PTSelect: A post-transcriptional technology that enables rapid establishment of stable CHO cell lines and surveillance of clonal variation

Vandhana Muralidharan-Chari[1], Zachary Wurz[2], Francis Doyle[1], Matthew Henry[3], Andreas Diendorfer[4], Scott Tenenbaum[1], Nicole Borth[5], Edward Eveleth[2], and Susan T. Sharfstein[1,1]

[1]SUNY Polytechnic Institute
[2]Hocus Locus
[3]The University of Queensland
[4]University of Natural Resources and Life Sciences
[5]University for Applied Live Sciences

April 28, 2020

Abstract

Currently, stable Chinese hamster ovary cell lines producing therapeutic, recombinant proteins are established either by antibiotic and/or metabolic selection. Here we report a novel technology, PTSelect that utilizes an siRNA cloned upstream of the gene of interest (GOI) that is processed to produce functional PTSelect-siRNAs, which enable cell selection. Cells with stably integrated GOI are selected and separated from cells without GOI by transfecting CD4/siRNA mRNA regulated by PTSelect-siRNAs and exploiting the variable expression of CD4 on the cell surface. This study describes the PTSelect principle and compares the productivity, doubling time and stability of clones developed by PTSelect with conventionally developed clones. PTSelect rapidly established a pool population with comparable stability and productivity to pools generated by traditional methods and can further be used to easily monitor productivity changes due to clonal drift, identifying individual cells with reduced productivity.

Introduction

Chinese hamster ovary (CHO) cells are preferred for biologics production for their safety as production hosts (*Biological Approvals by Year—2017 Biological License Application Approvals* , n.d.;*Biologics-revolution-in-the-production-of-drugs.pdf* , n.d.;*Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use – ACTIP* , n.d.; Research, n.d.), high specific productivity (Q_p), human-like protein glycosylation, and adaptability to grow in suspension in animal component (e.g. serum)-free growth media (Fischer et al., 2015; J. Y. Kim et al., 2012). Current technology for establishing stable CHO cell lines utilizing antibiotic/metabolic selection is time-consuming and expensive. In addition, metabolic selection requires expensive, auxotrophic cell lines that are either*DHFR* (dihydrofolate reductase) or *GS* (glutamine synthetase) negative (Bebbington et al., 1992; Gallagher & Kelly, 2017; Kaufman & Sharp, 1982). Finally, production instability caused by clonal drift affects the productivity of clones (Bandyopadhyay et al., 2018; Lee et al., 2018; Vcelar et al., 2018; Wurm & Wurm, 2017). While clonal variation is inevitable in rapidly proliferating immortalized cell lines, the dearth of tools to monitor clonal drift impacts productivity during CHO cell line expansion (Frye et al., 2016; Li et al., 2010; Mirasol, n.d.).

To overcome these issues, we developed PTSelect technology. It employs the cellular post-transcriptional

machinery and utilizes an siRNA to select and establish stable cell populations, replacing the need for selection markers. Here, we describe the principle of PTSelect technology and demonstrate its ability to establish stable CHO cell lines producing either EpoFc (erythropoietin-conjugated to immunoglobulin Fc region) (Lattenmayer et al., 2007) or the monoclonal antibody, adalimumab (Choi et al., 2014). In parallel, we established stable CHO cell lines using conventional antibiotic (EpoFc) and/or metabolic selection (EpoFc and adalimumab). Productivity, doubling time and stability were compared between clones produced by both methodologies.

We demonstrate that PTSelect can generate a stable cell pool within 11 days, increase the fraction of positive clones, and generate clones with greater stability compared to methotrexate (MTX)-selected clones, with no significant difference in the productivity. Most importantly, PTSelect technology provides a rapid, simple solution to monitor clonal drift; correlation between productivity and PTSelect-siRNA activity enables real-time, single-cell monitoring of productivity and population drift due to clonal variation.

Materials and Methods

Plasmid design and construction. All EpoFc-expressing clones were made from the parent plasmid, pCMV-EpoFc-pA (Lattenmayer et al., 2007). EpoFc-siRNA1 (ES) plasmid (Supplementary Figure 1a) was constructed by cloning the siRNA1/BART10 cassette between the*SacI* and *XbaI* sites upstream of the EpoFc protein.*D* HFR- *E* poFc-*N* EO (EDN) plasmid (Supplementary Figure 1b) expressing EpoFc along with DHFR and *NeoR* (aminoglycoside 3'-phosphotransferase) genes was constructed by inserting a cassette containing the DHFR and NeoR genes (under the control of SV40 promoters along with their respective poly A sequences) into the *EcoRI*site. EGFP-siRNA1 plasmid (Figure 2a) was constructed by cloning EGFP and the siRNA1/BART10-intron cassette between the *XbaI* and*EcoRV* sites, replacing EpoFc. Plasmid containing EpoFc along with siRNA1 and Puromycin resistance (ESP, Supplementary Figure 1c) was generated by inserting *PurR (* puromycin N-acetyl-transferase*)* into the EcoRI site and cloning the siRNA1/BART10 cassette into the *XbaI* site. Finally, HC1 (heavy chain along with siRNA1/BART10) -LC2 (light chain along with siRNA2)-GS plasmid (Figure 4a) expressing adalimumab and glutamine synthetase was cloned by inserting siRNA1-HC at *XbaI* , LC at *HindIII* , siRNA2 cassette at *KpnI* , and *GS* gene at *EcoRV* sites of pCMV-EpoFc-pA. All cloning was performed by Genscript (Piscataway, NJ) and confirmed by sequencing. Individual clones were delivered as an industrial grade plasmid DNA with a minimum seven stringent QC parameters analyzed (https://www.genscript.com/industrial-grade-plasmid.html).

Host cell lines and culture maintenance. CHO-K1 cells adapted to grow in suspension were used as host cell lines for both EpoFc and mAb expression and have been described previously (Bort et al., 2010). Cells were cultured in CD CHO medium (Thermo Fisher Scientific), supplemented with 8mM Glutamax and anti-clumping agent (Thermo Fisher Scientific). Cultures were routinely passaged twice a week at 1.5 x 10^5 cells/mL in 125 mL culture flasks (Thomson Instrument Company, Oceanside, CA) and incubated in a humidified 5% CO_2/air mixture at 37 °C, on a shaker at 120 rpm. Viable cells were distinguished from dead cells using trypan blue dye exclusion, and the viable cell concentration (VCC) was quantified using a Countess II® FL automated cell counter (Thermo Fisher Scientific).

Functional mRNA synthesis. All mRNAs containing siRNAs and all control mRNAs were generated by *in vitro* transcription. Plasmids expressing appropriate inserts were linearized using a unique restriction enzyme. The DNA was purified using QiaQuick PCR Purification kit (Qiagen, Germantown, MD), as per manufacturer's instructions. From the linearized DNA, RNA was transcribed using MEGAscript T7 transcription kit (Thermo Fisher Scientific) and then purified using the MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific) as per manufacturer's instructions. The capping (ScriptCap m7G Capping System) and tailing (Tailing – A-Plus Poly(A) Polymerase Tailing Kit) was then performed sequentially using kits from CELLSCRIPT (Madison, WI). The RNA was then purified again using the MEGAclear Transcription Clean-Up Kit.

Stable cell line development by PTSelect. CHO-K1 (80 x 10^6cells) were electroporated with plasmid DNA using a Maxcyte-ATX (MaxCyte, Inc., Gaithersburg, MD), according to the manufacturer's protocol.

After transfection, cells were seeded into pre-warmed CD-CHO media at 200,000 cells/mL and grown under normal growth conditions. After 48 hours, at least 80 x 10^6cells were transfected with 100-1000 ng of CD4/siRNA mRNA per million cells and incubated in pre-warmed CD-CHO media under normal growth conditions. After 4 to 6 hours, cells expressing CD4 on their cell surface (without GOI) were isolated using EasySep Release Human CD4 Positive Selection kit by STEMCELL Technologies (Vancouver, Canada), as per manufacturer's instructions with a key modification of keeping the supernatant (cells with GOI and not expressing CD4 on their cell surface). The cells in supernatant were incubated in CD-CHO media under normal growth conditions. This depletion process was typically performed on days 3, 5 and 10 after transfection with GOI or when the cells had reached 80 x 10^6 in number.

Stable cell line development by conventional methods. CHO-K1 cells were electroporated with plasmid DNA using a Maxcyte-ATX (MaxCyte, Inc., Gaithersburg, MD), according to the manufacturer's protocol. After transfection, cells were seeded at 1.5 x 10^5 cells/ml in 30 mL of CD-CHO medium in 125 mL shaker flasks. Selection was performed by adding either G418 (400 µg/mL) or Puromycin (10 µg/mL). For generating mAb-producing cell lines, selection was performed in a mixture of 20% PowerCHO-2 CD (Lonza Bioscience, Walkersville, MD) and 80% ExCell CHO cloning medium (Sigma-Aldrich, St. Louis, MO) with GS expression medium supplement (GSEM, Sigma-Aldrich), and MSX (25 µM) (Sigma-Aldrich, St. Louis, MO). Cell counts and viability were monitored twice a week, and a stable pool population was obtained when the viability reached greater than 90 percent. To obtain single cell-derived clones, cell pools were subjected to limiting dilution using growth media in 96-well plates (Supplementary Figure 5). After three weeks, media from established clones were harvested and ELISA (EpoFc, R&D Systems, Minneapolis, MN and Human IgG, STEMCELL Technologies, Vancouver, Canada) was performed, as per manufacturer's protocol. The top forty clones were expanded and frozen.

Short-term productivity assay and titer measurements. Four-day batch cultures were performed to determine specific productivity of both Epo-Fc and IgG (adalimumab). Cells were seeded in duplicate, at 1.0 x 10^5 cells/mL in 10 mL of CD CHO medium supplemented with 8 mM Glutamax and anti-clumping agent in 50 mL bio-reaction tubes on a shaker set at 195rpm. VCC and viability were measured daily as described above. On day 4, media were harvested from duplicate tubes, combined, centrifuged, and stored in aliquots at -20 °C. The EpoFc fusion protein and human IgG expression levels in CHO cell culture supernatant were measured as described above. Q_p(pg/cell/day) was calculated by dividing titer obtained from ELISA by integrated viable cell density (IVCD). IVCD was calculated as, $IVCD_1+(VCD_1+VCD_2)/2*(t_2-t_1)/24$, wherein VCD (cells/day/mL) is viable cell density and t1 and t2 are two different time points in hours.

Analysis of clone stability. The six highest producing EpoFc-clones generated by PTSelect and Neo/DHFR amplification were chosen. Cells were seeded at 1.5 x 10^5 cells/mL in 30 mL of CD CHO medium supplemented with 8mM Glutamax and anti-clumping agent in 125 mL shaker flask. Cells were passaged every 3-4 days and cultured for three months/12 weeks. Cells from weeks 0, 4, 8 and 12, as counted from the start of the long-term cultivation were analyzed for protein expression levels by ELISA as described above and for clonal variation. To test for clonal drift, cells from each time point were transfected with a plasmid expressing dTomato cloned with sequences that can be regulated by the siRNA in the EpoFc-plasmid and GFP-mRNA (transfection control). Parental CHO-K1 cells were also transfected with either dTomato/siRNA1 or GFP/siRNA2 to serve as control for compensation of the fluorochromes. Flow cytometry analysis of the transfected cells was performed using FACSAria II (BD Biosciences) at the Neural Stem Cell Institute (Rensselaer, NY). Five hours after transfection, cells were analyzed by flow cytometry; GFP signals were collected in the FITC channel and dTomato signals in the PE channel. Experiments were done in duplicate. A total of 50,000 events were collected in the analysis. Data analysis was performed using FlowJo 10 (FlowJo LLC).

Results

PTSelect-siRNA design strategies

PTSelect technology has two vital components; an siRNA cassette designed to contain the PTSelect-siRNA

cloned upstream of the GOI (Figure 1a) and CD4/siRNA mRNA containing an engineered RNA sequence that is complementary to the PTSelect-siRNA, and thus targeted by the PTSelect-siRNA. The siRNA cassette comprises three components: (I) 486 bases from intron-2 of *HBG* gene, which has been shown to improve transgene expression in CHO cells (Haddad-Mashadrizeh et al., 2009)·(S.-Y. Kim et al., n.d.). (II) The PTSelect-siRNA sequence that is co-transcribed with the GOI is a BART transcript of Epstein-Barr virus (EBV) (Kang et al., 2015). We used the highly expressed miR-BART10-3p (BART10), previously employed in the development of a related technology(Doyle et al., 2017). A single copy of the siRNA was used in the cassette, with an option to increase copies to reduce false positives. (III) For precursor sequences that enable siRNA processing and maturation, we tested 100-bp sequences (5' flank and 3' flank, Figure 1a) flanking both sides of the BART10 sequence in the EBV viral genome and two synthetic precursor sequences. Since the wild type sequences exhibited the most suppression of a reporter mRNA with a corresponding siRNA target site (data not shown), they were selected.

Principle of PTSelect technology in establishment of stable cell population

The expression of PTSelect-siRNA serves as a proxy for GOI expression, eliminating the need for drug resistance gene(s). To select stable cells, CD4/siRNA mRNA, which contains RNA sequences complementary to the PTSelect-siRNA is electroporated into the cells three days after the transfection of GOI. mRNA electroporation is highly effective with ˜99% transfection efficiency (Supplementary Figure 2a). In cells that express the GOI and therefore produce the PTSelect-siRNA, CD4 expression is downregulated, preventing CD4 expression on the cell surface. Conversely, cells that do not contain the GOI, do not produce PTSelect-siRNA and consequently express CD4 on the cell surface (Figure 1c). Maximum CD4 expression from the electroporated mRNA occurs at ˜10 hours after electroporation (Supplementary Figure 2b). To separate CD4-positive (no GOI) from CD4-negative (expressing GOI) cells, 6 hours after electroporation, the cells are incubated with anti-CD4-conjugated magnetic beads. Exposing the cells to a magnetic field, the GOI-negative cells attached to the beads adhere to the inner walls of the tube, while the CD4-negative (GOI positive) cells remain suspended and are removed (Figure 1d). From electroporation of CD4/siRNA mRNA to separation constitutes one round of depletion and is repeated on days 3, 7 and 10 after initial transfection of GOI to obtain an enriched pool of stable cells.

Establishment of EGFP-stable pool using PTSelect

As a proof of concept, a stable cell pool expressing EGFP was generated by PTSelect. PTSelect-siRNA1 was cloned into the plasmid containing EGFP (Figure 2a) and transfected into CHO-K1 cells by electroporation. On days 3, 7, 10, 17 and 19 after EGFP plasmid transfection, CD4/siRNA1 mRNA and dTomato/ siRNA1 mRNA were introduced by electroporation, followed by depletion. At each depletion, cells in the isolate, supernatant and cells not subjected to depletion (stock) were analyzed by flow cytometry (Figure 2b). The fraction of cells recovered in each round of depletion is shown in Table 1. On day 3, 4.8% of the cells were recovered in the supernatant from 160 x 10^6 cells; the recovered cells were expanded to 80 x 10^6 cells. On day 7, the recovery was 30% due to the expansion of enriched cells. However, on day 10, the recovery was only 2.4%, possibly due to the instability of the integrated sequences, resulting in loss from the genome (Wurtele et al., 2003). When this population was expanded and enriched on day 17, 80% of the cells were recovered, indicating that the integrated sequences have stabilized in the population. The percentage of recovered cells did not change on day 19, further confirming the stability of the cell population. Thus, it appears that stable cell establishment occurs by day 10, and expansion happens after day 10. Therefore, it is possible to proceed with limited dilution cloning (LDC) on day 10.

The presence of EGFP signals in the supernatant fraction was confirmed by flow cytometry (Supplementary Figure 3). The progressive enrichment of stable cells expressing EGFP in the supernatant was corroborated by a concomitant increase in the number of cells exhibiting decreased dTomato (PE) signal (Figure 2c, top panel). Conversely, the dTomato (PE) signal from the cells in the isolate progressively moved towards the control population signal (Figure 2c, bottom panel). Overall, the results confirm that PTSelect can establish a stable cell pool expressing EGFP.

Comparison of EpoFc-stable clones generated by PTSelect and conventional methods

Next, we compared individual clones producing a secreted product generated by PTSelect and by conventional methods (antibiotic selection and antibiotic selection with MTX amplification) (Supplementary Table 1, comparison 1) for their productivity, doubling time and stability. For this study, three plasmids were generated – EpoFc_siRNA1 (ES), EpoFc_DHFR_Neo (EDN), and EpoFc_siRNA1_Puro (ESP) (Supplementary Figure 3), and transfected into CHO-K1 cells by electroporation. Cells transfected with EDN were selected with neomycin followed by MTX amplification (Supplementary Figure 4a); limited dilution cloning (LDC) was performed on day 31. Cells transfected with ESP were selected with puromycin (Supplementary Figure 4b); LDC was performed on day 24. Cells transfected with ES were depleted on days 3, 7, 10, 13, 15, 18, 24, and 30 with 1000 ng of CD4/siRNA1 mRNA. LDC was performed on a fraction of depleted cells obtained from days 18, 24 and 30. Clonal selection and expansion were performed as outlined in Supplementary Figure 5 and described in materials and methods.

Three weeks after plating, EpoFc levels in expanded clones were assessed by ELISA (Figure 3a). From 80 clones per plate, PTSelect generated more positive clones (55 +- 15) compared to antibiotic selection (ESP) (6 +- 4) and antibiotic/MTX amplification (EDN) (10 +- 8). Productivity was assessed for 40 clones from each stable line (Figure 3b). Although the highest producer was generated by PTSelect technology (2,755,721 mlU/mL), the next ten highest producers were obtained from clones generated by traditional methods (Supplementary Table 2, Top 5 clones in comparison 1). The average doubling time was 18.6 +- 1.3h for clones generated by PTSelect, 19.5 +- 3.9h for ESP clones generated by puromycin selection, and 20.5 +- 2.6h for EDN clones generated by neomycin and MTX amplification. The doubling time in PTSelect-clones is significantly shorter ($p < 0.05$) compared to the EDN-clones, which could provide a substantial advantage during scale-up.

Next, we evaluated whether the amount of CD4/siRNA1 mRNA used in depletion had any effect on improving the productivity and doubling time of the generated clones (Supplementary Table 1, comparison 2). EpoFc_siRNA1_Puro was introduced into CHO-K1 cells by electroporation. Having the puromycin N-acetyltransferase gene in the plasmid also allowed us to evaluate the effects of the antibiotic resistance gene on the depletion process. Cells were depleted on days 3 and 8 using 500 ng of CD4/siRNA1 mRNA. On day 11, cells were depleted with 500 ng of CD4/siRNA1 mRNA, and a fraction of cells obtained was subjected to LDC. The remaining depleted cells were allowed to expand until day 21, depleted with either 500 ng or 1000 ng of CD4/siRNA1 mRNA, and then subjected to LDC. Expanded clones were analyzed by ELISA after three weeks. All three depletion approaches produced a similar number of EpoFc-positive clones (55 +- 10, out of 80, Supplementary Figure 6). Productivity was tested for 25 clones per condition (Figure 3c). The highest producer occurred in cells depleted on day 21 with 500 ng of CD4/siRNA1 mRNA (1,119,926 mlU/mL). Overall, there was no significant difference in productivity of the top clones between groups depleted on day 21 (f -ratio = 0.4; p= 0.67, one-way ANOVA). There was no significant difference in the average doubling time of 25 clones generated in each group (Supplementary Table 2). Thus, the amount of CD4/siRNA1 mRNA used in depletion had no significant effect on productivity or doubling time. Furthermore, stable clones can be established as early as day 11, and the presence of an antibiotic gene does not affect stable cell generation by PTSelect.

To compare clonal stability, we selected the six highest producing clones generated by both PTSelect and neomycin selection with MTX amplification. Clonal stability was assessed for a 12-week period as described in Methods. Based on the titer, 3 out of 6 clones generated by PTSelect passed the stability criterion with productivity remaining above 70% of initial productivity after 12 weeks (Figure 4a). However, only 1 out of 6 EDN clones passed the same stability criterion (Figure 4b). Thus, PTSelect technology generated clones with greater stability than those generated by antibiotic selection and MTX amplification.

Comparison of stable adalimumab clones generated by PTSelect and conventional methods

We generated plasmid mAb_siRNA1_siRNA2_GS that expresses both adalimumab heavy chain (HC) and light chain (LC) with siRNA1 and siRNA2 cassettes, respectively, cloned upstream. The glutamine synthetase

(*GS*) gene was also cloned into the same plasmid (Figure 5a) to test whether the presence of *GS* had any effect on PTSelect depletion. For this study, stable clones were established and compared as shown in Supplementary Table 1, comparison 3. Plasmid with mAb_siRNA1_siRNA2_GS was introduced into CHO-K1 cells by electroporation; a fraction of cells was taken for MSX selection, and the rest were selected using PTSelect technology. Depletion was done on days 3, 10, and 14 with 150 ng of each of CD4/siRNA1 mRNA and CD4/siRNA2 mRNA and on day 19 with 250 ng of each mRNA (mAb-500) or 500 ng of each mRNA (mAb-1000). LDC for both depleted samples was performed on day 19. For the traditional selection method, cells were selected with 25 mM MSX in glutamine-free medium. Selection was completed on day 24 (Supplementary Figure 7) and LDC was performed. After three weeks, the number of clones producing mAb was 4 +- 2 out of 80 for MSX-generated clones and 9 +- 6 out of 80 for PTSelect-generated clones (data not shown). Among the forty positive clones from both methods tested for productivity, the highest producer was generated by the conventional method (1.69 pg/cell/day), while the top producers from PTSelect had specific productivities of ˜ 1 pg/cell/day (Figure 5b, Supplementary Table 2). Interestingly, there was no difference in the average doubling times (Supplementary Table 2) between the forty clones generated by both methods.

Silencing activity of PTSelect-siRNA correlates with the expression of GOI

To evaluate the relationship between silencing efficiency and productivity, we chose high, medium and low productivity EpoFc PTSelect-clones, electroporated them with GFP/siRNA1 mRNA, and analyzed them by flow cytometry (Figure 6a). The cell populations that exhibited maximum GFP (FITC) signal suppression were analyzed by calculating the percentage of cells with low to no fluorescence, located at 10^0 on the x-axis (Figure 5a, insert). The percentage of cells with maximum GFP suppression (10^0 on the x-axis) correlates with their respective GOI expression levels (low producers, 3.7 and 5%; medium producers, 69 and 64.2%; high producers, 89 and 81%). Thus, there is a correlation between the PTSelect-siRNA silencing activity and the GOI expression.

Monitoring clonal productivity by measuring the silencing activity of PTSelect-siRNA

To demonstrate that PTSelect-siRNA can monitor clonal stability, we selected three clones previously subjected to stability assay (Figure 3d, Clone ES4, Clone ES21 and ES24). Clones ES4 and ES21 passed the stability test while clone ES24 failed. At selected times during the stability study (0, 4, 8 and 12 weeks), cells were frozen. Samples from all four time points from each of the three cell lines were transfected with dTomato/siRNA1 mRNA and analyzed by flow cytometry. Notably, both clones that passed the productivity test (Clones ES4 and ES21) maintained a significant number of cells at 10^0 on the x-axis (i.e., with little to no dTomato (PE) fluorescence) through week 12, indicating the presence of cells with high GOI expression. This cell population with negligible dTomato expression is absent in the clone that failed the productivity test (Figure 6b). These results further validate the correlation between the PTSelect-siRNA activity and GOI expression and demonstrate that the PTSelect-siRNA silencing activity can be utilized as a marker to monitor the productivity of PTSelect-generated clones.

Enrichment of high producers within a clone using PTSelect technology

We next tested whether PTSelect-siRNA activity could be used to enrich a clone by selecting its high producers. We used an EpoFc-clone (clone 15), which when electroporated with dTomato/ siRNA1 mRNA, showed three different sub-populations based on their PTSelect-siRNA1 activity (Figure 6c, Clone 15-pre-sorted). From this clone, the three distinct populations were separated. Sorted cells were expanded for four days for High-PE and Med-PE and two weeks for Low-PE, due to the differing numbers of sorted cells, and subjected to productivity assay along with unsorted clone 15. While, there was no significant difference in their doubling times (Low-PE-22h; Med-PE-20h; High-PE-18h; pre-sorted clone 15-23h), their specific productivities (Low-PE-653; Med-PE-397; High-PE-0; pre-sorted clone 15-506 mlU/day/cell) correlated with their ability to suppress the siRNA, as expected. Thus, by enriching clone 15 with cells showing maximum siRNA activity (Low-PE), the productivity of clone 15 was increased from 506 to 653 mlU/day/cell. The uniformity of the sorted Low-PE clone was determined (Figure 6c, 15-Low-PE-post-sorted). The cells that

exhibit most silencing activity (10^0 on the x-axis) had been enriched from 0.2 to 13.2%. Interestingly, clonal drift was also noticed within three weeks due to the presence of two other cell populations with decreased siRNA activity (populations 2 and 3). Overall, the results confirm that PTSelect technology can facilitate enrichment of high producers from a clone, and that it is a sensitive method to monitor single-cell clonal drift within a cell population.

Discussion

Here, we describe the principle of PTSelect technology and demonstrate its ability to establish stable CHO-K1 clones producing either EpoFc or adalimumab (Figure 3b and 5b). A stable cell pool expressing EpoFc was established in 11 days (Figures 2c and 3c, a significant time reduction compared to current antibiotic or metabolic selection methods. Notably, the PTSelect-siRNA activity correlates with the relative GOI expression (Figure 6a). Thus, PTSelect can be employed to monitor production instability due to clonal drift by simple flow cytometric analysis (Figure 6b) as well as to enable clonal enrichment (Figure 6c).

A critical pre-requirement for FDA approval of a clonal line is to demonstrate that the entire cell line population is derived from a single cell progenitor (ICH Q5D) (Plavsic, 2017; Welch & Arden, 2019), given the high propensity of CHO cells to undergo clonal variation or clonal drift(Ko et al., 2018). In addition to techniques that were developed to monitor clonal drift by the analysis of sub-clones (Aebischer-Gumy et al., 2018; Tharmalingam et al., 2018; Vcelar et al., 2018) and cell surface staining(Pilbrough et al., 2009), strategies have been explored to minimize clonal drift by introducing transgenes into certain target genomic sites identified to be stable(J. Y. Kim et al., 2012; Sakuma et al., 2015; Zhang et al., 2015; Zhou et al., 2010). The target sites were identified by utilizing CHO genome-based multiomics and *in silico* models (Ghorbaniaghdam et al., 2014; Lee et al., 2019). These approaches are time-consuming, expensive, laborious, or require expertise in a specific field. PTSelect technology enables monitoring productivity variation in a single cell in 6 hours, requiring simple transfection of cells with fluorescent/siRNA-mRNA, followed by flow cytometry analysis. Thus, PTSelect technology provides a breakthrough to monitor clonal drift that has been long sought in the biotechnology industry, single-cell productivity monitoring that is simple, rapid, and inexpensive.

To establish stable clones using PTSelect– first, the PTSelect-siRNA cassette is cloned into an existing plasmid containing the GOI. Our data indicate that the presence of antibiotic/metabolic selection genes does not interfere with PTSelect technology (Figures 3c & 5b). After transfecting cells with the plasmid-GOI-siRNA, performing three depletions is recommended (days 3, 7 and 10). On day 10, cells with the lowest fluorescent signal (lowest 1% or 0.5% based on stringency) can be plated using flow cytometry-based sorting into ten 96-well plates. High producers from the 96-well plates (at 4 weeks) can be enriched by FACS two to three times in a 3-week period, as demonstrated in Figure 6c. Thus, a clone enriched for high producers can be generated in 7 to 8 weeks.

PTSelect-siRNA activity also offers the convenience and serves as an indicator for GOI expression for those GOIs that lack antibodies for detection. Thus, PTSelect accelerates stable pool establishment, permits enrichment of high producers, and provides an alternative to screen for GOIs without an antibody. Overall, we have described an innovative siRNA-based technology to establish stable cell lines more rapidly compared with existing technologies. This technology further offers a simple and a reliable solution to monitor single-cell productivity and population drift in productivity due to clonal variation.

Acknowledgements

Funding for this work was provided by the National Institutes of Health, Phase II STTR grant (R42GM114935). We would like to thank Nicholas St. John for his assistance with mRNA synthesis and cell culture maintenance.

Author Contributions

Vandhana M-Chari (designed and performed experiments, analyzed data, wrote manuscript), Zachary Wurz (performed experiments), Francis Doyle (early conceptualization of PTSelect technology and designed PT-

Select siRNA cassette), Matthew Henry (performed and analyzed experiments), Andreas Diendorfer (performed experiments), Scott A Tenenbaum (early conceptualization of PTSelect technology), Nicole Borth (conceptualized PTSelect technology), Edward Eveleth (designed experiments and provided funding), Susan T Sharfstein (analyzed data, provided funding and wrote manuscript).

Competing Interests

Edward Eveleth is President of HocusLocus, a company commercializing the PTSelect technology. Susan Sharfstein was the recipient of an NIH STTR grant with HocusLocus related to this technology. Zachary Wurz was an employee of HocusLocus, and he and Francis Doyle hold an equity position in the company. Nicole Borth and Edward Eveleth have applied for a patent related to the technology described in this publication.

References

Aebischer-Gumy, C., Moretti, P., Little, T. A., & Bertschinger, M. (2018). Analytical assessment of clonal derivation of eukaryotic/CHO cell populations. *Journal of Biotechnology* , *286* , 17–26. https://doi.org/10.1016/j.jbiotec.2018

Bandyopadhyay, A. A., O'Brien, S. A., Zhao, L., Fu, H.-Y., Vishwanathan, N., & Hu, W.-S. (2018). Recurring genomic structural variation leads to clonal instability and loss of productivity: BANDYOPADHYAY et al. *Biotechnology and Bioengineering* . https://doi.org/10.1002/bit.26823

Bebbington, C. R., Renner, G., Thomson, S., King, D., Abrams, D., & Yarranton, G. T. (1992). High-Level Expression of a Recombinant Antibody from Myeloma Cells Using a Glutamine Synthetase Gene as an Amplifiable Selectable Marker. *Bio/Technology* , *10* (2), 169. https://doi.org/10.1038/nbt0292-169

Biological Approvals by Year—2017 Biological License Application Approvals . (n.d.). [WebContent]. Retrieved March 8, 2019, from https://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/BiologicalApprovals

Biologics-revolution-in-the-production-of-drugs.pdf . (n.d.). Retrieved March 7, 2019, from https://www.fraserinstitute.org/si revolution-in-the-production-of-drugs.pdf

Bort, J. A. H., Stern, B., & Borth, N. (2010). CHO-K1 host cells adapted to growth in glutamine-free medium by FACS-assisted evolution.*Biotechnology Journal* , *5* (10), 1090–1097. https://doi.org/10.1002/biot.201000095

Choi, D.-K., Bae, J., Shin, S.-M., Shin, J.-Y., Kim, S., & Kim, Y.-S. (2014). A general strategy for generating intact, full-length IgG antibodies that penetrate into the cytosol of living cells. *MAbs* ,*6* (6), 1402–1414. https://doi.org/10.4161/mabs.36389

Doyle, F., Lapsia, S., Spadaro, S., Wurz, Z. E., Bhaduri-McIntosh, S., & Tenenbaum, S. A. (2017). Engineering Structurally Interacting RNA (sxRNA). *Scientific Reports* , *7* (1). https://doi.org/10.1038/srep45393

Fischer, S., Handrick, R., & Otte, K. (2015). The art of CHO cell engineering: A comprehensive retrospect and future perspectives.*Biotechnology Advances* , *33* (8), 1878–1896. https://doi.org/10.1016/j.biotechadv.2015.10.015

Frye, C., Deshpande, R., Estes, S., Francissen, K., Joly, J., Lubiniecki, A., Munro, T., Russell, R., Wang, T., & Anderson, K. (2016). Industry view on the relative importance of "clonality" of biopharmaceutical-producing cell lines. *Biologicals* ,*44* (2), 117–122. https://doi.org/10.1016/j.biologicals.2016.01.001

Gallagher, C., & Kelly, P. S. (2017). Selection of High-Producing Clones Using FACS for CHO Cell Line Development. *Methods in Molecular Biology (Clifton, N.J.)* , *1603* , 143–152. https://doi.org/10.1007/978-1-4939-6972-2_9

Ghorbaniaghdam, A., Chen, J., Henry, O., & Jolicoeur, M. (2014). Analyzing clonal variation of monoclonal antibody-producing CHO cell lines using an in silico metabolomic platform. *PloS One* ,*9* (3), e90832–e90832. PubMed. https://doi.org/10.1371/journal.pone.0090832

Haddad-Mashadrizeh, A., Zomorodipour, A., Izadpanah, M., Sam, M. R., Ataei, F., Sabouni, F., & Hosseini, S. J. (2009). A systematic study of the function of the human beta-globin introns on the expression of the

human coagulation factor IX in cultured Chinese hamster ovary cells. *The Journal of Gene Medicine* , *11* (10), 941–950. https://doi.org/10.1002/jgm.1367

Kang, D., Skalsky, R. L., & Cullen, B. R. (2015). EBV BART MicroRNAs Target Multiple Pro-apoptotic Cellular Genes to Promote Epithelial Cell Survival. *PLOS Pathogens* , *11* (6), e1004979. https://doi.org/10.1371/journal.ppat.10

Kaufman, R. J., & Sharp, P. A. (1982). Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary dna gene. *Journal of Molecular Biology* ,*159* (4), 601–621. https://doi.org/10.1016/0022-2836(82)90103-6

Kim, J. Y., Kim, Y.-G., & Lee, G. M. (2012). CHO cells in biotechnology for production of recombinant proteins: Current state and further potential. *Applied Microbiology and Biotechnology* , *93* (3), 917–930. https://doi.org/10.1007/s00253-011-3758-5

Kim, S.-Y., Lee, S., Lee, Y.-K., Lee, J., Shin, H.-S., & Kim, Y.-S. (n.d.). *Hυμαν β-Γλοβιν Σεϛονδ Ιντρον Ηγηλψ Ενηανϛες Εξπρεσσιον οφ Φορειγν Γενες φρομ Μυρινε ῾ψτομεγαλοιρυς Ιμμεδιατε-Εαρλψ Προμοτερ* . 7.

Ko, P., Misaghi, S., Hu, Z., Zhan, D., Tsukuda, J., Yim, M., Sanford, M., Shaw, D., Shiratori, M., Snedecor, B., Laird, M., & Shen, A. (2018). Probing the importance of clonality: Single cell subcloning of clonally derived CHO cell lines yields widely diverse clones differing in growth, productivity, and product quality. *Biotechnology Progress* , *34* (3), 624–634. https://doi.org/10.1002/btpr.2594

Lattenmayer, C., Trummer, E., Schriebl, K., Vorauer-Uhl, K., Mueller, D., Katinger, H., & Kunert, R. (2007). Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters. *Journal of Biotechnology* , *128* (4), 716–725. https://doi.org/10.1016/j.jbiotec.2006.12.016

Lee, J. S., Kildegaard, H. F., Lewis, N. E., & Lee, G. M. (2019). Mitigating Clonal Variation in Recombinant Mammalian Cell Lines. *Trends in Biotechnology* , *37* (9), 931–942. https://doi.org/10.1016/j.tibtech.2019.02.007

Lee, J. S., Park, J. H., Ha, T. K., Samoudi, M., Lewis, N. E., Palsson, B. O., Kildegaard, H. F., & Lee, G. M. (2018). Revealing Key Determinants of Clonal Variation in Transgene Expression in Recombinant CHO Cells Using Targeted Genome Editing. *ACS Synthetic Biology* ,*7* (12), 2867–2878. https://doi.org/10.1021/acssynbio.8b00290

Li, F., Vijayasankaran, N., Shen, A. (Yijuan), Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production.*MAbs* , *2* (5), 466–477. https://doi.org/10.4161/mabs.2.5.12720

Mirasol, F. (n.d.). *The Challenge of Building Better Biologic Drugs* . Retrieved March 7, 2019, from http://www.pharmtech.com/challenge-building-better-biologic-drugs

Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use – ACTIP . (n.d.). Retrieved March 8, 2019, from http://www.actip.org/products/monoclonal-antibodies-approved-by-the-ema-and-fda-for-therapeutic-use/

Pilbrough, W., Munro, T. P., & Gray, P. (2009). Intraclonal protein expression heterogeneity in recombinant CHO cells. *PloS One* ,*4* (12), e8432–e8432. PubMed. https://doi.org/10.1371/journal.pone.0008432

Plavsic, M. (2017). Q5D Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products. In *ICH Quality Guidelines* (pp. 375–393). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118971147.ch13

Research, C. for B. E. and. (n.d.). *Biological Approvals by Year—2018 Biological License Application Approvals* [WebContent]. Retrieved March 8, 2019, from https://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalPr

Sakuma, T., Takenaga, M., Kawabe, Y., Nakamura, T., Kamihira, M., & Yamamoto, T. (2015). Homologous Recombination-Independent Large Gene Cassette Knock-in in CHO Cells Using TALEN and MMEJ-Directed Donor Plasmids. *International Journal of Molecular Sciences* ,*16* (10), 23849–23866. PubMed. https://doi.org/10.3390/ijms161023849

Tharmalingam, T., Barkhordarian, H., Tejeda, N., Daris, K., Yaghmour, S., Yam, P., Lu, F., Goudar, C., Munro, T., & Stevens, J. (2018). Characterization of phenotypic and genotypic diversity in subclones derived from a clonal cell line. *Biotechnology Progress* ,*34* (3), 613–623. https://doi.org/10.1002/btpr.2666

Vcelar, S., Melcher, M., Auer, N., Hrdina, A., Puklowski, A., Leisch, F., Jadhav, V., Wenger, T., Baumann, M., & Borth, N. (2018). Changes in Chromosome Counts and Patterns in CHO Cell Lines upon Generation of Recombinant Cell Lines and Subcloning. *Biotechnology Journal* ,*13* (3), 1700495. https://doi.org/10.1002/biot.201700495

Welch, J. T., & Arden, N. S. (2019). Considering "clonality": A regulatory perspective on the importance of the clonal derivation of mammalian cell banks in biopharmaceutical development.*Biologicals: Journal of the International Association of Biological Standardization* , *62* , 16–21. https://doi.org/10.1016/j.biologicals.2019.09.006

Wurm, F., & Wurm, M. (2017). Cloning of CHO Cells, Productivity and Genetic Stability—A Discussion. *Processes* , *5* (4), 20. https://doi.org/10.3390/pr5020020

Würtele, H., Little, K. C. E., & Chartrand, P. (2003). Illegitimate DNA integration in mammalian cells. *Gene Therapy* , *10* (21), 1791–1799. https://doi.org/10.1038/sj.gt.3302074

Zhang, L., Inniss, M. C., Han, S., Moffat, M., Jones, H., Zhang, B., Cox, W. L., Rance, J. R., & Young, R. J. (2015). Recombinase-mediated cassette exchange (RMCE) for monoclonal antibody expression in the commercially relevant CHOK1SV cell line. *Biotechnology Progress* ,*31* (6), 1645–1656. https://doi.org/10.1002/btpr.2175

Zhou, H., Liu, Z., Sun, Z., Huang, Y., & Yu, W. (2010). Generation of stable cell lines by site-specific integration of transgenes into engineered Chinese hamster ovary strains using an FLP-FRT system.*Journal of Biotechnology* , *147* (2), 122–129. https://doi.org/10.1016/j.jbiotec.2010.03.020

Figure Legends

Figure 1: Principle of PTSelect technology. a. PTSelect-siRNA design consisting of BART-10 siRNA precursor (90bp) flanked by 100bp of its native genomic sequence on both 5' and 3' flanks. This construct is situated within sequence taken from the human β-globin (HBG) intron (486bp). The entire cassette is cloned upstream of the GOI. **b.**Expected sequences of processed PTSelect-siRNAs of siRNA1 (3P segment) and siRNA2 (3P segment) in the context of precursor stem loop sequences.**c.** PTSelect-siRNA is processed within cells that contain GOI. When CD4/siRNA mRNA is introduced by electroporation into cells containing the GOI, PTSelect-siRNAs bind to the complementary sequences on CD4/siRNA mRNA and degrade the mRNA, eliminating exogenous cell-surface CD4 expression (core concept of PTSelect technology).**d.** Depletion is possible due to the variable expression of CD4 on the cell surface (present on cells that do not contain GOI and absent from the cell surface in cells that contain GOI), enabling the separation of cells with GOI from cells without GOI (steps 1 through 4).

Figure 2: Establishment of GFP-stable pool population as a proof of concept: a. Map of EGFP-siRNA1 plasmid with the GOI and the PTSelect-siRNA1 cassette. **b.** In each depletion experiment, CD4/siRNA mRNA and dTomato/siRNA1 mRNA are introduced into stock cells by electroporation. Both mRNAs contain sequences complementary to the PTSelect-siRNA1. At each depletion, cells retained by the magnetic beads (isolate, no EGFP), cells in the supernatant (expressing EGFP) and cells not subjected to separation (stock) were analyzed by flow cytometry analysis **c.** Flow cytometry analysis of depletion experiments performed on days 3, 10, and 19. The dTomato (PE) signals are suppressed by the PTSelect-siRNA1, which is expressed by cells that contain EGFP (supernatant) and not by cells that do not contain EGFP (isolate). Top panel shows the dTomato (PE) signals from the supernatant (cells with EGFP and PTSelect-siRNA), in which the dTomato (PE) signal is suppressed by the PTSelect-siRNA and from parental CHO cells (control), in which the dTomato (PE) signal remains unaffected. On day 10, the cell population has stabilized. It is possible to sort the fraction of cells with low dTomato signals (circled) and proceed with limited dilution cloning. Bottom panel shows the dTomato (PE) signal in the isolate fraction, in which there is no suppression of dTomato (PE) signal).

Figure 3: Comparison of EpoFc clones generated by PTSelect and conventional technologies.

a. EpoFc-ELISA of clones three weeks after limited dilution cloning. Significantly more EpoFc-positive clones were generated by PTSelect, compared to clones generated by puromycin selection or neomycin/MTX amplification. First two columns in each plate are ELISA standards in duplicate. **b.** Productivity of EpoFc clones (mIU*/cell/day) generated using, PTSelect (EpoFc_siRNA1), puromycin selection (EpoFc_siRNA1_-Puro) or neomycin selection followed by MTX amplification (EpoFc_DHFR_Neo). Each point represents an individual clone selected as indicated by the legend. **c.**Productivity of EpoFc clones generated by varying the amount of CD4 mRNA (CD4-500ng; depletion on day 11, CD4-500ng and 1000ng; depletion on day 21). Each point represents an individual clone selected as indicated by the legend. (*mlU definition: The standards in the EPO kit were assays against WHO standard and the conversion between IU (International Units) and mass is ˜125 IU/µg).

Figure 4 : **Productivity over 12 weeks of top 6 EpoFc-producing clones generated by PTSelect.** **a.** Clones ES24 (top 1), ES25 (top 2) and ES22 (top 6) dropped below 70% of their initial productivity, while the ES11 (top 3), ES4 (top 4) and ES21 (top 5) maintained productivity. **b.** Productivity over 12 weeks of top six EpoFc clones generated by neomycin selection followed by MTX amplification. Clones DEN43 (top 1), DEN44 (top 2), DEN30 (top 3), DEN39 (TOP 5) and DEN53 (top 6) dropped below 70% of their initial productivity, while the DEN31 (top 4) maintained productivity.

Figure 5: Comparison of adalimumab clones generated by PTSelect and conventional technologies. **a.** HC_siRNA1-LC_siRNA2_GS plasmid expressing adalimumab was generated with siRNA1 under the control of the same promoter as HC and siRNA2 under the control of the same promoter as LC. In addition, glutamine synthetase was cloned into this vector to enable stable cell generation by MSX selection. The presence of siRNA1 and siRNA2 along with GS enables this plasmid to be used for both conventional (MSX-selection) and PTSelect technologies. **b.**Productivity of adalimumab clones generated by PTSelect (mAb-500 and mAb-1000) using 500 ng or 1000 ng of CD4/siRNA1 mRNA and CD4/siRNA2 mRNA or MSX (25mM) selection (mAb-MSX-25mM). Each point represents an individual clone selected as indicated by the legend.

Figure 6: Monitoring clonal productivity by measuring silencing activity of PTSelect-siRNA. **a.** Two high, medium, and lower productivity EpoFc clones generated by PTSelect were electroporated with GFP/siRNA1 mRNA with sequences complementary to PTSelect-siRNA1. The number of cells with high suppression of GFP (FITC) signals (10^0 on x-axis) correlated with the productivity.**b.** PTSelect-generated EpoFc clones that passed (ES4 & ES21) and failed the stability assay (ES24) were electroporated with dTomato/siRNA1 mRNA and GFP-mRNA (transfection control). For control, parental CHO-K1 cells were transfected with either dTomato/siRNA1 or GFP-mRNA. Clones that passed the stability test show significant suppression of dTomato (PE) signals (10^0 on x-axis) even during week 12, correlating with high expression of EpoFc, while this is absent in the clone that failed the test (clone 24). **c.**Enrichment of clone 15 for high producers by cell sorting. **Left panel,** EpoFc-clone-15 showed three different populations (Clone 15-pre-sorted) when electroporated with dTomato/siRNA1 mRNA, based on the inhibition exerted by PTSelect-siRNA1. From this clone, the three distinct populations were selected by sorting (low, medium and high PE corresponding to high, medium and low expression of EpoFc, respectively). **Right panel,** three weeks after expansion, sorted Low-PE clone was transfected with GFP-mRNA (transfection control) and dTomato/siRNA1 mRNA. The appearance of population 2 and 3 indicates variation and/or drift in the sorted population. Since GFP was used, signals were compensated in 6b and 6c.

Figure 1a

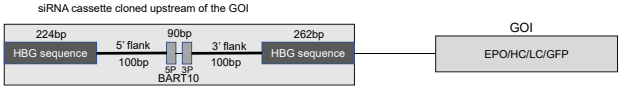
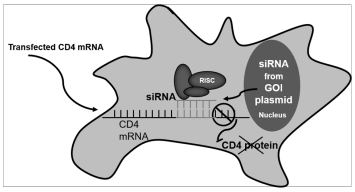


Figure 1b

5P	LOOP	3P	
GCCACCTCTTTGGTTCTGTACA	TATTTTGTTATTG	TACATAACCATGGAGTTGGCTGT	siRNA1
CGGACCAGTTTCCATCTCAACA	TATTTTGTTATTG	TTGATATGGATGCTGTTCCGTGT	siRNA2

Figure 1c



Hosted file

`Main figures April 19.pdf` available at https://authorea.com/users/313685/articles/444158-ptselect-a-post-transcriptional-technology-that-enables-rapid-establishment-of-stable-cho-cell-lines-and-surveillance-of-clonal-variation