PacBio

Extracting HMW DNA from human whole blood with RBC lysis using the Nanobind® PanDNA kit

Procedure & checklist

103-377-500 REV01 MAR2024

This protocol describes the extraction of HMW (50–300+ kb) DNA from 400 µL of human whole blood with RBC lysis. It is recommended for PacBio® HiFi sequencing. This protocol requires the Nanobind PanDNA kit (103-260-000).

Required equipment and materials

Equipment/reagent	Manufacturer (part number)
Nanobind® PanDNA kit	PacBio® (103-260-000)
Magnetic tube rack	Thermo Fisher Scientific 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)
2 mL Protein LoBind microcentrifuge tubes	Eppendorf (022431102)
1.5 mL Protein LoBind microcentrifuge tubes	Eppendorf (022431081)
1x PBS	Any major lab supplier (MLS)
Ethanol (96–100%)	Any MLS
Isopropanol (100%)	Any MLS
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

Before you begin

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

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: For the PanDNA kit, not all buffers are used in every procedure.

Kit storage

RNase A, Buffer CT, and Buffer RBC 10X 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 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 PanDNA kits are intended for research use only.

Input requirements

Amount: 400 µL of human whole blood

- Yield for human whole blood will vary from 3–25 µg based on donor WBC concentration.
- 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 anticoagulant. Samples stored in sodium heparin (NaHep) and citrate (NaCit) also performed well in limited testing.
- No systematic difference has been observed in DNA QC or sequencing results between fresh and frozen blood samples.

- Eppendorf Protein LoBind tubes are required for high purity. DNA LoBind tubes are less effective in preventing carryover contamination and are not recommended.
- 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.**

Procedure and checklist

1. HMW DNA extraction – 400 µL human whole blood with RBC lysis

1. Prepare 1X Buffer RBC by mixing 120 µL of Buffer RBC 10X and 1080 µL of nuclease-free water. 1X Buffer RBC should be used at RT. Excess 1X Buffer RBC should be stored at 4°C.
    - The volume of 1X Buffer RBC can be scaled as long as the dilution ratio is 10:1.
2. Thaw blood at 37°C for 15 min. Inversion mix 10X and spin 2 s in a mini-centrifuge to remove blood from the cap.
3. Add 400 µL blood to a 2 mL Protein LoBind tube.
4. Add 1200 µL 1X Buffer RBC. Inversion mix 5X and incubate at RT for 10 min. Inversion mix 5X every 5 min.
5. Centrifuge for 2 min at 2,000 x *g* and RT to pellet the white blood cells. Discard the supernatant.
    - Orient the tube in the centrifuge such that the location of the WBC pellet will be known after centrifugation, since the supernatant will be dark and the WBC pellet may be difficult to see.
6. Add 1 mL of 1X PBS. Vortex for 10 s (max setting) to resuspend the cells.
    - Cells from frozen blood can be more difficult to resuspend than those from fresh blood. This is completely normal and usually disappears during the step 14 incubation.
7. Centrifuge for 2 min at 2,000 x *g* and RT to pellet the white blood cells. Discard the supernatant.
8. Add 100 µL of 1X PBS. Vortex for 10 s (max setting) to resuspend the cells.
    - Cells from frozen blood can be more difficult to resuspend than those from fresh blood. This is completely normal and usually disappears during the step 14 incubation.
9. Add 20 µL of Proteinase K.

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.

Vortexing is your friend

Quick tip

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

10. Add 20 µL of RNase A, pulse vortex for 1 s x 10 times (max setting), and incubate at RT (18–25°C) for 3 min.
11. Add 150 µL of Buffer BL3 and pulse vortex for 1 s x 10 times (max setting).
    - A white precipitate may form after addition of BL3. This is completely normal and usually disappears during the step 12 incubation.
    - Insufficient mixing in step 8, step 10, and step 11 will result in very large DNA but also lower purity, lower yield, higher heterogeneity, and difficult elution.
12. Incubate on a ThermoMixer at 55°C and 2000 rpm for 20 min.
    - If a ThermoMixer is not available, a heat block or water bath can instead be used with periodic agitation to ensure lysis.
13. Pulse vortex 1 s x 10 times (max setting). Spin the tube 2 s in a mini-centrifuge to remove cell lysate from the cap.
14. Transfer cell lysate to a 1.5 mL Protein LoBind tube using a standard P1000 pipette tip.
15. Add the Nanobind disk to cell lysate and add 250 µL of isopropanol. Inversion mix 5X.
    - The Nanobind disk must be added before isopropanol.
16. Mix on tube rotator at 9 rpm at RT for 10 min.
    - Some white precipitate might be attached to the DNA as it binds to the disk. This is normal.
17. Place tubes on the magnetic tube rack.
    - Use the method described in the kit Guide & overview “Magnetic rack handling procedure” section.
18. 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.
19. 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.
20. 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.
21. Repeat step 20.
22. Spin the tube on a mini-centrifuge or 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.

23. Repeat step 22.
24. Remove tube from tube rack and add 100 µL of Buffer LTE (formerly Buffer EB). Incubate at RT for 10 min.
25. 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.
26. 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 25. **This clear gel is DNA!** The spin in step 26 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 more than 1–2 spins.
27. 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.
28. Let eluate rest overnight at RT to allow DNA to solubilize.
    - Visible "jellies" should disperse after resting.
29. Following overnight rest, pipette mix 10 times with a standard P200 pipette and analyze the recovery and purity as described in the **QC procedure** section.
    - 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 8, step 10, step 11, and step 13. 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 procedure

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 a NanoDrop UV/VIS measurement to determine total nucleic acid concentration as well as purity (A260/A280, A260/A230).
    - If the DNA is very heterogeneous or contains large amounts of unsolubilized "jellies", refer to the **kit Guide & overview "Heterogeneity and viscosity"** section for more information.
2. Perform a Qubit dsDNA BR assay measurement to determine DNA concentration.
    - 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 Qubit RNA BR assay measurement to determine RNA concentration (optional).
    - 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. Finger tap to mix.
    - Avoid mixing with a standard pipette. This will shear the DNA. Always use a wide-bore pipette when making dilutions.
    - Use the Genomic DNA 165 kb Kit (Agilent Technologies) for unsheared gDNA.

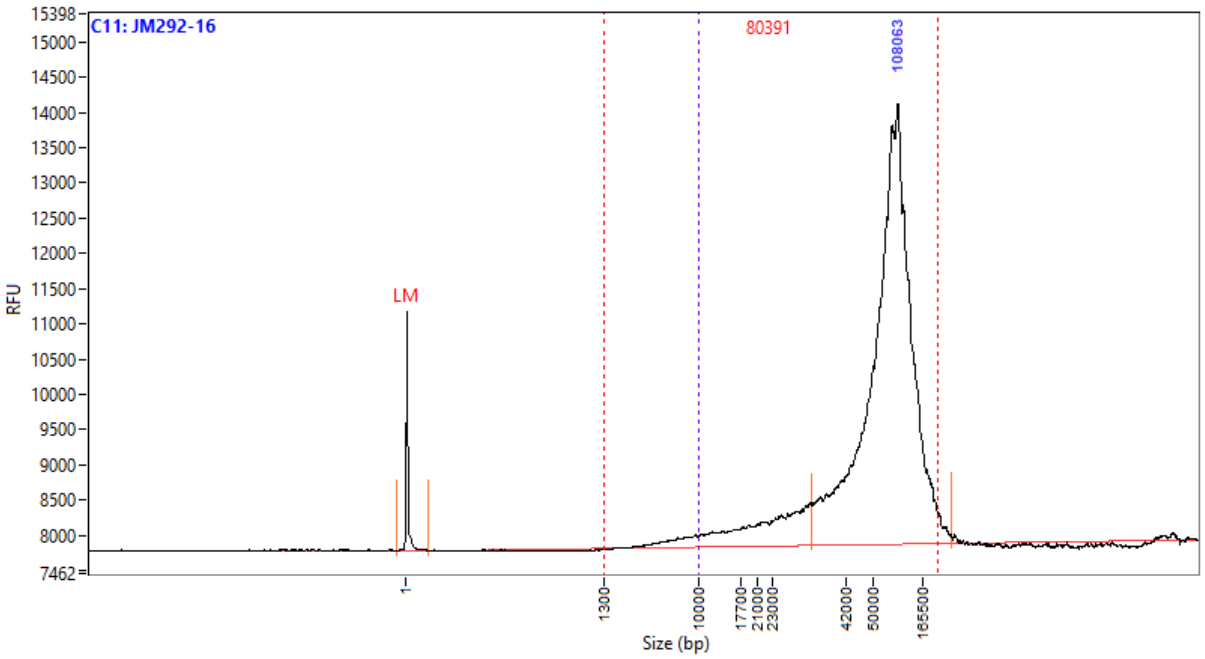
Storage of DNA

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 extracted from 400 µL human whole blood with RBC lysis will yield ~3–20 µg depending on donor white blood cell count.
- 260/280 ratios should consistently be 1.8–2.0.
- 260/230 ratio can vary from 1.9–2.3.
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process.
- The mode of DNA extracted from human whole blood with RBC lysis measured on the Femto Pulse system (Agilent Technologies) is typically 100 kb+.

Sample	WBC count (10^9 cells/L)	260/280	260/230	Nanodrop (ng/µL)	Qubit DNA yield (µg)
Fresh blood	5.3	1.87	2.13	125.1	9.80
Frozen blood	5.3	1.86	2.21	100.6	8.97



DNA size distribution of unsheared gDNA isolated from a 400 µL human whole blood sample with RBC lysis 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 longer the DNA, the more heterogeneous it will be.
   - Homogeneity can be improved by mixing 5–10 times 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 measurements to 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 need 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 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 3–25 µg per 400 µL of RBC lysed whole human blood.
   - If the sample is heterogeneous, you may be sampling from an area of the eluate that is less concentrated. Take measurements from the top, middle, and bottom of the eluate to get an average concentration.
   - Occasionally, samples can yield lower DNA recoveries than expected. This is typically due to factors beyond the control of the protocol, such as donors with very low WBC count. If the DNA yield is insufficient for sequencing and additional blood sample remains, the protocol can be repeated and the eluates pooled. Contact PacBio for further support.

4. **Why are the purities lower than expected? Is 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 if it is within the expected range for that particular sample type. Generally, human whole blood DNA with RBC lysis results in UV purities of 260/230 >1.9 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.
- DNA purity 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.
- 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	March 2024