Generation of Stable Chinese Hamster Ovary Pools Yielding Antibody Titers of up to 7.6 g/L Using the piggyBac Transposon System

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Chinese hamster ovary (CHO) cells remain the default production host for many biopharmaceutical drugs, particularly monoclonal antibodies (mAb). Production of gram and kilogram quantities of protein typically requires the generation of stable CHO clones. Unfortunately, this process takes several months, significantly slowing down the drug discovery and development process. Therefore, improved technologies are needed to accelerate biopharmaceutical drug discovery and final drug substance manufacturing. In this study, we describe the generation of stable CHO pools using the piggyBac transposon system. We evaluated the system using four model antibody molecules (3 mAbs and 1 bispecific Ab). Stable CHO pools were isolated in 7–12 days. Using a simple 16-day fed-batch process, we measured titers ranging from 2.3 to 7.6 g/L for the four model antibodies. This represented a 4- to 12-fold increase relative to the controls. Additionally, we isolated stable CHO clones. We found that the stable CHO clones isolated from the piggyBac transposon pools yielded titers two to threefold higher relative to the control clones. Taken together, these results suggest that stable CHO pool and clone generation can be significantly improved by using the piggyBac transposon system. © 2016 American Institute of Chemical Engineers *Biotechnol. Prog.*, 32:1301–1307, 2016
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Introduction

Biopharmaceutical drug discovery is a lengthy and iterative process. During a typical antibody drug discovery effort, several antibody variants are expressed, purified and extensively characterized. Information gathered during the characterization process is used to design improved molecules. The iterative process continues until a final drug candidate molecule has been identified. Due to the significant time and effort required, new and improved methods for recombinant protein production are needed to produce protein for all the necessary biological and physicochemical characterization assays.

Chinese hamster ovary (CHO) cells remain the default expression host for the manufacture of complex biomolecules.[1–3] The two most commonly used methods for protein expression in CHO cells are transient gene expression (TGE) and stable gene expression (SGE). TGE is the method of choice for rapid generation (1–3 weeks) of small quantities of recombinant protein. Recent improvements in TGE in CHO cells have resulted in recombinant protein titers of up to 2 g/L.[4–6] Unfortunately, TGE is not always practical for the generation of gram quantities of protein due to the large amount of DNA and host cells required. Therefore, SGE is typically used to generate gram quantities of recombinant protein. However, SGE is both time consuming and laborious. It usually takes 3–9 months to generate clonal CHO cell lines. Moreover, this process typically requires the isolation and extensive characterization of hundreds or thousands of individual stable CHO clones.

Several research groups have recently reported the generation and use of stable CHO cell pools, instead of stable CHO clones, to speed up the generation of gram quantities of recombinant protein. These CHO pools are heterogeneous populations of recombinant cells obtained by gene transfer followed by genetic selection but without the subsequent clone isolation steps.[7–10] This approach reduces the time required to produce gram quantities of protein to approximately 4–6 weeks (instead of 3–9 months). Unfortunately, stable CHO pools suffer from several limitations including

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lower expression titers and lower stability relative to clonal CHO cell lines.[9,10] In order to address the limitations of stable CHO pools, Balasubramanian et al. recently reported that the use of transposon systems for the generation of CHO-DG44 cell pools led to a significant increase in protein expression with titers reaching 1 g/L from a simple fed-batch process.[8,11] Additionally, the use of stable CHO pools offers several advantages over TGE. These include small amounts of plasmid DNA required for transfection, ease of volumetric scale-up and the flexibility of performing multiple production runs over an extended period of time using a frozen cell bank.

Transposon systems have previously been used to generate cell lines in both CHO and human embryonic kidney (HEK293) cells for protein production.[7,8,11–13] The piggyBac transposon system (PB system) represents one of several transposons that has been modified to function in mammalian cells.[14,15] The system consists of a helper plasmid vector coding for piggyBac transposase (PBase) gene and a donor plasmid vector containing the 5′ and 3′ inverted repeat elements (5′IR and 3′IR) flanking the ends of the artificial transposon.[8,12] The expression cassette coding for the gene of interest and the selection marker is introduced between the two IR elements. Following co-transfection of the helper and donor vectors, transiently expressed PBase catalyzes the excision of the artificial transposon (containing recombinant genes of interest) from the donor vector and mediates its integration into the host genome.

To our knowledge, PB system has not yet been applied to the industrially relevant GS (glutamine synthetase) CHO cell line for the generation of cell pools or clones. Therefore, the goal of this study was to evaluate the PB system for generation of cell pools and clones in a previously described CHO K1SV GS Knockout cell line (referred to as CHO hereafter).[10,16] In this study, we demonstrated that we could generate stable cell pools yielding antibody titers of up to 7.6 g/L using the PB system. This is the highest reported titer from a heterogeneous CHO cell population to the best of our knowledge.

Additionally, we applied the PB system to generable stable CHO clones. We also showed that the PB system significantly enhanced the frequency of high titer clone isolation relative to the control condition. This in turn suggests that a smaller screen size can be used to isolate high producing stable CHO clones, thus streamlining the stable CHO cell line generation process.

Materials and Methods

Cell culture

CHO cells were maintained in a proprietary DMEM-based medium with 8–12 mM L-glutamine (LM-Growth) (Cat. 59202C-100, SAFC, St. Louis, MO) in shake flasks at 37°C and 8% CO_2. Cells were passaged at a seeding density of 0.5 to 1.5 $\times$ 10^6 cells/mL, every 2–3 days. Stock cells were maintained in shake flasks under conditions of 37°C, 7% CO_2, and 160 rpm at a 25 mm shaking diameter in 80% humidified incubator.

Plasmid construction

Plasmid vector pB007 from Transposagen Biopharmaceuticals (SKU: PB007; Lexington, KY) was used as the shuttle vector. This vector has a multiple cloning site (MCS) flanked by proprietary insulator elements and inverted repeats. Expression cassettes for the four model proteins tested (mAb1, mAb2, mAb3, and BsAb1) were cloned into the MCS of pB007. Expression of the light chain (LC) and the heavy chain (HC) genes was controlled by the cytomegalovirus promoter and the GS gene was controlled by SV40E promoter. The entire expression cassette (LC, HC, and GS) was cloned into a single pB007 vector. Helper plasmid vector (coding for the PBase) was also obtained from Transposagen Biopharmaceuticals (SKU: sPBo-25; Lexington, KY).

PEI-transfection and cell pool generation

Two days prior to transfection, CHO cells were seeded at 1.5 $\times$ 10^6 cells/mL in LM-Growth medium. On the day of transfection, cells were recovered by centrifugation at 450*g* for 3 min and re-suspended in LM-TNX medium (identical to LM-Growth medium minus ferric ammonium citrate) at 4 $\times$ 10^6 cells/mL. DNA (3.2 mg/L) and PEI (8 mg/L) were sequentially added to the cells as previously described.[17] Cultures were fed at 4 h post-transfection. Cells were co-transfected with donor and helper plasmid DNA as described in the Results section. The transfected cultures were maintained at 37°C in an incubator with 6–8% CO_2 and 80–90% humidity. At 2 days post-transfection, cells were centrifuged and re-suspended in LM-selection medium (identical to LM-Growth medium plus insulin minus L-glutamine) at 1 $\times$ 10^6 cells/mL with different amounts of L-Methionine sulfoximine (MSX) as described in Results section. Cells were passaged three times a week under selection pressure until complete recovery (>90% viability). Viability of cultures was measured using the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter, Inc., Fullerton, CA). Selection pressure was maintained at all times in culture (during selection and production). For fed-batch production, cells were seeded at 1 $\times$ 10^6 cells/mL in proprietary LM-Production media (LM-Growth medium plus proprietary Lilly feeds minus L-glutamine) and incubated at 37°C. At Day 2 postinoculation, proprietary feeds were added followed by a temperature shift to 32°C. Cultures were fed again on Days 7 and 10, and harvested on Days 14–16. Both selection and production were performed in square shaped, pyramid bottom 24 deep well plate (DWP) with vented lids at a final volume of 4 mL unless stated otherwise.[18] All cultures were maintained under conditions of 6–8% CO_2 and 80–90% humidity and with shaking at 250 rpm with 19 mm shaking diameter.

CHO clone generation

Non-PB clones for mAb2 and BsAb1 were recovered from control pools generated using electroporation. CHO cells were electroporated using a Gene Pulser XCell (BioRad Laboratories, Hercules, CA). Electroporated CHO cells were given 48 h to recover in a LM-Growth medium. After recovery, cells were re-suspended in LM-Selection medium at a concentration of 0.5 $\times$ 10^6 cells/mL. Cultures were passaged twice a week under selection until complete recovery. For PB, the pools generated as described in the previous section were used. The clonal cell lines were recovered from cell pools by limiting dilution. Briefly, the cells were re-suspended at a density of 2.5 cells/mL in a mixture of 90% fresh medium and 10% conditioned medium. Aliquots of 200 μL were transferred to each well of a 96-well plate to give an average of 0.5 cells per well and incubated at 37°C under conditions of 6–8% CO_2 and 80–90% humidity. After

approximately 3 weeks, all wells showing colony growth were consolidated into a fresh 96-well plate containing 200 μL per well of a proprietary production medium and placed back in the incubator for 4 days (static conditions). After 4 days, the titer in each well was measured. The best 24 clones were identified for each molecule based on expression levels and were scaled up to 24 DWP and subject to 14-day fed-batch production. In brief, cells were seeded at 1×10^6 cells/mL in 4 mL proprietary production medium in square shaped, pyramid bottom 24 DWP with vented lids at a final volume of 4 mL.[18] All cultures were maintained at 37°C in an incubator with 6–8% CO_2 and 80–90% humidity, followed by a temperature shift to 32°C on Day 2 postinoculation. Cultures were fed on Days 2, 7, and 10. All cultures were harvested after 14 days.

ForteBio titer measurements

Titers were measured using ForteBio Protein A biosensors in conjunction with a ForteBio Octet QK Red (Pall Life Sciences) platform as previously described.[6] Titers for mAb1, mAb2, mAb3, and BSAb1 were determined by using four different molecule-specific standard curves previously generated and validated for the molecule in question.

Table 1. Transfection and Selection Conditions for Evaluation of piggyBac Transposon System for GS CHO KO Pool Generation

Donor pDNA (%)	Donor pDNA Type	Helper pDNA (%)	MSX Selection (μM)
100	Without IR's and insulators	0	0, 10, 20, and 50
100	With IR's and insulators	0	0, 10, 20, and 50
95	With IR's and insulators	5	0, 10, 20, and 50
90	With IR's and insulators	10	0, 10, 20, and 50
75	With IR's and insulators	25	0, 10, 20, and 50
50	With IR's and insulators	50	0, 10, 20, and 50

Results

Optimization of helper pDNA amount and selection stringency

Recently published results by Balasubramanian et al. showed that a wide range of helper vector coding for PBase (1–50% of total transfected pDNA) yielded similar protein levels from CHO-DG44 cell pools. Ideally, we wanted to minimize the amount of helper vector to reduce the frequency of transposase gene integration. Hence, we tested different ratios of donor (coding for mAb1) to helper pDNA (coding for PBase) to determine the optimal conditions for the generation of stable CHO cell pools. We also tested each co-transfection condition with multiple selection stringencies. The experimental design is outlined in Table 1.

CHO cells were transfected with the donor and helper pDNA as indicated in Table 1. Two days post-transfection, the cells were subjected to different selection conditions as shown in Table 1. Cells were passaged three times a week (under selection condition) until the recovery process was completed (cell viability >90%). Figure 1 shows the viability profile of the various pools tested. For conditions with 0 μM MSX (selection pressure mediated only by the lack of L-glutamine in the selection media), all PB pools (except when the helper vector accounted for 50% of the total DNA), showed a small

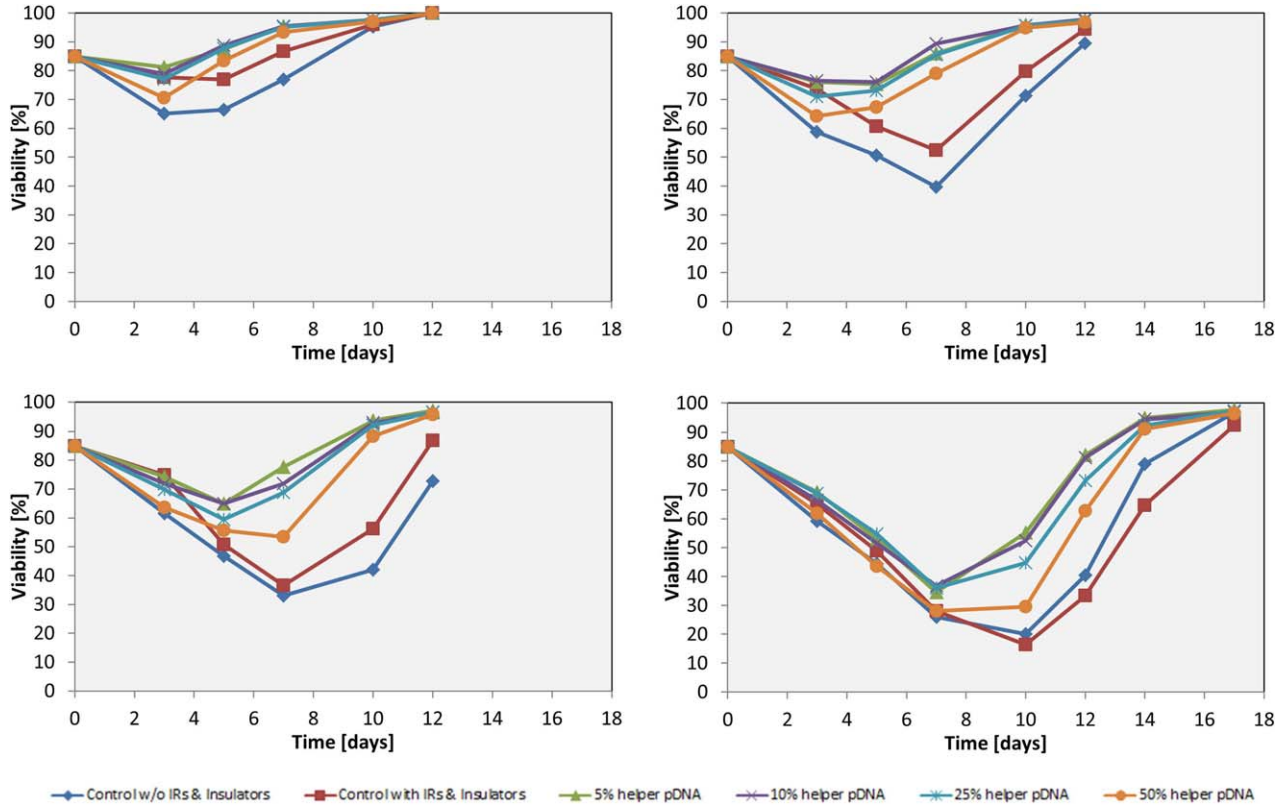

Figure 1. Effect of percent helper pDNA and selection stringency on GS CHO pool recovery.

CHO cells were transfected with the donor and helper pDNA as indicated in Table 1. Two days post-transfection (Day 0 in the figures), cells were subject to (A) 0 μM MSX, (B) 10 μM MSX, (C) 20 μM MSX, and (D) 50 μM MSX selection. Viability was measured using Vi-CELL XR Cell Viability Analyzer on the days shown in the figure.

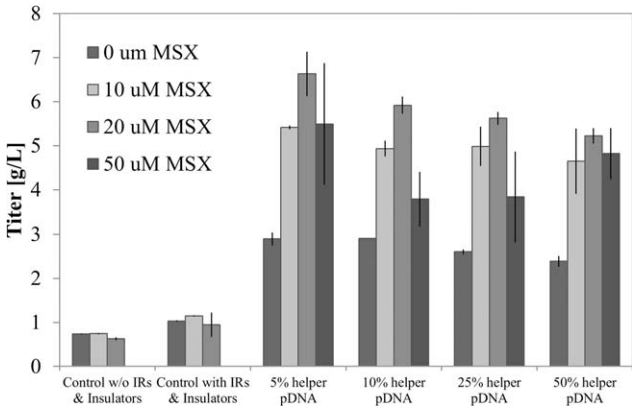

Figure 2. Effect of percent helper pDNA and selection stringency on CHO pool fed-batch expression titer.

CHO cells were transfected with the donor and helper pDNA as indicated in Table 1 and subject to 0, 10, 20, and 50 μM MSX selection. Once CHO pools fully recovered, they were subject to 2 week fed-batch process as described in the methods section. Titers were measured on Day 14 by ForteBio Octet.

decrease in viability. These pools all had viability greater than 85% by Day 4 and reached greater than 95% viability by Day 7. In contrast the control pools took 10 days to recover completely. Interestingly, the pools with donor pDNA containing IR and insulator elements, showed a smaller decrease in viability during selection relative to the control pools with donor pDNA lacking the IR and insulator elements.

Under stronger selection pressure (10 μM and 20 μM MSX), all the PB pools recovered in 7–10 days. In contrast, both control pools required approximately 12 days to recover. As expected, 50 μM MSX was the most stringent condition with the viability decreasing below 40% for the PB pools and below 20% for the control pools prior to recovery. Despite these decreases in viability during the selection process, all pools successfully recovered in 14–16 days after transfection.

Once recovered, all the stable CHO cell pools were subjected to a standard 2-week fed-batch process in 24 DWP before harvest and titer quantification. Results are summarized in Figure 2. The highest pool titer of 6.6 g/L on Day 14 was obtained for PB pool generated with 5% helper pDNA co-transfection and selection under 20 μM MSX. This represented an almost 10-fold improvement in expression titer as compared to the control pool with donor pDNA lacking the IRs and insulators. Under all the selection conditions, the highest titers were obtained with 5% helper pDNA co-transfection. We observed a trend of decreasing titers with increasing amount of helper pDNA co-transfection. There were no significant differences in growth or viability during productions (data not shown). Interestingly, we also observed a 40–50% increase in expression titer in the control pools with donor pDNA containing IRs and insulators in comparison to control pools with donor pDNA lacking the IRs and insulators. This is consistent with previous reports where the presence of insulator elements such as matrix attachment regions (MAR) or ubiquitous chromatic opening element (UCOE) in the expression cassette has been shown to improve protein expression.[19,20]

Evaluation of the PB system for the expression of multiple proteins

Next, we wanted to evaluate if the use of the PB system increased stable CHO pool productivity for multiple molecules. For this study, we chose a small panel of four model antibodies: three monoclonal antibodies and one bispecific antibody. One of the antibodies for this experiment was the same antibody as above (mAb1). We included this control to evaluate reproducibility.

Table 2. Selection Conditions for piggyBac and Control CHO Pools Used in This Study

Molecule	SV40E Promoter Type	Selection for piggyBac Pool (μM MSX)	Selection for Control Pool (μM MSX)
mAb1	Wild Type	20	10
mAb2	Wild Type	20	10
mAb3	Engineered	0	0
BSAb1	Engineered	0	0

Our colleagues have recently described the use of an engineered SV40E promoter for GS gene expression to increase the selection stringency allowing pool generation in absence of any MSX.[10] Plasmids coding for mAb1 and mAb2 contained a wild-type SV40E promoter controlling GS gene expression (the selectable marker). Plasmids coding for mAb3 and BSAb1 contained the engineered SV40E promoter controlling GS gene expression. Selection conditions for the different pools are listed in Table 2. All PB pools were generated with 5% helper pDNA. For this study, the control pools were generated with donor pDNA containing IR and insulators in the absence of helper pDNA.

We observed similar viability trends under selection with PB pools recovering faster than the respective control and as expected, the stringency was higher with the engineered SV40E promoter with viabilities dipping lower than those observed for the pools with the wild-type SV40E promoter controlling GS gene expression (data not shown). Titers ranged between 2.3 and 7.6 g/L by Day 16 for the PB pools. This represented a 4- to 12-fold increase in expression titer relative to the control pools (Figure 3A). The improved productivities in PB pools were due to an increase in specific productivity of the cells with the fold increase for specific productivity and volumetric productivity being similar (Figures 3A,B). This is consistent with literature reports that the PBase integrates the expression cassette in transcriptionally active regions of the host genome.[14,15] It is important to note that the PB pools reached specific productivity (picogram per cell per day) levels of 25–70 p/c/d which are typically only observed in manufacturing cell lines.[1,21,22] The PB pools for mAb2 and BsAb1 were scaled up to 100 mL in a shake flask. Very similar titers were measured in shake flasks and 24 DWPs (data not shown) confirming scale up of 24 DWPs to shake flasks.

Evaluation of clonal cell lines derived from CHO pools

We selected mAb2 and BsAb1 for the clonal CHO cell line evaluation. For this study, the control pools were generated from plasmid DNA vector lacking the IR and insulators. Also, transfection of cells was performed by electroporation with linearized plasmid DNA for generation of the control pools. The PB pools were generated as described above with 5% helper pDNA co-transfection. Selection conditions for all the pools were same as above. The schematic for the clonal cell line generation and evaluation is shown in Figure 4. At Step 4, supernatant titers were measured and the results are shown in Figure 5.

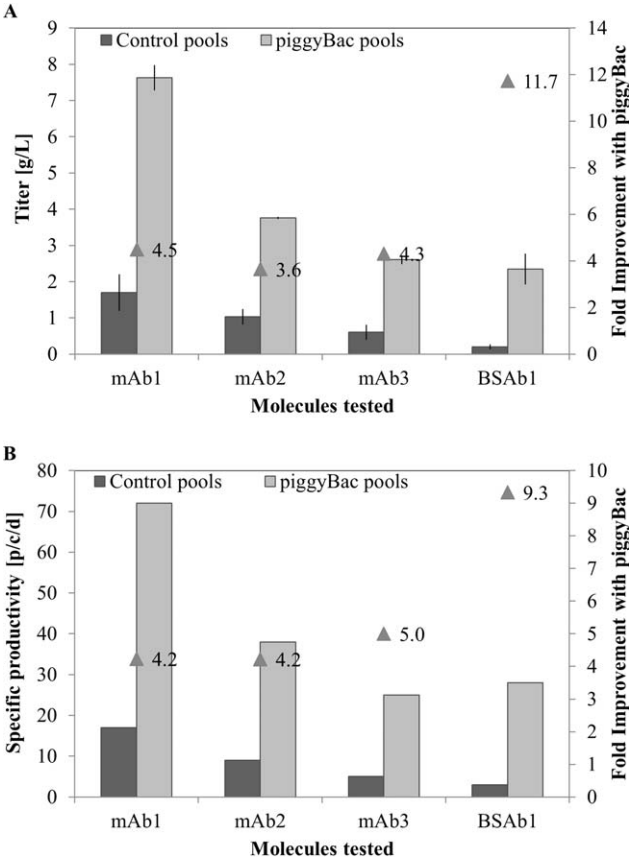

Figure 3. (A) Expression and (B) specific productivity comparison of control and piggyBac pools for the four molecules tested.

CHO cells were transfected with the 5% donor and 95% helper pDNA and subject to selection conditions shown in Table 2. Once CHO pools fully recovered, they were subject to 2 week fed-batch process as described in the methods section. Titers were measured on Day 14 by ForteBio Octet. Specific productivity was calculated based on integral viable cell density over the 2 week expression period.

The titer distribution of clones from the control pool was very different relative to clones from the PB pool. For mAb2, approximately 66% of the PB clones fell into the high expression category as compared to only 18% for the control clones. Interestingly, 7–15% of these clones did not express any detectable levels of antibody. The majority (66%) of the control clones fell into the medium expression category (Figure 5A). For BsAb1, 53% of the control clones fell in the nonproducer category and only 4% of the clones showed medium level of expression. In contrast, only 3% of the PB clones were nonproducers and 36% were medium producers. Moreover, 26% of the PB clones were high producers while none of the control clones were classified as high producers (Figure 5B).

The best 24 clones from each condition were transferred to suspension in two 24 DWPs. After two to three passages, the pools were subjected to a 14-day fed-batch production process in 24 DWPs as described in the Methods section. For mAb2, control clone titers ranged from 0.6 to 1.8 g/L while the PB clone titers ranged from 1.8 to 4.2 g/L (Figure 6A). Interestingly, the lowest titer PB clone had the same expression level as the highest titer control clone. For BSAb1, control clone titers ranged from 0.2 to 1.3 g/L while the PB clone titers ranged from 1.0 to 4.4 g/L (Figure 6B).

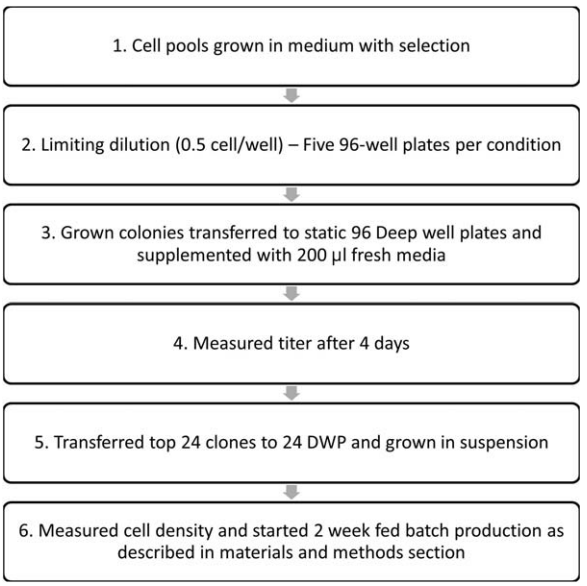

Figure 4. Schematic for clonal cell line generation and expression assessment from control and piggyBac pools for mAb2 and BSAb1.

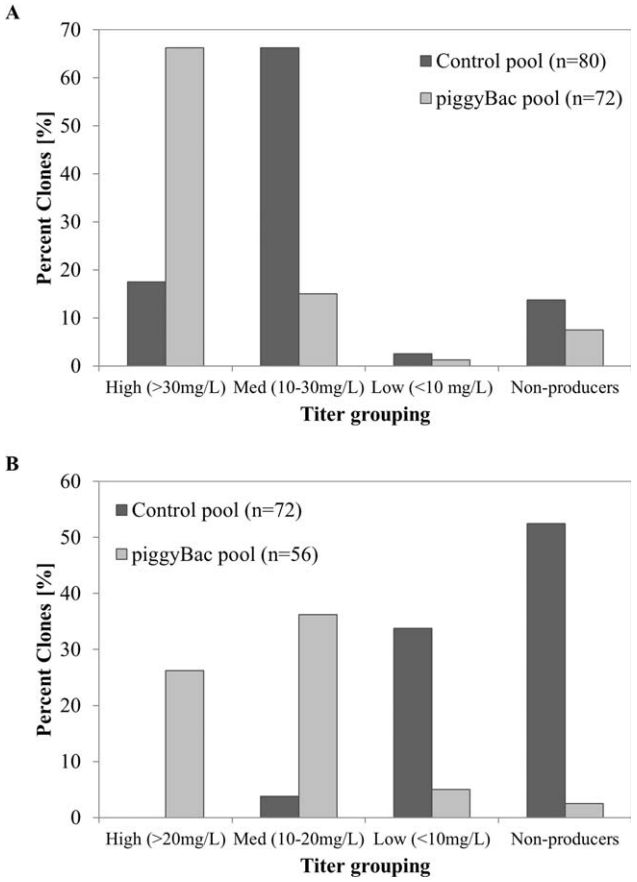

Figure 5. Titer distribution of clones isolated from control pools vs. piggyBac pools for (A) mAb2 and (B) BSAb1.

Volumetric protein titer of each clone was measured in a 4-day batch culture by ForteBio Octet and clones were grouped into categories as shown in the figure. Number of clones analyzed for each condition in listed in the figure.

Only six of the 24 PB clones had lower expression level than the highest titer control clone. Overall, the average increase in PB clone titer was 2.3- to 3.0-fold higher relative

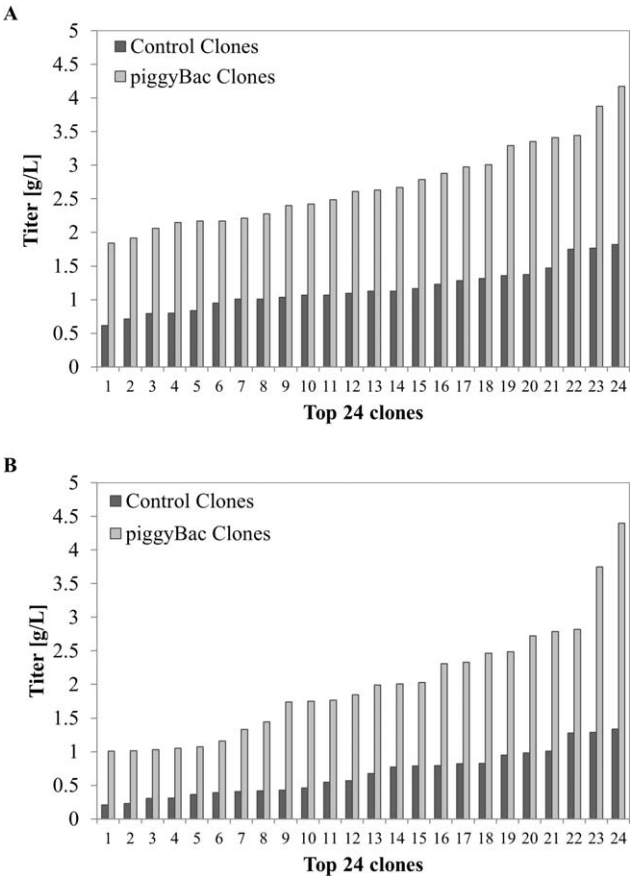

Figure 6. Expression titer comparison for the highest titer clones isolated from the control pool and the piggyBac pool for (A) mAb2 and (B) BSAb1.

The best 24 clones based on the 4-day batch screen were scaled up to suspension and subject to 2 week fed-batch production process as described in methods section. Titers were measured on Day 14 by ForteBio Octet.

to the control clones. These results demonstrate that we were able to isolate high titer clones with higher frequency with PB system relative to the clones generated from control pools.

Discussion

TGE in CHO and HEK cells has been the method of choice for rapid generation of recombinant protein for preclinical studies in the biopharmaceutical industry. It offers the advantage of speed (1-3 weeks). Unfortunately, the large amount of DNA and host cells required limits its application to producing gram and kilogram quantities of protein. Recently, improvements in titers obtained from stable CHO cell pools have allowed this approach to be used as an alternative to TGE. Stable CHO pool generation requires much less DNA and host cells relative to TGE and is easily scalable to large volumes. In 2015, Balsubramanian et al. reported the use of PB transposon-based gene delivery for the generation of both stable CHO cell pools and stable CHO clonal cell lines (titers of up to 1 g/L for stable CHO pools).[8,11]

The goal of this study was to evaluate the PB system in the context of an industrially relevant CHO cell line (CHO K1SV GS Knockout) to generate stable CHO cell pools and clones for the production of recombinant proteins. Under optimized conditions, we were able to generate cell pools with expression titers of up to 7.6 g/L. Based on the speed of PB pool recovery observed in this study and the time needed to scale up the cells, transfection to harvest of a large fed-batch production culture (>1 L) can be completed in less than 5 weeks. Additionally, this represents the highest reported titer from a stable CHO cell pool population that we are currently aware of. Traditionally, such high expression levels have only been obtained from stable CHO clonal cell lines following a lengthy and laborious clone selection and clone characterization process.

We also observed that PB-pools were able to recover 3–5 days faster than the control pools. This was likely due to the higher expression of the GS marker in these pools, ultimately leading to faster recovery times under selection conditions. We found that 10–20 μM MSX for the wild-type SV40 promoter controlling GS gene expression represented the optimal conditions for stable CHO pool generation. For the engineered SV40 promoter controlling GS expression, selection in the absence of glutamine alone was optimal. Interestingly, the PB pool for mAb1 selected with 0 μM MSX was able to recover in less than 5 days and still yield an expression titer of 2.8 g/L. Under these conditions, the entire process of selection and production was completed in less than 3 weeks while obtaining high levels of expression. Using multiple model proteins, we measured 4–10-fold higher expression titers for PB pools relative to pools generated in absence of PBase. Specific productivities of 25–75 p/c/d were achieved in PB pools which are traditionally only obtained from final manufacturing cell lines.

There are several factors contributing to the differences in titer seen in this study compared to those published by Balasubramanian et al. They used wild-type PBase in their study, while a hyperactive version of the enzyme was used in this study. Additionally, the host cell line, expression cassettes, specific antibody gene sequences and selection methods are all different. Moreover, the production scheme used in this study is an optimized fed-batch process using multiple feeds, while Balasubramanian et al. employed a simple, nonoptimized process using a commercially available medium.

Finally, we also evaluated the PB system for the generation of stable CHO clones. We found that the use of the PB system substantially increased the frequency of isolating high titer CHO cell lines. The average volumetric productivity of stable CHO cell lines isolated from PB derived cell pools was two to threefold higher than that for the cell lines recovered from the control pools. Overall, these results demonstrate the superiority of PB transposon system relative to the control method typically used for the generation of CHO cell pools and clones. However, a detailed genetic stability and product quality assessment will be necessary before PB-derived pools and clones can be used for therapeutic protein production.

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