Nanobind UHMW DNA Extraction – Dounce Homogenizer Tissue Protocol

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For UHMW (50 kb – 1+ Mb) DNA extraction from animal tissues using Dounce homogenizer for ultra long nanopore sequencing

Equipment and Reagent List

Equipment/Reagent	Manufacturer (Part Number)
Nanobind Tissue Big DNA Kit	Circulomics (NB-900-701-001)
Nanobind UL Library Prep Kit	Circulomics (NB-900-601-01)
Magnetic Tube Rack	Thermo Fisher DynaMag-2 (12321D)
Wheaton 1 mL Dounce Tissue Grinder with Tight and Loose Pestles	Fisher Scientific (06-434)
Surgical Scalpel	Fisher Scientific (22-079-712)
Heat Block (or Water Bath)	Fisher Scientific (11-715-125DQ)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
Platform Rocker or Mini-Tube Rotator	Thermo Scientific (M48725Q) or Fisher Scientific (05-450-127)
14 mL Round Bottom Tubes	Fisher Scientific (14-956-3B)
2.0 mL Protein LoBind Microcentrifuge Tubes	Eppendorf (022431102)
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%)	
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 #022431102 and #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 BL3 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 animal tissues using Dounce homogenizer 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 1) Nanobind Tissue Big DNA Kit (NB-900-701-01) and 2) Nanobind UL Library Prep Kit (NB-900-601-01).

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.

Sample Input Requirements

Amount: 25 mg of animal tissue. Input will vary by tissue type.

- The UHMW TissueRuptor and Dounce homogenizer tissue protocols differ only in the upfront tissue homogenization method. The two protocols result in similar DNA quality and size.
- See the **Nanobind Tissue Big DNA Kit Handbook Sample Information** section or the **Circulomics Support Page** (https://www.circulomics.com/support-nanobind) for recommendations and a list of tissues that require modifications to this protocol.
- Tissue samples should be dissected to avoid repeated freeze-thaws.
- This protocol has been validated on kidney.
- This protocol is suitable for tissues that are fresh frozen, ethanol preserved, and RNAlater preserved. Ethanol and RNAlater preserved tissue samples should be pre-treated as described in the **Nanobind Tissue Big DNA Kit Handbook Preservation Methods** section.

UHMW DNA Extraction – Dounce Homogenizer Tissue Protocol

1. **Place the Dounce homogenizer and tight pestle on ice. Chill the centrifuge to 4 °C.**
2. **Place ~25 mg of tissue on a clean, chilled surface and finely mince to ≤1 mm^3 pieces with a scalpel.**
    - A plastic weigh boat cleaned with 70% EtOH can be placed on an upside-down aluminum dry bath incubator heat block sitting in ice.
    - Fine mincing is particularly important for tissues with a rubbery texture (*e.g.* fibroid, skin, connective tissue).
    - For some tissue types, input can be increased 1.5- to 2-fold if the yield is low; however, larger increases may overwhelm the chemistries and adversely affect purity.
3. **Transfer minced tissue to the chilled Dounce homogenizer. Keep the Dounce homogenizer on ice during the entire disruption process.**
4. **Add 750 µL of cold Buffer CT.**
    - Buffer CT should be kept on ice when removed from refrigerator.
5. **Gently homogenize the tissue with the pestle 10X.**
    - Push the tissue with the pestle firmly into the bottom of the Dounce chamber with each stroke (Down + Up = 1X).

Quick Tip

Thorough tissue disruption is key to efficient lysis. It is also important to keep the tissue cold during the entire disruption process.

Quick Tip

See the **Nanobind Tissue Big DNA Kit Handbook Tissue Disruption Strategies** section for Dounce Homogenization Tips and Tricks.

- Keep the tissue between tip of pestle and the bottom of the Dounce chamber for thorough homogenization.
- Homogenate may become foamy, but this is not a cause for concern. In the next step, transfer any foam that forms.

6. **Transfer homogenate to a 2 mL Protein LoBind microcentrifuge tube.**
    - Transfer any undisrupted tissue chunks and any foam that forms.

Quick Tip
The 2 mL tube is essential for efficient lysis.

7. **Pellet homogenate by centrifuging at 3,000 x *g* and 4 °C for 5 min. Discard supernatant.**
    - For some tissue types, such as brain, the pellet may not be visible. In these cases, carefully remove the supernatant and avoid pipetting from the very bottom of the tube.
    - Recovery from certain tissue types, such as brain and liver, may be enhanced by increasing the spin speed to 6,000 x *g*; however, this may adversely affect purity.
8. **Add 1 mL of cold Buffer CT and pipette mix 10X with a wide bore P200 pipette to resuspend tissue.**
9. **Pellet homogenate by centrifuging at 3,000 x *g* and 4 °C for 5 min. Discard supernatant.**
    - For some tissue types, such as brain, the pellet may not be visible. In these cases, remove the supernatant carefully and avoid pipetting from the very bottom of the tube.
    - Recovery from certain tissue types, such as brain and liver, may be enhanced by increasing the spin speed to 6,000 x *g*; however, this may adversely affect purity.
10. **Pulse vortex for 1s x 5 times (max setting) to dislodge pellet.**
11. **Add 20 µL of Proteinase K to the previous pellet.**
12. **Add 150 µL of Buffer CLE3. Pipette mix 10X with a wide bore P200 pipette.**
13. **Incubate at 50 °C for 90 min. Pipette mix 3X with a wide bore P200 pipette every 30 min.**

Quick Tip
If there are still visible, undigested tissue pieces after step 13, the incubation may be extended up to 3 h. However, if tissue is appropriately disrupted in steps 1–5, then 90 min should be sufficient.

14. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
15. **Add 20 µL of RNaseA and pipette mix 3X with a wide bore P200 pipette.**
16. **Incubate at 50 °C for 30 min.**
17. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
18. **Add 60 µL of Buffer SB and inversion mix 20X.**
19. **Centrifuge at 10,000 x *g* and RT (18–25 °C) for 5 min.**

Quick Tip
The narrow taper of the 1.5 mL tube is essential for proper removal of wash buffer in steps 31 & 32 and for thorough recovery of eluate in step 36.

20. **Transfer up to 300 µL of supernatant to a new 1.5 mL Protein LoBind microcentrifuge tube using a wide bore P200 pipette. (Discard the 2 mL Protein LoBind microcentrifuge tube containing the precipitated pellet.)**
    - Volume of supernatant will vary with tissue type.
    - Some sample types may not result in a visible pellet after this spin.
    - If there is no visible pellet, transfer supernatant as if there were a pellet present and avoid pipetting from the very bottom of the tube.
    - For tissues with high DNA content and often for UHMW DNA extractions, a gel-like matrix may form. The gel-like matrix contains all the DNA – transfer the entire gel-like matrix even if this results in transferring the pellet as well.

Quick Tip
In UHMW DNA extractions, a diffuse, gel-like matrix may form that contains all the DNA – transfer the entire gel-like matrix.

- Some sample types may result in a waxy or opaque residue that rests atop the liquid after the spin; avoid transferring this substance. (Typically, this residue adheres to the outside of the pipette tip used to transfer supernatant; do not allow the pipette tip to touch the new tube in order to avoid transferring this residue.)

21. **Add 50 µL of Buffer BL3 to the previous supernatant and inversion mix 10X.**
    - Solution may turn cloudy but will become clear in step 24.
22. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
23. **Add Nanobind disk to lysate first and then add 350 µL of isopropanol. Inversion mix 10X.**
    - The Nanobind disk must be added before isopropanol.
    - A large, cloudy mass may appear upon addition of isopropanol and inversion mixing; this will adhere to the Nanobind disk and will become clear during the next step.
24. **Mix on a platform rocker at 20 rpm for 15 min at RT.**
25. **Place tube rack on the magnetic base.**
    - Use the method described in the **Nanobind Tissue Big DNA Kit Handbook Magnetic Rack Handling Procedure** section.
26. **Discard supernatant with a pipette, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**
    - Refer to the **Nanobind Tissue Big DNA Kit Handbook Pipetting** section for details.
    - Remove excess liquid from the tube cap to minimize carryover contamination.
27. **Add 500 µL of Buffer CW1, remove tube rack from magnetic base, inversion mix 4X, replace the tube rack on the magnetic base, and discard the supernatant.**
    - Remove excess liquid from the tube cap to minimize carryover contamination.
28. **Repeat step 27.**
29. **Add 500 µL of Buffer CW2, remove tube rack from magnetic base, inversion mix 4X, replace the tube rack on the magnetic base and discard the supernatant.**
    - Remove excess liquid from the tube cap to minimize carryover contamination.
30. **Repeat step 29.**
31. **Spin the tube on a mini-centrifuge for 2 s. With the tube rack already on the magnetic base and right-side-up, place tube on tube rack and remove 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.
32. **Repeat step 31.**
33. **Remove tube from tube rack.**
34. **Add 200–320 µL of Buffer EB+ directly onto the Nanobind disk and incubate at RT for 20 min.**

- We recommend starting with 200 µL. If the concentration exceeds 400 ng/µL or if the sample is very viscous, the elution volume can be increased to 320 µL.

35. **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.**
36. **Some liquid will remain on the Nanobind disk after pipetting. Spin the tube containing the Nanobind disk at 16,000 x *g* for 15 s. 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.
    - Samples with high DNA content may result in a dense, clear gel that adheres strongly to the Nanobind disk. **This clear gel is DNA!** For these tissue types, this spin step is critical for recovering all the DNA. Repeat until all the clear gel has spun off the Nanobind disk.
37. **Pipette mix the sample 2X with a wide bore P200 pipette.**
38. **Let sample rest at RT overnight to allow DNA to solubilize.**
39. **Following overnight rest, pipette mix 2X with a wide bore P200 pipette and analyze the recovery and purity as described in QC Procedures.**

Quick Tip

This spin step is **CRITICAL** for recovering the DNA. We do not recommend a 2nd elution.

Quick Tip

Resting overnight at RT will help the DNA solubilize. Mixing with a wide bore P200 pipette will also help.

QC Procedures

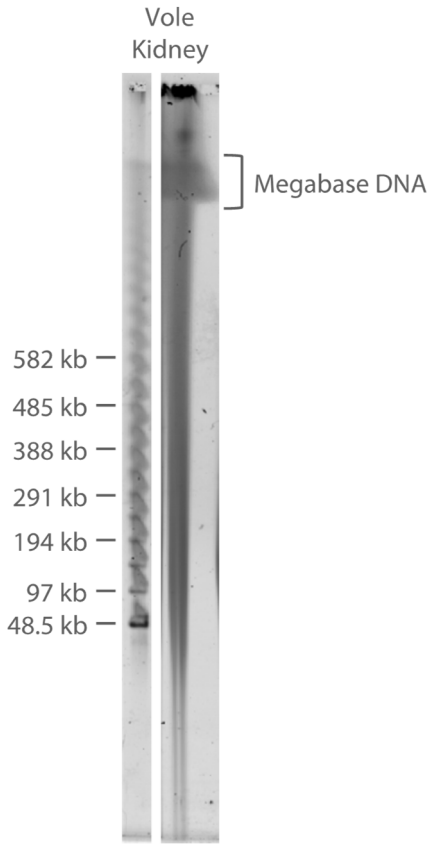
It is recommended that QC is performed after the DNA has been allowed to rest at RT overnight and appears homogenous under visual examination and when pipetting.

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 20 mg of frozen vole kidney.
- Individual Nanodrop measurements should be in the 100–450 ng/µL range. The average of triplicate measurements on a single extraction should be in the 150–350 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)
Vole Kidney (20 mg)	1.90	2.60	346.1	336.1	362.6	348.3	210	25.4



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

Troubleshooting FAQ

1. **During the spins in steps 7 & 9, why isn't my homogenate pelleting?**

   - Some tissues may result in pellets that are very small or pellets that are translucent and thus difficult to see.
   - If the pellet is not visible, remove the supernatant carefully and avoid pipetting from the very bottom of the tube.
   - Also, there are a small number of tissue types, such as mammalian brain and fish skeletal muscle, that may yield higher recovery with a faster spin speed in steps 7 & 9. Please refer to the Tissue Big DNA Kit Handbook for additional information on these tissues.

2. **After spinning in step 19, why don't I see a pellet?**

   - Some samples will not result in a visible pellet. In these cases, transfer the supernatant as if there were a pellet, avoiding the very bottom of the tube.

3. **After spinning in step 19, why don't I see a pellet and why is the supernatant a dense, gel-like substance?**

   - Some tissues with very high DNA content may result in a diffuse or indistinct pellet in step 19. In these cases, there is so much DNA that it creates a gel-like matrix in the supernatant which interferes with formation of a pellet during centrifugation. A common mistake is to avoid this gel-like matrix. Instead, be sure to transfer all of the gel-like matrix if it is present.
   - For very high DNA content samples, tissue input may be reduced to 15 mg in subsequent extractions.

4. **Is there enough Buffer EB to cover the Nanobind disk?**

   - **THE NANOBIND DISK DOES NOT HAVE TO BE SUBMERGED IN BUFFER EB**. Rather, it only needs to be wetted by Buffer EB. Just be sure to pipette Buffer EB directly onto the Nanobind disk in step 34, and it will be fine.
   - The tube may incubated on its side to encourage more of the buffer to remain on the disk.

5. **I transferred the eluate, but there is still liquid or a gel-like material stuck to the Nanobind disk – should I do a second elution?**

   - **DO NOT DO A SECOND ELUTION**. The gel attached to the Nanobind disk is HMW DNA and you can recover it by spinning for 15 seconds at 16,000 x *g* in step 36. The disk will be wedged in the taper of the 1.5 mL tube and the DNA will spin off of the disk to the bottom of the tube. You may repeat this step until all the DNA is spun off. Typically, this spin step only needs to be repeated once.

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

   - We do not recommend heating during the elution step.

7. **The eluate is exceedingly viscous and heterogeneous – did I do something wrong?**

   - **YOU DID A GREAT JOB! YOU HAVE HMW DNA!** HMW is inherently difficult to work with. The bigger it is, the more heterogeneous it tends to be. Homogeneity can be improved by mixing with a standard pipette to disrupt any particularly viscous regions. Overnight incubation at RT will then allow the HMW DNA to relax back into solution.
   - In addition, some tissues with very high DNA content can yield so much DNA that the eluate is exceedingly difficult to pipette even when eluting into 320 µL of Buffer EB+. For these tissues, we recommend decreasing the tissue input to 15 mg.

- Alternatively, you may add more Buffer EB+ to the eluate after you have measured the concentration and found it to be very high.

8. Why is the DNA yield is lower than expected?

- Make sure that all the DNA is recovered from the Nanobind disk by spinning for 15 seconds at 16,000 x *g* in step 36. The disk will be wedged in the taper of the 1.5 mL tube, and the DNA will spin off of the disk to the bottom of the tube. You may repeat this step until all the DNA is spun off. Typically, the spin step only needs to be repeated once.
- If the eluate is very heterogeneous, you may be sampling from an area of the eluate that is a substantially less concentrated. Take measurements from the top, middle, and bottom of the eluate to assess the heterogeneity and measure the %CV

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

- This is not uncommon for extractions from tissues with very high cellularity that can result in DNA that is very high concentration and heterogeneous. We recommend mixing the eluate 5X with a standard pipette, resting the DNA overnight at RT, and then performing triplicate Nanodrop measurements.