Using the BD Accuri™ C6 Cytometer for Rapid and Accurate Analysis of the Nuclear DNA Contents of Flowering Plants

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White Paper

Contents

1 Summary
2 Introduction
2 Materials and Methods
5 Results
8 Discussion and General Troubleshooting
10 Notes
10 Author Contact Information
10 References

Summary

The nuclear DNA content (C-value) is a fundamental parameter of eukaryotic cells. Flow cytometry provides the most convenient and accurate way to determine C-values of plants, but sampling of the approximately 480,000 species of angiosperms (flowering plants) remains very limited, only about 1% being reported. For plant species for which C-values are available, these values span an extraordinary range, approximately 1250-fold (0.2 pg to 254.8 pg). For plants for which C-values are unknown, this complicates analysis using flow cytometry, when the range of possible C-values exceeds the dynamic range of the flow cytometer. Various instrument settings (amplification values, PMT high voltages, and laser outputs) require adjustment to optimally position the 2C peak values for the unknowns and the appropriate controls within the fluorescence frequency distributions. Although this can be done empirically, it considerably slows down the sample throughput.

The BD Accuri™ C6 flow cytometer is ideally suited for determining C-values for new plant species. The unusually high dynamic range of the ADC allows simultaneous analysis of species for which C-values span almost the entire range of the described angiosperms. This white paper describes the way in which C-value determinations are done, including technical details for avoiding contribution from cellular debris, and includes typical measurements from plant species to illustrate the dynamic range of the measurements.

Introduction

Flow cytometry is the simplest way to determine plant nuclear DNA contents.[1] To allow the nuclei to pass through the flow cell, the plant tissues are homogenized by chopping, using a razor blade, in a suitable buffer. After filtration of the homogenate to remove large particulate materials, the samples are stained with DNA-specific fluorochromes and the fluorescence is quantified.

The absolute values for the amounts of the plant nuclear DNA are found by comparison to cells or nuclei of known DNA content, such as chicken red blood cells[1] or plant standards[2,3] (see also hyperlink c in Table 1). Standardization is complicated by base-pair specificity inherent to some fluorochromes (DAPI and Hoechst 33257 being AT-specific,[3] and mithramycin and chromomycin A3 being GC-specific). The fluorescence produced by intercalating fluorochromes, of which propidium iodide (PI) is the most popular, is not biased by base-pair composition, and staining in the presence of RNAse eliminates interference by double-stranded RNA.

In this white paper, we provide detailed information about how to use the BD Accuri C6 flow cytometer for the analysis of plant genome sizes. The DNA histograms produced by the BD Accuri C6 display excellent peak CVs, and this instrument, uniquely, can provide simultaneous linear estimates of nuclear DNA content spanning at least two orders of magnitude (a range of 0.34 to 80.9 pg). This covers most of the C-values encountered in the flowering plants found across the world. Galbraith[4] provides a fuller discussion.

Materials and Methods

Materials

The seeds mentioned in this paper are available from Lehle Seeds (Round Rock, TX 78680-2366 USA) and from J. Dolezel (Olomouc, Czech Republic).

Plant Production

We germinated all seeds, except those of *Arabidopsis*, in 3-inch plastic pots containing Scotts Metro-Mix 3000 (The Scotts Miracle-Gro Company, Marysville, Ohio) in a growth room at 25°C under a 12/12 light dark cycle with a light intensity of 150 to 175 µeinstein/m^{-2}/sec^{-1}. Plants were watered daily. Samples were generally taken after four weeks of plant growth.

For *Arabidopsis*, we germinated seeds and grew plants under sterile conditions using an established procedure.[5] In brief, seeds were sterilized by immersion for 5 minutes in a freshly prepared solution of commercial bleach diluted 1:1 with distilled water containing 10 µL/mL of Triton®-X100. The seeds were then rinsed three times in sterile distilled water and planted on Murashige and Skoog (MS) agar plates supplemented with 2% sucrose and 1.2% agar. The seeds on the plates were vernalized by incubation at 4°C for 2 days before transfer to a Conviron growth chamber under a 12-h day/12-h night illumination regime, with an incident light flux of 150 to 175 µm/m^{-2}/sec^{-1} and a temperature of 22°C (day) and 20°C (night).

The plates were kept in a vertical orientation so that the roots grew on the surface of, rather than within, the agar medium. Samples were harvested the day of the flow analyses.

We purchased plants of *Astroemeria aurea* (Lily of the Incas) from a local supermarket. The plants were supplied by Inter-American Products, Cincinnati, Ohio.

Propidium iodide was obtained from Calbiochem (EMD Chemicals, Gibbstown, NJ; Cat. No. 537059), and a stock solution (1 mg/mL) in deionized water was prepared and stored in aliquots at -20°C. RNAse A (10 mg/mL in water) was from Fermentas (Glen Burnie, MD; Cat. No. EN0531) and was stored at 4°C. Nylon filters were from Partec (Swedesboro, NJ; Cat. No. 04-0042-231). They have a notional porosity of 30 µm. Razor blades were obtained from VWR (VWR International, West Chester, PA; Cat. No. 55411-050).

Preparation of Plant Homogenates and Labeling of Nuclei

All procedures were done at 4°C or on ice. Approximately 50–100 mg of plant tissue was excised and placed in a plastic 60-mm petri dish standing on a pre-chilled metal plate placed on ice in a rectangular plastic tray. We recommend that the procedures all be done in a walk-in cold room. Galbraith's buffer supplemented with 0.1% w/v Triton® X-100[1] was added in the proportion of 1.5 mL of buffer per 100 mg of tissue. Tissues were chopped using a new razor blade for 2 to 3 minutes. Each slicing action of the razor blade should cut cleanly and without bruising the tissue. The razor blade was discarded after a single use. The homogenate was filtered through the 30-µm nylon filter. The homogenate (0.5 mL) was added to a labeled tube containing 2.5 µL of 10 mg/mL of DNAse-free RNAse A and incubated on ice for 15 minutes. Propidium iodide was then added to a final concentration of 50 µg/mL. The stained samples were incubated on ice in darkness for 30 minutes prior to analysis.

Flow Cytometry

The BD Accuri C6 flow cytometer was switched on and allowed to warm up for 15 minutes before analyses were begun. Waste and sheath fluid bottles were checked during this time, and emptied or refilled as required. BD Accuri™ C6 software was started. Performance validation of the BD Accuri C6 was then done using 6- and 8-peak fluorescent bead mixtures (Spherotech) according to instructions (*BD Accuri C6 Software User Guide*). Finally, the Plant DNA Analysis Template was opened. This template consists of the following plots and histograms: FSC-A vs SSC-A, FL1-A vs FL2-A, FL3-A vs FL2-A, and a univariate histogram of FL2A. The fluorescence signals (pulse area measurements) were screened by the following filter configurations: (a) FL-1: a 530/14-nm bandpass filter (b) FL-2: a 585/20-nm bandpass filter, and (c) FL-3: a 670-nm longpass filter. Analysis of the homogenates was based on light-scatter and fluorescence signals produced from 20-mW laser illumination at 488 nm.

Plant homogenate samples were introduced to the SIP, and the RUN tab was clicked in BD Accuri C6 software to initiate data acquisition. Threshold levels were set empirically to eliminate from detection the large amounts of irrelevant debris that are found in plant homogenates. The default threshold values on the BD Accuri C6 are set at 80,000, and this setting excludes from analysis very small nuclei. We set the threshold at 10,000 for FSC, with a secondary threshold of 1,000 for FL-2. These values were adjusted while acquiring data and observing the position of the nuclei on the bivariate dot plots such that all the nuclei were on scale with the least amount of debris appearing in these plots. The flow cytometer was routinely operated at the Slow flow rate setting (14 µL of sample per minute), and data acquisition for a single sample should typically take 3 to 5 minutes.

The PI-stained nuclei appeared on the bivariate plots as a region of dots clustered around a diagonal line. This region contained two populations of nuclei for non-endoreduplicated species. Additional populations are seen if somatic endoreduplication occurs. A polygonal gate was drawn to enclose the nuclei, and univariate histograms of PI fluorescence (FL2-A) were accumulated based on this

gate. The zoom tool was used to locate and isolate the peaks appearing within this univariate histogram. Regions of identification were then placed across the lowest peaks to export values representing peak positions and CVs. The data was used to calculate the DNA content of the nuclei.

Troubleshooting

Occasionally, low yields of intact nuclei from the chopping procedure are encountered. This is usually due to the poor condition of the source tissue. Source tissues should be fresh, young, and well hydrated. Old or dried-out tissues do not generally provide histograms of adequate quality. Grasses are often problematic, and one may need to use more than the 200-mg sample size suggested in the protocol to provide sufficient nuclei for analysis. The tissues of some species can be refrigerated in damp paper towels for up to two days prior to analysis, should this be necessary. Freezing tissues is not recommended.

Some plants may release mucilaginous compounds into the chopping buffer, resulting in slime, which should not be run through the instrument. Other species can release secondary plant products that reduce fluorochrome fluorescence. Careful observation of the samples during chopping is recommended; any changes in homogenate color and consistency are contraindications. If these are observed, alternatives are to employ different parts of the plant for analysis, and/or to include additives to the chopping buffer to absorb secondary products. Dolezel et al[3] provide additional suggestions for sample preparation in these situations.

Poor CVs may be the result of excessive chopping, or chopping with dull razor blades. Many sources exist for satisfactory blades (VWR Scientific, Fisher Scientific, Personna, America Safety Razor Company), which generally should have an edge thickness of 0.22 mm or finer. Double-edged shaving blades are ideal, except that one must find or devise suitable holders for them to avoid accidental injury. Unstable (drifting) peak positions may be due to insufficient periods of staining. In this white paper, we have recommended 15 minutes, but it is possible that some species may require longer staining times. This can be easily monitored by flow analysis over time of successive samples taken from a single stained homogenate.

Table 1. Names of plant species, source of seeds, 2c.

Species	Common name	2C DNA content (pg)	DNA content attribution	Source of plants and hyperlinks
Arabidopsis thaliana ecotype Columbia	Thale cress	0.32	Kew C-value Database (Bennett and Leitch, 2004)	Lehle Seeds[a]
Raphanus sativus cv. Saxa	Radish	1.11	Olomouc website (Dolezel et al, 2007)	J. Dolezel[b]
Medicago sativa L. cv. Cimarron	Alfalfa	3.5	Kew C-value Database (Bennett and Leitch, 2004)	Lehle Seeds[a]
Pisum sativum L. cv. Ctirad	Garden pea	9.09	Olomouc website (Dolezel et al, 2007)	J. Dolezel[b]
Secale cereale L. cv. Dankovske	Rye	16.19	Olomouc website (Dolezel et al, 2007)	J. Dolezel[b]
Triticum aestivum L. line 812	Wheat	34.65	Kew C-value Database (Bennett and Leitch, 2004)	Lehle Seeds[a]
Alstroemeria aurea Grah.	Lily of the Incas	80.9	Kew C-value Database (Bennett and Leitch, 2004)	Inter-American Products[c]

[a] http://www.arabidopsis.com/main/cat/seeds/teaching/Crop/!cps.html
[b] http://lmcc.ieb.cz/research/protocols.php?protocols=dna
[c] http://www.interamericanproducts.com

Results

The plants used in this study are identified in Table 1, along with their 2C DNA content values. A particularly useful resource is the C-value database maintained by Drs. Leitch and Bennett at The Royal Botanical Gardens, Kew.[6]

Figure 1A shows the biparametric frequency distribution (FL2-A vs FL3-A) of a PI-stained homogenate prepared from pea (*Pisum sativum*) leaf tissue, as analyzed by the BD Accuri C6 flow cytometer. Pea has a reasonably large nuclear genome, and is known to produce high quality uniparametric DNA histograms. Two clusters are seen within this frequency distribution. One roughly star-shaped cluster (arrow), which comprises most of the objects in the homogenate, corresponds to subcellular debris. No correlation is seen between the FL2 and FL3 signals within this cluster. The second cluster, boxed in red, forms a very minor proportion (1.8%) of the objects detected by the flow cytometer. Objects in this population exhibit a very strong correlation between the FL2 and FL3 signals, as would be predicted for PI-stained nuclei. If you place gates around this population (region P), the gated objects provide a very nice uniparametric DNA distribution with clearly defined G0/G1 and G2 peaks. The peak positions in this case are 533,898.3 and 1,065,516.0 fluorescence units (a ratio of 1:1.996). The CVs for the G0/G1 peak are 2.1% (when determined using BD Accuri C6 Software, after manually positioning windows at the 50% points on either side of the peak), and 2.6% (when determined using SigmaPlot® software, with fitting of a Gaussian function to the extracted data). This histogram is typical of the nuclear DNA contents found within non-endoreduplicated plant tissues.[1,7]

"The BD Accuri C6 cytometer and BD Accuri C6 software have far exceeded our expectations. The BD Accuri C6 cytometer provides important additional data that we have previously been missing. One is the highly accurate volume determination, so that absolute cell counts are automatically recorded for each experiment. Secondly, the six-log scale means that we can collect all data without compensation, so that runs from different days can be more easily compared. Overall, we are extremely pleased with the BD Accuri C6 cytometer, and would highly recommend it."

- Associate Professor of Immunology and Pediatrics

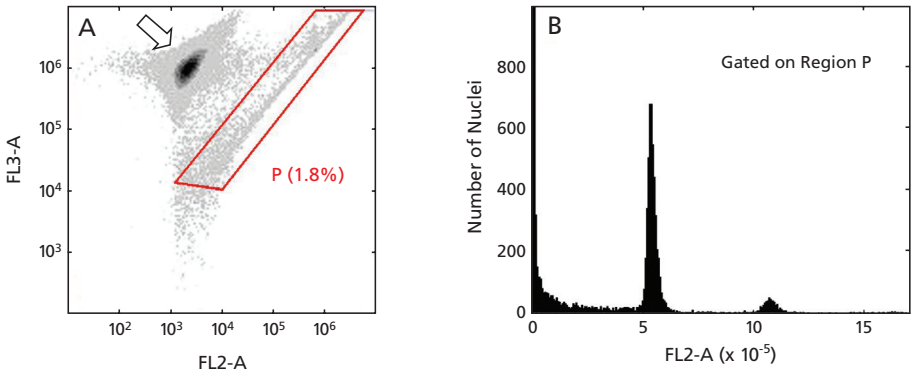

Figure 1. Flow analysis of homogenates from pea seedling tissue.
(A) *Biparametric dot plot of FL2-A (585/40 nm) vs FL3-A (>670 nm) fluorescence emission.* ***(B)*** *Uniparametric histogram of FL2-A fluorescence, gated on region P1 of panel A.*

Figure 2A illustrates the corresponding biparametric frequency distribution obtained from leaf homogenates of *Arabidopsis thaliana*, which contains one of the smallest genomes of flowering plants (~157Mb,[8] or a 2C value of 0.32 pg). *Arabidopsis* exhibits endoreduplication within most of its somatic tissues and organs,[7] and this produces multiple clusters within the frequency distributions, corresponding to an endoreduplicative series (2C, 4C, 8C, 16C, etc). These clusters fall on the characteristic PI-DNA diagonal, which becomes particularly obvious following electronic expansion of the region in which it falls (Figure 2B). Gating around this expanded region (P1, ~1.2% of the detected signals) provides uniparametric distributions with well-defined peaks (Figure 2C) typical of *Arabidopsis thaliana*.[7] The mean fluorescence values of the peak positions for the nuclei are 24,541.9, 47,446.1, 91,698.5, and 179,634.1, which fit a straight line almost perfectly ($r^2 = 0.9999$). The CVs are also very acceptable (3.6%, 2.7%, 2.2%, and 2.4%). Similar results are seen for *Arabidopsis* root homogenates (Figures 2D, 2E). The nuclei comprise a slightly larger proportion of the detected signals (region P1), probably due to the absence of chloroplasts from roots. Gating on region P1 again provides uniparametric histograms of excellent quality and linearity (Figure 2F): the fluorescence values of the peaks are 22,773.6, 44,930.8, 87,703.1, and 171,246.2 ($r^2 = 0.9999$). The CVs were 3.5%, 2.8%, 3.1%, and 3.4%.

Day-to-day reproducibility of the C-value measurements on the BD Accuri C6 flow cytometer is excellent. For seven repetitions on separate days, using different *Arabidopsis* seedlings as the source of nuclei, the mean FL2-A value of the 2C peak positions was 21,633.6 ±1,588.5 (mean ±SD).

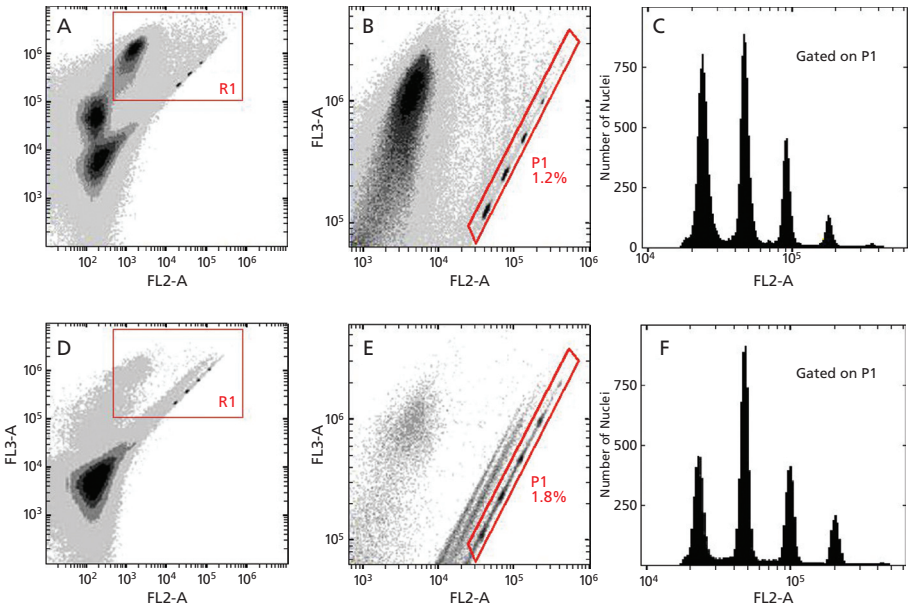

Figure 2. Flow analysis of homogenates from *Arabidopsis* leaf (A-C) and root (D-F). ***(A)*** *Biparametric dot plot of FL2-A versus FL3-A fluorescence emission.* ***(B)*** *Enlargement of the square region-of-interest (R1) containing the nuclei. The nuclei are indicated by polygonal region P1.* ***(C)*** *Uniparametric histogram of FL2-A fluorescence, gated on region P1 of panel B.* ***(D)*** *As for panel A, except using roots.* ***(E)*** *As for panel B, except using roots. (F) As for panel C, except using roots.*

The next experiment extended the observed linearity of PI-based C-value measurements to a dynamic range that spans most of the values reported for flowering plants. Plant tissues were taken from four species (*Arabidopsis thaliana*, *Pisum sativum*, *Triticum aestivum*, and *Alstroemeria aurea*), chopped, filtered, and stained separately, then mixed. The composite biparametric frequency distribution contains the diagonal region characteristic of PI-stained nuclei (Figures 3A, 3B). On magnification of the region, it is clear that the *Alstroemeria* nuclei produce FL3-A signals that are off-scale, but these could be captured using a suitably placed polygonal window (P2), and the resultant FL2-A uniparametric distributions had well-defined peaks corresponding to the nuclei of the different species (Figure 3C). Assignment of the six peaks to nuclei of individual species was done from the results of accumulation of histograms produced for analyses of the unmixed homogenates of individual species. For Figure 3C, the individual peaks and their positions and CVs are: *Arabidopsis* 2C, 20,868.2, 4.2%; *Arabidopsis* 4C, 40,333.2, 3.5%; *Arabidopsis* 8C, 77,941.5, 2.8%; *Pisum* 2C, 453,340.4, 2.1%; *Triticum* 2C, 1,584,795.7, 2.8%; *Alstroemeria* 2C, 3,486,157.8, 2.4%. The peak position values are strongly correlated with the reported nuclear DNA content values (Figures 3D, 3E; r^2 >0.99).

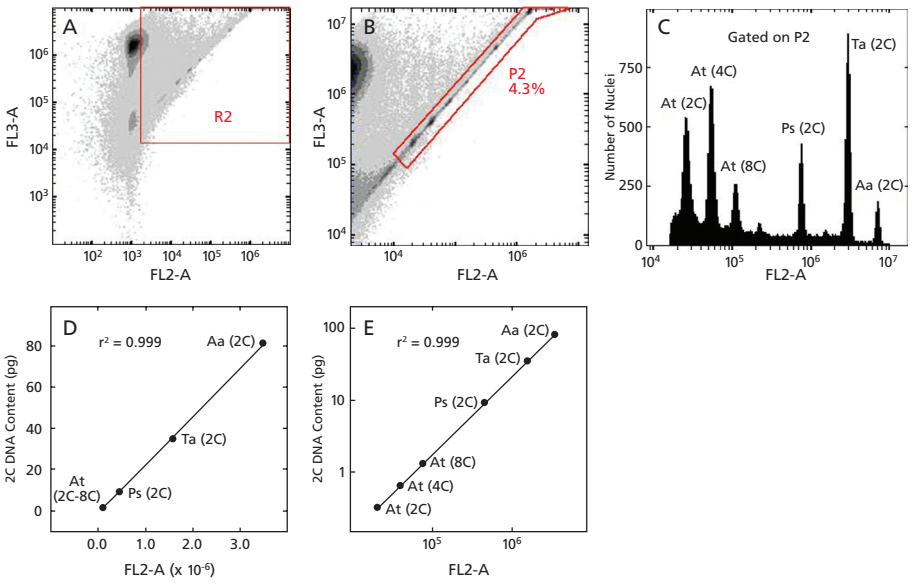

Figure 3. Simultaneous analysis of mixtures of plant homogenates.
(A) *Biparametric dot plot of FL2-A vs FL3-A fluorescence emission.* ***(B)*** *Enlargement of the square region-of-interest (R2) containing the nuclei; the nuclei are indicated by polygonal region P2.* ***(C)*** *Uniparametric histogram of FL2-A fluorescence, gated on region P2 of panel B.* ***(D)*** *Plot of DNA content vs the mean fluorescence values of the 2C peak psitions for the four species.* ***(E)*** *As for panel D except employing a log scale, with identification of the six DNA content peaks. Abbreviations: At,* Arabidopsis thaliana*; Ps,* Pisum sativum*; Ta,* Triticum aestivum*; Aa,* Alstromeria aurea*. 2C, 4C, 8C designate the C-values for the individual peaks within these species.*

Discussion and General Troubleshooting

The nuclear DNA contents (C-values) of higher plants span a remarkable range of values,[6,9] from 0.2 pg (*Fragaria viridis* Duch.) to 254.8 pg (*Fritillaria assyriaca* Baker[10]), or about three decades of DNA content. Nevertheless, C-value measurements are available only for about 2% of the species comprising the angiosperms. The BD Accuri C6 flow cytometer is optimally suited for the flow cytometric determination of plant nuclear DNA contents. This is for two main reasons: first, the BD Accuri C6 employs a 24-bit analog-to-digital converter (ADC) for signal processing, which provides an exceptional seven-decade dynamic range. This dynamic range is larger than that of the described 2C values for the flowering plants.

Second, the BD Accuri C6 is equipped with lasers that optimally excite PI for use as a DNA fluorochrome. This avoids base-pair bias inherent to DAPI, the Hoechst dyes, and mithramycin/chromomycin,[3] and also provides a convenient way to deal with the large amounts of debris released from plant tissues by the chopping process.[1] In comparison to situations typical in flow analysis of animal cell suspensions, in which the object of interest (the cell) represents most of the population, for analysis of C-values using plant homogenates, the objects of interest (the nuclei) are a very minor population. Plant debris both scatters light and can be autofluorescent. Since light scatter signals are generally used for triggering flow cytometric measurements, careful adjustment of thresholds is required to allow visualization of the nuclei. Without appropriate thresholding, auto-rescaling obscures the presence of the nuclei on frequency distribution displays, and sample acquisition, based on total counts of scattering particles, stops prior to accumulation of data for adequate numbers of nuclei. Autofluorescence produced by the photosynthetic organelles of aerial tissues can also overlap the emission of DNA-specific fluorescent signals and, since chloroplasts greatly outnumber nuclei within the cells of green tissue, this also can obscure the nuclei.

Using the BD Accuri C6, it is very simple to define the region of interest within biparametric distributions of FL2-A vs FL3-A, since these detection channels roughly split the fluorescence emission of PI-DNA into shorter and longer wavelength spectral components (Figures 1–3). Correlation between the fluorescence emissions within these two spectral bands produces an angled linear region within the biparametric contour plots containing discrete peaks of fluorescence, the lowest representing the 2C nuclei within G0/G1 cells (Figures 1–3). Gating around this region provides very clean uniparametric histograms. Debris from different tissue sources, for example shoots (Figures 2A, 2B) and roots (Figures 2D, 2E) can be readily excluded for consideration, and mixing homogenates from different plant species (Figures 3A, 3B) does not impede nuclear analysis.

An additional general problem in C-value determinations relates to the choice of a standard against which to determine the 2C value for the unknown species. This conventionally has been an internal standard, ideally using a plant species having a C-value similar to that of the unknown, but not overlapping. Experience with other flow cytometers indicates that data accumulation under conditions of linear signal amplification is preferred over logarithmic amplification, due to historical performance limitations of the latter. Given the extreme ranges of 2C values encountered in the plant kingdom, it can therefore be tricky to establish appropriate conditions and standards for accurate flow analysis of nuclear DNA contents.

This problem is solved by the BD Accuri C6 flow cytometer in two ways. First, and remarkably, the linearity of measurement of plant genome sizes based on PI fluorescence extends over a dynamic range from 0.32 to 80.9 pg of DNA (Figures 3D, 3E). This is the first time this relationship has been demonstrated over such a large range. The upper limit to C-value measurements using the BD Accuri C6 will be about 370 pg of DNA, based on the observed mean position for the 2C peak of *Alstroemeria* (80.9 pg; channel 3,486,157.8) and the highest bin value available on the instrument (~16 x 10^6). This exceeds the largest record in the Kew C-value database by a factor of 1.5-fold. Extending polygonal gate P2 (Figure 3) around the PI-DNA region downward below the *Arabidopsis* 2C peak revealed little noise from debris for FL2-A values as low as 2,000. This value would correspond to a nuclear 2C DNA content of 0.032 pg, which is about six-fold smaller than the smallest record for the flowering plants.[6,11]

Second, the BD Accuri C6 displays a very high level of reproducibility in day-to-day measurements of the fluorescence of PI-stained plant nuclei. The variability in the position of the *Arabidopsis* 2C peak had a CV of 7.3%. This essentially obviates the need for internal standardization of DNA content values. Caution should be observed, nevertheless, since a number of trivial issues can alter the fluorescence emission detected by the BD Accuri C6, including issues relating to experimental manipulations (the staining time and the presence of secondary products, for example), and those relating to the cytometer itself (deposition of murky substances on the flow cell walls). Appropriate controls can readily handle these issues.

Overall, this white paper indicates the BD Accuri C6 should be able to measure any plant 2C value without modification to the instrument, and without the requirement for even simple adjustments such as insertion of neutral density filters. The accuracy of measurement of the nuclear DNA contents, reflected by the low CVs, was consistently high across the entire range of measurements. We conclude that, for routine analysis of plant nuclear DNA contents, ploidy, or investigations of other issues requiring C-value determinations, the BD Accuri C6 provides an excellent measurement platform.

Notes

This white paper is abstracted in part from Galbraith,[4] with the permission of the author.

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