

Nanopore Sequencing Training

3rd June 2024, Pune, India

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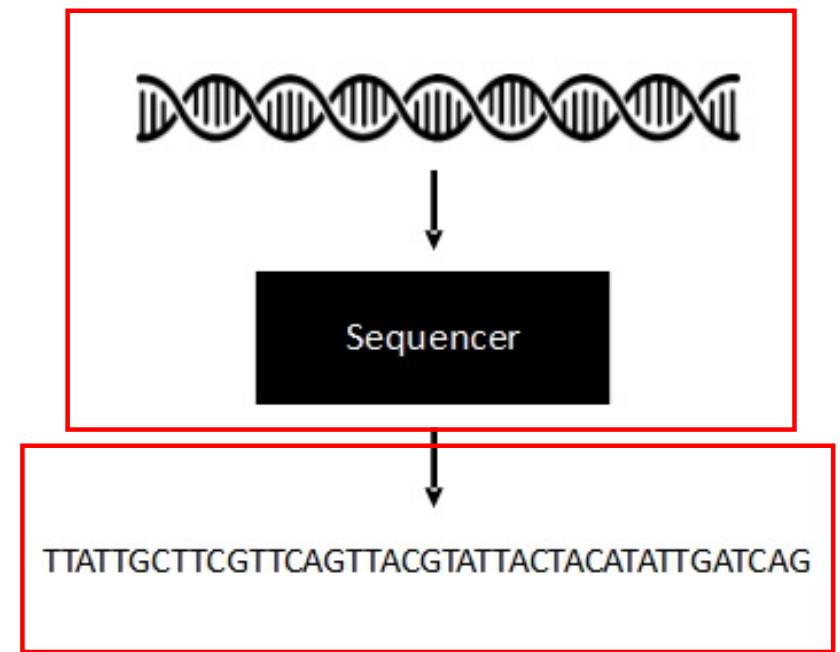
A joint venture between The University of Melbourne and The Royal Melbourne Hospital



WHO Collaborating Centre
for Reference and
Research on Influenza
VIDRL

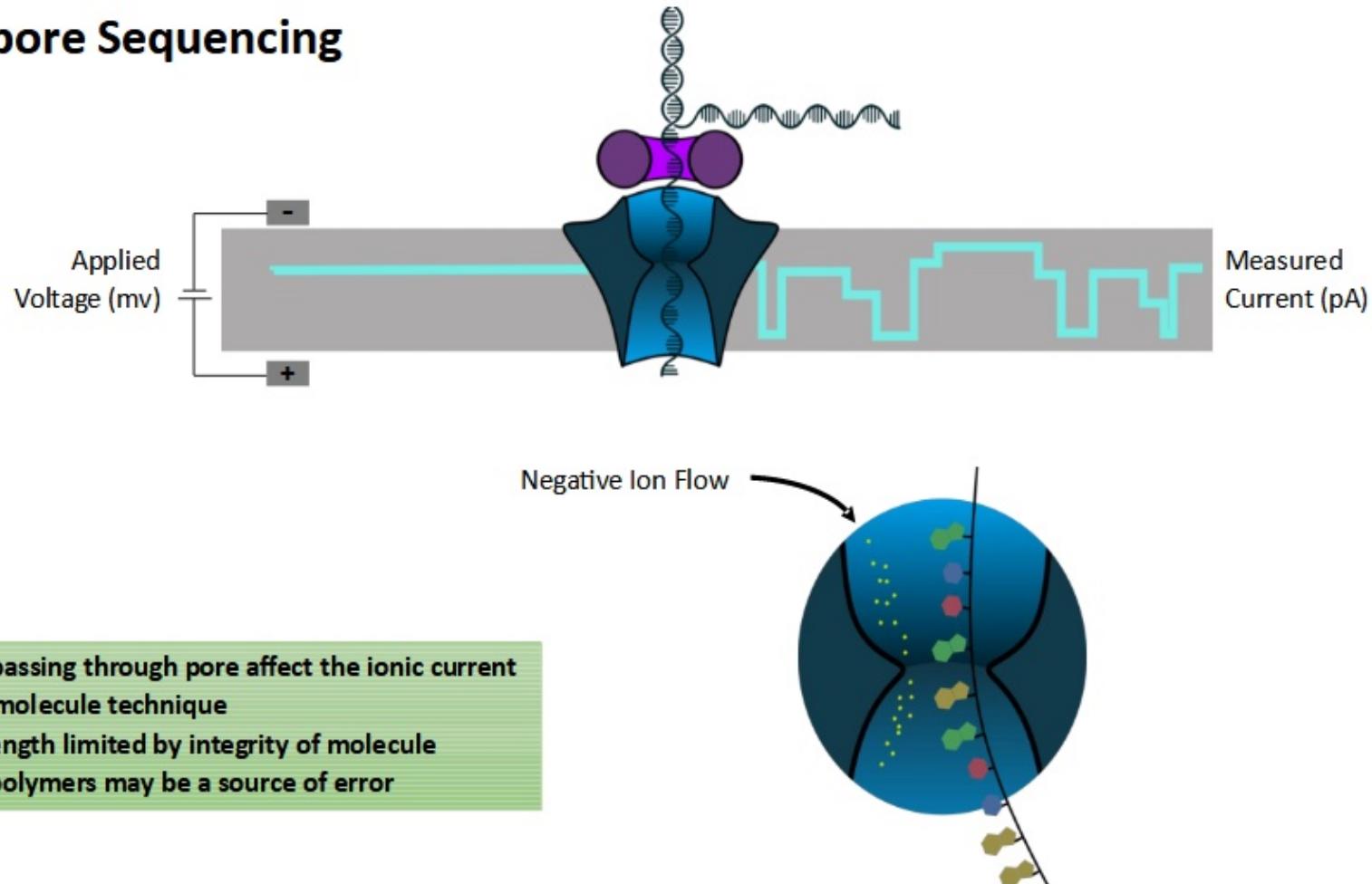
Overview

- Genomic material input
 - RT-PCR products
 - Specific amplicons for Flu or RSV
- Sequencer
 - MinION Mk1b/Mk1c
- Obtain sequence via bioinformatics analysis

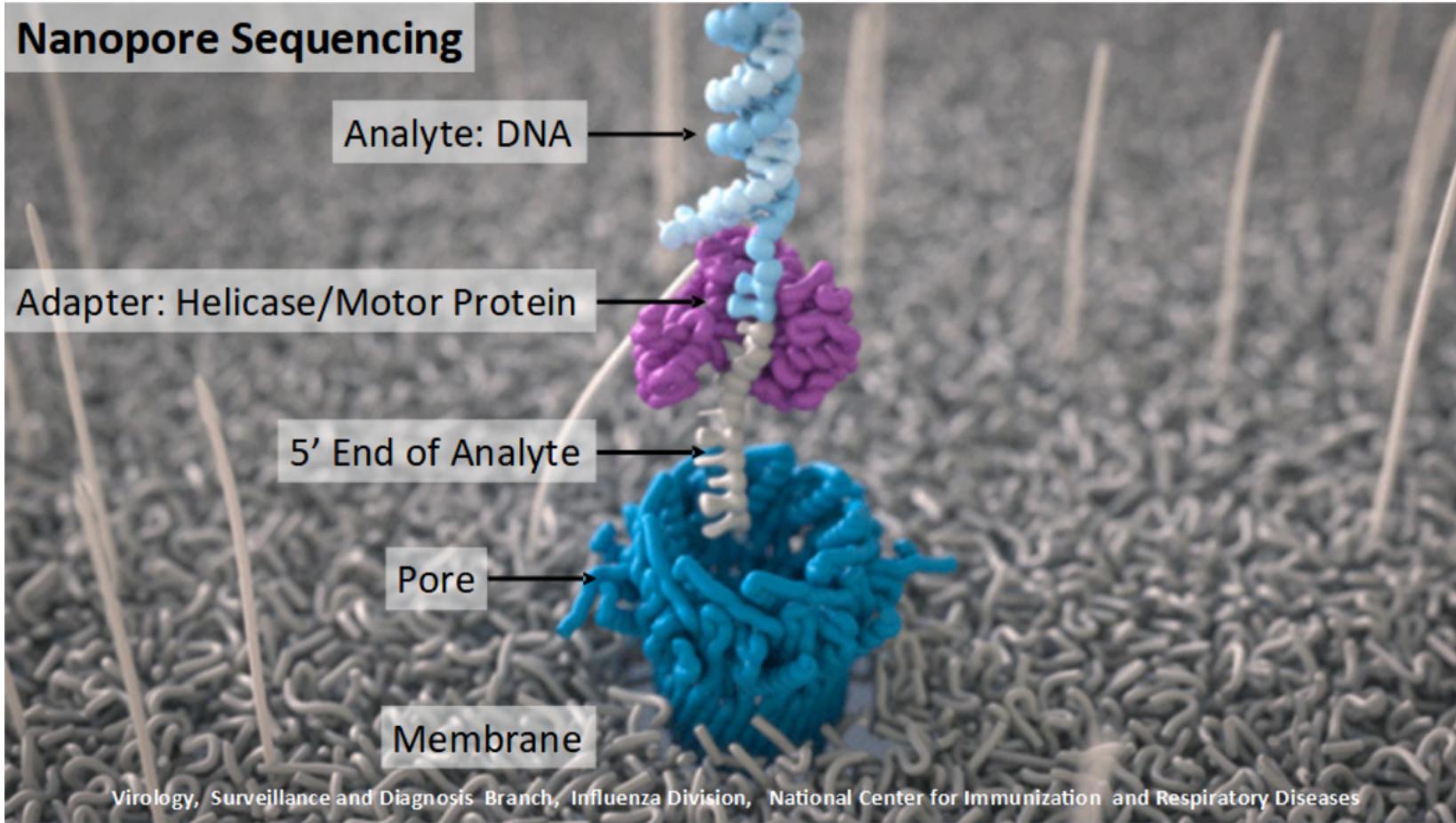


3rd Generation Sequencing

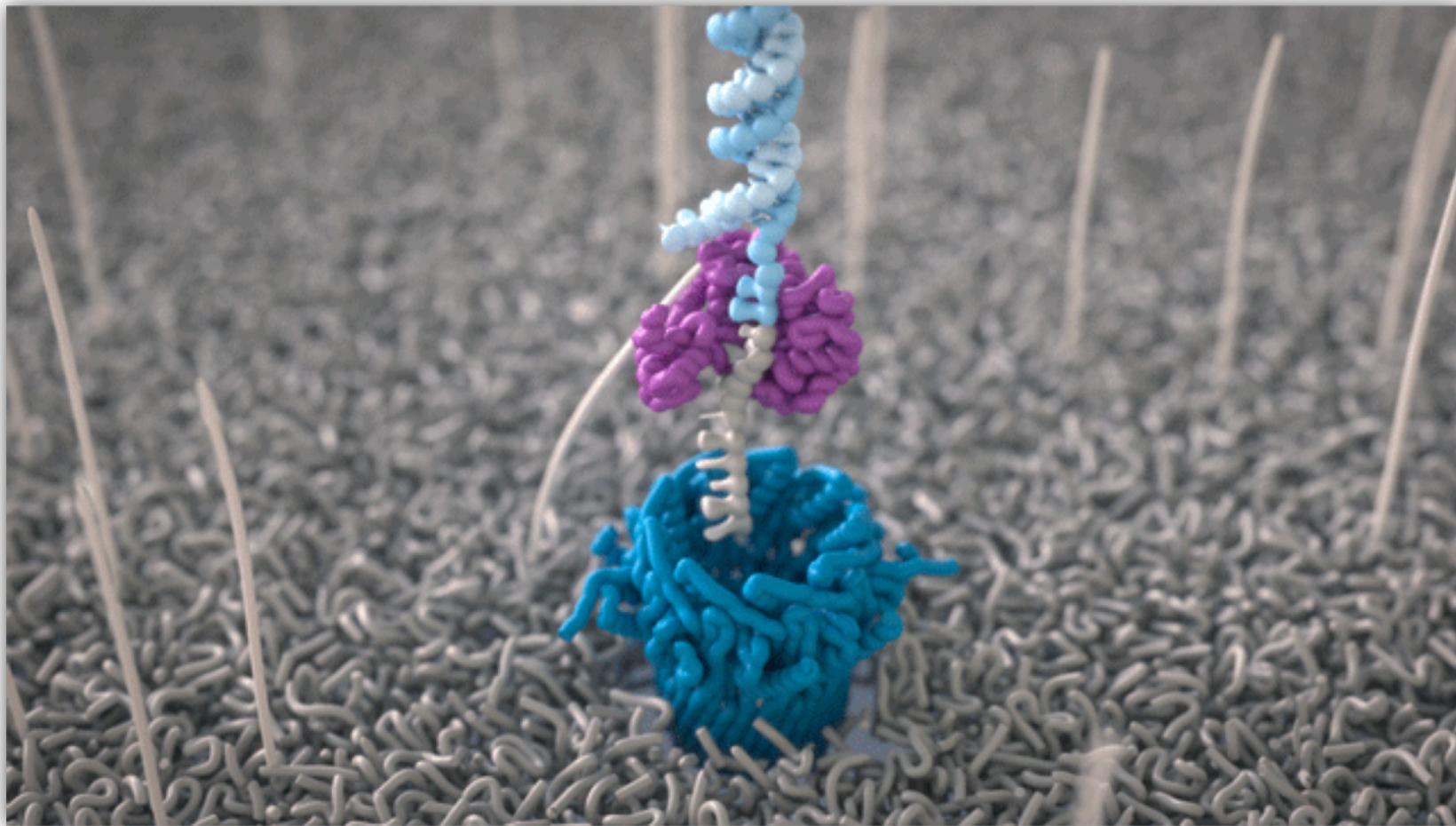
Nanopore Sequencing



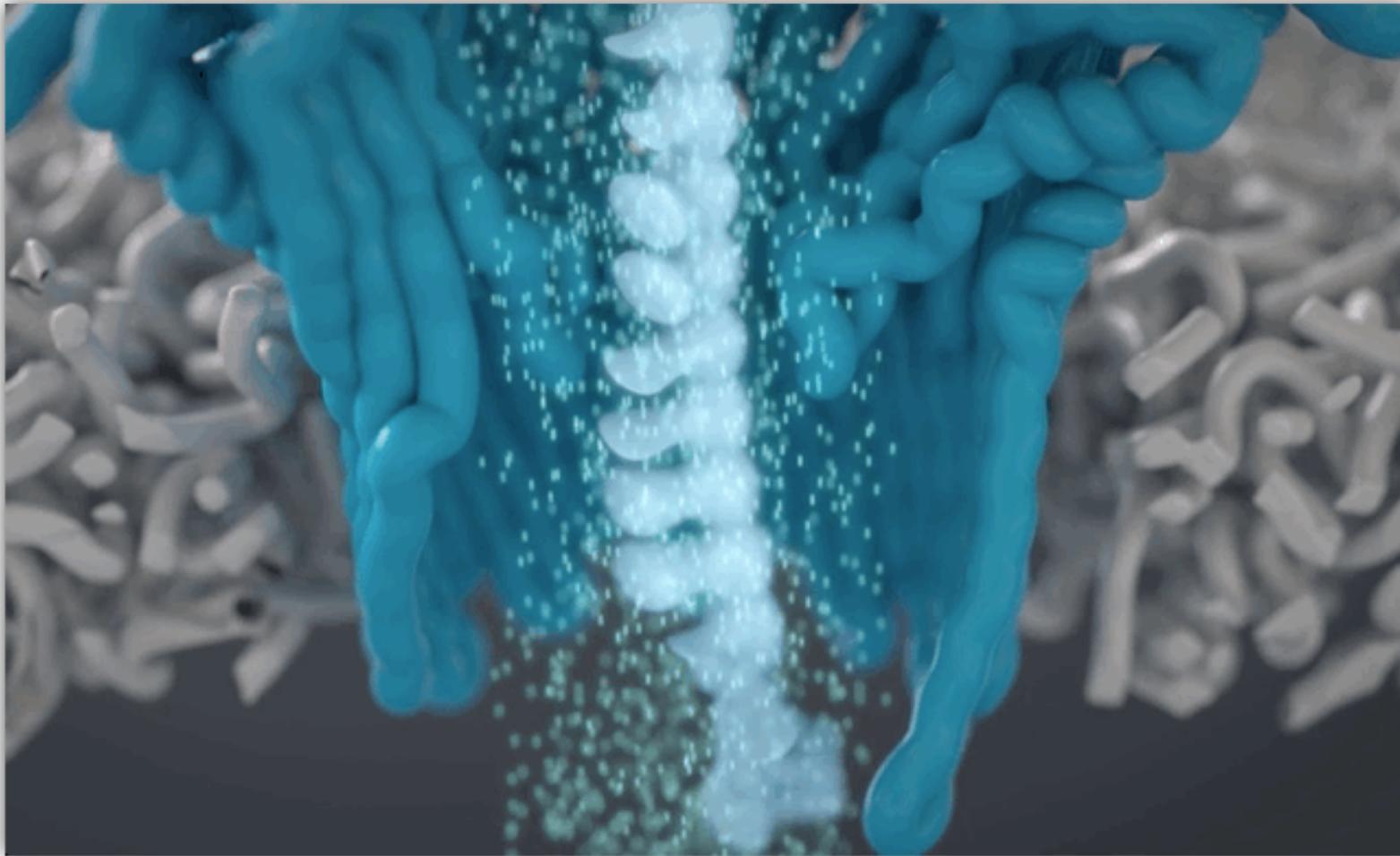
Nanopore Sequencing



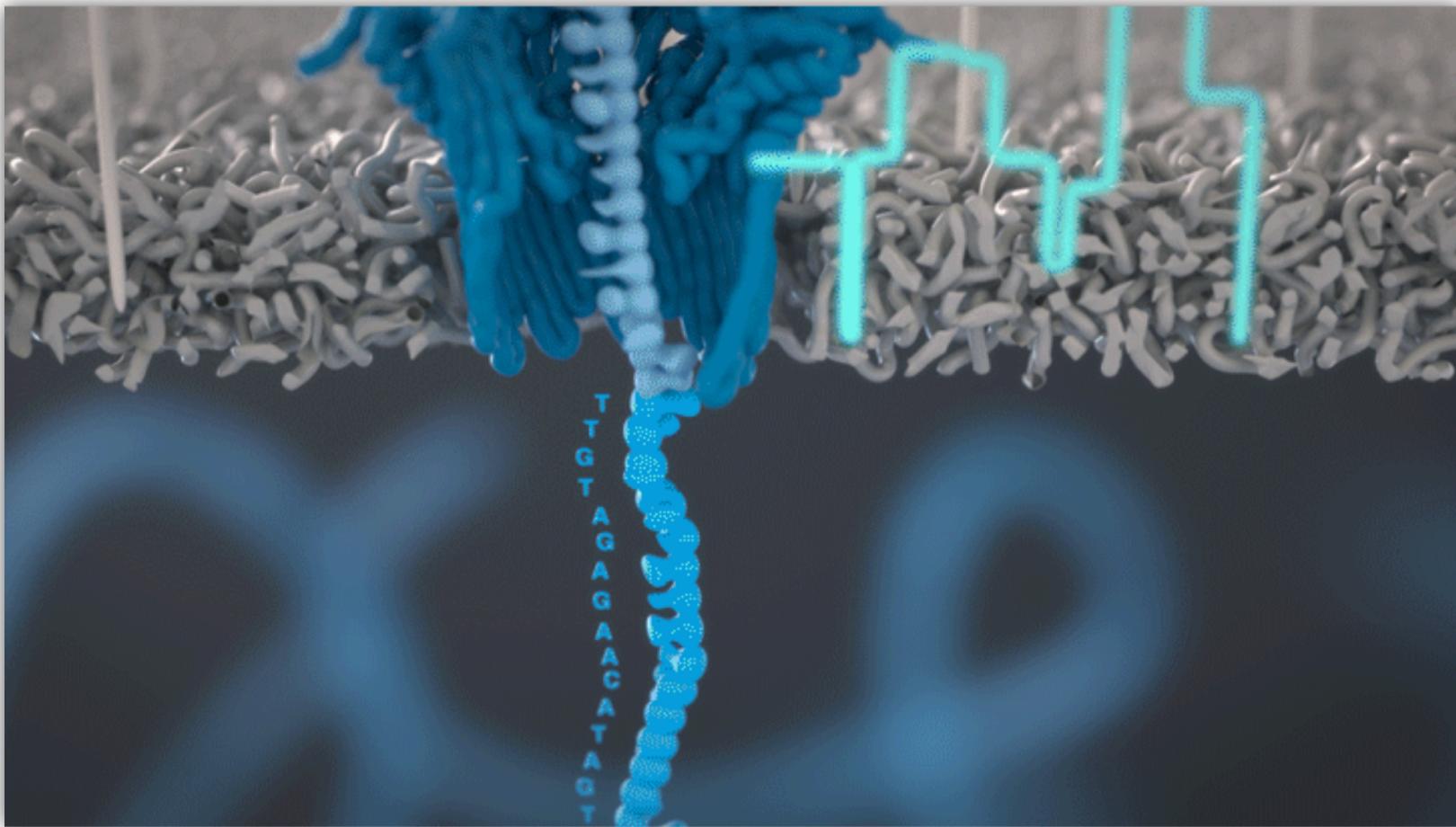
Motor Protein Action



Interruption of the Ion Movement



Generation of Electrical Signal



Wet Lab Workflow

- RNA extraction
- RT PCR set up
- RT PCR quality control (QC)
- Normalisation
- Library preparation
- Library QC
- Library loading
- Primary analysis
- Secondary analysis



MinION Mk1B

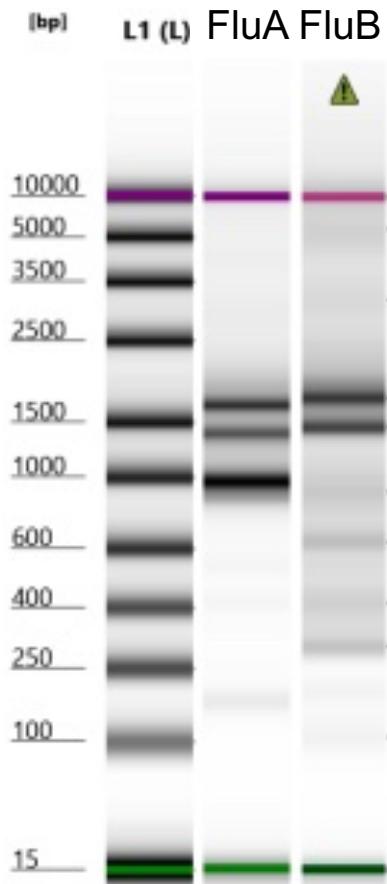


MinION Mk1C

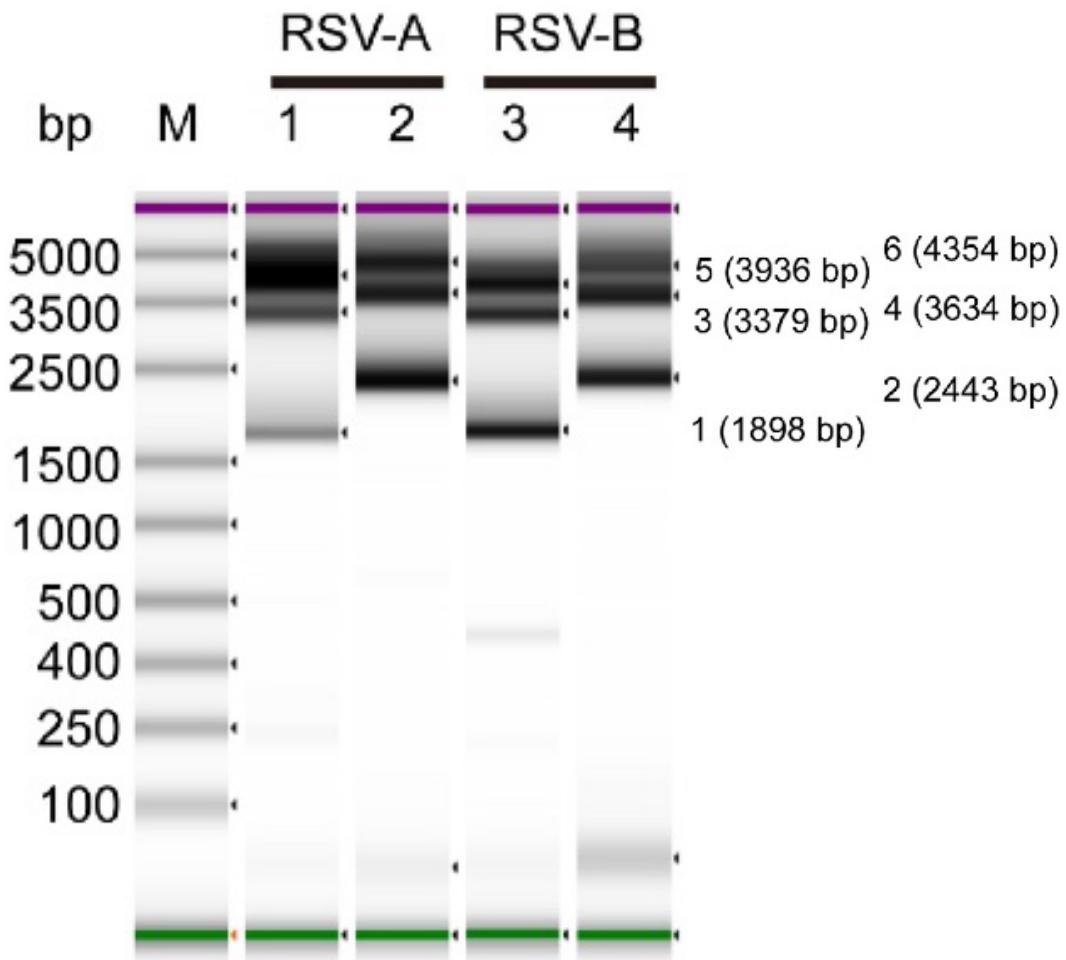
Reverse Transcriptase (RT) PCR

- Influenza and RSV are negative sense RNA viruses
- RT-PCR to amplify specific amplicons
- Influenza Flu A/B mRT-PCR in one reaction
 - Flu A HA, NA, MP
 - Flu B HA, NA
- RSV: 6 fragments in 2 reactions

RT PCR – Influenza 3 gene (NGS)



- Visualize the bands present
- Quantify each sample
- Use concentrations to normalize each sample to 200ng in 10 µl



RT PCR- QC

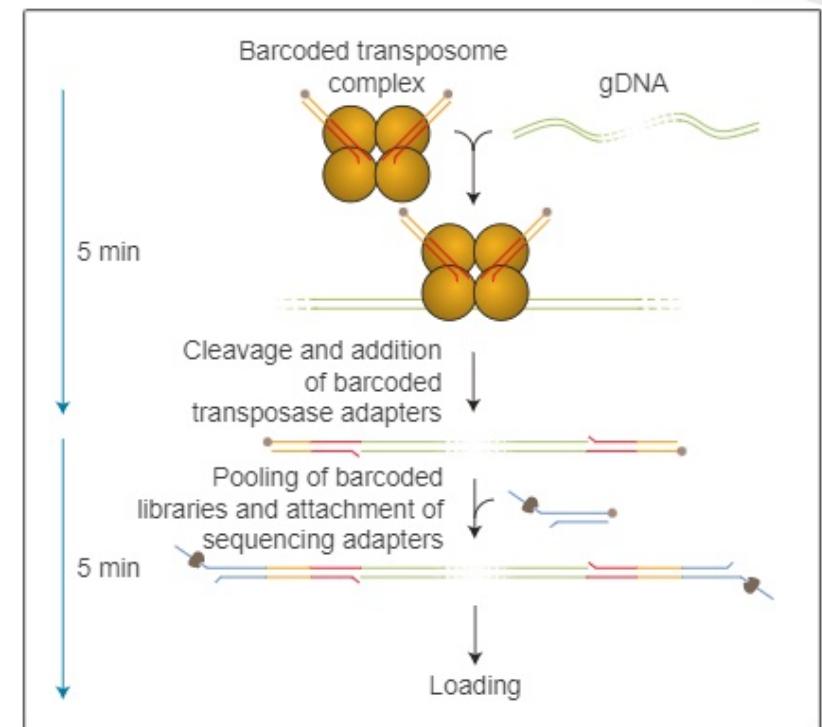
- Visualize the bands present
- Quantify each pool
- Use concentrations to normalize each sample to 200 ng in 10 μ l
- Combine 5 μ l of tube 1 and 2 of the same sample

Normalisation

- Use the concentrations of the amplified RT PCR product to dilute each sample to the same input concentration
- Uses the formula: $C_1V_1 = C_2V_2$
- Where
 - C_1 = Sample Concentration (ng/ μ L)
 - $C_2 \times V_2 = 200\text{ng}$
 - V_1 = Unknown Volume (μ L)
- Simplified: $V_1 (\mu\text{L}) = \frac{200\text{ng}}{\text{Sample conc (ng}/\mu\text{L})}$
- Volume of Diluent = Total volume (10 μ L) – V_1

Library preparation

- Amplified DNA fragments are simultaneously fragmented and tagged with biological “barcodes”
 - Known oligonucleotides used to identify each sample in a mixture
- Individual barcode for each sample in the same batch
- Write down which barcode used for each sample
- Samples are then pooled and cleaned using Beads



To determine the concentration of the library
Library QC

QuBit check using BR Qubit kit

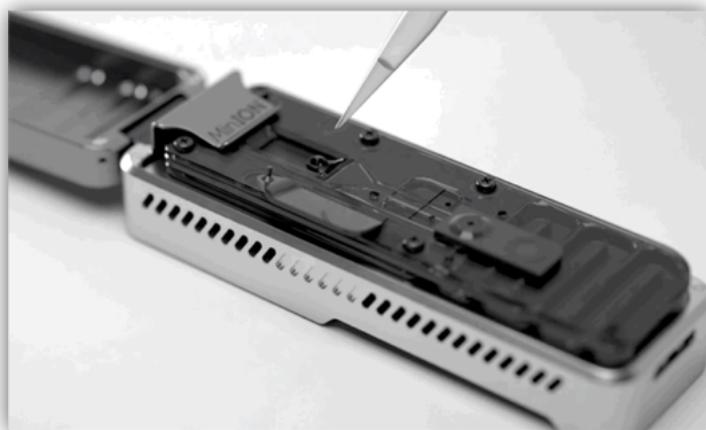
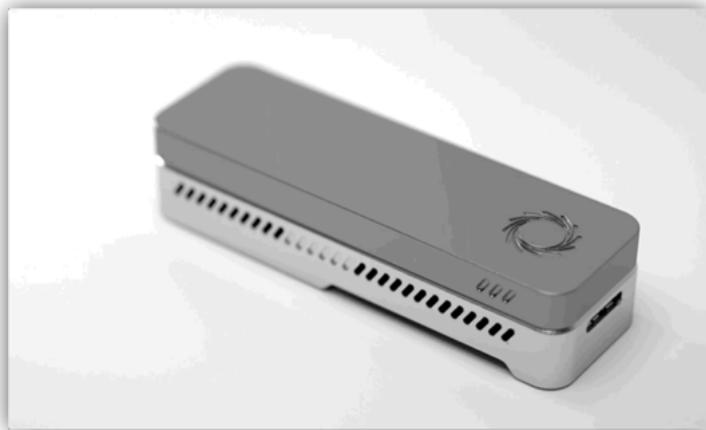
Calculate library concentration and dilute to
100ng/ μ l in 11 μ l

Combine 5.5 μ l of Flu library and 5.5 μ l of RSV
together

Add 1 μ l of rapid adapter and Adapter buffer

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Flow cell priming & Library loading



- Load 800 µl flow cell priming mix into the priming port, incubate for 5 minutes
- Add sequencing buffer and beads to the library.
- Load 200 µl of low cell priming mix into the priming port.
- Immediately load 75 µl of library mix dropwisely to the SpotON port.
- Data is obtained in real-time and stopped whenever enough data has been achieved

Analysis

- Primary analysis
 - Sequencing reads are de-multiplexed based on the barcode present
 - Sequencing reads are aligned to a reference genome using statistical analyses
 - Generates a consensus sequence based on these read alignments
- Secondary analysis
 - Consensus sequences are manually analysed for accuracy and error with context in mind
 - For example: SNPs, insertions and deletions

Questions?