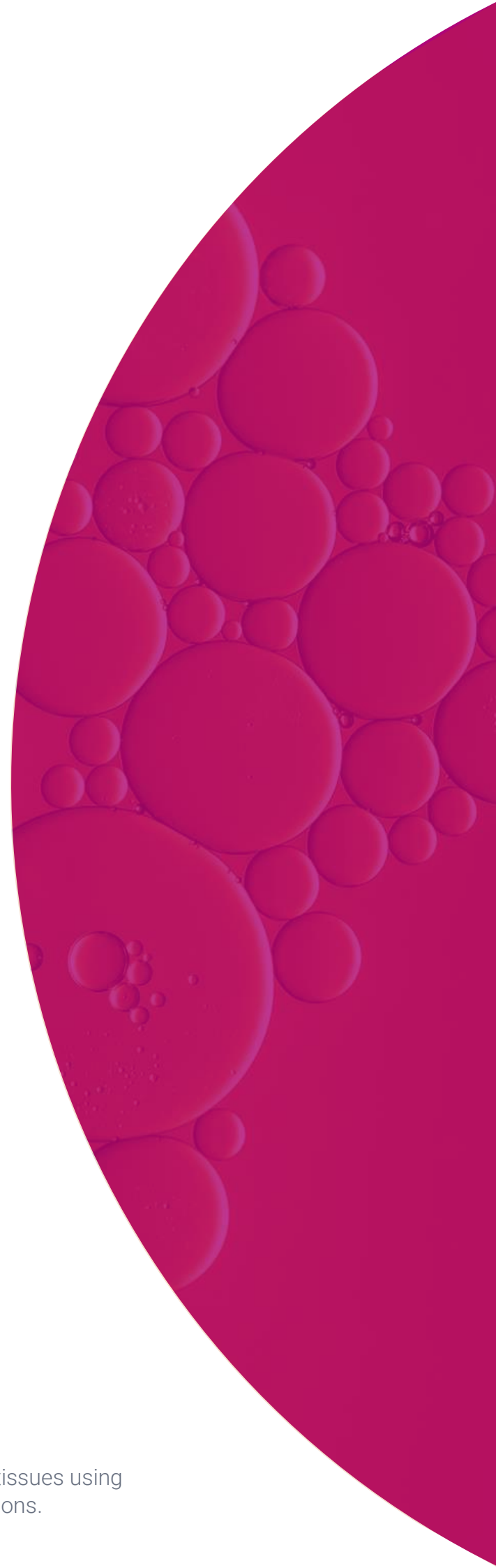
PacBio

Nanobind® HMW DNA extraction – standard TissueRuptor tissue

Procedure & checklist



For HMW (50 kb – 300+ kb) DNA extraction from animal tissues using TissueRuptor for standard long-read sequencing applications.

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind tissue kit	PacBio® (102-302-100)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
TissueRuptor II	Qiagen (9002755)
TissueRuptor disposable probes	Qiagen (990890)
Surgical scalpel	Fisher Scientific (22-079-712)
ThermoMixer	Eppendorf (5382000023)
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 micro-centrifuge tubes	Eppendorf (022431102)
1.5 mL Protein LoBind micro-centrifuge 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

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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 tissue kits are intended for research use only.

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HMW (50 kb – 300+ kb) DNA extraction protocol

This protocol describes the extraction of HMW DNA from animal tissues using TissueRuptor. It is recommended for HiFi sequencing applications.

Sample input requirements

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

- The standard 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 kit Guide & overview "Sample information"** section 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 for many mammalian, avian, and fish tissue types including heart, liver, spleen, kidney, and colon.
- 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 kit Guide & overview "Preservation methods"** section.

HMW DNA extraction – standard TissueRuptor tissue protocol

1. Place a 14 mL round bottom tube on ice and chill the centrifuge to 4 °C.
2. Place ~25 mg of tissue on a clean, chilled surface, and finely mince to ≤1 mm3 pieces using 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 14 mL round bottom tube. Keep the tube 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. Submerge the TissueRuptor probe tip in the buffer and blend at max speed for 10 s.
   - Homogenate may become foamy, but this is not a cause for concern. In the next step, transfer any foam that forms.

Quick Tip

The 2 mL tube is essential for efficient lysis because of its shape; the narrow taper of a 1.5 mL tube prevents proper mixing of the lysate during subsequent thermomixing

Quick Tip

If there are still visible, undigested tissue pieces after step 13, the incubation may be extended up to 2 h. However, if tissue is appropriately disrupted in steps 1-5, then 30 min should be sufficient.

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.

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

For high DNA content tissues, a diffuse, gel-like matrix forms that contains all the DNA – transfer the entire gel-like max.

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6. Transfer homogenate to a 2 mL Protein LoBind microcentrifuge tube.
    - Transfer any undisrupted tissue chunks and any foam that forms.
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 2 times (max setting) to dislodge pellet.
11. Add 20 µL of Proteinase K to the previous pellet.
12. Add 150 µL of Buffer CLE3 and pipette mix 10X with a wide bore P200 pipette.
13. Incubate on a ThermoMixer at 55 °C and 900 rpm for 30 min.
14. Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.
15. Add 20 µL of RNaseA.
16. Incubate on a ThermoMixer at 55 °C and 900 rpm 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 pulse vortex for 1s x 5 times (max setting) to mix.
19. Centrifuge at 10,000 x g and RT (18–25 °C) for 5 min.
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, such as spleen and gonad, a gel-like matrix may form. The gel-like matrix contains all the DNA – transfer the entire gel-like matrix.
    - Formation of this gel-like matrix may be mitigated by reducing the tissue input.
    - 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 and 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 **kit Guide & overview "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 **kit Guide & overview "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.
    - 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 75 µL of Buffer EB directly onto the Nanobind disk and incubate at RT for 10 min.
35. Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.
    - A standard P200 pipette can be used to aid in the removal of residual liquid after the majority of the eluate has been removed with a wide-bore pipette
36. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5s. Use a standard P200 pipette to combine any additional liquid that comes off the disk with the previous eluate. Repeat if necessary.
    - 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.

Quick Tip

The Nanobind disk only needs to be wetted in the elution step: THE DISK DOES NOT NEED TO BE FULLY SUBMERGED IN BUFFER EB.

37. Pipette mix the sample 5X with a standard P200 pipette to homogenize and disrupt any unsolubilized "jellies" that may be present.
    - Take care to disrupt any regions that feel more viscous than other regions.
    - Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.
38. Let sample rest at RT overnight to allow DNA to solubilize.
    - Visible "jellies" should disperse after resting.
39. Following overnight rest, pipette mix 5X with a standard P200 pipette and analyze the recovery and purity as described in QC Procedure.
    - If the concentration %CV exceeds 30% or if perceptible "jellies" remain, pipette mix 10X with a standard P200 pipette or needle shear 5X with a 26g needle and allow DNA to rest at RT for 2 hours. Take care to disrupt any regions that feel more viscous than other regions. Remeasure with NanoDrop.

Quick Tip

This 5 s spin is **CRITICAL** for recovering the DNA. We do not recommend a 2nd elution.

Quick Tip

The DNA will solubilize after resting at RT or by coaxing it into solution using standard P200 pipette mixing. For samples that need to be used immediately, we recommend needle shearing.

QC procedures

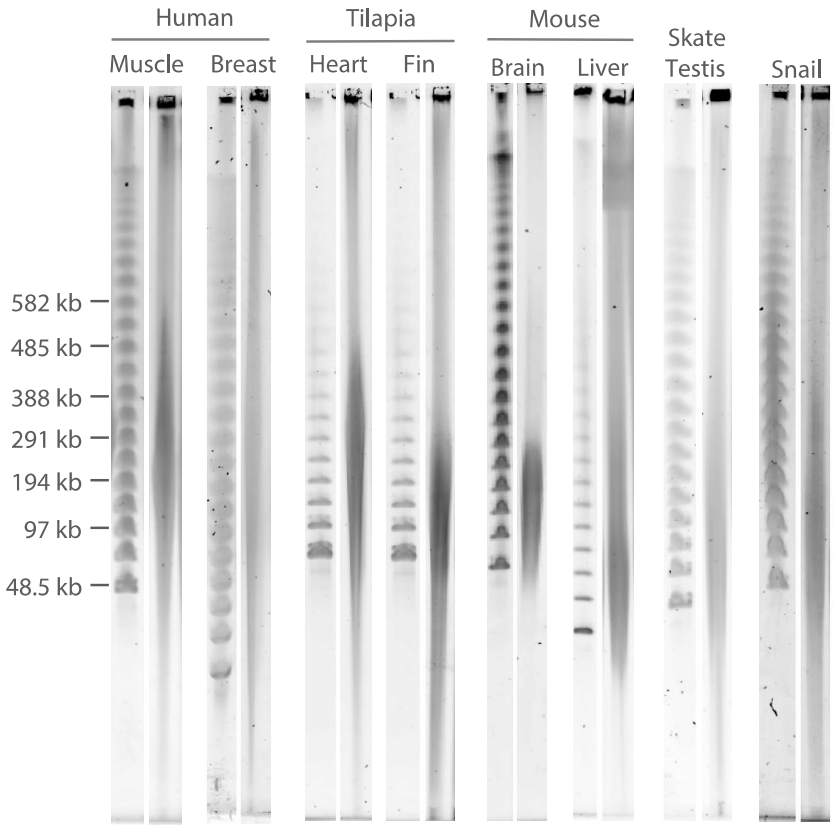
It is recommended that QC is performed after the DNA has been allowed to rest at RT overnight and appears homogeneous under visual examination and when pipetting.

1. Perform triplicate NanoDrop UV/VIS measurements from top, middle, and bottom of tube to determine total nucleic acid concentration as well as purity (A260/A280, A260/230).
    - HMW DNA is inherently difficult to work with as viscosity and inhomogeneity are often issues. We recommend taking at least three measurements, sampling from the top, middle, and bottom of the tube, to get an accurate concentration reading. We typically see concentration %CV values of <20%. However, if the DNA is very large, the %CV can exceed 30–40%.
    - If the DNA is very heterogeneous or contains large amounts of unsolubilized "jellies", refer to the **kit Guide & overview "Heterogeneity and viscosity"** and **"Troubleshooting FAQ"** sections.
2. Perform triplicate Qubit dsDNA BR Assay measurements from top, middle, and bottom of tube to determine DNA concentration.
    - We recommend taking the average of multiple measurements to ensure an accurate 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.
3. Perform a single Qubit RNA BR Assay measurement to determine RNA concentration (optional).
    - 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.
    - Use Agilent Femto pulse for HMW DNA size QC.

Results

- DNA was extracted from various animal tissues
- 260/280 ratios should consistently be in 1.8–2.0 range
- 260/230 ratio can vary from 2.0–2.3
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process

Sample	Input amount	260/280	260/230	Nanodrop top (ng/µL)	Nanodrop middle (ng/µL)	Nanodrop bottom (ng/µL)	Nanodrop avg (ng/µL)	Qubit DNA yield (µg)
Human skeletal muscle	25	1.85	2.04	119.9	125.6	126.8	124.1	8.3
Tilapia heart	25	1.86	2.18	252.6	240.3	230.5	241.1	13.1
Mouse brain	22	1.85	2.03	296.2	54.1	65.6	138.6	5.3
Human breast	20	1.88	2.33	579	596.3	549.8	575	60.6



22 hour Pulsed Field Gel Electrophoresis (PFGE) image of HMW DNA extracted from tissue samples

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Storage of DNA

DNA can be stored in Buffer EB at 4 °C for several months. Long term storage at -20 °C or -80 °C can be used if necessary. Avoid freeze/thaw cycles since this can degrade high molecular weight DNA.

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 **Nanobind tissue kit Guide & overview** for additional information on these tissues.

2. **After spinning in step 18, 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 18, 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 **18**. 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 such 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 5 full seconds 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 performed 1–2 times.
   - If a couple repeats of the step **36** spin are still not sufficient to pull all the DNA off the disk, you may spin the tube with the Nanobind disk at 16,000 x g for 15 seconds.

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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 75 µL eluate is exceedingly difficult to pipette. 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 lower than expected?**
    - Make sure that all the DNA is recovered from the Nanobind disk by spinning for 5 full seconds 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 a couple repeats of step **36** spin are still not sufficient to pull all the DNA off the disk, you may spin the tube with the Nanobind disk at 16,000 x g for 15 seconds.
    - 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.