Screening and selection strategy for the establishment of biosimilar to trastuzumab-expressing CHO-K1 cell lines

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Abstract

The high prices of biopharmaceutics or biologics used in the treatment of many diseases limit the access of patients to these novel therapies. One example is the monoclonal antibody trastuzumab, successfully used for breast cancer treatment. An economic alternative is the generation of biosimilars to these expensive biopharmaceutics. Since antibody therapies may require large doses over a long period of time, robust platforms and strategies for cell line development are essential for the generation of recombinant cell lines in a short period of time with higher levels of expression. Here, we obtain trastuzumab-expressing CHO-K1 cells through a screening and selection strategy that combined the use of host cells pre-adapted to protein free media and suspension culture and lentiviral vectors. The results shown that early screening strategy allowed to obtain recombinant CHO cell populations with higher enrichment of IgG-expressing cells. Moreover, the measurement of intracellular HC polypeptides by flow cytometry was a useful tool to characterize the homogeneity of cell population and our results suggest that might be used to predict the expression levels of monoclonal antibodies in early stages of cell line development process. Furthermore, using T-flask approach the assessment of the expression levels was studied in a setting more similar to that of a final production process. Finally, trastuzumab-expressing CHO-K1 clones were generated, characterized by batch experiments and preliminary results related to HER2-recognition capacity were successful. Further improvements in up-stream and down-stream steps related to this strategy will improve specific productivities and volumetric productivities of these clones.

Introduction

Trastuzumab is a humanized antibody (IgG1 isotype) specific for the human epidermal growth factor receptor 2 (HER2). It was generated by Genentech in a Chinese hamster ovary (CHO) cell line (Dillman, 1999; Heffner et al., 2015; McDonnell, 2015) and is currently commercialized by Roche for the treatment of HER2-positive breast cancer in the adjuvant and metastatic setting, and HER2-positive metastatic gastric or gastroesophageal junction adenocarcinoma (Genentech, 1998). The extraordinary achievements of trastuzumab in clinical setting have made history in the systematic treatment of breast cancer. Unfortunately, it is not uniformly available for routine use owing to its prohibitively high cost (Pivot and Petit, 2018). The expiration of the European Union (EU) patent of trastuzumab in 2014, and the last version of US patent in 2019 (Nelson, 2014), has encouraged the development of biosimilars to this antibody (Curigliano et al., 2016). Price competition in the form of less expensive biosimilars, predicted to be 20%-30% cheaper than the reference product, is expected to have a positive effect in terms of cost-effectiveness and increased availability and accessibility of targeted therapies (Paplomata and Nahta, 2018). In this scenario, the Center of Molecular Immunology (CIM; Havana, Cuba) decided to develop a biosimilar to trastuzumab, to guarantee the access of Cuban patients to this highly cost therapy. To achieve this goal, the generation of trastuzumab-expressing CHO cells was necessary.

The use of CHO cells for therapeutic proteins production has advantages as easier adaptation to grow in serum free or chemically defined media, capability of reaching high cell density in suspension culture (Bandaranayake and Almo, 2014; Kim et al., 2012; Kuystermans and Al-Rubeai, 2015a), among others;

which is very important for scaling up to production processes. Today, the CHO cell line development platforms use cells pre-adapted to grow in these culture conditions to decrease time and efforts to adapt the recombinant CHO cell lines (Jostock, 2011; Kim et al., 2012). On another hand, these cells can be easily transduced using lentiviral vectors (LVs) (Gaillet et al., 2010; Oberbek et al., 2011). This transfection method takes advantage of LV mechanisms for stable integration within the chromosome of the cells, specifically into transcriptionally open chromatin, which makes them excellent tools to obtain cell lines with high expression levels of recombinant proteins (Gödecke et al., 2018; Mursi and Masuda, 2018). In addition, the approach of LVs as gene transfer method is suitable because it allows the adjustment of the ratio of virus/cell (multiplicity of infection, MOI) and, therefore, a cha;nge on transgene copy number and/or the percentage of infected cells (Oberbek et al., 2011; Gödecke et al., 2018).

Once the cells are transfected the next crucial step is to identify desirable variants from the heterogeneous transfectant pool (Priola et al., 2016). In order to accomplish this purpose several methods have been used, from automated cloning techniques as ClonePix FL™ and Cello™ system, to manual techniques as limiting dilution cloning (Kuystermans and Al-Rubeai, 2015b). Moreover, several reports have demonstrated a positive correlation between productivity (volumetric or specific) and levels of mRNA or intracellular light chain (LC) or heavy chain (HC) polypeptides using flow cytometry. Despite the contrasting results on this issue (Borth et al., 1999; Chusainow et al., 2009; Dorai et al., 2006; Edros et al., 2013; Jiang et al., 2006; Lattenmayer et al., 2007; Lee et al., 2009; Park and Ryu, 1994; Roy et al., 2017), it would be interesting to verify whether these variables could predict the expression levels on early stage of the cell line development process; which could enable a decreasing in the time to obtain the recombinant cell lines.

In the present work we generated several trastuzumab-expressing cell lines following a strategy different to that used for obtaining other biosimilar candidates at CIM, which were expressed in both NS0 cells and CHO-K1 cells (unpublished data). In those cases, all the cell line development process and productivity assessments were performed in serum supplemented medium and adherent culture followed by a serum-free suspension adaptation step.

Here, we combined the use of CHO-K1 cells pre-adapted to grow in protein free media and suspension culture, transduction with lentiviral vectors, an early screening step and the early adaptation of recombinant cell lines generated to the afore mentioned conditions. Furthermore, the assessment of the expression levels was studied in a setting more similar to that of a final production process. Additionally, our results suggest that the content of intracellular HC polypeptides measured by flow cytometry might be used to predict the expression levels of monoclonal antibodies in early stages, which would be an advantageous approach to select precursor pool of cells of high producing clones. Biosimilar to trastuzumab-expressing clones were characterized by a growth kinetics study. Finally, the capacity of the recombinant antibody to recognize the HER2 molecule expressed in tumor cell lines was assessed by flow cytometry techniques.

Materials And Methods

Cell lines and cell culture conditions

Human embryonic kidney 293T cells (HEK-293T) were used as packing cell line for the production of LVs. These cells were grown in DMEM/F12 medium (Gibco, USA) with 5% FBS (HyClone, GE Healthcare, USA) (DMEM/F12-FBS) at 37°C in 5% CO_2.

CHO-K1 cells previously adapted to grow in suspension culture and protein free media were grown in CP-CHO medium (Merck, Germany) with 5 mg/L of insulin (Novo Nordisk, Denmark) and supplemented with 3 g/L of HyClone Cell Boost 5 (CB5) (GE Healthcare, USA) (CP-CHO-CB5), a chemically-defined and protein-free medium. The culture conditions were agitation (120 rpm) at 37°C in 5% CO_2.

The medium DMEM/F12-FBS was used for cell transduction and cloning procedures of recombinant CHO cells. Re-adapting of the obtained cell lines was done directly in CP-CHO-CB5 medium or in step-wise approach using CP-CHO-CB5 medium with 1% FBS (CP-CHO-CB5-FBS) and reducing the percent of serum. The drug Geneticin (G418) (Gibco, USA) was used as selection agent at a final concentration of 0.6 mg/mL.

For HER2-recognition assays were used the following tumor cell lines. SKBR3 cells (HER2+, CD20-) were cultured in McCoy’s5A medium (Gibco, USA) with 10 % FBS. SKOV3 cells (HER2+, CD20-) were cultured in DMEM/F12-FBS medium with 10 % FBS. Ramos cells (HER2-, CD20+) were cultured in RPMI medium (Gibco, USA) with 10 % FBS. All the cells were incubated at 37°C in 5% CO_2.

All cell lines used in this work were obtained from ATCC.

Plasmids

The plasmids used in this work correspond to the third generation HIV-1-based LV packaging system and included three helper plasmids (Invitrogen, USA): 1) pLP1 (contains gag and pol genes), 2) pLP2 (contains rev gene) and 3) pLP VSV-G (contains VSV G glycoprotein gene). For trastuzumab expression, LC and HC, carried by different lentiviral plasmids, were expressed by the same human cytomegalovirus promoter (CMV).

Trastuzumab gene sequences were previously obtained from publicly available databases (DrugBank and patent 5,821,337). The plasmid pLW-CMV-trastuzumab-LC (courtesy of Chimeric Protein Group of CIM) bears the gene encoding for trastuzumab LC and there is no selection marker (Fig.1A). To construct plasmid pLV-CMV-trastuzumab HC-IRES-Neo (Fig. 1B), variable region of trastuzumab HC gene was amplified by PCR from pLW-CMV-HCH-IRES-AcGFP1 (courtesy of Chimeric Protein Group of CIM), which bears variable region of HC of trastuzumab, obtaining fragment 1. The forward and reverse oligonucleotide primers were 5´-TACTTAGGATCCCACCATGGAATGCAGC-3´ (THA1) and 5´-TGGGCCCTTGGTGCTAGC-3´ (THA2). In parallel, the constant region of HC gene was amplified by PCR from pFUSEss-CHIg-hG1 (InvivoGen, USA). The forward and reverse oligonucleotide primers were 5´-GCTAGCACCAAGGGCCCA-3´ (THA3) and 5´-GTACAGCTCGAGTCATTTACCCGGAGACAGGGA-3´ (THA4). Finally, fragments 1 and 2 were joined by an overlapping PCR using oligonucleotide primers THA1 and THA4, to obtain the gene encoding for trastuzumab HC. The PCR product was digested with BamHI and XhoI and ligated upstream of an IRES (internal ribosome entry site) sequence in plasmid pLV-CMV-IRES-Neo (courtesy of Chimeric Protein Group of CIM) digested with the same two restriction enzymes. This plasmid bears a gene encoding for neomycin phosphotransferase selection marker (Neo) downstream of an IRES sequence.

Monoclonal antibodies

Trastuzumab (trade name Herceptin), a humanised mAb that recognizes human HER2 molecule, was purchased from Roche (Argentine). A biosimilar to trastuzumab candidate, named 5G4 and obtained from murine NS0 myeloma cells, was provided by Development Department of CIM (Havana, Cuba).

Quantification of human IgG-expression levels by ELISA

The human IgG-expression levels in supernatant were determined by sandwich ELISA. 96-wells plates (High Binding, Costar, USA) were coated with 3 µg/mL of a goat anti-human IgG (γ chain specific) antibody (Sigma-Aldrich, USA) using coating buffer (Na_2CO_3/$NaHCO_3$ 0.1 M, pH 9.6). After a step of incubation at 4°C during 16 hours, the

plates were washed three times with washing buffer (phosphate buffered saline (PBS); Tween 20 at 0.05%, pH 7.5). The samples, diluted in blocking buffer (washing buffer and bovine serum albumin (BSA) at 0.25%), were applied to the plates and incubated at 37°C during 1 hour. Then, the plates were washed three times with washing buffer and an alkaline phosphatase (AP)-conjugated goat anti-human IgG (γ chain specific) antibody (Sigma-Aldrich, USA) was added. After another step of incubation at 37°C during 1 hour, the plates were washed again and substrate was added (5 mg of p-Nitrophenyl phosphate diluted in 5 mL of diethanolamine, pH 9.8). 30 minutes later, the reaction was stopped with NaOH 3M and absorption was measured at 405 nm on a microplate reader (Dialab, Austria). To quantify the expression levels, commercial trastuzumab was used as a standard (standard curve ranges from 3.9 to 125 ng/mL). Samples were analyzed in triplicate.

In addition, another type of sandwich ELISA was used, allowing detection and quantification of a whole molecule of antibodies. In this case, the samples were diluted in a different blocking buffer (washing buffer and FBS at 5%) and it was used a horse-radish peroxidase (HRP)-conjugated goat anti-human kappa light chain antibody (Sigma-Aldrich, USA). The substrate was 5 mg of o-phenylenediamine dihydrochride (OPD) in 10 mL of citrate-phosphate buffer (pH 4.2) and 20 µL of H2O2 at 30%. Absorption was measured at 490 nm on a microplate reader (Dialab, Austria). Samples were analyzed in triplicate.

Production and quantification of LVs

LVs were produced by transfection of HEK-293T using lineal PEI (Sigma-Aldrich, USA) as previously described (Toledo et al., 2009) with some modifications. HEK-293T cells were cultured in a 75cm^2 T-flask in DMEM/F12-FBS medium until cells reached up to 70–80% confluence. The cells were co-transfected with one of the lentiviral transfer plasmids (pLW-CMV-trastuzumab-LC or pLV-CMV-CP trastuzumab-IRES-Neo) and helper plasmids: pLP1, pLP2 and pLP VSV-G at a ratio of 2:1:1:1 (w:w:w:w) for 30 µg of total DNA. Prior to transfection, supernatant was removed, the cells were washed with DMEM/F12 medium and 10 mL of this medium was added. In parallel, a mix of DNA, PEI and DMEM/F12

medium was prepared and added directly to the culture. After 6 hours of incubation at 37°C in the presence of 5% CO_2, 1 mL of FBS was added to the culture and the supernatant was harvested at 72 hours post-transfection. The supernatant was centrifuged at 290 g for 5 min, filtered (0.45 mm membrane) and stored to 4°C for immediate use, or -80°C for long periods of time. The Lenti-X™ Concentrator kit (Clontech, USA) was used to purify the LVs following manufacturer's instructions. The LVs pellet was diluted in 200-600 µL of DMEM/F12 medium and stored at -80°C. An ELISA for detection of HIV p24 capsid protein (DAVIH-Ag p24, LISIDA, Cuba) was used for titration of the concentrated LVs stocks.

Transduction of CHO cells using LVs and generation of trastuzumab-expressing cell pools, mini-pools and oligoclones

Day prior transduction 5 x 10^3 CHO cells, pre-adapted to grow in protein free media and shaking culture, were seeded in 100 µL of DMEM/F12-FBS medium in 96-wells plate and incubated at 37°C in 5% CO_2. After 16 hours, transductions were performed by incubating LVs with cells in a final volume of 100 µL of DMEM/F12 supplemented with 10 µg/mL of polybrene (Sigma-Aldrich, USA). For co-transduction, two different multiplicity of infection (MOI) ratios were used: (400:400) and (600:200) (MOI for LVs bearing LC: MOI for LVs bearing HC) (MOI-LC: MOI-HC). Eight hours post-transduction, the medium was replaced with 100 µL of fresh DMEM/F12-FBS medium and 0.6 mg/mL of G418 (selection medium). A second round of co-transduction was performed in the same conditions as outlined above. The supernatant was harvested at 72 hours post-transduction and human IgG-expression was assessed by ELISA (whole molecule of antibody).

To obtain cell mini-pools, after the second round of transduction, cells were cloned in 96-wells plate (strategy 1 or early screening) (Fig. 2). The cells were seeded in 10 plates at 100 cells/well in 200 µL of DMEM/F12-FBS medium and 0.6 mg/mL of G418. The plates were incubated at 37°C in 5% CO2 during 10 days. The ten cell mini-pools with the highest IgG expression levels, for each co-transduction ratio, were expanded to suspension culture in 25cm^2 T-flasks in 6 mL of CP-CHO-CB5-FBS medium and 0.6 mg/mL of G418. T-flasks were incubated in vertical position at 37°C in 5% CO_2 and shaking culture (120 rpm)

(Infors HT, Switzerland). After 21 days under drug pressure in presence of low content of FBS (1%), cells were cultured in previously mentioned conditions without G418.

To generate cell pools, transduced cells were expanded to 24-wells plate in 600 µL of DMEM/F12-FBS medium and 0.6 mg/mL of G418 (strategy 2) (Fig. 2). The plate was incubated at 37°C in 5% CO_2 until cells reached up to 90% confluence. Then, cells were expanded to suspension culture in 25cm^2 T-flasks in 5 mL of CP-CHO-CB5-FBS medium and 0.6 mg/mL of G418. T-flasks were incubated in vertical position at 37°C in 5% CO_2 and shaking culture (120 rpm) (Infors HT, Switzerland). After 26 days under drug pressure in presence of low content of FBS (1%), cells were cultured as outlined above without G418.

To obtain cell oligoclones, the two cell mini-pools with the highest IgG expression levels were cloned in 96-wells plates. The cells were seeded in ten plates at ten cells/well in 150 µL DMEM/F12-FBS medium and were incubated at 37°C in 5% CO_2. After 10 days, supernatant was removed to quantify human IgG-expression levels by ELISA. The oligoclones with the highest IgG expression levels in this stage, were expanded first to 24-wells plate in CP-CHO-CBS-FBS medium in static culture and later to 25 cm^2 and 75 cm^2 T-flasks in shaking culture.

Limiting dilution cloning

The three oligoclones with the highest IgG expression levels were cloned by limiting dilution in 96-wells plate. The cells were plated in ten plates at 0.5 cells/well in 150 µL of DMEM/F12-FBS medium and were incubated at 37°C in 5% CO_2. At day 10 and 14, the plates were analyzed and those wells with only one colony of cells were marked. The supernatant of these selected wells was harvested in different times, taking into account that culture medium was metabolized, and IgG expression levels were quantified by ELISA. Then, the relation IgG concentration/time of supernatant harvest (ng/(mL*día)) was used to select the clones with the highest IgG expression levels. 52 clones with levels of expression above 180 ng/(mL*day) were expanded, first to 24-wells plate and later to 25 cm^2 T-flasks in CP-CHO-CB5 medium. Although, the cells were cultured directly from a

medium supplemented with FBS 5% to a serum free medium, without an adaptation step; cell viability and growth of clones was not affected negatively, with only few exceptions.

Assessment of IgG expression levels in supernatant in 24-wells plate and shaking T-flask

To assess the IgG expression levels in supernatant in static culture, cells were seeded in 24-wells plates at 0.4 x 10^6 cells/well in 1 mL of CP-CHO-CB5-FBS medium. The experiment was performed in triplicates. Plates were incubated at 37°C in 5% CO_2 and after 9-10 days supernatant was removed to quantify human IgG-expression levels by ELISA.

To evaluate the IgG expression levels in supernatant in shaking culture, cells were seeded in 25 cm^2 T-flasks at 0.4 x 10^6 cells/mL in 10 mL of CP-CHO-CB5 medium. The experiment was performed in duplicates. T-flasks were incubated at 37°C in 5% CO_2 and shaking culture (120) (Infors HT, Switzerland) in vertical position. After 10 days, supernatant was removed to quantify human IgG-expression levels by ELISA.

Assessment of intracellular LC and HC polypeptides content by flow cytometry

The intracellular LC and HC polypeptides content of CHO-K1 cells, wild type or antibody expressing, was measured by flow cytometry. The cells were fixed and permeabilized using ethanol, as previously described (Lee et al., 1993). Intracellular HC and LC polypeptides were stained with a fluorescein-isothiocyanate (FITC)-labeled goat anti-human IgG (γ chain specific) antibody (Sigma-Aldrich, USA) and FITC-labeled goat anti-human kappa light chain antibody (Sigma-Aldrich, USA), respectively. Cells were analyzed on a Gallios flow cytometer (Beckman Coulter, USA) and data were processed with FlowJo 7.6.1 software (Tree Star Inc., USA).

Batch experiments

In order to perform batch experiments, shaking flasks with 60 mL of CP-CHO-CB5 medium (in duplicates) were inoculated with 0.3 x 10^6 cells/mL and incubated at 37°C in 5% CO_2 and shaking culture (120) (Infors HT, Switzerland). Every 24 hours, samples of cell culture

were collected and cell density and viability were determined by the trypan blue exclusion method. Supernatant was harvested to quantify human IgG-expression levels by ELISA (whole antibody detection). The maximum growth rate (μmax) in h^{-1} was calculated as the slope of the following Equation 1:

$$VCD(t) = VCD(t0) + \mu max \times t$$

where VCD(t0) and VCD(t) are viable cell density at times 0 and t in the exponential phase of cell growth, respectively. The specific productivity (q_{IgG}) in pg/cell/day (pcd) was determined in the exponential phase of cell growth according to Equation 2:

$$q_{IgG} = \frac{1}{VCD} \times \frac{d[IgG]}{dt}$$

where VCD(t) is the viable cell concentration at time t (h), concentration of IgG [IgG] in μg/mL. To calculate the time integral of viable cell concentration (IVCC) (∫Xv dt) (10^6 cells*hour/mL) was used trapeze method.

SDS-PAGE and Western Blotting analysis

SDS-PAGE was performed as described (Laemmli, 1970). Supernatant samples; 2.5 μg of commercial trastuzumab (anti-HER2; Roche, Argentine) and 2.5 μg of biosimilar to trastuzumab obtained in NS0 cells (named 5G4) (CIM, Cuba), were prepared under reducing conditions with sample buffer (containing beta-mercaptoethanol) for 1 min at 95°C or under non-reducing conditions (sample buffer without reducing agent) for 10 min at 65°C. In all cases, 15 μL of the sample supernatant were used and IgG concentration was not taken into account. A Color Prestained Protein Standard (NEB, UK) was used as molecular weight marker. Samples and controls under non-reducing conditions were loaded in 7.5% SDS-PAGE gel and for reducing conditions were loaded in 12 % SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes (Whatman, USA) by electric field in semi-humid conditions using a Semiphor Transphor Unit (Pharmacia Biotech, USA).

Antibody detection was carried out with a HRP-conjugated goat anti-human kappa light chain antibody (Sigma-Aldrich, USA) or an AP-conjugated goat anti-human IgG (γ chain specific) antibody (Sigma-Aldrich, USA). A TMB reagent for western blotting (Sigma-Aldrich, USA) and Color development kit (BIO-RAD, USA) were used, respectively, as substrate.

HER2-recognition assay

The recognition of human HER2 molecule by biosimilar to trastuzumab was evaluated by flow cytometry. Tumoral cell lines overexpressing HER2 molecule: SKBR3 and SKOV3 were used. Ramos cells (CD20+) were also used as a negative control of HER2 expression and recognition. Cells were stained with 10 µg/mL of produced antibody contained in supernatants or commercial trastuzumab (anti-HER2; Roche, Argentine) for 30 min at 4°C. A biosimilar to rituximab (anti-CD20) antibody contained in supernatant (10 µg/mL) was added as isotype-matched control. Cells were washed with PBS and the binding of the antibodies was detected by incubation with a FITC-labeled rabbit anti-human IgG antibody (F0315, Dako, USA) for 30 min at 4°C. Cells were analyzed on a Sysmex flow cytometer (Germany) and data were processed with FlowJo 7.6.1 software (Tree Star Inc., USA).

Statistical analysis

The results are expressed as mean ± standard deviation (SD). When necessary, data were analyzed by Student's T test, one-way ANOVA, Tukey test or Pearson correlation using Minitab 16.1.0 software (Minitab Inc., USA). The difference between the means was considered statistically significant at $p<0.05$.

Results

Obtaining trastuzumab-expressing CHO-K1 cell pools and mini-pools

We first obtained the transfer plasmids bearing the genes encoding for heavy chain (HC) and light chain (LC) of Trastuzumab. Gene sequences were obtained from publicly available databases (DrugBank and patent 5,821,337). HC gene was assembled by overlapping PCR

with fragments encoding variable and constant region. Next, it was cloned into the bicistronic vector pLV-CMV-IRES-Neo, which contains neomycin transferase gene for further selection (Fig. 1B). In the case of LC, the variable region gene was cloned into pLW, which bears human C kappa gene and an intron to enhance the expression of gene of interest (Fig. 1A).

Then, we produced LVs in HEK293 cells to transduce CHO-K1 cells previously adapted to grow in protein free media and suspension culture. Taking into account that ratio of LC and HC copy number inside the host cell genome can affects productivity (Chusainow et al., 2009; Jiang et al., 2006; Schlatter et al., 2005); the cells were co-transduced with two different ratios of lentiviral particles: (400:400) and (600:200) (MOI-LC: MOI-HC). The presence of antibody in the supernatants of transfected cells was verified by ELISA (data not shown). Then, two different strategies were followed in parallel (Fig. 2). The strategy 1 was based on early screening, in which recent co-transduced cells were distributed and cultured in 96-well plates under selective conditions during 10 days. This strategy generated 19 cell mini-pools: ten cell mini-pools from co-transduction ratio (400:400) (MOI-LC: MOI-HC) and nine cell mini-pools from co-transduction ratio (600:200) (MOI-LC: MOI-HC). On the other hand, strategy 2 rendered two different cell pools, for each co-transduction ratio. All these producing cells were cultured in suspension and shaking conditions in a protein free medium supplemented with 1% FBS (CP-CHO-CB5-FBS), showing good growth rate and cell viability above 90%, during at least three weeks, even under drug pressure.

We next evaluated the antibody production in such conditions to select the best candidates to be used in further steps of limiting dilution cloning. First, we assessed cell population homogeneity through measurements of intracellular content of LC and HC polypeptides, by flow cytometry. For the analysis, it was used a ratio of mean fluorescence intensity of trastuzumab-expressing cells/ mean fluorescence intensity of CHO-K1 wild type cells ($MFI/MFI_{CHO\text{-}K1}$). The figure 3 shows that all trastuzumab-expressing cells present a homogeneous expression of intracellular LC polypeptides and $MFI/MFI_{CHO\text{-}K1}$ values with a

coefficient of variation of 49.88% (CV=ratio of standard deviation to the mean). In case of intracellular HC polypeptides, heterogeneous expression is appreciated with MFI/MFI_{CHO-K1} values showing a CV of 94.12%. In addition, it can be appreciated that most of cell mini-pools present higher MFI/MFI_{CHO-K1} values related to intracellular HC polypeptides than cell pools (Fig. 3). Therefore, those cell mini-pools populations obtained by using the early screening strategy, are more enriched in trastuzumab-expressing cells.

Previous reports have shown that an excess of intracellular LC polypeptides may improve mAb expression and quality (Ho et al., 2012). Here, we use two different ratios of LVs: (400:400) and (600:200) (MOI-LC: MOI-HC) to transduce cells, one of them with higher MOI values for LV encoding LC. However, we did not observe significant differences in the intracellular expression of LC or HC polypeptides between cell pools and mini-pools that were obtained from cells infected with different ratios of LVs (Fig. 3).

Another approach to choose the best type of trastuzumab-expressing cells (mini-pools or pools) to be used in further experiments, was the measurement of IgG levels in supernatant from cultures with CP-CHO-CB5-FBS medium in 24-wells plate during 10 days. The average level of IgG concentration in supernatant for the 21 cell groups evaluated was around 2 µg/mL and only 7 cases were above this value (Student’s T test). Those producing cells were mini-pools (early screening strategy) and four of them were obtained from (400:400) (MOI-LC:MOI-HC) co-transduction condition (Fig. 4).

Several researchers have reported a correlation between intracellular IgG content with specific productivity (Borth et al., 1999; Park and Ryu, 1994; Roy et al., 2017) or volumetric productivity of antibody producing cell lines (Edros et al., 2013; Roy et al., 2017). Therefore, the measurement of intracellular LC and HC polypeptides could be used to identify early in the selection process the potential of primary transfected cell lines for high productivity (Roy et al., 2017). Here, it was found a positive correlation between MFI/MFI_{CHO-K1} values of intracellular HC polypeptides content and IgG concentration in 24-wells plate of cell mini-pools ($r_P=0.737$ and $p<0.01$) (Fig. 5). In the case of intracellular

LC polypeptides content it was not observed a correlation with the secreted antibody (r_P=-0.259 and p=0.284).

Obtaining trastuzumab-expressing CHO-K1 cell oligoclones and clones

Taking into consideration the highest MFI/MFI_{CHO-K1} values of intracellular HC polypeptides content and IgG concentration in 24-wells plate assay, we selected minipools 5B5 and 6G9 coming from early screening strategy (400:400, MOI-LC: MOI-HC) and adapted to grow in CP-CHO-CB5-FBS, for further cloning steps. In order to enrich the population of antibody producing cells and increase the probability to pick high producing clones, we next performed a cell cloning at 10 cells/well in 96-wells plate.

The 24 oligoclones with the highest IgG expression levels in 96-wells plate, were selected and further cultivated in suspension and shaking culture in CP-CHO-CB5-FBS medium: 6 oligoclones were obtained from parental cell mini-pool 6G9 and 18 from parental cell mini-pool 5B5. Once more, we evaluated cell population homogeneity through measurements of intracellular content of LC and HC polypeptides, by flow cytometry. It was observed that all oligoclones presented a homogeneous expression of intracellular LC polypeptides and less variable MFI/MFI_{CHO-K1} values (CV of 28.22%) while more heterogeneous for intracellular HC polypeptides content (CV of 50.56%) (data not shown). We also measured IgG levels through a 24-wells plate assay in CP-CHO-CB5-FBS medium and static culture (data not shown). The average level of IgG concentration in supernatant was around 7.9 µg/mL and 11 oligoclones were above this value (Student's T test). Due to an assessment of productivity in static culture may not be predictive of behavior in suspension culture and, therefore, the final production process (Porter et al., 2010). It was decided to measure the levels of expression of oligoclones in suspension and shaking culture, before a limiting dilution cloning at low cell density. Seven oligoclones were selected, including the five ones with higher expression levels at 24-wells plate assay, and further adapted to grow directly in CP-CHO-CB5 medium without serum supplementation. Thus, 25 cm^2 T-flasks were used to simulate small-scale bioreactors that could provide more precise information about the expression potential of these producing cells. The highest secreted IgG levels were exhibited

by oligoclones 10B10 (56.1 µg/mL) and 2F3 (51.5 µg/mL) (from parental cell mini-pool 5B5) and 10D3 (54 µg/mL) (originated from parental cell mini-pool 6G9).

After a step of limiting dilution cloning at low cell density (0.5 cells/well) in DMEM/F12-FBS, 52 clones were obtained and re-adapted to grow directly in suspension culture and protein free media. Then, we assessed only intracellular HC polypeptides content, by flow cytometry, because in previous experiments, it showed more variability and, therefore gives more information about cell population homogeneity. Figure 6 shows that some clones presented a homogeneous expression of intracellular HC polypeptides while others, two subpopulations. Afterwards, clones with a homogeneous cell population were selected. This criterion is based on some evidences that point out a relation between a decline in titer of antibody-expressing cell lines and appearance of a second lower expressing population (Dorai et al., 2012; Krebs et al., 2018). 17 clones were selected and their levels of expression in suspension culture and protein free media were assessed using the T-flasks approach previously mentioned. The average level of IgG concentration in supernatant on this experiment was around 62.8 µg/mL and 10 clones were above this value (Student's T test) (Fig. 7).

Batch experiments

From those clones with levels of expression above the average in suspension culture and protein free media in T-flasks we selected 6C8, 1B6 and 5B12 for additional batch experiments using shaking flasks, in the same conditions. Moreover, these cells showed a good morphology after 10 days of culture in shaking T-flaks experiments, while other clones showed macroscopic cell aggregates. In shaking flasks the growth profiles of these clones were very similar, with a cell viability above 90% during 6 days, however cells died abruptly from day 7 to 8 (Fig. 8). Clone 5B12 showed the higher values of viable cell density (VCD) and time integral of viable cell concentration (IVCC), which are related to cell growth; while those parameters were practically the same for clones 6C8 and 1B6. Besides, specific productivity was very similar among the three clones and significant

differences in IgG concentration were observed at the end of the experiment. Table 1 summarizes productivity and growth characteristics of these three clones in batch.

Table 1. Productivity and growth characteristics of selected trastuzumab-expressing CHO-K1 clones in batch experiments (protein free medium and shaking culture).

Clones	VCD_{max} (10^6cell/mL)	IVCC (10^8cell*h/mL)	μ_{max} (h^{-1})	Duration (days)	$[IgG]_{max}$ (µg/mL)	q_{IgG} (pcd)
5B12	7.7	811.59	0.0237	8	58.88±3.55	3.8
6C8	6.925	729.97	0.0247	8	69.01±2.24	3.86
1B6	7.1	723.01	0.0196	8	80.08±3.68	4.05

VCD_{max}: maximum viable cell density. IVCC: time integral of viable cell concentration. μ_{max}: maximum growth rate. : q_{IgG}: specific productivity.

Western Blotting and HER2-recognition assay

The supernatants of the selected clones (1B6, 6C8, 5B12) grown in batch experiments, were used to verify identity by western blotting. In the figure 9C it is shown that in non reducing conditions recombinant antibody exhibited an immunoreactive bands profile similar to that of commercial trastuzumab, indicative of similar molecular weight. Moreover, some bands around 50 kDa were observed, possibly corresponding to LC dimers. The western blotting results in reducing conditions corroborated the expected migration pattern for heavy (Fig. 9A) and light (Fig.9B) chains, corresponding to 25kDa and 50kDa, respectively.

Finally, we evaluated the binding specificity of the produced antibody by flow cytometry. The HER2 binding assay demonstrated that the recombinant antibody contained in supernatants of clones 1B6, 6C8 and 5B12 can recognize HER2 molecule overexpressed in

SKOV3 and SKBR3 cells in a similar way as commercial trastuzumab (Fig. 10). In addition, there was no recognition in the case of Ramos cells (HER2-).

Discussion

This work focused on the generation of biosimilar to trastuzumab-expressing CHO cells through a screening and selection strategy. The recombinant CHO cells were obtained using LVs. One advantage of LVs, is the possibility to influence the transgene copy number and/or the percentage of infected cells by adjusting the ratio of virus/cell (Gödecke et al., 2018; Oberbek et al., 2011). In this way, an increase of MOI values could mean higher copy number of DNA of interest inside the genome of host cells and, therefore, higher levels of expression of recombinant CHO cells. On another hand, it has been reported that ratio of copy number of the genes of LC and HC can affect the productivity of the recombinant cell lines and the quality of the antibody (Chusainow et al., 2009; Ho et al., 2013; Jiang et al., 2006; Schlatter et al., 2005). Moreover, some authors have shown that an excess of LC polypeptides can improve productivity and decrease protein aggregation (Ho et al., 2012; Ho et al., 2013; Lee et al., 2009; Schlatter et al., 2005), so it is possible to improve expression levels tuning gene doses (Jiang et al., 2006). Taking into account both advantages, in this work higher values of MOI and two different ratios of LC: HC were used: (400:400) and (600:200) (MOI-LC:MOI-HC). However, expected levels of expression were not obtained (Fig. 8) and no significant differences in intracellular content of LC and HC polypeptides were observed in cell mini-pools or pools (Fig. 3).

Once cells are transfected, subsequently one or more selection steps are applied to selectively kill cells that did not stably integrate the expression vector in their genome (Agrawal et al., 2012; Hacker and Balasubramanian, 2016; Jostock, 2011). Those selection steps could be either performed on entire transfected population (pools) or on small pools (minipools) of cells distributed in 96-well plates around 100–2000 cells/well (Agrawal et al., 2012; de la Cruz Edmonds et al., 2006; Kober et al., 2013; Noh et al., 2018). Here, both strategies were used and cell populations obtained were assessed taking into account the levels of secreted antibody and the intracellular content of LC and HC polypeptides by flow cytometry. The results shown that early screening strategy (or cell mini-pool approach) allowed to obtain recombinant CHO cell populations with higher enrichment of IgG-expressing cells.

Due to cell line screening is a time and resources-consuming and labor-intensive process, several researching have focused to find methods to identify high-producing clones with good product quality early in the cell line development screening process. Some researchers have found positive correlations between specific productivity and HC mRNA levels in antibody producing CHO cells (Edros et al., 2013; Jiang et al., 2006; Lee et al., 2009) or IgG titers and HC mRNA in myeloma cells (Dorai et al., 2006). However, a disadvantage of these assessments is that they not take into account or provide information on the heterogeneity of the population and only describes the average characteristics of the cells in a cell line. In contrast, the measurement of intracellular HC and LC polypeptides by flow cytometry, offers a direct method of assessing the antibody content of single cells within a cell culture (Roy et al., 2017). On

this way, Edros et al., found an association between specific productivity HC intracellular polypeptides using flow cytometry (Edros et al., 2013). Furthermore, Roy et al., measured intracellular HC polypeptides content to identify the best antibody-expressing NS0 cell clones in early stages of selection process. They observed a positive correlation between intracellular HC polypeptides content and specific productivity, and also final volumetric productivity (Roy et al., 2017). According to these observations, our results shown a positive correlation between MFI/MFI_{CHO-K1} values of intracellular HC polypeptides content and IgG titers in 24-wells plate assay of evaluated cell mini-pools (Fig. 5). Therefore, mentioned results support the measure of intracellular HC polypeptides content by flow cytometry as a useful tool to identify the higher expressing candidates in early stages of process and significantly reduce the timeline and efforts towards final clone selection (Roy et al., 2017).

On another hand, the measure of intracellular polypeptides content is also an effective tool to evaluate the homogeneity and stability of producing cell populations (Dorai et al., 2012; Krebs et al., 2018). Here, we also measured intracellular LC and HC polypeptides contents for IgG-expressing oligoclones and clones (data not shown). The results shown that in the case of intracellular LC content homogeneous and uni-modal distributions were observed, besides all these cells populations (cell pools, mini-pools, oligoclones and clones) presented different expression levels. However, cell pools and mini-pools shown a more heterogeneous distribution of intracellular HC polypeptides content and variable MFI/MFI_{CHO-K1} values compare to cell oligoclones and clones. Those last ones, presented a homogeneous or bi-modal distributions related to intracellular HC polypeptides content (Fig. 3, 6 and data not shown). Several authors have described that many Ig-producing cell lines in nature secrete an excess of LC which is not associated to HC and this have been appreciated in the field of biopharmaceutical industry in the generation of IgG-expressing cell lines (Krebs et al., 2018). This phenomenon can be explained due to glycosylation of HC apparently blocks its transduction (Bergman et al., 1981; Krebs et al., 2018). Therefore, internal measurement of the HC portion of an antibody therapeutic protein proved empirically to be most reliably representative of ultimate intact protein expression levels (Krebs et al., 2018).

Moreover, several authors have found a decreasing in antibody titles during time is associated to arising of secondary cell population with a low intracellular HC polypeptides content and, therefore, with instability of these cell lines expressing recombinant antibodies. So, this methodology could be implemented to identify what cell clones will have a better behavior in the production setting (Dorai et al., 2012; Krebs et al., 2018). Taking into account these antecedents, we used homogeneous distribution as a criterion for selection of cell clones for further analyzes.

The use of CHO-K1 cells pre-adapted to grow in protein free media and suspension culture as cell host, considerable decreased time and efforts needed to adapt the recombinant CHO cells to grow in mentioned conditions (Kim et al., 2012). Indeed, we culture recombinant cells directly to a protein free medium after a step limiting dilution cloning with 5% of FBS and cells shown good growth and cellular viability above 90%. So, a final step of adaptation to protein free media and suspension culture was not needed.

According to Porter et al., a challenge in the selection of cell lines destined for cGMP manufacture is that behavior of a cell line early on in development may not reflect behavior of that cell line in the final production process (Porter et al., 2010). Here, we used shaking 25 cm^2 T-flasks approach to assess a great number of candidates in conditions closer to a final production process and increase the flow work. As 24-wells deep plates, this is less expensive alternative to microbiorrectors to evaluated cell lines in suspension and stirred culture (Mora et al., 2018). Moreover, batch experiments in shaking flasks (erlenmeyers) of producing clones selected from shaking 25 cm^2 T-flasks approach, shown similar values of IgG expression levels compared to 25 cm^2 T-flasks approach.

The higher expression levels reached during shaking 25 cm^2 T-flasks assays or batch experiments are 3 to 4 times lower than higher results reported in literature for recombinant proteins-expressing CHO cells using LVs (Gaillet et al., 2010; Oberbek et al., 2011). Furthermore, a comparison between our batch results from trastuzumab-expressing cell clones and other CHO cell clones reported in literature proves our expression levels are lower (4–10 times) than expected. For example, LeFourn et al. generated several trastuzumab-expressing CHO-K1 cell clones through electroporation, selection with puromycin and limiting dilution cloning. The kinetics features of these clones were studied by fed-batch experiments in mini-bioreactors (50 mL) during 7 days. The maximum IgG concentration was 300–800 µg/mL, average specific productivity was 22.8 pcd and maximum viable cell concentration was $2–8 \times 10^6$ cells/mL (Le Fourn et al., 2014). In spite of results obtained in this work, we believe an increase of trastuzumab-expressing levels is possible through optimization of culture media composition and conditions and parameters of fermentation (Bandaranayake and Almo, 2014; Li et al., 2010; Wurm and de Jesus, 2016). Preliminary results related to HER2-recognition capacity of biosimilar to trastuzumab candidate were successful, besides all the procedures and culture media changes implemented during the generation of the trastuzumab-expressing cell lines adapted to grow in protein free media and suspension culture. Nevertheless, further experiments to assess antibody dependent cell-mediated cytotoxicity (ADCC) activity must be done, as well an extensive analytical characterization (Hutterer, 2019; Kim, 2017).

In conclusion, trastuzumab-expressing CHO-K1 cell lines were obtained through a strategy which combined LVs, CHO-K1 cells pre-adapted to protein free media and suspension culture, shaking 25 cm^2 T-flasks approach and assessment of intracellular heavy chain polypeptides by flow cytometry and its potential as early stage-productivity predictor. This strategy is very useful when low throughput techniques as limiting dilution cloning are used to select high producing cells and there is no access to automatized technology as CellCelector™ and ClonePix FL™ or even flow cytometric cell sorter instruments or micro-bioreactors. Indeed, IgG-expressing CHO-K1 cells, adapted to protein free media and suspension culture, would be generated in 2.5-4 months. Therefore, this strategy can be used as a platform for obtaining another biosimilars to monoclonal antibodies in CHO cells. Besides low expression levels of trastuzumab-expressing CHO-K1 cells generated in this work, we believe if there is an improvement in up-stream (tricistronic vectors, UCOEs, MARs, codon optimization, signal peptide optimization, amplification/selection systems, transfection/transduction procedures) and down-stream

steps (media composition optimization) related to this strategy, specific productivities around 20–30 pcd and volumetric productivities above 10 g/L would be reached.

Abbreviations

CHO
Chinese hamster ovary
HC
heavy chain
HER2
human epidermal growth factor receptor 2
IVCC
time integral of viable cell concentration
LC
light chain
LVs
lentiviral vectors
MFI
mean fluorescence intensity
MOI
multiplicity of infection
q_{IgG}
specific productivity.
VCD_{max}
maximum viable cell density
μ_{max}
maximum growth rate

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Thailin Lao Gonzalez: the conception and design of the work, acquisition of data, analysis and interpretation of data, writing and revising the article critically for important intellectual content, final approval of the version to be submitted.

Alexi Bueno Soler: acquisition of data, analysis and interpretation of data

Arnelys Duran Hernandez: acquisition of data, analysis and interpretation of data

Luis Eduardo Hinojosa Puertas: acquisition of data, analysis and interpretation of data

Katya Sosa Aguiar: acquisition of data

Tays Hernandez Garcia, Kathya Rashida de la Luz Hernandez, Julio Palacios Oliva, Tammy Boggiano Ayo: the conception and design of the work, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be submitted.

All authors read and approved the submitted version.

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Endnotes

Not applicable.

References

Agrawal V, Slivac I, Perret S, Bisson L, St-Laurent G, Murad Y, Zhang J, Durocher Y (2012) Stable expression of chimeric heavy chain antibodies in CHO cells. In: Saerens D, Muyldermans (eds) Single Domain Antibodies: Methods and Protocols, Methods in Molecular Biology, vol 911. Springer, New York, p 287-303.

Bandaranayake A D, Almo S C (2014) Recent advances in mammalian protein production. FEBS letters 588: 253-260.

Bergman L, Harris E, Kuehl W (1981) Glycosylation causes an apparent block in translation of immunoglobulin heavy chain. J Biol Chem 256: 701-706.

Borth N, Strutzenberger K, Kunert R, Steinfellner W, Katinger H (1999) Analysis of changes during subclone development and ageing of human antibody-producing heterohybridoma cells by northern blot and flow cytometry. J Biotech 67: 57-66.

Curigliano G, O'Connor D P, Rosenberg J A, Jacobs I (2016) Biosimilars: extrapolation for oncology. Critical reviews in oncology/hematology 104: 131-137.

Chusainow J, Yang Y S, Yeo J H, Toh P C, Asvadi P, Wong N. S, Yap M G (2009) A study of monoclonal antibody-producing CHO cell lines: What makes a stable high producer? Biotech and Bioeng 102: 1182-1196.

de la Cruz Edmonds M C, Tellers M, Chan C, Salmon P, Robinson D K, Markusen J (2006) Development of transfection and high-producer screening protocols for the CHOK1SV cell system. Mol biotech 34: 179-190.

Dillman R O (1999) Perceptions of Herceptin®: A monoclonal antibody for the treatment of breast cancer. Cancer biother & radiopharm 14: 5-10.

Dorai H, Corisdeo S, Ellis D, Kinney C, Chomo M, Hawley-Nelson P, Moore G, Betenbaugh M J, Ganguly S (2012) Early prediction of instability of chinese hamster ovary cell lines expressing recombinant antibodies and antibody-fusion proteins. Biotech and bioeng 109: 1016-1030.

Dorai H, Csirke B, Scallon B, Ganguly S (2006) Correlation of heavy and light chain mRNA copy numbers to antibody productivity in mouse myeloma production cell lines. Hybridoma 25: 1-9.

Edros R Z, McDonnell S, Al-Rubeai M (2013) Using molecular markers to characterize productivity in Chinese hamster ovary cell lines. PLoS One 8: e75935.

Gaillet B, Gilbert R, Broussau S, Pilotte A, Malenfant F, Mullick A, Garnier A, Massie B (2010) High-level recombinant protein production in CHO cells using lentiviral vectors and the cumate gene-switch. Biotech and bioeng 106: 203-215.

Genentech (1998). Herceptin prescribing.

Gödecke N, Hauser H, Wirth D (2018) Stable Expression by Lentiviral Transduction of Cells. In: Hacker D (ed) Recombinant Protein Expression in Mammalian Cells. Methods and Protocols. Springer, New York, p 43-55.

Hacker D L, Balasubramanian S (2016) Recombinant protein production from stable mammalian cell lines and pools. Curr Opin Struct Biol 38: 129-136.

Heffner K, Kaas C S, Kumar A, Baycin-Hizal D, Betenbaugh M (2015) Proteomics in Cell Culture: From Genomics to Combined 'Omics for Cell Line Engineering and Bioprocess Development. In Animal Cell Culture. Springer, New York, p 591-614.

Ho S C, Bardor M, Feng H, Tong Y W, Song Z, Yap M G, Yang Y (2012) IRES-mediated Tricistronic vectors for enhancing generation of high monoclonal antibody expressing CHO cell lines. J Biotech 157: 130-139.

Ho S C, Koh E Y, van Beers M, Mueller M, Wan C, Teo G (2013) Control of IgG LC: HC ratio in stably transfected CHO cells and study of the impact on expression, aggregation, glycosylation and conformational stability. J Biotech 165: 157-166.

Hutterer K M, Polozova A, Kuhns S, McBride H J, Cao X; Liu J (2019) Assessing Analytical and Functional Similarity of Proposed Amgen Biosimilar ABP 980 to Trastuzumab. BioDrugs. DOI: 10.1080/19420862.2017.1305530.

Jiang Z, Huang Y, Sharfstein S T (2006) Regulation of Recombinant Monoclonal Antibody Production in Chinese Hamster Ovary Cells: A Comparative Study of Gene Copy Number, mRNA Level, and Protein Expression. Biotechn Prog 22: 313-318.

Jostock T (2011) Expression of antibody in mammalian cells. In: Al-Rubeai M (ed) Antibody Expression and Production. Cell Engineering, vol 7. Springer, p 1-24.

Kim J Y, Kim Y G, Lee G M (2012) CHO cells in biotechnology for production of recombinant proteins: current state and further potential. App Microbiol Biotech 93: 917-930.

Kim S, Song J, Park S, Ham S, Paek K, Kang M, Chae Y, Seo H, Kim H, Flores M (2017) Drifts in ADCC-related quality attributes of Herceptin®: Impact on development of a trastuzumab biosimilar. mAbs 9: 704-714.

Kober L, Zehe C, Bode J (2013) Optimized signal peptides for the development of high expressing CHO cell lines. Biotech and Bioeng 110: 1164-1173.

Krebs L E, Bowden D M, Bray C M, Shaw M M, Frye C C (2018) Effective and efficient characterization of Chinese hamster ovary production cell lines using automated intracellular staining and statistical modeling. Biotechn Prog 34: 570-583.

Kuystermans D, Al-Rubeai M (2015a) Biopharmaceutical products from animal cell culture. In: Al-Rubeai M (ed) Animal Cell Culture, vol 9. Springer, New York, p 717-757.

Kuystermans D, Al-Rubeai M (2015b) Mammalian cell line selection strategies for high-producers. In: Al-Rubeai M (ed) Animal Cell Culture, vol 9. Springer, New York, p 327-372.

Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lattenmayer C, Trummer E, Schriebl K, Vorauer-Uhl K, Mueller D, Katinger H, Kunert R (2007) Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters. J Biotech 128: 716-725.

Le Fourn V, Girod P A, Buceta M, Regamey A, Mermod N (2014) CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. Metabolic Engin 21: 91-102.

Lee C J, Seth G, Tsukuda J, Hamilton R W (2009) A clone screening method using mRNA levels to determine specific productivity and product quality for monoclonal antibodies. Biotech and Bioeng 102: 1107-1118.

Lee G M C, Alice S, Palsson B (1993) Effect of fixation temperature on flow cytometric measurement of intracellular antibody content of hybridomas during batch culture. Biotech Techn 7: 271-276.

Li F, Vijayasankaran N, Shen A, Kiss R, Amanullah A (2010) Cell culture processes in monoclonal antibody production. mAbs 2: 466-479.

McDonnell, S. (2015). Production of antibodies in hybridoma and non-hybridoma cell lines. In: Al-Rubeai M (ed) Animal Cell Culture, vol 9. Springer, New York, p 65-88.

Mora A, Zhang S, Carson G, Nabiswa B, Hossler P, Yoon S (2018) Sustaining an efficient and effective CHO cell line development platform by incorporation of 24-deep well plate screening and multivariate analysis. Biotech Prog 34: 175-186.

Mursi, I. F. A., and Masuda, S. (2018). The Production of Recombinant Proteins from Mammalian Cells Using RNA Element. In: Masuda S, Izawa S (eds) Applied RNA Bioscience. Springer, Singapore, p 131-150.

Springer Nature Singapore Pte Ltd. 2018

S. Masuda, S. Izawa (eds.), Applied RNA Bioscience

Nelson K G, PC (2014) Biosimilars lining up to compete with Herceptin–opportunity knocks. Expert Opin Ther Pat 24: 1149-1153.

Noh S M, Shin S, Lee G M (2018) Comprehensive characterization of glutamine synthetase-mediated selection for establishment of recombinant CHO cells producing monoclonal antibodies. Scientific reports 8: 1-11.

Oberbek A, Matasci M, Hacker D L, Wurm F M (2011) Generation of stable, high-producing cho cell lines by lentiviral vector-mediated gene transfer in serum-free suspension culture. Biotech and Bioeng 108: 600-610.

Paplomata E, Nahta R (2018) ABP 980: promising trastuzumab biosimilar for HER2-positive breast cancer. Expert Opin Biol therapy 18: 335-341.

Park S H, Ryu D D (1994) Cell cycle kinetics and monoclonal antibody productivity of hybridoma cells during perfusion culture. Biotech and Bioeng 44: 361-367.

Pivot X, Petit T (2018) Can we establish a hierarchy among trastuzumab biosimilar candidates? British J Cancer 119:263–265.

Porter A J, Racher A J, Preziosi R, Dickson A J (2010) Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: improving the efficiency of cell line generation. Biotech Prog 26: 1455-1464.

Priola J J, Calzadilla N, Baumann M, Borth N, Tate C G, Betenbaugh M J (2016) High-throughput screening and selection of mammalian cells for enhanced protein production. Biotech J 11: 853-865.

Roy G, Miro-Quesada G, Zhuang L, Martin T, Zhu J, Wu H, Marelli M, Bowen M A (2017) Sequential screening by ClonePix FL and intracellular staining facilitate isolation of high producer cell lines for monoclonal antibody manufacturing. J Immunol Methods 451: 100-110.

Schlatter S, Stansfield S H, Dinnis D M, Racher A J, Birch J R, James D C (2005) On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotech Prog 21: 122-133.

Toledo J R, Prieto Y, Oramas N, Sánchez O (2009) Polyethylenimine-based transfection method as a simple and effective way to produce recombinant lentiviral vectors. App Biochem Biotech 157: 538-544.

Wurm F M, de Jesus M (2016) Manufacture of Recombinant Therapeutic Proteins Using Chinese Hamster Ovary Cells in Large-Scale Bioreactors. History, Methods, and Perspectives. In: Liu Ch, Morrow J (eds) Biosimilars of Monoclonal Antibodies: A Practical Guide to Manufacturing, Preclinical, and Clinical Development, John Wiley & Sons, Inc., p 327-353.

Figures

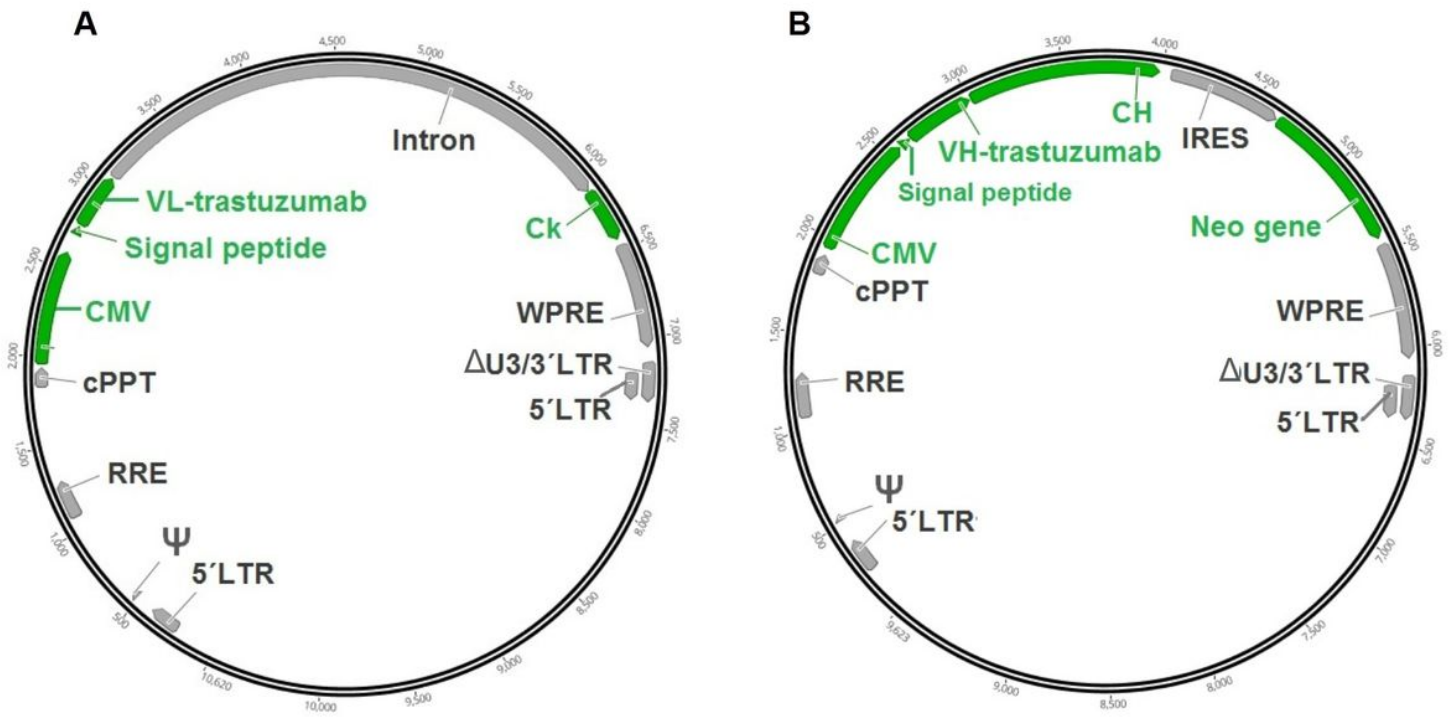


Figure 1

Schematic diagram of the LV transfer plasmids used. (A) pLW-CMV-trastuzumab-LC and (B) pLV-CMV-trastuzumab HC-IRES-Neo, encoding light chain (LC) and heavy chain (HC) of trastuzumab, respectively. 5´LTR: HIV-1 truncated 5' long terminal repeat. Ψ: HIV-1 psi packaging signal. RRE: Rev response element. cPPT: central polypurine tract. CMV: human cytomegalovirus promoter. VL: variable region of LC. Ck: constant region of LC (kappa). VH: variable region of HC. CH: constant region of HC. IRES: ribosome entry site. Neo gene: neomycin phosphotransferase gene. WPRE: woodchuck hepatitis virus posttranscriptional regulatory element. ΔU3/3´LTR: HIV-1 truncated 3' long terminal repeat.

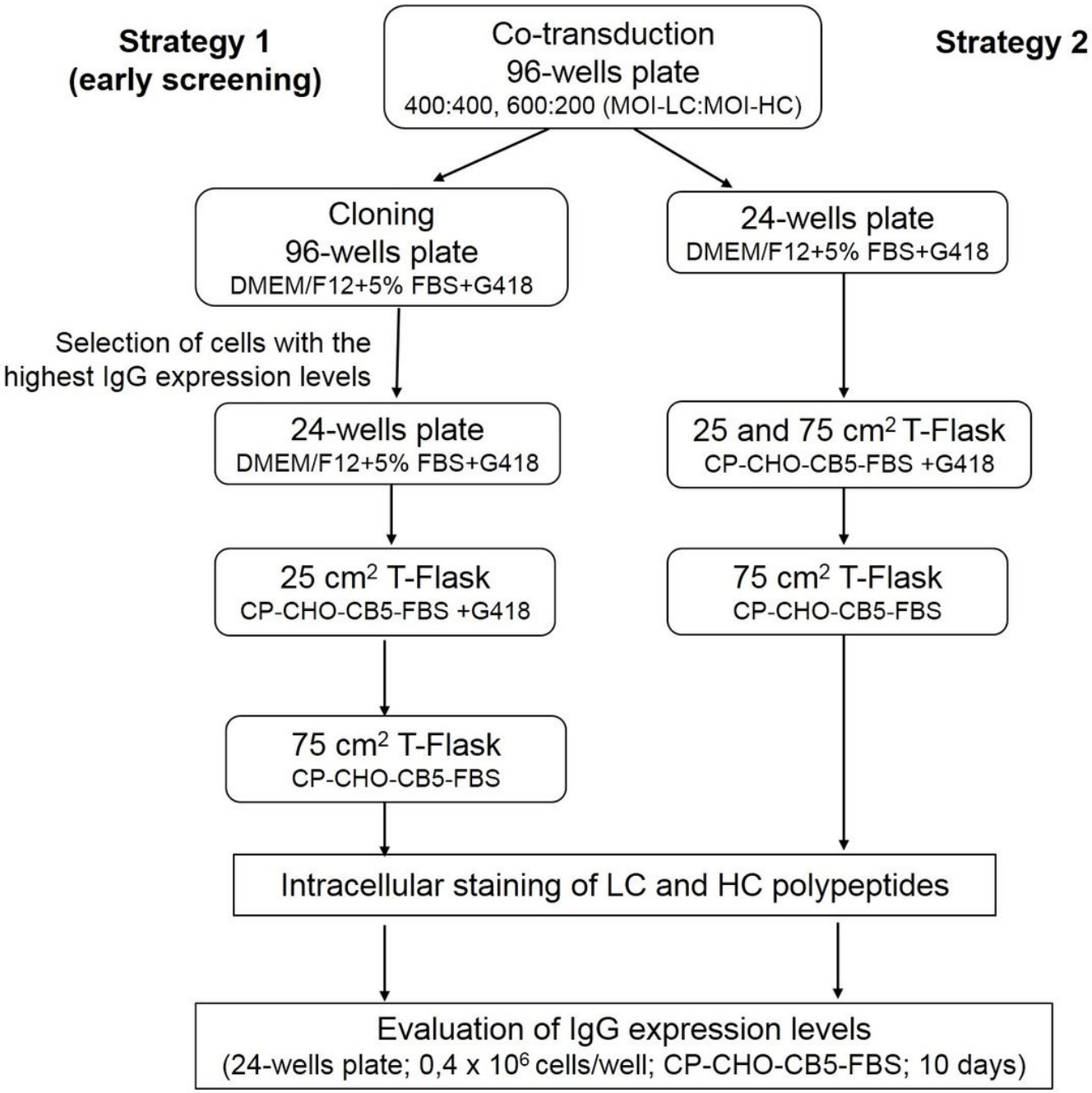


Figure 2

Strategies for obtaining trastuzumab-expressing CHO-K1 cells. FBS: fetal bovine serum. CP-CHO-CB5-FBS: protein free medium CP-CHO supplemented with 3 g/L of CB5 and 1% FBS. LC: light chain. HC:

heavy chain.

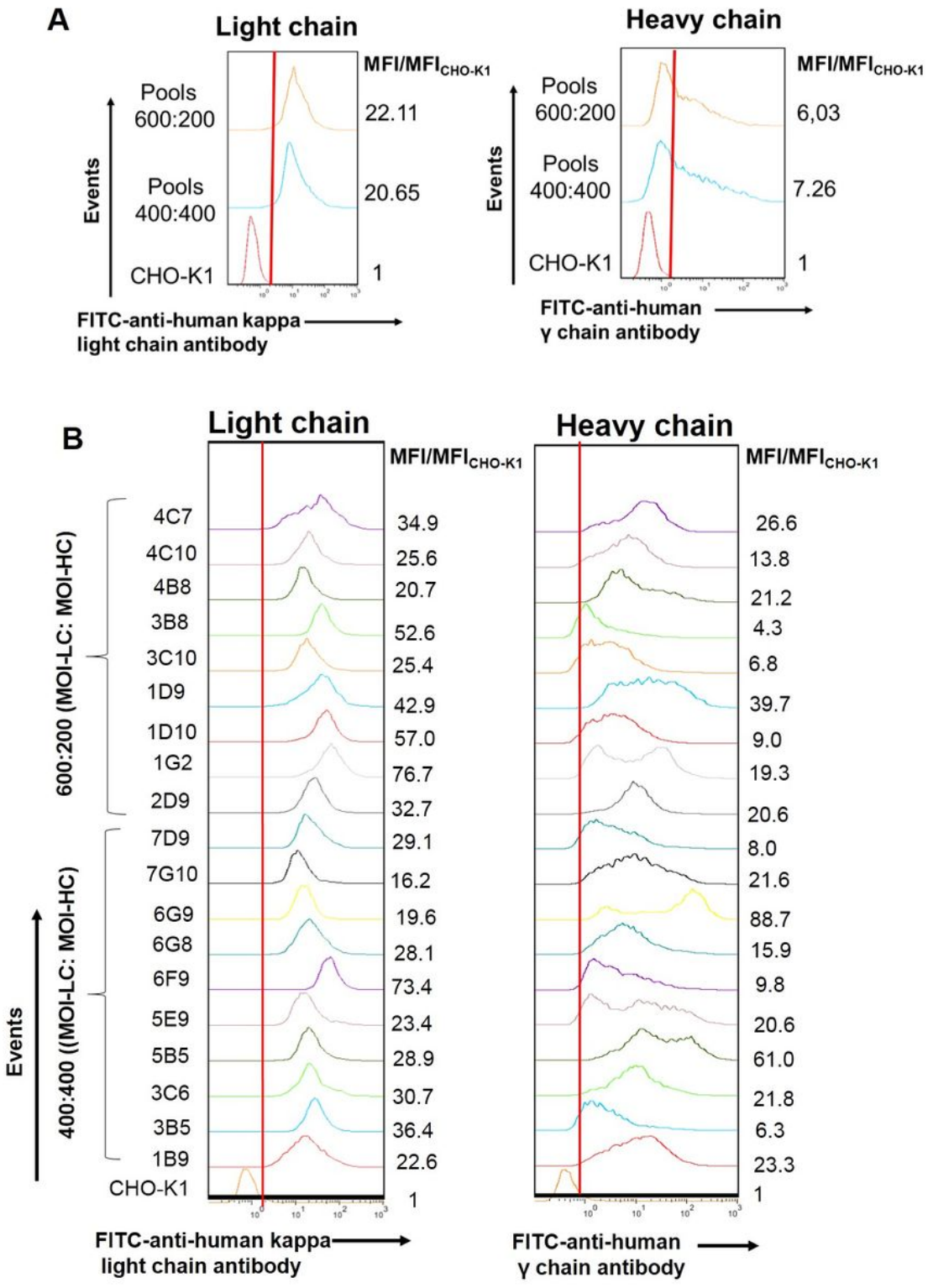


Figure 3

Assessment of intracellular light and heavy chain polypeptides content by flow cytometry. A: cell pools. B: cell mini-pools. Intracellular light and heavy chains polypeptides of ethanol fixed cells were measured using a FITC-labeled goat anti-human kappa light chain antibody and a FITC-labeled goat anti-human IgG

(γ chain specific) antibody, respectively. MFI/MFICHO-K1: relation of mean fluorescence intensity of trastuzmab-expressing cells/ mean fluorescence intensity of CHO-K1 wild type cells.

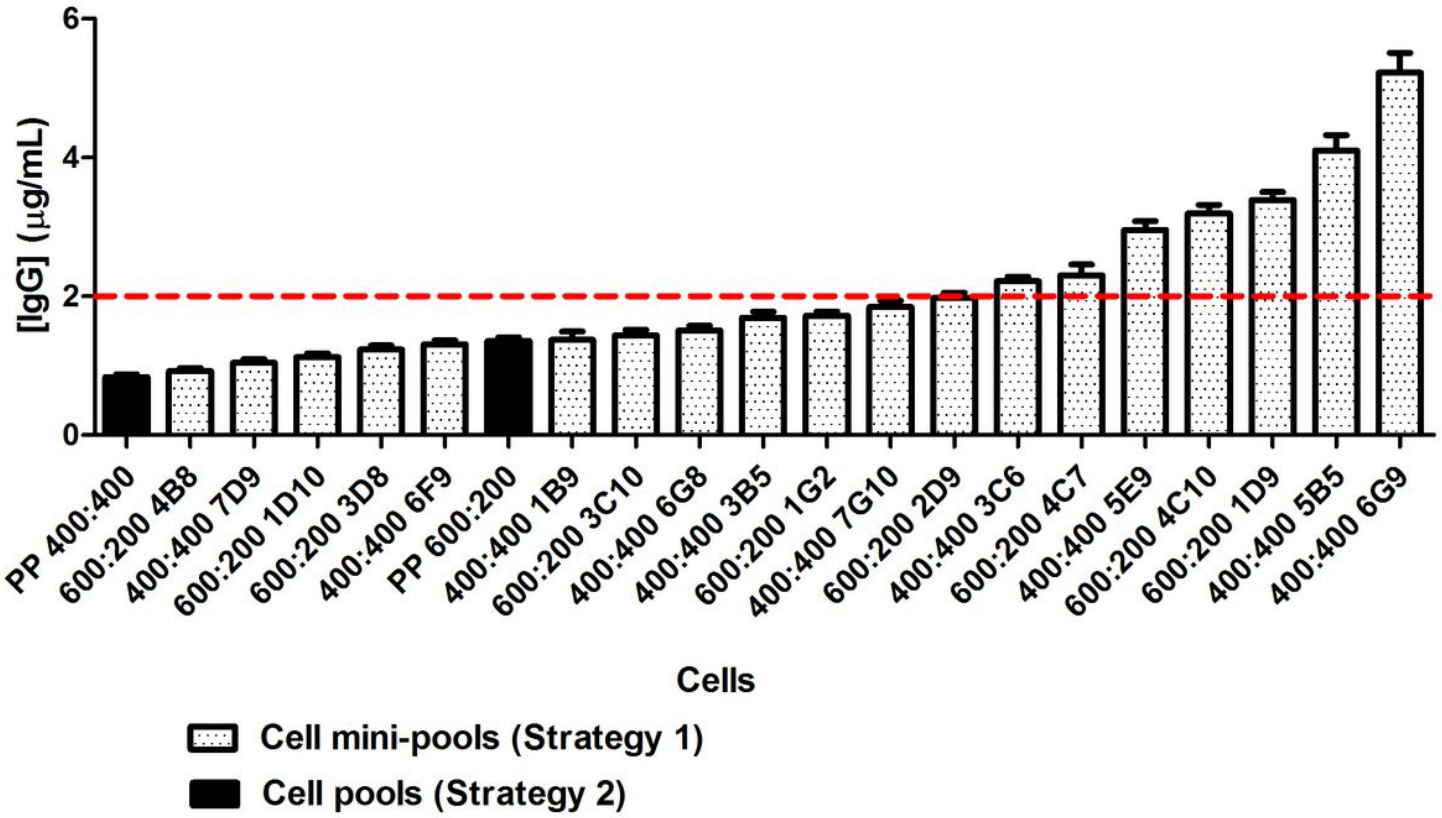
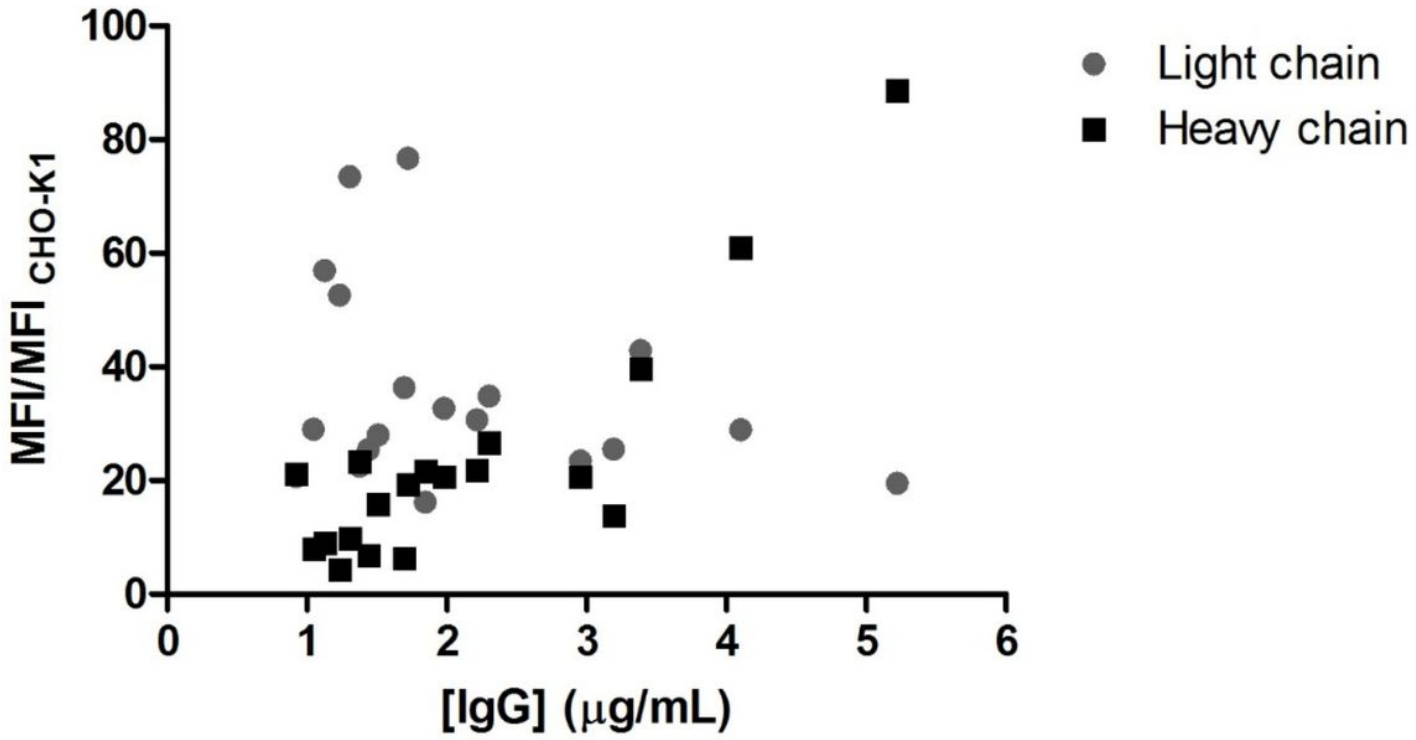


Figure 4

Evaluation of IgG expression levels of cell pools and mini-pools in 24-wells plate assay. Producing cells were seeded in 24-wells plates, at 0.4 x 106 cells/well in 1 mL of CP-CHO-CB5-FBS medium. The experiment was performed in triplicates. Plates were incubated at 37°C in the presence of 5% CO2. The concentration of IgG in supernatants after 10 days, was measured by ELISA (whole antibody detection). To quantify the expression levels, a standard curve was made using known amount of trastuzumab. The data correspond to mean ± SD. The discontinuous line indicates the average level of IgG concentration for all the evaluated supernatants.



Correlation	r_P	p
MFI/MFI_{CHO-K1}-HC and [IgG]	0.737	<0.01
MFI/MFI_{CHO-K1}-LC and [IgG]	-0.259	0.284

Figure 5

Correlation between intracellular antibody polypeptides content and IgG concentration in supernatants. Cell mini-pools were grown in 24-wells plate in CP-CHO-CB5-FBS medium. After 10 days IgG concentration was quantitated in supernatants and intracellular HC and LC polypeptides of ethanol fixed cells were assessed by flow cytometry. MFI/MFICHO-K1: relation of mean fluorescence intensity of trastuzmab-expressing cells/ mean fluorescence intensity of CHO-K1 wild type cells. The correlation between variables was computed using a parametric test based on Pearson correlation.

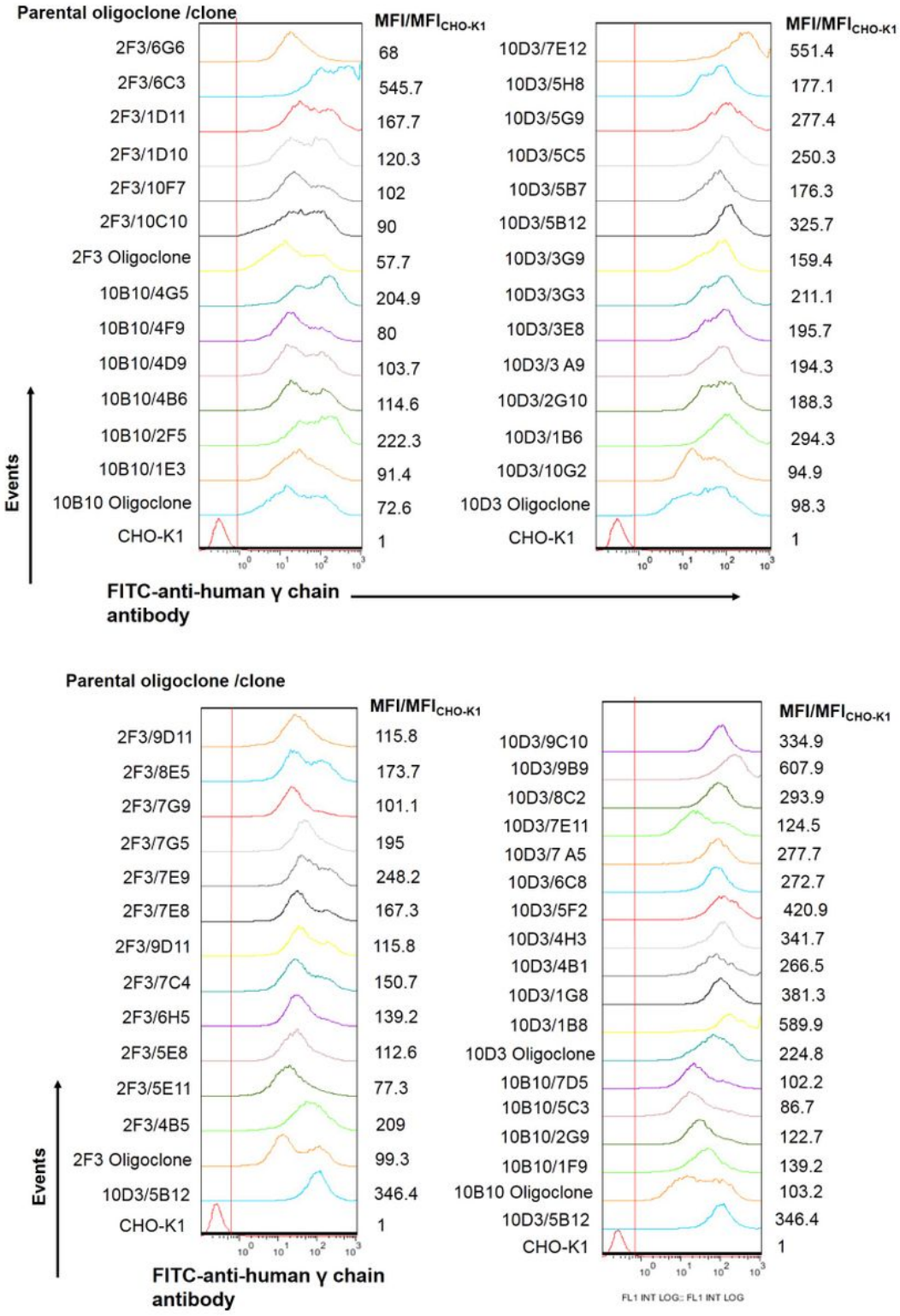


Figure 6

Assessment of heavy chain polypeptides content of clones by flow cytometry. Intracellular light and heavy chains polypeptides of ethanol fixed cells were measured using a FITC-labeled goat anti-human IgG (γ chain specific) antibody. MFI/MFICHO-K1: relation of mean fluorescence intensity of trastuzmab-expressing cells/ mean fluorescence intensity of CHO-K1 wild type cells.

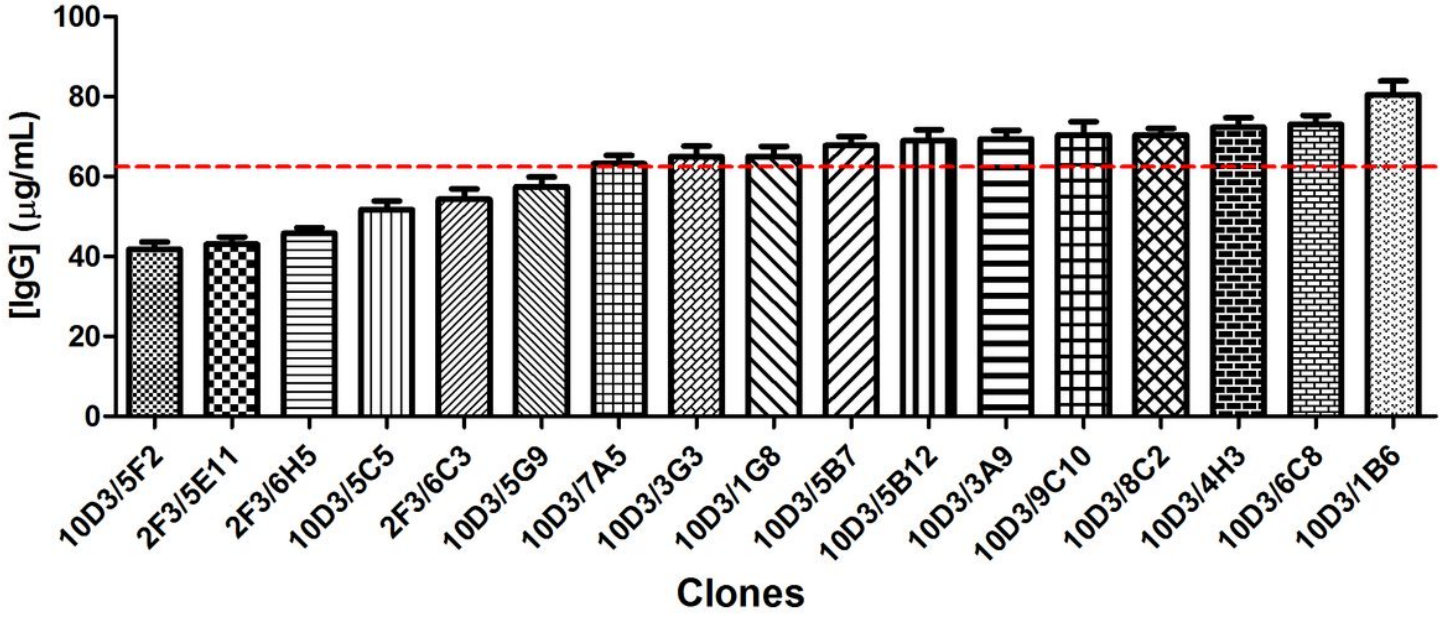


Figure 7

IgG expression levels of trastuzumab-expressing clones in protein free medium and shaken T-flasks. Producing cells were seeded in T-flasks 25 cm2 at 0.4 x 106 cells/well in 10 mL of CP-CHO-CB5 medium. The experiment was performed in duplicates. T-flasks were incubated at 37°C in the presence of 5% CO2 in vertical position and after 10 days supernatant was removed to quantify human IgG-expression levels by ELISA. To quantify the expression levels, a standard curve was made using known amount of trastuzumab. The data correspond to mean ± SD. The discontinuous line indicates the average level of IgG concentration for all the evaluated supernatants.

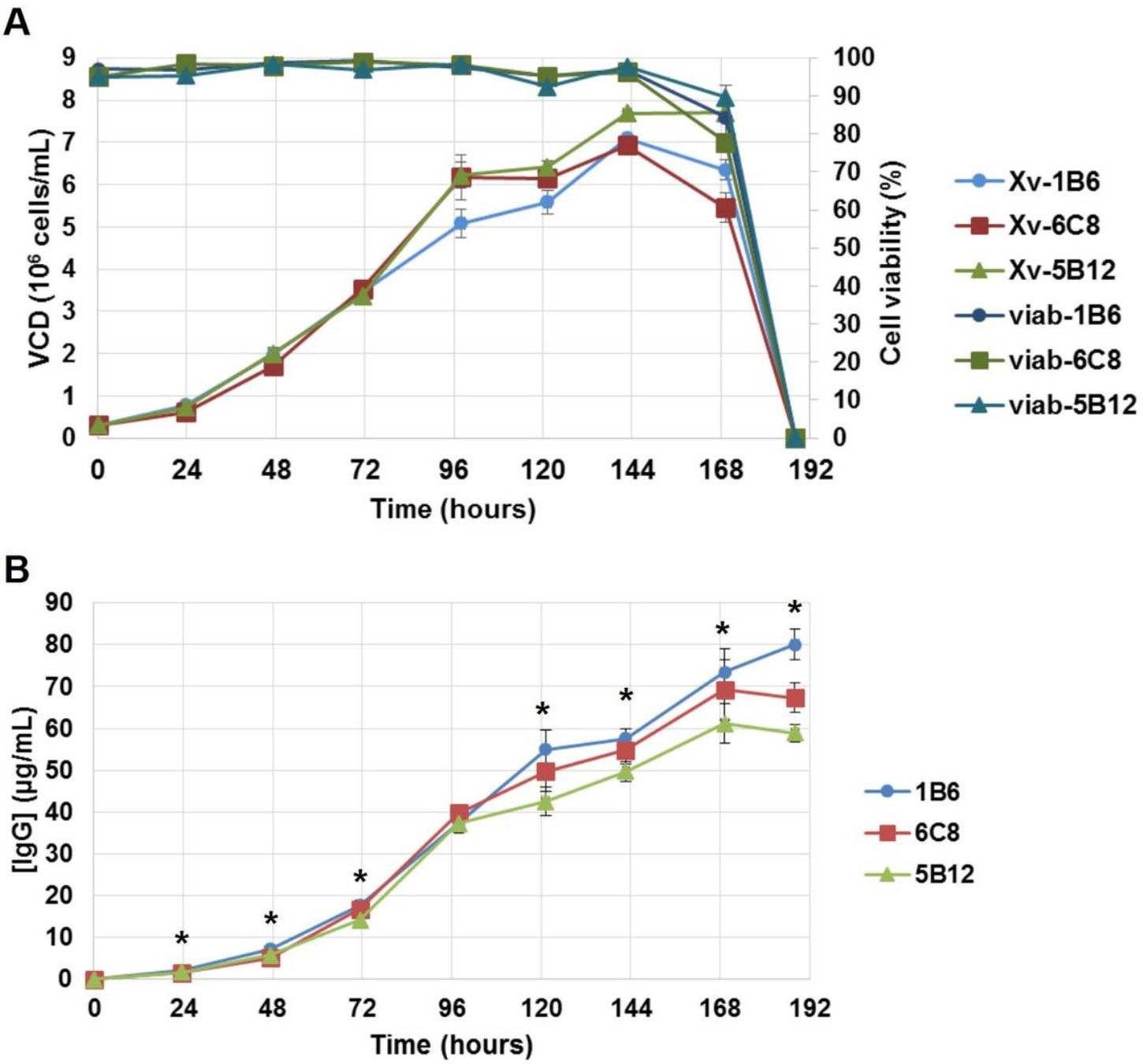


Figure 8

Batch experiments for selected trastuzumab-expressing clones cultivated in protein free medium and shaking culture. A: Growth profile. B: Titer of clones. Shaking flasks with 60 mL of CP-CHO-CB5 medium were inoculated with 0.3 x 106 cells/mL. The experiment was performed in duplicates. These flasks were incubated at 37°C in the presence of 5% CO2 and shaking culture (120) (Infors HT, Switzerland). VCD: viable cell density. All the clones are originated from parental cell oligoclone 10D3. The data correspond to mean ± SD. (*) indicates significant difference between means of almost two clones.

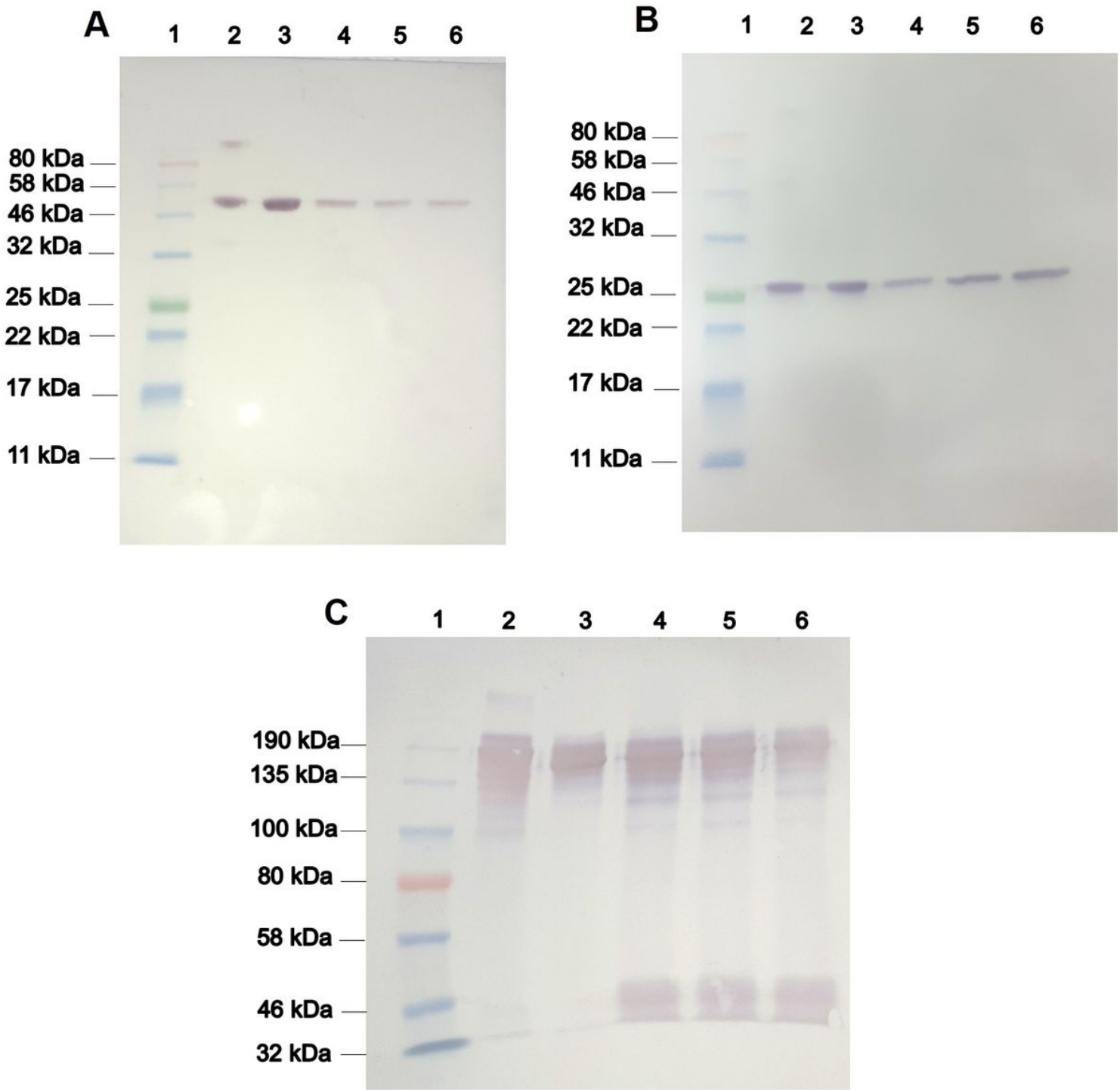


Figure 9

Analysis of trastuzumab biosimilar candidate by Western blotting. Supernatants of cells growing in protein free medium and shaking culture were loaded in 12% SDS-PAGE/reducing conditions (A and B) and 7.5% SDS-PAGE/non reducing conditions (C). Immunodetection of heavy chain (A) was performed with an AP-conjugated goat anti-human IgG (γ chain specific) antibody and light chain (B) and whole antibody (C) with HRP-conjugated goat anti-human kappa light chain antibody. 1: Pre-stained molecular weight marker (NEB, UK). 2: biosimilar to trastuzumab obtained in NS0 cells (2.5 µg) (named 5G4). 3:

commercial trastuzumab (2.5 µg). 4: Supernatant of 5B12 clone. 5: Supernatant of 1B6 clone. 6: Supernatant of 6C8 clone.

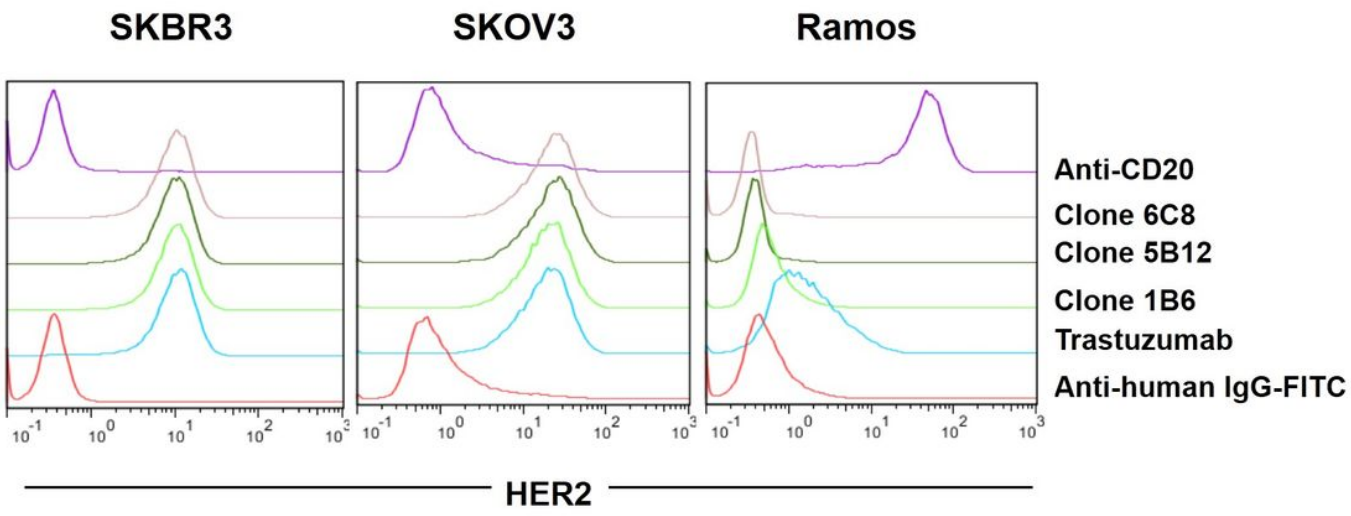


Figure 10

Recognition of HER2-expressing tumor cell lines. SKOV3, SKBR3 and Ramos cell lines were stained with 10 µg/mL of produced antibody contained in supernatants of 1B6, 6C8 and 5B12 clones, followed by a FITC-labeled rabbit anti-human IgG antibody. Commercial trastuzumab was used as positive control and supernatant of anti-CD20 expressing clone, as isotype matched control. Ramos cells (CD20+) were used as control of non-HER2 expressing cells. Results are representative of two independent experiments.