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Nuclei extraction protocol for flow cytometry based genome size estimation

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We use this protocol and it's working

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Abstract

Flow cytometry (FCM) is widely opted to estimate genome size in plants. The sample preparations include chopping a fresh plant material in a suitable buffer to release intact nuclei. The nuclear integrity is a vital component of flow cytometry. However, due to logistic constraints and lack of immediate lab facilities, fresh plant material becomes unsuitable for flow cytometry purposes. Plant material is frozen for genome size sequencing purposes, and it is common to extract nuclei from frozen plant material for nuclei acid extractions. The method presented here is for extracting nuclei from frozen plant material which can be used to estimate FCM-based genome size.

Reagents

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1. Liquid Nitrogen
2. spermidine trihydrochloride (Sigma,catalogue number S2501),
3. spermine tetrahydrochloride (Sigma, cat. no. S1141),
4. sucrose (Sigma, cat. no. S9378),
5. Triton X-100 (Chem Supply, cat. No. TL125-P),
6. polyvinylpyrrolidone-360(Sigma, cat. no. PVP360),
7. Trizma Base (Sigma, cat. no. T1503),
8. potassium chloride (Scharlau, cat. no. 0401)
9. 2-mercaptoethanol, 14 M (Sigma, cat. no. M3148),
10. 0.5 M ethylene diamine tetra acetic acid (Biobasic Inc., Product: EB0185)

Equipments

2

1. Sterilised mortar and pestle
2. refrigerated centrifuge equipped with fixed angle rotor (Sigma Model 4-16K),
3. Steriflip vacuum-driven filtration system with 20 µm nylon net filter (Merck Millipore, Cat. no. SCNY00020),
4. 40 µm polypropylene framed cell strainers (Biologix, Product ID 15-1040),
5. Pasteur pipettes (20 µl, 200 µl, 1000 µl, 5000 µl),
6. 50 ml conical bottom centrifuge tubes (Corning, Product ID 430304),
7. 1.5 ml microfuge tubes,
8. 5 ml (12 × 75 mm) polystyrene round bottom tubes (Falcon, Product ID PID0587866)

Buffer Preparation

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- 10x Homogenisation buffer (HB): Trizma Base (0.1 M), Potassium chloride (0.8 M), ethylene diamine tetra acetic acid (0.1 M), spermidine (17 mM), spermine (17 mM), 10 M NaOH to adjust pH to 9. The solution can be stored in a glass bottle at 4 °C for up to one year.
- 100 ml Triton sucrose buffer (TSB): Triton X-100 (20 %), 10x HB (10 %), sucrose (0.5 M), Volume was made up to 100 ml with distilled water. The solution can be stored in a glass bottle at 4 °C for up to one year.
- 1000 ml 1x Homogenisation buffer (HB): 10x HB (10%), Sucrose (0.5 M), Volume was made up to 1 L with distilled water
- 50 ml/sample Nuclei Isolation Buffer (NIB): 1x HB (48.75 ml), TSB (1.25 ml), polyvinylpyrrolidone-360 (0.5 gm), Add 125 µl of 2-mercaptoethanol before use and keep NIB on ice.

Nuclear Isolation Protocol

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1. Before starting the procedure of nuclei extraction, 50 ml of NIB per 2 gm sample was prepared fresh and stored at 4 °C.
2. 1.8 gm of the frozen leaf tissue of sample species and 0.2 gm of frozen leaf tissue of internal standard were taken in a sterilised, precooled mortar with liquid N_2. Leaf material was submerged in liquid N_2.
3. The plant material was pulverised **[1]** in a sterilised mortar and pestle in liquid N_2. Hard leaves took longer to grind; therefore, jabbing converted big leaf parts into smaller pieces. After removing large chunks, small pieces were crushed into powder form with circular round motions of the pestle. This is a temperature-sensitive step; therefore, keep adding liquid Nitrogen to avoid thawing.
4. Homogenisation and nuclear isolation: Using a precooled spatula, leaf powder was quickly transferred to a precooled 50 ml falcon tube prefilled with 7.5 ml of NIB. Falcon tubes with 7.5 ml NIB were kept on ice before starting the procedure.
5. With 4-5 swirls, the powder was submerged in the NIB that no clumps were visible. Another 7.5 ml
of NIB was added to the solution. The solution was gently mixed with the occasional end-to-end mixing for 2-3 min for 20 min. The solution was kept on ice to reduce the enzymatic activity of nucleases. After 20 min, a homogenate consisting of thousands of intact nuclei was ready.
6. Homogenate was filtered through 20 µm vacuum filtration system, and the filtrate was transferred in an empty 50ml falcon tube and kept on the ice.
7. Tubes were centrifuged at 7000 g and 4 °C for 20 min as the genome size was below 1000 Mbp. For large genomes (>1000 Mbp), centrifuge the tubes at 3000 g at 4 °C for 20 min. After centrifugation nuclei pellet was visible on the side or bottom of the tube; carefully discard the supernatant. At this stage, the pellet was green, representing contamination in the form of plant cell debris or secondary metabolites.
8. First wash: 7.5 ml of the NIB was added to the tube, and the pellet was mixed gently with a 10 ml
pipette by pipetting out 7-10 times. Another 7.5 ml of NIB was added to the tube. The solution was kept on ice for 10 min with occasional gentle mixing, as in step 5. Tubes were centrifuged as indicated in step 6. After discarding the supernatant pellet should have a light colour.
9. Second wash: Added 10 ml of NIB and mixed the pellet gently with a 10 ml pipette by pipetting out 7-10 times. The tubes were kept in ice for 10 min with occasional gentle mixing. Tubes were centrifuged, as mentioned in step 7. The supernatant was discarded carefully after the centrifugation.
10. Final wash and aliquots: In the final wash, 7.5 ml NIB was added to the tube, and the pellet was mixed with a pipette, as indicated in step 8. After mixing, the homogenate was equally allocated to five 1.5 ml microfuge tubes. Microfuge tubes were centrifuged at 7000 g for 10 min. The supernatant was discarded carefully. A white

nuclear pellet was visible at the bottom of the tubes. Nuclei pellets were snap-frozen in liquid N_2 after discarding the supernatant (no need to dry the tubes). Tubes were stored at -80 °C freezer until processed for flow cytometry measurement.

[1] Very fine grinding will damage the intactness of the nuclei, whereas too coarse will yield fewer
nuclei. For larger genomes, keeping the powder coarsely ground without any chunks or small leaf pieces is recommended. After grinding, secure the plant material at or below -80°C until processed further.

References

5 **Workman R, Timp W, Fedak R, Kilburn D, Hao S, Liu K**. 2018. High molecular weight DNA extraction from recalcitrant plant species for third generation sequencing.

Protocol references

Workman R, Timp W, Fedak R, Kilburn D, Hao S, Liu K. 2018. High molecular weight DNA extraction from recalcitrant plant species for third generation sequencing.