High-Throughput Screening of Antibody-Expressing CHO Clones Using an Automated Shaken Deep-Well System

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Biopharmaceutical protein manufacturing requires the highest producing cell lines to satisfy current multiple grams per liter requirements. Screening more clones increases the probability of identifying the high producers within the pool of available transfectant candidate cell lines. For the predominant industry mammalian host cell line, Chinese hamster ovary (CHO), traditional static-batch culture screening does not correlate with the suspension fed-batch culture used in manufacturing, and thus has little predictive utility. Small scale fed-batch screens in suspension culture correlate better with bioreactor processes but a limited number of clones can be screened manually. Scaled-down systems, such as shaken deep well plates, combined with automated liquid handling, offer a way for a limited number of scientists to screen many clones. A statistical analysis determined that 384 is the optimal number of clones to screen, with a 99% probability that six clones in the 95th percentile for productivity are included in

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the screen. To screen 384 clones efficiently by the predictive method of suspension fed-batch, the authors developed a shaken deep-well plate culturing platform, with an automated liquid handling system integrating cell counting and protein titering instruments. Critical factors allowing deep-well suspension culture to correlate with shake flask culture were agitation speed and culture volume. Using our automated system, one scientist can screen five times more clones than by manual fed-batch shake-flask or shaken culture tube screens and can identify cell lines for some therapeutic protein projects with production levels greater than 6 g/L. © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 34:1460–1471, 2018
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Introduction

More than 200 recombinant protein pharmaceutical products have been licensed in the U.S. and Europe, with new approvals occurring at a rate of 5–20 per year.[1,2] These biologics include recombinant blood factors, hormones, vaccines, monoclonal antibodies, growth factors, and enzymes. For companies to produce these drugs economically, the productivity of the cell culture process must be sufficiently high. Desirable product quality traits (e.g., appropriate glycosylation profiles or high purity) are also important considerations. The expression characteristics of each molecule, as well as the recombinant cell line, media and bioreactor production process are also key factors in determining productivity and product quality.

Cell line development encompasses stable cell line generation and screening. Typically, hundreds or thousands of stable cell lines are created by transfection and selection for integration of the protein coding sequence into the genome. The diversity of the transfected cell lines is a consequence of both the location of the random integration and the genetic heterogeneity already existing within the population of CHO host cells.[3] Successive screening steps narrow the initial pool of cell lines to a small number for evaluation in bioreactors to choose the final clone for production of clinical and commercial therapeutic protein product.[4] Production cell lines are clonally-derived from single cells to meet regulatory expectations and require detailed characterization of the cloning process to determine probability of monoclonality.[5–9]

The screening process plays an important role in identifying diverse and predictably productive cell lines for evaluation in bioreactors. In the conventional process, stable transfected cell clones are expanded from the original transfected cells into progressively larger-volume culture vessels. The earliest stage at which the culture medium volume is adequate for product titer screening is usually the 96-shallow well (96w) plate. Screening is often conducted in 96w and then 24-shallow well (24w) plates incubated in static culture. In contrast, most large-scale production is performed in suspension culture, with the transition from static to suspension often following the 24w stage. Once in suspension, an aliquot of the cells can be cultured for a period to generate the desired protein (batch culture). The cultures can also be fed concentrated nutrients (fed-batch), resulting in higher cell densities, longer survival, and the highest overall productivity. Suspension fed-batch is typically the ultimate production process, so prediction of productivity in that process is the most desirable for screening.

A comparison of the top cell lines from four screening steps in a model CHO cell line development process showed poor correlation between the final fed-batch suspension process and the static 96w, 24w, or suspension batch screening steps.[10] The lack of correlation may be due to differences in culturing conditions including static vs. shaken culture and fed vs. non-fed conditions.[11,12] It can be difficult to match the final fed-batch suspension conditions in the early screening steps when the number of clones is large.

Much effort has been devoted to developing new platforms combining cloning and screening steps to increase the screen size, including the use of colony pickers[13–16] and fluorescence-activated cell sorting, or FACS.[17–19] However, many of these screening platforms are based on a version of batch secretion in static culture and are subject to the same lack of correlation described by Porter et al.,[10] so are best used for eliminating non-expressing cell lines before starting a more predictive suspension fed-batch screen. Newer, more highly automated cell culture screening systems have been developed,[20–27] including the recently reported implementation of nanofluidics.[28] These methods can enable high throughput fed-batch screening for application in cell line development as well as for process development and media evaluation. In this manuscript, the authors describe our methodology for developing an improved cell line screening process, starting with a novel approach to calculate the appropriate number of cell lines to evaluate in predictive suspension fed-batch screens. Using the outcome of these calculations, we developed a practical, automated method for screening the required number of cell lines (384) in four 96 deep well (96DW) plates in the first predictive fed-batch screen.

Materials and Methods

Cell culture

The recombinant molecules used in this study included IgG1 and IgG2 format human monoclonal antibodies and a fusion protein. These recombinant molecules recognize human cytokines, chemokine receptors, signaling proteins, or growth factors. Cell lines expressing MedImmune monoclonal human antibodies A–D, F and G, and fusion protein E were generated using CHO-K1 cells adapted to suspension growth in animal-component free media. The cells were cultured using commercially available, chemically defined medium (CD CHO, GIBCO ThermoFisher Scientific, Grand Island, NY, USA) as well as proprietary in-house media and feed solutions. Cell lines were generated by electroporation with plasmids containing the genes for the desired proteins as well as the hamster glutamine synthetase gene, and selected by growth in medium containing methionine sulfoximine. Suspension cultures were passaged every 3–4 days in shake flasks at inoculation cell densities of $0.3–0.5 \times 10^6$ viable cells/mL. Fed-batch cultures were performed by feeding cells every 2 days with an in-house feed solution. For the 96DW fed-batch cultures, the inoculation and feeding processes were performed using the Hamilton robot (Hamilton, Reno, NV, USA), with nutrient feed being diluted with water to counteract reductions in volume due to evaporation.[22] Only one sample was removed for titer or cell number determination during the fed-batch in the 96DW plates and one sample was removed at the end.

The 96DW culturing utilized sterile 96DW MASTERBLOCK plates (Greiner Bio-One North America Inc., Monroe, NC, USA) with μ-Flask sandwich covers (Duetz, EnzyScreen B.V., Heemstede, The Netherlands) designed to allow gas exchange but exclude microbial contaminants and minimize evaporation, especially on the edges of the plate. The working volume in these plates was 350 μL/well. Unless otherwise noted, the culturing conditions for passaging cells were 37°C, 350 rpm with a 25 mm orbital diameter, 80% relative humidity, and 6% CO_2. Fed-batch conditions were identical to the passaging process except that the temperature was 35.5°C. The inoculation cell densities were 0.3–0.5 × 10^6 viable cells/mL for cell passaging and 0.8 × 10^6 total cells/mL for fed-batch.

Shake flask cultures used 250 mL polycarbonate Erlenmeyer flasks (Corning Life Sciences, Corning, NY, USA) with working volumes of 50–60 mL. The shaken culture tubes were CultiFlask 50 mL disposable bioreactors (Sartorius Stedim, Gottingen, Germany) with a 5 mL working volume. The shake flask and shaken culture tube incubator conditions were identical to the 96DW cultures except that the shake speeds were 120 rpm for the flasks and 160 rpm for the culture tubes. All shaken flask and tube cultures were incubated in Multitron 2 (Infors HT, Bottmingen, Switzerland) shakers using a 25 mm orbital diameter, the same used for the 96DW cultures. The static cultures of shallow 96 and 24 well plates (Greiner) were maintained in Heracell (Thermo Fisher Scientific, Waltham, MA, USA) incubators.

Measurements and sample preparation

For the Hamilton/96DW process, cell count and viability were measured using cell culture samples in a 384 well black optical bottom plate (Corning) on a Cellavista (Synentec, GmbH, Elmshorn, Germany). These plates contained diluted samples mixed 1:1 with 0.2% trypan blue. All dilutions were made using the culture medium. The dilution varied based on the number of cells expected in the original sample. Cell confluence of shallow 96w plates was determined by directly measuring the plates using the Cellavista. The monoclonal antibody and fusion protein titers were measured using an Octet QK384 (ForteBio, Menlo Park, CA, USA) and Protein A biosensors. The binding rates measured by the Octet were converted to protein concentrations using a standard curve. All dilutions were performed with the culture medium.

Cell counts and viabilities from shake flask and shaken culture tube samples were measured on a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA). Protein titer measurements were performed on an Agilent 1100 Series HPLC with a Protein A column.

Results and Discussion

Our original cell line generation process is illustrated in Figure 1. Suspension-adapted animal-component-free CHO-K1 cells were transfected with the genes coding for our monoclonal antibody or fusion protein products and the hamster glutamine synthetase gene for selection. These transfected cells were then diluted to the cloning density and grown in static culture in 96w plates under methionine sulfoximine selection pressure. A target of 800 wells was evaluated for static titer at the end of 14–21 days. A target of the 400 "top" cell lines was chosen and expanded into 24w plates, and the static titer screening process was repeated after 7 days. The "top" 160–200 cell lines were then expanded into shaken culture tubes[29] for simplified fed-batch screening in which each cell line was fed a bolus of nutrient feed and identical amounts of glucose. The top 20 cell lines were then transferred into shake flasks for a second, larger-scale evaluation that was more representative of the final bioreactor process: each culture was supplemented every 2 days with aliquots of nutrient feed with glucose added as needed, and measurements were performed frequently to determine cell counts, metabolite concentrations, titers, and product quality. This was the final screen in the cell line development process that identified the top six cell lines, which were cryopreserved as research cell banks for evaluation in bioreactors.

This methodology was limited in two ways: first, as shown in Figure 2a, the initial high-throughput screening steps for clones expressing monoclonal antibody A were based on static batch titers and had low correlation to shake flask fed-batch titers. This observation is like that which was previously reported by Porter et al.[10] Second, while the shaken culture tube fed-batch titers correlated better with shake flask fed-batch titers (as shown in Figure 2b), one scientist could only process 80–100 shaken culture tubes with the simplified feeding program. Two scientists were needed to screen 160–200 shaken culture tubes. To address these issues, the authors decided to improve their cell line development process by focusing on two areas: determination of the number of cell lines that should be screened in the predictive fed-batch process to find sufficient numbers of high-expressing clones, and then automation of the process to allow for efficient screening of those cell lines.

Determination of optimal screen sizes to identify high expressing clones

Screen size investigations were conducted to determine how many clones to evaluate. For the initial predictive fed-batch screen size, two sets of calculations were performed. The first set estimated the screen size without prior knowledge of the cell line diversity. The second set consisted of an in-silico model based on the historical cell line development campaign screening process.

The first set of calculations used a binomial distribution to estimate the minimum screen size to ensure that a chosen number (six) of cell lines from a selected top percentile (95th) of the population are sampled and included in the screen. The formula for a binomial distribution is given by:

$$\Pr(X \geq k) = 1 - \sum_{i=0}^{k-1} \binom{n}{i} P^i (1-P)^{n-i} \qquad (1)$$

where:

P is the probability of picking a clone in the top 1-Pth percentile; n is the screen size; i is the number of cell lines from the top 1-Pth percentile that we would like to contain within the screen.

$\Pr(X \geq k)$ is the probability that a screen size of size n will contain at least i cell lines from the top 1-P^{th} percentile.

Our requirement for an effective (predictive) first screening process was to achieve greater than 99% probability that six cell lines from the top 95th percentile for productivity would be included among the cell lines tested. A greater than 99% probability provides a high level of confidence that the screen size selected is adequate. To do this we found the value of

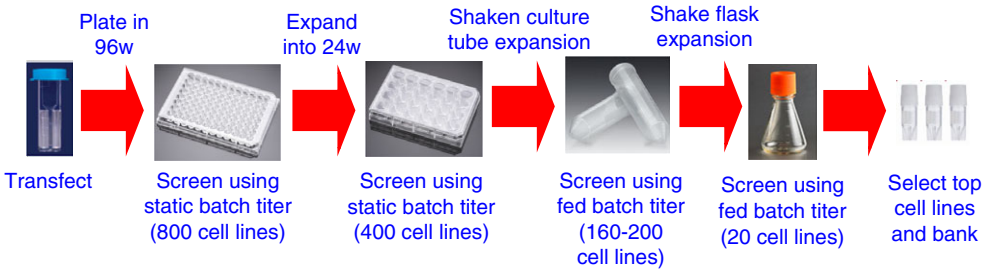

Figure 1. Overview of standard cell line development process. Initial screens were 96 and 24 shallow well (96w and 24w) static batch titers and shaken culture tubes. Final shake flask screen identified top six cell lines for banking.

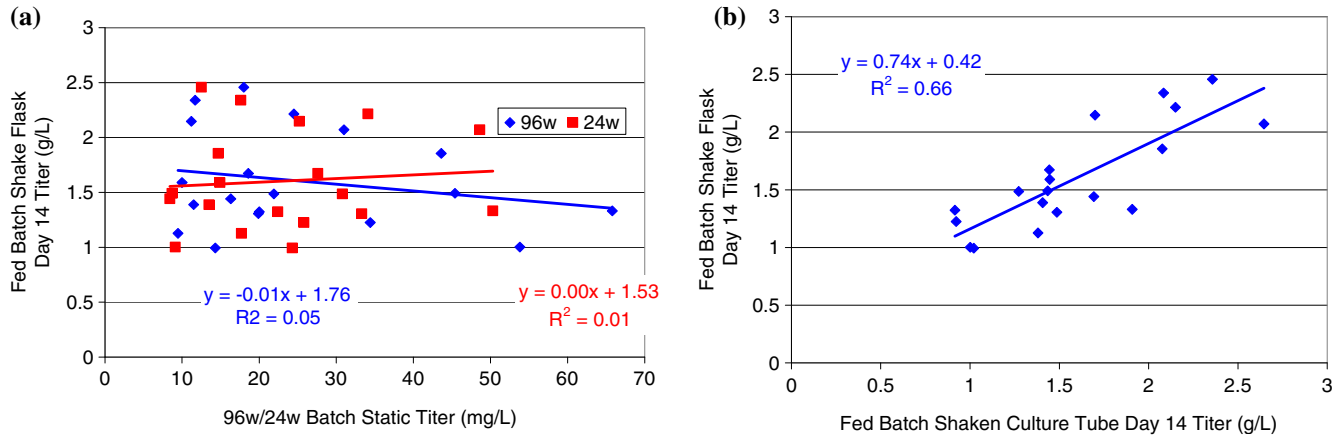

Figure 2. (a) Correlation graph of fed-batch shake flask titers with 96w and 24w static batch titers for monoclonal antibody A (n = 20). (b) Correlation graph of fed-batch shaken culture tube titers with fed-batch shake flask titers for monoclonal antibody A (n = 20).

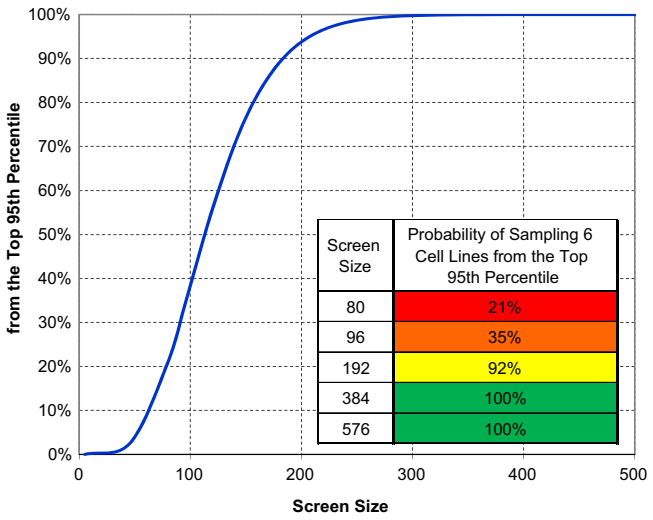

Figure 3. Probability of sampling six cell lines within the top 95th percentile for titer from a randomly distributed cell line population, for different screen sizes. Inset lists probabilities of selected screen size values in graph in a tabular format.

n in Eq. 1 that gives a $\Pr(X \geq k) \geq 0.99$ with $i = 6$ and $P = 0.05$.

Figure 3 shows the probability that six cell lines would be included in the tested sample is directly proportional to the screen size. These clones were chosen without prior knowledge of clone productivity from the set of stable clones that grew out during the 3 weeks of selection. If only 80 cell lines were sampled for testing, the probability that the top six cell lines in the tested sample were from the 95th percentile is 21%. When 192 cell lines are sampled, the probability that six cell lines from the 95th percentile are included increases to 92%. When 384 cell lines are sampled, the probability that six of them are from the 95th percentile reaches almost 100%. The approach discussed above gives a > 99% probability that the required number of cell lines from a chosen top percentile are included in the screen. However, this approach does not consider the potential increase in productivities resulting from increasing the chosen percentile, for example, 95th, to a higher percentile. Figure 4 and Table 1 contain an analysis of potential titer increases resulting from targeting higher percentiles based on four population distributions. Distribution #1 is almost symmetrical and represents the least amount of skewness of clone fed-batch productivity typically observed during cell line development. Distributions #2 and #3 represent the typical levels of clone productivity skewness observed during cell line development. Distribution #4 represents an extreme level of clone productivity skewness which far exceeds what is typically seen during cell line development. This last distribution is included for illustration purposes.

The results on the left side of Table 1 list the expected fold titer increases in the expression level of the lowest-expressing clone of the top six clones for selected percentiles compared with that for the 95th percentile for the four distributions. The right side contains the probability of sampling six cell lines in the corresponding percentile for various screen sizes. It is seen that, for the distributions of clone productivity typically found during cell line development, the increase in titer of the lowest-expressing clone among the top six that would be

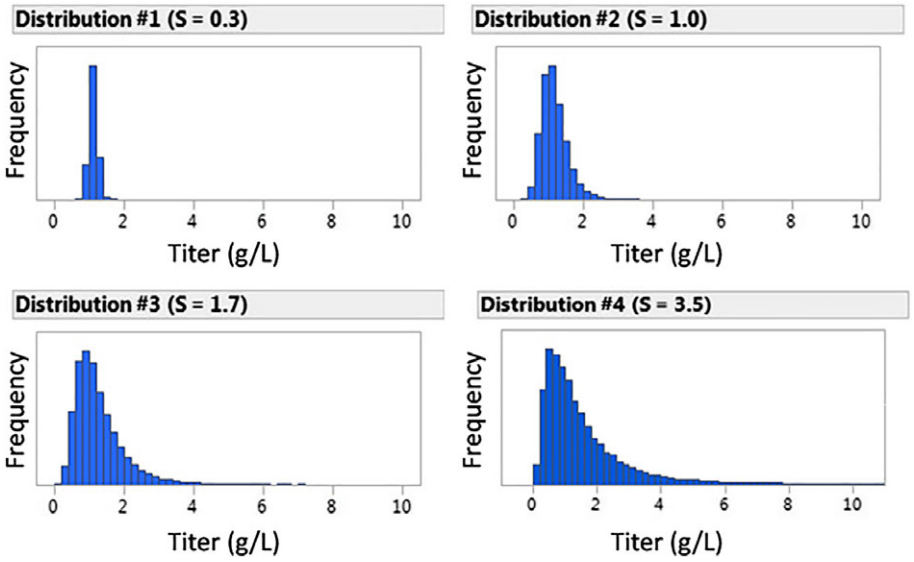

Figure 4. Examples of four fed-batch titer distribution shapes with varying degrees of skewness (S) ranging from almost symmetrical (Distribution #1, S = 0.3) to heavily right-skewed (Distribution #4, S = 3.5).

obtained by choosing a 98th percentile instead of the 95th percentile is 1.14 or less. However, to obtain a high probability that those increases are realized, the screen size would have to be larger. For example, a screen size of 576 will give a moderately high probability of 97% that six cell lines within the 98th percentile are among the 576 screened, while a screen size of 960 cell lines will provide a 100% (rounded to whole numbers) chance that six cell lines within the top 98th percentile are included in the 960 sampled. Choosing a screen size sufficiently large to include six cell lines within the 99th percentile would yield more meaningful titer increases of up 1.24 to 1.31-fold for the lowest expressing of the six clones in the productivity distributions typically observed in populations of stably transfected cells. The screen size necessary to achieve this improvement is approximately three times larger than the 384 needed to provide 99% probability that six cell lines in the 95th percentile are included. Thus, this trade-off is not deemed necessary for most cell line development needs, but it can be pursued should higher productivities be required. Finally, the analysis for the inclusion of the six cell lines in the 99.9th percentile shows that it can result in significantly larger titer increases for the type of distributions most commonly seen, however even a screen size of 1440 cell lines has 0% probability of containing six cell lines within this percentile. The sensitivity analysis provided in Figure 4 and Table 1 indicates that a 95th percentile with a screen size of 384 cell lines provides a sufficiently high confidence of sampling six high-producing cell lines for the productivity distributions typically observed from our transfections.

The optimal size of the second, larger-volume but smaller number fed-batch screen was also determined. This analysis used a Monte Carlo simulation that sampled titer values from a probability distribution with a shape like the ones typically observed in the primary screen (Figure 4, Distributions 2 and 3). The simulation model then generated enough variability to reproduce a target correlation level (R^2) based on the range of correlations typically seen during cell line development for the fed-batch titers used in the two sequential screening steps. Figure 5 contains an example of the simulated correlation plots. Correlations with R^2 values in the range of 0.5–0.8 are typically seen between titers of individual clones determined in primary (small scale fed-batch) and secondary (larger scale fed-batch) screens in cell line development projects. The data from the first screen for each clone was compared to the productivity for the same clone evaluated in the second screen. The results of the Monte Carlo simulations for sample sizes 20 and 40 are shown in Figure 6. In Figure 6, the average number of clones in the second screen that were in the 95th percentile of the initial 384 clone screen is plotted *vs.* the R^2. The results indicate that for the cases of no correlation (R^2 ~0) the number of high producers included in the secondary screen is very low. This is expected as it is equivalent to randomly selecting either 20 or 40 cell lines from the 384 cell lines in the primary screen, resulting in a secondary screen of 20 and 40 cell lines containing 1 and 2 high producers, respectively. For the cases of greater-than-zero correlations between the screens, the gain in numbers of clones in the 95th percentile for productivity obtained due to increasing the secondary screen size from 20 to 40 is greater for the R^2 values between 0.25 and 0.8. The improvement in numbers of clones in the 95th percentile for productivity then decreases as the R^2 approaches 1.0. The results indicate that with a sample size of 20–40 clones, a sufficiently large number of cell lines in the 95th percentile (12 or more on average) are included among those chosen for the typical level of correlation observed during cell line development (0.5–0.8, see Figure 2b). Based on this calculation, we chose to evaluate 20–40 clones by fed batch in the second screen.

Development of a high throughput fed-batch screening platform

For a scientist to evaluate multiple clones for selection of a manufacturing cell line, it is important to choose an appropriate screening method, one that predicts relative cell line performance in the final production process. For evaluating 384 clones in parallel, the screening method should be compatible with an automated liquid handling system. Because suspension fed-batch is the final culture process and titers using that process correlate well across different vessel types and scales, as demonstrated by Figure 2b, a small-scale

Table 1. The left side of the table lists the fold increase in the expression level of the lowest-expressing clone among the top six clones for selected percentiles compared with that for the 95th percentile for the distributions shown in Figure 4. The right side of the table contains the probability of sampling six cell lines in the corresponding percentile for various screen sizes

	Distributions with varying diversity (S = skewness)									
	Distribution #1 (S = 0.3)	Distribution #2 (S = 1.0)	Distribution #3 (S = 1.7)	Distribution #4 (S = 3.5)	Screen size					
					n = 192	n = 384	n = 576	n = 960	n = 1440	
Percentile	Fold increase relative to 95% percentile				Probability of sampling six cell lines in percentile					
95th	1.00	1.00	1.00	1.00	92%	100%	100%	100%	100%	
98th	1.04	1.14	1.14	1.42	19%	78%	97%	100%	100%	
99th	1.07	1.24	1.31	1.78	1%	19%	52%	78%	100%	
99.9th	1.16	1.55	1.91	3.03	0%	0%	0%	0%	0%	

culturing system was devised which keeps cells in suspension and facilitates addition of nutrient feeds. While 24 deep- or shallow-well shaken plates would also fit these criteria, we chose the 96DW plate platform[30,31] because it gives higher throughput and has better compatibility with automated liquid handling systems. A vented lid and clamping system was used that allows for uniform gas transfer and low evaporation rates across a shaken plate while maintaining sterility.[31]

Volumes and shaking speeds in the 96DW culturing system were optimized for cell growth to ensure a high correlation to the shake flask process. These parameters were initially evaluated by conducting a fed-batch with a single bolus feed on day 3 in 96DW plates, using different conditions, and measuring viable cell density. The growth of those cultures was compared with growth of parallel shake flask cultures. The starting volumes tested in the 96DW cultures were 350, 500, and 650 μL and the shaking speeds were 250, 300, and 350 rpm. As shown in Figure 7, the peak viable cell densities increased with lower volumes and higher speeds, and the growth of the 350 μL and 350 rpm condition in 96DW culture was similar to that for the shake flask culture. Volumes lower than 350 μL were not tested because evaporation affects cell viability at lower volumes (data not shown). Three hundred and fifty revolutions per minute was the maximum speed of our shaking incubator.

To quantify the correlation in titer between the 96DW and shake flask fed-batch processes, a 14-day fed-batch was performed in both systems using an identical set of cell lines in parallel. In this fed-batch experiment, feeds were added six times during the culture. The shake flasks were monitored for cell counts and titer frequently during the 14 days, but because of evaporation and the low starting volume, the 96DW cultures were only monitored once during the run and once at the end. The vented lid and clamping system decreased the amount of evaporation during shaking incubation to 10 μL per day. To address the evaporation during the 14-day fed batch, the feed for the 96DW cultures was diluted with water, and the resulting final volume per well at the end of the run, including the sampling, was 106% of the starting volume. For the shake flasks, however, with less proportional evaporation and multiple samples taken, the final volume was 143% of the starting volume.

Figure 8 shows a correlation factor (R^2) of 0.7 between the 96DW and shake flask fed batch runs, demonstrating predictive utility of the 96DW system. The slope is less than one (96DW plate titers were higher than the shake flask titers). Table 2 summarizes various additional experiments run for 96DW and shaken culture tube systems to evaluate their correlation with titers generated in fed batch shake flasks. The correlation coefficients for fed batch titers of both 96DW and shaken culture tubes with fed batch shake flasks were all greater than 0.5, compared to the correlations of 0.05 and 0.01 observed for our original static batch titer screens in Figure 2a. These results show that the fed-batch data from the 96DW system correlate to fed-batch shake flask titers as well as was observed with our previous platform shaken culture tube system (Figure 2b).

Development of an automated system for clone culturing and evaluation of productivity in 96DW plates

In passaging as well as fed-batch suspension, it is preferable to start all cultures at an identical cell density.[32,33] To initiate cultures of multiple clones at the same cell density, the cell

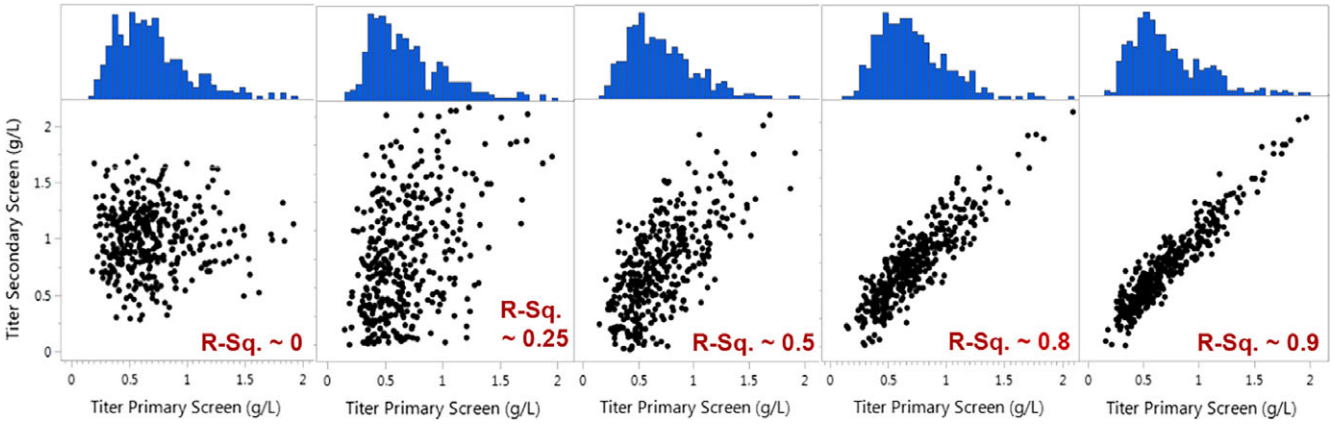

Figure 5. Example of data generated by the Monte Carlo simulation model. The distribution used for the primary fed-batch screen titers is based on the level of skewness typically observed during cell line development (see Figure 4, Distributions 2 and 3). Sufficient variability is added to generate different levels of correlation with resulting R^2 values from 0 to 0.9.

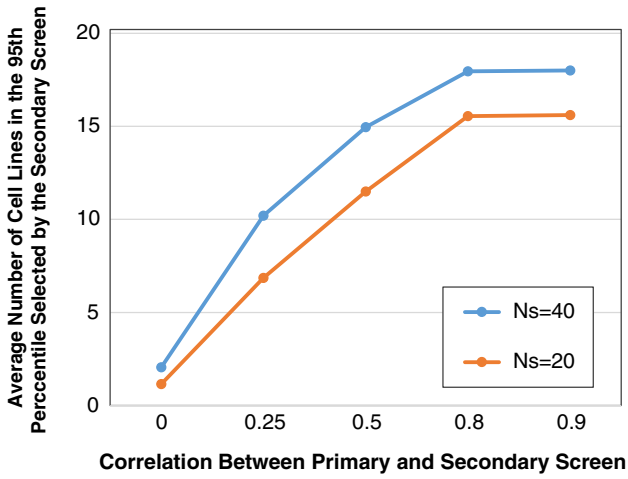

Figure 6. Results of the Monte Carlo simulation performed to determine the average number of cell lines in the 95th percentile in secondary screens as a function of the correlations (R^2) between the first and second screens. Ns denotes the size of the secondary screen.

suspensions, and fresh medium volumes for each clone will likely be different, making a manual process for 384 wells prohibitive. Therefore, we integrated the 96DW culture system with a high throughput automated liquid handling system, enabling a single scientist to maintain and passage the cell lines in suspension and then conduct fed-batch analysis using four 96DW plates. We designed an integrated high-throughput system composed of a Hamilton STARplus automated liquid handler, a Cellavista cell imaging system, and an Octet QK384 (Figure 9). The Cellavista is used to measure the cell density, viability, and confluence of cultures and the Octet QK384 measures recombinant protein titer.[34,35] The Hamilton STARplus liquid handler includes HEPA filters to maintain sterility, a liquid handling system with a 96-channel head to sample or feed all 96 wells in a plate simultaneously with one volume, as well as eight independent channels to pipette different volumes into each of eight wells. The system also contains a gripper to transport plates and lids and load them into the Octet and Cellavista, a media fill station which automatically refills a reservoir, and an integrated Liconic plate storage system to read barcodes and supply culture plates onto the deck of the Hamilton system. As all the tools are integrated, data from the Cellavista and Octet is used automatically to make liquid handling decisions. For example, the Hamilton system uses cell counts from the Cellavista to passage wells, inoculating different calculated volumes of media and cell suspensions into the wells of the fresh plate, achieving the same cell density for each well, without any user intervention other than specifying the inoculation cell density. All culture and measurement plates have barcodes that result in all pipetting and measurement data being tracked and stored in a SQL database. This computerized sample tracking reduces errors and saves the data in a format easily extracted for detailed data analysis.

Characterization of analytical measurement and process variation in the automated 96-DW fed-batch system

Before conducting a cell line development campaign with the 96DW plates and the integrated Hamilton system, experiments were performed to quantify the pipetting and analytical measurement variations of the cell counting and titer in this system. A goal of less than 10% variation was chosen so that cell line productivity differences greater than that amount could be accurately detected. As the automated system had multiple modules, we decided to characterize them individually.

First, the authors tested the cell counting accuracy and repeatability and focused mainly on the mixing capabilities of the Hamilton STARplus pipetting system and the Cellavista cell counting measurement, which used trypan blue staining for identifying nonviable cells. In this experiment (Table 3), cells at a specific cell density were manually pipetted into a 96DW plate. Then, the Hamilton sampled from each of these wells, diluted the sample, and added trypan blue. Cells from 28 wells were also counted using trypan blue on a Vi-CELL, which is our automated cell counting reference tool, and a well-to-well comparison was performed. The total cell count for the Cellavista matched that for the Vi-CELL. The viability measurement from the Cellavista was 10% lower than seen on the Vi-CELL, and the measurements had slightly higher variation (data not shown). The lower viability values observed with the Cellavista most likely reflect a limit of the resolution of the assay above 90% viability owing to the characteristics of imaging process and algorithms. The slightly higher variation may be due to several differences between the Cellavista and the Vi-CELL methods, including the use of a plate instead

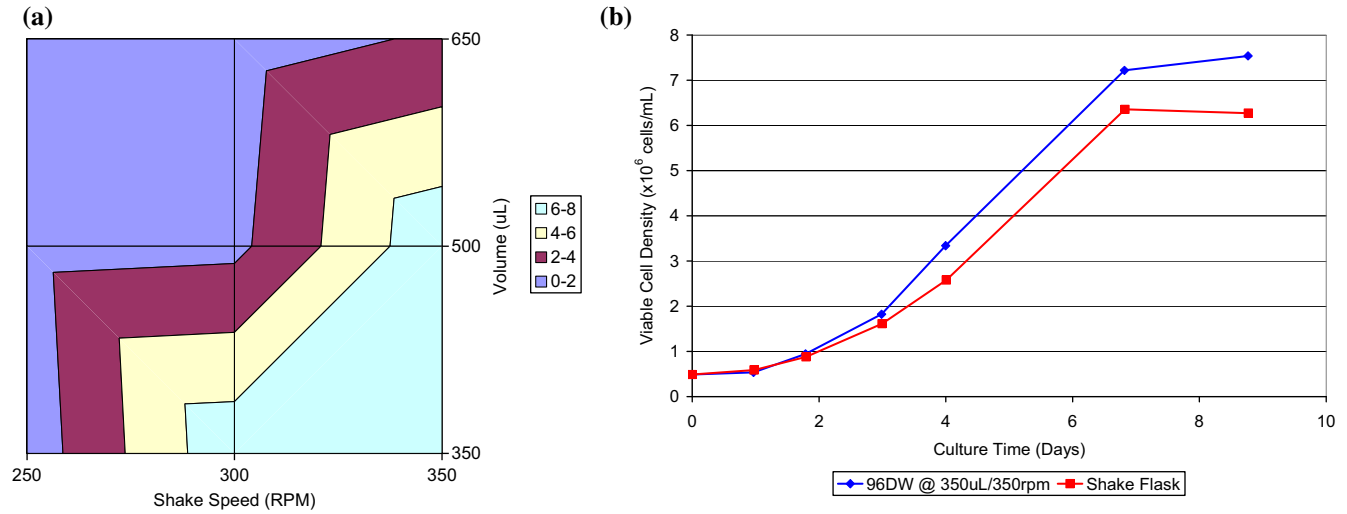

Figure 7. **(a) Viable cell density (× 10^6 per mL) surface plot of design of experiment with shake speeds of 250, 300, and 350 rpm and 96DW volumes of 350, 500, and 650 µL. (b) Comparison of growth in 96DW (350 µL and 350 rpm) with a 250 mL shake flask (60 mL working volume) shaken at 120 rpm. Data was generated from a single cell line; a pool of 8DW was counted on the Vi-CELL for each 96DW point.**

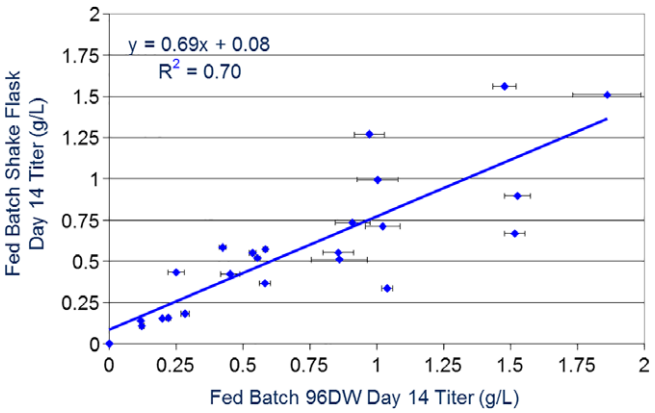

Figure 8. **Correlation of fed-batch shake flask day 14 titers vs. fed-batch 96DW day 14 titers. Ninety-six deepwell fed-batches of clones expressing monoclonal antibody B were run in duplicate and shake flasks were run in singlet. The error bars are one standard deviation.**

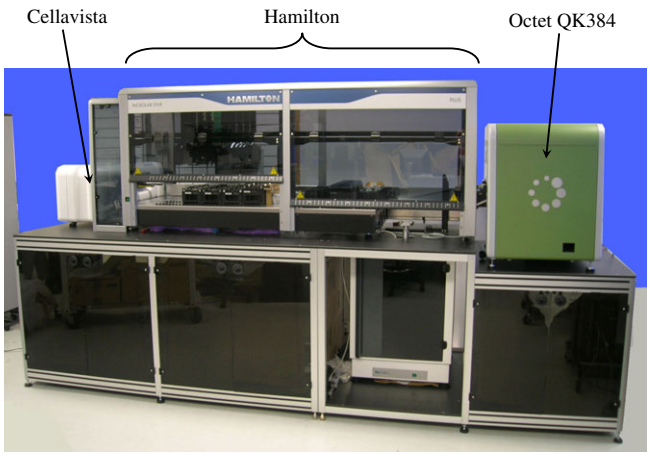

Figure 9. **Integrated high throughput screening tool consisting of a Hamilton STARplus liquid handler, Cellavista cell counter, and Octet QK384 protein quantitation tool.**

Table 2. Coefficient of correlations (R^2) for fed-batch shake flask day 14 titers vs. fed 96DW or shaken culture tube day 14 titers for various products. A#1, F and G are 96DW pooled from four wells for each data point; A#2 = 8 wells per data point

Product	Hamilton/96DW	Shaken culture tube
Antibody A #1	0.98	0.75
Antibody A #2	0.70	0.66
Antibody F	0.86	0.95
Antibody G	0.83	0.57

of a flow cell, a lower magnification, and the collection of fewer images.[35,36] However, high throughput viability measurement with the Cellavista still has utility as a relative gauge for determining the health of cells in a well, especially during initial transition to suspension culture and passaging.

These results were used to formulate a protocol for inoculating new cultures. For the passaging steps, cells were inoculated at specified viable cell densities. By using the Cellavista viability measurement, which read lower than the Vi-CELL viability, the new cultures would be seeded at a slightly higher cell density than would be the case with seeding based on Vi-CELL data. This was acceptable, as the risk of longer doubling times increases at lower, not higher inoculation densities. For the fed-batch analysis, in order to make the measurements more predictive we started the cultures for each cell line at the same density. Also, cells were inoculated at a specified total cell density rather than viable cell density because by the time the fed batch was initiated, the wells had been passaged multiple times to ensure that the cells had adapted to shaking culture. After these multiple passages, only the healthy cultures (e.g., ones with viability >90%) survived (unpublished data). Viability differences between wells would be minimal as a result. Determination of the total cell density is also more efficient than determination of viable cell density using the Cellavista.

The authors next quantified the variability of the integrated Octet protein titer measurement. Four 96w plates were created from a single sample of medium containing product B at a titer of 800 mg/L. Using the same two-step dilution that would be used in a fed-batch experiment (in this case, 1/10 and

Table 3. Total cell counts (TCD) and viability measured on the Cellavista and Vi-CELL from identical wells

	Cellavista		Vi-CELL	
n = 28	TCD ($\times 10^6$ cells/mL)	Viability (%)	TCD ($\times 10^6$ cells/mL)	Viability (%)
Average	1.5	89.5	1.7	97.0
Std. dev.	0.6	2.3	0.7	0.3
CV (%)	3.9%	2.6%	4.2%	0.3%

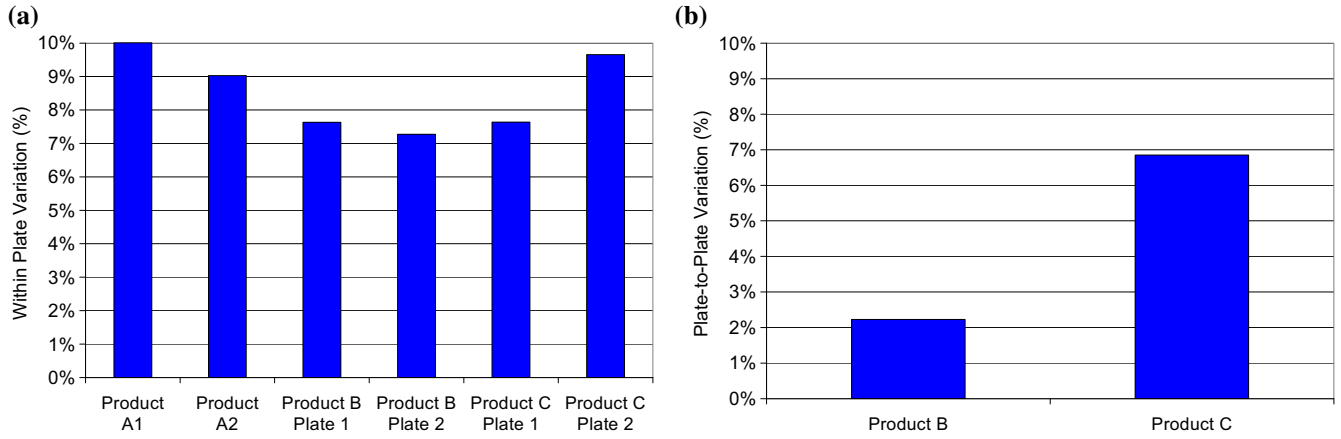

Figure 10. (a) Within plate titer variation (one standard deviation) for the Hamilton/96DW process for six plates (n = 96). (b) The plate-to-plate variation for monoclonal antibodies B and C (n = 192). Titer in diluted culture medium samples was measured using an Octet QK384.

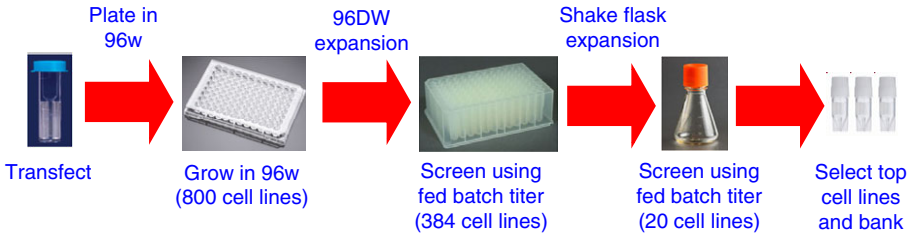

Figure 11. Overview of cell line development process with the Hamilton/96DW system.

1/1.6), four plates were prepared and measured on the Octet QK384. The overall coefficient of variation for all four plates was 3.6% (data not shown).

The within-plate and plate-to-plate variations of the 96DW fed-batch were also quantified. In this experiment, four clonal cell lines were used. Two were secreting antibody A (A1 and A2) and two others were producing two different antibodies (B and C). To simulate the actual cell line generation process, cultures of each cell line were plated into shallow 96w plates. One whole plate was plated for each cell line for antibody A, and two plates each for antibodies B and C were made. These cultures were transferred into 96DW plates, repeatedly passaged in those plates until they had good growth and viability, and then tested in fed-batch culture to quantify the productivity of the cells. The results of this experiment are shown in Figure 10. This work demonstrated that the integrated high throughput screen system achieved an average within-plate harvest titer variation of 8.5% and plate-to-plate variation below 7%. Thus, the titer variations were within the 10% goal that had been chosen to enable differentiation of productivity differences among clones.

Implementation of 96DW automated screening in the cell line development process

Having achieved our goal of developing an automated 96DW culturing system with sufficiently low variability, we modified our cell line development process to replace the static 24w plates and shaken culture tubes with the 96DW plates as shown in Figure 11. Like the standard process (Figure 1), cells are initially transfected with an expression plasmid encoding our product. These transfected cells are then selected and grown in static culture in 96w plates. The target of 800 cell lines are grown at this stage, to allow for low outgrowth rates. Then, the Hamilton system is used to passage 384 cell lines from the 96w static plate wells that are above the confluence threshold of 40% (determined on the Cellavista) into four 96DW plates. The Hamilton system is used to expand and culture these plates and then performs the first fed-batch screen. The top 20 cell lines identified in this screen are expanded for the second fed-batch evaluation, in a manual shake flask process. This larger volume shake-flask fed-batch screen allows for more frequent monitoring of the process, with glucose measurement and feeding as needed. The measurement of metabolites such as lactate and other culture characteristics can also be used to select the final clones.

Two production cell line generation projects were performed by running both the standard and new cell line generation processes in parallel (Figure 12). In both projects, the incoming clones for the parallel cell line screening arms were from the same transfection. For the first-round fed-batch screening of 437 total clones for antibody D, 76 clones were screened in shaken culture tubes while 361 were screened in

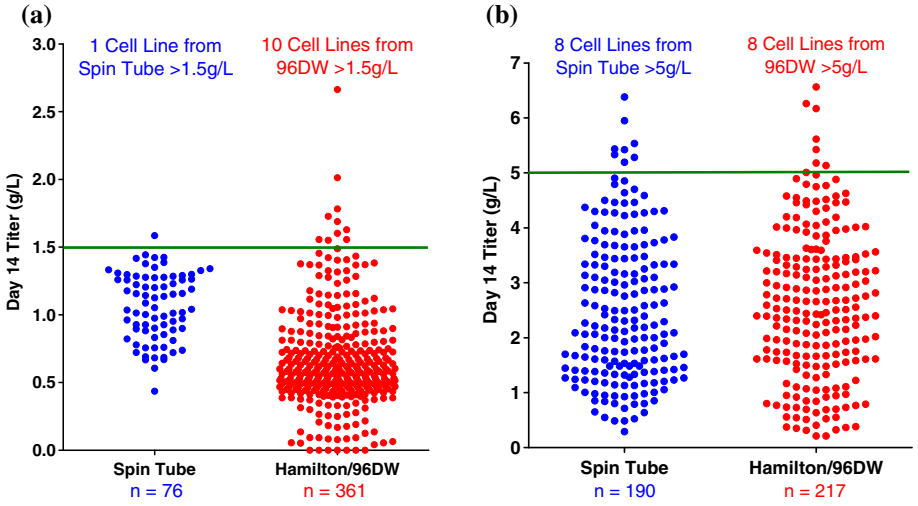

Figure 12. (a) Day 14 titers for clones expressing monoclonal antibody D in which the Hamilton/96DW process had a five times larger screen size than the standard process using shaken culture tubes. (b) Day 14 titers for fusion protein E in which both spin tube and Hamilton/96DW processes had similar screen sizes.

96DW (Figure 12a). The screen size of the 96DW process was five times larger than the shaken culture tube process and 10 times more cell lines were identified with day 14 titers greater than 1.5 g/L. This demonstrates the power of screening larger numbers of cell lines. For fusion protein E, approximately the same number of clones were evaluated in parallel by the first fed-batch screen in both cell line generation processes: 190 in shaken culture tubes and 217 in 96DW (Figure 12b). Each process independently identified eight cell lines producing titers greater than 5 g/L. These data confirm that the dynamic range of the titers from the cell lines screened in the automated 96DW screening process is the same as that obtained in the standard process.

Conclusion

The objective of cell line development for biopharmaceutical production is to generate a clonal cell line expressing maximal amounts of the protein of interest with the desired characteristics. To this end, often multiple scientists work together on a project for a single protein, performing multiple transfections, selecting and screening hundreds or even thousands of clones, as many as possible in the space and time available. These activities deliver a panel of high-yielding cell lines which are then evaluated for bioreactor performance and stability of productivity and product quality over long term culture to select the manufacturing clone. Increasing the number of clones evaluated will increase the likelihood of identifying rare high titer clones,[28] however we used a rational approach to determine how many clones to screen based on the target expression levels of a final panel of six clones and the desired probability of achieving that target. The authors chose to screen adequate clone numbers for a 99% confidence that six clones in the top 95th percentile for productivity were included in every predictive screening step. The authors confirmed that reliable prediction of relative productivity of CHO clones could best be achieved by using fed-batch as the screening step.[10] Two screening steps were planned: a first screen of a large number of clones in small volumes, and a second screen of a smaller number of clones in higher volumes and with more control of the culture process. Statistical methods were used to determine the screen sizes, with binomial distributions as well as asymmetric model populations based on historical data. The impact of increasing stringency was examined, both from the point of view of the increase in productivity of the chosen clones, and the labor required. To achieve the greater than 99% probability of sampling six cell lines from the top 95th percentile, an initial screen size of 384 clones was required. The size of the second, larger volume screening step was then determined to be between 20 and 40 to ensure that sufficient clones from the top 95th percentile are retained. The analysis showed that an average of 12 clones from the top 95th percentile will be among the 20–40 retained, given a sufficiently good correlation between the first small volume and the second large volume fed-batch screens of R^2 = 0.5–0.8.

The goal of a 384-clone screen determined by our rational approach was achieved with a predictive, high throughput, 96DW fed-batch screening platform that was enabled by incorporating automation into our process. A high-throughput automated liquid handling system for cell line development was reported by Lindgren[21] that was used to screen static batch cultures, which were then evaluated manually by fed-batch. Other automated systems, reviewed by Estes,[27] include the ambr microscale bioreactor system, which functions as a scale-down model of fed-batch bioreactors,[37] testing up to 48 cell lines in parallel, an excellent option for the second-round screen. Two publications describe plate-based automation for use in bioreactor process development.[25,26] Haines et al.[24] presented on an automated liquid handling system to maintain cell lines during evaluation but did not integrate titer and cell density determination into the platform. Automation is also being introduced earlier in the cell line development process for imaging single cells deposited into wells of multi-well plates to satisfy regulatory requirements to demonstrate clonal-derivation of cell lines.[5–9] Furthermore, miniaturization of the cell line screening processes with advances in microfluidic and nanofluidic instruments is underway to allow evaluation of large numbers of clones.[28]

Using shaken 96DW plates and an integrated automation system consisting of a Hamilton STARplus, Cellavista, and Octet QK384, we increased the number of cell lines that could be screened simultaneously by a single scientist in a predictive

fed-batch test to 384. This system also was shown to have the ability to identify cell lines with titers greater than 6 g/L for antibody and fusion protein projects, and could support four simultaneous 384 well evaluation projects, provided they had staggered initiations. The 96DW plate format we chose was designed for automated liquid handling. Using the 96-well format resulted in a smaller footprint than would the equivalent number of wells in 24-well plates. The square shape of the Greiner plate wells enhanced aeration at any given speed,[30] and the Duetz lids[31] decreased evaporation at the edges of the plates, compared with other shaking plate lids tested. A shaking speed of 350 rpm and volume of 350 μL were optimal for 96DW plate cell growth to correlate with shake flask cultures. These settings were also the maximum shaking speed of the Multitron incubator and the minimum volume in the 96DW plates to avoid decreased viability from evaporation. Pipetting and analytical measurement accuracy were tested using cell counts and titers, demonstrating that variabilities within and between plates were less than the 10% threshold. Automation allowed automatic data tracking, eliminating data transcription errors. A SQL database with Visual Basic or SAS queries provided more efficient data analysis and visualization.

Several parallel cell line development trials demonstrated that a single operator could maintain 384 different clones, expanding them in suspension culture and subjecting them to a predictive fed-batch screen, finally choosing the best 20–40 to take forward to larger volume screening procedures. The system we developed is modular and allows for integration with other automation-friendly analytical tools to identify cell lines with low product aggregation, optimal product glycosylation and desirable metabolite profiles, or other important properties.[25,26,38,39] These additional screening parameters increase the power of the cell line generation process, eliminating less desirable clones earlier. The automated culturing and screening system described here has widespread applications across the industry due its increased predictive screening capacity as well as the flexibility to handle future challenges in biopharmaceutical cell culture.

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