Extracting HMW DNA using the Nanobind® HT CBB kit for mammalian cultured cells 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 mammalian cultured cells using the Thermo Fisher KingFisher Duo Prime robotic instrument. This protocol requires the Nanobind HT CBB kit (102-762-700) and is recommended for HiFi sequencing.

Required materials and equipment

Equipment/reagent	Manufacturer (part number)
Nanobind HT CBB kit	PacBio® (102-762-700)
KingFisher Duo Prime System	Thermo Fisher Scientific (5400110)
KingFisher 96 deep-well plates	Thermo Fisher Scientific (95040450)
KingFisher 12-tip comb, for 96 deep-well plate	Thermo Fisher Scientific (97003500)
KingFisher elution strip for 12-pin magnet	Thermo Fisher Scientific (97003520)
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

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Before you begin

Prior to starting

Buffer CW1 and CW2 are supplied as concentrates. This kit uses CW1 with a 60% final ethanol concentration and = 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

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

Product use

Nanobind kits are intended for research use only.

Headspace

The automation script for the following protocol 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 protocol

Input requirements

Amount: $1x10^6$ diploid human cells or equivalent

- Cell counts should be accurately determined using a hemocytometer or cell counter.
- For non-diploid or non-human cells, the cell input should be scaled appropriately to contain 5–25 µg of DNA.
  Warning: >25 µg inputs can cause Nanobinds to be "dropped" in the Lysis/Binding solution and/or cause well-to-well contamination.
- This protocol has been validated on cell lines including GM24385, GM12878, and MCF-7.
- No systematic difference has been observed in 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.

Prior to beginning protocol

- Ensure the proper protocols have been installed on the KingFisher instrument (see **kit Guide & overview "Programs"**)

HMW DNA extraction – cultured cells

This procedure describes the workflow for automated HMW DNA extraction from cultured mammalian cells on the Thermo Fisher KingFisher Duo Prime instrument. This protocol uses a KingFisher 12-pin deep-well magnetic head, 12-tip comb, 96 deep-well plate, and 12-pin elution strip. This protocol cannot be run with the 6-pin deep-well head and 24 deep-well plates.

✔	Step	Instructions
	1.1	Collect a KingFisher 96 deep-well plate and prepare as indicated in the following table. **Add the components to Row A (Lysis/Binding) only after all other rows and the elution strip have been prepared.**

Row	Well name	Reagent	Volume per well
A	Lysis/Binding	Sample + reagents from Step 1.5	
B	Tip Comb	KingFisher 12-tip comb	
C	Nanobind Storage	One 3 mm Nanobind Disk per well*	
D	empty		
E	CW1 Wash 1	Buffer CW1	700 µL
F	CW2 Wash 1	Buffer CW2	700 µL
G	CW2 Wash 2	Buffer CW2	700 µL
H	empty		

***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.**

1.2 Prepare a 12-pin elution strip as indicated in the following table.

Row	Well name	Reagent	Volume per well
N/A	Elution Strip	Buffer EB	100 µL

1.3 Prepare sample: Harvest cells and centrifuge at 500 *x g* for 3–5 min at 4°C to pellet cells; remove the supernatant.

- Frozen cell pellets may also be substituted here.
- Use 1x10^6 diploid cells or equivalent.
- For non-diploid or non-human cells, the cell input should be scaled appropriately to contain 5–25 µg of DNA.
- Warning: >25 µg inputs can cause Nanobinds to be "dropped" in the Lysis/Binding solution and/or cause well-to-well contamination.

1.4 Add 50 µL of 1x PBS and pipette-mix 10 times with a standard P200 pipette to resuspend cells.

- Mix until cell pellet is fully resuspended without visible lumps. 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.

1.5 Prepare sample in Row A of the 96 deep-well plate:

- Add the samples prepared in Steps 1.3–1.4 to individual wells.
- Add 20 µL of Proteinase K to each well.
- Add 5 µL of Buffer CLE3 to each well.
- Add 20 µL of RNase A to each well. Let rest on benchtop for 5 minutes.
- Add 150 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.

1.6 Ensure the instrument is set up with the 12-pin deep-well magnetic head and heating block.

1.7 Select the **Cell_Nanobind_HT_DUO** script (103-416-500) on the KingFisher Duo Prime instrument and press 'Start'.

1.8 Insert the Elution Strip into the KingFisher Duo Prime instrument.

1.9 Insert the prepared 96 deep-well plate into the KingFisher Duo Prime instrument. The protocol will start when the plate is loaded and the 'OK' button is pressed.

1.10 When prompted by the instrument (~21 minutes after start), remove the plate from the instrument and add 250 µL 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.

1.11 At the end of the run (~65 minutes after start), remove the 96 deep-well plate and the elution strip from the KingFisher Duo Prime instrument.

1.12	Transfer eluates from the Elution Strip to a new storage plate or storage tubes. • The protocol is designed to leave the Nanobind disks in the Elution Strip. On occasion, the disk may be transferred back to the tip comb storage row (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. • **Note: If the Nanobind is stuck to the side of the elution strip (i.e., not resting in Buffer EB), use a pipette tip to push the Nanobind down into solution and swirl 10x.**
1.13	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.
1.14	Let eluate rest overnight at room temperature to allow DNA to solubilize. • Visible "jellies" should disperse after resting.
1.15	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 room temperature 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 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 1x10^6 GM24385 cells should yield ~4–10 µg.
- DNA extracted from 1x10^6 MCF-7 cells should yield ~12–18 µg.
- 260/280 ratios should consistently be 1.8–2.0.
- 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.
- The mode of extracted cultured cell DNA measured on the Femto Pulse system (Agilent Technologies) is typically 100 kb+.

Sample	Input Amount	260/280	260/230	Nanodrop (ng/µL)	Qubit DNA yield (µg)
GM12878 (diploid)	1 x 10^6 cells	1.84	2.01	101.3	7.32
GM24385 (diploid)	1 x 10^6 cells	1.86	1.98	98.1	8.29
MCF-7 (tetraploid)	1 x 10^6 cells	1.87	2.18	202.9	15.9

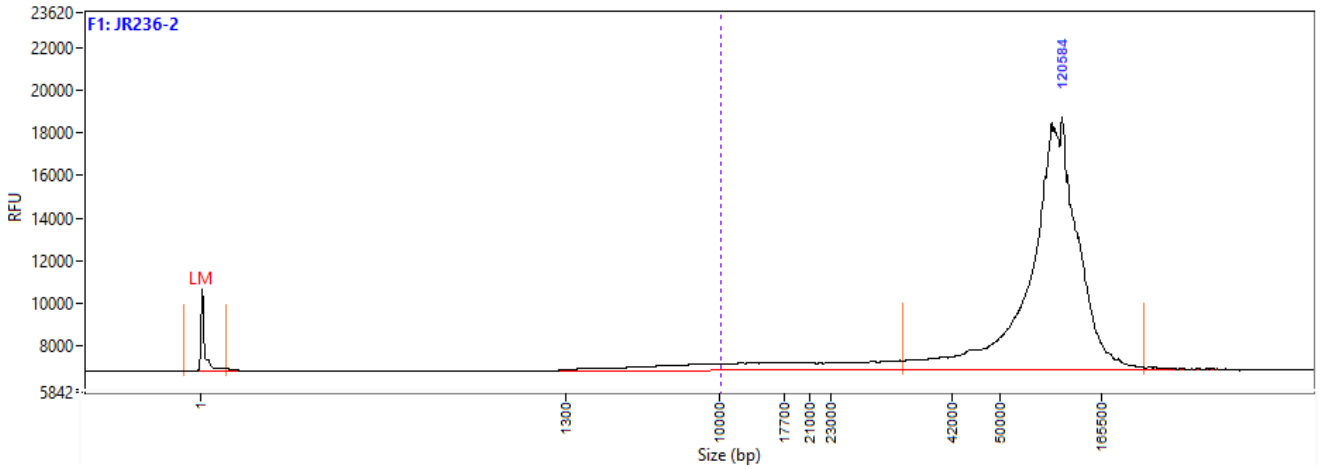

Figure 1. DNA size distribution of unsheared gDNA isolated GM12878 cells using the KingFisher Duo on the Femto Pulse system (Agilent Technologies).

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 RT will then allow the HMW DNA to relax back into solution.
   - High heterogeneity can be caused by insufficient mixing during the cell preparation steps. Ensure the cells have been fully resuspended in PBS before adding to the Lysis/Binding Row.

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 Step #2 for more information.
   - The cell input could be too low. For cultured human cells, we recommend $1x10^6$ – $2x10^6$ cells. For example, $1x10^6$ GM24385 cells should recover ~4-10 µg of DNA.
   - 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.
   - The lysis could be inefficient due to improper resuspension of the cell pellet prior to lysis. Make sure the cell pellet is completely resuspended during the cell preparation steps and 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 if it is within the expected range for that particular sample type. Generally, DNA extracted from cultured mammalian cells results in 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 $1x10^6$ – $2x10^6$ cells. Inputs greater than this can overwhelm the lysis chemistry, resulting in lower recoveries and lower purity.
   - If purities are generally low across all samples, ensure the isopropanol was added as recommended in Step 1.10 (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 protocol. What does this mean?**

- The Nanobind disks occasionally remain on the tip comb after elution and are returned to Row B (Tip Comb storage) at the end of the protocol. If the Nanobind corresponding to the sample in question has been returned to 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 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 Row (Row A). This is a rare occurrence but can happen, especially at high cell inputs (>25 µg yields). We recommend rerunning the protocol at a lower cell input if additional sample remains.
- If the Nanobind disk for the eluate in question is not on the tip comb in Row B and not in the Lysis/Binding Row (Row A), contact PacBio for further instruction.

6. Why isn't the protocol running or giving 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 block are installed. The instrument will give an error message if the correct magnet head for a given protocol is not 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, please contact Thermo Fisher.

Revision history (description)	Version	Date
Initial release	01	April 2023
Updated to correct script part number	02	September 2024