Bionano Prep High Polysaccharides Plant Tissue DNA Isolation Protocol

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Bionano Prep Plant DNA Isolation Kit Contents (Part # 80003, 5 preps)

Name	Volume	Part Number	Storage
Bionano Prep Plant Tissue Homogenization Buffer	2 x 500 mL (concentrate)	20283	4°C
Bionano Prep 5x Fixing Buffer	250 mL	20284	Room temp (15-25°C)
Bionano Prep Density Gradient	35 mL	20281	Room temp (15-25°C)
Bionano Prep Density Gradient Buffer	25 mL	20280	Room temp (15-25°C)
Bionano Prep 5x Wash Buffer	90 mL	20256	Room temp (15-25°C)
Bionano Prep Lysis Buffer	30 mL	20255	Room temp (15-25°C)
Bionano Prep Triton	40 mL	20285	Room temp (15-25°C)

User-Supplied Materials

	Item	Description
Tissue homogenization -Day 1. Plug Lysis - Day 1 and 2	Rotor-stator homogenizer (+ disposable probes)	QIAGEN, cat. # 9001271 (990890) or equivalent
	Refrigerated centrifuge	Eppendorf™ 5804R or equivalent
	Formaldehyde 36.5%-38% in H_2O	Sigma-Aldrich, cat. # F8775 or equivalent
	Spermidine trihydrochloride	Sigma-Aldrich, cat. # S2501 or equivalent
	Spermine tetrahydrochloride	Sigma-Aldrich, cat. # S1141 or equivalent
	β-mercaptoethanol (βME)	Sigma-Aldrich, cat. # M6250 or equivalent
	40 µm and 100 µm cell strainers	VWR cat. # 21008-949 and 21008-950 or equivalent
	50 mL conical tube(s)	VWR cat. # 21008-951 or equivalent
	15 mL conical tube(s)	VWR cat. # 21008-918 or equivalent
	Razor blades and Petri dish (square, 12 cmx 12 cm)	General lab supplier
	Plastic spatula	Corning catalog # 3005 or equivalent
	Filtered 200 µL tip	VWR cat. # 46620-642 or equivalent
	Small children paint brush, plastic	General supplier - i.e. Walmart
	Heat blocks or water baths set to 43°C and 70°C	General lab supplier
	Thermomixer C	Eppendorf cat. # 5382000023 or equivalent
	50 mL SmartBlock	Eppendorf cat. # 5365000028 or equivalent
	Proteinase K enzyme, 5 mL	QIAGEN, cat. # 158920
	RNase A solution, 5 mL	QIAGEN, cat. # 158924 or equivalent
	Green screened caps for 50 mL conicals	Bio-Rad, cat. # 1703711 or equivalent
	Agarose plug mold and plug mold plunger	Bio-Rad, cat. # 1703713 or equivalent
	Agarose, 2% CleanCut Low Melting Point	Bio-Rad, cat. # 1703594 or equivalent
	Filtered wide-bore pipette tip, 200 µl	VWR, Catalog # 46620-642 or Rainin equivalent
	TE Buffer, pH 8.0	ThermoFisher Scientific, cat. # AM9849 or equivalent
DNA Recovery -Day 2	Metal spatula	VWR cat. # 82027-530 or equivalent
	Orbital shaker, 12 in x 12 in Platform	Cole-Parmer, catalog # EW-51820-30 or equivalent
	Agarase (0.5 U/µL)	ThermoFisher Scientific, cat. # EO0461
	0.1 µm Dialysis membrane	Millipore cat. # VCWP04700 or equivalent
	Forceps for Membrane Placement	TDI International Catalog # TDI-2A-SA or Equivalant
	Petri dish, 6 cm x 6 cm	VWR cat. # 28384-092 or equivalent
	Non-filtered 200 µL tip	USA Scientific, cat. # 1111-1810 or equivalent
	Pipet Tips Wide-Bore, Filter, Aerosol, 200 µl	VWR, Catalog # 46620-642 or Equivalent for Rainin
	TE Buffer, pH 8.0	ThermoFisher Scientific, cat. # AM9849 or equivalent
Quant -Day 3	Bath sonicator	General lab supplier
	Vortexer	General lab supplier
	Qubit Fluorometer 3.0	ThermoFisher cat. # Q33216 or equivalent
	Qubit BR Assay Kit	ThermoFisher cat. # Q32850 or equivalent
	Qubit Assay Tubes	ThermoFisher cat. # Q32856 or equivalent

Bionano Prep High Polysaccharides Plant Tissue DNA Isolation Protocol

Introduction

The Bionano Prep Plant DNA Isolation Kit enables the isolation of megabase-sized genomic DNA from plant tissue for next-generation mapping. The kit provides critical reagents for high molecular weight (HMW) DNA isolation.

Overview

The Bionano Prep High Polysaccharides Plant Tissue DNA Isolation protocol facilitates the recovery of high-quality HMW DNA from plant species rich in polysaccharides. However, because of the diverse nature of polysaccharides, and varying amount of complex metabolites in different plant species, this protocol may not work on some polysaccharide-rich plants, requiring further optimization. The Bionano Prep High Polysaccharides Plant Tissue DNA protocol is a four step process that involves: 1) homogenization of plant material with a rotor-stator after a brief fixing in formaldehyde to protect nuclei/DNA against mechanical shearing needed to break tough plant cell wall, 2) purification of a fraction of the homogenate by extensive washes to produce clean nuclei without density gradient purification, 3) embedding of the purified nuclei into agarose plugs for subsequent DNA purification, and 4) recovery of HMW DNA.

Input Plant Tissue

The recommended starting amount of plant tissue is 0.5 grams of fresh young leaf tissue, preferably from seedlings, or new sprouts from mature plants. Mature tissues are not recommended, as they tend to accumulate a large amount of metabolites and waste product, which compromises DNA quality. We have successfully purified sufficient high-quality HMW DNA prepared from soybean, diploid strawberry, maize and tomato. Results may vary depending on the source tissue and plant species.

Estimated DNA Yield

Plant Species	Input Amount	Tissue Type	Tissue Storage	Tissue / Plug	# plugs	DNA Yield / Plug
Maize	0.25 g*	Young leaves	Fresh	50 mg	5	3.6 µg
Tomato	0.25 g*	Young leaves	Fresh	50 mg	5	4.0 µg
Soybean	Blend 0.5 g purify 0.1 g	Young leaves	Fresh	33 mg	3	3.5 µg
*Blend and purify 0.25 g.						

Important Notes

- Protocol Summary: Start with 0.5 g fresh young plant leaves, fix in formaldehyde, and homogenize tissue by rotor-stator. Carry forward 0.1-0.3 g equivalent of starting tissue for nuclei purification without a density gradient: perform a 2-minute low speed spin at 60xg, and clean nuclei by four extra washes until pellet is fresh yellow/light green and the supernatant is free of color & debris. Purified nuclei are embedded in agarose for HMW DNA purification.
- Start with 0.5 grams fresh young plant leaves even though only 0.1-0.3 g tissue will be needed for nuclei purification since there is potential tissue lose during the chopping blending process. Carrying forward more than 0.3 g of plant tissue will not only require a series of dilutions before embedding into agarose but also reduce the purification efficiency thus resulting in low-quality DNA.
- Before starting, make sure all reagents and equipment are available for disrupting tissue and embedding purified nuclei in plug(s) for subsequent DNA purification - see the 'User-Supplied Materials' section above.
- Avoid using mature plant tissue as it is rich in metabolite and waste product; this can complicate DNA recovery.
- Chop plant tissue into ~2x2 mm pieces and blend for up to 2 minutes on ice (20 seconds of blending followed by resting 30 seconds rest on ice) to get the desired puréed consistency of homogenate.
- This protocol is not recommended for plant species rich in polyphenols.
- DNA recovered by this protocol requires 7-10 units of nicking enzyme Nt.BspQI, and 20-50 units of nicking enzyme Nb.BssSI, for efficient nick-labeling of 300 ng DNA. Other nickases may require optimization. This does not apply to direct labeling with DLE-1. Follow the standard protocol.

Abbreviations

βME:	β-mercaptoethanol	FS:	Fixing Solution
DGB:	Density Gradient Buffer	HB_+:	Plant Homogenization Buffer *plus*
FB:	Fixing Buffer	PK:	Proteinase K

Bionano Prep High Polysaccharide Plant DNA Isolation Protocol (0.5 g)

Day 1: Tissue Homogenization / Embedding in Agarose / Proteinase K Digestion

Before First Use

1) Bionano Prep Plant Homogenization Buffer is supplied as a concentrate (2 bottles of 500 mL at 1.5x). Before first use add 250 mL molecular biology grade water per bottle and thoroughly mix by inverting gently until a homogenous solution is obtained. Store at 4°C for up to 1 year.

Before Each Use

Prepare the following solutions fresh for each prep and place on ice.

2) 250 mL 1x Fixing Buffer: 50 mL 5x Bionano Prep Fixing Buffer + 200 mL molecular biology grade water.
3) 60 mL Fixing Solution: 3.2 mL formaldehyde + 56.8 mL 1x Fixing Buffer.
4) Plant Homogenization Buffer *plus*: 300 mL Bionano Prep Plant Homogenization Buffer + 0.6 mL βME + 3.0 mL 100 mM spermine-spermidine solution (final concentration: 0.2% βME, 1 mM spermine/spermidine).
   Note: βME, spermine and spermidine are user supplied. Prepare a spermine-spermidine solution containing both 100 mM spermine and 100 mM spermidine in molecular grade water. Aliquot into 1.5 mL microfuge tubes and store at -20°C. Discard after 3 months and make a fresh stock.

Set Up

5) Gather materials (see 'User Supplied Material' section above).
6) Set a heat block or water bath to 70°C. Set another heat block or water bath to 43°C.
7) Melt 2% agarose at 70°C for 15 minutes and then equilibrate to 43°C for at least 15 minutes.
8) Pre-chill to 4°C: Bionano Prep Density Gradient Buffer, plug mold(s)/plunger, centrifuge.
9) Equilibrate a Thermomixer fitted with 50 mL adapter to 50°C for Proteinase K digestion.

Tissue Fixation (~1 hour)

Tissue Fixation is carried out in a fume hood. Fixing tissue protects DNA/nuclei against mechanical shearing needed to disrupt the tough plant cell wall.

10) If plant tissue is muddy, rinse with distilled water and gently pat dry with a paper towel without bruising the tissue.
11) Using a razor blade, remove the petiole and midrib, if applicable.
12) Weigh 0.5 grams, transfer to a 50 mL conical tube, and attach a green screened cap (Bio-Rad).
    Note: Large plant leaves should be cut to fit in 50 mL conical tube.
13) Add 60 mL Fixing Solution (containing formaldehyde), remove air bubbles by tapping/stirring the tube (keep upright) between the palms of your hands. Incubate 20 minutes on ice.
    Note*:* The Fixing Solution should come up halfway into the screened cap, and completely submerge the plant tissue throughout the fixation process.
14) Decant Fixing Solution through the screened cap. Wash tissue 3 times with 1x Fixing Buffer to remove remaining formaldehyde:
    a. Add 60 mL ice-cold 1x Fixing Buffer.
    b. Incubate on ice for 10 minutes.
    c. Decant Fixing Buffer through the screened cap.

Tissue Disruption with Rotor-stator and Nuclei Purification (~2.5 hours)

15) Transfer fixed tissue with a spatula to a 12x12 cm Petri dish that is pre-cooled on ice. Add ~4 mL ice-cold Plant Homogenization Buffer *plus* to wet the plant material.
***Note**:* Adding too much Plant Homogenization Buffer *plus* could result in splashing during chopping.
16) Manually chop plant tissue into ~2x2 mm pieces with a sharp razor blade:
    a. First cut leaves into ~2 mm wide strips by holding tissue down with spatula and slicing with razor blade along the veins.
    b. Hold the Petri dish with one hand and chop with an up-and-down motion using the other hand.
    c. Periodically gather the plant pieces at the center of the Petri dish and continue chopping to get ~2x2 mm pieces.

    ***Note**:* When chopping tough plant tissues replace the razor blade as needed. Manual chopping into ~2x2 mm pieces satisfies rotor-stator requirement for effective blending by ensuring that material to be blended is not greater than half the diameter of the rotor-stator probe.
17) Transfer the chopped tissue to a 50 mL conical tube on ice by first piling tissue in a corner of the Petri dish using the razor blade and then transferring to the 50 mL conical with a spatula.
18) Add 7.5 mL ice-cold Plant Homogenization Buffer *plus.*
19) Place the conical tube in a 250 mL beaker containing ice and ~20 mL water, creating an ice-water bath for effective cooling of the sample during the blending process.
    ***Note**:* The conical tube should be stationary in the ice-water bath; the 250 mL beaker permits holding the tube-beaker combination with one hand during blending.
20) Immobilize the rotor-stator on a vertical stand. Hold the tube-beaker in one hand and submerge the probe tip in the buffer-plant mixture as per manufacturer instruction.
21) Blend for 20 seconds at top speed while keeping the conical tube in the ice-water bath with the probe submerged at all times. Move the tube-beaker in a circular motion during blending to increase the efficiency of homogenization.
22) Rest the tube in the ice-water bath for 30 seconds. If the mixture is too dense (i.e., the probe is clogging), add an additional 5 mL ice-cold Plant Homogenization Buffer *plus* before continuing. Repeat blending up to five times, for a maximum of 2 minutes, cooling for 30 seconds between each blending step to protect DNA from heat damage.
    ***Note**:* It usually takes at least three blending of 20 seconds each to generate the desired puréed consistency of homogenate (no visible particles bigger than 0.5x0.5mm) for young maize leaves and tomato leaves/flowers.
    Note: During blending, if the probe becomes clogged, change or clean the probe.
    ***Note*:** If foaming occurs in the conical tube, either let the sample sit on ice for 5 minutes or centrifuge at 1,000xg at 4°C for 3 minutes before further blending. Under most circumstances, foaming occurs when the probe tip is not fully submerged in the buffer during blending.
23) Add Bionano Prep Plant Homogenization Buffer *plus* to a final volume of 40 mL. Swirl on ice and mix well.
24) Transfer a defined amount of homogenate equivalent to 0.1-0.3 g of starting tissue to a new 50 mL tube. Add Bionano Prep Plant Homogenization Buffer *plus* to a final volume of 40 mL.

Tissue	Input Amount	Amount to Carry Forward
Soybean	0.5 g	0.1 g
Maize/tomato	0.25 g	0.25 g

25) Filter plant homogenate first through a 100 µm cell strainer, followed by filtering through a 40 µm cell strainer:
    a. Chill 50 mL conical tube on ice. Firmly seat a 100 µm cell strainer on top of the tube. Transfer the homogenate to the cell strainer and collect crude nuclei suspension by gravity flow; squeeze plant material in filter with plastic spatula.
    b. Wash plant material trapped in the cell strainer 3 times, with 2 mL ice-cold Plant Homogenization Buffer *plus* each time, and collect in the same tube.
    c. Chill a new 50 mL conical tube on ice. Firmly seat a 40 µm cell strainer on top of the tube. Transfer the crude nuclei suspension to the cell strainer and collect the filtrate.

    ***Note**:* Bubble formation can block flow during filtration; to minimize bubbles, periodically lift the cell strainer by the tab and place back on the tube.
26) Bring filtrate to a final volume of 45 mL by adding ice-cold Plant Homogenization Buffer *plus*, cap the tube and swirl gently by hand for 30 seconds while the tube is touching ice.
27) Pellet nuclei by spinning filtrate for 20 minutes at 4°C using a swing bucket rotor. Set g force based on genome size:

g force	Genome Size
1,200 x g	> 6 Gbp (i.e. wheat ~17 Gbp)
2,000 x g	2 - 6 Gbp
2,500 x g	300 Mbp - 2 Gbp
3,500 x g	< 300 Mbp

    ***Note**:* If plant species contain extensive chloroplasts and polysaccharides the pellet usually is dark green color (Fig. 1 in Appendix of this protocol).
28) Discard the supernatant by decanting and set back on ice for 30 seconds to allow the remaining supernatant to accumulate at the bottom of the tube.
    Note: Usually there is approximately 0.5 mL remaining supernatant at the bottom.
29) Re-suspend the pellet with the assistance of a small paintbrush pre-soaked in ice-cold Plant Homogenization Buffer *plus*. Once re-suspended, add 3 mL ice-cold Plant Homogenization Buffer *plus* and swirl gently by hand for 30 seconds while the tube is touching ice.
30) Perform a low speed spin followed by a filtration step to clean the nuclei suspension:
    a. Add 40 mL ice-cold Plant Homogenization Buffer *plus*, cap tube, and mix by gently inverting back and forth several times.
    b. Spin at 60xg at 4°C for 2 minutes with no deceleration (i.e. acceleration=9, deceleration=1) to remove intact cells, cell debris, unbroken tissue and other residues.
    c. Collect the supernatant through a 40 µm cell strainer on top of a new chilled 50 mL tube.
    d. Repeat steps 27-29 to pellet, decant supernatant, and re-suspend nuclei in 3 mL Plant Homogenization Buffer *plus*.

    ***Note**:* The low speed spin removes tissue residues, unbroken cells and cell debris. Refer to Fig. 1 in Appendix for pellet sizes. Fig. 2 shows the debris from the low speed spin.
    ***Note**:* When performing the 40 µm cell strainer filtration, slowly transfer the supernatant to the strainer and avoid transferring the very loose pellet at the bottom of the tube, which contains tissue residues, unbroken cells and cell debris.
31) Perform four washes with Plant Homogenization Buffer *plus* to harvest high-quality nuclei (steps a-c below).
    a. Bring the total volume of 35 mL by slowly adding Homogenization Buffer *plus* to the nuclei suspension from step 30, swirl gently to mix, and pellet as in step 27.

b. Discard supernatant and re-suspend the nuclei pellet in residual wash buffer, with the assistance of a wet paint brush.
c. Repeat steps a-b 3 times until supernatant is clear and pellet is yellow/light green look (See Fig. 3 in Appendix). After the final wash, resuspend the nuclei pellet in residual wash solution, add 3 mL ice-cold Plant Homogenization Buffer *plus* and swirl gently by hand for 30 seconds while the tube is touching ice. Transfer the nuclei suspension to a new 15 mL conical tube and continue to step 32.

Note: Washes can remove the 'darker' green color from the plant tissue, especially those rich in polysaccharides/metabolites, such as spinach, soybean and diploid strawberry.
Note: Color of the resulting nuclei suspension should look fresh yellow/light green or white.

32) Add ice-cold Plant Homogenization Buffer *plus* to a total volume of 14 mL. Cap the tube, invert to mix and spin at 2,500xg for 10 minutes at 4°C with no deceleration (i.e., acceleration=9, deceleration=1).
33) Evaluate the clarity of the supernatant immediately after centrifugation stops before the loose nuclei pellet starts diffusing:
    a. If a clear supernatant is obtained and a pellet is observed, continue to step 34.
    b. If a cloudy supernatant is obtained and a pellet is observed, immediately discard the supernatant by carefully decanting without disturbing the pellet, add 10 mL ice-cold Plant Homogenization Buffer *plus*, cap the tube and re-suspend the pellet by inverting the tube several times. Centrifuge at 3,000xg for 10 minutes at 4°C with no deceleration (i.e., acceleration=9, deceleration=1) and continue with step 34.
    c. If there is no pellet or pellet is very loose (i.e. cloudy on the bottom of the tube), re-centrifuge at 3,000xg for 10 minutes at 4°C with no deceleration. Continue to step 34.

Note*:* The pellet at this step is greenish/yellowish and often very loose.
Note: if no pellet is observed even after step c, sample prep has failed - repeat with fresh young leaves or consult customer support.

34) Discard the supernatant by carefully decanting, and gently tapping the tube on a paper towel to drain excess liquid. Check the pellet size (usually spreading out on the bottom of the tube, about 3-6 mm diameter):
    a. If the pellet is small (e.g. size of a sesame seed), resuspend the nuclei pellet in 50 µL of ice-cold Bionano Prep Density Gradient Buffer (enough for one plug), and transfer 50 µL to a 1.5 mL microfuge tube using 200 µL wide-bore tip.
    b. If the pellet is large (e.g. size of a soybean) and very young plant tissue containing Gigabase pairs genome is used for nuclei isolation, titration may be needed. See Appendix III for one such titration scheme.
    c. Otherwise, resuspend nuclei pellet in 200 µL of ice-cold Bionano Prep® Density Gradient Buffer (enough for about 3 plugs), and transfer 200 µL to a 1.5 mL microfuge tube using 200 µL wide-bore tip.

Note: Transferring exactly 50 and 200 µL to a new tube is important to ensure the proper final agarose concentration in plugs upon addition of a fixed volume of agarose at step 35.
Note: Nuclei in 200 µL buffer can be further concentrated, if desirable, by centrifuging at 5,000 rpm for 1 minute at 4°C, pipetting out the supernatant, and replacing with desired volume of Density Gradient Buffer.
Note: Under most circumstances, titration is not necessary when following the Bionano Prep Plant Tissue DNA Isolation protocol and if using appropriate amount of young plant tissue (blending 0.5 g, purifying 0.1-0.25 g). Titration makes sense when nuclei pellet is big (e.g. soybean size) and the

input tissue is very young, with Gigabase pairs genome indicating a high DNA content. As plant tissue mature, cell size increases and larger pellets may be observed that have a lower DNA content compared to younger tissue. Titrating pellets resulting from the more mature tissue is not recommended as DNA concentration following melt/Agarase treatment can be compromised (recommended >35 ng/µL to support DNA labeling).

Embedding in Agarose (~30 minutes)

35) Pre-warm the nuclei suspension at 43°C for 3 minutes. Add appropriate volume of agarose depending on nuclei suspension volume as per table below to achieve a final agarose concentration of 0.82% :

Nuclei Suspension	43°C Agarose
50 µL	35 µL
100 µL	70 µL
200 µL	140 µL

36) Gently pipet mix three times with a 200 µL wide-bore pipet tip, taking care to avoid bubble formation. Immediately transfer 85 µL per plug into pre-cooled plug molds using the wide-bore tip.
37) Place plug molds at 4°C for 15 minutes to solidify the agarose.

Proteinase K Digestion (~overnight)

Up to five plugs can be processed simultaneously per 50 mL conical tube of the same sample from same homogenate input per plug. Ensure all plugs are fully submerged with Proteinase K solution throughout processing. For maximum workflow flexibility, two Proteinase K digestion options may be employed: 2 hours of Proteinase K digestion followed by overnight digest with a fresh Proteinase K solution, or overnight Proteinase K digestion followed by 2 hours digest with a fresh Proteinase K solution the following day.

38) Prepare fresh Proteinase K digestion solution by mixing 200 µL of Proteinase K enzyme with 2.5 mL of Bionano Prep Lysis Buffer per 1-5 plugs to be processed in the same 50 mL conical tube.
39) Transfer up to five plugs per conical tube containing Proteinase K digestion solution by first removing the tape from bottom of the plug mold(s) followed by dislodging the plug(s) with the plug mold plunger.
Note: Use a blunt end metal spatula to submerge plugs if they stick to walls of the conical tube.
40) Cap each tube and incubate in Thermomixer (fitted with adaptor for 50 mL conical tubes) for 2 hours or overnight at 50°C with intermittent mixing (mixing cycle: 10 seconds at 450 rpm followed by 10 minutes at 0 rpm).
41) Near the end of the incubation, prepare fresh Proteinase K digestion solution by mixing 200 µL of Proteinase K enzyme with 2.5 mL of Bionano Prep Lysis Buffer per 1-5 plugs to be processed per tube.
42) Remove each tube from the Thermomixer and equilibrate to room temperature for 5 minutes. Remove cap and attach a screened cap. Drain the Proteinase K digestion solution through the screened cap, and tap the bottom of the tube on the bench surface several times with strong repetitive force to localize the plugs at the bottom of tube.
43) Remove the screened cap, and add freshly mixed Proteinase K digestion solution. Account for all plugs and ensure that they are completely submerged in the Proteinase K digestion solution. Tightly cap each tube with its original cap. Incubate in Thermomixer with intermittent mixing as before for 2 hours or overnight depending on which Proteinase K digestion workflow was chosen.
Note: Additional 2-hour PK treatment may be needed when plugs have remaining fresh green color in agarose plug(s).

Day 2: RNase Digestion, DNA Stabilization Washes, DNA Recovery, Drop Dialysis (7 hours)

Set Up

1. Gather materials (see 'User Supplied Materials' section above).
2. Set up heat blocks/water baths. Fill wells with water and verify temperature with thermometer:
    a. Set a heat block or water bath to 70°C for melting of plugs.
    b. Set another heat block or water bath to 43°C for Agarase treatment of plugs.

RNase Digestion (1 hour)

3. Following the second Proteinase K digestion, remove each tube from the Thermomixer. Gently tap the tube to collect any condensation and incubate at room temperature for 5 minutes.
4. Remove cap, add 50 µL of RNase A Solution (Qiagen), recap and incubate in the Thermomixer for 1 hour at 37°C with intermittent mixing as described above.

Washing Plugs to Stabilize DNA (1 hour 30 minutes)

5. During RNase digestion, prepare 70 mL of 1x Wash Buffer for each 50 mL conical tube, using the Bionano Prep® 5x Wash Buffer and nuclease-free water. Mix thoroughly and store at room temperature until use.
6. After RNase digestion, replace original cap with screened cap, drain RNase digestion solution, and tap the bottom of each tube on the bench surface several times with a strong repetitive force to localize plugs at bottom of the tube.
7. Rinse each tube 3 times by:
    a. Adding 10 mL of 1x Wash Buffer through the screened cap.
    b. Swirling tube gently for 10 seconds.
    c. Discarding wash solution through the screened cap.
    d. Tapping plugs to bottom of tube before next rinse.
8. Wash each tube 4 times by:
    a. Adding 10 mL of 1x Wash Buffer through screened cap and capping tube.
    b. Gently shaking tube for 15 minutes on an orbital platform shaker with continuous mixing at 180 rpm.
    c. Discarding wash solution through the screened cap.
    d. Tapping plugs to bottom of tube before next wash. Do not discard the last wash.

    ***Note**:* Plugs can be stored in 1x Wash Buffer for up to 2 weeks at 4°C without significant degradation of DNA quality.

DNA Recovery (2 hours)

9. Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
10. TE Wash plugs 5 times by:
    a. Adding 10 mL of TE Buffer (pH 8.0) per wash through the screened cap and capping tube
    b. Continuous shaking for 15 minutes on a orbital platform shaker at 180 rpm.
    c. Discarding the wash through the screened cap
    d. Tapping plugs to bottom of tube before adding the next wash.
11. Discard the last TE wash through the screened cap and tap plugs to bottom of tube.
12. Scoop one plug at a time from conical tube with a metal spatula and drain excess liquid from plug by touching the bottom of the spatula to a clean KimWipe, being careful to not make contact with the gel plug. Place each semi-dried plug into a 1.5 mL microcentrifuge tube.
13. Pulse spin each microcentrifuge tube briefly to collect the plug at the bottom of the tube.
14. Melt each agarose plug in a water bath or heat block set at 70°C for 2 minutes.
15. Immediately transfer each tube to a water bath or heat block set at 43°C, incubate 5 minutes.
16. Process one tube at a time: add 2 µL of 0.5 U/µL Agarase (Thermo Fisher) enzyme to tube and stir mixture gently with a pipet tip for 10 seconds.
17. Incubate tubes at exactly 43°C for 45 minutes.
    Note: A temperature difference of +/-3°C can inactivate the Agarase enzyme.

Drop Dialysis to Clean DNA (1 hour)

18. Place 15 mL of TE Buffer into 6 cm Petri Dish (Thermo Fisher/VWR) per each DNA sample.
19. Float a 0.1 µm Dialysis Membrane (Millipore) on the surface of the TE Buffer. Place a cover on the Petri dish and let the membrane hydrate for 10 minutes.

    Note: Use forceps to grip the membrane by the edge and gently float it on the surface of the TE Buffer horizontally to prevent dipping or sinking during application. According to the manufacturer, both sides perform equivalently; either side can face up.
20. Pipet mix the entire volume of DNA solution 2 times with a wide bore tip and add as a single drop on the center surface of the dialysis membrane.

    Note: In order to avoid submersion of the membrane and loss of sample, it is critical to place the entire volume of DNA in the center of the membrane and avoid excessive downward force when applying or withdrawing DNA from membrane surface.
21. Place cover on the Petri dish and let the sample dialyze for 45 minutes at room temperature.
22. Transfer DNA to a 1.5 mL microcentrifuge tube with a wide bore tip.

    Note: Do not pipet mix the DNA while on the membrane.

Homogenization of DNA Solution (10 minutes)

23. Test DNA viscosity by slowly withdrawing the entire DNA volume into a non-filtered 200 µL tip while slowly releasing pipet plunger. If the DNA is not viscous, do not pipet mix. If the DNA is

extremely viscous, pipet mix up to 9 strokes (stroke = 1 up stroke + 1 down stroke) until entire DNA sample is taken up in a continuous flow. The DNA should aspirate evenly without lag.

Note: If DNA uptake stalls due to high viscosity, it may be necessary to stir gently while slowly releasing the plunger to withdraw the DNA. Viscous samples should get easier to pipet mix as the number of strokes increases.

24. Allow the DNA to equilibrate overnight at room temperature (25°C) for better homogeneity.

Day 3: Quantitation

Qubit Quantitation - BR dsDNA Assay (30 minutes)

Refer to the Qubit dsDNA BR Assay Kit user manual for kit details and follow the methods described in the "Pipetting Viscous Genomic DNA" section, to ensure accurate pipetting of viscous gDNA.

1. Equilibrate gDNA and Qubit BR Assay Kit Standards to room temperature.
2. Add Qubit BR Buffer to 0.5 ml Qubit Assay Tubes:
    a. For each sample, add 18 µl of Qubit BR Buffer to 3 separate Assay Tubes.
    b. For the Qubit Standards, add 10 µl Qubit BR Buffer to 2 separate Assay Tubes.
3. Using a 200 µl pipette with a wide bore tip, gently mix the entire gDNA sample volume by pipetting up and down 5 times, being careful not to generate bubbles.
4. Using a fresh standard bore pipette tip or positive displacement pipette tip for each draw:

    Remove 2 µl aliquots from the top, middle, and bottom of each sample and dispense into BR Buffer of corresponding Qubit Assay Tube, rinsing tip. Place Assay Tubes in a floating rack and sonicate in a bath sonicator for 10 minutes. Perform steps 5 and 6 during sonication.
    a. If a bath sonicator is not available, vortex for at least 30 seconds at maximum speed, then spin down briefly for 2 seconds.
5. Prepare Working Solution by diluting the Dye Assay Reagent into BR Dilution Buffer (1:200):
    a. 200 µl Working Solution for each of the two standards (400 µl total).
    b. 200 µl Working Solution for each sample aliquot (600 µl for each sample).
6. For the Qubit DNA standards, add 10 µl of Standards 1 and 2 to the Assay Tubes containing BR Buffer from Step 2b.
7. Once sonication is complete, retrieve assay tubes and pulse spin briefly. Vortex tubes for 5 seconds at maximum speed, then pulse spin again.
8. Add 180 µl of Working Solution to each sonicated DNA aliquot and Qubit DNA Standard aliquot. Vortex for 5 seconds, and pulse spin tubes.
9. Incubate samples in the dark for at least 3 minutes, then read on the Qubit Fluorometer.
10. Coefficient of Variation (CV = standard deviation/mean) from three readings should be < 0.25.

    ***Note*:** If CV > 0.25, gently pipette-mix the entire volume of gDNA with one additional stroke (1 stroke = 1 up stroke + 1 down stroke) using a non-filtered 200 µL tip (i.e. USA Scientific, #1111- 1810 or equivalent), pipette mix the entire volume of gDNA 5 times with wide bore tip. Let the gDNA rest at least overnight at room temperature before repeating quantitation.

Labeling

DNA is ready for labeling. See "Kits and Consumables" section at https://bionanogenomics.com/support/ for applicable kits and protocols.

Appendix

I. Pipetting Viscous DNA

To draw viscous gDNA, hold the stock DNA tube for close-up visualization, depress the pipet plunger until the first stop, submerge the pipet tip toward the top, middle, or bottom of the viscous solution, and carefully release the plunger, as **slowly** as possible, to start drawing the viscous DNA into the tip while carefully monitoring DNA uptake. Keep the tip submerged even after the viscous DNA solution stops moving upward and levels off. Viscous DNA can take ~30 seconds to fill the tip to the 2 µL mark. Releasing the plunger too fast can produce a bubble in the tip leading to under-sampling (start over if this occurs). After the solution in the pipet tip has leveled off and while the tip is still submerged in the DNA solution, scrape the tip against the bottom of the tube 3-5 times using a circular motion. Remove the tip from the DNA solution and visually inspect to confirm that it is filled to the 2 µL mark (a positive displacement pipette may be used as an alternative).

To deposit the entire volume of viscous gDNA, hold tube containing 18 µL Qubit buffer for close-up visualization, insert the pipet tip such that it contacts buffer and deliver the viscous DNA solution by gently pressing the plunger until the last bit of DNA has left the tip without introducing bubbles.

***Note*:** Removing the pipet tip from the gDNA solution too early, or ineffectively scraping the tip to break continuity of the viscous solution between the inside and outside of the tip, can produce a bubble at the tip of the pipet tip indicating under-sampling (start over if this happens).

II. Photo Reference of Key Steps

Fig. 1 Pellet nuclei using a swing bucket rotor on soybean (0.1 grams) as described in step 27.

Half of a gram young soybean leaves were used as initial input tissue, 0.1 g of equivalent homogenate was carried forward for nuclei purification. Pellet size is reflecting 0.1 g equivalent soybean young leaves. The pellet color is dark green due to high level of chloroplasts and polysaccharides in young soybean leaves.

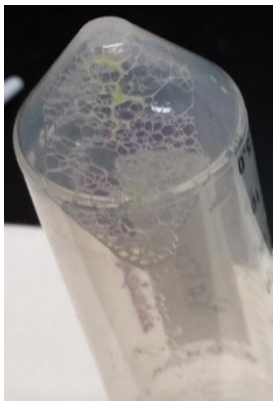

Fig. 2 Residuals left after the low speed spin (step 30).

Fig. 3 Ideal pellet should have yellow/light green look (step 31).

III. Titrating scheme if large pellet with high DNA content following density gradient purification is observed

a. Resuspend large (e.g. soybean size) nuclei pellet in 200 µL of ice-cold Bionano Prep® Density Gradient Buffer.
b. Transfer 100 µL with a wide-bore tip to 1.5 mL microfuge tube on ice and label 1x (enough for 2 plugs). Transfer another 100 µL to a new 1.5 mL tube containing 100 µL of ice-cold Bionano Prep® Density Gradient Buffer, pipet mix using 200 µL wide-bore tip and label 0.5x (enough for 3-4 plugs).
c. To embed nuclei, add 35 µL of 43°C equilibrated agarose per 50 µL of nuclei suspension and cast plugs.

High Polysaccharides Plant DNA Isolation - Experienced User Guide

Day 1 ----------

1) Prepare solutions for 1 prep and place on ice:
   - 250mL 1x Fixing Buffer (FB)
   - Fix Sol. (FS): 3.2mL formaldehyde + 56.8mL FB
   - HB_+: 300mL Plant Homogenization Buffer + 0.6mL βME + 3.0 mL spermine-spermidine
2) Melt agarose at 70°C & equilibrate to 43°C.
3) Chill centrifuge and plug mold to 4°C.
4) Set Thermomixer to 50°C.

Tissue fixation

5) Rinse tissue. Remove petiole & mid rib.
6) Weigh 0.5g and transfer to 50mL tube.
7) Fix in 60mL FS 20min on ice.
8) Wash tissue 3x with 50mL cold FB.

Tissue disruption with a rotor-stator and nuclei purification

9) Chop tissue into ~2x2mm, transfer to 50mL tube. Add 7.5mL HB_+.
10) Blend 20sec & ice 30sec. Repeat up to 5x.
11) Add HB_+ buffer to 40mL and transfer a defined amount of homogenate equivalent to 0.1-0.3g of starting tissue to a new 50 ml tube.

Tissue	Input amount	Amount to carry forward
Soybean	0.5g	0.1g
Maize/tomato	0.25g	0.25g

12) Add HB_+ to 40mL. Filter through 100µm & 40µm cell strainers. Add HB_+ to 45mL.
13) Pellet nuclei 20min at 4°C:

Genome size (Gbp)	>6	2-6	0.3-2	<0.3
X g	1,200	2,000	2,500	3,500

14) Decant the supernatant. Set 30sec on ice. Resuspend nuclei pellet with wet paint brush in residual HB_+. Add 3mL HB_+ & mix by swirling while touching ice.
15) Add 40mL HB_+, spin 2min 60xg 4°C, decant through 40µm strainer. Repeat 13-14.
16) Wash the pellet 4x with 35mL HB_+. Re-suspend nuclei pellet as in 14. After last wash, transfer nuclei suspension (~3mL) to 15mL tube.
17) Add HB_+ to 14mL. Spin 2,500g 10 min 4°C.
    -If pellet at bottom & clear sup: Go to 18.
    -If pellet at bottom & cloudy sup: Decant sup. resuspend pellet in 10mL HB_+, spin at 3,000g 10min, decant sup, and go to 18. If pellet is loose re-spin at 3,000g 10min.

Embedding in agarose

18) Discard sup. & check pellet size.
    If small (e.g. sesame seed) re-suspend in 50µL DGB & transfer 50µL to 1.5mL tube. If big (e.g. soybean seed), titration may be required. Otherwise resuspend in 200µL DGB & transfer 200µL.
19) Incubate 3 minutes at 43°C.
20) Add 35µL agarose if 50µL nuclei suspension or 140µL agarose if 200µL nuclei suspension.
21) Pipet mix 3x, cast plugs & solidify 15min 4°C.

Proteinase K (PK) digestion - starts day 1 ends day 2

22) Transfer up to five plugs per 50mL tube.
23) Add 2.5mL Lysis Buffer + 200µL PK per tube.
24) Set 2hrs, or overnight at 50°C (+mixing).
25) Replace PK sol. Set 2hrs, or overnight.

Day 2 ----------

1) Set heat sources to 70°C & 43°C.
2) Add 50µL of RNase A. Incubate 1hr at 37°C.

Washing plugs to stabilize DNA

3) Prepare 70mL 1x Wash Buffer.
4) Rinse 3x with 10mL 1x wash buffer.
5) Wash 4x with 10mL 1x wash buffer by shaking 15min at 180rpm. Do not discard last wash. Plugs are stable in 1x wash at 4°C.

DNA recovery

6) Wash plugs 5x with 10mL TE by shaking 15min at 180rpm.
7) Discard last TE wash. Scoop one plug, drain excess liquid & transfer to 1.5mL tube.
8) Melt 2min at 70°C. Equilibrate 5min at 43°C.
9) Add 2µL of 0.5U/µL Agarase & stir gently with pipet tip for ~10sec. Incubate 45min at 43°C.

Drop Dialysis / DNA homogenization

10) Dialyze DNA 45min on floating membrane in 15mL TE & transfer to 1.5mL tube.
11) If DNA is viscous, pipet mix up to 9x until entire sample is taken up in a continuous flow. Incubate overnight at room temp.

Day 3----------

Qubit Quantitation - BR dsDNA Assay

1) Pipet mix DNA 5x with wide bore tip, & transfer 2µL from top, middle & bottom to Qubit tubes containing 18µL BR Buffer.
2) Sonicate 10min in water bath, briefly spin, vortex 5sec at max speed & briefly spin.
3) Add 180µL Qubit BR Dye Reagent + Buffer mix. Vortex tubes continuously at max speed for 5 sec & briefly spin.
4) Set 5min at room temp & read on Qubit.
5) DNA ready for labeling.

Bionano Genomics, Inc.
9540 Towne Centre Drive, Suite 100
San Diego, CA 92121
Phone: (858) 888-7600
www.bionanogenomics.com