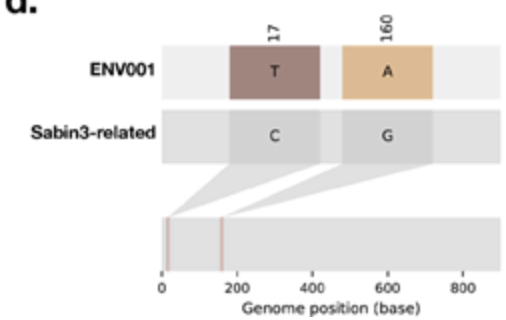
b. VP1 sequences

```
>ENV001|barcode01|Sabin3-related|Poliovirus3-Sabin_AY184221|2|17:CT;160:GA|date=2022-03-01
GGTATTGAAGATTTGATTTCTGAAGTTGCACAGGGCGCCCTAACTTTGTCACTCCCGAAGCAACAGGATAGCTTACCTGATACTAAGGCCAG
TGGCCCGGCGCATTCCAAGGAGGTACCTGCACTCACTGCAGTCGAGACTGGAGCCACCAATCCTCTGACACCATCCGACACAGTTCAAA
CGCGCCACGTAGTCCAACGACGCAGCAGGTCAGAGTCCACAATAGAATCATTCTTCGCACGCGGGGCGTGCGTCGCTATTATTGAGGTGG
ACAATGAACAACCAACCACCCGGGCACAGAAACTATTTGCCATGTGGCGCATTACATACAAAGATACAGTGCAGTTGCGCCGTAAGTTGGA
```

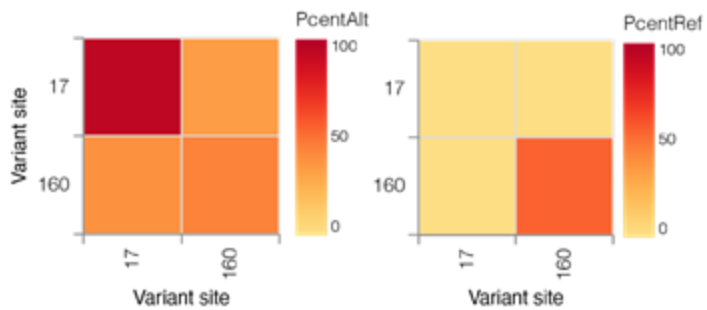
c.



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

Polio Sequencing Consortium

- 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	postive_control	barcode05		E01		
7	negative_ext	barcode06		F01		
8	negative_RTPCR	barcode07		G01		

Links your barcodes and your samples.

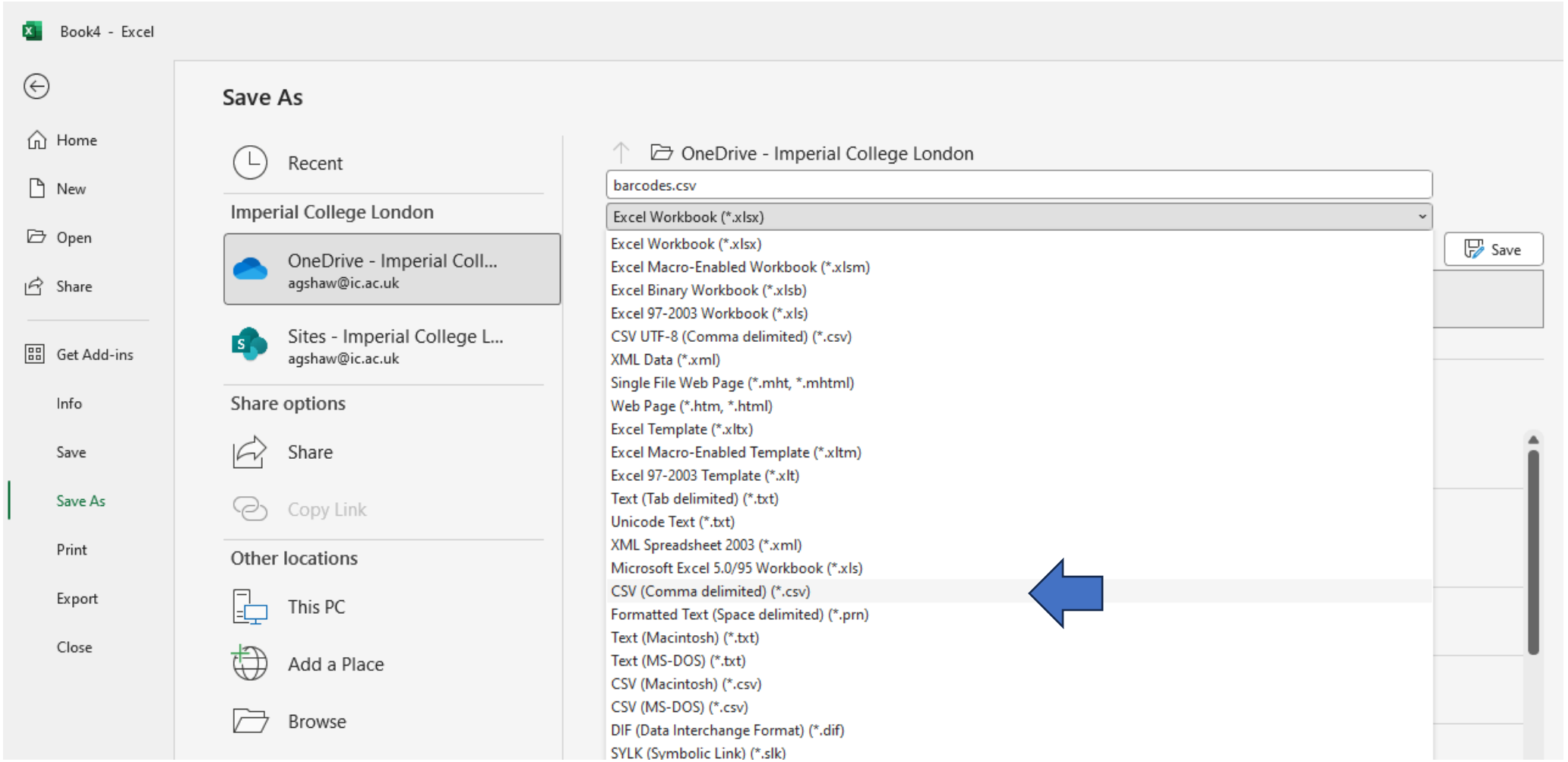
Use the headers “sample” and “barcode”

Barcodes must be entered as “barcode##”

More metadata columns can be added

Polio Sequencing Consortium

Saving as a .csv from excel



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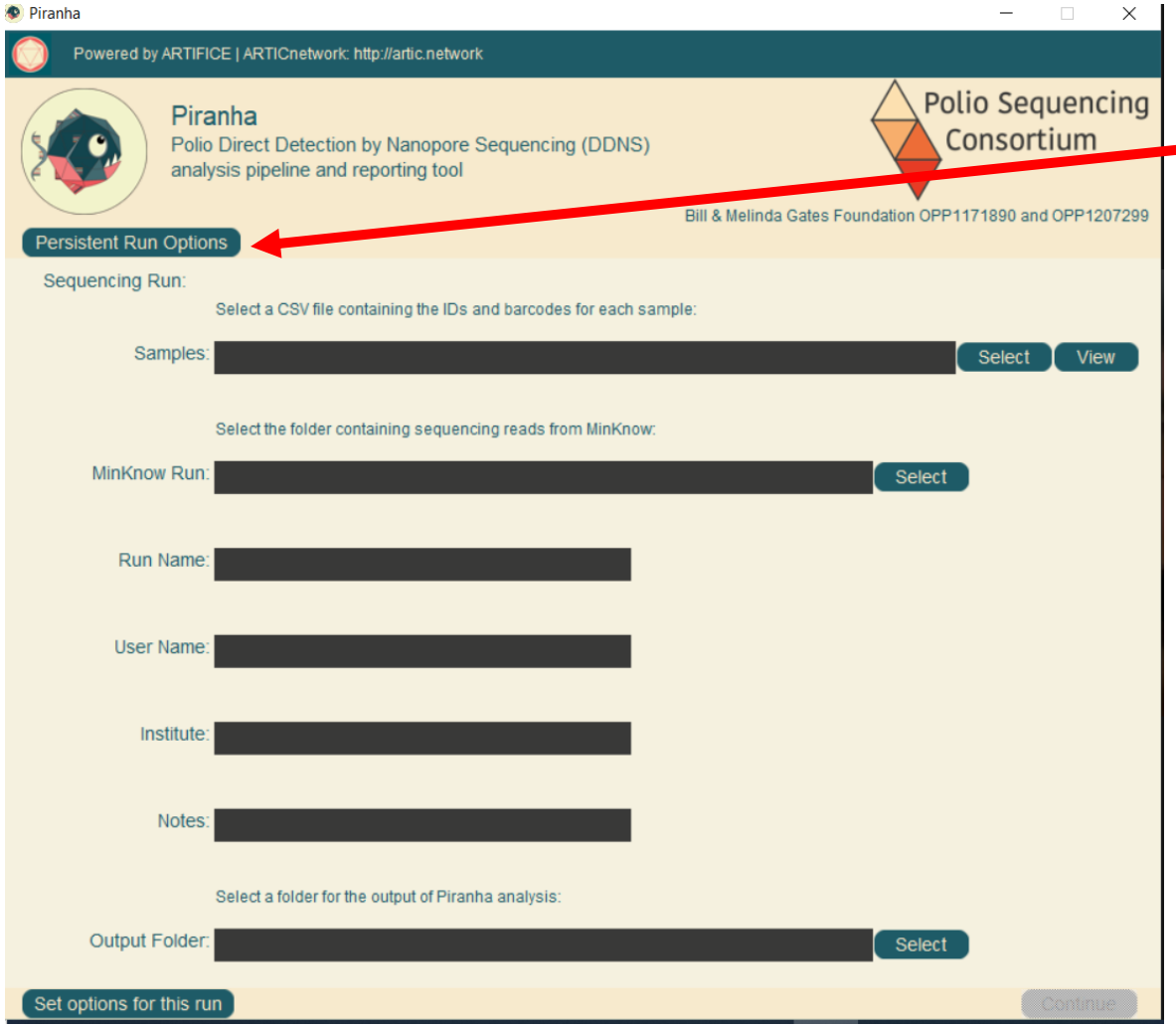
Appearance of a .csv file

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,
postive_control,barcode05,,E01,,
negative_ext,barcode06,,F01,,
negative_RTPCR,barcode07,,G01,,
```

Running PiranhaGUI



- Click here to set up the phylogenetic module

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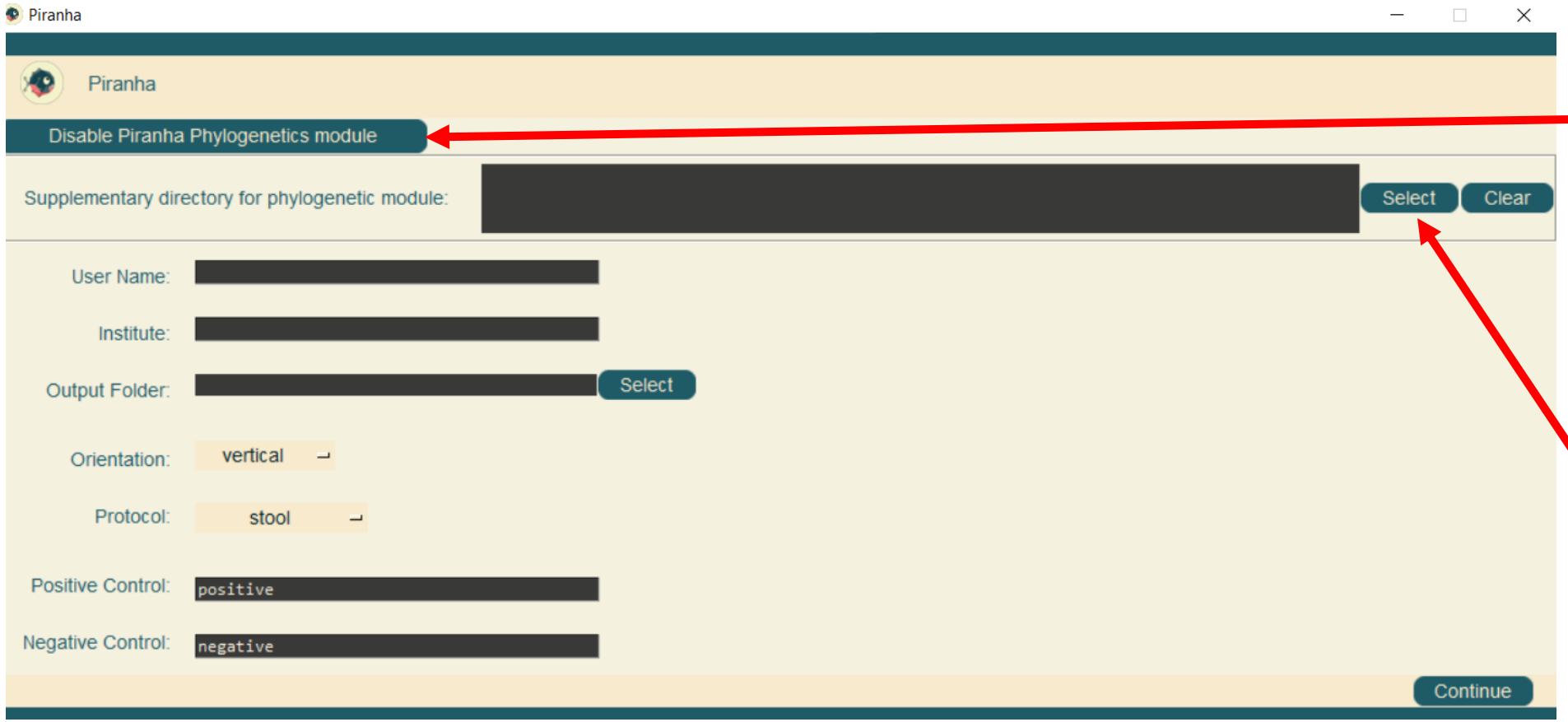
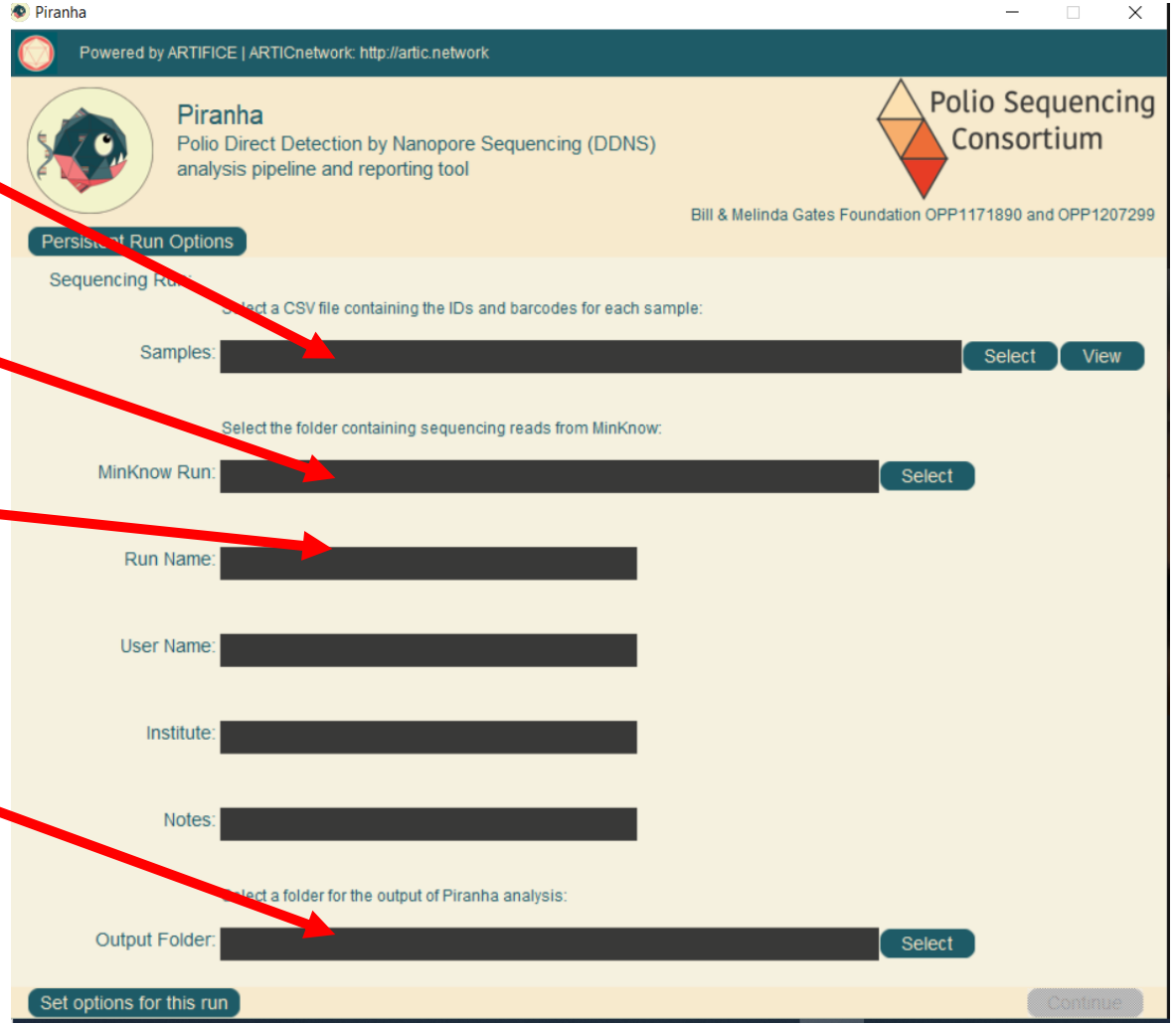
Running PiranhaGUI



- 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”

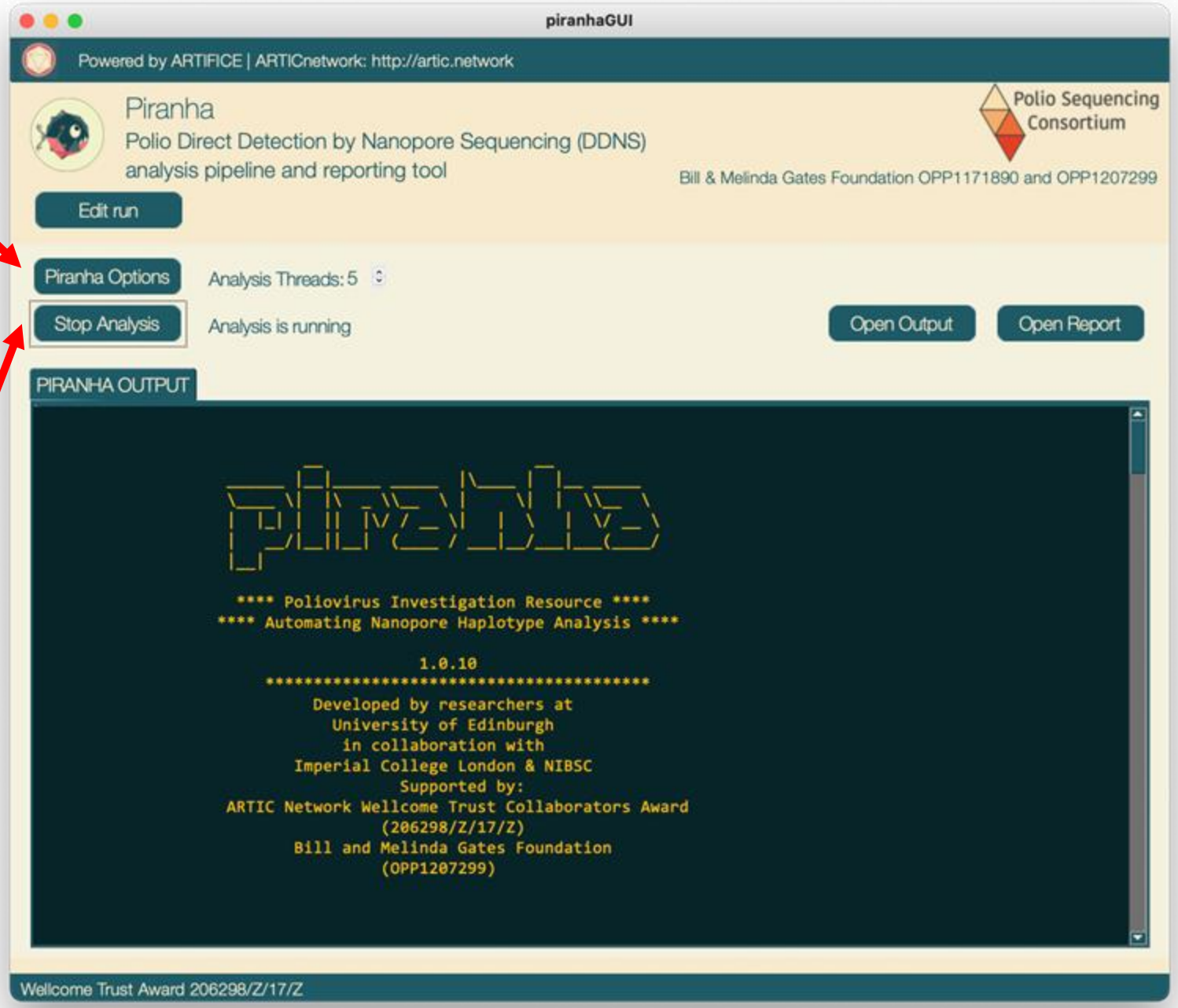


- Set run options. For DDNS stool testing:

Minimum length - 1000 bp
Maximum length - 1300 bp
Minimum read depth – 50
Minimum read percentage – 0

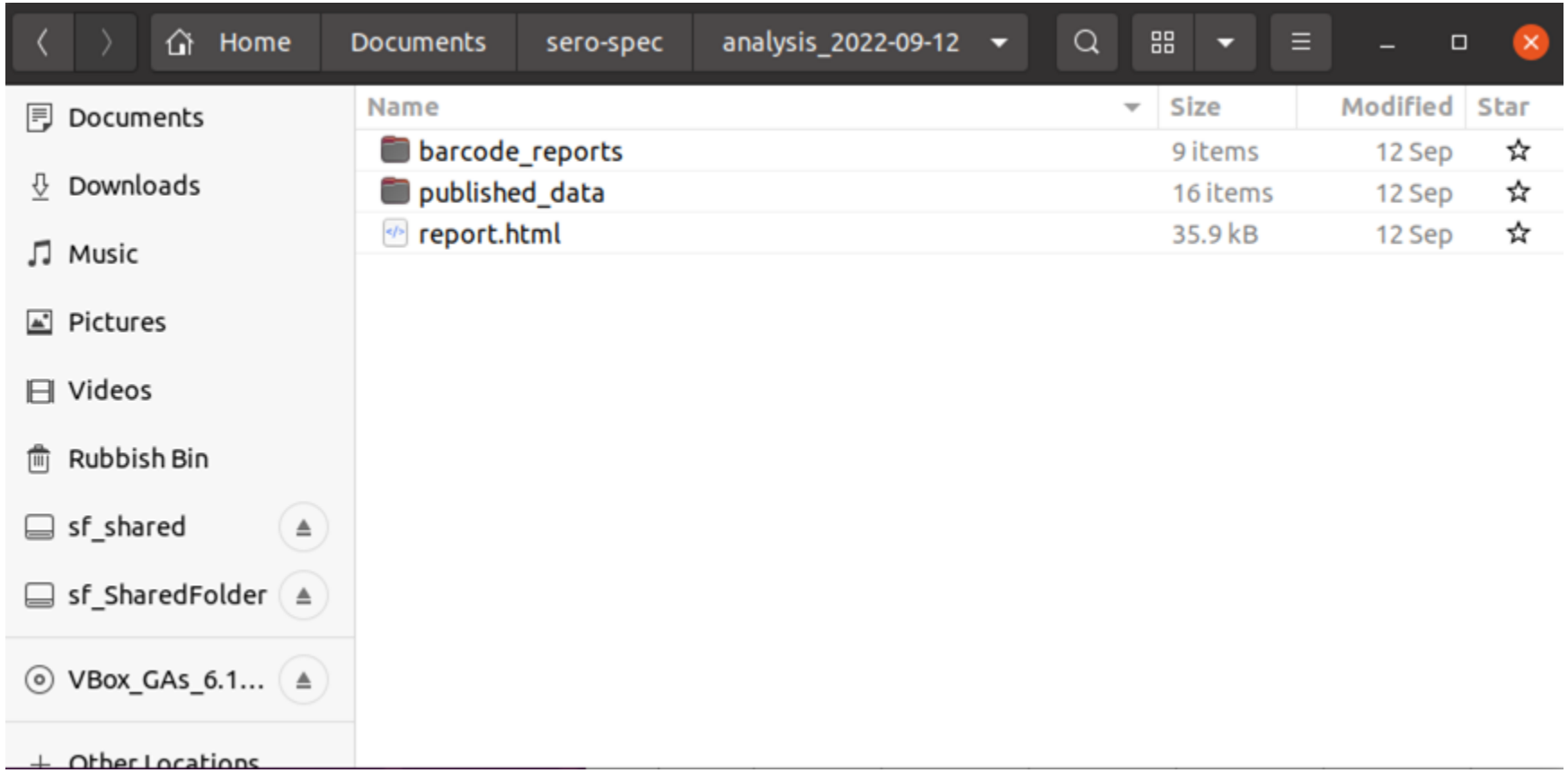
(see QC document)

- Start analysis



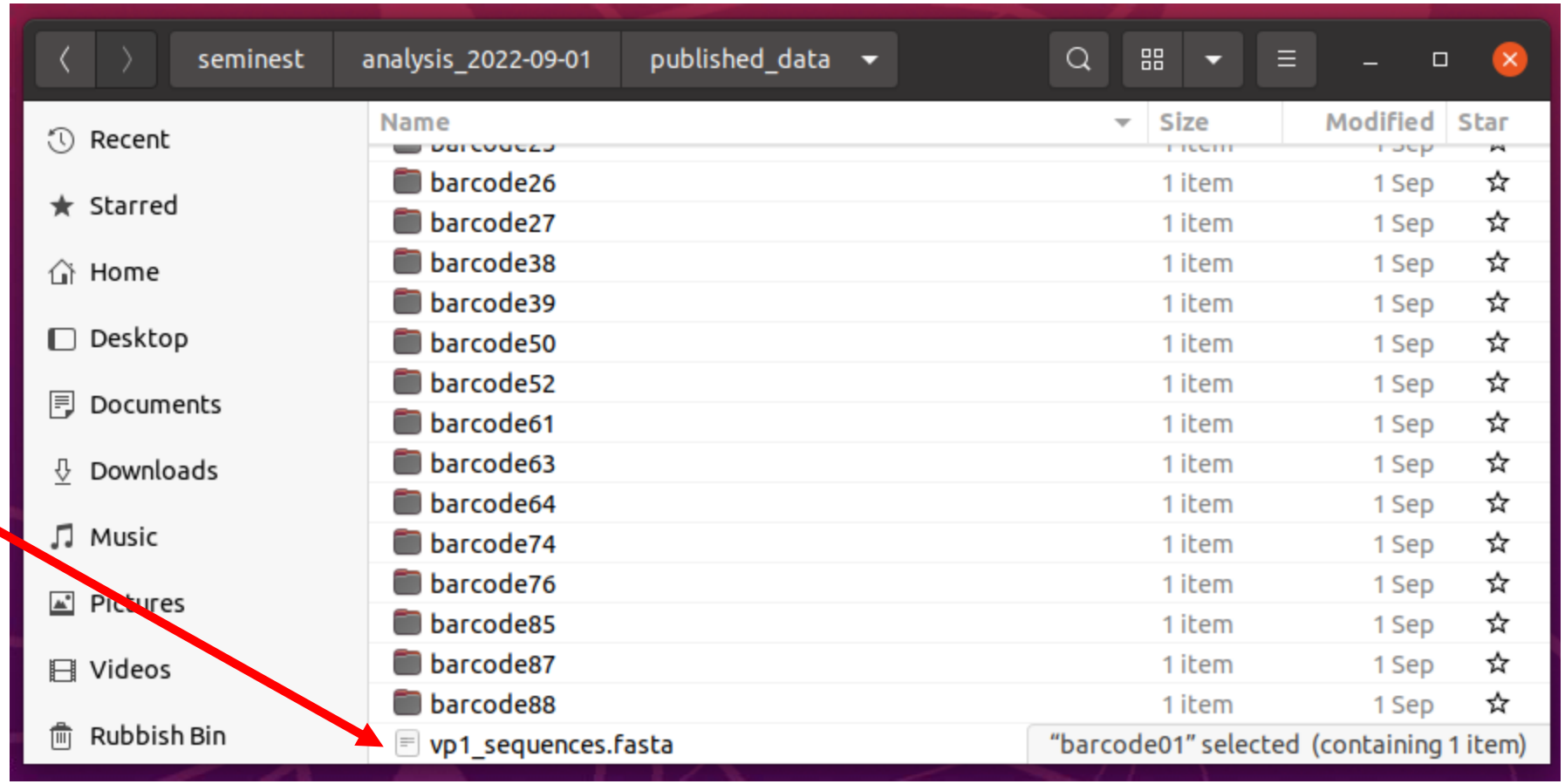
PIRANHA - output

- 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



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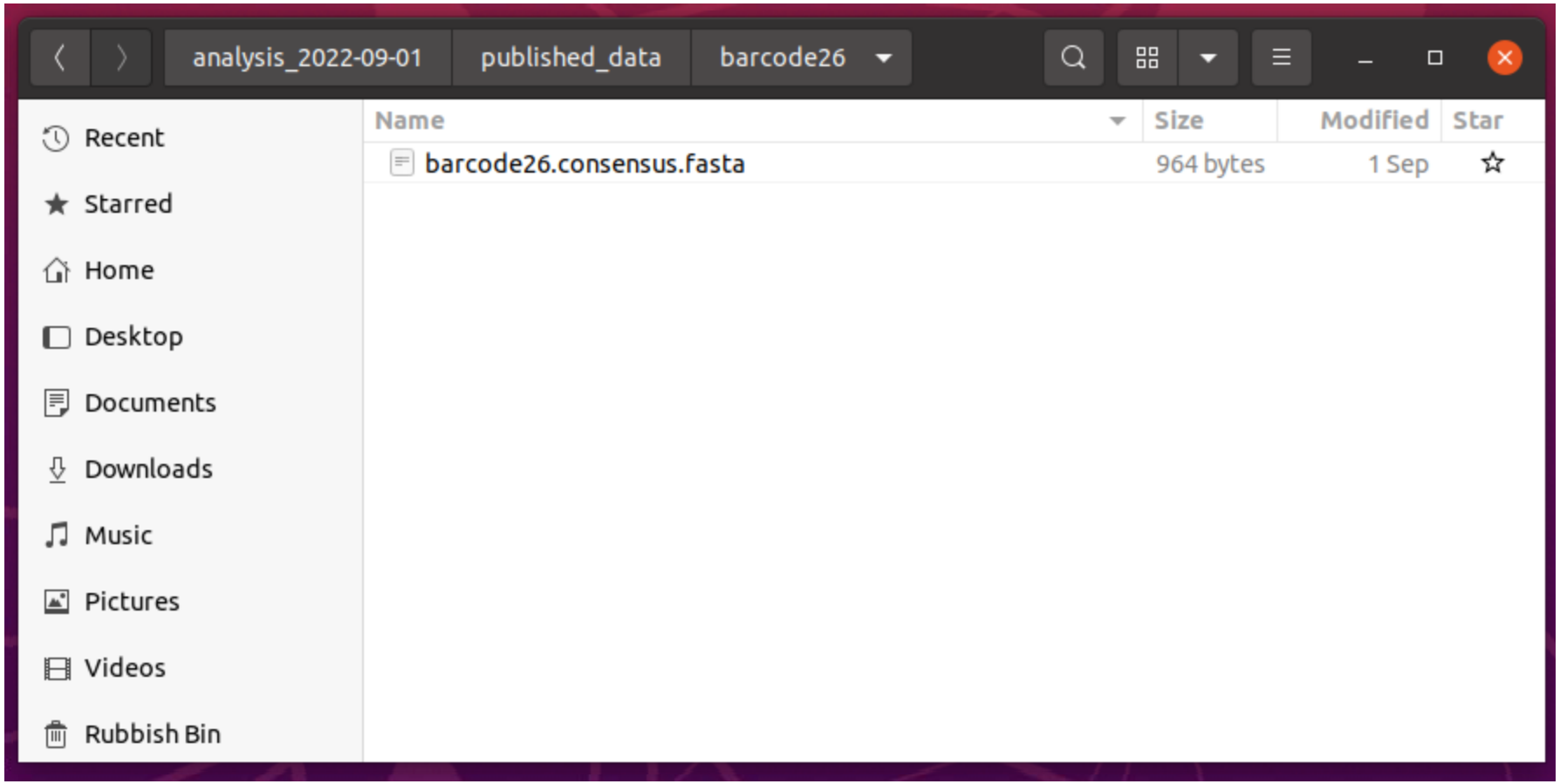
PIRANHA fasta output



Sample contents overview

Polio Sequencing Consortium

Navigate to individual sample reports

Download fasta files of consensus sequences

Search:

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

Export table

Search:

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

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
GGGTTAGGTCAGATGCTTGAAAGCATGATTGACAACACAGTCCGTGAAACGGTGGGGGCGGCAACGTCTAGAGACGCTCTCCCAAACACTGAAGCCAGTGGACCAGCACACTCCAAGGAAATTCCGGCACTCACCGCAGTGGAAACTGGGGCCAC
AAATCCACTAGTCCCTTCTGATACAGTGCAAACCAGACATGTTGTACAACATAGGTCAAGGTCAGAGTCTAGCATAGAGTCTTTCTTCGCGCGGGGTGCATGCGTGGCCATTTTAACCGTGGATAACTCAGCTTCCACCAAGAATAAGGATAAGC
TATTTACAGTGTGGAAGATCACTTATAAAGATACTGTCCAGTTACGGAGGAAATTGGAGTTCTTCACCTATTCTAGATTTGATATGGAATTTACCTTTGTGGTTACTGCAAATTTCACTGAGACTAACAATGGGCATGCCTTAAATCAAGTGTAC
CAAATTATGTACGTACCACCAGGCGCTCCAGTGCCCGAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTTACACCTACGGAACAGCTCCAGCCCGGATCTCGGTACCGTATGTTGGTATTTCGAACGCCTATTCACA
CTTTTACGACGGTTTTTCCAAAGTACCACTGAAGGACCAGTCGGCAGCACTAGGTGACTCCCTCTATGGTGCAGCATCTCTAAATGACTTCGGTATTTTGGCTGTTAGAGTAGTCAATGATCACAACCCGACCAAGGTCACCTCCAAAATCAGAG
```

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

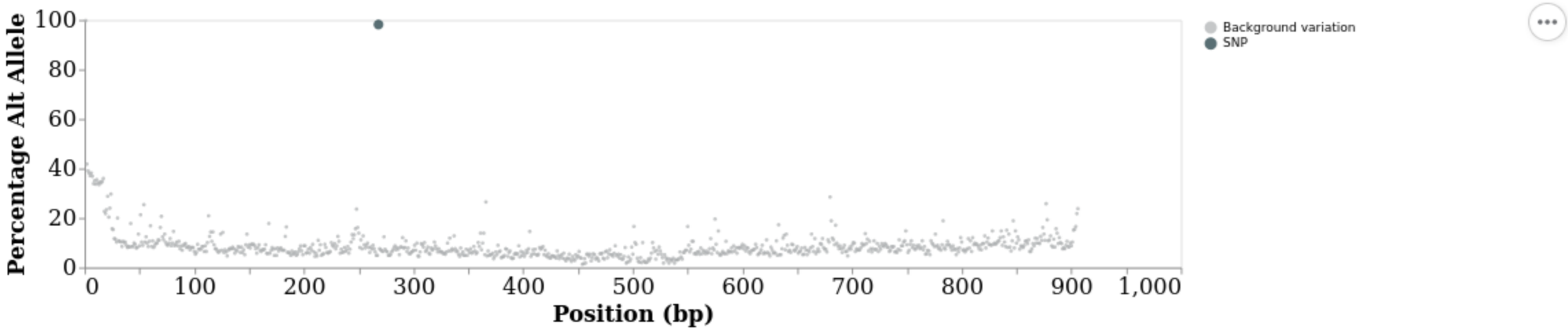


Figure 1 | Variation (errors + mutations) across Sabin1-Related reference in sample08

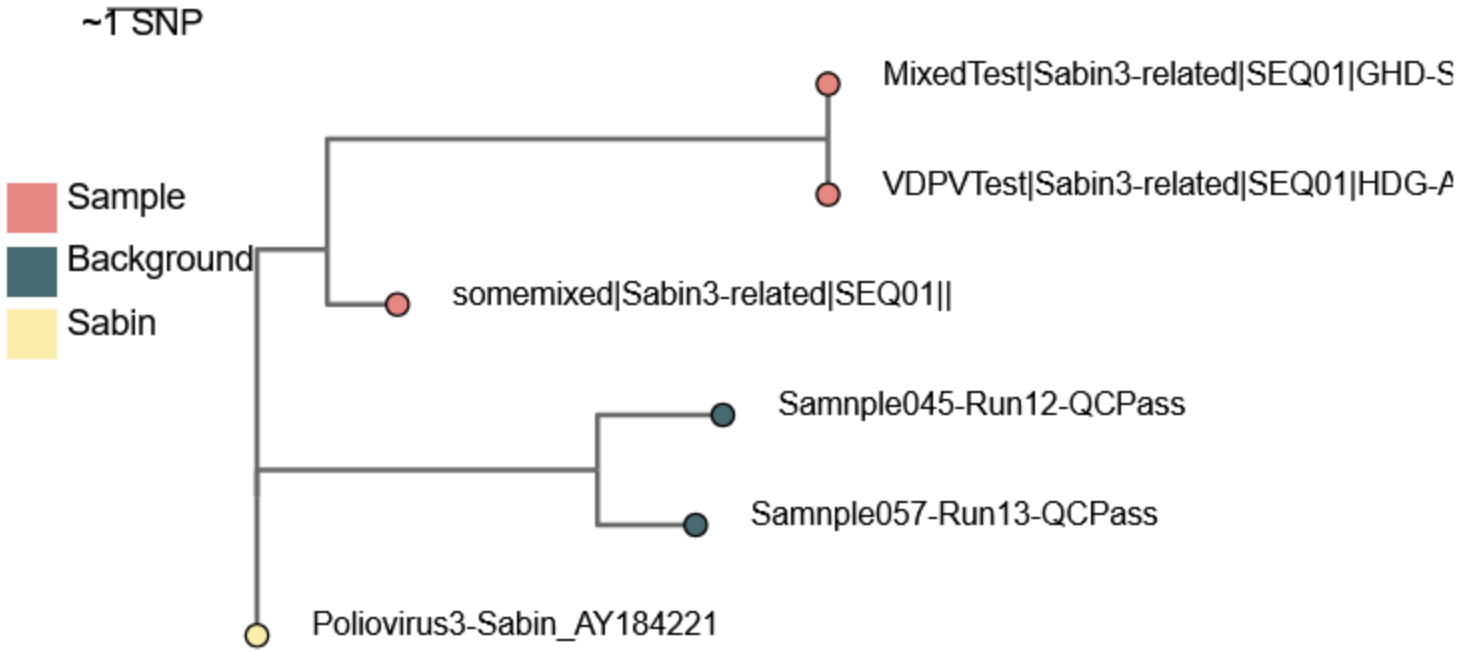


Figure 3 | Sabin3-related phylogeny

- 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	somemixe	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
	Poliovirus2	708	10	98.89	VDPV
		1			
		0			
		0			
		0			
	Poliovirus2	137	0	100	Sabin-like

- 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)

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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

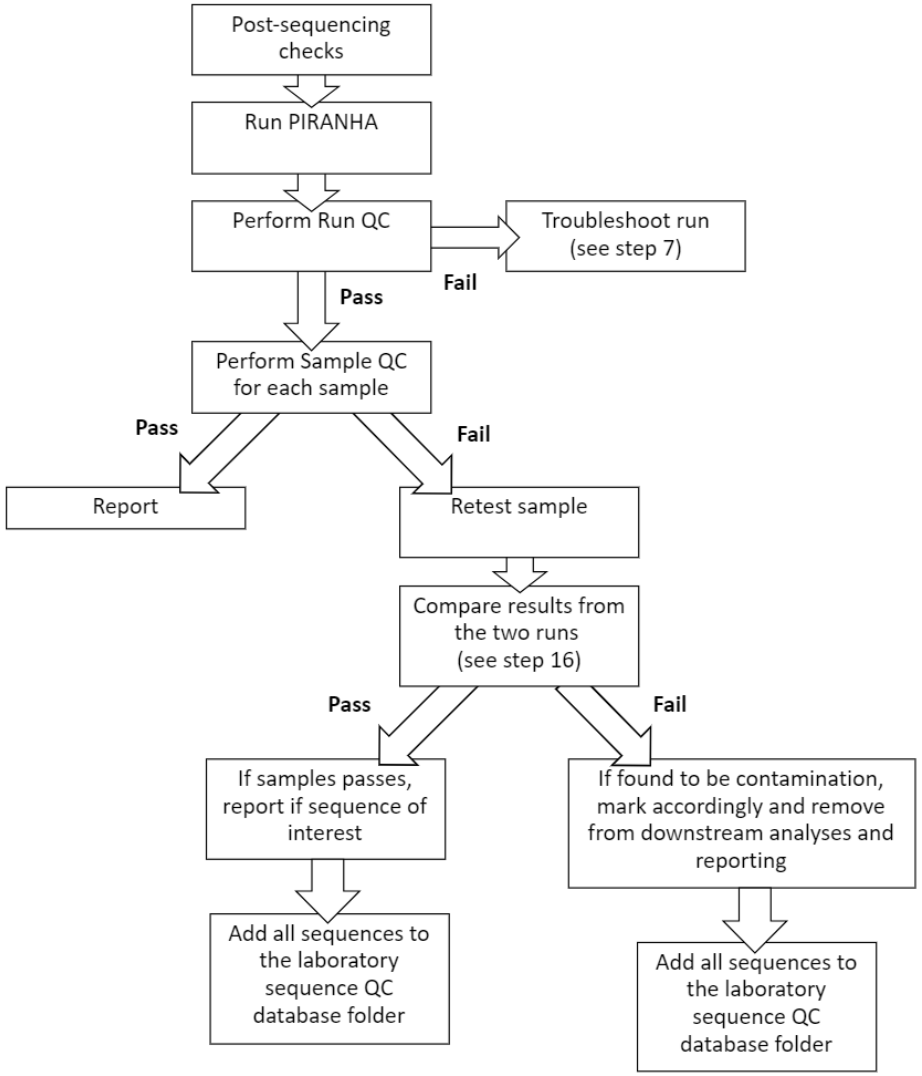
- Dates: always use the ISO 8601 date format: 2023-07-26

Polio Sequencing Consortium

7. Quality Control and Reporting

Presentation and discussion

Post-run QC for DDNS stool testing

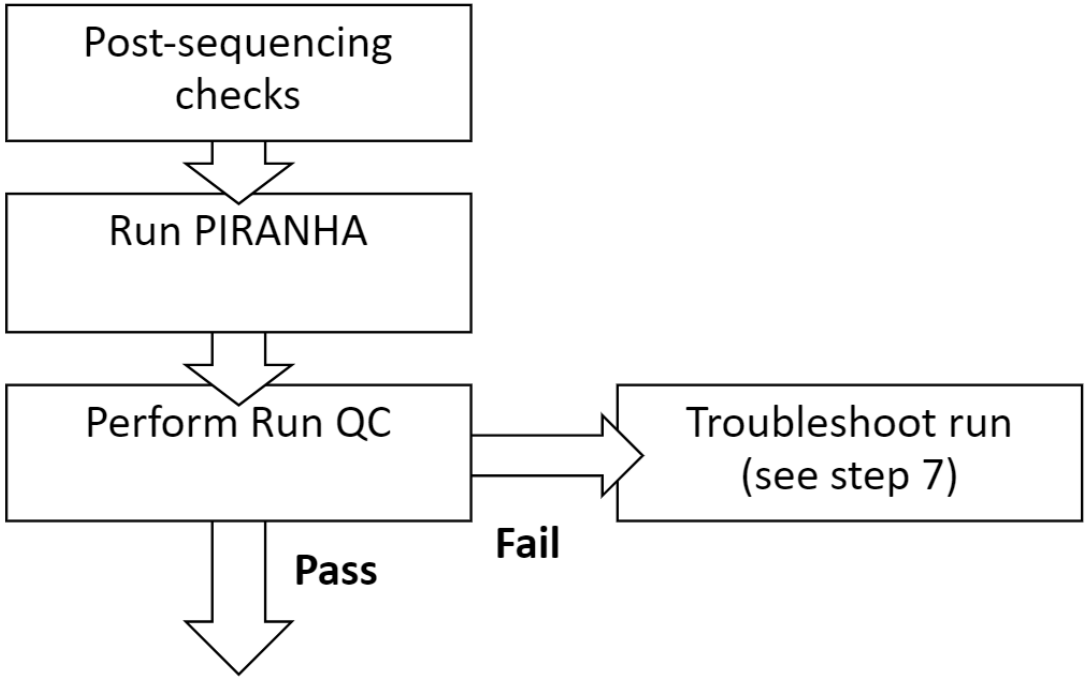


- QC process documented in:

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 fluoremeter.

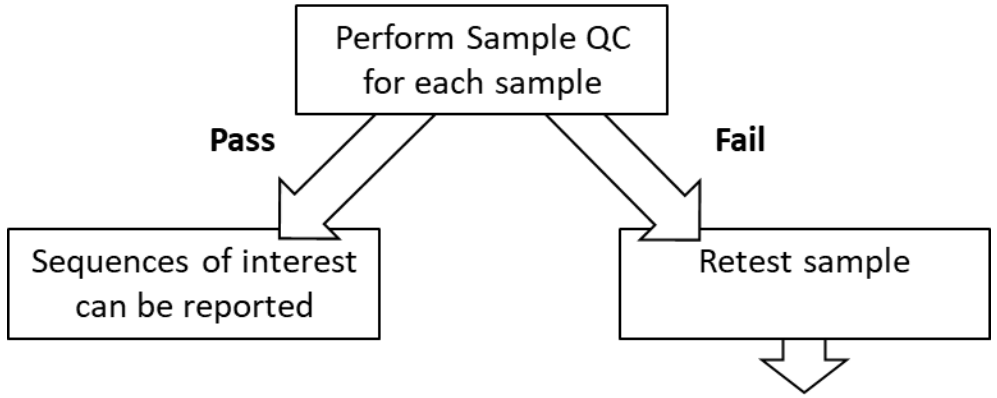
Check that you are ligating the correct adaptor (LA) and are using the short fragment buffer (SFB) during library preparation.

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

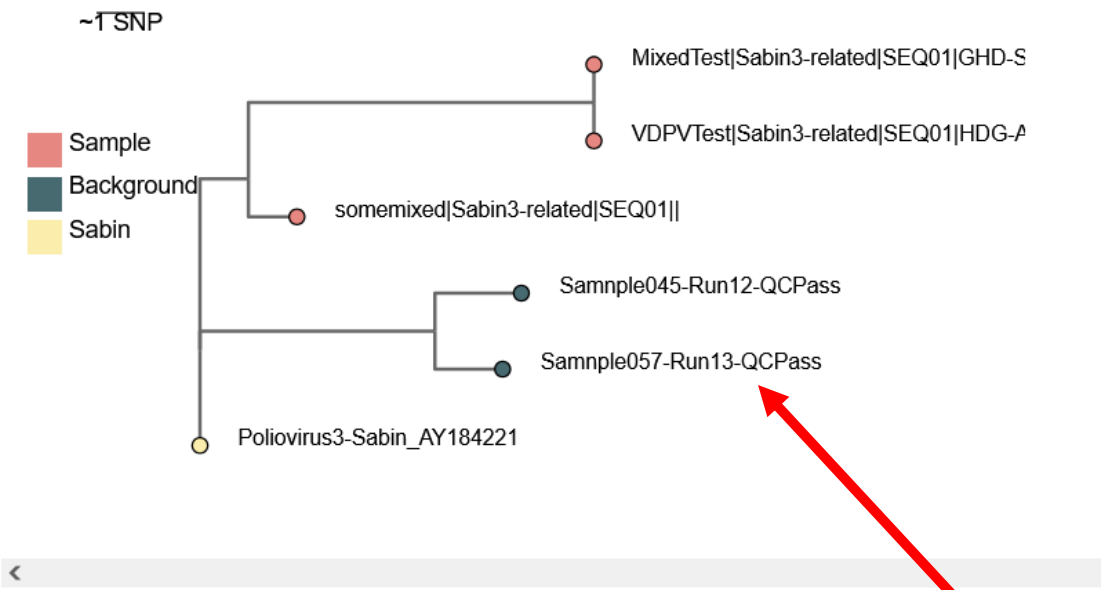
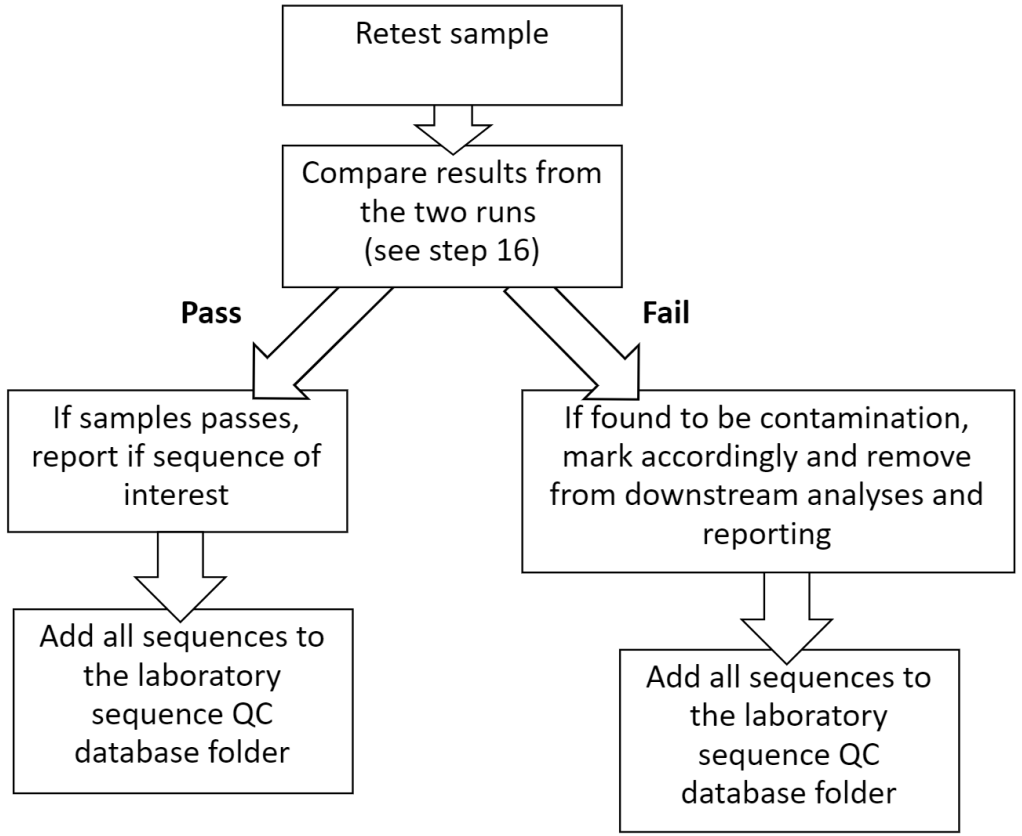


Figure 3 | Sabin3-related phylogeny

- 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

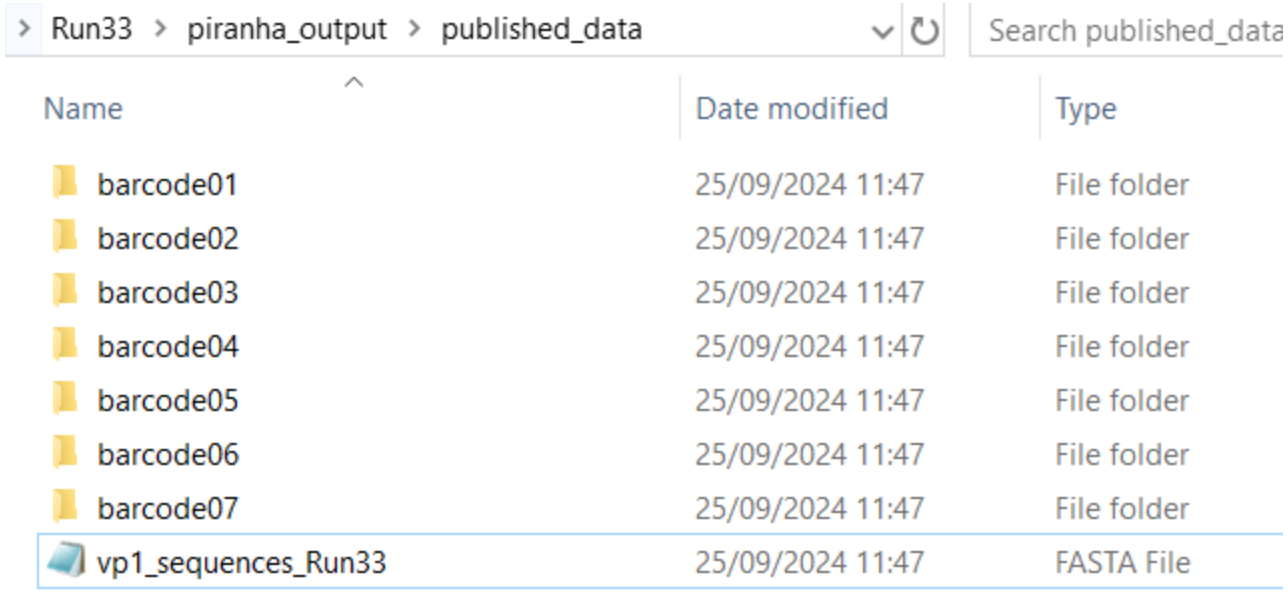
Sample retesting



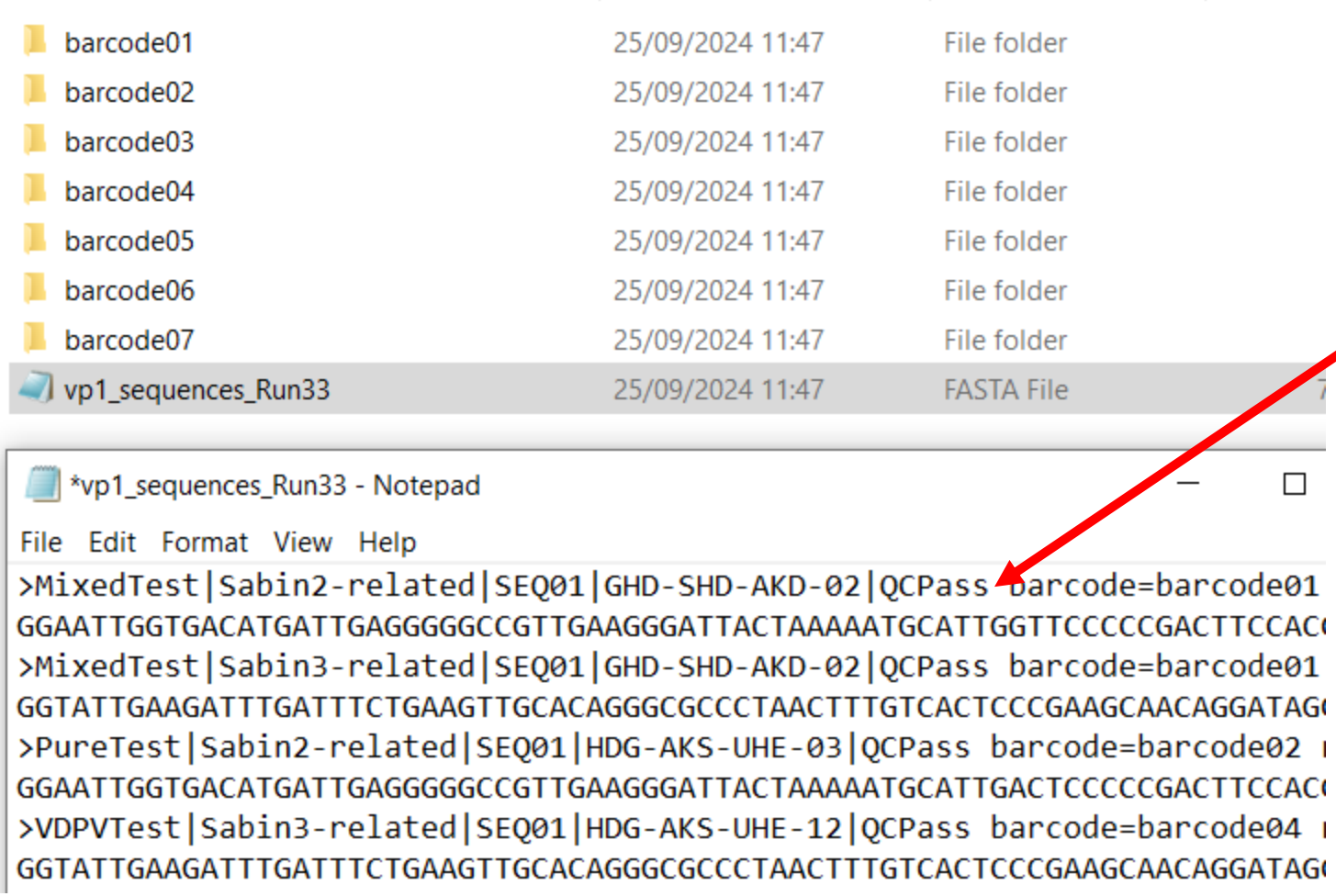
- Try to avoid using the same layout if checking a group of samples.
- Flag as a QC-check on the new sequencing run.
- Annotate the original run depending on the result. Report the sample if it passes the repeat.
- Talk to the appropriate person to arrange the retesting

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



Laboratory Sequence QC Database



Sequences passing QC can be annotated

Polio Sequencing Consortium

8. Preparation of the positive control for DDNS

Presentation and discussion

The positive control

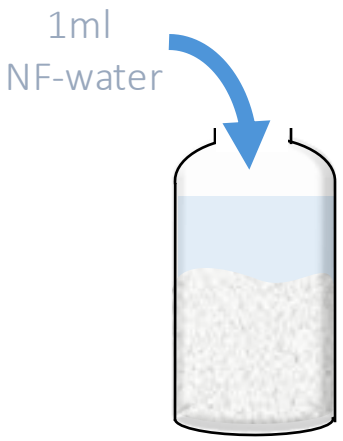
- Positive control: Coxsackie virus A20 (CVA20) Contact Dr. Erika Bujaki to order
- Supplied as pre-measured, lyophilised virus
- Must be reconstituted before use in extraction step

Lyophilised CVA20 positive control

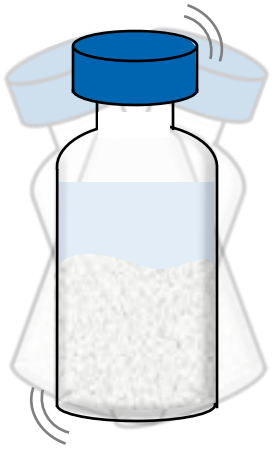
Preparation of positive control - stocks

BIOLOGICAL HAZARD
Must be handled in
Class 2 safety cabinet

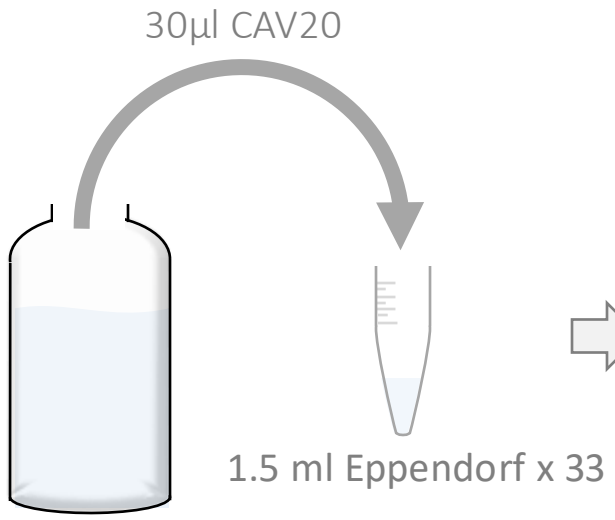
Step One : Add 1ml of nuclease-free water



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

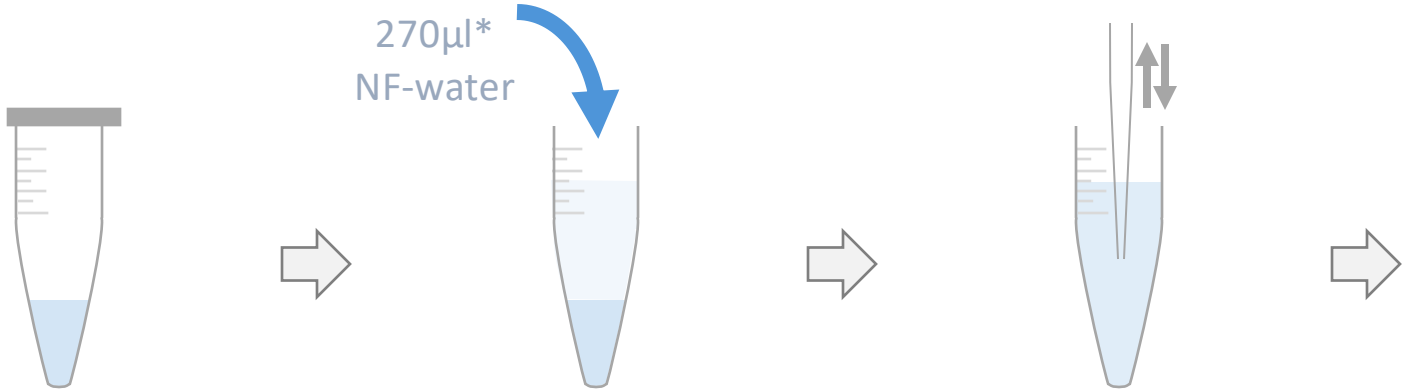
Preparation of positive control for extraction

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



** Volume for preparation of DDNS controls*

Proceed to RNA extraction

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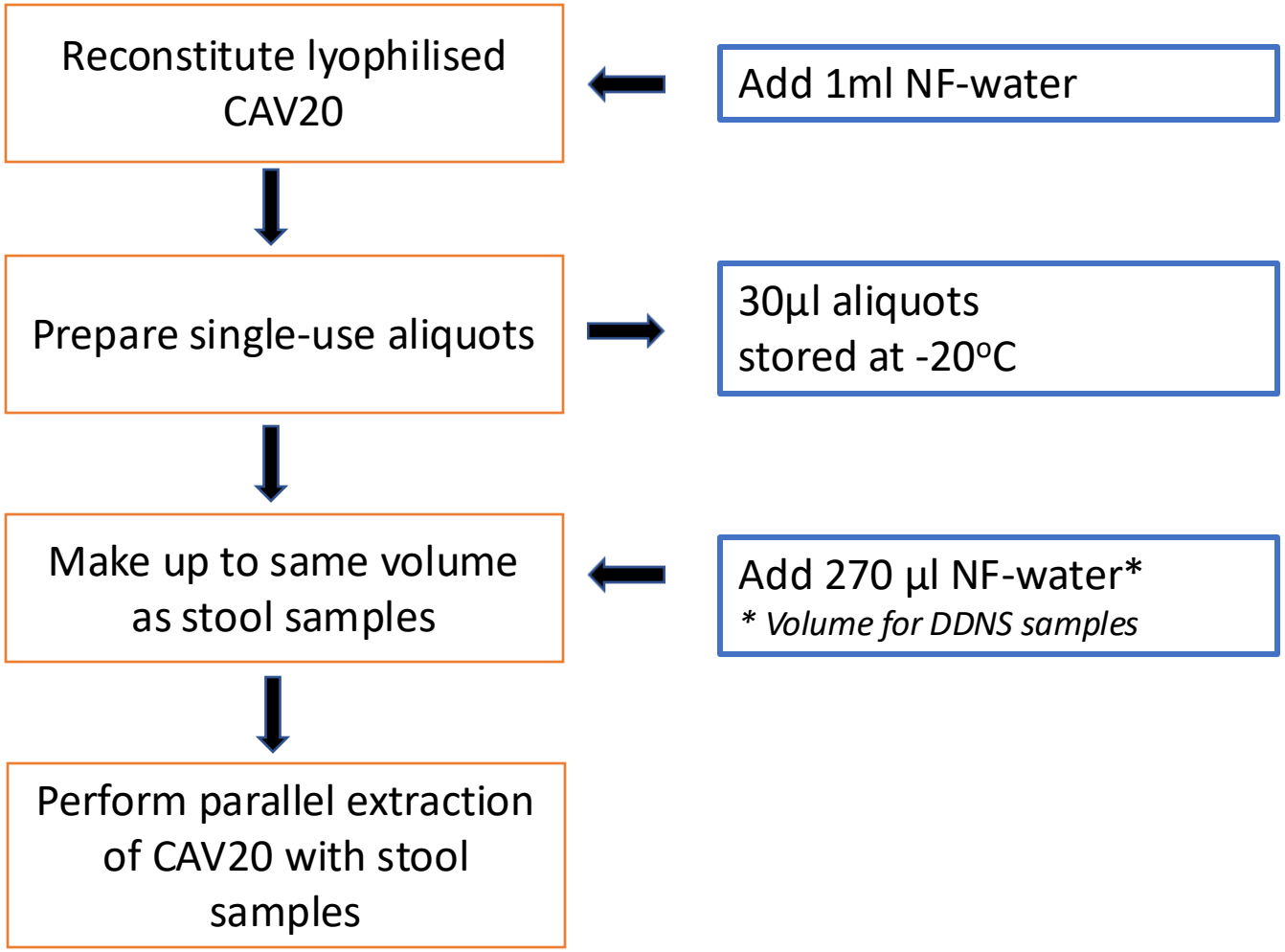
Preparation of positive control - overview



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9. In-house Verification

Presentation and discussion

In-house verification

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❖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.

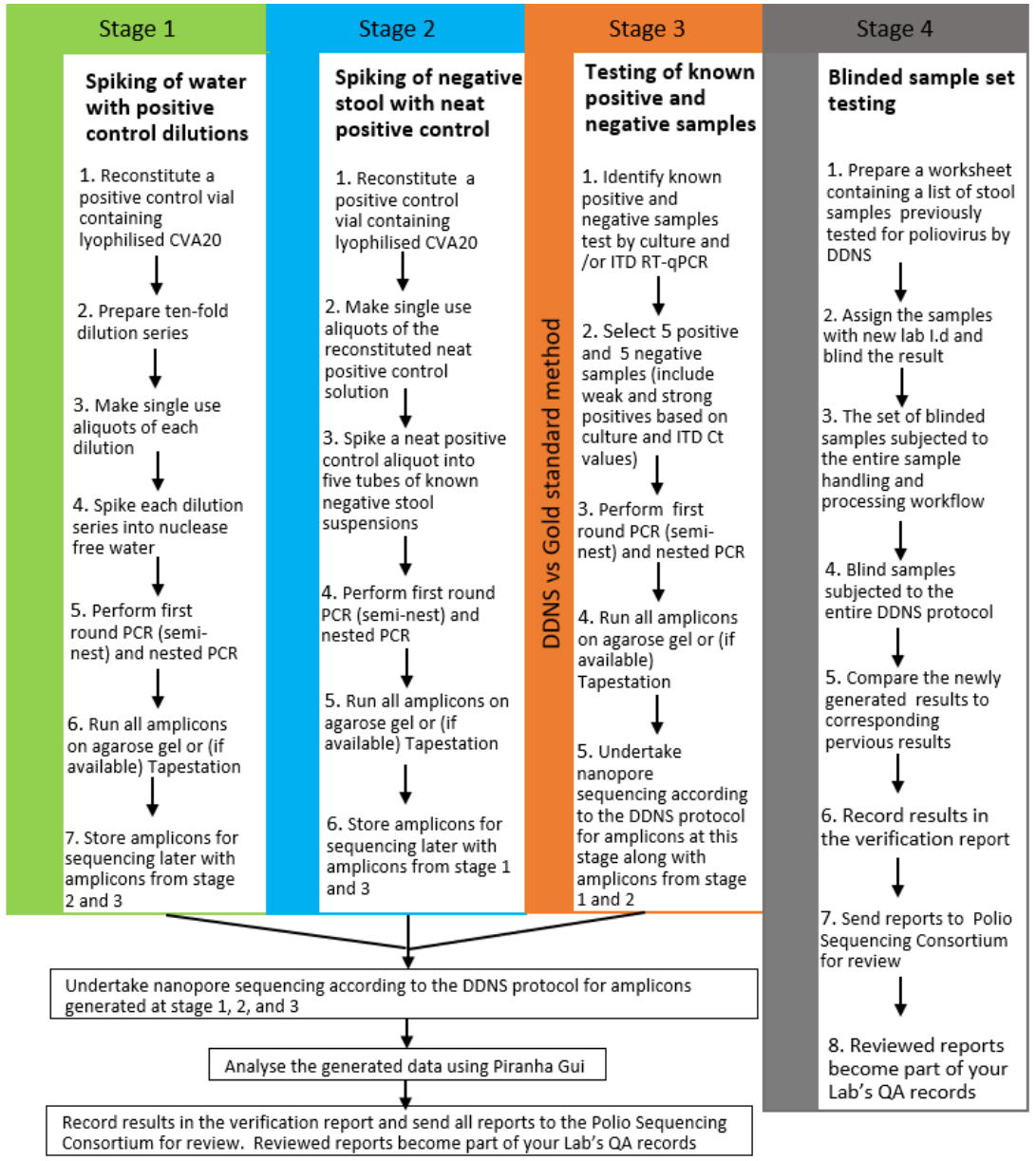
❖Positive controls – supplied by MHRA

- ✓ 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

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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		Pass	Fail
	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		Pass	Fail
	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):

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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	

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End