CASE STUDY

Cloning of Three Cell Types Comparing the Namo™ Single Cell Dispenser to Flow Cytometry and Limited Dilution Cloning

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INTRODUCTION

The FDA requires commercial biotherapeutic manufacturing to use an expression cell line derived from a single cell or clone [1]. Limiting dilution cloning (LDC) has been the standard method established for generating clonal cell lines, based on statistical data [2,3], however it is time-consuming and can be inefficient. Recent advances in imaging technology have provided the ability to directly visualize proof of clonality, however LDC, when used with imaging technology, can result in too few or too many cells in each of the wells. A Fluorescence-activated Cell Sorter (FACS) can dispense a single cell in each well of a plate [3], but high fluidics pressure can reduce cell viability and FACS devices are very costly. The Namo™ Single Cell Dispenser was designed by Namocell® to circumvent some of these disadvantages by seeding a single cell into each well of a plate, using much lower fluidics pressure [4], resulting in a higher number of wells containing viable single cells. Here we compared each of these methods, using different cell lines, to determine which method would deliver the highest number of viable clones. The results of our study show the Namo™ Single Cell Dispenser delivered the highest number of wells containing viable single cells when compared to LDC or FACS for all cell lines included.

MATERIALS AND METHODS

Figure 1. The Namo™ Single Cell Dispenser.

Cell Culture. *Experiment 1.* Freedom™ CHO-S™ cells, ExpiCHO-S™ cells, and Hybridoma cells were provided for this study by Cytovance Biologics. Each cell line was thawed and cultured for at least 2 to 3 passages in shake flasks prior to cloning plate seeding. Cells were maintained between 0.5-3.0 x 10^6 viable cells/mL. The culture and cloning media for each cell line is presented in Table 1. All culture media was supplemented with 8 mM L-glutamine. For plating, 3 x 384-well plates were pre-filled with cloning medium and allowed to equilibrate for 10 minutes prior to seeding. Post-plating, seeded plates were returned to the CO_2 incubator for 2 hours prior to Day 0 imaging. Imaging was performed on days 1, 2, 3, 7, 10, and 14 to track clonal outgrowth.

Experiment 2. A monoclonal antibody (mAb)-producing clone derived from the Freedom CHO-S host cell line itself was utilized in this experiment. Both cell lines used the same medium (Table 1). Plates were seeded as described in *Experiment 1*, with the exception that the cells were seeded in a 96-well plate and a FACS Aria Fusion™ was used for the FACS method. Working volume for each well was 200 µL. Post-plating, seeded plates were returned to the CO_2 incubator for 2 hours prior to imaging. Imaging schedule was the same as in *Experiment 1*.

Cell Plating: Once cells were in log phase for at least two passages, each culture was counted using the ViCell-XR™ and split into three separate tubes for each plating method. For plating, plates were pre-filled with cloning medium and allowed to equilibrate for 10 minutes prior to seeding. Post-plating, seeded plates were returned to the CO_2 incubator for 2 hours prior to imaging.

Namocell Method: Cells were run through a Millipore™ Steriflip™, then stained with Calcein AM (CAM) for 10 minutes at room temperature to sort by viability. Stained cells were diluted to 5,000 cells/mL and 700 µL of the diluted suspension was loaded into a Namocell microfluidics chip. The chip was loaded into the Namocell instrument and cells were processed at approximately 2.5 cells per second to seed one cell/well.

LDC Method: Cells were diluted 1:100 to target a seeding density of 0.5 cell/well. A total of 576 viable cells were taken from the 1:100 dilution and loaded

Table 1: Culture and Cloning Media for Each Cell Type

Cell Type	Culture Medium	Cloning Medium
Freedom™ CHO-S™	FortiCHO™	Sigma's ExCell Cloning Medium™ Supplemented with 10% Conditioned growth medium and 6mM L-glutamine
ExpiCHO-S™	ExpiCHO Expression Medium™	ExpiCHO Expression Medium Supplemented with 10% Conditioned growth medium and 6mM L-glutamine and 1x ClonaCell™ Supplement
Hybridoma	CD Hybridoma™	CD Hybridoma Supplemented with 10% Conditioned growth medium and 6mM L-glutamine
mAb Clone	FortiCHO™	Sigma's ExCell Cloning Medium™ Supplemented with 10% Conditioned growth medium and 6mM L-glutamine

into a media bottle containing 90 mL of cloning medium. The cell suspension was lightly mixed and then loaded into a sterile trough for transfer into cloning plates.

FACS Method: Cells were run through a Millipore Steriflip, then transferred, along with the pre-filled plates, to the Oklahoma University Health Science Center Flow Cytometry Core Facility. The FACS device(s) were pre-set to dispense one cell per well using no stain. After seeding, plates were transferred back to Cytovance. Of technical note, the Aria Fusion operates under a slightly lower fluidics pressure (20psi) than the FACS Jazz (27psi).

Imaging: Following seeding, cells were imaged using the Solentim CellMetric™ on Days 0, 1, 2, 3, 7, 10, and 14. Final images on Day 14 represent the time point when clones would be assayed and expanded.

RESULTS

Experiment 1: As shown in Figures 2 and 4, plating and cloning efficiency varies greatly between cell types and the cloning method employed.

Freedom CHO-S had an expected high cloning efficiency compared to the other two cell lines in *Experiment 1*. Using the Namocell, Freedom CHO-S exhibited a two-fold increase in cloning efficiency compared to the other two cell types (Figure 4). Namocell outperformed the other two methods in the Freedom CHO-S cells with a more than three-fold increase in efficiency over LDC and a 25-fold increase over FACS (Figure 4).

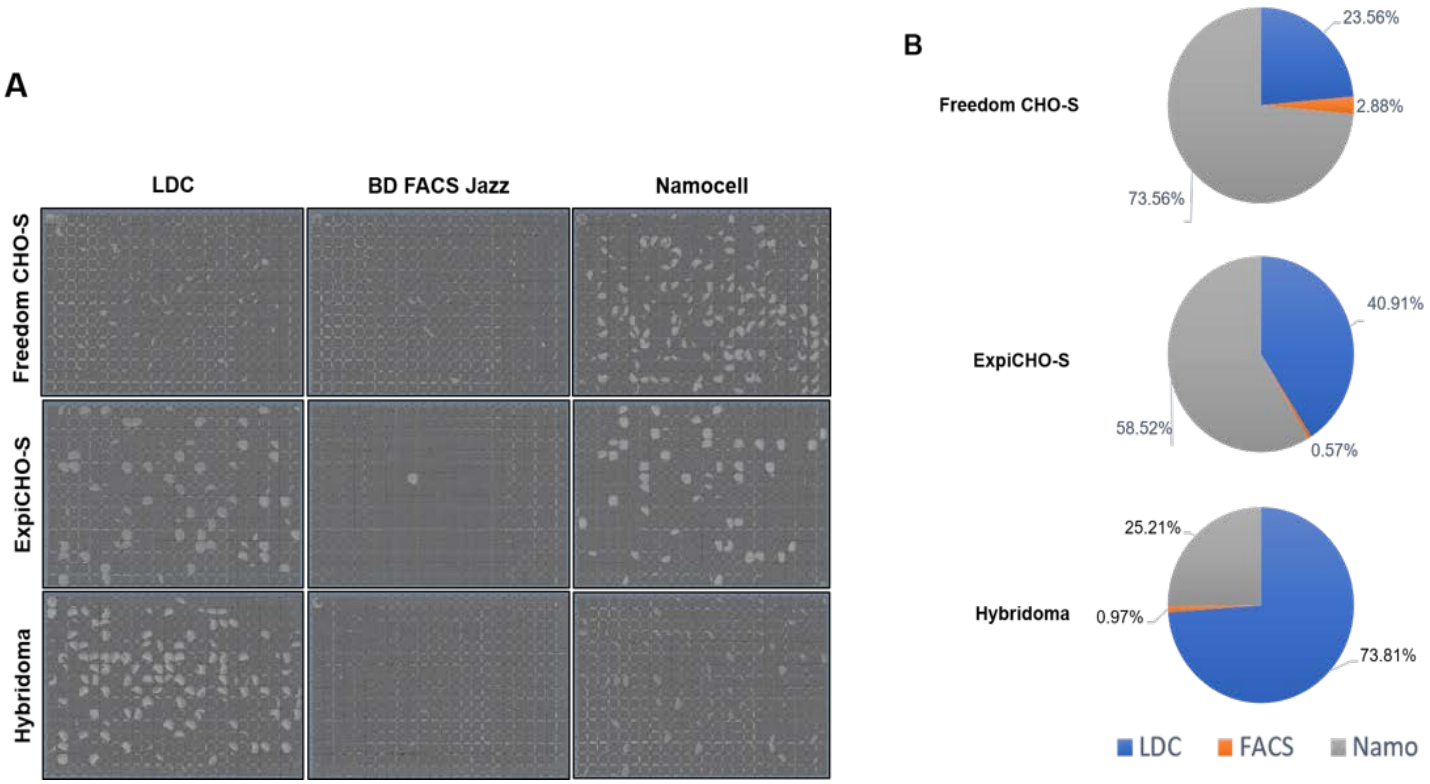


Figure 2. Clonality tracking for *Experiment 1*. **A.** Images were obtained using a Solentim CellMetric™ on Day 14 to track clonality for each of the methods and cell lines indicated. **B**. The percentage of clones derived from each of the methods on Day 14.

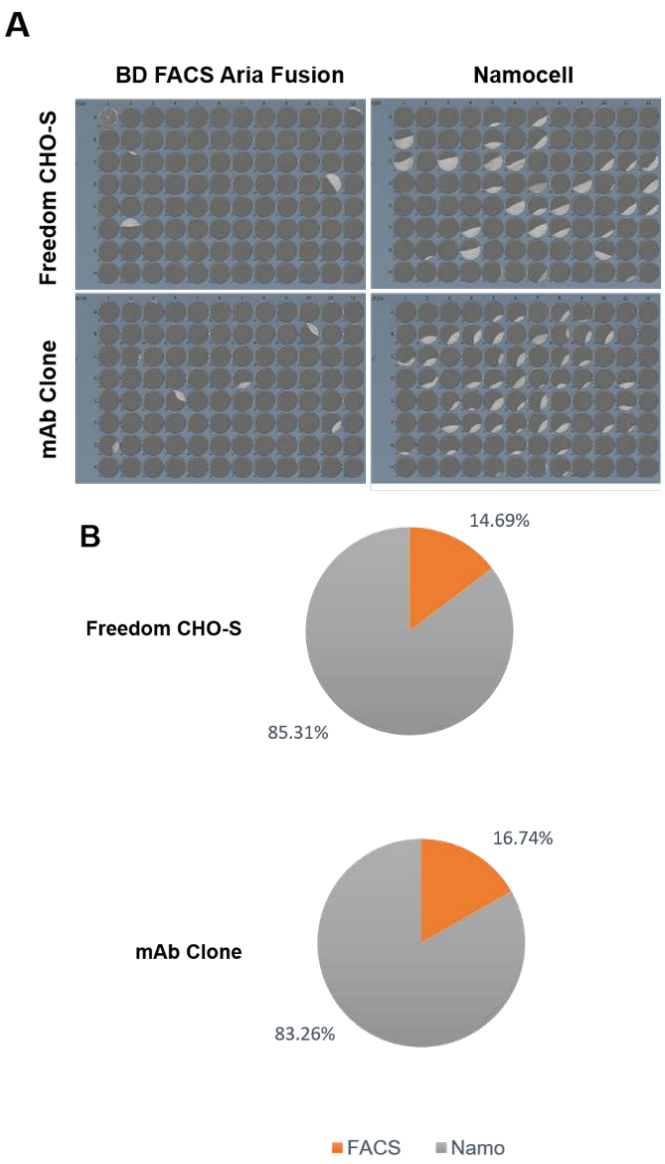


Figure 3. Clonality tracking for Experiment 2. **A.** Images were obtained on Day 14 to track clonality for each of the methods and cell types indicated. **B**. The percentage of clones derived from each of the methods.

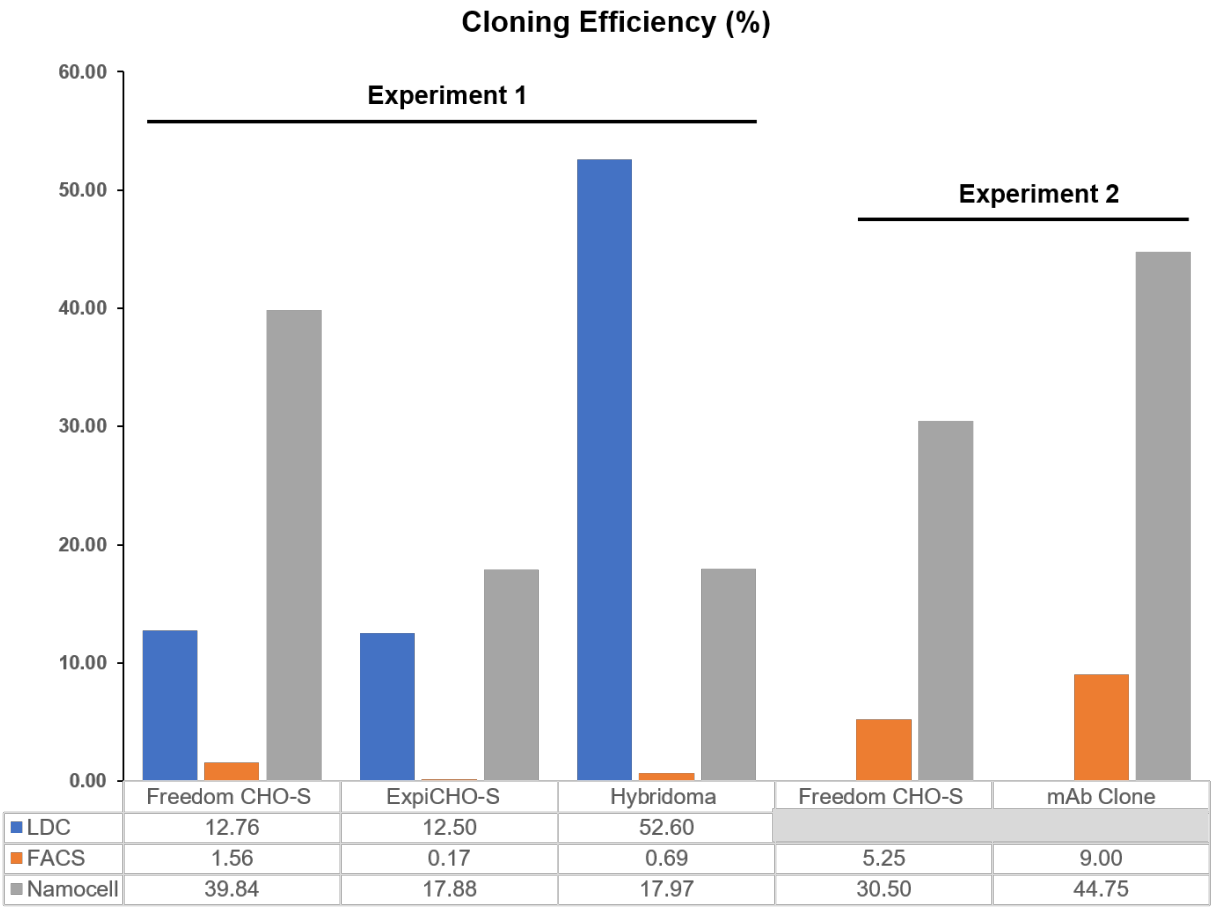
The ExpiCHO-S cells that are difficult to grow from clonal densities, had a cloning efficiency of 17.88% with the Namocell compared to 12.50% by LDC and 0.17% by FACS (Figure 4). As indicated in Table 2, the single cell seeding efficiency was comparable between the automated methods at 62.76% and 68.84% respectively. However, the single cell plating efficiency was low for the LDC method at 26.91%, indicating a possible count or dilution error.

The Hybridoma cells had a cloning efficiency of 17.97% with the Namocell compared to 61.28% by LDC and 0.69% by FACS (Figure 4). Single cell seeding efficiency was comparable between all methods at 73.78%, 59.46%, and 75.00% respectively, however it was unusually high for the LDC method, at 61.3%, indicating the possibility of an under-count or dilution error (Table 2).

Experiment 2: In the second experiment, the two cell lines tested are more closely related to each other than the lines tested in *Experiment 1* with the mAb clone being derived from the Freedom CHO-S parent line. Both cell lines had similar plating efficiencies (Figure 3). The only notable difference, as seen in Figure 3A, is the parent cell grew at a faster rate than the mAb clone. The cloning efficiency for the FACS Aria plates yielded far fewer viable clones than the Namocell (Figure 4). The total number of viable clones can be seen in Table 2. In general, the cloning efficiencies observed in *Experiment 2* were higher than the first trial with Namocell having a cloning efficiency range of 30.50% to 44.75%, while FACS Aria ranged from 5.25% to 9.00%. FACS Aria outperformed FACS Jazz with the Freedom CHO-S cell line by approximately 3-fold. Conversely, the Namocell was consistent between experiments with a 39.84% cloning efficiency value in *Experiment 1* and 30.50% in *Experiment 2* for the same cell line (Figure 4).

CONCLUSIONS

The Namo™ Single Cell Dispenser outperformed both FACS and LDC with respect to cell recovery and overall clonal cell numbers for each cell type tested and in multiple experiments regardless of plate size. LDC yielded a useful number of single cell clones in all three cell types but lacked the consistency of automated systems like Namocell. As illustrated by the large swing in cloning efficiency values from 12.5% to 52.6%, limiting dilution cloning depends strongly on cell counts and extensive mathematical factors which can introduce a significant amount of



	Freedom CHO-S	ExpiCHO-S	Hybridoma	Freedom CHO-S	mAb Clone
LDC	12.76	12.50	52.60		
FACS	1.56	0.17	0.69	5.25	9.00
Namocell	39.84	17.88	17.97	30.50	44.75

Figure 4. Cloning efficiency. Cloning and single-cell efficiencies were determined using the day 0 images on the Solentim CellMetric within two hours of seeding. *Experiment 1* utilized a FACS Jazz instrument and *Experiment 2* utilized a FACS Jazz instrument.

Table 2: Cloning Efficiency Summary

Cell Type	Plating Method	# Single Cell Depositions	# Multi-Cell Depositions	Clones Recovered	Single Cell Efficiency %
Freedom CHO-S	LDC	249	84	147	21.61
	FACS	647	135	18	56.16
	Namocell	816	144	459	70.83
ExpiCHO-S	LDC	310	75	144	26.91
	FACS Jazz	723	145	2	62.76
	Namocell	793	169	206	68.84
Hybridoma	LDC	750	303	606	65.10
	FACS Jazz	685	174	8	59.46
	Namocell	864	129	207	75.00
Freedom CHO-S	FACS Aria	30	3	21	7.50
	Namocell	141	57	122	35.25
mAb Clone	FACS Aria	45	2	36	11.25
	Namocell	197	49	179	49.25

error. This error can be inflated depending on how the cell counts are performed. The FACS Jazz and Aria yielded very little clonal outgrowth during the normal two weeks of incubation time from seed to assay. This low output is most likely due to the higher fluidics pressure (fixed at 27 psi for Jazz and 20 psi for Aria) that the cells must tolerate during the flow process. Cells are likely subject to a high level of shear force during the sorting and dispensing steps. The Namo™ Single Cell Dispenser utilizes a fluidics pressure of approximately 2 psi allowing for a much gentler seeding of cloning plates [4]. Overall, The Namo™ Single Cell Dispenser provides an easy-to-use platform, for single cell dispensing, that generates superior clonal recovery over a range of cell types compared to other methods.

REFERENCES

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