**Spekboom: Adapted Oxford Nanopore Technologies gDNA Library Preparation and Sequencing Protocol**

**Updated February 2023**

**Kits:**

* ONT Ligation Sequencing Kit (SQK-LSK110)
* NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing

***Library Preparation:***

***DNA repair and end-prep***

1. Prepare the DNA in Nuclease-free water: 1.2 μg (or more) genomic DNA into a 1.5 mL Eppendorf DNA LoBind tube. Adjust the volume to 47 μL with Nuclease-free water. Mix thoroughly by flicking the tube.

*\*\*With Spekboom a lot of DNA is lost during library preparation so we start with more DNA than stated in ONT protocol.*

1. In a 0.2 mL thin-walled PCR tube, mix the following:
   * 47 μL DNA.
   * 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.

**\*\*\**No need for DNA CS.***

1. Ensure the components are thoroughly mixed by pipetting using a wide bore pipette tip and gently flicking the tube and very brief spin down (on/off).
2. Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

***AMPure Bead Clean-up***

1. Take out AMpure XP beads and allow to reach room temperature.
2. Resuspend the AMPure XP beads by vortexing.
3. Transfer the DNA sample using wide bore pipette tips to a clean 1.5 mL Eppendorf DNA LoBind tube.
4. Add 60 µL of resuspended AMPure XP beads to the end-prep reaction and mix by gently flicking the tube until the solution is homogenous.
5. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
6. Prepare 500 μL of fresh 70% ethanol in Nuclease-free water.
7. Quickly spin down quick (on/off) the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant (±10 minutes).
8. Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 70% ethanol. Continuously pipette ethanol off and over beads 4X.
9. Remove the ethanol using a pipette and discard.
10. Repeat the previous step.
11. Quickly spin down with beads against wall of centrifuge and away from lid.
12. 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.
13. Remove the tube from the magnetic rack and resuspend the pellet in 65 µL Nuclease-free water by gently flicking the tube.

\*\*\*Use more water in order to lose less DNA.

1. Incubate for 2 minutes at RT.
2. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.
3. Remove and retain 63 µl of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube using wide bore pipette tips.
4. Quantify 1 µL of eluted sample using a Qubit fluorometer (~6-8 ng/μl).

***Adapter Ligation***

1. Spin down the Adapter Mix F (AMX-F) and Quick T4 Ligase, and place on ice.
2. Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
3. Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice.
4. In a DNA containing Eppendorf DNA LoBind tube, add the following:

* 25 µL Ligation Buffer (LNB).
* 10 µL NEBNext Quick T4 DNA Ligase.
* 5 µL Adapter Mix F (AMX-F).

1. Ensure the components are mixed by gently pipetting using a wide bore pipette tip and gently flicking the tube. Perform a quick spin down (on/off).
2. Incubate the reaction for 10 minutes at RT.

***AMPure bead clean-up***

1. Thaw Elution buffer (EB) and Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB).
2. Resuspend the AMPure XP beads by vortexing.
3. Add 40 µL of resuspended AMPure XP beads to the reaction and mix by gently flicking the tube.
4. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
5. Quickly spin down(on/off) the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
6. Wash the beads by adding either 250 μL Long Fragment Buffer (LFB) or 250 μL Short Fragment Buffer (SFB). Gently flick the beads to resuspend, quickly spin down(on/off), then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.
7. Repeat the previous step.
8. Quickly spin down (on/off) 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.
9. Remove the tube from the magnetic rack and resuspend the pellet in 30 µL Elution Buffer (EB). Quickly spin down (on/off) and incubate for 10 minutes at 37°C.
10. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.
11. Remove and retain 25 µL of eluate containing the DNA library using a wide bore pipette tip into a clean 1.5 ml Eppendorf DNA LoBind tube.
12. Quantify 1 µl of eluted sample using a Qubit fluorometer (~5-6 ng/μl).
13. Store the library on ice until ready to load.

***\*\*\*If final library concentrations are low (~3 ng/ μl), combine two libraries for loading.***

***Priming and loading the SpotON flow cell***

1. Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing and spin down at RT.
2. To 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 vortexing at RT.
3. Open the MinION device lid and slide the flow cell under the clip.
4. Slide the priming port cover clockwise to open the priming port.
5. 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):
   1. Set a P1000 pipette to 200 µl
   2. Insert the tip into the priming port
   3. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip
   4. Note: Visually check that there is continuous buffer from the priming port across the sensor array.
6. 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. During this time, prepare the library for loading by following the steps below.
7. Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.
8. n a new tube, prepare the library for loading as follows:
   1. 37.5 µL Sequencing Buffer II (SBII).
   2. 25.5 µL Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using
   3. 12 µL DNA library.
9. Start sequencing run on instrument.

***\*\*\*This will ensure that preliminary sequencing processes are started and none of your library is lost and all that is loaded will be sequenced.***

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. 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.
3. Mix the prepared library gently by pipetting up and down using a wide bore pipette tip just prior to loading.
4. 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.
5. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.