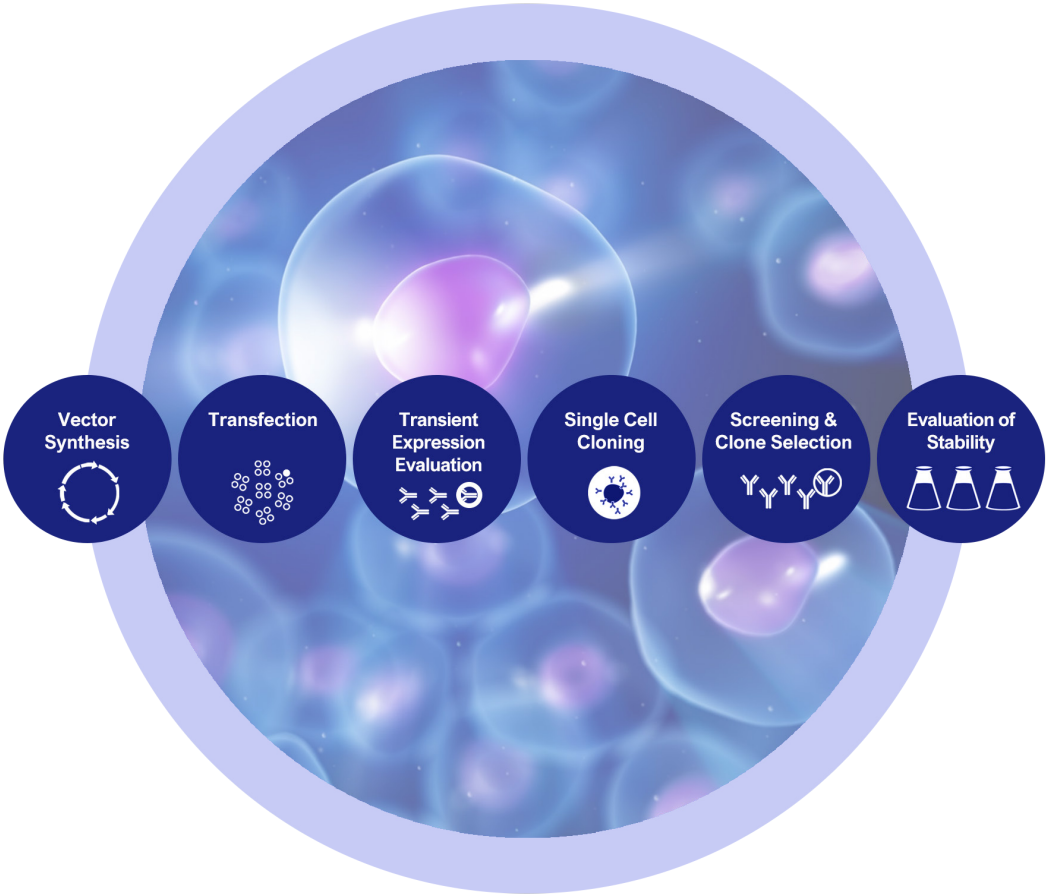

Overcoming challenges in the Cell Line Development process

Advances in cell line engineering and microfluidic technologies provide significant opportunities to reduce timelines in cell line development (CLD). As with any change, optimizing the CLD processes can introduce new challenges and uncertainties. Some of the key challenges include: 1) selection bias in the cell line; 2) unpredictable gene expression; 3) issues with automated cloning techniques; 4) varying cell growth rates.

This article provides an overview of these four challenges and how you can address each scenario.

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Host Cell Line Selection Bias

During cell line development, it is imperative to avoid selection bias in the cell line. For example, if clones are iteratively enriched and selected for stability, or chosen based on the level of antibody secretion at the time of measurement, this results in a highly-selected subset of the cell population that has not undergone thorough evaluation.

Consequently, picking the wrong parameter early on may prove to be a mistake in the later development stages. For example, early screening and selection of clones with high productivity may be found to be overly optimistic as early productivity measures do not guarantee future productivity, production, or growth stability, and the cells may die off due to reduced viability. Selection bias is, therefore, a vital issue to keep in mind if trying to predict the commercial performance of a clone, being careful to select cells based on their future potential.

The most common method to avoid selection bias in CLD is to choose a variety of high- and medium-producing clones to take on to further evaluation stages, to account for variations in product quality, stability, and growth. Clones can then be observed on how they perform during expansion, and with further validation, cell lines with improved characteristics can be identified.

Gene Expression

Despite trying to optimize the host cell line, possibly using directed evolution or the development and adaptation to a chemically-defined, animal-origin free medium, bottlenecks still exist at the initial stage of gene expression. A variety of techniques have been applied in CLD to control and improve gene expression in order to optimize protein yields. For example, site-specific integration and careful design of the expression vector have been investigated as important techniques for engineering the host cell line to maximize the amount of active product produced and to overcome issues faced with traditional processes.

Site-Specific Integration

The reliance on expressing a gene of interest (GOI) using the method of random integration can hamper the expression of the transgene. The random integration of the GOI into the genome can result in the unpredictable and unreliable expression of recombinant proteins due to the position of integration. This method, therefore, still requires screening many clones to identify cell lines with the desired expression levels.

In contrast to the ‘shotgun approach’ of random integration, targeted integration specifies a ‘landing pad’ in the cell genome and inserts the gene into that specific region during transfection, to reduce the likelihood of position effects affecting expression levels. With the advances in genome engineering and the development of engineered nucleases such as CRISPR/Cas9 RNA guided nucleases, transcription activator-like effector nucleases (TALENS), and zinc-finger nucleases (ZFNs), targeted integration has become a significant area of development. By harnessing these new technologies, researchers can focus on regulating the location of insertion and, consequently, recombinant gene expression resulting in a reduction of clonal variation.

When choosing targeted integration, researchers in CLD can opt for even more reliable expression by exploiting ‘safe harbour’ sites within the cell’s genome. These specific regions prevent the dampening or silencing of the transgene by the host cell genome or the context of the location of gene insertion. Two common ‘safe harbour’ sites have been tested for use in mammalian cell lines, *Hipp11* (11) and *Rosa26.* By precisely inserting a GOI into these defined loci of HEK293 and CHO cells, studies have shown an increase in gene copy number integration and stable gene expression which may result in increased and consistent protein production (Zboray *et al*., 2015 and Chi *et al*., 2019).

Targeted integration can help to improve product titer by increasing the reliability of gene expression for optimized gene expression with high accuracy. Although some cons come with using methods such as CRISPR, as they can generate off-target effects and create mutations in the host genome. CHO cells, for example, are uncooperative when it comes to applying the more reliable method of DNA repair, homology-directed repair (HDR). CHO cells are more amenable to DNA repair using the non-homologous end-joining (NHEJ) repair pathway, which is not as stable and can result in indel mutations (Lee *et al*., 2015). However, as the technology of CRISPR advances, more and more improvements are being made to increase functional capabilities, reduce off-target effects, and minimise mutation events.

Ultimately, targeted integration enables a more iterative and predictive way of making cell lines that follows a much more engineering design-build-test approach. This technique has been used for clinical and commercial production but has not been widely adopted.

Expression Vector Design

The transfection of a transgene very rarely results in a high producing cell line, and researchers in CLD often utilise gene amplification to increase protein expression. Consequently, the careful design of the expression vector can boost the specific productivity of the cell line to increase product yields.

The expression vector will traditionally contain the antibody gene and selection marker genes plus genes that allow expression in the desired cells. The incorporation of a selection marker enables high-producing clones to be easily selected from the cell population. Classical selection marker systems used for CHO cells (the most widely used cell line in CLD) are glutamine synthetase (GS) and dihydrofolate reductase (DHFR). These metabolic enzymes are inhibited by specific drugs to enable selection, GS is inhibited by methionine sulfoximine (MSX), preventing glutamine

production, and DHFR is inhibited by methotrexate (MTX) blocking RNA and DNA synthesis. CHO cell lines deficient in these metabolic enzymes have been developed to accompany these selection methods and are widely commercially available. Selection occurs when transfected cells are cultured in a single concentration of the specific drug, either MTX or MSX depending on which system is being used, and the GOI is situated close to either GS or DHFR on the expression vector. Amplification relies on a stepwise increase in drug concentration. Consequently, cells with higher levels of gene amplification for GS or DHFR, which can function in higher concentrations of MTX or MSX, are selected as having increased copies of the gene. These cells then undergo single cell cloning to further characterise expression and productivity.

The selection markers can be utilised further for a more robust method of cell selection, by creating expression vectors with a weakened promoter for the selection marker gene; this is called selection marker attenuation. In weakening the expression of the selection marker, the stringency of selection will be greater, resulting in only the very select few high-producing clones progressing for further characterisation. Higher stringency can also be achieved by simply increasing the concentration of the MSX or MTX. However, these drugs slow cell proliferation, making selection marker attenuation a more appealing option than ramping up toxic drug concentrations and extend timelines (Lai *et al*., 2013).

Usually, expression of the GOI is driven 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. Several companies and academic groups are designing and constructing completely synthetic promoters to help overcome the challenges that endogenous and viral promoters present (Yohari *et al*., 2019). By matching the promoter sequence to the host cell's transcription machinery, enhanced expression can be achieved. Compared to viral promoters, synthetic promoters perform predictably and provide spatial and temporal control over transgene expression to facilitate biopharmaceutical manufacturing developments.

Engineering of the host cell line to alter gene expression can result in a highly heterogeneous population of cells, single cell subcloning from the cultured population can alleviate the heterogeneity caused by random integration, gene amplification and the inherent genetic instability of cell lines. Subcloning reduces a large portion of the variation, but cell productivity and growth rate can still vary dramatically. High-producing cells have a greater metabolic need owing to the demand of protein production, so consequently, the higher-producing cells have a slower growth profile. With heterogeneity still present in the subcloned population, the lower-producing cells can outgrow and overtake the population of the slower growing high-producers. In order to retain the majority of high-producers, the number of sub-cloning events and length of time in culture should be limited so that the lower-producing cells do not have time to outgrow and overpower the population (Browne & Al-Rubeai, 2007).

The application of automated technologies within CLD workflows has helped to decrease overall timelines and speed up subcloning.

Automated Techniques

As manual cloning introduces a bottleneck because it's slow, low-throughput, prone to contamination, and expensive, these traditional approaches are being substituted or combined with more sophisticated high-throughput techniques and automated platforms to accelerate the CLD process.

Flow cytometry or fluorescence-activated cell sorting (FACS™) has become widely used for high-throughput isolation of high-producing clones to save time, energy, and money. However, one of the leading challenges experienced when trying to optimize and automate CLD workflows is the variation in cell viability and cell growth after sorting. In fact, limited cell viability is a significant issue encountered when using flow cytometry.

Considering this issue, it can seem like there is a trade-off between automation and cell viability. But this perceived trade-off can be overcome by carefully evaluating what technique you should use for single cell processing and dispensing.

Novel microfluidic systems, such as the Sphere Fluidics' Cyto-Mine® Single Cell Analysis Platform, offer new opportunities to address these issues. Unlike flow cytometry, Cyto-Mine® offers rapid yet gentle cell processing. The automated instrument utilizes picodroplet technology to encapsulate and process up to 2 million picodroplets, or up to 40 million cells in pools, in just a few hours. This approach enables the isolation of high-value cells of interest and dispenses with high viability so that CLD can be accomplished more effectively and efficiently (Figure 1).

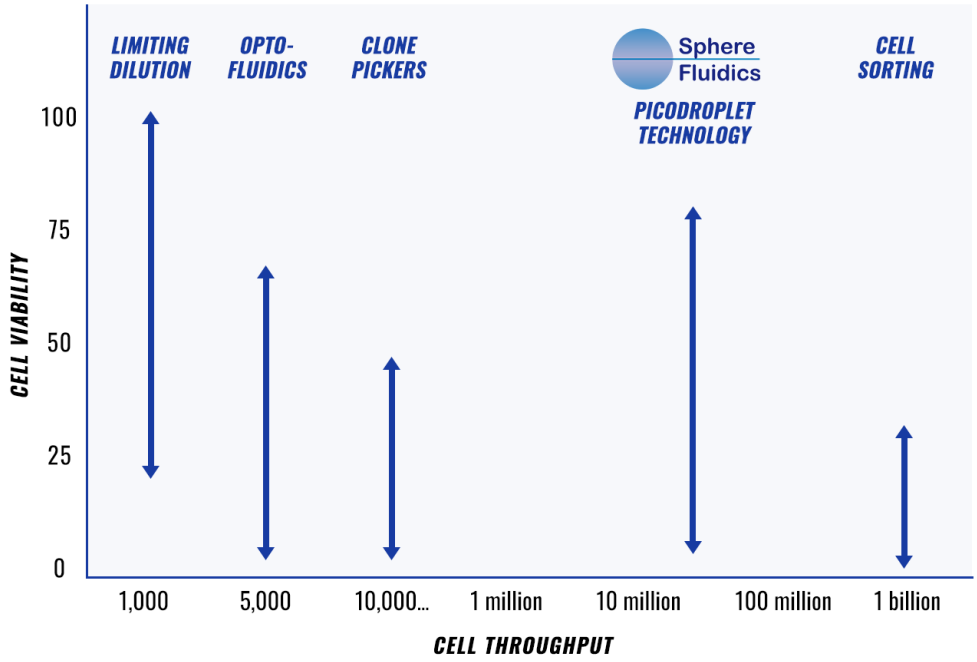


Figure 1. illustrates the high-throughput and high viability provided by Cyto-Mine® compared to alternative technologies.

Alternative methods that replace like for like, manual for automated, include cell printers, colony pickers and, cell-in-well imagers, each of these automate steps of the incredibly manual process of limiting dilution.

Seeding single cells using the limiting dilution technique is very low-throughput, as approximately only 1,000 cells can feasibly be seeded in one working day. Each well that the cells are seeded into must be imaged to trace monoclonality from day zero and throughout culture. Once the cells have grown into colonies, they need to be screened to identify antibody-secreting cells. This whole process is repeated once, and often twice, with a selection of colonies to fully ensure that the antibody-secreting cells can be shown statistically to be derived from a single progenitor. This multi-step method results in a lengthy, tedious process that can take many months to complete before the top clones can be progressed. As mentioned above, this traditional approach can exacerbate cell line heterogeneity and, over time, interfere and reduce the likelihood of finding the highest-producing clones.

Both liquid handling and single cell printers offer an easy, simple substitute to manual single cell seeding. The processing power of single cell printers enables more cells to be seeded and screened in less time, although imaging each well is still a limiting factor. This is where the development and introduction of cell-in-well imagers are beneficial as they can continuously monitor the whole well and track cells from day zero up until colony picking. Newer technologies are combining both single cell seeding and whole well cell imaging, which consequently accelerates the initial stages of CLD before titer screening.

Further automated instruments that replace manual steps include colony pickers, and these can be introduced to remove the initial single cell seeding by enabling scientists to culture a single cell suspension in a 6 well plate in semi-solid agar. In well imagers track each single cell from seeding up until colony formation. These colonies are assayed for antibody secretion *in situ*, and then high secreting colonies are automatically picked and dispensed into microtiter plates. Colony pickers can screen 10,000 cells in approximately 3 weeks, significantly improving throughput and reducing handling time by incorporating continuous single cell imaging, productivity screening, and then colony picking.

Each of these automated technologies replaces individual manual steps in the lengthy process of CLD, marginally making the workflow less labor intensive. Integrated platforms, like Sphere Fluidics' Cyto-Mine®, enable further process optimization by streamlining the workflow to make it more automatable, rather than automating the existing workflow.

Cyto-Mine® offers an innovative solution to extensively manual, and time-consuming workflows, the novel picodroplet technology ultimately enables single cells to be encapsulated into picolitre volume droplets, called picodroplets, where their secreted molecules can be analysed. The picodroplet, simply put, offers a micro reaction chamber in which single cells can be assayed for productivity of protein production. Cyto-Mine® can then rapidly analyse the assay results using fluorescence detection and select out the picodroplets containing the cells of interest. These selected picodroplets are then imaged multiple times to provide evidence of a single cell before

being dispensed into a multi-well microtiter plate. Cyto-Mine® overcomes many challenges faced in CLD by enabling the rapid screening for cells that produce the most antibodies, select out, image and dispense those 'hit' high producers all in a one-day workflow (Figure 2). Even without considering the vast time reduction or the reduction in manual handling processes that this technology can offer, it provides an additional attribute by reducing overall culture time and the need for multiple rounds of single cell subcloning. Consequently, Cyto-Mine® can help negate cell line heterogeneity caused by continued culture and subcloning.

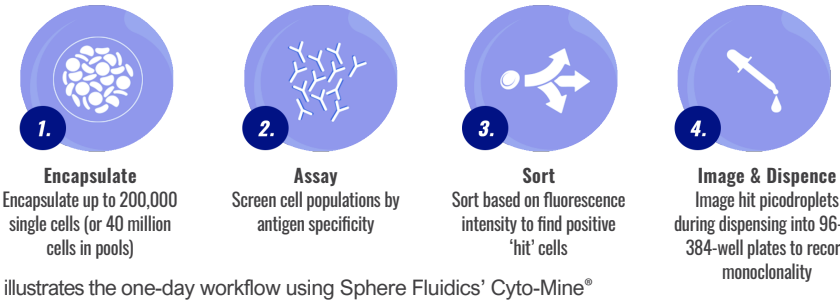


Figure 2. illustrates the one-day workflow using Sphere Fluidics' Cyto-Mine®

Microfluidic picodroplet technology offers an innovative and revolutionary pathway that can streamline workflows by changing how CLD is carried out. By combining multiple manual, labor-intensive processes into one streamlined, automated machine timelines can be significantly reduced, leading to an acceleration of finding the perfect clone.

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 48 or 96 clones can be screened at one 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, labor 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.

Cell Growth

One of the most significant challenges faced in CLD is colonising cells from a single cell progenitor. Cells require a range of supportive growth factors and nutrients to promote proliferation and survival, usually obtained from the neighbouring cells. It is important to remember that at the heart of the challenge is the fact that cells are not single entities and nor do they like being cultured as individual cells.

Optimizing the cell culture medium to support your cell line specifically is a great way to enhance single cell survival and support colony outgrowth. Some of the key considerations when creating and optimizing the medium formula are the cell line, the medium components themselves, and the method used for optimization.

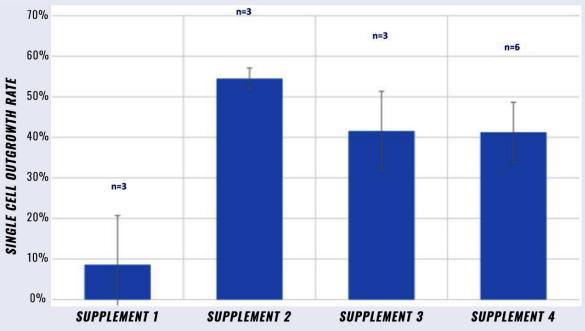
Cell Line

Due to the complexity of cellular metabolism, each cell line requires a unique composition of media. CHO cells, for example, have been shown to have a high degree of variation in their nutritional requirements, even if derived from the same parental host. As a result, each transfected CHO cell line can have a unique set of nutritional needs to support cell growth and protein production. So, it is essential to develop a medium formulation to match these needs as fully as possible.

Components

Standard components in CHO cell medium include water, energy sources such as carbohydrates, amino acids, salts, growth factors, lipids, vitamins, polyamines, and non-nutritional components like buffers. Each of these components can be adjusted and balanced to optimize cell growth, productivity, and viability, consequently finding the right formula can be time-consuming and tedious.

In CLD, there is a large demand that all media components must be chemically defined, to provide consistency across different batches, and free of animal components, to prevent any contamination of transmissible spongiform encephalopathies (TSEs) from animal products. This means that typical cell culture media containing animal sera must be modified, and the cells adapted to serum-free conditions. The culture of cells at high densities can mitigate the effects of removing serum from the medium; however, when cells are subcloned for monoclonality, they struggle to survive. Therefore, alternatives to derived animal components are required, these can be made recombinantly or be sourced from plants, like hydrolysates. Hydrolysates are peptides derived from plant protein such as soy, wheat, or cottonseed, that provide nutrient supplements to the media. A frequently used hydrolysate is Hy-Soy™, produced by enzymatic hydrolysis of soy. Hydrolysates are a well-proven replacement for serum and animal-derived components. Still, they can also be a source of contaminants and are much criticized for batch inconsistency and for not being chemically defined.



There are many commercial culture media available for supporting single cell culture, so we compared 4 commercially available cloning media to see the effect on single cell outgrowth after dispensing from Cyto-Mine®. After 2 weeks in culture, there were vast differences in the percentage of cell outgrowth, highlighting just how sensitive CHO cells are to different medium components.

In addition to recombinant or plant-based proteins, alternatives to animal-derived supplements can be sourced from the cells themself. Cells are known to secrete autocrine factors that are either auto-stimulatory or stimulate the growth and proliferation of surrounding cells. When cells are isolated during CLD, a large part of their supportive environment is removed, consequently disrupting the growth profile of the cell.

Conditioned media is an excellent source of autocrine growth factors from the cells' culture environment, which can help to stimulate single cell growth. Conditioned media can be obtained by culturing the host cells in parallel, concentrating the medium, and then using this as a supplement to support the growth of single cells.

One of the disadvantages of using conditioned media is that, despite being animal origin free, it is undefined, and the components will vary between each harvest depending on the condition of the host cell culture. To classify and define key components of CHO cell-conditioned media, Lim *et al.* (2013) analysed conditioned media from CHO cells using a proteomics approach. Once the key growth factors had been identified, they were able to find suitable recombinant replacements and supplement these into the single cell cloning medium. This extensive study found four autocrine growth factors; fibroblast growth factor 8 (FGF-8), hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), and vascular endothelial growth factor-c (VEGF-C), three of which promoted (FGF-8, HGF, and VEGF-C) and one which inhibited cell growth (LIF). When CHO-K1 cells were treated with basal medium containing recombinant FGF-8, HGF, and VEGF-C cloning efficiency was up to 30%, which was a 2-fold increase in comparison to the non-supplemented control. Lim *et al.* (2013) were able to create a chemically defined, serum-free single cell cloning medium optimized for the growth of their CHO-K1 cell line by mimicking CHO cell-conditioned media.

In addition to autocrine growth factors, paracrine factors such as insulin growth factor-1 (IGF-1), insulin, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), are noted to promote cell survival and growth, these can all be supplemented into culture media to stimulate single cell growth. Although these factors are beneficial, using them alone is not enough to conclusively increase cloning efficiency. There are many supplementary factors that researchers have manipulated to promote good cell outgrowth. Still, it is paramount that this is optimized for each unique cell line to support the survival of the best-isolated clones.

Methods

Traditional methods commonly used in the biopharma industry for medium development follow a step by step approach, changing only one factor at a time (OFAT). This approach is very time-consuming and arduous but has been successful in finding key components that are beneficial for the cell lines used. Other methods, such as media blending and automated screening, have a much higher throughput. Media blending is a simple and efficient approach to medium development. By using already manufactured media, issues such as pH and solubility do not need to be factored. Also, it considers the important point that each of the medium components interacts with each other; this is one disadvantage of the OFAT approach. The metabolism of CHO cells is so delicately balanced that when one component is changed, it can alter the requirement of another. The statistical design of media mixing is crucial to the success of this method. Researchers can draw on lower grade media, which are deficient in key components and blend with media that are significantly different, to effectively identify the best blend that will maximise cell proliferation and titers. Media blending provides a rapid method for finding an optimal medium that supports single cell outgrowth.

Another approach to medium optimization is to investigate spent medium and study the metabolic flux and energy consumption of the cells. With this method, nutritional usage can be calculated when compared to fresh culture medium. Using this stoichiometric approach, the usage rate of essential components such as glucose, glutamine, and amino acid can be calculated and adjusted in the medium formulation to increase cell growth and product titer.

Medium development can be a labor-intensive and time-consuming process as the optimal medium will be bespoke for every cell line. But, an integrated platform like Cyto-Mine® can accelerate the traditionally arduous medium development process. Using Cyto-Mine®, you can run multiple cloning experiments in a short amount of time, to identify the key growth factors that work best for your cell line. Overall many methods that can be used to try to optimize culture media, and it is important to remember that there is no one size fits all.

Sphere Fluidics' Cyto-Mine®

Conclusion

While CLD optimization and process innovation may be necessary for Pharmaceutical and Biopharmaceutical companies working in highly-competitive environments, these changes are likely to introduce new challenges in the CLD process. This article provides an overview of common challenges that may be encountered and how you can avoid them when implementing CLD optimization. The key recommendations include:

- Ensure robust validation of clones to offset selection bias.
- Use targeted integration to reducing clonal variation during cell line engineering.
- Leverage picodroplet technology for high-efficiency, high-throughput, and high viability.
- Design statistical experiment to develop the optimal medium formulation to support cell growth.

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