Bionano Prep Plant Tissue DNA Isolation Protocol – Liquid Nitrogen Grinding

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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	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 with caps, or ThermoFisher Scientific Nalgene® 3117-0160 16 mL Round-Bottom Polycarbonate Centrifuge Tube with closure 18 mm, ThermoFisher Scientific DS3111-0018 (VWR Cat No 21010-911 or equiv.); Kimble® 45550-15 Centrifuge Tube Rubber Adapter Sleeve
	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 cm x 12 cm)	General lab supplier
	Plastic spatula	Corning Cat No 3005 or equiv.
	Filtered 200µl tip	VWR Cat No 46620-642 or Rainin equiv.
	Small children paint brush, plastic	General supplier
	Heat blocks or water baths set to 43°C and 70°C	General lab supplier
	Thermomixer C	Eppendorf Cat No 5382000023 or equiv.
	50 mL SmartBlock	Eppendorf Cat No 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.
	Liquid nitrogen	General lab supplier
	Mortar and pestle	VWR Cat No 89038-148 and 89038-164 or equiv.
	Metal spatula	VWR Cat No 82027-530 or equiv.
	Filtered wide-bore pipette tip, 200 µl	VWR, Catalog # 46620-642 or Rainin equivalent

	Item	Description
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
	0.1 µm Dialysis membrane	Millipore Cat No VCWP04700 or equiv.
	Petri dish, 6 cm x 6 cm	VWR Cat No 25384-092 or equiv.
	Non-filtered 200µl 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.

Introduction

The Bionano Prep Plant 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 with the liquid nitrogen grinding method for the extraction of DNA from plants.

Overview

The Bionano Prep Plant Tissue DNA Isolation Protocol - Liquid Nitrogen Grinding (LNG protocol) is designed to meet customers' needs for high-quality HMW DNA purification from a wide variety of plant species. The protocol is a four step process that involves: 1) homogenization of plant material with liquid nitrogen grinding, 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 of plant tissue is 0.5 grams (max. 1.0 gram) of fresh young leaf tissue, or pre-weighed snap-frozen young leaf tissue, from seedlings or new sprouts from mature plants. Mature tissues are not recommended, as they tend to accumulate large amounts of metabolites and waste products, which compromise DNA quality.

Previously frozen tissue is best used within 3 months storage at -80 °C (plant species and tissue type dependent).

Estimated DNA Yield

Plant Species	Input Amount	Tissue Type	Tissue Storage	Tissue / Plug	# of Plugs	DNA Yield/Plug
Cotton	Grind 0.5 g, purify 0.1 g	Young leaves	Fresh	50 mg	2	2.5 µg
Cotton	Grind 0.5 g, purify 0.2 g	Young leaves	Frozen	66 mg	3	3.4 µg
Soybean	Grind 1 g, purify 1 g	Young leaves	Fresh	142 mg	7	11.9 µg
Soybean	Grind 0.5 g, purify 0.5 g	Young leaves	Fresh	71 mg	7	6.9 µg
Soybean	Grind 0.5 g, purify 0.2 g	Young leaves	Fresh	66 mg	3	5.2 µg

Important Notes

- Make sure that you have proper training in handling liquid nitrogen (LN) and proper protective equipment in accordance with your organization's Standard Operating Procedures.

- A Bionano Prep Plant Tissue DNA Isolation, Liquid Nitrogen Grinding Video Tutorial demonstrating proper grinding technique is available at: https://bionanogenomics.com/support-page/plant-dna-isolation-kit/

- 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. Pre-chill a small mortar (70 mm x 90 mm) and pestle in a -80°C freezer for at least 1 hour. Add Triton (part #20285) to increase Triton concentration if there is known high polyphenols content.

- Avoid using mature plant tissue as it is rich in metabolites and waste products; this can complicate DNA recovery.

- Start with 0.5 – 1.0 g fresh young plant leaves and carry forward (as little as 0.1 g equivalent) for nuclei purification. Pellet the nuclei by centrifugation based on genome size. Clean up nuclei by a total of four washes to get clear supernatant and yellow/light green/white pellet. Perform density gradient purification and embed nuclei into agarose. Homogenizing 0.5g or 1.0 g of tissue and carrying forward all of the tissue may not only require a series of dilutions before embedding into agarose, but also reduce the purification efficiency thus resulting in low-quality DNA.

- Grind the tissues (previous fresh weighed and snap-frozen young leaves may be used) with liquid nitrogen for 15-30 minutes until fine powder is obtained. For better grinding results, make sure to apply downward pressure (1.5 – 2.0 kg) to the pestle while grinding. Add liquid nitrogen frequently to avoid complete evaporation. Transfer the fine powder into a 50 mL conical tube and fully dissolve the powder into the Plant Tissue Homogenization Buffer *plus* (HB_+).

- One important indicator of high polyphenols content is a reddish/brown color starting after homogenization. However, it may be difficult to spot without a control prep (i.e. a low polyphenols content maize sample prep). A reddish/brown color of the plug during plug lysis is a strong indicator of high polyphenols content.

- 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 the 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 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, 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 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
HB_+:	Plant Homogenization Buffer *plus*
HMW:	High Molecular Weight
LN:	Liquid nitrogen
LNG:	Liquid nitrogen grinding
PK:	Proteinase K
βME:	β-mercaptoethanol

Plant Tissue DNA Isolation – Liquid Nitrogen Grinding – Reference Card

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

1) Prepare HB_+ & place on ice: 300.0mL HB Buffer + 0.6mL βME + 3.0mL spermine-spermidine + optional 7.5ml Triton if rich in polyphenols.
2) Pre-chill mortar & pestle in -80°C freezer for at least 1h.
3) Melt agarose at 70°C & equilibrate to 43°C.
4) Chill centrifuge and plug mold to 4°C.
5) Set Thermomixer to 50°C.

Liquid Nitrogen Grinding

6) Rinse fresh tissue. Remove petiole & mid rib.
7) Weigh 0.5g tissue and snap-freeze by liquid nitrogen. Previously frozen tissue (weighed before freezing) may be used if the tissue did not sustain cell damage when frozen.
8) Grind in LN for 15-30 min until fine powder.
9) Transfer powder to 50mL conical tube and re-suspend in 40mL HB_+.
10) Carry forward a fraction of suspension per table below to avoid overloading density gradient. Add HB_+ to 40mL. Input may need to be titered for other species.

Soybean	0.2 g eq.
Cotton	0.1 0.2 g eq.

11) Filter through 100µm & 40µm cell strainers.

Nuclei recovery / washes

12) Add HB_+ to 45mL and 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

13) Decant supernatant and set pellet on ice for 30sec. Resuspend nuclei with wet paint brush in residual HB_+ (~0.5ml). Add 3mL HB_+ & mix by swirling while touching ice.
14) Add 40mL HB_+, spin 2min 60xg 4°C and decant through 40µm strainer. Pellet and resuspend nuclei in 3mL HB_+.
15) Perform a total of 4 washes with 35mL HB_+ each time: 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).

If no band at DGI-II, with some debris in DGI/II or pellet at bottom, collect DGI-II interface. Go to 19.
If band only at DGI-sample interface with no debris in DGI/II & no pellet at bottom, prep failed – consult customer support.

19) Add HB_+ to 14mL. 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. If still no pellet, prep failed – consult customer support.

Embedding in agarose

20) Discard sup. & check pellet size. If small (e.g. sesame seed) resuspend in 50µL DGB & transfer 50µL to 1.5mL tube. If big (e.g. soybean seed), titration may be required. Otherwise re-suspend 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 Pipet mix 3x, cast plugs & solidify 15min 4°C.

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 45 min 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: Plant Tissue DNA Isolation Protocol – Liquid Nitrogen Grinding

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) Plant Tissue Homogenization Buffer *plus*: 300.0 mL Plant Tissue Homogenization Buffer + 0.6 mL βME + 3.0 mL 100 mM spermine-spermidine
Optional: + 7.5 mL Triton only if the plant species is known to be high in polyphenols. If you are not sure, omit the addition of the 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

3) Gather materials (see 'User Supplied Material' section above).
4) Set a heat block or water bath to 70°C. Set another heat block or water bath to 43°C.
5) Melt 2% agarose at 70°C for 15 minutes and then equilibrate to 43°C for at least 15 minutes.
6) Pre-chill to 4°C: Density Gradient Buffer, plug mold(s)/plunger, centrifuge with swinging bucket rotor and adapters. Pre-chill the mortar and pestle in a -80°C freezer for at least 1 hour.
Note: Use aluminum foil to wrap the cleaned and dried mortar and pestle before putting them into the -80°C freezer. You could pre-chill them the night before the nuclei isolation. Avoid using detergent to clean the mortar and pestle – clean them with brush under tap water and rinse with DI water.
7) Equilibrate a Thermomixer fitted with 50 mL adapter to 50°C for Proteinase K digestion.

Liquid Nitrogen Grinding (~0.5 hours, reference Bionano Prep Plant Tissue DNA Isolation, Liquid Nitrogen Grinding Video Tutorial, located here: https://bionanogenomics.com/support-page/plant-dna-isolation-kit/)

Wear protective cryogenic liquid handling gloves, protective goggles, lab coat and closed toe shoes, referencing your organization's Standard Operating Procedures, while handling liquid nitrogen experiments.

8) If plant tissue is muddy, rinse with distilled water and gently pat dry with a paper towel without bruising the tissue.
9) Using a razor blade, remove the petiole and midrib, if applicable.
10) Prepare ~3 L liquid nitrogen for easy access as frequent additions during the grinding process are required to keep tissue frozen to prevent DNA degradation.
11) Take out the mortar and pestle from -80 °C. Secure mortar in fresh ice by submerging it half way through the ice to anchor mortar when grinding. Put the pestle in the mortar and slowly pour liquid nitrogen into the mortar to ~2/3 volume; let liquid nitrogen evaporate. Repeat this chilling process 2-3 times to reach LN temperature.
12) Weigh 0.5 grams fresh young leaves and snap-freeze in the mortar containing liquid nitrogen. Add liquid nitrogen to fully submerge and freeze the input tissue.
Note: Previously weighed and snap-frozen fresh young leaves may be used. Clean and process tissue as in steps 9 - 11 followed by pre-weighing the tissue before snap-freezing. Frozen tissue can be stored up to 3 months at -80 °C (plant species and tissue dependent - tissue must not sustain cell damage when frozen). If frozen tissue is used, make sure the tissue is immediately submerged in liquid nitrogen in the mortar immediately after removing it from the -80 °C freezer.
13) To avoid liquid nitrogen spilling, slowly crush tissue by gently pounding the sample with the pestle. Gently rock the pestle across the bottom of the mortar, and move the pestle using a circular motion until the tissue pieces are smaller than 5 mm x 5 mm. Add liquid nitrogen frequently to keep all the tissue submerged in liquid nitrogen.
14) Apply pressure on the pestle (1.5 – 2.0 kg) while grinding the tissue until the liquid nitrogen is completely evaporated – when the tissue becomes loose and lighter in color, add liquid nitrogen to 1/3 – 1/2 mortar volume and resume the same steady grinding until the liquid nitrogen completely evaporates again. Repeat at least 3 times until "wheat flour like" very fine tissue powder is obtained. (See Fig. 1 in Appendix II). The whole grinding process takes about 15 - 30 minutes depending on the grinding pressure applied to the pestle.
Note: Add liquid nitrogen frequently to avoid tissue thawing –if the dry light green powder turns back to dark green muddy powder after the liquid nitrogen completely evaporates, the tissue is thawing. Restart from step 8.
Note: When adding liquid nitrogen, leave the pestle in the mortar. Carefully and slowly pour the liquid nitrogen on the pestle to a) rinse the pestle and b) avoid flushing dry powder out of the mortar.
15) Add 40 mL Plant Tissue Homogenization Buffer *plus* to a 50 mL conical tube and sit on ice.
16) Scrape the dry powder off the pestle with a liquid nitrogen pre-chilled metal spatula. Dislodge and pile the powder from the mortar. Carefully and slowly transfer the fine tissue powder to the 50 mL conical tube prepared in step 15.
Note: The mixture tends to bubble vigorously and spew out the powder when the tissue is added too quickly.

Note: Put the mortar and pestle at room temperature for 0.5 hours before cleaning. Do not use detergent. Brush the mortar and pestle under tap water and rinse with DI water. Air-dry and wrap with aluminum foil.

17) Gently swirl the tube while placing on ice with a specular and make sure the tissue powder is fully dissolved in Plant Tissue Homogenization Buffer *plus*.
18) Transfer a defined amount of homogenate, for example, equivalent to 0.1- 0.2 g of starting tissue, to a new 50 mL tube. Add Plant Tissue Homogenization Buffer *plus* to a final volume of 40 mL.

Tissue	Input amount	Amount to carry forward
Soybean	0.5 g	0.2 g
Cotton	0.5 g	0.1 g or 0.2 g

Nuclei Recovery / Washes (~2 hours)

19) 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.
20) 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.
21) 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

22) 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: There is usually approximately 0.5 mL remaining supernatant at the bottom.
23) Resuspend the pellet with the assistance of a small paintbrush presoaked in ice-cold Plant Tissue Homogenization Buffer *plus*. Once resuspended, add 3 mL ice-cold Plant Tissue Homogenization Buffer *plus* and swirl gently by hand for 30 seconds while the tube is touching ice.

24) 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 21-23 to pellet, decant supernatant, and resuspend nuclei.

    ***Note**:* The low speed spin removes tissue residues, unbroken cells, and cell debris. Please refer to Fig. 2 and Fig. 3 in Appendix II for pellet size and the debris from the low speed spin.

    ***Note**:* When performing the 40 µm cell strainer filtration at step 24c, 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.
25) 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 previous step, swirl gently to mix, and pellet as in step 21.
    b. Discard supernatant and resuspend the nuclei pellet in residual buffer, with the assistance of a pre-chilled wet paint brush.
    c. Repeat steps a-b 3 times to get the yellow/light green or white look (See Fig. 4 in Appendix II). After the final wash, resuspend the nuclei pellet in residual buffer, 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: The 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)

26) 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.
27) Prepare a step density gradient by transferring 4.5 mL of Density Gradient II to the bottom of a clean 15 mL clear glass centrifuge tube or 16 mL polycarbonate tube without introducing bubbles. By using a P1000 pipet tip, carefully and slowly lay 4.5 mL of Density Gradient I on top of Density Gradient II without disturbing the interface or generating bubbles.

28) Using a cut P1000 pipet tip (~1cm cut off from the tip head), carefully lay the ~3.5 mL nuclei suspension (from step 25) on top of Density Gradient I without disturbing the interface or generating bubbles. (See Fig. 5 in Appendix II)
29) 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**:* Make sure the glass tube is properly seated in its rubber adapter and is firmly supported at the bottom to prevent cracking during centrifugation.
30) After the centrifuge has completely stopped, examine the tube for a band at the interface between Density Gradient I and II. (See Fig. 6 in Appendix II)

Options	Visible Band at DGI-DGII interface	Additional requirements	Action
1	Yes (Fig. 6a)	None	Collect band (go to step 31).
2	No (Fig. 6b)	Pellet at bottom of tube and/or debris in DGI or DGII layers.	Collect DGI-DGII interface (go to step 31).
3	No (Fig. 6c)	Band at sample-DGI interface No pellet at bottom of tube No debris in DGI & DGII layers.	Prep failed – contact Customer Solution team.

31) To recover nuclei band at DGI-DGII interface:
    a. Remove and discard the sample layer by a P1000 pipet tip.
    b. Remove and discard the DGI layer by a P1000 pipet tip, 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 an ice-chilled 15 mL conical tube.
32) 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).
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 Tissue Homogenization Buffer *plus*, cap the tube and resuspend 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 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 whitish/yellowish and often very loose.
    Note: if no pellet is observed even after step 33c, prep failed - 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 Density Gradient Buffer (enough for one plug), and transfer 50 µL to a 1.5 mL microfuge tube using P200 wide-bore tip.
    b. If the pellet is large (e.g. size of a soybean seed) 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 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: 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 matures, 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 should be executed with caution as DNA concentration following melt/Agarase treatment can be compromised (recommended >35 ng/µL to support DNA labeling).

Embedding in Agarose (~0.5 hours)

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

36) Gently pipet mix three times with a P200 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 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 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.

Day 2: RNase Digestion / DNA Stabilization Washes / DNA Recovery / Drop Dialysis

Set Up

1) Gather materials (see 'User Supplied Materials' section above).
2) Set up heat blocks/water baths:
   a. Set a heat block or water bath to 70°C for melting of plug(s).
   b. Set another heat block or water bath to 43°C for Agarase treatment of plug(s).

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, 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)

5) During RNase digestion, prepare 70 mL of 1X Wash Buffer for each 50 mL conical tube, using the 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 three 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 four times by:
   a. Adding 10 mL of 1x Wash Buffer through the screened cap and capping tube.
   b. Gently shaking tube for 15 minutes on a horizontal platform mixer 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 4 months at 4°C without significant degradation of DNA quality.

DNA Recovery (~2.5 hours)

9) Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
10) Wash plugs **5 times**, by adding 10 mL of TE Buffer per wash through the screened cap, capping tube, gently shaking for 15 minutes on a horizontal platform mixer with continuous mixing at 180 rpm, discarding the wash through the screened cap, and 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 microfuge 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 and incubate for 5 minutes.
16) Processing one tube at a time: add 2 µL of 0.5U/µL Agarase enzyme to tube and stir mixture gently with a pipet tip for ~10 seconds.
17) Incubate tube(s) at 43°C for 45 minutes.

Drop Dialysis to Clean the DNA (~1 hour)

18) Place 15 mL of TE Buffer into 6cm Petri dish per each DNA sample.
19) Float a 0.1µm dialysis membrane on the surface of the TE Buffer. Place a cover on the Petri dish and let the membrane wet for 10 minutes.
    Note: Use forceps to grip the membrane by the edge and gently place it on the surface of the TE Buffer while keeping the membrane in a horizontal position to prevent dipping or sinking of membrane during application. According to the membrane manufacturer, applying either side of the membrane on the surface of TE Buffer should not impact performance.
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 microfuge tube with a wide bore tip.
    Note: Do not pipet mix the DNA while on the membrane.
    Note: DNA solution should be free of visible debris (See Fig. 7 in Appendix II)

Homogenization of DNA Solution (~overnight)

23) Test DNA viscosity by slowly withdrawing the entire DNA volume into a non-filtered P200 tip while slowly releasing pipet plunger. If the DNA is not viscous, do not pipet mix. If the DNA is 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.

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: DNA Quantitation / DNA labeling

Qubit Quantitation - BR dsDNA Assay (~30 minutes)

1) Equilibrate Qubit BR Assay kit standards to room temperature from 4°C.
2) Aliquot 18 µL of Qubit BR Buffer per Qubit assay tube, preparing three tubes per sample.
3) Carefully pipet mix the entire volume of DNA 5 times with a wide bore tip to avoid bubble formation.
4) Using a standard bore tip, pipet 2 µL from the top, middle and bottom of each DNA and dispense each draw into separate Qubit assay tubes (from step 2 above) following the methods described in 'Pipetting Viscous DNA' (see Appendix section below) to ensure accurate pipetting of viscous DNA for quantitation. Use a different standard bore tip for each draw.
5) Sonicate tubes in sonicating bath for 10 minutes and then briefly spin to collect solution at bottom of tubes.
6) Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin to collect solution at bottom of tubes.
7) Add 180 µL of diluted Qubit BR Dye Reagent + Buffer mix. Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin tubes to collect solution at bottom of tubes.
8) Incubate samples on bench top for 5 minutes and proceed with quantitation on Qubit reader.
9) Coefficient of variation (CV) from the three separate readings should be <25%.
   ***Note*:** If CV > 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 P200 tip (e.g. USA Scientific, Cat No 1111-1810 or equivalent), pipet mix the entire volume of DNA 5X 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 pipet 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 Fine powder of 0.5 grams of fresh young cotton leaves by liquid nitrogen grinding (Day 1, step 14).

Fig. 2 Pellet nuclei using a swing bucket rotor on grapevine fresh young leaves (0.2 g, Day 1, step 21).

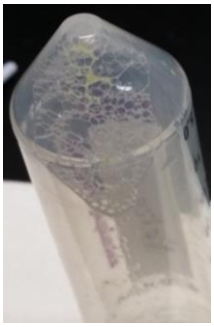


Fig. 3 Residuals left after the low speed spin (Day 1, step 24).

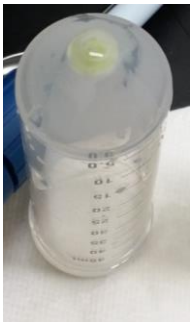
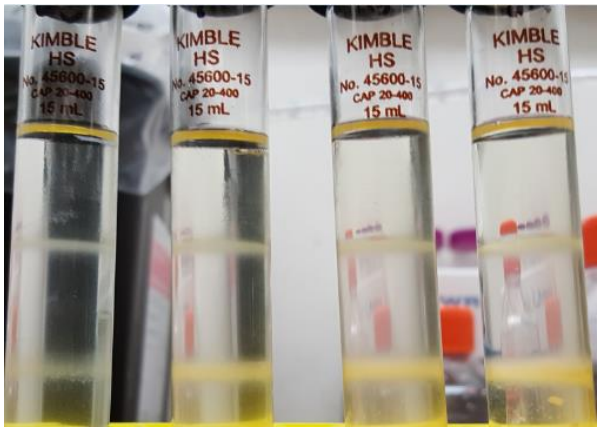


Fig. 4 Ideal pellet should have yellow/light green or white look before starting density gradient purification (Day 1, step 25)

Fig. 5 Density gradient assembly for nuclei purification (Day 1, step 28)

Nuclei suspensions are from four fresh young cotton leaf samples.



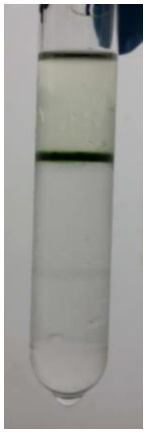


Fig. 6a Fig. 6b Fig. 6c

Fig. 6 Nuclei purification by density gradient (Day 1, step 30).

After the 4,500g 40 minutes centrifugation has completely stopped, examine the tube for a band at the interface between Density Gradient I and II. Fig. 6a. Ideal interface bands (from cotton samples in Fig. 5, for demonstration purposes only) – collect the bands; Fig. 6b. Band is not visible but have residuals and white pellet on the bottom – collect the band; Fig. 6c. Band is not visible, no residuals in DGI/II nor pellet on the bottom and one band is observed above Density Gradient I – prep failed and consult customer support.

Fig. 7 Purified DNA should be free of visible debris (Day 2, step 22). If debris is observed, consult customer support

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 the rest of 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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