

Comparison of Limiting Dilution and CellRaft Technology for Cell Line Development

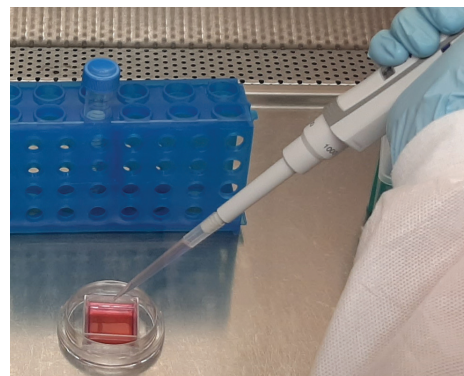
Summary

Therapeutic proteins play an essential role in the biological pharmaceutical market and are used in the treatment of many diseases, such as diabetes, cancer, and anemia. One of the main goals in recombinant protein development is the establishment of high quality monoclonal cell lines that consistently express large amounts of the given protein. Chinese hamster ovary (CHO) cell lines have dominated the industry as commercial hosts for recombinant protein production; however, the process of generating a homogenous CHO cell line is not trivial. Single cell cloning is both a regulatory requirement and technically necessary in the process of recombinant protein production because the productivity of individual cells varies within the population due to random integration of the transfected gene of interest.

Traditionally, a limiting dilution method is used to isolate and generate single cell-derived clones, which is both time and cost prohibitive due to low efficiency of success and the manual labor input required to successfully generate a useful cell line. Several automated technologies exist that aim to improve the efficiency of single cell or colony isolation, such as FACS, colony pickers, and droplet dispensers, but they often degrade cell viability due to harsh manipulation and stress on cells forced to grow alone in a large volume of media.

CellRaft® Technology allows for streamlined and efficient cell line development by leveraging the combined power of flask-like cell culture conditions of the CellRaft Array cell culture consumable with our automated isolation and imaging instrument, the CellRaft AIR® System. The CellRaft AIR System offers an automated solution to an otherwise labor-intensive workflow and supports cell health, time-course imaging for clonal verification, and automated isolation for downstream propagation, altogether providing an alternative that is more time, labor, and cost-efficient for cell line development.

To demonstrate the value of the CellRaft Technology for use in cell line development, we have compared the traditional limiting dilution method with the CellRaft Array using the CellRaft AIR System, evaluating both methods for single cell seeding efficiency, cloning efficiency, and clonal outgrowth.



Seeding a CellRaft Array Single Reservoir

Challenges with Limiting Dilution:

- 1) Lack of viable clones
- 2) Lack of proof of monoclonality
- 3) Requires significant lab supplies and equipment
- 4) Hands-on time requirements
- 5) Limited throughput

Questions Answered by this RaftNote:

How can I increase the number of monoclonal colonies I get for cell line development?

Can I save time when generating colonies from single cells?

How can I know if my colony came from a single cell?

Challenges and Limitations with Traditional Methods of Clonal Cell Line Development

Arguably one of the greatest challenges and investments of time and resources in recombinant protein production is in the development of monoclonal cell lines. Limiting dilution, the traditional approach to achieve monoclonality, entails diluting cells to a level at which there is approximately one cell per well. This process involves significant time and consumable resources, as the efficiency of plating at single cell density is low and often reduces cell viability in the process (Figure 1, Table 1).

The low efficiency of limiting dilution has driven the recent development of alternative technologies – such as cell sorting, automated clone picking, and single cell dispensers – to improve the process of single cell selection, delivery, and recovery, but all involve extensive cell manipulation and plating methods that can negatively impact cell viability. There remains an unmet need for an automated system that supports cell viability, efficiently generates monoclonal cell lines, and is affordable for academic and small industry labs. improve throughput.

Monoclonal Cell Line Development using CellRaft Technology

The CellRaft AIR System and CellRaft Array offer a novel approach to single cell isolation and recovery that is ideally suited for generating monoclonal cell lines. Cells are first seeded on the CellRaft Array – a disposable microwell array substrate for culturing and imaging. While the cells settle and adhere to the polystyrene CellRafts at the bottom of each microwell, they share a contiguous media reservoir, allowing for exchange of growth factors and other cell secretions that improves cell viability and proliferation, a unique benefit not available in single cell plating methods such as limiting dilution, FACS, and droplet dispensers.

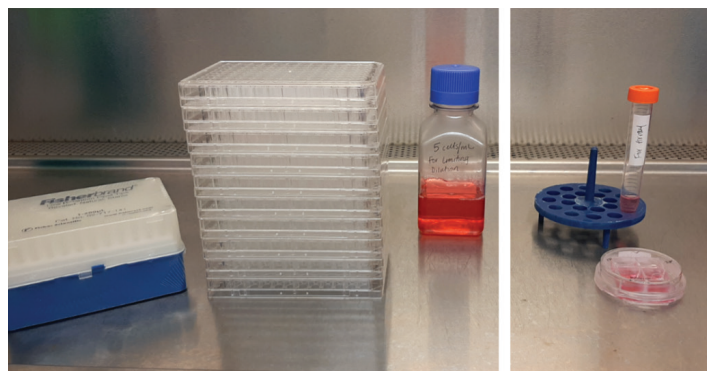


Figure 1. Inefficient limiting dilution workflows (left) require significantly more consumables and media to generate monoclonal cell lines compared to the CellRaft Array (right) when seeding equal total cell counts.

Attributes	Limiting Dilution	FACS	CellRaft Technology
High Cell Viability	X	X	✓
Monoclonal Verification	X	X	✓
Time, Labor, and Environmental Savings	X	✓	✓
Complete Growth Record	X	X	✓
Automated Isolation of Colonies	X	✓	✓
Single-cell Identification with Image	X	X	✓
Software Guided Clone Selection	X	X	✓

Table 1. Comparing limiting dilution and FACS to the CellRaft Technology.

After cell adherence, the CellRaft Array is scanned and imaged using the CellRaft AIR System in as little as 10 minutes, initially to identify rafts with a single cell, then periodically throughout the experiment to monitor colony formation on the array from those single cells (Figure 2). The CellRaft AIR System contains an optical system capable of brightfield and three-channel fluorescent imaging, and the software conveniently stores scans in a central file for each array, allowing for phenotypic analysis of cells of interest and easy tracking of individual CellRafts and colony growth over time.

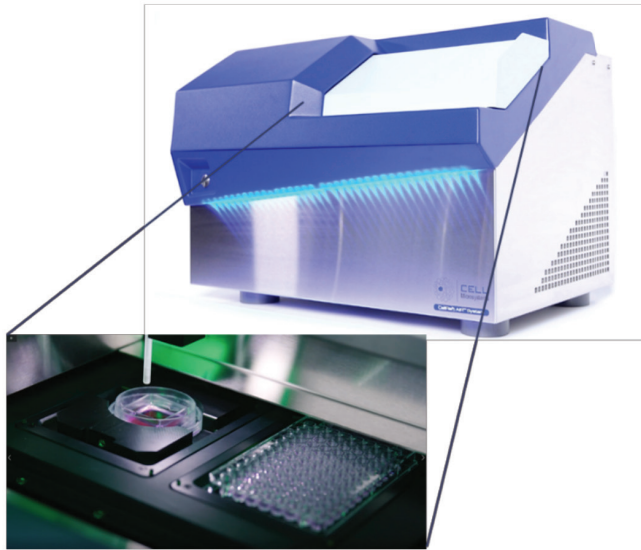
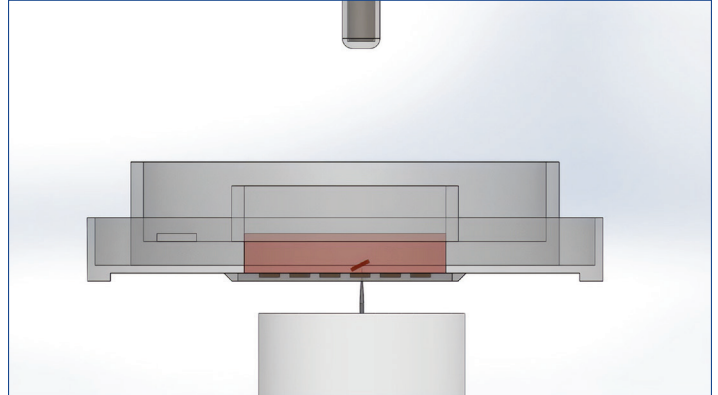


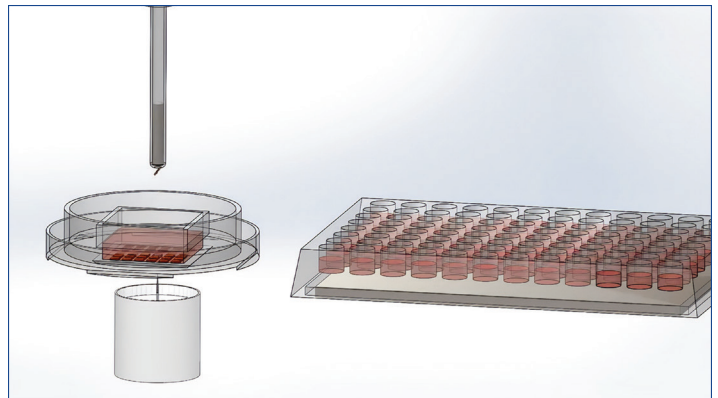
Figure 2. The CellRaft AIR System and CellRaft Array provide fully integrated brightfield and 3-channel fluorescent imaging, automated release and transfer, and optional stage-top incubation.

CellRafts with monoclonal colonies can be selected, released from the CellRaft Array, and transferred into a 96-well plate for further expansion. A motorized needle penetrates the resealable elastomeric substrate of the CellRaft Array to dislodge the individual CellRaft from its microwell. The CellRaft material is loaded with magnetic nanoparticles, allowing it to be retrieved with a magnetic wand. The cells remain attached to the CellRaft through the transfer, obviating trypsin or other dissociation reagents, which preserves cell viability and phenotype when combined with isolation at the small-colony stage. The overall workflow reduces hands-on time and dramatically improves the output, providing an alternative that is more time, labor, and cost-efficient for cell line development.

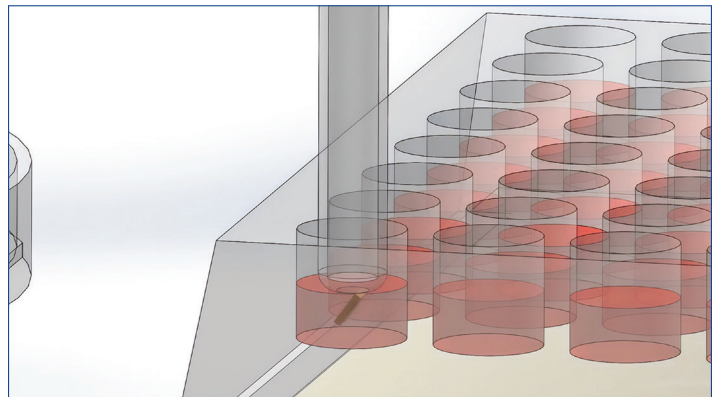
The desired microwell with cells is dislodged from the array



The wand picks up the microwell using a magnet



The wand places the microwell with the cells in the 96-well plate



Comparing Limiting Dilution and CellRaft Array Workflows

To demonstrate the ease of workflow, efficacy, and high cell viability that labs can expect when using the CellRaft Technology in single cell clonal expansion workflows, we directly compared cell seeding efficiency and clonal outgrowth between the CellRaft Array and CellRaft AIR and limiting dilution, using CHO-K1 cells, commonly used in biopharmaceutical applications. Two formats of the single-reservoir CellRaft Array – with 100x100µm (100S) and 200x200µm (200S) CellRafts – were evaluated to determine if the size of the CellRaft and corresponding colony impacted outgrowth of the colonies after isolation and transfer to the 96-well plate.

For each format (Limiting Dilution, 100S, and 200S), n=3 96-well plates were evaluated for colony outgrowth. Each workflow is described below.

For limiting dilution, 2,000 cells were seeded in well A1 of each 96-well plate, followed by a two-series serial dilution. From well A1, cells were serially diluted (1:2) down the first column, then subsequently serially diluted (1:2) across each row of the plate (Figure 3). Plates were incubated at 37 °C, 5% CO₂ and monitored for clonal outgrowth.

For cell seeding on the CellRaft Arrays, two arrays were prepared, one 100S and one 200S, and seeded according to standard procedures (see CellRaft Array user guide) with 20,000 cells and 5,000 cells, respectively. After allowing the cells to settle into the micro cells and adhere to the CellRafts, each array was scanned on the CellRaft AIR System to identify CellRafts containing a single cell.

Full array scans were serially performed every 24 hours to monitor clonal propagation of single cell CellRafts until isolating and transferring them to three 96-well plates per array. Selected clones were isolated from the 100S array 48 hours after seeding, while clones were

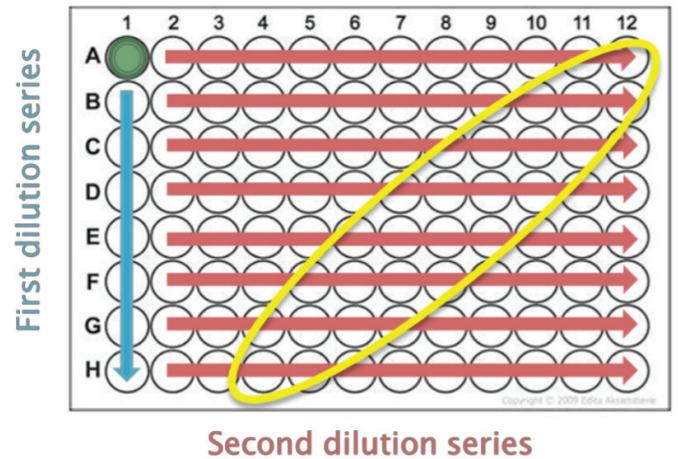


Figure 3. Two-series serial dilution for limiting dilution plates used to generate single cells/well. The region of expected single cell clones is identified in yellow.

isolated from the 200S array after 72 hours to allow for more complete colony coverage on the larger CellRafts.

Monitoring Clonality on the CellRaft AIR

The most essential component of cell line development is ensuring propagation from a single cell. The CellRaft workflow surpasses traditional limiting dilution in its ability to provide visual confirmation of single cells and conveniently monitor thousands of them on a single consumable. Using the CellRaft AIR System, all CellRafts on each array were imaged with the resolution necessary to track them from a single cell to a small colony, with the data stored in a single database to view individual CellRafts over time (Figure 4). then monitored for viability over the course of a week. In contrast, limiting dilution requires the seeding and manipulation of dozens of 96-well plates in which it is hard to confirm a single cell deposit and verify clonality without specialized imaging equipment or scanners.

Using the proprietary CellRaft AIR System software, we identified more than 5,400 single cell-derived clones on the 100S array and 1,100 single cell-derived clones on the 200S array (Figure 5), all available for isolation into 96-well plates.

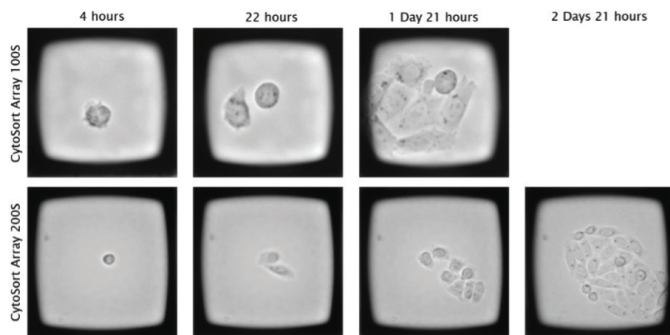


Figure 4. CHO cells seeded on the CellRaft Array are imaged and traced over time to monitor clonal outgrowth prior to isolation.

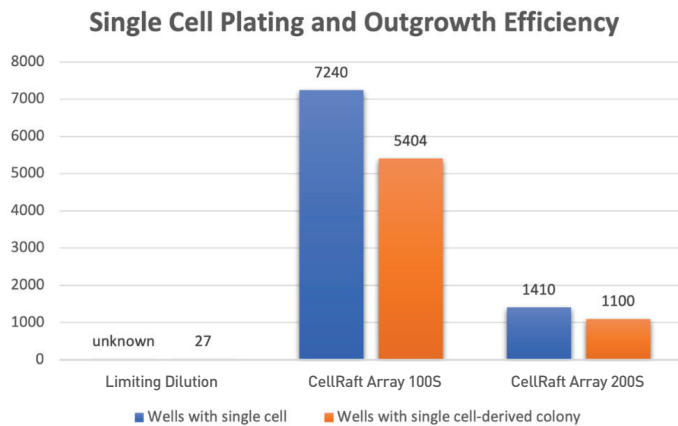


Figure 5. Seeding CHO cells on the CellRaft Arrays results in thousands of microwells with a single cell that can be reliably imaged, tracked, and phenotypically characterized as they grow into clonal colonies ahead of isolation into 96-well plates for further expansion. Seeding n=3 limiting dilution plates revealed 27 wells with colony growth, which cannot be verified as starting from a single cell.

Clonal Outgrowth using Limiting Dilution versus the CellRaft AIR System

Two hundred eighty-eight CellRafts with colonies derived from a single cell – which represent only 5% and 26% of all available clonal colonies on the 100S and 200S CellRaft Arrays – were isolated into three 96-well tissue culture plates for each array format. Of the total wells populated from the 100S and 200S arrays, 256 (89%) and 275 (95%), respectively, exhibited continued outgrowth (Figure 6), vastly out numbering the 27 colonies (9.4% of total wells seeded) in the three LD plates that had yet to be genotypically confirmed as clonal. Based on these numbers, using limiting dilution for single cell cloning would require on the order of thirty 96-well plates to achieve the equivalent number of clones as populating three 96-well plates with the CellRaft AIR System, resulting in a ten-fold reduction in plastic ware. Additionally, if more clones were necessary for evaluation, hundreds of single cell-derived colonies were still available and growing on the CellRaft Arrays.

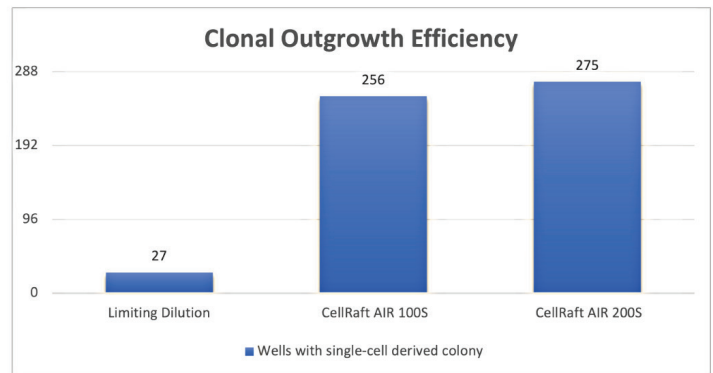


Figure 6. Clonal outgrowth efficiency of CHO-K1 cells in n=3 96-well plates using Limiting Dilution, CellRaft AIR 100S and 200S formats.

Seven days post-isolation into 96-well plates, colonies were dissociated for expansion using 0.25% trypsin-EDTA to disperse cells in the 96-well plates (Figure 7). After an additional three days of colony growth, wells were stained with Crystal Violet, which identifies viable cells and colonies, to visually demonstrate the improved cloning efficiency of the CellRaft AIR System compared to Limiting Dilution (Figure 8).

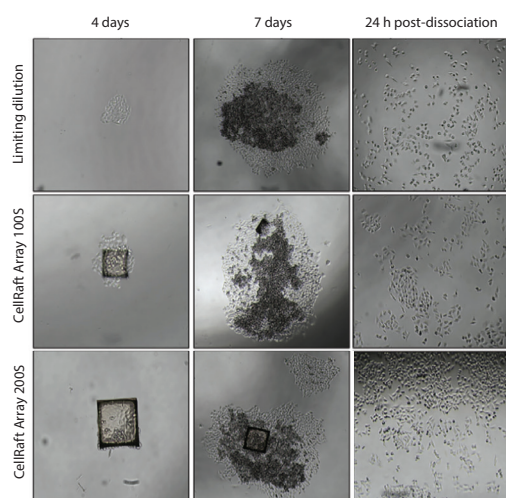


Figure 7. Representative images of CHO-K1 colony formation using Limiting Dilution, 100S, and 200S cell-seeding formats in 96-well plates 4 days and 7 days after initial cell seeding and 24-hours after dissociating colonies using 0.25% trypsin-EDTA.

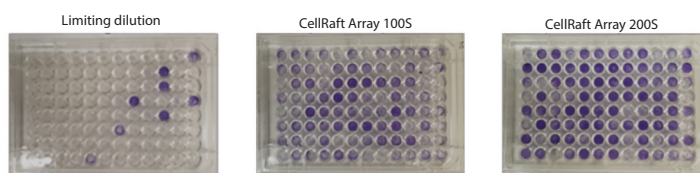


Figure 8. Representative 96-well plates of single cell derived CHO-K1 clones stained with Crystal Violet 10 days post-seeding.

Conclusions

The CellRaft AIR System and CellRaft Array vastly outperformed limiting dilution for monoclonal CHO-K1 cell line development. The technology offers an automated solution to an otherwise labor-intensive workflow, with a consumable uniquely designed to support cell health, high-resolution imaging for clonal verification at the single cell stage, time-course imaging for growth monitoring into small colonies, and automated isolation for downstream propagation. Switching to our technology for monoclonal cell line development methodologies gets your lab to the right clone faster for your application by reducing your effort, cost, and waste and increasing your efficiency and success rate.

For more information on the presented data or CellRaft Technology, visit cellmicrosystems.com