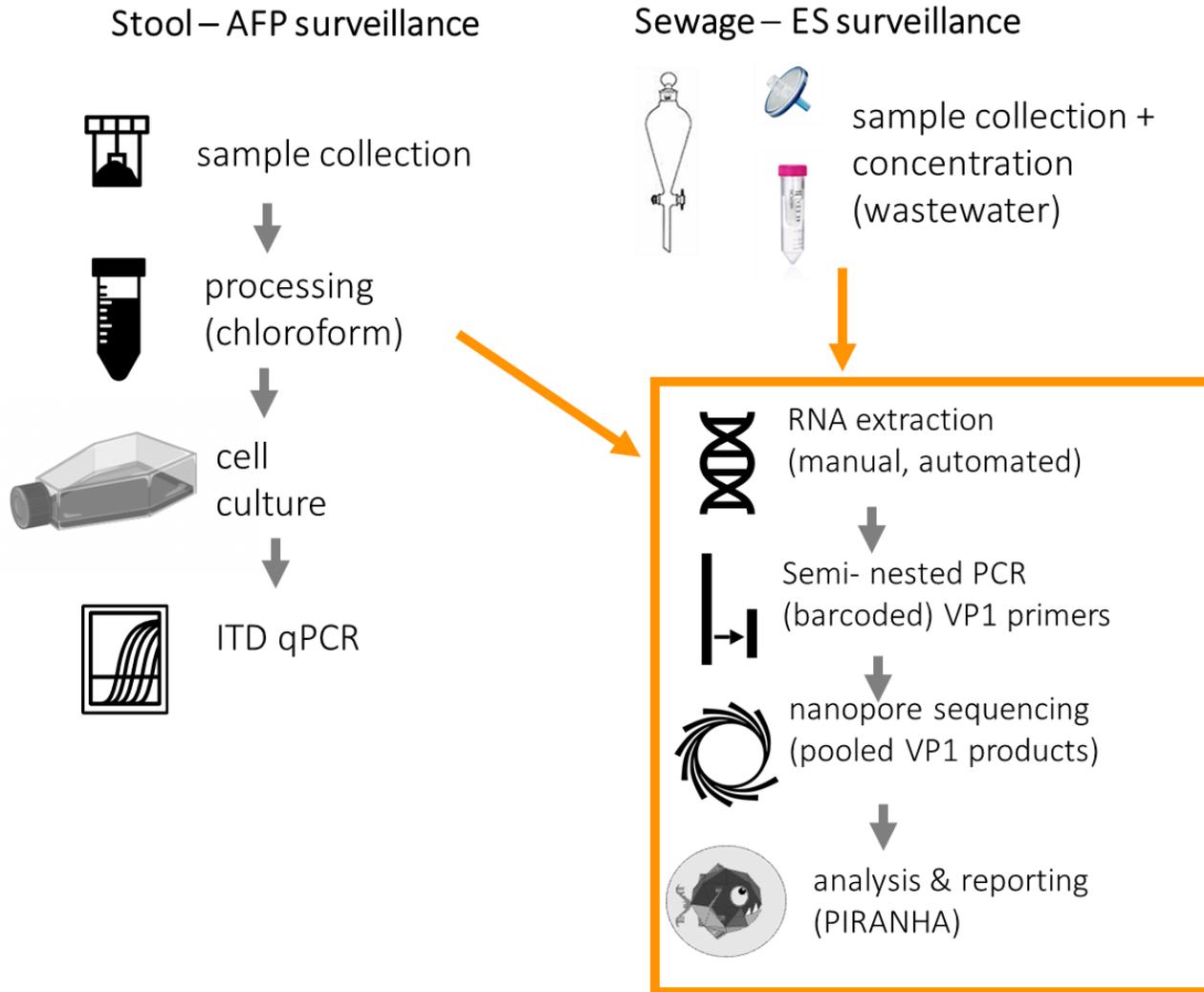
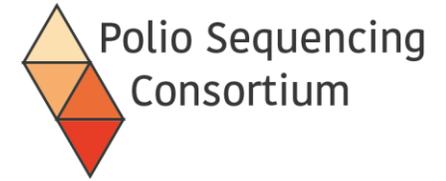


# 1. Overview of Direct molecular Detection and Nanopore Sequencing (DDNS) for Poliovirus

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

```
>sample08|barcode33|Sabin1-related|Poliovirus1-Sabin_AY184219|1|268-AT
GGGTTAGGTCAGATGCTGAAAGCATGATTGACACACAGTCCGTAACGGTGGGGCGGCAAGCTCTAGAGACGCTCCCAACACTGAAGCCAGTGGACAGCACACTCCAAAGAAATCCGGCACTCACCAGTGGAACTGGGGCCAC
AAATCCACTAGTCCCTTCTGATACAGTGCACACAGACATGTTGTACACATAGTCAAGGTCAGAGTCTAGCATAGAGTCTTCTCCGGCGGGGTGCATGGCTGGCATTTTAACCGTGGATAACTAGCTCCACCAAGAAATAGGATAAGC
TATTTACAGTGGGAAGTCACTTATAAAGTACTGTCCAGTTACGGAGAAATGGAGTCTTCCACTATTCTAGATTGATATGGAAATTTACTTTTGGTTACTGCAAAATTCACAGGACTAAATGGGCACTAAATCAAGTATGAC
CAAAATATGTACGTACCACAGGCGCTCCAGTGGCCGAGAAATGGGACGACTACACATGGCAAACTCATCAATCCATCAATCTTTTACACCTACGGAACAGCTCCAGCCGGATCTGGTACCGATATGGTATTTTGGACCGCTATTCA
CTTTTACGACGGTTTTTCCAAAGTACCCTGAAGGACAGTGGGACGACTAGGTGACTCCCTCTATGGTGCAGCATCTCTAAATGACTCTGGTATTTGGCTGTAGAGTAGTCAATGATCAACCCGACCAAGGTCACCTCCAAATCAGAG
```

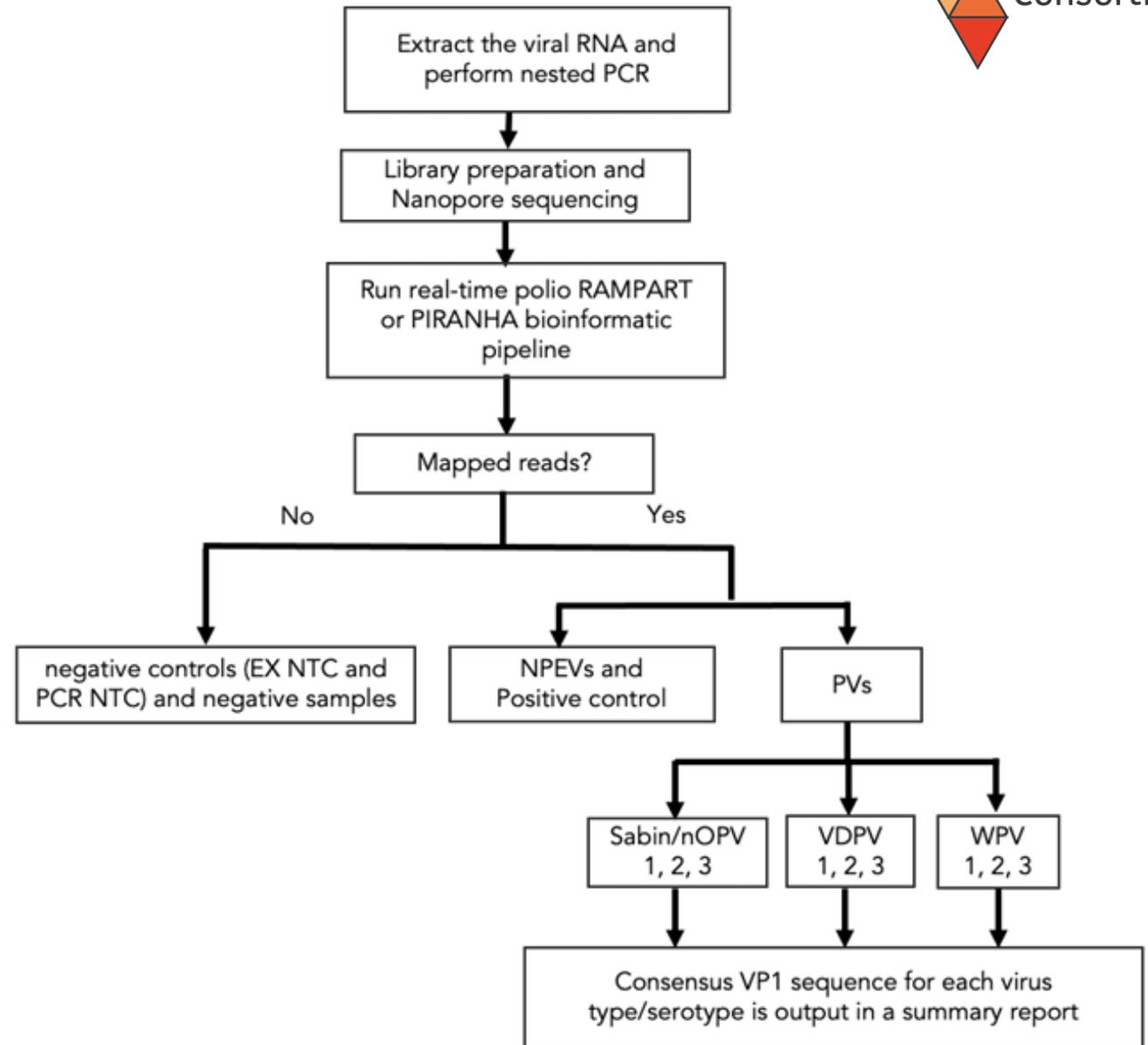
[Download](#)

# 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>

- Forums on the protocols.io site to raise any queries you have about the protocol
- New version of the protocols will be published here.

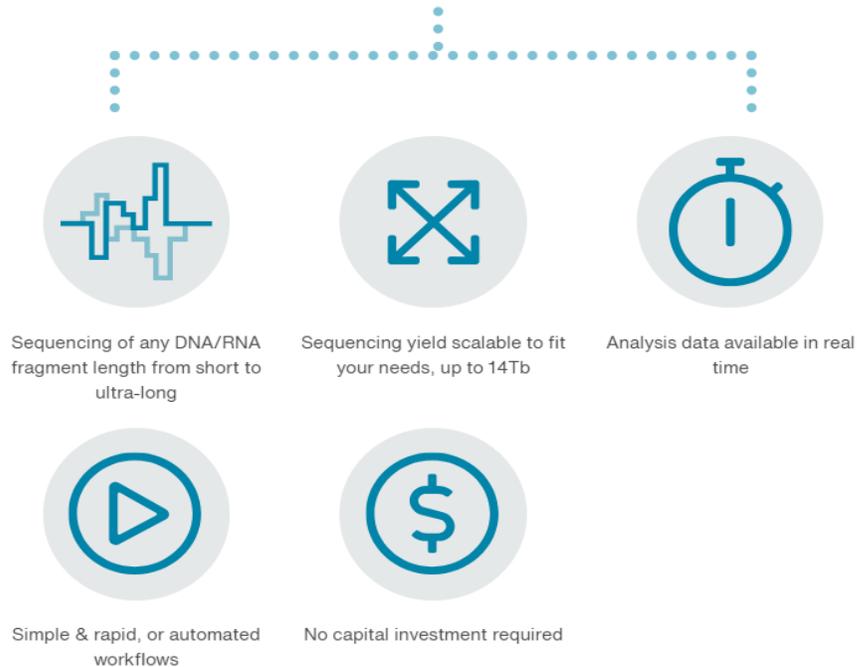


## Free, open source protocols and software for poliovirus detection and sequencing using nanopore.

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.

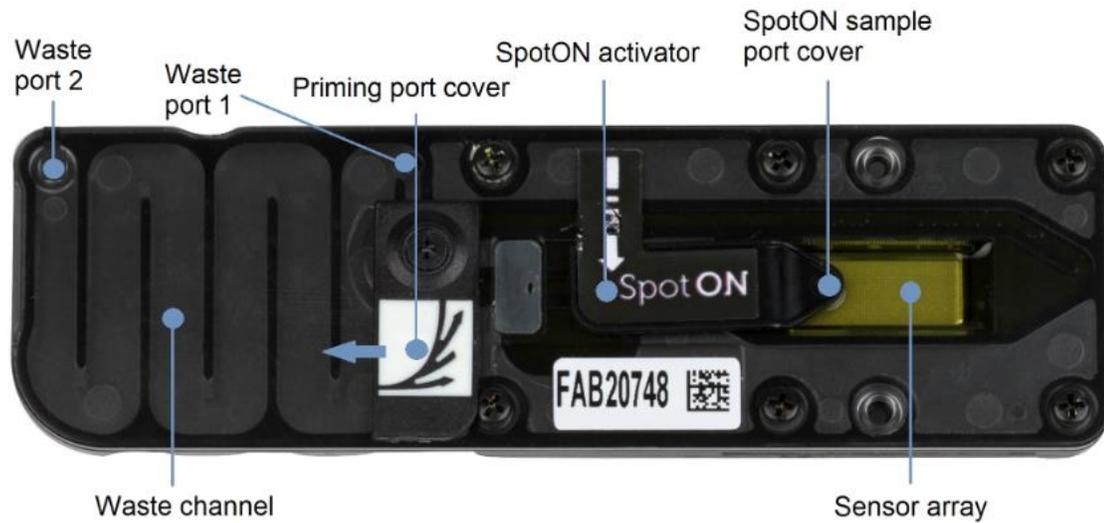


# Nanopore (MinION) Sequencing



- 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



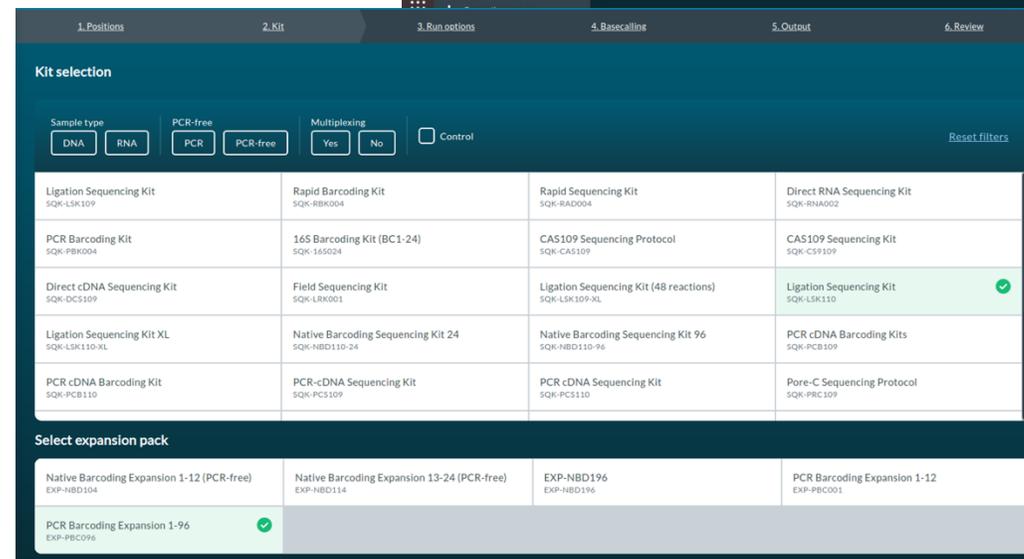
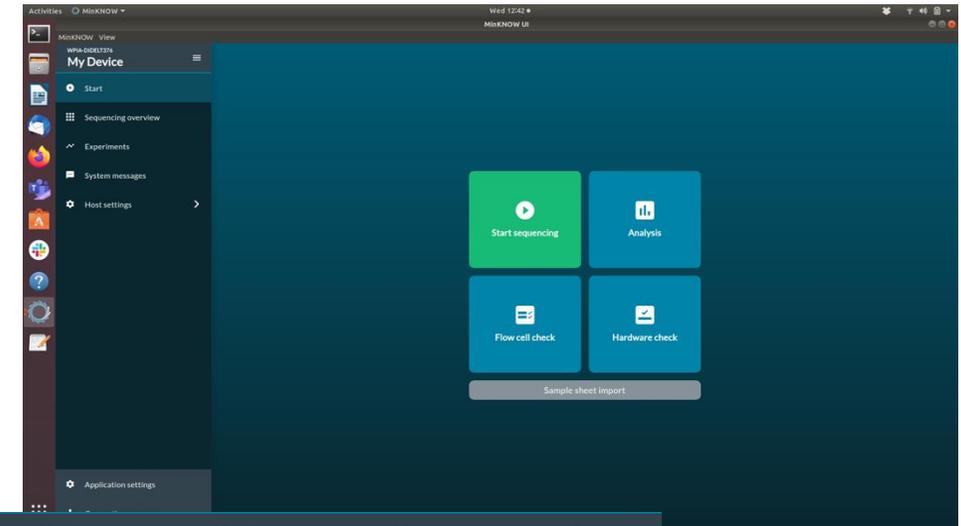
In this picture both the priming and sample loading ports are closed.



- 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

 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.

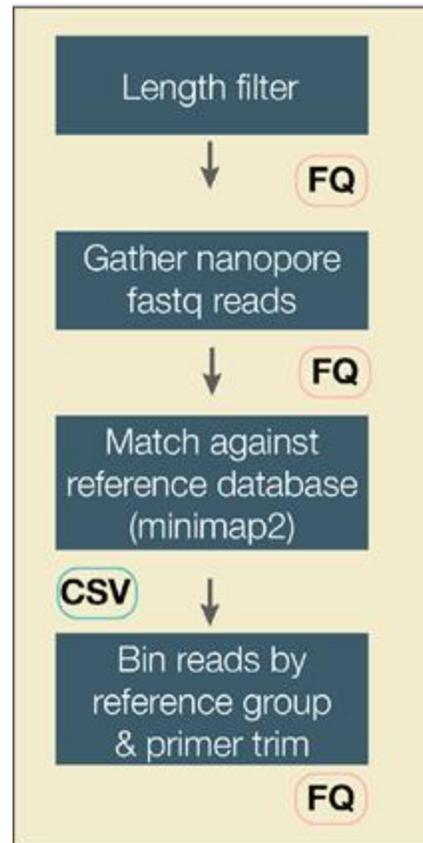
An internet connection and a Docker install is required to install or update software

Continue

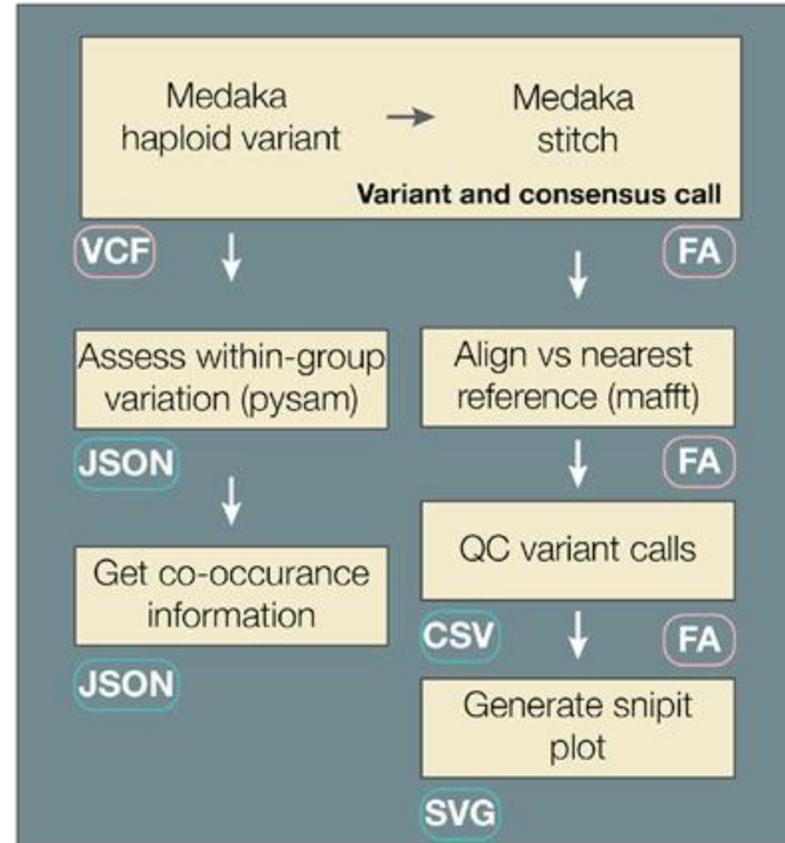
Wellcome Trust Award 206298/Z/17/Z

# Workflow schema

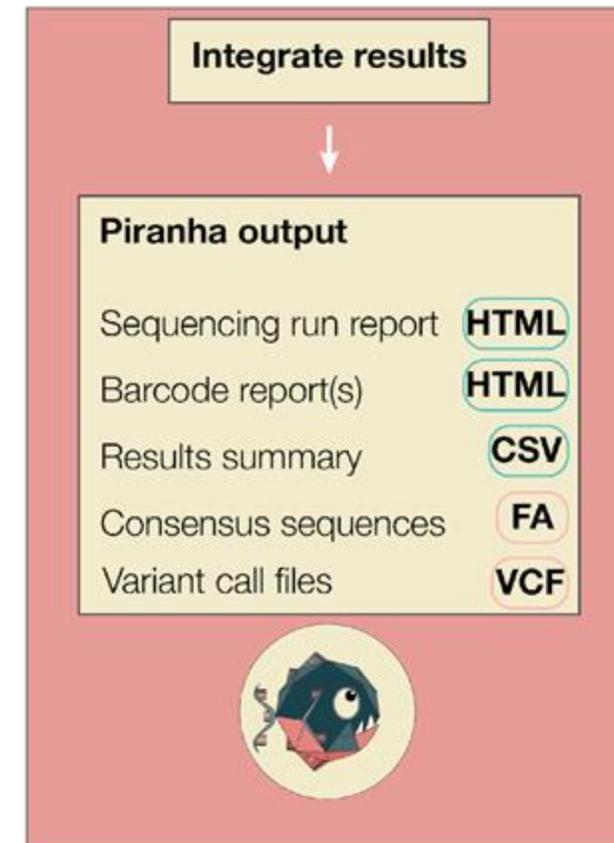
## A. Barcode analysis



## B. Reference group analysis



## C. Results reporting





# PIRANHA Outputs- Report

Search:

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

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
sample12	barcode37	0	4458	4	0	4400	0	578	1

## sample08 report 2022-09-12

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
GGGTTAGGTCAGATGCTTGAAGCATGATTGACAAACAGTCCGTGAAACGGTGGGGCGGCAACGCTAGAGAGCTCTCCAAACACTGAAGCCAGTGGACAGCACACTCCAAGGAAATTCGGCACTCACCGAGTGGAAATCGGGCCAC
AAATCCACTAGTCCCTTCGATACAGTGCAAACAGACATGTTGACAACATAGGTCAGGTCAGAGTCTAGCATAGAGTCTTCTTCGCGCGGGGTGCATGCTGGCCATTTAACCCTGGATAACTCAGCTTCCACAGAATAAGGATAAGC
TATTTACAGTGTGGAAAGATCACTTATAAAGATACTGTCCAGTTACGGAGGAAATTTGGAGTCTTCCACATTTAGATTTGATATGGAATTTACCTTTGGTGTACTGCAAAATTCAGTGAGACTAAACATGGGCATGCCTTAAATCAAGTGTAC
CAAATTTATGACGTACCACAGGCGCTCCAGTGGCCGAGAAATGGGACGACTACACATGGCAAACTCATCAAATCCATCAATCTTTTACCTACGGAAACAGTCCAGCCGGATCTGGTACCGTATGTTGGATTTTCGAAAGCCTAATTCACA
CTTTTACGACGGTTTTTCAAAGTACCACTGAAGGACAGTCCGGCAGCAGTCTAGGTGACTCCCTCTATGTTGTCAGCATCTCTAAATGACTTCGGTATTTGGCTGTTAGAGTAGTCAATGATCACAACCCGACCAAGGTACCTCCAAAATCAGAG
```

[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

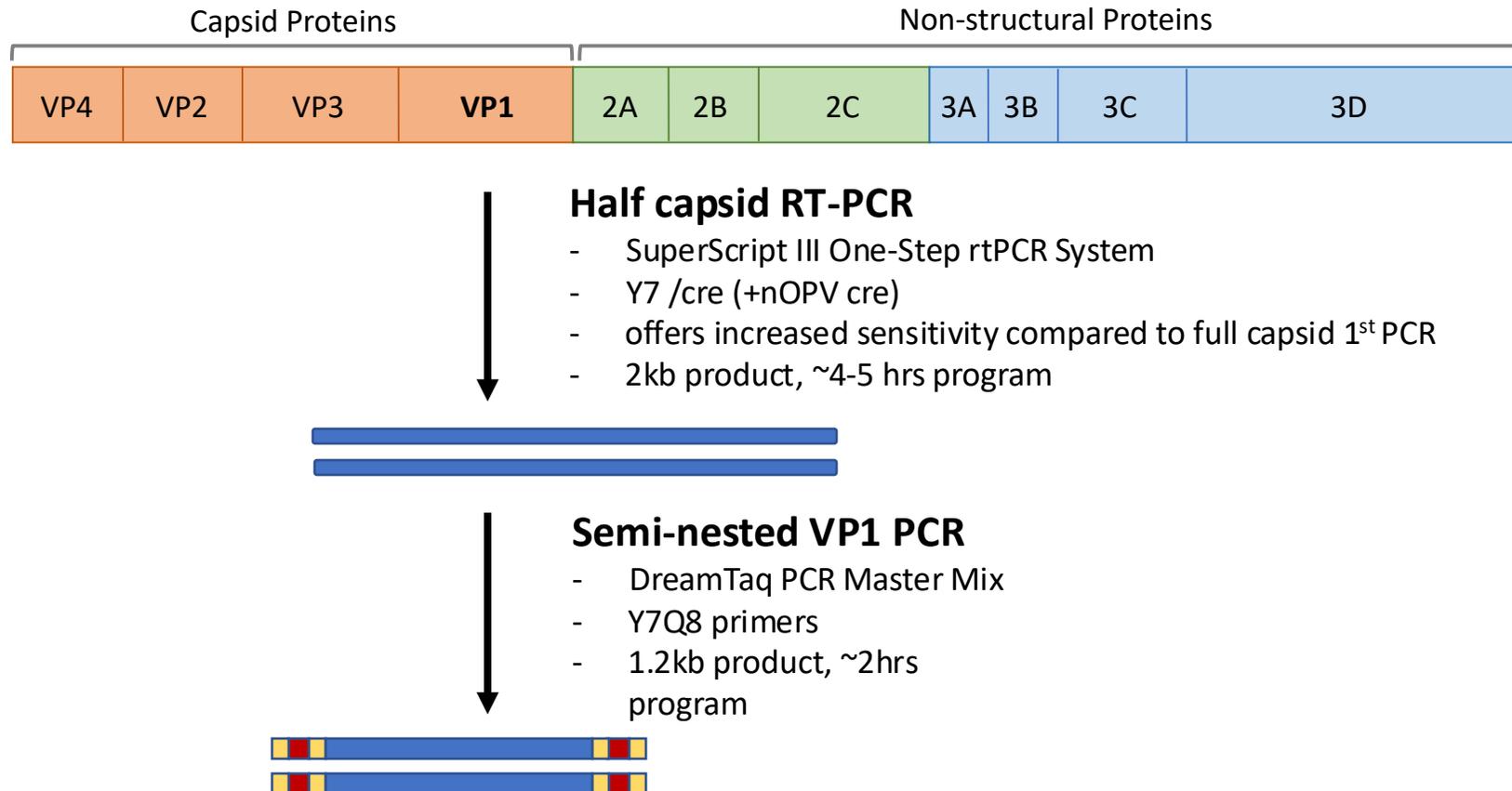
- All sample metadata can be entered into a csv file and each sample assigned a barcode.
- The analysis software (PIRANHA) will append the sequencing results to each sample.

	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

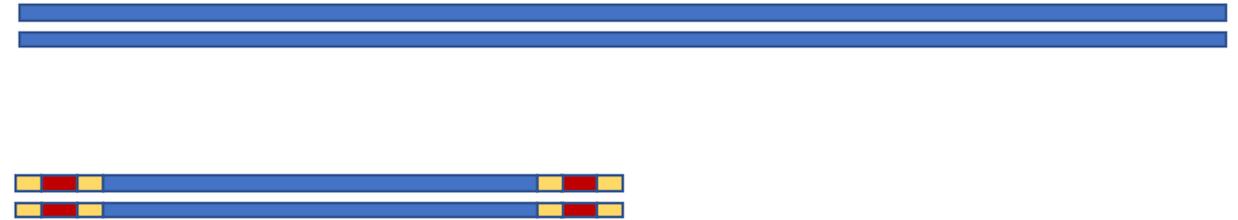
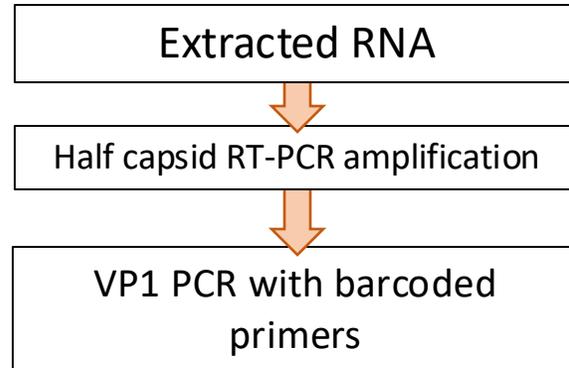
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40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

# Our PCR strategy in direct detection

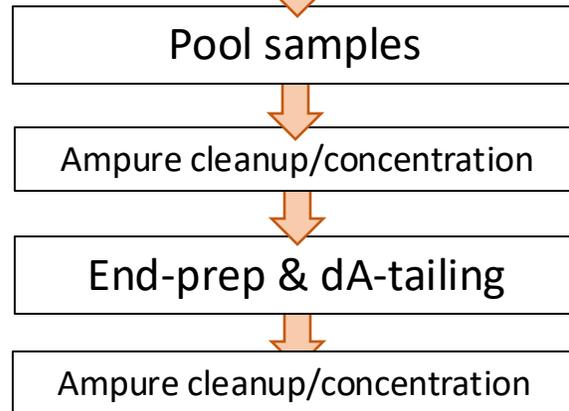


# Library preparation overview – stool DDNS

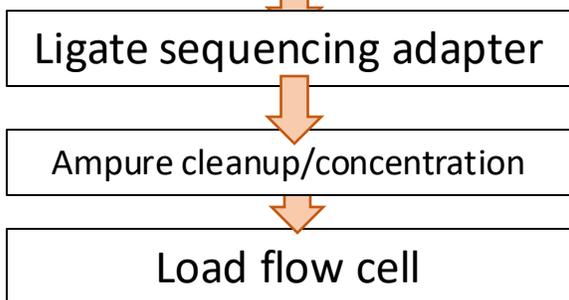
Part 1:  
RNA  
extraction,  
RT-PCR  
amplification



Part 2:  
Pooling,  
cleaning and  
end-prep



Part 3:  
Preparation  
for  
sequencing



# Run controls

---

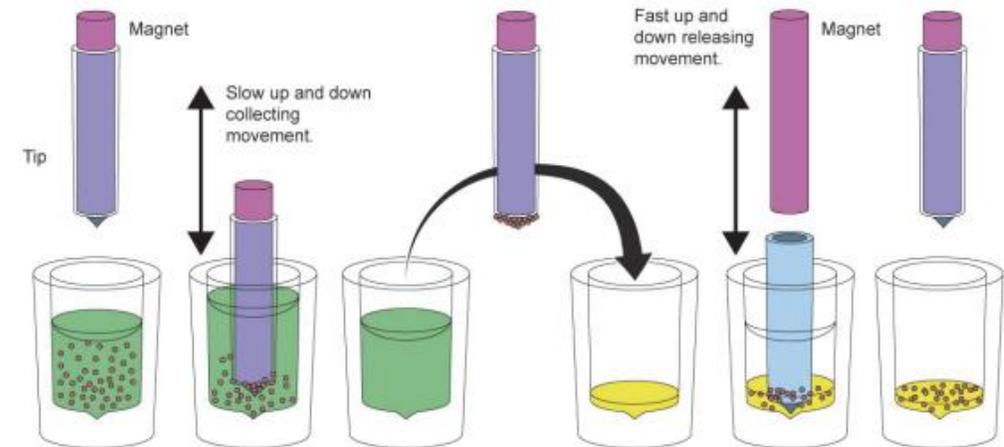
- Both negative and positive controls go through the whole process from RNA extraction to sequencing
- Negative run control: Nuclease free water used for RNA extraction
- Positive run control: CVA20 formulated by NIBSC, distributed freeze-dried and known to amplify in both PCR steps
- Instructions for 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

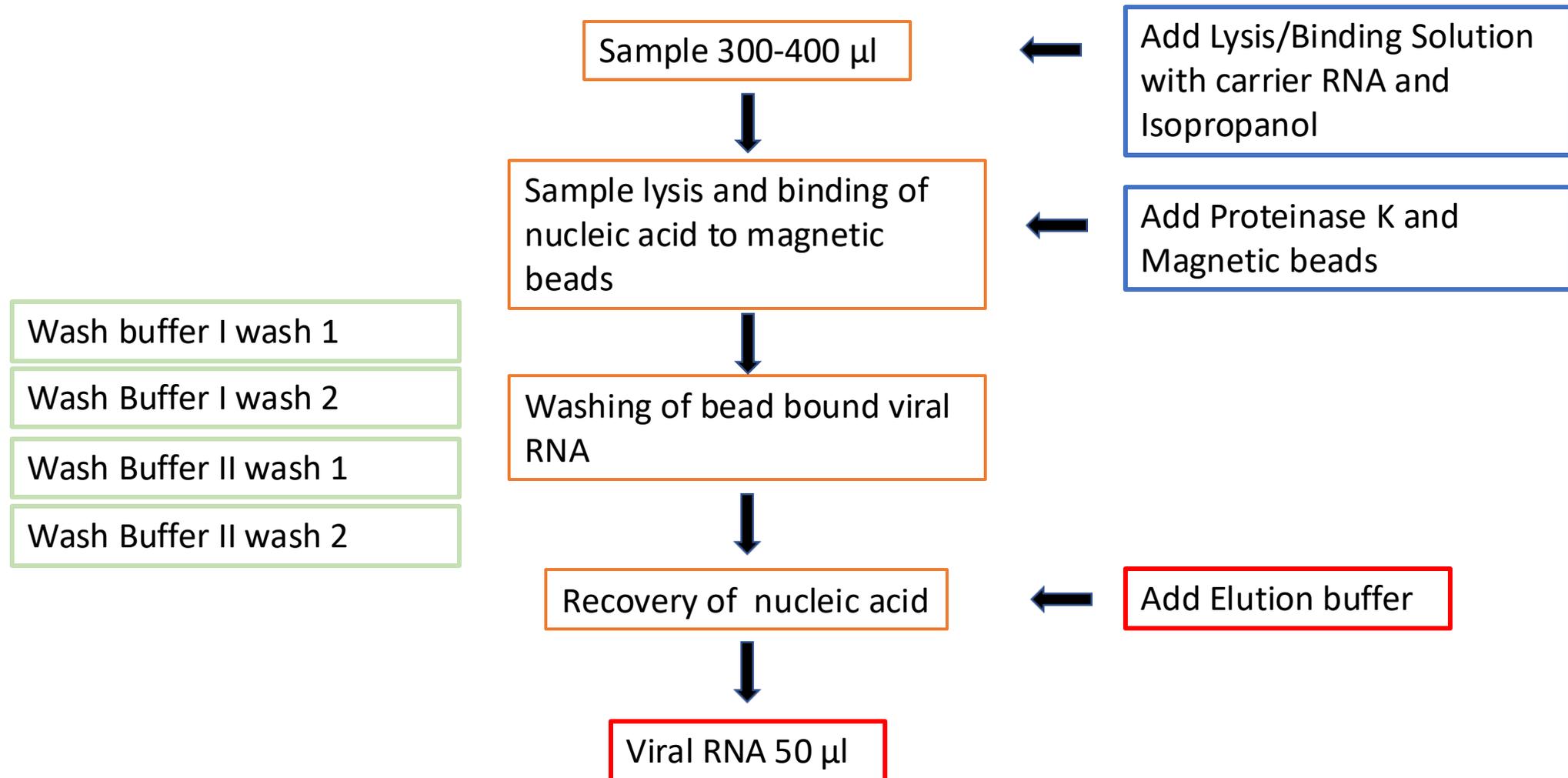
- Recommended kit: MagMAX Viral RNA Isolation kit Cat no: AM1939
- Magnetic bead based method
- Can be performed manually in microcentrifuge tubes or in deep-well plates using KingFisher equipment



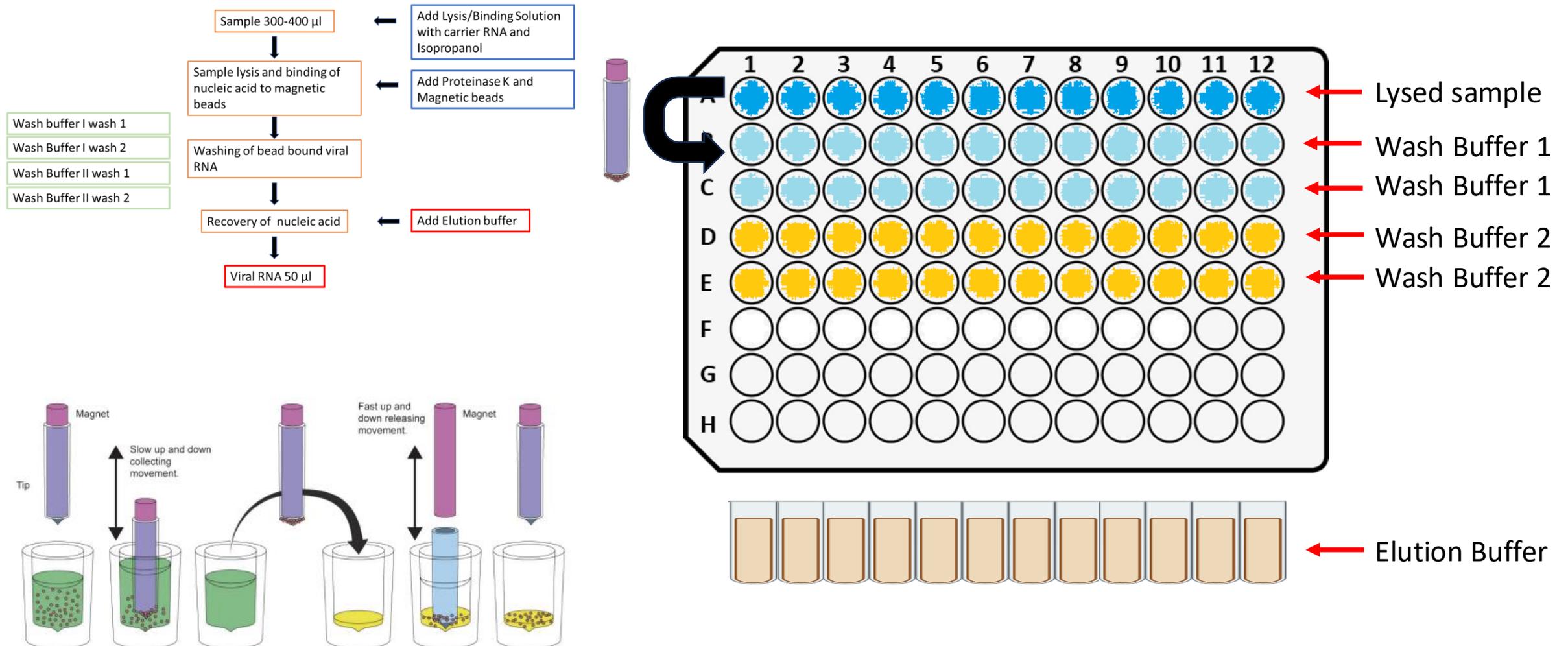
or



# RNA extraction - MagMAX Viral RNA Isolation Kit



# RNA extraction - Automated extraction (Kingfisher Duo)



# Setting up the half-capsid RT-PCR

Mastermix plate  
(19  $\mu$ l reaction mix per well)

	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

← Add 5  $\mu$ l

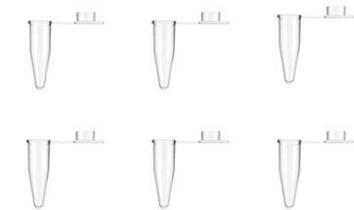
Automated

	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

Extracted RNA

Manual

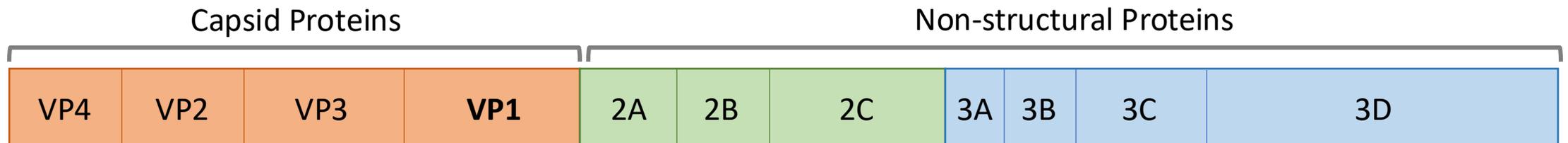
or



	1 Reaction ( $\mu$ l)
2x Master Mix	12.5
SSIII Platinum Taq mix	1
Reverse Primer (10 $\mu$ M, Cre/nOPV2-MM-R mix)	1
Nuclease free Water	4.5
<b>Total</b>	<b>19</b>

- 30 minutes of incubation at 50  $^{\circ}$ C (reverse transcription)
- Add 1  $\mu$ l of 10 $\mu$ M forward primer (Y7) for the PCR

# Half-capsid RT-PCR



**Half-capsid RT-PCR – Cre/nOPV2-MM-R (rev) and Y7 (for)**  
 - SuperScript III One-Step rtPCR System



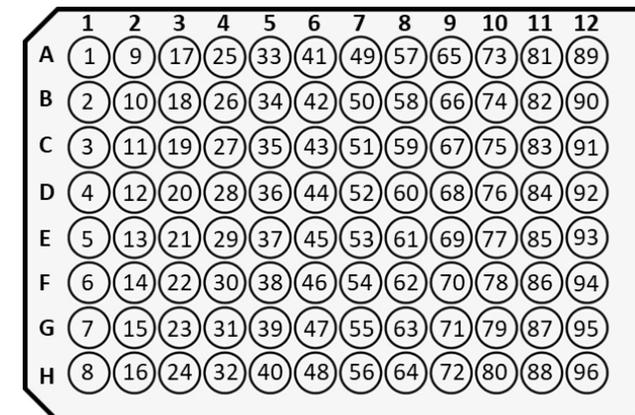
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	+	25	+	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	+	66	74	82	90
C	+	11	19	27	+	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	+
E	5	13	21	29	37	45	53	+	69	77	85	93
F	6	+	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	+	40	48	56	64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

- 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  $\mu\text{l}$  of each of the 1<sup>st</sup> 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



	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

# VP1 PCR primers

---

Flanking sequence

Y7 **GGTGCTGACCGAGATCCTACGAATGGAGTGTTAACCTGGGTTTGTGTCAGCCTGTAATGA**

Barcode sequence

Primer sequence

**TACACCTTRTCTCTGGAGAATCCAATTACCGAGATCCTACGAATGGAGTGTCGTGG** 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  $\mu$ l reaction mix per well)

	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
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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

	1 Reaction ( $\mu$ L)
Water	8.5
DreamTaq 2x master mix	12.5
<b>Total volume</b>	<b>21</b>

Add 2  $\mu$ l



Q8/Y7 barcoded primer plate (10 $\mu$ M)

	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

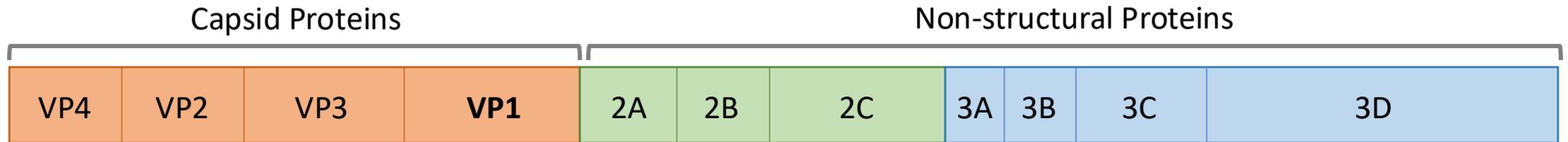
Add 2  $\mu$ l



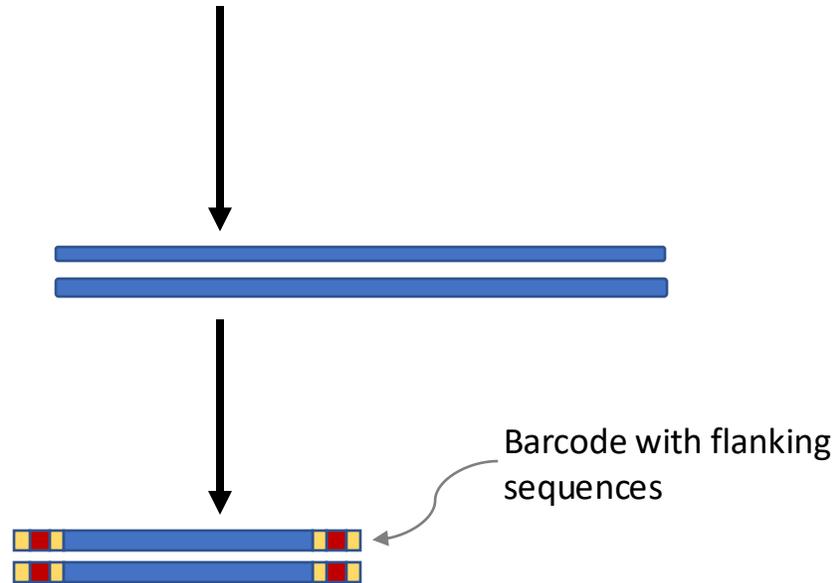
1<sup>st</sup> reaction plate

	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

# 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



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

# Library Pooling

- Barcoded samples equally pooled by volume (2 $\mu$ l 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	+	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	+	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	+
E	5	13	21	29	37	45	53	+	69	77	85	93
F	6	+	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



# 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/ $\mu$ 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  $\mu$ l of water
  - Use magnet to pull the beads out of solution
  - Retain 50  $\mu$ l of the eluate (the concentrated pool).



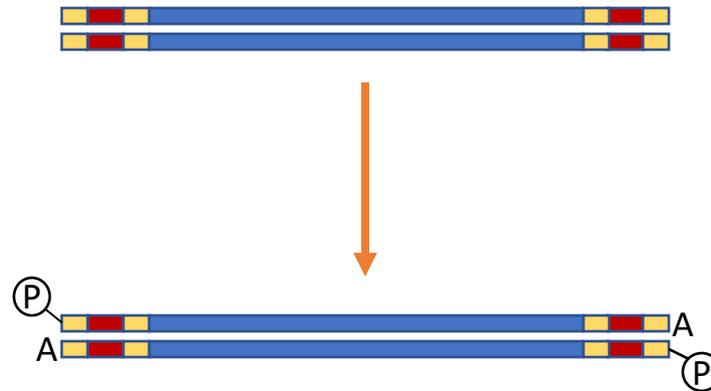
Ampure beads,  
Beckman Coulter

Pause point – store library at 4°C

# End preparation

---

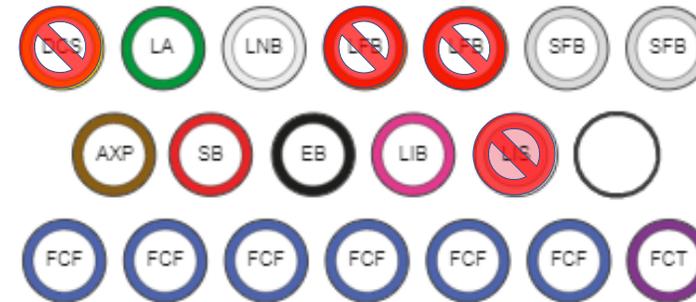
- Treatment of DNA with Ultra II End-prep
- Ensures DNA has 3' dA tailed and 5' phosphorylated ends making them sticky for ligation of the sequencing adapter



- 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)

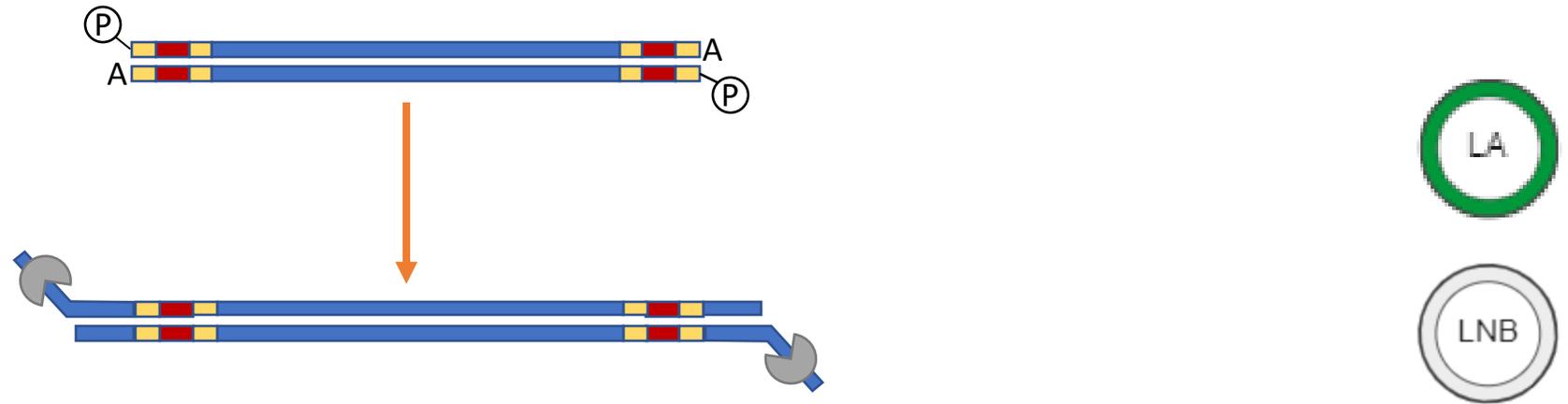
DCS : DNA Control Strand  
 LA : Ligation Adapter  
 LNB : Ligation Buffer  
 LFB : Long Fragment Buffer  
 SFB : Short Fragment Buffer  
 AXP : AMPure XP Beads

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)

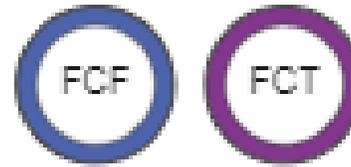
- SFB used for washing where no size selection is required.
- Long fragment buffer (LFB) can be used to select for DNA >3kb



# Priming of the flow cell

---

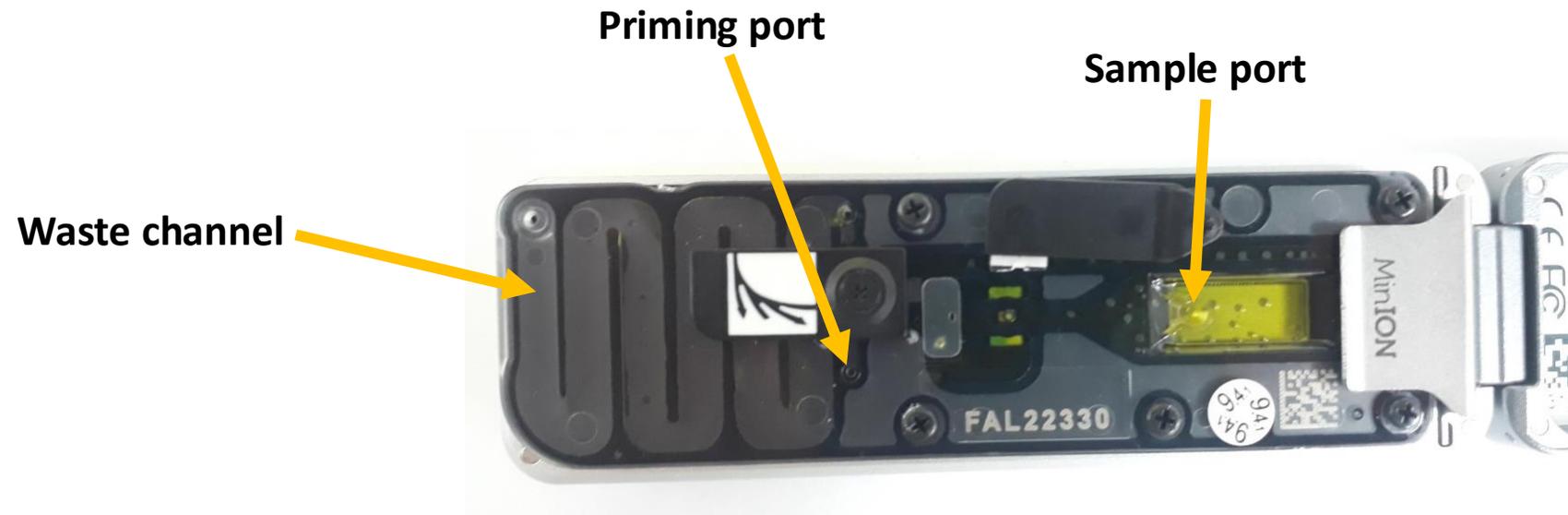
- Flowcell priming – Flow Cell Flush (FCF), Flow Cell Tether (FCT), and BSA



- 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

---

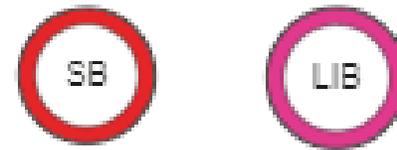


In this picture both the priming and sample loading ports are open.

# 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

---



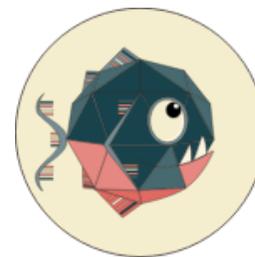
- Before flowcell priming/loading any small air bubbles are removed from beneath the priming port ( )
- Waste reagents are removed from the waste port ( ) with both priming port and sample port covers **closed**
- *Video guide: See [“Loading the MinION”](#)*

## 3. Nanopore sequencing

Presentation and discussion

# Library Preparation and Nanopore Sequencing

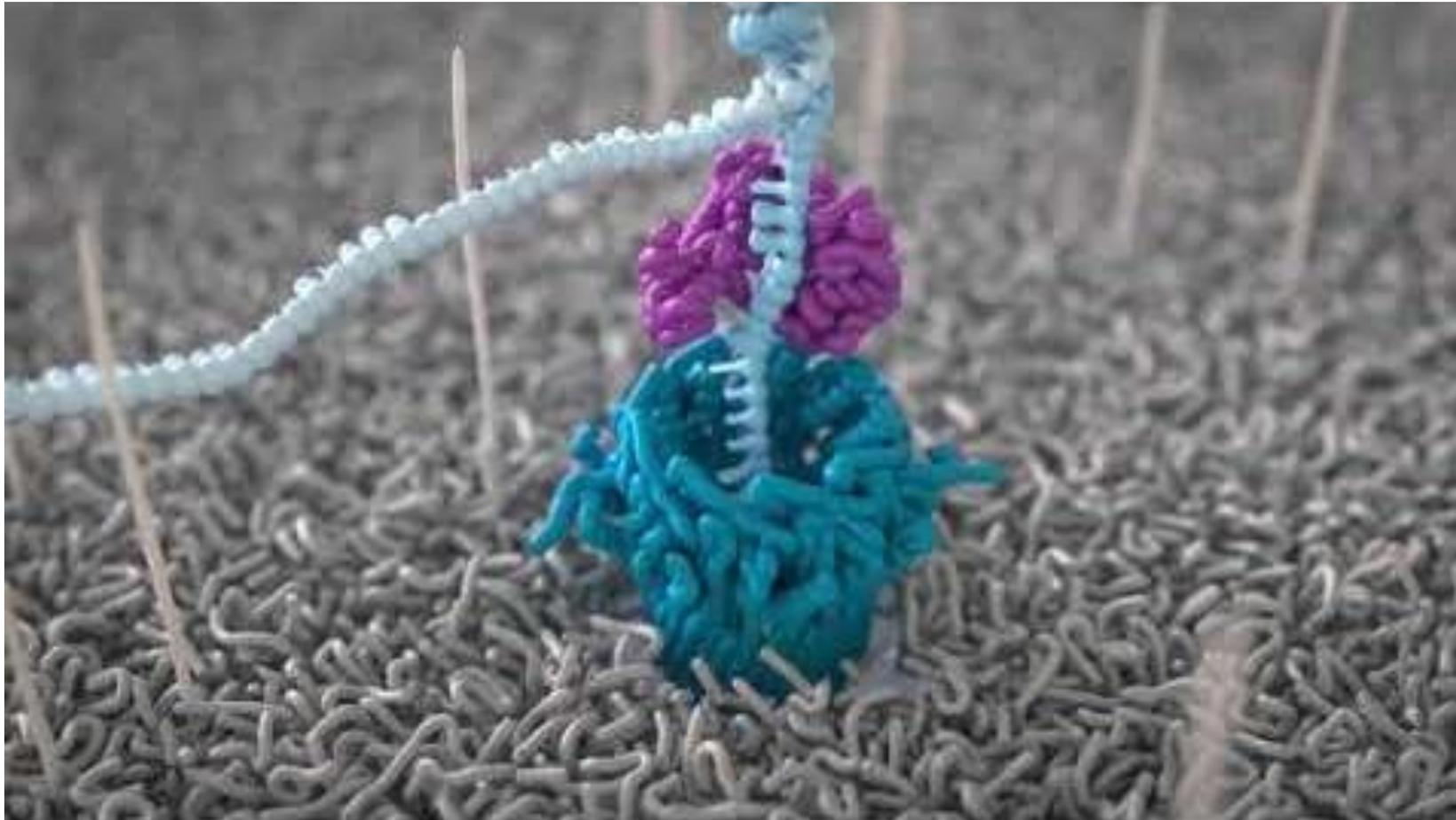
---



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

# Nanopore Sequencing

---



<https://youtu.be/RcP85JHLmnl>

# 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*

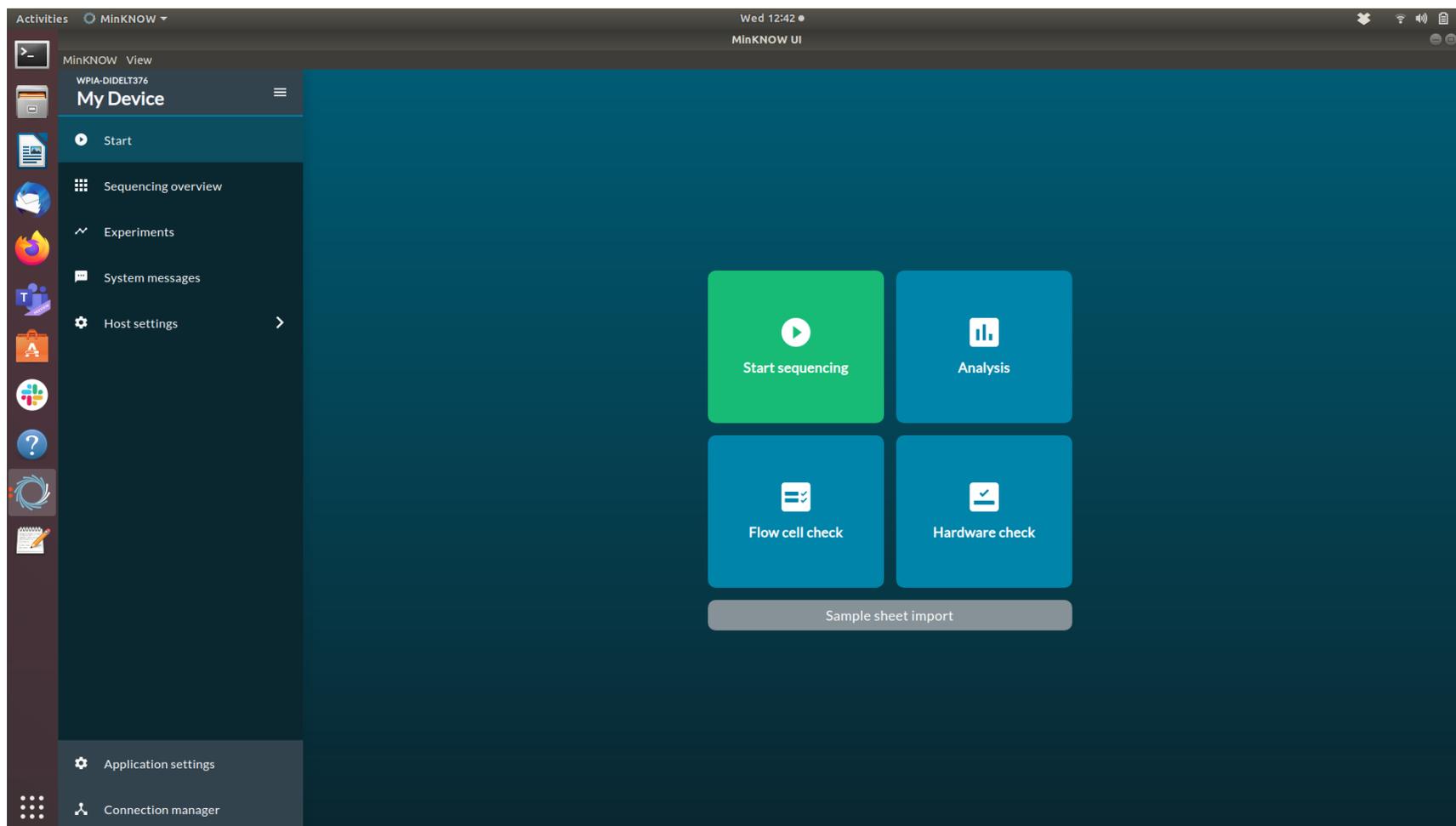
- Error rate per base (~0.5% with current highest accuracy settings)
  - can still give accurate consensus sequences.

# MinKNOW

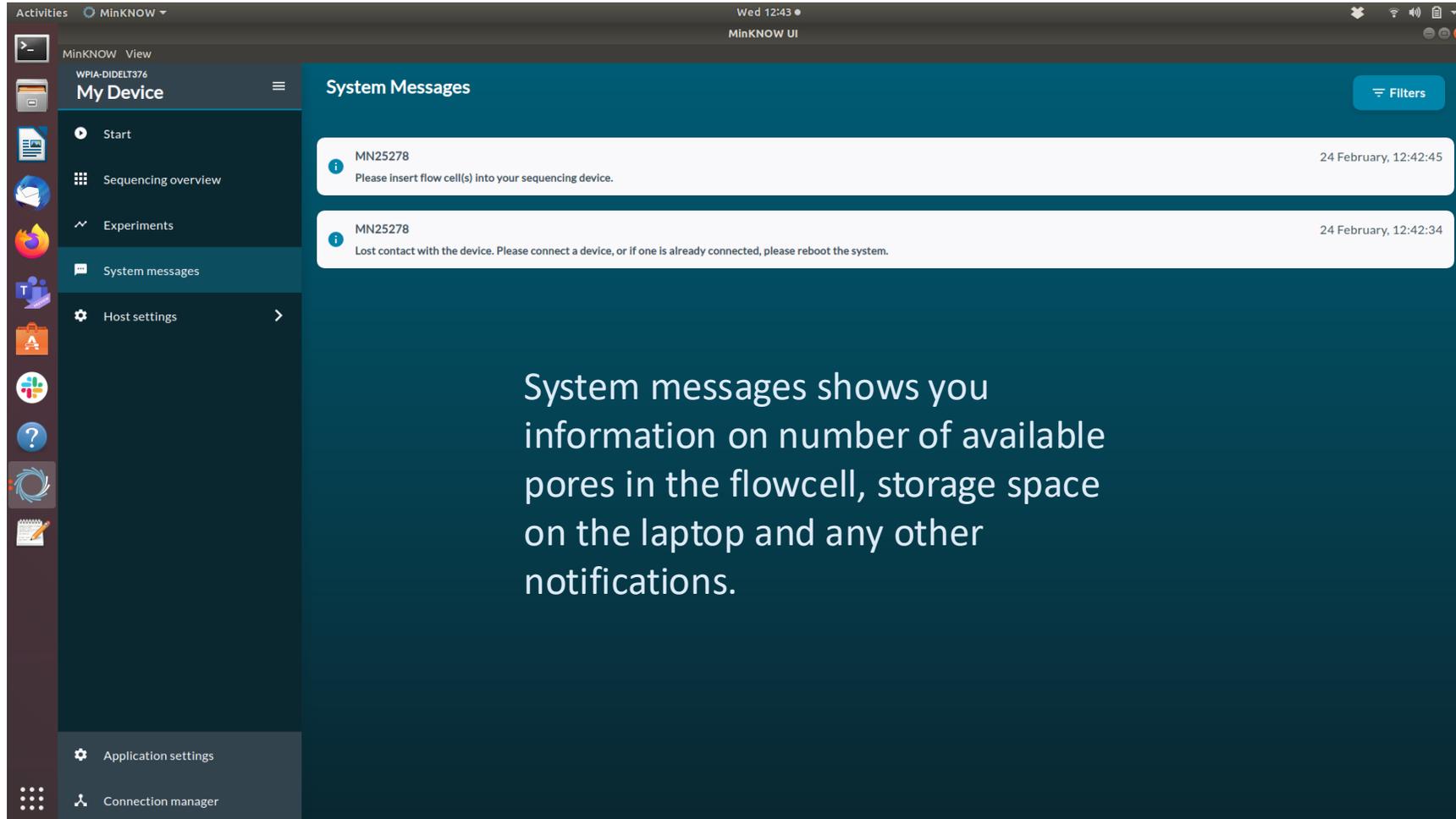
---

- 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

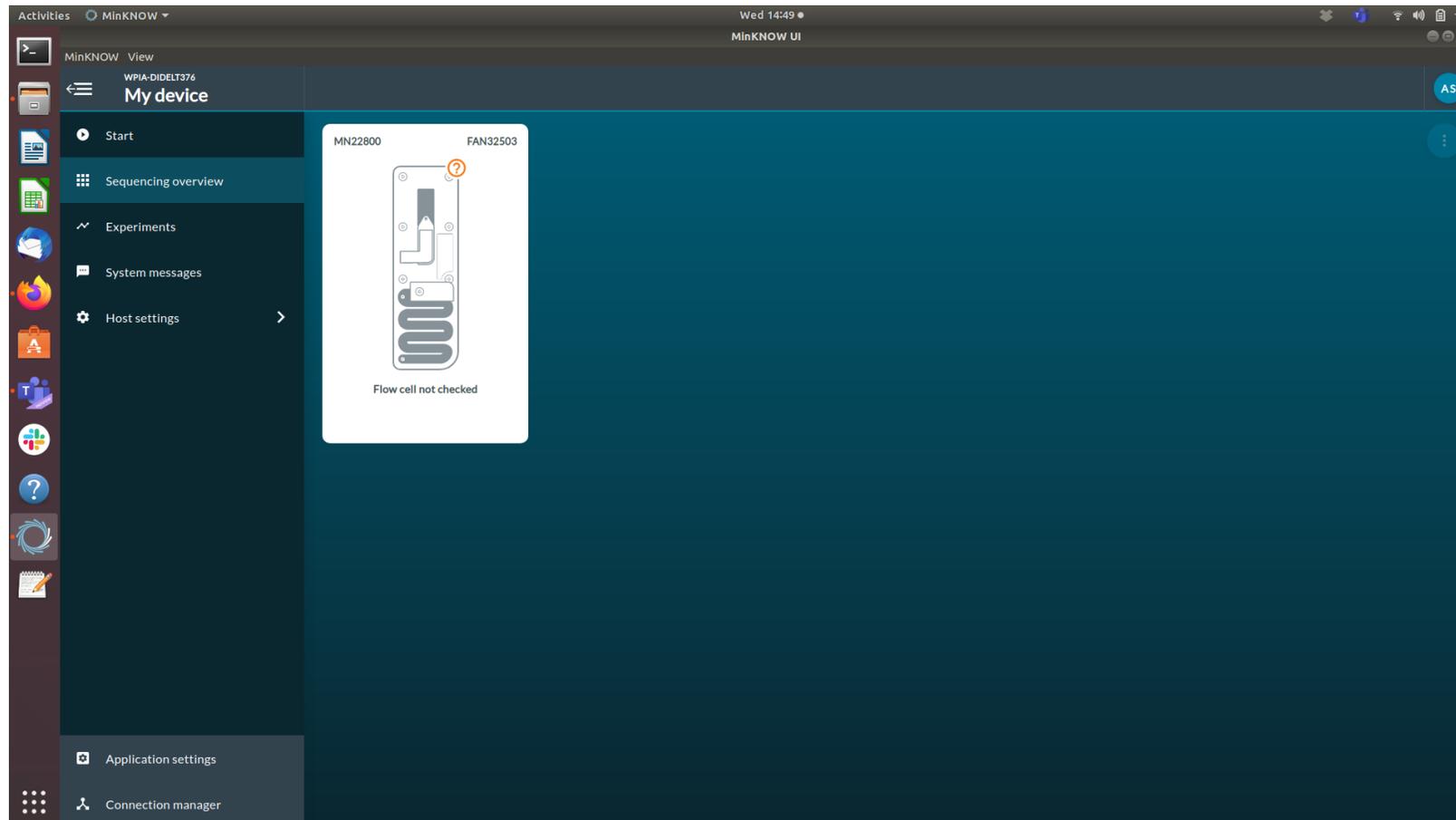


The screenshot shows the MinKNOW UI interface. The top bar displays 'Wed 12:43' and 'MinKNOW UI'. The left sidebar contains navigation options: 'My Device' (WPIA-DIDELT376), 'Start', 'Sequencing overview', 'Experiments', 'System messages' (selected), 'Host settings', 'Application settings', and 'Connection manager'. The main area is titled 'System Messages' and contains two messages:

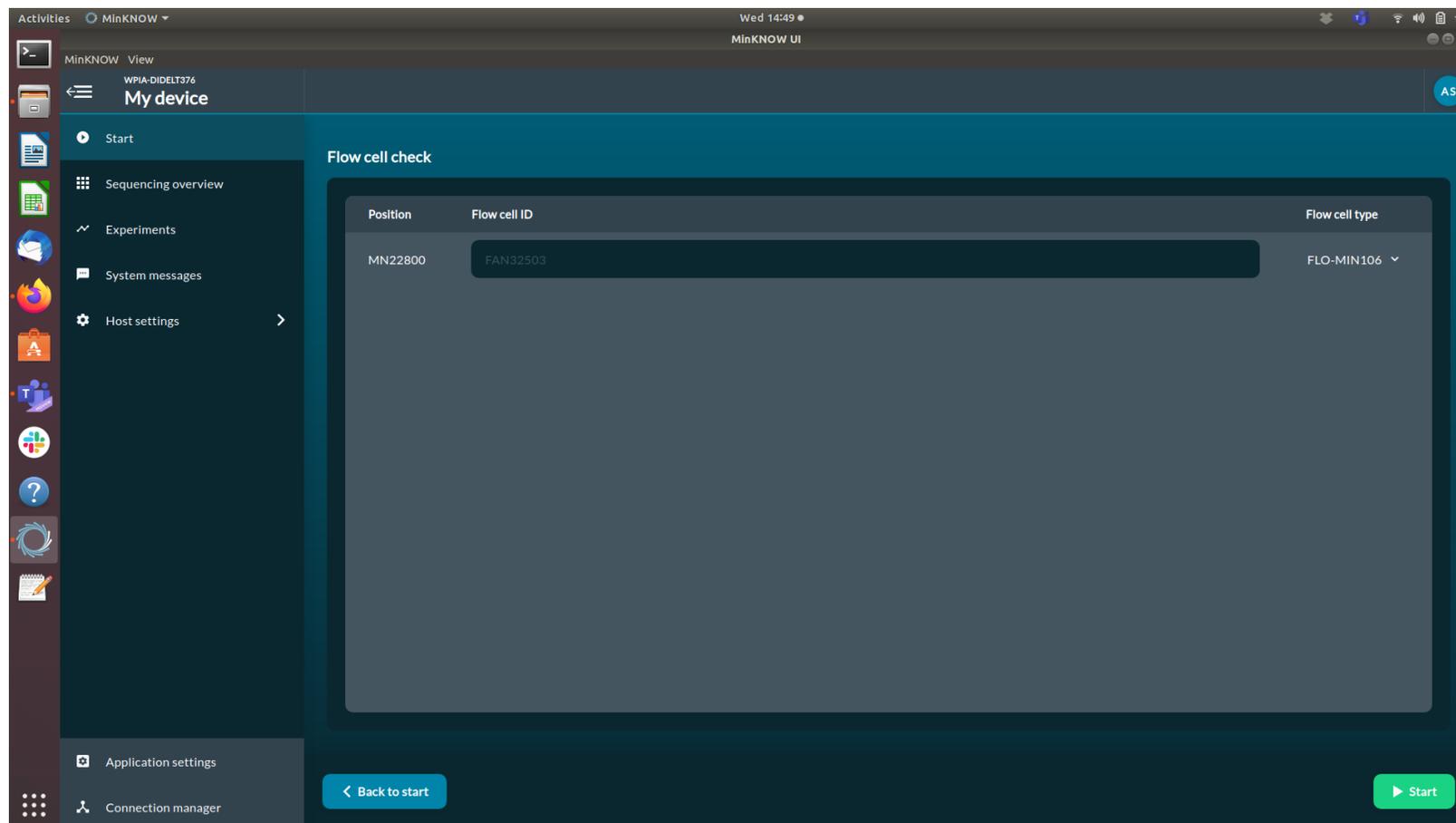
- MN25278** (24 February, 12:42:45): Please insert flow cell(s) into your sequencing device.
- MN25278** (24 February, 12:42:34): Lost contact with the device. Please connect a device, or if one is already connected, please reboot the system.

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

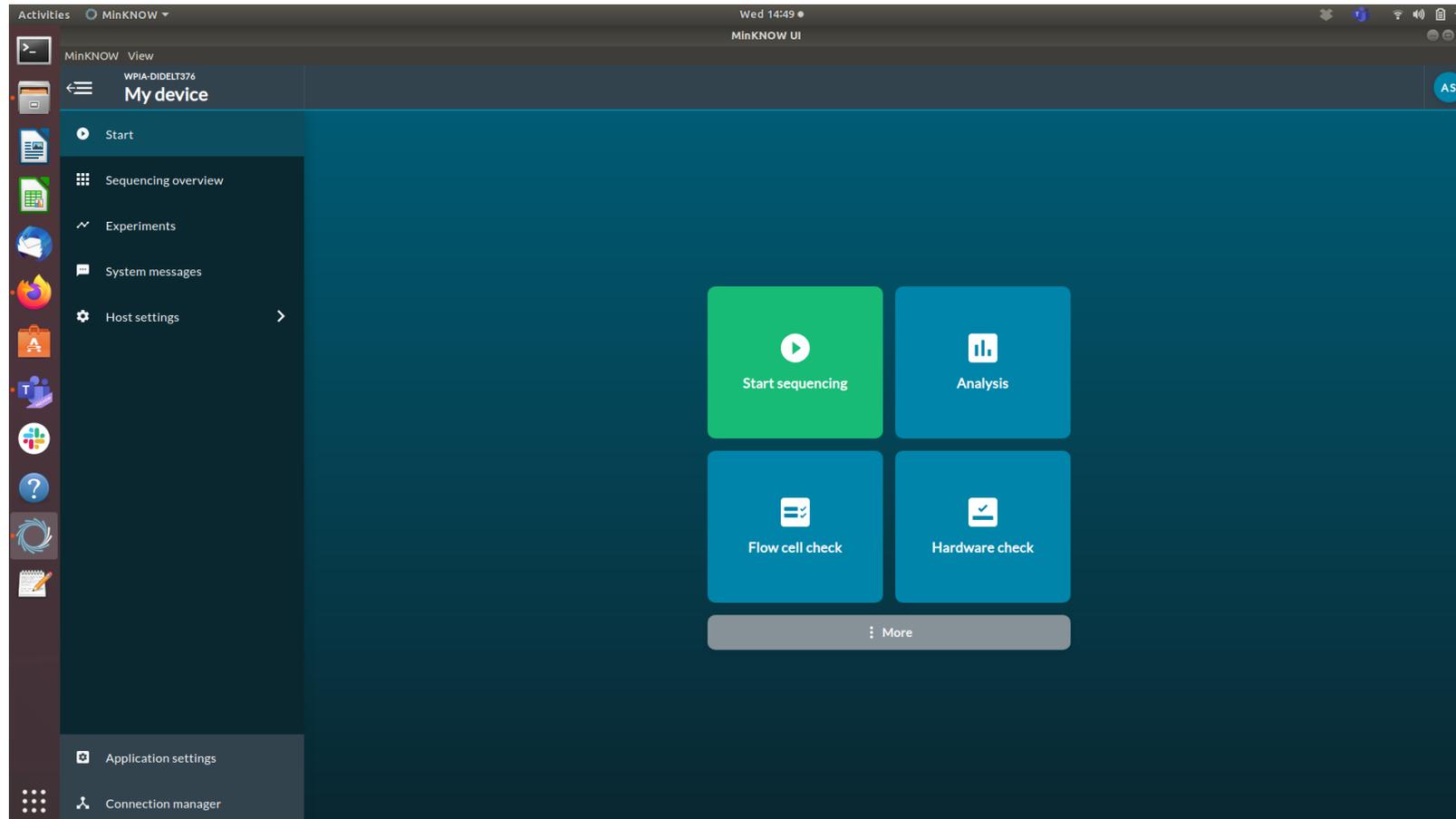


The screenshot displays the MinKNOW user interface. The top status bar shows 'Wed 14:49' and 'MinKNOW UI'. The main window title is 'MinKNOW View' with the device ID 'WPIA-DIDELT376' and 'My device' label. A sidebar on the left contains navigation options: Start, Sequencing overview, Experiments, System messages, Host settings, Application settings, and Connection manager. The central area is titled 'Flow cell check' and features a table with the following data:

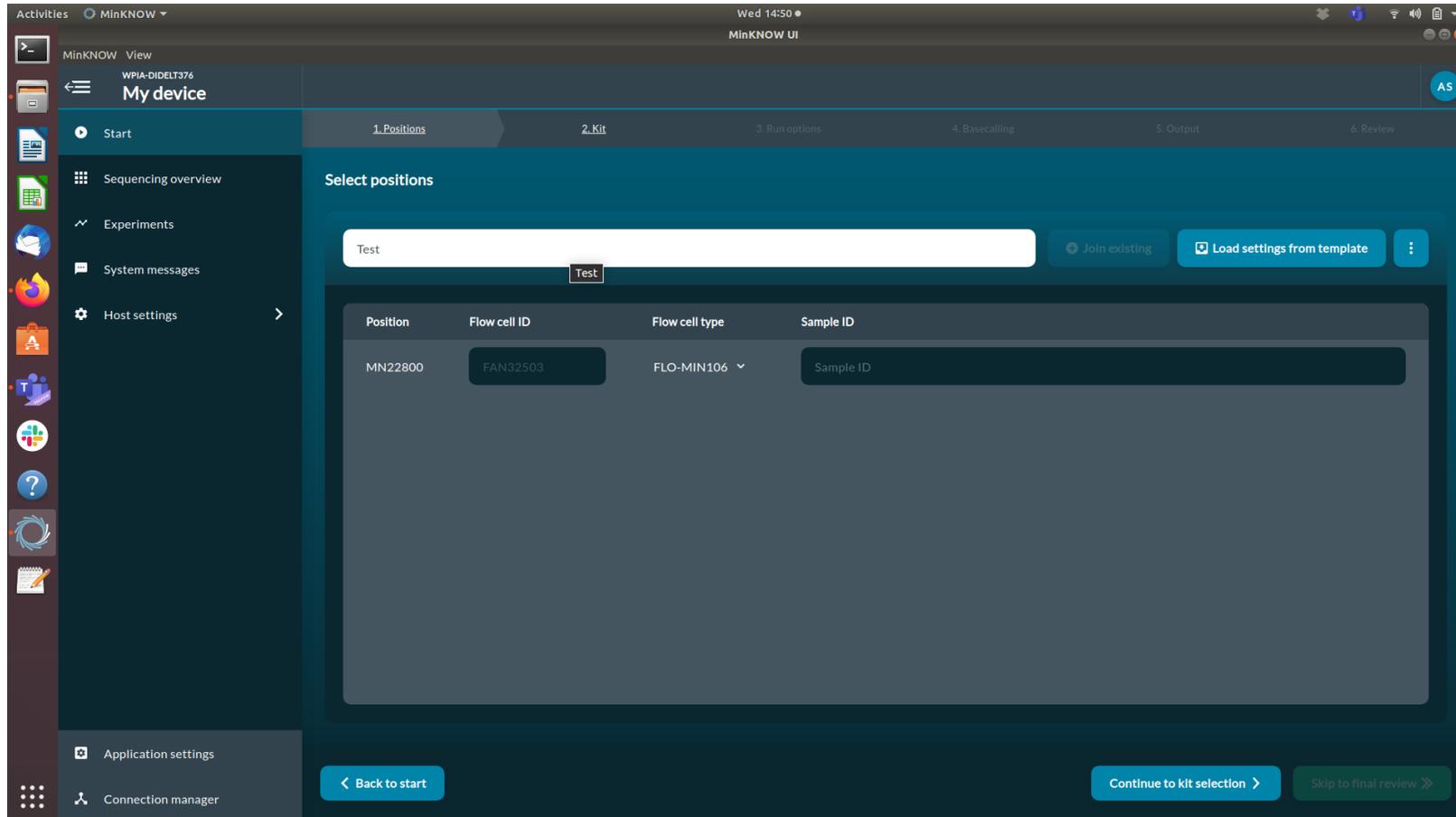
Position	Flow cell ID	Flow cell type
MN22800	FAN32503	FLO-MIN106

At the bottom of the screen, there are two buttons: '< Back to start' and '▶ Start'.

# Starting a sequencing run

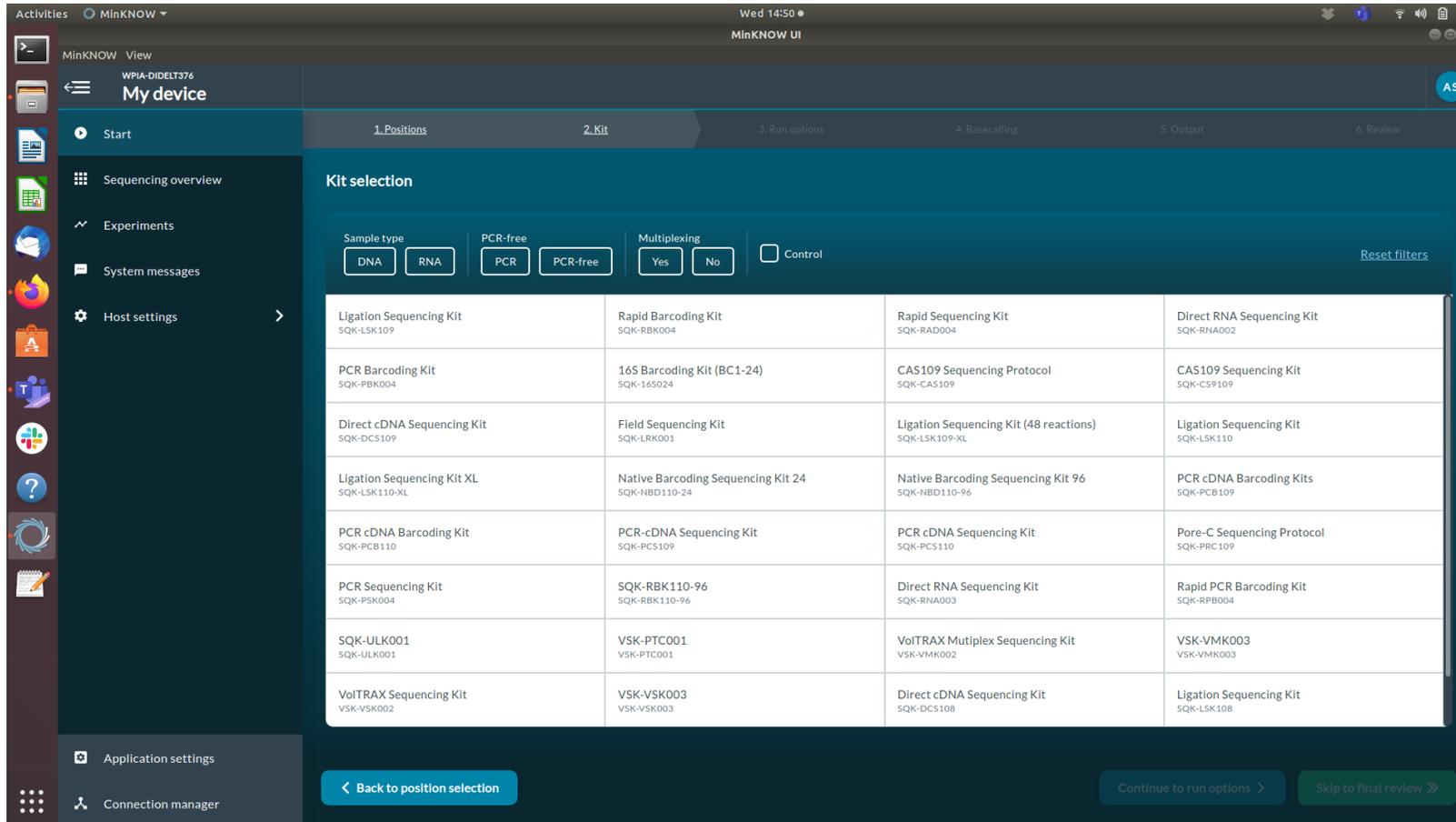


# Starting a sequencing run



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

# Selecting the sequencing kit



Kit selection

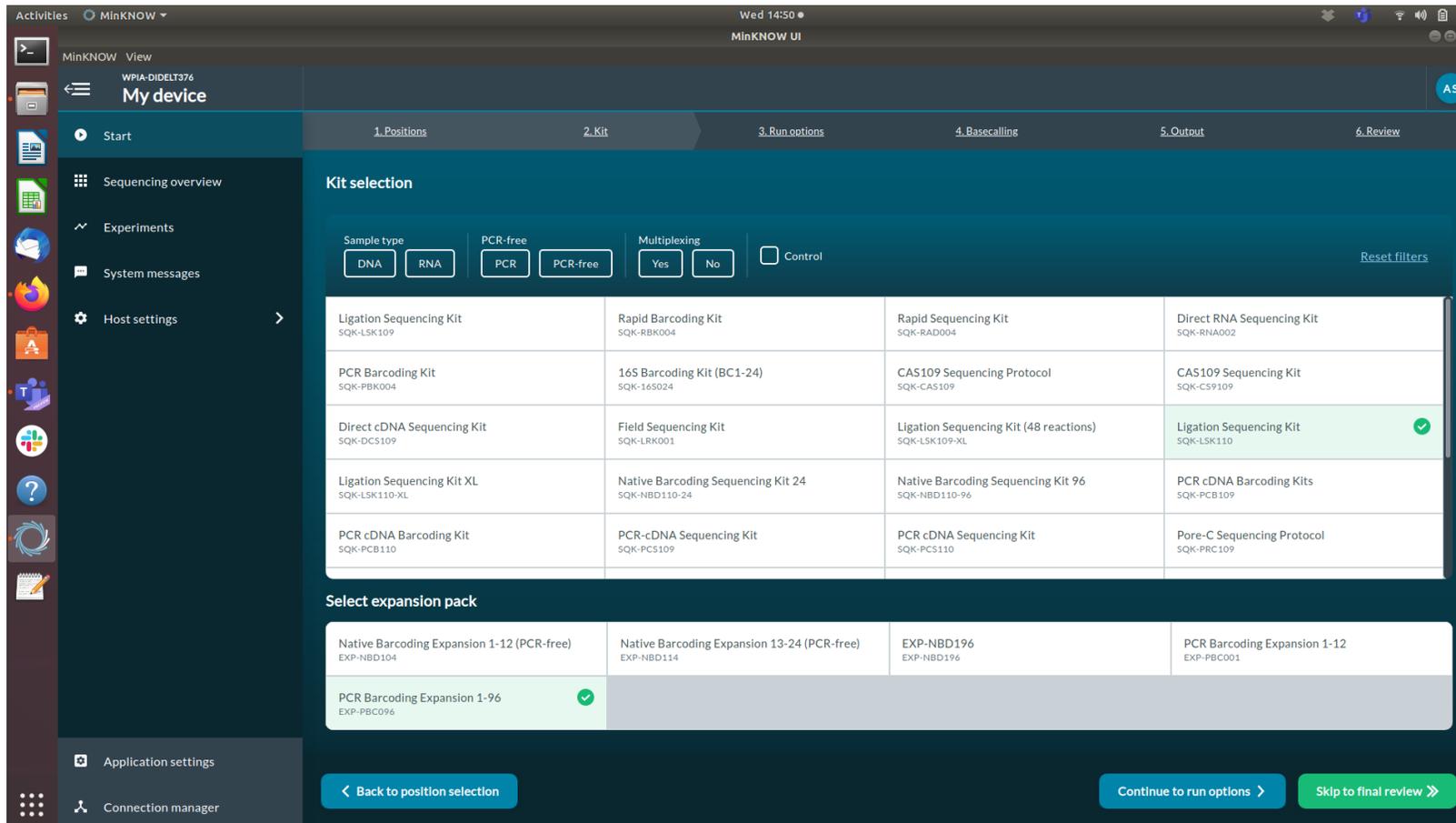
Sample type:   PCR-free:   Multiplexing:    Control [Reset filters](#)

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-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

[← Back to position selection](#)
[Continue to run options >](#)
[Skip to final review >>](#)

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

# Selecting barcodes



Activities MinKNOW Wed 14:50 MinKNOW UI

WPIA-DIDELT376 My device AS

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

### Kit selection

Sample type:   PCR-free:   Multiplexing:    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 <input checked="" type="checkbox"/>
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

### Select expansion pack

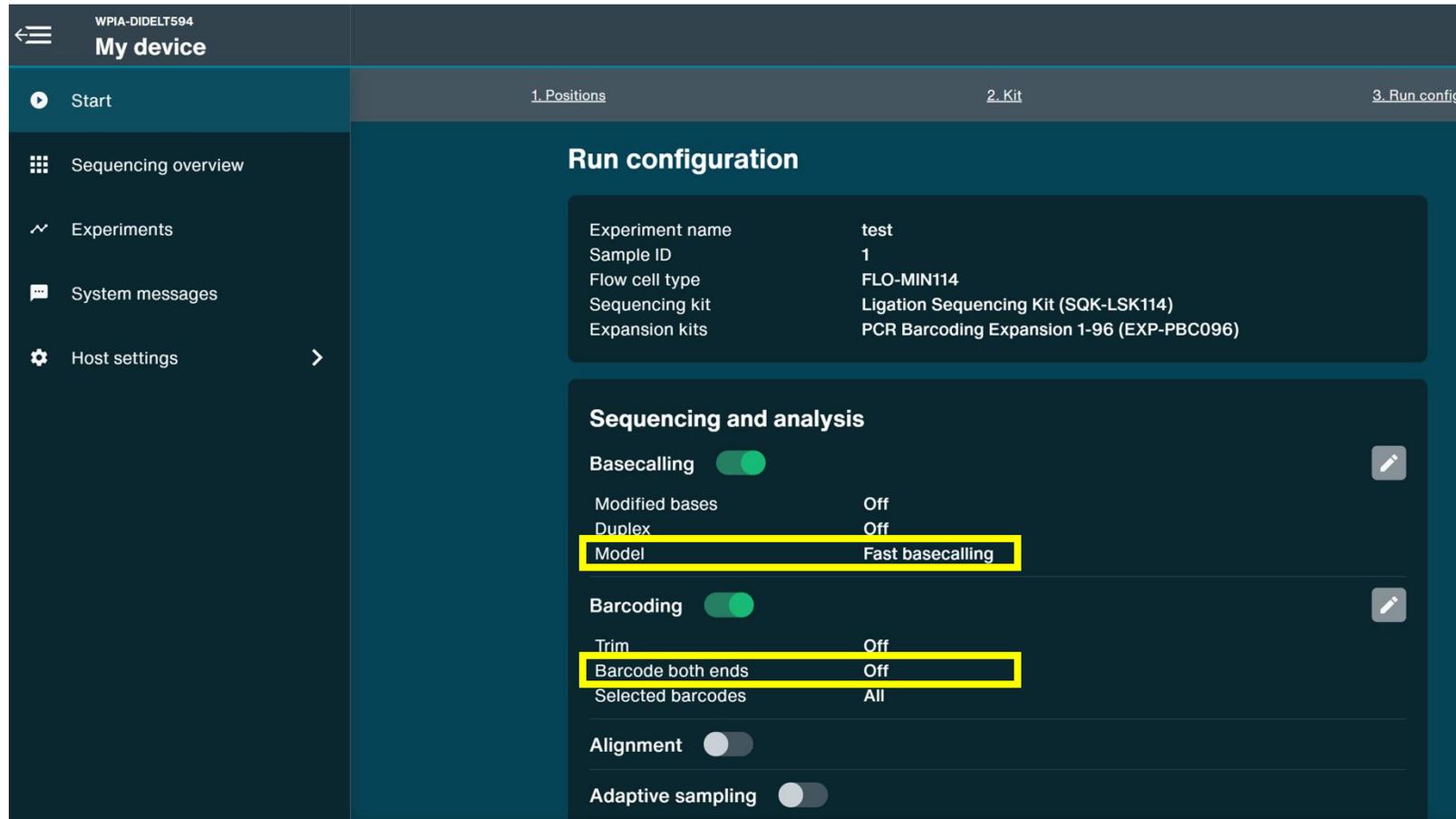
Native Barcoding Expansion 1-12 (PCR-free) EXP-NBD104	Native Barcoding Expansion 13-24 (PCR-free) EXP-NBD114	EXP-NBD196 EXP-NBD196	PCR Barcoding Expansion 1-12 EXP-PBC001
PCR Barcoding Expansion 1-96 EXP-PBC096 <input checked="" type="checkbox"/>			

[Back to position selection](#)
[Continue to run options](#)
[Skip to final review](#)

For the barcoded VP1 primers select EXP-PBC096

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

# Run Configuration



WPIA-DIDELTS94  
My device

Start 1. Positions 2. Kit 3. Run configuration

### Run configuration

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

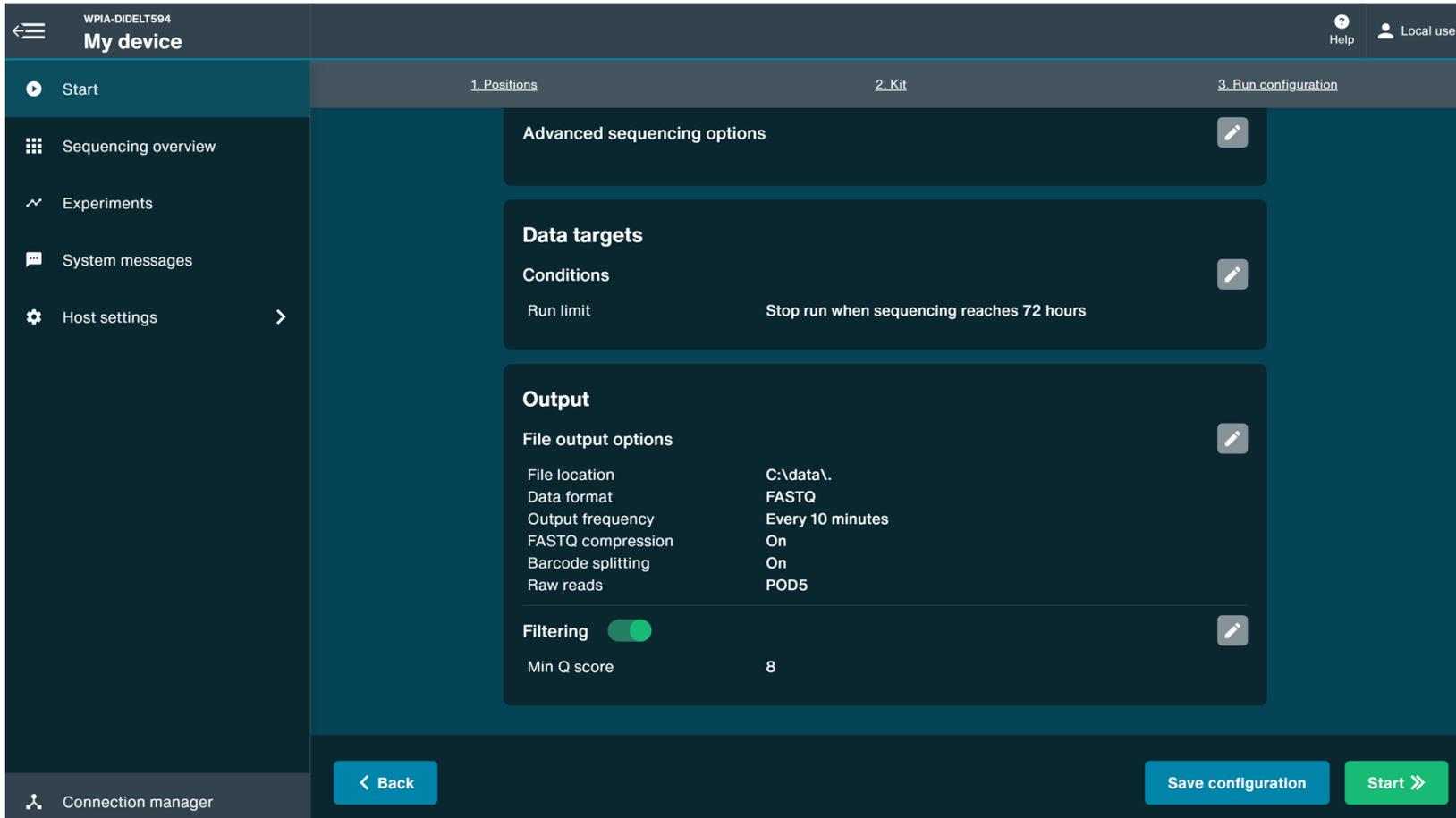
Basecalling	<input checked="" type="checkbox"/>	
Modified bases	Off	
Duplex	Off	
Model	Fast basecalling	
Barcoding	<input checked="" type="checkbox"/>	
Trim	Off	
Barcode both ends	Off	
Selected barcodes	All	
Alignment	<input type="checkbox"/>	
Adaptive sampling	<input type="checkbox"/>	

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 configuration' screen in the Polio Sequencing Consortium software. The interface is dark-themed with a sidebar on the left containing navigation options: Start, Sequencing overview, Experiments, System messages, and Host settings. The main content area is divided into three sections: 1. Positions, 2. Kit, and 3. Run configuration. The 'Run configuration' section is active and contains three main panels: 'Advanced sequencing options', 'Data targets', and 'Output'. The 'Data targets' panel shows a 'Run limit' set to 'Stop run when sequencing reaches 72 hours'. The 'Output' panel shows 'File output options' with a table of settings: File location (C:\data\), Data format (FASTQ), Output frequency (Every 10 minutes), FASTQ compression (On), Barcode splitting (On), and Raw reads (POD5). Below this is a 'Filtering' section with a toggle switch turned on and a 'Min Q score' set to 8. At the bottom of the screen, there are three buttons: '< Back', 'Save configuration', and 'Start >>'. The top right corner shows 'Help' and 'Local user'.

File location	C:\data\.
Data format	FASTQ
Output frequency	Every 10 minutes
FASTQ compression	On
Barcode splitting	On
Raw reads	POD5

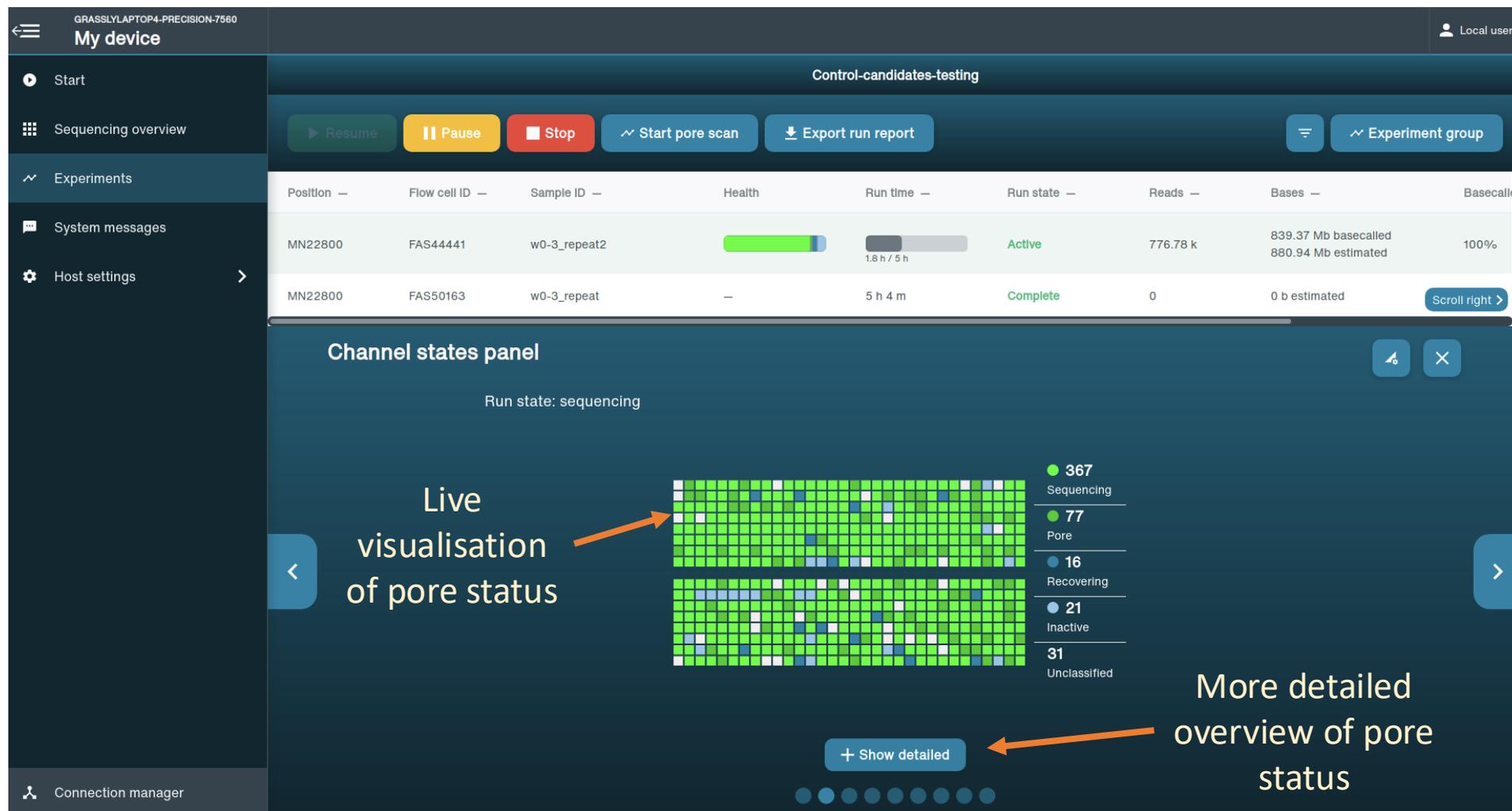
Run can be extended or stopped earlier as required.

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 Local user

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	Basecall
MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%; background-color: green;"></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	Scroll right >

Channel states panel

Run state: sequencing

Live visualisation of pore status

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

+ Show detailed

More detailed overview of pore status

Connection manager

# Sequencing Run

GRASSLYLAPTOP4-PRECISION-7560 Local user

**My device**

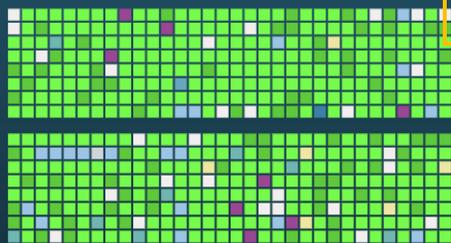
Control-candidates-testing

▶ Resume
⏸ Pause
■ Stop
⌵ Start pore scan
⬇ Export run report
⌵ Experiment group

Position	Flow cell ID	Sample ID	Health	Run time	Run state	Reads	Bases	Basecall
MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%; background-color: #00ff00;"></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	<span>Scroll right &gt;</span>

**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

GRASSLYLAPTOP4-PRECISION-7560
Local user

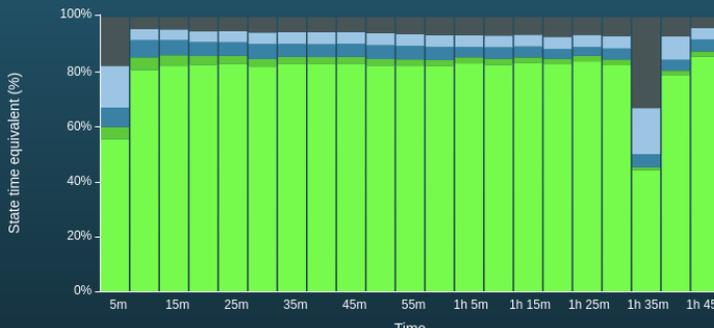
## My device

### 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	Basecall
MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%; height: 10px; background-color: #4caf50;"></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	<span style="float: right;">Scroll right &gt;</span>

### Pore activity



- sequencing ✓
- pore ✓
- recovering ✓
- inactive ✓
- unclassified ✓

+ Show detailed
⚙ Display settings

Connection manager



# Sequencing Run

GRASSLYLAPTOP4-PRECISION-7560
Local user

My device

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

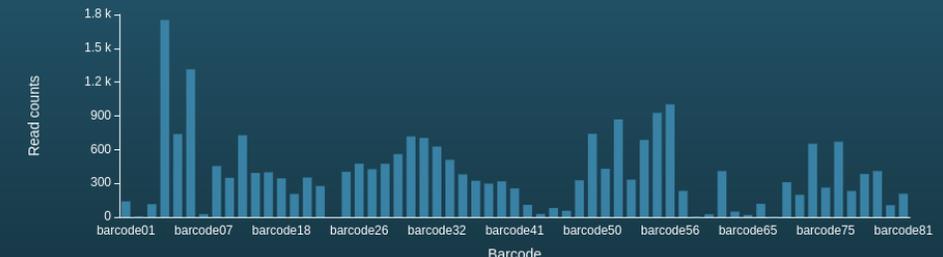
### Control-candidates-testing

Resume
Pause
Stop
Start pore scan
Export run report

Position	Flow cell ID	Sample ID	Health	Run time	Run state	Reads	Bases	Basecall
MN22800	FAS44441	w0-3_repeat2	<div style="width: 80%; height: 10px; background: linear-gradient(to right, #4caf50, #ccc);"></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	Scroll right >

### Barcode hits

● Passed reads



Read counts

Barcode

Sort

Reads Bases

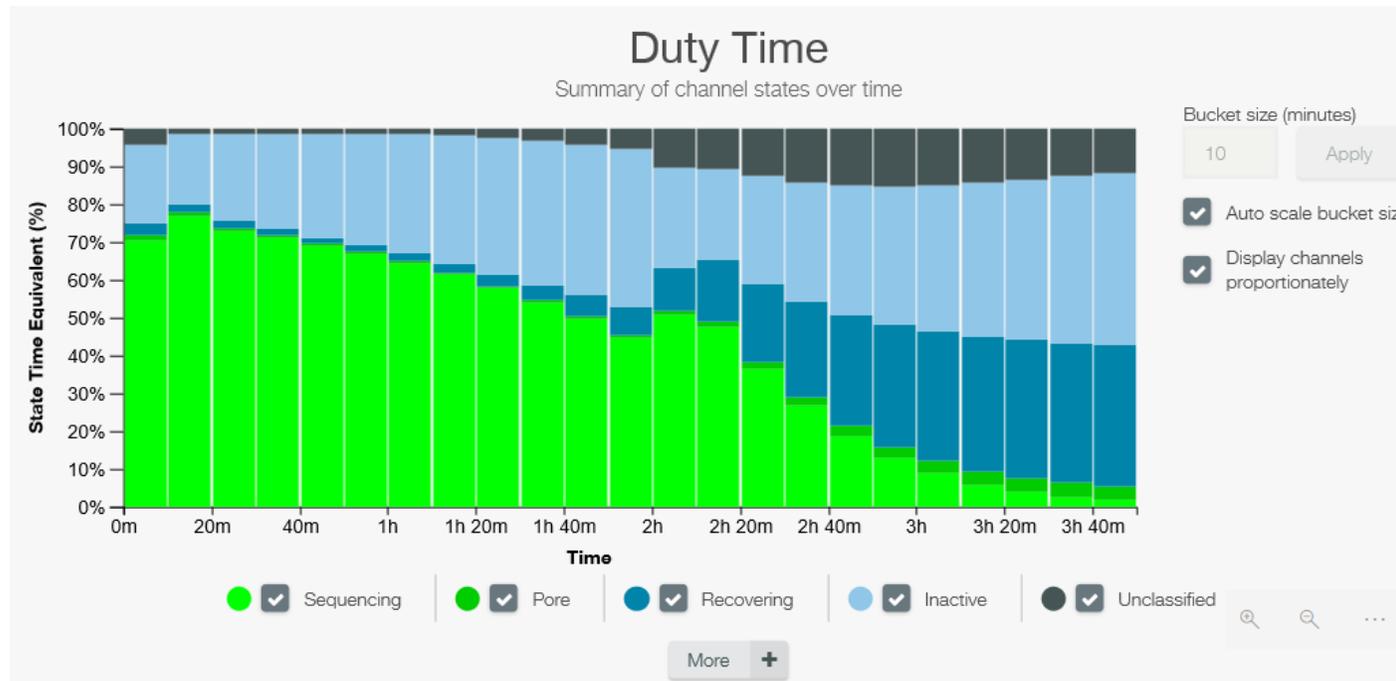
Display failed  
 Display unclassified  
 Hide zero values

Reset selection

A-Z  
 Z-A  
 Ascending  
 Descending

See which barcodes  
have been detected and  
how many reads each

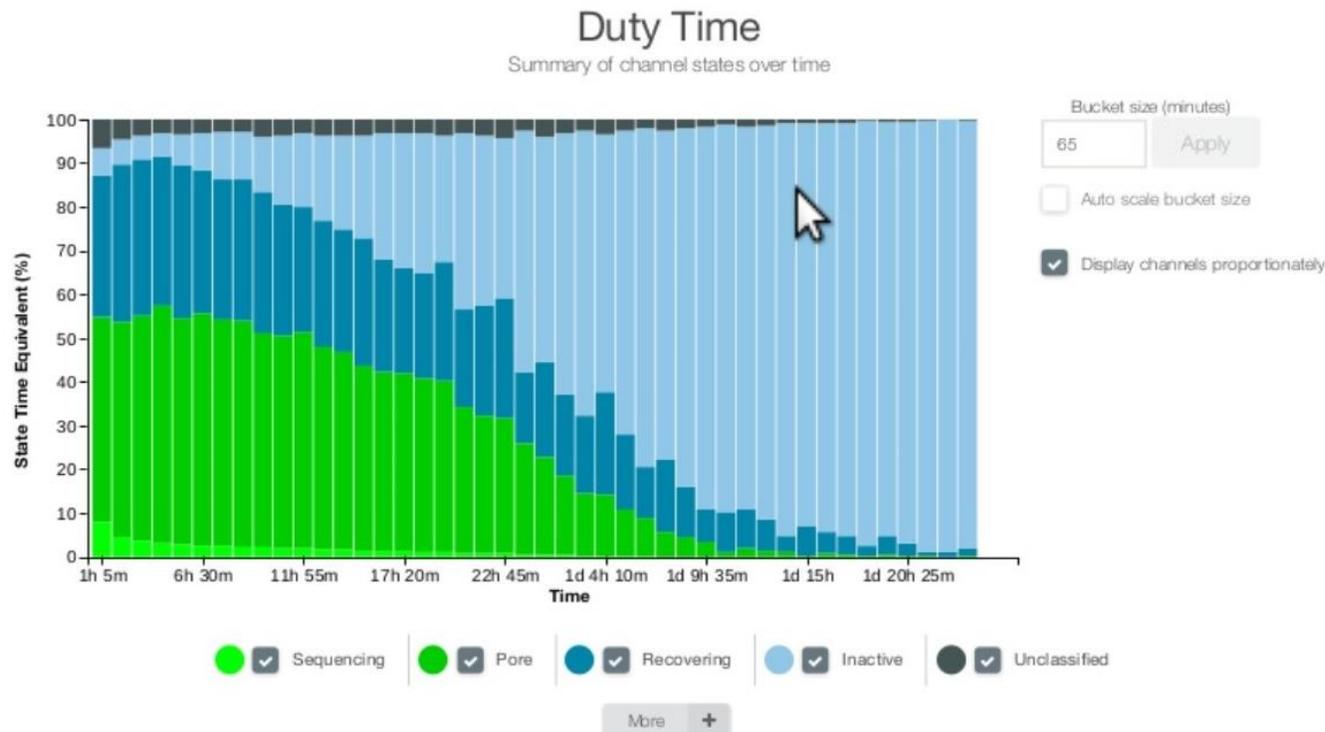
# Examples of a bad start



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:

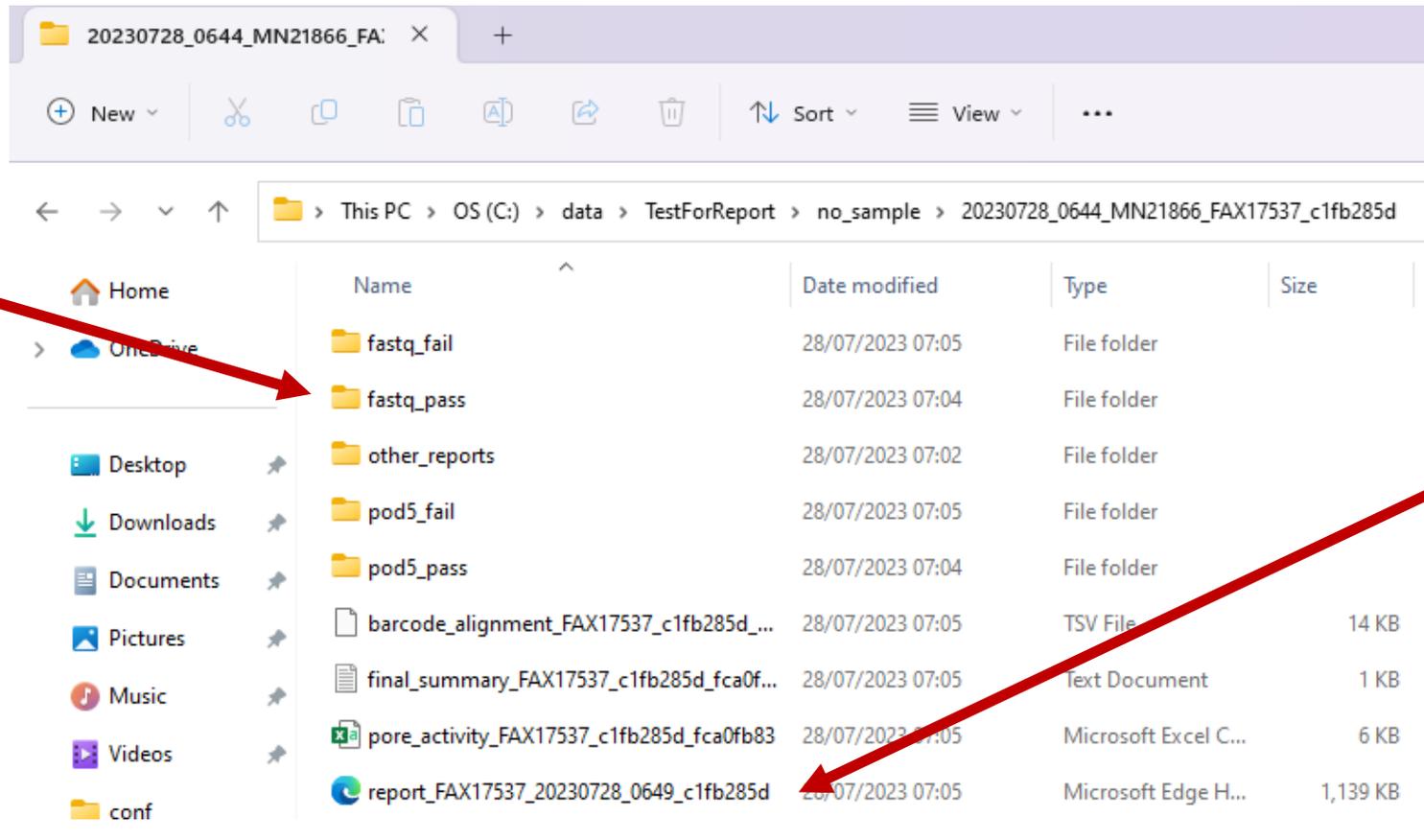
- Not loading enough sample onto the flowcell
- Failed adaptation for sequencing

## 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



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

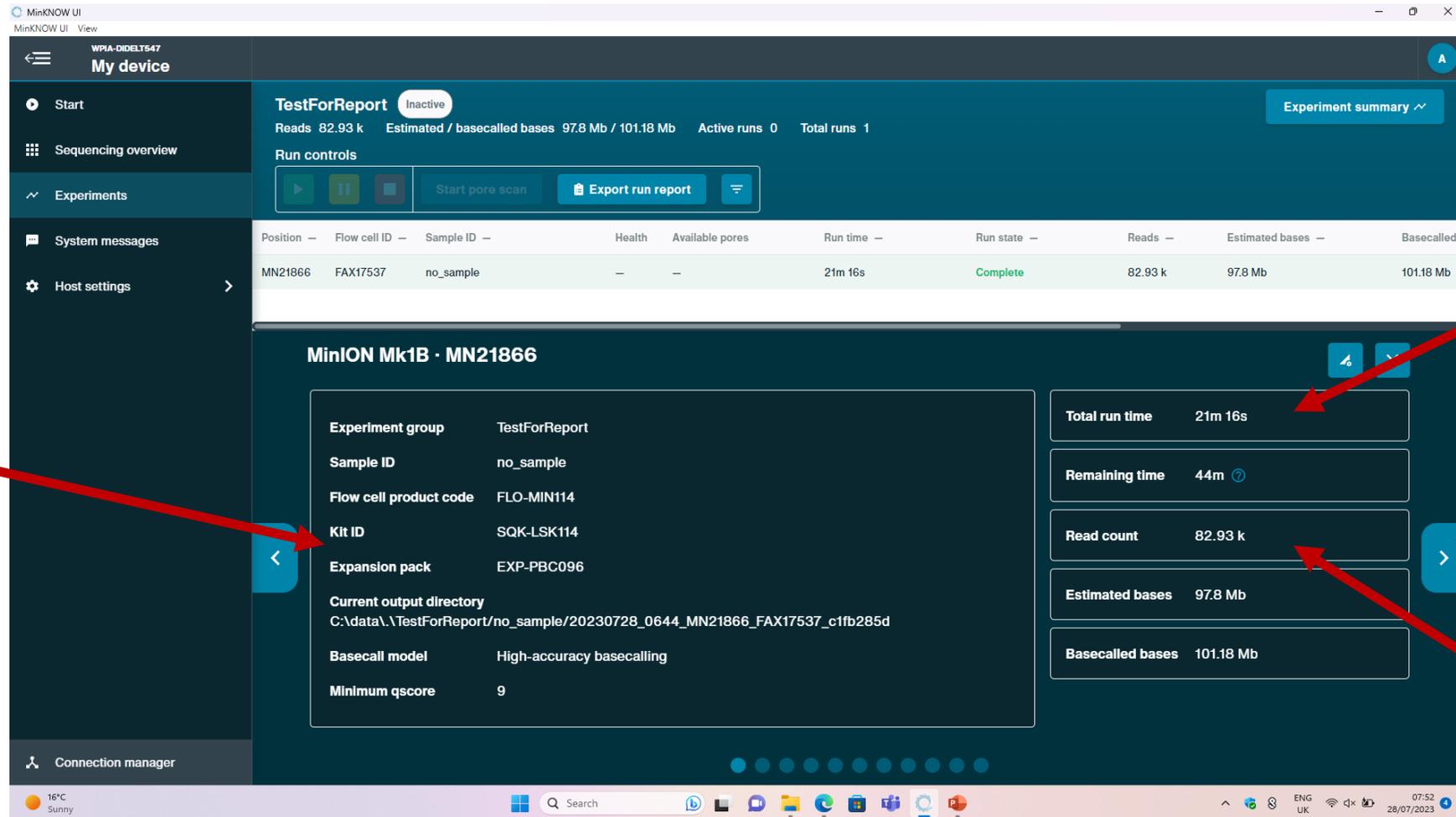
The screenshot shows the MinKNOW UI interface. The top navigation bar includes 'My device' and 'Experiments (13)'. Below this, there's a search bar and a list of experiments: 'TestForReport', 'groupe\_1\_ddns2', 'groupe\_2\_ddns2', and 'Test4\_FAX17330'. The 'TestForReport' experiment is selected and shows 'Inactive' status. It has 82.93 k reads, 97.8 Mb estimated / 101.18 Mb basecalled bases, 0 active runs, and 1 total run. The 'Run controls' section includes buttons for 'Start pore scan', 'Export run report', and a filter icon. Below this is a table of runs:

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

A red arrow points to the 'no\_sample' entry in the 'Sample ID' column. A green box highlights the 'Complete' status in the 'Run state' column.

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

### 3. Check your run performance



The screenshot shows the MinKNOW UI interface for a MinION Mk1B device (MN21866). The main display area is titled 'TestForReport' and shows the following metrics:

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

The 'Run controls' section includes buttons for 'Start pore scan' and 'Export run report'. Below this is a table of runs:

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

The 'MinION Mk1B · MN21866' section displays the following settings:

- Experiment group: TestForReport
- Sample ID: no\_sample
- Flow cell product code: FLO-MIN114
- Kit ID: SQK-LSK114
- Expansion pack: EXP-PBC096
- Current output directory: C:\data\TestForReport\no\_sample\20230728\_0644\_MN21866\_FAX17537\_c1fb285d
- Basecall model: High-accuracy basecalling
- Minimum qscore: 9

On the right side, a summary of key metrics is shown:

- Total run time: 21m 16s
- Remaining time: 44m
- Read count: 82.93 k
- Estimated bases: 97.8 Mb
- Basecalled bases: 101.18 Mb

The interface also includes a left sidebar with navigation options (Start, Sequencing overview, Experiments, System messages, Host settings) and a bottom status bar showing connection manager, weather (16°C Sunny), and system time (07:52 28/07/2023).

Your run settings

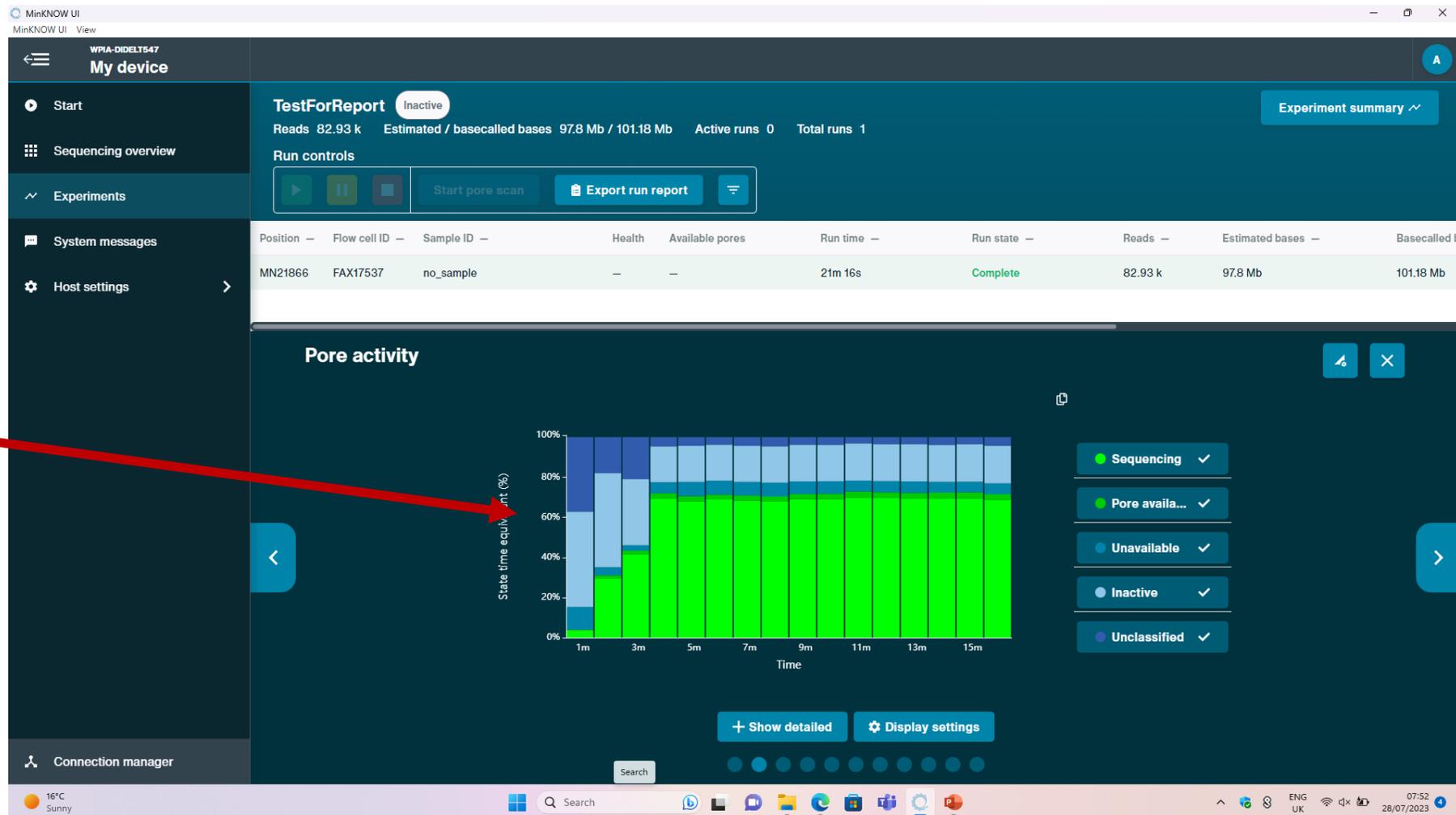
Confirm your run length (4 hours for routine stool testing by DDNS)

Click for more figures

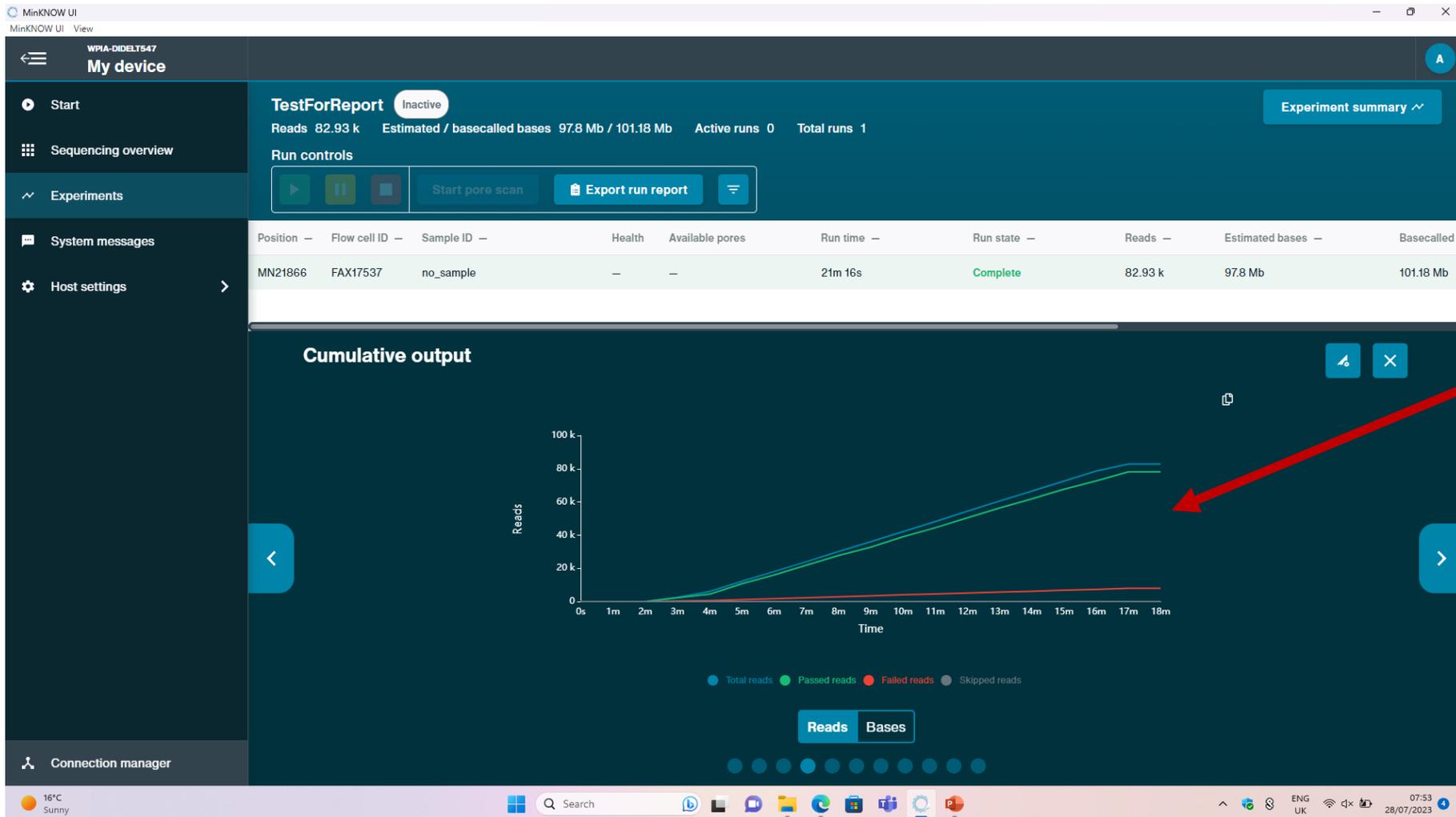
The total number of sequencing reads generated

## 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.

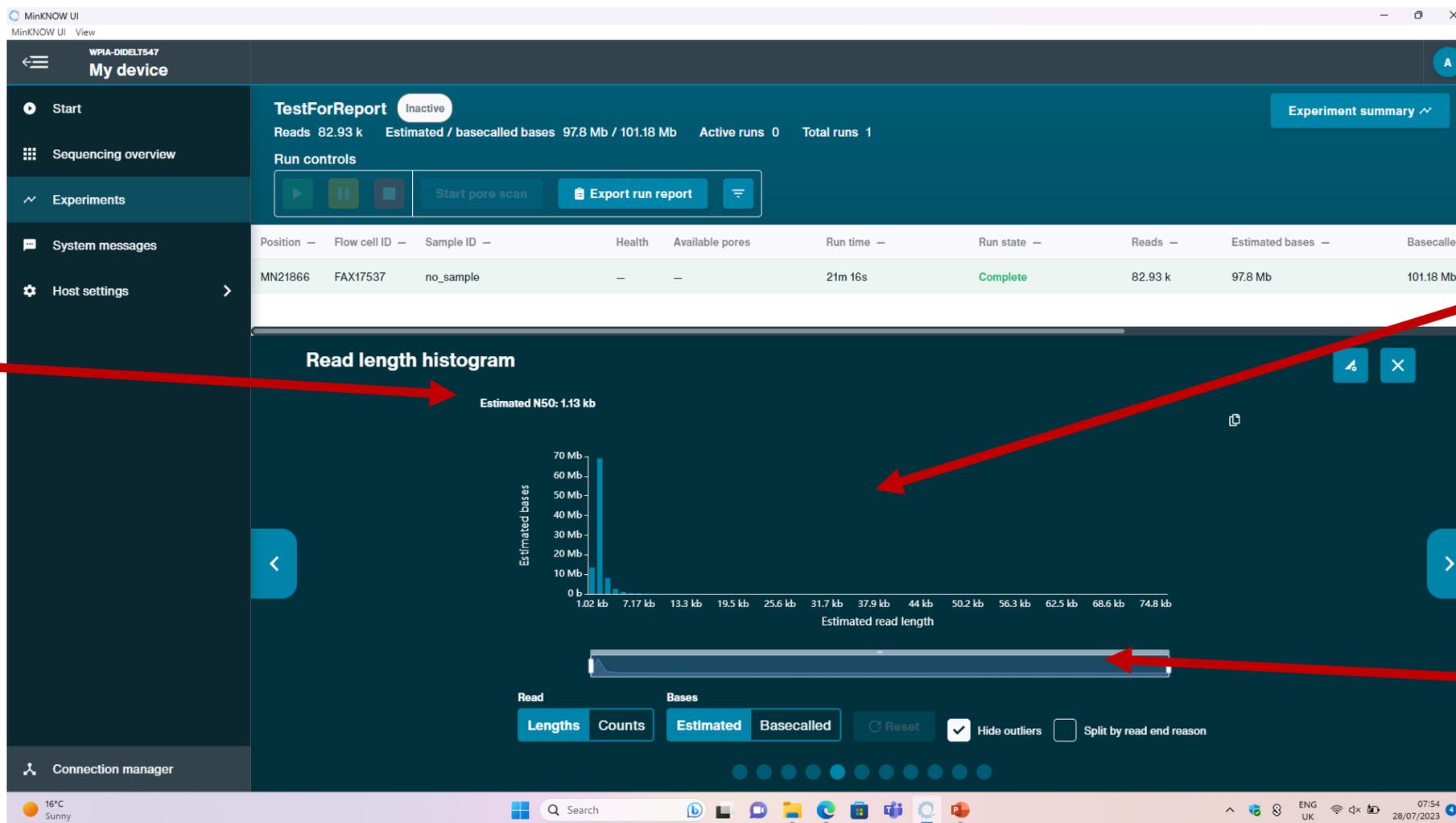


## 5. Check the quality of your data



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

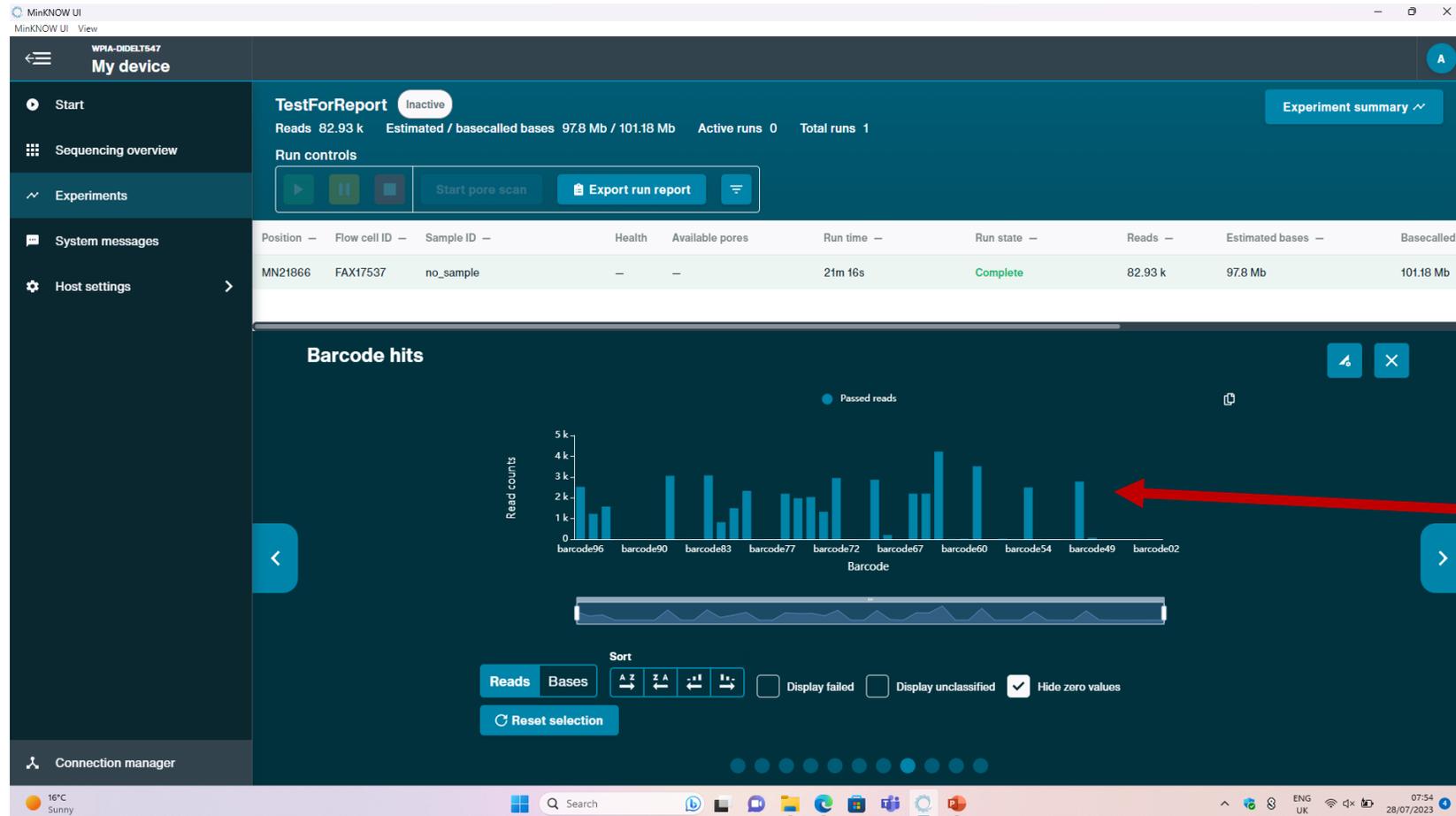


Check the average read length (N50)- for VP1 by DDNS this should be ~ 1.2 kb

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



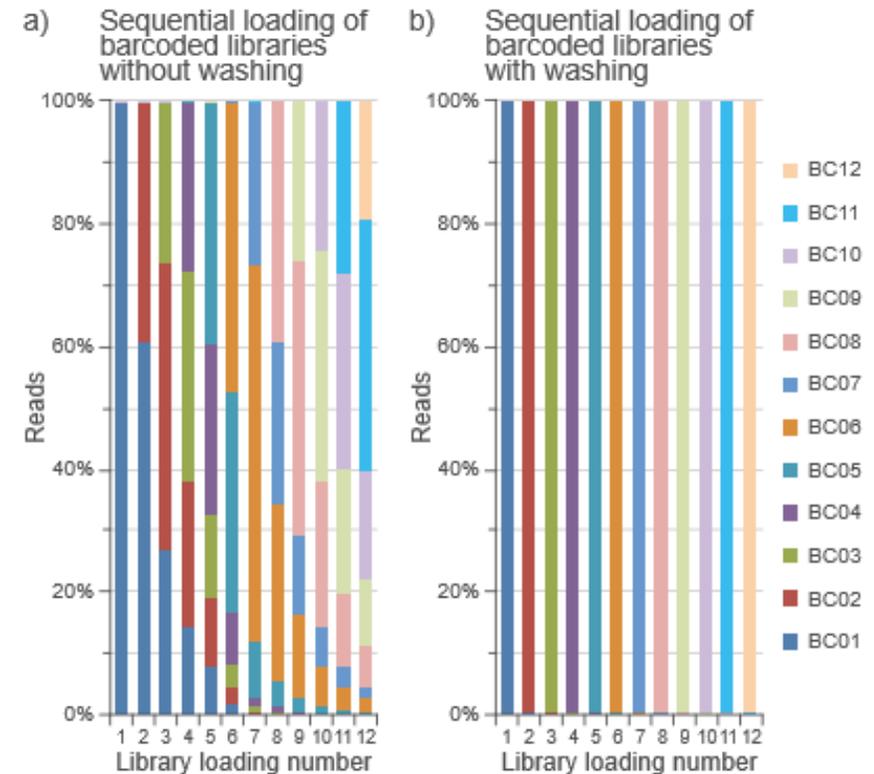
If your data has not been demultiplexed you can go to “Start”, “Analysis” and “Barcoding” in MinKNOW to perform the demultiplexing.

## 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

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### Affiliations

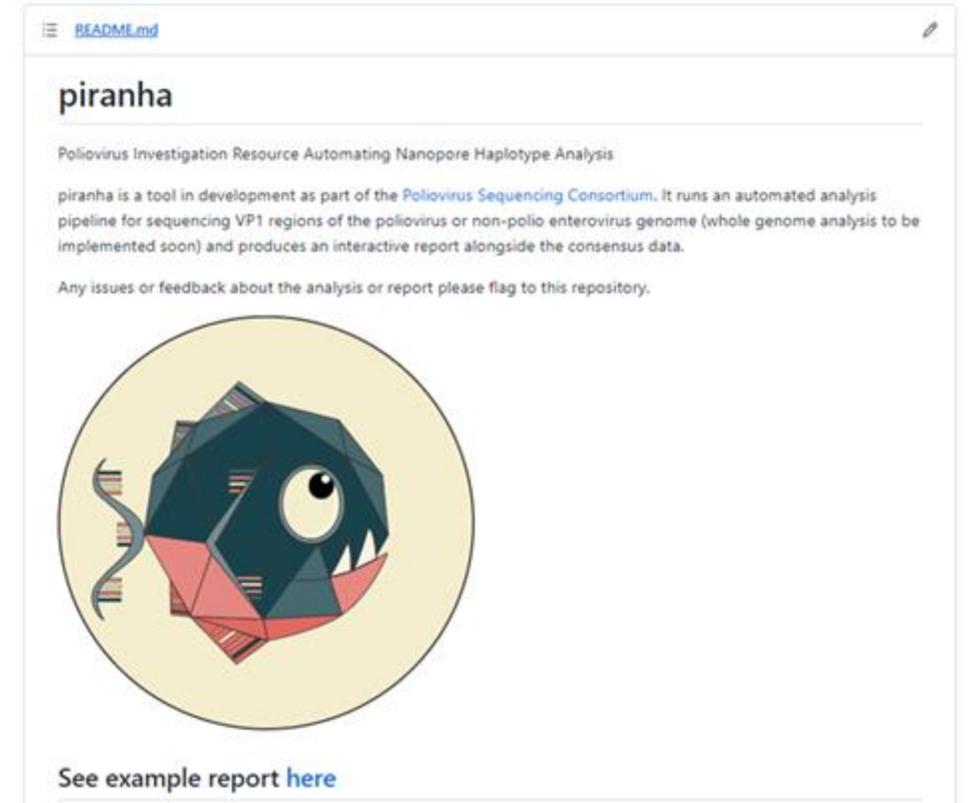
1. Institute of Ecology & Evolution, University of Edinburgh, United Kingdom
2. Department of Infectious Disease Epidemiology, Imperial College London, United Kingdom
3. Division of Virology, National Institute for Biological Standards and Control (NIBSC), Hertz, United Kingdom
4. Department of Virology,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



README.md

## piranha

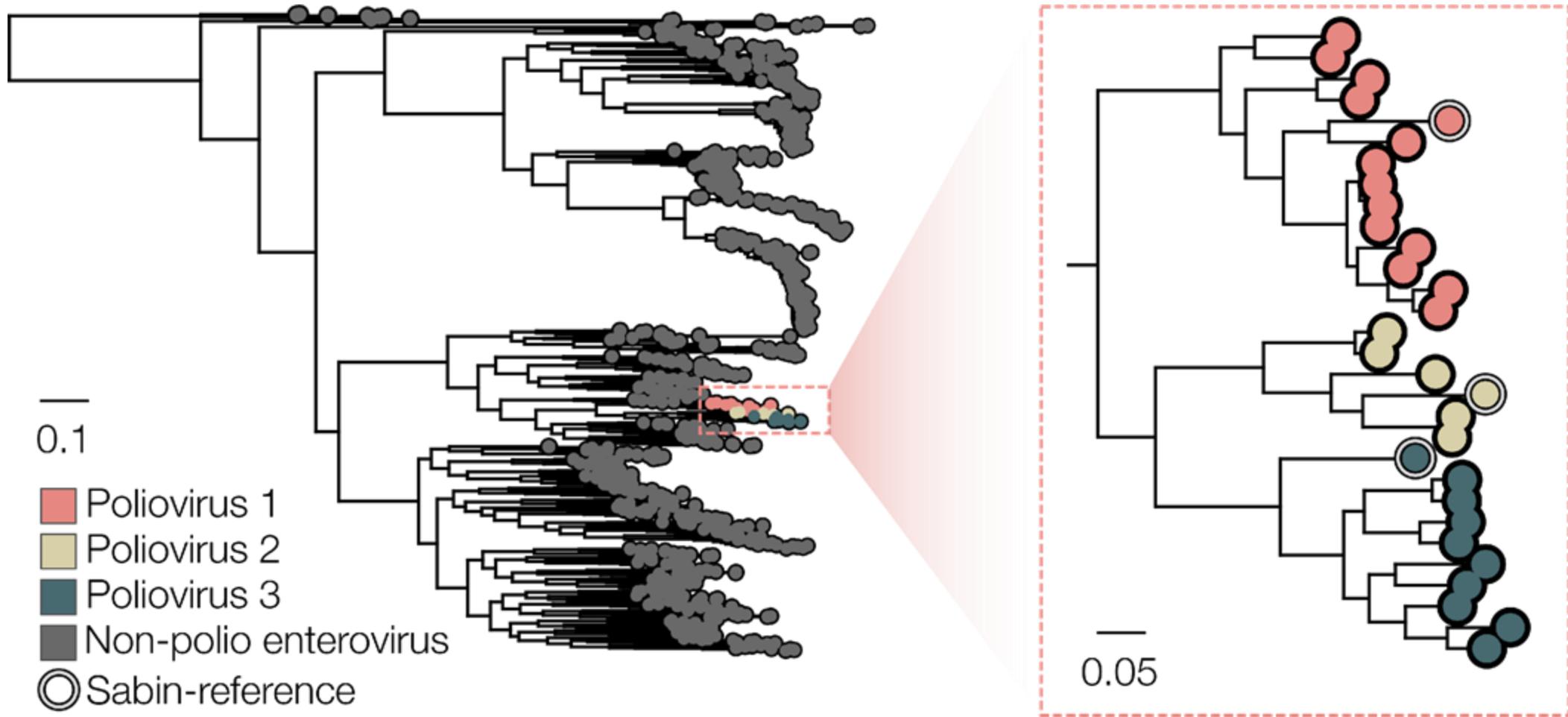
Poliovirus Investigation Resource Automating Nanopore Haplotype Analysis

piranha is a tool in development as part of the [Poliovirus Sequencing Consortium](#). It runs an automated analysis pipeline for sequencing VP1 regions of the poliovirus or non-polio enterovirus genome (whole genome analysis to be implemented soon) and produces an interactive report alongside the consensus data.

Any issues or feedback about the analysis or report please flag to this repository.

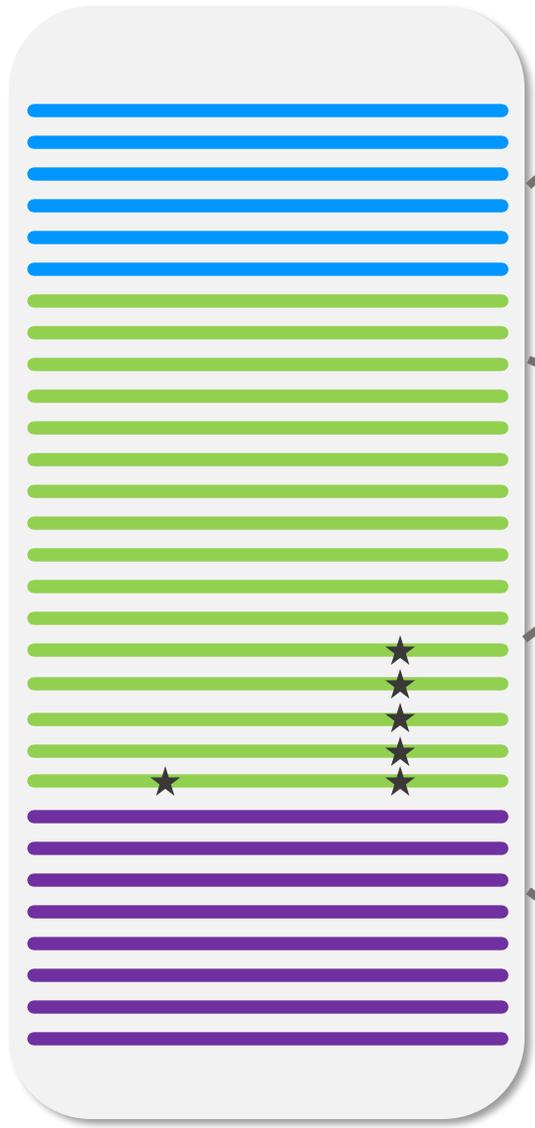
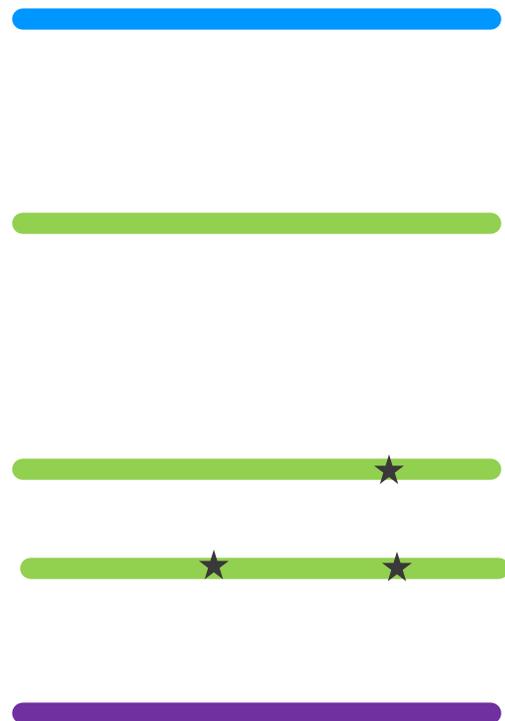


See example report [here](#)

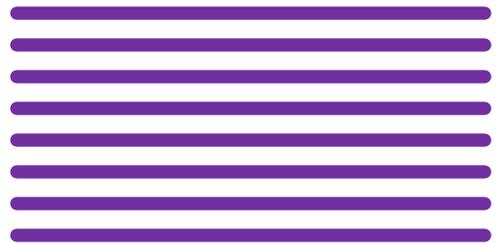
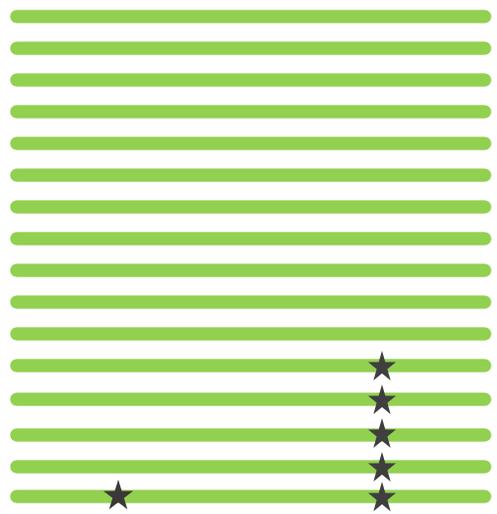


Background database: 959 VP1 sequences

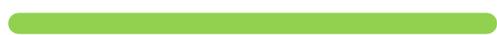
Mixed Sample



Reference groups



Consensus/ haplotype

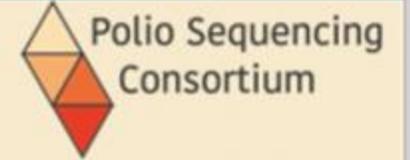




## Piranha

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

About



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

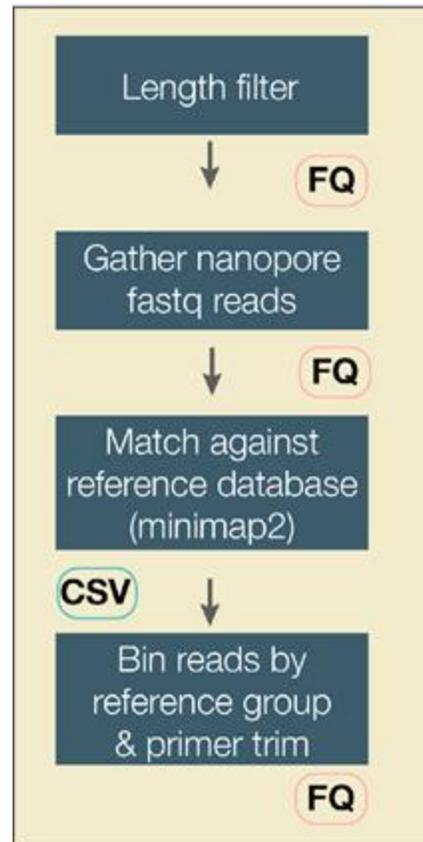
Piranha is the primary analysis pipeline for the DDNS polio detection platform.

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

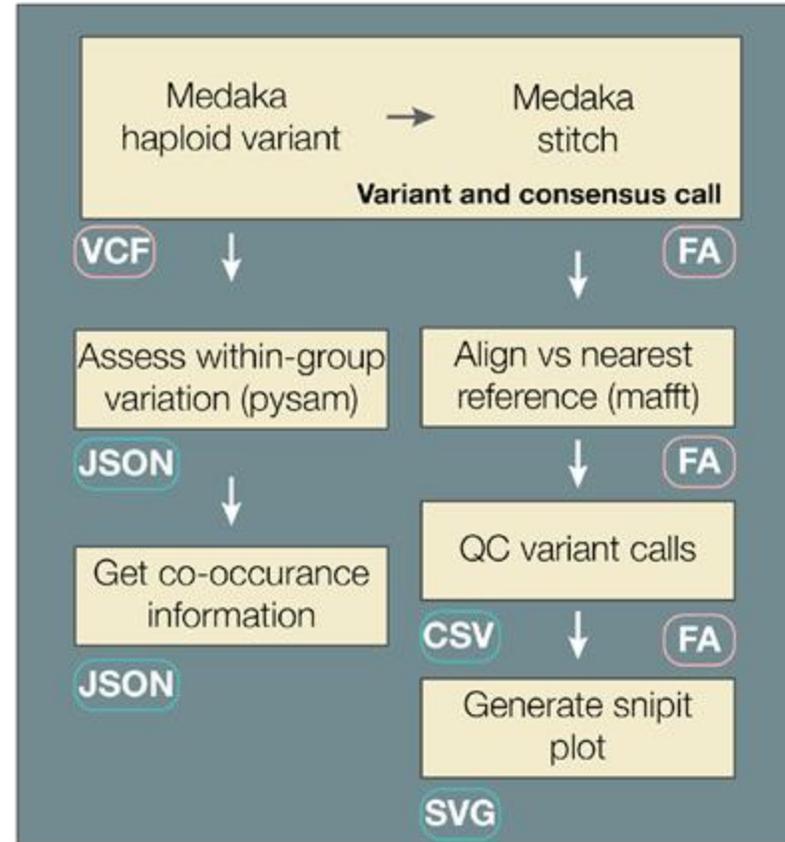
Continue

# Workflow schema

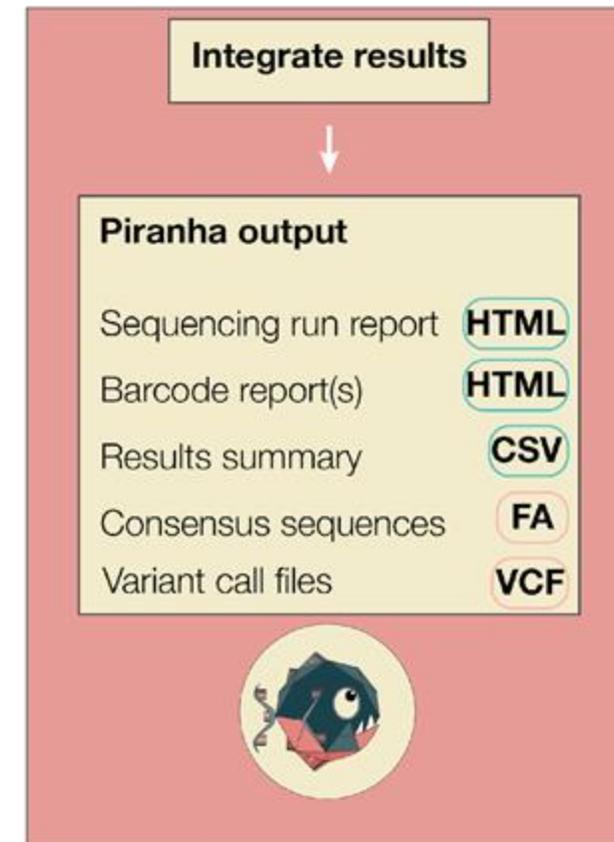
## A. Barcode analysis



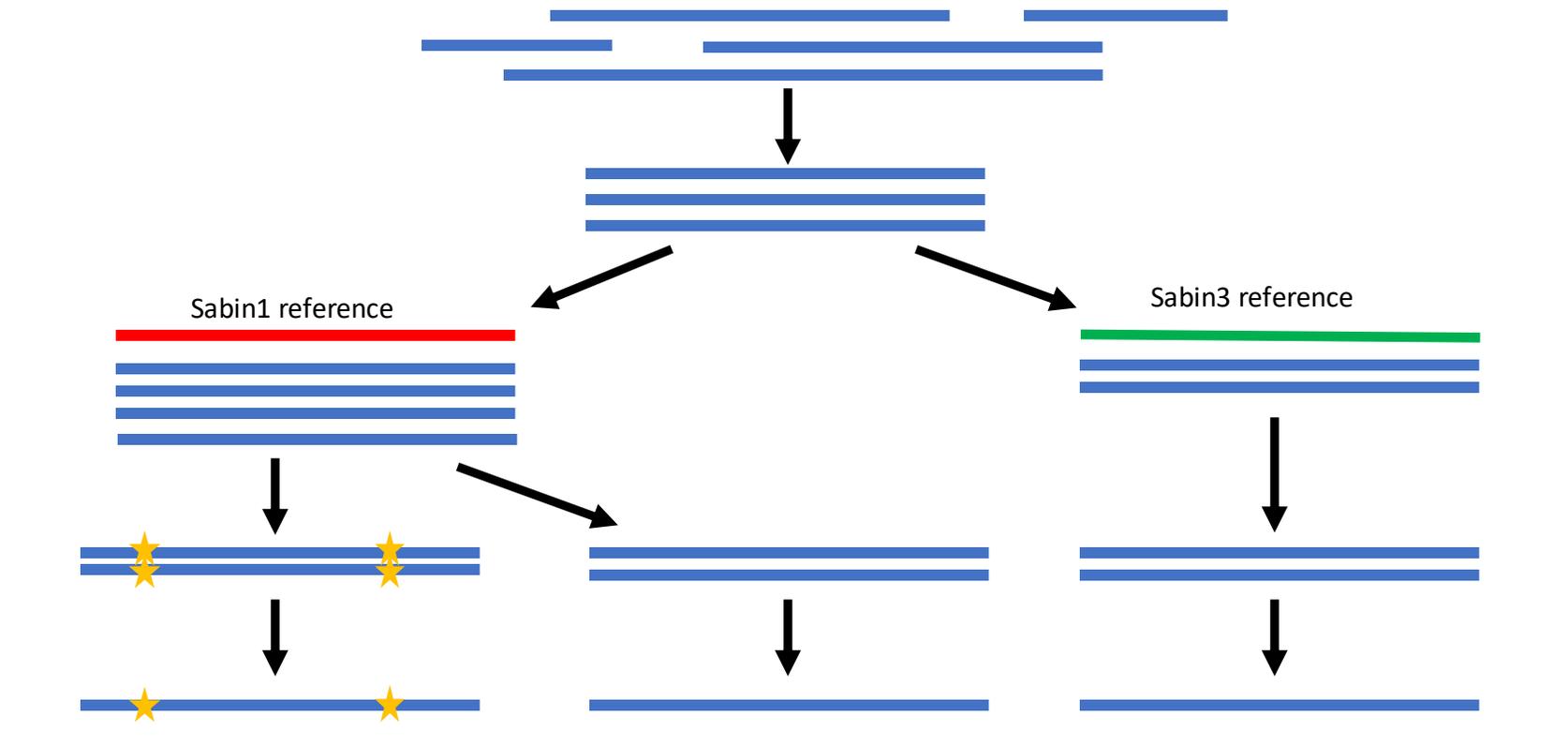
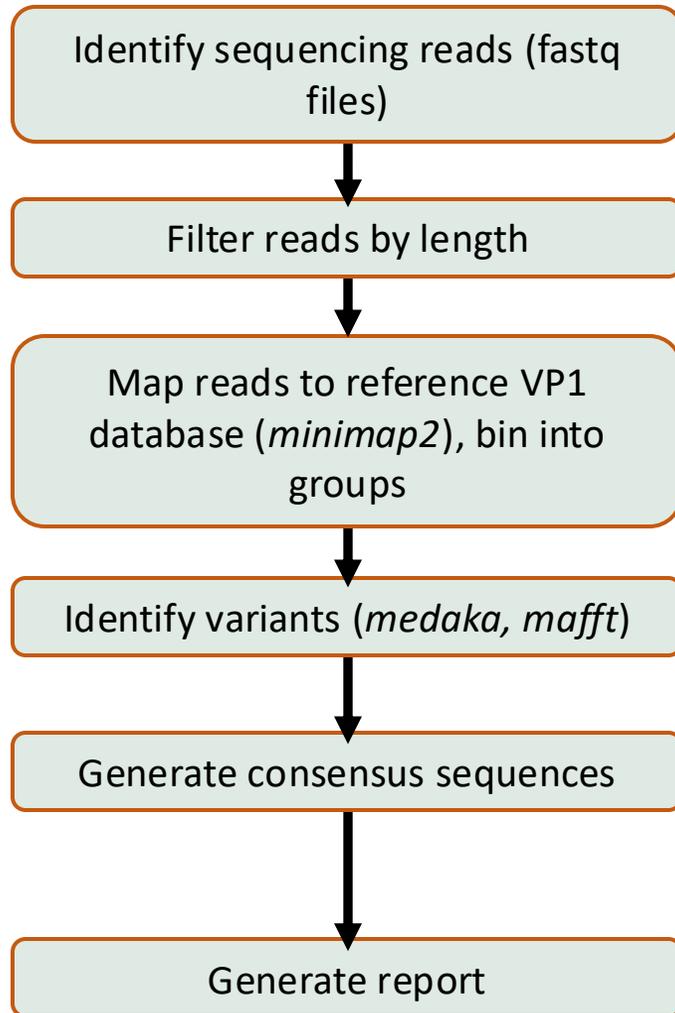
## B. Reference group analysis



## C. Results reporting

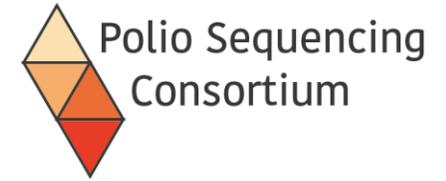


# Graphic workflow



Sample	Barcode	Sample classification	Reference Group	Number of Mutations	Sequence (VP1)
Sample01	barcode01	Sabin-like	Sabin1-related	2	<a href="#">Download FASTA</a>
Sample01	barcode01	Sabin-like	Sabin1-related	0	<a href="#">Download FASTA</a>
Sample02	barcode02	Sabin-like	Sabin3-related	0	<a href="#">Download FASTA</a>

# Example report



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

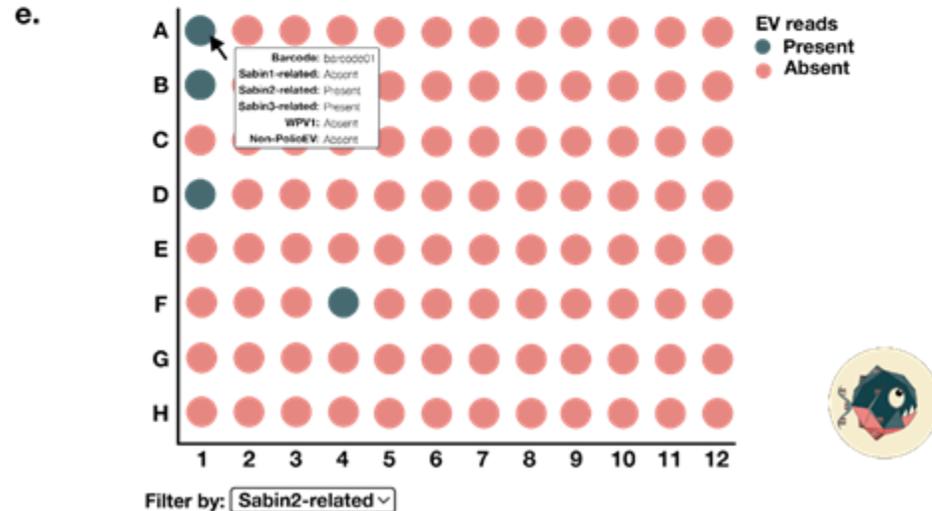
Showing 5 of 96 entries

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

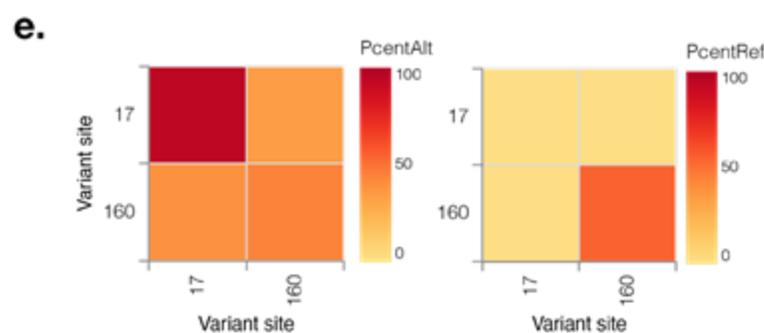
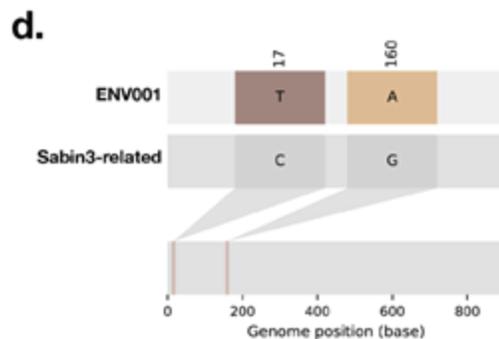
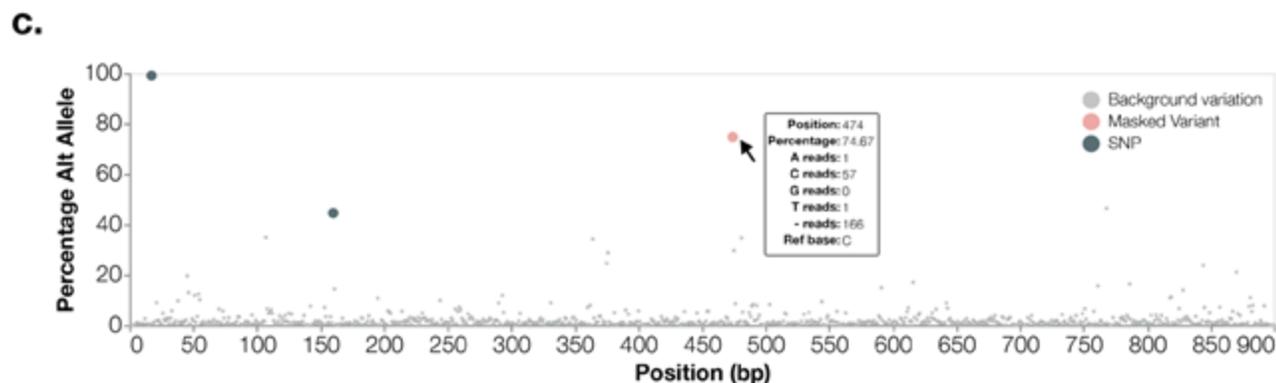


**a.**

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
TGGCCCGGCGCATTCCAAGGAGGTACTGCACTCACTGCAGTCGAGACTGGAGCCACCAATCCTCTGACACCATCCGACACAGTTCAAA
CGCGCCACGTAGTCCAACGACGCAGCAGGTCAGAGTCCACAATAGAATCATTCTTGGCAGCGGGGGCGTGCCTGCTATTATTGAGGTGG
ACAATGAACAACCAACCCCGGGCACAGAACTATTTGCCATGTGGCGCATTACATACAAGATACAGTGCAGTTGCGCCGTAAGTTGGA
```



# 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	postive_control	barcode05		E01		
7	negative_ext	barcode06		F01		
8	negative RTPCR	barcode07		G01		

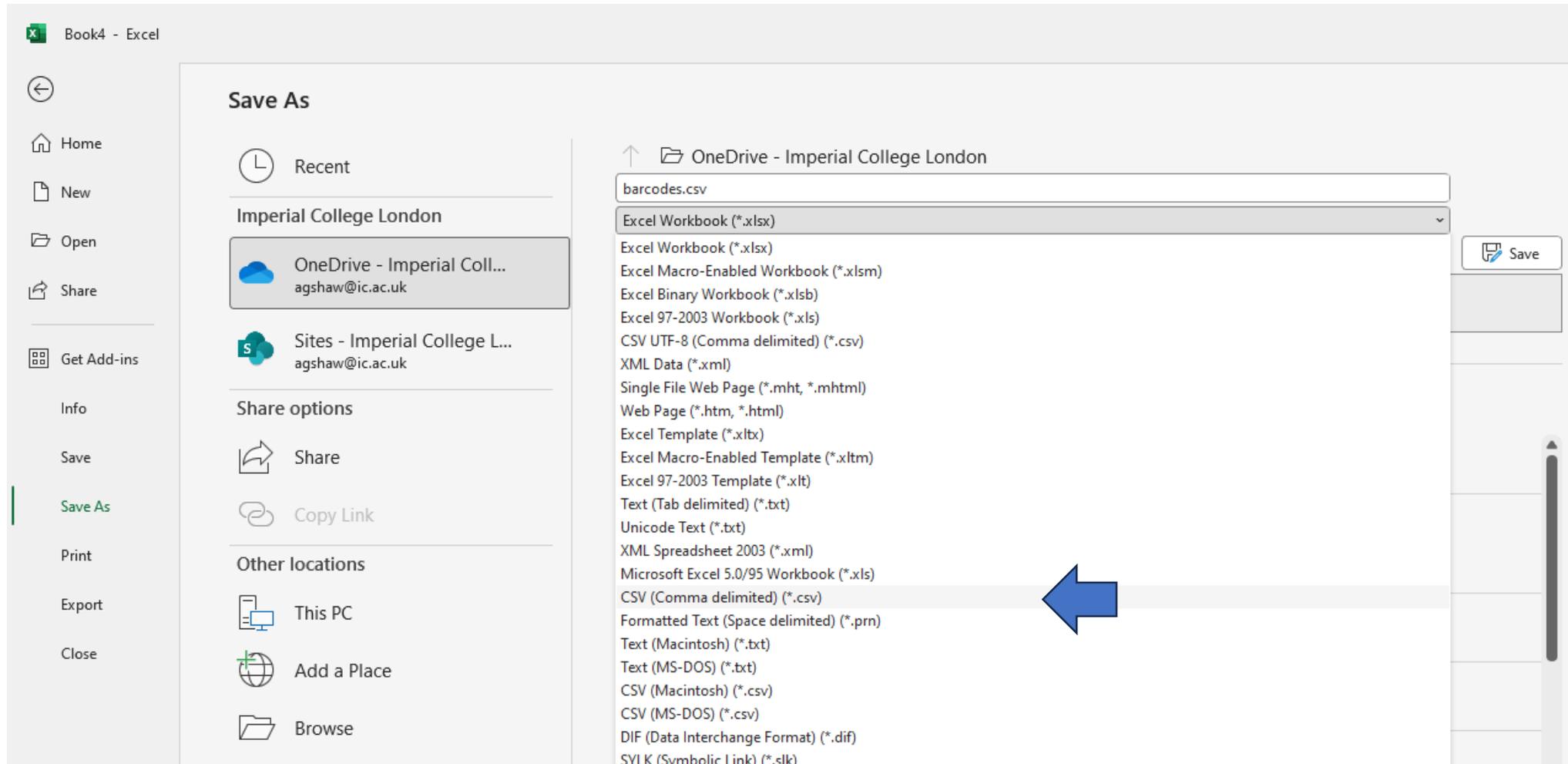
Links your barcodes and your samples.

Use the headers “sample” and “barcode”

Barcodes must be entered as “barcode##”

More metadata columns can be added

# Saving as a .csv from excel



The screenshot shows the 'Save As' dialog box in Microsoft Excel. The file name is 'barcodes.csv'. The file format dropdown menu is open, showing various options. A blue arrow points to the 'CSV (Comma delimited) (\*.csv)' option, which is highlighted. The 'Save' button is visible in the top right corner.

**Save As**

Recent

Imperial College London

- OneDrive - Imperial Coll...  
agshaw@ic.ac.uk
- Sites - Imperial College L...  
agshaw@ic.ac.uk

Share options

- Share
- Copy Link

Other locations

- This PC
- Add a Place
- Browse

OneDrive - Imperial College London

barcodes.csv

Excel Workbook (\*.xlsx)

- Excel Workbook (\*.xlsx)
- Excel Macro-Enabled Workbook (\*.xlsm)
- Excel Binary Workbook (\*.xlsb)
- Excel 97-2003 Workbook (\*.xls)
- CSV UTF-8 (Comma delimited) (\*.csv)
- XML Data (\*.xml)
- Single File Web Page (\*.mht, \*.mhtml)
- Web Page (\*.htm, \*.html)
- Excel Template (\*.xltx)
- Excel Macro-Enabled Template (\*.xltm)
- Excel 97-2003 Template (\*.xlt)
- Text (Tab delimited) (\*.txt)
- Unicode Text (\*.txt)
- XML Spreadsheet 2003 (\*.xml)
- Microsoft Excel 5.0/95 Workbook (\*.xls)
- CSV (Comma delimited) (\*.csv)
- Formatted Text (Space delimited) (\*.prn)
- Text (Macintosh) (\*.txt)
- Text (MS-DOS) (\*.txt)
- CSV (Macintosh) (\*.csv)
- CSV (MS-DOS) (\*.csv)
- DIF (Data Interchange Format) (\*.dif)
- SYLK (Symbolic Link) (\*.slk)

Save

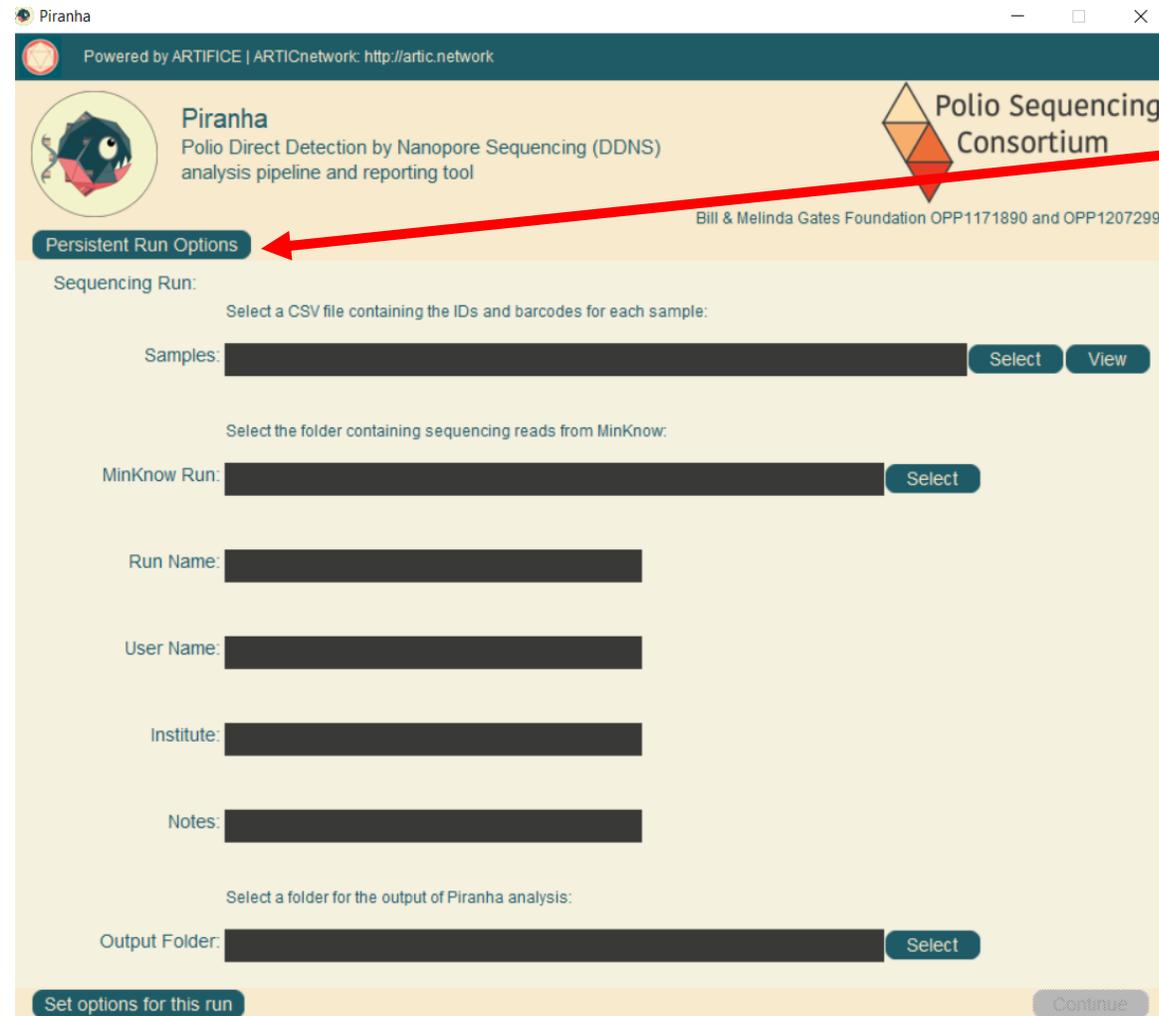
## Appearance of a .csv file

---

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



The screenshot shows the Piranha GUI interface. At the top, it says "Powered by ARTIFICE | ARTICnetwork: http://artic.network". Below that, there is a header with the Piranha logo and the text "Piranha Polio Direct Detection by Nanopore Sequencing (DDNS) analysis pipeline and reporting tool". To the right of the header is the Polio Sequencing Consortium logo and the text "Polio Sequencing Consortium". Below the header, there is a section titled "Persistent Run Options" with a red arrow pointing to it. The main content area is titled "Sequencing Run:" and contains several input fields and buttons:

- Sequencing Run:** Select a CSV file containing the IDs and barcodes for each sample:  
Samples:
- MinKnow Run:** Select the folder containing sequencing reads from MinKnow:  
MinKnow Run:
- Run Name:**
- User Name:**
- Institute:**
- Notes:**
- Output Folder:** Select a folder for the output of Piranha analysis:  
Output Folder:

At the bottom left, there is a button "Set options for this run" and at the bottom right, there is a button "Continue".

- Click here to set up the phylogenetic module

# Running PiranhaGUI

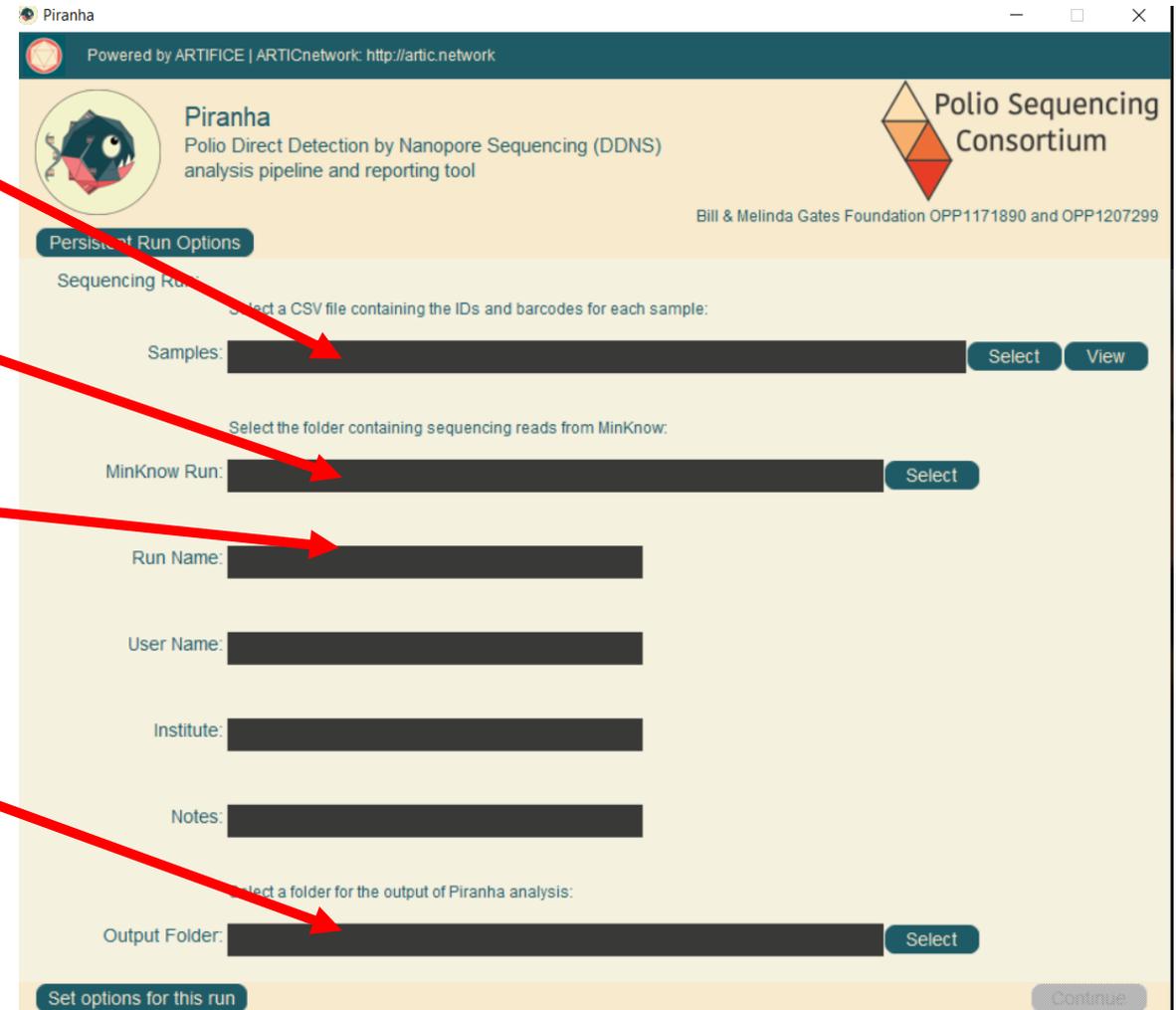


The screenshot shows the Piranha GUI interface. At the top, there is a header bar with the Piranha logo and the text 'Piranha'. Below this, there is a button labeled 'Disable Piranha Phylogenetics module'. A red arrow points from this button to the right. Below the button, there is a text input field for 'Supplementary directory for phylogenetic module:' followed by a 'Select' button and a 'Clear' button. A red arrow points from the 'Select' button to the right. Below the text input field, there are several form fields: 'User Name:', 'Institute:', 'Output Folder:' (with a 'Select' button), 'Orientation:' (with a dropdown menu set to 'vertical'), 'Protocol:' (with a dropdown menu set to 'stool'), 'Positive Control:' (with the text 'positive'), and 'Negative Control:' (with the text 'negative'). At the bottom right, there is a 'Continue' button.

- 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”



Piranha  
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

**Persistent Run Options**

Sequencing Run Options

Select a CSV file containing the IDs and barcodes for each sample:

Samples:

Select the folder containing sequencing reads from MinKnow:

MinKnow Run:

Run Name:

User Name:

Institute:

Notes:

Select a folder for the output of Piranha analysis:

Output Folder:

- Set run options. For DDNS stool testing:

Minimum length - 1000 bp

Maximum length - 1300 bp

Minimum read depth – 50

Minimum read percentage – 0

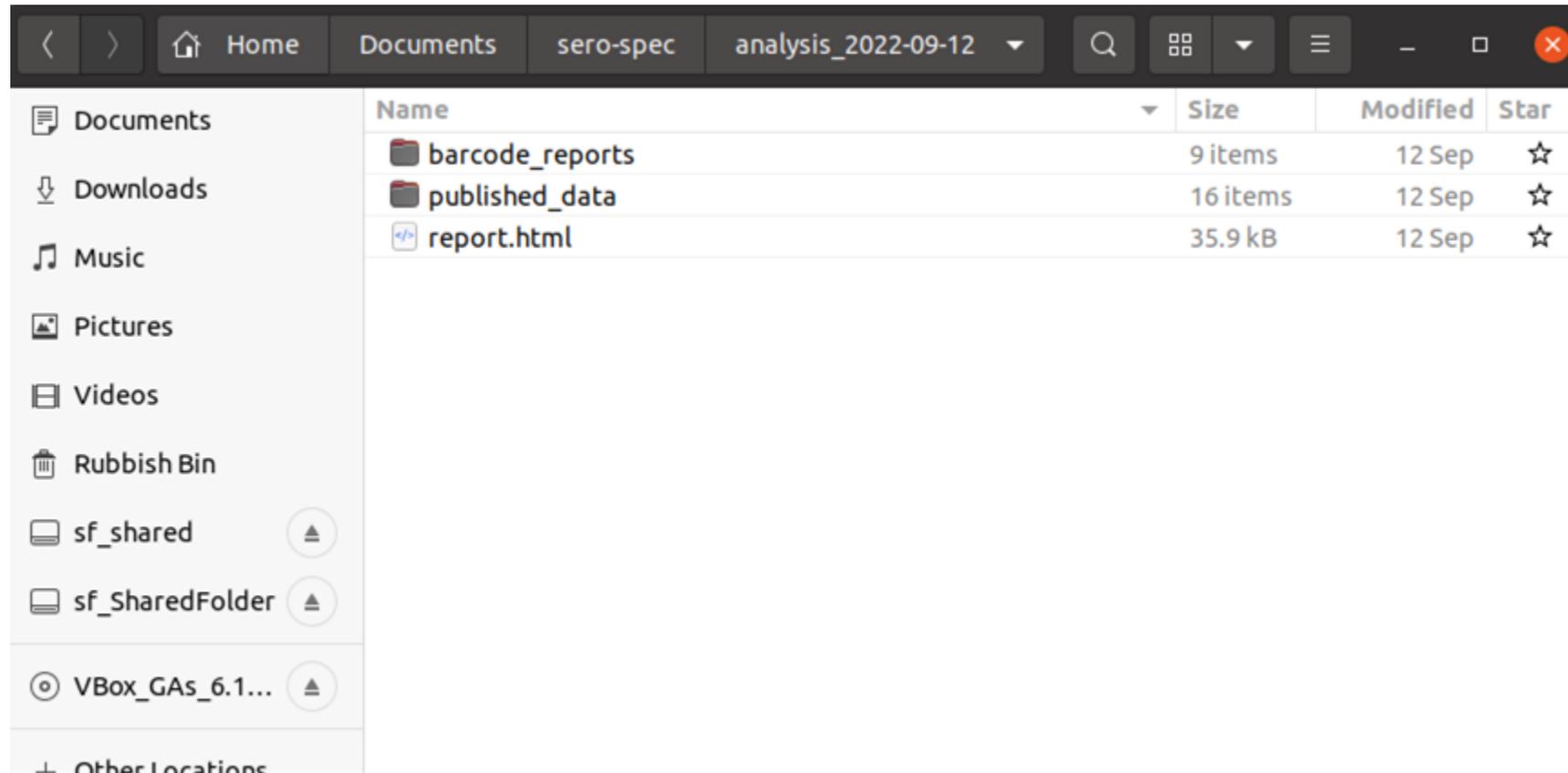
(see QC document)

- Start analysis

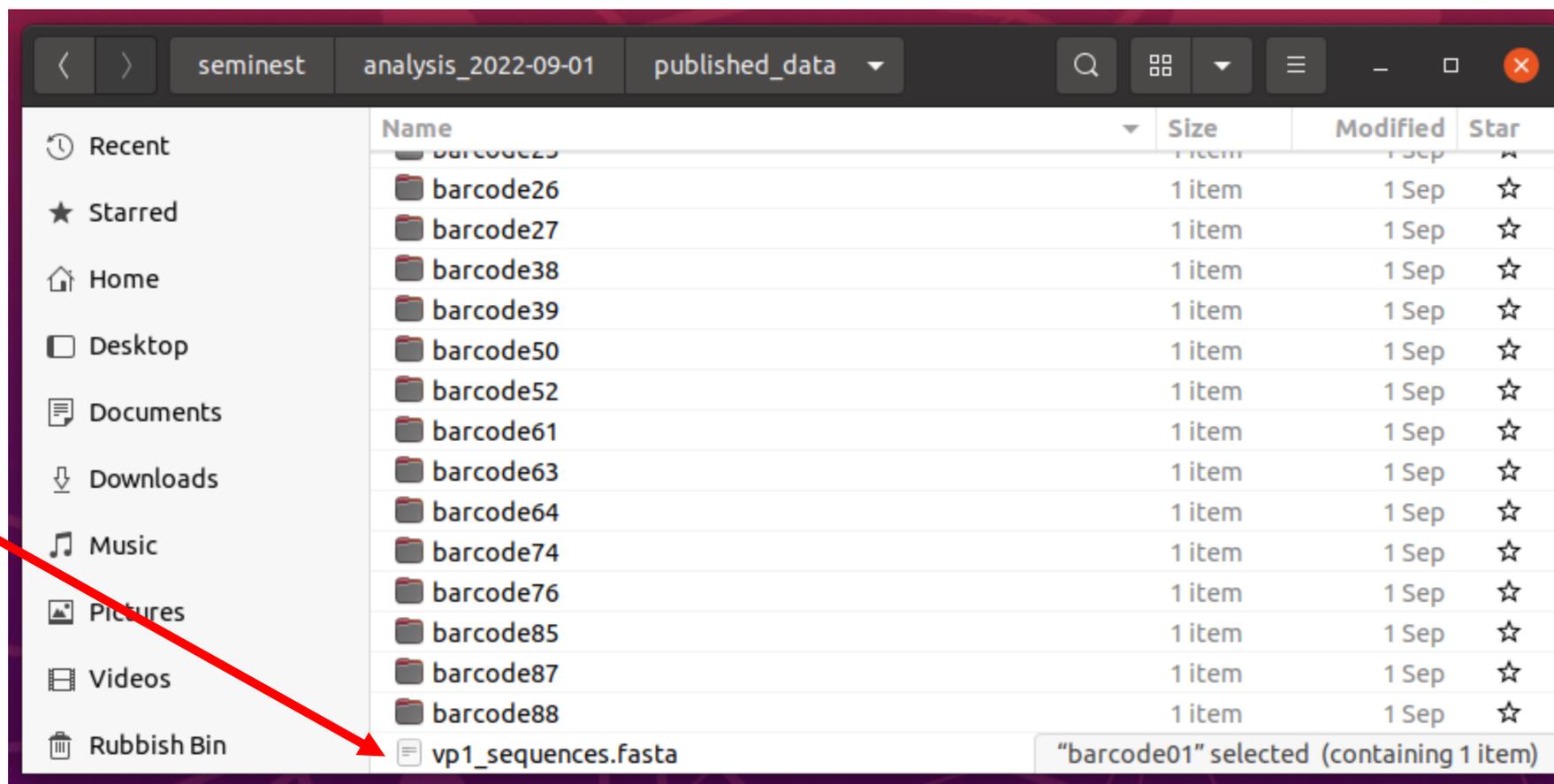
The screenshot displays the Piranha GUI interface. At the top, it says "piranhaGUI" and "Powered by ARTIFICE | ARTICnetwork: http://artic.network". Below this, there's a header for "Piranha Polio Direct Detection by Nanopore Sequencing (DDNS) analysis pipeline and reporting tool" with the Polio Sequencing Consortium logo and funding information from the Bill & Melinda Gates Foundation. The main control area includes buttons for "Edit run", "Piranha Options" (with "Analysis Threads: 5" set), "Stop Analysis" (highlighted with a red box), "Open Output", and "Open Report". The status indicates "Analysis is running". The "PIRANHA OUTPUT" section shows a stylized logo and text: "\*\*\*\* Poliovirus Investigation Resource \*\*\*\*", "\*\*\*\* Automating Nanopore Haplotype Analysis \*\*\*\*", "1.0.10", and credits to the University of Edinburgh, Imperial College London & NIBSC, supported by the ARTIC Network Wellcome Trust Collaborators Award (206298/Z/17/Z) and the Bill and Melinda Gates Foundation (OPP1207299). A footer at the bottom mentions "Wellcome Trust Award 206298/Z/17/Z". Two red arrows point from the text on the left to the "Piranha Options" and "Stop Analysis" buttons.

# PIRANHA - output

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

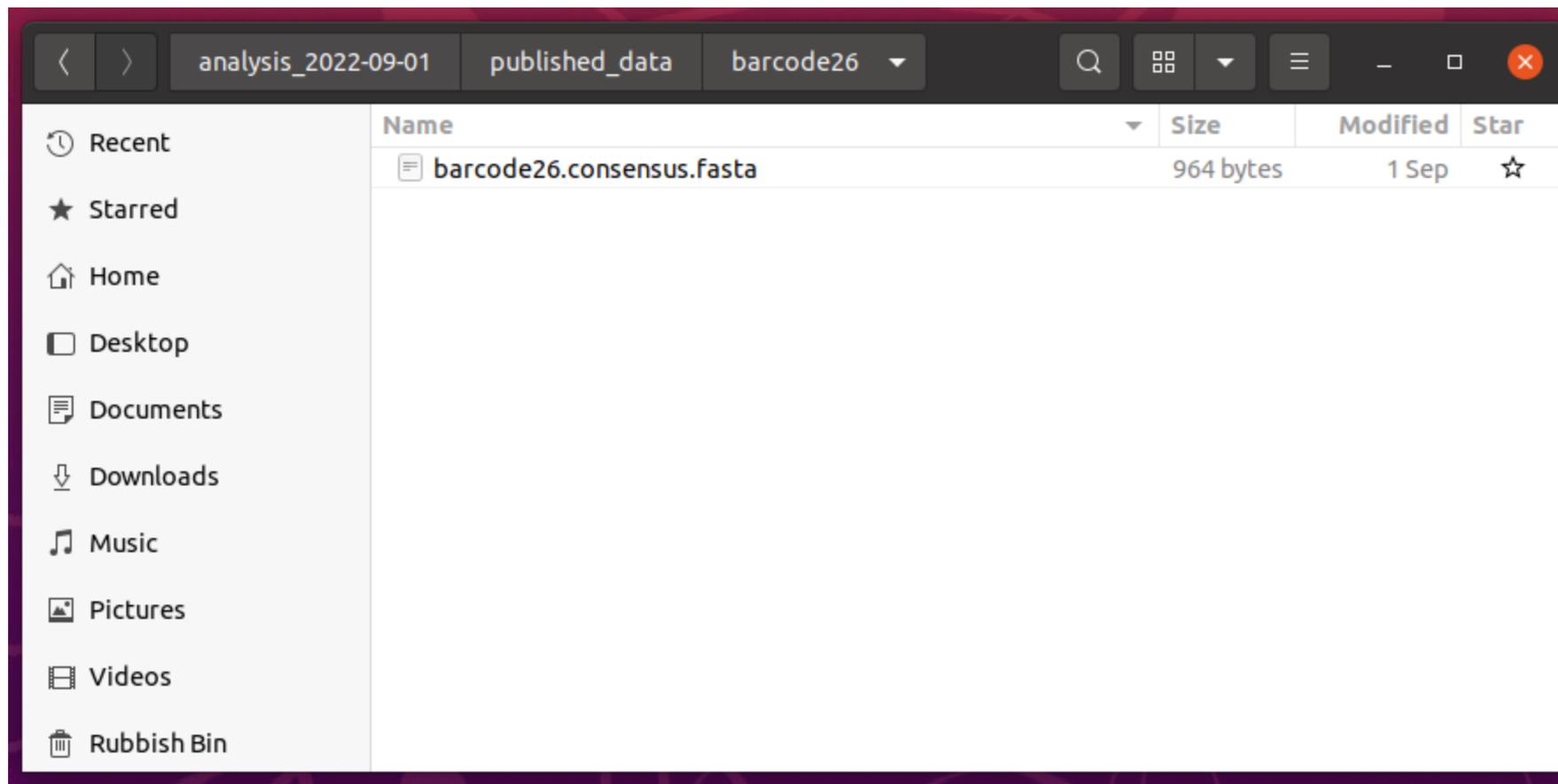


# PIRANHA fasta output



All VP1  
sequences  
for the  
sequencing  
run

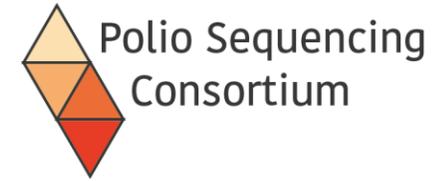
# PIRANHA fasta output



# Sample contents overview

Search:

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



Navigate to individual sample reports

Download fasta files of consensus sequences

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
sample12	barcode37	0	1134	4	0	1188	0	370	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
GGGTTAGGTCAGATGCTTGAAAGCATGATTGACAACACAGTCCGTGAAACGGTGGGGGCGGCAACGTCTAGAGACGCTCTCCAAACACTGAAGCCAGTGGACCAGCACACTCCAAGGAAATTCGGCACTCACCGCAGTGGAACTGGGGCCAC
AAATCCACTAGTCCCTTCTGATACAGTGCAAACAGACATGTTGTACAACATAGGTCAAGGTCAGAGTCTAGCATAGAGTCTTTCTTCGCGCGGGGTGCATGCGTGGCCATTTTAACCGTGGATAACTCAGCTTCCACCAAGAATAAGGATAAGC
TATTTACAGTGTGGAAGATCACTTATAAAGATACTGTCCAGTTACGGAGGAAATTGGAGTCTTTCACCTATTCTAGATTTGATATGGAATTTACCTTTGTGGTTACTGCAATTTCACTGAGACTAACAATGGGCATGCCTTAAATCAAGTGTAC
CAAATTATGTACGTACCACCAGGCGCTCCAGTGCCCGAGAAATGGGACGACTACACATGGCAAACCTCATCAAATCCATCAATCTTTTACACCTACGGAACAGCTCCAGCCCGGATCTCGGTACCGTATGTTGGTATTTTGAACGCCTATTCA
CTTTTACGACGGTTTTTCCAAAGTACCCTGAAGGACCAGTCGGCAGCAC TAGGTGACTCCCTCTATGGTG CAGCATCTCAAATGACTTCGGTATTTTGGCTGTTAGAGTAGTCAATGATCACAACCCGACCAAGGTCACCTCCAAAATCAGAG
```

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

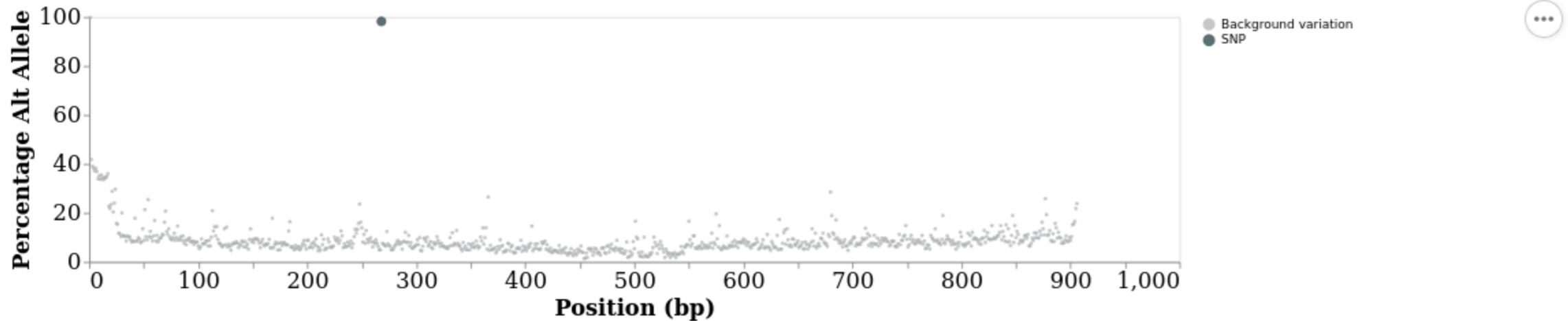
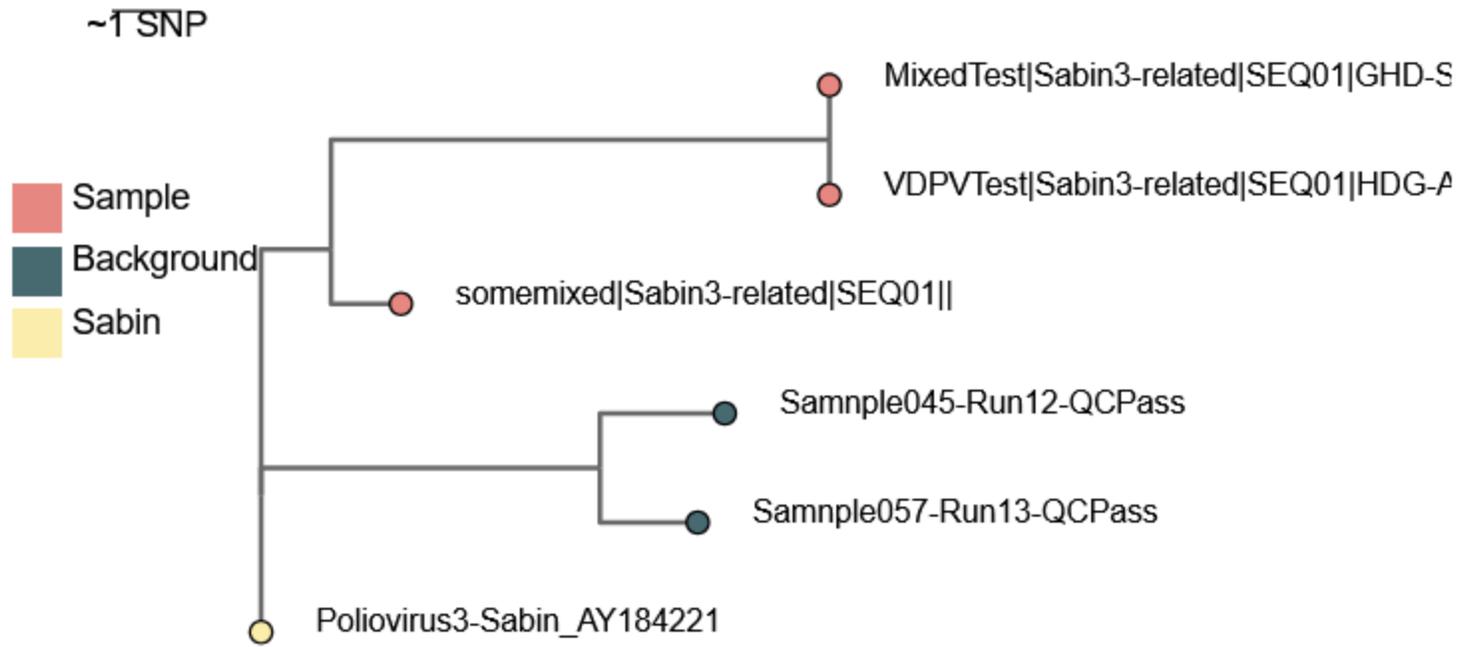


Figure 1 | Variation (errors + mutations) across Sabin1-Related reference in sample08



- 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.

<

**Figure 3** | Sabin3-related phylogeny

# 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)

# Things to look out for

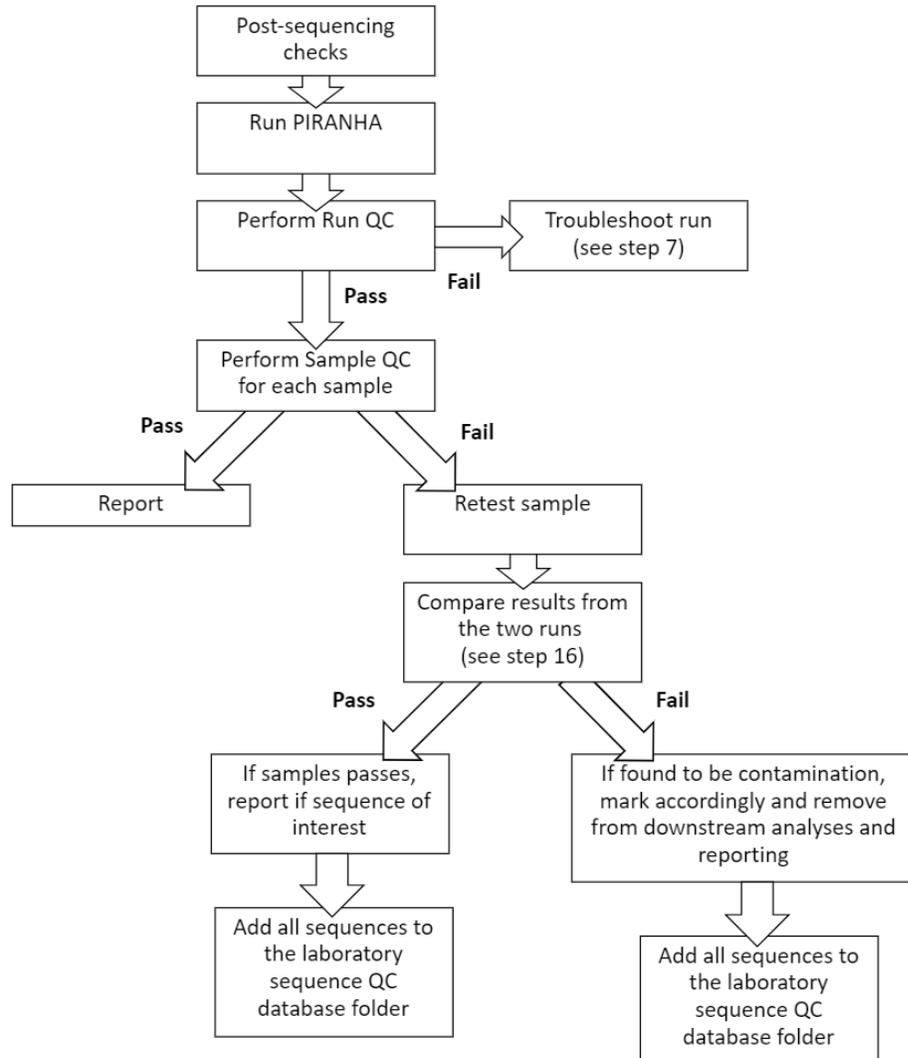
---

- 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_barcode.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

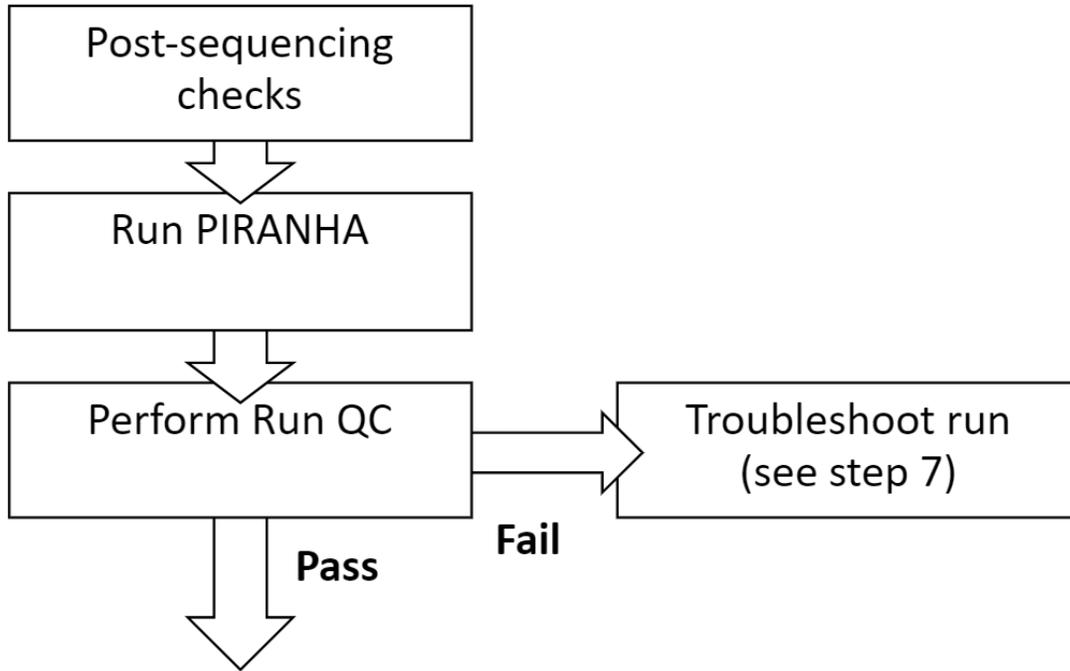
Presentation and discussion

# 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 fluoremeter.

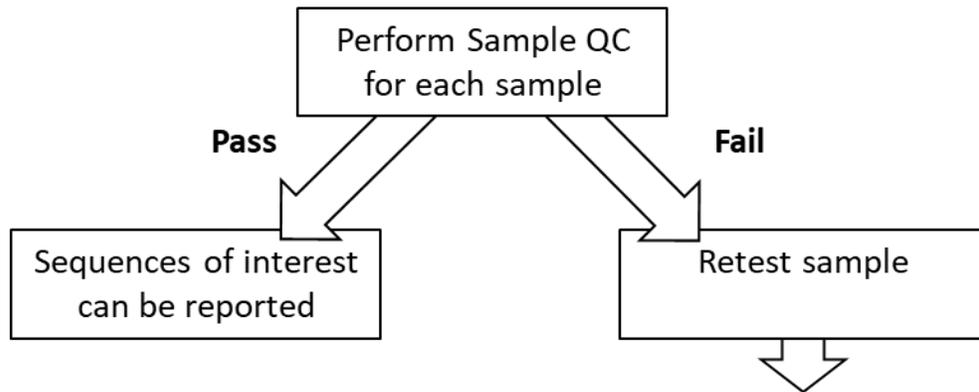
Check that you are ligating the correct adaptor (LA) and are using the short fragment buffer (SFB) during library preparation.

## **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

- 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.

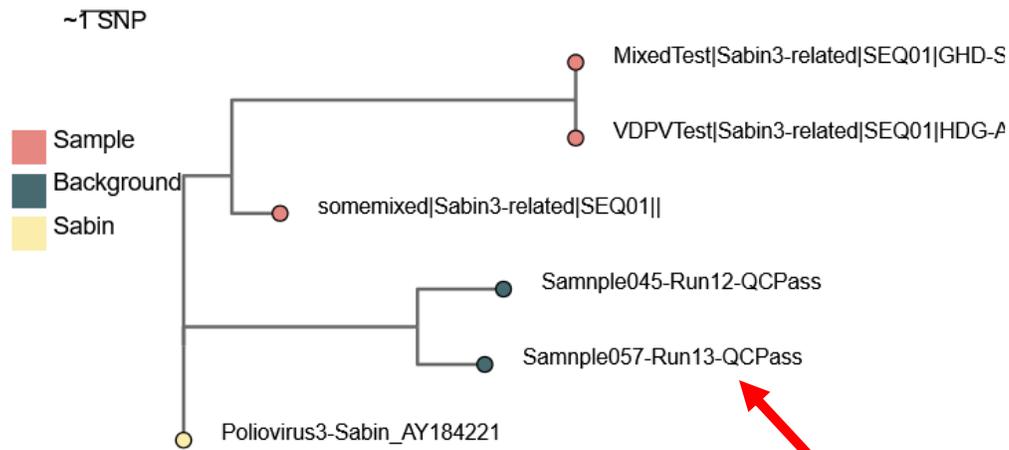
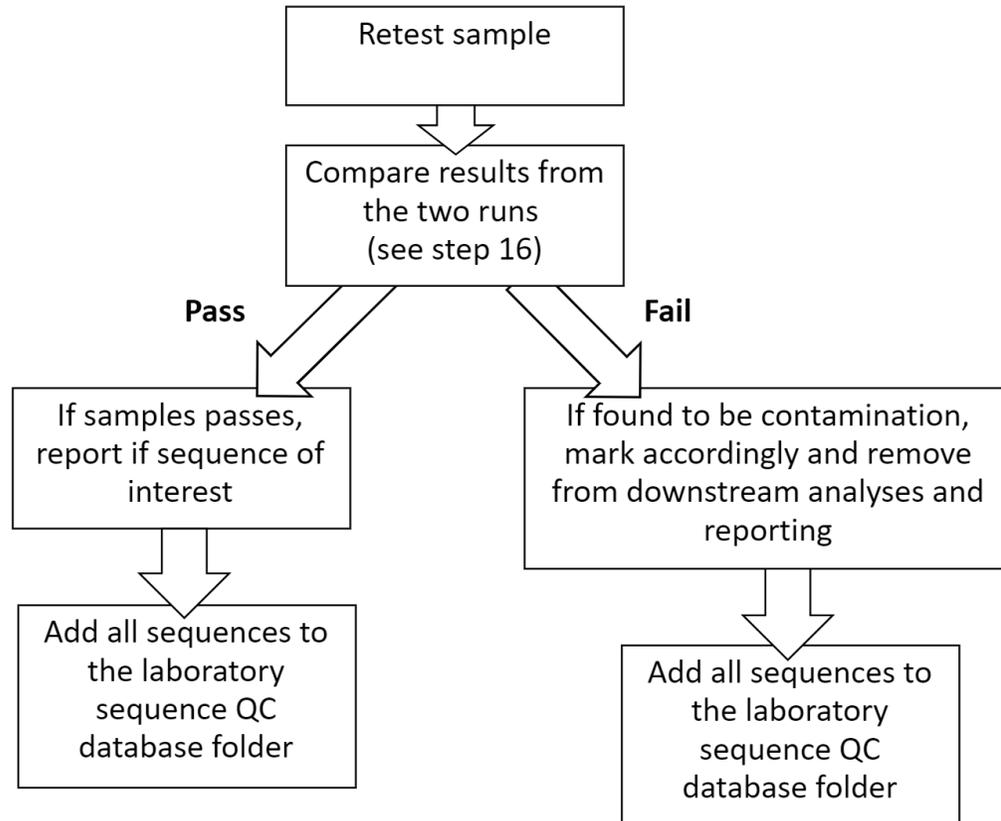


Figure 3 | Sabin3-related phylogeny

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`

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

# 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

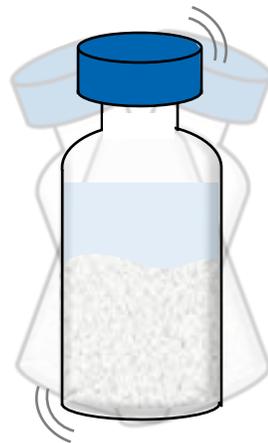
1ml  
NF-water



Lyophilised CAV20



Step Two:  
Vortex briefly



Step Three: Make 33  
single-use aliquots

30µl CAV20



1.5 ml Eppendorf x 33



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

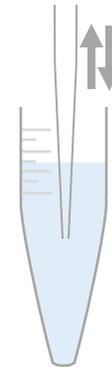


Step Two: Add nuclease-  
free water

270 $\mu$ l\*  
NF-water



Step Three: Pipette to  
mix

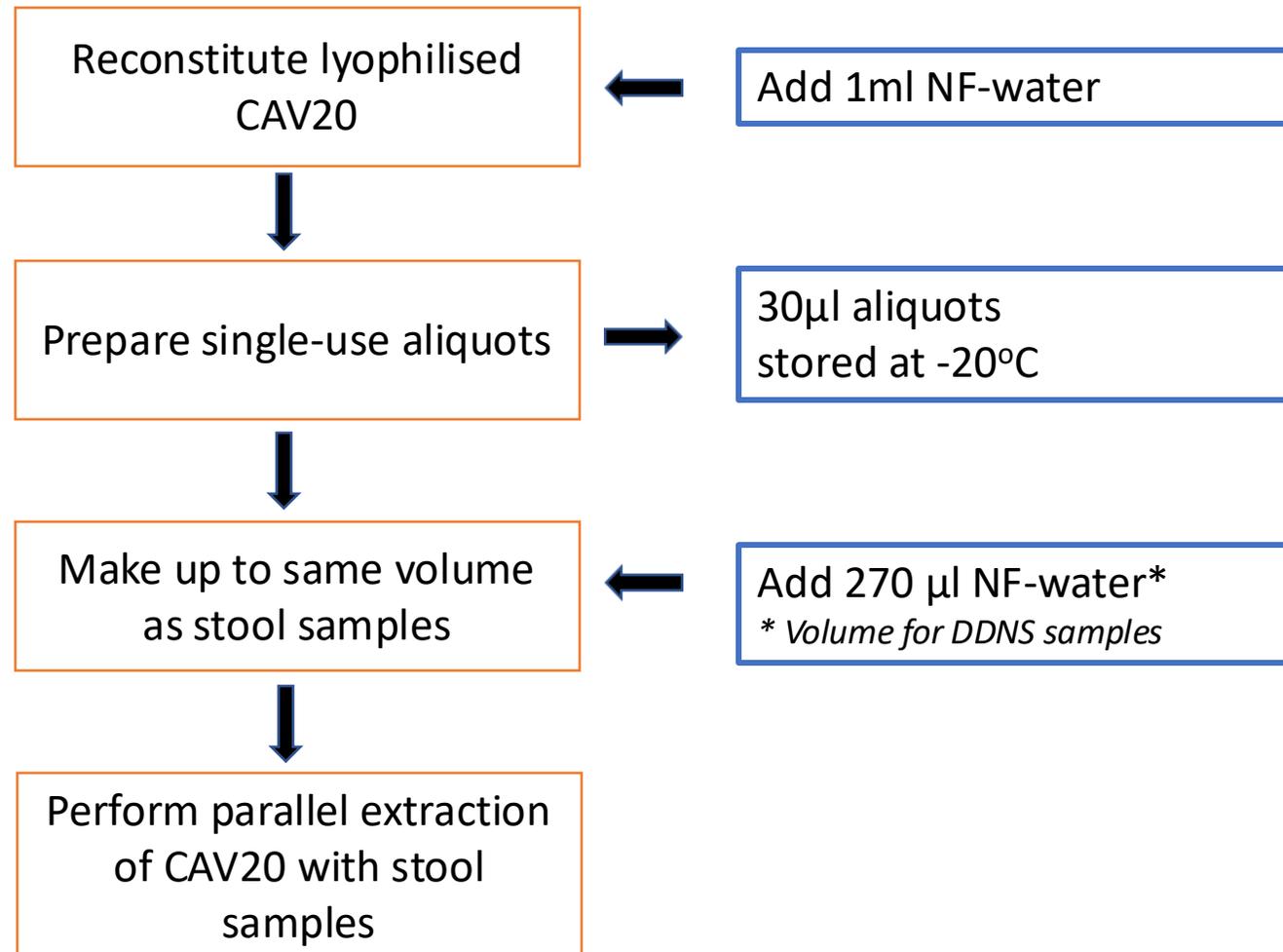


Proceed to RNA  
extraction

\* Volume for preparation  
of DDNS controls

# Preparation of positive control - overview

---



## 9. In-house Verification

Presentation and discussion

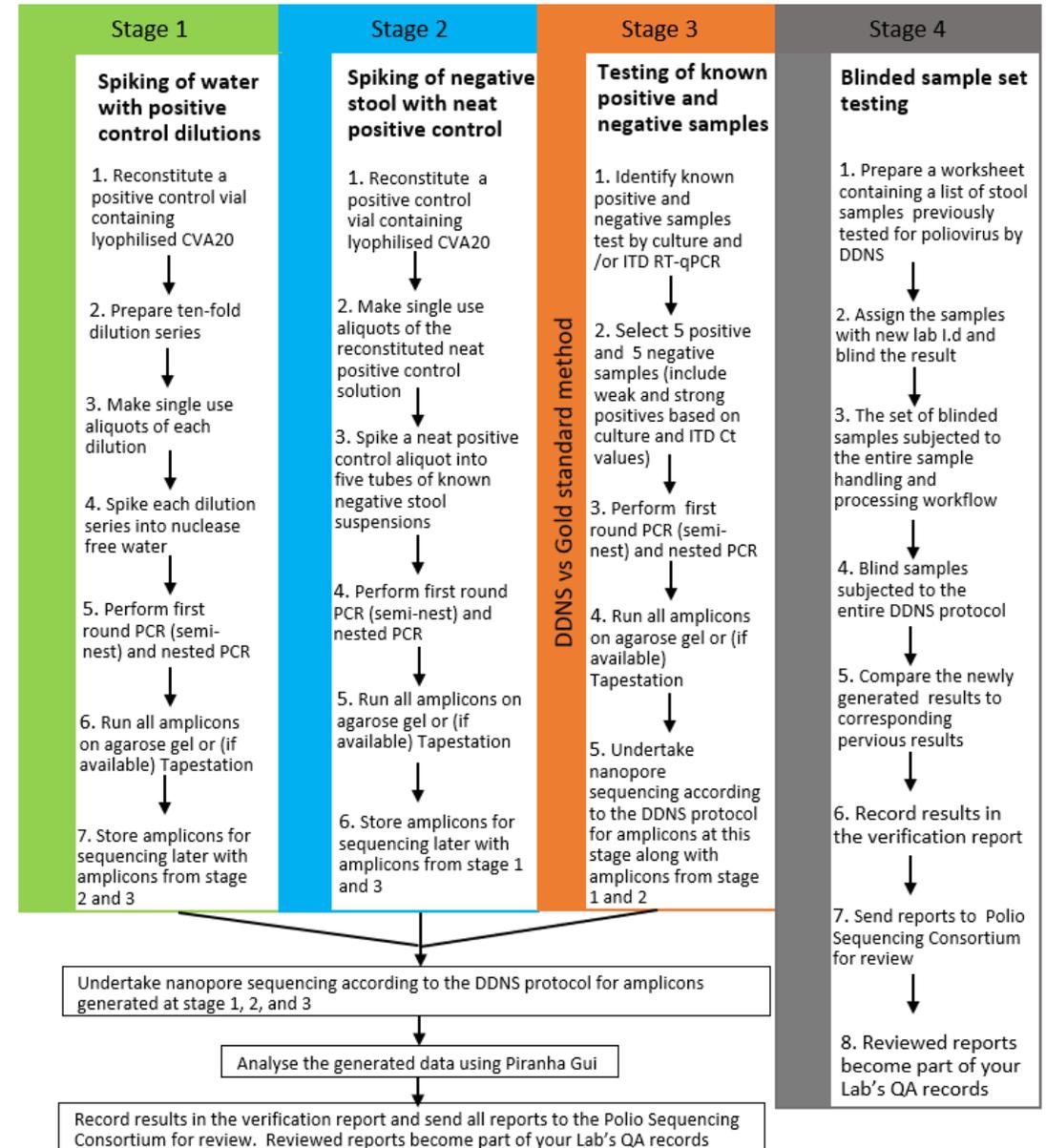
# In-house verification

- ❖ 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	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	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):



## DDNS In-House Verification Report

Stage 3 Testing of known positives and negative samples	Samples	Expected	Observed	For Official Use Only Outcome	
				Pass	Fail
Aim: DDNS vs gold standard method result comparison.	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	Samples	Expected	Observed	For Official Use Only Outcome	
				Pass	Fail
Aim: Lab to test its entire quality system and provide a real time assessment of the lab's proficiency	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