CHAPTER VII

STABLE EXPRESSION OF H1C2 MONOCLONAL ANTIBODY IN NS0 AND CHO CELLS USING pFUSE AND UCOE EXPRESSION SYSTEM

This chapter was submitted to *Cytotechnology* journal for consideration of publication.

7.1 OBJECTIVE

From chapter 6, it was found that the H1C2 mAb developed using deimmunization method had reduced immunogenicity compared to H2C2 mAb which was developed using logical approach method. Therefore, it was concluded that the deimmunization method is superior and should be applied for the development of humanized mAbs. Nevertheless, the overlapping-PCR mutagenesis used to humanize amphipathic mouse amino acids to their corresponding human amino acids is laborious and time-consuming. This is due to the high-error rate of *Taq* DNA polymerase which introduces undesired mutations in the DNA of mouse anti-C2 variable regions thus requiring large number of DNA samples for DNA sequence determination. Besides that, the use of monocistronic vectors, pAH4602 and pAG4622, in previous chapters to express H1C2 mAb in NS0 mammalian cells showed productivity of only 6 mg/L in static-based culture vessels. In addition to the relatively low yield, NS0 mammalian cells are less preferred for large-scale production compared to the more robust Chinese hamster ovary cells (CHO). Therefore, in this chapter, H1C2 mAb was redeveloped by deimmunization method but using synthetic DNA coding the variable regions. Besides that, two expression vectors, pFUSE (monocistronic) and UCOE (monocistronic and bicistronic), were used to express H1C2 mAb in NS0 cells and also in CHO cells. The productivity of H1C2 mAb in both cell lines using the two different expression vectors were then compared.

7.2 INTRODUCTION

In chapters 3-5, (Dharshanan et al. 2011a; Dharshanan et al. 2011b), the development and production of humanized anti-C2 monoclonal antibodies (H1C2 and H2C2 mAbs) using two different humanization methods had been reported. In chapter 6, it was found that deimmunization method (H1C2 mAb) elicited the least immunogenic response in monkey and therefore was most suitable for the development of humanized mAbs. However, there is a disadvantage in the use of overlapping-PCR mutagenesis to humanize potential immunogenic mouse residues in the deimmunization method as it frequently results in undesired mutations.

Besides that, despite the fact that significant improvements have also been made to increase mAbs yields through optimization of culture conditions and media formulations, more extensive understanding of cellular metabolism and processes that enable transfectoma cells to grow at higher densities for extended periods, mammalian cell culture still remains an expensive process with long development time (Browne & Al-Rubeai, 2007). An important reason to this is because the protein expression levels in mammalian cells are relatively low and often unstable over time, resulting in high costs and long duration for the development and production of therapeutic proteins. Hence, it is crucial to optimize the *in vitro* mammalian cell production system in order to increase culture yields and stability while also to decrease the duration for the development and production of humanized mAbs.

While there are other various expression systems available for the production of therapeutic proteins, including bacterial, yeast, plant and insect cells, nevertheless mammalian cells, despite lower yields, continue to be the system of choice owing to

their similarity to human cells with respect to post-translational modifications and glycosylation patterns that cannot be adequately reproduced in alternative systems (Browne & Al-Rubeai, 2007).

This is an important factor because lack of fidelity can affect protein stability and antigen binding and could lead to increased immunogenicity and rapid clearance especially if these mAbs are intended for repeated use as therapeutic agents in humans (Wurm, 2004). As a result, most marketed biopharmaceutical products have been produced in mammalian cells such as Chinese hamster ovary (CHO) cells, murine myeloma lymphoblastoid-like (NS0 and Sp2/0-Ag14) cells, human embryonic kidney 293 cells and baby hamster kidney cells (Zhu, 2011). Of these mammalian cells, two have been most commonly used for the production of recombinant antibodies: CHO and NS0 cell lines.

In this chapter, 3 modifications from previous experiments were employed with the view of finding the optimum conditions for the production of mAbs in terms of yield and cost. First, instead of the time-consuming and laborious overlapping-PCR mutagenesis used previously to humanize potential immunogenic mouse residues to its corresponding human residues, it was replaced with the use of synthetic DNAs which were pre-designed to contain all the desired humanized residues in its DNA sequences. Second, instead of the monocistronic pAH4602 and pAG4622 expression vectors used in previous chapters, two different monocistronic expression vector systems, pFUSE (pFUSE-CHIg-hG1 and pFUSE-CLIg-hk) and UCOE (CET1019AS) were used. In addition, a bicistronic UCOE expression vector system (CET1019AD) was also employed. The pFUSE vectors contain human antibody constant regions, while the UCOE expression vectors contain ubiquitous chromatin opening elements (UCOE) but

lack the human antibody constant regions. Third, aside from NS0 cells, CHO cells were also transfected with the pFUSE and UCOE expression vector systems. The productivity of the two expression vectors in both cell lines were compared, and the combination of expression vector and cell line that resulted in highest and most stable production of H1C2 mAbs were chosen for large-scale production. Although NS0 and CHO cell lines are the two commonly used cell line for the production of recombinant proteins, nevertheless CHO cells are more robust and therefore preferred for large-scale production compared to NS0 cells.

7.3 MATERIALS AND METHODS

In this experiment, various expression vectors containing synthetic genes were constructed using pFUSE and UCOE expression vectors. These vectors were transfected into NS0 and CHO cells as summarized in Table 7.1.

Table 7.1: Summary of combinations of vectors used for the expression of H1C2 mAbs in NS0 and CHO cells. For pFUSE vectors both linearized and unlinearized vectors were employed whereas for UCOE vectors only linearized vectors were used

	Expression vector system used		
Cells	**pFUSE monocistronic vectors**	**UCOE monocistronic vectors**	**UCOE bicistronic vectors**
NS0	• Linearized • Unlinearized	• Linearized	• Linearized
CHO	• Linearized • Unlinearized	• Linearized	• Linearized

7.3.1 Construction of expression vectors with synthetic genes coding the variable region of HIC2 mAb

7.3.1.1 Monocistronic pFUSE expression vectors

Monocistronic expression vectors, pFUSE-CHIG-hG1 and pFUSe2-CLIg-hk (Invivogen, USA) which contain the human IgG1 constant regions and the human kappa constant region, respectively, were used to co-express the heavy and the light chain of H1C2 mAb. However, instead of using the physical DNA coding humanized variable regions developed in chapter 3, the exact synthetic copies of DNA were designed and ligated into the expression vectors.

The physical DNAs, humanized-VH4A and humanized-VL3A from chapter 3, were substituted with the corresponding synthetic copies, syn-VH4A and syn-VL3A. Both synthetic genes were synthesized and ligated into pIDTSMART vectors at Integrated DNA Technologies, USA. The resulting vectors were named pSMART-hVH and pSMART-hVL.

The cloning vectors containing the synthetic genes were transformed in *E. coli* and the recombinant plasmids were isolated as described in sections 3.3.1.8-10. The expression vectors, pFUSE-CHIG-hG1 and pFUSe2-CLIg-hk were also transformed and isolated as described in section 3.3.1.8-10 but with minor modifications.

The LB-agar and LB-broth containing ampicillin were substituted with *E. coli* Fast-Media agar and broth containing zeocin (cat. no.: fas-zn-s and fas-zn-l, InvivoGen, USA) for pFUSE-CHIG-hG1 vectors and *E. coli* Fast-Media agar and broth containing

blasticidin (cat. no.: fas-bl-s and fas-bl-l, InvivoGen, USA) for pFUSe2-CLIg-hk. To facilitate the cloning of the synthetic genes into its respective expression vectors, the synthetic genes were also pre-designed and flanked with DNA sequences recognized by specific restriction enzymes as shown below:

1. syn-hVH4A: *EcoR*V (5') and *Nhe*I (3')
2. syn-hVL3A: *Age*I (5') and *BsiW*I (3')

For the subcloning of syn-hVH4A into pFUSE-CHIG-hG1, double-digestions were performed by adding 3.5 µl of each pSMART-hVH and pFUSE-CHIG-hG to 46.5 µl of digestion mixture 1 (section 3.3.3.1.2) and incubated at 37ºC for 1 hour. Similarly, for the subcloning of syn-hVL3A into pFUSe2-CLIg-hk, 3.5 µl of each pSMART-hVL and pFUSe2-CLIg-hk were added to 46.5 µl of digestion mixture 4 and incubated at 37ºC for 1 hour. Then, the digested DNA fragments of pSMART-hVL and pFUSe2-CLIg-hk were purified and 44.0 µl each of the purified digested vectors were added to 6 µl of digestion mixture 5 and incubated at 55ºC for 1 hour.

The digestion mixture 4 consists of 5.0 µl of buffer 1, 0.5 µl of BSA (100x), 0.5 µl of *Age*I and 40.5 µl sterile water, while digestion mixture 5 is made up of 5.0 µl of buffer 3, 0.5 µl of BSA (100x), and 0.5 µl*BsiW*I. After each digestion, the restriction enzymes were inactivated by incubating the mixtures at 80ºC for 20 minutes and the digested DNA fragments were subsequently purified using QIAquick PCR purification kit (Qiagen, Germany) following protocols recommended by the manufacturer.

The ligation of syn-hVH4A into pFUSE-CHIG-hG1 and syn-hVL3A into pFUSe2-CLIg-hk, were performed as described in section 3.3.3.1.3. Briefly, 6.0 µl of double-

digested syn-hVH4A and syn-hVL3A fragments were added to 2.0 µl of double-digested pFUSE-CHIG-hG1 and pFUSe2-CLIg-hk, respectively, in 0.6 ml centrifuge tubes. Then 1.0 µl of T4 DNA ligase and 1.0 µl of ligase buffer (10X) were added to both ligation mixtures.

The mixtures were incubated at 15ºC for 16 hours. The ligation products were then transformed in *E. coli* and the resulting recombinant vectors were named pFUSE-hVH (containing syn-hVH4A) and pFUSE-hVL (containing syn-hVL3A). The pFUSE-hVH vectors were isolated using *E. coli* Fast-Media agar and broth containing zeocin, while pFUSE-hVL vectors were isolated using *E. coli* Fast-Media agar and broth containing blasticidin as described in section 3.3.1.8. The vectors were then extracted using QIAprep Miniprep kit as described in section 3.3.1.9-10 and analyzed by agarose gel electrophoresis as described in section 3.3.1.5.

The bacteria clones having the vectors of desired size were subjected to large-scale vector extraction using QIAGEN Maxiprep kit as described in section 3.3.3.1.4 and were then quantified using a NanoPhotometer (Implen, Germany). The pFUSE-hVH and pFUSE-hVL expression vectors were then linearized using digestion mixture 3 as described 3.3.3.1.5 but the *Pvu*I enzyme was substituted with *Not*I restriction enzyme. The linearized vectors were then purified also as described in section 3.3.3.1.5 and were used for co-transfection in NS0 and CHO cells.

7.3.1.2 Monocistronic UCOE expression vectors

Unlike monocistronic pFUSE vectors, the monocistronic UCOE vector (CET1019AS) (Milipore, USA) does not contain the human antibody constant regions. Therefore, in

addition to the synthetic DNA variable region of HIC2 mAb, synthetic DNA coding the human antibody constant regions from pFUSE vectors were also designed.

The resulting synthetic genes designed are designated as follows:

(i) syn-hVH4A-hCHm: this is the gene sequence coding the humanized heavy region of H1C2 mAb (hVH4A) and the human IgG1 constant region of heavy chain (hCHm),

(ii) syn-hVL3A-hCL: this is the synthetic gene sequence coding the humanized light region of H1C2 mAb (hVL3A) and the Ig Kappa constant region (hCL).

The syn-hVL3A-hCL and syn-hVH4A-hCHm synthetic genes were synthesized and ligated into pIDTSMART and pUC vectors, respectively, at Integrated DNA Technologies, USA. The resulting vectors were named pSMART-hVL-hCL and pUC-hVH-hCHm. Both synthetic genes were separately cloned into monocistronic UCOE vector, CET1019AS. To facilitate the cloning of the synthetic genes into the expression vector, the genes were flanked with DNA sequences recognized by specific restriction enzymes shown below:

1. syn-hVH4A-hCHm: *NgoMI*V (5' and 3')
2. syn-hVL3A-hCL: *NgoMI*V (5') and *Nhe*I (3').

For the subcloning of syn-hVL3A-hCL in CET1019AS, digestions were performed by adding 3.5 µl of each pSMART-hVL-hCL and CET1019AS to 46.5 µl of digestion mixture 6 and incubated at 37ºC for 2 hours. Similarly, for the cloning of syn-hVH4A-hCHm in CET1019AS, 3.5 µl of each pSMART-hVH-hCHm and CET1019AS were added to 46.5 µl of digestion mixture 7 and incubated at 37ºC for 2 hours. Digestion

mixture 6 consists of 5.0 µl of buffer 4, 0.5 µl of BSA (100x), 0.5 µl of *Nhe*I, 0.5 µl of *NgoMIV* and 40.0 µl sterile water, while digestion mixture 7 contains the same components as digestion mixture 6, with the exception that 0.5 µl of *Nhe*I was substituted with sterile water.

After each digestion, the restriction enzymes were inactivated by incubating the mixtures at 80ºC for 20 minutes and the digested DNA fragments were subsequently purified using QIAquick PCR purification kit (Qiagen, Germany). Since the CET1019AS, pIDTSMART and pUC vectors all have the same ampicillin resistance gene, therefore to prevent self-ligations, calf intestinal alkaline phosphatase (CIAP) (Life Technologies, USA) were used to remove the phosphate from the 5'-end of the digested fragments of pSMART-hVL-hCL and pUC-hVH-hCHm.

The phosphate removal was executed by adding 1.0 µl of CIAP and 5.0 µl of CIAP buffer to 44.0 µl each of the purified digested fragments of pSMART-hVL-hCL and pUC-hVH-hCHm. The reactions were performed at 37ºC for 2 hours and the DNA fragments lacking the 5' phosphoryl termini were again purified using QIAquick PCR purification kit (Qiagen, Germany).

For ligation of syn-hVL3A-hCL into CET1019AS vector, 6.0 µl of the purified digested fragments of pSMART-hVL-hCL lacking the 5' phosphoryl termini, 1.0 µl of T4 DNA ligase and 1.0 µl of T4 DNA ligase buffer (10x) were added to 2.0 µl of the digested CET1019AS vectors purified from digestion mixture 6. Similarly for the ligation of syn-hVH4A-hCHm into CET1019AS vector, 6.0 µl of the purified digested fragments of pSMART-hVH-hCHm lacking the 5' phosphoryl termini, 1.0 µl of T4 DNA ligase

and 1.0 µl of T4 DNA ligase buffer (10x) were added to 2.0 µl of the digested CET1019AS vector purified from digestion mixture 7.

All ligations were then performed at 15ºC for 16 hours. The transformation and vector extraction were done as described in sections 3.3.1.8-10 and the resulting vectors were named pAS-hVL-hCL (containing syn-hVL3A-hCL) and pAS-hVH-hCHm (containing syn-hVH4A-hCH). The vectors were then transformed in *E. coli* and were isolated as described in section 3.3.1.8. The vectors were extracted using QIAprep Miniprep kit as described in section 3.3.1.9-10 and analyzed by agarose gel electrophoresis as described in section 3.3.1.5.

Only the pAS-hVH-hCHm vectors were sent for DNA sequencing (1st BASE Laboratories, Malaysia) for the determination of the bacterial clones containing the pAS-hVH-hCHm with the proper orientation (5' to 3') of syn-hVH4A-hCHm. This was because the double-digestion of pSMART-hVH-hCHm and CET1019AS from digestion mixture 7 were performed using the same restriction enzyme, and therefore it could result in two possible orientation of syn-hVH4A-hCHm in pAS-hVH-hCHm; 5' to 3' or 3' to 5'.

The bacterial clones having the desired size and orientation of vectors were subjected to large-scale vector extraction using QIAGEN Maxiprep kit as described in section 3.3.3.1.4 and were then quantified using a NanoPhotometer (Implen, Germany). The pAS-hVH-hCHm and pAS-hVL-hCL expression vectors were then linearized using digestion mixture 3 as described 3.3.3.1.5 but the *Pvu*I was substituted with *I-Sce*I homing endonuclease enzyme. The linearized vectors were then purified also as described in section 3.3.3.1.5 and were used for co-transfection in NS0 and CHO cells.

7.3.1.3 Bicistronic UCOE expression vectors

Like the monocistronic UCOE vector, the bicistronic UCOE vector (CET1019AD) (Milipore, USA) does not contain the human antibody constant regions. Therefore synthetic gene syn-hVL3A-hCL from section 7.3.1.2 was used for the expression of the light chain of H1C2 mAb. However, the syn-hVH4A-hCHm from section 7.3.1.2 was replaced with syn-hVH4A-hCHb which has the exact DNA sequence except for its restriction enzyme sequences. While syn-hVH4A-hCHm has *NgoMI*V restriction enzyme sequences at both 5' and 3' end of the gene, syn-hVH4A-hCHb has *BstB*I and *Not*I restriction enzyme sequence at the 5' and 3' end, respectively, to facilitate the cloning of syn-hVH4A-hCHb into CET1019AD vector. Like syn-hVH4A-hCHm, syn-hVH4A-hCHb synthetic gene was also synthesized and ligated into pUC vector at Integrated DNA Technologies, USA and the resulting vector was named pUC-hVH-hCHb.

The cloning of syn-hVL3A-hCL into CET1019AD was performed as described in section 7.3.1.2 and the resulting recombinant vector was named pAD-hVL-hCL. For the cloning of syn-hVH4A-hCHb into pAD-hVL-hCL, double-digestions were performed by adding 3.5 µl of each pUC-hVH-hCHb and pAD-hVL-hCL to 46.5 µl of digestion mixture 8 and incubated at 37ºC for 2 hours.

Then, the digested DNA fragments of pUC-hVH-hCHb and pAD-hVL-hCL were purified using PCR purification kit (Qiagen, Germany) and 44.0 µl of the purified digested vectors were added to 6 µl of digestion mixture 9 and were incubated at 65ºC for 2 hours. The digestion mixture 8 consists of 5.0 µl of buffer 3, 0.5 µl of BSA

(100x), 0.5 µl of *Not*I and 40.5 µl sterile water, while digestion mixture 9 is made up of 5.0 µl of buffer 4, 0.5 µl of BSA (100x), and 0.5 µl *BstB*I.

Since both pAD-hVL-hCL and pUC vectors have the same ampillicin resistance gene, thus, to prevent self-ligations, the phosphate from the 5'-end of the digested fragments of pUC-hVH-hCHb was removed by adding 1.0 µl of CIAP and 5.0 µl of CIAP buffer to 44.0 µl of purified digested fragments of pUC-hVH-hCHb. The reactions were performed at 37ºC for 2 hours and the DNA fragments lacking the 5' phosphoryl termini were again purified using QIAquick PCR purification kit (Qiagen, Germany).

For ligation of syn-hVH4A-hCHb into pAD-hVL-hCL vector, 6.0 µl of the purified digested fragments of pUC-hVH-hCHb lacking the 5' phosphoryl termini, 1.0 µl of T4 DNA ligase and 1.0 µl of T4 DNA ligase buffer (10x) were added to 2.0 µl of the digested pAD-hVL-hCL purified from digestion mixture 9. The resulting vector was named pAD-hVL-hCL-hVH-hCH and was transformed, extracted in large-scale and linearized as described in section 7.3.1.2. The linearized vector was then purified as described in section 3.3.3.1.5 and was used for transfection in NS0 and CHO cells.

7.3.2 Transfection, selection and qualitative ELISA

The transfection of NS0 and CHO cells were performed using a 6-well plate format and the cells were transfected using Lipofectamine 2000 (cat. no.: 11668027, Life Technologies, USA). NS0 cells were grown in hybridoma-SFM (cat. no.: 12045, Life Technologies, USA) containing 1% (v/v) synthechol (cat. no.:. S5442, Sigma-Aldrich, USA) and 1% glutamax (Life Technologies, USA). CHO cells were grown in HyClone

SFM4CHO media (cat. no.: SH30518, Thermo Scientific, USA), supplemented with 1% glutamax.

For the expression of H1C2 mAb using monocistronic pFUSE vectors in NS0 and CHO cells, the cells were co-transfected with linearized pFUSE-VH and linearized pFUSE-VL vectors. Since pFUSE vectors are relatively small, both cell lines are also co-transfected with unlinearized pFUSE-VH and unlinearized pFUSE-VL vectors.

However for the expression of H1C2 mAb using monocistronic UCOE vectors, NS0 and CHO cells were co-transfected only with linearized pAS-hVL-hCL and linearized pAS-hVH-hCHm vectors. Similarly, for the expression of H1C2 mAb using bicistronic UCOE vector, NS0 and CHO cells were transfected with only linearized pAD-hVL-hCL-hVH-hCH vector. This is because the UCOE vectors are large in size compared to that pFUSE vectors, hence only linearized form of vectors were used for transfection. For each well, a total of 4.0 μg of vector DNA were added. Thus for co-transfection, 2.0 μg of each monocistronic vector were used.

For transfection, each vector DNA was diluted in 250 μl of Opti-MEM reduced-serum medium (cat. no.: 51985034, Life Technologies, USA) using 1.5 ml centrifuge tubes. Using different 1.5 ml centrifuge tubes, 10 μl of lipofectamine were diluted into 250 μl of Opti-MEM reduced-serum medium and incubated at room temperature for five minutes. After that, the diluted vector DNA and lipofectamine were combined, gently mixed and incubated again at room temperature for 20 minutes.

For transfection, approximately 10^6 serum-free adapted NS0 or CHO cells were pelleted by centrifugation at 1000 rpm for 5 minutes and resuspended in 1 ml of Opti-MEM

reduced-serum medium into each well. Then, 500 µl of the lipofectamine-DNA complexes were added drop-wise to the cells in each well and the plates were then incubated at 37ºC incubator with CO_2 for 4 hours before the addition of 4 ml of the respective growth medium for NS0 and CHO cells.

For controls ("mock transfections") cells were similarly treated except that the no DNA was added to the Opti-MEM reduced-serum medium.

To remove non-transfected cells, selective media containing specific antibiotics were added 72-hours post-transfection. For cells transfected with pFUSE vectors, 200 µg/ml of zeocin and 2 µg/ml of blasticidin were added while for cells transfected with UOCE vectors, 5 µg/ml of puromycin were added. The concentrations of zeocin, blasticidin and puromycin antibiotics used had been pre-determined earlier and it was the lowest concentration of each antibiotic which killed the untransfected NS0 and CHO cells in 5 days. The antibiotic selections were then performed until all the mock transfected NS0 and CHO cells were non-viable. The viable parental transfectomas were then evaluated for H1C2 mAb productivity using qualitative ELISA as described in chapter 4 (section 4.3.5) (Dharshanan et al. 2011a).

7.3.3 Isolation of high producer transfectomas using ClonePix FL system and characterization of H1C2 mAb

The screening and selection of NS0 and CHO transfectomas secreting high levels of H1C2 mAb were done as described in chapter 4 with minor modifications (Dharshanan et al. 2011a). In order to grow clones from individual separate transfected cells, 5000

parental transfectomas cells secreting H1C2 mAb were added to 100 ml of semi-solid growth media.

For NS0 transfectomas, the semi-solid growth media consisted of 90 ml semi-solid media for hybridomas/myelomas (cat. no.: K8600, Molecular Devices, USA), 1 ml of glutamax, 1 ml of anti-human capture antibody conjugated to fluorescein isothiocyanate (FITC) (cat. no.: K8200, Molecular Devices, USA) and 8 ml of sterile water. For CHO cells, the semi-solid growth media for CHO cells (cat. no.: 8712, Molecular Devices, USA) was used instead.

The cells and media were mixed vigorously and 2 ml of each combination were transferred to each well of 6-well plates (cat. no.: 3516, Sigma Aldrich, USA). The plates were then incubated at 37ºC, 5% CO_2 with high humidity for 14 days.

The transfectomas in semi-solid media were then analyzed using ClonePix FL system and high producer clones were identified and isolated as described in chapter 4 (Dharshanan et al. 2011a). The isolated clones were scaled up and at passage ten, qualitative and quantitative ELISAs were performed to evaluate the H1C2 mAb productivity as described in chapter 4 (section 4.3.4). The functionality of the H1C2 mAbs secreted were characterized using SW1116 cells, a colorectal carcinoma expressing C2-antigen as described in chapter 6 (section 6.3.1.2).

7.4 RESULTS

7.4.1 Construction of expression vectors

7.4.1.1 Monocistronic pFUSE expression vectors

Monocistronic expression vectors; pFUSE-CHIG-hG1 (Figure 7.1) and pFUSe2-CLIg-hk (Figure 7.2) which contain the human IgG1 constant regions and the human kappa constant region, respectively, were used for the expression of complete H1C2 mAb. For the expression of the heavy chain of H1C2 mAb, pSMART-hVH (~3.0 kb) (Figure 7.3a) were double-digested to isolate the synthetic gene syn-hVH4A (~500 bp) (Figure 7.3b) which were then subcloned into pFUSE-CHIG-hG1 vector (~4.5 kb) (Figure 7.3). When the resulting recombinant vector pFUSE-hVH was analysed by agarose gel electrophoresis, a band was seen at approximately 5.0 kb, which corresponded to the expected size (4.5 kb + 0.5 kb) (Figure 7.4).

Similarly, for the expression of the light chain of H1C2 mAb, pSMART-hVL (~2.9 kb) (Figure 7.3a) were double-digested to isolate the synthetic gene syn-hVL3A (~400 bp) (Figure 7.3b) which was subcloned into pFUSE2-CLIg-hk vector (~3.9 kb) (Figure 7.3). The resulting recombinant vector pFUSE-hVL was approximately 4.3 kb when analyzed by agarose gel electrophoresis (Figure 7.4).

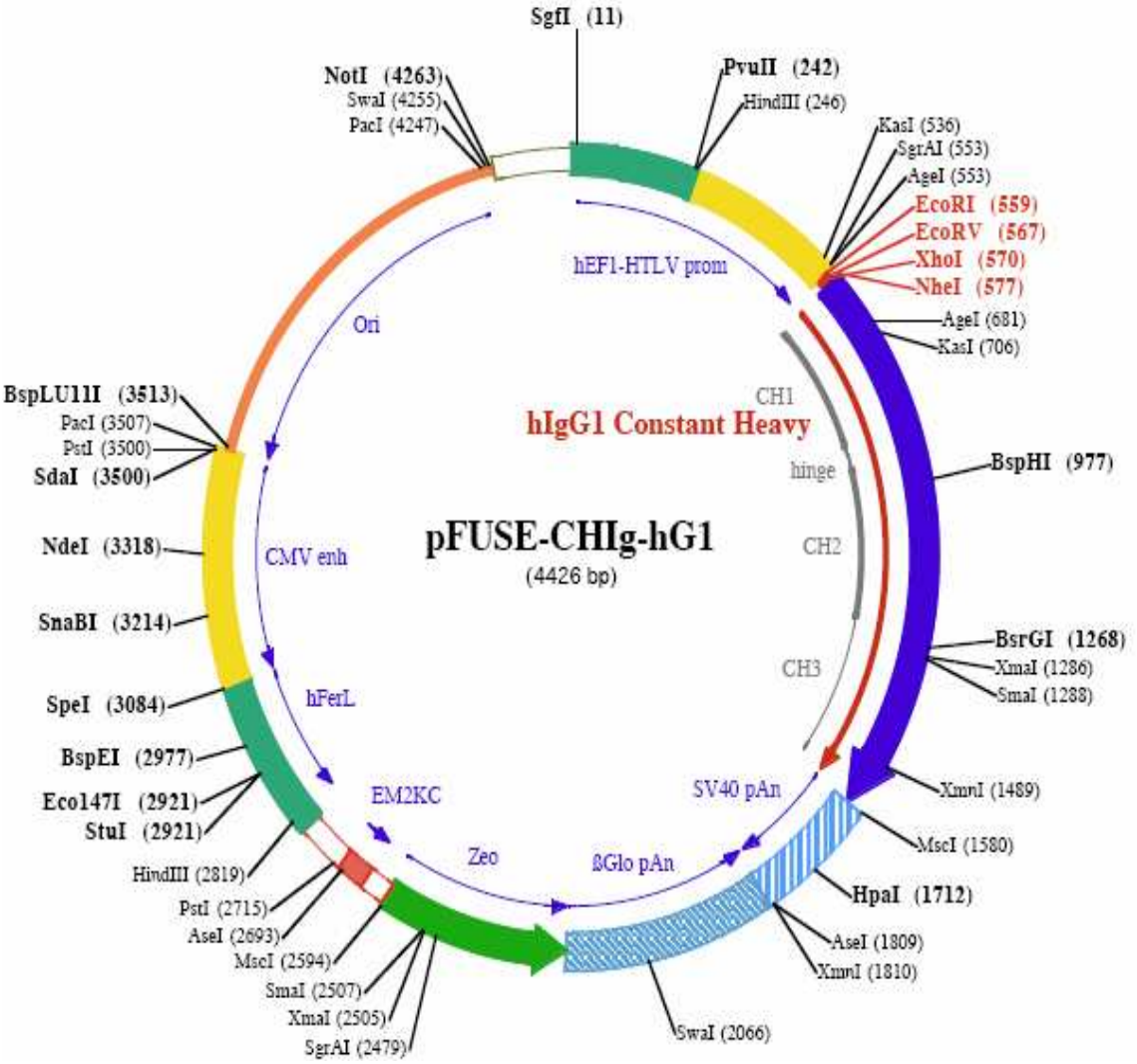


Figure 7.1: pFUSE monocistronic expression vector; pFUSE-CHIg-hG1 for the expression of heavy chain of antibody. The vector already contains the gene for the human constant heavy chain (encompassed by the red arrow line shown).

Source: InvivoGen, USA.

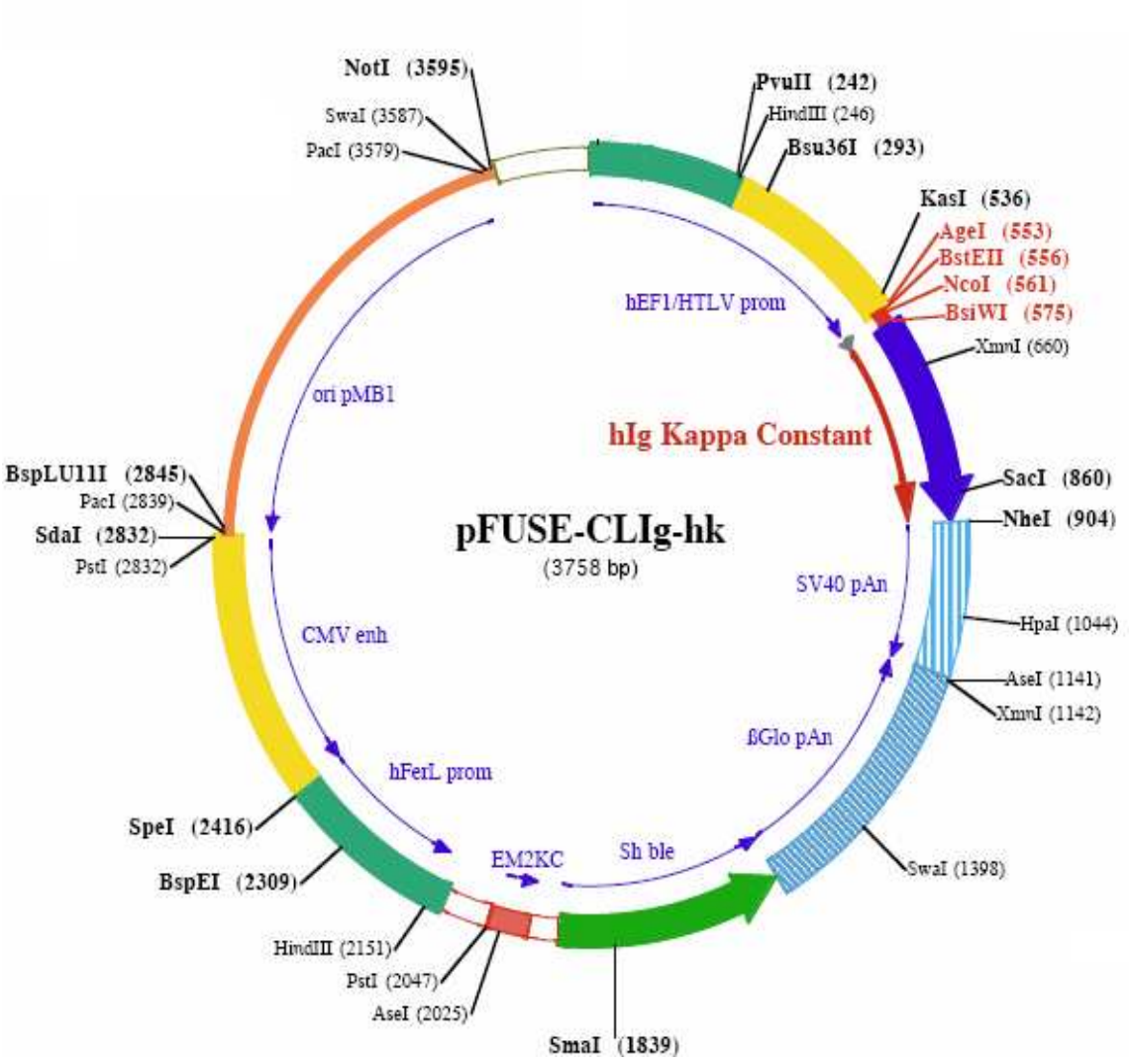


Figure 7.2: pFUSE monocistronic expression vector; pFUSE-CLIg-hk for the expression of light chain of antibody. The vector already contains the gene for the human light chain kappa constant (encompassed by the red arrow line shown).

Source: InvivoGen, USA.

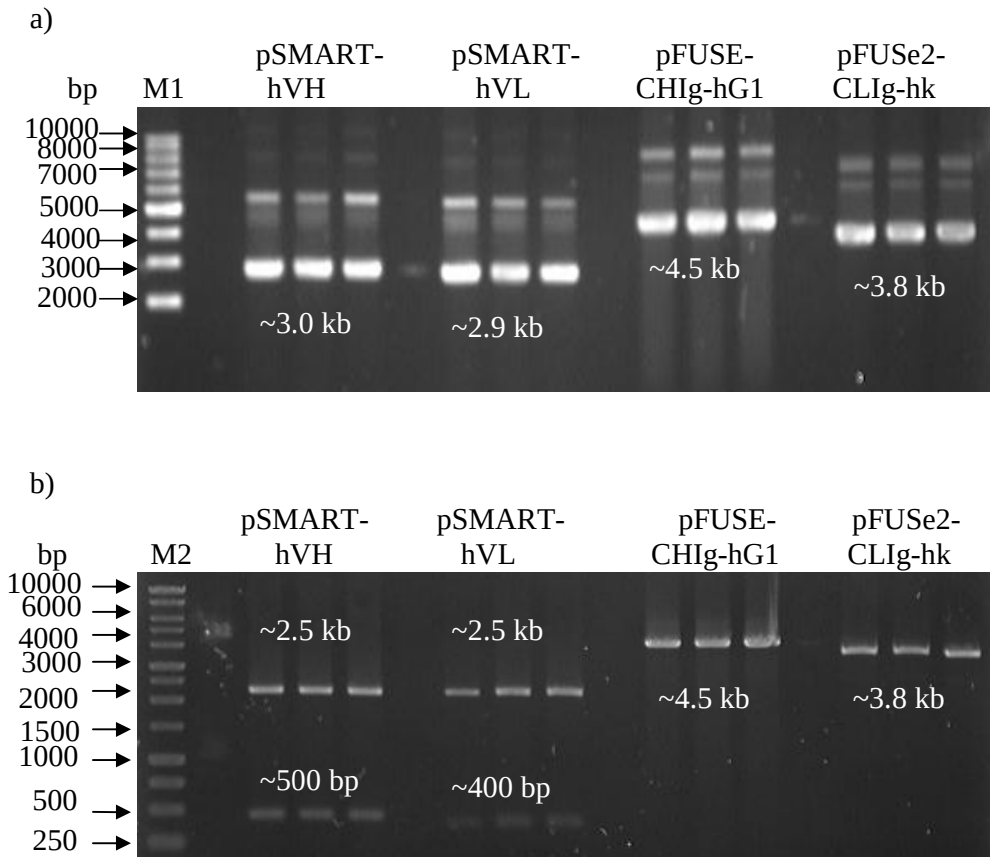
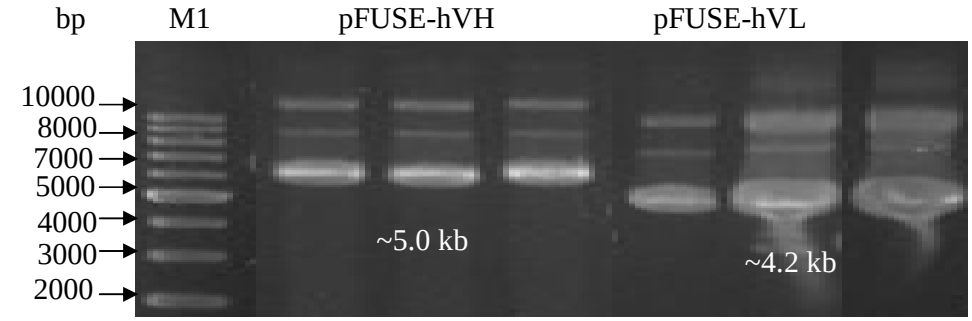


Figure 7.3: Agarose gel electrophoresis profiles of cloning vectors containing synthetic variable genes of H1C2 mAb and pFUSE expression vectors. Gel (a) was run with vectors in undigested form; gel (b), after double-digestion of the vectors. Lanes were run in triplicate with: pSMART-hVH (~3.0 kb) & pSMART-hVL (~2.9 kb), pFUSE expression vectors; pFUSE-CHIg-hG1 (~4.5 kb) and pFUSE2-CLIg-hk (~3.9 kb). M1 is supercoiled DNA ladder and M2 is 1 kb DNA ladder.

a)



b)

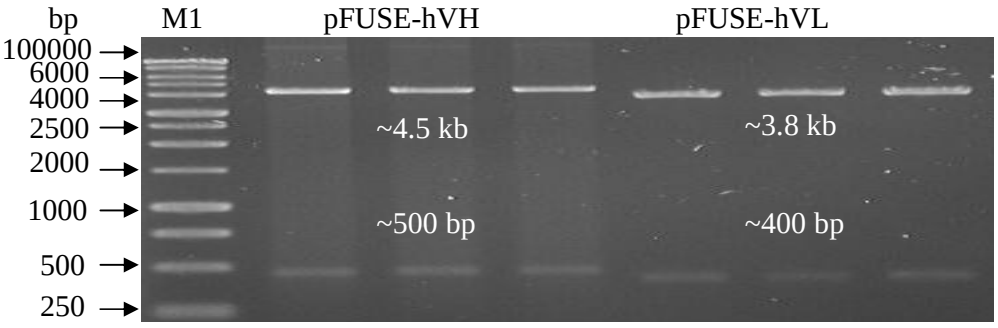


Figure 7.4: Agarose gel electrophoresis profiles of recombinant pFUSE expression vectors pFUSE-hVH (~5.0 kb) and pFUSE-hVL (4.3 kb) containing the synthetic humanized variable regions. Gel (a) was run with vectors in undigested form; gel (b), after double-digestion of the vectors. The double-digestions performed confirmed the presence of syn-hVH4A (~500 bp) and syn-hVL3A (~400 bp) in pFUSE-hVH and pFUSE-hVL, respectively. M1 is supercoiled DNA ladder and M2 is 1 kb DNA ladder. All samples were run in triplicate.

7.4.1.2 Monocistronic UCOE expression vectors

The monocistronic UCOE expression vector (CET1019AS) (Figure 7.5), does not contain the human antibody constant regions. Therefore synthetic DNA copies of human IgG1 constant region (hCHm) and the human kappa constant region (hCL) from pFUSE vectors were added to synthetic genes syn-hVH4A and syn-hVL3A, respectively. For the expression of the light chain of humanized H1C2 mAb, pSMART-hVL-hCL (~3.2 kb) (Figure 7.7c) was double-digested to isolate the synthetic gene syn-hVL3A-hCL.

The syn-hVL3A-hCL was then cloned into double-digested CET1019AS (~8.1 kb) (Figure 7.7a). After subcloning, the resulting recombinant vector pAS-hVL-hCL was approximately 8.8 kb (Figure 7.8a) and the double-digestion performed confirmed the successful insertion of the synthetic gene syn-hVL3A-hCL (~700 bp) in CET1019AS (~8.1 kb) (Figure 7.8b).

Similarly, for the expression of the heavy chain of H1C2 mAb, pUC-hVH-hCHm (~5.0 kb) (Figure 7.7b) was double-digested to isolate the synthetic gene syn-hVH4A-hCHm (~1.5 kb) which was then subcloned into double-digested CET1019AS (~8.1 kb) (Figure 7.7a) and the resulting recombinant vector pAS-hVH-hCHm was approximately 9.6 kb (Figure 7.9a). The double-digestion performed confirmed the successful insertion of the synthetic gene syn-hVH4A-hCHm (~1.5 kb) in CET1019AS (~8.1 kb) (Figure 7.9b).

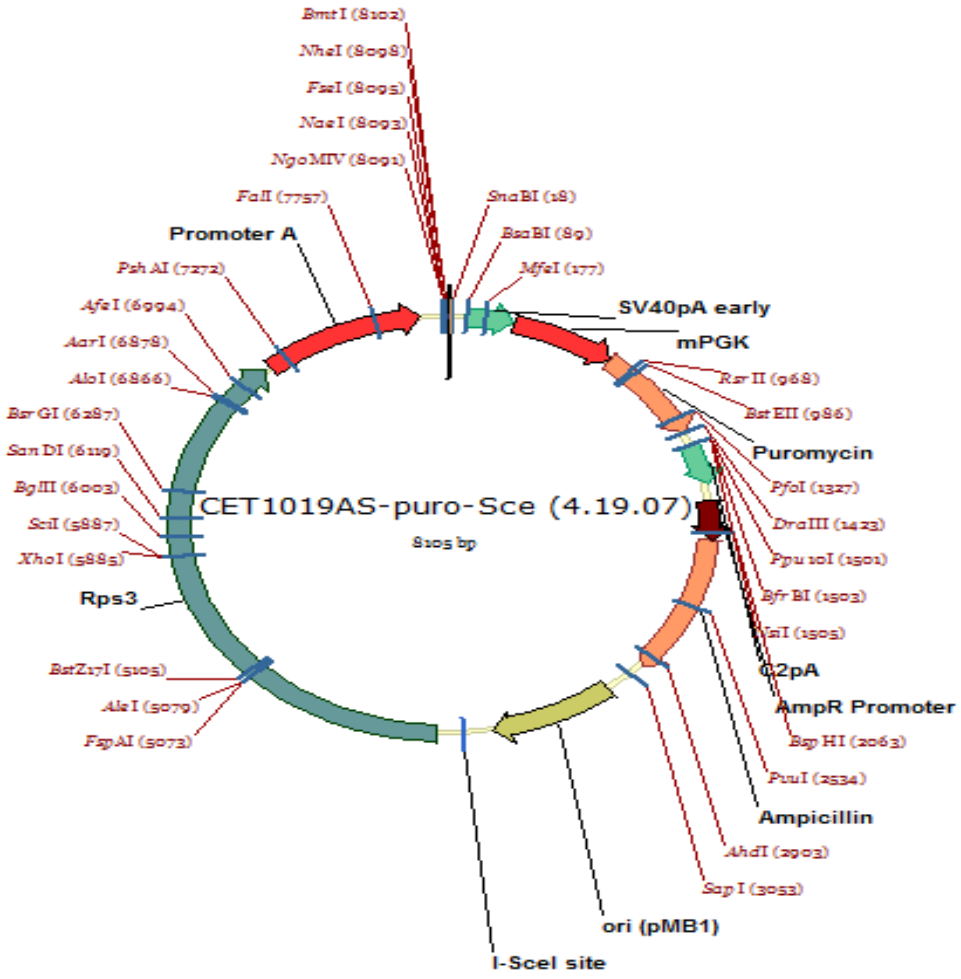


Figure 7.5: UCOE monocistronic expression vector CET1019AS for the expression of heavy or light chains of antibody. It does not contain genes for the heavy and light chain constant regions, and so genes for these constant regions have to be inserted.

Source: Millipore, USA.

7.4.1.3 Bicistronic UCOE expression vectors

Like monocistronic CET1019AS expression vector, bicistronic UCOE vector, CET1019AD (Figure 7.6), also does not contain the human antibody constant regions. Therefore synthetic DNA copies of human IgG1 constant region (hCHb) and the human kappa constant region (hCL) from pFUSE vectors were added to synthetic genes syn-hVH4A and syn-hVL3A, respectively. For CET1019AD bicistronic vector, sequential subclonings were performed whereby syn-hVL3A-hCL was first subcloned followed by syn-hVH4A-hCHb.

Agarose gel electrophoresis profiles, confirmed the sizes of the various vectors and synthetic genes before and after subcloning. For the first subcloning, pSMART-hVL-hCL (~3.2 kb) (Figure 7.7c) was double-digested to isolate the synthetic gene syn-hVL3A-hCL (~700 bp) which was subcloned into CET1019AD (~11.0 kb) (Figure 7.7a). The resulting recombinant vector pAD-hVL-hCL was approximately 11.7 kb (Figure 7.8a). The double-digestion performed confirmed the successful insertion of the synthetic gene syn-hVL3A-hCL (~700 bp) in CET1019AD (~11.0 kb) (Figure 7.8b).

For the second subcloning, pSMART-hVH-hCHb (~5.0 kb) (Figure 7.7b) was double-digested to isolate the synthetic gene syn-hVH4A-hCHb (~1.5 kb) which was subcloned into pAD-hVL-hCL (~11.7 kb) (Figure 7.8a). The resulting recombinant vector pAD-hVL-hCL-hVH-hCH was approximately 13.2 kb (Figure 7.10a). The double-digestion performed confirmed the successful insertion of the synthetic gene syn-hVH4A-hCHb (~1.5 kb) in pAD-hVL-hCL (~11.7 kb) (Figure 7.10b).

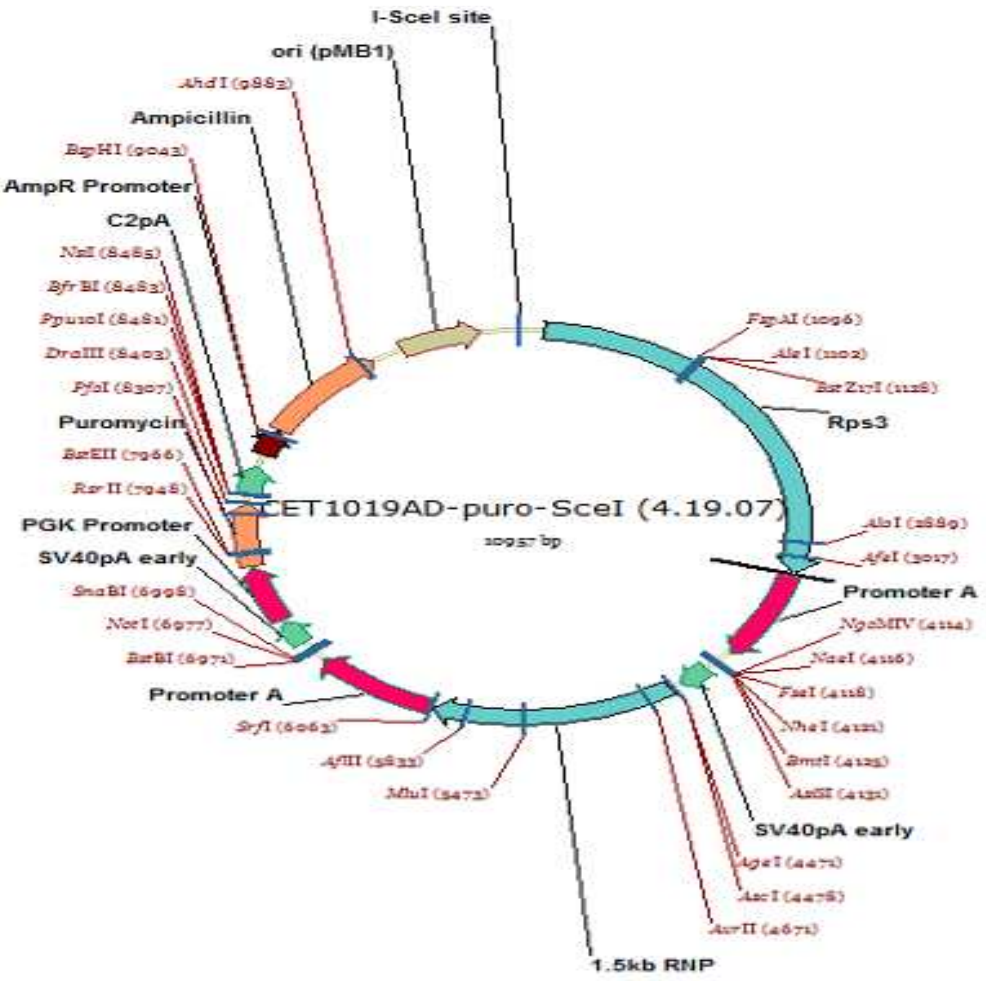


Figure 7.6: UCOE bicistronic expression vector: CET1019AD for the expression of both heavy and light chains of antibody. Similar to CET1019AS, it does not contain genes for the heavy and light chain constant regions, and so genes for these constant regions have to be inserted.

Source: Millipore, USA.

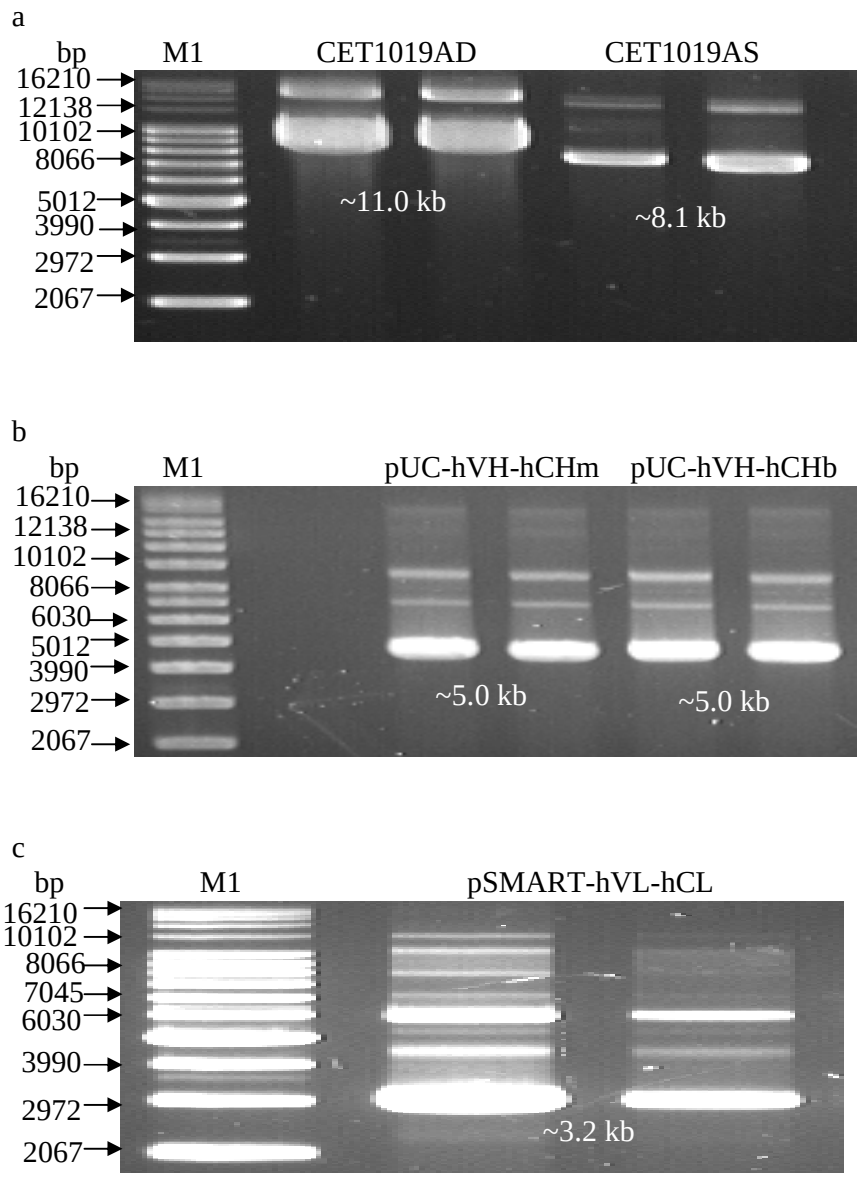


Figure 7.7: Agarose gel electrophoresis profiles of cloning vectors containing synthetic variable genes of H1C2 mAb and UCOE expression vectors. Expression vectors were (a): CET1019AD (~11.0 kb) and CET1019AS (~8.1 kb), cloning vectors containing synthetic humanized variable regions with human constant regions of heavy chain (b): pUC-hVH-hCHm (~5.0 kb) & pUC-hVH-hCHb (~5.0 kb) and light chain (c): pSMART-hVH-hCL (~3.2 kb). All samples were run in duplicate. M1 is supercoiled DNA ladder.

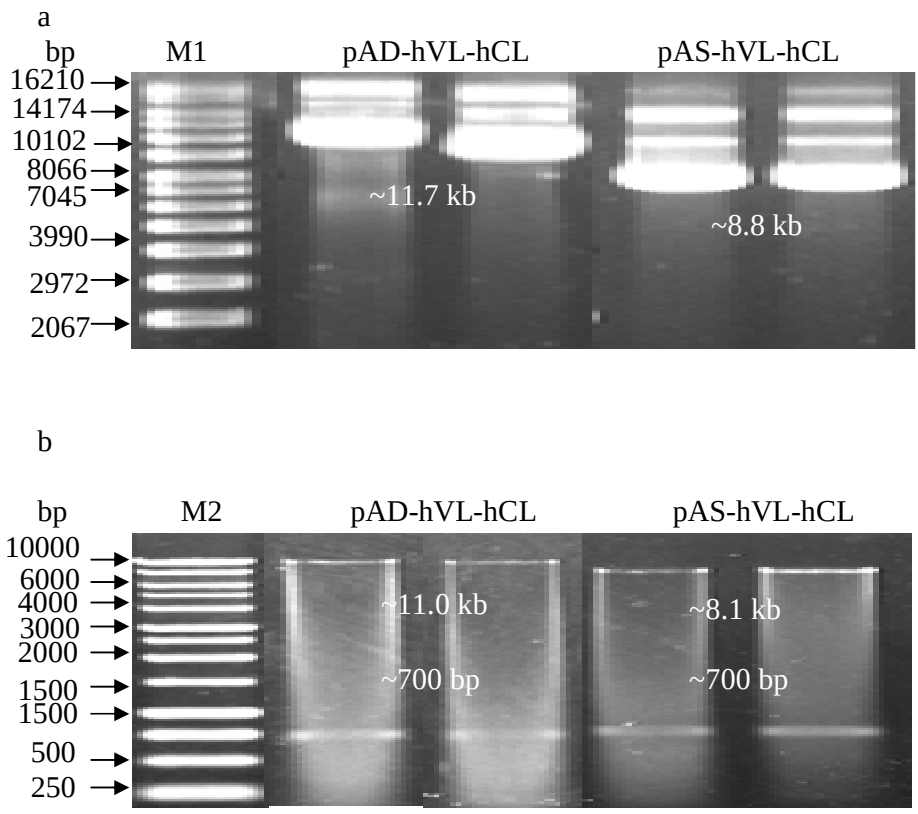
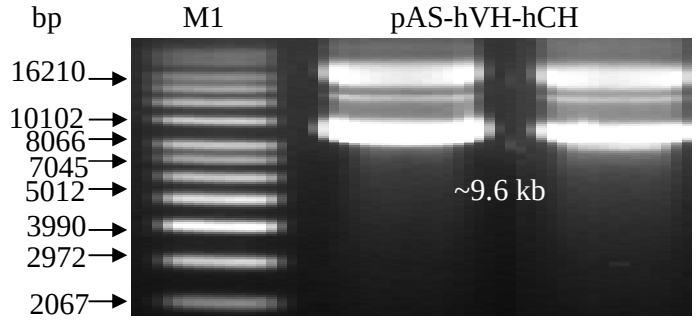


Figure 7.8: Agarose gel electrophoresis profiles of recombinant monocistronic and bicistronic UCOE expression vectors containing the synthetic syn-hVL-hCL. The recombinant expression vectors were pAD-hVL-hCL (~11.7 kb) & pAS-hVL-hCL (~8.8 kb) in undigested (a) and double-digested (b) forms. The double-digestions performed confirmed the presence of syn-hVL3A-hCL in both recombinant expression vectors (~700 bp) (b). All samples were run in duplicate. M1 is supercoiled DNA ladder and M2 is 1 kb DNA ladder.

a



b

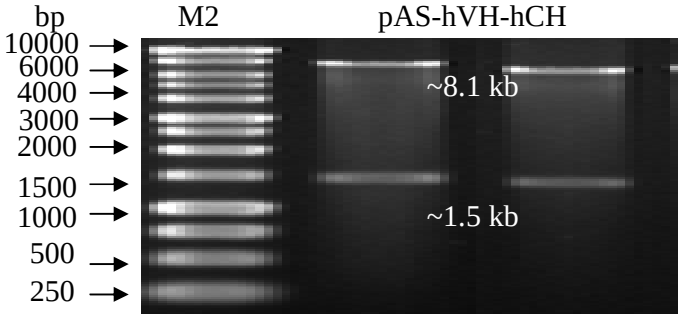
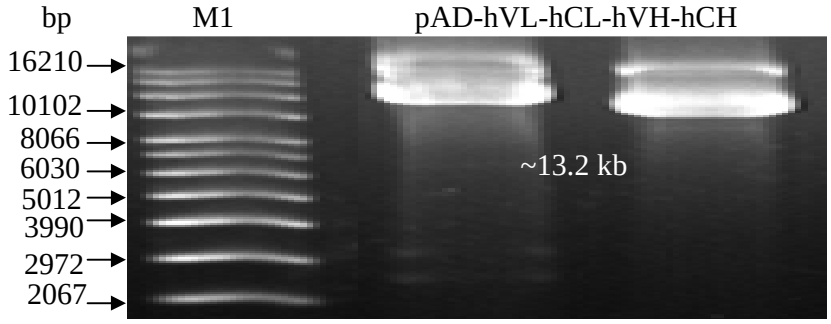


Figure 7.9: Agarose gel electrophoresis profiles of recombinant monocistronic UCOE expression vectors containing the synthetic syn-hVH-hCHm. The recombinant expression vectors were pAS-hVH-hCH (~9.6 kb) in undigested (a) and double-digested (b) forms. The double-digestions performed confirmed the presence of syn-hVH4A-hCHm (~1.5 kb) (b). All samples were run in duplicate. M1 is supercoiled DNA ladder and M2 is 1 kb DNA ladder.

a



b

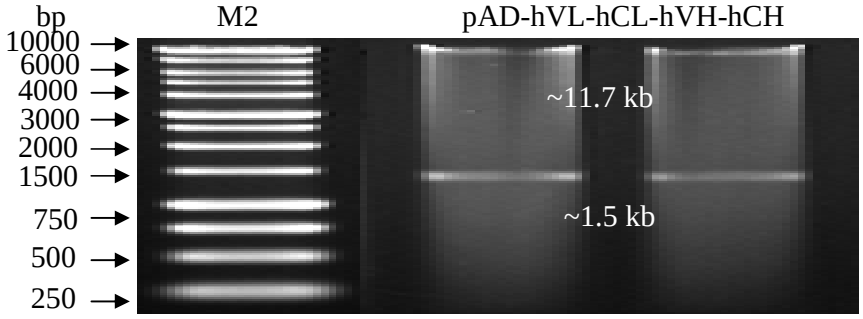


Figure 7.10: Agarose gel electrophoresis profiles of recombinant bicistronic UCOE expression vectors containing the synthetic syn-hVH-hCHb. The recombinant expression vectors were pAD-hVL-hCL-hVH-hCH (~13.2 kb) in undigested (a) and double-digested (b) forms. The double-digestions performed confirmed the presence of syn-hVH4A-hCHb (~1.5 kb) (b). All samples were run in duplicate. M1 is supercoiled DNA ladder and M2 is 1 kb DNA ladder.

7.4.2 Transfection and expression of H1C2 mAbs

Since the size of the vector DNA was reported to be inversely proportional to the transfection efficiency (Kreiss et al. 1999) and the fact that pFUSE expression vectors are relatively small in size (4.2 and 5.0 kb) compared to expression vectors used previously (12.0 and 14.5 kb), therefore transfection using pFUSE vectors were performed using both linearized and unlinearized form. The efficiency of both forms of vectors was then compared.

On the other hand, for the UCOE system, since both monocistronic (8.8 and 9.6 kb) and bicistronic (13.2 kb) vectors are large vectors, therefore only linearized vectors form was used for transfection.

The unlinearized and linearized monocistronic pFUSE vectors were transfected in both NS0 and CHO cells in triplicate. Similarly, the linearized monocistronic and bicistronic UCOE vectors were also transfected in both NS0 and CHO cells in triplicate.

With linearized pFUSE vectors, all NS0 cells were viable (resistant to the antibiotic used in the selective media). However, with unlinearized pFUSE vectors only 1 of the triplicate of NS0 cells transfected was viable (Table 7.1). On the other hand, all CHO cells transfected with linearized monocistronic and bicictronic UCOE vector were viable after antibiotic selection (Table 7.1). However for NS0 cells transfected with UCOE vectors and CHO cells transfected with pFUSE vectors, no viable cells were obtained. Therefore it may be concluded that pFUSE expression vectors work well for NS0 cells, while UCOE expression vectors work well for CHO cells.

Table 7.2: Viability of stable parental transfectomas after antibiotic selection.

For the selection of stable transfectomas, zeocin and blasticidin were used for cells transfected with pFUSE vectors, while puromycin was used for cells transfected with UCOE vectors. All transfections were performed in triplicate. M denotes monocistronic vector while B denotes bicistronic vector

Expression vector systems	**Number of viable transfectomas (out of 3 transfections)**	
	NS0	**CHO**
Linearized pFUSE-M	3	0
Unlinearized pFUSE-M	1	0
Linearized pUCOE-M	0	3
Linearized pUCOE-B	0	3

7.4.3 Productivity of H1C2 mAb in NS0 cells using pFUSE expression vectors

The viable parental NS0 transfectomas (pFUSE vectors) were then analyzed for HIC2 mAb productivity. As shown in Figure 7.11, all three parental NS0 transfectomas transfected with linearized pFUSE vector (NS0-pFUSE-M) produced H1C2 mAb. However, the one viable NS0 transfectoma (transfectoma-2) transfected with unlinearized pFUSE vectors, failed to produce or produced at low levels of H1C2 mAb despite the fact that they were resistant to the antibiotics used for the selection of the heavy and light chain of H1C2 mAb.

7.4.4 Productivity of H1C2 mAb in CHO cells using UCOE expression vectors

The viable parental CHO transfectomas (UCOE vectors) were also analyzed for HIC2 mAb productivity. From Figure 7.11, even though both monocistronic and bicistronic UCOE vectors were linearized and all the triplicates parental CHO transfectomas were viable after antibiotic selection, the H1C2 productivities were low in two of the triplicate cells transfected with monocistronic UCOE expression vectors (CHO-UCOE-M).

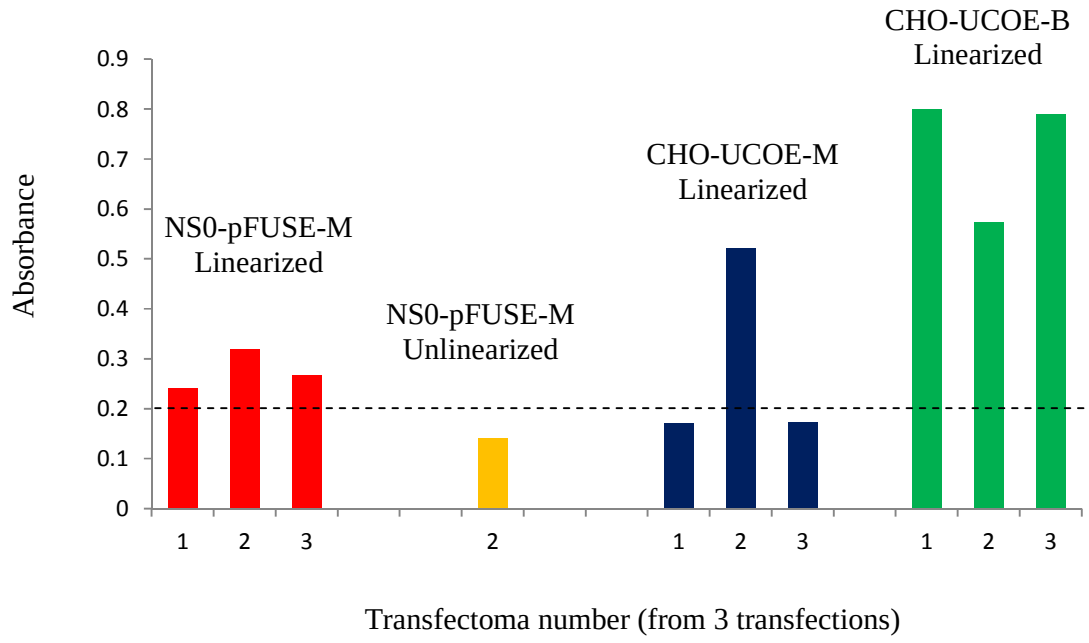


Figure 7.11: Qualitative ELISA of antibiotic resistant parental transfectomas.

All viable transfectomas were analyzed for H1C2 productivity. All transfections in triplicate had 3 viable clones each except for the parental NS0 cell transfected with unlinearized pFUSE vectors, which had only 1 (transfectoma 2) viable parental clone. Cells were considered low-producing if the absorbance value was below 0.2 units. Although, all CHO-UCOE-M were viable, however two of its transfectomas (transfectoma 1 and 3) were low producers.

7.4.5 Selection of high H1C2 mAb producing NS0 and CHO transfectomas using ClonePix FL system

All transfectomas were then screened using ClonePix FL system and ranked according to their sum total fluorescence intensities. Since the detection antibody (conjugated to FITC) used is specific to the human Fc region of human antibody, therefore the sum total fluorescence intensities of each transfectomas represents the complete H1C2 mAb productivity. From the dot-blot data (Figure 7.12), it was found that all parental NS0-pFUSE-M transfectomas (Figure 7.12a) were low producers as their productivities were less than 50 000 FU. The sum total intensity of the highest NS0-pFUSE-M producer was only 19 898 FU.

On the other hand, more than 90% of both CHO-UCOE-M (Figure 7.12b) and CHO-UCOE-B (Figure 7.12c) had productivity of greater than 50 000 FU. The sum total intensities of the highest producer of CHO-UCOE-M (Figure 7.12b) and CHO-UCOE-B (Figure 7.12c) were 3 985 878 and 5 357 028 FU, respectively. It was also found that CHO-UCOE-B (Figure 7.12c) had a higher number of transfectomas between 2 x 10^6 and 4 x 10^6 FU compared to that of CHO-UCOE-M (Figure 7.12b). In addition, CHO-UCOE-B had 6 clones with the sum total intensities greater than 4 x 10^6 FU.

A total of 90, 27 and 122 monoclonal transfectomas of NS0-pFUSE-M, CHO-UCOE-M and CHO-UCOE-B, respectively, were isolated, scaled-up and evaluated for H1C2 productivity using quantitative ELISA at passage ten (Figure 7.13). Clones were categorized as non-producers and low producers if the productivity was less than 1.0 μg/ml and between 1.0-10.0 μg/ml, respectively. Clones producing H1C2 mAbs

between 10.0-50.0 μg/ml were categorized as average producers and those above 50.0 μg/ml as high producers.

At passage 10, almost half of the NS0-pFUSE-M clones were no longer producing H1C2 mAbs, while only 14.3% and 7.5% of CHO-UCOE-M and CHO-UCOE-B clones, respectively, were non-producers. The remaining NS0-pFUSE-M (54.2%) clones were all low producers, while 50.0% and 24.1% of CHO-UCOE-M and CHO-UCOE-B clones, respectively, were low-producers. In contrast, 35.7% and 40.5% of CHO-UCOE-M and CHO-UCOE-B clones, respectively, were high producers which secreted more than 50.0 μg/ml H1C2 mAbs.

Quantitative ELISA performed also showed that the highest productivity of NS0-pFUSE-M clones was approximately 0.6 μg/ml, while the productivity of high producers of CHO-UCOE-M and CHO-UCOE-B were between 50-110 μg/ml. No significant difference in H1C2 mAb productivities were found between CHO-UCOE-M and CHO-UCOE-B transfectomas, but both CHO-UCOE-M and CHO-UCOE-B transfectomas had at least 100 times superior productivity compared to NS0-pFUSE-M.

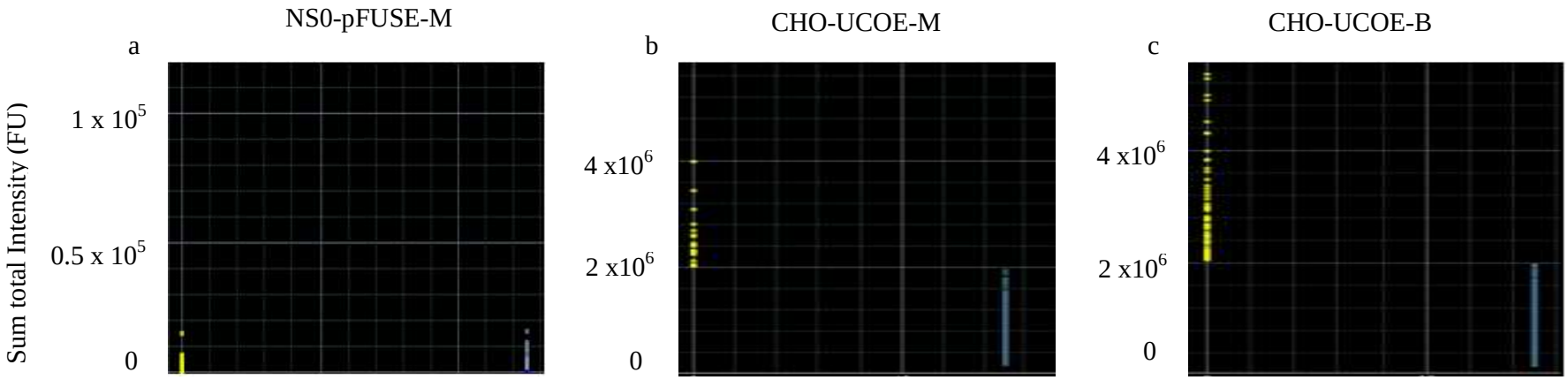


Figure 7.12: Dot-blot analysis of transfectomas using ClonePix FL system: Sum total intensity (FU).

From the fluorescence data whereby each colored dot represents one monoclonal transfectoma, NS0 cells transfected with linearized pFUSE vector (a) had lower fluorescence intensity compared to CHO cells transfected with either monocistronic (b) and bicistronic (c) UCOE vectors. Yellow dots and blue dots represent the isolated and non-isolated transfectomas, respectively.

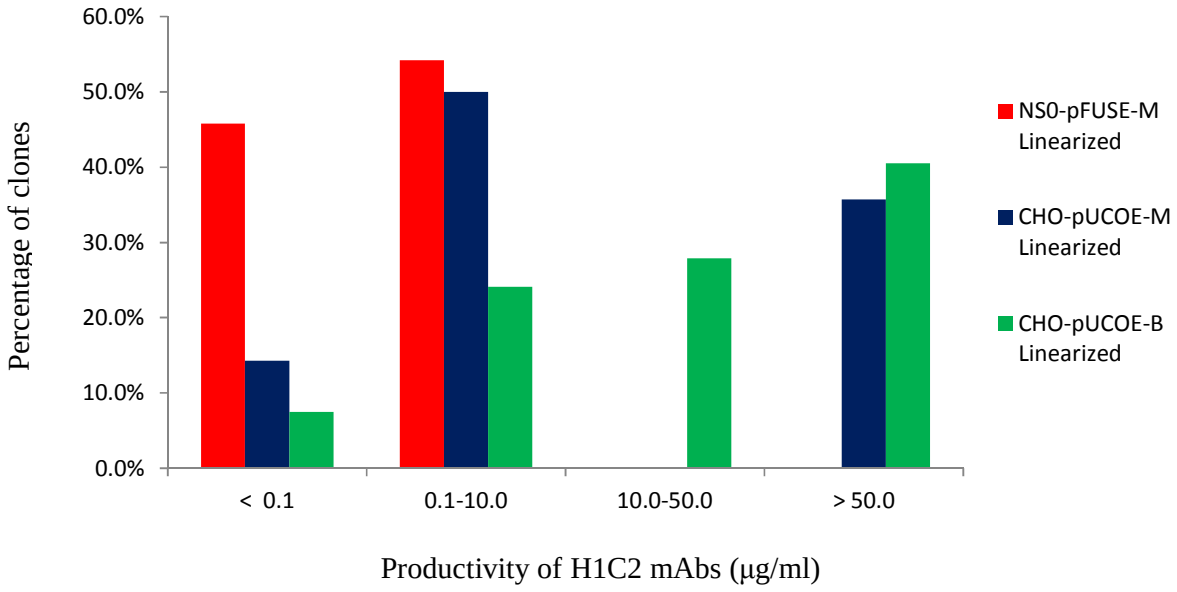


Figure 7.13: Quantitative ELISA of monoclonal high-producer transfectomas isolated using ClonePix FL system. A total of 90, 27 and 122 of the high-producing clones from each transfectomas were isolated, scaled-up and after ten passages, were evaluated for their stability and productivity. Around 45.8% of the NS0-pFUSE-M were no longer producing H1C2 mAbs (<0.1 µg/ml) and the remaining clones were low producers (0.1-10.0 µg/ml). For CHO-UCOE-M, 14.3% were non-producers, 50.0% were low producers and 35.7% were high producers (>50.0 µg/ml). For CHO-UCOE-B, 7.5% were non-producers, 24.1% were low producers, 27.9% were moderate producers (10.0-50.0 µg/ml) and 40.5% were high producers.

A cell-based ELISA performed as described in chapter 6 (Figure 6.2), also confirmed that H1C2 mAbs secreted by NS0-pFUSE-M, CHO-UCOE-M and CHO-UCOE-B were all able to bind to the C2-antigen expressed on the surface of colorectal cancer cells. Therefore, even with the use of synthetic DNA coding for variable antibody regions, the H1C2 mAb were still functional like the physical DNA developed using overlapping-PCR mutagenesis in chapter 3.

7.5 DISCUSSION

7.5.1 Synthetic DNA

In previous chapters, humanized anti-C2 mAbs (H1C2 mAb) were developed using a computer algorithm to predict amphipathic sequences (potential T-cell epitopes) in mouse variable regions and judicious replacements of a few amino acids residues located in the potential immunogenic epitopes with the corresponding residues from the highest homologous human sequences were done.

Although the specific conversion from mouse to its corresponding human residues could be executed by site-specific overlapping-PCR mutagenesis, however this results in the deimmunization method being time-consuming. This largely is due to the high error-rate of recombinant *Taq* DNA polymerase, which requires a large number of PCR products to be sequenced as the probability of the humanized PCR products having the desired DNA sequence is often low. Consequently, the increase in the number immunogenic mouse residues to be humanized will also result in the increase of the duration of obtaining the desired PCR products.

Synthetic genes on the other hand, are becoming increasingly popular for the production of recombinant proteins for many reasons (Roytrakul et al. 2001), and the increase in speed of developing synthetic genes and decrease in cost of synthetic DNA has provided us an alternative convenient route for the desired genes. Synthetic copies of the DNA encoding the VH and VL of humanized variables region of H1C2 mabs were pre-designed to contain all the desired humanized residues in its DNA sequences. This has significantly reduced the duration to develop humanized mAbs compared to overlapping-PCR mutagenesis procedure.

The use of synthetic genes also allows the convenient introduction of specific restriction enzyme recognition sites without the requirement to perform overhang-PCRs for the addition of the desired restriction enzyme sequences. Besides that, unlike the physical DNA which are routinely cloned in cloning vectors for sequence confirmation prior to cloning into expression vectors, the synthetic humanized variable genes can be directly cloned into the expression vectors and transfected into mammalian cells.

7.5.2 Comparison between linearized and unlinerized pFUSE vectors

Although supercoil, open circular and linear forms of vector DNA all have the same primary structure, nevertheless the transfection efficiency of each topology had been reported to vary. It may be possible that the unlinearized vector used for transfection was linearized by a random cut within the cell and it might be that the resistance gene or the gene of interest was destroyed upon random linearization (Stuchbury & Münch, 2010).

This random linearization may have caused the loss of viability and productivity of the NS0 cells transfected using unlinearized pFUSE vectors (Figure 7.11). On the other hand, linearization of vector DNA before transfection by a single digestion with a selected restriction enzyme in a non-coding area of the gene may have the advantage of ensuring the integrity of all necessary gene elements of the vector, hence improving the survival and productivity of the transfectomas in selective medium (Stuchbury & Münch, 2010).

7.5.3 Comparison between monocistronic and bicistronic UCOE expression vector

Although both monocistronic and bicistronic UCOE vectors were linearized and all the triplicates parental CHO transfectomas were viable after antibiotic selection, the H1C2 productivity was undetected in two of the triplicate cells transfected with monocistronic UCOE expression vectors (Figure 7.11). This could be due to the way that the IgG is processed and also the antibiotic used for selection of the transfectoma producing H1C2 mAbs.

H1C2 mAb is an IgG molecule, which consists of two identical heavy chains and two identical light chains. During production of the antibody in eukaryotic cells, both chains first enter endoplasmic reticulum as unfolded polypeptides, which are then modified and assembled in their export-competent conformation. However, free heavy chains are not exported, unless they are first combined with light chains to form complete antibody molecules (Li et al. 2007). In contrast, most light chains can be secreted as free monomers or homodimers.

In the ELISA for the detection of antibodies secreted by the transfectomas, the secondary antibody used recognizes the heavy chain, therefore any measurement made in the media will represent the level of the whole humanized IgG. Conversely the light chain is not detected and therefore the cells secreting only light chain of H1C2 mAb are regarded as non-producing cells. The existence of viable cells that are not secreting the whole IgG could be due to the selection process used, which only employs one antibiotic.

During the co-transfection of two monocistronic expression vectors (pAS-hVH-hCH and pAS-hVL-hCL) for the expression of heavy and light chain simultaneously, cells could be transfected with both vectors, which produce transfectomas that secrete the whole IgGs, or with only one of either vector, which then generate transfectomas that synthesize only the light chains as free heavy chains not exported. Since the antibiotic used select for cells that have been successfully transfected, therefore there will be viable transfectoma cells present that produce the entire IgG (secreted) and cells that produce only the light chain (secreted but not detected). This may explains the fact that why a certain percentage of the CHO-UCOE-M transfectomas are resistant to the antibiotic in the selective medium but secrete no detectable levels of H1C2 mAbs into the media (Figure 7.11).

On the other hand, for CHO cells transfected with a single bicistronic UCOE vector (pAD-hVL-hCL-hVH-hCH) (CHO-UCOE-B), all of the triplicate parental CHO-UCOE-B clones were producing H1C2 mAb (Figure 7.11). This is to be expected since every successful transfection using bicistronic vector will produce a cell that is resistant to the antibiotic used and also is able to synthesize the whole IgG. Hence, it is recommended that for co-transfection using two monocistronic vectors, two different

antibiotics should be used for selection of the heavy and light chains. However if a single bicistronic vector is utilized, then it is possible to use the same antibiotic for the selection of both the heavy and light chains.

7.5.4 Stable expression of H1C2 mAb

Transfection which is the introduction of foreign DNA into mammalian cells can be divided into two types: transient and stable transfection. Transiently transfected cells are cells that harbor foreign DNA but they are not incorporated into the chromosomes. Thus the foreign DNA is able to be transcribed, but cannot be copied and therefore will be degraded over time and diluted during mitosis. In stably transfected cells, on the other hand, the foreign DNA is incorporated in the chromosomes and therefore they can be copied during mitosis which enables the continuous expression of the foreign gene.

Although transient transfections have points in their favor due to short development time (Zhu, 2011), cell lines that continually express the foreign DNA of interest, as in stable transfection are much preferred. This is because stable production is more cost-effective compared to transient production, as the stably transfected pools can be frozen, providing working stocks for further production without the need for re-transfection which subsequently minimizes the quantities of vector DNA and save on the use of costly transfection agents.

Stable transfection relies on the insertion of the foreign DNA into the genome, a process which occurs infrequently, and this results in low numbers of stable clones. While the use of monocistronic pAH4602 and pAG4622 expression vectors (Dharshanan et al. 2011a) and monocistronic pFUSE expression vectors allows the expression of H1C2

mAbs, but due to substantial variation and unpredictable stability of expression among transfectomas, extensive clone screening is required to identify suitable high producers.

The low number of stable and low producer cells may be due to various factors:

i) The large genomic re-arrangements that frequently occur during amplification, thus resulting in high-producing clones that can be unstable as seen in NS0-pFUSE-M (Figure 7.13). Here almost half of the transfectomas were found to be non-producers. This instability of a transfectoma may involve silencing of the exogenous gene resulting from modifications such as methylation of CpG DNA sequences (Zhang et al. 2010), histone deacetylation and chromatin condensation (Kim et al. 2011).

ii) Efficient expression of the transgene is also highly dependent on the site of integration (Kwaks & Otte, 2006). In the genome, genes reside in chromatin which is the complex structure of DNA and two forms of chromatin, euchromatin and heterochromatin, can be distinguished. Integration of a transgene into, or close to, heterochromatin will result in a silenced or repressed state of the transgene, whereas integration into actively transcribed euchromatin usually leads to transgene expression. Because a large proportion of the genome is in the form of heterochromatin, the chance that a transgene integrating into, or close to, heterochromatin is high, and the chance that the gene is silenced or repressed is also high. Importantly, once genes are in a repressed heterochromatic state, this status is transmitted to daughter cells.

With conventional vectors, most integration is most likely to suffer from position effects and chromatin shutdown, which leads to gene-silencing and unproductive integration events. As a result, pools of stably-transfected cells generally show low productivity and stability, as only a few cells might be good producers, and gene silencing and overgrowth of non-producers rapidly diminish the overall productivity of the pool. This also makes it very difficult and time-consuming to find high-expressing stable clones needed for large-scale manufacturing.

Recently, many reports have also demonstrated that the inclusion of ubiquitous chromatin opening elements in expression vectors lead to increased production and stability of transgene expression in CHO cells (Barnes & Dickson, 2006). Ubiquitous chromatin opening elements are elements derived from the promoters of housekeeping genes which are usually transcriptionally active owing to a significant extent of histone acetylation. Therefore, UCOE elements incorporated expression vectors were reported to show major improvements in gene expression in stably-transfected mammalian cells through the effects on the structure of chromatin, preventing transgene silencing and giving consistent, stable and high-level gene expression irrespective of the chromosomal integration site.

However from our study, even with the use of UCOE vectors, non-producer monoclonal CHO transfectomas were still obtained (Figure 7.13) but it was at much lower percentage compared transfectomas with the conventional pFUSE vectors. The use of UCOE had dramatically increased the proportion of high-expressing cells within a stably-transfected pool of cells (Figure 7.13). Inclusion of UCOE in the expression construct may have ensured that the integration transgene is in an ‘open’ conformation,

maximizing the potential of each integration event, and ensuring more consistent high-level expression.

7.5.5 Advantages of CHO cells over NS0 cells

A majority of mAbs are currently produced using CHO cells instead of NS0, for several reasons:-

(i) CHO cell line, similar to other mammalian cells used for production, has the ability to modify its protein products with glycosylation patterns similar to those in humans.

(ii) CHO cells have the ability to grow to high densities in bioreactors.

(iii) CHO cells pose a low risk for the transmission of human viruses.

(iv) CHO cell line is known to have an unstable genome relative to other candidate cell lines considered for production, making them a good candidate for gene amplification and other genetic manipulations.

(v) CHO cells, similar to other cell candidates, can be easily transfected with gene vectors that are expressible to produce therapeutic proteins of choice.

(vi) Industrial CHO production process involving gene transfection and selection are well characterized (Cacciatore et al. 2010).

To our knowledge, H1C2 mAb has been only produced using NS0 cells and this will be the first time this antibody was produced in CHO cells.

Although the use of pFUSE vectors in NS0 cells gives low yield, the pFUSE vectors nevertheless are less costly compared to UCOE vectors. Therefore, it will be more cost-

effective to use pFUSE vectors and NS0 cells to expresses novel humanized mAbs for initial characterization studies. Once these novel humanized mAbs are validated to have significant therapeutic effect, and if high quantities of the humanized mAbs are required, then the production of humanized mAbs should be switched to CHO cells using UCOE expression vectors. Nevertheless, the reason why H1C2 mAb could not be expressed in NS0 cells using UCOE expression vectors or in CHO cells using pFUSE vectors could not be determined.

7.6 CONCLUSION

From the experiments described in this chapter several conclusions may be drawn. First, the use of synthetic DNA coding the variable regions of H1C2 mAbs provides a convenient and fast route to develop H1C2 mAb which are still functional. Second, irrespective of the vector DNA size, the linearization of vectors increases the transfection efficiency; however the restriction enzyme must be chosen with careful consideration. Third, pFUSE expression vectors work well in NS0 cells and UCOE expression vectors work well in CHO cells. Fourth, it is advisable to use two different selection antibiotics for co-transfection performed using two monocistronic vectors, in order to minimize the probability of obtaining transfectomas expressing only the light chain of the antibody. Finally, the use of UCOE vectors in CHO cells results in more high-producing and stable clones compared to use of pFUSE vectors in NS0 cells. Because the majority of the cells are stable high producers, by using UCOE vectors it is much easier and faster to find high-yielding clones with the productivity and stability required for manufacturing, as opposed to the ‘needle-in-a-haystack’ approach using conventional vectors. In addition, this is the first time H1C2 mAb was expressed in

CHO cells which is the preferred cells for manufacturing. Therefore these H1C2 mAbs could now be conveniently produced at large-scale at a reduced cost.