An optimized protocol for high-molecular-weight DNA extraction in plant genomic studies

Note: This protocol starts with 2 g of fresh and young leaves. Usually, the end product of one extraction process is enough for 4-5 libraries for sequencing with MinION or GridION of Oxford Nanopore Technologies (Oxford, United Kimdom).

I. PREPARATION OF SOLUTIONS

1. Preparation of nuclei isolation buffer (IB) (for 10 reactions)

- For 200 ml of nuclei isolation buffer, dissolve the following in ca. 100 ml water

  Tris-HCl (1 M stock; ph 9.5) 3 ml (Final conc.: 15 mM)

  EDTA (0.5 M stock) 4 ml (Final conc.: 10 mM)

  KCl 1.94 g (Final conc.: 130 mM)

  NaCl (5 M stock) 0.8 ml (Final conc.: 20 mM)

- Gradually add 16 g of PVP-10 while rapidly stirring the solution with a magnetic stir bar.
- Fill with water to 200 ml.
- Add 0.05 g of spermine and 0.07 g of spermidine (Store IB at 4 ℃).
- After mixing the IB with the ground sample, add Triton X-100 and B-mercaptoethanol to final concentrations of 0.1% and 7.5%, respectively (this constitutes IBTB). (e. g., add 40 ul of Triton X-100 and 3 ml of B-mercaptoethanol to 40 ml of IB).

Note: Store in a 4 ˚C refrigerator until use. Two weeks will be safe.

2. Preparation of Carlson lysis buffer (Carlson et al., 1991)

- Carlson lysis buffer = 2X CTAB (Cetyltrimethylammonium Bromide) buffer + 1% PEG 6000
- For 100 ml of Carlson lysis buffer

  Tris-HCl (1 M stock ph 9.5) 10 ml (Final conc.: 100 mM)

  EDTA (0.5M stock) 4 ml (Final conc.: 20 mM)

  NaCl 8.2 g (Final conc.: 1.4 M)

  CTAB 2 g (Final conc.: 2 %)

  PEG 1 g (Final conc.: 1 %)

Note: Store at RT until use. Two weeks will be safe.

3. Tris-EDTA buffer (TE) (1 X)

- TE buffer = Tris-HCl (pH 8.0) 10mM + EDTA 1mM

Note: Store at 4 ˚C until use.

II. GRINDING AND NUCLEI ISOLATION (modified from Peterson et al., 1997)

1. Chill mortar and pestle at a -80 ℃ before beginning extraction procedure. Grind 2 g of fresh young leaves in liquid nitrogen until they become a fine powder.
2. Add 2 g of ground leaf powder to 20 ml of IB in a 50-ml conical tube and mix by inverting.

   Note: Over-grinding is a negative factor in extracting HMW DNA. Grinding until the temperature increases is fine: additional liquid nitrogen is not necessary.

   Note: Increase the sample amount for succulent plants and increase the volume of IB when the mixture becomes viscous.
3. Immediately add 20 ul of Triton X-100 and 1.5 ml of B-mercaptoethanol and mix by inverting.
4. Keep on ice for 10 min.

   Note: This step should be conducted inside a fume hood because the IBTB contains B-mercaptoethanol, which is toxic.
5. Filter mixture through a vacuum-aid cell strainer (pore size: 100 ㎛) seated on a 50-ml conical tube to collect nuclei suspension (Fig. 1).

   Note: To aid filtration, gently scrape away plant tissue from the filter with the top of a 1,000-ul pipette (blue) tip. The filtrate should look light green.
6. Repeat the filtering step with 40-㎛ pore sized cell strainer.
7. Add 200 ul Triton X-100.

   Note: This is a step to lyse cell and organelle membranes (except nuclear membrane).
8. To pellet the nuclei, centrifuge for 10 min at 3,000 g at 4 ℃.
9. Discard the supernatant.

III. EXTRACT NUCLEAR DNA USING CTAB BUFFER (modified from Doyle and Doyle, 1987)

1. Add 5 ml of Carlson lysis buffer and 12.5 ul of B-mercaptoethanol to the tube and resuspend nuclei pellet with brief tapping.

   Note: Incomplete resuspension could reduce yield since many nuclei are not lysed by

CTAB. Briefly pipetting the pellet with an end-cut 1,000-ul pipette tip and gentle vortexing may help resuspension.

2. Incubate at 65 ℃ for 15 min. (maximum 2 hours).

Note: If the pellet is not completely resuspended after incubation, a brief centrifugation (3,000 g for 5 min) followed by only the use of the supernatant will help speed up processing.

3. Transfer suspended nuclei pellet to 15-ml polypropylene tube and add equal volume (5 ml) of chloroform:isoamyl alcohol (24:1) solution.

4. Invert several times to mix them.

5. Centrifuge (3,000 g) for 10 min. at 4 ℃.

6. Transfer the aqueous upper phase to a new tube using P1000 pipette.

Note: Taking only 9/10 of the supernatant is safe to avoid inclusion of cellular debris. This step is highly correlated with the quality of extracted DNA.

Note: If it is viscous, pipetting slowly will help avoid sucking up the plant tissue.

7. Repeat 3-6 (optional but highly recommended).

8. Add 1/10 volume of 3 M sodium acetate, mix gently, add the same volume of isopropanol (room temperature), and gently invert several times.

Note: For 4.5 ml of supernatant, add 0.45 ml of 3 M sodium acetate and 4.95 ml of isopropanol. 9. Precipitate at -20 ℃ for more than an hour.

Note: In case aggregates are visible, moving to the next step is possible for faster processing.

10. Centrifuge (3,000 g) for 10 min. at 4 ℃.

11. Discard supernatant.

12. Wash pellets with 70% cold ethanol (ca. 20 ml per tube).

13. Centrifuge (3,000 g) for 10 min. at 4 ℃.

14. Discard supernatant.

Note: Keep the tube inverted for 1 min., and wipe out the tube wall with a Kimwipe.

15. Dry the pellet completely.

Note: This step is very important for the quality of DNA. The smell of alcohol is a good indicator of incomplete drying.

IV. RNASE A AND PROTEASE K TREATMENT

1. Dissolve pellet with 2 ml of TE buffer.

   Note: If the pellet is difficult to dissolve, incubate in 50 ℃ water bath for up to 10 min.

   Note: Gently crushing pellet with pipette tip might be helpful for faster resuspension, but never vortex the sample. If the pellet is not completely resuspended after incubation, a brief centrifugation (3,000 g for 5 min.) followed by only the use of the supernatant will help speed up processing.

2. Add 20 ul (10 ul/ml) of RNase A (10 mg/ml conc.).

3. Incubate at 37 ℃ for 5 min.

4. Add 20 ul (10 ul/ml) of proteinase K (>600 units/ml conc.).

5. Incubate at 50 ℃ for 15 min.

6. Add equal volume (2 ml) of chloroform:isoamyl alcohol (24:1).

7. Invert several times to mix.

8. Centrifuge (3,000 g) for 10 min. at 4 ℃.

9. Transfer aqueous upper phase to a new 15-ml tube.

   Note: Taking only 9/10 of the supernatant is safe to avoid inclusion of cellular debris. This step is highly correlated with the quality of extracted DNA.

10. Repeat 6-9 (optional).

11. Add 1/10 volume of 3 M NaOAc, mix gently, add equal volume of room-temperature isopropanol, and gently invert several times.

    Note: For 3.5 ml of supernatant, add 0.35 ml of 3 M NaOAc and add 3.85 ml of isopropanol.

12. Precipitate at -20 ℃ for more than 1 hour.

    Note: If aggregates are visible, moving to the next step is possible for fast processing.

14. Centrifuge (3000 g) for 10 min. at 4 ℃.

15. Discard supernatant.

16. Wash pellets with 70% cold ethanol (ca. 5 ml per a tube).

17. Centrifuge (3000 g) for 10 min. at 4 ℃.

18. Discard supernatant.

    Note: Keep the tube inverted for 1 min., and wipe out the tube wall with a Kimwipe.

19. Completely drying.

    Note: This step is very important for the quality of DNA. The smell of alcohol is a good indicator of incomplete drying.

20. Add 50-500 ul of water in each tube to dissolve pellet.

    Note: If it is difficult to dissolve, incubate in 50 ℃ water bath for up to 10 min.

    Note: Crushing pellet with pipette tip might be helpful for faster resuspension, but never vortex the sample. If the pellet is not completely resuspended after incubation, a brief centrifugation (3,000 g for 5 min.) followed by only the use of the supernatant will help speed up processing.

21. Check the quality (A260/280 and A260/230) and quantity of extracted DNA using the Nanodrop (ND2000USCAN; Thermo Fisher Scientific, Waltham, USA) and the Qubit.

22. Check length distribution using the Femto Pulse System (Agilent, Santa Clara, USA).

V. SPECIAL REAGENTS AND CONSUMABLES

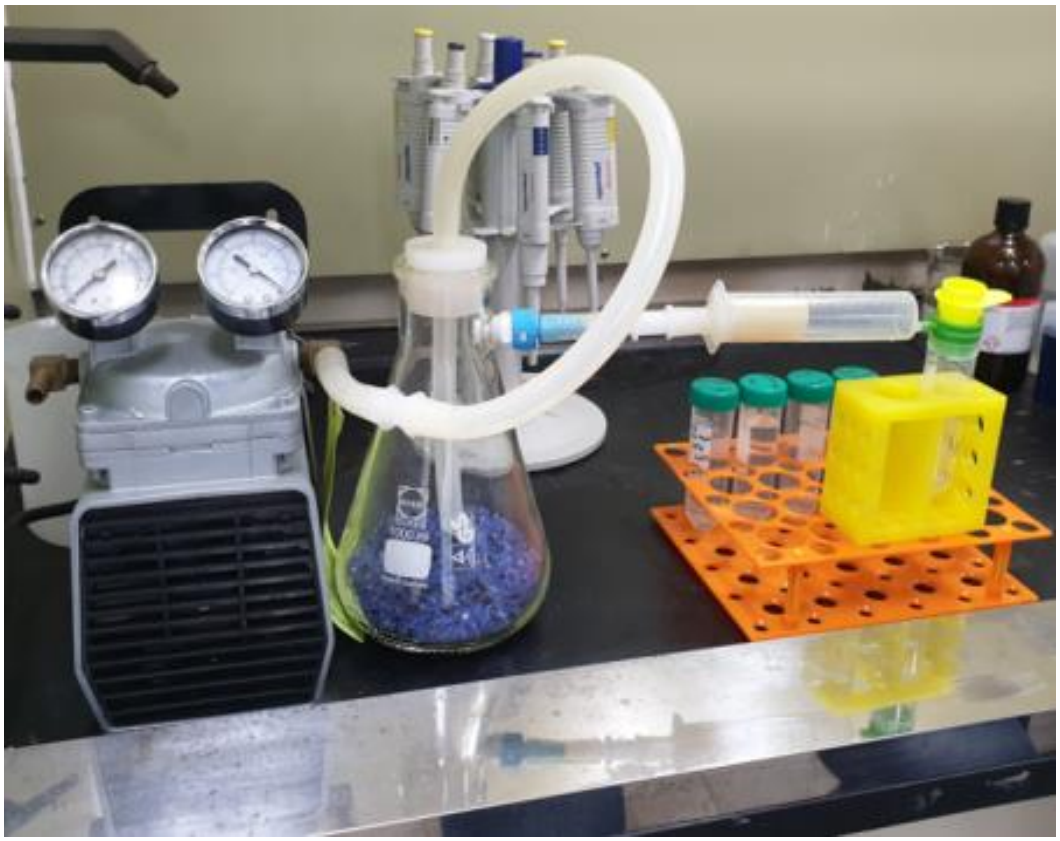
1. Reagents

- PVP-10: Sigma-Aldrich CAS 9003-39-8
- spermine: Sigma-Aldrich S2876
- spermidine: Sigma-Aldrich S2501
- Triton X-100: Sigma-Aldrich T8787
- PEG 6000: Sigma-Aldrich 81260
- RNase A: Sigma-Aldrich R6513
- Proteinase K: Sigma-Aldrich P2308

2. Consumables

- Vacuum-aid cell strainer (100 μm): pluriSelect 43-50100-51 yellow 100 μm
- Vacuum-aid cell strainer (40 μm): pluriSelect 43-50040-51 blue 40 μm
- Connector ring: pluriSelect 41-50000-03

Fig. 1. Use of vacuum-aid filtration can shorten extraction times.



References

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