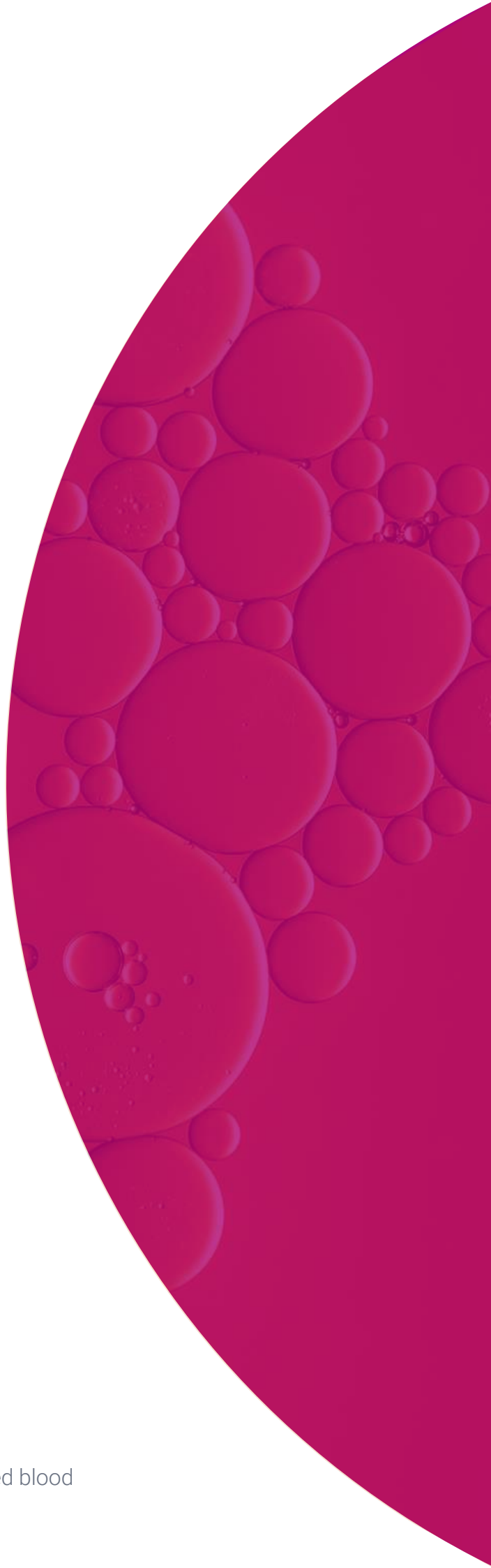
PacBio

Nanobind® HMW DNA extraction – nucleated blood

Procedure & checklist



For HMW (50 kb – 300+ kb) DNA extraction from nucleated blood for standard long-read sequencing applications.

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind CBB kit or Nanobind tissue kit	PacBio® (102-301-900 or 102-302-100)
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)
ThermoMixer	Eppendorf (5382000023)
1.5 mL Protein LoBind micro-centrifuge tubes	Eppendorf (022431081)
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
26g blunt end needle	SAI Infusion (B26150)
1 mL Syringe	Fisher Scientific (14-823-30)

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 CT (Nanobind tissue kit, 102-302-100, only) 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+ Mb) DNA extraction protocol

This protocol describes the extraction of HMW DNA from nucleated blood. It is recommended for HiFi sequencing applications.

Sample input requirements

Amount: 5–30 µL of nucleated blood

- Nucleated red blood cells are found in most vertebrate animals, with the exception of mammals. Nucleated blood samples contain 10–40X more DNA per volume of blood.
- Use 5–30 µL to prevent overloading the chemistry.
- Ethanol preserved nucleated blood can be processed by first centrifuging at 10,000 x g to pellet the RBCs and proceeding at step **2**. Begin with the equivalent of 5–30 µL of blood.
- No systematic difference has been seen in either DNA QC or sequencing results between fresh and frozen blood samples.
- Eppendorf Protein LoBind tubes (Eppendorf #022431081) are required for high purity. DNA LoBind tubes are less effective in preventing carryover contamination and are not recommended.

HMW DNA extraction – nucleated whole blood

1. Add 10 µL of nucleated whole blood sample into the bottom of a 1.5 mL Protein LoBind tube.
   - In general, 5–30 µL of nucleated whole blood yields enough DNA for most applications. We recommend starting with **10 µL**. Some species may require different inputs.
2. Add 190 µL of 1X PBS for a total volume of 200 µL.
   - If using an input volume of nucleated blood other than 10 µL, adjust the volume of 1X PBS accordingly so that the total volume of blood plus 1X PBS is 200 µL.
3. Add 20 µL of Proteinase K.
   - If removal of RNA is not necessary, proceed directly to step **5**.
4. Optional: add 20 µL of RNase A.
5. Pulse vortex for 1s x 10 times (max setting) and then spin on a mini-centrifuge for 2 s to remove liquid from the tube cap. Incubate at RT (18 – 25 °C) for 3 min.
   - Spinning the tube at each step prevents carryover of contaminants on the tube lip and cap surfaces.
6. Add 200 µL of Buffer BL3 and pulse vortex for 1 s x 10 times (max setting).
   - Insufficient vortexing in step **5** and step **6** will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
7. Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.
8. Incubate on a ThermoMixer at 55 °C and 900 rpm for 10 min.
9. Pulse vortex the lysate for 1 s x 3 times (max setting) and then spin on a mini-centrifuge for 2 s to remove liquid from the tube cap.
10. Add Nanobind disk to lysate and add 350 µL of isopropanol. Inversion mix 5X.

Quick tip

The Protein LoBind tubes will improve UV 260/230 ratios by up to 0.1 – 0.4 by preventing carryover of contaminants stuck to the tube surfaces.

Quick tip

Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length.

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- The Nanobind disk must be added before isopropanol.

11. Mix on tube rotator at 9 rpm at RT for 15 min.
12. Place tubes on the magnetic tube rack.
13. Discard the 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.
14. 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.
    - Use the method described in the **kit Guide & overview "Magnetic rack handling procedure"** section to ensure that the disk is captured near the top of the magnetic rack.
    - Remove excess liquid from the tube cap to minimize carryover contamination.
15. 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.
    - Use the method described in the **kit Guide & overview "Magnetic rack handling procedure"** section to ensure that the disk is captured near the top of the magnetic rack.
    - Remove excess liquid from the tube cap to minimize carryover contamination.
16. Repeat step 15.
17. 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 towards the magnet.
18. Repeat step 17.
19. Add 75–200 µL of Buffer EB directly onto the Nanobind disk and incubate at RT for 10 min.
20. Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube with a standard P200 pipette. Repeat until all eluate is transferred.
21. 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.
    - A small amount of liquid or gel-like material may remain on the Nanobind disk after transferring the eluate in step **Error! Reference source not found. This clear gel is DNA!** The spin in step **20** forces DNA to slide off the Nanobind disk into the bottom of the tube, after which it can be pipetted out and combined with the previously transferred eluate.
    - This should not require any more than 1–2 spins.
22. Pipette mix the sample 10X 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.

Vortexing is your friend

Quick tip

Pipette from the liquid interface rather than the bottom of the tube to avoid pipetting any dangling DNA

Quick tip

The DNA should appear free of color after washing. If DNA remains colored, repeat washes in a more aggressive fashion.

Quick tip

This 15 s spin is CRITICAL for recovering the DNA. We do not recommend a 2nd elution.

- Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.

23. Let eluate rest at RT overnight to allow DNA to solubilize.
    - Visible "jellies" should disperse after resting.
24. Following overnight rest, pipette mix 10X 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.
    - Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.
    - Heterogeneity can result from insufficient vortexing in step **5**, step **6**, and step **9**. Use aggressive mixing until familiar with the protocol.

Quick tip

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

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.

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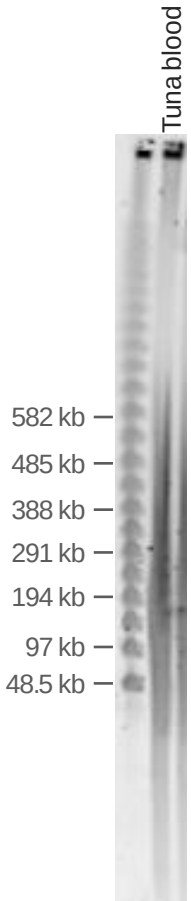
Results

- DNA was extracted from 20 µL of tuna blood.
- 260/280 ratios should consistently be in 1.8 range.
- 260/230 ratio can vary from 1.7 – 2.1.
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process.
- Higher input levels can be used with appropriate optimization of buffer volumes and enzyme levels.

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)
Tuna blood	20 µL	1.9	2.1	697.3	716.5	702.4	709.5	65



22 hour Pulsed Field Gel Electrophoresis (PFGE) image of HMW DNA extracted from nucleated blood

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Troubleshooting FAQ

1. **What do I do if the DNA is heterogenous and/or contains visible insoluble "jellies"?**

   - HMW DNA is inherently difficult to work with. The bigger it is, the more heterogeneous it tends to be.
   - Homogeneity can be improved by mixing 5–10X with a standard P200 pipette. Take care to disrupt any particularly viscous regions. Overnight incubation at RT will then allow the HMW DNA to relax back into solution.
   - High heterogeneity can be caused by insufficient mixing during the lysis steps. Many users will tend to be too gentle during the mixing steps. The resulting DNA will be bigger but will be difficult to handle and will tend to have lower purity. It is important to follow the vortexing steps outlined in the protocols. We recommend erring on the side of being overly aggressive. Even with all the vortexing, the DNA will still be 50-300 kb in length.
   - We recommend doing triplicate NanoDrop ensure accurate concentration readings and triplicate Qubit dsDNA BR assay measurements to ensure accurate DNA concentration readings.

2. **I transferred the eluate, but there is still liquid or a gel-like material on the Nanobind disk. What do I do?**

   - This is perfectly normal. The remaining DNA can be recovered by spinning the tube containing the Nanobind disk on a micro-centrifuge at 10,000 x g for 15 s. The disk will be wedged in the taper of the 1.5 mL tube, and the DNA will spin off 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.
   - We do not recommend a second elution. This is usually unnecessary and will result in a diluted, less-concentrated DNA sample.

3. **Why is my DNA yield lower than expected?**

   - Make sure that all the DNA is recovered from the Nanobind disk by centrifuging the tube containing the Nanobind disk at 10,000 x g for 15 sec.
   - If the sample is heterogeneous, you may be sampling from an area of the eluate that is a lot less concentrated. Take measurements from the top, middle, and bottom of the eluate to get an average concentration.
   - Your input could be too low. For nucleated blood, we recommend starting with 10 µL of blood, but some samples may require up to 30 µL of blood to get enough DNA for downstream applications.

4. **Why are the purities lower than expected? Is this this a problem?**

   - We do NOT see a correlation between UV purity and sequencing performance and do not pay particular attention to the UV purity as long as it is within the expected range for that particular sample type. Generally, nucleated blood gives purities of 260/230 >1.7 and 260/280 >1.8. Samples with UV purity slightly outside of this range will likely still sequence well. Samples with UV purity far outside this range should be treated with caution.
   - The purity could be lower due to insufficient lysis resulting from too high of a cell input. We recommend 10 µL of nucleated blood, but some samples may only require 5 µL. Higher inputs can yield more DNA but can overwhelm the lysis chemistry, resulting lower purity.
   - Lower purity can also be caused by insufficient mixing during the lysis steps. Many users will tend to be too gentle during the mixing steps. The resulting DNA will be bigger but will be difficult to handle and will tend to have lower purity. It is important to follow the vortexing steps outlined in the protocols. We recommend

erring on the side of being overly aggressive. Even with all the vortexing, the DNA will still be 50–300 kb in length.