A Review on Current Trends of High Titer Producing Mammalian Cell Line Generation And Use of HTP Tools For Cell Culture Process Optimization

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Abstract- *The complexities at various levels places great challenges to biopharmaceutical industry for manufacturing safe and effective biosimilar product. Over the last few years, the industry has shifted its production towards mammalian expression system along with gradual evolution from conventional technology towards advancements to improve quality & production of therapeutics proteins, industrial enzymes and hormones. Advances in cell engineering giving advanced expression platforms have proved to be stepping stone for improving titer in difficult to express proteins. These advances include OSCAR system, GS system, TALEN, CRISPR, FACS, ClonePIX and helps to reduce the required time of top clone selection as compared with conventional processes. Moving forward with top clones encompasses vigorous media and feed screening processes along with scale up studies which in conventional methods requires multiple shake flasks experiments and small reactors with more human interventions. The Fastest growing technology has given us various new advancements like AMBR 15 allowing us to perform number of experiments in one stretch, AMBR 250 as a scale down model and performing process characterization with integrated DOE. Drug development has to be regulatory compliance which has new amendment of QbD, many process analytical tools (PATs) are rolling in market for giving more sound precise understanding of process parameters. These tools include different DoE softwares such as MODDE, JMP for experiment design and SIMCA for Multivariate Data Analysis which enables to enhance the creation of Design Space for drug development. This review article throws light on the broader spectrum of drug development covering most of the critical phases of development.*

***Keywords*-** Biosimilar, Cell lines, Expression platforms, CRISPR, DoE, Glycosylation, Mammalian process, TALEN, QbD

I. INTRODUCTION

The biosimilar industry has entered into a very interesting and competitive phase as patents of blockbuster molecules have been expiring. The sales revenue for biosimilars in US stands at $1.9 billion in 2015 and anticipated to be $11 billion by 2020[1]. Commercial success of biosimilar is highly dependent on the development of high yielding stable cell line with quality target product profile, as it results in consistent, uniform product yield and quality, which have impact on the cost of goods (COGs). Development of high yielding stable cell line with quality target product profile is multifactorial and dependent on expression host system, expression vector design, transfection, ratio of heavy chain and light chain constructs, signal peptides, selection methods, Cell Engineering, media and feed selection and process monitoring.

I. EXPRESSION HOST SYSTEM

Various cell lines including NS0, SP2/O, BHK, HEK 293, CAP, HT-1080, Per.C6 are being used for large scale production, Chinese Hamster Ovary (CHO) cell line remains the foremost choice for the production of monoclonal antibody (mAb) and non-mAb glycoproteins. Nearly 70 % of all recombinant therapeutic proteins are produced in CHO cells[2]. The reasons for the wide usage of CHO cells are multifold and are as follows: CHO cells have been demonstrated as a safe host as pathogenic human viruses like HIV, influenza and polio do not replicate in CHO cells greatly increasing the safety of the protein therapeutics and might become easier to get regulatory approvals for marketing; adaptability and plasticity with respect to the phenotypic characteristics that are necessary for the industrial production; growth in suspension, easily adaptation to number of steadily improving chemically defined media and production of proteins of high quality suitable for safe use in humans with low occurrence of immunologic reactions.

There are associated drawbacks of the CHO cells with above mentioned advantages: low specific productivity; large number of clones need to be screened for each new production cell line to identify the few clones with high productivity with desirable quality, CHO cells have the advantage of the presence of high percentage of Neu5Ac, but still include some Neu5Gc which is immunogenic in humans.

CHO cell were established in the laboratory of Dr. Theodore T. Puck in 1957. CHO-DUKX-B11 and CHO-DG44, are DHFR deletion mutants, were isolated from different subline of original CHO cells by Chasin at University of Columbia, New York[3]. These cell lines are used efficiently with DHFR vector system. CHOK1SV is a suspension variant developed by Lonza, is a GS deletion mutant, used with GS vector system. CHOZN and HD-BIOP3 are other two GS null cell lines available for bioproduction from SAFC and Horizon respectively.

A new host cell line for the production of recombinant antibodies has been developed through collaboration between Lonza and BioWa. The new cell line, Potelligent® CHOK1SV, combines the advantages of Lonza's proprietary GS Gene Expression System™ with the power of BioWa's engineered glycosylation technology. This cell line produces afucosylated antibody which have enhanced ADCC activity[4].

II. DESIGN CONSIDERATIONS FOR EXPRESSION VECTOR

The expression of heterologous proteins in mammalian system requires the dedicated vectors to transfer the gene of interest into the host cells. The vectors should have following feature: the expression levels of target protein should not be dependent on the integration site in the host genome, so that expression levels of target gene could be linked with the number of transgenic copies integrated into the host genome[5]. Cell line development starts with transfection of a mammalian cell line with expression vectors or expression construct carrying gene(s) of interest. The expression of biosimilar molecules in mammalian cells for commercial production requires use of specialized vectors having strong viral or non-viral promoters, the Kozak sequence, polyadenylation sequence and metabolic selection marker gene and/or antibiotic selection marker and specialized genetic elements. These vectors are usually shuttle vectors contain elements to facilitate clone preparation and propagation in bacteria.

2.1 **Promoters and Enhancers**

The promoters and enhancers are the genetic elements that facilitate the expression of the recombinant gene. The promoter plays an important role in helping to correctly position the beginning of transcription, the enhancer elements help to increase the level of transcription. These two elements most widely used are derived from the Cytomegalovirus (CMV) and Simian virus 40 (SV40)[6]. Other sequences are also available for rodent-derived cell lines, such as Myeloproliferative sarcoma virus (MPSV), Rous sarcoma virus (RSV), Visna virus and CHO elongation factor-alpha (EF1-a)[7].

Promoters are prone to transcriptional silencing and randomness of their integration into the host genome remains a problem that results in inaccurate control of the relative expression of the genes. The development of synthetic promoters could solve many problems but their application would probably be cell-line specific[8].

2.2 The Kozak Sequence

Sequences immediately upstream of an initiation start codon play a significant role in the initiation of translation[9]. The 6-9 bases proximal to an initiation codon AUG referred to as the Kozak (GCCG/ACCAUGG) sequence facilitates start codon recognition by ribosomes to begin translating the DNA sequences into polypeptides. Purine at -3 and a G at +4 within the coding sequence can influence translation efficiency.

2.3 Polyadenylation Sequence

Polyadenylation signal plays important roles in proper transcription termination, extends half-life of mRNA in the cytoplasm and enhances the efficient translation. Despite their simple sequence composition, terminal polyadenosine have critical roles in multiple aspects of a transcript's life cycle.

The poly(A) tail is important for mediating the translocation of a completely processed mRNA to the cytoplasm, in addition, can play a key regulatory roles in enhancing translation efficiency. The most important poly(A) tails are vital for regulating the efficiency of mRNA quality control and degradation[10]. SV 40 and Bovine growth hormone (BGH) polyadenylation sequences are predominantly used for CHO cells.

2.4 The Expression System

The expression vector for stable cell line development also carries, metabolic selection marker gene in addition to antibiotic selection marker on the same or different vector. The metabolic selection genes increase the stringency of selection and amplify their copy number along with a gene of interest when the inhibitor concentration is increased gradually in the selection medium. For CHO cells, two powerful gene amplification systems are available: 1. DHFR (Dihydrofolate reductase) expression system and 2. GS (Glutamine synthetase) expression system.

2.4.1 DHFR Expression System

The DHFR expression systems carry DHFR coding gene under a separate promoter or fused to downstream of gene of interest (GOI) with internal ribosome entry site (IRES) at the N terminus. DHFR catalyzes the formation tetrahydrofolate from folic acid which is required for glycine, purines and thymidine. The availability of DHFR negative CHO-DUXB11 and CHO-DG44 allowed selection stringency in the medium lacking hypoxanthine and thymidine and an approach to amplify genes with the help of DHFR antagonist, (methotrexate, MTX). The selection of recombinant cell lines using stepwise increases in MTX concentration in the culture medium results in amplified copies of the transfected DHFR gene together with the GOI[11].

2.4.2 GS Expression System

The GS expression systems carry GS gene under weak promoter and exploits the glutamine metabolism in mammalian cells. GS is a dominant selectable marker that can be used with GS-negative NS0 cells as well as CHO cells that contain an active endogenous GS gene. Since GS gene catalyzes the formation of glutamine from glutamate and ammonia, this offers the best option for selection in the absence of endogenous GS gene and glutamine free selection media. In addition, the application of a GS inhibitor (methionine sulfoximine, MSX) allows either an increase of the stringency of selection or gene amplifications using increasing levels of MSX like DHFR/MTX system[12]. The GS selection system which is now out of patent protection, different gene editing tools like ZFN, CRISPR and rAAV mediated have been used successfully to delete GS genes from CHO cells[13].

The GS/MSX expression system has advantages over DHFR/MTX system in development timelines as it doesn't require repeated amplifications and very few copies of GOI are required to attain required productivity[14]. (Figure 1)

2.4.3 OSCAR Expression System

Using this system, selection and amplification occurs in single step, providing rapid selection of clones producing high levels of protein. Developers of this expression system claim that, the higher yield, time, growth rate, and stability are better than the prevailing expression systems. OSCAR™ is based on a series of partially disabled minigene vectors that encode for hypoxanthine phosphoribosyltransferase (HPRT). HPRT is essential for purine synthesis via the normal cellular salvage pathway. These minigenes have varying degrees of expression disability, allowing their use in a range of cell types that have different requirements for HPRT expression. HPRT-deficient mammalian cells transfected with one of these minigenes and a gene of interest are placed in selective HAT medium that blocks de novo purine synthesis, making cell survival reliant on the salvage pathway using a disabled HPRT enzyme. Large amounts of the disabled HPRT enzyme are required for cell survival, thus, driving gene amplification. In addition, the more disabled the minigene, the more amplification is required and, generally, the higher the expression levels achieved[15].

2.4.4 Specialized Genetic Elements

Stable cell line development involves transfection with linearized expression construct. Inside the cell, construct integrates into the host genome randomly and site of integration influences the transcription rate. These gives rise to considerable heterogeneity in the pools of transfected cells with varying degree of expression of GOI; this phenomenon is called the position effect[16]. To overcome the position effect, various approaches and strategies have been developed which include incorporating specific elements like scaffold or matrix attachment regions (S/MARs), ubiquitous chromatin opening elements (UCOE), expression augmenting sequence elements (EASE), stabilizing and anti-repressor (STAR) elements[17].

An additional element that has been included in expression construct is the IRES, is used to link expression of multiple genes in a Cap independent translation mechanism. It is a RNA sequence that forms a complex secondary structure that allows the initiation of translation from any position within an mRNA immediately downstream from where the IRES is located. The main IRES sequence used for the expression of heterologous genes are derived from Foot and Mouth Disease virus (FMDV) and Encephalomyocarditis virus (EMCV)[18]. The EMCV virus sequence is much more frequently used and consistently delivers slightly higher expression in the cell types. Placement of the selection marker gene at downstream of the gene of interest confirms that expression of selection marker is dependent on the successful transcription of the gene of interest. There are several advantages to linking the expression of multiple genes through the use of IRES. Steven C. L. Ho et al (2013) had used IRES to control the intracellular expression ratio of light chain and heavy chain in CHO cells to study antibody expression, aggregation, glycosylation and conformational stability. It has been found that more than 97% of secreted IgG was monomers when the LC: HC ratio was ≥ 1[19].

In a recent study, the application of IRES has also allowed for high recombinant protein production from a

methotrexate amplified cell pools, without limiting dilution of cloning[20].

The other approach for overcoming the position effect is to target site-specific integration. Recombinase enzymes are used to exchange the transfected DNA into the donor hot spots in the pre-established host cell line. Three examples include the Cre recombinase mediated recombination at loxP site[21], Flp recombinase mediated recombination at Flp recombination target (FRT) site[22] and zinc finger nuclease mediated recombination[23,24]. Effectiveness of these elements for increasing protein expression and stability is largely dependent on the design of expression vector, the selection system and experimental conditions.

2.4.4.1 SINEUPs

These are antisense lncRNAs which work to promote translation of partially overlapping sense protein coding mRNAs. Name SINEUPs denotes that inverted SINE B2 element is required to UP regulate the translation. (Figure 2)

Table 1 illustrates the comparison among different cloning platform.

III. SIGNAL PEPTIDES

Efficient expression of the HC and LC requires appropriate signal peptides for the transport of the HC and the LC polypeptides into the ER for proper folding, assembly and post-translational modification. Several studies have shown that protein production can be enhanced through the use of alternative signal peptides[25]. Native and nonnative signal peptides for expression of antibody molecules have been in use and the best approach to decide on the signal peptides and their combinations would be based on trial and error approach[26].

IV. TRANSFECTION AND DNA DELIVERY SYSTEM

Efficient DNA delivery is one of the most important and critical procedures for generation of high yielding cell lines[27]. Stable Cell line development involves integration of expression construct containing GOI and selection markers into the host system. Unlike transient transfection, in the stable transfection the expression construct is linearized with a suitable enzyme and delivered into cells by chemical or physical methods. Positive transfectants are selected for their drug resistance or growth advantage in the selection medium. Chemical transfection involves usage of cationic polymers (DEAE dextran, PEI, Dendrimer), Calcium phosphate, and cationic lipids (Lipofectin, Lipofectamine). The transfection efficiency of chemical methods is largely dependent on the factors such as nucleic acid/chemical ratio, solution pH, cell membrane conditions and it is also cell type specific. These methods have merits of relatively low cytotoxicity, no mutagenesis and no size limitations.

Electroporation is the most widely used physical method. This is accomplished by applying the electric field to solution containing cells and DNA. This method is less cell type specific and better efficiency can be obtained by working on the voltage, pulse length and number of pulses. This method results in low post transfection viability[28,29].

The efficiency of transfection and level of gene expression after non-viral DNA delivery remain low. It has been observed that DNA delivery approaches system that must be considered depending on cell type and purpose. The ideal method should have high transfection efficiency, low cell toxicity, minimal effects on normal physiology of the cells, and easy to use and reproducible.

4.1 PiggyBac transposon system

Conventional gene transfer relies on random integration of transgene into host genome which requires screening large number of clones to get high producing cell line. Various dissimilarities have been observed in clonal cell lines, reason behind lies integration sites and copy number. Naturally occurring “mobile genetic elements” can be harnessed for integrating transgene into host genome.

PiggyBac, a DNA transposon from Cabbage looper moth, Trichoplusia ni, can efficiently transpose in human, mouse and mice cell lines[30]. Also, PB has shown stable gene expression with more integration promoting clonal expansion in comparison to Sleeping Beauty (SB-11) & Tol2 transposons in primary T cells derived from Peripheral Blood Lymphocytes (PBL)[31].
(Figure 3)

V. CLONE SELECTION

The random integration of the expression construct into the host genome after transfection generates transfectant pools which are heterogeneous w.r.t. owing to integration at different sites, copy numbers and transgene expression. This heterogeneity is further enhanced by selection and amplification procedures. High producing clones rarely occur in the heterogeneous cell populations after transfection and gene amplifications. So, it becomes necessary to isolate the

high producers to have maximum protein productivity, consistency in production process, quality and to meet regulatory approval criterion. Limit dilution cloning is a traditional method of isolating high producer cells where the amplified pool is diluted to low concentration and plated 0.5 to 1 cell per well in 96 well plates. This method is a time consuming labor-intensive and requires multiple rounds of cloning are performed to attain the monoclonality. As a result, there has been increasing use of efficient high throughput cell screening systems such as fluorescence-activated cell sorting (FACS), the ClonePix System, the LEAP system and the CellCelector system[32,33]. (Figure 4)

Clones are selected on the basis of their high expression levels, and their performance is further tested in shake flask suspension fed-batch culture, which is most widely used for large-scale commercial production of therapeutic antibody.

VI. CELL ENGINEERING

The quantity of recombinant protein expressed in a cell culture is dependent on the time integral of viable cell density (IVCD) and specific protein productivity of the cells (qP). To improve IVCD, cell line engineering strategies focus on extending the longevity of cell culture, accelerating the specific growth rate and increasing the maximum viable cell density. These technologies are used in an attempt to modify specific features of the host cells to enhance the desired product yield as well as the quality[34].

Several methods for targeted genetic modification of cells have been developed. Some of those are homologous recombination, RNA interference and zinc-finger nucleases. ZFN technology have recently been reported providing an efficient yet stringent approach for the development of 'tailor-made' cell lines previously too laborious or difficult to generate. Genentech has utilized ZFN technology in conjunction with Sangamo Biosciences to create a cell line deficient in α-1,6-fucosyltransferase8 (FUT8)[35]. The absence of FUT8 allows for the production of mAbs with either reduced or absent fucosylation, thereby increasing ADCC of the molecule, potentially providing more efficacious treatments with a significant reduction in cost[36,37]. Genentech and Sangamo have also used ZFNs to delete the proapoptotic genes, BAX and BAK, in CHO cells to create a cell line with increased resistance to apoptosis, prolonging the production phase in batch culture and leading to a two- to five-fold increase in mAb titer[38,39]. Recently, for targeted genome editing Transcription Activator-like Effector Nucleases (TALENs) have become an alternative approach to ZFNs which relies on the use of engineered nucleases to induce double strand breaks (DSBs) into specific DNA sites. Repairing mechanisms of these DSBs used to create sequence alteration at cleavage site. (Figure 5)

To improve expression levels further through cell engineering requires an understanding of both the host organism and the biology of protein expression for which CRISPR-CAS9 enzyme mechanism has been setting remarkable stage for genomic manipulations experiments. Distinct from protein guided TALEN & ZFN, CRISPR/CAS9 system depends on small RNA sequence for specific cleavage rendering it easily applicable to vast majority of experimentations.[41,42]
(Figure 6)

A goal for the future is robust cell factories generated through a holistic approach that considers all the bottlenecks in the protein expression process such as transcription, translation, protein folding, and secretion and cell viability and engineer these through an integrative process to enable high-level expression of a variety of target proteins.

VII. MEDIA & FEED OPTIMIZATION

Media development is a key factor in improving productivity and growth behavior of cells but it also influences product quality[43]. The most significant improvements to CHO culture so far have resulted from the optimization of media, feeding strategies and processes.

Current development in cell culture technology for a recombinant CHO cells has led to substantial enhancements in target protein's production[44]. The formulation of media and feeds is an important phase of the process development. In the year of 1980s or in early 1990s, average titer at commercial started out at < 0.5 g/L. Over the years, undergone repeated cycles of technical production and upgrades, with titers and yields increasing incrementally, even for the oldest product. BioPlan estimates that ≥ 3.0 g/L is now the current industry standard titer for new bioprocess being developed, with ≤ 7.0 g/L now presumed to be general industrial top end titer level, while not unusual, is not often achieved[45]. Companies devote extensive efforts and time on the optimization of culture media and feeds as a basic development process for each cell line, however, no generic procedure exists for cell culture medium and feed optimization, and reports on the optimization of culture media or feeding strategies for CHO cell cultures are limited in number.

A valuable start to optimizing the medium or feed is to focus on basic groups of ingredients composing mammalian cell culture media. Traditional optimization methods, such as

the titration of single components, are reliable, but can be labor intensive and time consuming. Engineering of cell lines has resulted in improved product quality and some improvements in productivity.

Production of therapeutic monoclonal antibodies in CHO cells in optimized bioprocesses typically reaches 2.75 g/L titers and specific productivity of 50-90 PCD resulting in production costs accounting for only 1 to 5 % of the sales price[46].

Most biopharmaceutical production platforms are based on fed-batch cell culture protocols, which can support high volumetric productivity while maintaining low operational complexity. In a fed-batch process, a basal medium support initial growth and production, and a feed medium prevents depletion of nutrients and sustains the production phase[47].

Optimization of a fed-batch process can be achieved by development efforts by addressing three major elements: basal medium, feed medium and process settings. Media development needs to be optimized for each cell line individually and includes: literature survey to identify possible growth requirements based on related cell line/constructs, component swapping and titration to improve performance in a simple and straight forward way, and medium blending to select the best composition in an evolutionary manner. Approaches for feed media development include variations in the concentration of the basal medium, nutrient consumption, accumulation of impurities and balance of cell growth and volumetric productivity[48].

Bioproduction process involves monitoring of temperature, pH, dissolved oxygen, gas streams, and secondary metabolites and product. Media and feed optimization have direct impact on product quality, glycosylation, culture viability, higher specific productivity and enhanced volumetric production[49]. N-Linked glycan composition is a critical quality attribute for mAbs. N-linked glycosylation profile is essential to ensure the physicochemical, biological and clinical properties of mAbs, including structure stability, solubility, serum half-life, effector function, efficacy, immunogenicity[50].

It was observed in the CHO cells expressing EG2 antibodies that glucose limitation decreased site occupancy, galactosylation and sialylation of mAb. Glucose starvation at a stationary phase of fed-batch culture reduced specific productivity and improved maturation of glycans of mAb. Glucose concentration from 15 – 25 mM was found to be optimal for glycosylation of all the mAb molecules and Galactose Index value changed from 0.35 to 0.72. Addition of 20 mM Galactose resulted in an increase of galactosylated IgG from 14 % to 25 %. The UMG supplements are used to enhance galactosylation which consisted of uridine (4 mM), manganese chloride (MnCl2, 8 μM) and galactose (20 mM)[51,52].

Glutamine limitation increased Man5 glycan in mAb by limiting UDP-GlcNAc synthesis. Glutamine addition lead to high ammonium accumulation, thus, decreased galactosylation and sialyation of a recombinant protein[53,54]. Supplementing amino acids (cysteine, isoleucine, leucine, tryptophan, valine, asparagine, aspartate, and glutamate) that have been depleted in the cell culture increased sialylation of EPO[55]. High specific amino acid consumption rate increased NH4+ concentration in cell culture, thus, increased Man5 glycan of mAb[52].

VIII. PROCESS DEVELOPMENT

In the last 2 decades, volumetric yields from recombinant mammalian cell lines have increased dramatically. In addition to the generation of high-specific-productivity cell lines and formulation of media and feeds to support high-density cell cultivation, as described above, there have also been significant advances in understanding cell culture processes and in sustaining cell viability in high density cultures for optimal production.

Enhancements in a recombinant mAb production on a per cell basis are often the result of combined improvements of cell line, culture medium, and culture conditions. The almost 10-fold improvement in the titer every decade since 1980 has been partially due to improvements in expression technology and clone selection, as noted above, and partially due to the optimization of cell culture processes, which include media and feeds and culture conditions.

There are some important considerations in developing mammalian cell culture processes, including (i) cell generation number, affecting product yield and quality (ii) production bioreactor seeding age and density, affecting productivity during the given cycle time (iii) medium performance, making a direct difference in product titer or even quality (iv) feed rate, determining how long cell growth and production will be sustained and (v) parameters such as dissolved oxygen (DO), dCO2, pH, osmolality, lactate and ammonium level, etc. A step by-step process development approach is always performed, which led to titer progression by 30-fold to 4.3 g/liter for antibody. (Table 2)

Bioreactor process parameters influence the growth behavior and metabolism of mammalian cell culture, and the quality of the produced monoclonal antibodies (mAbs). A systematic assessment of their impact allows for a better link between process-related parameters and cellular processes, such as N-linked glycosylation, lactate metabolism and cell cycle transition. Hence, understanding the effect of process parameters is a prerequisite for providing better controllability over cell growth, productivity and mAb critical quality attributes[56].

Approaches in Upstream Process Development

Due to worldwide competition for making biosimilar molecules, each company has utmost objective of understanding the biotherapeutic molecule thoroughly and developing rapid and robust process. This is compounded by indigenous time, difficulties and expenses though there is need to hit market as quickly as possible with cost effective product and fulfilling all regulatory requirements. To meet these requirements, recent advances in bioprocessing include improvements in increasing adaptability to single-use systems and components, processes automation and Quality by Design approach[57]. This can be supported by robust analytical methods to characterize a product, risk assessment and developing reliable manufacturing process.

Incorporation of advanced automated tools in the course of clone selection and cell culture process optimization for producing high titre producing cell line are now in fashion to reduce time and cost. Tools like FACS, ClonePix, OctetQKe (PALL life sciences), mAb Glyco Chip-Kit (Thermo) has outweighed the advantages to conventional methods of clone and process screening. These cost effective automated system have provision to analyze the samples at micro scale with higher accuracy value.

Advancement in Upstream Process

Single use automated ambr15 micro bioreactor has replaced shake flask and classical 1L/2L bioreactors and mimics the process at micro scale. Ambr15 comprises DoE and fully controlled system to understand the process parameters and its effect on quality attributes. Ambr 250 is the single use scale down model for large scale bioreactor. Advanced automated system software and BioPAT system for online measurements of cell count (Vi-CELL XR, Beckman), metabolites (Bioprofile FLEX analyzer, Nova Biomedical) have been facilitated with its controlled bioprocess which reduces human errors. The integrated DoE software helps analyzing data and understanding the process thoroughly. This helped not only to control process, also to control the factors affecting critical quality attributes (CQAs). Design space can be optimized to develop and characterize the process. Aspects for robustness, scalability and reproducibility up to commercial scale is served by these high throughput tools. Figure 7 demonstrate the advanced HTP tools and process analytics tools used in processes from clone to commercial scale.

The advent of biosimilars is driving a desire to achieve lower CoGs and globalize biologics manufacturing. Use of advanced high throughput tools are not abnormal nowadays. HTP tools have significantly contributed in achieving high titer mAbs. In recent times, to meet the demand of product and to lead the market, manufacturers are moving to the productive alternative to achieve high titre with the best quality using Perfusion Technology. In contrast to the fed batch, perfusion process can be run over much longer period, even months, by continuously feeding the cells with fresh media and removing spent media while keeping cells in culture. This fresh media exchange replenishes nutrient levels for optimal growing conditions and cell waste product is removed to avoid toxicity[58], furthermore, the product is regularly removed before being exposed to excessive waste that leads to protein degradation. This supports unstable product to be harvested and purified much quickly to avoid product loss. Perfusion bioreactors outweighs the advantage over fed batch in footprint, labor, utilities, capital cost, efficient, product stability in culture, process convenience, flexible scalability and market demand, ultimately ending up with cost effective drug production[59]. Figure 8 explains the application of perfusion in seed train and production time line reduction.

Interestingly, an efficient fed-batch process with significantly reduced seed train and production duration was successfully demonstrated using ATF perfusion technology. The improved process involved the preparation of High Density Cryo-Seed-Intermediates (HDCSI) and n-1 seed expansion using ATF, and high seeding-density fed-batch production bioreactor. This method not only reduced the overall process time, but also decreased the number of seed expansion, which could provide a substantial throughput improvement and operating/equipment cost saving[60]. Concentrated fed-batch and concentrated perfusion are two production techniques based on the ATF System, which simultaneously nourishes the culture and concentrates the product within the bioreactor. These manufacturing methods permit great increases in cell and product concentrations as compared with fed-batch and perfusion.

Data analytics

Regulatory agencies, patents and science & technology are forcing rapid change on pharmaceutical manufacturing. Most important agenda for giant pharmaceutical manufacturing are the innovation and efficiency of the facility. These factors are most critical to sustain in upcoming years of development. The ICH Q8(R2) Guidelines describes Quality by Design as a systematic approach to development that begins with predefined objectives and emphasizes a product and process understanding and process control, based on intense science and quality risk management. The integrated MODDE DoE for design of experiments (DoE) and SIMCA software for multivariate data analysis (MVDA) enables the fast and straightforward implementation of a QbD approach to drug development. These tools empower easy and safe recipe transfer across scales, furthermore, monitoring and analysis of critical process parameters (CPPs) and critical quality attributes (CQAs) during upstream processing are possible with the PAT toolbox of sensors, controls tools and MVDA software[61].

These rapid developments of high-yielding and robust manufacturing processes for monoclonal antibodies is an area of significant focus in the biopharmaceutical landscape. Advances in mammalian cell culture have taken titres to beyond the 10g/l mark and expected to increase in the future.

IX. SUMMARY

Biopharmaceuticals, predominantly mAbs, have been growing at a much faster rate than the traditional pharmaceutical industry in recent years, and this trend is continuing. Mammalian cells are the favorite production system due to their capacity to produce human like post translational modifications to complex protein therapeutics. High productivities and desired product qualities have been achieved by the development of high yielding and stable cell lines, animal free and productive media, high density fed-batch cell culture conditions, and successful scale-up to large manufacturing bioreactors. Novel technologies such as enhanced expression systems, automated screening methods, cell line engineering, improved process monitoring, and disposable apparatuses are leading to more productive and efficient production of biopharmaceuticals by mammalian cell culture. Incorporation of HTP devices and different softwares leads to improve the timeline for generation of biotherapeutics. Utilization of single use systems in cell culture process minimizes the human born errors and facilitates the reduced timeline and complete utilization of facility. Adapting the QbD approach during drug development emphasizes the product and process understanding and process control, based on intense science and quality risk management.

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Figure 1

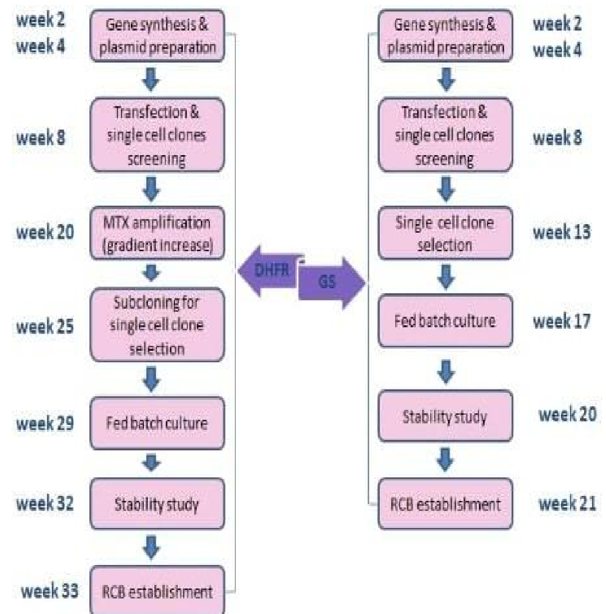


Figure 2

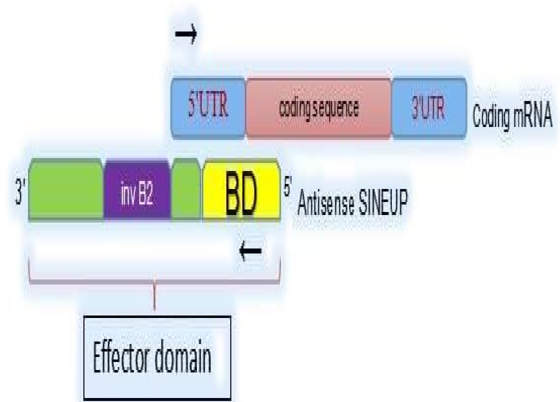
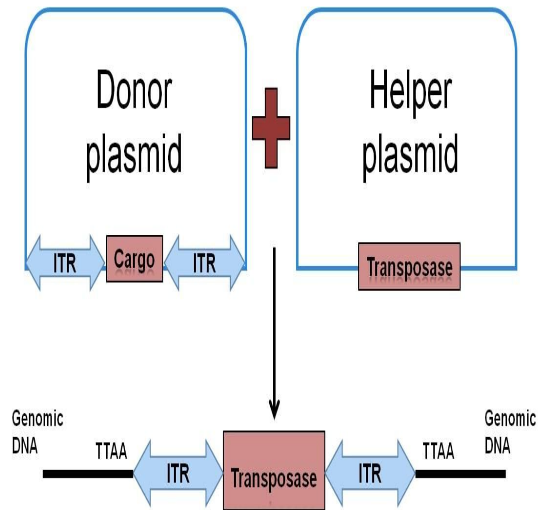


Figure 3



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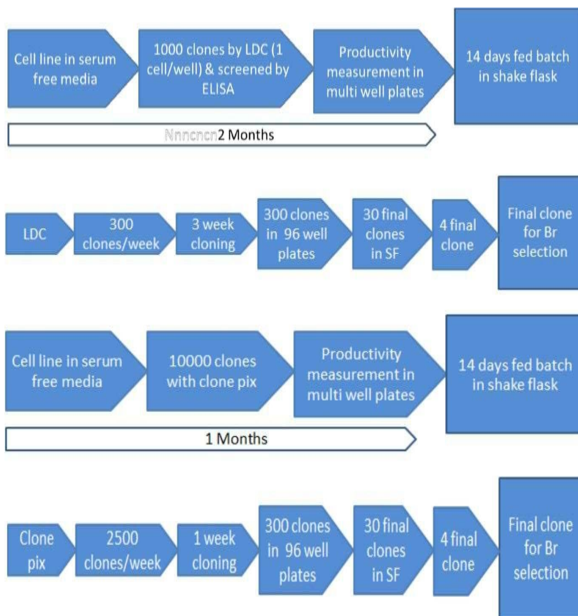

Figure 5

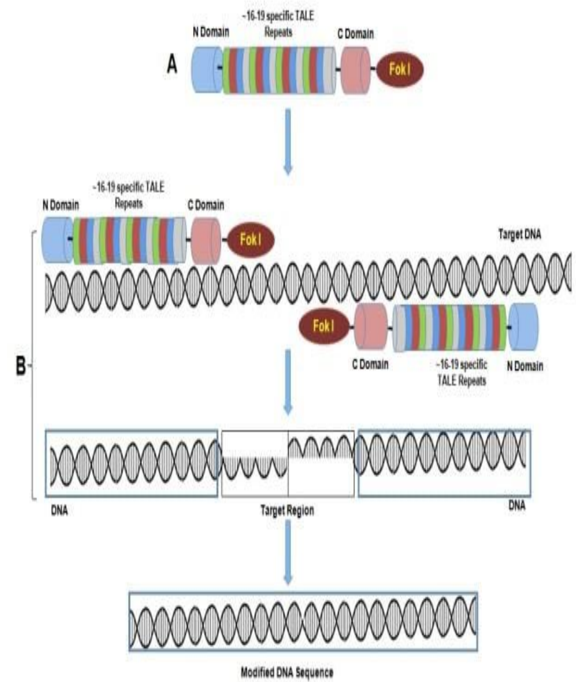

Figure 6

Figure 7

Figure 8

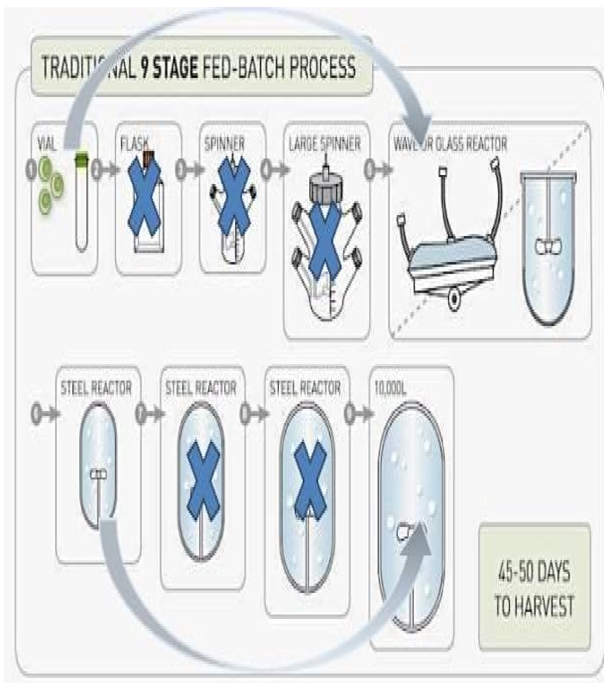

Figure 1. Schematic presentation of cell line development timelines with DHFR and GS expression systems

Figure 2. SINEUP structure and principle- SINEUPs contain two functional domain 1. Binding domain (BD) for target specificity 2. Effector domain (ED) for activation of protein synthesis

Figure 3. Diagramatic representation of gene transfer by PiggyBac Transposon.

Figure 4. Comparision between traditional limit dilution cloning and high throughput ClonePix for screening and isolation of high producer clones

Figure 5. Overview of TALENs. A) TALEN Modular structure containing TALE Repeats sandwiched between N-Terminal domain and C-Terminal domain. FokI nucleases is attached to Carboxy terminal. B) Dimerization of TALEN monomer is the key behind whole process, producing double stranded cut at the spacer sequence between two monomer by FokI cleavage.

Figure 6: Application of CRISPR-Cas9

Figure 7: Advanced HTP tools and process analytics tools for clone to commercial scale

Figure 8: Perfusion application in seed train and production time line reduction

Source: Perfusion and Beyond The XCell™ ATF System, Earl Pineda, May 9, 2018

Table 1. Comparision of clonning platforms

Characteristics	DHFR-system	GS-system	OSCAR system (HPRT)
Selection marker	MTX	MSX	HAT
Amplification	Required	Partial required	Not required
Single cell clone selection	~6 months	6-8 weeks	3-5 weeks
Clone stability	Less stable	Stable	Highly stable
Gene copy number	Less	High	High
Product titer	Low	High	Very high
Cost of goods	Costly	Costly	Cheaper
Commercialization purpose	Yes	Yes	Still under evaluation

Table 2: Effect of cell culture process variables on Glycosylation

Variables	Effect on glycosylation
Bioreactor pH	Galactosylation, sialylation, micro heterogeneity vary with pH set points (6.8–7.8)
Dissolved Oxygen (DO)	Variable effect on glycosylation that is cell line specific and/or protein specific, typically 10–100% DO will allow consistent glycosylation
pCO2	Polysialylation decreases with increasing pCO2 and decrease in NGNA when pCO2 is increased
Temperature	Low temperatures (30◦C) causes a decrease in sialic acid
pH and Temperature	Shifting pH to <7.0 while reducing temperature maintains productivity and sialylation