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

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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. Using this protocol, 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.

Keywords

HMW DNA, plants, mangoes, senecio, neptunia, long-read sequencing, DNA extraction

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 RT 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

Before start:

Prepare the following buffers and solutions before starting the experiment:

1. **Lysis Buffer** [100mM Tris-HCl, 20mM EDTA, 4% CTAB (w/v), 1.4M (NaCl), 1% PVP 360k (w/v), 2% β-mercaptoethanol (add just before use)]:
   Combine 5ml 1M Tris-HCl (pH = 8), 2ml 0.5M EDTA (pH = 8), 2g CTAB powder, 0.5g PVP, and 4g NaCl. Adjust the final volume to 50ml with Nuclease free water/lab grade water. Store at room temperature up to 3-4months.
2. **High-salt TE buffer** [2mM EDTA, 10mM Tris-HCl, and 1M NaCl]:
   Combine 581mg NaCl, 40µl 0.5M EDTA (pH=8), 100µl 1M Tris-HCl (pH=8). Complete to 10ml with Distilled water Ultra-Pure. Autoclave it for long-term (1 year) storage.
3. **Binding buffer (**20% PEG8000 and 3M NaCl):
   Add 2g PEG 8000 and 1.75g NaCl in 10ml nuclease free water and mix well until it turns as a clear solution and store at cold room or 4-7°C.

4. **Beads solution:** (4% Dynabeads™ M-270 Carboxylic Acid, 18%PEG8000, 1M NaCl, 10mM Tris-HCl pH-8, 1mM EDTA pH-8):
    - First prepare the required volume of the solution except Dynabeads.
    - Keep the Dynabeads at RT for at least 15min. 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 RT for at least 15min and mix well before using it.

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.

Materials and consumables

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	0030108051	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
Liquid Nitrogen (LN_2)	-	-
Isoamyl alcohol (>98%)	W205702-1KG-K	Sigma Aldrich
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-HCI, pH-8	15568025	Life Technologies
β-mercaptoethanol	M6250-100mL	Sigma Aldrich

Equipment

- Benchtop centrifuge
- Centrifuge for 15ml falcon tube
- Esky/insulated 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)

Additional Notes: NA

Procedures

Tissues preparation and lysis

1. Take 10ml lysis buffer and warm it at 60°C for 15-20min.
2. Take ~1L of LN2 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 500-1000mg healthy young fresh/snap frozen/frozen tissues in mortar and pestle chilled with Liquid Nitrogen (LN_2) to fine powder. It may require topping up 2-3 times LN_2 while grinding the plant tissues.
5. Keep a 15ml falcon tube on the dry ice for 5min then swirl the ground powder with LN_2 and pour directly into the falcon tube while keeping the falcon tube on the dry ice.
6. Keep the lid half-opened and let LN_2 to evaporate.
7. Take out the tube and add 10ml prewarmed lysis buffer (at 60°C) with freshly added 200µl β-mercaptoethanol.
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 a whiteish clumps (it is normal) and incubate at 60°C in thermomixer at 300rpm for 30 min.
9. Add 200µl Proteinase K (stock conc=20mg/ml) after 20 min of incubation.
10. Mix well by inverting the tube (15-20 times) and continue the incubation.
11. Spin the solution at RT for 5min at 3000×g. If any clump formed during the incubation pellet would be large.
12. Take an equal volume of the supernatant in two fresh 15ml falcon tubes using P1000 wide bore pipette tips.

Extraction of raw HMW DNA:

13. Add an equal volume of Chloroform:Isoamyl alcohol (24:1) into the solution.
14. Mix the solution by inverting the tube until a milky colour appears (~100 times) and centrifuge at RT for 10min at 3000×g.
15. Transfer the aqueous phase to a new 15ml falcon tube without disturbing interface layer.
16. Add an equal volume of Chloroform:Isoamyl alcohol (24:1) into the solution.
17. Mix the solution by inverting the tubes ~100 times and centrifuge at 3000×g for 10min.
18. Transfer the aqueous phase to a new 15 ml falcon tube without disturbing the interface layer (much thinner than the first extraction).
19. Dispense 1 ml aqueous solution in a 2 ml LoBind tube and add half volume of Ammonium acetate (7.5M) and mix well by inverting the tubes.
20. Centrifuge at 13000×g for 10 min.
21. Transfer the supernatant in fresh 2 ml LoBind tube and add an equal volume of Isopropanol, mix well, and incubate at RT for 10 min.
22. Centrifuge at 13000×g for 10 min.
23. Resuspend the pellet with 1ml 70% ethanol (freshly prepared) using a wide bore P1000 pipette tip and transfer all into a 2ml LoBind DNA tube.
24. Incubate 2ml tubes for 5min at RT in a HulaMixer at 9 rpm.
25. Spin the tube at 13000×g for 5min and discard the supernatant.

26. Repeat washing step once with 2ml 70% ethanol.
27. Keep the tubes under fume hood for 5min to remove any traces of ethanol.
28. Resuspend the DNA pellet in 200µl of prewarmed (60°C) High-salt TE buffer.
29. Add 4µl RNaseA and incubate at 37°C for 15-20min.

Beads Purification of HMW DNA:

30. Add 100µl ammonium acetate (7.5M) mix well and incubate it for 10 min at RT. Shake it once every 5min.
31. Spin the tube at 13000×g for 3min and transfer the supernatant using wide-bore pipette tips into a fresh tube.
32. Add equal volume of binding buffer and mix well by inverting the tube.
33. Add 150µl beads solution (8-9 million beads) and incubate it for 30min at RT in a HulaMixture.
34. Place the tube in magnetic rack for 2-3min and remove the supernatant and wash the beads with 500µl 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 500µl freshly prepared 70% ethanol.
36. Add 75µl prewarmed (at 50°C) 10mM Tris-HCl (elution buffer) pH-8 and incubate at RT for 15min.
37. Place the tube back in the magnetic rack and leave it for 5min.
38. Remove the supernatant in the fresh 1.5ml LoBind DNA tube (if the eluate is very viscus and beads could not pellet either add more elution buffer or centrifuge 13000×g for 5min).
39. Assess DNA quality in NanoDrop, Qubit, and TapeStation/PFGE.

Citations

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