nanocellect Biomedical, Inc.		Playbook for a successful plant protoplast demo
9525 Towne Centre Drive, Suite 150 San Diego, CA 92121 Tel: (858) 614-6400	Version: 1.0	**Title: Playbook for a successful plant protoplast demo**

PLANT PROTOPLAST DEMO DO'S AND DON'TS

DO'S

☐ **USE CHLOROPHYLL AUTOFLUORESCENCE AS A VIABILITY INDICATOR**

The presence of chlorophyll in a sample is a direct indication of viable plant or algal cells. It is also directly correlated with cell size. If the chlorophyll signal drops over time it indicates that the cell population is stressed and/or dying. It is suggested to use chlorophyll autofluorescence as a viability marker for protoplasts derived from leaves, shoots, or callus exposed to light. The first density plot can be chlorophyll autofluorescence vs. FSC. Protoplasts derived from dark grown cell suspensions, callus, or roots can be viewed with a FSC vs BSC plot.

☐ **USE PROTOPLAST CULTURE MEDIUM OR WASH BUFFER AS SHEATH**

Protoplasts are osmotically sensitive; they lack a cell wall. Use a culture medium or wash buffer that maintains a stable osmolarity; ~600 mOsm is a good starting point but will vary depending on crop type and protoplast source material. An appropriate osmolarity supports high viability pre and post sort. Culture media may include growth factors and nutrients to further aid in downstream growth.

NOTE: DO NOT CULTURE PROTOPLASTS IN WASH BUFFER

☐ **KEEP SHEATH BUFFER AT ROOM TEMPERATURE**

Plant protoplasts are sensitive to cold solutions. They will clump and settle. Keep all sorting solutions at room temperature. The only exception to this is if the customer has a functioning workflow that supports improved protoplast viability by keeping protoplasts cold in addition to clumping prevention.

☐ **ADJUST THRESHOLD & PMT VOLTAGE SETTINGS**

Threshold off FSC parameter and aim for a threshold of 10,000. Higher thresholds hide the protoplast populations.
Start all voltages at 200 mV and adjust as needed.

OVERNIGHT. PROTOPLASTS WILL SWELL. JUST USE CULTURE MEDIUM OR ASK CUSTOMER TO MAKE A WASH BUFFER WITH A HIGHER OSMOLARITY.

☐ **BE STRINGENT WITH GATING**
Be sure to gate on appropriate cell populations, removing debris and doublets from the target population.

☐ **FILTER SAMPLES**
Use a 40 µm nylon filter (or 50 µm max) just prior to sorting to remove clumps of protoplasts that could clog cartridge.

☐ **USE WIDE BORE SEROLOGICAL AND MICRO PIPET TIPS**
Protoplasts are fragile and large. They will lyse when using regular-sized tips.

☐ **PRIME & CALIBRATE WOLF WITH CULTURE MEDIUM.**
It works.
Exceptions: media containing ≥1% of Triton X-100 or ≥ Xanthan Gum. Avoid these on WOLF in general.

☐ **DEPOSIT PROTOPLASTS INTO CULTURE MEDIUM**
Instruct customers to add 100 µl of culture medium into each 5.0 ml FACS tube prior to sorting. This cushions the protoplasts as they are sorted into the tubes. If the customer is performing a single-cell sort, then add a minimum of 100 µl to each well of a 96-well plate. N1 should be set up to deposit cells in the center of the well. Always check plate calibration on N1 prior to sorting.

☐ **KNOW THE SIZE DISTRIBUTION OF THE PROTOPLAST SUSPENSION**
There is a direct correlation between protoplast diameter, size distribution, and sorting cell density. The smaller the protoplast diameter and distribution, the easier the sort will be. If most protoplasts are on the larger end (40-50 µm), decrease the cell density to support a successful sort. Protoplasts within 15-50 µm in diameter have been successfully bulk sorted on WOLF using a cell density between 250,000 and 500,000 protoplasts per milliliter culture medium. Stick to 100,000 protoplasts per milliliter for single-cell sorting. Sorting at higher protoplast densities increases risk of coincidence, which reduces sort purity.

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☐ **ADD A LOW LEVEL OF CELLULASE TO PROTOPLAST CULTURES PRIOR TO SORTING**

Plant protoplasts are known for clumping aggressively immediately after transfection due to stress or when they are generating new cell walls. For a successful protoplast sort, it is recommended to maintain a low level of digestive enzyme in the culture medium during the workflow to prevent cell clumping and cartridge clogging. The customer will need to test this as the concentration used will vary from crop to crop and if too much is used it may negatively impact callus formation and plant regeneration. A good starting point is 0.01-0.5% cellulase.

Clarification: if sorting same day as protoplast isolation, ensure the customer keeps a low level of cellulase in the culture medium. If sorting the following day (after a transfection event, for example), ensure that cellulase is included in the recovery culture medium overnight. **Cellulase will not function optimally if added the morning of the sort when protoplasts are already clumping.**

☐ **SET APPROPRIATE EXPECTATIONS AND ASK FOR MICROSCOPE VERIFICATION OF PROTOPLAST QUALITY PRE-SORT**

Sorting success relies on good quality samples. If viability is low or protoplasts are clumping, then the WOLF cannot function optimally. If cell viability is low communicate the impact of sample quality on the success of the sort to the customer. If the target is a transfected cell population (GFP+ for example), ensure that GFP fluorescence is detected under a fluorescence microscope prior to placing the samples on the WOLF. The same goes for protoplast integrity – do the protoplasts look healthy before the sort? Healthy protoplasts are spherical and cytoplasmically dense. The more information you have pre-sort, the better expectations you can set!

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DO NOT

☐ **USE PHOSPHATE BUFFER SALINE**
PBS has a low osmolarity (~200 mOsm). It will cause plant protoplasts to lyse. Raising the osmolarity of a PBS solution is also not recommended as the overall composition of PBS is not suitable for protoplast maintenance.

☐ **USE ANTIBIOTICS IN MEDIA**
To ensure sterility of the sort, the WOLF should be placed in a laminar flow hood or biosafety cabinet and the individual running the experiment should be versed in aseptic technique. The use of antibiotics to prevent microbial contamination may lead to pleiotropic effects downstream – for example: reduced cell division, microcallus formation, or regeneration of plants from calli. If the customer is adamant on using antibiotics in their workflows, and have successfully tested it, then allow it but suggest they do an antibiotic negative control to compare.

☐ **CHILL THE SAMPLES**
Plant protoplasts are very sensitive to temperature. Cold temperatures will induce aggregation, clumping, and settling (even with low levels of cellulase in the medium). The result is a cartridge clog. If a customer has had success with sorting of cold-treated protoplasts

☐ **VORTEX, PIPET VIGOROUSLY, OR STIR PROTOPLASTS TO KEEP THEM SUSPENDED**
Plant protoplasts are very sensitive to osmotic and manual stresses as they do not have cell walls. If the protoplasts start to settle during a sort, it is recommended to pause the sort, cap the tube and gently/slowly invert the tube a few times to resuspend the cells. Then continue the sort.

☐ **PLACE ALL THE FILTERED PROTOPLASTS INTENDED FOR SORTING INTO FACS TUBES ALL AT ONCE**
Protoplasts become stressed if stored in FACS tubes for extended periods of time before being sorted. It is suggested to move a maximum of 3.0 ml of protoplast suspension to a FACS tube at one time. Clients pursuing gene-editing workflows with protoplasts may need to perform longer sorts (like 3+ hours) depending on the percentage of their target population. Protoplast suspension that has not yet been used for sorting should stay in their original petri dish/culture dish to maintain the surface area ratio they are happiest in.

☐ **COMPARE RESULTS TO ANOTHER CELL SORTER DONE ON A DIFFERENT DAY**
Any comparison of the WOLF to other instrumentation or protocols should be performed concurrently with the same protoplast sample as the WOLF sort.

without clumping, suggest they run a room temperature control during the demo to compare.

☐ **ATTEMPT TO SORT PROTOPLASTS GREATER THAN 50 µm IN DIAMETER**
Our current cartridges can sort up to 50 µm. The only work around is for the customer to manipulate the osmolarity of their culture medium IF (and ONLY IF) the size is bordering acceptable. For example: 60-70 µm with adjustments to media osmolarity is possible. Greater than 70 um is not possible.

☐ **USE VIABILITY DYES FOR WORKFLOWS THAT INCLUDE REGENERATION OF PLANTS FROM PROTOPLASTS**
The compatibility of live/dead cell staining should be evaluated for effects due to long-term exposure. Literature states that viability dyes can have negative effects on downstream cell division, microcallus formation and plant regeneration. In addition, cell-permeant dyes, such as Fluorescein Diacetate (FDA) will bleed into all living and dead cells over time. Sorts that include FDA must be done quickly otherwise there will be no difference in live/dead identification.

REVISION HISTORY:

	ISSUED DATE	Description of Change	Author
(A)	(02/09/2023)	Playbook for a successful plant protoplast demo – V1.0	D.Moellering
(B)	(10/26/2023)	Playbook for a successful plant protoplast demo – V1.0	D.Moellering