Protocol for Sequence-Independent Single Primer Amplification (SISPA) and Nanopore Sequencing of RNA Viruses

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1. General Workflow

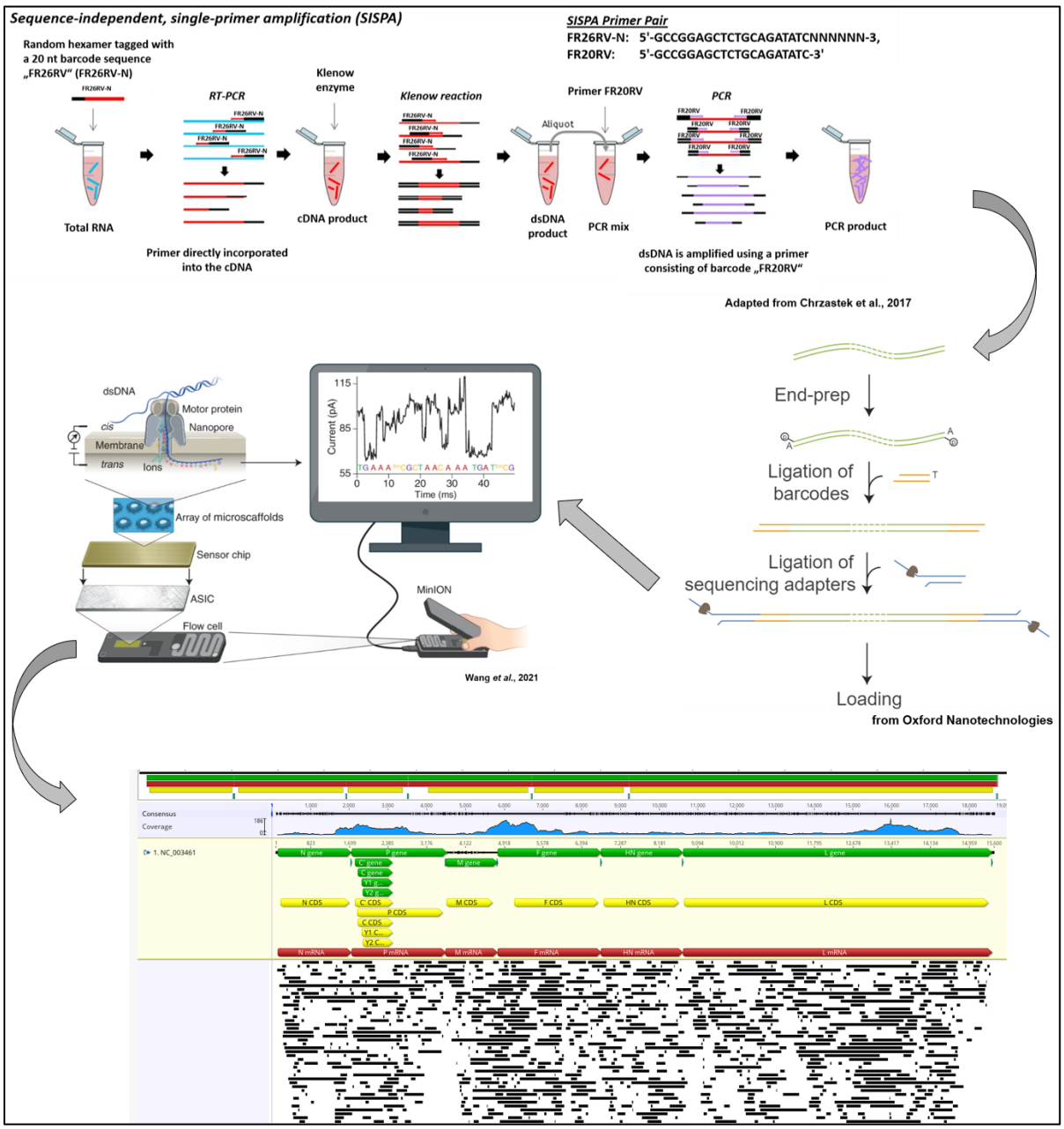


Figure 1: Schematic overview of the workflow for sequencing RNA viruses based on Sequence-independent single primer amplification (SISPA). In the first step the viral genome is reverse transcribed and amplified based on SISPA primers. Afterwards barcodes are attached to the DNA fragments and sequencing adapters ligated before loading the samples on the MinION nanopore sequencer. Adapted from Chrzastek et al., 2017 and Oxford Nanotechnologies.

2. Reagents and Material

2.1. Reagents / Kits / Chemicals

- *SISPA Primer*
  - 1st SISPA Primer: 5'-GCCGGAGCTCTGCAGATATCNNNNNN-3' (FR26RV-N)
  - 2nd SISPA Primer: 5'-GCCGGAGCTCTGCAGATATC-3' (FR20RV)
- *Klenow Polymerase* (DNA Polymerase I, Large (Klenow) Fragment, NEB #M0210S)
- *Super Script Reverse Transcriptase IV* (Invitrogen, #18090050)
  - SuperScript IV RT
  - 5x RT Buffer
  - 0.1 M DTT
- *RNaseOUT™ rekombinanter Ribonuklease-Hemmer* (Invitrogen, #10777019)
- *dNTP-Mix* (Thermo Scientific, #R0192)
- *PfU Ultra II Fusion HS DNA Polymerase* (Agilent, #600670)
  - PfU II HS DNA Polymerase
  - 10x PfU Ultra II reaction buffer
- *AMPure XP magnetic beads* (Beckman Coulter, #A63881)
- *Ligation Sequencing Kit* (Oxford Nanopore Technologies, #SQK-LSK109)
  - Adapter Mix (AM)
  - Ligation Buffer (LNB)
  - Long & Short Fragment Buffer (LFB & SFB)
  - Sequencing Buffer (SB)
  - Elution Buffer (EB)
  - Loading Beads (LB)
- *Native Barcoding Expansion 1-12* (Oxford Nanopore Technologies, #EXP-NBD104)
  - Adapter Mix II (AMII)
  - NB01 – NB12
- *Flow Cell Priming Kit* (Oxford Nanopore Technologies, #EXP-FLP002)
  - Flush Buffer (FB)
  - Flush Tether (FLT)
- *Flow Cell Wash Kit* (Oxford Nanopore Technologies, #EXP-WSH004)
  - Wash Mix (WMX)
  - Diluent (DIL)
- *NEBNext® Ultra™ II End Repair/dA-Tailing Module* (New England Biolabs, #E7546)
  - NEBNext Ultra II End Prep Enzyme Mix
  - NEBNext Ultra II Reaction Mix
- *NEBNext® Quick Ligation Module* (New England Biolabs, #E6056)
  - Quick T4 DNA Ligase
  - NEBNext Quick Ligase Reaction Buffer
- *NEBNext® Ultra™ II Ligation Module* (New England Biolabs, #E7546)
  - Ligation Enhancer
  - Ligation Master Mix
- *Ethanol*
- *Nuclease-free water*
- *Flow Cells R9.4.1* (Oxford Nanopore Technologies, # FLO-MIN106D)

2.2. Material

- MinION sequencer (e.g. Mk1C or Mk1B)
- Thermal cycler
- Optional: Thermoblock/ Thermomix
- Magnetic rack
- Benchtop centrifuge
- Vortex mixer
- Pipettes
- Pipette-Tips
- Racks
- Cooling block/ice
- 1.5 mL DNA LoBind Tubes
- 0.2 mL PCR-tubes
- External Drive / USB-Stick

3. SISPA and Library Preparation

Before using reagents make sure to spin them down shortly to avoid spill-over from the lids!!

3.1. cDNA Synthesis

Purpose: In the first step of the protocol, the isolated viral RNA is reverse transcribed in the presence of the first SISPA Primer, containing a barcode sequence followed by random hexamers. The random hexamers of the SISPA Primer anneal to the viral RNA during reverse transcription and the barcode sequence becomes integrated into the cDNA.

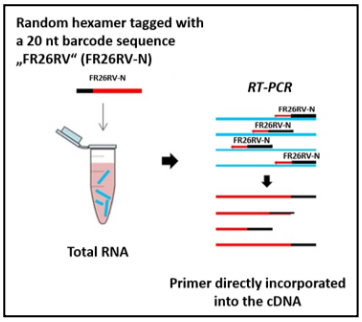


Figure 2: Reverse Transcription with the first SISPA Primer; adapted from Chrzastek et al, 2017.

Practical steps:

- Mix in a clean 0.2 ml PCR tube:
  - 1 µl dNTP Mix (10 mM)
  - 1 µl FR26RV-N (Stock = 50 µM)
    - For the preparation of primer stock, see point 6 in the supplements of the protocol **(p. 18)**
  - 11 µl Viral RNA (ideally the Cq should be <23-26, depending on the virus)
- *Incubate for 5 min at 65 °C in the thermal cycler. Place on ice for 1 min.*

- *Pre-warm 5x SSIV Buffer at 37 °C for 10 minutes before use, mix by vortexing and spin down.*
- Add per sample:
  - 4 µl SSIV buffer 5x
  - 1 µl DTT 100 mM
  - 1 µl RNase Inhibitor (RNase OUT; 40 U/µl)
  - 1 µl SSIV Reverse Transcriptase (200 U/µl)
- Close lids thoroughly, spin down quickly using bench top centrifuge
- Place in the thermal cycler: *23 °C for 10 min, 50 °C for 50 min, 80 °C for 10 min, Hold at 4 °C*

3.2. Double-strand synthesis

Purpose: After reverse transcription, the cDNA is single-stranded. The Klenow polymerase is used for the synthesis of double-stranded cDNA. As the first SISPA Primer is still in the reaction tube, the random hexamers will anneal to the single-stranded cDNA and serve as primers for this reaction. At the same time, the barcode sequences of the primer will be integrated also in the second strand. The Klenow polymerase lacks the (5'→3')-exonuclease activity and is thereby not able to displace the annealed primers during synthesis.

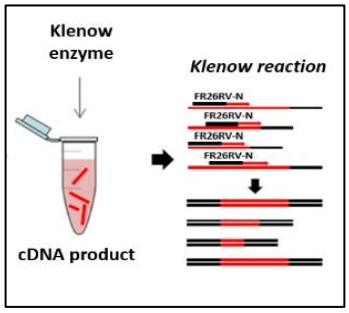


Figure 3: Klenow reaction to synthesize double-stranded cDNA; adapted from Chrzastek et al, 2017.

Practical steps:

- Add 1 µl of Klenow polymerase (5 U/µl) per sample directly to the tube.
- Close lids thoroughly, spin down quickly using bench top centrifuge
- Place in the thermal cycler: *37 °C for 60 min, 75 °C for 10 min*

3.3. cDNA Amplification

Purpose: The double-stranded cDNA is amplified in this step, in order to have sufficient genomic material for sequencing. With the barcode sequences of the first SISPA primer integrated into the cDNA in the previous steps, the second SISPA primer (only barcode sequence, no random sequence) is used for the amplification in a standard PCR.

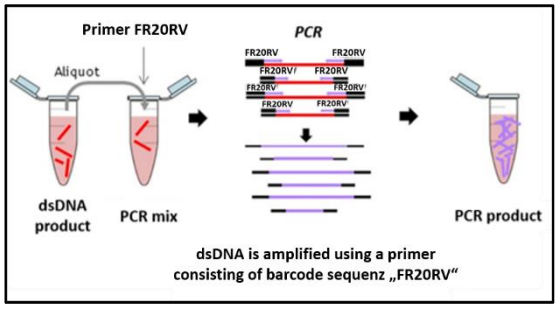


Figure 4: Amplification of cDNA using the second SISPA Primer; adapted from Chrzastek *et al*., 2017.

Practical steps:

- Mix:
  - 5 µl dsDNA
  - 1 µl FR20RV (Stock 40 µM, final concentration 0.8 µM)
  - 5 µl 10 X PfU Ultra II reaction Buffer
  - 1.25 µl dNTP Mix 10 mM
  - 1 µl PfU Ultra II Fusion HS DNA polymerase (keep on cooling rack)
  - 36.75 µl Nuclease-free Water
- Close lids thoroughly, spin down quickly using bench top centrifuge

- Place in the thermal cycler:

Denaturation	*95 °C*	*1 min*
45 Cycles	*95 °C*	*20 sec*
	65 °C	*20 sec*
	72 °C	*3 min*
Final Extension	*72 °C*	*3 min*
Hold	*4 °C*	

3.4. Cleanup of cDNA Amplicons

Purpose: Enzymes, salts and excess primers are removed by cleaning up the amplified cDNA with magnetic beads. The cDNA binds to the magnetic beads and is washed with ethanol, before the elution of the cDNA in nuclease-free water for subsequent library preparation.

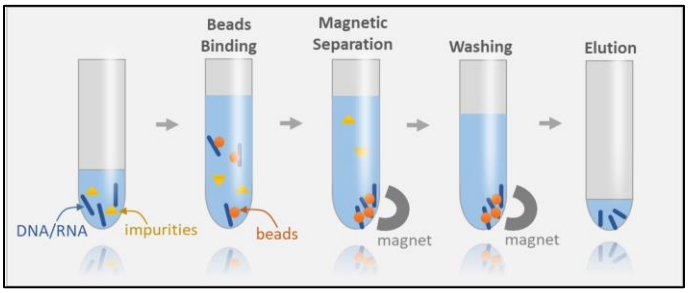


Figure 5: General procedure for the purification of DNA with magnetic beads. From biodynamic.com.

Practical steps:

- Transfer 25 µl of the dsDNA to a 1.5 mL DNA LoBind tubes; the spare dsDNA should be stored at 4 °C as backup.
- Bring AMPure beads to room temperature. Mix the beads thoroughly before use! Add 1.8 x (sample volume) beads, e.g. for 25 µl sample add 45 µl beads;
- Mix sample thoroughly by pipetting up and down at least 15 x
- Incubate at room temperature for 7 min on a thermomix (room temperature, 550 rpm)
- Quickly centrifuge
- Place tubes in the magnetic stand and leave tubes in the magnetic rack until elution **(!)**
- After 5 minutes remove the supernatant slowly; hold the tube with one hand while opening the cap!
- Add 1 ml 80 % ethanol and incubate for 30 sec at room temperature
- Slightly rotate the tubes in the magnetic rack to concentrate the beads
- Slowly remove the supernatant and discard the ethanol
- Add another 1 ml 80 % ethanol and incubate for 30 sec at room temperature
- Slowly remove the supernatant and discard the ethanol
- Remove all remaining ethanol with a 10 µl pipet and a clean tip and let air dry for 10 min with the tube opened
- Elution
    - Take the tube from the magnetic stand and add 25 µl nuclease-free water directly onto the beads. Resuspend the beads by pipette mixing.
    - Incubate 2 min at room temperature
    - Place tube on magnetic stand and incubate for 10 min
    - Transfer the eluate containing the DNA into a clean reaction tube

3.5. End Prep and Barcode Ligation

Purpose: To be able to sequence different samples within a single run, a unique barcode sequence is adapted to the ends of the amplified cDNA for each sample. Therefore, a 3'- adenine overhang is added to the blunt ends in the first step, while the barcodes hold a complementary thymine overhang. Thereby, the barcodes are ligated to the corresponding cDNA. After the barcodes are ligated, the samples can be identified after sequencing and hence all samples can be pooled for the later steps.

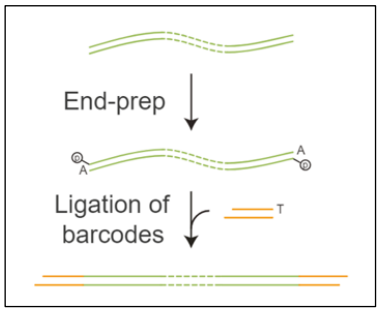


Figure 6 End-Prep and Ligation of Barcodes after the clean-up of amplified cDNA; taken from Oxford Nanotechnologies

Practical steps:

- Mix in a clean 1.5 ml DNA LoBind tube:
  - 10 µl DNA
  - 1.4 µl Ultra II End Reaction Buffer
  - 0.6 µl Ultra II End Prep Enzyme Mix (keep in cooling rack or on ice)
- Incubate using thermomix: *10 min Room Temperature, 5 min 65 °C, Place on ice 1 min.*

- **Directly add:**
  - 2.5 µl NB(XX) barcode
    - e.g. Sample 1 = Barcode 06; Sample 2 = Barcode 07; Sample 03 = Barcode 08
    - **Note down which barcode was used for which sample (!)**
  - 14,5 µl Ultra II Ligation Master Mix
  - 0,5 µl Ligation Enhancer
- Incubate using thermomix: *20 min Room Temperature, 10 min 70 °C, Place on ice 1 min*

- **POOL ALL SAMPLES** in a single tube!

3.6. Cleanup of barcoded DNA

Purpose: Similar to step 3.4., enzymes, salts and excess nucleotides are removed by cleaning up the amplified cDNA with magnetic beads.

Practical steps:

- Add 29.5 µl AMPure XP beads **per sample** [equal volume (1:1) sample:beads]
- Mix by pipetting and quickly centrifuge
- Incubate for 5 min at room temperature
- Place the sample on the magnetic rack
- Incubate for at least 2 min until the supernatant is completely clear
- Carefully remove the supernatant
- Add 300 µl 70% ethanol to the tube still on the magnetic rack
- Incubate 30 secs
- Carefully remove the supernatant
- Repeat last three steps (*2nd ethanol wash*)
- Spin down and remove residual ethanol with the tube placed on the magnetic rack using a clean filter tip and a pipette.

- Air dry 1 min
- Resuspend in 30 µl EB by pipetting
- Incubate off the magnetic rack for 2 min
- Replace on magnetic rack
- Transfer eluted DNA to a fresh 1.5 mL DNA LoBind tube
- Label tube accordingly with an ethanol-resistant labmarker

3.7. Sequencing Adapter Ligation

Purpose: After purification of the barcoded DNA, sequencing adapters are ligated at each side of the fragment. The sequence of the adapter is also the binding site for the motor protein that translocates the DNA through the nanopores during sequencing.

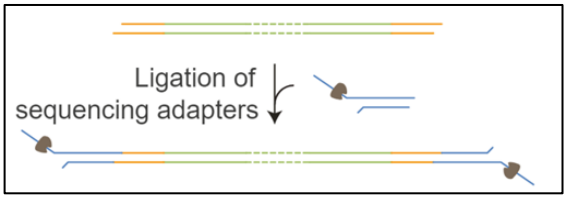


Figure 7 Ligation of the sequencing adapter to the barcoded and purified DNA; from Oxford Nanotechnologies.

Practical steps:

- Mix in a clean 1.5ml DNA LoBind tube for Adapter Ligation:
  - 30 µl Barcoded and purified DNA
  - 10 µl NEBNext Quick Ligation Reaction Buffer
  - 5 µl Adapter Mix II (from EXP-NBD104)
  - 5 µl NEBNext Quick T4 Ligase (keep on cooling rack)

- Flick tube, spin down an *incubate at RT for 20 min*

3.8. Cleanup of ligated DNA

Purpose: The cDNA library is now prepared and purified one last time to remove salts, enzymes and excess nucleotides. In contrast to the previous washing steps, the provided fragment buffer is used instead of ethanol. Please note, that short as well as long fragment buffer is provided in the sequencing kit (SFB vs. LFB). For DNA fragments shorter than ~3 kb the short fragment buffer (SFB) is recommended. SISPA typically produces fragments shorter than 1 kb hence SFB is used in this protocol.

- Add 50 µl AMPure XP beads to the sample and mix by pipetting
- Spin down quickly
- Incubation at room temperature for 5 min
- Place tube on the magnetic stand
- Incubate for 2 min or until the supernatant is completely clear at room temperature
- Slowly remove the supernatant
- Add 200 µl Short Fragment Buffer (SFB; in *Ligation Sequencing Kit*) and remove tube from magnetic stand, mix by pipetting, and quickly spin down)
- Place tube on the magnetic stand
- Remove supernatant after 2 minutes
- Repeat the steps for a *second wash*
- Remove remaining SFB with a 10 µl pipette
- Remove tube from magnetic stand and add 15 µl Elution Buffer (EB; in *Ligation Sequencing Kit*)
- Mix by pipetting

- Incubation at room temperature for 2 min
- Place tube on the magnetic stand for 2 mins
- Transfer eluted DNA to a fresh 1.5 mL DNA LoBind tube

4. Sequencing

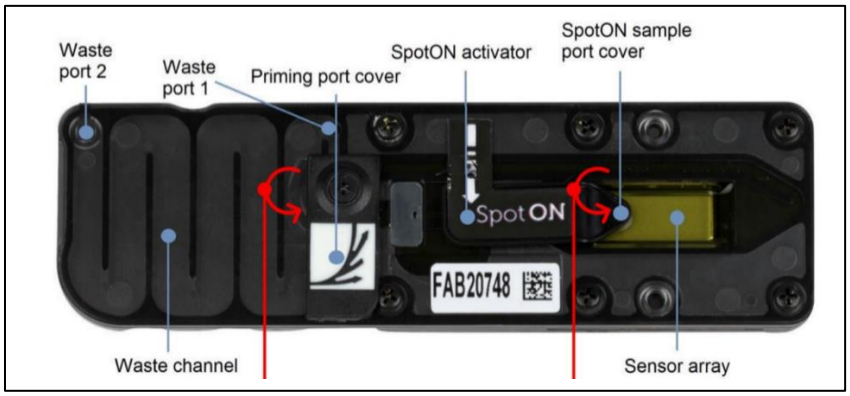
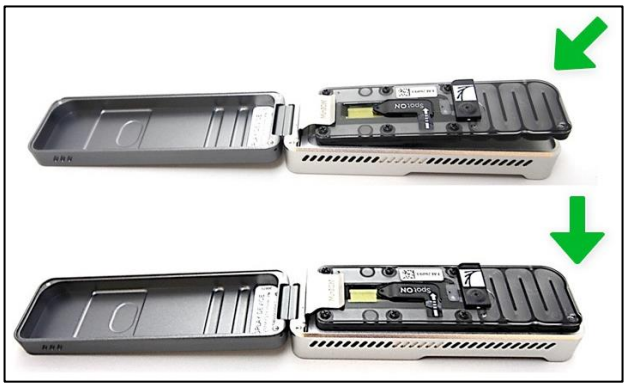


Figure 8: Schematic overview of the flow cell structure; from Oxford Nanotechnologies.

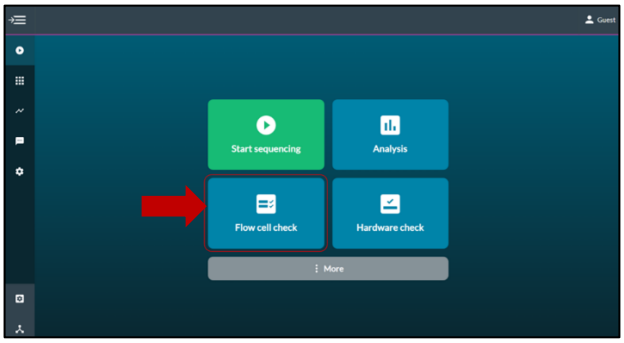
4.1. Flow Cell Check

Purpose: Before every sequencing run, the flow cell needs to be checked for the number of active pores available for sequencing. Within the warranty period (3 month after purchase), the minimum number of active pores should be 800. If there are less pores, the flow cells can be exchanged for new flow cells within this period of warranty!

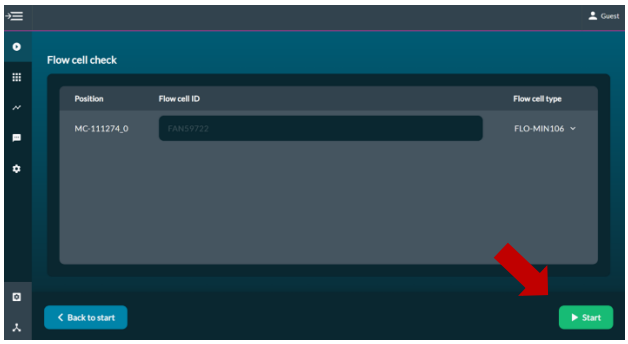
- Open the MinION and place the flow cell under the clip:



- On the Start page of the software, select “Flow Cell Check”



- The flow cell type and ID should be automatically recognized, click “Start”

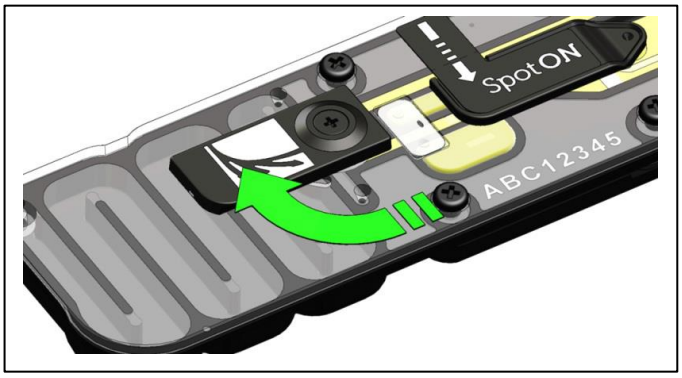


- A loading bar will be displayed under the flow cell during the check on the Sequencing Overview page

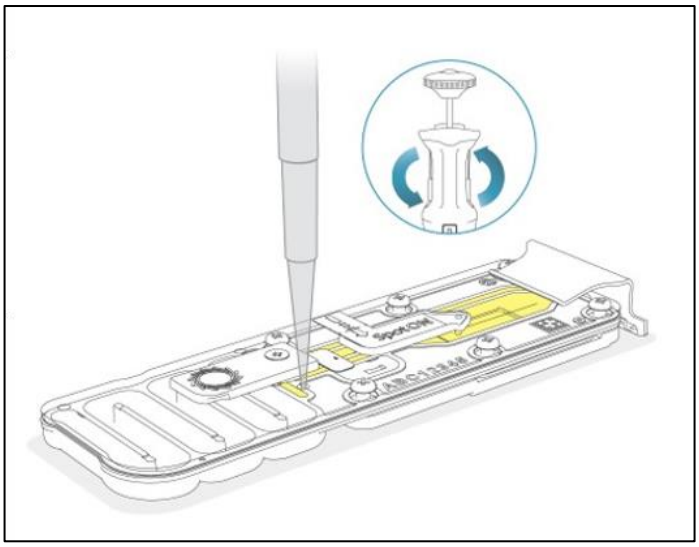
4.2. Priming and Loading of the Flow Cell

*****Please note: if you're unsure about handling flowcells, take an old flowcell instead and spend a few minutes practicing with water. Once you're comfortable with the steps, you can load the real samples. ******

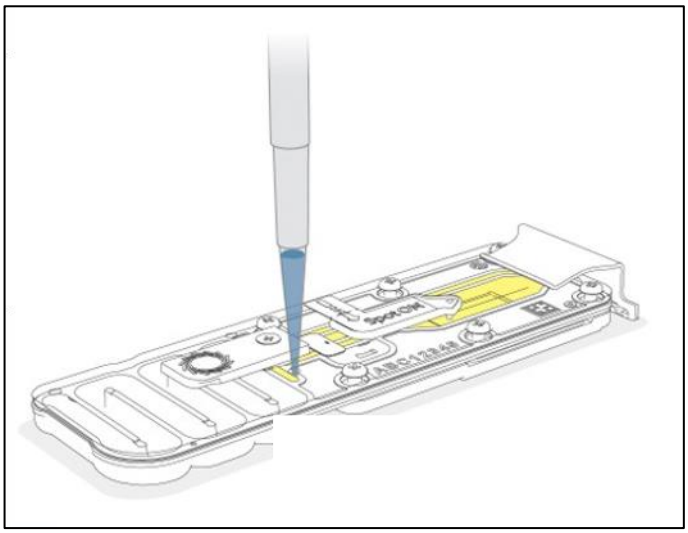
- From the *Ligation Sequencing Kit* thaw the Sequencing Buffer (SQB) & Loading Beads (LB)
- From the *Flow Cell Priming Kit* thaw the Flush Buffer (FB) & Flush Tether (FLT)
- Mix the SQB, FLT, and FB by vortexing and quickly centrifuge
- Prepare the flow cell **priming mix**:
    - Add 30 µl FLT to one complete tube of FB
    - Mix by vortexing and quickly centrifuge
- Slide the priming port clockwise **(!)** to open the priming port:



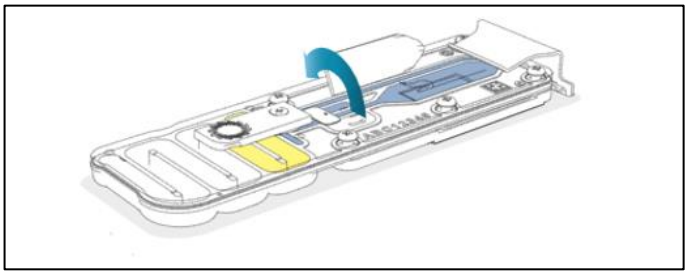
- To avoid the introduction of air bubbles, draw back any air from the priming port:
    - Set a 1000 µl pipette to 200 µl
    - Insert the tip vertically (!) into the priming port
    - Draw back around 20 – 30 µl until you can see a small amount of buffer entering the tip



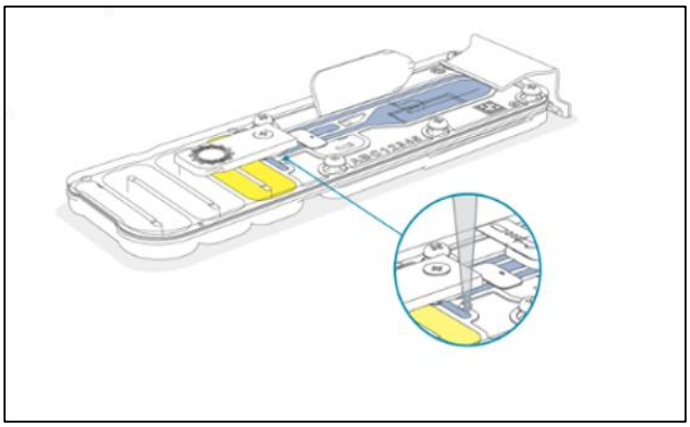
- Load 800 µl of the priming mix (FB + FLT) into the flow cell through the **priming port**. Take care not to introduce any air bubbles into the flow cell:



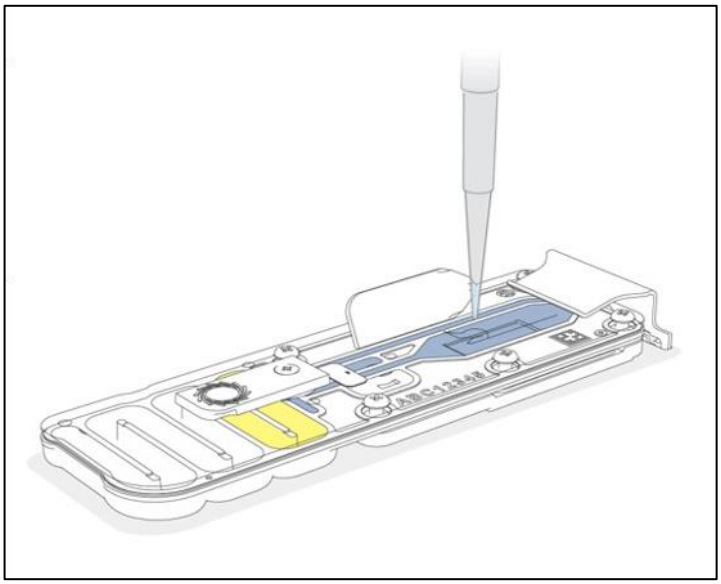
- Incubate the flow cell for 5 min
- Meanwhile, prepare the library for sequencing; mix:
  - 37.5 µl Sequencing Buffer (SQB)
  - 25.5 µl Loading beads (LB); mix thoroughly by vortexing/pipetting before use
  - 12.0 µl DNA Library from **step 3.8**
- Open the **sample port cover**



- Load another 200 µl of priming mix via the **priming port** as described above:



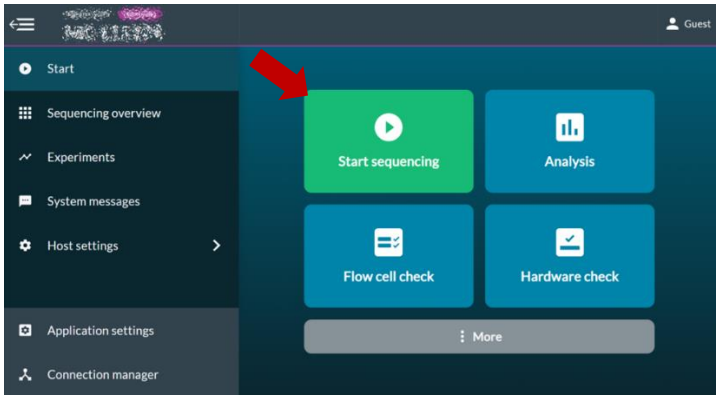
- Mix the prepared library by pipetting and add 75 µl of the library directly to the **sample port** in a dropwise fashion:



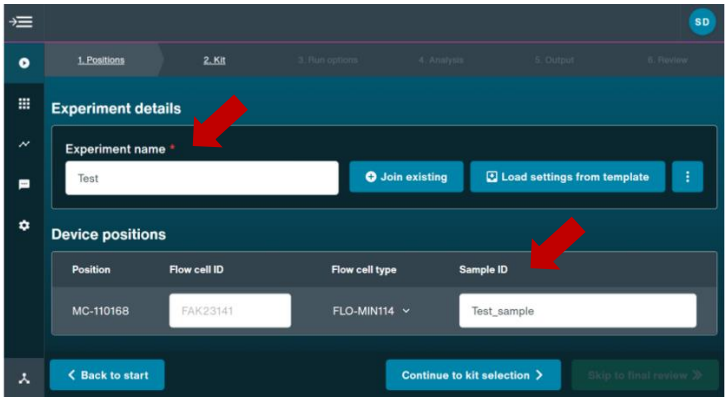
- Cover the **sample port** with the sample port cover
- Close the **priming port** and MinION lid

4.3. Start of Sequencing Run

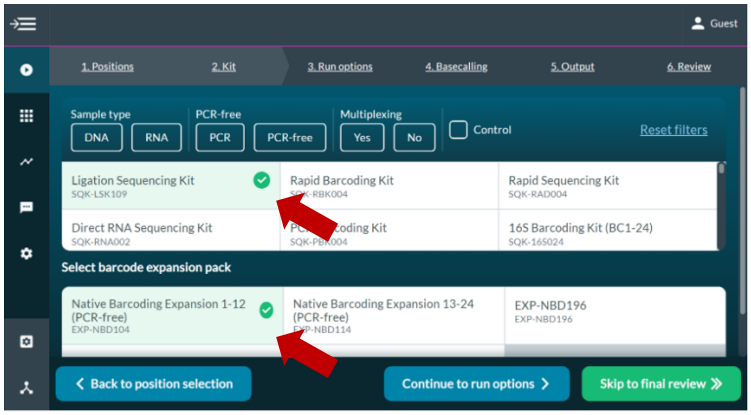
- On the start homepage, click “Start sequencing”



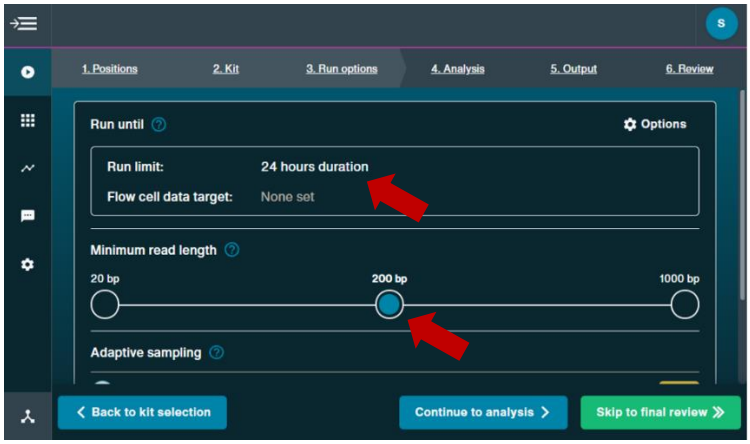
- Type in the experiment name and sample ID: **Choose an experiment name that clearly allows to identify the samples at later timepoints**: e.g. Date_Experiment_Virus, 20230919_IMBTraining_RFV. In the next window, then press “Continue to kit selection”



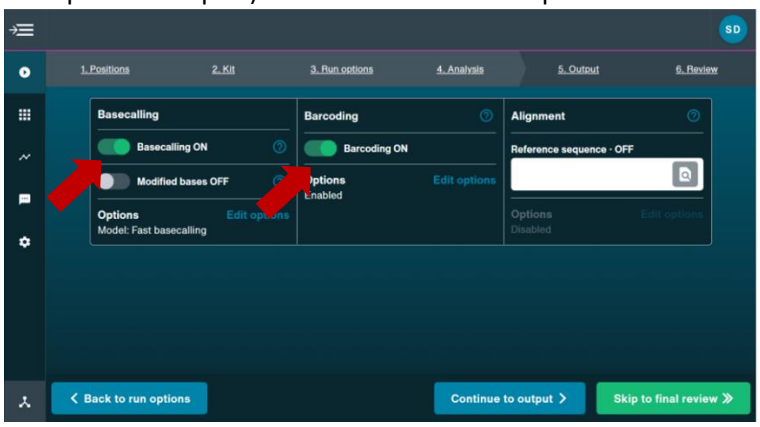
- In the next tab, select the sequencing and barcode expansion kit used during library preparation; e.g. **for this protocol: SQK-LSK109 and EXP-NBD104**. Afterwards, click “Continue to run options”



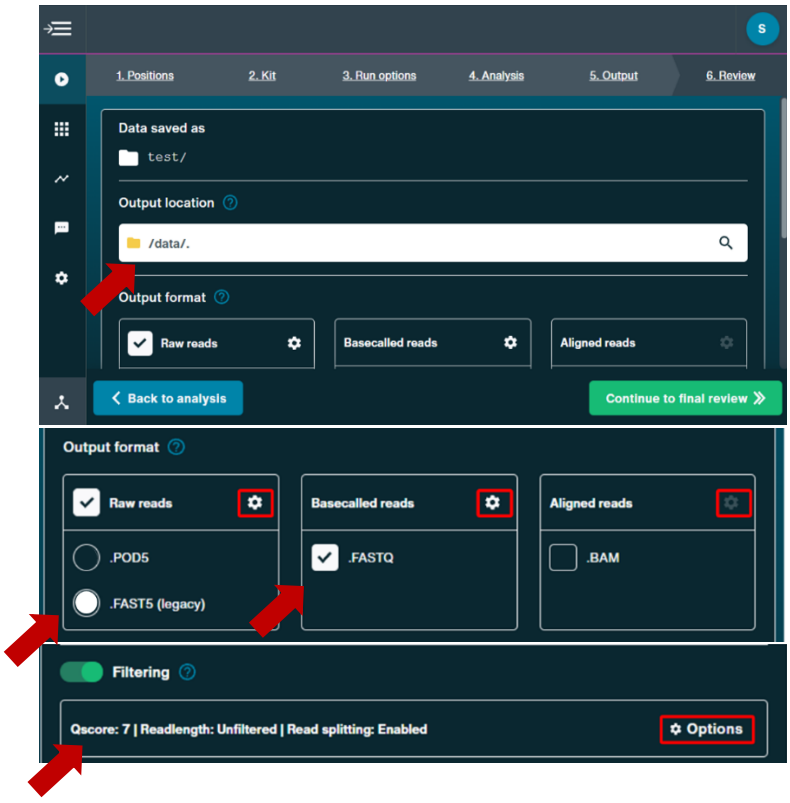
- Set the run options, including run time and minimum read length. For this protocol, you can use a run time for **72 hours** and a minimum read length of **200 bp**.
  Please ensure a continuous power supply for this time. Regarding the run time, the run can be stopped earlier at any time point, for example if no more reads are generated. Press “Continue to analysis”.



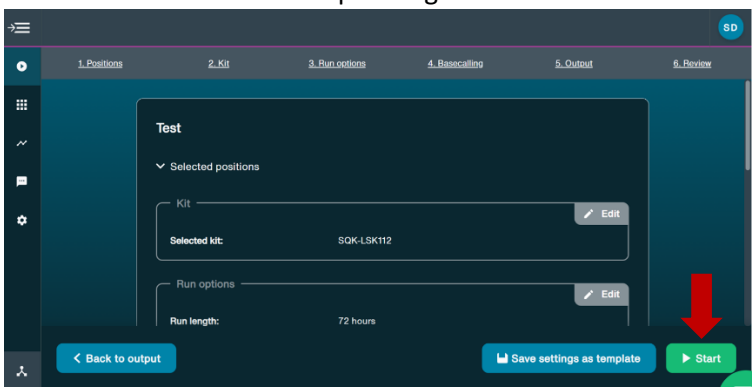
- In the analysis window, choose “Basecalling ON” (Fast basecalling) and “Barcoding ON” (for multiplexed samples). Press “Continue to output”.



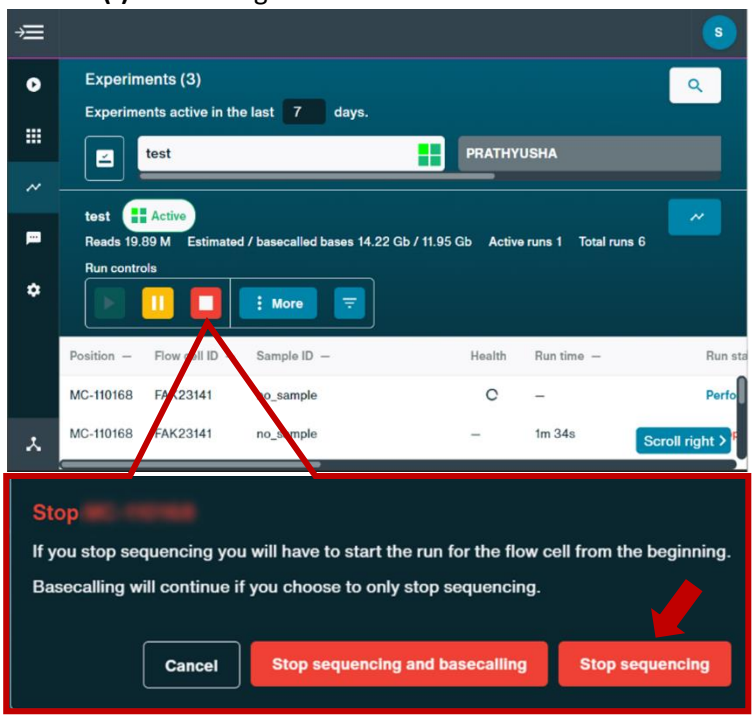
- Select output data location, format and filtering options in the output tab. Select both raw reads as **.FAST5** as well as basecalled reads as **.FASTQ** as output formats. The number of **reads per file** can be set to **4,000** (default) and the **quality score** to **7** (default). Afterwards, click “Continue to final review”.



- On the Review page, re-check all settings. Click “Edit” in case something needs to be changed. Press “Start” to start the sequencing run.



- When sequencing starts, the Sequencing Overview is displayed. Here, the flow cell’s health, passed and failed reads, assignment to barcodes etc. can be checked, while sequencing progresses.
- In case you want stop a run before the end of the set run time, navigate to the experiments page and press the stop button on the running experiment. Take care to only stop sequencing but not **(!)** basecalling.



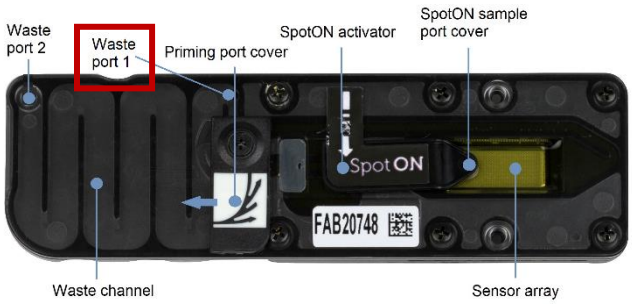
- **Wash the flow cell and add storage buffer (4.4. & 4.5.) if the flow cell should be used for a second sequencing run!**

4.4. Flushing of Flow Cells

Purpose: Whenever not all pores were depleted during the sequencing run, the flow cells can be re-used for sequencing. Therefore, it is however necessary to wash the flow cell and remove the waste. Storage buffer is added, allowing the storage of the flow cell at 4- 8 °C. As also performed for "new" flow cells, a flow cell check (4.1.) is mandatory before starting a new sequencing run!

Practical steps:

- Place a tube of Wash Mix (WMX, in *Flow Cell Wash Kit*) on ice. Do not vortex the tube.
- Thaw one tube of Wash Diluent (DIL, in *Flow Cell Wash Kit*) at room temperature.
- Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, spin down briefly and place on ice.
- In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix:
  - 2 µl Wash Mix (WMX)
  - 398 µl Wash Diluent (DIL)
- Mix well by pipetting, and place on ice. Do not vortex the tube.
- Stop or pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.
- Before removing the waste fluid, ensure that the **flow cell priming port cover** and **SpotON sample port cover** are **closed**.
- Using a P1000, remove all fluid from the waste channel through **Waste port 1 (see picture on next page)**. As both the flow cell priming port and SpotON sample port are closed, no fluid should leave the sensor array area.



As described in 4.2.:

- Rotate the flow cell priming port cover clockwise so that the priming port is visible.
- To avoid the introduction of air bubbles, draw back any air from the priming port:
  - Set a 1000 µl pipette to 200 µl
  - Insert the tip vertically (!) into the **priming port**
  - Draw back around 20 – 30 µl until you can see a small amount of buffer entering the tip
- Load 400 µl of the prepared Flow Cell Wash Mix into the flow cell **priming port**, avoiding the introduction of air.
- Close the flow cell priming port and incubate for 60 min
- Remove the waste fluid a second time through Waste port 1 while the priming port cover and sample port cover are closed

4.5. Storage of MinION Flow Cell

- Thaw one tube of Storage Buffer (S, in *Flow Cell Wash Kit*) at room temperature.
- Mix contents thoroughly by pipetting and spin down briefly.
- Rotate the flow cell priming port cover clockwise so that the priming port is visible.

- After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
  - Set a P1000 pipette to 200 µl.
  - Insert the tip into the flow cell priming port.
  - Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer/liquid entering the pipette tip.
  - Visually check that there is continuous buffer from the flow cell priming port across the sensor array.
- Slowly add 500 µl of Storage Buffer (S) through the flow cell priming port.
- Using a P1000 pipette, remove all fluid from the waste channel through **waste port 1**. As both the flow cell priming port and SpotON sample port are closed, no fluid should leave the sensor array area
- The flow cell can now be stored at 4-8 °C

4.6. Data Management

Purpose: For the analysis of the samples, data needs to be transferred to the corresponding working station. For Mk1B sequencing devices, the sequencer is already connected to the computer, and data can be copied directly to an external drive. The default storage folders are:

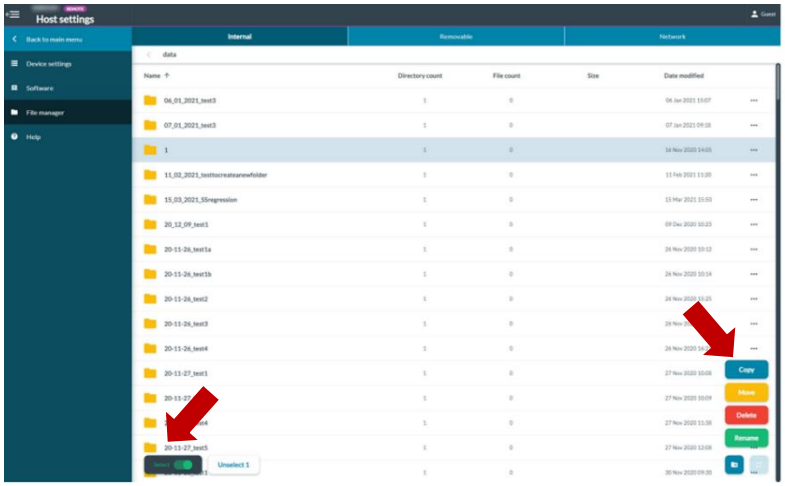
Windows: C:\data\"Experiment Name"

Mac OS X: /Library/MinKNOW/data

Linux: /var/lib/MinKNOW/data

An external drive as well as a USB-stick with sufficient storage capacity can be plugged into the Mk1C sequencer and copied as follows:

- Navigate to the "File manager" in the device settings, toggle on "Select" and select the folder with the corresponding experiment.
- Click "Copy" to open a new window with a list of available external drives



- Choose a drive and destination for the files and click "Copy" to confirm.

5. Literature

5.1. Publications

- Original publication with SISPA primers used in this protocol: Peserico A, Marcacci M, Malatesta D, Di Domenico M, Pratelli A, Mangone I, D'Alterio N, Pizzurro F, Cirone F, Zaccaria G, Cammà C, Lorusso A. *Diagnosis and characterization of canine distemper virus through sequencing by MinION nanopore technology*. Sci Rep. 2019 Feb 8;9(1):1714. doi: 10.1038/s41598-018-37497-4. PMID: 30737428; PMCID: PMC6368598.
- Publication on sequencing of arboviruses in Africa using SISPA and MinION: Schulz, Ansgar, Balal Sadeghi, Franziska Stoek, Jacqueline King, Kerstin Fischer, Anne Pohlmann, Martin Eiden, and Martin H. Groschup. 2022. "*Whole-Genome Sequencing of Six Neglected Arboviruses Circulating in Africa Using Sequence-Independent Single Primer Amplification (SISPA) and MinION Nanopore Technologies*" Pathogens 11, no. 12: 1502. https://doi.org/10.3390/pathogens11121502
- SISPA for the identification of avian RNA viruses: Chrzastek K, Lee DH, Smith D, Sharma P, Suarez DL, Pantin-Jackwood M, Kapczynski DR. *Use of Sequence-Independent, Single-Primer-Amplification (SISPA) for rapid detection, identification, and characterization of avian RNA viruses*. Virology. 2017 Sep;509:159-166. doi: 10.1016/j.virol.2017.06.019. Epub 2017 Jun 21. PMID: 28646651; PMCID: PMC7111618.
- Nanopore sequencing for Ebola surveillance in the field laboratory: Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, Cowley L, Bore JA, Koundouno R, Dudas G, Mikhail A, Ouédraogo N, Afrough B, Bah A, Baum JH, Becker-Ziaja B, Boettcher JP, Cabeza-Cabrerizo M, Camino-Sanchez A, Carter LL, Doerrbecker J, Enkirch T, Dorival IGG, Hetzelt N, Hinzmann J, Holm T, Kafetzopoulou LE, Koropogui M, Kosgey A, Kuisma E, Logue CH, Mazzarelli A, Meisel S, Mertens M, Michel J, Ngabo D, Nitzsche K, Pallash E, Patrono LV, Portmann J, Repits JG, Rickett NY, Sachse A, Singethan K, Vitoriano I, Yemanaberhan RL, Zekeng EG, Trina R, Bello A, Sall AA, Faye O, Faye O, Magassouba N, Williams CV, Amburgey V, Winona L, Davis E, Gerlach J, Washington F, Monteil V, Jourdain M, Bererd M, Camara A, Somlare H, Camara A, Gerard M, Bado G, Baillet B, Delaune D, Nebie KY, Diarra A, Savane Y, Pallawo RB, Gutierrez GJ, Milhano N, Roger I, Williams CJ, Yattara F, Lewandowski K, Taylor J, Rachwal P, Turner D, Pollakis G, Hiscox JA, Matthews DA, O'Shea MK, Johnston AM, Wilson D, Hutley E, Smit E, Di Caro A, Woelfel R, Stoecker K, Fleischmann E, Gabriel M, Weller SA, Koivogui L, Diallo B, Keita S, Rambaut A, Formenty P, Gunther S, Carroll MW. *Real-time, portable genome sequencing for Ebola surveillance*. Nature. 2016 Feb 11;530(7589):228-232. doi: 10.1038/nature16996. Epub 2016 Feb 3. PMID: 26840485; PMCID: PMC4817224.
- Review on Nanopore sequencing and applications:Wang, Y., Zhao, Y., Bollas, A. et al. *Nanopore sequencing technology, bioinformatics and applications.* Nat Biotechnol 39, 1348–1365 (2021). https://doi.org/10.1038/s41587-021-01108-x

5.2. Online Resources

- The Oxford Nanopore Community offers a wide variety of information and also detailed protocols for sequencing of different specimen. Furthermore, you can find online tutorials and videos about the techniques and theoretical background. You need to create a free account to get access: https://nanoporetech.com/community

- Online version of the protocol for library preparation (barcoding, adapter ligation): https://www.protocols.io/view/one-pot-native-barcoding-of-amplicons-e6nvw6617gmk/v1

- Database for viral reference genomes: https://rvdb.dbi.udel.edu/

- User Manual for MinION Mk1C (you need to create a free account to access): https://community.nanoporetech.com/docs/prepare/library_prep_protocols/minion-mk1c-user-manual/v/mkc_2005_v1_revae_27nov2019

6. Supplements

Preparation of primers:

- The lyophilized Primer contains 20 nmol and should be dissolved to 100 µM in nuclease-free water:
    - c= concentration (100 µM), n= substance quantity (20 nmol); V= the volume of nuclease free water for dissolvement
    - $c = \frac{n}{V}$
    - $V = \frac{n}{c}$; $V = \frac{20\ nmol}{100\ \mu mol/l}$; $V = \frac{20\ nmol}{100000\ nmol/l}$; $V = 0{,}0002\ l = 200\ \mu l$
    - Dissolve the lyophilized primers in *200 µl nuclease free-water*
    - Prepare aliquots of *10 µl à 100 µM*
- If the working concentration is e.g. *50 µM*
    - $c1 * V1 = c2 * V2; V1 = \frac{c2*V2}{c1} = \frac{100\ \mu M*10\ \mu l}{50\ \mu M} = 20\ \mu l$
    - Add 10 µl nuclease-free water to the 10 µl aliquots ($20\ \mu l - 10\ \mu l = 10\ \mu l$)
- If the working concentration is e.g. *40 µM*
    - $c1 * V1 = c2 * V2; V1 = \frac{c2*V2}{c1} = \frac{100\ \mu M*10\ \mu l}{40\ \mu M} = 25\ \mu l$
    - Add 15 µl nucleas-free water to the 10 µl aliquots ($25\ \mu l - 10 \mu l\ =\ 15\ \mu l$)