What's in Your Media?

The Critical Role of Cell Culture Media Analysis in Biotherapeutics Development

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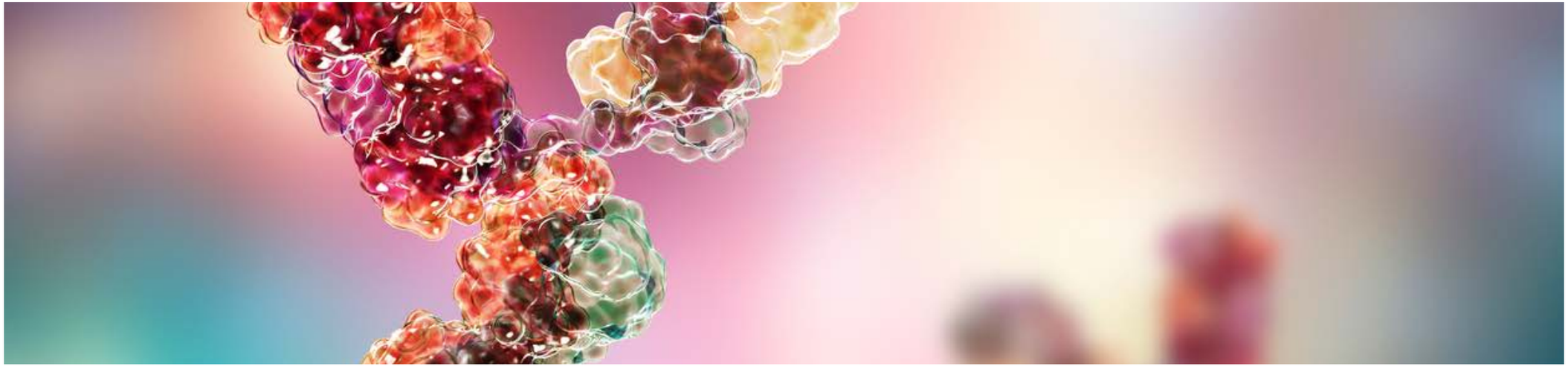
The Critical Role of Cell Culture Media Development and Optimization

Cell culture media remains a key component of biologics-based therapeutics manufacturing. As such, cell culture media development and optimization continue to be important focuses for the biopharmaceutical industry. Increased analytics capabilities have provided new insights into the relationship between cell culture media, the cells they support, and ultimately, the outcome of the final biologics product. That greater insight offers more opportunities to create products faster, more cost effectively, and with greater therapeutic success.

Media development and optimization are critical when looking to streamline biotherapeutic process development. Analyzing how cells consume medium components provides valuable information about the health and productivity of the overall production process. Analysis of key media components — such as amino acids, vitamins, and the metabolites produced as a result of cell growth — is beneficial at all stages of process development, including cell line development, media development, and process optimization. This information provides insight not just on media composition and performance, but also on clone selection, optimal harvest time, potential product quality issues, purification strategies, and more.

Diversity of Biotherapeutic Manufacturing Media

Biotherapeutics is a broad term that encompasses more traditional protein-based biologics like monoclonal antibodies, and also newer therapeutics including cell and gene therapies. These biotherapeutics represent different medical modalities and can address different therapeutic indications as well. They also employ distinct therapeutic vehicles, thus requiring separate manufacturing practices. For instance, with protein-based biologics like antibodies, the final product is not the cells, but the proteins that the cells produce. For cell therapies, the cell is the therapeutic, and for viral



vector-based gene therapies, viral vectors are the key component.

Producing these diverse therapeutics, requires that various cells be used based on the specific application. Since various cells have different requirements, the medium used to culture them must be designed for optimal growth and/or productivity of each cell type. When the composition of the cell culture medium does not match the cells' requirements, manufacturing productivity and product quality attributes can suffer. Therefore, medium must be developed and optimized for each cell type and cell clone.

In this publication, we will look at the different media requirements and optimization strategies for three types of biologics: protein-based biologics, cell therapies, and gene therapies.

Protein-based Biologics Media

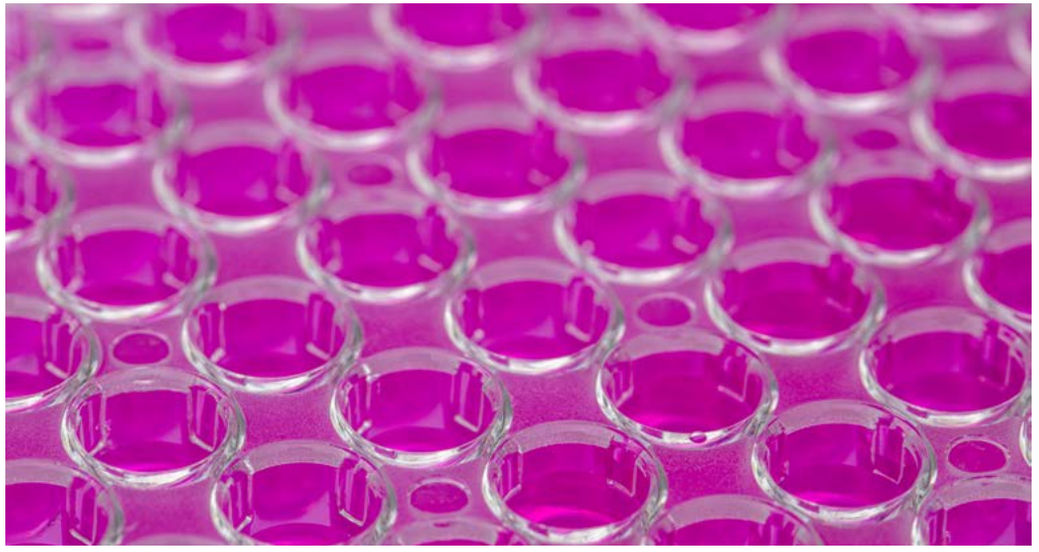
Bioproduction media has been under development and optimized for decades. Chinese hamster ovary (CHO) cells are the most popular choice for commercial production of protein therapeutics, such as monoclonal antibodies (mAbs). Other cell lines such as HEK293 and Vero, also play an important role in the therapeutic space and they are commonly used to produce non-mAb products such globular proteins, viral therapeutics, and vaccines.

Bioproduction media has traditionally been refined by first removing serum, then removing animal-components and other undefined components such as hydrolysates, and finally developing chemically-defined formulations. Our increased understanding of the relationship between cells and the cell culture environment has resulted in vastly improved cell productivity, growth and viability. For instance, mAb titer has been increased from ~ 1g/L to greater than 10 g/L in some instances. In addition, we now know that mAb product quality attributes such as glycosylation patterns, aggregation, charge variants and stability are affected by media composition. Thus, media development and optimization is critically important and can have a dramatic impact on the manufacturability of a product for commercialization.

A typical bioproduction medium optimization effort involves multiple levels of optimization through analysis of spent media and monitoring the effect of supplementation or reduction of individual components on the desired outcome of the culture. Optimization can be a complex and a time-consuming experience due to the large number of possible medium components and the even greater number of possible concentration-dependent combinations. Design of experiment (DOE) can be utilized to reduce the workload, however analysis of each of the possible combinations is still required.

Cell Therapy Media

There are multiple cell types used for cell therapy production. Since each cell type has unique requirements that impact their growth, viability, and functionality, and since the cell itself is the therapy, careful consideration of media components is necessary. For instance, removing serum from media formulations is a particular concern due to the increased risk associated with animal-derived components in media for clinical or commercial



manufacturing. The use of any animal-derived product in medium, including serum, increases the risk of contamination, supply chain instability, and variability. In addition, there is the possibility of introducing adventitious agents, including viruses or prions that may be present in the cells for the final product. However, developing a media without serum or optimizing a media to remove or reduce serum is complex and requires extensive knowledge, time, and resources to complete. Since timelines for cell and gene therapies are frequently constrained compared to protein-based biologics, it makes the task that much more challenging.

The need to develop and optimize a medium specifically designed for that application is universal.

Unlike protein-based biologics manufacturing where established, clonal cell lines are utilized, cell therapies using primary and stem cells present unique challenges particularly in autologous settings. The degree of *ex vivo* cell expansion, differentiation, and functional activation can vary significantly from patient to patient where the starting cells are limited and are often in poor health. Because cell culture media formulations determine cell characteristics (i.e., growth kinetics, health status and functional properties), there is renewed interest in metabolomics to better understand the impact of various media additives such as glucose, amino acids, vitamins, and dipeptides, on critical quality attributes (CQAs) that influence therapeutic efficacy.

Viral Vector Production Media for Gene Therapies

Gene therapies treat diseases through genetic modification, most often taking the form of genetically engineered viral vectors used to deliver a genetic payload to the cells. There are non-viral methods in development; however, viral vectors are still the most popular approach.

Efficient viral vector production is a key element for gene therapy manufacturing success and there is currently a push to meet the high demand for viral vectors. Thus, the industry must transition from small to large-scale commercial manufacturing and increase viral titer production, while still maintaining critical quality attributes. The complexity of viral vectors, significantly more complex than recombinant proteins, has posed biomanufacturing challenges both upstream and downstream including scalability, productivity, and overall lack of robustness. To develop long-term solutions to these problems, manufacturers are looking to optimize both the producer cells and the culture media they are grown in.

Understanding cell metabolism and the impact of medium components, like amino acids and glucose, on titer levels can be done using media analysis studies and is key to increasing productivity. Ideally, the media selected should provide the essential nutrients to facilitate high productivity, consistency and quality as well as being amenable to support scale up to larger manufacturing platforms.

Media Analysis to Enable Development and Optimization

What each of these therapeutic manufacturing processes have in common is the need for in-depth analysis of the impact that the medium has on cells in culture, and consequently, the final product. While each may have different process challenges, productivity targets, and manufacturing requirements, the need to develop and optimize a media specifically designed for that application is universal.

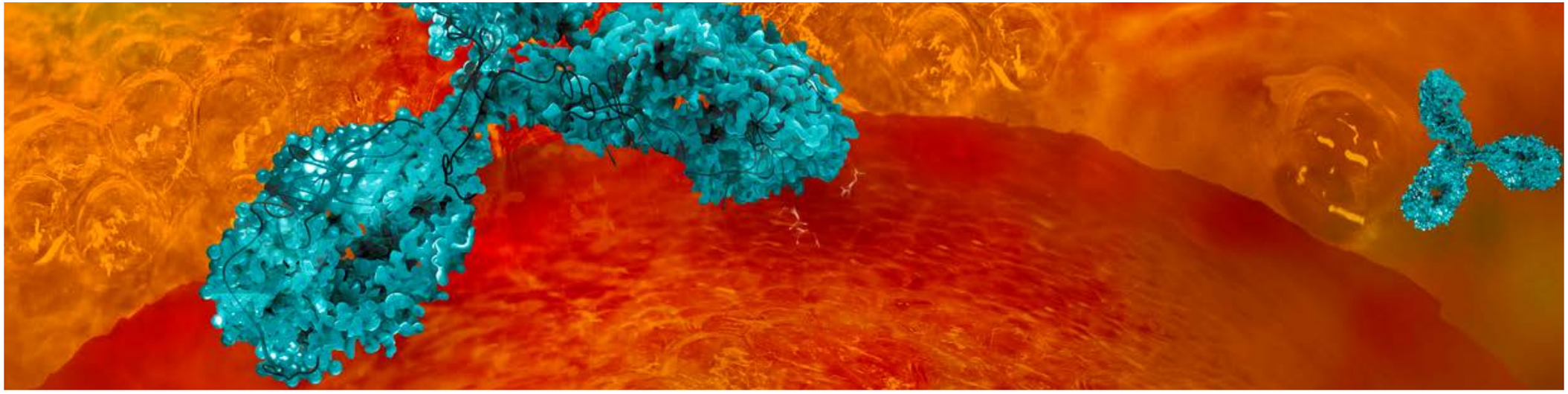
In each scenario, product quality is critical when examining the relationship between media components, how those components are consumed, metabolites produced, and the overall impact on growth, and productivity. While spent media analysis data is vital to moving media development and optimization forward, it can be difficult and expensive to obtain in a timely

matter. Frequently, process development groups do not have the analytical tools or resources in their lab to engage in the in-depth analysis required. This means sending samples to on-site or off-site labs for analysis. Outsourcing can take days to weeks to obtain results and can be quite costly. This results in PD groups having to make difficult decisions about the number and frequency of samples that can be sent for analysis. Inevitably, this creates delays in process development and an incomplete picture of what is happening in culture.

A better solution is at-line spent media analysis. Real-time analysis also supports process analytics technology (PAT) initiatives through monitoring and maintaining critical quality attributes to ensure product quality and batch-to-batch consistency. Waiting until the end of a culture run for spent media analysis results does not inform operators about the day-to-day process nuances. Running this analysis in real-time speeds process development and opens up the possibility to affect change in real-time instead of having to wait for the next run to implement changes.

The large number of samples generated when using a microbioreactor system can also put a strain on analytics resources. The development of scaled-down bioreactor models for media development has been a breakthrough in that they permit simultaneous testing of many different culture conditions in a fraction of the time. However, the number of samples generated is substantial, and the volume of these microbioreactors is small, thus creating another bottleneck and limiting the ability to run samples daily.

To address the challenge of obtaining at-line media analysis, microfluidic analysis technologies have been developed to allow for measurement next to the bioreactor. One such device is the REBEL analyzer which combines capillary electrophoresis with high-pressure mass spectrometry. An analyzer like the REBEL provides several advantages over classical analytical methods by providing near-instantaneous readings at-line, and with a simple-to-operate interface within a small footprint. The near real-time monitoring of CQAs has direct implications on the therapeutic efficacy, safety, product quality, and batch-to-batch consistency. These advances in medium analysis should support the industry “into the future with expanded capabilities in process modeling and predictability to not only ensure high productivity with consistent product quality but also provide process control.



New microfluidic technologies, **like the REBEL, have advanced over classical analytical methods** by providing near-instantaneous readings, at-line, with a simple-to-operate device within a small footprint.

Cell Culture Medium Development and Analysis for Bioproduction

Over the past two decades, the protein biologics market has exploded and now comprises the fastest growing segment in therapeutics. Chinese hamster cells (CHO) have become the host cell line of choice for the majority of commercial production of protein therapeutics, such as monoclonal antibodies (mAbs), on the market.[1] Other cell lines such as HEK 293 and Vero also play an important role and they are commonly used to produce non-mAb products such globular proteins, viral vector therapeutics, and vaccines.[2]

The impact of cell culture medium optimization: productivity, quality, and overall success

Early work by Harry Eagle first demonstrated the importance in medium composition in the growth of cells in culture.[3,4] Among his findings, Eagle reported that 13 amino acids are essential for cell growth. Richard Ham reinforced the importance of component selection and optimization in development of F12, a fully synthetic, chemically defined, and serum-free medium for single cell cultivation and expansion of CHO cell cultures.[5] F12 medium, however, was not well suited to support growth of cell to densities above 10^5 cell/mL.[6] Work by Sato and colleagues created the improved formulation DMEM/F12 via supplementation with hormones and growth factors and by blending 50:50 with Dulbecco's MEM (DMEM) which provided increased levels of key amino acids and vitamins and additional trace metals.[7]

Since these early achievements, serum-free medium designed for bioproduction has further advanced and has resulted in vastly improved cell viability, growth, and productivity. A large amount of knowledge has now accumulated, particularly for CHO cells, that demonstrates the importance of medium composition. These efforts have played a major role in increasing

mAb titer production from ~ 1 g/L to greater 10 g/L, in some cases.

Media studies have shown that ingredient composition and their respective and relative concentrations can dramatically improve or decrease medium performance. The effect is not limited to energy components (Glucose, glutamine, others) as amino acids, and other ingredients have a key role as well. Groups from MIT and U. Minn. pioneered the concept of stoichiometric analysis of cell growth and amino acid utilization in basal and feed formulations according to nutrient demand; thus preventing depletion of key nutrients while minimizing toxic metabolic waste.[8-10] Other approaches such as metabolic, gene analysis, and others have been developed as well. These studies show that both essential and non-essential amino acid composition can have a dramatic effect on cell metabolism as pathways can shift due to the levels of amino acids in the medium.[6,11]

There are numerous examples in the literature of the importance of amino acid concentration on CHO cell metabolism and production that demonstrate that maintenance of most amino acids at specific concentration ranges is very important for CHO culture [for review see (6)]. Thus, it is not the total amount of amino acids that is critical but rather a balanced composition that meets the needs of the individual cell line[6,12,13] or individual cell clone.[14]

Amino acids can also play a role in other cellular function outside of protein synthesis and energy source. Reports indicate that some amino acids can act as signal molecules, and may influence the rate of apoptosis in cells and other cellular parameters.[15] Researchers have also identified that some amino acids at certain concentrations can offer some protection from elevated ammonia[6,12] or osmolality.[12,16] Chen and Harcum reported that Thr, Pro, and Gly at a level of 20 mM are able to mitigate the negative effects of ammonia.[17] McAtee Pereira showed Gln, Glu, Asn, Asp, and Ser levels can reduce ammonia production while preserving cellular carbon flux[23]. Others have found that care must be taken to avoid excessive amounts of some amino acids such as Asn and Gln, to avoid excess production of detrimental waste.[6,11] Note that some amino acids such as Cys, Tyr, Trp, can have low solubility and others can be unstable in some formulations (Gln, Cys), and these need to be carefully monitored in media [for review on solubility see (18)].

Studies have also shown that mAb quality attributes, such as glycosylation pattern, aggregation, and charge variant can be affected by media composition as well.[2,13,19-21] Beyond amino acids, additional examples include vitamins, biogenic amines, and others. For example, depletion of the vitamin choline can have a dramatic effect on both mAb titer and mAb aggregation.[22,23] Thus, there are ongoing efforts to optimize media formulations to enable production of biotherapeutics at high levels and of high quality. Investment in medium optimization, and the control of media composition, can have a dramatic impact on the overall outcome of a bioproduction effort-as well as shortening the developmental pathway

The important role of medium component analysis

Current chemically defined production media typically contain 50 -100 ingredients, which include sugars, amino acids, vitamins, biogenic amines, metals, buffers, and others.[6] A typical medium optimization effort usually involves multiple rounds of optimization through analysis of spent media and monitoring the effect of supplementation or reduction of individual components on the desired outcome of the culture. Optimization can be a complex and a time-consuming experience due to the large number of medium components and the even greater number of possible concentration-dependent combinations. Design of experiment (DOE) can be utilized to reduce the workload somewhat.[6,13,24] Approaches to develop a mathematical model (digital twin) will further help in decreasing the number of physical experiments but also require input from the cell culture system.[33] Nevertheless, a medium development effort started from scratch can require months to achieve a highly optimized formulation.

Complete optimization of production extends beyond the base medium. Development of a feed-based strategy that presents components at optimized concentrations during the exponential growth phase and

production phase can result in significant improvement that sometimes reaches several-fold [13,22,25]. Thus, medium composition, medium optimization, and the degree of monitoring and control[26] can have profound effects on cell metabolism, cell health, and product production and quality.

The medium also needs to support scale up efforts from single cell cloning, to clone selection to expansion in mini-bioreactor[27] (Ambr© type), to bench-top bioreactor, to large scale. This can include perfusion bioreactors at the N-1 or in the production vessel, which use medium exchange to achieve increased cell densities and output by removal of toxic metabolites.[28,29] The effort to optimize and monitor media throughout development and production phases can result in large benefit at each step of the process development.

The need for at-line and efficient analytical capability to monitor medium composition is central to the effort to develop, optimize, and control media formulations. This desire for robust analytical methods is compounded by the fact that each cell line/clone can a have different metabolic demand, and thus compounds the need in a multi-line production environment. Sadly, much of current bioproduction is accomplished without fully optimized formulations due to poor workflows, cost, or time constraints. Formulations are also often under-optimized due to sampling media from only one or two time points in the cell growth curve. Examples of such are end-of-growth-phase or end-of-culture, both of which typically lie outside of the most productive phase of the cell growth curve. Early phase development is focused on speed and often uses a ready-to-go commercial formulation. Late phase development focuses on further optimization but cost and lack of accessible analytics can prevent a full optimization. Thus, many media currently in use are not fully optimized to due to lack of a robust analytical solution.

The limitations of classical analytical methods

The most common method of quantitating medium components is by classical methods; typically, HPLC. This method requires experienced operators, optimization of conditions, the use of amino acid labelling reagents, and specialized columns for each type of component. While HPLC methods are effective they can be expensive as dedicated facilities may be required. Often sample turn-around is less than desired due the complexity of the task or because of a backlog at the testing facility. These delays, in turn, negatively impact the process development timeline. Moreover, the sample size requirements for these classical methods may not allow frequent analysis of samples from smaller-scale developmental platforms such as microbioreactors.

Outsourcing quantification is an option, however it is expensive and time consuming as it involves shipping of frozen samples and ultimately analysis is performed using the same time-consuming classical methods. The additional time for shipping further extends the timeline of media optimization. Thus, these classical strategies typically do not provide actionable data, as it can take days to weeks to receive testing results.

The REBEL can analyze a broad panel with multiple amino acids, vitamins, and bioactive amines in minutes.

To address the current testing problems, the REBEL analyzer, by 908 Devices, uses microchip capillary electrophoresis coupled with high pressure mass spectrometer (CE-HPMS) to streamline media analysis. The REBEL can analyze a panel with multiple amino acids, vitamins, and bioactive amines in minutes. This new, affordable, easy-to-use technology provides multiple benefits and can substantially reduce timelines for medium and feed development. The affordability of this analytical method available at the point of need supports analysis of more time points within a production time course. In addition, the near real-time capability, and small footprint of the Rebel enables at-line analysis, that in turn, can support process analytical

technology (PAT) strategies and the potential employment of mitigation strategies, if used at-point during a production campaign.

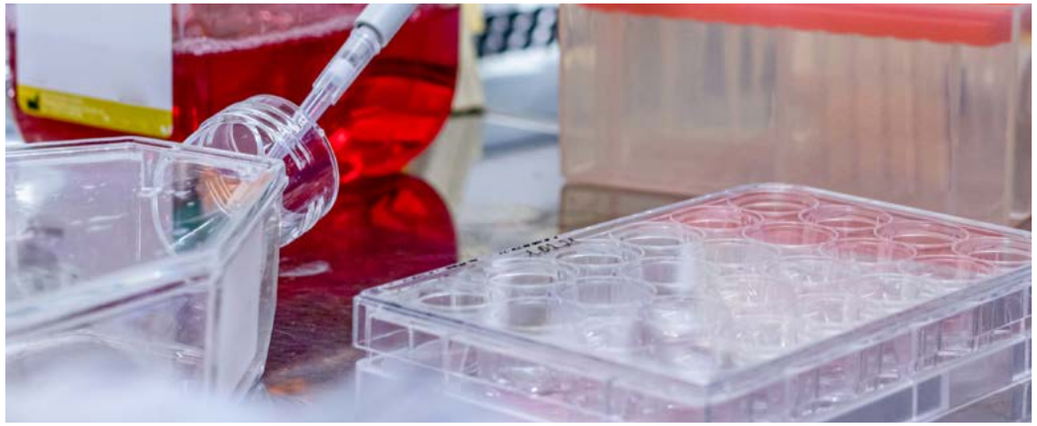
The three general methods available for media development

Generally, there are three options to implement a media development effort for bioprocessing applications which consists of: 1) use of-the-shelf commercial media and feeds, 2) outsourcing media development, or 3) in-house media development.

Off the shelf media

There are numerous off-the-shelf media and feeds available on market. These have the limitation in that they were developed using the cell line/clone of the vendor and vendor's developmental system (bioreactor and process). Thus, commercial formulations are optimized to someone else's cell line, rather than your own.[13]

This can result in vast underperformance of these formulations in your own production line(s) and may require extensive screening of media from several vendors to identify an appropriate formulation. Furthermore, the variation in nutrient demand between clones of the same cell line (example CHO) can further complicate the robustness of off-the-shelf solutions. It has been shown that amino acids can be easily depleted in some off-the-shelf media.[13]



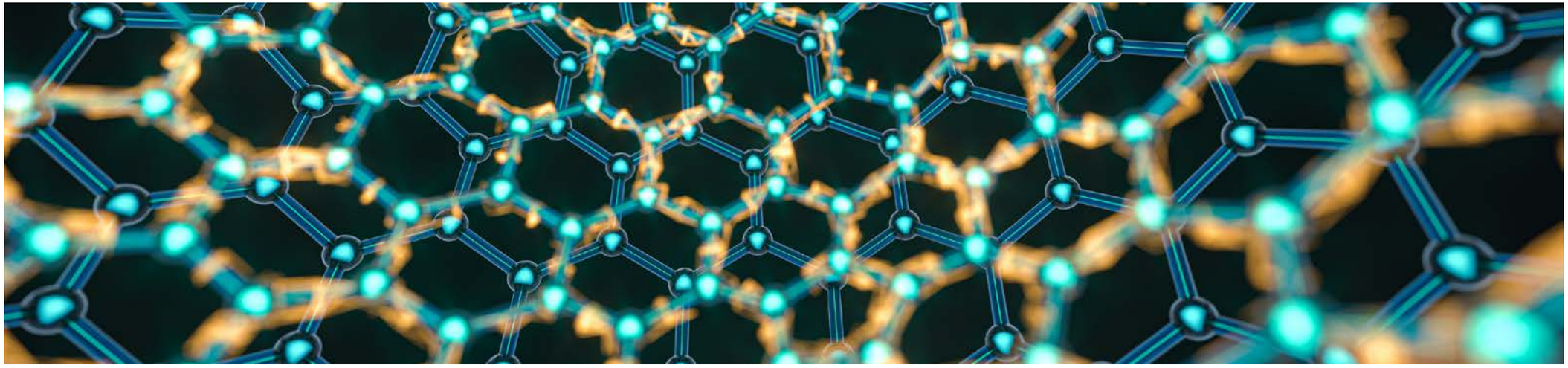
Using off-the-shelf media does not eliminate developmental efforts, as typically several media and feeds must be screened to maximize desired outcome.[13] Another limitation with this approach is that you do not know the complete formulation and list of ingredients. Thus, therapeutic producers lack comprehensive knowledge of their cell system and their process, and cannot be certain that their production is fully optimized and controlled. Sometimes, a therapeutic producer will find that there are no satisfactory off-the-self options that provide the desired outcome.

The main advantage of utilizing off-the-shelf strategies is savings of time and expense. Use of pre-formulated solutions can reduce time to market through a shortened developmental timeline. They can also reduce costs, particularly short-term cost, with possible consequence of greater overall cost.

Outsourcing media optimization

Outsourcing media optimization has resulted in the advancement of custom institution-specific "platform media" that can offer increased performance over off-the-shelf-solutions. However, this solution also has limitations in that the medium is only optimized for the cell line/clone that is sent. Full optimization of a medium may take months, even with experienced service providers. Furthermore, the therapeutic producer will not be able to gain first-hand knowledge of their cell line from the developmental process.

Since these outsourced formulations are developed to a clone that is provided to them, in their production vessel, there are often problems with use during culture scaling. Cells early in the selection and amplification process can have a vastly different nutrient demand than small-scale culture, and again from cells grown in high-density multi-1000 liter bioreactors. This problem can be mitigated somewhat by development of specific media for each phase of need. It is not uncommon for producers to develop several formulations and feeds for the production phase, which are then screened and further optimized, similar to off-the-shelf solutions, in order to mitigate in-house clonal variation.



In-house media development

In-house development has distinct advantages over other options as the therapeutic producer gains knowledge of their cell line, will know the formulation, and can be reasonably certain that an optimization technique is carried out to a high level. However, this approach has limitations, such as the need to acquire expert personnel and analytical capability. The timeline can be longer than with other solutions if a complex build out of equipment is required. Nevertheless, acquiring medium analytical equipment with at-line capability can provide long-term benefits, as it can be employed during product production as part of a process analytical technology (PAT) strategy.[26,30]

Combination Approach

Often a combination of strategies can be considered such as outsourcing development with a limitation for off-the-shelf components, rather than the creation of a highly optimized custom media. Other combination strategies may include using off-the-shelf solutions early in production to hasten speed to market and later using fully optimized media to improve efficiency and lower long-term costs.

Media QC testing

QC testing of cGMP media is usually performed by the service provider and QC of non-GMP developmental media can usually be performed for an additional fee. However, it is a smart approach to self-validate any acquired media, so possible mistakes in development or bioproduction can be avoided. Studies have found wide variation in expected levels of ingredients in off-the-shelf media. Some results showed as much as 2.5x expected levels and others were not present at all.[31] For this purpose, the REBEL analyzer is also of great value as it can analyze a robust panel of dozens of media ingredients onsite in just a few minutes. This provides the upmost confidence that the media purchased is composed as promised and that additional lots will perform as expected, thereby reducing any possible variability in performance.

Cell culture media analysis and process analytical control (PAT)

The drive towards an in depth-understanding of a production process effect on product quality is partly driven by the US Food and Drug Administration's (FDA) process analytical technology (PAT) initiative that was introduced in their Guidance to the Industry document in 2004.[32] The PAT framework seeks to ensure the quality of pharmaceutical products through real-time monitoring of the process.[26] Identifying the sources of variability in a process,

variability management, and determining whether product quality may be affected are components of PAT.[26]

Many bioreactor conditions such as DO, temperature, conductivity, pH, p02, pC02, metabolites, cell viability, density, and productivity can currently be measured by either in-line sensors or near-line analysis of culture fluids with bioanalyzers. For a more in-depth analysis of medium conditions, media analyzers such as the REBEL can provide near real-time analysis (7 minutes) of >30 different amino acids, vitamins and biogenic amines.

The small footprint of the REBEL also enables use at-line where the analyzer can be placed on bench or countertops along with other analytical equipment or placed on a cart for movement to different locations. Furthermore, this device requires a sample volume of only 10 µL, which is just a fraction of the sample already taken for other biochemical analyses. Other aspects that support integration into a PAT initiative include: simple operation (load and push a button), 24/7 availability (unlike many in-house analytical facilities), software that is designed for cGMP compliance with support of 21 CRF Part 11 for digital documentation, and .CSV file format for integration into other software systems.

Additional benefits of at-line medium analysis

The advancement of optimized media and feed strategies for fed-batch culture and optimized media for perfusion bioreactors has played a large role in advancing volumetric productivity into the multi-gram/L range. A properly constructed medium strategy can potentially increase productivity several fold. One reason these strategies work so well is because they ensure that media components are present and maintained at an optimal concentration. Thus, near real-time, at-line analysis can be used to monitor whether your feed or perfusion strategy is operating as expected.

In addition to monitoring and control, at-line medium analysis may enable a mitigation pathway for adverse events due to unexpected medium or feed imbalance. This in turn, could increase the overall efficiency of the production campaign.

Summary

Much of the increase in productivity since the start of the bioproduction era decades ago has been due to optimization of cell culture media to support cellular health and to maximize product productivity and quality. With this advancement in media formulation and feed strategies, there is increased need for robust analytical tools. Many of the classical methods of media analysis were burdensome, slow, expensive, and limiting.

New microfluidic technologies, like the REBEL, have advanced over classical analytical methods by providing near-instantaneous readings, at-line, with a simple to operate device within a small footprint. These advances in medium analysis should support the industry into the future with expanded capabilities that ensure high productivity and consistent product quality.

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New analytical platforms like the **REBEL have broad utility across cell therapies** from development through to commercial manufacture.

Cell Culture Media Analysis for Cell Therapy Applications

In the field of regenerative medicine, cell therapies are increasingly becoming viable therapeutic alternatives to traditional treatments for a wide range of diseases. Most recently, T-cells have been the focus of much clinical investment with chimeric antigen receptor (CAR) T-cell immunotherapies for blood cancers, but other cell types including mesenchymal stem cells (MSCs) and other immune cells (i.e., NK cells, macrophages) have also been investigated for their therapeutic potential. Regardless of the cell type, understanding the best way to culture the cells *in vitro* to achieve optimal cell activation and expansion, while maintaining functionality is key to the success of these "living medicines".

One of the keys to success lies in the cell culture media used to culture these cells after isolation. Since each cell type has unique requirements that impact their growth, viability, and functionality, and the cell itself is the therapy additional scrutiny of the media used during cell therapy development is necessary. There is a wide range of cell culture media available from do-it-yourself recipes to commercially available one-size-fits-all complete formulations. Because of the diversity of compositions and a multitude of options for each cell type, it can be a challenging landscape to navigate when identifying the optimal culture media for your cell type.

Additionally, many companies developing cell therapies are looking to lower the very high COGs for current autologous cell therapies by developing allogenic cell therapies which will allow better scalability and standardization but still have safety and efficacy issues. There are rigorous safety and regulatory guidances that restrict certain additives that could pose a health risk to patients, from inclusion in cell culture media. This has resulted in increased investment in developing xeno-free and/or chemically defined media alternatives. Again, highlighting the importance of cell culture media in the development, manufacture and commercialization of any cell-based therapy. Overlooking this aspect and failing to plan appropriately can result in inconsistent performance and quality of the cell therapy and present roadblocks in moving it successfully through regulatory approval. These challenges can ultimately reduce speed to market, which is particularly critical to cell therapies.

Cell therapies are increasingly becoming viable therapeutic alternatives to traditional treatments for a wide range of diseases

Rise Of Metabolomics Analysis To Improve Clinical Success

Unlike traditional biopharmaceutical manufacturing where established, clonal cell lines are utilized, cell therapies using primary and stem cells present some unique challenges particularly in autologous settings. The degree of *ex vivo* cell expansion, differentiation and functional activation can vary significantly from patient to patient where the starting cells are limited and are often in poor health. Because cell culture media formulations can influence cell characteristics (i.e., growth kinetics, health status and functional properties), there is renewed interest in metabolomics to better understand the impact of various media additives such as glucose, amino acids, vitamins and dipeptides, on critical quality attributes (CQAs) that

influence therapeutic efficacy[1,2]. The effects of metabolism on CAR T-cell efficacy have important implications for improving the quality of T-cells used in adoptive immunotherapy. The potency of therapeutic CAR T-cells has been found to be enhanced by extrinsic factors present in the medium during the manufacturing process[3]. A thorough understanding of metabolic mechanisms that regulate gene transfer, metabolic fitness, effector function, and persistence may facilitate stepwise improvements in the cell culture media used for *ex vivo* T cell production. The variety of media formulations intended for therapeutic applications merits comparative metabolic profiling to gain deeper insights into the contribution to exogenous nutrient levels that impact cell metabolism for each specific target.

Another area where metabolic profiling has utility is in the process development stage to inform design of experiment (DOE) optimization of cell culture media formulations to improve overall manufacturing success. Capillary electrophoresis coupled to mass spectrometry (CE-MS) is a rapidly emerging analytical tool for metabolomics investigations to gain more insight into cell culture media, able to separate analytes with high efficiency and at high speed. The at-line CE–MS device, REBEL from 908devices allows rapid quantification of amino acids and vitamins and has the potential to quantify other media components much faster than conventional HPLC-based analytics. Both untargeted methods capable of fingerprinting the complete metabolite profile and also targeted methods enabling the precise and accurate determination of a selected group of metabolites have been developed to analyze the media efficacy[4].

As an example, arginine has been identified as a key amino acid for T-cell proliferation and thus its inclusion in media formulations for *ex vivo* expansion in CAR T workflows is required to obtain the necessary cell numbers for activation and genetic manipulation[5,6]. However, as shown in Figure 1, a wide range of arginine concentrations can be found across a panel of commercially available media formulations, which can have a significant impact on T-cell performance.

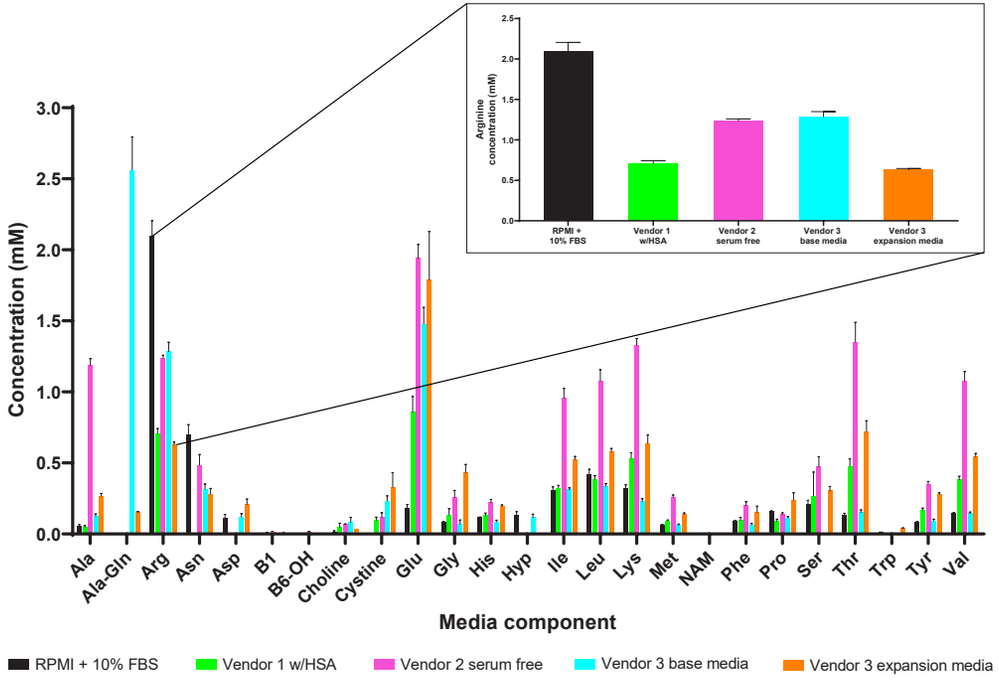


Figure 1. Amino acid analysis across 4 different commercial T-cell formulations shows a wide concentration range for the amino acid arginine, which is essential for T-cell activation and proliferation. Error bars are from the standard deviation of n=5 replicates. All media samples diluted 25x before analysis on the REBEL with no additional sample preparation.

The unprecedented efficacy of CAR T-cell therapies to treat hematologic malignancies has also led scientists to investigate this approach for solid tumor cancers. However, the immunosuppressive tumor microenvironment has proven to be difficult for T-cells to overcome. As such, strategies are being developed to improve T-cell metabolic fitness to overcome the hostile tumor microenvironment (TME) by pre-adapting the cells *in vitro* in culture media that is more reflective of the oxygen and nutrient status of the TME to prime them towards more effective and robust anti-tumor response once reinfused back into the patient[6,7]. Metabolic analysis of commercially available

T-cell media formulations suggests they are not reflective of physiological conditions, and thus may require fine-tuning to ensure CAR T-cell readiness *in vivo*.

Similar conclusions have been made with human mesenchymal stem cells (hMSCs) for clinical applications. MSCs are multipotent adult stem cells that have promising therapeutic potential for tissue engineering applications because of their ability to both differentiate into distinctive mesenchymal phenotypes and to induce a regenerative microenvironment by secreting bioactive chemical compounds. However, clinical success has been hampered by poor cell survival and engraftment upon transplantation into the site of injury. The transplanted MSCs experience a hostile, injured microenvironment of ischemia (lack of oxygen and nutrients), inflammation, and oxidative stress, which negatively imposes metabolic stress on the cells, affecting their ability to exert a positive therapeutic effect. Scientists are investigating metabolomics strategies to prime the MSCs *in vitro* prior to transplantation to improve their survival to increase the overall therapeutic potential of MSCs[8].

Fresh Media Analysis

Serum Removal

For clinical applications, there is a shift in the industry to cell media platforms that exclude serum and other animal-derived raw material components to establish more defined growth parameters to improve consistency, mitigate contamination and immunogenic risk, and reduce COGS.

Conventional media used for isolation and expansion often include supplementation with fetal bovine serum (FBS) at 5–20% (v/v). FBS has been a widely utilized media additive because it contains a high levels of nutritional and physiochemical compounds required for attachment and growth. However, safety and regulatory concerns raised by the use of animal serum for clinical use of autologous or allogeneic human blood-derived materials has increased interest in identifying defined serum-free media formulations. Identifying critical factors and their concentrations with an eye towards designing an ideally formulated, defined serum-free medium should be carried out using rational and systematic approaches. In-depth global analysis of serum-containing media can facilitate reformulate efforts to replace serum with more defined components or to provide insight into commercially available, proprietary formulations (Figure 2)[9,10]. The REBEL has a sensitive dynamic range of 5-100uM of detection to detect low levels of amino acids and other nutrients accurately, without interference from serum proteins, unlike other quantitative methods.

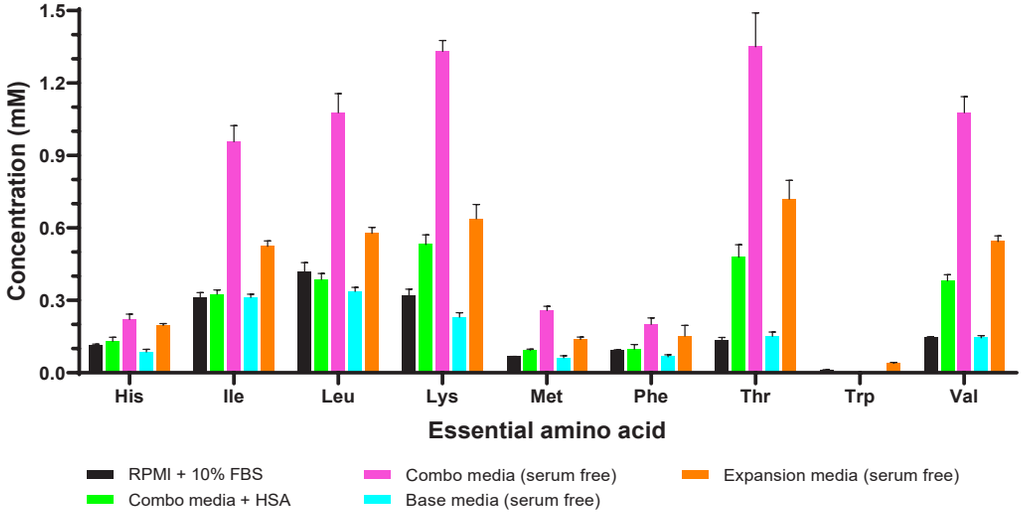


Figure 2. Amino acid profile across five media formulations with and without serum for T-cell expansion. Error bars are from the standard deviation of n=5 replicates. All media samples diluted 25x before analysis on the REBEL with no additional sample preparation. With this quick insight from the REBEL, researchers can make informed decisions about which media platform is best for growing CAR T-cells.

Implementing defined media formulations into cell therapy manufacturing would enhance consistency across cell bioprocessing protocols with the provision of a more uniform and controllable environment, which may be crucial to boost clinical efficacy.

Media Optimization

Another reason to focus on media development is to optimize the formulation to maximize the expansion of your cell type to increase output while reducing cost of goods (COGS). In an autologous setting, each patient's health status and demographics translates to a unique cell population, which imparts variability to the subsequent cell therapy manufacturing process emphasizing the importance of in-process controls to ensure a consistent drug product is produced in each and every manufacturing run even in the presence of such variability. Process analytical technologies (PATs) can provide visibility into and information on critical quality attributes (CQAs) throughout the manufacturing process and QC release testing where speed, accuracy and throughput are needed to provide real-time process feedback for optimization efforts and to speed product release. The focus of PAT within the Quality by Design (QbD) framework has highlighted a need for better analytical tools that can allow rapid and frequent monitoring to inform process development in real-time. Current analytical methods suffer from long lag time between sampling and data generation ranging from days to weeks, which is prohibitive to effective design of experiment (DOE) methodology. Additionally, these assays may lack the resolution needed to show subtle changes that may result from incremental process optimization steps.

Spent Media Analysis

Targeted metabolomics-based PAT allows for metabolic profiling of the T-cells throughout the manufacturing process through spent media analysis. Particularly with the adoption of more automated and closed processes for large-scale commercial manufacturing, analytical tools that can be operated at- or on-line to minimize manual handling time are desirable. Traditionally, groups involved in process development do not have access to the necessary analytical tools or resources required for this type of analysis. Outsourcing analysis to core labs or CROs can take days to weeks from sample-to-result, which can severely delay process development and PAT implementation efforts.

The REBEL is built for purpose, enabling frequent, at-line measurements from the bioreactors for active, near real-time monitoring of CQAs, which has direct implications on the therapeutic efficacy and safety, to ensure product quality and batch-to-batch consistency. Its small, self-contained footprint fits alongside bioreactors and has the ability to rapidly analyze samples to provide quantitative identification and analysis on a panel of >30 analytes including all the key amino acids, biogenic amines, water soluble vitamins and dipeptides with accuracy and reproducibility (Figure 3). Integrating data reporting capabilities with portable report formats and automated performance qualification and 21 CFR Part 11-compliant software make the REBEL easy to integrate into cGLP/cGMP environments.

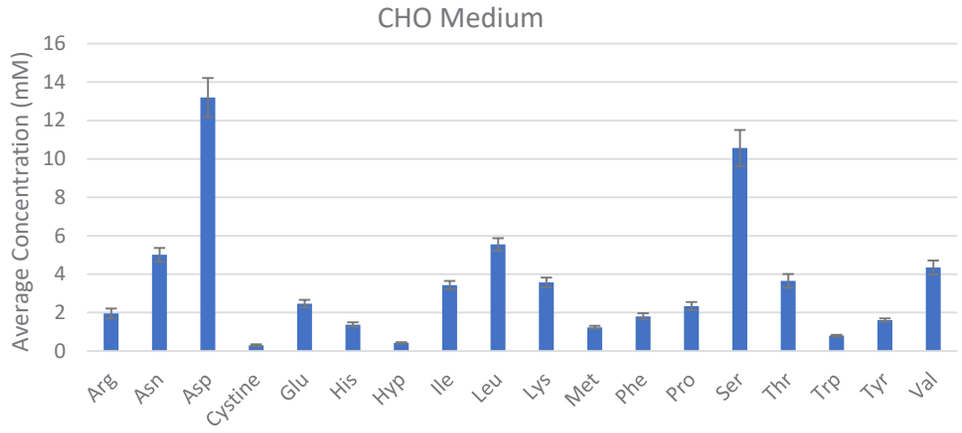


Figure 3. Average concentration of amino acids measured on the REBEL analyzer in chemically-defined media. 6 samples of the medium were prepared, aliquoted 6 times into a 96 well plate and the REBEL performed 5 replicate measurements for each sample (n=180). Average concentrations and standard deviations for each amino acid detected are shown.

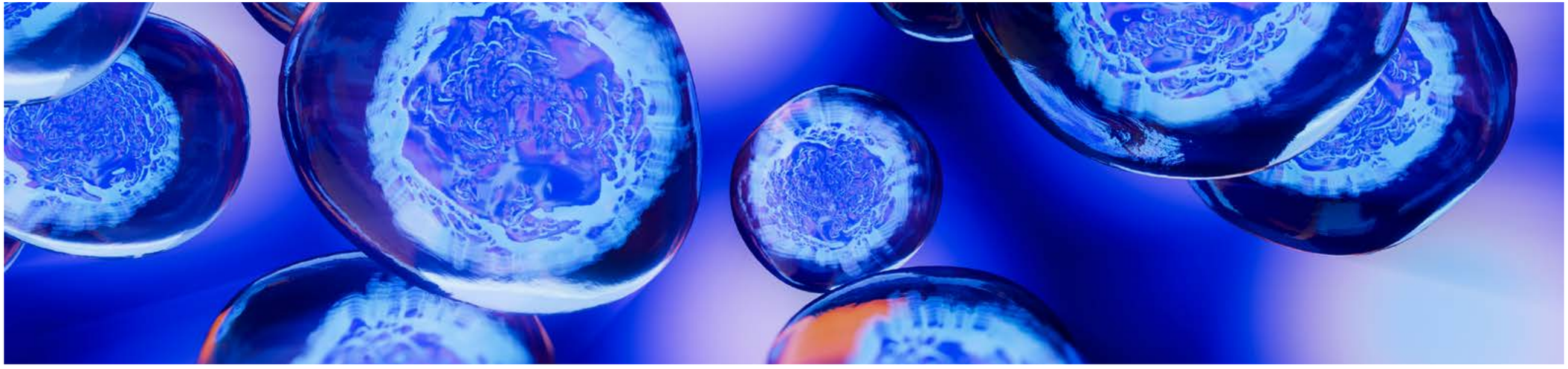
Scale out and scale up are two paradigms to increase production for cellular therapies, each with their own distinct challenges. Scaling out involves increasing the number of operation units in parallel to increase capacity whereas, scale up increases the size of the operation unit, which typically

means moving to larger culture vessels that are different than what was used in small-scale production. Scale out options are easy to implement since the culture parameters do not change but inefficiencies in the scale out model may result in increased COGS that doesn't meet long-term product needs. During scale up to larger bioreactor platforms, the change in vessel size, agitation and other physiological parameters can affect growth and cellular performance, which can require extensive process optimization to ensure that CQAs are still met. In some cases, it may be necessary to select a different cell culture media or the addition of additional factors to maintain phenotype control and optimize cell growth without inducing other changes that could result in a loss of functionality.

A Perspective On Outsourcing

In efforts to bring therapies to market sooner, many companies partner with CMOs and CDMOs to aid in the movement of their cell therapy products through all phases of clinical development. However, with regards to outsourcing, the developer may have less oversight over the manufacturing process, and disruptions at the CMO or CDMO can create bottlenecks for the developer, particularly if they rely on a single CMO or CDMO to fulfill their needs[11]. Because many of the current generation of regenerative medicine products are unique and highly specialized, requiring customized components and non-standard technology approaches that need specially trained staff, traditional CMOs/CDMOs struggle to adapt to these bespoke requirements. This can ultimately delay tech transfer and extend timelines that negatively impact the developer and their patient population especially when speed to market is so important. Additionally, industry experts recommend that every developer should keep some aspect of manufacturing in-house even when partnering with a contract organization for certain aspects of the manufacturing process to build process knowledge and understanding, advantageous when seeking regulatory approvals[12].

New analytical platforms like the REBEL have broad utility across cell therapies from development through to commercial manufacture. The speed and throughput of CE–MS based approaches to deliver in-depth spent and fresh media analysis can be leveraged to improve the process understanding and efficiency and maintain visibility and control throughout all stages of cell therapy production with the ultimate goal of bringing these life-saving therapies to patients faster.



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The small footprint of the REBEL analyzer allows it to reside in the process development space or manufacturing suite alongside the bioreactors

Cell Culture Media Analysis for Gene Therapy

The genes within our cells contain the necessary information to direct the production of proteins and enzymes to support normal functions in the body. However, when a gene or part of a gene is defective, mutated or missing, this can disrupt normal functions manifesting in a number of health problems and diseases. Gene therapy aims to treat diseases through genetic modification, most frequently genetically engineered viral vectors are used to deliver a genetic payload to the cells. There are non-viral methods in development, however, viral vectors are still the most popular approach with two-thirds of the clinical trials to date delivered via viral vector.[1] It has the potential to transform medicine and create new therapeutic options for patients who are living with difficult, and even incurable, diseases. The gene therapy can be delivered directly *in vivo* to affected cells through either providing the cell with working copies of the gene or by silencing mutated genes that function improperly and cause disease. Another gene therapy strategy aims to reengineer cells *ex vivo* to increase their therapeutic efficacy against a target population. This is the principle behind CAR T-cell therapy where the patient's own immune cells are collected and modified with viral vectors in a laboratory before being reintroduced to the patient where they can exert their effect against the target cancer cells *in vivo*.

Delivering and expressing a gene of interest typically relies on viral vector delivery systems derived from lentivirus (LV), adenovirus (AV) or adeno-associated virus (AVV). Of these, AAVs have become the vector of choice for *in vivo* gene therapies owing to their safety profile, high gene transfer efficiency, stable long-term expression, and selective tissue tropism. The most common way of producing AAV for gene therapy involves transient transfection of three different plasmids into the cells – the transfer plasmid containing the gene of interest, the second with the *rep-cap* sequences for viral replication and

capsid assembly, and the final helper plasmid contains the adenovirus helper functions.

The majority of approved gene therapies are targeted to rare diseases with niche patient populations but the hope is to design targeted therapies for more prevalent disease indications that affect larger patient populations. The increased investment and innovation in the gene therapy space holds great promise for patients but brings with it challenges for manufacturers to meet the high clinical and commercial demand. cGMP plasmid DNA is an essential starting material for viral vector production but current manufacturing strategies based on methods adapted from the basic research laboratory lack the efficiency and consistency to meet yield requirements. These same issues extend to viral vector manufacturers, resulting in high cost of goods (COGS) and bottlenecks along the entire supply chain for gene therapies. Manufacturers are working actively to simultaneously scale up manufacturing processes and increase host cell productivity aimed at addressing these challenges.

Plasmid DNA

As more cell and gene therapies advance to FDA approval, it is essential to scale up current production processes to increase plasmid DNA productivity economically, and at the scale needed to meet industry demand. Plasmid production is commonly achieved through recombinant *Escherichia coli* fed batch fermentation, during which the appropriate genetic sequences are amplified, harvested, purified, and tested for safety. There can be issues with lot-to-lot consistency in the fermentation media which contains hydrolysates, which in turn imparts lot-to-lot variability in plasmid yield and purity. Added to this is the desire in the industry for costly GMP-compliant manufacturing since plasmid DNA is an upstream raw material for viral vectors in clinical applications, which has imposed increasing levels of GMP stringency where more defined cell culture media/growth conditions without animal-derived components, better in-process monitoring and overall process control are required[2].

Typical fermentation media is composed of undefined components, like peptone or hydrolysate mixtures with a wide range of amino acid concentrations (Figure 1) resulting in yield, quality and purity inconsistencies in manufacturing. Here, the REBEL analyzer can be leveraged to check media conformity across different lots of fermentation media and between vendors to mitigate inconsistencies during manufacturing.

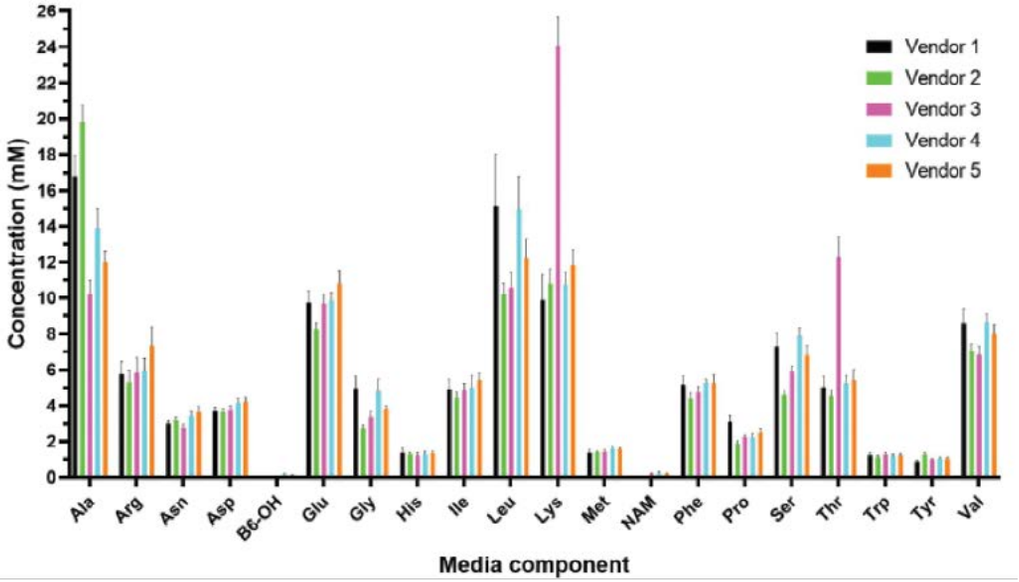

Figure 1. Amino acid concentrations across five different vendors of Terrific broth for E. coli fermentation. Media samples were diluted 250X prior to analysis on the REBEL analyzer. Error bars are the standard deviation of n=5 replications.

Additionally, in-depth media analysis can aid in the movement towards chemically defined fermentation media to meet increased regulatory standards and to achieve optimal productivity and plasmid quality necessary for downstream viral vector production.

Viral Vectors

Efficient viral vector production is a key element for gene therapy manufacturing where improving overall yield of functional full capsids containing the desired genetic payload is still the biggest hurdle. Typical workflows begin upstream where adherent HEK293 cells, cultured in serum-containing medium, undergo transient transfection with plasmids encoding the main building blocks of the desired viral vector. Viral particles are produced by the HEK293 cells, harvested and put into downstream purification processes. Broadly speaking, one of the main challenges in meeting the high titers required by the industry is how to effectively move from small to large scale commercial manufacture in a reliable and reproducible manner while still maintaining critical quality attributes (CQAs). The complexity of viral vectors —significantly more complex than recombinant proteins, has posed biomanufacturing challenges both upstream and downstream including scalability, productivity, and overall lack of robustness.[3] To develop long-term solutions to these problems, manufacturers are looking to optimize both the producer cells and the culture media they are grown in and the mode of culture (adherent vs suspension, fed-batch vs perfusion).[14]

Scalability

In order to meet market demands in gene therapies, there is a need to improve production efficiencies, which will reduce manufacturing costs and improve patient access. As an example, a phase 1/2 trial for hemophilia B required over 400 ten-layer cell stacks to generate sufficient material for six patients[2]. While the trial was a success, this study highlights the need for new methods to improve vector generation especially if gene therapies are adopted for larger disease indications.

The ability to culture the production cells at higher densities than is possible in adherent monolayers is one way to increase titers. Adapting adherent HEK293 cells to grow in suspension in a bioreactor system provides a more robust and scalable process enabling higher cell densities to increase productivity as well as reducing overall labor and materials costs[3]. However, adapting a cell line to grow in suspension requires medium and process development time that the industry can ill-afford. As an alternative, microcarrier technology for adherent cell lines has also been considered for scale up. This offers advantages of providing a larger surface area for cell growth and the ability to utilize bioreactor platforms that are more suited for large-scale production without the need to adapt the cells to non-adherent conditions[4]. In stark contrast to conventional bench-scale static cultures, large-scale suspension bioreactor cultures impart complex hydrodynamic forces on cells due to the fluid agitation required to aid in the transport of nutrients and gasses within the culture volume[5,6] The environmental perturbations and increased cell densities that the cells experience in bioreactor environments may alter their nutrient requirements or require additional additives to serve as a protectant against shear stress[7] (i.e., Pluronic F68, methylcellulose or dextran) and thus may require the optimization of the cell culture media in process development.

The use of any animal derived product in medium, including serum, increases the risk of contamination, supply chain instability and variability. However, removing serum and optimizing media for production of viral vectors does require extensive media development and additional time and resource commitment. HEK 293 has been used extensively for transient protein expression, and thus, commercially available chemically defined media for HEK293 may not be optimized for production of viral vectors. Viral vector production is more complex than single protein production, such as monoclonal antibodies. In addition, transfection media may be optimized for high growth, but not for producing high viral titers, therefore significant optimization is required to meet the specific needs of viral vector production culture. Understanding cell metabolism and the impact of medium components, like amino acids and glucose, on titer levels can be done using media analysis studies and is key to increasing productivity.

Additionally, the FDA has expressed concern over the use of HEK293T cells owing to the inclusion of the SV40 large T antigen DNA sequence. This

oncogenic construct could pose the risk of tumor development in patients, which has prompted manufacturers to investigate other HEK293 clones and other cell platforms. The baculovirus- *Spodoptera frugiperda* insect cell (Sf9) platform is an attractive alternative to mammalian HEK293T cells and holds potential as a GMP-compatible viral production system.[8] In implementing insect cell lines, the selection of the appropriate media, in particular, the amino acid content, is important because many amino acids required for their growth and/or protein production are not synthesized by insect cells, and must be supplied by the culture media. As shown in Figure 2, the amino acid profile across several commercial formulations of insect cell media varies across a wide range. This global analysis of amino acid content coupled with an understanding of insect cell metabolism can inform media optimization efforts during process development to modulate critical parameters like cell growth/viability and productivity.

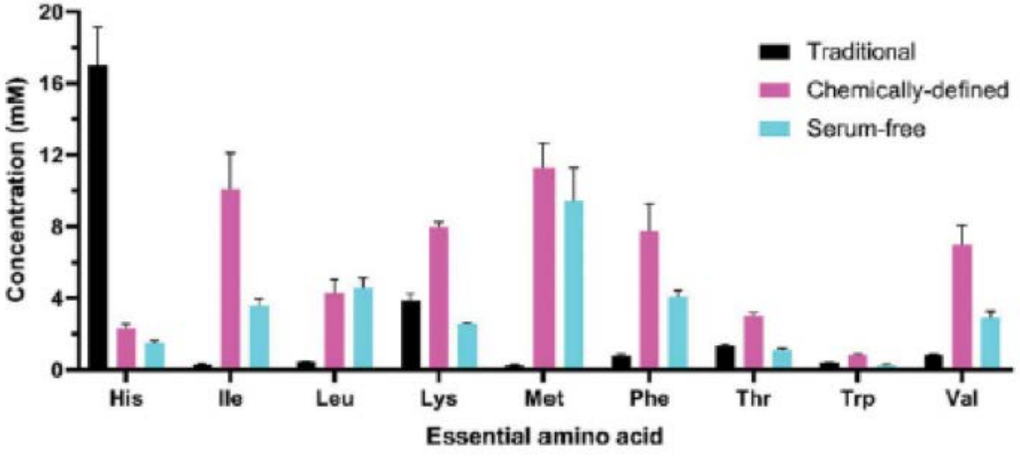

Figure 2. Essential amino acid profile from traditional, chemically-defined and serum-free insect cell media formulations. Error bars are from standard deviation of n=5 replicates.

Productivity

The biggest hurdle in viral vector production is yield, not only to increase overall viral titer, but also a need to maximize the full capsids that contains the gene of interest as opposed to partial or empty capsids. Simply put, the more empty capsids there are, the less overall yield. However, the impact of cell culture medium on empty vs. full capsid production is not well defined or understood.

In order to meet market demands in gene therapies, there is a need to improve production efficiencies

Particularly with AAV vectors, there is a propensity to package any available DNA sequence, which results in capsids that contain illegitimate DNA sequences from the host cell DNA and plasmid DNA or fragmented pieces of the gene of interest. Grimm and colleagues[9] first reported that only a small percentage (1.7–20%) of the AAV particles generated in cell culture by transient transfection actually contain vector genomes, the majority corresponding to empty capsids. While we have made significant progress in our understanding of viral capsids as vector technology becomes more mature, the impact of different manufacturing processes on capsid processing, such as post-translational (PTM) modifications are still evolving. Identification of molecular features required for efficient packaging, and modification of production plasmids and the specific culture conditions that can increase the proportion of full capsids can help enhance productivity as well[10]. The inefficiencies in viral vector production and the noticeable productivity gap compared to other recombinant proteins like monoclonal antibodies is that bioproduction processes for these mature therapeutics have been subjected to years of intense cell culture process development and optimization to define improved media, nutrients, and conditions[10]. Similar efforts using these approaches that have been effective for other biotherapeutics may substantially improve AAV specific productivity in cell culture.

Safety

As with cell therapy manufacturing, there is a movement towards more defined, animal-free and/or chemically-defined medium that mitigates any potential risk of transfer of viruses or prions into the final product that could induce an undesirable immunogenic reaction in the patient. In addition, it is important to consider whether the vector bulk can be sterilized. For instance, viral clearance is used to some degree in both AAV and Lentivirus processes, which confers a level of viral safety.[9] Furthermore, as viral vector demand climbs with more products reaching late-stage large-scale manufacture, supply limitations of certified serum are a real possibility[9]. In turn, the reduced reliance on serum, the improved lot-to-lot consistency, and the need for more defined mammalian cell growth conditions have driven the development of chemically defined media formulations that are both serum-free and protein-free. Commercially sourced chemically defined formulations are available but many of these formulations are proprietary and thus pose hurdles in optimization for a specific vector product because the components and their concentrations are unknown[12], which is where the REBEL can provide valuable intel and insight to drive optimization (Figure 3).

As in monoclonal antibodies where the cell culture medium can also impact PTM and other CQAs, the medium also could have an impact on the capsid PTM and associated CQAs as capsid proteins are glycosylated[13]. Also medium optimization needs to be taken into consideration during scale up as cell nutrient requirements can change as the scale of the bioreactor increases.

When developing a new AAV or LV process, decisions have to be made on cell line, mode of culture, and which cell culture medium and feeding strategy to use for optimal productivity and desired CQAs. One common strategy is to perform a screening of commercially available cell culture media. For example there are multiple commercial media available for HEK 293 cells, one of the most used cell lines in viral vector production. As shown in Figure 3, these media can have similar or widely different compositions in terms of amino acids. Is it beneficial to screen as diverse set of medium as possible to maximize the chance to identify a medium that provide the right nutrients to a specific HEK293 cell line. There are many sub-types of HEK 293 also available and each one may need a different balance of AA and vitamins.

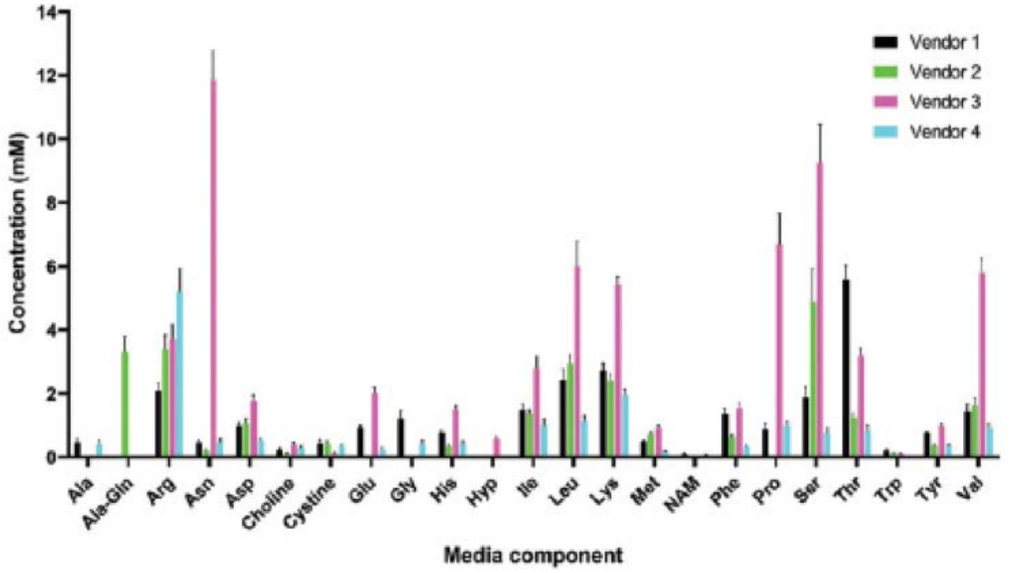

Figure 3. Comparison of the media components in 4 different vendors of chemically-defined HEK293 media demonstrates the diversity of formulations. Error bars are from standard deviation of n=5 replicates.

Culture Media Characterization & Optimization

It is evident throughout these discussions that there are many opportunities to improve manufacturing efficiencies for both plasmid DNA and the viral vectors where the cell culture media used for the production cell lines is vitally linked to their productivity and is a critically important aspect of any manufacturing platform. In-depth characterization could aid in optimization efforts to decrease COGS and improve the productivity of host cell lines through better understanding of nutrient requirements or to facilitate process development efforts such as adapting adherent cells to suspension culture

and more. Ideally, the media selected should provide the essential nutrients to facilitate high productivity, consistency and quality as well as being amenable to support scale up to larger manufacturing platforms.

At-line and frequent spent media analysis can enable a faster in-house process development pace since there is no need to wait for samples to be processed at a core lab or through outsourced contract services especially with the BSL2 safety requirements associated with viral vectors for transport and handling. The small footprint of the REBEL analyzer allows it to reside in the process development space or manufacturing suite alongside the bioreactors. Leveraging CE-MS technology, the REBEL can produce a quantitative list of amino acids, dipeptides, water soluble vitamins and amines in under ten minutes with very small sample volumes that can facilitate near the real-time decision making necessary to drive forward progress in a rapidly advancing industry.

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