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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

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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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 = ΔVP/ΔIVCC. 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 ΔIVCC 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 ΔIVCC 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 μl of activation buffer (0.1 M NaH_2PO_4, pH 6.2) and then activated for 20 min at RT by addition of 20 μl of 50 mg/ml Sulfo-NHS (Thermo Fisher Scientific) and 20 μl 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 μl of activation buffer before addition of the antibody (5 μg 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 μl 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 μl of standards or cell supernatants diluted with PBS-TBN were mixed with 50 μl of capture beads (2,000 beads per well) and incubated for 2 hr at room temperature. Beads were then washed twice with ×1 phosphate buffered saline/tween (PBST) on a Tecan HydraSpeed plate washer equipped with a magnetic plate carrier (Tecan). Beads were incubated with 100 μl of 4 μg/ml selected biotinylated mAbs in PBS-TBN for 1 hr and after two washes followed by 30 min incubation with 100 μl of 2 μg/ml streptavidin-PE (Thermo Fisher Scientific) at RT. All solutions were then removed, and beads were suspended in 150 μl 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 μg/ml of 4I22 (100 μl per well in PBS) overnight at 4°C. The plate was washed twice with 300 μl of wash buffer containing 0.05% Polysorbate 20 (PS20) in PBS. Blocking buffer (1% BSA in PBS, 200 μl 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 ×1 PBS w/o Ca^{2+}/Mg^{2+}) were added at 100 μl per well in triplicates. After 1 hr incubation, plates were washed four times and 100 μl per well of biotinylated 8I21 antibody (0.25 μg/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 μl per well and incubated for 30 min at RT. Following four additional washes, TMB (3,3′,5,5′-tetramethylbenzidine) substrate (100 μl per well) was added. Color development was stopped by adding 100 μl 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 μg/ml in sample diluent buffer (ForteBio). CMV Pentamer standard dilutions were prepared at 2.5–150 μg/ml range. Aliquots of 200 μl 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-quad (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 electrophoresys (SDS-PAGE) and Western blot analysis under boiled and reduced condition. For SDS-PAGE, 8 μg 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 (Genscript) 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 μl 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 × 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 Pentamer[7] 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 μg/ml analyte levels are required for BLI quantification. In our Octet assay, the limit of quantification (LOQ) was determined at 2.5 μg/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

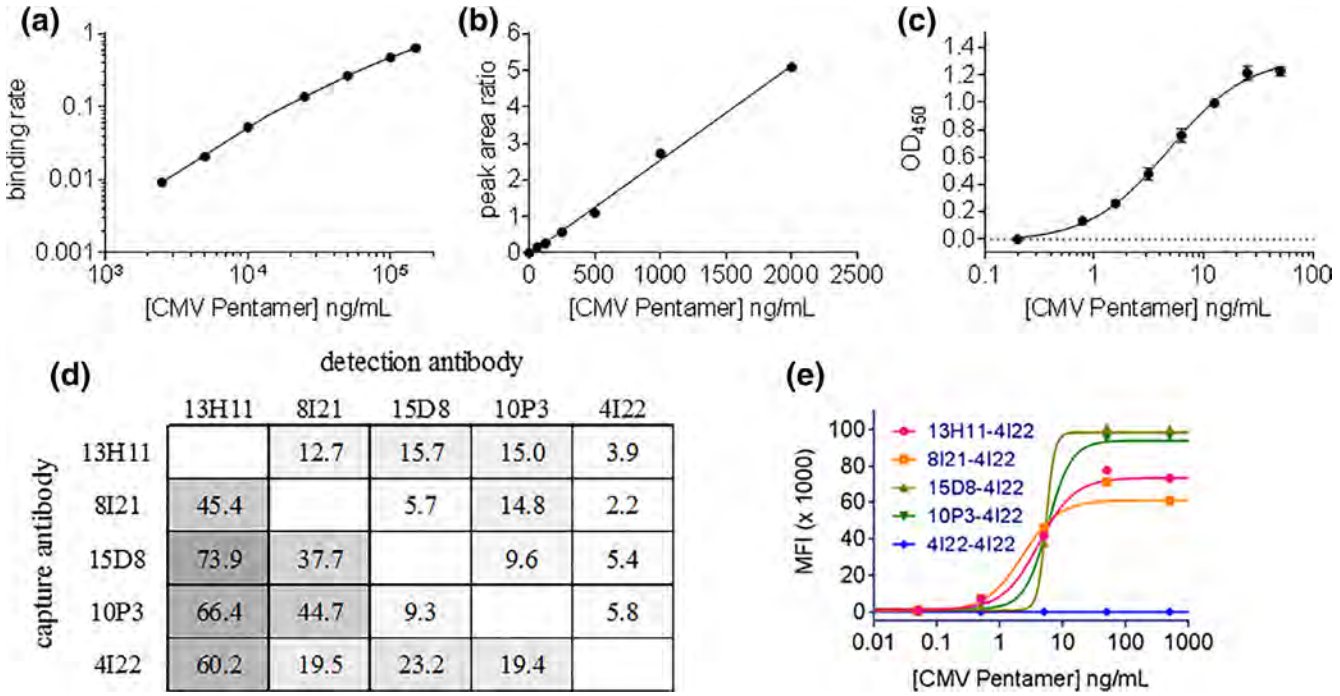

FIGURE 1 The assessment of quantitation assays for CMV Pentamer. CMV Pentamer standard curves were generated with four different assays. (a) Bio-layer interferometry (BLI) assay—Octet biosensors coated with 4I22 capture antibody; (b) Immuno-capture MS assay with 4I22 antibody; (c) Sandwich ELISA assay 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 μL 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 μg/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 μg/ml ranges; whereas, BLI assay was only applicable for the samples at μg/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

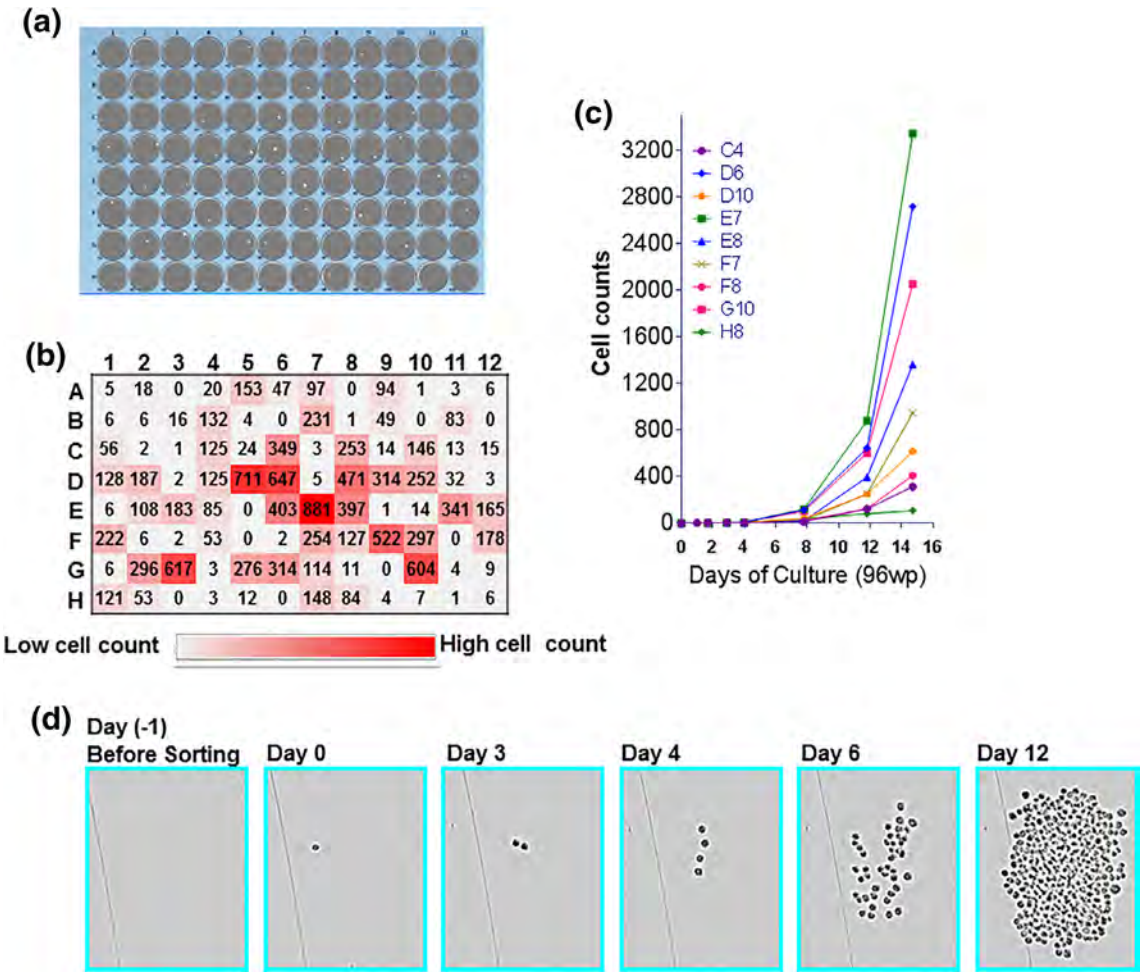

FIGURE 2 Cell counting with Cell Metric CLD. (a, b) Thumbnail views of colony outgrowth and cell count for individual clones of a representative 96-well plate at Day 12 after single cell cloning; (c) growth curves of selected clones (N = 10) with distinct growth rates; (d) a representative monoclonality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

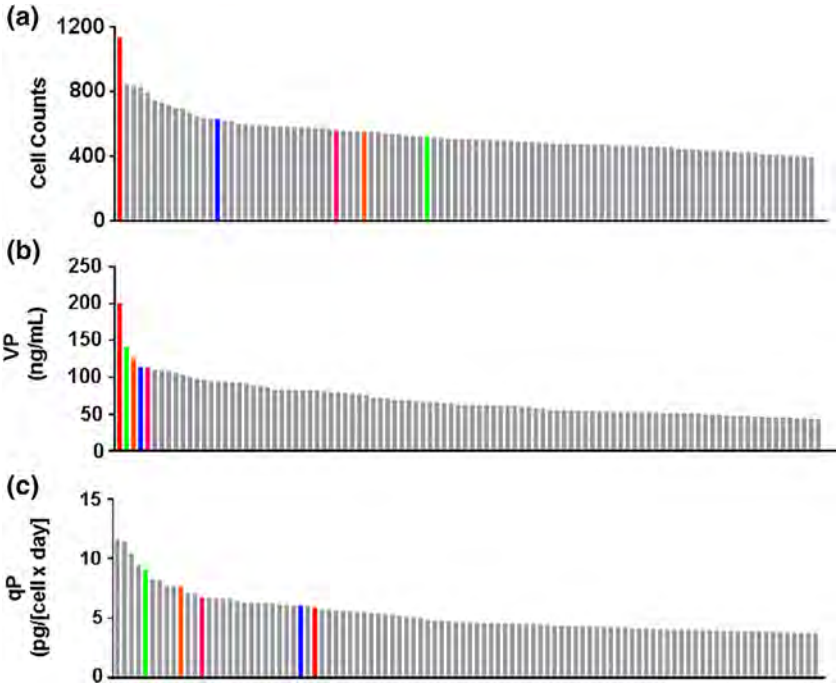

FIGURE 3 Early clone characterization: cell count, volumetric productivity (VP), and specific productivity (qP). Individual clones were generated with FACS enrichment and single-cell sorting, and seeded in 96-well plate with 200 μl of expansion medium per well. The 96-well plates were kept in static incubator for 14 days allowing clone outgrowth and monoclonality evidence generation. Cell culture of 100 μl 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 = ΔVP/ΔIVCC. 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

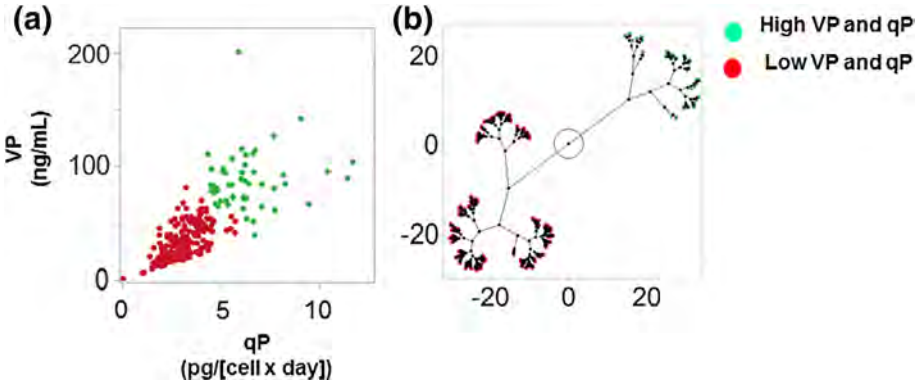

FIGURE 4 Multivariate analysis of clone productivity. Recovered clones (N = 269) at 96-well plate culture stage were clustered based on their volumetric productivity (VP) and specific productivity (qP) value. (a) Scatter plot and (b) Constellation plot

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

	Site7	gH	Site1	Site4	Site5
Clone ID	8I21	13H11	15D8	10P3	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

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

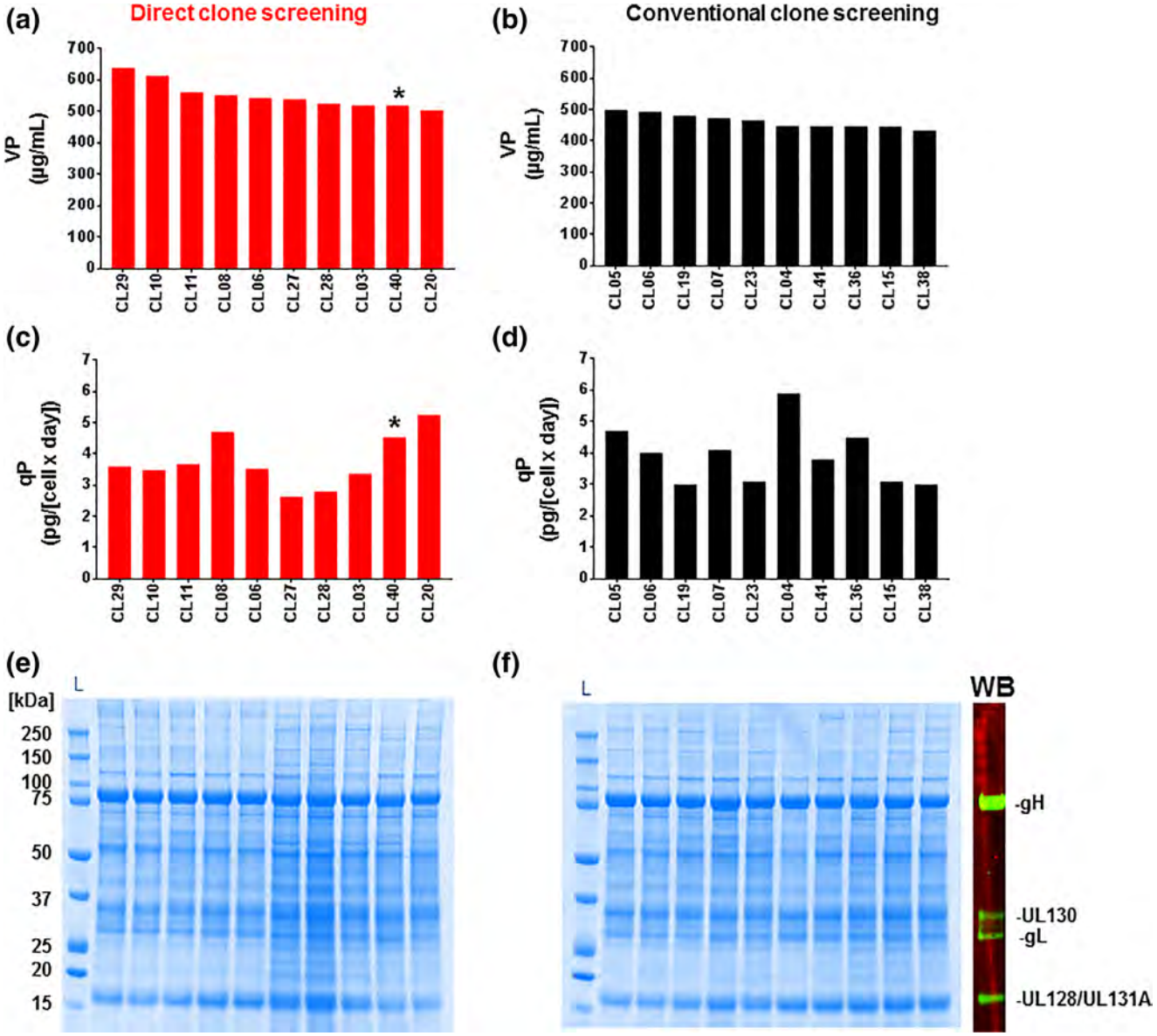

FIGURE 5 The productivity and product quality comparison of top clones selected by conventional and direct early clone screening processes. Volumetric productivity (VP) and specific productivity (qP) were determined by Octet assay using anti-CMV Pentamer mAb 4I22 for fed-batch cell culture supernatants. 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 multivariant 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-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.

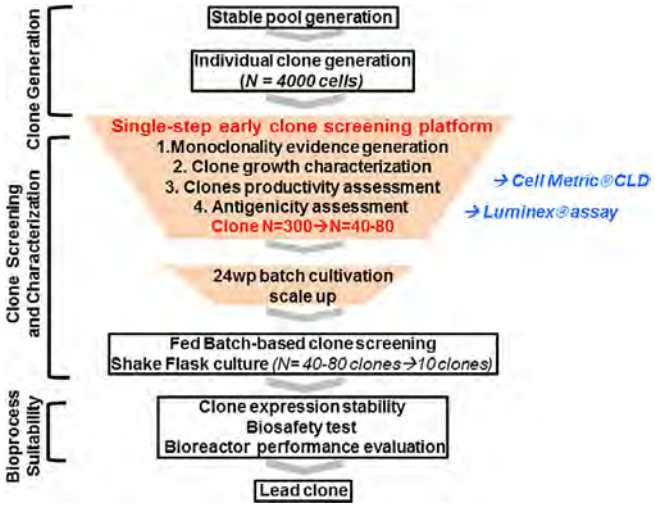

FIGURE 6 Proposed single-step early clone screening process with the integration of Luminex/Cell Metric CLD platform

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.

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CONFLICT OF INTEREST

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REFERENCES

1. 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.
2. 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.
3. 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.
4. 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.
5. Wen Y, Monroe J, Linton C, et al. Human cytomegalovirus gH/-gL/UL128/UL130/UL131A complex elicits potently neutralizing antibodies in mice. *Vaccine*. 2014;32(30):3796-3804.
6. 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.
7. 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.
8. 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 potently neutralizing human antibodies. *PLoS Pathog*. 2015;11(10):e1005230.
9. 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.
10. Chen LZ, Roos D, Philip E. Development of immunocapture-LC/MS assay for simultaneous ADA isotyping and semiquantitation. *J Immunol Res*. 2016;2016:7682472.
11. Chandramouli S, Malito E, Nguyen T, et al. Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. *Sci Immunol*. 2017;2(12):eaan1457.
12. Petersen RL. Strategies using bio-layer interferometry biosensor technology for vaccine research and development. *Biosensors (Basel)*. 2017;7(49):1-15.
13. 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.
14. Grav LM, Lee JS, Gerling S, et al. One-step generation of triple knockout CHO cell lines using CRISPR/Cas9 and fluorescent enrichment. *Biotechnol J*. 2015;10(9):1446-1456.
15. 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.
16. Lekomtsev S, Aligianni S, Lapao A, Burckstummer T. Efficient generation and reversion of chromosomal translocations using CRISPR/Cas technology. *BMC Genomics*. 2016;17(1):739.
17. Alvin K, Ye J. Generation of cell lines for monoclonal antibody production. *Methods Mol Biol*. 2014;1131:263-271.
18. 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.
19. 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.
20. 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.
21. 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.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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.

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