



ELSEVIER

Contents lists available at ScienceDirect

Biologicals

journal homepage: [www.elsevier.com/locate/biologicals](http://www.elsevier.com/locate/biologicals)

## Review

# Considering “clonality”: A regulatory perspective on the importance of the clonal derivation of mammalian cell banks in biopharmaceutical development

Joel T. Welch\*, N. Sarah Arden

U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Office of Product Quality, Office of Biotechnology Products, Silver Spring, MD, USA

## A B S T R A C T

There has been much recent focus on the regulatory emphasis and the relative importance surrounding clonal derivation of mammalian production cell lines used in the manufacture of recombinant DNA-derived biopharmaceuticals. This interest has led to an ongoing discussion between regulators and industry on how this topic is evaluated and the role it plays in the development of a new biopharmaceutical. Herein the authors describe that the clonal derivation of the production cell line is a factor with potential impact on product quality, and thus should not be considered separately from, but rather in the context of all elements comprising the control strategy necessary to support approval of a regulatory application. Considerations for how clonal derivation of cell banks and clonal variation thereof may be viewed during the lifecycle of a biopharmaceutical product is provided.

## 1. Introduction

There has been much recent focus [1–4] on the regulatory emphasis and scientific practices that surround the clonal derivation of mammalian production cell lines used in the manufacture of recombinant DNA derived biopharmaceuticals. This interest has led to an ongoing discussion between regulators and the regulated industry on how this topic is evaluated. The evaluation of clonal derivation can be viewed as either a stand-alone consideration or as an aspect informing the understanding of risk in the manufacturing process. Herein the authors describe that clonal derivation of the production cell line is a factor that has potential impact on process consistency and/or product quality, and consequently should not be considered separately, but rather in the context of an assessment of the control strategy necessary to support licensure. Additionally, the authors seek to further extend that idea to its logical conclusion: that as clonal derivation has the potential to impact the manufacturing process as well as the critical quality attributes (CQAs) of a product, sufficient detail should be provided to evaluate the details surrounding how it was performed. Moreover, when such cloning methods are considered high risk for product quality (e.g., less rigorous in scientific practice, including documentation) the resulting risk should be necessarily mitigated by an augmentation of the control strategy. Recent work in support of intentionally using non-clonal cell lines for toxicology, non-clinical and first in human studies are also described herein. Finally, key remaining unanswered questions about the potential impact of clonal derivation on cell line stability are

highlighted.

## 2. Background

### 2.1. Current practices

The use of mammalian cell lines has become the most common platform to produce commercial recombinant protein therapeutic products in recent decades [5]. Other cell substrates are potentially viable choices as well, and have been used to a lesser degree, such as bacterial, yeast, plant, and insect cell lines. Despite the relatively lower yield and slower development timelines compared to some of these alternative systems, mammalian cell lines (in particular, Chinese hamster ovary [CHO], and to a lesser degree NS0) are the most frequently selected host cell lines given their unique characteristics to produce relatively large amounts of correctly folded, assembled, and appropriately post-translationally modified product. Moreover, CHO cells are chosen by product developers over other mammalian cells lines given their durability and capacity to grow as single-cell suspensions in culture allowing for easier manipulation during cell line development [6]. Additionally, several other subtle features and nuances associated with CHO cell lines have made them a particularly desirable choice as a commercial cell line. These include: straightforward adaptation to chemically defined, well-controlled and serum free media making them readily amenable to process scaling, relatively low susceptibility to viral infection, and the relatively mature gene selection and amplification

\* Corresponding author.

E-mail address: [Joel.Welch@fda.hhs.gov](mailto:Joel.Welch@fda.hhs.gov) (J.T. Welch).<https://doi.org/10.1016/j.biologicals.2019.09.006>

Received 12 July 2019; Received in revised form 13 September 2019; Accepted 16 September 2019

1045-1056/ © 2019 Published by Elsevier Ltd on behalf of International Alliance for Biological Standardization.

platforms that aid in selection and screening of the initial “clone” [7]. As a consequence, recent research and development activity has explored strategies to allow these cell lines to improve productivities of production cell lines during all phases of development [8],

The original CHO cell line, from which all current commercial CHO cell lines are derived, was generated from the ovaries of an outbred female Chinese hamster. As an immortal cell line, it is inherently unstable, and has resulted in a genetically and phenotypically diverse family, reflected by many single nucleotide polymorphisms (SNPs), copy number variations, and karyotypes. This has led some authors to describe CHO host cell lines as a “quasi species” [9]. Recent studies of the CHO genome, potentially informative for cell line engineering, also demonstrate significant chromosomal heterogeneity among different CHO cell lineages and genomic landscapes [10–12]. Moreover, host cell line development efforts often introduce additional genetic, environmental or chemical factors intended to optimize desirable production cell attributes. Additionally, these factors may increasingly apply selective pressures, cellular –omics and growth kinetics which, when subsequently combined with the demands placed on cells during cell screening and cell culture operations, may increase the potential of volatility in bioreactor performance of a commercial cell line.

Despite rapidly emerging technologies, the most common industry practice for cell line development has remained relatively unchanged: delivery of a genetically manipulated plasmid, and subsequently, random integration of the plasmid into the genome. Selection is usually facilitated by the plasmid to overcome a deficiency inherent to the parental cell line, thereby improving selection and survival. Typically, either dihydrofolate reductase (DHFR) or glutamine synthetase (GS) are used as selection agents. Recent developments suggest that some selection approaches, in particular amplification methods using DHFR and methotrexate may be particularly susceptible to instability of the transgene upon further culturing [13].

The generation of a typical cell bank typically includes a “cloning” step. This step serves not only to help identify stable, highly productive cell populations, but also to perform the critical function which is the focus of this publication: **creation of a single cell precursor or progenitor at the inception of the cell bank in order to minimize cell bank heterogeneity**. Screening of putative production cell lines and cell bank establishment remains a major bottleneck in upstream cell line and process development. Thus, interest exists in developing and refining the methods used for selection of high expressing cell lines for development [8,14]. Despite advances in technology, limiting dilution, one of the oldest and most time consuming methods to isolate a single precursor cell, is still frequently used for at least one round, and occasionally all rounds of selection and generation of clonally-derived cell lines [15]. This method relies strictly on a probabilistic approach, where dilution achieves an extremely low concentration of cells, with subsequent plating resulting in  $< 1$  cell per well, followed by expansion from each individual well. The method can provide a relatively high statistical probability of “clonal derivation” (namely, deriving from a single original cell) but suffers from being time consuming, slow, and low in throughput. Microscopic evaluation is typically performed for the purposes of providing visual confirmation of a single cell. In this method, cell lines must be expanded prior to characterization. This process may limit true screening to a few hundred choices, and consequently may result in failing to identify a more desirable candidate.

Flow cytometry was successfully adapted from a research tool allowing selection of cell lines based on cell surface characteristics and has been used to select cells using specific interactions with antibodies. This method can screen millions rather than a few hundred potential cells, and stringent selection criteria may be established and captured that further support the technique to place cells within a multiplate system. Moreover, this strategy offers tremendous versatility in identification strategies for cell lines of interest, including labeling, conjugates or the use of reporter genes. However the selection process itself introduces stress to the cells and may serve to reduce outgrowth [16].

Another group of technologies that has played a significant role in the advancement of cell line derivation and selection are automated clone screening and selection systems such as Clonepix and CellCelector [17–20]. These systems immobilize the cell within a semi-solid media matrix. A detection antibody provides a fluorescent identifier in the vicinity of the resulting colony. Such systems suffer from a relatively long duration of time required for expansion of the clones prior to selection and introduce limitations for the evaluation and control of antibodies used for selection. Frequently, these techniques may be used for initial screening as a mechanism to remove poor producers rather than serving for the identification of final candidates. This initial screening approach derives from the potentially limited correlation between behavior within the semi-solid matrix and within liquid matrices, as well as the potential need to further adapt the cell line based on buffer modifications.

Finally, imaging systems are frequently combined with other cloning tools, be it limiting dilution, FACS, ClonePix, etc., providing a visual evaluation of the techniques’ “success” in real time. For this reason, from a development perspective, imaging technology offers an attractive way of providing supportive data to assure clonal derivation of production cell lines in lieu of additional laboratory work. However, their utility (and “accuracy”) may be affected by factors, such as calibration, focus, illumination, focal depth, and/or resolution issues. In addition, these imaging systems present other, non-optical based, considerations as well, such as the probability that the cell is on the bottom of the well, and the probability of locating the cell within the well during imaging.

## 2.2. Exploration of statistical evaluation of clonal derivation

Recent perspectives on statistical and probabilistic methods in assessing the assurance of clonal derivation of recombinant cell lines have attempted to probe the level of accuracy in estimating monoclonality. The original statistical evaluation of monoclonality results from simplifying the infinite series proposed by Coller and Coller [21]. Recent work by Zhou et al. discusses the use of the Poisson distribution and highlights its underestimation of the probability of monoclonality in the presence of a selection agent [22]. These experiments challenge the inferences that cell lines derived from a single round of limiting dilution follow the Poisson distribution as an estimate of monoclonality [23,24]. Still others have proposed the use of confidence intervals to assess monoclonality after a single round of limiting dilution rather than the use of point estimates [23]. Data collection for such an approach requires that both the number of empty wells and the total number of wells are captured for evaluation. The use of confidence intervals accounts for the level of uncertainty inherent in assuring monoclonality after a single round of limiting dilution.

Substantial interest has emerged on the potential to use new selection platforms with an eye toward the assessment of likelihood of clonal derivation. For example, fluorescence activated cell sorting (FACS), given sufficient information related to for the analysis performed, such as a detailed description of how the cells were sorted, process parameters, images, training of analysts has been proposed to provide probabilities of greater than 98% for isolating a single cell [25]. With respect to solid media selection platforms, sponsors have provided widely different estimates of probability of single-clone selection. This is not surprising, given that the likelihood of success of the clone picking (and with it, monoclonality upon expansion) can be heavily influenced by the size of the colonies at the time of selection. Indeed, a colony’s relative distance from other potential colonies, and moreover the plating density selected at the time of plating have been proposed to play a large role in this techniques feasibility [26].

## 2.3. Intentional use of non-clonally derived cell lines

As novel technologies impact our perspective on the approaches

used to support development of clonally-derived cell lines for drug development, entirely different approaches are also being proposed for development of cell banks for early pharmaceutical development. For instance, multiple recent industry publications propose the use of stable bulk cell pools which are developed by selective pressure on a non-clonal pool of cells stably expressing transgenes as part of a recombinant expression platform technology (e.g., DHFR). This is in contrast to the traditional approach of developing clonally-derived cell lines as a mechanism to rapidly deploy drug product in early drug development stages for the purposes of toxicology and non-clinical studies, and even potentially for first-in-human studies [27,28]. A comparative study of two different therapeutic antibody candidates derived from small stable pools (mini-pools) and clonal cell lines evaluated the cell culture performance in a 14-day fed-batch culture in terms of growth profiles, productivity, viable cell densities and viabilities. Product quality attributes between the drug substance generated from mini-pool cells were similar to drug substance generated from the clonally-derived production cell lines derived from the same mini-pool (in some instances) with some differences noted in glycosylation, charge variants, and growth kinetics in some instances. Where differences in quality attributes have been observed, study authors discuss how one may reduce or eliminate the observed product quality differences, by consideration of the potential impact to product performance, by either screening additional clonal cell lines derived from the mini-pool or tightening process parameters as alternative approaches to their conclusion. This discussion highlights the feasibility of using the mini-pools and bulk pools for toxicological and first-in-human studies [29]. Others have also proposed to use pools for toxicology studies followed by single cell cloning from the specific pool used in the toxicology study. This approach bridges the pool used in toxicology studies to the clonally-derived production cell line selected from a given pool based on product quality attributes [30].

### 3. Regulatory background

There are both scientific and regulatory considerations regarding the clonal derivation of the cell line used to create the master cell bank. Though no one single regulatory guidance speaks definitively to the need of having a demonstrably “clonally derived” cell line, multiple guidance documents allude to this general expectation and underscore it as a basic expectation. These include, but are not limited to:

- ICH Q5D Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products, “For recombinant products, the cell substrate is the transfected cell containing the desired sequences which has been cloned from a single cell progenitor” [31],
- US FDA Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use notes that “The MCB

is defined as a collection of cells of uniform composition derived from a single tissue or cell” [32], and

- EMA/CHMP guidance [33] which notes “The cell substrate to be used for the production of the monoclonal antibodies should be a stable and continuous monoclonal cell line that has been developed by means of recombinant DNA and/or other suitable technologies”.

A recent WHO technical report [34] provides far more granularity on the topic. Though they do note that “For proteins derived from transfection with recombinant plasmid DNA technology, a single fully documented round of cloning is sufficient,” and also note that alternatively, or in addition to limiting dilution steps, the cloning procedure can include “more recent technology such as single-cell sorting and arraying or colony-picking from dilute seeds into semi-solid media”. Understandably, these recommendations do reflect the scientific reality of the cloning process, namely that it does not “necessarily guarantee derivation from single cells” and part of their underlying basis is that the cloning procedure should be “fully documented, with details of imaging techniques and/or appropriate statistics.” Thus, though consensus is needed to clarify what the specific expectations are (e.g., how many rounds of limiting dilution are sufficient), little incongruity appears to exist on the concept that the manner in which the cell bank was created, and the supporting data thereof, is important.

The choice to perform cloning steps is not exclusive to regulatory expectation, but rather also driven by scientific necessity. Indeed, the primary purpose is to allow for the discrimination and ultimate identification of individual cell populations that possess the unique profile of being stable, producing large quantities of protein, and producing protein that possesses the unique characteristics and CQAs suitable for assuring safety and efficacy of the product. Indeed, identifying this “sweet spot” is a relatively rare phenomenon, as high-producing candidates comprise a small fraction of the large number of relative choices [14]. Additionally, there is widespread agreement that additional cell passaging ultimately corresponds to an increase in the rates of low protein producing or non-protein producing clones as high producing clones suffer from lower growth rates associated with consumption of metabolic energy and with protein production at the expense of growth [35]. Indeed, this can pose incredible difficulty on the selection of an appropriate cell line for development where the range of appropriate attributes is further restrained based on the need to target a very specific range of critical quality attributes (CQAs) for the resulting protein based on characterization demands (e.g., in biosimilar development).

The basis for regulatory concern for the steps within clonal derivation is reflected by hypothetical cell banks as shown in Fig. 1 below. Depicted in the figure is a non-clonally derived cell bank on the left (A) and a clonally-derived cell bank on the right (B), where the non-clonally derived cell bank is generated by unintentional seeding of two cells per well during development of the cell bank. The y-axis depicts a particular attribute (e.g., a CQA) that is sensitive to cellular phenotype,

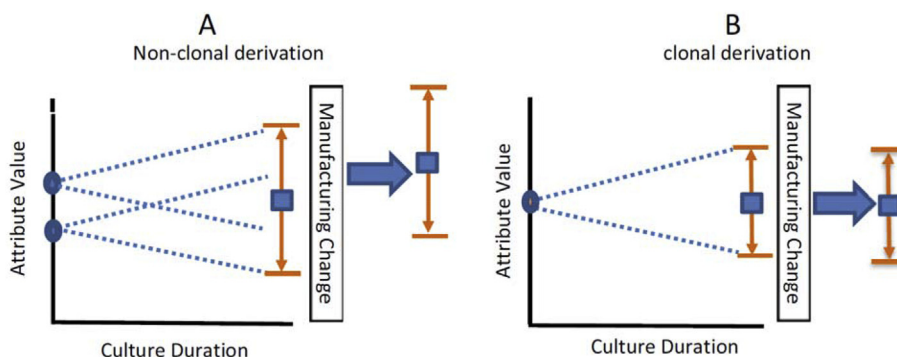


Fig. 1. Variability of unintentionally non clonally-derived cell bank and potential effect on manufacturing change (orange arrows depict range of values at time of harvest, blue squares depict mean of range). Time zero on y-axis reflects single cell isolation.

reflecting a single value at time zero (on the y-axis) for single cell isolation, for the clonally derived cell bank and two values for the non-clonally derived cell bank. The x-axis depicts time. During culturing, phenotypic drift occurs, impacting the genetic population mean at the time of harvest which results in that population having a mean (depicted as a blue square), and a range (depicted by orange arrows) for a given attribute. Critically, though the nonclonally derived bank may result in the same CQA mean value at the time of harvest as the clonally derived bank, its divergent starting point during cloning could potentially result in greater variability of one or more CQA's, and potentially higher susceptibility to drift, shift, and unforeseen selective pressures during manufacturing lifetime, depicted by the manufacturing change shown as the blue arrow. Such variations within a cell bank may have impacts that could be observed (if quantifiable) in product performance.

One counter-consideration provided by an industry whitepaper [4] is that a definitive demonstration of “clonality” is never truly possible (with some residual, albeit small probability of non-clonal derivation remaining regardless of scientific practice), and that the control strategy and development activities should be the focus in order to ensure product quality. However, creating a final control strategy under such circumstances can prove challenging, especially when process capability and knowledge gained in development activities are sought for leverage to support control strategy flexibility or reduced regulatory commitments to perform testing and establish process control parameters (or reduced requirements to report changes thereof), circumstances where stability and consistency of cell bank performance are paramount.

It is acknowledged that the control strategy, and particularly analytical testing does play a key role to ensure minimum drift over time for CQAs. However, the establishment of a control strategy with indifference to the clonal derivation of the cell bank and sufficient information for how the bank was created is inherently problematic for several additional key reasons. First, while testing may prove successful in identifying changes or drifts in quality attributes in real time, identifying drift is distinct from controlling it and may not be possible for all CQAs for each batch. Second, quality systems are not necessarily well configured to identify ‘clonal derivation’ as the root cause for a change observed in one or more quality attributes. As a hypothetical example, should a new lot of media result in a change due to selective pressure of a cell line, the OOS investigation would likely begin its investigation of a confirmed atypical result by posing the question, “What is Different?”. Indeed, given that the cell bank itself in this example is likely unchanged, it may well be excluded from consideration as a root cause from the outset.

Finally, it is acknowledged that the testing strategy does play an integral part in maintaining quality over time. However, demonstration of an “unsuitable” cell line through product testing may itself not be a guarantee that the cell bank will be able to support the lifecycle of the product. Indeed, the cell bank must assure product quality against the background of an emerging 21st century biotechnology landscape: with an ever increasingly complex manufacturing reality, frequently with different manufacturing scales and variable manufacturing processes, raw materials and geographic locations. This is crucial given the potential for cell banks to be exposed to future manufacturing changes such as scaling or introduction into perfusion cultures with their prolonged culture periods for which impact and risk is harder to predict. Much work from industry has demonstrated that other factors can contribute to potential heterogeneity and thus, pose questions regarding the scientific and practical necessity of attempting to demonstrate well-controlled and highly probable clonal derivation. Recent efforts have examined the heterogeneity inherent to single cells expanded from clonally derived lines [36] and demonstrated significant phenotypic variation across both productivity and product quality. Recent work by Scarcelli et al. [37] included subcloning of a clonally derived cell line to evaluate the potential origins of sequence

heterogeneity. This work not only corroborated expected phenotypic variability within the cell bank, but also strongly suggests that observed sequence heterogeneity likely derives from events associated with culturing post cloning. In support of this inherent genetic flux, He and Frye [38] proposed a risk based transgene characterization strategy for recombinant CHO cell lines as a risk mitigation approach. Another study by He et al. [39] analyzed transgene copy number distribution in single recombinant CHO cells to find that cell lines which maintain high levels of productivity over time displayed more consistent and homogenous transgene copy number distributions than cell lines which exhibited a loss of productivity over time. Additional efforts [40] to probe the role of epigenetic factors including cytosine methylation and its relative role in cell line behavior are ongoing. Finally, work by Vcelar [41] has demonstrated that karyotypic stability may not impact cell line performance.

Nevertheless, a demonstration that even clonally derived cell lines possess tremendous heterogeneity (or clonal variation) and that non-clonally derived pools can in some cases produce drug substance with CQAs matching those of drug substance produced by a clonally derived line fails to address key unresolved questions. Moreover, answers to these questions are necessary to allow for the extrapolation of these results to the ambitious ultimate outcome of rendering concerns around “clonal derivation” immaterial: that in spite of the fact that clonally derived cell lines possess heterogeneity, there is no need for clonal derivation of cell banks. Examples of questions that need to be addressed, include:

- Does a cell bank from a non-clonally derived line, generated by an unintentional introduction of an additional single cell (possessing different production characteristics) during generation, *potentially* result in greater lot to lot variability?
- Does a cell bank from a non-clonally derived line, generated by an unintentional introduction of an additional single cell (possessing different production characteristics) during generation, *potentially* result in greater likelihood that small changes in manufacturing conditions lead to a drift/shift for one or more quality attributes?
- Finally, if either of the first two questions have an answer of yes, is there a screening or characterization technique(s) in early development that could potentially address the risk?

#### 4. Regulatory discussion

Tremendous recent discussion has emerged on the topic of the clonal derivation of mammalian production cell lines used in the production of the recombinant DNA-(rDNA) derived biopharmaceuticals. Several recent publications [3,4] have provided an industry and academic perspective that this topic perhaps has been overemphasized by regulators. These publications have provided areas where additional discussion and even further clarification is useful. These areas include but are not limited to: 1). *The semantical*: The use of the term “clonality” being a scientific misnomer because clonally-derived cell banks can still be genetically and phenotypically heterogeneous, 2). *The practical*: A lack of clonal derivation of the production cell line that would impact CQAs would very likely have been detected and the potential risk assessed during early process development, 3). *The appropriate area of regulatory emphasis*: that regulatory emphasis should be primarily placed on ensuring quality of the product that is administered to patients, and the adequacy of the final control strategy 4). *The hypothetical*: challenging the underlying assumption that the production cell line and process stability (with respect to product CQAs) are linked to assurance of “clonality”. Indeed, this has led authors of a recent white paper [4] to propose a pair of potential inferences: “(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”.

This perspective is valuable, and we acknowledge that a population of mammalian cells, generated from an immortal cell line, subject to conventional manufacturing and cultured for any meaningful duration of time, will demonstrate substantial genotypic and phenotypic heterogeneity. Thus, a master cell bank created under even the strictest conditions possible may never be truly “clonal.” And therefore, the suggestion of the term “clonally-derived population” to describe the original cell bank is preferable and scientifically more accurate [4,42].

Secondly, though we do acknowledge the potential for cell line-related problems to be detected and risk thereof assessed during early process development (as well as process validation), such activities reflect merely a brief snapshot in time and are unsuited for identifying the possible risks (e.g., unanticipated shifts in CQAs) that may develop during the lifecycle of the product. Additionally, it is important to note that the 21st century manufacturing reality of expedited timelines and breakthrough therapy designations may further decrease the possibility of activities to identify issues related to the clonal derivation of the host cell line. Specifically, such compressed product development timelines result in not only the back-loading of CMC development activities, but also, potentially diminishing and streamlining some process development activities that would occur within a “standard” development program. Therefore, management of the risks associated with reduced CMC development timelines inherent to rapid product development programs is an area where quality risk management frameworks are of regulatory concern.

Additionally, environmental conditions in cell culture necessary for maintaining stability and minimizing genetic drift of host cell lines are poorly understood, and have only recently been discussed [9]. Critically, the recommendation for minimizing such heterogeneity, including minimizing growth-restricting (selective) conditions, and consistent bioreactor design across all production environments is largely incompatible with the nature of modern biopharmaceutical production.

Thirdly, it is acknowledged that regulatory emphasis should be placed on ensuring the quality of the product administered and, with it, ensuring the continued availability of medically necessary drugs to patients (e.g., reducing the risk of drug shortages). Moreover, a determination of the probability of a master cell bank reflecting “a clonally-derived population” and the nature of the corresponding risk is not a determination made in a vacuum, rather it is considered in the context of the overall control strategy proposed by the sponsor.

Because the clonal derivation approach used for a product, and the corresponding documentation matter, regulators should request sufficient details to evaluate the process and procedures associated with the clonal derivation. Data, such as images in support of what happened during cell bank generation should be available for review and consideration. Finally, steps in cell bank development (including adaptation), storage, handling procedures and the control of these procedures, or changes to storage or handling that may impact the cell bank, are critical points of potentially high risk. This is especially true in instances where the original bank was expanded from an unintentional “pool” of non-clonal cells that reflect different production characteristics and express protein reflecting slightly different quality attributes. Additionally, it follows that in the absence of robust and well-documented procedures, there are risks associated with the use of a non-clonal cell line in routine manufacture of a product and throughout the product life cycle. Thus, the control strategy necessitates additional elements be included and documented in the regulatory filings to reflect this reality.

To this end, two potential considerations emerge: (1) that without a sufficient description and details for the creation of the cell bank, it is impossible to assess the residual risk associated with nonclonal derivation, and that (2) in instances where insufficient controls were documented or included within the cell bank derivation process, the resulting control strategy necessary to support licensure will need to address the associated risks.

## 5. Future perspective and conclusion

Herein, we have provided a discussion, rationale, and context which describes “clonal derivation” of a mammalian cell line: one factor that influences reproducible manufacture of biopharmaceuticals, control of its CQAs, and consequently the ability to turn a cell bank into a product used by patients around the world. Indeed, future technological advances may render this area of discussion moot as new techniques offer the potential for substantially improved genetic homogeneity compared to the current random integration approach. Additional information on the complex interplay of cell bank characteristics, process parameters and process controls forming the dynamics and behavior of mammalian cells in culture may well lead to risk assessments and control strategies that are far more effective in avoiding unintended selective pressure or increased rates of clonal variation that lead to one or more changes in product quality over time. However, clonal derivation of production cell lines is a factor that has real and tangible potential impact on reproducible manufacture and product quality attributes. For circumstances where the assessment of an application is that the clonal derivation results in residual uncertainty, additional elements of the control strategy may be proposed to aid in the long-term commitment to providing high quality medicines to patients.

The steps chosen for cell line derivation and the technologies that support them can vary so long as they are scientifically sound and there is sufficient information to support that the final control strategy ensures the safety and efficacy of the product. While industry and regulators continue to seek and identify opportunities aimed at reducing the time of advancing novel products to market, what must be demonstrated is a clear understanding and comprehensive assessments of associated risks, in order to garner a level of confidence that early stage development results are accurate and that product quality is unlikely to be adversely impacted by new proposed approaches. Indeed, discourse on this topic is critical to understanding the impacts upon product quality from a scientific perspective, even if further development work [43–46] demonstrates clonal derivation to be a less relevant concern even for commercialization. Thus, ongoing process and product quality research with the critical factor of clonal derivation in mind will help advance the field so long as rigorous and comprehensive data representing an array of variables including differing cell substrates, platforms, manipulations, and manufacturing changes are considered.

## Declarations

This publication reflects the views of the authors and should not be construed to represent FDA's views or policies.<sup>1</sup>

## References

- [1] Kennett S. Establishing clonal cell lines – a regulatory perspective. Presentation, CASSS WCBP. 2014. Washington, D.C.
- [2] Novak R. Regulatory perspective on the evaluation of clonality of mammalian cell banks. Presentation, CASSS WCBP. 2017. Washington, D.C.
- [3] Wurm F, Wurm M. Cloning of CHO cells, productivity and genetic stability – a discussion. *Processes* 2017;5(20).
- [4] Frye C, et al. Industry view on the relative importance of “clonality” of biopharmaceutical-producing cell lines. *Biologicals* 2016;44(2):117–22.
- [5] Zhu J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol Adv* 2012;1158–70.
- [6] Abidin S, Anuar N. Comparison of the production of recombinant proteins in suspension culture of CHO cells in spinner flask and shake flask systems. *IJUM Eng J* 2011;43–9.
- [7] Dumont J, et al. Human Cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit Rev Biotechnol* 2016;1110–22.

<sup>1</sup> Commercial equipment, instruments, and materials are identified throughout this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by FDA nor does it imply that the equipment, instruments, or materials are necessarily the best available for the intended purpose.

- [8] Hansen H, et al. Improving the secretory capacity of Chinese hamster ovary cells by ectopic expression of effector genes: lessons learned and future directions. *Biotechnol Adv* 2017;35(1):64–76.
- [9] Wurm F. CHO quasispecies—implications for manufacturing processes. *Processes* 2013;296–311.
- [10] Xu X, et al. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat Biotechnol* 2011;29(8):735–41.
- [11] Wurm F, Hacker D. First CHO Genome. *Nat Biotechnol* 2011;29:718–20.
- [12] Lewis N, et al. Genomic landscapes of Chinese hamster ovary cell lines as revealed by the *Cricetulus griseus* draft genome. *Nat Biotechnol* 2013;31(8):759–65.
- [13] Fan L, et al. Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. *Biotechnol Bioeng* 2012;1007–15.
- [14] Misaghi S, et al. Slashing the timelines: opting to generate high-titer clonal lines faster via viability based single cell sorting. *Biotechnol Prog* 2016;32(1):198–207.
- [15] Lefkovits I, Waldmann H. Limiting dilution analysis of cells in the immune system. London and New York: Cambridge University Press; 1979.
- [16] Burke J, Mann C. Rapid isolation of monoclonal antibody producing cell lines. *Bioprocess Int* 2006;48–51.
- [17] Shi S, et al. A high-throughput automated platform for the development of manufacturing cell lines for protein therapeutics. *J Vis Exp* 2011;Sep(55).
- [18] Roy G, et al. Sequential screening by ClonePix FL and intracellular staining facilitate isolation of high producer cell lines for monoclonal antibody manufacturing. *J Immunol Methods* 2017;Dec(451):100–10.
- [19] Caron A, et al. Fluorescent labelling in semi-solid medium for selection of mammalian cells secreting high-levels of recombinant proteins. *BMC Biotechnol* 2009;May(9):42.
- [20] Contie M, et al. IL-17F co-expression improves cell growth characteristics and enhances recombinant protein production during CHO cell line engineering. *Biotechnol Bioeng* 2013;1153–63.
- [21] Collier H, Collier B. Statistical analysis of repetitive subcloning by the limiting dilution technique with a view toward ensuring hybridoma monoclonality. *Hybridoma* 1983;2(1):91–6.
- [22] Zhou Y, et al. Beating the odds: the Poisson distribution of all input cells during limiting dilution grossly underestimates whether a cell line is clonally-derived or not. *Biotechnol Prog* 2017;34(3):559–69.
- [23] Quiroz J, Tsao Y. Statistical analysis of data from limiting dilution cloning to assess monoclonality in generating manufacturing cell lines. *Biotechnol Prog* 2016;32(4):1061–8.
- [24] Staszewski R. Murphy's law of limiting dilution cloning. *Stat Med* 1990:457–61.
- [25] Fieder F, et al. A single-step FACS sorting strategy in conjunction with fluorescent vital dye imaging efficiently assures clonality of biopharmaceutical production cell lines. *Biotechnol J* 2017;12(6).
- [26] Hutchinson G. The impact of using an imaging system to provide the regulatory evidence that a cell line has a high probability of being clonally derived. *Solentim*; 2016 [www.trio-biotech.com](http://www.trio-biotech.com).
- [27] Munro T, et al. Accelerating patient access to novel biologics using stable pool-derived product for non-clinical studies and single clone-derived product for clinical studies. *Biotechnol Prog* 2017;33(6):1476–82.
- [28] Rajendra Y, et al. Evaluation of piggyBac-mediated CHO pools to enable material generation to support GLP toxicology studies. *Biotechnol Prog* 2017;33(6):1436–48.
- [29] Fan L, et al. Comparative study of therapeutic antibody candidates derived from mini-pool and clonal cell lines. *Biotechnol Prog* 2017;33(6):1456–62.
- [30] Hu Z, et al. A strategy to accelerate protein production from a pool of clones in Chinese hamster ovary cells for toxicology studies. *Biotechnol Prog* 2017;33(6):1449–55.
- [31] International Conference for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Q5D: Derivation and characterization of cell substrates used for production of biotechnological/biological products. 1997.
- [32] US Food and Drug Administration. Points to consider in the manufacture and testing of monoclonal antibody products for human use. 1997.
- [33] European Medicines Agency: committee for medicinal products for human use. Guideline on development, production, characterisation, and specification for monoclonal antibodies and related products. 2016.
- [34] World Health Organization. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. 2013.
- [35] Browne S, Al-Rubeai M. Selection methods for high-producing mammalian cell lines. *Trends Biotechnol* 2007;25(9):425–32.
- [36] Ko P, et al. Probing the importance of clonality: single cell subcloning of clonally derived CHO cell lines yields widely diverse clones differing in growth, productivity and product quality. *Biotechnol Prog* 2017;33(3):624–34.
- [37] Scarcelli J, et al. Analytical subcloning of a clonal cell line demonstrates sequence heterogeneity that does not impact process consistency or robustness. *Biotechnol Prog* 2018;34(3):602–12.
- [38] He L, F C. A risk-based genetic characterization strategy for recombinant CHO cell lines used for clinical and commercial applications. *Biopharm Int* 2016;29(3):32–40.
- [39] He L, et al. Transgene copy number distribution profiles in recombinant CHO cell lines revealed by single cell analyses. *Biotechnol Bioeng* 2012;109(7):1713–22.
- [40] Patel N, et al. Antibody expression stability in CHO clonally-derived cell lines and their subclones: role of methylation in phenotypic and epigenetic heterogeneity. *Biotechnol Prog* 2018;34(4):635–49.
- [41] Vcelar S, et al. Karyotype variation of CHO host cell line over time in culture characterised by chromosome counting and chromosome painting. *Biotechnol Bioeng* 2017;115(1):165–73.
- [42] Kim N, Kim S. Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: stability in the absence of selective pressure. *Biotechnol Bioeng* 1998;60(6):679–88.
- [43] Orellana C, et al. RNA-seq highlights high clonal variation in monoclonal antibody producing CHO cells. *Biotechnol J* 2018;13(3).
- [44] Ghorbaniaghdam A, et al. Analyzing clonal variation of monoclonal antibody-producing CHO cell lines using an in silico metabolomic platform. *PLOS* 2014;9(8).
- [45] Kang S, et al. Metabolic markers associated with high mannose glycan levels of therapeutic recombinant monoclonal antibodies. *J Biotechnol* 2015(203):22–31. June.
- [46] Yusufi F, et al. Mammalian systems biotechnology reveals global cellular adaptations in a recombinant CHO cell line. *Cell Syst* 2017;4(5):530–42.