Faster, cheaper and under control: de-risking CMC development with transposon-derived manufacturing cell lines

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ABSTRACT

The development of highly productive, genetically stable manufacturing cell lines is on the critical path to IND filing for protein based biologic drugs. Here we describe Leap-In Transpoasase® platform, a novel transposon-based mammalian (e.g. CHO) cell line development system that produces high titer stable pools with productivity and product quality attributes that are highly comparable to clones that are subsequently derived therefrom. The productivity distributions of clones are strongly biased towards high producers and both genetic and expression stability is consistently high. By avoiding the poor integration rates, concatemer formation, detrimental transgene recombination, low average expression level, unpredictable product quality and inconsistent genetic stability characteristic of non-homologous recombination methods, Leap-In provides several opportunities to de-risk programs early and reduce timelines and resources.

INTRODUCTION

The development of robust, stable, high-producer cell lines is critical for commercial manufacturing of therapeutic proteins, vaccines, and for gene therapy modalities, yet this step it is often rate-limiting. The labor-intensive process of isolating stable, high-producer cell lines is compounded as next generation biologics frequently require the expression of multiple subunits at optimal ratios (Klein et. al., 2012, Spiess et. al., 2015). The limitations of traditional stable cell line generation approaches using viral or plasmid-based vectors include low integration rate, limited cargo size, genetic instability (Kim et al 2011), and diminishing expression due to gene silencing (Moritz et. al., 2015). These all hamper the standardization of efficient stable cell line development workflows.

Stable genomic integration mechanisms are frequently categorized into two groups: non-homologous recombination based random integration, and site-specific recombinase mediated

integration processes (Carver et. al., 2020). However, there is a third category of stable integration mechanism, which combines the high copy numbers obtained through random integration with the intact transgene structure and open chromatin target sites characteristic of site-specific landing pads. This third category is a transposon-based mechanism, which has emerged as a DNA transfer tool for gene discovery and gene delivery applications in vertebrates. Several transposon-based systems have been characterized, including the natural medaka fish hAT gene family element Tol2 (Kawakami et. al., 2000), the engineered Tc1/mariner transposons named *Sleeping Beauty* (SB) (Mikkelsen et. al., 2003) and *Frog Prince* (Miskey et. al., 2003), and the insect-derived natural element *PiggyBac* (Yusa, 2015).

More recently, a novel transposon-transposase system, Leap-In Transposase®, has been engineered from a frog transposon (Balasubramanian et. al., 2018,) Here we describe the characteristic structural and functional features of Leap-In mediated integrations and illustrate how those unique features enable a more efficient, robust approach to manufacturing stable cell lines.

RESULTS AND DISCUSSION

The Leap-In Transposase® system

The Leap-In transposon® system utilizes transposon-based expression vectors and a cognate transposase enzyme. As is typical of transposase based gene transfer, the Leap-In transposon vectors contain two inverted terminal repeat (ITR) sequences recognized by the transposase enzyme. The genetic elements between these ITRs are completely customizable, with no size or sequence limitations. For manufacturing cell line development applications, transposons are typically configured to contain open reading frames to express the target biomolecule, a gene encoding a selectable marker, and all the associated regulatory elements

needed for highly efficient expression in the host cell. These elements are flanked by left and right inverted terminal repeats and the target site duplication TTAW (TTAT or TTAA) (Figure 1).

The Leap-In enzymes belong to the DDE/D integrase family. (Nesmelova and Hackett 2010). The DDE/D integrases mediate stable integration by a two-step, cut and paste, mechanism. (Mitra et al, 2008). To set a time limit for their intracellular presence, the enzymes are introduced to the host cells by co-transfecting Leap In mRNA with the transposon based expression construct. First, upon binding to the specific left and right ITRs, the enzyme initiates double stranded breaks in the flanking TTAW integration site signatures, Than short lived TT/AA hairpin intermediates are formed at the ends of the released transposon .The ITR bound transposase enzyme than recognizes a suitable genomic target site characterized with signatures associated with open chromatin and the presence of a TTAW sequence. Once an appropriate genomic site is found, the enzyme makes double stranded break at the TTAW site resolves the hairpins and integrates the transposon (Figure 1).

Characteristics of transposon-mediated integration of expression constructs into a target cell genome include (i) all sequences between the ITRs are faithfully integrated without deletions, insertions or structural rearrangement and (ii) when multiple copies of a transposon are introduced into a cell's genome, each insertion occurs at a separate location so that no concatemeric structures are introduced that could be prone to rearrangement or silencing. To confirm that transposition by the Leap-In system inserts multiple independent copies of structurally intact transposons, we analyzed clonal cell lines isolated from three uniquely different Leap-In cell pools. Each cell pool was produced by co-transfection of the glutamine synthetase knockout HD BioP3 CHO host (provided by Horizon Discovery UK) with a different

DNA plasmid borne transposon and Leap-In transposase mRNA. Each transposon contained genes encoding an antibody heavy and light chain, regulatory elements directing their high level expression, and a glutamine synthetase selectable marker. Forty-eight hours after transfection the cells were placed into glutamine-free media and incubated under static conditions until cell viability was >95%. In order to evaluate the genomic structure of the transposition events, one monoclonal line isolated from each pool was analyzed by targeted locus amplification (TLA) performed by Cergentis (Hottentot et al., 2017).

Transposition events were identified by looking for the characteristic sequences (TTAA and ITR's) at each end as shown for two representative examples in Figure 2. Transposition of the transposon to the genome results in a duplication of the 5'-TTAA-3' target site (black letters in Figure 2) within the CHO genome (blue letters in Figure 2) on either side of the transposon. Between the target site duplications are the sequence of the two ITRs (red letters in Figure 2) and between them the entire contents of the transposon. Bacterial elements from the plasmid (the kanamycin gene and bacterial origin of replication) are not present in a transposon and hence not present in the transposition mediated integration sites in the CHO genome. In contrast, if a transposon has been integrated by random fragmentation and non-homologous integration, the ITRs will still be within their bacterial context, there will be a break at some location within the plasmid, and the two ends of that break will be adjacent to CHO genomic sequences.

We identified 108 transposon integrations in the three clonal cell lines (Table 1). Out of the 108, only one integration in each cell line did not have the structure shown in Figure 2 indicating that under the applied transfection and selection conditions >97% of the integrations were Leap-In mediated transpositions.

In addition, the TLA analysis revealed that the transposed Leap-In transposons integrated as single copy transgenes, as shown in **Figure 2**. The integrated sequences included the entire segment of the expression construct located between the ITRs. The sequences, integrated via transposition, were without any deletion, truncations or transgene-transgene fusions and maintained the original configuration of the expression construct. In contrast, all three sites where integration occurred by non-homologous recombination contained transgene-transgene fusions.

The Leap-In mediated integration mechanism results in multiple transposons in the genome. Each integration site contains only a single copy of the transposon and the transposons are structurally intact at the nucleotide level. This is in stark contrast to transgenes integrated by non-homologous end joining, which are frequently rearranged (Tharmalingam et. al., 2018, Lattenmayer et. al., 2006).

DHFR based selection of Leap-In mediated stable DG44 pools.

Chinese Hamster Ovary (CHO) cells are commonly used for biologic manufacture. Two popular selection systems employ host cells that are incapable of synthesizing a critical metabolic intermediate. Hosts such as the DG44 cell line lack a functional gene for the dihydrofolate reductase (DHFR) enzyme, which is required for the synthesis of purine and thymidylate (Florin et. al., 2011). An alternative system uses CHO cells lacking a functional glutamine synthetase (GS) gene, the only enzyme catalyzing glutamine synthesis (Fan et. al., 2012) glutamine is required for cells to make several amino acids and pyrimidines as well as serving as a major energy source. The newer glutamine synthetase system has become increasingly popular, largely because when random-integration methods are used with DHFR selection, an amplification step is frequently required to increase production titers to acceptable

levels (Cacciatore et. al. 2010). By using a transposon containing a DHFR gene driven by an attenuated promoter, we expect to be able to select pools with high transgene copy numbers in a single step.

A Leap-In transposon construct was designed to contain an antibody heavy and light chain as well as associated expression regulatory elements together with a selection cassette. Stable pools were established by co-transfecting transposon DNA in plasmid for and transposase mRNA into CHO DG44 DHFR$^{-/-}$ host and selecting cells for survival with different concentrations of the DHFR inhibitor methotrexate (MTX) in a single step following transfection. Once cell pools had reached >95% viability (14-21 days depending on selection stringency), genomic DNA was prepared from samples and the average number of transposon integrations per genome were determined using ddPCR. Figure 3 shows that with increasing concentrations of methotrexate a linear increase in the average transposon copy number was observed. Thus, the average number of transposons integrated per genome can be controlled by the stringency of selection.

Several additional pools were generated, using transposons with further attenuated DHFR expression. Average transposon copies per cell were measured for these pools also. The specific productivity of each pool was then determined in 25mL shake-flask–scale fed batch experiments. A strong linear correlation (R^2= 0.84) was observed between the average number of integrated transposons per cell and the corresponding specific productivities (Figure 4).

Increasing the number of transposons integrated into the CHO DG44 genome results in a proportional increase in the specific productivity of the pool, a feature of a copy-number dependent expression system where the transgene expression cassettes are faithfully integrated into and insulated from the surrounding genome elements. It also demonstrates that, on average,

each integration via transposition maintains the functional integrity of the transposon (LC, HC, regulatory elements and selection cassette) without deleterious recombination or silencing.

Leap-In transposase mediated stable pools established from GS$^{-/-}$ CHO cells

We designed Leap-In transposons with five different glutamine synthetase cassettes which result in GS expression levels designated as h+(low),ht (medium),ht+ (medium-high),hxt (medium-high) and hxt+ (high). Open reading frames encoding the same heavy and light chain of an antibody were synthesized and cloned into these five transposons. The heavy and light chains were under the control of identical EF1 promoter based regulatory element and flanked by the same HS4 and D4Z4 insulators, hence, the five transposons differed only in the glutamine synthetase selection cassette. Transposons were co-transfected with Leap-In Transposase mRNA into GS knockout cells. Stable pools were subsequently established by transferring the cells into glutamine-free media 48 hours post transfection. No additional selection pressure (e.g. MSX addition) was used during the selections. Figure 5a shows the viability selection curves for the five pools.

Following recovery, each pool was analyzed for average transposon copy number and grown in a 10-day fed batch culture. Specific productivities, and copy numbers are shown in Table 2.

The pool with the transposon conferring the least stringent (h+) selection recovered to >95% viability in less than a week. Pools with three intermediate stringencies (ht, hxt and ht+) had reached 95% viability within about twelve days, but the most stringent (hxt+) selection took around twenty days to recover. Similar to the DHFR based selection (Fig 4), there is a correlation between the selection stringency, the integrated transgene copy numbers and the specific productivities of the pools (Fig 5b).

This data differentiates the Leap-In mediated stable GS pools from pools established by random integration where such correlation cannot be established. (Noh et. al., 2018). Strikingly, the volumetric productivity values correlated strongly (R2=0.97) with the copy number (Fig 5c) further distinguishing the copy number dependent Leap-In mediated expression from random integration systems.

Clonal distributions from Leap-In pools are biased towards high-expressing clones.

Single cells were deposited into 96 well plates (one plate for each pool) and monoclonal lines were derived from the four most productive pools presented in Table 2. We observed that under identical cloning conditions, we obtained gradually fewer viable clones, from more stringently selected pools (Table 3). After expansion, the productivity of these clones was measured in 7-day fed batch cultures in 24 deep-well plates. The distribution of productivities is shown in Figure 6.

The productivity of the highest expressing clones from the first three pools was similar, ~ 2g/Lin the 7 day fed batch cultures. Interestingly, the highest expressing clone from the most stringent (hxt+) selection produced just over 1.5 g/L, significantly less than the top clone from the other three pools. Based on these observations, in our current platform selection system, Leap-In stable pools established by medium-high stringency selections are the preferred choice to isolate clonal cell lines.

When the clonal productivity ranges of the four pools were divided into four equal quartiles and the clones sorted into these quartiles by their productivities, selection stringency dependent trends can be observed (Table 3). The selection stringency directly correlates with the Q1 fractions. The relatively small Q3 and Q4 fractions show a small inverse correlation with the

selection stringency. The combined Q1+Q2 fractions represent >75% of all clones, a remarkable characteristic bias towards high producer cells in Leap-In generated stable pools.

The data in Table 3 and Figure 6 demonstrate that less than 100 monoclonal stable clones are sufficient to isolate high producer clones from Leap-In mediated stable pools even when established at medium stringency selection. In contrast, from random integration pools many thousands of clones need to be screened in a successful cell line development campaign (Le et al 2018). The unique clonal distributions, of the Leap-In mediated stable pools, decrease clone screening requirements by one to two orders of magnitude, resulting in drastically reduced resource requirement.

The Leap-In transposons are stable in the CHO genome

Genetic stability is a critical quality attribute of commercial manufacturing cell lines, yet the current mainstream random integration mechanisms can't control or even predict genetic stability. The frequently rearranged tandem transgene integrants, often exacerbated when gene amplification methods are used, result in genetically unstable recombinant loci.

We have analyzed the genetic stability of more than 80 clones derived from multiple external and internal R&D programs. To do such, the cells were passaged for between 60 and 90 population doublings with and without selective pressure. Assessment of integrated copy numbers by ddPCR, and productivity assessments from representative production cultures demonstrate that >90% of the clones established by Leap-In transposons maintain the T0 productivity and copy number levels (Figure 7a, b). In the remaining <10% clones, productivity is decreased by less than 30% of the T0 value, a value generally considered as acceptable stability within the industry. In short, in Leap-In mediated cell line development programs, genetic stability is not a clone ranking parameter. This eliminates the need to compensate for

instability during the ranking process and further reduces the number of clones required to handle and triage during a development program.

We also made a more in-depth assessment of the genetic stability of one clone over 90 population doubling. Nucleotide sequence level data was derived from the recombinant transgene insertions using TLA technology performed by Cergentis (Hottentot et. al., 2017). There were 58 transposase-mediated integration sites detected in the cells analyzed at the T0 and the PD90 timepoints. Importantly, their flanking genomic sequences were identical at the two timepoints **(Figure S1)**. The integration sites were mapped to the host genome scaffolds and were found at the exact same positions at both timepoints demonstrating the consistent structural stability of Leap-In transposase-mediated stable integrations.

Product from Leap-In pools and clones are highly comparable

The more homogeneous distribution of clonal productivities means that pool titers and clone titers are very comparable. With other words, the Leap In pools reliably predict their derivative clonal productivities. Data from nine different programs are shown in Table 4. This high degree of correlation means that pools can in principle be used for early process development work, even before single cell cloning has begun. This also means that one can screen Leap-In pools established using various vector elements, chain ratios, coding sequences and so on, and be assured that the performance in the pools is predictive for the derivative clones.

The comparability of stable pools and clones is not limited to productivity. Three pools (ht, hxt and ht+) described in Table 2 and clones from Figure 6 were used as models. We looked at two global physicochemical critical quality attributes: charge profile (Figure 8a), and N-linked

glycan distribution (Figure 8b) in the same monoclonal antibody produced by the three Leap-In mediated stable pools and randomly isolated high producer derivative clones from each.

The data in Figure 8b presents the N-linked glycan distributions in the three stable pools and derivative clones. While the two higher stringency pools produce comparable glycosylation profile to their derivative clones, the clones isolated from the lower stringency pool show more diversity in N glycan composition. This observation may guide selection stringency choice depending on how homogeneous or diverse glycan structures are preferred.

Leap-In pools are stable enough for process development and biological product DS manufacturing

Based on their productivity and product quality comparability, the Leap-In mediated stable pools can be considered as representative cell substrates to the derivative final clones. This suggests the pools to support process development, analytical development and IND enabling tox manufacturing. These activities can be initiated while the final clones are being identified, shortening the CMC development timelines.

As discussed, the individual Leap-In clones demonstrate remarkable genetic stability. On the other hand, the inherent clonal growth differences, driving population dynamics, may change the clonal distribution of the stable pools over time. To evaluate whether the population dynamics changes permit using the pools for representative drug substance manufacturing, the same three stable pools presented in Figure 5 were subjected to a standard stability passage study in glutamine-free formulation for 30 population doublings. At the end of the passages, fed batch production runs were performed using the Time 0 and the PD30 pools. The results are shown in Figures 9a, b. and c.

As expected, the volumetric productivities are lower in the PD30 pools compared to their T0 counterparts. The degree of productivity decrease inversely correlates with the fraction of clones in Quartile 1 encompassing the top producers (R^2=0.99, data not shown). Nevertheless, the productivity change, observed in all three pools, is less than 25%. This is below the conventionally recognized ~30% productivity loss acceptance criteria for clonal stability.

Analytical comparability was established for charge profile and N-linked glycosylation of the products made by the T0 and the PD30 pools (Figure 9b, c)

The data presented in Figures 9 b and c demonstrates a high degree of product quality comparability between T0 and PD30 pools. 30 population doublings, starting from a vial of 10E7 cells, is sufficient to seed a 5000L bioreactor which, at multi-gram per liter would produce kilogram quantities of bulk drug substance. The data endorse the Leap-In system as a viable alternative to other approaches aiming to utilize stable pools to shorten CMC development timelines (Hu et. al., 2017; Rajendra et al 2017; Scarcelli et. al., 2017).

Summary

Compared to conventional random integration-based technologies, the Leap-In transposase-mediated stable cell line development provides an array of valuable features, from genetically stable integrations through high expression levels of consistent ratios of multi-cistronic units to robust pool-to-clone productivity and product quality comparability.

The structural and functional integrity of the Leap-In mediated stable integrants leads to more homogeneous stable pools where ~97% of the recombinant integration sites represent single and exact copies of the expression construct. As a consequence, there is a strong productivity and product quality comparability between Leap-In mediated stable pools and their derivative clones.

This characteristic pool to clone comparability enables early de-risking of the development programs. This is accomplished by triaging a number of representative predictive stable pools for optimal productivity and product quality, thus changing the traditional manufacturing cell line development paradigm. The productivity and product quality decisions and selections at pool ranking stage reliably predict the productivity and product quality spectrum of the final clones. Once the optimal stable pool is identified, only a small (<100) number of clones need to be screened and ranked to isolate genetically stable clones with the desired productivity and critical quality attribute (CQA) profile. The observed high producer clone enrichment is more prominent in the Leap-In mediated pools than by the alternative, industrially relevant epigenetic regulatory elements including UCOEs, insulators and MARs that have been described elsewhere (Saunders et. al., 2015).

Unlike the pools established by traditional expression technologies, the Leap-In stable pools maintain genetic, productivity and product quality stability This unique stability of pools enables the early and efficient manufacturing of representative drug substance for analytical and formulation development studies as well as material for toxicology studies or even Phase I clinical trials. Also, based on their productivity and robust genetic stability, the Leap-In mediated stable clones are attractive candidates to support perfusion based manufacturing processes where in addition to solving engineering and logistical challenges, the development of production clones best suited for extended operation modes is also a mission critical task (Bielser et. al., 2018)

In summary, the Leap-In mediated manufacturing cell line development workflow results in high productivity, predictable product quality, robust genetic stability, and shortened CMC development timelines with significant resource reduction.

MATERIALS AND METHODS

- **Recombinant DNA methods**

  Vector construction

  The recombinant genes were synthesized and the expression constructs were assembled in ATUM's laboratories using proprietary technologies. The sequence of the assembled constructs was confirmed using Sanger sequencing.

  The expression of codon optimized human IgG1 heavy and light chains were driven by the human and the murine EF1α promoters respectively. The variable expression levels of the codon optimized glutamine synthetase gene were achieved by combinations of 5' and 3' regulatory elements, and coding sequence attenuation. The transcription units were flanked by the HS4 and the D4Z4 insulator elements. In the constructs, the segment containing the bacterial selection marker and replication origin was separated from the mammalian expression units by the left and right Leap-In1 boundary elements. The selection in the GS experiments was performed only by glutamine deprivation. No methionine sulfoximine (MSX) was used.

  The constructs designed for the DG44 experiments were essentially the same with the exception of the selection cassette. The murine dhfr gene was linked to a puromycin N-acetyl transferase gene by various IRES sequences of different strengths. The transfected DG44 cells were selected in HT^- media supplemented with different amounts of methotrexate (MTX)

  Leap-In mRNA was manufactured using an AOF process by TriLink. The mRNA lots were released per ATUM's specifications

- **Cell culture.**

HD BIOP3 (Horizon Discovery) and DG44 (Prof. Lawrence Chasin) cell derived stable pools and clones were used in the study. All cell culture procedures were performed in chemically defined media formulations. The cells were maintained by routine passages two-three times a week. Cells were counted by ViCell (Beckman USA). The cells were transfected using the Neon electroporation system (Thermo/Invitrogen) equipped with 100 ul tips. Expression construct (transposon) DNA and Leap-In transposase mRNA were co-transfected. Stable pools were selected under glutamine-free condition for the BIOP3 lines and with HT^- formulation supplemented with various MTX concentrations for DG44. Single cell cloning was performed by the VISP system (Solentim, Dorset UK). Monoclonality was confirmed by the VISP and Cell Metric (Solentim) instruments. Volumetric productivities were assessed in fed batch production runs following ATUM's standard feeding protocols in either 24 deep well plates, tube spins or shake flasks.

- **Protein purification**

  The product from model antibody cultures grown for product quality characterization were purified using protein A capture. The concentration of the purified protein was determined by A280 absorbance and using molecular extinction coefficients.

- **Analytical methods**

  <u>Protein</u>

  Productivities were estimated by an Octet HTX (Pall, USA) using protein A sensors. Protein charge variants were separated on Caliper chips (Perkin Elmer, US) following the manufacturers recommendation. PNGAse F released N-linked glycan structures were identified and quantified by hydrophobic interaction chromatography and mass spectrometry.

  <u>Molecular biology</u>

Droplet digital PCR (ddPCR) was performed using a BioRad QX200 system (BioRad, US) following the manufacturers protocol and using transposon specific primers. The primers and probes were synthesized by IDT (US). Transgene structure integrity characterization and genomic integration site identification was performed using targeted locus amplification (TLA) by Cergentis (Utrecht, The Netherlands).

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REFERENCES

1) Klein C, Sustmann C, Thomas M, Stubenrauch K, Croasdale R, Schanzer J, Brinkmann U, Kettenberger H, Regula JT, Schaefer W: Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies
   mAbs 4:6, 653-663, 2012
2) Spiess C, Zhai Q, Carter PJ: Alternative molecular formats and therapeutic applications for bispecific antibodies
   Mol Immunology 67:2 (part A), 95-106, 2015
3) Kim M, O'Callaghan MO, Droms KA, James DC: A mechanistic understanding of production instability in CHO cell lines expressing recombinant monoclonal antibodies
   Biotechnology and Bioengineering 108:10, 24342446, 2011
4) Moritz B, Becker PB, Gopfert U: CMV promoter mutants with a reduced propensity to productivity loss in CHO cells
   Scientific Reports 5:16952, 2015

5) Carver J, Ng J, Zhou M, Ko P, Zhan D, Yim M, Shaw D, Snedecor B, Laird MW, Lang S, Shen A, Hu Z: Maximizing antibody production in targeted integration host by optimization of subunit gene dosage and position
Biotechnology Progress 2020 Jan 21,:e2967 (Epub ahead of print)
6) Kawakami K, Shima A, Kawakami N: Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish ger lineage
Proc.Natl.Acad.Sci 97:21, 11403-11408, 2000
7) Mikkelsen JG, Yant SR, Meuse L, Huang Z, Xu H, Kay MA: Helper-independent Sleeping Beauty transposon-Transposase vectors for efficient non-viral gene delivery and persistent gene expression in vivo
Molecular Therapy 8:4, 654-665, 2003
8) Miskey Cs, Izsvak Zs, Plasterk RH, Ivics Z: The Frog Prince: a reconstructed transposon from Rana pipiens with high transpositional activity in vertebrate cells
Nucleic Acids Research 31:23, 6873-6881, 2003
9) Yusa K: piggyBac transposon
Microbiol Spectrum 3(2):MDNA3-0028-2014 , 2015
10) Balasubramanian S, Peery RB, Minshull J, Lee M, White R, Kelly RM, Barnard GC: Generation of high expressing Chinese hamster ovary cell pools using the Leap-In transposon system
Biotechnology Journal 13:10,, 2018
11) Nesmelova IV, Hackett PB: DDE transposases: structural similarity and diversity
Adv Drug Deliv Rev. 62:12 1187-1195, 2010

12) Mitra R, Fain-Thornton J, Craig NL: PiggyBac can bypass DNA synthesis during cut and paste transposition
The EMBO Journal 27:7, 1097-1109, 2008

13) Hottentot QP, van Min M, Splinter E, White SJ: Targeted Locus Amplification and next generation sequencing
In Genotyping: Methods and Protocols (eds White SJ and Cantsilieris S). Methods in Molecular Biology vol 1492 pp:185-196, 2017

14) Florin L, Lipske C, Becker E, Kaufmann H: Supplementation of serum free media with HT is not sufficient to restore growth properties of $DHFR^{-/-}$ cells in fed batch processes-Implications for designing novel CHO-based expression platforms
J Biotechnology 152:4, 189-193, 2011

15) Fan L, Kadura I, Krebs LE, Hatfield CC, Shaw MM, Frye CC: Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells
Biotechnology and Bioengineering 109:4, 1007-1015, 2012

16) Cacciatore JJ, Chasin LA, Leonard EF: Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production using the Dhfr-based CHO cell selection system
Biotechnology Advance 28:6, 673-81, 2010

17) Noh SM, Shin S, Lee GM: Comprehensive characterization of glutamine synthetase-mediated selection for the establishment of recombinant CHO cells producing monoclonal antibodies
Scientific Reports 8:5361, 2018

18) Boldog F: A genomic case of structure-function relationship: The Leap-In transposase system
Proceedings of Cell Line Development and Engineering US 2019
19) Saunders F, Sweeney B, Antoniou MN, Stephens P, Cain K: Chromatin function modifying elements in an industrial antibody production platform-comparison of UCOE, MAR, STAR and cHS4 elements
PLOS ONE 10(4): e0120096, 2015
20) Le K, Tan C, Gupta S, Guhan T, Barkhordarian H, Lull J, Stevens J, Munro T: A novel mammalian cell line development platform utilizing nanofluidics and optoelectro positioning technology
Biotechnology Progress 34:6, 1438-1446, 2018
21) Bielser J-M, Wolf M, Souquet J, Broly H, Morbidelli M: Perfusion mammalian cell culture for recombinant protein manufacturing-A critical review
Biotechnology Advances 36, 1328-1340, 2018
22) Hu Z, Hsu W, Pynn A, Ng D, Quicho D, Adem Y, Kwong Z, Mauger B, Joly J, Snedecor B, Laird MW, Andersen D, Shen A: A strategy to accelerate protein production from a pool of clones in Chinese hamster ovary cells for toxicology studies
Biotechnology Progress 33:6, 1449-1455, 2017
23) Rajendra Y, Balasubramanian S, McCracken NA, Norris DL, Lian Z, Schmitt MGT, Frye CC, Barnard GC: Evaluation of piggyBac-mediated CHO pools to enable material generation to support GLP toxicology studies
Biotechnology Progress 33:6, 1436-1448, 2017

24) Scarcelli JJ, Shang TQ, Iskra T, Allen MJ, Zhang L: Strategic deployment of CHO expression platforms to deliver Pfizer's monoclonal antibody portfolio Biotechnology Progress 33:6, 1463-1467, 2017

25) Lattenmayer C, Loeschel M, Steinfellner W, Trummer E, Mueller D, Schriebl K, Vorauer-Uhl K, Katinger H, Kunert R: Identification of transgene integration loci of different highly expressing recombinant CHO cell lines by FISH Cytotechnology 51:171-182, 2006

26) Tharmalingam T, Barkhordarian H, Tejeda N, Daris K, Yaghmour S, Yam P, Lu F, Goudar C, Munro T, Stevens J:Characterization of phenotypic and genotypic Diversity in subclones derived from a clonal cell line Biotechnology Progress 34:613-623, 2018l

FIGURE LEGENDS

Figure 1. Schematic representation of transposition, the Leap-In transposase mediated cut and paste transgene integration mechanism**.** (explained in the text). For simplicity only the TTAA recognition signature example is shown.

Figure 2. The nucleotide level structures of two Leap-In mediated integration junctions from one recombinant cell**.** Blue: CHO genome sequence, black: target site duplication, pink: transposon.

Figure 3. Correlation between selection stringency and integrated Leap-In transposon copy number in stable DG44 pools selected in HT^- media. The stringencies were controlled by the indicated MTX concentrations during the entire duration of pool selections without any stepwise increase.

Figure 4. Correlation between transgene copy number and cell specific productivity in CHO DG44 stable pools established under different selection stringencies

Figure 5a. Viability of GS KO cells transfected with different selection stringency transposons during stable pool selection in glutamine-free media.

Figure 5b and c. Correlation between the integrated transposon copy number and specific productivity (5b) and volumetric productivity (5c) in the five Leap-In mediated stable pools established at different selection stringencies.

Figure 6. Productivity of clones isolated from four stable pools expressing the same monoclonal antibody but established under different selection stringencies. Productivities were measured by Octet at the end of 7-day long 24 deep-well plate scale fed batch cultures.

Figure 7a, b. Stability of copy number and productivity in Leap-In stable clones.

Figure 8a. The distribution of basic, main and acidic charge groups produced by the three stable pools, established at different stringencies, and from their derivative clones.

The data indicates that there is strong charge profile comparability between the stable pools and the derivative clones.

Figure 8b. The N-linked glycan distribution in the three stable pools and derivative clones.

Figure 9 a. The volumetric productivity in Leap-In stable pools. Grey bars: Time 0, black bars: PD30. The arrows indicate the productivity change expressed in % of the T0 value.

Figure 9b. Charge profile comparability between T0 and PD30 stable pools.

Figure 9c. N-linked glycosylation comparability between the T0 and PD30 stable pools.

TABLE LEGENDS

Table 1 The fraction of transposition based stable integrations in three Leap-In mediated stable, MAb producing CHO clones.

Table 2. Day 10 volumetric productivities, calculated specific productivities and integrated transgene copy numbers in the five Leap In pools established at different selection stringencies.

Table 3. Data shown in Figure 6 was analyzed to show the distribution of productivity. For each pool, Q1 is the number of clones producing between 75% and 100% of the amount of antibody made by the most productive clone. Similarly, Q2 is the number of clones producing between 50% and 75%, Q3 is the number of clones producing between 25% and 50%, and Q4 is the number of clones producing between 0% and 25% of the amount of antibody made by the most productive clone.

Table 4. Comparison of Leap-In mediated stable pool and derivative clonal productivities in various cell line development (CLD) programs.