Extracting HMW DNA from cultured suspension cells using Nanobind® kits

Procedure & checklist

This protocol describes the extraction of HMW (50–300+ kb) DNA from cultured suspension cells. It is recommended for PacBio® HiFi sequencing. This protocol requires the Nanobind CBB kit (102-301-900) or the Nanobind PanDNA kit (103-260-000).

Required materials and equipment

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%)	Any major lab supplier (MLS)
Isopropanol (100%)	Any MLS
1X PBS	Any MLS
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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Before you begin

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 below 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.

Cell input requirements

Amount: 1 x 10^6–5 x10^6 diploid human cells or mammals with similar genome sizes

- Cell counts should be accurately determined using a hemocytometer or cell counter.
- For non-diploid cells, the cell input should be scaled appropriately to contain 5–30 µg of DNA (e.g., tetraploid cells will require half the cell input as diploid cells for the same target amount of DNA).
- This protocol has been validated on cell lines including HG001 and HG002.
- No systematic difference has been seen in either DNA QC or sequencing results between fresh and frozen cell samples.
- Cell pellets should be frozen dry with as much liquid removed as possible. No cryoprotectant is needed.

Procedure and checklist

1. HMW DNA extraction – cultured suspension cells

✔	Step	Instructions
	1.1	Harvest cells and centrifuge at 500 x *g* for 3–5 min at 4°C to pellet cells in a 1.5 mL Protein LoBind tube; remove the supernatant. • Frozen cell pellets may also be substituted here.
	1.2	Add 100 µL of 1x PBS and pipette mix 10X with a standard P200 pipette to resuspend cells. • Mix until cell pellet is fully resuspended without visible lumps. Complete resuspension of the cell pellet is critical for ensuring efficient lysis. Sticky cell types may require additional pipette mixing or vortexing. • Aggressive mixing at this step will not affect DNA size. However, incomplete resuspension will result in inefficient lysis and digestion which will lead to low yield, low purity, and high heterogeneity.
	1.3	Add 20 µL of Proteinase K.
	1.4	Pulse vortex for 1 s x 10 times (max setting). **Note:** Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length
	1.5	Add 20 µL of RNase A, pulse vortex for 1 s x 5 times (max setting), and incubate at RT (15–30°C) for 3 min.
	1.6	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 Buffer BL3. This is completely normal and usually disappears during the step **7** incubation. • Insufficient mixing in step **2**, step **4**, step **5**, and step **6** will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
	1.7	Incubate on a ThermoMixer at 55°C and 2000 rpm for 10 min. • If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
	1.8	Pulse vortex for 1 s x 10 times (max setting).
	1.9	Add Nanobind disk to cell lysate and add 250 µL of isopropanol. Inversion mix 5X. • The Nanobind disk must be added before isopropanol.
	1.10	Mix on tube rotator at 9 rpm at RT for 10 min. • Some white precipitate may be seen attached to the DNA as it binds to the disk. This is normal.
	1.11	Place tubes on the magnetic tube rack. • Use the method described in the **kit Guide & overview "Magnetic rack handling procedure"** section
	1.12	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. • Pipette from the liquid interface rather than the bottom of the tube to avoid pipetting any dangling DNA.
	1.13	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. • Remove excess liquid from the tube cap to minimize carryover contamination.
	1.14	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. • Remove excess liquid from the tube cap to minimize carryover contamination.

1.15	Repeat step **14**.
1.16	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.
1.17	Repeat step **16**.
1.18	Remove tube from tube rack and add 100–200 µL of Buffer LTE (formerly Buffer EB). Incubate at RT for 10 min.
1.19	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.
1.20	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. • This 15 s spin is **critical** for recovering the DNA. We do not recommend a second elution. • A small amount of liquid or gel like material may remain on the Nanobind disk after transferring the eluate in step **19**. **This clear gel is DNA!** The spin in step **20** 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.
1.21	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.
1.22	Let sample rest at RT for overnight to allow DNA to solubilize. • Visible "jellies" should disperse after resting.
1.23	Following overnight rest, pipette mix 10X with a standard P200 pipette and analyze the recovery and purity as described in QC Procedure. • 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. • 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**, step **6**, and step **8**. Use aggressive mixing until familiar with the protocol.

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 for more information.

1. 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.

2. 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.

3. 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.

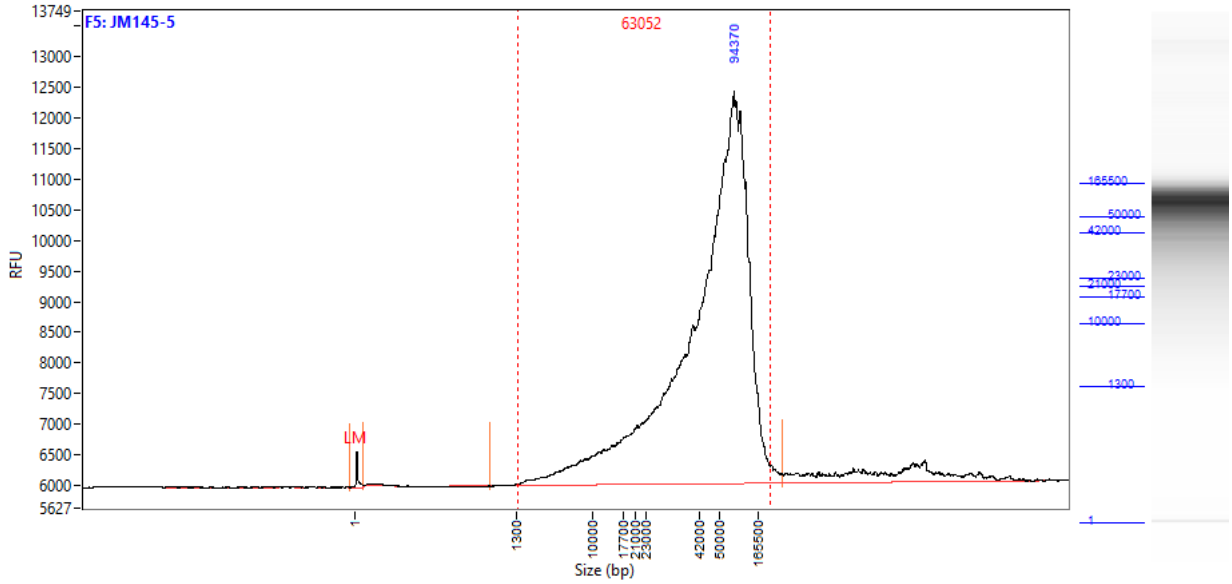
Storage of DNA

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 HG001 and HG002 cells.
- 260/280 ratios should consistently be in the 1.8–2 range.
- 260/230 ratio can vary from 1.7–2.2.
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process.
- 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)
HG002 (diploid)	$2x10^6$ cells	1.88	2.23	232.5	233.0	233.9	233.1	22.3



DNA size distribution of unsheared gDNA isolated from $2x10^6$ HG002 cells 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 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 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.
   - 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 cultured human cells, we recommend $1x10^6$–$5x10^6$ cells. For example, $5x10^6$ HG002 cells should recover ~30 µg of DNA.
   - The lysis could be inefficient due to improper resuspension of the cell pellet. Make sure the cell pellet is completely resuspended in step **2** and that no visible cell clumps remain. We recommend being overly aggressive at this step.

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 as long as it is within the expected range for that particular sample type. Generally, cultured human cells give UV 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 $1x10^6$–$5x10^6$ cells. Inputs greater than this can overwhelm the lysis chemistry, resulting in lower recoveries and 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.

Revision history (description)	Version	Date
Initial release	01	February 2024