CHAPTER III

CONSTRUCTION OF EXPRESSION VECTORS WITH HUMANIZED ANTI-C2 VARIABLE REGIONS USING DEIMMUNIZATION AND LOGICAL APPROACH METHODS

3.1 OBJECTIVE

The main objective of this chapter is to use recombinant DNA technologies to generate expression vectors harboring humanized anti-C2 variable regions for transfection into mammalian cells to produce specific humanized antibodies. Two methods of antibody humanization; a deimmunization method and a logical approach method were applied in this chapter to identify potential immunogenic mouse amino acids in the variable regions. Judicious replacements of those few amino acids residues by the corresponding residues from the highest homologous human sequences were performed using overlapping-PCR mutagenesis. The DNA sequences coding the humanized heavy and light anti-C2 variable regions were cloned into pAH4602 and pAG4622 expression vectors respectively and were co-transfected in NS0 cells. The expression, production and characterization of humanized anti-C2 mAbs developed using deimmunization (H1C2 mAb) and logical approach method (H2C2 mAb) are described in the subsequent chapters.

3.2 INTRODUCTION

The use of monoclonal antibodies (mAbs) generated from hybridoma technology for diagnosis and treatment of human cancers has been the subject of intense research for many years (Zafir-Lavie et al. 2007). Unfortunately, since mouse splenocytes are used, the resulting mouse mAbs are likely to induce an anti-antibody response (AAR), known as human anti-mouse antibody (HAMA) response when administered in humans (Hwang et al. 2005).

Via DNA recombinant technologies, it is possible to produce chimeric and humanized mAbs having more desirable properties. Although the use of chimeric mAbs may reduce the AAR, however, chimeric mAbs still contain one third of antibody of mouse origin and could still induce a significant AAR, known as human anti-chimeric antibody (HACA) response. On the other hand, humanized mAbs which are commonly developed by complementary-determining regions (CDR)-grafting method may have negligible AAR but are often non-functional due to the loss of antigen-binding function.

This is because some non-CDR mouse residues in the framework region are still required for the effective binding of humanized mAbs and injudicious substitution of critical residues that maintain the CDR conformation required for specific binding to antigen may cause loss of binding ability (Mateo et al. 2000). Consequently, tedious and laborious back mutations are required to restore their functionality.

Therefore, in the process of humanization it would be desirable to substitute mouse amino acid residues in the variable region as judiciously as possible. A high degree of homology in the mouse and human framework will ensure that the CDR will have the

greatest chance of retaining the binding properties. However, there will still be a certain degree of amino acid mismatch in these frameworks between human and mouse antibodies, which may give rise to AAR. Thus careful consideration of strategic substitution of these mismatched residues is needed for the humanized mAb to maintain binding affinity and at the same time induce minimum AAR when being administered in humans.

In this chapter, we describe the generation of two humanized mAbs using deimmunization method and logical approach method against the C2-antigen, which is specifically expressed in colorectal carcinoma cells, from mouse anti-C2 mAbs. Here, only limited residues in the mouse framework region were considered for humanization. Two versions of humanized anti-C2 mAbs were developed; H1C2 mAb using deimmunization method and H2C2 mAb using logical approach method. The functionality of both humanized mAbs were characterized *in vitro* using cell-based assay. The immunogenicity were characterized *in vivo* using *Macaca fascicularis* monkeys and is presented in chapter 6.

3.3 MATERIALS AND METHODS

All materials required for the construction of monocistronic expression vectors harboring humanized VH and VL, unless otherwise mentioned, were provided in the deimmunization technology transfer. The procedures involved in this technology were done as described by Roque-Navarro et al. (2003). The molecular techniques were performed according to Sambrook and Russell (2001) and unless otherwise stated,

centrifugation were all performed using MiniSpin plus (Eppendorf, Germany) at 14 500 rpm.

The quantification of RNA, DNA fragments and vectors DNA were done using a Nanophotometer (Implen, Germany), while all recombinant *Escherichia coli* containing desired vector DNA were preserved for long-term storage by adding 0.15 ml glycerol to 0.85 ml of the recombinant bacteria culture which were grown for 12-14 hours at 250 rpm at 37ºC. The mixture was mixed by pipetting and stored at -80ºC. All primers were synthesized at 1st BASE Laboratories, Malaysia. All DNA sequencing reactions were also performed at 1st BASE Laboratories, Malaysia. All restriction enzymes, ligase, DNA markers were purchased from Promega, USA. Phosphate buffers used were 1M with pH 7.4. All other consumables and chemicals used in this thesis were acquired from Life Technologies, USA and Sigma-Aldrich, USA.

3.3.1 Amplification, cloning and sequence analysis of mouse anti-C2 variable regions

3.3.1.1 Culture of hybridoma cells secreting anti-C2 mAb

Hybridoma cells secreting anti-C2 mouse monoclonal antibodies were kindly provided by Dr. Cristina Mateo from Centre of Molecular Immunology, Havana, Cuba. The hybridoma cells were developed by the method described by Kohler and Milstein (1975) using a colorectal cell line, SW1116, which expresses the C2-antigen. The cryopreserved hybridomas were thawed by adding 1 ml of freshly prepared growth media (GM) and mixed by pipetting. The GM was made up of Dulbecco's modified eagle media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (cat. no.:

S0615, Biochrom, Germany), 1% (v/v) glutamax (cat. no.: 35050, Life Technologies, USA) and 1% (v/v) antibiotic/antimycotic (cat. no.: 15240, Life Technologies, USA). Then, the mixture was transferred to a 15 ml centrifuge tube containing 9 ml of GM and cells were pelleted by centrifugation at 1000 rpm at 25ºC for 5 minutes. The cells were then dissolved in 1 ml of GM and transferred to T75 flasks (cat. no.: 156472, Thermo Fisher Scientific, USA) containing 19 ml of GM and incubated in CO_2 incubator (Binder, Germany) for 24 hours. Then, cell supernatant was removed and 20 ml of fresh GM were added.

Cells were observed daily under an inverted microscope (Nikon, USA) and when cells were 80-90% confluent, cells were dislodged from the surface of the flask by 'tapping' and 10 ml of media containing cells were transferred to new T75 flask, followed by the addition of 10 ml of fresh GM to both flasks. For long term storage, cells with 80-90% confluency were similarly dislodged and centrifuged at 1000 rpm at 4ºC for 5 minutes. The cells were resuspended in 1 ml cryopreservation media which was made up of GM containing 10% DMSO (v/v) and were transferred to cryotubes (cat. no.: 377224, Thermo Fisher Scientific, USA). The cryotubes were placed in Coolcell (Biocision, USA) at -80ºC overnight. Coolcell allowed the gradual freezing of cells at -1ºC/minute before the vials containing frozen cells were transferred to liquid nitrogen.

3.3.1.2 RNA extraction

Prior to the extraction of RNA, cells in T-75 flasks with 80-90% confluency were dislodged and transferred to a 50 ml centrifuge tube. Cells were pelleted at 1000 rpm at 25ºC for 5 minutes and resuspend in 10 ml phosphate buffer. The cells were pelleted and resuspended with phosphate buffer again. Then, the volume containing 10^6 of viable

cells which were calculated using Trypan Blue dye exclusion assay (Dharshanan et al. 2011b) were resuspended in 1 ml phosphate buffer using 1.5 ml centrifuge tube.

The total RNA was extracted using RNeasy Mini Kit (cat. no.: 74104, Qiagen, Germany) according to the manufacturer's instructions. Briefly, cells in 1 ml phosphate buffer were centrifuged for 10 minutes and resuspended in 600 µl of buffer RLT by pipetting. Cells were then homogenized by passing the lysate through blunt 21-gauge needles fitted to a 1 ml syringe at least 10 times. Then, 600 µl of 70% ethanol were added to the homogenized lysate and were mixed by pipetting before 600 µl of the mixture were transferred to an RNeasy spin column placed in a 2 ml collection tube. The mixture was centrifuged for 15 seconds and the flow-through was discarded. The process was repeated with the remaining 600 µl mixture, followed by the addition of 700 µl of buffer RWI to the column and centrifugation for 15 seconds.

The flow-through was discarded; the column was washed by adding 500 µl of buffer RPE and centrifuged for 15 seconds. The flow-through was discarded and the washing was repeated using 500 µl of buffer RPE and was centrifuged for two minutes. The column was further centrifuged for one minute to remove any possible carry-over of buffer RPE. The RNeasy spin column was then placed in a 1.5 ml centrifuge tube and 50 µl of RNase-free water were added directly to the membrane of the column. The RNA was eluted by centrifugation for one minute and was quantified as mentioned in section 3.3.

3.3.1.3 cDNA synthesis of VH and VL

The cDNA synthesis of VH and VL was done using Superscript III First strand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (cat. no.: 1800080-051, Life Technologies, USA). For each synthesis, 7.0 µl (5 µg) of purified total RNA from section 3.3.1.2, 1.0 µl of dNTPs (10 mM) and 2.0 µl of primer H (10 mM) for VH or primer L (10 mM) for VL (Table 3.1) were used. The mixtures were incubated at 65ºC for 5 minutes and then placed on ice for 1 minute. Then, 10 µl of cDNA mix were added into each mixture and incubated at 50ºC for 50 minutes followed by incubation at 85ºC for 5 minutes. The mixtures were then incubated on ice for 1 minute prior to the addition 1 µl of RNase H and incubated at 37ºC for 20 minutes. The cDNA mix is made up of 2.0 µl RT buffer (10X), 4.0 µl MgCl (25 mM), 2.0 DTT (0.1 M), 1.0 µl RNaseOUT and 1.0 µl SuperScript III RT. The resulting cDNA of VH and VL were named mouse-cDNA-VH and mouse-cDNA-VL, respectively.

3.3.1.4 PCR amplification of mouse VH and VL

The cDNAs from section 3.3.1.3 were amplified using *Taq* DNA Polymerase Recombinant kit (cat. no.: 11615-010, Life Technologies, USA). For each VH and VL amplification, 5.0 µl buffer (10X), 1.0 µl dNTPs (10 mM), 3.0 µl MgCl (25 mM), 1.0 µl *Taq* DNA Polymerase Recombinant, and 2.0 µl of cDNA, 2.0 µl of forward primers (10 mM), 2.0 µl of reverse primers (10 mM) and sterile water were added to a final volume of 50.0 µl.

For the amplification of VH, mouse-cDNA-VH, primer HF0 and HR0 were used while for amplification of VL, mouse-cDNA-VL, primer LF0 and LR0 were used (Table 3.1). Primers HF0 and LF0 are forward primers while primers HR0 and LR0 are reverse primers. Both amplifications were executed by PCR using Peltier Thermal Cycler-100, (MJ Research, USA) and according the following steps: 1) 94ºC for 3 minutes, 2) 94ºC for 40 seconds, 3) 55ºC for 30 seconds, 4) 72ºC for 90 seconds, 5) repeat steps 2-4 for 30 cycles, 6) 72ºC for 10 minutes, and finally cool to 4ºC**.** The resulting PCR products were named mouse-PCR-VH for VH and mouse-PCR-VL for VL.

3.3.1.5 Agarose gel electrophoresis

Both PCR products were analyzed via agarose gel electrophoresis using Mini 50 VP model (Major Science, USA).

Agarose gel 1% (w/v), was prepared by adding 0.4 g of agarose (1st BASE Laboratories, Malaysia) in 40 ml of TAE buffer (1 L of sterile water containing 0.372 g EDTA, 4.84 g Tris Base and 1.142 ml of acetic acid glacial) using a 100 ml conical flask. The mixture was then heated using microwave for two minutes, and allowed to cool before transfer to gel cast.

The comb was inserted and gel was allowed to cool for 30 minutes before the comb was removed. Then, 5.0 µl of mouse-PCR-VH and mouse-PCR-VL were each mixed with 1.0 µl loading dye and transferred into designated wells. Similarly, 100 bp DNA ladder was also loaded in a separate well for estimation of the size of the PCR-products. The electrophoresis was performed at 75 V for 50 minutes and the PCR products were stained by soaking the gel in with DNA staining solution (1 L of sterile water containing

0.002% (v/v) ethidium bromide) for 5 minutes and visualized using AlphaImager 2200 (ProteinSimple, USA).

3.3.1.6 Purification of VH and VL from agarose gel

The DNA fragments corresponding to mouse-PCR-VH and mouse-PCR-VL described in section 3.3.1.5 were purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Germany) following procedures outlined by the manufacturer. The DNA fragments which were visible under UV light were excised using scalpel and gels containing the DNA fragment were then transferred to pre-weighed 1.5 ml centrifuge tubes and reweighed. For every mg of the gel, 3 µl of buffer QG were added and the tubes were incubated at 50ºC for 10 minutes. Once the gels had dissolved, 1 µl of isopropanol was added to each mg of gel and the dissolved gel was transferred to QIAquick columns and the columns were centrifuged for 1 minute.

The flow-through was removed, 0.5 ml of buffer QG were added to the QIAquick columns and the columns were centrifuged for 1 minute. The flow-through was removed and, 0.75 ml buffer PE were added to columns and centrifuged for 1 minute. The flow-through was discarded and the columns were centrifuged for an additional 1 minute to remove residual buffer PE. The columns were then placed in new 1.5 ml centrifuge tubes and 50 µl of elution buffer was added to the membrane of the columns. The columns were allowed to stand at room temperature for 1 minute before they were centrifuged for 1 minute. The flow-through containing the purified mouse-PCR-VH and mouse-PCR-VL were then used for section 3.3.1.7.

3.3.1.7 Cloning of mouse-VH and mouse-VL into cloning vectors

The purified mouse-PCR-VH and mouse-PCR-VL from section 3.3.1.6 were then ligated to pCR2.1-TOPO cloning vectors using TOPO-TA cloning kit (cat. no.: K4500-01, Life Technologies, USA). For ligation, 1.0 µl of salt solution, 1.0 µl of pCR2.1-TOPO vectors and 2.0 µl of sterile water were added to 2.0 µl of purified mouse-PCR-VH or mouse-PCR-VL. The ligations were performed at room temperature for 30 minutes. The resulting vectors were named mouse-pVH for VH and mouse-pVL for VL.

3.3.1.8 Transformation

For the propagation of vectors, One Shot Mach1-T1 Chemically Competent *E. coli* (cat. no.: C8620-03, Life Technologies, USA) was used. Vials containing competent *E. coli* were thawed on ice before 5.0 µl of the ligation mixture from section 3.3.1.7 were added. The vials were incubated on ice for 30 minutes and were then heat-shocked for 30 seconds at 42ºC. Vials were then immediately placed on ice for 2 minutes. Next, 250 µl of S.O.C media were added into each vial and the mixtures were incubated at 37ºC for 90 minutes at 225 rpm. Fifty µl of the transformation mix were spread on pre-warmed LB-agar plates containing 100 µg/ml of ampicillin and 40 µg/ml of X-gal. The plates were then inverted and incubated at 37ºC for 16 hours.

3.3.1.9 Isolation and culture of bacterial colonies

For each mouse-pVH and mouse-pVL vector, 24 white bacterial colonies were isolated using sterile tips and transferred to 15 ml centrifuge tubes containing 5 ml of LB-broth

with 100 µg/ml ampicillin. The recombinant bacteria were propagated at 37ºC for 16 hours at 250 rpm.

3.3.1.10 Vector extraction (small-scale)

Mouse-pVH and mouse-pVL vectors were purified in small-scale using QIAprep spin Miniprep kit (cat. no.: 27106, Qiagen, Germany) according to the manufacturer's instructions. First, 1.5 ml of each bacterial cultures prepared as described in section 3.3.1.9 were transferred to 1.5 ml centrifuge tubes and centrifuged for 5 minutes. The supernatants were discarded and another 1.5 ml of bacterial cultures were added to the same tubes and centrifuged again.

The bacterial pellets were then resuspended in 250 µl of buffer P1 before 250 µl of buffer P2 were added. The buffers were mixed by inverting the tubes 6-8 times prior to the addition of 350 µl of buffer P3. The buffers were mixed by inverting the tubes 6-8 times. The mixtures were then centrifuged for 10 minutes and the supernatants were transferred to QIAprep spin columns. The columns were centrifuged for 1 minute, then the flow-through was discarded and 0.5 ml of buffer PB was added. The columns were again centrifuged for 1 minute, the flow-through was discarded and the columns were washed by adding 0.75 ml buffer PE and centrifuged for 1 minute. The resulting flow-through was discarded and the columns were centrifuged again for another 1 minute to remove residual buffer PE. The QIAprep spin columns were then placed in 1.5 ml centrifuge tube, 50 µl of buffer EB were added and allowed to stand at room temperature for 1 minute. The vectors were then eluted by centrifuging the mixture for 1 minute. The eluted vectors were analyzed by agarose gel electrophoresis as described in

section 3.2.1.5 using supercoiled DNA ladder (cat. no.: 15622-012, Life Technologies, USA) as reference.

3.3.1.11 DNA sequencing and multiple alignments

Ten µl of each eluted mouse-pVH and mouse-pVL vector from section 3.3.1.10 were transferred to 0.6 ml centrifuge tubes and sent to 1st BASE Laboratories, Malaysia for DNA sequence analysis using M13 forward and reverse primers. The DNA sequences of mouse-VH and mouse-VL sequence were determined and compared with the reference sequences provided in the technology transfer. The comparison was executed using ClustalW multiple alignment program in Bioedit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.zip). This software was also used to convert the DNA sequences to its corresponding amino acids sequence.

3.3.2 Humanization of mouse anti-C2 mAbs

To determine the amino acids of mouse anti-C2 VH and VL obtained from section 3.3.1.11 that differ with its homologous human amino acids, 100 most homologous human sequences for each VH and VL were obtained from the IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/). The amino acid sequences of the mouse mAb variable regions were compared with the corresponding sequences of human antibody variable regions using IgBLAST. Only selected mouse amino acids that differed from the homologous human amino acids residues were considered to be humanized using deimmunization method and logical approach method.

3.3.2.1 Deimmunization method

For the development of humanized anti-C2 mAb using deimmunization method (H1C2 mAb), another software known as AMPHI (Margalit et al. 1987) was used to identify the amphipathic α helix sequences which are putative T-cell epitopes existing in both VH and VL amino acids sequences of the mouse anti-C2 antibodies acquired from section 3.3.1.11. These amphipathic mouse α helix sequences were predicted to be immunogenic to humans.

The mouse amino acid residues in the amphipathic α helix structure that differed from the residue present at the same position in the human sequence were considered candidates to be substituted. The mouse amino acids were substituted with the residue at the same position in the human sequence. These amino acid substitutions were performed by overlapping-PCR mutagenesis using primers pre-designed to contain mutations in its DNA sequence to substitute the original mouse DNA sequence to the sequence that codes for corresponding homologous human amino acid residue.

3.3.2.2 Logical approach method

For the development of humanized anti-C2 mAb using logical approach method (H2C2 mAb), no additional software aside from IgBLAST was used. From the mouse-human amino acids comparison from section 3.3.2, the mouse framework with the highest homology to its corresponding human framework was determined. Then, the mismatched mouse-human residues (if present) were considered candidates for substitution only if the corresponding human amino acid was present in 90% of the total human variable regions analyzed in section 3.3.2. The substitution was also executed by

overlapping-PCR mutagenesis using primers pre-designed to contain mutations in its DNA sequence.

3.3.3 Humanization and subcloning of humanized variable regions into expression vectors

3.3.3.1 Deimmunization method

3.3.3.1.1 Overlapping-PCR mutagenesis

Only the mouse-pVH and mouse-pVL vectors from section 3.3.1.10 that were confirmed to have the exact DNA sequence (as the reference sequences provided in the technology transfer) were used as templates to develop humanized-pVH4A and humanized-pVL3A. Humanized-pVH4A and humanized-pVH3A are vectors containing mouse-pVH and mouse-pVL that have 4 and 3 humanized amino acid residues, respectively. The humanization was executed by overlapping-PCR mutagenesis using primers shown in Table 3.1.

Primers prefixed H and L were used for VH and VL, respectively. For both VH and VL, primers HF0-3 and LF0-4 were used as forward primers, while primers HR0-3 and LR0-4 were used as reverse primers.

Primers numbered 1-4 were mutagenic primers containing nucleotide substitution (letters underlined and bolded) in its DNA sequence to introduce the specific amino acid conversion at the desired residues. From the IgBLAST and AMPHI software analysis, it

was found that 7 amino acids; residues 10, 17, 44 and 45 on mouse VH and residues 15, 50 and 68 on mouse VL should be humanized to its corresponding human residues, in order for the resulting H1C2 mAb to have reduced immunogenicity and still able to bind to the C2-antigen.

A) Humanization of residue 10 of mouse VH and residue 15 of mouse VL

Each of the site-specific mutagenesis was done by three sets of PCRs outlined below.

(i) PCR-1: the mixture contained 48.0 µl of master-mix components and 1.0 µl each of primers HF0/LF0 and HR1/LF1.

(ii) PCR-2: contained the exact components as PCR-1 except that primers HF1/LF1 and HR0/LF0 were used instead.

The master-mix was made up of 10.0 µl of reaction buffer (10X), 2.0 µl of dNTPs (10 mM), 2.0 µl of recombinant *Taq* DNA polymerase, 3.0 µl of MgCl, 73.0 µl of sterile water and 6.0 µl of mouse-pVH/mouse-pVL vectors. The two PCR products were then purified as described in section 3.3.1.6 before being merged using overlapping-PCR (PCR3).

(iii) PCR-3 (overlapping-PCR): the mixture was similar to the master-mix of PCR-1 and 2 with the following exception, 76.0 µl of sterile water, 1.0 µl of recombinant *Taq* DNA polymerase, 1.0 µl of each primer HF0/LF0 and HR0/LR0 were used and the mouse-pVH/mouse-pVL vectors were substituted with 3.0 µl of each purified PCR-1 and PCR-2 products for a final volume of 100 µl.

Table 3.1: List of primers and their DNA sequences. Primers with underlined and bold nucleotide are mutagenesis primers designed to introduce mutations at specific mouse residues to its homologous human residues

Primer name	Primer sequence
H	5' GGTCTAGAACCTCCACACACAGCAACCAGTGGATAGAC-3’
L	5'-GCGTCTAGAACTGGATGGTGGGAAGATGG-3’
HF0	5'-GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT-3’
HR0	5'-GGGGCTAGCTGCAGAGACAGTGACCAGAGT-3’
HF1	5'-GAGTCAGGACCTG**G**CCTGGTGAAACCT-3’
HR1	5'-AGGTTTCACCAGG**C**CAGGTCCTGACTC-3’
HF2	5'-GTGAAACCTTCTCAG**A**CACTTTCACTCACC-3’
HR2	5'-GGTGAGTGAAAGTG**T**CTGAGAAGGTTTCAC-3’
HF3	5'-CGGCAGTTTCCAGGAAA**AGG**ACTGGAATGG-3’
HR3	5'-CCATTCCAGT**CCT**TTTCCTGGAAACTGCCG-3’
LF0	5'-GGGGATATCCACCATGAGGTCCCCTGCTCAGCTC-3’
LR0	5'-AGCGTCGACTTACGTTTTATTTCCAGCTTGGTCCC-3’
LF1	5'-TTGTCGGTTACC**C**TTGGACAACCAGCC-3’
LR1	5'-GGCTGGTTGTCCAA**G**GGTAACCGACAA-3’
LF2	5'-GGCCAGTCTCCAA**G**GCGCCTAATCTAT-3’
LR2	5'-ATAGATTAGGCGC**C**TTGGAGACTGGCC-3’
LF3	5'-CCTGACAGATTCA**G**TGGCAGTGGATCA-3’
LR3	5'-TGATCCACTGCCA**C**TGAATCTGTCAGG-3’
LF4	5'-GATTTCGCACTGAAAATCAG**C**AGAGTG-3’
LR4	5'-CACTCT**G**CTGATTTTCAGTGCGAAATC-3’

All PCR reactions were executed as described in section 3.3.1.4. The products of PCR-3 were purified, ligated to cloning vectors and were sent for DNA determination as described in sections 3.3.1.6-11. Vectors with the desired sequences were determined and named humanized-pVH1A and humanized-pVL1A respectively.

B) Humanization of residue 17 of mouse-VH and residue 50 of mouse-VL

The humanization was performed as described in section 3.3.3.1.1A with the following differences:

- mouse-pVH and mouse-pVL (in both PCR-1 and PCR-2) were replaced with humanized-pVH1A and humanized-pVL1A, respectively.
- Primers HF1 and HR1 were replaced with HF2 and HR2, respectively.
- Primers LF1 and LR1 were replaced with LF2 and LR2, respectively.

Vectors with the desired sequences were determined and named humanized-pVH2A and humanized-pVL2A.

C) Humanization of residues 44 & 45 of mouse-VH and residue 68 of mouse-VL

These final mutations were performed as described in 3.3.3.1.1A with the following differences:

- mouse-pVH and mouse-pVL (in both PCR-1 and PCR-2) were replaced with humanized-pVH2A and humanized-pVL2A, respectively.
- Primers HF1 and HR1 were replaced with HF3 and HR3, respectively.
- Primers LF1 and LR1 were replaced with LF3 and LR3, respectively.

Vectors with the desired sequences were determined and named humanized-pVH4A and humanized-pVL3A.

3.3.3.1.2 Double-Digestion

To facilitate the cloning of humanized variable regions into its respective expression vectors, the cloning vectors harboring the humanized variable genes were double-digested to release the relevant genes coding for VH and VL. The humanized-pVH4A and mouse-pAH4602 vectors were digested with digestion mixture 1 while humanized-pVL3A and mouse-pAG4622 vectors were digested with digestion mixture 2. Digestion mixture 1 consisted of 5.0 µl buffer 2, 0.5 µl BSA (100X), 0.5 µl *Eco*RV, 0.5 µl *Nhe*I, and 40.0 µl sterile water, while digestion mixture 2 consisted of the same components as digestion mixture 1, except that the 5.0 µl buffer 2 and 0.5 µl *Nhe*I were replaced with 5.0 µl buffer 3 and 0.5 µl *Sal*I.

To start the digestion, 3.5 µl of humanized-pVH4A and mouse-pAH4602 vectors or humanized-pVL3A and mouse-pAG4622 vectors were added to their respective digestion mixtures. Both digestions were performed at 37ºC for 1 hour, followed by the inactivation of the restriction enzymes at 80ºC for 20 minutes. The digested vectors were then analyzed by agarose gel electrophoresis using 1 kb DNA ladder as reference marker. Fragments corresponding to humanized-VH4A, humanized-VL3A, pAH4602 and pAG4622 were then purified from agarose gel as described in section 3.3.1.6.

3.3.3.1.3 Ligation

For ligation of the humanized-VH4A and humanized-VL3A into pAH4602 and pAG4622, respectively, purified digested fragments obtained from section 3.3.3.1.2 were used. Briefly, 6.0 µl of humanized-VH4A and humanized-VL3A fragments were added to 2.0 µl of purified digested pAH4602 and pAG4622 in 0.6 ml centrifuge tubes.

Then 1 µl of T4 DNA ligase and 1 µl of ligase buffer (10X) were added to both ligation mixtures.

The mixtures were then incubated at 15ºC for 16 hours. The ligation products were then transformed in *E. coli* and were plated on LB-agar containing 100 µg/ml of ampicillin. Small-scale vector extractions were performed as described in section 3.3.1.10 and 3.0 µl of the extracted vectors were again double-digested with its respective restriction enzymes to confirm the presence of humanized variable regions. The recombinant vectors were named humanized-pAH4602A for humanized-VH4A and humanized-pAG4622A for humanized-VL3A.

3.3.3.1.4 Vector extraction (large-scale)

For transfections, humanized-pAH4602A and humanized-pAG4622A vectors were purified in large-scale using QIAfilter Plasmid Maxi kit (cat. no.: 12263, Qiagen, Germany) according to the manufacturer's instructions. Fifty µl of bacterial cultures from section 3.3.3.1.3 having the desired recombinant vectors were transferred to 15 ml centrifuge tubes containing 5 ml of LB-broth with 100 µg/ml amplicilin and cultured at 37ºC for 8 hours at 300 rpm.

For each vector, the procedure described next is applied. First, 200 µl of the 8 hour bacterial culture were transferred to conical flask containing 100 ml LB-broth with 100 µg/ml ampicilin and incubated at 37ºC for 16 hours at 300 rpm. Bacterial cells were then harvested by transferring 40 ml of the 16 hours of bacterial culture to 50 ml centrifuge tubes and centrifuged at 6000 rcf for 15 minutes at 4ºC. The centrifugation was repeated for the remaining 60 ml of the bacterial culture. Then the bacterial pellets

were resuspended in 10 ml buffer P1, before 10 ml of buffer P2 were added. The buffers were mixed by inverting the tubes 6-8 times and were then incubated at room temperature for 5 minutes. Ten ml of chilled buffer P3 were added to the lysate and the contents were mixed immediately by inverting the tubes 6-8 times. The lysate were then transferred into QIAfilter cartridge and incubated at room temperature for 10 minutes.

As the incubation proceeded, QIAGEN-tip 500 column was equilibrated by applying 10 ml of buffer QBT, and the column was allowed to empty by gravity flow. Then, the lysate in the QIAfilter cartridge was transferred into the equilibrated QIAGEN-tip 500 column and allowed to be filtered by gravity flow. The QIAGEN-tip 500 column was then washed twice with 30 ml of buffer QC before 15 ml of buffer QF was added to elute the DNA by placing the column in a clean 50 ml centrifuge tube.

DNA was then precipitated by adding 10.5 ml isopropanol to the elution and the contents were mixed and immediately centrifuged at 20 000 rcf for 30 minutes at 4ºC. The supernatant was carefully discarded without disturbing the pellet and 5 ml of 70% ethanol were added to wash the DNA. The sample was centrifuged again at 20 000 rcf for 15 minutes at 4ºC. The supernatant was carefully discarded and the pellet was air-dried for 15 minutes before being dissolved in 100 µl of sterile water. The vector was then analyzed by agarose gel electrophoresis as described in section 3.3.1.5.

3.3.3.1.5 Linearization of vectors

The vectors were linearized before transfections were done. To perform the linearization, 25.0 µg of each vector were added to digestion mixture 3 which consisted of 5.0 µl buffer 3, 0.5 µl BSA (100X), 1.0 µl*Pvu*I and sterile water was added to a final

volume of 50 µl. The digestions were then performed at 37ºC for 16 hours. To purify the linearized vectors, 200 µl of 100% ethanol were added and mixed by pipetting and the mixtures were incubated at -80ºC for 20 minutes. Then the samples were centrifuged at 13 000 rpm at 4ºC for 20 minutes, the supernatant was discarded and the pellet was washed with 200 µl of 70% ethanol. The mixtures were centrifuged at 13 000 rpm at 4ºC for 15 minutes; the supernatant carefully discarded and the pellets air-dried for 10 minutes before being resuspended in 1.2 ml of sterile water.

3.3.3.2 Logical approach method

From the IgBLAST analysis in section 3.3.2, it was found that the amino acids in framework 4 of both VH and VL had 100% homology between mouse and human. Thus no further manipulation in this framework was necessary. Therefore framework 1 of VH and 3 VL, which had the second highest degree of homology to that of human frameworks were chosen for humanization. Due to the high degree of homology between mouse and human residues, it was felt that humanization on these frameworks would result in H2C2 mAb that would still be able to bind to the C2-antigen on colorectal carcinoma and also have a reduced immunogenicity when applied in humans. In framework 1 of mouse VH, residue 10 was targeted for substitution and for framework 3 of mouse VL, residues 68 and 81 were targeted for substitutions.

These residues were chosen because the corresponding human amino acids are present in 90% of all the human antibody variable regions analyzed and substituting these residues would have the best chance of reducing immunogenicity while retaining reactivity.

3.3.3.2.1 Overlapping-PCR mutagenesis

Similar to the deimmunization method, only the mouse-pVH and mouse-pVL vectors from section 3.3.1.10 that were confirmed to have the exact DNA sequence as that provided in the technology transfer were used as templates to develop humanized-VH1B and humanized-VL2B vectors.

A) Humanization of residue 10 of mouse VH

Since this residue was also humanized with the same corresponding human amino acid in the deimmunization method, humanized-pVH1A vector from section 3.3.3.1.1A which contains this humanized residue was used, and named humanized-pVH1B instead.

B) Humanization of residue 68 of mouse VL

The mutations of these residues were performed as described in 3.3.3.1.1A with the following differences:

- mouse-pVL (in both PCR-1 and PCR-2) was used.
- Primers LF1 and LR1 were replaced with LF3 and LR3, respectively.

Vectors with the desired sequences were determined and named humanized-pVL1B.

C) Humanization of residue 81 of mouse VL

The mutations of these residues were performed as described in 3.3.3.1.1 A with the following differences:

- humanized-pVL1B (in both PCR-1 and PCR-2) was used.
- Primers LF1 and LR1 were replaced with LF4 and LR4, respectively.

Vectors with the desired sequences were determined and named humanized-pVL2B.

3.3.3.2.2 Cloning into expression vectors.

The humanized-VH1B and humanized-VL2B were cloned into pAH4602 and pAG4622 by methods described in section 3.3.3.1.2-4. The recombinant vectors were named humanized-pAH4602B for humanized-VH1B and humanized-pAG4622B for humanized-VL2B. Both recombinant plasmids were linearized and purified as described in section 3.3.3.1.5.

3.4 RESULTS AND DISCUSSION

3.4.1 Amplification, cloning and sequence analysis of mouse variable regions

Cytoplasmic total RNA was extracted from hybridoma cells expressing mouse anti-C2 mAbs and was converted to cDNA of mouse VH and VL by reverse transcription polymerase chain reaction (RT-PCR) using specific primers for VH and VL regions. Then, PCR was used to amplify the mouse variable regions. The PCR-amplified mouse VH and VL regions were found to be of approximately 500 bp and 400 bp, respectively, as illustrated in the agarose gel electrophoresis profile in Figure 3.1.

Cloning of mouse-PCR-VH and mouse-PCR-VL into pCR2.1-TOPO vectors by TA cloning method yielded vectors of approximately 3.4 kb (Figure 3.2a) and 3.3 kb (Figure 3.2b), respectively, which consisted of pCR2.1-TOPO vectors (2.9 kb) plus mouse VH (500 bp) or mouse VL (400 bp). Mouse-pVH and mouse-pVL vectors purified from 24 bacterial clones were sequenced in forward and reverse direction to double-confirm the DNA sequences. From the 24 DNA sequences analyzed, only 2 bacterial clones had the identical mouse VH DNA (Figure 3.3) and 6 clones had identical mouse VL DNA (Figure 3.4) sequence, when compared with the reference sequences provided in the technology transfer. The remaining clones had undesired mutations in their DNA sequences.

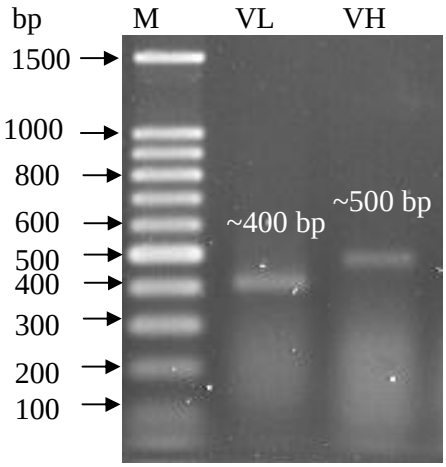
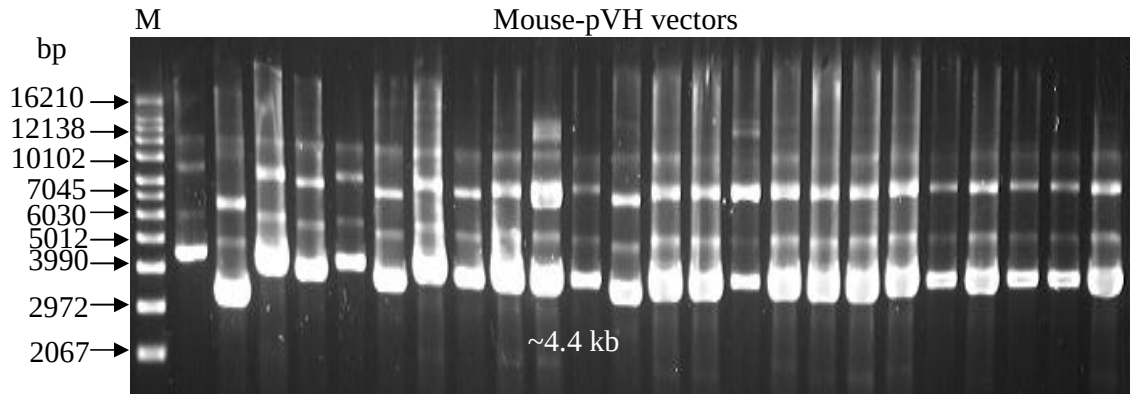


Figure 3.1: Agarose gel electrophoresis profile of PCR-amplified cDNA of mouse variable regions. Hybridoma cells secreting mouse anti-C2 monoclonal antibodies were used for the extraction of total RNA. Then, the RNA was used to synthesize cDNAs of mouse VH and VL of anti-C2 mAb, which were both amplified using PCR. The amplified mouse-PCR-VH (VH) and mouse-PCR-VL (VL) were approximately 500 bp and 400 bp, respectively. 100 bp DNA ladder (M) was used as reference.

a



b

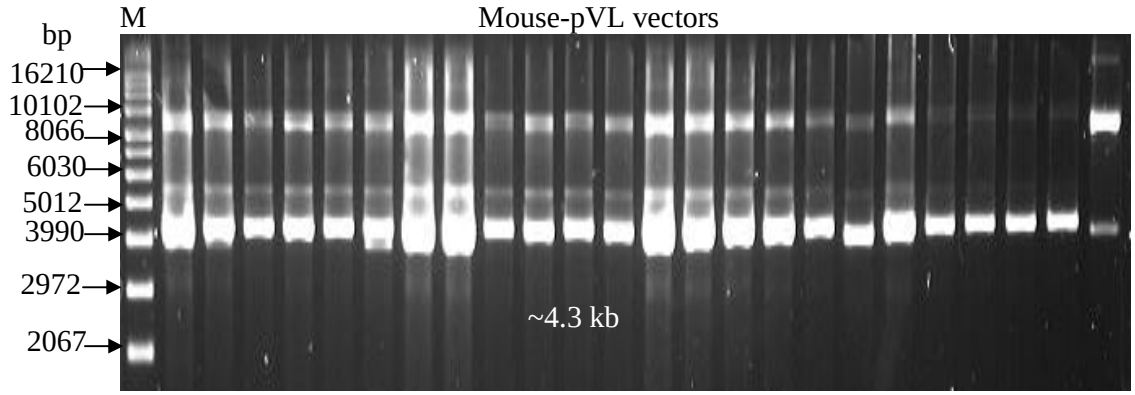


Figure 3.2: Agarose gel electrophoresis profiles of recombinant vectors containing mouse-PCR-VH (a) and mouse-PCR-VL (b). For each mouse variable region, vectors from 24 bacterial clones were extracted and analyzed. With the exception of one clone, mouse-pVH vectors from the remaining 23 clones were approximately 4.4 kb which consisted of pCR2.1-TOPO vectors (3.9 kb) and VH (500 bp). All 24 clones of mouse-TA-VL vectors were approximately 4.3 kb, which was made up of pCR2.1-TOPO vectors (3.9 kb) and mouse-VL (400 bp). Supercoiled DNA ladder (M) was used as reference.

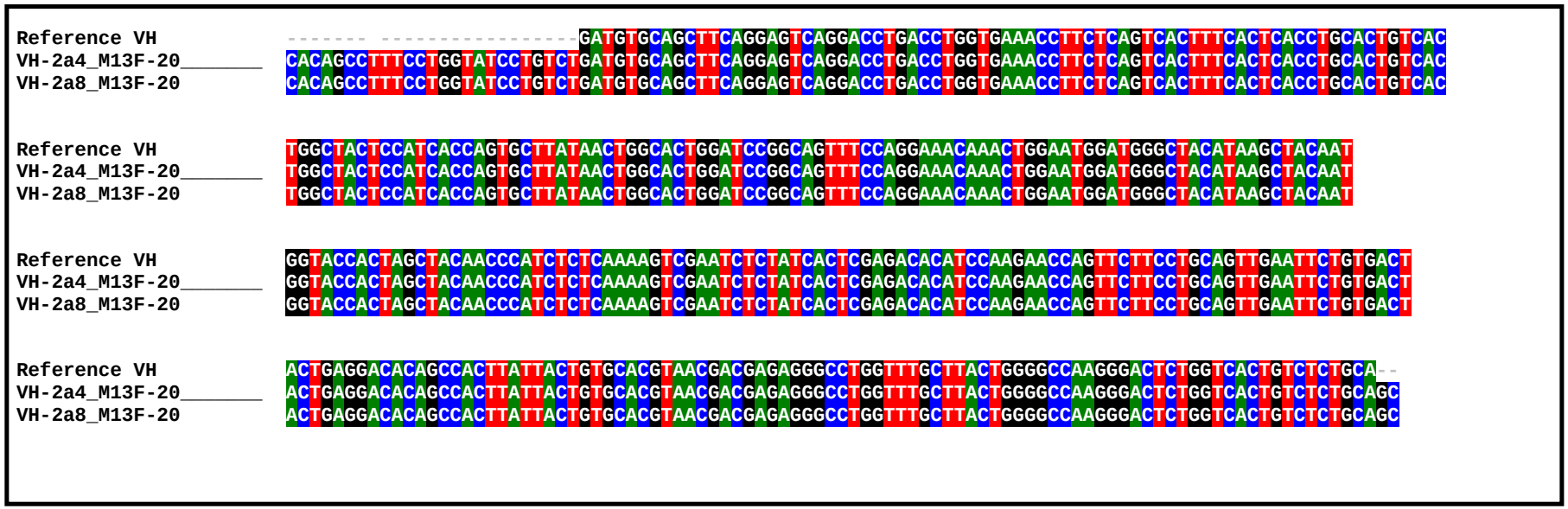


Figure 3.3: Profile of DNA sequence of mouse VH compared with reference sequence provided. From the DNA sequencing of 23 bacterial clones having mouse-pVH vectors, two clones (VH-2a4_M13F-20 and VH-2a8_M13F-20) had the exact DNA sequences as reference sequence (Reference VH).

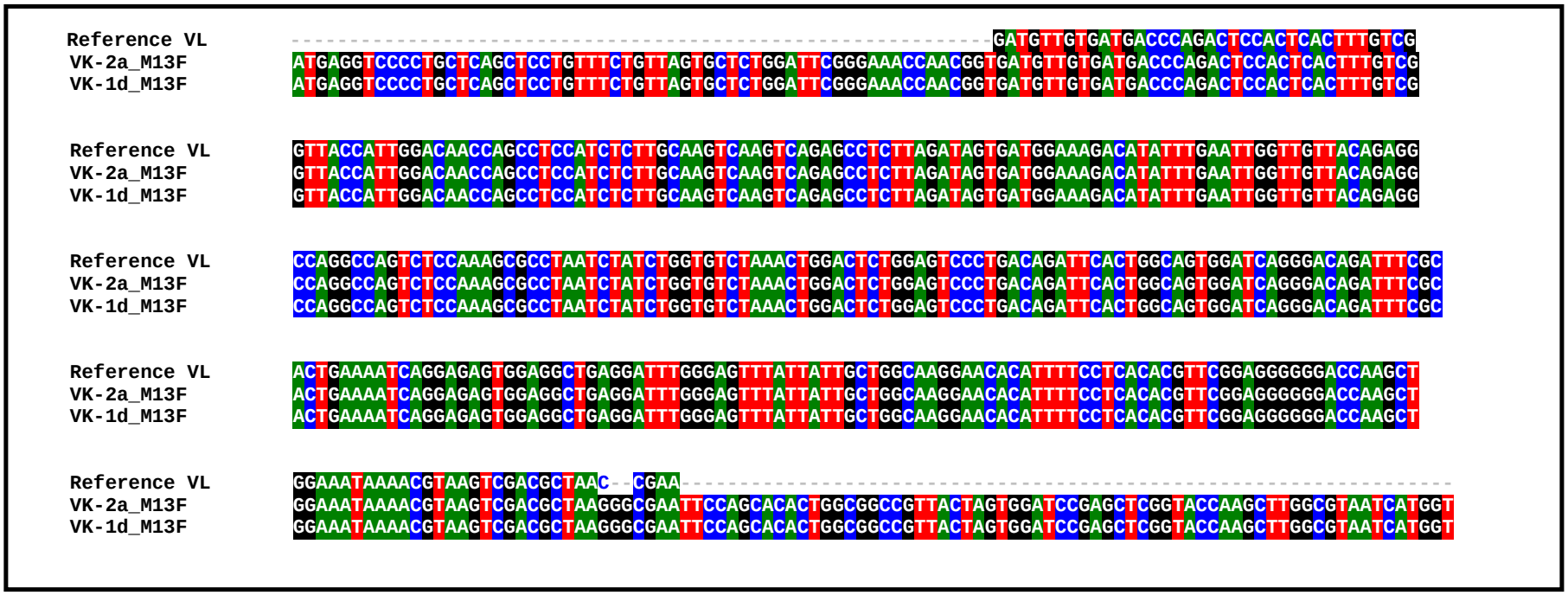


Figure 3.4: Profile of DNA sequence of mouse VL compared with reference sequence provided. From the DNA sequencing of 24 bacterial clones having mouse-pVL vectors, six clones had the desired DNA sequences as reference sequence (Reference VL). Only 2 clones are shown (VK-2a_M13F and VK-1d_M13F) in this diagram.

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3.4.2 Development of humanized anti-C2 mAbs

3.4.2.1 Deimmunization method

The DNA sequences of mouse VH and VL were converted to their corresponding amino acid sequences and were compared with their homologous human VH and VL. From the IgBLAST analysis (excluding the CDR regions), it was found that 21 (Figure 3.5) and 11 (Figure 3.6) residues differed between mouse heavy and light variable regions and their respective homologous human VH and VL sequences. The mouse amino acids were then analyzed for potential immunogenic epitopes using AMPHI.

From the output results of mouse VH using AMPHI software (Appendix A), five mid-points of amphipathic block were identified: residues 8-9, 11-16, 33-38, 47-52 and 106-110. Similarly, from the output results of mouse VL using AMPHI software (Appendix B), four mid-points of amphipathic block were identified: residues 17-18, 46-49, 59-70 and 99-104. Since five amino acids residues before and after the mid-points blocks were also classified amphipathic (Roque-Navarro et al. 2003), therefore three amphipathic segments were identified in VH (Figure 3.5): residues 3-21, 28-57 and 101-115. Likewise, three amphipathic segments were also identified in mouse VL (Figure 3.6): residues 12-23, 41-75 and 94-109.

Nevertheless, from the IgBLAST analysis, only 10 residues in VH (Figure 3.5) and 6 residues in VL (Figure 3.6) that differed between mouse heavy and light variable regions and their respective homologous human VH and VL sequences are located in the amphipathic regions. Therefore, only these residues were considered to be replaced by the respective residue present at the same position in the human sequences.

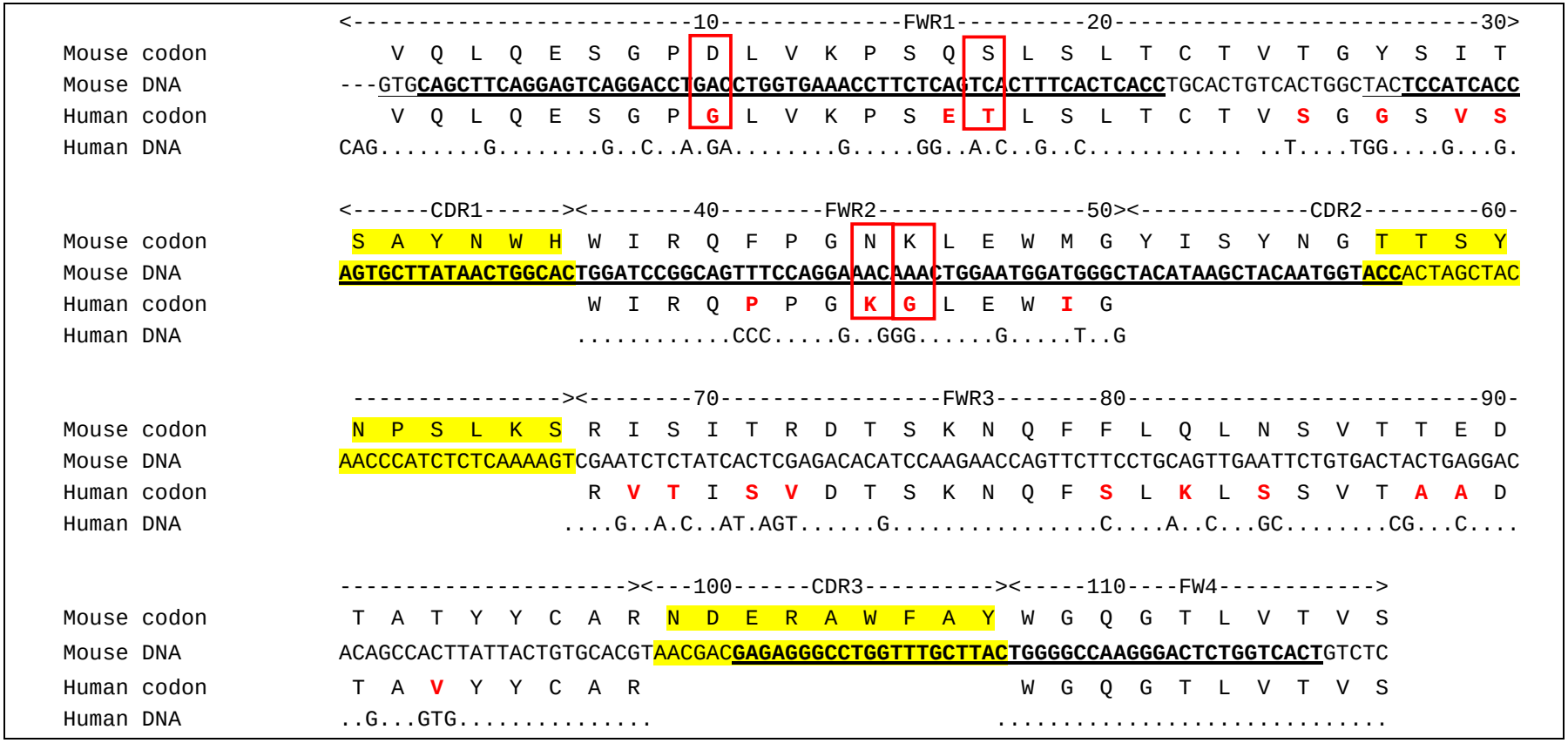

Figure 3.5: Profile of DNA and amino acid sequence comparison between mouse and human VH. Based on the amphipathic region residues (underlined) that differ (red color), only four residues (in red boxes) were humanized to the homologous human residues. Residues in the CDR regions are highlighted in yellow.

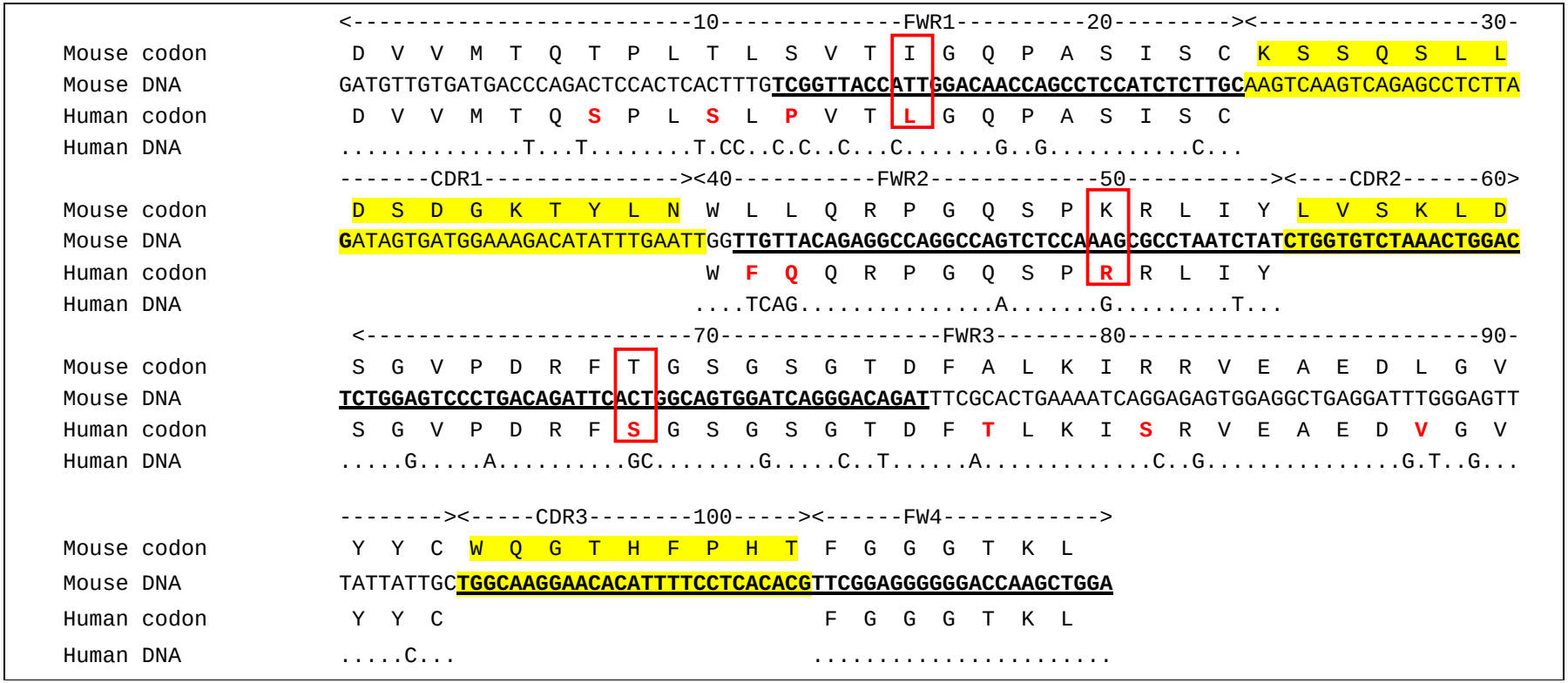

Figure 3.6: Profile of DNA and amino acid sequence comparison between mouse and human VL. Based on the amphipathic regions residues (underlined) that differ (red color), only three residues (in red boxes) were humanized to the homologous human residues. Residues in the CDR regions were highlighted in yellow.

From the 10 residues that differed in the amphipathic regions of mouse VH, three residues (27-Y, 29-I, 30-T) were located in the vernier zone (Table 3.2). While for mouse VL, from the 6 residues that differed in the amphipathic regions, 2 residues were present in vernier zone (41-L & 42-L) (Table 3.2) (Almagro & Fransson, 2008).

Residues present in both CDR and the vernier zone as summarized in Table 3.2 were not replaced because these residues contribute to the conformation of the binding site (Roque-Navarro et al. 2003, Almagro & Fransson, 2008). Although the remaining 7 and 4 residues of VH and VL may be humanized, nevertheless only four residues of mouse VH (10-D, 17-S, 44-N and 45-K) and 3 residues of mouse VL (15-I, 50-K, 68-T) were humanized.

The remaining residues of mouse VH (16-Q and 41-F) and VL (12-S) were not humanized because these amino acids are found frequently at these positions in human framework sequences (Roque-Navarro et al. 2003). The remaining 41-F of mouse VH was not humanized due to the structural constraints on the binding site confirmation, which do not allow the replacement of these residues (Roque-Navarro et al. 2003).

Hence, in the final proposed humanization for VH (Figure 3.5), the following mouse residues were substituted to the corresponding human residue: residue 10 from aspartic acid (D) to glycine (G); residue 17 from serine (S) to threonine (T); residue 44 from asparagine (N) to lysine (K); and residue 45 from lysine (K) to glycine (G). While for VL (Figure 3.6), the residues substituted were: residue 15 from isoleucine (I) to leucine (L); residue 50 from lysine (K) to arginine (R); and residue 68 from threonine (T) to serine (S).

Table 3.2: Amino acid residues of VH and VL that are located in the CDR and vernier regions

Source: Roque-Navarro et al. 2003; Almagro & Fransson, 2008

	VH (Residues)	VL (Residues)
CDR	31- 36	24- 39
	51-66	55- 60
	99-107	94- 102
Vernier Zone	2	4
	27-30	27-28
	51-52	30
	75	41-42
	79	52-53
	84	55
	101-102	74-75
	114	77
		79
		84
		106

To introduce the specific amino acids conversion from the mouse to the homologous human residue, mutagenesis primers were designed and used. Since it is also possible for more than one codon to code for one amino acid, the codon that requires the least number of nucleotide substitution was chosen as summarized in Table 3.3. Also, since residues 44 and 45 are next to each other on VH, the same set of mutagenesis primers were designed to include both mutations.

The proposed mutations were carried out for both VH and VL using three sets of sequential overlapping-PCR for both VH and VL for each amino acids conversion. Each set of overlapping-PCR mutagenesis consisted of two mutagenesis-PCRs (PCR-1 & PCR-2) and one overlapping-PCR (PCR-3).

PCR-1 resulted in the synthesis of the 5' of the VH and VL until the point of amino acid (s) substitution, while PCR-2 resulted in the synthesis of the 3' of the VH and VL from the point of amino acid (s) substitution. PCR-3 then overlapped both products of PCR-1 and PCR-2 for a complete DNA synthesis from the 5' to 3' of VH and VL identical to mouse VH and VL with the exception of the substitution of the nucleotides sequences giving rise to the desired human amino acid residues.

From Figure 3.7 which shows the set of PCRs for the conversion of residue 10 of VH, products of PCR-1 and PCR-2 (Figure 3.7a) were approximately 150 bp and 350 bp while PCR-3 product which is a combination of both PCR-1 and 2 products was 500 bp (Figure 3.7b). Similarly for the conversion of residue 68 of VL, PCR-1 and PCR-2 products (Figure 3.7c) were approximately 300 bp and 100 bp and thus combination of both products in PCR-3 (Figure 3.7d) was 400 bp.

Table 3.3: Humanization of amphipathic mouse residues to their homologous human residues using deimmunization method. The original codon that codes for mouse residues were substituted to that of human that required the least nucleotide for substitution (underlined)

Residue	Mouse amino acid	Human amino acid	Possible human codons
10 (D)-VH	Aspartic acid (GAC)	Glycine (G)	GGT, GGC, GGA, GGG
17 (S)-VH	Serine (TCA)	Threonine (T)	ACT, ACC, ACA, ACG
44 (N)-VH	Asparagine (AAC)	Lysine (K)	AAA, AAG
45 (K)-VH	Lysine (AAA)	Glycine (G)	GGT, GGC, GGA, GGG
15 (I)-VL	Isoleucine (ATT)	Leucine (L)	TTA, TTG, CTT, CTC, CTA, CTG
50 (K)-VL	Lysine (AAG)	Arginine (R)	CGT, CGC, CGA, CGG, AGA, AGG
68 (T)-VL	Threonine (ACT)	Serine (S)	TCT, TCC, TCA, TCG, AGT, AGC

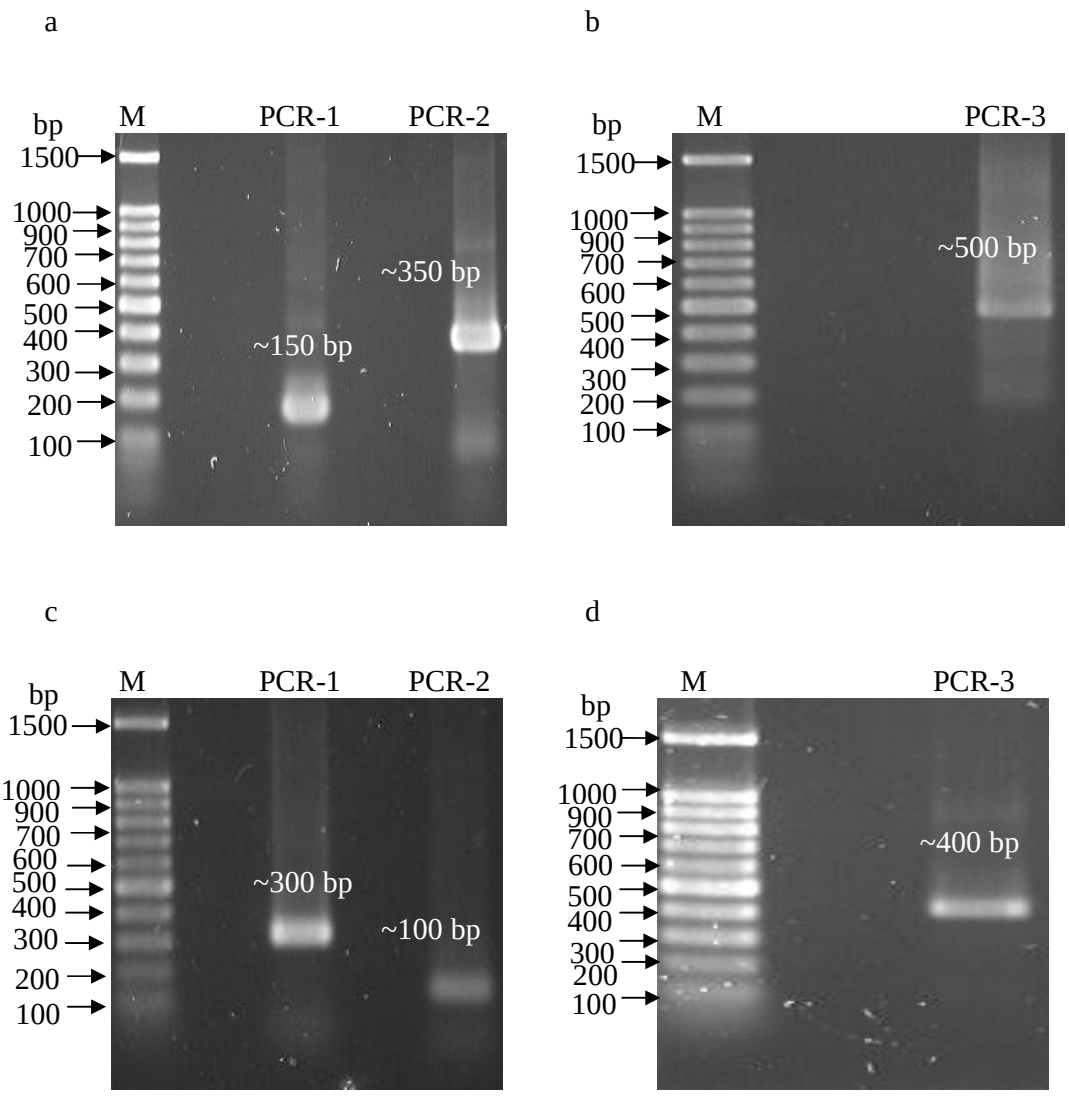

Figure 3.7: Agarose gel electrophoresis profiles of humanization of residue 10 of mouse VH (a & b) and 68 of VL (c & d). PCR-1 and PCR-2 are mutagenesis-PCR which introduces the specific amino acid humanization. PCR-3 is an overlapping-PCR which overlaps both PCR-1 and PCR-2 products. The products of PCR-1 and PCR-2 of VH (a) were approximately 150 bp and 350 bp respectively while that for VL (c) were approximately 300 bp and 100 bp. The products of PCR-3 of VH (b) and VL (d) were 500 bp and 400 bp, respectively. 100 bp DNA ladder (M) was used as reference.

After each set of overlapping-PCR mutagenesis, the products of PCR-3 were purified, cloned into TOPO-vectors and vectors from 24 bacterial clones were sent for DNA sequence determination. For the development of humanized-pVH4A, the first set of overlapping-PCR mutagenesis was performed to develop humanized-pVH1 for the conversion of aspartic acid (GAC) to glycine (GGC) at residue 10 as illustrated in Figure 3.8. The humanized-pVH1 vector was then used as a template for the development of humanized-pVH2A for the conversion of serine (TCA) to threonine (ACA) at residue 17, in addition to previous mutation at residue 10 as shown in Figure 3.9. Finally, the humanized-pVH2A vectors having both mutations at residues 10 and 17 was used as a template for the development of humanized-pVH4A for the conversion of asparagine (AAC) to lysine (AAA) and lysine (AAA) to glycine (GGA) at residues 43 and 44, respectively (Figure 3.10).

Similarly, for the development of humanized-VL3A, the first set of overlapping-PCR mutagenesis was performed to develop humanized-pVL1A for the conversion of isoleucine (ATT) to leucine (CTT) at residue 15 (Figure 3.11). The humanized-pVL1A vector was then used as a template for the development of humanized-pVL2A by the conversion of lysine (AAG) to arginine (AGG), at residue 50 in addition to previous mutation at residue 15 as shown in Figure 3.12. Finally, the humanized-pVL2A having both mutations at residues 15 and 50 was used as a template for the development of humanized-pVL3 by the conversion of threonine (ACT) to serine (AGT) at residue 68 in addition to previous mutations at residue 15 and 50 (Figure 3.13).

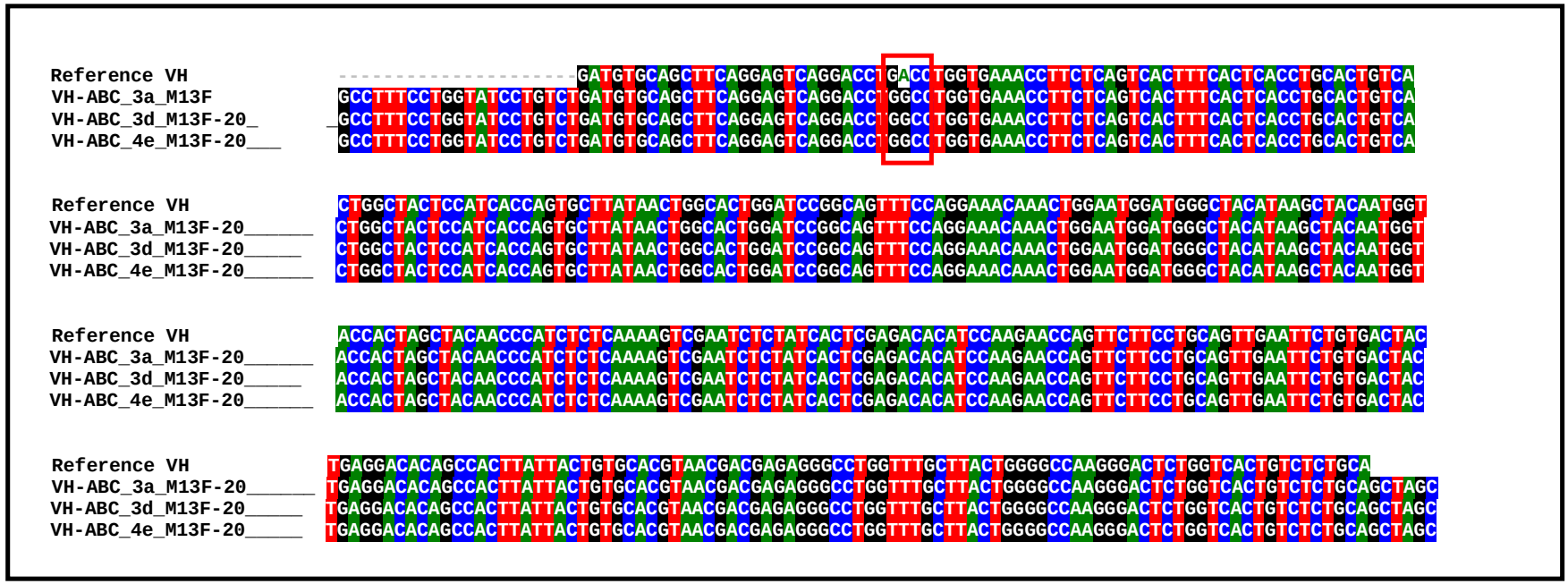

Figure 3.8: Humanization of residue 10 of VH from aspartic acid (GAC) to glycine (GGC). From the DNA sequencing of 24 bacterial clones having humanized-pVH1A vectors, three clones (VH-ABC_3a_M13F-20, VH-ABC_3d_M13F-20 and VH-ABC_4e_M13F-20) had the desired DNA humanization at residue 10 (depicted in red box), compared to the original mouse VH DNA sequence (Reference VH).

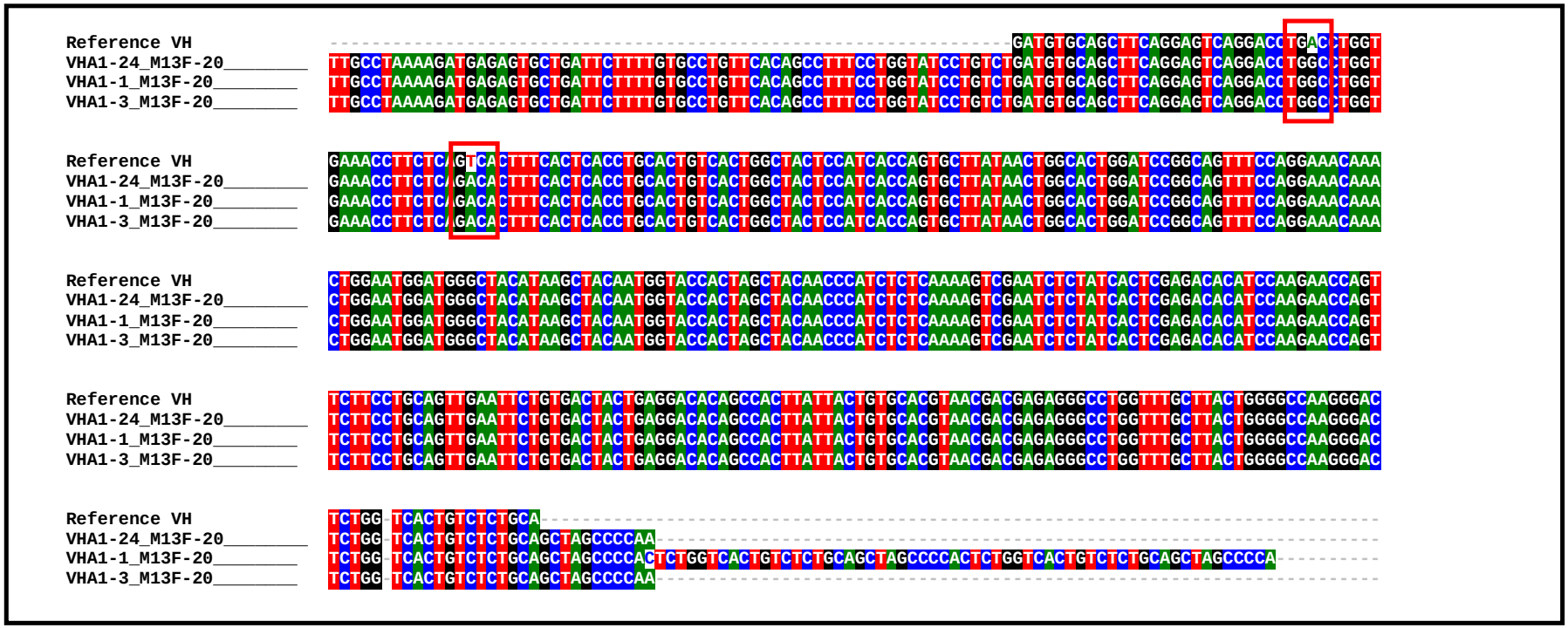

Figure 3.9: Humanization of residue 17 of VH from serine (TCA) to threonine (ACA). From the DNA sequencing of 24 bacterial clones having humanized-pVH2A vectors, eight clones had the desired DNA humanizations at residue 17 in addition to residue 10 which was humanized previously (depicted in red boxes), compared to the original mouse VH DNA sequence (Reference VH). Only 3 clones are shown (VHA1-1_M13F-20, VHA1-3_M13F-20 and VHA1-24_M13F-20) in this diagram.

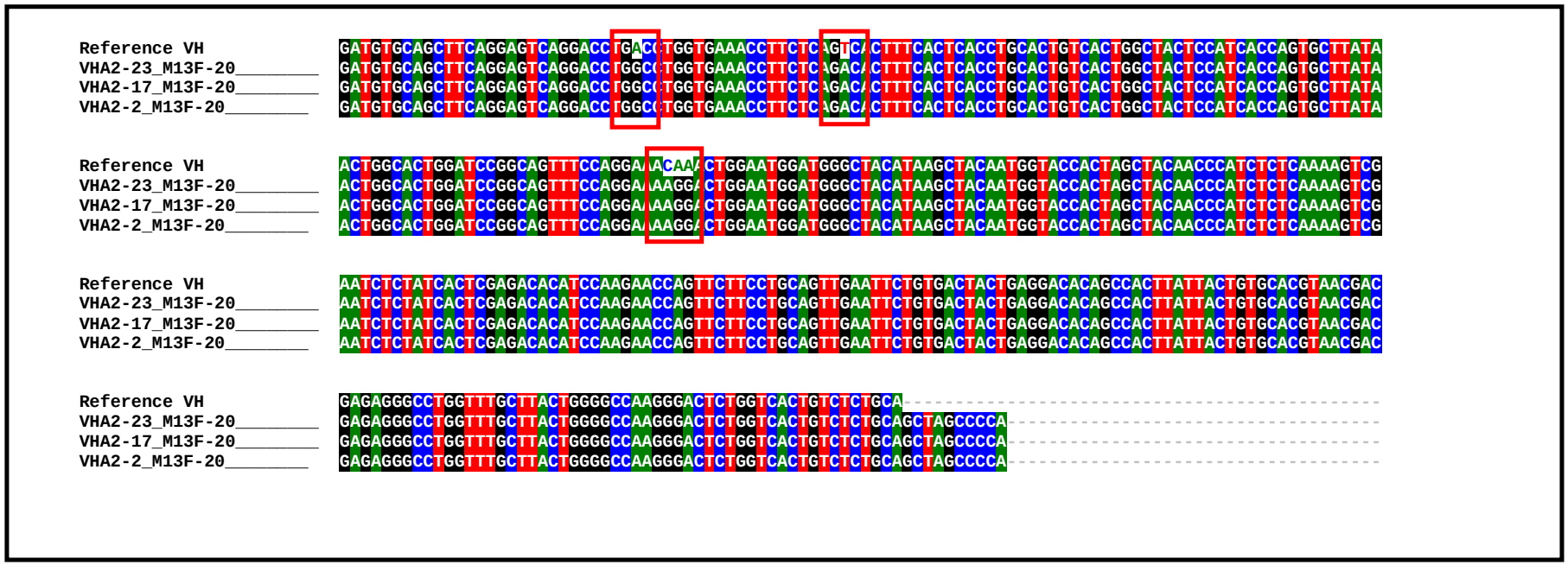

Figure 3.10: Humanization of residues 44 and 45 of VH from asparagine (AAC) to lysine (AAA) and from lysine (AAA) to glycine (GGA) respectively. From the DNA sequencing of 24 bacterial clones having humanized-pVH4A vectors, eight clones had the desired DNA humanizations at residue 44 and 45, in addition to residues 10 and 17 which were humanized previously (depicted in red boxes) compared to original mouse VH DNA sequence (Reference VH). Only 3 clones (VHA2-2_M13F-20, VHA2-17_M13F-20 and VHA2-23_M13F-20) are shown.

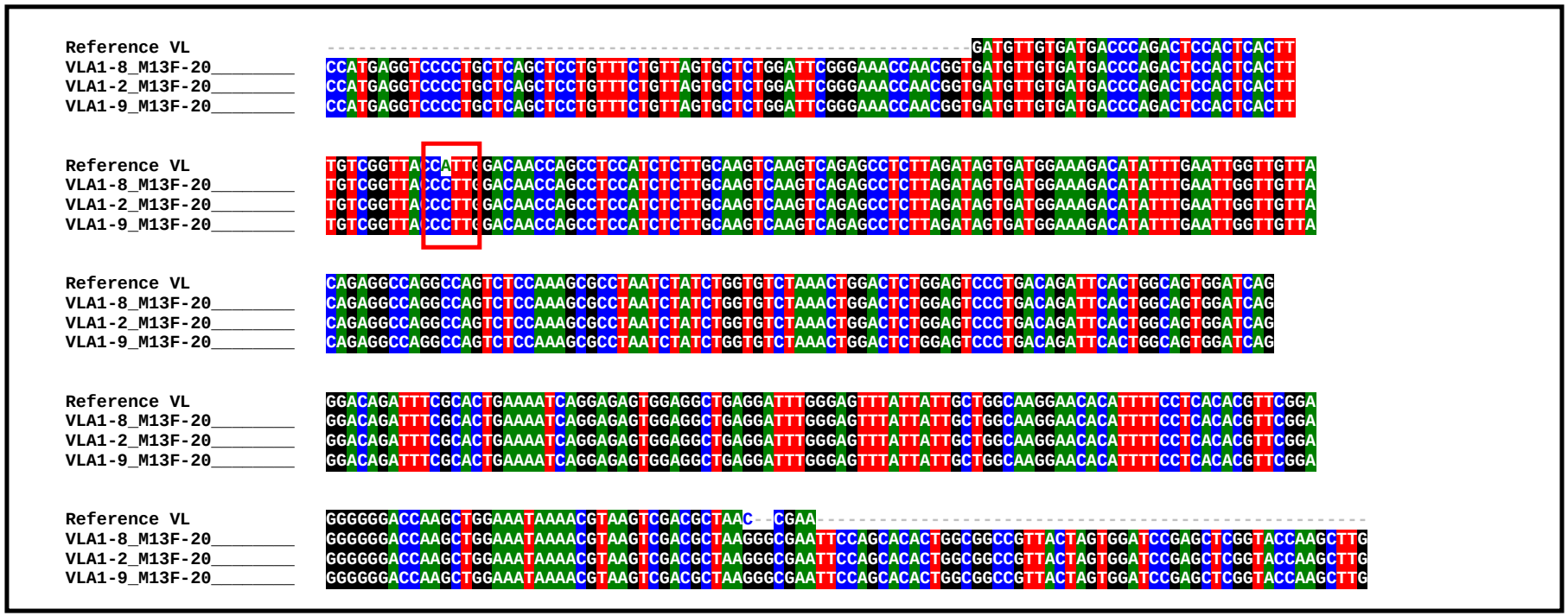

Figure 3.11: Humanization of residue 15 of VL from isoleucine (ATT) to leucine (CTT). From the DNA sequencing of 24 bacterial clones having humanized-pVL1A vectors, five clones had the desired DNA humanization at residue 15 (depicted in red box) compared to original mouse VL DNA sequences (Reference VL). Only 3 clones (VLA1-2_M13F-20, VLA1-8_M13F-20 and VLA1-9_M13F-20) are shown.

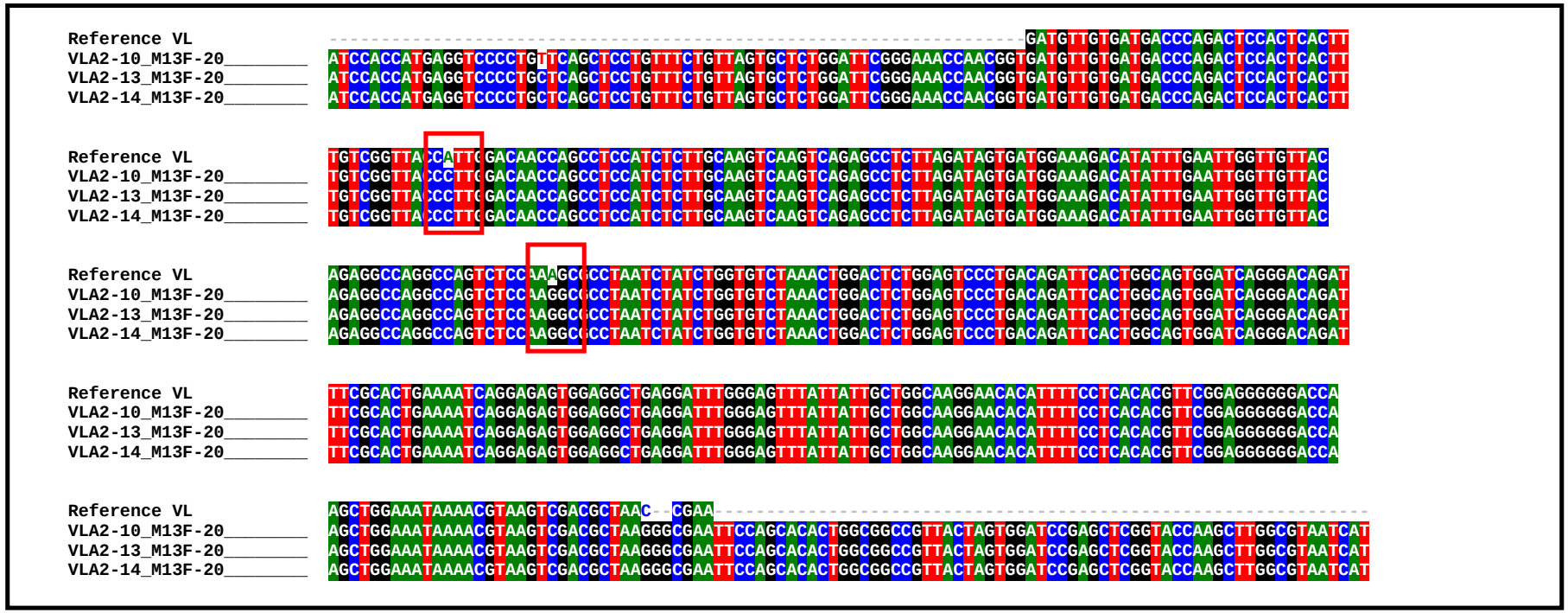

Figure 3.12: Humanization of residue 50 of VL from lysine (AAG) to arginine (AGG). From the DNA sequencing of 24 bacterial clones having humanized-pVL2A vectors, six clones but only 3 clones are shown (VLA2-10_M13F-20, VLA2-13_M13F-20 and VLA2-14_M13F-20) had the desired DNA humanizations at residue 50 in addition to residue 15 which was humanized previously (depicted in red boxes) compared to original mouse VL DNA sequences (Reference VL).

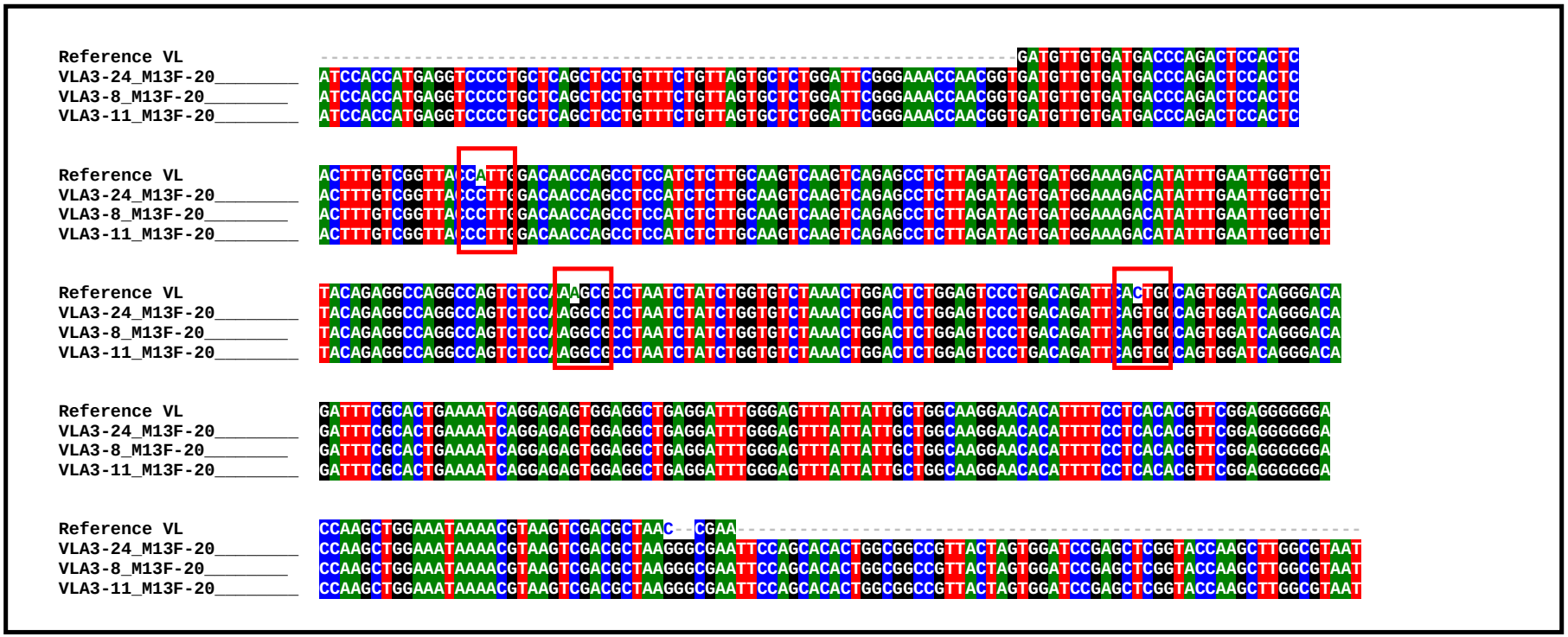

Figure 3.13: Humanization of residue 68 of VL from threonine (ACT) to serine (AGT). From the DNA sequencing of 24 bacterial clones having humanized-pVL3A vectors, six clones had the desired DNA humanizations at residue 68 in addition to residues 15 and 50 which were humanized previously (depicted in red boxes), compared to original mouse VL DNA sequence (Reference VL). Only 3 clones (VLA3-8_M13F-20, VLA3-11_M13F-20 and VLA3-24_M13F-20) are shown.

For the expression of humanized heavy and light chain of H1C2 mAb, two monocistronic expression vectors were used. The pAH4602 expression vector (Figure 3.14) which contains the human IgG1 constant regions was used to express the heavy chain of the mAb, while pAG4622 expression vector (Figure 3.15) which contains the human κ constant region was used to express the light chain of mAb.

The cloning vectors; pVH4A and pVL3A which contain humanized VH4A and humanized VL3A, respectively, were double-digested to isolate the humanized variable regions. From the agarose gel electrophoresis profiles (Figure 3.16), two DNA bands were obtained: bands of approximately 3.9 kb which corresponded to the size of cloning vector (pCR 2.1 TOPO), and bands which corresponded to the size of humanized VH4A (~500 bp) (Figure 3.16a) or humanized VL3A (~400 bp) (Figure 3.16b).

Likewise, expression vectors: mouse-pAH4602 and mouse-pAG4622 which initially contained mouse VH and mouse VL, respectively, were similarly double-digested with appropriate restriction enzymes (Figure 3.16). This resulted in the detection of DNA bands corresponding to the size of pAH4602 (~11.5 kb) (Figure 3.16a) and pAG4622 (~14.0 kb) (Figure 3.16b). The smaller band which corresponded to mouse VH (~500 bp) (Figure 3.16a) and mouse VL (~400 bp) (Figure 3.16b) were also detected.

Humanized VH4A and humanized VL3A fragments were then ligated into pAH4602 and pAG4622 vectors and named humanized-pAH4602A and humanized-pAG4622A, respectively. The detection of bands of approximately 12.0 kb (Figure 3.17a) shows the success of the ligation of humanized VH4A (~500 bp) to pAH4602 (~11.5 kb). Likewise, the detection of bands of approximately 14.4 kb (Figure 3.17a) also shows the success of the ligation of humanized VL3A (~400 bp) to pAG4622 (~14.0 kb). To

increase the transfection efficiency of the expression vector into NS0 mammalian cells, both expression vectors were linearized by single digestion (Figure 3.17b).

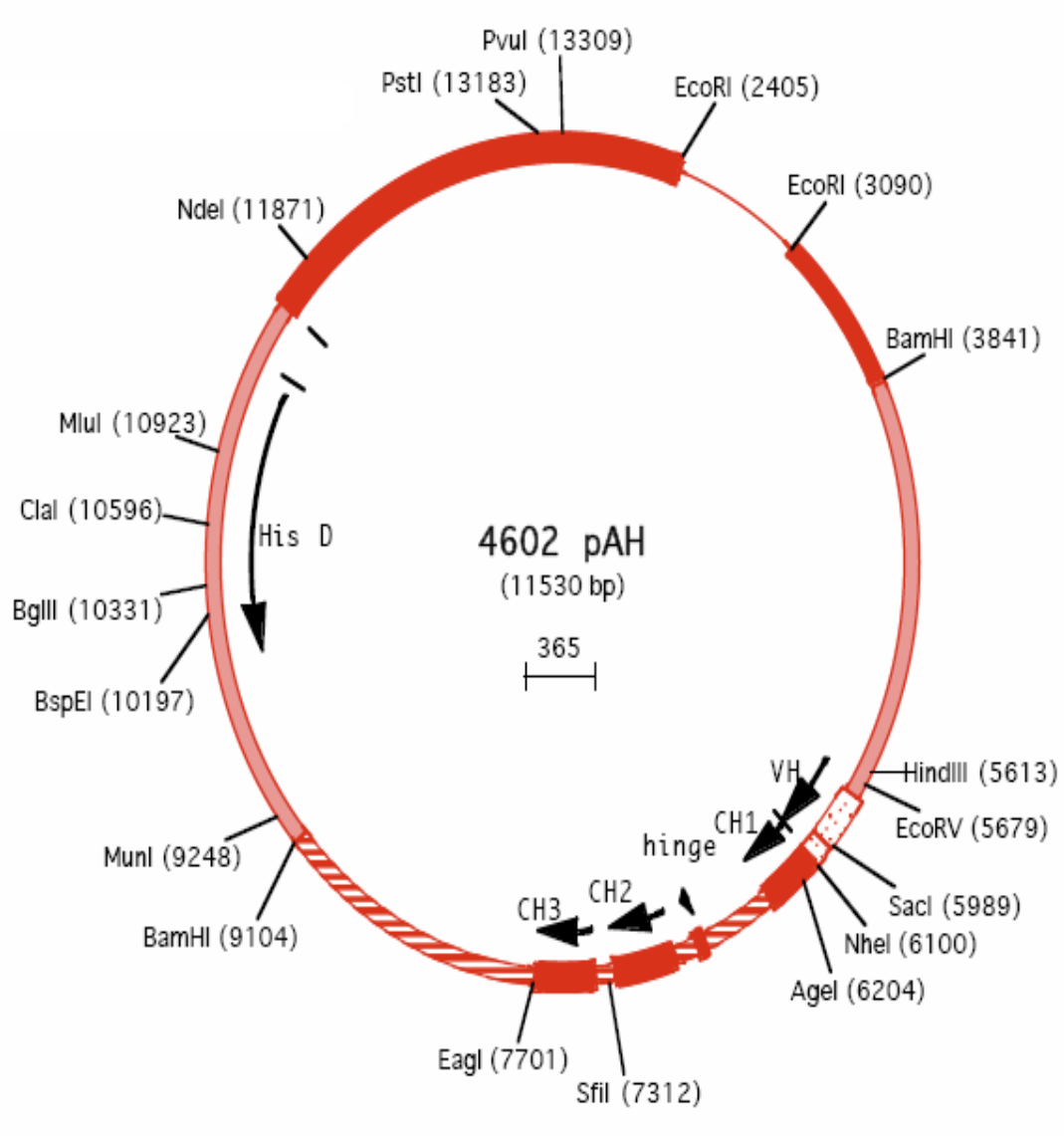

Figure 3.14: Monocistronic expression vector pAH4602 for the expression of heavy chain of antibody.

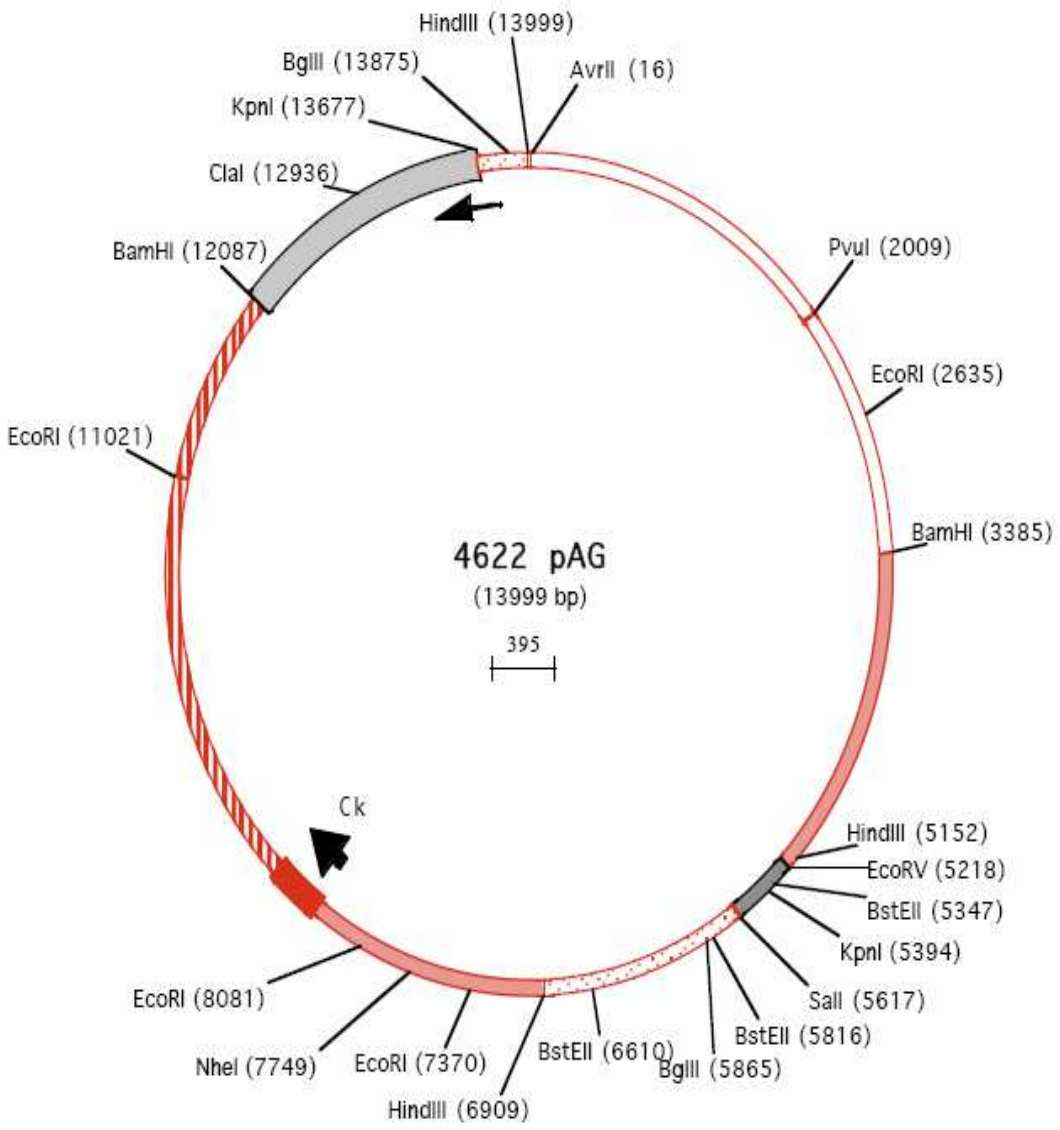

Figure 3.15: Monocistronic expression vector pAG4622 for the expression of light chain of antibody.

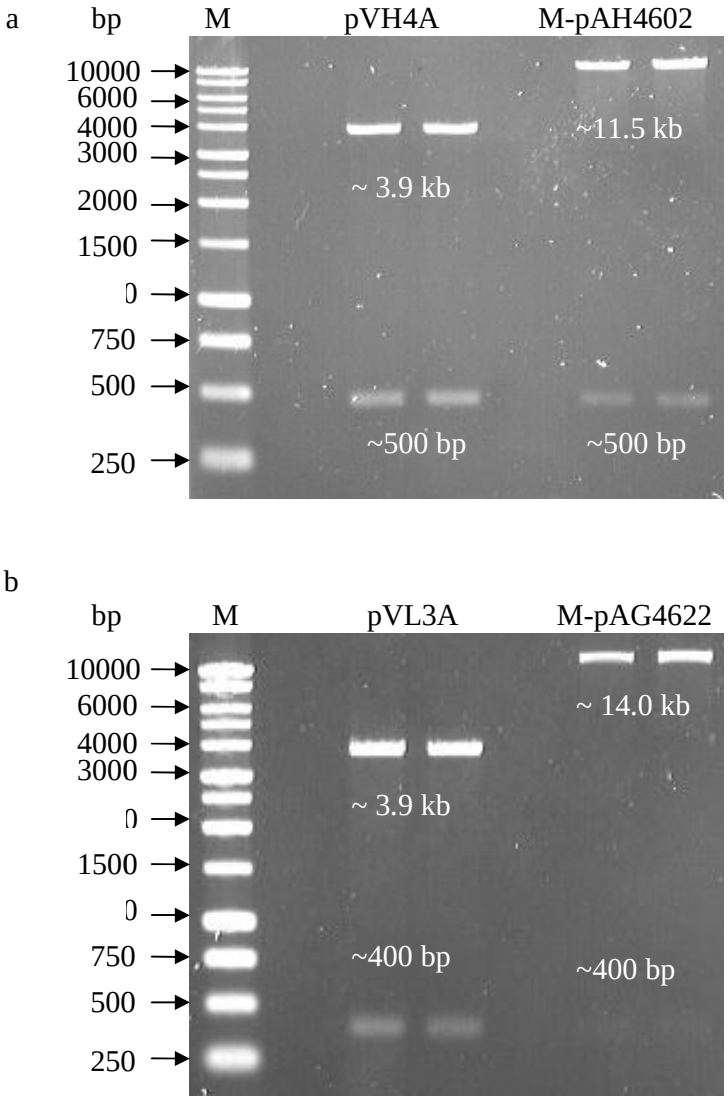

Figure 3.16: Agarose gel electrophoresis profiles of double-digested recombinant cloning and expression vectors containing VH (a) and VL (b). Double-digestion of humanized-pVH4A (pVH4A) resulted in pCR2.1 vector (~3.9 kb) and humanized VH (~500 bp) while that of mouse-pAH4602 (M-pAH4602), resulted in pAH4602 vector (~11.5 kb) and mouse VH (~500 bp). Likewise, double-digestion of humanized-pVL3A (pVL3A) resulted in pCR2.1 vector (3.9 kb) and humanized VL (400 bp) while that of mouse-pAG4622 (M-pAG4622), resulted in pAG4622 vector (14.0 kb) and mouse VL (400 bp). Supercoiled DNA ladder was used (M).

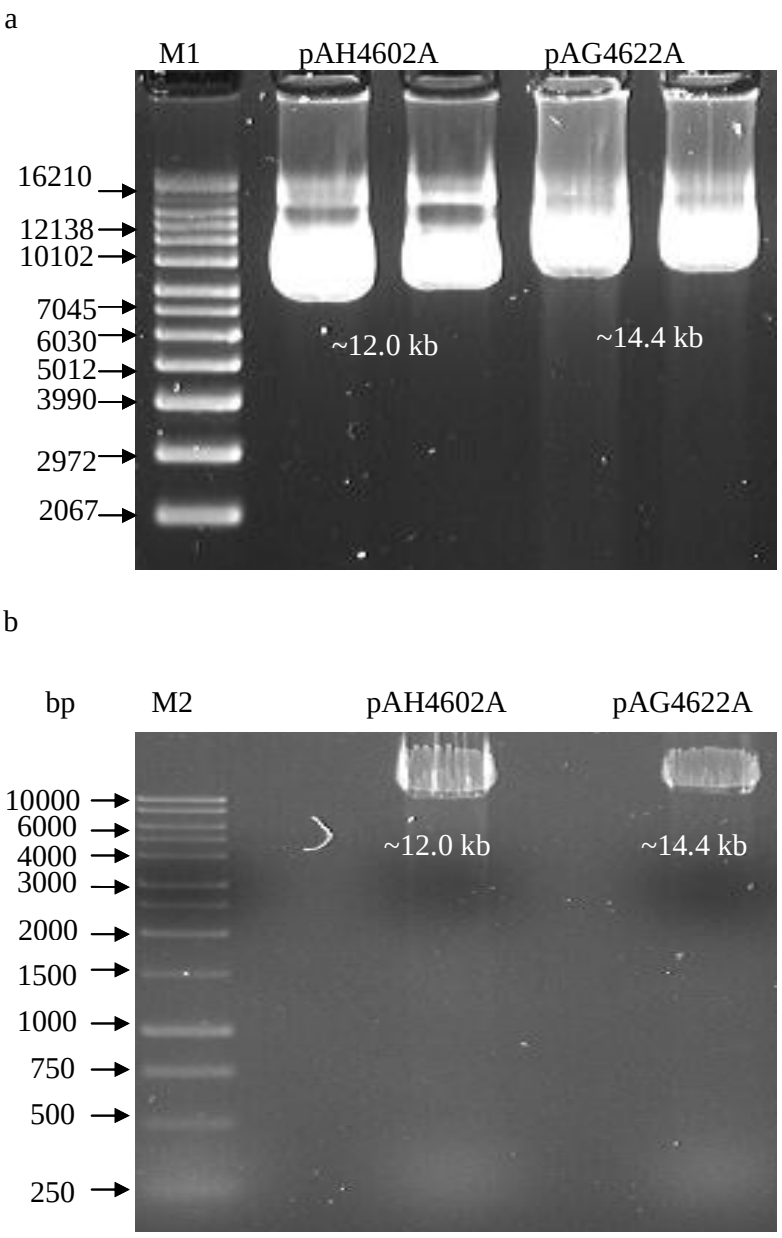

Figure 3.17: Agarose gel electrophoresis profiles of recombinant humanized-pAH4602A (pAH4602A) and humanized-pAG4622A (pAG4622A) purified in large-scale in (a) non-linearized (duplicates) and (b) linearized forms. Both forms of humanized-pAH4602A were approximately 12.0 kb, while that of humanized-pAG4622A were approximately 14.4 kb. Supercoiled DNA ladder (M1) and 1 kb DNA ladder (M2) were used.

3.4.2.2 Logical approach method

Compared to deimmunization method, in logical approach method no additional software was used to predict the immunogenic mouse anti-C2 amino acids residues. From IgBLAST analysis (section 3.2.2), it was found that 21 (Figure 3.5) and 11 (Figure 3.6) residues differed between mouse heavy and light variable regions and their respective homologous human VH and VL sequences. From the IgBLAST analysis, the frameworks that had the highest homology between human-mouse VH and VL were determined.

From the comparison (Figures 3.5 and 3.6), it was found that framework 4 of both VH and VL had 100% homology between mouse and human (Table 3.4a). Therefore no further humanization was performed in these frameworks. Hence, the frameworks with the second highest degree of homology were chosen for humanization. As seen in Table 3.4a, for VH, framework 1 (75.9%) and for VL, framework 3 (87.8%) had the second highest degree of homology. Due to the high degree of homology between mouse and human residues, it was felt that humanization on these frameworks would least likely to affect the functionality of H2C2 mAb.

Table 3.4: Comparison of percentage homology between mouse and human framework regions (a) and percentage of mismatched mouse-human residues on VH (b) and VL (c) of anti-C2 monoclonal antibody obtained using IgBLAST. The mismatched mouse-human residues were humanized with the corresponding residues that are present in at least 90% of all the human residues analyzed (*****)

a)

Frameworks	Percentage homology (%)	
	VH	VL
1	75.9	82.0
2	71.0	80.0
3	67.7	87.8
4	100.0	100.0

b)

Framework 1-VH

Mismatched residue	Mouse	Human (%)
*10	D	G (91.2)
		T (6.8)
		V (1.0)
		I (1.0)
16	Q	E (44.7)
		Q (29.1)
		R (14.6)
		D (6.8)
		G (4.8)
17	S	T (85.4)
		S (14.6)
25	T	S (86.4)
		Y (12.6)
		T (1.0)

c)

Framework 3-VL

Mismatched residue	Mouse	Human (%)
*68	T	S (100.0)
*81	R	S (90.3)
		G (5.8)
		C (1.9)
		R (1.0)
		N (1.0)
88	L	F (64.1)
		V (24.3)
		E (5.8)
		A (2.9)
		I (1.9)
		T (1.0)

Using logical approach method to further minimize AAR, within each framework, the mismatched residues was humanized only if the corresponding human amino acid was present in at least 90% of all the homologous human variable regions analyzed. In framework 1 of VH, seven mismatched mouse-human residues were present (Figure 3.18), however three of the residues (27-Y, 29-I and 30-T) are located in the vernier zone and were not considered for humanization (Table 3.2).

Hence, from the analysis of the remaining four mismatched mouse-human residues, (Table 3.4b), only aspartic acid at residue 10 was targeted for substitution to the corresponding human amino acid, glycine, which is the most abundant (91.2%) on the human framework. Coincidently, this residue was also humanized to serine in the deimmunization method. The other three mouse residues (16-Q, 17-S and 25-T) were not humanized because the corresponding human amino acids were present at less than 90% frequency (Table 3.4b).

Similarly, four mismatched mouse-human residues were found in the framework 3 of VL (Figure 3.19). However, mismatched residue 77-A was not humanized as it is located in the vernier zone (Table 3.2), while residue 88-L was not humanized because corresponding human amino acids were present with less than 90% frequency (Table 3.4c). Therefore the remaining two of the mismatched residues, 68 (threonine) and 81 (arginine) in mouse, were substituted with the corresponding most abundant amino acid in the human framework, i.e. serine (100%) and serine (90.3%), respectively. Coincidently, the residue 68 was also humanized to serine in the deimmunization method.

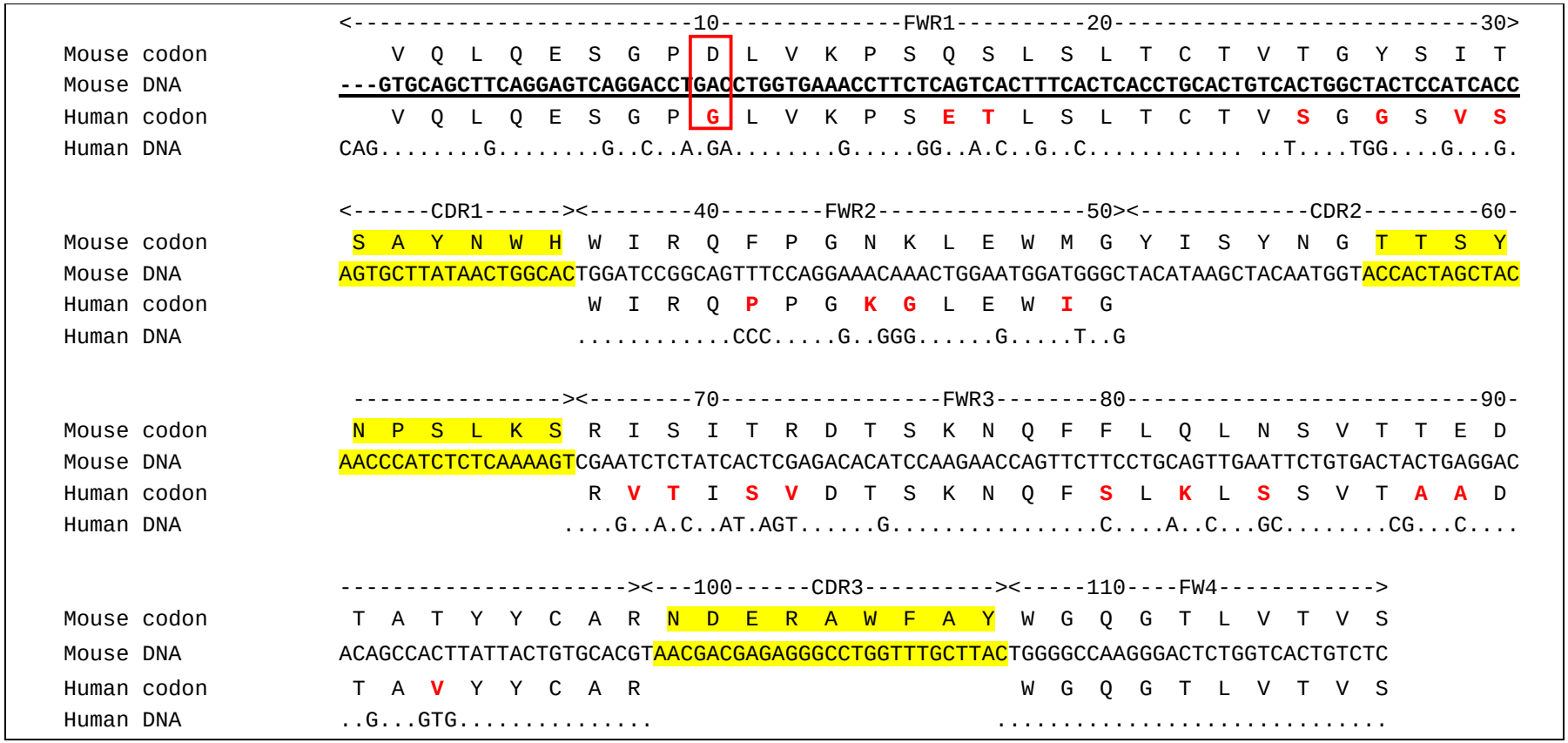

Figure 3.18: Profile of DNA and amino acid sequence comparison between mouse and human VH. From framework 1, (FWR1-underlined), only one residue (in red box) was humanized.

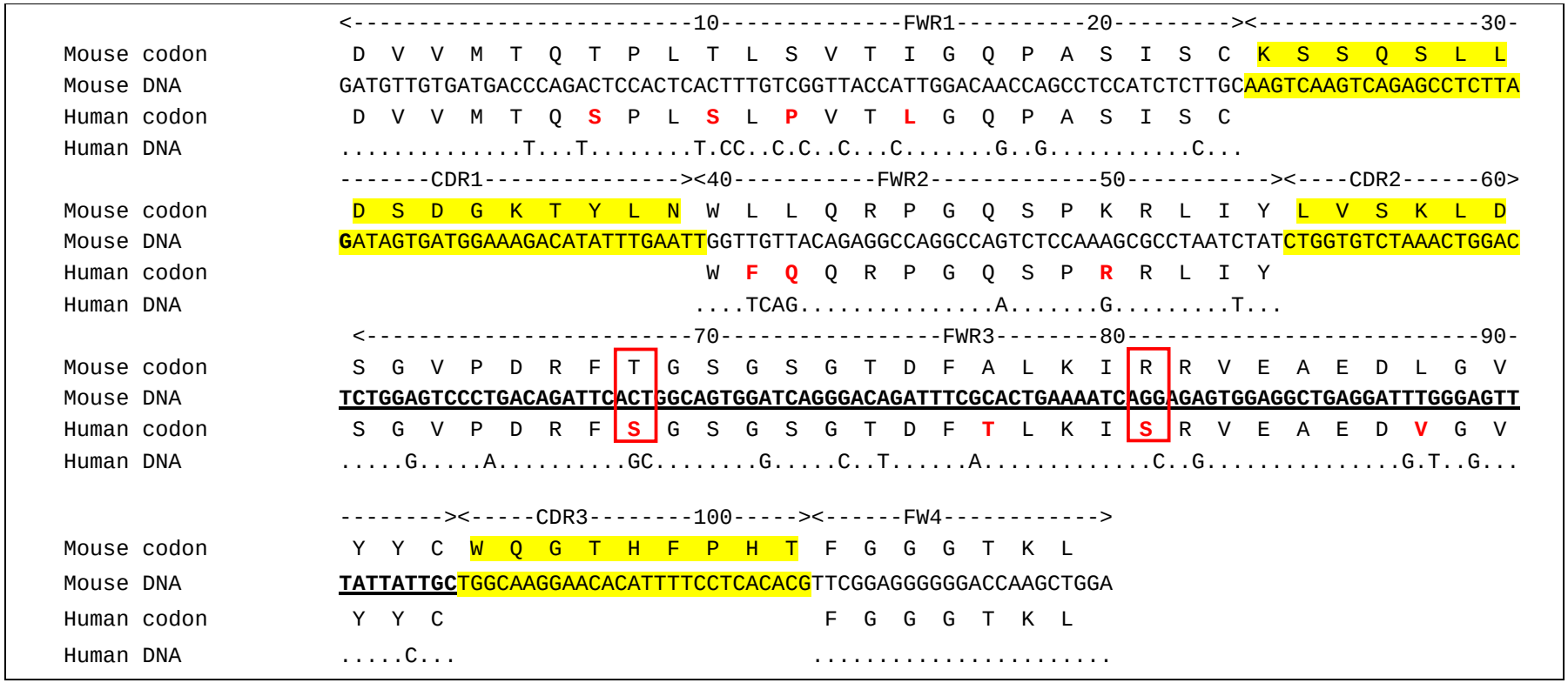

Figure 3.19: Profile of DNA and amino acid sequence comparison between mouse and human VL. From framework 3, (FWR3-underlined), only two residues (in red boxes) were humanized.

Just like the humanization in the deimmunization method, the conversion from the mouse amino acid to homologous human residue for the logical approach method was performed using overlapping-PCR mutagenesis with pre-designed mutagenic primers. Mutagenic primers that have the DNA mutation that code for the corresponding human codons that require the least number of nucleotide substitution was chosen, as summarized in Table 3.5. Since the proposed humanization for mouse VH was the same as performed in deimmunization method, therefore humanized-VH1A vectors were used directly as humanized-VH1B without additional modifications.

For the development of humanized-pVL2B, the first set of overlapping-PCR mutagenesis was performed to develop humanized-pVL1B for the conversion of threonine (ACT) to serine (AGT) at residue 68 as illustrated in Figure 3.20. The humanized-pVL1B vector was then used as a template for the development of humanized-pVL2B for the conversion of arginine (AGG) to serine (AGC) at residue 81 (Figure 3.21). Humanized VH1B and humanized VL2B were then ligated into pAH4602 (containing human IgG1 constant regions) and pAG4622 (containing human κ constant region) and the resulting vectors; humanized-pAH4602B and humanized-pAG4622B were used for heavy and light chain expression, respectively.

Table 3.5: Humanization of mismatched mouse-human residue to its homologous human residue using logical approach method. The original codon that codes for mouse residues were substituted with the human codon that required the least nucleotide substitution (underlined)

Residue	Mouse amino acid	Human amino acid	Possible human codons
10 (D)-VH	Aspartic acid (GAC)	Glycine (G)	GGT, <u>GGC</u>, GGA, GGG
68 (T)-VL	Threonine (ACT)	Serine (S)	TCT, TCC, TCA, TCG, <u>AGT</u>, AGC
81 (R)-VL	Arginine (AGG)	Serine (S)	TCT, TCC, TCA, TCG, AGT, <u>AGC</u>

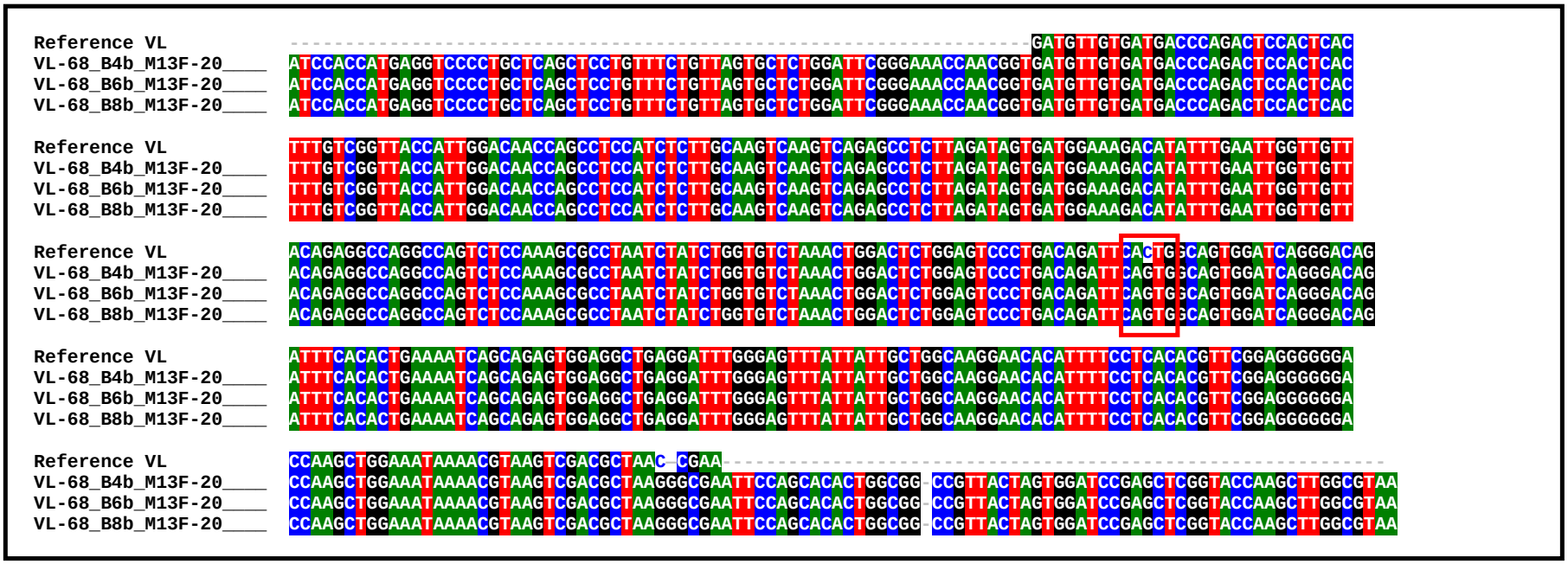

Figure 3.20: Humanization of residue 68 of VL from threonine (ACT) to serine (AGT). From the DNA sequencing of 24 bacterial clones having humanized-pVL1B vectors, three clones (VL-68_B4b_M13F-20, VL-68_B6b_M13F-20 and VL-68_B6b_M13F-20), had the desired DNA humanizations at residue 68 (depicted in red boxes) compared to original mouse VL DNA sequence (Reference VL).

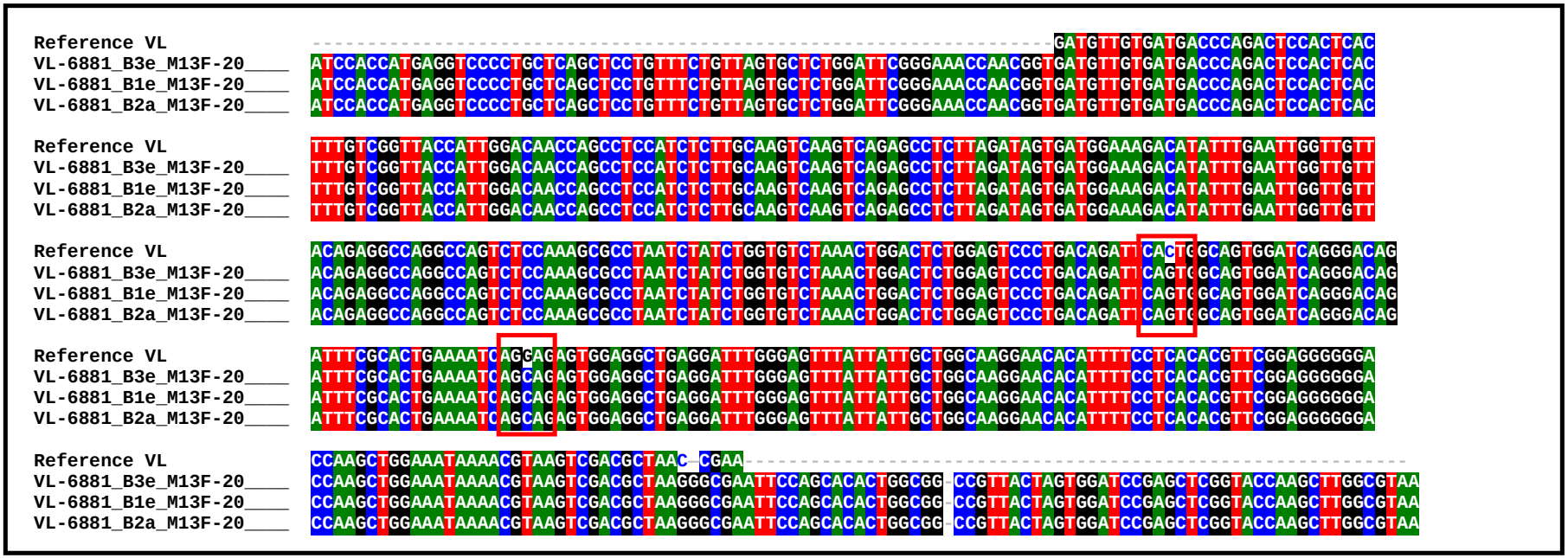

Figure 3.21: Humanization of residue 81 of VL from arginine (AGG) to serine (AGC). From the DNA sequencing of 24 bacterial clones having humanized-pVL2B vectors, five clones, had the desired DNA humanization at 81 in addition to residue 68 which were humanized previously (depicted in red boxes) compared to original VL DNA sequence (Reference VL). Only 3 clones (VL-6881_B1e_M13F-20, VL-6881_B2a_M13F-20 and VL-6881_B2a_M13F-20) are shown.

3.5 CONCLUSION

In summary, monocistronic vectors for the expression of two humanized anti-C2 mAb; H1C2 (humanized-pAH4602A & humanized-pAG4622A) and H2C2 (humanized-pAH4602B & humanized-pAG4622B) mAbs had been developed using recombinant DNA technologies. The H1C2 mAb was developed using deimmunization method while that of H2C2 mAb using logical approach method. In both methods, the predicted immunogenic mouse amino acid residues were substituted to its corresponding human resides using overlapping-PCR mutagenesis.