Nanobind Library Prep – Ultra Long Sequencing

Document ID: LBP-ULN-001

Release Date: 03/24/2021

For ultra long sequencing library preparation on Oxford Nanopore MinION, GridION, and PromethION using a 3X or 6X scale library

Equipment and Reagent List

Equipment/Reagent	Manufacturer (Part number)
Nanobind UL Library Prep Kit	Circulomics (NB-900-601-01)
Ultra-Long DNA Sequencing Kit	Oxford Nanopore Technologies (SQK-ULK001)
Flow Cell Wash Kit	Oxford Nanopore Technologies (EXP-WSH004)
Flow Cell Priming Kit	Oxford Nanopore Technologies (EXP-FLP002)
MinION Flow Cell (R9.4 or R10.3)	Oxford Nanopore Technologies (FLO-MIN106D or FLO-MIN111)
PromethION Flow Cell (R9.4 or R10.3)	Oxford Nanopore Technologies (FLO-PRO002 or FLO-PRO111)
MinION, GridION, or PromethION Sequencer	Oxford Nanopore Technologies
Magnetic Tube Rack	Thermo Fisher DynaMag-2 (12321D)
Mini-Tube Rotator	Fisher Scientific Mini-Tube Rotator (05-450-127)
Heat Block (or Water Bath)	Fisher Scientific Isotemp Dry Bath Incubator (11-715-125DQ)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
1.5 mL DNA LoBind Microcentrifuge Tubes	Eppendorf (022431048)
Wide Bore 200 µL Pipette Tips	USA Scientific (1011-8410)
Wide Bore 1000 µL Pipette Tips	Thermo Scientific (2079G)
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

Kit Storage

Nanobind disks and all other buffers should be stored at room temperature (18–25 °C).

Follow instructions from Oxford Nanopore Technologies for correct storage of kits, buffers, and consumables.

Product Use

Nanobind UL Library Prep Kits are intended for research use only.

Ultra Long Library Preparation Protocol

This protocol describes the preparation of a 6X (MinION/GridION) or 3X (PromethION) scale library for generating ultra long (100 kb – 1+ Mb) reads on Oxford Nanopore MinION/GridION/PromethION using Nanobind disks for library cleanup. To maximize sequencing throughput, each 1X library fraction is sequenced for 24 hours, after which a nuclease wash is used to remove the library and recover the pores so that a subsequent 1X library fraction can be loaded and sequenced. This process is repeated using 3 libraries for a total of 72 hours of sequencing. This protocol requires the 1) Circulomics UL Library Prep Kit (NB-900-601-01), 2) Oxford Nanopore Ultra-Long DNA Sequencing Kit (SQK-ULK001), and 3) Oxford Nanopore Flow Cell Wash Kit (SQK-WSH004). The appropriate Circulomics Nanobind Big DNA Kit should be used for UHMW DNA extraction before beginning this protocol.

Please refer to the Circulomics Support Page (https://www.circulomics.com/support-nanobind) for the latest version of this protocol and the appropriate Nanobind Kit Handbook for additional data and guidance.

Oxford Nanopore Technology has also released detailed ultra long library preparation protocols that may be used (https://community.nanoporetech.com/protocols/ultra-long-reads-ULK001). Their protocols contain detailed guidance regarding flow cell priming and loading and should be referred to for such.

Nanobind UHMW DNA Extraction

This protocol requires exceptionally clean and large UHMW (50 kb – 1+ Mb) DNA. It has been validated across a variety of sample types including human cell lines, Gram-negative and Gram-positive bacteria, human blood, nucleated blood, animal tissues, and plants extracted using the appropriate Nanobind Big DNA Kit. We recommend using only the validated Nanobind UHMW DNA extraction protocols to minimize sequencing variability from poor quality DNA. ***The latest Nanobind UHMW DNA extraction protocols and Nanobind Big DNA Kit Handbooks can be found on the Circulomics Support Page.***

We recommend performing extraction at least 1 day before sequencing and allowing the DNA to rest at room temperature overnight before performing QC and library preparation.

DNA Input Requirements

Size: Significant fractions of 100 kb – 1+ Mb UHMW DNA as verified by Bio-Rad CHEF gel.

Amount: Nominally 40 µg of UHMW DNA in 750 µL of Buffer EB+

- Reducing DNA input to 35 µg has been tested and may not negatively impact sequencing.
- Reducing DNA input to 20 µg will generate higher throughputs at slightly shorter read lengths.
- Increasing DNA input will increase read length N50 but may cause reduced throughput.

Recommend Input Method: Because the measurement CV for UHMW DNA is significantly greater than the extraction variability, we recommend going into DNA extraction with an accurately controlled input and then using the entire eluate in the subsequent library prep.

- For diploid human cells, we recommend extracting from $6x10^6$ cells and using the entire elute. For non-diploid or non-human cells, adjust the cell input accordingly to contain 40 µg of gDNA.

- For human blood, we recommend extracting from 1.5 mL of whole blood and using the entire eluate. This input is suitable for samples with healthy WBC counts. For samples with very low WBC counts, the blood input may need to be adjusted to generate 40 µg of DNA if read lengths are unacceptably short.
- For bacteria, we recommend extracting from $5x10^9$ – $5x10^{10}$ cells and using the entire eluate. The bacterial input should be adjusted based on genome size to contain 40 µg of gDNA.
- **See individual extraction protocols for detailed input recommendations and expected DNA recoveries.**

Alternative Input Method: For sample types where the extraction yield is not easily estimated based on the input amount (*e.g.* animal tissues and plants), we recommend using ~40 µg of UHMW DNA based on the average of replicate Nanodrop measurements (n=3–5).

- Determine DNA concentration by taking the average of replicate (n=3–5) UV/Vis absorbance measurements from top, middle, and bottom of the tube (*e.g.* Nanodrop UV/Vis). Concentration measurement CVs can exceed 100% for some sample types. Take additional measurements if necessary.
- **Minimum limit: At least one of the Nanodrop measurements should be >30 ng/µL.**
- **Maximum limit: (Mean – SD) should be <100 ng/µL, where Mean and SD are the mean and standard deviation of the Nanodrop concentration measurements, respectively.**
- If 260/280 and 260/230 ratios deviate significantly from 1.8, Nanodrop nucleic acid concentration measurements may need to be adjusted accordingly to account for contamination.
- We have found that Qubit DNA measurements underestimate UHMW DNA concentration and should only be used in a supplementary fashion. Nanodrop concentrations should take precedence.
- If Qubit RNA measurements indicate high RNA (>50%), Nanodrop nucleic acid concentration measurements may need to be reduced to account for RNA.

Elution Buffer: DNA must be eluted in Circulomics Buffer EB+.

- If an alternate elution buffer is used, please repeat the extraction using the correct elution buffer.

Processing Tips

For new users, always err on the side of being too aggressive with mixing rather than too gentle. Undermixing will result in low throughput and poor sequencing performance.

Gentle mixing should take 4–10 s per 1X pipette cycle (up + down).

DNA Tagmentation / FRA Reaction

Reagent	Volume (µL)
FRA Dilution Buffer (FDB)	244
Fragmentation Mix (FRA)	6
Total	250

1. **Add 244 µL of ONT FRA Dilution Buffer (FDB) and 6 µL of ONT Fragmentation Mix (FRA) to a 1.5 mL Eppendorf DNA LoBind tube. Mix by vortexing and spin down using a mini-centrifuge.**
2. **Add 750 µL of Nanobind-extracted UHMW DNA to a new 1.5 mL Eppendorf DNA LoBind tube using a wide bore P1000 pipette. Then add the 250 µL of diluted ONT FRA from step 1 to the DNA. Stir the reaction with the pipette tip whilst expelling the diluted FRA to ensure an even distribution.**
3. **Immediately vortex the reaction for 5 s at the lowest setting able to generate a gentle vortex in the fluid. Visually check the reagents are thoroughly mixed. If necessary, complete the mixing by gentle pipetting with a wide bore P1000 tip.**
    - It is important to thoroughly mix the solution immediately upon addition of the diluted FRA to the DNA.
    - Longer reads are obtained without vortexing and using only gentle pipette mixing. However, poor mixing will result in reduced pore occupancy and throughput.
4. **Incubate at RT for 5 min.**
5. **Incubate in a heat block at 75 °C for 5 min.**
6. **Remove from heat block and incubate at RT for a minimum of 10 min.**
    - The reaction must cool to room temperature before adding RAP F to prevent denaturing the enzyme.

Quick Tip

For many samples, this can take 5–10 pipette cycles.

Adapter Attachment / RAP Reaction

Reagent	Volume (µL)
DNA from above	1000
Rapid Adapter F (RAP F)	5
Total	1005

7. **Add 5 µL of ONT Rapid Adapter F (RAP F) to the DNA with a standard pipette tip. Mix gently using a wide bore P1000 pipette set to 1000 µL. The mixture should appear homogeneous when pipetting. Inversion can be used to aid mixing.**
8. **Incubate at RT for 30 min.**

Quick Tip

Thorough mixing of RAP F and DNA usually takes 5–10 pipette cycles.

Library Cleanup with Nanobind

9. **Add a Nanobind disk to the DNA library and invert to ensure Nanobind disk is fully wetted.**
10. **Add 500 µL of Buffer NAF. Gently invert 20–30X to mix until all the reagents are thoroughly mixed. Incubate at RT for 10 min.**
    - You should see a DNA "jelly fish" attached to the Nanobind disk.
11. **Invert tube again 10X to ensure all DNA has precipitated and is tightly bound to the Nanobind disk.**
    - A DNA "cloud" should be clearly visible on Nanobind disk.
12. **Place tube on a magnetic rack. Remove and discard the binding solution.**
13. **With tube still on magnetic rack, add 1000 µL of ONT Long Fragment Buffer (LFB) and gently invert once. Keep tube on magnetic rack and incubate at RT for 5 min.**
14. **With tube still on magnetic rack, remove and discard supernatant, taking care not to disturb DNA precipitate. Remove any supernatant in the lid of the Eppendorf tube.**
15. **Repeat steps 13 and 14.**
16. **Add 225 µL of ONT Elution Buffer (EB) to the tube. Incubate overnight at RT, for minimum of 12 hours. Gently aspirate and dispense the eluate over the Nanobind disk at regular intervals with a wide bore pipette tip to aid elution.**
17. **Gently aspirate/dispense the elute over the Nanobind disk 4X with a wide bore P200 pipette before removing the elute from tube and transferring to a new 1.5 mL Eppendorf DNA LoBind tube using a wide bore P200 pipette. The library should be viscous.**

18. **Spin the tube containing the Nanobind disk on a micro-centrifuge at 10,000 x g for 10–15 s and combine any additional liquid that comes off the disk with the previous eluate. Repeat until no more eluate is present at bottom of tube after spin.**
    - This centrifuge step is critical to recover all the library.
    - A standard P200 tip can be used to retrieve the final 5–10 µL of eluate from the bottom of the tube if it is difficult to remove with a wide bore pipette tip.
    - This should not require any more than 1–2 spins.
19. **Gently pipette mix the eluate 5X with a wide bore P200 pipette. Incubate for 2 hours at RT. The library should be viscous.**
20. **Optional: measure the concentration using Nanodrop. The concentration CVs of UHMW DNA are usually large. This step is mainly used as a sanity check for the presence of DNA rather than to accurately determine concentration. If the eluate is viscous and/or the DNA 'strings' away from the solution when the pipette is removed, then DNA is present and Nanodrop measurements are unnecessary.**
21. **The prepared library is used for loading into the flow cell. Store at room temperature to allow DNA to homogenize before loading. Store the library at 4 °C if sequencing is not carried out the same day as library preparation. Library can safely be stored in this way for 3 days.**

MinION Flow Cell Loading and Sequencing

Reagent	Volume (µL)
DNA library	37.5
Sequencing Buffer (SQB)	37.5
Total	75

1. **Add 37.5 µL of ONT Sequencing Buffer (SQB) to 37.5 µL of the DNA library from above. Gently pipette mix with a wide bore P200 pipette set to 75 µL until the sample is homogeneous. Incubate at RT for 30 min.**
    - Do not use loading beads.
    - Store the remaining library at 4 °C until ready to use.
2. **Gently pipette mix with a wide bore P200 pipette set to 75 µL. Confirm visually that the sample is homogeneous. Library is now ready to load.**
3. **Before loading the library, prime the flow cell following instructions from Oxford Nanopore Technologies.**
4. **Add the library to the flow cell via SpotON port using a wide bore P200 pipette. It should be quite viscous but should load smoothly in <1 min.**
    - If the library is too viscous and does not readily flow into the SpotON port, negative pressure can be applied by gently aspirating from the lower adjacent port using a pipette: 1) Cover Waste Port 2 and the Priming Port with clean gloved fingers; 2) Using a P200 pipette, insert the tip in Waste Port 1; 3) Very slowly aspirate to pull the library into the SpotON sample port. Closely watch the library on the SpotON sample port. Completely remove the pipette as soon as the library starts to be pulled into the port; 5) Ensure there are no air gaps between subsequent loads of library.
    - If the library does not load with negative pressure on the waste port, remove it and pipette it into to a 1.5 mL Eppendorf DNA LoBind tube. Slowly pipette mix 5X with a standard P200 pipette set to 75 µL. Then, try re-loading onto the flow cell using a wide bore P200 pipette.
    - If it still does not load, the DNA extraction and library prep will need to be repeated.
5. **Start sequencing run with sequencing kit set to SQK-ULK001 and the re-mux time set to 6 hours.**
6. **After 24 hours, pause sequencing run and wash flow cell using the Flow Cell Wash Kit (EXP-WSH004). Follow Flushing a MinION/GridION Flow Cell in the Flow Cell Wash Kit protocol. To run another library straight away, follow the modified method: To run another library of ultra-long DNA on a MinION/GridION flow cell straight away in the SQK-ULK001 MinION protocol.**
7. **Re-load library following steps 1–6.**
8. **Un-pause sequencing run to resume sequencing. Manually trigger mux scan immediately.**

Quick Tip

Thorough mixing of SQB and library in step 1 of MinION Flow Cell Loading and Sequencing usually takes 10 pipette cycles.

Quick Tip

For MinION loading: Try to load the DNA onto the flow cell in one continuous stream rather than in drops.

Quick Tip

Do not use EXP-WSH003. EXP-WSH004 results in improved read length and pore recovery.

Quick Tip

To run a second library of ultra-long DNA straight after flushing a flow cell, we recommend removing all fluid from the waste channel after each priming step.

PromethION Flow Cell Loading and Sequencing

Reagent	Volume (µL)
DNA library	75
Sequencing Buffer (SQB)	75
Total	150

1. **Add 75 µL of Sequencing Buffer (SQB) to 75 µL of the DNA library from above. Gently pipette mix with a wide bore P200 pipette set to 150 µL until the sample is homogeneous. Incubate at RT for 30 min.**
    - Do not use loading beads.
    - Store the remaining library at 4 °C until ready to use.
2. **Gently pipette mix with a wide bore P200 pipette set to 150 µL. Confirm visually that the sample is homogeneous. Library is now ready to load.**
3. **Before loading the library, prime the flow cell following instructions from Oxford Nanopore Technologies.**
4. **Add library to flow cell by dropping 30 µL at a time onto the inlet port. It should form a droplet on top of the port and get drawn in slowly. It should be quite viscous, but each 30 µL should load smoothly in about 1–2 min.**
    - If the library is too viscous and does not get drawn into the inlet port, negative pressure can be applied by gently aspirating from Port 2 using a pipette: 1) Set a P1000 pipette to 200 µL; 2) Insert the tip into Port 2; 3) Turn the volume setting wheel higher to aspirate and create negative pressure in the channel, pulling the library into the inlet port (Port 1). Closely watch the library on the inlet port. Completely remove the pipette as soon as the library starts to be pulled into the port; 5) Ensure there are no air gaps between subsequent loads of library.
    - If the library does not load with negative pressure on Port 2, remove it and pipette it into a 1.5 mL Eppendorf DNA LoBind tube. Slowly pipette mix 5X with a standard P200 pipette set to 150 µL. Then, try re-loading onto the flow cell using a wide bore P200 pipette.
    - If it still does not load, the DNA extraction and library prep will need to be repeated.
5. **Start sequencing run with sequencing kit set to SQK-ULK001 and the re-mux time set to 6 hours.**
6. **After 24 hours, pause sequencing run and wash flow cell using the Flow Cell Wash Kit (EXP-WSH004). Follow Flushing a PromethION Flow Cell in the Flow Cell Wash Kit protocol. To run another library straight away, follow the modified method: To run another library of ultra-long DNA on a PromethION flow cell straight away in the SQK-ULK001 PromethION protocol.**
7. **Re-load library following steps 1–6.**
8. **Un-pause sequencing run to resume sequencing. Manually trigger mux scan immediately**

Quick Tip

Thorough mixing of SQB and library in step 1 of PromethION Flow Cell Loading and Sequencing usually takes 10 pipette cycles.

Quick Tip

Do not use EXP-WSH003. EXP-WSH004 results in improved read length and pore recovery.

Quick Tip

To run a second library of ultra-long DNA straight after flushing a flow cell, we recommend removing all fluid from the waste channel after each priming step.

Troubleshooting FAQ

1. **Why is my recovery from the library preparation low?**

   - UHMW DNA will be clearly visible on the Nanobind disk during binding with Buffer NAF. If you do not see DNA at this step and do not recover DNA, then either the input mass is too low or the DNA size is too small.
   - It is possible to aspirate very loosely bound DNA. To avoid this and subsequent DNA loss, nudge the Nanobind disk up the side of the tube using the pipette tip while the tube is on the magnetic rack and take care not to aspirate DNA.
   - Ensure all eluate is removed from the disk and transferred to another tube after Nanobind cleanup. Some liquid may remain on the Nanobind disk after pipetting. Spin the tube containing the Nanobind disk on a micro-centrifuge at 10,000 x g for 15 s and transfer any additional liquid that comes off the disk to the previous eluate. Repeat as necessary until all DNA is removed. Complete elution of UHMW DNA can take patience. This centrifuge step is critical but should not take more than 1–2 spins.

2. **The library will not load onto the flow cell.**

   - For MinION loading: If the library is too viscous and does not readily flow into the SpotON port, negative pressure can be applied by gently aspirating from the lower adjacent port using a pipette: 1) Cover Waste Port 2 and the Priming Port with clean gloved fingers; 2) Using a P200 pipette, insert the tip in Waste Port 1; 3) Very slowly aspirate to pull the library into the SpotON sample port. Closely watch the library on the SpotON sample port. Completely remove the pipette as soon as the library starts to be pulled into the port; 5) Ensure there are no air gaps between subsequent loads of library.
   - For PromethION loading: If the library is too viscous and does not get drawn into the inlet port, negative pressure can be applied by gently aspirating from Port 2 using a pipette: 1) Set a P1000 pipette to 200 µL; 2) Insert the tip into Port 2; 3) Turn the volume setting wheel higher to aspirate and create negative pressure in the channel, pulling the library into the inlet port (Port 1). Closely watch the library on the inlet port. Completely remove the pipette as soon as the library starts to be pulled into the port; 5) Ensure there are no air gaps between subsequent loads of library.

3. **I've tried using negative pressure to load the flow cell, and it doesn't work. What can I do now?**

   - If the library does not load with negative pressure on the flow cell described above, remove it and pipette it into to a 1.5 mL Eppendorf DNA LoBind tube. Slowly pipette mix 5X with a standard P200 pipette set to 75 µL (MinION) or 150 µL (PromethION). Then, try re-loading onto the flow cell using a wide bore P200 pipette.

4. **I've tried everything, and the library still won't load.**

   - It is likely that the mass concentration of DNA is too high. Try 1) reducing the amount of library loaded onto the flow cell by diluting the library with EB before adding SQB and loading or 2) reducing the amount of DNA going into the library preparation.
   - It's possible the extraction was performed too gently, resulting in compromised lysis efficiency and overly viscous DNA. Repeat the extraction with more aggressive mixing, particularly at any steps that involve resuspension of cell pellets.
   - You may also try combining more 1) reduced library prep input and 2) more aggressive extraction.

5. **The sequencing throughput is low. How can I increase it?**

   - Vortex and pipette mix more aggressively after adding the diluted FRA to the DNA to break up the largest fragments.
   - Use lower masses of DNA input for the library preparation.

6. The read lengths are not long enough. How can I increase them?

- Increase the mass of DNA used as input for the library preparation. This will increase read lengths but may also increase library viscosity.
- Check length of extracted UHMW DNA using pulsed-field gel electrophoresis. Other DNA sizing instruments do not have sufficient resolution at 500+ kb to determine the presence of UHMW DNA.