

MinION Genomic Sequencing

Ligation Sequencing Kit

SQK-LSK109

Originally crafted by Riccardo Racicot 11/5/2018

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The information found in this protocol is adapted from or directly taken from Oxford Nanopore 1D Lambda Control Experiment (SQK-LSK109) Version: CDE_9062_v109_revC_23May2018 Last update: 10/15/2018. Background information on the flow cell is adapted from Lannoy et al. 2017 doi: 10.12688/f1000research.12012.1. Background information on DNA repair and End Prep is adapted from NEB product information for the respective reagents and mixes.

Some notes from New England Biolabs:

-MagAttract HMW DNA kit by Qiagen Cat#67563 is recommended for DNA extraction.

-As a final step of extraction they recommend putting the DNA through a Monarch DNA cleanup column. You will lose 40-75% of your sample but helps library prep a lot. (We started with 2µg of DNA in 24µl and added 48µl of binding buffer. Ended up with .6µg according to Qubit, 1µg according to nanodrop). They have a genomic DNA version coming out in January or February of 2019 that may reduce this loss - within this protocol, do an extra, quick spin after step 4 to eliminate residual EtOH, and use 20µl in Step 6

Check the Flow Cell

Background

MinION SpotON R9.4 flow cells consist of 512 pores, each containing 4 wells for up to 2048 active nanopores. Wells suitable for sequencing have intact membranes and exactly one inserted, properly functioning, nanopore. Checking the flow cell for quality is necessary as: 1. Not all pores will contain functional wells and 2. The signal quality from each functional well must be ranked. These attributes will be checked during this step to provide the highest quality signal and thus the best quality data. To differentiate between cells that contain wells that are suitable for sequencing versus those that do not, DNA strands present in the shipping buffer are sequenced. Next switches in each cell, known as multiplexers, rank each functional well for signal quality. The best quality well is used to begin readout. After 8 hours readout is obtained from the second-best quality nanopore as signal quality declines over the course of a run. After another 8 hours the third-best quality is used.

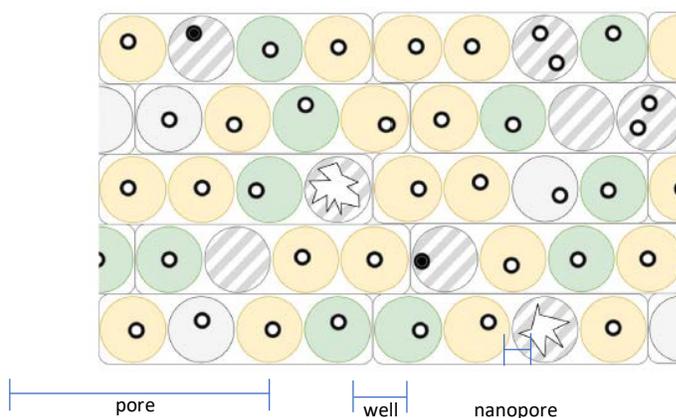


Fig. Wells determined to be unusable are denoted by hash marks. Top ranked wells are denoted in green. Second and third ranked are denoted in yellow. Unused wells are denoted in white. Adapted from Lannoy 2018.

Materials

MinION sequencer
Host computer
SpotON R9.4 Flow cell

Procedure

1. Set up the MinION, flow cell and computer.
 - a. You will see a light and hear a fan if the MinION is set up successfully.
2. Open the MinKNOW GUI.
3. Choose your flow cell type from the selector box. Mark the flow cell as selected.
4. Click “Check flow cells” at the bottom of the screen. Select the appropriate flow cell.
5. Click “Start Test”.
6. Check the number of active pores available for the experiment, reported in the System History Panel when the check is completed.
7. Record the number of active pores. This information will be used during run start up.

DNA Repair and End Prep

Background

Obtaining a high-yield library product for DNA sequencing allows for high quality data. High yield is particularly important for Nanopore sequencing as sequencing is continuous. This means the more DNA you have, the more data you are able to extract and a higher percentage of genome coverage is obtained. Prior to sequencing, DNA repair increases library yield. In this workflow, DNA repair is accomplished using NEBNext FFPE DNA Repair Mix. This repair mix contains enzymes that repair deamination of cytosine to uracil, nicks and gaps, oxidized bases and blocked 3' ends. Damage that is not repaired by this mix includes DNA fragmentation and DNA-protein crosslinks. NEBNext Ultra II End Repair/dA-Tailing Module is also used at this step to repair the 3' and 5' ends of the DNA. This module converts fragmented DNA to repaired DNA that has 5' phosphorylated and 3' dA-tailed ends. This allows fragmented DNA strands that were not repaired by the FFPE mix to be sequenced. DNA CS is included in this reaction to act as a positive control. DNA CS consists of a standard DNA sequence that can be used to provide quality control for sequencing and alignment. After performing DNA repair, it is necessary to purify your DNA to have a pure sample free of contaminants. Purification is accomplished using magnetic AMPure XP beads. Excess nucleotides, oligonucleotides, salts and enzymes must be removed from the DNA product as they may interfere with downstream reactions. AMPure XP beads selectively bind RNA and DNA and a magnetic stand is used to separate bead-bound DNA from contaminants left behind in the buffer. The DNA product is then eluted in nuclease-free water and can be used in downstream steps.

Materials

DNA CS (DCS)
Experimental gDNA
NEBNext FFPE Mix
NEBNext End Repair/ dA-Tailing Module
AMPure XP beads
70% nuclease free ethanol, molecular grade
Nuclease free water
Magnetic 1.5 mL tube rack
Rotating mixer

Notes

- When taking tubes from a bag, do not reach into the bag.
- Pick up each tube by the bottom and cap it carefully without touching the mouth or the inside of the cap.
- Use nuclease-free filtered pipet tips.
- Never vortex enzymes.
- Always keep enzymes on ice and place back into -20°C as soon as possible.

Before starting – now NEB has sample purification beads too if prefer over AMPure – cat#E7104S. May have to call to get them.

- Clean your work surface and pipets thoroughly with **regular, bench grade 70% ethanol**.
- For the AMPure wash steps, make up fresh 70% ethanol using molecular biology grade ethanol and nuclease-free water.
- Preheat thermal cycler.

Procedure - Finger flick only for all mixing to preserve long fragments - Use only Lo Bind tubes for better recovery

1. Thaw DCS at room temperature, spin down, mix by pipetting and place on ice.
2. In a 0.2 mL thin-walled PCR tube, combine the following:
 - 27 μL Nuclease Free water
 - 20 μL Experimental gDNA
 - 1 μL DNA CS
 - 3.5 μL FFPE DNA Repair Buffer
 - 2 μL FFPE DNA Repair Mix
 - 3.5 μL Ultra II End Prep Reaction Buffer
 - 3 μL Ultra II End Prep Enzyme Mix

3. Mix gently by flicking, and spin down.
4. Incubate on thermal cycler:

Step 1	5 minutes	25°C
Step 2	5 minutes	65°C

5. Resuspend AMPure beads by thoroughly vortexing. Quick spin and place on ice.
6. Transfer the end prep reaction product to a clean 1.5 mL tube if not already in one.
7. Add 60 μL AMPure beads to the end prep reaction product. Mix by pipetting.
8. Incubate for 5 minutes at 25°C in a Thermomixer at 350rpm.
9. Quick spin down sample and pellet on magnet. Allow the pellet to separate for 5 minutes.
10. Pipet off the supernatant.
11. Keeping the tube on the magnet, wash the beads with 200 μL of 70% ethanol. Tip rack away from pellet, then run EtOH down opposite side from pellet and suck back up with same tip. No waiting.
12. Repeat step 11
13. Quick spin down the tube and place back on magnet.
14. Remove residual ethanol using a small-volume pipet.
15. Allow the pellet to air dry for 30 seconds exactly.
 - a. Do not dry the pellet to the point of cracking.

16. Remove the tube from the magnetic rack and resuspend the pellet in 61 μL of nuclease-free water. Incubate off of the magnetic rack for 2 minutes. (Can do 5 minutes at 30°C to try to get more off).
17. Quick spin and place the tube back on the magnet rack for 2 minutes.
18. Remove 61 μL of eluate and place into a clean 1.5 μL tube.
 - a. Leaving behind a few μL of eluate okay. It's better to lose volume than to contaminate the sample with magnetic beads.

Adapter Ligation

Background

Adapters need to be ligated to the freshly prepped ends of the DNA fragments. These adapters help the DNA find and bind to the nanopores on the flow cell to initiate sequencing.

Materials

Adapter Mix (AMX)
Ligation buffer (LNB)
T4 Ligase from NEBNext Quick Ligation Module
Elution buffer (EB)
L Fragment buffer or S Fragment buffer
AMPure XP beads

Before Starting

- Spin down AMX and T4 Ligase and place on ice.
- Thaw LNB, spin down, mix by pipetting and place on ice.
- Thaw EB, vortex, spin down and place on ice.
- Thaw either L fragment or S Fragment buffer, vortex, spin down and place on ice.

Procedure

1. In a 1.5 mL tube combine the following in the given order:

60 μL	DNA sample from end repair step
25 μL	LNB
5 μL	AMX -add then finger mix
10 μL	T4 DNA Ligase -add then finger mix
2. Mix gently by flicking the tube, and spin down.
3. Incubate at room temperature for 10 minutes. Can start warming up and checking flow cell-screen shot check results then bring flow cell back to bench to load when ready.
4. Resuspend AMPure beads by thoroughly vortexing.
5. Add 40 μL AMPure beads to the end prep reaction product. Finger mix.
6. Incubate for 5 minutes on a rotating mixer at 25°C in Thermomixer at 350rpm.
7. Spin down sample and pellet on magnet. Allow the pellet to separate for 5 minutes.
8. Pipet off the supernatant.
9. Wash the beads with 250 μL of either L Fragment or S Fragment Buffer. Flick to resuspend the beads.
10. Quick spin and return the tube to the magnet and allow the beads to pellet for 2 minutes.
11. Pipet off and discard the supernatant.
12. Repeat steps 9-11.
13. Spin down the tube, place back on rack and pipet off any residual buffer using a small volume pipet. Count to 30.

14. Remove the tube from the magnetic rack and resuspend the pellet in 15 μL EB. Incubate off of the magnetic rack for 10 minutes.
15. Place the tube back on the magnet rack for 2 minutes.
16. Remove 12.5-15 μL of eluate and place into a clean 1.5 μL tube.
17. Run on Qubit. Can also run on Fragment Analyzer or Bioanalyzer to check average size maintained.

Priming and Loading the Flow Cell

Materials

Sequencing buffer (SQB)
 Loading Beads (LB)
 Flush Buffer (FLB)
 Flush Tether (FLT)

Before Starting

- Thaw SQB, LB, FLB, FLT and place on ice.
- Vortex SQB and FLB, return to ice.
- Spin down the FT, pipet to mix, return to ice.

Procedure

1. Open the lid of the MINION and slide the flow cell's priming port cover clockwise so that the priming port is visible.
2. Check the priming port for bubbles. If there are any present, remove them with a pipet.
 - a. **DO NOT** remove more than 20-30 μL or you risk damaging the pores.
3. Draw back 20-30 μL of buffer from the priming port:
 - a. Set a P1000 pipet to 500. μL .
 - b. Insert the tip into the priming port.
 - c. Turn the wheel until the dial shows 525 μL or until you can see a small volume enter the pipet tip.
4. Prepare the flow cell priming mix by adding 30 μL of FLT directly to the tube of FLB. Mix by pipetting up and down.
5. Load 800 μL of flow cell priming mix into the flow cell priming port. Avoid introducing air bubbles by reverse pipetting - going past the first stop before drawing up the 800 μL , then only go to the first stop to emit.
6. Wait 5 minutes.
7. Thoroughly mix the LB tube by pipetting.
 - a. The beads settle very quickly so mix them immediately before use.
8. Prepare the library for loading by mixing the following in a new tube:

37.5 μL	SQB
25.5 μL	LB (freshly mixed)
12 μL	DNA library

No mixing – place immediately on ice
9. Lift the SpotON sample port cover and make sure the sample port is accessible.
10. Complete flow cell priming by loading 200 μL of the priming mix into the flow cell via the priming port (**NOT** the sample port). Avoid introducing air bubbles again by reverse pipetting.
11. Mix the prepared library gently by pipetting 5X and load 75 μL into the sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. Should see beads diffuse all the way across nanopores.

12. Replace the sample port cover, making sure the bung enters the port. Close the priming port and replace the lid.

Starting a Run and Protocol Script

Procedure

1. Double-click the MinKNOW icon on the desktop to open the MinKNOW GUI.
2. Choose the flow cell type from the selector box.
3. Mark the flow cell as selected.
4. Click “New Experiment” at the bottom of the GUI.
5. On the New Experiment pop up screen, select the running parameters for your experiment. Choose for ‘enable active pores’ to be on.
6. Click “Start Run”
7. Monitor messages in the Message panel.
8. To view the progression of the script, click the “Jump to run”. On the dropdown menu click on the run that you just started. Take a screen shot of metrics immediately and again after 5 minutes.
9. Check that the active pores reported in the MUX scan are similar (within 10-15%) to those that are reported at the end of the Flow Cell Check.
 - a. If there is a significant reduction, restart MinKNOW.
 - b. If the numbers are significantly different, restart the computer.
 - i. Once the numbers are similar to those reported in the Flow Cell Check, restart the experiment on the Connection page.
10. Monitor the pore occupancy. A good library will be indicated by a higher proportion of light green channels in Sequencing than are in Pore. The combination of Sequencing and Pore indicates the number of active pores at any one time. A low proportion of Sequencing channels will reduce the throughput of the run. Looking for ~400 Sequencing and that should hold for a day.
11. Other metrics to look at. Click on ‘More’ – Read length histogram should be ~6kb, active feedback should be <20, otherwise indicating fragments are too long. Adaptor should be in the single digits-higher numbers indicates too many free adaptors.

To stop the experiment: press “Stop Run” at the top of the screen. Data acquisition will stop but the software will continue to basecall unless you click “Stop Basecalling”. Check that the temperature has reached 34°C.