Nanobind CBB Big DNA Kit - Beta

Handbook v1.7 (10/2018)

For extraction of HMW (50 kb – 300+ kb) and UHMW (50 kb – 1+ Mb) genomic DNA from cultured cells, cultured bacteria, and human whole blood

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Kit Specifications

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Nanobind CBB Big DNA Kit Part Number Number of Samples	Beta Version NB-900-001-01 20
Nanobind Disks	20
Proteinase K	0.45 mL
RNase A	0.45 mL
Buffer CLE3	0.45 mL
Buffer BL3	4.5 mL
Buffer CW1 Concentrate – Dilute to 60% final ethanol concentration as indicated on the bottle.	6.5 mL (16.5 mL after EtOH)
Buffer CW2 Concentrate – Dilute to 60% final ethanol concentration as indicated on the bottle.	10 mL (25 mL after EtOH)
Buffer EB	5 mL

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 (18–25 °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 CBB Big DNA Kits are intended for research use only.

Version History

See Nanobind Big DNA Kit Version History Document (www.circulomics.com/support-nanobind) for a list of kit and protocol changes.

User Supplied Equipment and Reagent List

Equipment	Model
Magnetic Tube Rack	Thermo Fisher DynaMag-2 (12321D)
Mini-Tube Rotator	Fisher Scientific Mini-Tube Rotator (05-450-127)
Heat Block (or Water Bath)	Fisher Scientific Isotemp Dry Bath Incubator (11-715-125DQ)
ThermoMixer	Eppendorf (5382000023)
Mini-Centrifuge	Ohaus Mini-Centrifuge (FC5306)
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.

For Pre-Digestion of Gram-Positive Bacteria

- Lysozyme (MP Biomedicals #100831)
- STET Buffer:
    - 8% sucrose
    - 50 mM Tris-HCl, pH 8.0
    - 50 mM EDTA, pH 8.0
    - 5% Triton X-100
    - Add lysozyme immediately before use (10 mg/mL final concentration)

For some bacteria, such as *Staphylococcus aureus*, lysostaphin (Sigma-Aldrich #L7386) should be substituted for lysozyme at 0.15 mg/mL final concentration.

Introduction

Nanobind is a novel magnetic disk covered with a high density of micro- and nanostructured silica (**Figure 1**). The Nanobind disk can be used for rapid extraction of high quality DNA and RNA. It uses a standard lyse, bind, wash, and elute procedure that is common for silica DNA extraction technologies (see **Workflow**). However, unlike magnetic beads and silica spin columns which shear large DNA, Nanobind binds and releases DNA without fragmentation, resulting in high purity, high molecular weight (HMW) and ultra high molecular weight (UHMW) DNA. The high surface area and unique binding mechanism give it an extraordinary binding capacity, allowing isolation of >200 µg of DNA in a 1.5 mL format.

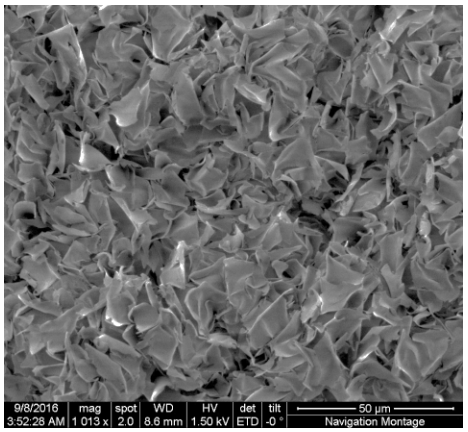


Figure 1. SEM image of Nanobind's silica surface structure.

The Nanobind CBB Big DNA Extraction Kit can be used for the extraction of HMW and UHMW genomic DNA from cultured cells, cultured bacteria (gram negative and gram positive), and human whole blood. Process time is approximately 60 minutes depending on sample type. Protocols are provided for extraction of HMW (50 kb – 300+ kb) and UHMW (50 kb – 1+ Mb) DNA (**Figure 2**).

The extracted DNA has been used on a variety of genomics platforms including PacBio RSII/Sequel, Oxford Nanopore MinION/GridION/PromethION, Bionano Genomics Irys/Saphyr, and 10X Genomics Chromium.

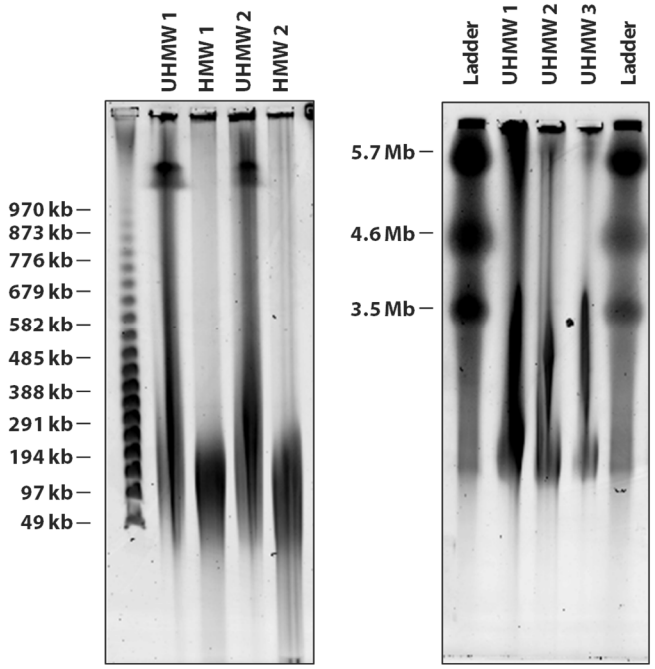


Figure 2. Pulsed Field Gel Electrophoresis (PFGE) comparison of HMW and UHMW DNA extracted using Nanobind. The HMW protocols typically result in DNA from 50 – 300+ kb. The UHMW protocols are capable of obtaining megabase DNA surpassing 5.7 Mb in length.

Sample Information and Expected Yields

Yields of HMW genomic DNA will vary depending on the sample being processed. The following table provides suggested input ranges and expected yields for the validated sample types. Each sample has been validated by long read sequencing.

Nanobind CBB Big DNA Kit – Expected Yields					
Sample	**Suggested Input[1]**	**Example Input**	**Example 260/280**	**Example 260/230**	**Example Yield (µg)**
MCF-7 cells (tetraploid)[2]	0.5x10^6 - 10x10^6 cells	1x10^6 cells	2.0	1.9	14.1
MCF-10A cells (diploid)	0.5x10^6 - 10x10^6 cells	1x10^6 cells	2.0	1.7	6.8
GM12878 cells (diploid)[2]	0.5x10^6 - 10x10^6 cells	5x10^6 cells	1.9	2.1	34.1
Escherichia coli[2]	5x10^8 - 5x10^9 cells	2x10^9 cells	1.8	1.4	22.0
Shigella sonnei[2]	5x10^8 - 5x10^9 cells	1x10^9 cells	1.8	1.4	27.7
Salmonella enterica[2]	5x10^8 - 5x10^9 cells	1x10^9 cells	1.8	1.5	23.4
Listeria monocytogenes[2]	5x10^8 - 5x10^9 cells	1x10^9 cells	1.8	1.9	21.7
Human Whole Blood[2]	200 µL	200 µL	1.9	1.9	5.6

[1]Higher input levels can be used with appropriate optimization of buffer volumes and enzyme levels.
[2]Samples have been validated by long read sequencing.

Nanobind CBB Big DNA Kit – Blood Anticoagulant Validation			
Sample	**260/280**	**260/230**	**DNA Yield (µg)**
Whole Blood – K2 EDTA	1.9	2.0	5.5
Whole Blood – Sodium Citrate	1.9	2.0	5.0
Whole Blood – Heparin	1.9	1.9	5.1
Whole Blood – ACD	1.9	1.8	4.4

Nanobind was used to extract HMW DNA (up to 300 kb) from 200 µL of fresh human whole blood (n=3). All four samples showed similar yields and UV purity. Validation by long read sequencing likewise performed similarly, with EDTA stabilized blood displaying small advantages in read length.

DNA Size

The size of the genomic DNA will vary depending on sample type, the quality of the starting material, and processing parameters. The HMW DNA Extraction Protocols typically yield DNA in the 50 kb – 300+ kb size range (**Figure 3)**. Alternatively, megabase-sized DNA (50 kb – 1+ Mb) can be obtained by following the UHMW DNA Extraction Protocols. For most long-read sequencing applications, superior sequencing yields will be obtained using the HMW DNA Extraction Protocols. UHMW DNA is inherently heterogeneous and highly viscous and can cause problems with many downstream applications. The UHMW DNA Extraction Protocols are only recommended for optical mapping and when trying to obtain ultra-long reads (>300 kb).

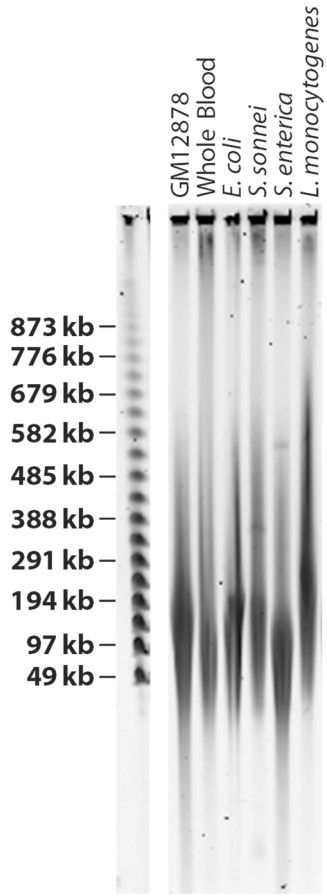


Figure 3. Pulsed Field Gel Electrophoresis (PFGE) comparison of DNA extracted using the HMW DNA Extraction Protocol from various sample types.

PacBio Sequencing

Sequencing validation of HMW DNA extracted from several sample types using the Nanobind CBB Big DNA Kit was performed using the PacBio® Sequel® long read sequencing platform. The following tables show typical sequencing results.

Nanobind CBB Big DNA Kit – PacBio Sequel Sequencing			
Sample	**Subread Length N50 (bp)**	**Max Subread Length (bp)**	**Total Data (Gb)**
GM12878 cells	32,813	95,946	10.4
Escherichia coli	29,611	80,713	6.8
Shigella sonnei	35,251	83,705	9.0
Salmonella enterica	33,420	93,614	10.5
Listeria monocytogenes	32,010	95,382	10.2
Human Whole Blood (200 µL)	28,443	95,679	6.7
Human Whole Blood (1 mL)	26,325	89,360	6.4

HMW DNA (up to 300 kb) was extracted using the Nanobind CBB Big DNA Kit, needle sheared, and used with the PacBio SMRTbell® Express Template Prep Kit to prepare >30 kb libraries. The libraries were then sequenced on the PacBio Sequel® System (10-hour movie, Sequel® Sequencing Kit 2.0, Sequel® Binding Kit 2.1). Data generated in collaboration with PacBio®.

Nanobind CBB Big DNA Kit – PacBio RSII Sequencing Blood Anticoagulant				
Sample	**260/280**	**260/230**	**DNA Yield (µg)**	**Subread Length N50 (bp)**
Whole Blood – K2 EDTA	1.88	2.04	5.5 ± 0.3	26,906
Whole Blood – Sodium Citrate	1.87	1.98	5.0 ± 0.1	25,610
Whole Blood – Heparin	1.87	1.93	5.1 ± 0.1	23,152
Whole Blood – ACD	1.85	1.81	4.4 ± 0.1	20,862

HMW DNA (up to 300 kb) was extracted from 200 µL of fresh human whole blood using the Nanobind CBB Big DNA Kit, needle sheared, and used with the PacBio SMRTbell® Express Template Prep Kit to prepare >30 kb libraries. The libraries were then sequenced on the PacBio RS II® System (6-hour movie). All four samples showed similar yields and UV purities with small differences in read length. Data generated in collaboration with PacBio®.

Oxford Nanopore Sequencing

Sequencing validation of HMW DNA extracted from several sample types using the Nanobind CBB Big DNA Kit was performed using Oxford Nanopore GridION and MinION long read sequencing. The following tables show typical sequencing results. UHMW DNA is recommended primarily for the Rapid Sequencing Kit. Contact us for more information.

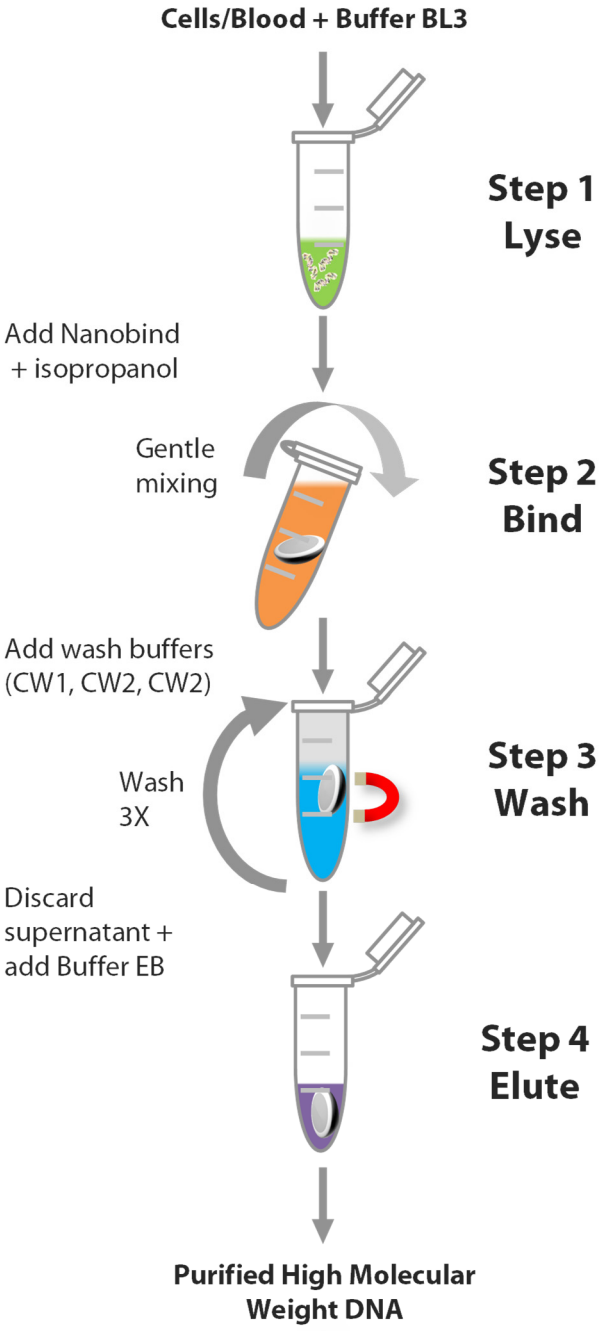
Nanobind CBB Big DNA Kit – ONT MinION/GridION Sequencing				
Sample	**Library Prep**	**Read Length N50 (bp)**	**Longest Read (bp)**	**Total Data (Gb)**
GM12878 Cells	5X needle shear, BluePippin (30 kb)	42,997	215,259	2.9
Escherichia coli	5X needle shear, BluePippin (30 kb)	46,156	191,836	6.8
Listeria monocytogenes	5X needle shear, BluePippin (30 kb)	48,391	254,985	8.0
Human Whole Blood	No shear, BluePippin (30 kb)	43,105	204,757	2.8

Typical sequencing metrics from HMW DNA extracted using the Nanobind CBB Big DNA Kit. The HMW DNA was prepared using the Oxford Nanopore Ligation Sequencing Kit 1D (SQK-LSK108) and sequenced on either Oxford Nanopore MinION or GridION (FLO-MIN106). Data generated in collaboration with Timp Lab at Johns Hopkins University.

Nanobind CBB Big DNA Kit – ONT Ultra Long Read Sequencing						
Sample	**Nanobind Protocol**	**Library Prep**	**Read Length N50 (bp)**	**Longest Read (bp)**	**Reads >100 kb**	**Total Data (Gb)**
GM12878 Cells	Std HMW Protocol	Modified SQK-RAD004	30,893	644,937	2,388	2.9
GM12878 Cells	Heated UHMW Protocol	Modified SQK-RAD004	39,962	661,590	4,004	3.1
GM12878 Cells	Std UHMW Protocol	Modified SQK-RAD004	50,014	765,479	3,079	1.9

Typical sequencing metrics from HMW and UHMW DNA extracted using Nanobind CBB Big DNA Kit. The extracted DNA was prepared with a modified version of the Oxford Nanopore Rapid Sequencing Kit 1D (SQK-RAD004). The samples were then sequenced on Oxford Nanopore GridION (FLO-MIN106). Data generated in collaboration with Winston Timp at Johns Hopkins University.

Workflow


Cells/Blood + Buffer BL3
Step 1
Lyse
Add Nanobind
+ isopropanol
Gentle
mixing
Step 2
Bind
Add wash buffers
(CW1, CW2, CW2)
Wash
3X
Step 3
Wash
Discard
supernatant +
add Buffer EB
Step 4
Elute
Purified High Molecular
Weight DNA

Processing Tips

Magnetic Rack Handling Procedure

To capture the Nanobind disk and enable simple processing, the 1.5 mL tubes are placed in a tube rack that is used with a magnetic base. Although DNA is bound quite robustly, proper pipetting and handling will ensure thorough washing and minimize disturbances of the bound DNA. For best results, the Nanobind disk should be captured near the top of the tube so that fluid can be easily removed from the bottom of the tube. The following procedure (**Figure 4**) is recommended.

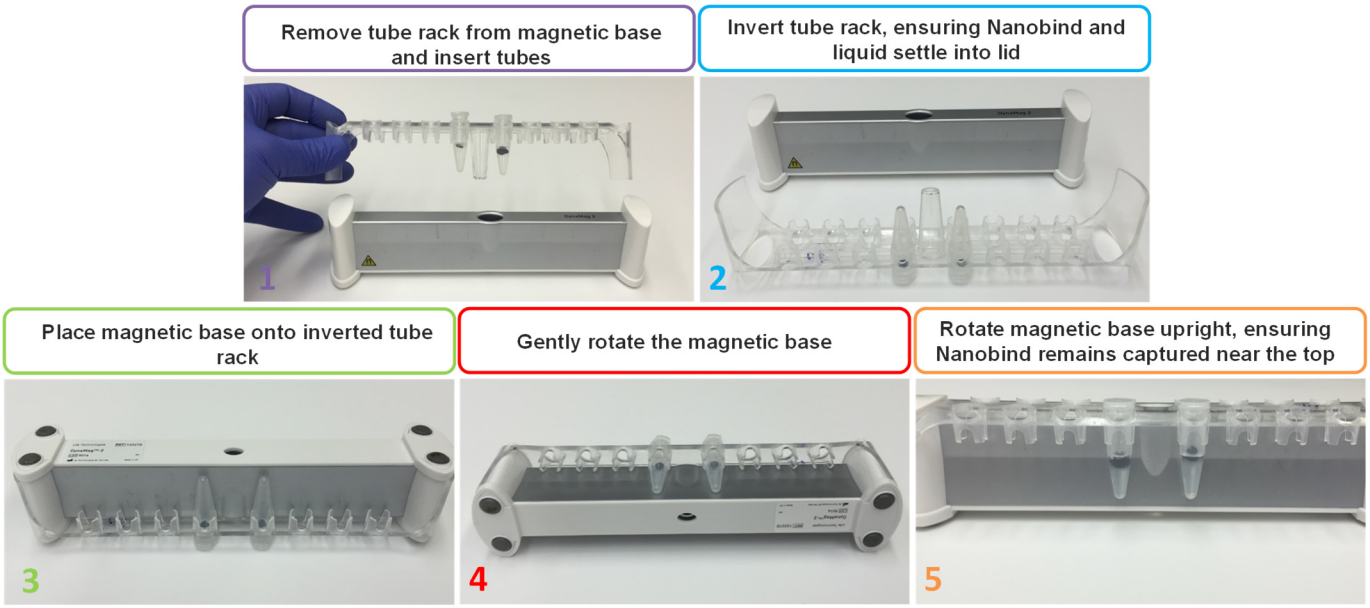


Figure 4. Recommended procedure for capturing Nanobind disk on a tube rack and magnetic base. This procedure ensures that the Nanobind disk is captured near the top of the liquid interface which minimizes the chances for disturbing the bound DNA and facilitates processing.

Pipetting

When removing liquid from the 1.5 mL microcentrifuge tube, the Nanobind disk should not be disturbed. Carefully insert the pipette tip against the wall opposite the Nanobind disk and remove liquid by pipetting from the liquid surface (**Figure 5**). This will minimize the chances of accidentally pipetting bound DNA. Likewise, when adding liquid, dispense against the wall opposite the Nanobind disk.

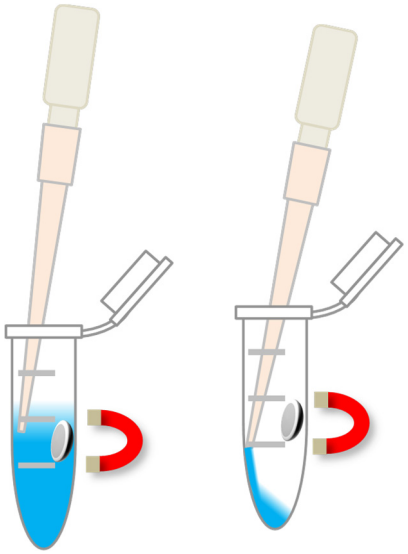


Figure 5. Pipetting procedure for removal (left) and addition (right) of liquid during wash steps. Avoid disrupting the Nanobind disk and bound nucleic acids.

Heterogeneity and Viscosity

The heterogeneity and viscosity of the DNA eluate will vary depending on sample type, DNA size, sample input, and processing parameters. More gentle processing will yield larger DNA size but will also result in higher heterogeneity and larger amounts of highly viscous, unsolubilized "jellies." Processing that is too gentle can dramatically reduce DNA purity and yield. Our standard protocols provide reliable means to obtain HMW (50 kb – 300+ kb) and UHMW (50 kb – 1+ Mb) DNA. The table below illustrates how various processing steps affect the final DNA size and heterogeneity.

	HMW Protocols	UHMW Protocols
Mixing Steps	Thermomixing Vortexing	Pipetting with wide-bore tips Inversion mixing
General Pipetting	Standard tips	Wide-bore tips
Homogenization of DNA Eluate	Pipette mixing Needle shearing Vortexing	Extended resting at RT
Typical DNA Size	50 kb – 300+ kb	50 kb – 1+ Mb
Sample Heterogeneity	Low Heterogeneity <10% after resting	Higher Heterogeneity <30% CV after resting

If the extracted DNA is very heterogeneous and contains large amounts of unsolubilized "jellies," we first recommend:

More aggressive mixing during lysis – The most common reason for high sample heterogeneity and low purity is insufficient mixing during lysis. More aggressive mixing will result in samples with improved purity due to more efficient lysis and digestion. Improved sample purity will lead to improved DNA homogeneity. Aggressive mixing during lysis will not significantly impact DNA length. Mixing may be carefully scaled back by skilled users to obtain UHMW DNA, as necessary.

If the extracted DNA is still heterogeneous after more aggressive processing, we recommend:

Pipette mixing the extracted DNA and RT resting – The extracted DNA can be mixed 1–10X using a standard P200 pipette tip to coax the "jellies" into solution. The pipette mixed DNA should then be left to rest at RT for 1 hour to overnight. Moderate amounts of pipette mixing will not significantly impact DNA length. This is our standard DNA homogenization method. It is appropriate for both HMW and UHMW DNA. We routinely use it for all long-read sequencing and optical mapping applications.

If the extracted DNA needs to be used immediately after extraction, we recommend:

Needle shearing the extracted DNA – The extracted DNA can be sheared 5X using a 26g blunt stainless steel needle and 1 mL syringe. The needle-sheared DNA can be used immediately for library preparation. Moderate amounts of needle shearing will not significantly impact DNA length. This method is appropriate for HMW DNA. We routinely use this method for long-read sequencing applications.

Vortexing the extracted DNA – The extracted DNA can be vortexed for 10–30 s on high. The vortexed DNA should then be left to rest at RT for a few hours until homogenous. This method is appropriate for HMW DNA.

HMW (50 kb – 300+ kb) DNA Extraction Protocols

The following protocols detail extraction of HMW DNA from cultured mammalian cells, bacteria, and whole blood. RNase treatment is described as an option to obtain pure DNA. Recommended QC procedures are also provided.

Cultured Mammalian Cells - HMW

This protocol describes the procedure for HMW DNA extraction from cultured mammalian cells. This protocol has been validated for several cell types including GM12878, MCF-7, and MCF-10A.

1. **To harvest cells, 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.**
    - Standard extractions use $1x10^6$–$5x10^6$ cells, but up to $25x10^6$ cells can be processed if particular care is taken during the wash steps (steps 11–15) and drying steps (steps 17–18)*;* use caution to avoid accidental pipetting of bound DNA, as high cell input will result in large DNA that hangs from the Nanobind disk.
2. **Add 20 µL of 1x PBS and pipette mix to resuspend cells.**
    - Mix until cell pellet is resuspended without visible lumps. Poor resuspension will result in inefficient lysis and digestion which lead to low yield, low purity, and high heterogeneity.
3. **Add 20 µL of Proteinase K.**
4. **Add 20 µL of CLE3 and pulse vortex 10X.**
5. **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.
6. **Optional for removal of RNA: add 20 µL of RNase A, pulse vortex 5X, and incubate at RT (18–25 °C) for 3 min.**
7. **Add 200 µL of Buffer BL3 and pulse vortex 10X.**
    - A white precipitate may form after addition of BL3. This is completely normal and usually disappears during the step 8 incubation.
    - Insufficient mixing in step 4 and step 7 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
8. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 10 min.**
    - Cell inputs greater than $5x10^6$ may require longer incubation times to ensure complete lysis. If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
9. **Add Nanobind disk to cell lysate and add 300 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
10. **Mix on tube rotator at 9 rpm at RT for 10 min.**
11. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
12. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**

Quick Tip

Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

13. **Add 700 µL of Buffer CW1, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
14. **Add 500 µL of Buffer CW2, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Repeat step 14.**
16. **Remove liquid from the tube cap.**
17. **Spin the tube on a mini-centrifuge for 2 s and remove the 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. **Add 75–200 µL Buffer EB and incubate at RT for 10 min.**
20. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.**
21. **Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - A small amount of white or gel-like material may remain on the Nanobind disk after transferring the eluate in step 20. This is HMW DNA that has not fully solubilized. The spin in step 21 will allow the 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.
22. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted HMW DNA can be heterogeneous. This is normal and is one of the challenges of working with HMW DNA. The bigger the DNA, the more this will be apparent. See **Heterogeneity and Viscosity** section for detailed tips.
    - Heterogeneity can result from insufficient vortexing in step 4 and step 7. Use aggressive mixing until familiar with the protocol.
    - If the extracted HMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a standard P200 pipette and then let the sample rest at RT for 1 hour to overnight.
    - Alternatively, the DNA sample can be needle sheared 5X using a 26g needle and used immediately.
    - Vortexing the eluted DNA 10-30 s can also be used with minor effects on DNA size.
    - All of the above suggestions will only have minor to unnoticeable effects on DNA size and sequencing read lengths.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

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 or needle shearing.

Gram-Negative Bacteria - HMW

This protocol describes the procedure for HMW DNA extraction from Gram-negative bacteria. This protocol has been validated for *E. coli, S. sonnei,* and *S. enterica*.

1. **To harvest cells, centrifuge at 16,000 x *g* for 1 min at 4 °C to pellet cells in a 1.5 mL Protein LoBind tube; remove the supernatant.**
    - Standard extractions use $5x10^8$–$5x10^9$ cells without modification of the protocol. Larger cell inputs can be extracted with modifications to the protocol (contact Circulomics for more details).
2. **Add 20 µL of 1x PBS and pipette mix to resuspend cells.**
    - Mix until cell pellet is resuspended without visible lumps. Poor resuspension will result in inefficient lysis and digestion which lead to low yield, low purity, and high heterogeneity.
3. **Add 20 µL of Proteinase K.**
4. **Add 20 µL of CLE3 and pulse vortex 10X.**
5. **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.
6. **Optional for removal of RNA: add 20 µL of RNase A, pulse vortex 5X, and incubate at RT (18–25 °C) for 3 min.**
7. **Add 200 µL of Buffer BL3 and pulse vortex 10X.**
    - A white precipitate may form after addition of BL3. This is completely normal and usually disappears during the step 8 incubation.
    - Insufficient mixing in step 4 and step 7 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
8. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 10 min.**
    - Cell inputs greater than $5x10^9$ may require longer incubation times to ensure complete lysis. If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
9. **Add Nanobind disk to cell lysate and add 300 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
10. **Mix on tube rotator at 9 rpm at RT for 10 min.**
11. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
12. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**
13. **Add 700 µL of Buffer CW1, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
14. **Add 500 µL of Buffer CW2, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Repeat step 14.**

Quick Tip

Increased Proteinase K incubation times may be necessary for some bacteria.

Quick Tip

Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired

16. **Remove liquid from the tube cap.**
17. **Spin the tube on a mini-centrifuge for 2 s and remove the 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. **Add 75–200 µL Buffer EB and incubate at RT for 10 min.**
20. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.**
21. **Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 20. This is HMW DNA that has not fully solubilized. 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.
22. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted HMW DNA can be heterogeneous. This is normal and is one of the challenges of working with HMW DNA. The bigger the DNA, the more this will be apparent. See **Heterogeneity and Viscosity** section for detailed tips.
    - Heterogeneity can result from insufficient vortexing in step 4 and step 7. Use aggressive mixing until familiar with the protocol.
    - If the extracted HMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a standard P200 pipette and then let the sample rest at RT for 1 hour to overnight.
    - Alternatively, the DNA sample can be needle sheared 5X using a 26g needle and used immediately.
    - Vortexing the eluted DNA 10-30 s can also be used with minor effects on DNA size.
    - All of the above suggestions will only have minor to unnoticeable effects on DNA size and sequencing read lengths.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

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 or needle shearing.

Gram-Positive Bacteria - HMW

This protocol describes the procedure for HMW DNA extraction from Gram-positive bacteria, which includes incubation with lysozyme to disrupt their cell walls before DNA isolation. This protocol has been tested with *L. monocytogenes*.

- Before starting, prepare STET buffer as described in **User Supplied Equipment and Reagents**.
- For some gram positive-bacteria, such as *S. aureus*, lysostaphin (0.15 mg/mL final concentration) is recommended in place of lysozyme.
- For difficult to lyse bacteria, enzymatic cocktails or bead beating may be necessary.

1. **To harvest cells, centrifuge at 16,000 x *g* for 1 min at 4 °C to pellet cells in a 1.5 mL Protein LoBind tube; remove the supernatant.**
    - Standard extractions use $5x10^8$–$5x10^9$ cells without modification of the protocol. Larger cell inputs can be extracted with modifications to the protocol (contact Circulomics for more details).
2. **Add 20 µL of 1x PBS and pipette mix to resuspend cells.**
    - Mix until cell pellet is resuspended without visible lumps. Poor resuspension will result in inefficient lysis and digestion which lead to low yield, low purity, and high heterogeneity.
3. **Add 100 µL of STET Buffer + lysozyme and pulse vortex 10X.**
4. **Incubate at 37 °C for 30 min.**
5. **Add 20 µL of Proteinase K and pulse vortex 10X to mix.**
6. **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.
7. **Optional for removal of RNA: add 20 µL of RNase A, pulse vortex 5X to mix and incubate at RT (18–25 °C) for 3 min.**
8. **Add 100 µL of Buffer BL3 and pulse vortex 10X.**
    - Insufficient mixing in step 3, step 5, and step 8 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
9. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 10 min**
    - Cell inputs greater than $5x10^9$ may require longer incubation times to ensure complete lysis. If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
10. **Add Nanobind disk to cell lysate and add 400 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
11. **Mix on tube rotator at 9 rpm at RT for 10 min.**
12. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
13. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**

Quick Tip

Increased lysozyme and Proteinase K incubation time up to 2 hours may be necessary for some bacteria.

Quick Tip

Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

14. **Add 700 µL of Buffer CW1, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Add 500 µL of Buffer CW2, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
16. **Repeat step 15.**
17. **Remove liquid from the tube cap.**
18. **Spin the tube on a mini-centrifuge for 2 s and remove the 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.
19. **Repeat step 18.**
20. **Add 75–200 µL Buffer EB and incubate at RT for 10 min.**
21. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.**
22. **Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 21. This is HMW DNA that has not fully solubilized. The spin in step 22 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
23. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted HMW DNA can be heterogeneous. This is normal and is one of the challenges of working with HMW DNA. The bigger the DNA, the more this will be apparent. See **Heterogeneity and Viscosity** section for detailed tips.
    - Heterogeneity can result from insufficient vortexing in step 3, step 5, and step 8. Use aggressive mixing until familiar with the protocol.
    - If the extracted HMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a standard P200 pipette and then let the sample rest at RT for 1 hour to overnight.
    - Alternatively, the DNA sample can be needle sheared 5X using a 26g needle and used immediately.
    - Vortexing the eluted DNA 10-30 s can also be used with minor effects on DNA size.
    - All of the above suggestions will only have minor to unnoticeable effects on DNA size and sequencing read lengths.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

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 or needle shearing.

Whole Blood - HMW

This protocol describes the procedure for HMW DNA extraction from whole blood. The protocol is optimized for extraction from **200 µL** of whole blood.

- Other volumes can also be used with modification to the protocol. Contact Circulomics for more information.
- 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.
- This protocol has been validated with K2 EDTA, sodium citrate, heparin, and ACD anti-coagulants.

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 sample.**
    - The addition of blood to Proteinase K rather than vice versa increases lysis efficiency and purity through improved mixing.
    - If removal of RNA is not necessary, proceed directly to step 4.
3. **Optional for removal of RNA: add 20 µL of RNase A.**
4. **Pulse vortex 10X. Incubate at RT (18–25 °C) for 3 min.**
5. **Add 200 µL Buffer BL3 and pulse vortex 10X.**
    - 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.
6. **Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.**
    - Keeping the lip of the tube and the cap clean is necessary to minimize contaminant carryover.
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 3X and spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
9. **Add Nanobind disk to lysate and add 350 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
10. **Mix on tube rotator at 9 rpm at RT for 15 min.**
11. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
12. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk. Avoid placing the pipette tip near the bottom of the tube to prevent aspirating the DNA bound to the Nanobind.**
13. **Add 700 µL of Buffer CW1, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**

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. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

Quick Tip

Pipette from the liquid interface rather than the bottom of the tube to avoid pipetting any dangling DNA.

14. **Add 500 µL of Buffer CW2, vigorously inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Repeat step 14.**
16. **Remove liquid from the tube cap.**
17. **Spin the tube on a mini-centrifuge for 2 s 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. **Add 75–200 µL Buffer EB and incubate at RT for 10 min.**
20. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.**
21. **Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 20. This is HMW DNA that has not fully solubilized. 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.
22. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted HMW DNA can be heterogeneous. This is normal and is one of the challenges of working with HMW DNA. The bigger the DNA, the more this will be apparent. See **Heterogeneity and Viscosity** section for detailed tips.
    - Heterogeneity can result from insufficient vortexing in step 4, step 5, and step 8. Use aggressive mixing until familiar with the protocol.
    - If the extracted HMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a standard P200 pipette and then let the sample rest at RT for 1 hour to overnight.
    - Alternatively, the DNA sample can be needle sheared 5X using a 26g needle and used immediately.
    - Vortexing the eluted DNA 10-30 s can also be used with minor effects on DNA size.
    - All of the above suggestions will only have minor to unnoticeable effects on DNA size and sequencing read lengths.

Quick Tip

The DNA should appear free of color after washing. If DNA remains colored, repeat washes in a more aggressive fashion.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

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 or needle shearing.

UHMW (50 kb – 1+ Mb) DNA Extraction Protocols

The following protocols detail extraction UHMW DNA from cultured mammalian cells, gram negative bacteria, and whole blood. RNase treatment is described as an option to obtain DNA only. Recommended QC procedures are also provided.

Cultured Mammalian Cells - UHMW

This protocol describes the procedure for UHMW DNA extraction from cultured mammalian cells. This protocol has been validated for several cell types including GM12878, MCF-7, and MCF-10A.

1. **To harvest cells, 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.**
    - Standard extractions use $1x10^6$–$5x10^6$ cells, but up to $25x10^6$ cells can be processed if particular care is taken during the wash steps (steps 11–15) and drying steps (steps 17–18)*;* use caution to avoid accidental pipetting of bound DNA, as high cell input will result in large DNA that hangs from the Nanobind disk.
2. **Add 20 µL of 1x PBS and pipette mix to resuspend cells.**
    - Mix until cell pellet is resuspended without visible lumps. Poor resuspension will result in inefficient lysis and digestion which lead to low yield, low purity, and high heterogeneity.
3. **Add 20 µL of Proteinase K.**
4. **Add 20 µL of CLE3 and pipette mix 5X with a P200 wide-bore pipette.**
5. **Incubate at RT (18–25 °C) for 15 min.**
6. **Optional for removal of RNA: add 20 µL of RNase A, pipette mix 5X with a P200 wide-bore pipette, and incubate at RT for 3 min.**
7. **Add 200 µL of Buffer BL3 and pipette mix 10X with a P200 wide-bore pipette.**
    - A white precipitate may form after addition of BL3. This is completely normal and usually disappears during the step 8 incubation.
    - Insufficient pipette mixing in step 4 and step 7 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
8. **Incubate at RT for 15 min.**
    - Cell inputs greater than $5x10^6$ may require longer incubation times to ensure complete lysis. If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
9. **Add Nanobind disk to cell lysate and add 300 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
10. **Mix on tube rotator at 9 rpm at RT for 10 min.**
11. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
12. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**

Quick Tip

We recommend that new users follow the protocol as closely as possible until they are familiar with the system, particularly on all mixing steps. Scaling back the mixing steps will result in very large DNA but purity and extraction efficiency could suffer. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

13. **Add 700 µL of Buffer CW1, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
14. **Add 500 µL of Buffer CW2, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Repeat step 14.**
16. **Remove liquid from the tube cap.**
17. **Spin the tube on a mini-centrifuge for 2 s and remove the 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. **Add 75–200 µL Buffer EB and incubate at RT for 20 min.**
    - Elution with smaller volumes will yield higher concentration nucleic acid but may result in UHMW DNA that is difficult to resolubilize.
20. **Collect DNA by transferring eluate with a P200 wide-bore pipette to a new 1.5 mL microcentrifuge tube.**
21. **Some liquid will remain on the Nanobind disk after pipetting. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - Complete elution of UHMW DNA can be more challenging than HMW DNA.
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 20. This is UHMW DNA that has not fully solubilized. 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.
    - A standard P200 pipette can be used to aid in the removal of residual liquid after the majority of the eluate has been removed with a wide-bore pipette.
22. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted UHMW DNA will be even more heterogeneous than HMW DNA. This is normal and one of the tradeoffs of using megabase-sized DNA. See **Heterogeneity and Viscosity** section for detailed tips.
    - If the extracted UHMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a P200 wide-bore pipette and then let the sample rest at RT for 1 hour to overnight. This will only have slight impact on DNA length.
    - Homogeneity will improve with more mixing in step 4 and step 7; however, this will decrease the length of the extracted DNA.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

Quick Tip

The DNA will solubilize after resting at RT or by coaxing it into solution using gentle mixing. We recommend patience if megabase-sized DNA is needed.

Gram-Negative Bacteria - UHMW

This protocol describes the procedure for UHMW DNA extraction from Gram-negative bacteria. This protocol has been validated for *E. coli*.

1. **To harvest cells, centrifuge at 16,000 x *g* for 1 min at 4 °C to pellet cells in a 1.5 mL Protein LoBind tube; remove the supernatant.**
    - Standard extractions use $5x10^8$–$5x10^9$ cells without modification of the protocol. Larger cell inputs can be extracted with modifications to the protocol (contact Circulomics for more details).
2. **Add 20 µL of 1x PBS and pipette mix to resuspend cells.**
    - Mix until cell pellet is resuspended without visible lumps. Poor resuspension will result in inefficient lysis and digestion which lead to low yield, low purity, and high heterogeneity.
3. **Add 20 µL of Proteinase K.**
4. **Add 20 µL of CLE3 and pipette mix 5X with a P200 wide-bore pipette.**
5. **Incubate at RT (18–25 °C) for 15 min.**
6. **Optional for removal of RNA: add 20 µL of RNase A, pipette mix 5X with a P200 wide-bore pipette, and incubate at RT for 3 min.**
7. **Add 200 µL of Buffer BL3 and pipette mix 10X with a P200 wide-bore pipette to mix.**
    - A white precipitate may form after addition of BL3. This is completely normal and usually disappears during the step 8 incubation.
    - Insufficient pipette mixing in step 4 and step 7 will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.
8. **Incubate at RT for 15 min.**
    - Cell inputs greater than $5x10^9$ may require longer incubation times to ensure complete lysis. If cellular aggregates are visible, extend lysis time by 10 min (up to 30 min).
9. **Add Nanobind disk to cell lysate and add 300 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should be added before isopropanol.
10. **Mix on tube rotator at 9 rpm at RT for 10 min.**
11. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
12. **Discard the supernatant with a pipette using the procedure described in Figure 5, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**
13. **Add 700 µL of Buffer CW1, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
14. **Add 500 µL of Buffer CW2, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.**
15. **Repeat step 14.**
16. **Remove liquid from the tube cap.**

Quick Tip

Increased Proteinase K incubation times may be necessary for some bacteria

Quick Tip

We recommend that new users follow the protocol as closely as possible until they are familiar with the system, particularly on all mixing steps. Scaling back the mixing steps will result in very large DNA but purity and extraction efficiency could suffer. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

17. **Spin the tube on a mini-centrifuge for 2 s and remove the 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. **Add 75–200 µL Buffer EB and incubate at RT for 20 min.**
    - Elution with smaller volumes will yield higher concentration nucleic acid but may result in UHMW DNA that is difficult to resolubilize.
20. **Collect DNA by transferring eluate with a P200 wide-bore pipette to a new 1.5 mL microcentrifuge tube.**
21. **Some liquid will remain on the Nanobind disk after pipetting. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - Complete elution of UHMW DNA can be more challenging than HMW DNA.
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 20. This is UHMW DNA that has not fully solubilized. 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.
    - A standard P200 pipette can be used to aid in the removal of residual liquid after the majority of the eluate has been removed with a wide-bore pipette.
22. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted UHMW DNA will be even more heterogeneous than HMW DNA. This is normal and one of the tradeoffs of using megabase-sized DNA. See **Heterogeneity and Viscosity** section for detailed tips.
    - If the extracted UHMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a P200 wide-bore pipette and then let the sample rest at RT for 1 hour to overnight. This will only have slight impact on DNA length.
    - Homogeneity will improve with more mixing in step 4 and step 7; however, this will decrease the length of the extracted DNA.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 21 will remove the remaining DNA quickly without diluting the eluate.

Quick Tip

The DNA will solubilize after resting at RT or by coaxing it into solution using gentle mixing. We recommend patience if megabase-sized DNA is needed.

Whole Blood - UHMW

This protocol describes the procedure for UHMW DNA extraction from whole blood. The protocol is optimized for extraction from **200 µL** of whole blood.

- Other volumes can also be used with modification to the protocol. Contact Circulomics for more information.
- 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.

1. **Dispense 50 µL of Proteinase K into the bottom of a 1.5 mL Protein LoBind tube.**
2. **Add 200 µL of whole blood sample.**
    - The addition of blood to Proteinase K rather than vice versa increases lysis efficiency and purity through improved mixing.
    - If removal of RNA is not necessary, proceed directly to step 4.
3. **Optional for removal of RNA: add 20 µL of RNase A.**
4. **Inversion mix 5X. Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap. Incubate at RT (18–25 °C) for 3 min.**
5. **Add 200 µL Buffer BL3 to sample. Pipette mix 15X with a P200 wide-bore pipette.**
    - 1X pipette mix is defined by one full aspiration through the pipette tip (*i.e.* 1 stroke up and 1 stroke down).
    - 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.
6. **Mix on tube rotator at 9 rpm at RT for 15 min.**
7. **Spin tube on a mini-centrifuge for 2 s to remove liquid from the tube cap.**
    - Keeping the lip of the tube and the cap clean is necessary to minimize contaminant carryover.
8. **Add Nanobind disk to lysate and add 350 µL of isopropanol. Inversion mix 5X.**
    - For best results, the Nanobind disk should always be added before isopropanol.
9. **Mix on tube rotator at 9 rpm at RT for 15 min.**
    - Do not exceed 15 minutes. Longer binding times will result in DNA that is more difficult to elute and has lower purities.
10. **Place tubes on the magnetic tube rack using the procedure described in Figure 4.**
11. **Carefully discard 700 µL of the lysate with a pipette using the procedure described in Figure 5 to avoid disrupting the Nanobind. Avoid placing the pipette tip near the bottom of the tube to prevent aspirating the DNA bound to the Nanobind.**
    - Leave approximately 100 µL of lysate in the bottom of the tube to ensure that the DNA bound to the Nanobind is not accidentally discarded with the lysate.
12. **Add 700 µL of Buffer CW1, inversion mix 4X, and replace tube rack on the magnetic base. Discard the supernatant, avoiding the bottom of the tube to prevent aspirating the DNA bound to the Nanobind.**

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

We recommend that new users follow the protocol as closely as possible until they are familiar with the system, particularly on all mixing steps. Scaling back the mixing steps will result in very large DNA but purity and extraction efficiency could suffer. Once users are familiar with the system, they can carefully reduce mixing to increase DNA size if desired.

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.

13. **Add 500 µL of Buffer CW2 and inversion mix 4X. With tubes inverted, vigorously shake the samples for 10 s to ensure thorough mixing. Replace tube rack on the magnetic base and discard the supernatant.**
    - Vigorous shaking of the tubes at this step does not damage the DNA and is necessary for proper washing. Inadequate washing will decrease DNA purity and will yield inhomogeneous DNA.
14. **Repeat step 13.**
15. **Remove liquid from the tube cap.**
16. **Spin the tube on a mini-centrifuge for 2 s 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.
17. **Repeat step 16, ensuring side walls are clear of any residual wash buffer.**
18. **Add 75–200 µL Buffer EB. Spin tube on a mini-centrifuge for 2 s and incubate at RT for 20 min.**
19. **Collect DNA by transferring eluate with a P200 wide-bore pipette to a new 1.5 mL microcentrifuge tube.**
20. **Some liquid will remain on the Nanobind disk after pipetting. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s and transfer any additional liquid that comes off of the disk to the previous eluate. Repeat if necessary.**
    - Complete elution of UHMW DNA can be more challenging than HMW DNA.
    - A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in step 19. This is UHMW DNA that has not fully solubilized. 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.
    - A standard P200 pipette can be used to aid in the removal of residual liquid after the majority of the eluate has been removed with a wide-bore pipette.
21. **Analyze the recovery and purity of the DNA by NanoDrop and Qubit as described in QC Procedure.**
    - The extracted UHMW DNA will be even more heterogeneous than HMW DNA. This is normal and one of the tradeoffs of using megabase-sized DNA. See **Heterogeneity and Viscosity** section for detailed tips.
    - If the extracted UHMW DNA has large amounts of unsolubilized “jellies”, pipette mix 1-10X with a P200 wide=- bore pipette and then let the sample rest at RT for 1 hour to overnight. This will only have slight impact on DNA length.
    - Homogeneity will improve with more mixing in step 5; however, this will decrease the length of the extracted DNA.

Quick Tip

We do not recommend a 2nd elution or heated elution. The spin in step 20 will remove the remaining DNA quickly without diluting the eluate.

Quick Tip

The DNA will solubilize after resting at RT or by coaxing it into solution using gentle mixing. We recommend patience if megabase-sized DNA is needed.

QC Procedures

It is recommended that QC is performed after samples have been allowed to rest and appear homogenous under visual examination and when pipetting.

1. **Use NanoDrop UV/VIS 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," see **Troubleshooting Guide** and **Heterogeneity and Viscosity** section.
    - For cultured mammalian cells, we routinely see A260/A280 in the range of 1.8–2.0 and A260/A230 in the range of 1.9–2.2
    - For bacteria, we routinely see A260/A280 in the range of 1.8–2.0 and A260/A230 in the range of 1.2–1.8
    - For whole blood, we routinely see A260/A280 in the range of 1.8–2.0 and A260/A230 in the range of 1.8–2.0
    - Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process. See **Troubleshooting Guide**.
2. **Use Qubit to determine DNA concentration.**
    - We recommend making multiple measurements to ensure an accurate DNA concentration reading.
    - We use the Qubit 3.0 (Thermo Fisher Scientific) with either the dsDNA BR Assay or HS Assay kits.
3. **Run pulsed field gel electrophoresis to determine size.**
    - The size of the extracted genomic DNA can be determined using pulsed field gel electrophoresis (PFGE) (**Figure 6**). We recommend loading 200 ng of DNA per well. For analysis of 50 kb –1000 kb DNA, we recommend the following PFGE conditions:
        - Instrument: Bio-Rad CHEF-DR III Variable Angle System or CHEF Mapper XA System
        - Agarose: 1.0% Certified Megabase Agarose (Bio-Rad #1613109)
        - 30-well comb (Bio-Rad #1703628)
        - Buffer: 0.5X TBE
        - Lambda DNA Ladder: Bio-Rad #1703635, Lonza #50401, or NEB #N0341S
        - 6X gel loading dye (NEB #B7021S)
        - Temperature: 14 °C
        - Initial Switch Time: 35 seconds
        - Final Switch Time: 90 seconds
        - Run Time: 22 hours
        - Angle: 120°
        - Voltage Gradient: 5.5 V/cm

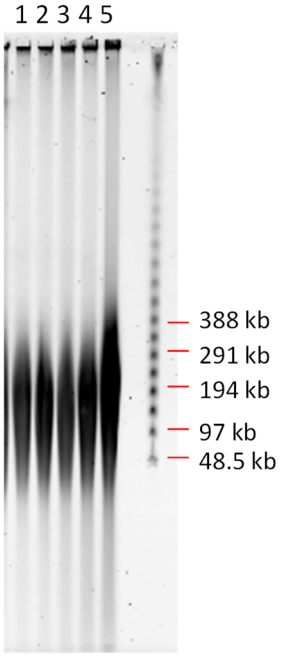


Figure 6. PFGE of replicate extractions of 2x10^6 MCF7 cells on a Nanobind disk along with a lambda ladder (Lonza).

Storage of DNA

DNA can be stored in Buffer EB at 4 °C for up to a month. Avoid freeze/thaw cycles since this can degrade high molecular weight DNA.

Troubleshooting Guide

1	**Extracted DNA is heterogeneous and/or contains visible insoluble "jellies"**		
	a)	Sample contains HMW and UHMW DNA	HMW and UHMW DNA is inherently difficult to work with. The bigger it is, the more heterogeneous it tends to be. Homogeneity can be improved by overnight incubation at RT or 4 °C without affecting DNA length. The sample can also be pipette sheared or needle sheared to improve homogeneity in a more time efficient manner. However, this will result in minor reductions of DNA length. See **Heterogeneity and Viscosity** for details. Minor amounts of DNA "jellies" can often be left in the sample without affecting downstream analysis.
	b)	Poor mixing during lysis and digestion	Increase the amount of vortexing and pipette mixing during the lysis and digestion steps prior to binding. This will not affect DNA length much but will improve sample homogeneity by improving digestion and lysis efficiency.
	c)	Poor sampling of DNA	HMW DNA will be less homogeneous and more viscous than low molecular weight DNA, which can lead to measurement errors that appear as large %CV in NanoDrop or Qubit measurements. In addition, measurement and sampling errors can occur by pipetting an incorrect volume of sample or by pipetting a concentrated glob of DNA gel. For highly viscous samples, the tip of the pipette can be gently scraped along the bottom of the tube to aid in sample pipetting. We recommend allowing the sample to rest before QC and taking at least three measurements, sampling from the top, middle, and bottom of the eluate, to get an accurate concentration reading.
	d)	High sample input and/or small elution volume	HMW DNA that is high concentration can be particularly viscous and challenging to handle. To improve homogeneity, a larger elution volume can be used. Alternatively, a smaller input can be used.
	e)	Insufficient elution (time, temperature, volume)	Ensure that the correct temperature and time are used so that DNA completely elutes from the Nanobind disk. If large cell inputs are used, elution volumes greater than 200 µL may be necessary. Increasing the time can also help to improve elution of bound DNA. Elution at temperatures higher than room temperature is not recommended since it can degrade HMW DNA.
	f)	Binding time exceeded 10–15 min	Binding time should not exceed 10–15 min as this can lead to DNA that is inhomogeneous and difficult to solubilize.
2	**DNA remains on Nanobind after elution or is difficult to remove**		
	a)	Sample contains HMW and UHMW DNA	Solubilizing HMW and UHMW DNA can be inherently challenging. In some cases, DNA in the form of a white or gel-like material may remain on the Nanobind disk after elution. It is important to remove the DNA to ensure a high yield of HMW DNA. The DNA can be eluted by spinning the tube with a mini-centrifuge for 5–10 s. This spin can be repeated multiple times to ensure full elution. We do not recommend 2nd elutions or heated elution as these methods dilute the eluate and can damage DNA.
	b)	Extracted DNA is difficult to pipette	HMW DNA is inherently very viscous and difficult to pipette. When pipetting the highly viscous sample, the tip of the pipette can be gently scraped along the bottom of the tube to aide in sample pipetting. Wide-bore pipette tips can also be used.
	c)	Binding time exceeded 10–15 min	Binding time should not exceed 10–15 min as this can lead to DNA that is inhomogeneous and difficult to elute.

d)	High sample input and/or small elution volume	High sample input and high DNA concentration can result in DNA that is difficult to completely solubilize. For large cell inputs (i.e. >5x10^6 mammalian cells or >5x10^9 *E. coli* cells), a larger elution volume can be used to improve elution of the DNA. Consider smaller cell inputs if the problem persists.
e)	Wrong volume or concentration of isopropanol used during binding	Ensure that the correct volume of 100% isopropanol was added after inserting the Nanobind into the cell lysate. Addition of too much isopropanol can lead to DNA that is difficult to elute from the Nanobind.
f)	Nanobind disk was overdried	Over-drying the Nanobind disk after the wash steps will lead to DNA that is difficult to elute from the Nanobind. Do not allow bound DNA to over-dry after wash steps. Proceed to elution immediately after washing.
g)	Buffers CW1 or CW2 prepared incorrectly	Ensure that the proper amount of 100% ethanol was added to the buffers (page 2).

3 Low yield

a)	Poor mixing during lysis and digestion	Increase the amount of vortexing and pipette mixing during the lysis and digestion steps prior to binding. This will improve yield by improving digestion and lysis efficiency.
b)	DNA remains on Nanobind after elution	For maximum recovery of HMW DNA, any DNA remaining on the Nanobind after elution should be removed by spinning on a mini-centrifuge. This step can be repeated as necessary until all DNA is removed. Heat elution can be used as a last resort.
c)	Insufficient lysis time or enzymatic treatment	For larger cell inputs (>5x10^6 mammalian cells or >5x10^9 *E. coli* cells), increased lysis time may be necessary to ensure complete lysis. Increase lysis time by 10 min increments until complete lysis is observed. Some bacteria and cell types may require more aggressive lysis protocols and/or additional enzymes.
d)	DNA lost during removal of binding and wash buffers	DNA can be lost during the binding and wash steps by accidental pipetting of the bound DNA. Use the technique illustrated in **Figure 4** to capture the Nanobind disk near the top of the tube. Pipette near the top of the liquid interface away from the disk to prevent accidental removal of DNA.
e)	Extracted DNA sample is inhomogeneous	It is possible that the yield is fine but sample heterogeneity is skewing concentration measurements. For accurate quantification of HMW DNA using NanoDrop or Qubit, take multiple measurements per tube, sampling from the top, middle, and bottom of the liquid. A portion of the sample can also be sheared if necessary.
f)	DNA does not bind to Nanobind	Ensure that the correct volume of Buffer BL3 was added prior to adding Nanobind disk to lysate.
g)	Buffers CW1 and CW2 prepared incorrectly	Ensure that the correct volumes of 100% ethanol were added to wash Buffers CW1 and CW2.

4 Yield too high

a)	Extracted DNA sample is inhomogeneous	For accurate quantification of HMW DNA using NanoDrop or Qubit, take multiple measurements per tube, sampling from the top, middle, and bottom of the liquid.
b)	RNA contamination	Use the optional RNase A addition step to minimize RNA contamination.

5 A_{260}/A_{280} or A_{260}/A_{230} signals are low

a)	Insufficient mixing during lysis	Mix more aggressively during lysis steps. This is the primary reason for low purity. Poor mixing will result in very large DNA

		with accompanying high heterogeneity and low purity due to poor digestion efficiency and poor removal of contaminants.
b)	Insufficient mixing during washes	When performing the washes, it is important not only to completely wash the Nanobind disk but also to completely wash all surfaces of the 1.5 mL tubes. Inversion mixing 5 times is typically sufficient, but in some cases more mixing may be necessary. No noticeable decrease in DNA length has been observed from gentle inversion mixing.
c)	Insufficient number of washes	For some samples, an additional CW1 wash or CW2 wash may be necessary to remove all contaminants. In some cases, transferring the Nanobind disk to a clean 1.5 mL tube after the CW2 wash may be necessary.
d)	Buffers CW1 and CW2 prepared incorrectly	Ensure that the proper amount of 100% ethanol was added to Buffer CW1 and CW2.
e)	Buffers CW1 and CW2 used in wrong order	Ensure that Buffer CW1 is used first, followed by CW2. Buffer CW1 contains a chaotropic salt that can cause low A_{260}/A_{230} signals. Buffer CW2 is used to ensure that all salts are completely washed away.

6 Large DNA "bundle" interferes with washing

a)	Large number of cells used	With a large input of cells (>$5x10^6$ mammalian cells or >$5x10^9$ E. coli cells), a large DNA bundle can form that hangs from the Nanobind disk. Care should be taken to avoid accidental pipetting of the bound DNA. In some cases, it is not possible to completely remove all of the fluid during one or more of the wash steps without disrupting the bound DNA. This should not dramatically impact the extraction results.
b)	Nanobind not captured near top of tube when placed on magnet	Capturing the Nanobind disk near the top of the 1.5 mL tube when placing the tube rack on the magnetic base will help minimize accidental pipetting of the bound DNA. Follow the procedure outlined in **Figure 4** or tilt the magnetic rack during pipetting.

7 Nanobind not held in place by magnet

a)	Large number of cells used	With a large input of cells (>5 x 10^6), a large DNA bundle can form that hangs from the Nanobind disk. The extra weight of the bound DNA can cause the Nanobind disk to slide towards the bottom of the tube. Care should be taken to capture the disk at the top of the tube to minimize this effect and to remove supernatant slowly during the wash steps.
b)	Buffers CW1 and CW2 prepared incorrectly	Ensure that the proper amount of 100% ethanol was added to Buffers CW1 and CW2.