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

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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 enrichment. 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.

1. Introduction

Chinese hamster ovary (CHO) cells are preferred for biologics production for their safety as production hosts, (U. S. Food and Drug Administration. U. S. Food and Drug Administration. (CBER), 2017 (U. S. Food and Drug Administration. (CBER), 2018) (U. S. Food and Drug Administration. (CBER), 2019) 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; Kim et al., 2012). Current technologies for establishing stable CHO cell lines utilizing antibiotic/metabolic selection are time-consuming and expensive. While accelerating cell line and process development has always been of interest in the biopharmaceutical industry, the current COVID-19 pandemic has increased the impetus for rapid manufacturing of biopharmaceuticals. 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 and Kelly, 2017; Kaufman and Sharp, 1982). Finally, production instability caused by clonal drift affects the productivity of clones (Bandyopadhyay et al., 2019; Lee et al., 2018; Vcelar et al., 2018; Wurm and 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, 2018).

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 in 10

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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.

2. Materials and methods

2.1. 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 was constructed by cloning the siRNA1/BART10 cassette between the *SacI* and *XbaI* sites upstream of the EpoFc protein. DHFR- EpoFc-NEO (EDN) plasmid 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 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) 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 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).

2.2. 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 8 mM Glutamax and anti-clumping agent (Thermo Fisher Scientific). Cultures were routinely passaged twice a week at 1.5×10^5 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).

2.3. 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.

2.4. Stable cell line development by PTSelect™

CHO-K1 (80×10^6cells) were electroporated with 160 µg plasmid DNA (2 µg/million cells for a 5 kb plasmid or ~650 fmol/million cells) 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 72 h, at least 80×10^6 cells 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 4–6 h, 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 80×10^6 in number.

2.5. 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 1.5×10^5 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. 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.

2.6. 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 1.0×10^5 cells/mL in 10 mL of CD CHO medium supplemented with 8 mM Glutamax and anti-clumping agent in 50 mL bioreaction tubes on a shaker set at 195 rpm. 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 t_1 and t_2 are two different time points in hours.

2.7. Analysis of clone stability

The six highest producing EpoFc-clones generated by PTSelect™ and Neo/DHFR amplification were chosen. Cells were seeded at 1.5×10^5 cells/mL in 30 mL of CD CHO medium supplemented with 8 mM Glutamax and anti-clumping agent in 125 mL shaker flask. Cells were passaged every 3–4 days and cultured for 12 weeks. Samples were taken weekly and analyzed for protein expression levels by ELISA as described

above. Cells from weeks 0, 4, 8 and 12, as counted from the start of the long-term cultivation were frozen and analyzed for clonal variation/drift at the end of the experiment. To test for clonal drift, cells from each time point were thawed and 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).

3. Results

3.1. PTSelect™-siRNA design strategies

PTSelect™ technology has two vital components; (1) an siRNA cassette designed to contain the PTSelect™-siRNA cloned upstream of

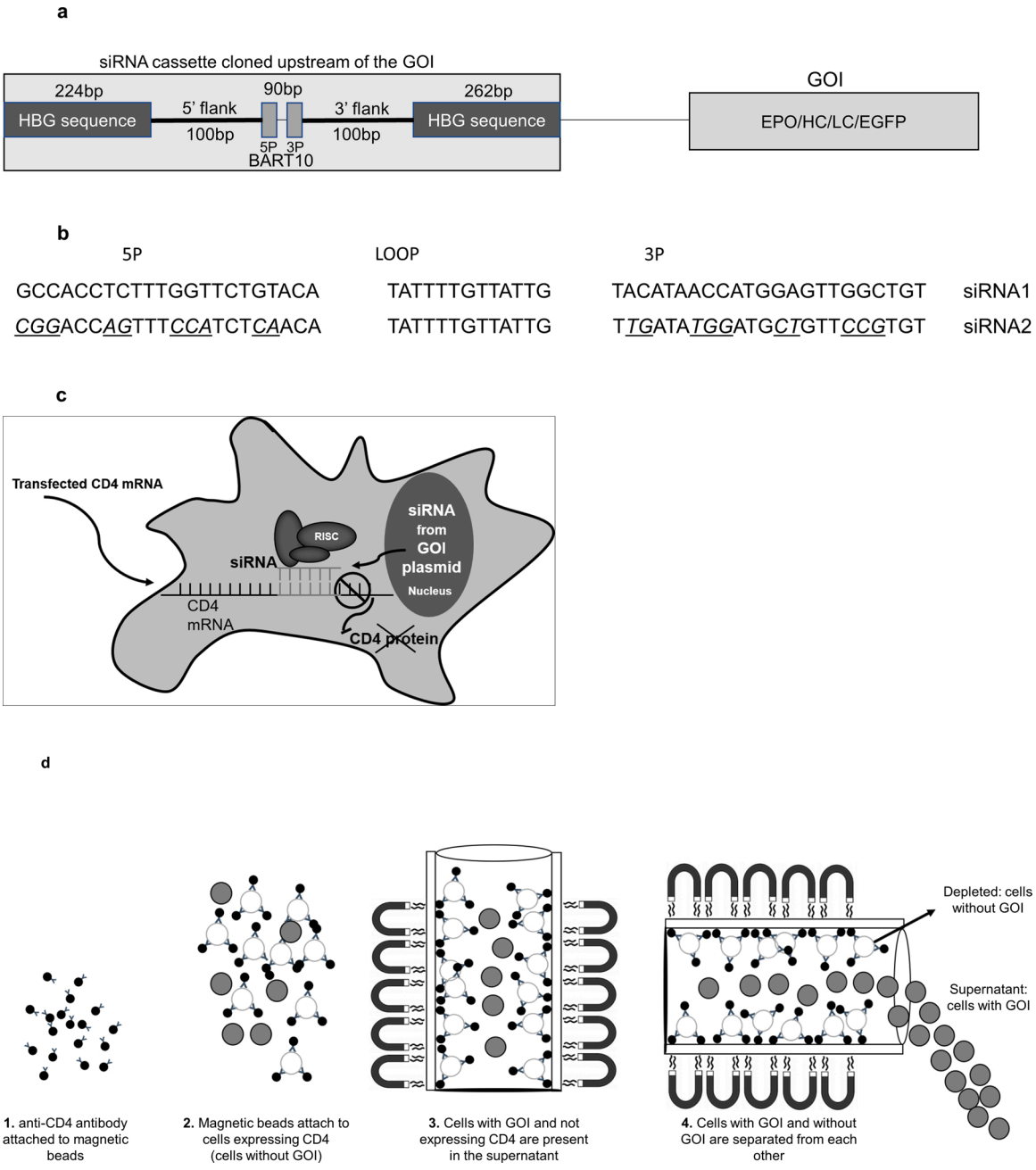

Fig. 1. Principle of PTSelect™ technology. (a) PTSelect™-siRNA design consisting of BART-10 siRNA precursor (90bp) flanked by 100bp of its native genomic sequence on both 5′ and 3′ flanks. This construct is situated within sequence taken from the human β-globin (HBG) intron (486bp). The entire cassette is cloned upstream of the GOI. (b) Expected sequences of processed PTSelect™-siRNAs of siRNA1 (3 P segment) and siRNA2 (3 P 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).

the GOI (Fig. 1a) with both the siRNA cassette and the GOI regulated by the same promoter, and (2) a CD4/siRNA mRNA containing an engineered RNA sequence that is complementary to the PTSelect™-siRNA, and thus, targeted by the processed PTSelect™-siRNA (Fig. 1b). The siRNA cassette is comprised of 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; Kim et al., 2005), was used to flank the siRNA construct; (II) The PTSelect™-siRNA sequence that is co-transcribed with the GOI is a sequence from the BART segments (*Bam*HI A rightward transcripts) of the Epstein-Barr virus (EBV) (Edwards et al., 2008; Kang et al., 2015), a region from which a group of miRNAs originate (Edwards et al., 2008). We used the

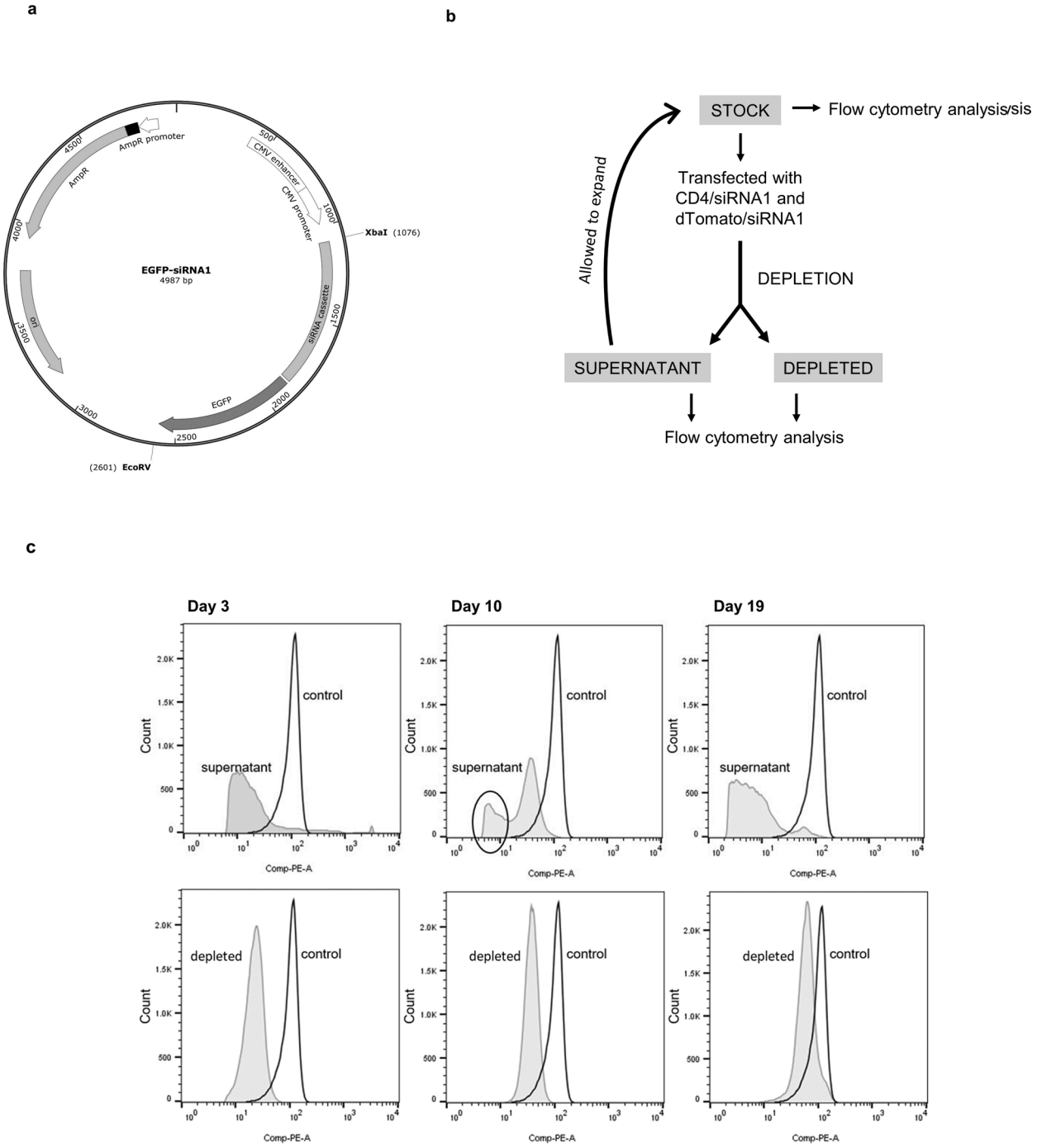

Fig. 2. Establishment of GFP-stable pool population as a proof of concept: (a) Map of EGFP-siRNA1 plasmid with the GOI and the PTSelect™-siRNA1 cassette. (b) In each depletion experiment, CD4/siRNA mRNA and dTomato/siRNA1 mRNA are introduced into stock cells by electroporation. Both mRNAs contain sequences complementary to the PTSelect™-siRNA1. At each depletion, cells retained by the magnetic beads (depleted, 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 (depleted). 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 depleted fraction, in which there is no suppression of dTomato (PE) signal).

highly expressed miR-BART10-3p (BART10), previously employed in the development of a related technology (Doyle et al., 2017), which is an identified miRNA (miRbase accession MIMAT0003420). A single copy of the DNA for the precursor sequence of BART-10 was cloned into the siRNA 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, Fig. 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. We expect the processed BART-miRNA made from the PTSelect™-siRNA cassette to target the synthetically designed target sequences in CD4/siRNA mRNA complementary to the BART-miRNA, to elicit siRNA-like action (Aldred et al., 2011).

3.2. 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 Fig. 1a). 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 (Fig. 1c). Maximum CD4 expression from the electroporated mRNA occurs at ~10 h after electroporation (Supplementary Fig. 1b). To separate CD4-positive (no GOI) from CD4-negative (expressing GOI) cells, 6 h 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 (Fig. 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. Given that the mRNA electroporation efficiency is not consistently 100%, the less than 1% untransfected cells that are CD4 negative will not bind anti-CD4-conjugated magnetic beads and thus, will remain among the CD4-silenced stable cells. We therefore recommend performing at least three to four cycles of depletion to reduce the false positives in the enriched stable cell pool.

3.3. 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 (Fig. 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 depleted fraction, supernatant and cells not subjected to depletion (stock) were analyzed by flow cytometry (Fig. 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 160×10^6 cells; the recovered cells were expanded to 80×10^6 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 (Würtele 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 abundance of EGFP signals in the supernatant fraction, which on day 10, comprised ~40% of the total population was confirmed by flow cytometry (Supplementary Fig. 2). 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 (Fig. 2c, top panel). Conversely, the dTomato (PE) signal from the cells in the depleted fraction progressively moved towards the control population signal (Fig. 2c, bottom panel). Overall, the results confirm that PTSelect™ can establish a stable cell pool expressing EGFP.

3.4. 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 Fig. 3), and transfected into CHO-K1 cells by electroporation. Cells transfected with EDN were selected with neomycin followed by MTX amplification (Supplementary Fig. 4a); limited dilution cloning (LDC) was performed on day 31. Cells transfected with ESP were selected with puromycin (Supplementary Fig. 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 supernatant cells obtained from days 18, 24 and 30. Clonal selection and expansion were performed as outlined in Supplementary Fig. 5 and described in Materials and Methods.

Three weeks after plating, EpoFc levels in expanded clones were assessed by ELISA (Fig. 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 (Fig. 3b).

Table 1
Progressive establishment of stable pool population.

Time after initial transfection	Stock (10^6 cells)	Supernatant (10^6 cells)	Percentage recovered
3 days	160	7.2	4.5
7 days	80	24	30
10 days	80	1.9	2.4
17 days	80	65	81
19 days	80	66	83
Note that the depleted cells maintain a healthy doubling time			

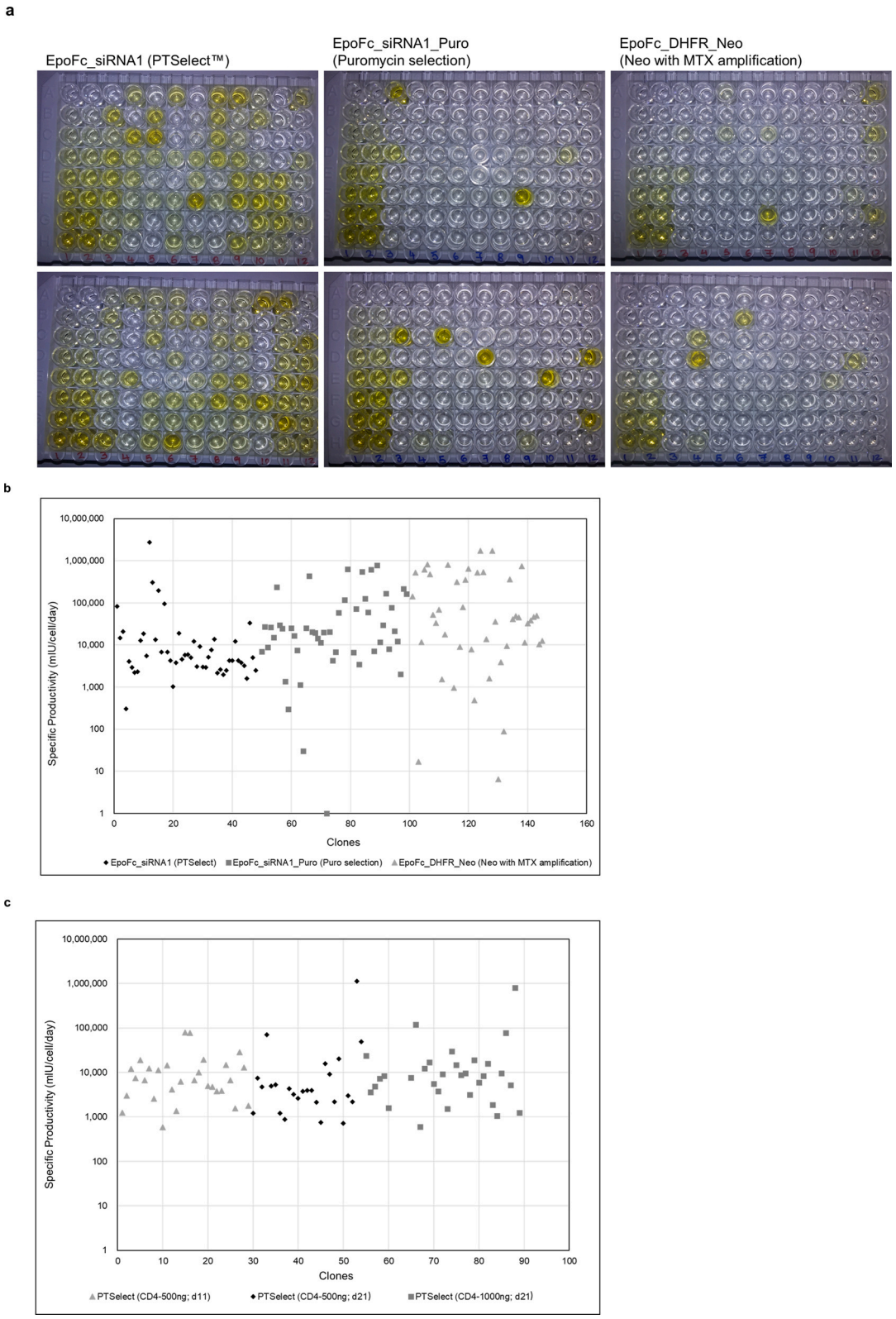

Fig. 3. Comparison of EpoFc clones generated by PTSelect™ and conventional technologies. (a) EpoFc-ELISA of clones three weeks after limited dilution cloning. Significantly more EpoFc-positive clones were generated by PTSelect™, compared to clones generated by puromycin selection or neomycin/MTX amplification. 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 (CD4-500 ng; depletion on day 11, CD4-500 ng and 1000 ng; depletion on day 21). Each point represents an individual clone selected as indicated by the legend. (*mIU 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).

Although the highest producer was generated by PTSelect™ technology (2,755,721 mIU/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 ± 1.3 h for clones generated by PTSelect™, 19.5 ± 3.9 h for ESP clones generated by puromycin selection, and 20.5 ± 2.6 h 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-acetyl-transferase 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 the supernatant cells obtained was subjected to LDC. The remaining supernatant 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 Fig. 6). Productivity was tested for 25 clones per condition (Fig. 3c). The highest producer occurred in cells depleted on day 21 with 500 ng of CD4/siRNA1 mRNA (1,119,926 mIU/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 10, 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 (Fig. 4a). However, only 1 out of 6 EDN clones passed the same stability criterion (Fig. 4b). Thus, PTSelect™ technology generated clones with greater stability than those generated by antibiotic selection and MTX amplification.

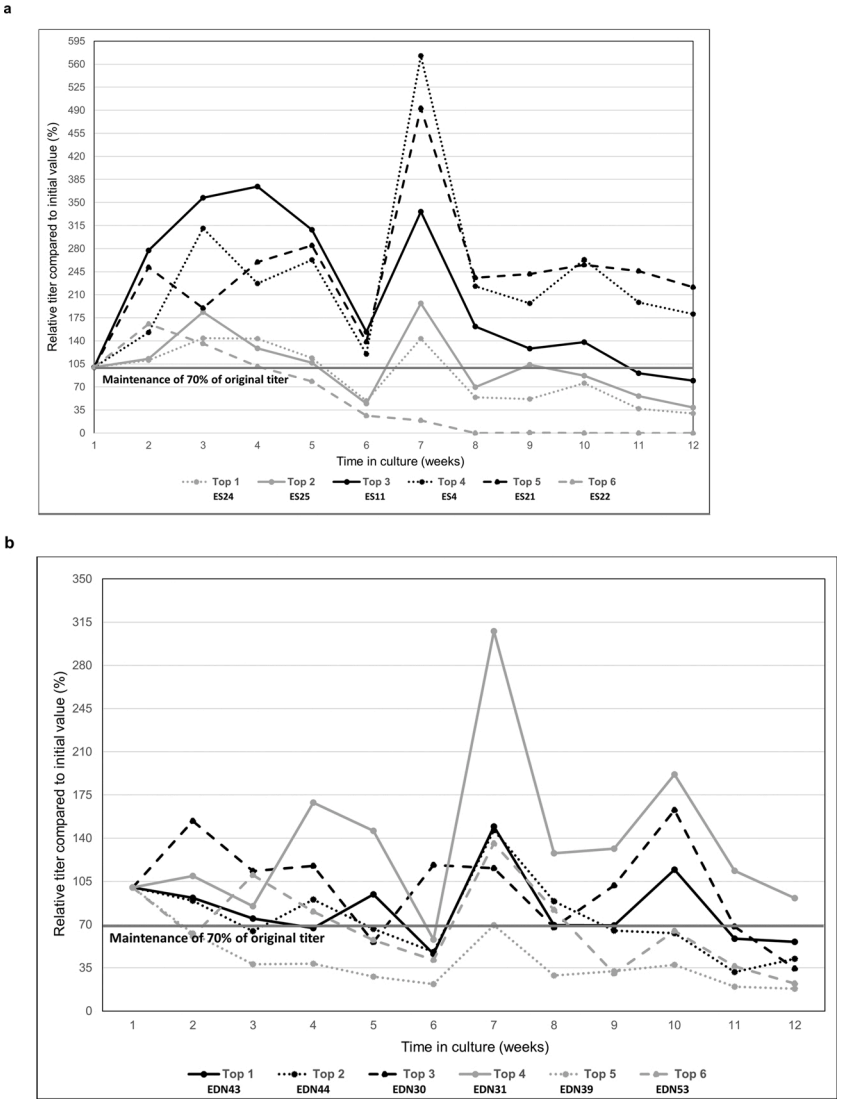

Fig. 4. Productivity over 12 weeks of top 6 EpoFc-producing clones generated by PTSelect™. (a) Clones ES24 (top 1), ES25 (top 2) and ES22 (top 6) dropped below 70% of their initial productivity, while the ES11 (top 3), ES4 (top 4) and ES21 (top 5) maintained productivity. (b) Productivity over 12 weeks 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.

3.5. 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 (Fig. 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 supernatant 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 (Fig. 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.

3.6. Silencing activity of PTSelect™-siRNA correlates with the expression of GOI

To evaluate the relationship between silencing efficiency and productivity, we chose high (>100,000 mIU/day/cell), medium (between 1000 and 50,000 mIU/day/cell) and low (<1000 mIU/day/cell) productivity EpoFc PTSelect™-clones, electroporated them with GFP/siRNA1 mRNA, and analyzed them by flow cytometry (Fig. 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° on the x-axis (Fig. 5a, insert). The percentage of cells with maximum GFP suppression (10° 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.

3.7. 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 (Fig. 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), cell aliquots were frozen. Frozen samples from all four time points from each of the three cell lines were revived, 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° 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 (Fig. 6b). However, the number of cells at 10° on the x-axis does not directly correlate with the productivity of these clones (ES4 and ES21 in Fig. 4a). A possible explanation can be given by the movement of the cells in the second peak closer to the 10° on the x-axis (8 w and 12 w of ES21) and by the presence of the long tail (8 w, 12 w of ES4 and 4 w of ES21). 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.

3.8. Enrichment of high producers within a clone using PTSelect™ technology

We next tested whether PTSelect™-siRNA activity could be used to

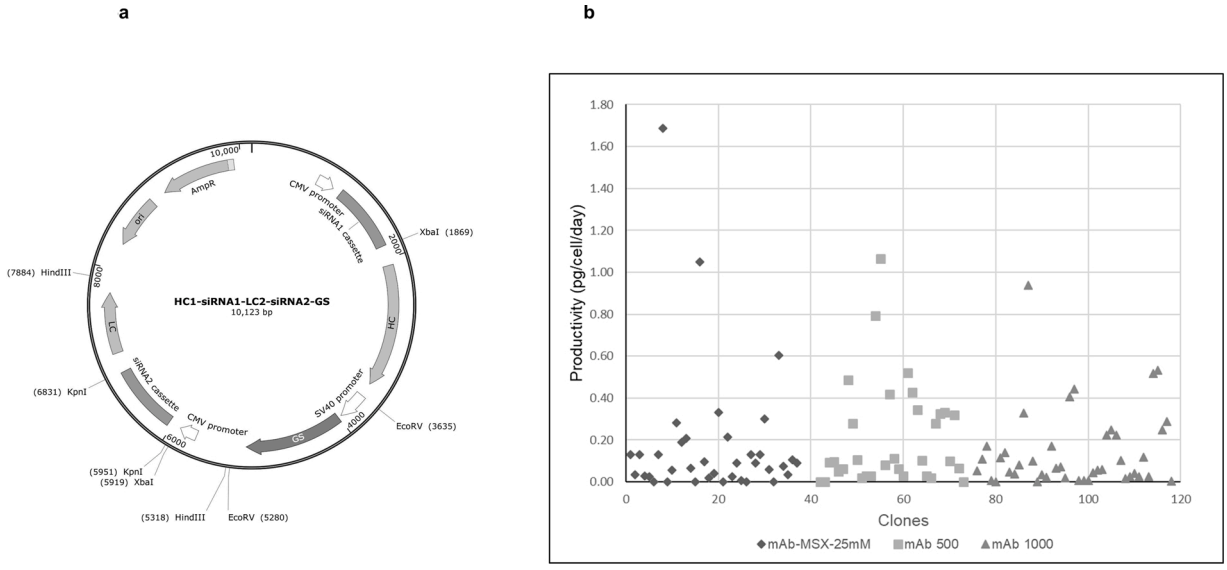

Fig. 5. Comparison of adalimumab clones generated by PTSelect™ and conventional technologies. (a) HC_siRNA1-LC_siRNA2_GS plasmid expressing adalimumab was generated with siRNA1 under the control of the same promoter as HC and siRNA2 under the control of the same promoter as LC. In addition, glutamine 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 (25 mM) selection (mAb-MSX-25 mM). Each point represents an individual clone selected as indicated by the legend.

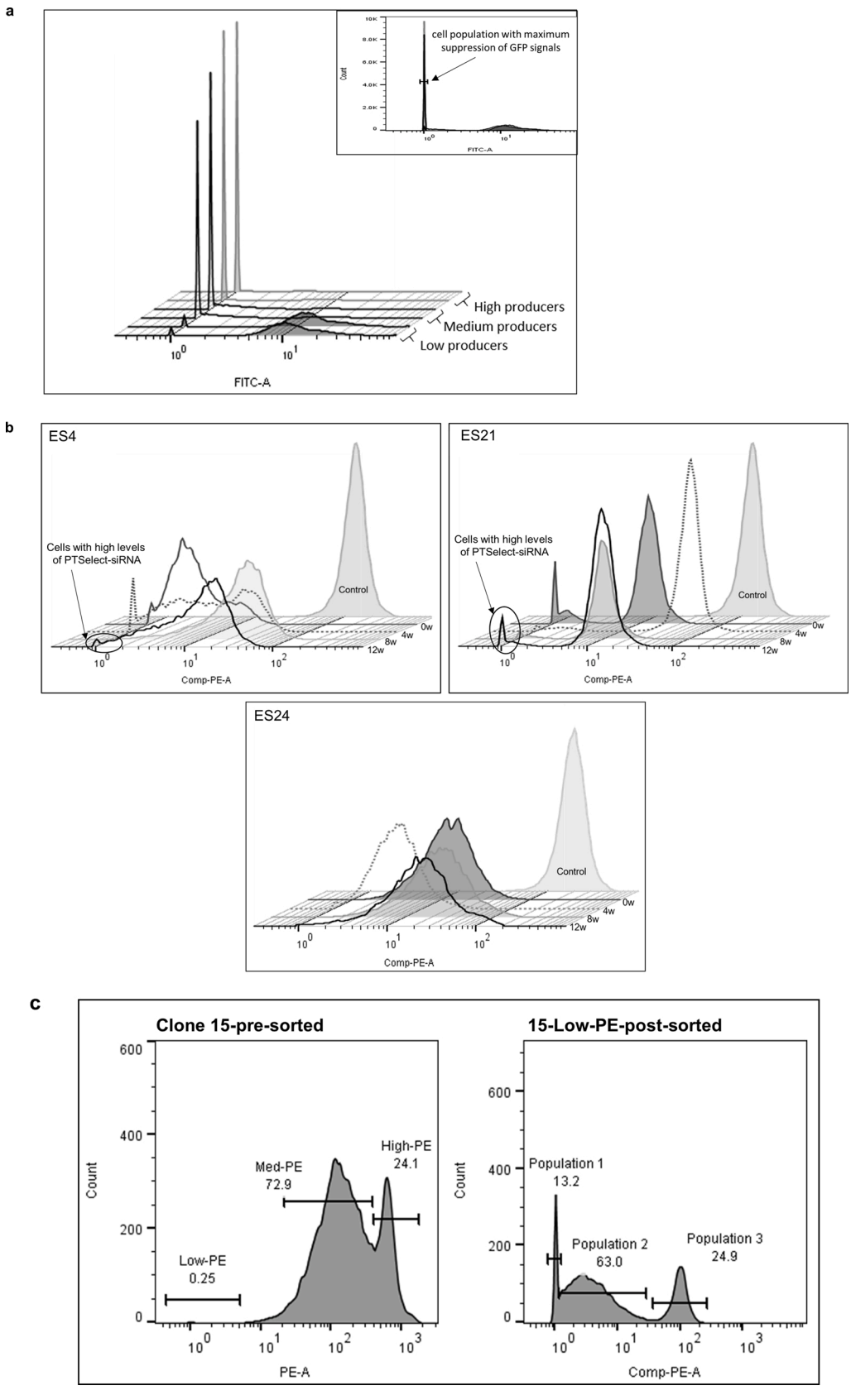

Fig. 6. Monitoring clonal productivity by measuring silencing activity of PTSelect™-siRNA. (a) Two high, medium, and lower productivity EpoFc clones generated by PTSelect™ were electroporated with GFP/siRNA1 mRNA with sequences complementary to PTSelect™-siRNA1. The number of cells with high suppression of GFP (FITC) signals (10° 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° 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.

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 (Fig. 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-22 h; Med-PE-20 h; High-PE-18 h; pre-sorted clone 15−23 h), their specific productivities (Low-PE-653; Med-PE-397; High-PE-0; pre-sorted clone 15-506 mIU/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 mIU/day/cell. The uniformity of the sorted Low-PE clone was determined (Fig. 6c, 15-Low-PE-post-sorted). The cells that exhibit most silencing activity (10° on the x-axis) had been enriched from 0.2–13.2%. 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.

4. Discussion

Here, we describe the principle of PTSelect™ technology and demonstrate its ability to establish stable CHO-K1 clones producing either EpoFc or adalimumab (Figs. 3b and 5 b). A stable cell pool expressing EpoFc was established in 10–11 days (Figs. 2c and 3 c), a significant time reduction compared to current antibiotic or metabolic selection methods. In addition, the decrease in the doubling time of PTSelect-clones (Supplementary Table 2) points to the healthy, optimal, favorable conditions under which the enrichment of cells takes place using the PTSelect method, compared to the sub-optimal and less favorable selection conditions that occur amidst dead cells and the presence of toxic selection agents in both antibiotic and metabolic selection methods. Notably, the PTSelect™-siRNA activity correlates with the relative GOI expression (Fig. 6a). Thus, PTSelect™ can be employed to monitor production instability due to clonal drift by simple flow cytometric analysis (Fig. 6b) as well as to enable clonal enrichment (Fig. 6c).

While other selection processes based on sorting cells that display a secreted protein of interest on their surface exist (Carroll and Al-Rubeai, 2005; Droz et al., 2017; Kuhne et al., 2014; Lang et al., 2016), there are several unique features of the PTSelect™ technology. The limitations of the other FACS-based or magnetic selection of cells are that there is a strict necessity for the GOI to be expressed on the cell surface and for the availability of an antibody to detect the GOI. This limitation is also seen in the existing techniques to monitor the expression stability of cell clones that use an antibody followed by flow cytometry to detect cell surface protein expression levels. Again, PTSelect™ offers the advantage of monitoring GOI expression by mRNA transfection and flow cytometry under circumstances when there are no antibodies available for the GOI or when the GOI is not a cell-surface protein.

The ClonePix™ technology is a popular selection tool that guarantees monoclonality of cell lines derived from a stable cell pool, by culturing the mammalian cell lines in a semi-solid medium, to obtain discrete colonies each from a single parent cell. Similarly, a variety of microfluidic technologies (e.g., Beacon®, Cyto-Mine®) have been introduced into the bioprocessing workflow in lieu of traditional LDC. We believe that stable cell pools generated by PTSelect™ technology could be channeled to the ClonePix™ or microfluidic technologies for clonal isolation, rather than performing LDC. Hence, PTSelect™ is agnostic to clonal isolation technology.

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 and 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. In contrast, PTSelect™ technology enables monitoring of productivity variation in a single cell population in 6 h, requiring simple cell transfection with mRNA coding for an siRNA-regulated fluorescent protein, 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 (Figs. 3c and 5b). After transfecting cells with the plasmid-GOI-siRNA, performing three depletions is recommended (days 3, 7 and 10). The depletion steps can be automated, requiring less hands-on time and effort. On day 10, cells with the lowest fluorescent signal (lowest 1% or 0.5% based on stringency) can be plated using automated 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 Fig. 6c. Thus, a stable cell pool can be obtained in 10 days, and high producer-clones can potentially be obtained in 4 weeks, with further enrichment for productivity in an additional 3 weeks.

This study clearly demonstrates that PTSelect™ accelerates stable pool establishment, permits enrichment of high producers, and provides an alternative approach to screen for GOIs without an antibody. We had initially anticipated that a significant increase in productivity and titer would occur with this technology, as the cells are relieved of expressing drug-resistance proteins (Kallehauge et al., 2017), but that appears not to be the case. We were able to achieve similar productivities as with antibiotic selection, but not dramatically higher values. Future studies will elucidate the mechanisms driving PTSelect™ technology and may provide opportunities for further improvement. For example, the CD4 message is clearly repressed adequately to enable stable cell selection based on differential expression of CD4. However, it is not clear if the reduction in the CD4 expression occurs due to message degradation or by simple stoichiometric repression upon binding of PTSelect™-siRNA to the CD4/siRNA mRNA. It is notable that miRNA target sequences are widely used to reduce ectopic gene expression (https://switchgeargenomics.com/resources/publications). Another issue may be that as PTSelect™-siRNA and the GOI are regulated by the same promoter and transcribed as a single transcript, it is not clear what proportion of the transcripts are excised to generate GOI-mRNA and what fraction are processed to produce PTSelect™-siRNA. Optimization of this ratio by engineering the siRNA cassette may provide another avenue for improved expression of the GOI.

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, providing tremendous potential to accelerate cell line development with rapid clonal screening and identification of potentially unstable cell lines. Innovations in cell line development such as PTSelect™ will hasten novel biopharmaceutical production, including those poised to treat emerging infectious diseases.

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 PTSelect™ 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).

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.09.025.

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