Plant nuclear genomic DNA preps

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Abstract

The isolation of nuclear genomic DNA of high molecular weight is becoming a crucial step for obtaining long read sequencing data produced by the PacBio and Oxford Nanopore platforms. Although it involves a few more steps than a standard total genomic DNA preparations, it is worth optimizing the protocol to avoid wasting valuable sequence data on organellar DNA. The current protocol has been successfully applied to kiwifruit, apple, mānuka, *Nicotiana benthamiana*, some solanaceas, and two insects (pupae of the light brown apple moth (*Epiphyas postvittana*) and hind leg of a weta (*Deinacrida* spp)). Three alternative methods to extract DNA are also presented.

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Guidelines

Each plant poses a different challenge regarding the best approach to extract good quality DNA. The following review gives a very thorough introduction on this topic: Kasem S, Rice N, Henry R (2008) DNA extraction from plant tissue. Plant Genotyping II: SNP Technology. pp. 219-271.
The method presented here has been applied in a mid-throughput manner using a blender to homogenize the plant tissue instead of the traditional freeze/grind method with liquid nitrogen. However if the leaf tissue is tough (for example mānuka or rosemary) it would be best to use liquid nitrogen and a precooled mortar and pestle. The same would apply to very precious samples where nothing should be wasted.

Before start

Solutions and Reagents

- NEB (Nuclei Extraction Buffer) 0.5 M Mannitol, 10 mM PIPES-KOH, 10 mM $MgCl_2$ $6H_2O$, 2% PVP K40, 200 mM L-lysine monohydrochloride, 6 mM EGTA, pH 6

Reagent	Sigma Aldrich cat #	1 L
Mannitol	M4125-1KG	91 g
PIPES-KOH	P7643-100G	3.78 g
$MgCl_2$ $6H_2O$	M9272-100G	2.03 g
PVP K40	PVP40-500G	20 g
L-lysine monohydrochloride	L5626-1KG	36.52 g
EGTA	E3889-100G	2.28 g

Heat up 800 mL of deionized water, 2-4 min in microwave. Pour into a glass beaker with a magnetic stirrer. Add PVP K40, three spoonfuls at a time, stir vigorously. To speed up this process you can use two flat spatulas and “cut” the PVP blobs as if it was a steak.
Add mannitol and dissolve completely before adding PIPES, magnesium, lysine, and EGTA. Adjust

the pH to 6.0 with 10 M NaOH. Adjust the volume to 1 L. Split the buffer into two 1 L Schott bottles. Autoclave and cool down to room temperature. Store at 4°C until ready to use.

- NEB complete and NEB –no b-mercaptoethanol

Just before starting the nuclei isolation add 0.9 g sodium meta-bisulphite (Sigma Aldrich cat # 255556-100G) to each Schott bottle. Close the bottle and shake until it is completely dissolved. The final concentration is 10 mM.
Add 0.2 mL β-mercaptoethanol to one Schott bottle only. Label this one as NEB complete buffer. The other flask is NEB – no β-mercaptoethanol. Keep on ice.

- 25% Triton X-100 in NEB complete

Transfer 7.5 mL of NEB complete buffer to a 50 mL Falcon tube. Add 2.5 mL of Triton X-100, use a wide bore tip. Aspirate the detergent very slowly from the stock bottle until you don't see any change in the liquid level. Do not dip the tip too far into the stock bottle to avoid carrying extra detergent into the Falcon tube. Dispense the detergent into the 7.5 mL of buffer, do it very slowly. Aspirate some buffer to clean up the inside of the wide bore tip until there is no more detergent lumps visible. Close the tube and vortex thoroughly until fully dissolved. Spin down ~ 10 min at 3000 rpm at room temperature to pop all the bubbles. Keep the tube on ice.

- Tris-borate/K-acetate/SDS Lysis buffer: 0.5% SDS, 5 mM EDTA, 150 mM Tris-borate pH 7.4

To prepare this solution the Tris buffer is titrated with boric acid, instead of the usual HCl. Autoclave and keep at room temperature.

- CTAB/NaCl lysis buffer: 2% CTAB (hexadecyltrimethyl ammonium bromide), 2% PVP K40 (polyvinyl pyrrolidinone K40), 2 M NaCl, 25 mM EDTA, 100 mM Tris-HCl pH 8.0. To dissovle the PVP lumps, use the same technique as described above for NEB. Split the buffer into two 1 L Schott bottles and autoclave. Keep at room temperature.
- 5 M potassium acetate pH 7.0. Prepare according to Green and Sambrook 2012 and sterilize by filtration.
- 100% Ethanol
- 70% Ethanol
- Isopropanol
- Chloroform:isoamyl alcohol (24:1)
- 3 M sodium acetate pH 5.2 (see Green and Sambrook 2012)
- β-mercaptoethanol (Sigma Aldrich cat # 63689)
- TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA pH 7.5)
- RNase A 100 mg/mL (Qiagen cat # 19101)
- Proteinase K 20 mg/mL (Qiagen cat # 19133)
- Qiagen DNeasy Plant Maxi kit (Qiagen cat # 68163)
- SybrSafe (Invitrogen cat # S33102)
- Lambda DNA (Invitrogen cat # SD0011)
- 1 kb+ ladder (Invitrogen cat # 10787018)
- Lambda ladder (BioRad cat # 170-3635)
- Standard agarose (Invitrogen cat # 16500500)
- PFGE grade agarose (BioRad cat # 1613108)
- 1X TAE gel running buffer

Equipment

- Waring commercial blender or a standard kitchen blender (for example, Sunbeam model PB7950)
- Miracloth
- Cheese cloth, sterile
- Two Large funnels
- Two 500 mL cylinder
- Parafilm
- Wide bore tips: 200, 1000 and 5000 µL, sterile, to pipette the purified nuclear genomic DNA and the Triton X-100 stock solution
- Kimwipes
- Qubit system with HS dsDNA kit or DeNovix (DS-11 FX, FX+ or QFX) with dsDNA High Sensitivity kit
- Microvolume spectrophotometer Nanodrop or DeNovix (DS-11, 11+, FX or FX+)
- Sorvall centrifuge or similar with SLA 600 TC rotor or SS34 rotor
- Nalgene centrifuge tubes Nalgene™ Oak Ridge (cat # 3119-0050)
- Bench top centrifuge with plate rotor (for example, Eppendorf SKU 5810000084 Rotor A-4-62, with 4 MTP buckets; Cat. # 5810711002)
- Falcon tubes, sterile, 50 mL
- Microcentrifuge
- 1.5 mL microcentrifuge tubes, sterile
- Gel electrophoresis box and power supply
- CHEF-DR III variable angle system for PFGE analysis (BioRad cat # 1703702)
- Water bath with shaker

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Protocol

Nuclei isolation

Step 1.

Pour 300 mL ice-cold NEB complete buffer in a blender. Work inside a fume hood.

Step 2.

Weight 20 g of kiwifruit leaves, cut any long stems and transfer to the blender

Step 3.

Homogenize for 30 sec in low setting

NOTES

(Note: If your blender is powerful, use low setting, but if using a kitchen blender set it on High)

Do not use a stick hand-held blender because the sample won't be homogenized all at the same time.

Step 4.
Filtrate homogenate through 4 layers of cheesecloth into a sterile glass cylinder

NOTES

It is best to use sterile glassware, but if you are confident your washing techniques are thorough, use non-sterile glassware.

If you can't find good quality cheese cloth, you can use disposable kitchen cloth (for example CHUX superwipes)

Step 5.
Filter again through 4 layers of miracloth into another 500 mL sterile glass cylinder

NOTES

Stack the miracloth layers against the fabric grain, i.e., perpendicular to each other

Step 6.
Adjust the volume to 294 mL with NEB complete buffer

Step 7.
Add 6 mL of 25% Triton X-100 in NEB complete buffer by placing the tip of the pipette on the inside wall of the cylinder, close to the top. Dispense the solution slowly and rotate the cylinder with your other hand. You will see the syrup-like solution smear downwards towards the homogenate.
Seal cylinder with parafilm and mix very gently by inversion 10-20 times. Rotate the cylinder every time you mix it

Step 8.
Aliquot the homogenate into six 50 mL Falcon tubes

NOTES

You can also use Nalgene centrifuge tubes. And instead of the SLA-600 TC rotor you will need the SS34

Step 9.
Spin down at 588 rpm (57 g) using the SLA-600 TC rotor at 4-10°C, for 2 min only. A tiny pellet might be visible. If no pellet is observed, continue with next step without changing tubes.

DURATION

00:02:00 Additional info: Low speed centrifugation

NOTES

If using the SS34, adjust the speed to correspond to the same g values used on the SLA-600 TC

Step 10.
Transfer the supernatant to a new set of 50 mL Falcon tubes and spin down at 3308 rpm (1800 g), same

rotor, 10 °C, 15 min

TEMPERATURE

10 °C Additional info:
Centrifugation
temperature

DURATION

00:15:00 Additional info: Nuclei pellet centrifugation

NOTES

The centrifugal force depends on the genome size but also on the medium's density. The osmoticum used in this buffer (mannitol) is at 0.5 M and seems to work well with genomes upto 3 Gb (N. benthamiana), at 1800 x g, but please see recommendations here. Other cellular material such as starch might contribue to the easy sedimentation of large genomes such as the N. benthamiana.

Step 11.

Transfer supernatant to a waste container for proper disposal later.

SAFETY INFORMATION

Check the waste disposal regulations for b-mercaptoethanol solutions and solid waste

Step 12.

Resuspend each pellet with 50 mL NEB –no β-mercaptoethanol. Mix gently by inversion until the pellet is resuspended. Spin down again as before.

GOTO

Nuclei pellet centrifugation -> go to step #10

Step 13.

Transfer supernatant to the waste container for proper disposal

SAFETY INFORMATION

This supernatant does not contain β-mercaptoethanol and could be poured down the drain unless you are working with regulated biological material

Step 14.

Resuspend each pellet with 5 mL NEB –no β-mercaptoethanol and collect all pellets into one Falcon tube previously weighted. Add more NEB –no β-mercaptoethanol to final volume of 50 mL

Step 15.
Collect the nuclei by centrifugation

GOTO

Nuclei pellet centrifugation -> go to step #10

Step 16.
Weight the tube to find out the total amount of nuclei isolated. From 20 g of kiwifruit fully developed leaves expect about 0.2 g of nuclei, but this will vary depending on the species and genotype. Keep the tube on ice

NOTES

If you can't continue with the nuclear genomic DNA extraction, store the nuclei pellet at -80°C until ready to extract the DNA

DNA extraction options

Step 17.
Depending on the nature of the tissue and the budget, select one of the following methods to isolate the nuclear genomic DNA.

DNA extraction I Tris-borate/K-acetate/SDS

Step 18.
Resuspend 0.2 g of nuclei pellet in 14 mL lysis buffer, mix by inversion, do not vortex

NOTES

A modified version of the method published by Lopez-Gomez and Gomez-Lim (1992) HortScience 27(5):440-442. This protocol is suitable for plants with a high carbohydrate content.

Step 19.
Add 7 µL of RNase A 100 mg/mL. Mix by inversion

Step 20.
Incubate 10 min at 37°C with gentle shaking

TEMPERATURE

37 °C Additional info: RNA digestion

DURATION

00:10:00 Additional info: Lysis and RNA digestion

Step 21.
Add 1.4 mL 5 M K-Acetate pH 7, mix by inversion

Step 22.

Add 3.5 mL 100% Ethanol, vortex at maximum speed for 30 s

Step 23.
Extract the sample with an equal volume of chloroform:isoamyl alcohol (24:1). Mix by inversion 10 times, then place the tube horizontally in orbital shaker at room temperature and shake for 5 min, gently

TEMPERATURE

20 °C Additional info:
Chlorform extraction

DURATION

00:05:00 Additional info: Chloroform extraction

Step 24.
Spin down at 3000 rpm 10 min at room temperature using a swing bucket rotor

DURATION

00:10:00 Additional info: Chloroform extraction centrifugaion

Step 25.
Transfer the aqueous phase to a new 50 mL Falcon tube. Add an equal volume of ice cold isopropanol. Mix by inversion and store at -20°C for 30 min

TEMPERATURE

-20 °C Additional info:
Isopropanol precipitation

DURATION

00:30:00 Additional info: Isopropanol precipitation

NOTES

If the aqueous phase is not clear, repeat the chloroform extraction (steps 23-24)

Step 26.
Spin down at 3000 rpm for 25 min at room temperature or 10°C

DURATION

00:25:00 Additional info: DNA precipitation centrifugation

Step 27.
Discard supernatant and add same volume of 70% ethanol to wash the pellet. Let it stand at room temperature for 10 min

TEMPERATURE

20 °C Additional info:
Elute salts from DNA
pellet

DURATION

00:10:00 Additional info: Elute salts from DNA pellet

Step 28.
Collect DNA by centrifugation

GOTO

Centrifuge DNA pellet -> go to step #26

Step 29.
Discard the supernatant. Remove all traces of ethanol with a kimwipe without touching the pellet.
Air dry the DNA pellet at room temperature for 20-30 min and add 100-200 µL TE buffer pH 7.5. Tap the bottom of the tube to detach the pellet and let it diffuse slowly at room temperature for a few hours or in the refrigerator overnight. Avoid vortexing the DNA solution at high speed.

NOTES

If you are concerned the DNA will bind to the tube irreversible, add the TE buffer immediately and leave the tube open, at room temperature. Any traces of ethanol will evaporte from the solution.

Step 30.
Store the DNA at 4°C. For long term storage, make numbered aliquots and store at -20°C. Use DNA aliquots sequentially and avoid freeze/thaw cycles

DNA Extraction II CTAB/NaCl/Proteinase K protocol

Step 31.
Add 3.5 mL of CTAB/NaCl extraction buffer and 80 µL Proteinase K to the nuclei pellet and vortex gently to resuspend

Step 32.
Incubate at 56°C for 1-2 hour without shaking or until all solution is clear

Step 33.
Add 10 µL RNase A 100 mg/mL, mix and incubate at room temperature for 5 min

DURATION

00:05:00 Additional info: RNase A digestion

Step 34.
Add 3.5 mL of chloroform:isoamyl alcohol (24:1) and vortex until an emulsion is obtained

Step 35.
Separate the phases by centrifugation using a swing bucket rotor, 3000 rpm, at room temperature for 15 min

DURATION

00:15:00 Additional info: Chloroform extraction centrifugation

Step 36.

If the interphase between the organic and aqueous solutions is not tight, you could centrigue the sample for another 15 min or transfer the aqueous phase to a new tube and add equal volume of chloroform:isoamyl alcohol

GOTO

Chloroform extraction centrifugation -> go to step #35

GOTO

Chloroform extraction centrifugation -> go to step #35

Step 37.

Transfer aqueous phase to a new Falcon tube and add 2 volumes of 100% ethanol. Mix gently. Incubate at -20°C for at least 2 h

TEMPERATURE

-20 °C Additional info:
Ethanol precipitation

DURATION

02:00:00 Additional info: Etahnol precipitation

Step 38.

Collect the DNA by centrifugation as explained before (Step 26-30)

DNA extraction III Qiagen DNeasy Plant Maxi Kit

Step 39.

Follow the Qiagen DNease Plant Maxi kit instructions with the following modifications:

- The final pellet of nuclei should be divided into 2 tubes and treat each one as the starting material for one extraction using this kit
- To elute the DNA incubate the binding column with 1 mL of AE buffer for 1 h. Spin down as described in the kit instructions. Add another mL of AE buffer to the binding column and incubate at room temperature for 30 min. Spin down again. Each column/tube will have 2 mL of eluted DNA
- Combine both samples (4 mL) and add 1/10 volume of 3 M sodium acetate pH 5.2, mix, and add 2 volumes of 100% ethanol. Mix. Store at 4°C or -20°C ovenight
- Collect, wash and resuspend the pellet as described in steps 26-30

The fragment size distribution obtained with this kit is 20-40 kbp

DNA quality check

Step 40.

Check the quality of the ngDNA by absorbance ratios at 260/280 nm and 260/230 nm. Aim for 1.8 and >2.0, respectively.

Step 41.

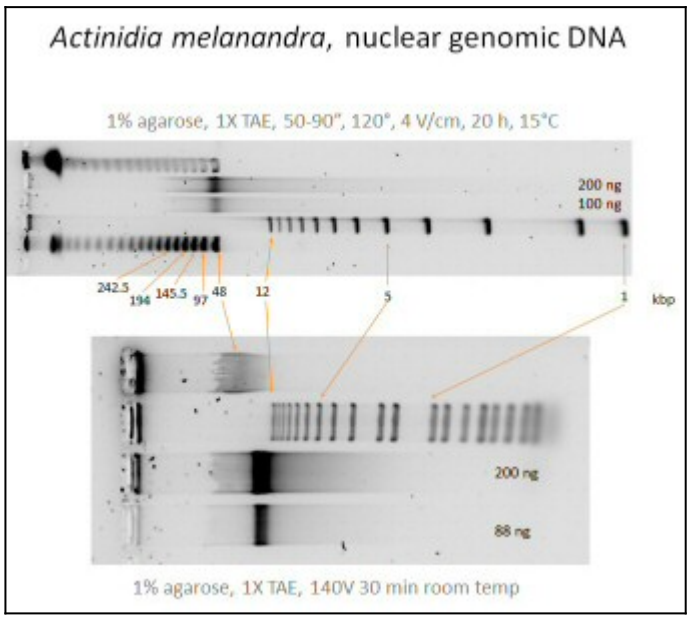
Quantify the ngDNA by fluorescence (Qubit or DeNovix) using a high sensitivity kit. Make 10-fold serial dilutions and quantify 1-5 µL

Step 42.

Run 100- 200 ng of ngDNA in a standard 1% agarose gel, stained with Sybr safe. Use lambda DNA (48.5 kbp) as control.

If you have access to a pulse field gel electrophoresis system, run 200 ng against a lambda ladder, intact lambda DNA and 1 kb+ ladder.

EXPECTED RESULTS



Warnings

The protocol requires β-mercaptoethanol, chloroform and guanidine hydrochloride (Qiagen DNeasy Plant Maxi kit only). Dispose these chemicals according to your safe method of use and local regulations.