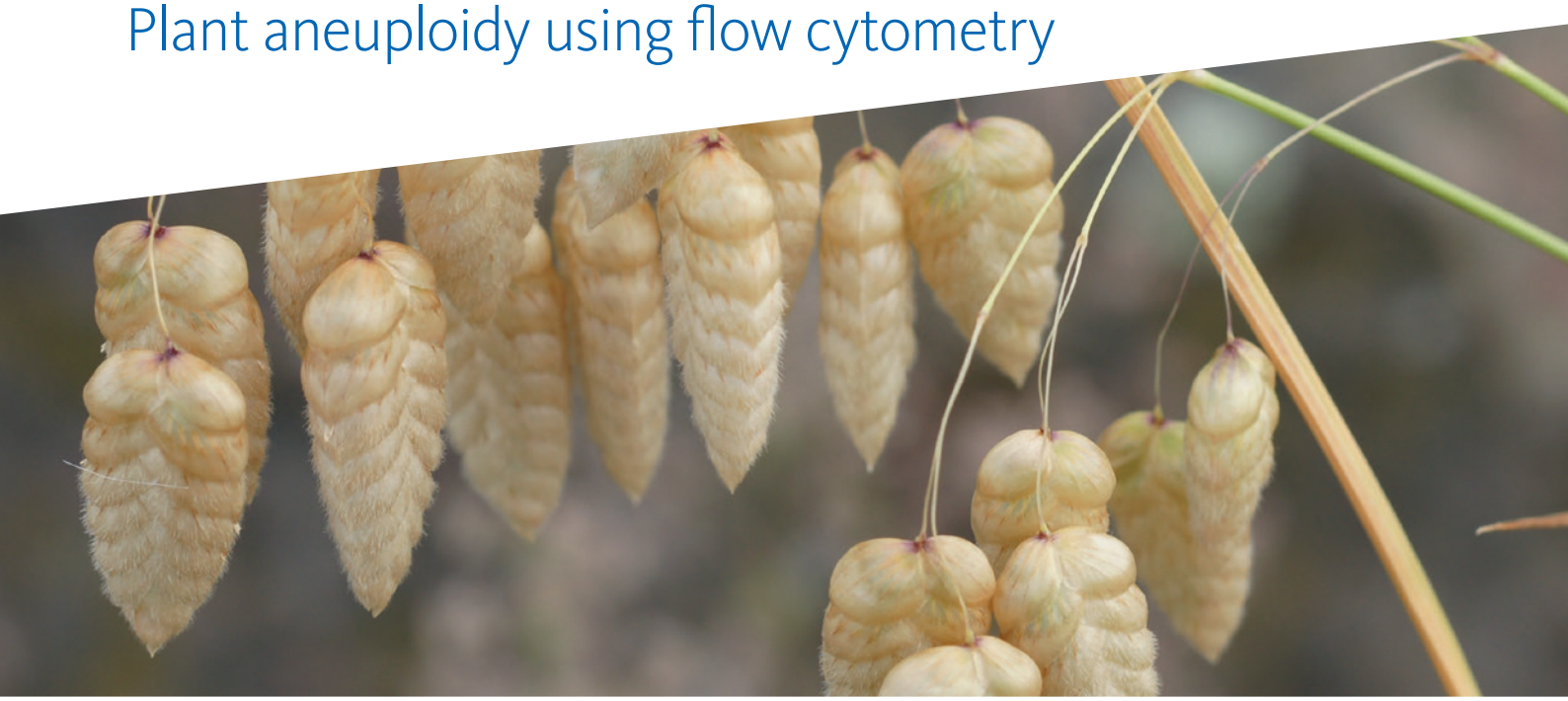
APPLICATION NOTE

Plant aneuploidy using flow cytometry



Background

Aneuploidy is defined as the addition or deletion of chromosomes compared to the 'normal' number of chromosomes as a multiple of the haploid chromosome number. Aneuploidy is probably the most common type of chromosomal aberration in the plant kingdom.

Traditionally, detecting aneuploidy in plants has been carried out by microscopic chromosome counting of preparations of metaphase chromosomes. In many cases metaphase chromosomes are difficult or almost impossible to obtain or count due to the high number and/or small size of the chromosomes. The procedure of counting chromosomes is time-consuming and involves handling unpleasant reagents, such as strong acids.

Flow cytometry, on the other hand, is fast since it does not count chromosomes but rather quantifies true amounts of DNA. And since the reagents are system-contained, there is less interaction with biohazardous materials.

Sysmex offers a number of flow cytometry-based **high-resolution DNA analysis** systems, such as CyFlow® PA, CyFlow® Space & CyFlow® Space Autoloading Station. These analysers detect variations in the number of chromosomes by measuring the amount of DNA of each individual nucleus. The method compares a standard, known ploidy (of the same species) with the sample of interest (the suspected aneuploid). Additions or reductions to the chromosome number necessarily lead to more or less DNA per nucleus, and therefore provide an indication of the existence or absence of aneuploidy.

Please note that it is highly impractical to use a normal ploidy standard of species X for analysing an aneuploid sample from species Y (as in different species the chromosome number might be identical but the amount of DNA – or chromosome size – can differ substantially). It is not possible to count chromosomes directly using flow cytometry.

Basic procedure of a different species

Ploidy analysis standard determination

Individual samples are prepared together with an internal reference plant of a different species. The internal reference should have a similar DNA content as the sample plant so that, in the DNA histogram, the sample peak and internal reference peak are close. For instance, if the internal reference DNA peak is positioned on channel 100, the sample plants DNA peak should be located in a range of +/- 50 channels from that internal reference peak.

The mean position (x-axis value) of both reference and sample has to be defined. The standard ratio ($ratio_{std}$) between a sample peak position (e.g. of a diploid standard (chs_{td}) and a reference peak position (ch_{ref}) is a constant value. To obtain information on the mean value and standard deviation of this standard ratio ($ratio_{std} = ch_{std} / ch_{ref}$), five to ten repetitive analyses of the standard and reference have to be performed.

Table 1: *Example of standard calculation $ratio_{std}$ mean and $ratio_{std}$ standard deviation with X as reference and Y as sample*

Sample No.	ch_{std}	ch_{ref}	$ratio_{std}$
01	76.4	100.2	0.762
02	74.8	101.0	0.740
03	75.3	101.3	0.743
04	74.6	100.1	0.745
05	76.0	099.9	0.761
Mean			**0.750 +/- 0.010**

After analysing the ploidy standard mixed with the reference for establishing both $ratio_{std}$ mean and $ratio_{std}$ standard deviation, individual samples can be investigated for aneuploidy.

In this previous example, the $ratio_{std}$ mean is 0.750 with a standard deviation of +/- 0.010.

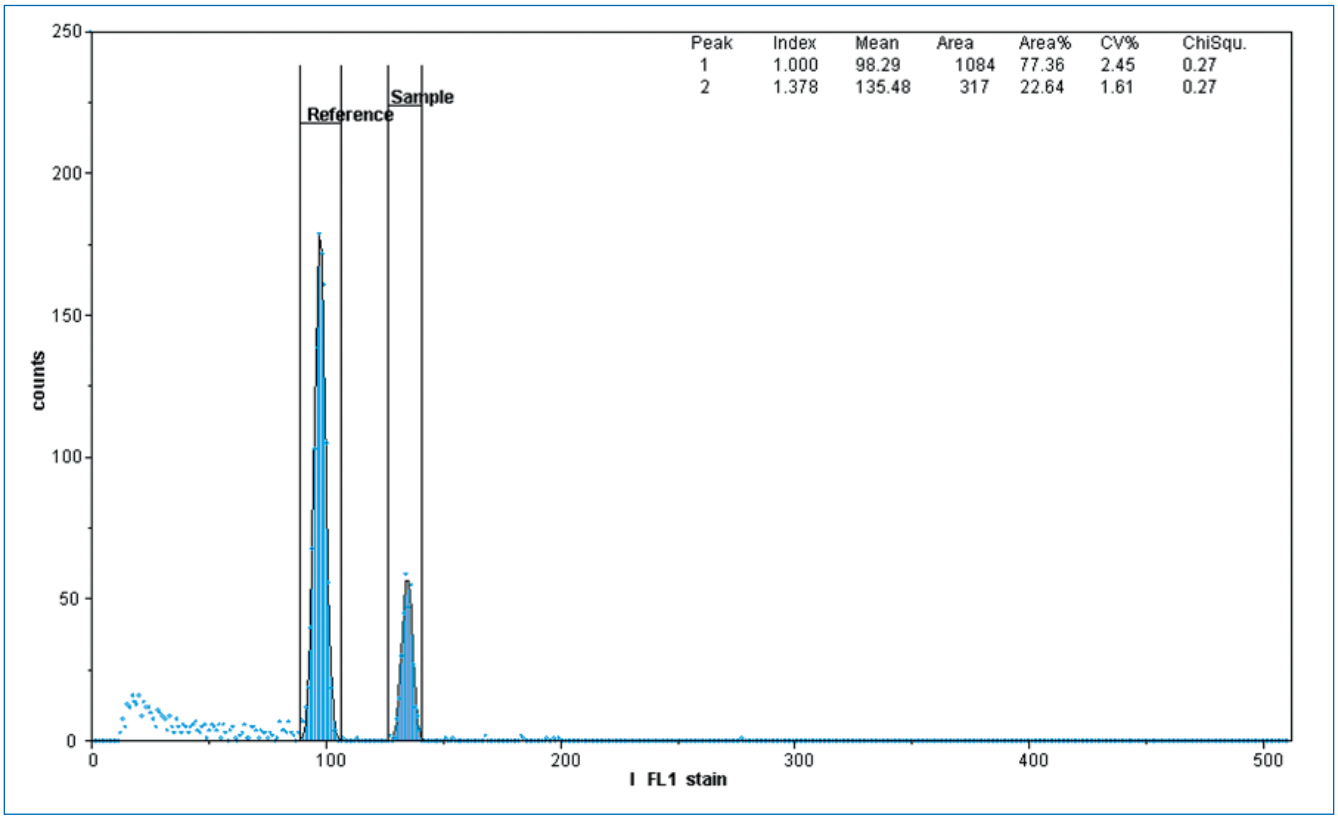


Figure 1: *Nuclear DNA content analysis using a known plant as an internal reference. DNA peak is positioned on channel 100. Ideally, the sample plants DNA peak is located within +/- 50 channels from that internal reference peak.*

Material solutions for aneuploidy analysis

Flow cytometry-based analysis for aneuploidy needs analyser configurations and reagents that are suited specifically to the complexity and characteristic of the sample DNA in question. Most samples can be analysed using the basic reagent (CyStain® UV Precise P). The remaining samples are analysed using more specialised, targeted solutions.

Table 3: *Reagents*

Reagent	Sysmex Order Codes	Fitting configuration
CyStain® UV Precise P	05-5002	for UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) ■ CyFlow® Ploidy Analyser (CY-S-3039_V1)
CyStain® UV Precise T	05-5003	for UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) ■ CyFlow® Ploidy Analyser (CY-S-3039_V1)
CyStain® UV Precise P automate	05-5002-a	for UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) with Autoloading Station
CyStain® UV Precise T automate	05-5003-a	for UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) with Autoloading Station
CyStain® PI Absolute P	05-5022	optimal for flow cytometer configurations with green (532 nm) or suitable with blue (488 nm) laser excitatins for example ■ CyFlow® Space (CY-S-3001R_VS08) ■ CyFlow® Ploidy Analyser (CY-S-3039_V2)
CyStain® PI Absolute T	05-5023	optimal for flow cytometer configurations with green (532 nm) or suitable with blue (488 nm) laser excitations for example ■ CyFlow® Space (CY-S-3001R_VS08) ■ CyFlow® Ploidy Analyser (CY-S-3039_V2)

Sysmex also provides other consumables for the analysis process.

Table 4: *Consumables*

Material	Sysmex Order Codes
Petri dishes	04-2005
30 µm mesh CellTrics® filter	04-0042-2316
3.5 mL polystyrene sample tube	04-2000
DNA Control UV	05-7302
DNA Control PI	05-7303

Literature

[1] ***Doležel J, Goehde W.*** *(1995): Sex determination in dioecious plants Melandrium album and M. rubrum using high-resolution flow cytometry. Cytometry, , 19: 103 – 106.*

[2] ***Doležel J, Greilhuber J, Suda J.*** *Flow Cytometry with Plants: an overview. Flow Cytometry with Plant Cells. Copyright® 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, ISBN: 978-3-527-31487.*

Example aneuploidy analysis procedure

Each individual sample preparation mixed and stained with the reference plant has to follow the identical staining protocol as used to define the standard ratio. For each sample, calculate the sample ratio ($ratio_{sample} = ch_{sample} / ch_{ref}$) and Δ ratio = $ratio_{sample}$ - $ratio_{std}$.

Samples with a Δ ratio exceeding the standard deviation of the $ratio_{std}$ are suspected of aneuploidy. The higher the value of Δ ratio, the higher the probability of the sample being aneuploid.

Table 2: *Example of sample calculation $ratio_{sample}$ and Δ ratio with X as reference and Y as sample. Δ ratio values shown in red exceed the $ratio_{std}$ standard deviation of +/- 0.010.*

Sample No.	ch_{sample}	ch_{ref}	$ratio_{sample}$	$ratio_{std}$	Δ ratio	% DNA
s1	74.9	101.0	0.742	0.750	-0.008	98.9
s2	74.3	99.8	0.745	0.750	-0.005	99.3
s3	79.1	100.4	0.788	0.750	+0.038	105.1
s4	74.0	100.5	0.736	0.750	-0.014	98.1
s5	75.1	99.8	0.753	0.750	+0.003	100.4

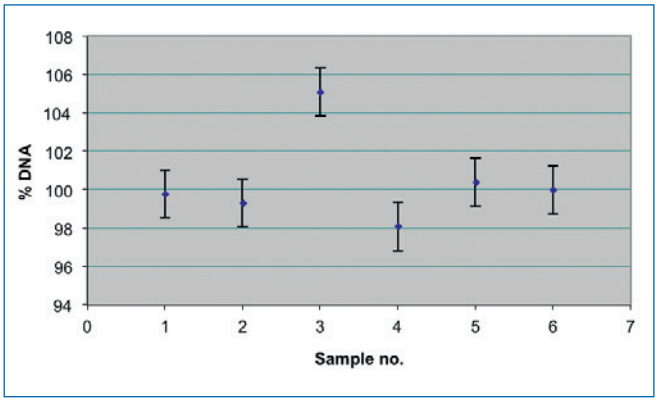


Figure 2: *Chart displaying the percentage variation of DNA for every sample.*

Case interpretation

Given a chromosome number of 24 chromosomes in a 2n plant (diploid), a statistical mean DNA difference of about 4.1% can be expected for the deletion or addition of a single chromosome (as one average chromosome represents 4.1% of total DNA). Therefore, 4.1% variation of the $ratio_{std}$ would correspond to 0.750 +/- 0.031. When Δ ratio strongly exceeds the $ratio_{std}$ standard deviation of 0.010, aneuploidy would be detected with high accuracy.

Naturally, the standard sample can be a different ploidy level (3n, 4n, 6n). If aneuploidy occurs in a different level of ploidy, it could also be detected.

For this case, in our previous example the $ratio_{sample}$ of sample s3 and s4 differs significantly from the analysis of the diploid standard and from the rest of the samples.

These results indicate that sample s3 (Δ ratio = +0.038) encloses 25 chromosomes instead of 24 with similar mean standard deviation of a chromosome (+/- 0.031). Sample s3 can be considered aneuploid by the addition of a single chromosome (s3 = 2n + 1). Sample s4 shows (Δ ratio = -0.014) a less significant deviation from the diploid standard. It might be considered aneuploid due to the deletion of one chromosome.

The result of such individual analysis should be verified by repeating the analysis of the suspect aneuploid plant.

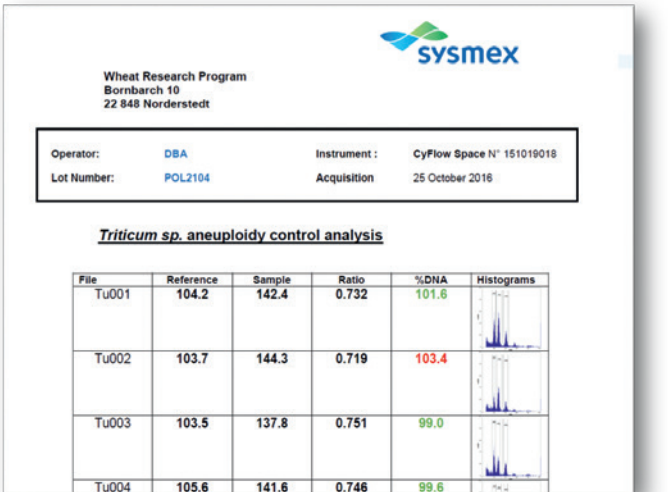
Since the system does not *count* chromosomes, the final scientific verification can only be carried out by light microscopy and counting chromosomes. Aberrations in the DNA content can also occur by the addition of DNA during DNA exchange between single chromosomes without increasing their total number.

Please note that, if the total number of chromosomes is very high and/or the samples cannot be analysed with an acceptable level of reproducibility, detecting aneuploid plants with one chromosome more or less is uncertain. The $ratio_{std}$ standard deviation of repetitive analysis would be higher than the Δ ratio standard deviation, systematically leading to the interpretation of the addition/deletion of chromosomes.

Report examples

CyFlow® Space for aneuploidy analysis

Example: wheat sample aneuploidy analysis report with FloMax® software displaying fluorescence mean intensity of the sample and reference as well as a calculation of the Ratio and % DNA.



sysmex

Wheat Research Program
Bornbarch 10
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Operator:	DBA	Instrument :	CyFlow Space N° 151019018
Lot Number:	POL2104	Acquisition	25 October 2016

Triticum sp. aneuploidy control analysis

File	Reference	Sample	Ratio	%DNA	Histograms
Tu001	104.2	142.4	0.732	101.6	
Tu002	103.7	144.3	0.719	103.4	
Tu003	103.5	137.8	0.751	99.0	
Tu004	105.6	141.6	0.746	99.6	

Figure 3: *Wheat sample aneuploidy analysis – tube Tu002 is likely to be an aneuploid sample*

CyFlow® Space Autoloading Station for aneuploidy

Example: aneuploidy analysis with CyPAD® software of a sample of cabbage. The report generated by Excel using data produced by a system with autoloading station conveniently offers the option of selecting different variables to adequately select undesirable specimens in regenerants from in vitro plant culture for instance.

In this example, three outliers in the graph are clearly aneuploid samples.

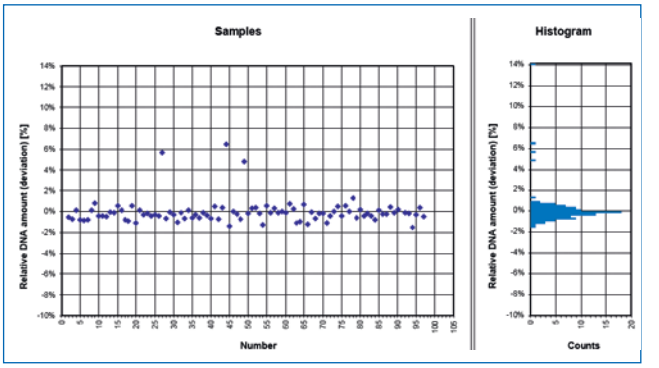
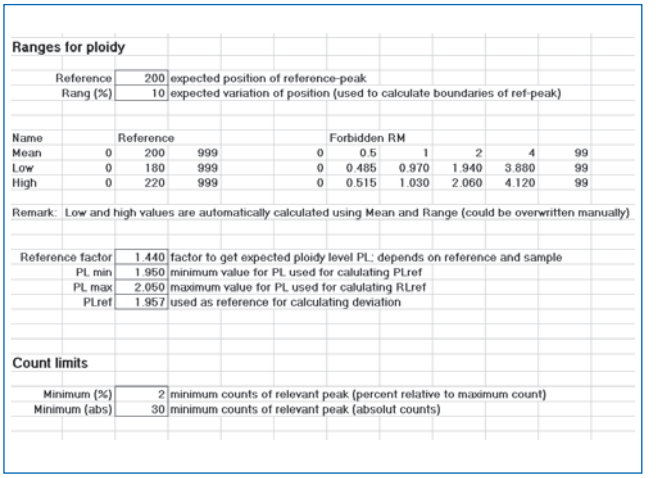


Figure 4: *Cabbage sample aneuploidy analysis – histogram page clearly shows three outliers (aneuploid samples).*

In the following image, open fields are used to fine-tune the aneuploidy experiments: position of the reference and expected variation as well as the calculated deviation of the reference.



Ranges for ploidy

Reference	200	expected position of reference-peak
Rang (%)	10	expected variation of position (used to calculate boundaries of ref-peak)

Name	Reference			Forbidden RM				
Mean	0	200	999	0	0.5	1	2	4	99
Low	0	180	999	0	0.485	0.970	1.940	3.880	99
High	0	220	999	0	0.515	1.030	2.060	4.120	99

Remark: Low and high values are automatically calculated using Mean and Range (could be overwritten manually)

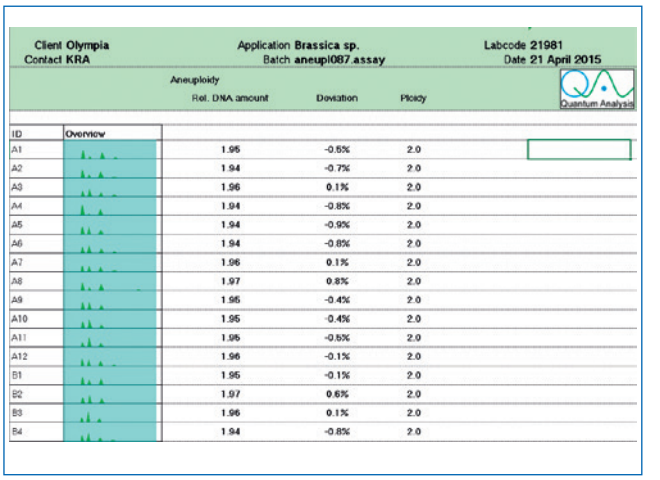
Reference factor	1.440	factor to get expected ploidy level PL; depends on reference and sample
PL min	1.950	minimum value for PL used for calulating PLref
PL max	2.050	maximum value for PL used for calulating RLref
PLref	1.957	used as reference for calculating deviation

Count limits

Minimum (%)	2	minimum counts of relevant peak (percent relative to maximum count)
Minimum (abs)	30	minimum counts of relevant peak (absolut counts)

Figure 5: *Cabbage sample aneuploidy analysis – user-definable page to input and fine-tune aneuploidy analysis report criteria*

A general aneuploidy report including the 96-well cabbage samples is also generated.



Client Olympia
Contact KRA

Application Brassica sp.
Batch aneupl087.assay

Labcode 21981
Date 21 April 2015

Quantum Analysis

ID	Overview	Aneuploidy Rel. DNA amount	Deviation	Ploidy
A1		1.95	-0.5%	2.0
A2		1.94	-0.7%	2.0
A3		1.96	0.1%	2.0
A4		1.94	-0.8%	2.0
A5		1.94	-0.9%	2.0
A6		1.94	-0.8%	2.0
A7		1.96	0.1%	2.0
A8		1.97	0.8%	2.0
A9		1.95	-0.4%	2.0
A10		1.95	-0.4%	2.0
A11		1.95	-0.5%	2.0
A12		1.96	-0.1%	2.0
B1		1.95	-0.1%	2.0
B2		1.97	0.6%	2.0
B3		1.96	0.1%	2.0
B4		1.94	-0.8%	2.0

Figure 6: *Cabbage sample aneuploidy analysis – result page displaying individual histograms, DNA amount and ploidy level*

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