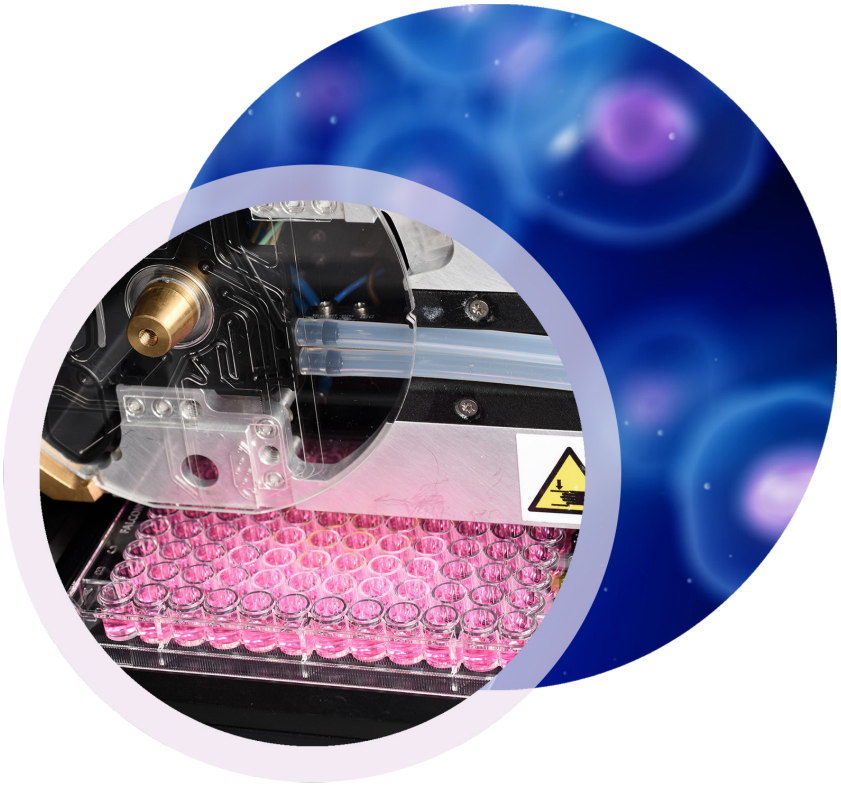

Best practices for optimizing Cell Line Development processes

Cell line development (CLD) follows a little changed process from that used in the early days of biopharmaceutical development. The steps to create a stable, manufacturing cell line include host cell line selection, engineering of the expression vector, transfection, cloning and cell expansion, as well as various screening steps to select the clone with the highest cell viability, growth, expression level, stability and product quality. However, within this process, there are many different strategies that companies can adopt to optimize their workflow.

In this article, we spoke with Dr Jon Dempsey about his thoughts regarding best practices for optimizing CLD processes.

Jon Dempsey is the CEO and Founding Partner of Pathway Biopharma. Jon has spent nearly 30 years working in the Biopharmaceutical industry, bringing expertise in biologics chemistry, manufacture and control (CMC) including cell line development, cell therapy and process development, analytics and protein chemistry, CDMO selection and manufacturing. During this time, Jon has been involved in the development of several commercial biotherapeutics.

We asked Jon what his advice would be for those researchers/ scientists starting out and for those currently in CLD, what are the best strategies for improvement?

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Insights from Jon Dempsey

As CLD and the development of the first clinical batch is on the critical path for advancing a novel therapeutic to a clinical trial, companies are looking to do anything they can to make the development process faster. If I were building a CLD system today, these are the four stages I would first address:

1. Design the workflow, then design the cell line to fit this workflow using directed evolution or clonal selection in the process.

2. Use synthetic vector technologies to improve expression.

3. Use automation, like Cyto-Mine®, to remove manual techniques and to speed up the process by eliminating rounds of single cell cloning.

4. Focus on getting the cells to grow as fast as possible by reducing cell doubling time.

Design the cell line to fit your workflow

Chinese Hamster Ovary (CHO) Cell Lines

Antibodies for therapeutic use are almost entirely produced within CHO cells. This is because CHO cells represent an established regulatory pathway approved by the FDA. The first approved recombinant biopharmaceuticals were mostly produced using CHO cells, so most biopharmaceutical companies follow this regulatory pathway as it provides an established route to market. As a result, >70% of all recombinant proteins are manufactured using CHO cells.

In addition to being an FDA approved regulatory pathway, there are several scientific benefits for using CHO cells. They can grow in suspension culture and in serum-free, animal-origin-free, chemically-defined medium that enables large-scale production and reproducibility with improved performance across different cultures. CHO cells often exhibit high gene expression levels, leading to increased protein yield and specific productivity. Expression systems based upon CHO cells can be more robust and tolerant of changes in culture conditions. Plus, and perhaps most importantly, CHO cells provide a “human-like” glycosylation profile that can prevent the activation of a patient’s immune response that can lead to unwanted side effects or loss of product efficacy.

Yet, despite the preference for CHO cells, there is no single, well-defined cell line used by the industry, or even a standard CHO host cell line. Most companies will derive their own CHO host cell line from a parental line obtained from a cell culture collection. Cells will be serum and suspension adapted to the company's process and be stored in a cell bank. Ultimately, this results in a divergence in cell phenotypes and differences in cell performance. The predominant use of CHO cells does also make new industry entrants more conservative when it comes to choosing a cell line to utilise. Some companies have used less traditional cell lines, such as the Per. C6® or other human cells, but they have not been successful in generating products for market as they are still relatively new technologies. When it comes to which cell lines to use the main objectives are to ensure that the end clone can colonise from a single cell, produce high yields and provide high product quality. Most companies have adapted their cell line and culture medium to improve cell outgrowth during single cell cloning. In some CLD strategies, companies start by optimizing their cell line using directed evolution to isolate a cell line that performs well in their workflow.

Directed Evolution

Typically, during the development of cell lines, multiple screening stages that assess cell characteristics, such as productivity and growth, are used to manage cell line heterogeneity and reduce the number of cell lines progressed to further assessment stages. However, directed evolution provides a far more efficient approach to generate improved mammalian cell hosts, that can be applied to optimize your CLD process.

Figure 1. Representation of the directed evolution process.

Rather than using a traditional approach of taking a cell line through the development stages and making choices throughout the process as would be done traditionally, directed evolution offers a way to control the heterogeneity of cells during CLD right from the beginning by pre-adapting the cells to the development process. For example, directed evolution can involve enriching and sorting the cell line to isolate subclones that exhibit desirable attributes for production, then using these subclones as the host parental cell line ensuring that they are capable of growing during the development process (Figure 1).

Leverage synthetic promoter technologies

During transfection, specialized expression vectors that contain the product (therapeutic protein) of interest are inserted into the host cell to develop a recombinant cell line. These expression vectors can be constructed to include elements that improve protein expression levels to enable more effective clone selection.

The expression vector is essentially a piece of DNA that has elements, such as the antibody gene and the selection marker genes plus genes which allow expression in CHO cells. The primary purpose of the selection marker gene is to expedite the expression of the genetic vector in the host cell line. Classical selection markers used for CHO cells include glutamine synthetase (GS) and dihydrofolate reductase (DHFR). Usually, the selection marker gene is regulated by a promoter which is most commonly viral or endogenous. However, viral and endogenous promoters can provide unpredictable and uncontrollable functionality that is suboptimal for industrial-scale processes. As such, this introduces an opportunity to optimize protein expression by exploring synthetic promoters.

Several companies and academic groups are designing completely synthetic promoters and other genetic elements for CHO cell engineering. By matching promoter sequence to the host cell's transcription machinery enhanced expression can be achieved. Compared to viral promoters, synthetic promoters perform predictably and provide much more transcriptional control to facilitate biopharmaceutical manufacturing developments. For example, you can design a promoter for CHO which would operate differently in a gene therapy vaccine. Each promoter would express only in specific conditions or locations, such as only expressing in specific tissues or under specific conditions.

Use automation to remove manual techniques

Techniques to automate single cell cloning

In the development of any production cell line, it is essential to assure monoclonality to meet FDA guidelines. However, due to variations in cell recovery, single cell cloning to select for high expressing and stable clones has been a universal challenge. Particularly as the technique chosen for single cell processing and dispensing can further affect single cell viability.

Traditionally, manual single cell cloning has been a very time consuming and labour-intensive process. For example, during the early part of my career cell cloning involved manual dispersal of cells into multi-well plates and the identification and selection of a single cell by visually screening with a microscope. Most workflows have advanced beyond this cell selection method, but the primary strategy is unchanged.

Limiting dilution is a widely used method for single cell cloning. This method involves diluting cells to a low concentration (often as low as a quarter of a cell per well), plating the cells out, and waiting for the cells to proliferate. While providing a gentle and straightforward cloning method, limiting dilution is low-throughput, prone to contamination and is expensive. As limiting dilution relies on statistical distribution, this iterative clone screening technique requires at least two rounds to improve monoclonality and requires a considerable amount of time. To get around this, some companies perform only a single round of cloning for early phase trials, and the additional resource required for a second round of cloning will not be spent if the drug is not successful.

Another popular single cell cloning method is flow cytometry or more commonly know as fluorescence-activated cell sorting (FACS™). Flow cytometry offers high throughput screening and sorting capabilities to isolate cells based on high cell surface protein expression levels. However, it is complex to use and can be damaging to cells due to high shear forces. Cell damage inevitably impairs cell viability and integrity during the cell isolation process.

There have been several automation developments in the field of CLD. One of them is ClonePix™, an automated colony picking and imaging instrument which involves culturing the cells in a semi-solid media. fluorescence imaging is then used to identify the best candidates for expansion. However, cell isolation in semi-solid media may impede cell outgrowth. CHO cells are thought to release autologous growth factors that can stimulate cell growth from single cells. When single cells are isolated using ClonePix™, the semi-solid media may prevent diffusion of growth factors into the surrounding culture media. To overcome this, some companies add specially optimized cloning media or cell conditioned medium to support cell growth in the ClonePix™. The throughput is about 10,000 cells per three week campaign.

Microfluidic systems, such as Sphere Fluidics' Cyto-Mine® single cell analysis platform, are a significant area of interest that may offer a different and innovative perspective for the improvement of traditional methods for isolating single cells. Using picodroplet technology, Cyto-Mine® rapidly isolates hundreds of thousands of single cells into individual picolitre volume droplets called picodroplets. These picodroplets enable each cell to be assayed in their own microenvironment. The cells can be assayed and selected based on which single cell exhibits the highest specific productivity. At the same time, the picodroplet shields the cells from shear stress to produce a high number of valuable, viable clones. Additionally, the Cyto-Mine® platform integrates high-speed imaging to provide visual evidence of monoclonality and to support confidence of a single progenitor. This process is undertaken using one automated instrument and takes less than one day - resulting in significant time and cost savings (Figure 2).

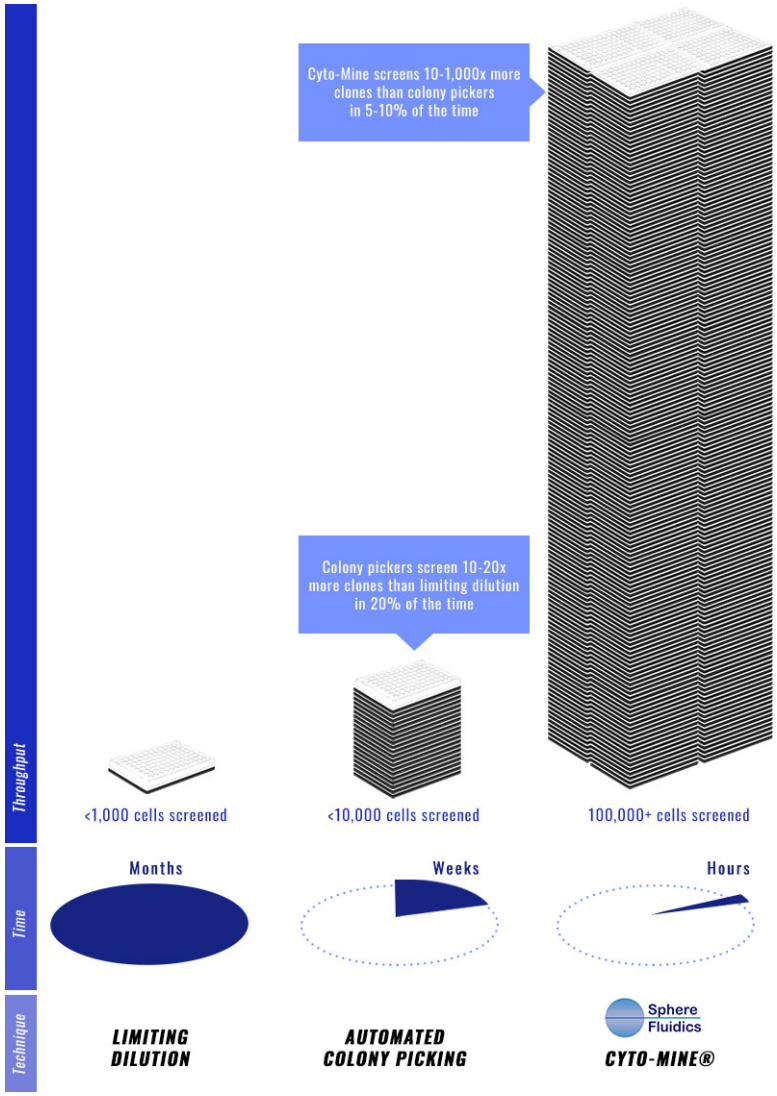

Figure 2. Comparison of various approaches to isolate single cells producing high levels of valuable biotherapeutics.

Following single cell cloning, the highest-producing clones are subject to further characterization to ensure they can be scaled for production. Shake flasks were traditionally used for clone expansion and productivity screening. However, this method does not allow users to monitor or control environmental parameters, such as pH, oxygen and temperature. As a result, this approach may not be representative of the production process which uses bioreactors with highly controlled conditions in a very defined system.

Another significant advancement in the last five years that has enhanced clone productivity screening has been the development of miniaturized, automated bioreactors. One innovative instrument has been the AMBR® 250 system (Sartorius) - an automated bioreactor that provides sophisticated control of culture conditions in a miniature system so you can screen 48 or 96 clones at a time. The use of automated bioreactor systems also allows clone screening in multiple 'platform' production processes. Many combinations of media, feed and process can be evaluated on tens of clones to identify the most process ready clone. This can also allow for improved clone selection.

Rather than simply automating the existing CLD workflow- unchanged for many years, perhaps now is the time to rethink and to overhaul manual, labour intensive steps by the reconstruction and optimization of the workflow to make it more automated. You can achieve this streamlined optimization by using instruments such as Cyto-Mine®. The latter automates cell isolation, titer determination and clonality assurance providing a robust one-step cloning strategy. In doing so, the fully-integrated platform enables productivity assessment to be done earlier and more quickly, with less effort. Cyto-Mine® also eliminates the need for two rounds of limiting dilution cloning, greatly saving time and resource required to demonstrate monoclonality.

Sphere Fluidics' Cyto-Mine®

Focus on getting the cells to grow-out as fast as possible

It is worth remembering that cells are usually not single entities and evidence suggests that they do not like being isolated as single cells. Consequently, single cell processing and dispensing can greatly impact cell recovery from a single clone. In order to promote single cell out-growth cells require a supportive environment to flourish. So, whether you have optimized single cell cloning or not, outgrowth is often problematic and is a hurdle that scientists in CLD must face.

As briefly mentioned, when discussing single cell cloning, cell growth can be influenced by autocrine growth factors that mammalian cells secrete. In bulk culture cells are surrounded by molecules that influence their growth. These molecules may promote or inhibit cell proliferation. For example, CHO cells are thought to secrete signalling compounds which are auto-stimulatory, but which can also act as an external stimulus for overall cell growth.

The lack of these stimulatory compounds when cells are isolated as single cells can have a significant impact on outgrowth. Additionally, cell biologists have known for many years that mammalian cells can have incredibly elegant ways of sensing their environment. Therefore when you isolate a single cell, you can change a large part of their supportive environment, and this disruption can prevent them from growing (Wang & Lei., 2018).

Unfortunately, there is currently no one size fits all approach to optimizing single cell outgrowth. Different CHO cell types and clones have different nutrient requirements. Many kinds of cloning media have been designed to help improve volumetric productivity and product titer. Some scientists use hydrolysates. Hydrolysates are peptides derived from plant protein that provide nutrient supplements to the media. They are a well-proven replacement for serum and animal-derived components, but they can also be a source of contaminants. For example, a frequently used hydrolysate is Hy-Soy™, produced by enzymatic hydrolysis of soy. Others use conditioned medium developed by growing host cell cultures in parallel, concentrating the medium, then using this as a supplement to support cell growth. Conditioned medium is assumed to contain growth factors that stimulate cell growth. Many cell line developers also closely monitor the primary nutritional conditions, and environmental conditions such as carbon dioxide concentration, to ensure optimal conditions for CHO cell growth. There are many supplementary factors which researchers have manipulated to promote good cell outgrowth, but it is paramount that this is optimised for each unique cell line in order to support the survival of the best isolated clones.

Conclusion

Advancements in the field are enabling CLD processes to become increasingly optimized. The key steps to integrate for optimized CLD workflows include:

1. Using directed evolution to preadapt your cell line to your workflow.

2. Use synthetic promoter technologies to make more sophisticated vectors to improve expression.

3. Rethink manual and partially automated protocols and incorporate automated integrated technologies like Cyto-Mine® to accelerate the CLD process.

4. Formulate optimized cloning medium to improve single cell growth.

It is essential to note that not one solution fits all cells. Each stage in your CLD workflow will require optimization for your specific clone. It is understandable that individuals working with traditional workflows, based on historical perspectives, may not have the opportunity to go back and design or incorporate new processes such as directed evolution. However, the availability of accessible, easy-to-use automation and cell biology platforms can help facilitate process optimization in a stage-by-stage way. This offers low risk and minimal effort with the potential return of accelerated CLD and a reduction in labour-intensive processes.

References: Wang, Y.-P.; Lei, Q.-Y. Metabolite sensing and signaling in cell metabolism. Signal Transduct. Target. Ther. 2018, 3, 30.

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