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Research paper

Sequential screening by ClonePix FL and intracellular staining facilitate isolation of high producer cell lines for monoclonal antibody manufacturing

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ABSTRACT

Screening and characterization of cell lines for stable production are critical tasks in identifying suitable recombinant cell lines for the manufacture of protein therapeutics. To aid this essential function we have developed a methodology for the selection of antibody expressing cells using fluorescence based ClonePix FL colony isolation and flow cytometry analysis following intracellular staining for immunoglobulin G (IgG). Our data show that characterization of cells by flow cytometry early in the clone selection process enables the identification of cell lines with the potential for high productivity and helps to eliminate unstable cell lines. We further demonstrate a correlation between specific productivity (qP) and intracellular heavy chain (HC) content with final productivity. The unique combination of screening using ClonePix FL and the flow cytometry approaches facilitated more efficient isolation of clonal cell lines with high productivity within a 15 week timeline and which can be applied across NS0 and CHO host platforms. Furthermore, in this study we describe the critical parameters for the ClonePix FL colony based selection and the associated calculations to provide an assessment of the probability of monoclonality of the resulting cell lines.

1. Introduction

The generation of stable cell lines that express therapeutic monoclonal antibodies (mAbs) for manufacturing purposes is a time and labor intensive process. Since cell line development is on the critical path to clinical testing, timely generation of highly productive manufacturing cell lines is essential to remain competitive in the biopharmaceutical industry. Cell line development (CLD) for NS0 (Bebbington et al., 1992) and Chinese hamster ovary cells (CHO), the leading mammalian cell line platforms in the biopharmaceutical industry for the production of antibodies, generally requires the screening of hundreds of primary cell lines to identify high producers. This process is labor intensive and may take months to complete (de la Cruz Edmonds et al., 2006; Fussenegger et al., 1999; Wurm, 2004). A number of metrics have been used to predict the potential of primary transfectant cell lines for high productivity early in the clone selection process. The methods aimed at increasing screening efficiency and discriminating high producing cells include FACS-based high throughput cell sorting (DeMaria et al., 2007), measurement of heavy chain (HC) and light chain (LC) mRNA or gene copy levels (Wurm, 2004), nucleic acid capture assay (Tsai et al., 2003), and QuantiGene Plex (QGP) assay to measure HC or LC mRNA levels (Lee et al., 2009) as well as by ClonePix FL (Molecular Devices, CA) (Nakamura and Omasa, 2015; Serpieri et al., 2010). However, none of these methods takes into account early cell line instability of mAb expression, an important criterion that could be used as an elimination tool during the clone selection process. Because of this, we sought to develop a method that was both efficient and reliable in generating manufacturing cell lines. In antibody expressing cells this can be readily achieved by intracellular (IC) staining of antibody HC and LC followed by flow cytometric analyses (Strutzenberger et al., 1999).

In this paper, we describe a dual screening method to reliably identify high producers early in the CLD process. First, ClonePix FL was used for the isolation of high titer mAb-expressing NS0 primary transfectants. The isolation is based on the fluorescence intensity of a halo formed upon precipitation of secreted antibody around colonies that are grown in a semisolid medium (Caron et al., 2009; Contie et al., 2013). We were able to pick secreting colonies and discard non-expressing ones. We further screened picked colonies by measuring early qP to determine the levels of secretion and also measured HC and LC content

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by intracellular staining (IC) followed by flow cytometry. A lack of correlation between HC and LC expression and IgG secretion has been previously reported (Kang et al., 2014; Meilhoc et al., 1989). However, here we demonstrate a strong correlation between early qP and intracellular HC and also with end-of-run fed-batch titers. Together, these methods allowed us to discard low expressing and/or unstable primary colonies from consideration as early as 4 weeks after transfection, to focus on higher potential cell lines.

An important consideration in the engineering of manufacturing cell lines is the clonality of the cell lines (Frye et al., 2016). Limiting dilution and FACS sorting are proven methods for the clonal isolation of mammalian cell lines (Hulett et al., 1969; Coller and Coller, 1983). But prior attempts to prove the probability of monoclonality using ClonePix FL were limited in scope (Ahmed et al., 2009) Here, we present a statistically validated probability of monoclonality (*P(clonality)*) equation and defined critical parameters to ensure clonality of stable cell lines isolated using ClonePix FL. Taken together the process described here enables the rapid generation of cell lines for high titer clinical development enabling stable expression from NS0 cell lines for high titer clinical development with a high probability of monoclonality enabling stable expression. This dual approach is equally effective in CHO host platform resulting in a significant reduction of workload.

2. Materials and methods

2.1. Cell culture and isolation of IgG expressing primary isolates by ClonePix FL

NS0 cells growing in animal protein-free (APF) proprietary media were transfected by nucleofection (Lonza, Slough) using linearized plasmid DNA containing single copy genes of antibody HC, LC, and the glutamine synthetase (GS) selection marker. Expression of HC and LC was driven by CMV promoters, whereas GS expression was under the control of an SV40 promoter.

Transfected cells were grown in the presence of 10% FBS and GS supplement (SAFC Biosciences, KS, USA) on ClonaCell TCS semisolid media (Stem Cell Technologies, Vancouver) or CloneMatrix (Molecular Devices, CA) that contained a fluorophore-labeled antibody cross-linking reagent. IgG producing colonies were imaged using a ClonePix FL instrument (Molecular Devices, Sunnyvale, CA), ranked by sum total intensity (STI) and picked into 96-well plates containing glutamine-free IMDM (SAFC Biosciences, KS) supplemented with 10% dialyzed FBS and 1x GS supplement, and then cultured in a humidified environment at 37 °C with 5% CO_2. During cell expansion stage cells were gradually weaned off of serum and scaled up in shake flasks in APF production medium and shaken at 100 rpm at 37 °C with 5% CO_2 and 80% humidity in a Multitron incubator (Infors, Switzerland). Cell viability and viable cell densities (VCD) were determined by ViCell (Beckman Coulter, CA), and IgG titers were measured by Octet (Pall Corporation, NY) using Protein A sensors or by Protein A HPLC.

For a 14-day fed-batch suspension assay, cells were seeded at 1×10^6/mL in APF medium, sampled for cell viability and IgG titer, and fed with a nutrient-rich feed formulation on days 2, 4, 6, 8, 10, and 12.

Suspension CHO cells were grown in CD CHO (Thermo Fisher Scientific, CA) medium (Shridhar et al., 2017).

2.2. Intracellular staining of heavy and light chains and analysis by flow cytometry

Intracellular staining was performed on cells growing in log phase at the beginning of the fed-batch suspension assay. 1×10^6 cells were resuspended in FACS buffer consisting of 1x D-PBS with 5% FBS (Thermo Fisher Scientific, Carlsbad, CA). The cells were fixed and permeabilized using Fix and Perm reagents A and B according to the manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA).

Table 1
Isolation of IgG Expressing Primary Isolates by ClonePix FL.

Screening criteria	Number of colonies
Fluorescence based colony screening	106,889
Colony picking	601
Live colonies screened for IgG titer	487
Colonies expressing > 10 μg/ml	66
Fed-suspension assay	26
Primary isolates selected for cloning	7

Table 2
Comparison in colony ranking using different metrices.

Samples	Titer (mg/L)	Fed-batch Titer	Early qP	HC MFI	HC MFI CV
10A1	4600	1	2	4	1
13D1	4400	2	1	5	5
3B3	3100	3	9	11	12
2B3	2860	4	5	6	4
12A10	2800	5	3	3	3
7F8	2500	6	11	2	2
13C1	2300	7	10	9	13
7A5	2100	8	6	16	14
12B5	2000	9	7	12	11
8A5	1800	10	16	13	15
13B4	1300	11	15	14	10
11C1	1090	12	13	1	8
12A5	1000	13	20	24	11
4B1	1000	14	12	20	19
5B1	982	15	22	17	17
13A4	971	16	8	15	16
12C10	921	17	18	23	21
4B2	890	18	21	19	20
7B11	758	19	4	10	9
12A1	751	20	19	18	18
6B1	678	21	14	7	6
15A1	657	22	17	8	7
15B1	575	23	24	21	23
7B3	544	24	25	26	26
4C2	426	25	23	22	22
7D1	0	26	26	25	25

Intracellular HC and LC were stained with 5 μg/mL of each of Alexa-488 labeled anti-human Fc specific and APC-labeled anti-human light chain specific (Thermo Fisher Scientific, Carlsbad, CA) antibodies. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), a standard instrument for flow cytometry analysis. The sensitivity for the FACSCalibur is at 750 molecules of equivalent soluble fluorochrome. The purpose of our study was to qualitatively, compare clone expression patterns by CV and mean fluorescence intensity (MFI) where a correlation could be determined between expression of HC and LC alone or in combination with IgG secretion and colony production capability. Data from these studies was found to be qualitatively similar with that acquired for other studies on newer, more sensitive instruments such as LSRII (BD Biosciences, San Jose, CA) with fluorescence sensitivity of less than 80 molecules of equivalent soluble fluorochrome. The flow cytometry data were processed with FlowJo software (Tree Star Inc., Ashland, OR).

2.3. Determination of specific productivity rate (SPR)

Cells were seeded in an APF proprietary medium in a shake flask at a density of 1×10^6/mL. At 48 to 72 h, IgG titers and cell counts were measured as described above. Specific productivity rate (SPR = qP), a function of both titer and growth rate, was expressed in terms of pg $cell^{-1}$ day^{-1} (pcd) using the calculation as described by (Brezinsky et al., 2003). qP was determined at early passage during the initial colony expansion stage and was used as a tool to detect early colony instability.

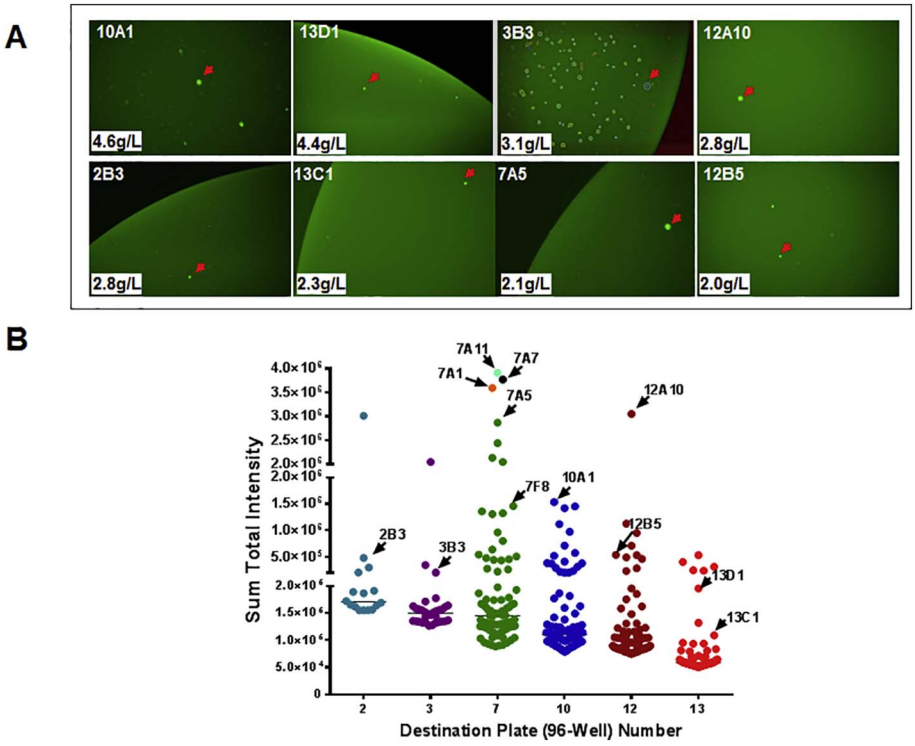

Fig. 1. Identification of high expresser colonies picked by ClonePix FL. **A**: Fluorescent ClonePix images of colonies formed on solid medium directly from a transfection. Arrows depict isolates retrospectively identified as highly productive. Fed suspension productivity determined for each colony is shown in boxes with the figures. **B**: Statistics of colonies picked into multiple 96 well destination plates based on sum total intensity (combined fluorescence intensity of the halo inside and outside of the colony) and subsequently evaluated for IgG titer. The top ranking clones determined by fed-batch suspension titers are shown by the arrows. The best producers from each destination plate contain high sum total fluoresce intensity and are among the top 8% of clones that were picked. Three highly fluorescent colonies, marked by arrows, such as 7A1 and 7A7 did not survive expansion to suspension growth while 7A11 was eliminated due to instability.

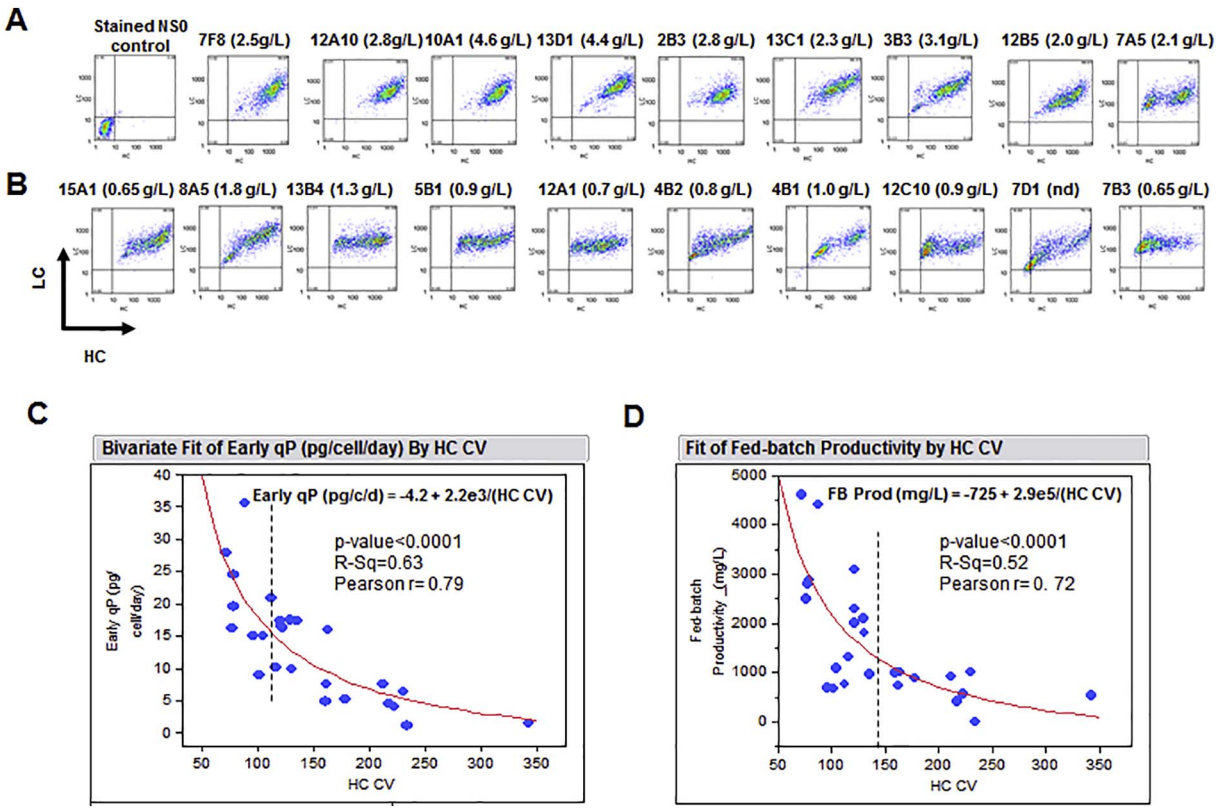

Fig. 2. Qualitative and quantitative flow cytometry scatter analyses of primary isolates intracellularly stained for antibody HC and LC. **A**: Flow cytometry scatter plots of primary isolates with homogeneous distribution of HC and LC expressing greater than 2.0 g/L of mAb A. **B**: Flow cytometry scatter plots of primary isolates with a heterogeneous distribution of cells expressing HC and LC with final IgG productivity less than 2 g/L **C**: Inverse correlation between early qP and the CV of HC mean fluorescence intensity of flow cytometry scatter plots derived from individual cells. The mean fluorescence intensity is a measure of HC expression in primary isolates stained for intracellular antibody HC. **D**: Inverse correlation between final fed-batch productivity and the CV of flow cytometry scatter plots derived from individual cells. The mean fluorescence intensity is a measure of HC expression in primary isolates stained for intracellular antibody HC.

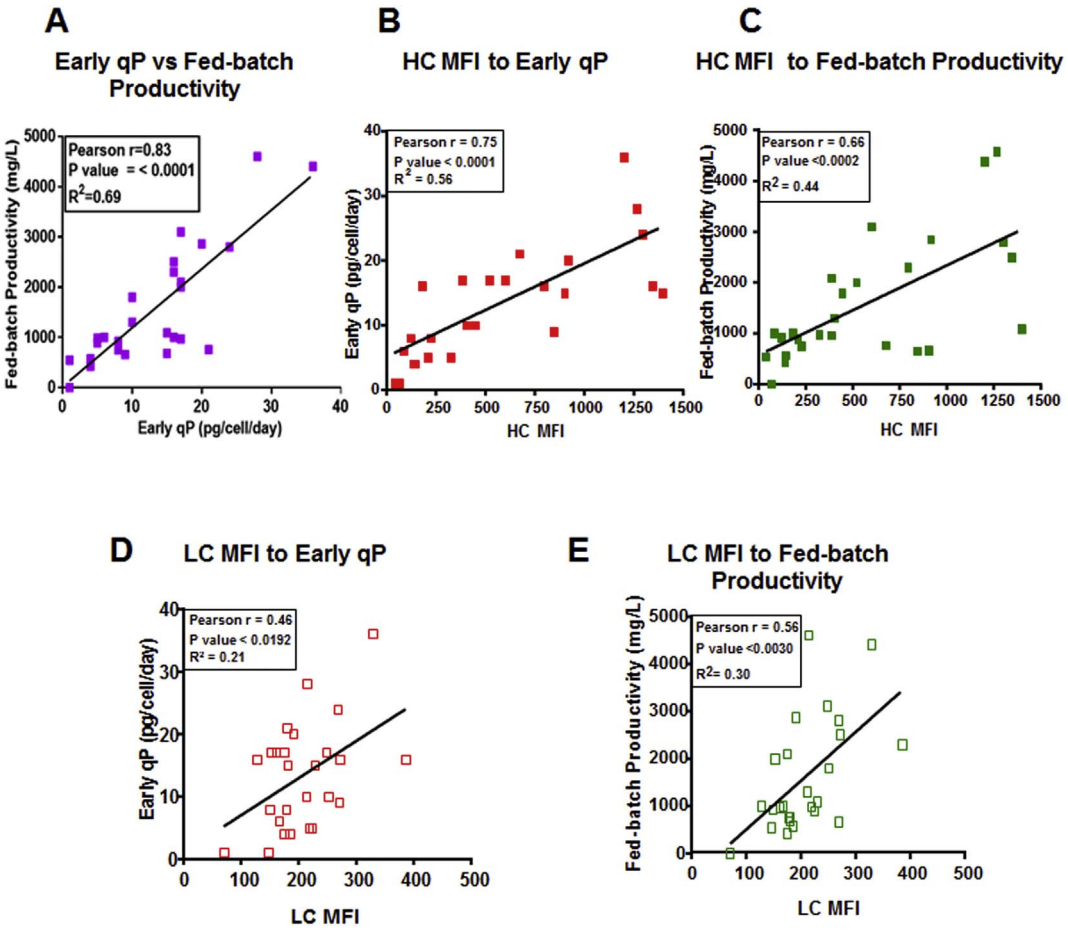

Fig. 3. Correlation between fed-batch productivity, early qP and HC mean fluorescence intensity as a measure of HC expression leading to IgG productivity. **A** Linear correlation between early qP and 14-day fed-batch productivity. **B**: Correlation between HC MFI (mean fluorescence intensity) derived from FACS-based IC staining assay, to early qP. **C**: Correlation between HC MFI and 14-day fed-batch productivity. **D**: Correlation between LC MFI and early qP. **E**: Correlation between LC MFI and 14-day fed-batch productivity.

2.4. Generation of GFP and RFP expressing stable pools in a CHO Flp-In host for determination of probability of monoclonality

A suspension-adapted CHO cell line was transfected with a plasmid containing a CMVpromoter-FRT-hCD4 cassette and a SV40promoter-GS cassette, subjected to GS selection, and followed by single cell cloning. One of these clonal cell lines was then co-transfected with a Flp recombinase expression vector (Thermo Fisher Scientific, Carlsbad, CA) and a promoterless-FRT-hygroR/CMV promoter-GFP or RFP vector, followed by selection with Hygromycin B, which yielded isogenic GFP or RFP positive homogeneous stable pools. GFP and RFP expressing cells in suspension were mixed and plated in semisolid CloneMedia for CHO (Molecular Devices, Sunnyvale, CA) and incubated for 14 days before they were picked by a ClonePix FL into 96 well plates. At 7–10 days post picking, clones were further screened by flow cytometry in high throughput mode using appropriate lasers for GFP and RFP using a BD LSRII instrument (BD Biosciences, San Jose, CA). Optimal compensation was applied to minimize overlap in wavelengths for better separation of green and red fluorescent proteins for accurate detection of mixed colonies.

2.5. RNA isolation and quantitative PCR (qPCR)

Total RNA was isolated from primary isolates using an RNeasy plus mini kit (Qiagen), and reverse transcription (RT) was performed using TaqMan Reverse Transcription Reagents (Life Technologies, Grand Island, NY, USA) with 2 μg RNA according to the manufacturer's protocol. LC, HC and GAPDH mRNA expression levels were measured using TaqMan technology on the 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA). The probes and primers were generated by assays-by-design (Applied Biosystems); the probes contain a 6-carboxy-fluoresceinphosphoramidite (FAM dye) label at the 5′ end of the oligo and non-fluorescent quencher at the 3′ end. qPCR was carried out by denaturing at 95 °C for 20 s and then cycling at 95 °C for 1 s and 60 °C for 20 s, for 40 cycles. Rodent glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) was used as the endogenous RNA control (Applied Biosystems). Expression levels of LC and HC were normalized to endogenous rGAPDH.

2.6. Statistical methods

The results obtained from intracellular staining (FACS), the fed-batch culture studies and the qPCR RNA expression were subject to correlation analyses using simple regression. Linear regression was performed with either JMP v 8.0.1 or GraphPad Prism v 6.0. In the case of Fig. 2, an inverse transformation was used to develop the relationship between specific productivity and expression (titer), and the coefficient of variability (CV) for HC observed in the FACS analysis. Regression analyses were accompanied by the R-Sq coefficient, Pearson "r" coefficient and *p*-values for showing statistical significance, that is, slope ≠ 0.

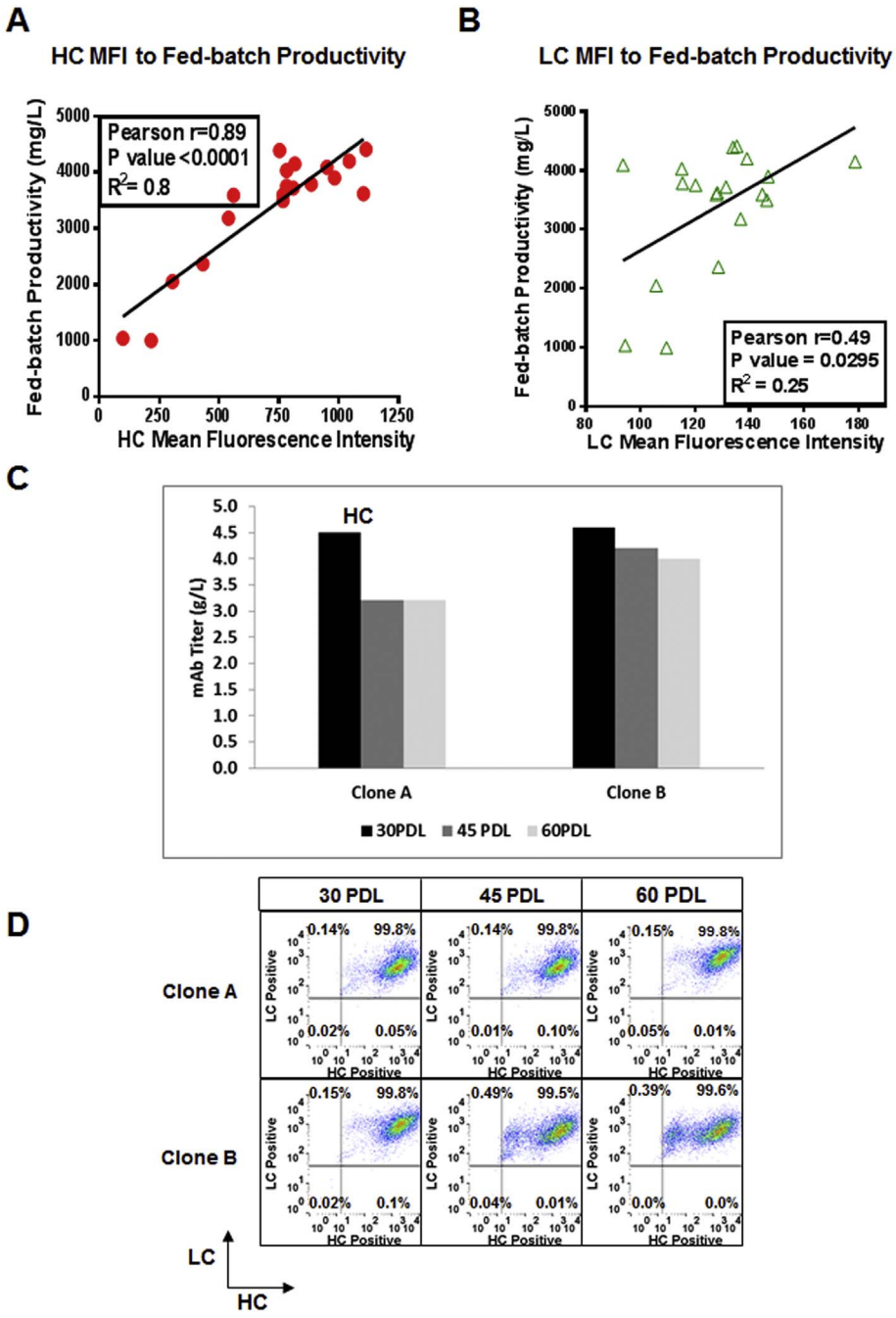

Fig. 4. A. Correlation between HC MFI and 14-day fed-batch productivity in clonally derived stable IgG cell lines. **B**. Correlation between LC MFI and 14-day fed-batch productivity in clonally derived stable IgG cell lines. **C**. IgG expression over 60 generations in top 2 clones isolated by dual screening methods using ClonePix FL and intracellular staining of HC and LC followed by flow cytometry. **D**. Corresponding flow cytometry scatter plots of intracellular HC and LC measured over 60 generations for Clone A and Clone B.

3. Results and discussions

3.1. Isolation of IgG expressing primary isolates by ClonePix FL

ClonePix FL is an automated robotics-based screening and picking system which detects Ab expressing colonies by measuring the fluorescent intensity of a halo of immunoprecipitated IgG around colonies of cells as a marker of antibody secretion (Nakamura and Omasa, 2015). In this study, NS0 mAb-A producing primary transfectants were isolated by applying selection to cells suspended in a semisolid matrix in the presence of a fluorophore-labeled antibody cross-linking agent. As the cells secrete antibody, the fluorescent detection reagent cross-links the secreted antibodies in the vicinity of the colony, forming an insoluble immune complex. The accumulated protein is referred to as a halo, which the ClonePix FL quantifies based on fluorescence intensity. Image analysis, by Clonepix software considers a number of parameters including halo size and intensity, shape (axis ratio or compactness), and distance between nearest neighbor colony (proximity) to allow decisions on colony isolation based on parameters chosen by an operator. As halo size and intensity are related to the amount of target protein secreted by the cells, this can be used to select cell colonies with the highest productivity.

We surveyed approximately 100,000 GS-NS0 primary transfectant colonies using a ClonePixFL, of which 601 were picked based on sum total intensity (combined fluorescence intensity of the halo inside and outside of the colony). While expanding in 96-well plates under selective conditions, some cell lines lose IgG expression due to early stage gene instability. Of the 601 picked colonies, 487 were capable of establishing viable cultures that were then assessed for mAb production in a 96 well screen. The top 26 expressing isolates surviving the serum weaning process were chosen for further evaluation in a fed-batch culture assay. The screening cascade is summarized in Table 1.

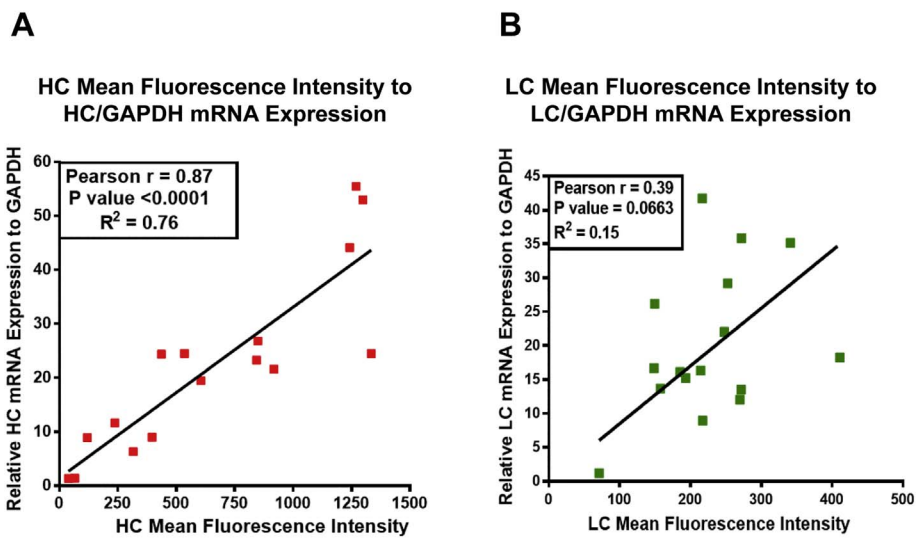

Fig. 5. Correlation between mRNA and protein expression levels for HC and LC. **A**. Correlation between HC mRNA expression measured and expressed by real-time PCR as ratio to GAPDH and the HC protein levels determined by flow cytometry-based intracellular staining. **B**. Correlation between LC mRNA expression and the LC protein levels.

Expression levels in these selected colonies ranged from 0 to 4.6 g/L (Table 2). ClonePix images of the top eight producers (Fig. 1A), showing expression levels above 2 g/L, were then cross referenced with the sum total fluorescence intensity data derived from the ClonePix colony selection (Fig. 1B). We observed that these top isolates were among the top 8% of brightly fluorescent colonies (Fig. 1B). This confirms the use of ClonePix colony picking based on fluorescent intensity of colonies grown in a static culture as a means to enrich for high producing cell lines. It is noteworthy, that some of the colonies such as 7A11, 7A7 and 7A1 with higher Sum Total Intensity were not pursued any further as they lost viability during clonal expansion either at the 96-well (7A1) or 24-well stage (7A7) or due to loss of titer during prescreening stage (7A1), possibly due to early instability.

3.2. Intracellular staining of HC and LC, staining patterns, and correlation with early specific productivity and final titer

Production cell lines have routinely been characterized by means of productivity assessments by measuring IgG titer, transgene copy number analysis, HC and LC RNA expression or by qRTPCR, all of which provide essential information on the attributes that contribute to a cell line's expression potential. However, all of these metrics describe the average characteristics of the cells in a cell line and do not take into account or provide information on the heterogeneity of the population. In contrast, simultaneous staining of intracellular HC and LC followed by flow cytometry, offers a direct method of assessing the Ab content of single cells within a cell culture. Flow cytometry data presented as scatter plots provide a data-point for each cell of the entire population and provides a visual assessment of population diversity. Scatter plots for a variety of mAb-expressing isolates were analyzed and we show that high and low expressers had distinct staining profiles (Fig. 2). Cultures with higher relative expression of secreted mAb tend to show more homogeneous scatter plots (Fig. 2A). Some cell lines were heterogeneous, clearly showing differential staining (Fig. 2B) that may be the result of unstable cells in the culture that were losing HC or LC expression, resulting in lower overall mAb expression. The loss of HC and LC expression is usually irreversible and heterogeneous cell lines rarely settle back into a homogeneous, high HC and LC staining pattern. In a statistical assessment of these data, we found that the tighter and more homogeneous staining patterns, which have a lower Coefficient of Variation ≤ 150 (CV = ratio of standard deviation to the mean) for HC, correlated with higher early qP ($p < 0.0001$ and R-Sq = 0.63) as well as with higher overall productivity ($p < 0.0001$ and R-Sq = 0.52) (Fig. 2C and D, respectively). In contrast, cultures with a HC CV ≥ 150 represented very heterogeneous and potentially unstable cell lines with much lower qPs and overall productivity. Based on the correlations that we established above, cell lines with higher HC CV and lower early qP could be eliminated during the CLD process prior to scaling up to measure the final titer from the 14-day fed-batch suspension assay. In this example, only isolate 7A5 showed heterogeneity by flow cytometry and high productivity. This represents a single outlier in the dataset and would likely not be selected for further development due to heterogeneity on flow cytometry.

In an effort to develop additional metrics for cell line characterization we also quantified intracellular HC and LC content in terms of geometric mean of the fluorescence intensity (MFI) as a measure of expression, and compared the ranking of cell line HC and LC MFI to other measurements, including fed-batch harvest titer, early qP, and HC MFI CV (Fig. 3 and Table 2). As seen in Table 2 for mAb-A, the cell specific productivity (qP) determined for cell lines during the expansion stage in shake flasks can be used to identify top producers at an early stage in the cell line generation process. Indeed cell lines with a higher qP represent the higher productive cell lines identified by the fed-batch suspension assay (Table 2 and Fig. 3A). Fig. 3B and C demonstrate a high correlation between intracellular HC content and early qP and final productivity in a 14-day fed-batch suspension assay, respectively. We found that cell lines with the highest early qP and higher intracellular HC content generally had the highest final productivity, while the cells with the both lowest early qP and intracellular HC had the lowest productivity. In contrast, intracellular LC content correlates poorly with either early qP or final productivity (Fig. 3D, E), probably because the cells can synthesize and secrete free LC without assembling with HC (Reddy et al., 1996; Shapiro et al., 1966; Feige et al., 2010).

The advantage of identifying high producing primary isolates for cloning at a very early stage significantly reduces the timeline towards final clone selection. This strategy can also be applied to screening subclones. The primary isolates were plated on the semisolid matrix for a second round of cloning in the presence of the fluorescently labeled detection reagent for isolation of subclones based on secretion in the static environment. We compared intracellular HC and LC content of 20 clonal cell lines by flow cytometry against final productivity. A strong correlation was found between intracellular HC MFI and the final productivity (Fig. 4A) in the clonally derived cell lines. However, similar to the primary isolates, intracellular LC of the clonal cell lines shows poor correlation to the final titer in fed-batch culture (Fig. 4B).The high throughput screening tools combining the ClonePix FL and flow cytometry-based analysis of intracellular staining of HC and LC, presented here are not only applicable for efficient identification of cell lines with high productivity potential, but also allows elimination of most low expressers before significant effort is invested

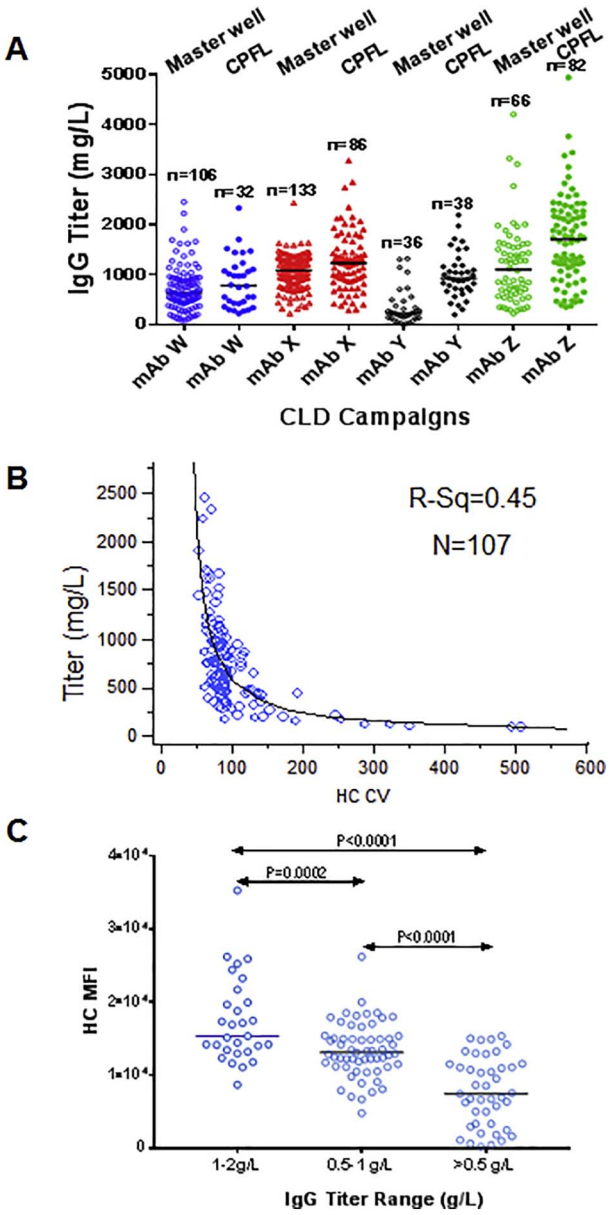

Fig. 6. A: Titer comparison of IgG expressing colonies from four different CLD campaigns for antibodies X, Y and Z. Colonies were selected using dual screening by ClonePix FL and IC staining of HC and LC or conventional cell screening in master well format. **B**: Inverse correlation between final productivity of primary isolates of mAb W and the CV of HC mean fluorescence intensity of flow cytometry scatter plots. **C:** Clustering of colonies based on titer and HC mean fluorescence intensity, for mAb W expressing cells. The medians are indicated and compared between each data set. Each data point represents an individual antibody expressing colony.

in the process, which combined leads to a significantly reduced timeline and the work load.

To ensure the stability and consistency in product quality of subclones isolated by the strategy described above, two top NS0 subclones were evaluated for long-term stability for 60 generations followed by IgG productivity evaluation in fed-batch in 3 L bioreactor (Fig. 4C). Despite, the fact that the intracellular HC and LC expression represented by the flow scatter plots remained homogeneous between 30-60PDL for Clone A (Fig. 4D), there was a 29% drop in titer between 30 and 45 PDL. However, consistent productivity was maintained between 45 and 60 PDL. On the other hand, a slight drop in titer between 30 and 45 PDL and between 45 and 60 PDL were consistent with simultaneous loss in HC population in Clone B.

3.3. Comparison of intracellular staining to real-time QPCR

We performed a screening method comparison between RTPCR-based methods to measure HC and LC mRNA levels and flow cytometric analysis of intracellularly stained HC and LC protein levels and assessed both methods as prediction tools for identifying high expressing cell lines. A comparison of the data suggests HC staining and HC QPCR correlate better to final productivity than LC staining and LC QPCR (Fig. 5A and B). However, the flow cytometry of intracellular HC and LC offers a direct measure of expression on a single cell basis whereas QPCR measures the bulk characteristics of the cell line. Moreover, the flow scatter plots of the stained HC and LC of an IgG expressing cell line represent its homogeneity which can be expressed quantitatively by CV and can readily be used as a predictor of instability (Dorai et al., 2012). A stronger correlation (R-sq. = 0.76) was found between HC expression at the protein and mRNA levels (Fig. 5A), whereas the protein and mRNA expression of LC showed statistically insignificant correlation with an R-Sq of 0.15 (Fig. 5B).

3.4. Application of a dual screening strategy for effective isolation of high expressers in GS-CHO CLD campaigns using ClonePix FL and intracellular staining of HC and LC

Similar to NS0 described above, GS-CHO cell lines expressing recombinant mAb can also be isolated by plating fresh transfectants on the semisolid matrix in presence of methionine sulphoximine (MSX) for selection (Nakamura and Omasa, 2015). We compared the dual screening method using ClonePix FL followed by cell line characterization by IC staining of HC and LC in four different CLD campaigns for mAb W, mAb X, mAb Y and mAb Z using a GS-CHO platform, with a conventional master well process by plating and selecting transfectants in 96-well plates. We consistently achieved higher median titers in the dual screening arm compared to the conventional master well arm (Fig. 6A). Moreover, cell line characterization by IC staining of HC and LC provided a similar (Fig. 2D) inverse correlation with HC CV and final productivity. Data from mAb W campaign is represented in Fig. 6B. However, CHO being a more robust host than NS0 can be selected with relatively higher concentration of MSX that offers a more stringent selection pressure on fresh transfectants resulting in overall reduction of colony heterogeneity. Additionally, we observed that CHO cells are more susceptible to response to feed and culture conditions that influences growth, viability and ultimately the final titer in shaking fed batch culture. All of these may result in a weaker correlation between HC CV and fed-batch titers (Fig. 6B) compared to NS0 (Fig. 2D). However, the HC MFI, a quantitative measurement of intracellular HC expression showed a direct impact on the final IgG titer as represented by titer distribution of primary isolates from mAb W campaign (Fig. 6C). In summary, the dual screening approach by ClonePix FL followed by colony characterization by IC staining of HC and LC was an effective means of reducing the screen size by eliminating low to non-expressers at a very early stage in the CHO CLD process.

3.5. Determination of probability of monoclonality by ClonePix FL

During the development of a production cell line for manufacturing, the clonality of the cell line is a major consideration to assure predictable productivity as well as consistency of product quality (Frye et al., 2016). The colony based selection of expressers by ClonePix FL allows the isolation of clonal populations and led us to develop a method to evaluate the probability of obtaining clonal cell lines by using this tool. To do this, isogenic CHO cells expressing either a GFP or RFP reporter, with equivalent growth characteristics either in routine suspension culture or their ability to grow on the semi-solid matrix, were mixed and plated onto semisolid media at different densities. Fluorescent colonies were then isolated using the robotic capabilities of the system (Fig. 7A). The accuracy of picking single clones was tested

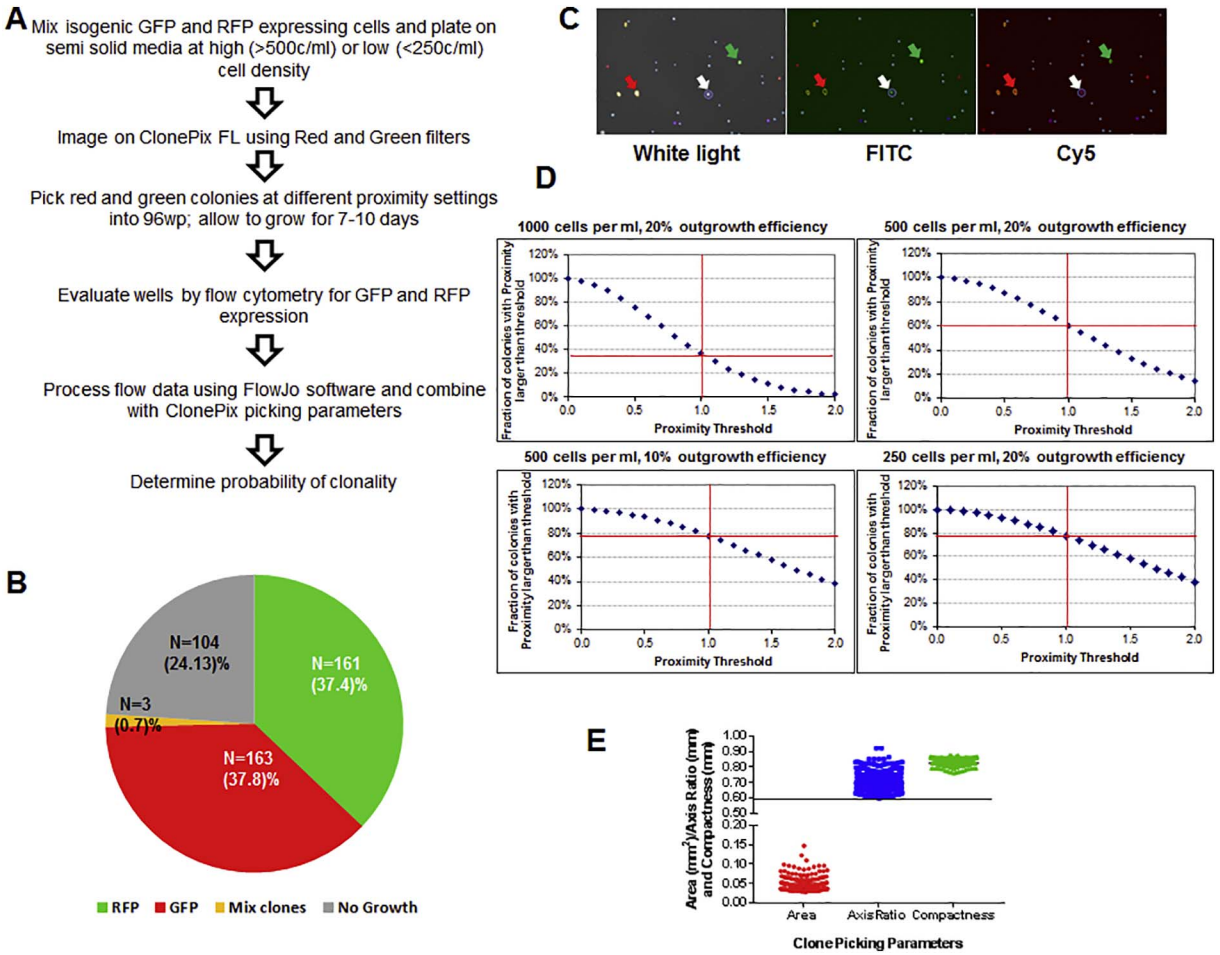

Fig. 7. A: Description of the steps performed for the determination of probability of monoclonality. **B**: Distribution of RFP, GFP and mixed GFP/RFP expressing clones picked by ClonePix. 104 of 431 picked clones did not grow and were included as *nd* during calculation of probability of monoclonality. **C:** CPFL image showing field of clones expressing RFP (red arrow), GFP (green arrow) or RFP/GFP (white arrow) captured under white light, FITC or Cy5 filters. The white arrow indicates one out of three undesirable mixed clones that were evaluated in the monoclonality test. **D**: Examples of the influence of proximity thresholds on cell plating and outgrowth efficiencies. The probability of achieving a given proximity threshold decreases with higher cell densities. **E**: Distribution of colony area (mm^2), axis ratio (mm) and compactness (mm) for picked colonies in the monoclonality test.

Table 3
Summary of probability of monoclonality *P*(*clonality*) with varying Proximity ranges.

Proximity (mm)	P(clonality)
0.5–0.7[a]	44%
0.8-0.9	61%
0.9–1.0	71%
1.0–1.2	93%
> 1.25	> 99%

[a] No clonality should be claimed if proximity is < 0.5 mm.

by assessing fluorescent GFP or RFP signal in the isolated colonies by flow cytometry and counting the frequency of colonies expressing GFP or RFP, and both GFP and RFP which can be a result of co-picking of overlapping clones in the matrix and also due to the closeness of clones defined by the parameter 'Proximity'. These are critical parameters that impact the probability of monoclonality (*P*(*clonality*)) and were incorporated in the mathematical equations. A total 431 colonies were picked into 96 well plates, after 7–10 days growth was seen in 327 wells, among which 161 colonies were GFP positive and 163 colonies were RFP positive. The distribution of colonies in different categories is charted in in Fig. 7B. We found 3 colonies that were positive for both GFP and RFP (Fig. 7C). In estimating the *P*(*clonality*), it is necessary to double the number of mixed-colonies to account for those colonies that were generated from two cells of the same type, since the probability of two GFP expressing cells plus the probability of two RFP expressing cells equals the probability of mixed colonies. The above is true as long as there is a similar proportion of RFP and GFP expressing colonies growing out on the semisolid medium and getting picked into 96 well plates. Thus the probability of monoclonality *P*(*clonality*) was determined by:

$$P(clonality) = 1 - \frac{2n_{RG}}{n - n_d}$$ where,

n = Total number of clones picked

n_{RG} = Number of colonies showing both green and red fluorescence

n_d = Number of clones that did not grow in 96-well plate.

In order to determine the influence of colony proximity contributing to *P*(*clonality*), we increased the cell plating density of the isogenic GFP and RFP expressing pools to 500 cells/ml. Colonies were then picked from a range of proximity settings starting from 0.5 mm. We found that there is a higher propensity of co-picking of mixed colonies at when colonies are separated by a distance of < 1.0 mm, while > 99% *P*(*clonality*) can be achieved for proximities larger than 1.25 mm (Table 3). Based on the above findings, we found that more than one round of cloning is necessary to achieve > 99.5% *P*(*clonality*) by using ClonePix FL. We further determined the rounds of cloning that are necessary to achieve > 99.5% *P*(*clonality*) by using the following equation.

$$P_N = P_{N-1} + (1 - P_{N-1})P_n$$ where,

P_N: is the cumulative probability of clonality including the n^{th} step, i.e., the last step

Table 4a
ClusterSizeData from ViCell XR for parent primary isolates and final clones for CLD#1 and #2.

Criteria	Parent primary isolate (CLD#1)	Final clone (CLD#1)	Parent primary isolate (CLD#2)	Final clone (CLD#2)
ViCell images analyzed	15	15	15	15
Total cells	253	893	475	1155
Cluster of 2 cells	17	66	31	52
Cluster of 3 cells	4	16	5	2
Cluster of 4 cells	1		4	0
Cluster of 5 cells	1		1	1
Cluster of 6 cells	1		1	
Cluster of 7 cells			1	
Cluster of 8 cells			0	
Cluster of 9 cells			1	
Cluster of 10 cells			0	
Cluster of 11 cells			0	
Cluster of 12 cells			1	
Total cells in cluster	55	180	132	115
Single cells	198	713	343	1040
%single				
% single + double				
CFU	222	795	388	1095
% single cells	89%	90%	88%	95%

Table 4b
ClonePix FL Characteristics of Parent Primary Isolates and Final Clones of CLD#1 and CLD#2.

ClonePix FL Attribute	Primary isolate (CLD#1)	Final clone (CLD#1)	Primary isolate (CLD#2)	Final clone (CLD#2)
Area (mm^2)	0.054	0.119	0.115	0.029
Axis ratio	0.752	0.841	0.661	0.768
Compactness	0.821	0.828	0.838	0.814
Proximity (mm)	1.57	2.182	2.8	1.088

Table 4c
Clonality calculations combining cell aggregation and ClonePix FL criteria.

Probability cloning round	CLD campaign	ViCell single (%)	ClonePix clonality	Final clonality
Pc1	CLD#1	90%	99%	0.90 * 0.99 = 0.89
Pc2	CLD#1	90%	99%	0.90 * 0.99 = 0.89
Pc1 + Pc2				98.8%
Pc1	CLD#2	88%	99%	0.88 * 0.99 = 0.87
Pc2	CLD#2	95%	93%	0.95 * 0.93 = 0.88
Pc1 + Pc2				98.4%

$P_{N\text{-}1}$: is the cumulative probability of clonality before the n^{th} step
P_n: is the probability of clonality achieved by the n^{th} step only

For example if during the first round of cloning a probability of 95% is achieved and a second round results in 98%, then the total probability of clonality will be:

$P_2 = 0.95 + (1 - 0.95) \times 0.98 = 0.998.$

Therefore, there is 99.8% probability that the cell line is clonal after the second round of cloning. The proximity threshold required to obtain assurance of monoclonality depends on the aspiration volume used. The studies reported here were performed with 10 μL aspiration volume. If a different aspiration volume is used a suitable proximity threshold needs to be developed. The cell density has an indirect effect via the likelihood of finding colonies with proximities greater than the threshold. The higher the cell density, the fewer colonies with proximities greater than the threshold. However, the actual distribution of colonies is random and cannot be fully controlled. The distribution of colonies in the semi-solid medium can be modelled by a homogeneous Poisson point process. Poisson point processes are frequently used to model the random distribution of particles over a surface or volume and have been used successfully to model the distribution of bacteria on solid surfaces (Lagido et al., 2003). This model allows us to predict the probability distribution of the proximities as a function of the average number of colonies per unit area:

$$Prob(Proximity \geq x) = e^{-\lambda \pi x^2}$$

Where λ denotes the number of cells per unit area and x denotes the target proximity threshold. Given that typically the number of cells plated is sufficiently large, e.g., > 1000, the equation effectively predicts the proportion of colonies that will have a proximity of greater or equal to x. To use this equation the cell density or number of cells per unit volume needs to be converted to cells per unit area. Below we present an example of the calculations:

Cells per mL = 1000 cells/mL

Volume Plated = 16 mL

Total Cells = 16,000 cells

Plate width = 128 mm

Plate length = 86 mm

Plate Area = 11,008 mm^2

Cells per mm^2 = 1.45 cells/mm^2

Assumed Outgrowth efficiency = 20%

Colonies per mm^2 = 0.29 cells/mm^2

Using λ = 0.29 cell/mm^2 and x = 1.0 mm we obtain that the probability of colonies being at least 1.0 mm apart is 40%:

$$Prob(proximity \geq 1.0\ mm) = e^{-0.29 \times 3.1416 \times 1.0^2} = 40\%$$

Fig. 7D shows how the proportion of colonies with proximities greater than a threshold changes as a function of the threshold for various combinations of cell density and colony outgrowth efficiency. It is to be noted, that the colony outgrowth efficiency was calculated based on the experimental data obtained from a number of stable IgG expressing CHO cell lines and their efficiency to grow on CloneMedia K8725. The other picking parameters such as colony area (> 0.03 mm^2), axis ratio (> 0.6 mm) and compactness (> 0.6 mm) were followed as recommended by the manufacturer. A distribution of such parameters of the picked colonies in the monoclonality test experiment are summarized in Fig. 7E.

It should be noted that the ClonePix clonality analysis can be used to determine probability of clonality only if the suspension of cells plated in the semi-solid medium consists of single cells. However, a recent study (Klottrup et al., 2017) shows that the cell aggregation can have a minor impact on the probability of monoclonality after two-rounds of cloning by ClonePix FL. Probability of monoclonality data obtained from two independent cell line development campaigns are summarized in Table 4a, b and c. Table 4a describes the ClusterSizeData data from ViCell XR analysing the single cells, doublets, triplets, quadruplets at post transfection cell plating and also at the plating of the primary isolates prior to the subcloning. Table 4b summarizes the ClonePix FL characteristics including colony area, axis ratio, compactness and proximity of the top primary isolates and the top clones. Whereas, Table 4c describes the probability of monoclonality combining the ClusterSizeData and the ClonePix FL data.

4. Conclusions

In the manufacture of biologic drugs in mammalian cell lines, better

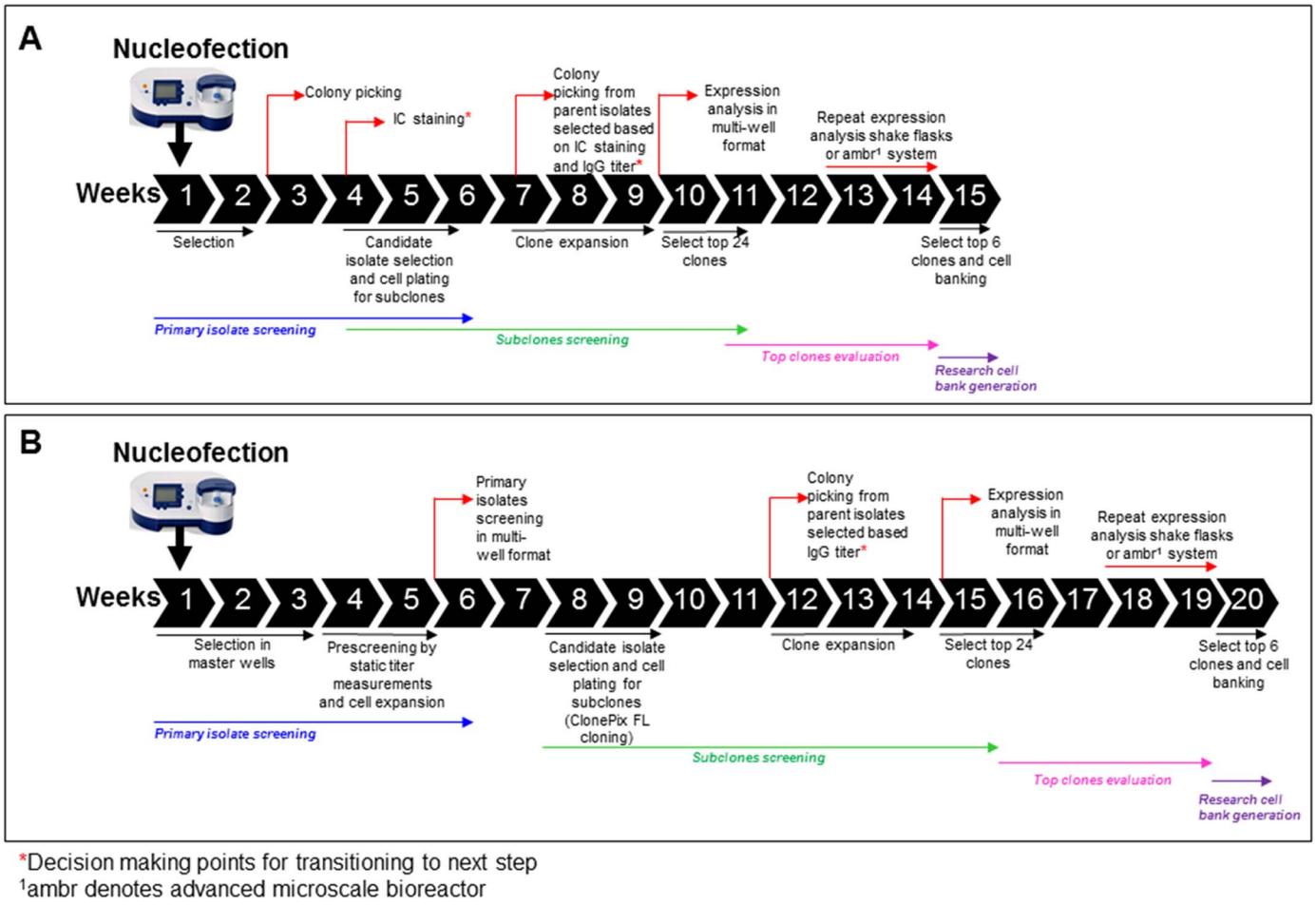

Fig. 8. Workflow for ClonePix/IC staining (A) and conventional (B) CLD campaigns. ClonePixFL and IC staining enables earlier identification of high producing cells, while avoiding lengthy titer-based cell culture assays for primary isolates. This enables a 15 week CLD workflow for the generation of high producing clones. Decision making points for transitioning to next step are marked with red asterisks, 'ambr' denotes advanced microscale bioreactor system.

predictability of cell line productivity early in the development process could help reduce time and labor, and therefore would expedite transitioning therapeutics into the clinic for testing to treat human disease. To address this challenge, we developed a process that combines the cell line screening potential of both the ClonePix FL and measurement of intracellular HC and LC content by flow cytometry during CLD campaigns. ClonePix FL enabled the isolation of rare events by screening tens of thousands of colonies based on the secretion of product in a semi-solid matrix suspension (Serpieri et al., 2010; Dharshanan and Hung, 2014; Hou et al., 2014). This technology is very effective in visualizing high expressers from a cell population even when the frequency of expressers is very low. The cell line generation campaign for mAb-A in the NS0 platform was typical, where the frequency of high expressers, as determined by fluorescence intensity in and around the colony on the semisolid matrix, was very low. When utilizing ClonePix FL to isolate high producing cell lines from fresh transfectants, many of the isolates with high fluorescence lose expression after picking. In this case, the bright halo may be formed from transient IgG expression that is lost over time. Nevertheless, the top ranking highly fluorescent-halo containing clones that were picked to destination plates were enriched for higher productivity when final titers were determined. Our results also demonstrate the advantage of ClonePix FL for efficient elimination of low and non-expressers. Instability in NS0 and CHO recombinant cell lines utilizing the GS and DHFR systems in short and long-term culture is well documented in the literature (DeMaria et al., 2007; Kaufman and Schimke, 1981; Barnes et al., 2004; Kim et al., 1998). Therefore, the detection of early instability would benefit from a second line of screening to avoid pursuing heterogeneous or unstable colonies in subsequent cell cloning process and other downstream manipulations. To that goal, screening cells by a flow cytometry-based assay for intracellular (IC) HC and LC (Dorai et al., 2012; Prieto et al., 2011; Lee et al., 1996; Ohlin and Borrebaeck, 1994) allowed for the identification of high producing, stable isolates, and established a strong correlation between intracellular HC expression with early qP and final productivity of primary isolates (Jiang et al., 2006; Schlatter et al., 2005). Using IC staining as a predictive tool, we were able to identify the top expressers as good candidates for cloning early in the clone selection process, which enabled us to generate high expressers from a smaller screen size in a significantly shorter timeline. Additionally, using IC staining, we can measure intracellular protein content to predict protein secretion, while RNA quantitation, being an indirect measurement of protein levels, has its limitations when predicting protein production.

Previously published work demonstrated that neither surface or intracellular expression show a strong correlation with the productivity of cells (Kang et al., 2014; Meilhoc et al., 1989). It is noteworthy, that IgG expression is a complex process that is affected by multiple factors including HC/LC expression ratio, site of gene integration, stability of recombinant genes, and post-translational processes such as proper assembly of HC/LC. Inefficiencies at any of these steps can result in low expression yields. The dual screen outlined here takes into account protein expression and secretion (ClonePix FL) as well as early antibody gene stability determined by IC staining of HC and LC to identify colonies with potential for high levels of antibody expression. It is

important to note that IC staining is used primarily to identify candidate clones that show homogenous expression of HC and LC. This homogeneity is indicative of early stability of IgG expression and can distinguish suitable candidates for subcloning for generation of high expressing stable clones suitable for manufacturing.

The clonality of cell lines is often associated with safety and efficacy over a lifetime of a biological product (Frye et al., 2016). We successfully introduced the probability of monoclonality by ClonePix FL by using a robust experimental approach using isogenic RFP and GFP stable cells generated in Flp-In CHO host that offered identical growth properties on the semisolid matrix for statistical validation of the biological experiments. We established that 2-rounds of cell cloning are necessary to obtain > 99.5% clonal stable hosts. Additionally, we established that cell proximity is one of the major criteria for improving probability of monoclonality and determined the Proximity brackets of > 1.25 mm that offers reduced chances of colony co-picking.

The dual screening clone selection methods described here are widely applicable across host platforms such as NS0 and CHO resulting in highly productive clonal stable manufacturing cell lines in shorter timelines with significant reduction in workload and resource burden. Implementation of the dual screening approach helped us to achieve highly productive stable IgG expressing cell lines within 13–15 weeks; while screening significantly fewer candidate colonies and still ensuring the > 99% probability of clonality of cell lines as well as maintaining consistent productivity and product quality over 3 months in long-term stability studies. A detailed workflow comprising of two rounds of ClonePix FL cloning with the timeline and necessary steps has been summarized in Fig. 8. New methods such as the single-cell printer (Cytena, Freiburg, Germany) showing an image based trace for clonality have emerged that allow us to follow an individual cell from the dispensing capillary into the deposited well of a multiwell plate (Gross et al., 2013; Stumpf et al., 2015). However, colony isolation using ClonePix FL offers an expression based method for the selection of high producers and enables a significantly enriched population for high producers. Furthermore, IC staining allows for a rapid means to monitor the quality, integrity, and stability of expresser cell lines. Overall the high-content methods described herein can be used in whole or in part when developing robust and efficient CLD processes.

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