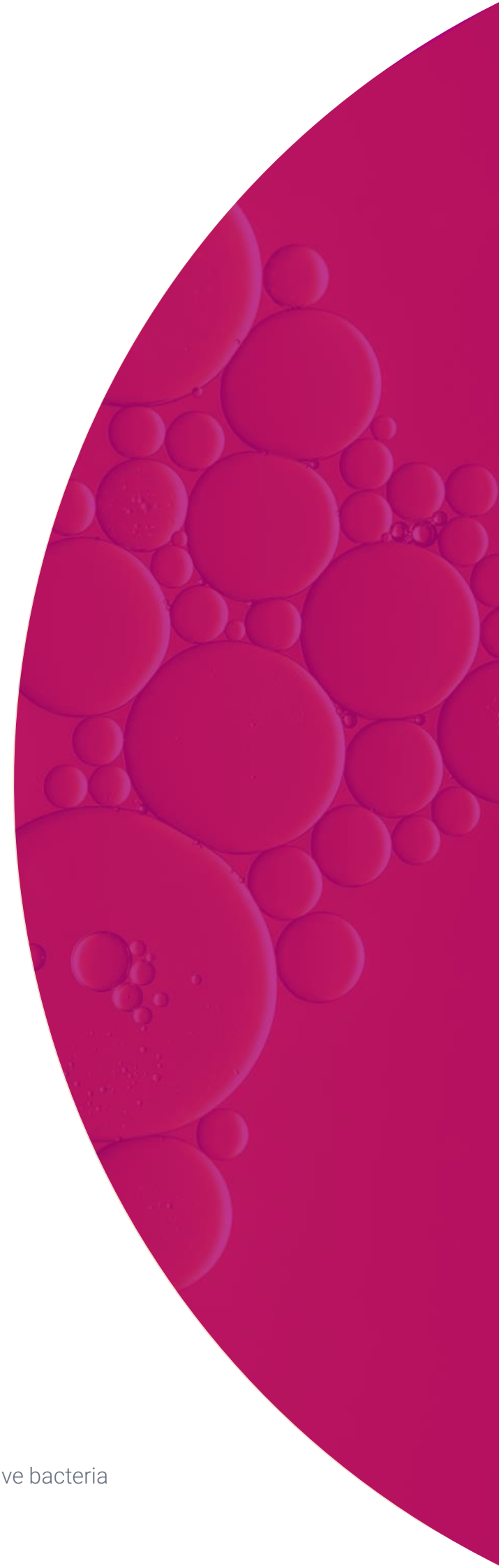
PacBio

Extracting HMW DNA from Gram-positive bacteria using Nanobind® kits

Procedure & checklist



For HMW (50–300+ kb) DNA extraction from Gram-positive bacteria for standard long-read sequencing applications.

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind CBB kit or Nanobind tissue kit	PacBio® (102-301-900 or 102-302-100)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
Mini-tube rotator	Fisher Scientific Mini-Tube Rotator (05-450-127)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
ThermoMixer	Eppendorf (5382000023)
1.5 mL Protein LoBind micro-centrifuge 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
Tris-HCl, 1 M, pH 8.0	Invitrogen (15568025)
Ethylenediaminetetraacetic Acid (EDTA), 0.5 M, pH 8.0	ThermoFisher (15575020)
Sucrose	Fisher Scientific (BP220)
Triton X-100	Sigma-Aldrich (X100)
Lysozyme	MP Biomedicals (100831)
Lysostaphin	Sigma-Aldrich (L7386)
26G blunt end needle	SAI Infusion (B26150)
1 mL syringe	Fisher Scientific (14-823-30)

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

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 and Buffer CT (Nanobind tissue kit, 102-302-100, only) should be stored at 4°C upon arrival. Nanobind disks and all other buffers should be stored at room temperature (15–30°C).

Safety precautions

Buffer BL3 and Buffer CW1 contain guanidine hydrochloride. Warning! Guanidine hydrochloride is harmful if swallowed or inhaled and causes skin and eye irritation. DO NOT mix with bleach or acidic solutions.

Product use

Nanobind CBB kits and Nanobind tissue kits are intended for research use only.

Buffer preparation

Prepare the following buffer prior to beginning DNA extraction.

STET buffer

Reagent	Final concentration
Tris-HCl	50 mM
EDTA	50 mM
Sucrose	8% (m/v)
Triton X-100	5% (v/v)
Lysozyme[1,2,3]	10 mg/mL

[1]Lysozyme should be added immediately before use.

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

[3]For difficult to lyse bacteria, enzymatic cocktails may be necessary.

HMW (50–300+ Mb) DNA extraction protocol

This protocol describes the extraction of HMW DNA from Gram-positive bacteria. It is recommended for HiFi sequencing applications.

Cell input requirements

Amount: $5x10^8$–$5x10^9$ Gram-positive bacteria.

- Input should be titrated to yield 2.5–25 µg of DNA per extraction.
- For *L. monocytogenes*, this was 1 mL of 1 OD600 culture (5 µL of an overnight culture was used to inoculate a 50 mL culture and grown to 1 OD, taking approximately 5 hours).
- Overloading the input will result in reduced purity and flocculates during lysis.
- Larger cell inputs can be extracted with modifications to the protocol.
- Underloading will result in reduced recovery efficiency.
- This protocol has been validated for *L. monocytogenes*.
- No noticeable difference is seen between fresh and frozen cells.

HMW DNA extraction – Gram-positive bacteria

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 used with modifications to the protocol.
2. Add 10 µL of 1x PBS and pipette mix 10X with a standard P200 pipette 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.
    - Aggressive missing at step **2** will not impact the size of the extracted DNA.
3. Add 50 µL of STET Buffer + lysozyme and pulse vortex for 1s x 10 times (max setting).
4. Incubate at 37°C for 30 min.
5. Add 20 µL of Proteinase K.
6. Add 20 µL of Buffer CLE3 and pulse vortex for 1s x 10 times (max setting).
7. Incubate on a ThermoMixer at 55°C and 2000 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. Add 20 µL of RNase A, pulse vortex for 1s x 5 times (max setting), and incubate at RT (15–30°C) for 3 min.
9. Add 100 µL of Buffer BL3 and pulse vortex for 1s x 10 times (max setting).

Quick tip

Overloading the chemistry with too much bacteria will result in low purity. If purity is low, reduce bacterial input.

Quick tip

Complete resuspension of the cell pellet is critical for ensuring efficient lysis.

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.

- Insufficient mixing in step **2**, step **3**, step **6**, and step **9** will result in very large DNA but also low purity, low yield, high heterogeneity, and difficult elution.

10. Incubate on a ThermoMixer at 70°C and 2000 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).
11. Add Nanobind disk to cell lysate and add 220 µL of isopropanol. Inversion mix 5X.
    - The Nanobind disk must be added before isopropanol.
12. Mix on tube rotator at 9 rpm at RT for 10 min.
13. Place tubes on the magnetic tube rack.
    - Use the method described in the **kit Guide & overview magnetic "Rack handling procedure"** section.
14. 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.
15. 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.
16. 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.
17. Repeat step **16**.
18. Remove liquid from the tube cap.
19. 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.
20. Repeat step **19**.
21. Add 75–200 µL of Buffer EB and incubate at RT for 10 min.
22. Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube with a standard P200 pipette. Repeat until all eluate is transferred.
23. Spin the tube containing the Nanobind disk on a micro-centrifuge at 10,000 x *g* for 15 s and combine any additional liquid that comes off the disk with the previous eluate. Repeat if visible DNA remains on the disk.
    - A small amount of liquid or gel like material may remain on the Nanobind disk after transferring the eluate in step **22**. **This clear gel is DNA!** The spin in step **23** 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.
24. 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.

Vortexing is your friend

Quick tip

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

Quick tip

This 15 s spin is **CRITICAL** for recovering the DNA. We do not recommend a 2nd elution.

- Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.

25. Let sample rest at RT for overnight to allow DNA to solubilize.
    - Visible “jellies” should disperse after resting.
26. Following overnight rest, pipette mix 10X with a standard P200 pipette and analyze the recovery and purity as described in QC Procedure.
    - If the concentration %CV exceeds 30% or if perceptible “jellies” remain, pipette mix 10X with a standard P200 pipette or needle shear 5X with a 26g needle 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 **3**, step **6**, and step **9**.

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.

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.
2. 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.
3. 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.
4. 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.

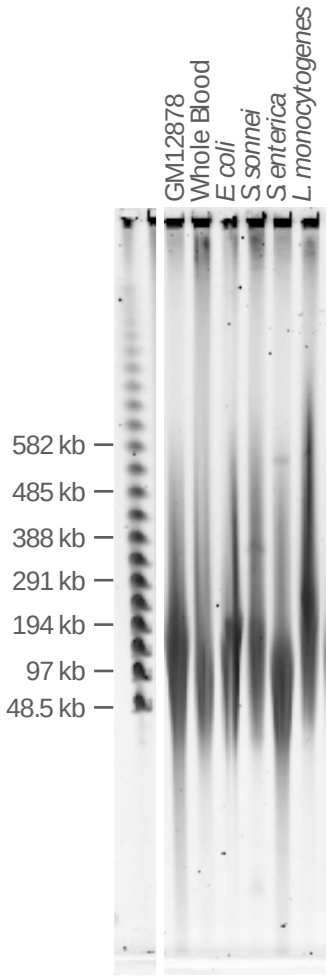
Storage of DNA

DNA can be stored in Buffer 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.

DNA extraction

- DNA was extracted from the stated volumes of 1 OD600 *L. monocytogenes* culture.
- 260/280 ratios should consistently be in 1.8 range.
- 260/230 ratio can vary from 1.2–1.8.
- 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)
Listeria monocytogenes	1 mL	1.8	1.9	246	249	248	248	21.7



22 hour Pulsed Field Gel Electrophoresis (PFGE) image of HMW DNA extracted from Gram-positive bacteria compared to other sample types

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 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 Gram-positive bacteria, we recommend $5x10^8$–$5x10^9$ cells. For example, we use 1 mL of 1 OD600 culture of *L. monocytogenes* and recover >20 µg of DNA.
   - The lysis could be inefficient due to improper resuspension of the bacterial 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.
   - Some bacteria are difficult lyse and could require additional reagents, other enzymes, or even mechanical lysis. Contact PacBio for questions about specific bacteria.

4. **Why are the purities lower than expected? Is this 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 bacteria give purities of 260/230 = 1.2–1.8 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 $5x10^8$–$5x10^9$ 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	July 2022
Protocol changes and minor updates throughout	02	December 2022

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