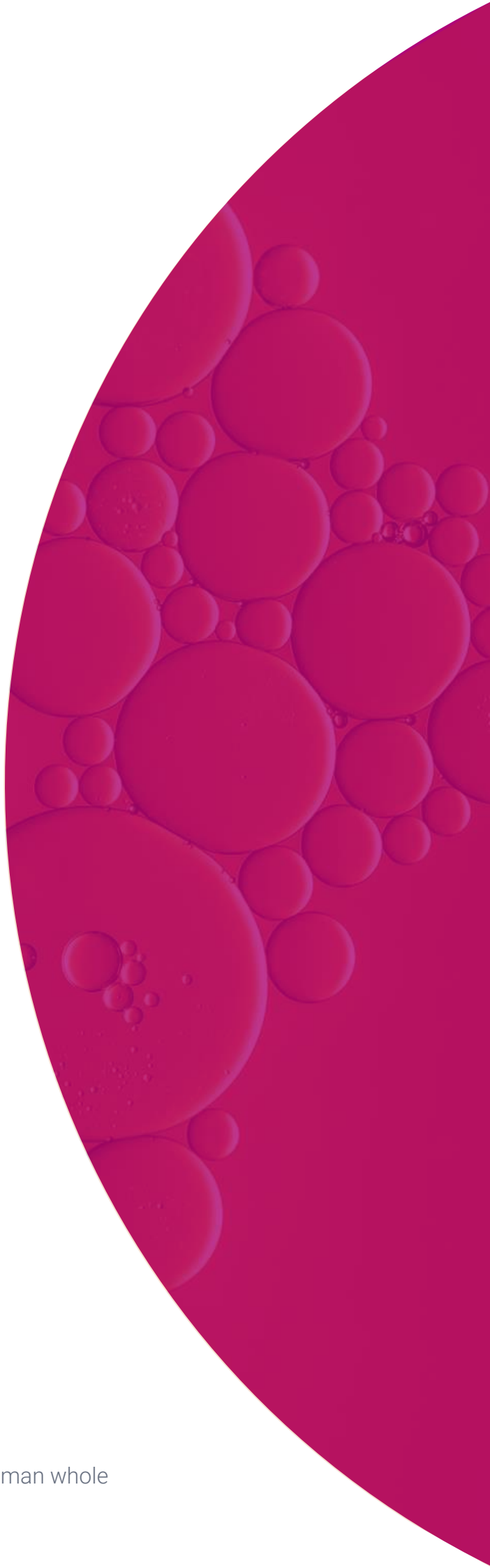
PacBio

Extracting HMW DNA from human whole blood using Nanobind® kits

Procedure & checklist



For HMW (50–300+ kb) DNA extraction from 200 µL of human whole blood for standard long-read sequencing applications.

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind CBB kit or Nanobind PanDNA kit	PacBio® (102-301-900 or 103-260-000)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
Platform rocker or mini-tube rotator	Thermo Scientific (M48725Q) or Fisher Scientific (88-861-051)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
ThermoMixer	Eppendorf (5382000023)
1.5 mL Protein LoBind microcentrifuge 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

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

The PanDNA kit contains 3 wash buffers (CW1, CW2 and, PW1) to extract various sample types. The CBB kit only contains 2 wash buffers (CW1 and CW2). Buffers CW1, CW2, and PW1 are supplied as concentrates. CW1 and CW2 are used with a 60% final ethanol concentration. PW1 is used with a 70% final ethanol concentration. Before using, add the appropriate amount of ethanol (96–100%) to Buffers CW1, CW2, and PW1, as indicated on the bottles.

Note: Not all buffers are used in every procedure.

Kit storage

Buffer CT and Buffer RBC 10X (Nanobind PanDNA kit, 103-260-000, only) and RNase A should be stored at 4°C upon arrival.

Nanobind disks and all other buffers should be stored at room temperature (15–30°C).

Buffer NPL (Nanobind PanDNA kit, 103-260-000, only) may form precipitates if stored cooler than room temperature. If this happens, precipitates will return to solution when stored at room temperature. Alternatively, the buffer can be warmed in a water bath to re-dissolve precipitates.

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Product use

Nanobind CBB and Nanobind PanDNA kits are intended for research use only.

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

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

Input requirements

Amount: 200 µL of human whole blood

- Yield for human whole blood will vary from 3–10 µg depending on donor WBC concentration.
- Blood samples need to be ≥ 4 X 10^6 WBC cells/L to give ≥ 3 µg HMW DNA yield.
- Blood samples should be frozen as quickly as possible after being drawn.
- Storage at 4°C should be limited to 2 days or fewer to prevent sample degradation.
- Blood samples should be aliquoted to avoid repeated freeze-thaws.
- K2 EDTA is the recommended anti-coagulant. Samples stored in sodium heparin (NaHep) and citrate (NaCit) also performed well in very limited testing.
- No systematic difference has been seen in either DNA QC or sequencing results between fresh and frozen blood samples.
- Frozen blood must be thawed at 37°C for 10–15 min and then thoroughly inversion mixed prior to extraction.
- 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.

Prior to beginning protocol

- If using frozen blood, incubate in a water bath or dry block heater at 37°C for 15 minutes to thoroughly thaw the sample. Mix the sample by inverting the tube >15 times immediately prior to use. **Improperly thawed and mixed samples may result in inconsistent DNA yield and purity.**

HMW DNA extraction – human whole blood

1. Dispense 20 µL of Proteinase K into the bottom of a 1.5 mL Protein LoBind tube.
2. Add 200 µL of whole blood.
   - The addition of blood to Proteinase K rather than vice versa increases lysis efficiency and purity through improved mixing.
3. Add 20 µL of RNase A.
4. Pulse vortex for 1 s 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 (15–30°C) for 3 min.
   - Spinning the tube at each step prevents carryover of contaminants on the tube lip and cap surfaces.
5. Add 200 µL of Buffer BL3 and pulse vortex for 1 s x 10 times (max setting).
   - Insufficient mixing in step 4 and step 5 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.

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.

6. Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.
7. Incubate on a ThermoMixer at 55°C and 900 rpm for 10 min.
   - If a ThermoMixer is not available, a heat block or water bath can instead be used with periodic agitation to ensure lysis.
8. 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.
9. Add Nanobind disk to lysate and add 350 µL of isopropanol. Inversion mix 5X.
   - The Nanobind disk must be added before isopropanol.
10. Mix on tube rotator at 9 rpm at RT for 15 min.
11. Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.
12. Place tube on the magnetic tube rack taking care to keep the tube cap and lip clean.
    - To prevent getting blood on the tube cap and lip, do not invert the tube at this step. Gently tilt the magnetic tube rack to allow Nanobind disk to be captured high up along the tube wall.
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 tube.
    - 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 tube.
    - 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. 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. Remove tube from tube rack and add 100–200 µL of Buffer LTE (formerly Buffer EB) directly onto the Nanobind disk. Incubate at RT for 10 min.
20. Collect DNA by transferring eluate to a new 1.5 mL Protein LoBind microcentrifuge tube with a standard P200 pipette. Repeat until all eluate is transferred.

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.

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 **20**. **This clear gel is DNA!** The spin in step **21** will allow 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.
    - 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 sample 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 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.
    - Heterogeneity can result from insufficient vortexing in step **4**, step **5**, and step **8**. Use aggressive mixing until familiar with the protocol.

Quick tip

This 15 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 gentle mixing. For samples that need to be used immediately, we recommend pipette mixing

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 **Nanobind CBB kit Guide & overview** **"Heterogeneity and viscosity**" and **"Troubleshooting FAQ "** sections for more information.
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.
4. Use Agilent Femto Pulse for HMW DNA size QC.
   - We recommend diluting the sample to 250 pg/µL.
   - Follow Agilent instructions for diluting the sample.
   - Use the Genomic DNA 165 kb Kit (Agilent Technologies) for unsheared gDNA.

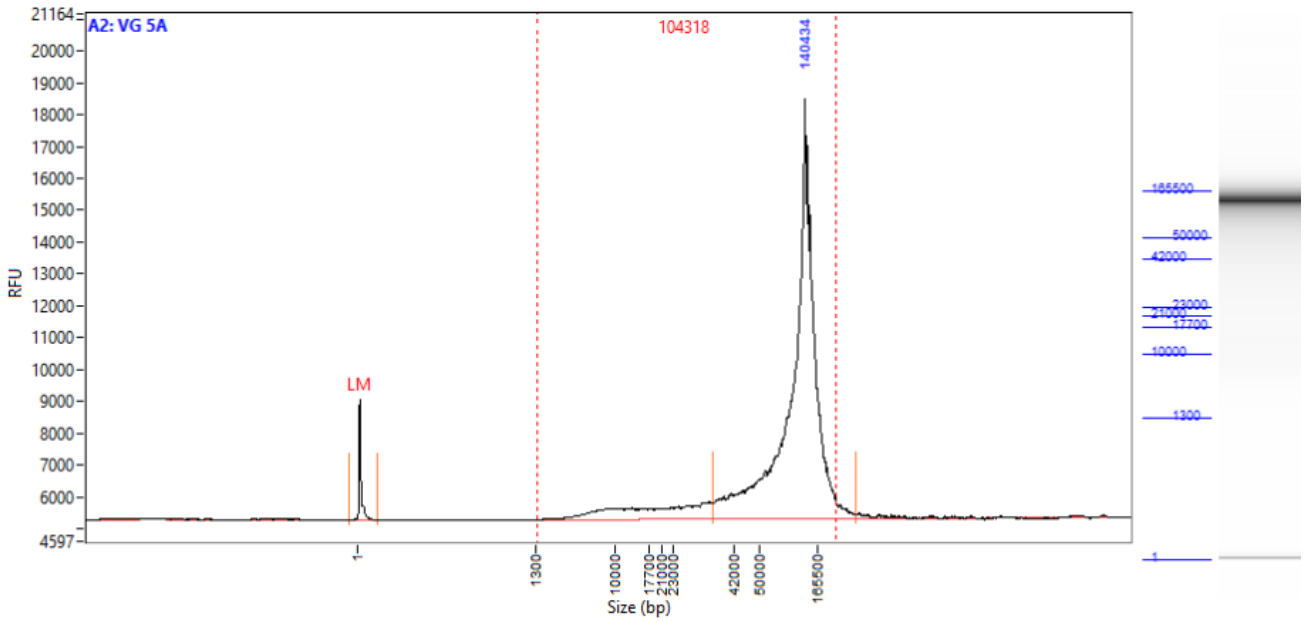
Storage of DNA

DNA can be stored in Buffer LTE (formerly 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.

Results

- DNA was extracted from 200 µL of fresh and frozen human whole blood.
- 260/280 ratios are consistently ≥1.7.
- 260/230 ratios are consistently ≥1.5.
- Samples with UV purities within the expected range should sequence well. UV purities below 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)
Fresh blood	200 µL	1.90	1.84	77.2	77.4	76.9	77.2	5.2
Frozen blood	200 µL	1.90	1.88	68.0	67.7	69.1	68.3	4.8



DNA size distribution of unsheared gDNA isolated from 200 µL of frozen human blood on the Femto Pulse system (Agilent Technologies).

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 s.
   - DNA yield can be affected by improper thawing and/or mixing of the blood immediately prior to beginning the protocol. Refer to the recommendations in the **Prior to beginning protocol** section to properly prepare the sample.
   - With blood, the DNA recovery is highly dependent on the WBC concentration of the donor. We typically get 3–10 µg per 200 µL of whole blood but can get as low as 1 µg and as high as 14 µg of DNA, depending on the donor.
   - For donors with low WBC concentration, the blood input can be increased.
   - 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.
   - During the removal of the binding solution in step **13**, it is possible to accidentally pipette bound DNA. To remove the binding solution, carefully insert the pipette tip against the wall opposite the Nanobind disk and remove liquid by pipetting from the liquid surface. To avoid pipetting bound DNA, a small amount of the binding solution can be left in the bottom of the tube if necessary.

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,

whole blood gives purities of 260/230 >1.5 and 260/280 >1.7. 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.

- It is important to keep the cap and the lip of the tube clean. This is the main source of sample contamination and low purity. Make sure to spin down the tube after all mixing steps.
- We highly recommend the use of Eppendorf Protein LoBind tubes. These tubes are effective in reducing contamination from protein carryover on the tube surfaces.

Revision history (description)	Version	Date
Initial release	01	July 2022
Minor updates	02	December 2022
Updated for new Nanobind CBB kit and Nanobind PanDNA kit	03	March 2024