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IDENTIFICATION OF ANTIBODY TARGETS USING PHOTOACTIVATED CROSS-LINKERS LOCATED IN THE BINDING SITE

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ABSTRACT

We converted the monoclonal antibody (STRO-1), into a recombinant antibody fragment and subsequent alteration of the complementarity determining region (CDR 3) of the variable region of the heavy chain (V_H) by site-directed mutagenesis (SDM). We modified the nucleotide codon (TAT) which encode the amino acid tyrosine (Y), to an amber codon (TAG) in DNA or (UAG) in mRNA in the CDR3 of the heavy chain variable region (V_H) and was transformed into *E.coli* (TOP10, XL1-Blue) cells. The amino acid sequence of both the heavy and light chains obtained from US patent no: 8,101,155 B2 (Simmons & Torok-Storb, 1991) were translated to the corresponding nucleotide sequence. The variable region of the heavy chain (V_H) and light chain (V_L) were amplified by PCR after 30 cycles reaction. However, we were completely unable to clone the native STRO-1 genes that encode for the V_H and V_L fragments into the pHenIX cloning vector after several attempts. The DNA fragment which contains the sequence that encode the (V_H) gene was then cloned into a TA cloning vector (pCR 2.1, 3.9kb) after PCR amplification of the altered target of interest. Following ligation reaction, we transformed *E.coli* (TOP10) cells, grown overnight, purified, and sequenced for determination of the incorporation of the modified nucleotide sequence. Comparison of the mutated CDR3 of the variable heavy (V_H) chain with the native sequence after sequencing was carried out showed that mutation has not

occurred at the desired positions due to mismatch of some codons possibly due to incorrect primer design.

KEYWORDS: Strol-1, monoclonal antibodies, site directed mutagenesis, sequencing, cloning, expression vectors, recombinant fragment.

INTRODUCTION

Antibodies are commonly defined as glycoproteins of the immunoglobulin (Ig) superfamily produced by B lymphocytes to recognise and neutralize foreign intruders and consist of two heavy and light chains classified into distinct isotypes reliant on the type of heavy chain they constitute (Buss *et al*., 2012). Monoclonal antibodies (MAbs) of the therapeutic importance belong to the immunoglobulin G (IgG) isotype. The antigen binding site (ABS) known as the Fab region is formed by the combination of the hypervariable regions of the heavy (V_H) and light (V_L) chains while the two constant domains constitute the crystallisable fragment (Fc) which determines the effector function of the antibody.

Subsequently, monoclonal antibodies (MAbs) can be produced and isolated with the capacity of identifying unique cells independent of the particular antigen to be targeted. Also, pathogenic antibody types can be isolated from patients reliant on the bound cell type independent of the antigen, and even though it is essential to know the precise specificity, the identification of the recognised epitope is usually difficult even for antibodies produced against specific antigens.

Cloning at the molecular level and the sequencing of antibody variable domains is fundamental in modelling of antibodies, antibody manipulation, and antibody structural determination via experimental approach (Ress *et al*., 1994; Nilsson, 1995; Freund *et al*., 1994) using nuclear magnetic resonance (NMR). Also, the cloning of antibody's variable region genes enhances the manipulation of the antibody's domain in different ways resulting in antibody types with decrease immunogenicity (Gussow and Seeman, 1991), increased affinity (Deng *et al*., 1994), mutated antigenic specificity (Ohlin *et al*., 1996), and improved stability. More so, gene fusion of the single chain fragment variable (scFv) into effector proteins is important in molecular and therapeutic research (Huston *et al*., 1993). Cloning the variable domains of both heavy (V_H) and light (V_L) chains of native antibody unique to a particular antigenic epitope is of immense benefits in antibody engineering. Molecular cloning and antibody sequencing result in the maintenance and immortalisation of specific

features of monoclonal antibodies (MAbs) and their binding affinity for a particular antigenic epitope.

Techniques, including immunoprecipitation which depends on the interaction between antibodies and antigens being sustained during purification, their usefulness is however, in part, limited by the antibody's affinity. Known techniques that cross link antibodies chemically to structures near specific targets are by nature relatively non-specific. Also, physically linking an unknown antigen to an antibody heavy or light chains will subsequently permit the purification and identification of the antigen but these techniques are also limited in their specificity to certain antigens (Sallach *et al*., 2009). Recombinant techniques can therefore be employed to specifically incorporate modified amino acid residues at specific sites of interest with photoactivated crosslinked molecules such as p-benzoyl-L-phenylalanine (*pBpa*) into proteins expressed in *E.coli*, though this technique is in part limited to *in vitro* studies of polypeptides (Farrell *et al*., 2005). A novel approach which favours both *in vitro* and *in vivo* incorporation of photoactivated cross linkers at specific sites is based on the use of bio-orthogonal amino-acyl tRNA synthetase as a result permitting protein crosslinking in *E.coli* (Chin and Schultz, 2002; Schlieker *et al*., 2004). When such unnatural amino acids are placed in close proximity to the binding site of an antibody, it can be permanently bound to a specific antigen within the antigenic epitope. STRO-1, a low affinity monoclonal antibody is suitable for this purpose since minor changes alterations in its binding site can be tolerated without the loss of its specificity.

The goal of this project was to convert a monoclonal antibody (STRO-1) to a recombinant fragment, site directed mutagenesis of the antibody sequence to allow the incorporation of the unnatural amino acid residue whose side chain (R-group) can be crosslinked to amino groups in close proximity following exposure to ultraviolet (UV) light, expression and evaluation of both natural and unnatural antibody fragments to their specific binding sites and subsequent characterisation of the antigenic epitope recognised by the antibodies.

MATERIALS AND METHODS

Strains, Culture media, Plasmids and Reagents

E.coli strains XL1-Blue and TOP10 were used in the transformation reaction. Plasmids (pCR 2.1 of 3.9kb or pHenIX of 4.5kb) were used in the study. LB (Luria-Bertani) medium, LB agar, SOB (Super Optimal Broth), SOC (Super Optimal broth with Catabolite repression) medium of appropriate composition were used in the study. We purchased DNA marker, T4

DNA ligase, restriction endonucleases, *Taq* polymerase, Pfu DNA polymerase from New England Biolabs (NEB). All medium used in the study were sterilised by autoclaving for 15 minutes at 121^{o}C and 50% glycerol stock was sterilised by 0.5µm filtration used for the preparation of competent *E.coli* cells.

Nucleotide Sequence of V_H and V_L Chains

The amino acid sequence of both the heavy (V_H) and light (V_L) chains were obtained from US patent no: 8,101,155 B2 (Simmons & Torok-Storb, 1991) and were translated to the corresponding nucleotide sequence using an online reverse translation tool available at http://www.bioinformatics.org/sms2/rev_trans.html. Primers were designed from the nucleotide sequence and used in the amplification of both the V_H and V_L chains during the study.

Preparation of competent E.coli (XL1-Blue) cells

A single colony of *E.coli* cell from previously cultured cells was inoculated into 10ml of lysogeny broth and incubated overnight at 37^{o}C with shaking at 250rpm. The overnight 10ml culture was then used to inoculate 200ml of LB medium and incubated at 37^{o}C with shaking at 250rpm for 90 minutes until the optical density at 600nm (OD_{600}) reached 0.46. 0.1M $MgCl_2$, 0.1M $CaCl_2$, and 50% glycerol stock were used to make the cells chemically competent following standard procedures as described by Chung *et al*., (1989) and used for the study.

Amplification of the V_H and V_L Chains

Both the V_H and V_L chains were amplified in a PCR setup of 20µl final reaction volume using a 10pm/µl of mV_{HSfiI} and mJ_{HSalI} were used as the forward and reverse primers respectively for the V_H gene while mV_{LApaLI} and mJ_{LNotI} were used as the forward and reverse primers respectively for the V_L gene. The thermocycling reaction condition is as shown in Table 1. Analysis of the DNA fragments of both the V_H and V_L chains that migrated the expected length were done with 0.8% TBE agarose gel electrophoresis and visualised using UV light as shown in Figure 1.

Purification of V_H and V_L chains and plasmid DNA (vectors)

The PCR products of the variable regions of the V_H and V_L chains were purified using QIAquick PCR purification Spin protocol while the plasmid DNA were purified using QIAprep Spin Miniprep and the QIAGEN plasmid Midi Kit protocols and used for the study.

Cloning of Amplified of V_H and V_L chains

After amplification and purification of the V_H and V_L chains, the PCR products were digested with restriction endonucleases SalI or XhoI and SfiI for genes encoding forthe V_H region while NotI and ApaLI were used to digest the genes encoding the V_L fragment. However, the V_L fragment was not clone into the plasmid due to time constrain. After the digestion and subsequent purification, the V_H fragment was ligated into cloning vectors (pCR 2.1 or pHenIX) and then introduced into competent *E.coli* cells (XL1-Blue or TOP10).

Transformation of Competent E.coli (XL1-Blue or TOP10) cells

Competent *E.coli* (XL1-Blue or TOP10) were transformed following the ligation reaction and inoculated in an LB (Luria-Bertani) agar which contains carbenicillin (100µl/ml) following standard protocol as described by Hanahan, (1983). Plates were incubated overnight at 37^{o}C and transformed cells were used in the study.

Colony PCR

Ten large colonies of *E.coli* (TOP10) cells from the transformed insert plate and a single colony from the transformed vector only plate were picked at random after an overnight incubation and were used to performed colony PCR for the screening of the amplified V_H fragment with 2µl of M13 (F) and M13 (R) as forward and reverse primers respectively in two separate tubes of varying concentrations of 25mM $MgCl_2$ (1 in 25 and 1 in 50) in a 10µl final reaction volume using MJ Mini^{TM} Thermocycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA) PCR. The thermocycling reaction condition is as shown in Table 2. Following the initial denaturation step for 5minutes at 95^{o}C, the PCR reaction was carried out for 30 cycles with denaturation at 95^{o}C for 1 minute, annealing of M13 (F) and M13 (R) primers at 48^{o}C for 1 minute, polymerisation of the product at 72^{o}C for 1 minute and final extension at 72^{o}C for 10 minutes and 10 minutes incubation at 10^{o}C.

TA Cloning and Ligation

After several failed attempts to clone the variable region of the heavy (V_H) chain fragment into plasmid DNA (pHenIX, 4.5kb), we decided to clone the purified V_H fragment into pCR 2.1 vector (3.9kb) which is far easy to manipulate, following the ligation of the purified V_H and digestion of the pCR 2.1 vector with EcoRI-SalI and EcoRI-SfiI restriction endonucleases and analysed with 0.8% TBE agarose gel electrophoresis after PCR under UV light as shown in Figure 3.

PCR Mutagenesis and mutagenic primer design

Replacement of the tyrosine (Y) residues at position 295 and 307 with the amber codon (TAG) in the complementarity determining region (CDR 3) of the variable region of the heavy (V_H) chain was carried out following the QuikChange Site Directed Mutagenesis protocol by Strategene (La Jolla, CA) (Zheng *et al*., 2004). Primers were designed following the QuikChange II Site-Directed Mutagenesis Kit protocol.

Sequencing

After the amplification of the altered complementarity determining region (CDR3) of the variable region of the heavy (V_H) chain with four mutagenic primers, MUT1 (forward), MUT1 (reverse), MUT2 (forward), and MUT2 (reverse) of 2.5pmoles/µl containing, 0.25µl dNTP (10 mM), 0.25µl Pfu, and 1µl of purified DNA template (2ng/µl) in a final reaction volume of 12.5µl in a 18 cycles thermocycling reaction condition as shown in Table 3, the PCR product was electrophoresed with 0.8% TBE agarose gel and visualised with UV light (figure not shown). The PCR products were then digested with 0.25µl DpnI (20 units/µl, New England Biolabs) which were used to transformed competent *E.coli* (TOP10) cells. A single colony from each transformed plate was used to inoculate 3ml of LB agar, grown overnight, purified, and sequenced.

RESULTS

Amplification of the V_H and V_L Chains

The amplification of the variable regions of the heavy (V_H) and light (V_L) chains were carried out in a PCR reaction condition as shown in Table 1. The analysis of the V_H and V_L chains fragments migrating the expected length is as shown in Figure 1.

Table 1: Thermocycling condition for amplification of the (V_H) and (V_L) chains.

Steps		Temperature	Time
Initial denaturation		95^{o}C	5 minutes
30 cycles	Denaturation	95^{o}C	1 minute
	Annealing	58^{o}C	1 minute
	Extension	72^{o}C	1 minute
Final extension		72^{o}C	10 minutes
Hold		10^{o}C	to end

The initial amplification of the variable (V) region of the heavy (V_H) and light (V_L) chains with a 10pm/µl of mV_{HSfiI} and mJ_{HSalI} as forward and reverse primers respectively for the V_H gene, mV_{LApaLI} and mJ_{LNotI} as the forward and reverse primers respectively for the V_L gene.

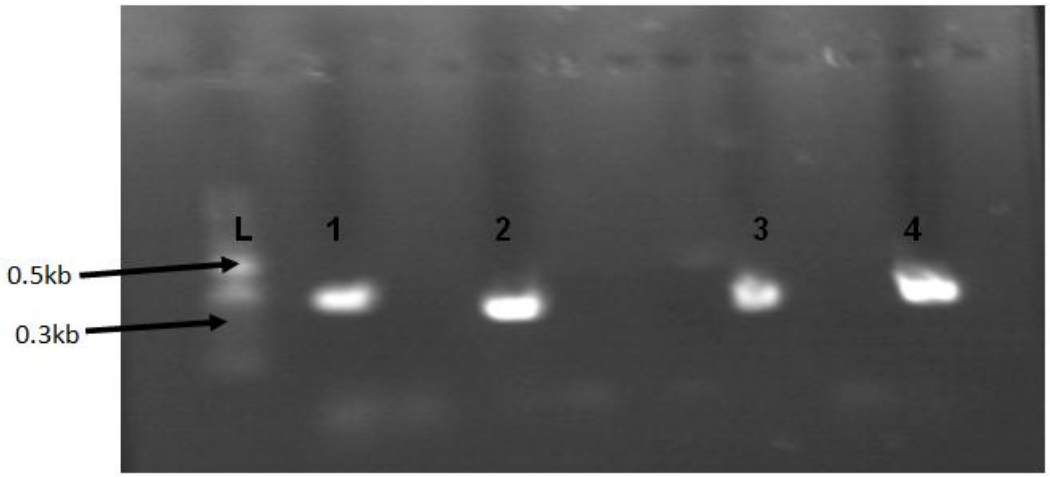


Figure 1: 0.8% TBE agarose gel electrophoresis of the PCR products for the amplification of the V_H and V_L chains. Lane L, DNA molecular ladder (2-log DNA ladder). Lane 1, primers used for the amplification of the light chain (V_L) $mV_{LApaLIForward}$ and $mJ_{LNotIReverse}$, 360bp; lane 2, primers used for the amplification of the heavy (V_H) chain $mV_{HSfiIForward}$ and $mJ_{HSalIReverse}$, 342bp while lane 3 and 4 were CDR3 regions used in another study. Standard DNA sizes are given in kb.

Table 2: Thermocycling Condition for Screening of Insert with M13 primers.

Steps		Temperature	Time
Initial denaturation		95°C	5 minutes
30 cycles	Denaturation	95°C	1 minute
	Annealing	48°C	1 minute
	Extension	72°C	1 minute
Final extension		72°C	10 minutes
Hold		10°C	to end

The thermocycling condition for screening of insert with M13 (F) and M13 (R) as forward and reverse primers respectively to check the potency of the heavy (V_H) chain primers ($mV_{HSfiIForward}$ and $mJ_{HSalIReverse}$).

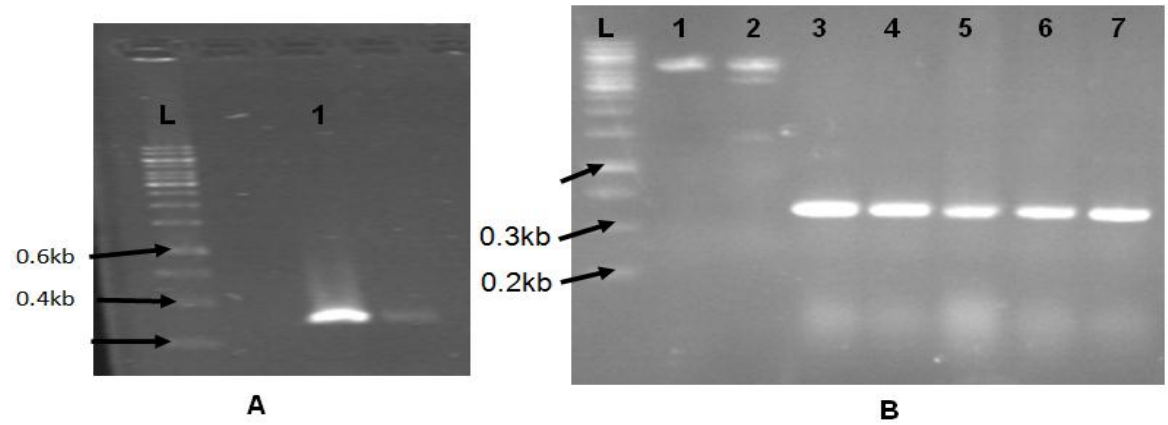


Figure 2: 0.8% TBE agarose gel electrophoresis of the heavy (V_H) chain and visualisation under ultraviolet (UV) light. (A) Amplification of the heavy (V_H) chain with $mV_{HSfiIForward}$ and $mJ_{HSalIReverse}$ as forward and reverse primers. Lane L, DNA molecular ladder (2-log DNA ladder); lane 1, the heavy (V_H) chain fragment migrating the expected distance, 342bp. (B) PCR screening of overnight culture with M13For and M13Rev as forward and reverse primers respectively after TA cloning of the heavy (V_H) chain, 342bp into plasmid DNA (pCR 2.1, 25ng/µl). Lane L, DNA molecular ladder (2-log DNA ladder), lanes 1 & 2 are empty pHenIX vector, lanes 3-5 are overnight cultures from ligation plates containing the heavy (V_H) chain. Standard DNA sizes are given in kb.

Table 3: Thermocycling Condition for MUT1 and MUT2 primers.

Steps		Temperature	Time
Initial denaturation		95°C	5 minutes
18 cycles	Denaturation	95°C	50 seconds
	Annealing	60°C	50 seconds
	Extension	68°C	5 minute
Final extension		68°C	1 min+1min/kbp
Hold		10°C	to end.

The thermocycling conditions for four mutagenic primers MUT1For & Rev (2.5pmoles/µl) as forward and reverse primers for the first mutation; MUT2For & Rev (2.5pmoles/µl) as forward and reverse primers for the second mutation of the CDR3 region. Each reaction tube contains 0.25µl dNTP (10 mM), 0.25µl Pfu, and 1µl of purified DNA template (2ng/µl) in a final reaction volume of 12.5µl in 18 cycles PCR thermocycling reaction.

Colony PCR of Inserts with M13 primers

We selected ten large colonies of *E.coli* (TOP10) cells at random from the insert overnight culture plate and a single colony from the vector only plate to perform a colony PCR as depicted in Figure 3.

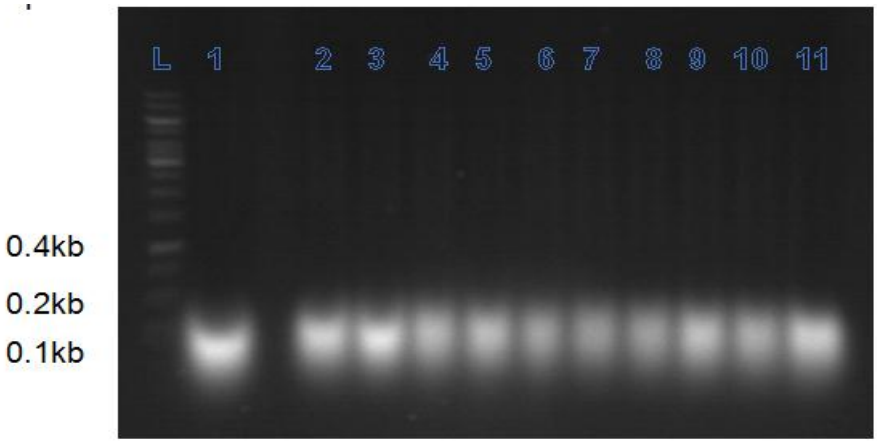


Figure 3: Colony PCR of insert electrophoresed with 0.8% TBE agarose gel and visualised by UV light. Lane L, DNA molecular ladder (2-log DNA ladder); lane 1, vector only, lanes 2 – 11 are random colonies selected from insert plate. This shows that the insert has not been cloned into the pHenIX vector. The colony PCR was carried out with M13 primers. The reaction mix contains varying concentrations of $MgCl_2$ (25mM, Promega Madison, W1, USA). Standard DNA sizes are given in kb.

Generation of mutations and mutagenic primer design

The underlined tyrosine (Y) residues of the complementarity determining region (CDR3) of the variable (V) region of the heavy (V_H) chain was replaced with the amber codon (TAG) which was introduced at the specific site of interest using PCR.

V Y Y C A T Y A M D Y W G Q G T S V GTG TAT TAT TGC GCG ACC TAT GCG ATG GAT TAT TGG GGC CAG GGC ACC AGC GTG

The complementarity determining region (CDR3) of the V region of the heavy (V_H) chain and its corresponding amino acid residues. Altered codon of interest are underlined.

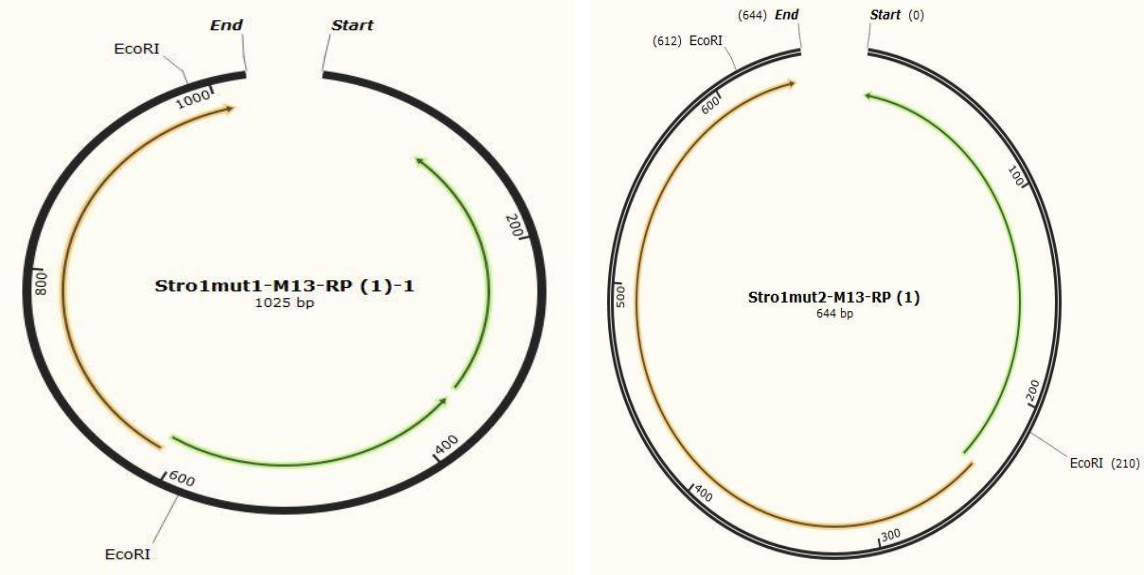
Table 4: Primers for V_H and V_L amplification & Mutagenic oligonucleotide primers.

Primer code	Sequence	Function
Mut1For	5’-GTGTATTATTGCGCGACC**TAG**GCGAT*ggatt*ATTGGG-3’	Mutant1 sequencing
Mut1Rev	5’-CCCAATAATCCATCGC**CTA**GGTCGCGCAATAATACAC-3’	
Mut2For	5’-CGACCTATGCGAT*ggatt***AG**TGGGGCCAGG-3’	Mutant2 sequencing
Mut2Rev	5’-CCTGGCCCCA**CTA**ATCCATCGCATAGGTCG-3’	
mV_{HSfiI}	5’-AACTGCGGCCCAGCCGGCCATGGCCGAGGTCCAGCTGCAGCAG-3	V_H amplification
mJ_{HSalI}	5’-CTCGTGTCGACACGGTGACTGAGGTTCCTTG-3’	
mV_{LApaLI}	5’-CACAGTGCACTCCAGGCTGTTGTGACTCAGGA-3’	V_L amplification
mJ_{LNotI}	5’-ACTTGCGGCCGCTAGGACAGTGACCTTGGTTC-3’	

Mutagenic primers used the study. Mut1 (For & Rev) are primers used for sequencing of the first mutation in the CDR3 region, while Mut2 (For & Rev) are primers used for the sequencing of the second mutation in the same region. Mutation positions are bold and underlined, EcoRI restriction site is in lower case and italicised. mV_{HSfiI} and mJ_{HSalI} (For & Rev), mV_{LApaLI} and mJ_{LNotI} (For & Rev) are primers used for the amplification of the V_H and V_L genes respectively.

Sequencing

The generated mutagenic complementarity determining region (CDR3) of the variable region of the heavy (V_H) chain was amplified and sequenced with four mutagenic primers MUT1 (forward), MUT1 (reverse), MUT2 (forward), and MUT2 (reverse) of 2.5pmoles/µl for the first and second tyrosine (Y) residues respectively. Comparing the altered CDR3 sequence with the native nucleotide sequence after sequencing was carried out revealed a mismatch in the sequence due to some missing codons. Thermocycling reaction conditions is as shown in Table 3. The sequencing result is as shown in Figure 4 and 5.



A **B**

Figure 4: Sequence analysis of altered CDR3 region of the heavy (V_H) chain. (A) and (B) SnapGene map view of the altered first (Stro1mut1) and second (Stro1mut2) tyrosine (Y) residues respectively of the CDR3 region with EcoRI recognition sites.

CtcgganCcaCTAGTAaCGGCCGCCagTGTGCTGGAATTCGGCTTCTCGTGTCgacacGGTGACTGAGGTTCCTTGACCCCAG
TAGTCCATAGCATCCCCACGCCCGTAACCATCGGCACAGTAATAGACCGCAGAGTCCTCAGATGTCAGGCTGCGGAGCTCCAT
GTAGGCTGTGCTGGATGACTTGTCTACAGTTAATATGGCCTTGCCCTTGAACTTCTGGTTGTAGCTAGTACCACCATTGTTAG
GATTAACACGTCCAATCCACTCAAGGCTCTTTCCATGGCTCTGCTTCACCCAGTGCATGTAGTAGCCAGTGAATGAGTAACCA
GAAGCCTTGCAGGATATCTTCACTGAAGCCCCAGGCTTCACCAGGTCAGGTCCAGACTGCTGCAGCTGGACCTCGGCCATGGC
CGGCTGGGCCGCAGTTAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTC
GCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTA
ATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC
AGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACA
CTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCT
AAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAaAanCTTGATtanGgTGATGGTTCAC
GTAGTGGGCCATCGCCCTGATAGACGgTTTTTcgCCcTTTGACGTTGGaGTCCacGTTCTTTAatannggnaCTcttGTtccA
AACTGgAaaCAACACTCAACCCTATCtng

A

CcnCtaGTAaCGGCCGCCAGTGTGCTGGAATTCGGCTTCTCGTGTCnaCncGgtGaCTGAGGTTCCTTGA
CCCCAGTAGTCCATAGCATCCCCACGCCCGTAACCATCGGCACAGTAATAGACCGCAGAGTCCTCAGATG
TCAGGCTGCGGAGCTCCATGTAGGCTGTGCTGGATGACTTGTCTACAGTTAATATGGCCTTGCCCTTGAA
CTTCTGGTTGTAGCTAGTACCACCATTGTTAGGATTAACACGTCCAATCCACTCAAGGCTCTTTCCATGG
CTCTGCTTCACCCAGTGCATGTAGTAGCCAGTGAATGAGTAACCAGAAGCCTTGCAGGATATCTTCACTG
AAGCCCCAGGCTTCACCAGGTCAGGTCCAGACTGCTGCAGCTGGACCTCGGCCATGGCCGGCTGGGCCGC
AGTTAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATT
CGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGtcgtGACTGgnAAAnCCCTgg
CGTTACCcAaCTTAAtCGCCTtgCAGCACATCCCCcTTTcnCcAGCTGGcgtnntagcgaacAGGcccGC
ACCGATCGCCcttc

B

Figure 5 (A & B): Sequenced analysis of the CDR3 region of the heavy (V_H) chain nucleotide. Nucleotide sequence of the first altered tyrosine (Y) residue (A) and the second tyrosine (Y) residue (B) to an amber codon (TAG). Comparing these sequences with the native nucleotide sequence revealed a mismatch of the codons at the positions of alteration.

DISCUSSION

Historically, Stro-1 monoclonal antibody have been significant in the identification of both mesenchymal precursor cells and its lineage since it was first discovered and the binding of Stro-1 to nascent pluripotent mesenchymal precursor cells (MPCs) remains the cornerstone in the field of MPCs research (Fitter *et al*., 2017). Even with it much great details in the definition of mesenchymal stem cells, the definitive antigen to which Stro-1 binds to was not completely understood until recently, even though much work is still needed in this promising field. In this study, report the conversion of the monoclonal antibody (Stro-1) into a recombinant fragment and mutated the complementarity determining region (CDR3) of the variable heavy (V_H) chain to allow the incorporation of the amber codon (TAG) in DNA and (UAG) in messenger RNA (mRNA). We initially amplified both the variable (V) region of the heavy (V_H) and light (V_L) chains in order to clone the genes encoding the CDR3

fragments in pHenIX vector. After the successful amplification of the heavy (V_H) and light (V_L) chains, we aim to introduce the V_H and V_L genes into pHenIX vector, alter the genes before introducing both mutated genes into an expression vector such as the pUC119 vectors or the Drosophila expression system vectors to generate mutant libraries. Several attempts to achieve this with the pHenIX vector was however unsuccessful. This in part, could be as a result of errors due to PCR priming, PCR reaction mix, and incomplete or partial digestion from restriction endonucleases such as SalI or SfiI used in digesting the vector. Also, since the colonies from the transformation reaction after the ligation of insert were selected at random, there is a probability that selected colonies did not contained insert, even though this is not completely understood. The native CDR3 region of the heavy (V_H) chain was then cloned into a TA cloning vector (pCR 2.1, 3.9kb) after digestion with DpnI restriction enzyme with the intension of altering the fragment encoding the CDR3 region, before transferring the altered sequence into an expression vector such as the Drosophila expression vector. The cloning of the CDR3 region into TA vector (pCR 2.1, 3.9kb) was successful as shown in figure 3 as analysed by 0.8% TBE agarose gel electrophoresis under UV light.

Mutagenic oligonucleotides primers were designed from the CDR3 region (italicised) of the heavy (V_H) at the specific sites of interest (underlined) as shown in Figure 6.

GAAGTGCAGCTGCAGCAGAGCGGCCCGGATCTGGTGAAACCGGGCGCGAGCGTGAAAATT
AGCTGCAAAGCGAGCGGCTATAGCTTTACCGGCTATTATATGCATTGGGTGAAACAGAGC
CATGGCAAAAGCCTGGAATGGATTGGCCGCGTGAACCCGAACAACGGCGGCACCAGCTAT
AACCAGAAATTTAAAGGCAAAGCGATTCTGACCGTGGATAAAAGCAGCAGCACCGCGTAT
ATGGAACTGCGCAGCCTGACCAGCGAAGATAGCGCGGTGTATTATTGCGC*ACCTATGCG*
ATGGATTATTGGGGCCAGGGCACCAGCGTGACCGTGAGCAGC

Figure 6: Native nucleotide sequence of the variable heavy (V_H) chain. CDR3 region in italics and position of interest italicised and underlined.

The primer pairs Mut1 (For) 5'-TGTATTATTGCGCGACCTAGGCGATGGATTATTGGG-3' and Mut1 (Rev) 5'-CCCAATAATCCATCGCCTAGGTCGCGCAATAATACAC-3', Mut2 (For) 5'-CGACCTATGCGATGGATTAGTGGGGCCAGG-3' and Mut2 (Rev) 5'-CCTGGCCCCACTAATCCATCGCATAGGTCG-3') were used in the amplification of the target of interest before sequencing was carried out. The sequencing result reveals that mutation has not occurred at the different positions of alteration of the first and second tyrosine (Y) residues replaced with the amber codon (TAG) in the complementarity

determining region (CDR3) of the variable heavy (V_H) chain. We compared the mutated CDR3 region (Figure 5 A & B) of the variable heavy (V_H) chain after sequencing with the native sequence and observed a mismatch at the altered tyrosine (Y) residues of the CDR3 region replaced with the amber codon (TAG). This could be as a result of deletion of two or more amino acid residues of the heavy chain during reverse translation of the amino acid sequence to the corresponding nucleotide sequence using sequence manipulation site (SMS) reverse translation tool available at http://www.bioinformatics.org/sms2/rev_trans.html. Subsequently, since the primers designed for the amplification of the mutated target of interest were incorrect, a possibility is that these primers had lower annealing temperatures (Tm) < 68^{o}C which explain why there was no visible PCR product after analysis with agarose gel electrophoresis (figure not shown). The transformed cells therefore are likely not to contain the plasmid with the desired mutation at these sites hence were not digested with the DpnI restriction enzyme. Previous studies have shown that the amber codon (TAG in DNA is scarcely employed by *E.coli* and often does not result in the termination events of critical genes (Nakamura *et al*., 2000; Xie and Schultz, 2005), during translation. In conclusion, it is necessary to point out that this is not a conclusive study as certain parts of the research were not carried out due to time constrain. Therefore, much detailed study is needed to unravel the incorporation or replacement of glycine residues at these positions.

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