Laboratory Practical

Date started: _____

Group ID: _____

Over the next 2-days you will collectively prepare a library for nanopore sequencing. The samples you will use are rabies virus samples from Tanzania, which have been pre-prepared to amplicon stage, i.e. cDNA synthesis and multiplex PCR have already been done. This step is too time-consuming for the workshop!
(Abbreviations: NFW, nuclease free water; RT, room temperature; NTC, negative template control*)*

Samples

Work in your assigned group to prepare a set of samples for sequencing. Note that each group will prepare a NTC (label your group id), which is NFW. Record sample ids and other information in the table below:

Table 1. List of samples		
Sample ID	**Sample handler**	**Barcode**
NTC-gp___		

Part I: Amplicon preparation

Amplicons have been prepared in advance for you. We will use a protocol that does not include a sample normalisation step, i.e. a quick and dirty version. It still works!

1. For each sample, combine Pool A and B into one tube
2. Take a Quantus measurement to get the concentration of each sample. This should be >2ng/ul to continue with library prep.

3. Prepare 1:10 dilutions:
    a. Prepare a fresh set of PCR tubes (one per sample) and label
    b. Aliquot 45µl nuclease-free water to each
    c. Add 5µl of pooled amplicons
4. Proceed to nanopore library prep

Part II: Nanopore library preparation

These steps are specific to nanopore library preparation, based on using the specific kit SQK-LSK114. This library method is called sequencing by ligation with native barcodes.

A. One-pot end-preparation and barcoding

In this version of the protocol (relative to nanopore's) we perform a "one-pot" reaction, which does not have a clean-up step between end repair and barcoding. This makes things faster and is widely used. If you have particularly precious samples and/or you want to optimize the results as much as possible, you might want to consider nanopore's version instead of a one-pot.

End repair and dA-tailing

1. Set up an end-prep reaction for each sample. Calculate and prepare a master mix according to the number of samples (plus 10% excess). Take care when pipetting as reagents are viscous.

(e.g. if one has 10 samples, the mastermix calculation for NFW will be 3.5 x 10.1=35.4)

Table 2. End-prep reaction

Component	Volume per reaction (µL)	Master mix volume (µL)
NFW	3.5	
End Prep Reaction buffer	1	
End Prep enzyme mix	0.5	

TOTAL	5	

2. Add 5 µL of master mix into each tube of pooled DNA (5 µL). The total reaction mix should be 10 µL. Change tips each time and only have one tube open at a time.
3. Incubate in a thermal cycler under the conditions below:

Table 3. Thermal cycler conditions for End-prep reaction	
Temp	**Time**
20°C	5 min
65°C	5 min
4°C	1 min

Barcoding

4. Collect your group's barcodes, assign each to a sample and record in Table 1.
5. Prepare a fresh set of PCR tubes (one per sample) and label
6. Aliquot 1.25 µL of each barcode into the corresponding labelled tube (according to Table 1)
7. Add 0.75 µL of the end-prepped sample to its assigned barcode aliquot.
8. Prepare a ligation master mix according to the number of samples (plus 10% excess) (**Table 4**).

Table 4. Master mix for Barcoding		
Component	**Volume per reaction (µL)**	**Mastermix volume (µL)**
NFW	3	
Ligation Master Mix	5	
TOTAL	**8**	

9. Add 8 µL of ligation master mix to the tube that contains the end-prepped sample + barcodes, giving a total reaction of 10 µL.
10. Incubate at 20 °C in a thermal cycler for 20 min
11. Add 2 µL of EDTA to each reaction tube, leave for 2mins.
12. Put tubes on ice for 5mins

B. SPRI bead clean up and DNA quantification

At this point we ask two groups to combine their samples. This is to ensure we can elute in a large enough volume after clean-up. Join your groups as follows:

- Groups 1&2
- Groups 3&4

1. Pool all the barcoded samples together in a 1.5 mL lobind microcentrifuge tube. The total volume should be 120 µL, i.e. the number of samples in your combined group x final reaction volume in Step B.
2. Add a 0.4x volume (i.e. 48 µL) of AMPure XP Beads (AXP) to the barcoded pool. Mix gently (flicking or pipetting) and incubate at RT for 5 min
   Make sure to fully resuspend the AXP beads before using.
3. Place the tubes on a magnetic rack until the beads have pelleted and the supernatant is completely clear (~2 min). Remove and discard the supernatant. Take care not to disturb the beads.
4. Wash twice with 250 µL of SFB (note: during workshop we used 125 µL because we didn't have enough SFB).
   a) Remove the tube from the magnet and completely resuspend the pellet in 250 µL of SFB. Incubate for 30s, pulse centrifuge, and return to the magnet.
   b) Remove the supernatant and discard.
   c) Repeat a-b to perform a second SFB wash
5. Pulse centrifuge and remove any residual SFB.

6. Add 200 µL of 80% (RT) ethanol to bathe the pellet. Remove and discard the ethanol, being careful not to disturb the bead pellet. Air-dry for 30 s or until the pellet has lost its shine.
7. Resuspend in 18 µL of NFW and incubate at 37°C for 10 min.
8. Place on the magnet, leave to settle for ~2 min, then carefully remove the solution and transfer to a clean 1.5 mL microcentrifuge tube. Make sure to label with your collective group ID.
9. Quantify 1 µL on the Quantus machine and record below

Pool (ng/ul) [ ]

Optional pause point: At this point, the library can be stored at 4 °C for up to 1 week or -20 °C for longer-term storage, but it is preferable to continue with adapter ligation and sequencing.

C. Adapter ligation

As we are preparing only one library we have combined 15 µL from each mega-group into one tube. This one tube now contains all of the pooled, barcoded, cleaned samples. The final step is an adapter ligation.
To allow each group to go through the motions of the adapter ligation step, one group will be assigned the “real” library and the others will process “fake” libraries. You will not be told who has the real library so continue to prepare with care and attention!

1. Pulse centrifuge the adapter mix and ligation enzyme and place on ice.
2. Mix elution buffer (EB), SFB, and ligation buffer at RT by vortexing or flicking the tube, pulse centrifuge, and place on ice.
3. Calculate the volume of barcoded amplicon pool required to obtain 200fmol of final library and add to the table below. If required make up to 30 µL with NFW.
4. Prepare the adapter ligation master mix (**Table 5**), combining reagents in the specified order in a lobind tube. With each addition to the tube pipette mix 10-20 times.

Table 5. Adapter ligation reaction	
Component	**Volume per reaction (µL)**
Barcoded amplicon pool	____*
NFW (to make up to 30 µL)	____*
Adapter Mix	5
NEBNext Quick Ligation Reaction Buffer (5X)	10
Quick T4 DNA Ligase	5
TOTAL	**50**
* 100–200 fmol of final library is optimal	

5. Mix by gentle flicking and pulse centrifuge. Incubate at RT for 20 min.

D. Clean up using SPRI beads (do not use ethanol as in earlier clean ups).

1. Add 20 µL volume of AMPure XP Beads (AXP) to the completed adaptor ligation reaction. Incubate at RT for 10 min, gently flick intermittently to aid mixing.
2. Place on the magnet until the beads and solution have fully separated (~5 min). Remove and discard the supernatant; take care not to disturb the bead pellet.
3. Wash twice with 125 µL of SFB.
    a) Resuspend the pellet completely with 125 µL of SFB by mixing with a pipette. Leave to incubate for 30 s.
    b) Pulse centrifuge to collect liquid at the tube base and place on the magnet.
    c) Remove the supernatant and discard.
    d) Repeat step a-c to wash the pellet a second time
4. Pulse centrifuge and remove excess SFB.
5. Resuspend in 15 µL of EB and incubate for 10 min at 37°C.

6. Return to the magnet for ~2 min until the beads have pelleted and the supernatant is clear. Then carefully transfer the supernatant to a clean 1.5 mL microcentrifuge tube.
7. Quantify 1 µL of the eluted library

NOTE: For best results, proceed directly to MinION sequencing; however, the final library can be stored in EB at 4 °C for up to 1 week if needed.

E. Run a flow cell quality check (demo).

1. Connect the sequencing device to a laptop and open the sequencing software.
2. Select flow cell type, and then click **Check Flow Cell** and **Start Test**.
3. Once complete, the total number of active (i.e., viable) pores will be displayed. A new flow cell should have >800 active pores; if it does not, contact the manufacturer for a replacement.

F. Priming and loading the flow cell (these steps will be demonstrated)

1. Collect the reagents for this step: Sequencing Buffer **(SB),** Flow Cell Tether (FCT), Flow Cell Flush (FCF), and Library Beads (LIB), Bovine Serum Albumin (BSA)
2. Vortex SB and FCF, pulse centrifuge, and place on ice.
3. Pulse centrifuge FCT and mix by pipetting; then place on ice.
4. Prepare the flow cell priming mix as indicated below. Mix by pipetting at RT.

Table 6. Priming mix

Component	**Volume per flow cell** (µl)
Flow Cell Flush (FCF)	1,170
Bovine Serum Albumin (BSA) at 50 mg/ml	5
Flow Cell Tether (FCT)	30
Total volume	**1,205**

5. Flip back the sequencing device lid and slide the priming port cover clockwise so that the priming port is visible

6. Remove air bubbles carefully by setting a P1000 pipette to 200 µL, insert the tip into the priming port, and turn the wheel until a small volume entering the pipette tip is seen (max turn to 230 µL).
7. Load 800 µL of flow cell priming mix with BSA into the flow cell via the priming port, taking care to avoid bubbles.
8. Leave for 5 min. During this time, prepare the library for loading by following the steps below.
9. Calculate the volume of DNA required for 50fmol of final library and add to the table below. If required make up to 12 µL with Elution Buffer (EB).
10. In a fresh tube, prepare the final library dilution for sequencing, as indicated in Table below.

Mix the library beads by pipetting immediately prior to use as they settle quickly.

Table 6. Final library dilution	
Library mix reagents	**Volume per reaction (µL)**
Sequencing Buffer (SB)	37.5
Library Beads (LIB)	25.5
DNA library	_____*
Elution Buffer (to make up DNA library volume)	_____*
TOTAL	75
* 20fmol of final library is optimal.	

11. Gently lift open the sample port cover. Load 200 µL of flow cell priming mix with BSA into the flow cell via the priming port using a P1000 pipette. You will see buffer bubbling out of the sample port- this is normal. The flowcell is now fully primed and ready to load!
12. Pipette the library mix up and down prior to loading, ensuring loading beads in the master mix are resuspended before loading.

13. Load 75 µL of library mix to the flow cell via the sample port in a dropwise fashion. Ensure that each drip flows into the port before adding the next.
14. Replace the sample port cover gently, making sure the bung enters sample port.
15. Close the priming port and replace the sequencing device lid.

G. Sequencing run

1. Connect the sequencing device to the laptop and open the sequencing software.
2. Click **start** and then click **Start Sequencing**.
3. Click **New Experiment** and follow the sequencing software graphical user interface (GUI) workflow to set up the parameters for the run.
4. Type in the experiment name and sample ID (e.g., rabv_run1), and choose the **Flow Cell Type** from the drop-down menu.
5. Continue to kit selection and choose the relevant kit used (SQK-NBD114.24).
6. Continue to **Run** options. Keep the defaults, unless it is desired for the run to stop automatically after a certain number of hours (runs can be stopped manually at any time).
7. Continue to **Basecalling**. Choose to turn **Basecalling On** or **Off** according to the computing resources. Choose **Edit Options** under barcoding and ensure **Barcode Both Ends** is turned on. Save and continue to the output section.
8. Accept the defaults and continue to final review, check the settings and click **Start**.
9. Record the initial active channels-if this is significantly lower than the quality control (QC) check, restart the sequencing software. If still lower, then reboot the computer.
10. Record the initial channels in strand versus single pore to give an approximate pore occupancy. This number will fluctuate, so give an approximation.
11. Monitor the run as it progresses.