Standard Operating Procedure (SOP) for Ploidy Analysis in Banana

Authors & Contributors

V. Akech*, T. Oparok(T.OparokAcgiar.org), N. Nyanzi(N.Nyanzi@cgiar.org), R.Simba(R.Simba@cgiar.org), C. Nasuuna(C.Nasuuna@cgiar.org), C. Nabukenya(C.Nabukenya@cgiar.org), Ivan. K. Arinaitwe, B.Uwimana(B.Uwimana@cgiar.org)
*Correspondence: V.Akech@cgiar.org

1. Introduction

Banana is a natural multi-ploid crop. Wild bananas are seeded diploids (2x), while banana cultivars are seedless, mostly triploid (3x) and sometimes diploid. Because of the sterility of triploid bananas, improvement entails the generation of tetraploid intermediate hybrids (4x) which are fertile. Therefore, the determination of ploidy level is important for informative use and management of banana germplasm for different breeding objectives and targets. It guides the breeder on the choice of parents to include in a breeding scheme on the basis of ploidy among other considerations. Upon hybridization, bananas produce hybrids of mixed ploidy levels such as diploids, triploids and tetraploids. So it is very important to determine their ploidy level along with their performance in field trials so that hybrids are compared within their ploidy levels. Ploidy analysis happens at all stages of banana improvement, either on the products of hybridization for pre-breeding and breeding (figure 1), or on any newly acquired germplasm from genebanks or other banana breeding programs.

Stage Title	Product Design		Trait Discovery		Trait Deployment		Crossing & screening		Early Testing		Late Testing		Pre-commercial Testing and Product Registration		Product Introduction
Description in banana	Market research, Crop Strategy Review, Product Profile Review		Evaluation of germplasm sources, trait validation, inheritance and molecular discovery		Introgression of trait into improved diploid and/or tetraploid parents		Product development, seed production, clonal multiplication		Clone development from selected EET, small plot testing with 2-5 clones/genotype		Selected clones in replicated multi-site representing TPE (yield/resistance stability)		National Performance Trials and On-Farm trials		Official release and product launch
Short name in banana	Product profile		Prebreeding		Parent development		Generating EET (Early evaluation trial)		PYT: Preliminary Yield Trial		AYT: advanced yield trial (multilocational testing)		By TARI, NARO, etc		By TARI, NARO, etc

Figure 1. Stages and gates for Musa improvement.

Ploidy can be determined by several methods with the most consistent ones being chromosome counting and DNA flow cytometry. Chromosome counting is accurate but very laborious. DNA flow cytometry has been demonstrated to be rapid and reliable for ploidy screening in *Musa* (Dolezel *et al*., 1997). A critical aspect for obtaining accurate, reliable, and high-resolution estimates of nuclear DNA content is the release of nuclei from the cytoplasm in sufficient amounts, while maintaining their integrity throughout the analysis, protecting their DNA from degradation by endonucleases, and enabling stoichiometric DNA staining (Loureiro *et al*., 2021).

2. Purpose

The purpose of this SOP is to describe a series of activities, processes, data capture and interpretation carried out during ploidy analysis of banana using the DNA flow cytometry method based on the operation protocol of the Sysmex ploidy analyzer machine (see details in the list of equipment and apparatus below).

3. Scope

The SOPs apply to all breeding activities implemented under the IITA banana breeding programme in Namulonge, Uganda in collaboration with the National Banana Programme of NARO, Uganda. It aims to reach the broader banana breeding community in Uganda and for use as a blue-print in other banana breeding programmes in Africa.

4. Definition of terms

- **Ploidy**

This is the number of sets of chromosomes in a cell or an organism.

- **Diploid**

This is the presence of complete sets of chromosomes in an organism’s cells, with each parent contributing a chromosome to each pair.

- **Triploid**

The presence of 3 sets of haploids (single) chromosomes in an organism or cell. This is termed as 2n = 3x = 33, where n is the haploid chromosome number for the species concerned.

- **Tetraploid**

This is the presence of four homologous sets of chromosomes in an organism's cells, expressed as 2n = 4x = 44.

- **Standards**

These are organisms or species or genotypes with known chromosome numbers used as controls in calibrating the ploidy analyser machine before running samples with unknown ploidy level to determine their chromosome numbers. Examples of standards used in ploidy analysis is Calcutta 4 (diploid), East African highland banana (triploid) and 1201K-1 (tetraploid).

5. Roles and responsibilities

- **Molecular banana breeder:** Responsible for managing and overseeing all molecular breeding activities, data production, analysis and reporting as pre-breeding stage, leading to trait discovery for the banana breeding programmes of IITA in East Africa.
- **Research associate:** To support the molecular banana breeder by coordinating molecular breeding activities including but not limited to design and management of trials and experiments, supervising of field and laboratory operations, data collection, data entry and analysis, synthesis and writing, coordinate and supervise field and laboratory labour of research assistants, technicians and trainees.
- **Molecular lab technician:** Perform the ploidy analysis activities from field sample collection, sample and reagents preparation, analysis, reporting the results and ploidy machine maintenance.

6. Procedure

6.1 Requirements

6.1.1 Equipment and apparatus

- Ploidy Analyser Machine (the CyFlow® ploidy analyser, Software-CyView™ 1.6, REF-CY-S-3039).

A ploidy analyser is a fully equipped desktop Flow cytometer (FCM) and features a modular optical concept that allows the use of different lasers as light sources and the detection of up to 6 optical channels (parameters). It operates with an internal PC (personal computer), and data acquisition, instrument control, and data analysis are controlled and performed by the CyView™ software (CyView™ 1.6)

- Waste bottle
- Sheath fluid bottle
- Sterile falcon tubes
- Cuvettes
- Filter paper (Whatman paper)
- Stirring machine
- CellTrics® sieves
- Glass Petri dishes
- Forceps
- Sharp razor blades
- Scissors
- Marker pen
- Fridge (+4°C)
- Deionized water
- Paper Towels

- Android device installed with ODK Collect App and a QR bar code reader App.

6.1.2 Buffers and solutions

- Otto I buffer: Citric acid Monohydrate (0.1M). This is used to break the nucleic membrane to release he nucleic contents from the plant cell nucleus.
- Tween 20: This is a surfactant that helps in the spreading of reagents reducing the tendency of nuclei and debris to aggregate.
- Otto II buffer: 0.4M Anhydrous Sodium hydrogen phosphate (Na_2HPO_4) added to the isolated nuclei to enable staining.
- DAPI (4',6-diamidino-2-phenylndole): This is a fluorescent dye used to stain the nucleic acid.
- $Na_2S_2O_5$ (Sodium Metabisulphite). This is an anti oxidant.
- 70% Ethanol.

6.1.3 Software and Apps

- CyView™ software (CyView™ 1.6)

This is the software installed in the ploidy analyser that controls data acquisition, instrument control, and data analysis. It is purchased together with the machine.

- ODK Collect App

This is the software that runs the banana tracking tool (BTracT) used to create the bar codes for the samples from the tissue culture laboratory and to record the ploidy analysis results. This can be downloaded from google play services using the link https://play.google.com/store/apps/details?id=org.odk.collect.android&hl=en&gl=US&pli=1.

- QR barcode reader

This is needed for scanning to read the QR barcodes to identify the samples for analysis and recording.

6.2 Buffer preparation

6.2.1 Otto I Buffer (500ml)

- Weigh 10.5g of Citric acid Monohydrate (0.1M) and dissolve into 400ml of deionized water.
- Add 2.5ml of Tween 20 to the solution.
- Stir the solution using a magnetic stirrer to dissolve solids.
- Filter the solution using Whatman filter paper to remove small particles.
- Adjust the volume of the solution to 500ml with deionized water.
- Store the solution at 4°C.

6.2.2 Otto II Buffer (500ml)

- Weigh 28.4g of Anhydrous Na_2HPO_4(0.4M) and dissolve in 400ml of deionized water.
- Heat the mixture while stirring until Na_2HPO_4 dissolves.
- On cooling, filter the solution with filter paper to remove small particles.
- Adjust the volume to 500ml with deionized water.
- Store the solution at room temperature in the dark.

Note:

- At the time of running the samples, add 4µl of DAPI (4',6-diamidino-2-phenylindole) for every 10 ml of Otto II buffer, (20µl of DAPI for every 50 ml of Otto II buffer).
- Add 1ml of $Na_2S_2O_5$ (Sodium Metabisulphite) to 50 ml of Otto II buffer.

6.3 Ploidy machine set up

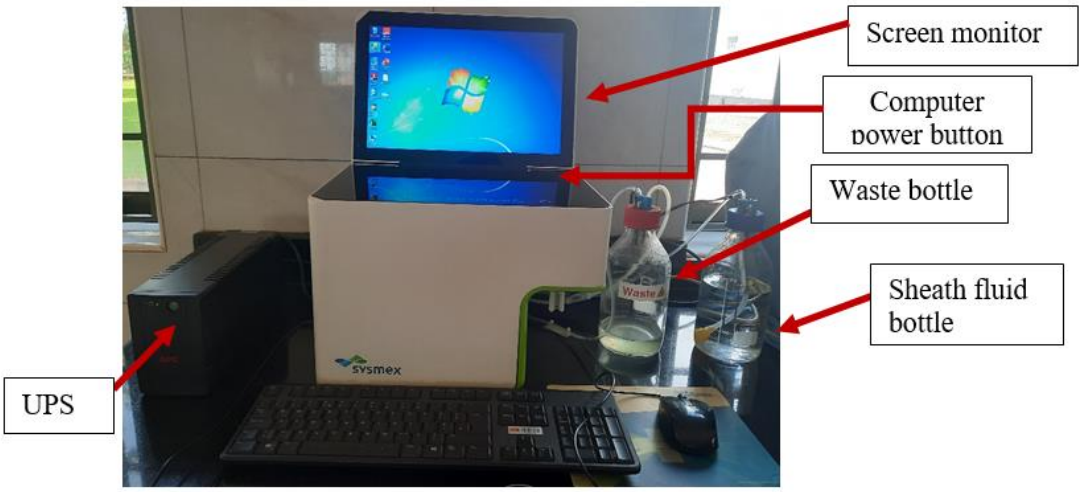


Figure 2. The CyFlow® ploidy analyser machine

6.3.1 Starting the machine and analyzer software

- Switch on the uninterrupted power supply (UPS).
- Plug the ploidy analyzer into the UPS (UPS protects the machine from unstable power supply).
- Switch on the ploidy analyzer at the power button found at the lower back panel of the machine.
- Lift open the screen monitor of the machine.
- Start up the computer of the machine by the pressing the computer power button shown in figure 2 above.
- Click open the ‘Cube 1.6’ icon to the desktop to start running the CyView ™ software as seen in figure 3 below.

Figure 3. Ploidy machine display screen with cube 1.6 software icon highlighted.

- Login into the software using the details; Login: **USER**, Password: **Cube1** as shown in figure 4 below. Both username and password are case sensitive.

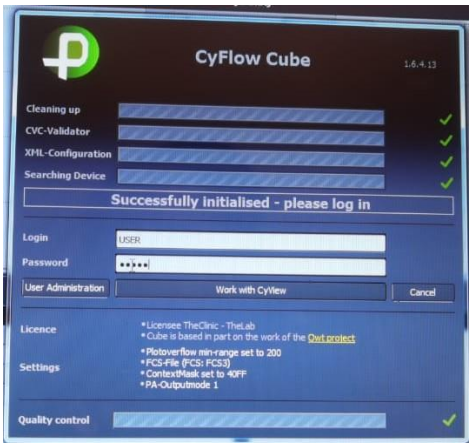
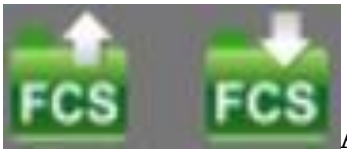
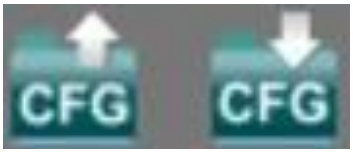


Figure 4. Display of the CyFlow® Cube 1.6 soft ware login details page.



A



B

Figure 5. Icons for loading and saving data files (A) and for saving and retrieving the configuration files (B).

6.3.2 Priming the machine

Priming is the initial process carried out to not only get the machine ready but also to clean the entire flow cytometry system of the machine like the tubes, getting rid of blocked air and stabilize the system. Priming is done when starting the instrument for the first time (daily initialization), after the Sheath fulid bottle is filled and as a trouble shooting procedure like when there is no signal or bad signal and blockage.

- Load the configuration script Cfg-file for priming (this script is preset) from the CFG folder as showed in figure 5 B above.
- Select the initialization icon highlighted in figure 6 below and click ‘play’.

Figure 6. Icons for Prime (highlighted for use during priming), work, and clean mode.

- This will start the priming process and a series of guide dialogue to follow;
- Connect a sample tube with the Decontamination solution (violet solution), press “continue” and wait until the system has finished running.

Figure 7. Step 1 of the priming process.

- Disconnect the sample tube, press "Continue" and wait until the system has finished running.

Figure 8. Step 2 of the priming process.

- Reconnect the sample tube with the Decontamination solution, press "Continue".

Figure 9. Step 3 of the priming process.

- Connect the sample tube containing sterile distilled water or sheath fluid and press "Continue".
- When the the machine stops running, it is ready for use. A message to confirm end of priming process will be displayed as in figure 10 below.

Figure 10. Step 4 and the final stage of the priming process.

6.4.3 Calibration of the machine

Calibration is done using fish DNA as it is of known ploidy as a diploid, UV calibration beads to ensure that the UV lamps are working, and banana cultivars of known ploidy called standards.

- Switch from Prime to the "WORK" mode by clicking on work icon as shown in figure 11 below.

Figure 11 Work mode activated.

- Load the calibration files from the CFG folder by clicking on the icon highlighted in figure 12 below.

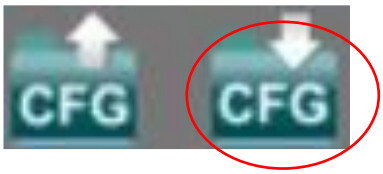


Figure 12. Icon for loading configuration files.

- Select the DNA calibration script.
- Put 1 ml of fish DNA in a cuvette and load it in the sample chamber.
- Run the sample by clicking 'play' in work mode.
- Adjust the gains to set the control peak at absorbance frequency of 200.
- Then select the UV calibration beads script from the CFG folder.
- Mix 1 drop of the UV calibration beads solution into 50 ml of sterile de-ionized water and shake gently, to mix well.
- Add l ml of the solution into a cuvette and run it.
- Adjust the gains to set the control peak at absorbance frequency 200 in region 1 as seen in figure 13 below.

Figure 13. Peak at absorbance frequency 200 in region 1 indicating well calibrated machine.

- Once both fish DNA and UV beads have peaked at 200, the machine has been calibrated.

- Further calibration can be done using accessions with known ploidy like Calcutta 4 as a diploid, any of the East African highland bananas as triploid and 1201K-1 as tetraploid.

6.5 Sample collection, preparation and analysis

6.5.1 Sample collection

6.5.1.1 Tissue culture laboratory sampling

- Select plantlets in the tissue culture laboratory before the rooting stage.
- Pick 2-3 leaves from each plantlet using disinfected surgical blade.
- Wrap the leaves in aluminum foil and stick the respective sample ID barcode on the sample as seen in figure 14 below.
- Take the samples to the molecular laboratory.
- The samples can be kept in the + 4^0c refrigerator for a maximum of three days, if they are not going to be worked on immediately.

Figure 14. Example of a sample labelled with a barcode.

- Sterilize the blade by wiping it with a paper towel soaked in ethanol before taking the next sample.

6.5.1.2 Field sampling

This is done for plants that are already established in the field (not in the tissue culture laboratory) but whose ploidy level needs to be determined as needed.

- Identify a healthy clean green cigar leaf (the cigar leaf is the youngest leaf on the plant, still rolled like a cigarrete) on the banana plant.
- Scan the bar code on the plant to record the sample to be collected using the Coordinate App.
- Cut about 6 cm of the leaf from the leaf apex (tip of the leaf) using sterile scissors.
- Wrap the leaf sample in aluminium foil and stick the sample ID barcode.
- Sterilize the scissors by wiping the cutting edges with a paper towel soaked in ethanol before sampling the next plant.

6.5.2 Sample preparation in the laboratory

- Hold the fresh leaf sample with a pair of forceps in a Petri dish containing 1 ml of cold Otto I buffer and chop the midrib into very fine pieces, using a sharp razor blade taking care not to crush the sample (figure 15). This is to release nuclei into the isolation buffer.
- Mix the homogenate in the Petri dish and then filter it to remove leaf debris, using a cellTrics® sieve into a cuvette.
- Incubate the solution for 1-5 minutes to allow the cell suspension to thoroughly pass through the cellTrics® sieve.

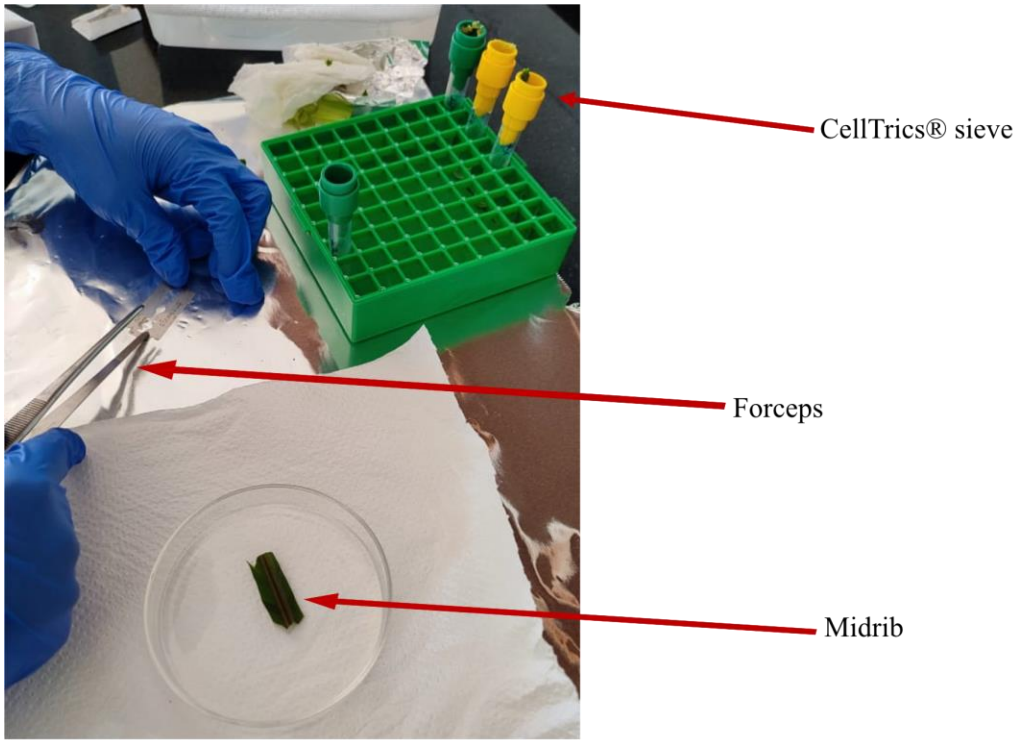


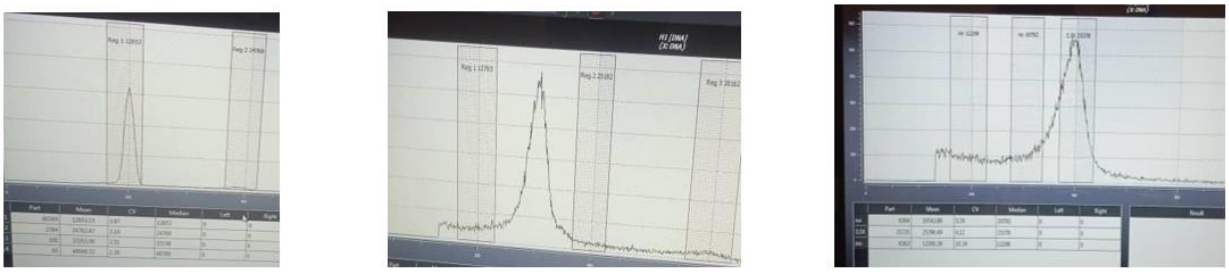
Figure 15. Sample preparation for ploidy analysis.

- Since the samples are stable in Otto I buffer, several samples can be prepared while still fresh, cell suspension in cuvette tubes covered and stored in the at +4°C in the fridge.

6.5.3 Sample analysis

- Choose the work mode as in figure 11 in section 6.4.3 to measure and acquire data from samples measured.
- Add 1ml of Otto II buffer (containing DAPI) into the cuvette tube containing the sample.

- Attach the cuvette tube to the ploidy analyzer machine and run the sample.
- Observe the peak formed . Samples of different ploidy will show corresponding peaks as shown in figure 16 below.



A B C

Figure **16**. Peaks showing ploidy level diploid (A), triploid (B) and tetraploid (C).

- Record the ploidy level results as they appear using the ODK collect app as illustrated in steps 1 to 5 in figure 17 below.
- Save the sample data results (peaks) by clicking the FCS icon shown in figure 5A above, to save the fcs files on the computer.

Figure 17. The process (Step 1-5) of data recording of ploidy results using BTracT system.

6.5.4 Clean mode (Shutdown process)

This mode is activated to clean the sample port, tubes and shut down the process. It follows the steps below;

- Select the "CLEAN" mode by clicking on the "CLEAN" icon highlighted in figure 18 below.

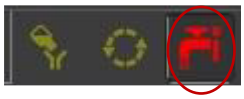


Figure **18**. Activated clean mode icon.

- Press start (play icon), the system will start the priming process automatically and guide you through the program

- Connect a sample tube with the cleaning solution (green solution) showed in figure 19 below and press continue.

Figure 19. Decontamination solution (violet) and cleaning solution (green).

Figure 20. Step 1 of the cleaning process.

- Connect a sample tube with decontamination solution (violet solution) and press “Continue”.

Figure 21. Step 2 of the cleaning process.

- Connect a sample tube with water (sterile distilled water or sheath fluid) and press "Continue".

Figure 22. Step 3 of the cleaning process.

- After completing the cleaning process, CyView™ will be shut down automatically. Laser sources will be switched off.
- Switch off the PC, close windows (start-Exit-switch computer off).
- To power off completely, use the main power switch at the back panel.
- Close the machine and cover it with dustproof protection.

6.6 Critical control points to note and guide when troubleshooting.

6.6.1 Sample preparation

- In the chopping procedure, it is important that the razor blade is sharp, and the material is chopped into very fine pieces and not just crushed.

6.6.2 Levels of waste and water bottles

- Always check the levels of the sheath fluid and waste bottles before switching on the machine. The sheath bottle should be filled with at least 700ml of sheath fluid and closed with a screw top. Please note that a higher level of sheath fluid could lead to unstable sample flow. The level of waste in the waste bottle and sheath fluid in the sheath fluid bottle should not be the same at any given time to avoid a buildup of pressure in either bottle.
- When filling up the sheath fluid bottle, ensure that no air bubbles are trapped in the yellow filter unit inside the bottle. Get rid of any air bubbles that appear.
- It is recommended to replace the sheath fluid at least once a week or before any daily use.
- The waste bottle must be emptied after and before each user session and the screw top tightly closed.

6.6.3 Prime mode should be performed in the following cases.

- Starting the machine for the first time (Daily initialization).
- After the sheath fluid bottle was refilled.
- As troubleshooting procedure (when no/bad signals were observed, blocking, etc).
- In case of need to perform a daily checkup, please select the “Calibration Beads” configuration file for the correct settings for the calibration beads (Very crucial settings for the correct verification of the performance of the machine).

6.6.4 Intermediate cleaning process (cleaning between samples)

- After running a sample, load the sample chamber with a cuvette containing deionized sterile water.
- Click the "clean" mode icon to clean the system between samples.

6.6.5 Maintenance of the Ploidy Analyser machine

- The machine should be placed on a flat surface, free from shaking forces.
- Clean the machine casing regularly and carefully with a soft dry cloth. Water must not enter the machine or peripheral devices or come into contact with electric conditions and switches.
- Use a special screen cleaner and a soft cloth for cleaning the screen. **Do not use any organic solvents, nitro thinner, benzol, alcohol, highly concentrated bleach, etc.** to clean the ploidy analyser or its screen.
- Do not use tools to clean the flow cuvette. In case it is blocked, enquire Partec for rapid exchange
- Regularly empty the waste bottle and clean it with a warm detergent solution and a brush
- Clean the sheath bottle (reservoir) with distilled water and a clean brush and flush with clean distilled water several times. A sheath bottle is critical for the optimum operation of the machine.
- The machine should be primed once a week when not used regularly as a form of routine maintenance. That is to say: clean the flow system by using distilled water.
- Always keep the electrodes of the sample chamber dipped in distilled sterile water or sheath fluid at the sample port.
- The machine should also be serviced by certified technicians accordingly as a form of routine maintenance.

References

Dolezel, J. (1997). Application of flow cytometry for the study of plant genomes. *Journal of applied Genetics*, 38, 285–302.

Loureiro, J., Kron, P., Temsch, E.M., Koutecký, P., Lopes, S., Castro, M. & Castro, S. (2021). Isolation of plant nuclei for estimation of nuclear DNA content: overview and best practices. *Cytometry Part A*, 99, 318–327.