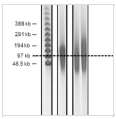

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High Molecular Weight DNA Extraction from Recalcitrant Plant Species for Third Generation Sequencing

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1 *Works for me* dx.doi.org/10.17504/protocols.io.4vbgw2n

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ABSTRACT

Single molecule sequencing requires optimized sample and library preparation protocols to obtain long-read lengths and high sequencing yields. Numerous protocols exist for the extraction of DNA from plant species, but the genomic DNA from these extractions is either too low yield, of insufficient purity for sensitive sequencing platforms, e.g. nanopore sequencing, too fragmented to achieve long reads, or otherwise unattainable from recalcitrant adult tissue. This renders many plant sequencing projects cost prohibitive or methodologically intractable. Existing protocols are also labor intensive, taking days to complete. Our protocol described here yields micrograms of high molecular weight gDNA from a single gram of adult or seedling leaf tissue in only a few hours, and produces high quality sequencing libraries for the Oxford Nanopore system, with typical yields ranging from 3-10 Gb per R9.4.1 flowcell and producing reads averaging 5-8 kb, with read length N50s ranging from 6-30 kb depending on the style of library preparation (details in sequencing outcomes section), and maximum lengths extending up to 200 kb+.

EXTERNAL LINK

www.circulomics.com/support-nanobind

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

a. Y. Zhang, Y. Zhang, J. M. Burke, K. Gleitsman, S. M. Friedrich, K. J. Liu, and T. H. Wang, A Simple Thermoplastic Substrate Containing Hierarchical Silica Lamellae for High- Molecular-Weight DNA Extraction. Adv Mater (2016). PubMed PMID: 27862402

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GUIDELINES

Introduction

The assembly of high quality conifer genomes can benefit many fields of research from conservation and restoration efforts, to disease and stress studies, and evolutionary history. However, these tree genomes present unique assembly challenges; they are large (10-30+ Gb haploid), repetitive, and can have high ploidy. While long read sequencing, e.g. Oxford Nanopore, 10X, or PacBio, can greatly improve assembly contiguity, extracting large amounts of high quality, high molecular weight (HMW) DNA from adult trees presents a unique challenge. Although many extraction methodologies exist for recalcitrant plant species, most yield either DNA of quality “fit for PCR” and not for sensitive nanopore sequencing applications, or DNA too fragmented to obtain sequencing reads of sufficient length to improve assembly contiguity. Obtaining 60 kb+ and "nanopore clean" DNA places higher demands on sample extraction and preparation than existing methodology can provide in adult trees.

We have combined several techniques to develop HMW, "nanopore clean" DNA extraction methodologies from conifer species Sequoiadendron giganteum (giant sequoia) and *Sequoia sempervirens* (coast redwood) and generated sequence data on the Oxford Nanopore MinION. Our method integrates nuclei isolation and Nanobind DNA isolation (Circulomics) to improve purity and recovery 10-fold and reduce extraction time from 2-3 days to a single day. We also detail sequencing library preparation methodology and demonstrate extension of our methodology to Maize tissue.

Timing

Nuclei extraction: 2-3 hours
Nanobind gDNA extraction: 1-2 hours
Elution: 30 minutes
DNA relaxation (optional): overnight
Sequencing library preparation: 15 minutes - 2 hours

Oxford Nanopore Technologies Protocol Modifications

The ligation protocol was carried out as described for the LSK108 1D nanopore library preparation, with the exception of the following steps:

- Input to ligation protocol was **1.5 µg** gDNA sheared to 8 kb with the Diagenode Megaruptor. Shearing to 10 kb with Covaris G-tubes (**5000 x g** for **00:01:00** each way) achieves similar results.

Alternatively, for longer reads it is recommended not to shear and perform the ligation protocol as recommended, or include a size selection step with the Blue Pippin (Sage Sciences). Sequencing outcomes for all options are given in table below.

- End repair (NEBNext Ultra II) volumes were doubled over protocol recommendations (**100 µl** buffer, **14 µl** enzyme), and the reaction was incubated at **20 °C** for **00:20:00** and **65 °C** for **00:20:00**.

Troubleshooting table

Issue	Recommendations
gDNA is brown	Typically due to polyphenolic oxidation. Sample is generally not sequenceable. Ensure you are using the correct concentration of reducing agent, that your buffers are at the correct pH, and that your reaction prior to nuclear lysis was carried out at 4°C.
gDNA yield is low (<5ug per gram of tissue)	Generally from loss at the nuclear prep stage - this could be due to insufficient sample grinding in liquid nitrogen, to unoptimized Triton lysis, or to sub-optimal spin speed during differential centrifugation steps.
gDNA fragments short (mean <50kb)	Incorrect buffer pH can degrade DNA. Ensure pH of homogenization buffer (HB) is 8.5-9. Excessive pipetting/vortexing can fragment DNA. Ensure that wide bore pipette tips are used during DNA elution (step 17 of the Nanobind protocol).
Sequencing yield poor (<5Gb)	Residual impurities can affect sequencing library preparation, delivery of library molecules to the pore (or ZMW) array, or effective molecule sequencing. Sample can be ran through an Amicon 100K ul Ultra Centrifugal filter or re-extracted.

Anticipated results

	Giant Sequoia	Coast Redwood	Maize (MSU)
Input	1 gram leaf tissue	1 gram leaf tissue	1 gram leaf tissue*
Mean gDNA yield (ug)	13.4 ± 1.1 µg (11.5-15.1 ug)	11.5 ± 2.5 µg (7.9-14.8 ug)	5.8 ± 0.9 µg (4.6-6.5 ug)
Mean PFGE sizing+	35-150 kb	45-250 kb	45-300 kb
Nanodrop (260/280)	1.77 ± 0.07 (1.70-1.82)	1.77 ± 0.03 (1.73-1.83)	1.85 ± 0.01 (1.83-1.87)
Nanodrop (260/230)	1.41 ± 0.27 (1.12-1.65)	1.40 ± 0.16 (1.20-1.69)	1.87 ± 0.20 (1.48-2.13)

*Maize tissue was etiolated shoot tissue.
+PFGE sizing provided as the size range at which the majority of gDNA sample was present.

Oxford Nanopore sequencing outcomes

Sample	Giant Sequoia	Coast Redwood A	Coast Redwood B
Shearing	Megaruptor (8 kb)	Covaris G-tube (8 kb)	26G Needle shear (5X)
Nanopore chemistry	LSK108, R 9.4	LSK108, R 9.4	LSK108, R 9.4
Seq yield	6.4 Gb	10.10 Gb	3.3 Gb
Mean read length	5.5 kb	5 kb	6.8 kb
Max read length	121 kb	78 kb	227 kb
Read length N50	6.9 kb	6.6 kb	29 kb

MATERIALS

NAME	CATALOG #	VENDOR
Liquid nitrogen		
200 proof ethanol		
ddH20		
Potassium Chloride	P9541	Sigma Aldrich
0.5M EDTA solution	15575020	Thermo Fisher Scientific
14 M ß-mercaptoethanol	M3148	Sigma Aldrich
Triton X-100	X100	Sigma Aldrich
Trizma® base	T4661	Sigma Aldrich
Spermidine trihydrochloride	S2501	Sigma Aldrich
Spermine tetrahydrochloride	S1141	Sigma Aldrich
Sucrose molecular biology grade	S0389	Sigma Aldrich
10 N NaOH	72068	Sigma Aldrich
PVP 360K	PVP360	Sigma Aldrich
Nanobind Plant Nuclei Big DNA Kit - Alpha Kit	SKU NB-900-801-01	Circulomics

MATERIALS TEXT

Reagents

- 14 M ß-mercaptoethanol (Sigma-Aldrich, M3148-100ML)
- Triton X-100 (Sigma-Aldrich, X100-100ML)
- Trizma base (Sigma-Aldrich, T4661-100G)
- ddH20
- Potassium chloride (Sigma-Aldrich, P9541-500G)
- 0.5 M EDTA pH 8.0 (ThermoFisher, 15575020)
- Spermidine trihydrochloride (Sigma-Aldrich, S2501-5G)
- Spermine tetrahydrochloride (Sigma-Aldrich, S1141-5G)
- Sucrose, molecular biology grade (Sigma-Aldrich, S0389-1KG)
- 10 N NaOH (Sigma-Aldrich, 72068-100ML)
- PVP 360K (Sigma-Aldrich, PVP360-100G)
- 200 proof ethanol
- Liquid nitrogen

Nanobind Plant Nuclei Big DNA Kit - Alpha Kit (Circulomics Inc)

- Nanobind disks
- Proteinase K
- RNase A
- Buffer PL1 - Lysis/Binding buffer
- Buffer PW1 Concentrate - Wash buffer concentrate
- Buffer EB - Elution buffer

Equipment

- Refrigerated centrifuge
- Paintbrushes
- Miracloth (Millipore Sigma, 475855-1R)
- Mortar and pestle (Fisher Scientific, 12-947-1)
- Conical vials (15 mL and 50 mL)
- 250 mL capped bottle
- 100 mL beaker
- End over end mixer (optional)
- Magnetic stir plate + stir bars
- pH meter or strips
- Fume hood
- Funnel
- NanoDrop and/or Qubit Fluorometer (Thermo Fisher)
- ThermoMixer (Eppendorf)
- HulaMixer (Thermo Fisher)

Reagent setup

HB (homogenization buffer) stock 10X (100 mL)

1.21 g	Trizma Base
5.96 g	KCL
20 mL	0.5 M EDTA
0.255 g	Spermidine
0.348 g	Spermine
Fill to 100 mL	ddH20

Adjust pH to 9-9.4 with 10M NaOH Drops. Can store at 4 °C in a glass bottle for up to 1 year

HB 1X solution (1000 mL)

100 mL	10X HB
171.2 g	Sucrose
Fill to 700 mL	ddH20 (stir until dissolved)

Bring to final volume of 1L. Can store at 4 °C in glass bottle for 3 months.

Triton X-100 (20% vol/vol, 100 mL)

20 mL	Triton X-100
10 mL	10X HB
17.15 g	Sucrose
Fill to 60 mL	ddH20 (stir until dissolved)

Bring to final volume of 100 mL Can store at 4 °C in a glass bottle for up to 1 year.

Prepare day of isolation:

NIB (nuclear isolation buffer)

Make 10 mL NIB per gram of tissue, plus an additional 50 mL for washes. Example recipe for **5 g** of tissue:

97.5 mL	1X HB
2.5 mL	Triton X-100 mix
250 µl	ß-mercaptoethanol
1 g	PVP

Stir until mixed

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

BEFORE STARTING

See "Materials" for reagent setup.

Nuclei Isolation

1 Grind **1 g** of tissue, preferably fresh or snap frozen, into fine powder in liquid nitrogen with a mortar and pestle. Immediately transfer ground tissue to capped 250 mL bottle containing **10 ml** NIB.

Cap bottle and attach to end over end mixer, rotating at max speed for **00:15:00** at **4 °C**.

1.1 Grind **1 g** of tissue, preferably fresh or snap frozen, into fine powder in liquid nitrogen with a mortar and pestle.

1.2 Immediately transfer ground tissue to capped 250 mL bottle containing **10 ml** NIB.

1.3 Cap bottle and attach to end over end mixer, rotating at max speed for **00:15:00** at **4 °C**.

Alternatively, lay bottles on their side on a shaker (150 rpm), or transfer ground tissue to a beaker capped with foil and mix on stir plate with stir bar at 4 °C for 00:15:00 .

2 Using a funnel, gravity filter homogenate through 5 layers of Miracloth into a 50 mL conical tube. Cap tube and centrifuge at 4 °C for 00:20:00 .

2.1 Using a funnel, gravity filter homogenate through 5 layers of Miracloth into a 50 mL conical tube.

2.2 Cap tube and centrifuge at 4 °C for 00:20:00 .

Speed of centrifugation is dependent upon size of genome:

For larger genomes such as redwood (30 Gb), we spun down at 1900 x g.

For smaller genomes like walnut (1 Gb) we spun down at 2900 x g.

For maize (2.5 Gb) was spun down at 2500 x g.

3 Decant the supernatant and add 1 ml cold NIB to pellet. Resuspend pellet with paint brush pre-soaked in NIB.

3.1 Decant the supernatant and add 1 ml cold NIB to pellet.

3.2 Resuspend pellet with paint brush pre-soaked in NIB.

Pipetting up and down with wide bore tips works for some species (for example maize) but for many species the pellet is too sticky to allow for sufficient resuspension by pipet.

4 Transfer 1 ml nuclei suspension to a 15 mL conical.

5 Bring volume up to 15 ml with ice-cold NIB. Centrifuge at 4 °C for 00:10:00 .

5.1 Bring volume up to 15 ml with ice-cold NIB.

5.2 Centrifuge at 4 °C for 00:10:00 .

6 If after centrifugation supernatant is clear, decant supernatant and take pellet into **step 7**.

If coloration remains in the supernatant after centrifugation, dispose of supernatant and resuspend nuclei pellet in NIB, bringing volume up 10 ml - 15 ml, and go to step #5.2 .

For conifer tissue it was important to repeat this step 3-5X; however, in maize tissue subsequent washes have been unnecessary.

7 Remove supernatant and resuspend in 1 ml 1X HB.

8 At this point, you can either snap freeze nuclei or proceed to lysis.

- Please select 'Snap Freezing' or 'Lysis' to proceed.

step case

Snap Freezing

no description provided

Snap Freezing

9 Spin down your nuclei suspension in a 1.5 mL tube at 5000 x g - 7000 x g for 00:05:00 , remove supernatant and snap freeze in liquid nitrogen, then store at -80 °C.

Lysis Preperation

step case

Lysis

and **Nanobind-assisted DNA Purification**

9 Spin down your nuclei suspension in a 1.5 mL tube at 5000 x g - 7000 x g for 00:05:00 , remove supernatant, and proceed to lysis.

Nanobind-assisted DNA Purification

10 Resuspend isolated plant cell nuclei with 30 µl of Proteinase K. Vortex on high until fully resuspended. Spin tube on mini-centrifuge for 00:00:02 to remove liquid from cap.