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High Molecular Weight Total DNA Extraction from plant tissues for Long Read Sequencing

Forked from Total High Molecular Weight DNA Extraction from plant tissues for Long Read Sequencing

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ABSTRACT

This protocol was developed as a research within GIH collaborative projects for a sample where a species of Neptunia leaves sample was problematic in our previously developed protocol. At step 21 of previous protocol, the solution forms either a brownish mark on the top layer of the solution or the whole solution depending on the starting sample amount which resulted brown CTAB-DNA complex. In this protocol, the problematic steps for a particular sample is improved to extract high quality High Molecular Weight (HMW) DNA >60kb. The DNA quality was assessed in Qubit, NanoDrop, TapeStation, and Oxford Nanopore Technologies. Using a LSK109 ligation chemistry and R9.4 flow cell, a total yield of 24gb with N50 29kb was generated in MinION sequencing platform.

ATTACHMENTS

High Molecular Weight Total DNA Extraction from plant tissues for Long Read Sequencing - Forked.pdf

DOI

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PROTOCOL CITATION

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FORK NOTE

FORK FROM

Forked from Total High Molecular Weight DNA Extraction from plant tissues for Long Read Sequencing, Subash Rai

KEYWORDS

High Molecular Weight DNA, Plant HMW DNA extraction, Neptunia HMW DNA, long read sequencing, HMW total DNA

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GUIDELINES

Starting materials:

- Young and healthy tissues are ideal samples for HMW DNA extraction. The amount of sample required depends on the plant genome size. More material is required for small genome plants when compared to bigger genome plants (of equivalent sample quality).

Handling of HMW DNA:

- Always use wide-bore pipette tips as recommended in the protocol.
- Allow the DNA to stand in elution buffer **Overnight** at **Room temperature** or tap the tube gently.

NO vortexing at all!!

- Avoid repeated cycle of freezing and thawing. Aliquot the required amount of DNA in multiple tubes before storing at **-20 °C** / **-80 °C** .

Citations

Xin, Z., & Chen, J. (2012). A high throughput DNA extraction method with high yield and quality.. Plant Methods. https://doi.org/10.1186/1746-4811-8-26

Mayjonade, B., Gouzy, J., Donnadieu, C., Pouilly, N., Marande, W., Callot, C., Langlade, N., & Muños, S. (2018). Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules. Biotechniques. https://doi.org/10.2144/000114460

MATERIALS TEXT

Materials and consumables

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A	B	C
Items description	**Catalogue number**	**Suppliers/Manufacturers**
Ammonium Acetate 7.5M Solution	A2706-100ML	Sigma Aldrich
Chloroform:Isoamyl alcohol (24:1)	ACR327155000	Thermo Fisher
CTAB	52365-50G	Sigma Aldrich
Distilled water Ultra-Pure	10977015	Thermo Fisher Scientific
DNA LoBind tubes 1.5ml	30108051	Eppendorf
Dry ice	-	-
Dynabeads M-270 Carboxylic Acid	14306D	Thermo Fisher
EDTA (0.5M), pH-8, Nuclease-free	AM9260G	Life Technologies
Ethanol (>98%)	US015017	Thermo Fisher Scientific
Falcon tube 15ml	FAL352096	In Vitro Technologies
Isoamyl alcohol (>98%)	W205702-1KG-K	Sigma Aldrich
Liquid Nitrogen (LN2)	-	-
P1000 wide bore pipette tips	2079GPK	Thermo Fisher Scientific
P200 wide bore pipette tips	LC1152-965	Adelab Scientific
PEG 8000	V3011	Promega
Proteinase K (PK) Solution	MC5005	Promega
Qubit 1× dsDNA HS Assay Kit	Q33231	Life Technologies
RNase solution	A7973	Promega
Sodium Chloride	71580-500G	Sigma Aldrich
UltraPure 1M Tris-HCl, pH-8	15568025	Life Technologies
β-mercaptoethanol	M6250-100 mL	Sigma Aldrich

☒ Ammonium acetate solution for molecular biology, 7.5 M **Millipore Sigma Catalog #A2706**

☒ CTAB (Hexadecyltrimethylammonium bromide) **Sigma Aldrich Catalog #52365-50G**

☒ UltraPure Distilled Water **Thermo Fisher Scientific Catalog #10977015**

☒ DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108051**

☒ Dynabeads™ M-270 Carboxylic Acid **Thermo Fisher Catalog #14306D**

☒ EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G**

☒ Falcon® 15 mL Polystyrene Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Bag **Invitrogen Catalog #FAL352095**

☒ ART™ Barrier Specialty Pipette Tips, 1000, wide bore **Thermo Fisher Catalog #2079GPK**

☒ PEG-8000 **Promega Catalog #V30111**

☒ Proteinase K (PK) Solution, 4ml **Promega Catalog #MC5005**

☒ Qubit™ 1X dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231**

☒ RNase A Solution, 4mg/ml **Promega Catalog #A7973**

☒ 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #15568025**

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1% β-mercaptoethanol

Sigma Catalog #M6250

isoamyl

alcohol **Sigma Catalog #W205702**

Equipment

- Benchtop centrifuge
- Centrifuge for 15ml falcon tube
- Esky/container for dry ice
- Flask Dewar or equivalent to transport LN2
- Heat block
- HulaMixer
- Magnetic rack
- Mini centrifuge
- Mortar and pestle
- NanoDrop
- Qubit
- TapeStation or equivalent
- Thermomixer (with adapter for 15ml tubes)

SAFETY WARNINGS

- Chloroform: Isoamyl alcohol (24:1) waste should be collected in a separate waste container.
- Experiment should be performed under fume hood after adding β-mercaptoethanol in lysis buffer during the extraction step.
- Follow the standard Liquid Nitrogen handling procedures.
- Consult MSDS for each required reagent and handle accordingly.

BEFORE STARTING

Prepare the following buffers and solutions before starting the experiment:

Lysis Buffer

A	B
Tris-HCl	100 mM
EDTA	20 mM
CTAB (w/v)	4%
NaCl	1.4 M
PVP 360k (w/v)	1%
β-mercaptoethanol (add just before use)]	2%

Combine the reagents given in the table below.

A	B
1M Tris-HCl (pH = 8)	5 ml
0.5M EDTA (pH = 8)	2 ml
CTAB powder	2 g
PVP	0.5 g
NaCl	4 g

Adjust the final volume to **50 mL** with Nuclease free water/lab grade water. Store at **Room temperature** for up to 3-4months.

High-salt TE buffer

A	B
EDTA	2 mM
Tris-HCl	10 mM
NaCl	1 M

Combine the reagents given in the table below

A	B
NaCl	581 mg
0.5M EDTA (pH=8)	40 µl
1M Tris-HCl (pH=8)	100 µl

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Complete to **10 mL** with Distilled water Ultra-Pure. Autoclave it for long-term (1 year) storage.

Binding buffer (20% PEG8000 and 3M NaCl):
Add **2 g** PEG 8000 and **1.75 g** NaCl in **10 mL** nuclease free water and mix well until it turns as a clear solution and store at cold room or 4-7°C.

Beads solution:

A	B
Dynabeads™ M-270 Carboxylic Acid	4%
PEG8000	18%
NaCl	1 M
Tris-HCl pH-8	10 mM
EDTA pH-8	1 mM

- First prepare the required volume of the solution except Dynabeads.
- Keep the Dynabeads at RT for at least **00:15:00** . Mix well by vortexing, then take 4% of the beads solution (v/v) immediately.
- Wash the beads with nuclease free water 3 times. Resuspend the beads pellet completely while washing.
- Add the beads solution and store the beads solution at **4 °C** .
- Keep the beads solution at **Room temperature** for at least **00:15:00** and mix well before using it.

Tissues preparation and lysis 1h 12m

1 Take **10 mL** **lysis buffer** and warm it at **60 °C** for 15-20 min. 20m

2 Take ~ **1 L** of liquid nitrogen (LN_2) in Dewar Flask that requires for chilling mortar and pestle and grinding the tissues.

3 Take dry ice in an esky/insulated container for later steps.

4 Grind **500 mg** to **1000 mg** healthy young fresh/snap frozen/frozen tissues in mortar and pestle chilled with LN_2 to fine powder. 15m

> It may require topping up 2-3 times LN_2 while grinding the plant tissues.

5 Keep a 15 ml falcon tube on the dry ice for **00:05:00** then swirl the ground powder with LN_2 and pour directly into the falcon tube while keeping the falcon tube on the dry ice. 5m

6 Keep the lid half-opened and let LN_2 to evaporate. 10m

7  1m

Take out the tube and add **10 mL** prewarmed **lysis buffer** (at **60 °C**) with freshly added **200 µL** β-mercaptoethanol.

 30m

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8

Mix well by inverting the tubes (~100 times) until the solution become more homogenous. In some sample, solution may not be homogenous but form whiteish clumps (it is normal) and incubate at **60 °C** in thermomixer at **300 rpm** for **00:30:00** .

9 1m

Add **200 µL** Proteinase K (stock conc= **20 mg/mL**) after 15 min of incubation.

10 1m

Mix well by inverting the tube (15-20 times) and continue the incubation.

11 5m

Spin the solution at **3000 x g, Room temperature, 00:05:00** .

If any clump formed during the incubation pellet would be large.

12 3m

Take an equal volume of the supernatant in two fresh 15 ml falcon tubes using P1000 wide bore pipette tips.

Extraction of raw HMW DNA 2h 37m

13 30s

Add an equal volume of Chloroform:Isoamyl alcohol (24:1) into the solution.

14 10m

Mix the solution by inverting the tube until a milky colour appears (~100 times) and centrifuge at **3000 x g, Room temperature, 00:10:00** .

15 2m

Transfer the aqueous phase to a new 15ml falcon tube without disturbing interface layer.

16 30s

Add an equal volume of Chloroform:Isoamyl alcohol (24:1) into the solution.

17 10m

Mix the solution by inverting the tubes ~100 times and centrifuge at **3000 x g, 00:10:00** .

18 2m

Transfer 1 ml aqueous phase to 2 ml LoBind tube without disturbing the interface layer (much thinner than the first extraction). It requires multiple 2 ml LoBind tube.

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transferring aqueous phase to 2 ml tube is for making things easy for centrifugation but can be done in a single tube if the centrigugation set up is available for a bigger volume tube.

19 12m

Add half volume of Ammonium acetate (7.5 M). Mix well by inverting the tubes and incubate for **00:10:00** at **Room temperature**

20 10m

Centrifuge at **13000 x g, Room temperature, 00:10:00** .

21 Transfer the supernatant in a fresh 2ml LoBind tube and add equal volume of Isopropanol (>98%), mix well, and incubate for **00:10:00** at **Room temperature** . 10m

22 1m

Resuspend the pellet with **1 mL** 70% ethanol (**freshly prepared**) using a wide bore P1000 pipette tip and transfer all into a 2ml LoBind DNA tube.

23 1m

Rinse the tube with additional **1 mL** 70% ethanol to collect remaining CTAB-DNA complex. Perform the same for another 15 ml falcon tube.

24 5m

Incubate 2 ml tubes for **00:05:00** at **Room temperature** in a HulaMixer at **9 rpm** .

25 5m

Spin the tube at **13000 x g, 00:05:00** and discard the supernatant.

26 10m

Repeat washing steps & once with **2 mL** 70% ethanol.

27 Keep the tubes under fume hood for **00:05:00** to remove any traces of ethanol. 5m

28 Resuspend the DNA pellet in **200 µL** of prewarmed (**60 °C**) **High-salt TE buffer**. 5m

29 20m

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Add **4 µL** RNaseA and incubate at **37 °C** for 15-20 min.

Beads Purification of HMW DNA 1h 11m

30 10m

Add **100 µL** ammonium acetate (**7.5 Molarity (M)**) mix well and incubate it for **00:10:00** at **Room temperature** . Shake it once every 5 min.

31 3m

Spin the tube at **13000 x g, 00:03:00** and transfer the supernatant using wide-bore pipette tips into a fresh tube.

32 1m

Add equal volume of **binding buffer** and mix well by inverting the tube.

33 30m

Add **150 µL** beads solution (8-9 million beads) and incubate it for **00:30:00** at **Room temperature** in a HulaMixture.

34 3m

Place the tube in magnetic rack for 2-3 min and remove the supernatant and wash the beads with **500 µL** freshly prepared 70% ethanol (clumping of beads may appear but try to dislodge by inverting the tube several times).

35

Wash the pellet once again with **500 µL** freshly prepared 70% ethanol.

35.1 Take out the tubes from magnetic rack and add **500 µL** freshly prepared 70% ethanol. 30s

35.2 Dislodge the beads by inverting the tubes. 30s

35.3 Place the tubes back to the magnetic rack for 2 min and remove the supernatant. 2m 30s

36 15m

Add **75 µL** prewarmed (at **50 °C**) **10 millimolar (mM)** Tris-HCl (elution buffer) **8** and incubate at **Room temperature** for **00:15:00** .

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37 Place the tube back in the magnetic rack and leave it for **00:05:00** . 5m

38 Remove the supernatant in the fresh 1.5ml LoBind DNA tube. 30s

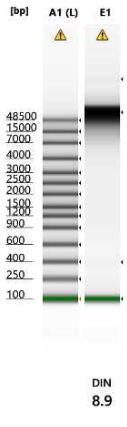
If the eluate is very viscus and beads could not pellet either add more elution buffer or centrifuge **13000 x g, 00:05:00** .

39 Assess DNA quality in NanoDrop, Qubit, and TapeStation/PFGE

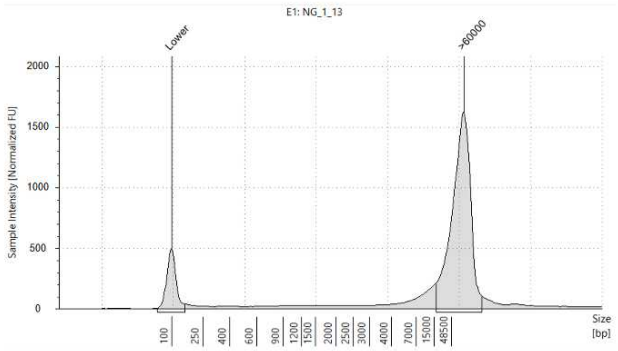
Worked Results

40

General Quality check:



Qubit (ng/µl)	NanoDrop (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}	DIN
150	185	1.86	1.7	8.9



QC check in Oxford Nanopore Sequencing:

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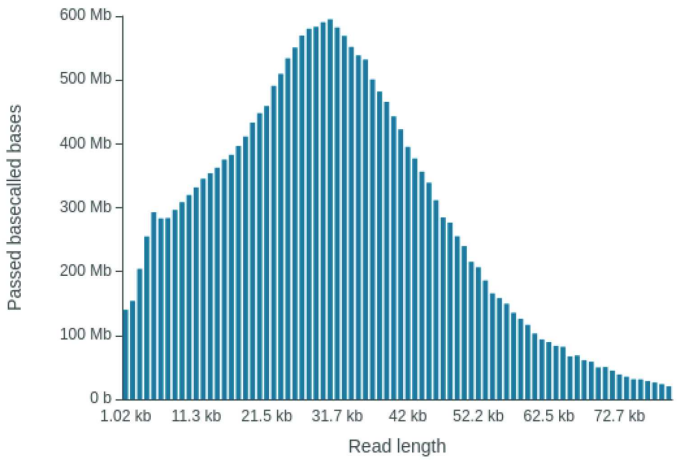
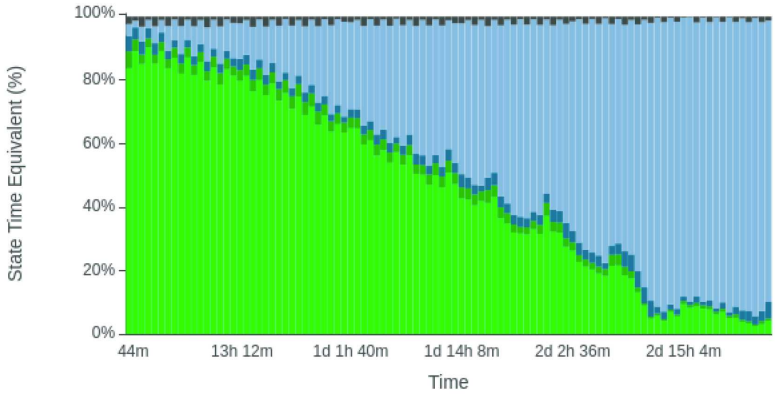
Estimated N50: 29.54 kb



Read length distribution (estimated N50 = 29.5kb) in MinION Nanopore sequencing. DNA was extracted from frozen leaves sample. The size selection was performed with standard SRE kit (circulomics) prior to the library preparation using ONT LSK109 kit.

Duty Time Grouped



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