Nanobind UL Library Prep Kit Handbook

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For ultra long sequencing library preparation on Oxford Nanopore MinION, GridION, and PromethION

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Kit Specifications

Contents

Nanobind UL Library Prep Kit Part Number Number of Samples	v1 NB-900-601-01 6
Nanobind Disks	6
Buffer NAF	3.3 mL
Buffer WAF	13.2 mL
Buffer EB+	10 mL

Kit Storage

All reagents should be stored at room temperature (18–25 °C).

Shelf Life

Nanobind UL Library Prep Kits are guaranteed for 1 yr from the date of purchase.

Product Use

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

UHMW DNA Aux Kit Part Number Number of Samples	v1 NB-900-101-01 10
Buffer RBC 10X	10 mL
Proteinase K	0.25 mL
RNase A	0.25 mL
Buffer CLE3	0.45 mL
Buffer ULL	5 mL
Buffer CS	0.5 mL

Kit Storage

RNase A and Buffer RBC10X should be stored at 4 °C upon arrival.

All other reagents should be stored at room temperature (18–25 °C).

Shelf Life

UHMW DNA Aux Kits are guaranteed for 1 yr from the date of purchase.

Safety Precautions

Buffer ULL contains guanidine hydrochloride. Warning! Guanidine hydrochloride is harmful if swallowed or inhaled and causes skin and eye irritation. DO NOT mix with bleach or acidic solutions.

Product Use

UHMW DNA Aux Kits are intended for research use only.

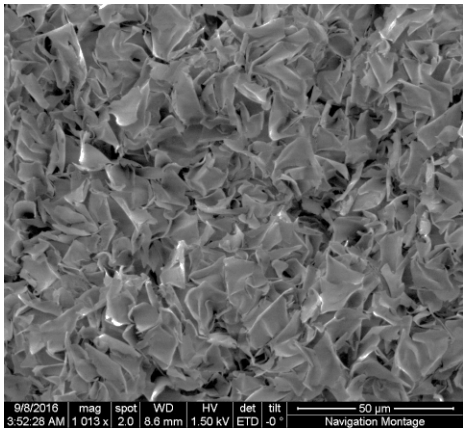
Equipment and Reagent List

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

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

Introduction

Nanobind is a novel magnetic disk covered with a high density of micro- and nanostructured silica that can be used for rapid extraction and purification of high-quality DNA and RNA. The high surface area and unique binding mechanism give it an extraordinary binding capacity, allowing isolation of high purity, high molecular weight DNA in a microcentrifuge tube format. It uses a standard lyse, bind, wash, and elute procedure that is common for silica DNA extraction technologies. A single disk is used in each tube. However, unlike magnetic beads and silica spin columns which shear large DNA, Nanobind binds and releases DNA without fragmentation, to yield DNA up to megabases in length.



SEM image of Nanobind's silica surface structure.

Kit Overview

The Nanobind UL Library Prep Kit is used in Nanobind Ultra Long Sequencing to generate ultra long (100 kb – 1+ Mb) reads on Oxford Nanopore MinION/GridION/PromethION instruments. This method is designed to maximize production of 100+ kb and 200+ kb reads and generates data throughputs that are 10–100X greater than previous ultra long sequencing methods. It generates read length N50s of 50–100+ kb with throughputs of 10–25+ Gb per flow cell on MinION and 40–125+ Gb per flow cell on PromethION. Runs will typically have a long-tail of reads extending to 1–2+ Mb in length. The current record read length generated using this kit is 4.2 Mb.

First, the appropriate Nanobind Big DNA Kit (CBB, Tissue, or Plant) is used to extract 40 µg of UHMW (50 kb – 1+ Mb) DNA. The DNA must be exceptionally clean and contain substantial portions of 500+ kb DNA for best results. New Nanobind UHMW DNA extraction protocols were specifically developed to generate longer and less viscous, megabase-sized DNA. The UHMW DNA Aux Kit is used in conjunction with the Nanobind Big DNA Kits to run these new protocols.

Then, the library is prepared for sequencing using the Oxford Nanopore Ultra-Long DNA Sequencing Kit (SQK-ULK001). This is a transpose library preparation that has been optimized to efficiently adapt ultra long DNA while minimizing fragmentation.

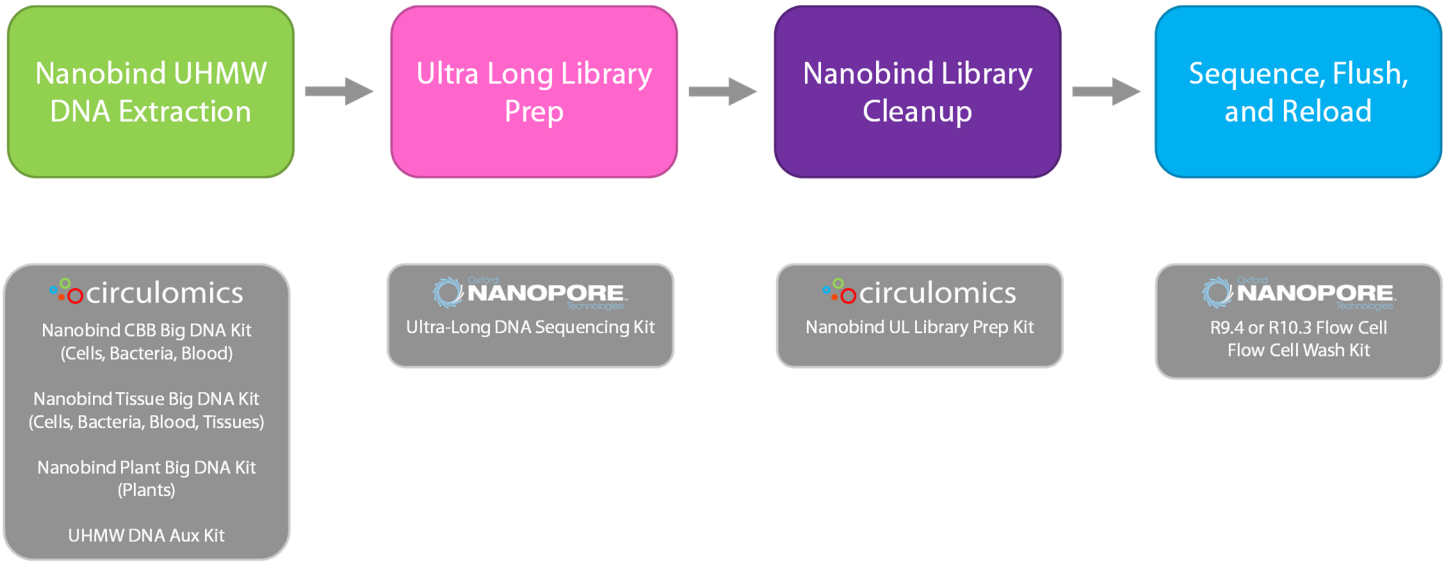
Next, the library is cleaned up using the Nanobind UL Library Prep Kit. The Nanobind disks enable purification of megabase length libraries without shearing. The purification chemistries are fully compatible with all Oxford Nanopore sequencing platforms and kits.

Finally, sequencing is performed on any Oxford Nanopore instrument using either R9.4 or R10.3 flow cells. The standard library preparation protocol generates a 6X scale library for MinION/GridION or a 3X scale library for PromethION that is split into 1X fractions. Each 1X library fraction is loaded sequentially onto the same flow cell every 24 hours, interspaced by nuclease wash steps, for a total of 72 hours of sequencing. The entire workflow from extraction through library prep and sequencing has been optimized to generate ultra long reads and high pore occupancy. Maintaining high pore occupancy is the key to high sequencing throughput!

A single library prep protocol is provided that can be used for either MinION or PromethION sequencing. The only difference lies in the flow cell loading steps.

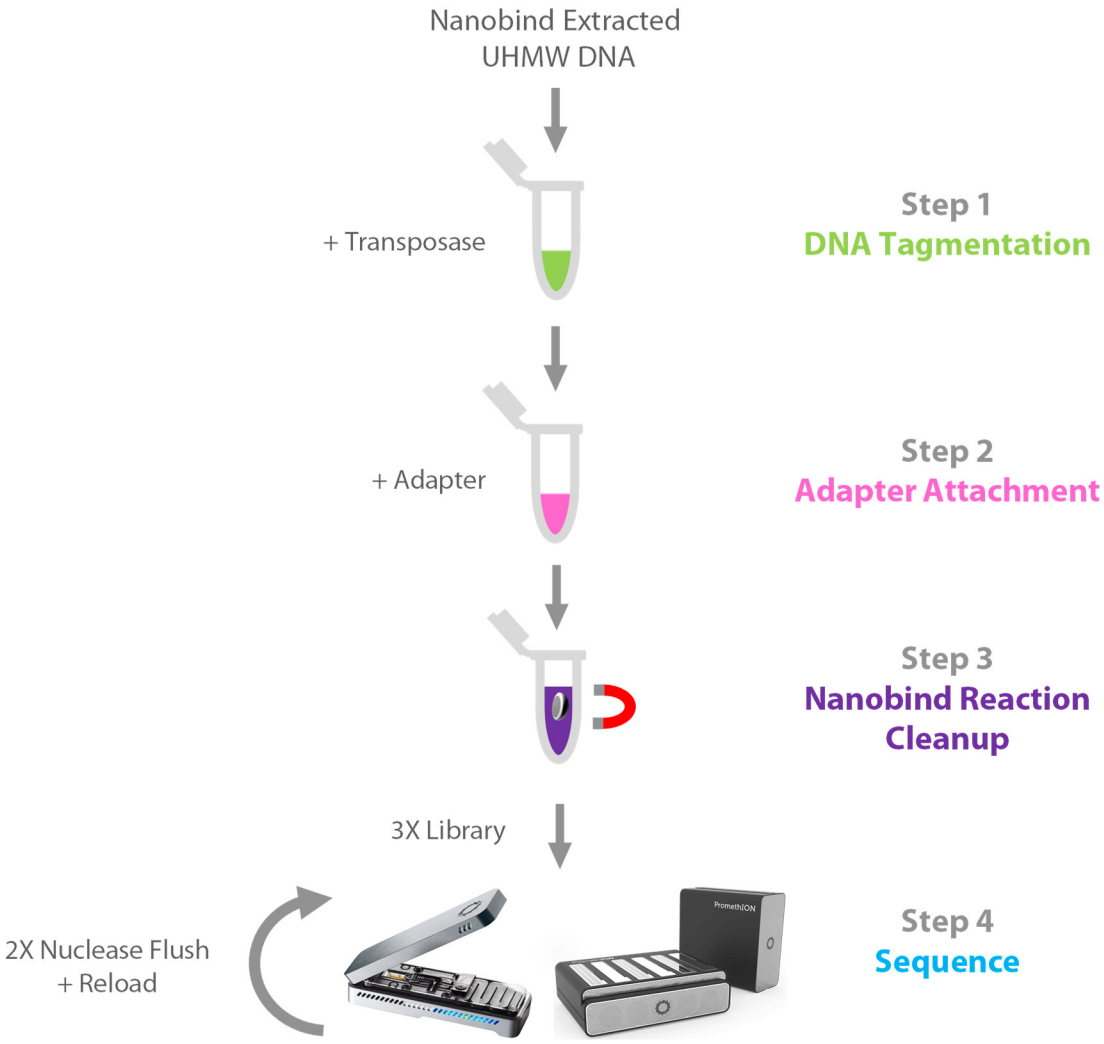
UHMW DNA Extraction protocols for a variety of validated sample types are also provided.

Protocols are updated and added frequently so please check the Nanobind Support page for the most up-to-date list and for the current version of the protocol (http://www.circulomics.com/support-nanobind).



Nanobind Ultra Long Sequencing uses a combination of kits for UHMW DNA extraction, library preparation, library cleanup, and sequencing to generate large numbers of ultra long reads (>100 kb).

Workflow



Nanobind Ultra Long Sequencing library preparation and sequencing workflow.

Sequencing Data

The following pages illustrate sequencing data generated by first performing Nanobind UHMW DNA extractions using the appropriate Nanobind Big DNA Kit and then library prep and sequencing using the Nanobind UL Library Prep Kit and Oxford Nanopore Ultra-Long DNA Sequencing Kit. A summary is provided in the tables below. Please check our website for the most recent data.

Samples generate nearly identical read length distributions on MinION and PromethION with the main difference being that total data throughput is 3–5X higher on PromethION. Libraries are compatible with both R9.4 and R10.3 flow cells.

R9.4 Sequencing Summary

Nanobind Ultra Long Sequencing – R9.4 Sequencing Metrics							
Sample	**Instrument**	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
HG02723 Human Cell Line	MinION	24.5	96.8	11.9	4.7	6	1.2
HG02723 Human Cell Line	PromethION	129.6	93.1	60.5	20.3	0	0.99
Human Blood	MinION	18.6	104.6	9.9	4.2	11	1.5
Human Blood	PromethION	67.6	84.4	28.4	8.1	6	1.3
E. coli	MinION	25.9	66.4	8.6	2.7	1	1.1
E. coli[1]	PromethION	77.4	128	47.1	22.5	133	3.3
L. monocytogenes	MinION	22	50	4.8	1.3	1	1.2
L. monocytogenes	PromethION	58	53	13.2	3.6	3	1.0
Fish Blood	MinION	16.8	98.6	8.3	3.4	1	1.0
Shingleback Lizard Blood[2]	PromethION	62.8	78.4	24.0	5.8	3	1.6
Bovine Lung[3]	PromethION	38.5	51	8.9	1.9	3	0.9
Pepper Leaf[4]	MinION	9.0	51.5	1.9	0.3	0	0.52

[1]Data generated in collaboration with Oxford Nanopore Technologies
[2]Data generated in collaboration with Garvan Institute
[3]Data generated in collaboration with USDA
[4]Data generated in collaboration with Keygene

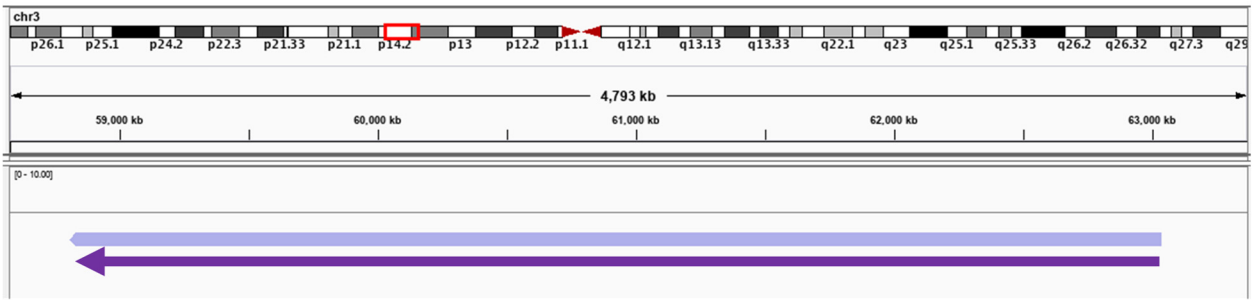
R10.3 Sequencing Summary

Nanobind Ultra Long Sequencing – R10.3 Sequencing Metrics							
Sample	**Instrument**	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
HG02723 Human Cell Line	PromethION	89.7	83.3	37.2	11.0	1	1.1
Human Blood	PromethION	43.3	90.3	19.5	5.9	14	1.6

Longest Reads

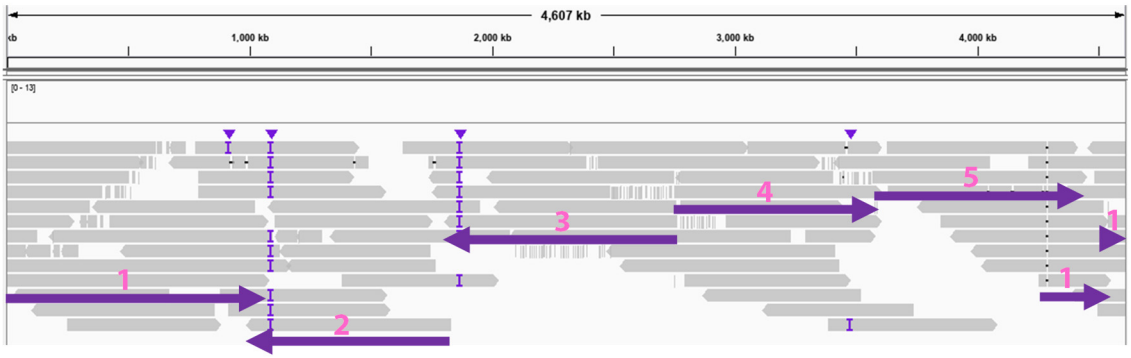
Nanobind Ultra Long Sequencing has been used to generate the longest sequencing reads ever. To date, the record is a 4.2 Mb read mapping to human chromosome 3. This read was generated from cell lines by Oxford Nanopore. We routinely see longest reads of 1–2+ Mb in each run.

4.2 Mb Read Aligning to Human Chr. 3



Below are 600+ kb reads from a single MinION run on *E. coli*. Miniasm was used to assemble a complete genome using only 5 reads (contig N50 = 4,597,548)

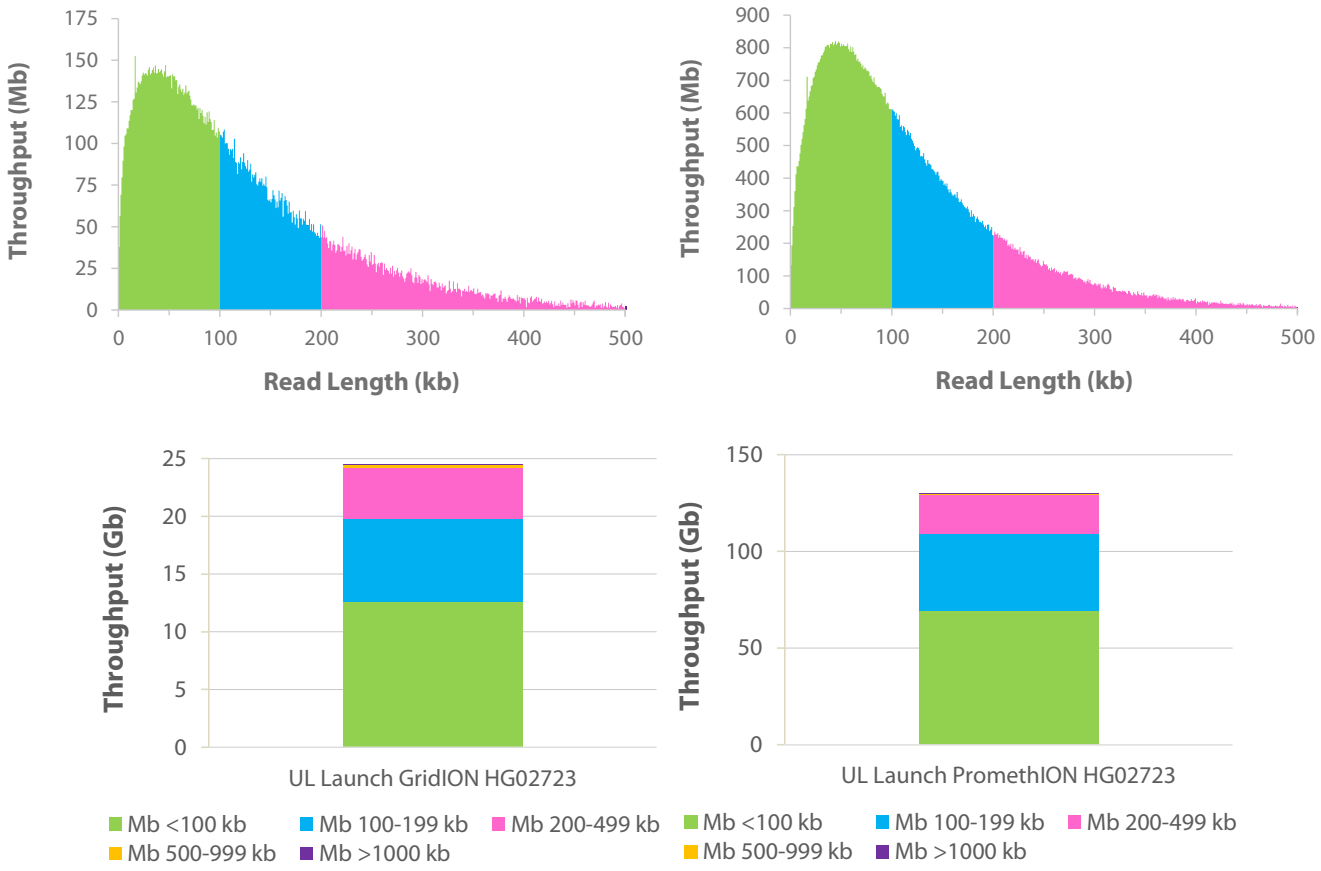
Complete *E. coli* Assembly Using Only 5 UL Reads



Cell Line Sequencing

Ultra long libraries were prepared from HG02723 cells and sequenced in parallel on MinION and PromethION, respectively.

- UHMW DNA was extracted from $6x10^6$ cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Cultured Cells Protocol.
- 3X libraries were prepared and sequenced on R9.4 MinION (FLO-MIN106D) and PromethION Flow Cells (FLO-PRO002) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- MinION and PromethION generated similar read length distributions with PromethION generating 5X more data.
- Cell lines typically result in read length N50 of 75–100+ kb and throughputs of 15–25+ Gb on MinION and 40–125+ Gb on PromethION.
- If read length N50 is lower than expected, please increase DNA input.



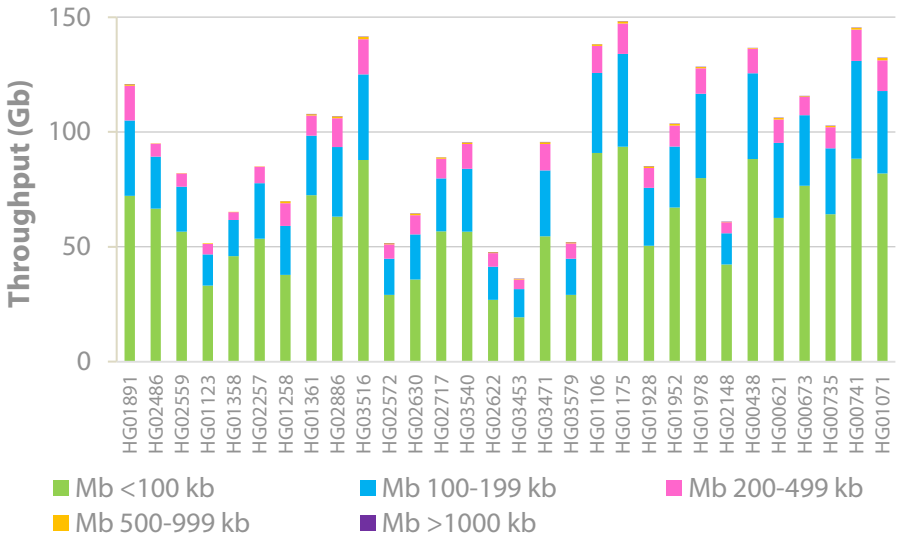
Nanobind Ultra Long Sequencing – HG02723 Human Cell Line						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
MinION (left)	24.5	96.8	11.9	4.7	6	1.2
PromethION (right)	129.6	93.1	60.5	20.3	0	0.99

Human Pangenome Reference Ultra Long Sequencing Beta Test

A beta version of Nanobind Ultra Long Sequencing was tested on the first 30 genomes for the Human Pangenome Reference Program in collaboration with UC Santa Cruz Genomics Institute and Oxford Nanopore Technologies.

- UHMW DNA was extracted from $10x10^6$ cells in triplicate using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Cultured Cells Protocol.
- 3X libraries were prepared and sequenced on R9.4 PromethION Flow Cells (FLO-PRO002) using a beta version of the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- 3 flow cells were run per cell line (91 flow cells total).
- The runs resulted in an average read length N50 of 77.9 kb with throughput of 20–50+ Gb per flow cell and an average of 17 megabase reads per flow cell. The longest read generated was 3.5 Mb.
- Nanobind Ultra Long Sequencing protocol required just 3 PromethION flow cells to generate 148 Gb of total data for HG01175.

Nanobind Ultra Long Sequencing – Human Pangenome Beta Test						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
Average Per Genome	95.3	77.5	35.9	9.7	50.2	3.5
Average Per Flow Cell	31.4	77.9	11.8	3.2	16.6	3.5

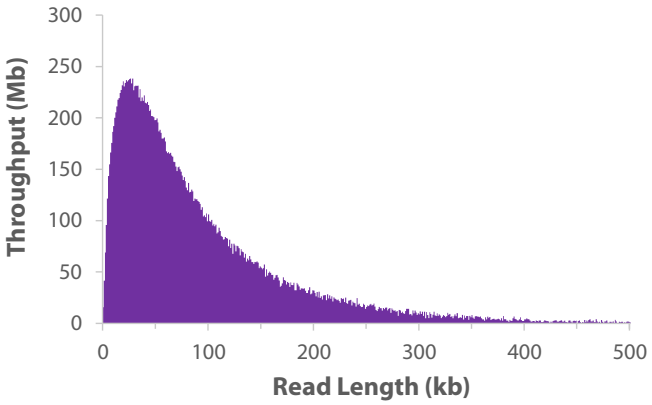


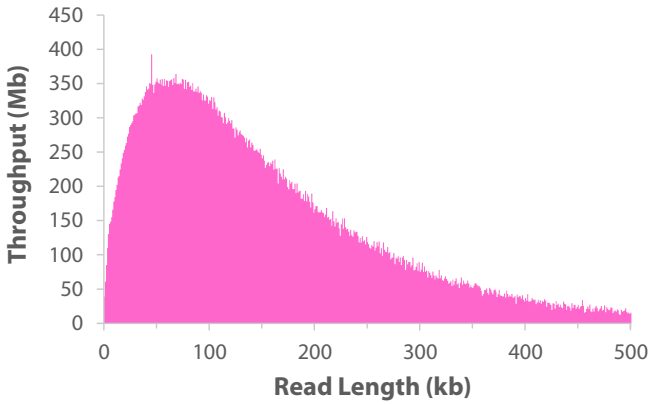
Data throughput categorized by read length for each genome.

Bacteria Ultra Long Sequencing

Ultra long libraries were prepared from cultured *E. coli* and *L. monocytogenes* pellets and sequenced on MinION and PromethION

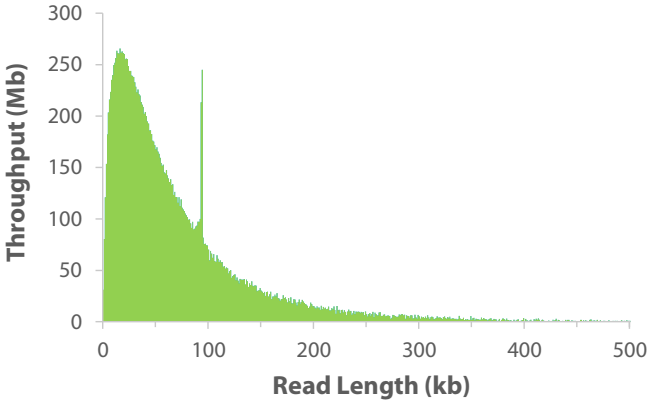
- UHMW DNA was extracted from 1x10^9 *E. coli* cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Gram-Negative Protocol.
- UHMW DNA was extracted from 5x10^9 *L. monocytogenes* cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Gram-Positive Protocol.
- *E. coli* data was generated in collaboration with Oxford Nanopore.
- A 3X library was prepared and sequenced on R9.4 MinION (FLO-MIN106D) and PromethION Flow Cells (FLO-PRO002) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- Bacteria typically result in read length N50 of 50–100+ kb and throughputs of 15–25+ Gb on MinION and 40–125+ Gb on PromethION.
- If read length N50 is lower than expected, please increase DNA input.

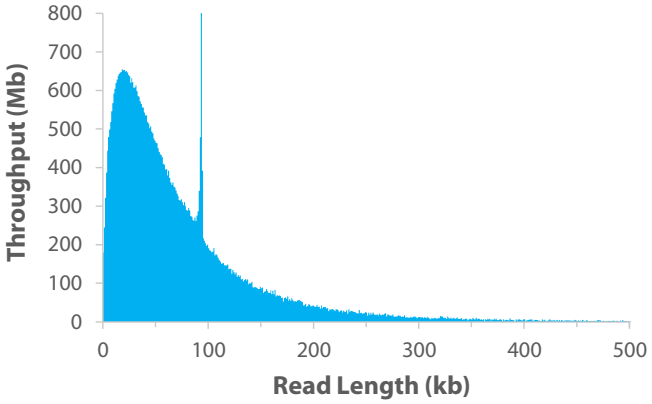




Nanobind Ultra Long Sequencing - Gram-negative bacteria, *E. coli*						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
MinION (purple)[1]	25.9	66.4	8.6	2.7	1	1.1
PromethION (pink)[2]	77.4	128	47.1	22.5	133	3.3

[1]Sequenced using a single load for 72 h
[2]Data generated in collaboration with Oxford Nanopore Technologies. Sequenced using a single load for 72 h.



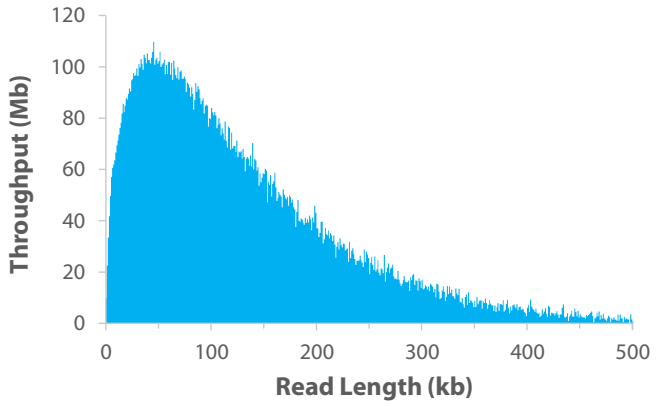


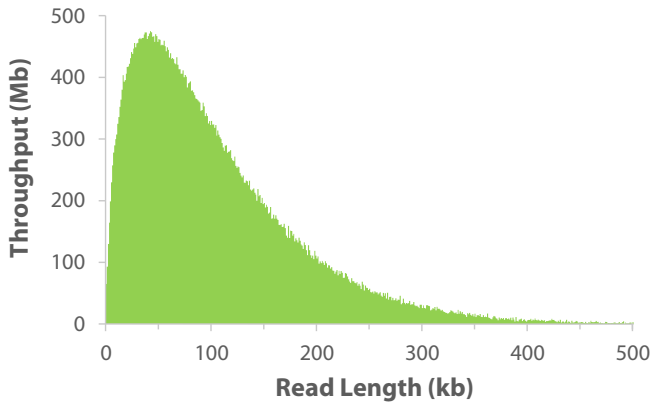
Nanobind Ultra Long Sequencing – Gram-positive bacteria, *L. monocytogenes*						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
MinION (green)	22	50	4.8	1.3	1	1.2
PromethION (blue)	58	53	13.2	3.6	3	1.0

Human Blood Ultra Long Sequencing

Ultra long libraries were prepared from human whole blood samples and sequenced on MinION and PromethION

- UHMW DNA was extracted from 1.5 mL of frozen human whole blood using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Human Blood Protocol.
- 3X libraries were prepared and sequenced on R9.4 MinION (FLO-MIN106D) and PromethION Flow Cells (FLO-PRO002) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- Human blood typically results in read length N50 of 75–100+ kb and throughputs of 15–25+ Gb on MinION and 40–125+ Gb on PromethION.
- If read length N50 is lower than expected, please increase DNA input.



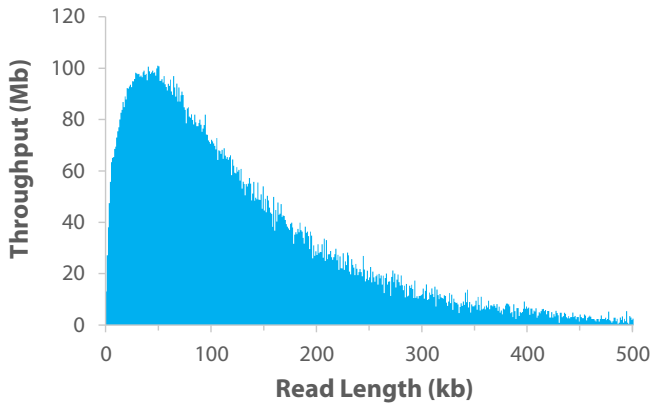


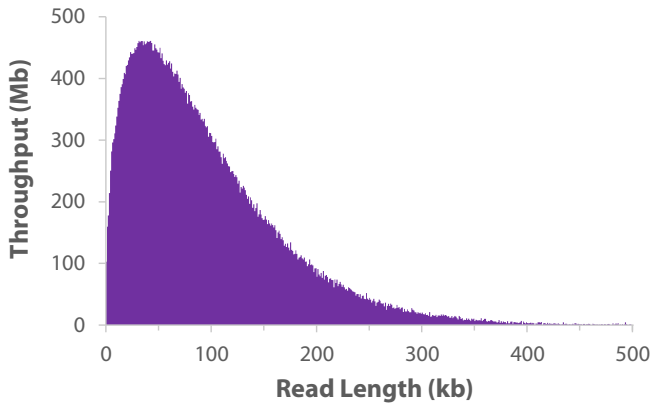
Nanobind Ultra Long Sequencing – Human Blood						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 k**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
GridION (blue)	18.6	104.6	9.9	4.2	11	1.5
PromethION (green)	67.6	84.4	28.4	8.1	6	1.3

Nucleated Blood Ultra Long Sequencing

Ultra long libraries were prepared from nucleated blood samples from fish and lizard and sequenced on MinION and PromethION

- UHMW DNA was extracted from 10 µL of frozen fish blood or frozen lizard blood using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Nucleated Blood Protocol.
- Lizard blood was data generated in collaboration with Garvan Institute.
- 3X libraries were prepared and sequenced on R9.4 MinION Flow Cells (FLO-MIN106D) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- Nucleated blood typically results in read length N50 of 75–100+ kb and throughputs of 15–25+ Gb on MinION and 40–125+ Gb on PromethION.
- If read length N50 is lower than expected, please increase DNA input.



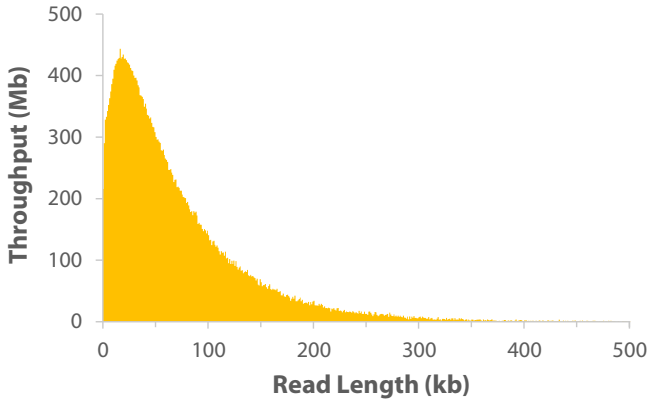


Nanobind Ultra Long Sequencing – Nucleated Blood						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
Fish Blood (GridION, blue)	16.8	98.6	8.3	3.4	1	1.0
Australian Sleepy Lizard (PromethION, purple)	62.8	78.4	24.0	5.8	3	1.6

Tissue Ultra Long Sequencing

An ultra long library was prepared from a bovine fetal lung tissue and sequenced on PromethION. These data are preliminary and shown for reference purposes.

- UHMW DNA was extracted from 34 mg of bovine fetal lung tissue using the Nanobind Tissue Big DNA Kit and Nanobind UHMW DNA Extraction –TissueRuptor Tissue Protocol.
- Data generated in collaboration with USDA.
- A 3X library was prepared and sequenced on a R9.4 PromethION Flow Cell (FLO-PRO002) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- Development is underway to enhance read length and throughput.

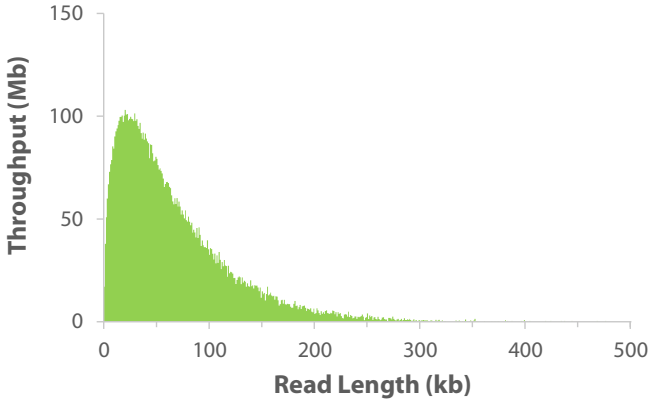


Nanobind Ultra Long Sequencing - Bovine Fetal Lung						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
PromethION	38.5	51	8.9	1.9	3	0.9

Plant Ultra Long Sequencing

An ultra long library was prepared from a pepper leaf sample and sequenced on GridION. These data are preliminary and shown for reference purposes.

- UHMW DNA was extracted from 5 g of pepper leaf using the Nanobind Plant Nuclei Big DNA Kit and Nanobind HMW DNA Extraction – Plant Nuclei Protocol.
- Data generated in collaboration with Keygene.
- A 3X library was prepared and sequenced on a R9.4 MinION Flow Cell (FLO-MIN106D) using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- Development is underway to enhance read length and throughput.

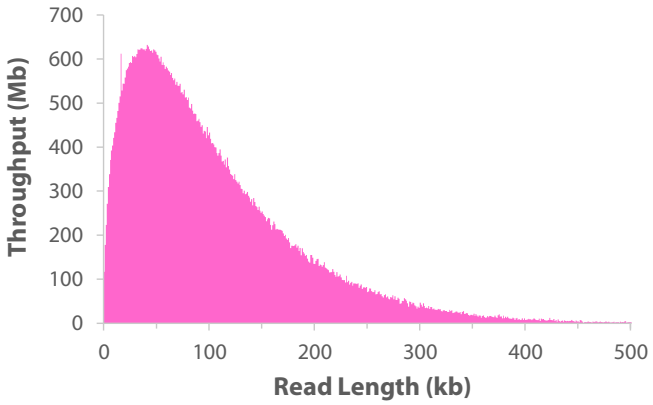


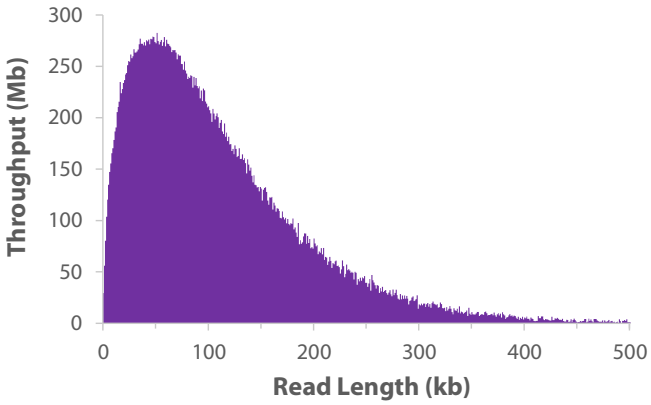
Nanobind Ultra Long Sequencing – Pepper						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
MinION	9.0	51.5	1.9	0.3	0	0.52

R10.3 Cell Line Ultra Long Sequencing

Ultra long libraries were prepared from HG02723 cells and human whole blood and sequenced on PromethION using an R10.3 flow cell.

- UHMW DNA was extracted from 6x10^6 cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Cultured Cells Protocol.
- UHMW DNA was extracted from 1.5 mL of frozen human whole blood using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Human Whole Blood Protocol.
- 3X libraries were prepared and sequenced on R10.3 PromethION Flow Cells using the Nanobind Library Prep – Ultra Long Sequencing Protocol.
- R10.3 PromethION flow cells have resulted in read length N50 of 75–100+ kb and throughputs of 40–90 Gb.





Nanobind Ultra Long Sequencing – Pepper						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
HG02723 (pink)	89.7	83.3	37.2	11.0	1	1.1
Human Blood (purple)	43.3	90.3	19.5	5.9	14	1.6

Processing and Sequencing Tips

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 (https://www.circulomics.com/support-nanobind).***

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.

Always recover all eluate from a Nanobind extraction/ cleanup. Use a narrow bore P200 to facilitate recovery of all liquid after spinning at 10,000 x g. It is more important to recover everything than to use wide bore pipettes for handling. Significant reduction of sequencing throughput will occur if not all DNA/library is recovered, whereas we have found that the use of standard pipettes reduces the number of Mb+ reads but does not significantly impact read N50.

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.

If the library does not load with negative pressure on Waste Port 2 then the library/SQB mixture can be removed and added to a 1.5 mL Eppendorf DNA LoBind tube. Slowly pipette mix 5x with a narrow-bore P200 pipette set to 75 µL and try re-loading to the flow cell using a wide bore P200 pipette.

PromethION Loading

Try to keep the area around the loading port clear and dry after performing nuclease wash and flush buffer loading. There are two reasons for this: 1) excess nuclease should be removed before loading DNA to avoid contamination of nuclease that will degrade sample and reduce read lengths; 2) when applying sample in 30 µL drops it should form a tightly localized droplet above the port, excess residual liquid around the port will spread the droplet out and impair efficiency of sample 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.

If the library does not load with negative pressure on the waste port then the library/SQB mixture can be removed and added to a 1.5 mL Eppendorf DNA LoBind tube. Slowly pipette mix 5x with a narrow-bore P200 pipette set to 75 µL and try re-loading to the flow cell using a wide bore P200 pipette.

Read Splitting

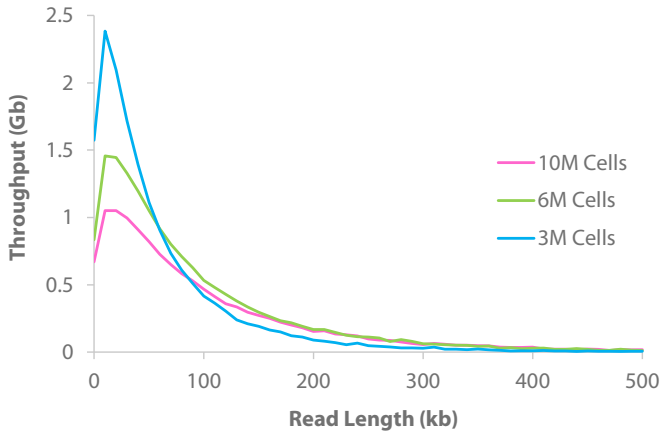
MinKNOW can sometimes inadvertently split ultra long reads. Please select the SQK-ULK001 kit when starting sequencing run to minimize read splitting and increase re-mux time to 6 hours.

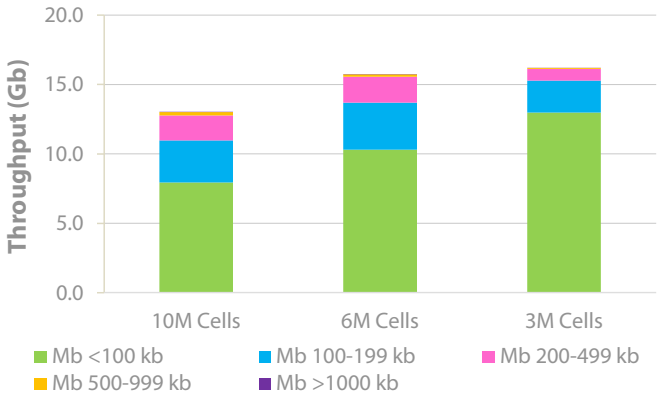
If a suitable reference exists, split reads can be identified using scripts from Payne *et al*. Bioinformatics 2018 (https://doi.org/10.1093/bioinformatics/bty841).

DNA Input Titration

Ultra long libraries were prepared from $3x10^6$, $6x10^6$, and $10x10^6$ HG02723 cell pellets and sequenced on MinION.

- UHMW DNA was extracted from $3x10^6$, $6x10^6$, and $10x10^6$ cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Cultured Cells Protocol.
- All DNA from each extraction was brought forward into library prep.
- 3X libraries were prepared and sequenced on R9.4 MinION (FLO-MIN106D) Flow Cells using the Nanobind Library Prep – Ultra Long Sequencing Protocol. Other than cell input, no other parameters were changed.
- As DNA input is reduced, fragmentation increases and read length N50 drops. However, overall sequencing yield increases. Further reduction in DNA input will likely cause decreases in throughput.
- The highest absolute amount of UL data (*i.e.* Gb in reads >100 and >200 kb) is maximized at moderate read length N50 where fragmentation and pore occupancy are balanced.
- If read lengths are too short, increase DNA input.
- If throughput is too low, decrease DNA input.



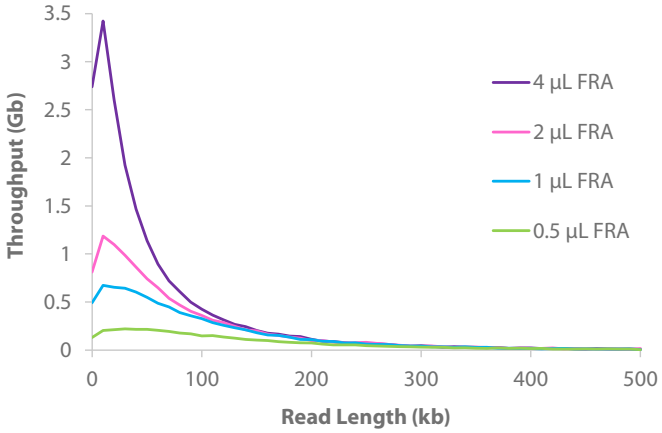


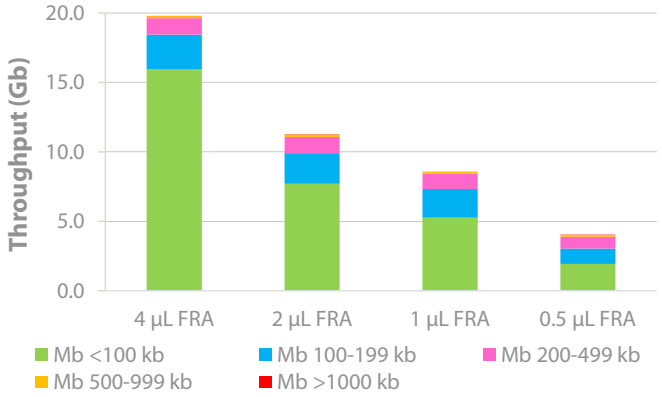
Nanobind Ultra Long Sequencing – ONT MinION DNA Input Titration Sequencing Metrics						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
$10x10^6$ Cells (10M)	13.0	74.4	5.1	2.1	13	1.3
$6x10^6$ Cells (6M)	15.8	66.1	5.4	2.0	6	1.6
$3x10^6$Cells (3M)	16.2	42.2	3.2	0.9	1	1.3

FRA Input Titration

Ultra long libraries were prepared from HG02723 cells and sequenced on MinION.

- UHMW DNA was extracted from 10x10^6 cells using the Nanobind CBB Big DNA Kit and Nanobind UHMW DNA Extraction – Cultured Cells Protocol.
- 3X libraries were prepared and sequenced on R9.4 MinION (FLO-MIN106D) Flow Cells using the Nanobind Library Prep – Ultra Long Sequencing Protocol. In each of the libraries, different amounts of FRA were used during tagmentation. No other parameters were changed.
- Read lengths can be increased by reducing FRA but this will often reduce overall throughput.
- The highest absolute amount of ultra long data (*i.e.* Gb in reads >100 and >200 kb) is maximized at moderate read length N50 where fragmentation and pore occupancy are balanced.





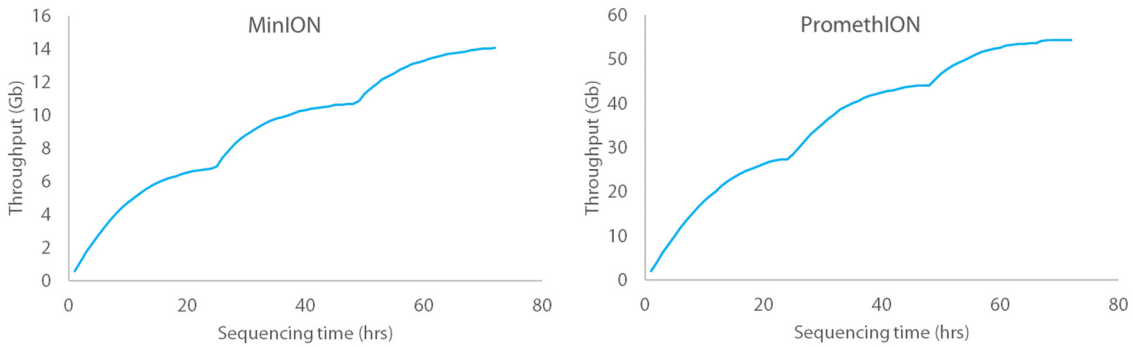
Nanobind Ultra Long Sequencing – ONT MinION DNA Input Titration Sequencing Metrics						
Instrument	**Total Bases (Gb)**	**N50 (kb)**	**Gb >100 kb**	**Gb >200 kb**	**Number Reads > 1 Mb**	**Longest Read (Mb)**
4 µL FRA	19.8	35.5	3.8	1.3	4	1.1
2 µL FRA*	11.3	59.2	3.5	1.3	5	1.2
1 µL FRA	8.6	74.0	3.3	1.2	4	1.2
0.5 µL FRA	4.0	105.2	2.1	1.0	11	1.4

* only two libraries were loaded.

Nuclease Flush Throughput and Pore Recovery

The standard protocol generates a 6X (MinION/GridION) or 3X (PromethION) scale library that is split into 1X fractions and sequenced sequentially on the same flow cell. Each fraction is sequenced for 24 h and then washed from the flow cell using nuclease flush until 72 hours of sequencing is completed.

- Under ideal conditions, each 1X fraction generates approximately 1/3 of the total data.
- The earlier loads usually generate slightly more data than the subsequent loads.
- If subsequent loads generate much lower data throughput or lower N50 than previous loads, something has gone wrong in the extraction or library preparation. See troubleshooting tips.
- It is important to use the EXP-WSH004 kit and accompanying protocol when performing nuclease wash, rather than previous wash kits or protocols.



Data throughput over time of ultra long HG02723 sequencing on MinION (left) and PromethION (right). Each 3X library is sequenced in thirds for 24 h, the flow cell is washed, and a new third is loaded to maximize throughput per flow cell.

UHMW (50 kb – 1+ Mb) DNA Extraction Protocols

As of the document release date, the following protocols are available for UHMW (50 kb – 1+ Mb) DNA extraction. These protocols generate large amounts of megabase sized DNA and are only recommend for ultra long sequencing on Oxford Nanopore instruments and for optical mapping. They have been specifically optimized for Nanobind Ultra Long Sequencing and should take precedence over previous UHMW DNA extraction protocols. They are not recommended for use in the standard long-read sequencing applications. For standard long-read sequencing applications, superior results will be obtained using the **HMW (50 – 300+ kb) DNA Extraction Protocols**. Please contact us if you'd like to use the **UHMW (50 kb – 1+ Mb) DNA Extraction Protocols** in applications other than Nanobind Ultra Long Sequencing.

Protocols are updated and added frequently. ***Please refer to the Circulomics Support Page (https://www.circulomics.com/support-nanobind) for the latest versions and the appropriate Nanobind Kit Handbook for additional data and guidance.***

Contact us if you have any questions about which protocol should be used (support@circulomics.com).

UHMW DNA Extraction – Cultured Cells (EXT-CLU-001)

This protocol describes the extraction of UHMW DNA from cultured cells. This protocol has been validated on several cell types including GM12878 and HG02723. This protocol requires the 1) Nanobind CBB Big DNA Kit (NB-900-001-01) or Nanobind Tissue Big DNA Kit (NB-900-701-01), 2) Nanobind UL Library Prep Kit (NB-900-601-01), and 3) UHMW DNA Aux Kit (NB-900-101-01).

UHMW DNA Extraction – Gram-Negative Bacteria (EXT-GNU-001)

This protocol describes the extraction of UHMW DNA from Gram-negative bacteria. This protocol has been validated on *E. coli*. This protocol requires the 1) Nanobind CBB Big DNA Kit (NB-900-001-01) or Nanobind Tissue Big DNA Kit (NB-900-701-01), 2) Nanobind UL Library Prep Kit (NB-900-601-01), and 3) UHMW DNA Aux Kit (NB-900-101-01).

UHMW DNA Extraction – Gram-Positive Bacteria (EXT-GPU-001)

This protocol describes the extraction of UHMW DNA from Gram-positive bacteria. This protocol has been validated on *L. monocytogenes*. This protocol requires the 1) Nanobind CBB Big DNA Kit (NB-900-001-01) or Nanobind Tissue Big DNA Kit (NB-900-701-01), 2) Nanobind UL Library Prep Kit (NB-900-601-01), and 3) UHMW DNA Aux Kit (NB-900-101-01).

UHMW DNA Extraction – Mammalian Whole Blood (EXT-BLU-001)

This protocol describes the extraction of UHMW DNA from mammalian whole blood. The protocol is optimized for extraction from 1–3 mL of whole blood. It has been validated on human and bovine blood. This protocol requires the 1) Nanobind CBB Big DNA Kit (NB-900-001-01) or Nanobind Tissue Big DNA Kit (NB-900-701-01), 2) Nanobind UL Library Prep Kit (NB-900-601-01), and 3) UHMW DNA Aux Kit (NB-900-101-01).

UHMW DNA Extraction – Nucleated Blood (EXT-NBU-001)

This protocol describes the extraction of UHMW DNA from nucleated blood. The protocol is optimized for extraction from 5–30 µL of nucleated blood. It has been validated on frozen fish and lizard blood. This protocol requires the 1) Nanobind CBB Big DNA Kit (NB-900-001-01) or Nanobind Tissue Big DNA Kit (NB-900-701-01), 2) Nanobind UL Library Prep Kit (NB-900-601-01), and 3) UHMW DNA Aux Kit (NB-900-101-01).

Ultra Long Library Preparation Protocols

As of the document release date, the following protocols are available for ultra long library preparation. These protocols are recommended for generating ultra long reads (100 kb – 1+ Mb) on Oxford Nanopore using the Nanobind Ultra Long Sequencing Protocol. Protocols are updated and added frequently. ***Please refer to the Circulomics Support Page (https://www.circulomics.com/support-nanobind) for the latest versions and the appropriate Nanobind Kit Handbook for additional data and guidance.***

Contact us if you have any questions about which protocol should be used (support@circulomics.com).

Nanobind Library Prep – Ultra Long Sequencing (LBP-ULN-001)

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.

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.

QC Procedures

Accurate quantification of UHMW DNA is challenging due to sample inhomogeneity, often leading to concentration measurements with CVs >100%. For UHMW DNA extraction, we recommend performing replicate Nanodrop UV/Vis, Qubit BR DNA Assay, and Qubit BR RNA Assay measurements. For library preparation, we recommend performing Nanodrop UV/Vis measurements only.

See individual UHMW DNA extraction and library preparation protocols for detailed guidance.

Storage of Libraries

6X or 3X scale libraries are eluted from the Nanobind disk overnight at room temperature, then mixed, and left to rest for 2 hours before sequencing the first load. The rest of the library should be stored at 4 ºC before use. Library can be stored up to three days at 4 ºC before commencing with first load.

Troubleshooting FAQ

See individual UHMW DNA extraction and library preparation protocols for details.