

Sample-sequence-interpretation pipeline

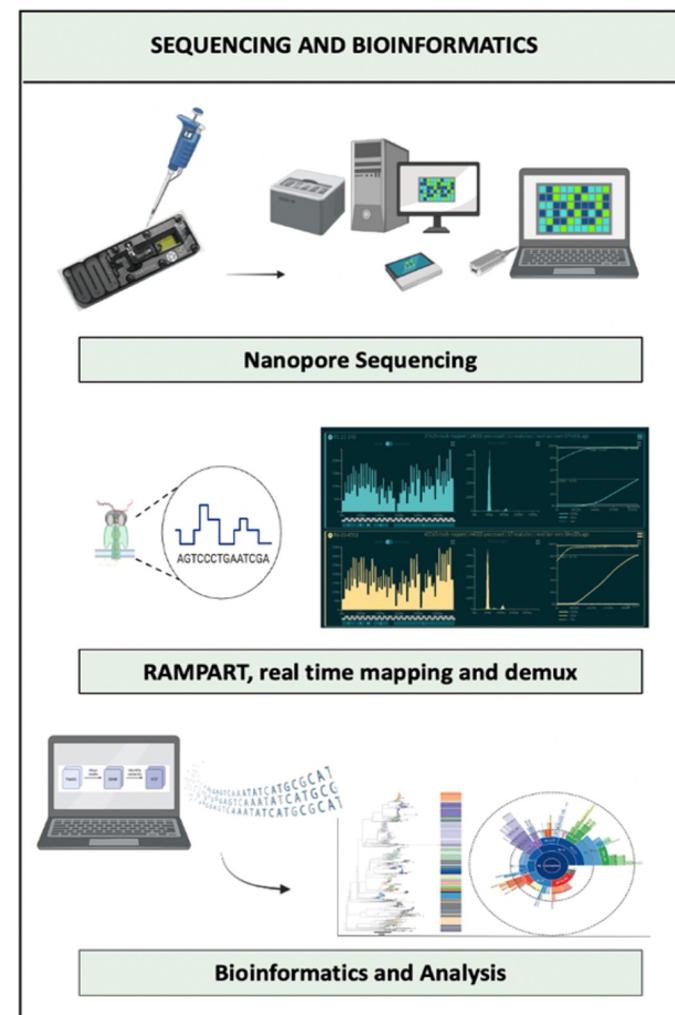
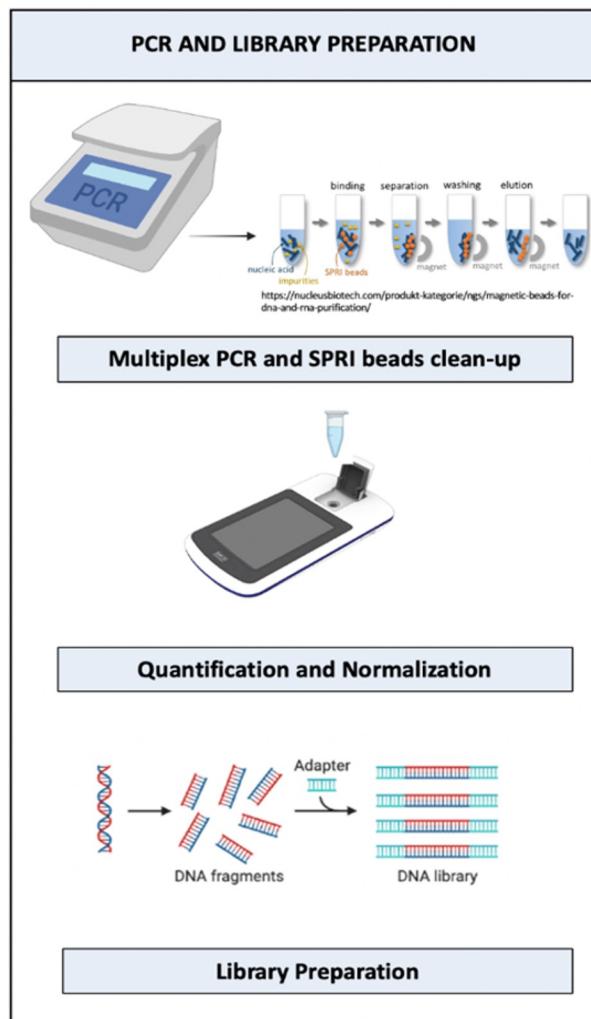
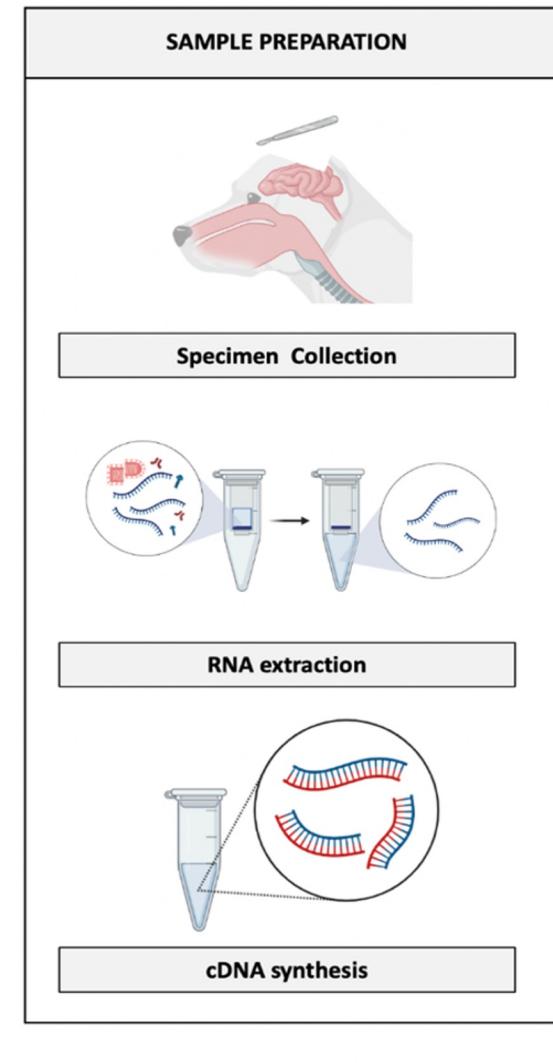


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Sample-sequence-interpretation workflow



Sample Preparation

Sample collection (brain sample) +
rapid test is performed in the field



Biosafety Requirements

1. Pre-Exposure Prophylaxis
2. Proper PPE
3. Proper disposal of dog's head

Sample Preparation

Sample Quality Check

1. Use RNA stabilization/ preservation buffer
2. Ensure good quality samples
 - a. Properly labelled samples
 - b. Triple packaging system
 - c. Good transport conditions: at least 4°C



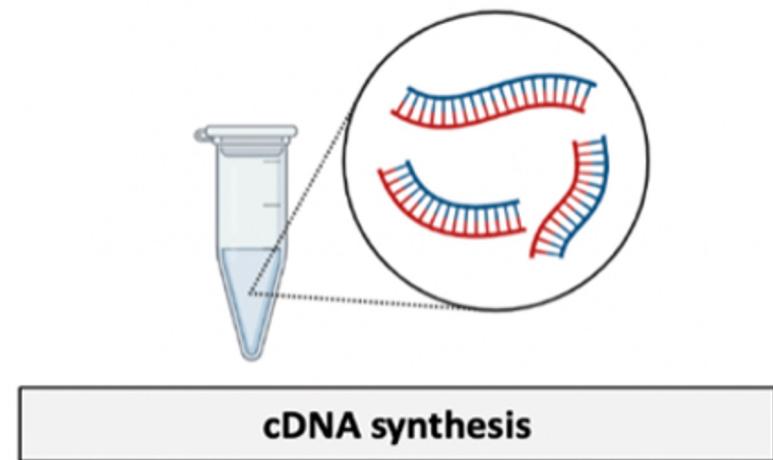
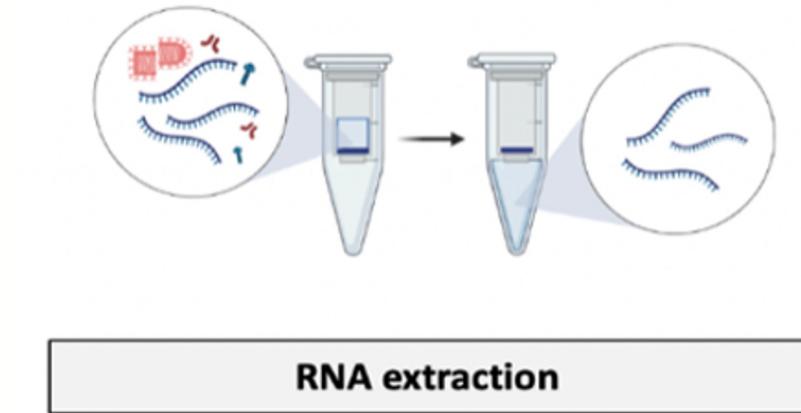
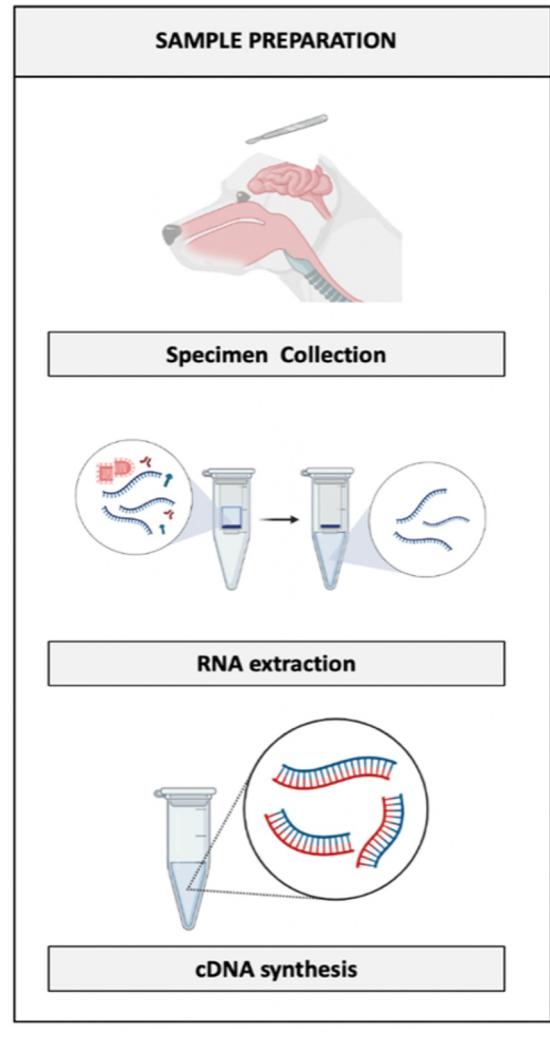
Sample storage

1. Ultra low Freezer -70 / -80
2. Sample in RNA shield - room temp for months

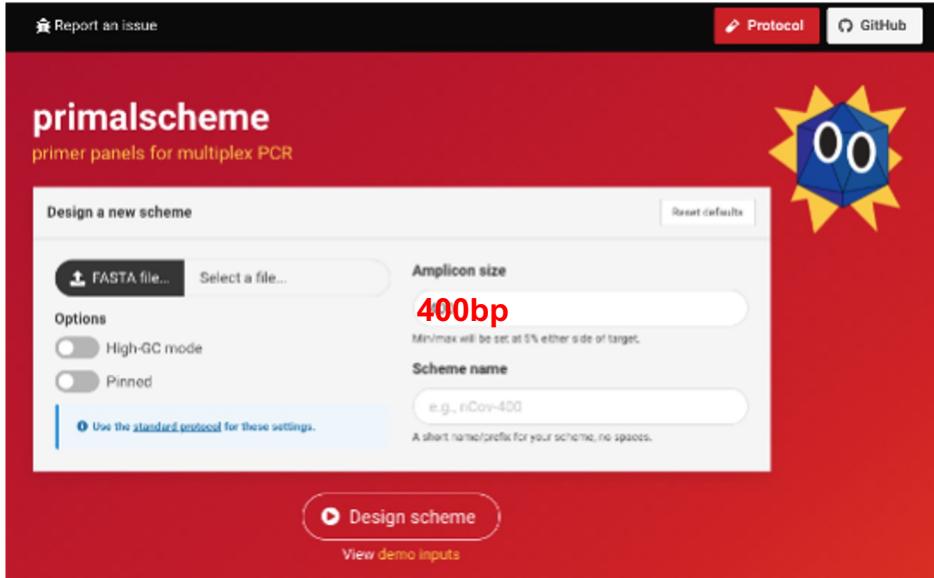




RNA extraction - cDNA extraction



Multiplex PCR



primalscheme
primer panels for multiplex PCR

Design a new scheme

FASTA file... Select a file... Amplicon size **400bp**

Options

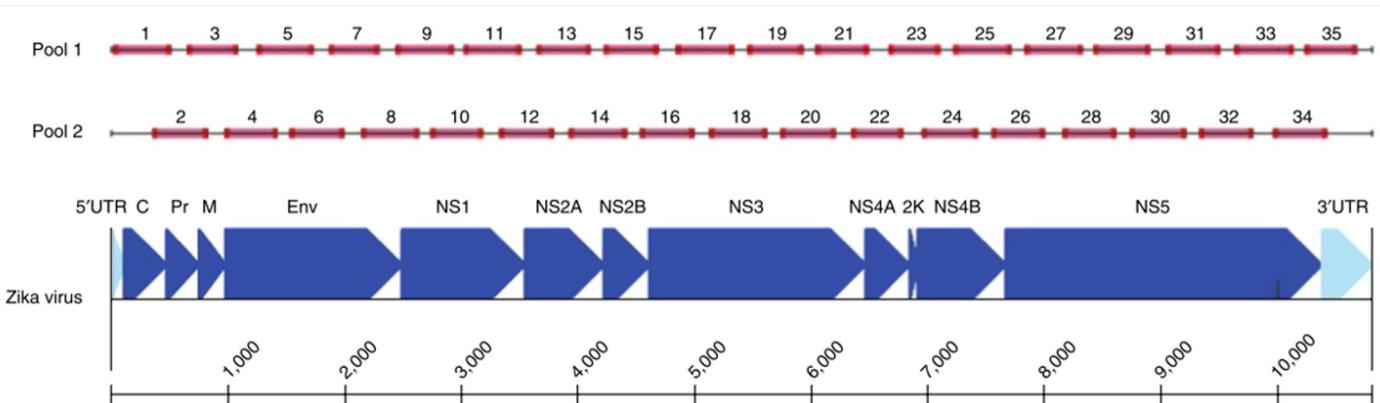
- High-GC mode
- Pinned

Use the standard protocol for these settings.

Design scheme

View demo inputs

Multiplex tiling PCR approach



- References: From Genbank, East Africa/Peru/Philippines/Nigeria/Malawi rabies virus WGS
- Short fragments (~400bp)
- Initially developed for Zika sequencing (high Ct clinical samples)
- Generates tiling amplicons for near complete genome coverage

Multiplex PCR

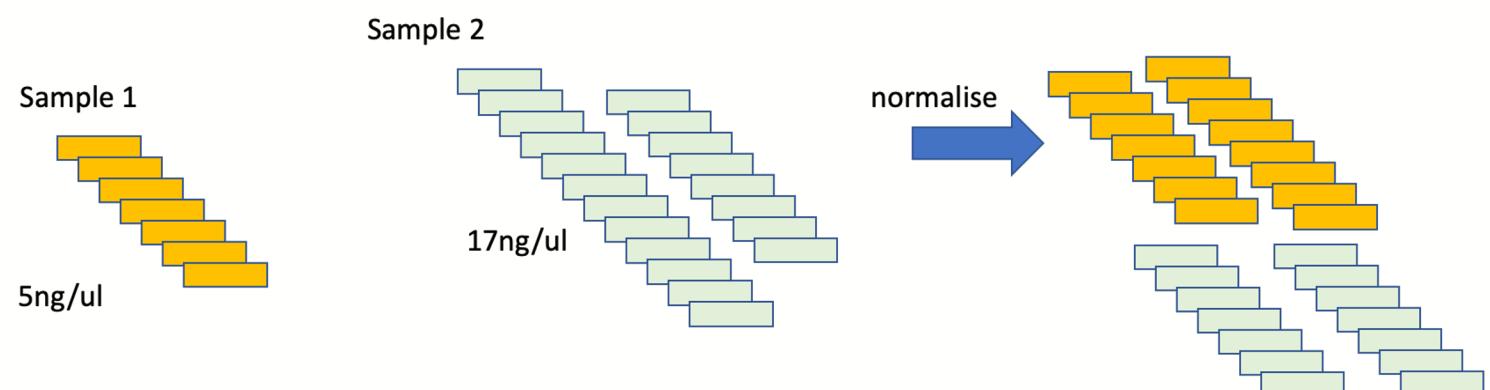
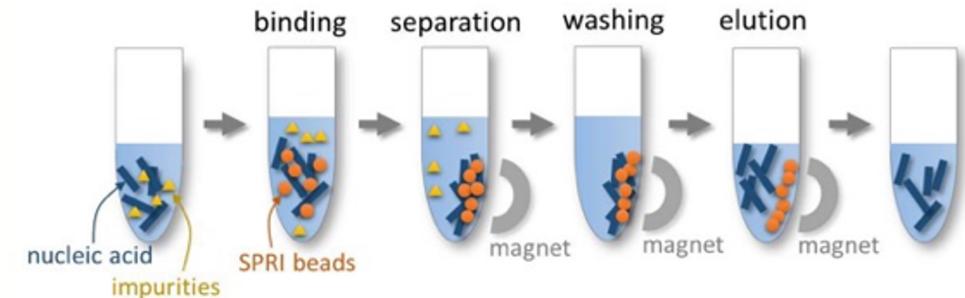
- Q5® Hot Start High-Fidelity DNA Polymerase
 - Must be used, other polymerase won't work
 - Has a higher Tm than other polymerases
- Mastermix contains all PCR components
 - Just combine with cDNA and water

- Require 2 reactions per sample:
 - Pool A and Pool B



SPRI Beads Clean-up, Quantification & Normalisation

- SPRI – Solid Phase Reversible Immobilisation
- Quantification using fluorometer
- Variation between samples
- Normalisation to even the sequencing coverage across samples



Library preparation

1. End repair/dA tailing

- Add a 3'-dA tail to dsDNA

2. Barcoding and barcode ligation

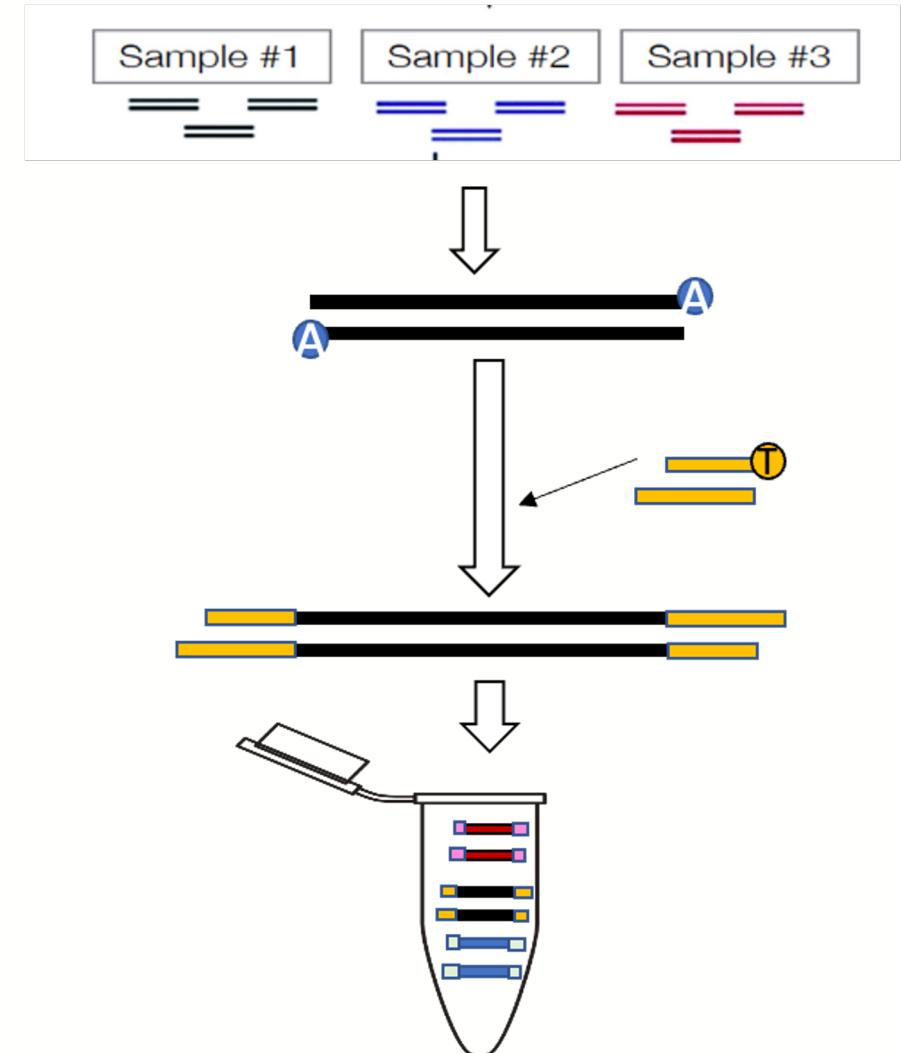
- In order to multiplex samples on one run, we need to BARCODE

3. Pool together barcoded DNA

4. Clean-up with SPRI beads

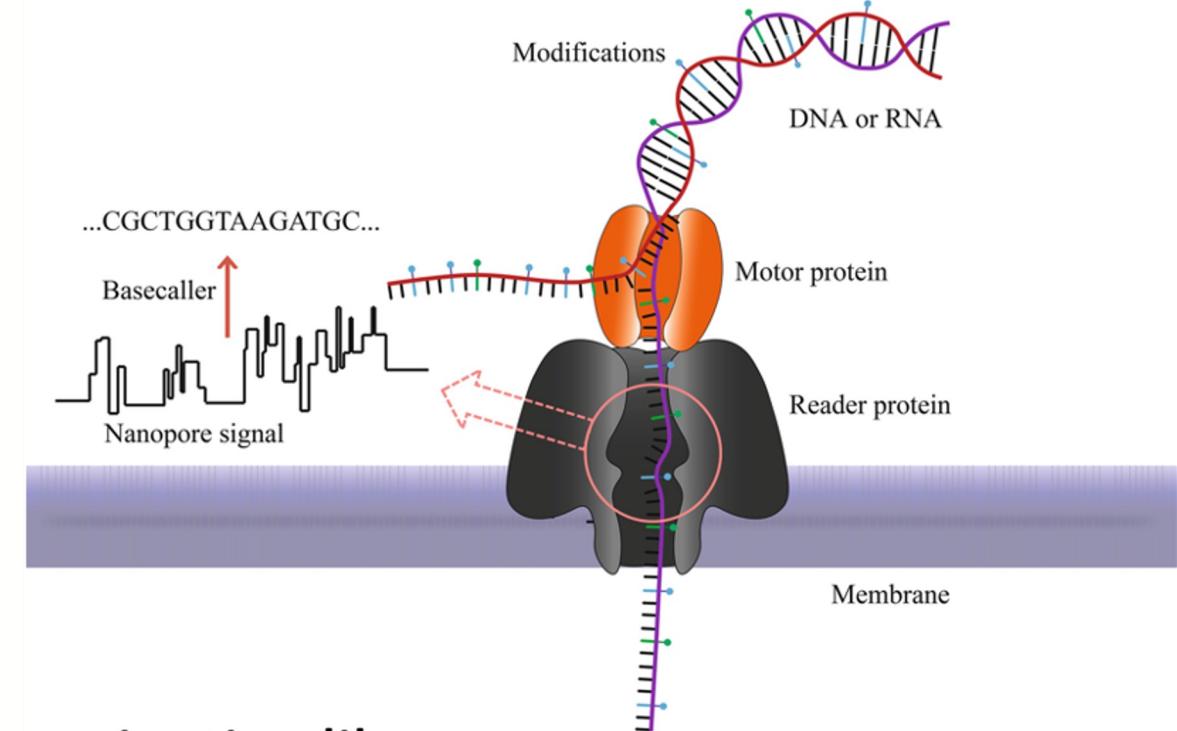
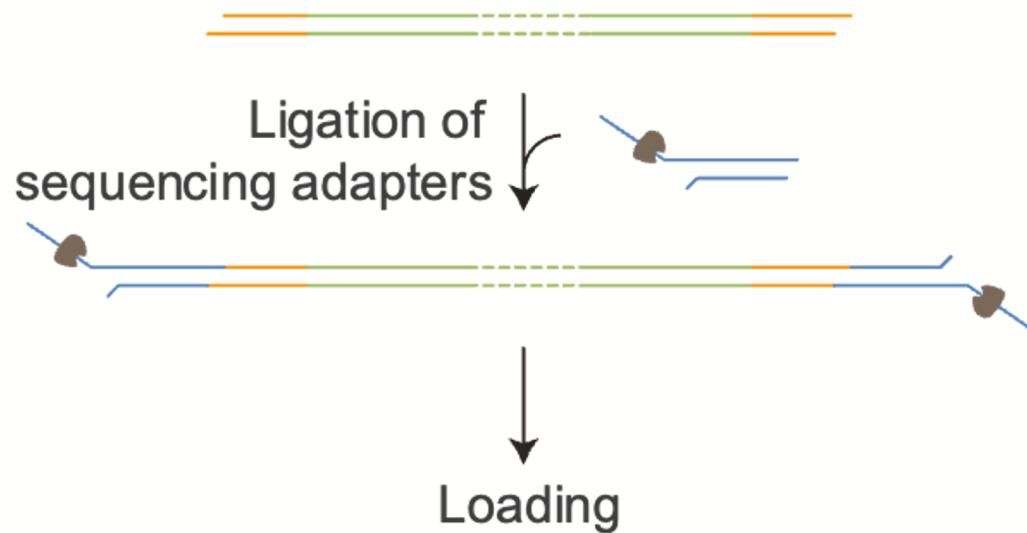
5. Quantify with Quantus Fluorometer

6. Adaptor Ligation



Adaptor Ligation

- Must attach a **nanopore-specific adaptor** to the DNA in order for it to sequence



- Final clean-up to get rid of loose adaptor contaminating library



Sequencing: Minknow and RAMPART interpretation - to be shown during the actual run

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

Output

Data saved as: Sequencing/

Output location: /data/

Output format: FAST5 (checked), FASTQ (checked), BAM (unchecked)

Filtering: Qscore: 8 | Readlength: Unfiltered | Read splitting: Enabled

Advanced options

FAST5 options

Compression: VBZ, Reads per file: 4000

Filtering options

Pass / fail filtering: Setting a minimum and maximum read length will determine the cut-off of reads for the pass and fail folders.

Qscore: 8, Min readlength (kb): 8, Max readlength (kb): Max readlength

FASTQ options

Compression: Gzip, Reads per file: 4000

Read splitting

Enable read splitting: Gzip compression

Cancel, Save, Continue to final review

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

Sequencing

Selected positions

Kit: Selected kit: SQK-LSK109, Expansion packs: EXP-NBD104

Run options

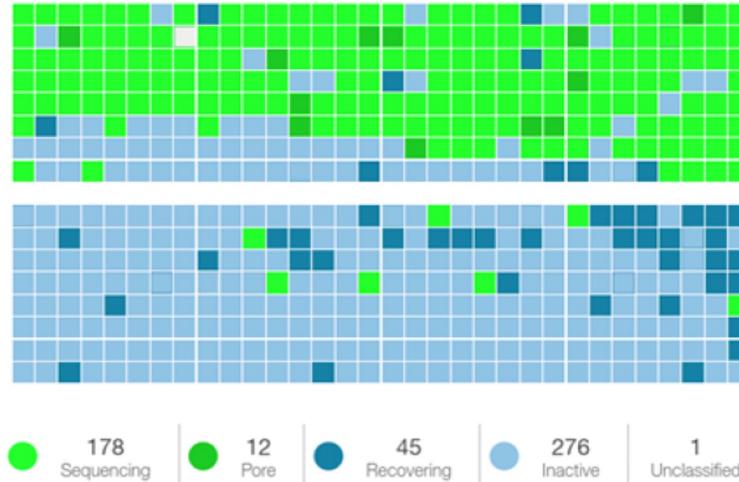
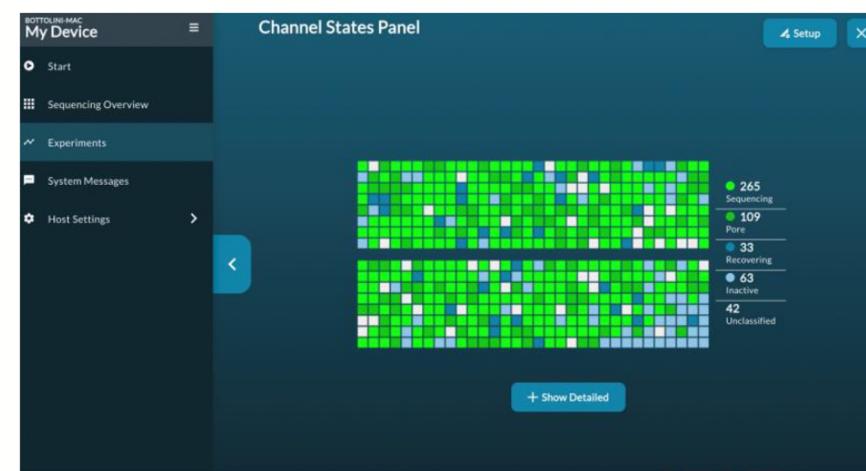
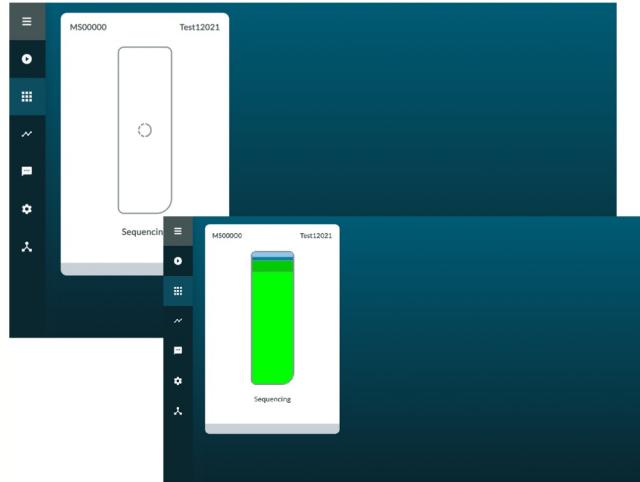
Run length: 72 hours, Minimum read length: 200 bp, Adaptive sampling: Off

Advanced run options

Back to output, Save settings as template, Start



Sequencing: Minknow and RAMPART interpretation - to be shown during the actual run



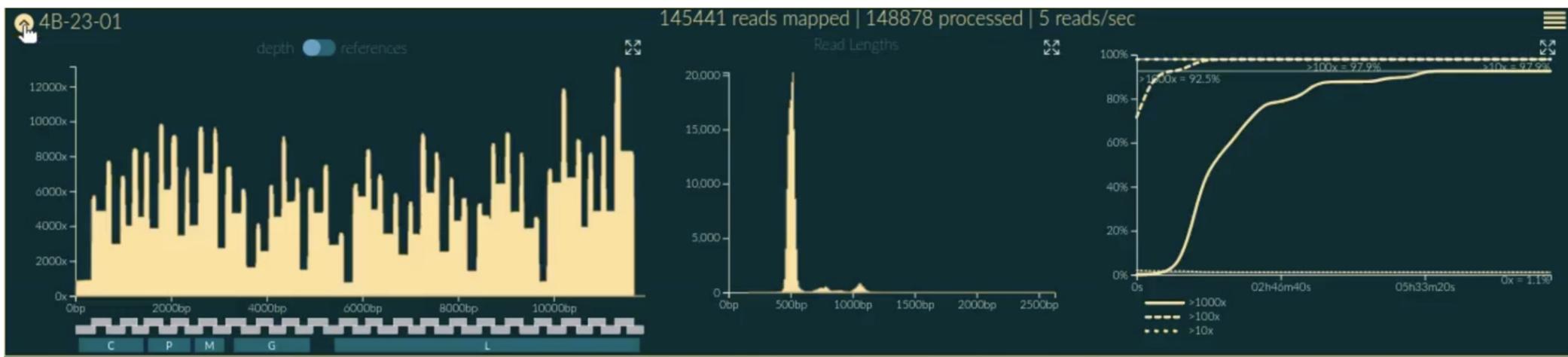
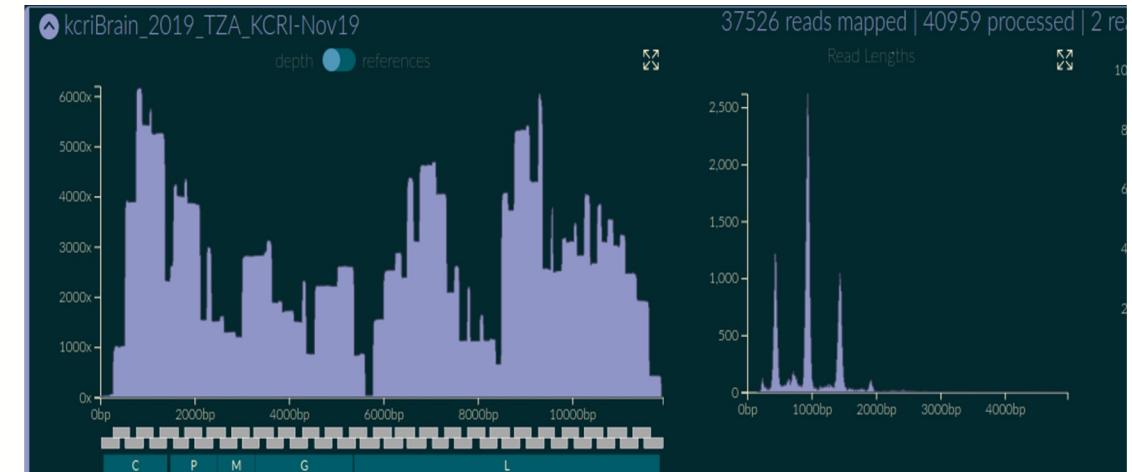
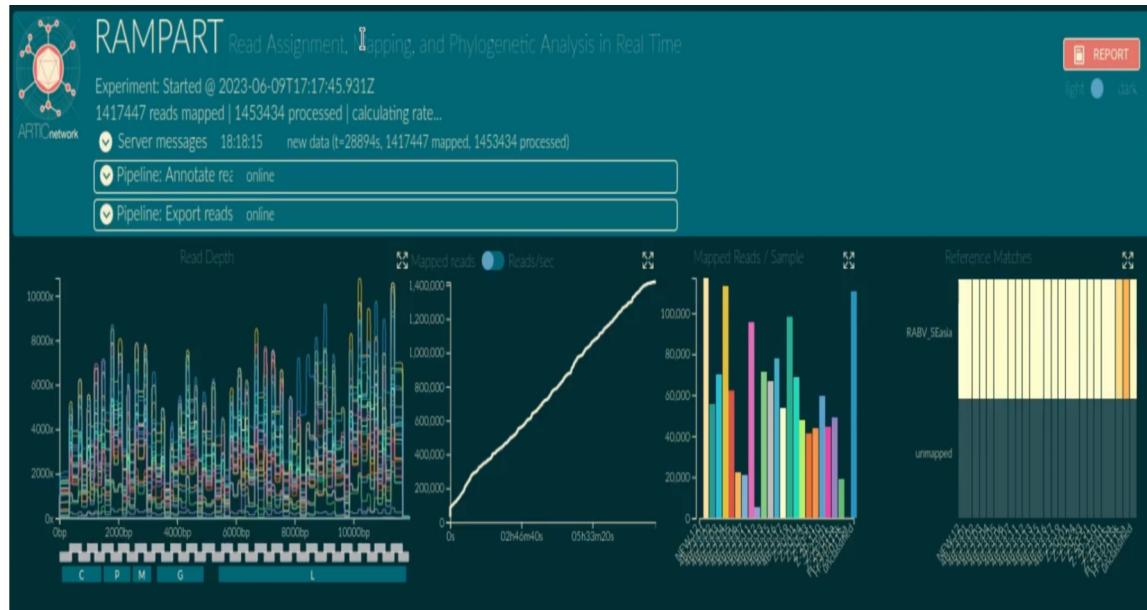
High number of inactive channels

Clear spatial pattern

Indicates an air bubble has been introduced onto the sensor array



Sequencing: Minknow and RAMPART interpretation - to be shown during the actual run



Consensus generation

- **Artic-rabv pipeline**
- **Demultiplexing**
- **Read Filtering**
- **Medaka consensus pipeline**



Summary

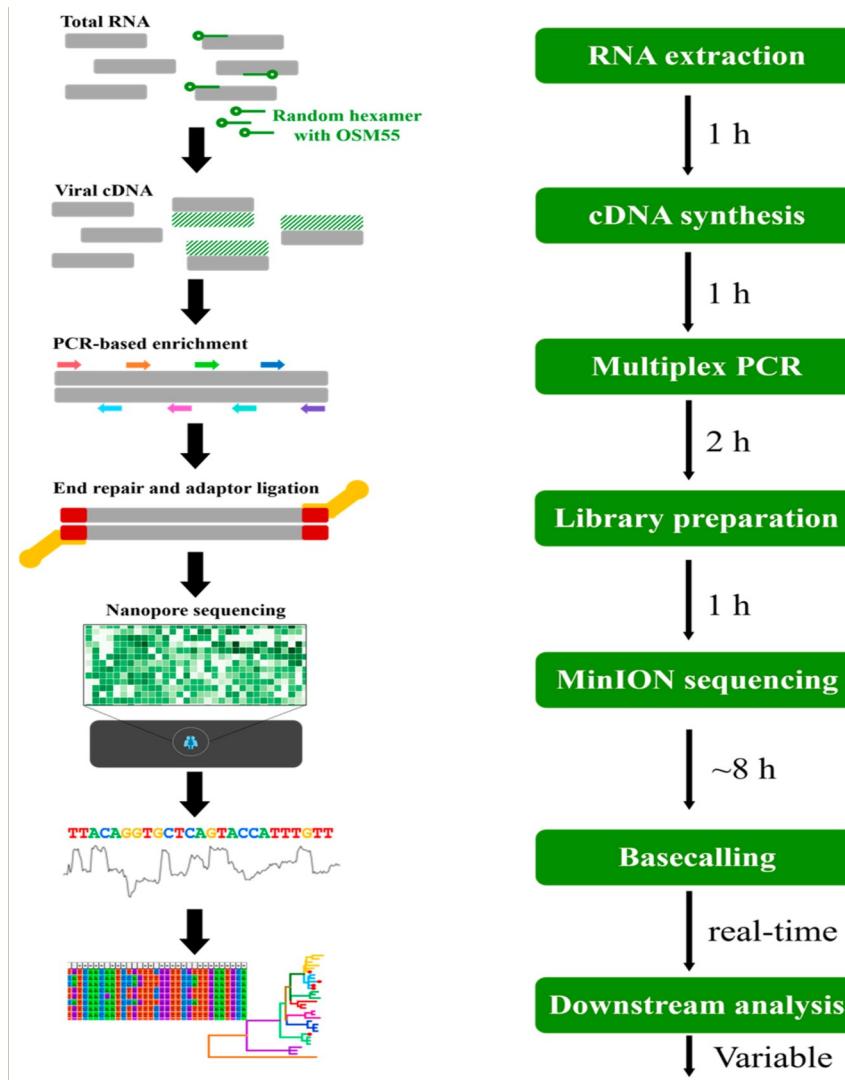
Pause point

Pause point

	Process	Timing	Equipment	Reagents
Day 1	CDNA SYNTHESIS	1hr	Thermocycler <i>Optional:</i> centrifuge	LunaScript RT SuperMix Kit
	MULTIPLEX PCR	4-5hr	Thermocycler <i>Optional:</i> centrifuge	SARS-CoV-2 ARTIC primers Q5® Hot Start HF Polymerase 2x mastermix
	(<i>Optional:</i> GEL, PCR CLEAN UP, QUANTIFICATION & NORMALISATION)	(4hrs)		
	POOL AND DILUTE AMPLICONS + QUANTIFY	1hr	Quantus Fluorometer	Quantus DNA reagents
Day 2	ONE POT: END-REPAIR/DA-TAILING AND BARCODE LIGATION	1.5hr	Thermocycler (or heating block), magnetic rack	NEBNext® UltraII™ End-prep kit Blunt/TA mastermix SPRI beads Nanopore Native Barcoding Expansion Kit (1-24)
	ADAPTER LIGATION	1hr	Thermocycler (or heating block), magnetic rack	NEBNext Quick Ligation Module SPRI beads Nanopore library reagents
	PRIME FLOWCELL & SEQUENCING	20min (prime) Sequence until...(max 48h)	MinION Mk1c <i>Optional:</i> laptop	Nanopore library reagents Flowcell (R9.4.1)



Summary



Multiplex PCR-Based Nanopore Sequencing and Epidemiological Surveillance of Hantaan orthohantavirus in *Apodemus agrarius*, Republic of Korea

by Kyungmin Park ^{1,2} , Seung-Ho Lee ² , Jongwoo Kim ^{1,2} , Jingyeong Lee ² , Geum-Young Lee ² , Seungchan Cho ² , Seung Ho Lee ² , Kkothanahreum Park ² , Jin Sun No ³ , Shailesh Budhathoki ⁴ , Yu-Jin Kim ⁵ , Young-Su Kim ⁶ , Heung-Chul Kim ⁷ , Terry A. Klein ⁷ , Won-Keun Kim ^{4,8} and Jin-Won Song ^{1,2,*}

Your advice on how to fit protocol into working day

- ❖ We have three are pause points in protocol to allow breaks!
- ❖ Take your time before and when doing the sequencing work - make sure you have all what is needed
- ❖ You can do this work in two days, not so intense
- ❖ Think what you want at the end of the project



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