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Nanobind UHMW DNA Extraction – Mammalian Whole Blood Protocol

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For UHMW (50 kb – 1+ Mb) DNA extraction from 1.5 mL of mammalian whole blood 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	
Nuclease-Free Water	
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 and Buffer RBC10X 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 human whole 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: 1.5 mL of human whole blood

- This extraction protocol is designed to yield ~40 µg of gDNA that can be used immediately for ultra long nanopore library preparation.
- 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.
- White blood cell counts should be accurately determined using a HemoCue WBC System or similar instrument.
- The whole blood input should be scaled to contain 40 µg of gDNA based on WBC counts.
- If white blood cell counts cannot be accurately determined *a priori*, begin with 1.5 mL of whole blood and scale the input up or down based on the sequencing results.
- This protocol has been validated on 1–3 mL of fresh and frozen human and bovine blood.
- No systematic difference has been seen in either DNA QC or sequencing results between fresh and frozen 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.

RBC Lysis – Human Whole Blood

1. **Prepare 1X RBC lysis buffer by mixing 0.45 mL of Buffer RBC10X and 4.05 mL of nuclease-free water. 1X RBC lysis buffer should be used at RT. Excess 1X RBC lysis buffer should be stored at 4 °C.**
    - The mixture can be scaled as long as 10:1 dilution is performed.
    - If frozen blood is used, thaw before extraction by incubating in heat block at 37 ºC for 4–8 min.

2. **Add 1.5 mL of blood to a 15 mL tube. Add 4.5 mL of 1X RBC lysis buffer to lyse the red blood cells. Inversion mix 5X and incubate at RT for 10 min. Inversion mix every 5 min.**
    - The reaction can be scaled as long as a 3:1 ratio of 1X RBC lysis buffer:whole blood is maintained.
3. **Centrifuge for 2 min at 2,000 x g at RT to pellet the white blood cells.**
4. **Remove and discard the supernatant.**
5. **Resuspend cells in 1 mL of 1X PBS, taking care to pipette mix well to ensure proper resuspension. Transfer to a 1.5 mL Eppendorf Protein LoBind tube.**
    - Cells from frozen blood can be more difficult to resuspend than those from fresh blood. We recommend first resuspending in 200 µL of PBS and using a standard P200 pipette to break up the cell aggregates. Then, add the remaining 800 µL of PBS and pipette mix further before transferring to the 1.5 mL Eppendorf Protein LoBind tube.
6. **Centrifuge for 2 min at 2,000 x g at RT to pellet the white blood cells.**
7. **Remove and discard the supernatant using a standard P200 pipette.**

Quick Tip

Insufficient resuspension will compromise sequencing results. Mix and resuspend extremely thoroughly.

UHMW DNA Extraction – White Blood Cells

8. **Add 40 µL of 1X PBS to the pelleted white blood cells. Pipette mix 10X with a standard P200 pipette to resuspend cells.**
    - Mix until cell pellet is fully resuspended without visible lumps. Cells from frozen blood may require additional pipette mixing or vortexing.
    - Aggressive mixing at this step will not affect DNA size. However, incomplete resuspension will result in inefficient lysis and digestion which will lead to low yield, low purity, and high heterogeneity.
    - WBC pellets from 1–3 mL of blood have been tested with this protocol. Other volumes may be processed by scaling the reactions. Contact Circulomics for details.
9. **Add 40 µL of Proteinase K. Gently pipette mix 10X with a wide bore P200 pipette set to 80 µL.**
    - Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.
10. **Add 40 µL of Buffer CS. Gently pipette mix 10X with a wide bore P200 pipette set to 120 µL.**
    - Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.
11. **Add 40 µL of Buffer CLE3. Gently pipette mix 10X with a wide bore P200 pipette set to 160 µL.**
    - Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.
12. **Incubate at RT for 30 min.**
13. **Add 40 µL of RNase A. Gently pipette mix 10X with a wide bore P200 pipette set to 200 µL, and incubate at RT for 3 min.**

Quick Tip

If extracting multiple samples, perform steps 9–11 in groups of two, i.e. perform steps 9–11 for pellets #1 and #2, then steps 9–11 for pellet #3 and #4, etc.

We use 2 pipettes for steps 9–11. Set the first to 40 µL to add Proteinase K, Buffer CS, and CLE3. Use the second with a wide bore pipette set to mix the full volume at each step.

- Setting the pipette to the full volume of the solution ensures complete mixing. Ensure the sample is visually homogeneous with no lumps.

14. **Add 400 µL of Buffer ULL. Gently pipette mix 10X with a wide bore P1000 pipette set to 600 µL. Incubate at RT for 10 min.**
    - A white precipitate may form after addition of Buffer ULL. This is completely normal.
15. **Gently pipette mix 5X with a wide bore P1000 pipette set to 600 µL. Incubate at RT for 10 min.**
16. **Repeat step 15.**
    - Larger precipitates are sometimes seen, particularly when extracting from frozen blood. Repeat step 15 until these precipitates are broken up.
17. **Add Nanobind disk to cell lysate first and then add 600 µL of isopropanol. Inversion mix 5X.**
    - The Nanobind disk must be added before isopropanol.
18. **Mix on tube rotator at 9 rpm at RT for 10 min.**
    - Some white precipitate may be seen attached to the DNA as it binds to the disk. This is normal.
19. **Place tubes on the magnetic tube rack.**
    - Use the method described in the **Nanobind Big DNA Kit Handbook Magnetic Rack Handling Procedure** section.
20. **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.
21. **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.**
22. **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.**
23. **Repeat step 22.**
24. **Remove liquid from the tube cap.**
25. **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.
26. **Repeat step 25.**
27. **Add 760 µL of Buffer EB+ and incubate at RT overnight.**
28. **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.**
29. **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.**

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 29 will remove the remaining DNA quickly without diluting the eluate.

- This centrifuge step is critical. A standard P200 pipette 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.

30. Gently pipette mix the sample 5X with a wide bore P1000 pipette set to 760 µL to homogenize sample.

31. Let sample rest at RT for 2 hours to allow DNA to solubilize.

- Incubation at 4 °C is not effective.

32. 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 29 ng/µL and 150 ng/µL. UHMW DNA extracted from blood can be very inhomogeneous with CVs from these triplicate measurements often exceeding 100%.
- The Qubit dsDNA concentration should be between 20 ng/µL and 180 ng/µL.
- The Qubit RNA concentration should be <50% of the Qubit dsDNA concentration.

33. 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 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.

34. Proceed to library preparation. If not proceeding immediately, store the DNA at 4 ºC and prepare libraries within one week.

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

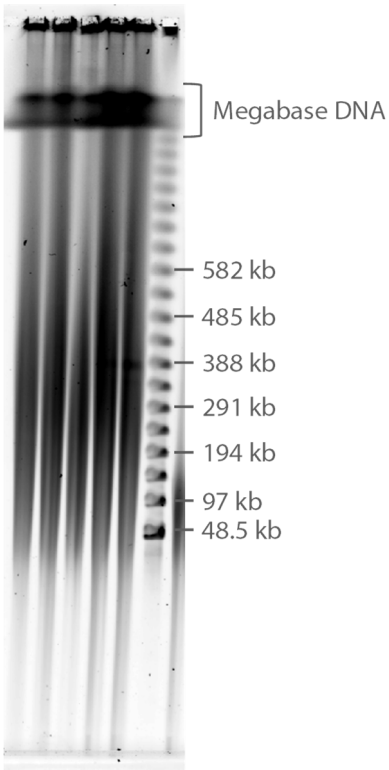
It is recommended that QC is performed after the DNA has been allowed to rest for at least 2 hours following overnight elution and appears homogenous under visual examination and when pipetting.

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.

Results

- DNA was extracted from 1.5 mL of human blood containing 4.5x10^6 white blood cells/mL. Individual Nanodrop measurements should be in the 7–360 ng/µL range. The average of triplicate measurements on a single extraction should be in the 29–150 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 Nanodrop 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)
Human Blood	2.2	3.0	7.7	72.2	17.9	32.6	143	7.1



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

Troubleshooting FAQ

1. **Why do I see large precipitate chunks during lysis?**

   - This often results when the cells are not properly resuspended and lysis efficiency is compromised. Ensure that the cells appear entirely homogeneous during resuspension in PBS. The DNA will not be sheared at this step so don't be afraid to aggressively pipette mix a few extra times with a standard P200 pipette.
   - After each addition of Proteinase K, Buffer CS, and Buffer CLE3, mix immediately and thoroughly. Ensuring the solution is homogeneous after each of these mixing steps will prevent precipitates from appearing later.
   - If large, visible precipitates still persist after the Buffer ULL mixing steps, we recommend repeating step 15 until the precipitates are gone.

2. **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.

3. **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.

4. **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.

5. **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, 4 extractions were performed from 1.5 mL frozen human 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 12 Nanodrop measurements in total. The minimum and maximum individual measurements were 7.7 and 120 ng/µL; the minimum and maximum of the averages of the triplicates were 32 and 56 ng/µL.

6. **My cells are still pink/red after RBC lysis. Should I repeat the RBC lysis step?**

   - We've tested performing 1 and 3 RBC lysis steps. Both seem to work.