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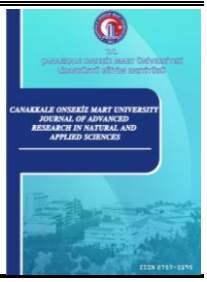

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Evaluation of Recombinant Antibody Production Efficiency in CHO Cells with Sleeping Beauty Transposon Vector System

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Abstract − Chinese hamster ovary (CHO) mammalian cell lines are widely used as cell platforms in biopharmaceutical productions. Different transfection systems are employed for the integration of the target gene cassette into the cell genome and have limitations, such as (i) the integration region in the genome, (ii) the size of the target cassette, and (iii) long selection periods for stable expression. Transposon systems can be utilized to overcome the limitations mentioned in the efficient production of commercially significant recombinant proteins. This study aims to demonstrate the differences in production potential and selection periods by using a specially designed vector system for random genome integration in CHODG44 DHFR -/- cells and the Sleeping Beauty (SB) transposon system. In this context, the optimal transfer ratio between the donor and the helper plasmid was determined for the most efficient co-transfection in the SB transposon system. According to the results, the pools obtained using the SB transposon system had titers ranging from 1300 to 2600 mg/L in 13-day fed-batch studies, while the pool obtained using the random transfer system had a titer of 0.056 mg/L. Additionally, stable cell pools obtained using the transposon system underwent selection in a short period of 52 days, compared to over 100 days for the pool obtained through random transfer. Considering all these results together, it is demonstrated that stable CHO pools obtained using the optimal SB transposon system can achieve high-efficiency monoclonal antibody production in a short period, making it an optimal production platform in the biopharmaceutical field.

Keywords − *Sleeping Beauty transposon, gene integration, expression level, CHO cells, industrial production*

1. Introduction

Immunological surveillance and tumoricidal activity towards newly formed tumor cells are the primary defense against cancer formation. Thus, cancer immunotherapy started appealing to attention for therapeutic purposes. One such therapeutic strategy involves the blockade of the PD1 - PDL1/2 axis. Antibodies directed to either T-cell receptor PD1 or its ligand PDL-1 are gaining popularity to overcome this mode of immune evasion in various malignancies, including metastatic melanoma, non-small cell lung cancer (NSCLS), renal cell carcinoma and bladder cancer [1].

In the last 30 years, over 170 therapeutic monoclonal antibodies have received approval from administrative authorities worldwide. Most of these complex recombinant proteins are produced in mammalian expression systems. Chinese hamster ovary (CHO) cells have become one of the most favored platforms to express recombinant mAbs, providing protein folding and processing capabilities comparable to human cells. Owing

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to established cell lines, vectors, and broad experience, it is not unusual to reach several grams per liter yields [2]. Development and characterization of stable cell lines with desired production capacity and product quality are the most critical parameters of this workflow. The mode of gene transfer is closely related to the expression efficiency. Conventional methods for transformation usually involve chemicals like polycationic substances or calcium phosphate. These methods typically provide a random integration of the target gene into chromosomal sites, which may be transcriptionally inactive. Intense effort is required to select productive clones that retain a high expression profile from a heterogeneous pool of cells. Low and variable yields with long-term stability issues are not uncommon for these random integrants.

On the other hand, naturally occurring vertebrate transposons have been engineered for efficient gene delivery systems. PiggyBac (PB), Tol2, and Sleeping Beauty (SB) are the most popular commercialized transposon-based gene delivery systems. Despite the diverse ancestral origins, all three systems are highly efficient in generating producer cell pools.

Therefore, creating low-cost production platforms with high quality and production capacity is increasingly necessary to meet the growing demand in the biopharmaceutical market. The first stage of protein-specific production involves transferring the target protein gene to the host cell using gene transfer systems. However, this process can affect the physicochemical and functional quality of the monoclonal antibody and product production efficiency, so pharmaceutical R&D needs to make applications to increase efficiency at this stage. The target gene transfer systems used at these stages can be divided into viral or non-viral systems. While viral vectors provide effective integration, deoxyribonucleic acid (DNA)-based plasmids, usually non-immunogenic and can carry large amounts of target genes, are used despite their limitations in transferring large genes due to their poor transfection and integration abilities compared to viral vectors [3]. Techniques mainly involve optimizing CHO cells and expression vectors by promoting the expression of the target gene to ensure efficient expression of the transgene. However, inefficient stable integration occurs due to the random integration of one to five copies into the host genome [4-6]. Consequently, protein expression in producer cell lines generated by random integration results in unpredictable, variable, and often unstable protein levels [7]. Transposon vector systems, which enable efficient integration of the target gene to overcome this problem by inserting it into a transcriptionally active region, can allow the production of expression-challenging recombinant proteins such as monoclonal antibodies in stable cell pools suitable for large-scale production [8].

Transposons are genetic elements primarily consisting of transposase genes and inverted repeat (ITR) sequences. Transposases bind to directed repeat sequences in the ITR and function by cutting the transposon sequence into the host DNA genome to insert these transposon fragments. Non-homologous end-joining, a DNA double-helix repair system, is used to repair the resulting open DNA ends [9]. The most used transposon systems in mammalian cells are PiggyBac (PB), Tol2, and Sleeping Beauty (SB). While all three systems are effective, PB and SB transposon vector systems are more successful than Tol2 transposon systems in obtaining stable and efficient producer cell lines [10-12]. Therefore, these transposon systems offer a valuable alternative to random genome integration for generating stable and efficient producer cell lines, providing an effective and practical approach to creating a producer cell platform. In order to enhance the proficiency of the dual-vector transposon system in delivering the target gene into the host genome, it is necessary to implement optimizations such as determining the optimal proportions of the two vectors and the transposase enzyme, which can be developed through engineering technologies. In this study, the utilization of optimized transposase enzymes, such as SB100X, demonstrated 100-fold greater transposition activity compared to the wild-type SB transposase [13, 14].

Moreover, the effectiveness of the transposase and transposon plasmid ratio on transposition efficiency is not the sole determinant of success. Direct optimization of the transposon donor also plays a significant role in transposition. The circular or linear form of the target DNA can impact the efficiency of SB and PB transposon systems and the duration of subsequent cell pool selections [8, 15, 16].

This research study primarily aimed to evaluate the pool selection times and production performance of SB100X transposon-generated anti-PD-1 hIgG producer CHODG44 cell pools. We sought the optimal donor

and helper plasmid ratio and determined whether linear or circular transposon donor plasmid worked better. We compared the overall efficiency of the transposon system to a transgene random integration strategy.

2. Materials and Methods

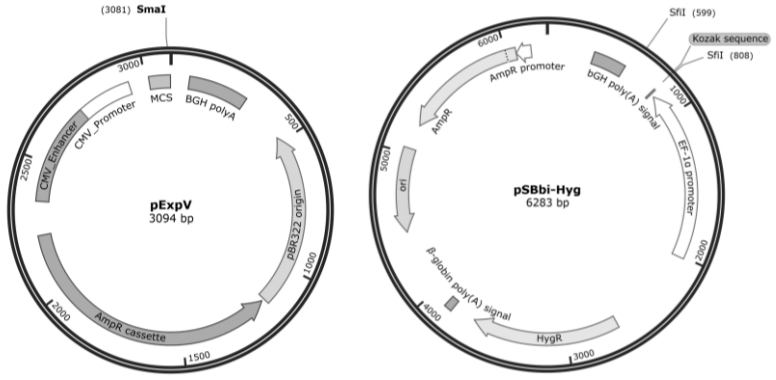
2.1. Construction and Transformation of Expression Vectors

Mammalian expression vectors pExpV-antiPD-1 and pSBbiHYG-antiPD-1 were constructed to investigate the impact of random and transposon-mediated integration strategies, respectively. Both vectors carried a tricistronic design in which anti-PD-1 hIgG4 light chain (LC), heavy chain (HC), and dihydrofolate reductase (DHFR) open reading frames were joined by intervening FMDV internal ribosomal entry sites (IRES).

Insert DNA segments in this study were generated by using PCR amplification. Reaction mixtures contained 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM potassium chloride, 0.2 mM dNTP, 2 mM magnesium chloride, 0.5 µM of each oligonucleotide primer, 1 unit of Phusion high-fidelity DNA polymerase and 1 ng of template DNA. Following the initial denaturation at 98°C for 2 min, the amplifications were performed for 30 cycles of denaturation at 98°C for 10 s, annealing at 70°C for 30 s, and extension at 72°C. The amplicons were analyzed on 1.5% (w/v) agarose gels stained with SYBR Safe (Invitrogen, S33102). Before proceeding to the subsequent steps, products were spin column purified from agarose gels (Macherey Nagel, 740609)

Exp-Cassette-F and Exp-Cassette-R oligonucleotide primer pair was used to amplify a codon-optimized synthetic DNA segment anti-PD-1 hIgG4 LC-IRES (wt)-HC-IRES (att) (Geneart, Thermo). The mammalian selection marker DHFR open reading frame was amplified using pGEM-T-DHFR-ORF (SinoBiological, HG14365-G) DNA template with DHFR-F and DHFR-R oligonucleotide primers. The primer sequences are outlined in Table 1.

The random integration vector backbone pExpV (Figure 1A) was custom-designed to allow CMV promoter-driven expression of the insert DNA (Geneart, Thermo). The SLiCE cloning method was used to assemble and ligate the amplicons into the SmaI linearized pExpV DNA with the specified order described by Zhang et al. [17].



A. B.

Figure 1. Vectors used in expression vector construction: A) pExpV vector map, B) pSBbiHYG vector map

Transposon mediated integration vector has been built on backbone pSBbi-Hyg (AddGene #60524). For this purpose, we used SfiI-Anti-PD-1-F and SfiI-DHFR-R oligonucleotide primer pair to amplify LC-IRES (wt)-HC-IRES (att)-DHFR insert from pExpV-antiPD-1 (in this study) plasmid DNA template. Following digestion with SfiI endonuclease, insert DNA and pSBbi-Hyg segments were ligated using T4 DNA ligase (Thermo Scientific, K1422). We transformed *Escherichia coli* Stbl3 cells with pExpV-antiPD-1 and pSBbiHYG-antiPD-1 ligation products using the TSS method, as described by Chung et al. [18]. We performed a screening PCR following overnight incubation to determine insert positive colonies were present. Each 25 µl reaction tube included 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM potassium chloride, 0.2 mM dNTP, 2 mM magnesium chloride, 0.5 µM of each primer, 0.5 U Taq DNA polymerase and 5 µl of colony suspension. We used CMV-

F and BGH-R or EF1a-F and BGH-R primer pairs to scan pExpV-antiPD-1 and pSBbiHYG-antiPD-1 transformants, respectively. Following initial denaturation at 95°C for 5 mins, target DNAs were amplified for 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 minute, and extension at 68°C for 5 min. The primer sequences are depicted in Table 1. Midiprep plasmid isolation was performed to insert positive colonies using a kit (NucleoBond Xtra Midi Columns-Macherey Nagel, 740410).

Table 1. Sequences of the primers for construction of pExpV-antiPD-1 and pSBbiHYG-antiPD-1

Primer Name - Gene	Sequences (5′ – 3′)
Exp-Cassette-F	5′-CGGGCCCGGGATCCACCGGTGCCGCCACCATGAAATGG -3′
Exp-Cassette-R	5′-AGTTCAATGGTCGAACCATGGAATTCGATCCTCATC-3′
DHFR-F	5′-GATGAGGATCGAATTCCATGGTTCGACCATTGAACT-3′
DHFR-R	5′-CTAGAGTCGCGGCCGCTTTATTAGTCTTTCTTCTCGTAGACTTC-3′
SfiI-Anti-PD-1-F	5′-GCTGGCCTCTGAGGCCGCCACCATGAAATGGGTCACCTT-3′
SfiI-DHFR-R	5′-GCTTGGCCTGACAGGCCTTATTAGTCTTTCTTCTCGTAGACTTC-3′
CMV-F	5′-CGCAAATGGGCGGTAGGCGTG-3′
EF1a-F	5′-TCAAGCCTCAGACAGTGGTTC-3′
BGH-R	5′-TAGAAGGCACAGTCGAGG-3′

2.2. Cell Culture and Standard Cultivation

Serum free-suspension culture adapted CHO DG44 DHFR -/- cells were a kind gift from Dr. Lawrence Chasin (Columbia University, NY). The cells were propagated in either 6-well plates (TPP, 92106) or 125 ml shaking flasks (Thermo Scientific, 4115-0125). The media used for routine subculturing, transfection, and selection is listed in Table 2. After every 2 - 3 days of cultivation, the cells were transferred to a fresh media at a 3 - 5 x 10^5 cells/ml density. All cultures were incubated at 36.8°C with 7.5% CO2 (v/v) (HERAcell 240, Thermo Scientific, US). Flask cultures were shaken at 110 rpm (Celltron, Infors HT shaker, Denmark).

Table 2. Cell culture media compositions

Composition of media for CHO DG44 (DHFR -/-) wild-type cells (Media A1)	
96.8 %	eCHO Basal Media (Lonza, BEBP12-933Q)
3 %	L-Glutamine (200 mM) (Sigma Aldrich, G7513)
0.2 %	HT Supplement 100X (Thermo Scientific, 11067030)
Composition of media for CHO DG44 cells transfection (Media A2)	
97 %	BalanCD Transfectory CHO Media (Irvine Scientific, 91147)
3 %	L-Glutamine (200 mM) (Sigma Aldrich, G7513)
10 % (after 24 Hours)	Transfectory Supplement (Irvine Scientific, 91148)
Composition of media for CHO DG44 cells transfected with semi-targeted integration system and CHO DG44 cells selection	
97 %	eCHO Basal Media (Lonza, BEBP12-933Q)
3 %	L-Glutamine (200 mM) (Sigma Aldrich, G7513)
Composition of media for CHO DG44 cells transfected with random integration system and CHO DG44 cells selection	
97 %	eCHO Basal Media (Lonza, BEBP12-933Q)
3 %	L-Glutamine (200 mM) (Sigma Aldrich, G7513)
25 nM	Methotrexate Hydrate (Sigma Aldrich, M8407)

2.3. Transfection and Sub-Cultivation

Stable pools of anti-PD-1 hIgG4 producer CHO cells were generated by random integration vector pExpV-antiPD-1 or transposon vector pSBbiHYG-antiPD-1. Prior to the transfection process, 60 µg of pExpV-antiPD-1 vector was linearized using restriction enzyme digestion with SspI (NEB, R3132S) and PvuI (NEB, R3150S) and then purified using a plasmid isolation/purification kit (Macherey Nagel, 740490).

Logarithmic phase 2x10^6 CHO DG44 cells were transfected with 2 µg of linearized plasmid DNA by using a 4D Nucleofector system (Core and X Unit, Lonza Amaxa) with X kit L (Lonza, V4XC-2012) and DU-158 program, following the manufacturer's instructions. The transfection procedure was carried on by seeding the cells in 6-well plates at 2x10^6 cells/well in media A2. After 24 hours, 10% (v/v) transfection supplement was added to each well to improve the transfection efficiency. Cells were counted 48 hours after transfection using

Vicell XR (Beckman Coulter). To establish stably transfected cell populations, the cell cultures were transferred to media A3 and incubated under standard conditions with media changes every 2-3 days until cell viability reached 95% (30 days of hypoxanthine and thymidine (HT) selection). Following HT selection, methotrexate (MTX)-driven gene amplification was performed by seeding and passaging at a concentration of 0.5 - 0.8x10^6 cells/ml according to standard conditions in media A4 supplemented with 50, 100, 200, 400, and 800 nM MTX, respectively. The concentration of metotreksat (MTX) in the culture media increased after cell viability reached above 80%. To ensure genetic stability, the decision was to employ selective media A4 containing 25 nM MTX rather than discontinuing MTX treatment in subsequent passages of selected cell cultures. This served to maintain selection pressure.

In order to establish a semi-integrated transfection system based on the transposon system, 50 µg of the pSBbiHYG-antiPD-1 vector was linearized through digestion with BamHI and then isolated using a gel and PCR isolation kit (Macherey Nagel, 740609). The linearized and circular forms of the donor plasmid encoding the target gene and the helper plasmid encoding the transposase (SB100X) were then transfected into CHO cells at three different ratios (1:2, 1:3, and 1:4) using media A2. This was done to determine the optimal transposon condition. After 24 hours post-transfection, a 10% (v/v) transfection supplement was added to each well to enhance transfection efficiency. To establish stable transfected pools 48 hours after transfection, cell populations were seeded in HT-free media A3 under standard conditions until cell viability reached at least 90%. The cell populations were then passaged every 2-3 days using the fresh medium at 0.5 - 0.8x10^6 cells/ml concentration. All media compositions are outlined in Table 2.

2.4. Fed-Batch Cultivation and Protein Quantification

For the fed-batch study, 25 ml of Excell Advanced CHO Fed-Batch media (Sigma Aldrich, 14366C) supplemented with 6.0 mM of L-glutamine (Sigma Aldrich, G7513) were inoculated into shaking flasks at a concentration of 300,000 viable cells/ml. The cultures were maintained under standard conditions using nutrient media consisting of CHO Feed 1 - with glucose (Sigma Aldrich, 24367C) and CHO Feed 1 - without glucose (Sigma Aldrich, 24368C) at a concentration of 28.0 g/L, as well as glucose (Thermo Scientific, A2494001) prepared according to the manufacturer's instructions. The daily glucose concentration was adjusted to between 4.5 and 7.0 g/L, and when the daily Feed-1 supplementation exceeded 10% by volume, the remaining glucose requirement was supplemented with 200 g/L of glucose solution. The cell concentration and viability were determined using the Vi-Cell XR (Beckman Coulter).

The Anti-PD-1 Human IgG4 protein was quantified in the cultures using ACQUITY UPLC (Waters) Protein A chromatography with a Bio-Monolith Protein A column (Agilent, 5069-3639). The analysis parameters are listed in Table 3, and the gradient information is listed in Table 4.

Table 3. Protein A chromatography quantitative analysis parameters

Autosampler Temprature: 10°C	
Mobil Phase A	50 mM Sodium Phosphate pH: 7,4
Mobil Phase B	100 mM Citrate pH: 2,8
Colomn	Agilent Bio-Monolith Protein A, 5.2 mm x 4.95mm
Colomn Temprature	25°C
UV	280 nanometer
Injection Volume	50 µL

Table 4. Protein A chromatography gradient system parameters

Time (Minute)	Flow rate (µL/dk)	Solvent A (%)	Solvent B (%)	Curve
0.00	0.5	100	0	Initial
2.00	0.5	100	0	6
2.01	0.5	0	100	6
6.00	0.5	0	100	6
6.01	0.5	100	0	6
8.00	0.5	100	0	6

2.5. Statistical Analysis

The results were analyzed by applying Student's t-test or One-Way ANOVA, nonparametric Mann-Whitney U, Tukey and Dunnet tests according to the suitability of the parameters. For this purpose, the GraphPad (version 8) program was used, and $p < 0.05$ values were considered statistically significant.

3. Results and Discussion

3.1. Construction of A Random Integration Vector Expressing Anti-PD-1 Human IgG4

Random integration vector pExpV-anti-PD-1 carried a CMV promoter-driven tricistronic expression cassette. We used PCR to generate the 3367 bp long LC-IRES (wt)-HC-IRES (att) and 601 bp long DHFR gene (Figure 3B). SLiCE ligated these segments with SmaI linearized (Figure 3C) pExpV. We performed a colony PCR to screen the insert positive bacterial transformants. Plasmid isolation was performed from insert positive colonies (Figure 3.D). Restriction mapping was done to confirm pExpV-anti-PD-1 constructs. Eco321/XhoI and BamHI/NotI enzyme pairs generated 2539/4431 bp and 3890/3080 bp long bands, respectively (Figure 3E). The band observed around 6900 bp was likely caused by the incomplete digestion of one of the enzymes.

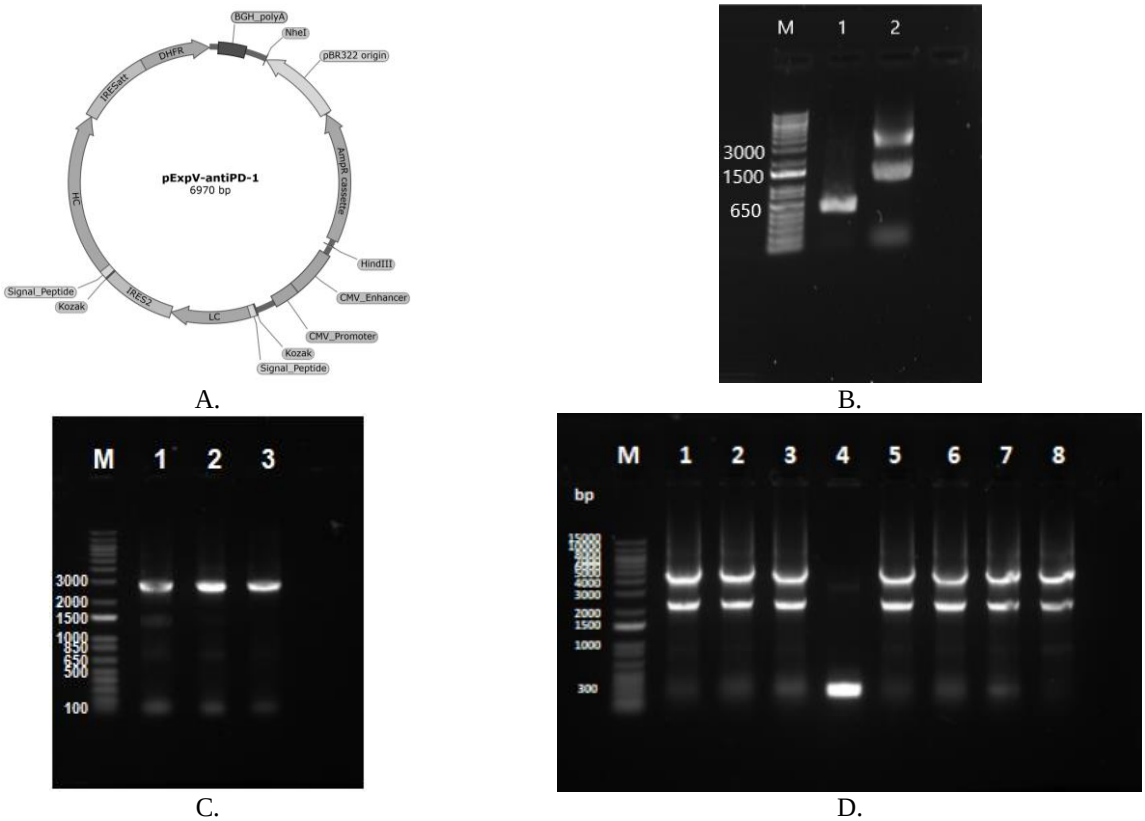
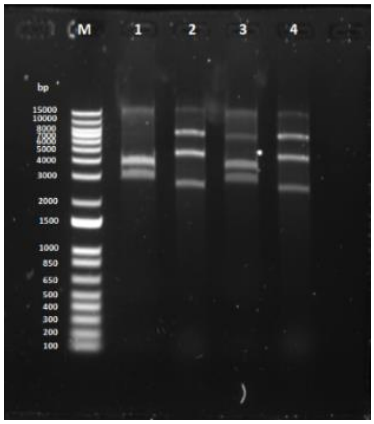


Figure 2. Design and construction of pExpV-antiPD-1. A) Random integration vector pExpV-antiPD-1 map. B) Expression cassette gene segments, Lane M: MW standard, Lane-1: DHFR amplicon (601 bp), Lane-2: LC-IRES-HC-IRES segment (3367 bp) C) SmaI linearized pExpV vector, Lane M: MW standard, Lane 1-3: SmaI treated pExpV backbone (3094 bp). D) Colony screening PCR products, M: Marker, 1–8: 4166 and 300 bp products amplified from insert positive and negative colonies, respectively. E) Restriction endonuclease mapping of pExpV-antiPD-1 vector, Lane M: MW Standard, Lane 1: Colony-1 BamHI/NotI digestion, Lane 2: Colony-1 Eco321/XhoI digestion, Lane 3: Colony-2 BamHI/NotI digestion, Lane 4: Colony-2 Eco321/XhoI digestion. Expected fragment sizes for BamHI/NotI are 2539/4431 bp, and Eco321/XhoI are 3890/3080 bp. F) 1 kb DNA MW Standard. Expected fragment sizes for BamHI/NotI and XhoI/Eco32I digestion are 3077/3893 and 2538/4432 bp, respectively



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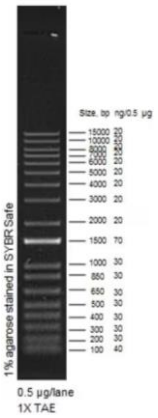
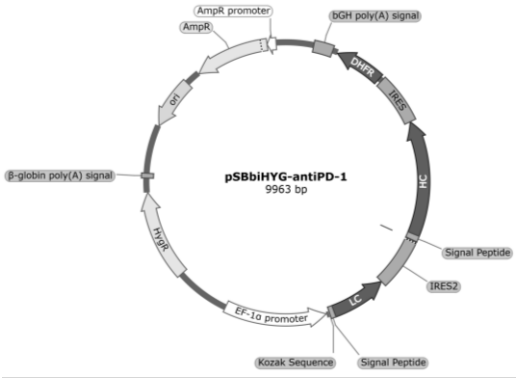


F.

Figure 3. (Continued) Design and construction of pExpV-antiPD-1. A) Random integration vector pExpV-antiPD-1 map. B) Expression cassette gene segments, Lane M: MW standard, Lane-1: DHFR amplicon (601 bp), Lane-2: LC-IRES-HC-IRES segment (3367 bp) C) SmaI linearized pExpV vector, Lane M: MW standard, Lane 1-3: SmaI treated pExpV backbone (3094 bp). D) Colony screening PCR products, M: Marker, 1–8: 4166 and 300 bp products amplified from insert positive and negative colonies, respectively. E) Restriction endonuclease mapping of pExpV-antiPD-1 vector, Lane M: MW Standard, Lane 1: Colony-1 BamHI/NotI digestion, Lane 2: Colony-1 Eco321/XhoI digestion, Lane 3: Colony-2 BamHI/NotI digestion, Lane 4: Colony-2 Eco321/XhoI digestion. Expected fragment sizes for BamHI/NotI are 2539/4431 bp, and Eco321/XhoI are 3890/3080 bp. F) 1 kb DNA MW Standard. Expected fragment sizes for BamHI/NotI and XhoI/Eco32I digestion are 3077/3893 and 2538/4432 bp, respectively

3.2. Construction of SB Transposon Vector Expressing Anti-PD-1 Human IgG4

Previously constructed pExpV-antiPD-1 served as a template for the LC-IRES (wt)-HC-IRES (att)-DHFR segment. Agarose gel purified amplicon (Figure 5B) was ligated to the SfiI digested transposon donor vector (Figure 5C) in order to build pSBbiHYG-antiPD-1 (Figure 5A). We screened bacterial transformants for insert positive colonies (Figure 5.D) and performed a restriction endonuclease mapping using BamHI/FseI and SalI/FseI enzyme pairs to confirm the constructs. Agarose gel electrophoresis revealed the expected band pattern of 2015/2063/4985 and 2500/7463 bp for respective digestion products (Figure 5E).



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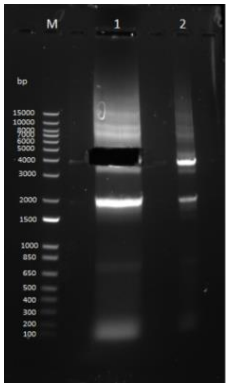
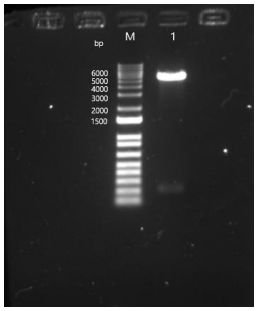


B.

Figure 4. Design and construction of pSBbi-HYG-antiPD-1. A) Transposon-mediated integration vector pSBbi-HYG-antiPD-1 map. B) LC-IRES (wt)-HC-IRES (att)-DHFR amplicon, agarose gel electrophoresis. Lane M: MW standard, Lane 1: 3903 bp band excised from agarose gel, Lane 2: amplification products. C) SfiI digest of pSBbiHYG. D) Colony screening PCR products. Lane M: MW standard, Lane 1-8: 4025 bp products amplified from insert positive colonies. E) Restriction endonuclease mapping of pSBbiHYG-antiPD-1 vector. Lane M: MW standard, Lane 1: BamHI/FseI digestion products with expected band profile of 2015/2063/4985 bp, Lane 2: SalI/FseI digestion products with expected 2500/7463 bp band profiles. F) 1 kb DNA MW Standard



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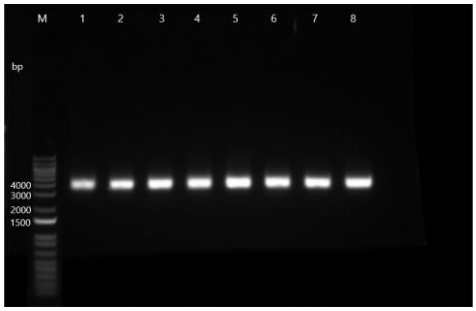

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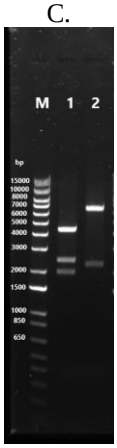

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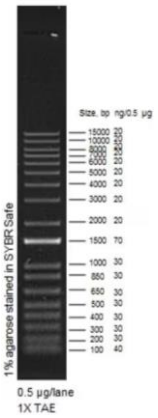


F.

Figure 5. (Continued) Design and construction of pSBbi-HYG-antiPD-1. A) Transposon-mediated integration vector pSBbi-HYG-antiPD-1 map. B) LC-IRES (wt)-HC-IRES (att)-DHFR amplicon, agarose gel electrophoresis. Lane M: MW standard, Lane 1: 3903 bp band excised from agarose gel, Lane 2: amplification products. C) SfiI digest of pSBbiHYG. D) Colony screening PCR products. Lane M: MW standard, Lane 1-8: 4025 bp products amplified from insert positive colonies. E) Restriction endonuclease mapping of pSBbiHYG-antiPD-1 vector. Lane M: MW standard, Lane 1: BamHI/FseI digestion products with expected band profile of 2015/2063/4985 bp, Lane 2: SalI/FseI digestion products with expected 2500/7463 bp band profiles. F) 1 kb DNA MW Standard

3.3. Generation and Evaluation of CHO-DG44 Cell Pools Stably Expressing Anti PD-1 Human IgG4

3.3.1. Producer Pools Generated with Sleeping Beauty Transposon System

We digested pSBbiHYG-antiPD-1 with BamHI to cut out the 2015 bp long ampicillin resistance cassette. The linearized transposon donor vector segment was agarose gel purified and recovered at a concentration of 0.862 µg/µl (Macherey Nagel, 740609) (Figure 6).

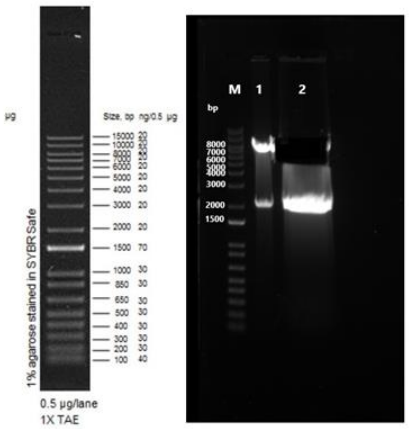
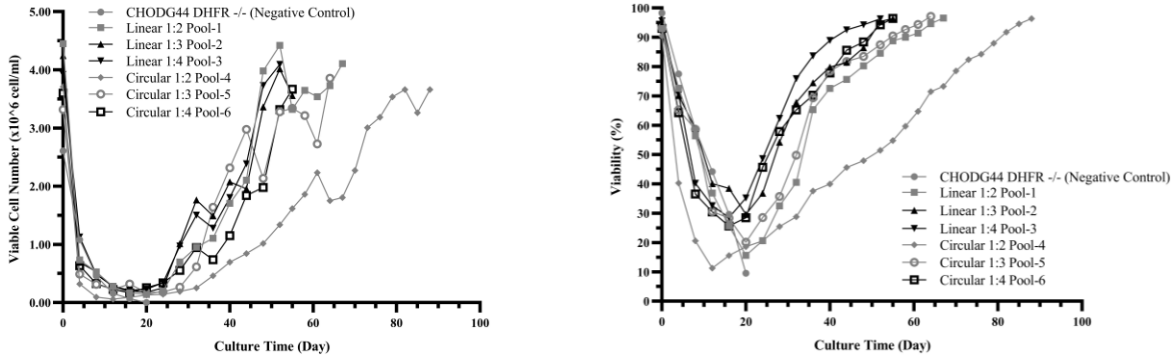


Figure 6. Agarose gel electrophoresis of BamHI digested pSBbiHYG-antiPD-1. M: MW standard, Lane 1: 7948/2015 bp digestion products, Lane 2: 7948 bp transposon donor segment excised from the gel

We determined the optimal transposon: transposase vector ratio. CHO DG44-DHFR-/- cells were co-transfected with transposon encoding vector pSB100X and either with linearized or circular pSBbiHYG-antiPD-1 transposon donor vector DNA at ratios 1:2, 1:3, and 1:4. Upon hypoxanthine and thymidine depletion, cell viability rapidly declined for the first 20 days of incubation. Under selective pressure, pool-3 (linear vector, 1:4 ratio) reached 90% cell viability as early as day 44. This was followed by pool-2 (linear vector, 1:3 ratio) and pool-6 (circular vector, 1:4) on day 52 (Figure 7) ($p<0.01$). Comparing all six cell pools, linear donor plasmid favors faster recovery of the cell viability, regardless of the transposon: transposase vector ratio.

In this study, we also showed that increasing the transposon donor vector ratio positively affected the selection times and production potentials of the cell pools. This may be attributed to more efficient transposition. As an autoregulatory control, an overabundance of mariner transposase drastically decreases the excision activity over its target. Specifically, keeping SB transposase expression constant and low is critical to avoid the phenomenon of overproduction inhibition (OPI), as previously reported [19-21]. Our findings were consistent with other studies, which favored a higher transposon donor vector over the SB transposase ratio [22, 23].



A. B.

Figure 7. Selection of SB transposon-generated cell pools by HT depletion A) percent cell viability (%), B) viable cell count (x10^6 cells/ml) over days of selection

3.3.2. Producer Pool Generated by Random Integration

The random integration vector pExpV-antiPD-1 was digested by SspI/PvuI restriction endonucleases (Figure 8). Linearized product was used to nucleofector CHO DG44 DHFR-/- cells.

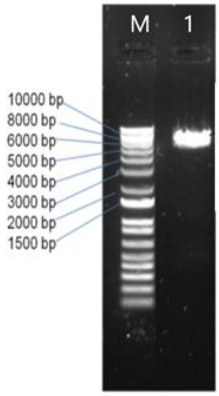


Figure 8. Agarose gel image of linearized random integration vector. M: MW standard, Lane 1: SspI/PvuI digestion product of pExpV-antiPD-1

The cells were allowed to recover for the first 48 hours. Subsequently, we transferred the cells to the HT-depleted selection medium until the non-integrants were killed, and later, the cell viability was restored. By day 30, the cell viability reached 90%, and the pool was subjected to gradually increasing MTX concentrations up to 800 nM to pick up cells with amplified copy numbers of the expression cassette. After 85 days of

consequent selection and amplification, a stable cell pool expressing human antiPD-1 was obtained (Figure 9). Generating stable producer cells through random integration vectors is well-known for being labor-intensive and time-consuming. Similar workflows and time courses were described in previous studies [23].

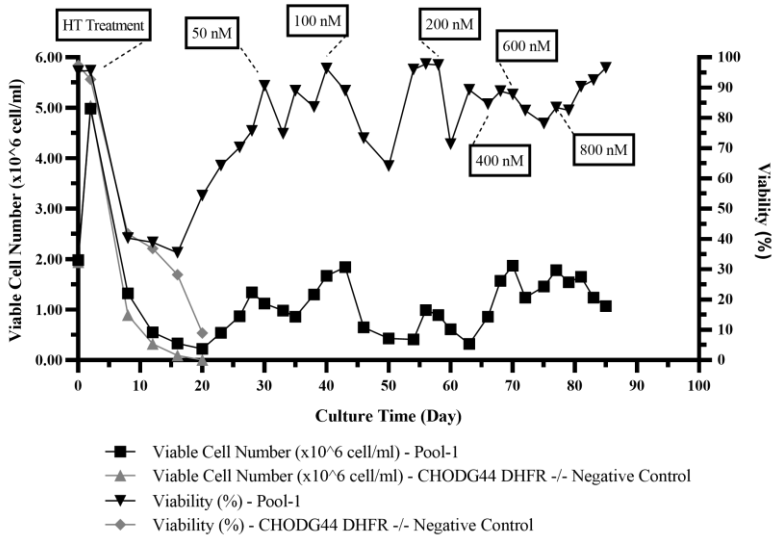


Figure 9. Viable cell counts (x10^6 cells/ml) and percent viability (%) of random integration cell pool subjected to consequent HT selection and MTX-mediated gene amplification

3.3.3. Fed-Batch Culture Study in Pools and Evaluation of Anti-PD-1 Human IgG4 Production Performance

A fed-batch culture was conducted with random and transposon-generated pools to investigate the performance of biomass throughput, cell viability, essential metabolic parameters, and protein production. The random integration pool reached a peak density of 9.5x10^6 cells/ml, with a steady decrease in cell viability. By day 10, this pool had lost >20% viability and entered the death phase. The cell counts of transposon-generated pools ranged between 12 - 20x10^6 cells/ml with >90% viability at the end of the active growth phase. Among these, pool 3 was the best-performing pool, reaching the plateau phase on day 6 with 19.7x10^6 cells/ml and the highest overall cell viability. Pools 4, 5, and 6 showed similar growth kinetics with marginally lower cell counts and cell viability ratios (Figure 10AB). Transposon-generated pools 1 and 2 behaved like a random integration pool, entering the death phase immediately after reaching a peak cell density. We also observed a premature decline in cell viability in those pools.

We evaluated glucose utilization and lactate accumulation of random integration and transposon-generated pools. We observed gradually increasing glucose uptake in the exponential phase of the cultures (Figure 10.D). CHO cells are renowned for utilizing glucose as a carbon and energy source to sustain rapid growth rates and release lactic acid into the medium. Later, the cells start to consume lactic acid to drive the tricarboxylic acid cycle (TCA) cycle to supply anabolic intermediary substrates and energy for the biosynthesis of the recombinant product. Lactic acid builds up had also occurred in our pools. Upon reaching the stationary phase, we observed decreased lactic acid concentrations in certain cell pools (Figure 10C). The pools following the sequential glucose and lactic acid metabolism pattern displayed high recombinant product yields (Figure 11). Random integration pool formed the smallest cell mass with sub-optimal glucose utilization. Concerning the biomass size, this pool over-accumulated lactic acid in the rapid growth phase without valorizing in the stationary phase. Random integration vectors may cause insertional silencing of certain critical host genes and become detrimental to cellular functions.

Furthermore, subsequent MTX gene amplification may form repeated arrays of transgene and trigger epigenetic silencing of the expression cassette. Once seeded, epigenetic reprogramming may spread hundreds

of thousands of bases away from the initial target. Most probably, these contagious modifications that result in heterochromatin formation may have a negative impact on the overall robustness of the cell physiology.

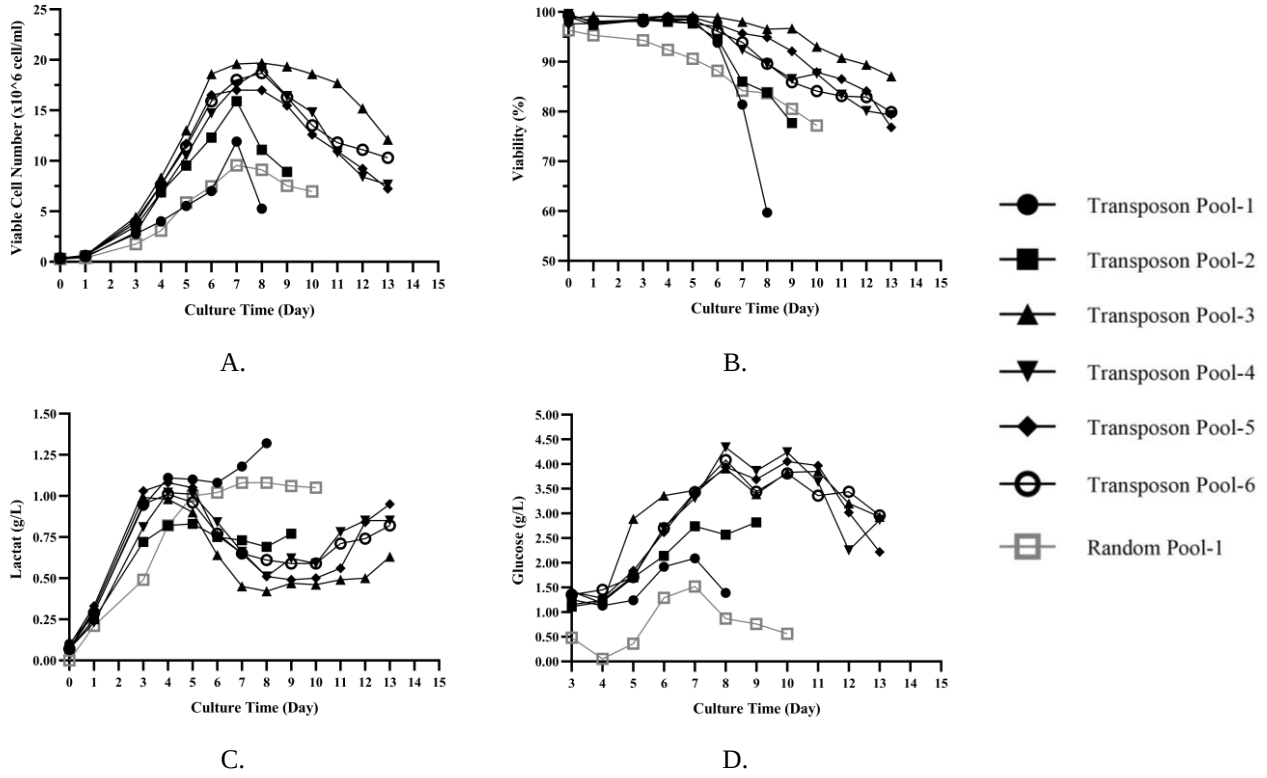


Figure 10. Growth and metabolic characteristics of fed-batch cultures of random and transposon integration pools. A) Viable cell count (x10^6 cells/ml). B) Cell viability ratio (%). C) Lactate production (g/l). D) Glucose consumption (g/l)

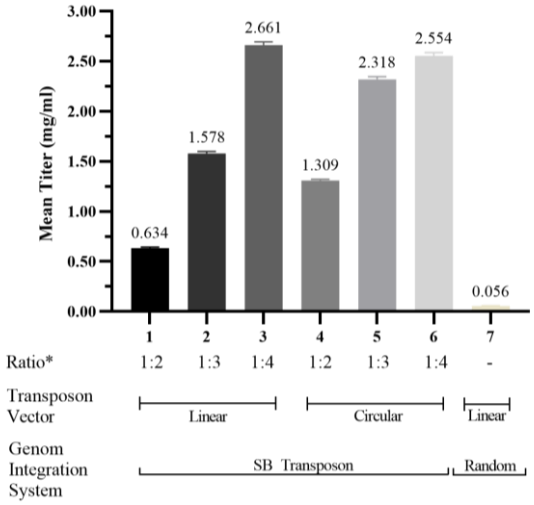


Figure 11. Titer of antiPD-1 human IgG4 production in fed-batch pools (mg/ml)

Transposon vector technology, which is employed to create recombinant cell lines utilized in the industrial production of biological drugs, vaccines, and viral vector particles, presents a more advantageous delivery system than random integration techniques [8]. Random integration systems have the potential to produce unforeseeable and occasionally controversial effects on gene expression, such as gene silencing and activation or disruption of endogenous genes. However, using random integration for industrial purposes can result in unpredictable cell line behavior, unbalanced and inefficient gene expression, and potentially irreproducible outcomes [24]. In contrast, the transposon-mediated cut-and-paste style of gene delivery ensures a single copy of the transgene per insertion site. Therefore, transgene concatemer-induced epigenetic silencing is much more

unlikely. Nevertheless, all transposon systems are subject to events such as undesired integration, continuous transposition, or insufficient transposition due to the continuous expression of the transposase gene as a result of stable transfection of the transposase vector and the suboptimal ratio between the transposase vector and transposon vector [12, 25]. Consequently, genetic instability may occur, leading to inadequate or loss of production in recombinant cell lines obtained and selected using the transposon system.

Transposon vector systems have successfully been used for creating industrial cell lines to manufacture biological drugs, vaccines, and viral vector particles. Commonly used transposases PiggyBac, Sleeping Beauty, and Tol2 are engineered to operate in non-native mammalian host cells, such as CHO, HEK293, and HeLa. An enhanced version of Sleeping Beauty transposase, SB100X can transpose 10% of transfected mammalian cells. This is 10 times more efficient than Tol2 and approximately 3 times more efficient than PiggyBac [19]. Contrastingly, transfection via random integration vectors tends to stabilize at several magnitudes of lower frequencies [24, 26]. Therefore, transposase-mediated integration could offer a starting blend of cell populations that may evolve through the adaptation landscape to reach a new fitness maxima. Consequently, more productive cell pools become generated in much shorter time frames. In our study, the top producer cell pool was achieved in the shortest selection time of 44 days.

In this research study, we sought to rapidly produce high-potential cell pools using the transposon system, increasing both productivity and efficiency in commercial production. We applied effective optimization techniques to develop producer cell lines to achieve this. Specifically, we used host CHO-DG44 recombinant cell pools and evaluated their potential for target protein production using random transfers and the SB transposon system. Additionally, we examined the impact of optimal vector ratios on anti-PD-1 human IgG4 expression through the batch culture of stable cell pools selected from transfection of the donor vector with the transposase vector in either linear or circular form at various ratios within the SB transposon system.

Tests were conducted to compare the performance of the optimized transposon with that of the random transfer method in the SB transposon system. Various transposase and transposon vector ratios were used to thoroughly examine their effects on productivity. In our study, transposon-generated pools 3, 6, and 5 yielded the best antiPD-1 hIgG4 titers (Figure 11). However, in the fed-batch process, pools 1 and 2 abruptly lost viability following the exponential growth phase (Figure 10B). Nonetheless, these underperforming pools were still 10 - 30 times more productive than the random integration pool. The results also revealed that the 1:4 ratio (transposase to transposon) was the optimal ratio, with a protein content of 2.6 mg/ml (Figure 11), regarding production efficiency and the shortest selection time of 44 days. In our findings on different transfer rates, we observed that increasing the transposon vector (donor vector) ratio positively impacted production potential and selection due to efficient transposition. Specifically, we kept the ratio of the donor vector expressing the SB transposon enzyme constant and low to avoid the phenomenon of overproduction inhibition, as stated in previous studies [20, 21]. The high production amount was compatible with the low lactate level and high glucose consumption observed in fed-batch studies of the pools. Studies have shown that 90% of the glucose taken into cell metabolism is used for glycosylation due to the high amount of precursor glycans required, especially in producing glycoproteins such as monoclonal antibodies [2, 27]. Additionally, the low amount of lactate measured may indicate that the pools utilize the lactate produced to replenish the TCA cycle and meet the energy demand from high production [28-30].

Circular and linear donor vector transfections were performed on cell pools to evaluate the effect of donor vector transfection on production and selection. The results indicated that the average production trend was higher in pools obtained with circular donor vectors, regardless of the ratios (Figure 11). This finding is consistent with previous literature on the PB system, which showed that transposition on linear substrates is less efficient than on circular ones in the SB system. Studies have found that linear donor molecules impair transposition. However, there are examples where linear donor DNA can be transformed into circular form using recombinases inside the cell [16, 31, 32]. Additionally, the position of the donor DNA in the chromosome, whether in a circular or linear form, can affect the frequency of transposition due to chromatin status [33].

The results of the current study demonstrated that random transformation, compared to the transposon system with a low protein production amount measured as 0.056 mg/ml, can obtain more efficient results with cells developed with the transposon system, especially in accordance with industrial productions. CHO cell systems, frequently preferred in industrial production, are highly prone to genetic instability (chromosomal instability, haploidy/polyploidy, gene loss, etc.) [34-36]. Due to this genetic plasticity, the structural instability and uncertain chromosomal location of transgenes with random integration leads to unstable production when using CHO cell lines, requiring extensive and time-consuming colony screening during cell line development to identify a small number of stable colonies from an inefficient pool. Considering these findings, the transposon system is more advantageous than random integration.

The utilization of transposon vectors in this study has demonstrated the potential to enhance the production of recombinant proteins in CHO cells when transfected at optimal rates. The amount and form of the donor vector employed directly influence the efficiency of the process. These findings offer valuable insights for future system optimization, particularly to produce complex proteins such as monoclonal antibodies using the SB transposon system. In addition, the genome-wide distribution of insertion sites for transposon and retroviral systems shows varying bias. SB transposase exhibits limited preference towards specific chromatin states and insertional hot spots compared to other systems. SB-mediated insertions' near-random nature may have the least detrimental effect on nearby genes [37].

4. Conclusion

In this study, we aimed to generate stable and highly productive antiPD-1 hIgG4-expressing CHO cell pools. We compared the performance of SB100X transposase to random integration in terms of stable pool selection time, metabolic robustness, biomass, and recombinant protein yields.

SB transposase is well known for the overproduction inhibition effect (OPI). Our data confirmed that keeping the transposase vector constant at a minimum concentration and co-transfecting it with an increased transposon donor vector favors the selection of better-performing producer cell pools in a shorter period. SB100X transposase enabled us to select the most productive stable cell pool in as early as 44 days (Figure 7). In contrast, a random integration vector required 85 days of selection with HT depletion and MTX amplification (Figure 9). Despite the lengthy selection time, most transposon-generated pools surpassed this pool regarding growth potential, glucose, and lactic acid metabolic profile. Moreover, the recombinant protein yields of transposon pools were far superior to the random integration pool in the 1 - 2 orders of magnitude range (as illustrated in Figure 10 and Figure 11). However, it is notable that transposon pools 1 and 2 experienced an abrupt loss of viability before the random integration pool in batch culture conditions (Figure 10B).

Transposons have proved useful as an expansion to the molecular biology toolbox. Functional genomics studies, gene therapy, and cell factory technologies have benefited greatly from transposon-mediated gene delivery.

The versatility of the SB transposon system may give superpowers to researchers and industrial cell line developers. Highly productive, stable cell pools and lines can be developed with proper optimization in considerably shorter periods. Moreover, the resulting cell pools have exceptionally high recombinant product yields compared to traditional cell factory engineering techniques like random integration vectors. Taking advantage of these benefits, the SB transposon system offers an accelerated research and development phase and a more efficient and cost-effective upstream process.

Author Contributions

The third author directed the project and supervised this study's findings. The second and first authors devised the main conceptual ideas and performed the experiment and statistical analyses. The first author wrote the manuscript with support from the second and third authors. All authors reviewed and edited the paper. All

authors read and approved the final version of the paper. This paper is derived from the first author's doctoral dissertation thesis supervised by the third author.

Conflicts of Interest

All the authors declare no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

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