Bionano Prep High Polyphenols 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	2x 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 No 9001271 (990890) or equiv.
	Refrigerated centrifuge + swinging bucket - 4500xg	Eppendorf™ 5804R equipped with A-4-44 Rotor or equiv.
	High-strength glass centrifuge tubes, 15-16 mL, closures and adapter sleeves (for density gradient centrifugation only)	Kimble® 45600-15 High Strength Glass Class B 15 mL Centrifuge Tube + caps, or ThermoFisher Scientific Nalgene® 3117-0160 16 mL Round-Bottom Polycarbonate Centrifuge Tube 18 mm, ThermoFisher Scientific DS3111-0018 (VWR Cat No 21010-911 or equiv.); Kimble® 45550-15 Centrifuge Tube Rubber Adapter.
	Formaldehyde 36.5%-38% in H_2O	Sigma-Aldrich, Cat No F8775 or equiv.
	Spermidine trihydrochloride	Sigma-Aldrich, Cat No S2501 or equiv.
	Spermine tetrahydrochloride	Sigma-Aldrich, Cat No S1141 or equiv.
	β-mercaptoethanol (βME)	Sigma-Aldrich, Cat No M6250 or equiv.
	40 µm and 100 µm cell strainers	VWR Cat Nos 21008-949 and 21008-950 or equiv.
	50 mL conical tube(s)	VWR Cat No 21008-951 or equiv.
	15 mL conical tube(s)	VWR Cat No 21008-918 or equiv.
	Razor blades and Petri dish (square, 12 cmx 12 cm)	General lab supplier
	Plastic spatula	Corning Cat No 3005 or equiv.
	Filtered 200 µL tip, wide bore	VWR Cat No 46620-642 or Rainin 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 and 50 mL SmartBlock	Eppendorf Cat No 5382000023 and 5365000028 or equiv.
	Proteinase K enzyme, 5 mL	QIAGEN, Cat No 158920
	RNase A solution, 5 mL	QIAGEN, Cat No 158924 or equiv.
	Green screened caps for 50 mL conicals	Bio-Rad, Cat No 1703711 or equiv.
	Agarose plug mold and plug mold plunger	Bio-Rad, Cat No 1703713 or equiv.
	Agarose, 2% CleanCut Low Melting Point	Bio-Rad, Cat No 1703594 or equiv.
	Metal spatula	VWR Cat No 82027-530 or equiv.
DNA Recovery -Day 2	Agarase (0.5 U/µL)	ThermoFisher Scientific, Cat No EO0461
	Orbital shaker, 12 in x 12 in Platform	Cole-Parmer, catalog # EW-51820-30 or equivalent
	0.1 µm Dialysis membrane	Millipore Cat No VCWP04700 or equiv.
	Forceps for Membrane Placement	TDI International Catalog # TDI-2A-SA or Equivalant
	Petri dish, 6 cm x 6 cm	VWR Cat No 25384-092 or equiv.
	Non-filtered 200 µL tip	USA Scientific, Cat No 1111-1810 or Rainin equiv.
	TE Buffer, pH 8.0	ThermoFisher Scientific, Cat No AM9849 or equiv.
	Bath sonicator	General lab supplier
Quant -Day 3	Vortexer	General lab supplier
	Qubit Fluorometer 3.0	ThermoFisher Scientific Cat No Q33216 or equiv.
	Qubit BR Assay Kit	ThermoFisher Scientific Cat No Q32850 or equiv.
	Qubit Assay Tubes	ThermoFisher Scientific Cat No Q32856 or equiv.
	Metal spatula	VWR Cat No 82027-530 or equiv.

Introduction

The Bionano Prep Plant Tissue DNA Isolation Kit enables the isolation of megabase containing genomic DNA from plant tissue for next-generation mapping using the Irys® or Saphyr™ System. The kit provides critical reagents for high molecular weight (HMW) DNA isolation.

This protocol explains how to use the Bionano Prep Plant Tissue DNA Isolation Kit for the extraction of DNA from plants with high polyphenol content.

Overview

The Bionano Prep High Polyphenols Plant Tissue DNA Isolation protocol facilitates the recovery of high-quality HMW DNA from plant species rich in polyphenols. However, because of the diverse nature of polyphenols, and varying amount of complex metabolites in different plant species, this protocol may not work on some polyphenol-rich plants, requiring further optimization. The Bionano Prep High Polyphenols Plant Tissue DNA Isolation 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, which is needed to break tough plant cell wall, 2) density gradient purification of the resulting nuclei, 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 for most polyphenol-rich plant tissues is 0.5 grams of young fresh leaves, 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 products, which compromise DNA quality. The Bionano Prep High Polyphenols Plant Tissue DNA Isolation protocol can process up to 1 gram of leaf tissue rich in polyphenols. We have successfully purified sufficient high-quality HMW DNA from fresh young cotton leaves, *Eucalyptus* and grapevine leaves. Results may vary depending on the source tissue and plant species.

Estimated DNA Yield

Plant Species	Input Amount	Tissue Type	Tissue Storage	Tissue per Plug	# of Plugs	DNA Yield per Plug
Cotton	0.5 g	Young leaves	Fresh	167 mg	3	6.3 µg
Eucalyptus	1.0 g	Young leaves	Frozen	200 mg	5	4.8 µg
Grapevine	0.5 g	Young leaves	Fresh	167 mg	3	3.0 µg
Desired DNA output for optimal plug lysis performance is 3 - 6 µg. Adjust input tissue per plug accordingly.						

Important Notes

- Protocol Summary: Start with 0.5 g of fresh young leaves (unless otherwise specified in the previous table), fix in formaldehyde, and homogenize tissue by a rotor-stator homogenizer. Purify nuclei by a density gradient after performing a 2-minute low speed spin at 60xg, and cleaning nuclei by four extra washes until pellet is fresh yellow/light green and the supernatant is free of color & debris. Purified nuclei are then embedded in agarose for HMW DNA purification.
- Before starting, make sure all reagents and equipment are available for disrupting tissue and embedding homogenate in plug(s) for subsequent HMW DNA purification - see the 'User-Supplied Materials' section above. Be sure to add appropriate amount of Triton (part #20285) to the Plant Tissue Homogenization Buffer *plus*.
- Avoid using mature plant tissue as it is rich in polyphenols, other metabolites and waste products, which can complicate DNA recovery. Avoid using ≥ 1 gram of young leaf tissue rich in polyphenols as it will overload the density gradient system and reduce the purification efficiency thus resulting in low-quality DNA.
- 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.
- Perform 4x mandatory washes using Plant Tissue Homogenization Buffer *plus* (HB_+) to get a nuclei suspension that looks fresh yellow/light green or white. Additional washes may be needed when the pellet's color is dark green or the supernatant is colored and/or cloudy (i.e., debris) after the 4^{th} wash/spin-down.
- For density gradient purification a swinging bucket rotor capable of 4,500xg is essential to ensure proper nuclei banding in the density gradient. A fixed angle rotor is not recommended because centrifugation force has to be empirically determined assuming that plant debris, which are pushed to the side, do not collapse when tube is held upright to recover nuclei band, if any.
- Following density gradient purification, a nuclei band should be visible at the interface between the density gradients - Density Gradient I and Density Gradient II. A clear centrifuge tube is strongly recommended for the density gradient centrifugation.
- Recover the nuclei band in 0.5 - 1 mL after discarding the sample layer and Density Gradient I.
- DNA recovered by the Bionano Prep Plant Tissue DNA Isolation Kit requires 7-10 units of nicking enzyme Nt.BspQI, or 20-50 units of nicking enzyme Nb.BssSI, for efficient nick-labeling of 300ng DNA. This does not apply to direct DNA labeling with DLE-1. Follow the standard protocol.

Abbreviations

DG:	Density Gradient
DGI:	Density Gradient I
DGII:	Density Gradient II
DGB:	Density Gradient Buffer
FB:	Fixing Buffer
FS:	Fixing Solution
HB_+:	Plant Tissue Homogenization Buffer *plus*
HMW:	High Molecular Weight
PK:	Proteinase K
βME:	β-mercaptoethanol

High Polyphenols Plant Tissue DNA Isolation – Reference Card

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

Reagent preparation /setup

1) Prepare solutions for 1 prep and place on ice:
   - 250mL 1x FB
   - FS: 3.2mL formaldehyde + 56.8mL FB
   - HB+: 300.0mL Plant Tissue Homogenization Buffer + 0.6mL βME + 3.0mL spermine-spermidine + 7.5mL Triton
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 fresh 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 ice-cold FB.

Tissue disruption with a rotor-stator

9) Chop tissue into ~2x2mm, transfer to 50mL tube. Add 7.5mL HB_+ per 0.5g tissue, max. 15mL HB_+ for 1g tissue.
10) Blend 20sec & ice 30sec. Repeat up to 5x.
11) Filter through 100µm & 40µm cell strainers. Add HB_+ to 45mL.
12) Pellet nuclei 20min at 4°C:

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

13) Decant sup. & set 30sec on ice. Resuspend nuclei pellet with wet paint brush in residual HB_+. Add 3mL HB_+ & mix by swirling while touching ice.
14) Add 40mL HB_+, spin 2min 60xg 4°C, decant through 40µm strainer. Repeat 12-13.
15) Wash 4x with 35mL HB_+: after each wash, spin as per step 12, decant sup, resuspend nuclei pellet in residual buffer, add HB_+ to 35mL and repeat wash. After final wash resuspend nuclei in 3mL HB_+ as in step 13.

Density Gradient nuclei purification

16) Form a two-step density gradient:
    DGI: 2.6mL DG + 2.4mL DGB - top layer
    DGII: 3.6mL DG + 1.4mL of DGB – bottom layer
17) Add nuclei suspension. Spin 4,500g 40min 4°C.
18) Collect nuclei band at DGI-II interface (~1mL) to a 15mL conical tube and continue to step 19.

    If no band at DGI-II, with some debris in DGI/II or pellet at bottom, collect DGI-II interphase. Go to 19.

    If band only at DGI-sample interphase with no debris at DGI/II & no pellet at bottom, prep failed - contact Customer Solution team.

19) Add HB_+ to 14mL and spin 2,500g 10 min 4°C.
    If pellet at bottom & clear sup: Go to 20.
    If pellet at bottom & cloudy sup: Decant sup. resuspend pellet in 10mL HB_+, spin at 3,000g 10min, decant sup, and go to 20.
    If no pellet or pellet is loose re-spin at 3,000g 10min. Go to 20 if pellet is observed.
    If still no pellet, re-start with 1g tissue or contact Customer Solution team.
20) 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.
21) Incubate 3 minutes at 43°C.
22) Add 35µL agarose if 50µL nuclei suspension or 140µL agarose if 200µL nuclei suspension.
23) Pipet mix 3x, cast plugs & solidify 15min 4°C.

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

24) Transfer up to five plugs per 50mL tube.
25) Add 2.5mL Lysis Buffer + 200µL PK per tube.
26) Set 2hrs, or overnight at 50°C (+mixing).
27) 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 is ready for labeling.

Getting Started: High Polyphenols Plant Tissue DNA Isolation Protocol (0.5 g)

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

Before First Use

1. Plant Tissue 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 the expiration date.

Before Each Use

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

2. 250 mL 1x Fixing Buffer: 50 mL 5x 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 Tissue Homogenization Buffer *plus*: 300.0 mL Plant Tissue Homogenization Buffer + 0.6 mL βME + 3.0 mL 100 mM spermine-spermidine + 7.5 mL Triton.

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: Density Gradient Buffer, plug mold(s)/plunger, centrifuge with swinging bucket rotor and adapters.
9. Equilibrate a Thermomixer fitted with 50 mL adapter to 50°C for Proteinase K digestion.
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.

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.

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 (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 (~0.5 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 Tissue Homogenization Buffer *plus* to wet the plant material.
    ***Note**:* Adding too much Plant Tissue 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 ~2mm wide strips by holding tissue down with a 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 Tissue Homogenization Buffer *plus* per 0.5 grams of input plant tissue.
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 Tissue Homogenization Buffer *plus* before continuing. Repeat blending up to five additional 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 rounds of blending of 20 seconds each to generate the desired puréed consistency of homogenate (no visible particles bigger than 0.5x0.5 mm) for young leaves.
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 1,000 x g 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.

Nuclei Recovery / Washes (~2 hours)

23. Filter the 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 a plastic spatula, if necessary.
    b. Wash the material trapped in the cell strainer 3 times, with 2 mL ice-cold Plant Tissue 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.
24. Bring filtrate to a final volume of 45 mL by adding ice-cold Plant Tissue Homogenization Buffer *plus*, cap the tube and swirl gently by hand for 30 seconds while the tube is touching ice.
25. Pellet nuclei by centrifuging 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
2,000 x g	2 - 6 Gbp
2,500 x g	300 Mbp - 2 Gbp
3,500 x g	< 300 Mbp

26. 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.
27. Re-suspend the pellet with the assistance of a small paintbrush pre-soaked in ice-cold Plant Tissue Homogenization Buffer *plus*. Once re-suspended, add 3 mL ice-cold Plant Tissue Homogenization Buffer *plus* and swirl gently by hand for 30 seconds while the tube is touching ice.
28. Perform a low speed spin followed by a filtration step to clean the nuclei suspension:
    a. Add 40 mL ice-cold Plant Tissue 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 25-27 to pellet, decant supernatant, and re-suspend nuclei.

    ***Note**:* The low speed spin removes tissue residues, unbroken cells and cell debris. Please refer to Fig. 1 and Fig. 2 in Appendix II for pellet size and 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.

29. Perform a total of 4 washes using Plant Tissue Homogenization Buffer *plus*
    a. Slowly add 35 mL Plant Tissue Homogenization Buffer *plus* to the nuclei suspension from step 28, swirl gently to mix, and pellet as in step 25.
    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 to get the yellow/light green or white look (See Fig. 3 in Appendix II). After the final wash, resuspend the nuclei pellet in residual wash solution, add 3 mL ice-cold Plant Tissue Homogenization Buffer *plus* and swirl gently by hand for 30 seconds while the tube is touching ice.

Note: Color of the resulting nuclei suspension should look fresh yellow/light green or white. Additional washes are needed when the pellet's color is dark green and the supernatant is colored or cloudy after the 4th wash.

Nuclei Purification by Density Gradient (~1 hour)

30. Prepare the density gradient solutions (Density Gradient I and II):
    a. Before each use, gently mix Density Gradient by inverting back and forth several times to ensure a uniform suspension.
    b. To prepare Density Gradient I (DGI): Transfer 2.6 mL Density Gradient to a 15 mL tube, add 2.4 mL of Density Gradient Buffer, gently invert to mix until homogeneous and place on ice.
    c. To prepare Density Gradient II (DGII): Transfer 3.6 mL Density Gradient to a 15 mL tube, add 1.4 mL of Density Gradient Buffer, gently invert to mix until homogeneous and place on ice.
    d. Place Density Gradient Buffer on ice for resuspending pellet in step 38.
31. Prepare a step density gradient by transferring 4.5 mL of Density Gradient II to the bottom of a clean 15 mL clear centrifuge tube or 16 mL polycarbonate tube without introducing bubbles. Carefully and slowly lay 4.5 mL of Density Gradient I on top of Density Gradient II without disturbing the interface or generating bubbles.
32. Using a cut P1000 pipet tip (~1cm cut off from the tip head), carefully lay the ~3.5 mL nuclei suspension (from step 29) on top of Density Gradient I without disturbing the interface or generating bubbles. (See Fig. 4 in Appendix II)
33. Centrifuge at 4,500xg for 40 minutes at 4°C using a swinging bucket rotor with no deceleration (i.e., acceleration=9, deceleration=1).
    ***Note**:* If using the Kimble 15 mL glass centrifuge tube, make sure the glass tube is properly seated in its rubber adapter and is firmly supported at the bottom to prevent cracking during centrifugation.
34. After the centrifuge has completely stopped, examine the tube for a band at the interface between Density Gradient I and II.

Option	Visible Band at DGI-DGII Interface	Additional requirements	Action
1	Yes (Fig. 5a)	None	Collect band (go to step 35).
2	No (Fig. 5b)	Pellet at bottom of tube and/or debris in DGI or DGII layers.	Collect DGI-DGII interface (go to step 35).
3	No (Fig. 5c)	Band at sample-DGI interface No pellet at bottom of tube No debris in DGI & DGII layers.	Prep failed – contact Customer Solution team.

35. To recover nuclei band at DGI-DGII interface:
    a. Remove and discard the sample layer.
    b. Remove and discard the DGI layer leaving 2-3 mm on top of the nuclei band at DGI-DGII interface.
    c. Collect the nuclei band in about 0.5 mL to 1.0 mL using a cut P1000 pipet tip, and transfer to a pre-chilled 15 mL conical tube.
36. Add ice-cold Plant Tissue Homogenization Buffer *plus* to a total volume of 14 mL. Cap the tube, invert to mix and centrifuge at 2,500xg for 10 minutes at 4°C with no deceleration (i.e., acceleration=9, deceleration=1).
37. 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 38.
    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 Tissue 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 to step 38.
    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 38.

    Note*:* The pellet at this step is whitish/yellowish and often very loose.

    Note: if no pellet is observed even after step c - consider to re-start the isolation using 1 g fresh young leaves or consult customer support.
38. 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 1/8 – 1/4 inch diameter):
    a. If the pellet is small (e.g. size of a sesame seed), resuspend the nuclei pellet in 50 µL of ice-cold 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 Density Gradient Buffer (enough for about 3 plugs), and transfer 200 µL to a 1.5 mL microfuge tube using P200 wide-bore tip.

Note: Transferring exactly 50 or 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 39.

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 and not recommended when following the Bionano Prep High Polyphenols Plant Tissue DNA Isolation protocol and if using appropriate amount of young plant input tissues. Titration makes sense when nuclei pellet is big 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, following density gradient purification 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)

39. 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 2% Agarose
50 µL	35 µL
100 µL	70 µL
200 µL	140 µL

40. 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.
41. 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 in the 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.

42. Prepare fresh Proteinase K digestion solution by mixing 200 µL of Proteinase K enzyme with 2.5 mL of Lysis Buffer per 1-5 plugs to be processed in the same 50 mL conical tube.
    Note: Optional- adjust the Lysis Buffer to pH 9.0 by adding sodium hydroxide solution and add 5 µL βME to 2.7 mL Lysis Buffer containing Proteinase K during Proteinase K digestion process in the fume hood.
43. 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.

44. 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).
45. 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 Lysis Buffer per 1-5 plugs to be processed per tube (See note in step 42).
46. 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.
47. 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.

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

1. If gDNA was stored at 4°C, equilibrate to room temperature before use.
2. Equilibrate Qubit BR Assay kit standards to room temperature from 4°C.
3. Make 200 µl BR Working Solution for each sample assay tube (3 per sample) and each Qubit BR Assay Kit Standard (2 total) by diluting Qubit dsDNA BR Reagent in BR Buffer (1:200 dilution).
4. Aliquot 18 µL of Qubit BR Buffer per Qubit assay tube, preparing three tubes per sample.
5. Carefully pipet mix the entire volume of DNA 5 times with a Wide Bore Tip, avoid bubble formation.
6. Using a standard bore tip or positive displacement pipet, aspirate 2 µL from the top, middle and bottom of each DNA sample and dispense each draw into separate Qubit assay tubes (from step 2 above) following the methods described in the "Pipetting Viscous Genomic DNA" section, to ensure for accurate pipetting of viscous DNA for quantitation. Use a different tip for each draw.
7. Sonicate tubes in sonicating bath for 10 minutes to fragment DNA and then briefly spin to collect solution at bottom of tubes.
8. Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin to collect solution at bottom of tubes.
9. Add 180 µL of diluted Qubit BR Working Solution (Dye Reagent + Buffer). Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin tubes to collect solution at bottom of tubes.
10. Incubate samples, light-protected, for 2 minutes and proceed with quantitation on Qubit reader.
11. Coefficient of variation (CV) from the three separate readings should be < 0.25. (CV = standard deviation/mean).

    ***Note*:** If CV > 0.25, gently pipet mix the entire volume of DNA 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), pipet mix the entire volume of DNA 5 times with wide bore tip and let the DNA rest overnight at room temperature. Repeat quantitation of DNA the next day.

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 grapevine fresh young leaves (0.5 g, Day 1, step 25).

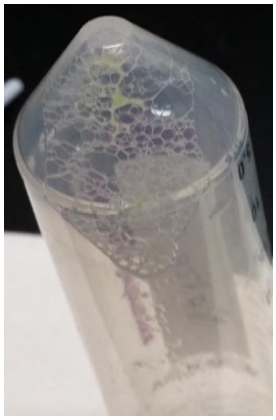

Fig. 2 Residuals left after the low speed spin (Day 1, step 28).

Fig. 3 Ideal pellet should have yellow/light green or white look before starting density gradient purification (Day 1, step 29).

Fig. 4 Density gradient assembly for nuclei purification (Day 1, step 32)

Nuclei suspensions are from four fresh young cotton leaf samples (left to right: 0.5 g, 1.0 g, 1.5 g and 2.0 g of input tissue).

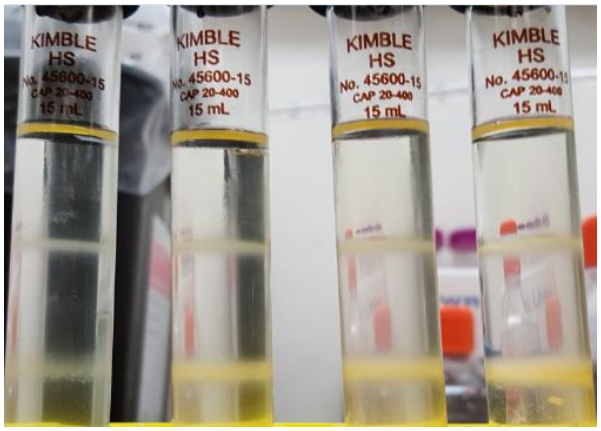

Fig. 5a

Fig. 5b

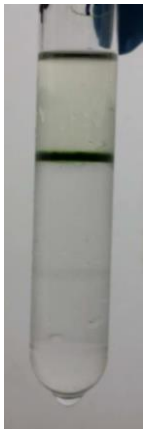

Fig. 5c

Fig. 5 Nuclei purification by density gradient (Day 1, step 34).

After the 4,500g 40 minutes centrifuge has completely stopped, examine the tube for a band at the interface between Density Gradient I and II (see Figure 5) a. Ideal interface bands (from cotton samples in Fig. 4, for demonstration purposes only) – collect the bands; b. Band is not visible but have residuals and white pellet on the bottom – collect the band; c. Band is not visible, no residuals nor pellet on the bottom and one band is observed above Density Gradient I.

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 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 Density Gradient Buffer, pipet mix using P200 wide-bore tip and label 0.5x (enough for 4 plugs).
c. To embed nuclei, add 35 µL of 43°C equilibrated agarose per 50 µL of nuclei suspension and cast plugs.

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