CHAPTER IV

RAPID AUTOMATED SELECTION OF NS0 MAMMALIAN CELL LINE SECRETING HIGH LEVEL OF H1C2 MONOCLONAL ANTIBODY USING CLONEPIX FL SYSTEM

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4.1 OBJECTIVE

The main objective of this chapter is to evaluate and optimize the ClonePix FL system in selecting NS0 transfectomas producing H1C2 mAb developed by the deimmunization method. The monocistronic humanized-pAH4602A and humanized-pAG4622A expression vectors coding the heavy and light chain of this mAb from chapter 3 were co-transfected in NS0 cells. The data obtained from the ClonePix FL system would be used for future procedures involving selection of rare transfectomas producing high levels of mAb using this system. The NS0 transfectoma secreting the highest level of H1C2 mAb was chosen for serum-free adaptation as described in chapter 5. Although not presented in this chapter, the expression of anti-C2 mAb developed using logical approach method (H2C2 mAb) and the selection of high producer NS0 transfectomas secreting H2C2 mAb was also similarly performed.

4.2 INTRODUCTION

Production of complex recombinant proteins often requires the use of mammalian cells, which is an expensive process with often disappointingly low yields and long development times (Wurm, 2004; Butler, 2005). Due to the growing demand for therapeutic proteins especially monoclonal antibodies, generation of high-producing clones is a perquisite for achieving antibody yields suitable for biopharmaceutical production (Carroll & Al-Rubeai, 2005).

However, in many industrially important cell lines used to produce recombinant proteins such as Chinese hamster ovary (CHO), mouse myeloma cell line (NS0) and

hybridomas, only a minority of clones show productivity significantly above average (Browne & Al-Rubeai, 2007). Thus, in order to have a reasonable probability of finding rare high-producing clones, a large number of clones need to be screened.

Limiting dilution cloning (LDC) is the most commonly used traditional method, owing to its relative simplicity and low cost. In this method, transfected cells are diluted into microtiter plates at an average of less than one cell per well. Antibody producing wells containing clones presumably derived from single cells are selected for further analysis. However, due to the obvious low density, the viability of single cells is very low even in the presence of feeder cell layers. This will increase the risk of losing high-producing clones.

Besides that, the low-throughput nature of this method significantly limits the number of clones that can feasibly be screened. The use of liquid media in this method also makes the selection of monoclonal colonies to be labor-intensive and time-consuming (Underwood & Bean, 1988). Thus, the chances of obtaining high-producing monoclonal colonies through this method are extremely low.

Fluorescence activated cell sorters (FACS) have been used to screen several million cells and single clones could be isolated in shorter time (Carroll & Al-Rubeai, 2004; Kacmar & Srienc, 2005; DeMaria et al. 2007). Although FACS is higher throughput compared to LDC, FACS is more suited for the selection of high-producers of non-secreted proteins (Meng et al. 2000; Yoshikawa et al. 2001).

This is because FACS measures productivity based on cell-surface expression and secreted proteins such as monoclonal antibodies are quickly dissociated from cells when

liquid media is used. It was also reported that through a kinetic analysis of hybridoma clones, there is no correlation between the amount of cell surface antibody and the level of antibody in cell culture supernatants, which therefore limits the use of FACS for isolation of secreting clones.

In order to retain secreted recombinant proteins in the vicinity of the secretary clones, semi-solid media has been employed. Semi-solid media which has high viscosity and minimizes diffusion of secreted proteins is used in three popular selection methods, matrix-based selection assay (MBSA) (Frykman & Srienc, 1988), gel microdrop technology (GMT) (Akselband et al. 2003) and laser-enabled analysis and processing (LEAP) (Koller et al. 2004).

Using MBSA method, clones with a five-fold superior productivity compared to the highest producing clone selected by LDC method have been isolated (Borth et al. 2000). Furthermore, using MBSA method, it took only 480 subpopulations to identify high-producing clones compared to 4320 subpopulations when done by LDC method.

Via GMT method, the isolation of rare high-producing mammalian cells with a two- to five-fold increase in specific productivity has also been achieved. Similarly, through LEAP method an increase of five- to twenty-fold in productivity, a significant decrease in the level of heterogeneity within selected cell lines and higher levels of secreted protein have also been shown (Hanania et al. 2005).

Nevertheless, the disadvantages of MBSA, GMT and LEAP methods have also been reported. The MBSA method is unsuitable for fragile cell types and requires laborious optimization. Likewise, GMT method is described as being user-unfriendly, needing

extensive optimization of conditions for each cell line used and requiring dedicated equipment.

Additionally, due to low density seeding of cells used to ensure single cell occupancy in beads, only 10-15% of the beads contain single cells, which means that up to 90% of the beads analyzed are empty and wasted. Also, GMT method is reported to reduce the viability of some cell types commonly used for expression of recombinant proteins, most notably NS0 cells (Barnes et al. 2001). Finally the use of laser technology in LEAP method to eliminate unwanted clones may damage high-producing clones (Browne & Al-Rubeai, 2007).

Roque-Navarro et al. (2003) had used the limiting dilution method to isolate monoclonal NS0 cells secreting high levels of humanized-C2 monoclonal antibodies (hum-C2 mAbs). In this project, we have used the method described by Roque-Navarro et al. (2003) to humanize mouse-C2 mAbs; however, instead of the limiting dilution method, the ClonePix FL system was employed in order to minimize the duration of clone selection and increase the probability of obtaining high-producing clones with high monoclonality.

We also evaluated the correlation between exterior fluorescence intensity and antibody productivity using quantitative enzyme-linked immunosorbent assay (ELISA). The hum-C2 mAbs were engineered to have lower immunogenicity compared to its precursor which was developed in the mouse through hybridoma technology (Mateo et al. 2000; Yazaki et al. 2004).

4.3 MATERIALS AND METHODS

4.3.1 Expression vector construction

Expression vectors coding for humanized heavy and light antibody chains were developed using the deimmunization method as described in chapter 3.

4.3.2 Transfection of NS0 mammalian cells

One day prior to transfection, 1 x 10^5 NS0 cells (American Type Culture Collection (ATCC), USA) were seeded into each well of a 6-well plate (Corning, USA). The cells were grown in growth media consisting of Dulbecco's modified eagle medium (DMEM) (Biochrom, Germany), 1% glutamax (Life Technologies, USA), 1% antibiotic/antimycotic (Life Technologies, USA) and 5% fetal bovine serum (FBS) (Biochrom, Germany) and at 37ºC, 5% CO_2 and high humidity.

The humanized-pAG4622A and humanized-pAH4602A were purified in large-scale using Qiafilter plasmid maxi kit (Qiagen, Germany), linearized by *Pvu*I and purified again using QIAEX gel extraction kit (Qiagen, Germany). Purified linearized humanized-pAG4622A and humanized-pAH4602A vectors were each diluted in sterile water to a final concentration of 2 μg/100 μl. Transfection complexes were formed by adding 16 μl of FuGENE HD transfection reagent (Roche, Germany), 100 μl of humanized-pAG4622A (2 μg) and 100 μl of humanized-pAH4602A (2 μg) in 1.5 ml centrifuge tubes. The mixtures were then vortexed vigorously for 5 seconds and incubated at room temperature (15-20ºC) for 15 minutes.

Transfection complexes were added to NS0 cells drop-wise while the plate was being swirled to ensure even distribution. Cells were then incubated and after 48 hours, 3 ml of growth media containing 5 mM of L-histidinol dihydrochloride (Sigma-Aldrich, USA), were added every 48 hours for a duration of 6 days to inhibit the growth of non-transfected cells. Although electroporation and liposome-based reagents are the two most common approaches for transfection, we decided to use FuGENE HD because of its higher efficiency and lower cytotoxicity to mammalian cells (Jacobsen et al. 2009).

4.3.3 Seeding of transfected NS0 cells in semi-solid media

In order to grow clones from individual separate transfected cells, 1 x 10^5 transfected cells pre-treated with L-histidinol dihydrochloride were added to 100 ml of semi-solid growth media comprising of 90 ml semi-solid media for hybridomas/myelomas (cat. no.: K8600, Molecular Devices, USA), 1 ml antibiotic/antimycotic (Life Technologies, USA), 1 ml glutamax (Life Technologies, USA), 7 ml FBS (Biochrom, Germany) and 1 ml of anti-human capture antibody conjugated to fluorescein isothiocyanate (FITC) (cat. no.: K8200, Molecular Devices, USA). The mixture was mixed vigorously and 2 ml of the mixture were transferred to each well of equiglass 6-well plate (Molecular Devices, USA). The plates were then incubated at 37ºC, 5% CO_2 with high humidity for 7 days.

4.3.4 Isolation of single high producing clones

Before each procedure to isolate clones, the ClonePix FL system (Molecular Devices, USA) was sanitized and calibrated according to the manufacturer's instructions to ensure that the selected colonies were accurately isolated and contamination free. The clones in the semi-solid media were then imaged under white light and also under

fluorescence (Lee et al. 2006). Both images were superimposed and the colonies were then sorted according to exterior fluorescent intensities.

The individual clones with high exterior fluorescent intensities were aspirated with micro-pins controlled by the ClonePix FL system and dispersed automatically in a 96 well plate containing 160 µl of growth media/well. The selected clones could then be transferred for downstream applications. The whole process of sanitization, calibration, imaging of 1 x 10^5 clones and subsequently selection of high-producers could be completed in 1 hour. The clones were then expanded in T75 flasks (Thermo Fisher Scientific, USA).

4.3.5 Quantitative ELISA to determine the antibody productivity of clones

To evaluate the correlation between the exterior fluorescence intensity and antibody productivity, quantitative ELISA were performed using supernatant from selected clones with exterior fluorescence intensity of less than 1000 fluorescence units (FU), between 1000-2000 FU, between 2000-3000 FU and all 3 clones with more than 3000 FU.

First, immuno 96 micro well solid plates (cat. no.: 442404, Thermo Fisher Scientific, USA) were coated with capture antibodies: anti-human IgG antibody (AHIgG) which is specific to the Fc region of hum-C2 mAbs (cat. no.: I2136, Sigma-Aldrich, USA). AHIgG were diluted 1:2500 in coating buffer (0.05 M of sodium carbonate and 0.05 M of sodium bicarbonate, pH of 9.6).

One hundred µl of the diluted AHIgG were added to each well. Plates were incubated at 4ºC for 16 hours and subsequently washed three times with 200 µl of washing buffer (sterile water containing 0.09% sodium chloride and 0.05% tween-20) to remove excess antibodies. Plates were blocked by the addition of 200 µl of blocking buffer (phosphate buffer containing 3% bovine serum albumin (BSA) and 0.1% tween-20) into each well and incubated at 37ºC for 90 minutes. Wells were then washed 3 times with washing buffer.

For ELISA, 100 µl of sample (undiluted supernatants from each clone with cell densities of 3 x 10^6 cells/ml) or a standard antibody, immunopure human IgG (cat. no.: 31154, Thermo Fisher Scientific, USA) with concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200 ng/ml in phosphate buffer were added in triplicate to designated wells. The plates were incubated at 37ºC for 90 minutes. Wells were washed 3 times with washing buffer and 100 µl of diluted secondary antibody, anti-human IgG conjugated to peroxidase enzyme (cat. no.: A6029, Sigma-Aldrich, USA), were added to each well. The secondary antibody used is specific to the gamma chain (γ-chain) of hum-C2 mAbs and was diluted to a ratio of 1:2500 in phosphate buffer before use. The mixtures were again incubated at 37ºC for 60 minutes.

Unbound secondary antibodies were removed by washing and 100 µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate solution (cat. no.: 11112422001 and 11112597001, Roche, Germany) were added. The mixtures were incubated at room temperature in the absence of light for 30 minutes. Finally, the enzyme-substrate reaction was stopped by adding 50 µl of 0.5 normality (N) of sulfuric acid and absorbance was measured at 405 nanometer (nm) using a Synergy HT multi-mode microplate reader (BioTek, USA). A standard curve was plotted and was used to

determine the concentration of secreted hum-C2 mAbs from the selected clones. Supernatants from the untransfected clones, immunopure Mouse IgG (cat. no.: 31202, Roche, Germany) and phosphate buffer (1 M with a pH 7.4) were also used as negative controls.

4.4 RESULTS

4.4.1 Transfection and antibiotic selection

Theoretically, a population of transfected cells should be genetically and phenotypically identical; however, in practice, significant deviations in growth rate and specific productivity are present (Kim et al. 2001; Barnes et al. 2006). Usually, the population consists of an abundance of non- and low-producing clones and only a small number of high-producing clones.

The growth rates of high-producing clones are relatively lower compared to its low- and non-producing counterparts (Hammill et al. 2000). This is because in high-producing clones, a large part of their metabolic resources are utilized for antibody production, thus explaining the lower growth rate. Conversely, the non- and low-producing clones are able to utilize more of their metabolic resources for growth. Hence it is vital to select and isolate the high-producing clones before they are overgrown by their non- and low-producing counterparts.

The elimination of non-transfected clones was done by the addition of 5 mM of L-histidinol dihydrochloride, which inhibits the protein synthesis process of non-

transfected clones. In theory, increasing the concentration of L-histidinol will eliminate the low-producing clones, however higher concentrations of selective antibiotics were observed to be detrimental to all clones, including the high-producers.

Thus, cloning is required to identify and isolate the high-producing cells. The conventional method of cloning uses liquid media which has many drawbacks (Bailey et al. 2002). Due to the nature of liquid media, it is physically impossible to separate the clones individually, and low- and high-producing cells growing together will eventually lead to the clones to be dominated by low-producing cells. Therefore, semi-solid media has been used to immobilize clones and a high-throughput, automated colony picker (ClonePix FL), was used to efficiently isolate monoclonal high-producing clones secreting humanized-C2 monoclonal antibodies. The fluorescence labeling in semi-solid media also allows rapid visualization and discrimination of the rare high secretors from a majority of low producers (Caron et al. 2009).

4.4.2 Screening and selection of transfectomas using ClonePix FL system

Seven days after seeding into semi-solid media, discrete colonies derived from transfected cells could be observed. Secreted hum-C2 mAbs were retained in the vicinity of their associated colonies because of the semi-solid media's high viscosity. Anti-human-Fc capture antibodies conjugated to FITC added to the semi-solid growth media would bind to secreted hum-C2 mAbs to form immunoprecipitates around the colonies. In order to identify high-producing clones, the visible clones were imaged using ClonePix FL system and data from white light and fluorescent images were merged (Figure 4.1).

After screening of all clones, a dot-plot of fluorescence unit (exterior fluorescent intensity) against area of colonies was generated (Figure 4.2). From the plot, it was obvious that the majority of the colonies had intensities of less than 1000 fluorescence units (FU) which served as the threshold for our purposes. From a total of 1×10^5 clones possible, only 271 (~0.271%) clones were above the threshold value of 1000 (FU).

Furthermore, of the 271 significantly producing clones, 217 (~0.217%), 51 (~0.051%) and only 3 (0.003%) clones had readings of between 1000-2000 FU, 2000-3000 FU and 3000-4000 FU, respectively. The extremely low percentage of high producers clearly shows the rationale of using a high-throughput selection method to screen large number of clones. The fact that clones with low fluorescence intensity occupy larger areas, reinforces the fact that non- or low-producing clones had greater growth rates and so it is important to separate them from the high-producers at as earlier a point as possible to prevent the latter from being outcompeted.

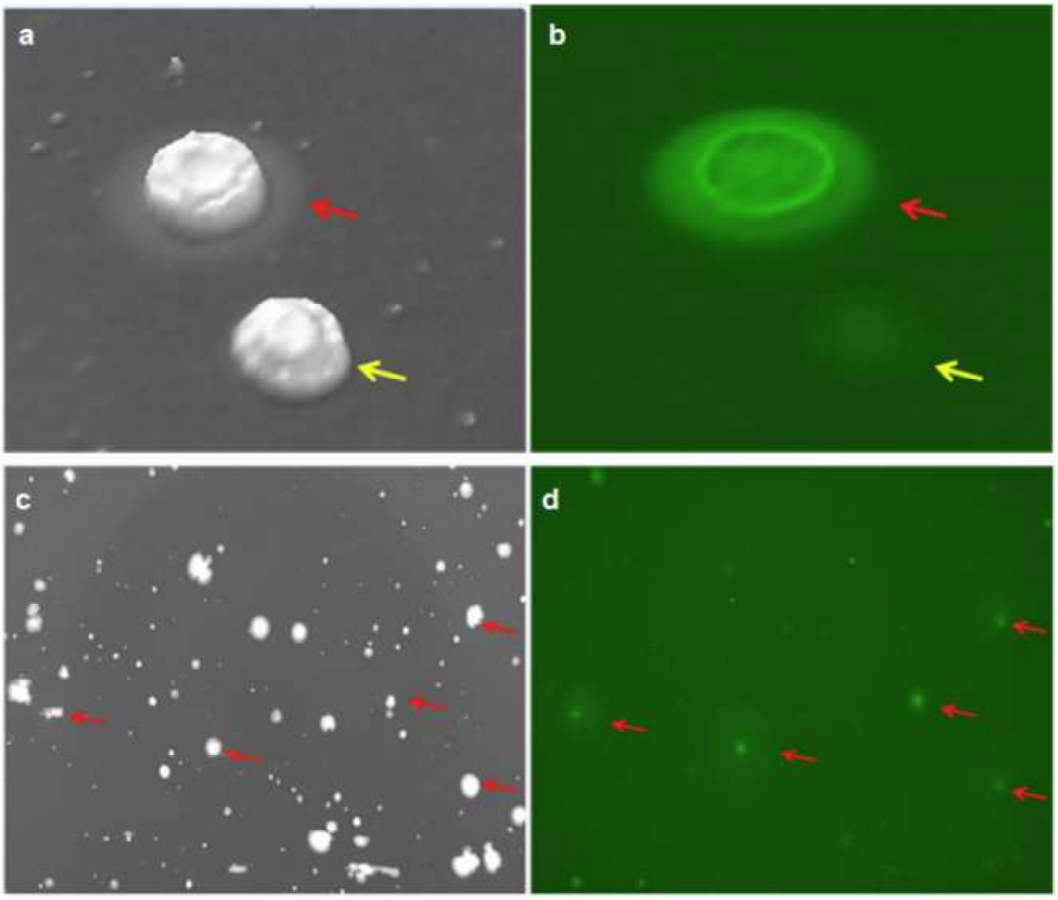

Figure 4.1: Screening of colonies under white light and under fluorescence. Cells which were plated in semi-solid media and incubated to form discrete colonies were imaged under white light (a & c) and fluorescence (b & d), respectively. High- and low-producer clones are depicted by the red and yellow arrows, respectively. In monoclonal antibody producing colonies, precipitations were formed around the respective colonies due to the interaction between secreted humanized anti-C2 monoclonal antibodies from the clones and the capture antibodies conjugated to FITC. Therefore, the greater the quantity of antibodies secreted, the higher the exterior fluorescent intensity displayed (b & d). In fact, the precipitation around the high producer could be visible even under white light (a).

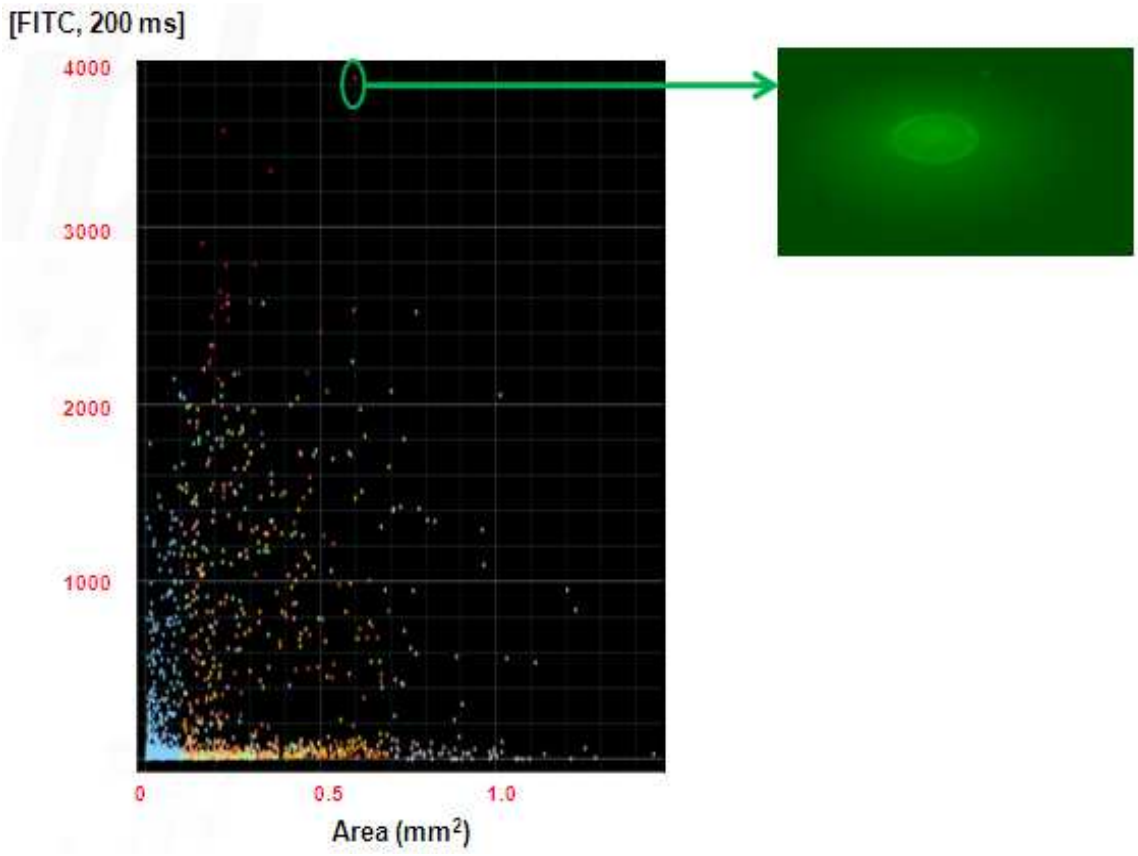

Figure 4.2: Dot-plot analysis of fluorescence unit (FITC, 200 ms) against colony size (Area, mm2). After colonies were imaged, a dot-plot was generated for all the colonies. Each colored dot represents a particular colony, e.g. the dot with highest exterior fluorescent intensity, 3840 FU can be identified as a single discrete colony as shown above. From a total of 1 x 10^5 clones, only 217, 51 and 3 clones were between 1000-2000 FU, 2000-3000 FU and more than 3000 FU, respectively. Almost 99.73% of the clones were low-producers with less than 1000 FU. Another significant trend observed was the decreasing colony size with the increase in fluorescent intensity. Thus, an incubation period of 7-10 days is optimum for high viability of isolated high producers.

4.4.3 Correlation between exterior median intensities of transfectomas and antibody productivity

The correlation between fluorescence intensity and antibody productivity was evaluated in selected clones using ELISA method to measure the levels of antibodies in the supernatants of cells at a concentration of 3 x 10^6 cells/ml. Clones with less than 1000 FU had low productivity of less than 1.0 μg/ml. In clones with 1000-2000 FU, 2000-3000 FU and greater than 3000 FU, there appeared to be no difference in antibody productivity with the increase in fluorescence intensity (Table 4.1). Theoretically, at equivalent cell densities, clones with higher exterior fluorescence intensities should have higher concentration of antibodies being secreted. Furthermore, it has been reported that secreted recombinant protein levels correlated well with their fluorescence intensities (Caron et al. 2009).

However, our results show that at equivalent cell densities of 3 x 10^6 cells/ml, the exterior fluorescence intensities and antibody productivity of our humanized cells does not correlate well for clones above 1000 FU. Some clones having low exterior fluorescence intensity such as clone B4 with 1749 FU had a higher antibody productivity of 11.0 μg/ml compared to 8.6 μg/ml secreted by clone D3 which had the highest fluorescence intensity of 3840 FU.

It was also noticed that clone D2 with fluorescence intensity of 3545 FU, secreted only 1.4 μg/ml. This could be due to instability of the clone which accounts for the subsequent loss of antibody productivity upon further culturing.

Table 4.1: Comparison of exterior fluorescence intensity (FU) and antibody productivity (μg/ml) of clones selected by ClonePix FL system. In general, clones above 1000 FU secretes humanized anti-C2 antibody with at least five times superiority compared to that of clones below 1000 FU. However, no correlation was observed between exterior fluorescence intensity and antibody productivity for clones with more than 1000 FU

Clones	Exterior fluorescence intensity (FU)	Antibody productivity (μg/ml)
	<1000	
Clone A1	273	0.6
Clone A2	508	0.8
Clone A3	573	0.6
Clone A4	870	0.4
Clone A5	937	0.6
	1000-2000	
Clone B1	1107	8.3
Clone B2	1511	6.0
Clone B3	1648	11.4
Clone B4	1749	11.0
Clone B5	1968	7.4
	2000-3000	
Clone C1	2173	11.2
Clone C2	2479	8.8
Clone C3	2786	11.0
Clone C4	2910	7.2
Clone C5	2935	5.0
	>3000	
Clone D1	3318	8.7
Clone D2	3545	1.4
Clone D3	3840	8.6

4.5 DISCUSSION

In general, it can be concluded that among all the clones secreting hum-C2 mAbs, clones with readings of greater than 1000 FU would be considered high producers. However, clones with the highest FU readings do not necessarily mean that they would be the highest producers, and clones chosen for further development would need to be evaluated further to confirm their productivity. Of all the 1 x 10^5 transfected cells seeded, 217 (0.217%) clones had fluorescence readings of greater than 1000 FU and 3 (0.003%) had FU of greater than 3000. Thus it is possible to identify rare high producing cells at a rate of as low as 0.003% of the whole population with a great degree of confidence of monoclonality within 7 days of cloning for further evaluation, which is impossible with other commonly used methods.

Although LDC method is comparably high-throughput in comparison with other traditional selection methods, only a few hundred clones can realistically be characterized, thus increasing the chance of missing out on high-producers due to the relatively low number of cells screened. This method may also take a significantly longer time (1-2 months) to screen for significantly producing cells. A significant problem of LDC method and other conventional methods is that it makes assumptions that high-producing clones obtained are monoclonal, i.e. derived from a single cell. Statistical analysis of the LDC method shows that even after repeated rounds, monoclonality still is not guaranteed (Underwood & Bean, 1988).

Monoclonality is important because in a heterogenous population, non-producing cells will overgrow producing cells and this presents a barrier to the enhancement of product yields. A growth advantage of as little as 9% is sufficient for overgrowth by low- or

non-producers to occur after 25 passages. Thus, the requirement for the production of monoclonal antibodies in industrial cell culture is that the producing cell line is monoclonal (Barnes et al. 2006).

With liquid media, it is impossible to be entirely certain that the cell line generated is derived from a single cell, and cell lines can only be said to have a probability of being monoclonal (Staszewski, 1990). However using ClonePix FL system (Mann, 2007), growing colonies in semi-solid medium allows the clonal progeny of a single cell to stay together. The colonies are therefore monoclonal from the start. In addition, to isolate the high-producing monoclonal clones for downstream applications, an automated colony picker, ClonePix FL system was used. Using pre-programmed software and micro-pins, the desired clones were selected without contamination from neighboring clones. Hence, the clones which were initially monoclonal, remained monoclonal during incubation and even after isolation.

Nevertheless, there are a few other issues of using ClonePix FL system that need to be addressed. First, although by using this method for our hum-C2 mAbs transfected cells, we can separate high-producing colonies from their low producing counterparts, it not advisable to seed more than 1000 cells/ml in semi-solid growth media. This is because the use of more than 1000 cells/ml will increase the risk of overlapping of precipitation of secreted proteins between two or more colonies, hence giving a false positive high fluorescent reading. Second, it is also vital to incubate the clones in semi-solid media for at least 7 days but not more the 10 days prior to picking. This is to ensure that the high-producing clones will have reached a viable size and can be detected. From Figure 4.2, most of high producing clones have small areas compared to low-producers; therefore isolation of clones before 7 days might lead to low viability after isolation.

However, incubation of more than 10 days is discouraged as this can lead to overgrowth of low-producing clones and subsequently the loss of producing clones. Thus the optimum cell concentration and duration of incubation should be between 800-1000 cells/ml and 7-10 days depending on cell type.

4.6 CONCLUSION

The use of semi-solid growth media and the high-throughput ability of ClonePix FL system enable the screening of large number of clones and increase the probability of finding high-producer clones which are extremely rare. These high-producing clones can be isolated in just 7 days are also monoclonal which is important for large-scale production of monoclonal antibodies. In addition, the rapid and fully automated nature of ClonePix FL system is also more time- and labour-efficient and has a lower risk of cross clone contamination. However, because there is no linear proportionality between exterior fluorescence intensity obtained by ClonePix FL system to antibody productivity for clones above 1000 FU, downstream analysis is vital to determine the ‘actual’ and stable high producer clones.