

Original Research

Strategy for Developing a Stable CHO Cell Line that Produces Large Titters of Trastuzumab Antibody

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Abstract

Background: Trastuzumab (Herceptin®) is currently the main treatment option for breast cancer patients that overexpress the human epidermal growth factor receptor 2 (HER2). This antibody binds specifically to HER2, blocks cancer cell growth, and promotes effective cell death. In the present study, we sought to develop a robust and efficient process for the development of a stable Chinese hamster ovary (CHO) cell line with high trastuzumab expression and production. **Methods:** We adapted a process that combines transposon system-based vector construction, suspension cell culture, and a high selection process. The latter, involved enhanced green fluorescent protein (eGFP) expression, fluorescence-activated cell sorting (FACS), and semi-solid methylcellulose media. **Results:** The construction of trastuzumab as a humanized monoclonal antibody was achieved by subcloning the synthesized light and heavy chain sequences into a suitable piggyBac expression vector. The optimized piggyBac vector used for the expression of trastuzumab in CHO cells resulted in the production of trastuzumab and reached 4.24 g/L in the T1A7 clone after a 7-day batch culture. The T1A7 clone was selected after screening over 1500 clones. **Conclusions:** The current simple workflow ensures strict monoclonality and relatively high production of trastuzumab. This workflow could potentially be implemented in Research and Development (R&D) laboratories, including in developing countries for the production of recombinant monoclonal antibodies in a cost-effective manner.

Keywords: batch-mode; CHO; FACS/Methylcellulose media (MCM); PiggyBac; trastuzumab

1. Introduction

Trastuzumab (Herceptin®), a humanized immunoglobulin G1 (IgG1) that targets the human epidermal growth factor receptor (HER2), is one of the top six selling monoclonal antibodies (mAbs) worldwide. It was the first monoclonal antibody (mAb) to be approved by the Food and Drug Administration (FDA) for the treatment of early breast cancer, advanced breast cancer, and gastric cancer, where HER2, a transmembrane protein encoded by the HER2/neu, is overexpressed [1–6]. Unfortunately, access to trastuzumab has been hampered by its high cost. Hence, the development of trastuzumab biosimilars has been stimulated by the expiration of the European Union (EU) patent on trastuzumab. Thus, to facilitate the availability and accessibility of this expensive therapeutic class, the development of biosimilars is predicted to be between 20% and 30% less expensive than the reference molecules [7].

In the biopharma industry, expressing therapeutic mAbs that are high quality and titters in an economically viable process is vital. Achieving a robust and controllable process for antibody production is a highly complex and sensitive pathway that requires careful consideration of many factors and parameters, which includes choosing the

host, gene amplification strategy, expression vector types, and clone screening techniques [8]. Nowadays, 95% of the therapeutic mAbs are produced in mammalian cell lines [9]. Chinese hamster ovary (CHO) cells are currently the most widely used host cells in medical Research and Development (R&D) laboratories and the biopharmaceutical industry in producing mAb-based biologics. They can ensure the correct protein assembly, folding, and sophisticated post-translational modifications (i.e., glycosylation, phosphorylation), the exact same as human cells [10,11]. CHO cells are also of interest to pharmaceutical companies owing to their growth rate in serum-free media, high yield (up to 10 g/L), scalability, manufacturability, and utility in conjunction with selective markers, such as specific antibiotics, methotrexate (MTX), or glutamine synthetase (GS) [12,13].

While the high production capacity of a recombinant protein from a selected cell clone is economically important, consistency and stability over generations of cell cultures and fed-batch process cultures are critical for biopharmaceutical companies. Therefore, the choice of expression vector used to transfect the cell lines is a key factor in maintaining high productivity. Several studies have shown that random integration of the transgene into the genome of cells



is the main factor limiting the stability of protein expression [14–17]. Unlike conventional transfection methods, which typically use vector-based random integration of foreign genes into chromosomes, site-specific recombination strategies offer effective alternatives for developing productive and stable clones [18]. Thus, this represents an alternative approach to obtaining constant protein expression of transgenes in transposon systems [19–21]. The Piggy-Bac transposon system (PB system) is one of several transposons that has been engineered to be functional in mammalian cells. The system combines a helper plasmid, which encodes the transposase gene (PBase), and a donor vector featuring the 5' and 3' inverted terminal repeat elements (5' ITR and 3' ITR) that flank the donor ends. The expression cassette encoding the gene of interest is inserted between the two ITR elements. Following co-transfection, the transiently expressed PBase catalyzes the expression cassette excision from the donor vector and mediates its integration into the host genome [20,22]. In addition, the selection of the clones with desirable characteristics, such as protein productivity, antigen specificity, and cell survival, is a key backbone of the process. For decades, labor-intensive manual handling (limiting dilution) and semi-automated methods (FACS) have been used for this purpose [23]. Today, fully automated systems that combine integrated robotics and sophisticated software, such as the ClonePix picker system and Cell Xpress™ technology (Molecular Devices, CA, USA), can enable the screening of over 10,000 clones an hour, with high purity and monoclonality assurance [24,25].

In the present study, the PiggyBac system was used for the purpose of mAb expression. A helper vector and a donor containing trastuzumab heavy- and light-chain sequences were synthesized. These sequences consisted of optimized signal sequences to ensure the proper antibody folding and post-translational processing. Using puromycin as a selection pressure, the expression vector was stably expressed in the CHO-S cell line. Thereafter, the ability of the CHO-S cells to produce the target antibody was assessed. By outlining a simple workflow, the main objective of this work was to establish the first Moroccan biotechnology platform to produce recombinant proteins, including mAbs. This could strongly encourage the Moroccan pharmaceutical industry to adopt such platforms and produce biologics, which are currently imported. This will result in long-term savings on costs in the Moroccan healthcare system, thereby improving their affordability and accessibility to Moroccan patients.

2. Materials and Methods

All cell lines were validated by Short Tandem Repeat (STR) profiling; Mycoplasma testing was performed in all cell lines every two weeks using the MycoSEQ™ Mycoplasma Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.1 Gene Synthesis and Plasmid Construction

The trastuzumab expression cassette was designed in-house. Indeed, the trastuzumab heavy-chain (Tras-HC) and light-chain (Tras-LC) sequences were obtained from the Protein Data Bank (PDB) (1N8Z) and Drug Bank (DB00072) databases. Cytomegalovirus (CMV) promoter was selected to drive Tras-HC and Tras-LC expression. Then, the dual reporter gene for enhanced green fluorescent protein (eGFP) and puromycin was inserted under the control of the CMV promoter, using HindIII and SpeI restriction sites and terminated by BGH pA. The gene encoding the IL-2 secretory signal sequence was inserted upstream of Tras-HC and Tras-LC and three different poly A elements were inserted upstream of the CMV promoter. Then, the expression cassette was cloned into the donor vector (Piggy-Tras), and while the Piggy-Tras construct was generated by commercial synthesis (Vector Builder, NJ, USA).

Next, *Escherichia coli* DH5- α cells were transformed using the Piggy-Tras and Piggy-Tras plasmids. Sufficient plasmids were prepared for transfection. Following transformation, clones corresponding to each of the vectors were randomly selected for purification. Recombinant plasmids were purified from bacterial cells using the Pure-Link HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The purified Piggy-Tras were amplified by PCR for Tras-HC and Tras-LC using primers (forward-HC: 5'-CAGATCCAAGCTGTGACCGG-3', reverse-HC: 5'-TCTTATCATGTCTGGCCAGC-3') and (forward-LC: 5'-ACAGATCCAAGCTGTGACC-3', reverse-LC: 5'-CTGCATTCTAGTTGTGGTTT-3'), respectively. Afterward, gel electrophoresis was performed on the amplicons using a 1% agarose gel. The sequences of the Piggy-Tras construct were confirmed by means of DNA sequencing.

2.2 Cell Culture

The suspension Freestyle CHO-S cells (Thermo Fisher Scientific, Waltham, MA, USA) were maintained in FreeStyle™ CHO expression medium (Thermo Fisher Scientific, Waltham, MA, USA). The chemically defined, protein-free medium was supplemented with 8 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 2 mM Pen-Strep (Invitrogen, Carlsbad, CA, USA), and an anti-clumping agent (Invitrogen, Carlsbad, CA, USA). The cells were kept in a humidified incubator on a shaker at 37 °C with 8% CO₂. The CHO-S cells were cultivated in shake flasks and sub-cultured twice a week at a density of 2×10^6 cells/mL. Cell viability was evaluated using the Trypan blue exclusion method and cell number was measured using an automated cell counter (Eve Automatic Cell Counter, NanoEnTek, Seoul, Korea).

2.3 Cell Transfection

Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) was used to co-transfect the FreeStyle CHO-S cells in triplicate. Twenty-four hours prior to transfection, 30 mL of FreeStyle™ CHO expression medium containing 6×10^5 cells/mL was seeded into 125 mL shaking flasks. Three molar ratios were tested to optimize protein expression: 1:3, 1:5, and 1:10 helper/donor (Piggy-Trans/Piggy-Tras). On the day of transfection, 37.5 µg of helper/donor DNA was diluted in 600 µL of FreeStyle™ CHO expression medium. In a separate tube, 37 µL Lipofectamine 3000 reagent was diluted in 600 µL FreeStyle™ CHO expression medium. Diluted Lipofectamine reagent was added to the diluted DNA, incubated for 10 min at room temperature, and then, added dropwise to the cells. The cells were maintained at 37 °C with 8% CO₂ in a humidified incubator. After 6 hours of incubation, the medium was replaced with a fresh medium containing puromycin. Trastuzumab expression was evaluated on day 4 post-transfection using a human IgG enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, St. Louis, MO, USA).

2.4 Stable Pool Generation

Before transfection, an enhanced stringency selection of the CHO-S cells was performed using a killing curve, which used puromycin concentrations ranging from 20 to 100 µg/mL (Sigma-Aldrich, St. Louis, MO, USA). Following, 48 hours of transfection, the cell media were changed and supplemented with the optimal puromycin concentration. Thereafter, the selective medium was replaced every three days for a month to obtain a stable pool, the stability of which was evaluated by fluorescence microscopy and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

2.5 ELISA

The level of mAb expression in the cell culture supernatants (plates and shake flasks) was determined by indirect ELISA. The concentration of the secreted mAb was determined using the Herceptin Trastuzumab/Anti-HER2 Humanized IgG ELISA Kit (Alpha Diagnostics International, San Antonio, TX, USA). Briefly, 8-well strips were used, which were pre-coated with HER2 antigen and post-coated with stabilizers. Standards and culture supernatants were diluted, loaded into each well, and treated with anti-human IgG-horseradish peroxidase (HRP) conjugate. TMB (tetramethylbenzidine) (Sigma-Aldrich, St. Louis, MO, USA) and HRP substrate were added to the plates. After incubating for approximately 10–20 min at room temperature in the dark, sulfuric acid (H₂SO₄) (Merck, New York, NY, USA) at a concentration of 2N was added to stop the reaction. The absorbance was measured at 450 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). Washing procedures were

performed between all steps using a PBS buffer containing 0.05% (v/v) Tween 20. Human IgG with a defined concentration was applied to draw a standard curve, with the trastuzumab quantitation range from 0.5 ng/mL to 10 ng/mL.

2.6 Fluorescence Microscopy

Fluorescence microscopy was used to monitor the stability of the cell pool in the shake flask during a one-month culture without selection pressure via the eGFP expression. The cells were washed twice with 1 mL of PBS, and fixed in 1 mL of ice-cold 100% methanol for 10 min before being washed again with 1 mL of PBS. The coverslips were mounted in ProLong Diamond Antifade Mountant and DAPI was applied (Life Sciences, Cambridge, MA, USA). The images were acquired using an EVOS® FL Cell Imaging microscope.

2.7 Fluorescence-Activated Cell Sorting (FACS) Analysis

Since the donor vector was harboring eGFP, the encoding sequence transfection rates in the cells were determined 48 hours post-transfection. The cells were cultivated for two days at 37 °C before being resuspended in PBS. The FACSCalibur™ system (BD Biosciences, San Jose, CA, USA) was used to estimate the green fluorescence intensity of various clones. Green fluorescence at 525 nm was detected through an FL1 set at a PMT voltage of 400, with a logarithmic gain. Ten thousand cells were analyzed for each sample.

2.8 Picking and Expansion

The methylcellulose-based semi-solid medium was prepared in-house using a minimum essential medium (MEM) 10X (Gibco, Waltham, MA, USA), 2% methylcellulose 4000 pentose (Sigma-Aldrich, St. Louis, MO, USA), 1% non-essential amino acids (Gibco, Waltham, MA, USA), 14 mM NaHCO₃ (Sigma-Aldrich, St. Louis, MO, USA), 0.5% penicillin/streptomycin. The prepared medium was used to grow FACS-sorted CHO-S cells in isolated colonies. Briefly, the CHO-S population expressing eGFP was prepared by aseptic FACS sorting and plated in 60 mm Petri dishes at a range of cell densities to achieve a final density of 150 colonies per dish. After plating, single-cell separation was assessed under-inverted microscope. Then, the plates were incubated at 37 °C with 8% CO₂. After 12 days of undisturbed incubation, the clones were selected based on the established picking parameters, including colony size, the distance between clones, and colony compactness. Well-dispersed colonies were picked manually and transferred to individual wells in a 96-well plate pre-filled with the Freestyle CHO expression liquid medium. Clones showing high mAb production in supernatants were subsequently expanded in 24-well plates.

2.9 Trastuzumab Production and Quantification

The high clone producer T1A7 was cultured in triplicate in a batch culture mode, in a 125 mL Erlenmeyer flask containing 30 mL of FreeStyle™ CHO expression medium (Thermo Fisher, Waltham, MA, USA) supplemented with 8 mM glutamine (Invitrogen, Carlsbad, CA, USA), and 1% anti-clumping agent (Invitrogen, Carlsbad, CA, USA). Flasks were inoculated with 1×10^6 viable cells/mL and kept in a humidified incubator shaker at 37 °C with 8% CO₂. Samples were collected every day and the mAb concentration was quantified using a Herceptin Trastuzumab/Anti-HER2 Humanized IgG ELISA Kit (Alpha Diagnostics International, San Antonio, TX, USA).

2.10 Antibody Purification

After 7 days of batch culture, clarification was carried out on the T1A7 clone by centrifuging the cells at 5000 ×g for 15 min, followed by filtering the supernatants. Then, the clarified supernatants were dialyzed with the binding buffer, consisting of 20 mM sodium phosphate, and 0.15 M NaCl, at pH 7.2. Subsequently, protein purification was performed by fast protein liquid chromatography (FPLC) (Cytiva, Uppsala, Sweden). Five column volumes of 20 mM sodium phosphate buffer, at pH 7.2, were used to equilibrate the HiTrap MabSelect SuRe column (GE Healthcare Life Sciences, Danderyd, Sweden) before loading the concentrated supernatants. Then, the column was washed with 10 column volumes of binding buffer to remove any contaminants. Subsequently, elution was conducted using an elution buffer consisting of 0.1 M sodium citrate, at pH 3.3. The collected fractions were analyzed by SDS-PAGE.

2.11 Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified mAb produced from the T1A7 clone was analyzed by SDS-PAGE under reducing conditions. Samples were heated at 95 °C for 5 min and loaded into the gel wells. As a negative control, 10 µL of non-transfected CHO-S cells supernatant was loaded alongside 10 µL of the three purified samples. Coomassie blue staining was performed to visualize the resulting protein bands.

2.12 SEC-HPLC

The protein aggregates were analyzed using an Agilent HPLC system (1260 Infinity II series). For that purpose, an Agilent AdvanceBio size exclusion chromatography (SEC) column was used with the following characteristics: 7.8 × 300 mm, 300 Å pore size, and 2.7 µm particle size. The column was previously equilibrated in PBS 1 × (0.8 mL/min) with the oven set to 30 °C. The isocratic program was set to analyze approximately 5–15 µg of each sample via a 10 µL injection at 0.8 mL/min for 20 min. A series of proteins from the Sigma-Aldrich protein standard mix were used to calibrate and test the SEC columns. The proteins used included ribonuclease A (13,700 Da), ovalbu-

min (44,300 Da), gamma globulins (150,000 Da), and thyroglobulin (670,000 Da). The mass of the mAb secreted by the T1A7 clone was determined with an error of less than 15%. After SEC analysis, 214 and 280 nm chromatograms were extracted from the raw data and analyzed by peak integration.

2.13 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (version 9.2.0 (283), GraphPad Software, Inc., San Diego, CA, USA), with two-tailed *p*-values below 0.05 being considered statistically significant, and a 95% confidence interval (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001). For cell pool generation, a one-way ANOVA test was performed. Normality tests were also performed in order to evaluate the population distribution.

3. Results

3.1 Construction of Trastuzumab Recombinant Vector

The generated Piggy-Trans and Piggy-Tras vectors were obtained from Vector Builder, Englewood Cliffs, NJ, USA. The expression cassette designed to express the full-length trastuzumab (Tras-HC sequence encoding 451 amino acids and the Tras-LC encoding 215 amino acids) was inserted between the 5' ITR and the 3' ITR, thereby flanking the ends of the Piggy-Tras (Fig. 1a(1)). Considering that PB transposons can carry large cargo [26,27], we designed a large expression cassette in which the choice of each element defining the expression cassette was investigated with the goal of achieving high protein expression, including cis-regulatory elements and enhancers [28], CMV promoters, SV40 polyA signal, polyadenylation signal of bovine growth hormone (BGH pA), and Kozak sequences. Other genes enabling plasmid replication and antibiotic resistance in bacteria and mammalian cells were also investigated. Indeed, the Piggy-Trans plasmids included the transposase sequence under the control of the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (GAG) promoter (Fig. 1a(2)). The insertion of the Tras-HC and Tras-LC was evaluated by PCR. Agarose gel electrophoresis (1%) of the Tras-HC and Tras-LC PCR products confirmed the presence of a 1356 bp fragment that encoded Tras-HC and a 648 bp fragment that encoded Tras-LC (Fig. 1b).

3.2 Cell Pool Generation

To generate a stable CHO-S cell line, a puromycin kill curve representing a dose-response experiment was performed to determine the minimum concentration of puromycin that can kill all the cells during a specific period. Twenty-four hours after culturing the non-transfected CHO-S cells, the growth medium was replaced with a selection medium that had been supplemented with a range of puromycin concentrations (20 µg/mL–100 µg/mL). A

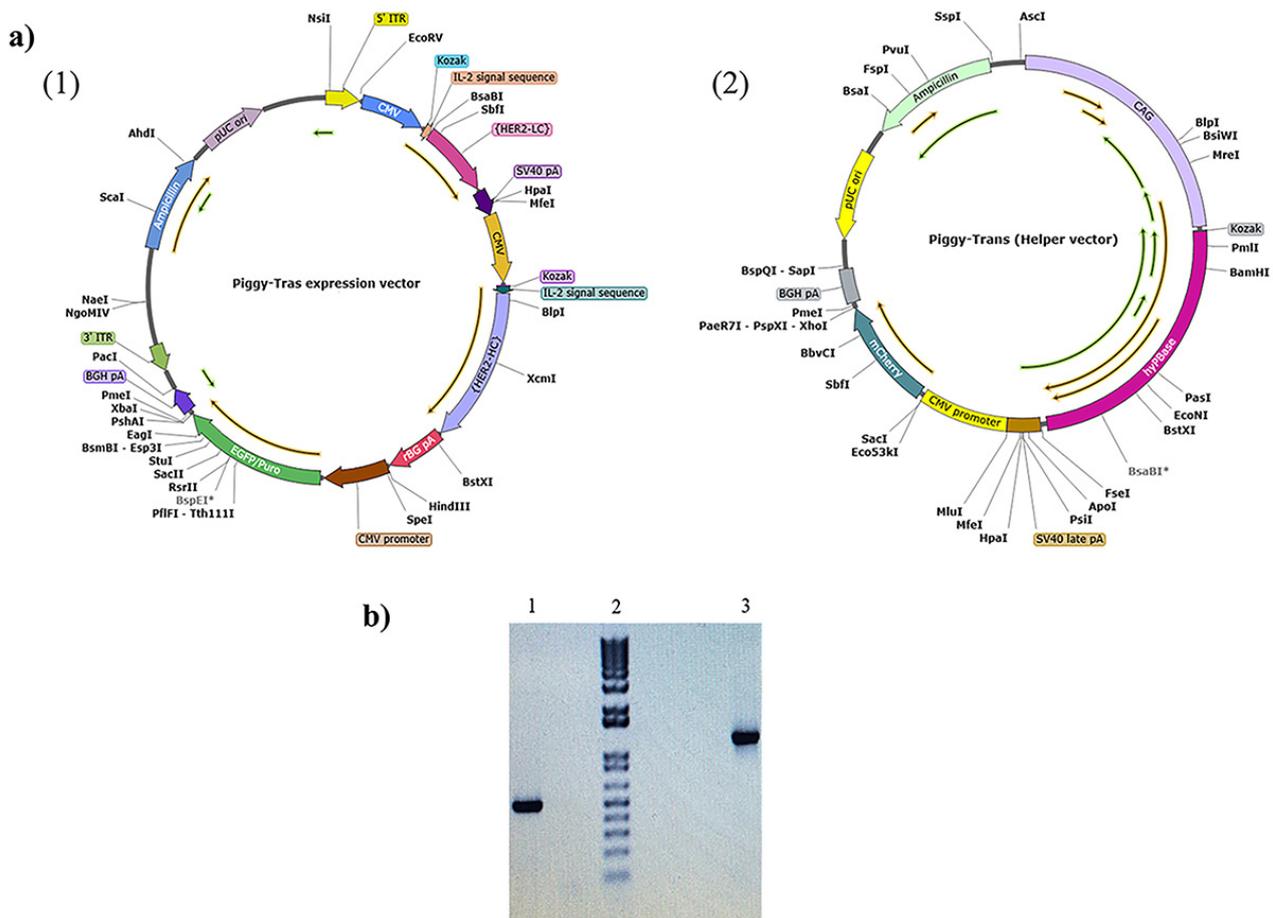


Fig. 1. Representation of the PiggyBac vector maps for Piggy-Tras and Piggy-Tras and agarose gel illustrating Tras-HC and Tras-LC insertion. (a) Piggy-Tras and Piggy-Tras maps. (1) Piggy-Tras vector containing the expression cassette encoding the full-length trastuzumab (Tras-HC and Tras-LC) under the control of the cytomegalovirus promoter (CMV). It also harbors the pressure selection genes (puromycin and ampicillin) and enhanced green fluorescent protein (eGFP). (2) Piggy-Tras encoding the transposase under the control of the enhancer-chicken β -actin hybrid (GAG) promoter. (b) Agarose gel electrophoresis of the Tras-HC and Tras-LC PCR products. The gel confirms the presence of a 1356 bp fragment that encodes Tras-HC (band in column 1) and a 648 bp fragment that encodes Tras-LC (band in column 3). Column 2 corresponds to the ladder.

concentration of 50 $\mu\text{g}/\text{mL}$ was determined as the minimum concentration that would kill all the cells within 9 days (Fig. 2).

The impact of the Piggy-Tras:Piggy-Tras ratio on the generation of a stable CHO-S pool was also investigated. For that purpose, cells were co-transfected in triplicate with three Piggy-Tras/Piggy-Tras ratios (1:3, 1:5, and 1:10) to determine the optimal conditions for efficient insertion of the gene of interest (GOI) into the host cell genome, and to generate an efficient cell pool with a high mAb expression and production [29,30]. The transfection supernatants of the three ratios were collected and the secreted antibodies were evaluated using the human IgG ELISA kit. mAb quantification showed that a 1:3 transfection ratio of Piggy-Tras/Piggy-Tras was optimal in CHO cells to obtain higher titers of secreted antibodies (5.1 $\mu\text{g}/\text{mL}$), compared to 0.58 $\mu\text{g}/\text{mL}$ for the 1:5 ratio and 0.38 $\mu\text{g}/\text{mL}$ for

the 1:10 ratio (Fig. 3). Subsequently, the cells from the 1:3 ratio pool were maintained in the selection medium (50 $\mu\text{g}/\text{mL}$ of puromycin) for one month. The cell viability was constantly monitored during the 1-month generation of stable cells. During the first days of culturing under selective conditions, the proportion of viable cells was very low (12%) compared to the non-transfected cells (88%). Gradually, the cell viability of the transfected cells improved and reached 98% on day 31 (Fig. 4a). Fluorescence microscope showed that most of the CHO-S cells in the stable pool expressed GFP (Fig. 4b(B)), while non-transfected cells (Fig. 4b(A)) show no expression of GFP. In addition, SDS-PAGE, under reducing conditions, showed that the migration of the cell pool supernatant (in triplicate) was characteristic of an antibody, with the HC and LC bands observed at the expected sizes of 50 kDa and 25 kDa, respectively (Fig. 4b(C)).

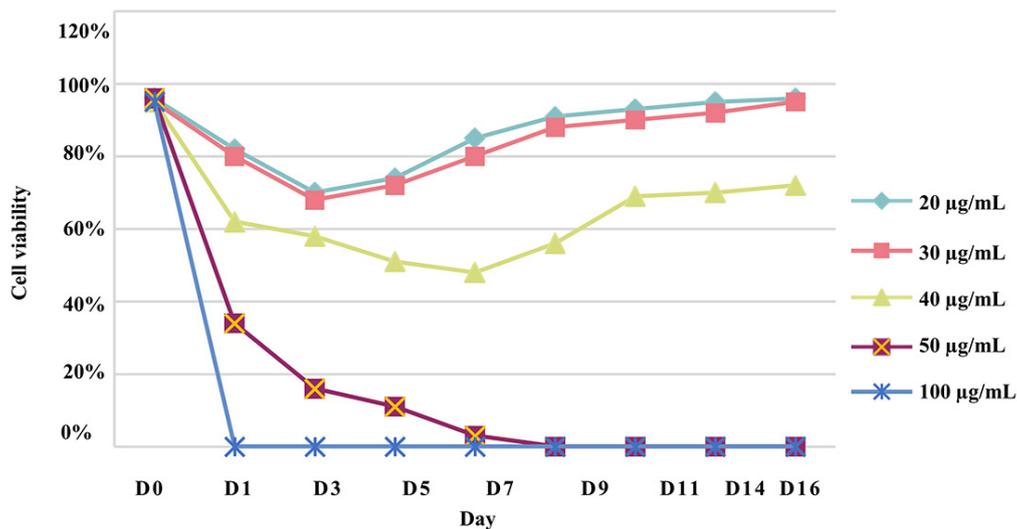


Fig. 2. Chinese hamster ovary (CHO)-S cell puromycin kill curve. To determine the optimal concentration for selecting transfected cells, a puromycin concentration range of 20 µg/mL to 100 µg/mL was used on CHO-S cells for two weeks and performed in triplicate.

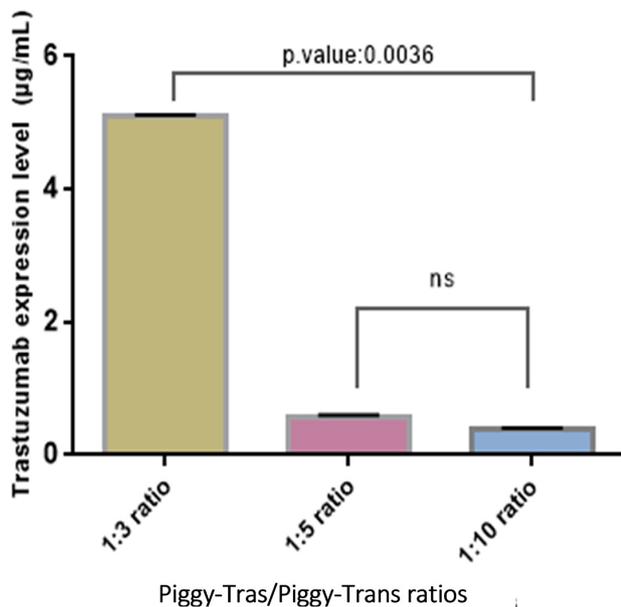


Fig. 3. Impact of Piggy-Trans/Piggy-Tras ratio on antibody expression. Three pools of CHO-S cells were generated (in triplicate) at three different ratios: 1:3, 1:5, and 1:10. Following 4 days of co-transfection, mAb titers were measured using a human IgG enzyme-linked immunosorbent assay (ELISA) kit. Error bars represent the average SD from triplicate measurements. ANOVA (Kruskal–Wallis test) was used to detect statistically none significant (ns) and significant differences between the generated pools.

3.3 Clone Screening Strategy

The strategy of isolating stable CHO-S clones was based on the combination of fluorescence-activated cell sorting (FACS) followed by methylcellulose semi-solid media. The FACS Calibur system was used to purify the stable

cell pool based on the eGFP expression. The eGFP fluorescence was detected in the FL1 channel, and the population of interest was gated to sort the sterile selected CHO-S population (Fig. 5a). The cells were collected for subsequent culturing in 2% MCM. The medium formulation was optimized in-house and included basal 10× MEM, glutamine, vitamins, amino acids, and sodium bicarbonate. The optimized 2% MCM was used for the expansion of the FACS-purified CHO-S population into 60 mm Petri dishes in a humidified 8% CO₂/air mixture at 37 °C. After 12 days of controlled incubation, 1500 clones were selected based on size, compactness, and inter-colony distance (Fig. 5b). Thereafter, 100 clones were picked and expanded. Every single clone was cultured separately in 96-well plates containing Freestyle expression medium for approximately 4 days and observed under an optical microscope. The supernatants were analyzed using the ELISA Herceptin Kit, as shown in Fig. 5c. The three top clones T1A1, T1A7, and T1B7 showed the best antibody expression levels at 26.87 ng/mL, 34.16 ng/mL, and 28.30 ng/mL, respectively.

3.4 Batch Production and Quantification

The T1A7 clone was chosen for the triplicate batch culture investigation. Indeed, 30 mL of Freestyle CHO expression medium was inoculated with the clones seeded at a density of 1×10^6 cells/mL and incubated at 37 °C with 8% CO₂ and shaken at 125 rpm. The cell density and viability of each clone were monitored daily during the 7 days of batch culturing (Fig. 6(1)). The trastuzumab in the T1A7 supernatant was quantified every day during the batch-culture period and was observed to reach its maximum on day 5, with 4.24 g/L of the antibody being secreted (Fig. 6(2)). Then, trastuzumab was purified by FLPC using the HiTrap MabSelect SuRe column as described previously.

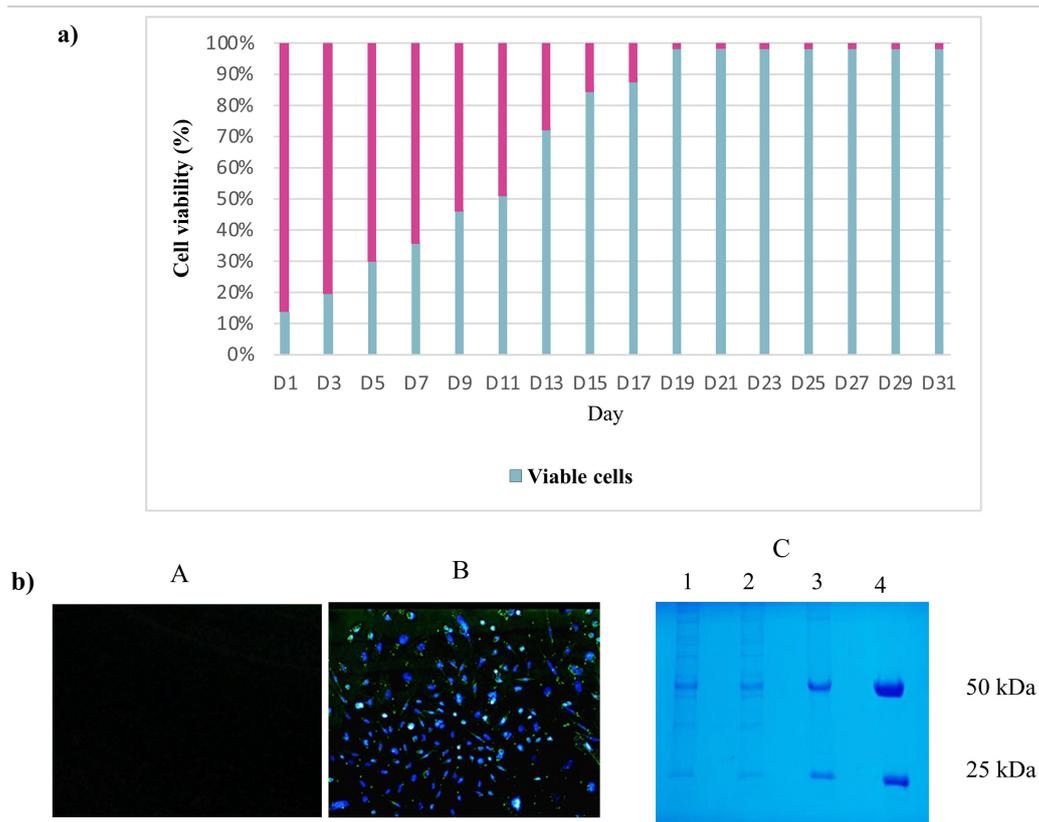


Fig. 4. Fluorescence microscopy and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) evaluation of stable pool generation. (a) Stable cell pool generation: During one month of cell culture under puromycin selection, the CHO-S cell viability was measured. Indeed, the CHO-S cell viability was low (12%) during the first days but increased to 98% by day 23. (b) Fluorescence microscopy and SDS-PAGE evaluation of the stable pool. (A) The supernatant of non-transfected CHO-S cells was visualized by fluorescence microscopy and used as a negative control. (B) The CHO-S stable pool shows the expression of eGFP protein, which is proportional to the expression of the mAb. (C) SDS-PAGE analysis following Coomassie blue staining: migration of the CHO-S stable cell pool supernatants (in triplicate: lane 1, 2, 3) in SDS-PAGE (10%) under reducing conditions showing the expected bands, 50 kDa for Tras-HC and 25 kDa for Tras-LC. Lane 4 refers to the positive control: commercial Herceptin® (Roche, Basel, Switzerland).

3.5 Analysis of Protein Size and Aggregates

The purity and size of Herceptin® (Roche, Basel, Switzerland) and the mAbs secreted by our T1A7 clones were analyzed by SDS-PAGE and SEC-HPLC. Indeed, the molecular size of Herceptin® and the purified mAbs secreted from the T1A7 clones were also analyzed by SDS-PAGE, under reducing conditions, and showed that the protein bands of both molecules matched, while there was also an absence of any other low or high molecular weight protein bands (Fig. 7b).

According to the calibration curve, the purity of the mAbs secreted from the T1A7 clone using SEC-HPLC was 95%, with a retention time of 8.691 min for the main peak, and a mass of 165,000 g/mol. In addition, 3% high molecular weight (HMW) aggregates were present (Fig. 7a), without any low molecular weight (LMW) aggregates.

4. Discussion

A renewed focus on rapid, cost-effective cell line development and predictable product quality attributes is required in the biosimilar market. Traditional stable cell line development involves selective pressure for clone isolation and random integration of the gene of interest (GOI) into the host cell genome, to potentially provide heterogeneity and unstable expression. Therefore, high and sustained antibody expression are certainly two key attributes in the selection of a CHO cell line development strategy. To this end, several studies have recently been conducted on the use of PiggyBac technology in CHO cells for the production of proteins of interest [31–34].

In the present study, we used PiggyBac technology to develop, for the first time in Morocco, a cell line that produces trastuzumab antibodies. This technology specifically targets stable regions of the genome with highly expressed genes. It is based on mobile genetic elements that can be transposed between genomic sites by a “cut and paste”

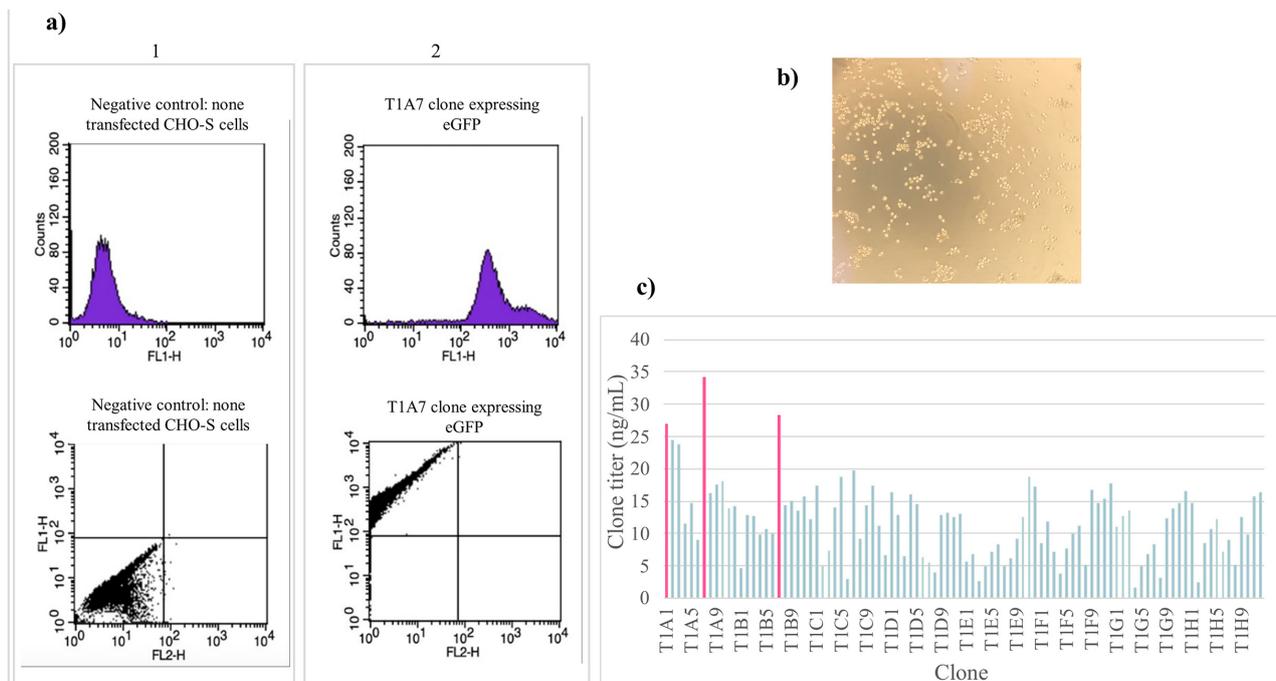


Fig. 5. CHO-S cell sorting and clone generation. (a) Cell sorting analysis was performed on the stable pool of CHO-S cells. GFP fluorescence was detected in the FL1 channel and the population of interest with high eGFP expression was gated for sterile sorting. (b) Following the cell sorting, the cells were cultured in optimized 2% MCM, the figure shows the clones generated after 12 days of incubation in a humidified 8% CO₂ air mixture at 37 °C. (c) The selected clones were transferred to 96-well plates and the mAb expression was determined after 4 days using the ELISA Herceptin Kit.

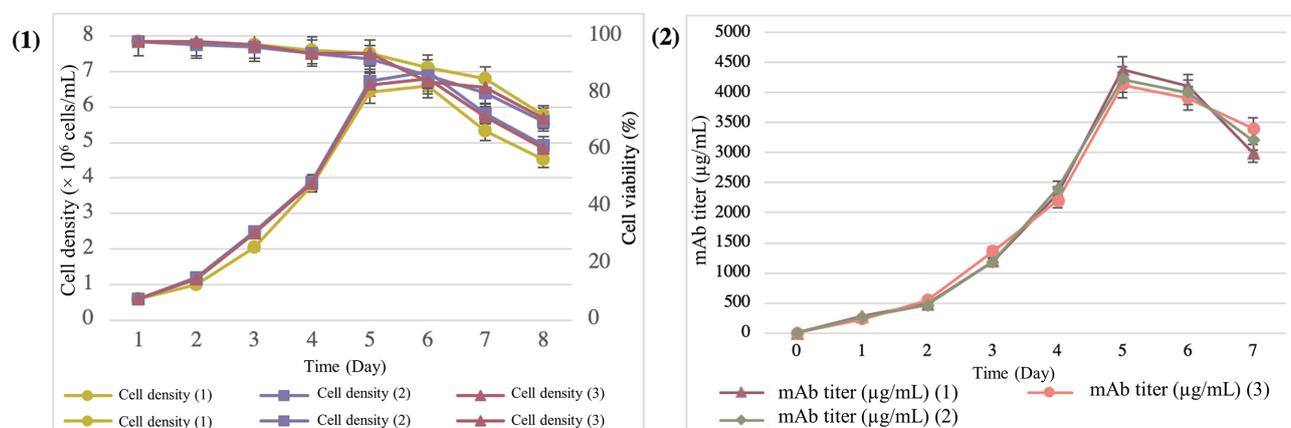


Fig. 6. Expression and quantification of mAbs from the supernatant of the T1A7 clone during the 7-day batch-culture mode. (1) Cell density and viability during the batch-culture mode. (2) Determination of the trastuzumab titer by the ELISA Herceptin Kit.

mechanism, allowing the GOI to integrate into the tetranucleotide TTAA chromosomal sites distributed throughout the CHO cell genome [20,22]. To achieve high levels of antibody expression, vector design requires the selection of promoters, signal peptides, and codon optimization elements. Therefore, the Piggy-Tras possessed the strong CMV promoter used to drive the expression of Tras-HC and Tras-LC. To increase the export of the transcribed messenger RNA (mRNA) from the nucleus to the cytoplasm, an IL-2 intron sequence was inserted upstream of the Tras-

HC and Tras-LC. The IL-2 intron sequence is also responsible for transporting the expressed antibody to the endoplasmic reticulum for folding, assembly, and post-translational modification before being secreted into the extracellular environment. The BGH pA and SV40 late polyadenylation signal sequence was also inserted to promote mRNA polyadenylation and splicing and to maximize mRNA levels. A consensus Kozak sequence was inserted upstream of the first translation initiation codon to enhance the initiation of translation (Fig. 1a) [35]. The transposase generated

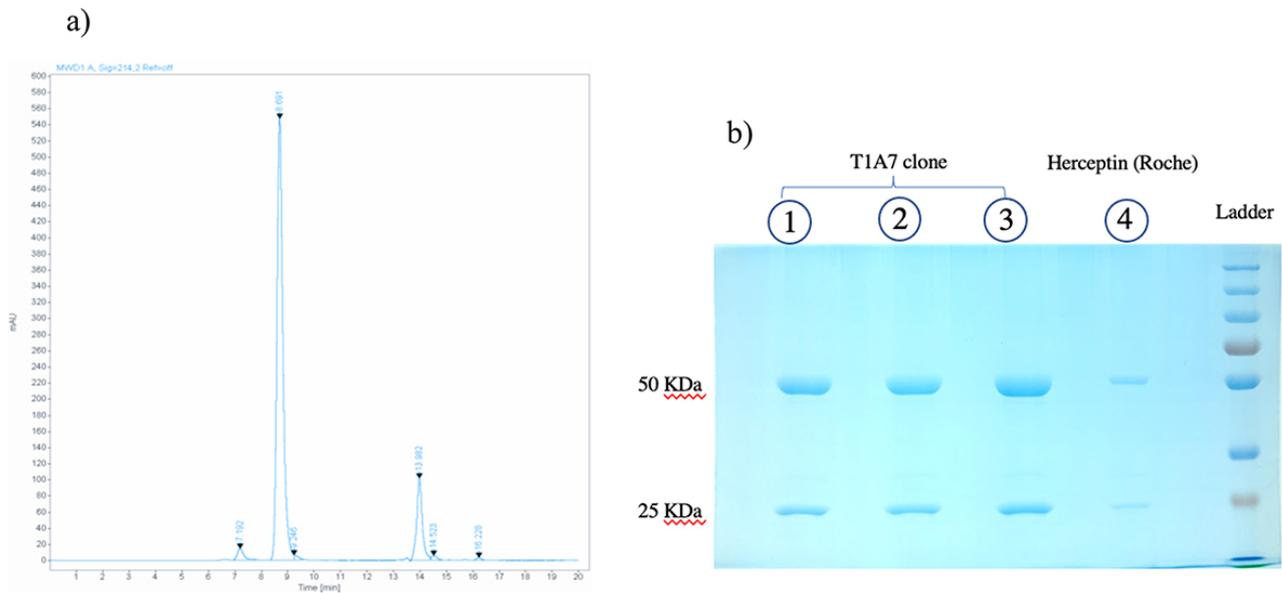


Fig. 7. Size and aggregate analyses. (a) SEC chromatogram obtained from SEC-HPLC showed a main peak with a mass of 165,000 g/mol that was eluted at 8.691 min and a mass of 3000 g/mol that was eluted at 13.982 min. (b) SDS-PAGE analysis of the purified mAbs from the T1A7 clone following Coomassie blue staining: after protein A purification of mAbs from the T1A7 clone supernatant, mAb migration (in triplicate: lane 1, 2, 3) in SDS-PAGE (10%) under reducing conditions showed the expected bands, 50 kDa for Tras-HC and 25 kDa for Tras-LC. Lane 4 refers to the positive control: commercial Herceptin® (Roche).

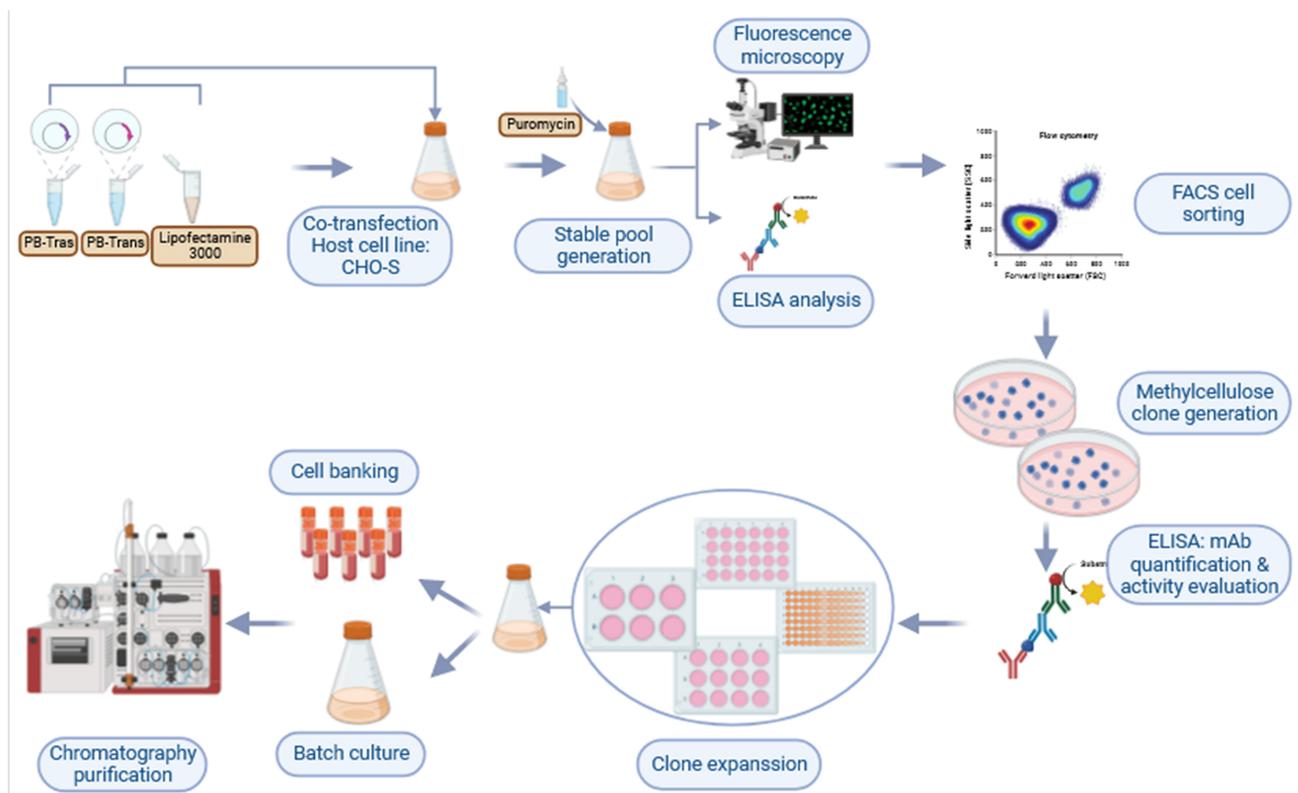


Fig. 8. Graphic representation showing the workflow used to develop a CHO cell line that produces high titers of trastuzumab.

from the Piggy-Tras (Fig. 1a(2)) would recognize the two ITRs on the Piggy-Tras and insert the flanked region into the host genome. Integration typically occurs at sites on the

host chromosome that contain the TTA sequences that are duplicated on both flanks of the integrated cassette [36].

In addition to PB technology, the major goal of this study was to establish a stable CHO-S cell line capable of maintaining expression levels over time. To this end, a stable pool of cells was generated under puromycin pressure selection for one month. Despite the advent of highly efficient transient transfection protocols, generating stable mammalian cell lines remains critical for further investigating mAb functions and structures. Protein production using stable cell lines can be easily scaled up to achieve high product yields [32,37,38]. Fluorescence microscopy and Coomassie gels were used to verify the stability of the generated cell pool. Several studies have demonstrated a tight expression relationship between eGFP and the gene of interest (GOI), which is achieved when the GOI is cloned into a bicistronic vector together with a fluorescent protein [39]. As shown in Fig. 4b(B), the CHO-S cell pool expressed GFP on the 31st day, and the migration of the cell pool supernatant in SDS-PAGE, under reducing conditions, revealed the characteristic antibody bands at the expected sizes—50 kDa for HC and 25 kDa for LC (Fig. 4b(C)).

The isolation and screening strategy for stable CHO-S clones was based on the combination of two sequential steps: fluorescence-activated cell sorting (FACS) followed by a semi-solid methylcellulose medium. FACSCalibur was used to purify the stable cell pool based on eGFP expression (Fig. 5a), while our optimized 2% MCM was used to successfully expand the FACS-purified CHO-S population. Indeed, MCM has the advantage of restricting the movement of individual cells and preventing them from re-aggregating. At the same time, it is soft enough to support the growth of 3D-CHO cells *in vitro* [40].

Once the clones were generated, they were examined under the microscope to confirm that they successfully met the selection parameters (size, compactness, and distance between colonies). Amongst the 100 clones selected, T1A1, T1A7, and T1B7 clones showed the best titers with mAb concentrations of 26.87 ng/mL, 34.16 ng/mL, and 28.30 ng/mL, respectively (Fig. 5c). These results demonstrate that using the above sequential screening workflow, including cell population purification by FACS followed by our optimized MCM (2%) expansion, can provide high assurance of monoclonality.

The T1A7 clone, which produced the highest mAb titer, was selected and seeded for 7 days in a batch culture. In terms of productivity, we observed an average peak cell density of 6.79×10^6 and good overall mAb expression, which reached an average of 4.24 g/L on day 5 (Fig. 6a(2)). However, Z Li *et al.* [41] provided a PB-based expression system for the generation of stably transfected mammalian cell cultures for large-scale protein production. They reported a maximum production of 30 mg/L. In addition, Lao-Gonzalez T *et al.* [42] reported an early screening and selection strategy for trastuzumab expression that combined the use of CHO-K1 pre-adapted to protein-free media and suspension culture with lentiviral vectors. Us-

ing batch-mode cultures, they were able to demonstrate a high titer of mAb, 80.08 mg/L, against the 1B6 clone [41]. In a similar study, Y. Rajendra *et al.* [43] reported the generation of stable pools of CHO cells using a fed-batch process, yielding antibody titers up to 7.6 g/L in the same system, whereas the best PB clone titers ranged from 1.8 to 4.2 g/L. Hence, our approach yielded higher titers, up to 4.24 g/L. This is due to the high efficiency associated with PB-mediated DNA integration, which potentially opens up new applications that have been difficult to achieve with other approaches [41,43]. These applications could be applied to gene therapy, protein expression enhancement, and gene transfer, given the ability to combine PB systems with other technologies [44]. A recent study reported a case of RNA-driven insertion of the PiggyBac genome into human cells by fusing the transposase with the Cas9 endonuclease Dead [45]. Cooney *et al.* [46] explored new *in vivo* gene delivery tools to increase the efficiency of therapeutic gene delivery by combining DNA-PB vectors with the technology used to deliver adenoviral and adeno-associated viruses. PB systems can also be used to genetically modify stem cells to effectively monitor the expression of transgenes during the differentiation process [47].

Regarding protein size and aggregates, we noted the presence of 3% high molecular weight (HMW) aggregates without any low molecular weight (LMW) (Fig. 7a). In a related study, Joshi *et al.* [48] reported the presence of 2.20% HMWS of a biosimilar compared to 0.90% aggregates of the trastuzumab originator molecule. Cation exchange chromatography (CEX) could be applied to increase the mAb purity and eliminate mAb aggregates, especially following the optimization of the NaCl concentration and pH [49]. Indeed, protein aggregation is a common issue that can occur during various stages of bioprocessing, including cell culture, protein A purification, and formulation. Implementing appropriate strategies to control the development process and prevent aggregates would be of critical interest to minimize the risk of immunogenicity. Several studies have reported the need to explore strategies based on process optimization and understanding the aggregation mechanisms to prevent mAb aggregation [50–53].

Ultimately, in addition to the simple and efficient cell line development described herein (Fig. 8), the T1A7 clone is a promising candidate for producing high yields of trastuzumab.

5. Conclusions

The current study was the first attempt in Morocco to develop stable mammalian cells that produce recombinant mAbs. Our first established biotechnology platform allowed us to apply a simple strategy for the rapid development of CHO clones that produce good trastuzumab titers up to 4.24 g/L, which can be highly improved in a future pilot fed-batch process.

Our workflow combines PiggyBac-based vector technology, CHO suspension cell line, GFP expression monitoring, and dual FACS/MCM (2%) selection processes. It can be implemented in R&D laboratories, including in low- and middle-income countries (LMIC) for the development of cost-effective monoclonal antibodies and biosimilars with strict monoclonality assurance in a short and controlled timeframe (Fig. 8).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HAB conceptualized the study. HAB, HS, YC, and MYAF designed the study and secured funding. HB, NB and YA participated in designing & performing the experiments. HB wrote the original draft and final manuscript. HAB reviewed and edited the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Balazs M. Characterization of a novel trastuzumab resistant human breast cancer cell line. *Frontiers in Bioscience*. 2010; E2: 627–640.
- [2] Stecklein SR, Jensen RA, Pal A. Genetic and epigenetic signatures of breast cancer subtypes. *Frontiers in Bioscience-Elite*. 2012; 4: 934–949.
- [3] Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. *MAbs*. 2015; 7: 9–14.
- [4] Lo H. EGFR and her2 signaling in breast cancer brain metastasis. *Frontiers in Bioscience*. 2016; 8: 245–263.
- [5] Barbier L, Declercq P, Simoens S, Neven P, Vulto AG, Huys I. The arrival of biosimilar monoclonal antibodies in oncology: clinical studies for trastuzumab biosimilars. *British Journal of Cancer*. 2019; 121: 199–210.
- [6] Palavecino LA, Rodrigues CR, Bello ML, Vasconcellos AG. Inventive step assessment of top selling monoclonal antibodies in Brazil. *Expert Opinion on Therapeutic Patents*. 2021; 31: 193–202.
- [7] Barrios CH, Reinert T, Werutsky G. Access to high-cost drugs for advanced breast cancer in Latin America, particularly trastuzumab. *Ecancermedicalscience*. 2019; 13.
- [8] Zhang JH, Shan LL, Liang F, Du CY, Li JJ. Strategies and considerations for improving recombinant antibody production and quality in Chinese hamster ovary cells. *Frontiers in Bioengineering and Biotechnology*. 2022; 10: 856049.
- [9] Frenzel A, Hust M, Schirrmann T. Expression of recombinant antibodies. *Frontiers in Immunology*. 2013; 4: 217.
- [10] Kuystermans D, Al-Rubeai M. Mammalian Cell Line Selection Strategies for High-Producers. *Cell Engineering*. 2015; 377: 327–372.
- [11] Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. *Applied Microbiology and Biotechnology*. 2016; 100: 3451–3461.
- [12] Derouazi M, Martinet D, Besuchet Schmutz N, Flaction R, Wicht M, Bertschinger M, *et al*. Genetic characterization of CHO production host DG44 and derivative recombinant cell lines. *Biochemical and Biophysical Research Communications*. 2006; 340: 1069–1077.
- [13] 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*. 2012; 109: 1007–1015.
- [14] West AG, Fraser P. Remote control of gene transcription. *Human Molecular Genetics*. 2005; 14: R101–R111.
- [15] Lai T, Yang Y, Ng SK. Advances in mammalian cell line development technologies for recombinant protein production. *Pharmaceuticals*. 2013; 6: 579–603.
- [16] Frye C, Deshpande R, Estes S, Francissen K, Joly J, Lubiniecki A, *et al*. Industry view on the relative importance of “clonality” of biopharmaceutical-producing cell lines. *Biologicals*. 2016; 44: 117–122.
- [17] Tihanyi B, Nyitray L. Recent advances in CHO cell line development for recombinant protein production. *Drug Discovery Today: Technologies*. 2020; 38: 25–34.
- [18] Huang Y, Li Y, Wang YG, Gu X, Wang Y, Shen BF. An efficient and targeted gene integration system for high-level antibody expression. *Journal of Immunological Methods*. 2007; 322: 28–39.
- [19] Balasubramanian S, Rajendra Y, Baldi L, Hacker DL, Wurm FM. Comparison of three transposons for the generation of highly productive recombinant CHO cell pools and cell lines. *Biotechnology and Bioengineering*. 2016; 113: 1234–1243.
- [20] Matasci M, Baldi L, Hacker DL, Wurm FM. The PiggyBac transposon enhances the frequency of CHO stable cell line generation and yields recombinant lines with superior productivity and stability. *Biotechnology and Bioengineering*. 2011; 108: 2141–2150.
- [21] Alattia J, Matasci M, Dimitrov M, Aeschbach L, Balasubramanian S, Hacker DL, *et al*. Highly efficient production of the Alzheimer’s γ -Secretase integral membrane protease complex by a multi-gene stable integration approach. *Biotechnology and Bioengineering*. 2013; 110: 1995–2005.
- [22] Yusa K. piggyBac transposony. In Chandler M, Gellert M, Lambowitz AM, Rice PA, Sandmeyer SB (ed.) *Mobile DNA III* (pp. 873–890). American Society for Microbiology. 2015.
- [23] Song M, Raphaelli K, Jones ML, Aliabadi-Zadeh K, Leung KM, Crowley D, *et al*. Clonal selection of high producing, stably transfected HEK293 cell lines utilizing modified, high-throughput FACS screening. *Journal of Chemical Technology & Biotechnology*. 2011; 86: 935–941.
- [24] Hou JJC, Hughes BS, Smede M, Leung KM, Levine K, Rigby S,

- et al.* High-throughput ClonePix FL analysis of mAb-expressing clones using the UCOE expression system. *New Biotechnology*. 2014; 31: 214–220.
- [25] Hanania EG, Fieck A, Stevens J, Bodzin LJ, Palsson BØ, Koller MR. Automated in situ measurement of cell-specific antibody secretion and laser-mediated purification for rapid cloning of highly-secreting producers. *Biotechnology and Bioengineering*. 2005; 91: 872–876.
- [26] Li MA, Turner DJ, Ning Z, Yusa K, Liang Q, Eckert S, *et al.* Mobilization of giant piggyBac transposons in the mouse genome. *Nucleic Acids Research*. 2011; 39: e148.
- [27] Troyanovsky B, Bitko V, Pastukh V, Fouty B, Solodushko V. The Functionality of Minimal PiggyBac Transposons in Mammalian Cells. *Molecular Therapy - Nucleic Acids*. 2016; 5: e369.
- [28] Wang XY, Du QJ, Zhang WL, Xu DH, Zhang X, Jia YL, *et al.* Enhanced Transgene Expression by Optimization of Poly A in Transfected CHO Cells. *Frontiers in Bioengineering and Biotechnology*. 2022; 10: 722722.
- [29] Fonager J, Franke-Fayard BM, Adams JH, Ramesar J, Klop O, Khan SM, *et al.* Development of the piggyBac transposable system for *Plasmodium berghei* and its application for random mutagenesis in malaria parasites. *BMC Genomics*. 2011; 12: 155.
- [30] Alessio AP, Fili AE, Garrels W, Forcato DO, Olmos Nicotra MF, Liaudat AC, *et al.* Establishment of cell-based transposon-mediated transgenesis in cattle. *Theriogenology*. 2016; 85: 1297–1311.e2.
- [31] Huang Z, Lee D, Yoon S. Quantitative intracellular flux modeling and applications in biotherapeutic development and production using CHO cell cultures. *Biotechnology and Bioengineering*. 2017; 114: 2717–2728.
- [32] Oller-Salvia B, Kym G, Chin JW. Rapid and Efficient Generation of Stable Antibody–Drug Conjugates via an Encoded Cyclopropene and an Inverse-Electron-Demand Diels–Alder Reaction. *Angewandte Chemie International Edition*. 2018; 57: 2831–2834.
- [33] Lee JS, Kildegaard HF, Lewis NE, Lee GM. Mitigating Clonal Variation in Recombinant Mammalian Cell Lines. *Trends in Biotechnology*. 2019; 37: 931–942.
- [34] Lee M, Rice-Boucher PJ, Collins LT, Wagner E, Aulisa L, Hughes J, *et al.* A Novel Piggyback Strategy for mRNA Delivery Exploiting Adenovirus Entry Biology. *Viruses*. 2022; 14: 2169.
- [35] Ho SC, Tong YW, Yang Y. Generation of monoclonal antibody-producing mammalian cell lines. *Pharmaceutical Bioprocessing*. 2013; 1: 71–87.
- [36] Fueyo R, Judd J, Feschotte C, Wysocka J. Roles of transposable elements in the regulation of mammalian transcription. *Nature Reviews Molecular Cell Biology*. 2022; 23: 481–497.
- [37] Hamaker NK, Lee KH. Site-specific integration ushers in a new era of precise CHO cell line engineering. *Current Opinion in Chemical Engineering*. 2018; 22: 152–160.
- [38] Hunter M, Yuan P, Vavilala D, Fox M. Optimization of Protein Expression in Mammalian Cells. *Current Protocols in Protein Science*. 2019; 95: e77.
- [39] Freimark D, Jérôme V, Freitag R. A GFP-based method facilitates clonal selection of transfected CHO cells. *Biotechnology Journal*. 2010; 5: 24–31.
- [40] Nasatto PL, Pignon F, Silveira JL, Duarte MER, Noseda MD, *et al.* Methylcellulose, a cellulose derivative with original physical properties and extended applications. *Polymers*. 2015; 7: 777–803.
- [41] Li Z, Michael IP, Zhou D, Nagy A, Rini JM. Simple piggyBac transposon-based mammalian cell expression system for inducible protein production. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110: 5004–5009.
- [42] Lao-Gonzalez T, Bueno-Soler A, Duran-Hernandez A, Sosa-Aguir K, Hinojosa-Puerta LE, Hernandez-Garcia T, *et al.* Screening and selection strategy for the establishment of biosimilar to trastuzumab-expressing CHO-K1 cell lines. *AMB Express*. 2022; 11: 1.
- [43] Rajendra Y, Peery RB, Barnard GC. Generation of stable Chinese hamster ovary pools yielding antibody titers of up to 7.6 g/L using the piggyBac transposon system. *Biotechnology Progress*. 2016; 32: 1301–1307.
- [44] Hiltensperger M, Krackhardt AM. Current and future concepts for the generation and application of genetically engineered CAR-T and TCR-T cells. *Frontiers in Immunology*. 2023; 14: 1121030.
- [45] Hew BE, Sato R, Mauro D, Stoytchev I, Owens JB. RNA-guided piggyBac transposition in human cells. *Synthetic Biology*. 2019; 4: ysz018.
- [46] Cooney AL, Singh BK, Sinn PL. Hybrid Nonviral/Viral Vector Systems for Improved piggyBac DNA Transposon in Vivo Delivery. *Molecular Therapy*. 2015; 23: 667–674.
- [47] Park MA, Jung HS, Slukvin I. Genetic Engineering of Human Pluripotent Stem Cells Using PiggyBac Transposon System. *Current Protocols in Stem Cell Biology*. 2018; 47: e63.
- [48] Joshi S, Rathore AS. Assessment of Structural and Functional Comparability of Biosimilar Products: Trastuzumab as a Case Study. *BioDrugs*. 2020; 34: 209–223.
- [49] Scheffel J, Isaksson M, Gomis-Fons J, Schwarz H, Andersson N, Norén B, *et al.* Design of an integrated continuous downstream process for acid-sensitive monoclonal antibodies based on a calcium-dependent Protein a ligand. *Journal of Chromatography a*. 2022; 1664: 462806.
- [50] Li W, Prabakaran P, Chen W, Zhu Z, Feng Y, Dimitrov DS. Antibody Aggregation: Insights from Sequence and Structure. *Antibodies (Basel)*. 2016; 5: 19.
- [51] Pang KT, Yang YS, Zhang W, Ho YS, Sormanni P, Michaels TCT, *et al.* Understanding and controlling the molecular mechanisms of protein aggregation in mAb therapeutics. *Biotechnology Advances*. 2023; 67: 108192.
- [52] Mason DM, Friedensohn S, Weber CR, Jordi C, Wagner B, Meng SM, *et al.* Optimization of therapeutic antibodies by predicting antigen specificity from antibody sequence via deep learning. *Nature Biomedical Engineering*. 2021; 5: 600–612.
- [53] Li F, Lee B, Zhou J, Tressel T, Yang X. Current Therapeutic Antibody Production and Process Optimization. *BioProcessing Journal*. 2006; 5: 16–25.