Methodology

Isolation of plant nuclei

We recommend (unless specified otherwise) working under cold conditions (i.e. keep all solutions, buffers and prepared samples for analysis on ice, and do the chopping step in a Petri dish resting on a bed of ice. Together this helps to inhibit the negative effect of many cytosolic compounds that may be present (e.g. DNase, phenols, tannins etc.) and it can be especially helpful when working with recalcitrant samples.

One-step protocol

- Place a small amount of the plant tissue plus the reference standard (usually about 1 cm^2 or 20 mg of each one) in a 6 cm Petri dish.
- Add 1 mL of ice-cold isolation buffer of choice to the Petri dish.
- Chop the tissues up in the buffer using a new razor blade or sharp scalpel.
- Add another 1 ml of the same ice-cold buffer.
- Mix the crude suspension by gently shaking the Petri dish.
- Filter the homogenate through a 30-80 µm nylon mesh filter into a labelled flow cytometry tube.
- Add the appropriate volume of fluorochrome to the suspended nuclei and vortex gently. For a typical sample which is c. 2 ml the amount of stock propidium iodide (PI) added is 100 µl while for DAPI 80 µl should be added.
- Incubate samples on ice until ready to analyse.
- Proceed to assess the nuclear DNA content, vortexing the sample before putting it on the flow cytometer.

Two-step protocol

- Place a small amount of the plant tissue plus the reference standard (usually about 1 cm^2 or 20 mg of each one) in a 6 cm Petri dish.
- Add 1 ml of ice-cold Otto's I or ice-cold Baranyi's solution I.
- Chop the tissues up in the buffer using a new razor blade or sharp scalpel.
- Mix the crude suspension by gently shaking the Petri dish.
- Filter the homogenate through a 30-42 µm nylon mesh filter into a labelled 1.5 ml tube.
- Pellet the nuclei by centrifuging at 150 g for 5 min.
- Carefully remove the supernatant leaving approximately 100 µl of the buffer.
- Resuspend the pellet by gently shaking and add a further 100 µl of the buffer used in step 2.
- Add 1 ml of room temperature buffer, either Otto's II or Baranyi's solution II.
- Add the appropriate volume of the fluorochrome to the suspended nuclei (if not already in the buffer) and vortex gently. For a typical sample which is c. 1.2 ml the amount of stock PI added is 60 µl while for DAPI 50 µl should be added.
- Incubate the samples at room temperature for few minutes in the dark (this time needs to be optimised).
- Proceed to assess the nuclear DNA content, vortexing the sample before putting it on the flow cytometer.

Simplified two-step protocol

- Place a small amount of the plant tissue plus the reference standard (usually about 1 cm^2 or 20 mg) in a 6 cm Petri dish.
- Either (i) add 0.5 mL of ice-cold Otto's I or ice-cold Baranyi's solution I buffer, or (ii) add 0.2 ml of ice-cold Mishiba's solution A.
- Chop the tissues up in the buffer using a new razor blade or sharp scalpel.
- Mix the crude suspension by gently shaking the Petri dish. If using Mishiba's buffer, incubate for 5 min at room temperature.
- Either (i) add 2 ml of Otto's II or Baranyi's solution II buffer, or (ii) add 1 ml of Mishiba's solution B.
- Filter the homogenate through a 30-42 µm nylon mesh filter into a labelled flow cytometry tube.
- Add the appropriate volume of the fluorochrome to the suspended nuclei (if not already in the buffer) and vortex gently. (i) For a typical sample using either Otto's or Baranyi's buffers the volume is usually c. 2.5 ml thus the amount of stock PI added is 125 µl while for DAPI 100 µl should be added.
- (ii) For a typical sample using Mishiba's buffer, the volume is usually c. 1.2 ml thus the amount of stock PI added is 60 µl while for DAPI, 50 µl should be added.
- (i) For samples in the Otto's or Baranyi's buffer incubate at room temperature for few minutes in the dark.
- (ii) For samples in Mishiba's buffer incubate at room temperature in the dark for 20 min.
- Proceed to assess the nuclear DNA content, vortexing the sample before putting it on the flow cytometer.

Assessment of the absolute nuclear DNA content of a sample using a reference standard

To ensure the estimate of nuclear DNA content in absolute units is as accurate as possible, FCM researchers have adopted several best-practice approaches (e.g. three plant specimens are collected per population/taxon and three independent replicates are processed per sample, or five specimens are collected per population/taxon and two independent replicates are processed per specimen). Only intercalating fluorochromes (e.g. PI) should be used, base-specific fluorochromes such as DAPI are not suitable for estimating nuclear DNA content.

- Load the sample which contains a suspension of stained nuclei of both the target species and the selected internal reference standard onto the flow cytometer sample port and run for a few seconds at low speed until the flow has stabilised through the tubing system
- Adjust the flow rate to a speed of 10-25 nuclei/second.
- Once the sample is running through the flow cytometer, a flow histogram with peaks will start to appear. The peak positions can then be adjusted, if necessary, using the instrument gain settings to move the peaks within the histogram.
- Check to see if there is any evidence of negative effects caused by the presence of cytosolic compounds which can affect the accuracy of the C-value estimation.
- When this situation arises, alternative isolation methods should be tested, otherwise proceed with the next step.
- Measure 5,000-10,000 particles.
- Use the software provided by the flow cytometer manufacturer to assess the quality of histograms, assuming the quality of the histograms is suitable (i.e. CVs < 5%).

- Calculate the nuclear DNA amount (2C-value) of the target plant in each replicate as follows:

$$\text{2C DNA content target (pg)} = \frac{\text{target sample mean } G_1 \text{ peak}}{\text{standard sample mean } G_1 \text{ peak}} \times \text{2C DNA content standard (pg)}$$

- Calculate the mean nuclear DNA content and the standard deviation for the species (to convert between picograms (pg) and megabase pairs (Mbp) use: 1 pg = 978 Mbp; Doležel *et al.*, 2003).

Reference

Doležel J, Bartoš J, Voglmayr H, Greilhuber J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry Part A* 51A: 127-128.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.

Doležel J, Greilhuber J, Suda J. 2007. Flow Cytometry with Plant Cells Analysis of Genes, Chromosomes and Genomes. Weinheim: *Wiley-VCH Verlag GmbH & Co. KGaA*.