



Bionano Prep™ Plant Tissue DNA Isolation Base Protocol

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Experienced User Card – Base Plant Protocol

DAY 1

- 1) Prepare solutions for 1 prep and chill on ice:
 - 250 ml 1x Fixing Buffer (FB)
 - 60 ml Fixing Solution (FS): 3.2 ml formaldehyde + 56.8 ml FB
 - *HB₊*: 150 ml Plant Homogenization Buffer + 0.3 ml β ME + 1.5 ml 100 mM spermine-spermidine + optional 3.75 ml Bionano Prep Triton
- 2) Melt agarose at 70°C and equilibrate to 43°C.
- 3) *Chill centrifuge, adaptors, square petri dish, density gradient buffer, and plug mold.*
- 4) *Set Thermomixer to 50°C.*
- 17) Lay nuclei suspension on top of DGI and spin 4,500 x g 40min 4°C.
- 18) Collect nuclei band at DGI-II interface, ~1 ml.

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, no debris in DGI/II & no pellet at bottom, recover band, add *HB₊* to 40ml, and swirl on ice to mix. Transfer 0.2g equivalent to new 50ml tube, add *HB₊* to 35 ml and pellet as in 12. Repeat wash for a total of 4 washes including optional washes at 15. After final wash resuspend nuclei pellet in 3ml *HB₊* & go to 19.*

Tissue fixation

- 5) Rinse tissue. Remove petiole and mid rib.
- 6) Weigh 1-2 grams of tissue and transfer to 50 ml tube.
- 7) Fix in 60 ml FS 20 min on ice.
- 8) Wash tissue 3 times with 50 ml cold FB.

Tissue disruption with a rotor-stator

- 9) Cut tissue into approx. 2 x 2 mm, transfer to 50 ml tube. Add 7.5 ml *HB₊* per gram of tissue.
- 10) Blend 20 sec and then, ice 30 sec. Repeat up to 5 times.
- 11) Filter through 100 μ m and 40 μ m cell strainers. Add *HB₊* to 45 ml.
- 12) Pellet nuclei 20 min at 4°C:

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

- 13) Decant supernatant and stand tube on ice for 30 sec. Resuspend nuclei pellet with pre-wet paint brush in residual *HB₊*. Add 3 ml *HB₊* and swirl to mix on ice.
- 14) *Add 40 ml *HB₊*, spin 2min 60 x g at 4°C, decant through 40 μ m strainer. Repeat steps 12-13.
*Skip this step if <0.5 g of very young tissue, very small pellet at step 12, and no high polyphenols-polysaccharides-metabolites content;
- 15) **Optional:** Wash up to 4 times with 35 ml *HB₊* \pm Triton. Re-suspend nuclei pellet as in 13.

Density Gradient purification of nuclei

- 16) *Setup a two-step density gradient:*
DGI (top layer): 2.6 ml DG + 2.4 ml DGB
DGII (bottom layer): 3.6 ml DG + 1.4 ml DGB

- 19) Add *HB₊* to 14 ml. Spin 2,500 x g 10 min 4°C. If pellet at bottom with clear supernatant: Go to 20. If pellet at bottom with cloudy supernatant: Decant supernatant, resuspend pellet in 10 ml *HB₊*, spin 3,000 x g 10 min, decant supernatant, and go to step 20. If no pellet or loose pellet re-spin 10 min at 3,000 x g. If no pellet, restart using Bionano Prep High Polysaccharides Plant Tissue DNA Isolation Protocol.

Embedding in agarose

- 20) Discard supernatant & check pellet size. If small (e.g. sesame seed) re-suspend in 50 μ l DGB & transfer 50 μ l to 1.5 ml tube. If big (e.g. soybean seed), titration may be required. Otherwise resuspend in 200 μ l DGB & transfer 200 μ l.
- 21) *Incubate 3 minutes at 43°C.*
- 22) Add 35 μ l agarose per 50 μ l nuclei suspension.
- 23) Pipette mix 3x, cast plugs & solidify 15 min 4°C.

Proteinase K (PK) digestion

- 24) For each sample, make PK solution (up to 5 plugs): 200 μ l Proteinase K + 2.5 ml Lysis buffer.
- 25) Dislodge plugs into PK Solution, cap and incubate for 2 hours at 50°C with intermittent mixing (or overnight first if desired).
- 26) After 2 hours, replace PK solution with freshly made PK solution, incubate overnight at 50°C with intermittent mixing (or overnight incubation followed by 2 hours).

DAY 2

- 1) Set heat sources to 70°C & 43°C.
- 2) Add 50 µl of RNase A to PK solution. Incubate 1hr at 37°C with intermediate mixing.

Washing plugs to stabilize DNA

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

DNA recovery

- 6) Wash plugs 5 times with 10 ml 1x TE by shaking 15 min at 180 rpm.
- 7) Discard last TE wash. Scoop one plug, drain excess liquid & transfer to 1.5 ml tube.
- 8) Incubate 2min at 70°C and then equilibrate 5min at 43°C.
- 9) Add 2 µl of 0.5U/µl Agarase & stir gently with pipet tip for 10sec.
- 10) Incubate 45min at 43°C.

Drop Dialysis / DNA homogenization

- 11) Dialyze DNA for 45 min (room temp.) on floating membrane in 15 ml 1x TE & transfer to 1.5 ml tube.
- 12) If DNA is viscous, pipet mix up to 9 times until entire sample is taken up in a continuous flow. Incubate overnight at room temp.

DAY 3

Qubit Quantitation - BR dsDNA Assay

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

Bionano Prep™ Plant Tissue DNA Isolation Kit Contents RE-014-05 (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 handling and homogenization – Day 1; Plug lysis – Day 1 and 2	
Rotor-stator homogenizer (+ disposable probes)	QIAGEN, cat. # 9001271 (990890) or equivalent
Refrigerated centrifuge + swinging bucket - 4500xg	Eppendorf™ 5804R equipped with A-4-44 Rotor or equivalent
High-strength clear 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 included), Nalgene® 3117-0160 16 ml Round-Bottom Polycarbonate Centrifuge Tube with closure 18, VWR cat. # 21010-911 or equivalent; Kimble® 45550-15 Centrifuge Tube Rubber Adapter Sleeve or equivalent
Formaldehyde 36.5%-38% in H ₂ O	Sigma-Aldrich, cat. # F8775 or equivalent
Spermidine trihydrochloride	Sigma-Aldrich, cat. # S2501, 85578, 85580 or equivalent
Spermine tetrahydrochloride	Sigma-Aldrich, cat. # S1141, 85605, S2876 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
Petri dish (square, 12 x 12 cm)	General lab supplier
Razor blades	General lab supplier
Plastic spatula	Corning catalog # 3005 or equivalent
Filtered pipette tip, 200 μl	VWR cat. # 46620-642 or equivalent
Small children paint brush	General supplier
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 molds and plug mold plungers	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 equivalent
DNA Recovery – Day 2	
Metal spatula	VWR cat. # 82027-530 or equivalent
GELase™ Agarose Gel-Digesting Preparation, 0.2U/μL	Epicentre, Catalog # G31200
Agarase (0.5 unit/μl) (alternative to GELase)	ThermoFisher, Catalog # EO0461
0.1 μm Dialysis membrane, 47 mm diameter	Millipore cat. # VCWP04700 or equivalent
Petri dish, 60 x 15 mm	VWR cat. # 28384-092 or equivalent
TE Buffer, pH 8.0	ThermoFisher Scientific, cat. # AM9849 or equivalent
Non-filtered, beveled pipette tip, 200 μl	USA Scientific, cat. # 1111-1810 or equivalent

DNA Quantification – Day 3	
Bath sonicator	VWR cat. # 82027-530 or equivalent
Vortexer	ThermoFisher, Catalog # EO0461
Qubit Fluorometer 3.0	Millipore cat. # VCWP04700 or equivalent
Qubit BR Assay Kit	VWR cat. # 28384-092 or equivalent
Qubit Assay Tubes	USA Scientific, cat. # 1111-1810 or equivalent

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.

Overview

The Bionano Prep 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 of plant tissue is 1 - 2 grams of fresh young leaf tissue, preferably from seedlings, or new sprouts from mature plants. Fully mature tissues are not recommended, as they tend to accumulate a large amount of metabolites and waste product, which compromises DNA quality. The Bionano Prep Plant Tissue DNA Isolation protocol can process up to 3 grams of leaf tissue starting material; however, this amount is not recommended for species like trees and woody plants that are rich in metabolites. We have successfully purified sufficient high-quality HMW DNA prepared from as low as 0.5 grams of fresh young leaves of maize seedlings, new sprouts or flower petals from mature tomato plants. 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	1.3 g	Young leaves	Fresh	400 mg	3	9.5 µg
Tomato	0.7 g	Young leaves	Fresh	150 mg	4	5.8 µg
<i>Eucalyptus</i>	2.0 g	Young leaves	Frozen	600 mg	3	9.3 µg
<i>Arabidopsis</i>	1.0 g	Flower	Frozen	300 mg	3	4.4 µg

Important Notes

- 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 tissues. They are rich in metabolites and waste products that can complicate DNA recovery.
- Cut plant tissue into approximately 2 x 2 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.
- After blending, pellet the nuclei by centrifugation based on genome size.
- To minimize loss of DNA yield, skip the 2-minute low speed spin at step 28 if (1) starting material is young tissue < 0.5 g, (2) very small nuclei pellet (half of the soybean seed size, 1/8 inch in diameter, or smaller), **and** (3) tissue is not known to contain high levels of polyphenols, polysaccharides, or metabolites.
- Perform additional washes using Plant Homogenization Buffer *plus* (HB₊) if the pellet is dark green in color, or if the supernatant is colored or cloudy.
- If plant tissue is rich in polyphenols, add Bionano Prep Triton in HB₊ at Step 5 and/or Step 29 and perform up to four additional washes to get a nuclei suspension that is light greenish-yellow to white in color.. One important indicator of high polyphenols content is a reddish/brown color 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 plug during plug lysis is also a strong indicator of high polyphenols content. Abandon the prep if a reddish/brown color is observed and re-start the prep using plant polyphenol protocol.
- For density gradient purification a swinging bucket rotor capable of 4,500 x g is essential to ensure proper nuclei banding in the density gradient. Fixed angle rotors are strongly not recommended because centrifugation force has to be empirically determined assuming that the plant debris, which are pushed to the side, do not collapse when tube is held upright to recover nuclei band.
- The use a clear centrifuge tube during the density gradient purification steps is strongly recommended so as to ascertain the nuclei band (if any) at the interface between the density gradients, Density Gradient I and Density Gradient II.
- 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 will require optimization.

Abbreviations

βME:	β-mercaptoethanol	FS:	Fixing Solution
DG:	Density Gradient	HB ₊ :	Plant Homogenization Buffer <i>plus</i>
DGI:	Density Gradient I	PK:	Proteinase K
DGII:	Density Gradient II	Spm:	Spermine
DGB:	Density Gradient Buffer	Spd:	Spermidine
FB:	Fixing Buffer		

Getting Started: Base Plant Tissue Protocol (up to 3g)

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

Before First Use

- 1) Bionano Prep **Plant Homogenization Buffer (HB)** 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.
- 2) Prepare a **spermine-spermidine (Spm-Spd)** stock solution containing both 100 mM spermine and 100 mM spermidine in molecular biology grade water. Aliquot into 1.5 ml tubes and store at -20°C. Discard after 3 months and make a fresh stock.

Note: For 10 ml 100 mM Spm-Spd stock solution, weigh & dissolve 255 mg spermine (Sigma, cat. #S2501) + 348 mg Spermine (Sigma, cat. #S1141) in 8 ml molecular biology grade water. Top up to 10 ml. Discard after 3 months and make a fresh stock.

- 3) For first-time use of agarose included in Bio-Rad kit, melt entire bottle in microwave-boiled water for 15 minutes. Then aliquot melted agarose into 500 µl aliquots in microfuge tubes that can be stored at 4°C. Use one or more 500 µl aliquots for each new procedure.

Before Each Use

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

- 4) 250 ml **1x Fixing Buffer**: 50 ml **5x Bionano Prep Fixing Buffer** + 200 ml molecular biology grade water.
- 5) 60 ml **Fixing Solution**: 3.2 ml **formaldehyde** + 56.8 ml **1x Fixing Buffer**.
- 6) **Plant Homogenization Buffer plus (HB₊)**, as follows:
 - 150 ml **Bionano Prep Plant Homogenization Buffer**
 - + 300 µl **βME**
 - + 1.5 ml **100 mM Spm-Spd** stock solution (prepared in Step 2)
 - Optional:* + 3.75 ml **Bionano Prep Triton**

Note: Add 3.75 ml **Bionano Prep Triton** to Plant Homogenization Buffer *plus* only if the plant species is known to be high in polyphenols. If you are not sure, omit the Bionano Prep Triton.

Set Up

- 7) Set a heat block or water bath to 70°C and another to 43°C.
- 8) Melt 2% agarose at 70°C for 15 minutes and then equilibrate to 43°C for at least 15 minutes.
- 9) Pre-chill to 4°C: Bionano Prep Density Gradient Buffer, 12 x 12 cm square petri dish, plug mold(s)/plunger, centrifuge with swinging bucket rotor and adapters.
- 10) 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.

- 11) If plant tissue is muddy, rinse with distilled water and gently pat dry with a paper towel without bruising the tissue.
- 12) Using a razor blade, remove the petiole and midrib, if applicable.

- 13) Weigh 1-2 grams of tissue, 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.
- 14) 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.
- 15) 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)

- 16) Transfer fixed tissue with a spatula to a pre-chilled 12 x 12 cm **square Petri dish**. Add 4 ml ice-cold **Plant Homogenization Buffer plus (HB+)** to wet the plant material.
Note: Adding too much *Plant Homogenization Buffer plus* could result in splashing during chopping.
- 17) Manually cut plant tissue into approximately 2 x 2 mm pieces with a sharp razor blade:
 - a. First cut leaves into strips (approximately 2 mm wide) 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 until desired size achieved.
Note: When cutting tough plant tissues replace the razor blade as needed. Manual cutting into 2 x 2 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.
- 18) Transfer the chopped tissue into 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.
- 19) Add 7.5 ml ice-cold **Plant Homogenization Buffer plus per gram of input plant tissue**.
- 20) 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.
- 21) 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*. Refer to manufacturer's instruction/ manual for proper operation of instrument.
- 22) 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.
- 23) 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 cycles of 20 seconds each to generate the desired puréed consistency of homogenate (no visible particles bigger than 0.5 x 0.5 mm) for young maize leaves and tomato leaves/flowers.

Note: 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 (1 - 2 hours)

24) 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, if necessary.
- 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.

- 25) 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.
- 26) Pellet nuclei by spinning filtrate for 20 minutes at 4°C using a **swing bucket rotor**. Set *g* force based on genome size:

<i>g</i> force	Genome Size
1,200xg	>6 Gbp
2,000xg	2-6 Gbp
2,500xg	300 Mbp - 2 Gbp
3,500xg	<300 Mbp

Note: Often nuclei pellet has two layers: a white/opaque layer on the bottom and a green layer on top. See Fig. 1a in Appendix II.

- 27) Check the size of the pellet and clarity of the supernatant. Without disturbing the pellet, carefully discard the supernatant by decanting and set back on ice for 30 seconds to allow the remaining supernatant (approximately 0.5 ml) to accumulate at the bottom of the tube.
- 28) Re-suspend the pellet in residual supernatant with 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.
- 29) Only skip this step if following criteria are met:
- I. Starting material was less than 0.5 gram of very young tissue;
 - II. The pellet in Step 26 was 3 mm or smaller in diameter; **AND**
 - III. The plant is not known to have high levels of polyphenols, polysaccharides, or metabolites.

Otherwise, 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 60 x g at 4°C for 2 minutes with lowest or no deceleration (e.g. acceleration=9, deceleration=1) to pellet intact cells, cell debris, unbroken tissue and other residues.
- c. Without disturbing the pelleted debris, pour the supernatant through a **40 µm cell strainer** and collect the filtrate in a new chilled 50 ml tube.
- d. Repeat steps 25-27 to pellet, decant supernatant, and re-suspend nuclei.

Note: Refer to Fig. 1a and 1b in Appendix II for pellet sizes from Step 26. For larger pellet similar to Fig. 1a, it is highly recommended to perform the low speed spin; for smaller pellet as in Fig. 1b, skip this step if starting material is <0.5 gram of very young leaves and is not known to have high levels of polyphenols, polysaccharides, or metabolites. 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.

Note: The resulting nuclei suspension should be light yellow, light green, or white in color.

Optional Washes:

- 29) If the supernatant is not clear from step 26, or the pellet and/or nuclei suspension from steps 27 or 28 is 'darker' green, or the plant species is known to have high polyphenol content such as wild cotton varieties, perform up to four washes with **Plant Homogenization Buffer plus** (with or without **Bionano Prep Triton** added) to clarify the supernatant or remove the darker green color (steps a-d below).

For plants with high polyphenol content such as cotton and *Eucalyptus* addition of Bionano Prep Triton is recommended, bringing final Triton concentration to 1% (original concentration in plant homogenization buffer is 0.5%). Excessive or indiscriminate use of Triton can damage nuclear membrane and is not recommended.

- a. *To carry out additional washes, prepare up to 140 mL (for four 35 ml washes) of Plant Homogenization Buffer plus as in step 4, and place on ice. If Triton is to be added follow the table below.*

# washes	Plant Homogenization buffer plus	Bionano Prep Triton
1	35 mL	0.9 mL
2	70 mL	1.8 mL
3	105 mL	2.7 mL
4	140 mL	3.6 mL

- b. Slowly add 35 ml *Homogenization Buffer plus* (with optional Bionano Prep Triton) to the nuclei suspension from step 27 or 28, swirl gently to mix, and pellet as in step 25.
- c. Discard supernatant and re-suspend the nuclei pellet in residual wash buffer, with the assistance of a wet paint brush.
- d. Repeat Step b-c up to 3 times until supernatant is clear and pellet is yellow, light green, or white (See Fig. 3 in Appendix II). After the final wash, re-suspend the nuclei pellet in residual liquid, add 3 mL ice-cold *Plant Homogenization Buffer plus* and swirl gently by hand for 30 seconds while the tube is touching ice. Proceed to Step 30.

Nuclei Purification by Density Gradient (~1 hour)

- 30) Prepare the density gradient solutions (Density Gradient I and II) and chill both on ice:
- Before each use, gently mix **Bionano Prep Density Gradient** by inverting back and forth several times to ensure a uniform suspension.
 - To prepare **Density Gradient I (DGI)**: Transfer 2.6 ml **Bionano Prep Density Gradient** and 2.4 ml of **Bionano Prep Density Gradient Buffer** into a 15 ml tube. Mix thoroughly by gently inverting the tube and place on ice.
 - To prepare **Density Gradient II (DGII)**: Transfer 3.6 ml **Bionano Prep Density Gradient** and 1.4 ml of **Bionano Prep Density Gradient Buffer** into a 15 ml tube. Mix thoroughly by gently inverting the tube and place on ice.
 - Place **Bionano Prep 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 1,000 µl pipette tip (~1cm cut off from the tip head), carefully lay the ~3.5 ml nuclei suspension (from step 27, 28, or 29) on top of Density Gradient I without disturbing the interface or generating bubbles. (See Fig. 4 in Appendix II)
- 33) Spin at 4,500 x g for 40 minutes at 4°C using a swinging bucket rotor with low or no deceleration (e.g., acceleration=9, deceleration=1).
- Note:** Ensure that the clear tube is properly seated in its rubber adapter and is firmly supported at the bottom to prevent cracking during centrifugation.
- 34) After the centrifugation, examine the tube for a **nuclei band** at the interface between Density Gradient I and II. Refer to the table below to determine the next step:

Options	Visible Band at DGI-DGII Interface	Additional Observations	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	If maximum number of washes were performed at step 29, continue with steps b-g skipping step f.
			If no washes, or minimal washes, at step 29, continue with steps a-g to complete a total of 4 washes.
<p>a. Prepare 40 ml Plant Homogenization Buffer <i>plus</i> per wash, such that the total number of washes here and at step 29 do not exceed 4 washes, and place on ice.</p> <p>b. Retrieve the band (about 0.5-1 ml in volume) above DGI layer using P1000 wide-bore tip and transfer to a new 50 ml conical tube on ice. Bring the final volume to 40 ml by adding HB₊ buffer.</p> <p>c. Transfer a fraction of the nuclei suspension (<i>i.e.</i>, equivalent to 200 mg plant tissue) to a new 50 ml conical tube.</p> <p>d. Bring the final volume to 35 ml by adding HB₊ buffer, cap and mix well by inverting gently. Pellet the nuclei as described in step 25.</p> <p>e. Discard the supernatant by decanting, set on ice for 30 seconds, and re-suspend the pellet in residual buffer with the assistance of a wet paint brush. Add 3 ml ice-cold Plant Homogenization Buffer <i>plus</i> and swirl gently by hand for 30 seconds while the tube is touching ice.</p> <p>f. Repeat steps d-e to reach a total of 4 washes (including Optional Washes at step 29).</p> <p>g. Transfer the nuclei suspension (~3 ml) to a new 15 ml conical tube. Continue with step 36.</p>			

- 35) To recover nuclei band at DGI-DGII interface:
- Remove and discard the sample layer.
 - Remove and discard the DG I layer leaving 2-3 mm on top of the nuclei band at DGI-DGII interface.
 - Collect the nuclei band (volume range from 500 μ l to 1,000 μ l) using a cut 1,000 μ l pipette tip, and transfer to a pre-chilled 15 ml conical tube.
- 36) 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 low or no deceleration (e.g., acceleration=9, deceleration=1).
- 37) Evaluate the clarity of the supernatant immediately after centrifugation stops before the loose nuclei pellet starts diffusing:
- If a clear supernatant is obtained and a pellet is observed, continue to step 38.
 - 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 3,000 x g for 10 minutes at 4°C with low or no deceleration (e.g., acceleration=9, deceleration=1) and continue with step 38
 - If there is no pellet or pellet is very loose (i.e. cloudy on the bottom of the tube), re-centrifuge at 3,000 x g for 10 minutes at 4°C with low or 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, sample prep has failed - repeat with fresh young leaves using **Bionano Prep High Polysaccharides Plant Tissue DNA Isolation Protocol**, or contact customer support for troubleshooting.

- 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 3-6 mm in diameter):
- If the pellet is small (<3 mm in diameter), resuspend in 50 μ l of ice-cold **Bionano Prep Density Gradient Buffer** using a 200 μ l **Wide-Bore Tip** and transfer 50 μ l to a 1.5 ml microfuge tube.
 - If the pellet is large (>6 mm in diameter) and very young plant tissue with genome size greater than 1 Gbp was used, titration is recommended. See Appendix III for titration example.
 - Otherwise, resuspend nuclei pellet in 200 μ l of ice-cold **Bionano Prep Density Gradient Buffer** using a 200 μ l **Wide-Bore Tip** and transfer 200 μ l to a 1.5 ml microfuge tube.

Note: Transferring exact volumes 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 μ l 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 (e.g. > 6 mm in diameter) 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/ μ L to support NLRS labeling).

Embedding in Low Melting Agarose (~0.5 hours)

- 39) Pre-warm the nuclei suspension at 43°C for 3 minutes. Add appropriate volume of agarose to corresponding nuclei suspension volume as shown in table below (final agarose concentration of 0.82%):

Nuclei suspension	43°C 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 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)

- 42) Prepare fresh **Proteinase K digestion solution** by mixing 200 µl of **Proteinase K (PK) enzyme (Qiagen)** with 2.5 ml of **Bionano Prep Lysis Buffer** to be processed in the same 50 ml conical tube. **Note:** If polyphenols are suspected, adjust the Lysis Buffer to pH 9.0 by adding sodium hydroxide solution, before adding 200 µl Proteinase K enzyme and 5 µl βME to 2.5 ml Lysis Buffer.
- 43) Transfer plugs to 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. Use the blunt end of a metal spatula to submerge plugs if they stick to walls of the conical tube. **Note:** Up to five plugs can be processed simultaneously per 50 ml conical tube if each plug is from the same sample with the same homogenate input. Ensure all plugs are fully submerged with Proteinase K Solution throughout processing. Exceeding the recommended number of plugs can reduce the efficiency of Proteinase K digestion.
- 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). **Note:** 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.
- 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 **Bionano Prep Lysis Buffer** per 1-5 plugs to be processed per tube.
- 46) Remove each tube from the Thermomixer and equilibrate to room temperature for 5 minutes. Remove cap and attach a **green 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

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 (Qiagen)**, recap and incubate in the Thermomixer for 1 hour at 37°C with intermittent mixing (mixing cycle: 10 seconds at 450 rpm followed by 10 minutes at 0 rpm).

Washing Plugs to Stabilize DNA (~1.5 hours)

- 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 **three times** with **1x Wash Buffer**:
 - a. Add 10 ml of **1x Wash Buffer** through the screened cap.
 - b. Gently swirl tube for 10 seconds.
 - c. Discard wash solution through the screened cap.
 - d. Tap plugs to bottom of tube before next rinse.
- 8) Wash each tube **four times** with **1x Wash Buffer** :
 - a. Add 10 ml of **1x Wash Buffer** through the screened cap and capping tube.
 - b. Shake tube for 15 minutes on a horizontal platform mixer with continuous mixing at 180 rpm.
 - c. Discard wash solution through the screened cap.
 - d. Tap plugs to bottom of tube before next wash.

Note: Do not discard last wash if storing plugs. 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. Ensure plugs are at the bottom of the tube.
- 10) If processing all plugs continue with step 11. Otherwise, transfer plugs destined for DNA recovery to a new 50 ml conical tube and continue with step 11. Add 1x wash buffer to remaining plugs for storage
- 11) Wash plugs **five times** with **1x TE Buffer**:

- a. Add 10 ml of **1x TE Buffer** through the screened cap and cap tube.
 - b. Shake tube for 15 minutes on a horizontal platform mixer with continuous mixing at 180 rpm.
 - c. Discard wash solution through the screened cap.
 - d. Tap plugs to bottom of tube before next wash.
- 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 either **.2 U/µl Gelase (Epicenter)** or **0.5U/µl Agarase (ThermoFisher)** to tube and stir mixture gently with a pipet tip for 10 seconds. Repeat the same for the remaining tubes.
 - 17) Incubate tube(s) for 45 minutes at 43°C.

Drop Dialysis to Clean the DNA (~1 hour)

- 18) Aliquot 15 ml of **1x TE Buffer** into a **6 cm Petri dish (ThermoFisher/VWR)** per plug.
- 19) Float a **0.1 µm dialysis membrane (Millipore)** on the surface of the 1x TE Buffer. Place a cover on the Petri dish and let the membrane equilibrate 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 1x TE Buffer should not impact performance.
- 20) Pipette 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 pipetting DNA onto or 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 pipette mix the DNA while on the membrane.

Homogenization of DNA Solution (overnight)

- 23) Test DNA viscosity by slowly withdrawing the entire DNA volume into a **Non-Filtered Beveled 200 µl Tip** while slowly releasing pipet plunger. If the DNA is not viscous, do not pipette mix. If the DNA is viscous, pipette 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 pipette mix with each added stroke.
- 24) Allow the DNA to equilibrate overnight at room temperature (25°C) for better homogeneity.

Day 3: DNA Quantitation / NLRS labeling

Qubit Quantitation - BR dsDNA Assay (~0.5 hours)

- 1) Equilibrate Qubit BR Assay kit standards to room temperature from 4°C.
- 2) Prepare 200 µl **BR Working Solution** for every sample assay tube (3 assay tubes per DNA sample) and every Qubit BR Assay Kit Standard (2 BR standards in total) by diluting **Qubit dsDNA BR Reagent** in **Qubit BR Buffer** (1 : 200 dilution).
- 3) Aliquot 10 µl of **Qubit BR standards** to individual Qubit assay tube, containing 10 µl of Qubit BR Buffer.
- 4) For each sample – Prepare three Qubit assay tubes and aliquot 18 µl of Qubit BR Buffer to each tube.
- 5) Carefully pipette mix the entire volume of DNA five times with a **Wide Bore Tip** to avoid bubble formation.
- 6) Using a **Narrow Bore Tip** or **Positive Displacement Pipet**, pipet 2 µl from the top, middle and bottom of each DNA sample and dispense each draw into separate Qubit assay tubes (from step 4 above) following the methods described in 'Pipetting viscous DNA' (see Appendix I below) to ensure accurate pipetting of viscous DNA for quantitation. Use a **new tip** for each draw.
- 7) Sonicate tubes in sonicating bath for 10 minutes 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** (BR Dye Reagent + BR 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 in the dark for 5 minutes and proceed with quantitation on Qubit reader.
- 11) Check that the **coefficient of variation (CV)** from the three separate readings obtained for each sample is **< 0.25**.
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 on the following day.

NLRS Labeling

Proceed to use DNA for NLRS procedure. Refer to Bionano Prep Labeling NLRS Protocol (30024) for details.

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 (to the top, middle, or bottom of the DNA sample), and carefully release the plunger, as **slowly** as possible, to start drawing the viscous DNA into the pipet 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 up to 30 seconds to fill the tip to the 2 μ l 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 μ l mark. A positive displacement pipette may be used as an alternative.

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 with a new tip if this happens).

To deposit the entire volume of viscous gDNA, hold tube containing 18 μ l 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.

II. Photo Reference of Key Steps

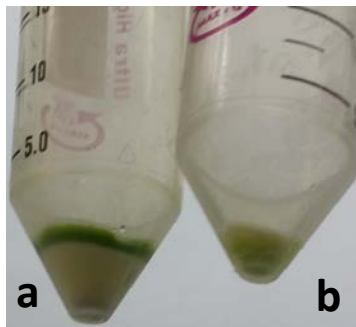


Fig. 1 Pellet nuclei using a swing bucket rotor on tomato (a, 1.6 grams) and maize (b, 0.5 grams) as described in step 25.

a. 1.6 grams of new sprouts from tomato plants were used as input tissue. Nuclei pellet may show two distinct layers: a white/opaque layer on the bottom and a green layer on top. It is strongly recommended to perform the low speed spin for such size of the pellet.

b. 0.5 gram of young maize leaves were used as input tissue, the pellet size was much smaller (about 1/8 inch in diameter); and the low speed spin may be skipped in this case.

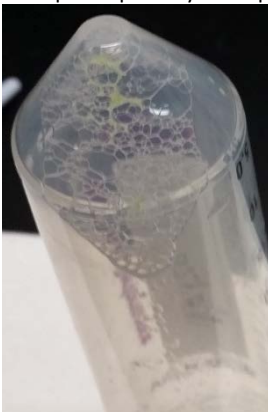


Fig. 2 Residuals left after the low speed spin (step 28).



Fig. 3 Ideal pellet should be yellow/light green or white before starting density gradient purification (step 29).



Fig. 4. Density gradient assembly for nuclei purification (step 32; left: tomato; right: maize)

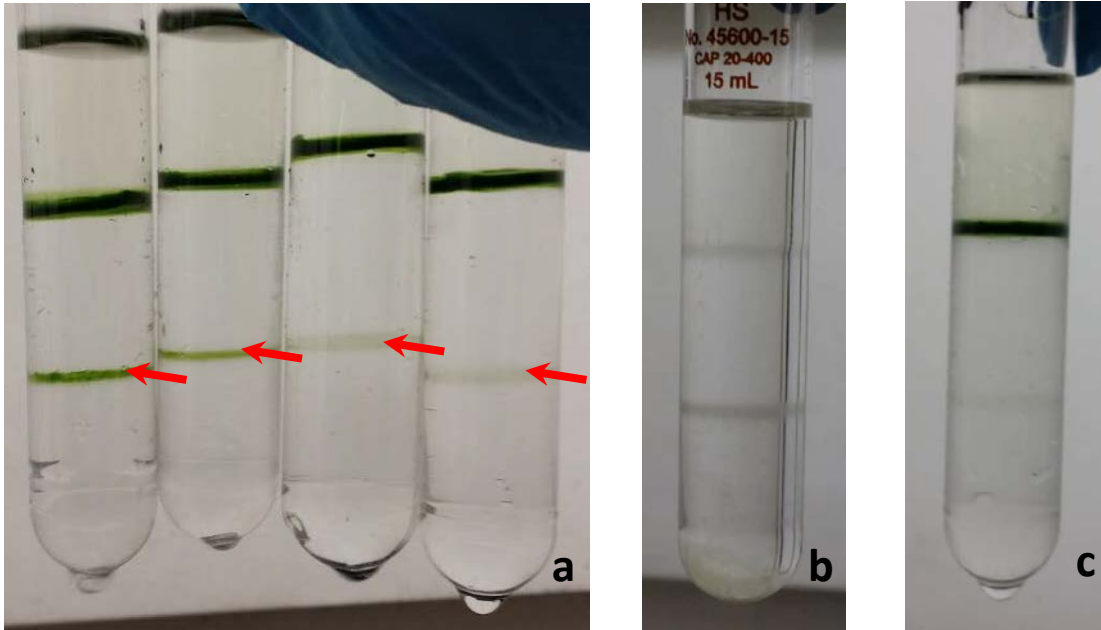


Fig. 5 Nuclei purification by density gradient (step 34).

After centrifugation at 4,500 x g for 40 minutes at 4°C, examine the tube for a band at the interface between Density Gradient I and II (see Figure 5; red arrows) a. Ideal interface bands – 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 – additional washes may be required.

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

- Resuspend large (e.g. soybean size) nuclei pellet in 200 μ l of ice-cold Bionano Prep Density Gradient Buffer.
- Transfer 100 μ l with a **Wide Bore Tip** to a 1.5 ml microfuge tube on ice and label 1x (enough for 2 plugs). Transfer another 100 μ l to a new 1.5 ml tube containing 100 μ l of ice-cold Bionano Prep Density Gradient Buffer, pipet mix using **Wide Bore Tip** and label 0.5x (enough for 3-4 plugs).
- To embed nuclei, mix 35 μ l of 43°C equilibrated agarose per 50 μ l of nuclei suspension and cast plugs.

Technical Assistance

For technical assistance, contact Bionano Genomics Technical Support.

You can retrieve documentation on Bionano products, SDS's, certificates of analysis, frequently asked questions, and other related documents from the Support page or by request through e-mail and telephone.

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Email	support@bionanogenomics.com
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