Nanopore Sequencing Library Prep Using the MinION Flongle and the Ligation Sequencing Kit V14 (SQK-LSK114)

Nanopore is a third-generation sequencing platform that produces genomic sequence data in real time. Nanopore sequencing has become popular because the sequencers are very inexpensive, making it possible for even the smallest labs to conduct sequencing in-house. Nanopore technology can also sequence both long fragments (as long as you can isolate), as well as short fragments, making it quite flexible.

*This protocol describes library prep for sequencing using the **MinION and the Flongle flow cell**. The MinION is Nanopore's portable DNA sequencer and the Flongle flow cell is a low capacity and low-cost sequencing flow cell. It costs under $100 and can generate up to ~**2.5 Gb of sequence data** (for reference, the human genome is about 3.1 Gb). Note that depending on the application, you would have previously isolated the DNA, PCR amplified barcoding genes (if conducting DNA barcoding) and quantified the DNA using the Qubit.*

*The protocol assumes that you are starting with **500 ng of DNA**. In our experience, you want to see strong PCR bands on a gel for best results. We also typically add **1000-2000ng of DNA** at the start, that is, 2X to 4X the recommended amount, because much of the DNA is lost during clean up. These elevated amounts of DNA tend to produce better results in our experience. However, the official protocol warns that too much DNA may be detrimental to the sequencing reaction.*

Section a. DNA repair, end-prep, and cleanup:

The library prep officially begins here. Library prep involves the steps that are required to get our PCR products (assuming you have done PCR prior to library prep) in optimal shape for sequencing on a genomic sequencer. In this section, we will repair the ends of the PCR products and prepare them for the adapter attachment. Attaching adapters (next section) is necessary for the DNA to be pulled through to nanopores on the flow cells.

1. Thaw the reagents in the table below and place on ice once thawed.
    a. Flick and/or invert the reagent tubes to ensure they are well mixed.
    b. Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix.
    c. Always spin down tubes before opening for the first time each day.

Reagent	Volume
DCS	0.5 µl
DNA	23.5 µl
NEBNext FFPE DNA Repair Buffer	1.75 µl
NEBNext FFPE DNA Repair Mix	1 µl
Ultra II End-prep Reaction Buffer	1.75 µl
Ultra II End-prep Enzyme Mix	1.5 µl
Total	30 µl

2. The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilize any precipitate.
    a. Note: It is important the buffers are mixed well by vortexing.
    b. The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.
3. Thoroughly mix the reaction by gently pipetting and briefly spinning down.
4. Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.
5. Resuspend the **AMPure XP Beads (AXP)** by vortexing.
    a. It is important to vortex the beads immediately prior to each application because the beads settle quickly. You will not be getting the appropriate amount of beads if you fail to vortex just prior to using them every time you do.
6. Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA **LoBind tube**.
    a. These are special tubes, not the ordinary tubes we use in lab. Check the tube bag label to make sure you have the right tubes.
7. Add 30 µl of resuspended AMPure XP beads (AXP) to the end-prep reaction and mix by flicking the tube.
8. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
9. Prepare 500 µl of fresh **80% ethanol** in nuclease-free water.
10. Spin down the sample and pellet on a magnet until supernatant is clear and colorless.
    a. Note that we have a special rack with magnets for this step.
11. Keep the tube on the magnet, and pipette off the supernatant.
12. Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
13. Repeat the previous step.
14. Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
15. Remove the tube from the magnetic rack and resuspend the pellet in 31 µl nuclease-free water. Incubate for 2 minutes at room temperature.
16. Pellet the beads for at least 1 minute on a magnet until the eluate is clear and colorless.
    a. You may need to leave it on longer if the eluate is not clear.
17. Remove and retain 31 µl of eluate into a clean, labeled 1.5 ml Eppendorf DNA **LoBind tube**.
    a. This is your DNA!!!

Section B. Adapter ligation and clean-up:

In this section, we will attach the adapters to the ends of the PCR products. The adapters are necessary for the DNA to be pulled through to nanopores on the flow cell.

1. Spin down the **Ligation Adapter (LA)** and **Quick T4 Ligase**, and place on ice.
2. Thaw the **Ligation Buffer (LNB)** at room temperature, spin down and mix by pipetting. Due to its viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
3. Thaw the **Elution Buffer (EB)** at room temperature and vortex. Spin down and place on ice.
4. Thaw the **Short Fragment Buffer (SFB)** at room temperature and mix by vortexing. Then spin down and place on ice.
    a. The SFB is used for fragments that are smaller than 3000bp, which is typically what we are working with for barcoding applications. If the fragments are longer than 3000 bp, use 125 µl of the Long Fragment Buffer (LFB).
5. In a 1.5 ml Eppendorf DNA **LoBind tube**, mix in the following order:

Reagent	**Volume**
DNA sample from the previous step	30 µl
Ligation Buffer (LNB)	12.5 µl
NEBNext Quick T4 DNA Ligase	5 µl
Ligation Adapter (LA)	2.5 µl
Total	50 µl

6. Thoroughly mix the reaction by gently pipetting. Briefly spin down.
7. Incubate the reaction for 10 minutes at room temperature.
8. Resuspend the **AMPure XP Beads (AXP)** by vortexing. Add 20 µl of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube.
    a. It is important to vortex the beads immediately prior to each application because the beads settle quickly. You will not be getting the appropriate amount of beads if you fail to vortex just prior to using them every time you do.
9. Incubate on a **Hula mixer (rotator mixer) for 5 minutes** at room temperature.
10. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colorless.
    a. Note that we have a special rack with magnets for this step.
11. Wash the beads by adding 125 µl **Short Fragment Buffer (SFB)**. Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.
12. Repeat the previous step.
13. Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

14. Remove the tube from the magnetic rack and resuspend pellet in 7 µl **Elution Buffer (EB).** Incubate for 10 minutes at room temperature.
15. Pellet the beads on a magnet for at least 1 minute, until the eluate is clear and colorless.
    a. You may need to leave it on longer if the eluate is not clear.
16. Remove and retain 7 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube. This is your DNA!!!
17. Dispose of the pelleted beads
18. Make up your library to 5 µl at 5-10 fmol.

Section C. Quantify the DNA concentration using the Qubit 4 and *1X HS (High Sensitivity) kit*:

Preparation of standards.-

The Qubit requires standardization only once per working session.

1. Take a 0.5 mL Qubit tube and label it STD 1; add 190 µL of working solution and 10 µL of the Standard #1 from the assay kit. Set aside and keep at room temperature for 2 min.
2. Take a 0.5 mL Qubit tube and label it STD 2; add 190 µL of working solution and 10 µL of the Standard #2 from the assay kit. Set aside and keep at room temperature for 2 min.
3. Mix each sample vigorously by vortexing for 3–5 seconds, then quick spin.
4. Calibrate the Qubit using the standards. From the initial home screen, select "DNA", then select the assay type "dsDNA 1X High Specificity protocol". Select the "Read Standards" option.
5. Insert the tube STD 1 into the chamber. Close the lid and select "Read standard." When reading is complete, remove the tube from the chamber and set aside.
6. Insert the tube STD 2 into the chamber. Close the lid and select "Read standard." When the reading is complete, remove the tube from the chamber and set aside.

Preparation of Samples and Reading Concentrations.-

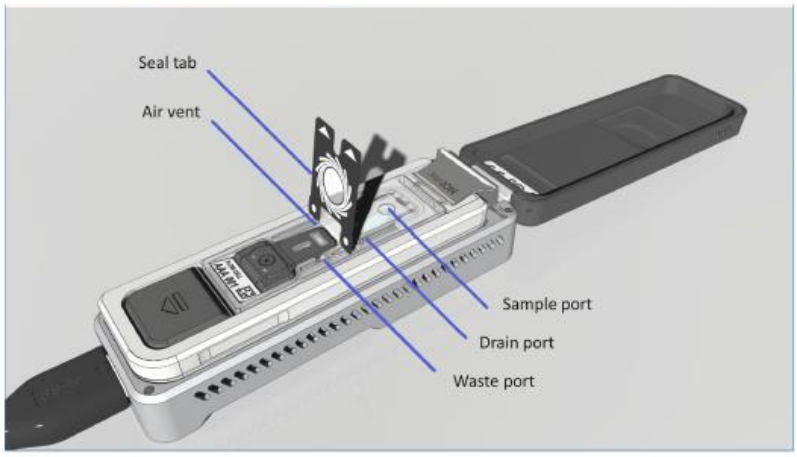
1. To each 0.5 mL Qubit sample tube, add 198 µL of working solution and 2 µL of the diluted DNA sample. Close the lid, vortex 2-3 sec and quick spin. Set aside and keep at room temperature for 2 min.
    a. *Total volume in sample tube must be 200 µL.*
    b. *Any amount of DNA between 1 and 20 µl is OK, just adjust water to a final volume of 200ul.*
2. After the 2 min incubation, read the DNA samples. From the current screen (post calibration), select "Run samples". Select the sample DNA volume (typically 2 µL) using the +/- button and the units (ng/µL) from the dropdown menu. Insert the first DNA sample tube into the chamber. Close the lid and select "Read sample."
3. Repeat for the remaining DNA sample tubes.
    a. Note that the Qubit 4 has a USB drive so you can download the data after reading the samples.

Section D. Loading the sequencing library on the Flongle flow cell:

Now that we have our sequencing library prepared, we will load it on the Flongle flow cell and conduct the sequencing reaction.

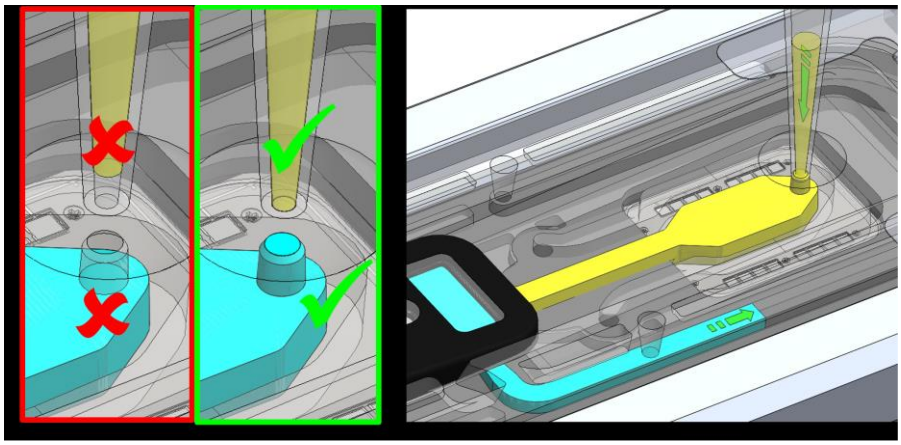
1. Thaw the **Sequencing Buffer (SB), Library Beads (LIB)**, **Flow Cell Tether (FCT),** and one tube of **Flow Cell Flush (FCF)** at room temperature before mixing by vortexing. Then spin down and store on ice.
2. In a fresh 1.5 ml Eppendorf DNA LoBind tube, add 117 µl of Flow Cell Flush (FCF) with 3 µl of Flow Cell Tether (FCT) and mix by pipetting.
3. Place the **Flongle adapter** into the MinION.
    a. The adapter should sit evenly and flat. This ensures the flow cell assembly is flat during the next stage.
4. Place the **flow cell** into the Flongle adapter and press the flow cell until you hear a click.
    a. The flow cell should sit evenly and flat inside the adapter, to avoid any bubbles forming inside the fluidic compartments.
5. Peel back the **seal tab** from the Flongle flow cell, up to a point where the sample port is exposed, as follows:
    a. Lift up the seal tab:
    b. Pull the seal tab to open access to the sample port:
    c. Hold the seal tab open by using adhesive on the tab to stick to the MinION lid.

The diagram below shows the components of the Flongle flow cell:



The seal tab, air vent, waste channel, drain port and sample port are visible here. The sample port, drain port and air vent only become accessible once the seal tab is peeled back.

6. Prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier. Ensuring that there is no air gap in the sample port or the pipette tip, place a P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell by slowly pipetting down.
    a. You can twist the pipette plunger down to avoid flushing the flow cell too vigorously.



7. Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows:

Reagents	**Volume**
Sequencing Buffer (SB)	15 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using. LIS can be used instead of LIB when preparing libraries with the Ligation Sequencing Kit V14 (SQK-LSK114)	10 µl
DNA library	5 µl
Total	**30 µl**

8. To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip.
9. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down.
    a. Again, you can twist the pipette plunger down to avoid flushing the flow cell too vigorously, although the smaller volume makes this less critical at this step.
10. Seal the Flongle flow cell using the adhesive on the seal tab, as follows:
    a. Stick the transparent adhesive tape to the sample port.
    b. Replace the top (Wheel icon section) of the seal tab to its original position.
11. Close the lid on the MinION sequencer and set up the sequencing run on the computer or screen of the sequencing device. You will need to login to our lab's Nanopore account for the sequencing software to work.
    a. We will run the sequencing reaction overnight and into the next day to generate as many sequences as possible.
    b. Sequences will begin to be produced in the data folder in files with 1000 sequences each. The first files should appear within a few minutes of the sequencing reaction starting and you can use these to begin exploring the data.
    c. Enjoy!!!