CHAPTER V

APPLICATION OF CYTOTECHNOLOGY TECHNIQUES: A CASE STUDY FOR PRODUCTION AND PURIFICATION OF H1C2 MONOCLONAL ANTIBODY SECRETED BY NS0 TRANSFECTOMA

This chapter was published in *Asia Pacific Journal of Molecular Biology and Biotechnology* (2011), volume 19, issue 2, page 63-71. http://www.msmbb.org.my/apjmbb/html192/192b.pdf.

.

5.1 OBJECTIVE

The main objective of this chapter is to obtain purified form of humanized anti-C2 mAbs developed by deimmunization method (H1C2 mAb). The NS0 transfectoma secreting the highest level of H1C2 mAb from chapter 4 was subjected to serum-free adaptation in this chapter. The serum-free adapted NS0 transfectoma was then cultured in triple flasks for small-scale production of H1C2 mAb. An automated liquid chromatography system, Äktaprime Plus was used to purify H1C2 mAb from the serum-free adapted NS0 cell culture supernatant. Although not presented in this chapter, the NS0 transfectoma secreting the highest amount of H2C2 mAb (logical approach method) from chapter 4, was adapted to serum-free medium by a similar procedure. The H2C2 mAb were also purified using the same methodologies as the H1C2 mAb. In addition, although also not included in this chapter, the NS0 transfectomas and hybridomas cells secreting chimeric and mouse anti-C2 mAbs, respectively, were similarly adapted to serum-free media and the mAbs were similarly purified. Both cell lines secreting chimeric and mouse anti-C2 mAbs were provided in the technology transfer. The functionality and immunogenicity of both H1C2 and H2C2 mAbs and also the chimeric and mouse anti-C2 mAbs were characterized and compared as described in chapter 6.

5.2 INTRODUCTION

In chapter 4, (Dharshanan et al. 2011a), the isolation of monoclonal NS0 transfectomas secreting humanized anti-C2 monoclonal antibodies (H1C2 mAbs) using the ClonePix FL system was reported.

Initially, these H1C2 mAb producing NS0 transfectoma cells were cultured and maintained in cell culture media containing fetal bovine serum; however the use of serum involves ethical, scientific and safety complications (Even et al. 2006). Therefore, it is crucial to adapt and maintain the transfectoma cells in serum-free growth media. Unfortunately, the versatile function of serum makes its removal from cell culture media a challenging task especially in non-static conditions such as in spinner flasks.

Besides that, the purification of mAb is usually done using conventional affinity chromatography method, owing to its low cost. However, this method has two main disadvantages. First, being non-automated in nature, it is labor-intensive, time-consuming and increases the risk of errors, such as spillage or misplacing of tubes. Second, the lack of real-time monitoring in the traditional method often requires subsequent downstream analysis to determine the exact fractions which contain the purified antibodies.

Hence, in this case study, we report on the application of cytotechnology techniques to circumvent the disadvantages and limitations associated with the use of serum, spinner flasks and conventional antibody purification method. First, the use of serum and its attendant complications were eliminated by directly adapting the serum-dependent NS0

transfectoma cells to a commercial serum-free media for hybridoma cells containing a synthetic cholesterol supplement. Then, the low viability of the serum-independent NS0 transfectomas during the production in spinner flasks was overcome by using triple flasks instead. Third, an automated liquid chromatography system, Äktaprime Plus, was used to purify antibodies, replacing the conventional method which is time-consuming, laborious and prone to errors.

5.3 MATERIALS AND METHODS

5.3.1 Isolation of monoclonal NS0 transfectoma secreting H1C2 mAb

Monoclonal NS0 transfectomas secreting humanized anti-C2 mAb (H1C2 mAb) were developed using methods described in chapter 3 and chapter 4 (Dharshanan et al. 2011a).

5.3.2 Adaptation of NS0 transfectoma in serum-free media

For subsequent downstream applications, monoclonal NS0 A33-transfectoma (NS0-TFA33) was chosen due to its high H1C2 mAb productivity compared with other monoclonal transfectoma clones. To circumvent the problems associated with the use of serum, NS0-TFA33 cells were directly adapted into serum-free media, SGFM. Briefly, NS0-TFA33 cells growing at the exponential phase in serum-supplemented media were dislodged from the surface of a T75 flask by tapping and transferred to a 50 ml centrifuge tube.

The cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 25ºC. The supernatant was discarded and the cells were resuspended in a sterile pH 7.4 phosphate buffer (1 M) (containing a mixture of 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of K_2HPO_4 and 0.24 g of KH_2PO_4 dissolved in 1 L of sterile water) and centrifuged again at 1000 rpm for 5 minutes at 25ºC.

The washing step was repeated at least twice to ensure that all previous media which contained serum were completely removed. After that, approximately 2×10^6 of NS0-TFA33 cells were inoculated into new T75 flasks containing 20 ml of SFGM which was made up of hybridoma-SFM (cat. no.: 12045, Life Technologies, USA) and 1% (v/v) synthechol (cat. no.: S5442, Sigma-Aldrich, USA).

The transfectomas were then incubated at 37ºC with 5% CO_2 to allow the adaptation and growth in a serum-free environment. The viable cell concentration and percentage cell viability in SFGM were determined every 24 hours by Trypan blue dye exclusion assay. In this assay, 10 µl of serum-independent NS0-TFA33 cells were taken and transferred to a 0.6 ml centrifuge tube containing 90 µl of Trypan blue dye (Biochrom, Germany). After the mixture was briefly vortexed, 10 µl of the mixture were then loaded onto a haemocytometer and using an inverted microscope, the number of viable and the non-viable cells were determined.

Once the serum-independent NS0-TFA33 cells in T75 flasks had reached a concentration of 3×10^6 cells/ml with $\geq$ 90% viability in SFGM even after five passages, the cells were harvested and cryopreserved. For cryopreservation, approximately 3×10^7 of the serum-independent NS0-TFA33 cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 25ºC and then resuspended in a serum-free cell freezing media

containing DMSO (cat. no.: C6295, Sigma-Aldrich, USA). The mixture was cooled gradually at -80ºC using CoolCell (Biocision, USA) and then transferred to liquid nitrogen for long term storage.

5.3.3 Production of NS0 transfectoma in serum-free media

Initially, spinner flasks were used for the production of 1 L of the serum-independent NS0-TFA33 cells but despite repeated trials, the viability of the transfectoma decreased rapidly after 48 hours. As a result, 5 triple flasks (Thermo Fisher Scientific, USA), with a working volume of 200 ml each were used instead. A total of 2×10^7 serum-independent NS0-TFA33 cells were suspended in 200 ml of SFGM and then transferred to a triple flask. The triple flasks were then incubated at 37ºC with 5% CO_2 to allow cell multiplication and H1C2 mAb secretion for seven days. The viable cell concentration, percentage cell viability and H1C2 mAb concentration in SFGM were determined every 24 hours using Trypan blue dye exclusion assay and quantitative ELISA as described in chapter 4 (Dharshanan et al. 2011a). At the end of seven days, all the supernatants were pooled.

5.3.4 Purification of H1C2 mAb

The mAb in the pooled supernatant was purified by chromatography using the Äktaprime Plus system (GE Healthcare, USA). First, the cell debris was removed by centrifuging the supernatant in 50 ml centrifuge tubes at 6000 rcf at 4ºC for 10 minutes. The clarified supernatant was subjected to tangential flow filtration (TFF) using Vivascience Vivaflow 200 (Sartorius, Germany). During TFF, the supernatant was

filtered through a membrane with a molecular weight cut-off (MWCO) value of 50 kDa and also was simultaneously concentrated from 1 L to approximately 50 ml.

Protein in the concentrated supernatant was precipitated with ammonium sulfate ($(NH_4)_2SO_4$). Fifty ml of 80% (w/v) concentrated $(NH_4)_2SO_4$ were added in drop-wise manner to 50 ml of filtered and concentrated cell culture supernatant. The mixture was then incubated at 4ºC for 60 minutes with constant stirring.

After 60 minutes, the mixture was centrifuged at 20 000 rcf at 4ºC for 15 minutes and the supernatant was carefully discarded. The pellet was dissolved in 3 ml of pH 7.4 phosphate buffer (1 M). The $(NH_4)_2SO_4$ was removed by transferring the mixture to dialysis tubing with MWCO value of 50 kDa and was dialyzed against 3 changes of 2 L of sterile water. Water was changed at 3 hour intervals. Later, the H1C2 mAb was conditioned to pH 7.4 phosphate buffer (1 M) by substituting the sterile water with 3 L of the phosphate buffer and dialysis was continued at 25^{o}C against 6 changes of buffer, with 3 hours between each change.

Prior to the purification of H1C2 mAb, the HiTrap Protein A HP 1 ml column (GE Healthcare, USA) and Äktaprime Plus system were flushed and calibrated according to the manufacturer's instructions. The H1C2 mAb was then purified through a pre-programmed protocol in Äktaprime Plus system using pH 7.4 phosphate buffer (1 M) as the binding buffer, sterile water as the washing buffer and a commercial IgG Elution Buffer with a pH of 2.8 (Cat. no. 21004, Thermo Fisher Scientific, USA) as the elution buffer. Finally, purified H1C2 mAb was immediately conditioned back to pH 7.4 phosphate buffer (1 M) by dialysis and stored at -20ºC.

5.4. RESULTS AND DISCUSSION

5.4.1 Adaptation of NS0 transfectoma in serum-free media

Fetal bovine serum is a complex mixture containing a large number of components such as growth factors, hormones, transport proteins, detoxifying agents, attachments factors and protease inhibitors. It also provides nutrients, purines, pyrimidines, vitamins, trace elements, inorganic and shear protective compounds which are all essential for the growth and support of NS0 cells (Keenan et al. 2006). However, the use of serum is problematic for a number of reasons. First, the collection of serum involves animal welfare issues as it is obtained from the unborn calf (Valk et al. 2004). Second, the qualitative and quantitative variation of the serum composition results in batch-to-batch variations, which explains the lack of reproducibility of experiments (Kannan et al. 2009).

Third, being a chemically undefined mixture, serum poses a potential source of contaminants and pathogens such bacterial endotoxins, fungi, mycoplasma, prions and viruses (Even et al. 2007; Falkner et al. 2006), thus rendering any mAbs produced unsuitable for human use. In fact, Wessman and Levings (1999) reported that between 20-50% of commercial serum were virus-positive. Fourth, although only 5-10% (v/v) of serum is usually used for mammalian cell culture, it is still very costly as serum accounts for up to 70% of the cost of overall media formulation.

Another major drawback with the use of serum for the production of H1C2 mAbs is the high protein content especially from bovine polyclonal IgG antibodies (bIgGs) (Rasmussen et al. 2005). These contaminating bIgGs and other exogenous proteins will

subsequently be introduced into the pool of expressed H1C2 mAbs and may exceed the concentration of the H1C2 mAbs product by five- to several thousand-fold (Even et al. 2007).

Using protein A affinity chromatography (Swinnen et al. 2007), it is possible to purify the H1C2 mAbs from most contaminating exogenous proteins except for the bIgGs. Protein A has a high affinity to the Fc-region of H1C2 mAb but also a low undesired affinity to the Fc-region of bIgG (Hober et al. 2007; Huse et al. 2002). Thus, even after protein A affinity chromatography, the use of serum during NS0-TFA33 production may still result in H1C2 mAbs being contaminated with bIgGs.

Several methods could be employed to circumvent this problem. One method is to perform a two-step chromatography, with protein A as the capture step and ion-exchange (Jiskoot et al. 1991) or hydrophobic interaction (Grunfeld & Moore, 1997) as the second step. Although it is possible to obtain H1C2 mAb with reduced bIgGs contamination by this method (Aybay & Imir, 2000), the increase in the number of purification steps will subsequently lower the yield of H1C2 mAb. Another approach is to employ a single-step chromatography procedure using protein L. This method has been used to purify H1C2 mAb from NS0-TFA33 cells cultured in growth media containing 5% (v/v) serum.

Unlike protein A, protein L only binds to the kappa light chains of human antibodies and does not bind to the Fc-region of human nor bovine antibodies. As a result, even with the use of serum-supplemented media, it should be possible to obtain pure H1C2 mAbs without the contamination of exogenous proteins and even bIgGs. Unfortunately, although H1C2 mAb consists of two kappa light chains, we were unable to purify H1C2

mAbs using protein L affinity chromatography. This may be due to the fact that protein L only recognizes certain subclasses of kappa light chains and not that of H1C2 mAb.

The next possible option was to culture the serum-dependent NS0 transfectomas using commercial serum with depleted bIgGs, however this option was unfavorable because serum with depleted bIgGs is costly and issues concerning contamination with hazardous substances associated with the use of serum are still present. In order to overcome these concerns it would be most advantageous to adapt the NS0-TFA33 cells to serum-free culture conditions.

In this study, we have successfully adapted NS0-TFA33 directly from serum-supplemented media into serum-free media, SFGM, which consists of a serum-free media (SFM) for hybridoma cells with the addition of 1% (v/v) synthechol. The cell growth and viability in both original serum supplemented medium as well as SFGM were similar. The NS0-TFA33 initially inoculated at 0.1×10^6 cells/ml, was able to reach to a concentration of 3.25×10^6 cells/ml in just 5 days even after several passages. The fact the serum-dependent NS0-TFA33 could be directly switched from a serum-supplemented medium to a serum-free medium clearly demonstrates the analogy in nutrient components and composition in serum-supplemented media and SFGM required for the growth and support of NS0-TFA33 cells.

Synthechol, which is synthetic cholesterol, was added to SFGM because unlike hybridoma cells, NS0 cells are cholesterol-auxotrophic due to the demethylation of lanosterol to C-29 sterols (Seth et al. 2006). While antibiotics had been used previously in serum-supplemented media, it was not added in SFGM because the use of antibiotics may result in a decrease in cell viability of serum-independent NS0-TFA33 cells, most

likely due to the absence of certain protective components available in serum (Valk et al. 2010). Furthermore, the addition of antibiotics in SFGM was not necessary because compared to serum-supplemented media, the SFGM is less susceptible to microbial contamination.

5.4.2 Production of NS0 transfectoma in serum-free media

For small-scale production, initially, spinner flasks were employed. Three spinner flasks containing 50 ml of SFGM were inoculated with 0.1×10^6 cells/ml of serum-independent NS0-TFA33 cells and one spinner flask containing 50 ml of serum-supplemented media was inoculated with serum-dependent NS0-TFA33 cells. After 48 hours, the cell viability in all three flasks containing SFGM had decreased to approximately 30%, but the cells cultured in serum-supplemented media had 94% of cell viability. Since all other parameters were constant, SFGM obviously lacked the shear protective compounds present in serum. It was also observed that the use of spinner flasks often resulted in the undesired increase of the temperature in the CO_2 incubator which could be caused by the frictional-heat effect during magnetic agitation.

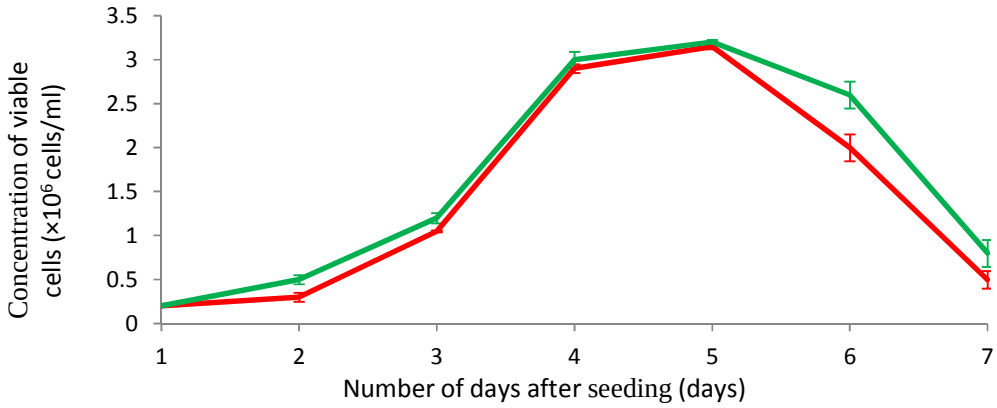
Therefore, triple flasks which are disposable cell culture vessels each having three growth surfaces totaling 500 cm^2, were used instead. Since triple flasks are static cell culture vessels similar to T75 flasks, the shear effect and undesired temperature increase associated with the use of spinner flasks were no longer problems.

As expected, the serum-independent NS0-TFA33 cells had only slightly lower viable cell concentration, percentage of viable cells and H1C2 mAb productivity compared to their serum-dependent counterparts when both were cultured in triple flasks (Figure

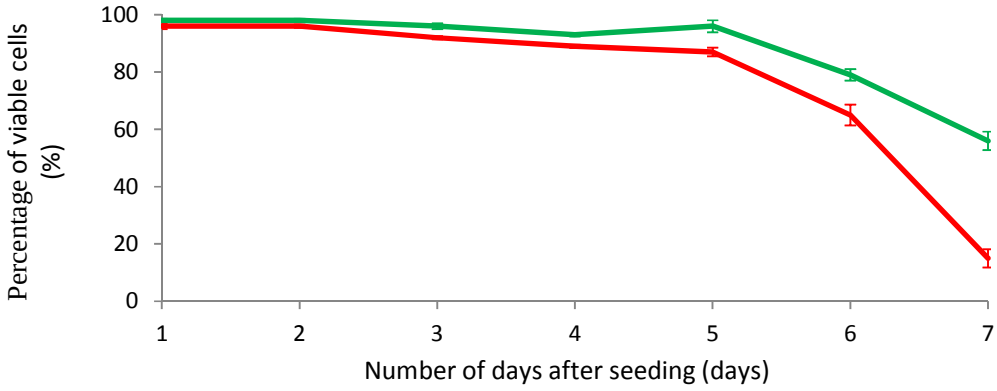
5.1). After 7 days, the supernatant from serum-independent NS0-TFA33 were collected because the viable cell concentration and percentage of viable cells had decreased to 0.4 $\times 10^6$ cells/ml and 14% , respectively (Figure 5.1a and 5.1b).

It was felt that further incubation would not significantly increase the concentration of H1C2 mAb in the cell culture supernatants (Figure 5.1c). Although the use of triple flasks might have overcome the shear effect present with the use of spinner flasks, triple flasks are more suitable for small-scale production, and for medium- and large-scale production, stirrer-based bioreactors might still have to be used. Therefore, for medium and large-scale production, pluronic, a shear protective compound might be used to combat the shear effect of spinner flasks on NS0 transfectomas (Whitford, 2003).

a)



b)



c)

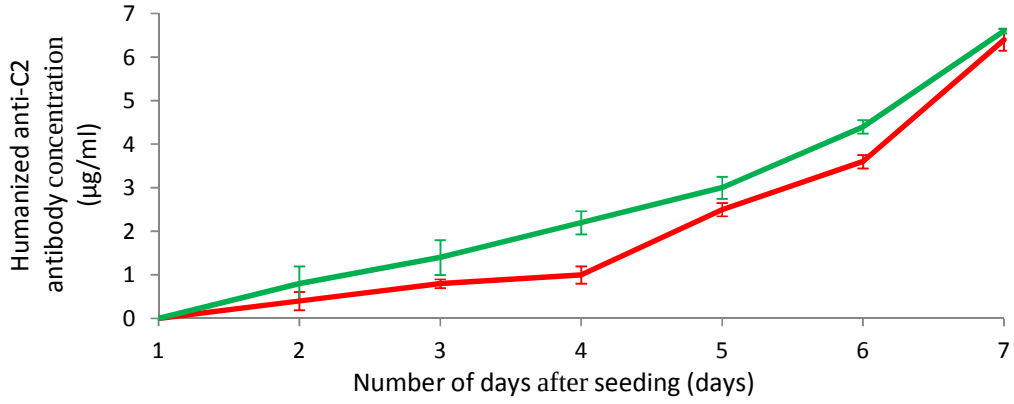


Figure 5.1: Comparison of growth characteristics and productivity of NS0 A33-transfectomas cultured in the absence (red line) or presence of serum (green line) using triple flasks. The viable cell concentrations (a), percentage of viable cells (b) and concentration of humanized anti-C2 monoclonal antibody (H1C2 mAb) in cell culture supernatant (c) of NS0 A33-transfectomas cultured in SFGM were all slightly lower compared to that of cells cultured in serum-supplemented media.

5.4.3 Purification of H1C2 mAb

Ammonium sulfate precipitation was done prior to the purification of H1C2 mAbs using protein A affinity chromatography. To reduce the total $(NH_4)_2SO_4$ needed the supernatant was concentrated to 50 ml from the original 1 L using TFF. Thus the quantity of $(NH_4)_2SO_4$ required was reduced from 800 g to 40 g.

Äktaprime Plus system was used to purify the H1C2 mAbs. Using this system and a HiTrap Protein A HP column, H1C2 mAbs in 10 ml of phosphate buffer was purified in less than 30 minutes (Figure 5.2), rather than around 6 hours if conventional method were used instead. The significant reduction in the duration was possible because of the use of a built-in pump in Äktaprime Plus that contributed to the high flow-rate, whereas the absence of a pump in the conventional method meant that the flow-rate was entirely dependent on gravitational force. The built-in fraction collector in Äktaprime Plus also allowed the automated collection of fractions during the elution process which minimized the risk of spillage or misplaced tubes which are common during conventional method of antibody purification.

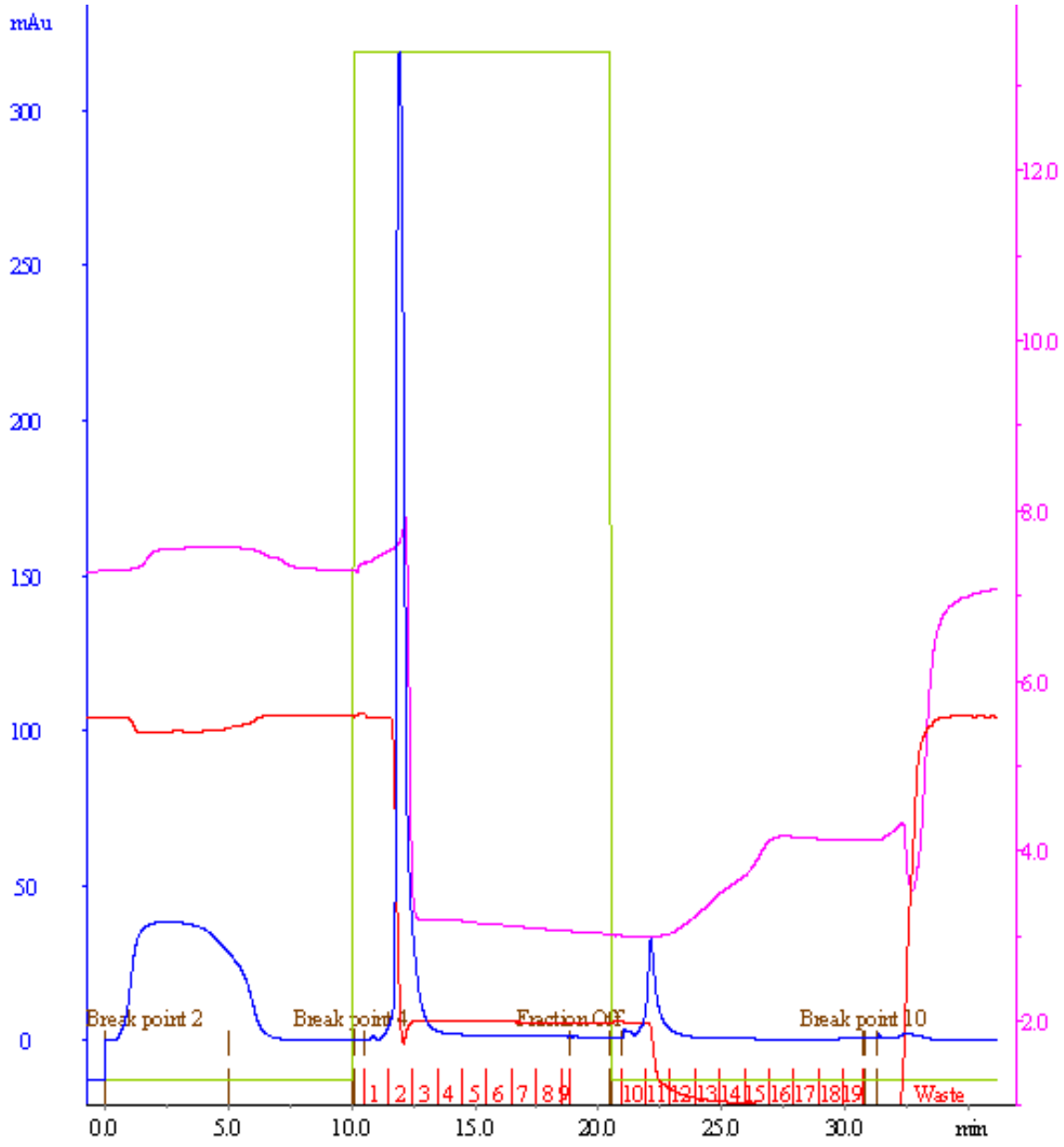


Figure 5.2: Real-time chromatograms obtained during purification of humanized anti-C2 monoclonal antibodies (H1C2 mAb) using Äktaprime Plus. Various parameters such as pH (pink line), conductivity (red line) and UV absorbance at 280 nm (blue line) were displayed by using PrimeView software. The green line depicts the start to end of elution process and together with the built-in pump and fraction collector; the humanized antibodies were purified in just 30 minutes. From the real-time monitoring, the increase in UV absorbance shows that the H1C2 mAb are eluted in fraction 2 and 3.

The PrimeView software of Äktaprime Plus system allowed real-time monitoring of the H1C2 mAb as well as providing vital information such as the pH, temperature, conductivity and pressure in the column during the purification process. During purification of H1C2 mAb, the increase in UV absorbance at 280 nm (Figure 5.2), directly confirmed that H1C2 mAb was eluted in fractions 2 and 3 only: therefore a downstream analysis to determine the antibody containing fractions was not required as in the conventional method.

5.5 CONCLUSION

In conclusion, we have demonstrated that it is possible to directly adapt a serum-dependent mAb secreting NS0 cells into serum-free environment using SFGM. Although the serum-independent NS0 transfectomas were able to grow reasonably well in SFGM using T75 flasks, it failed to grow in spinner flasks. Therefore, for small-scale production of H1C2 mAbs, the serum-independent NS0 transfectomas were cultured using triple flasks. The use of Äktaprime Plus system also enabled the purification of H1C2 mAbs to be carried out smoothly, effectively and in much shorter time compared to the conventional method of antibody purification. We have also applied the same technologies to grow cells producing H2C2, chimeric and mouse anti-C2 mAbs, which were all purified similarly.