

# C.BIRD™ | Improving the single-cell cloning workflow through increasing cell growth rate by C.BIRD mixing culture

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## Abstract

Cell line development (CLD) is a critical step in biologics development. To generate a stable cell line for protein production, single-cell cloning (SCC) is an essential but labor-intensive and time-consuming step. Optimization of SCC procedures can send new biologics to regulatory approval sooner and at reduced costs. In this study, we established an optimized SCC workflow of the monoclonal antibody

(mAb)-producing Chinese Hamster Ovary (CHO) cell line, CHO-S cells, using CYTENA's F.SIGHT™ 2.0 and the C.BIRD microfluidic reactor. The C.BIRD can improve cell growth in 96-well and 24-well plate cultures compared to static cultures. In summary, the C.BIRD significantly improved the cell growth rate in the early clonal expansion stage compared to traditional SCC workflows with static culture, and has great potential in shortening CLD workflows overall.

## Introduction

The market for therapeutic biologics has grown rapidly over the past few decades. The global market is expected to generate approximately \$300 billion in revenue by 2025 with mAb alone [1]. About three-quarters of approved biologics are produced from mammalian cells and more than 80% of those are produced from CHO cell lines. The key features of CHO cell lines include ease of culture, high productivity, efficient post-translational modification, human compatible glycol-forms of recombinant proteins and approval from the U.S. Food and Drug Administration (FDA), making CHO cell lines mainstream producer cells in the biologics industry.

The development of a new biologic starts from the product development stage to identify the target, the optimal sequence and the preclinical studies. Once the lead is characterized, the next step is to find the best producing cell line through CLD. The following stage is process development which aims to find the optimal manufacturing condition for biologic production. Among these steps, CLD is the primary and critical process aimed at finding a high-producing cell line from SCC. However, the CLD workflow is a labor-intensive and time-consuming step and can take months to obtain desirable cell lines. Therefore, researchers in the biopharmaceutical industry are seeking methods to accelerate the CLD timeline so that new biologics can gain faster regulatory approval and be produced at reduced costs. After the process development stage, the new biologic is qualified to proceed to clinical development and final commercialization stages.

In this application note, we focused on CLD in the process development stage, especially in the SCC procedure. The biopharmaceutical company must prove the clonality of the producer cell line according to the regulations of the FDA and the European Medicines Agency [2, 3]. The most used SCC methods include limiting dilution, fluorescent-activated cell sorting (FACS) and single-cell dispensing. In this study, we used the F.SIGHT 2.0 single-cell dispenser to dispense single cells into 96-well and 384-well plates. The single-cell dispenser not only assured monoclonality during cell dispensing [4], but also achieved high cell viability and cloning efficiency compared to other methods.

We have confirmed in previous application notes that the C.BIRD microbioreactor can improve CHO cell growth in 96-well and 24-well plate cultures compared to static culture [5, 6]. We further confirmed in this study that the C.BIRD microbioreactor can also improve cell growth from cultures with a low initial cell number. Therefore, we applied the C.BIRD microbioreactor to the SCC workflow when the colonies grew up to 5% confluence in 96-well plates and 40% confluence in 384-well plates. Compared to traditional static culture scale-up methods, the C.BIRD not only accelerated cell growth but showed great potential in shortening CLD workflows.

## Materials and methods

### Comparison of C.BIRD and static culture for cell growth

The mAb-expressing CHO-S cell lines were used in this study. The cells were routinely cultured in chemically defined and animal-component-free CD Hybridoma Medium (Gibco, #11279-023) supplemented with 8 mM L-Glutamine (Gibco, #25030-081), Penicillin-Streptomycin (Corning, #30-0020-CI), Cholesterol (Gibco, #12531-018) and Anti-Clumping Agent (Gibco, #01-0057AE). Standard 96-well plates (Corning, #3599) and 24-well plates (Greiner, #662102) were used for the C.BIRD and static culture with 200  $\mu$ L and 1,600  $\mu$ L total volume per well, respectively. The cells in the C.BIRD device were cultured at 37°C in a 5% CO<sub>2</sub> incubator for 4 days of continuous culture. The 96-well comparison tests were performed with cells cultured in standard static culture and C.BIRD culture. The initial cell densities were 1 x 10<sup>4</sup>, 1.5 x 10<sup>4</sup>, 2 x 10<sup>4</sup> and 3 x 10<sup>4</sup> cells/well. The C.BIRD mixing mode was set up to continuous mode with a 50 seconds/cycle mixing rate. The 24-well comparison tests were performed with static culture and C.BIRD culture. The initial cell densities were 5 x 10<sup>4</sup>, 1 x 10<sup>5</sup>, 2 x 10<sup>5</sup> and 5 x 10<sup>5</sup> cells/well. The C.BIRD mixing mode was set up to continuous mode with a 25 seconds/cycle mixing rate. Cell numbers and cell viability were counted with an automated cell counter (CURIOSIS, #FACSCOPE B) after 4 days of culture in each test. 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  $\pm$  SD.

Single-cell dispensing

In this study, we used the F.SIGHT 2.0 to isolate single cells into 96- and 384-well plates for further scaling-up growth tests. The SCC medium used in this study was EX-CELL CHO Cloning Medium (Merck, #C6366). The volume per well was 200  $\mu$ L for the 96-well plates and 80  $\mu$ L for the 384-well plates. The F.SIGHT 2.0 is a single-cell dispenser that enables fully automated isolation

and fluorescent sorting of single cells using high-resolution imaging. The CHO-S cells were diluted to  $5 \times 10^5$  cells/mL, and 50  $\mu$ L of this cell suspension was loaded into the cartridge. The dispenser chip rapidly generated small droplets and the nozzle region was imaged continuously during operation to confirm the droplet was a single cell with correct parameters. These verified cells were then allowed to be dispensed into selected wells (Figure 1).

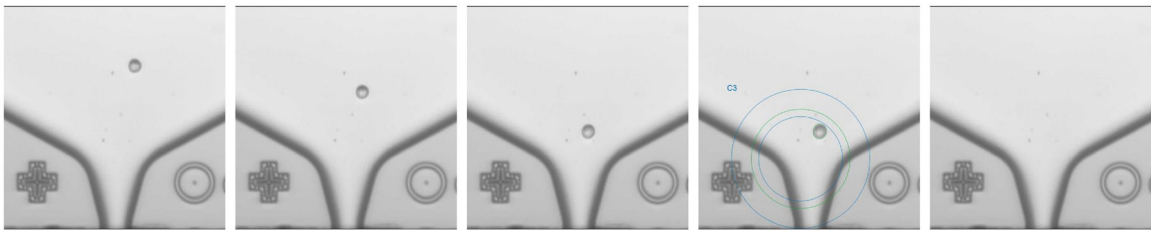


Figure 1. A series of nozzle images during single-cell dispensing with the F.SIGHT 2.0 to prove monoclonality.

Clonal expansion workflow

The comparative experiment was designed to evaluate the cell growth rate between static,

shake plate and C.BIRD culture methods (Figure 2). All groups were cultured at 37°C in a 5% CO<sub>2</sub> incubator.

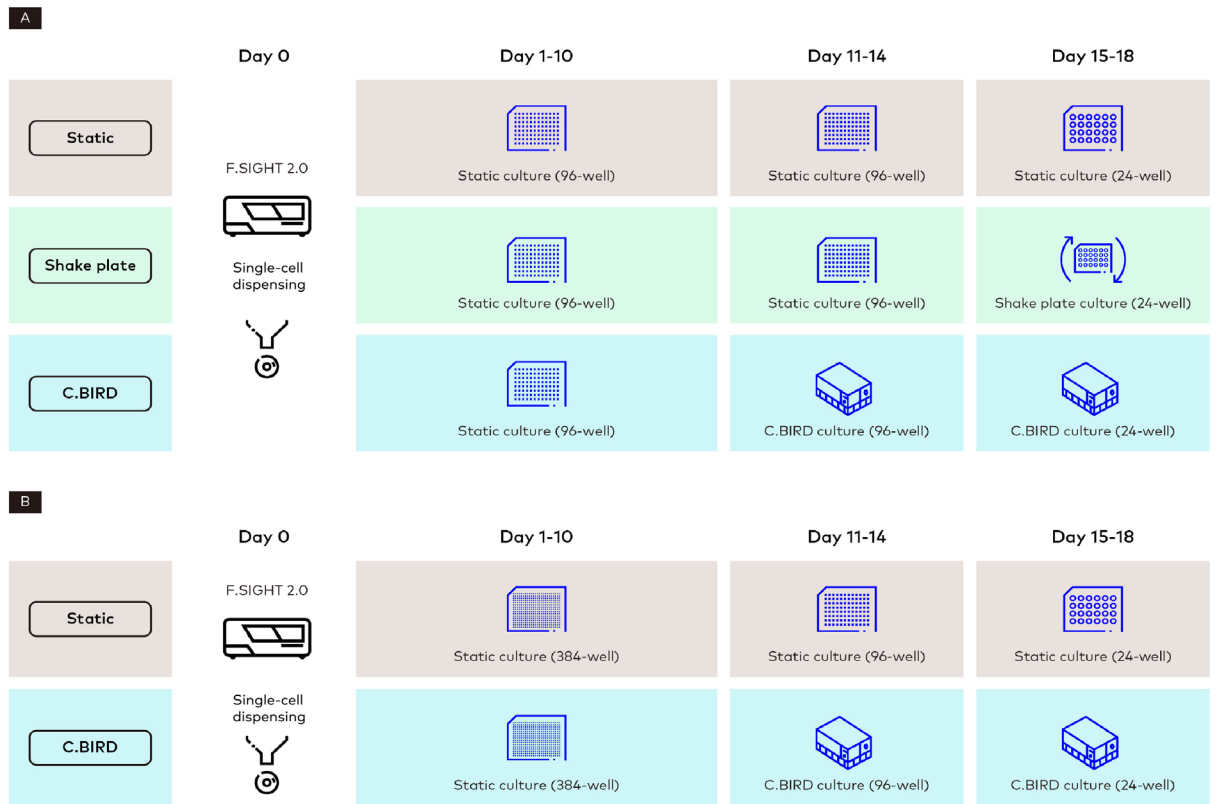


Figure 2. Diagram of clonal expansion experiments design. (A) The workflow of single-cell cloning starts from 96-well plate culture. (B) The workflow of single-cell cloning starts from 384-well plate culture.

For the 96-well traditional static culture group, cells were cultured in 96-well plates for 14 days after single-cell dispensing. The cells in selected wells were transferred to 24-well plates containing 1,400  $\mu\text{L}$ /well CD Hybridoma medium and cultured for another 4 days. For the 96-well shake plate culture group, cells were also static cultured in 96-well plates for 14 days and the cells in selected wells then transferred to 24-well plates with 130 rpm shaking. For the C.BIRD culture group, cells were static cultured in 96-well plates for 10 days, applied to the C.BIRD microbioreactor and cultured for 4 days with 50 seconds/cycle continuous mixing mode. After the 96-well C.BIRD culture, cells in selected wells were transferred to 24-well plates, applied to the C.BIRD microbioreactor and cultured for 4 days. Cell density and cell viability were counted with a cell counter after 14 days and 18 days following single-cell dispensing.

For the 384-well traditional static culture group, cells were static cultured in 384-well plates for 10 days after single-cell dispensing. The cells in selected wells were transferred to 96-well plates containing 120  $\mu\text{L}$ /well EX-CELL CHO Cloning Medium and cultured for 4 days. Then, the cells were transferred to 24-well plates containing 1,400  $\mu\text{L}$ /well CD Hybridoma Medium and cultured for 4 days. For the 384-well C.BIRD culture group, cells were static cultured in 384-well plates for 10 days after single-cell dispensing. Cells in selected wells were then transferred to 96-well plates containing 120  $\mu\text{L}$ /well EX-CELL CHO Cloning Medium, applied to the C.BIRD microbioreactor and cultured for 4 days. After being cultured in the C.BIRD for 4 days, the cells in each well were transferred to 24-well plates and cultured with the C.BIRD microbioreactor for 4 days. Cell density and cell viability were counted with a cell counter after 14 days and 18 days following single-cell dispensing. Statistics were performed by 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  $\pm$  SEM.

## Results and discussion

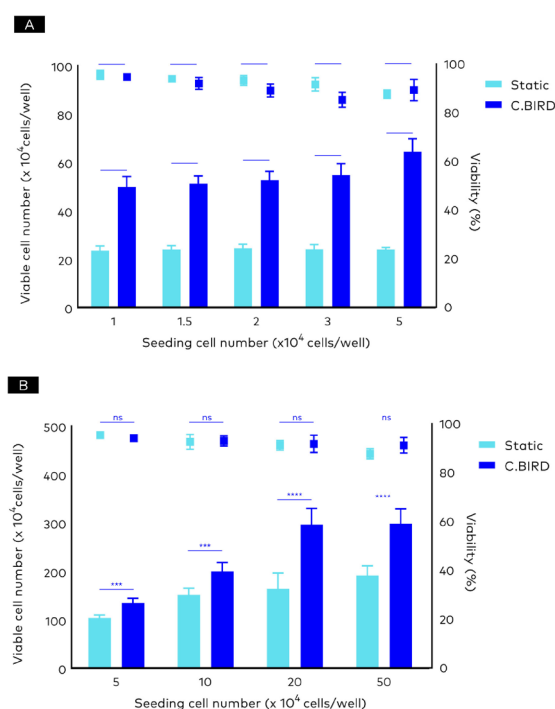
### Comparison of C.BIRD and static culture for cell growth at low seeding density

We have previously confirmed that the C.BIRD microbioreactor system can improve CHO cell

growth in 96-well and 24-well plate culture compared to static culture. In this study, we compared the cell growth from static and C.BIRD culture methods at lower seeding densities.

For the 96-well plate culture, the seeding densities were  $1 \times 10^4$ ,  $1.5 \times 10^4$ ,  $2 \times 10^4$ ,  $3 \times 10^4$  and  $5 \times 10^4$  cells/well. The cells were cultured for 4 days and counted. On day 4, the C.BIRD culture achieved a viable cell number of  $4.98 \times 10^5$ ,  $5.11 \times 10^5$ ,  $5.26 \times 10^5$ ,  $5.46 \times 10^5$  and  $6.43 \times 10^5$  cells/well, while the static culture only reached  $2.34 \times 10^5$ ,  $2.39 \times 10^5$ ,  $2.46 \times 10^5$ ,  $2.41 \times 10^5$  and  $2.39 \times 10^5$  cells/well as the seeding cell number increased. The cell viabilities of each group were all maintained above 85% (**Figure 3A**).

For the 24-well plate culture, the seeding densities were  $5 \times 10^4$ ,  $1 \times 10^5$ ,  $2 \times 10^5$  and  $5 \times 10^5$  cells/well. The cells were cultured for 4 days and counted. On day 4, the C.BIRD culture achieved a viable cell number of  $1.42 \times 10^6$ ,  $2.15 \times 10^6$ ,  $3.22 \times 10^6$  and  $3.27 \times 10^6$  cells/well, while the static culture only reached  $1.42 \times 10^6$ ,  $1.62 \times 10^6$ ,  $1.80 \times 10^6$  and  $2.18 \times 10^6$  cells/well as the seeding cell number increased. The cell viabilities of each seeding density in the C.BIRD culture were maintained above 90%, while the static culture group starting from  $5 \times 10^5$  cells/well were dropped to 87% (**Figure 3B**).



**Figure 3** The viable cell number and cell viability of static culture and C.BIRD culture. **(A)** For the 96-well plate culture, the C.BIRD mixing condition was continuous with 50 seconds/cycle. **(B)** For the 24-well plate culture, the C.BIRD mixing condition was continuous with 25 seconds/cycle.

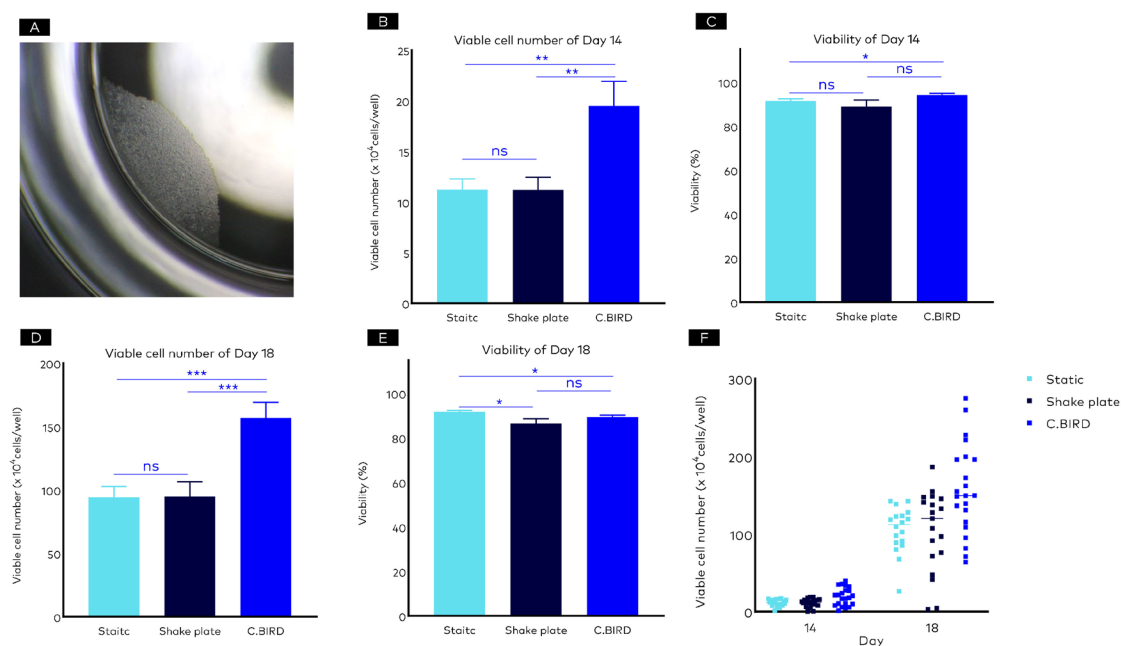
These results show that the C.BIRD microbioreactor improved cell growth at low seeding densities by approximately 2.1 to 2.7 times in 96-well plates compared to static culture. Likewise, cell density was improved by approximately 1.3 to 1.8 times in 24-well plates compared to static culture. Meanwhile, cell viability was not affected.

### Improvement of clonal expansion workflow

In this study, we confirmed that the C.BIRD microbioreactor can improve cell growth at a low seeding density. Therefore, we applied the C.BIRD microbioreactor to optimize the clonal expansion workflow of SCC. The C.BIRD microbioreactor was applied to the 96-well and 24-well plate culture steps.

For groups that started in the 96-well plates, we used the F.SIGHT 2.0 to seed one cell per well in 96-well plates and checked the clonality on day 0. All plates were static cultured in an incubator for 10 days. The confluences were observed by microscope on day 10, and the wells with cells that reached 5% confluence were selected for further analysis (**Figure 4A**). For the static and shake plate culture groups, the cells remained incubated in 96-well plates for the next 4 days, while the C.BIRD culture group applied the C.BIRD microbioreactor to the

96-well plate and was set to continuous mixing mode with a 50 seconds/cycle mixing rate. On day 14, the cell density and viability of each group were counted with a cell counter. The average viable cell numbers on day 14 of the static culture and shake plate culture groups were  $1.12 \times 10^5$  and  $1.11 \times 10^5$  cells/well, while the C.BIRD culture group reached  $1.94 \times 10^5$  cells/well (**Figure 4B**). The average viable cell number of the C.BIRD culture group was about twice of the static culture. The cell viability of the static, shake flask and C.BIRD culture groups on day 14 were 91.4%, 88.8% and 94.1%, respectively (**Figure 4C**). After 14 days of cell culture in 96-well plates, the cells in the static, shake plate and C.BIRD culture groups were scaled up to 24-well plates with static, shake plate and C.BIRD culture for an additional 4 days, respectively. The average viable cell number of the static culture and shake plate groups were  $9.42 \times 10^5$  and  $9.48 \times 10^5$  cells/well. The average viable cell number of the C.BIRD group on day 18 was  $1.56 \times 10^6$  cells/well, which was about 1.6 times higher than the other two groups (**Figure 4D**). The cell viability of the static, shake flask and C.BIRD culture groups on day 18 were 91.7%, 86.4% and 89.3%, respectively (**Figure 4E**). The cell number of individual clones on day 14 and day 18 is shown in **Figure 4F**.

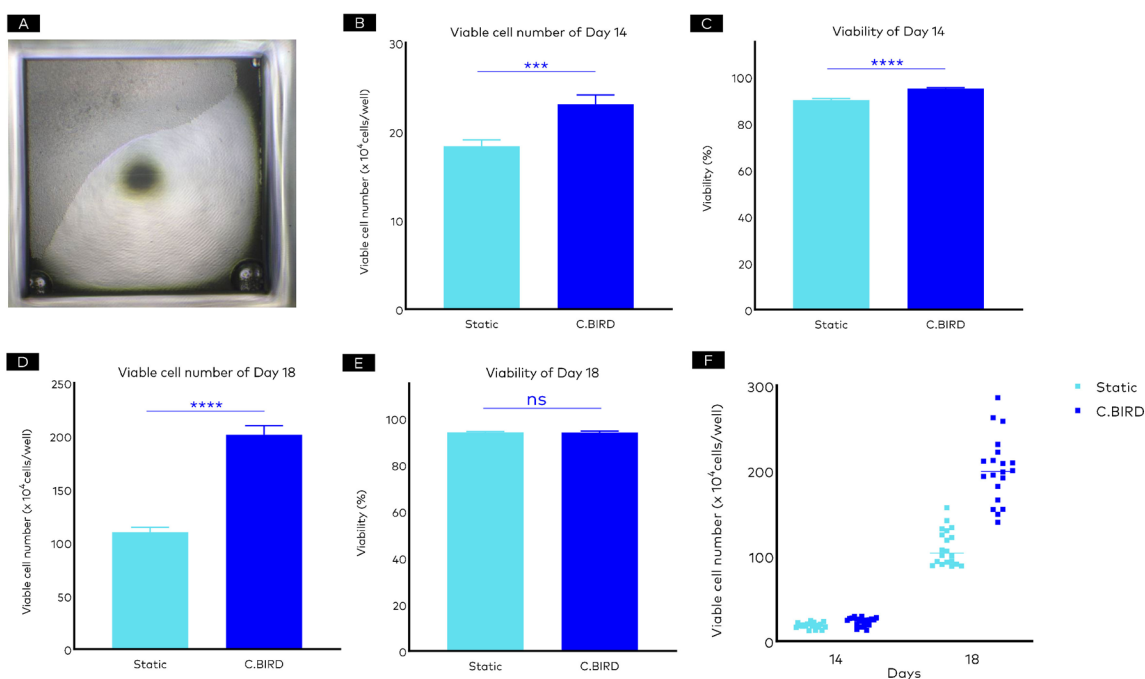


**Figure 4.** (A) Schematic photo of the cell confluency (5%) of selected wells in clonal expansion workflow starts from 96-well plate culture. (B-E) The viable cell number and cell viability of clonal expansion workflow started from 96-well plate. (F) The cell number of individual clone of day 14 and day 18.

For groups initiated from 384-well plates, the cells were static incubated for 10 days after single-cell dispensing and clonality confirmation by the F.SIGHT 2.0. The confluences were observed by microscope on day 10, and wells with cells at 40% confluency were selected for further analysis (**Figure 5A**). The cells from selected wells were transferred to 96-well plates. The cells in the static culture group remained in static culture, and the cells in the C.BIRD culture group were cultured with the C.BIRD microbioreactor for 4 days. After the 4 days, the average viable cell number of static culture was  $1.83 \times 10^5$  cells/mL, while the C.BIRD culture was  $2.30 \times 10^5$  cells/well (**Figure 5B**). The cell viability of the static and C.BIRD culture groups on day 14 were 90.1% and 95.0%, respectively (**Figure 5C**). On day 14, the cells were transferred to 24-well plates with static culture or C.BIRD culture for 4 days and cell density and viability were measured. The final average viable cell numbers of the static and C.BIRD group after the 18-day culture were  $1.09 \times 10^6$  and  $2.01 \times 10^6$  cells/well, an approximately 1.8-time difference in cell number between the C.BIRD culture and static culture (**Figure 5D**). The

cell viability of the static and C.BIRD culture groups on day 18 was the same at 93.8% (**Figure 5E**). The cell number of individual clones of day 14 and day 18 is shown in **Figure 5F**.

SCC is a critical yet time-consuming step in the CLD process. Here, we demonstrated how the application of the F.SIGHT 2.0 single-cell dispenser and the C.BIRD microbioreactor in the early stages of SCC optimizes the process. The F.SIGHT 2.0's patented technology uses high-resolution optics and image recognition to dispense single-cell-containing, free-flying small droplets in an inkjet-like process, significantly improving single-cell efficiency and outgrowth efficiency compared to limiting dilution or FACS methods. After dispensing single cells into 96-well or 384-well plates and culturing until an appropriate amount of cell growth, the C.BIRD microbioreactor provides optimal conditions for maximizing cell growth in 96-well and 24-well plates compared to static culture. This study supported that using the C.BIRD microbioreactor in the early stages of SCC could accelerate the timeline of collecting enough cells to scale up.



**Figure 5.** (A) Schematic photo of the cell confluency (40%) of selected wells in clonal expansion workflow starts from 384-well plate culture. (B-E) The viable cell number and cell viability of clonal expansion workflow started from 384-well plate. (F) The cell number of individual clone of day 14 and day 18.



## Conclusion and future direction

We have previously confirmed that the C.BIRD microbioreactor can improve suspension CHO cell growth in 96-well and 24-well plates. In this study, we further proved that the C.BIRD microbioreactor can increase the cell number from a relatively low seeding density compared to the static culture method in the same culture period without compromising cell viability. Based on this phenomenon, we applied the C.BIRD microbioreactor to accelerate the clonal expansion process of SCC using the CHO-S cell line. We found that applying the C.BIRD microbioreactor

in the SCC workflow starting from a 96-well plate can increase the average viable cell number in the 96-well culture scale by 1.74 times and following 24-well culture scale by 1.65 times compared to traditional static culture. Likewise, applying the C.BIRD microbioreactor in the SCC workflow starting from a 384-well plate can increase the average viable cell number in the 96-well culture scale by 1.25 times and following 24-well culture scale by 1.84-times compared to traditional static culture. Accordingly, we have provided a new SCC workflow with C.BIRD culture in the early clonal expansion stage to shorten the timeline required to obtain enough cell numbers to scale up and ultimately reduce the steps required for scale up to 48-well, 12-well or 6-well plate culture.

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