Industry view on the relative importance of "clonality" of biopharmaceutical-producing cell lines

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ARTICLE INFO

ABSTRACT

Recently, several health authorities have requested substantial detail from sponsor firms regarding the practices employed to generate the production cell line for recombinant DNA-(rDNA) derived bio-pharmaceuticals. Two possible inferences from these regulatory agency questions are that (1) assurance of "clonality" of the production cell line is of major importance to assessing the safety and efficacy of the product and (2), without adequate proof of "clonality", additional studies of the cell line and product are often required to further ensure the product's purity and homogeneity. Here we address the topic of "clonality" in the broader context of product quality assurance by current technologies and practices, as well as discuss some of the relevant science and historical perspective. We agree that the clonal derivation of a production cell line is one factor with potential impact, but it is only one of many factors. Further, we believe that regulatory emphasis should be primarily placed on ensuring product quality of the material actually administered to patients, and on ensuring process consistency and implementing appropriate control strategies through the life cycle of the products.

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1. Introduction

Recently, several health authorities have requested substantial detail from sponsor firms regarding the practices employed to generate the production cell line for recombinant DNA-(rDNA) derived biopharmaceuticals. Two possible inferences from these regulatory agency questions are that [1] assurance of "clonality" of the production cell line is of major importance to assessing the safety and efficacy of the product and [2], without adequate proof of "clonality", additional studies of the cell line and product are often required to further ensure the product's purity and homogeneity. Here we address the topic of "clonality" in the broader context of product quality assurance by current technologies and practices, as well as discuss some of the relevant science and historical

perspective. We agree that the clonal derivation of a production cell line is one factor with potential impact, but it is only one of many factors. Further, we believe that regulatory emphasis should be primarily placed on ensuring product quality of the material actually administered to patients, and on ensuring process consistency and implementing appropriate control strategies through the life cycle of the products.

1.1. Historical perspective

Mammalian cells have been used to produce rDNA-derived human therapeutic proteins for over 25 years. ICH and analogous regional guidelines were developed and orthogonal control strategies have been applied to ensure consistent product safety and efficacy during clinical development and commercialization. These guidelines and control strategies include but are not limited to I) development and validation of appropriate and robust

manufacturing processes, starting with generation of the production cell line and II) monitoring of those manufacturing processes and resulting biopharmaceutical products through pertinent control strategies and characterization. Collectively, these control principles have worked well to provide a steady stream of over 100 therapeutic biopharmaceuticals which have significantly benefited public health, while building an admirable record from the standpoint of product consistency and safety over this period of time [1,2].

An important characteristic of any biopharmaceutical manufacturing process is consistent cell culture performance, in turn delivering consistency in product quality attributes. Cell culture performance can be impacted by many factors, including the production cell line. However, it is critical to recognize that a culture of any production cell line consists of a population of cells and absolute genetic homogeneity, whether of the transgene or at the genomic level, is not achievable given the genomic plasticity inherent to immortalized mammalian cell lines [3]. This situation is especially true in the case of immortalized cell lines typically employed for production (e.g. CHO and NS0) and the generational span encompassed from introduction of product-encoding transgene to the End of Production Cells at the limit for *in vitro* cell age [4]. Therefore, referring to a production cell line as a "clone" or to the "clonality" of a manufacturing cell bank is misleading, as any population of these types of cells cultured for a length of time will accumulate genetic and phenotypic heterogeneity [5]. In the strictest sense, a more accurate description would be that these cell lines can have a high probability of being clonally-derived. That is, the cells can be grown from what is likely to be a single cell through a laboratory manipulation (termed "cloning"). One can subsequently obtain a population of cells that are derived from that cloning event. Post-cloning, during cell growth and expansion, a variety of factors create genomic heterogeneity including inherent DNA replication errors, error-prone SOS DNA repair processes of immortal cell lines and Darwinian selection. These factors combine to introduce, amplify, and select for genetic variation within the cell population. To better understand the potential impact of these types of cells on biopharmaceutical manufacturing, it is important to first understand their underlying nature.

The ability to grow mammalian cells for rDNA technology in culture has relied on the selection of cell populations that have escaped normal control of cell division. This attribute is inherently aided by genetic perturbations that cause impairment of cell cycle checkpoints, and early attempts to grow cells in culture for extended periods were only successful when they were isolated from neoplastic tissue or were spontaneously "transformed". Many cell lines generated in this way displayed increased genetic drift and chromosomal instability. For the biotechnology industry, this research fortuitously involved the isolation of an immortalized cell line from an ovarian biopsy of a juvenile female Chinese hamster [6,7]. This work was part of much broader studies in which careful techniques were honed to isolate human cell lines from a variety of tissues. It would have never been imagined by Theodore Puck and others that these hamster-derived cell lines would go on to become the production system underlying our ability to produce a multitude of protein therapeutics which have changed the lives of so many patients and helped create a biotechnology revolution.

The commercial need to grow mammalian cells outpaced the fundamental scientific understanding and for many years successfully culturing these cells relied on the presence of complex, undefined additives such as serum or embryo extracts. Advancement in bioreactor engineering, characterization, automation, and the development of chemically-defined media in addition to the ability to adapt cells to suspension growth has enabled the

biotechnology industry to expand cells rapidly from a cryopreserved state into culture volumes ranging from static microwell plate with culture volumes of <1 mL to >20,000 L in stirred bioreactors. Despite these advances, the fundamental nature of these cells do not allow for control of the genetic and phenotypic drifts that occur whenever such mammalian cells are grown in culture. Though this genetic drift can present potential challenges, it is important to place this in context as these inherent characteristics underpin the ability of these cells to accept transgenes and to adapt readily to process conditions. For example, it allows adaptation of cells to a variety of basal culture media and growth under demanding process conditions. These changes occur at an individual cell level within a population even within clonally-derived populations [5]. This point is fundamentally important as it underscores the potential genetic and phenotypic changes that occur within a cell population irrespective of the origin of the cells.

It is also important to recognize the complex and varied origin of the cell lines used within industry and the inherent genetic perturbations that are present. For example, the commonly used dihydrofolate reductase (DHFR) deficient DXB11 and DG44 CHO cell lines were generated via multiple rounds of chemical and radiation-induced mutagenesis [8,9]. This has resulted in CHO cell lines whose karyotypes are very different from that of the parental hamster. A recently published CHO cell line karyotype demonstrated that, in contrast to the 22-chromosome diploid genome of the hamster, even the non-mutagenized CHO-K1 cell line had only 21 chromosomes, of which only eight were cytogenetically similar to the hamster and the remaining 13 showed extensive changes via deletions, reciprocal and nonreciprocal translocations and pericentric inversions [10]. The results of research in this area clearly demonstrate the genetic plasticity that occurs when these types of cell lines are cultured over time, and provides additional technical justification that the primary focus for any biopharmaceutical manufacturing process should be on the product being produced rather than on the "clonality assurance" of the cell line used for its derivation. Similarly, the product is ultimately highly purified, and the focus should be on product and process consistency rather than on uncontrollable aspects of the cell lines used to produce the product. As will be discussed in more detail later, it is also important to bear in mind that the product, not the cells, is administered to patients.

In spite of the perceived negative view of the genetic plasticity inherent to these types of cells, it is a critical factor that the biopharmaceutical industry has been dependent upon for the past three decades. Cellular genetic plasticity fundamentally underpins our ability to genetically engineer cell lines to be an appropriate substrate for biopharmaceutical manufacturing and improve their performance by directly impacting productivity and product quality. A cornerstone of the biotechnology industry has been the ability to readily insert a foreign transgene into the host cell genome and expect a reasonable number of cells to accommodate this manipulation. Transgene incorporation relies on a method to facilitate expression plasmid delivery, typically via electroporation or lipid-mediated reagents, and subsequent random integration of the plasmid DNA into the genome. The use of random integration as the current standard approach results in significant heterogeneity in which the transgene location within a cell will vary across the selected population. The level of genetic heterogeneity is further complicated by the methods employed to select for cells which have integrated the transgene and which produce acceptable yields of protein product. Within industry these methods typically rely on the use of metabolic markers as selective pressure in which the plasmid encodes a gene which complements a deficiency of the cell line used, typically DHFR or glutamine synthetase (GS) mutant CHO cells under conditions where expression of those genes is favored

in terms of growth or survival [8,11]. Some of these selection methodologies, especially DHFR with methotrexate, can be associated with genetic rearrangements and cell survival, and may lead to instability of the transgene which is only apparent after extended cell cultivation. Further, the pre-existing levels of genetic heterogeneity in host cell lines prior to adaptation, transfection, or selection is typically not known or studied. For all these reasons, the perceived ability to control heterogeneity partially while obtaining high productivity cell lines in these initial transfected cell populations was perhaps the early driver to incorporate a single-cell cloning step as part of cell line development programs supporting clinical and commercial use.

1.2. The purpose of cloning

As a means to harmonize technical standards for the manufacture and control of therapeutic proteins, the ICH guidelines were established beginning about 20 years ago. In the intervening two decades, the industry and regulators have gained considerably more experience and there has been significant progress in the technologies and methods employed to generate and engineer recombinant microbial strains and mammalian cell lines, and more importantly to characterize and control the products they produce. Yet, inherent complexities continue to exist in developing bioprocesses, especially those leveraging mammalian cell-based systems. As a means of attempting to counter the potential challenges presented by the nature of these types of cells and to facilitate meeting guidelines released by the FDA (Points to Consider, 1997) and ICH (Q5D, 1998), a cloning step is required as part of the overall cell line generation process. Q5D specifically states in Section 2.1.3 "For recombinant products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor." The language cited is consistent with clonal derivation but not an expectation that the cell substrate is truly clonal. Additionally, the World Health Organization published in 2013 a Technical Report Series (TRS 978 *Recommendations for the Evaluation of Animal Cell Cultures as Substrates for the Manufacture of Biological Medicinal Products and for the Characterization of Cell Banks*) that "For proteins derived from transfection with recombinant plasmid DNA technology, a single, fully documented round of cloning is sufficient provided product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process". Incorporation of this cloning step helps to serve two core purposes: I) minimizing, but not eliminating, cell population heterogeneity (by removing genetic variation from the cell population which pre-existed prior to transfection) and II) isolation of highly-productive, stable cell populations. Again recognizing the intent of these guidelines and the value of a cloning step, nevertheless, it should be reiterated that incorporation of such a step cannot prevent genetic and phenotypic heterogeneity within the population of cells that arise post-cloning as these events are inherent to the use of immortalized mammalian cell lines.

There are numerous approaches to cloning that can be used to generate a commercial manufacturing cell line. The traditional approach is the use of limiting dilution [12]. In some instances, more than one round of limiting dilution can be carried out to further increase the probability of clonal derivation of the resulting cell lines. However, an additional round of limiting dilution cloning does not provide an absolute assurance of clonal derivation of a stable cell line, and may actually increase the theoretical risk of increasing heterogeneity due to additional cell divisions, while also lengthening the timeframe to the ultimate delivery of potential life-saving therapeutics. Each round of cloning only provides a probabilistic estimate of genetic homogeneity, never achieving complete assurance. As described above, the concept of clonality is theoretical and cannot be experimentally determined to certainty.

Recently, there have been major advances in instrumentation that have significantly enhanced the isolation of high-expressing cell lines in a manner that gives documentation of the likelihood of clonal derivation. Flow cytometry was a research tool that was adopted to facilitate the isolation of high-expressing cell lines either by assessing cell surface association of the therapeutic protein as it is being secreted or alternatively measuring expression of a surrogate reporter genetically linked to the gene of interest such as a fluorescent protein or a cell surface marker [13,14]. By defining stringent acceptance criteria for the instrument's interrogation of what cell containing droplets are sent to waste and which are collected, one can establish a high level of confidence in the clonal derivation of cells deposited by the FACS into multi-well plates. An alternative approach that has been widely embraced by industry has been the use of semi-solid matrices for cell colony isolation and screening. While having limitations for establishing clonality, the ability to assess secretion of the therapeutic protein from colonies growing in semi-solid media in a reasonably high throughput manner has been a powerful tool to enrich for high-producing cell lines from a starting population containing a wide range of expression levels [15]. A third technology, which can be leveraged in conjunction with other cloning methods to further support clonal derivation, has been the introduction to the market of plate-based imaging instruments that allow high-throughput scanning of microwell plates and generate images of the freshly plated cells. However in all of these approaches, there are challenges associated with each technology and, at best, they can only enhance the probability of generating clonally-derived cell lines.

Although the purpose of a cloning step is to facilitate the isolation of stable, highly-productive cell populations, and to minimize the genetic and phenotypic diversity within a cell line population, the key to ultimately identifying an appropriate production cell line is the genotypic and phenotypic (including the expressed product) characterization of the chosen cell lines post-cloning. The approach begins with phenotypic screening of prospective production cell lines for "stable" productivity and desirable product quality characteristics, which serves as an initial means to eliminate cell lines which do not demonstrate required consistent performance [3,16,17]. Given the significant number of generations that a cell line will accumulate after cloning (in some cases, 90 or more from a clonal derivation step to the end of production at manufacturing scale), it is important to note that the characteristics of candidate cell lines are evaluated in small-scale studies over cell generation numbers that are representative of a commercial manufacturing "window" as part of identifying a suitable final production cell line. This "suitability" screening process results in the elimination of cell lines which do not demonstrate stability in the expression of products with acceptable product quality profiles. Ultimately, the performance of the chosen production cell line is thoroughly assessed at larger scales representative of the eventual commercial manufacturing process.

Though the focus of this manuscript is placed on therapeutic proteins, it would be an oversight to not also note that traditional biologicals, such as viral vaccines, are also manufactured using cell culture. These therapies have an extensive track record of safe and effective use that extends decades longer than even the earliest protein therapeutics, in spite of their being manufactured using processes that rarely, if ever, originate from cell substrates which are "clonal" in any sense. Whether the cell substrates for the production of these vaccines are derived from primary cell sources (chick embryo fibroblasts obtained directly from eggs) or from serially propogated cell sources (e.g. Vero cells), none of these cell substrates are clonally-derived. Recognized as diverse cell

populations, "clonality" was not an expectation for the cell substrates utilized to produce these traditional biologicals, yet they have been used for many decades to consistently produce safe and effective vaccines, providing protection from life-threatening diseases in patient populations from new-born children to the elderly.

2. Product quality & process development, control and validation

2.1. Product quality complexity & advancements in product characterization

Beyond recognizing that the types of cells used in biopharmaceutical manufacturing processes are inherently prone to genotypic and phenotypic drift, it is as important to acknowledge that the protein-based products derived from these cells are themselves highly complex. In light of these realities, there is an expectation that companies develop a comprehensive understanding and control of each product, and the potential impact of the manufacturing process on that product. Process and product understanding result from knowledge gained through the application of sound scientific approaches and rigorous quality risk management, and is applied during clinical development, presented fully in the marketing application, and further updated during the life cycle of the product based on new knowledge gained. While clonal derivation of the cell line that will eventually be utilized to manufacture clinical and commercial protein therapeutics can potentially reduce heterogeneity of the cell population, it is only one of many steps in the development of robust manufacturing processes and as part of a control strategy ensuring a constant, consistent supply of safe and efficacious products. Since it is the product, not the production cell line which is used to treat the patient, primary emphasis should be placed on the elements that comprise holistic integrated process development such as risk assessments, control strategies, process characterization and process validation.

Recombinant proteins produced by living cells are highly complex and inherently heterogeneous molecules. The innate cellular processes that govern expression, secretion and post-translational modification of proteins, such as signal peptide removal, glycosylation, proteolysis, and other enzymatic, as well as chemical modifications, contribute to heterogeneity of all protein products. These modifications can be even more variable based on specific growth and expression characteristics of the production cell line. Additional layers of complexity that can influence heterogeneity in product quality attributes are the impact of cell culture conditions and, to a lesser extent, purification process conditions. It is recognized that residual process and host-related impurities such as phospholipases and proteases have the potential to impact the quality attributes or shelf life of the drug product, but these are generally product or expression host dependent rather than related to a specific production cell line [18–20]. These factors dictate that a therapeutic protein derived from a recombinant gene stably integrated into the CHO genome can produce potentially millions of chemically unique molecules that possess an acceptable safety profile, biological activity and therapeutic efficacy [21].

Given the complexity of biologics, analytical capabilities to characterize proteins are foundational to the development of biopharmaceutical products. In the last 20 years, analytical methods used to characterize protein products have significantly improved in sensitivity, selectivity and robustness. Within the last five years, improvements in analytical and characterization technologies, specifically in mass spectrometry instrumentation and analytical software, have enabled more efficient and sensitive characterization of proteins. As a result, considerable detail, involving micro-

heterogeneity (resulting from in some cases extremely small mass unit differences) existing within the product quality profile have been revealed that were not previously accessible. This is exemplified by recent advancements in the detection of very low level sequence variants, as well as other low level modifications [22–24]. Some of these sequence variants result from mutations in the product-encoding transgene, while others result from the misincorporation of amino acids during translation. These techniques have also been used to characterize the low level sequence variants that occur within native proteins [23]. Beneficially, these improved and advanced analytical and characterization methods can be applied to the screening process used to identify the production cell line during early process development. Quality attributes which are cell line dependent, and possibly sensitive to "genetic drift", can be thoroughly characterized within and beyond the manufacturing window by orthogonal methods (e.g., LC-MS, peptide mapping, transgene copy number, DNA sequencing etc.) during process development. Although low level sequence variants have only recently been observed, probably this micro-heterogeneity has always existed and probably can be found in current commercial recombinant protein products that have been administered to millions of patients safely for decades [25]. These improved analytical capabilities, in conjunction with orthogonal approaches to product characterization, are the underpinning of a product's control strategy. It is the total control strategy, developed hand-in-hand with the risk assessment and process characterization, which is a centerpiece of process development and key to ensuring product safety and efficacy.

In addition to the continued advancements made in analytical methodologies and protein characterization technologies, a cornerstone of the bioanalytical control strategy is potency assessment. Advances in the ability to develop very sensitive cell-based assays, which often directly reflect the mechanism of action of the protein therapeutic being assessed, provide a key orthogonal means to assure the biological effectiveness of the protein-based therapeutic. These assays are utilized to differentiate product-related substances (modified forms of the therapeutic protein retaining biological effectiveness) from product-related impurities (modified forms of the therapeutic protein with altered or lost biological effectiveness), thus helping inform the risk assessment as a part of the holistic integrated control strategy.

2.2. Risk-based product & process development and control

For all manufacturing processes, risk assessment and process characterization studies are extensively used in guiding development of the process, applied throughout clinical development and continually assessed through the life cycle of the product. These approaches and tools are effectively utilized to evaluate the potential impact of process parameters and raw materials, including the production cell line, and on product quality attributes. Risk assessments consider process materials, operational parameters, facilities and both process-related, as well as product-related substance/impurity factors based on activity or potential as safety risk (e.g. potential immunogenicity). Central factors inherent to the process and product-related substance/impurity component of the risk assessment include detectability, occurrence and severity. The list of quality attributes, risk assessment and experimental studies can be iteratively updated throughout the life cycle as process understanding increases further, ensuring the appropriateness of the manufacturing process. Production data, including product quality profiles, are collected and evaluated using statistical methods (e.g. trend analysis, control charts etc.) to ensure that the process is in control. Critical quality attributes, some of which might be impacted by heterogeneity within the cell line population,

are used to guide process design using QbD principles and manufacturing experience, validated at commercial scale, and monitored throughout the life cycle. A lack of clonal derivation of the production cell line that would impact critical quality attributes would very likely have been detected and the potential risk assessed during early process development. Ultimately, licensure of a biological product requires comprehensive process characterization and in-process controls to ensure that product quality attributes derived from cells across the manufacturing window, including cells at the limit of *in vitro* cell age, are characterized with representative process and scale to demonstrate integrity of the expression construct, consistent transgene copy number, product and process consistency, and freedom from adventitious agents. The latter which can represent both a safety challenge from the perspective of potential impurities but also from the consistency of product perspective given adventitious agents can alter gene expression or protein processing.

2.3. Process validation & life cycle management

Following early risk assessment and laboratory-scale characterization studies, the manufacturing process, including production cell line, is evaluated at commercial scale during process validation to ensure that the process is robust, delivering product quality consistency. After process validation, the process is ensured to be compliant, consistent, and capable of continued verification and potentially improving throughout its life cycle. Process validation at the time of licensure requires demonstration of consistent product quality attributes from multiple batches at scale generated from multiple cell bank containers. Any requested process improvement change is required to have no impact (within an agreed range) on product quality attributes that have already been established and tested in clinical trials. These changes typically require both comparability studies as well as repetition of relevant process validation studies, potentially including product stability. If upstream or downstream process changes result in significant differences in product attributes, additional pre-clinical or clinical studies may be required to verify that these differences are determined to be comparable, having no impact to product efficacy and safety. Comparability assessments are also applied to changes made to processes, scale, and facility during clinical development. If the production cell line is sensitive to process changes, which are commonly introduced during clinical development, the impact of these changes would have been assessed through analytical and/or pre-clinical and clinical comparability. At the time of product licensure, orthogonal analytical approaches to process and product characterization provide a high level of assurance that the recombinant protein product will be safe, efficacious and that the manufacturing process is consistent and robust. Subsequently, cell line robustness, product characterization, manufacturing process understanding, and required process revalidation, and supporting clinical experience ensure the consistency of product post-registration.

3. Future perspectives & concluding remarks

Looking forward, from a cell line engineering perspective, a potential paradigm shift is being enabled by the explosive growth in genomic and transcriptomic sequencing along with genome editing capabilities which may reduce the genetic heterogeneity that is the byproduct of current cell line engineering methods. These disruptive technologies enable the cell line engineer to identify within a host, one or more predefined "landing pads" within the chromatin environment that is predisposed to sustain high level expression of a gene. This coupled with precise targeting via a recombinase or precision nucleases such as CRISPR, ZFNs,

meganucleases or TALENs, enables the confident delivery of a gene payload to the exact destination within the genome with minimal off target effects. The end result is a population of recombinant cells with potentially far greater genetic homogeneity than that obtained via the traditional method of random integration [26–29]. In addition, these types of host engineering capabilities are also providing means to address product quality challenges such as eliminating trace levels of host cell impurities (e.g., host cell proteins), or to improve the efficacy of protein therapeutics through glycan profile alteration. These advances represent important opportunities to further improve the processes that industry develops and demonstrate its commitment to continue to deliver safe and efficacious products. Even if these potential benefits are realized, and reduce variation due to multiple/random integration sites, they will not alter inherited characteristics of these immortal cells. Genetic plasticity means continued genetic changes due to DNA mutation or the effect of phenotypic selection in highly selective environments. It is also possible that as our understanding of the interplay between growth requirements and the dynamics of production cell line populations deepens, improvements in cell culture media will continue to provide an opportunity to positively impact our ability to reduce heterogeneity within the production cell line population. This ability could prove even more valuable as industry moves to the production of more and more "toxic" proteins as biotherapeutic products.

From a process characterization perspective, ever-improving analytical and protein characterization technologies continue to provide further insight into product quality characteristics further supporting risk assessments and development of control strategies. This insight can also potentially guide the application of host engineering capabilities. Advancements in enhanced at-line analytics will perhaps provide opportunity for real-time monitoring of processes and process feedback control. Thus, technological improvements may provide possible paths to limit genetic variation after cloning, as well as allow improved characterization and monitoring of manufacturing processes, but none of these improvements will provide a means of limiting therapeutic protein complexity or provide an absolute guarantee of lack of variation after cloning during the time frame of cell banking and use of the cells in a validated production process.

Scientific evidence and rationale has been presented in this manuscript to substantiate the authors' view that "clonality" of mammalian host cells, such as CHO and NS0 which are widely used for commercial manufacturing of biopharmaceuticals, does not exist at the time of product manufacture. The chemical complexity innate to protein therapeutics and the very nature of the mammalian cells utilized for biopharmaceutical manufacturing leads to an inherent heterogeneity that will exist in any production cell line population and, in fact, is the underpinning of these types of cell's utility for such purposes. These realities remind us that descriptions such as "clone" are misleading; nevertheless, incorporation of a cloning step is a useful component of the holistic cell line development process serving to isolate high-producing cell lines while helping limit population genetic heterogeneity. However, the value of definitive demonstration of clonal derivation of a production cell line population is small relative to the significance of those aspects such as ensuring appropriate risk assessments, control strategies, process characterization and process validation, which are part of the holistic integrated process development design, and are the foundation of ensuring that commercial manufacturing processes are delivering products of appropriate quality and consistency to ensure their safety and efficacy. Even in light of the complexity involved in the development of bioprocesses, one aspect is clear — that any manufacturing process which is not developed on these principles and which does not

demonstrate robustness and an ability to consistently deliver acceptable product quality would not be a viable commercial manufacturing process, and would face significant regulatory issues at market application review.

Acknowledgment

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