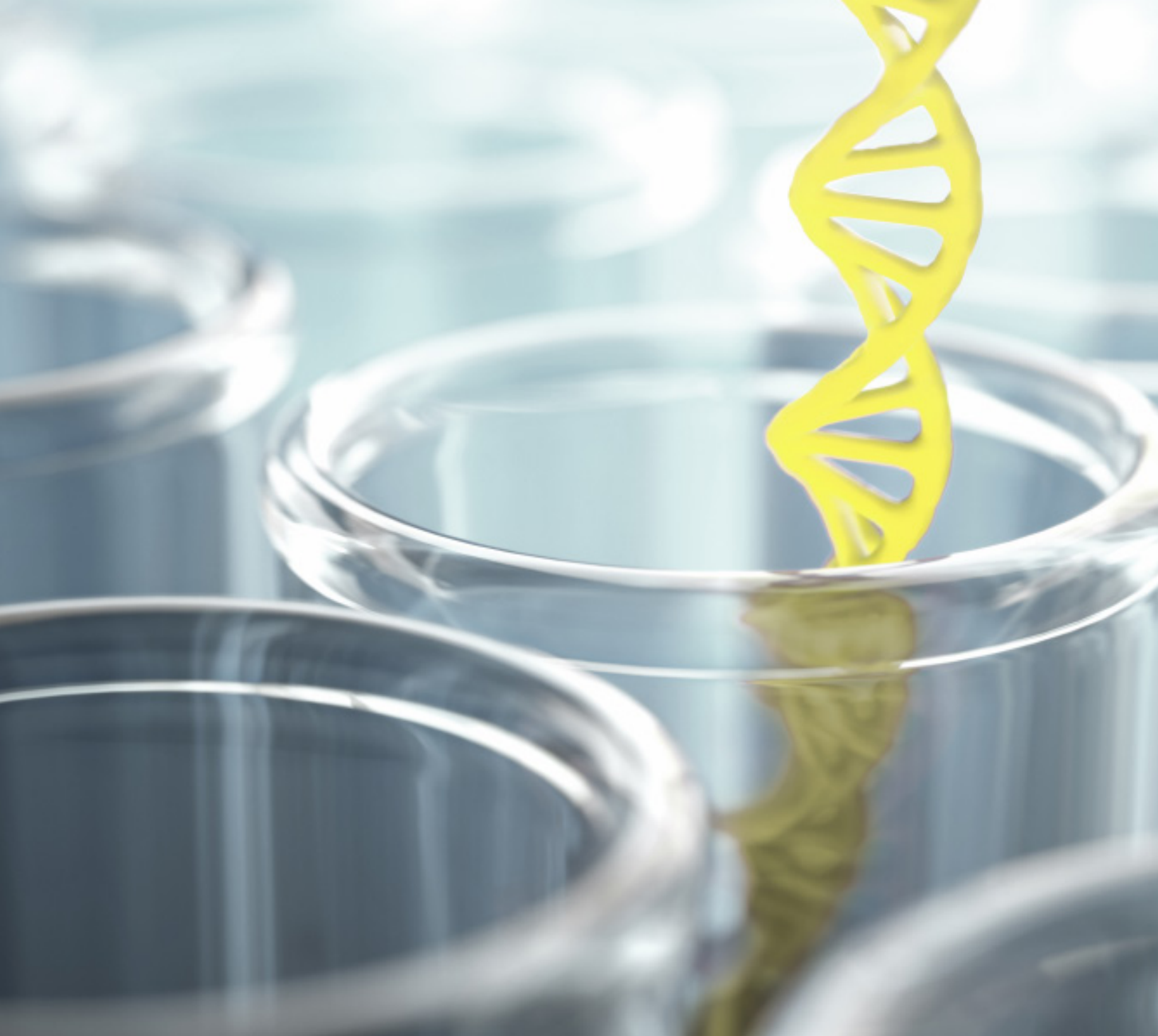
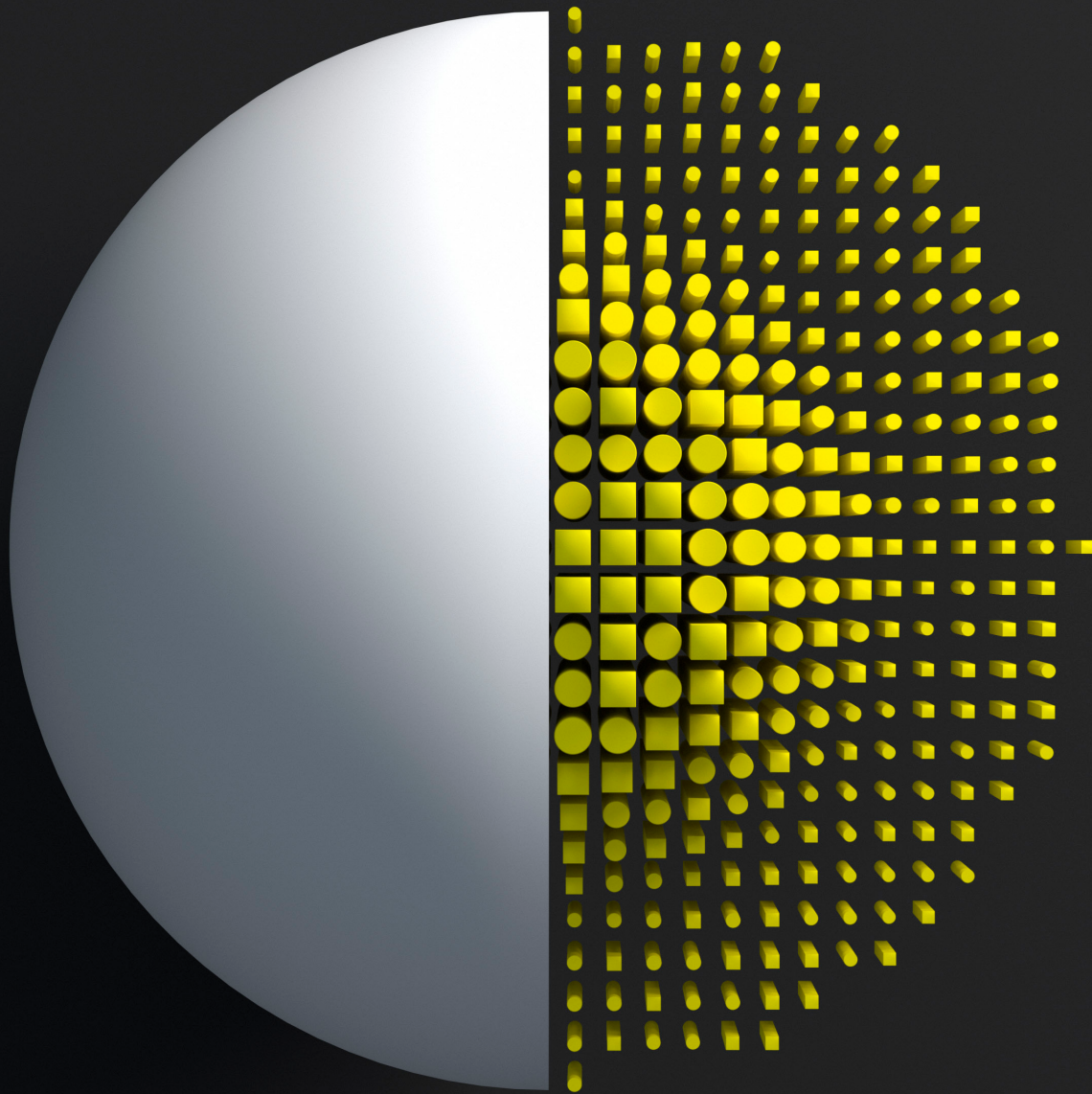




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Introduction

The advent of engineered recombinant proteins for use as therapeutic agents, termed biologics, has led to an explosion in the number of treatments available in the healthcare industry. To generate these biologics, vast quantities of host cell lines are used to express the protein in question, which is then isolated and purified. Generating these lines is known as cell line development, which encompasses the process of engineering a host cell line to express a designed therapeutic molecule for the purpose of commercial production, a process which can take months to years to accomplish.

Cell line development for a novel biologic typically follows an industry-standardized workflow in five stages. First, a host mammalian cell line is engineered to express the recombinant protein of interest. This involves creating the DNA construct encoding the protein, incorporating it into the cells, and conducting an initial identification of recombinant cell lines. Second, clones are further screened and selected based on qualifications such as high titer expression of the biologic and culture growth curves. Third, selected clonal lines go through expansion and extensive characterization for expression stability and to ensure the produced biologic meets the design criteria. Fourth, clonal lines undergo tertiary expansion to generate the capacity needed for commercial production, with extensive testing of media and incubation conditions to optimize growth and biologic production. Lastly, once clonal lines are established, they undergo cell banking, a process for generating high-density cultures used for continued cell line turnover and biologic manufacturing.

For any cell line development pipeline, key considerations include accurate measurements of cell line growth and productivity, biologic product quality, and costs associated with reagents and manual effort to maintain the cultures required for commercial production. Technologies that enable positive development outcomes and reduce the time and investment required are constantly being evaluated. In this article collection, we present recent advances in cell line development for biological-based therapeutics. These new technologies and methodologies are revolutionizing our ability

to generate, screen and characterize large number of cell lines in parallel with great accuracy and fidelity than ever before.

First, Huhn et al. (2021) details a method for generating a novel CHO host cell line for biopharmaceutical production. They engineer cell lines using a more efficient zinc finger nuclease (ZFN) approach for generating a glutamine synthetase knockout cell line with enhanced transgene production and growth characteristics. Their ZFN methodology can also be employed in creating other CHO-null lines with different characteristics based on production needs. Next, Kuhn et al. (2020) describes a protocol for identifying monoclonal cell populations using high throughput DNA sequencing and statistical analysis pipelines to detect single nucleotide variants. This approach allows for highly sensitive detection of low-percentage clonal cell fractions present in non-clonal samples by directly characterizing the genome of the clonal cells instead of the more standard method of evaluating the cloning procedure. This methodology stands to reduce the number of cloning rounds needed, thereby decreasing the overall cell line development timeframe.

Moving further into the cell line development pipeline, Markert et al. (2019) seeks to improve the clone selection process. They developed an automated high-throughput clone screening workflow that performs continual monitoring of growth and engineered biologic expression of 96 clonal lines, a significant increase in the parallel screening capacity over manual batch processing. Further, this high-throughput workflow displayed greater sensitivity and identification of optimal clones through the continual monitoring of selection criteria to isolate the best performing clones. Next, Li et al. (2019) describes an accelerated and adaptable clone selection workflow designed to identify productive and high-quality clones within 14 days of the single-cell cloning stage. This automated process incorporates single cell sorting by flow cytometry, cell imaging and multiplex immunoassays conducted in a 96-well format, with a reported four-to-seven-fold reduction in the complexity of the clonal screening process and significantly reduced timelines.

Lastly, Kreye et al. (2019) evaluates an automated microbioreactor, a reduced culture system requiring significantly fewer reagents and operational costs, for the productivity and quality of produced biologic agents. They reported the novel finding that this automated bioreactor system could be applied to cell cultures grown in suspension, without loss of the product and quality prediction power of larger cell culture approaches.

Collectively, these articles illustrate how advances in genetic editing, high-throughput sequencing, automated

monitoring, and reduced culture systems can significantly improve the process of creating and characterizing cell lines used in biologics development. By utilizing these advanced techniques, biologic manufacturers can reduce their development time and bring novel therapies to market faster and with greater effectiveness for public health.

by Jeremy Petravic, PhD.
Senior Editor, *Current Protocols*

References

Huhn, SC, Ou, Y, Tang, X, et al. Improvement of the efficiency and quality in developing a new CHO host cell line. *Biotechnology Progress*. 2021; 37(5):e3185. <https://doi.org/10.1002/btpr.3185>

Kuhn, A, Le Fourn, V, Fisch, I, Mermod, N. Genome-wide analysis of single nucleotide variants allows for robust and accurate assessment of clonal derivation in cell lines used to produce biologics. *Biotechnology and Bioengineering*. 2020; 117: 3628–3638. <https://doi.org/10.1002/bit.27534>

Markert, S., Musmann, C., Hülsmann, P. and Joeris, K. (2019), Automated and enhanced clone screening using a fully automated microtiter plate-based system for suspension cell culture. *Biotechnology Progress*., 35: e2760. <https://doi.org/10.1002/btpr.2760>

Li, X, Zhang, Y, Jing, L, et al. Integration of high-throughput analytics and cell imaging enables direct early productivity and product quality assessment during Chinese Hamster ovary cell line development for a complex multi-subunit vaccine antigen. *Biotechnology Progress*.. 2020; 36:e2914. <https://doi.org/10.1002/btpr.2914>

Kreye, S, Stahn, R, Nawrath, K, Goralczyk, V, Zoro, B, Goletz, S. A novel scale-down mimic of perfusion cell culture using sedimentation in an automated microbioreactor (SAM). *Biotechnology Progress*. 2019; 35:e2832. <https://doi.org/10.1002/btpr.2832>

Genome-wide analysis of single nucleotide variants allows for robust and accurate assessment of clonal derivation in cell lines used to produce biologics

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Funding information

Université de Lausanne; Swiss Government Commission for Technology and Innovation, Grant/Award Number: 17196.1 PFLS-LS; Selexis SA, Switzerland

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Abstract

A clonally derived (or “monoclonal”) cell line is a cell population derived from a single progenitor cell. Clonally derived cell lines are required for many biotechnological applications. For instance, recombinant mammalian cells used to produce therapeutic proteins are expected by regulatory authorities to be clonally derived. Assurance of clonal derivation (or “clonality”) is usually obtained from the characterization of the procedure used for cell cloning, for instance by assessing the success rate of single-cell sorting but not by assessing the cell line itself. We have developed a method to assess clonal derivation directly from the genetic makeup of cells. The genomic test of clonality is based on whole-genome sequencing and statistical analysis of single nucleotide variants. This approach quantifies the clonal fractions present in nonclonal samples and it provides a measure of the probability that a cell line is derived from a single cell. Upon experimental validation of the test, we show that it is highly accurate and that it can robustly detect minor clonal fractions of as little as 1% of the cell population. Moreover, we find that it is applicable to various cell line development protocols. This approach can simplify development protocols and shorten timelines while ensuring clonal derivation with high confidence.

KEYWORDS

biologic, cell line development, clonal derivation, genomics, high-throughput sequencing, monoclonality

1 | INTRODUCTION

Recombinant cell lines used to produce biologics for therapeutic use should be clonally derived (i.e., “monoclonal”), that is they should derive from a single progenitor cell. Specifically, regulatory authorities expect that master cell banks (i.e., the cell line used for manufacturing) be thoroughly documented for their clonal derivation. The rationale for this requirement is that such a cell bank is

genetically more homogeneous, which can improve the consistency and robustness of recombinant protein. In contrast, if several clonal populations are present within the cell line, potential changes in manufacturing conditions could put selective pressure on the cells, possibly resulting in modified clonal composition and variations or heterogeneity of the final product (Welch & Arden, 2019).

Most efforts geared at ensuring clonal derivation (or “monoclonality”) have relied on characterizing single-cell cloning

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procedures. Hence, from the classical limiting dilution method to the recent development of microfluidic chips to sort single cells individually, every new technology has improved the effectiveness of single-cell cloning. In contrast, direct assessment of clonal derivation based on the analysis of the recombinant cell line itself has witnessed few improvements. Indeed, until recently, molecular genetic methods that could help to assess clonality directly were very laborious (e.g. analyzing transgene integration sites) or had poor resolution (e.g. Southern blots). The advent of high-throughput sequencing, however, has opened new possibilities.

High-throughput DNA sequencing-based methods can now readily identify transgene integration sites. When transgene integration is random, integration sites can be used as a unique genetic feature of the cell line, that is as a clonal signature. The clonal derivation of a cell line can thus be established by verifying that the specific integration site is homogeneously present in the population. For instance, Aebischer-Gumy, Moretti, Little, and Bertschinger (2018) have assessed cell line clonality by generating individual subclones and by verifying that a large number of these subclones contained the same specific genome-transgene junction.

Over the last years, however, it has become increasingly clear that even clonally derived cell lines can gradually become genetically heterogeneous. Indeed, several studies have shown that subclones from the same cell line can display different phenotypic behaviors (Ko et al., 2018; Patel et al., 2018; Tharmalingam et al., 2018). In addition, clonally derived cell lines have been shown to display genetic evolution affecting their genome at various scales (from the accumulation of point mutations to chromosomal rearrangements), as well as epigenetic changes (Feichtinger et al., 2016; Vcelar et al., 2018). However, a systematic understanding of how genetic and epigenetic changes relate to phenotypic characteristics of a cell line is lacking.

Here, we show that non-clonal cell lines can be efficiently detected based on the genome-wide analysis of its single nucleotide variants (SNVs). Building on this principle, we developed and validated a formal statistical procedure for testing clonal derivation named genomic test of clonality (GTC). We derived the minimal requirements needed to ensure the high sensitivity of the test. We also show that this procedure is very robust and that it does not depend on details of either the sequencing technology or the bioinformatic analysis used for the identification of SNVs for instance. Importantly, we demonstrate that it can be efficiently integrated in the context of commercial cell line development, even in the case of procedures involving multiple successive subcloning steps.

2 | MATERIALS AND METHODS

2.1 | Cell lines, whole-genome sequencing and detection of SNVs

All cell lines used here were derived from the Chinese hamster ovary (CHO) host cell line HCB-2 (Selexis SA), except for the cell lines SG

and FG that were derived from the host cell line HCB-1 (Selexis SA). Both HCB-1 and HCB-2 lines were obtained from the CHO-M (Selexis SA) cell line, originally derived from a CHO-K1 line. Construction of expression vectors, transfection conditions, and cell culture conditions were previously described (Grandjean et al., 2011; Le Fourn, Girod, Buceta, Regamey, & Mermoud, 2014). The details of sample preparation for all experiments presented here are provided in the Supporting Information.

Sample-specific SNVs are SNVs that can be detected in the tested cell line (e.g. with a frequency >0.05 , i.e., 5%) but that have very low frequency in the parental population. We performed whole-genome sequencing using Illumina technology and detected sample-specific SNVs using standard bioinformatics methods (see the Supporting Information), including the R/Bioconductor package VariantTools (Lawrence, Degenhardt, & Gentleman, 2019). Restricting to SNVs that are very rare in the parental population allows us to measure clonal fractions in the tested population. Specifically, it ensures that if there are two progenitor cells (in the event of failed cell cloning), the probability that they will each contain a different, specific set of SNVs is very high (see Section 2.2.1). Specifically, we selected all SNVs that were present in the tested cell line and undetected in the parental cell population (i.e., no variant allele observed). Considering the high sequencing depth used for HCB-2, this corresponds to SNVs with variant allele frequencies <0.05 . Moreover, we restricted sample-specific SNVs located in regions that were present in a single copy in the genomes of both the tested cell population and the parental cell line. As fixation of such SNVs results in allele frequencies of one (whereas fixation of an SNV located in a 2-copy region results in an allele frequency of 0.5), this facilitates the estimation of clonal fractions from the parametric model (see Section 2.2.2).

2.2 | Genomic test of clonality

2.2.1 | Parametric model

Let us consider SNVs that are specifically detected in the cell population tested for clonal derivation. Each SNV is characterized by the coverage c_d (i.e., the total number of sequencing reads covering the genomic position) and the number of sequencing reads carrying the variant allele a_d (also referred to as the alternative allele count). The frequency of a variant is defined as the ratio a_d/c_d . We assume that each SNV is specific to a given clone, that is a given SNV cannot be present in two clonal subpopulations in the case of a clonal mixture (see “Verification of model specification” in the Supporting Information for the assessment of this assumption). For a given SNV in a 1-copy region of the genome, a_d is modeled as a variable from a Binomial distribution with parameters c_d (number of trials) and f (probability, corresponding to the fraction of the clone in the cell population). In other words, a_d is distributed as the number of “successes” (i.e., reads carrying the variant allele) in a series of c_d independent experiments (i.e., the total number of reads covering

that genomic location), each with probability f to “succeed” (i.e., the read originates from the clonal population carrying the variant allele). In addition, the coverage c_d varies across SNVs and it can be modeled as a variable from a Poisson distribution (Lander & Waterman, 1988) with parameter C (true average coverage). Thus, considering the set of SNVs corresponding to a given clone, the alternative allele count a_d can be modeled as a Binomial distribution (i.e., the probability of observing the alternative allele count for a fixed coverage) compounded by the Poisson-distributed coverage (see e.g. Ocerin & Pérez, 2002). This compound distribution is another Poisson distribution with parameter $C * f$ (true average coverage times clonal fraction). Finally, if we assume that the cell population is composed of two clones, we can model the variant allele count for all SNVs in the population as a mixture of two Poisson distributions (with parameters $C * f$ and $C * (1 - f)$, respectively, see Figure S1a).

2.2.2 | Parameter estimation

We aim to estimate f from the parametric mixture model described above. In the present case, directly fitting a mixture of Poisson distributions is unpractical, however, because the alternative allele count distribution is usually left truncated (at the minimal number of reads set to detect SNVs reliably, e.g. here four reads) or contaminated by artefactual SNVs. To circumvent this problem, we developed a 2-step procedure: We obtain an initial estimate of f that allows for the detection of balanced to moderately unbalanced (e.g. $f = 0.5$ – 0.8) clonal mixtures without making any assumption. Based on this initial measure, we next determined a second, very accurate measure of f for mixtures with larger major clonal fractions (and correspondingly smaller minor fractions). This refined measure of f provides the high sensitivity needed to detect highly unbalanced samples.

The initial estimate of f is obtained by fitting the upper component of the Poisson mixture only. Specifically, we fit a single, truncated Poisson distribution, whose truncation level is given by half the mean coverage (i.e., $\bar{c}_d/2$), allowing to estimate $C * f$ (Figure S1b). The fraction of the majority clone f is given by $f_{\text{pois}} = (C * f) / \bar{c}_d$. This initial measure of f allows us to efficiently and safely reject monoclonality in the cases of clonal mixtures that are not too unbalanced. When clonal ratios are greater than approximately 80/20, however, we can obtain a more accurate measure of f by identifying SNVs from the majority clone and fitting them specifically, hence removing the mixture problem. We define the two Poisson component distributions as “separable” when their overlap is small enough such that the probability of making an error when assigning each allele count (i.e., SNV) to a clonal subpopulation has a fixed upper bound (specifically, if there is a count value such that at least 95% of counts from the majority clone are strictly greater and at most 5% of counts from the minority clone are strictly smaller, see Figure S1a). Separability depends on SNV coverage: the higher the coverage, the greater the separability (conversely, if coverage is low, e.g. <5 , even two clones with a very unbalanced clonal ratio, e.g. 90/10, will not be separable).

If the initial Poisson-based measure f_{pois} is greater than the minimal fraction ensuring separability (for the observed average coverage $\bar{c}_d$) (Figure S1c), we identify SNVs from the majority clone and use them to calculate the more accurate, Binomial-based measure of f . For a typical average coverage of 13, the minimum (true) f allowing separability is 0.81.

SNVs from the majority clone are identified as follows (Figure S1d): for each SNV coverage value, we determine the count value such that when most of the allele counts (i.e., at least 99%) of the distribution from the majority clone (modelled as Binomial with parameters c_d and f_{pois}) are included, the probability of having an allele count of the distribution of the minority clone (modelled as Binomial with parameters c_d and $1 - f_{\text{pois}}$) above this count value is upper bounded (at most 5%). Thus, SNVs with counts above this boundary are confidently assigned to the majority clone (Figure S1d). When restricting to SNVs identified as originating from the majority clone, we obtain a more accurate measure of f as follows: as the probability parameter of these Binomial variables is identical, their sum ($\sum a_d$) follows a Binomial distribution with parameters $\sum c_d$ and f , and f is thus given by $f_{\text{binom}} = \sum a_d / \sum c_d$. We finally used the Agresti-Coull method to calculate a confidence interval for this binomial fraction estimate. A sample is called (clonally) pure only if the lower limit of the Agresti-Coull confidence interval for f_{binom} is >0.99 (see “Operational threshold for clonal purity” in the Supporting Information). All statistical procedures were implemented using the R software (R Core Team, 2020).

2.2.3 | p-value for clonal derivation

For a cell population that is deemed clonally pure, we can obtain a p -value corresponding to the hypothesis that it is derived from a single progenitor cell (i.e., clonal derivation, or monoclonality). For the sake of illustration, let us first consider a single individual SNV that is rare in the parental population and becomes fixed upon cell cloning. If single-cell cloning fails and there are two progenitor cells instead of 1, we will wrongly call the population clonally derived if, and only if, the second cell bears the same SNV. If we assume that the two progenitor cells are randomly drawn from the population, the probability that the second cell bears the same SNV is given by its population frequency. This frequency thus formally corresponds to a statistical p -value for clonal derivation (i.e., the probability of making an error if we accept the null hypothesis that the population is clonally derived).

If we now consider more than 1 fixed SNV in the derived population, the probability that the exact same set of SNVs is carried by the second progenitor cell is bound to be lower than the probability obtained with a single SNV, such that the confidence of clonal derivation is higher. Formally, the p -value corresponding to the smallest SNV frequency observed in the parental population corresponds to the upper bound (i.e., conservative estimate) of the true p -value.

In practice, the measurement of SNV frequency via standard sequencing library preparation and sequencing has a limited

resolution. Owing to the intrinsic per-base error in the current best standard sequencing technologies, the limit of rare variant detection is (conservatively) estimated at 0.05. This limit can be reached with a sequencing depth $>50\times$ – $100\times$ and higher sequencing depths do not improve the sensitivity of detection. Here, we thus performed deep sequencing of the host cell population (i.e., $150\times$ for HCB-2) and considered that if we did not observe a single alternative allele in the parental population (i.e., for an SNV fixed in the derived population), it had a frequency <0.05 , yielding a p -value for clonal derivation <0.05 . Importantly, we have shown that SNVs fixed in derived cell lines are at least $\times 10$ rarer than this (i.e., <0.005 , see “Verification of model specification” in the Supporting Information). This implies again that in the present application of GTC, the true p -value for clonal derivation is significantly less than the upper bound of 0.05.

3 | RESULTS

3.1 | Characterization of the genetic diversity in parental host cells and in clonally derived cell lines

In the process of producing a new cell line for recombinant protein production, a cell population (i.e., the “host” or “parental” population) is transfected with a vector directing the expression of the recombinant protein. A single cell is isolated from that population and

gives rise to the new cell population (i.e., the derived cell line) upon cell divisions (Figure 1a). If there is abundant genetic diversity in the host cell line, the single-cell cloning step is expected to result in a dramatic decrease in the genetic diversity of the derived cell line as compared to the host cell line: mutations contained in the progenitor cell will be inherited by daughter cells, whereas other genetic variants present in the host cell line will be absent from the derived cell line. In contrast, if cell cloning fails and two progenitor cells lead to a non-monoclonal cell line, the derived population will contain two cell subpopulations that each inherited from the genetic variants contained in the corresponding progenitor cell (Figure 1c).

Here, we specifically consider the genetic diversity provided by SNVs but the argument holds true for other types of mutations as well. The difference in the genetic makeup of a population originating from one or two progenitor cells is reflected in the SNV frequency spectrum of the derived population. In the case of a single progenitor cell, all SNVs are fixed and thus have a population frequency of 1 (Figure 1b) whereas if there are two progenitor cells and each cell bears specific SNVs, the SNVs in the derived population are present in only half of the cells and thus have a frequency of 0.5 (Figure 1d). We will show that this difference can provide the basis for a formal test of clonality.

To verify our assumptions, we first characterized SNVs in a standard host cell line (CHO-K1 derivative) that is routinely used to generate cell lines for recombinant protein production, as well as in a

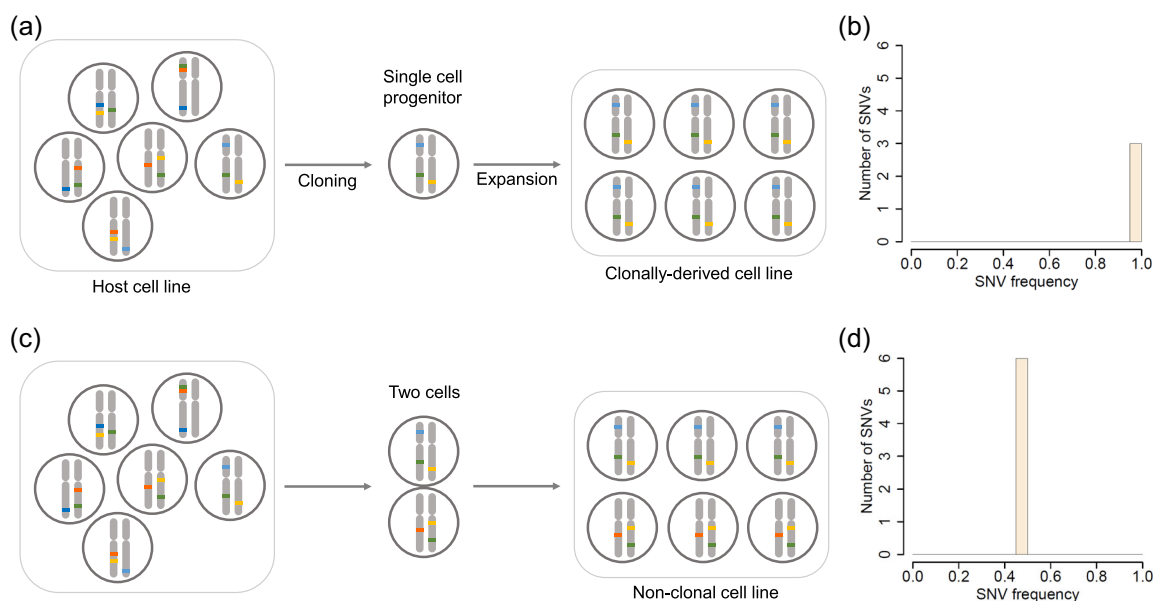


FIGURE 1 Schematic representation of genetic diversity in a host cell line and derived cell lines. (a) Cells in the host cell line contain a variety of rare mutations (specifically single nucleotide variants (SNVs), represented as color ticks on the chromosomes). The genome is depicted as a single pair of chromosomes for simplicity. Upon cell cloning SNVs harbored by the progenitor cell (and represented by the blue, green, and yellow ticks) are inherited by all daughter cells and thus become fixed in the clonally derived cell line. (b) Population frequency of SNVs in the clonally derived cell line (represented in panel a) showing that three SNVs are detected with a population frequency of 1 (i.e., present in all cells). (c) Non-monoclonal cell line derived from two progenitor cells. Each progenitor cell contains specific SNVs and the derived cell line is composed of two subpopulations that are each derived from one of the progenitor cells. (d) Population frequency of SNVs in the derived non-monoclonal cell line (represented in panel c) showing six SNVs each with a population frequency of 0.5 (i.e., present in half of the cells) [Color figure can be viewed at wileyonlinelibrary.com]

clonally derived cell line. Both samples were subjected to whole-genome sequencing and SNVs were detected using standard bioinformatics methods. The vast majority of SNVs detected in the host cell line were low-frequency (<0.2 , i.e., 20%) mutations whereas the derived cell line indeed contained many newly fixed mutations (Figure S2), as anticipated.

3.2 | Cell lines derived from one or from two progenitor cells display different SNV frequency spectra

We, then, explored the detection of clonality based on the analysis of SNV frequencies using clonal mixtures generated in silico. Specifically, we subjected two clonally derived cell lines to whole-genome sequencing and generated mixed samples by combining their sequencing data. To obtain a sample mimicking a balanced (50/50) clonal mixture (corresponding to the hypothetical case illustrated in Figure 1c,d), we pooled equal proportions of reads from each of the two clonally derived cell lines (Figure 2a). The analysis of this mixed sample revealed that sample-specific SNVs had frequencies centered around 0.5, as anticipated.

We repeated the mixing procedure to generate samples mimicking clonal mixtures of varying ratios (80/20 and 95/5 in Figure 2b and 2c, respectively). Upon detection of SNVs, we observed fewer SNVs with frequency around 0.5 and relatively more SNVs with lower (<0.5) or higher (>0.5) frequencies. This reflects the underlying presence of two SNV clusters: The lower cluster originates from SNVs fixed in the cell line representing the minor clonal

fraction, whereas the higher SNV cluster contains SNVs fixed in the cell line representing the majority clone (see also Figure S3). Moreover, the greater the fraction of the majority clone, the greater the number of high-frequency SNVs. In conclusion, the analysis of SNVs can reveal the presence of two clonal populations even when unbalanced and their frequencies provide quantitative information on clonal fractions.

3.3 | The genomic test of clonality (GTC) has high accuracy and sensitivity

Building on the statistical analysis of SNVs, we developed an accurate measure of the clonal fractions (potentially) present in a cell population and derived a measure of the probability that the cell line is clonally derived (i.e., p -value for clonal derivation, or “monoclonality”) (see Section 2 and Figure S4). The procedure relies on the whole genome sequencing of the cell line and its corresponding host cell population. Subsequent bioinformatic analysis allows us to identify SNVs that are specific to the derived cell line (i.e., SNVs that have very low frequency in the parental population). The detected SNVs are then used to run GTC, which involves two steps: First, we fit a parametric model that yields measures of clonal fractions. If the cell population is deemed pure (clonal homogeneity), the second step calculates a p -value for clonal derivation. Specifically, we test the hypothesis that the cell population is derived from a single progenitor cell. In the terms of statistical inference, the p -value thus is the probability that the cell population is not clonally derived and actually derives from two progenitor cells bearing the same SNVs (indeed,

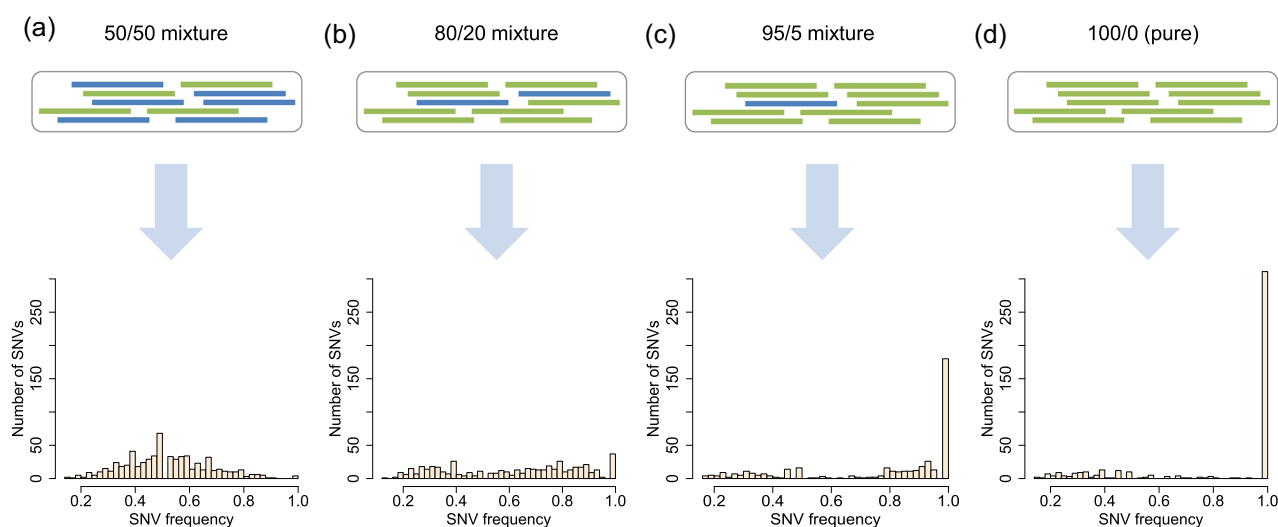


FIGURE 2 Simulation of mixed clonal populations and analysis of their single nucleotide variant (SNV) frequency spectra. Sequencing reads originating from whole-genome sequencing of two clonally derived cell lines (represented by blue and green rectangles) were combined to simulate clonal mixtures presenting varying ratios of the two cell lines. Each mixture was subjected to SNV analysis and the corresponding SNV frequency spectrum is represented at the bottom. The ratio of the two cell lines in each artificial sample is as follows (percentage of green cell line/percentage of blue cell line): (a) 50/50, (b) 80/20, (c) 95/5, (d) 100/0 (monoclonal population) [Color figure can be viewed at wileyonlinelibrary.com]

if the two progenitor cells do not carry the same SNVs, clonal heterogeneity is detected in the first step of GTC).

We tested the accuracy of the clonal fractions measured by GTC and the sensitivity of the method using a cellular mixing experiment. In short, cells from two clonally derived cell lines were mixed in various ratios (Figure 3a) to determine what was the lowest minor clonal fraction that GTC could robustly detect. We analyzed the following mixed populations (percentage of major clone/percentage of minor clone): 95/5, 98/2, 99/1, and 100/0 (i.e., a pure clone). To independently verify the actual clonal proportions in the mixed samples, one of the two clonal populations was stained beforehand so that sample composition could be independently measured using flow cytometry. Finally, each mixed sample was subjected to whole-genome sequencing and analyzed using GTC in a blinded fashion. It is important to highlight that the identity of SNVs fixed in each of the two clones was not known a priori to GTC.

Analysis of the mixed samples showed that SNVs yield a strong and robust signal for clonality assessment (Figure 3b). As the fraction of the major clone increased from 95% (Sample 1) to 100% (Sample 4), the distribution of SNV frequencies gradually shifted to the right towards 1. Based on these SNVs, GTC could infer measures of clonal

fractions (along with tight confidence intervals, as shown at the top of each barplot in Figure 3b). Samples 1–3 were deemed clonally heterogeneous and thus non-monoclonal. Sample 4 presented mostly fixed SNVs and was deemed (clonally) pure and clonally derived ($p < .05$). Comparison with parallel flow cytometry-based measurements showed that the clonal fractions obtained with GTC were at least as accurate (Figure 3c). Notably, GTC could differentiate between samples composed of 99% of a clone (Sample 3) and the corresponding pure sample (Sample 4), demonstrating that it can robustly detect minor fractions of contaminating cells as low as 1%.

3.4 | Validation of GTC in the context of cell line development

We asked if GTC could be applied to assess clonality in the context of routine cell line development. Our standard platform is based on the use of the ClonePix FL (Genetix). We thus performed a standard single-cell cloning procedure involving plating a cell suspension in semi-solid medium and automated colony picking following strict imaging criteria. Instead of a transfected cell population, however, we

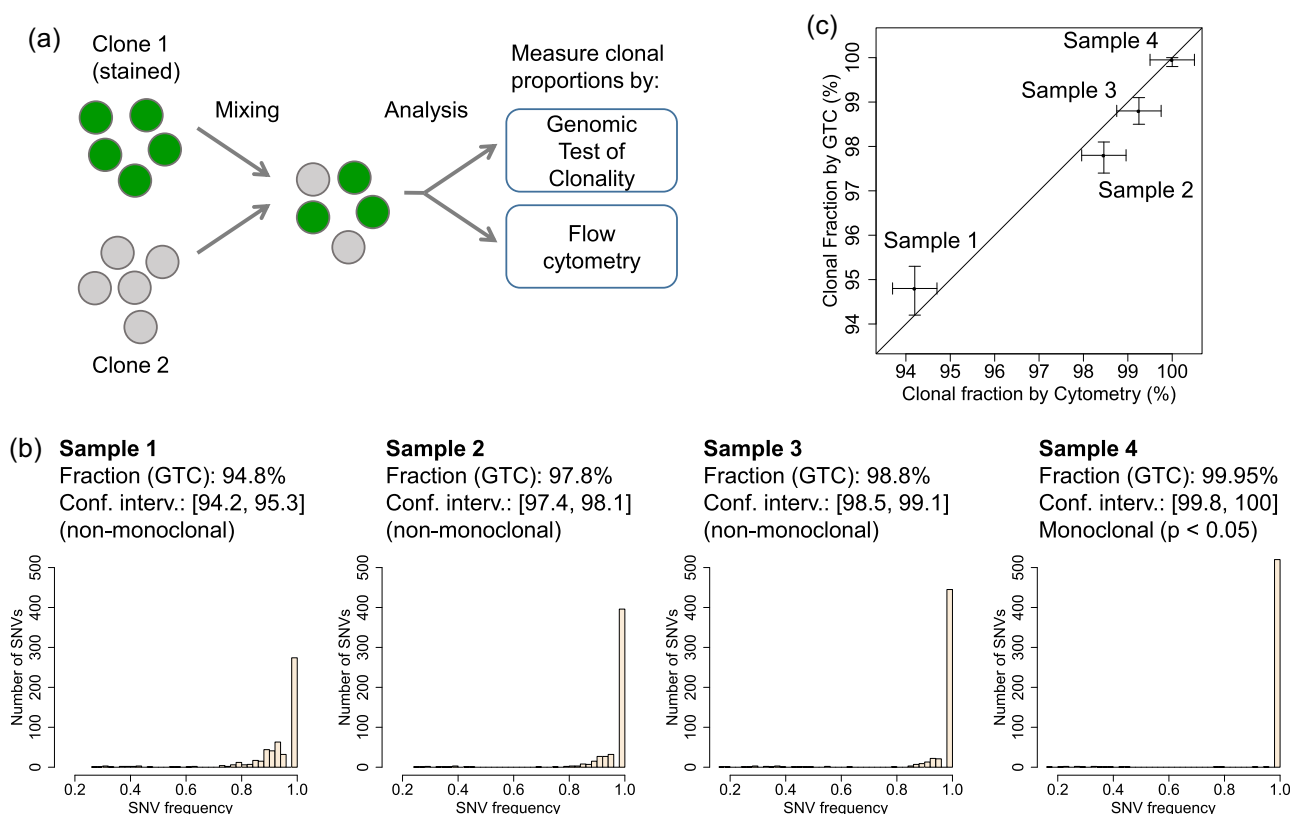


FIGURE 3 Experimental validation of clonal fractions measured by the genomic test of clonality (GTC). (a) We experimentally mixed two clonally derived cell lines in varying cellular proportions and applied GTC to measure clonal fractions. Cells from one cell line were stained beforehand and the actual cellular composition in each mixture was measured by flow cytometry as well. (b) Single nucleotide variant (SNV) frequency spectra, clonal fractions, corresponding confidence intervals, and p value for clonal derivation provided by GTC analysis for three highly unbalanced samples and a pure sample. GTC deemed Samples 1–3 as clonally heterogeneous (non-monoclonal) and Sample 4 as clonally pure and clonally derived ($p < .05$). (c) Comparison of clonal fractions measured by GTC and by flow cytometry. The bars represent confidence intervals [Color figure can be viewed at wileyonlinelibrary.com]

used a mixture of two clonally derived cell lines selected for growing at extremely fast and slow rates (referred to as fast grower, or FG, and slow grower, or SG, see "Preparation and detection of non-clonal colonies during cell line development" in Supplementary Methods). This was performed to mimic the dilution of slow-growing cells cultured within a population of fast-growing ones, to ascertain that a mix of two clones growing at highly different speeds could effectively be distinguished using GTC (Figure 4a). Using previously established diagnostic PCR assays designed to identify each of the FG and SG clones, we screened the picked colonies for non-clonal samples showing both the FG- and SG-specific amplicons (Figure 4b), as opposed to clonal samples showing either the FG- or SG-specific

amplicon. Given the very rare occurrence of non-clonal colonies under standard operating conditions used for cell cloning, we intentionally increased the cell density used to seed semi-solid medium to decrease the number of colonies that needed to be screened to find non-clonal ones. Finally, we subjected several clonal and non-clonal samples to whole-genome sequencing and GTC in a blinded manner.

Without a priori information about the FG and SG cell lines, GTC correctly identified clonal (i.e., pure) and non-clonal (i.e., mixed) samples, in line with the PCR screen (Figure 4c). Given that the FG cell line had a shorter doubling time, we expected to obtain a much greater fraction of FG cells compared to SG cells in the case of mixed

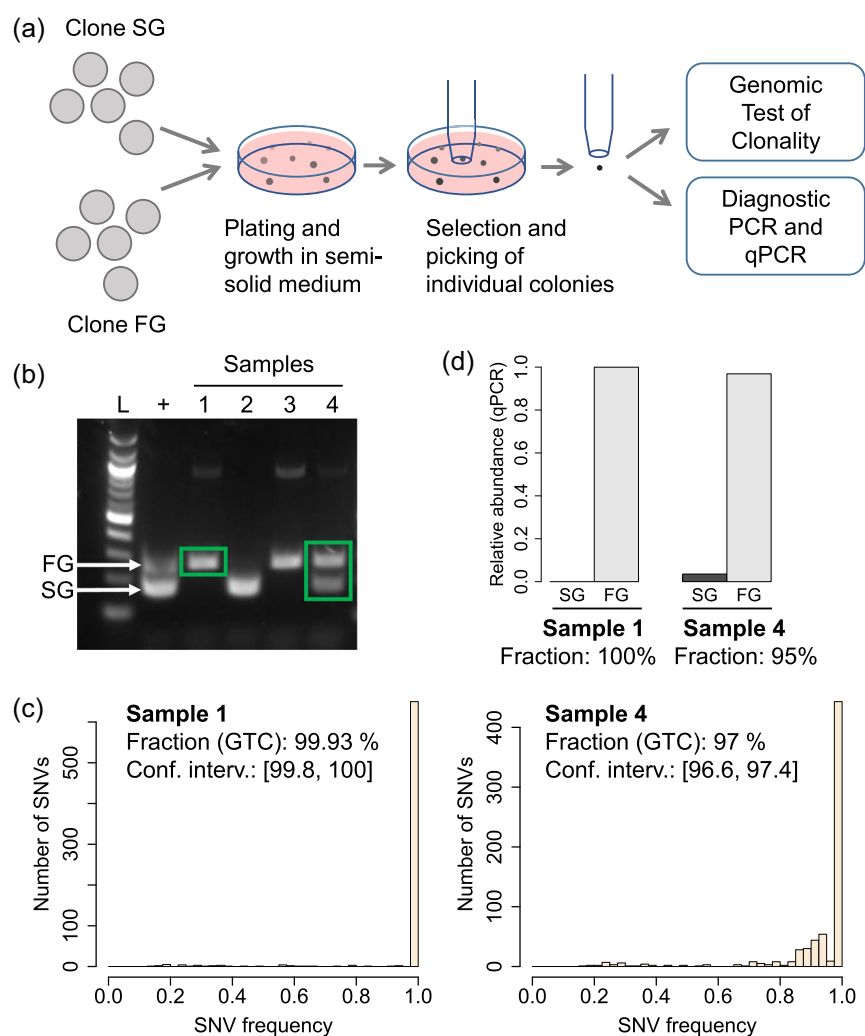


FIGURE 4 Validation of genomic test of clonality (GTC) in the context of cell line development using an automated cloning and imaging system. (a) A cell suspension composed of two clonally derived cell lines (named FG and SG) was plated in semi-solid medium. Ten days later, individual colonies were picked according to standard imaging criteria. Colonies were screened by polymerase chain reaction (PCR) to identify clonal and non-clonal colonies. Selected clonal and non-clonal samples were subjected to GTC and quantitative PCR (qPCR). (b) Result of the PCR screen. Gel electrophoresis analysis of four samples showing FG-specific amplicon only (Samples 1 and 3), SG-specific amplicon only (Sample 2) or both amplicons simultaneously (Sample 4), hence revealing a colony composed of both clones. Green rectangles highlight samples displayed in panel c. (c) Single nucleotide variant (SNV) frequency spectra and results of GTC applied to Samples 1 and 4. Sample 1 was deemed clonally pure. It could not be called clonally derived because the sequencing depth of the parental cell line HCB-1 was insufficient to reach a significance threshold of 0.05. Sample 4 was found to be clonally heterogeneous (and thus non-monoclonal). (d) Fractions of the FG and SG clones measured by qPCR in Samples 1 and 4, in line with the clonal fractions measured by GTC [Color figure can be viewed at wileyonlinelibrary.com]

samples. In line with our expectation, the major clonal fraction of the non-clonal sample was measured by GTC to comprise 97% of the sample. As a validation, we independently measured clonal proportions using qPCR assays which confirmed the accuracy of GTC (Figure 4d). These results show that GTC provides an agnostic and accurate method that can be used for the assessment of clonality in the context of standard cell line development procedures, even in the very unfavorable case of mixed clones bearing highly different doubling times.

3.5 | GTC can be applied in cell line development procedures involving successive subcloning steps

Current cell line development procedures can involve multiple transfection and cell cloning steps. For instance, a workflow involving two successive transfection-and-cloning rounds may be applied to increase cell line productivity (Figure 5a). Moreover, an additional, final round of cell cloning can be used to ensure the clonal derivation of a cell line designed for therapeutic protein production. In that case,

applying GTC to the cell line obtained after the second transfection-and-cloning round (SCC1 in Figure 5a) would eliminate the need for a second cloning step, thereby saving time and resources.

The application of GTC, however, requires that sample-specific SNVs be identified. In the present case of two successive rounds of cloning, the parental population to consider is the cell line obtained after the first transfection-and-cloning round (i.e., SCC0 in Figure 5a), not the original host cell line. Indeed, if sample-specific SNVs are detected by comparison to the host cell line, SNVs identified as fixed in SCC1 may be already fixed in SCC0. Such SNVs will confound the estimation of clonal fractions in the case of failed cell cloning because two potential progenitor cells (from the SCC0 population) will carry the same SNVs.

In such a cell line development protocol containing multiple cloning rounds, the parental population is thus freshly clonally derived and its level of genetic diversity is expected to be low. GTC, however, requires that the parental population contains enough genetic diversity to distinguish potential subclonal populations (Figure S5a,b). Thus, to determine if GTC may be used, we first characterized the genetic diversity in cell populations obtained from successive

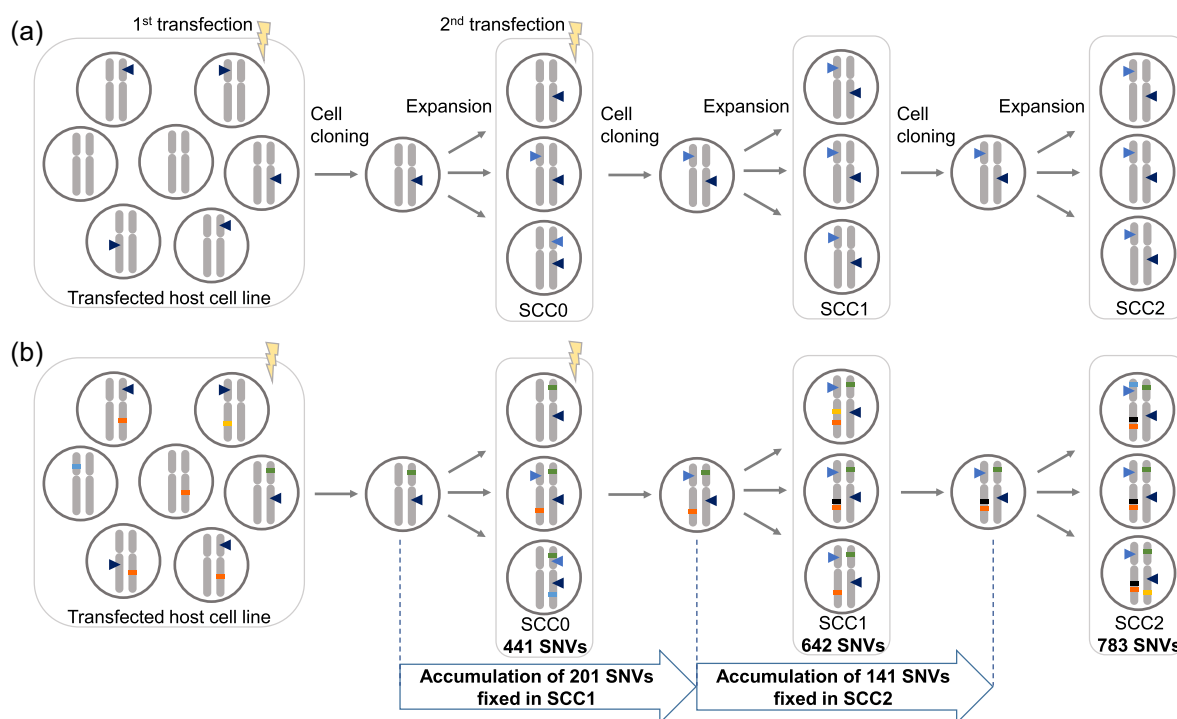


FIGURE 5 Schematic representation of a multistep process used for commercial cell line development (comprised of two transfections and three rounds of cell cloning) and the genetic evolution of successive subclones. (a) A host cell line is transfected and the transgene (dark blue triangle) randomly integrates into the genome. Upon cell cloning and expansion (SCC0), a second transfection is performed, resulting in additional transgene integration sites (light blue triangle) that increase transprotein production. Two final consecutive rounds of cell cloning are performed to obtain high assurance of clonal derivation for the final cell line (SCC2). (b) Schematic representation of the measured genetic diversity and evolution during successive rounds of single-cell cloning and expansion. Single nucleotide variants (SNVs) harbored by the progenitor cell selected upon the first transfection (green tick) are fixed in the derived cell line (SCC0). However, new SNVs appear during expansion (orange and blue ticks). The new SNV contained in the next progenitor cell (orange tick) becomes fixed upon expansion of SCC1. Whole-genome sequencing revealed 207 such SNVs in the SCC1 cell line. The final round of cell cloning selects a progenitor cell that contains an SNV (black tick) that appeared during SCC1 expansion and that becomes fixed in SCC2. Whole-genome sequencing revealed 150 such SNVs in the SCC2 cell line [Color figure can be viewed at wileyonlinelibrary.com]

subcloning steps. Specifically, we sequenced three samples from the same direct lineage (Figure 5a): the cell line obtained after initial transfection and cell cloning (SCC0), the cell line (SCC1) obtained from SCC0 re-transfection and subcloning and the final cell line (SCC2) obtained from the second consecutive cell cloning. We identified SNVs fixed in each of the three samples by comparing it to the host cell line (Figure S6a). We observed that the number of fixed SNVs steadily increased along this cell lineage, as expected from several rounds of mutation occurrence and fixation during cloning, starting at 441 fixed SNVs in SCC0, and increasing to 642 and 783 fixed SNVs in SCC1 and SCC2, respectively (Figure 5b, and see “Genetic diversity in cell populations obtained from successive sub-cloning steps” in the Supporting Information and Figure S6b,c).

These results indicated that a newly cloned population subjected to 6–8 weeks of culture already shows a significant genetic diversity. We then asked how rare new SNVs were in the parental population, to make sure that GTC would be able to detect two clonal sub-populations in the case of failed cell cloning (as each of the two progenitor cells that would be selected should carry a specific set of SNVs). Instead of measuring SNV frequency by deep sequencing of SCC0 cells, we reasoned that if new SNVs contained in SCC0 were indeed rare, two different SCC1 cell lines obtained from the same transfection should show very few common, newly fixed SNVs. We thus sequenced an additional SCC1 cell line obtained from the same transfection (see “Verification of model specification: potential presence and influence of shared SNVs” in the Supporting Information and Figure S5c). This SCC1 sister line displayed 634 fixed SNVs, comprised of 425 SNVs found to be fixed in SCC0 and 209 newly appeared SNVs. Importantly, all newly fixed SNVs in the sister SCC1 cell line were different from the newly fixed SNVs identified in the original SCC1 cell line (Figure S5d). This demonstrates that if single-cell cloning performed upon the second transfection had failed (and resulted in the selection of two progenitor cells), each of the two cells would contain hundreds of SNVs that would be absent from the other progenitor cell and that would yield a strong signal for GTC to detect non-clonality and provide a sensitive measure of clonal fractions. In conclusion, GTC can also be efficiently applied to development protocols involving multiple successive cloning steps where the parental cell line is a recently cloned population.

4 | DISCUSSION

We report the development of a novel method to assess cell line clonality based on the genome-wide analysis of SNVs. This genomic test of clonality requires the genome sequencing of the cell line to be tested as well as its parental cell line. We validated the clonal fractions measured by GTC using mixtures of two clonal cell lines and showed that the test is accurate and highly sensitive as it can robustly detect minor clonal fractions of 1%. Interestingly, GTC does not require deep sequencing of the test cell line as it leverages the pooled information gathered over many SNVs. Thus, relatively shallow sequencing (i.e., 25× coverage) yields high-resolution measures of

clonal fractions (i.e., 1% resolution, as fractions of 98% and 99% can be distinguished) and correspondingly tight confidence intervals. GTC is a statistically safe procedure as built-in calculations verify beforehand that the number and coverage of detected SNVs provide enough statistical power. Specifically, it ensures that a minor clonal fraction of 1% can be detected if it is present (see “Operational threshold for clonal purity” in the Supporting Information).

Cell lines can undergo clonal and genetic evolution, even upon culturing under constant conditions. For instance, two clonal sub-populations that originated from the presence of two progenitor cells at cloning might gradually change in relative abundance over time (as assessed in our experiment using two clones with very different growth rates). We cannot exclude, for instance, that a minor clone having an initially very low growth rate might represent a sub-threshold fraction (i.e., <1%) at the time of analysis but might later increase in relative abundance upon subsequent culturing. On the other hand, a clonally derived cell line might spontaneously become heterogeneous upon culturing. To avoid potential issues of genetic evolution, it is thus generally advisable to use GTC on cell lines that have not been subjected to extended culturing times.

The statistical procedure used in GTC, however, is robust against SNVs that would newly appear in the cell population after cloning. Specifically, our method avoids relying on low-frequency SNVs to infer clonal fractions. This point was illustrated by applying GTC to two clonally derived research cell banks (RCBs). Both RCBs were deemed (clonally) pure (major clonal fractions $f = 99.95\%$ and 99.94%). After further development into master cell banks (MCBs), which involved culturing the two cell populations for several additional weeks, they were sequenced and subjected to GTC again: The major clonal fractions estimated by GTC from MCBs ($f = 99.96\%$ and 99.85% , respectively) were unchanged compared to what was obtained from the analysis of the RCBs, despite the new SNVs that had inevitably appeared during further culturing.

For cell populations deemed clonally pure, GTC can provide a p -value for clonal-derivation (“monoclonality”), that is they are derived from a single progenitor cell. Specifically, the confidence for clonal derivation is inversely related to the frequencies of SNVs in the parental cell line. The latter can be measured by deep sequencing of the parental population (e.g. here we used 150× coverage), yielding significant p -values for clonal derivation. Alternatively, an approach combining shallower whole-genome sequencing of the parental cell line (which is sufficient for the accurate measurement of clonal fractions in the derived cell line, see Methods) followed by deeper, targeted resequencing of a small set of loci might be used to confirm that at least a small subset of SNVs are very rare in the parental cell line and thus provide high assurance of clonality. Importantly, we found, however, that most of the specific SNVs fixed in a clonally derived cell line are at least 10-fold rarer than what can be measured by standard high-throughput sequencing methods, indicating that true p -values for clonal derivation are even much smaller than the conservative estimate provided by GTC. Thus, currently, the overall assurance of clonal derivation provided by GTC is effectively conditioned by the sensitivity of GTC to detect minor

clonal fractions (conservatively estimated at 1%), rather than by the *p*-value for clonal derivation as it is comparatively much lower. A future, formal assessment of LOD (limit of detection) may thus bring the overall assurance of clonal derivation beyond 99%.

Current methods used for the routine assessment of clonal derivation during cell line development are usually based on the technical characterization of the cell cloning procedure. When performing limiting dilution, for instance, the probability of selecting two progenitor cells instead of one can be estimated from the density of the cell suspension and appropriate probability calculations. Typically, if one-third of the wells of the limiting dilution vessel contains cells, then at least one-third of the cell populations derived from wells containing cells are non-clonal. Imaging cells during the cloning process can significantly increase the assurance of clonal derivation (see e.g. the review by Chen et al., 2020). It is not devoid of technical difficulties though, ranging from optical performance to automated cell recognition. To date, no method can provide definitive, 100% assurance of clonal derivation. Furthermore, minor changes in a process previously characterized to yield clonal populations with a given probability may also alter statistical confidence, which would not be detected by the experimenter or regulator *a posteriori*. The genetic analysis performed by GTC is the first method that provides direct characterization of a cell line. Here, we also show an example application of GTC in the context of cell line development using an automated imaging and cloning system (Figure 4), which allowed us to detect non-clonal samples that were not detected by imaging alone.

Furthermore, GTC can be applied to protocols involving multiple cloning steps, such as the subcloning of a recently derived clonal cell line (e.g. when performing successive rounds of transfection and cloning). Indeed, our analysis showed that clonal evolution proceeds at a rate that is sufficient for genetic diversity to reestablish upon weeks of cell culture only. We hypothesize that the underlying origin of these mutations corresponds to the known low mutagenesis background of mismatched bases spontaneously introduced by DNA polymerase during DNA replication, most of which may be neutral from an evolutionary and selection perspective. Indeed, we observed in subclones of recent clonal cell lines that the number of fixed SNVs was proportional to the culturing time preceding cell cloning (Figure 5b and “Genetic diversity in cell populations obtained from successive subcloning steps” in Supplementary Results). Moreover, SNV frequency spectra measured in host cell lines showed exponentially decreasing distributions (Figure S2), in line with the theoretical prediction made for dividing cell populations undergoing a fixed mutation rate. Note also that this type of mutation frequency spectrum, with the rarest mutations belonging to the most frequent mutation type, is ideal for GTC as it ensures with high probability that two random cells in the population contain a majority of different SNVs thereby allowing for highly sensitive detection of two clonal subpopulations in the derived cell line.

In conclusion, GTC can be flexibly implemented in the context of many current cell line development protocols. Given its performance, this direct genetic assessment could eliminate the need for a

second round of cell cloning that is often needed to obtain high final assurance of clonal derivation. GTC thus has the potential to allow for faster development timelines. The case of routine cell line development based on a single transfection of a host cell line could particularly benefit from the application of GTC: As the same host cell line is used repeatedly as a parental line, it must be sequenced only once. Subsequently, shallow sequencing of derived cell lines combined with GTC can provide a cost-effective method to obtain highly characterized cell lines with a high assurance of clonal derivation.

ACKNOWLEDGMENTS

We thank Samuel Neuenschwander for the bioinformatic analysis of transgene integration sites in the SG and FG cell lines, Alexandre Regamey for providing the corresponding diagnostic PCR assays, and Myriam Adam for the management of cell line development projects. This study was supported by the Swiss Government Commission for Technology and Innovation (grant number 17196.1 PFLS-LS), Selexis SA, and the University of Lausanne.

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REFERENCES

- Aebischer-Gumy, C., Moretti, P., Little, T. A., & Bertschinger, M. (2018). Analytical assessment of clonal derivation of eukaryotic/CHO cell populations. *Journal of Biotechnology*, 286, 17–26. <https://doi.org/10.1016/j.jbiotec.2018.08.020>
- Chen, C., Le, K., Le, H., Daris, K., Soice, N., Stevens, J., & Goudar, C. T. (2020). Methods for estimating the probability of clonality in cell line development. *Biotechnology Journal*, 15(2), e1900289. <https://doi.org/10.1002/biot.201900289>
- Feichtinger, J., Hernández, I., Fischer, C., Hanscho, M., Auer, N., Hackl, M., ... Borth, N. (2016). Comprehensive genome and epigenome characterization of CHO cells in response to evolutionary pressures and over time. *Biotechnology and Bioengineering*, 113(10), 2241–2253. <https://doi.org/10.1002/bit.25990>
- Grandjean, M., Girod, P.-A., Calabrese, D., Kostyrko, K., Wicht, M., Yerly, F., ... Mermoud, N. (2011). High-level transgene expression by homologous recombination-mediated gene transfer. *Nucleic Acids Research*, 39(15), e104. <https://doi.org/10.1093/nar/gkr436>
- Ko, P., Misaghi, S., Hu, Z., Zhan, D., Tsukuda, J., Yim, M., ... Shen, A. (2018). 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. *Biotechnology Progress*, 34(3), 624–634. <https://doi.org/10.1002/btpr.2594>
- Lander, E. S., & Waterman, M. S. (1988). Genomic mapping by fingerprinting random clones: A mathematical analysis. *Genomics*, 2(3), 231–239. [https://doi.org/10.1016/0888-7543\(88\)90007-9](https://doi.org/10.1016/0888-7543(88)90007-9)
- Lawrence, M., Degenhardt, J., & Gentleman, R. (2019). *VariantTools: Tools for Exploratory Analysis of Variant Calls* (R package).
- Le Fourn, V., Girod, P.-A., Buceta, M., Regamey, A., & Mermoud, N. (2014). CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. *Metabolic Engineering*, 21, 91–102. <https://doi.org/10.1016/j.ymben.2012.12.003>
- Ocerin, J. M. C. y, & Pérez, J. D. (2002). Point and interval estimators in a binomial-Poisson compound distribution. *Statistical Papers*, 43(2), 285–290. <https://doi.org/10.1007/s00362-002-0101-3>

- Patel, N. A., Anderson, C. R., Terkildsen, S. E., Davis, R. C., Pack, L. D., Bhargava, S., & Clarke, H. R. G. (2018). Antibody expression stability in CHO clonally derived cell lines and their subclones: Role of methylation in phenotypic and epigenetic heterogeneity. *Biotechnology Progress*, 34(3), 635–649. <https://doi.org/10.1002/btpr.2655>
- R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.
- Tharmalingam, T., Barkhordarian, H., Tejada, N., Daris, K., Yaghmour, S., Yam, P., ... Stevens, J. (2018). Characterization of phenotypic and genotypic diversity in subclones derived from a clonal cell line. *Biotechnology Progress*, 34(3), 613–623. <https://doi.org/10.1002/btpr.2666>
- Vcelar, S., Jadhav, V., Melcher, M., Auer, N., Hrdina, A., Sagmeister, R., ... Borth, N. (2018). Karyotype variation of CHO host cell lines over time in culture characterized by chromosome counting and chromosome painting. *Biotechnology and Bioengineering*, 115(1), 165–173. <https://doi.org/10.1002/bit.26453>
- Welch, J. T., & Arden, N. S. (2019). Considering “clonality”: A regulatory perspective on the importance of the clonal derivation of mammalian cell banks in biopharmaceutical development. *Biologicals*, 62, 16–21. <https://doi.org/10.1016/j.biologicals.2019.09.006>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

RESEARCH ARTICLE

Integration of high-throughput analytics and cell imaging enables direct early productivity and product quality assessment during Chinese Hamster ovary cell line development for a complex multi-subunit vaccine antigen

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Peer Review

The peer review history for this article is available at <https://publons.com/publon/10.1002/btpr.2914>.

Abstract

Mammalian cell line generation typically includes stable pool generation, single cell cloning and several rounds of clone selection based on cell growth, productivity and product quality criteria. Individual clone expansion and phenotype-based ranking is performed initially for hundreds or thousands of mini-scale cultures, representing the major operational challenge during cell line development. Automated cell culture and analytics systems have been developed to enable high complexity clone selection workflows; while ensuring traceability, safety, and quality of cell lines intended for biopharmaceutical applications. Here we show that comprehensive and quantitative assessment of cell growth, productivity, and product quality attributes are feasible at the 200–1,200 cell colony stage, within 14 days of the single cell cloning in static 96-well plate culture. The early cell line characterization performed prior to the clone expansion in suspension culture can be used for a single-step, direct selection of high quality clones. Such clones were comparable, both in terms of productivity and critical quality attributes (CQAs), to the top-ranked clones identified using an established iterative clone screening approach. Using a complex, multi-subunit antigen as a model protein, we observed stable CQA profiles independently of the cell culture format during the clonal expansion as well as in the batch and fed-batch processes. In conclusion, we propose an accelerated clone selection approach that can be readily incorporated into various cell line development workstreams, leading to significant reduction of the project timelines and resource requirements.

KEYWORDS

CMV Pentamer, early clone screening, epitope profiling, monoclonality, Quality by Design

Xiangming Li and Yujian Zhang contributed equally to this work.

1 | INTRODUCTION

Mammalian cell lines, particularly Chinese Hamster Ovary (CHO) cells, represent a well-established recombinant protein expression platform. Generation of stable cell lines which support high quantity and quality of target protein production is one of the first and most critical steps of process development for biologics. Conventional mammalian cell line development workflow includes: (a) stable pool generation; (b) single cell cloning with documented monoclonality to meet regulatory requirements; (c) clone screening based on protein yield and quality and (d) cell line bioprocess suitability testing (e.g., expression and genetic stability, and fed-batch bioreactor process compatibility and scalability).^{1,2}

The early clone screening stages are typically quite challenging. Multiple clones, usually hundreds to thousands, are actively maintained in culture and several rounds of screening are performed to identify the top performance clones. Ideally, the clone ranking should be based on the combinational assessment of volumetric productivity (VP) and cell culture density normalized productivity readout such as specific productivity (qP), which are both important indicators of the cell line productivity under batch and fed-batch culture conditions. Due to the variability in clone growth rates and their adaptation to suspension culture, the iterative rounds of clone expansion and productivity screening often introduce a bias towards faster growing clones. It is usually impractical to measure cell densities during the early stage clone screening stages. Therefore, the initial clone productivity ranking is typically based on the assessment of VP only.

Analytical methods used for early clone screening require high-throughput and rapid turnaround time. Concurrently, high sensitivity and low matrix interference are needed to quantify relatively low levels of target proteins. In most clone selection strategies, multiple screening rounds are performed to allow for a margin of error in the clone productivity ranking. Multi-step down-selection of the top clone candidates minimizes the risk that high productivity clones would be randomly eliminated from the top candidate set based on variability of the small-scale cell culture samples or from low quality analytical data.

Additional challenges are associated with the selection of cell lines producing complex recombinant vaccine antigens with multiple protein subunits. The protein complexes are required to be correctly assembled with multiple conformational epitopes preserved in the vaccine product in order to induce neutralizing antibodies. Multiplex immunoassays are optimal to simultaneously evaluate the quality and quantity of epitopes associated with multi-subunit antigens. In line with Quality by Design (QbD) approach, antigen variant-, clone- and process-dependent differences in antigenicity profiles could be identified and monitored using such methods.

In this study, we present a new cell line development strategy used for CHO expression of the human cytomegalovirus (HCMV) pentameric complex (Pentamer), a model multi-subunit recombinant protein. CMV Pentamer is an envelope glycoprotein complex consisting of five protein subunits (gH, gL, UL128, UL130, and UL131A). It is required for the infection of endothelial and epithelial cells.^{3,4}

CMV Pentamer is a major target of neutralizing antibodies against HCMV and is therefore a promising vaccine candidate.^{5,6} Multiple B cell epitopes have been identified and mapped onto CMV Pentamer using isolated neutralizing monoclonal antibodies (mAbs).^{7,8}

To select the CMV Pentamer expressing clones, we developed a single-step clone screening process that incorporated a comprehensive characterization of candidate production cell lines within 14 days of the single cell cloning: monoclonality, clonal growth curves, VP and qP as well as antigenicity profiles of the expressed pentameric complex were determined for several hundred tested clones. The early clone screening strategy yielded top cell lines with comparable or improved productivity when compared to clones selected by a conventional iterative cell line development workflow including two additional titer screening rounds. We estimate that the new approach leads to four- to sevenfold reduction in clone screening complexity achieved within shorter development timelines while minimizing clone contamination or misidentification risks. In summary, this study demonstrates an accelerated and efficient way to select top performing expression cell lines for complex recombinant proteins.

2 | MATERIALS AND METHODS

2.1 | Monoclonal antibodies

Monoclonal antibodies against CMV Pentamer—4I22, 8I21, 10P3, 13H11, and 15D8 were isolated from immortalized human B cells.⁷ Antibodies used in this study were expressed by Expi293 cells and harvested 6 days after transient transfections. The supernatant was purified with protein A columns (Thermo Fisher) and stored in phosphate buffered saline (PBS) buffer.

2.2 | CMV Pentamer stable pool and clone generation

The CMV Pentamer expressing pools were generated according to the published procedures.² Briefly, internally sourced CHO-K1 cells were transfected by electroporation with Amaxa nucleofection system (Lonza, CH) with linearized CMV Pentamer expression plasmid including DHFR selectable marker. After MTX selection and recovery, the stable pools were used for fluorescence activated cell sorting (FACS) enrichment and individual clone generation as reported previously.² Briefly, the stable cell pools that co-express cell membrane-associated and secreted CMV Pentamer variants were immunolabeled with FITC-conjugated 4I22 or 8I21 antibodies that recognize conformational epitopes of CMV Pentamer. Top 5% brightest cells were FACS sorted into individual clones and subcultivated in 96-well plates. For the workflow of single-step early clone screening strategy, refer to Figure 6. For the conventional iterative clone screening strategy, after 14-day culture in 96-well plate, recovered clones ($N = 260$, by random selection) were transferred to 24-well plates and evaluated by two rounds of VP-based batch culture screening to eliminate the low producing clones. Top $N = 40$ clones were transferred to the shake flasks for the subsequent fed-batch study.

2.3 | Clone-specific productivity assessment

Clone-specific productivity was calculated by the equation: $qP = \Delta VP / \Delta IVCC$. Integrated viable cell count (IVCC) was calculated according to the equation: $IVCC_t = IVCC_{t-1} + \Delta t(x_t + x_{t-1})/2$, where $x_t + x_{t-1}$ are viable cell counts at time t and $t - 1$. For qP assessment of clones expanded in 96-well plate culture format, cell counts from two time points were used for $\Delta IVCC$ calculation: Day 0 (the day for individual cell deposition) and Day 12. For qP assessment of clones cultured in shake flask fed-batch format: cell counts from six time points were used for $\Delta IVCC$ calculation: Day 4, Day 6, Day 8, Day 10, Day 12, and Day 14.

2.4 | Fed-batch culture and productivity assessment

Fed-batch procedures were carried out in line with the published procedure.² Briefly, selected clones were grown in 60 ml working volume cultures (250 ml shake flask format) with a starting seeding cell density of 0.4×10^6 viable cells/ml. Addition of feeding solutions started on Day 4 and the temperature shift from 36.5 to 33°C was on Day 5. Viable cell densities were measured by Vi-Cell (Beckman-Coulter, Fullerton, CA) every other day from Day 4 onward. To establish the clone productivity ranking, CMV Pentamer VP and qP were determined on Day 14 or when the culture viability dropped below 70%.

2.5 | Luminex assay

The capture antibodies 4I22, 8I21, 10P3, 13H11, and 15D8 were individually coupled to magnetic carboxylated microspheres (Luminex Magplex-C beads) using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (MilliporeSigma). In brief, 5×10^6 Luminex beads were washed twice and suspended in 160 μ l of activation buffer (0.1 M NaH_2PO_4 , pH 6.2) and then activated for 20 min at RT by addition of 20 μ l of 50 mg/ml Sulfo-NHS (Thermo Fisher Scientific) and 20 μ l of 50 mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Thermo Fisher Scientific) dissolved in activation buffer. The activated beads were washed three times in 250 μ l of activation buffer before addition of the antibody (5 μ g in PBS) in 0.5 ml of activation buffer. After coupling for 2 hr at RT beads were washed with activation buffer. Beads were blocked by incubating with 0.5 ml of PBS-TBN (PBS containing 0.05% Tween 20, 1% bovine serum albumin and 0.1% sodium azide, pH 7.4) at RT for 30 min. After two washes with PBS-TBN, beads were stored in 0.5 ml of the same buffer. The antibody-coupled beads were counted using a Countess II (Thermo Fisher Scientific) and stored at 4°C in the dark.

mAbs used for the detection of the captured CMV Pentamer was biotinylated with EZ-Link NHS-PEG4-Biotin (Thermo Fisher Scientific). Briefly, 0.5 ml of antibody (2 mg/ml in PBS) was mixed with 6.5 μ l of 20 mM biotin solution and incubated at 4°C for 2 hr. To remove free biotin, buffer exchange was carried out on PBS equilibrated Zeba column (5 ml) (Thermo Fisher Scientific).

For Luminex assays, 50 μ l of standards or cell supernatants diluted with PBS-TBN were mixed with 50 μ l of capture beads

(2,000 beads per well) and incubated for 2 hr at room temperature. Beads were then washed twice with $\times 1$ phosphate buffered saline/tween (PBST) on a Tecan HydraSpeed plate washer equipped with a magnetic plate carrier (Tecan). Beads were incubated with 100 μ l of 4 μ g/ml selected biotinylated mAbs in PBS-TBN for 1 hr and after two washes followed by 30 min incubation with 100 μ l of 2 μ g/ml streptavidin-PE (Thermo Fisher Scientific) at RT. All solutions were then removed, and beads were suspended in 150 μ l of PBS-TBN for analysis in a Luminex FlexMap 3D instrument (Luminex). Median fluorescence intensities (MFIs) and measured concentrations were reported by the build-in instrument software.

2.6 | Sandwich ELISA assay

A 96-well microtiter plate (Nunc-Immuno Plate, MaxiSorp, Thermo Fisher Scientific) was coated with 0.0195 μ g/ml of 4I22 (100 μ l per well in PBS) overnight at 4°C. The plate was washed twice with 300 μ l of wash buffer containing 0.05% Polysorbate 20 (PS20) in PBS. Blocking buffer (1% BSA in PBS, 200 μ l per well) was then added and the plates were incubated for 1 hr at RT. Prior to the assay, plates were washed four times with 0.05% PS20 in PBS. Standards and samples diluted in sample buffer (1% BSA, 0.1% Triton X-100 in $\times 1$ PBS w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$) were added at 100 μ l per well in triplicates. After 1 hr incubation, plates were washed four times and 100 μ l per well of biotinylated 8I21 antibody (0.25 μ g/ml in sample buffer) was added for 1 h incubation. After four washes, avidin-HRP (1:35,000 in sample buffer) was added at 100 μ l per well and incubated for 30 min at RT. Following four additional washes, TMB (3,3',5,5'-tetramethylbenzidine) substrate (100 μ l per well) was added. Color development was stopped by adding 100 μ l of 1 M H_2SO_4 . Optical density was measured at 450 and 650 nm (background) with SpectraMax Plus 384 (Molecular Devices).

2.7 | Bio-layer interferometry assay

BLI experiments were performed on Octet RED384 (ForteBio, Menlo Park, CA). Antibodies (4I22 and 13H11) were prepared at 20 μ g/ml in sample diluent buffer (ForteBio). CMV Pentamer standard dilutions were prepared at 2.5–150 μ g/ml range. Aliquots of 200 μ l from each sample and standard were placed in the wells of a 96-well microplate and measured using anti-human IgG Fc Biosensors (ForteBio). Sensor tips were pre-hydrated in sample diluent buffer for 5 min, followed by 20-s dips in sample diluent buffer, and then transferred to the mAb-containing wells for a 60-s loading. After a 20-s baseline dip in sample diluent buffer, measurements were performed by dipping mAb-coated sensors into the sample wells.

2.8 | Immuno-capture mass spectrometry

Immuno-capture MS was performed using Protein G coated 96-well plates (ThermoFisher) according to the manufacturer's manual. Briefly, antibodies (4I22 and 8I21) were coated on the plates. Standard protein and diluted culture media were incubated at 37°C for 2 hr with

shaking (300 rpm) for immuno-capture. The captured proteins were digested with trypsin. Isotopically labeled peptides with known concentration were spiked into the digested peptides and were then subjected to multiple reaction monitoring (MRM) analysis (Skyline, version 3.6). The MRM assay was performed on TSQ Endura triple-quadrupole (ThermoFisher) mass spectrometer equipped with an Ultimate 3000 UHPLC (ThermoFisher) system. At least one peptide from each subunit of CMV Pentamer was analyzed. The ratios of digested peptides to their isotopically labeled synthetic peptide counterparts were used for quantification.

2.9 | Monoclonality evidence generation and 96-well plate cell counts

The monoclonality evidence generation was conducted by Cell Metric CLD (Solentim, UK) as per manufacturer's instructions. Briefly, 96-well plates were imaged prior to the single cell sorting by Becton Dickinson FACS Aria III (BD Biosciences) in order to capture the background images for individual wells (Day 1). Time course images were then taken on Day 0 (individual cell deposition), and then on Days 1, 2, 3, 4, 8, 12, and 14.

The total cell count for individual clones was estimated by utilizing cell counting function of Cell Metric CLD using colony images collected for monoclonality evidence generation. Briefly, cell counting function parameters were predefined: sensitivity at 32 and edge exclusion at 0.322 mm. To minimize the background, the false positive cell counts on Day 1 were subtracted from the corresponding total cell counts on Days 0 to 14 (Figure S2).

2.10 | SDS-PAGE and Western blot

Day 14 fed-batch cell culture supernatants from two sets of top 10 clones (derived from both clone screening strategies) were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis under boiled and reduced condition. For SDS-PAGE, 8 μ g of CMV pentamer (based on Octet titer determination) was loaded per lane for each clone and Coomassie staining was conducted with eStain Protein Staining System (Genescript). For Western blot, 200 ng of CMV Pentamer was loaded. PVDF membrane transfer was conducted with iBlot2 (Thermo Fisher). CMV Pentamer was detected by a rabbit anti-CMV Pentamer polyclonal antibody (Genescript) as the primary antibody and IRDye 800CW donkey anti-rabbit IgG secondary antibody (LI-COR Biosciences). SDS-PAGE gel and Western blot images were acquired by Odyssey 9120 Imaging system (LI-COR Biosciences).

2.11 | Statistical analysis

2.11.1 | Multivariate statistical analysis

Hierarchical cluster analysis was performed to partition multivariate data into significant subgroups or clusters. To obtain clusters of clones which are similar to one another and different from clones in other

clusters, Z-score standardization of the variables and Ward's method using Euclidean distances as a measure of similarity were used. The number of clusters was chosen based on the practicality of the outputs.

2.11.2 | Assay agreement analysis

The data from Luminex and Octet assays were used to calculate the concordance correlation coefficient. The readouts of Luminex and Octet assays were ranked independently, and then dichotomized using the cutoff of top 30% to assess the agreement between two measurements for binary clone screening selection evaluation. The kappa coefficient and concordance correlation coefficient were calculated.

Multivariate statistical analysis and assay agreement analysis were completed using software JMP version 12.0.1 and SAS version 9.2, respectively (SAS Institute Inc., Cary, NC).

3 | RESULTS

In line with QbD concept, assessment of the product quality attributes and cell substrate performance attributes at the early clone screening phase would accelerate the bioprocess development and enhance the final product quality. For the recombinant protein-based vaccine development, the antigenicity profile as well as the product yield represent two most critical quality attributes while the monoclonality and growth rate of the production cell line are examples of the key performance attributes. In this study, we describe high-throughput analytic and cell imaging tools that allowed us to evaluate these critical production cell line characteristics at the very early clone screening phase.

3.1 | Assessment of bio-layer interferometry, immuno-capture mass spectrometry, ELISA, and Luminex assay formats for early clone screening

To enable the product yield assessment at the early phase for clone screening, analytical assays need to meet stringent throughput and sensitivity requirements, and optimally with multiplex capability to enable antigenicity profile assessment. The assays should be able to process a few hundred clones per day and deliver the results in a timely manner. The sensitivity of the assays should be sufficient to quantify secreted protein by a few hundred cells expanded in a 96-well plate format (200 μ l per well working volume). On average, 12 days after a single cell cloning, a CHO colony contains 500 cells. With the expected specific productivity (qP) of 1–10 pcd (pg/[cell $\times$ day]), we estimated the corresponding secreted protein concentrations in the supernatants to be in the 30–300 ng/ml range.

As the first step of the study, we evaluated four different immunodetection methods in terms of their throughput and sensitivity. We used 4I22, the human high affinity mAb that recognizes a conformational epitope present on CMV Pentamer⁷ in all assay formats evaluated for the purpose of this study. Bio-layer interferometry (BLI) quantitates antigen levels based on the binding kinetics of the analyte

to a biosensor coated with an antigen-specific antibody. The binding rate is antigen concentration dependent. The Octet 384 instrument is capable of 96- or 384-well plate sample analysis with 8- or 16-channel biosensors. The results can be delivered within 1–2 hr. Due to its throughput, short assay turnaround time and high consistency, Octet-based BLI assays have been widely used to monitor product expression levels during process development. However, this assay has relatively less sensitivity and typically $\mu\text{g/ml}$ analyte levels are required for BLI quantification. In our Octet assay, the limit of quantification (LOQ) was determined at $2.5 \mu\text{g/ml}$ (Figure 1a) and therefore lacked ng/ml range sensitivity needed for early clone screening applications.

In immuno-capture mass spectrometry (MS) assay,^{9,10} the 4I22 mAb was used to enrich the antigen in the cell culture supernatant. Following a protease digestion step, a previously identified peptide specific to CMV Pentamer was measured by MS. Quantitation was based on the standard curve generated with isotopically labeled synthetic subunit-specific peptides with known concentrations. The assay results were very consistent with those of BLI technology. The application of this assay to the early clone screening was, however, limited by low assay throughput and insufficient sensitivity—an LOQ of 62.5 ng/ml (Figure 1b); although the sensitivity level can be substantially improved with nano-flow LC, nanospray ionization, and a mass spectrometer with higher sensitivity.

Sandwich ELISA is a commonly used immunoassay applied to product titer determination due to its high specificity, sensitivity, and throughput. In a capture/detection format using two noncompeting mAbs, 4I22/8I21,⁷ the CMV Pentamer ELISA assay achieved LOQ of 1 ng/ml (Figure 1c). This assay was therefore suitable for the early clone screening applications.

Finally, we explored multiplex Luminex-based sandwich immunoassay. Luminex is essentially a bead-based two-color flow cytometry assay with one color identifying a Luminex bead ID (and therefore the capture antibody conjugated to the bead) and a second color showing the concentration-dependent signal of analyte elicited by a detection antibody. An advantage of Luminex assay is that multiple analytes (or multiple epitopes associated with an individual analyte) can be measured simultaneously in a single well. Since the CMV Pentamer molecule is composed of five subunits and exposes several distinct epitopes,^{7,11} to determine the best antibody pair for clone productivity assessment during early clone screening, beads were individually labeled with five noncompeting previously identified CMV pentamer neutralizing mAbs (4I22, 13H11, 8I21, 10P3, and 15D8) targeting the major epitopes on CMV Pentamer molecule. Combinations of these beads with individual detection antibodies of the same set were tested. EC_{50} values derived from individual standard curves are shown in Figure 1d. The best sensitivity, demonstrated as low EC_{50} values, were observed when 4I22 was used as a detection antibody. The

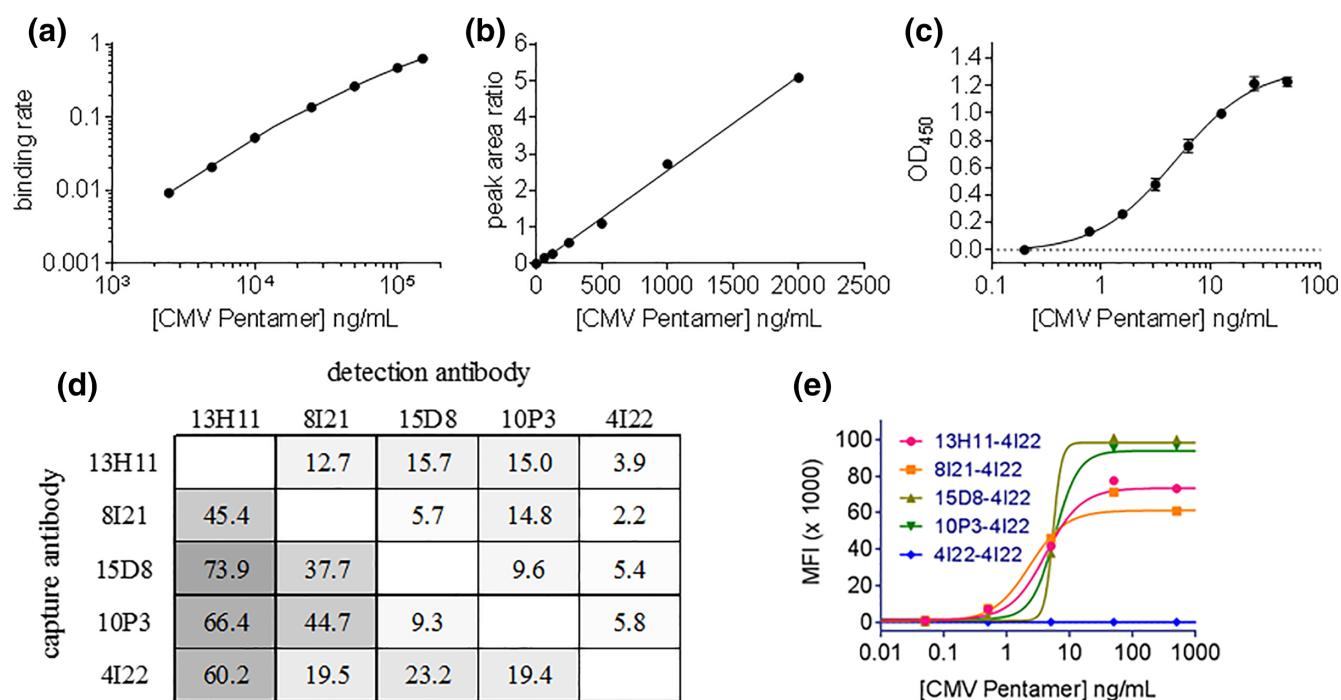


FIGURE 1 The assessment of quantitation assays for CMV Pentamer. CMV Pentamer standard curves were generated with four different assays. (a) Bio-layer interferometry (BLI) assay—Octet biosensors coated with 4I22 capture antibody; (b) Immuno-capture MS assay with 4I22 antibody; (c) Sandwich ELISA assay with 4I22 capture antibody/8I21 detection antibodies; (d, e) Luminex sandwich assays—MagPlex beads coupled with different capture antibodies (13H11, 8I21, 15D8, 10P3, and 4I22), respectively, were mixed at equal quantity and were incubated with CMV Pentamer of various concentrations. Ten thousand beads were used per reaction in $100 \mu\text{L}$ volume. Five different detection antibodies were tested separately (13H11, 8I21, 15D8, 10P3, and 4I22). EC_{50} in ng/ml were calculated with GraphPad shown in panel (d). The standard curves with 4I22 as detection antibody was shown in panel (e)

assay format with 8I21 labeled beads and 4I22 as a detection antibody had a LOQ of below 1 ng/ml (Figure 1e), comparable to the sandwich ELISA. Furthermore, coefficient of variation of the Luminex assay was generally less than 5%, significantly lower than that of a conventional ELISA (10–20%), and therefore was more reliable when measuring low levels of antigen molecules. In addition, the Luminex assay had a similar assay throughput to ELISA with the added advantage of a multiplex format, which allows simultaneous evaluation of multiple epitope quality.

Since BLI assays are an established platform with demonstrated performance for quantitative analysis of therapeutic molecules,^{12–14} we further evaluated whether the results from Luminex assay were comparable to those from BLI in the $\mu\text{g/ml}$ range. Supernatants from CMV Pentamer fed-batch cultures ($N = 54$) were measured by both BLI and Luminex sandwich assays. Clones were then ranked based on Luminex assay results. As revealed by correlation analysis (Figure S1), both methods achieved similar titers for the same set of samples. Thus, the Luminex sandwich assay was selected for sample analysis in both ng/ml and $\mu\text{g/ml}$ ranges; whereas, BLI assay was only applicable for the samples at $\mu\text{g/ml}$ levels, which was used as a complementary or

preferred analytical platform at later clone screening stage due to its simpler sample processing (e.g., lower sample dilution) and higher throughput.

In summary, for our current assay formats, the ranking of the assay throughput for immunodetection assays is BLI > ELISA = Luminex > Immuno-capture MS, and the ranking of the assay sensitivity is Luminex > ELISA > Immuno-capture MS > BLI. Considering the assay throughput, sensitivity and multiplex capability of Luminex sandwich assay, we decided to use its simplex assay format (8I21 beads, detection antibody 4I22) for early clone productivity assessment and its multiplex format (multiple mAbs on beads, detection antibody 4I22) for the antigen epitope profiling. BLI assay (detection antibody 4I22) was chosen for clone productivity assessment at later stages.

3.2 | Early clone growth characterization by Cell Metric CLD imager

Clone ranking based solely on VP introduces a selection bias toward clones with higher growth rates. In order to determine qP during the very early stage of clone selection, there is a need for implementing a

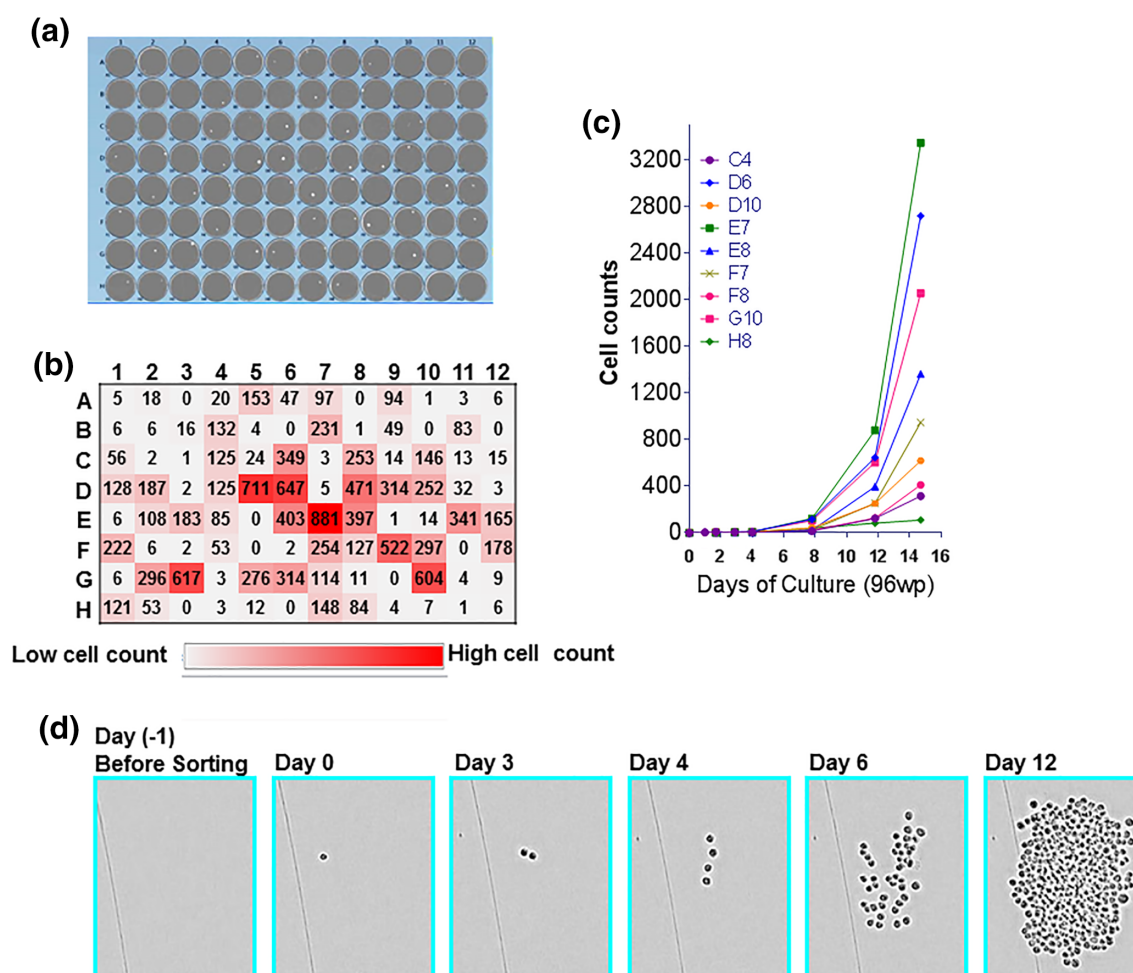


FIGURE 2 Cell counting with Cell Metric CLD. (a, b) Thumbnail views of colony outgrowth and cell count for individual clones of a representative 96-well plate at Day 12 after single cell cloning; (c) growth curves of selected clones ($N = 10$) with distinct growth rates; (d) a representative monoclonality report generated by Cell Metric CLD

high-throughput cell counting platform for evaluating individual clones. Cell Metric CLD is an automated, high resolution, high-throughput system providing fast whole well imaging capability. In conjunction with other similar optical systems, it has been widely used for monoclonality evidence generation (Figure 2d),^{15–18} which is required for the cell lines intended for the recombinant protein production in the GMP environment.¹⁹ Here, we developed a cell counting application based on Cell Metric CLD imaging analysis software for determining total cell count and growth patterns of individual clones. As revealed by Figure 2a–c, this cell counting application was used to generate growth curves of individual clones during the entire clonal recovery phase (Day 0 to Day 15) that distinguished “fast” and “slow growing” clones. The total cell numbers within each individual colony can be estimated 10–14 days after single cell cloning step.

In conclusion, Cell Metric CLD imaging platform has the capability of assessing clone growth characteristics for individual clones in a 96-well plate format. Along with VP results measured with Luminex assay, Cell Metric CLD data can be used to determine qP values of expression cell lines within 10–14-day window after the single cell cloning.

3.3 | Early clone screening by clone productivity and antigen quality

To demonstrate the feasibility of utilizing high-throughput Luminex sandwich assay and Cell Metric CLD imaging for early clone screening, a proof of concept experiment was conducted: ~4,000 single cells from two CMV Pentamer stable pools were deposited in 96-well plates via FACS single cell sorting (one cell per well). The recovery and growth of cell clones was monitored with Cell Metric CLD imager. After 12-day culture, colonies of ~200 cells or more were detected in

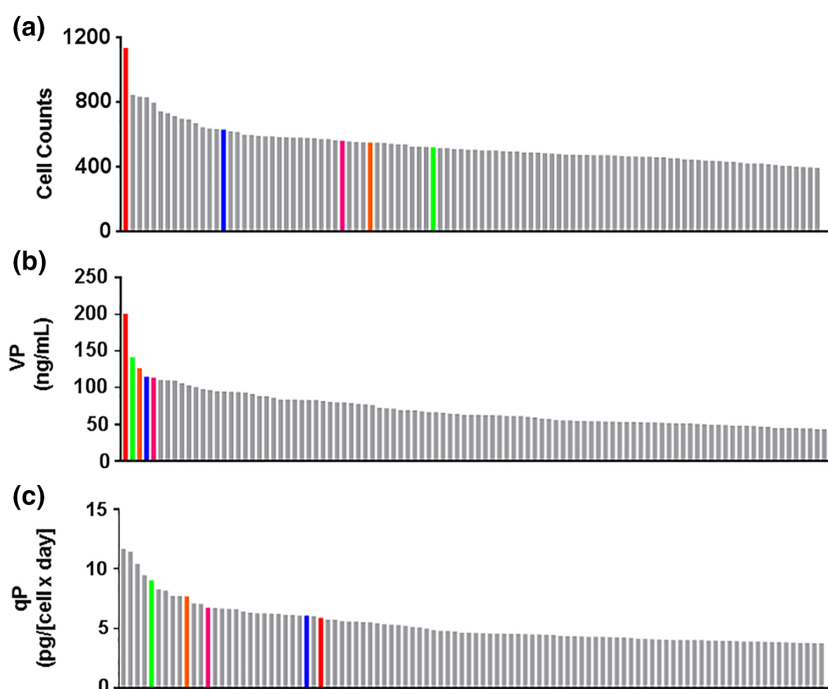
approximately 800 wells, demonstrating a clonal recovery rate of ~20%, which is typical for the CHO platform used in the experiment. Conditioned culture medium aliquots of 100 μ l were harvested from a randomly selected subset of recovered clones ($N = 269$) and used in Luminex sandwich assay for antigen titer determination based on 8I21/4I22 mAb pair (Figures 3 and 4) and the multiplex epitope profiling (Table 1). As shown in Figure 3, the recovered clones represented a wide range of growth rates and VP/qP productivities. Multivariate VP and qP analysis was used to cluster the recovered clones into two different groups: (a) high VP and qP clones (~20%; $N = 51$) and (b) low VP and qP clones (~80%; $N = 218$) (Figure 4).

The Luminex epitope profiles were further used to evaluate the quality of the expressed CMV Pentamer molecules. The levels of five distinct epitopes (Table 1) were individually quantified in a multiplex Luminex sandwich assay and their relative ratios were calculated. A relative epitope ratio of 1:1:1:1:1 was assigned for the reference standard samples. Four clones that expressed CMV Pentamer molecules with significantly altered antigenicity profile (as indicated by a dramatic change of the relative epitope ratios) were eliminated from the subsequent screening (Tables 1 and S1).

3.4 | Fed-batch culture-based productivity and epitope quality evaluation for selected clones

In order to evaluate whether the single-step early clone screening strategy could yield cell lines with comparable productivity to the ones identified with conventional iterative clone screening strategy (refer to Section 4), representative clones from both high ($N = 27$) and low ($N = 11$) VP/qP clusters were scaled up to grow in shake flask and evaluated in a platform fed-batch culture format. Clone growth parameters, productivity, product quality, and epitope quality were

FIGURE 3 Early clone characterization: cell count, volumetric productivity (VP), and specific productivity (qP). Individual clones were generated with FACS enrichment and single-cell sorting, and seeded in 96-well plate with 200 μ l of expansion medium per well. The 96-well plates were kept in static incubator for 14 days allowing clone outgrowth and monoclonality evidence generation. Cell culture of 100 μ l from recovered clones was harvested for Luminex sandwich assay. VP was determined with Luminex sandwich assay using 8I21 as capture antibody and 4I22 as detection antibody. qP was derived from the equation: $qP = \Delta VP / \Delta IVCC$. Ranking of 100 clones were shown (from high to low). (a) Cell count ranking; (b) VP ranking; (c) qP ranking. Top 5 clones based on VP ranking were highlighted with color



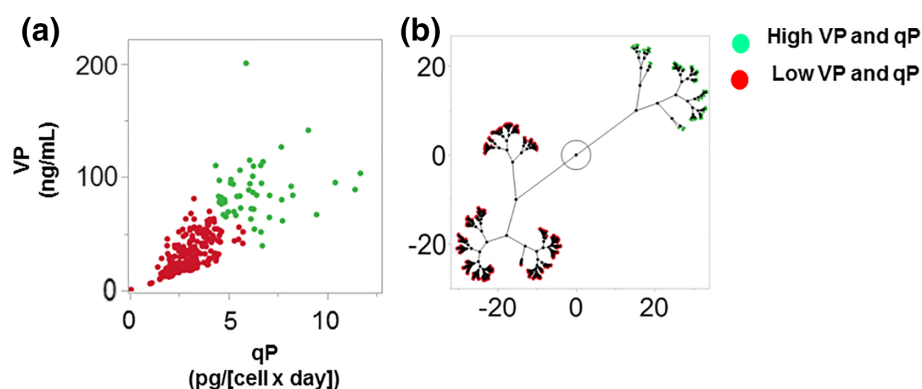


FIGURE 4 Multivariate analysis of clone productivity. Recovered clones ($N = 269$) at 96-well plate culture stage were clustered based on their volumetric productivity (VP) and specific productivity (qP) value. (a) Scatter plot and (b) Constellation plot

Clone ID	Site7 8I21	gH 13H11	Site1 15D8	Site4 10P3	Site5 2C12
453_B06	1	1.4	1.3	1	0.9
453_B12	1	1.3	1.2	1	0.9
453_C03	1	1.4	1.2	1.1	1
453_C04	1	1.5	1.2	1.1	0.9
453_C05	1	1.4	1.2	0.9	0.9
453_D05	1	1.3	1.1	1	0.9
453_D06	1	1.4	1.1	1.1	1
453_D10	1	1.4	1.1	0.9	0.8
453_E03	1	1.2	1.2	0.9	0.8
453_E10	1	1.3	1.1	1	1.1
453_F02	1	1.4	1.3	1	1
453_F07	1	0.5	1	0.4	1
453_G03	1	1.5	1.2	1.1	0.9
453_G05	1	1.3	1.1	0.9	0.9
453_G08	1	1.3	1.1	1.1	0.9
453_H07	1	1.3	1.1	1	0.9
453_H09	1	1.2	1.2	1.1	1
454_C07	1	1.3	1.1	0.9	0.8
454_D03	1	1.4	1.2	1.1	0.9
454_D07	1	1.4	1.3	1	0.9

TABLE 1 The cell supernatant from representative clones (20 out of total 269 clones) were subjected to epitope profiling analysis by Luminex sandwich assay

Note: For epitope profiling data of all 269 clones, refer to Table S1. Five anti-CMV Pentamer mAbs that recognize conformational epitope Sites 1, 4, 5, 7 and gH were used as the capture antibodies, and 4I22 that recognizes Site 2 was used as the detection antibody.¹¹ For the comparison, titer values of different epitopes were normalized against Site 7 (8I21) which was set to 1. Clones with ratios >1.5 or ≤ 0.5 were highlighted in red.

evaluated (see Figure 5 and Figure S3). Nine out of 10 clones with highest VP in the fed-batch culture were derived from the high VP/qP cluster defined based on the single-step early clone screening process (refer to Figure 4). Furthermore, the productivity and product quality of the top 10 clones in fed-batch culture were on a par with the top 10 clones selected independently by the conventional iterative clone screening process from the same CMV Pentamer stable clone pools. Finally, the epitope profiling with multiplex Luminex assay was carried out for batch, early (Day 6), middle (Day 10) and late (Day 14) fed-

batch supernatant samples. Tested material demonstrated a stable antigenicity profile, with minimal clone-to-clone variability and minor process-related epitope ratio shifts (Figure S3).

4 | DISCUSSION

In the current study, we described a QbD driven early clone screening strategy broadly applicable to mammalian cell line development. We

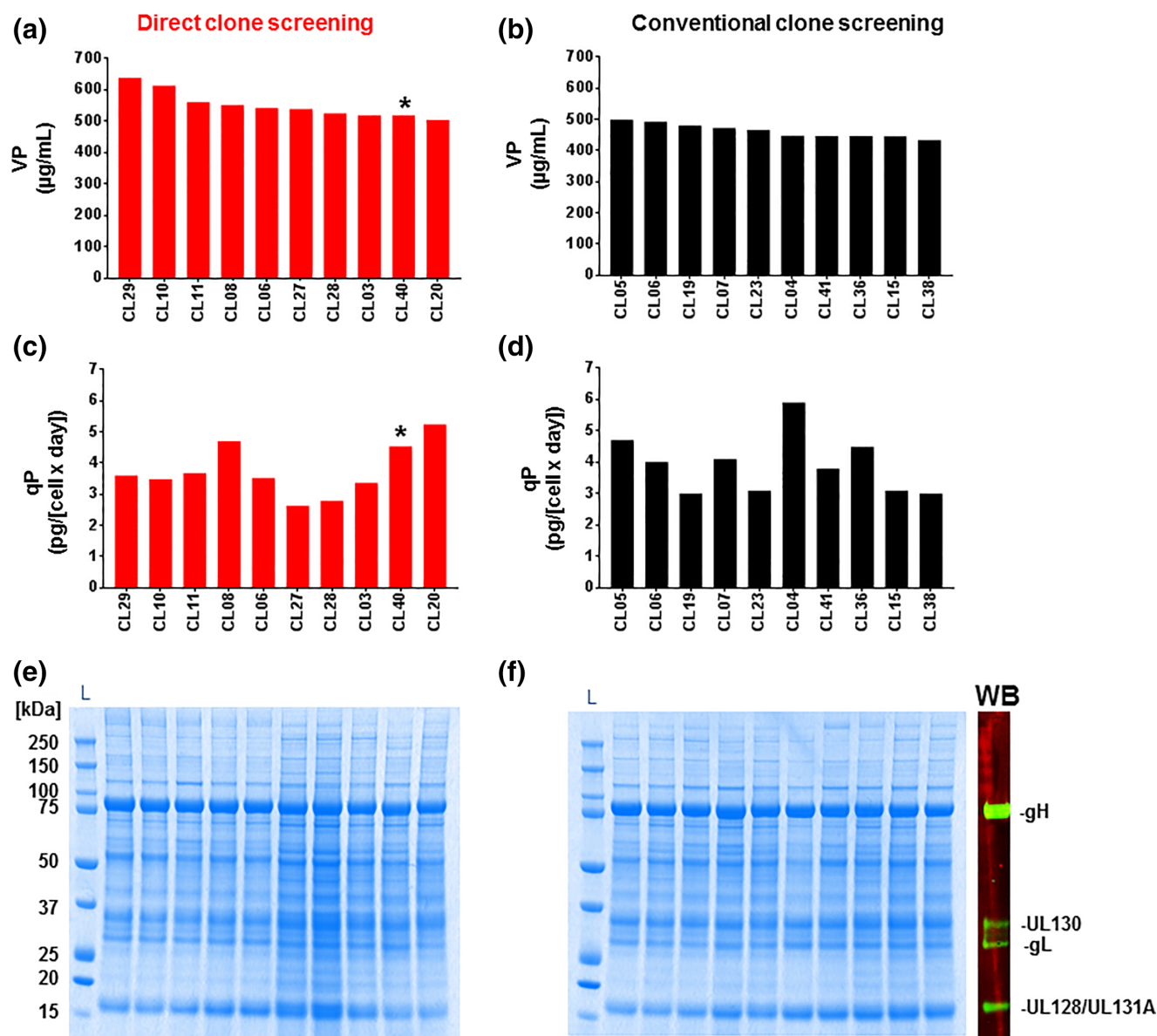


FIGURE 5 The productivity and product quality comparison of top clones selected by conventional and direct early clone screening processes. Volumetric productivity (VP) and specific productivity (qP) were determined by Octet assay using anti-CMV Pentamer mAb 4I22 for fed-batch cell culture supernatants. Top 10 clones were ranked based on the Day 14 fed-batch VP. Product quality of the clones was evaluated by SDS-PAGE with Coomassie blue staining and Western blot. (a, c and e) VP, qP, and SDS-PAGE/Western blot analysis for top 10 clones derived from direct early clone screening process; (b, d and f) VP, qP, and SDS-PAGE/Western blot analysis for top 10 clones derived from conventional clone screening process. *Clone 40 (CL40) was from the low VP/qP cluster defined by multivariate VP and qP analysis. WB, Western blot; L, protein ladder

demonstrated the feasibility of performing early comprehensive expression clone characterization by application of complementary high-throughput analytical technologies. The cell substrate and expressed product analytics included: flow cytometry coupled with single cell sorting, automated cell imaging and a highly sensitive multiplex immunoassay. The clones were evaluated at the 1 to ~2,000 cell stage during the initial 14-day colony expansion window. The following clone characteristics were established: (a) single-cell FACS sorting and automated 96-well plate imaging were used as orthogonal high-throughput approaches to ensure the clone monoclonality, with

probabilities exceeding 99%^{2,15,17,20,21}; (b) total cell counts were estimated between Day 0 (a single cell deposition) and Day 14 providing growth rates of the clones; (c) VP and qP were estimated for each clone; (d) the epitope profiling of the secreted antigen was performed using multiplex Luminex platform. The quantitative cell growth, productivity, and product quality data obtained in 96-well plate culture format allowed us to rapidly narrow down the lead candidate clone set and proceed directly to the clonal expansion and fed-batch evaluation. We refer to this approach as direct or single-step clone selection strategy.

In addition to the approach described here, other high-throughput early clone characterization strategies have also been developed. ClonePix (Molecular Devices, Sunnyvale, CA) was able to automatically image, select, and pick mammalian cell colonies expanded in semi-solid media, based on a number of parameters such as colony size and yield of a recombinant secreted product.²²⁻²⁴ However, the ClonePix readouts are not quantitative. VPs are estimated as the total fluorescence of the product diffusion pattern around each colony. Moreover, for colonies in 3D semi-solid media system, it is difficult to acquire the clone growth rate data and to conclusively demonstrate monoclonality in parallel with the primary clone screening. Another emerging technology, Beacon, utilizes nanofluidics and OptoElectro position technology to achieve single cell cloning and high cell density culture conditions, potentially providing an advantage for selecting clones compatible with upstream bioreactor processes. However, similarly to ClonePix technology, the productivity is estimated indirectly, based on diffusion-based staining of the secreted antibody with a fluorophore-tagged small molecule binding human IgG Fc. Therefore, current Beacon platform is limited to the antibody projects. While it represents a very promising approach for cell line development, the flexibility, performance and regulatory acceptability of the system (e.g., monoclonality evidence) remains to be proven.

Our study provides a proof-of-concept for a single-step early clone selection leading to the identification of the top 40–80 clone candidates that can be further characterized in the fed-batch culture format. The direct comparison between the top clones identified by our single- and multi-step (iterative) selection workflows executed using the same starting stable cell pools showed overlapping productivity ranges (VP and qP ranges, see Figure 5) and growth characteristics. These ranges, particularly VPs of the top clones derived using both selection workflows, are very narrow, indicating comparable high selection stringencies of both approaches. In this context, it is important to point out that our clone selection platform incorporates single cell selection (with FACS-based enrichment sorting) and clone selection steps (in 96- or 24-well plate format), both contributing to overall very low selection background seen at the top 10–40 clone stage.

Another notable observation is that most clone selection procedures do not show direct statistical correlations between the clone characteristics at the 96-well plate format (static batch culture, colony expansion from 1 to 2,000 cells), versus the shake-flask fed-batch format. The apparent absence of such correlations can be related to the impact of the cell culture format on clone performance. Indeed, the cell line productivity assessment (VP and qP) at early batch cultivation stage may not be a perfect predictor for bioreactor-scale process performance.^{25,26} Fed-batch-based clone screening, followed by expression stability assessment is required for the lead clone selection (Figure 6). On the other hand, such correlations might exist but may have been difficult to demonstrate, for example, due to the lack of quantitative cell growth and productivity data. Interestingly, our study shows the strongest correlation between VPs in the 96- and 24-well plate formats (Figure S4). The observed correlation further validates the direct clone selection strategy that by-passes the 24-well plate small-scale culture screening step(s). Further refinement of the single-

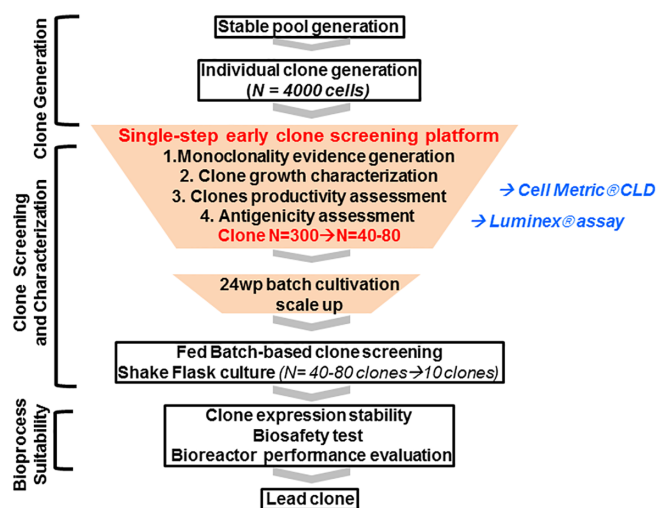


FIGURE 6 Proposed single-step early clone screening process with the integration of Luminex/Cell Metric CLD platform

step selection approach will likely lead to the in-depth assessment of the high VP selection bias inherent to the majority of the clone selection strategies. High sensitivity of the Luminex assay enables early identification of slower growing clones with high qP, which may represent an interesting wider-range phenotype candidate pool that could be compared against the high VP/qP cluster.

Cell line development approach described here is based on the comprehensive clone and product characterization. Since the early clone-specific analytics is available prior to the colony isolation and suspension culture adaptation, the overall clone screening effort can be significantly reduced, both in terms of duration and complexity. The single-step clone selection approach eliminates the 2–3 week long peak workload window typically associated with the clone expansion and productivity screening in the 24-well plate small-scale culture format. Conservatively, the direct selection of 40–80 top clones based on the quantitative data versus the random selection of 300 clones in the traditional workflow results in the four- to sevenfold reduced clone screening complexity at this clone generation step. In practice, peak work demand is further reduced since our strategy does not require maintaining any back-up cultures. Furthermore, the small-scale culture format experiments are also operationally challenging as they require additional 3–6 passages prior to the cell bank cryopreservation which introduces additional risks of clone miss-identification, microbial contamination, and cross-contamination. The top clone selection presented in this study is based on statistical analysis of quantitative data and integrates VP/qP multivariate clustering as a clone ranking tool. As a result, we demonstrate the feasibility of replacing the cell line development “clone down-selection” paradigm with the positive identification of the top clone candidates performed in parallel with the colony formation in the 96-well plate format.

In conclusion, in the present study, we provide an example of a QbD-driven clone selection approach that supports shorter development timelines and simplified workflow. We expect that this approach can be further refined and extended to other CHO platforms and, more broadly, to other mammalian cell expression systems.

ACKNOWLEDGMENTS

We thank Amin Khan, Sumana Chandramouli and Ying Huang for critically reviewing our manuscript.

CONFLICT OF INTEREST

All authors were employed by GSK group of companies at the time for the completion of the work and the research was sponsored by GSK.

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REFERENCES

- Fischer S, Marquart KF, Pieper LA, et al. miRNA engineering of CHO cells facilitates production of difficult-to-express proteins and increases success in cell line development. *Biotechnol Bioeng*. 2017; 114(7):1495-1510.
- Hofmann I, Wen Y, Ciferri C, et al. Expression of the human cytomegalovirus pentamer complex for vaccine use in a CHO system. *Biotechnol Bioeng*. 2015;112(12):2505-2515.
- Ryckman BJ, Chase MC, Johnson DC. HCMV gH/gL/UL128-131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors. *Proc Natl Acad Sci U S A*. 2008;105(37):14118-14123.
- Hahn G, Revello MG, Patrone M, et al. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J Virol*. 2004;78(18):10023-10033.
- Wen Y, Monroe J, Linton C, et al. Human cytomegalovirus gH/gL/UL128/UL130/UL131A complex elicits potentially neutralizing antibodies in mice. *Vaccine*. 2014;32(30):3796-3804.
- Wang D, Freed DC, He X, et al. A replication-defective human cytomegalovirus vaccine for prevention of congenital infection. *Sci Transl Med*. 2016;8(362):362ra145.
- Macagno A, Bernasconi NL, Vanzetta F, et al. Isolation of human monoclonal antibodies that potently neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex. *J Virol*. 2010;84(2):1005-1013.
- Ciferri C, Chandramouli S, Leitner A, et al. Antigenic characterization of the HCMV gH/gL/gO and pentamer cell entry complexes reveals binding sites for potentially neutralizing human antibodies. *PLoS Pathog*. 2015;11(10):e1005230.
- Dubois M, Fenaille F, Clement G, et al. Immunopurification and mass spectrometric quantification of the active form of a chimeric therapeutic antibody in human serum. *Anal Chem*. 2008;80(5):1737-1745.
- Chen LZ, Roos D, Philip E. Development of immunocapture-LC/MS assay for simultaneous ADA isotyping and semiquantitation. *J Immunol Res*. 2016;2016:7682472.
- Chandramouli S, Malito E, Nguyen T, et al. Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. *Sci Immunol*. 2017;2(12):eaan1457.
- Petersen RL. Strategies using bio-layer interferometry biosensor technology for vaccine research and development. *Biosensors (Basel)*. 2017;7(49):1-15.
- Dysinger M, King LE. Practical quantitative and kinetic applications of bio-layer interferometry for toxicokinetic analysis of a monoclonal antibody therapeutic. *J Immunol Methods*. 2012;379(1-2):30-41.
- Grav LM, Lee JS, Gerling S, et al. One-step generation of triple knock-out CHO cell lines using CRISPR/Cas9 and fluorescent enrichment. *Biotechnol J*. 2015;10(9):1446-1456.
- Evans K, Albanetti T, Venkat R, et al. Assurance of monoclonality in one round of cloning through cell sorting for single cell deposition coupled with high resolution cell imaging. *Biotechnol Prog*. 2015;31(5):1172-1178.
- Lekomtsev S, Alianni S, Lapao A, Burckstummer T. Efficient generation and reversion of chromosomal translocations using CRISPR/Cas technology. *BMC Genomics*. 2016;17(1):739.
- Alvin K, Ye J. Generation of cell lines for monoclonal antibody production. *Methods Mol Biol*. 2014;1131:263-271.
- Breinin A, Taylor I. Paradigm shift for cell-line development: increasing workflow productivity and assuring clonality for IND and BLA submissions to regulators. *Genet Eng Biotechnol News*. 2016;36:24-25.
- Food and Drug Administration (FDA). Q5D Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products. <https://www.fda.gov/media/71463/download>. Updated September, 1998. Accessed October 1, 2019.
- DeMaria CT, Cairns V, Schwarz C, et al. Accelerated clone selection for recombinant CHO CELLS using a FACS-based high-throughput screen. *Biotechnol Prog*. 2007;23(2):465-472.
- Sleiman RJ, Gray PP, McCall MN, Codamo J, Sunstrom NA. Accelerated cell line development using two-color fluorescence activated cell sorting to select highly expressing antibody-producing clones. *Biotechnol Bioeng*. 2008;99(3):578-587.
- Dharshanan S, Hung CS. Screening and subcloning of high producer transfectomas using semisolid media and automated colony picker. *Methods Mol Biol*. 2014;1131:105-112.
- Nakamura T, Omasa T. Optimization of cell line development in the GS-CHO expression system using a high-throughput, single cell-based clone selection system. *J Biosci Bioeng*. 2015;120(3):323-329.
- Roy G, Miro-Quesada G, Zhuang L, 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;451:100-110.
- Porter AJ, Dickson AJ, Racher AJ. Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: realizing the potential in bioreactors. *Biotechnol Prog*. 2010;26(5):1446-1454.
- Porter AJ, Racher AJ, Preziosi R, Dickson AJ. Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: improving the efficiency of cell line generation. *Biotechnol Prog*. 2010;26(5):1455-1464.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

RESEARCH ARTICLE

Improvement of the efficiency and quality in developing a new CHO host cell line

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Abstract

Chinese hamster ovary (CHO) cells are a ubiquitous tool for industrial therapeutic recombinant protein production. However, consistently generating high-producing clones remains a major challenge during the cell line development process. The glutamine synthetase (GS) and dihydrofolate reductase (DHFR) selection systems are commonly used CHO expression platforms based on controlling the balance of expression between the transgenic and endogenous GS or DHFR genes. Since the expression of the endogenous selection gene in CHO hosts can interfere with selection, generating a corresponding null CHO cell line is required to improve selection stringency, productivity, and stability. However, the efficiency of generating bi-allelic genetic knockouts using conventional protocols is very low (<5%). This significantly affects clone screening efficiency and reduces the chance of identifying robust knockout host cell lines. In this study, we use the GS expression system as an example to improve the genome editing process with zinc finger nucleases (ZFNs), resulting in improved GS-knockout efficiency of up to 46.8%. Furthermore, we demonstrate a process capable of enriching knockout CHO hosts with robust bioprocess traits. This integrated host development process yields a larger number of GS-knockout hosts with desired growth and recombinant protein expression characteristics.

KEYWORDS

Chinese hamster ovary cell, glutamine synthetase, knockout, transfection efficiency, zinc finger nuclease

1 | INTRODUCTION

Chinese hamster ovary (CHO) cells are the most commonly used mammalian hosts for the manufacture of industrial therapeutic recombinant proteins.^{1,2} These lineages have unique advantages such as high growth rates, genetic plasticity, are amenable to growth in protein-free medium, and produce desired product quality attributes.^{3–5} Although significant improvements have been achieved, developing stable, high-producing clones over a stringent timeline remains a major challenge in cell line development.

The most ubiquitous expression systems in industry are the Dihydrofolate Reductase (DHFR)-based methotrexate (MTX), Glutamine

Synthetase (GS)-based methionine sulfoximine (MSX), as well as antibiotic selection systems. Whereas antibiotic selection (referred to as positive selection) relies on transfer of an antibiotic resistance gene coupled to antibiotic containing growth media for selection, both the DHFR and GS function through auxotrophy (negative selection). In negative selection, cell enrichment occurs in the absence of an essential supplement, which can then be rescued by the expression construct.⁶ Auxotrophic selection affords higher titers, decreased off-target effects, enhanced stability, and lower environmental footprint when compared with positive selection methods, but generally requires validated and specialized cell lines.^{7,8}

The DHFR expression system represents the biopharmaceutical work horse of recombinant therapeutics, and occupies a market share of hundreds of billions annually.² This system uses the DXB11 and CHO-DG44 DHFR null cell lines which rely glycine, hypoxanthine, and thymidine (GHT) for growth.⁹ Accordingly, the deficiencies of these hosts are exploited by transfecting cells with a gene of interest (GOI) coupled to DHFR and then selecting GHT-free medium. This system allows high expression of recombinant proteins due to its unique gene amplification mechanism and thus has been widely used in biotherapeutics production.^{2,10–12} Alternatively, the GS/MSX expression system demonstrates a reduced propensity for gene amplification and has been used as alternative platform during bioprocess.^{13,14} Unlike DHFR, the GS selection is based on controlling the balance between the expression of the endogenous GS gene, the exogenously provided GS expression vector, and the concentration of GS inhibitor, MSX.¹⁰ Since the canonical GS system is reliant on wild-type CHO hosts, interference from the endogenous GS enzyme can often result in high amounts of “pseudo positive cells” following selection. These phenomena can lead to significant quantities of MSX-resistance clones that either do not contain exogenous transgene or produce appreciable titer.¹⁵ Therefore, the disadvantage of the canonical GS selection system is significant when a well-defined auxotrophic host is not supplied, as opposed to the well-established DHFR methodology.^{2,10} To avoid these issues, knocking-out the endogenous glutamine synthetase gene (GS) is the ultimate approach to improve selection stringency, remove non-producers, and stabilize recombinant clones in the context of large-scale commercial of manufacturing.

There are multiple approaches for genome engineering in mammalian cells, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas systems.^{16–18} Each approach has unique advantages and pitfalls; e.g. efficiency, process complexity, and IP landscape.¹⁹ The most commonly described knockout approaches thus far have used ZFNs; a class of engineered DNA-binding proteins capable of binding to specific gene locus. When fused to a nuclease to form a chimeric ZFN molecule, these constructs induce a double-strand break (DSB), which can be repaired by endogenous DNA repair processes inside the cell.²⁰ These repair processes can lead to the generation of targeted genomic edits resulting in a cell line with specific gene deletions, insertions, or mutations.²⁰ Individually designed ZFNs consist of two functional domains: a DNA-binding domain comprised of chain of zinc finger proteins and a DNA-cleaving domain comprised of the nuclease domain of FokI.²¹ Notably, the endonuclease domain of FokI has been reengineered to function as an obligate heterodimer in order to cleave DNA, and thus ensures specificity.²¹ However, the efficiency of generating bi-allelic knockout clones for targeted endogenous gene is usually very low (~2% for GS gene knockout using standard protocols), making the screening process both labor-intensive and time-consuming.²²

In this study, we use ZFN-mediated GS knockout to improve cell engineering efficiency in the context of recombinant protein production. After systematic improvement of several critical process

procedures, including gene delivery/transfection method optimization, posttransfection recovery, and clone expansion we significantly improved ZFN mediated GS knockout efficiency from 2% to 46.8%. The ultimate GS-knockout host line generated by our methodology demonstrates significantly higher productivity and growth characteristics as compared with those published protocols and commercial alternatives. This process can also be applied to other gene knockout CHO host line development.

2 | MATERIALS AND METHODS

2.1 | Cell culture

CHO host cells were cultured in CD-CHO medium (Gibco) containing 1× HT Supplement (Thermo Fisher) and 8 mM L-Glutamine (Gibco). Recombinant GSKO cells were selected and grown in MSX-containing (12.5 μM, Sigma Aldrich) CD-CHO medium (Gibco) containing 1× HT Supplement (Gibco) and 400 μg/ml G418 (Thermo Fisher). Recombinant wild type cells were selected in identical media with increased MSX (25–50 μM, as indicated in figures).

2.2 | CompoZr® ZFN mRNA and CRISPR, preparation and transfection

CompoZr® ZFN mRNAs were prepared from two plasmids (ZFNGSA9075 and ZFNGSB9372, Sigma, ZFNGS) expressing a pair of ZFNs targeting CHO GS. The two plasmids were first linearized by XbaI, followed by purification and In Vitro transcription using HiScribe™ T7 ARCA mRNA Kit (NEB). The two paired-ZFN mRNAs were purified using MegaClear Kit (Ambion), combined, and used for transfection.

CRISPR guide RNAs (gRNAs) were designed according to procedures already outlined, and the sequence 5' TAGCACCAAGGCCA TGCGGG 3' was synthesized.^{23,24} CRISPR RNAs (crRNAs) were synthesized by IDT and were combined at a 1:1 molar ratio with ATTO-550 labeled (Excitation: 560 nm, Emission: 575 nm) trans-activating crRNA (tracrRNA; IDT, Cat: 1075928) in 30 mM HEPES, pH 7.5; 100 mM Potassium Acetate. The RNAs were then heated to 95°C for 5 min on a heat block and allowed to reach room temperature. The duplexed gRNAs were complexed with purified Cas9 protein (NEB) at room temperature for 20 min before transfection.

2.3 | Transfection and process description

In Process-1, 5×10^6 CHO cells were collected and mixed with ZFN mRNA or RNP mixture (see text for amounts) and processed for electroporation with the Neon transfection system (Thermo Fisher) using the manufacturer's CHO protocol (voltage: 1700, Width [ms]: 20, Pulse Number: 1) Following electroporation, transfected cells were cold-shocked in a static incubator at 30°C for 2 days, and then placed

at 37°C for 2 days. Cells were then cloned into 96-well plates through limiting dilution at 0.8 cell/well. Cells were then monitored by imaging (Cellvista, Synentec) until 40%–50% confluency was scored by Cellvista software (~10–14 days). Cells were then expanded in a continuous and stepwise manner based on growth (40%–50% confluency) from plates to spin tubes, for ~20 days.

Process-2 followed the protocol described previously.²² Briefly, 2×10^6 cells were collected and mixed with ZFN mRNA or RNP mixture (see text for amounts) and processed for electroporation with Gene Pulser XCell (BioRad Laboratories) using parameters previously published, as per the manufacturer's instructions (Voltage = 115, Capacitance = 950 μ F, Resistance = ∞).²² Recovery culture and cloning proceeded identically to Process-1. However, the cells were cultured in a 96 well plate for 3 weeks, until wells were outgrown. Null cell lines were then transferred deep well plates, as previously reported.²²

2.4 | GS knockout clone screening

We followed an identical approach to published procedures.²⁴ After ~2 weeks, each well with colony was screened for GS gene disruption (knockout) through Sanger sequencing. For Sanger sequencing, genomic DNA from each clone was extracted by 50 μ l of Quick Extract solution (Epicenter), followed by heating at 65°C for 15 min and then 95°C for 5 min.

2.5 | PCR

PCR reactions in this study used AccuPrime Pfx DNA Polymerase (Invitrogen) and an ABI Veriti thermocycler. The reactions proceeded identically to the manufacturer's recommendations, except that 100 μ l of reaction volume was used per reaction with 100 ng of input gDNA, using an annealing temperature of 68°C. Each reaction did not exceed 30 cycles. The GS gene fragment from each clone was amplified from its genomic DNA through PCR using forward primer (5'-GGGTGGCCCGTTTCATCT-3') and reverse primer (5'-CGTGACAACCTTCCCATATCACA-3'). The PCR products were sent for PCR cleanup followed by Sanger sequencing using the reverse primer.

2.6 | T7 Endonuclease assay

PCR products were purified using the PCR purification kit (Qiagen) and eluted in molecular biology grade water. They were then adjusted to 1X NEB Buffer 2.1 (NEB). Products were then boiled for 10 min, allowed to cool to room temperature, divided in two, and treated with 1.5 μ l of T7 Endonuclease (NEB) or water. The reaction proceeded for 1 h, until it was deactivated by adjusting the mixture with 1X Purple loading dye (NEB). The reaction was then run at 120 V for 65 min on a 1.5% agarose gel. The band intensities were quantified by densitometry using image-j according to previously published procedures.^{24,25} For fragment analysis we utilized the method by Ran et al.²⁵

2.7 | Minipool generation

Cells were transfected using the Process-1 or Process-2 transfection methods. Immediately after transfection, 5000 cells were seeded into 96 well plates containing selection media. Titer was assessed 17 days posttransfection.

2.8 | Octet screening

Following 10–17 days of minipool or clone outgrowth, 100 μ l of media from the culture plate of was transferred to 96-well black tilted-bottom plate (Pall, ForteBio). The octet HTX system was used to analyze samples using the Protein A Dip and Read™ Biosensors to bind IgG directly from the media (ForteBio). Probes were first hydrated with basal alpha MEM (Thermo) media supplanted with 10% FBS and regenerated with 10 mM glycine, pH 1.7. Wells were neutralized with basal media.

2.9 | ELISA screening

96-well plates were coated with 100 μ l diluted Capture Ab (Jackson Labs) in carbonate buffer (Sigma) overnight at four degrees. The next day, plates were washed thrice in TBST (Sigma) for 5 min each. The plates were incubated with 100 μ l cell supernatant for 2 h at room temperature, washed as above, and then incubated with secondary antibody (ThermoFisher, Cat: 31413) in TBST +5% BSA for 1 h. Following an additional wash series (as above) plates were developed by adding 100 μ l ABTS substrate solution (ThermoFisher) for 10–20 min. The reaction was stopped by adding 100 μ l SDS Termination Reagent (ThermoFisher) to each well. Plates were then measured at 405 nm using a SpectraMax Plus (Molecular Devices).

2.10 | Next-generation sequencing

Amplicons were first assayed for quality using an Invitrogen Quant-iT dsDNA (Thermo Fischer) assay and gel electrophoresis to determine DNA concentration and DNA quality. Samples were then used to generate libraries using the Illumina TruSeq Nano DNA kit (Illumina, Cat: 20015964). The concentration and size range of the generated libraries were later determined using the Quanti-iT dsDNA Assay kit. The libraries were sequenced using the Illumina MiSeq platform, with read length of 2×150 bp. 4.5Gb of sequencing data was generated per DNA sample.

Prior to data analysis, samples were demultiplexed using bcl2fastq-v.1.8.4, and adapter sequences were trimmed using trim_galore_v0.3.3. Following CRISPRESSO,²⁶ BBDuk (<http://jgi.doe.gov/data-and-tools/bb-tools/>) was utilized for an additional cleanup step. Briefly, right end adapters with a 27-Kmer length were trimmed with a maximum substitution setting of 1. Low quality reads were trimmed from both ends with a minimum quality of 30 and short reads less

than 70 base pairs were discarded. Duplicates were removed, and adapters were trimmed based on paired read overhangs with a minimum overlap of 24.

For CRISPRESSO, the amplicon sequence was utilized as a reference and reads with a phred quality of less than 30 were discarded.

2.11 | Glutamine dependency assay

Clones with GS mutations (knockouts) were verified using a Glutamine Dependency Assay. Both Wild-type cells and GS-knockout clones were cultured in basal media (see Cell Culture above) with glutamine (+Gln) and without (−Gln). Viable cell density and cell viability were measured using a Guava® easyCyte flow cytometer (Millipore).

2.12 | Evaluation of mAb production

Host cell lines were transfected with mAb-containing expression vectors and selected with MSX-containing culture medium. The selected

stable pools were then evaluated by fed-batch production assay following Merck in-house production process conditions. The measurement of growth, viability, osmolarity, glucose, and lactate were collected daily. Cell density and viability were measured a Vi-CELL cell counter (Beckman Coulter). Glucose and lactate levels were measured by using the RANDOX RX imola chemistry analyzer (Crumlin) or D-Fructose/D-Glucose Assay Kit (Megazyme). mAb production levels were determined by Protein-A HPLC.

3 | RESULTS

3.1 | The pitfalls of the GS selection in wild-type hosts and developing an improved methodology for null-cell line development

In order to better characterize the GS system, we transfected commercially available wild-type CHO hosts with mAb producing vector in combination with a GS-minigene or empty control. Recombinant cells in addition to mock-transfected hosts were then selected in

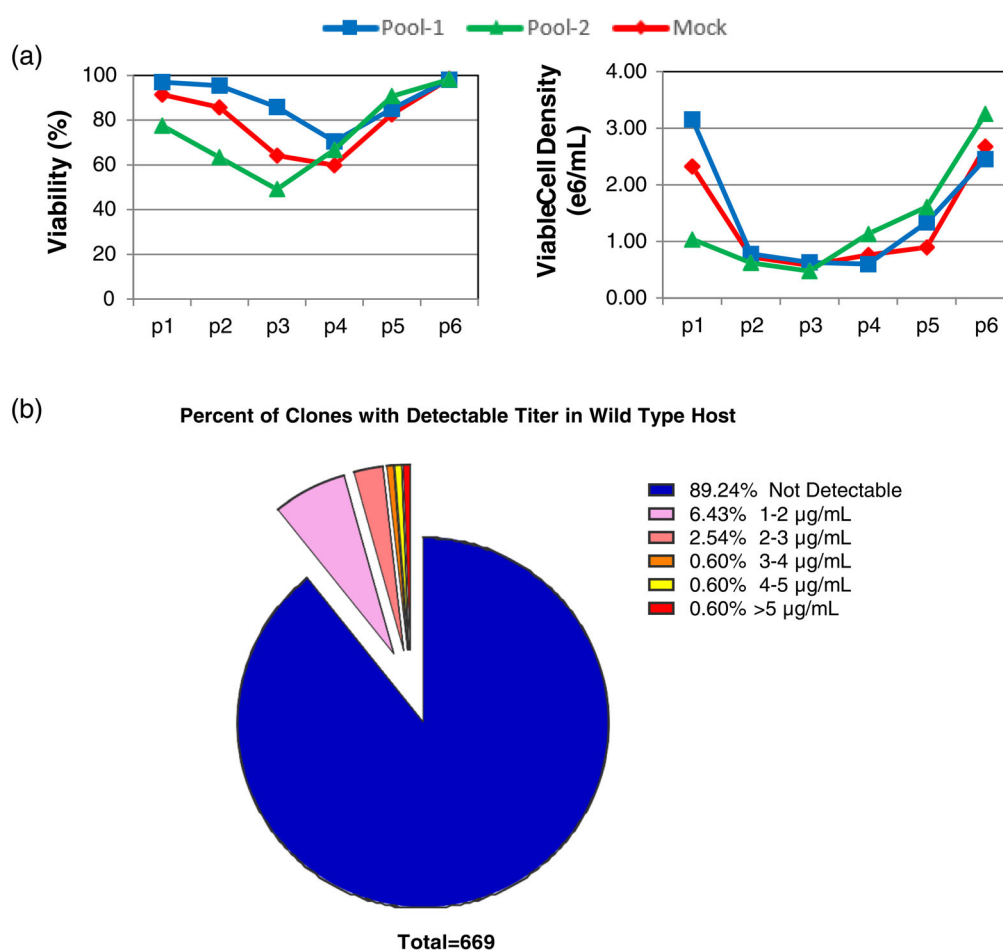


FIGURE 1 Prevalence of false-producers when using GS-selection combined with a wild-type host. (a) Cells were transfected with mAb expressing vector (green and blue lines) or vehicle control (red lines) and then selected in glutamine free media with MSX. The viability (left panel) and VCD (right panel) is depicted after each 3-day passage during clone selection. (b) Transfected pools were cloned, and colonies were evaluated for titer using ELISA. The resultant titers of the clones identified were binned and plotted ($N = 669$)

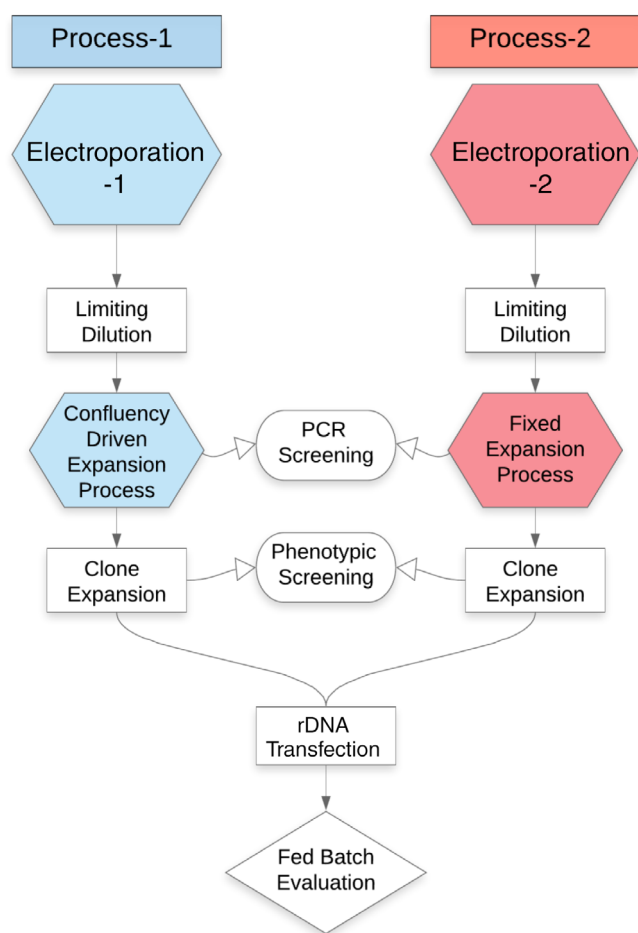


FIGURE 2 Flow chart of typical GS-knockout host development schema and evaluation. A typical cell line development workflow to generate auxotrophic CHO hosts is depicted, with the major areas for improvement we optimized highlighted in red/blue. GS null lines are first generated by gene editing, which can diverge in delivery (lipofection, electroporation, etc.), modality (i.e., CRISPR, ZFN, or TALEN), and molecule (i.e., RNP, mRNA, or cDNA). Resultant pools are allowed to recover, and then cloned. The pools are then expanded, screened via genetic methods (i.e., PCR) and positive hits are assessed by a functional assay. Null cell lines are finally assessed for performance via transfection with mAb and fed-batch production. We developed combinatorial improvements to host cell line development and combined them into a new process, denoted “Process-1.” In Process-1, cells are transfected with the Neon Transfection apparatus, single cell cloned, and then scaled with continuous media replenishment (see material and methods, “Transfection and Process Description”). In Process-2 we closely followed the benchmark protocol.²² Cells were transfected with the Biorad Genepulser transfection apparatus and single cell cloned. The cells were allowed to reach confluency, scaled to deep-well plates, and again grown until culture saturation

glutamine-free medium with the addition of 25 μ M MSX, which is thought to be stringent.^{13,14,27} In agreement with previous reports, we observed rapid recovery of the transfected cell pools, but unexpectedly, we also observed near complete recovery of the mock transfected cell line (Figure 1a). Furthermore, when these recombinant pools were combined were cloned, we observed that the vast majority

of colonies (89%, $N = 669$) demonstrated non-detectable mAb expression via ELISA screening (Figure 1b). Further increasing the concentration of the GS inhibitor, MSX, resulted in a near-disappearance of both mock cell lines and producers in an independent experiment (Figure S1A). These data suggest that the endogenous GS gene is sufficient to support cell survival under the selection pressure whereas the transgenic GS gene is dispensable.¹⁵

We therefore directed our efforts to optimize GS-null cell line development, which suffers from low efficiency [$\sim 2\%$].²² GS knockout involves the transfection of the genetic modification agent into the cells followed by recovery, single cell cloning through limiting dilution, and expansion (Figure 2). Single cells are then screened for GS knockout genetically and functionally before recombinant protein expression. By analyzing previous approaches, we identified several major bottlenecks vs. previously published methods: the gene delivery and the clone expansion procedures (Figure 2, colored flow-chart components). We deemed the improved and optimized process “Process-1” (shown in blue) whereas the process derived from the literature was deemed “Process-2” (shown in red). We set forth an ultimate goal of greater than 2% isolation of bi-allelic knockout clones, as previously described.

3.2 | Transfection efficiency markedly impacts downstream cellular traits

Delivery of expression constructs through electroporation has gained extensive utility in the context of gene-editing.²⁸ However, optimization of electroporation platforms and parameters significantly varies among cell and tissue types.²⁹ In order to fully optimize genetic knockout in CHO, we first assessed electroporation efficiency by transfecting cells with GFP mRNA in in-house and commercial CHO cell lines using two different platforms (see Figure 2). The electroporation described in Process-1 was established in-house, while the method in Process-2 was chosen as a comparative benchmark to previous results.²² As shown in Figure 3, electroporation Process-1, resulted in $\sim 90\%$ GFP-positive cells in both cell lines. However, electroporation through published procedures (Process-2) only generated 65% and 30% GFP-positive cells in the in-house and commercial hosts, respectively. Next, when GFP mRNA was replaced by GS exon-5 directed ZFN mRNA (see cartoon, Figure 4a), Process-1 resulted in a maximal gene modification at 19.9% while Process-2, yielded efficiencies of only $\sim 9\%$, despite payloads of up to 25 μ g of ZFN mRNA (Compare left and right gel images in Figure 4a and blue vs. red histograms in Figure 4b). These results were confirmed by next-generation sequencing, which demonstrated that 68% of alleles generated by Process-1 were mutant, representing an improvement compared with previous methods (Figure 4c²²). Alternative gene editing technologies such as CRISPR RNPs demonstrated an identical trend, albeit at higher efficiencies ($\sim 81\%$ GS gene disruption; Figure 4a–c). Altogether, these data suggest that ZFNs delivered by Process-1 result in higher editing efficiency as compared with Process-2. Therefore, we conclude that electroporation parameters have a significant impact on impact cargo delivery and/or stability.

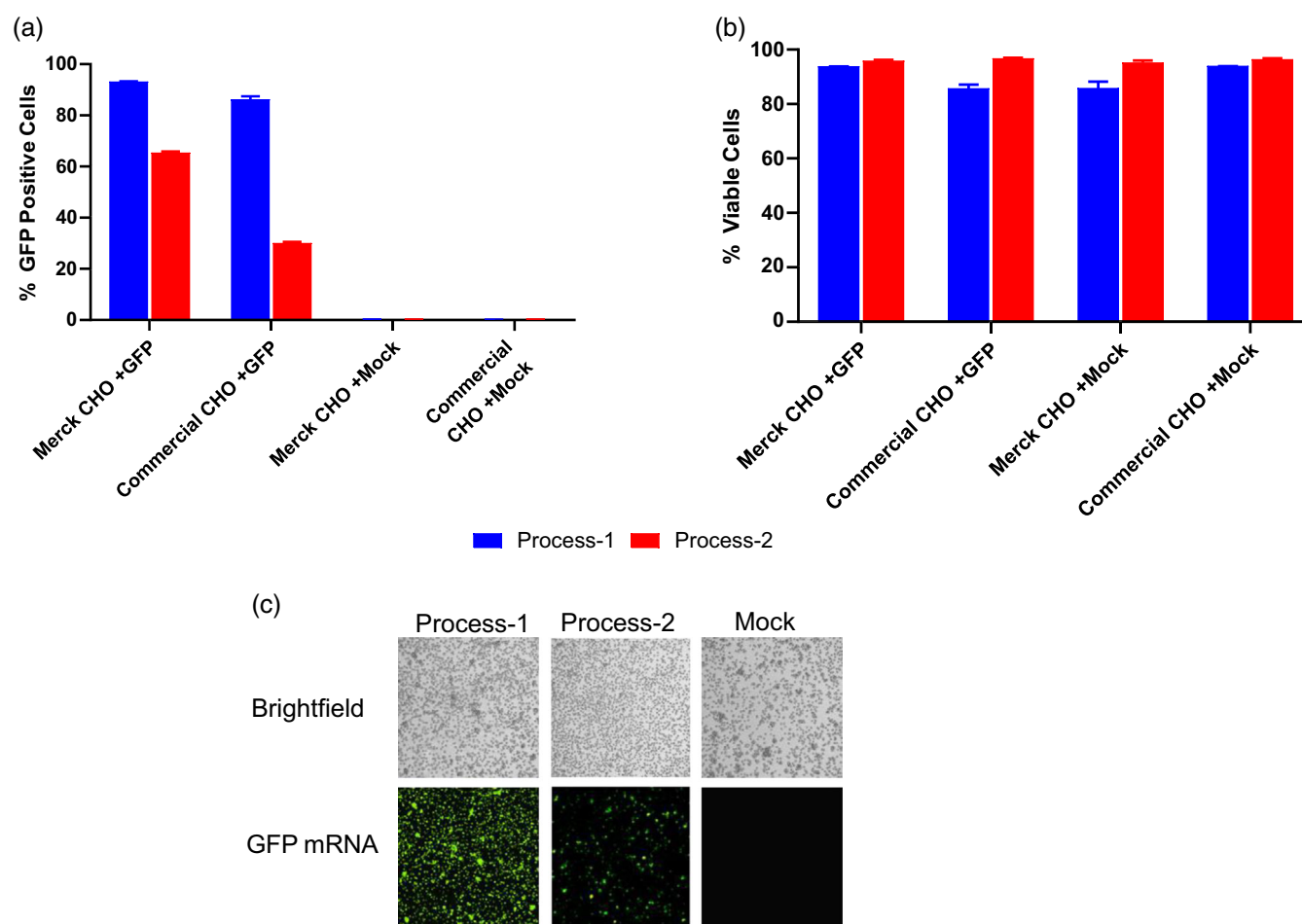


FIGURE 3 Comparison of transfection efficiency between two electroporation methods. In-house developed hosts (Merck CHO) and commercial suspension CHO (Commercial CHO) cell lines were transfected with GFP mRNA using Process-1 and Process-2 (two different electroporation methods). The percentage of GFP-positive live cells (a, left hand panel) and their viability (b, right hand panel) was assessed by flow-cytometry 3 days after transfection. Representative images from Commercial-CHO posttransfection were acquired by a fluorescent microscope are depicted in (c)

3.3 | Implementation of a framework to circumvent clone development associated stress

Conventional single-cell growth is typically accomplished by up to 3 weeks of proliferation within the same culture vessel without changing media, which may result in significant ER Stress.³⁰ These cellular phenotypes may result in long-term epigenetic reprogramming that persists in the ultimate cell hosts.³⁰ Therefore, we next sought to optimize the clone development process to improve the yield and quality of GSKO clones. Accordingly, the maximally edited ZFN mRNA-transfected bulk pools from both Process-1 and Process-2 were cloned by limiting dilution. Following different clone expansion procedures, (see Material and Methods), clones from different processes were analyzed for the frequency of GS-knockout using Sanger sequencing (Figure 5a). The representative electropherograms are shown in Figure 5b and demonstrate that the more edited Process-1 bulk pools also yielded more GS null clones. Notably, the total yield of knockout clones was improved 2.5-fold in Process-1 in comparison to the conventional Process-2, with similar fold enrichment in

homozygous knockout cells. Next, to mitigate stress associated with outgrowth, cells from Process-1 were continuously expanded based on confluency from 96-well plates until transfer to shake-flasks. As a comparison, previously reported procedures were followed in Process-2, and wells were fully synchronized and completely outgrown before transfer to deep-well plates.²² When the growth phenotype of knockout clones from both procedures were analyzed, we observed that cells from Process-1 demonstrated superior growth characteristics to those from Process-2, as indicated as the average doubling time (Figure 6a). To verify that the changes resulting from the growth strategy were not a result from the transfection methods, we next transfected cells with recombinant DNA using both electroporation methods, performed limiting dilution, and completely outgrew the wells as in Process-2. This experiment demonstrated that the yield of producers was markedly different between both transfection approaches, mirroring our gene editing results. Despite this, no changes were observed in doubling times, suggesting that the observed growth characteristics were resultant from our scaling, but not the transfection strategy (Figure S1B). These data suggest that

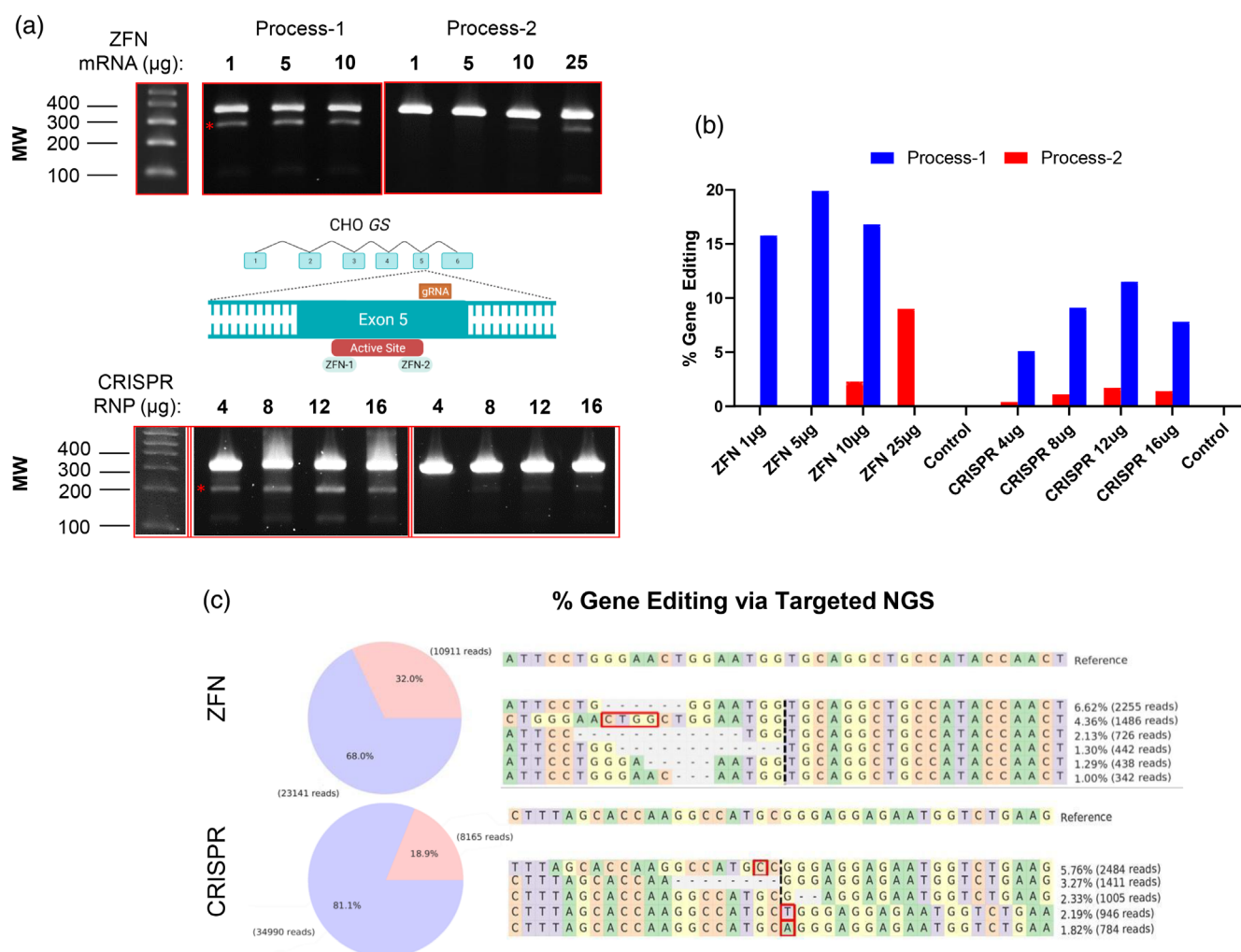


FIGURE 4 Generation of GS-knockout Pools. In-house developed CHO hosts (Merck CHO) were transfected with the indicated amount of ZFN mRNA or CRISPR RNP for the GS locus using two electroporation methods. (a) The GS target region (see schematic cartoon, middle panel) was PCR amplified from genomic DNA 3 days from ZFN mRNA (top-left hand panel) or CRISPR RNP (top right-hand panel) after transfection. Samples were then digested with T7 Nuclease and run on a 4% agarose gel. Red asterisks indicate observed cleavage bands. (b) The percent edited alleles in were then assessed by densitometry and plotted for the two different processes. (c) Samples from Process-1 demonstrating maximal gene editing efficiency (CRISPR and ZFN) were assessed by next-generation sequencing. The percentage of mutant alleles is shown to the left, while the identity of the top mutated alleles is shown to the right

process design, including transfection method, posttransfection recovery, and clone expansion procedures, result in combinatorial improvements to cell health and performance.

3.4 | Evaluating recombinant pools generated from differential processes

The GS selection system is more readily leveraged in GS-null CHO hosts, which demonstrate significantly higher productivity as compared with their wild type counterparts.¹⁴ Having demonstrated that the yield and growth characteristics from Process-1 clones was superior to Process-2, we next sought to evaluate the GSKO phenotype from both processes. To accomplish this, cells were subjected to growth with or without glutamine and passaged over

a 6-day period. Here, we observed rapid decline in viability after continuous passaging (Figure 6b, compare red and blue curves for the GS-KO cells). No differences were observed in glutamine sensitivity between hosts from the two processes (data not shown) suggesting that both processes result in a robust sensitization to glutamine deprivation.

In order to evaluate the performance of the genetic engineered host cells for recombinant protein production, the top five clonally derived hosts from either Process-1 and Process-2 as well as a wild type progenitors were transfected with mAb-containing expression vector containing a GS selection marker and selected in glutamine-free medium containing 12.5 μM MSX. The selected stable pools were evaluated for mAb production by using a 10-day fed-batch production assay. In addition, a recombinant commercial control pool, expressing an identical mAb and developed using identical methodology was

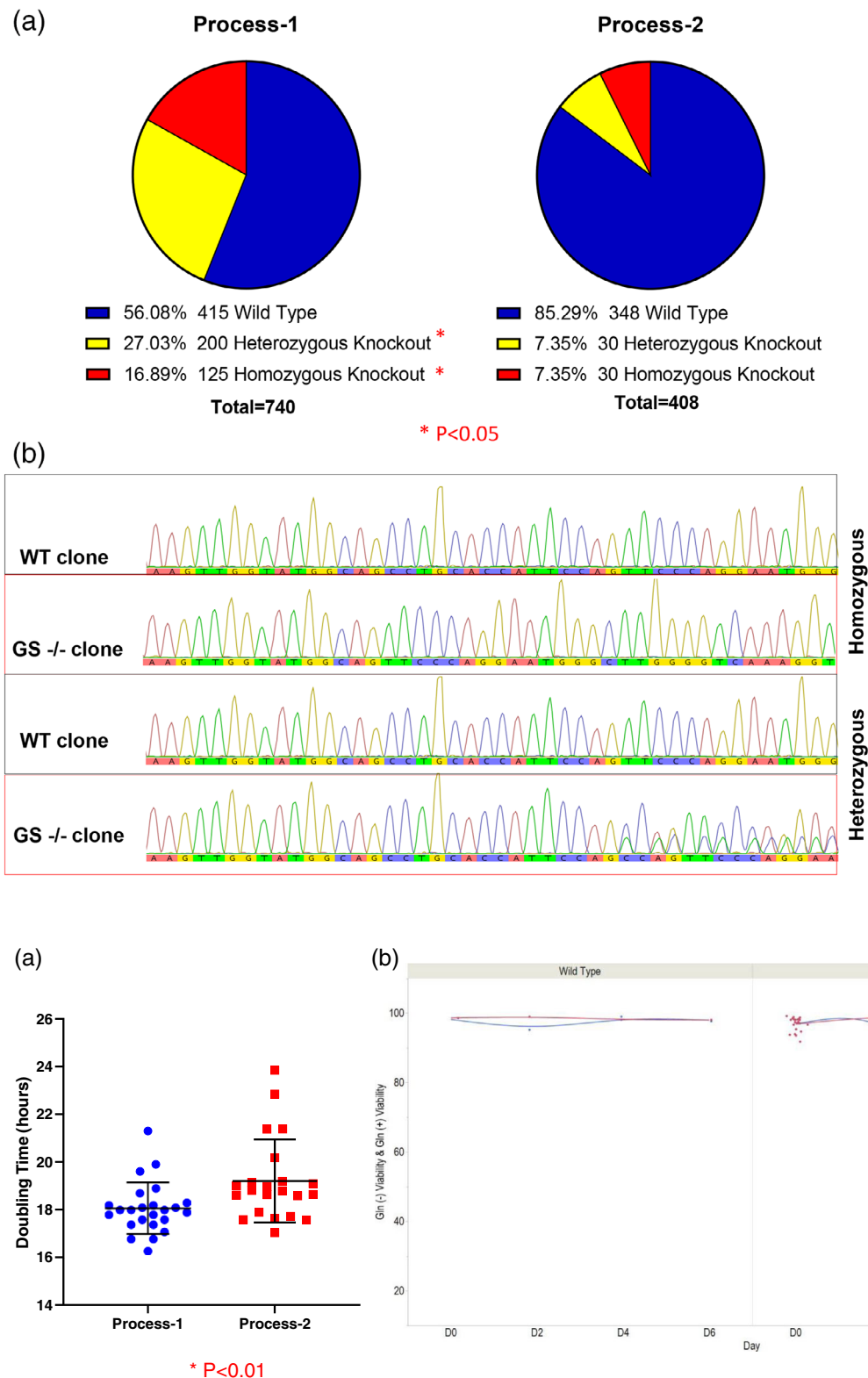


FIGURE 5 Comparison of GS-knockout hosts generated from two protocols. Process-1 and Process-2 derived ZFN-edited bulk pools were cloned by limiting dilution. Colonies demonstrating outgrowth were assessed by PCR and Sanger sequencing. (a) The percentage of wild type, heterozygous, or homozygous knockout clones obtained from each electroporation condition is represented in the pie graph form. Wild type alleles are indicated in blue, while heterozygous knockouts are shown in yellow. Homozygous knockouts are presented in red. Significant changes between processes are noted with a red asterisk. (b) Representative Sanger traces from homozygous (top panel) and heterozygous (bottom-panel) GS-null clones

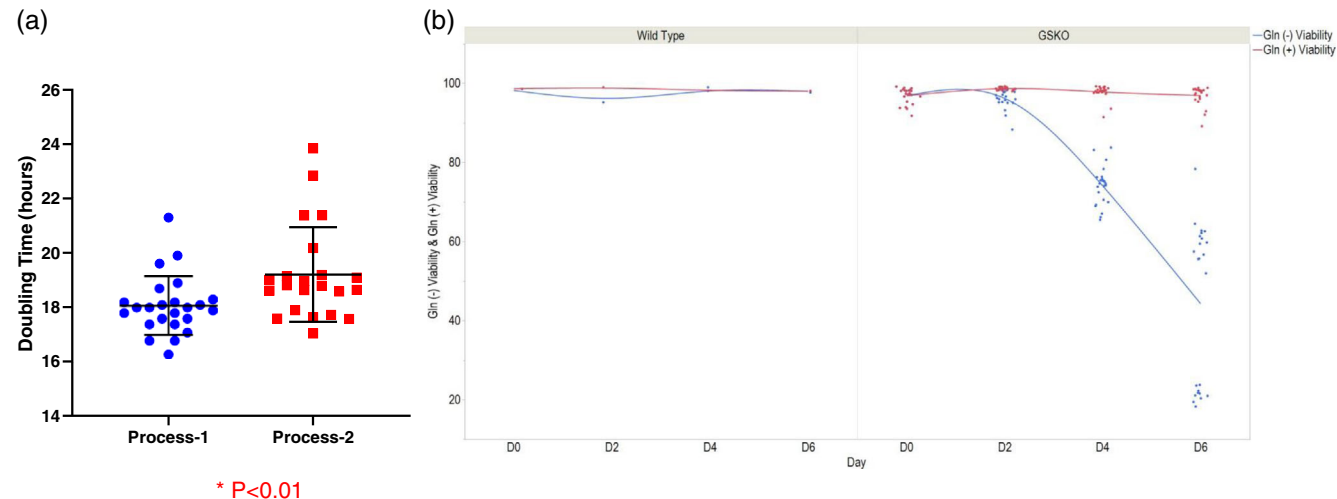


FIGURE 6 Phenotypic Assessment of GS Knockout Hosts. GS knockout clones from both Process-1 and Process-2 were expanded and cellular phenotypes were assessed. (a) The average doubling time of confirmed null clones obtained from each process was plotted in dot plot form (b) the viability of either wild type cells (left hand panel) or GS-null clones (right hand panel) with (blue lines and dots) and without (red lines and dots) glutamine was plotted following a six-day outgrowth. Each data point represents an individual clone, while curves represent confidence of fit

included as benchmark control. As shown in Figure 7a, the GS-knockout pools generated from Process-1 demonstrated superior cell growth and health characteristics performance as compared with

Process-2. Notably, top performing GS-null pools were observed to grow to higher densities (Figure 7a) and were able to maintain high viability for the duration of the fed-batch production. As a result, the

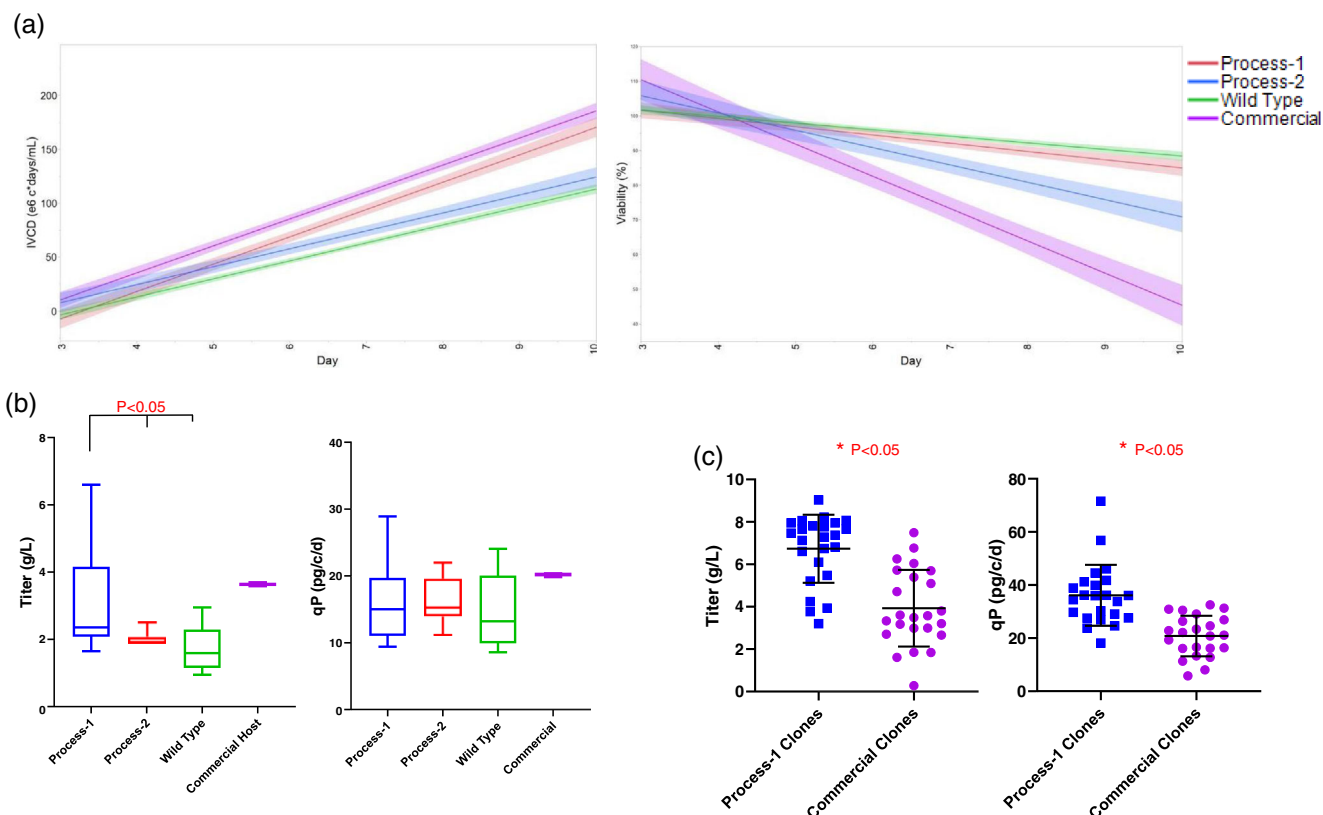


FIGURE 7 Evaluation of Bulk Pool and Clone Production from GS Knockout Hosts. GS knockout, Wild-type hosts, and a commercial GSKO control were transfected with mAb expressing vector and pools were established following a 2-week selection in media without glutamine supplemented with 12.5 μ M MSX. Stable pools were then subject to a 10-day fed batch production assay. (a) The IVCD (left-hand) and viability (right hand) of cells from Process-1 (blue curves and histograms), Process-2 (red curves and histograms), Wild Type (green curves and histograms), or Commercial GSKO pool (purple curves and histograms), were plotted and the (b) final titer and qP of the resulting pools is shown in box-plot form. (c) Represents the titer and qP of the top producing clones (blue dots) obtained from the highest producing Process-1 pool and the commercial pool (purple dots)

productivity of the selected stable pool derived from Process-1 reached 6.7 g/L on with a specific productivity of 28.7 pg/cell/day; while the top Process-2 derived pool reached 2.5 g/L of titer with specific productivity of 21 pg/cell/day (Figure 7b). The robustness of the GSKO host cells was further validated by cloning the top performing recombinant pool via FACS. Following subsequent outgrowth and evaluation with fed-batch production, we identified that the recombinant GSKO clones were robust producers, and outpaced cloned commercial comparators (Figure 7c). This procedure yielded an abundance of high expressing clones, notably reaching a maximal titer of 9 g/L and a qP of 71 (Figure 7c).

4 | DISCUSSION

Host cell engineering is important in biopharmaceutical industry to improve productivity and product quality. This process is both laborious and time-consuming, involving many steps including gene editing method design, gene delivery/transfection method optimization, cell recovery and cloning, clone expansion, genotyping evaluation, and phenotypic evaluation with recombinant protein production. These seemingly unlinked steps play a synergistic effect on host cell robustness in recombinant protein manufacturing. In this study, we

established a new process by combining improved procedures in transfection, cell recovery, and clone expansion. Compared with conventional methods, the newly developed process demonstrated superior advantages in supporting host cell growth and recombinant protein production.

ZFN-mediated GS knockout was also used as a model to demonstrate the improved cell engineering efficiency. Our efforts were stymied by the low efficiency of ZFN technology commonly in published benchmarks. Though ZFNs have been successfully applied to multiple CHO hosts, low efficiency remains a serious drawback.³¹ In the context of highly time sensitive cell line development projects, low efficiency adds yet another labor-intensive and time-consuming step. Alternative gene editing modalities, such as the CRISPR/Cas9 system, which cleaves a target sequence by complementary pairing using a guide RNA (gRNA) have largely resolved these issues.³⁰ Nevertheless, commercial application of CRISPR/Cas9 is stymied due to intellectual property disputes.¹⁹ We therefore sought to improve the parameters associated with the ZFN platform. Accordingly, we used ZFN-mediated GS knockout to demonstrate that the knockout efficiency of ZFN can be easily increased from 2% to as high as 68%, which is comparable to TALENs and CRISPR/Cas9 technologies. The improved efficiency warrants improved screening of GS-knockout clones to obtain robust host lines. In addition to changing

electroporation method we optimized posttransfection recovery and clone expansion procedure of single cell clones, resulting in shorter doubling time and higher robustness. The optimized process resulted in a 23-fold increase in knockout efficiency and clone titers up to 9 g/L during a 14-day Fed-batch production process. Our results demonstrate that both gene editing and culture process are closely linked to cell performance, such as growth, survival, and protein expression.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/btpr.3185>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

- Werner RG, Noe W, Kopp K, Schluter M. Appropriate mammalian expression systems for biopharmaceuticals. *Arzneimittelforschung*. 1998;48(8):870-880.
- Andersen DC, Krummen L. Recombinant protein expression for therapeutic applications. *Curr Opin Biotechnol*. 2002;13(2):117-123.
- Hacker DL, De Jesus M, Wurm FM. 25 years of recombinant proteins from reactor-grown cells - where do we go from here? *Biotechnol Adv*. 2009;27(6):1023-1027.
- Rita Costa A, Elisa Rodrigues M, Henriques M, Azeredo J, Oliveira R. Guidelines to cell engineering for monoclonal antibody production. *Eur J Pharm Biopharm*. 2010;74(2):127-138.
- Kremkow BG, Lee KH. Glyco-mapper: a Chinese hamster ovary (CHO) genome-specific glycosylation prediction tool. *Metab Eng*. 2018;47:134-142.
- Sun T, Kwok WC, Chua KJ, et al. Development of a proline-based selection system for reliable genetic engineering in Chinese hamster ovary cells. *ACS Synth Biol*. 2020;9(7):1864-1872.
- Diego Schmidhalter H-PM. *Industrial Scale Suspension Culture of Living Cells*. Weinheim, Germany: Wiley Blackwell; 2014.
- Zhang Q, Jiang B, Du Z, Chasin LA. A doubly auxotrophic CHO-K1 cell line for the production of recombinant monoclonal antibodies. *Biotechnol Bioeng*. 2020;117(8):2401-2409.
- Urlaub G, Kas E, Carothers AM, Chasin LA. Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. *Cell*. 1983;33(2):405-412.
- Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol*. 2004;22(11):1393-1398.
- Cacciatore JJ, Chasin LA, Leonard EF. Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production using the Dhfr-based CHO cell selection system. *Biotechnol Adv*. 2010;28(6):673-681.
- Cacciatore JJ, Leonard EF, Chasin LA. The isolation of CHO cells with a site conferring a high and reproducible transgene amplification rate. *J Biotechnol*. 2012;164(2):346-353.
- Lianchun Fan CCF, Andrew JR. The use of glutamine synthetase as a selection marker: recent advances in Chinese hamster ovary cell line generation processes. *Pharm Bioprocess*. 2013;1(5):487-502.
- Noh SM, Shin S, Lee GM. Comprehensive characterization of glutamine synthetase-mediated selection for the establishment of recombinant CHO cells producing monoclonal antibodies. *Sci Rep*. 2018;8(1):5361.
- Sanders PG, Wilson RH. Amplification and cloning of the Chinese hamster glutamine synthetase gene. *EMBO J*. 1984;3(1):65-71.
- Gupta SK, Shukla P. Gene editing for cell engineering: trends and applications. *Crit Rev Biotechnol*. 2017;37(5):672-684.
- Valdmanis PN, Kay MA. Future of rAAV gene therapy: platform for RNAi, gene editing, and beyond. *Hum Gene Ther*. 2017;28(4):361-372.
- Gaj T, Gersbach CA, Barbas CF 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31(7):397-405.
- Cloney R. Patent law and genome engineering: a short guide to a rapidly changing landscape. *Mol Ther*. 2016;24(3):419-421.
- Carroll D. Genome engineering with zinc-finger nucleases. *Genetics*. 2011;188(4):773-782.
- Carroll D, Morton JJ, Beumer KJ, Segal DJ. Design, construction and in vitro testing of zinc finger nucleases. *Nat Protoc*. 2006;1(3):1329-1341.
- Fan L, Kadura I, Krebs LE, Hatfield CC, Shaw MM, Frye CC. Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. *Biotechnol Bioeng*. 2012;109(4):1007-1015.
- Doench JG, Fusi N, Sullender M, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol*. 2016;34(2):184-191.
- Huhn SC, Ou Y, Kumar A, Liu R, Du Z. High throughput, efficacious gene editing & genome surveillance in Chinese hamster ovary cells. *PLoS One*. 2019;14(12):e0218653.
- Ran FA, Hsu PD, Lin CY, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*. 2013;154(6):1380-1389.
- Pinello L, Canver MC, Hoban MD, et al. Analyzing CRISPR genome editing experiments with CRISPResso. *Nat Biotechnol*. 2016;34(7):695-697.
- Lin PC, Chan KF, Kiess IA, et al. Attenuated glutamine synthetase as a selection marker in CHO cells to efficiently isolate highly productive stable cells for the production of antibodies and other biologics. *MAbs*. 2019;11(5):965-976.
- Fajriat AK, He QQ, Wirusanti NI, Slansky JE, Ding X. A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing. *Theranostics*. 2020;10(12):5532-5549.
- Laustsen A, Bak RO. Electroporation-based CRISPR/Cas9 gene editing using Cas9 protein and chemically modified sgRNAs. *Methods Mol Biol*. 1961;2019:127-134.
- Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339(6121):819-823.
- Liu PQ, Chan EM, Cost GJ, et al. Generation of a triple-gene knockout mammalian cell line using engineered zinc-finger nucleases. *Biotechnol Bioeng*. 2010;106(1):97-105.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Automated and Enhanced Clone Screening Using a Fully Automated Microtiter Plate-Based System for Suspension Cell Culture

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DOI 10.1002/btpr.2760

Published online January 9, 2019 in Wiley Online Library (wileyonlinelibrary.com)

Recently, we established an automated microtiter plate (MTP)-based system for suspension cell culture for high-throughput (HT) applications in biopharmaceutical process development. In the present report, the new system was evaluated regarding its potential to improve clone screening by allowing high-throughput fed-batch cultivation at an early stage. For this purpose, a fully automated procedure was compared to a mainly batch mode-based manual standard process. The new system performed daily measurements of viable cell density and product concentration for a total of 96 clones in biological duplicates that were evaluated for final clone selection. This resulted in a more than fivefold increase in sample throughput and 4 weeks of time saving compared to the reference process. The top clone characterized by the highest cell specific productivity was identified only by the new process. In contrast, this clone was lost in the expansion phase of the reference procedure. Overall, the new system identified more high-productive clones, offering more alternatives and flexibility for process development. In-process monitoring of glucose and lactate levels representing crucial secondary selection criteria further enhanced top clone identification. Clone characterization at an early stage was further extended by linking the MTP-based cell culture system to additional HT-analytic systems for N-glycosylation analysis as well as gene expression analysis by reverse transcriptase-quantitative polymerase chain reaction. These powerful tools connected to the automated MTP-based cell culture system lead to considerably advanced quality and speed of clone screening, and increase the probability of selecting the most suitable clone. © 2018 American Institute of Chemical Engineers Biotechnol. Prog., 35: e2760, 2019.

Keywords: automated clone screening, fed-batch CHO suspension culture, early stage clone characterization, clone evaluation parameters, in-process monitoring, glycosylation analysis, RT-qPCR analysis

Introduction

Recently, we reported the establishment of an automated MTP-based system for suspension cell culture to improve screening and optimization of applications in biopharmaceutical process development.¹ The generic system uses off-the-shelf commercial laboratory automation equipment and MTPs in orbital shaken mode to accomplish fully automated fed-batch cell cultivation with integrated process analytics. The gained process understanding supports the development of reproducible and robust processes from product yield as well as product quality point of view.^{2,3} In addition, a modular set-

up allows for adaptability and flexible extension of analytical parameters.

Earlier in the development of biopharmaceuticals, the step of suitable clone selection for recombinant protein expression represents a significant challenge to achieve satisfactory product yield and quality.^{4,5} Huge numbers of transfected cells have to be screened and evaluated to identify the clones with the most favorable product formation profile as well as product quality and growth characteristics. These approaches usually start with cells cultivated in batch mode using static microtiter plates (MTPs), followed by a phase in shaken MTP requiring many manual steps of pre-cultures and preselection based on product concentration. The final clone evaluation is performed under representative fed-batch conditions (2 L bioreactors, shake flasks) which is the major process form for the

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production of recombinant proteins.^{6,7} These common selection procedures are laborious as well as time consuming and show further shortcomings. First of all, the number of clones needs to be narrowed down from a few thousand at the beginning of the screening process to a handful that can be evaluated in the final fed-batch stage in bioreactors or shake flasks. In addition, batch mode screening commonly used in the pre-selection process mostly focuses on overall titer without cell density measurements. Thus, high product yield may result from high growth rates, but the cell-specific productivity of the clones is not available at an early stage. The standard procedures bear the risk to miss promising, high productivity clones during the preselection.

Furthermore, clones may show different characteristics regarding growth, production rate, and product quality when comparing batch and fed-batch cultivation.^{8–11} Limitations start to affect metabolism, growth, and product accumulation after approximately 7 days in a batch cultivation. In contrast, fed-batch conditions compensate for exhausted nutrients and cell growth as well as production can be significantly extended and increased to achieve maximal product concentration required for full-scale production.⁷ Due to these differences, batch cultivation appears less appropriate for clone screening and hence an use of fed-batch at earlier stages of selection is favorable.¹² Besides screening the clones in a fed-batch process, early availability of product quality data will improve the chances to select the most suitable clone and to reduce risks during later development stages. In addition to a variety of growth parameters, glycosylation, which is a major posttranslational modification of therapeutic mAbs may be of interest as well as the mRNA expression level of individual product components, such as mAb heavy chain (HC) and light chain (LC).

The fully automated MTP-based cell culture system was already successfully used to handle large experimental setups using fed-batch cultivations with simultaneous process analytics. Up to 288 cultures can be performed in duplicate at the same time using the 24-well MTP format.¹ The purpose of the present study was to evaluate the automated MTP-based system

for clone screening and selection. The procedure using the automated system was compared to a standard procedure of process development to identify and rank the selected 18 top clones in a side by side clone screening experiment. In addition, the performance of the new system was extensively evaluated regarding its potential for extended clone characterization at an early stage in order to increase reliability of clone selection. This included real-time in-process monitoring of metabolites crucial for cell growth and product formation. Moreover, the automated MTP-based cell culture system offered the possibility to link to additional HT-analytical systems and to enable *N*-glycosylation analysis as well as reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) expression analysis for mAb products of cell culture samples which becomes even more important when expressing complex molecule formats. The fully automated process and the extended clone characterization show considerable potential to accelerate clone selection and to increase the probability for selecting the most suitable clone.

Materials and Methods

Materials and disposables

The following sterile MTPs and shake flasks used in the standard screening process were sourced from Corning Life Sciences (Corning, NY): 384-well MTPs, clear, polystyrene, used for the initial static batch experiment, 96-well MTPs, flat bottom, polystyrene, tissue culture treated for the second static cultivation step, 24-well MTPs, flat bottom, polystyrene, tissue culture treated and six-well MTPs, flat bottom, polystyrene, tissue culture treated used for the shaken batch experiments, and 125 mL shake flasks w/vented cap for the final fed-batch cultivation step.

The following MTPs were used in the automated MTP-based system: sterile 24-square deep well MTPs, polypropylene, obtained from Porvair Sciences were used for cell culture. BD Falcon™ 96-well MTPs, U-Bottom, polystyrene, nonsterile supplied by Thermo Scientific were used for taking samples. 96-MicroWell™ plates, Nunc™, flat, polystyrene, clear used for automated cell counting as well as miniaturized glucose and

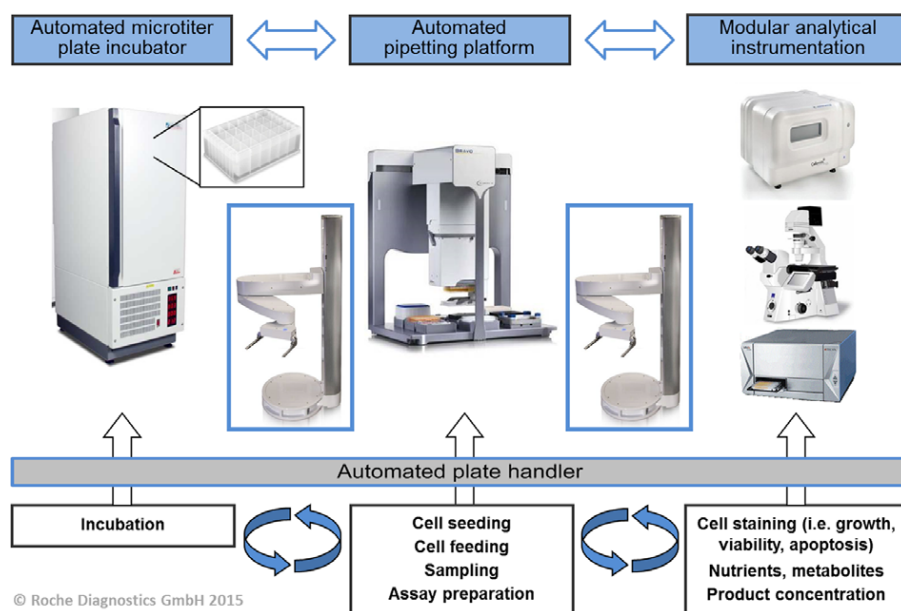


Figure 1. Composition of the automated MTP-based cell culture system. The core system consists of a robotic microtiter plate handler as key device connecting shaken cultivation, processing and analytical evaluation (according to Markert and Joeris, 2017).

lactate assays were from Thermo Scientific. Greiner bio-one PP microplate black, 96 well, F shape were applied for automated product concentration measurement using the Forte Bio Octet.

Cell culture

Chinese Hamster Ovary clonal cell lines (CHO-K1) engineered to produce a mAb were generated in-house and used under standard culture conditions according the Roche in-house media and process platform. Cells were seeded in 384-well plates 2 days after transfection and cultured at 37 °C in a humidified 7% CO₂ incubator. 96 clones were evaluated in a shaken fed-batch experiment using the automated MTP-based cell culture system. Feed media were added once a day as a bolus feed. The system operates sterile throughout the whole process making any additions of antibiotics to media unnecessary.

No feeds were added under batch conditions. Cells were transferred manually to the next MTP at the end of the growth phase. Undefined seeding was performed only in the batch culture of the reference process because the low sample volume combined with the high throughput did not allow determination of cell density by the Cedex HiRes Analyzer (Roche Diagnostics GmbH). This seeding mode uses a fixed dilution factor per plate not considering the individual cell density in each well. In contrast, a defined seeding using a fixed volume per clone based on the determined viable cell density (VCD) was applied in the last batch experiment and in the final fed-batch mode (please see also Figure 2). In the

manual process, cell banks were created from aliquots taken from cultures at the six-well stage.

The automated microtiter plate-based screening system for suspension cell culture

The cell culture system used for the clone selection is shown in Figure 1 and was recently described in detail elsewhere.¹ In brief, the system consists of three functional modules for incubation, liquid handling, and analytical evaluation. It enables a fully automated workflow under sterile conditions under a biological safety cabinet. The control of all components is carried out by the software VWorks Automation Control (Agilent Technologies, Waldbronn, Germany). Due to the modular and flexible system structure, the integration of new system components or the extension of the analytical portfolio is easy to accomplish.

Computational fluid dynamics (CFD) simulations were used to establish optimized shaking conditions, 24-deep well plates were filled with 4.5 mL per well and shaken with a 3 mm orbital radius at 350 rpm.

Miniaturized analytical methods integrated in the automated MTP-based system

The automated MTP-based system performed daily sampling and a volume of approximately 50 µL–100 µL was taken for analytical evaluation. Standard analytical methods were adapted for low sample volume and high throughput to

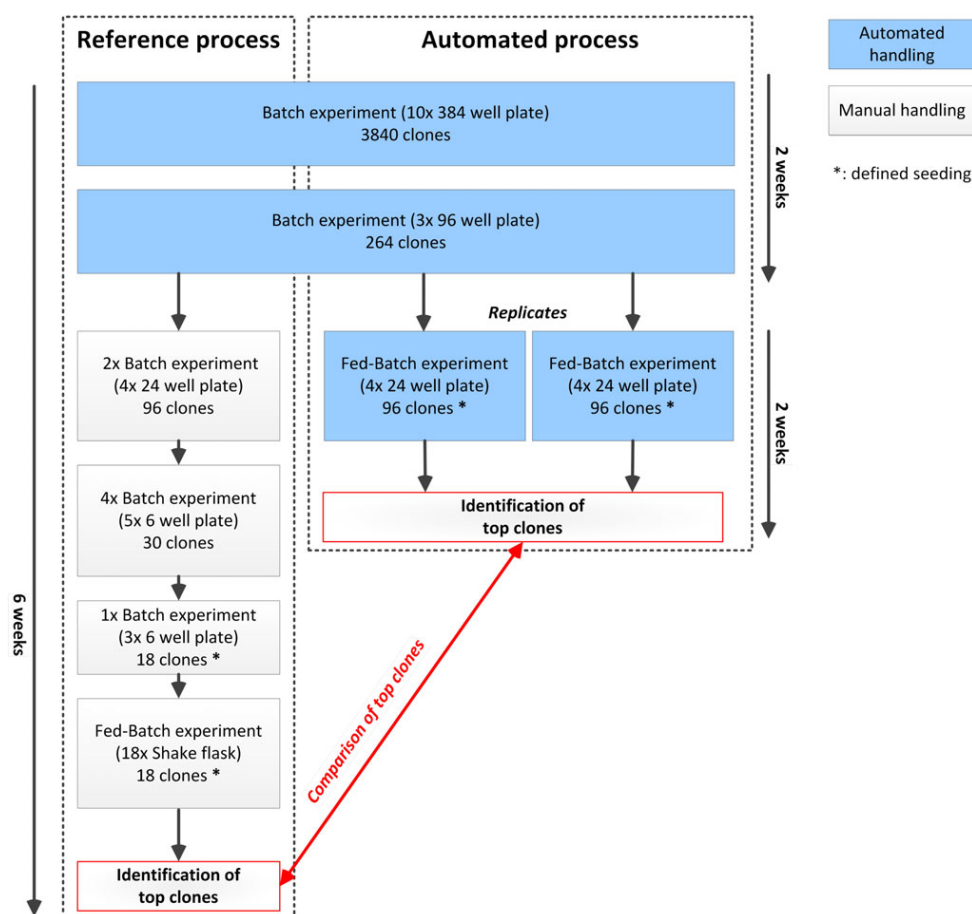


Figure 2. Flowchart comparing the new process including the fully automated MTP-based cell culture system with the standard reference process.

enable their application in the automated cell culture system. The resulting miniaturized assays for VCD (LIVE/DEAD® cell Vitality Assay Kit, Molecular Probes), glucose and lactate (based on Cedex Bio reagents, Roche Diagnostics GmbH) were performed daily by the automated and integrated analytical module as previously described.¹

For the determination of mAb product concentration, samples automatically taken by the liquid handling module were transferred to the in-house analytical department for routine processing using an Octet System (Pall ForteBio Corp., Menlo Park).

Based on VCD, daily growth rate and product concentration (P), the cell-specific productivity of a clone was determined according Clarke et al¹³:

$$qP \left[\frac{pg}{cell*day} \right] = \left(\frac{P2-P1}{VCD2-VCD1} \right) * \left(\frac{\ln(VCD2) - \ln(VCD1)}{time2-time1} \right) / 24$$

Setting up the clone screening and selection case study

Clone selection started with automated screening in a static 384-well MTP batch experiment (Figure 2). In the next step, the top quartile of clones was transferred to a 96-well MTP based on product concentration. These clones were then manually transferred to shaken 24-well MTPs in batch mode with undefined seeding cell density to run the reference process. In addition, the same clones were manually transferred to shaken 24-well MTPs in duplicates to run the automated process in fed-batch mode and defined seeding cell density.

Clone screening and selection performed by the reference procedure

The top 30 of these 96 clones were manually transferred to 5 × 6-well MTPs in batch mode with undefined seeding density. At that stage, cell density measurement was feasible and the selected 18 top clones were manually transferred with equal seeding density to 3 × 6-well MTP. For the final fed-batch experiment, these 18 clones were then manually transferred with equal seeding density to 250 mL shake flasks. Final selection of clones in shake flasks with fed-batch mode was based on the parameters product concentration, VCD, and cell-specific productivity.

Clone screening and selection performed by the automated MTP-based cell culture system

The clones were manually transferred in duplicate to the automated MTP-based system for fed-batch cultivation in 8 × 24-well MTPs (Figure 2). Automated cell culture lasted for 14 days (336 h) and final clone evaluation was performed for all 96 clones in biological duplicates. Primary parameters were product concentration and VCD to calculate cell-specific productivity. Lactate and glucose concentrations determined by in-process analytics were available as secondary selection criteria. The 18 top clones identified by the new process were compared to the 18 top clones selected by the standard procedure.

High-throughput N-glycosylation analytics

Cell culture samples were taken at the end of the fed-batch culture and transferred to 96-well U-bottom plates (BD Falcon™) which were transferred to the internal analytical

department. A minimum of 10 µg of mAb was required to execute a fully automated high-throughput method established for glycosylation profiling of immunoglobulin G forms (IgGs) from harvested cell culture fluid.¹⁴ Quantitative assessment of glycans was based on glycopeptide analysis using hydrophilic interaction solid-phase extraction (HILIC) and electrospray mass spectrometry in the positive-ion mode (ESI-MS).

High-throughput RT-qPCR expression analysis

RT-qPCR was based on the establishment of a reference gene for relative quantification, and normalization of expression levels of genes for mAb LCs and HCs. The housekeeping gene eIF3i was found suitable as it showed constant and robust expression, and regulatory effects were not observed (data not shown).

RNA was isolated using the MagNAPure LC 2.0 System or the RealTime ready Cell Lysis Kit (both Roche Diagnostics GmbH, Penzberg, Deutschland) on the last day of the fed-batch experiment and cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH) together with random hexamer primers and standard incubation at 55 °C in a Thermo Cycler (MJ Research PTC-200 Thermo Cycler). cDNA was stored at -20 °C until use. The mRNA quality was checked to ensure isolating meaningfully intact mRNA.

For qPCR, the LightCycler® 480 Probes Master Mix was prepared adding 5 µL of cDNA to achieve a sample volume of 20 µL. Samples were amplified by the LightCycler® 480 instrument (Roche Diagnostics GmbH) using 96-well MTPs (LightCycler® 480 Multiwell Plate 96) and 45 cycles. Amplification products were detected by hydrolysis probes labeled with fluorescein (Universal Probe Library, Roche Diagnostics GmbH). Data were automatically processed to obtain product concentration and respective expression levels. Finally, regression analysis was performed using results of expression levels and cell-specific productivity.

Results and Discussion

Clone screening and selection performed by the fully automated MTP-based system in comparison to a semi-automated reference procedure

Clone screening aims to identify the most suitable clone that delivers optimum mAb yield and quality in the final scale-up production process. Clone screening using the automated MTP-based system was performed with fed-batch cultivation of high clone numbers selected from the initial static phase as illustrated in Figure 2. An early implementation of the fed-batch mode during the selection process is expected to improve predictivity for the large-scale production.⁶ The whole clone selection process was accomplished fully automatically within 14 days. Applying the standard procedure, clones grown in the batch mode required multiple manual seeding and pre-cultures and only the two final clone evaluation steps were then performed in the fed-batch mode.

A comparison between the standard procedure and the fully automated procedure using the MTP-based cell culture system was performed. The focus was on the identification of the 18 top clones based on the maximal final product concentration. The comparison of the two procedures showed that 15 of the 18 top clones were identical, but top candidates were

Table 1. The Top 18 Clones Identified by the Procedure Implementing the Automated MTP-Based Cell Culture System in Comparison to the Standard Process

Rank Order	Automated Process		Manual Process	
	Clone No.	Max. Product Concentration (mg/L)	Clone No.	Max. Product Concentration (mg/L)
1	5	2417	8	1740
2	83	2103	52	1474
3	91	1813	66	1474
4	52	1716	34	1305
5	8	1668	57	1233
6	34	1523	91	1209
7	15	1354	15	1136
8	64	1354	60	1112
9	9	1329	59	1112
10	35	1305	83	1088
11	94	1305	94	1063
12	65	1305	16	1015
13	59	1209	35	967
14	38	1160	38	918
15	49	1136	65	894
16	71	1136	49	822
17	57	1136	64	628
18	60	1112	81	435

different as shown in Table 1. Overall, the ranking of the top clones was quite different. The automated MTP system identified clone 5 as top clone, whereas this clone was not among the 18 top clones of the standard procedure. The top clone identified in the standard procedure (clone 8) was ranked on position 5 in the MTP-based system.

Differences in the ranking of top clones are most likely caused by the differences in the cultivation protocol, namely batch vs. fed-batch mode. The higher levels of product concentrations obtained for the clones identified by the MTP-based system (Table 1) also demonstrate the optimized cultivation conditions of the simplified culturing protocol. Differences in the seeding cell densities may have also affected the final outcome of clone selection. It was not possible to perform cell counts in the initial phase in static 384-well and 96-well MTPs because the available sample volume was not sufficient. Therefore, a constant dilution ratio was applied during the transfer of the clones resulting in undefined seeding density of the evaluated 96 clones. The manual standard procedure continued with these undefined seeding densities throughout the pre-culture phase and only switched to controlled seeding densities for the fed-batch cultivation of the last 18 clones. The MTP-based cell culture system compensated for the effects of the undefined seeding densities in the used pre-cultures at least partly by daily measurement of VCD. Further advancement for future clone selection approaches may come from using the automated system in the static, early screening phase to establish cell density measurement and defined seeding density from the beginning of the screening process.

These factors may have also contributed to the fact that clone 5 was not identified by the standard selection procedure. Backtracking clone 5 in the standard process revealed that the clone was lost at the 24-well stage and not transferred to the first six-well stage. Up to the 24-well stage, the transfer was performed with a constant dilution rate that favors faster growing clones and selection is only based on titer measurements. Clones with a lower growth rate keep starting at lower seeding densities, despite of a high productivity. Multiple repetitions of this constant dilution splitting reinforced this effect. This situation most probably occurred for clone 5 and explained why it was lost within the standard process. Thus, this standard procedure tended to miss slower growing clones, although their cell-specific productivity might have been appropriate or even superior. Using the

automated system also for the first steps of the selection process would solve the problem.

Clones 5 and 8 were reevaluated in six-well plates under conditions equivalent to shaker cultivations and the ranking obtained by the new automated process was confirmed. This highlights the risk of missing a suitable clone at early stages by using the standard procedure. Clone stability is another crucial and well-known criterion for successful clone selection. In this context, it is worth mentioning that the new system is also suitable to test the stability of clones in long-term culture in order to identify instability as early as possible. Clone 5 was confirmed to be stable for at least 56 days by comparison of the re-evaluation experiments and the initial fed-batch screening.

The comparison of top clone selection indicates that the biggest potential of the automated MTP-based cell culture system for this application lies in early fed-batch cultivation making multiple seeding and pre-culturing steps unnecessary while increasing overall efficiency. Differences in growth and production characteristics or in product quality resulting from different cell behavior under batch and fed-batch conditions can be largely excluded to support predictability of early screening results for later development and production processes.¹⁰

A further advantage is related to culture capacity. In our study, the automated MTP-based cell culture system evaluated 96 clones in biological duplicates (192 samples) in a fed-batch process. This was a more than fivefold increase as compared to the 18 clones finally evaluated by the standard procedure without replicates. The MTP-based system is able to handle a total of 288 clones (in biological duplicates) when using 24-well plates and full capacity. A significant further capacity increase would come from the introduction of the 96-well MTP format for cultivation (1152 clones in biological duplicates). Thus, the new system provides significant improvement for early stage high-throughput fed-batch cultivation in the clone selection process. Clones can be cultivated in triplicates or even higher replicate numbers to increase reproducibility and improve reliability of evaluation and selection.

The overall clone selection process was also significantly accelerated from 6 weeks to 4 weeks by using the MTP-based system. The resulting cell age is lower compared with the standard process offering the advantage of increased flexibility in later production regarding the cell age. Further time and cost savings can be achieved by collecting detailed data during the clone selection process that can be used for process optimization in late stage. Also, convenience and robustness of

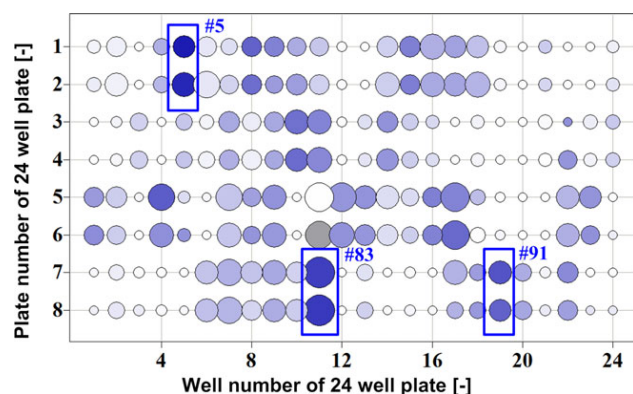


Figure 3. Comprehensive depiction of the screening results obtained by the automated MTP-based cell culture system for the total of 96 clones studied in biological duplicates. Product concentration and maximal VCD are correlated to color intensity and spot size, respectively. Some clones showed no growth and product formation most probably due to insufficient seeding density (see explanation of undefined seeding density in the text).

clone selection are considerably increased, as the complete process is free of manual processing.

The use of the automated MTP-based system enabled the selection of the most suitable clone, which was not identified by the standard procedure. In addition, the new system provides increased information density that helps to increase clone selection quality based on process requirements. These results clearly demonstrate the importance of early stage fed-batch cultivation for clone selection and indicate that the application of the new system greatly improves the probability to find the top clone.

Performance of the automated MTP-based system for clone screening and selection

Figure 3 shows product concentration (intensity of color) as well as maximum VCD (symbol size) for all 96 clones in biological duplicates. Clones 5, 83, and 91 appeared to be the most promising ones regarding cell-specific productivity as calculated from product concentration and VCD. The results also show satisfactory reproducibility of the biological replicates for the majority of the clones investigated.

The time courses of VCD and product accumulation identified clone 5 as top clone with the highest cell-specific productivity of all clones ($qP_{\max}$ 35 pg/[cell*day]) (Figure 4). For this clone, the overall highest productivity in combination with a moderate cell growth was observed. Moderate cell growth is also favorable for the harvest procedure and further purification regarding the removal of cell debris, host cell proteins, and DNA. In contrast, clone 83 showed the highest VCD with lower product concentration than clone 5. Clone 91 showed a cell density similar to clone 5 but with a lower product concentration. The time courses of product concentration and cell growth are very helpful tools to identify stagnation, plateau phases or continuous increase and offer improved characterization of the product accumulation kinetics as compared to end-point determination. The additional information can also be used to optimize the cultivation conditions (e.g., feed amount and timing). The new system facilitates an estimation regarding the performance of clones in the used fed-batch process which in many cases will be a platform process.

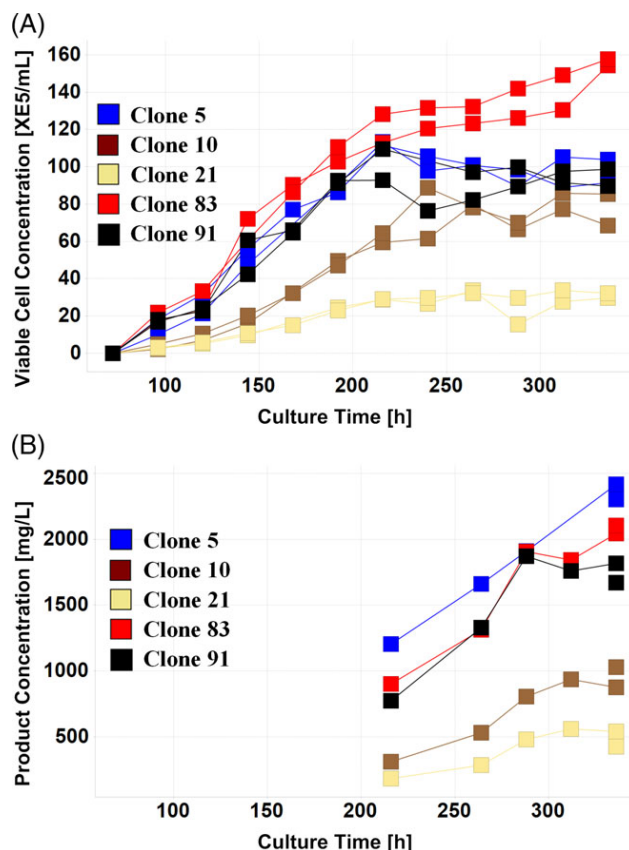


Figure 4. In-process monitoring of (A) VCD and (B) product concentration displayed for selected clones representative for high, moderate, and low cell growth and productivity.

In-process analytics for advanced metabolic clone characterization

Besides productivity and growth characteristics, metabolic behavior may be essential for successful clone selection and often serves as secondary selection criteria. The MTP-based system has the capability for in-process monitoring of various metabolites during the whole cultivation process. The time courses of glucose and lactate concentrations represent essential standard parameters for cell growth, product accumulation, and quality.^{10,11} Glucose monitoring is advantageous to detect and prevent limitations of cell growth, while the lactate profile of a clone is primarily important for later process development. The daily in-process control of the glucose and lactate levels performed for all 96 clones provided useful information for the selection process. The three top clones 5, 83, and 91 displayed acceptable and reliable profiles for both metabolites as shown in Figure 5. Moderate glucose levels, for example, are indicative for clones that fit well into the fed-batch process platform. In contrast, steep glucose increases resulting in high osmolality are generally unfavorable for cell growth, whereas steep decreases indicate special nutritional requirements and support clone exclusion. Similarly, the lactate profiles of the three clones were adequate showing a moderate increase and reaching a plateau. A successive decrease indicative for lactate re-metabolization is desirable, whereas steep and continuous increases in lactate might negatively affect product accumulation.^{15,16} In contrast to clones 5, 83, and 91, double measurement for clone 8 and clone 52 appeared

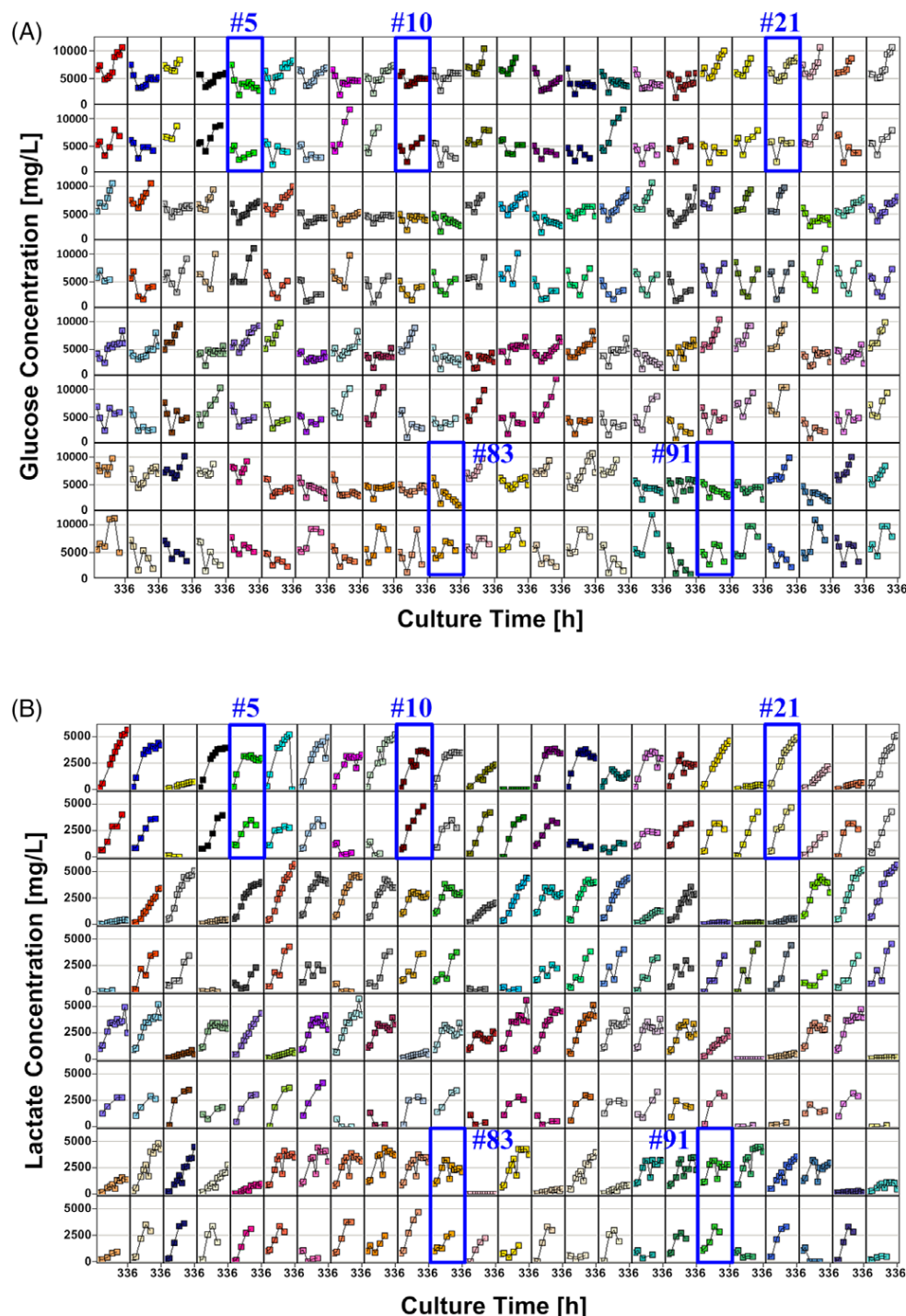


Figure 5. (A) In-process monitoring of glucose concentration as important secondary parameter for clone evaluation displayed for the total of 96 clones studied. Biological duplicates are given in same colors. The selected clones shown in Figure 4 are highlighted. (B) In-process monitoring of lactate concentration as important secondary parameter for clone evaluation displayed for the total of 96 clones studied. Biological duplicates are given in same colors. The selected clones shown in Figure 4 are highlighted.

less consistent making these clones less suitable for selection. The real-time metabolite monitoring allowed more detailed clone evaluation in order to confirm the selection based on the major criteria product yield. The detection of inappropriate metabolic conditions would warrant the exclusion of a clone at this step. In this way, the automated MTP-based system significantly supports robustness and reliability of the clone selection process and increases the probability to identify the

top clone that fits as well as possible to the process. The modular setup of the new system makes it highly flexible and allows for the integration of further analytical units to extend real-time measurement to additional parameters, such as glutamine, glutamate, or ammonium. The new process supports the principle that an increased data density in the early phase of clone selection will lead to overall reduced development effort and robust processes.

Interface for further early stage high-throughput analytics 1: glycosylation analysis

The second focus of the present study was to investigate the potential benefits of coupling further HTS analytics to the automated MTP-based system. The system was interfaced with external analytics to generate quality attribute data already during early stage clone selection. The *N*-glycosylation analysis is of particular interest as therapeutic mAbs may display a high degree of heterogeneity in their glycosylation pattern.^{17,18} The glycosylation pattern depends on cell culture and media conditions and the glycosylation pattern was shown to differ between batch and fed-batch cultivation but not during process scale-up.^{8,10,11} Glycosylation is known to have a strong impact on the Fc (constant, crystallizable fragment) effector functions affecting efficacy and safety as well as pharmacokinetics and pharmacodynamics.^{19–22} Therefore, the glycosylation profile of a mAb is a further important parameter of product quality and product performance.^{8,18,23} This confirms the strategic value of quality data for the clone selection process.

Samples taken by the automated MTP-based system from all clones were analyzed for *N*-glycosylation (Figure 6).¹⁴ It was crucial for the project presented in this study to determine the fraction of high mannose species as early as possible. Clone 5 could be confirmed as the most suitable clone for further process development because of an acceptable and reproducible ratio of the relevant glycan species. The big advantage over the standard process is the earlier availability of product quality data (e.g., information on glycosylation) which can be generated for a significant larger number of clones.

Interface for further early stage high-throughput analytics 2: RT-qPCR expression analysis

Apart from product assessment at the protein level, mRNA expression levels may serve as an additional parameter to

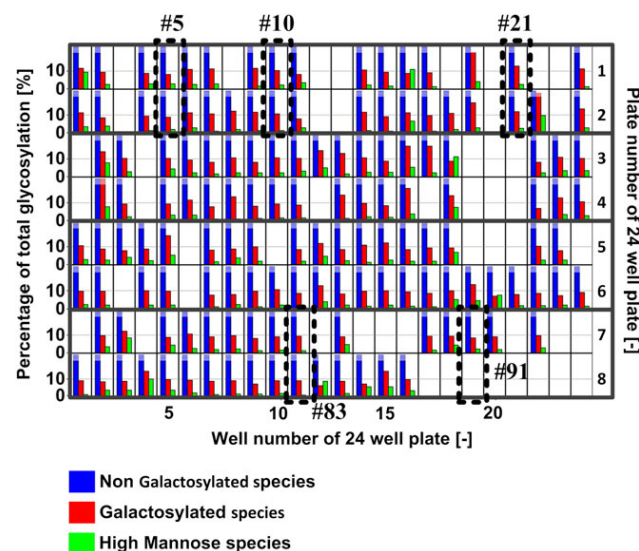


Figure 6. Results of *N*-glycosylation analysis of mAb product displayed for the total of 96 clones investigated. Samples produced by the automated MTP-based cell culture system were subjected to linked external glycopeptide analysis. For some clones, no results were available due to limited mAb amount (low product concentration). The selected clones shown in Figure 4 are highlighted.

evaluate product formation. High-throughput RT-qPCR analysis can provide further useful data to assess the productivity of a large set of clones at a very early time point. The automated MTP-based cell culture system was linked to gene expression analysis by performing RT-qPCR analysis of cell culture samples using the LightCycler[®] instrument (Roche Diagnostics GmbH, Penzberg) technology in combination with RealTime ready (Roche Diagnostics GmbH, Penzberg) assays. The sample preparation for RT-qPCR analysis was done directly on the MTP-system, making full use of its flexibility. DNA amplification could be executed by an integrated LightCycler[®] instrument by extending the system configuration. The results showed that top clone 5 displayed the highest mRNA expression level for the HC of the examined mAb (Figure 7) which correlated with the highest specific productivity recorded at the protein level. The studies on 37 clones also revealed that their cell-specific productivity correlated with the gene expression level of the HC of the mAb, whereas a correlation with the expression of the LC was not observed in our study (Figure 7). With these results, we successfully demonstrated the connection of high-throughput fed-batch cultivation with high-throughput RT-qPCR analysis for the first time.

RT-qPCR analysis may also be beneficial to monitor the expression of stress markers like chaperones and factors related to endoplasmic-reticulum stress potentially correlating with product concentration or epigenetic expression effects.^{24–26} Moreover, RT-qPCR may be applied to monitor

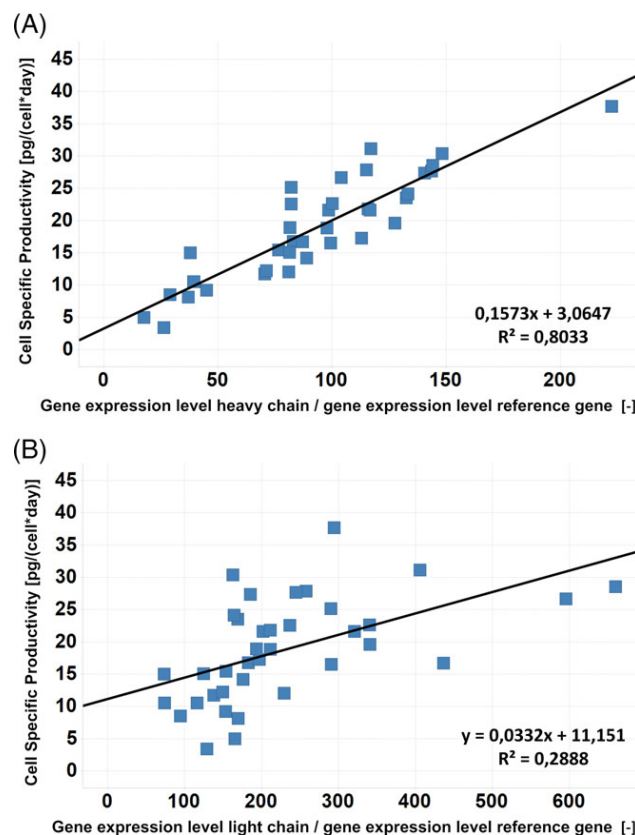


Figure 7. Regression analysis performed on expression levels of (A) HC and (B) LC of the mAb product obtained by RT-qPCR and on the cell-specific productivity of the respective clones. In total, 37 clones were included in this study. Resulting correlation coefficients (R^2) indicate a correlation of the cell-specific productivity with the expression level of the HC, but not with the expression level of the LC of the mAb.

the expression of potential marker genes recently identified to correlate with stable expression of recombinant proteins in CHO clones.²⁷ In a similar way, qPCR may be applied to improve prediction of clone stability attributes related to promoter methylation or transgene copy number.^{28–30} In general, clones with a high product expression level resulting from low gene copy numbers are more preferred for process development than clones with high gene copy numbers because the latter usually display unfavorable stability. The establishment of these useful RT-qPCR or qPCR assays was beyond the scope of the present study and is presented as an outlook.

In summary, we showed the feasibility of connecting the automated MTP-based cell culture system to external analytical systems, such as automated gene expression analysis and glycan analysis. The use of these technologies at an early stage of clone screening provides further important information for reliable clone selection in order to minimize the risks and required effort in later process development. This approach also suggests that the new MTP-based system opens up almost unlimited possibilities to implement and establish new clone evaluation criteria in order to advance the clone selection to the next level.³¹

Conclusions

Using the MTP-based cell culture system for clone screening and selection offers substantial potential for improvement and acceleration: the new system provides a fully automated process and enables fed-batch cultivation at an early stage which is of high importance for successful clone selection. It considerably increases the number of clones that can be evaluated and speeds up the processing. The potential to integrate real-time monitoring of metabolites and other secondary selection criteria crucial for productivity and product quality further supports reliable clone selection. Moreover, the MTP-based system establishes an interface for advanced HT analytics to identify additional parameters for clone evaluation. Thus, we demonstrated the technical feasibility to couple the MTP-based cell culture system with glycosylation analysis as well as the LightCycler[®] technology to perform automated RT-qPCR gene expression analysis for a large number of cell culture samples. This particular advancement combined with the high flexibility of the system opens up future perspectives for optimizing the selection process at an early stage, for example, by using multiple selection criteria that are especially tailored for each product. The acceptability of the quality profile of a molecule is driven by the quality target profile which is based on indication, mode of action, target dose and patient population, among others and will vary very significantly between projects. The new system appears most suitable to enhance efficiency and robustness of the clone screening procedure as well as the quality of the selected clones.

Acknowledgments

The authors would like to thank the Roche Pharma Technical Development nonGMP lab and the genetic characterization group, our colleagues from Roche Pharma Research and Early Development (Klaus Neff, Silke Hansen), the Roche Pharma analytical department (Markus Habegger), and our colleagues from Roche Diagnostics division for the RT-qPCR support (Olaf Kaiser, Martin Wiesinger). In addition, we would like to

thank Heidemarie Peuker (BISs—Biomedical Investigation Services) for diligent preparation of the manuscript.

Literature Cited

- Markert S, Joeris K. Establishment of a fully automated microtiter plate-based system for suspension cell culture and its application for enhanced process optimization. *Biotechnol Bioeng*. 2007; 114(1):113–121.
- Chen A, Chitta R, Chang D, Amanullah A. Twenty-four well plate miniature bioreactor system as a scale-down model for cell culture process development. *Biotechnol Bioeng*. 2009;102:148–160.
- Kelley B. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. *MAbs*. 2009;1:443–452.
- Browne SM, Al-Rubeai M. Selection methods for high-producing mammalian cell lines. *Trends Biotechnol*. 2007;25:425–432.
- Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. *Appl Microbiol Biotechnol*. 2016;100:3451–3461.
- Wlaschin KF, Hu WS. Fedbatch culture and dynamic nutrient feeding. *Adv Biochem Eng Biotechnol*. 2006;101:43–74.
- Templeton N, Dean J, Reddy P, Young JD. Peak antibody production is associated with increased oxidative metabolism in an industrially relevant fed-batch CHO cell culture. *Biotechnol Bioeng*. 2013;110:2013–2024.
- van Berkel PH, Gerritsen J, Perdok G, Valbjørn J, Vink T, van de Winkel JG, Parren PW. N-linked glycosylation is an important parameter for optimal selection of cell lines producing biopharmaceutical human IgG. *Biotechnol Prog*. 2009;25:244–251.
- Hegde S, Pant T, Pradhan K, Badiger M, Gadgil M. Controlled release of nutrients to mammalian cells cultured in shake flasks. *Biotechnol Prog*. 2012;28:188–195.
- Fan Y, Jimenez Del Val I, Müller C, Wagtberg Sen J, Rasmussen SK, Kontoravdi C, Weilguny D, Andersen MR. Amino acid and glucose metabolism in fed-batch CHO cell culture affects antibody production and glycosylation. *Biotechnol Bioeng*. 2015a;112:521–535.
- Fan Y, Jimenez Del Val I, Müller C, Lund AM, Sen JW, Rasmussen SK, Kontoravdi C, Baycin-Hizal D, Betenbaugh MJ, Weilguny D, Andersen MR. A multi-pronged investigation into the effect of glucose starvation and culture duration on fed-batch CHO cell culture. *Biotechnol Bioeng*. 2015b;112:2172–2184.
- Hemmerich J, Adelantado N, Barrigón JM, Ponte X, Hörmann A, Ferrer P, Kensy F, Valero F. Comprehensive clone screening and evaluation of fed-batch strategies in a microbioreactor and lab scale stirred tank bioreactor system: application on *Pichia pastoris* producing *Rhizopus oryzae* lipase. *Microb Cell Fact*. 2014;13:36.
- Clarke C, Doolan P, Barron N, Meleady P, O'Sullivan F, Gammell P, Melville M, Leonard M, Clynes M. Predicting cell-specific productivity from CHO gene expression. *J Biotechnol*. 2009;151:159–165.
- Reusch D, Habegger M, Selman MH, Bulau P, Deelder AM, Wührer M, Engler N. High-throughput work flow for IgG fc-glycosylation analysis of biotechnological samples. *Anal Biochem*. 2013;432:82–89.
- Lao MS, Toth D. Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. *Biotechnol Prog*. 1997;13:688–691.
- Li J, Wong CL, Vijayasankaran N, Hudson T, Amanullah A. Feeding lactate for CHO cell culture processes: impact on culture metabolism and performance. *Biotechnol Bioeng*. 2012;109:1173–1186.
- Beck A, Wagner-Rousset E, Bussat MC, Lokteff M, Klinguer-Hamour C, Haeuw JF, Goetsch L, Wurch T, Van Dorselaer A, Corvaia N. Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and fc-fusion proteins. *Curr Pharm Biotechnol*. 2008;9:482–501.
- Ling N, Zhang P, Song Z, Bardor M. The sweet tooth of biopharmaceuticals: importance of recombinant protein glycosylation analysis. *Biotechnol J*. 2012;7:1462–1472.
- Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends Pharmacol Sci*. 2006;30:356–362.
- Abès R, Teillaud JL. Impact of glycosylation on effector functions of therapeutic IgG. *Pharmaceuticals (Basel)*. 2010;3:146–157.

21. Liu L. Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and fusion proteins. *J Pharm Sci.* 2015;104:1866–1884.
22. Quast I, Peschke B, Lünemann JD. Regulation of antibody effector functions through IgG fc N-glycosylation. *Cell Mol Life Sci.* 2017;74(5):837–847.
23. Alt N, Zhang TY, Motchnik P, Taticek R, Quarmby V, Schlothauer T, Beck H, Emrich T, Harris RJ. Determination of critical quality attributes for monoclonal antibodies using quality by design principles. *Biologicals.* 2016;44:291–305.
24. Kober L, Zehe C, Bode J. Development of a novel ER stress based selection system for the isolation of highly productive clones. *Biotechnol Bioeng.* 2012;109:2599–2611.
25. Prasad K, Mehra S. Dynamics of unfolded protein response in recombinant CHO cells. *Cytotechnology.* 2015;67:237–254.
26. Krebs LE, Bowden DM, Bray CM, Shaw MM, Frye CC. Effective and efficient characterization of Chinese hamster ovary production cell lines using automated intracellular staining and statistical modeling. *Biotechnol Prog.* 2018;34(3):570–583.
27. Jamnikar U, Nikolic P, Belic A, Blas M, Gaser D, Francky A, Laux H, Blejec A, Baebler S, Gruden K. Transcriptome study and identification of potential marker genes related to the stable expression of recombinant proteins in CHO clones. *BMC Biotechnol.* 2015;15:98.
28. Yang Y, Mariati, Chusainow J, Yap MG. DNA methylation contributes to loss in productivity of monoclonal antibody-producing CHO cell lines. *J Biotechnol.* 2010;147:180–185.
29. Osterlehner A, Simmeth S, Göpfert U. Promoter methylation and transgene copy numbers predict unstable protein production in recombinant Chinese hamster ovary cell lines. *Biotechnol Bioeng.* 2011;108:2670–2681.
30. Moritz B, Becker PB, Göpfert U. CMV promoter mutants with a reduced propensity to productivity loss in CHO cells. *Sci Rep.* 2015;5:16952.
31. Traylor MJ, Tchoudakova AV, Lundquist AM, Gill JE, Boldog FL, Tangarone BS. Comprehensive discovery and quantitation of protein heterogeneity via LC-MS/MS peptide mapping for clone selection of a therapeutic protein. *Anal Chem.* 2016;88:9309–9317.

Manuscript received Aug. 28, 2018, and revision received Nov. 28, 2018.

RESEARCH ARTICLE

A novel scale-down mimic of perfusion cell culture using sedimentation in an automated microbioreactor (SAM)

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Abstract

Continuous upstream processing in mammalian cell culture for recombinant protein production holds promise to increase product yield and quality. To facilitate the design and optimization of large-scale perfusion cultures, suitable scale-down mimics are needed which allow high-throughput experiments to be performed with minimal raw material requirements. Automated microbioreactors are available that mimic batch and fed-batch processes effectively but these have not yet been adapted for perfusion cell culture. This article describes how an automated microbioreactor system (ambr15) can be used to scale-down perfusion cell cultures using cell sedimentation as the method for cell retention. The approach accurately predicts the viable cell concentration, in the range of about 1×10^7 cells/mL for a human cell line, and cell viability of larger scale cultures using a hollow fiber based cell retention system. While it was found to underpredict cell line productivity, the method accurately predicts product quality attributes, including glycosylation profiles, from cultures performed in bioreactors with working volumes between 1 L and 1,000 L. The spent media exchange method using the ambr15 was found to predict the influence of different media formulations on large-scale perfusion cultures in contrast to batch and chemostat experiments performed in the microbioreactor system. The described experimental setup in the microbioreactor allowed an 80-fold reduction in cell culture media requirements, half the daily operator time, which can translate into a cost reduction of approximately 2.5-fold compared to a similar experimental setup at bench scale.

KEYWORDS

cell culture, high throughput, microbioreactor, perfusion, scale-down

1 | INTRODUCTION

Continuous processing has regained popularity in both upstream and downstream applications due to technological developments and a greater focus by the biopharmaceutical industry on product quality as well as productivity.¹ Continuous upstream processing allows higher maximum cell densities and viabilities to be achieved and can lead to processes that are more productive. Continuous perfusion cultivation

can deliver consistently high quality product because cells are sustained within in their optimum environment for growth.^{2,3} While technological innovations are still required to allow continuous downstream processing, continuous upstream applications are already well developed.^{4,5} The production of recombinant proteins using hybridoma cell lines grown in perfusion systems was relatively common before the late 1990s. The technology was ideal for the production of unstable proteins such as blood factors that would be degraded if

exposed to long bioreactor residence times. The method, however, decreased in popularity as cell culture scientists became familiar with high-yielding fed-batch CHO (Chinese hamster ovary) cells processes for products with sufficient stability and limited requirements for posttranslational modifications and other complex glycosylation. The broader application of perfusion cell culture was limited due to the lack of small-scale models that would allow multivariate experiments to be performed, too high failure rates associated with unsophisticated process equipment and the difficulties of scaling-up cell retention devices.⁶

Cell retention device technology has improved in recent years. The alternating flow filtration system (ATF) (Repligen, MA) and CARR Centritech separation system (Pneumatic scale angelus, OH) have become established as the most commonly used methods.⁷⁻⁹ These new cell retention devices in combination with other technologies like single-use processing or advanced process control by PAT technology also decreased the technical complexity and lowered the associated failure rate.¹⁰ The lack of appropriate scale-down systems is addressed within this work.

The ability of cell retention devices to retain cells within a bioreactor can be quantified by the separation efficiency. Spin-filter, hollow fiber, cross-flow devices including alternating flow filtration cell retention systems can retain 100% of all the cells within the bioreactor. Centrifugal methods have been found to retain 95–100%^{9,11,12} while laboratory-scale experiments with acoustic cell separation yields 90–98% cell retention.¹³⁻¹⁵ The separation efficiency of cell retention devices based on gravity like external settlers or sedimentation inside the culture vessel as presented in this work can be a function of the perfusion rate and reactor volume. A larger reactor volume, a higher perfusion rate, and potentially higher cell density may yield lower separation efficiency since the flowrate inside the cell retention devices increases and in turn impairs cell separation.¹⁶

1.1 | Perfusion scale-down models

Reliable scale-down models allow efficient and fast bioprocess development because they allow multiple experiments to be run in parallel and can be automated to increase throughput. Cell culture media optimization, process optimization and the screening of clones are fast and inexpensive to perform in high-throughput models when compared to the process-scale, which could be a bioreactor of several cubic meters in volume. To have reliable results for process and cell line development, those activities should be ideally performed under conditions that are representative of the production-scale.^{17,18} Development at final production scale is usually not economic due to a high resource demand.

Cell retention devices for perfusion operation have thus far proved difficult to miniaturize and can have significant dead-volumes. This means that scale-down models of perfusion bioreactor systems have typically required greater working volumes than those used for batch and fed-batch cultures. Most early stage studies of perfusion cultures rely on laboratory-scale bioreactors ranging from 0.5 to 2 L.^{19,20} These have a limited throughput, high media consumption,

and a high labor requirement. This makes clone screening or DoE (design of experiment) studies cumbersome to perform.

Spinner flasks with 100 mL working volume have also been used in a semicontinuous mode to create pseudo-steady states and study the effect of temperature shifts on tPA (tissue plasminogen activator) producing CHO cells. The method successfully predicted the increase in t-PA production at lower temperatures though the authors expressed the need to exercise caution when interpreting the results from such a system to avoid erroneous conclusions regarding actual productivity and growth characteristics.²¹

To mimic perfusion cultivation at an even smaller scale, shake flasks with a 30 mL working volume have been used to simulate perfusion by centrifuging the suspension every day of the culture and resuspending the cells with fresh media. Although useful as an indicative screening tool, this mimic has failed to reproduce media optimization results obtained in 4 L lab scale bioreactor, most likely because of the lack of pH and DO control in shake flasks.²² Spin tubes, also with daily manual centrifugation and spent media replacement, have also been popular approaches to mimic perfusion bioreactors. It has been shown that cell growth, peak viable cell density as well as titer are lower in this approach than in standard bench-scale bioreactors. In terms of product quality, glycosylation was comparable but other attributes like deamidation and C-terminal lysine clipping were found to be lower.²³ Another study using this application of spin-tubes showed a similar growth profile in spin-tubes and a steady-state perfusion bioreactor but higher lactate levels were observed. Charge-variants of the produced antibody were comparable but differences in glycosylation were noted.²⁴ Both studies suggest a lack of pH, DO, and pCO₂ control in combination with discontinuous daily media exchanges as the cause of the observed differences.

The advanced microbioreactor system (ambr[®]) is widely accepted as an effective scale-down model of large-scale bioreactors but has thus far been used primarily to mimic batch, fed-batch and chemostat cultures.²⁵⁻²⁸ The ambr15 system allows reasonable throughput (24–48 parallel vessels) and low liquid handling volumes, high automation, and little need for manual user interventions. It has been used to perform DoE (Design of Experiments) studies in fed-batch mode,²⁹ to compare a fed-batch CHO process performed in an ambr system to bench-top bioreactor and shake flasks²⁶ and to successfully predict the performance of 3, 15, and 200 L³⁰ as well as 15,000 L manufacturing scale³¹ cultures. These studies show that a good alignment between the ambr system and larger scale bioreactors can be achieved in a fed batch mode with respect to cell growth, productivity, and product quality even though some physical characteristics of the ambr system differ from production-scale equipment.²⁷ The system is scalable and outperforms other small scale systems in regard to controlling culture pH and DO thus displaying a good starting point for the development of a perfusion small-scale system.

To mimic perfusion cultures in the ambr systems, a semi-continuous chemostat mimic was developed for media screening experiments.^{28,32,33} A chemostat-based approach allows certain culture parameters such as minimum cell specific perfusion rate to be determined. However, there is very little evidence that chemostat

cultures can predict the effect on product quality of media optimization or other process development activities. Continuous operation of the ambr system with cell retention has, to date, only been possible with cells grown on microcarriers.³⁴ The use of the ambr system for the perfusion culture of cells growing in suspension has not been described so far.

In this article, we describe how the ambr 15 microbioreactor system can be used as a novel scale-down mimic of perfusion cell cultures in stirred tank vessels. A novel cell retention principle is established in the ambr system where cells are retained by sedimentation in the absolute absence of agitation. This sedimentation in an automated micro bioreactor approach (SAM) presents a mimic to fully continuous perfusion cultures that retain cells via an external cell retention device.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

2.1.1 | CHO cell cultures

CHO DG44 cells were cultivated with FortiCHO media (Gibco) supplemented with 1× Glutamax (Gibco), 1× insulin (Gibco) at 37°C and 8% CO₂ in shake flasks at 150 rpm with an orbital throw of 25 mm. The cell concentration was adjusted to $3\text{--}4 \times 10^5$ viable cells per mL every 2–3 days. Antifoam (Sigma) was used as required for bioreactor cultivations.

2.1.2 | mAbExpress cell culture

GlycoExpress® (GEX®) (Glycotope, Germany) cell lines, which have been used in this study, have been designed and glyco-engineered for the production and screening of glycoproteins in order to achieve fully human glycoproteins and to optimize protein properties. Cell lines of the glyco-engineered proprietary human cell line portfolio mAbExpress were cultivated in the proprietary GTM cell culture medium from Glycotope. In total three cell lines were used (mAbExpress cell line A expressing mAb 1 and B expressing mAb 2 are optimized for low mAb fucosylation, whereas mAbExpress cell line C expressing mAb 3 is optimized for high mAb fucosylation). The cells were expanded by subcultivation every two to three days in T-flasks (TPP, Switzerland) or spinner flasks (Integra Biosciences IBS, Cellspin, Switzerland) agitated at 60 rpm. These were placed in incubators (Integra Biosciences IBS, Biosafe plus, Switzerland or Thermo/Heraeus BBD 6220, Germany) at 37°C, 8% CO₂ atmosphere and 95% relative humidity. Typical cell densities in the logarithmic growth phase were 0.15 to 1.5×10^6 cells/mL.

2.1.3 | General perfusion cell culture parameters

All cell cultures were inoculated with 2.0×10^5 cells/mL. Continuous operation was initiated by feeding GTM medium at a perfusion rate of 0.5 V/d when glucose concentration dropped below 2.5 g/L and then

adjusted to cell concentration and glucose concentration. Maximum perfusion rate was 2 V/d unless otherwise indicated. When the maximum perfusion with GTM was achieved, the feed medium was replaced stepwise in 25% increments by a modified GTM medium (GTM 2x) with twice or increased concentration of key nutrients with adjusted osmolality. The pH was maintained at pH 7.0 unless otherwise indicated. The pH was adjusted by adding 0.5 M sodium hydroxide or by sparging carbon dioxide. The dissolved oxygen concentration was set to 40% and controlled by oxygen sparging. Temperature was set to 37°C. The cultures were terminated when the cell viability dropped under a certain threshold (e.g., 70%) or when the intended run time (e.g., 40 days) was reached. Specific power input was comparable for the 1 L system and the ambr15 system.

2.1.4 | Ambr perfusion cell cultures

Small-scale perfusion cell cultures were performed in the ambr® 15 system (Sartorius Stedim Biotech, Germany). Spargeless microbioreactor vessels were used for the cultivation of GlycoExpress® (GEX®) cells. GEX® cultures were agitated at 830 rpm while the CHO cell line between 830 and 1,300 rpm depending on the dissolved oxygen concentration. The working volume of each microbioreactor vessel was 12 mL (10 mL minimum, 15 mL maximum). Daily samples of between 250 and 450 µL were taken to determine the cell concentration and measure metabolite concentrations. The pH sensors were calibrated every 3–4 days using offline measurements.

A cell sedimentation method was used to retain cells with the microbioreactor vessels during perfusion cell cultures. Ten minutes prior to agitation being switched off to allow settling to begin the pH control was stopped to prevent the addition of sodium hydroxide directly before stirring stopped. The DO control was stopped at the same time that stirring was stopped. Cells were allowed to settle for 30–45 min before 3 mL of harvest was removed using the ambr 15 liquid handler. The sampling depth of the ambr 15 system was configured to ensure removal of a cell-free harvest. Stirring was continued after the harvest sample was removed and the volume of the culture restored to 12 mL by the addition of fresh medium. DO and pH control was continued after the addition of the fresh medium. All steps were performed by the ambr 15 system automatically. The cell sedimentation and harvest sample removal take approximately 1 hour. For a perfusion rate of one reactor volume a day (Vr/d) four times 3 mL harvests are required every 6 hours. User interaction is only required for daily sampling, reloading of single-use tips, removal of harvest and the replenishment of feed media. The maximum perfusion rate that could be achieved was 2 Vr/d.

2.1.5 | 1 L ATF perfusion culture

1 L laboratory-scale cultures were performed in Sartorius Biostat B-DCU Quad 2 L bioreactors fitted with 3-blade segment impellers. Dissolved oxygen and pH were measured with InPro 6,800 and 405-DPAS-SC-K8S Mettler Toledo electrodes respectively (Mettler Toledo, Switzerland). An ATF2 cell retention device (Repligen) with a

60 cm PES membrane (0.2 µm pore size and 0.15 m² membrane area, Spectrum, USA) was used with a flow rate at the inlet of 0.9 L/min. Membranes were changed when clogging was indicated by a decrease in the product concentration in the permeate stream below that of the retentate. Maximum perfusion rate was two reactor volumes a day, as described above.

2.1.6 | Determination of sedimentation efficiency

Separation efficiency is used commonly to express the performance of cell retention in perfusion culture and calculated as follows:

$$SE = \frac{C_{\text{cells, reactor}} - C_{\text{cells, harvest}}}{C_{\text{cells, reactor}}}$$

A separation efficiency of 100% corresponds to no cell at all in the harvest while 0% means the harvest shows the same cell concentration as the cell suspension in the bioreactor.

2.2 | Analytical methods

2.2.1 | Cell concentration and viability

Cell concentration and viability were determined by Cedex HiRes (Roche, Switzerland) using the trypan blue exclusion principle. The sample volume was 300 µL and high cell density samples were diluted up to 1:10 with PBS.

2.2.2 | Glucose/lactate and glutamine/glutamate

Glucose/lactate and glutamine/glutamate were measured in 15–30 µL cell-free supernatant samples using a YSI2700 Select Biochemical Analyzer (Yellow Springs Instruments).

2.2.3 | IgG concentrations

IgG concentrations were determined by ELISA. Multisorp 96 well plates (Nunc, Denmark) were coated overnight with Mouse antihuman Igκ light chain. Blocking was performed by adding 200 µL per well of 5% BSA (Roth, Germany). A POD conjugated goat-anti human IgG Fc was used as secondary antibody. Staining was performed with TMB (Tebu-Bio Laboratories, Germany). The reaction was stopped using 2.5 N H₂SO₄ (Sigma, Germany). Color formation was measured using a Tecan Infinite F200 (Tecan Group Ltd., Switzerland) at 450/620 nm. Each sample was measured at three different dilutions and in duplicate. Alternatively, to ELISA IgG quantification, the Octet QKe system (Forte Bio) was used. IgG concentration was determined using protein tips provided by forte bio. Using a standard curve with known concentration (3.91–500 µg/mL, protein A HPLC determined) the concentration of the samples was quantified.

2.2.4 | Size exclusion chromatography

Low and high molecular weight species were measured using size exclusion chromatography (SEC). 250 µL in PBS diluted samples (50–200 µg) were separated on a Superdex 200 10/300GL column (GE Healthcare) installed in an Äkta prime system (GE Healthcare). PBS was used as running buffer with flowrate of 0.5 mL/min. Quantification was done by peak integration.

2.2.5 | SDS-page

SDS-PAGE was done in the Mini-Protean Tetra cell (Bio-Rad) using Mini-ProteanTGX precast gels (Bio-Rad). 0.5 to 3 µg sample amount was transferred into the wells Staining was done with Coomassie Brilliant Blue.

2.2.6 | Antigen ELISA

To check for correct antigen binding of the antibody, an antigen ELISA was performed. The antigen was coated on a Multisorp 96-wellplate (Nunc, Denmark) and blocked with BSA to saturate unspecific binding. Detection was done using a POD rabbit antihuman IgG Fc fragment or F(ab)₂ specific antibody. Staining, stopping of color formation and signal quantification is analogous to standard ELISA. A standard curve with known concentration was used to determine the concentration of the samples. Since the sample concentration is known, the concentration determined by the ELISA should be ±25% of the known concentration.

2.2.7 | FcγRIIIa alpha screen

To check for correct binding of the produced antibodies to the FcγRIIIa an Alpha Screen assay (Perkin Elmer) was used where Nickel donor beads are coupled to a His-tagged FcγRIIIa. When human IgG is present in the sample the human antibody displaces a rabbit IgG leading to a decreased signal. The decrease of the signal depends on the human antibody concentration. Samples and FcγRIIIa were diluted to the indicated concentration in AlphaLisa® Universal Buffer. Donor (AlphaScreen® Nickel Chelate Donor-Beads) and acceptor (AlphaScreen® Rabbit-anti-mouse Acceptor-Beads) beads were diluted in AlphaLisa® Universal Buffer. Chemoluminescence was measured using an EnSpire 2,300 Plate reader (PerkinElmer) with excitation of donor-beads at 680 nm and emission of acceptor-beads at 520–620 nm. Chemiluminescence signals were plotted against logarithmic concentration and fitted with sigmoidal dose-response variable slope regression. EC₅₀ values (concentration for half-maximal binding) for control and samples were calculated and the relative potency, defined as EC₅₀ of reference divided by EC₅₀ of sample was calculated. Relative potency must be in the range 0.8 to 1.2 for comparability.

2.2.8 | Peptide map fingerprinting (for determination of deamidation, C-terminal lysine clipping, oxidation)

Samples were dried in a SpeedVac evaporator (Eppendorf, Germany) and sample was digested with ProteoExtract® All-In-One Trypsin Digestion Kit following the manufacturer's protocol. Peptide digest was separated using UPLC chromatography (Acquity UPLC I-Class, Waters) with a Waters CSH C18 (1.7 μ m, 2.1 mm $\times$ 150 mm) column, water +0.033% trifluoroacetic acid as solvent A and acetonitrile 0.03% trifluoroacetic acid as solvent B. Detection was done via UV detection (Diode array detector 210 nm). ESI-Q-TOF mass spectrometry was used for determination and quantification of deamidation, C-terminal lysine clipping, and oxidation.

2.2.9 | Antibody-dependent cellular cytotoxicity assay

Target cells (DU145) were resuspended in europium buffer, placed on ice and then electroporated using amaxa nucleofector (Amaxa Biosystems, acquired by Lonza Group, Switzerland). Cells were washed six times. 5,000 to 10,000 cells were used per well. As effector cells Fc γ RIIIa (F-variant) stably, transfected KHYG cells were used. The ratio of effector to target cells (E:T ratio) was 80:1. Analysis was done in triplicate wells, control in six identical wells. Spontaneous release was tested without effector cells, maximal release with 1% Triton-X-100 without effector cells and basal release without target and effector cells. 96-well well plate was then placed in an incubator (37°C) for 5 hr. Supernatant was mixed with enhancement solution (PerkinElmer) and incubated. Fluorescence was measured using Tecan Infinite F200 (Tecan Group Ltd., Switzerland) at 340 nm excitation and emission at 610 nm (400 μ s delay). Specific lysis is plotted versus antibody concentration and fitted using a nonlinear 4-parameter logistic plot. EC50 and relative potency were calculated (analogous to Fc γ RIIIa Alpha Screen). Samples are regarded as comparably active if the relative potency is in the range of 0.5 to 2.0.

2.2.10 | N-glycan profiling

The GlycoPrep™ Instant AB™ Kit (Prozyme) was used for glycoprofiling. Briefly, intact N-glycans are released from the protein core by the action of PNGase. Free N-glycans are labeled with the fluorescence™ Instant AB™. The purified sample of N-glycans is separated by means of hydrophilic interaction chromatography (GlykoSep™N-Plus, Prozyme) with fluorometric detection on an HPLC 1200 series (Agilent). The assignment of N-glycans employs comparison of retention times with a standard chromatogram for which structure mapping is based on MALDI-TOF MS (MicroFlex, Bruker, Germany).

2.3 | DoE analysis

DoE studies were done with BioPAT® MODDE Version 10.1.1 (Umetrics AB, Sweden).

3 | RESULTS AND DISCUSSION

The efficiency of cell retention with the sedimentation technique described in this work can be adjusted by varying the time of sedimentation (Figure 1). This experiment was performed in the ambr system with typical process cell densities of 1×10^7 cells/mL and confirmed at lower cell densities (data not shown) but not verified at high or very high cell densities ($4\text{--}10 \times 10^7$ cells / mL). For optimal perfusion operation 40 min of sedimentation can be used to obtain an almost cell free harvest (99% separation efficiency) for both GEX® and a CHO cell line, which is comparable to other cell retention methods.^{9,11–15} Shortening the sedimentation time allows cell bleeding to occur and the control of the maximum cell density. For example, when using sedimentation time of 30 min corresponding to a separation efficiency of around 80%. This approach cannot be performed at larger scale bioreactors for comparison as the ratio of cell free supernatant volume to total bioreactor volume decreases with scale.

To study the effect of sedimentation on productivity and metabolite profiles, two sets of cultures were run using the ambr 15 scale-down mimic. Typical cell densities of 10^7 cell/mL were achieved in these cultures. A control culture was stirred continuously while vessels in which sedimentation was allowed to occur were discontinuously mixed with four breaks for 40 min of stirring without media replacement. Sampling volume was kept as low as possible and was identical for all cultures. Cell growth (Figure 2a) and viability (not shown) were unaffected by the sedimentation method. Figure 2c,e,f shows that glucose, glutamine and glutamate levels do not differ between the culture conditions with continuous stirring and sedimentation. However, differences in titer and lactate levels can be observed (Figure 2b,d). The cells with regular sedimentation show higher lactate levels than continuously stirred cells. When stirring and gassing is stopped to initiate sedimentation, oxygen transfer rapidly decreases leading to conditions that are potentially hypoxic. This effect is likely to be greater for accumulated cells at the

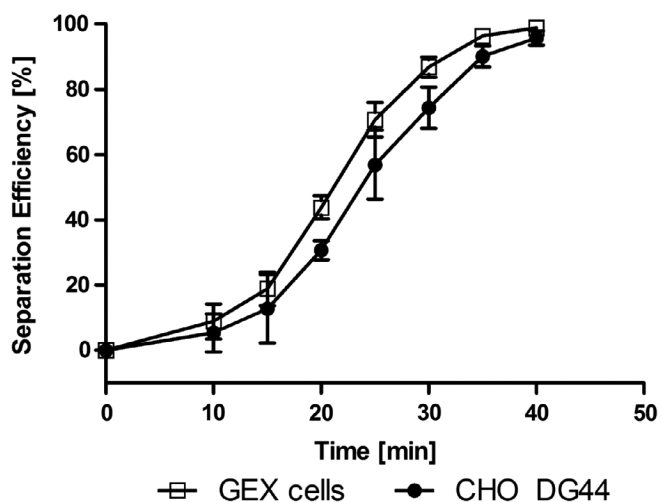


FIGURE 1 Sedimentation of GEX® mAbExpress cell line A and CHO DG44 cell line in the ambr microbioreactor system at a sample height corresponding to 9 mL volume (working volume = 12 mL) (error bars are one standard deviation, $n = 3$)

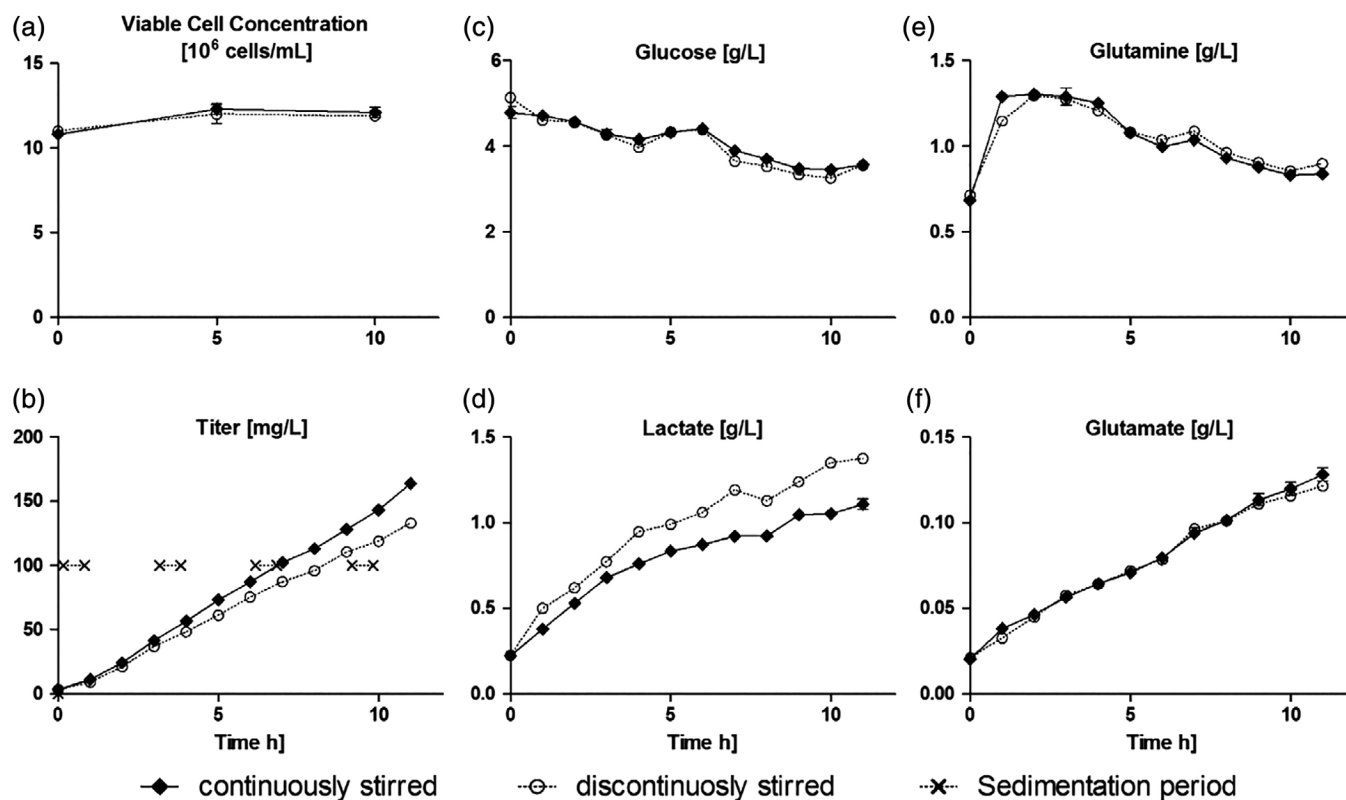


FIGURE 2 Comparison of metabolites from GEX® mAbExpress cell line A expressing monoclonal antibody 1 grown in the ambr 15 microbioreactor with continuous stirring and discontinuous stirring allowing cell sedimentation. (a) viable cell concentration, (b) product titer, (c) glucose, (d) lactate, (e) glutamine, and (f) glutamate (error bars are standard deviation, $n = 3$). In Panel (b), lines mark the sedimentation times

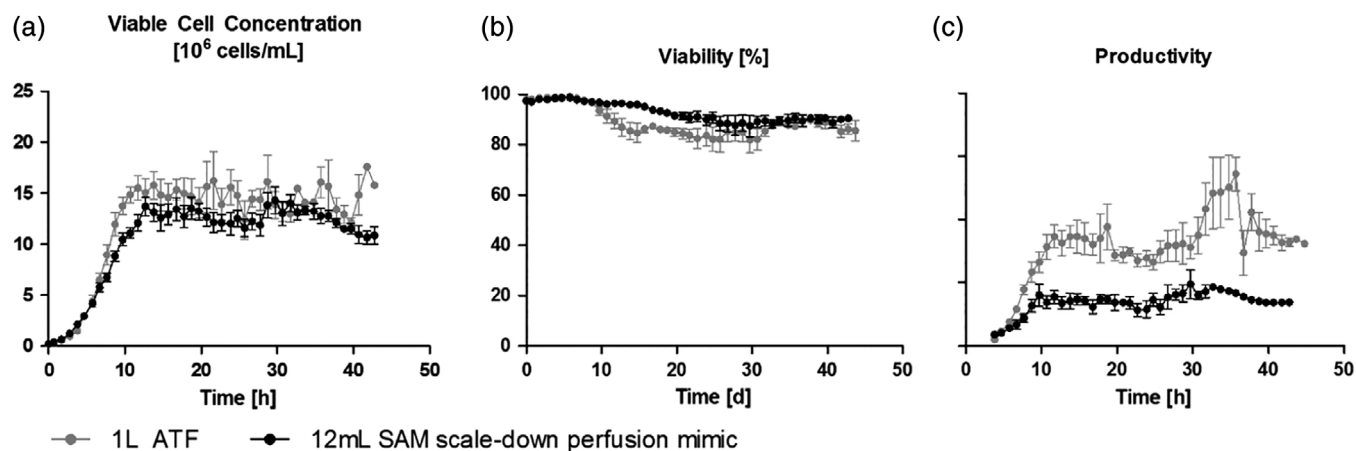


FIGURE 3 A comparison of the 12 mL SAM scale-down perfusion process mimic with the standard 1 L Biostat B-DCU with ATF over 45 days for GEX® mAbExpress cell line A expressing monoclonal antibody 1. Process parameters were (a) viable cell concentration, (b) cell viability, and (c) volumetric productivity (e.g., in g/L d). (error bars are SEM)

bottom of the vessel where the local cell concentration is much higher than that under well mixed conditions. Cells rely more on anaerobic energy production under hypoxic conditions and convert glucose to lactate³⁵ meanwhile reducing protein production.³⁶ Increased lactate levels were also found in a study using spin tubes with daily centrifugation as perfusion mimic, probably due to a lack of pH control and discontinuous media exchange.²⁴ During the sedimentation of the here described SAM method pH as well as DO is also not controlled.

Thus hypoxic conditions during cell settling may result in increased lactate production and decreased cellular productivity. The effect of product quality (e.g., glycosylation) was not studied for this data set, but was investigated in the following experiments.

Figure 3a–c shows a comparison of the 12 mL SAM scale-down perfusion process mimic with 1 L cultures performed in the Biostat B-DCU with ATF over 45 days. This 1 L setup is the current standard perfusion approach for process development used at Glycotope and is

the benchmark for comparison. An average of eight SAM perfusion runs was compared to seven 1 L runs. The Glycotope mAbExpress cell line A expressing monoclonal antibody 1 was used for these experiments. There was good agreement between the SAM perfusion mimic and the 1 L benchmark system with respect to viable cell concentration and cell viability and highlights the ease of scale-up between the ambr 15 operated with the SAM method and Biostat B-DCU operated with an ATF cell retention device. Viable cell concentration and cell viability do not appear to be affected by the sedimentation process, during which the agitation and the pH and DO controls are stopped. Indeed Figure 3b shows that the cell viability is actually higher than was achieved in the 1 L biostat B-DCU with ATF which is surprising as lactate levels, as shown in Figure 2c, are higher with the settling approach in the ambr. This is also the case for the experiment shown in Figure 3 (data not shown). Higher shear rates of the hollow fiber cell retention device or different gassing strategies may explain lower viability in the 1 L scale. Figure 3c shows that the productivity from the SAM mimic is lower than that of the 1 L benchmark system. Analysis of metabolites from cells grown in the SAM mimic have shown glucose, glutamine and glutamate concentrations are unaffected by discontinuing stirring but that lactate levels increase due to a mild hypoxia effect causing anaerobic respiration and a decrease in recombinant protein production.

Table 1 shows that general product quality characteristics produced at the 1 L-scale are predicted by the SAM perfusion mimic. The

TABLE 1 A comparison of product characteristics of monoclonal antibody 1 (expressed in GEX[®] mAbExpress cell line A) produced in the 12 mL SAM scale-down perfusion process mimic with the 1 L Biostat B-DCU with ATF corresponding to the reference

Assay/parameter	Assay results
SEC	Fully comparable to reference Multimer/dimer/fragment $\leq 1\%$
SDS-PAGE	Fully comparable to reference Clear bands for HC and LC chains at correct MW
IEF/WCX	Slightly weaker basic bands/peaks for ambr samples (C-terminal lysine clipping 98.7% compared to 90.9% for reference)
Antigen ELISA	Fully comparable to reference
Fc γ RIIIa AlphaScreen	Fully comparable to reference, 1.01 potency
Oxidation (methionine)	Fully comparable to reference, $\leq 0.8\%$
Deamidation	Fully comparable to reference, $\leq 0.1\%$
Further PTMs (e.g., glycation, phosphorylation, isomerization; N-terminal acetylation, hydroxylation)	Not detected for GEX [®] monoclonal antibodies incl. SAM and Reference
ADCC	Fully comparable to reference, 0.96 potency

antibody product from the SAM perfusion mimic is fully comparable by the size exclusion chromatography, SDS-PAGE, Antigen ELISA, Fc γ RIIIa alpha screen, oxidation and ADCC activity. Only the IEF profile and weak cationic-exchange chromatography profiles differed between the antibodies produced from the different systems. The results show that the charge variants of antibody from the SAM mimic were less basic and C-terminal lysine clipping was more prevalent, which might be due to a lower specific productivity of the SAM perfusion meaning that antibody post translational processing (in this case C-terminal lysine clipping) is more complete at low cellular protein productivities. A study with spin-tubes and daily centrifugation plus media exchange as perfusion mimic reported as well decreased C-terminal lysine levels compared to bench-top bioreactor cultivations.²²

Figure 4 shows a comparison of the glycosylation profiles from monoclonal antibody1 produced in the microscale and laboratory-scale perfusion systems. The relative abundances of fucosylated species (F), species with bisecting glucosamine structures (B), sialylated species (S > 0) and species with galactosylation structures (G > 0) show a high degree of consistency between the 1 L Biostat B-DCU with ATF and the SAM scale-down mimic.

Figure 5 shows the high level of comparability between the glycosylation profiles of two different monoclonal antibodies across a range of scales. In both cases, the product quality was conserved across scales. The SAM mimic predicted the glycosylation pattern of the large-scale up to either the 200 L perfusion bioreactor scale (larger scale data not available) or the 1,000 L perfusion bioreactor scale. These results give confidence that the SAM mimic is an effective tool for selecting cell lines that will deliver the desired product quality during GMP manufacturing, even with slight differences, for example, in cell concentration or titer across scales (data not shown).

Studying the effect of four different media formulations on cell growth and productivity was attempted in the ambr 15 system operated in batch, chemostat and SAM perfusion modes. A control media

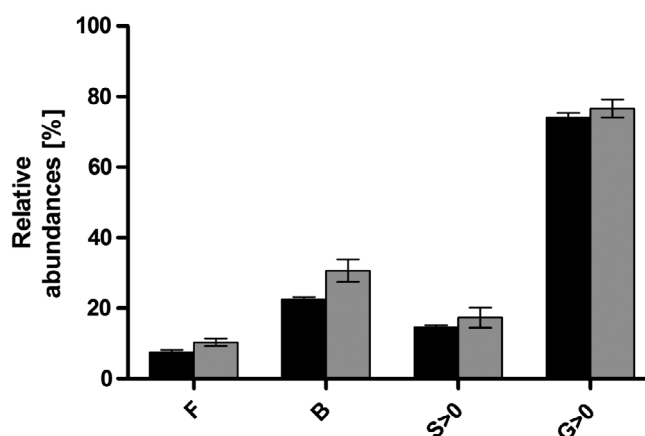


FIGURE 4 A comparison of the glycosylation profiles (F: fucosylation, B: bisecting GlcNac, S > 0: sialylation, G > 0: galactosylation) of monoclonal antibody 1 expressed in GEX[®] mAbExpress cell line A and produced in either the 12 mL SAM scale-down perfusion process mimic (black) or in the 1 L Biostat B-DCU with ATF (grey) (mean ± 1 SD, $n \geq 6$)

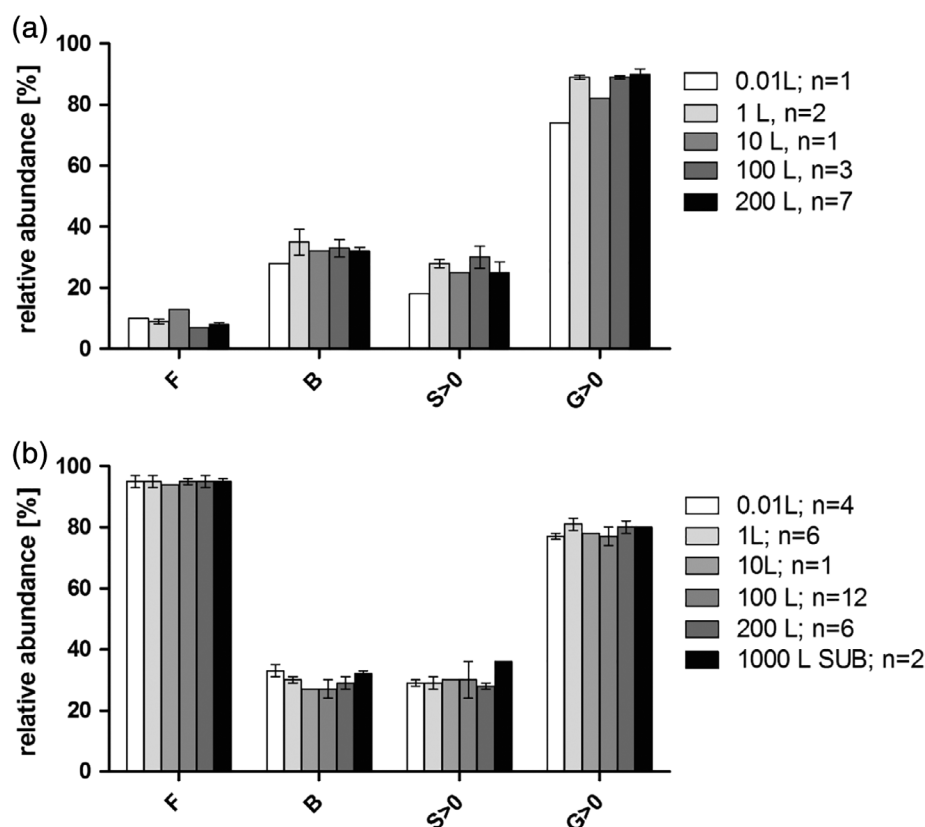


FIGURE 5 Comparisons of glycosylation profiles (F: fucosylation, B: bisecting GlcNAc, S > 0: sialylation, G > 0: galactosylation) for two different monoclonal antibodies produced across a range of perfusion bioreactor scales. (a) Monoclonal antibody 1 expressed in GEX[®] mAbExpress cell line B (optimized for low fucose): glycosylation profiles from the SAM mimic to a 200 L bioreactor. (b) Monoclonal antibody 2 expressed in GEX[®] mAbExpress cell line C (optimized for high fucose): Glycosylation profiles across scales from the SAM mimic to a 1,000 L bioreactor—SUB = single use bioreactor (error bars are one standard deviation)

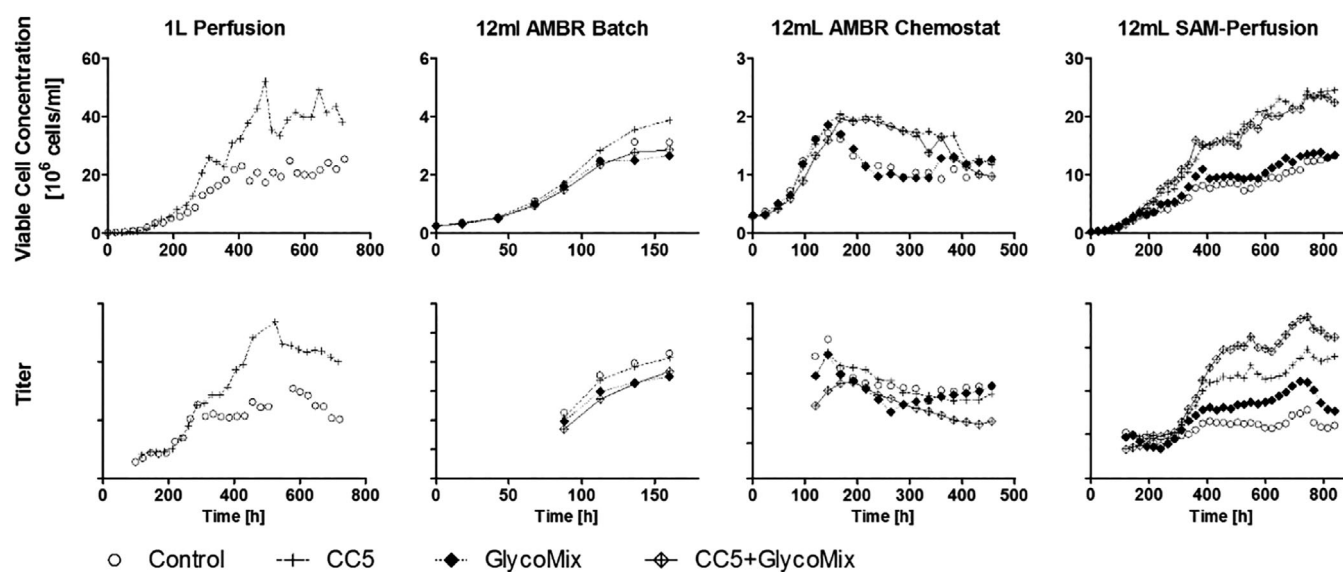


FIGURE 6 Comparison of the SAM perfusion mimic with ambr batch and chemostat mimics for the ability to predict the effect of four different cell culture media formulations on process performance for GEX[®] mAbExpress cell line C. Batch and chemostat experiments were performed in duplicate, also in the ambr system (12 mL ambr batch and 12 mL ambr Chemostat). 1 L ATF-perfusion panels show data from a 1 L process with alternating tangential flow as cell retention device

was used to benchmark performance. CC5 is a proprietary media supplement that increases maximum viable cell density. Supplement CC5 was added to the control media to create a second media formulation. A third formulation was created by adding GlycoMix, another proprietary supplement, which increases culture productivity. A final and

fourth media formulation was created by adding both supplements to the control media. The results are shown in Figure 6.

The influence of the two supplements, either individually or together, cannot be detected when performing the experiment in the ambr 15 system operated in batch mode (Figure 6 second column).

When operating the system in the chemostat mode, the benefit of adding the CC5 supplement on viable cell concentration can be detected but not the impact of adding GlycoMix on productivity (Figure 6 third column). The chemostat also showed the lowest cell concentration density compared to batch or perfusion as the semi-continuous mode of the chemostat was initiated below the maximum cell density to avoid potential limitation. The decline of viable cell concentration of CC5 supplemented chemostat culture might be due to starting nutrient limitation caused by the semicontinuous operation of the ambr. The SAM perfusion mimic, however, detects the anticipated improvements as they are seen at the 1 L scale (Figure 6 first column; only control and CC5/GlycoMix supplementation available) in both viable cell concentration and productivity when both supplements are added simultaneously to the control media (Figure 6 fourth column). Due to the longer run time and higher cell densities usually achieved in perfusion culture compared to batch or chemostat mode, differences in process performance are generally better detected. For example, cell culture media is generally a larger contributor to process improvement in perfusion than in batch cultures as perfusion has higher requirements for cell culture media due to, for example, larger cell densities in perfusion culture. Therefore, the results show that the SAM mimic is a more effective tool for performing media optimization experiments than the ambr 15 system used in batch or chemostat modes. The chemostat and batch modes were not found to be representative of the performance of 1 L perfusion cell cultures when assessing the influence of media composition. This is a significant finding because the ambr 15 has previously been used in the chemostat mode to mimic perfusion performance.^{28,32,33} The difference in the predictive ability of the two scale-down mimics might be explained by differences in cell metabolisms when grown in chemostat and perfusion cultures. In chemostat culture cells grow to lower cell density and are kept in exponential growth phase during the entire cultivation time. Perfusion cultures grow to much higher cell densities and as no bleeding strategy is pursued cells grow under nonsteady-state conditions. Experiment run time for perfusion culture

in this specific experiment was also longer compared to batch or chemostat.

The SAM perfusion mimic is well suited to performing optimization studies using Design of Experiments (DoE) methodologies due to being able to operate many perfusion bioreactor runs simultaneously and with a limited amount of materials. The ambr platforms are fully automated and significantly reduce the amount of operator intervention required for performing cell cultures. In this way, a maximum amount of information can be acquired in a minimum amount of time and with minimal need for resource. Figure 7 and Table 2 show the results from a DoE experiment in which the effect of cell culture media pH and the concentration of the supplements CC5 and GlycoMix on cell viability, viable cell density, and productivity were studied. The model coefficients as shown in Table 2 suggest a statistically valid model as expected from a highly parallelized system. As the experiment has been run in the SAM perfusion mode, results are likely to mimic the responses of larger scale perfusion cultures. The coefficient plots in Figure 7 indicate a significant influence of CC5 concentration and pH on the three dependent variables. The model describes a significant amount of the variation observed in the responses. The exemplary contour plot for IVCD (integral viable cell density) shows how the system can be used for process optimization. As also seen in the coefficient plots, GlycoMix does not have a large influence while pH and CC5 concentration both reveal their optimal settings indicated by the red area of the contour plots. The output of the same experimental setup in shake flasks or spin tubes or at the bench-scale remains to be seen.

TABLE 2 Summary of DoE fit for USP parameters. All model coefficients indicate a good model fit

Response	R ²	Q ²	Validity	Reproducibility
Product	0.949	0.831	0.758	0.950
IVCD	0.972	0.845	0.950	0.930
Viability	0.928	0.749	0.975	0.785

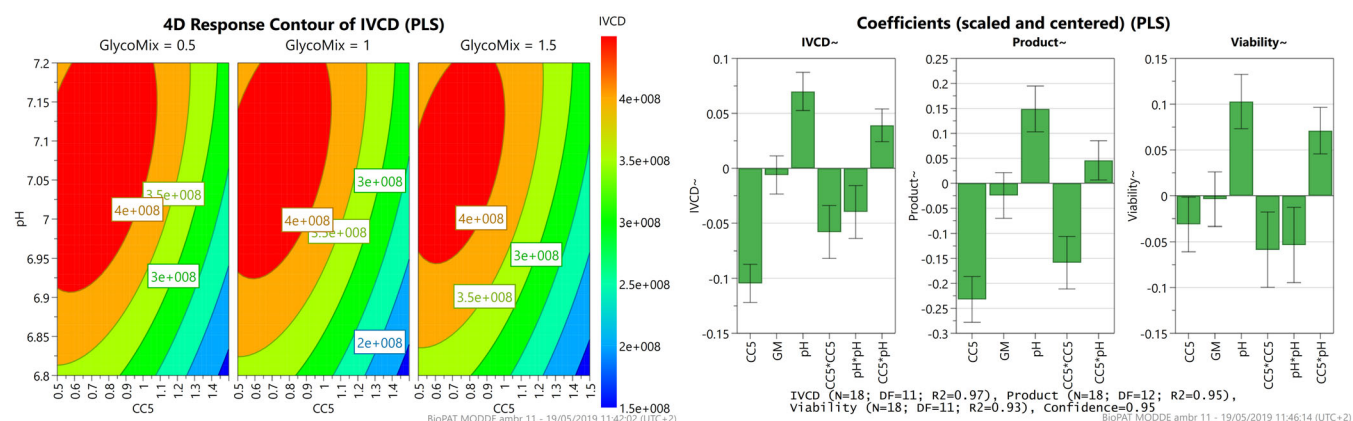


FIGURE 7 Contour plots generated from an experiment with a central composite design using GEX[®] mAbExpress cell line A. The design had four center points. The independent variables were the cell culture media pH and the concentrations of the supplements CC5 and GlycoMix. The dependent variables were cell viability, viable cell density, and productivity

The experiment had a central composite design and required the operation of 18 parallel perfusion bioreactors. To perform the equivalent experiment in the 1 L Biostat B-DCU with ATF system would have required 80-fold more cell culture media, double the amount of operator time each day and would have increased the cost by approximately 2.5-fold. This significant resource saving should translate for using the SAM system compared to, for example, benchtop or larger-scale perfusion.

In general, it was shown that the cell retention principle of sedimentation can be applied to microbioreactors enabling them to mimic larger scale perfusion cultures for upstream process characteristics as well as product quality. Compared to other cell retention devices the separation efficiency can be adjusted by the sedimentation time. However, it has to be noted that prolonged sedimentation times lead to increased lactate levels and decreased titer. In terms of representation of larger-scale perfusion data, it was shown that the system mimics well the main characteristics of 1 L scale ATF based perfusion cultivation. However, it remains to be shown how the SAM system responds to processes with other cell lines and also how the system performs at high (e.g., 5×10^7 cells/mL) or very high (e.g., 1×10^8 cells/mL) cell densities. It can be hypothesized that separation efficiency as shown in Figure 1 is lower at higher cell densities. The effect of higher cell densities on cell metabolism as well as product quality might also be altered. So far, the system has been successfully used with GEX[®] cells at cell densities of 2×10^7 cells/mL and with CHO cells at 3×10^7 cells/mL (data not shown). Product quality especially glycosylation was found to be very well comparable to even 200 L or 1,000 L perfusion runs. The system offers process development experts a tool to perform media optimization studies in a high throughput, small scale perfusion system with little resource use early on in process development or even cell line development that is more representative than batch or chemostat-based approaches. The option to run DoE studies in highly parallelized manner in scale-down perfusion setup might facilitate QbD (Quality by design) approaches for continuous bioprocessing.

The system has the potential to replace, for example, shake flask or spin tube based scale-down approaches with manual user interaction as perfusion mimic. It may also contribute to a broader implementation of perfusion processes in the industry.

In the future, this perfusion scale-down mimic should be verified with a greater number and more diverse set of cell lines. Furthermore, the DoE experiments that investigated the effect of cell culture media pH and the concentration of the supplements CC5 and GlycoMix on cell viability, viable cell density, and productivity need to be verified in depth at the bench-scale.

The ambr 250 is an automated bioreactor system for 100 to 250 mL cell cultures. The bioreactor vessels used with the ambr 250 have greater geometric similarity to large-scale cell culture bioreactors and may offer further advantages for scale-down mimics of large-scale perfusion processes than the ambr 15. The development of an ambr 250 perfusion scale-down mimic alongside the ambr 15 perfusion mimic may provide a comprehensive set of development tools to allow the biopharmaceutical industry to develop highly productive, continuous upstream processes.

4 | CONCLUSION

New process development tools are needed to allow the optimization and characterization of perfusion cell culture processes. A scale-down mimic has been developed that allows the ambr 15 microbioreactor system to be operated in perfusion mode. Cells are retained by periodically allowing sedimentation in the absence of agitation then withdrawing supernatant and replacing with fresh media.

The automated method gives comparable results to that of a 1 L bioreactor culture with ATF retention device at cell density of about 1×10^7 cells/mL for a human cell line although culture lactate concentrations were higher in the microscale mimic and the cell productivity lower. Furthermore, the antibody produced in the microscale mimic showed less basic variants. Cell concentration and viability however were equivalent with the 1 L mimic as were all other product quality attributes. In addition, these product qualities were also equivalent to according perfusion runs in 200 L and 1,000 L bioreactor scales demonstrating the full comparability over all relevant production scales. The sedimentation method allows cell culture development scientists to accurately predict the performance of large-scale bioreactors up to 1,000 L at producing antibody with the required glycosylation profiles as well as optimizing various process parameters. This article proves that the SAM-method is more effective than using the ambr system in batch or chemostat mode for predicting the effects of media optimization trials on large-scale perfusion performance. The automated and high throughput nature of the ambr technology allows the optimization of media and feeding strategies using DoE experiments allowing biomanufacturers to develop more optimized perfusion processes in less time and therefore increase the speed with which new biologic drugs can reach the clinic.

ACKNOWLEDGMENTS

Steffen Goletz is a shareholder of Glycotope GmbH. Steffen Kreye, Rainer Stahn, and Karina Nawrath were employees of Glycotope GmbH. Vicky Goralczyk is an employee of Glycotope GmbH. Barney Zoro is an employee of Sartorius Stedim Biotech.

REFERENCES

1. Konstantinov KB, Cooney CL. White paper on continuous bioprocessing. May 20–21, 2014 continuous manufacturing symposium. *J Pharm Sci*. 2015;104(3):813–820.
2. Goldman MH, James DC, Rendall M, Ison AP, Hoare M, Bull AT. Monitoring recombinant human interferon-gamma n-glycosylation during perfused fluidized-bed and stirred-tank batch culture of CHO cells. *Biotechnol Bioeng*. 1998;60(5):596–607.
3. Lipscomb ML, Palomares LA, Hernández V, Ramírez OT, Kompala DS. Effect of production method and gene amplification on the glycosylation pattern of a secreted reporter protein in CHO cells. *Biotechnol Prog*. 2005;21(1):40–49.
4. Hernandez R. Continuous manufacturing: a changing processing paradigm. *BioPharm Int*. 2015;28(4):20–27.
5. Zydney A. Perspectives on integrated continuous bioprocessing—opportunities and challenges. *Curr Opin Chem Eng*. 2015;10:8–13.

6. Voisard D, Meuwly F, Ruffieux P-A, Baer G, Kadouri A. Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. *Biotechnol Bioeng.* 2003;82(7):751-765.
7. Karst DJ, Serra E, Villiger TK, Soos M. Characterization and comparison of ATF and TFF in stirred bioreactors for continuous mammalian cell culture processes. *Biochem Eng J.* 2016;110:17-26.
8. Clincke M-F, Molleryd C, Zhang Y, Lindskog E, Walsh K, Chotteau V. Study of a recombinant CHO cell line producing a monoclonal antibody by ATF or TFF external filter perfusion in a WAVE Bioreactor™. *BMC Proc.* 2011;5(8):105.
9. Kim BJ, Oh DJ, Chang HN. Limited use of Centrtech Lab II centrifuge in perfusion culture of rCHO cells for the production of recombinant antibody. *Biotechnol Prog.* 2008;24(1):166-174.
10. Mercier SM, Rouel PM, Lebrun P, Diepenbroek B, Wijffels RH, Streefland M. Process analytical technology tools for perfusion cell culture. *Eng Life Sci.* 2016;16:25-35.
11. Johnson M, Lanthier S, Massie B, Lefebvre G, Kamen AA. Use of the centrtech lab centrifuge for perfusion culture of hybridoma cells in protein-free medium. *Biotechnol Prog.* 1996;12(6):855-864.
12. Takamatsu H, Hamamoto K, Ishimaru K, Yokoyama S, Tokashiki M. Large-scale perfusion culture process for suspended mammalian cells that uses a centrifuge with multiple settling zones. *Appl Microbiol Biotechnol.* 1996;45(4):454-457.
13. Mercille S, Johnson M, Lanthier S, Kamen AA, Massie B. Understanding factors that limit the productivity of suspension-based perfusion cultures operated at high medium renewal rates. *Biotechnol Bioeng.* 2000;67(4):435-450.
14. Ryll T, Dutina G, Reyes A, Gunson J, Krummen L, Etcheverry T. Performance of small-scale CHO perfusion cultures using an acoustic cell filtration device for cell retention: characterization of separation efficiency and impact of perfusion on product quality. *Biotechnol Bioeng.* 2000;69(4):440-449.
15. Zhang J, Collins A, Chen M, Knyazev I, Gentz R. High-density perfusion culture of insect cells with a BioSep ultrasonic filter. *Biotechnol Bioeng.* 1998;59(3):351-359.
16. Wen ZY, Teng XW, Chen F. A novel perfusion system for animal cell cultures by two step sequential sedimentation. *J Biotechnol.* 2000;79(1):1-11.
17. Tescione L, Lambropoulos J, Paranandi MR, Makagiansar H, Ryll T. Application of bioreactor design principles and multivariate analysis for development of cell culture scale down models. *Biotechnol Bioeng.* 2015;112(1):84-97.
18. Tsang VL, Wang AX, Yusuf-Makagiansar H, Ryll T. Development of a scale down cell culture model using multivariate analysis as a qualification tool. *Biotechnol Prog.* 2014;30(1):152-160.
19. Shimoni Y, Goudar C, Jenne M, Srinivasan V. Qualification of scale-down bioreactors: validation of process changes in commercial production of animal-cell-derived products, part 1—concept. *BioProcess Int.* 2014;12:38-45.
20. Subramanian G. *Continuous processing in pharmaceutical manufacturing.* Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2015.
21. Fernandez D, Femenia J, Cheung D, et al. Scale-down perfusion process for recombinant protein expression. *Anim Cell Technol: Basic Appl Aspects.* 2009;1:59-65.
22. Angepat S, Gorenflo VM, Piret JM. Accelerating perfusion process optimization by scanning non-steady-state responses. *Biotechnol Bioeng.* 2005;92(4):472-478.
23. Gomez N, Ambhaikar M, Zhang L, et al. Analysis of Tubespins as a suitable scale-down model of bioreactors for high cell density CHO cell culture. *Biotechnol Prog.* 2017;33:490-499.
24. Wolf MKF, Lorenz V, Karst DJ, Souquet J, Broly H, Morbidelli M. Development of a shake tube-based scale-down model for perfusion cultures. *Biotechnol Bioeng.* 2018;115:2703-2713.
25. Lewis G, Lugg R, Lee K, Wales R. Novel automated micro-scale bioreactor technology: a qualitative and quantitative mimic for early process development. *BioProcess J.* 2010;9(1):22-25.
26. Hsu WT, Aulakh RP, Traul DL, Yuk IH. Advanced microscale bioreactor system: a representative scale-down model for bench-top bioreactors. *Cytotechnology.* 2012;64(6):667-678.
27. Nienow AW, Rielly CD, Brosnan K, et al. The physical characterisation of a microscale parallel bioreactor platform with an industrial CHO cell line expressing an IgG4. *Biochem Eng J.* 2013;76:25-36.
28. Poulsen BR. *Scale-down tools for the evaluation of perfusion processes.* Munich: Cell Culture World Congress; February 2013.
29. Moses S, Manahan M, Ambrogelly A, Lin WLW. Assessment of ambr as a model for high-throughput cell culture process development strategy. *Adv Biosci Biotechnol.* 2012;3:918-919.
30. Rameez S, Mostafa SS, Miller C, Shukla AA. High-throughput miniaturized bioreactors for cell culture process development: reproducibility, scalability, and control. *Biotechnol Prog.* 2014;30(3):718-727.
31. Janakiraman V, Kwiatkowski C, Kshirsagar R, Ryll T, Huang Y-M. Application of high-throughput mini-bioreactor system for systematic scale-down modeling, process characterization and control strategy development. *Biotechnol Prog.* 2015;31(6):1623-1632.
32. TAP Biosystems. (2013). Implementation of a micro bioreactor model for perfusion cell culture using a chemostat approach. Application note TAP Biosystems.
33. Ho D, Traul D, Park S, et al. A simplified microbioreactor culture model to mimic perfusion. Poster. Integrated Continuous Biomanufacturing. Oct 20–24, 2013. Castelldefels, Barcelona, Spain.
34. Zoro B. Optimizing vaccine process scale-up of attachment dependent cells'. *Pharm Process.* 2014;29(7):34-36.
35. Draoui N, Feron O. Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments. *Dis Model Mech.* 2011;4(6):727-732.
36. Qian Y, Xing Z, Lee S, et al. Hypoxia influences protein transport and epigenetic repression of CHO cell cultures in shake flasks. *Biotechnol J.* 2014;9(11):1413-1424.