Extracting HMW DNA using the Nanobind® HT 1 mL blood kit for mammalian whole blood on the KingFisher Duo Prime system

Procedure & checklist

This procedure describes the workflow for high-throughput automated extraction of HMW (50–300 kb) DNA from 1 mL of whole mammalian blood using the Thermo Fisher KingFisher Duo Prime robotic instrument. This procedure requires the Nanobind HT 1 mL blood kit (102-762-800) and is recommended for HiFi sequencing.

Required materials and equipment

Equipment/reagent	Manufacturer (part number)
Nanobind HT 1 mL blood kit	PacBio® (102-762-800)
KingFisher Duo Prime System	Thermo Fisher Scientific (5400110)
KingFisher 24 deep-well plates	Thermo Fisher Scientific (95040470)
KingFisher 6-tip comb, for 24 deep-well plate	Thermo Fisher (97003510)
Ethanol (96–100%)	Any major lab supplier (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

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 (15–30°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 kits are intended for research use only.

Headspace

The automation script for the following procedure includes "headspace" volume in each plate. The "headspace" volumes are virtual volumes added to the automation script to improve the retention of the Nanobind on the magnetic rod and do not interfere with the extraction efficiency or performance.

Procedure and checklist

Automated HMW (50– 300 kb) DNA extraction procedure

Input requirements

Amount: 1 mL of mammalian whole blood

- The yield for human whole blood will vary from 3–70 µg based on donor white blood cell 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 and reduced DNA recovery.
- 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.

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 15–20 times immediately prior to use. **Improperly thawed and mixed samples may result in inconsistent DNA yield and purity.**
- Ensure the proper script has been installed on the KingFisher instrument (see **Nanobind HT kit Guide & overview "Programs"**).
- This protocol has been validated on human whole blood. No systematic difference has been seen in either DNA QC or sequencing results between fresh and frozen blood.

HMW DNA extraction – mammalian whole blood (1 mL)

This procedure describes the workflow for automated HMW DNA extraction from 1 mL of mammalian whole blood on the Thermo Fisher KingFisher Duo Prime robotic instrument. This procedure uses a KingFisher 6-pin magnetic head, 6-tip comb, and 24 deep-well plates. This procedure cannot be run with the 12-pin magnetic head and 96 deep-well plates.

✔	Step	Instructions
	1.1	Prepare Plate 2: collect a KingFisher 24 deep-well plate and prepare as indicated in the following table.

Row	Row name	Reagent	Volume per well
A	CW2 Wash 1	Buffer CW2	2 mL
B	CW2 Wash 2	Buffer CW2	2 mL
C	empty		
D	Elution	Buffer EB	200 µL

1.2 Prepare Plate 1: collect a KingFisher 24 deep-well plate and prepare it as indicated in the following table. **Add the components to Row A (Lysis/binding) only after all other rows have been prepared.**

Row	Row name	Reagent	Volume per well
A	Lysis/Binding	Sample + reagents from Step 1.3	
B	Tip Comb	KingFisher 6-tip comb, for 24 deep-well plate	
C	Nanobind Storage	One 5 mm Nanobind disk per well*	
D	CW1 Wash 1	Buffer CW1	2 mL

***Nanobind disks do not need to be perfectly centered in the wells, but ensure they are at the bottom of the well and not stuck to the side walls.**

1.3 Prepare sample in Row A of Plate 1:

- Add 100 µL of Proteinase K to each well.
- Add 1 mL of whole blood to each well.
  - Thaw frozen whole blood at 37°C using a water bath or dry block heater for 15 minutes. Invert-mix >15 times to thoroughly mix immediately prior to use.
- Add 100 µL of RNase A to each well.
- Add 750 µL of Buffer BL3 to each well.

Note: add BL3 gently against the side of the well into the Lysis/Binding solution. Adding BL3 directly to the Lysis/Binding solution may affect extraction performance.

Note: the sample and reagents MUST be added to the wells in the order described above.

1.4 Ensure the instrument is set up with the 6-pin magnetic head and heating block.

1.5 Select the **1mL_Blood_Nanobind_HT_DUO** (102-998-200) script on the KingFisher Duo Prime instrument and press 'Start'.

1.6	Insert plates into the KingFisher Duo Prime instrument as indicated on the display and press 'OK' after every plate to confirm position. The protocol will start when the final plate is loaded and the 'OK' button is pressed.
1.7	When prompted by the instrument (~50 minutes after start), remove Plate 1 from the instrument and add 1.5 mL (750 µL x 2) of isopropanol to the lysate in Row A. Re-insert the plate and press 'OK' to resume the protocol. **Note: add isopropanol gently against the side of the well into the Lysis/Binding solution. Adding isopropanol directly to the Lysis/Binding solution may affect extraction purity.**
1.8	At the end of the run (~105 minutes after start), remove both 24 deep-well plates from the KingFisher Duo Prime instrument.
1.9	Transfer eluates from Row D of Plate 2 to a new storage plate or storage tubes. • The protocol is designed to leave the Nanobind disks in Row D (Elution). On occasion, the disk may be transferred back to the tip comb storage row (Plate 1 Row B) after elution. This does not affect extraction performance. • When transferring eluate, a small amount of liquid may remain on the Nanobind disk. Use a P200 pipette tip to transfer any liquid remaining on the Nanobind disk.
1.10	Pipette mix the sample 10 times 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.
1.11	Let eluate rest overnight at room temperature to allow DNA to solubilize. • Visible "jellies" should disperse after resting.
1.12	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.

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 **Nanobind HT 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 inconsistent.
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 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

See the example results table and Figure 1 reflecting typical parameters as listed below.

- DNA extracted from 1 mL human whole blood will yield ~3–70 µg depending on donor white blood cell count.
- 260/280 ratios should consistently be 1.8–2.0.
- 260/230 ratio can vary from 1.3–2.2.
- 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 extracted human whole blood DNA 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)
Donor #1	4.6	1.87	2.02	90.3	14.7
Donor #2	6.3	1.87	2.11	174.3	28.2
Donor #3	9.6	1.89	2.10	278.9	55.2

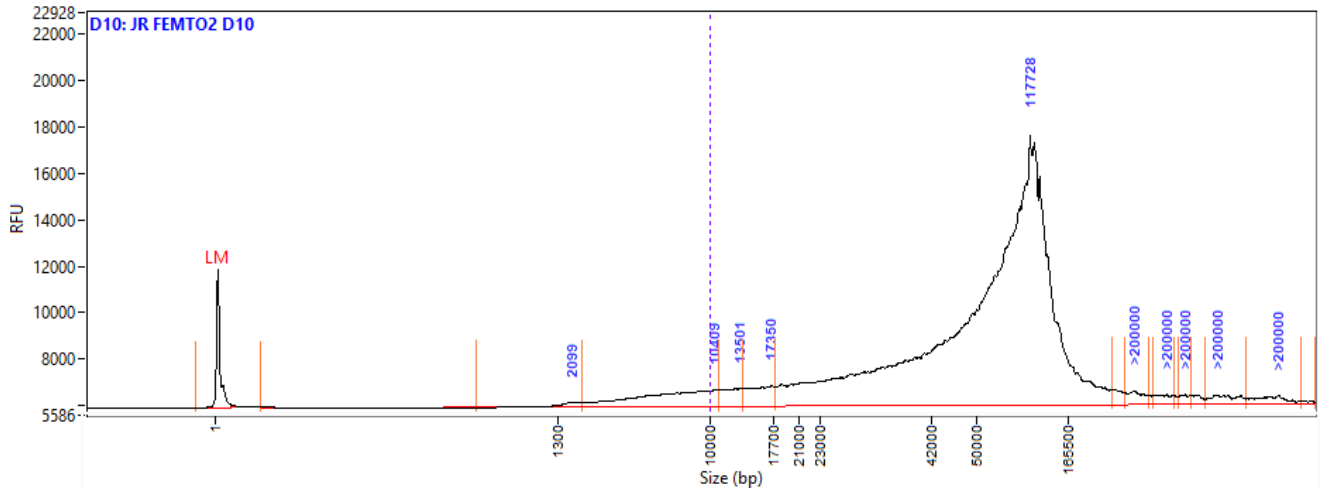

Figure 1. DNA size distribution of unsheared gDNA isolated from a 1 mL human whole blood sample using the KingFisher Duo on the Femto Pulse system (Agilent Technologies).

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 room temperature will then allow the HMW DNA to relax back into solution.

2. **I transferred the eluate, but there is still liquid or a gel-like material on the Nanobind disk. What do I do?**
   - Ensure all the DNA is recovered from the sample by visually inspecting the Nanobind disk after the eluate has been transferred. The Nanobind disk should appear mostly free of any substances. If any material remains on the Nanobind after elution, remove as much as possible using a P200 pipette. Leaving a small amount of DNA/liquid on the Nanobind disk should not have a large impact of DNA yield.
   - 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?**
   - Ensure all the DNA was recovered from the Nanobind disk. See FAQ #2 for more information.
   - DNA yield can be affected by improper thawing and/or mixing of the blood immediately prior to beginning the KingFisher Duo Prime protocol. Refer to the recommendations in the **Prior to beginning protocol** section to properly prepare the sample.
   - 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 inherent sample inhomogeneity from certain donors or low white blood cell count. If the DNA yield is insufficient for sequencing and additional blood sample remains, rerun the protocol and contact PacBio for further steps.

4. **Why are the purities lower than expected? Is this a problem?**
   - A correlation has NOT been seen between UV purity and sequencing performance and no particular attention to the UV purity is needed if it is within the expected range for that particular sample type. Generally, human whole blood DNA results in UV purities of 260/230 >1.3 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 KingFisher Duo Prime procedure. Refer to the recommendations in the **Prior to beginning protocol** section to properly prepare the sample.
   - If purities are generally low across all samples, ensure the isopropanol was added as recommended in Step 1.7 (add isopropanol so it gently trickles down the side of the well into the Lysis/Binding solution). Adding isopropanol directly to the Lysis/Binding solution can result in decreased purities.

5. **One or more of my eluates do not contain a Nanobind disk after completion of the procedure. What does this mean?**

   - The Nanobind disks occasionally remain on the tip comb after elution and are returned to Plate 1 Row B at the end of the procedure. If the Nanobind corresponding to the sample in question has been returned to Plate 1 Row B, move forward with sample QC as this should not have significant effects on DNA recovery.
   - If a Nanobind disk for the eluate in question is not on the tip comb in Plate 1 Row B, this sample will likely not contain any DNA. Occasionally, the disk can become dislodged from the magnet during binding and remains in the Lysis/binding well (Plate 1, Row A). This is a rare occurrence but can happen. We recommend rerunning the protocol if additional blood sample remains.
   - If the Nanobind disk for the eluate in question is not on the tip comb in Plate 1 Row B and not in the Lysis/Binding well (Plate 1 Row A), contact PacBio for further instruction.

6. **One or more of my eluates exhibit a slight red/orange tint. What does this mean?**

   - Occasionally some of the blood coloration carries through the extraction. This can result in a slight decrease in 260/230 purity; however, it should not affect DNA yield or sequencing performance.

7. **Why isn't the protocol running and/or why is there an error message?**

   - Check to ensure the correct script is installed. See **Nanobind HT kit Guide & overview "Programs."**
   - Check to ensure the correct magnet head and heat blocks are installed.
   - Check to ensure all consumables are in the correct positions. The instrument will give an error message if it does not detect the tip comb (i.e., the tip comb is not in the correct position).
   - For other on-instrument error messages, contact Thermo Fisher.

Revision history (description)	Version	Date
Initial release	01	April 2023