Nanobind UHMW DNA Extraction – Nucleated Blood Protocol

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For UHMW (50 kb – 1+ Mb) DNA extraction from cultured cells for ultra long nanopore sequencing

Equipment and Reagent List

Equipment/Reagent	Manufacturer (Part number)
Nanobind CBB Big DNA Kit or Nanobind Tissue Big DNA Kit	Circulomics (NB-900-001-01 or NB-900-701-001)
Nanobind UL Library Prep Kit	Circulomics (NB-900-601-01)
UHMW DNA Aux Kit	Circulomics (NB-900-101-01)
Magnetic Tube Rack	Thermo Fisher DynaMag-2 (12321D)
Mini-Tube Rotator	Fisher Scientific Mini-Tube Rotator (05-450-127)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
1.5 mL Protein LoBind Microcentrifuge Tubes	Eppendorf (022431081)
Wide Bore 200 µL Pipette Tips	USA Scientific (1011-8410)
Wide Bore 1000 µL Pipette Tips	Thermo Scientific (2079G)
Ethanol (96–100%)	
Isopropanol (100%)	
1X PBS	
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

For All Protocols

Eppendorf Protein LoBind tubes (Eppendorf #022431081) are highly recommended for all extractions to reduce protein contamination from tube carryover. Protein LoBind tubes are more effective in reducing carryover contamination than DNA LoBind tubes or other tubes and will result in improved UV purity.

Prior to Starting

Buffer CW1 and CW2 are supplied as concentrates. This kit uses CW1 with a 60% final ethanol concentration. This kit uses CW2 with a 60% final ethanol concentration. Before using, add the appropriate amount of ethanol (96–100%) to Buffer CW1 and Buffer CW2 as indicated on the bottles.

Kit Storage

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

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

Safety Precautions

Buffer ULL and Buffer CW1 contain 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

Nanobind Big DNA Kits are intended for research use only.

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

This protocol describes the extraction of UHMW DNA from nucleated blood for Nanobind Ultra Long Sequencing on Oxford Nanopore MinION/GridION/PromethION. This protocol has been specifically optimized for ultra long nanopore sequencing and should take precedence over previous UHMW DNA extraction protocols. It is not recommended for use with the Ligation Sequencing Kit. For ligation sequencing, superior results will be obtained using the HMW DNA Extraction Protocol. 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).

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.

Blood Input Requirements

Amount: 5–20 µL of nucleated blood

- This extraction protocol is designed to yield ~40 µg of gDNA that can be used immediately for ultra long nanopore library preparation.
- The nucleated blood input should be scaled to contain 40 µg of gDNA. 5-20 µL is typically sufficient.
- Nucleated blood contains much more DNA than mammalian blood.
- This protocol has been validated on fish blood.
- We have only tested frozen nucleated blood.
- We recommend using blood collected in K2 EDTA tubes.

Processing Tips

Unless otherwise noted, always pipette solutions containing UHMW DNA using a wide bore pipette tip.

We recommend setting the pipette to the full volume of solution to be mixed to ensure thorough mixing.

Gentle mixing should take 2–10 s per 1X pipette cycle (up + down). For new users, always err on the side of being too aggressive with mixing rather than too gentle.

UHMW DNA Extraction – Nucleated Blood

1. **Add 10 µL nucleated blood to a 1.5 mL Protein LoBind tube. Top up to 200 µL by adding 190 µL of 1X PBS. Mix by tapping.**
    - If frozen blood is used, thaw quickly at RT and use immediately.
    - If possible, thaw blood by adding correct volume of PBS for use in protocol and pipette to mix and thaw. Use immediately.
    - If starting volume of nucleated blood is different from 10 µL, scale volume of 1X PBS added such that total volume of nucleated blood + 1X PBS is 200 µL.
    - Mix until fully homogenized without visible lumps.
    - Aggressive mixing at this step will not affect DNA size. However, incomplete mixing will result in inefficient lysis and digestion which will lead to low yield, low purity, and high heterogeneity.
2. **Dispense 40 µL of Proteinase K into the bottom of a new 1.5 mL Protein LoBind tube.**

3. **Add the 200 µL of diluted nucleated blood to tube containing Proteinase K.**
4. **Add 200 µL of Buffer BL3 to the sample followed by 20 µL of Buffer CS.**
5. **Pipette mix 15X with a wide bore P1000 pipette set to 450 µL.**
    - Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.
    - Insufficient mixing in step 5 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
6. **Mix on a tube rotator at 9 rpm at RT for 30 min.**
    - Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.
7. **Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.**
    - Keeping the lip of the tube and the cap clean is necessary to minimize contaminant carryover.
8. **Add Nanobind disk to cell lysate first and then add 350 µL of isopropanol. Inversion mix 5X.**
    - The Nanobind disk must be added before isopropanol.
9. **Mix on tube rotator at 9 rpm at RT for 30 min.**
    - Do not exceed 30 minutes. Longer binding times will result in DNA that is more difficult to elute and has lower purities.
10. **Spin in tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.**
    - Keeping the lip of the tube and the cap clean is necessary to minimize contaminant carryover.
11. **Place tubes on the magnetic tube rack.**
12. **Discard the supernatant with a pipette, taking care to avoid pipetting the DNA.**
    - Refer to the **Nanobind Big DNA Kit Handbook Pipetting** section for details.
    - The UHMW DNA often hangs down from the disk along the side of the tube. Extra care must be taken to prevent pipetting it up. To avoid this, the Nanobind disk and bound DNA can be nudged up the side of the tube using the pipette tip and away from the supernatant.
13. **Add 700 µL of Buffer CW1, remove tube rack from magnetic base, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
    - Remove excess liquid from the tube cap to minimize carryover contamination.
14. **Add 500 µL of Buffer CW2, remove tube rack from magnetic base, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
    - Remove excess liquid from the tube cap to minimize carryover contamination.
15. **Repeat step 14.**
16. **Remove liquid from the tube cap.**
17. **Spin the tube on a mini-centrifuge for 2 s and remove the residual liquid.**
    - If the Nanobind disk is blocking the bottom of the tube, gently push it aside with the tip of the pipette. At this stage, DNA is tightly bound to the disk and gently manipulating the disk with a pipette tip should not cause any damage.
18. **Repeat step 17.**
19. **Add 760 µL of Buffer EB+ and incubate at RT overnight.**

20. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube with a wide bore P1000 pipette. Repeat until all eluate is transferred.**
21. **Some liquid will 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 combine any additional liquid that comes off the disk with the previous eluate. Repeat if visible DNA remains on the disk.**
    - This centrifuge step is critical. 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.
22. **Gently pipette mix the sample 5X with a wide bore P1000 pipette set to 760 µL to homogenize sample.**
23. **Let sample rest at RT for 2 hours to allow DNA to solubilize.**
    - Incubation at 4 °C is not effective.
24. **Analyze the recovery and purity of the DNA by Nanodrop and Qubit as described in QC Procedures.**
    - After resting overnight, the average of triplicate Nanodrop concentration readings, sampling from top, middle and bottom of the tube should be between 30 ng/µL and 150 ng/ µL.
    - The Qubit dsDNA concentration should be between 6 ng/ µL and 100 ng/µL.
    - The Qubit RNA concentration should be <50% of the Qubit dsDNA concentration.
25. **Confirm triplicate Nanodrop measurements are within range to give optimal sequencing results.**
    - 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 the concentration measurements are outside the given ranges, pipette mix the sample 5X with a wide bore P1000 pipette and measure again.
    - If the measurements are still out of range, then the sequencing is likely to be not optimal. Repeat the extraction.
26. **Proceed to library preparation. If not proceeding immediately, store the DNA at 4 ºC and prepare libraries within one week.**

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

Quick Tip

The DNA will solubilize after coaxing it into solution with gentle pipette mixing and resting. We recommend patience if megabase-sized DNA is needed. Do not skip these steps. Do not heat DNA.

QC Procedures

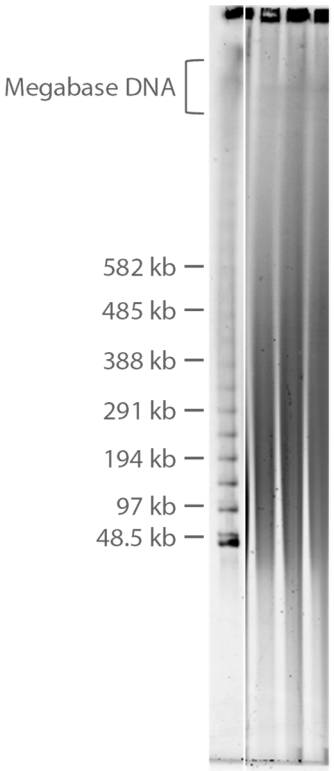
It is recommended that QC is performed after the DNA has been allowed to rest for at least 2 hours following overnight elution and appears homogenous under visual examination and when pipetting.

1. **Perform triplicate NanoDrop UV/Vis measurements from top, middle, and bottom of the tube to determine total nucleic acid concentration as well as purity (A260/A280, A260/230).**
    - UHMW DNA will be viscous and inhomogeneous. Take 3–5 measurements, sampling from the top, middle, and bottom of the tube, to get an accurate concentration reading. We typically see concentration %CV values of <50%. However, if the DNA is very large, the %CV can exceed 100%. In this case, take additional measurements to ensure that the concentration is accurate. Homogenization of the sample to reduce measurement CV is not required.
    - 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. UV ratios outside of this range do not necessarily indicate that sequencing will be poor.
2. **Use Qubit dsDNA BR Assay to determine DNA concentration.**
    - We recommend taking a single measurement to get an approximate DNA concentration reading.
    - We recommend the Qubit 3.0 (Thermo Fisher Scientific) with the dsDNA BR Assay kit. We do not recommend the dsDNA HS Assay kit as we have found the concentration measurements to be unreliable
    - If Qubit DNA measurements disagree with Nanodrop measurements, we recommend using the Nanodrop data as we have found the Qubit DNA measurements to provide inconsistent readings with UHMW DNA.
3. **Use Qubit RNA BR Assay to determine RNA concentration.**
    - We recommend taking a single measurement to get an approximate RNA concentration reading.
    - We recommend the Qubit 3.0 (Thermo Fisher Scientific) with the RNA BR Assay kit.
    - If Qubit RNA measurements indicate high RNA (>50%), Nanodrop nucleic acid concentration measurements may need to be reduced to account for RNA.
4. **Run pulsed field gel electrophoresis to determine size.**
    - The size of the extracted genomic DNA can be determined using pulsed field gel electrophoresis (PFGE). We recommend loading 200 ng of DNA per well. For analysis of 50 kb – 1+ Mb DNA, we recommend the following PFGE conditions:
        - Instrument: Bio-Rad CHEF-DR III Variable Angle System or CHEF Mapper XA System
        - Agarose: 1.0% Certified Megabase Agarose (Bio-Rad #1613109)
        - 30-well comb (Bio-Rad #1704344)
        - Buffer: 0.5X TBE
        - Lambda DNA Ladder: Bio-Rad #1703635, Lonza #50401, or NEB #N0341S
        - 6X gel loading dye (NEB #B7021S)
        - Temperature: 14 °C
        - Initial Switch Time: 35 s
        - Final Switch Time: 90 s
        - Run Time: 22 hours
        - Angle: 120°
        - Voltage Gradient: 5.5 V/cm
    - Stain for 1 hr using SYBR Gold and image immediately.
    - No other DNA sizing method has sufficient sizing resolution to characterize Mb sized DNA.

DNA Extraction

- DNA was extracted from 10 µL of fish blood. Individual Nanodrop measurements should be in the 6 –350 ng/µL range. The average of triplicate measurements on a single extraction should be in the 30-150 ng/µL range. If measurements fall outside of these ranges, the samples should be pipette mixed 5X with a wide bore P1000 pipette and re-measured.
- Qubit dsDNA measurements are used as a sanity check only. We find that the numbers do not accurately reflect UHMW DNA concentration even when replicate measurements are performed and go by Nanodrop instead.
- Qubit RNA measurements are used to verify that high RNA levels will not throw off Nanobind DNA concentration measurements.
- Example measurements from an extraction are shown below.

	260/280	260/230	Nanodrop Top (ng/µL)	Nanodrop Middle (ng/µL)	Nanodrop Bottom (ng/µL)	Nanodrop Avg (ng/µL)	Qubit dsDNA (ng/µL)	Qubit RNA (ng/µL)
Fish Blood	1.91	2.56	14.8	148.4	9.3	57.5	28.0	5.5



22 hour Pulsed Field Gel Electrophoresis (PFGE) image of fish blood gDNA. UHMW DNA can be identified through 1) streaking up to 1 Mb and 2) compression zone banding.

Troubleshooting FAQ

1. **How do I prevent accidentally pipetting the bound DNA when removing the binding and wash solutions?**

   - Some sample types generate UHMW DNA that dangles down from the Nanobind disk. If a lot of DNA is bound, it can appear as a large globule extending from the Nanobind disk. In this instance, the Nanobind and globule of bound DNA can be pushed up the side of the tube using the pipette tip before removing the remaining solution in the bottom of the tube. The magnetic rack and tube can be held at a shallow angle to prevent the Nanobind disk from slipping back down to the bottom of the tube.
   - Typically, the DNA is bound progressively tighter with each wash. A small amount of binding solution can be carried over between washes if this helps to avoid pipetting the DNA.

2. **The DNA is very viscous and doesn't come away from the Nanobind disk.**

   - There is sometimes a small amount of DNA that remains tightly bound to the Nanobind disk. First, we recommend removing as much as possible using a wide bore P1000 pipette. If some DNA remains attached to the disk (<20 µL), centrifuge the tube at 10,000 x *g* for 15 s and then use a standard P200 pipette to remove the eluate that should have gathered at the bottom of the tube. Repeat until no more DNA comes away from the Nanobind disk. This should not take more than 1–2 spins.

3. **Can I heat the Nanobind to help elute the DNA?**

   - We don't recommend heating and have never done so in any of our runs.

4. **My Nanodrop measurements are all over the place. How do I know how much DNA is in sample?**

   - This is normal for UHMW DNA. We recommend using the average of triplicate Nanodrop measurements. These measurements should be taken after the DNA has rested for at least 2 hours at RT following overnight elution. The triplicate measurements will have large CVs, often exceeding 100% due to the sample inhomogeneity. If desired, additional measurements can be taken to get a more accurate average. Homogenization of the sample to reduce measurement CV is not required.
   - As an example, 3 extractions were performed from 10 µL of fish blood and eluted into 760 µL of Buffer EB+. Triplicate Nanodrop measurements were performed on each extraction, measuring at the top, middle and bottom of the eluate, giving 9 Nanodrop measurements in total. The minimum and maximum individual measurements were 5.8 and 301.4 ng/µL; the minimum and maximum of the averages of the triplicates were 58 and 139 ng/µL.