OXFORD NANOPORE LIBRARY CONTRUCTION AND MINION SEQUENCING

Objectives

- Generate a nanopore sequencing library from a mangrove sediment DNA sample
- Run library on a MinION sequencer, analyzing read length distribution and metagenomic composition

Principle: Nanopore sequencing is fundamentally different than other traditional and next-generation sequencing strategies. It relies on passing single-stranded nucleic acids through small pores that separate negatively and positively charged sides of a flow cell. The nucleic acid limits the flow of ions through the pore, and the different nucleotides in a nucleic acid differ in the magnitude of this blocking effect. As such, by measuring changes in current as a DNA or RNA strand passes through a nanopore, it is possible to infer its sequence. Oxford Nanopore sequencers contain hundreds or thousands of pores on each flow cell, each with an imbedded transporter protein complex. To work with these sequencers, DNA samples must have added adapters with an attached motor protein, which unwinds the double-stranded molecule and feeds a single strand into the pore. We will use a rapid library construction technique, in which a transposase enzyme simultaneously introduces breaks into the DNA molecules and attaches the first part of the adapter to the ends of the fragmented DNA molecules. This approach has the advantage of speed and simplicity, but it also fragments the DNA sample, so alternative end-ligation methods should be considered if your goal is to maximize read length. After the initial transposon-mediated step, the remaining portion of each sequencing adapter (which also carries the motor protein) are connected via non-enzymatic “click chemistry”. The libraries can then be loaded and run on a MinION or other Oxford Nanopore Sequencer.

Image source: Oxford Nanopore Technologies

Materials and Equipment

- DNA sample (we will be using a mangrove sediment DNA sample provided by Adan Silva Flores)
- Oxford Nanopore MinION Sequencer (Mk1B; MIN-101B) and flow cell (FLO-MINSP6)
- Oxford Nanopore Rapid Sequencing Kit (SQK-RAD004)
- Oxford Nanopore Flow Cell Priming Kit (EXP-FLP002)
- Suitable computer with MinKNOW and EPI2ME software installed

- PCR tubes
- Thermal cycler
- Microcentrifuge
- Ice bucket and ice
- dH_2O
- Pipet set and tips

Protocol

You can find a full protocol from the manufacturer here.

1. Place the flow cell in the MinION sequencer and perform a pore check by connecting the MinION to a computer and using the MinKNOW software. The functional pore count on a new flow cell should be >800.

2. Thaw kit components at room temperature, spin down briefly using a microcentrifuge and mix by pipetting as indicated by the table below. Put thawed reagents on ice.

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix (FRA)	Not frozen	✓	✓
Rapid Adapter (RAP)	Not frozen	✓	✓
Sequencing Buffer (SQB)	✓	✓	✓*
Loading Beads (LB)	✓	✓	Mix by pipetting or vortexing immediately before use
Flush Buffer (FLB) - 1 tube	✓	✓	✓*
Flush Tether (FLT)	✓	✓	✓

*Vortexing, followed by a brief spin in a microfuge, is recommended for Sequencing Buffer (SQB) and Flush Buffer (FLB).

3. Add ~400 ng of DNA sample in a volume of 7.5 µl (diluting as necessary with nuclease-free dH_2O) to a PCR tube.

4. Add 2.5 µl of the Fragmentation Mix (FRA). Mix gently by flicking the tube, and spin down if needed.

5. To perform transposase-mediated fragmentation and adapter ligation, incubate at 30 ºC for 1 min followed by 80 ºC for 1 min. Place the tube on ice briefly to cool.

6. Add 1 µl of the Rapid Adapter (RAP). Mix gently by flicking the tube, and spin down if needed. Incubate the tube for 5 min at room temperature to incorporate the remaining portion of the adapter, which carries the motor protein. Then store the library on ice while preparing the flow cell.

7. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice as soon as thawing is complete.

8. Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.

9. Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

10. Open the lid of the MinION flow cell and slide the flow cell's priming port cover clockwise so that the priming port is visible.

11. Use a P1000 pipet to remove the air bubble in the priming port. Insert the tip, and slowly dial the pipet wheel back until you see a very small volume (a few µl) of green fluid enter the tip. Remove the tip and this small volume of liquid from the flow cell and discard. Do not remove more than 20 or 30 µl, as this can introduce air into the array and damage the sequencer.

12. Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB) and mix by pipetting up and down.

13. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

14. Thoroughly mix the contents of the SQB and LB tubes by pipetting (the beads must not settle before adding to the subsequent library step).

15. In a new PCR tube, prepare the library as follows:

Reagent	Volume
Sequencing Buffer (SQB)	34 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
Nuclease-free water	4.5 µl
DNA library	11 µl
Total	**75 µl**

16. Gently lift the SpotON sample port cover on the MinION flow cell to make the SpotON sample port accessible.

17. Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

18. Mix the prepared library gently by pipetting up and down just prior to loading.

19. Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

20. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

21. Start the sequencing run, using the MinKNOW software interface. Once the sequencing run is initiated, launch a real-time metagenomic analysis, using the “What’s in My Pot” (WIMP) workflow in EPI2ME.