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C.NEST | A novel microbioreactor shortens the single-cell cloning timeline with the single-cell dispenser UP.SIGHT

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Abstract

Generating the best stable cell lines for biologics production is crucial for biopharmaceutical and biotech companies. High-producing stable cell lines can ensure the quality and quantity of the products and efficiently reduce costs. To establish the finest stable cell lines, researchers must perform single-cell cloning (SCC) to assure the cell line originated from a single parental cell. However, SCC is a time-consuming and labor-intensive procedure when performed manually. Here, we have designed a novel SCC workflow using CYTENA's UP.SIGHT single-cell dispenser along with the C.NEST microbioreactor to significantly increase throughput and reduce the timeline, thereby helping researchers find better clones more efficiently and at lower costs.

Introduction

Single-cell cloning (SCC) is a critical step in generating stable cell lines, which are widely used for producing therapeutic biologics. As the global market for therapeutic biologics has grown rapidly [1], the demand for efficient SCC devices has also increased. Optimal solutions for SCC offer higher throughput, proof of clonal derivation, better single-cell dispensing efficiency and outgrowth efficiency, as well as shorter expansion time [2]. These solutions allow researchers to increase the chance of establishing high-producer cell lines that are ready for regulatory review [3, 4]. In this study, we have introduced the UP.SIGHT single-cell dispenser integrated with the C.NEST microbioreactor as an optimal solution for SCC.

The UP.SIGHT is a single-cell dispenser that enables cell isolation based on cell morphology and/or fluorescence using high-resolution imaging of the dispensing nozzle. With its gentle single-cell isolation technology, high single-cell dispensing efficiencies and outgrowth efficiencies can be achieved. The UP.SIGHT also provides a double proof of clonal derivation by confirming single-cell dispensing through nozzle images and 3D Full Well images acquired directly after cell dispensing **(Figure 1A)**. Using these two independent imaging systems, the UP.SIGHT provides a probability of clonal derivation of >99.99% (Application Note-UP.SIGHT single cell dispenser) [5, 6]. After single-cell dispensing, the UP.SIGHT can also be used as plate imager to easily track colony growth **(Figure 1B)**.

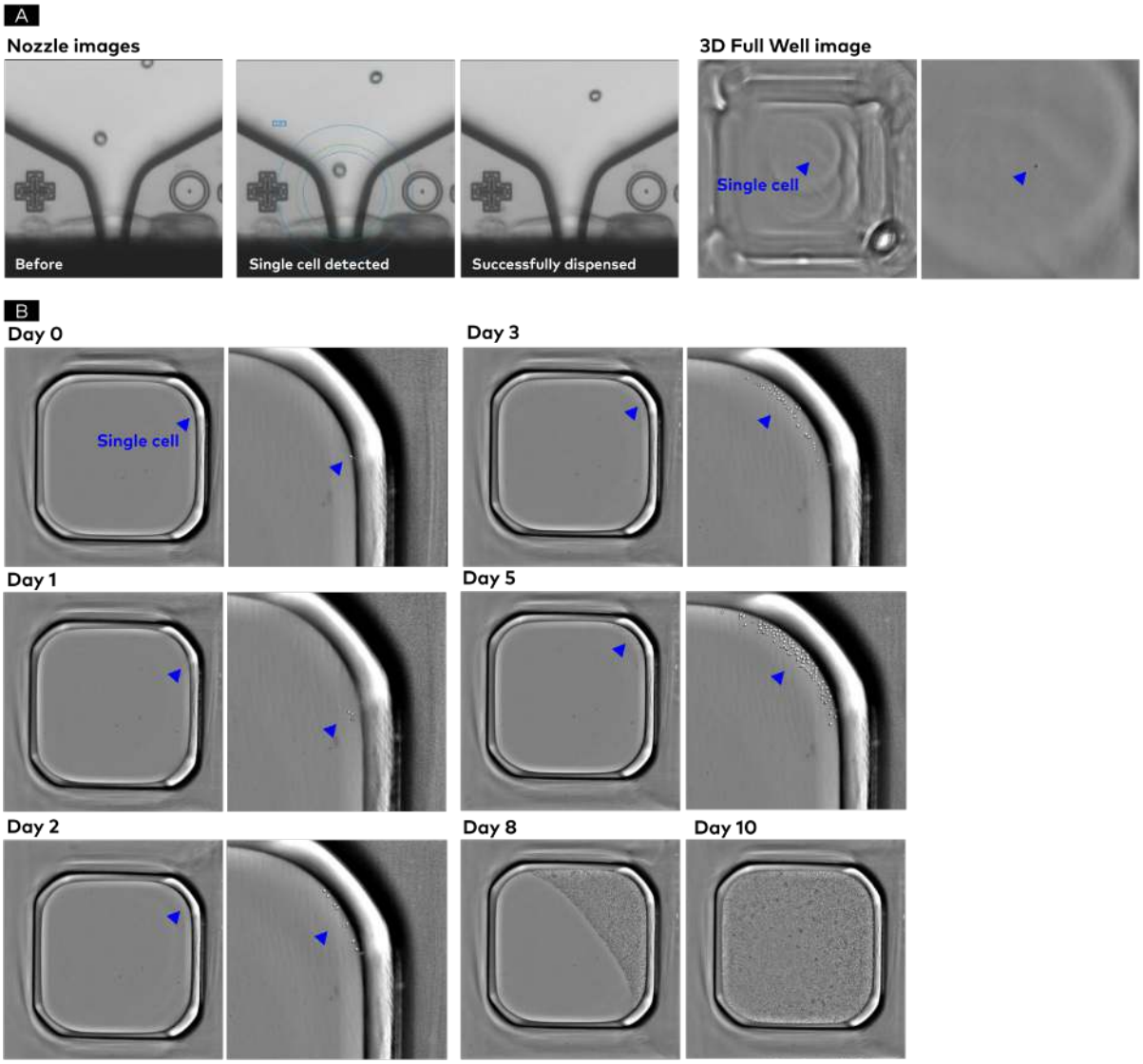

Figure 1. *(A) The UP.SIGHT provides a double proof of clonal derivation by confirming single-cell dispensing through nozzle images and 3D Full Well images acquired directly after cell dispensing. (B) Colony tracking images taken by the UP.SIGHT.*

The C.NEST is a microbioreactor that contains four independent chambers for cell culture **(Figure 2A)**. Each chamber contains UV lights for sterilization, CO_2 and humidity sensors for environmental monitoring, and heating modules on the top and bottom of the chambers for temperature adjustment **(Figure 2B)**. The C.NEST provides multiple options for cell culture with microplates. The standard microplate with a standard lid or a C.NEST lid can undergo static or mixing culture in the system **(Figure 2C)**. All the culture conditions can be set through the device's intuitive software, which can control up to four system customized C.NEST **(Figure 2D)**. The C.NEST's mixing culture improves the oxygen transfer rate and homogenizes the medium composition in each well. Our previous application notes have demonstrated that mixing culture can enhance cell growth and protein production and has great potential to shorten SCC workflows (C.BIRD Improving the Single-Cell Cloning Workflow) [7].

In this application note, Chinese Hamster Ovary (CHO-S) cells served as the model cell line and were dispensed by the UP.SIGHT or through a limiting dilution method and further cultured with the C.NEST or other culture methods to compare the timelines of different workflows. We demonstrated single-cell dispensing using UP.SIGHT can significantly improve the outgrowth efficiency compared to the limiting dilution method. Moreover, the UP.SIGHT-C.NEST culture workflow can save seven days, which is about 30% time compared to other SCC workflows.

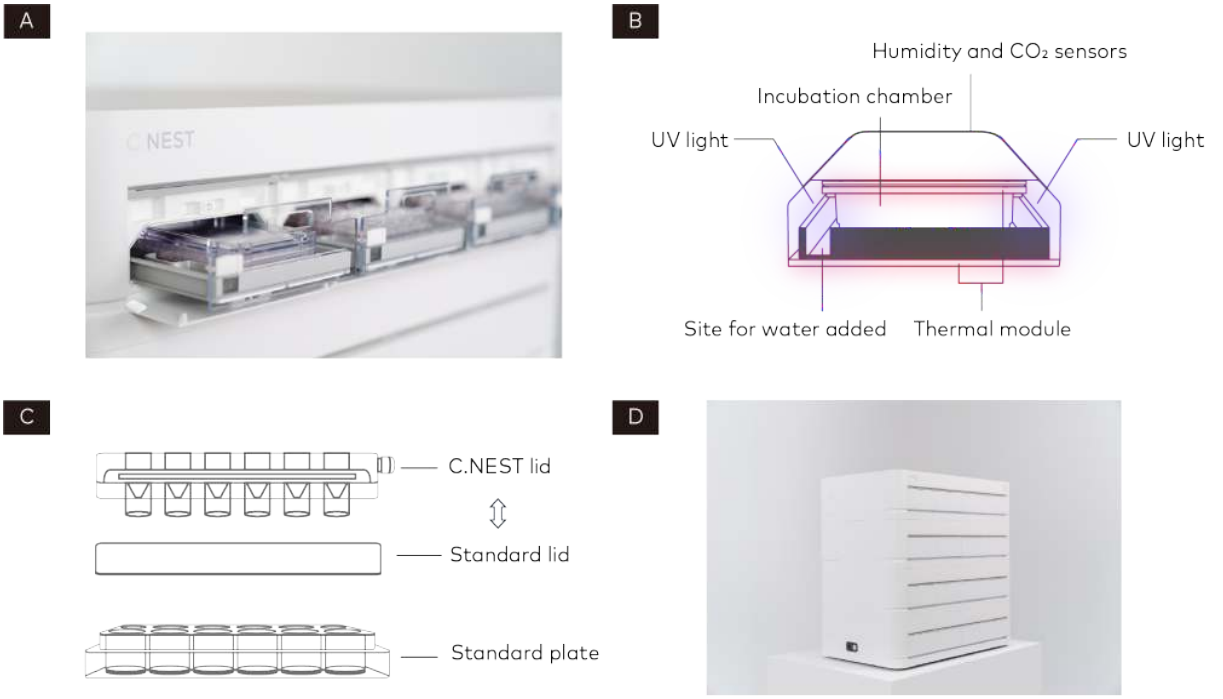

Figure 2. (A) *The C.NEST culture system contains 4 independent chambers.* ***(B)*** *A schematic diagram of environmental settings in the C.NEST.* ***(C)*** *The C.NEST supports static culture with a standard lid and mixing culture with the C.NEST lid.* ***(D)*** *The C.NEST software can control up to four-system settings in the instrument at the same time.*

Materials and methods

Cell, medium and culture conditions

A monoclonal antibody (mAb)-expressing CHO-S cell line was used in this study. The cells were cultured in CD FortiCHO medium (Gibco, #A1148301) supplemented with Penicillin-Streptomycin (Gibco, #15140122) and 4 mM L-glutamine (Corning, 25-005-CI) except for the single-cell dispensing step in 384-well plate culture. The cells were cultured in either a humidified standard incubator or the C.NEST with 5% CO2 at 37°C. In this study, the volume of 384-well plate (Corning, #3680), 96-well plate (Corning, #3599), 24-well plate (Greiner, #662160), 12-well plate (Corning, #3513), 6-well plate (Corning, #3516), and the TubeSpin (TPP, #Z761028) were 80 µl, 200 µl, 1.4 ml, 2 ml, 3 ml, and 15 ml, respectively. The shaking rate of the shake plate culture and the TubeSpin culture were 130 rpm and 220 rpm with an orbital shaker (AS ONE, COSH6), respectively. The shaking diameter was 19 mm.

Scaling-up workflow design

In this study, we compared the required time of different single-cell cloning workflows. The experiment was designed as shown in **Figure 3**. In the UP.SIGHT-C.NEST culture workflow, single cells were dispensed by the UP.SIGHT and statically cultured in the C.NEST for 10 days. The cells were then transferred to the 96-well plates, lidded with the C.NEST 96-well lid and cultured in the C.NEST with 50 seconds/cycle of continuous mixing until the cell number was sufficient to scale up to 24-well plates. Once transferred to 24-well plates (Greiner, #662160) and lidded with the C.NEST 24-well lid, the cells were cultured in the C.NEST with 10 seconds/cycle of continuous mixing until the cell number was sufficient to scale up to the TubeSpin.

Both the UP.SIGHT-static culture workflow and the UP.SIGHT-shake plate culture workflow were initiated from the single cell dispensed by the UP.SIGHT into 384-well plates. The cells were then statically cultured in the standard incubator for 10 days and then transferred to 96-well plates and kept in static culture until the cell number was sufficient to scale-up to 24-well plates. In the UP.SIGHT-static culture workflow, the cells were statically cultured in 24-well plates and scaled-up to 6-well plates until the cell number was enough to scale up to the TubeSpin. In the UP.SIGHT-shake plate culture workflow, cells in the 24-well plates were switched to shaking condition (130 rpm, diameter: 19 mm) and then scaled up to 12-well plates with the same shaking condition until the cell number was enough to scale up to the TubeSpin.

In the traditional approach, we used the limiting dilution method to seed single cells into 384-well plates and statically cultured in the standard incubator. Cells were then further scaled up to the 96-well plates, 24-well plates and 6-well plates with the same static culture condition until the cell number was enough to scale up to the TubeSpin.

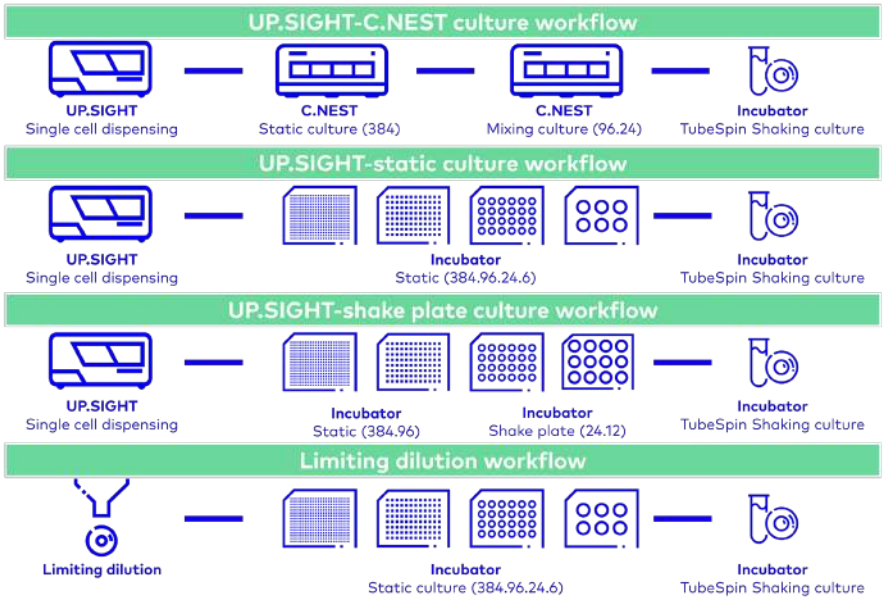

Figure 3. *Experimental design of the four tested single-cell cloning workflows.*

Single-cell dispensing and limiting dilution

In this study, we used the UP.SIGHT single-cell dispenser and limiting dilution method to obtain single cells in 384-well plates (Corning, #3680) and compared the outgrowth efficiency after 10 days of static culture for each method. Furthermore, we performed growth tests during the upscaling process for each tested workflow. The single-cell cloning medium was composed of EX-CELL CHO Cloning medium (Merck, #C6366) supplemented with 20% conditioned medium and 4 mM L-glutamine (Corning, 25-005-CI). The volume was 80 µl per well. Cells were diluted in the single-cell cloning medium at a concentration of 5 x 10^5 cells/ml. Single cells were dispensed into 384-well plates using the UP.SIGHT. Another single-cell isolation method in this study was the limiting dilution method. We diluted the cell suspension to 0.5 cells per well with single-cell cloning medium and seeded into 384-well plates. The single cells dispensed by the UP.SIGHT were confirming through nozzle images and 3D Full Well images. Furthermore, the plates were briefly centrifuged, and all wells were imaged with the UP.SIGHT to confirm clonality for further analysis after single-cell seeding.

Cell imaging and single-cell outgrowth analysis

After seeding the cells into 384-well plates with the UP.SIGHT or the limiting dilution method, the plates were incubated either in the standard incubator or the C.NEST. One of the plates cultured with the C.NEST was taken out on days 1, 2, 3, 5, 8, 9 and 10 to take cell images and demonstrate the UP.SIGHT's colony tracking function. On day 10, the 384-well plates from all groups were imaged using the UP.SIGHT. The cell confluency on day 10 was classified as level 1, 2, 3 and 4, which represent less than 10%, 10-40%, 40-70% and greater than 70% confluency, respectively. The cell numbers of each confluency level were calculated using an automated cell counter (CURIOSIS, #FACSCOPE B). All wells in all groups were observed, classified, and analyzed following the confluency level. The outgrowth efficiency was calculated by dividing the number of wells containing a single-cell derived colony on day 10 with the total number of eligible wells (wells used for single-cell dispensing).

Single-cell cloning workflow analysis

The comparison test for the required time of each single-cell cloning workflow was previously described. The cell numbers and viabilities were counted at indicated days using the automated cell counter. The criteria for transferring the cells to the TubeSpin with 15 ml culture medium was 2.25 x 10^6 viable cells per clone, and the minimum initial cell density of the TubeSpin culture was 1.5 x 10^5/ml. Afterwards, the cells were transferred to the TubeSpins and cultured for 4 days. Cell density and viability were analyzed with an automated cell counter and the doubling time was further calculated based on the analysis. Statistics were performed by multiple unpaired t-tests. Significance of *p* value is listed as follows: > 0.05 (ns), < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****). Data are shown as mean ± SD.

Results and discussion

Outgrowth efficiency and colony analysis

The CYTENA single-cell dispenser had proved that it has a significantly higher clonal outgrowth rate compared to the limiting dilution method. In this study, we replicated this result using the UP.SIGHT. We compared the outgrowth efficiency between three single-cell cloning workflows: The UP.SIGHT-C.NEST culture workflow, the UP.SIGHT workflows (UP.SIGHT-static workflow/ UP.SIGHT-shake plate workflow) and the limiting dilution workflow. The outgrowth efficiency was calculated by dividing the number of wells containing a single-cell derived colony on day 10 with the total number of eligible wells. The outgrowth efficiency was 62.9%, 51.9% and 6.7% in the UP.SIGHT-C.NEST culture workflow, the UP.SIGHT workflows and the limiting dilution workflow, respectively **(Table 1)**.

We further classified the colonies into 4 levels according to the cell confluency **(Figure 4A)**, chose some wells in each cell confluency level and counted the cell number and viability. The average viable cell numbers were 2.6 x 10^3, 8.9 x 10^3, 1.94 x 10^4 and 4.96 x 10^4 cells/well of cell confluency levels 1 to 4, respectively **(Figure 4B)**. The average cell viabilities were 82.2%, 85.5%, 95.0% and 95.1% of cell confluency in levels 1 through 4, respectively **(Figure 4B)**. Table 2 shows the cell growth percentage of each level. Several wells from cell confluency level 3 and level 4 were chosen to transfer to 96-well plates and cultured following the workflow comparison test design.

Overall, The UP.SIGHT-C.NEST culture workflow group had the highest outgrowth efficiency in level 3 and 4 **(Table 2)** likely because of more stable conditions provided by the C.NEST compared to the incubator, which was opened frequently by other users.

Dispensing method	Culture method	Outgrowth efficiency (%)
UP.SIGHT	C.NEST	62.9%
UP.SIGHT	Incubator	51.9%
Limiting dilution	Incubator	6.7%

Table 1. *The outgrowth efficiency after single-cell dispensing using the UP.SIGHT or limiting dilution and 10 days of static culture in the C.NEST (UP.SIGHT-C.NEST workflow) or standard incubator (UP.SIGHT-static workflow, UP.SIGHT-shake plate workflow and Limiting dilution workflow). The outgrowth efficiency was calculated by dividing the number of wells containing a single-cell derived colony on day 10 with the total number of eligible wells.*

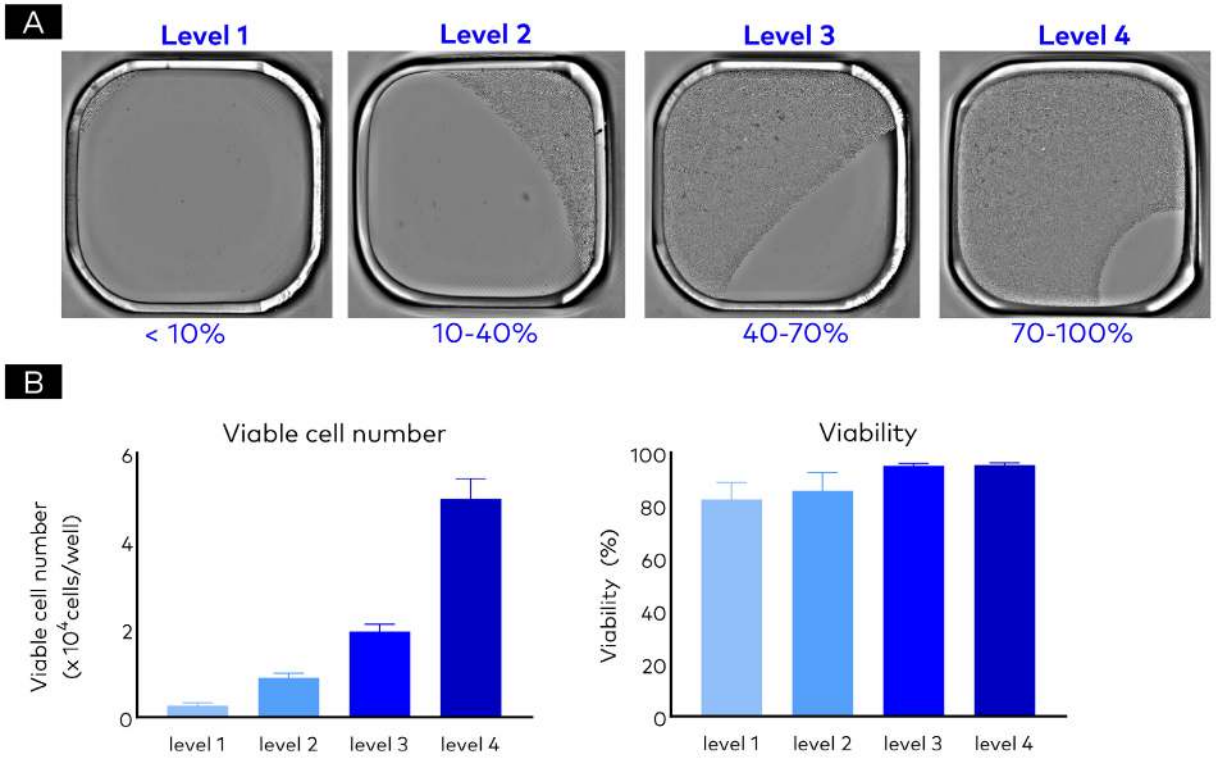

Figure 4. *Cell confluency, corresponding viable cell number and viability of colonies in 384-well plates after 10 days of static culture of all tested workflows.* ***(A)*** *Representative images of different cell confluency levels.* ***(B)*** *The average viable cell numbers and viability of the clones from different cell confluency levels.*

Outgrowth efficiency (%)						
Dispensing method	Culture method	Confluency level				Total
		Level 4	Level 3	Level 2	Level 1	
UP.SIGHT	C.NEST	19.4%	15.4%	19.5%	8.6%	62.9%
UP.SIGHT	Incubator	7.2%	11.8%	20.8%	12.1%	51.9%
Limiting dilution	Incubator	2.1%	1.8%	1.0%	1.8%	6.7%

Table 2. *The outgrowth efficiency of the clones with respective confluency levels in different workflows. Confluency was determined after single-cell dispensing using the UP.SIGHT or limiting dilution and 10 days of static culture in the C.NEST (UP.SIGHT-C.NEST workflow) or standard incubator (UP.SIGHT-static workflow, UP.SIGHT-shake plate workflow and Limiting dilution workflow)*

Scale-up process analysis

96-well plate culture

For all tested workflows, single cells were seeded into 384-well plates using the UP.SIGHT or limiting dilution and then cultured for 10 days in static conditions. Colonies with a confluency level of 3 or 4 were chosen to be scaled up to 96-well plates

In the UP.SIGHT-C.NEST culture workflow, the cells were cultured in 96-well plates with the C.NEST 96-well lid. The mixing condition was set to continuous mixing mode with a mixing rate of 50 seconds/cycle and cultured for 4 days. After culturing with the C.NEST, the average viable cell number and viability was 5.20 x 10^5 cells/clone and 90.3% **(Figure 5, green bar)**. In both the UP.SIGHT-static culture and UP.SIGHT-shake plate culture workflows, the 96-well plate culture was set up in static condition. Therefore, the two workflows shared the same average viable cell number and viability, which was 2.78 x 10^5 cells/clone and 88.8% **(Figure 5, blue bar)**. As for the limiting dilution workflow, the average viable cell number and viability of 96-well plate cultures was 2.53 x 10^5 cells/clone and 89.5% **(Figure 5, dark blue bar)**.

24-well plate culture

In the UP.SIGHT-C.NEST culture workflow, the cells were transferred to 24-well plates after 4 days of 96-well plate culture, covered with the C.NEST 24-well lids and further cultured for 3 days. The C.NEST culture condition was set to continuous mixing mode with a mixing rate of 10 seconds/cycle. After 3 days of culture in 24-well plates (Day 17), the average viable cell number and viability were 2.46 x 10^6 cells/clone and 89.4%. In this group, the viable cell numbers were enough to transfer to TubeSpin culture with 15 ml culture medium and the seeding density of TubeSpin cultures were all above 1.5 x 10^5 cells/ml. On the same day, the cell number and viability of the UP.SIGHT-static culture workflow, UP.SIGHT-shake plate culture workflow and limiting dilution workflow groups were also counted. Their average viable cell numbers and viability were 2.8 x 10^5, 4.7 x 10^5 and 4.3 x 10^5 cells/clone **(Figure 6A)** and 75.6%, 75.6% and 78.2% **(Figure 6B)**, respectively. The viable cell numbers in these groups were not enough to transfer to TubeSpin, hence the cells were cultured in 24-well plates for additional 4 days to obtain enough cell numbers to scale up.

On day 21, after another 4 days of culturing in the 24-well plates, the cell number and viability were counted for all workflows except for the UP.SIGHT-C.NEST workflow. The average viable cell number and viability of the UP.SIGHT-static culture workflow, the UP.SIGHT-shake plate culture workflow and the limiting dilution workflow groups were 1.08 x 10^6 cells/clone and 86.0%, 9.7 x 10^5 cells/clone and 84.6%, and 1.04 x 10^6 cells/clone and 85.1%, respectively **(Figure 6C, 6D)**. Since the average viable cell number of these three groups had not reached cells/clone required for TubeSpin scale up, the cells needed to be scaled up to other large-scale plates. Therefore, we transferred the cells from the UP.SIGHT-static culture workflow group and the limiting dilution culture workflow group to 6-well plates and kept culturing. The cells from the UP.SIGHT-shake plate culture workflow group were transferred to 12-well plates for further culturing.

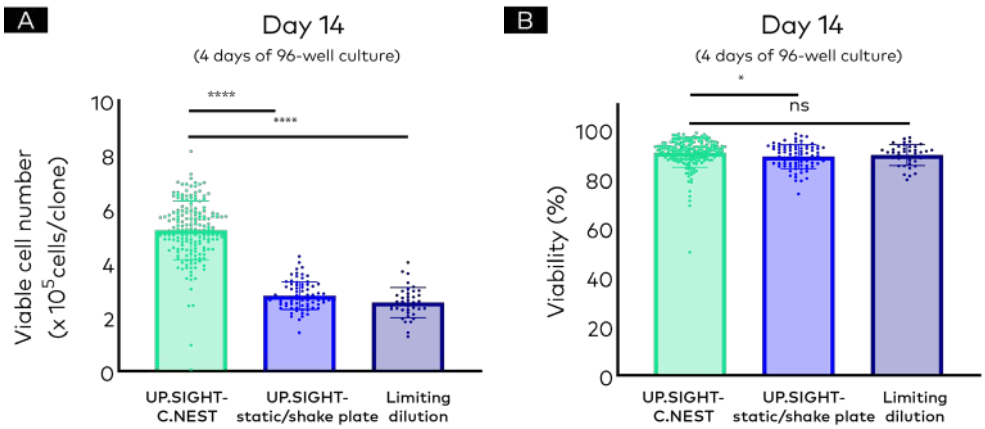

Figure 5. *The* ***(A)*** *viable cell number and* ***(B)*** *cell viability of different workflows on day 14, after upscaling from 384-well plates to 96-well plates, and further cultured for 4 days in 96-well plates.*

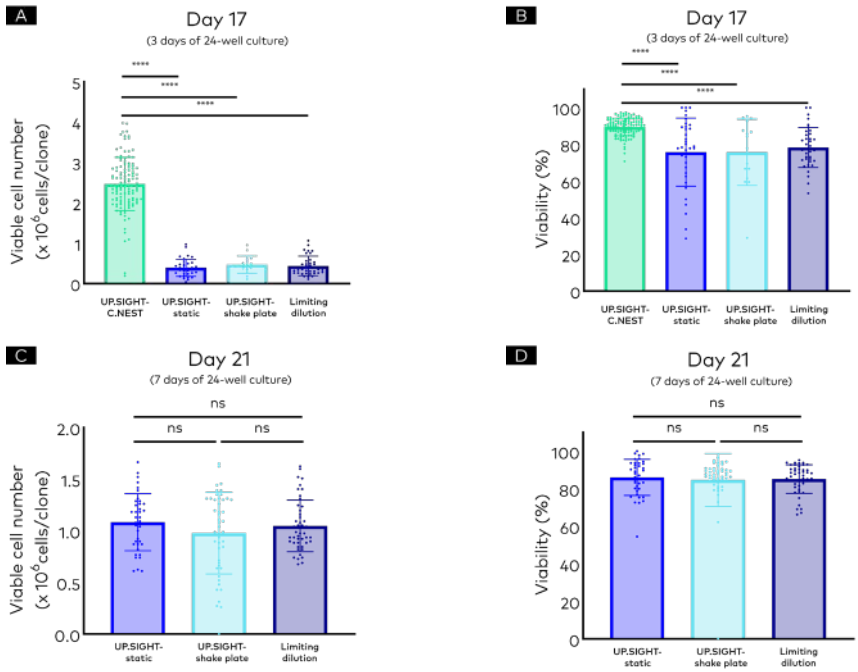

Figure 6. *The viable cell number and cell viability of different workflows on* ***(A, B)*** *day 17, after upscaling from 96-well plates to 24-well plates and further cultured for 3 days in 24-well plates and* ***(C, D)*** *day 21, after upscaling from 96-well plates to 24-well plates and further cultured for 7 days in 24-well plates.*

6 or 12-well plate culture

Since the cell numbers in the UP.SIGHT-static culture workflow group, the UP.SIGHT-shake plate culture workflow group and the limiting dilution group were not sufficient to scale up to the TubeSpin, the cells in these three groups were transferred to either 6- or 12-well plates and cultured for another 3 days. On day 24, the cell number and viability were determined. The average viable cell number of these three groups were 3.93 x 10^6, 7.25 x 10^6 and 4.14 x 10^6 cells/clone, respectively **(Figure 7A)**. The average cell viability of these three groups was 83.9%, 94.4% and 88.7%, respectively **(Figure 7B)**. The cells were then scaled up to the TubeSpin culture.

TubeSpin culture

In this study, all workflow groups were finally transferred to the TubeSpin and cultured for 4 days. To evaluate the cell growth ability of each group, we recorded the initial viable cell densities and the final viable cell densities after 4 days of culture in the TubeSpin. Based on the cell densities, we calculated the doubling time of each group. The doubling time of the UP.SIGHT-C.NEST culture, the UP.SIGHT-static culture, the UP.SIGHT-shake plate culture and the limiting dilution workflow group were 21.36, 21.34, 22.90 and 21.93 hours, respectively **(Figure 8)**.

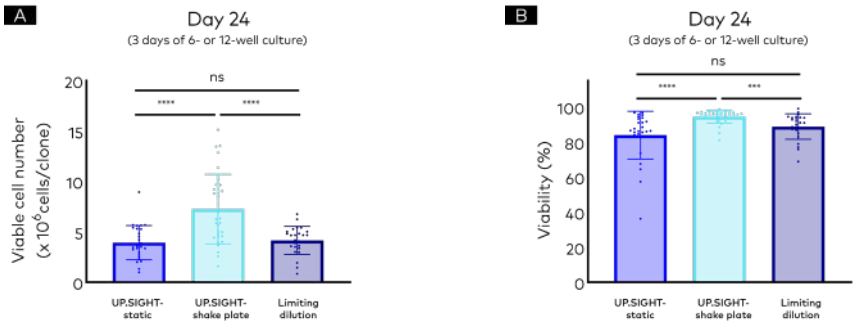

Figure 7. *The* ***(A)*** *viable cell number and* ***(B)*** *cell viability of different workflows on day 24, after upscaling from 24-well plates to 6- or 12-well plates and further culturing for 3 days in 6- or 12-well plates.*

There were no significant differences between the UP.SIGHT-C.NEST culture workflow group and the other three workflow groups. This suggests that the C.NEST culture not only can accelerate the timeline of single-cell cloning, but also does not negatively affect the cell growth ability after the upscaling process.

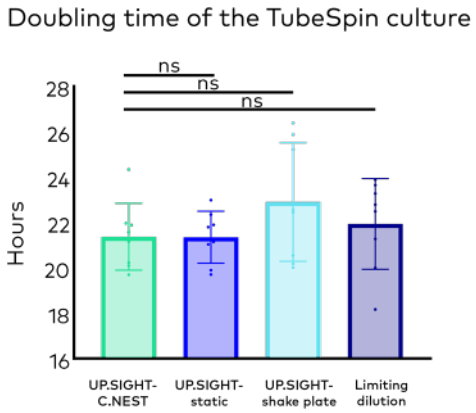

Figure 8. *Doubling time in the TubeSpin culture comparing different workflows. The doubling time was calculated based on the initial cell densities in the TubeSpin and the final cell densities after 4 days of culture.*

Conclusion

Stable cell lines are widely used to produce therapeutic biologics such as monoclonal antibodies and recombinant proteins. As the global market for therapeutic biologics has grown rapidly over the past few decades, biopharmaceutical companies are searching for optimal methods to obtain the best stable cell lines in less time to save costs. Here, we provide an ideal solution for single-cell cloning, the workflow using the UP.SIGHT single-cell dispenser and the C.NEST microbioreactor.

We had already proven that the UP.SIGHT single-cell dispenser can significantly increase the single-cell isolation efficiency compared to the limiting dilution or FACS method. Furthermore, the probability of clonal derivation is >99.99% using the nozzle images and the 3D Full Well images generated by the UP.SIGHT [6], which is a great advantage for biopharmaceutical companies to fulfill regulatory needs. In this study, we demonstrate that the outgrowth efficiency of single cells dispensed by the UP.SIGHT is about 7-9x higher compared to the outgrowth efficiency after single cell seeding using limiting dilution. The higher outgrowth efficiency allows researchers to characterize more clones, increasing the throughput and reducing time for finding the best cell clones.

The UP.SIGHT provides an ideal single-cell dispensing method and the C.NEST microbioreactor further provides the optimal conditions for cells to grow in 96-well and 24-well plates. In this study, after the single cells grew in the 384-well plates, we transferred the cells to 96-well plates and then 24-well plates with mixing culture conditions. The mixing culture provides the cell with more favorable growth conditions, such as homogenous media composition and a higher oxygen transfer rate compared to static culture. After only 4 days of 96-well plate mixing culture and 3 days of 24-well plate mixing culture, the average viable cell number in the UP.SIGHT-C.NEST workflow group was ready for scale up to the TubeSpins for further characterization and cell banking. Compared to the UP.SIGHT-static culture, UP.SIGHT-shake plate culture, and limiting dilution workflow, the viable cell number of the UP.SIGHT-C.NEST culture only needed 17 days to transfer to TubeSpin from a single cell, which saved 7 days on expanding clones **(Figure 9)**.

In conclusion, we demonstrate that the UP.SIGHT single-cell dispenser in combination with the C.NEST microbioreactor provides an optimal solution for single-cell cloning. This workflow may allow researchers to prove clonal origin, track colony growth and improve outgrowth efficiency by the UP.SIGHT. The C.NEST microbioreactor increases the cell growth rate and helps researchers obtain enough cell numbers for clone characterization and banking faster. Meanwhile, the mixing culture condition in 96- and 24-well plates does not affect the cell growth ability after the upscaling process to SpinTube cultures. These advantages highlight that the UP.SIGHT in combination with the C.NEST provides an optimal solution for single-cell cloning and upscaling in cell line development workflows, from which biopharmaceutlical companies can clearly benefit.

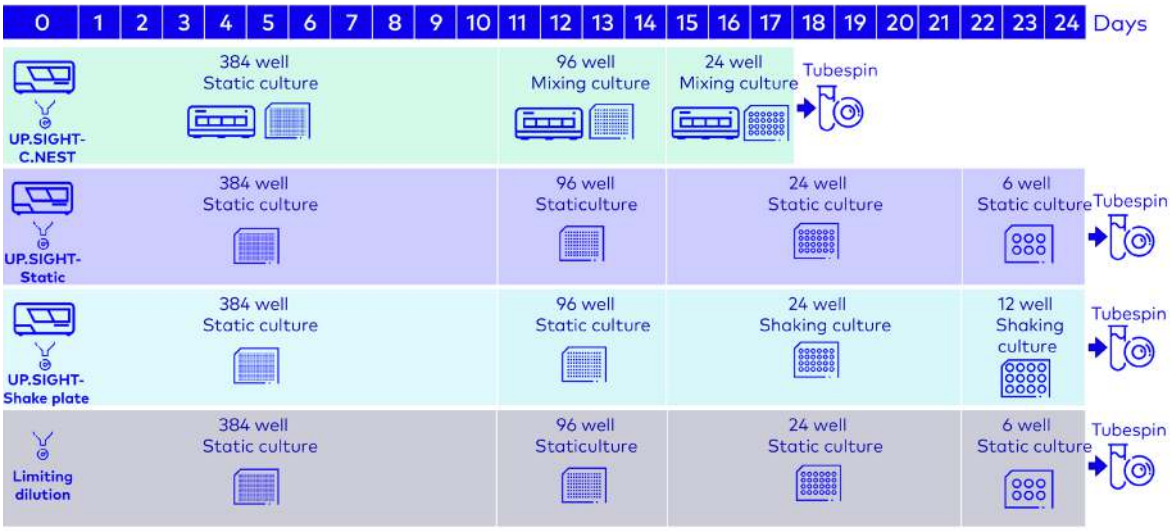

Figure 9. *Summary diagram of required time for each workflow.*

References

1. Lu RM, Hwang YC, Liu IJ, et al. Development of therapeutic antibodies for the treatment of diseases. Journal of Biomedical Science. 2020; 27(1). DOI: 10.1186/s12929-019-0592-z
2. Tejwani V, Chaudhari M, Rai T, Sharfstein ST. High-throughput and automation advances for accelerating single-cell cloning, monoclonality and early phase clone screening steps in mammalian cell line development for biologics production. Biotechnology Progress. 2021; 37(6): e3208. DOI: 10.1002/btpr.3208.
3. U.S. Food and Drug Administration. Supplement to the points to consider in the production and testing of new drugs and biologic & produced by recombinant DNA technology: nucleic acid characterization and genetic stability. fda.gov. April 6, 1992. https://www.fda.gov/media/70898/download
4. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Derivation and characterisation of cell substrates used for production of biotechnological/biological products Q5D. database.ich.org. July 16, 1997. https://database.ich.org/sites/default/files/Q5D%20Guideline.pdf
5. Sinkala E, Scherzinger J, Lautscham L, Riba J. Combined Single-cell Dispensing and 3D Full Well Imaging for Cell Lines with >99.99% Probability of Monoclonality. CYTENA. 2021. (CYTENA Application note-UP.SIGHT)
6. Scherzinger J, Türk D, Aprile-Garcia F. An optimized and validated workflow for developing stable producer cell lines with >99.99% assurance of clonality and high clone recovery. bioRxiv. 2022; 520697. DOI: https://doi.org/10.1101/2022.12.16.520697
7. Yu N, Chiu S, Lin SP. C.BIRD™ | Improving the single-cell cloning workflow through increasing cell growth rate by C.BIRD mixing culture. CYTENA-BPS. 2022. (CBSAPP13)

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