The survival-promoting Bcl-2 family of proteins is generally believed to exist at mitochondria to block cytochrome c release. However, Bcl-2 family proteins have emerging roles in other cellular processes at various subcellular localizations including the endoplasmic reticulum (ER) and outer nuclear membranes. Thus, it is not unreasonable to speculate that the localization of Bcl-2 family proteins determines their functioning. Since a great deal of research has focused on Bcl-2, we investigate Bcl-xL, an anti-apoptotic homologue of Bcl-2, and its localization and role at the ER. We found here by confocal microscopic examinations that heterologous Bcl-xL exists in Chinese hamster ovary (CHO) cells in three distinct patterns: (1) some Bcl-xL distribute throughout the outer mitochondrial membrane (OMM); (2) about 43% of Bcl-xL clusters on the OMM adjacent to the ER-mitochondrion interface; and (3) interestingly, approximately 20% of Bcl-xL are juxtaposed to mitochondria in the cytosolic region where Bcl-xL colocalize with proteins specific to mitochondrion-associated ER membrane (MAM), such as sigma-1 receptor, BiP (binding immunoglobulin protein), IP3R3 (type 3 IP3 receptor), and mitofusion-2. The MAM is a specialized ER subdomain physically associated with mitochondria to regulate the direct Ca\textsuperscript{2+} transmission from ER to mitochondria. A cell fractionation study with standard markers confirms that about 45% of Bcl-xL localizes to the mitochondria with 15% of Bcl-xL at MAM-enriched membranes whereas a small fraction of Bcl-xL resides at the bulk ER. The BH4 domain of Bcl-xL was also shown to physically interact with IP3R3 residing at the MAM and upon cellular stress induced by thapsigargin, Bcl-xL translocates from the OMM to MAM to further interact with IP3R3. Since IP3R3 regulate ER Ca\textsuperscript{2+} efflux at the MAM, it is speculated that Bcl-xL binds IP3R3 to regulate direct Ca\textsuperscript{2+} transmission from the ER to mitochondria. Indeed, preliminary data from our Ca\textsuperscript{2+} signaling study found that overexpression of Bcl-xL decreases thapsigargin-induced Ca\textsuperscript{2+} efflux from the ER into the cytosol, whereas Ca\textsuperscript{2+} efflux from the ER into mitochondria is enhanced. When taken together, the above results suggest that MAM localized Bcl-xL may alter communications between the mitochondria and the ER membrane via interaction with type 3 IP3 receptors with a potential role in Ca\textsuperscript{2+} signaling and bioenergetics. Future efforts will explore the effects of Bcl-xL on cellular energetics with the end goal of altering cell production of useful biotherapeutics.
PRO-DOMAIN MUTATION LEADS TO INCREASED BMP-2 EXPRESSION AND REDUCED ACTIVITY

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Background: Bone morphogenetic proteins (BMPs) are key regulators of bone growth and differentiation. Their osteoinductive ability has led to the use of recombinant human BMPs (rhBMPs) for bone regeneration. Due to the structural complexity of BMPs, their manufacturing has been problematic. Although eukaryotic systems are able to synthesize active rhBMPs, the typical yield is very low compared to other proteins produced in similar cell culture systems. BMP-2 is synthesized as a precursor protein, proBMP-2, which undergoes proteolytic cleavage to form mature BMP-2. ProBMP-2 contains two proprotein convertase (PC) recognition sites (S1 and S2) and is postulated to be cleaved by PCs at these sites. Several studies on members of the BMP family have showed that pro-domain cleavage can affect intracellular processing and activity of the mature proteins.

Objective: To investigate the role of pro-domain cleavage on BMP-2 expression and activity.

Methods: The S1 cleavage site of the human BMP-2 gene was silenced (REKR→IEGR), making it resistant to PC cleavage. The mutated (mS1) or wild type (wt) gene was stably transfected into the Flp-In™ human embryonic kidney (HEK-293) and a suspension Chinese hamster ovary (CHO) cells lines. Secreted BMP-2 proteins were characterized by Western blot and the cleavage sites were verified by N-terminal sequencing. The amount of pro- and mature BMP-2 secreted was quantified by sandwich ELISA. Mature BMP-2 was further purified and its biological activity was evaluated in vitro by assessing the osteoblastic differentiation of C2C12 cells.

Results: Whereas the wt cells secreted a mixture of 18 and 20 kDa rhBMP-2 isoforms, mS1 expressed only the 20 kDa form. The N-terminal sequences showed that the 18 and 20 kDa BMP-2 began with the sequences QAKHK and TFGHD, respectively. The cells expressing mS1 rhBMP-2 produced 15-fold (Student’s t test, \( p < 0.001 \)) and 1000-fold (Student’s t test, \( p < 0.001 \)) more mature and proBMP-2, respectively, compared to cells expressing wt rhBMP-2. The mutant-secreted rhBMP-2 induced dose-dependent differentiation of myogenic C2C12 cells, suggesting it was biologically active; however, it was less active than wt rhBMP-2 at its optimal concentration (ANOVA, Tukey’s, \( p < 0.0001 \)).

Conclusions: By mutating the S1 cleavage site, cells secreted mature BMP-2 cleaved at an alternative site upstream. Cells expressing mS1 secreted greater amounts of both pro- and mature BMP-2 compared to the wt, in spite of the genes being inserted into the same integration site. Together with the biological activity exhibited by mS1-secreted BMP-2, these findings suggest mutation of the S1 site can modulate mature BMP-2 expression and activity.

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POLYSACCHARIDE DERIVED FROM RAKKYO IS EFFECTIVE FACTOR AGAINST FREEZING STRESS OF MAMMALIAN CELLS

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[Background]
Cryopreservation is an important technique for long term maintenance of mammalian cells. Serum is usually used as the protective factor to improve survival of the cells after freezing but it also has various disadvantages including variation and an infective risk by virus. In order to eliminate the several risks of serum, serum-free formulations are developed for cryopreservation but universal freezing reagent has not been yet.

[Purpose]
In this study, we developed new serum-free freezing reagents using rakkyo fructan obtained from rakkyo, Allium bakeri. Rakkyo fructan consists of poly-D-fructose residues in 1,2- and 2,6-glycosidic linkage. It functions as a mitogenic factor of mammalian cells and anti-freezing factor of some plants. In addition, we attempted replacement of dimethyl sulfoxide (DMSO), which is powerful cryoprotectant agent, by other compound such as ethylene glycol (EG) and propylene glycol (PG), because DMSO injures the cells after thawing. First, we examined whether rakkyo fructan prevented cells from injury by freezing. Next we examined the effect of three fructan freezing reagents on freezing stress of cells.

[Materials and Methods]
Serum-free cultured mouse hybridoma 2E3-O cells and CHO-DP12 cells were used in this study. They were suspended by 30 wt% fructan-phosphate buffered saline (PBS) with DMSO, EG or PG. Then they were transferred to freezing tubes, placed in a BIOCELL® container and frozen at −80 °C for several days. Then, these cells were thawed and the rakkyo fructan solution was removed and then the cells were cultured in serum-free medium. At the third and sixth day after thawing, the cultured cells were collected, stained with trypan blue exclusion method and both of viable and dead cells were counted with hemocytometer.

[Results]
When thawed, the viabilities of cells preserved in rakkyo fructan-PBS were similar to that of cells preserved in fetal bovine serum. Furthermore, the cells frozen in all of the rakkyo fructan freezing regents kept the ability to proliferate and to produce antibody. These results indicate that rakkyo fructan protect cells against freezing stress and we succeed in serum-free and DMSO-free freezing regents.
Phase contrast microscopy (PCM) is commonly used to monitor cell cultures. However, in opposition to fluorescence imaging, PCM is much less amenable to automated image analysis. We have developed software sensors to segment and analyze PCM images from a long-term, large-field automated live cell imaging platform. An IX81 Olympus microscope equipped with an automated stage was used to monitor myoblast cell cultures grown in either serum supplemented or serum-free medium (SFM). A total of 500,000 cell culture images were acquired using Metamorph® (Olympus) and analyzed using custom Matlab scripts to measure the total surface occupied as well as some textural features of these cells, as a function of time. Kinetic growth models were fitted using the surface recovered by the cells in each well. Textural features computed using wavelets were used to distinguish between cells grown in serum supplemented medium and cells grown in SFM. A classification was performed using principal component data analysis. This method allowed the identification of morphological features that were directly related to the culture conditions. The proposed algorithms can therefore be used for online monitoring and quality control of cell cultures, such as the screening of serum replacement factors.
A pool of recombinant CHO DG44 cells (transfected with an IgG construct) was characterized throughout shake flask batch culture in CD OptiCHO™ in terms of growth, immunoglobulin production and extracellular metabolite profile. When seeded at 2x10^5 cells/ml, based on growth profile, the cell culture could be defined into four stages (Phase I – initial growth, from 0-60 hours; Phase II – an enhanced, exponential period of growth from 60-150 hours; Phase III – stationary phase, from 150-190 hours; Phase IV – decline phase, from 190 hours onwards). Maximum cell density (achieved in Phase III) was 6x10^6 cells/ml and viability at the start of Phase III was 95%, dropping to 85% at the end of that phase of culture. In Phase IV, cultures took about 70 hours to reach 50% viability. Antibody production increase was greatest in Phase II followed by a leveling off in Phase IV. Metabolites in the extracellular compartment were assessed by GC-MSa. Depletion of a group of metabolites in the medium was associated with entry into Phase III (stationary phase). These included asparagine, glutamine, lysine, serine, and threonine. Extracellular organic acids accumulated through Phases II and III, with citrate and succinate declining during Phase IV. Intracellular malate and fumarate declined during the growth phase (II). Potentially osmoprotective glucose metabolites (sorbitol and treitol) were generated during Phases I, II and III followed by leveling or decline in Phase IV, while glycerol release occurred mostly during Phases III and IV. Cells retained reasonable viability throughout Phase III but the entry into decline phase was associated with complete depletion of glucose. Metabolite utilization exhibited Phase-specific changes during growth (Phases I and II). For example, Phase II (characterized by higher growth rate) was associated with a cessation in lactate accumulation, despite continued high rates of glucose utilization. Overall, the time courses and patterns of depletion or accumulation of metabolites highlight metabolic states associated with growth and maintenance of cells in batch culture, reflecting of differential intracellular events that support biosynthetic and maintenance activities. Such information offers potential towards design of media and feeds that will favor specific cellular phenotypes related to growth and productivity.

VOLUME DISTRIBUTIONS IN CHO CELL POPULATIONS DURING ADAPTATION TO CHEMICALLY DEFINED MEDIUM

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Introduction: The distribution of cell volumes within a cell population measured by electronic impedance using a flow-based cell counter is a quick and robust measurement which allows monitoring of population changes during adaptation to chemically defined medium, scale up to bioreactor conditions and changes in culture conditions. From a simple and single measurement, the entire distribution of volumes of cells within the population is measured. Changes in cell volumes can indicate changes in growth rates of cells and/or changes in division times. Population volumes can change over time when, for example, the original population is being overtaken by a rapidly growing subpopulation. Using a model previously developed (Halter et al., 2009) the average rates of cell growth and division and the biological variation in those rates throughout a population of cells can be estimated from the shape of the volume distribution. In this study, we have used cell volume measurements to monitor the changes during adaptation to chemically defined medium of CHO DP-12 clone 1934 cells.

Methods: Cells were adapted to chemically defined medium (CDM) by sequential media substitution from 25% to 100% CDM. At each passage, volume distributions of single cell suspensions were measured using a Coulter particle counter, and the growth rate was calculated. Evaluation of uncertainty in the measurement and development of methods to generate single-cell (non-aggregated) suspensions of cells, were required to perform these measurements.

Results: CHO DP-12 clone 1934 cells adapted to 100% CDM showed a 76% increase in median volume compared to cells grown in standard medium containing 10% fetal bovine serum (FBS). This occurs because the average division time for cells in the population decreases substantially. Our model predicts that the average growth rate is not significantly changed for cells adapted to serum free conditions.

Conclusions: The fact that cell growth is similar, but division is slower for cells in chemically defined medium, may indicate that protein synthesis per cell is not compromised due to the removal of serum. Further studies with a CHO line that is over-expressing GFP will assist in the development of models for understanding and predicting how CHO cell populations change during adaptation to production scale culture.
Mammalian cells are widely used for recombinant protein production; therefore, extensive research and development efforts have been conducted to improve the growth and production capabilities of these cells. So far these efforts have concentrating on developing efficient growth strategies and genetic modifications aimed at increasing specific growth rate, reducing apoptosis and improving nutrients utilization by altering the expression of a single gene or a single pathway. The latter approach may not be sufficient for producing desirable phenotypes and, therefore, regulation of global gene expression may be a better approach for the engineering of high-producing mammalian cell lines. This approach may be accomplished by the utilization of microRNAs (miRNAs) which are global regulators of gene expression that are involved in multiple cellular processes such as apoptosis, cell development, differentiation, metabolism, and proliferation. Simultaneous alteration of multiple pathways to improve cells performance may be possible by the modification of miRNA expression profiles. Since one of these pathways is apoptosis, an understanding of the mechanisms involved in the regulation of endogenous miRNA(s) expression in response to apoptotic stimuli in mammalian cells will be useful for the creation of stress-resistant cell lines. Another advantage of using miRNAs as engineering targets for cells improvement is that manipulation of miRNA expression profiles does not burden the translational machinery of the cell and, therefore, cellular metabolic resources are allocated to recombinant protein production. In our studies we showed that miR-466h was involved in induction of apoptosis in CHO cells by simultaneously targeting the bcl2l2, birc6, dad1, stat5a and smo genes. We also showed that inhibition of miR-466h expression caused a delay in apoptosis initiation has demonstrated shown by increased cell viability and decreased Caspase-3/7 levels. This suggests the pro-apoptotic role of mmu-miR-466h and its ability to modulate the apoptotic pathway in mammalian cells. miR-466h can be explored as an engineering target in mammalian cells, and the mechanisms of its activation and involvement in other relevant industrial pathways should be investigated.
Several transcriptomics and proteomics studies have been conducted to uncover the molecular characteristics of optimal growth and recombinant protein production in animal cell cultures. However, considerably less effort has been put into comprehensively profiling the metabolome of producer cell lines. In particular, extracellular metabolite concentrations in the culture supernatant are sensitive markers of cellular physiology, thus contributing to a better knowledge of what characterizes a hyper productive phenotype. Understanding how cells use medium nutrients and secrete by products through exometabolome measurements will allow designing more efficient culture media and feeds to support cell growth as well as higher recombinant protein yields.

Nuclear magnetic resonance (NMR) spectroscopy has been one analytical platform pushing forward the field of metabolomics. $^1$H-NMR takes advantage of proton natural abundance to virtually detect and quantify most metabolites in biological samples. Being non destructive, it allows performing multiple tests on the same sample and sample preparation is relatively minimal when compared to mass spectrometry-based methods.

In this work, we analyzed and compared the exometabolome from batch cultures of two CHO-K1 cell clones expressing different amounts of a monoclonal antibody (IgG). $^1$H-NMR was performed in a 500 MHz Avance Bruker spectrometer, equipped with a 5 mm QX1 inverted probe and spectra were analyzed with the software Chenomx NMR Suite 7.1. The developed protocol allowed monitoring the consumption or accumulation of approximately 40 cell culture compounds throughout culture time, with experimental error in general below 5%. Besides the well known accumulation of lactate in the supernatant, the build-up of other by-products was also observed, including formate, acetate, isovalerate, glycerol, among others. A comprehensive comparison of specific metabolite consumption/production rates at different productive states will be presented, and possible markers that may determine these states will be identified.
The generation of comparable samples for physiological analyses such as transcriptomics or proteomics is a delicate task. Gene expression can vary significantly during batch cultivation where the conditions change continuously due to nutrient consumption and the accumulation of metabolites and biomass. Furthermore, the cumulated biomass is generated in different phases of the batch. Hence, steady-state cultivation of mammalian cells would be a huge benefit.

In this study, high and low producing Chinese hamster ovary (CHO) cell lines for two model proteins (human serum albumin and the anti-HIV-1 single chain Fv-Fc fusion antibody 3D6scFv-Fc) will be compared on a physiological level using various state-of-the-art omics technologies. Additionally, these data should also be comparable to those generated with *Pichia pastoris* strains expressing the same two model proteins. They will then be the basis for a genome-wide cross-species comparison of these two commonly used expression systems. From the collected data we expect to identify cellular components that are crucial for high growth rates or efficient protein production and secretion. Furthermore, genes and pathways that are correlating to the specific strengths of the individual production systems will be identified (e.g. growth and cell density in yeast or secretion capacity of CHO cells). Identified targets will then be functionally validated for their effect on cellular physiology in order to finally increase productivity, process robustness, etc. of the individual production systems.

For obvious reasons, cultivation strategies have to be comparable for CHO cells and *Pichia pastoris* which are generally different. Among individual cultivation parameters, controlling cell growth is the most critical issue and has to be adjusted accordingly. This can be achieved by a substrate limited chemostat process which is very commonly used for microbial expression systems. Here, we demonstrate that chemostat cultivation can also be used for mammalian cell cultures in order to generate comparable samples for this purpose. Using glucose as the growth limiting substrate allows the establishment of steady-state conditions, whereas the specific growth rate can be controlled by the dilution rate. In our experiment, cultivations were performed in 800 mL cell culture bioreactors (DS0700TPS, DASGIP) and the dilution rate was set to 0.5 d⁻¹ for all CHO cell lines which will be compared in this study. Thereby, using 0.9 g L⁻¹ glucose in the feed medium was sufficient to achieve growth limitation. Our results show that the viable cell concentration could be maintained at a constant level during chemostat cultivation. Additionally, the concentrations of the main nutrients glucose and glutamine as well as the metabolites lactate and ammonium were stable as well. Also the specific productivities of the model proteins remained constant during steady-state cultivation.

We believe that it is very important to use samples produced under such defined and constant conditions in order to generate high quality transcriptomics and proteomics data for comparative physiological analyses.
DEVELOPMENT AND IMPLEMENTATION OF A HIGHLY AUTOMATED CELL LINE DEVELOPMENT PLATFORM

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The development of mammalian biotherapeutic production cell lines is both a time and resource intensive process industry-wide, and represents one of the most critical components of the drug development process. In order to ensure the selection of an optimal biotherapeutic protein possessing superior clinical performance together with good physicochemical and manufacturing characteristics, Amgen utilizes a molecule assessment (MA) as part of our early molecule development workflow. The need for the rapid generation of high productivity cell lines in conjunction with this evaluation of multiple molecular candidates represents a difficult balance with limited FTE resources. The cell line development workflow needs to support sufficient molecule diversity in conjunction with the resource intensive activities needed to generate high performance stable production CHO lines.

In order to significantly increase both the potential number of candidate molecules assessed and the number of distinct cell lines that can be generated per program, we have developed innovative systems to establish a highly automated cell line development process. We have successfully optimized high throughput plate based electroporation systems in combination with suspension plate based pool selection, amplification and recovery; enabling high levels of automation support for the generation of stable CHO production pools. In order to maintain the resulting large numbers of distinct cell populations, we have developed automated systems capable of maintaining and passaging these cultures and implemented systems to facilitate the rapid cryopreservation of these large sample numbers.

To enable the rapid evaluation of the large number of pools or clones we have also established automated scaledown production models in suspension plates. This has enabled a large increase in assay throughput, which we have supported by pairing these assays with high throughput, high content analytics to rapidly derive productivity and product quality metrics for these large sample numbers. In order to ensure verifiable clonality for each individual subclone from the earliest stages of the cell line development process, we have also introduced a highly automated clonality verification system that provide the capability for us to develop detailed growth timecourse for up to thousands of individual clones.

We will present data on multiple components of this highly automated, highly integrated workflow that has enabled us to significantly increase throughput without sacrificing timelines or increasing FTE resource requirements. This new integrated system enables us to increase the number of cell lines and molecular candidates assessed, while also facilitating improved performance outcomes via the significant increase in the scalability of both our pool and clone screening capabilities.
IMPLEMENTATION OF AUTOMATED MINIATURE BIOREACTORS FOR RAPID PROCESS OPTIMISATION AND DEVELOPMENT

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The drive in biopharmaceutical production to reduce time to market for new products means that research and development processes must constantly be updated through the introduction of new technologies. Two key activities during development that must be undertaken before a new indication can become a product are cell line selection and process optimization. These activities can be both time consuming and costly. A number of strategies have been implemented across the industry to ensure that these processes are addressed in an efficient manner, e.g. high throughput cell line screening and platform technologies, however there can still be a disconnect between the optimal conditions identified in the early stages of development and those eventually used in large-scale manufacture. In addition to these issues, the drive to fully characterize processes in a QbD manner means that further time and resource pressures are placed on the development program. Here we present data from studies using automated miniature bioreactors for screening and development that demonstrate the implementation of this technology can both improve the information gathered during early stage product development whilst reducing the impact on full time employee requirement.
FLUX BALANCE ANALYSIS (FBA) FOR QUANTIFYING CHO CELL PHYSIOLOGICAL RESPONSE DURING A PERFUSION CULTIVATION SCREENING DOE STUDY

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Metabolic flux analysis provides valuable quantitative information on cell physiology which can augment the conventional data set that comprises of prime variables and their specific rates. Application of metabolic flux analysis to mammalian cell cultures continues to increase with the majority of studies focused on early-stage process development activities. Here we demonstrate the extension of flux analysis to a screening DOE study where the underlying process was well characterized at set-points. Specifically, CHO cells were cultivated in a laboratory-scale perfusion system and bioreactor DO, temperature, and pH were varied over the course of the experiment. Each set-point change was maintained for ~10 days and data from the last 3 days were used to characterize the system for that condition. In addition to prime variable measurement and specific rate computations, metabolic fluxes were first estimated using the metabolite balancing approach because adequate experimental measurements were made to ensure that the system was over-determined. Subsequently, the same experimental data were used for FBA analysis using multiple optimization criteria which corresponded to different assumptions regarding cell physiology and metabolism. Comparison of FBA results with those from the metabolite balancing approach helped evaluate the appropriateness of the various assumptions made during flux estimation by FBA. Typical applications of FBA have been large bioreaction networks with limited experimental data necessitating assumptions regarding cell behavior. By limiting FBA application to a smaller, over-determined network, a direct comparison of FBA results with the more representative flux values from metabolite balancing was possible and the results from this study can guide future applications of FBA to more comprehensive mammalian cell bioreaction networks.
MIXING ISSUES IN CELL CULTURE BIOREACTORS USING MICROCARRIERS

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Though in the early 1980s animal cell culture was generally based on attaching cells to microcarriers, little work was done to optimize their suspension. Partly this was because around this time the ability to grow cells in free suspension was established under which conditions cells were much less likely to be damaged by fluid dynamic stresses. For example, Croughan et al (1987) showed that mean specific energy dissipation rates, W/kg, had to be < ~1 x 10^{-3} W/kg to prevent damage with 180 mm microcarriers. However, work was not undertaken to search for vessel/agitator configurations that would achieve this end. Indeed, some geometric recommendations (for example, the use of hemispherical bioreactor bases to aid suspension (van Wezel, 1985)) were counter to the general findings on particle suspension (Nienow, 1985). Now with the increasing importance of regenerative medicine and the need to grow stem cells on microcarriers at large scale for allogeneic usage, it is essential to establish efficient bioreactor geometries for microcarrier suspension. Clearly, it is essential for the microcarriers to be suspended if the important well-mixed features of the stirred bioreactor are to be achieved. The minimum speed, NJS, and mean specific energy dissipation rate at that speed, W/kg, has been measured for many geometries but a review of the literature still shows little work has been done on microcarriers. The most relevant study (Ibrahim and Nienow, 2004) investigated these parameters using Cytodex 3 microcarrier beads using a range of different diameter Chemineer HE-3 hydrofoils, a pair of Ekato InterMIG impellers and a six-blade, 45-pitch turbine impeller in a baffled vessel of 19.2 L operating volume containing phosphate buffer saline solution. Flat and modified tank bases were used and NJS values were observed to be in the range of 50 to 90 rpm. The use of Zwietering’s correlation using existing literature geometric suspension parameters, S, to predict NJS would have given values up to 50% higher. The low NJS values obtained were attributed to the very small particle–liquid density difference (40 kg/m^3), which eased the lifting of the particles from the tank bottom, compared to those used in non-microcarrier studies. With these microcarriers, the three-blade hydrofoil HE-3 impeller of D/T = 0.39 in a cone-and-fillet based tank was marginally the most efficient; that is, it had the lowest W/kg, ~ 0.5 x 10^{-3} W/kg. However, mean specific energy dissipation rate was < ~1 x 10^{-3} W/kg in most cases with the different size HE-3 hydrofoils, which implies that these geometries would probably be suitable for application in shear-sensitive cell culture systems using such microcarriers. If cells have to be attached to microcarriers, then damage due to this energy dissipation rate might occur to cells from impeller-microcarrier, microcarrier-microcarrier and microcarrier-vessel internals impacts or due to cells being stripped off them; plus direct stress from turbulent eddies, so it is important to minimise it. However, it must also be remembered that mean specific energy dissipation rate is a critical parameter and needs to be sufficient to achieve the required rate of oxygen and carbon dioxide transfer and give an adequate quality of homogenization. Thus, it is important to consider all these parameters which are dependent on W/kg when developing the stirred bioreactor.
GLYCOSYLATION OF MONOCLONAL ANTIBODIES FOR CLINICAL TRIALS AND TRANSLATIONAL CANCER RESEARCH

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The Clinical Program of the Ludwig Institute for Cancer Research (LICR) aims to translate basic laboratory discoveries into early phase clinical trials in cancer patients. The LICR Antibody Program has developed six recombinant humanized antibodies into human trials, and has a pipeline of novel antibodies in progress. Through the combined efforts of the global LICR laboratories and GMP production facilities, LICR sponsored clinical trials have been conducted in sites in the US, Europe, Australia and Japan. A key component of the LICR approach has been to focus on the identification of antibodies selectively targeting antigens preferentially expressed in tumor tissue, and the molecular engineering of chimeric or humanized antibodies to these targets. The development of high producing cell lines is crucial in the development of each antibody construct with an emphasis of maintaining the highest possible product quality. One important component of antibody product quality is the nature of the complex glycan located in the N-glycosylation site (Asn 297) of the IgG Fc fragment. This study aims to compile a baseline glycan analysis of monoclonal antibodies produced by the LICR in various cell lines and cell culture production systems.
The estimation of cell density and cell viability of mammalian cell lines in cell culture has traditionally been performed using the exclusion dye trypan blue that stains “dead” cells when their cell membrane is damaged. In large scale cell cultures using bioreactors this estimation is performed off-line. The online biomass probe is based on the principle that under the influence of an electric field between two electrodes, ions in suspension migrate toward the electrodes. The cell plasma membrane is non-conductive so that the cells with intact plasma membranes are polarized and act as tiny capacitors and it has been shown that capacitance increases as the cell concentration does. The measurement is based on the linear relationship between the permittivity difference $\Delta \varepsilon_1 - \Delta \varepsilon_2$ and the viable biomass concentration. This study compares the data obtained using the biomass probe at different frequencies with measurement of rhodamine-123 and pan-caspase activation by flow cytometry for a number of mammalian cell lines.
Protein glycosylation is an inherently variable process and understanding the factors which contribute to this variability is critical to developing robust cell culture processes. This study details the product quality changes observed when adapting a non-antibody CHO cell culture process developed by a third party manufacturer to our CHO platform expression system and process. Further improvements to the process were also characterized for their impact on the glycosylation profile of this recombinant fusion protein.

In-house characterization of the original process identified several limitations, including poor cell growth and insufficient productivity levels. Efforts were therefore initiated to develop a new cell line using our GS-CHO platform expression system. Early characterization of GS-CHO material revealed that the host cell change had resulted in differences in terminal sialic acid content and had also impacted the amount of Man6 species detected.

We next sought to identify process parameters which could modulate similar changes in glycosylation profiles. One potential parameter was viability at harvest. The original process contained a narrow harvest criterion based on cell viability, and we wanted to better characterize the impact of this parameter on glycosylation profiles. We found that sialic acid content decreased with decreasing cell viability, although this trend was more pronounced in the original cell line than in GS-CHO cells. The impact of additional process parameters on glycosylation, including pH and temperature shifts, as well as alternative basal media and feeds, was also investigated. To complement these studies, we also examined the product quality changes observed between primary and secondary clones.

These investigations provided an early characterization of the process design space and allowed us to assess the impact of key process parameters on product quality. The final process was successfully scaled-up and transferred for clinical material production.
APPLICATION OF RNAI IN BIOPROCESSING TO IMPROVE PRODUCT QUALITY AND BIOLOGIC FUNCTIONALITY

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RNA interference (RNAi) represents a powerful new tool available in bioprocessing to generate biologics with specific characteristics that improve efficacy, potency, and safety. RNAi offers many advantages over traditional cell line development strategies for modifying cellular pathways including all genes can be targeted, selectable markers are not required, no cell line development is needed, ability to tailor mRNA silencing as desired, simultaneous silencing of multiple target genes, and avoidance of off-target effects. RNAi is achieved by adding small interfering RNA (siRNA) duplexes directly to manufacturing cell lines grown in bioreactors or shake flasks as a media supplement to silence target gene(s) mRNA expression.

To examine the potential of RNAi for bioprocessing, potent siRNA duplexes targeting well known genes in the de novo fucosylation pathway were designed using proprietary design algorithms and dosed based on a proprietary dosing strategy into a bioprocess that generates an anti-CD20 monoclonal antibody. Dose response studies revealed siRNA can be introduced to the bioprocess, up to 50 picomoles per 10^6 cells, without deleterious effects on cell growth and productivity. Furthermore, these studies established that by appropriately choosing the siRNA concentration, a desired amount of silencing of gene expression can be achieved. In addition, anti-CD20 monoclonal antibodies produced from siRNA-treated cells growing in shake flasks or bioreactors exhibited improved functional characteristics compared to control material including reduced fucosylation, improved FCαRIIIa receptor binding, and increased antibody dependent cellular cytotoxicity (ADCC). No significant differences were observed when other key product quality attributes were compared between purified antibody from siRNA-treated bioreactors and controls. This further demonstrates the specificity of RNAi in bioprocessing and the minimal impact on other product quality attributes due to siRNA addition. Thus, the use of RNAi provides a powerful method for modifying expression of cellular pathways in cell lines used in bioprocesses to improve biologics.
BI-HEX® – OPTIMIZING PRODUCT QUALITY ATTRIBUTES THROUGH HOST CELL ENGINEERING AND UPSTREAM PROCESS OPTIMIZATION

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Boehringer Ingelheim’s Biopharma Process Science is dedicated to developing cell lines and manufacturing processes both to a diverse panel of customer novel biological entities (NBEs) as well as for internal products including biosimilars. Our CHO-based BI-HEX® platform combines state-of-the-art technologies within vector design, cell line generation, process and media optimization in one concept enabling us not only to meet the growing demands for fast CMC development times but importantly also to manipulate molecule properties and product quality attributes. The aspect of tailoring product quality through CMC process optimization is getting increasingly important, not only for development of efficacious NBE functionalities, but also to allow fast and successful development of biosimilars with the adequate properties.

Product mechanism of action and efficacy are at least for monoclonal antibodies frequently linked to the glyco-profile of the molecule. We will show how key platform tools such as expression vector elements, use of genetically modified CHO subclones as well as DoE-based approaches to media and process optimization have been used at BI to manipulate and obtain distinct glyco-patterns. High-throughput glycoanalysis technologies have been established as very important tools in this regard and will be described. Furthermore, the presentation will focus on molecule property manipulation through the use of host cell evolution to obtain subclones with different glyco-profiles, and we will also show data from the combined use of BI-HEX tools in conjunction with the GlymaxX glyco-engineering technology.
MICROENGRAVING: AN EMERGING TECHNOLOGY FOR CLONAL SELECTION OF HIGHLY PRODUCTIVE CELL LINES

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Therapeutic proteins, including antibodies, account for more than $99 billion of drug revenues annually worldwide and constitute a quarter of all new drugs for the treatment of diseases ranging from autoimmunity to cancer. Fermentation titer remains a dominant cost determinant in the production of recombinant proteins, and thus, identifying clonal master cell lines that maximize protein expression and secretion is critical for bioprocess development. Additionally, since therapeutic efficacy and biological function of biologic drugs in vivo is significantly affected by factors like antigen binding capability and glycosylation pattern, early selection of highly productive clones secreting proteins with a particular product quality profile would improve production economics and further shorten process development timelines.

Advances in robotics, automated liquid handling and high-throughput imaging of microtiter plates have enabled new technologies for clonal selection of highly producing cell lines. For example, the Genetix ClonePix instrument can pick ~1000 clones per day, but requires highly sophisticated and costly equipment, and does not allow for the direct screening of cells for secreted proteins. Furthermore, cells must be incubated in semi-solid medium, and cannot be screened immediately following cultivation by fermentation.

Previously, we have reported a method that used microengraving for clonal selection to screen production-ready yeast hosts for the secretion of desired recombinant proteins. Microengraving is a bioanalytical process that isolates and quantitatively measures the rates of protein secretion for thousands of single cells simultaneously. Cells are deposited into an array of picoliter wells at a density of ~1 cell per well, and the array is the sealed to a glass slide uniformly coated with a reagent to capture specific secreted products of interest, for example antibodies. Following a short incubation of the sealed array (1 to 2 h), the slide is removed to yield a protein microarray comprising the secreted proteins captured from each individual well. Cells with desired phenotypes are identified and recovered in less than 24 hours using automated micromanipulation. Multiplexing of this process allows for cells to be analyzed for multiple desirable characteristics concurrently, including productivity, antigen binding and glycosylation pattern. The miniaturized format of each microengraving assay also helps conserve reagents—-a typical screen costs less than a single enzyme-linked immunosorbent assay (ELISA) using a 96-well microtiter plate.

Here, we extend the use of our technology to perform clonal selection of CHO cells. Analysis of CHO cell populations using microengraving allows for rapid identification of highly productive clones secreting antibodies that demonstrate antigen-specific binding. This population may be further refined to select for clones capable of yielding desired glycosylation patterns. Cells are screened immediately following cultivation in conditions designed to promote secretion (e.g., fermentation) using any media composition. Additionally, microengraving may be used as a process analytical technology to monitor single cell productivity following selection and outgrowth, enabling early identification of clonal cell lines with stable secretion profiles.
Therapeutic and market values of monoclonal antibodies (Mabs) have been dramatically increased over the past few years. The intracellular assembly and glycosylation of Mabs is very important in ensuring consistent glycan profiles which are essential for efficacy and effectiveness. Differing theories have been proposed for how Mabs are assembled and this assembly mechanism may play a role in glycosylation events. The two main models have secondary intermediates of either a heavy chain dimer (100kDa) or heavy chain-light chain (75kDa). The research here highlights the association between IgG1 intracellular intermediates and glycan profiles by examining the temporal relationship between glycosylation and disulfide bond formation between the individual chains of a Mab produced from a murine cell line (NS0). Using a non-cholesterol dependent NS0 cell line in serum free media containing radioactive isotopes of 35S labeled cysteine and methionine, Mabs were labeled, produced and examined at various time points under varying reducing media conditions to extract information regarding this association. Using HILIC-HPLC methods a 33% downward shift in GI (Galactosylation Index) was observed when reducing agents were introduced. The autoradiographs of the protein A purified intracellular IgG1 and its fragments provided two results. Firstly, the assembly pathway of this IgG1 followed published reports that low galactosylation was favored in situations where heavy chain dimers (100kDa) formed as opposed to heavy chain-light chain (75kDa) intermediates. Secondly, the ratio of heavy chain dimer to heavy chain monomer increased over time within the reducing agent cultures. The increase in heavy chain dimers and lower GI appear to be correlated, possibly due to disruption of the disulfide bonds at the higher levels of assembly. A change in the assembly pathway may alter the final IgG glycan pattern and possibly lead to control mechanisms that influence glycan profiles of monoclonal antibodies.
Cell age can vary significantly between campaigns for Phase 1 and commercial launch as a result of scale-up to larger production bioreactors as well as the need to have manufacturing flexibility in the seed train design. It is therefore critical to choose a phenotypically stable cell line that consistently produces the expected quantity and quality of an antibody product within a large manufacturing cell age window. During our cell line screening process, cell lines are expanded to an age beyond that expected at the commercial scale, and a fed-batch production experiment is performed comparing cells at various population doublings to assess cell line phenotypic stability. In this poster, we present the impact of the choice of media used to maintain cell cultures on phenotypic stability. Furthermore, we will demonstrate how these results have allowed us to identify an optimal maintenance medium which dramatically improves phenotypic stability.
Recombinant CHO cell lines are the workhorses for the production of protein therapeutics. The generation of these stable cell lines typically involves random integration of the genes into the genome and a selection of stable high producers follows. These processes typically take at least 6 months using high through-put methods. There is a lack of understanding of the physiological adaptive mechanisms that occur during cell line development. To address these questions, we compared the genomic, transcriptome, glycomic and metabolic profiles of parental CHO-K1 and SH87, a recombinant CHO-K1 anti-Her 2 producing cell line. The recombinant clone was generated using an in-house tricistronic vector using standard cell-culture transfection methods and was isolated within two months (Ho et al., 2011). The high expressing clone was then adapted back into suspension in protein-free medium with G418 selection. Productivity and growth characterization of the parental and recombinant cell lines were performed in shake-flasks.

The genomic profile and transcriptome profile of the CHO-K1 and SH87 cell lines were obtained using next-generation sequencing technology. The CHO-K1 genome was reassembled and compared with the recently published genomic data (Xun et al., 2011). As expected, high degree of similarity has been found between the two genomes. The genome of CHO-K1 and SH87 were scanned for structural variances, point mutations and integration sites. Interestingly, initial results show multi-copy integration of vector into the recombinant genome. RNA-Seq and full-length RNA-PET analysis of the clone reveal potential transcriptome hotspots, functionally enriched pathways and all possible transcript isoforms present in CHO-K1 and SH87.

Liquid chromatography-mass spectrometry (LC-MS) based metabolomics analysis was also carried out to identify key metabolic differences between the two cell lines. Preliminary results suggest these differences include molecules involved in lipid metabolism and the removal of reactive oxygen species. Additionally, the glycosylation profiles of the two cell lines are currently under investigation to evaluate whether the development of such recombinant cell line result in a different N- and O-linked glycosylation capabilities.

This comprehensive study yields valuable insights into the cellular physiological mechanisms of recombinant cell line development and suggest strategies to optimize future cell line development work.

References


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Perfusion cell culture process development is a time consuming and labor intensive activity. With the rising pressure to increase the speed and throughput of the drug development pipeline, efficiencies of the upstream process development component require ongoing analysis and improvement. Additionally, regulatory expectations have increased with respect to process design space definition using Quality-by-Design principles. All this demands greater efficiency from a Process Development organization if it is to be scalable. With the absence of suitable smaller-scale models of bioreactor processes, the bench-top bioreactor remains the work horse for much of the development effort. Typically, these laboratory reactors are limited in number requiring a judicious choice of experiments. The experimental approach has traditionally been by trial and error and dependent on statistical comparison of results. There have been many publications on design of experiments (DOE) being applied to process development but DOE remains a largely empirical approach and does not reveal the underlying structure of the performance contours. This study will examine the application of basic biochemical engineering principles in a rational manner that will minimize the number of experiments needed to define the process design space.

Through the measurement and determination of the kinetics of basal biological and biochemical processes and the sequence of experiments, perhaps the ultimate number of experiments can be decreased as well as yielding more structure to the design space topography. We examine the use of fundamental cell growth and product formation and degradation kinetics as the basis of choosing an optimal bioreactor operating mode. Using a directed approach combining DOE, where it is best suited, with a more fundamental approach may improve the outcome and the efficiency of bioprocess development. We also deconstruct previous cell culture development projects to see if a different approach may have led to a reduced effort overall.
Cultured mammalian cells consume large amounts of glucose and divert most of it towards lactate, whose accumulation is inhibitory to growth and product synthesis. In fedbatch cultures, fortuitous metabolic shifts to low lactate production or lactate consumption lead to sustained viability and higher productivity of recombinant proteins. The mechanisms governing this phenomenon are still not clearly understood. Analysis of such shifts through a systems biology approach revealed that various pathways, both metabolic and signaling, play key roles in inducing such shifts.

Our experimental data as well as the process data of over two hundred manufacturing runs revealed that lactate consumption occurs in slow growth stage and is accompanied by low glucose uptake rates. Using a mechanistic model for the central metabolic network, high lactate concentration, low glucose consumption rate and growth regulation were identified as key factors for such a metabolic shift. However, overwhelming experimental evidence demonstrate that under the same set of glucose and lactate concentrations, a culture could be in a lactate production state or in a lactate consumption state, strongly suggesting that the concentrations of lactate and glucose are not the only controlling variables.

The glycolysis pathway is subject to complex allosteric regulations that impart non-linear behavior to its activity. Model analysis demonstrates that multiplicity of steady states is present under some culture conditions. The results also imply that the metabolism of the culture is affected by its history in addition to its present state.

The model was employed to simulate the transient behavior of lactate metabolism under different culture conditions, and it indeed predicted vastly different lactate profiles even under the same culture conditions. In the metabolically shifted culture, lactate consumption may proceed to its exhaustion as observed experimentally.

Our model prediction suggested that robust lactate consumption can be attained by restricting glucose consumption while the growth rate is decreasing. Such shift to lactate consumption was experimentally illustrated, both by diminishing sugar consumption in late stage of fedbatch culture and by the modulation of signaling pathways regulating glycolysis, such as AKT-mTOR, in the late stages of culture.

The insights from the mechanistic model enhances our understanding of the physiology of metabolic shifts in mammalian cells and is likely to contribute to the design of strategies for process enhancement through manipulation of cellular energy metabolism.
Bend Research Inc. is currently exploring the use of dielectric spectroscopy (DS) in cell culture applications, which may be a particularly promising technology for providing cell-level process information. DS may provide valuable data not accessible with other technologies because of its non-invasive nature and potential to report cell-level properties. The generation of bioreactor process data at the cellular level could result in more informative design space models by directly measuring the biological machinery (cells) instead of inferring the same from measurements of the cell environment. Cell-level observations are more likely to enable predictions of the observed product attributes.

Historically, cellular level data sets generated using dielectric spectroscopy in mammalian cells have been used to detect changes in cell populations (e.g., cell death, cell morphology, organelle content, and other biophysical properties) for study of different disease states. The current poster is focused on the fundamental biophysics of DS, and describes how DS may allow unique observability of cellular-level information within a cell culture process. This observability, coupled with models and data-analysis techniques, is discussed in context of how it may be leveraged to: 1) aid in development scale bioreactor experiments for design space generation, 2) increase process understanding at the cell level, 3) guide media development, and 4) as a monitoring tool for larger scale bioreactors.
Glycan structure is a key product quality attribute of monoclonal antibody biologics. A decrease in galactosylated product was observed during the transition from hydrolysate-containing media to chemically defined (CD) media based processes for Chinese Hamster Ovary (CHO) cell cultures. The distribution of major glycan species varies with the activity of manganese-dependent galactosyltransferases. Galactosylation profiles were restored with the addition of manganese sulfate (MnSO4) to chemically defined media. This study found that increasing Mn2+ level was positively correlated with an increase in galactosylated species for several molecules. Modulating Mn2+, by itself, may offer a potential means to control galactosylation of mAbs in chemically defined CHO cell culture processes.
The potential for monoclonal antibody (MAb) disulfide reduction, and eventual product loss, during harvest operations warrants a lab-scale method to assess the risk associated with cell culture production processes. A cell line’s susceptibility to lysis has become an important characteristic as cell lysis in high cell density processes can lead to MAb reduction in harvested cell culture fluid. A working hypothesis is that as cells lyse they release cellular components including macromolecules (e.g. reducing enzymes) and active proton carriers (e.g. NADPH) into the cell culture fluid (CCF). Released cellular components in turn partner to hydrolyze the interchain disulfide bonds of the MAb. Lysis susceptibility can be screened by subjecting cultures to a flow contraction device (FCD) in which variable degrees of lysis between cell lines are achieved at known energy dissipation rates (EDR). The EDRs generated by the FCD are between 10E5 – 10E8 W/m3 across flow rates of 10 – 100mL/min. These EDRs are sufficient to cause lysis in CHO cells. In the complete method shown here, lysis susceptibility screening is integrated with analysis of MAb reduction in the resulting lysates. Part of this method includes screening for the effect of cell-size on lysis where cell-size is modulated through the use of a salt-shock technique just prior to the lysis event. The method has been used to characterize lysis susceptibility and MAb reduction risk in numerous cell lines and processes at Genentech.
One of the challenges of cell culture process scale-up is to maintain a consistent physical and chemical environment in the bioreactors as vessel size increases. Fast mixing and high gas mass transfer rates in bioreactors are often achieved by using high agitation and gas flow rates, resulting in high shear stress levels. A pneumatic mixing mechanism has been developed using the Air-Wheel™ which converts the buoyancy of sparge gas bubbles into rotational energy, achieving efficient liquid and gas mixing without any external mixing device. This mixing system is scalable from 2L to 5,000L with mixing times of 20 to 60 seconds, respectively, using gas flows that are compatible to those in stirred-tank bioreactors. High mass transfer rates (kLa > 20 hr⁻¹) are achieved by delivering pure oxygen through a micro-sparger. Impeller wall shear stress (τ_avg, in Pa) and turbulent kinetic energy dissipation rate (ε, in m²/s³) of the system were calculated from computational fluid dynamics (CFD) modeling and compared to stirred-tank bioreactors. The average level of shear stress of the pneumatic mixing system (< 0.3 Pa) is significantly lower than in conventional stirred tank bioreactors (1.0–2.4 Pa) and remains constant during scale-up from 3L to 2,500L. The turbulent kinetic energy dissipation rate (ε < 0.01 m²/s³) also remains the same over the broad range of working volumes. These results indicate that the cell culture environment in pneumatic mixing bioreactors is consistent across scales, which will make the process scale up from laboratory to production stage much more predictable and reliable.

Cell culture performance of the pneumatically-driven single-use bioreactor system (PBS) was evaluated using various cell types and processes. Human alveolar adenocarcinoma (A549) cells were cultured on Cytodex-1 micro-carriers for production of oncolytic adenovirus. Cells attached much faster and more evenly on the surface of micro-carriers in the PBS bioreactor, resulting in significantly higher cell growth and virus titers. Cell density (1.2 x 10⁶ cells/mL vs. 0.6 x 10⁶ cells/mL) at infection time and maximum virus productivity measured by TCID50 (9.9 x 10⁶ IP/mL vs. 5.3 x 10⁶ IP/mL) in the pneumatic mixing bioreactor were nearly double those of a conventional stirred bioreactor. CHO cells expressing a monoclonal antibody in PBS bioreactors with 2L, 50L, and 250 L working volumes showed comparable results to stirred bioreactors with peak cell densities up to 10⁷ cells/mL with > 95% viability and similar antibody productivity. Final purification yield and product quality profiles were also comparable between the bioreactor systems. In addition, high peak cell densities of 2.5 x 10⁷ cells/mL and 3.0 x 10⁷ cell/mL were achieved in the PBS bioreactor using CHO cells cultured in a fed-batch mode and in a perfusion mode, respectively, both of which demand high mass transfer rates for oxygenation. In conclusion, PBS offers more homogenous mixing with lower shear stress than conventional stirred bioreactors and may offer improved performance in potential applications such as cell therapy, personalized medicine, viral production, and adherent cell culture on micro-carriers, in addition to traditional suspension cell culture processes.
Recombinant glycoproteins represent a large part of the drug market: For example, in 2008 in the United States, monoclonal antibodies sales exceeded $20 billion with growth perspectives estimated at 14% per year. Industrial production of these molecules is mainly performed with Chinese Hamster Ovary (CHO) stable cell lines. These cell lines are not adapted to produce material dedicated to fundamental and pre-clinical studies because their establishment is long and very expensive. To facilitate and accelerate the discovery of new biotherapeutic products, we currently develop and optimize new transient expression systems based on the infection of CHO cells with baculovirus. For that, we propose: 1) to modify the virus tropism in order to increase CHO cells infection efficiency, 2) to use the new powerful promoter/transactivator system referred to as the cumate gene-switch, 3) to insert a set of anti-apoptotic genes in CHO cells to extend the viability of infected cells. Some preliminary data of this original concept of recombinant protein expression in mammalian cells will be presented.
Chinese hamster ovary (CHO) cells are frequently used for the commercial expression of recombinant therapeutic antibodies. As a consequence of their initial derivation by mutagenesis and the divergence into distinct cell lines, the CHO cell is a misnomer. Clonally-derived cell lines are phenotypically distinct. Molecular understanding of the features that determine the properties of a CHO clone (and consequent cell line) is fundamental to the optimization of cell environment and, potentially, engineering or selecting CHO clones with the “best” phenotype.

The profile of the endoplasmic reticulum (ER) environment (with a specific complement of chaperones, co-chaperones, and sensors) is important for cell growth and maximal recombinant protein secretion. We have addressed how the modulation of two components in the ER, XBP-1(s) and Ero1α, influences CHO cell function. XBP-1(s) is generated by a novel mRNA splicing mechanism in response to ER stress and is the key regulator factor for the development of professional secretory cells. Ero1α plays a critical role in setting the redox state of foldases (such as PDI) and is also known to be induced by ER stress. In our studies, CHO S cells were doubly transfected with human XBP-1(s) and human Ero1α constructs to generate a series of CHO cell lines that overexpressed each gene. These novel engineered host cell lines are referred to as CHO SXE.

The CHO SXE cell lines exhibit a series of improvements in desirable phenotypes compared to the non-engineered CHO S cell line. For example, CHO SXE cell lines have an increased resistance to oxidative stress (as measured by glutathione recycling) and display a different profile of endogenous ER components (chaperones and co-chaperones). Despite over-expressing human XBP-1(s) the activation of endogenous CHO XBP-1 splicing still occurs in response to ER stress imposition. Intriguingly, in parallel assessments of the transfection of CHO S and CHO SXE with recombinant antibody vectors, CHO SXE gave greater numbers of clones with suitable expression. Our interpretation is that the engineering of cells to over-express XBP-1(s) and Ero1α generates a more favorable phenotype for cells to handle the stresses that result from protein transit in the ER. Whether this is a direct effect of XBP-1(s) and Ero1α or due to a secondary consequence of their over-expression on the ER chaperone complement remains unclear. However, our data identify important combinations of regulatory factors that influence ER function and, consequently, the ability to define improved CHO cell phenotypes for expression of different types of protein products.
ENHANCED ADCC ACTIVITY FOR AN FC-CONTAINING PROTEIN PRODUCED IN A GLCNAC T1 DEFICIENT CHO HOST

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For recombinant Fc proteins, N-glycan moieties are critical to the activation of downstream effector mechanisms. Absence of the core fucose molecule has been shown to increase Antibody-Dependent Cellular Cytotoxicity (ADCC) activity by over 100-fold. In an attempt to increase ADCC activity, expression of N-acetylglucosaminyltransferase 1 (GlcNAc T1), a key enzyme in the glycosylation progression from high mannose glycan structures to complex glycan forms and an enzyme upstream of the fucose addition, was targeted for elimination. The absence of GlcNAc T1 was expected to halt the glycosylation pathway prior to the formation of the complex oligosaccharide form (a substrate necessary for fucose addition) and result in protein with N-linked glycans of the Man5 form. The Man5 N-glycans lack fucose and therefore an increase in the percentage of Man5 species in the final product yield may exhibit increased ADCC activity typical of proteins with reduced % fucosylation. To demonstrate the ability of proteins containing Man5 Fc glycans to increase ADCC activity, a novel Lec1-CHO-dukx host deficient for GlcNAc T1 activity was stably transfected with either an IgG1, or a recombinant Fc fusion protein containing a B-cell surface receptor-binding domain. Protein produced from these transfected Lec1 cells was purified and glycan analysis performed; nearly100% of the product for both molecules was found to be of the Man5, a-fucosylated form. The recombinant Fc Man5 protein was further purified and analyzed for target binding and effector function. While no difference in target binding was observed, a decrease in complement-dependent cytotoxicity was seen for the Man5 species compared to the complex N-linked glycan protein. In contrast, the Man5 protein demonstrated a significant increase in ADCC activity over protein containing complex N-glycosylation on the Fc gycan. These data support the hypothesis that modulating GlcNAc T1 activity influences antibody Fc glycan structure and specifically leads to increased ADCC activity of antibody produced in Lec1 CHO cells.
DEVELOPMENT OF A CHO-S TRANSIENT EXPRESSION SYSTEM TO RAPIDLY GENERATE PRECLINICAL MATERIAL SUPPLY

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For antibody programs in preclinical stage, it is desirable to generate material for testing in a significantly faster timeframe than is typically possible using stable transfection methods. One such alternative is to produce antibodies in transiently transfected HEK 293 cells, which can yield purified protein in as little as 1-2 weeks. Historically, HEK293 cells have been used in transient expression systems because they are easy to transfect and they express high levels of proteins. Because the lead antibody will eventually be expressed in a stable CHO line, however, it would be desirable to perform preclinical assessments of candidate clones using the same cell type. Indeed, several reports describe that post-translational modifications affecting effector function or PK (i.e. glycosylation) can be dramatically different in antibodies generated from CHO vs HEK293 cells. Unfortunately, transient expression in CHO cells has not been widely used historically, mainly due to its reported low expression levels. We have therefore performed a systematic optimization of a transient transfection method to improve expression levels using a commercially available CHO-S cell line. Initially, different DNA:PEI ratios were screened for optimal transfection efficiency, with 1:4 chosen as the optimal ratio. The use of a Takeda proprietary vector and the addition of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) were also discovered to improve CHO transient transfection expression levels. Finally, transfection at high cell densities (2 x 106 cells /mL) and a 2 week fed-batch process under hypothermic conditions were observed to further increase production yield. Overall, our preliminary optimization efforts were successful at increasing antibody titers from 3 µg/mL to 60 µg/mL, a range well within what has been reported for HEK293 systems. In addition we have demonstrated that the CHO system is amenable for expression of soluble antigens, which are typically more difficult to express than antibodies. Moving forward, this transient CHO-S expression system represents a versatile platform that is not only capable of yielding preclinical quantities of material in a short timeframe, but will also help ensure that key quality attributes of lead antibodies are captured and preserved through their various phases of development.
EFFECT OF GROWTH MEDIUM EXCHANGE AND DISSOLVED OXYGEN CONCENTRATION ON THE IN VITRO PROLIFERATION AND METABOLISM OF HUMAN MESENCHYMAL STEM CELLS: A QUANTITATIVE APPROACH.

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For the potential of human mesenchymal stem (hMSC) cell-based therapeutics to be realized, the ability to culture hMSCs at sufficient scale in vitro is vital. It is recognized that today hMSC culture is where CHO cell culture was 20-30 years ago indeed current measurements taken during standard T-flask culture are usually restricted only to cell number, viability, confluency and those related to functionality. Here we argue that in order to take an informed approach to the development of any larger scale production process for hMSC’s either by scale up or scale out methods, it is essential to understand the processes occurring in the bioreactor.

This work provides a quantitative analysis of the changes in concentration of the main metabolites (glucose, lactate and ammonium) with time during human mesenchymal stem cell (hMSC) monolayer culture over 4 passages where a 100%, 50% and 0% growth medium exchange was performed after 72h in culture. The work demonstrates that a lower dO2 tension, in this case 20% dO2, impacts hMSC proliferation resulting in lower cell yields in comparison to hMSCs cultured under 100% dO2 conditions. This is in contrast to other studies which demonstrate an increase in hMSC yield under lower dO2 conditions and the hypothesis that hMSC proliferation would increase under physiologically similar dO2 levels. That there is disparity in the literature with regards to the effect of dO2 on hMSC proliferation highlights the variability that exists between cell lines and is likely to be a result of donor-to-donor variability and/or medium formulation. This finding also highlights the importance of implementing a quantitative approach and measuring as many parameters as possible in an attempt to understand internal cellular mechanisms and processes. Whilst hMSC yield decreased under a lower oxygen tension, the rate of glucose consumption and lactate and ammonium production increased, therefore suggesting different metabolic pathways being employed as the hMSCs adapt to low oxygen conditions. The percentage of medium exchange was found to also have an effect cell proliferation and metabolism, with a 100% medium exchange resulting in the greatest cumulative viable cell number over the series of four passages, followed by the 50% and then 0% respectively. Based on the metabolite data, this is likely to be due to the accumulation of inhibitory concentrations of lactate and ammonium or other unknown waste products. There was a clear correlation between the concentration of these known metabolites and the percentage of medium exchange performed, whereby the lower percentage of medium exchange performed, the higher the concentration of both ammonium and lactate at the end of each passage. This demonstrates that where possible, a complete medium exchange would aid cell proliferation through the replacement of lost nutrients, and more importantly perhaps through the removal of inhibitory waste products. Despite the variation in experimental conditions, there was no discernible effect on cell quality as the cells retained both their immunophenotype and multi-lineage differentiation capability throughout.
RAPID, LARGE-SCALE MANUFACTURE OF IMMUNOTHERAPEUTICS

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The manufacture of immunotherapeutics, such as monoclonal antibodies, is of great interest not only for treatment of cancer, arthritis, and other medical needs, but for the production of medical countermeasures. Production systems for these molecules are well established, yet tradeoffs in platforms exist between speed, cost, and product quality. Bacterial, fungal, plant and insect cells are capable of rapid manufacture, yet suffer from non-human-like glycosylation or no glycosylation, and thus are used for few if any of the approved antibody therapies to date. In contrast, mammalian cells, especially Chinese Hamster Ovary (CHO) cells, produce high-quality immunotherapeutic with appropriate glycosylation for necessary effector function, and provide products with over $60 billion in annual sales. However, industrial processes utilizing these systems apply stable cell lines for protein expression, wherein the plasmid coding for the protein of interest is stably integrated into the host cell chromosomes. Preparation of the associated Master Cell Bank, including cloning, expansion, selection, cryopreservation, and testing, typically takes at least 4 months, usually much longer. The development time required for this traditional approach is not compatible with very rapid manufacture, as required for emergency medical countermeasures. We propose to use large-scale, transient transfection of CHO cells as a superior approach for very rapid production of antibodies with human-like glycosylation and full effector function. Transient transfection is an established system and a routine approach to make small scale, research-grade material of recombinant proteins where expression occurs from plasmid DNA maintained extra-chromosomally. Transient transfection is capable of generating gram quantities of material within weeks of lead target identification. Despite being scaled up to the 400-liter scale, with some current attempts at the 1000-liter scale, these processes are not able to economically produce sufficient amounts of proteins for use in surge capacity, mass-casualty purposes due to low specific productivity, low volumetric yields and the high cost of DNA. With improvements in titer performance and economies of scale using the largest industrial bioreactors (12,500 to 25,000L), transient transfection could provide a turn-key platform manufacturing system capable of producing on-demand, high quality immunotherapeutics for emergency scenarios. There is currently a substantial excess capacity of such large bioreactors in the U.S. In order to increase titer and determine scalability, it is necessary to characterize some of the key physio-chemical parameters involved in the transfection process through scale down experiments in shaker flasks and stirred tank bioreactors of various scales, geometries, and compositions. Our approach builds off of existing studies and expands the variables tested in CHO cell transfections to achieve record transient titers for monoclonal antibody production, in the hundreds of mg/L range. Transport phenomena, including temperature, mass transfer, and transfection time and media composition were analyzed for increases in specific and volumetric productivity.
As the workhorse of biopharmaceutical production, Chinese Hamster Ovary (CHO) cells are constantly being pushed to achieve greater productivity and higher titers of valuable life saving drugs. A number of methods have been developed toward this end including process control optimization, feeding strategy, genetic engineering, and media supplementation. Here, we tested two animal-free media supplements: recombinant lactoferrin (rL), a milk protein with antimicrobial properties that can also aid in iron absorption by cells, and recombinant human serum albumin (rHSA), an anionic serum protein that aids in cell uptake of nutrients. We evaluated their effect on CHO cell growth and product yield. We tested various concentrations and combinations of the two and identified 250 mg/L of rHSA as giving optimum results. Shake flask experiments indicated all supplement formulations supported higher viable cell densities (VCD), with rHSA supplementation yielding a peak VCD 28% greater, on average, than that achieved in unsupplemented control. Product titer improvement was in line with VCD data with cells grown in rHSA showing a 40% increase, on average, in mAB concentration at harvest. Results varied on a process to process basis but supplementation was observed to consistently improve performance. Compared to unsupplemented controls, peak VCD improvement ranged between 2 - 65% and product titer improvement ranged between 2 - 80%. Similar results were obtained in preliminary bioreactor experiments, with titers up 75% using 250 mg/L rHSA. Our experiments suggest that the improvement in product titer may be attributed to increased VCD as specific productivity was unchanged in supplemented versus control cells. Increased VCD may in turn be attributed to higher specific net growth rate at later stages of the growth phase, often due to reduced death rates. Specific net growth was comparable in supplemented versus control cells at the early stages of growth but diverged around days 4-7, leading to significant increases in cell density in rHSA supplemented samples. We have found that rHSA supplementation consistently improves specific net growth rate of CHO after day 4 in many fed batch cultures.
Chemically defined (CD) media and CD Supplements have been presented as an alternative to overcome challenges in the pharmaceutical industry regarding the use of protein hydrolysates. Hydrolysates might convey to inconsistent process performance, which is attributed to lot-to-lot variability in raw material as well as the presence of unknown components. In this work we present a systematic evaluation of CD-supplements, including a proprietary formulation (BD-Recharge). Cell culture performance has been validated in three different culture vessels: shake flasks, wave bioreactors and two different stirred tanks (New Brunswick and Dasgip). Results indicate that cell growth, protein production and product quality were similar using CD-Supplement and protein hydrolysates.
Stress proteins are expressed in response to different environmental stresses such as heat, nutritional deficiency, oxidative stress and inhibitory chemicals. While it has been speculated for years, to our knowledge, the expression of stress proteins in response to hydrodynamic stress in bioreactors has not been demonstrated. Complementary to stress proteins, speculation exists with respect to a link between production of recombinant proteins by mammalian cells with a particular cell cycle growth phase. Understanding the relationship between cell cycle phase and productivity could contribute to optimization of large scale processes. In this work we explored the expression of stress proteins under different hydrodynamic conditions in different culture vessels, including static cultures, spinners and bioreactors. Cell cycle profiles were evaluated in 2 L working volume bioreactors with different impeller/sparger configuration in order to determine the potential effect of different hydrodynamic conditions on cell cycle profile and recombinant protein production. Significant difference in stress protein expression between bioreactors and T-Flasks was observed. In contrast, with cell cycle studies, specific productivity seems to be associated with G1 phase and no significant differences were observed when culturing environments (vessels) were changed.
Rapid screening for stable, high producing cell lines using a representative scale-down cell culture process is a critical step in drug development and facilitates more rapid progression from the research phase to the clinical proof-of-concept phase. Cell line development has been hindered by a number of factors making application of smaller-scale, higher-throughput screening methodologies nearly impossible. These factors include: 1) the disparity between productivity results from small scale screening assays and larger scale bioreactors, 2) the expense of multiple, conventional stirred-tank bioreactors, their control units and the manpower and expertise to operate such systems, and 3) the lack of robustness of small-scale, inline pH and dissolved oxygen sensor technology. In this study, we present data from a side-by-side evaluation of four 5 L stirred-tank bioreactors and the advanced microscale bioreactor, ambr™, workstation equipped with twenty-four 15 mL bioreactors. Both systems were used to screen multiple recombinant CHO cell lines in a fed-batch process. The ambr™ system is a completely automated system which utilizes disposable stirred culture vessels, a robotic liquid handler, and fluorimetric pH/DO sensor technology to screen 24 or 48 cell lines in an environment that mimics 5L stirred-tank bioreactors typically used in cell culture development. Moreover, the automated system can be easily operated by a single user.

Our results demonstrated the ambr™ system to be an efficient tool to identify highly productive cell lines (i.e. = 2-3g/L) in a manner representative of our scaled-up processes. Ambr™ is a representative scaled-down screening method that will identify high producing cell lines and predict their performance in larger bench-top and production scale bioreactors. Advances such as ambr™ could significantly reduce costs and eventually lead to replacement of more traditional larger bioreactor systems at this critical stage of drug development.
Cardiovascular disease remains the main cause of mortality worldwide. Several cell-based treatments for severe vascular disease and heart failure have progressed to clinical trials, many of which target an enhanced neovascularisation of ischemic tissue. Endothelial progenitor cells are thought to participate in vasculogenesis in adults. However, there is a lack of adequate in vitro vascular regeneration models, and hence a lack of suitable platforms to test therapeutic cells or drugs that could influence vasculogenesis.

This work describes a novel live cell imaging system that comprises four flow chambers to visualize endothelial progenitor cell adhesion and proliferation in real time. The flow paths to each chamber are separate, allowing testing of different shear rates (ranging between 0.05 and 2.5 Pa), cell types or soluble molecules in parallel. Cell adhesion and spreading rates can be quantified by time lapse phase contrast and fluorescence imaging. Different test surfaces can be inserted into each chamber, providing control over the vascular model being examined.

Primary human saphenous vein endothelial cells were used to validate the performance of this system. Static control cultures maintained in an incubator with controlled temperature and CO2 leading to low and high rates of endothelial cell expansion were developed and are being compared to the flow chamber system. Positive controls with cells cultured on surfaces with micropatterned peptides that promote cell adhesion and migration led to $3.4 \pm 0.7$ fold higher cell yields than unpatterned surfaces. During the first 3 hours of adhesion, the maximum surface area covered by cells was increased by 2-fold on micropatterned surfaces compared to untreated surfaces, whereas the time required to reach half-maximal spreading was not increased. The performance of these control surfaces is now being assessed in the flow chamber system. The multi-well flow chamber system will eventually serve as a cell and drug discovery tool to advance cardiovascular regenerative medicine.
Two experiments were performed to evaluate the suitability of the ambr® micro reactor system (TAP Biosystems) for use in cell culture process development and clone screening. The ambr® is a micro-reactor system with pH, temperature, and dissolved oxygen control. It also has automated liquid handling for feed and base additions, and sample gathering. To assess its performance in process development, a Response Surface Methodology (RSM) experiment was performed in both bench top bioreactors, as well as in the ambr® system. Factors included in the design were temperature, pH, feed volume, feed timing, seed density, and glucose feeding strategy. A total of 72 micro-reactors were run and the growth, titer and product quality were compared to that obtained from reactors. While the confidence intervals around the resulting model were larger for the ambr® data than for the benchtop reactor data, the magnitude and shape of the trends for cell growth, viability, and titer were similar.

The second experiment was a simple clone screen where clones were run in reactors, plates, and benchtop reactors and the results compared. Product quality from the ambr® system was found to be similar for some, but not all, product quality attributes.
Real-time bioprocess monitoring is fundamental for maximizing yield, improving efficiency and process reproducibility, minimizing costs, optimizing product quality, and full understanding of how a system works. The FDA's Process Analytical Technology initiative (PAT) encourages bioprocess workflows to operate under systems that provide timely, in-process results. At the same time the demand for ever increasing supplies of biological pharmaceuticals, such as antibodies and recombinant proteins, has fueled interest in streamlined manufacturing solutions. Bioreactors that are monitored continuously and in real-time offer the advantage of meeting current and future supply demands with biological product of the utmost quality and safety, achieved at the lowest overall cost and with least risk. Continuous multivariate monitoring, performed by combining multiple single variable parameters and through scanning multi-frequency dielectric spectroscopy, offers the opportunity to comparatively profile multiple bioreactor runs and elucidate fine details concerning cell viability and mechanism of cell death. In this study, the simultaneous application of radiofrequency impedance for cell culture monitoring is shown to be complementary and augment off-line data, providing a unified description for the full growth and death profiles of Chinese Hamster Ovary (CHO) cells. The robust models developed here through the use of partial least squares and genetic algorithms provide an approach to harmonize discrepancies between off-line permeability-based VCD measurements and VCD changes observed by in-line capacitance-based probes. In addition, scanning multi-frequency dielectric spectroscopy reveals additional information on the mechanisms of membrane permittivity changes as a function of cell state. By fusing in-line and off-line data streams deviations for VCD, root causes for bioprocess deviation can be better modeled and streamline the integration of the PAT initiative in a bioreactor workflow to improve product quality.
The use of RF Impedance or capacitance to monitor cell culture processes is well established in biopharmaceutical applications. With the increasing trend toward using disposable bioreactors, there is now a need for single use biomass probes suitable for these systems. This will ultimately enhance the opportunity to use these bioreactors in both process development and ultimately in cGMP manufacturing. Single-use biomass probes are now available for use with capacitance based instruments for measuring the live cell concentration and publications to date have been based on bioreactors using agitators. Adoption has been slower than expected but in many cases the initial evaluation of the disposable technology has been at the small to mid-scale where traditionally there has been a very low level of automation and instrumentation. In this paper, we show how a biomass probe has been optimized for use with disposable bioreactors In order to demonstrate the performance of the disposable biomass probes, data will be presented on the growth of SF-9 insect cells in a 25L prototype Sartorius Stedim Biotech rocking platform bag. The study shows how the positioning of the probe within the vessel is critical for performance and the way the probe deals with varying levels of fluid due to the motion of the bag and increasing volumes during fed batch culture. The poster will also show how the disposable biomass probe is able to track the viable SF-9 cell density of the culture before the addition of a Baculouivirus and how it successfully detected the infection phase of the culture.
Keratinocytes are used extensively for the clinical treatment of severe burn patients in intensive care burn units all around the world. To reconstruct epidermis sheets from a patient biopsy, cells presently need to be expanded in fetal bovine serum containing medium over a layer of irradiated mouse irradiated cells. To our knowledge, no defined media currently available allow the production of usable skin sheets. We have used statistical design of experiment (DOE) to screen the individual as well as synergistic effects of a large number of defined culture medium additives. Responses such as growth rate and growth extent, but also cell morphology were used to screen the effective mixture of factors. Through this approach, we have developed a completely defined culture medium that allows short and long-term expansion of human keratinocytes to a level and a quality comparable to serum-containing and feeder layer supported culture. Cells grown with this new medium are fully functional, as they can re-form epidermis.
Orbitally shaken bioreactors have proven to support the efficient cultivation of mammalian cells in suspension. For small-scale cultures, disposable tubes with ventilated caps and nominal volumes of 50 mL (TubeSpin® bioreactor 50 or TubeSpin) and, recently, 600 mL (TubeSpin® bioreactor 600 or MaxiTubeSpin) are commercially available. Both bioreactors have a conical bottom designed to fit into standard swinging bucket rotors facilitating the centrifugation of cells. These bioreactors have mass transfer coefficient (kLa) values ranging from 7 to 50 hr⁻¹ compared to values below 7 hr⁻¹ for orbitally shaken Erlenmeyer flasks and cylindrical glass bottles. Mixing times of less than 10 sec were observed for the TubeSpins and MaxiTubeSpins at shaking speeds suitable for cell cultivation. In order to define the optimal working conditions for the MaxiTubeSpin, we measured cell growth, recombinant protein production, and environmental conditions of the culture (pH, pO₂, pCO₂) over a range of kLa values and mixing times using four CHO- and HEK-derived cell lines stably expressing either a recombinant IgG antibody or a tumor necrosis factor receptor: Fc fusion protein. Each cell line was cultivated in 1-L cylindrical glass bottles, 1-L Corning Erlenmeyer flasks, and MaxiTubespin tubes at a working volume of 300 mL. Growth of the two CHO-derived cell lines was comparable in all three vessels. Cultures of the two HEK-derived cell lines exhibited higher cell densities and volumetric protein productivities in MaxiTubeSpins than in the other two vessels. Oxygen levels remained above 80% air saturation in the MaxiTubeSpins, but fell to 35% in the other two vessels. In MaxiTubeSpin tubes the CO₂ level remained at 5% throughout the cultivation period. However, in the other two vessels the CO₂ levels were greater than 5%, indicating insufficient removal of carbon dioxide. The pH profiles were similar in all vessels. This study shows the utility of MaxiTubeSpins for cell culture applications with suspension-adapted cells at volumes ranging from 100 to 500 mL.
In general, the process flow chart for the development of a stable cell line has involved transfection of a CHO cell line with the gene of interest and a selectable marker, direct selection for cells that contain the desired insert and then exposure of the selection+ cells to higher concentration of the selection reagent to amplify number of copies of the insert. Clonal selection is performed on this amplified cell pool to screen for the highest producers. This is followed by further screening for the highest productivity, growth characteristics and product quality. Subcloning is typically employed at this stage to identify cell lines produced from a single cell. Stability studies of the top 1-3 cell lines are an essential step in choosing a master cell bank candidate. At what step of the cell line development process a stability study is implemented can be different depending on the company’s platform process. GPEx® cell line development technology utilizes replication incompetent retroviruses to insert the gene of interest into any active regions of the genome as a single insert of any mammalian cell line. Transduction efficiency is close to 100% eliminating the need for any selection or amplification. Because the gene insertion targets different genomic locations, the cell lines are inherently stable. The elimination of antibiotic selection and stability studies during cell line development process significantly shorten the timeline for a master cell bank candidate. Case studies on the development of three different antibody-producing cell lines produced by both a traditional amplification process and the GPEX® system will be presented. Overall, comparisons of both the productivity and stability (over 100 generations) from clones identified from each cell line development process consistently favored the GPEX®-produced clones.
A fusion protein produced in CHO was found to have significant protease clipping between an agrinine and leucine. The protease was determined to be in the serine protease family with “trypsin like” clipping but was not inhibited by soybean trypsin inhibitor. Protease identification work continues pointing towards the thrombin group of the serine protease family but is incomplete. A significant effort was made to screen different media, feeds, hydrolysates, and yeastolates with no significant impact. Competitive inhibitors known to help reduce “trypsin like” clipping in cell culture were tested with no effect. Small molecule inhibitors known to inhibit serine proteases have been tested in cell free broth with success but with no success in cell culture due to their toxic impact (AEBSF, Benzamidine). Process controls such as duration, temperature, and pH, were found to impact the level of protease clipping. These process conditions were optimized decreasing the level of clipping and increasing the overall intact titer.
CHARACTERIZING HESC METABOLISM BY SYSTEMS BIOLOGICAL APPROACH

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Human embryonic stem cells (hESC) offer new potentials for treating various diseases due to their unique regenerative capability (1). One of the key aims of this emerging discipline of research is to understand the effect of various nutrient/environmental/cultural conditions on the growth of undifferentiated stem cells that are characterized by the secretion of undesired toxic metabolites such as lactate and ammonia leading to reduced pluripotent and self-renewal properties. Therefore, it is imperative to explore the intracellular metabolism of hESC and elucidate the mechanism behind the toxic secretion. In this regard, systems level modeling and in silico analysis of hESC metabolism can be crucial for characterizing the phenotypic behaviors and metabolic states during stem cell cultures. Similar analysis has been successfully applied and proven useful in understanding the physiological characteristics of well-known microbial and mammalian model organisms such as E. coli, S. cerevisiae, mouse and CHO cells (2). In this work, we have first attempted to develop a metabolic model for hESC based on recently available human genome-scale metabolic network (3). This generic network model was fine-tuned by constraining hESC-specific conditions which were inferred from the literature and our experiments (4). Subsequently, the network model was validated by simulating the cell growth rate during exponential phase in 2D colony and microcarrier cultures using different media (conditioned medium and 3 serum-free medium); the results showed that the simulated growth rate was highly consistent with the experimental observations, revealing unique features of hESC compared to other mammalian cells. While detailed investigation awaits exploring energy metabolism and waste secretion in hESCs, together with omics data analysis in future, we anticipate that combined experimental and in silico analysis would serve as a promising approach for optimal media development and potential cell engineering targets for developing pluripotent stem cells and stem cell based therapies within the context of systems biology.

Keywords: systems biology, human embryonic stem cells, genome-scale metabolic network, constraint-based flux analysis.

References


Microline is a fully disposable GMP manufacturing facility for production of small quantities of antibodies for exploratory IND studies. Single use systems were selected over traditional SS system to provide a cheaper, faster, flexible option for exp IND studies. The disposable manufacturing facility provides the flexibility to operate with quick turn around times without too much capital investment, facility modifications, or long construction timelines. Additionally, pre-assembled, gamma-irradiated assemblies are generally faster, easier and cheaper to set-up than their steel and fixed-pipe counterparts. Disposable shake flasks, WAVE, Single Use Bioreactors were used for upstream processing whereas GE Akta systems were used for downstream processing. The use of disposable technology and aseptic connectors/welding allowed for a fully closed system making it feasible to do bioprocessing (upstream and downstream) in one suite. The project focuses on comparison of the product quality, limitations/benefits and economic analysis of MAb manufactured in disposable manufacturing facility (Microline) compared to a stainless steel facility production. The product quality and titers for product produced in Microline facility were similar to historical data generated using SS equipment. The cell growth and nutrient metabolites were also similar to historical data. Additionally, quality control samples were taken during the run to measure the contaminant level. The tests completed were Mycoplasma DNAF and Culture, Rodent Parvovirus, and General Viral Screening. All samples were negative for the contaminants tested. No contaminations occurred throughout the entire production campaign. The production results show that a fully disposable production line from thaw to formulated bulk product, and in a single manufacturing suite can be accomplished under GMP guidelines while meeting all product quality specifications.
PROTEIN EXPRESSION IN DEFINED CHROMOSOMAL LOCI OF SF9 INSECT CELLS: A VALUABLE ALTERNATIVE TO BACULOVIRUS INFECTION

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Insect cells, in particular the Spodoptera frugiperda Sf9 cell line, have been widely used for the production of biologically active recombinant proteins. However, the current technology uses baculovirus infection which has two main disadvantages: firstly, the recombinant gene is only expressed transiently during the infection cycle, after which cells die; secondly, due to the lytic nature of this system, the cellular protein processing machinery is severely compromised at the end of the infection cycle, affecting the correct formation of recombinant proteins whose expression is usually controlled by very late baculovirus promoters. Stably transfected insect cell lines represent an alternative system for continuous protein production. However, their establishment is laborious, requiring the identification of cell clones that display the right expression properties due to random integration of the heterologous gene.

To overcome these issues, we developed a Sf9 cell line using targeted integration by recombinase-mediated cassette exchange (RMCE), allowing repeated use of the same locus to produce different proteins. RMCE technology consists of the replacement of a genetic cassette anchored in a well characterized chromosomal locus by another encoding a gene of interest, a process mediated by a recombinase enzyme. Different promoters and transfection protocols were evaluated to define optimal conditions for gene expression from single copy integration of the tagging cassette. Relevant cell clones were identified and co-transfected with reporter gene and flippase containing vectors. After three weeks in antibiotic selection, successful flippase mediated cassette exchange was confirmed for the first time in Sf9 cells. Reporter protein expression levels in batch cultures of the best performing clones were comparable to those obtained with baculovirus infection. These results open promising perspectives for the expression platform developed here as it combines the advantageous growth properties of Sf9 cells with continuous protein production and the possibility of re-using a well characterized locus for targeted gene-of-interest integration.

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OPTIMISATION OF CHO TRANSIENT TRANSFECTIONS TO OBTAIN HIGH TITRE ANTIBODY EXPRESSION

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Transient gene expression (TGE) systems have recently advanced and now provide a rapid and scalable (up to 100L) method for generating gram quantities of recombinant proteins. In HEK293 cells titres of 1g/L have been achieved, (Backliwal et al., 2008) but TGE in the more commercially relevant CHO has lagged behind despite reports of yields in excess of 300mg/L being achieved. As TGE methods require the delivery of large quantities of DNA to the host cells this can create bottlenecks (during transcription, translation, protein folding and/or secretion) in the cellular production of recombinant protein including MAbs. We have evidence that in a TGE system it is protein synthesis and subsequent secretion that are the predominant rate-determinant steps resulting in accumulation of nascent polypeptides and unfolded proteins within the cell (manuscript in preparation). In order to eliminate secretory bottlenecks that may be imposed on the cells during TGE we set out to generate a CHO host cell line with an increased secretory capacity and an improved ER environment. Here we report on the establishment of an engineered CHOS cell line which has been developed to stably over express the UPR master regulator, XBP1-S, and an ER oxidation protein, ERO1-Lá.in order to improve expression in our TGE platform. The resultant cell line, CHOS-XE has an enlarged ER and improved recovery from oxidative stress, differences that can be functionally attributed to the effects of the over-expression of XBP1S and ERO1-Lá. The cell line following transient transfection with various antibody genes also exhibited up to a 6-fold improvement in yields of MAb expression when compared to CHOS as a host in a TGE platform, whilst retaining equivalent product quality. We have also assessed the performance of the CHOS-XE cell line in a number of commercial media and have identified a medium that can further enhance product generation.
EVOLUTION FROM THE CONVENTIONAL STIRRED TANK BIOREACTOR VESSEL: CULTIVATION OF MAMMalian CELL LINES USING A DISPOSABLE GRADIENT-FREE CELL-TRAP BIOREACTOR TO ACHIEVE HIGH CELL GROWTH POTENTIAL WITHOUT THE USE OF EXTERNAL MEMBRANE DEVICE IN PERFUSION MODE

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After decades of product development, the cultivation of mammalian cell in bioreactor remains a challenge. And the methods of cultivation in bioreactor have not changed since its inception. The traditional stirred tank reactor (STR) vessel design is limited by mass transport because of damaging effect of agitation and sparging which restrict cell growth potential in both batch and fed-batch modes. And in perfusion mode, high cell density is achieved only by using external membrane device, which can be both expensive and cumbersome to setup and operate. In addition, the conventional bioreactor control strategy lack advanced sensing capabilities beyond the conventional temperature, pH, and Dissolved Oxygen (DO) monitoring. This severely hampers the automation implementation which leaves the current bioreactor operations overly reliant on labor-intensive and error-prone “sample-as-you-go” control schemes.

From an engineering perspective, the challenge is to combine de-bottlenecking mass transport limitations which restricts cell’s access to oxygen and nutrients set by the low shear requirement of mammalian cell, with a built-in cell trap in the same vessel to increase both cell growth potential and protein titer. And from an operational perspective, the challenge is to integrate advance sensing into the bioreactor monitoring to allow for automation and minimize manual operations.

With these challenges in mind, we present here a novel way to cultivate mammalian cell lines in the industry’s first fully disposable gradient-free bioreactor system (CerPOD). Built around an even number of envelops with permeable wall, cells are trapped in a cell supporting and selectable porous scaffolding. This design mimics the pack bed bioreactor, and removes the limits on gas transfer and mass transfer with the traditional STR design. And the pre-woven porous fiber traps cell in “micro pockets” that through the action of centrifugal pump, keeps the suspension cell lines well-mixed and at the same time, provides large surface area for anchorage cell lines to attach.

Furthermore, the vessels are completely disposable with built-in single use pH, DO, and biomass sensors, which make the vessel automation ready. And finally, the dielectric-scanning spectroscopy of the capacitive sensors allow the users not only to monitor viable cell density (VCD) and cell viability online, but also track cell physiological changes, model cell apoptosis events, and predict protein titer plateau for optimal time of harvest all in real time.

Lab testing of the CerPOD system with 2 CHO and 1 HEK293 cell lines showed high cell growth potential in perfusion mode, reaching peak cell density of over 1.2x10^8 cells/mL and 1.5x10^8 cells/mL in the CHO cell cultures, and 5.0x10^7 cells/mL in HEK293 cell culture using commercially available media without the use of extra equipment such as an external membrane-based cell retention device. In addition, metabolic and productivity profiles such as glucose, glutamine, and glutamate consumption rates, lactate and ammonium production rates, as well as protein titer will also be presented.
Ambrx is in the field to expand genetic code to incorporate novel amino acid into proteins, such as mAbs and recombinant proteins. One of applications for Ambrx’s technology is for antibody drug conjugation (ADC), which can precisely load toxic small molecules on mAb antibodies. We have demonstrated feasibility of our ADC platform in in-vitro and in-vivo models. However, the challenge is to demonstrate manufacturability for this technology to produce proteins containing Ambrx’s novel amino acids under conventional fed-batch process, such as ~g/L for mAbs in CHO cells. In this presentation, we describe our efforts in address this issue. We focused our efforts on identifying basal media and feeds by screening commercially available chemical defined media and feeds. Our approach for basal media is to identify several media candidates and then mix them to find a blended media which gave highest titer. With improved stable cell lines, we developed a robust fed- process, which achieved over g/L for multiple CHO cell lines for mAb production. Furthermore, the process was successfully scaled up in 5L bioreactors.
Current methods for the testing of cell line identity are based on molecular approaches such as STR (short tandem repeat)-typing or sequencing technologies. These methods are time consuming and rather expensive. MALDI-TOF mass spectrometry is an emerging tool for fast and reliable identification of insect and mammalian cell lines within minutes. In addition, MALDI-TOF MS allows the direct characterization of recombinantly expressed proteins in the mass range of m/z 2 – 50 kD from the same sample preparation.

Baculovirus-infected insect cells, transiently transfected Hek293 cells and stable recombinant cell lines were analyzed for their expression profiles. It was possible to determine the precise masses of overexpressed proteins as compared to control samples. No further sample preparation was necessary for these additional experiments.

We propose this comparatively simple and inexpensive method to be used routinely for ensuring the identity of cell lines employed and determining the quantity and quality of proteins expressed.
LARGE-SCALE EXPERIENCES WITH THE HIPDOG (HI-END PH-CONTROLLED DELIVERY OF GLUCOSE) TECHNOLOGY IN CHO FED-BATCH CULTURE

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The HIPDOG technology has now been effectively employed for either the re-supply (process II) or the Phase I CHO fed-batch process at the 2,500-liter scale for three clinical programs, resulting in a more robust cell culture process and multi-fold increases in titer above conventional fed-batch processes.

We will describe the differences in terms of process performance and product quality (including glycosylation pattern analysis) that result from the exploitation of this lactate suppression technology at the large scale. Lessons learned during the implementation of the technology at the large scale will also be discussed.

Long term development of highly enriched medium formulations and concentrated feed solutions at Pfizer enabled the full exploitation of the technology, allowing for the regular attainment of cell densities exceeding 30 or 35 million cells/ml in fed-batch, and titers from 5-10 grams per liter from 12-18 day cultures. The technology enables processes to be less affected by minor perturbations in the health of the incoming inoculum and other process parameters. The technique allows the reconsideration of cell lines (or clones) that while highly productive under ideal culture conditions, would normally be unacceptable in a conventional fed-batch process due to high rates of lactic acid production.

Data from small-scale experiments exploring the robustness of the technology and its general applicability to multiple glycosylated and non-glycosylated protein constructs (Ab’s, Fc fusion proteins, small protein therapeutics) and expression systems with multiple cell lines will also be presented. All experiments were performed in chemically defined, low-protein medium.
Scale-up of 10L to 250L scale bioreactor for fed-batch process producing monoclonal antibody using CHO cell line in chemically defined medium.

As part of process development scale-up of a fed-batch process utilizing a CHO monoclonal producing cell line in animal derived component free, chemically defined, medium was performed from 10L lab scale bioreactors to 250L production scale using disposable bioreactors. During early process development a small scale model was developed based on the intended yet unknown final manufacturing process, in order to reflect the intended commercial process and operation in the scale-down model as early as possible. Scale-up from 10L to 250L bioreactor scale was performed using engineering scale-up principles including mixing, mass transfer and process controls. Evaluation of scale-up and down included viable cell density, viability, metabolites, amino acid and product quality analyses and potential Critical Process Parameter assessments.
Rapamycin, a specific mTOR inhibitor, has been used as a chemical activator in autophagy research both in vitro and in vivo. Recently, autophagy has received attention as an anti-cell death engineering target in addition to apoptosis in the Chinese hamster ovary (CHO) cell engineering field. Here, the effect of rapamycin and the subsequent autophagy induction is investigated on two CHO cell lines, DG44 and an antibody-producing recombinant CHO (rCHO), in a serum-free suspension culture. In both cell lines, the rapamycin treatment delayed the viability drop and apoptosis induction. In particular, the improved cell viability of the antibody-producing rCHO cell line resulting from the rapamycin treatment led to a 21% increase in the maximum antibody concentration. From observations that a rapamycin derivative, everolimus, demonstrated similar positive effects, but not FK-506, which forms the same complex as rapamycin, but does not inhibit mTOR, it was demonstrated that the positive effects of rapamycin appear to be mTOR-dependent. In addition, the cultivation with rapamycin and/or an autophagy inhibitor, bafilomycin A1, indicated that the autophagy induction is related to the positive effects of rapamycin. The genetic perturbation of the autophagy pathway through the regulation of the expression level of Beclin-1, an important autophagy regulator, resulted in a delayed autophagy induction and apoptosis inhibition in response to the rapamycin treatment. Taken together, the results obtained in this study imply a positive role for autophagy and predict the usefulness of pro-autophagy engineering in CHO cell cultures.
Human cell lines are important host organisms for the production of biologics and therapeutic proteins. For these applications, mammalian cell lines often require sustained, high level expression of heterologous DNA. However, the industry standard for developing stable recombinant clones is an arduous, random, and inconsistent process of library creation and clone selection. Genomic integration locus, transgene structure, and pool selection can play a significant role in defining success in cell line development. Here we describe recent successes in (1) improving library efficiency through transgene structure and choice of selection marker, (2) identifying putative genomic hot-spots for high level transcriptional activity, and (3) improving the utility of Cre-recombinase for site-specific swapping. Each of these facets greatly impacts the efficiency of cell line development. First, we report on progress towards characterizing the impact of transgene structure and selection marker on cell library quality in the human cell line HT1080. Specifically, we find that zeocin and hygromycin are superior selection markers yielding marked improvements of library quality and stability compared with other antibiotics. Moreover, we find important relationships between transgene arrangement and gene expression profiles. Second, we report progress towards an alternative approach to cell line development—namely identifying re-targetable genomic “hot spot loci”. Hot spots are genomic locations that are supportive of stable, high-level transgene expression. Cell-based genomic libraries of hot spots were created in a human cell line using a dual antibiotic-GFP reporter construct and stable, high expression loci were identified. Compiling this information leads to a genomic map of transcriptionally active areas. Highly expressing clones from these libraries were characterized on the basis of stability, protein expression, and capacity for retargeting. Finally, in an effort to retarget these sites, it is essential to have site-specific recombinases (such as Cre) that can support high levels of swapping. However, the current efficiency of this recombinase (<1%) is too low for efficient cell line development applications. To address this issue, we sought to increase the propensity of Cre recombinase for swapping by undertaking a simultaneous, unbiased evaluation of several parameters found to influence Cre-mediated swapping and excision in human cells (HT1080). We identify parameters that bias total number of recombination events and a separate set of parameters that influence the ratio of swapping to excision events. In particular, a novel mutant lox site pairing and delayed introduction of Cre are two critical factors for achieving optimal swapping efficiency. By simultaneously optimizing these parameters, we obtain the highest Cre-mediated swapping frequencies without antibiotic enrichment reported in literature, between 8 and 12%. Collectively, these constructs and methodologies can lead to improved cell line development.
Biopharmaceutical manufacturing processes are generally comprised of a series of batch mode unit operations to produce therapeutic protein. Although the basic ideas behind the manufacturing process are easy to understand, the detailed processes of unit operations are complex and intricate. The complicated biological mechanism in upstream processes, the lot-to-lot variations of raw materials, and variability in medium preparation and bioreactor operations, result in batch-to-batch variations in upstream process performance and product quality attributes. In this poster, our strategies of process monitoring and control for a commercial upstream process are described. Examples are given to showcase the application of analytical tools in raw material characterization and control, and application of multivariate analysis in raw material screening and cell culture process monitoring. Process variation in yield and product quality attributes was observed across sites and among campaigns over a few years and hundreds of batches. Several efforts have been made to investigate root causes for the process variations. Continuous improvement of the process within change management requirements was carried out to improve process robustness against raw material variations and was proven successful.
RAPID DEVELOPMENT AND CHARACTERIZATION OF AN HTST PASTEURIZATION PROCESS FOR COMMERCIALLY-USED, SOY HYDROLYSATE-CONTAINING CELL CULTURE MEDIUM

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High-temperature, short-time (HTST) heat treatment of cell culture media has seen increasing acceptance as a simple, effective barrier against viral contamination of cell culture processes. We describe the rapid development of an HTST process for a complex, soy-containing culture medium from proof-of-concept to implementation in commercial-scale cell culture operations. Efforts were focused on investigating potential deleterious effects of HTST treatment on the culture medium. No significant impact on growth nor titer was observed at scales ranging from shake flask culture to 200L perfusion bioreactor at a target HTST temperature of 100°C and a corresponding hold time of 10 seconds. Product quality analysis at bioreactor scale also proved to be within pre-defined specifications. In addition, a set of assays were utilized to identify the impact of HTST treatment on specific, potentially heat-labile nutrients. HPLC assays for free amino acids and water soluble vitamins identified the amino acids: asparagine, glutamine, cysteine; and vitamins: B12, thiamine, nicotinamide, PABA as being the generally most heat-labile of those tested, but none of these species showed notable reductions due to relevant HTST treatments. A chromogenic assay was used to measure concentrations of Pluronic F-68, a surfactant used in the culture medium; no impact of HTST treatment was observed. A metals and salts analysis was performed and showed only calcium to have a significant temperature-dependent reduction (a maximal 20% reduction at an HTST temperature of 110°C). All other metals/salts showed reductions of approximately 10% or less. An in-house, cost-effective ELISA was also developed and used to measure concentrations of long R3-IGF I in the medium. A time and temperature-dependent reduction in long R3-IGF I concentration was observed in the medium irrespective of HTST treatment. The concentration of long R3-IGF I was reduced by 50% after 2 weeks of storage of untreated medium at 4°C. HTST treatment did accelerate the decline in IGF I levels; 50% of the starting concentration was observed after only 6 days of storage at 4°C when HTST treatment was employed (100°C/10 sec). However, no significant impact of long R3-IGF reduction was observed on primary cell culture parameters (growth, titer, product quality). Finally, nutrient analyses were performed for medium prepared with both pilot-scale and commercial scale HTST skids. No significant nutrient differences were observed from medium produced at the two scales, thus supporting scalability of the process.
NOVEL STRATEGY FOR A HIGH YIELDING MAB-PRODUCING CHO STRAIN (OVEREXPRESSION OF CYSTEINE SULFINIC ACID DECARBOXYLASE [CSAD] CAUSED BETA-ALANINE BIOSYNTHESIS AND IMPROVED MAB YIELD)

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Innovation in monoclonal antibody (mAb) production continues to be driven by cell engineering strategies to increase yield. In a previous study, to investigate the effectiveness of transporter overexpression strategies, we prepared a TAUT-overexpressing host cell line that produced a higher proportion of high-mAb-titer strains than did the parent host cell line, and we selected a single TAUT/mAb strain. This TAUT/mAb strain remained viable for longer (up to 1 month) under common fed-batch culture conditions, and the improvement in viability could be attributed to improved metabolic properties. It was also more productive (up to >100 pg/cell/day) and yielded more mAb (up to 8.1 g/L/31 days) than the parent cell line, and the mAb it produced was of comparable quality. Those results suggested that this host cell engineering strategy has unique potential for the improvement of mAb-producing CHO cells; for example, it may be appropriate for high-density culture. In the present study, our challenge was to achieve a high yield in a short culture period by applying a strategy for modulating cell metabolism by using a substrate that is rapidly synthesized by cysteine sulfinic acid decarboxylase (CSAD). The original function of CSAD is to catalyze the penultimate and rate-limiting step of taurine synthesis. Although TAUT takes up taurine and f-α-alanine with high efficiency, accumulation of these substrates is low in CHO cells, even if TAUT is overexpressed. Accordingly, we expected that overexpression of CSAD would cause taurine accumulation, and that it might play a pivotal role in various physiological functions including osmoregulation, antioxidation, detoxification and stimulation of glycolysis, and glycogenesis. To accomplish this, we genetically modified the TAUT/mAb strain to overexpress CSAD gene. The resulting co-overexpression strain (TAUT/mAb/CSAD strain) gave increased yield in a shorter culture period (up to 7.6 g/L/19 days) under common fed-batch culture conditions. Interestingly, however, this effect might be due to the accumulation of an unforeseen substrate in this case: f-α-alanine but not taurine is dramatically synthesized in cell lines overexpressing CSAD. The lyase activity of overexpressed CSAD can probably synthesize f-α-alanine in CHO cells in the same manner as the lyase activity of glutamate decarboxylase I (GAD1) does in the brain. Since f-β-alanine is the rate-limiting precursor of carnosine, the accumulation might scavenge reactive oxygen species (ROS).
Momenta has developed an analytical technology platform to facilitate detailed structural characterization of protein biologics and an understanding of process to product relationships. These technologies can be used to inform the development of a biogeneric process from cell line generation through scale-up. Clone selection is guided by high throughput analysis of multiple cell based and product attributes. The goal of these analytics is to identify clones with the greatest potential to reach a target profile for which a key factor to achieving objectives is proper selection of the host. Cell culture process development involves multiple steps to identify basal media, supplements and process conditions that favor the desired product attributes. These efforts can be used to improve the product profile obtained from the selected clone at early screening stages but gaps will likely remain. To address these gaps, process strategies have been developed to manage various parameters that require adjustment once a base process has been developed. Examples include the modulation of glycans such as G0F, G1F, G2F, HM5 and the level of sialylation. As the process is scaled-up, changes in the profile can also be managed with these ‘levers’ as required. These types of approaches have provided a strong link between process and quality and the extensive analytics enable a deeper understanding of the design space.
IMPLEMENTATION OF 3L DISPOSABLE REACTORS FOR USE AS A DIRECT SCALE-UP FOR CGMP MANUFACTURING

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CMC Biologics is a Contracting Manufacturing Organization with multiple cGMP manufacturing lines. One production facility has a fully disposable upstream suite including a 500L single use bioreactor (SUB). Currently, manufacturing scale-up proceeds directly from development in 15L glass bench-top bioreactors to cGMP manufacturing in the 500L SUB. Millipore 3L disposable reactors were investigated as an alternative to 15L glass bioreactors and as a potential direct scale-up vessel for the 500L production vessel. Different cell lines and production processes were compared side-by-side in 3L and 15L reactors. The 3L disposable reactors displayed similar growth, productivity and metabolic chemistry with some cell lines reaching cell densities greater than 10 million viable cells/mL. Control systems for dissolved oxygen and pH were able to be used “as-is” without requiring major system modifications. A cost model was also developed to determine the potential cost savings compared to the 15L glass bioreactor. Disposable systems offer the advantage of reduced turn-over time, reduced setup costs including labor and sterilization, and reliable consistency between reactor sets. The performance and cost-benefit advantages of disposable bioreactors make them a suitable alternative for bench-scale process development and potential scale up to manufacturing scale.
The N-glycosylation of therapeutic monoclonal antibody Fc domain impacts its quality attributes such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The modification of monoclonal antibody N-glycosylation has been reported through genetic manipulation of cell line or cell culture process changes of physical and chemical parameters. This presentation focused on the effects of cell culture process and media supplements on monoclonal antibody N-glycosylation. We investigated the impact of media supplements and/or physical parameters during cell culture process and demonstrated that N-glycan profile of target monoclonal antibody could be controlled to certain level.
Multivariate analysis of cell culture manufacturing data has recently emerged as a promising approach to gain fundamental understandings of process characteristics and to devise means of enhancing process performance. The time-series data of 134 process parameters acquired throughout the inoculum train and the production bioreactors of 243 runs at the Genentech’s Vacaville manufacturing facility were investigated in this study.

Two multivariate methods, kernel-based support vector regression (SVR) and partial least square regression (PLSR), were used to predict the process outcome of the final antibody concentration and the final lactate concentration. Both productivity and cellular metabolic state can be predicted accurately using data from the early stages of the production scale, suggesting that the history of the culture exerts significant influence on the final process outcome. Furthermore, the majority of parameters contributing significantly to the predictability were related to lactate metabolism, suggesting the important role of cellular metabolic activities on process characteristics. Lactate consumption, which occurred rather independently of the residual glucose and lactate concentrations, was shown to be a prominent factor in determining the final outcome of production-scale cultures.

This study presents pattern recognition as an important process analytical technology (PAT). Furthermore, the high correlation between lactate consumption and high productivity can provide a guide to apply quality by design (QbD) principles to enhance process robustness.
Dihydrofolate reductase (DHFR)-based amplification system is the most commonly used method for introducing a product gene into CHO cells for therapeutic protein production. The system has afforded the transformation of host cells which secrete almost no proteins to become professional secretors. However, three decades after the development of the method, we still have little understanding on the mechanisms of this process of transforming cells into hyperproducers.

To gain mechanistic insights, a parental CHO cell line deficient in DHFR activity was transfected with a vector expressing a DHFR gene, a hygromycin resistance marker, and transgenes encoding for an antibody product. A control process using the vector without the antibody transgenes was also carried out. Following transfection and initial selection in hygromycin, methotrexate (MTX) amplification was performed. Cell pools and clones at different stages were isolated, and representatives of varying productivities were subjected to transcriptome analysis.

The integration and amplification of the vectors under the pressure of MTX was confirmed by mRNA levels of the exogenous DHFR. Transcripts differentially expressed consistently as well as inconsistently amongst the clones at different stages were compared to reveal the effect of selection and amplification. Surprisingly, more profound differences between the IgG transfected cells and the control cells were seen after selection than upon gene amplification. Interestingly, the transcript level of the IgG transgene showed a similar trend of a tremendous surge upon selection and only a moderate or no increase upon amplification. Functional analysis of the candidate pivotal genes revealed signaling pathway enrichment upon amplification while gene classes related to mRNA and protein processing were enriched during selection. The results suggest that cellular machinery development for IgG production occurs even prior to amplification.

We hypothesize that the selection process, with the forced expression of a secretory protein IgG, enriched survivors with superior secretion machineries. The role of amplification process is less about further enhancement of the transgene’s transcript level than further reinforcement of other cellular characteristics which favor high productivity, including enhanced stress response, ROS balance, and protein processing. The mechanistic insights gained through such systems analysis will allow for a rational design of robust production cells and selection strategies for therapeutic proteins.
UNDERSTANDING TRANSCRIPTIONAL ENHANCEMENT IN MAB PRODUCING CHO CELLS

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The ever increasing demand for monoclonal antibodies has led to an interest in understanding productivity rates in CHO cells. To investigate whether differential transcriptional rates in parental and progeny cells are the result of altered interactions of transcriptional machinery with the CMV promoters, well characterized and commonly occurring transcription factors interacting with CMV promoter were selected. Using chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA), we were able to quantify the interactions and observe differences in post-translational modification of transcriptional factors Ap-2 and NfκB. Our results indicate a difference in binding of these transcriptional factors to the DNA in higher producer cell lines. Hence, the enhancement of transcriptional rates may be explained by improved accessibility of the transgene inserts to the transcriptional machinery. In most industrial applications, the strong, viral CMV promoter is used to drive recombinant protein expression. De-methylation of CMV promoters is known to improve accessibility to transcription. We demonstrated the loss of methylation along the promoter sequence of transgenes in amplified cell lines using methylated DNA immunoprecipitation. By carrying out methylated DNA immunoprecipitation and bisulphite sequencing, we were able to relate the transgene expression to the methylation state of the promoter region. Based upon our observations, we subjected the cells to known methyltransferase inhibitors and were able to increase the productivity of parental cell clones to the same level achieved by repeated rounds of MTX amplification. Identification of these factors indicative of higher producers will help develop selection methods and strategies for cell design that will bring down costs, reduce timelines for development, and help realize the conversion of candidate molecules to therapeutics benefitting patients.
ENGINEERING CHO CELLS AND VECTORS FOR IMPROVED TRANSGENE INTEGRATION AND ANTIBODY PRODUCTION

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Epigenetic regulatory DNA elements can be incorporated in expression vectors to yield stable and very high specific transgene expression from CHO cells and increased antibody production in the bioreactor. However, extremely high specific productivities reveal new cellular bottlenecks from CHO cell lines, encompassing transgene genomic integration, protein secretion and cell physiology. We have determined the sequence of the genome and transcriptome of a CHO sub-line used for the production of pharmaceuticals and have identified genes involved in transgene genomic integration and in recombinant protein processing and secretion. Cell engineering methods for increased genomic integration of the transgene by homologous recombination will be presented. Proper protein secretion and modifications is another bottleneck met with high level expression, especially for some difficult to express immunoglobulin variants. We will show how the faulty steps can be identified at the molecular level and how the screening and expression of a number of protein-folding, transport, secretion or modification pathway proteins can be used to solve processing and secretion limitations. This presentation will illustrate how the use of a systematic and multi-level approach can be used to generate improved gene transfer methods and recipient cells for more efficient expression of pharmaceutical proteins.
A CHO cell banking process was developed using disposable systems: cells are expanded in disposable shake flasks and Wave bioreactors, concentrated with disposable hollow fiber filters, and then formulated for cryopreservation. This work summarizes the approach and success in defining the cell expansion and cell concentration processes. For cell expansion in the Wave bioreactors, our goal was to achieve cell densities exceeding 18 million cells/mL without relying on pH and dissolved oxygen (DO) feedback controls. After identifying initial challenges, we characterized O₂ and CO₂ transfer in the system, and used these results to define the rock rate, rock angle, and gas flow rate for the Wave bioreactor process (without pH and DO control). When tested using six recombinant CHO cell lines, this process maintained pH and DO within our desired range—pH 6.8-7.2 and DO exceeding 20% of air saturation. When compared to the stirred-tank bioreactor process with online pH and DO control, this Wave bioreactor process also demonstrated comparable cell growth (attaining 20 million cells/mL after six days of perfusion culture) and viability (~95% at harvest). For cell concentration using tangential flow filtration in hollow fiber filters, our goal was to concentrate the Wave bioreactor cultures to ~100 million cells/mL in <30 minutes without adverse effect on viability. After considering and testing the limitations associated with shear, flux, and conversion, we identified a feasible operating space—defined by these three parameters—that enabled us to achieve our goal. When comparing cell banks generated by the two different concentration methods, i.e., centrifugation versus hollow fiber filtration, we observed comparable performance during thaw, seed train, and production for the three recombinant CHO cell lines tested.
Influenza virus vaccines have been produced in embryonated chicken eggs and widely used for over 60 years. The egg-based technology may not suffice to fulfill the high demand for flu vaccines in the case of a pandemic. The development of a cell-based platform as an alternative to egg-based technology is becoming unavoidable in order to rapidly achieve commercial production to meet the global demand for flu vaccines. The Baculovirus/Insect cell system is a reliable and low-cost technology and is becoming an attractive platform for the rapid production of vaccines. Protein Sciences Corporation (PSC) is a leader in developing and manufacturing therapeutic vaccines, having a GMP-compliant process that consists of a baculovirus vector system. PSC utilizes this platform to produce recombinant hemagglutinin (rHA) for the influenza vaccine FluBlok®, which is currently awaiting FDA approval. The PSC expression platform uses proprietary expresSF+® insect cells and Protein Sciences Formulary Medium® growth media. This platform is a simple, low-cost and scalable process for vaccine manufacturing in a very short time frame. The increasing demand for flu vaccines has triggered efforts to accelerate further development and improve the existing process for manufacturing vaccines at a large scale. Improvement to the current FluBlok process in order to maximize productivity is increasingly important to meet global demand for flu vaccines. A simple fed-batch strategy suitable for the production of recombinant proteins was developed with the aim of enhancing rHA yields by maximizing cell biomass and extending cell viability. A stable feeding solution was developed and optimized to yield high product titer in fed-batch culture compared to batch control. Several different rHA proteins have been produced using this fed-batch process; improvements in cell biomass and volumetric rHA production have varied among strains, but significant increases were observed in all cases when compared to a batch control, averaging increases of 60% and 80%, respectively. The performance and scalability of the fed-batch process have been demonstrated in shake-flasks and 2L- and 10L-bioreactors using over five different rHA strains.
PROCESS CHARACTERIZATION AND VALIDATION FOR CELL CULTURE PROCESSES: CHALLENGES AND OPPORTUNITIES

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Substantial efforts are expended on process characterization and validation prior to commercialization of cell culture processes. Requirements for process characterization and validation are continually evolving with ongoing developments on both process and product sides. On the process side, these are driven by accumulated experience and understanding of correlation of cell culture process parameters and their interactions on product quality, process analytical technologies, and introduction of risk-based characterization concepts of Quality by Design. On the product side, understanding of mechanism of action through detailed structure-function studies and application of better and orthogonal analytical techniques are the driving changes.

This presentation compares the earlier paradigm of process validation versus the newer approaches integrating process characterization based on design of experiments and multivariate analysis modeling. Discussion is carried out on aspects where the new paradigm needs to evolve further. Challenges can relate to integration of timing of activities, approach for standardization, and opportunities for process improvement as a corollary to process characterization. Proper timing and sequencing of activities is critical for a robust outcome. Scale-down modeling timing is particularly important when activities are shared across labs and final scale manufacturing is still to be done. Standardization of process characterization can lead to efficiency via application of a core one-size-fits-all approach and will be illustrated. Detailed process characterization can be valuable to identify improvements in consistency, robustness, and performance of a cell culture process. These improvements can be implemented immediately or else subsequent to commercialization based on a risk benefit analysis. Finally, industry and regulatory authorities need to continually evaluate the optimal point where efforts spent on process characterization and validation of cell culture processes are adequately balanced against the risk of product quality variation. The application of the pareto principle to define extent of process characterization will be discussed.
Developing a Phase III/commercial cell culture process presents many challenges including optimizing cell culture conditions in order to maintain quality and maximizing process performance. Development of a phase III/commercial process will be described for a CHO monoclonal antibody product through process optimization and scale-up. A new chemically defined medium (CDM) formulation was implemented for this Phase III process at large scale for the first time. The use of this CDM formulation resulted in highly consistent cell culture performance at the small scale and pilot scale. However, during scale-up for clinical manufacturing, additional challenges to optimal productivity were identified, and potential process improvements were investigated to improve productivity in future campaigns.
Eli Lilly and Company utilizes the GS-CHO expression technology and has developed a rapid and efficient process for the generation of clonally-derived cell lines in support of therapeutic protein clinical development. Recently, efforts have been undertaken in our lab to improve cell line generation efficiency and bulk culture productivity through alternative selection schemes, focusing primarily on selection stringency. These approaches include modifications to the selection procedure itself, as well as manipulation of the expression plasmids and expression host cell engineering. A GFP expression cassette has been developed that enables the monitoring of these changes on the selected bulk population for multiple therapeutic antibodies. The GFP profile of a bulk culture as related to its productivity has provided an insight into the selection process. The GFP profile has enabled the development of processes and molecular tools to increase the efficiency of improved cell productivity.
DISSECTING THE MECHANISMS OF PHENOTYPICAL INSTABILITY IN ANTIBODY PRODUCTION
CHO CELL LINES

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The generation of production cell lines which maintain high, stable production levels is critical for the successful development of therapeutic protein drugs. However, production instability occurs in a large percentage of cell lines. Since the cause(s) or mechanism(s) of instability are not clear, it is difficult to predict which cell lines will remain stable over the range of cell ages used during the clinical and commercial manufacturing process. To study factors which influence phenotypic stability, multiple manufacturing candidate CHO cell lines were monitored for recombinant protein productivity over a defined range of cell ages. In this study, we present several underlying causes for production instability in these cell lines. Understanding of these causes will increase our ability to develop methods for the early detection of unstable cell lines during the cell line development process and will aid in the design of resource efficient strategies to effectively counter this problem.
MECHANISTIC STUDIES ON THE IMPACT OF PGAM1 AND OTHER KEY GENES IN GLYCOLYSIS ON ENERGY METABOLISM AND PROTEIN GLYCOSYLATION IN IgG PRODUCING CHINESE HAMSTER OVARY (CHO) CELLS

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Energy metabolism in recombinant protein producing mammalian cells can profoundly affect productivity and product quality of secreted therapeutic proteins. Researchers aim to control glycolysis and direct pyruvate flux into the TCA cycle, thus increasing the net ATP generated in order to improve recombinant protein productivity. Our particular interest lies in understanding the potential role of energy metabolism in recombinant protein post-translational modification specifically N-linked glycosylation. In the present study, we focus on characterizing and manipulating four genes in the glycolytic pathway: Phosphoglycerate mutase 1 (Pgam1), Phosphofructokinase (Pfk), Phosphoglycerate kinase 1 (Pgk1) and Pyruvate Kinase (Pk) to study the impact on productivity and glycosylation.

Pgam1 has been reported to contribute to an alternative glycolytic pathway in rapidly proliferating cells via the conversion of phosphoenolpyruvate to pyruvate, independent of enolase activity. Transcriptional profiling studies using cDNA microarrays on a recombinant IgG producing CHO cell line, comparing non-targeting control (higher IgG expression) vs. an IgG Heavy and Light Chain siRNA knockdown culture (lower IgG expression levels), showed that Pgam1 was moderately down-regulated, Pfk significantly down-regulated and Pk moderately down-regulated in the higher IgG expressing cells. Additionally, differential gel electrophoresis (DIGE) studies on two IgG producing CHO (dhfr -/-) lines with different %Man5 glycans (indicating incomplete glycosylation) showed that Pgam1 protein level was down-regulated 1.66 fold in the cell line with lower %Man5. In order to further explore the link between energy metabolism and glycosylation efficiency, we conducted qRT-PCR differential expression studies of Pfk, Pgk1 and Pk on a panel of IgG producing SAFC CHOZN (gs -/-) cell lines with varying growth, productivity and glycosylation phenotypes.

Quantitative RT-PCR for expression levels of Pgam1 showed an inverse correlation between Pgam1 relative expression and IgG productivity. Furthermore, shRNA stable knock down of Pgam1 in a cell line from the panel with highest Pgam1 expression and lowest productivity indicated a mild increase in IgG productivity. The effect of Pgam1 on glycosylation was studied by the stable shRNA knockdown of Pgam1 in another model CHO cell line (dhfr -/-) producing antibodies with higher %Man5. Pfk, Pgk1 and Pk were also stably knocked down in selected IgG producing CHO cell lines, and the effects on N-glycan profiles, growth, IgG productivity, lactate production and ATP generation were studied.

The results from this study may suggest methods for engineering CHO host cell lines towards better energy metabolism and potentially resultant improved glycosylation profiles.
IMPACT OF AERATION STRATEGIES ON FED-BATCH CELL CULTURE KINETICS IN A SINGLE-USE 24-WELL BIOREACTOR

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The need to bring new biopharmaceutical products to market more quickly, and to reduce final manufacturing costs, is driving early stage, small scale bioprocess development and optimization. Monoclonal antibodies have been the most successful products due to the establishment of platform manufacturing processes. The ability to ‘scale down’ each operation in the platform process will enable early evaluation of new antibodies and their fit to platform as well as optimization of bioprocess conditions. The fundamental challenge is in creating a scale down model that accurately recreates the engineering environment experienced at large scale and which yield process relevant data.

The aim of this work is to establish a small scale cell culture platform, to enable the rapid selection of robust and scaleable cell lines expressing a range of antibody products. The results describe a comprehensive engineering characterization of a novel single-use 24-well parallel miniature bioreactor system (micro24 bioreactor) that enables control of key culture parameters like pH and DOT. Cell culture performance is then investigated, with particular focus paid to the aeration strategies adopted at this small scale (7 mL) using different plate designs; either by headspace sparging alone or by direct gas sparging into the culture medium.

Apparent kLa values determined using the static gassing out method in media, ranged between 3 – 22 hr^-1 and 4 – 53 hr^-1 for headspace aeration and direct gas sparging respectively. Similarly, mixing times were determined over a range of operating conditions for both plate designs. These were found to be less than 2 seconds at standard culture conditions for both plate types. Mixing times were generally in the range 1 - 13 seconds and decreased with increasing shaking frequency (500 - 800 rpm). Direct gas sparging also helped to reduce tm values. The impact of aeration strategies on fed batch cell culture kinetics of an industrial CHO cell line expressing a whole monoclonal antibody was next investigated. Cultures performed with head space aeration showed the highest VCD and antibody titers. These were greater than in conventional shake flask cultures due to the improved control of the micro24 bioreactor system. Cultures performed with direct gas sparging showed an approximately 25 – 45% reductions (dependent on gas aeration strategy) in VCD and similarly an approximate 44 – 70% reduction in antibody titer but were more comparable to those found in a conventional stirred bioreactor.

These results show the successful application of the miniature bioreactor system for performing industrially relevant fed-batch cultures and highlights the impact of the dispersed gas phase on cell culture performance at the small scale.
Insulin is a hormone that is important in the regulation of carbohydrate and fat metabolism in mammals and has an application in mammalian cell culture. Insulin \textit{in-vitro} acts as a mitogen for most cells and is involved in glucose and lipid metabolism, amino acid uptake and DNA synthesis. The supplementation of cell culture media with insulin stimulates growth and proliferation of a variety of somatic cells. It has therefore become a valuable supplement in many serum free media formulations.

In this study, the effect of 8 commercially available recombinant insulins (rInsulin) including Sheffield's rInsulin CC was studied on three industrially relevant cell lines MRC-5, MDCK and SP 2/0 Ag-14. The cells were examined in normal culture conditions, MRC-5 cells were maintained in EMEM medium with 20\% Fetal bovine serum (FBS), MDCK cells were maintained with 10\% FBS and SP 2/0 cells were maintained in a mixture of DMEM and Ham's F12 media in 10\% FBS. The effect of all eight rInsulins on the same cells was also examined in serum depletion study, where the FBS was lowered to 5\% for MRC-5 cells and 2\% for MDCK and SP2/0 cells. All the cell lines were treated with 10 ng/ml of each rInsulin. The cells were passaged for three successive subcultures and population doubling level and \(i\) were calculated. The results were compared against the same cells in medium untreated with insulin. Images of the cell lines were also taken to capture any morphological effects. For each of the cell lines limited seeding conditions were also assessed to determine the best environmental conditions of every cell media, and determine the most efficient rInsulins.

The PDL of the MRC-5, MDCK and SP 2/0 cells in normal conditions without serum was 47 hr, 24 hr and 35 hr respectively. The PDL of the MRC-5, MDCK and SP 2/0 cells in serum depleted conditions was longer at 59 hr, 30 hr and 38 hr respectively. The addition of all 8 insulin's led to a decrease in PDL in both normal and serum depleted conditions, with one exception. The average PDL in the rInsulin treated cell lines with serum depleted media dropped to a mean of 50 hrs in MRC-5 (max = 57 hr, min = 42 hr), 23 hr in MDCK (max = 27 hr, min= 21 hr) and 31 hr in SP 2/0 (max=39 hr, min = 26 hr). In all cases the biggest decrease in PDL was achieved with Sheffield's rInsulin CC. Examination of images taken of the MDCK cell lines showed that two of the rInsulins caused these cells to develop multiple nuclei. The limit seeding studies showed that, in every cell line, Sheffield's rInsulin CC and one other commercial rInsulin were the most efficient for stimulating cell growth.

The study shows an unexpected large range of \textit{in-vitro} efficacy across commercially available rInsulins in these 3 cell lines.
Trypsin is a serine protease found in the digestive system of most mammals. In cell culture, trypsin derived from porcine pancreases has historically been utilized. Trypsin is used to resuspend adherent cells attached to cell culture flasks. It is also used in primary cell culture/cell isolation techniques to break down clumps of tissue into singular cells. Trypsin also has an application in influenza vaccine production in MDCK cell lines, where it increases virus infectivity by cleaving haemagglutinin. Although trypsin for a variety of cell culture applications has historically been extracted from porcine pancreas, the trend toward animal component free (ACF) media ingredients has led to an increasing interest in recombinant trypsin (rTrypsin). In this study, the performance of four commercially available rTrypsins was assessed alongside a native trypsin (animal derived) in 6 different cell lines.

MRC-5, L929, C2C12, LLCPK1, MDCK and VERO cells were cultivated in FBS supplemented media for 5 successive passages. The cells were detached from flasks at each passage using a native trypsin as a control and 3 rTrypsins, TrypLE (Invitrogen), TrypZEAN (SIGMA) and r-Trypsin (Sheffield). The time for monolayer detachment was recorded and the action of each trypsin was filmed. The population doubling length (PDL) of each cell after passage with each trypsin was also calculated to see if any cumulative toxicity occurred.

All the products were efficient at removing the cell monolayers from flasks with the exception of the LLCPK1 and MDCK cells. None of the trypsins were capable of removing the tight intracellular junctions formed by these cells. In terms of time required to remove the monolayer, TrypZean and TrypLE were the fastest enzymes. However, these two enzymes were very aggressive on the cells and led to slight increases in PDL due to carryover toxicity. The Sheffield r-Trypsin was as efficient as native trypsin without causing any increase in PDL. The study shows, in a broad range of cell lines, that recombinant trypsin is an alternative to native trypsin in terms of effectiveness and ease of use. The study also shows that some recombinant trypsins can have toxic effects on the cells over time.
UPREGULATION OF HISTONE DEACETYLASE (HDAC) ACTIVITY IS ASSOCIATED WITH LONG TERM EXPRESSION INSTABILITY IN A BHK21 CELL LINE DURING CONTINUOUS PERFUSION CULTURE

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Mammalian cell lines, such as BHK21 and CHO cells, are widely used to produce recombinant therapeutic proteins. To the companies that manufacture these proteins, it is of great interest that production cell lines not only produce high titer of the recombinant protein but also maintain the titer during long term culture. Although expression stability is selected for during cell line development, it is not uncommon that titer decline is observed during the culture scale-up and production process. In order to better understand the mechanism of titer decline in BHK21 cells expressing a recombinant protein over 60-day continuous perfusion fermentation, we collected cells and product over the course of the culture and analyzed for product quality, gene copy number, integration status, mRNA, HDAC protein & activity, and the expression of selected ER chaperones. The results of this study indicate that the observed titer decline was not associated with changes in the recombinant protein quality attributes, genetic instability, a decrease in copy number or a change in selected chaperone expression but did correlate with a decrease in the mRNA coding for the protein of interest. On further investigation, this gene silencing was found to correlate with HDAC 1, 2 and 3 expression levels which were increased in older, lower titer cells. HDACs are involved in histone deacetylation which promotes condensation of chromatin structure, resulting in gene silencing. HDACs activity was also found to be elevated in these older, lower titer cells. Finally the effect of HDAC inhibitors was explored and found to improve mRNA expression in these older, lower titer cells. Our findings suggest that epigenetic pathways are involved in maintaining expression stability. It may be possible to use epigenetic tools to maintain active gene transcription during cell culture and avoid titer decline in future.
DEVELOPMENT OF THE EPI-CHO TRANSIENT EXPRESSION SYSTEM FOR IMPROVED MAB PRODUCTION

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Chinese Hamster Ovary cells (CHO) are commonly used as a production host for therapeutic molecules including monoclonal antibodies in the biopharmaceutical industry. Large-scale production of recombinant monoclonal antibodies or proteins is usually achieved in stably transfected CHO cells. However, creation of these cell lines is both time consuming and laborious. During early stage biologics development, mammalian cell-based transient gene expression (TGE) systems are routinely used for rapid production of pre-clinical material. Given the importance of stable CHO cell lines for biologics production, there is a need for high performing CHO based TGE systems.

Here we report the further development and characterization of the epi-CHO episomal-based transient expression system. This system enables increased and prolonged recombinant protein expression by utilizing elements from the Epstein Barr virus nuclear antigen 1 (EBNA-1) and Polyoma virus large T-antigen (Py-LT) to promote the replication and maintenance of plasmid DNA. Components of this system include the use of a host cell line (CHO-T) and an expression plasmid. We have shown that the epi-CHO system allows for improved and prolonged recombinant protein expression, achieved through the optimization of a series of key transfection parameters. These include the screening of various chemically defined and serum free media, transfection and feeding methods to increase cell densities, prolonged cell viability and enhance recombinant protein yields. Elevated and prolonged recombinant product yield was achieved using a transfection platform using serum-free and chemically defined media and incorporating a feeding strategy (Codamo et al. 2010; Codamo et al. 2011).

This paper describes the development of the system to create a second generation epi-CHO transient expression system. This development utilizes a modified CHO cell line, which stably expresses both EBNA-1 and PyLT to support episomal plasmid amplification and maintenance, and allows the expression plasmid to be smaller, resulting in improved protein expression. Using this modified expression system and an optimized transfection platform incorporating mild hypothermia and a simple feeding strategy, a further improvement in product yield is achieved in comparison to the existing epi-CHO system. Transfected cells also demonstrate higher maximal viable cell densities post-transfection.
Heparin is the most widely used pharmaceutical to control blood coagulation in modern medicine. A health crisis that took place in 2008 led to a demand for production of heparin from non-animal sources. Chinese hamster ovary (CHO) cells, commonly used mammalian host cells for production of foreign pharmaceutical proteins in the biopharmaceutical industry, are capable of producing heparan sulfate (HS), a related polysaccharide naturally. Since heparin and HS share the same biosynthetic pathway, we hypothesized that heparin could be produced in CHO cells by metabolic engineering.

Based on the expression of endogenous enzymes in the HS/heparin pathways of CHO-S cells, stable CHO cell lines expressing human N-deacetylase/N-sulfotransferase (NDST2) and mouse heparan sulfate 3-O-sulfotransferase 1 (Hs3st1) genes were established. An antithrombin III (ATIII) binding assay using flow cytometry, designed to recognize a key sugar structure characteristic of heparin, indicated that Hs3st1 transfection was capable of increasing ATIII binding. An anti-factor Xa assay, which affords a measure of anticoagulant activity, showed a significant increase in activity in the dual-expressing cell lines. Immunoassay following Golgi fractionation showed that NDST2 colocalized with the Golgi complex. Disaccharide analysis of the engineered HS showed a substantial increase in N-sulfo groups, but did not show a pattern consistent with pharmacological heparin, suggesting that further balancing of the expression of transgenes with the expression levels of endogenous enzymes involved in HS/heparin biosynthesis might be necessary. Critical issues currently under investigation are to determine biological activities of endogenous and introduced enzymes and whether overexpression of Exostosins, involved in polymerization of HP chains, will be necessary to obtain a sufficient yield of HP.
A human hybrid cell line named F2N78 was developed by somatic fusion of human embryonic kidney cells (HEK293) and Namalwa (Burkitt’s Lymphoma) cells. F2N78 cell line inherited advantageous phenotypes such as ease to suspension, high transfection efficacy, EBV genome insertion in chromosome, and human-specific glycosylation. For that reason, this cell line can be used for the production of human therapeutic antibody or vaccines. In order to be used in commercial processes, the formulation of the culture medium for F2N78 cell line has been investigated for optimal culture condition. Glucose and glutamine are the most commonly employed carbon and nitrogen sources and directly affect cell growth. However, their rapid depletion was found to cause the accumulation of lactate and ammonia during F2N78 cell culture. To support cell growth with the generation of minimal quantities of lactate and ammonium, amino acids were chosen as the alternatives for glucose and glutamine. Lysine, histidine, valine, cysteine, isoleucine and glycine were found to be critical amino acids for cell growth in previous studies. By calculating the consumption rate from amino acid analysis during previous studies, the amino acids which showed more than 50% consumption rates were selected. The objective of this study is to find the best ratio between the selected amino acids which mitigate the negative effects of byproducts and improve cell growth. The concentration range of selected amino acids was set 1-fold, 2-fold, 3-fold and the experiments were designed by using 2-level fractional factorial design. ANOVA statistical analysis results showed that cysteine and isoleucine were the most significant factors on specific growth rate and maximum viable cell density, and glycine showed the most negative results. Maximum viable cell density with fortification of selected amino acids was increased from $8.61 \times 10^6$ cells/ml to $9.7 \times 10^6$ cells/ml and accumulation of NH4+ was decreased from 2.44 mmol to 1.76 mmol.
USE OF HOMOLOGOUS RECOMBINATION BASED GENOME EDITING FOR CHO CELL LINE ENGINEERING

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Since the sequencing and draft genome assembly of the CHO-K1 cell line was published in Nature Biotechnology in July 2011, it has set the stage for routinely modifying the CHO genome to improve the production of recombinant antibodies. Certain key genes such as FUT8, which encodes α 1,6 fucosyltransferase, an enzyme that catalyzes the post-translational fucosylation of expressed proteins, have already been targeted using gene engineering techniques to prevent fucosylation of recombinant antibodies. Many other proteins encoded by the CHO genome, however, have yet to be explored for their potential impact on the efficacy, safety and half-life of recombinant proteins. Horizon Discovery’s proprietary gene targeting technology utilizes recombinant adeno-associated virus vectors (rAAV) to exclusively stimulate homologous recombination, a natural high-fidelity repair mechanism that exists to maintain the integrity of the genome during mitosis. This process can be easily piggy-backed to create targeted, in-frame and error free genome alterations, by providing DNA vectors with stretches of homology to a target locus, which at a certain frequency are seamlessly recombined with the endogenous sequence and can be used to insert, delete or substitute specific sequences with single nucleotide resolution. No DNA breaks are created by rAAV and there are no additional off-target integrations within targeted cell lines. The only drawback is that the actual targeting process can be slower, principally if one wants to create bi-allelic alterations, since alleles have to be targeted sequentially. In stark contrast, alternative endonuclease technologies i.e. Zinc-finger Nucleases (ZFNs) use a fundamentally different process to perform gene editing. Firstly, the engineering process to target your locus of interest is a complex task involving the alteration of the binding specificity of a protein, and thus in practice is often less than optimal. Even on well validated ZFN’s there are a large number of additional ‘off-target’ cuts as well as the intended cut, which means the process is inherently not a clean one. Secondly, dsDNA breaks are predominantly repaired by the ‘non-homologous recombination end-joining’ (NHEJ) pathway. NHEJ simply rejoins and ligates the DNA ends, usually after some chewing back of the free ends, frequently creating null alleles. The important thing to note is that none of these events involve targeted, in-frame, splicing events, which can only be achieved by eliciting homologous recombination. I will describe how Horizon’s technology has already been successfully applied to reconstitute hundreds of oncogenes in human cell lines, and how, due to its accuracy, rAAV based genome editing is well placed both to decipher the CHO genome sequence, and to modify the genomes of CHO cell lines and other host cell lines used for bioproduction. I will draw upon examples from Horizon’s bioproduction development program to demonstrate the applicability of the technology to the field of bioproduction. These include projects to knock-out metabolic genes (Glutamine Synthetase) and to generate ‘landing pad’ CHO cell lines that contain an antibody back-bone ‘constant region’, wherewith variable regions can be ‘knocked-in’ using an rAAV vectors to facilitate rapid product of antibody isoforms.
UNDERSTANDING INCREASED C-TERMINAL LYSINE IN A RECOMBINANT MONOCLONAL ANTIBODY PRODUCTION USING CHINESE HAMSTER OVARY CELLS WITH CHEMICALLY DEFINED MEDIA

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C-terminal lysine (C-K) variants are commonly observed in therapeutic monoclonal antibodies and recombinant proteins. Heterogeneity of C-K residues is believed to result from varying degree of proteolysis by endogenous carboxypeptidase(s) during cell culture production. The achievement of batch-to-batch culture performance and product quality reproducibility is a key cell culture development criterion. In this study, atypical high variation (from ~5% to ~31%) of C-K level at different scales and process conditions was observed during development of a monoclonal antibody produced by CHO cell line X. The specific CHO cell line was selected as the model cell line due to the exhibited sensitivity of its C-K level to the process conditions. A weak cation exchange chromatography (WCX) method with or without carboxypeptidase B (CpB) treatment was developed to monitor the C-K level for in-process samples. The heterogeneity of basic variant was contributed to cell culture process and no selective removal effect was observed through purification steps. Then, the three different parameters to generate high and low C-K levels of mAb X, were evaluated. They are a medium component (copper) and operation conditions (temperature and culture duration). Of those tested, the copper concentration in the cell culture medium was found to be the most significant parameter affecting the mAb C-K level. Further, the correlations between media copper and zinc concentrations, zinc/copper ratio, and carboxypeptidase(s) activity were examined in both shake flask and bioreactor cultures. The opposite effect of copper and zinc on C-terminal processing was observed. The existence of intracellular carboxypeptidase(s) in CHO was confirmed by Western blot. A hypothesis on C-K processing was proposed based on the analyses of C-K levels in bioreactor cultures with cell culture fluid (CCF) and harvested cell culture fluid (HCCF) samples by WCX and the Western blot results. Understanding the biological cause of the lysine removal and the process parameters affecting the extent of C-K may provide valuable insights to cell culture process development and control.
Novartis has developed a (proprietary) completely animal component free (ACF), CHO cell based, mammalian expression platform. Key components of the technology are a suspension growth adapted CHOK1 derivate and a chemically defined media platform. After transfection, selection is performed in suspension culture, in shake flask format. FACS based high throughput (HT) screening allows enrichment of pools for transfectants with highest expression of the gene of interest, and single cell cloning of “high-expressing” cells. Selection of potential candidate cell lines in suspension culture enables early initiation (3 to 4 months after transfection) of cell line stability testing and upstream cell culture development activities. This regulatory and upstream development friendly CHO platform is extensively used for generation of stable cell lines expressing therapeutic antibodies; titers of stable pools reach up to 0.5g IgG/L, antibody cell line candidates can demonstrate expression levels of 3g/L and above.

We used the CHO platform described above for generation of stable cell sources of a recombinant viral glycoprotein. More recently recombinant viral subunit proteins are discussed and investigated as alternative to classical vaccine formats e.g. attenuated viruses and virus like particles (VLPs). A viral surface protein, as the one expressed in this study, that is essential for entry of the virus into a cell, and a target of virus neutralizing antibodies appears to be a suitable vaccine candidate.

We transfected CHO cells with expression constructs of a soluble viral glycoprotein (vaccine candidate) or antibodies specific for said glycoprotein. A viral glycoprotein / vaccine candidate specific antibody prepared under ACF conditions is required to attempt FACS enrichment/sorting of stable CHO pools. After two subsequent selection steps (Geneticin, followed by Methotrexate) we obtained stable pools expressing human / mouse chimeric antibodies specific for the candidate protein with titers of 150-200mg/L; characterization of stable pools of the vaccine candidate is currently ongoing. Likewise, cloning and characterization of cell line candidates is work in progress and will be discussed.
Previous work has demonstrated synergies among plant-derived hydrolysates, yeast extracts and recombinant proteins for enhancement of CHO fed-batch cultures. Building on this knowledge, a CHO specific feed system was developed with the intent to increase biopharmaceutical process yield, improve culture health and reduce media-related cost.

A CHO-K1 cell line modified to produce secreted alkaline-phosphatase (SEAP) was used to develop the complex feed system. A series of shake flask experiments were performed using DMEM basal medium supplemented on days 5, 7, 9 and 12 with Sheffield’s complex CHO fed-batch supplement. The culture viability, density and nutrient profile was measured over time and compared to CHO-K1 cultures supplemented with a series of commercially available feed supplements. Supernatant was assayed on day 14 to assess the effect of each feed system on SEAP production. The efficacy of the developed feed supplement was then verified in a 500 ml WV Multifors Bioreactor system.

When supplemented into a cost-effective DMEM basal media, the complex feed system demonstrated equivalent or improved overall culture performance when compared to other chemically defined feed systems. Application of the complex feed system to a commercially available CDM realized even greater performance improvements.

Sheffield’s CHO fed-batch supplement is a cost effective and efficient alternative to other commercially available fed batch systems.
DESIGN OF EXPERIMENT (DOE) STUDIES TO EVALUATE PROCESS ROBUSTNESS IN HIGH DENSITY PERFUSION MAMMALIAN CELL CULTURES

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In the current regulatory environment, process robustness is, perhaps, the most defining attribute of a biopharmaceutical manufacturing process, because it relates to the consistency and quality of the drug product. Design space experiments are the foundations upon which a robust manufacturing process can be established and evaluated. Specifically, critical cell culture parameters are identified and a design of experiments (DOE) approach is used to evaluate the cellular response to changes in these parameters. In this study, we first performed a risk analysis to identify temperature, pH, dissolved oxygen, and perfusion rate as critical process variables in a high density BHK perfusion cell culture process. A full factorial experimental design was then used to characterize the cellular response to changes in these critical variables in an established scale-down model. Parameters pertaining to cell growth, metabolism, protein productivity, and protein quality were monitored over the course of perfusion cultivation. A response surface analysis of this data was used to examine the relationship between the critical process parameters. Findings from such analyses can pave a path for logical identification and verification of optimal operating ranges for process variables that would be appropriate in a commercial manufacturing setting.
SCALABILITY OF THE DISPOSABLE MOBIUS® CELLREADY STIRRED TANK BIOREACTORS

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EMD Millipore has developed a family of single-use bioprocess containers designed for mammalian cell growth and recombinant protein production. The Mobius® CellReady product offering includes bench-scale (3L), small-scale (50L) and pilot-scale (200L) bioreactors spanning early process development through clinical batch production. To enable the successful scale-up of a biomanufacturing process, a number of factors critical to efficient cell growth, viability and protein production were considered and used to develop an optimized design. These include mixing efficiency, gas transfer capability and a system design that minimizes cell shear. In this study, several key engineering parameters, such as power per unit volume, oxygen mass transfer coefficient (kLa), mixing time and tip speed were characterized for the three different sized single-use bioreactor containers. Based on these data, CHO cells were cultured in each of these bioreactors using based on the power per unit volume as the primary scaling parameter. These data provide evidence of cell culture scalability across the entire Mobius® CellReady bioreactor platform.
EXPLORING THE TRANSCRIPTOME SPACE OF RECOMBINANT BHK CELLS THROUGH NEXT GENERATION SEQUENCING AND MICROARRAY ANALYSIS

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Baby hamster kidney (BHK) cell lines, as well as the Syrian hamster from which they are derived, have for decades served as valuable model systems in many areas of biomedical research ranging from virus production to studies of prion diseases, cardiomyopathy, and metabolism. BHK cells are also employed in the pharmaceutical industry to produce several important recombinant protein therapeutics. BHK-specific resources would facilitate more comprehensive assessment of characteristic transcriptome profiles for these cells. We therefore set out to assemble and annotate a complete BHK transcriptome, starting with 40 Gbp of paired-end 90-100 bp reads obtained using Illumina high-throughput sequencing. This resource allowed us to establish a baseline profile against which future studies can more confidently identify and evaluate transcriptome dynamics to answer many physiological questions.

The cDNA libraries sequenced represented a recombinant protein-producing BHK cell line as well as two transcriptionally diverse Syrian hamster tissues, liver and brain, which were included to increase the number of genes detected. De novo assembly was performed using the Oases transcriptome assembler, followed by long-read assembly in which 6,000 previously obtained BHK Sanger ESTs were incorporated. The final assembly is comprised of ~221,000 contigs with an average length of 577 bp. Annotation performed by BLAST alignment yielded over 50,000 unique gene IDs representing more than 15,000 unique Ensembl mouse gene IDs. In particular, we were able to annotate 85% of contigs of at least 600 bp, and 94% of contigs at least 1000 bp in length. Comparison of the BHK assembly to an in-house CHO transcriptome revealed 94% sequence identity, while only 91% identity exists between BHK and Ensembl mouse transcripts. Well-annotated genes were used to design a 12-plex Nimblegen expression array to be used for further transcriptome analyses. Samples used for array performance evaluation included those used for sequencing, allowing us to assess the concordance between microarray intensities and RNA-seq abundance values.

Transcript abundance levels, determined by alignment and frequency computation for each library against assembled transcripts, revealed a wide dynamic range spanning five orders of magnitude. Visualization in a pathway context enables one to conjecture possible physiologically active pathways in these cultured cells. Comparative transcriptome analyses between different libraries provide insight into gene expression changes, such as isozyme preferences, which may occur in high-producing cell lines. The diversity of libraries sequenced also permitted exploration of single nucleotide variants in transcripts between cell line and tissue sources as well as within a single library. We also investigated more broadly how such variants can be distinguished from sequencing errors. These genomic resources should serve as valuable process diagnostic tools by providing further opportunities to fingerprint, engineer and enhance BHK cells in their role as recombinant protein producers.
Intracellular metabolites such as nucleotides and nucleotide sugars can give valuable insight into cell metabolism, particularly for the glycosylation processes in cells. Nucleotide sugars are the donor substrates of glycosyltransferases and their availability is known to have an impact on the glycosylation of recombinant proteins including monoclonal antibodies. In addition, nucleotides specifically the nucleotide triphosphates play a central role in metabolism serving as a chemical energy source and acting in cellular signaling. Thus, nucleotides can provide information about the physiologically state of the cell. However, fast turnover rates for some metabolites (e.g., ATP – 1-2 sec) make it difficult to obtain accurate data for all metabolites at a certain sampling point. Therefore, to obtain a representative picture of these intracellular metabolites during a culture the metabolism of the cell sample needs to be stopped immediately after sampling. Furthermore, it is important to use a method of extraction efficient in the recovery of the metabolites of interest.

In this study a matrix testing four different extraction methods combined with no quenching solution, quenching solution 1 (0.9% (w/v) NaCl, 0.5°C) and quenching solution 2 (60% methanol buffered with 0.85% ammonium bicarbonate, AMBIC, pH 7.4, -20°C) was developed. Each combination in the matrix was performed twice with one quenched sample being stored at -70°C for one week before performing the appropriate extraction testing its stability (each done in duplicate). Using high-pressure anion exchange chromatography (HPAEC) the intracellular nucleotide and nucleotide sugars were determined per extraction and compared among the combinations in the matrix as well as between the immediate extracted and stored cell samples. In addition, each combination in the matrix was tested for metabolite leakage and loss during quenching and extraction as well as efficiency of extraction.

Using this matrix we found that the quenching solution containing 60% methanol buffered with 0.85% AMBIC (pH 7.4, -20°C) was the most efficient to recover nucleotide triphosphates compared to the control and quenching solution containing 0.9% (w/v) NaCl (0.5°C) which showed signs of nucleotide triphosphate turnover during the sampling and extraction process. Of the four extraction methods tested, solid-phase extraction with a cartridge containing graphitized carbon showed the best recovery of nucleotide and nucleotide sugars from the CHO cell line used.
CHOGENOME.ORG – AN ONLINE RESOURCE FOR THE CHO GENOME

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The Chinese hamster genome database (www.CHOgenome.org) is an online resource for the Chinese hamster and Chinese hamster ovary (CHO) cell communities. A quarter of all FDA approved new drugs are biopharmaceuticals, most of which are produced in CHO cells. A variety of international efforts are focused on sequencing the CHO-K1 genome and the genomes of other related CHO cell lines and Chinese hamster tissues. The development of genomic resources for the Chinese hamster and CHO cell lines as a bioprocessing tool will facilitate cell line engineering and investigation of the mechanisms underlying cellular phenotypes such as high productivity or viral resistance. In addition, the CHO-K1 genome will serve as a reference for future genomic, transcriptomic, and proteomic studies. Incorporating features of other model organism databases, such as those developed for the Drosophila, mouse, and rat communities, the Chinese hamster genome database aims to provide a centralized resource for the development of genome-scale tools and technologies for CHO cells. Currently, the database contains sequence and annotation information for more than 24,000 protein-coding genes derived from the initial sequencing of the CHO-K1 genome. Details for each protein-coding gene, including genomic location, nucleotide and protein sequences, homologous proteins, and Gene Ontology (GO) terms, were compiled from public databases such as NCBI GenBank, EMBL-EBI and UniProt. A series of tools for searching, browsing, and viewing the genomic data are provided to facilitate access to the CHO genomic information. A CHO BLAST server was developed to allow for sequence similarity searching of the genome and protein databases. Future plans include expansion of the database to host a variety of genome-scale data from CHO cells including genomic, transcriptomic, microRNA, proteomic, metabolic, and microarray data. The integration of such -omic data sets will accelerate the identification of genetic engineering targets and the development of sequence-specific tools for use in CHO cell line engineering.
Getting novel therapeutic antibodies to patients with unmet medical needs necessitates the implementation of strategies to allow rapid preclinical development. Attaining a robust, highly efficient process for commercialization requires totally separate goals and strategies. Regeneron’s unique approach aimed at meeting these competing objectives will be described along with the technologies and strategies used to carry this out. Increased reliance on Quality by Design (QbD) principles have been incorporated into our approach to provide sound scientific rationale for changes in cell lines and processes. Regeneron’s VelociSuite™ technologies that drive this approach will also be described.
The cell line development group at Janssen is responsible for delivering high expressing, well-characterized, clonal cell lines that display sufficient production stability to support clinical development. To maximize our productivity, the creation and selection of cell lines needs to be performed as rapidly as possible without sacrificing cell line or product quality. One of the last and most time-intensive steps in the process is determining stability in protein expression over time. Stability testing generally requires culturing cells over 30-50 generations and assessing titer at various time points throughout the process to determine if antibody expression declines over time. We have developed a method to measure cell line stability early in the development process, thus allowing for both more efficient and cost-effective use of time and resources and the ability to shorten our already aggressive timelines. Using flow-cytometry to measure intracellular recombinant antibody expression levels in clonal cell lines we can detect heterogeneity in antibody expression even when occurring in a small subset of the cellular population. Importantly, we have found that this heterogeneity in expression is an indicator of cell line instability and appears to occur irrespective of underlying cellular cause. The rate of change of the peaks in a heterogeneous population over time correlates strongly with changes in cell line stability, thus effectively functioning as an indicator of future instability. Moreover, these changes can be detected early, when they have started to occur in only a small fraction of the cells and before antibody titer is appreciably affected. Implementation of this assay into our process platform has greatly increased our ability for early prediction of stability in monoclonal antibody expressing cells and, as a result, it has allowed for earlier key decisions in the cell line selection process.
The Keck Graduate Institute's Masters of Bioscience (MBS) is a two-year Professional Science Masters (PSM) program that includes science, engineering, and business course work. Students have the opportunity to focus their studies in one of the five career focus tracks: Medical Devices, Pharmaceuticals, Bioprocessing, Business of Bioscience or Clinical and Regulatory Affairs. Course offerings for the Bioprocessing focus track include two extensive lab classes: Mammalian Cell Biotechnology Laboratory and Bioseparations Laboratory. This poster will describe the Mammalian Cell Biotechnology Lab course in detail. This will include: 1. The required laboratory exercises undertaken by all the students. This includes flask cultures, shaker cultures, and bioreactor cultures, including high-density fed-batch cultures with up to 30 million cells per ml. 2. The equipment used, including Applikon bioreactors, Applikon controllers, a Nova Bioprofile 400 with auto sampling and data logging, and a Beckman Coulter Vi-Cell. 3. Representative data from the students. 4. The weekly lecture sessions and student presentations. 5. The resources required, in terms of both personnel and laboratory expenses, to have 25 students complete this course. Finally, we have BioXpert software that is tied into our bioreactor systems for real time data capture and trending, as well as supervisory control. Program can take all online (in-situ) and offline measurements (ex-situ) where interpolating data, graphing, predictive metabolic kinetics can be obtained; and can be hooked up to every reactor’s assist in feedback control. In total these tools allow us to measure or control a multitude of conditions that help us understand the physiological state of the cells during our experiments.
DEVELOPMENT AND APPLICATION OF AN AUTOMATED, MULTIWELL PLATE BASED SCREENING SYSTEM FOR SUSPENSION CELL CULTURE

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Rationale:
The drive to develop new processes faster and more efficient requires a streamlined workflow. Resource intensive approaches like the use of shake flasks limit the accessible design space for the development of highly productive processes or the characterization of established processes. Process automation provides the appropriate tools to address the following key points:
Increasing experimental throughput --> enable full factorial design of experiments
Increasing process information --> improve process understanding
Automate repetitive manual work --> gain efficiency, focus on high value tasks

Technology:
We developed an automated, multiwell plate based screening system for cell culture processes. The system is setup to be generic and can utilize multiwell plates of different configurations as bioreactors (6 to 48 wells per plate). The screening system is based on off-the-shelf commercial laboratory automation equipment. It is fully automated and handles plate transport, feeding and seeding of cells, daily sampling and preparation of metabolite assays. The integration of all required analytical instrumentation to perform these metabolic assays into the system enables a hands-off operation and renders the system independent from the analytical capabilities available in development thus preventing a potential bottleneck in sample processing.

Results:
1. Scale-up prediction
The comparability of results obtained with the multiwell plate based system and bioreactors had to be verified. It could be shown that 6 well plates were predictive for a scale-up to a 1,000 L stirred tank reactor (scale factor 1:200,000). The parameter profiles of viable cell density, lactate and product concentration were comparable in multiwell plates and bioreactors (2 L, 10 L and 1,000 L).

2. Media screening
An automated media blend screening was carried out to assess another main area of application. Two seed trains of a CHO cell line, media blends of two growth media and two feeding strategies were screened in 120 wells on 6 well plates. An increase in viable cell density and product titer of about 20% in comparison to the reference process was achieved. Several 2 L bioreactor runs using these optimized parameters later confirmed these predicted results.

3. Clone screening
Clone selection processes are another field of application. The standard manual protocol was compared to an automated protocol using 96 clones. The automated approach screened the clones in duplicates using shaken 24 well plates and fed-batch mode. Key metabolites were measured daily and the glycosylation pattern was analyzed at harvest for all clones. All duplicates showed reproducible performance and the overall top producer was initially identified only by the automated process but later confirmed in the manual process. The automation enabled the screening of a larger number of clones using a process very similar to the final manufacturing (platform) process while also providing metabolic profiles.

Conclusion and Outlook
The developed robotic screening system is capable of performing a fully automated workflow consisting of incubation, sampling, feeding and near real-time analytics. The robotic screening system has therefore been proven to be a suitable screening tool for process optimization. Ongoing work is focusing on extending the analytical portfolio. A second focus area is in the field of process characterization and process robustness studies.
ESTABLISHMENT OF A NOVEL GENE AMPLIFICATION PLATFORM BY ATR DOWN-REGULATION IN CHO CELL LINES

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The productivity of recombinant mammalian cell lines has increased dramatically through improvements in vectors, medium optimization, host cell engineering and process development. Among these strategies which contributed to the increase of productivity, dihydrofolate reductase (DHFR)-mediated gene amplification system has been used as a fundamental platform to establish high-producing cell lines. However, it is a time-consuming and laborious process, since many rounds of MTX selection and screening of several hundred individual clones are required to obtain cells with high gene copy numbers. It would therefore be highly desirable to establish a more efficient platform system, which can accelerate the extent of gene amplification in a whole pool of cells.

Ataxia-Telangiectasia and Rad3-Related (ATR) is one of the cell cycle checkpoint kinases which senses DNA damage and activates DNA repair pathway to maintain chromosome integrity. In this study, we propose a novel platform system to accelerate gene amplification more rapidly in CHO cells. The influences of ATR down-regulation by small interfering RNA (siRNA) in rCHO cells producing green fluorescent protein (GFP) and monoclonal antibody (mAb) were investigated. The changes of transgene copy number and productivity during early gene amplification steps were analyzed in a whole pool of cells.

ATR down-regulation in the GFP-producing CHO cell line induced fast transgene amplification compared to the control group. The copy number analysis of the GFP by quantitative real-time PCR showed that the average copy numbers of ATR down-regulated cells are 16, 28, and 61 at 100, 250, and 500nM MTX concentration, respectively. These numbers were 8 to 20-fold higher than those of control groups. The ratio of GFP-positive cells in the ATR down-regulated group was also 2 to 7-fold higher than those in control groups. This data suggested that the increased copy number of genomic GFP could increase the expression level of GFP. Analysis of the growth rate revealed that ATR down-regulated cells have a decreased growth rate during the gene amplification steps under MTX selection pressure. The effect of ATR down-regulation on gene amplification was confirmed through the construction process of the CHO cell line producing mAb. The copy number of the objective gene in the constructed cell lines were 2 to 5-fold higher at 100nM MTX concentration. The ATR down-regulated group also showed a significant increase in specific productivity, which was 4-fold higher than that of control group. Although the growth rate of the ATR down-regulated group during MTX treatment was slightly decreased as expected from the previous result in GFP producing cells, the volumetric productivity overcame this drawback, which has shown 3-fold higher than that of control group.

These results suggest that the ATR down-regulated host cell line can be used as one of the efficient platforms to induce gene amplification more rapidly for constructing highly productive cell lines. We hypothesized that ATR down-regulation may make some cells escape cell cycle checkpoint with damaged DNA, increase fragile site expression, and finally enhance the frequency of chromosome rearrangements. Through this study, we partially proved the relationship between cell cycle and gene amplification.
The objective of process characterization is to demonstrate robustness of manufacturing processes by understanding the relationship between key operating parameters and final performance; with the aim to maximize protein yield while ensuring the efficacy of the molecule. One of the major challenges during the scale up of a commercial cell culture process is the homogeneous mixing of the production bioreactor in order to increase cell biomass and improve productivity. When scaling up, it is important to consider factors such as mixing time, oxygen transfer, and carbon dioxide removal.

As a first step towards robust commercial manufacturing, cell-free mixing studies were performed in production 4000 L scale bioreactors to evaluate scale-up issues. Empirical equations and computerized models were developed to predict mixing time, oxygen transfer coefficient, and carbon dioxide removal rate under different mixing-related engineering parameters. The mixing studies suggest that the engineering parameters related to mixing and carbon dioxide removal may need optimization to mitigate the risk of different performance upon process scale-up.

When cell culture process was established at 4000L scale, further studies were executed to investigate the homogeneity of the culture as cells are continuously exposed to fluctuating conditions within different parts of bioreactors which can affect cell metabolism, yields, and quality of the product. These studies, among others, included stratification analysis, cone dip-tube positioning, sparger performance, mixing and kLa studies. Obtained data suggest that cell homogeneity could be improved by increasing the agitation rate stepwise. Additionally, it was demonstrated that a higher level of N2 sparging can contribute to the reduction of stratification and that reposition of the cone dip-tube play a role in reduction of microcarrier/cell loss to the harvest vessel. Further, comparison between standard and dynamic kLa study approach was estimated for different O2 and N2 sparge, and agitation rates with objective to establish historical datasets which are further used for process evaluation and contusions improvements initiatives.

Additionally, in the light of the FDA’s process analytical technology initiative, novel technologies are explored to further provide information in real time regarding substrate, biomass, product and metabolite concentrations in cell culture processes. These tools, prior implementation at scale, are assessed for their applicability at qualified small scale models. This knowledge is further applied to continuously verify and improve processes performance at commercial scale and will be discussed here.
A novel technology is emerging for real-time in situ monitoring of cell culture applications. This technology, Kaiser Raman, represents a technical evolution of the well understood Raman spectroscopy approach to deliver on the promise of a single probe, capable of multiple process measurements, so long sought after for cell culture applications. To date, those scalable measurements achieved from a single, 12mm, 316L S.S. Kaiser Raman probe in cell culture applications have included Glucose, Glutamate, Glutamine, Lactate, Ammonium, Osmolality, Viable Cell Density and Total Cell Density.

As a result of the success of this technology in measuring the process conditions associated with cell culture applications; coupled with its accuracy, repeatability and reliability, as well as its lack of an associated consumable and requirement for sampling, Kaiser Raman is gaining acceptance as the near term reference and likely eventual successor to the classic bioprocess analysis instruments.

In this presentation we will discuss the historical challenges associated with Raman spectroscopy in bioprocess applications, explain the technical evolutions found in the Kaiser Raman which address these challenges, discuss that which is required to deploy Kaiser Raman and present several case studies. These case studies of the use of Kaiser Raman in cell culture applications at industry leaders Amgen, BiogenIdec and others will include presentation of the associated data from their efforts. This data will illustrate the role of Kaiser Raman as a near term reference for the other types of cell culture process measuring instruments currently deployed and its likely progression to that of eventual successor.
TEMPORAL OPTIMIZATION OF VPA ADDITION DURING TRANSIENT EXPRESSION IN HEK293 CELLS INCREASES FINAL PROTEIN YIELD

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Transient expression in HEK293 cells is a quick and efficient way of producing proteins for use in research. Proteins are required for a multitude of applications e.g. for use as antigens, generation of antibodies and evaluating altered functions of engineered protein variants.

Addition of the transcriptional enhancer buturic acid to cell cultures has been employed for many years. More recently a similar agent, valproic acid (VPA) has been described. The mode of action of VPA, and buturic acid, is believed to include inhibition of histone deacetylases, leaving the histones acetylated and thereby inducing a form of increased global gene expression by opening of the compact chromatin structures. Importantly, VPA has also been shown to induce less apoptosis than buturic acid, resulting in healthier cell cultures and higher titers.

Based on data from the literature we have explored the use of VPA in more detail. However, employing addition of 4 mM VPA at 4 hours after transfection, as described in the literature (Backliwal et al., Biotechnol. and Bioeng. 2008;101 182-189), we only found a moderate increase or even a decrease of expression yield, depending on the protein. We noted that cell growth was severely impacted, and therefore we explored whether the yield could be improved by delaying the addition of VPA until the cell density had increased.

Our results show that the yield of transiently expressed recombinant proteins indeed can be significantly improved by delaying the addition of VPA until cell growth reached a certain level. We found that with careful optimization of the temporal addition of VPA, antibody yield was increased between 65-166% (n=3), FVIII yield 50-100% (n=4) and FVII yield 116-120% (n=2, also employing temperature shift). An important finding was the significance of increasing the cell density before addition of VPA. Also it is shown that in order to identify the optimal setting for VPA addition and temperature shift, the temporal details have to be empirically determined for each specific protein.
The selection of a culture medium is usually crucial for the success of a project, both in terms of product titers obtained and compliance with regulatory demands. Thus, in this work an accelerated program of cell adaptation and comparison of 19 different culture media was performed.

An adaptation protocol lasting 2.5 weeks was evaluated, and adaptation of cells was carried out in spin tubes with 5 mL working volume, using a shaker with 5-cm orbit at 185 rpm and 5% CO2. In total, cells were adapted from their original medium to 18 other media, which were all animal-component-free and were selected based on information found in the scientific literature or provided by the manufacturers. These culture media were proprietary formulations from 9 different suppliers (Hyclone, TeutoCell, Irvine Scientific, Gibco, Lonza, BD, PAA, SAFC and LECC-UFRJ) and were tested for several different recombinant CHO cell lines producing different proteins. Most media contained either recombinant insulin or recombinant IGF-1. Since the presence of hypoxanthine and thymidine (HT) and Pluronic F68 varied among the media, for facilitating comparison HT (1x) and Pluronic F68 (0.1% v/v) were added to those media that were originally devoid of these components.

From the results of adaptation of the first two cell lines to the whole range of media, it was possible to select 12 media for further evaluation. More detailed kinetic evaluations were then carried out with these media for a wider range of cell lines in batch mode in spin tubes with 10 mL working volume, monitoring not only cell growth, but also product formation. The 4 most promising media were further cultivated in small-scale stirred-tank bioreactors (0.7 L working volume), allowing the selection of the two most promising media, which are currently being tested in more detail under different culture conditions (temperature, pH, etc.).

In a joint activity with the supplier of one of the best media (TeutoCell), a further study on the need for supplementation with HT was carried out, as well as a comparative evaluation of their medium containing either recombinant insulin or recombinant IGF-1 or none of these. With these purposes, one of the cell lines previously tested was again adapted from cryotubes of the original cell bank to different versions of the medium, and the results showed that within less than 20 days from start of adaptation cell densities as high as 14 million viable cells per mL could be achieved in batch culture using the protein-free version of the medium.
In this study we compare the limits imposed on recombinant Monoclonal Antibody (MAb) synthesis by its gene coding sequence. For a panel of eight IgG1 MAbs (which only differ in respect to their variable domain sequence) we empirically analyze synthetic intermediate flux from recombinant mRNA to secreted protein, and analyze co-regulation between MAb synthesis and secretion and the unfolded protein response (UPR). These analyses are integrated into product-specific mathematical models of recombinant MAb manufacture which reveal that control of MAb specific productivity (qMAb) is inherently gene sequence specific. Difficult to express MAbs are limited by their folding and assembly rate, with cells exhibiting excess unfolded protein within the ER, leading to induction of a UPR and subsequent attenuation of global translation rates. In comparison, for easier to express MAbs, qMAb is more limited by HC availability. The accuracy of these model based predictions were evaluated by analyzing the effect of co-transfecting a chaperone inducing ER stress element, which confirmed that only difficult to express molecules exhibit a MAb folding and assembly bottleneck during cellular production. In summary, this study highlights that this systems procedure can be utilized to inform product specific cell engineering approaches to increase the production of recombinant proteins by mammalian cell factories.
A recent paradigm in biopharmaceutical production is the implementation of ‘quality by design’ into a process in order to ensure product consistency. This consistency is designed into the product by mapping out and building knowledge of the process operating space. Two case studies will be presented where Quality by Design concepts were applied for the characterization of mammalian cell culture processes used to manufacture recombinant proteins. Different process control strategies were required for the two processes in these cases studies. Process characterization for these case studies included identifying and mitigating process risks, developing an experimental bench scale model representative of the manufacturing process, and performing statistically designed experiments that define the operating space and points of failure. The work presented here will focus on 1) experiments performed to generate statistical models and 2) how these models were used to define the process design space. The experiments included fractional factorial, response surface method, and model augmentation studies. The statistical models generated from this work allowed identification of process optima, action limits, and process parameter criticality. Lessons learned from these cases studies were used to define a generalized approach for performing process characterization and defining the operating space and control strategies.
Eli Lilly and Company utilizes the GS-CHO expression technology and has developed a rapid and efficient process for the generation of clonally-derived cell lines in support of therapeutic protein clinical development. Recently, efforts have been undertaken in our lab to improve cell line generation efficiency and bulk culture productivity through alternative selection schemes, focusing primarily on selection stringency. These approaches include modifications to the selection procedure itself, as well as manipulation of the expression plasmids and expression host cell engineering. A GFP expression cassette has been developed that enables the monitoring of these changes on the selected bulk population for multiple therapeutic antibodies. The GFP profile of a bulk culture as related to its productivity has provided an insight into the selection process. The GFP profile has enabled the development of processes and molecular tools to increase the efficiency of improved cell productivity.
Optimization of bioreactor operation, media composition, and feed strategies typically involves a significant set of experiments based on limited, empirical, off-line data. Cell physiology dynamically affects the nutrient requirements of the culture, so it is critical to obtain the appropriate data over appropriate time intervals to assess the impact of process conditions on the cell population.

To accomplish continuous process improvements, achieve quality by design, and shorten development timelines, a new development paradigm is required. Here, we present an emerging process-development methodology that is based on applying novel and existing bioreactor monitoring technologies to existing bioreactor processes. We then interpret the resulting data so that it may be better understood and leveraged for enhanced process control.

This approach employs on-line dielectric spectroscopy and cell-based bioreactor models to enhance process understanding. These tools are used in conjunction with a simple, compact device that automatically obtains aseptic samples at specified intervals for off-line analysis. This automated aseptic sampling (AAS) system was developed by Bend Research Inc., in collaboration with Pfizer, to ensure bioprocess conditions remain within the desired operating space for maximum product production.

This methodology enables the better use of data to meet dynamic nutrient requirements of cell cultures and allows responsive control of the system, positively influencing the behavior of the cell population. The knowledge gained using this improved process development methodology supports a less-invasive monitoring and feedback system and can be implemented using a customized bioreactor control code. Additional process analytical technologies (PATs), coupled with cell-based bioreactor models, have also been developed to enhance process understanding.

This emerging process-development methodology holds promise to shorten development timelines and deliver a higher-quality process that significantly reduces the cost of goods.
Mammalian cells are used in the manufacture of therapeutic proteins because of their ability to synthesize these complex molecules with appropriate post-translational modifications. The conditions under which cells grow have been shown to directly impact product quality. Therefore it is critical to understand the hydrodynamic forces in bioreactors and how these forces affect the physiology and structure of mammalian cells.

Here we study the sub-lethal effects that aeration and a shear protectant agent have on Chinese hamster ovary cells during the production of monoclonal antibodies. Aeration was delivered into the culture in two ways: direct gas sparging, and via a silicone membrane gas permeable system to avoid the air-liquid interfaces and hydrodynamic forces created by bubbles. The two aeration systems were tested using a chemically defined media with and without shear protectant agent (Pluronic F-68). When direct gas sparging was utilized to provide aeration to the culture the maximum cell density and percent viability were observed to be higher in the culture operated with low flow rate, indicating that cells cultured in presence of fewer bubbles are more able to grow efficiently. However, the productivity (mass of product per batch) of the culture was higher with increased gas flow. The silicone membrane aeration system was an efficient method to deliver gas into the culture, the cell densities and percent viability were comparable to the ones obtained when direct gas sparging was utilized.

The importance of shear protectant agent was highlighted, as cells were unable to grow with direct gas sparging when a shear protectant free media was used. On the contrary when aeration was provided with the silicone membrane aeration system the cell density and percent viability obtained were similar to the ones obtained with direct gas sparging.

It was found that the culture length had an effect on the product concentration, which was accentuated in the media without shear protectant agent, showing that the bioreactor environment may have undesired effects for DSP and mAb efficacy. When cells were subjected to increased hydrodynamic stress they entered apoptosis earlier in the culture and presented variations in the F-actin, one of the main cytoskeleton proteins, intensity measured by flow cytometry. Confocal microscopy also revealed that the aeration method altered the cytoskeleton morphology, when cells were grown using the silicone membrane aeration system a less defined structure was observed. The impact these changes may have on downstream operations is discussed.
MaxCyte has developed a proprietary flow electroporation technology that enables transfection of up to 1x10^10 cells with DNA, mRNA, siRNA or protein in less than thirty minutes. Levels of transfection efficiency and viability exceed 90% with many cell types. Data will be presented to show that the MaxCyte technology enables gram-scale production of monoclonal antibodies in suspension-adapted CHO cells following large-scale, transient transfection. The effects of electroporation energy, DNA concentration, buffer composition and cell concentration during electroporation on protein titers will be discussed. It will be shown that a systematic approach to optimizing protein titers through the use of media additives, temperature shifts, and use of higher cell seeding density post electroporation can generate monoclonal antibody titers that exceed 200 mg/L with transiently transfected CHO cells. In addition to secreted proteins, the MaxCyte technology enables production of viral vectors and VLPs. Successful efforts to develop a large-scale, cGMP-compliant process for manufacturing lentiviral vectors in suspension HEK cells will be discussed, and data will be presented on use of MaxCyte technology to produce VLPs by transient transfection of vero cells with DNA and mRNA.
Chinese hamster ovary (CHO) cell are widely used in producing therapeutic proteins. The proline-requiring CHO K1 cell and the DHFR-deficient CHO DG44 which are derived as subclones from the parental CHO cell line, are the most widely used industrial cells. These subclones show chromosomal aneuploidy and the aneuploidy should be changed with cell passage culture.

In this study, the chromosome number distributions of CHO DG44 and CHO DR1000L 4N (CHO 4N) cell pools were investigated. CHO 4N was constructed by methotrexate (MTX) selection from a CHO DG44 cell line and contains more than 160 copies of exogenous DHFR. Several subclones which contain different number of chromosome were isolated from these parental cell pools and maintained. Possible relation of different chromosome number with the growth and production level of CHO cells was investigated during long-term cultivation. Chromosome number variation of the subclones was observed during a 3-4 months period. The stability of chromosome number and specific growth and production rates was studied during long-term cultivation. Moreover, the chromosome instability was investigated by fluorescence in situ hybridization imaging using BAC clones as hybridization probes (BAC-FISH) according to BAC-based physical map of CHO chromosomes [1, 2].

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INTEGRATING FUNCTIONAL GENOMICS TOOLS TO SURVEY RETROVIRUS PRODUCTION IN HUMAN CELLS

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Retrovirus derived particles found valuable biotechnological application in vaccinology and gene therapy, although challenging from the manufacturing viewpoint, due to the low titers and high content of contaminant defective particles. On the other hand, endogenous retrovirus contamination constitutes a major concern in large-scale biopharmaceuticals production, potentially compromising several-liter production plants. Thus, an integrated overview on the cellular determinants of retrovirus production can provide a valuable tool to either pursue enhanced productivities, in the case of the virus as a final product, or viral replication inhibition in the contamination scenario.

In this work, we analyzed transcriptome changes between retrovirus producers and the corresponding parentals using two different cell lines HEK 293 and Te 671 derived, and took a systems approach to identify biochemical networks overrepresented in the virus production state. Nearly 200 pathways were identified, majorly represented by signaling and metabolism. Lipid metabolism was among the most prominently altered pathways, including cholesterol, phospholipid synthesis and fatty acid mobilization. Amino acid degradation was notably up-regulated as well. Virus production appears to result in higher energy demands, evidenced by the strong up-regulation of the oxidative phosphorylation and electron transport chain. Oxidative stress, nucleotide and polyamine metabolism, protein synthesis and post-translation modification were also highlighted. Gene expression of enzymes and transporters related to biogenic amines metabolism and neurotransmitter-like molecules was additionally suggested to be a potential target to inhibit viral replication. We investigated particular transcriptome-fluxome correlations by HPLC and NMR spectroscopy. Amino acid catabolism profile, one of the most significantly enriched pathways in terms of gene expression, was found to be considerable increased in virus producer cells. Other suggested pathways are currently under study, some of which further substantiate pathways analysis results, including oxidative stress metabolism and lipid biosynthesis.

This work has a direct application in the field of retrovirus manufacture for vaccinology and gene therapy with the generated knowledge providing a framework to guide the rational design of cellular and process engineering. It can also proffer further insights on the cell-virus interaction, disclosing potential targets to inhibit viral replication.
IMPACT OF BIOREACTOR DESIGN ON THE PERFORMANCE OF MICROCARRIER CELL CULTURES

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Gaining from the pioneer work on microcarrier cell culture hydrodynamics of Matt Croughan and Dany Wang in the ‘80, at IBET we have been optimising use of MC for processes involving industrial cell lines used for protein or vaccine production (BHK, VERO, MRC-5...), cells for viral vector production for gene therapy (A549, TeFLY...) or primary and stem cells for cell therapy or preclinical/discovery studies. Hydrodynamics being of special concern, we have been evaluating some recently available disposable bioreactors, as they are becoming the option of choice in many processes, from small, hospital environmental or research lab scale up to thousand liters, industrial scale. Data obtained from tests with the low shear Air-Wheel PBS Biotech bioreactor using MC cell culture will be presented, showing superior cell attachment and growth on microcarries; when these are used to grow viruses, high viral productivities are also obtained.
One of the current commercial manufacturing challenges for legacy cell culture processes that were developed decades ago is the ability to improve process robustness and troubleshoot problems. To overcome this challenge, a qualified scale-down model is crucial in order to conduct studies which can be translated into the manufacturing scale. In order to better understand our legacy cell culture manufacturing processes and subsequently improve process robustness, scale-down model using 12-liter bioreactors was developed and subsequently qualified for a microcarrier-based perfusion cell culture process. Strategies for the development of the scale-down model will be discussed including optimization of several process parameters such as agitation speed and gas sparging strategy to match the manufacturing scale. In order to qualify the developed model, statistical equivalency needs to be established by comparing selected process performance parameters in the 12L scale to the manufacturing scale using a two one-sided test (TOST) analysis. In addition, product attributes such as aggregation level and specific activity were within the range of specification in the scale-down model.
More and more regulatory agencies, such as the FDA are forcing manufacturers to pay more attention to the quality of their production processes. FDA for instance does not only express its concern about stagnation in process development, it also made suggestions to the manufacturers. In its “process analytical technology (PAT)” initiative, FDA proposes concretely: (i) to make use of new measurement techniques for online supervision of the processes and (ii) to more exhaustively exploit the measurement data gathered from the processes to gain mechanistic understanding, predominantly knowledge about the interrelationships between the various process variables. According to the FDA-initiative, measurement data should (iii) be used online in order to recognize deviations from ‘in control situations’ before the processes run out of control. And (iv) in the case of significant deviations from the desired setpoint profiles, the processes must be drawn back to the predefined path by automatic feedback control in the engineering sense. In this presentation online monitoring techniques for process supervision and control of cell culture attributes will be addressed. In a first step a feasibility study with various regression techniques has been performed in order to estimate one of the most important variables of a cell culture process, the viable cell density. We will compare several possibilities for the concrete case of the large scale production of therapeutically proteins and perform a ranking. Data driven regression techniques, such as artificial neural networks (ANNs) and principle component analysis (PCA), as well as statistical correlations like multivariate regression techniques and simple model-based approaches will be introduced. A sensitivity analysis will assess the robustness of these indirect measurement techniques. All techniques examined are in line with the recommendations expressed in the PAT-initiative of the FDA.
Human embryonic stem cells (hESCs) are an attractive source for cell replacement therapies and in vitro toxicology studies due to their inherent self-renewal ability and pluripotency. However, the establishment of effective protocols for large-scale expansion, storage and distribution of hESCs is imperative for the development of high quality therapeutic products and functional screening tools. In fact, the current static colony cultures present high variability, lack of environmental control and low production yields, hampering the development of efficient, scalable and cost effective stem cell expansion systems [1]. The low cell recovery yields and the high rates of uncontrolled differentiation obtained after cryopreservation also limit their use in clinical or industrial applications [2].

In this study, cell microencapsulation in alginate was used to develop an integrated bioprocess for expansion and cryopreservation of pluripotent hESC. Different three-dimensional (3D) culture strategies were evaluated and compared: microencapsulation of hESC as single cells, cell aggregates and cells immobilized on microcarriers [3]. hESC-microcapsules were cultured in stirred tank bioreactors, aiming to establish a scalable and robust bioprocess.

Our results show that, the combination of cell microencapsulation and microcarrier technology resulted in a highly efficient protocol for the production and storage of pluripotent hESCs. This strategy ensured high expansion ratios (approximately 20-fold increase in cell concentration) and high cell recovery yields after cryopreservation (>70%). When compared to non-encapsulated cells, an improvement up to 3-fold in cell survival post-thawing was obtained without compromising hESC phenotype and pluripotency [3].

This work demonstrates, for the first time, that cell microencapsulation in alginate is a powerful tool to integrate expansion and cryopreservation of pluripotent hESCs. The 3D culture strategy developed herein represents a significant breakthrough towards the translation of hESCs to clinical and industrial applications.

The production of cardiomyocytes from induced pluripotent stem cells (iPSC) holds great promise for patient-specific cardiotoxicity drug testing, disease modeling and cardiac regeneration. The successful translation of iPSCs to these fields requires the development of robust bioprocesses for the production of cardiomyocytes in high purity, consistent quality and relevant quantities. However, existing protocols for the differentiation of iPSC to the cardiac lineage are still highly variable and inefficient, hampering their implementation in the clinic and industry.

The main aim of this study was to develop a robust and scalable platform for the efficient production of iPSC-derived cardiomyocytes. Our strategy consisted in designing an integrated bioprocess by combining cardiac differentiation and cell lineage purification steps in environmentally controlled stirred tank bioreactors, where the necessary conditions to control stem cell fate are perfectly tuned. A transgenic murine iPSC line, transfected with αPIG vector in which the promoter of the cardiomyocyte lineage marker, α-myosin heavy chain, drive both GFP and puromycin resistance gene expression, was used to establish the utility of this bioprocess. Different bioprocessing parameters were evaluated and the results showed the importance of controlling pH, pO2 and agitation regime to improve the final yields of cardiomyocytes. Cell characterization and monitorization of the differentiation/purification process was performed along culture time using phase contrast and fluorescence microscopy, flow cytometry and qRT-PCR analysis. In addition, novel cryopreservation strategies were developed aiming to guarantee an efficient storage of iPS-derived cardiomyocytes after “large-scale” production.

Another major challenge in stem cell bioprocessing is the establishment of novel and reliable methodologies for non-invasive and on-line monitoring of the differentiation process. To address this issue a fluorescence probe was incorporated in the bioreactor apparatus and the data generated was compared and validated taking into account the results obtained by flow cytometry analysis.

The integrated bioprocess and monitoring tools developed in this work provide important insights for the establishment of more robust iPSC production platforms, hopefully potentiating the implementation of novel cell-based therapies, in vitro disease modeling and drug screening.
Central Nervous System (CNS) disorders, such as neurodegenerative diseases, remain a formidable challenge for the development of new and efficient therapies. Gene therapy approaches are promising alternatives that can provide specific targeting and prolonged length of action, treating the causes rather than the symptoms. Hampering the fast translation of gene therapy-based treatments to the clinic is the lack of valid and reliable preclinical models that can contribute to evaluate feasibility and safety.

Preclinical research has traditionally relied on 2D \textit{in vitro} cell models, that fail to recapitulate the characteristics of the target tissue (cell-cell and cell-matrix interactions, cell polarity, etc) and genetically engineered animal models, which often diverge considerably from the human phenotype (developmental, anatomic and physiological). Human 3D \textit{in vitro} models are useful complementary tools towards more accurate evaluation of drug candidates in preclinical stages, as they present an intermediate degree of complexity between the traditional 2D monolayer culture conditions and the brain.

Herein, we describe a robust and reproducible methodology for the generation of 3D \textit{in vitro} models of the human CNS following a systematic technological approach based on stirred culture systems and using human neural stem cells (hNSC) as a scalable supply of neural-subtype cells. We took advantage of midbrain-derived hNSC commitment to the dopaminergic lineage to generate differentiated neurospheres enriched in dopaminergic neurons. Control of chemical and physical environmental parameters allowed for the differentiation into neural-subtype cells in reproducible ratios. Detailed cell characterization of differentiated neurospheres was performed along culture time using spinning disk confocal microscopy, field emission scan electron microscopy (FESEM), transmission electron microscopy (TEM), qRT-PCR and Western Blot.

The feasibility of using this novel 3D model to address viral vector safety was addressed using canine adenovirus type 2 (CAV-2) viral vectors, good candidate for CNS gene therapy applications due to their high cloning capacity, long-term transgene expression and low immunogenicity and tropism towards neurons. The perturbation caused by CAV-2 vectors in human CNS cells was evaluated, aiming at predicting adverse effects. CAV-2 transduction efficiency was optimized using a CAV-2 vector carrying eGFP reporter gene, creating a good reference for future clinical trials.

The model system developed in this work constitutes a practical and versatile new \textit{in vitro} approach for preclinical research of human CNS disorders and is expected to increase its relevance. Furthermore, this culture strategy may be extended to other sources of human neural stem cells, such as human pluripotent stem cells, including patient-derived induced pluripotent stem cells, broadening the applicability of these models even further.
SPEED UP PROCESS DEVELOPMENT AND CLINICAL MANUFACTURING USING DISPOSABLE STIRRING TANK REACTORS

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Single-use bioreactor offers advantages such as lower upfront capital investments, fewer requirements for process validation, significantly shorter time needed to build a cGMP manufacturing facility, and higher flexibility to be reconfigured for a new process, compared to traditional stainless steel reactors. An increasing number of biotech companies, especially new antibody developers and manufacturers are building large-scale clinical manufacturing facilities equipped with disposable Stirring Tank Reactors (STRs). Agensys is one of these companies that commit to use disposable STRs for its early-phase clinical manufacturing. Through a case study, this presentation will show how the disposable STRs were used to speed up process development and clinical manufacturing to meet our aggressive timeline at Agensys. Performance comparability will be assessed by comparing results from bench-scale disposable bioreactors and conventional glass bioreactors. Then process scalability will be evaluated using results from disposable STRs at different scales (2L, 50L, and 200L). In addition, cost analysis will be presented to illustrate cost-effective situations of applying the bench-scale disposable bioreactors over the conventional glass bioreactors.
ENGINEERING AUTOPHAGY IN CHO CELLS TO INCREASE PROTEIN PRODUCTION IN FED-BATCH PROCESSES

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Understanding the physiological factors that affect cell proliferation and productivity should assist the optimal design of robust high-performance bioprocesses. Autophagy is a cellular process that targets intracellular components for lysosomal degradation as part of a survival adaptation to nutrient limitations and other stresses. We investigated autophagy in serum-free fed-batch cultures using Chinese Hamster Ovary cells producing tissue plasminogen activator (t-PA). The mRNA levels of several autophagy genes increased 4-fold during the process. Glutamine deprivation did not affect cell viability but was followed by significant expansion of the lysosomal compartment, along with decreased mitochondrial mass, cell proliferation and cell-specific productivity. The formation of GFP-LC3 fluorescent puncta and a LC3 flux assay confirmed that there was increased autophagic activity upon glutamine withdrawal. Chemical inhibitors of autophagy as well as the timing of their addition were screened to determine if the process performance could be improved. Addition of 3-methyladenine (3MA) to the fed-batch process yielded an almost 3-fold increase in t-PA production (>0.5 g/L). Treatment with 3MA did not impair the glycosylation capacity of the cells, as treated cultures displayed the highest levels of complex sialylated glycans.

In order to evaluate the applicability of these findings to other cell lines and products, we investigated the interplay between autophagy and glutamine metabolism in CHO cells producing recombinant monoclonal antibodies. In all tested cell lines, glutamine deprivation decreased cell proliferation, glucose uptake and lactate production, without adverse effects on cell viability. However, mitochondrial and lysosomal changes varied between cell lines, suggesting that the susceptibility to autophagy was cell-line dependent. Consistent with these observations, the different cell lines did not respond in the same way to autophagy inhibition by 3MA. Combining inhibition of autophagy with a strategy of partial glutamine replacement in the glutamine-sensitive cells, we obtained up to an overall 4.5-fold increase of recombinant protein production compared to control fed-batch process.
There is a need for a predictive model describing CHO cells behaviour. Such model can eventually be useful to screen for high producing cell lines as well as for medium composition optimisation. But it can also be an efficient on-line monitoring tool to follow, fine-tune and control a fed-batch or perfusion bioreactor culture. A kinetic-metabolic model approach describing and simulating Chinese hamster ovary (CHO) cells behavior is presented. The model includes glycolysis, pentose phosphate pathway, TCA cycle, respiratory chain, redox state and energetic metabolism. Growth kinetics are defined as a function of the major precursors for the synthesis of cell building blocks. Michaelis-Menten type kinetics are used for metabolic intermediates as well as for regulatory functions from energy shuttles (ATP/ADP) and cofactors (NAD/H and NADP/H). The model structure and the parameters were first calibrated using data obtained from bioreactor cultures of a CHO cell line expressing recombinant t-PA. The model shows to simulate experimental data for extracellular glucose, glutamine, lactate and ammonium concentration time profiles, as well as cell oxygen consumption and energetic state. The model can also provide an estimate of the cell’s redox state. The modeling framework was then shown to be readily adaptable for studying the effect of sodium butyrate addition on CHO cells metabolism, either at mid-exponential growth phase (48 h) or at the early plateau phase (74 h). Analysis of cell behavior from model simulations further substantiates our previous findings that butyrate treatment at mid exponential phase caused a significant shift in cellular metabolism toward a sustained and more efficient energetic state. The application of the model to other CHO cell lines will also be presented and discussed.
A NOVEL METHOD OF GROUPING AMINO ACIDS FOR MEDIA OPTIMIZATION

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Amino acids are among the most critical components in cell culture media. They play multiple roles as energy sources, metabolic intermediates, and raw materials for the production of both recombinant and host cell proteins. As one of the major contributing factors in media development, optimization of amino acids has become even more important recently because of the trend towards chemically-defined media. Optimizing amino acids individually is complex, impractical, and many times ineffective. However, optimizing amino acids as groups can be a more effective approach, depending on the method of grouping. Traditional methods of grouping typically rely on their essential versus non-essential roles or physiochemical properties. Even these grouping rationales fall short in directing their optimization. Although several metabolic pathways have been elucidated, how the optimization of amino acids falls into this framework remains largely unclear. Individual clones may exhibit a wide range of metabolic behavior, even when derived from the same host cell. A more relevant grouping scheme might lead to more effective media optimization. In this case study, it was hypothesized that enriching those amino acids with the highest cell specific consumption rate (CSCR) would have the most positive impact on culture performance. In order to test this hypothesis, amino acids were prioritized based on their CSCRs. The amino acids were then enriched based upon their prioritization, in a cumulative fashion. While enrichment of the first nine amino acids showed a stepwise increase in performance, no further gains in culture performance were realized from enriching the remaining ten. This experiment successfully identified two groups of amino acids, those to be enriched and those which should be maintained, or possibly reduced in concentration. The result proved the initial hypothesis true. To see if this new finding could be exploited for amino acid optimization, an experiment was conducted in which amino acids were grouped as high, medium, and low with regards to their CSCRs. Optimal concentrations for each group were determined using a Design of Experiments (DoE) methodology. The results led to an increase in concentration of the high and medium groups and a slight reduction in concentration of the low group, supporting our initial findings. A significant gain in culture performance was achieved through this approach, ultimately proving its benefit for amino acid optimization. This novel, more relevant method of grouping amino acids has proven effective for media optimization. Being a data driven approach to optimization using CSCRs suggests that this method could be applied dynamically as a platform approach for media development.
An important paradigm in process development for recombinant biotherapeutics has been the platform approach. A platform generally employs a single host cell type and a well defined process for going from gene to stable clone to pilot scale (and eventually full-scale) production. The process often includes multiple media for different stages of the upstream process with a single basal medium and feed chosen for pilot scale and beyond. While the intent of a platform process is to reduce complexity and variability, using different media for different parts of the process may actually increase variability. Altering the nutrient environment of cultured cells can force an adaptation to the new medium and may result in selection for an undesirable phenotype that thrives in the new conditions. This may in turn require re-cloning to identify the right phenotype, additional stability testing and characterization, ultimately resulting in extended timelines and increased cost. To avoid the potential negative impacts of undesired selection we employed multiple analytical methods to characterize cellular metabolism. This information was used to develop a selectively fortified basal medium (CD FortiCHO™ medium) that is able to be used in all stages of process development and is manufacturable in both liquid and granulated (AGT™ medium) formats. When the CHO- S™ host cell line was pre-adapted to this basal medium, it was possible to conduct transfection, selection/recovery, cloning, expansion and production of recombinant protein using a single formulation. The use of this single medium formulation facilitated cell line development from gene to stable clone in approximately 4 months. Process development was further enabled by use of a feed supplement that was matched to CD FortiCHO™ medium resulting in >3 g/L recombinant monoclonal antibody titer in a fed-batch process. Additional analytical methodologies including liquid chromatography mass spectrometry (LC/MS) and capillary electrophoresis (CE) were used to characterize intra- and extracellular environments in an effort to assess cellular health and expressed product quality. Specific examples of the efforts to balance cell growth, recombinant antibody titer and galactosylation through rational design of the fed-batch process will be discussed.
Over the last 5-10 years, many biopharmaceutical companies have developed cell culture platform processes for the production of recombinant glycoprotein therapeutics, mainly antibodies (mAbs), in mammalian cells. The use of platform manufacturing processes for the production of clinical material has several advantages including lower development cost for process development and faster generation of clinical material, thus enabling a reduced timeline to entry into clinical studies.

Genentech, which became a member of the Roche group in 2009, has developed and optimized cell culture platform processes over the last 10 years. Both Genentech and Roche implemented their first platform cell culture process using fully chemically-defined media in 2009. However, since these platform processes were developed independently by different organizations, significant differences in process details were observed. These included the use of different (a) CHO host cell lines, (b) proprietary in-house chemically-defined media and feed formulations, and (c) different bioreactor processes. With the integration of the two process development organizations into one global organization, the decision was made to implement one single upstream platform at both Roche process development sites. Drivers for this strategic decision include increased flexibility with regard to process development and manufacturing: any process could be executed at any network facility without significant limitations (plant fit, raw material availability). Another benefit is that all future development efforts could be leveraged by the entire organization leading to more efficient use of resources (e.g., process characterization and validation).

This presentation will focus on the evaluation of the two legacy platform processes and the development of a single cell culture platform process with improved capabilities and increased yields.
MEDIUM CONDITIONS INFLUENCE THE TERTIARY STRUCTURE OF THE T-PA BY REDUCING / OXIDIZING THE CYS182-CYS313 DISULFIDE BOND

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The modified tissue-type plasminogen activator (t-PA) pamiteplase (PM) is known to have both an oxidized (PM^{ox}) and a reduced (PM^{red}) form at the disulfide bond linking Cys182 and Cys313. The ratio of the reduced form to total PM (PM^{total} = PM^{red} + PM^{ox}) in the cell culture medium was found to increase over culture time. The reducing or oxidizing influence of the culture supernatant itself on the t-PA decreased during the late phase of the culture, but recovered after adding adequate amounts of cysteine and cystine. Furthermore, the redox reaction rate in culture fluid depended on the concentration of cysteine and cystine. The ratio of PM^{red}/PM^{total} in the culture medium was considered to be influenced by the concentration of cysteine and cystine. A kinetic simulation study using cell-free culture fluid suggested that the ratio of PM^{red}/PM^{total} in culture medium was influenced not only by the cysteine/cystine ratio in the culture medium but also by the ratio of PM^{red}/PM^{total} synthesized through the secretion pathway. The redox state of Cys182-Cys313 upon secretion was considered to change depending on the cystine concentration in the culture medium which may influence the redox state of the endoplasmic reticulum. The construction of a tertiary structure model of PM molecule revealed that the Cys182-Cys313 disulfide bond is located on the surface of the PM molecule, which gives it easy access to medium components. To attain the low-level control of the ratio of PM^{red}/PM^{total}, copper-catalyzed oxidation (CCO) of cysteine to cystine was investigated. Three-liter bioreactors were used to optimize oxidization conditions, which were then applied to a 1,200-L bioreactor. The CCO system reduced the rate of specific cystine uptake into cells, which resulted in the maintenance of oxidative medium conditions. The cysteine/cystine CCO system was useful to control the tertiary structure of t-PA in long-term culture methods like one-month cell-recycle repeated-batch cultures.
Quality control of therapeutic antibodies is one of the most important topics in the manufacturing process [1]. Aggregation of therapeutic antibodies is the fundamental issues to be addressed because of reduced biological activity and immunogenicity after the administration. An addition of several co-solutes is known to suppress the protein aggregation. Trehalose, non reducing sugar formed from two glucose units with ƒ¿-1,1 linkage, is effective co-solute for anti-aggregation, and we assess the effects of trehalose on the antibody aggregation during the production process, especially cultivation process of Chinese hamster ovary (CHO) cell. In the present study, we show the case of humanized IgG-like diabody-type bispecific antibody. The antibody is the promising candidate for next-generation therapeutic antibody because of dual functionality, whereas it shows aggregation tendency. We investigated the effects of trehalose on biophysical properties of bispecific antibody. Circular dichroism (CD) measurement showed that the secondary structure was not disrupted by 200 mM trehalose. The aggregation temperature, assessed by thermal unfolding experiment, was raised from 60.8 to 63 degrees. In heat incubation, the aggregation percentage was significantly decreased from 80% to 40%. These results indicate that trehalose is effective on anti-aggregation of bispecific antibody due to enhanced thermal stability. Next, we investigated the effects of the addition of trehalose on cell cultivation process. Serum free-adapted CHO Top-H cell line, which produces bispecific antibody, was cultivated in suspension. Cell growth was largely affected by trehalose addition; the specific growth rate and maximum cell density were decreased. On the other hands, the lifetime was prolonged. Interestingly, the antibody production was largely enhanced. Trehalose may induce the suppressed cell growth and enhanced productivity in antibody producing CHO cell. Size exclusion chromatography (SEC) analysis showed that the formation of large aggregates was significantly suppressed by trehalose, suggesting that the addition of trehalose is available for suppressing antibody aggregation in cell culture process.

Mammalian cell culture processes for producing biotherapeutics have evolved significantly in the past 25 years. Initial product concentrations were on the order of 100 mg/L while it is now common to achieve concentrations upwards of 5 – 10 g/L in fed-batch cultures. A number of cell culture processes emerged as new culture methods to increase productivities were developed.

Batch culture, a relatively simple cell culture approach, can be limited by the substrate that is initially available. Fed-batch cultures incorporate concentrated feeds to prevent the depletion of limiting substrates. Continuous stirred tank bioreactors replenish spent media components while simultaneously removing waste products and cells. Further increases in productivity can be accomplished using perfusion techniques, whereby cells, the product protein or both are retained in the bioreactor as potentially toxic metabolites are flushed from the system and replaced with fresh medium. Ultimately, the process chosen should consider the balance among the productivity requirements, the product quality targets and the complexity of operations at the industrial scale.

An elegant process that increases volumetric productivities by over 40% will be presented. The semi-continuous fed-batch process retains all or a significant portion of the cells in culture for subsequent cultures. The higher volumetric productivity relative to conventional platform fed-batch processes is a result of both eliminating the growth phase of the fed-batch and collapsing the turnaround time between production batches within a clinical campaign. Furthermore, the semi-continuous process reduces the complexity of a continuous perfusion approach.
Eli Lilly has recently constructed and qualified a commercial biologics manufacturing facility in Kinsale, Ireland. This talk will focus on the transfer and validation of the inaugural product for manufacture in that facility. The product and process was developed using QbD principles. Laboratory model based studies have shown that the cell culture process parameters have the potential to impact product related Critical Quality Attributes (CQAs). The presentation will provide a detailed overview of the collaboration between manufacturing and development organisations to develop a robust cell culture control strategy using Design of Experiments (DoE) and statistical techniques. The focus will then switch to the application of this control strategy, including operational considerations between sending and receiving sites. The talk will conclude with a comparison of cell culture performance at sending and receiving sites.
Objective:
Several studies have investigated the effect of individual microRNAs (miRNAs) on Chinese hamster ovary (CHO) cell phenotype. The impact of global alterations in mature miRNA levels on CHO cells is, however, unknown. Therefore, we investigated the relevance of miRNA biogenesis by studying the expression of DICER, DROSHA and DCGR8 - the main responsible enzymes for miRNA biogenesis - under different settings, and subsequently performing a targeted knockdown of these genes in recombinant CHO dhfr (-) cells.

Results:
The initial approach is based on two conflicting observations: In fast growing cancer cell lines the overall miRNA biogenesis is downregulated, while the adaptation to slower, serum-free growth of 3 different CHO cell-lines was also accompanied by an almost exclusive downregulation of miRNA expression (Hackl, 2011). Therefore, the impact of reduced serum-levels on the expression of DICER, DROSHA and DGCR8 were analyzed using qRT-PCR. Indeed, mRNA levels of all three enzymes were found to be down-regulated upon serum removal, suggesting that the initial observation might be the consequence of reduced miRNA biogenesis. To further explore the functional role of miRNA processing, DICER and DROSHA mRNA levels were analyzed in five CHO cell lines that were grown in suspension in serum-free media and exhibited low, medium and high growth rates. qRT-PCR results show that DICER and DROSHA levels are elevated more than 2-fold in fast growing compared to slow growing cells. Based on recently published cDNA sequences, specific small-hairpin RNAs (shRNAs) were designed to knockdown expression of DICER, DROSHA and its binding partner DGCR8, and tested in transient transfections in recombinant CHO dhfr (-) cells. For DROSHA, transient knockdown of >70% was achieved, resulting in a significant reduction in the specific growth rate, which was further enhanced by simultaneous knockdown of DGCR8, and negatively correlated with cell specific productivity.

Conclusion:
Our data suggest, that in suspension cultivated CHO cells the expression levels of DICER and DROSHA correlate with cell growth, that a global reduction in miRNA levels by down-regulating DROSHA levels reduces the growth of CHO cells and that therefore miRNA expression signatures must exist that support fast growth of CHO cells. These observations lead us to look for miRNA signatures that support CHO cell growth using microarray analysis. As global downregulation of miRNA biogenesis is counteracting cellular growth, the more promising path is clearly a targeted approach to specifically knockdown individual, growth suppressing miRNAs.

References:
Objective:
The development of microRNA tools that allow targeted selection (biomarkers) or improvement (engimiRs) of Chinese hamster ovary cell phenotypes requires a profound knowledge of miRNA sequences and their genomic organization, which exceeds the currently available information of 398 conserved mature CHO miRNA sequences. Based on two independent CHO genome assemblies - the recently published CHO-K1 genome (K1-P, Xu et al.), as well as a CHO-K1 genome assembly generated at BOKU and Bielefeld University (K1-BB) - we here describe the computational identification of CHO miRNA genomic loci, the respective precursor miRNA sequences (pre-miRNAs) as well as practical applications of this knowledge.

Results:
Using BLAST alignment, a total of 370 out of 420 miRNAs were mapped to either reference genome. Sixteen miRNA alignments in genomic repeat regions were removed and the remaining 354 miRNA alignments were sorted into 230 distinct genomic miRNA loci, as approximately 50% of miRNAs represented matching pairs of 5’ and 3’ miRNAs. Despite the fact that in-house assembled draft genome K1-BB consisted only of a preliminary assembly, the use of both data sets proved valuable for two reasons. Although most miRNA loci (204 out of 230) were found in both genomes, a significant number was either found in the published assembly K1-P (16 loci) or in the alternative CHO-K1 genome K1-BB (10 loci). After extraction of precursor-miRNA (pre-miRNA) sequence information from the genomes, the availability of two genomes, however incomplete, also allowed sequence comparison of these 230 pre-miRNAs and revealed four pre-miRNAs harboring putative SNPs. As a proof-of-principle for the usability of the published genomic loci, four polycistronic miRNA cluster were chosen for PCR amplification using CHO-K1 and DHFR (-) genomic DNA as templates. Clusters were cloned into pcDNA 6.2 emGFP expression vector, transfected into a recombinant DHFR (-) CHO cell line and miRNA overexpression was confirmed by qRT-PCR.

Conclusion:
The presented information on the genomic context of miRNA expression in CHO cells will not only improve CHO-specific miRNA detection and quantification methods, but will also feed into the improvement of current miRNA engineering approaches: firstly, the use of endogenous miRNA hairpins sequences as basis for miRNA overexpression might prove to be superior to so far used artificial constructs harboring standardized loop and flanking regions, and secondly, cloning and engineering of entire miRNA clusters is likely to have a stronger impact on CHO cell phenotypes than individual miRNAs.

References:
MIXING UNIFORMITY CHARACTERIZATION OF 15,000L MAMMALIAN CELL CULTURE BIOREACTOR

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The mass transfer uniformity of a 15,000 liter mammalian cell culture bioreactor with modified pitched blade impellers was demonstrated by empirical methods and computational fluid dynamics (CFD) modeling. The gas dispersion, mass transfer and blending performance of the 15,000 L bioreactor were characterized empirically. Two empirical methods were used to evaluate the bioreactor blend times under different agitation speeds. A computational fluid dynamics (CFD) model was developed using ANSYS Fluent. The CFD model was verified by empirical blend time results. The flow pattern and mass distribution in the 15,000 liter bioreactor over a range of impeller operating speeds were characterized with CFD modeling. The results from empirical blend studies and CFD modeling indicated top/bottom blend time uniformity. Also, zonal uniformity of oxygen transfer was demonstrated by gas dispersion and mass transfer characterization studies. The study results and lessons learned will be discussed.
GENERATING A PRODUCTION CELL LINE FOR CLINICAL MANUFACTURING OF ANTIBODIES IS A TIME-INTENSIVE PROCESS THAT INVOlVES FUNNELING A LARGE NUMBER OF CANDIDATE CLONES THROUGH VARIOUS SCREENING STEPS BEFORE FINALLY SELECTING A LEAD. PREVIOUS STUDIES IN OUR LAB DEMONSTRATED LITTLE TO NO CORRELATION OF CELL GROWTH AND TITER (R² < 0.4) BETWEEN THESE INTERMEDIATE SCREENING STEPS (I.E. 96-Well Plates, 24-Well Plates, Batch Shake Flasks) AND FED-BATCH SHAKE FLASK CULTURES. THEREFORE, EVALUATING CLONES IN CONDITIONS SIMILAR TO A BIOREACTOR AS EARLY AS POSSIBLE IN THIS PROCESS IS CRUCIAL TO IDENTIFYING THE MOST SUITABLE, HIGHEST-EXPRESSING CELL LINES. INCORPORATION OF A HIGH-THROUGHPUT, DISPOSABLE BIOREACTOR SYSTEM WOULD GREATLY FACILITATE THIS PROCESS BY 1) ALLOWING A LARGER NUMBER OF CANDIDATE CELL LINES TO BE SCREENED IN A BIOREACTOR, 2) REDUCING THE AMOUNT OF TIME AND RESOURCES NECESSARY FOR BIOREACTOR SETUP AND OPERATION, AND 3) PROVIDING AN OPPORTUNITY TO PERFORM MULTI-FACtor PROCESS OPTIMIZATION AT A SMALL-SCALE. THEREFORE, WE EVALUATED THE ADVANCED MICROSCALE BIOREACTOR (AMBR™) SYSTEM AS A HIGHER-THROUGHPUT, DISPOSABLE ALTERNATIVE TO TRADITIONAL STIRRED TANK BIOREACTORS. EVALUATION PARAMETERS INCLUDED EASE OF OPERATION, REPRODUCIBILITY, TIGHTNESS OF THE pH, DISSOLVED OXYGEN (DO²), AND TEMPERATURE CONTROLLERS, AND COMPARISON OF CELL GROWTH AND ANTIBODY EXPRESSION TO TRADITIONAL SYSTEMS (I.E. STIRRED TANK BIOREACTORS AND SHAKE FLASKS). USING THREE PRODUCTION CELL LINES FOR A PRECLINICAL ANTIBODY PROGRAM, WE FOUND THE SYSTEM TO BE HIGHLY REPRODUCIBLE IN OUR HANDS, WITH R² > 0.9 WHEN COMPARING DUPLICATE BIOREACTORS ACROSS VARIABLE CELL DENSITY, PERCENT VIABILITY, AND ANTIBODY TITER. IN ADDITION, STRONG CORRELATIONS FOR ANTIBODY TITER COMPARING THE AMBR™ SYSTEM TO SHAKE FLASKS AND STIRRED TANK BIOREACTORS WERE OBSERVED (R²=0.89 AND R²=0.75, RESPECTIVELY). FINALLY, THE AMBR™ SYSTEM WAS ABLE TO ACCURATELY CONTROL TEMPERATURE AT TWO DIFFERENT SET POINTS (34.0°C AND 37.0°C) WITHIN ± 0.2 DEGREES, DO² AT A SETPOINT OF 50% WITHIN ± 5%, AND pH AT TWO DIFFERENT SET POINTS (6.8 AND 7.0) WITHIN ± 0.08pH UNITS. WHEN THE CULTURE pH WAS COMPARED TO OFFLINE MEASUREMENTS, THE ROOT MEAN SQUARE OF THE DIFFERENCE OVER THE ENTIRE PROCESS WAS 0.1pH UNITS. THE RESULTS OF THIS EVALUATION STRONGLY SUGGEST THAT THE 10-15 ML AMBR™ SYSTEM REPRESENTS A SUITABLE SMALL-SCALE MODEL OF A 3-5L STIRRED-TANK BIOREACTOR. IMPLEMENTATION OF THIS SYSTEM INTO A PRODUCTION CELL LINE DEVELOPMENT PLATFORM WILL THEREFORE FACILITATE IDENTIFICATION OF HIGH-EXPRESSING CLONES, DECREASING DEVELOPMENT TIMELINES BY UP TO 3 WEEKS AND INCREASING THROUGHPUT UP TO 6-FOLD OVER TRADITIONAL BIOREACTORS.
Glycosylation, a post-translational modification in which a carbohydrate chain is added to a protein, is an important quality attribute affecting monoclonal antibodies (MAbs). MAbs validated for use as human therapeutics have precise glycosylation patterns that must be accurately replicated for the MAb to function as intended in vivo. However, glycan formation and attachment are subject to variability and are often non-uniform. Consequently, regulatory agencies are now encouraging biopharmaceutical manufacturers to develop strategies to control glycosylation online during production. However, online glycosylation control is yet to be implemented in the biopharmaceutical industry.

Our goal is to develop—and validate experimentally—a comprehensive strategy for effective real-time, on-line control of glycosylation patterns, using a combination of multi-scale modeling, hierarchical control, and state estimation. To achieve this goal, however, it is imperative first to assess the controllability of glycosylation. Specifically, with \( x \) defined as the vector of the percentages of each glycoform present in the overall glycans pool from a batch of therapeutic protein, we must determine if protein glycosylation can be directed from any initial state \( x(0)=x_0 \) to any arbitrarily specified desired final state \( x_f \), in finite time, via admissible manipulations of available process variables and operating conditions. Such a controllability analysis allows one to determine the degree to which glycosylation can be controlled and provides a theoretical basis for determining the best achievable control possible. Once the achievable degree of glycosylation control has been determined, a real-time control strategy can be developed to achieve the identified best possible control on-line. Such an on-line glycosylation control strategy requires first the successful establishment of base regulatory control for the key process variables known to affect glycosylation, such as glucose and glutamine media concentrations, reactor temperature, dissolved oxygen (DO), pH, and agitation rate. Subsequently, the relationship between these process variables and the glycan distribution must be quantified in the form of appropriate process models that can be used for control system design and for on-line estimation of glycan distribution from infrequent measurements. Finally, glycosylation assays for determining actual glycan distributions during MAb production must be developed and integrated with the other components to complete the glycosylation control system.

In this presentation, we will discuss first a method for determining the controllability of glycosylation and demonstrate how this method, which is predicated upon employing statistical design of experiments to carry out glycosylation model simulations systematically in order to obtain a glycosylation process gain matrix, was used to determine the conditions under which glycosylation is controllable. We will then discuss the development of a novel bioreactor system equipped with an OPC interface that has made possible the implementation of on-line feedback control of glucose and glutamine concentrations. The challenges associated with nutrient control, such as inherent process nonlinearity, batch-to-batch variability, and measurement noise have been systematically addressed through the implementation of a PID controller with data filtering and gain scheduling. Experimental results demonstrating the bioreactor system’s ability to maintain desired nutrient set-points with this control strategy will be presented. In addition, progress to date on the development of an at-line assay for glycosylation macro-heterogeneity will be presented along with preliminary results from a simple proof-of-concept glycosylation controller.
THE CHANGING DIELECTRIC PROPERTIES OF CHO CELLS CAN BE USED TO DETERMINE EARLY APOPTOTIC EVENTS IN A BIOPROCESS

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Cell density and viability are two important parameters that are monitored during a mammalian cell bioprocess. The predominant method used to determine the viability of a cell population is trypan blue exclusion. This marks the loss of viability by the inability of cells to exclude the dye as a result of membrane damage. However, the loss in cell membrane integrity can be regarded as a late-stage event in the demise of cells and often marks the end of apoptosis. Early indicators of apoptosis are desirable in cell bioprocesses because the initial stages are reversible by appropriate intervention such as nutrient feeding.

In our study five different and independent methods were compared for the determination of viable and/or total cell density as well as cell viability. These included the particle counter (Coulter Counter), image analyser (CEDEX with trypan blue exclusion), an on-line capacitance probe (Aber-Instruments), an off-line flow cytometer (Guava Millipore) and a prototype dielectrophoretic (DEP) cytometer. The multiple techniques of cell monitoring were applied to the culture of a novel CHO cell line producing a human-llama chimeric antibody (EG2) grown in a bench-top bioreactor. This is a unique study which determined the relationship between these techniques and may be applied to elucidate some important properties of cells during metabolic changes in a bioprocess.

Our results show that the various on- and off-line techniques gave similar values during the exponential growth phase for cell densities but cell viability measurements diverged at the point of highest cell density. This divergence was investigated further by using a flow cytometer (Guava Millipore) to measure the process of cell apoptosis through the use of fluorescent cell labelling kits that measure enzymic and structural changes during early, mid, and late apoptosis. This includes a measure of phosphatidylserine translocation (early apoptosis), caspase activation (mid-apoptosis) and DNA laddering (late apoptosis). These stages were correlated with changes in the electrical properties of the cell as measured by a capacitance probe using a 25-point-frequency scan within the radio-frequency range (0.1 to 20 MHz). Further, dielectric properties were measured by a novel prototype instrument for single cell dielectric spectroscopy (dielectrophoretic cytometer) designed by the Thomson-Bridges group at the Department of Electrical and Computer Engineering at the University of Manitoba.

Dielectrophoretic (DEP) cytometry offers a novel and unique technology for analyzing populations of mammalian cells by their changing electric properties. The dielectric properties of the cells can be related both to their source of origin and metabolic status. For example, the cell cycle changes in the membrane composition and ionic content of cells are both known to influence their dielectric response. Through this new technique we have established that the trajectory of viable CHO cells in a narrow bore capillary and subjected to a radionfrequency actuator can be distinguished from nonviable cells by the change in the cellular dielectric polarization. This is an extremely important observation because it lays open the possibility of monitoring the incremental changes that occur during cell growth and death in bioreactors.

Poster Number 129
PHYTOPLANKTON EXTRACTS AS MEDIA SUPPLEMENTS SUPPORT GROWTH AND PRODUCTIVITY OF RECOMBINANT CHO CELLS

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The production of biotherapeutics such as recombinant proteins and monoclonal antibodies, require serum-free cell culture media to maintain high cell yields, high protein productivity and consistent quality. Historically, cell culture media has been enriched with animal products such as serum, and more recently with animal hydrolysates such as Primatone. The possibility of contamination of products with mycoplasma, prions and viruses has increased the pressure to eliminate animal-derived components from cell culture media, and has lead to the use of many plant and yeast hydrolysates as media supplements. In the study we have investigated extracts of marine phytoplankton as potential supplements for recombinant protein production from Chinese hamster ovary (CHO) cells. Three commercial phytoplankton preparations were tested for their effect on the growth and productivity of a CHO cell producing a marker recombinant protein (luciferase) in a base of Biogro-CHO media. One preparation had higher bioactivity for cell growth and productivity. This bioactivity was enhanced with further extractions of the slurry preparation. Supplementation of this extract into Biogro-CHO media allowed cells to be maintained over several culture passages. Treatment of the extract for 24 h at 37°C resulted in a browning reaction concomitant with a further increase in bioactivity. The bioactivity was shown to be thermally stable at varying exposures to high temperature. This novel protocol for the extraction of phytoplankton preparations produced a supplement for mammalian cell culture that resulted in a high cell yield (>2x10^6 cells/ml) and recombinant protein production equivalent to a positive control. The bioactivity has also been confirmed using another CHO cell line that produces a humanized camelid IgG. Further extraction procedures are in progress to isolate bioactive components that may be useful in the development of chemically defined media.
USE OF LIVE CELL MICROSCOPY AND IMAGE ANALYSIS TO FOLLOW THE TEMPORAL
REGULATION OF GENE EXPRESSION AND POTENTIAL APPLICATIONS TO PROTEIN
PRODUCTION IN CHO CELLS

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Introduction: Quantitative measurements of dynamic processes in single cells by live cell imaging are challenging, but present the opportunity to measure temporal patterns in gene regulation and correlations between gene expression and cell phenotype. For biomanufacturing in CHO cells, these measurements may provide new insight into how the population behaves during scale-up and production. Current population-averaged measurements do not provide information about which specific cells in a population of CHO cells survive methotrexate selection and become high expressors.

Methods: As a model system, we quantified the fluorescence intensity from individual NIH-3T3 fibroblasts stably transfected with a promoter (tenascin-C) driving a destabilized eGFP reporter. Cell images obtained during live cell imaging experiments lasting 62 hours were segmented using the phase contrast channel, and the fluorescence intensity from individual cells was quantified from individual cells throughout the cell cycle. Hundreds of individual cells were segmented and tracked both manually and by fully automated image analysis routines throughout the cell cycle during live cell imaging experiments lasting 62 hours. Using the time lapse imaging data, we developed a stochastic differential equation (SDE) that describes the fluctuations in single cell gene expression levels.

Results: The automated segmentation and tracking of cell objects in phase contrast images was validated against the results from manual segmentation. We find that individual cells vary substantially in their expression patterns over the cell cycle, but that on average this promoter activity increases during the last 40% of the cell cycle and that higher gene expression levels were correlated with shorter cell cycle times. Importantly, the time-dependent data allowed us to calculate parameters of the SDE. This model accurately predicted the results of a months-long experiment in which cells were sorted by their promoter activity, and allowed to relax back to the steady state distribution of activities.

Conclusions: Live cell imaging of cell populations with fluorescent protein reporters can be used to identify correlations between phenotypic properties and gene expression. The data also describe dynamic changes gene expression that can be used to model changes in the population with time. With regard to biomanufacturing in CHO cell populations, this information could provide useful criteria for selecting cells early in the scale up process that are likely to become high producers. Representative cell lines and experiments are currently being developed for this purpose.
A COMPARISON OF SHEAR STRESS INDUCED PLURIPOTENCY IN TWO-DIMENSIONAL AND THREE-DIMENSIONAL EMBRYONIC STEM CELL CULTURES

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Stirred suspension bioreactors provide several advantages over small-scale static cell and tissue culture vessels. These include improved oxygen transfer, scalability, reduced labour, and a well-mixed homogeneous environment. For these reasons, stirred suspension bioreactors have been studied for the expansion and differentiation of a number of stem cell types, either as single cells, as cell aggregates or on microcarriers. When scaling up from a static culture to a suspension bioreactor, murine embryonic stem cells transition from an adherent colony formation (mostly 2D) to a spherical aggregate morphology (3D). It is well known that the hydrodynamics within the bioreactor can affect the viability of the cell population, and can be used to control the size of cellular aggregates. What is less known is the effect of the shear forces on the phenotype of the cells. We have previously reported that cultures of murine ESCs in stirred suspension retain some of their pluripotent characteristics when undergoing directed differentiation to bone and cartilage lineages as aggregates in serum-containing media. Removal of serum, and protection of the aggregates from shear within macroporous microcarriers allowed the cells to differentiate and resulted in a complete loss of pluripotency markers. We hypothesized that the shear stress within the bioreactor was influencing the expression of pluripotency genes. We conducted studies exposing murine ESCs to different shear stress levels in both 2D (monolayer in parallel plate bioreactor) and 3D (cell aggregates in stirred suspension bioreactor) cultures and found a number of interesting parallels. These studies, combined with possible mass transfer limitations of oxygen and nutrients in larger aggregates, point to the need for more detailed bioengineering studies of stem cell culturing and the importance of including future clinical-scale needs in the design of bioprocesses for stem cell expansion and differentiation. In addition, the use of microcarriers (both micro and macroporous) may provide an interesting alternative combining adherent with 3D culture.
During large-scale production of a monoclonal antibody product, we observed significant reduction of the antibody’s interchain disulfide bonds. This reduction event culminated in lost product as the bulk failed to meet product quality specifications. Small-scale laboratory studies revealed that the cause of the reduction was due to mechanical shearing of the cells, which resulted in the release of cellular enzymes that in turn reduced the antibody product. To further investigate the mechanism, the cytosolic isoform of thioredoxin, TXN1, was knocked down by lentiviral-mediated RNAi to determine if inhibiting its expression and/or activity could attenuate (or prevent) antibody disulfide reduction. The results of these experiments provided the rationale to design a strategy to develop a host cell line that is devoid of antibody disulfide reduction and to further analyze how the thioredoxin pathway may be modulated during our CHO cell culture processes.
A NOVEL STRATEGY TO REDUCE BOTH LACTIC ACID AND AMMONIA PRODUCTION IN ANIMAL CELL CULTURE

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Industrially relevant animal cell cultures often produce high amounts of lactic acid even under well oxygenated conditions. Accumulation of this metabolic byproduct can undermine maximum cell growth and productivity through lactic acidosis or excessive osmolality increases in pH-controlled bioreactors. Many strategies that have been developed to reduce lactic acid production lead to an increase in ammonium production and/or are generally not considered robust enough for implementation into cGMP operations. There still exists a need to develop a strategy that restricts both lactic acid and ammonium production in a simple and robust manner.

We have recently developed a novel strategy to reduce lactic acid, as well as ammonium production, through the use of lactate-supplemented medium designed to shift the metabolic flux in favor of lactate consumption. Although it may seem ill-advised to supplement basal medium with a potentially inhibitory metabolite, we discovered that the typical negative effects on cell growth can be negated through the use of adapted cell lines. These findings were then extended toward the development of higher density, fed-batch cell cultures in pH-controlled bioreactors. Our approach successfully reduced lactic acid production and base additions by eight fold and allowed more concentrated nutrient feeds to be added without excessive increases in osmolality. We achieved viable cell densities of 35 million cells per ml, among the highest currently reported for a fed-batch animal cell culture. Furthermore, high viabilities were maintained for an extended period, resulting in an integral viable cell day of 273 million cell-days per ml, again among the highest currently reported for a fed-batch animal cell culture. Highlights from a metabolomics study will also be discussed to promote a better mechanistic understanding of this approach. Further investigation of the benefits of lactate supplementation will likely lead to improvements in culture performance far above current levels.
RAPID LARGE-SCALE PRODUCTION OF NOVEL INFLUENZA VIRUS LIKE PARTICLE VACCINES
USING THE SF9 - BACULOVIRUS EXPRESSION SYSTEM

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The World Health Organization estimates approximately forty to fifty thousand deaths occur per year within the United States alone from seasonal influenza virus. The most recent 2009 H1N1 pandemic caused nearly 14,000 deaths worldwide. The ability to respond effectively to such threats depends on manufacturing vaccines in a rapid and scalable fashion far beyond current levels. Titer production targets must be met while maintaining low production costs and ease of manufacture. Novavax’s unique manufacturing process of recombinant Virus Like Particle (VLP) vaccines using the SF9 - baculovirus expression system is a viable alternative to current low yielding egg-based production methods. A unique challenge for this expression system is reduction of specific VLP productivity at desired high cell densities. This is thought to be partially related to loss of host cell mitochondrial function upon viral infection. Understanding the differences in central carbon metabolism before and after infection may allow the design of feeding regimes to overcome this limitation. Application of bioprocess fundamentals and traditional scale-up principles toward the development of novel large-scale insect cell culture using disposable, and portable, platform technologies will also be discussed. These strategies allow trivalent vaccine supplies to be produced in a twelve week time frame without the need to culture highly pathogenic flu strains. These VLPs have shown to be immunogenic across a broad population group in phase II clinical trials.
Embryonic stem (ES) cell culture is currently a largely manual process, with major challenges to address in the methods used regarding scalability and variability. Process automation can be of great benefit to reduce operator-dependent variation, therefore improving cell yield and quality. This would be beneficial for production of defined cells for high throughput screening or definition of a robust cGMP process suitable for scaled-out production of cells for clinical application. This work describes the use of a fully automated Tecan platform for the hands-free expansion of mouse and human ES (mES and hES) cells, as well as directed neural differentiation of mES cells.

Key bioprocess variables were initially optimised to develop a Standard Operating Procedure (SOP) for the expansion and differentiation of mES. Comparisons between the manual and automated process were shown by expanding Oct-4-GiP mES cell line over eight passages followed by directed differentiation into neural precursors. Automated culture was shown to improve the consistency of cell yield up to 3-fold. Cells produced maintained their pluripotency and were able to form derivatives of all three germ layers. Using the platform’s ability to control oxygen tension, mES cells were further differentiated into neural precursors at 2% oxygen and results compared to manual differentiation at 2% and 20% oxygen. Use of the enclosed automated platform avoided changes in oxygen tension during media changes, as occurs in manual culture. A 3-fold increase was found in cells expressing βIII-tubulin at 2% oxygen (automated) compared to 20% oxygen, as well as a 16-fold increase in cells expressing MAP2 at 2% oxygen (automated) compared to 20% oxygen.

For hES culture, standard mechanical passaging of cells can introduce variability during a process and furthermore is not suitable for the automated microwell platform. Two new cell lines were derived from mechanically passaged hES cells by adaptation to TrypLE Express™, resulting in a karyotypically abnormal (Shef-3) and normal (Shef-6) cell line. Optimum growth conditions for each line were evaluated. Design of Experiments (DoE) was used to evaluate the effect of feeder cell and hES inoculation cell density (ICD). Results indicated that both TrypLE-adapted lines were capable of growing on a feeder layer ICD as low as 3,125 cells.cm⁻². hES ICDs between the two lines vary greatly, with that of the Shef-6 line almost double.

An SOP for passaging of Shef-3 cells was established and optimised. The dissociation step was found to be critical in maintaining cell yield over multiple passages on the platform. This step was improved by using a non gelatin-coated tissue culture surface and increasing the dissociation time to 30 min. Cells were passaged over 5 consecutive passages. Cell yield remained stable at approximately 2 x 10⁵ cells.well⁻¹ and cell viability did not drop below 98%. Cells were shown to express pluripotency marker Oct-4 throughout all 5 passages. Pluripotency was further confirmed by high expression of SSEA-4 (98% and 98%) and TRA-1-60 (95% and 82%) before and after processing respectively. Future work will involve passaging of the karyotypically normal Shef-6 line on the automated microwell platform.
THE MAMMALIAN UPR COMPONENTS ATF6 AND ERSE CAN BE USED TOGETHER TO ENHANCE PRODUCTION OF ‘DIFFICULT TO EXPRESS’ PROTEINS

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In this study we use two components of the mammalian unfolded protein response (UPR) (ATF6 – activating-transcription factor 6, and ERSE – ER stress response elements) to increase the specific productivity of CHO cells transiently expressing a recombinant protein.

UPR components such as XBP1 have already been used for this purpose but with mixed results. Results differed due to the difficulty of expressing some recombinant proteins and the cell culture platform used.

Our engineering strategy differs from studies utilizing XBP1 as we aim to improve recombinant protein production via a combination of two mechanisms.

1.) Over-expression of ATF6, inducing expression of endogenous UPR target genes important in protein processing and improving the protein synthesis capacity of the cell.

2.) ERSE inserted upstream of the promoter in recombinant protein production vectors and co-expression of their binding/activating factor, ATF6, leading to increased transcription from the production vector.

When inserted in front of an SV40 promoter, ERSE and co-expression of ATF6 increased the amount of SEAP (secreted alkaline phosphatase) mRNA and protein compared to controls.

We predict that our system will help in the expression of so called ‘difficult to express‘ proteins which suffer processing problems when moving through the protein synthesis machinery of a cell.
In an industrial antibody production fed-batch process, cell metabolism can change considerably over the production time course. We have performed 13C labeling experiments and metabolic flux analysis (MFA) to characterize cell metabolism throughout four separate phases of an industrial fed-batch production process. First, in spite of the fact that lactate has long been viewed as a wasteful byproduct of metabolism, this study found that high lactate flux corresponded with peak specific growth rates. In addition, during peak growth stages there was minimal TCA cycling as the culture was primarily glycolytic. Conversely, we found that as the culture transitioned from peak growth to peak antibody production, lactate production completely reversed. Instead, energy was primarily generated through a highly oxidative state of metabolism, which coincided with peak antibody production. Interestingly, as TCA cycling and antibody production reached their peak, specific growth rate continued to diminish and the culture entered stationary phase. In spite of the culture entering stationary phase, high TCA cycling remained even when viable cell density had begun to decline. Using MFA, we found that a highly oxidative state of metabolism corresponds with peak antibody production, whereas peak cell growth is characterized by a highly glycolytic metabolic state.
ANALYSIS OF THE SECRETOME OF CHINESE HAMSTER OVARY (CHO) CELLS

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Objective:
Chinese hamster ovary cells are the most commonly used mammalian host cell line for the production of protein therapeutics. The cell secretome represents an important source of information as cells secrete various proteins including growth factors and proteolytic enzymes into the medium that can affect the cell growth and product quality of the culture. Identification of these secreted host cell proteins will help both in identifying or creating appropriate production cell lines and in improving process performance.

Results:
The proteins secreted by a highly viable CHO cell culture (viability >98%) into the protein-free medium were analysed using 2D-PAGE and shotgun proteomics. A total of 138 spots were picked from coomassie stained 2D-PAGE gel, out of which 108 spots were identified using MALDI-ToF/ToF mass spectrometry and nano LC MS/MS. Many of the identified proteins are described in the literature to be involved in regulation of a variety of cellular phenotypes such as growth, apoptosis and protein degradation. For example, S100A13, a secretory protein, is involved in the regulation of cell cycle progression and secretion of other proteins such as fibroblast growth factors and chemokines. Similarly, Nucleobindin 1 (NUCB1), which is a downstream target for caspase-mediated cleavage during apoptosis was also identified in this study. Both proteins known to be secreted and proteins that are expected to be intracellular were found. Thus, proteins in the culture supernatant of CHO cells are a mixture of actively secreted proteins and proteins released from a small number of dying cells. Additional work on analysis of proteins by shotgun proteomics is currently in progress and will be presented.
The proteins identified are involved in process relevant cellular phenotypes such as growth, apoptosis and protein degradation, thus promise to be important markers for cell line behaviour and process state. A number of proteins were also identified at multiple locations on the gel, suggesting either degradation of the protein or varying post-translational modifications. More detailed analysis of changes in the secretome during batch or fedbatch culture will allow identification of potential markers for process control as well as of targets for engineering (such as knock-out of secreted proteases or glucosidases).
CAP: A PROTEIN AND VACCINE PRODUCTION PLATFORM BASED ON IMMORTALIZED HUMAN AMNIOCYTES

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Human CAP (CEVEC’s Amniocyte Production) cells allow for stable and high yield production of recombinant proteins, with excellent biologic activity and therapeutic efficacy, as a result of authentic posttranslational modification. Based on CAP cells a transient expression system has been developed, that enables extremely high production yields of recombinant proteins within a few days. Thus, CAP and CAP-T technologies offer the use of only one platform for early preclinical development through to clinical supply of recombinant biotherapeutics. Additionally, various human-pathogenic viruses could be propagated successfully on CAP cells, which underlines the potential of the cells as a broad production host for vaccine development. This talk will highlight recent progress in terms of producing various difficult to express proteins, antibodies and also viruses on the CAP technology platform.
CONTROLLING HIGH MANNOSE GLYCAN LEVEL AND OPTIMIZING TITER THROUGH A BALANCED MODULATION OF CELL CULTURE PROCESS AND MEDIUM CHANGES

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High mannose glycoform may be an important quality attribute that can affect the efficacy of therapeutic monoclonal antibodies. Meeting comparable high mannose glycan level (%HM) is often important when developing a commercial process to replace the early phase clinical process. Strategies were developed to tune the %HM in order to meet the comparability range while at the same time increasing titer for the commercial process. The strategies utilized a combination of process optimization and medium modification in order to produce the desirable %HM profile. In several process instances, titer had to be sacrificed in order to meet the desirable %HM level; however, this was overcome by modulation of certain medium components. Yet, precise balance between process and medium needed to be made in order to achieve the most desirable state for both titer and %HM. Overall strategies and learning on taking this dual approach to controlling the %HM while improving titer will be discussed.
CONTROL OF POLYPLEX MEDIATED TRANSFECTION OF CHO CELLS

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Transient gene expression is a rapid and inexpensive means to supply recombinant proteins for early stage testing. Over the last few years, scalable transient platforms have become increasingly productive, with reported titers surpassing 1 g L\(^{-1}\). However, as for stable production systems, high transient production is usually the result of process variable optimization (e.g. medium, feed, temperature), in fact very little is known about control of transient production at the sub-cellular level. In this project we dissect the molecular interactions and cellular processes that underpin polyethylenimine (PEI) mediated transfection of CHO cells to enable knowledge-based screening or cell-engineering strategies to improve CHO cell based transient production systems. All experiments described utilized CHO-S cells cultured in CD-CHO medium at 37°C, transfected with polyplexes composed of plasmid DNA condensed to linear 25 kDa PEI using a protocol previously optimized by Design of Experiments methodology.

Analysis of the transfection process by flow cytometry using fluoro-labeled polyplexes revealed that polyplex binding to the cell surface and subsequent intracellular internalization were both rapid, saturable (first-order) processes associated with cell surface heparan sulfate proteoglycans (HSPG). Polyplex internalization was effectively complete within one hour – representing less than 25% of total cell surface polyplex. During this transfection phase, the rate of polyplex internalization correlated with the rate at which cell surface HSPG levels declined (by over 80%). The cellular mechanism of polyplex uptake was investigated using chemical inhibitors of discrete endocytotic pathways. Both rottlerin (an inhibitor of fluid phase endocytosis via macropinocytosis) and methyl-\(\beta\)-cyclodextrin (which sequesters plasma membrane cholesterol) dramatically decreased reporter protein production in a dose dependent manner. Taken together, we suggest that lipid-raft associated HSPGs are clustered via polyplex binding, promoting non-specific endocytosis by macropinocytosis, itself lipid-raft dependent.

Lastly, an in-house library of clonally derived sub-populations of a CHO-S parent was used to investigate cellular mechanisms underpinning cell line specific variation in PEI-mediated transfection. CHO-S clones heritably varying in reporter production were identified, which exhibited differences in cell surface HSPG content and fluoro-polyplex binding and uptake. These studies identify the major cellular constraints on polyplex-mediated transfection and indicate that it is possible (as for stable transfectants) to utilize specific, high-throughput screening assays to identify variant CHO cells that are inherently suited to transient production.
Recombinant mammalian cell lines typically exhibit reduced growth and higher rates of nutrient utilization compared to parental cells, presumably to meet the increased demand for energy and precursors needed for heterologous protein synthesis, folding, modification and secretion. In order to quantitatively assess the impact of recombinant protein expression on the primary metabolism of CHO cells in culture, we have employed an efficient inducible expression system (named the 'cumate gene-switch') and performed an extensive metabolic characterization of the on and off states.

To this end, a comparative 13C-metabolic flux analysis was conducted, whereby cells were grown in parallel semi-continuous cultures containing various labeled glucose and glutamine tracers and the resulting mass isotopomer distributions of extracellular metabolites (three secreted amino acids and lactate) were measured by LC-QTOF-MS. This approach allowed us to obtain reliable estimates for the main intracellular fluxes, including pathways that cannot be observed from external rate measurements (e.g. the pentose phosphate pathway).

Upon addition of a non-toxic concentration of cumate and under mild-hypothermic conditions (30°C), the cell specific productivity was 23 pg/cell.d, corresponding to an on/off induction ratio of approximately 20. From this measured cellular productivity, it was estimated that recombinant proteins accounted for at most 15% of the total cellular protein mass. Accordingly, our study revealed that recombinant protein expression is correlated with small but significant variations in a number of key intracellular pathways related to ATP and NADPH formation, including the pentose phosphate pathway, the malic enzyme reaction and the TCA cycle. When expressing the recombinant antibody, the cells notably exhibited a more efficient utilization of glucose, characterized by a higher fraction of pyruvate entering the TCA cycle. Conversely, the catabolic rates of most amino acids, including glutamine, remained unaffected by the onset of protein expression.

Elucidating the alterations in central carbon metabolism caused by protein production is instrumental for the establishment and optimization of a productive mammalian cell expression platform. Such analysis can help guide the identification of robust biomarkers of productivity, the selection of a proper induction time, as well as rationalize the development of improved medium formulations and feeding strategies for biphasic processes.
EVALUATION OF CELL METABOLISM AS A HIGH THROUGHPUT INDICATOR OF THE IMPACT OF MEDIUM COMPONENTS ON AUTOLOGOUS CELLULAR IMMUNOTHERAPY PRODUCT ATTRIBUTES

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In 2010 Provenge® (sipuleucel-T), an autologous cellular immunotherapy (ACI), was approved by the FDA for the treatment of asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer. Sipuleucel-T consists of a patient’s own peripheral blood mononuclear cells (PBMC) collected by leukapheresis and cultured in the presence of a recombinant human fusion protein consisting of prostatic acid phosphatase, a prostate-specific antigen, and granulocyte-macrophage colony stimulating factor, an immune cell activator. The resulting cellular product contains antigen presenting cells (APC’s) that are infused back into the patient 3 days later.

Ex vivo cell culture is a key unit operation in the manufacturing of ACI; cell culture conditions can strongly influence APC activity. An investigation of the effects of cell culture medium components on process performance and robustness was undertaken. Given the high variability among healthy human donors, a scale-down model was developed to allow the screening of multiple culture conditions using PBMC from a single donor. In order to support increasing sample numbers, higher throughput assays are required. APC activity has traditionally been assessed in cell-based antigen presentation assays using HLA-restricted responder cell lines. Assay complexity limits throughput while HLA specificity restricts the size of the donor pool. It was hypothesized that the metabolic state of the cells could be used as a predictor of APC activity. A metabolic flow cytometry-based approach using C12-resazurin was investigated as an early high throughput development tool for cell culture medium optimization. Within the cell, C12-resazurin is reduced into fluorescent C12-resorufin which can be readily detected by flow cytometry. The C12-resazurin assay was combined with immunophenotyping, allowing the assessment of the impact of different medium formulations on specific cell populations including monocytes, B cells, and T cells. Within a common medium base and initial culture parameters, cell culture supplement combinations leading to increased APC metabolism were found to be a good predictor of APC activity measured by the low throughput cell-based bioassay. This method can be performed in less than 3 hours and is not limited by HLA haplotype-restrictions, thus allowing for near real-time results and access to a large eligible donor pool. This metabolic approach will facilitate the high throughput screening of media formulations and selection of promising candidates for additional APC activity analysis. While not a replacement for the APC-responder assay, this metabolic strategy has the potential to accelerate cell culture medium development for autologous cellular immunotherapies.
PERFUSION BIOREACTOR CULTURE OF HUMAN LIVER CELL SPHEROIDS FOR REPEATED-DOSE LONG-TERM DRUG TESTING

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Bringing a new drug to the market costs 900 million USD and takes 12 years, on average. Thus, failures in the drug development process impair significant costs to the pharmaceutical companies; it has been estimated that the probability of a given candidate drug to progress from phase II to phase III clinical trials was about 28% from 2006 to 2007 and 18% in the period from 2008-2009. More than 20% of the failures in phase II clinical trials were due to unpredicted toxicological effects; thus, a more efficient assessment of repeated dose, long-term drug metabolism-related toxicology, during the pre-clinical phase, would allow a more efficient selection of the lead drug candidate for the clinical trials phase. Since the liver is the main site for drug metabolization, primary cultures of human hepatocyte spheroids are a promising in vitro model for long term studies of drug metabolism and cytotoxicity. The lack of robust methodologies to culture cell spheroids, as well as a poor characterization of human hepatocyte spheroid architecture and liver-specific functionality have hampered a widespread adoption of this three-dimensional culture format. In this work, an automated perfusion bioreactor was used to obtain human hepatocyte spheroids with an average diameter of 80 μm within 3 days. These spheroids were further cultured for 3-4 weeks in serum-free conditions, sustaining their phase I enzyme expression and permitting repeated induction during long culture times; rate of albumin and urea synthesis, as well as phase I and II drug-metabolizing enzyme gene expression and activity of spheroid hepatocyte cultures, presented reproducible profiles, despite basal inter-donor variability (n=3 donors). Immunofluorescence microscopy of human hepatocyte spheroids after 3-4 weeks of long-term culture confirmed the presence of the liver-specific markers, hepatocyte nuclear factor 4α, albumin, cytokeratin 18, and cytochrome P450 3A. Moreover, immunostaining of the atypical protein kinase C apical marker, as well as the excretion of a fluorescent dye, evidenced that these spheroids resume a hepatic functional bile canaliculi network, extending from the surface to the interior of the spheroids, after 3-4 weeks of culture. In conclusion, perfusion bioreactor cultures of primary human hepatocyte spheroids maintain liver-specific activity and architecture and are thus suitable for drug testing in a repeated-dose, long term format. These cultures can be implemented as toxicological assessment platforms, contributing to increase the efficiency of the pre-clinical phase and accelerate the whole drug discovery process.
ENGINEERING THE ENERGY METABOLISM AND LACTATE PRODUCTION IN MAMMALIAN CELLS PRODUCING COMPLEX BIOPHARMACEUTICALS: DOWN-REGULATION OF THE WARBURG EFFECT

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Most mammalian cell lines used in the production of biopharmaceuticals present high glucose consumption rates accumulating significant amounts of lactate: the Warburg Effect (WE). While the WE seems to offer a proliferative advantage for in vitro cell survival, it is highly undesirable from the biotechnology viewpoint, as the accumulation of lactate reduces culture viability, shortens maximum production phase and significantly impacts final product titers and quality. Additionally, the reduced channeling of glycolytic intermediates into the tricarboxylic acid cycle (TCA) might decrease the availability of reducing power and biosynthetic building blocks crucial for a high productivity expression system. In this work, we present a proof-of-concept metabolic engineering study based on the down-regulation of two key molecular effectors of the WE, hypoxia inducible factor 1 (HIF1) and pyruvate dehydrogenase kinase 1 and 3 (PDK1/3), simultaneously entangling reduced lactate accumulation and enhanced channeling of glycolytic intermediates into the TCA. The effect of this down-regulation was evaluated in HEK 293 cells producing recombinant retroviruses, E1 transcomplementing MDCK cells for the production of canine adenovirus and telomerase immortalized RPE cells stably expressing monoclonal antibody.

The silencing of HIF1 in RPE cells reverted telomerase immortalization yielding senescent cells without proliferative capacity, further compromising additional evaluation of productivity improvements. In HEK 293 retrovirus producers the LDH activity was found to be decreased by 60% in the silenced clones. Glucose uptake and lactate production rates were also reduced by more than 2 fold. More importantly, a drastic increase in virus productivity was achieved, with 18 fold increase in total particles (T.P.) and 22 fold in infectious particles (I.P.) production, highlighting the improvement of the culture productive potential not only in quantity but also in quality (evaluated by the P.I. to P.T. ratio). Further silencing of PDK1/3 potentiated the effects of HIF1 down-regulation and resulted in an additional 3 fold improvement of the viral preparation quality. For MDCK E1 cells the effects were modest compared to those of HEK 293, although a 2 fold increase in infectious particles productivity was also obtained. This effect might be derived from a sub-optimal performance of the human U6 shRNA promoter in this cell line due to its canine origin. We are currently evaluating the metabolic fingerprints associated to these manipulations and characterizing the flux distribution of central carbon metabolism in silenced cells. A second retrovirus producer cell line is also being tested for further validation. These results highlight the manipulation of Warburg Effect as a potential metabolic engineering tool for the improvement of complex biopharmaceuticals production in cultured mammalian cell lines.
IMPLEMENTATION AND PERFORMANCE OF A HIGH-THROUGHPUT CELL CULTURE SYSTEM FOR PROCESS DEVELOPMENT

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Most process development efforts in cell culture including clone screening, media development and process optimization are currently performed at various scales of shaker flasks and controlled bioreactors. These efforts require significant resources, both equipment and personnel. In addition, the above listed cell culture systems allow only for low (bioreactor) to medium (shaker flasks) experimental throughput.

In an attempt to overcome these limitations, we have developed and implemented a novel high-throughput system for cell culture (HTS-CC) to improve the efficiency of cell culture process development. Our automated system is based on commercially available as well as newly developed equipment, and is used as a complementary tool to our existing shake flask and bioreactor culture systems.

A Biomek™ FX high-throughput liquid handling system is used for media preparation and dispensing, inoculation of cells and sampling. We currently employ shaken conical 50-mL SeptaVent™ tubes as the culture system of choice. High-throughput measuring technologies were developed that allow us to monitor cell growth and viability, pH, glucose, lactate, ammonia, osmolality, and product concentration. The assay results are integrated with the Biomek™ FX liquid handling system, enabling automatic osmolality and pH adjustment during media preparation, and pH and glucose adjustments during a cell culture run. This system allows us to increase our experimental throughput capability up to 10-fold compared to the typically used shake flasks (up to 400 cultures in parallel). The performance of the various analytics will be discussed in depth. The fed-back control of pH in particular has improved the comparability of the system to bioreactors when compared to shake flasks without control.

The system is routinely used to rapidly produce candidate proteins for evaluation in early development. It is also used for clone screening, media development, and process optimization. Case studies of these applications will be presented.
The application of systems biology tools – for instance to analyze intracellular flux patterns, metabolic control regimes or to identify regulatory circuits - is of utmost importance for the knowledge-based optimization of industrial bioprocesses using "simply structured" microbial (e.g. prokaryotic) cells. Based on these results optimization strategies for metabolic engineering and process engineering can be derived successfully.

In contrast, the application of the same tools for highly compartmented mammalian cells is somewhat hampered by the basic need for compartment-specific application of the same to achieve valuable results. Metabolic and regulatory network structures and metabolome patterns may differ significantly when e.g. mitochondrial and cytoplasmic conditions are compared. Consequently successful systems biology approaches are requested to carefully consider the differences for properly analyzing realistic scenarios of cellular states. In accordance with this goal, the contribution presents first steps for the quantification of the number of mitochondria in antibody producing CHO cells as the basis for ongoing systems biology studies.

An experimental approach has been developed that consists of the optimal permeabilization of CHO cells such that antibodies against the translocase of outer mitochondria membrane 22 (TOM 22) can bind with the related mitochondria. Further binding of a second isotype-specific antibody carrying the fluorophore phycoerythrin (PE) allows to determine fluorescence intensity as a signal for translocase presence. The later can be interpreted as the number of mitochondria per cells.

Results are presented indicating that this approach is highly suitable to monitor the cellular response after cells are exposed to osmotic stress. Biological replicates of 0.7-L cultivations analyzing stressed and non-stressed cells allow the conclusion that osmotically stressed CHO cells differ significantly from non-stressed ones with respect to the fluorescence signal obtained. Flow cytometric studies with propidium iodide (PI) stained cells showed that osmotically stressed cells were arrested in G1-phase. In accordance with previous findings G1-arrest and the reduced fluorescence intensity give rise to the hypothesis that osmotically stressed cells contain less mitochondria.

The experimental approach presented serves as a basis for further metabolic flux studies and metabolome analysis which both follow the strategy to separately analyze mitochondria and cellular cytoplasm.
RESOLVING PROCESS VARIABILITY WITH AN INCREASED UNDERSTANDING OF CELL METABOLISM

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We have developed a robust, scalable process platform using chemically defined medium that delivers over 3 g/L in monoclonal antibody concentration. Recently, large variations in metabolism were observed at extended cultivation times when applied to an antibody program. This variability is undesirable and limits process options. We investigated the causes of the metabolic variability and have identified several triggers which ultimately result in variation in metabolism. We have used lactate production rate as a marker of the metabolic variability as it demonstrates this metabolic variability most effectively. By implementing an intracellular fluorescent staining protocol, a likely mechanism inducing the variability was elucidated. Risk mitigation techniques were implemented taking direct advantage of the mechanistic knowledge, and these mitigation steps will also be discussed.
Poster Number 150

EXCHANGE FLOW AND CELL LATERAL MIGRATION IN ROTATING CYLINDRICAL FILTERS FOR ANIMAL CELL PERFUSION CULTURE: A CFD STUDY

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A Computational Fluids Dynamics model to calculate the particle dynamics and the fluid flow on a Rotating Cylindrical Filter, for animal cell perfusion application, was developed. In spite of the fully turbulent regime and the high permeability on these devices, the presence of counterrotating toroidal eddies typical of Taylor-Couette system was detected. The model predicts the occurrence of a radial flow, at the filter surface, on both directions consistent with the exchange flow described in the literature for internal spin-filters. The presence of Taylor vortices combined with the intrinsic high permeability of these filters provoke such an exchange flow that achieves values as high as 100 times the perfusion flow. Lateral particle migration was also predicted. Particle migration is responsible for a 10 % particle concentration reduction near the filter wall. Particle lateral migration and exchange flow are highly influenced by the filter rotation rate.
THE USE OF EXISTING ANIMAL CELL CULTURE FACILITIES TO MAKE INSECT CELL CULTURE EXPRESSED INFLUENZA VACCINE

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Traditional approaches to making vaccines are still used such as growth of attenuated virus in chicken eggs or on human cell lines such as MRC5 cells. Increasingly there is interest in a recombinant protein approach and an example described here is for an insect cell line combined with a baculovirus expression system. Recent examples of licensed products using insect cell culture include CERVARIX, a recently licensed HPV vaccine and also the protein antigen component of PROVENGE. The general challenge for making a vaccine antigen is to present the protein in such a way that it is immunogenic.

The challenge of growing insect cell culture in suspension is very similar to that for suspension adapted CHO cells. The cells can be propagated in large stirred tank bioreactors and protection by Pluronic F68 allows the use of oxygen or air sparging. Methods of optimization, such as the introduction of nutrient feeding, result in substantial productivity benefits with the added challenge of optimizing infection times and multiplicity of infection (MOI) as well as rate of flow of nutrients.

An example will be given here of such an approach applied to an HA protein antigen based influenza vaccine made by insect cell culture that is currently being reviewed for licensure in the USA (FluBlok) by CBER. Illustration will also be made of important differences to animal cell culture because the Insect cell line remains constant from product to product. A very rapid generation of the baculovirus expression system is required as the influenza vaccine component may change from year to year. A unique manufacturing approach will be described that allows for the timely response to frequent changes in product composition.

An important option to consider for the manufacture of FluBlok is the use of existing mammalian cell culture based manufacturing facilities such as those used for making antibodies. There is in fact a relatively good fit and this will be described along with minor differences and modifications that would be required. This flexibility could be invaluable in the event of a future pandemic ‘flu emergency.
The presentation provides an overview of our strategy regarding the implementation of the QbD (Quality by design) approach in upstream processing of therapeutic proteins. This approach consists of the identification of critical process parameters (CPPs) that have a statistically significant influence on the critical quality attributes (CQAs) of a specific process. By applying the acceptance criteria to the CQAs, proven acceptable ranges (PARs) for the critical process parameters can be deduced from experimental data. The multidimensional combination of these ranges form the design space and thus assures the quality of the product.

The QbD approach according to Q8, Q9 and Q10 may be subdivided in scale down model qualification, risk analysis, process characterization and range studies. The foundation of the QbD approach is represented by the scale down model. Several different scale down criteria were applied and adapted until a satisfactory match of scale down to commercial scale data was achieved. The scale down model is then used to investigate cause effect relationships between process parameters and quality attributes of the production process. Since a standard cell culture process from thawing of the vial up to the final production fermenter can comprise up to 100 process parameters, a risk based approach is helpful to filter the most important ones. Those parameters are then experimentally investigated to verify their criticality for the quality attributes of the process. This approach relies on design of experiment (DoE) to reduce the number of required experiments to a manageable number while maintaining meaningful results. During the range studies, those critical parameters will be investigated with the help of a high resolution DoE matrix so as to be able to reveal possible interactions and higher order effects.

The presentation will cover the above mentioned steps. Case study data will be shown and the benefit for future processes will be discussed.
A model antibody, expressed under various cell culture conditions in both a CHO DG44 cell line and a CHO-S cell line, was used to study the effects of process conditions on aggregate levels and to identify the level of free light chain expression as a possible indicator of product aggregation levels in expressed monoclonal antibodies (mAb). Light chain plays a significant role in the proper folding of intact antibody and facilitates release of the protein from the endoplasmic reticulum. Therefore it is possible that the expression level of free light chain in excess of intact antibody is an indicator of the status of cellular expression machinery. Furthermore, the state of the free light chain’s unpaired cysteine may be in indicator related to either expression or intracellular redox environment. To investigate these relationships a series of analytical methods were used to quantify free light chain expression levels in cell culture media, determine the structural status of the free light chain and assess the aggregation levels of the mAb. Size exclusion chromatography (SEC) was used to assess the protein aggregate levels in cell culture media. The high molecular weight peak from SEC was subjected to SDS-PAGE analysis confirming that this fraction contained mAb aggregates and low levels of host cell proteins. In addition, reverse-phase chromatography with on-line mass spectrometry (RP-HPLC-MS) was used to quantify the level of free light chain and to characterize the different free light chain variants (free light chain, light chain dimer, and light chain capped with either glutathione or cysteine).

By analyzing samples from different cell lines under identical culture conditions, as well as a single clone under varying culture conditions, we were able to establish a correlation between free light chain expression levels and protein quality. The harvest viability was found to be critical in managing aggregation levels and it was observed that allowing the harvest viability to drop below 80% caused the aggregation levels to increase to unacceptable levels. When the ratio of free light chain to mAb was plotted, the data could be approximated by two linear regions which intersect at the experimentally determined harvest viability.

Though harvest viability was found to be important in reducing the aggregation levels, harvest viability itself could not account for all of the aggregate level reduction. A temperature shift that was performed during one process was found to significantly reduce the level of aggregated antibody. Free light chain was tracked over the course of multiple bioreactor runs. A correlation was identified between free light chain concentration and aggregate levels that held across multiple cell lines and process conditions.
ANALYSIS OF THE ACTIVATION STATUS OF THE PI3K/AKT AND RAS/MAPK SIGNALLING PATHWAYS AND THEIR ROLES IN THE SERUM-FREE, SUSPENSION ADAPTATION OF CHO CELLS

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Industrial fed-batch culture of CHO cell lines for recombinant protein production utilises the ability of engineered CHO cells to grow in suspension in a synthetic environment free of many of the extracellular signals (e.g. growth factors and cell-to-cell contacts) that control growth \textit{in vivo}. This functional capability derives from extensive cell culture experimentation and adaptation to generate production cell lines. In this project we aim to develop an understanding of the underlying signalling pathway changes that facilitate cell adaptation to suspension growth in the synthetic growth environment.

Here we describe an initial study involving the growth characterisation of a parental adherent CHO cell line and a directly adapted suspension CHO cell line in different growth environments. Differences in the PI3K/Akt and Ras/MAPK signalling pathway activation status between the adherent and suspension phenotypes, were comparatively mapped by western blotting and immunoprecipitation/immunoblot analysis of various proteins with regions rich in serine and/or tyrosine phosphorylated residues. Confocal microscopy and flow cytometry were used to deduce how these signalling pathways interact with specific cell surface membrane transducers such as integrins and receptor tyrosine kinases (RTKs).

We have shown that a shift in signalling occurs when cells are taken from an adherent growth environment to a suspension growth environment. In adherent cells, integrin-mediated attachment to the extracellular matrix stimulates an up-regulation in signalling flux via the Ras/MAPK pathway. When integrin-mediated attachment is abolished, as in suspension cells, a shift in signalling flux is seen towards the PI3K/Akt pathway. Further experimental data using chemical inhibitors against specific signalling intermediates such as PI3K, Akt and MEK 1/2 suggests that activation of these signalling intermediates is vital for cell survival and proliferation in differing synthetic environments and therefore they are viable targets for genetic engineering strategies used to functionally substitute for extracellular signals.

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ADVANCE MULTIVARIATE MODELING: A COMPREHENSIVE TOOL FOR IGG PROCES DEVELOPMENT AND MANUFACTURING ACTIVITIES.

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Advance Multivariate Data Analysis (AdMVDA) is an important statistical Modeling tool to analyze complex data from (batch) processes from QbD Activities in Development throughout the manufacturing lifecycle for IgG production according to ICH/FDA. In this poster we present our approach in the Qualification of a small scale model representative for large scale.
In the current biopharmaceutical production landscape, substantially increased process development costs coupled with competitive pressure necessitate that new candidate molecules be advanced to the clinical proof of concept stage in the shortest possible time and at the lowest possible cost. Single use bioreactors have the potential to reduce both cost and time lines given their advantages over conventional stainless steel bioreactors which include substantially lower capital investment costs, rapid turn-around time between campaigns, and the elimination of cleaning validating studies during GMP production. In this study, we present data from the production of an Fc-fusion protein using a recombinant CHO cell line in a fed-batch process. Preliminary process development studies were performed in 5 and 10L glass/stainless steel bioreactors while the preclinical and clinical material producing campaigns were 100% disposable from vial thaw until the final bioreactor step. The working volume of the disposable bioreactor for the preclinical campaign was 200L while that for the phase I clinical campaign was 1000L. In addition, the 200L bioreactor served as the seed reactor for the clinical campaign. Multiple cell culture attributes were monitored over the course of the development, preclinical, and clinical campaigns and a comparison of these data sets will be presented. Overall, results from multiple experiments suggested a high degree of consistency between cell physiology and protein productivity attributes across the 5 – 1000L bioreactor scales. This similarity in performance was also associated with substantially reduced manufacturing time and overall cost and resulted in product with the desired quality attributes. Overall, our results validate the concept that disposable bioreactors can play a pivotal role in accelerating the advancement of a candidate molecule to human clinical trials with very favorable cost and time line attributes.
EVALUATION OF LONG-TERM CRYOBAg STORAGE OF MAMMALIAN CELLS FOR DIRECT BIOREACTOR INOCULATION

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The prevalent method of seed train expansion in commercial mammalian cell culture starts with thawing cryopreserved vials followed by shake flask cell culture. Subsequent cell growth in shake flasks over the course of several days results in adequate cell number for bioreactor inoculation. While robust, this approach involves open processing and also extends the manufacturing process. We have demonstrated in the past that these limitations can be overcome by cryopreserving a higher volume of cells in bags thereby allowing direct inoculation of a bioreactor. In this study, results from direct bioreactor inoculation using cryobags stored over a 9 year period are presented. Specifically, 50mL cryo-bags were prepared directly from a 15 L high cell density perfusion bioreactor. These 50 mL cryo-bags were then used at distinct time points over a 9 year period to directly inoculate a specially designed small bottom 12 L perfusion bioreactor to initiate the seed train expansion. A total of 9 cultures were evaluated during this time span. Bioreactor evaluation of these 9 cultures included the characterization of important cell culture attributes related to viability, growth, metabolism, and protein production. Comparisons were made between the original and all subsequent cultures and the results suggested no adverse impact on cell physiology due to extended cryopreservation in bags. We believe this is the first study which demonstrates the feasibility of long-term cryo-bag preservation of a biopharmaceutical producing mammalian cell line. Such cryo-bags are readily produced from a perfusion bioreactor where cells are typically in the exponential phase of growth and are characterized by both high density and viability. Once created, cryo-bags eliminate the need for open processing and reduce the duration of a manufacturing campaign thereby making them an attractive alternative for seed train expansion in commercial mammalian cell based biopharmaceutical manufacturing. References: Heidemann et al. (2002) Cytotechnology 38: 99–108, Heidemann et al. (2005) United States Patent 6,953,692 B2, Heidemann et al. (2010) Biotechnol. Prog. 26: 1154-1163
The pharmaceutical industry is facing challenges in the upcoming years. Decreasing R&D productivity and increasing development costs combined with increased competition, patent expiries, Biosimilars, etc., put significant pressure on the value and supply chain of global research-driven pharmaceutical companies. Since the 1950s 1222 NMEs have been approved - 1103 small molecules and 119 biologics. The trend towards Biopharmaceuticals products has been seen since the early 80s and recombinant antibodies produced by mammalian cell culture has clearly been the growth driver in the biotech industry creating a meaningful contribution to patients’ lives. However, the trend of NBE is stagnant/declining while the costs are increasing substantially in a strictly regulated environment\(^1\). In order to answer these threats the industry has launched several initiatives along the value and supply chain focusing on several areas, e.g.: Enhance success rate in R&D, increase speed to market, excellence in supply chain management, Operational Excellence (six sigma and lean), business process management, excellence in technology lifecycle management, etc.\(^2\)

For operations the major focus during the life cycle of a process is to guarantee uninterrupted supply of high quality products in compliance with regulatory expectations at best financial performance for a changing evolving product portfolio. Manufacturing binds the majority of assets in a company and therefore efficiency in operations is key to free up resources to invest in R&D. New technologies and their holistic management are crucial to the success and driving efficiencies in operations. Due to extensive process development work several new technologies have made their way into biotech manufacturing with new portfolio projects. Examples include disposable technologies, supply chain risk mitigation measures, and high temperature short time media treatment. In order to derive the full benefits of these mature technologies for existing manufacturing processes, supply chain risk mitigation and end to end technology lifecycle management is necessary. Driving incremental innovation in existing processes by applying well known and mature technologies is achievable and in alignment with the regulatory expectations given having an appropriate regulatory and quality strategy in place. This presentation includes a technology lifecycle management model and case studies of applying mature technologies to existing processes highlighting business considerations, regulatory strategies/aspects and product quality contributions.


CELL LINE DEVELOPMENT TOOL BOX FOR EXPRESSION: E.COLI, CHO, INSECT CELLS

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Cell Line development: The conditions for E.coli, CHO, HEK 293 and Insect Cell lines need to be maintained at small scale and within fermentation. Data will be presented on techniques and technology that allow for mimicking large scale fermentation with non-controlled devices from 1mL-2.5L. All of these techniques are proven technologies for protein production, structural biology, and can lead to successful transfer from cell line development to bioprocess group. Keywords: Cell Line Development, E.coli, fermentation, CHO, Hek293.
Recombinant therapeutic proteins play a vital role in treating diseases in this modern era of medicine. Increasing the production of these therapeutic proteins is of prime importance owing to their high demand. Proper folding by ER-resident chaperones is a prerequisite to the secretion of these proteins. The ER chaperones and enzymes in association with the UPR pathway ensure only properly folded proteins to be secreted. Misfolded proteins are either refolded by chaperones or degraded by the ubiquitin proteosome complex machinery. The UPR pathway also helps in homeostasis in the event of increasing load of unfolded proteins inside ER. Together, these pathways are believed to play a significant role in overall productivity.

In this study, the transcriptional response of recombinant CHO cells treated with ER stress modulators was investigated. A high producer cell line that secretes an Anti-Rhesus IgG antibody with a maximum productivity of 40 pg/cell-day in batch cultures was used for this work. Valproic acid, a cytoprotective drug used to treat epilepsy was used as a positive modulator of ER stress. It has been shown to increase productivity in transiently transfected CHO cells. Brefeldin A was chosen as a negative modulator of ER stress. It is a cytotoxic drug which inhibits the transfer of folded proteins from ER to Golgi. Cells were screened for growth and secreted antibody levels at various concentrations of both drugs. Optimal concentrations of both drugs were identified for further transcriptomic profiling of ER chaperones and UPR genes.

Treatment with valproic acid gradually increases productivity. The specific IgG productivity increased by about two-fold from 34 pg/cell-day to 63 pg/cell-day three days post treatment. At the transcriptional level, both heavy and light chain mRNA were transiently induced by over 4 fold with a maximum induction on day one post treatment with valproic acid. In contrast, treatment with brefeldin led to a decrease in productivity. Chaperones including GRP78, GRP94 and ERDj4 were also induced with a maximum induction observed one day post valproic acid treatment. Activation of PERK and the IRE1 pathway was also observed in both the treatments. Induction of chaperones and activation of PERK and IRE1 pathways in response to both positive and negative modulators suggests adaptation of these cells in an effort to maintain ER homeostasis. The results of this study are used to understand the role of ER stress in determining recombinant cell line productivity.
Various biologics including antibody therapeutics and erythropoietin are produced by mammalian cell culture. Into the culture media, mammal-derived factors, such as fetal bovine serum (FBS) and bovine serum albumin, are often supplemented to supply various growth factors. Although mammal-derived factors are effective, they also have disadvantages; an infective risk by virus and abnormal prion and lot variation. To overcome these disadvantages, various serum- and mammal-free culture media have been developed. But adaptation to the mammal-free medium is required before the culture. Therefore, mammal-free culture media should be improved. For the purpose, we have been focusing on bio-active factors derived from insect or plant. We report some of these factors are useful for expansion of cell number and production of biologics.
After successful development and implementation of a high intensity fed-batch process platform we turned our attention to development of enhanced process monitoring and control tools. The objectives of this program were, 1) to develop and implement additional tools for process monitoring in our pilot and manufacturing facilities and 2) to develop an on-line tool for feed-back based feeding of the high density production fed-batch culture. Several technologies were evaluated including automated sampling combined with metabolite analysis, culture biomass capacitance, off-gas sensor based OUR, NIR and Raman Spectroscopy. Among the tested technologies Raman spectroscopy turned out to be most promising. Using in-line Raman data and chemometric analysis, process models were built using partial least square analysis. Among the modeled parameters were metabolites (including glucose, lactate, glutamine, glutamate and ammonia), Mab product concentration, osmolality and viable cell density. Model consistency was evaluated for several high cell density Mab platform fed-batch processes. We also tested model consistency across scales (3L, 200L and 2,000L) for a representative process and results showed good alignment. Current work focuses on communication tools that will allow integration of Raman spectroscopy into a DeltaV based process control system as a step towards automated culture feeding at manufacturing scale.
Biotech processes still contain a number of open and at risk transfers steps. Cell culture from one vial to containers such as T-flasks or shake flasks is usually one of these open phases. This critical step may take several days or even weeks and thus can delay production timeline due to contamination. By coupling traditional technologies (bags) and new technologies (NovaCase®, Disposable bioreactor and Aseptic Transfer Cap), Merck Biodevelopment succeeded in freezing mammalian cells in bags with direct thawing in closed cell culture containers. A study on bags and freezing conditions was performed. The best parameters have been applied to different cell lines and media. With this technique, cell amplification and cross contamination were reduced. This first improvement was combined to single-use bioreactors that are now commonly used for seeding stainless steel bioreactors or for producing material. The profitability of these equipment has been well demonstrated on more that decade, however, only a few data on their scalability are published. During the period 2010-2011, we performed a study in order to evaluate the performances of disposable bioreactors. As different technologies were available, this study compared performances of several types of mixing in single-use bioreactors. The evaluation was performed both for seeding application and for clinical material production. A fed-batch process producing a highly glycosylated molecule was performed in 7 different types of disposable bioreactors. The quality of the molecule together with the molecule titer and the cell growth was compared between the different single use technologies. These process performances were also compared to glass and stainless steel bioreactors of different sizes ranging from 3.6L and 1.25kL. This study demonstrated the benefits of using disposable equipment in several key areas. The comparison of stainless steel, glass and disposable equipment showed how comparable they are. Finally, coupling cell freezing in bag and disposable bioreactors allowed us to develop a fully closed USP process.
In mammalian cell culture producing therapeutic proteins, one of the important challenges is the use of several complex raw materials whose compositional variability is relatively high and their influences on cell culture is poorly understood. Under these circumstances, application of spectroscopic techniques combined with chemometrics can provide fast, simple and non-destructive ways to evaluate raw material quality, leading to more consistent cell culture performance. In this study, a comprehensive data fusion strategy of combining multiple spectroscopic techniques is investigated for the prediction of raw material quality in mammalian cell culture. To achieve this purpose, four different spectroscopic techniques of near-infrared, Raman, 2D fluorescence and X-ray fluorescence spectra were employed for comprehensive characterization of soy hydrolysates which are commonly used as supplements in culture media. First, the different spectra were compared separately in terms of their prediction capability. Then, ensemble partial least squares (EPLS) was further employed by combining all of these spectral datasets in order to produce a more accurate estimation of raw material properties, and compared with other data fusion techniques. The results showed that data fusion models based on EPLS always exhibit best prediction accuracy among all the models including individual spectroscopic methods, demonstrating the synergetic effects of data fusion in characterizing the raw material quality.
The construction of a metabolic network and a constraint-based metabolic flux model for a cell culture process is discussed. Cellular uptake/production rates of various nutrients/products and compositions of biomass were analyzed and used for model development. Model construction involved bounds in the reaction fluxes, reversibility constraints, energy balance and lumped collinear reactions. A linear optimization approach combined with experimentally determined metabolite consumption and production rates was used to solve the fluxes through the reactions. Flux calculations also involved customized weighted matrix for each metabolite in the reaction network. The model-generated fluxes matched well with experimentally measured external fluxes. The model was also employed to successfully estimate metabolic fluxes for a different cell culture process, which used the same cell line but utilized different medium/feed compositions. Fluxes that were directly and indirectly correlated with process outputs such as lactate, biomass and antibody production were identified using multiple methods including PCA/PLS. Hypotheses were generated from these direct/indirect correlations and tested in experimental set-up to assess if these correlations can be used to predict the outcomes of the experiments. Model verification was carried out by performing internal metabolite measurements. Concentration trends of internal metabolites matched well with that calculated using the model. This metabolic model can be utilized for creating snap-shots of metabolic state of the cell culture and in aiding cell culture process development efforts.
COMPARABILITY STUDIES OF CELL CULTURE FOR MONOCLONAL ANTIBODY PRODUCTION IN MINIBIOREACTORS AND BENCH SCALE BIOREACTORS

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The main goal of the regulatory initiatives falling under the quality by design (QbD) and process analytical technology (PAT) umbrella is to achieve consistent production of biopharmaceutical products of predefined quality. The establishment of such a process necessitates extensive process development studies. Currently, these studies are largely being carried out in bench scale systems (1-10 L). However, bench scale bioreactor studies are costly, tedious to set up and time consuming. Novel high-throughput mini-bioreactor systems show a promising potential to enhance and streamline process development studies. However, in order to fully validate this system as a scale down model, sufficient comparability between the two scales needs to be established. Comparability studies of high-throughput minibioreactors versus 5L bioreactors focusing on gene profiling have been performed by our group and discussed in the past. However, protein quality aspects have not been addressed to date. Here, we further investigate cell culture comparability by comparing monoclonal antibody titers and N-glycan profiles produced by serum free mammalian cell culture in these two systems. We use a two-step downstream processing scheme modeled after industrial practice to purify a model murine IgG3 antibody from the cell culture broth. N-glycan analysis was carried out using high pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). Structure determination of the major N-glycans present on IgG3 was done using MALDI TOF mass spectrometry. It was found that although the relative concentration of major N-glycans was similar, a noticeable difference was seen in the product titers between the two scales. In order to further understand this difference, the influence of pCO2 and glutamine in both the systems was evaluated and compared. Finally, the effect of dissolved oxygen (DO) on titers and N-glycan profiles of a monoclonal antibody (IgG3) produced by mammalian cell culture in both minibioreactors and bench scale bioreactors was investigated and will also be discussed in my presentation.
Robust fed batch processes are required to leverage high cell-specific productivity and get the most out of bioreactor throughput. Current chemically defined fed batch platforms for CHO-based production of recombinant proteins involve additions of one or more concentrated nutrient solutions. Proportionately large amounts of liquid are often required to support the stoichiometrically balanced metabolic requirements of high density cell culture. Large feed additions can ultimately dilute the recombinant product and can present liquid handling, storage, and/or shipping issues during bioprocess scale up and manufacturing. As such it is desirable to minimize liquid volumes and concentrate components as much as possible. However, pH neutral nutrient solutions can’t be concentrated excessively without adversely impacting solubility and stability. Greater solubility can be achieved through use of acidic or basic solutions, but this can complicate pH control strategies in bioreactors and substantially increase culture osmolality. Getting around this difficulty traditionally involves separating components into multiple concentrated subgroups having high and low pH, but this method often produces nutrient solutions with very short shelf lives. Using a novel and proprietary technology, we have developed a prototype feed supplement that addresses these pervasive problems. The supplement is highly concentrated, single part, pH-neutral, stable in dry or liquid format for long periods of time, and is designed to “enhance” existing feeds and bolster fed batch productivity of current platforms. This approach has been demonstrated to be highly effective in several CHO cell lines producing IgGs. Without process optimization, supplementation with this prototype feed in relatively low proportions (approximately 2.5-10% of total culture volume) has resulted in 45-120% improvement in product titers in several CHO fed batch processes. Initial liquid format prototypes have been shown to be precipitate-free for over a year. The ability to develop highly concentrated, pH-neutral supplements that can be added to any CHO culture system represents a major advancement in fed batch technology.
Dendreon has developed an innovative and proven proprietary platform technology for various cancer treatments based on baculovirus/insect cell-derived antigen and autologous active cellular immunotherapy (ACI).

The insect cell-baculovirus expression vector system (IC-BEVS) has long been widely used to produce recombinant proteins for research use and has only recently been applied as a platform to produce biologics for therapeutic purpose. This presentation will discuss the complexity of the IC-BEVS, including growth of insect cells, amplification of viruses, virus infection of insect cells, and production of recombinant protein(s). Many critical factors (such as virus quality, medium components, process parameters, virus and protein assays, etc.) have been thoroughly investigated with aims to improve protein production, enhance process robustness, as well as reduce cost-of-goods. Current data indicate that critical medium components, nutrient consumption and supplementation, virus passage numbers and amplification methods, virus storage, and assay methods are among the challenges and opportunities in the production of an IC-BEVS-derived recombinant antigen for cancer immunotherapy, these data and conclusions will be shared in the presentation.
Microcarrier culture is widely used for the cultivation of anchorage-dependent mammalian cells, especially for human and veterinary vaccine production. The prospect of cell therapy and the possible need for a large quantity of cells has renewed the interest in microcarrier culture recently. An overriding concern in cell culture process development for cell therapy applications is the incompatibility of extensive cell purification and the possible inadequacy in contaminant removal. As a result the process design tends to opt for minimal cell manipulation to avoid the introduction of chemical and biological contaminant. While microcarrier culture offers the advantage of being a closed system as compared to traditional culture flasks, it also poses a challenge in serial propagation. Cell detachment based serial propagation method, e.g. employing trypsin or collagenase, offers operational simplicity and predictability. However, the attachment kinetics and cell distribution on microcarriers can profoundly affect culture performance. Microcarrier agglomeration and cell migration based serial propagation, on the other hand, eliminates the need of cell detachment, but may suffer from other factors which have not been brought to the attention of practitioners. To analyze and compare various process options, we developed mathematical models for cell attachment to microcarriers, microcarrier aggregation, cell migration and growth. Some kinetic parameters were quantified experimentally while others were order-of-magnitude estimates. The model was used to examine a number of process options and simulations were performed to provide insight into advantages and pitfalls of each option. The “optimal” method of serial propagation differs with microcarrier types and the kinetics of attachment, aggregation and migration. The strategy in selecting the optimal region in the design space will be discussed.
Recombinant antibody mixtures represent an important new class of antibody therapeutics where combinations of two or more antibodies show superiority compared to monoclonal antibodies for treatment of e.g. cancer and infectious diseases. Recombinant antibody mixtures can in principle be made in three different ways, i.e. as individual drug products that are simply administered to the patients as a combination, as individual drug substances that are mixed as one drug product and finally using a single batch manufacturing of drug substance and subsequently drug product. Simple mixtures, containing 2-3 antibodies, are typically produced, released, and characterized as individual drug substances and subsequently mixed as one drug product. One example of such a product is Sym004, composed of two antibodies targeting non-overlapping epitopes of the epidermal growth factor receptor (EGFR), that act in a synergistic manner to induce an efficient internalization of EGFR leading to subsequent receptor degradation. Sym004 exhibits superior anticancer efficacy as demonstrated in several preclinical in vivo models. At Symphogen A/S, we have developed an expression platform, Sympress™, for cost-efficient production of antibody mixtures. Rozrolimupab, composed of 25 anti RhD antibodies is like the vast majority of recombinant antibodies produced by a CHO expression platform, based on site specific integration using the Flp-In system in CHO-K1 cells. The Sympress™ technology has subsequently been optimized to achieve higher titers and the currently employed expression technology is based on expression in the ECHO cell line, a genetically modified version of the dihydrofolate reductase (DHFR) negative Chinese Hamster Ovary (CHO) cell line DG44. ECHO parental cells are transfected separately with each of the individual antibody expression vectors using standard transfection technology, after which cells are subjected to a methotrexate (MTX) selection schedule. The selected stable pools are single-cell cloned by FACS and high-expressing clones are expanded and frozen, still as individual research cell banks. The antibody mixtures are produced using a single-batch manufacturing approach where a polyclonal working cell bank (pWCB) prepared by mixing the individual stable cell lines producing all the desired antibodies is used as seed material for a bioreactor process. This technology has certain challenges in terms of cell banking strategy, manufacturing approach and strategies for the release and characterization of such types of products and this will be addressed in the presentations and compared to individual manufacturing approaches. Furthermore, a comparison in terms of development timelines, preclinical developmental costs, and manufacturing COGS between the two manufacturing approaches will be made in the presentation.
Cell lines for industrial pharmaceutical protein production processes need to be robust, fast growing and high producing. In order to find such cells we performed a high passage cultivation of monoclonal antibody producing Chinese hamster ovary (CHO) cells in shaking flasks for more than 420 days. Examinations of cell growth, productivity, intracellular protein and metabolite characteristics as well as product transcript and genomic integrate levels revealed substantial differences between subpopulations that were cryopreserved from long-term cultivation at different time points. Detected growth performance as well as intracellular adenylate energy charge (AEC) increased during high passage cultivation. In addition, proteome analysis indicated an augmented utilization of glycolysis with higher passage number and an enhanced robustness based on anti-stress proteins. Interestingly, the product formation increased at first, but decreased dramatically during the later subcultivations, although selection pressure was applied. Utilizing flow cytometry and quantitative real-time PCR, we further examined the translational, transcriptional and genomic basis for the observed phenotypes. The detected reduction of antibody expression, in particular of the heavy chain, was ascribed to a decrease of antibody transcript, caused by loss of gene copy number and assumedly a malfunctioning splicing mechanism of the dicistronic mRNA. To our knowledge, this is the first systematic approach using process analytics and targeted omic techniques to elucidate the effects of long-term cultivation of CHO cells expressing a therapeutic protein.
RNA INTERFERENCE OF COFILIN IMPROVES RECOMBINANT PROTEIN PRODUCTIVITY IN CHINESE HAMSTER OVARY CELLS

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CHO cells are the most commonly used mammalian cell line for production of biopharmaceutical proteins that require proper folding and glycosylation for full activity. Gene silencing using RNA interference (RNAi) technology is a recent approach to alter signaling and metabolic pathways in CHO cells to improve cell viability, enhance cellular productivity, and increase product efficacy. Previous proteomic characterization of a gene-amplified CHO cell line identified several proteins with altered expression including cofilin, a key regulatory protein of the actin cytoskeleton that was down-regulated in this cell line. Here, RNAi is used as a genetic approach to cell line engineering by reducing cofilin levels in CHO cells expressing the model protein human secreted alkaline phosphatase (CHO-SEAP) and the therapeutic protein tissue plasminogen activator (CHO-tPA). The transient reduction of cofilin by small interfering RNA (siRNA) enhanced specific productivity by up to 80% in adherent cells and by up to 55% in suspension cells. Stable reduction of cofilin by the expression of short hairpin RNA (shRNA) vectors in adherent CHO cells enhanced specific productivity by up to 65%. The effect of cofilin reduction on the actin cytoskeletal structure was investigated as previous work demonstrated that destabilization of the actin cytoskeleton is associated with enhanced productivity. CHO-SEAP cells expressing a cofilin-specific shRNA vector showed a decreased number of F-actin filaments compared to parental and control cell lines, suggesting that the destabilizing effects of cofilin silencing in this cell line may contribute to the enhanced specific productivity. Members of the cofilin protein family are emerging as regulators of cellular homeostasis that may control several processes often targeted in cell line engineering such as gene expression, apoptosis, and vesicular trafficking. The use of RNAi technology to alter cofilin levels may provide a means by which to alter multiple signaling and trafficking pathways in CHO cells to improve biopharmaceutical production.
Several prototype and development runs of a novel, single-use bioreactor system were conducted at the Keck Graduate Institute’s (KGI) Amgen Bioprocessing Center. Described as a “pneumatic bioreactor system” and supplied by the company PBS Biotech Inc., each bioreactor vessel contains a specialized air-wheel that utilizes sparged air to achieve efficient mixing. Early acid-base mixing studies were carried out to compare mixing times between the prototype system against traditional stir-tank bioreactors and wave bag bioreactor systems. Subsequent cell culture studies with Chinese Hamster Ovary (CHO) cell lines were carried out on the prototype systems. These included studies at the laboratory scale, which utilized stirred tank, wave-type, and PBS bioreactors all operated at identical mixing time conditions. It also included scalability studies of 3-L to 80-L PBS bioreactors. Finally, development runs were carried out on a progressive series of improved PBS prototypes, to optimize the detailed design and operation of the control and sampling systems. Each prototype was tested in duplicate against standard glass bioreactors (Applikons), using a high-density fed-batch process that achieves viable cell densities of 30 million cells per mL. These high-density cell cultures used the Croughan-Freund Process: a lactate-adapted CHO cell line, grown in suspension culture, in chemically-defined medium supplemented with lactate.
The effect of hydrodynamic forces on CHO cells used for the production of biopharmaceuticals has been studied by multiple academic and industrial researchers. Such studies have included evaluation of lethal forces, as well as non-lethal forces, for their effect(s) on cell growth and productivity as well as recombinant protein quality attributes. In this work, two different CHO production cell lines were studied in a repetitive defined elongational laminar shear field device, and in high turbulent shear field bioreactor cultures. Specific energy dissipation rates in the laminar flow in the shear device were tested at levels comparable to peak turbulent levels for typical bioreactor operating conditions for successful industrial operation and at significantly higher levels. Even specific energy dissipation rates much higher than those practically required do not show any adverse effects on performance or protein quality for both cell lines, consistent with previous results showing no significant effects on growth or productivity with multiple cell lines. This work will be summarized in the context of practical conditions in typical stirred tank bioreactors, and the likelihood that such hydrodynamic forces would significantly impact process performance or protein quality attributes.
Expression level variation among different antibodies produced in permanently transfected Chinese Hamster Ovary (CHO) cells is a well-established observation. Antibody expression issues can be mitigated through optimization of coding sequences, manipulation of cell culture conditions, and process optimization in bioreactors. In the course of developing CHO-K1 cell lines expressing human antibodies, we encountered an antibody which was poorly expressed in permanently-transfected cells and was not amenable to standard approaches for mitigating the expression issues. Transfectants expressing this antibody were developed using vectors containing genes encoding light and heavy chains, each under control of the CMV promoter, and a “sequential” transfection strategy. By this approach, cells were transfected with a vector containing the *neo* gene encoding resistance to G418 and then the top G418 – resistant transfectants were re-transfected with a vector containing the *hisD* gene encoding resistance to histidinol. Despite using codon – optimized V regions and screening large numbers of clones in both the first and second transfections, expression levels in shake flasks and bioreactors were lower than expected even with nutrient feeds. Furthermore, purified antibody from bioreactors contained unacceptable levels of high molecular weight aggregates and heavy chain dimer. Using a rapid and sensitive transient expression system with CHO-K1 cells and co-transfection with light and heavy chain on separate expression vectors, we established the light chain to be the source of the expression issues for this antibody. Thus, whereas other tested antibodies achieved optimal expression at 1:1 or 2:1 light chain (LC) to heavy chain (HC) ratios, this antibody required more than a 2:1 LC to HC ratio for maximal expression which was still up to 2-fold lower than that for other antibodies. In addition, the results of a light chain “swap” experiment in which the light and heavy chains from the poorly – expressed antibody were co-expressed with heavy and light chains, respectively, from a highly – expressed antibody demonstrated that the poor expression was associated with the light chain from the poorly-expressed antibody. Based on these results, light chain shuffling was performed with the heavy chain to select antibodies with new light chains which retained or exceeded the binding affinity of the original antibody. Co-transfection of these new light chains with the original heavy chain in our transient CHO-K1 expression system resulted in high antibody expression at 1:1 or 2:1 LC to HC ratios. Additional experiments with the new light chains co-expressed with the heavy chain in permanently – transfected CHO-K1 cells confirmed the transient expression results. Finally, purified antibodies produced by permanent transfectants expressing the heavy chain and the new light chains displayed normal product quality. These results demonstrate that the cause of at least some antibody expression issues can reside with the light chain and that engineering antibodies by pairing heavy chains with new light chains can greatly improve both expression levels and product quality without losing affinity.
For monoclonal antibodies and other biotherapeutic proteins, the primary point of control for many critical quality attributes (CQAs) is the production bioreactor step. The purification steps are designed to ensure the purity of the final drug substance through the removal of process- and product-related impurities. Other aspects of the protein, such as glycosylation and charge profile, are not typically modified via chromatography steps, although there are exceptions. The in-process hold points can also alter product quality (e.g. charge profile) depending on the conditions during the hold.

The goal of process characterization is to gain a thorough understanding of the impact of process inputs and their interactions so that an in-process control strategy can be developed that ensures quality of the drug substance. Systematically mapping the design space provides confidence that any combination of operating ranges contained within it will result in drug substance CQAs that meet requirements for safety and efficacy. Each unit operation is characterized independently or in groupings using scale-down models of the bioreactor, centrifuge and chromatography columns. Mathematical models are generated to predict the effects of input parameters on product quality at each step. The final control strategy, however, must encompass the combined impact of the entire process.

Several examples of integrated design space mapping and process improvements for better control of CQAs such as host cell protein, aggregate levels, glycosylation, and charge profile will be described. Data from laboratory and pilot-scale studies and manufacturing data will be presented. These case studies will highlight the linkage of production bioreactor and purification design spaces to demonstrate the approach we have taken at Bristol-Myers Squibb to develop control strategies for biologics manufacturing processes.
DIFFERENTIAL EFFECT OF REDUCED CULTURE TEMPERATURE ON THE EXPRESSION AND BIOPHYSICAL PROPERTIES OF MONOCLONAL ANTIBODY VARIANTS

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The use of reduced culture temperature is becoming an increasingly popular practice to improve recombinant protein yields in CHO cells. Recent studies have attributed the enhancement of protein titers at sub-physiological temperatures to increased mRNA levels and extended stationary phase. In this study, we observed that reducing the culture temperature resulted in arrest of cell growth, prolonged viability, and increased cell size. However, the reduced culture temperature had a differential effect on protein and mRNA expression of closely related antibody mutants from stable cell lines. The high-expressing mutant (277 Ala) exhibited similar or decreased specific productivity and decreased volumetric productivity over the culture lifetime at 32°C compared to 37°C. In contrast, the specific and volumetric productivity of the poorly expressing mutant (277 Gly) was enhanced when cultured at the lower temperature. The difference in specific productivity was reflected in the amounts of heavy and light chain mRNA. Analysis of the secondary and tertiary configurations of the purified antibodies by circular dichroism revealed fundamental structural differences imposed by the Ala to Gly mutation as well as reduced culture temperature. We propose that the effect of reduced culture temperature on expression is protein-dependent; protein-folding fidelity and assembly is improved at lower temperatures, therefore enhancing the expression of proteins that have a propensity to misfold.
This presentation describes a rapid comprehensive screening strategy for a commercial medium. Implementation of the strategy resulted in the resolution of significant growth and process variability issues observed in three different monoclonal antibody projects utilizing the medium. The issue of media instability and its quick resolution (less than a month) enabled the accelerated process technology transfer of a fed-batch monoclonal antibody production process to a contract manufacturing organization (CMO) for production of Phase III clinical supplies. The medium screening, coupled with in-depth physical, chemical, and stability characterization, facilitated the identification of key raw material components impacting process reproducibility and robustness. The strategy included chemical analysis (amino acid, vitamins), metabolic analysis, spectroscopy and growth assessment in shake flasks for light and/or temperature sensitivity.

The effect of storage of media at cold and elevated temperatures and under light/no light conditions was studied over time using the platform analytical methods. In the presence of room fluorescent light there was a pronounced effect on growth for all three monoclonal antibody programs. With prolonged light exposure (for example, during use of the medium in small scale glass bioreactors), culture growth was effectively halted. The results showed an extreme light sensitivity (less than 1 week exposure) of the media in terms of significantly reduced growth. This correlated with a decrease in vitamins: riboflavin and thiamine to near depletion levels and changes in spectral properties between 300 and 400 nm. Feedback addition of the vitamins into the medium prior to inoculation restored complete growth. There was also sensitivity of the media to elevated 37°C temperature storage condition. The results from spectral scans showed that the media was stable for up to 2 weeks at 37°C, although there was no effect on growth rates up to 5 weeks of storage. Decrease in certain amino acids like methionine, histidine, tryptophan and arginine started to occur from 5 weeks onwards. Increase in lactate and ammonia and decrease in pH over time was also observed for the samples which were stored at 37°C. Based on the results, a strict control of medium storage conditions was proposed, which resulted in eradication of the observed growth issues and a successful process technology transfer for production of clinical supplies.
This presentation describes a case study for an accelerated process technology transfer of a fed-batch monoclonal antibody production process to a contract manufacturing organization (CMO) for production of Phase III clinical supplies. Outsourcing to CMOs to supplement internal manufacturing capacity can provide a valuable opportunity to defer capital costs and decrease time to commercial launch. Formation of a strategic external partnership can provide further advantage by leveraging experience from multiple project transfers over time, allowing the two organizations to streamline interactions and take material production off of the critical path to licensure. However, successful technology transfer requires close coordination between the two organizations; the coordination can be even more challenging when the process unit operations and/or raw materials are new to the external facility. To facilitate the transfer and process implementation into the new facility, it is beneficial to augment normal process development efforts with process ranging, facility fit, and scale translation studies to test and confirm process robustness and identify the operational parameter(s) that may be impacted with scale-up.

For this case study, we describe a strategy for testing the process robustness of an antibody production process with respect to productivity and product quality, through targeted process ranging and scale-up studies. Late stage process development included DOE-based robustness studies at bench scale that were designed to address process fit and delineate robust operating ranges. Pilot production runs to confirm scale up parameters and produce pre-clinical material included four batches at the 500-L scale. The manufacturing process was run at 10,000+/-L scale to produce Phase III clinical material. One particular issue that was discovered during these studies was the sensitivity of the commercial growth medium to preparation and storage conditions, which led to significant upstream process variability. However after extensive internal investigation the medium issues were addressed and resolved before the start of clinical production.
DETAIL ANALYSIS OF CHROMOSOME REARRANGEMENTS IN CHO CELLS USING BAC-BASED PHYSICAL MAP

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CHO (Chinese hamster ovary) cells have frequently been used in biotechnology for many years as a mammalian host cell platform for cloning and expressing genes of interest. A detailed physical chromosomal map of the CHO DG44 cell line was constructed by fluorescence in situ hybridization (FISH) imaging using randomly selected 303 BAC clones as hybridization probes (BAC-FISH) [1,2]. The two longest chromosomes were completely paired chromosomes; other chromosomes were partly deleted or rearranged. The end sequences of 624 BAC clones, including 287 mapped BAC clones, were analyzed and 1,119 informative BAC end sequences were obtained.

Among 303 mapped BAC clones, 185 clones were used for BAC-FISH analysis of CHO K1 chromosomes and 94 clones for primary Chinese hamster lung cells. Based on this constructed physical map and end sequences, the chromosome rearrangements between CHO DG44, CHO K1, and primary Chinese hamster cells were investigated. Among 20 CHO chromosomes, 8 were conserved without large rearrangement in CHO DG44, CHO K1, and primary Chinese hamster cells. The longest two chromosomes are only conserved paired chromosomes in both the CHO DG44 and CHO K1 cell lines. We compared these chromosomes with the mouse genome for further detailed analysis. Eighty-two BAC clones were mapped on these two chromosomes. The end sequences of 48 BAC clones showed homology with mouse genome sequences. Twenty BAC clones had a 70 - 140 kb homology region with mouse genome contigs. It was estimated that these clones covered about 2.14 Mb of hamster genomes and were homologous with mouse genomes among 11 mouse chromosomes.

REFERENCE
The inoculum build process for one antibody product being tech transferred from Development to Manufacturing used doubling time (Dt) out of vial thaw as a criterion to indicate that the culture was suitable to progress into the inoculum building stages. During the transfer we encountered unacceptably high variation in this parameter. We executed several experiments to examine the impact and robustness of selected vial thaw process factors on Dt. Warming the medium appropriately showed itself to be the largest contributor to reducing long lag times, in both RCB and WCB thaws. A follow-up study indicated 3 additional factors - decant to resuspend time, centrifugation speed, and seeding density errors - that affected Vial thaw Dt in a statistically significant manner, but these three were not 'practically' significant. The process was quite robust on the other factors tested, including operator, cell pellet resuspension technique, and incubator conditions.
Continuous solid-liquid separation mostly represents one of the final steps in cell culture fermentation processes in the biopharmaceutical industry. Besides aspects like medium additives and cell disruption during fermentation it is of crucial importance when it comes to deliver constant and best possible starting conditions for the following purification process. In large scale production, continuously working disc stack centrifuges are mainly used for this separation process. Especially in mammalian cell culture processes producing antibody and recombinant proteins issues like cell disruption by shear stress and product loss during discharge of solid components have a detrimental effect on the rate of yield. Thus, detailed equipment characterization combined with suitable online and offline process monitoring are required for optimization of the solid-liquid separation step. Another challenge is the scale down of the large scale continuous separation using disc stack centrifuges from production to bench top scale. Bench top scale down models for bioreactors and most downstream processing steps are widely established. However, the separation step often represents the missing link to complete small scale platforms, thus, often preventing exploitation of the scale down approach in modern process development. Due to the harsh conditions during this process step adequate small scale systems are of crucial importance to prevent scale dependent issues during scale up. Physical limitations make the determination of adequate scale down and scale up parameters very challenging. Besides methods for the characterization of separation processes, online and offline monitoring techniques will be discussed in this presentation. Process optimization conducted after detailed equipment characterization will be presented. Moreover, possible scale down setups including scale down criteria on technological and cell physiological level are compared and discussed.
Novel orbital shaken bioreactors are getting more and more popular in the area of cell cultivation (human, animal, stem and plant cells), because they offer ease-of-use, increased flexibility and reduced costs. Recently, orbital shaken bioreactors of capacities from 1mL - 2500L have been employed for cultivation and are expected to become attractive alternatives to conventional stirred-tank bioreactors. To carry out a scale up fundamental parameters such as the oxygen transfer rate (OTR), aeration rate and mixing time have to be known.

This poster summaries the mixing times of several widely used shaken bioreactors depending on the shaking speed, shaking diameter, filling volume and baffles. Furthermore the oxygen transfer rate and the fermentation data (dissolved oxygen, cell count and viability) of cultivation of CHO cells are compared between baffled and unbaffled flasks.
The next generation of vaccines and therapeutic proteins are being developed today using a wide variety of expression systems and it is imperative that they are developed quickly and cost effectively to meet the demands of a commercial launch. It is beneficial to have a controlled scale-up/scale-down tool that is flexible enough to be used to develop a variety of expression platforms and products. There are options today for creating cell lines and developing upstream and downstream processes using high-throughput technologies. DoE experiments are often used to define the multivariate design space of the fermentation processes and this is done with greater efficiency using high-throughput bioreactors, such as the Micro-24 Microbioreactor, which exemplify the principles of Quality by Design (QbD). Case studies will be presented. The first study will show how Pfenex Inc. uses the latest technologies such as the Micro-24 Microbioreactor along with Pfenex’s Pseudomonas fluorescens-expression system to conduct rapid simultaneous production strain selection and development of highly productive fermentation processes and scale-up of the processes. A scale-up/scale-down study for a vaccine process will also be presented which will consist of industrial and small scale bioreactor data. The data will show the importance of identifying critical parameters early in development and then having the confidence in the models to scale up those findings. Another investigation will be presented which investigates different “engineering” aspects of the Micro-24 MicroReactor system compared to larger scale reactors such as pH control, dissolved oxygen control and temperature control for both microbial and mammalian applications.
The biotechnology industry has continued to make improvements in processing over the past several years. In leveraging the perfusion cell culture process, Genzyme is achieving a breakthrough advancement by integrating upstream and downstream operations for the continuous processing of enzymes and antibodies. In brief, continuous production results from the perfusion cell culture platform with steady-state achieved via cell mass control. Harvest material is then linked directly to a multi-column periodic counter-current chromatography (PCC) system operating through a series of UV-detection-based switching routines that allow for full resin binding capacity to be utilized. The resulting universal biomanufacturing platform, when linked with modern transfected cell lines, robust chemically-defined media, and appropriate capture columns enables high productivity, high throughput, reduced costs, and increased manufacturing flexibility.

This presentation will describe a case study in which the PCC system is linked to an enzyme-producing cell culture process operating at a steady-state near 40*10^6 cells/ml, over 60 days, and producing at volumetric productivity rates near 1 g/L-d. The consistency of PCC operation with respect to in-process indicators, in-process controls, and product critical quality attributes will be shown for up to 640 column operations, 160 cycles, and 30 days. These results show that integration of a high cell density and high producing perfusion cell culture process with the PCC collectively allowed for a 70% increase in the utilization of resin capacity, a 70% decrease in buffer usage, the elimination of the clarification unit operation and associated holding tanks, and a greater than 10-fold reduction in bioreactor size when compared to traditional processes. In summary, a significantly streamlined and simplified process was obtained. Similar integrated bioprocessing results in relation to antibody production will be shown. Overall the integrated bioreactor and capture system results in decreased operating and capital cost by allowing high efficiency and high volumetric productivity manufacturing process.
GENE EXPRESSION PROFILES IN ATF4-OVEREXPRESSING CHO CELL LINE

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Mammalian cells have a strict quality control system called unfolded protein response (UPR) to response to the stress of misfolded proteins in the endoplasmic reticulum (ER). Recently, some researchers reported the translational or secretory capacity improvements by the post-transcriptional and secretory pathways engineering. We focused on the signaling of UPR via PERK (protein kinase R-like ER kinase), such as elf2 alpha (alpha subunit of eukaryotic translation initiation factor 2) and ATF4 (activating transcription factor 4) [1, 2]. ATF4-overexpression in Chinese hamster ovary (CHO) 13D-35D cells improved, the recombinant human antithrombin III (hAT-III) production in 13D-35D cells. In this study, High Coverage Expression Profiling (HiCEP) was performed to investigate gene expression profiles in ATF4-overexpressing CHO 13D-35D cells. HiCEP could be performed without cDNA or genome sequence information and detect about 70% of all transcripts, including non-coding transcripts and unknown, known genes [3]. The expression of three genes were significantly different between ATF4-overexpressing and parental CHO 13D-35D cells. Among these genes, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (nfbiz) was identified. The expression of this gene is controlled by transcription factor nuclear factor-kappa B (NF-kappaB) and associate with cell proliferation and cell survival. We are now investigating the effect of NF-kappaB expression on hAT-III production.

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GLYCOMICS TO INVESTIGATE THE IMPACT OF PROCESS CHANGES ON PRODUCT QUALITY IN CELL CULTURE-BASED INFLUENZA VACCINE PRODUCTION

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Influenza viruses, major agents of respiratory diseases, are responsible for epidemics resulting in high mortality and morbidity every year. Manufacturers try to improve and increase worldwide vaccine manufacturing using different production systems, i.e. fertilized chicken eggs and mammalian cell lines. In all processes, the two viral membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA) are the most important components. In particular the HA of the viral envelope is able to induce a strong and protective immune response. With changes in existing production systems or introduction of new methods several key questions concerning the possible impact of antigen quality on vaccine quality have to be addressed. This includes, for example, the selection of host cells, virus strains, cultivation conditions, process parameters, and unit operations in up- and downstream processing.

In the work presented, glycomics was used to investigate the impact of changes in cultivation conditions, process parameters, and cellular physiology on the HA antigens at the molecular level. Therefore, several influenza virus strains were propagated in different cell lines and bioreactors and HA antigens were analyzed regarding their specific N-glycosylation pattern. The glycosylation of HA antigen was characterized utilizing a newly developed, sophisticated high performance glycoanalysis tool, based on multiplexing capillary gelelectrophoresis with laser induced fluorescence detection (xCGE-LIF) [1,2]. Results regarding the influence of the host cell line on complexity and composition of the HA N-glycosylation pattern, are presented. Furthermore, significant virus type and subtype dependence of HA N-glycosylation was found [3]. Virus-adaptation studies demonstrated a cell line-dependent selection of certain virus subpopulations out of the so-called viral quasispecies. Here, HA N-glycosylation pattern analysis was combined with in-depth pyrosequencing analysis of the viral genomic RNA [4]. Finally, the impact of the bioreactor on HA N-glycosylation pattern was investigated.

Overall, results clearly suggest that monitoring of antigens on the molecular level during process development in vaccine production could be a useful measure to evaluate the impact of process modifications on antigen quality, and to improve the significance of experimental designs concerning specific product properties, like immunogenicity and efficacy.


**CHO-ENGIMIRS: GROWTH ENHANCEMENT BY THE MIR-17-92 CLUSTER IN CHO CELLS**

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Objective:  
MicroRNAs have recently emerged as master regulators of post-transcriptional gene activity that affect a  
broad range of physiological functions. The molecular analysis of their effects might be the basis for  
novel, low-stress cell engineering strategies. We here present a standardized screening method for  
identification of miRNAs with beneficial effects on different bioindustrially relevant characteristics, such as  
growth, viability and productivity and demonstrate the benefits of miRNA engineering on the example of  
the miR 17-92 cluster.

Results:  
A standardized protocol for rapid functional screening of miRNA overexpression was established, using  
transient transfection into a recombinant EpoFc production cell line. For proof of concept, four CHO-  
specific miRNAs (cgr-miR-17, cgr-miR-221, cgr-miR-21, and cgr-miR-210) were cloned into small hairpin  
vectors including a GFP cassette. After transfection cells were analyzed for 4 days to observe growth and  
productivity. Cgr-miR-17 overexpression resulted in a 15% increase in specific growth rate which lead to a  
26% increase in viable cell density and a 14% higher titer. miR-17 is a member of the miR-17-92 cluster,  
a key regulator in cell cycle control and a known oncogene consisting of 6 distinct miRNAs. For further  
characterization of the effect of stable overexpression of this cluster, a construct expressing the entire  
cgr-miR-17-92 cluster was cloned. Upon transient transfection of the cluster the average specific growth  
rate was increased by 23%. Stable overexpressing subclones were established from the same  
transfection protocol by selection and cell sorting for GFP expression.

Discussion:  
The established protocol allows rapid screening of the effect of multiple miRNA candidates. A proof of  
concept study pinpointed miR-17 and the miR-17-92 cluster as prime candidates for CHO cell  
engineering to enhance growth rate. This is in accordance with its well described function in  
tumorigenesis, where it is amplified in several types of lymphomas and solid tumors. In addition to the  
flexibility of the screening protocol, that allows testing for multiple cellular phenotypes under the same  
experimental conditions, the same procedure can be used for establishment of stable overexpressing cell  
lines. These can now be used for detailed and long-term analysis of the resulting cellular phenotype and  
for target effects of the overexpressed miRNAs.
COMPARATIVE METABOLIC FLUX ANALYSES OF CULTIVATIONS WITH NOVEL AVIAN DESIGNER CELL LINES USED FOR VACCINE PRODUCTION

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The avian designer cells AGE1.CR and AGE1.CR.pIX (duck) were evaluated over the last years for production of influenza and vaccinia virus. With regards to vaccinia replication, the modified cell line AGE1.CR.pIX shows higher specific productivity\(^1,2\), which was expected because the pIX protein is known to stabilize the virus capsid. Both cells grow in suspension in a chemically defined medium. Along with the design of an efficient production process, efforts were made regarding media optimization for cell proliferation and production. Therefore, the metabolism during cell growth was examined carefully to reveal differences between both producer cell lines as well as differences between cultivation systems using systems biology approaches.

Here, we present results for cultivations of AGE1.CR and AGE1.CR.pIX cells in 1 L stirred tank reactor and 1 L wave bioreactor. Focus was on metabolic flux analysis during batch growth. Biomass and elemental composition of avian cells were determined experimentally and showed to be comparable to mammalian cells. On the basis of networks describing metabolism of other eukaryotic cells and several databases, a stoichiometric model of the central metabolism of duck cells was derived. It comprises 70 metabolites and 101 reactions, including the avian-specific uric acid synthesis. All reactions included were checked against annotated genomes of avian species in the KEGG database. Cell concentration, cell diameter and concentrations of extracellular metabolites and amino acids were measured in the exponential growth phase to characterize the metabolic profile of the cells. A Monte Carlo based approach was used to compute intracellular flux distributions based on extracellular rates taking into account standard deviations of assays\(^3\). The computed flux distributions were (despite some specifics of avian metabolism) comparable with published data of mammalian cell lines.

Comparison of wave bioreactor and stirred tank reactor cultivations revealed some differences in the flux distribution. In particular, shear stress and aeration conditions seem to lead to changes in metabolism. Differences between both cell lines cultivated in wave bioreactor were observed for example for the consumption rate of glucose which was higher in the modified AGE1.CR.pIX cells. The intracellular rates of these cells also reflected an overall increased metabolism. This indicates that the modified cell line needs slightly more substrates to constitutively produce the pIX protein. Therefore, when using the AGE1.CR.pIX cells, the higher metabolic activity has to be taken into account for medium design and process optimization.

References

The information, which can potentially arise from metabolic pathway modeling, is a better understanding of the pathways effectively used by the cells and a tool to force the cells to use more favorable pathways, e.g. leading to less toxic by-product production, given that the way to influence which pathways the cells are using, is known. For this purpose a model has to identify all the reactions that the cells can potentially use as well as their kinetics. One way to tackle this difficult task is to trigger all the reactions used by the cells by systematically varying the cell states. This can be achieved by varying the environmental conditions for instance by varying the availability of the nutrients, the growth factors, the precursors, or varying the environmental parameters.

The purpose of the present study was to develop a method generating a mathematical model able to predict the behavior of a cell culture system in varying environmental conditions while measuring the extracellular components only.

In the present study, in order to reduce the complexity of the task, a simple model including the reactions involving the glycolysis, the TCA cycle and the amino acid metabolism was considered. An antibody producing CHO cell line was cultured in defined medium. The amino acid concentrations in the cultivation medium were varied in pseudo-continuous cultivation systems and the resulting concentrations of the extracellular components, glucose, lactate and amino acids, were measured. A quasi steady-state assumption was adopted. A reduced model of macro-reactions was generated by elementary flux analysis approach using software Metatool 5.1 to algebraically eliminate the intracellular components. Michaelis-Menten models were adopted for the macro-reaction rate kinetics in which the maximal kinetic rates were unknown constants. The maximal kinetic rates were determined by non-negative least squares algorithm minimizing the error between the modeled macro-reaction rate kinetics and the measured fluxes of consumption or production of the extracellular components. Single values of the maximal kinetic rates were determined for the whole set of experimental data, i.e. in presence of varied concentration of amino acids resulting in a unique model valid for varying amino acid concentrations. Finally an excellent fitting was observed between the measured fluxes of consumption or production of the extracellular components and the values estimated by this model.
High cell density perfusion process of monoclonal antibody producing CHO cells was developed in disposable WAVE Bioreactor™ using tangential flow filtration by external hollow fiber filter (0.2 μm microfilter) as cell separation device. Either 'classical' Tangential Flow Filtration (TFF) or Alternating Tangential Flow system (ATF) equipment were used and compared for their process performances. Consistency and reproducibility of both TFF and ATF perfusion cultures were shown at 20 to 35 * 10^6 cells/mL cell density stabilized by cell bleeds. In order to minimize the nutrients deprivation and metabolites accumulation, a perfusion rate strictly correlated to the cell density was chosen: a Cell Specific Perfusion Rate (CSPR) controlled-feed strategy of 0.05 nL/cell/day was identified and applied onwards in this study to achieve higher cell densities. In this study, this strategy allowed maintaining the cells in growing state and at high viability as shown when the cell density was then stably maintained at 1 to 1.2 * 10^9 cells/mL by cell bleeds. Finally, with the settings used here, maximal cell densities of 2.14 * 10^8 cells/mL and 1.32 * 10^8 cells/mL were reached using TFF and ATF systems respectively. To our knowledge, it is the first time that a density of CHO cells larger than 2 * 10^8 cells/mL was achieved in a wave-agitated bioreactor.

The present perfusion process setting was then mounted with ultrafilter cartridges (UF) to evaluate the performances of this system. Cell densities up to 10^9 were obtained using UF TFF or UF ATF. Using ATF or TFF in perfusions by microfiltration (MF) or ultrafiltration, the cells produced comparable amounts of IgG. The IgG was partially retained by the MF cartridge using ATF or TFF but the retention was higher in the TFF system. The consequence of this retention was mainly an IgG loss when cell broth from the bioreactor was discarded in the daily bleeds while maintaining the cell density at a given level. The MF TFF was thus less favorable for the production of IgG in comparison with MF ATF. The production obtained by perfusion process was compared to fed-batch process. About 5.2 times more IgG could be harvested using perfusion by ATF or TFF, MF or UF, instead of fed-batch after 12 days of culture.

Furthermore, cell cryopreservations at 0.5 * 10^9 cells/mL and 10^9 cells/mL were performed using cells directly taken from perfusion at 10^9 cells/mL cell density. Cell thaw and expansion showed excellent cell resuscitation and expected IgG production for this cell line, leading to the conclusion that this system could be a reliable process for generation of cell banks. This paper gives fundamental information for the use of both industrially relevant disposable technologies, ATF and TFF, allowing reaching very high CHO cell densities.
Recombinant monoclonal antibody use for research, purification, diagnostic testing and therapeutic biologics is rapidly growing. Production cell line selection is typically the longest step in the development of a new mammalian cell antibody manufacturing process. To obtain stable high producers with acceptable growth rates, time-consuming and labor-intensive screens of hundreds or thousands of clones are performed. The reduced survival of mammalian cells at the low seeding density used to obtain clonal populations poses challenges to the selection process and may exclude cells that would otherwise perform well in bioreactor conditions. Methods for the quantification of secreted product using semi-solid cell culture further complicate the process and make it difficult to verify clonality.

To address these challenges, we have developed a microfluidic platform for the clonal culture and the antibody productivity assessment of suspension-adapted mammalian cells. The sequestration of single cells into 4 nl volume culture chambers provided $2.5 \times 10^5$ cells/ml seeding concentrations, enhancing cloning efficiency several-fold compared to limiting dilution in multiwell plates. The concentration of secreted antibody into the small volumes also allowed for the rapid identification (within 2 h) of high-producing clones using a bead immunocapture assay. The unique configuration of the device allowed clones to be isolated within separate chambers by an integrated system of microvalves to prevent diffusion between chambers during the secretion assay. Image analysis verified the clonality of the cells and was used to measure their proliferative capacity over 3-5 days of culture. Selected clones were then recovered and expanded to inoculate shake flask cultures within 3 weeks. We have applied this technology to screen hundreds of Chinese Hamster Ovary (CHO) cells simultaneously and generate cell lines producing a human IgG1 monoclonal antibody. The robustness, flexibility and scalability of this microfluidic platform provide unique advantages for the rapid generation of clonal cell lines.
Biomanufacturing processes using mammalian cells have evolved significantly in last two decades. It is gradually entering into mature technology stage. Meanwhile, interests in biosimilar products have surged significantly in recent years and will last for a while until regulatory and market perspective gets clear. Healthcare companies also face increasing regulatory pressure on the pricing of medicine. All these factors exert strong stimulus on improving manufacturing efficiency and lowering the cost in order to gain competitive edge. In the past, we have reported the increase of manufacturing volumetric productivity by pushing titer higher [1] and shortening seed train stages by implementing high density cell bank [2], which can make manufacturing campaign shorter and simpler. Since then, new initiatives haven been developed to further reduce the cost. The focus is on how to develop a flexible manufacturing system which can handle more variable program and process demands. The system will increase overall manufacturing throughput and lower overhead cost. This presentation will discuss this concept from three aspects. 1) Facility flexibility. It includes retrofitting a seed train bioreactor for intermediate manufacturing capability as well as facility design for GMP manufacturing with single use technologies. A case study about how to deal with unexpected bioreactor characterization data will be given. Design considerations about layout, architecture, environment, and operation will be shared also. 2) Process flexibility. Experience and future works will be discussed on how to meet unusual process demands such as high mass transfer demand, large feed percentage or continuous feed, especially for sensitive cell lines. 3) Operation flexibility. It includes a tailored Quality Assurance (QA) system for various program purposes and a workforce training and development system for cross-functional groups. Overall, the practices described above are necessary and helpful to keep us an industrial leader in biomanufacturing area and prepare for unprecedented industrial transformation in future. [1] Huang et al., 2010. Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. Biotech Prog. 26: 1400-1410 [2] Tao et al., 2011. Development and implementation of a perfusion-based high cell density cell banking process. Biotech Prog. 27: 824-829
CHARACTERIZATION AND SELECTION OF SUSPENSION CELL LINES FOR FUTURE VIRAL VACCINE PRODUCTION PLATFORMS

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In order to reduce the time required for development and production of viral vaccines, animal host cell line production platforms and processes for viral vaccines should be developed and optimized. As standardization is necessary, state of the art platform technology is required. This includes development of standard animal host cell lines and target processes as platform technology that is suitable for several (groups of) viruses with sufficient synergy. In this study, an initial screening is done to assess and compare cell lines for production of a wide variety of vaccines. A selection of candidate cell lines is made which will be matched with different groups of viruses in the course of the project. At this stage, a selection of cell lines was made, based on their previous application in virus propagation for vaccine development. Next to that, the CHO-K1 cell line, which is widely used in large scale protein production, was used as negative control. In virology a rule of thumb is that cells require adherence and an orientation to allow virus entry. However several single cell suspension cell lines are available in which viruses can replicate. Therefore, differences between adherent cell lines and their single cell suspension counterparts were studied to derive more accurate guidelines for cell line selection for virus replication. Six adherent cell lines, and six suspension cell lines, were assessed for their properties and qualities to propagate viruses: Sabin polio type 1, 2 and 3; Influenza H1N1 and H3N2; RSV A2. Furthermore, viable cell densities, CPE and organization of the actin cytoskeleton during infection were assessed. Finally, the ability to produce virus progeny of the viruses mentioned above was assayed for each cell line.
Chinese hamster ovary (CHO) cells are the main platform for production of biotherapeutics in the pharmaceutical industry. To acquire high-titer cell lines, CHO cells have been engineered in the past decades using a combination of genetic engineering and cell screening. However, relatively little is known about the metabolism of CHO cells at the intracellular metabolic level [1]. In this work, metabolic changes in CHO cell metabolism were studied at the growth phase and stationary phase of a cell culture using multiple $^{13}$C-isotopic tracers and mass spectrometry [2]. CHO cells were cultured over six days in fed-batch. On days 2 and 4, the isotopic tracer $[1,2-^{13}$C$]glucose$ was added and the $^{13}$C-labeling of intracellular metabolites was measured by gas chromatography-mass spectrometry (GC-MS), at 6, 12 and 24 hr after tracer addition. Intracellular metabolic fluxes were estimated from the measured extracellular rates and $^{13}$C-labeling dynamics of mass isotopomers of intracellular metabolites using isotopic non-stationary $^{13}$C-metabolic flux analysis ($^{13}$C-MFA). The flux results revealed significant rewiring of intracellular metabolic fluxes in the transition from growth to non-growth, including changes in energy metabolism, redox metabolism, oxidative pentose phosphate pathway and anaplerosis. At the exponential phase, CHO cell metabolism was characterized by a high flux of glycolysis from glucose to lactate, anaplerosis from pyruvate to oxaloacetate and from glutamate to $\alpha$-ketoglutarate, and cataplerosis though malic enzyme. At the stationary phase, the flux map was characterized by a reduced flux of glycolysis, net lactate uptake, oxidative pentose phosphate pathway flux, and reduced rate of anaplerosis. The fluxes of pyruvate dehydrogenase and TCA cycle were similar at the exponential and stationary phases. To further validate the flux results, multiple isotopic tracers were applied in additional fed-batch cultures. The flux observability was enhanced with the use of multiple isotopic tracers and cross-validated with previous $^{13}$C-tracer sets to estimate oxidative and non-oxidative pentose phosphate pathway fluxes, pyruvate cycling and citric acid cycle-related fluxes. Furthermore, lipid metabolism was analyzed using $^{13}$C-labeling of cellular lipids. Significant recycling of lipids to citrate pool was determined at the stationary phase, but not at the growth phase. Taken together, the results provide a solid foundation for future studies of CHO cell metabolism for applications such as cell line development and medium optimization for high-titer production of recombinant proteins.

References
EFFICIENT POLYMER-MEDIATED TRANSIENT GENE EXPRESSION IN SERUM-FREE SF9 CELLS IN TUBESPIN® BIOREACTORS

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Insect cells are a major host for recombinant protein production. Sf9 cells are typically grown in suspension in spinner or Erlenmeyer flasks. In this study, we instead used orbitally shaken TubeSpin® bioreactor 50 ("TubeSpins") and TubeSpin® bioreactor 600 ("Maxi-TubeSpins") for the growth and transfection of these cells at working volumes of 10 mL and 300 mL, respectively. Cells reached a maximal density of 11 x 10^6 cells/mL with a viability above 90% in both vessel types. Sf9 cells were transfected in TubeSpins and MaxiTubeSpins using an expression vector carrying the enhanced green fluorescent protein (EGFP) gene under the control of a baculovirus immediate early promoter (IE-1). DNA delivery was performed using polymer mediated transfections. By 2 d post-transfection, 50% of cells in TubeSpins and 40% of cells in Maxi TubeSpins were EGFP-positive. Transfections with a construct for tumor necrosis factor receptor-Fc fusion protein resulted in volumetric yields of 40-50 mg/L in the two vessels by 5 d post-transfection. These results highlight the utility of TubeSpins and Maxi TubeSpins for cell cultivation and transient recombinant protein production with Sf9 cells. Further studies to improve the volumetric yields are underway.
ESTABLISHMENT OF MAMMALIAN CELL LINE SUITABLE FOR PRODUCING RECOMBINANT PROTEIN USING MUTATION INDUCED BY HIGH ENERGY BEAM RADIATION.

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Mammalian cell culture is extensively performed in industry so as to produce various bio-pharmaceuticals such as erythropoietin and antibody therapeutics. Most of the cell lines for industry have infinite proliferative capacity, which is pivotal for industry, because abundance in cell number is strongly correlate with productivity. But the infinite cells continue to multiply in unregulated manner after cell density reaches sufficient levels. This excess proliferation wreaks aggravation of culture environment; ineffectual consumption of nutrients and accumulation of byproducts such as ammonia and lactic acid, resulting in shorter culture period and less productivity. Therefore, after the cell density reaches sufficient levels, down-regulation of the proliferation would effective for preventing the culture from aggravation and thereby prolong the culture period and improve the productivity. In order to realize suitable proliferation, we aimed to establish novel cell lines whose proliferation should be spontaneously down-regulated so as to prevent the over growth. To obtain such cell lines, we mutated the cells with high energy beam irradiation. CHO-DP12 cells, producers of recombinant humanized anti-interleukin-8 antibody, were irradiated Proton- or carbon-beam or X-ray, and then screened. Screening was performed using hydroxyurea and 5- fluorouracil one by one. Both of them are known to inhibit S-phase progression in different manner, so we expected to effectively concentrate desired mutants and to eliminate other cells. After irradiation of 2.5Gy X-ray and screening with hydroxyurea and 5- fluorouracil, six clones were survived. Among them, one colony, named CHO-M1, was selected and further studied. Cell cycle analysis using flow cytometry indicated that CHO-M1 cells were quickly accumulated in the G1 phase just before confluent and then did not progress the cell cycle, suggesting that CHO-M1 proliferate normally until confluent, and did not proliferate after they reached confluent. The specific antibody production rate of CHO-M1 was kept high after confluent, while that of parental CHO was drastically decreased soon. These results suggest that CHO-M1 cell line spontaneously down-regulates the proliferation after confluent and then continues high level production of antibody and that high energy beam irradiation could be an efficient mutagenic technique for breeding industrial cell lines.
Sodium butyrate (NaBu) is a media additive in CHO cell cultures that is widely used to increase the specific productivity. However, NaBu induces significant cell growth inhibition and apoptotic cell death, resulting in a limited increase of therapeutic proteins. In a previous study, it was determined that co-down-regulation of caspase-3/7 could not inhibit cell death during batch culture in the presence of a high concentration of NaBu (3 mM), whereas Bcl-2 overexpression could successfully inhibit the cell death and increase the culture longevity. To investigate the mechanistic differences between these two engineered cell lines, autophagy induction was investigated in this study. Autophagy is an evolutionarily-conserved intracellular catabolic process in which double membrane vesicles (autophagosomes) engulf a portion of cytoplasm, and then fuse with lysosomes for degradation. Basically, autophagy is an adaptive response to sublethal stress. Conversely, uncontrolled autophagy might be associated with a particular type of cell death (termed autophagic, or type II).

Herein, we evaluated whether and how autophagy is related to the different outcomes upon NaBu treatments in two apoptosis pathway engineered rCHO cells and suggest a more efficient anti-cell death engineering scheme to protect against cell death by NaBu treatment.

A high concentration of NaBu (3 mM) induced autophagy in all cell lines, which was evidenced by the accumulation of the autophagic marker protein, a 16 kDa form of LC3-II, on the autophagosomal membrane and autophagosome structures in cytosol. However, it was shown that basal and NaBu-induced autophagy levels in caspase-3/7 co-down-regulating cells were significantly higher than those levels in control cells. On the other hand, Bcl-2 overexpressing cells showed different profiles of autophagy induction. Until 24 hr exposure to 3 mM NaBu and control cultures, Bcl-2 overexpressing cells, in contrast to control cells, were able to efficiently inhibit the autophagy induction. After 48 hr exposure, however, autophagy was induced more rapidly than in the control cells, which process showed the fine control of Bcl-2 overexpression on autophagy induction in response to persistent stress conditions. However, treatment with an autophagy inhibitor, bafilomycin A1, induced apoptosis and rapid reduction in viability in all cells including Bcl-2 overexpressing cells, indicating that autophagy is not a per se cell death mechanism and is, rather, necessary for cell survival. In addition, by shRNA mediated down-regulation and co-immunoprecipitation of Beclin-1, which is the core autophagy pathway protein, it was shown that NaBu induces autophagy in a Beclin-1 independent manner.

Taken together, the results of our present study provide evidence that autophagy was differentially induced in two different types of apoptosis pathway engineered rCHO cells upon NaBu treatment, in which treatment the inhibition of lysosomal turnover of autophagosome induced drastic cell death even in the Bcl-2 overexpressing cell lines. In caspase-3/7 co-downregulating cell lines, the autophagy induction did not eventually block the NaBu-induced cell death, and these results suggest that controlled induction of autophagy will be able to effectively block cell death when coupled with anti-apoptosis engineering acting early in the apoptosis pathway, such as through Bcl-2 overexpression.
Industrial production of monoclonal antibody (mAb) is carried out by transfecting mammalian cells like the Chinese hamster ovary (CHO) cells either with two vectors, referred to as Co-transfection or with a Multi-promoter single vector for expression of the light chain (LC), heavy chain (HC) and a selection marker. Each gene is driven by its own promoter and transcribed in separate units. One disadvantage of this design is that vector fragmentation results in a significant proportion of non-expressing clones. Upon entering the cell after transfection, the vector can be fragmented before integration or have parts of it removed from the genome after integration as a result of DNA rearrangement. As each gene is independently expressed, if the expression units for the product genes are damaged while that of the selection marker remains intact, cells will not express any mAb product but can still survive the drug selection. It has been reported that up to 50% of non-expressing clones generated using a Multi-promoter vector could escape drug selection. Another disadvantage of having individual expression units is the lack of control of the relative expression of LC over HC. Variations in LC : HC expression ratios have been observed in clones generated using both the Co-transfection and Multi-promoter vector systems. This change in the ratios of LC over HC expression can affect both mAb expression level and quality. As a result, a large number of clones must be screened to obtain high-producing cell lines with good product quality.

We have developed a Tricistronic vector utilizing internal ribosome entry site (IRES) elements to express the light chain (LC), heavy chain (HC), and a neomycin phosphotransferase (NPT) selection marker from one transcript for generation of mAb expressing CHO cell lines. As compared to the commonly used vectors, benefits of this design include: (1) minimized non-expressing clones, (2) enhanced stable mAb productivity without gene amplification, (3) control of LC and HC expression at defined ratios, and (4) consistent product quality. After optimization of the LC and HC arrangement and increasing selection stringency by weakening the NPT selection marker, this Tricistronic vector is able to generate stably transfected pools with specific productivity (qmAb) greater than 5 pg/cell/day (pcd) and titers over 150 mg/L. 5% of clones from these pools have qmAb greater than 20 pcd and titers ranging from 300 to more than 500 mg/L under non-optimized shake flask batch cultures using commercially available protein-free medium. The mAb produced by these clones have low aggregation and consistent glycosylation profiles. The entire process of transfection to high-expressing clones requires less than 4 months. The IRES-mediated Tricistronic vector provides an attractive alternative to commonly used vectors for fast generation of mAb CHO cell lines with high productivity.
MULTI-DIMENSIONAL PROCESS MODELING FOR CHARACTERIZATION OF A CHO FED-BATCH PROCESS

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The ICH Q8 guideline encourages development of a design space based on an enhanced understanding of the manufacturing process. Characterizing the design space involves understanding the linkage between the process operating parameters and critical quality attributes and identifying a robust process window within which consistent quality can be achieved. A characterized design space not only enhances process knowledge but can also later be utilized for process validation and regulatory filings, saving the time and cost of additional filings if process changes are implemented in the future.

This work addresses characterization of a CHO fed-batch process using the Design of Experiment approach. The aim was to develop a design space for achieving robust process and consistent product quality. A failure modes and effects analysis (FMEA) was employed to determine the criticality of process operating parameters and to choose the parameters that require experimental characterization. During mapping of the process design space, operating parameters were studied using a qualified scale-down model. A screening study using a two level fractional factorial design of resolution IV was first carried out to evaluate the characterization ranges, to identify significant parameters, to assess parameter interactions and to identify potential edge of failure. Results from the factorial design studies were evaluated by multiple linear regression models and multivariate data analysis using the softwares Modde 9.0 and SIMCA, respectively, from Umetrics. Axial point and follow up experiments and worst case studies were then carried out to enhance the model resolution, to identify the process parameter that may have a curvature effect and to confirm the edge of failure. Seven runs, each typically consisting of ten 3 L bioreactors running in parallel, were carried out to characterize the seven process operating parameters chosen from the FMEA exercise. In each run two of the ten bioreactors were operated at the set-point to verify the process consistency (between run variations). Run to run variation and analytical variation made it challenging to develop a reliable model for quantifying the impacts of operating parameters on the process performance and product quality. One corner of the design space had bad processability. The design space was therefore modified to improve the process robustness. Initial evaluation showed that the modified design space enabled a reasonable product yield while ensuring acceptable product quality.
QUALIFICATION OF SCALE DOWN BIOREACTORS FOR VALIDATION OF PROCESS CHANGES IN COMMERCIAL PRODUCTION

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The biopharmaceutical industry increasingly invests in post regulatory-approval process improvements to reduce production cost, increase efficiency and/or improve product safety. However, before implementation in Manufacturing, many changes must be first validated. Validation of cell culture and fermentation process changes at full production scale is often not feasible, calling for the use of qualified scale down platforms.

Here we propose an approach, in line with current regulatory guidelines, to qualifying a small scale bioreactor in order to justify its use for validating process improvement changes. Data will be presented demonstrating the application of the principals of this approach in the scaling down of a commercial perfusion bioreactor at a Bayer HealthCare commercial manufacturing facility. Results demonstrating comparable performance between the scales will be discussed through: (1) scale down system design and operation; (2) cell culture metabolism, growth and productivity and (3) product quality attributes. The design of specific experiments to demonstrate comparability between the scale down and the commercial production scale will be covered as well. The scale down system can then be used in applications that include validation of future process changes for direct implementation in Manufacturing.
DEVELOPMENT OF A SCALE-DOWN MODEL OF THE INACTIVATED POLIO VACCINE PRODUCTION PROCESS

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Industrial scale inactivated polio vaccine (IPV) production dates back to the 1960s when at the “Rijks Institute voor de Volksgezondheid” (RIV) in Bilthoven a process was developed based on micro-carrier technology and primary monkey kidney cells. Starting in the 1990s, the process was improved by scale-up, and in a later stage, the introduction of Vero cells as replacement of the primary cells. The challenge of such long-running manufacturing processes is to keep the knowledge up to date and at the level required for future, or even inevitable, process changes.

To increase the knowledge on the IPV production process, data from over 50 production runs were analyzed using multivariate data analysis. The explorative analysis performed on single unit operations indicated consistent manufacturing. Although variation in the dataset was large, this method allowed to detect outliers and to set specifications for important variables like cell densities and product yield. The information obtained from this analysis is being applied in process development studies for which a scale-down model of the production line was established. Using this scale-down model, the process, the knowledge, and the design space can be further explored. It was aimed that this scale-down model would encompass the complete IPV production process, i.e. all unit operations were scaled down separately but when used in line they, as a whole, translate their large-scale counterpart.

Cell culture and subsequent virus production were scaled down from 750-L to 2.3-L bioreactor volume. Growth curves and metabolite consumption and production rates were largely in agreement. Further, a scale-down model of the following down-stream processing unit operations was setup successfully: clarification by means of dead-end filtration, concentration using tangential flow filtration, size exclusion chromatography (SEC), ion exchange chromatography (IEX) and formaldehyde inactivation. In this contribution practical results of this unique scale-down approach of a complete vaccine production process will be presented.

Unfolded protein response (UPR) is the primary signaling network activated in response to the accumulation of unfolded and/or misfolded protein in the endoplasmic reticulum (ER). The expression of high levels of recombinant proteins in mammalian cell cultures have been linked to increased UPR. However, the kinetics of different UPR-mediated events and their impact on cell performance and recombinant protein secretion during production are ill defined. We created an UPR-responsive, fluorescence-based reporter system to detect and quantify specific UPR-mediated transcriptional activation of different intracellular signaling pathways. We generated stable antibody-expressing clones containing this UPR responsive system and established FACS-based methods for continuous, real-time monitoring of endogenous UPR activation in cell cultures. We found that clones differed in their UPR induction pattern; both the timing and the degree of UPR-induced transcriptional activation were linked to the growth, viability, and productivity of the cells. In addition, endogenous UPR activation was significantly impacted by the cell culture environment, i.e. amino acid levels and osmolarity. We will discuss the role of UPR-mediated transcriptional activation of different signaling pathways on cell performance during recombinant protein production, and the use of an inducible system and UPR monitoring to engineer or improve control of recombinant protein production.
A RATIONALLY INTEGRATED APPROACH FOR FED-BATCH CELL CULTURE PROCESS OPTIMIZATION

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The majority of therapeutic proteins, including recombinant monoclonal antibodies, are produced in fed-batch cell culture processes which offer the advantages of high volumetric productivity and low operational complexity. Continuous interest in fed-batch process development and optimization is driven by increasing biopharmaceutical market demands. Fed-batch process optimization generally includes refining one or more of three elements: basal medium, feed medium, and process parameters. In a recent collaborative project, the goal was to upgrade a high-performing fed-batch platform process targeting titer improvement. The initial approach involved optimizing the basal medium while retaining the existing feed medium and process parameters. After evaluating a total of 46 different basal medium formulations in 3 rounds of design of experiment (DOE) studies, no titer improvement was achieved in fed-batch cell culture process. In an attempt to resolve the issue, we used an iterative approach to evolve to a new workflow. The new workflow included a combinatory optimization of basal and feed media (a total of 17 feed variants) followed by an integrated feed and process optimization (a total of 24 fed-batch conditions). Without impacting the overall project timeline, the new workflow resulted in an upgraded fed-batch process boosting the IgG titer by 40%. From the project, it is evident that the basal medium and feed have an interrelated impact on process outcomes ("pairing effect"). Therefore, a combined or integrated approach, addressing the networked nature between the basal medium and feed, should be considered to maximize the fed-batch process outcome. Furthermore, the concept of rationally integrated optimization is proposed based on the new approach utilized in this project. The rationally integrated approach would enable multiplexing optimization of basal medium, feed medium, and process parameters through a set of studies within a reasonable timeline, and thus can be applied to best serve the practices of fed-batch cell culture process optimization.
IMPROVING PRODUCTIVITY OF CHO CELLS CULTURES BY ENHANCING ENERGY METABOLISM DURING CELL GROWTH

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The importance of CHO cells in the pharmaceutical industry is given by their use as the standard host for therapeutic protein synthesis. Because of the high operating costs of these processes, many efforts have been made to improve their protein production yield. Previous works have shown that when metabolism is efficient, producing little lactate per glucose consumed, cultures are able to produce recombinant protein at higher specific rates. To decrease lactate production from pyruvate, several works have proposed the reduction of lactate dehydrogenase activity and enhancement pyruvate carboxylase and malate dehydrogenase II activity. These changes resulted in cultures which are capable of achieving higher cell densities, and in the first two cases superior protein synthesis. In an intracellular level these cells showed an improved energy metabolism because of higher fluxes and increased availability of pyruvate for the TCA cycle.

In this work we aim to engineer CHO cells by introducing changes in two key points of pyruvate metabolism. We propose to generate engineered CHO cell that overexpress PYC or MDHII genes while presenting decreased LDH activity. This should result in a redistribution of fluxes in the pyruvate node where most of it goes into the TCA cycle, therefore enhancing the cells’ energy metabolism. A comparison of the effect of these changes under similar culture conditions has not been shown to date. We present a comparative analysis of the impact of over-expressing one or two genes simultaneously on the culture’s productivity, for the same cell line and culture conditions.

IgG producing CHO cells were transfected using Lipofectamine 2000 with vectors pcDNA3.1-MDHII, pIRE-PSYC and/or LDH_KO and selected with the appropriate antibiotics. Growth curves were performed with each clone and wild-type CHO cells as a control to study the cultures' performance and metabolic state. Cell density, glucose, lactate and IgG concentrations were monitored and specific rates and metabolic efficiency, characterized by ΔL/ΔG, were calculated.

Comparison of cells that over express PYC and MDHII against control cells show great improvement in terms of cell density, reaching concentrations between 1.5 and 2 times higher than wild-type CHO cells. Metabolism was greatly enhanced reaching lower levels of ΔL/ΔG, reflecting a better use of glucose with lower lactate production. Finally, both modified cells outperformed control CHO cells in terms of IgG productivity, synthesizing approximately 50% more with specific rates that double those of the control. Comparing performance of both clones, overexpression of PYC in CHO cells has greater impact over cell density and metabolic efficiency than MDHII, but regarding recombinant protein production both reach similar concentrations and specific rates. Results show that cells over-expressing more than one key enzyme show greater improvement than cells with only one modification. We expect that combination of overexpression of a limiting step and underexpression of an undesirable pathway will improve the cells performance by enhancing their energy metabolism.

Poster Number 205
Chinese hamster ovary (CHO) cells are routinely used in the biopharmaceutical industry as factories for recombinant proteins. Recombinant protein expression in CHO cells could potentially be increased via microRNA (miRNA) manipulation. Recent reports of miRNA functional studies in CHO cells involve the perturbation of miRNA expression by varying culture conditions in order to investigate the roles of miRNAs in cellular processes. For example, temperature shift in CHO cell cultures to study miRNAs involved in proliferation and cell cycle arrest. To understand the role of miRNAs in conferring increased productivity in CHO cells, we carried out high throughput sequencing of four in-house generated IgG-expressing CHO subclones of varying productivities (5-20 pg/cell-day).

The reads were mapped to miRBase (release 17) to identify conserved miRNAs. Comparative analysis between the miRNAs expressed by the high and low producers revealed that 22 miRNAs were differentially expressed (>1.5-fold). Following that, the integrative web resource miRecords was used to identify the gene targets of these miRNAs in human, mouse and rat. Genes which are predicted by a minimum of three programs to be targeted by at least 3 differentially up/down regulated miRNAs were consolidated for pathway enrichment analysis using Metacore™.

Interestingly, the predicted targets of miRNAs upregulated in high producers are enriched in various apoptotic pathways, whereas predicted targets of downregulated miRNAs were found to be enriched in immune response signaling, cytoskeleton remodeling and clathrin coated vesicular transport. Our results suggest that miRNAs could regulate productivity in CHO cells via the repression of apoptotic or de-repression of protein secretory pathways. Assessment of the effects of overexpressing / knockdown of the differentially expressed miRNAs on these pathways is currently ongoing and will be reported.