Ploidy analysis of plants using the CyFlow® Ploidy Analyser

A fast and reliable method for DNA ploidy analysis in agroscience and botanical research

Introduction

The ploidy level describes the number of chromosome sets included in the cell nucleus. The analysis of DNA ploidy level is the most frequent application of flow cytometry (FCM) in plant breeding. From the early beginning Sysmex Partec GmbH contributed to the application of FCM in the field of plant genetics and plant breeding, leading to a wide-spread substitution of previously used methods of conventional chromosome counting (using microscopy) by FCM.

The analysis of plant ploidy became significantly faster, more accurate and more convenient by FCM and the fluorescent staining of plant nuclei. Due to its characteristics, it opened new research perspectives and breeding strategies allowing the analysis of nuclear DNA of hundreds of samples within short time at a minimum of manpower.

Using FCM technology (e.g. CyFlow® Ploidy Analyser) variations of the number of chromosomes can be detected indirectly by measuring the DNA amount of the individual nuclei.

This method is based on the comparison of a reference plant with known ploidy level (of the same species) with the plant of interest. Applications of ploidy analysis range from quality control in seed production over ploidy screening of individuals from *in-vitro* culture to biological research in taxonomy and geobotany with investigations on ploidy level distribution within population studies.

Based on our expertise in the analysis of nuclear DNA in plants, different staining kits for fast screening of ploidy (one step procedure) or high precision DNA analysis (two step procedure), are offered by Sysmex Partec GmbH. Nuclei containing material of plants like leaves, seedlings, roots, flowers or seeds can be used for flow cytometry, without the need for mitotically active cells as required for microscopic ploidy analysis.

For specific applications, particular tissues have to be used for the analysis, e.g. microspores, pollen, embryos and endosperm or protoplasts. Regardless of the source material, the preparation procedure is based on the extraction of the intact nuclei and their subsequent quantitative fluorescent DNA staining.

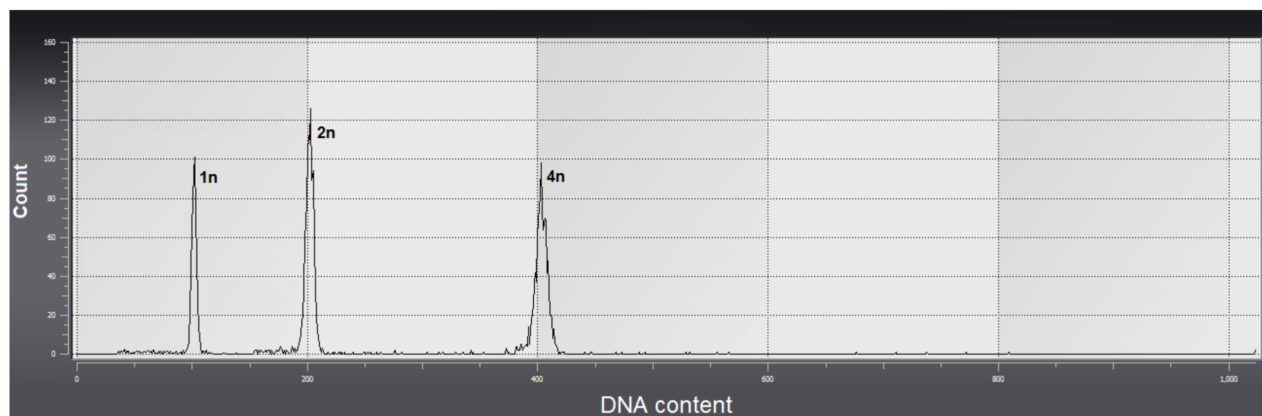


Fig. 1: Principle of ploidy analysis. The ploidy level is analyzed by comparing a reference peak (e.g. 2n) to the peak of interest. This example is a mixture of three samples of Rape: haploid, diploid and tetraploid. *Data acquisition and data analysis were performed on a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).*

DNA-specific fluorochromes

The selection of the used fluorochrome is depending on the experimental aim and of course is influenced by the instrumental configuration with respect to light sources and detection wavelength (*check the **Instrument requirements** paragraph for the DNA dye usable in your instrument*).

Propidium Iodide: This fluorochrome is a DNA/RNA intercalating dye. Thanks to its characteristics, it is suited for the determination of absolute DNA amount per nuclei (genome size) but also for ploidy analysis. It is optimally excited by a 532 nm green laser.

DAPI: we recommend the use of this fluorochrome for analysis of relative differences in DNA (ploidy analysis). DAPI is excited by UV light from a UV-LED or UV laser and emits blue fluorescent light. The use of a UV-LED instead of laser represents an advantage in terms of life-time, accuracy and price. In addition, DAPI is known to be less sensitive against staining artefacts caused by secondary plant metabolites, it is only staining ds DNA and no RNA and, it is less harmful to man compared to the PI fluorochrome.

		Wavelength (nm)	
Fluorescent Dye	Binding Mode	Excitation	Emission
Propidium iodide	Intercalation	540	615
DAPI	AT-selective	365	450

Table 1: Most frequently used **DNA-specific fluorochromes for Ploidy Analysis**

Instrument requirements

We recommend our dedicated CyFlow® Ploidy Analyser equipped with either green laser light excitation (532 nm, CyFlow® Ploidy Analyser V2, for Propidium Iodide), UV-LED (365 nm, CyFlow® Ploidy Analyser V1, for DAPI) or both light sources (CyFlow® Ploidy Analyser V3, for both DNA dyes) as needed for ploidy analysis via flow cytometry. In this case, we used a ***CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).***

Materials and Methods

For this application note we performed a ploidy analysis using a two-step DAPI procedure: nuclei extraction step and a staining step.

NOTE: *It could be possible to perform a single step procedure (nuclei extraction and staining) by using the appropriate reagents (e.g. CyStain® UV Ploidy,* article no. 05-5001).

Reagents and consumables

- CyStain® UV Precise P (article no. 05-5002)
- Cleaning Solution (article no. 04-4009_R)
- Hypochlorite Solution (article no. 04-4012_R)
- CellTrics® 50 µm (article no. 04-0042-2317)
- Petri dishes (6cm, article no. 04-2005)
- Plastic Pasteur pipettes or 1 ml pipette tips (widen orifice by cutting off 2-3 mm of the tip)
- Razor blades
- Reference plant of the test species with known ploidy level
- DNA Control UV (article no. 05-7302) for instrument calibration

Selection of a Reference sample

For a correct interpretation of ploidy level of a sample, the use of a reference sample is required, which is a sample with known ploidy level of the same species as the test plants. There are several ways of using this reference plant in the analysis.

One can distinguish between an ***internal*** and an ***external reference*** method:

- ***Internal Reference:*** The sample under study is mixed with a piece of material of a reference plant and both samples are prepared and analyzed together (Co-chopping procedure).

- ***External Reference:*** The reference plant material is prepared and analyzed separately from the samples and is used to calibrate the instruments scale.

At the end of the analysis it is possible to determine the ploidy level of the sample under study by comparing the DNA peak position of the reference

with the DNA peak position of the sample using this simple calculus:

$$\text{Sample Ploidy Level} = \frac{\text{Peak Position Sample}}{\text{Peak Position Reference}} \times \text{Ploidy Level Reference}$$

Sample preparation

For this example of ploidy analysis, we used the CyStain® UV Precise P kit (article no. 05-5002), a ready-to-use reagent kit for nuclei extraction and DAPI staining of nuclear DNA from plant tissues.

1. Cut a tissue fragment, preferably young leaf lamina, measuring approximately 0.5 cm^2 (or 10-20 mg)

2. Put the fragment in a plastic petri dish and add 1-2 drops of Nuclei Extraction Buffer on top of the tissue and chop with a sharp razor blade for 20 – 30 seconds

3. Add 500 µl of Nuclei Extraction Buffer to the chopped tissue and mix gently by pipetting few times (do not use narrow pipette tips). This procedure allows the release of nuclei from the tissue fragments

4. Transfer and filter the plant lysate in a sample tube using a CellTrics® 50 µm (article no. 04-0042-2317) filter

NOTE: *the filtration stage removes the tissue debris after release of the nuclei. It is essential to remove fragments to prevent clogging of the flow cytometers flow cuvette. Soluble substances which may interact with the staining of the DNA will not be retained on the filter!*

5. Add 2 ml of Staining Buffer to the filtered nuclei extraction

6. Analyze the sample. In this case, samples were analyzed using a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm) as described below.

Data acquisition

Start the flow cytometer and load a CFG-script **DNA-DAPI** for analysis of DAPI-stained samples or alternatively **DNA-PI** for analysis of samples stained with Propidium Iodide or any customized own configuration file. Saved instrument settings will be loaded.

1) Initial cleaning procedure

1.1 Use the initial 'Prime' mode of your instrument. Follow the instructions of the guided Prime cycle.

2) Instrument check with DNA Control UV (article no. 05-7302, or alternatively DNA Control PI (article no. 05-7303) when using propidium iodide as fluorochrome

2.1 Attach a tube with DNA Control UV/PI to the instrument and start the measurement

2.2 Adjust gain values for DNA fluorescence placing the signal peaks into the preselected regions as shown in Figure 2.

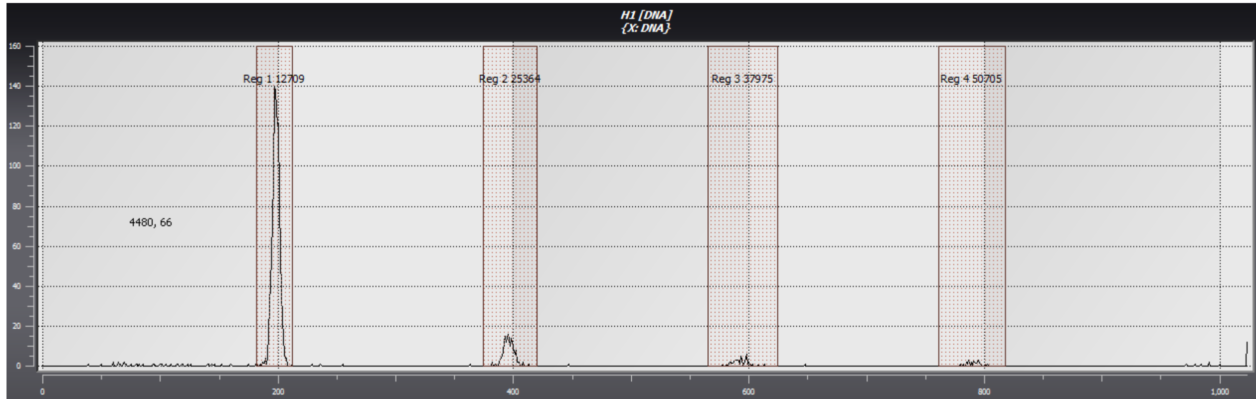


Fig. 2: Data acquisition – DNA control UV: gain values were adjusted on fluorescence signal of DNA Control UV in the specified linear regions. *Data acquisition and data analysis were performed on a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).*

3) Sample Analysis

In this example, we performed the analysis of the ploidy level of 2 samples of Rape (*Brassica napus*) by using a diploid External Reference (see Reference sample paragraph) of Rape.

3.1 Connect a sample tube with the reference ploidy sample to the instrument and start the measurement. Use a flow rate of 0.5 – 1.0 µl/s. Higher flow rates will speed up the analysis but reduce the accuracy.

3.2 Adjust gain values for DNA fluorescence placing the 2n signal peak on the 200 value of the linear scale as shown in Figure 3 Adjust the threshold to cut of debris signals showing up on the left side of the histogram. The amplitude of the debris peak must be smaller than the amplitude of the signal peak!

NOTE*: We recommend to use channel 200 of the linear scale as reference for the diploid peaks and to collect around 1000 events per sample.*

NOTE: *A sample can present more than one signal peak due to the natural presence of nuclei with increased number of chromosomes (G2 nuclei, nuclei after endoreduplication of chromosomes). The relevant signal peak is always the peak with the lowest signal on the x-axis.*

3.3 Stop the analysis by removing the test tube. An automatic cleaning will be initiated. Alternatively, you may push the end button first and remove the sample tube. In this case, no automatic cleaning will be performed. Hence connect a sample tube filled with Sheath Fluid and perform one cleaning step.

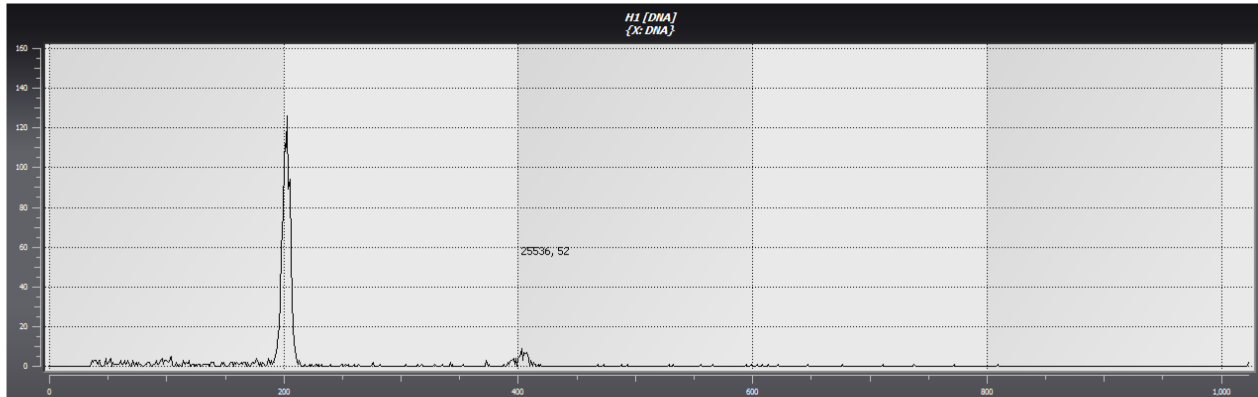


Fig. 3: Data acquisition – Sample analysis: gain values were adjusted on fluorescence signal of the reference sample in the specified linear regions (diploid = 200 channel). *Data acquisition and data analysis were performed on a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).*

3.4 To activate a Peak Analysis, use right mouse click on the 1-parameter histogram to open the histograms context menu. Peak Analysis options **CV Peak-Analyze** and **GAUSS Peak-Analyze** can be selected by a left mouse click.

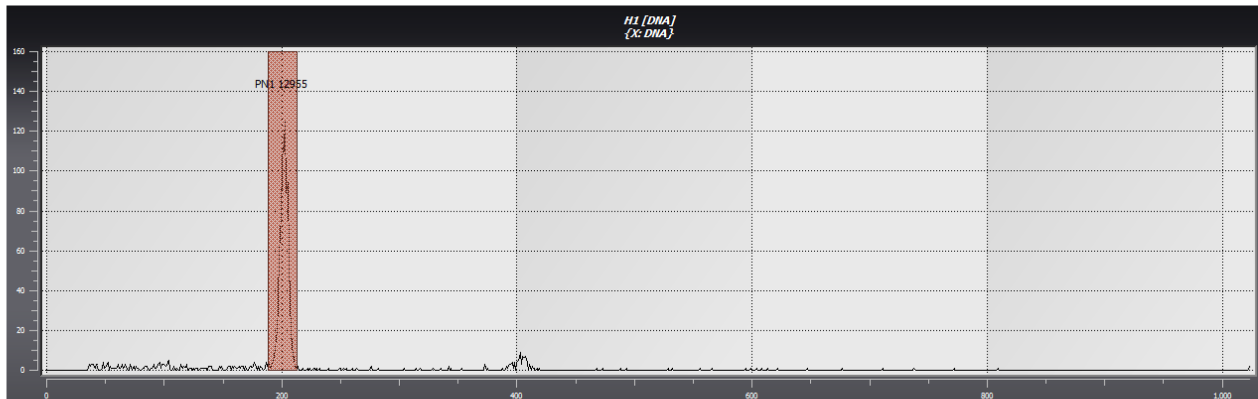


Fig. 4: Data acquisition – Sample analysis: activation of CV Peak-Analyze option. Peaks are automatically identified and labelled by a red region. *Data acquisition and data analysis were performed on a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).*

***NOTE:** It is possible to activate the automated CV peak detection by switching on **AutoPA** button in the register card Measure. The selected peak analysis mode (CV Peak-Analyze / GAUSS Peak- Analyze) will be automatically triggered after a measurement is started.*

*! In case of high background levels in your instrument, please check in the **Troubleshooting** paragraph. High background is caused by fragmented nuclei or unspecific stained debris particles. This could be due to a problem in the sample preparation !*

! Voltage settings for the fluorescence parameter is completed. Do not change voltage from now on during sample measurements !

3.5 Connect a sample tube with your sample of unknown ploidy and start the measurement (Figure 5).

***NOTE:** With this setting (diploid nuclei at position 200 of the linear scale), haploid nuclei (n) will appear around the position 100 of the linear scale while tetraploid nuclei (4n) will appear around the position 400 of the linear scale and so on.*

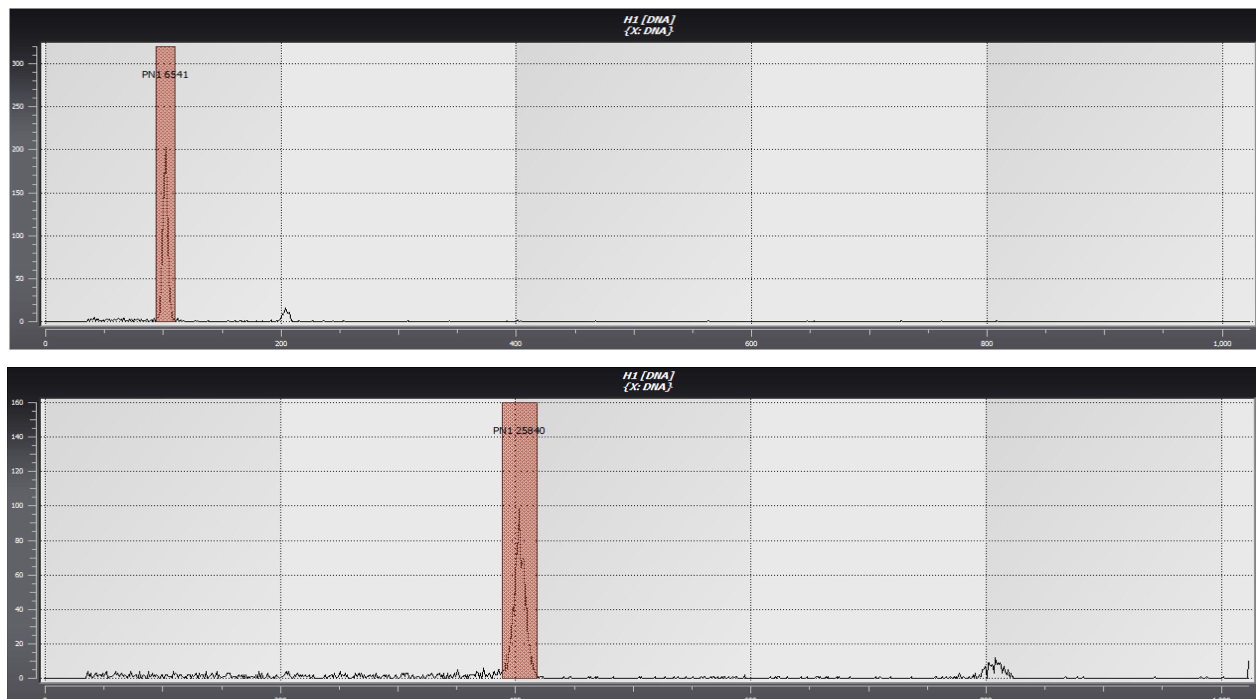


Fig. 5: Data acquisition – Sample analysis: measurement of unknown ploidy samples. **top)** With the pre-set settings, the labelled nuclei peak appears on the position 100 thus indicating that the sample under study presents a haploid phenotype (refer to formula above). **bottom)** measurement of a tetraploid (4n) sample. In this case, the labelled nuclei peak appears on the position of 400. Peaks are automatically identified and labelled by a red region. *Data acquisition and data analysis were performed on a CyFlow® Ploidy Analyser equipped with a UV-LED (365 nm).*

NOTE: *Small populations of G2 phase nuclei do occur at position 200 (Figure 5 top) and 800 (Figure 5 bottom). These nuclei show exactly the double x-axis position as the major peak and mimicry higher ploidy level. Similar effects can be caused by endo-reduplication of chromosomes (DNA replication without subsequent cell division). By multiple endo-reduplication cycles, nuclei with high ploidy levels can be generated.*

Data analysis

In this example, we performed a ploidy determination of 2 samples of Rape (*Brassica napus*) by using an external reference from the same species with known ploidy (**diploid**).

Once the data from a measurement are obtained as described in the previous chapter, two values need to be checked: **Mean** of the peak and **Coefficient of Variation in % (CV%)** of the peak.

The Mean represents the intensity of the fluorescent signals and is directly proportionate to the ploidy of the sample under study. The CV% of the fluorescent peak ideally should be low. With a high CV% value the accuracy of the Mean will decrease. For Ploidy Analysis, we do recommend CV values below 6 %, but even higher values may lead to reasonable ploidy determinations.

NOTE: *Too high CV% values could be due to problems with sample preparation. Please check the Troubleshooting paragraph.*

By the analysis of our external reference and our samples with unknown ploidy we obtained the following results:

Sample	Mean	CV
Reference	12949.13	2.23
Sample 1	6536.68	1.81
Sample 2	25840.02	1.29

In order to determine the ploidy level of our samples we use the formula shown in *Selection of a Reference sample* paragraph:

$$\textbf{Sample 1} = \frac{6536.68}{12949.13} \times 2 = \mathbf{1.00} = \boxed{\textbf{n}\text{ Haploid}}$$

$$\textbf{Sample 2} = \frac{25840.02}{12949.13} \times 2 = \mathbf{3.99} = \boxed{\textbf{4n}\text{ Tetraploid}}$$

Troubleshooting and general hints

Reference choice according to DNA application:

As previously described, we do distinguish an ***internal*** and an ***external*** reference method. Both are used for different analytical targets. **Table 2** summarizes the use of reference methodology according to the intended application.

Application	Reference	Nature of reference	Comment
Ploidy Anaysis	External	Same species as sample with known ploidy level	In case peak position is stable and allows safe determination of ploidy level
Ploidy Anaysis	Internal	Same species as sample with known ploidy level	In case peak position is not stable and does not allow safe determination of ploidy level
Aneuploidy	Internal	Plant with regular genome size	Mix the "normal" plant with the sample, co-chopping
Aneuploidy	Internal	Any plant with 1.2 – 4 fold genome size	Mix reference (RP) with normal plant (NP) and test plant (TP) by co-chopping procedure. Establish the DNA ratios and compare ratios of RP/TP and RP/NP. Deviations in ratios indicated aneuploidy of the test plant

Table 2: Reference choice according to DNA application: DNA determination and reference method.

Precautions for a good sample preparation:
Three criteria must be kept in mind during the sample preparation: *Incubation time*, *temperature* and *secondary metabolites* deriving from cells.

An aspect that may have strong influence on the staining result is the ***incubation time*** of the sample. For most plant species, short incubation periods of 30 to 120 seconds or up to 5 minutes are well suited whereby some species may require incubation of up to 2 hours.

Moreover, even the ***temperature*** at preparation could play a pivotal role. The preparation at 4°C leads to a reduction of DNAse activity and oxidation effects but also to a slower speed of fluorochrome diffusion and binding to the DNA. Often preparation with buffers at room temperature is more advantageous than on ice.

During tissue disruption and nuclei extraction ***secondary metabolites*** located in compartments of the cytoplasm and the cell vacuole (i.e. poly-phenolic substances, organic

acids, mucilage) might be released and getting into direct contact with the nuclei. Such substances disturb the binding of fluorochromes to the DNA or aggressively destroy nuclei integrity. In general, such effects may be inhibited or reduced by adding chelating and reducing agents. For example, nuclei oxidation leads to the degradation of DNA. Oxidation often becomes visible by a brownish or a greyish colour of the nuclei suspension developing after nuclei extraction. The effect creates broad DNA histograms with high CV% (peak with left shoulder) and instable (shifting) peak positions
Here we report a list (**Table 3**) of the possible chemicals to be added (single or in combination) to lysis and staining buffer in order to reduce DNA degradation.

Additive	Function	Concentration
EDTA	Chelating agent	2 mM
EGTA	Chelating agent	2 mM
Mercaptoethanol	Reducing agent	15 mM / 0.1 %
Dithiothreitol	Reducing agent	15 mM / 0.1 %
Polyvenylpyrelidone (PVP-10, PVP-20)	Complexing agent	0.5 – 2 %
Spermine	Chromatine stabilizer	0.5 mM

Table 3: Precaution for a good sample preparation: Additives and their respective concentration to be added to lysis and/or staining buffer.

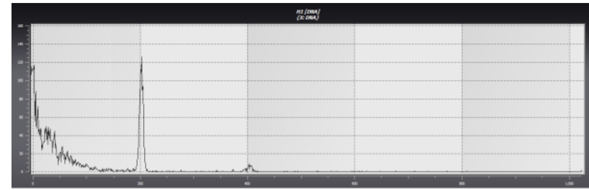
Background and wide peaks

Background signals can create a problem for the detection of ploidy and could be the result of different aspects related to the sample preparation and to the sample itself. When sample background is mixing with signals of nuclei, data interpretation can be impeded.

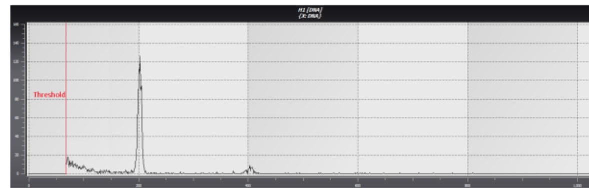
Factors influencing the quality of samples negatively are:

- Old tissues samples (use preferably young leaf material)
- Withered or stressed tissues
- Blunt razor blades (in this case cells are squeezed and not chopped)
- Exaggerated tissue chopping (fragmentation of nuclei)
- Non-suited buffer systems

Thus, during sample measurement increased background signals as shown in the figure below could be present. Background even could cover signal peaks:



To eliminate high background signals, the threshold can be increased.

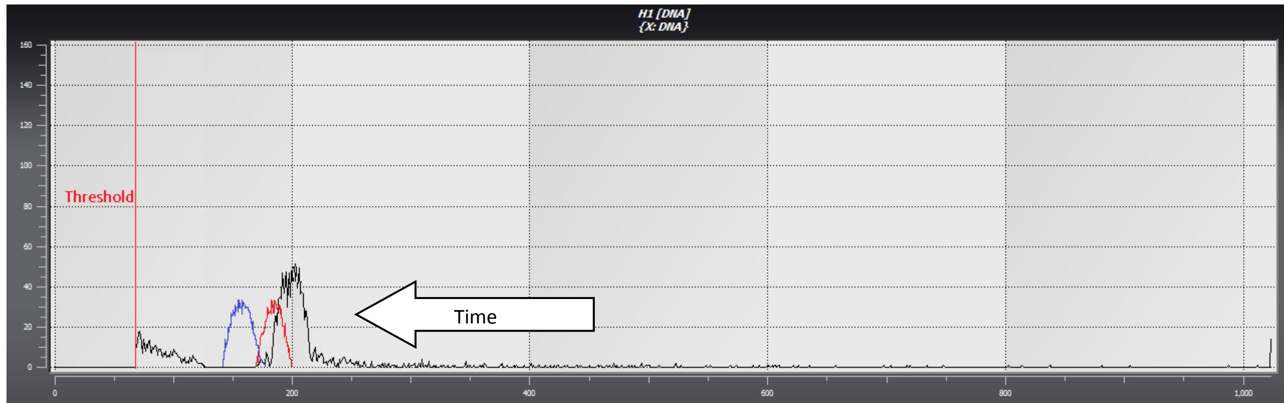


NOTE: *Rule of thumb: The background signals should not grow above the signal* peak. *But be sure to not increase threshold over the position of the lowest sample peak you expect in the experiment, e.g. if a diploid sample is positioned at 200 and you might have haploid samples, the threshold must be < 100.*

To reduce sample background:

- Change razor blade
- Select younger leaf tissue or change to a different tissue
- Reduce time of chopping (during sample preparation)
- Reduce the amount of tissue
- Change to a different buffer system
- Add EDTA (2 mM) and/or Spermine (0.5 mM)

Other factors that could create problems in the binding of fluorochromes to the DNA and/or aggressively destroy nuclear integrity are secondary metabolites potentially located in the cell vacuole or in compartments of the cytoplasm (i.e. organic acids, polyphenolic substances, mucilage). After lysis of the tissue nuclei are exposed to these substances. In this case, ploidy peaks become wider with time thus resulting in an increased CV. Nuclei oxidation leads to the "peak shifting phenomena" of the ploidy peaks during sample measurement and/or if it is analyzed longer time after sample preparation as shown in the figure below. Often such sample behavior is misinterpreted as instability of the analyzer.



In this case, the samples under study (2n) present a wide diploid peak with an increased CV value. In case of advancing nuclei degradation, a shifting of the peak (red and blue peaks) within the analysis or after a second analysis of the same sample can be observed.

NOTE: *Nuclei oxidation often becomes visible by a brown or grey/dark coloration of the nuclei suspension, the so called "sample browing".*

However, to avoid the increased CV and "peak shifting phenomena" we suggest repeating sample preparation with some of the following changes:

- Prepare the samples on ice, use ice-cold buffers (recommended)
- Reduce time of chopping (during sample preparation)
- Add antioxidant (reducing) agents, chelating/complexing agents and chromatine stabilizer (Table 3)
- Use an internal reference
  NOTE: *in this case both peaks will be shifted, but the ratio between them will remain similar*
- Reduce amount of plant material

Recommended settings:

Light source:	UV-LED (365 nm)/Green laser (532 nm)
FL Gain:	recommended range 200 – 700V
Speed:	0.5 – 1 µl/s
Flow rate:	approx. 20-100 events/s

References

1. Package insert: CyStain® UV Precise P article no. **05-5002**

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