04_04_Nanopore-sequencing-with-MinION

DIENSTAG, 13.7.2021

Goal-Setting

- Sequencing of DNA strands

Terms / abbreviations

- RT = Room temperature

Risk areas

- None

Required materials and / or information

- Agencourt AMPure XP beads
- DNA LoBind tube, Eppendorf
- Flow Cell Priming Kit, Nanopore Technologies
- Freshly prepared 70% ethanol in nuclease-free water
- Ligation Sequencing Kit, Nanopore Technologies
- Next <ffpe <repair Mix, NEB
- Next Quick Ligation Module, NEB
- Next Ultra II End repair/dA-tailing Module, NEB
- Nuclease-free water
- Original protocol:

  Genomic DNA by Ligation (SQK-LSK109)-minion.pdf

- Pipettes, Eppendorf
- Qubit fluorometer, ThermoFisher
- Solutions are stored in the freezer in Extension Lab in a green box

Templates, devices, software

- Flow Cell stored in black bag in 4 °C fridge in Extension Lab
- Ice bucket with ice
- Magnet rack in the first drawer in Extension Lab
- MinION, Nanopore Technologies in the first drawer in Extension Lab
- Software for analysis --> MinKNOW
- Thermocycler, ThermoFisher
- Vortex mixer, Scientific Industries Vortex Genie 2

Preliminary work

- 03_01_Thermofisher-protocol-for-TdT-tailing-reaction
- 03_04_PCR-for-ssDNA-samples

Operation

DNA repair and end-prep

- Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice
- Prepare the DNA in nuclease-free water
    - Measure the concentration of the DNA sample with the Qubit fluorometer
    - 1 µg DNA is needed for the MinION
    - Calculate the needed amount of DNA and fill it up to 48 µL with nuclease-free water
- In a PCR tube, mix the following:
    - 48 µL DNA (sample)
    - 3.5 µL NEBNext FFPE DNA Repair Buffer
    - 2 µL NEBNext FFPE DNA Repair Mix
    - 3.5 µL Ultra II End-prep reaction buffer
    - 3 µL Ultra II End-prep enzyme mix
- Mix gently by flicking the tube, and spin down
- Using a thermal cycler, incubate at 20 °C for 5 min, 65 °C for 5 min and cool down to 10 °C

AMPure XP bead clean-up

- Resuspend the AMPure XP beads by vortexing
- Transfer the DNA sample to a clean 1.5 mL Eppendorf DNA LoBind tube
- Add 120 µL of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube
- Incubate for 5 min at RT
- Prepare 500 µL of fresh 70 % ethanol in nuclease-free water
- Spin down the sample and pellet on a magnetic rack until eluate is clear and colourles. Keep the tube on the magnet, and pipette off the supernatant
- Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70 % ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard it.
- Repeat the previous step
- Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow it to dry for ~30 s, but do not dry the pellet to the point of cracking
- Remove the tube from the magnetic rack and resuspend the pellet in 61 µl Nuclease-free water. Incubate for 2 min at RT
- Pellet the beads on a magnet rack until the eluate is clear and colourless
- Remove and retain 61 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube
- Quantify 1 µL of eluted sample using a Qubit fluorometer

Adapter ligation and clean-up

- Spin down the Adapter Mix and Quick T4 Ligase, and place on ice
- Thaw Ligation Buffer at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing
- Thaw the Elution Buffer at RT, mix by vortexing, spin down and place on ice
- To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice
- In a 1.5 mL Eppendorf DNA LoBind tube, mix in the following order:
    - 60 µL DNA sample from the previous step
    - 25 µL Ligation Buffer
    - 10 µL NEBNext Quick T4 DNA Ligase
    - 5 µL Adapter Mix
- Mix gently by flicking the tube, and spin down
- Incubate the reaction for 10 min at RT

AMPure XP bead clean-up

- Resuspend the AMPure XP beads by vortexing

- Add 40 µL of resuspended AMPure XP beads to the reaction and mix by flicking the tube
- Incubate for 5 min at RT
- Spin down the sample and pellet on a magnet rack. Keep the tube on the magnet rack and pipette off the supernatant
- Wash the beads by adding 250 µL Short Fragment Buffer. Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard it.
- Repeat the previous step
- Spin down and place the tube back on the magnetic rack. Pipette off any residual supernatant. Allow it to dry for ~30 s, but do not dry the pellet to the point of cracking.
- Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB)
- Spin down and incubate for 10 min at RT
- Pellet the beads on a magnetic rack until the eluate is clear and colourless
- Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 mL Eppendorf DNA LoBind tube
- Quantify 1 µL of eluted sample using a Qubit fluorometer

Priming and loading the SpotON flow cell

- Thaw the Sequencing Buffer, Loading Beads, Flush Tether and one tube of Flush Buffer at RT
- Mix the Sequencing Buffer, Flush Tether and Flush Buffer tubes by vortexing and spin down at RT
- Open the MinION Mk1B lid and slide the flow cell under the clip
- Slide the priming port cover clockwise to open the priming port
- After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µL):
    - Set a P1000 pipette to 200 µL
    - Insert the pipette tip into the priming port
    - Turn the wheel until the dial shows 220-230 µL, or until a small volume of buffer entering the pipette tip can be seen
- To prepare the flow cell priming mix, add 30 µL of thawed and mixed Flush Tether directly to the tube of thawed and mixed Flush Buffer, and mix by vortexing at RT
- Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 min
- During this time, prepare the library for loading in a new tube as follows:
    - 37.5 µL Sequencing Buffer
    - 25.5 µL Loading Beads, mixed immediately before use
    - 12 µL DNA library
- Complete the flow cell priming by loading 200 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles
- Gently lift the SpotON sample port cover to make the SpotON sample port accessible
- Mix the prepared library gently by pipetting up and down just before loading
- Add 75 µL of the 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 --> do not put the top of the pipette tip into the opening!
- Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

Starting the sequencing with the Software MinKNOW

- Start sequencing
- There is no change of any settings
- Give the sample a name and a sample-ID
- Stop the run after roughly 20 min because these huge amounts of data are not needed

Analyse the results

- Upload the data to https://usegalaxy.org/
- There a lot of analysis methods can be choosen
- NanoPlot gives some histograms about the lenght and the length vs quality

- FastQC gives some general information about nucleotide content, adapter content, overrepresented sequences and quality score

Troubleshooting

- None

Follow-up work

- If the flow cell will be reused, follow the Wash Kit Instructions and store the washed flow cell at 2-8 °C
    - 04_06_Washing-of-the-Flow-Cell