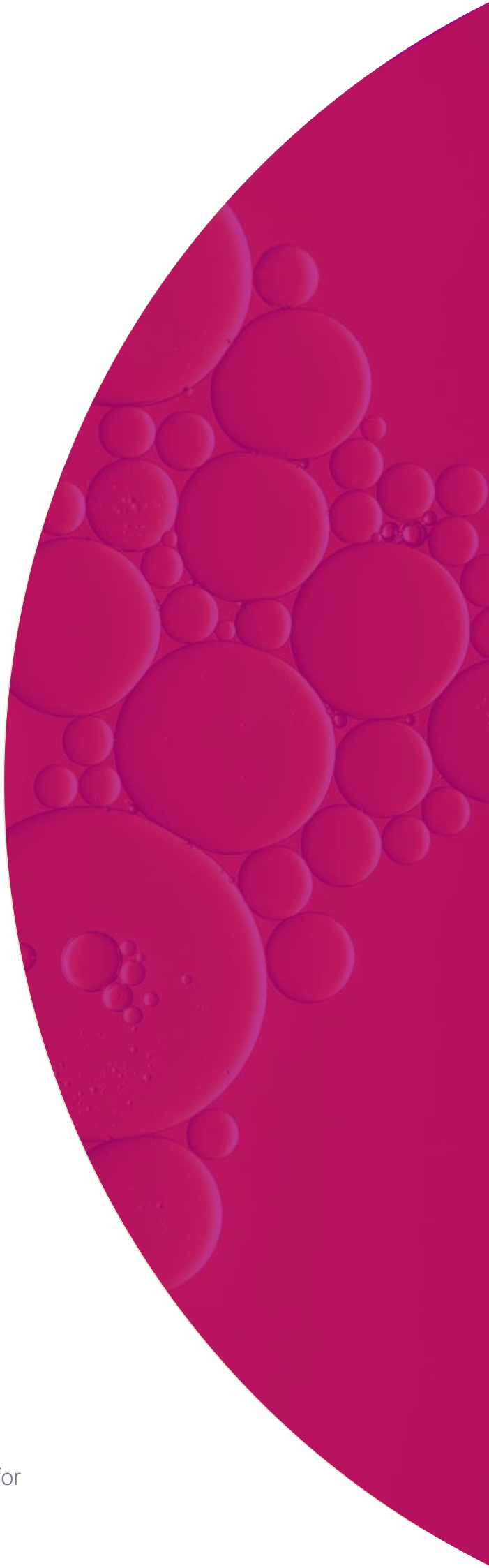
PacBio

Extracting HMW DNA from plant nuclei using Nanobind® kits

Procedure & checklist



For HMW (50–300+ kb) DNA extraction from plant nuclei for standard long-read sequencing applications

Equipment and reagent list

Equipment/reagent	Manufacturer (part number)
Nanobind plant nuclei big DNA kit	PacBio® (102-302-000)
HulaMixer	Thermo Fisher (15920D)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
Mini-centrifuge	Ohaus (FC5306)
Micro-centrifuge	Eppendorf (5404000413)
ThermoMixer	Eppendorf (5382000023)
1.5 mL protein LoBind microcentrifuge tubes	Eppendorf (022431081)
Isopropanol (100%)	
Ethanol (96–100%)	
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay kits
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 PW1 is supplied as a concentrate. This kit uses PW1 with a 70% final ethanol concentration. Before using, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

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).

Product use

Nanobind plant nuclei kits are intended for research use only.

HMW (50–300+ kb) DNA extraction protocol

This protocol describes the extraction of HMW DNA from plant nuclei. It is recommended for HiFi sequencing.

Input requirements

Amount: Nuclei pellet from 1 g of plant tissue. Input will vary by tissue type.

- Prepare a plant nuclei pellet with sufficient material for 5–20 µg of isolated DNA. This typically requires 1–10 g of plant tissue.
- Samples can be pooled or diluted to yield the appropriate amount of DNA for HiFi library preparation.
- The size of the nuclei pellet will vary based on the plant species, nuclei isolation method, and amount of starting material. Typical pellets range from 10–100 µL in volume. Smaller pellet volumes are acceptable but will likely result in lower DNA yields; larger pellet volumes may require additional Proteinase K and Buffer PL1.
- This protocol has been validated for a variety of plant species, including giant sequoia, lavender, holly, baby's breath, and rice.
- Young leaf material typically results in the highest quality DNA and the highest ratio of DNA to input tissue mass.

HMW DNA extraction – plant nuclei

1. Add 30 µL of Proteinase K to a 1.5 mL Protein LoBind tube containing plant nuclei from 1 g of tissue and vortex at maximum speed until the nuclei pellet is fully resuspended. Spin tube on a mini-centrifuge for 2 s to remove liquid from the cap.
   - Vortexing at this step is critical for high quality DNA and will not damage high molecular weight DNA.
   - If pellet is not thoroughly resuspended by vortexing, mixing with a P200 pipette will further homogenize the pellet without damaging high molecular weight DNA.
   - The input amount can be scaled from 0.5–5 g as necessary depending on plant species, age, and nuclei isolation efficiency.
2. Add 10 µL of RNase A and pulse vortex 1 s x 5 times (max speed). Spin tube on a mini-centrifuge for 2 s to remove liquid from the cap. Incubate at RT (15–30°C) for 3 min.
3. Add 80 µL of Buffer PL1 and pulse vortex 1 s x 10 times (max speed). Spin on a mini-centrifuge for 2 s to remove liquid from the cap.
   - Thorough mixing at this step is necessary to ensure complete lysis of the nuclei. Sample should appear cloudy and viscous but homogeneous.
   - If sample appears inhomogeneous, vortex continuously at the maximum speed for 10 s or until sample appears homogeneous. Mixing at this stage will only have minor effects on DNA length but is critical for yield and purity.
4. Incubate on a ThermoMixer at 55°C and 900 rpm for 30 min.
   - Pulse vortexing 1 s x 5 times after 15 min (mid-lysis) can be performed to enhance lysis efficiency. Pulse vortexing at this stage will still result in very large DNA.
   - If a ThermoMixer is not available, a heat block or water bath can be used instead. Inversion mix 5X every 5 min.
   - Lysis incubation time can be increased up to 2 h.

Quick tip

The nuclei suspension must be mixed with Proteinase K until it appears completely homogeneous. Insufficient mixing will result in low purity and poor yield. This typically takes 10-20 s of vortexing on max speed, Pipette mixing with a P200 can be used to aid homogenization.

Quick tip

Do not skip vortexing steps. Mix aggressively. Even with aggressive vortexing, the DNA will be hundreds of kilobases in length.

5. Centrifuge the lysate at 16,000 x *g* at RT (15–30°C) for 5 min.
6. Transfer the supernatant to a new 1.5 mL Protein LoBind tube.
   - The DNA should be handled carefully from this point forward to prevent shearing.
7. Add a Nanobind disk to the tube followed by ~120 µL (1X volume) of isopropanol. Gently invert the tube 5X, or until the supernatant-isopropanol mixture is homogeneous, and place onto a HulaMixer with the cap-side down.
   - If the volume of supernatant is greater or less than 120 µL, the volume of isopropanol should be adjusted accordingly.
   - The Nanobind disk must be added before the isopropanol.
8. Carefully mix with the tube oriented cap-side down, with all fluid and the Nanobind contained in the cap, at RT (15–30°C) for 20 min. Ensure that the fluid is continuously mixing and that the Nanobind disk remains submerged in the solution throughout the binding process.
   - We recommend the following HulaMixer settings:

Step	Setting	Time (s)
Rotation	9 rpm	OFF
Tilting	70°	12
Vibration	2°	1

   - If a HulaMixer is not available, manually mix the tube by inversion (e.g., 5X inversions every 2-3 min) to facilitate binding.
   - DNA binding is optimal when binding occurs with tube cap-side down to maximize mixing volume. A tube rotator is not recommended as there is not enough fluid for adequate mixing.
9. Place tubes on the magnetic tube rack.
   - Use the method described in the Guide & overview – Nanobind HMW plant nuclei kit **"Magnetic rack handling procedure"** section.
10. Discard the supernatant with a pipette**, taking care to avoid pipetting the DNA.**
    - Refer to the Guide & overview – Nanobind HMW plant nuclei kit **"Pipetting"** section for details.
11. Add 500 µL of Buffer PW1, remove tube rack from magnetic base, inversion mix 4X, replace tube rack on the magnetic base, and discard the supernatant.
    - Remove excess liquid from the tube cap to minimize carryover contamination.
12. Repeat step **11**.
13. Spin the tube on a mini-centrifuge for 2 s. With the tube rack already on the magnetic base and right-side-up, place tube on tube rack and remove residual liquid.

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.

- 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.

14. Repeat step 13.
15. Add 50–100 µL of Buffer EB and spin the tube on a mini-centrifuge for 2 s. Incubate at RT (15–30°C) for 10 min.
16. Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube.
    - A standard P200 pipette can be used to aid in the removal of residual liquid after most of the eluate has been removed.
    - Avoid Axygen tubes as these have been shown to interfere with PacBio sequencing.
17. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s. Use a standard P200 pipette to combine any additional liquid that comes off the disk with the previous eluate. Repeat if necessary.
    - A small amount of liquid or gel-like material may remain on the Nanobind disk after transferring the eluate in step **16**. **This clear gel is DNA!** The 5 s spin in step **17** 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.
18. Let eluate rest at RT overnight to allow DNA to solubilize.
    - Visible "jellies" should disperse after resting.
19. 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%, 5X pipette mix with a standard P200 pipette and allow DNA to rest at RT for 1 hour to overnight. Take care to disrupt any regions that feel more viscous than other regions. Remeasure with NanoDrop.
    - Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.

Quick tip

This 5 s spin is CRITICAL for recovering the DNA. We do not recommend a 2nd elution.

Quick tip

The DNA will solubilize after resting at RT or by coaxing it into solution using standard P200 pipette mixing. For samples that need to be used immediately, we recommend 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 for more information.
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.
    - Use the Genomic DNA 165 kb Kit (Agilent Technologies) for unsheared gDNA.

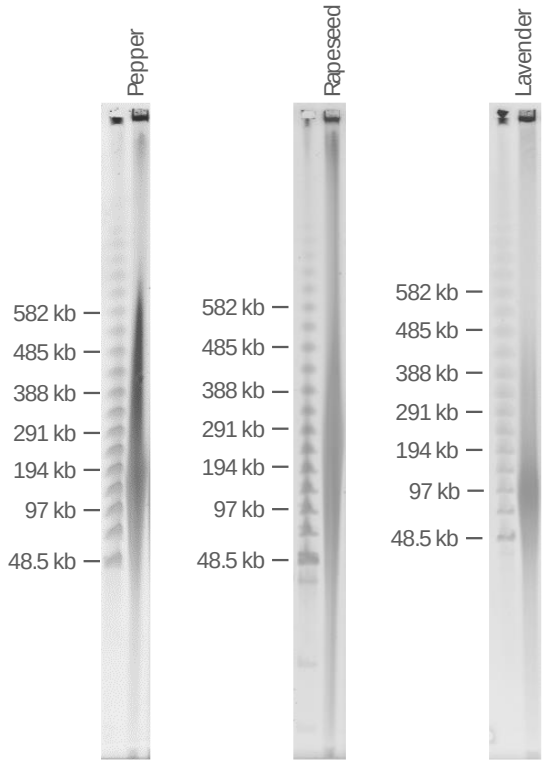
Storage of DNA

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

- DNA was extracted from 1 g of leaf tissues from Pepper, Rapeseed, and Lavender.
- 260/280 ratios should consistently be 1.7–2.0.
- 260/230 ratio can vary from 1.1–2.3.
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process.
- Example data is provided below.

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)
Pepper leaf	1 g	1.8	1.9	278.9	218.3	272.9	256.7	7.3
Rapeseed	1 g	1.8	1.9	445.0	494.8	526.5	488.8	12.5
Lavender	1 g	1.8	1.8	161.9	173.2	170.8	168.6	6.2



22 hour Pulsed Field Gel Electrophoresis (PFGE) image of gDNA from pepper leaf, rapeseed, and lavender.

Troubleshooting FAQ

1. **How can I increase DNA recovery?**

   - Adequately disrupt plant tissue prior to beginning the nuclei isolation protocol. If using LN2 grinding, grind the tissue for >20 min to maximize DNA recovery. If using TissueRuptor disruption, include 1 or 2 additional 30 s disruptions to further disrupt the tissue. Inadequate tissue disruption often leads to complete failure of the nuclei preparation and subsequent Nanobind DNA extraction.
   - Ensure that the starting nuclei pellet does not contain too much debris by selecting the proper size filter during nuclei isolation (<25 µm pore size). Improper filtration during nuclei isolation can lead to nuclei pellets which contain too much cellular debris and are exceedingly large in volume, causing poor lysis efficiency and failure of the downstream DNA extraction.
   - Thoroughly resuspend the nuclei pellet during both Proteinase K and Buffer PL1 additions (steps **1** and **3**). Pipette mixing during these steps will not substantially decrease the DNA size and can significantly increase DNA recovery.
   - Assess the homogeneity of the eluted DNA to ensure accurate quantification. If the CV of Nanodrop measurements is high (>50%), we recommend pipette mixing 5X with a P200 pipette and allowing the DNA to rest 1 h–overnight at RT before measuring again.
   - If above suggestions have been addressed, input tissue mass can be increased or multiple nuclei pellets can be extracted in parallel to increase DNA recovery. Some plant samples have a lower DNA to tissue ratio, particularly mature tissues; more than 1 g of starting material may be necessary to obtain adequate DNA for downstream applications.
     - If DNA recovery is <5 µg per gram of tissue, increase the input material to up to 5 g per nuclei pellet.
     - If DNA recovery is >5 µg per gram of tissue, prepare multiple nuclei pellets, perform DNA extraction in parallel, and combine the final eluates.

2. **Why are my A260/A280 or A260/A230 ratios low?**

   - Low UV ratios do not necessarily indicate that sequencing will be poor. As long as the UV ratios are close to the stated ranges (260/260 = 1.7–2.0, 260/230 = 1.1–2.3), sequencing performance should be good.
   - Poor mixing during nuclei resuspension and lysis can result in low purities. Ensure that the nuclei pellet is thoroughly resuspended during both Proteinase K and PL1 additions (steps **1**and **3**). Pipette mixing during these steps will not substantially decrease DNA size and can significantly increase DNA purity and recovery.
   - Insufficient washing during either nuclei isolation or DNA extraction (steps **11** and **12**) can result in low purities. An additional NIB wash during the nuclei isolation or an additional PW1 wash during DNA extraction (step **12**) can be included to increase DNA purity.
   - Tissue input may be too high. If DNA recovery is adequate, sample input can be decreased to avoid overwhelming the lysis chemistry, resulting in increased purity.

3. **How can I recover residual DNA from the Nanobind disk after elution?**

   - The DNA can be eluted by spinning the tube on a mini-centrifuge for 5–10 s or centrifuging the tube at 10,000 x *g* for 15 s. This spin can be repeated multiple times to ensure full elution. This should not take more than 1-2 spins. We do not recommend performing a 2nd elution or heated elution as these methods dilute the eluate and can damage DNA.

- Consider lower tissue inputs. High sample input and high DNA concentration can result in DNA that is difficult to completely solubilize. For high nuclei inputs, a larger elution volume can be used to facilitate elution of the DNA.
- Do not allow bound DNA to dry after wash steps. Over-drying the Nanobind disk will lead to DNA that is difficult to elute.
- Verify that the proper amount of 100% ethanol was added to Buffer PW1 (page **3**).

Revision history (description)	Version	Date
Initial release	01	July 2022
Protocol changes and minor updates throughout	02	December 2022