Myths, Risks, and Best Practices

Production Cell Line Development and Control of Product Consistency During Cell Cultivation

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Health authorities are requesting substantial details from sponsors regarding practices used to generate production cell lines for recombinant DNA–(rDNA) derived biopharmaceuticals. Authorities also are asking for information about the clonality of master cell banks (MCBs) and control strategies to minimize genetic heterogeneity. Such requests are prompted by recent reports indicating "nonclonality" for certain production cell lines. To address these and related issues, the CASSS CMC Strategy Forum on "Production Cell Line Development and Control of Product Consistency During Cell Cultivation: Myths, Risks and Best Practices," was held 23 January 2017 in Washington, DC. The overarching objective of this forum was to define myths about and risks to cell line development and product quality associated with cell cultivation. Forum participants identified current best practices to ensure that sponsors meet regulatory expectations when assessing and assuring the appropriateness of cell lines for biopharmaceutical production during development and commercialization.

This report summarizes considerations for development of production cell lines including

- The choice of expression system
- Strategies to minimize genetic heterogeneity of production cells
- The characterization of cell population genetic heterogeneity and potential approaches to improve cell line performance through host engineering
- Assurance of consistent production of desired product
- Approaches to ensuring appropriate control of product quality throughout a cell culture process, including advancements in analytical control strategies
- Strategies for accelerating early product development though the use of pool clones
- Lifecycle management of production cells.

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The forum offered introductory presentations by representatives of regulatory agencies including the FDA and the EMA and by industry representatives. Their presentations were followed by panel discussions of selected topics of interest.

Presentations: Clonality and Host-Cell Engineering

The meeting began with Anthony Lubiniecki (Janssen R&D, LLC, Malvern, PA USA) presenting on an **"Industry View on the Relative Importance of 'Clonality' of Biopharmaceutical-Producing Cell Lines."** He noted recent feedback from some regulators saying that without adequate proof that a cell bank is derived from a single cell, additional studies/controls of the cell line and product may be required to ensure a product's purity. Although Lubiniecki stated that developers can provide reasonable certainty that a cell bank is

derived from a single cell, they cannot conclude that all resulting cells are genetically identical. Indeed, mutations are an inherent property of DNA replication and will accumulate during expansion of a cell culture. Single-cell cloning cannot prevent genetic heterogeneity after cloning. Thus, assurance of product quality depends on developing an integrated control strategy that includes, but is not limited to control of starting materials (including a demonstration that end-of-production cells yield a product consistent with the desired product), raw materials, process conditions, and product testing, as appropriate. Lubiniecki finished by stating that emphasis should be placed on ensuring product quality of all materials administered to patients.

Lianchun Fan (Bristol-Myers Squibb Company) presented on **"Evolving Biological Product Expression Systems with Host Cell Engineering."** Several successful case studies demonstrated the power of host-cell engineering technology to drive development of new host cells with improvements on cell-line productivity, product quality, and/or cell-line development efficiency. Fan described improvements in host cell lines that include engineering cell lines by expressing an enzyme to increase afucosylation, enhancing ADCC activity, and switching to newer platforms. Of note was the use of targeted gene integration to develop more homogeneous cell populations, shorten selection processes, and enhance cell-line stability.

Luhong He from Eli Lilly and Company then presented the industry viewpoint for **"Characterization of Production Cell Lines."** He emphasized that production cell lines are clonally derived populations of cells exhibiting various levels of genetic heterogeneity, including aberrant splicing and sequence variants. Risks associated with genetic and phenotypic heterogeneity can be mitigated through extensive characterization of an expression construct's stability as well as product expressed by end-of-production cells. This characterization includes assessment of transgene integrity (by RT-PCR and Southern blots), copy number (by qPCR), and population drift (assessed by qPCR of single cell clones). Cell lines showing transgene heterogeneity are rejected. Such a characterization strategy enables identification of production cell lines that express appropriate and consistent product quality — even though the absolute genetic and phenotypic homogeneity of clonally derived Chinese-hamster ovary (CHO) production cell lines are not achievable due to low-frequency changes in the genetic composition of a cell (an inherent property of DNA replication). The promising potential for applications using targeted integration also was discussed.

Rachel Novak's (FDA CDER) presentation highlighted current **"Regulatory Expectations Regarding Characterization of Cell Substrates."** Assurance of clonality is expected to minimize

Acronyms Used Herein

ADCC: antibody-dependent cell-mediated cytotoxicity

CDC: complement-dependent cytotoxicity

CDER: Center for Drug Evaluation and Research

CHO: Chinese hamster ovary (cells)

CQA: critical quality attribute

EoP: end of production

FACS: fluorescence-activated cell sorting

FDA: Food and Drug Administration

FISH: fluorescence in-situ hybridization

EMA: European Medicines Agency

EoP: end of production

HCP: host-cell protein

ICH: International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IEF: isoelectric focusing

IND: investigational new drug

LC-MS/MS: liquid chromatography with tandem mass spectrometry

LoD: limit of detection

MAb: monoclonal antibody

MCB: master cell bank

MS: mass spectrometry

NGS: next-generation sequencing, also known as high-throughput sequencing

PDL: population doubling limit

PK: pharmacokinetics

qPCR: quantitative polymerase chain reaction

RP-HPLC: reverse-phase high-performance liquid chromatography

RT-PCR: reverse-transcription polymerase chain reaction

SDS-PAGE: sodium-dodecyl sulfate polyacrylamide gel electrophoresis

SEC: size-exclusion chromatography

WCB: working cell bank

CMC Forum Series

The CMC Strategy Forum series provides a venue for biotechnology and biological product discussion. These meetings focus on relevant chemistry, manufacturing, and controls (CMC) issues throughout the lifecycle of such products and thereby foster collaborative technical and regulatory interaction. The Forum strives to share information with regulatory agencies to assist them in merging good scientific and regulatory practices. Outcomes of the Forum meetings are published in this peer-reviewed journal to help assure that biopharmaceutical products manufactured in a regulated environment will continue to be safe and efficacious. The CMC Strategy Forum is organized by CASSS–Sharing Science Solutions (formerly the California Separation Science Society), and is supported by the US Food and Drug Administration (FDA).

genetic heterogeneity within a company's MCB because every change to an upstream process for a nonclonal cell bank presents a potential risk to select for a product variant that might alter a final drug product. Such assurance involves both a calculation of the probability of single-cell cloning and additional "supporting data," presumably on cell-bank growth parameters and homogeneity.

High probability that the MCB is derived from a single cell is a critical component of an integrated control strategy. It can be achieved through two rounds of limiting dilutions or appropriate FACS or clonal analysis. Imaging techniques can supplement the choice of cloning strategy. During an initial IND application, the cloning process should be described along with stage-appropriate cell-bank characterization.

Cells should be adapted to serum-free conditions before final cloning. A high probability of single-cell cloning would not require a heightened control strategy. Although a lower probability of clonality can be acceptable, either additional data supporting assurance of clonality or augmentation of an integrated control strategy to reduce the risk associated with cell line heterogeneity would need to be submitted. Enhancement of a control strategy could entail shortening the limit of in vitro cell age, adding additional critical process parameters, or adding specifications for minor amino acid variants or various glycoforms, even if a glycoform is not important for the mechanism of action. A more robust comparability analysis for qualification of a new working cell bank (WCB) also is expected when assurance of clonality is low.

Panel Discussion: Cell-Lines and Expression Systems

The first set of questions to the panel explored the nature of cell lines and expression systems currently leveraged for biopharmaceutical production along with their impact on bioprocess development. Attendees generally agreed that certain CHO lines have been used extensively and are well understood, but that introduction of other cell lines should include as much information as possible on the origins and processes for generating them. Cell lines of human origin were noted to pose a greater risk to viral safety, requiring more characterization work than is typical for common CHO lines. The host cell line can dictate a product's critical quality attributes (CQAs) and must be considered carefully. The potential impact of expression systems on bioprocesses had been described by Lianchun Fan (see above), but during discussions it also was noted that when a product has been found to be toxic to production cells, inducible expression systems have been used successfully by some manufacturers.

What types of information support the assurance of clonality? The FDA indicates that information required would be determined case by case but could include application of NGS, FISH, or subclone analysis, as appropriate. Stephan Gross from the Paul-Ehrlich-Institut indicted that clonality is not a big issue in Europe but that full genetic characterization of the expression construct is expected for marketing applications. That has generally included analysis by reverse transcription-polymerase chain reaction (RT-PCR, Southern blotting, and assessment of copy numbers. Full analytical characterization of a final product from end-of-production (EoP) cells also is expected. Most participants agreed that consistent quality of a final product is the primary concern when considering cell line stability; if a commercial process is in a demonstrated state of control, then extra controls are not needed. However, if changes to a process could select differentially for a product variant, then a comprehensive analytical comparability exercise should be performed. That exercise would evaluate lot-to-lot consistency of the product following a change in the cell production process, including a new WCB. The question was posed whether characterization of production cells would be different for implementation of a continuous culture process. The audience felt that there would

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be no special concern as long as data supported cell line stability and as long as attributes were well controlled.

If unexpected genetic heterogeneity is observed during genetic characterization of a production cell line, what additional work might be necessary? One company with this issue resorted to subclone analysis, demonstrating that although a couple of product variants were observed, all the subclones had the same genetic structure. That information was accepted by regulators for demonstrating consistency of the expression construct because the production cells were stable. In other cases, subclone analysis can show more genetic heterogeneity than desired and sometimes correlate with variable productively, but even singly derived clonal lines can show such variability. A decrease in copy number might be common, but what really matters is the quality of the final product. However, in one case, a significant loss of copy number (e.g., 25%) without an impact to product quality also raised a concern about the control of a process and a potential increased risk of an undesirable selection process. That resulted in a regulator's request to tighten the population doubling limit (PDL).

In all cases, control of CQAs should be demonstrated. In some cases, additional process controls could be warranted particularly if heterogeneity potentially introduces undesired product-related variants. Although recloning can yield a more homogenous cell population, the potential risk to product quality can be high — a factor to consider when planning to reclone an MCB.

What should a company do for cell lines for legacy products that typically are not up to today's expectations and may show some genetic heterogeneity? If clinical or biological characterization data support that a variant has no impact on safety and efficacy, then such justification should be acceptable. If an undesired variant is present, developers should monitor that variant whenever a process change might compromise the stability of production cells. Developers need to include additional routine testing to control that attribute. A variant sequence can be associated with a metabolic issue rather than genomic heterogeneity, so developers should think about the origin of variants and potential consequences to their control strategies. Forum participants agreed that the main concern is how it affects the quality of product that is administered to patients. Therefore, regardless of origin, the root cause must be understood and controlled.

Several participants noted that the use of cell-line pools for early clinical development can help accelerate product development and obtain information on proof of principle. Nonclonal lines have been used successfully for toxicology lots with a transition from "pool" to clonally derived production cell line. A thorough comparability study to assess the switch to a clonal cell line would be needed. However, the FDA does not recommend pooling for phase 1 studies. The use of transient mammalian expression systems was not viewed as a realistic approach to proof of concept for early clinical studies.

Regarding use of new analytical methods to assess clonality/stability of a cell line, participants saw no barrier to using new tools that provide value. New analytics for cell-line characterization are becoming powerful, but developers must balance understanding what a technique delivers and the utility of that information. For example, next-generation DNA sequencing can provide a great deal of information, much of which may not be relevant to a final product. Participants generally agreed that the higher the resolution of an analytical method, the greater are the chances that low-level events can be revealed that are unlikely to be meaningful. So developers must guard against overinterpreting risks to product quality.

Presentations: Screening, Characterization, and Product Consistency

The afternoon session started with a talk by Christopher Sellick (MedImmune Limited,

Cambridge, UK) on "**Screening Approaches for Product Quality to Enable Attribute-Driven Cell Line Development with an Eye Toward Commercialization."** The development of non-MAb entities has raised new challenges to cell-line development and requires upfront loading of a desired target product profile and titer together with distinguishing between cell-line–dependent and process-dependent attributes. Sellick described an analytical toolbox for monitoring product attributes (e.g., glycosylation, truncation, aggregation, and terminal clips) that permitted high throughput, required low sample volumes, was applicable to crude supernatants, and had a fast turnaround to enable timely decisions. The tools were successfully applied to the qualification of a new cell line during phase 2, the development of a highly glycosylated Fc-fusion protein, and a large multimeric Fc-fusion protein.

Jason Rouse (Pfizer, Inc., Andover, MA USA) then presented on **"Advances in Product Characterization During Cell Cultivation."** A best practice identified in characterization of cell production systems was through application of various ultrahigh-resolution MS-based methods at the clone selection stage and during cell culture process development. That was shown to yield vital product-quality information at the molecular level for C-terminal lysine, trisulfides, N-glycosylation patterns, aglycosylation, signal peptides, genetic sequence variants, and misincorporations. Such activities greatly enhanced assurance that the desired product quality was obtained and that the product was manufacturable.

Steffen Gross (Paul-Ehrlich-Institut, Germany) presented on **"Product Consistency During Cell Cultivation: Regulatory Expectations"** and started out discussing risks to product quality and the potential controls to ensure consistency of a production cell line and resulting product. He added that appropriate controls can be placed at different steps in a process depending on the issue at hand. For example, several MAb products showed a significant decrease in copy number over time and led to a tightening of the proposed limit of in vitro cell age. That was particularly critical if the levels of heavy and light chains were different. Genetic drift during cell-bank establishment is a recent observation, but apparently it is a widespread phenomenon possibly associated with implementation of more sensitive analytical methods that can detect low levels of variants. Observed variants are evaluated to assess the impact to safety or efficacy, and depending on the criticality, where controlled by establishing an in-process action limit or specification on the variant and/or reducing the limit of in vitro cell age. Emphasis was placed on the concern that posttranslational modifications such as glycosylation are sensitive to changes in a production system and can influence ADCC/CDC activity, antigenicity, and PK. Changes in the production system also can influence the HCP profile as shown by coelution of phospholipase-B–like activity that caused degradation of polysorbate 80 over time and would have restricted the shelf life of the product if the impurity were not eliminated. Gross described a case in which a 24-amino acid insertion in a production cell line affected up to 10% of the product. It was not detected by SDS-PAGE, SEC, or IEF but was detected by RP-HPLC with MS. His presentation concluded that the final purified protein (and final product) must be rigorously evaluated to ensure consistent quality of a DNA-derived product.

Juhong Liu (FDA CDER) presented **"Regulatory Expectations and Case Studies for Product Cell Line Development."** He stated that cell production systems are fundamental building blocks in production because multiple critical attributes are sensitive to both clonal selection and cell culture conditions. He discussed a phase-appropriate approach for development of a production cell line that allows for modifications to production cells during product development. The approach relies on a defined target product profile, prior knowledge of a product and process, well-qualified methods, and a robust comparability study. Under circumstances in which differences in CQAs are identified, additional clinical data also may be needed.

One case study Liu described involved a proposed new production cell line at the end of phase 2 that was evaluated by a strong analytical comparability package showing minor changes in critical glycans. A nonclinical PK study was requested and showed comparability sufficient to go into phase 3. Another case study showed that three WCBs derived from the same MCB yielded a comparable product but different productivity, illustrating that clonal variations can occur during expansion of a WCB. MCB changes along with changes in culture conditions have resulted in both noncomparable and comparable products, illustrating the risks associated with changes in cell-line production systems.

Disclaimer

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Panel Discussion: Sequence Variants and Regulatory Expectations

What are the expectations in terms of understanding and controlling sequence variants through a cell culture process? There was general agreement that understanding whether the root cause of a sequence variant is genetic or caused by a misincorporation event is important because that knowledge is needed to implement an effective control strategy. For example, if a variant is associated with a misincorporation with depletion of an amino acid during cell cultivation, then supplementation of the media with the appropriate amino acid is an easy fix. Genetic variants may need to be controlled by limiting population doublings, by monitoring the variant, or if early in the development process, selecting a new clone. It was noted that clones with different levels of sequence variants can be generated using the same expression system, so developers should screen for and select the most appropriate clone. In general, it was felt that the ideal situation is to eliminate all sources above a certain level for nucleotide variants even to the point at which one manufacturer stated that it will try to select a better clone if a silent nucleotide change occurs. Some regulators also worried about the potential impact of a nucleotide variant on protein translation, so developers must carefully consider the risk to product quality.

What is an appropriate limit of detection for sequence variants? Although no set LoD was identified, several participants mentioned that they have used methods with an LoD of 0.5% and that no regulator has questioned the adequacy of that detection limit. A robust discussion was held on what level of a variant would be a concern, particularly because some MS methods can quantitate sequence variants below 0.1%. There was some agreement that the level of a variant could in part depend on its biological significance, but participants generally agreed that if the observed level of a variant is linked to clinical data, then there should be few safety or efficacy concerns if the control strategy maintains that level. However, if a new variant is observed that was not present in material used in clinical trials, immunogenicity concerns could be raised that might warrant new immunogenicity studies. The level of a new sequence variety that would trigger an immunogenicity study would depend on the potential risks to product quality as it relates to safety.

What methods should be used to evaluate sequence variants? Two methods were discussed extensively aside from traditional methods: LC-MS/MS-based and NGS-based methods. Sequence variants can be reliably detected, identified, and quantitated by LC-MS/MS (with bioinformatics) down to very low levels and provide information about the primary structure of a product administered to patients. That is thus viewed as a powerful tool for characterizing sequence variants. However, some companies are moving to NGS at least as part of initial screening activities because it has a good sensitivity (0.4–0.5 %) and is relatively fast and cheaper than MS-based methods. Generally, there is good agreement between these two methods. Use of NGS during product development as a screening tool and MS during full characterization was viewed as a best practice.

What are regulatory expectations regarding use of these new technologies and applications? Regulators were cautious about providing detailed recommendations because they have limited experience with the newer technologies. Each situation may require different approaches, but in general, it is important to understand the limitations of such methods and obtain data supporting them to provide meaningful results.

Regarding changes to cell production processes, introduction of a new WCB generally does not require submission of a supplement or a variation if a company is following an approved protocol. Protocol changes require a submission for review and approval. Regulators in the United States and European Union noted that scale-up of a cell culture process has not been a significant issue unless the process changes the way cells interact with their environment (e.g., media changes, addition of wave-motion bags, and extension of the limit of cell culture age). For changes in a cultivation process, a comprehensive comparability study is warranted that might include in-process testing. Differences in CQAs must be justified.

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