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Determining Genome Size from Spores of Seedless Vascular Plants

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[Abstract] Seedless vascular plants, including ferns and lycophytes, produce spores to initiate the gametophyte stage and to complete sexual reproduction. Approximately 10% of them are apomictic through the production of genomic unreduced spores. Being able to measure the spore nuclear DNA content is therefore important to infer their reproduction mode. Here we present a protocol of spore flow cytometry that allows an efficient determination of the reproductive modes of seedless vascular plants.
Keywords: Apomixis, Bead-vortex, Fern, Flow cytometry, Lycophyte, Spore

[Background] In seedless vascular plants, sporogenesis features, such as meiotic chromosome counts, were traditionally used to infer nuclear DNA content as well as reproductive modes. However, these approaches are time-consuming, or can only provide indirect evidence. An efficient and reliable method to estimate spore nuclear DNA content of these plants had not been established until Kuo *et al.* (2017). Herein, we describe a protocol using flow cytometry to evaluate spore genome sizes of these plants based on the work of Kuo *et al.* (2017).

Materials and Reagents

1. Pipette tips (10, 100, and 1,000 µl)
2. 50-ml tube
3. 1.7-ml tubes with caps
4. 2.0-ml tubes with caps
5. 2.3-mm stainless steel beads (Bio Spec Products, catalog number: 11079123ss)
6. 30-µmnylon meshes (Sysmex, CellTrics®, catalog number: 04-0042-2316)
7. 20-µmnylon meshes (Sysmex, CellTrics®, catalog number: 04-0042-2315)
8. 10-µm nylon meshes (Sysmex, CellTrics®, catalog number: 04-0042-2314)
9. Glass Petri dish (Corning, PYREX®, catalog number: 423790)
10. Leaf tissue of C-value standard (*e.g.*, *Nicotiana tabacum* L. 'Xanthi'; 2C = 10.04 pg, Johnston *et al.*, 1999)
11. Spores of ferns or lycophytes (kept by dry storage and under < 4 °C)
12. PVP-40 (Sigma-Aldrich, catalog number: PVP40)
13. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
14. RNaseA solution (10 mg/ml in ddH_2O) (Sigma-Aldrich, catalog number: R5000-100MG)

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15. Triton X-100
16. Sodium sulfite (Na_2SO_3)
17. Tris-HCl (pH 7.5)
18. Propidium iodide
19. Backmen stock buffer (see Recipes)*
    **Note: LB01 buffer (Doležel et al., 2007) or GPB buffer (Loureiro et al., 2007) can be alternatively used depending on plant material properties.*
20. PI solution (see Recipes)

Equipment

1. Pipette (10, 100, and 1,000 µl)
2. Vortex (Scientific Industries, model: Vortex-Genie 2)
3. Razors and razor pen
4. Flow cytometer (BD, BD Biosciences, model: FACScan)*
   **Note: FACScan with a 15 microW blue argon ion laser of an emission wave length of 488 nm.*

Software

1. BD FACSCan system (BD Biosciences, Franklin Lake, NJ, USA)

Procedure

1. Prepare buffer for use
   a. Allocate appropriate amount of Backmen stock buffer to a 50-ml tube based on an estimation of 1-1.5 ml per sample.
   b. Add 0.04 g PVP-40, 5 µl 2-mercaptoethanol, 1 µl RNase per ml of buffer.
2. Extract spore nuclei by bead-vortexing
   a. For each sample, weigh ~0.007 g spores into a 1.7-ml tube. Green spores that usually have thin spore walls, 4 times the amount of spores are recommended (Kuo *et al.*, 2017)*.
      **Note: For bead-vortexing, the detailed process can be seen in the video supplied in Kuo et al. (2017): http://onlinelibrary.wiley.com/store/10.1111/nph.14291/asset/supinfo/nph14291-sup-0002-VideoS1.mov?v=1&s=dae6f0590d33413f1444bc0add857d285fb73cd6*
   b. Add 16 stainless steel beads into each 1.7-ml tube.
   c. Add 250 µl of buffer into each 1.7-ml tube.
   d. Vortex these tubes at a speed of 1,900 rpm for 1 min. For green spores, a speed of 3,200 rpm and a vortex duration of 0.5 min are recommended (Kuo *et al.*, 2017).
   e. Filter bead-vortexed samples into 2.0-ml tubes through nylon meshes. The size of nylon mesh is selected based on spore sizes to prevent spore being filtered through the mesh.

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f. Add additional buffer to the samples, and ensure each of filtered spore nuclei solutions is greater than 500 µl in volume.

3. Extract standard nuclei by chopping leaf tissue
   a. Add 500 µl of buffer to a glass Petri dish.
   b. Add a (~400 mm^2) piece of young leaf to the Petri dish, and chop it with a razor on ice until most tissue slices are less than 1 mm in size.
   c. Filter the chopped sample into a 2.0-ml tube through a 30-µm nylon mesh.
   d. Add additional buffer to the sample, and ensure that the filtered leaf nuclei solution is greater than 500 µl in volume or more depending on need.
4. Staining nuclei solutions
   a. Mix spore nuclei and standard leaf nuclei solutions into a 500-µl volume in 2.0-ml tubes.
   b. Add 10 µl PI solution into each of mixed nuclei solutions.
   c. Incubate in the dark at 4 °C for 1 h for staining.

Data analysis

1. Set up a histogram plot of particle count vs. linear value of relative fluorescence in BD FACSCan system.
2. After PI staining, measure nuclear DNA content of the samples in BD FACSCan system, and adjust the fluorescence laser voltage to visualize the nuclei peaks on the histogram plot.
3. Measure > 1,300 particles for each peak, and the coefficient variation for each peak should be lower 5% as the quality criteria suggested by Greilhuber *et al.* (2007).
4. Genome size of spore nuclei (pg or Mbp) = $\frac{Sporenuclei\ mean\ peak\ position}{Standard\ 2C\ leaf\ nuclei\ mean\ peak\ position}$ x standard 2C value (Figure 1).

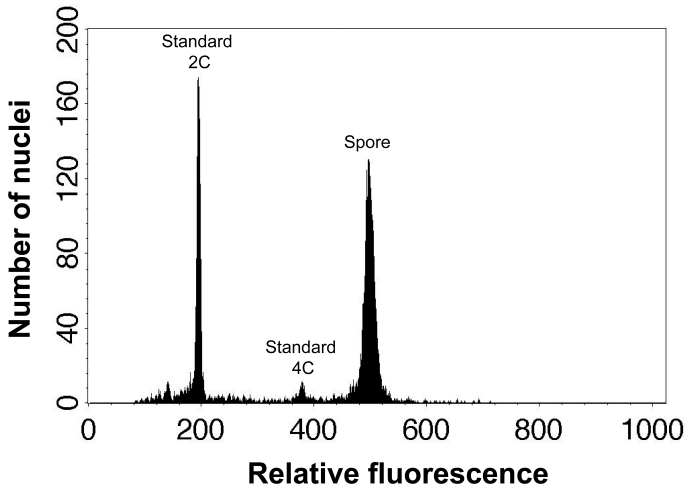

Figure 1. An example of flow cytometric result of spore and standard nuclei. These peaks are spore nuclei of *Dryopteris varia* and 2C and 4C leaf nuclei of *Nicotiana tabacum* L. 'Xanthi'.

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Notes

1. To dry fertile leaves to release and collect spores, air-drying process under room temperature for 2 to 3 days is recommended. For long-term storage, spore material is better stored in tubes without any solution at a temperature lower than 4 °C.

Recipes

1. Backmen stock buffer (Ebihara *et al.*, 2005)
   1.0% Triton X-100
   50 mM Na_2SO_3
   50 mM Tris-HCl (pH 7.5)
   ddH_2O (the solvent)
   Note: Store at 4 °C up to 1 year.
2. PI solution
   2.04 mg/ml propidium iodide
   ddH_2O (the solvent)
   Note: Store in the dark at 4 °C for long-term storage.

Acknowledgments

The bead-vortexing condition to extract spore nuclei of seedless vascular plants is accessed and constructed by Kuo *et al.* (2017). We thank Fay-Wei Li and three anonymous reviewers for providing comments on the draft of this manuscript.

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