Research Article

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Developing selection strategy for CHO-K1 cell line that secretes scfv-Fc fusion antibodies using ClonePix2

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ABSTRACT

In the pharmaceutical industry, biopharmaceuticals (biologics) are gaining market share. There has been a dramatic increase in the sale and market penetration of monoclonal antibodies in particular. Typically, therapeutic antibodies are produced using high-expression, clonal, or recombinant CHO cell lines. CHO cells dominate the market as a commercial production host due to their ease of use, built-in regulatory records, and security profiles. While traditional limiting-dilution and cloning-ring regulations are frequently used to select mammalian cell lines that produce high levels of proteins, they have a number of drawbacks. ClonePix2 is a fully automated, single cell-based clone selector that significantly increases the likelihood of rapidly selecting high-production clones with high monoclonality. Scfv-Fc recombinant antibody structures with a variety of therapeutic advantages have gained prominence in recent years. Single cell cloning of CHO cells expressing the scfv-Fc fusion protein, which differs from the classical immunoglobulin structure, was performed *in situ* using the ClonePix2 device using FITC-tagged anti-Fc and anti-H+L antibodies. The fluorescent intensity parameters of the resulting cell clones were analyzed. Additionally, ELISA was used to determine the production capacities of the best clones. As a result, it was established that anti-Fc antibody recognizes the scfv-Fc fusion protein in a semi-solid environment, enabling the identification of higher production clones.

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Introduction

A critical step in the production of commercial therapeutic antibodies (Ab) is the selection of clones that produce high levels of protein. Various methods are used to select a stable cell line that produces recombinant proteins. Although laborious and time consuming, the limiting-dilution and cloning-ring methods are still widely used due to their simplicity and low cost. These methods, however, do not allow for the separation of high, low, and non-productive clones prior to cell culture growth [1,2]. Recently, an alternative cell-cloning method that contains fluorescent-labeled antibodies against the interest protein secreted by cells, coated with a semi-solid medium, and then presented to an automated

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colony selector such as ClonePix2 has been developed to increase the likelihood of obtaining high protein-producing clones [3,4,5]. This robotic selection system can identify all productive colonies derived from single cells based on parameters such as colony size, roundness, and proximity to neighbors, as ascertained by bright-field and fluorescent images of relative protein expression [3,6] **.** The semi-solid environment keeps dividing cells in place during single-cell cloning, allowing control of the parameters mentioned above and increasing the likelihood of collecting high-producer clones. The most significant advantage of automatic clone collection is the capture of rare clones that perform high expression [2].

Single-chain variable fragment (scfv) is a class of engineered antibodies generated by the fusion of the heavy (VH) and light chains (VL) of immunoglobulins through a short polypeptide linker (10-25 amino acid). The benefits of scfv stem from their small size. Because they are smaller, they can be cleaned from the blood more quickly in treatment approaches, penetrate the tissues more effectively, and have low immunogenicity [7]. The scfv-Fc fusion protein is made up of a scfv linked to the CH2 and CH3 regions of the immunoglobulins (IgG) fixed region, which can then form a homodimer with another copy. Fragment crystallizable (Fc) parts of immunoglobulins are composed of the heavy chain fixed zone (Fc region) and mediate cellular effector functions. Fc contains common protein sequences for all IgG as well as class-specific determinants. These regions are known as fixed regions because they do not differ significantly between IgG molecules of the same class [3]. The fusion of scfv and Fc provides several advantages, including two valuable bindings to scfv structures, a longer half-life, and Fc-mediated effector functions. In addition, the scfv-Fc format can be used in the clinic for therapeutic purposes [4,5].

Many studies on the choice of single cell-based cloning of IgG in a semi-solid environment have been conducted using the ClonePix2 system. In these cases, the selection can be made by marking with antibodies conjugated with fluorescence provided by the vendor. Clone-Detect reagents, which each contain an antibody specific to different parts of immunoglobulins (Fc, H+L chain, etc.) or the antibody's subtype (such as Kappa), are among the antibodies offered by the vendor [6,7]. The Scfv-Fc fusion protein, however, differs from the classical IgG structure and has not been studied until now to identify these structures using Clone-Detect reagents unique to the Clonepix2 system.

Two antibodies supplied by the vendor were compared in this study to identify highly productive clones from the same stable cell pool.

Material and Methods

Cells, cell culture and transfection

CHO-K1 cells are transfected with using plasmid DNA containing single copies of the fusion of scfv and Fc genes. The CMV promoter directed the expression of the scfv-Fc fusion protein. The plasmid pTK-Hyg (Clontech, USA, 631750) was also used in transfection to create a stable cell line, which is a selection vector that confers hygromycin resistance in mammalian cells and is used for the selection of stably transformed cells. All cell counts were either performed using the trypan blue exclusion method via the Luna II™ cell counter (Logos biosystems, South Korea) or estimated based on well confluency using the Clone Select Imager (CSI, Molecular Devices, USA).

$3x10^4$ CHO-K1 cells (ATCC, CCL-61, USA) were seeded into each well of a 6-well plate one day before transfection. The cells were grown in Dulbecco's modified eagle and F12 medium (DMEM-F12) (Thermo Fisher, Gibco, USA), 1% Penicillin-Streptomycin, and 10% fetal bovine serum (FBS) (Cytiva HyClone, South America Origin) in a 37ºC, 5% CO_2 environment with high humidity. On the day of transfection, the culture medium was replaced with Opti-MEM Reduced-Serum Medium (Thermo Fisher, 31985062, USA). The Lipofectaime LTX (Thermo Fisher, A12621, USA) protocol was used to create transfection complexes with 3 µg scfv-Fc fusion and 0.3 µg pTK-Hyg plasmids. The mixture was then incubated for 5 minutes at room temperature (15-20 ºC). Drop by drop, the transfection complex was added to CHO-K1 cells. Cells were then incubated for 48 hours at 37ºC, 5% CO_2, and high humidity. The medium was then replaced with 2 ml of DMEM-F12 medium containing 10% FBS and 350 µg/ml Hygromycine (Thermo Fisher, 10687010, USA). Antibiotic selection was carried out for 10 days, and surviving cells were directly used for subsequent clonal isolation [8,9].

Clonal isolation

Methylcellulose-based semi-solid medium used in conceived with the ClonePix2 (Molecular Devices, USA) device, was used for the isolation of the resulting clones. CloneMatrix concentrate (2,5X) (Molecular Devices, K8600, USA) is diluted to 1X with DMEM-F12 medium containing 10% FBS and supported by anti-Fc CloneDetect™ (anti-Human IgG (Fc) Specific, Fluorescein antibody, 10000U/ml) (Molecular Devices,

K8205, USA) or anti-H+L CloneDetect™ (anti-Mouse IgG (H+L) Specific, Fluorescein antibody, 10000U) (Molecular Devices, K8220, USA). Following the 10-day selection period, the transfected, stable pools are plated at 200 cells/mL in 6-well tissue culture plates (Corning, 657185, USA) containing 2 ml 1X semi-solid media at each well. Before coating, the media and CHO cells are thoroughly mixed to ensure that all components are evenly distributed. After plating, plates were observed with CSI (Molecular Devices, USA) to verify single cells were well distributed. The plates were incubated at 37 ºC with 5% CO_2 for 12 days [6].

ClonePix 2 isolation of single high-producing clones

Prior to each cloning procedure, the ClonePix2 device was sanitized and calibrated according to the manufacturer's instructions to ensure that the selected colonies were isolated accurately and without contamination. After 12 days, individual 6-well plates were analyzed using ClonePix2™ software (Molecular Devices, USA). Each plate was divided into 36 sections; each section was imaged for 1 ms under white light and then fluorescence measured for 2000 ms with the LED intensity set to 81. A “local threshold” algorithm was used to detect each colony. Colonies were chosen based on their exterior mean fluorescence intensity (EMFI), their size, their compactness, and their proximity to neighboring colonies. Colonies that met the picking criteria were seeded into individual wells of a 96-well plate containing 150 µL of DMEM-F12 containing 10% FBS medium. Cell growth was monitored using the CSI device. Confluent wells appeared after 6–10 days, and cells were expanded to 6-well plates for titer testing [7,8].

Scfv-Fc expression control by Protein L/Fc capture ELISA

Scfv-Fc expression was monitored using the enzyme linked immunosorbent assay (ELISA). "Recombinant Protein L Type-1 Protein" (Novus Biologicals, USA) was coated onto an immunoplate (Corning, 3690, USA) overnight at a concentration of 250 ng/50 µl in 0.1 M $NaHCO_3$. Skim milk was used to block all plates, and PBS-T was used to wash them (0.1 % Tween 20 in Phosphate Buffered Saline). In designated wells, 50 µl of sample (undiluted supernatants) from each clone (top 25 colonies of anti-human IgG (Fc) specific (Fc-1 to Fc25) and anti-human (H+L) specific (HL1- to HL-25) with cell densities of 3 x 10^6 cells/ml were added in duplicate. For 90 minutes, the plates were incubated at 37ºC. Wells were washed three times with PBS-T before adding 50 µl of secondary antibody: anti-Human IgG conjugated to alkaline phosphatase enzyme (Sigma,

A9544, Germany) to each well. The secondary antibody used is specific to the Fc of human mAbs and was diluted to a 1:50.000 ratio prior to use. The mixtures were incubated for another 60 minutes at 37ºC. Washing was used to remove unbound secondary antibodies, and 50 µl of 4-Nitrophenyl phosphate disodium salt hexahydrate substrate solution (Sigma, N2765, Germany) was added. For 30 minutes, the mixture was incubated at room temperature in the absence of light. Finally, the enzyme-substrate reaction was stopped by adding 50 µl of 3 N NaOH, and absorbance at 405 nanometers was measured with microplate reader (Bio-Tech EIA Reader, USA) [10].

Results and Discussion

The VH and VL heavy- and light-chain variable domain constructs (scfv) used in this study were obtained from a mouse phage display library. The Fc region, where it is fused, contains the human IgG's second and third constant domains (CH2 and CH3), as well as a hinge region (H) that allows the formation of two disulfide bonds. After this structure is established, the two fusion proteins form a homodimeric structure with sulfide bonds, similar to IgGs. The resulting homodimeric fusion protein contains two scfv structures that can be recognized by the "anti-Mouse IgG (H+L) Specific" antibody (anti-H+L) and an Fc structure that can be recognized by the "anti-Human IgG (Fc) Specific" antibody (anti-Fc) (Figure 1). In this study, it was determined which of these antibodies recognizes the scfv-Fc fusion protein better in a semi-solid environment.

To ensure the stability of the construct, CHO-K1 cells are transfected with two plasmids, one containing the scfv-Fc construct conraining plasmid and the other containing the Hygromycin phosphotransferase gene. Following transfection, the cells were grown for 10 days in a selection medium containing Hygromycine, ensuring the death of unstable cells. A stable cell pool expressing untaged scfv-Fc fusion protein (Figure 1) is obtained in this manner. When the viability of cells reached % 65 in the post-transfection selection process, the selection was stopped and the cells were placed in a 6-well plate containing anti-Fc or anti-H+L CloneDetect^{TM}. After that, the plates were incubated to form distinct colonies. Four 6 well plates were studied for each antibody. After the fifth day, colony formations began to be controlled by the CSI device.

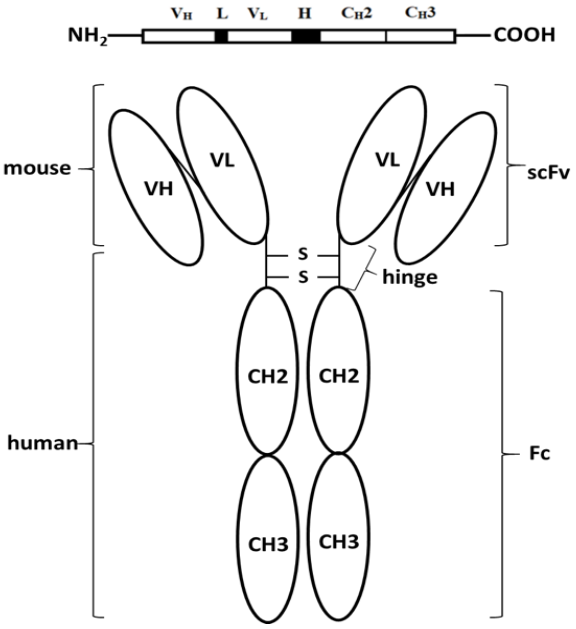

Fig 1 The scfv-Fc fusion protein is schematically displayed. VH and VL variable domains of mouse scfv; H hinge area of human IgG containing two disulfide bridges; and CH2 and CH3 second and third constant domains of human IgG, respectively

The colonies that formed on the 12th day were isolated clonally. Plates labeled with both antibodies were analyzed with CSI prior to isolation to ensure that the estimated cell numbers were in the same ranges. It has also been demonstrated that colonies in both groups are generally distributed evenly to plate wells, and that colony numbers are equally dense (**Figure 2**). Thus, it has been demonstrated that different CloneDetect^{TM} antibodies added to the environment have no effect on cell growth.

Because of the semi-solid environment, the secreted recombinant proteins remain around the colonies where they are secreted. Cells coated with semi-solid media with a high viscosity that reduces secretion diffusion and incubated to form discrete colonies were shown under white light and fluorescence, respectively. Precipitation occurs around the relevant colonies in colonies that produce fusion protein due to the interaction of Scfv-Fc antibodies secreted from colonies and the capture antibody conjugated to FITC. As a result, the higher the fluorescent density, the greater the amount of antibodies secreted [11].

Following imaging with Clonepix2, each colony is classified primarily based on criteria such as size, proximity, irregularity, and fluorescent intensity. Table 1 summarizes the colony groups that resulted from this classification.

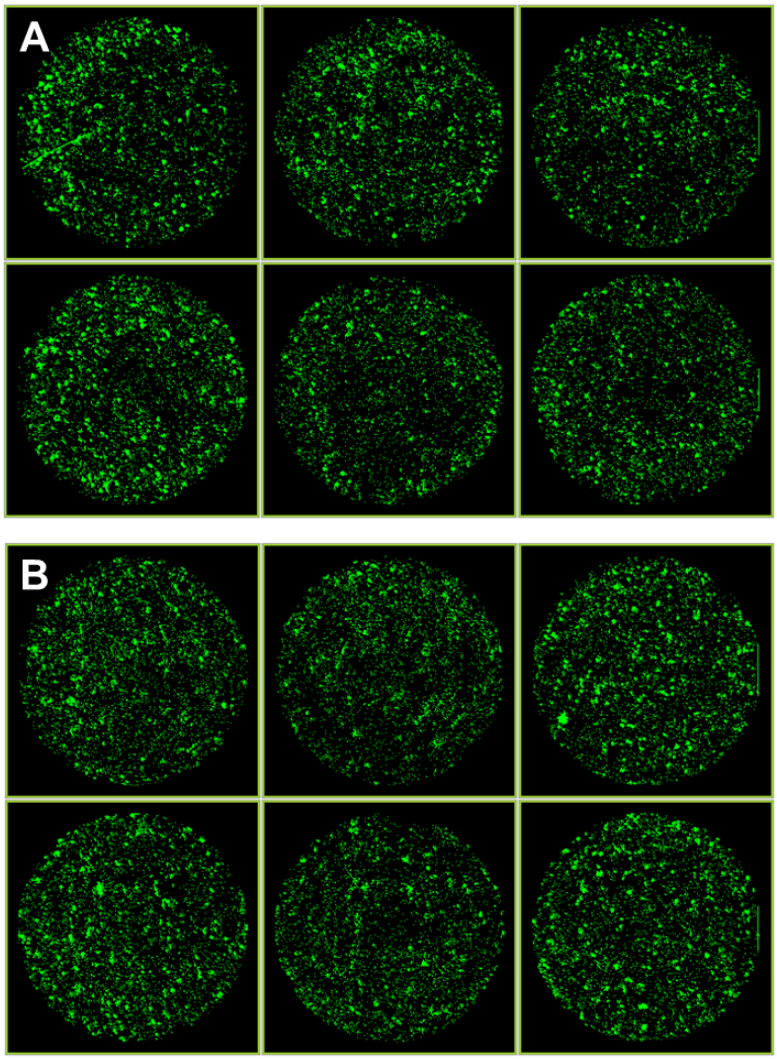

Fig 2 Images from the Clone Select Imager (CSI) grown in a 6-well plate with detection antibodies Anti-Human IgG (Fc) Specific **(A)** or Anti-Mouse IgG (H+L) Specific **(B)**

When compared to colonies grown in both antibodies, it has been determined that most colonies do not reach sufficient size, despite the passage of sufficient time. Colonies that are very close to each other and have irregular shapes have also been identified. Individual clones that are excluded from all of these clones and have the highest EMFI values are aspirated with the ClonePix2 system's micro-pins. Each of the 48 colonies identified by the anti-Fc and the 43 colonies identified by the anti-H+L antibody was transferred to a 96-well plate well. The fact that approximately 4000 clones were screened and approximately 50 colonies (~1%) exceeded the threshold indicates that the high number

of producers is extremely low. This clearly demonstrates the reasoning behind scanning a large number of clones with a highly efficient selection method.

Table 1 Colony group distribution of "Human IgG (Fc) Specific, Fluorescein antibody" and "Mouse IgG (H+L) Specific, Fluorescein antibody"

Conditions	**anti-Human IgG (Fc)**	**anti-Mouse IgG (H+L)**
Too small (excluded colonies too small)	3436	3966
Proximity (excluded colonies too close to each other)	187	186
Irregular colonies (roundness & axis ratio)	436	469
Screened and picked clones (best expressed)	48	43
Grown colonies after picked	31	33

The "Exterior Mean Fluorescence Intensity" (EMFI) values used in the selection of colonies expressing a high amount of protein are divided by the number of pixels in the exterior area (arithmetic mean). This statistic approximates how bright the area immediately surrounding a given colony is. Colonies' "Interior Mean Fluorescence Intensity" (IMFI) values can also be used to identify colonies that produce a high quantity of proteins. IMFI is calculated by dividing Interior Total Intensity by colony area (in pixels). It is the arithmetic mean of the 'average' brightness of all pixels in the colony [7,12]. When the EMFI values of the top 25 colonies from both selection groups were compared, it was discovered that the proteins expressed by the colonies identified by the anti-Fc antibody had a higher intensity (Figure 3A). Similarly, the IMFI values of the top 25 colonies were compared, and it was discovered that the scfv-Fc structure, which is also defined by the anti-Fc antibody, had a higher intesity (Figure 3B). The values after the 25th colony in both colony groups were low (for EMFI and IMFI<15) and were not included in the comparison results.

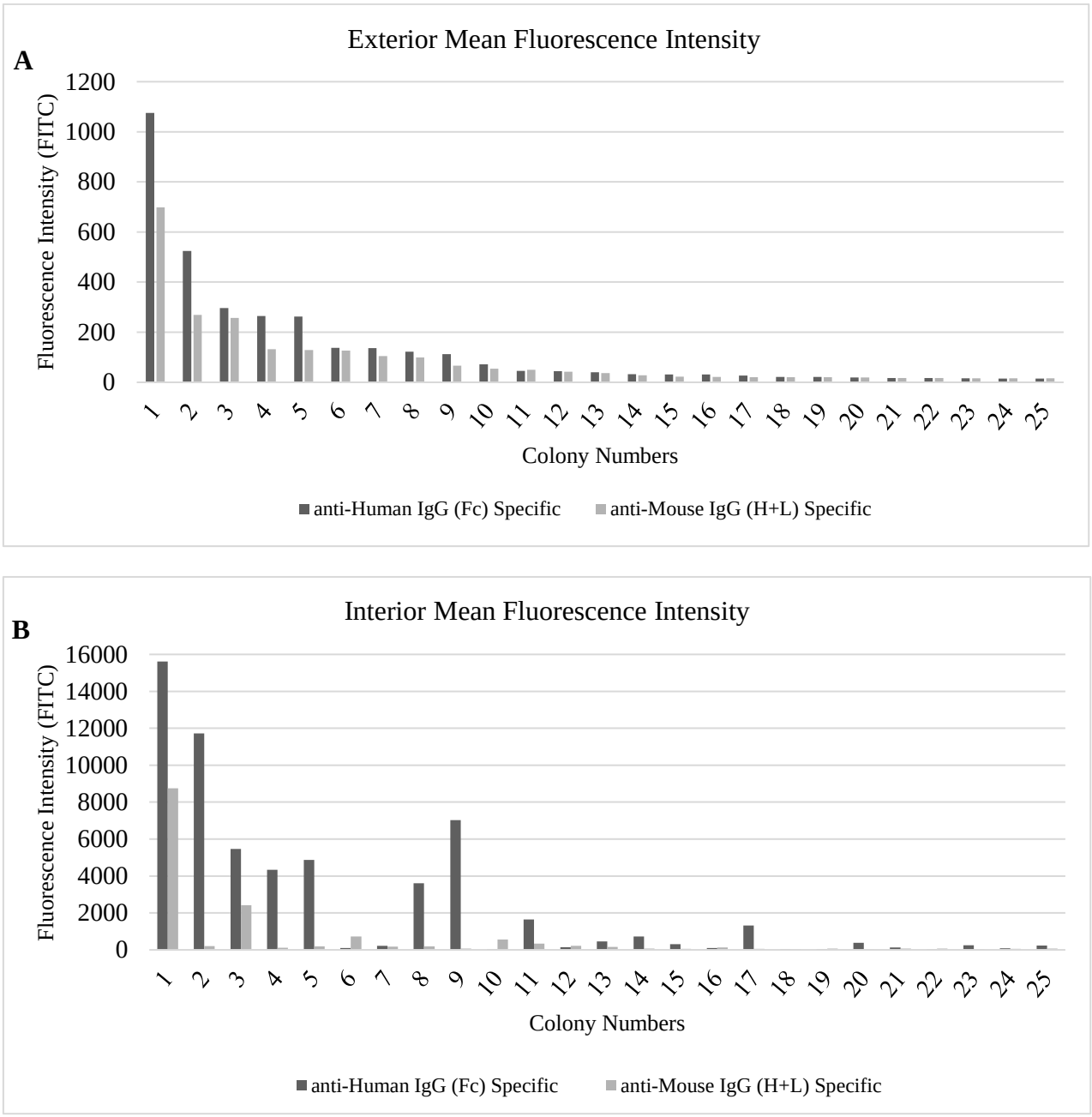

Fig 3 Comparison graphs of the top 25 colonies identified by the anti-Human IgG (Fc) Specific and the anti-Mouse IgG (H+L) Specific antibody's EMFI **(A)** and IMFI **(B)** values

Colonies were grown in 96 well plates for 7 days while being monitored for growth with a CSI device. However, when high-producer clones are detected and collected, they may change their production profiles or cease to grow when returned to their original environment. After being removed from the semi-solid environment, 31 of the 48 colonies identified by the Anti-Fc antibody and 33 of the 41 colonies identified by the Anti-H+L antibody were able to continue their development in liquid medium.

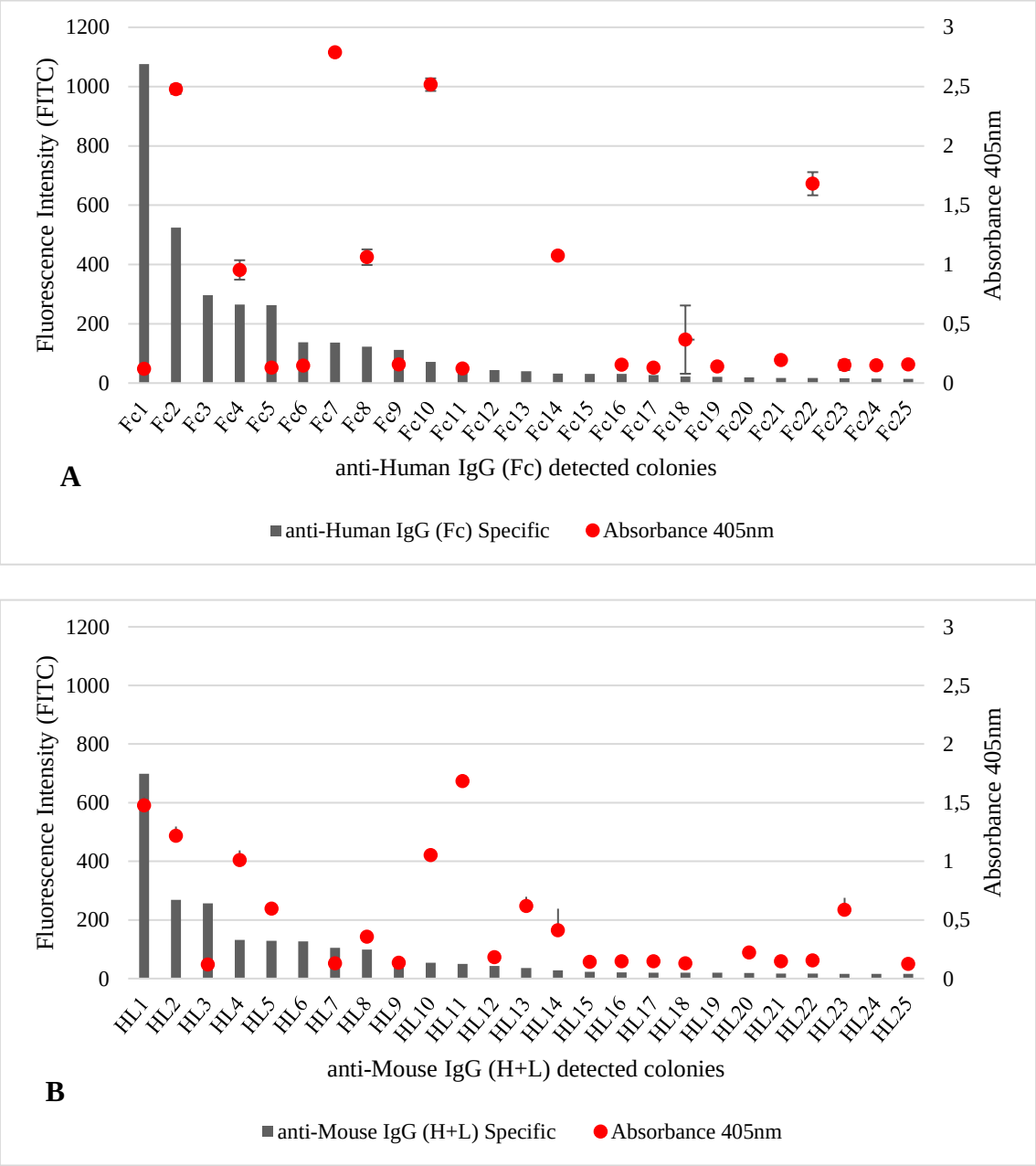

Fig 4 EMFI (column) and antibody productivity (red dot) (OD_{405}) graphs of top 25 colonies selected with anti-Human IgG (Fc) Specific antibody **(A)** and anti-Mouse IgG (H+L) Specific antibody **(B)**

The relationship between fluorescence intensity and scfv-Fc productivity was also investigated in the top 25 clones using the ELISA method to measure antibody levels in cell supernatants at a concentration of $3x10^6$ cells/ml. At equivalent cell densities, clones with higher exterior fluorescence intensities should have a higher concentration of

antibodies secreted [11]. This correlation was found in some colonies in this study. When protein expressions were evaluated using the EMFI value of the top 25 colonies in both groups, it was discovered that clones detected with the anti-H+L antibody had an absorbance value of 0.5 or less, with the exception of one clone less than 10 florescence intensity (Figure 4A). Except for one, all of the clones with less than 50 florescence intensity identified by the anti-Fc antibody had a value of 0.5 or less (Figure 4B). Seven of the remaining clones have an absorbance value greater than 0.9; the Fc2 clone has an absorbance of 2.47, the Fc7 clone has an absorbance of 2.78, and the Fc10 clone has an absorbance of 2.51, making them the highest producer clones obtained from all scans performed within the scope of this study. Despite the fact that the scfv structure in the scfv-Fc structure is compact and dimeric, it has a lower identifiability capacity. This could be due to scfv's conformation. Scfv molecules are held together by a flexible linker, which allows them to oscillate between open and closed states [13,14]. This study can also be done with scfv structures with different linker lengths to see if the scfv conformation differs in identifying with anti-H+L antibodies.

As a result, this study demonstrated that targeting Fc is more appropriate in identifying the best producing clone from colonies that secrete scfv-Fc in the semi-solid medium. This could be because the Fc structure is more stable.

Conclusion

It is critical to have an antibody that can detect scfv-Fc well secreted from colonies in a semi-solid environment. The ClonePix2 system was used to select the CHO-K1 cell line, which expresses a high level of the scfv-Fc protein. Two antibodies supplied by the vendor were used concurrently to identify highly productive clones from same stable cell pool. It was discovered that the antibody "anti-Human IgG (Fc) Specific" detects homodimeric scfv-Fc fusion antibody better and allows for the selection of higher-producing clones.

Abbreviations

CHO: Chinese Hamster Ovarian, scfv: Single-chain variable fragment, Fc: Fragment crystallizable, Ab: antibody, VH: heavy chain, VL: light chains, CH2: second constant domain, CH3: third constant domain, a.a: amino acid, IgG: immunoglobulin, H+L chain: heavy and ligth chain, anti-Fc: anti-Human IgG (Fc) Specific, Fluorescein antibody, FITC: Fluorescein, anti-H+L: anti-Mouse IgG (H+L) Specific, Fluorescein antibody, DNA: Deoksiribo nucleic acid, CMV: Cytomegalovirus, CSI: Clone Select Imager, FBS: fetal bovine serum, MEM: Modified Eagle Medium , DMEM-F12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, EMFI: exterior mean fluorescence intensity, IMFI: intyerior mean fluorescence intensity, ELISA: enzyme linked immunosorbent assay, PBS: Phosphate Buffered Saline, LED: light-emitting diode

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Availability of data and material
Please contact the corresponding author for any data request.

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