Generation and analysis of mutated clonal scFv antibody fragments against R7V epitope of HIV-1

by

Jiya George

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Biochemistry

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

South Africa

June 2012

Submission Declaration

I, Jiya George declare that the dissertation, which I hereby submit for the degree M.Sc. Biochemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Date:

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Acknowledgements

I am ever grateful to God, the Creator and the Guardian, for his richest blessings.

I would like show my gratitude to the following individuals for their valuable assistance in the preparation and completion of this study.

First and foremost, I would like to thank my supervisor, Dr. M. Beukes for his guidance and support through the duration of the study.

I would like to thank Prof. Jan Verschoor, Head of Department of Biochemistry, for his kind concern and consideration regarding my academic requirements.

I would like to thank Mr. Johan Schoombie for his advices and assistance through the duration of the study.

I would like to show my gratitude to Dr MZ Ahmed and Miss. Pelly Malebe for their encouragement and editing of this dissertation.

I would like to thank my colleagues and staff in the Biochemistry Department for their help and moral support.

Special thanks to my family and friends in India for their prayers, love and best wishes.

Last but not the least, I would like to show my deepest gratitude to my parents for their understanding and support to pursue postgraduate studies.

"Satisfaction lies in the effort, not in the attainment, full effort is full victory."

Mohandas Gandhi

Summary

Human immuno deficiency virus (HIV) incorporates host cellular protein, beta-2-microglobulin (β2m), into its surface envelope during budding. β2m is a cellular protein that belongs to the major histocompatibility complex (MHC) Class I molecules. Studies have shown anti-β2m monoclonal antibodies (mAbs) has the ability of to neutralize the virus. An epitope consisting of seven amino acids of the β2m protein designated as R7V produces antibodies that protect HIV infected people from progressing to AIDS. These protective antibodies, called anti-R7V antibodies, were able to neutralize different HIV isolates, despite their genomic variations, various cellular targets and geographic origin.

Anti-R7V antibodies in the format of single chain variable fragments (scFvs) were produced in our laboratory using the M13 phage display technology. These scFv antibody fragments were used during *in vitro* studies for the detection and neutralization of the R7V antigen by enzyme linked immune sorbent assay (ELISA). The scFv fragments produced against the R7V epitope showed interaction, however the antibody-antigen affinity was too weak for the virus neutralization assay. Hence, this project focused on the affinity maturation of the anti-R7V scFv fragments through random mutagenesis using the error prone (EP) PCR method.

The EP PCR method generated two mutated anti-R7V scFvs. The mutated clones were subcloned into the pAK400 expression vector. The computer-based models, created using the Swiss PDB Deep Viewer 4.02 software, were used to predict the antigen-binding site and affinity analysis of both parent and mutated scFv's.

Mutated clone 1 failed to bind to the R7V epitope whereas mutated clone 2 had similar binding pattern as the parent scFv. Mutated clone 2 was predicted to have a higher binding affinity compared to the parent scFv. The results obtained demonstrate the efficacy of EP PCR to generate high affinity antibodies. Future experiments using high affinity anti-R7V scFv's may lead to its potential use in diagnostics, therapeutics or vaccine development.

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Abbreviations

Units of Measurement

µl	microlitre	N	normality
µM	micromol	ng	nanogram
kDa	KiloDalton	nm	nanometre
M	moles/litre	pmol	picomol
mg	milligram	U	unit of enzyme
ml	millilitre	V	volt
mM	millimol	V/cm	volt per centimetre
w/v	weight per volume	v/v	volume per volume

Amino acids

A	Alanine	(Ala)	M	Methionine	(Met)
C	Cysteine	(Cys)	N	Asparagine	(Asn)
D	Aspartic acid	(Asp)	P	Proline	(Pro)
E	Glutamic acid	(Glu)	Q	Glutamine	(Gln)
F	Phenylalanine	(Phe)	R	Arginine	(Arg)
G	Glycine	(Gly)	S	Serine	(Ser)
H	Histidine	(His)	T	Threonine	(Thr)
I	Isoleucine	(Ile)	V	Valine	(Val)
K	Lysine	(Lys)	W	Tryptophan	(Trp)
L	Leucine	(Leu)	Y	Tyrosine	(Tyr)

a.a	amino acid
ADCC	antibody directed cell mediated cytotoxicity
AIDS	acquired immuno deficiency syndrome
amp	ampicillin
amp^R	ampicillin resistance gene
ARV	anti retroviral
AZT	3′-azido-2′,3′-dideoxythymidine
β2m	beta 2 microglobulin
BF	blocking buffer
bp	base pair
BSA	bovine serum albumin
C	constant region
$CaCl_2$	calcium chloride
cam	chloramphenicol
cam^R	chloramphenicol resistance gene
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CDC	Centre for Disease Control and Prevention
CDR	complementarity determining region
CDR3	CDR region 3
C_H	constant region of the heavy chain on antibody
C_L	constant region of the light chain on antibody
CTL	cytotoxic T lymphocyte
D	diversity gene segment
dNTPs	deoxynucleotide triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid

dsDNA	double stranded DNA
$dddH_2O$	double deionized distilled water
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immuno sorbent assay
EP PCR	error prone polymerase chain reaction
EtBr	ethidium bromide
Fab	antigen-binding fragment
FD	fast digest
FDA	Food and Drug Administration
FR	framework region
Ff	F-specific filamentous
g3	phage gene 3
g3p	gene3-protein
HAART	highly active anti-retroviral treatment
HAMA	human anti-mouse antibody
HCDR	complementarity determining region on heavy chain of antibody
HCl	hydrochloric acid
HIS	histidne
HIV	human immuno deficiency virus
HLA	human leukocyte antigen
hnmAbs	human neutralizing monoclonal antibodies
HRP	horseradish peroxidase
ICAM1	inter cellular adhesion molecule 1
IFNα	interferon alpha
Ig	immunoglobulin
IgG1	immunoglobulin type G subclass 1
IL-7	interleukin 7
IPTG	isopropyl-beta-D-thiogalactopyranoside

J_H	joining gene segment on heavy chain of antibody
J_L	joining gene segment on light chain of antibody
kb	kilobase
K_D	equilibrium dissociation constant
LB	Luria-Bertani media
LCDR	complementarity determining region on light chain of antibody
LFA1	lymphocyte function-associated antigen 1
mAbs	monoclonal antibodies
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
$MnCl_2$	manganese Chloride
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
MTCT	mother-to-child transmission
nAb	neutralizing antibody
NaCl	sodium chloride
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
OD	optical density
PBS	phosphate buffered saline
PBST	phosphate-buffered saline with Tween-20
PCR	polymerase chain reaction
PIs	protease inhibitors
PVDF	polyvinylidene fluoride
RE	restriction enzyme
RNA	ribonucleic acid
rpm	revolution per minute
RT	reverse transcriptase
scFv	single-chain variable fragment

SD	shine dalgarno sequence
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDT7g10	shine dalgarno sequence of T7 phage gene 10
ssRNA	single stranded RNA
ssDNA	single stranded DNA
SPR	surface plasmon resonance
TB	tuberculosis
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TBST	tris-buffered saline with Tween 20
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TES	tris(hydroxymethyl)-methyl-2-aminoethane sulfonate
tet^R	tetracycline resistance gene
T_h	T-helper cells
TNFα	tumor necrosis factor alpha
U.S.	United States
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	ultra violet
V	variable region
V_H	variable region of the heavy chain on antibody
V3	third hypervariable loop of HIV gp120
V_L	variable region of the light chain on antibody
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

Chapter 1

Literature Review

1.1 Introduction

Human Immuno Deficiency Virus (HIV) is a retrovirus that invades the human immune system by attacking the CD4 T-helper cells (T_h) causing the immune system to weaken. People infected with HIV have an increased susceptibility to opportunistic infections such as pneumonia and rare cancer because of their loss of normal immune responses (Campbell and Reece, 2008). HIV infection is the cause of Acquired Immuno Deficiency Syndrome (AIDS). AIDS is a devastating health disease that negatively affects the economic and social development of a country (Hecht *et al*., 2006).

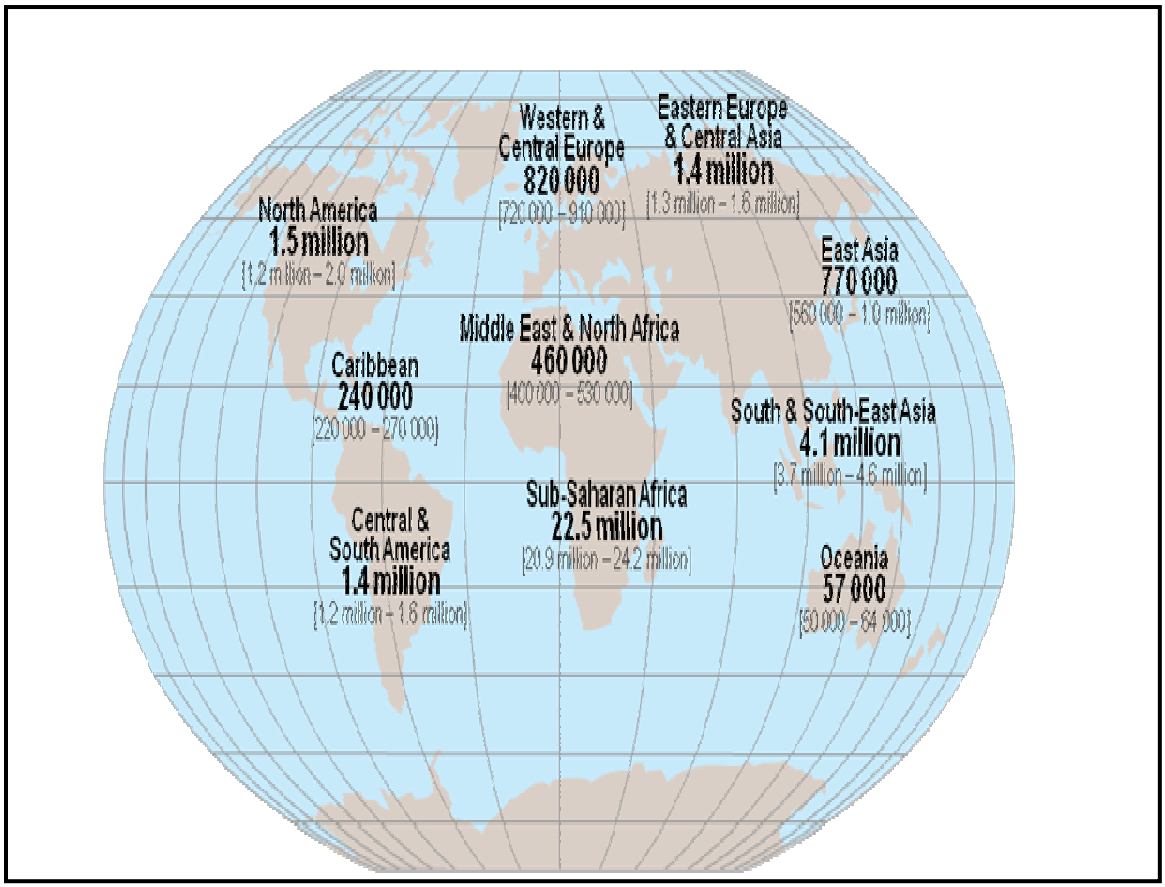


Figure 1: The estimated number of people (both adults and children) living with HIV around the world (UNAIDS, 2010)

Millions of people around the world, including young children, have become victim of this virus (Figure 1). According to U.S. Centre for Disease Control and Prevention (CDC), HIV-infected people with AIDS have a CD4 count less than 200 cells/µl or a CD4 percentage less than 14%, along with symptoms such as pulmonary tuberculosis (TB), pneumonia or invasive cervical cancer (Shirriff, 2000). According to the global statistics released by UNAIDS, around 2 million deaths occurred from AIDS-related diseases in 2009 (UNAIDS, 2010).

The first incidence of HIV infection was believed to have had happened back in 1960 through zoonotic transmission (Karim and Karim, 2005). Since then the virus have spread widely especially in developing countries. To date, about 33.3 million people are living with HIV worldwide (UNAIDS, 2010).

HIV is transmitted through unprotected sexual intercourse, use of HIV contaminated needles, transfusion of contaminated blood, and from mother to child during pregnancy, childbirth or breast-feeding. It takes 10 to 15 years for HIV-infected individuals to develop AIDS (WHO, 2011). Antiretroviral drugs have been developed to slow the HIV reproduction and progression to AIDS. HIV awareness campaigns and sex education at school level have also been implemented to control the spread of HIV (UNAIDS, 2010).

The biggest problem with the treatment of HIV infection is the high mutation rate of the virus, which results in the generation of different HIV mutant strains that are resistant to immune responses and antiretroviral (ARV) drugs. HIV changes its structure continuously within the same individual during infection, from one individual to another and during the ARV therapy treatment. Hence, the development of an effective treatment method against HIV has been unsuccessful (Haslin and Chermann, 2002; Zhang *et al*., 2003).

The problem of high mutation rate can be solved by identifying and targeting conserved HIV epitopes (Haslin and Chermann, 2002; Zhang *et al*., 2003). Polyclonal and monoclonal antibodies (mAbs) have been used as therapeutics in cancer treatment, diagnostics, autoimmune diseases and treating bacterial, viral and parasitic infections. The potential of mAbs for HIV-1 treatment have also been explored (Huber *et al*., 2008).

An effective antiviral vaccine should have the ability to elicit antibodies capable of neutralizing the viral infectivity. Hence, efforts have been taken to identify and characterize HIV neutralizing antibodies with the hope of developing an effective HIV vaccine.

1.2 Human Immuno Deficiency Virus (HIV)

There are two known types of HIV, namely HIV-1 and HIV-2. Both HIV-1 and HIV-2 causes clinically indistinguishable AIDS. The difference is that HIV-2 is less easily transmitted and the infection stage to AIDS is longer than in HIV-1. The predominant type is the HIV-1 virus whereas the HIV-2 is common only in West Africa and rarely found elsewhere. HIV referred in literature without subtype specification is generally the HIV-1 virus (AIDS, 2010).

HIV is a retrovirus, meaning it has the ability to reverse transcribe its genomic RNA into double stranded DNA (dsDNA) and integrate into host genome. The integrated dsDNA is called a provirus, as it serves as the template for DNA dependent RNA polymerase to produce new viral RNA genomes. These viral RNA genomes are then translated into proteins that make up the viral core proteins, envelope protein components and small regulatory proteins (Barré-Sinoussi, 1996).

1.2.1 Structure of HIV

HIV has an enveloped structure with external spikes (Figure 2). The viral envelope is a lipid bilayer membrane. The envelope spike consists of two viral encoded glycoproteins, gp120 (outer envelope protein) and gp41 (transmembrane protein). The glycoprotein, gp120 is a highly glycosylated protein. Sequence analysis of different HIV isolates revealed that gp120 consists of five variable (V1 – V5) and five constant (C1 – C5) regions. It has a CD4 receptor site and a chemokine receptor (CCR5 or CXCR4) site. The CD4 binding site and the co-receptor binding site are relatively conserved and inaccessible unless the CD4 binding triggers a conformational change to express the site to the antibody. The primary role of gp120 is to attach the virus to its target cells through receptor binding and bring the virus close to the target cell membrane. The transmembrane protein, gp41 is then responsible for the fusion of the viral and target cell membranes, allowing the flow of genetic information from the virus to the target cell. The glycoprotein, gp41 is well conserved and most of its surface is hidden from the antibody (Barré-Sinoussi, 1996).

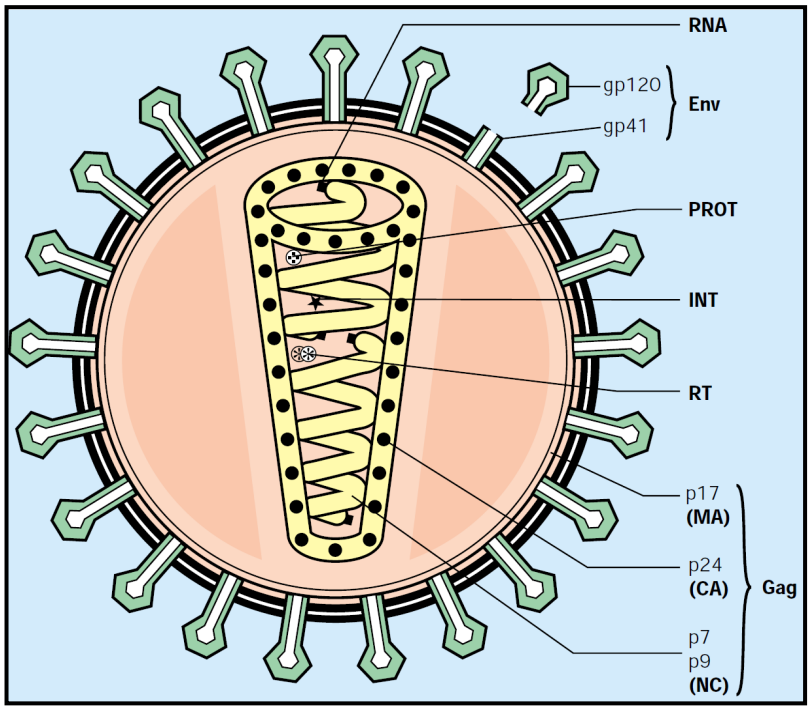


Figure 2: The structure of HIV (Barré-Sinoussi, 1996)

HIV contains a viral capsid, which consists of two copies of single-stranded RNA (ssRNA) genome, a major capsid protein (p24), a nucleocapsid protein (p7/p9) and the three enzymes, namely, protease (PROT), integrase (INT) and reverse transcriptase (RT). The RT enzyme enables the reverse transcription of the viral genomic RNA into dsDNA, which is then referred to as a proviral DNA. The proviral DNA integrates into the host genome and generate new viral RNA genomes using DNA dependent RNA polymerases. These viral RNA genomes are then translated into proteins that make up the viral core proteins, envelope protein components and small regulatory proteins. The viral capsid is surrounded by a matrix protein (p17), which is involved in the early stages of the replication cycle of the virus. The matrix protein also helps in the integration of the proviral DNA into the nucleus of the host cell (Barré-Sinoussi, 1996).

1.2.2 Life cycle of HIV

The main receptor of HIV is the CD4 molecule on the T_h cells. The virus enters the host cell through the surface receptor CD4 (Mishra *et al.*, 2009). There are three steps involved in the entry of HIV into the host cell: firstly, binding of the virus to the CD4 molecule through gp120; secondly, binding of the virus to the co-receptors, CCR5 and CXCR4 and thirdly, fusion between the virus and host cell membrane and penetration of virus into the cell, through gp41 (Haslin and Chermann, 2002).

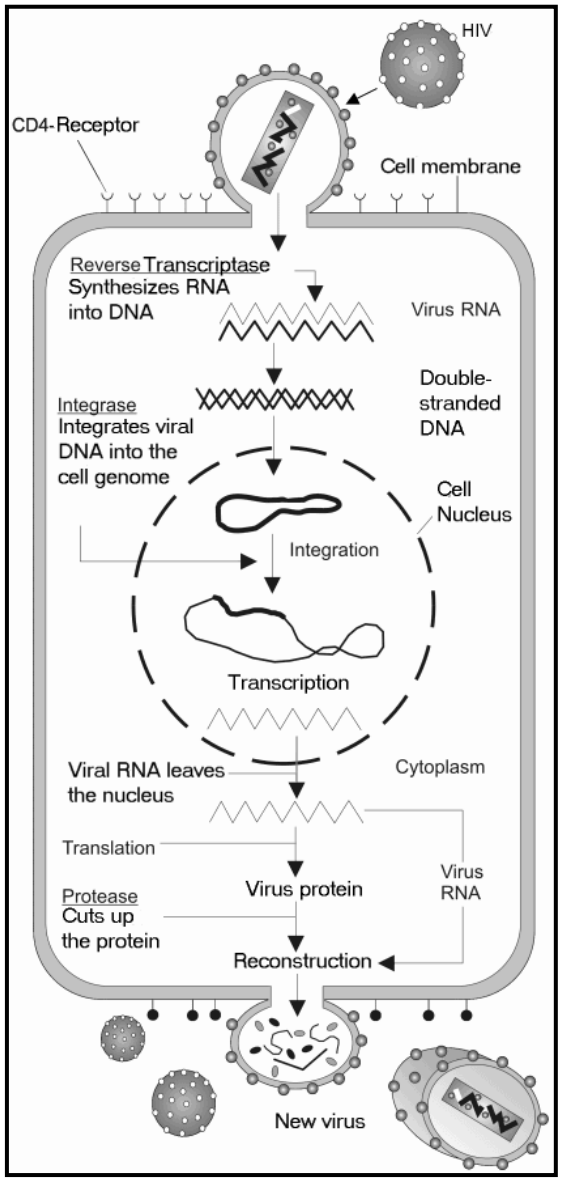


Figure 3: The life cycle of HIV (image taken from The History of AIDS © 2005, web link http://fohn.net/history-of-aids/)

Figure 3 illustrates the entry and replication of HIV in the host cell. Upon viral entry into the host's plasma membrane, the viral capsid releases its RNA, proteins and RT into the cytoplasm. The RT in the cytoplasm enable the synthesis of viral DNA through reverse transcription, which is then integrated into the host's chromosomal DNA. The host RNA polymerase transcribes the proviral DNA into RNA. This RNA functions as messenger RNA (mRNA) and produce viral proteins through translation, for the synthesis of new viruses (Campbell and Reece, 2008).

1.3 Antiretroviral (ARV) therapy

To date, there are approximately 30 antiretroviral products, used either singly or in combination, to treat HIV infected patients. The first type of antiretroviral drugs generated were the nucleoside reverse transcriptase inhibitors (NRTIs), in the form of dideoxynucleosides, which function by competitive inhibition and chain termination of HIV-1 DNA polymerase. Zidovudine (AZT) was the first NRTI ARV drug approved by the U.S. Food and Drug Administration (FDA). The discovery of AZT (3′-azido-2′,3′-dideoxythymidine) showed that it is possible to treat HIV-1 infection. AZT was first synthesized as potential anti-cancer agents, but it failed to create a beneficial impact as anti-cancer drugs. However, AZT was found to be highly active against HIV-1 *in vitro*, as it showed clinical activity in the first study of drug administration to AIDS patients. AZT is currently used in combination therapies (Broder, 2010).

The second-generation ARV drugs were the non-nucleoside reverse transcriptase inhibitors (NNRTIs). They act at a specific pocket-binding site within the RT of HIV-1 and the viral protease inhibitors (PIs). Other type of drugs have also been developed that target different phases of the HIV life cycle, such as the fusion inhibitors, entry inhibitors and HIV integrase function inhibitors (Broder, 2010).

ARV therapy has been successful in significantly decreasing the rate of mortality and morbidity and the rate of mother-to-child transmission (MTCT). Since ARV therapy does not eliminate the virus itself, people are required to be under chronic medication. This can be problematic due to the side effects of ARV drugs and possible evolution of drug resistant viral strains. Evolution of drug resistant strains towards the available drugs has been reported. Drug resistant HIV strains have the ability to mutate and replicate in the presence of ARV drugs. The consequences of drug resistance are treatment failure, economic loss, and spread of resistant strains and the need for a more costly second line treatment (Huber *et al*., 2008). World Health Organization (WHO) and its HIV ResNet group have developed a *Global strategy for prevention and assessment of HIV drug resistance* to analyze the scale of HIV drug resistance and also, to create awareness on it (WHO, 2011). Due to the evolution of drug resistant HIV strains, alternative treatments are essential to control HIV infection (Huber *et al*., 2008).

1.4 Current statistics on HIV

According to the official statistics released by UNAIDS at the end of 2009, HIV epidemic seems to have stabilized in most regions. In 2008, the number of new HIV infections was approximately 30% lower than that in 1996; the year in which the HIV spread was at its peak. Also, the number of AIDS-related deaths in 2008 was around 10% lower than in 2004 and the number of children newly infected were 18% lower than in 2001 (UNAIDS, 2010).

Globally, a significant progress has been achieved in preventing new HIV infections and in decreasing the number of AIDS-related deaths. Nonetheless, the number of people living with HIV continued to rise in 2008 because of new HIV infections and the beneficial effect of ARV therapy. Sub-Saharan Africa still remains the worst affected region, accounting for 67% of HIV infections worldwide, 68% and 91% of new HIV infections among adults and children, respectively, and 72% of AIDS-related deaths worldwide (Coster and Monaghan, 2002; UNAIDS, 2010).

Highly active anti-retroviral treatment (HAART) is the current standard of drug treatment for HIV. HAART is a combination prevention strategy to control HIV infection. HAART is a combination of two or three ARV drugs. HAART reduces the rate of development of HIV resistant strains, thereby making the treatment more effective. This treatment does not cure AIDS, but reduce the effect of infection and prolongs a healthy state. Most HIV infected people using HAART are able to continue to work and lead normal lives (Clayton and McQueen, 2011).

Also, new improved drugs based on the existing ARV drugs are being developed. An example is Festinavir drug, modified form of Stavudine. Festinavir is less toxic and has fewer side effects. Researchers are also looking into developing non-ARV drugs. The protein, interleukin 7(IL-7) is being investigated to increase the immune function in HIV-infected people. IL-7 proteins help the immune cells to develop and survive (Clayton and McQueen, 2011).

A new HIV drug, Rilpivirine (TMC278) has been approved by U.S. FDA on 21 May, 2011. The market name of this drug is Edurant. It is used along with the other ARV medications. This drug blocks the reproduction of HIV. In the clinical trial, 83% of HIV-infected people have shown suppression of HIV reproduction. This drug does not cure HIV infection and has to be taken everyday to contain HIV infection (Sanford, 2012).

1.5 HIV vaccine

The ARV therapy has been very effective in reducing HIV-related morbidity and mortality but not in preventing or eradicating the HIV infection. The ultimate goal would therefore be the development of a safe, effective and affordable HIV vaccine that can eliminate the HIV epidemic. A successful HIV vaccine has not been developed to date. The unique characteristics of HIV, such as high genetic variation, rapid replication and integration of proviral DNA into the host genome has caused tremendous challenges in the development of a successful HIV vaccine. The lack of understanding of which type of immune response will contain HIV infection, unavailability of useful small animal models, and the danger in using live, attenuated HIV are the other challenges faced in the development of HIV vaccine. Other vaccines (such as for small pox) have been developed from live viruses. The danger in using live attenuated HIV is that they can still cause AIDS (Bangham and Phillips, 1997; Gallo, 2005).

1.6 Therapeutic antibodies

Antibodies are natural molecules of high specificity that recognize and eliminate pathogenic antigens. Target specificity for treatment was attained through the development of mAbs. The mouse hybridoma technology developed by Kohler and Milstein (1975) was an important development of antibody technology. The emergence of therapeutic mAbs using hybridoma technology in the 1970 has revolutionized the medicinal field. The OKT3 (OrthoClone) mAb approved by the U.S. FDA in 1986 is an example of this revolution. OKT3 mAb is used against the antigen CD3, which is involved in acute kidney transplant rejection (Brekke and Løset, 2003).

1.6.1 Hybridoma technology

In the human immune system, there are two types of lymphocytes: B lymphocytes (B cells) and T lymphocytes (T cells). Both B and T cells help destroy foreign cells. Some T cells aid in the activation of other lymphocytes whereas other T cells detect and destroy infected host cells. B cells function by producing a soluble protein called the antibody. An antibody recognizes and binds to foreign particles from the invading pathogen, such as viruses, and initiates the killing of the invader or signals other cells in the immune system to bind and kill the invading pathogen. Foreign particle on the invading pathogen is called the antigen. An antibody-antigen interaction is highly specific. Antibodies generally recognize a small portion on the antigen called the epitope. A single antigen usually contains more than one epitope. When an antibody recognizes

its antigen, the B cell starts to proliferate making millions of copies of the B cell, which enables the production of large amounts of the antibody with the right specificity (Coster and Monaghan, 2002).

B cells can only divide a finite number of times in an artificial culture, thus limiting the production of antibodies for therapeutic purposes. Nonetheless, hybrid cells produced by the fusion of B cell and an immortal cancer cell such as myeloma cells can produce large amounts of the desired antibody. Such hybrid cells that produce both the desired antibody and are immortal are called hybridomas. Antibodies produced using this hybridoma technology are called mAbs. They are called mAbs because they are produced from one cell type and are specific for the same epitope on an antigen molecule (Coster and Monaghan, 2002).

Antibodies obtained from the sera are polyclonal meaning multiple antibodies recognize and bind specifically to a different epitope on an antigen, which results in cross reactivity and high background. Hybridoma technology was a landmark development as it avoids the problem of polyclonal antibodies by producing a large amount of mAb with defined specificity (Bradbury *et al*., 2003; Wu and Dall'Acqua, 2005).

Murine hybridoma technology has been used for the production of mAbs. These murine mAbs were of limited use in human therapeutics because of the human anti-mouse antibody (HAMA) immune responses in the host. Hence, new approaches have been developed to create human mAbs, such as chimeric antibodies, humanization of mouse mAbs and use of transgenic animals. Chimeric antibodies were generated by replacing the constant regions of the mouse antibodies with the human antibodies. Humanized antibodies were generated by transplanting the complementarity determining regions (CDR) from mouse to human antibodies. In transgenic animals, transgenes containing the human immunoglobulin (Ig) is inserted into the germline of the animal creating a transgenic animal that is capable of producing large amounts of fully human antibodies when challenged with an antigen (Coster and Monaghan, 2002; Torphy, 2002; Wu and Dall'Acqua, 2005).

Even though the new generation antibodies reduced the HAMA response, phage display system simplified the generation of fully human mAbs of high affinity and specificity (Hoogenboom, 1997). Phage display is a powerful technique used for the identification of ligands for peptide receptors, epitopes for mAbs, enzyme substrate selection and screening of cloned antibody from libraries. The most successful application of phage display technology has been the isolation of mAbs using large phage antibody libraries (Benhar, 2001).

1.6.2 Recombinant monoclonal antibody technology

Recombinant mAb technology has replaced the conventional hybridoma technology. This new technology was able to produce more and fully human mAbs using the phage display system. The recombinant mAb technology is a rapidly developing field involved in the study and improvement of antibody properties by means of genetic engineering. The FDA approved the first recombinant human mAb, Adalimumab®, in 2003. This antibody is used to treat Rheumatoid Arthritis. Adalimumab is a human immunoglobulin G subclass 1(IgG1), derived from phage library that has a high affinity for tumor necrosis factor-alpha (TNF-α). Studies have shown that Adalimumab has the ability to control signs and symptoms of Rheumatoid Arthritis. This mAb has a low immunogenicity, thus avoiding the need of using immunosuppressants such as methotrexate (Brekke and Løset, 2003).

Antibodies produced using hybridoma technology is in conventional antibody format whereas antibodies isolated using phage display are either as single-chain variable fragment (scFv) or antigen-binding fragment (Fab) (Figure 4). The smaller size of the antibody fragment reduces toxicity and increases the efficacy of antibodies (Wark and Hudson, 2006).

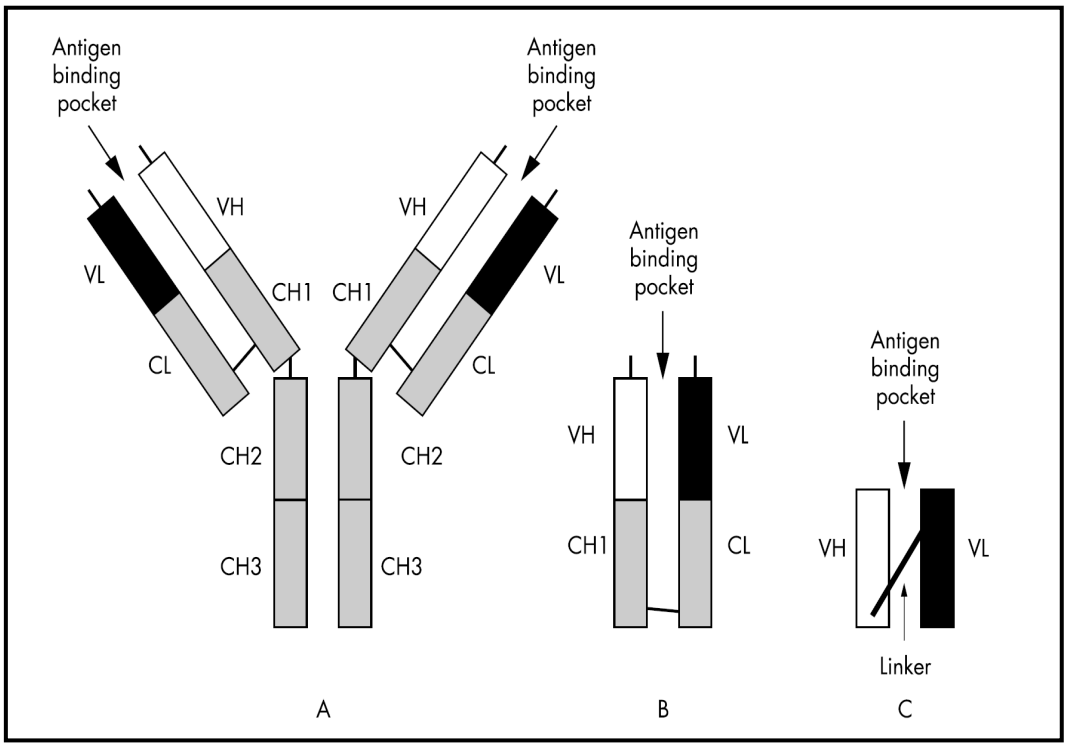


Figure 4: The structures of (A) whole antibody, (B) Fab fragment and (C) scFv fragment (Smith *et al.*, 2004)

***V_H – variable region of heavy chain, *V_L – variable region of light chain**
***C_H – constant region of heavy chain, *C_L – constant region of light chain**

The antibody molecule consists of two identical light chains and two identical heavy chains joined by disulfide bonds. Both heavy and light chains have a constant domain and a variable domain. The antigen-binding site is on the variable domain of the antibody gene (Kumagai and Tsumoto, 2001). The scFv antibody fragment consists of the variable domain of heavy (V_H) and light (V_L) chains and the antigen-binding site, whereas the Fab fragment additionally contains the constant domain of heavy (C_H) and light (C_L) chain of the antibody (Brekke and Løset, 2003). These fragments retains the antigen-binding activity of the parental whole antibody since the antigen-binding activity is determined by the CDRs located on the variable domain (V) of the antibody (Adams *et al*., 1998).

1.7 The scFv antibody fragment

The scFv molecules are the smallest single-chain variable antibody fragments that can specifically bind to their target antigen. They can be easily isolated from phage display libraries and have the ability to recognize conserved antigens. The scFv molecules are monovalent antibodies and are about 25 – 30 kDa in size (Adams *et al*., 1998; Azzazy and Highsmith, 2002). Even though Fab antibody fragment represents the natural antibodies better than the scFv fragments, scFvs are preferred for phage display because they are more stable and are smaller compared to Fab gene fragment. The complete scFv gene can be fused into the phage coat protein, thus enabling its expression on the phage surface (Davies *et al*., 2000).

Recombinant antibody constructs generally consists of scFv molecules, where the V_H and the V_L chain of the antibody are joined together using a polypeptide linker (Hudson, 1999). The linker that joins the V_H and V_L gene segments of the scFv fragment mostly consists of a combination of glycine (Gly) and serine (Ser) residues. These sequences provide flexibility, enhance the hydrophilicity of the peptide backbone, allowing hydrogen bonding with solvent molecules and are relatively protease resistant. Typically, the length of the linker sequence is 15 amino acids, which provides sufficient flexibility for the V_H and V_L regions to form scFv with a monovalent antigen-binding site (Kortt *et al*., 2001). The monomeric form of scFv can be dimerized or trimerised to diabodies or triabodies (Figure 5) which will increase the avidity and possibly cross link the target with effectors using bispecific diabody constructs (Brekke and Løset, 2003).

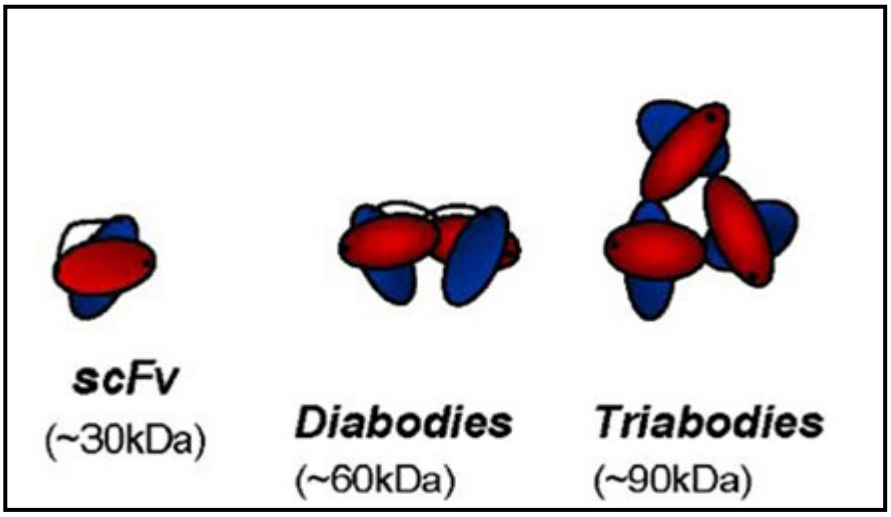


Figure 5: The structures of engineered scFv antibody fragments (Wark and Hudson, 2006)

The affinity of a scFv molecule for its target antigen can be improved by: i) modifying the complementarity determining regions (CDRs) ii) increasing the functional affinity by the production of multivalent species or iii) increasing the size of scFv (Adams *et al*., 1998; Adams and Schier, 1999). The most successful method for improving the affinity is by modifying the CDR regions (Hudson, 1999). The sequence and conformation of the amino acids of the CDRs, that are located on the V_L and the V_H chain of the antibody determines the antigen-binding activity (Adams *et al*., 1998; Adams and Schier, 1999).

1.8 Phage display technology

George P. Smith first introduced phage display in 1985. Phage display refers to the expression of proteins or peptides on the surface of filamentous phage (Azzazy and Highsmith, 2002). This technology allows the polypeptide/protein (with a particular function) to be displayed on the phage surface by inserting the gene encoding for the polypeptide into the phage genome (Nilsson *et al*., 2000).

Phages are viruses that infect gram-negative bacteria with pili as receptor. The most commonly used bacteria is the *Escherichia coli* (*E.coli*) (Smith and Petrenko, 1997). Filamentous phages such as M13, Fd and f1 are the commonly used strains that infect the *E.coli* via F pili. The phage particles infect the bacteria by attaching to the tip of the F pilus and translocate their genome into the bacterial cytoplasm (Nilsson *et al*., 2000; Azzazy and Highsmith, 2002). These phages

consists of a circular single stranded DNA (ssDNA) genome packed in a long tube composed of a single major coat protein (pVIII) and four minor coat proteins (pIII, pVI, pVII and pIX) at the tips. Of the five coat proteins, pVIII and pIII have been commonly used to display proteins or peptides. Filamentous phages are assembled in the cytoplasm and secreted from the bacteria without cell lysis as opposed to lytic bacteriophages such as T4 and T7, where the foreign protein is assembled and folded in the cytoplasm (Mullen *et al*., 2006).

1.8.1 Principle of phage display system

The foreign DNA encoding the desired protein or peptide is cloned into the phage genome. The foreign gene is fused with the gene encoding one of the coat proteins resulting in a hybrid fusion protein. Upon expression in *E.coli*, the hybrid fusion protein will incorporate into new phage particles that are released from the cell, resulting in the protein or peptide being presented on the outer surface of the phage and its genetic material remains within the phage particle (Smith and Petrenko, 1997; Hoogenboom *et al*., 1998). This link between the protein/peptide phenotype and genotype allows for the enrichment of a specific antibody, using an immobilized or labelled antigen. Phages that bind to a specific antigen are retained, while the unbound phages are washed away. The bound phages can be recovered and re-infected into the bacteria for further enrichment and affinity analysis (Hoogenboom *et al*., 1998). Figure 6 illustrates the steps involved in the selection and affinity maturation of antigen-specific antibodies using phage display technology.

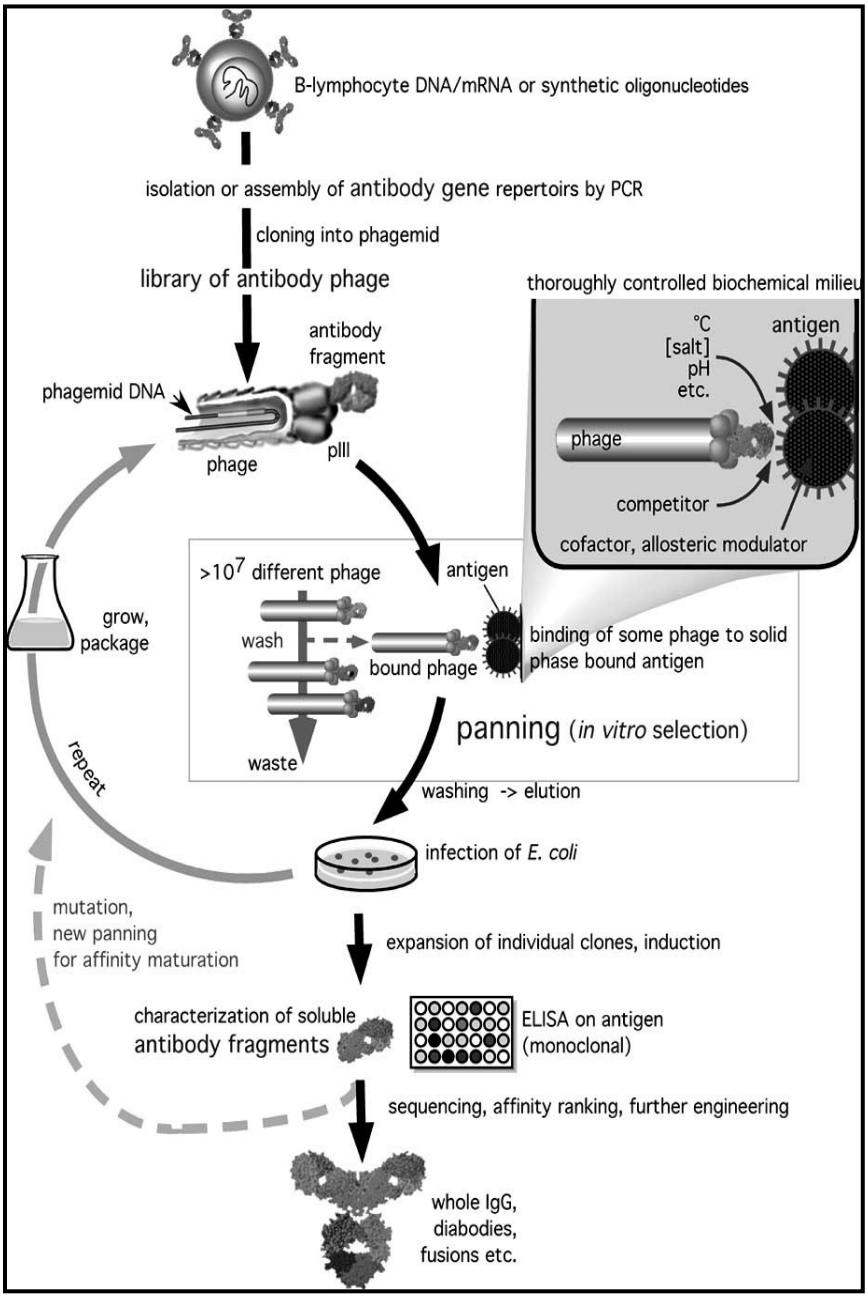


Figure 6: Principle of phage display system (Thie *et al.*, 2008)

1.8.2 Phage display libraries

One of the most successful applications of the phage display technology is the isolation of recombinant mAbs using large antibody phage display libraries. The phage display library is composed of a variety of antibody genes that is constructed *in vitro* using PCR (Hoogenboom *et al*., 1998). There are two main types of libraries, namely, immune and non-immune libraries. The non-immune libraries can be subdivided into naive and synthetic antibody libraries (Benhar, 2001). Phage antibodies can be obtained from any of the libraries mentioned below.

1. Immune libraries: In this library, antibody genes are created using the variable genes from the B cells of immunized donors. This type of library is enriched with antigen-specific antibodies, producing higher affinity antibodies even with small library size. The advantage of this library is that the antigen-specific antibodies have already undergone *in vivo* affinity maturation by the host immune system. The limitation of this library is that only antibodies against specific antigens are generated, they require library construction for each individual antigen and are labour intensive (Hoogenboom *et al*., 1998; Nilsson *et al*., 2000; Benhar, 2001; Azzazy and Highsmith, 2002).

2. Naive libraries: In this library, antibody genes are created using the variable genes from the B cells of non-immunized donors. Antibodies generated this way can recognize a variety of antigens, thus acting as a universal library for the detection of all antigens. The affinity of isolated antibodies is correlated with the library size. Therefore, the limitation of naïve library is that a large library size is required to obtain high affinity antibodies (Hoogenboom *et al*., 1998; Benhar, 2001; Azzazy and Highsmith, 2002).

3. Synthetic or semi-synthetic libraries: In this library, the antibodies are created artificially by *in vitro* assembly of variable genes with randomization in the CDR regions in order to obtain antibodies with high affinity. This library is also called single-pot library as they provide antibodies directed against multiple antigens (Hoogenboom *et al*., 1998; Benhar, 2001).

1.8.2.1 *Nkuku*® phage library

Rabbits (Ridder *et al*., 1995), cattle (O'Brien *et al*., 1999), camels (Ghahroudi *et al*., 1997), sheep (Li *et al*., 2000) and chickens (Davies *et al*., 1995) can be used as the source of antibodies (van Wyngaardt *et al*., 2004). For practical purposes, antibodies from chickens are preferred since it is easier to access the chicken antibody repertoire compared to the other antibody donors. All chicken variable regions have identical amino acid sequences at both ends, enabling the

naive antibody repertoire to be amplified using a single set of polymerase chain reaction (PCR) primers each, for the V_H and the V_L chain. Whereas, a number of PCR sets are required for the amplification of the V_H and V_L gene segments from the human or mouse antibody repertoire (van Wyngaardt *et al*., 2004). In addition, the production of mAbs against antigens that are highly conserved in mammalian species using the mammalian donors are difficult. Chicken is a favourable alternative for the production of these mAbs as they belong to the avian species and are different from mammals but still has similar immunological potency compared to the mammals (Yamanaka *et al*., 1996).

In mammalian species, diverse antibody library is created through somatic recombination of individual variable (V_H/V_L), diversity (D) and joining (J_H/J_L) gene segments for the heavy and light chains respectively. Both heavy and light chains of chicken Ig consist of a single functional variable region and joining region genes and are rearranged using conventional VDJ recombination method (Andris-Widhopf *et al*., 2000). Avian species uses an additional DNA recombination event called gene conversion to incorporate pseudo V region genes to create variability. The pseudo V regions are created in such a way that all V regions of the chicken Ig have identical ends, thus simplifying the amplification of the V region genes (Davies *et al*., 1995). The single variable genes in chicken Ig allows for simple antibody library construction, as only one set of primers are required for each antibody (Andris-Widhopf *et al*., 2000).

Davies *et al*. (1995) were the first to show the potential of chicken as a source of recombinant antibody fragments. They constructed a small naïve phage library and generated antibodies against three different proteins (Davies *et al*., 1995). The anti-R7V scFv gene fragment for this project is obtained from the chicken-based phage library, named *Nkuku*® phage library, constructed by Van Wyngaardt *et al*. (2004). The large non-immune combinatorial library constructed by Van Wyngaardt *et al*. (2004) yielded antibody fragments that recognize haptens, proteins and viruses. Antibodies produced using the *Nkuku*® library were used in enzyme linked immuno sorbent assay (ELISA) to detect African Horse sickness virus and blue tongue virus, demonstrating *Nkuku*® library as a potential source of *in vitro* diagnostic reagents (van Wyngaardt *et al*., 2004).

1.8.3 Phage display vectors

Proteins/peptides are displayed on the surface of the phage as fusions with the coat proteins, pIII or pVIII, using phage or phagemid vectors. A phage vector carries all the genetic information necessary for phage life. In a phage vector, a foreign gene is inserted directly into the phage

genome and proteins/peptides are displayed as fusion proteins with pIII or pVIII coat proteins. However, if the foreign gene disrupts the pIII or pVIII function, the phage becomes non-viable and does not display the foreign proteins. This problem is solved by the use of phagemids (Davies *et al*., 2000; Nilsson *et al*., 2000).

Phagemids are hybrids of the phage and plasmid vectors. Phagemid cloning vector contains an origin of replication (*ori*) for the filamentous (Ff) phage and *E.coli*, multiple cloning sites, gene III or gene VIII for fusion formation and an antibiotic resistance gene. In phagemid, the foreign gene is inserted into a separate plasmid that contains pIII or pVIII to display the foreign gene. They lack the other phage genes that are required for the production of a complete phage. The bacterium carries a helper phage to produce the complete phage particle. The helper phage (e.g. M13K07) contains a slightly defective *ori* and it provides all the structural proteins necessary for the production of a complete phage. This process is called phage rescue. Phagemids can be grown as plasmids in *E.coli* and packaged as recombinant Ff phage DNA with the aid of helper phage (Davies *et al*., 2000; Azzazy and Highsmith, 2002; Mullen *et al*., 2006).

1.8.4 Antibody selection from phage display libraries

The process of selection of antibodies from the phage libraries is called bio-panning. In bio panning, the phage displaying the scFv antibody fragment is incubated with immobilized antigens. The bound phages are eluted and amplified in *E.coli* while the unbound phages are washed away. This selection cycle is repeated 2 – 4 times for the enrichment of antigen-specific antibodies (Figure 6) (Azzazy and Highsmith, 2002).

There are a number of bio-panning selection strategies that can be adopted for the phage antibody selection. The different selection strategies are listed below.

1. Selection using immobilized antigens: In this case, the antigens are immobilized onto affinity columns, immunotubes, ELISA plates or chips of BIAcore sensors. The phage antibodies are passed over the immobilized antigens and the bound phages are eluted and amplified in *E.coli*. The bound phage antibodies can be eluted from their specific antigen with acidic (HCl or glycine buffer) solution, basic solution (triethylamine), enzymatic cleavage of the protease site or with excess antigen (Smith and Petrenko, 1997; Azzazy and Highsmith, 2002). The phage antibodies may not be able to recognize the native form of the adsorbed antigen which can be solved by indirect antigen coating using antigen-specific capture antibodies (Azzazy and Highsmith, 2002).

2. Selection using antigens in solution: This method overcomes the problem of conformational changes of the adsorbed antigens. Labelled soluble antigens are used for accurate quantification during selection. Phage antibodies are incubated with biotinylated antigen, and the bound phages are recovered with avidin or streptavidin-coated paramagnetic beads. Phages antibodies are then eluted from the antigen. A disadvantage of this method is the production of anti-streptavidin antibodies which can be removed by a depletion step using streptavidin-coated magnetic beads (Azzazy and Highsmith, 2002).

3. Selection on cells: This method involves direct selection of antibodies against cell surface markers on cells in suspension or monolayers. Unbound phages are washed away by centrifugation (cell suspension) or by washing the tissue culture flasks (monolayers) (Azzazy and Highsmith, 2002).

4. *In vivo* selection: In this method, the phage antibodies are injected into the animal (direct selection) and antibodies specific to certain cell markers are revealed (Azzazy and Highsmith, 2002).

1.8.5 Affinity maturation

Typically, antibodies selected from phage libraries are not suitable for direct therapeutic or reagent applications (Irving *et al*., 1996). High affinity antibodies are essential for the discovery and validation of biomarkers for human diseases, clinical diagnosis and therapeutic agents (Benhar, 2001). Affinity maturation involves the use of one or more low affinity clones to develop a secondary library from where higher affinity clones can be identified (Dall'Acqua and Carter, 1998). The affinity maturation method allows to understand the relationship between antigen-binding affinity and target localization *in vivo* (Dall'Acqua and Carter, 1998). Adams *et al*., (1998) showed that the specificity and extent of tumor localization was significantly enhanced by increasing the antigen-binding affinity (K_d) from 320 nM to 1 nM (Dall'Acqua and Carter, 1998; Adams *et al*., 1998). Random mutagenesis followed by selection using phage display provides an efficient method for affinity maturation of antibody fragments (Daugherty *et al*., 2000). Random mutation are introduced using different techniques such as DNA shuffling (Crameri *et al*., 1998), using a bacterial mutator strain (Low *et al*., 1996), error prone PCR (Cadwell and Joyce, 1992), site directed mutagenesis (Chowdhury and Pastan, 1999) and chain shuffling (Marks *et al*., 1992). The mutated genes are then expressed in a microbial host and functional clones with improved affinity are selected using phage display method (Daugherty *et al*., 2000).

1.8.5.1 Affinity maturation using Error Prone PCR

Error prone (EP) PCR mimics the *in vivo* process of somatic mutation that generates high affinity antibodies. Several groups have used EP PCR method to generate libraries with high mutation rate (Gram *et al*., 1992; Deng *et al*., 1994; Daugherty *et al*., 2000, Drummond *et al*., 2005; Depetris *et al*., 2008). Studies conducted by Daugherty *et al.* (2000) and Drummond *et al.* (2005) illustrate that an increase in mutation error rate is directly proportional to the generation of functional clones. Depetris *et al*. (2008) were able to produce a new antibody fragment with increased affinity and neutralizing activity against a wide variety of interferon alpha (IFN-α) subtypes using the standard error prone PCR method. In the study by Drummond *et al*. (2005), they show how to calculate the optimal EP PCR mutation rate for the generation of more improved and functional clones. They demonstrate that the optimal EP mutation rates are both protocol and protein-dependent (Drummond *et al*., 2005). Daugherty *et al*. (2000) quantitatively analyzed the effect of mutation frequency. They concluded that libraries with a moderate to high mutation error rate are effective for *in vitro* affinity maturation. High mutation frequency permits adjacent nucleotide substitution that result in amino acid change. However, two base changes in a single codon is rare (Daugherty *et al*., 2000). The advantage of using EP PCR method is that it does not require prior knowledge on the gene sequence and mutated clones with different affinities can be amplified in one PCR reaction.

1.8.5.2 Affinity maturation using chain shuffling

In chain shuffling, antigen binding specificity are increased by the recombining the V_H or the V_L chain from antibodies of defined specificity into a library of V_L and V_H chain, respectively (Kang *et al*., 1991). During shuffling, one of the V domains is kept unchanged. In light chain shuffling, the V_H gene region of the scFv molecule is recombined with the V_L domain of the phage library. The resulting scFv-phage library contains V_H gene region specific for the target antigen and random V_L chain domain. The new recombined library is then used for the selection and identification of clones with improved antigen-binding affinity(Azzazy and Highsmith, 2002). The opposite holds true for heavy chain shuffling. Azzazy and Highsmith (2002) were able to increase the affinity of human scFv against glycoprotein tumor antigen c-erbB-2 by 6-fold using the light chain shuffling method and by 5-fold using the heavy chain shuffling method (Azzazy and Highsmith, 2002). Affinity maturation is random but requires the prior knowledge of the gene sequence for functional recombination of V domains.

1.8.5.3 Affinity maturation using site directed mutagenesis

Site directed mutagenesis can be used to alter the nucleotide sequence of the desired gene using synthetic oligonucleotides (Carter, 1986). The amino acids in the CDR regions are replaced with other amino acids using mismatched oligonucleotides that will aid in improving the binding properties of the antibody. The mismatched oligonucleotides are used to create point mutation, multiple mutation, insertion or deletion (Carter, 1986; Azzazy and Highsmith, 2002). This method requires prior knowledge of the gene sequence, the molecular structure of antibody-antigen interaction and critical amino acid residues that favours a stronger binding affinity.

1.8.5.4 Affinity maturation using *E.coli* mutator strain

Affinity maturation using *E.coli* mutator strain allows for the introduction of random point mutations in a controllable manner and rapid selection of mutants with improved affinity or expression level. Specificity and rate of mutation is manipulated by the growth conditions (Coia *et al*., 2001). The *E.coli* mutD5 mutator strain is a conditional mutant and produces single point mutation at a rate 10^5 higher than the normal *E.coli* cells. The mutation rate of *E.coli* mutD5 is higher compared to other mutator stains such as XL-1 RED and mutT1. The advantage of using mutator cells is that the mutated clones do not need to be subcloned into a vector (Irving *et al*., 1996).

1.8.5.5 Affinity maturation using DNA shuffling

DNA shuffling involves random fragmentation of a pool of related genes and recombination in a self-priming polymerase reaction generating a library of chimeric genes. The reshuffled genes may produce antibodies with higher affinity. A study conducted by Crameri *et al*. (1998) showed that clones obtained from reshuffling a pool of four related genes yielded a 270 to 540-fold improvement as compared to the 8-fold improvement produced from clones obtained from reshuffling the four genes separately (Crameri *et al*., 1998).

1.9 Functional expression of antibody fragments in *Escherichia.coli (E.coli)*

E.coli is the primary microbial host that have been used for the production of recombinant therapeutic proteins (Andersen and Krummen, 2002). The protein expression in the bacterial system encounters problems such as intracellular protein stability, insolubility and cellular toxicity. However, the expression of recombinant proteins in *E.coli* using filamentous phage is an exception. Filamentous phage allows correct disulfide bond formation and expression of

functional antibody fragments because of the assembly of phage coat proteins on the inner bacterial membrane and the secretion of phage particles into the oxidizing periplasm (Pavoni *et al*., 2007).

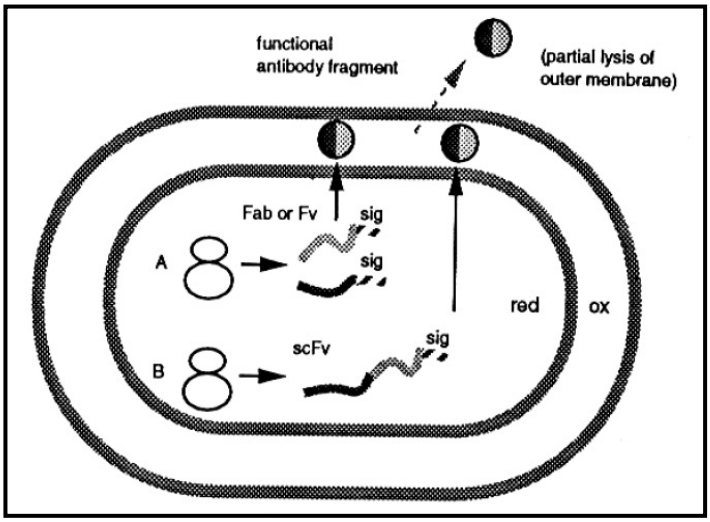


Figure 7: The expression of functional scFv fragment in *E.coli* Periplasm (Plückthun, 1994)

Recombinant antibodies and their fragments are obtained from *E.coli* either by producing inclusion bodies or by secreting the protein. The antibody fragments expressed in *E.coli* are usually secreted as soluble proteins into the bacterial periplasm. Bacterial periplasm is an oxidizing environment, containing enzymes that catalyze the formation and re-arrangement of disulfide bonds (Baneyx, 1999). *E.coli* contains secretion machinery that directs the protein, which carries a signal sequence, to the periplasm (Figure 7). The scFv fragments are targeted to the periplasm using secretion signals, *pel*B and *omp*A (Plückthun, 1994; Kortt *et al*., 2001).

Antibodies are secreted into the periplasm to avoid the reducing environment of the cytoplasm. The reducing environment of the cytoplasm prevents the disulfide bond formations, which are generally necessary for antibody stability. Periplasmic secretion of proteins allows for functional expression of a variety of antibody fragments and its production in sufficient amounts for studies (Plückthun, 1994). Soluble scFv molecules are produced by infecting a non-suppressor strain of *E.coli* (e.g. *E.coli* HB2151), since the bacterium can recognize the amber stop codon placed between the scFv gene and the gene3 (g3) in the phagemid vector and express the scFv without the g3-protein (g3p) (Azzazy and Highsmith, 2002).

For this study, the expression of scFv fragments are performed using two vector systems; recombinant pHEN1 and pAK400 vector system. Both *E.coli* TG1 and HB2151 strains were

used to express the protein. In the non-suppressor strain, *E.coli* HB2151, the amber stop codon is read as a stop codon and therefore a soluble scFv-tag fusion protein is produced. Whereas, in suppressor strain, *E.coli* TG1, the amber stop codon is read through and hence produces scFv-tag-g3p fusion protein. The expression of the scFv fragments are induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) and the soluble scFv antibody fragments are secreted into bacterial periplasm. The molecular weight of a scFv fragment is expected to be between 25 and 30 kDa (Huston *et al*., 1988; Adams *et al*., 1998; Azzazy and Highsmith, 2002).

The drawbacks of secretion in periplasm include insufficient secretion, misfolding of recombinant proteins or formation of inclusion bodies (Baneyx, 1999). The secretion of proteins cause stress in *E.coli*, which results in leakage of the outer membrane. The leakage depends on the temperature, primary sequence and type of the antibody fragment used. Growing bacterial cells at a low temperature (e.g. 25°C) can prevent leakage and formation of periplasmic inclusion bodies. The inclusion body formation is temperature-dependent and is induced at high temperatures (37°C or higher) (Plückthun, 1994).

1.10 Antibody-based Vaccine for HIV treatment

Virus-specific antibodies have the ability to block the entry of virus into cells and also promote antibody-directed cell mediated cytotoxicity (ADCC) (Ferrantelli and Ruprecht, 2002). The role of neutralizing antibodies (nAbs) in containing HIV infection has been under estimated due to several factors such as i) the decline in the peak viremia before the appearance of nAbs (Figure 8), ii) the efficacy of nAb in protection against different strains of HIV, since nAb responses are directed mainly against the highly variable loop of gp120, iii) the antibody neutralization induction limit due to trimeric structure and heavy glycosylation of the envelope spikes and iv) the high concentration of antibody titer required for controlling the HIV replication *in vivo* (Ferrantelli and Ruprecht, 2002). Nonetheless, passive immunization studies in primates showed that the human neutralizing monoclonal antibodies (hnmAbs) have the ability to contain HIV infection (Ferrantelli *et al*., 2002).

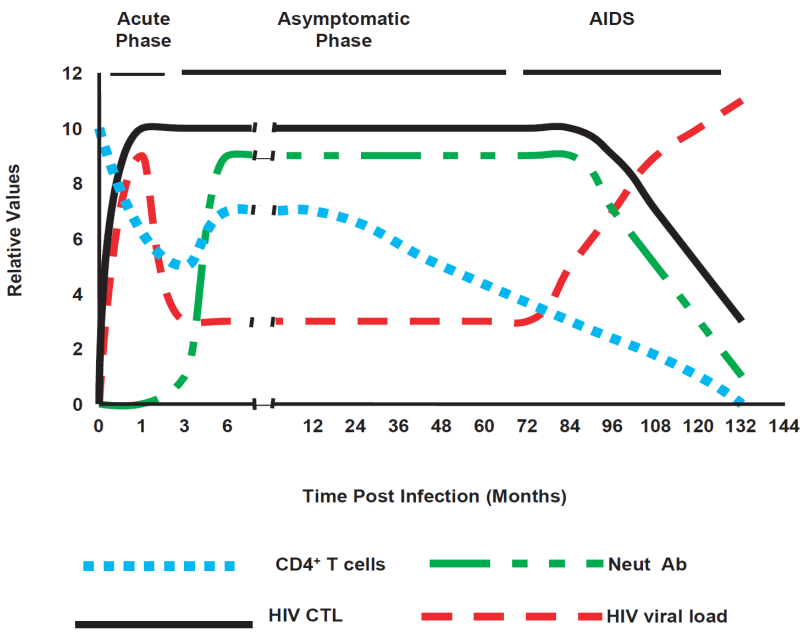


Figure 8: Time courses of viral load, CD4 count, CTL and nAb response during HIV infection and disease progression (Alimonti *et al*., 2003)

As from the above figure, it can be seen that n Ab responses are developed to suppress the HIV infection by lowering the viremia but only after a few months after infection. As the nAb response decreases, the viral load increases, which implies that the nAb influence the natural course of viral infection (Ferrantelli and Ruprecht, 2002). HIV infection also triggers the cytotoxic T lymphocyte (CTL) immune responses. The cytotoxic T lymphocytes (CTLs) help to eradicate the virus by lowering the rate of replication (Bangham and Phillips, 1997).

Neutralizing antibodies with right specificity and sufficient concentration evoke immune protection (Zolla-Pazner, 2004). The third hypervariable loop (V3) of the HIV gp120 subunit was identified as an important target of neutralization (Nunberg *et al*., 2000). Monoclonal antibodies targeted against the V3 loop were able to neutralize the HIV infectivity *in vitro*. The V3 loop of gp120 is highly variable and therefore neutralization was limited to related virus isolates. Also, the V3 loop specific mAbs were not effective against any primary HIV isolates, regardless of the relatedness. Neutralizing antibodies against different gp120 epitopes were also identified, but were effective only against laboratory strain viruses (Nunberg *et al*., 2000).

The complex structure of the gp120 envelope glycoprotein hinders the antibody from binding. The highly variable loops within the envelope subunits shield the important envelope epitopes from neutralizing antibodies. In addition, glycosylation and occlusion of gp120 subunit via

gp120 oligomerisation protect the epitopes from the antibodies. Also, the conformational change of gp120 during binding to the co-receptors constitutes an additional mechanism by which HIV escapes the antibody-mediated neutralization (Garber *et al*., 2004).

Despite the complexity of the HIV envelope glycoproteins, potent nAbs are present during the natural HIV infection. These mAbs have been characterized and were found to effectively target the conserved regions of the gp120 and gp41 subunits (Garber *et al*., 2004). Currently, there are only four broadly neutralizing human mAbs, namely - IgG1B12 and 2G12 (anti-gp120 antibodies), 2F5 and 4E10 (anti-gp41 antibodies). IgG1B12 is directed against the CD4 binding site of gp120 whereas 2G12 is raised against an epitope on gp120. Both 2F5 and 4E10 recognize a constant part of gp41 (Haslin *et al*., 2007).

IgG1B12 neutralizes primary HIV-1 isolates and is the only antibody that has access to the CD4 binding site on the viral envelope. This mAb has a CDR region 3 (CDR3) on the heavy chain that extends out like a finger. The N-linked glycan carbohydrate shield on gp120 protects the HIV from antibody recognition. The 2G12 mAb was shown to recognize these glycans exclusively (Burton *et al*., 2005).

The anti-gp41 antibody, 2F5 have broad neutralizing activity against diverse HIV-1 primary isolates. But 4E10 is the broadest neutralizing antibody as it is active against the diverse HIV-1 isolates with less potency than 2F5, 2G12 and IgG1B12 (Cardoso *et al*., 2005). The mAb 2F5 recognizes a 6 a.a core epitope (ELDKWA) (Zolla-Pazner, 2004). The mAb 4E10 recognizes a core epitope NWF(D/N)IT on gp41 adjacent to 2F5 epitope (Cardoso *et al*., 2005). Antibodies against this core epitope occur infrequently making it less immunogenic (Zolla-Pazner, 2004).

1.11 Anti-R7V Antibodies for HIV therapy

1.11.1 Beta-2 microglobulin

HIV is an intracellular pathogen as it acquires its envelope from the host cellular membrane through the process called budding. During this process, the virus non-randomly acquires and integrates some of the human cellular proteins such as beta-2 microglobulin (β2m), intercellular adhesion molecule 1 (ICAM1), human leukocyte antigen (HLA) class I and class II proteins and lymphocyte function-associated antigen 1 (LFA1), into their viral coat. These cellular antigens retain their functional activity and can affect the HIV infectivity and pathogenesis (Haslin and Chermann, 2002).

β2m is a cellular protein that belongs to major histocompatibility complex (MHC) class I molecules. MHC class I presents the antigen to CD8 cytotoxic cells, thereby playing a role in immune system. β2m is expressed in nucleated cells and all potential viral targets. The virus incorporates β2m into its surface envelope. Le Contel *et al.* (1996) showed that antibodies directed against β2m were able to neutralize HIV-1 virus indicating the presence of β2m on the surface of HIV virions (LeContel *et al.*, 1996). β2m is a highly conserved protein within the HIV species. Therefore, by targeting this cellular protein, the problem of variability of the viral glycoproteins can be solved. Anti- β2m mAbs have been shown to neutralize the virus and interfere in an early step of HIV replication (Haslin and Chermann, 2002).

The role of β2m as a potential vaccine target has been investigated (LeContel *et al.*, 1996). Anti-human β2m mAbs were used for the neutralization of different HIV-1 isolates. Overlapping 14 amino acid synthetic peptides from the human β2m sequence and 7 amino acid peptides derived from the overlapping synthetic peptides were used to determine which β2m epitope is responsible for the neutralization of HIV-1 virus. The studies showed that the 14 amino acid peptides were able to reverse the neutralization and they all share a common motif, PKI. The 7 amino acid peptides were shown to be more efficient, specifically the R7V, F7E and S7K peptides. Of these three peptides, R7V was found to be the most efficient in reversing the virus neutralization (LeContel *et al.*, 1996).

1.11.2 R7V epitope

R7V epitope was selected from a list of short, overlapping peptides derived from β2m as it was the most efficient epitope that was able to block the HIV neutralization when mixed with known nAbs (Haslin and Chermann, 2002). R7V epitope is found on the surface of HIV infected cells (Figure 9). This epitope is responsible for the production of protective antibodies that restrain the HIV positive individuals from progressing to AIDS. These protective antibodies are called the anti-R7V antibodies (Torphy, 2002). Studies conducted by other groups also revealed the presence of anti-R7V antibodies in HIV-infected patients (Galéa *et al.*, 1999ab; Haslin and Chermann, 2002; Sanchez *et al.*, 2008). Purified human anti-R7V antibodies were able to neutralize different types of HIV strains (primary HIV isolates, laboratory-based HIV strains, and HIV strains that are resistant to anti-retroviral drugs) (LeContel *et al.*, 1998). These studies conclude R7V epitope as a common neutralizing determinant found in all HIV strains. Antibodies against this epitope are naturally present in HIV infected people and contribute to the non-progression status making anti-R7V antibodies an efficient marker of HIV non-progression

(LeContel *et al*., 1998; Sanchez *et al*., 2008). Figure 10 is a side chain structure of a human β2m, drawn using the SWISS PDB Deep Viewer Software. The seven amino acids (RTPKIQV) that makes up the R7V epitope are highlighted in different colours.

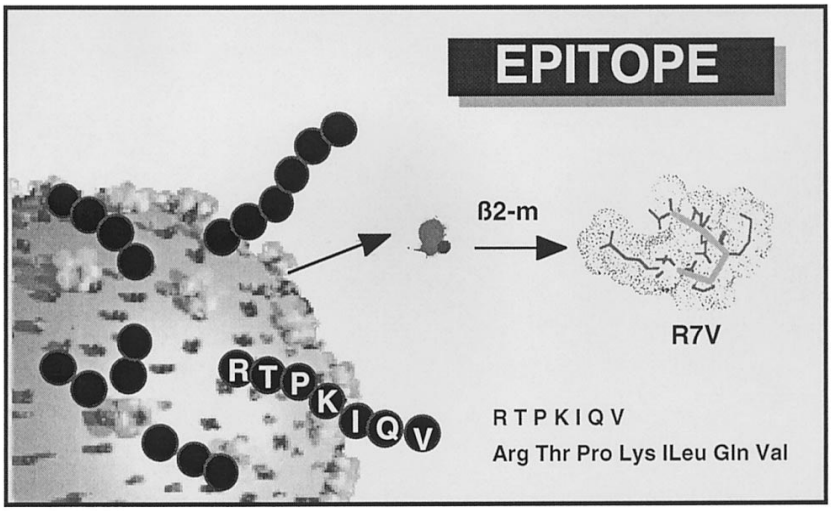


Figure 9: R7V epitope (Galéa *et al.*, 1999b)

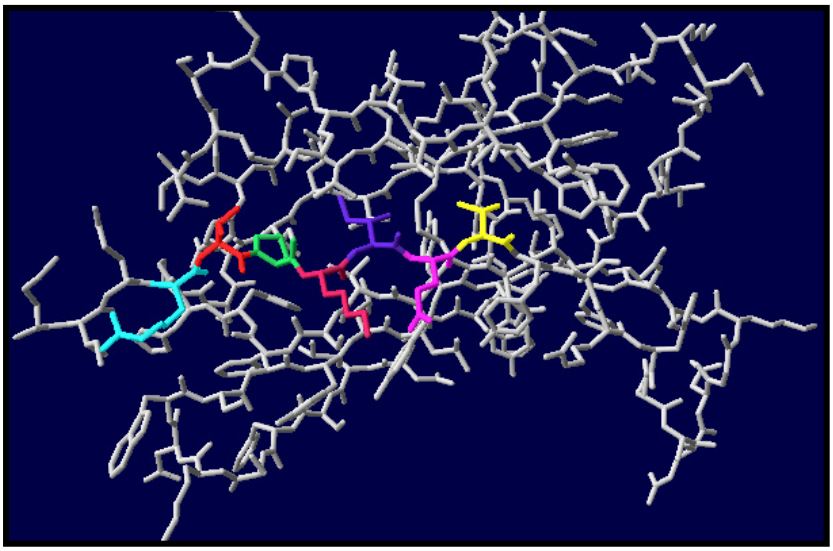


Figure 10: R7V epitope (RTPKIQV) in human β2m (PDB entry 1LDS) [Structure drawn using SWISS PDB DeepViewer 4.02]

1.12 Potential of R7V epitope as vaccine target

R7V peptide is present in all HIV viruses irrespective of their phenotype, genotype and tropism. R7V epitope is immunogenic *in vivo* and the antibodies targeted against this epitope do not cause autoimmune disease. All these characteristics make R7V epitope a good target as a vaccine against HIV (Galéa and Chermann, 1998; Galéa *et al*., 1999ab).

Some of the HIV-infected individuals do not develop AIDS and are called the non-progressors or late-progressors. Studies have suggested a relationship between the non-progression state and the presence of anti-R7V antibodies (LeContel *et al*., 1998) Anti-R7V monoclonal antibodies have the ability to neutralize the cell infection caused by the HIV. The production of anti-R7V antibodies could be a possible solution to fight HIV infection since purified anti-R7V antibodies were able to neutralize the different strains of HIV (Haslin and Chermann, 2007). Antibody-based therapy is being accepted, as several mAbs are currently being used as therapeutic agents or are in late-stage of clinical trials. Thus, R7V epitope represents an attractive target as a vaccine candidate to eliminate the HIV epidemic.

1.13 Motivation for the study

The anti-R7V antibodies have been produced by Haslin *et al*. (2007) in France, using the baculovirus expression system. The anti-R7V antibodies produced were able to neutralize the different HIV clades including subtype C. Anti-R7V antibodies were obtained from the B-cells of a non-progressor patient (Haslin *et al*., 2007).

In the study conducted by Bermanæs (2010), recombinant anti-R7V antibodies produced in our laboratory were used in an HIV-1 subtype C neutralization study. HIV-1 subtype C is the most commonly found HIV strain in South Africa (Karim and Karim, 2005). The recombinant anti-R7V antibodies were able to detect the R7V antigen in ELISA. However, the anti-R7V antibodies were not able to neutralize the HIV-1 subtype C virus (Bremnœs, MSc Thesis, 2010). The anti-R7V antibodies were obtained from the *Nkuku*® phage library (van Wyngaardt *et al*., 2004).

Antibodies obtained from some phage libraries have low affinity, making them unsuitable for direct therapeutic or diagnostic applications (Irving *et al*., 1996). The antigen-binding affinity of recombinant anti-R7V antibodies obtained from the *Nkuku*® phage library was low; hence they

were not able to neutralize the virus. This study thus focused on the affinity maturation of the anti-R7V antibodies in a HIV neutralization study.

1.14 Hypothesis

High affinity anti-R7V antibodies can be generated *in vitro* using EP PCR.

1.15 Aim

The aim of this project is to improve the affinity of an anti-R7V scFv antibody fragment for its possible future use in diagnostics and therapeutics.

In addition, to identify critical amino acids involved in the antigen-antibody interaction.

Chapter 2

Materials and Methods

2.1 Introduction

The anti-R7V scFv fragment was produced in our laboratory, using M13 phage display technology, from the chicken-based *Nkuku*® phage library. The *Nkuku*® library is a combinatorial phage display library. The combinatorial phage display libraries produces antibodies with high affinity that can be used in diagnostic and therapeutic studies (Rader and Barbas, 1997).

Some phage display libraries such as the phage library constructed by Vaughan *et al*. (1996) are able to produce scFv fragments with a high affinity as well as a biological activity (Vaughan *et al*., 1996; Dall'Acqua and Carter, 1998). Xie *et al.* (1997) were able to isolate 21 scFv fragments against the tyrosine kinase receptor, MuSK. Of these 21 scFv's, four bind MuSK with a high affinity (K_d < 10 nM) and activate the receptor. Affinity maturation is thus not required for scFv fragments isolated from this phage display library for *in vitro* applications (Xie *et al*., 1997; Dall'Acqua and Carter, 1998). However, most of the antibody fragments produced using phage display libraries has a lower affinity than required for *in vitro* or *in vivo* applications (Irving *et al*., 1996; Park *et al*., 2000). The equilibrium dissociation constant (K_d) of the binders are usually >100 nM. For research and therapy, antibody affinities in the lower nanomolar or sub nanomolar range are preferred (Thie, 2010).

The scFv fragments produced against the R7V epitope showed interaction but the antibody-antigen affinity was too weak for the neutralization assay. Hence, this project focused on the affinity maturation of the anti-R7V scFv fragment through random mutagenesis using an EP-PCR method.

The most common technique used for random mutagenesis is the EP PCR method (Martineau, 2002). The binding affinity of the antibody gene can be determined using the crude cell supernatants in ELISA (Friguet *et al*., 1985). The production of mAbs are performed in microtiter plates (Konthur *et al*., 2005; Hust *et al*., 2009). The antigen-antibody affinity can also be determined by kinetic analysis using surface plasmon resonance (SPR) (Wassaf *et al*., 2006) and the specificity can be determined using flow cytometry (Lou and Marks, 2010).

EP PCR introduces random point mutations in the DNA sequence using low fidelity *Taq* DNA polymerase. *Taq* DNA polymerase lacks 3′ - 5′ proofreading exonuclease activity. Thus, produces mutations greater than $5x10^{-3}$/base/PCR cycle. *Taq* DNA polymerase enzyme combined with other non-standard PCR conditions such as addition of manganese ions and the use of unequal concentrations of dNTPs, further enhances the mutation frequency to $9.8x10^{-3}$ mutation/base pair/PCR cycle (Wark and Hudson, 2006). The advantage of using EP PCR is that antibodies with different affinities are amplified in one PCR reaction.

For this study, the scFv fragments were expressed in small quantities using *E.coli* as the host organism. Both phage (pAK400) and phagemid (recombinant pHEN1 and pGE20) vector systems were used for the expression of the scFv antibody fragment.

The phagemid vector, pHEN1 is based on the vector pUC119 and contains unique restriction sites, *Sfi*I and *Not*I, for the cloning of the antibody fragment gene (Figure 11). The lacZ promoter induces transcription and the antibody-g3p fusion protein is targeted to the periplasm using the pelB leader sequence. A c-myc peptide tag and amber codon is introduced between the antibody and g3p, which allows production of antibody either for phage display by growth in suppressor strains of *E.coli* such as *E.coli* TG1 or as tagged soluble fragments by growth in non-suppressor strains such as *E.coli* HB2151. The pHEN1 phagemid vector contains an ampicillin resistance gene (amp^R) to distinguish between transformed and non-transformed cells (Hoogenboom *et al*., 1991).

The phagemid vector, pGE20 is a modified expression vector obtained from the combined λHC4/λC3 bacteriophage lambda vector system. PelB secretion signal sequence allows for the secretion of cloned antibody fragment into the bacterial periplasm. Insertional inactivation of the *lacZ′* gene allows for blue-white screening between non-recombinant and recombinant constructs. A 15 amino acid tag is introduced at the C-terminal of the antibody-coding region followed by two stop codons for protein purification. The pGE20 phagemid vector also contains ampicillin resistance gene (amp^R) for the selection of transformed cells (Orfanoudakis *et al*., 1993).

The phage vector, pAK400 is an optimized expression vector, which uses rare cutting *Sfi*I restriction enzyme for directional cloning of antibody fragments. The phage vector contains a tetracycline resistance (tet^R) stuffer cassette to monitor complete *Sfi*I digestion and selection of transformed cells, a chloramphenicol resistance (cam^R) cassette as a selective marker to distinguish between the recombinant and non-recombinant plasmids. The tetracycline resistance

(tet^R) stuffer cassette will be replaced by the antibody fragment gene sequence. The expression of cloned antibody genes are enhanced by replacing the Shine Dalgarno (SD) sequence with the stronger SDT7g10 (Shine Dalgarno sequence of T7 phage gene 10). A 6xHIS peptide tag is introduced at the C-terminal end of the antibody for purification as well as detection of the antibody using anti-HIS tag antibodies (Krebber *et al*., 1997).

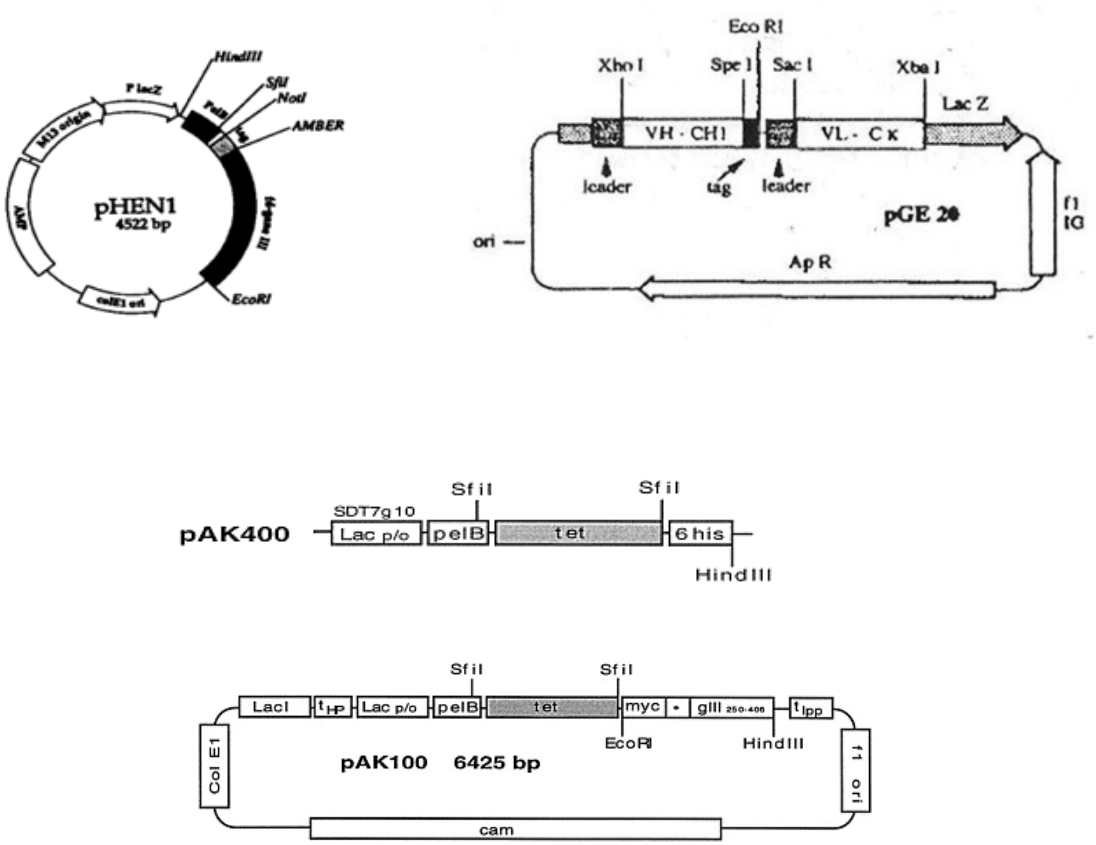


Figure 11: Phage and phagemid vectors used for expression of anti-R7V scFv fragments in *E.coli*
A. pHEN1 phagemid vector (Hoogenboom *et al.*, 1991)
B. pGE20 phagemid vector (Orfanoudakis *et al.*, 1993)
C. pAK100 and pAK400 vector series (Krebber *et al.*, 1997)

2.2 Experimental Methods

2.2.1 Production of recombinant anti-R7V antibody fragment

The anti-R7V antibody fragment was selected from the chicken-derived *Nkuku*® phage library (van Wyngaardt *et al*., 2004). The *Nkuku*® phage library comprising of combinatorial pairings of chicken V_H and V_L chain was screened for binding to the synthetic R7V peptide (GenScript Corporation, Piscataway, USA). The scFv fragments that recognized the R7V peptide were selected using M13 phage display technology. The scFv fragments obtained were named recombinant anti-R7V antibody fragments.

2.2.2 Preparation of $CaCl_2$ competent *Escherichia coli* (*E.coli)* cells

Pre-culture conditions for *E.coli* cells:

A glycerol stock of individual *E.coli* strains taken from -70°C was placed on ice and left to thaw gently. The culture was streaked onto a petri-dish containing Luria-Bertani agar (LB-agar) (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% w/v agar) medium, using a sterile inoculation loop. The cells were incubated overnight at 37°C.

Preparation of $CaCl_2$ competent *E.coli* cells:

A single colony was picked from an overnight plate and inoculated in 10 ml LB broth (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl), and grown overnight at 37°C in a shaking incubator at 200 rpm. From the overnight culture, 1 ml was inoculated into fresh 100 ml of LB broth and incubated at 37°C with shaking at 200 rpm, until the culture reached an optical density (OD_{600}) value of 0.6.

All the centrifuge tubes, eppendorf tubes and reagents were kept ice-cold to ensure efficient preparation of competent cells. Once the culture reached an OD_{600} value of 0.6, the cells were transferred to two clean sterile pre-chilled 50 ml centrifuge tubes and placed on ice for 10 min. The cells were then centrifuged at 4648 x g for 8 min at 4°C (Beckman, Avanti J-25 centrifuge, California, USA). The supernatant was discarded. Each cell pellet was re-suspended in 25 ml of ice-cold 0.1 M $CaCl_2$ and combined into a single tube. The re-suspended cell pellets were centrifuged at 4648 x g for 8 min at 4°C. The supernatant was discarded. The cell pellet was re-suspended in 50 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 4 - 6 hr. After incubation, the cell suspension was centrifuged at 4648 x g for 8 min at 4°C. The cell pellet was re-suspended in 10 ml of ice-cold 0.1 M $CaCl_2$ containing glycerol at a final concentration of 14%.

Cells were aliquoted (0.2 ml each) into sterile 1.5 ml eppendorf tubes and stored at -70°C (Ausubel, 1989; Schmauder, 1997) until needed.

2.2.3 Transformation using $CaCl_2$ competent *Escherichia coli (E.coli)* cells

Plasmid DNA (10 ng plasmid DNA) was added to $CaCl_2$ competent *E.coli* cells (50 µl) that have been thawed on ice. The solution was mixed gently and placed on ice for a further 30 min. The cells were then heat shocked at 42°C in a water bath for 2 min, and rapidly transferred onto ice for 1 – 2 min. Pre-warmed (37°C) LB broth (1 ml) was then added to the cells and incubated at 37°C in a water bath for 1 hr. The transformation mix (100 µl) was spread under aseptic conditions onto LB–agar plates containing the appropriate antibiotic [for pHEN1 and pGE20 vectors: ampicillin (100 µg/ml), for pAK400 vector: chloramphenicol (25 µg/ml)] and incubated overnight at 37°C (Sambrook *et al*., 1989)

2.2.4 Preparation of electrocompetent *Escherichia coli (E.coli)* cells

A single colony of *E.coli* cells was picked from an overnight plate and inoculated in 10 ml LB broth (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl), and grown overnight at 37°C with shaking (200 rpm). From the overnight culture, 2x 5 ml was inoculated into fresh 2x 500 ml pre-warmed LB broth and incubated at 37°C with shaking at 200 rpm, until the culture reached an optical density (OD_{600}) value of 0.6.

All the centrifuge bottles, eppendorf tubes and reagents were kept ice-cold to ensure efficient preparation of competent cells. Once the culture reached an OD_{600} value of 0.6, the cells were transferred to clean pre-chilled centrifuge bottles and placed on ice for 20 min. The cells were then centrifuged at 4648 x g for 10 min at 4°C (Beckman, Avanti J-25 centrifuge, California, USA). The supernatant was discarded. Pellets were kept on ice. Each cell pellet was re-suspended in 10 ml of ice-cold $dddH_2O$ with swirling. Another 240 ml of ice-cold $dddH_2O$ was added to the cell pellet and mixed gently. The cells were centrifuged at 4648 x g for 10 min at 4°C. The supernatant was discarded. The washing step was repeated by the addition of 240 ml of ice-cold $dddH_2O$. The cells were centrifuged as before. Each cell pellet was re-suspended in 10% glycerol (10 ml), transferred to pre-chilled 50 ml centrifuge tubes, and placed on ice for 60 min. The cells were centrifuged at 4648 x g for 10 min at 4°C. The supernatant was discarded. The final cell pellet was re-suspended in 500 µl of 10% glycerol. Resuspended pellets were pooled together into a pre-chilled 1.5 ml eppendorf tube. Cells were aliquoted (90 µl each) into sterile ice-cold 1.5 ml eppendorf tubes and stored at -70°C (Maritz-Olivier, 2005).

2.2.5 Transformation using electrocompetent *Escherichia coli (E.coli)* cells

Plasmid DNA (10 ng) was added to 90 µl electrocompetent *E.coli* cells that have been left to thaw on ice. The solution was mixed gently with a pipette tip. The 100 µl mixture was transferred into pre-chilled (at -20°C for 1 hr) Bio-Rad Gene-Pulser® Cuvette (Bio-Rad Laboratories, China) and electroporated (Electroporator 2510, Eppendorf, Germany) at 2000 V. Pre-warmed (37°C) LB-glucose medium (100 µl) was added to the electroporated cells in the cuvette and combined with the rest of the LB glucose medium (900 µl) in a 1.5 ml eppendorf tube. The cells were incubated for 1 hr at 37°C with shaking. The transformation mix (100 µl of 1:100, 1:50, 1:10 dilutions using LB-glucose medium) was spread under aseptic conditions onto LB–agar plates containing the appropriate antibiotic [for pHEN1 and pGE20 vectors: ampicillin (100 µg/ml), for pAK400 plasmid DNA: chloramphenicol (25 µg/ml)] and incubated overnight at 37°C.

2.2.6 Plasmid DNA isolation

A NucleoSpin® Plasmid Kit (Macherey-Nagel, Germany) or Zyppy™ Plasmid Miniprep Kit (Zymo Research, USA) was used to obtain pure plasmid DNA for further analysis. The principle for isolation of DNA is based on the traditional alkaline lysis method. With the NucleoSpin® Plasmid Kit, total cellular DNA is released from the bacterial cells using SDS/alkaline lysis method. RNA is removed by the addition of RNAse. Genomic DNA and protein are precipitated under high salt, low pH buffer conditions whereas plasmid DNA remains in solution. The genomic DNA together with the proteins and other cellular debris is removed by centrifugation. Plasmid DNA binds to the silica membrane in the presence of chaotropic salt guanidine-HCl. Contaminants like salts, metabolites and other soluble cellular components are removed by a simple wash step. Pure plasmid DNA is eluted under low ionic strength conditions. The protocol used for the plasmid DNA isolation using NucleoSpin® Plasmid Kit is explained below.

A single colony from the transformed plate was inoculated into 3 ml of LB broth supplemented with appropriate antibiotic [for pHEN1 and pGE20 vector: ampicillin (100 µg/ml), for pAK400 plasmid DNA: chloramphenicol (25 µg/ml)]. The culture was incubated overnight at 37°C with shaking at 250 rpm. Cells were collected from the overnight culture by centrifugation at 11 000 x g for 30 s in a standard benchtop microcentrifuge (MiniStar Plus, Super Mini Centrifuge, China). The supernatant was discarded. The cell pellet was re-suspended in 250 µl resuspension buffer A1 by thoroughly mixing the solution using a pipette. Lysis buffer A2 (250 µl) was added to the resuspension and mixed by inverting the tube 4 – 6 times for cell lysis. The cells were

incubated at room temperature for 5 min or until the lysate appeared clear. Neutralization buffer A3 (300 µl) was then added to the cells and mixed by inverting the tube 6 – 8 times. To ensure complete neutralization the tubes were inverted another 3 – 4 times. The cell debris was removed by centrifugation at 11 000 x g for 5 min. The supernatant was transferred to a NucleoSpin® plasmid column, placed in a 2 ml collection tube, and centrifuged for 1 min at 11 000 x g. The flow through was discarded. The DNA bound to the silica membrane of the column was washed with 500 µl wash buffer AW (pre heated to 50°C) and the column was centrifuged at 11 000 x g for 1 min. The washing step was repeated with 600 µl wash buffer A4 and the column was centrifuged at 11 000 x g for 1 min. The flow through was discarded. The column was centrifuged at 11 000 x g for 2 min to remove any residual buffer and to dry the silica membrane. The NucleoSpin® plasmid column was then placed in a sterile 1.5 ml microcentrifuge tube and 50 µl elution buffer AE (5 mM Tris-HCl, pH 8.5) was added to the silica membrane. The column was incubated for 2 min at room temperature and the bound DNA was eluted by centrifugation at 11 000 x g for 1 min. The concentration and purity of the eluted plasmid DNA was determined using a Nanodrop ND-1000 spectrophotometer (Inqaba Biotec, SA).

Zyppy™ Plasmid Miniprep Kit is also based on the traditional alkaline lysis method of plasmid DNA isolation but bypasses the bacterial culture centrifugation and resuspension steps. A single colony from the transformed plate was inoculated into 3 ml of 2xTY broth supplemented with the appropriate antibiotic [for pHEN1 and pGE20 vector: ampicillin (100 µg/ml), for pAK400 plasmid DNA: chloramphenicol (25 µg/ml)] and incubated overnight at 37°C with shaking (200 rpm). Cells were collected from the overnight culture by centrifugation at 11 000 x g for 30 s in a standard benchtop microcentrifuge (MiniStar Plus, Super Mini Centrifuge, China). The supernatant was discarded. The cell pellet was resuspended in 600 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA) buffer. The 7x lysis buffer (100 µl) was added to the resuspension and mixed thoroughly by inverting the tube 4 – 6 times. The solution change from opaque to clear blue indicates complete lysis. The next step was performed within 2 min. The cold neutralization buffer (350 µl) was then added to the cells and mixed thoroughly by inverting the tube 6 – 8 times. The formation of a yellow precipitate indicates complete neutralization. The tube was inverted an additional 2 – 3 times to ensure complete neutralization. The cell debris was removed by centrifugation at 11 000 x g for 4 min. The supernatant (~900 µl) was transferred to a Zymo Spin II column, placed in a 2 ml collection tube, without disturbing the cell debris and centrifuged at 11 000 x g for 20 sec. The flow-through was discarded and the column was

placed back into the same collection tube. Endo-wash buffer (200 µl) was added to the column and centrifuged at 11 000 x g for 20 sec. Zyppy wash buffer (400 µl) was then added to the column and centrifuged at 11 000 x g for 30 sec. The Zymo Spin II column was then placed in a sterile 1.5 ml microcentrifuge tube and 30 µl of Zyppy elution buffer (10 mM Tris-HCl, pH 8.5 and 0.1 mM EDTA), pre-warmed to 50°C, was added to the silica membrane. The columns were left to stand for 2 min at room temperature. The column was centrifuged at 11 000 x g for 20 sec to elute the bound DNA. Nanodrop ND-1000 spectrophotometer (Inqaba Biotec, SA) was used to determine the purity and yield of the isolated plasmid DNA. Agarose gel electrophoresis was used for the visualization of the plasmid DNA.

2.2.7 DNA concentration determination

The Nanodrop ND-1000 spectrophotometer (Inqaba Biotec, SA) was used to measure the concentration of DNA in ng/µl. The nanodrop pedestal was first cleaned with 1 µl dddH_2O. Elution buffer (1 µl), used to elute the DNA, was used as the blank. The concentration of DNA was determined by loading 1 µl of the sample to the pedestal.

2.2.8 Agarose gel electrophoresis

Plasmid DNA and PCR DNA products were analyzed on 1% (w/v) agarose gels. Agarose gel was prepared by adding agarose in 1X TAE (0.04 M Tris-acetate, 1 mM EDTA, pH 8) buffer. The agarose (Promega, USA) was melted by boiling the solution in a microwave. Once the agarose was dissolved completely, the solution was allowed to cool down to 50°C. Ethidium bromide (EtBr) was then added to the solution at a final concentration of 10 µg/ml and mixed thoroughly. The gel was casted in a casting tray and allowed to set for 30 min. The samples (approximately 0.72 µg, 10 - 30 µl each), containing 1X Orange DNA loading dye (Fermentas, USA) were then loaded onto the wells and the gel was run for 5.8 V/cm. O'Gene Ruler 1 kb DNA ladder (Fermentas, USA) was used as the molecular marker. The DNA bands were visualized under UV light at 312 nm, with the use of a Spectroline TC-312 AV transilluminator (Spectronics Corporation, USA). DNA gel pictures were captured using a CCD camera coupled to IC Capture Software (The Imaging Source Europe, Germany). The DNA gel pictures shown in this dissertation are presented as negative images.

2.2.9 Conventional PCR of recombinant pHEN1 phagemid DNA

Recombinant pHEN1 phagemid DNA was used as template DNA. PCR primers, Sfi1L and LCNOT1 (van Wyngaardt *et al*., 2004) (Table 1) were used for the amplification of the anti-R7V

scFv gene from the template DNA. PCR reaction mixture contained 1X *Taq* reaction buffer, complete (Jena Bioscience, Germany), 100 – 250 ng template DNA, 0.2 µM PCR primers (Inqaba Biotec, SA), 200 µM dNTP mix (Jena Bioscience, Germany), 2.5 U *Taq* DNA polymerase (Jena Bioscience, Germany) and water to a final reaction volume of 50 µl (Table 2). PCR was performed in a thermal cycler 2720 (PE Applied Biosystems, USA), using the following amplification reaction steps: Pre-heating of 1 min at 94°C, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, respectively and a final extension step of 72°C for 5 min (van Wyngaardt *et al*., 2004) (Table 3). The amplified DNA product was analyzed on a 1% (w/v) EtBr-stained agarose gel.

Table 1: Primers used for amplifying scFv gene from recombinant pHEN1 phagemid DNA

Primer	**Sequence (5′ – 3′)**
Sfi1L	GTCCTCGCAACTGCGGCCCAGCCGGCCCTGATGGCGGCCGTGACG
LCNOT1	TGATGGTGGCGGCCGCATTGGGCTG
pAKrev	TGGGCCCCCGAGGCCGCATTGGGCTG (own design)
HENUNIF1	CTGATGGCGGCCGTGACG (own design)
HENUNIR1	GAATTCATTGGGCTGGCCTAG (own design)

* Restriction enzyme sitesare underlined

Table 2: Reaction components for conventional PCR reaction

Reagent	**Concentration**
10X Taq reaction buffer complete	1x
Template DNA	100 - 250 ng
Forward Primer (Sfi1L)	0.2 µm
Reverse Primer (LCNOT1)	0.2 µm
dNTPs mix	200 µm
Taq DNA Polymerase (1 U/µl)	2.5 U
Final reaction volume (adjusted with water)	50 µl

Table 3: PCR cycling conditions for the amplification of scFv genes from recombinant pHEN I vector.

	Step	Temperature and Time
	Initial Denaturation	94°C for 1 min
25 cycles of	Denaturation	94°C for 1 min
	Annealing	60°C for 1 min
	Extension	72°C for 2 min
	Final Extension	72°C for 5 min

2.2.10 Purification of PCR amplicons

The NucleoSpin® Extract II purification kit (Macherey-Nagel, Germany) was used for the purification of amplified PCR products. The NucleoSpin® Extract II method is based on the binding of DNA to the silica membrane on the NucleoSpin® Extract II column, in the presence of chaotropic salts added by the binding buffer NT. Ethanolic wash buffer NT3 is used to remove the contaminants and pure DNA is eluted under low ionic strength conditions, using slightly alkaline elution buffer NE (5 mM Tris-HCl, pH 8.5). The protocol for PCR clean up provided in the user manual was followed with modifications.

For each volume of PCR sample, two volumes of binding buffer NT were added (1:2). Alternatively, if the DNA is purified from an agarose gel, 200 µl binding buffer NT was added for each 100 mg of agarose gel slice. The DNA band was excised from the agarose gel using a clean scalpel and the weight of the gel slice was determined. The gel slice was completely dissolved by incubating the sample at 50°C for 10 min.

The sample was then loaded onto NucleoSpin® Extract II column, placed in 2 ml collection tube, and centrifuged at 11 000 x g for 1 min. The flow through was discarded. Wash buffer NT3 (600 µl) was added to the column and centrifuged at 11 000 x g for 1 min. The flow through was discarded. To prevent carry-over of chaotropic salts, an additional 250 µl wash buffer NT3 was added to the column and centrifuged at 11 000 x g for 1 min. The residual ethanol from buffer NT3 was removed by centrifugation at 11 000 x g for 5 min. The column was then placed in a sterile 1.5 ml microcentrifuge tube and 50 µl elution buffer NE was added and the column was incubated for 2 min at room temperature to increase the yield of eluted DNA. The bound DNA

was eluted by centrifugation at 11 000 x g for 1 min. Nanodrop instrument (Inqaba Biotec, SA) was used to determine the concentration and purity of the eluted DNA.

2.2.11 Restriction enzyme digestion of recombinant pHEN1 phagemid DNA

The recombinant pHEN1 phagemid DNA was digested using *Sfi*I and *Not*I enzymes. The optimal activity of each enzyme is at different temperatures. The plasmid DNA is first digested with the one enzyme that requires a lower temperature and subsequently digested with the other enzyme that requires higher optimal temperature. The recombinant pHEN1 plasmid DNA was therefore digested first with *Not*I at 37°C in a water bath for 5 min and then with *Sfi*I at 50°C in a water bath for 15 min.

Table 4: Fast digest (FD) *Sfi*I and *Not*I restriction enzyme reaction set up for single and double digestion of recombinant pHEN1 DNA

Reagent	Reactions			
	Uncut DNA	**Not1**	***Sfi*I**	***Not*I+*Sfi*I**
DNA (µg)	0.72	0.72	0.72	0.72
10X FD buffer	1X	1X	1X	1X
FD *Not*I (1 U)	0	1	0	1
FD *Sfi*I (1 U)	0	0	1	1
Final reaction volume (µl) (adjusted with sterile $dddH_2O$)	20	20	20	20

*on left column, brackets are showing units; *FD – Fast digest

2.2.12 Sub cloning of anti-R7V scFv gene into pGE20 phagemid DNA

The anti-R7V scFv gene was inserted within the *Xho*I and *Eco*RI restriction enzyme sites of the pGE20 phagemid vector. The anti-R7V scFv gene was amplified from recombinant pHEN1 phagemid DNA using primers, HENUNIF1 and HENUNIR1 (Table 1) using the same PCR cycling conditions as mentioned above (Table 3). HENUNIR1 primer had an *Eco*RI recognition site at its 3′ end and HENUNIF1 primer had a blunt end at its 5′ end to allow for ligation into

*Xho*I/*Eco*RI digested pGE20 vector DNA. Agarose gel (1%, w/v) electrophoresis was performed to analyse the amplified PCR DNA.

Since the insert gene has an internal *Xho*I restriction enzyme site, the *Xho*I restriction enzyme site on the vector was removed by digesting the vector DNA first with fast digest *Xho*I restriction enzyme (1 U, Fermentas, USA) at 37°C for 1 hr. The *Xho*I digested pGE20 vector DNA was purified from the gel, followed by the addition of a Klenow polymerase enzyme (5 U, Fermentas, USA) at 37°C for 2 hr to produce a 5′ blunt end. Both anti-R7V PCR gene fragment (0.2 µg) and the purified 5′ blunt end pGE20 vector DNA (0.8 µg) were then digested with fast digest *Eco*RI enzyme (Fermentas, USA) at 37°C for 5 min and purified prior to ligation. A 1:3 vector:insert ratio was used for the ligation of digested vector and insert DNA. The 20 µl ligation reaction contained 100 ng vector DNA, 71 ng insert DNA, 1X T4 DNA ligase buffer and 3 Weiss units T4 DNA ligase (Promega, USA). The ligation reactions were incubated overnight at 4°C. The ligation mix (20 µl) was used to transform competent *E.coli* XL1-blue cells as described in section 2.2.3. The transformation mix (200 µl) was plated onto LB-agar (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% w/v agar) plates supplemented with ampicillin (100 µg/ml), isopropyl-beta-D-thiogalactopyranoside (IPTG) (0.2 mM) and X-gal (0.04 mg/ml) and incubated overnight at 37°C. The blue-white screening method allowed selection of transformants with recombinant plasmid DNA. The recombinant phagemid DNA, pGR7V2 was isolated using the Zyppy™ Miniprep Kit. The presence of the anti-R7V insert gene was confirmed using conventional PCR with HENUNIF1 and HENUNIR1 primers (Table 1) and using sequence analysis with the M13 reverse primer (Table 11).

To assist in the protein protein purification, a 6xHIS tag was inserted between the *Eco*RI and *Xba*I restriction enzyme sitesof pGE20 vector. For the construction of the HIS tag, complementary single stranded oligonucleotides were designed (Table 6). One oligonucleotide had a 5′ *Eco*RI and the second one had a 3′ *Xba*I restriction enzyme site. To generate a double stranded fragment, both complementary oligonucleotides were resuspended at equimolar concentration using oligo-annealing buffer (10 mM Tris, pH 7.8, 50 mM NaCl and 1 mM EDTA). The annealing of the complementary oligos took place by placing the reaction tube at 95°C for 4 min in a heat block. The tube containing the annealing reaction (along with the beaker of water) was removed from the heat block and allowed to cool down to room temperature for the single stranded oligos to anneal (Sigma-Aldrich, USA and KochLab Protocols).

A 1:6 vector to insert molar ratio was used for the ligation of vector and insert DNA. The 10 µl ligation reaction contained 100 ng vector DNA, 6 ng insert DNA, 1X T4 DNA ligase buffer and 3 Weiss units T4 DNA ligase (Promega, USA). The ligation reactions were incubated overnight at 4°C. The ligated DNA was transformed into $CaCl_2$ competent *E.coli* XL1-blue cells and plated onto LB-agar (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% w/v agar) plates supplemented with amp (100 µg/ml), IPTG (0.2 mM) and X-gal (0.04 mg/ml) for blue-white screening of transformants. The recombinant phagemid DNA, pGHE20 was isolated using Zyppy™ Miniprep Kit.

Table 5: Reaction composition for the construction of the HIS tag fragment

Reagent	Concentration
His-*Eco*RI	25 µM
His-*Xba*I	25 µM
10x Oligo Annealing buffer	2 µl
Final reaction volume (adjusted with sterile $dddH_2O$)	20 µl

Table 6: DNA sequence of the oligonucleotide primers designed for the HIS tag fragment

Primer	Sequence (5′ – 3′)
His-*Eco*RI	AATTCCATCATCATCATCATTAAG
His-*Xba*I	GGTAGTAGTAGTAGTAATTCAGATC

2.2.13 Sub cloning of anti-R7V scFv gene into pAK400 phage DNA

The anti-R7V scFv gene was amplified from recombinant pHEN1 phagemid DNA using gene specific primers, Sfi1L (van Wyngaardt *et al*., 2004) and pAKrev (Table 1). Gradient (touch down PCR) PCR (thermal cycler, Bio-Rad) with annealing temperature ranging from 60 to 69°C was performed to determine the optimum annealing temperature. Sequence of the amplicons at the different annealing temperatures was analyzed.

The optimum annealing temperature was determined to be 69°C. The PCR cycling conditions for this reaction were as follows: Pre-heating of 1 min at 94°C, followed by 25 cycles of 94°C for 1 min, 69°C for 1 min and 72°C for 2 min, respectively and a final extension step of 72°C for 5 min. The Sfi1L primer has a *Sfi*I recognition site at its 5′ end and the pAKrev primer were designed with *Sfi*I recognition site at its 3′ end. Agarose gel (1%, w/v) electrophoresis was performed to analyze the amplicons.

Both the anti-R7V PCR gene fragment (0.2 µg) and the pAK400 vector DNA (0.8 µg) were digested with conventional *Sfi*I (Fermentas, USA) restriction enzyme (1 U) at 50°C for 4 hr to produce cohesive ends required for ligation. A 1:3 vector to insert molar ratio was used for the ligation of digested vector and insert DNA. The 20 µl ligation reaction contained 100 ng vector DNA, 35 ng insert DNA, 1X T4 DNA ligase buffer and 3 Weiss units T4 DNA ligase (Promega, USA). The ligation reactions were incubated overnight at 4°C. The ligated DNA (20 µl) was transformed into electrocompetent *E.coli* HB2151 cells as described in section 2.2.5. The transformation mix (200 µl) was plated onto 2X TY-agar (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% w/v NaCl and 1.4% w/v agar) plates supplemented with chloramphenicol and glucose at a final concentration of 25 µg/ml and 1% (w/v), respectively and incubated overnight at 37°C. The plasmid DNA was isolated using the NucleoSpin® plasmid kit (Macherey-Nagel, Germany). The presence of the anti-R7V gene insert was confirmed using conventional PCR with Sfi1L and pAKrev primers (Table 1) and using sequence analysis with pAKseqF and pAKseqR primers (Table 11).

2.2.14 Error Prone PCR of anti-R7V scFv gene

Conventional PCR anti-R7V scFv amplicons was used as template DNA. PCR primers, Sfi1L and pAKrev (Table 1) were used for the amplification of the scFv gene from recombinant pAK400 phage DNA. Mutagenic PCR reaction mixture contained 1X *Taq* reaction buffer, incomplete (Jena Bioscience, Germany), 7 mM $MgCl_2$, 0.5 ng template DNA, 0.2 µM PCR primers (Inqaba Biotec, SA), 0.2 mM dATP, 0.2 mM dGTP, 1 mM dTTP and 1mM dCTP (Jena Bioscience, Germany), 0.5 mM $MnCl_2$, 5 U *Taq* DNA polymerase (Jena Bioscience, Germany) and water to a final reaction volume of 50 µl (Cadwell and Joyce, 1992) (Table 9). PCR was performed in a thermal cycler 2720 (PE Applied Biosystems, USA). PCR amplification reaction steps were as follows: Pre-heating of 1 min at 94°C, followed by 30 cycles of 94°C for 1 min, 69°C for 1 min and 72°C for 3 min, respectively and a final extension step at 72°C for 7 min (Table 10). Mutated DNA products were visualized on a 1% (w/v) EtBr-stained agarose gel.

The suboptimal [$MgCl_2$], co-factor of thermostable DNA polymerase for EP PCR was determined using different concentrations of Mg^{2+} ranging from 1.5 to 9 mM and visualized to determine which concentration would produce the desired product with minimal non-specific amplification. Conventional PCR reaction conditions (Table 2 and Table 3, section 2.2.9) were used with the exception of using a lower amount of template DNA (0.5 ng) and an increased number of cycles (30 cycles) to increase the mutation rate during amplification.

Table 7: Reagent requirements for [$MgCl_2$] PCR

Reagent	Concentration
10X Taq reaction buffer without $MgCl_2$	1x
$MgCl_2$	1.5 – 9 mM
Template DNA	0.5 ng
Forward Primer (Sfi1L)	0.2 µm
Reverse Primer (LCNOT1)	0.2 µm
dNTPs mix	200 µm
Taq DNA Polymerase (1 U/µl)	2.5 U
Final reaction volume (adjusted with water)	50 µl

Table 8: Conventional PCR Cycling Conditions

Step		Temperature and Time
Initial Denaturation		94°C for 1 min
30 cycles of	Denaturation	94°C for 1 min
	Annealing	60°C for 1 min
	Extension	72°C for 2 min
Final Extension		72°C for 5 min

Table 9: Reagent requirements for EP PCR reaction

Reagents	Concentration
10x *Taq* reaction buffer without $MgCl_2$	1x
$MgCl_2$	7 mM
Template DNA	0.5 ng
Forward Primer	0.2 µm
Reverse Primer	0.2 µm
dATP	0.2 mM
dGTP	0.2 mM
dTTP	1 mM
dCTP	1 mM
$MnCl_2$	0.5 mM
Taq DNA Polymerase (1 U/µl)	5 U
Final reaction volume (adjusted with sterile $dddH_2O$)	50 µl

Table 10: Error Prone PCR reaction conditions

Step		Temperature and Time
Initial Denaturation		94°C for 1 min
30 cycles of	Denaturation	94°C for 1 min
	Annealing	69°C for 1 min
	Extension	72°C for 3 min
Final Extension		72°C for 7 min

2.2.15 Automated Nucleotide sequencing

The nucleotide sequences of DNA samples were determined with the ABI PRISM® 3130xl Genetic Analyzer using Big Dye sequencing chemistry (Applied Biosystems, California, USA). The 10 µl sequencing reactions contained 2 µl Big Dye Reaction mix version 3.1, 1 µl Big Dye Sequencing buffer (400 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 3.2 pmol primer (Table 12) and 80 – 150 ng of template DNA. The amplification reaction steps were as follows: an initial denaturation step at 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, respectively (Table 13). Reactions were performed on a thermal cycler 2720 (PE Applied Biosystems, USA). The primers used for sequencing are listed in Table 11.

Table 11: Sequencing Primers

Primer	Sequence (5′ – 3′)
M13rev	CAGGAAACAGCTATGAC
pAKseqF	GTGGAATTGTGAGCG
pAKseqR	CAGTAGCGGTAAACG

Table 12: Reagents requirements for Sequencing Reaction

Reagents	Concentration
Template DNA	80 – 150 ng
Primer	3.2 pmol
Big Dye buffer	1 µl
Big dye 3.1	2 µl
Final reaction volume (adjusted with sterile $dddH_2O$)	10 µl

Table 13: Sequencing Reaction Cycling Conditions

Step		Temperature and Time
Initial Denaturation		96°C for 1 min
25 cycles of	Denaturation	96°C for 10 sec
	Annealing	50°C for 5 sec
	Extension	60°C for 4 min

The sequencing products were precipitated to remove any un-incorporated nucleotides, which would result in high background readings. The sequencing clean up process included the addition of 10 µl of sterile dddH_2O to the samples, followed by the addition of 2 µl sodium acetate (3 M, pH 4.8) and 64 µl absolute ethanol. The reaction mix was then centrifuged at 13 000 x g for 30 min at 4°C. The supernatant was discarded. The cell pellet was washed twice with 60 µl of 70% (v/v) ethanol and centrifuged at 13 000 x g for 10 min at 4°C. Supernatant was removed and the pellet was left to dry at room temperature for 15 min. The samples were run on the ABI PRISM® 3130xl Genetic Analyzer to obtain nucleotide sequences, which were then analyzed using the BioEdit Sequence Alignment Editor V 7.0.5.3 (Hall, 1999).

2.2.16 Protein expression of recombinant anti-R7V scFv protein

A single colony was selected from the freshly transformed plate and inoculated in 4 ml 2xTY (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% w/v NaCl) broth supplemented with appropriate antibiotic [for pHEN1: amp (100 µg/ml), for pAK400: chloramphenicol (25 µg/ml)] and 2% glucose. The cell culture was grown overnight at 37°C with shaking at 250 rpm. Following this, the overnight culture was diluted 1:100 in the same growth medium (0.2 ml culture in 20 ml 2x TY/G with the appropriate antibiotic) and further grown at 37°C with shaking (250 rpm) until the OD_{600} reached a value of 0.6. Cells were pelleted by centrifugation at 2 000 x g for 10 min at room temperature. The cell pellet was re-suspended in one-fifth volume of 2x TY (4 ml) containing appropriate antibiotic and 1 mM IPTG and the culture further grown for 4 hr at 30°C (4 hr at 24°C for pAK400 vector) with shaking at 250 rpm (van Wyngaardt *et al*., 2004).

The culture was then divided into two 15 ml batches in sterile centrifuge tubes and the cells pelleted by centrifugation at 2 000 x g for 10 min at room temperature. The supernatants were pooled together into a new 15 ml sterile centrifuge tubes and kept on ice. To isolate soluble

periplasmic proteins, the cell pellet was re-suspended in 500 µl ice-cold 1x TES buffer (30 mM Tris, 5 mM EDTA and 20% Sucrose). Another 750 µl of ice-cold 1x TES buffer (diluted 1:4 in $dddH_2O$) was added to the suspension, vortexed and incubated on ice for a further 30 min. The suspension was then transferred to a 1.5 ml sterile micro centrifuge tube and centrifuged at 2 000 x g for 10 min at 4°C. The supernatant (which contains the soluble protein from the periplasm) was transferred to a clean sterile 1.5 ml micro centrifuge tube and stored at -20°C until needed (EasyMatch Phage Display Manual).

2.2.17 Protein concentration determination

The QuickStart™ Bradford Protein Assay (Bio-Rad, USA) was used to determine the concentration of isolated proteins. Bovine serum albumin (BSA, Promega, USA) was used as the standard protein to construct a standard curve to determine the concentration of the unknown protein samples. A stock solution of BSA (100 µg/ml) was used to prepare a standard dilution series of following concentrations in µg/ml: 100, 50, 25, 12.5 and 6.25.The experiment was conducted in a 96-well ELISA plate (Sero-Wel®, UK). Bradford dye (50 µl) and 50 µl of each standard dilution sample or the protein sample (diluted 10x and 100x) was added onto the wells of the ELISA plate. The absorbance was read at 595 nm using a Multiscan Ascent Plate Reader (AEC Amersham, SA). The ELISA plate was incubated for 15 min at room temperature before measuring the absorbance. A Nanodrop (Inqaba Biotec, SA) was also used as an alternative to determine the concentration of protein. The Nanodrop spectrophotometer (Inqaba Biotec, SA) measured the protein concentration in mg/ml.

2.2.18 SDS-PAGE of recombinant anti-R7V scFv protein

Protein expression was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Protein gel consisting of 5% stacking gel (5% acrylamide, 0.5 M Tris-HCl, pH 6.8 and 0.4% w/v SDS) and 12% separating gel (12% acrylamide, 1.5 M Tris-HCl, pH 8.8 and 0.4% w/v SDS) were prepared from acrylamide (30% w/v acrylamide, 0.8% w/v N'N'- methylene bisacrylamide) stock solution. The acrylamide separating gel was polymerised by the addition of 200 µl 10% (w/v) ammonium persulphate (MP Biomedicals, France) and 10 µl TEMED (N,N,N',N'-Tetramethyl ethylenediamine, Merck, Germany). The stacking gel was polymerised by the addition of 80 µl 10% (w/v) ammonium persulphate and 5 µl TEMED.

The protein samples were diluted 2:1 in 1x SDS reducing sample buffer (60 mM Tris-HCl pH 6.8, 2% w/v SDS, 0.1% w/v glycerol, 0.05% v/v β-mercaptoethanol and 0.025% w/v

bromophenol blue) and boiled for 5 min at 95°C. Page Ruler Unstained Protein Ladder (Fermentas, USA) was used as molecular marker. Electrophoresis was performed in 1X SDS electrophoresis buffer (0.02 M Tris-HCl, 0.1 M glycine, 0.06% SDS, pH 8.3) using a Hoefer® mini VE vertical gel electrophoresis system (Amersham Pharmacia Biotech, USA). Proteins were visualized by staining overnight in 0.1% (w/v) Coomassie Brilliant Blue R-250 (40% methanol, 10% acetic acid) staining solution. The gel was then destained in destaining solution (50% methanol, 10% acetic acid) until the background of the gel was clear and the protein bands were clearly visible.

2.2.19 Acetone precipitation of protein

Protein samples with concentrations less than 1 µg/ml were concentrated using the acetone precipitation method to visualize their bands on SDS-PAGE gel (Hahn lab Protocol). Acetone was added to the protein samples in the ratio of 1: 4 (protein sample: acetone). The samples were left at -20°C for overnight. Protein samples were then centrifuged at 13 000 x g for 30 min at 4°C. Supernatant was discarded. After removal of acetone by air drying at room temperature, the pellet was resuspended in 10 µl 1x SDS reducing sample buffer and boiled for 5 min at 95°C before loading the samples onto the SDS-PAGE gel.

2.2.20 Western blot of recombinant anti-R7V scFv protein

Western blotting was used for the detection of the secreted proteins. The anti-R7V scFv fragments expressed in pAK400 vector were analyzed using HisProbe-HRP (Thermo Scientific, Pierce, USA). The HisProbe-HRP was used for direct detection of recombinant histidine-tagged proteins.

After running the his-tag protein samples on a SDS-PAGE, the gel was equilibrated in 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid, pH > 9, Sigma, USA) buffer for 5 min to ensure all proteins are positively charged. The polyvinylidene fluoride (PVDF) transfer membrane (BioTrace™ PVDF, Life sciences, USA) was activated by wetting the membrane in methanol for 15 sec and then equilibrated in 10 mM CAPS buffer (pH > 9). The proteins were electrophoretically transferred to PVDF membrane at 10 V for 40 min using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, USA). The membrane was then blocked in blocking buffer (TBS with 2.5% w/v BSA, 0.05% v/v Tween-20, pH 7.6) for 1 hr at room temperature with gentle agitation.

For HisProbe-HRP immunodetection, the membrane was incubated with working solution (10 ml blocking buffer and 5 µl of HisProbe (5 µg/µl)) for 2 hr at room temperature with gentle agitation. The membrane was washed four times with TBST (25 mM Tris pH 7.6, 0.15 M NaCl, 0.05% Tween-20) wash buffer for 10 min at room temperature with gentle agitation. The 1-Step™ TMB-Blotting (Thermo Scientific, Pierce, USA) solution was then added to the membrane and the development of a blue-purple precipitate was monitored. The reaction was stopped by rinsing the membrane with water.

2.2.21 Presence and binding activity of recombinant anti-R7V scFv antibody fragment

The anti-R7V scFv antibody fragments expressed using recombinant pHEN1 vector system was analyzed for functional expression by ELISA using mouse anti-c-myc:HRP antibody. The standard ELISA protocol was used (Walker, 1987). Briefly, a 96-well Nunc-Immuno™ Maxisorp™ ELISA plate (Nunc, Roskilde, Denmark) was coated with 100 µl/well of 50 µg/ml R7V peptide (GenScript Corporation, dissolved in 10 mM PBS, pH 7.4) and incubated overnight at 4°C. After overnight incubation, the excess antigen was removed by flipping over the ELISA plate and 300 µl of blocking buffer (5% w/v fat free milk powder in PBST) was added to each well. The plate was incubated at room temperature for 4 hr. The blocking buffer (BF) was discarded and each well was rinsed five times with 300 µl of wash buffer (0.5% v/v Tween-20 in PBS). Recombinant anti-R7V protein (100 µl/well) was then added and the plate was incubated overnight at 4°C. The washing step was repeated as before, after which 100 µl/well of diluted (1:1000, diluted in blocking buffer) mouse anti-c-myc:HRP conjugated secondary antibody (AbD Serotec, UK) was added and the plate was incubated at room temperature for 3 hr. The final washing step was followed by the addition of 50 µl/well of the 1-Step™ Ultra TMB-ELISA (Thermo Scientific, Pierce, USA) solution. The plate was incubated at room temperature until a blue colour developed. The reaction was stopped by the addition of 50 µl/well of 2 N sulphuric acid, and the colour of the solution changed from blue to yellow. The absorbance was read at 450 nm using a Multiscan Ascent Plate Reader (AEC Amersham, SA).

Chapter 3

Results and Discussion

3.1 Introduction

High affinity antibodies are very important in diagnostic and biological assay applications. Phage display technology used for the production of mAbs usually produces antibodies with lower affinity than desired (Thie, 2010). Random mutagenesis is an efficient method for affinity maturation of mAbs. Different techniques have evolved to introduce random mutations in DNA (section 1.8.5). This project used the EP PCR technique to introduce random point mutations. The occurrence of gain-of-function mutated clones determines the efficacy of EP PCR. The aim of this study was to produce anti-R7V scFv antibody fragments with higher antigen-binding affinity, to improve its performance in ELISA and HIV neutralization assays.

3.2 Isolation of anti-R7V scFv gene

3.2.1 Plasmid DNA isolation

Recombinant pHEN1 phagemid DNA transformed into $CaCl_2$ competent *E.coli* XL-1 blue cells was isolated using Zyppy™ Miniprep kit (Figure 12 A). The transformation efficiency was $1x10^5$ transformants/µg of DNA. The Zyppy kit gave a good yield of pure DNA (40 µg DNA). The concentration of DNA was determined using Nanodrop spectrophotometer. The Nanodrop spectrophotometer calculates both A_{260}/A_{280} and A_{260}/A_{230} ratio. Pure DNA has an A_{260}/A_{280} ratio of 1.7 – 2.0. The A_{260}/A_{230} should be greater than 1.5; the lower the ratio the higher the amount of carry-over salts present in the purified DNA. Purified DNA is important for downstream applications such as cloning and DNA sequencing.

Isolated plasmid DNA was screened using PCR and RE digestion to determine the presence of the insert gene. Conventional PCR using gene-specific primers Sfi1L and LCNOT1 (van Wyngaardt *et al*., 2004) (section 2.2.9 Table 1) were used for the amplification of anti-R7V scFv gene from recombinant pHEN1 phagemid DNA. EtBr-stained agarose gels (1%) of the PCR product confirmed the presence of scFv gene at the expected size (750 bp) (Figure 12 B).

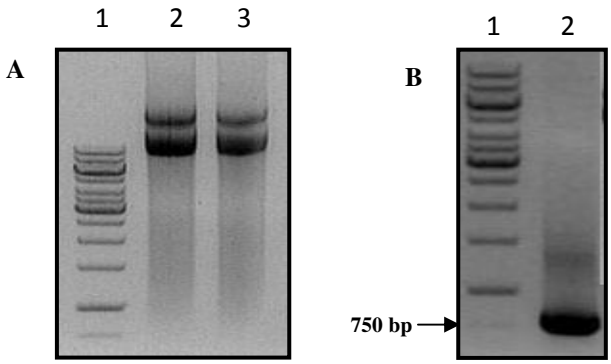


Figure 12: A: EtBr-stained agarose gel electrophoresis (1%) of recombinant pHEN1 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2 and 3: recombinant pHEN1 phagemid DNA] B: Agarose gel electrophoresis of amplified anti-R7V scFv gene from recombinant pHEN1 phagemid DNA using conventional PCR [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Amplified anti-R7V scFv gene at 750 bp]

The amplified DNA was purified from the agarose gel for further analysis. The PCR products were purified, from contaminants such as primer dimer, salts, enzymes and amplification primers, using the NucleoSpin® Extract II purification kit.

3.2.2 Restriction enzyme digestion of recombinant pHEN1 phagemid DNA

Restriction enzyme (RE) digestion of the recombinant pHEN1 phagemid DNA was performed, as a secondary method, to confirm the presence of the anti-R7V insert gene. The anti-R7V gene was inserted between the *Not*I and *Sfi*I restriction sites on the recombinant pHEN1 vector. RE double digestion was carried out using FD *Not*I and FD *Sfi*I enzymes.

Agarose gel of a double digested recombinant pHEN1 phagemid DNA was expected to show two bands, one at 4500 bp for digested linear plasmid DNA and second at 750 bp for digested insert DNA. However, the outcome was different. As can be seen in Figure 13 A, no DNA band was observed at 750 bp as expected, this indicates incomplete digestion of recombinant pHEN1 phagemid DNA. According to the manufacturer's (Fermentas) instruction, complete digestion of plasmid DNA is attained by incubating the DNA reaction mix with 1 U of FD *Not*I enzyme at 37°C for 5 min and 1 U of FD *Sfi*I enzyme at 50°C for 15 min.

In order to determine which restriction enzyme failed to completely digest the DNA, single digestion of recombinant pHEN1 phagemid DNA using each FD *Not*I and FD *Sfi*I RE was performed. From results preseted in figure 13 B, it can be concluded that the DNA double digestion was incomplete because of FD *Sfi*I RE. Only partial digestion was achieved using FD

*Sfi*I RE single digestion compared to the complete digestion attained by FD *Not*I RE single digestion (Figure 13 B).

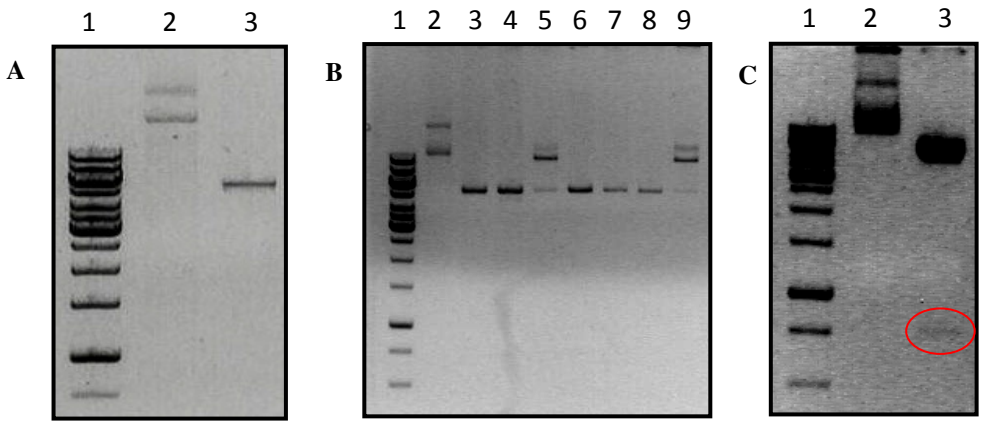


Figure 13: A: Agarose gel electrophoresis (1%) of FD *Not*I and *Sfi*I restriction enzyme double digested recombinant pHEN1 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Undigested recombinant pHEN1 DNA, Lane 3: *Not*I+*Sfi*I (1 U) digested recombinant pHEN1 DNA] B: Agarose gel electrophoresis (1%) of FD *Not*I and *Sfi*I restriction enzyme single and double digested recombinant pHEN1 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Undigested recombinant pHEN1 DNA, Lane 3: *Not*I (1 U) digested recombinant pHEN1 DNA, Lane 4: *Not*I+*Sfi*I (1 U) digested recombinant pHEN1 DNA), Lane 5: *Sfi*I (1 U) digested recombinant pHEN1 DNA, Lane 6: *Not*I (2 U) digested recombinant pHEN1 DNA, Lane 7: *Not*I+*Sfi*I (2 U) digested recombinant pHEN1 DNA, Lane 8: *Not*I+*Sfi*I (2 U) digested recombinant pHEN1 DNA), Lane 9: *Sfi*I (2 U) digested recombinant pHEN1 DNA] C: Agarose gel electrophoresis (1.5%) of FD *Not*I and *Sfi*I restriction enzyme double digested recombinant pHEN1 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Undigested recombinant pHEN1 DNA, Lane 3: *Not*I+*Sfi*I (1 U) digested recombinant pHEN1 DNA]

The incapability of fast digest *Sfi*I enzyme to completely digest the recombinant pHEN1 plasmid DNA can be correlated to its unique enzymatic properties. Most restriction enzymes have a recognition site that contains 4 or 6 consecutive specified base pairs. However, the *Sfi*I restriction endonuclease has a recognition site that contains 8 specified base pairs interrupted by a spacer of five unspecified base pairs (5′-GGCCNNNNNGGCC-3′). While most restriction enzymes are dimeric proteins and cut the DNA molecule at a single site, a study by Wentzell *et al.* (1995) showed that *Sfi*I enzyme is a tetrameric protein that interacts with two copies of its recognition site and cleaves the DNA molecule at two sites at once. The two *Sfi*I sites can be on the same or on separate DNA molecule. The rate of cleavage of DNA by *Sfi*I depends on the number of recognition site present on the molecule. The activity of *Sfi*I enzyme on DNA

molecules containing one *Sfi*I recognition site is much slower compared to DNA molecules with two *Sfi*I sites (Wentzell *et al*., 1995). The recombinant pHEN1 phagemid DNA contains only a single copy of the *Sfi*I recognition site, which can be the rationale for the incomplete digestion of the DNA by *Sfi*I enzyme.

The RE reaction conditions were modified by the addition of 2 U of each RE and the incubation time was increased by 1 hr. However, increasing the concentration of *Sfi*I enzyme did not have an effect on the digestion of recombinant pHEN1 phagemid DNA (Appendix A).

The *Sfi*I is a type II restriction enzyme and type II enzymes function catalytically rather than stoichiometrically (Wentzell *et al*., 1995). Hence, increasing the concentration of enzyme did not improve the efficiency of digestion. Krebber *et al.* (1997) used the pAK vector system that contains two *Sfi*I sites for their studies. The phage display vector pAK100 was digested with *Sfi*I enzyme for 3 to 4 hr at 50°C (Krebber *et al*., 1997). The restriction enzyme condition mentioned by Krebber *et al*. (1997) was adapted in this project.

The recombinant pHEN1 plasmid DNA was first digested with *Not*I for 1 hr at 37°C and then with *Sfi*I for 4 hr at 50°C. The new RE reaction condition resulted in the complete digestion of the recombinant pHEN1 phagemid DNA (Figure 13 C). The double digested recombinant pHEN1 phagemid DNA was purified from the gel and ligated with purified FD *Not*I/*Sfi*I double digested anti-R7V scFv gene. RE digested DNA was purified, from contaminants such as salts and enzymes, using the NucleoSpin® Extract II purification kit for efficient ligation. Sequence analysis of re-ligated recombinant pHEN1 phagemid DNA revealed the presence of anti-R7V insert gene in recombinant pHEN1 phagemid DNA in the correct orientation (Appendix B). The modified restriction enzyme reaction conditions, *Sfi*I enzyme was able to digest the recombinant pHEN1 DNA completely. Nonetheless, use of a different vector system was favoured for a simple ligation procedure and subcloning of mutated anti-R7V gene.

3.2.3 Sequence analysis of anti-R7V scFv gene

The sequence of anti-R7V scFv gene was analyzed using BioEdit Sequence Alignment Editor V7.0.5.3. The complete scFv gene sequence was obtained by aligning both forward and reverse sequences. The DNA sequence analysis of anti-R7V scFv gene (Figure 14 A) shows the *Not*I and *Sfi*I restriction sites within which the scFv gene was inserted correctly. The anti-R7V scFv gene has an internal *Xho*I restriction enzyme site, which can also be seen in the obtained sequence. The size of anti-R7V scFv gene was found to be 747 bp. The amino acid sequence analysis

(Figure 14 B) revealed the $(Gly_4S)_3$ linker that connects the V_H and the V_L chain of the scFv fragment.

A: Anti-R7V scFv nucleotide sequence:

GGCCCAGCCGGCCCTGATGGCGGCCGTGACGTTGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGA
GGAGCGCTCAGCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGCAGTTATGGCATGGGCTGGGTGCG
ACAGGCACCCGGCAAGGGGCTGGAATACGTCGCGGGTATTAGAAGTGATGGTAGTAACCCAAACTAC
GGGGCGGCGGTGAAGGGCCGTGCCACCATCTCGAGGGACAACGGGCAGAGCACAGTGAGGCTGCAGC
TGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGCGCCAAAAAGATGAATAGGGCTTATATT
ATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCCGGTGGAGGCGGTTCAGGTGGAGGTG
GCTCTGGCGGAGGCGGATCGGCGCTGACTAGCCGTCCTCGGTGTCAGCGAACCCGGGAGAAACCGTC
AAGCTCACCTGCTCCGGGGGTGGCAGCTATGGCTGGTACCAGCAGAAGGCACCTGGCAGTGCCCCTGT
CACTGTGATCTATGACAACACCAAGAGACCCTCGGACATCCCTTCACGATTCTCCGGTTCCACATCTGG
CTCCACACACACATTAACCATCACTGGGGTCCGAGCCGAGGACGAGGCTGTCTATTTCTGTGGGAGTG
CAGGCAGCAGTACTGGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGCCAGCCCAATGCGGCCG
GC

*Sfi*I – GGCCCAGCCGGCC *Xho*I – CTCGAG *Not*I - GCGGCCGC

B: Anti-R7V scFv amino acid sequence:

LMAAVTLDESGGGLQTPGGALSLVCKASGFTFSSYGMGWVRQAPGKGLEYVAGIRSDGSNPNYGAAVKG
RATISRDNGQSTVRLQLNNLRAEDTGTYYCAKKMNRAYIIDAWGHGTEVIVSSGGGGSGGGGSGGGGSAL
TQPSSVSANPGETVKLTCSGGGSYGWYQQKAPGSAPVTVIYDNTKRPSDIPSRFSGSTSGSTHTLTITGVRA
EDEAVYFCGSAGSSTGIFGAGTTLTVLGQPNAAA

$(Gly_4S)_3$ linker - GGGGSGGGGSGGGGS

Figure 14: A: Nucleotide sequence of anti-R7V scFv gene B: Amino acid sequence of anti-R7V scFv gene

3.3 Subcloning of anti-R7V scFv gene into pGE20 phagemid expression vector

3.3.1 Subcloning of anti-R7V scFv gene into pGE20 vector

In both recombinant pHEN1 and pGE20 vectors, the primary selection of transformed cells is through antibiotic selection. Both vectors confer resistance to ampicillin; hence, transformed cells are ampicillin resistant. Secondary selection is the blue-white selection, which will indicate the presence of an insert in the vector. If there is an insert, β-galactosidase do not break X-gal and thus no colour is obtained. This is referred to as insertional inactivation. Thus, transformed cells will produce white colonies. Non-transformed cells will form blue colonies as X-gal is broken and forms end product which is blue in color (Primrose and Twyman, 2006).

The pGE20 vector contains the β-galactosidase (*lacZ*) gene, which allows for the blue-white selection process to distinguish between non-recombinant and recombinant clones. The recombinant pHEN1 vector system also carries the *lacZ* gene. However, it could not be used for blue-white screening procedure since there was no parental pHEN1 plasmid DNA available in our laboratory. The pGE20 phagemid expression vector was hence chosen for the subcloning of mutated anti-R7V gene. Another reason for choosing pGE20 vector was the presence of unique antibody cloning sites that allows straightforward cloning and production of scFv fragments (Orfanoudakis *et al*., 1993).

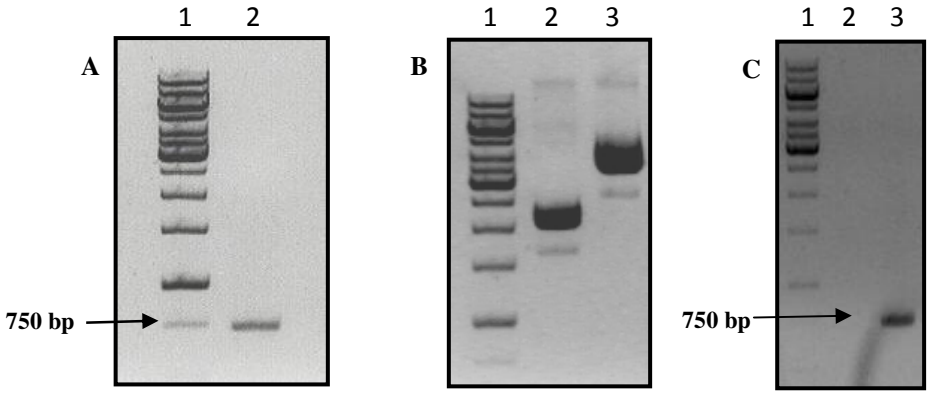


Figure 15: A: Agarose gel electrophoresis of amplified anti-R7V gene using HENUNIF1 and HENUNIR1 primers [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Amplified anti-R7V DNA] B: Agarose gel electrophoresis of ligated pGR7V2 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: pGE20 phagemid DNA, Lane 3: pGR7V2 phagemid DNA] C: Agarose gel electrophoresis confirming the presence of anti-R7V gene (750 bp) in the ligated pGR7V2 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Negative Control, Lane 3: Amplified anti-R7V DNA]

Gene-specific primers, HENUNIF1 and HENUNIR1 were designed using Primer3 software for the amplification of scFv gene and subcloning into pGE20 vector. The amplified anti-R7V gene (Figure 15 A) was inserted between *Xho*I and *Eco*RI restriction sites of the vector. Since the insert gene also contains an *Xho*I restriction site, the *Xho*I restriction site on the pGE20 vector was removed using DNA polymerase I Klenow fragment. The *Xho*I digested vector produced a 3′-overhang. The 3′-5′ exonuclease activity of the Klenow fragment was used to generate blunt ends from the 3′-overhang, thereby removing the *Xho*I site from the pGE20 vector (Promega Protocol, 2005).

The PCR primers, HENUNIF1 and HENUNIR1 were designed such that it amplifies the insert gene with a 5′ blunt end and a 3′ *Eco*RI site. *Eco*RI digested vector and insert DNAwere ligated and transformed to *E.coli* XL-blue cells. The transformed cells were grown on LB-Amp-X-gal-IPTG agar plates to allow for the blue-white screening of transformed cells. Zyppy™ Miniprep kit was used to isolate plasmid DNA and the recombinant DNA obtained was named pGR7V2 (Figure 15 B).Transformation efficiency of pGE20 and pGR7V2 DNA was calculated to be $1x10^5$ transformants/µg DNA and $1.2x10^2$ transformants/µg DNA, respectively. The presence of insert gene in pGR7V2 DNA was determined using conventional PCR with HENUNIF1 and HENUNIR1 primers (Figure 15 C).

In order to confirm the integrity of parental pGE20 phagemid DNA after ligation, *Xba*I RE digestion of pGR7V2 phagemid DNA was performed. Single RE digestion of the pGR7V2 DNA should show a fragment at 4000 bp on the agarose gel which will confirm the integrity of the parental pGE20 DNA. In addition, *Eco*RI digested pGR7V2 DNA should give a band at 4000 bp which will confirm the presence of the insert gene. *Xba*I and *Eco*RI single digested pGE20 was used as a positive control. *Xba*I and *Eco*RI single digestion of pGE20 vector would produce a band at 3200 bp.

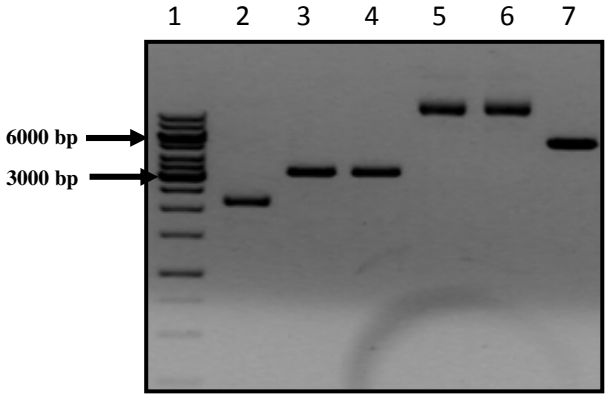


Figure 16: Agarose gel electrophoresis of *Xba*I digested and *Eco*RI digested pGR7V2 phagemid DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Undigested pGE20 DNA, Lane 3: *Xba*I digested pGE20 DNA, Lane 4: *Eco*RI digested pGE20 DNA, Lane 5: Undigested pGR7V2 DNA, Lane 6: *Xba*I digested pGR7V2 DNA, Lane 7: *Eco*RI digested pGR7V2 DNA]

In Figure 16, both *Xba*I and *Eco*RI single digestion of parental pGE20 vector DNA produced a single band at 3200 bp as expected. However, no digestion was observed in the pGR7V2 DNA using the *Xba*I RE. *Eco*RI single digestion of pGR7V2 gave a band of 6000 bp instead of the expected 4000 bp. This result implies a possibility of non-specific amplification of the anti-R7V scFv gene.

3.3.2 Sequence analysis of pGR7V2 phagemid DNA

Sequence analysis of pGR7V2 phagemid DNA was performed to confirm the correct anti-R7V scFv gene sequence (with 5′ blunt end *Xho*1 and 3′ *Eco*R1 RE site) has been amplified and subcloned into pGE20 vector. The sequence of pGR7V2 DNA was analyzed using BioEdit Sequence Alignment Editor V7.0.5.3. The complete scFv gene sequence was obtained by aligning both forward and reverse sequences.

The DNA sequence analysis of pGE20 vector (Figure 17 A) shows the *Xho1* and *EcoR1* RE site within which the anti-R7V scFv gene is to be inserted. However, the DNA sequence analysis of pGR7V2 vector (Figure 17 B) revealed non-specific amplification of anti-R7V gene since the HENUNIF1 and HENUNIR1 primers amplified parts of recombinant pHEN1 vector sequence together with the scFv gene. Only the bases highlighted in red were supposed to be ligated into pGE20 phagemid DNA (Figure 17 B).

A: Sequence of pGE20 vector DNA:

CTCGAGATTTCTGACTAGTCGTCCGAACTCCGATAATCGCCGTCAGGGCGGTCGCGAACGTTTATAAT
AGAATTCTAAACTAGCTAGTCGCCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGC
TGGATTGTTATTACTCGCTGCCCAACCAGCCATGGCCGAGCTCGTCAGTTCTAGAGTTAAGCGGCCGC
AATCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTT
TTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC
GCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGCAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATT
TTTT

XhoI – CTCGAG EcoRI – GAATTC XbaI – TCTAG

B: Sequence of pGR7V2 vector DNA:

CTGGAGGGACCCTCCCGGCCCAGCCGGCCCTGATGGCGGCCGTGAGAGGGGCGAGTCCGGGGGCGGC
CTCCAGACGCCCGGAGGAGCGCTCAGCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGCAGTTATGG
CATGGGCTGGGTGCGACAGGCACCCGGCAAGGGGCTGGAATACGTCGCGGGTATTAGAAGTGATGGT
AGTAACCCAAACTACGGGGCGGCGGTGAAGGGCCGTGCCACCATCTCGAGGGACAACGGGCAGAGCA
CAGTGAGGCTGCAGCTGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGCGCCAAAAAGAT
GAATAGGGCTTATATTATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCCGGTGGAGGCG
GTTCAGGTGGAGGTGGCTCTGGCGGAGGCGGATCGGCGCTGACTAGCCGTCCTCGGTGTCAGCGAACC
CGGGAGAAACCGTCAAGCTCACCTGCTCCGGGGGTGGCAGCTATGGCTGGTACCAGCAGAAGGCACC
TGGCAGTGCCCCTGTCACTGTGATCTATGACAACACCAAGAGACCCTCGGACATCCCTTCACGATTCTC
CGGTTCCACATCTGGCTCCACACACACATTAACCATCACTGGGGTCCGAGCCGAGGACGAGGCTGTCT
ATTTCTGTGGGAGTGCAGGCAGCAGTACTGGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGC
CAGCCCAATGCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAGACTG
TTGAAAGTTGTTTAGCAAAACCTCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACT
TTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGC

Figure 17: A: Nucleotide sequence of anti R7V scFv gene in pGE20 vector DNA
B: Nucleotide sequence of anti R7V scFv gene in pGR7V2 vector DNA

Several factors such as annealing temperature, Mg^{2+} concentration, primer concentration, primer design and cycle number can result in non-specific amplification during PCR (Sipos *et al.*, 2007). The results obtained here illustrates that even though the agarose gel shows a PCR DNA band at the expected size (Figure 15 C), it does not ensure that the specific gene with the correct sequence has been amplified. Analysis of the DNA sequence of the amplified product should be performed before further experiments, to confirm that the correct gene sequence was amplified.

Nested PCR was performed to solve the problem of non-specific amplification. Nested PCR primers re-amplify the desired product to eliminate production of any non-specific products. It is highly unlikely to amplify a non-specific product through a second round of PCR, using internally nested primers (Robert Lyons, DNA Sequencing Core, University of Michigan).

In order to minimize the non-specific amplification, the anti-R7V scFv insert gene was first amplified with Sfi1L and LCNOT1 primers and purified from the gel. A nested PCR using HENUNIF1 and HENUNIR1 was then used to re-amplify the scFv gene.

Annealing temperature is the most critical parameter that influences the specificity of the PCR. The gradient function in the PCR thermal cycler allows setting up a gradient of temperature in one single run (Vincent and Jahns, 2000). Since, the PCR thermal cycler in our laboratory at the time did not have the gradient function, three separate PCR reactions were run using three different annealing temperatures: 63, 66 and 68°C. The amplified DNA was sequenced and the sequencing results revealed poly-T tails at both ends of the amplified gene (Figure 18).

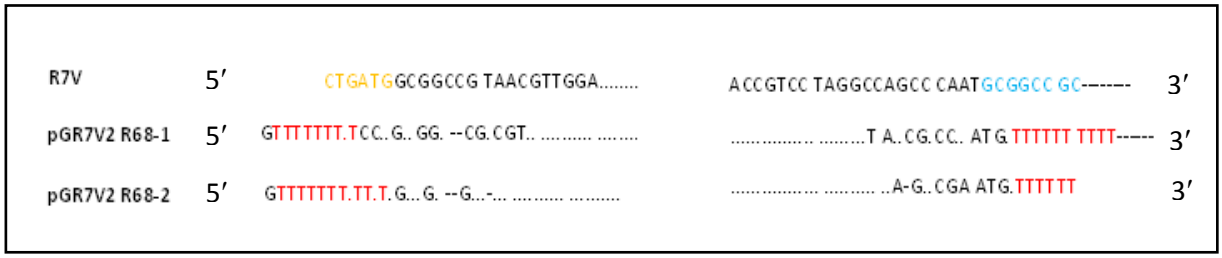


Figure 18: Poly-T tails at the 5′and 3′ end of a nested PCR optimized anti-R7V gene

Parallel to this experiment, attempts were made to insert a 6xHIS tag between the *Eco*RI and *Xba*I restriction site of the pGE20 vector, for the purification of the scFv gene after expression. The transformed pGE20-HIS ligated DNA (pGHE20 vector) gave few white colonies and few blue colonies on the LB-agar plate. The transformation efficiency was $2.5x10^{1}$ transformants/µg of DNA. The formation of white colonies indicates the presence of recombinant DNA. However, the sequence results of the isolated pGHE20 DNA (Figure 19 B) did not show 6xHIS sequence within the *Eco*RI and *Xba*I restriction site. As can be seen in Figure 19 B, *Eco*RI digestion of pGE20 vector caused star activity, deleting off a part of the vector sequence including the *Xba*I restriction site, thereby preventing the ligation of 6xHIS tag between the *Eco*RI and *Xba*I digested pGE20 DNA. Figure 19 A shows the sequence of the pGE20 vector DNA.

A: Sequence of pGE20 vector DNA:

CTCGAGATTTCTGACTAGTCGTCCGAACTCCGATAATCGCCGTCAGGGCGGTCGCGAACGTTTATAAT
AGAATTCTAAACTAGCTAGTCGCCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGC
TGGATTGTTATTACTCGCTGCCCAACCAGCCATGGCCGAGCTCGTCAGTTCTAGAGTTAAGCGGCCGC
AATCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTT
TTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC
GCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGCAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATT
TTTT

B: Sequence of pGHE20 vector DNA:

CTCGAGATTTCTGACTAGTCGTCCGAACTCCGATAATCGCCGTCAGGGCGGTCGCGAACGTTTATAAT
AGAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTT

*Xho*I – CTCGAG *Eco*RI – GAATTC *Xba*I – TCTAGA

Figure 19: A: Sequence of pGE20 vector DNA B: Sequence of pGHE20 vector DNA

The *Eco*RI star activity (*Eco*RI*) could have been caused either by i) the longer incubation time used for the *Eco*RI digestion of the template DNA or ii) the use of excess enzyme.

The *Eco*RI digestion of the plasmid DNA was carried out at 37°C for 30 mins. The template DNA becomes prone to *Eco*RI star activity at long incubation periods (Primrose and Twyman, 2006). In order to avoid the problem of *Eco*RI star acivity, it is recommended toshorten the incubation time for *Eco*RI restriction enzyme digestion to 5 min. The second reason was that the volume of the total enzyme added exceeded 1/10th the volume of the total reaction. Too high concentration of enzyme could also cause *Eco*RI star activity to occur.

*Eco*RI* is the non-specific digestion of the *Eco*RI restriction site. *Eco*RI* cleaves N/AATTN sequences, where N is any base. *Eco*RI* caused part of the plasmid DNA sequence including the *Xba*I restriction site to be cut out of the plasmid DNA. This resulted in the inability of 6xHIS tag to be ligated into pGE20, as there was no *Xba*I RE site. The white colonies obtained were as a result of the removal of a part of the sequence from the plasmid DNA that disrupted the *lac*Z gene function.

3.4 Subcloning of anti-R7V scFv gene into pAK400 phage expression vector

3.4.1 Subcloning of anti-R7V scFv gene into pAK400 vector

Since subcloning into pGE20 vector was not successful, a different expression vector system, pAK400, was chosen for the subcloning purpose. The anti-R7V scFv gene was subcloned from recombinant pHEN1 vector to phage expression vector, pAK400. Figure 20 A shows the agarose gel of pAK400 plasmid DNA. The pAK400 vector is an improved expression vector and contains unique *Sfi*I restriction sites that allows for unidirectional cloning (Krebber *et al*., 1997). The vector system confers resistance to chloramphenicol. Thus, only transformants containing the insert gene are able to grow in the presence of chloramphenicol, thus eliminating the growth of background transformants that do not contain an insert.

A new reverse primer, pAKrev containing 3′ *Sfi*I RE site (section 2.2.9 Table 1) along with Sfi1L primer was used to amplify the scFv gene. The annealing temperature was optimized using gradient PCR. Annealing temperatures ranging from 60 to 69°C was selected for amplification. Annealing temperature of 69°C amplified the specific region of the scFv gene with the 5′ *Sfi*I and 3′ *Sfi*I RE sites (Appendix B).

The RE digestion of both vector and scFv insert DNA was performed using conventional *Sfi*I enzyme. *Sfi*I is a rare cutting enzyme (Krebber *et al*., 1997). Majority of the vector DNA was digested when conventional *Sfi*I RE was used, as compared to the digestion performed by FD *Sfi*I RE (Figure 20 B). The purified *Sfi*I RE digested insert PCR DNA was ligated into purified *Sfi*I RE digested pAK400 vector DNA and transformed into electrocompetent *E.coli* HB2151 cells. The recombinant vector was named pAKR7V400 phage DNA. Transformation efficiency of pAK400 and pAKR7V400 DNA was calculated to be 3.2×10^5 transformants/µg of DNA and 8.0×10^0 transformants/µg of DNA, respectively.

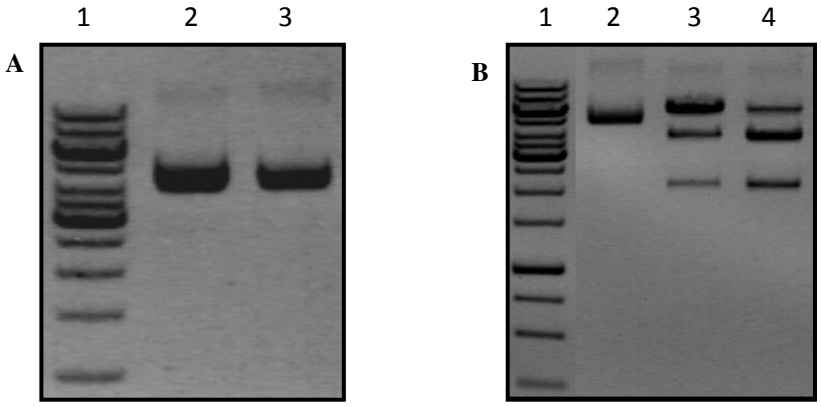


Figure 20: A: Agarose gel electrophoresis (1%) of pAK400 phage DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2 and 3: pAK400 phage DNA] B: Agarose gel electrophoresis (1%) of *Sfi*I restriction enzyme digested pAK400 phage DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Undigested pAK400 DNA, Lane 3: Fast digest *Sfi*I digested pAK400 DNA, Lane 4: Conventional *Sfi*I digested pAK400 DNA]. In lane 3 and 4, the first band from top is the partially undigested vector DNA, second band is the partially digested vector DNA (which was gel excised) and third band is the insert gene (tetracycline cassette)

The pAKR7V400 phage DNA was isolated using NucleoSpin® plasmid kit (Figure 21 A). The presence of insert gene (750 bp) was confirmed using conventional PCR (Figure 21 B).

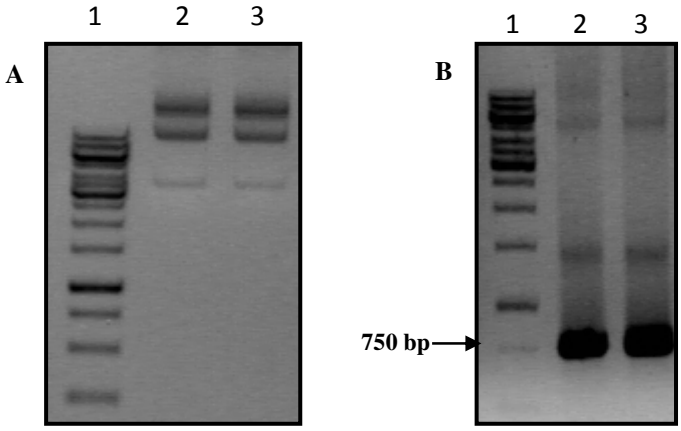


Figure 21: A: Agarose gel electrophoresis (1%) of pAKR7V400 phage DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2 and 3: pAKR7V400 phage DNA] B: Agarose gel electrophoresis (1%) confirming the presence of anti-R7V scFv gene (750 bp) in ligated pAKR7V400 phage DNA using conventional PCR [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2 and 3: Amplified anti-R7V gene]

3.4.2 Sequence analysis of pAKR7V400 phage DNA

DNA sequence analysis (Figure 22) confirmed the presence of anti-R7V insert gene in pAKR7V400 vector and in the correct orientation. Subcloning of anti-R7V scFv gene into pAK400 expression vector was successful.

GGCCCAGCCGGCCCTGATGGCGGCCGTAACGTTGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGA
GGAGCGCTCAGCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGCAGTTATGGCATGGGCTGGGTGCG
ACAGGCACCCGGCAAGGGGCTGGAATACGTCGCGGGTATTAGAAGTGATGGTAGTAACCCAAACTAC
GGGGCGGCGGTGAAGGGCCGTGCCACCATCTCGAGGGACAACGGGCAGAGCACAGTGAGGCTGCAGC
TGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGCGCCAAAAAGATGAATAGGGCTTATATT
ATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCCGGTGGAGGCGGTTCAGGTGGAGGTG
GCTCTGGCGGAGGCGGATCGGCGCTGACTAGCCGTCCTCGGTGTCAGCGAACCCGGGAGAAACCGTC
AAGCTCACCTGCTCCGGGGGTGGCAGCTATGGCTGGTACCAGCAGAAGGCACCTGGCAGTGCCCCTGT
CACTGTGATCTATGACAACACCAAGAGACCCTCGGACATACCTTCACGATTCTCCGGTTCCACATCTG
GCTCCACACACACATTAACCATCACTGGGGTCCGAGCCGAGGACGAGGCTGTCTATTTCTGTGGGAGT
GCAGGCAGCAGTACTGGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGCCAGCCCAATGCGGC
CTCGGGGGCC

*Sfi*I – GGCCCAGCCGGCC *Xho*I – CTCGAG *Sfi*I – GGCCTCGGGGGCC

Figure 22: Nucleotide sequence of anti R7V gene in pAKR7V400 vector

3.5 Error prone PCR of anti-R7V scFv gene

The anti-R7V scFv gene was subjected to random mutagenesis using EP PCR. The scFv gene was amplified from pAKR7V400 phage DNA using Sfi1L and pAKrev primers (Figure 23 A) under error prone conditions (section 2.2.15, Table 9).

The magnesium ion (Mg^{2+}) concentration is a crucial factor in a PCR, as it affects the specificity of a PCR reaction. A lower [Mg^{2+}] increases specificity whereas a higher [Mg^{2+}] decreases the specificity (Blanchard *et al*., 1993). For EP PCR reaction, a higher [Mg^{2+}] that would decrease the specificity but at the same time produce the specific product was required. The results from the EP PCR using different concentration of $MgCl_2$ showed that the [Mg^{2+}] at 7 mM produced the desired product without any non-specific amplification. Hence, the [Mg^{2+}] in EP PCR, for increasing the error rate without non-specific amplification was determined to be 7 mM.

The mutated PCR product was digested with conventional *Sfi*I enzyme and ligated into purified pAK400 vector cut with *Sfi*I. The ligated DNA was electroporated into *E.coli* HB2151 cells. The new recombinant vector was named pAKEPR7V400 phage DNA (Figure 23 B). Transformation efficiency was calculated to be $2.6x10^0$ transformants/µg DNA.

The recombinant pAKEPR7V400 phage DNA was isolated using NucleoSpin® plasmid kit. Conventional PCR confirmed the presence of the mutated insert gene in pAKEPR7V400 DNA (Figure 23 C).

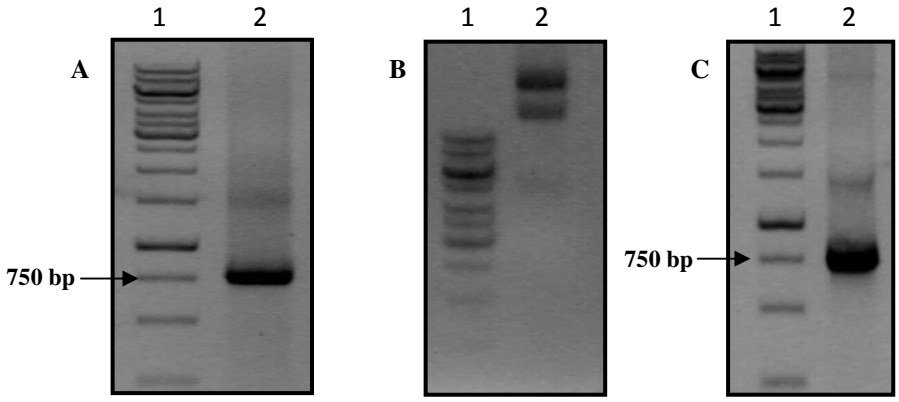


Figure 23: A: Agarose gel electrophoresis (1%) of mutated anti-R7V scFv gene using EP PCR [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Amplified mutated anti-R7V PCR gene] B: Agarose gel electrophoresis (1%) of mutated pAKEPR7V400 phage DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: pAKEPR7V400 phage DNA] C: Agarose gel electrophoresis (1%) confirming the presence of mutated anti-R7V scFv gene in ligated pAKEPR7V400 phage DNA [Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder), Lane 2: Amplified mutated anti-R7V PCR gene]

3.5.1 Analysis of mutated anti-R7V scFv gene

The mutation rate was determined by sequencing the mutated scFv genes in both forward and reverse direction. Figure 24 shows the nucleotide sequence of mutated clones. The alignment between the parent (non-mutated) and mutated clones revealed that there were 14 mutations in Clone 1 and 11 mutations in Clone 2. Amino acid (a.a) sequence analysis (Figure 25) revealed that there were 10 a.a substitutions in Clone 1 (MFO1 and MRO1) and 5 a.a substitutions in Clone 2 (MFO2 and MRO2). Amino acid (a.a) sequence was inferred from the nucelotide sequence. Majority of the substitutions were found in the framework regions (FR). Clone 1 had three substitutions in the CDR regions and Clone 2 had two substitutions in the CDR regions.

The effect of mutation in the V_H and V_L regions can be various. In some instances, a single a.a change may decrease or increase the affinity of antibody. In addition, mutations in the V region may affect the stability, assembly and secretion of the antibody. The FR region maintains the overall structure of the antibody. The CDR are directly involved in antigen binding activity. Mutations in the CDR regions are more susceptible to impaired binding as compared to mutations in the FR regions (Wiens *et al.,* 1997).

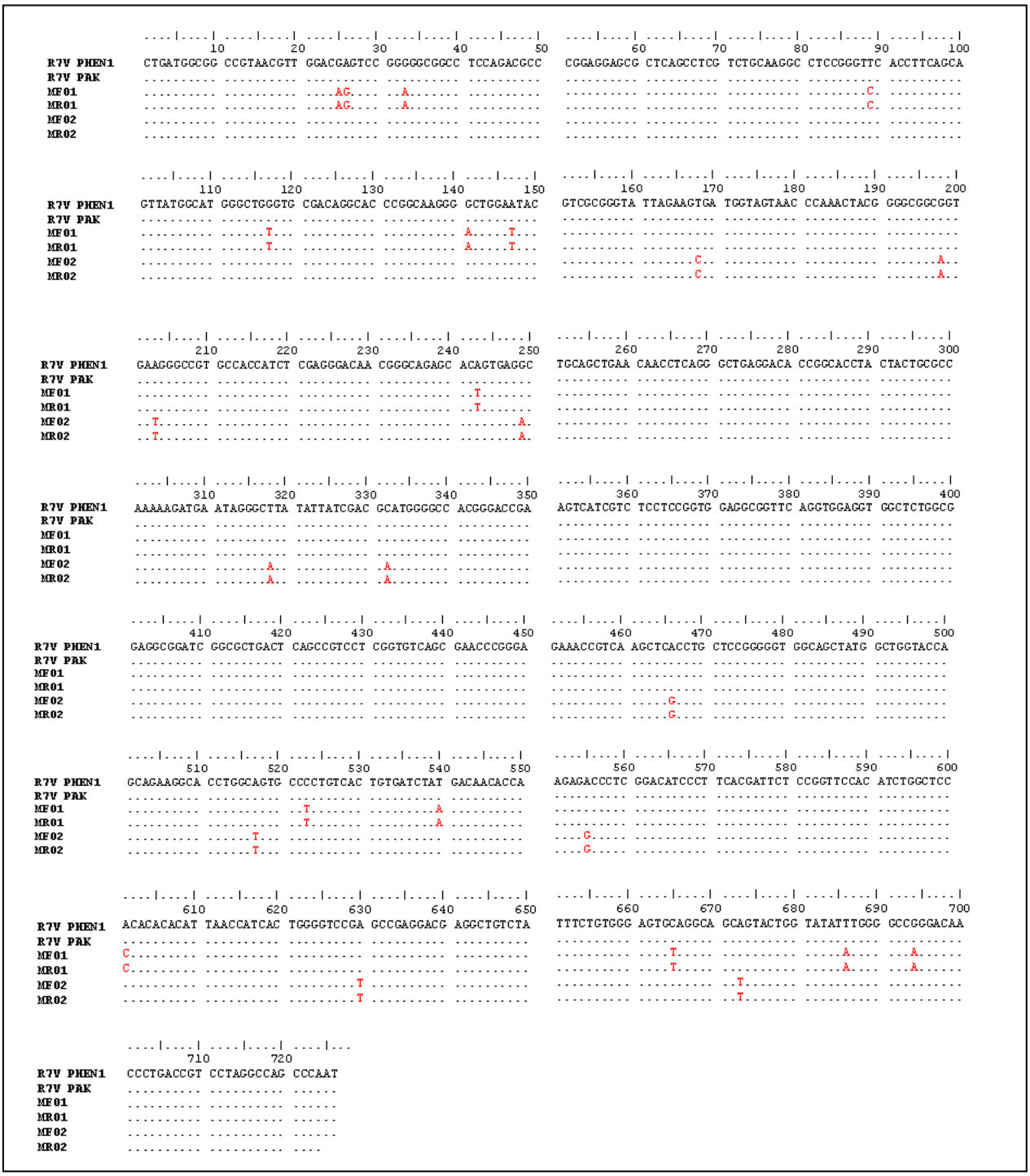


Figure 24: Nucleotide sequence of mutated anti-R7V scFv clones

***black dotes – same nucleotides as in parent gene**

*** nucleotides in red – mutated nucleotides**

R7V PHEN1 – Parent (non-mutated) anti-R7V DNA sequence in pHEN1 vector

R7V PAK – Parent (non-mutated) anti-R7V DNA sequence in pAKR7V400 vector

MF01/MR01 – Mutated clone 1 anti-R7V DNA sequence using forward and reverse primers

MF02/MR02 – Mutated clone 2 anti-R7V DNA sequence using forward and reverse primers

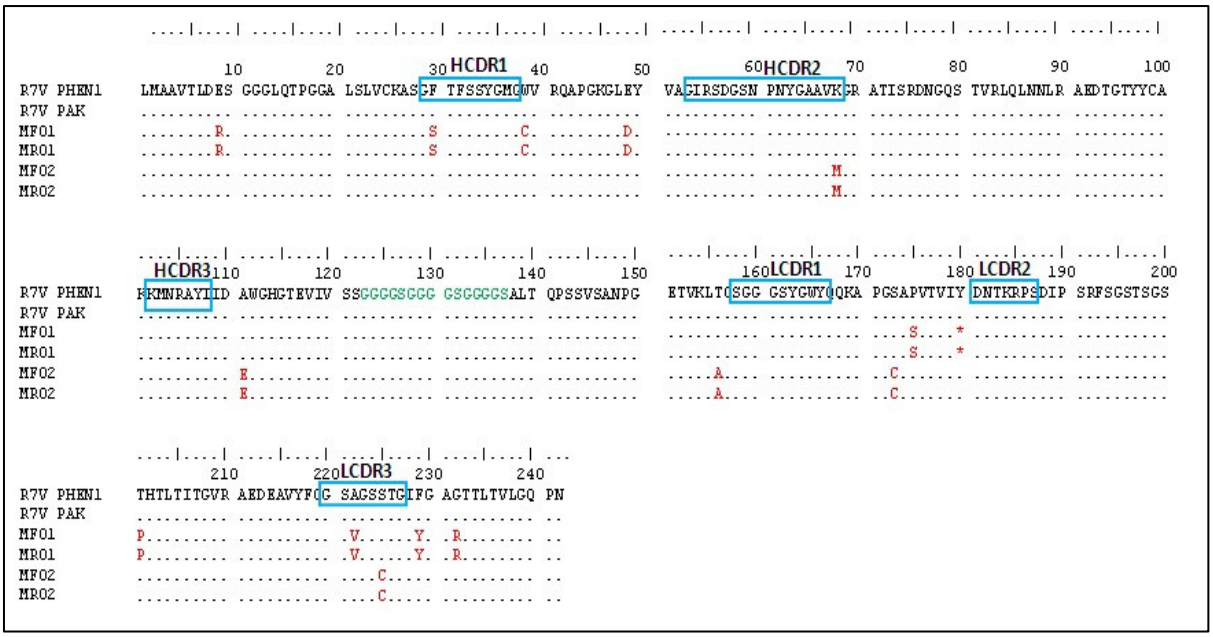
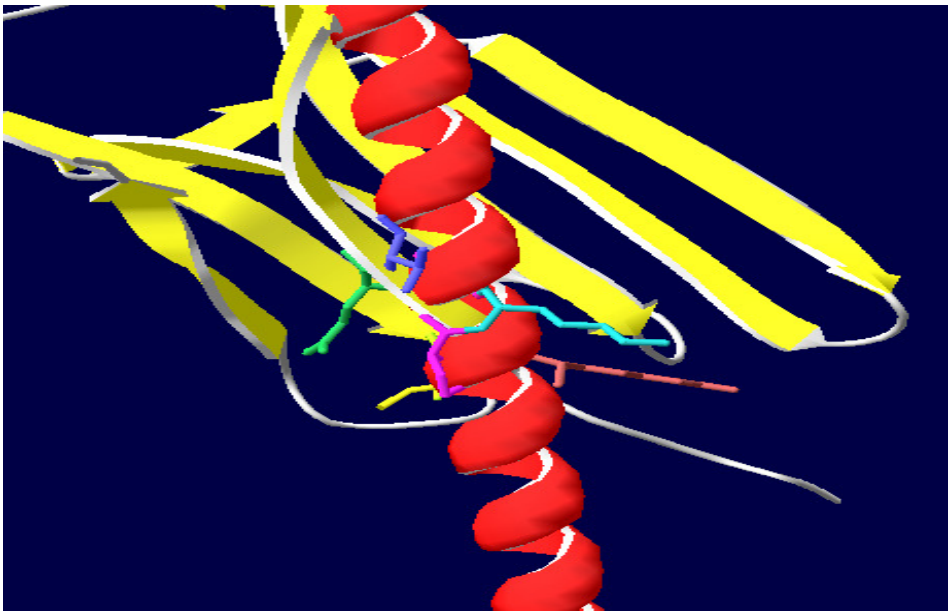


Figure 25: Amino acid sequence of mutated anti-R7V scFv clones [Desgnation of CDRs are as defined by Kabat database (Johnson and Wu, 2000)]

***black dotes**	**– a.a same as in parent gene**
*** a.a in red**	**– substituted a.a**
R7V PHEN1	– Parent anti-R7V a.a sequence in pHEN1 vector
R7V PAK	– Parent anti-R7V a.a sequence in pAKR7V400 vector
MF01/MR01	– Mutated clone 1 anti-R7V a.a sequence using forward and reverse primers
MF02/MR02	– Mutated clone 2 anti-R7V a.a sequence using forward and reverse primers

Swiss PDB Deep Viewer, 4.02 software was used to align the anti-R7V antibody fragment with the R7V epitope in human β2m (PDB entry 1LDS). The ribbon structure model of parent anti-R7V antibody (Figure 26 A) showed that the anti-R7V scFv binds to the R7V peptide at the N-terminal end of the template protein. Figure 26 B shows the interaction between the R7V epitope and the anti-R7V scFv antibody fragment using the backbone structure. Mutated clone 2 showed similar binding pattern as the parent anti-R7V scFv (Figure 28 A, 28 B) whereas mutated clone 1 did not bind to the peptide (Figure 27 A, 27 B).

A



B

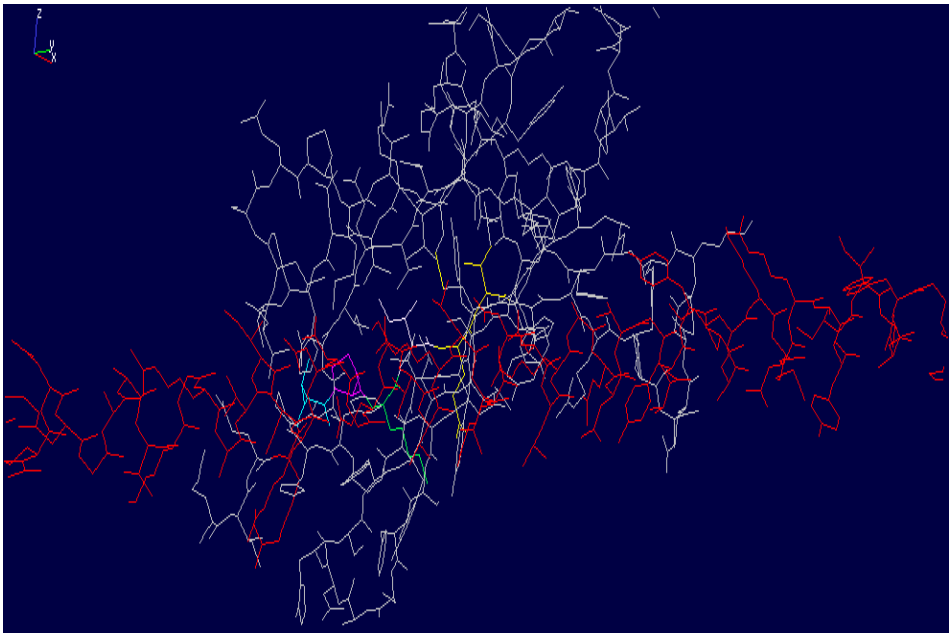
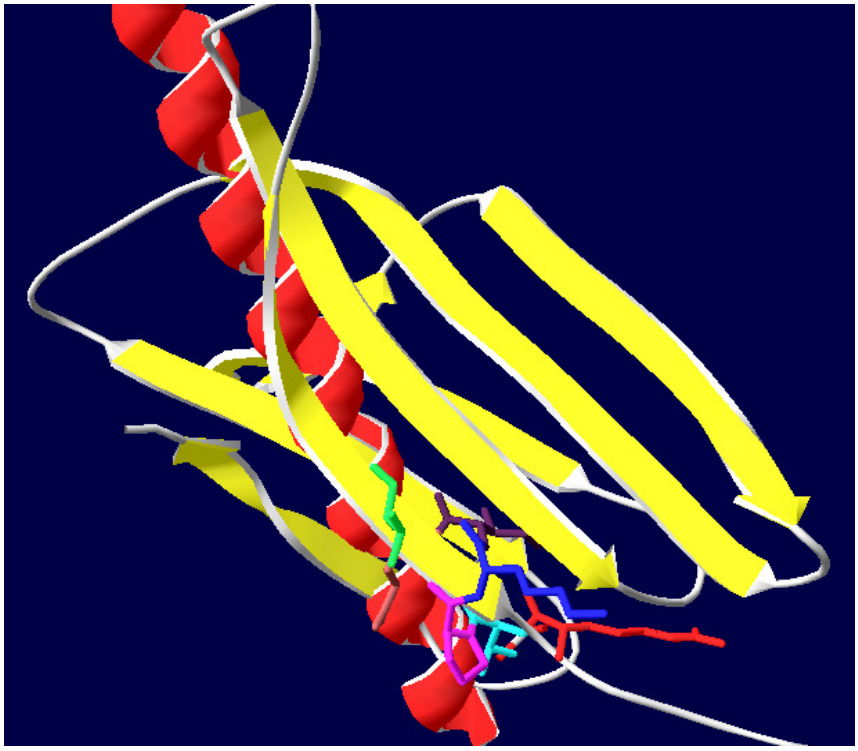


Figure 26: Anti-R7V antibody fragment (colored in red) interaction with R7V epitope (side chains with different colors) in 1LDS
A) Ribbon structure B) backbone + side chain structure

A



B

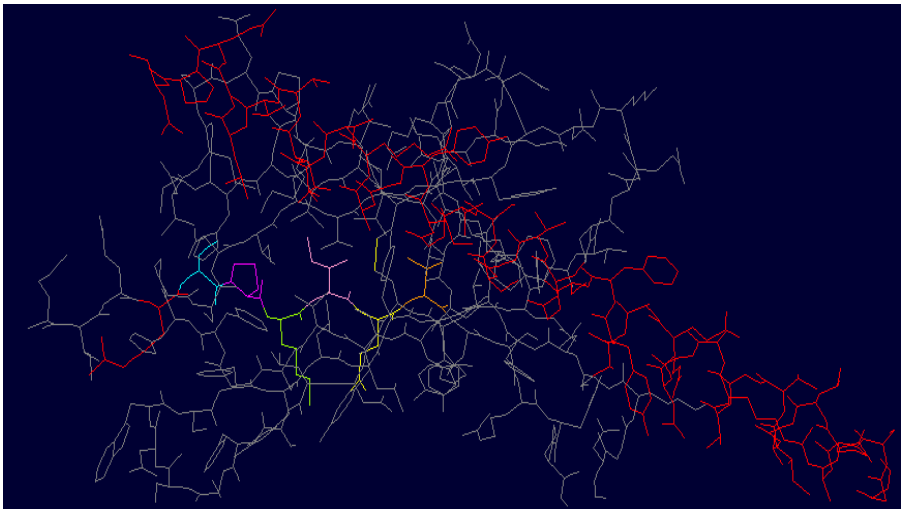
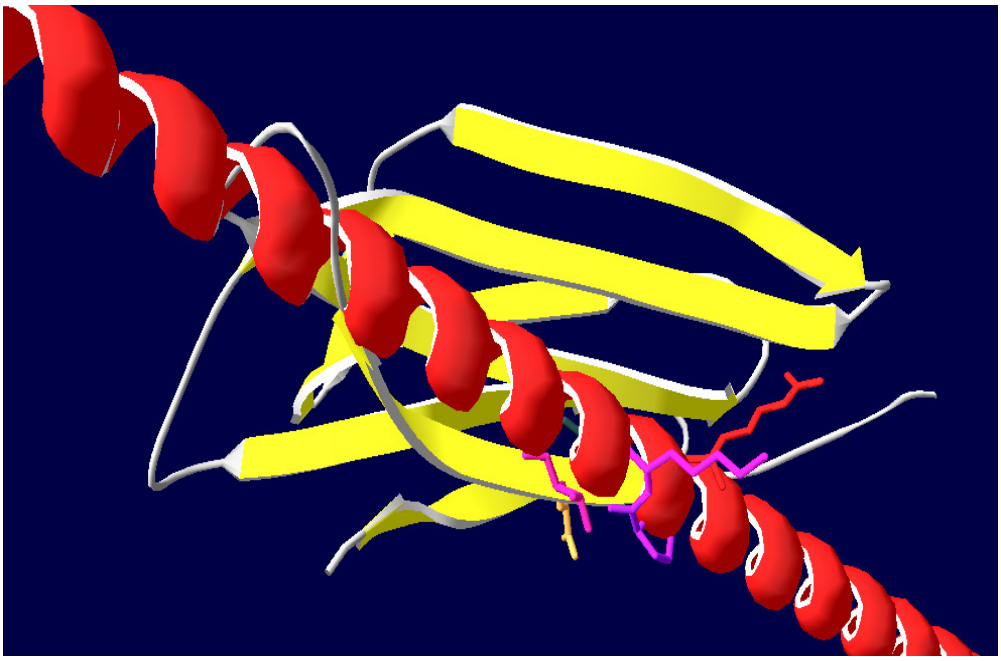


Figure 27: Mutated Clone 1 antibody fragment (colored in red) interaction with R7V epitope (side chains with different colors) in 1LDS
A) Ribbon structure B) backbone + side chain structure

A



B

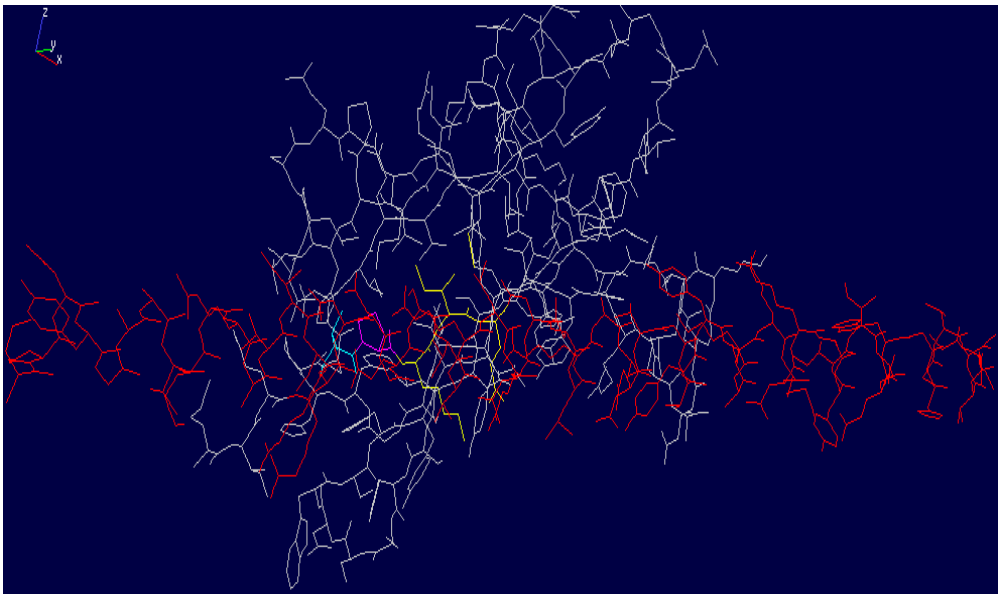
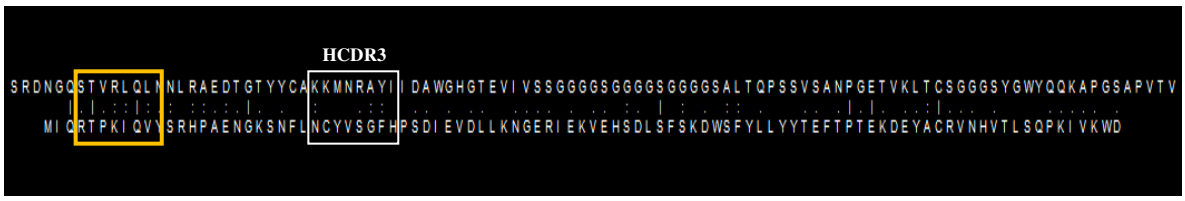


Figure 28: Mutated Clone 2 antibody fragment (colored in red) interaction with R7V epitope (side chains with different colors) in 1LDS
A) Ribbon structure B) backbone + side chain structure

Anti- R7V



Mutated Clone 1

Mutated Clone 2

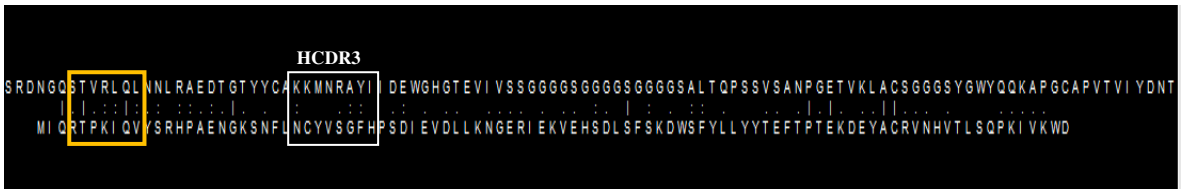


Figure 29: Antigen-binding site of anti-R7V and mutated scFv clones
*antigen-binding site: R7V epitope (RTPKIQV) - highlighted in orange box

The structural alignment results from the Swiss Deep Viewer 4.02 software (Figure 29) showed that the FR3 region on the heavy chain is involved in the interaction between the R7V epitope and the anti-R7V scFv antibody. The alignment results showed that mutated clone 2 binds at the same site (FR3 adjacent to HCDR3 region) whereas mutated clone 1 binds at an entirely different site (FR4 adjacent to LCDR1 region).

The different a.a substitutions in mutated clone 1 were as follows: Glu $(E)_9 \rightarrow$ Arg $(R)_9$, Phe $(F)_{30} \rightarrow$ Ser $(S)_{30}$, Trp $(W)_{39} \rightarrow$ Cys $(C)_{39}$, Glu $(E)_{49} \rightarrow$ Asp $(D)_{49}$ in the heavy chain domain and Pro $(P)_{175} \rightarrow$ Ser $(S)_{175}$, Tyr $(Y)_{180} \rightarrow$ stop codon $(*)_{180}$, Thr $(T)_{201} \rightarrow$ Pro $(P)_{201}$, Ala $(A)_{222} \rightarrow$ Val $(V)_{222}$, Phe $(F)_{229} \rightarrow$ Tyr $(Y)_{229}$, Gly $(G)_{232} \rightarrow$ Arg $(R)_{232}$ in the light chain domain (Figure 25).

The mutation of Arginine (Arg, R) in place of Glutamic acid (Glu, E) can cause steric hindrance due to the longer side chain of R group and also, electrostatic repulsion, caused by the oppositely charged amino acids. The R group of Arg is positively charged whereas the R group of Glu is negatively charged. A Serine (Ser, S) mutation instead of Phenylalanine (Phe, F) can cause

hydrogen bond formation with other side chains causing possible changes in the protein structure. Similar differences in protein structure can possibly be caused by the Trp→Cys mutation. Cysteine (Cys, C) would create a sulfhydryl bond in between the loops whereas Tryptophan (Trp, W) forms hydrogen bonds with neighbouring side chains causing a change in the protein structure. Trp are essential structural or contact residues (Lou and Marks, 2010). Mutation in position 180 was a stop codon, which truncates the protein making it non-functional. Since the stop codon mutation occurred outside of the antigen-binding site, this mutation may not have caused Clone 1 antibody to become non-functional. The mutation of Alanine (Ala, A) to Valine (Val, V) does not create a major impact as both amino acids are non-polar and form hydrogen bonds with other side chains. Glycine (Gly, G) to R can also cause steric hindrance due to the longer side chain of the R group (The structure of amino acids is provided in Appendix F). These mutations may have contributed to a conformational change in the CDR loops that prevented the mutated clone 1 from accessing the antigen-binding site.

Mutated clone 2 showed similar binding pattern as the parent scFv fragment. The binding affinity of mutated clone 2 was predicted from the structural alignment results. Three mutations were found in the antigen-binding site of mutated clone 2 namely; A_{110} → E_{110}, T_{156} → A_{156} and S_{173} → C_{173} (Figure 30).

Figure 30: Interaction of substituted and non-substituted amino acid residues with the target protein (using Swiss PDB Deep Viewer, 4.02)
*substituted a.a: highlighted in red boxes, antigen-binding site highlighted in orange box

In the parent anti-R7V antibody, Ala (A) interacts with Aspartic acid (Asp, D). A is a non-polar a.a whereas D carries a carboxyl group in their side chain and is negatively charged amino acid. The interaction between A and D would be weak due to their differences in binding properties (hydrogen bonding and electrostatic interactions). As can be seen in Figure 30, the single dot between A and D represents weak similarity and hence weak interaction between the two amino acids. In mutated clone 2, A is subsititued by E. Both E and D amino acids have carboxyl groups in their side chains and hence their interaction would be similar due to the hyrdogen bond formation between the two a.a. Figure 30 shows double dots between E and D, which implies there is stronger interaction between Clone 2 and the target protein as compared to the parent anti-R7V antibody fragment. The substitution of A to E can be considered as a gain-of-function mutation.

Second is the a.a substitution of A instead of Threonine (Thr, T). In the parent antibody, T binds to A on the target protein, which is a strong interaction as represented by the double dots between T and A (Figure 30). In mutated clone 2, A binds to A creating a strong hydrophobic interaction and hence interaction between the antibody and target protein will be stronger (implied by the single line between A and A, in Figure 30) compared to the T →A interaction in the parent R7V antibody. Thus, the substitution of T to A can also be considered as a gain-of-function mutation.

The third substitution of S to C can be considered as a loss-of-function mutation. In the parent R7V antibody, S binds to D on the target protein. S can from hydrogen bonds with the D, through its hydroxyl group thus creating a weak interaction between them as denoted by the single dot between S and D, in Figure 30. However, the C a.a contains a sulfhydryl group and cannot form hydrogen bonds with D and thus there is no interaction between the two amino acids (denoted by absence of a dot between C and D, in Figure 30).

Thus, from the structural alignment results it was predicted that mutated Clone 2 antibody fragment could have a higher antigen-binding affinity compared to the parent anti-R7V antibody fragment.

3.6 Protein Expression of anti-R7V scFv antibody fragment

The Gram-negative bacterium, *E.coli* is a reliable and cost-effective source of production of large quantities of functional protein. For laboratory research, *E.coli* is mostly the host of choice because of their ease of transformation and manipulation. Proteins expressed in bacteria are

either secreted into the cytoplasm, periplasm or culture supernatant (Charlton, 2004). In the study conducted by Depetris *et al*. (2008), they showed that scFv antibody fragments are mainly secreted into the bacterial periplasm during short-term expression periods (4 hr), while longer incubation time result in the secretion of the scFvs into the culture supernatant.

In this study, bacterial cells were incubated for 4 hr for efficient periplasmic protein expression. Generally, the expression of soluble antibodies are carried out at 30°C (Orfanoudakis *et al*., 1993; Depetris *et al*., 2008). For recombinant pHEN1 vector system, expression of the anti-R7V antibody fragments was carried out at 30°C. For pAK400 vector system, expression of the anti-R7V antibody fragments was carried out at 30°C and 24°C. The expression of soluble antibodies using pAK400 vector system was carried out at 24°C as stated in Krebber *et al.* (1997). Bacterial cells grown at a low temperature minimize periplasmic aggregation and increase the yield of the antibody fragments (Plückthun, 1994).

The expression of soluble scFv fragments were determined in crude periplasmic extracts using 12% SDS-PAGE analysis. Protein concentration of the samples was either determined using the Bradford assay (Appendix G) or using the Nanodrop spectrophotometer. Protein concentration of the samples was less than 1 µg/ml. This concentration was not sufficient for the visualization of the protein bands on SDS-PAGE using the Coomassie Brilliant Blue R-250 stain. Hence, the protein samples were concentrated using the acetone precipitation method. The advantage of precipitation method is that precipitation allows concentration as well as purification of protein sample from unwanted substances (Tech Tip #40 at www.thermo.com/pierce).

As can be seen in Figure 31 A, a prominent band was observed between 25 and 30 kDa for proteins expressed in *E.coli* HB2151 using recombinant pHEN1 vector (lane 2 and 3) as compared to the faint bands observed for proteins expressed in *E.coli* HB2151 using pAK400 vector (lane 6 and 7). The same holds true for proteins expressed in *E.coli* TG1 (Figure 31 B).

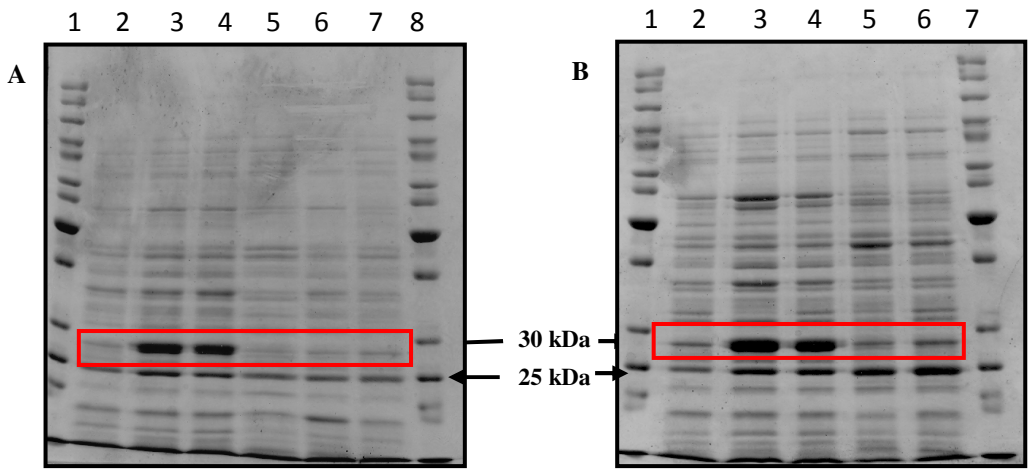


Figure 31: 12% SDS-PAGE gel of anti-R7V antibody fragment
A: Anti-R7V Protein Expresion in *E.coli* HB2151 [Lane 1: Molecular Mass Marker (Page Ruler Unstained Protein Ladder), Lane 2: *E.coli* HB2151 (total cell lysate), Lane 3: scFv expressed in pHEN1 (Induced), Lane 4: scFv expressed in pHEN1 (Uninduced), Lane 5: scFv expressed in pAK400 (Induced), Lane 6: scFv expressed in pAK400 (Induced), Lane 7: scFv expressed in pAK400 (Uninduced), Lane 8: Molecular Mass Marker (Page Ruler Unstained Protein Ladder)]
B: Anti-R7V Protein Expresion in *E.coli* TG1 [Lane 1: Molecular Mass Marker (Page Ruler Unstained Protein Ladder), Lane 2: *E.coli* TG1 (total cell lysate), Lane 3: scFv expressed in pHEN1 (Induced), Lane 4: scFv expressed in pHEN1 (Uninduced), Lane 5: scFv expressed in pAK400 (Induced), Lane 6: scFv expressed in pAK400 (Uninduced), Lane 7: Molecular Mass Marker (Page Ruler Unstained Protein Ladder)]

The western blot analysis was performed on anti-R7V antibodies expressed using pAK400 vector to verify the presence/absence of the protein. HisProbe-HRP was used to detect the presence of the desired protein.

The anti-R7V antibodies expressed using pAK400 vector carries a HIS tag at its C-terminal end. If the protein is expressed, the HIS probe would detect the tag and produce a single band between 25 and 30 kDa on the western blot. The pGEM HIS-tagged vector was used as a positive control in Western blot analysis. The expected size of HIS-tagged protein was 70 kDa. The western blot showed a positive band at 70 kDa for the HIS tag positive control. However, no protein bands were observed for the anti-R7V antibody fragments expressed using pAK400 vector system (Figure 32). The absence of the development of protein bands on the western blot confirmed that the pAK400 vector system failed to express the recombinant anti-R7V scFv fragments.

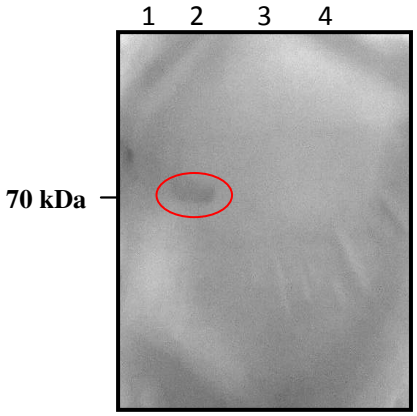


Figure 32: Western blot of anti-R7V scFv antibody fragment [Lane 1: Molecular Mass Marker (Page Ruler Unstained Protein Ladder), Lane 2: HIS tag positive control, Lane 3: Periplasmic extract of scFv protein in *E.coli* HB2151, Lane 4: Periplasmic extract of scFv protein in *E.coli* TG1]

An argument for no protein expression could be the use of the vector with strong transcription and translation initiation signals. Increased promoter strength does not increase the amount of soluble functional protein but increases the amount of insoluble functional protein. Protein folding plays an important role in the efficient expression of secreted proteins. The primary sequence of the antibody determines if the protein will fold to its native structure, aggregate or degrade. The expression of the soluble protein will be prominent if the sequence and structure of the antibody fragment allows for efficient folding (Plückthun, 1994; Kipriyanov, 2002).

Protein aggregation can be limited by growing the bacterial cells at low temperature. Bacterial cells grown at low temperature has been shown to decrease aggregation and increase the yield of soluble scFvs. Alternatively, the bacterial cells can be grown under osmotic stress, for example, addition of 0.4 M sucrose increases the yield of soluble scFvs by 80-fold. These conditions were followed during the protein expression study. However, the periplasmic expression of scFv fragment, using pAK400 phage vector at 24°C (Appendix D) or at 30°C (Figure 31), was not observed.

The bacterial growth medium used for protein expression was 2xTY media with 2% glucose. The 2xTY broth gives a 4-fold increase in the yield of soluble scFvs as compared to the LB broth. In future, 2xTY media with 0.2% glycerol may be used as the bacterial growth medium for the protein expression. Protein expression is enhanced by the addition of glycerol instead of glucose. Glycerol at a concentration of 0.2% (v/v) significantly increased the expression of scFv fragment as compared to glucose (0.2%, w/v). Also, the induction time should be monitored

every two hours to determine the optimal time required for better expression of protein (Su *et al*., 2003).

Other factors that may affect the protein expression include the stability of the scFv fragments and rate of protein secretion, which are temperature dependent. Proteolysis is another factor that can limit the expression of correctly folded soluble proteins. Transcription and translation steps can also limit the expression of the protein (Plückthun, 1994).

3.7 Presence and binding activity of anti-R7V scFv antibody fragment

Anti-R7V scFv antibody fragments expressed in *E.coli* TG1 and HB2151 strains using recombinant pHEN1 vector system were detected using SDS-PAGE. A prominent single band between 25 and 30 kDa were visible for both induced and uninduced samples. Proteins induced with IPTG are supposed to show a thicker band compared to the uninduced proteins. However, SDS-PAGE analysis showed similar bands for both induced and uninduced (Figure 31).

ELISA was performed to confirm that the proteins expressed were indeed the anti-R7V antibody fragments. Anti-R7V fragment carries a c-myc tag at its C-terminal end. An anti-c-myc:HRP conjugated antibody was used to detect the anti-R7V antibodies. The ELISA graph (Figure 33) clearly shows a binding signal for the protein sample, which is only possible if the expressed protein carries a c-myc tag. The signal obtained was higher compared to the signal obtained from the positive control (anti-c-myc antibody). This in turn confirms that anti-R7V protein was expressed. The antigen-binding affinity of the expressed protein could not be determined, as the R7V antigen had lost its activity. As compared to the signal obtained from the negative control (blocking buffer), no signal was observed with the antigen, which implies that the pure antigen was degraded/became inactive.

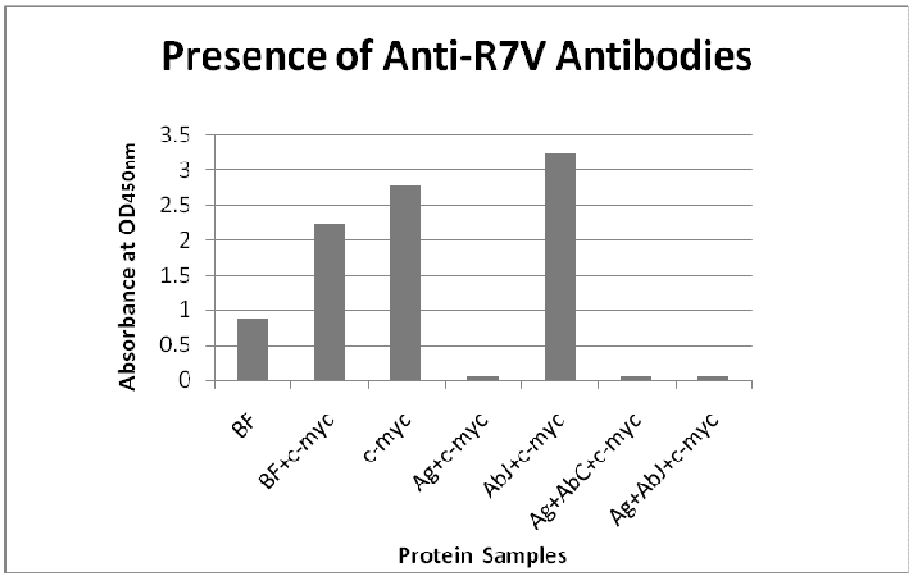


Figure 33: Graph showing the presence of anti-R7V antibodies in expressed protein samples

BF – blocking buffer (used as blank)
BF+c-myc – blocking buffer and c-myc tag (used as blank)
c-myc – anti c-myc tag (used as positive control)
Ag+c-myc – R7V antigen with anti c-myc (used as negative control)
AbJ+ c-myc – expressed anti-R7V antibody with anti c-myc
Ag+AbC+c-myc – R7V antigen with expressed anti-R7V antibody and anti c-myc
Ag+AbJ+c-myc – R7V antigen with expressed anti-R7V antibody and anti c-myc

The above graph shows that the antigen does not bind to the plate; confirmed by the absence of a signal in the antigen-antibody complex and antigen-c-myc complex (Ag+c-myc, Ag+AbC+c-myc and Ag+AbJ+c-myc). This implies the pure antigen was degraded/became inactive. However, the expressed anti-R7V antibody fragments binds to the anti-c-myc antibody. This confirms that the desired protein was expressed. Because of low resource, further experiments to determine the antigen-antibody affinity (K_d value) using a new active R7V antigen and the statistical significance of the ELISA results could not be conducted.

Chapter 4

Concluding Discussion

It has been almost 30 years since the discovery of the HIV virus, an invader of the human immune system. HIV infection remains a pandemic. HIV is a retrovirus that causes AIDS in humans. HIV virus attacks the CD4 T cells, impairing the immune response (Alimonti *et al*., 2003). To date, about 33 million people are living with HIV worldwide (UNAIDS, 2010).

Measures taken to contain the spread of HIV are successful to a certain extent. ARV drugs developed to slow the HIV reproduction and progression to AIDS are very expensive and unavailable to most infected people, especially in poor countries (UNAIDS, 2010). Recent economic crisis also has negatively affected the public health investment on HIV treatment. The evolution of drug resistant HIV strains has also paused challenges in the eradication of this virus. Due to its high replication, mutation and recombination rate, the virus is able to evolve rapidly within an individual and hence overcome the natural immune responses and immune responses caused by vaccine (Bangham and Phillips, 1997).

The main challenge for HIV treatment is to develop a HIV vaccine that can elicit both humoral and cellular mediated immune responses. However, it is difficult to elicit these responses with the already discovered antibodies. Nonetheless, β2m (the target cellular protein of study in this project) is believed to be useful because the antibodies directed against this protein can act both directly on the virus (humoral) and on the cells associated with β2m (cellular).

The β2m protein, found on the surface of the HIV, has many overlapping peptides. These peptides were tested for its ability to reverse the neutralizing action of mAbs directed against the protein. The heptamer, R7V (RTPKIQV) was found to be the most efficient in reversing the neutralization action of mAbs (LeContel *et al*., 1996). Antibodies directed to the R7V epitope (anti-R7V antibody) were naturally present in HIV infected individuals. Studies have shown that anti-R7V have a protective function (Galéa and Chermann, 1998; Haslin and Chermann, 2002; Sanchez *et al*., 2008). Anti-R7V antibodies were reported to be responsible for the non-progression state of HIV, making it an efficient marker of non-progression HIV infection (Galéa and Chermann, 1998; Sanchez *et al*., 2008). However, a study conducted by Margolick *et al.* (2010) showed there was no correlation between the presence of anti-R7V antibody and non-progression of HIV-1 infection. On the contrary, they found that individuals with anti-R7V

antibody had advanced HIV-1 infection, as evident from the lower CD4 count and high plasma viral loads (Margolick *et al*., 2010). Also, a study conducted by Bremnœs (MSc Thesis, 2010) showed there was no difference in the presence of anti-R7V antibodies between ARV-treated individuals compared to drug naive individuals or progressors compared to non-progressors. This study suggested the use of anti-R7V antibodies as diagnostic rather than prognostic markers. The use of anti-R7V antibody in therapeutics has also been suggested (Galéa *et al*., 1999a; Haslin and Chermann, 2002; Haslin *et al*., 2007). The anti-R7V antibody produced using the baculovirus expression system was able to neutralize all HIV-isolates, including the drug-resistant HIV strains. They also showed that anti-R7V antibodies do not cause an autoimmune response (Haslin *et al*., 2007). However, there has been no report to date on the use of anti-R7V antibodies in *in vivo* therapeutics studies (Bremnœs, MSc Thesis, 2010). The current literature on anti-R7V antibodies clearly shows that more extensive research are required to maintain optimism on its use as diagnostic or therapeutic tools.

This dissertation describes the production of high affinity anti-R7V scFv antibody fragments for the detection of R7V antigen in ELISA (diagnostic tool) and for the neutralizing activity against HIV-1 (therapeutic tool).

In this study, antibodies targeted against the conserved R7V epitope was selected from a combinatorial chicken-based phage library, called the *Nkuku*® phage library. The antibodies were selected using R7V antigen through a bio-panning process. The recombinant anti-R7V antibody fragments produced in the laboratory were used in the HIV-1 subtype C virus neutralization assay (Bremnœs, MSc Thesis, 2010). The recombinant anti-R7V antibody fragments were able to detect the R7V antigen by ELISA, but were not able to neutralize the virus. The antigen-binding affinity of the antibody fragments obtained was not sufficient for its use in neutralization assay.

In vitro display technologies such as phage and yeast display has been used for the selection of antibodies for over 20 years. Antibodies obtained using this technology illustrates properties that are difficult to obtain by immunizing animals. Many antibodies produced this way are in clinical use, with many more in the line (Bradbury *et al*., 2011).

Phage display technology involves the selection of antigen-specific antibody from a large library that consists of a variety of antibody genes constructed, *in vitro*, using PCR. Antibody against a specific antigen is selected by a process called biopanning. In biopanning, the phage vector carrying the library is incubated with immobilized antigens. Bound phages are eluted and

amplified in *E.coli* while the unbound phages are washed away. This selection cycle is repeated 3 - 4 times for obtaining high antigen-specific antibodies. Antigen-specific antibodies with low affinity are used to create a secondary library that contains higher affinity antibodies, through a process known as affinity maturation (Azzazy and Highsmith, 2002). Affinity maturation improves the function of antibody fragments for their various applications (Daugherty *et al*., 2000).

Orfanoudakis *et al*. (1993) compared the efficacy of antibody fragments derived from bacteria to that derived from hybridomas. They showed that bacterially produced antigen binding fragments (Fabs) were as effective as the hybridomas-derived mAbs. The production of large quantities of antibody fragments with potent neutralizing activity at low cost is a significant advance of phage display technology. These antibody fragments has the potential to be used in therapeutics (Orfanoudakis *et al*., 1993). Initially, phage display was used for the isolation of antibodies against diverse target molecules. This technology has since then developed into an efficient technique used for *in vitro* manipulation of antibody affinity, specificity and stability (Benhar, 2001).

In vitro phage display technology increases the antibody affinity by introducing random mutations into the variable antibody genes, thus mimicking the in vivo process of somatic hypermutation. Mutations are introduced by chain shuffling, EP PCR, DNA shuffling or use of mutator strain of *E.coli*. Mutations in the CDR regions are an effective way for increasing the antibody affinity. In *in vivo* somatic hypermutation, mutations usually occur in the CDR regions as compared to the FR regions (Loh *et al*., 1983). The V_H CDR3 and V_L CDR3 regions are involved in high-energy interactions with the antigen. Mutations in these regions may increase the affinity (by replacing the low affinity residue with a more favourable one) or abolish the antigen-binding activity (Novotny *et al*., 1989).

In this study, the EP PCR method was chosen for the affinity maturation of the anti-R7V antibody fragments. The EP PCR is a common technique used to introduce random point mutations in a gene. EP PCR utilizes the natural error rate of *Taq* DNA polymerase, along with suboptimal buffer conditions during amplification to introduce mutations (Thie, 2010).

The EP PCR method generated two defined mutated anti-R7V antibody clones. The mutated clones were subcloned into pAK400 vector. The pAK400 vector is an improved expression vector system that allows unidirectional cloning using unique *Sfi*I restriction sites and enhanced protein expression using the stronger SDT7g10 sequence (Krebber *et al*., 1997). The binding

affinity is determined using the crude cell supernatant in ELISA (Friguet *et al*, 1985). However, the mutated anti-R7V scFv fragments were not expressed using the pAK400 vector system.

Since the mutated clones could not be expressed using pAK400 vector (discussed in section 3.6), structural interpretation of the affinity improvement was performed based on the models created using the Swiss PDB Deep Viewer 4.02

Sequence analysis of mutated anti-R7V antibody clones revealed a low mutation rate (5 to 10 mutations). Majority of the mutations were found in the FR regions. FR regions are usually not involved in antigen binding. However, they are essential to maintain the integrity of the antigen-binding site (Kumagai and Tsumoto, 2001). Clone 1 had 10 a.a substitutions and Clone 2 had 5 a.a substitutions. From the structural alignment between the antibody and target protein, it was predicted that mutated Clone 2 would have a higher antigen binding affinity compared to the parent R7V antibody fragment.

Mutated Clone 1 antibody fragment did not bind to the epitope. Mutations in Clone 1 may have altered the confirmation of CDR loops, thereby preventing the antibody from accessing the antigen-binding site.

On the other hand, mutated Clone 2 antibody fragment were able to bind to the antigen-binding site in a similar pattern as the parent R7V antibody fragment. Three substitutions, Ala H111 Glu, Thr L156 Ala and Ser L173 Cys, were identified in close proximity with the peptide. These substitutions contributed to hydrogen bonding, electrostatic and hydrophobic interactions with the peptide.

The aim of this study was partially achieved. Since the periplasmic protein expression of anti-R7V scFv failed, the *in vitro* ELISA experiments using crude cell supernatant on antigen-binding affinity and HIV subtype C neutralization study could not be conducted.

High affinity antibodies are essential for the development of a simple and highly sensitive diagnostic tool to detect even small amounts of the target antigen. The future perspective for this study includes performing more rounds of EP PCR, followed by selection of high affinity binders using phage display technology. The antigen-antibody affinity can be measured using common ELISA or SPR.

HIV infection triggers antibody, cytotoxic T lymphocyte (CTL) and CD4 T helper cell immune responses. The cytotoxic T-lymphocytes (CTLs) help to eradicate the virus by lowering the rate

of replication. The CTLs and T_h cells are able to recognize the short peptide fragments on the antigen that binds to the MHC molecules. Thus, from the immune responses studies on viral infections, an effective vaccine would require antibodies, CTLs and T_h cells for optimum protection against the virus. However, vaccines that contain antibodies from recombinant proteins generally fail to elicit CTL response (Bangham and Phillips, 1997). More studies related to the efficacy of anti-R7V antibodies are required before it can be used as a vaccine.

For therapeutic purposes, human mAbs are desirable. However, human antibodies are not easily available and are limited to infected or vaccinated individuals. An alternative is to obtain antibodies from immunized animals and humanize them. Chickens are a viable source of humanized antibodies since many of the highly conserved proteins in mammalian species (human, mice and rabbits) are less conserved or absent in avian species. Therefore, the long-term goal of anti-R7V vaccine development can be attained using the *Nkuku*® chicken phage library. The choice of chicken as the source of antibody is beneficial as it provides human antigen specific antibodies which are difficult to obtain otherwise with mice or rabbits (Andris-Widhopf *et al*., 2000).

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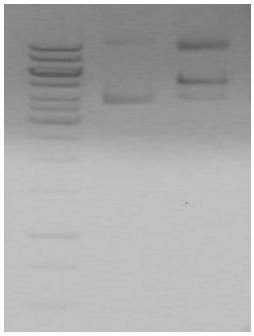
Appendix

A: Agarose gel electrophoresis (1%) of fast digest *Sfi*I restriction enzyme double digested recombinant pHEN1 phagemid DNA (using 4 U of the enzyme)

1 2 3



Lane 1: Molecular Mass Marker (O'Gene Ruler 1kb DNA ladder),

Lane 2: Undigested recombinant pHEN1 DNA

Lane 3: *Sfi*I (4 U) digested recombinant pHEN1 DNA

B: Re – ligated recombinant pHEN1 phagemid DNA sequence

GGCCCAGCCGGCCCTGATGGCGGCCGTAACGTTGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGA
GGAGCGCTCAGCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGCAGTTATGGCATGGGCTGGGTGCG
ACAGGCACCCGGCAAGGGGCTGGAATACGTCGCGGGTATTAGAAGTGATGGTAGTAACCCAAGCTAC
GGGGCGGCGGTGAAGGGCCGTGCCACCATCTCGAGGGACAACGGGCAGAGCACAGTGAGGCTGCAGC
TGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGCGCCAAAAAGATGAATAGGGCTTATATT
ATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCCGGTGGAGGCGGTTCAGGTGGAGGTG
GCTCTGGCGGAGGCGGATCGGCGCTGACTAGCCGTCCTCGGTGTCAGCGAACCCGGGAGAAACCGTC
AAGCTCACCTGCTCCGGGGGTGGCAGCTATGGCTGGTACCAGCAGAAGGCACCTGGCAGTGCCCCTGT
CACTGTGATCTATGACAACACCAAGAGACCCTCGGACATCCCTTCACGATTCTCCGGTTCCACATCTGG
CTCCACACACACATTAACCATCACTGGGGTCCGAGCCGAGGACGAGGCTGTCTATTTCTGTGGGAGTG
CAGGCAGCAGTACTGGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGCCAGCCCAATGCGGCC
GCAGAACAAAAACTCATCTCAGAAGAGGATCTGAAT

GGCCCAGCCGGCC = *Sfi*I CTCGAG = *Xho*I GCGGCCGC = *Not*I

GAACAAAAACTCATCTCAGAAGAGGATCTGAAT = c-myc tag

C: Sequence of anti-R7V scFv gene amplified using gradient PCR at an annealing temperature of 69°C

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                    ....|....|....|....|....|....|....|....|....|....|
                            410        420        430        440        450
R7V                 ---------- ---------- -----GGCCC AGCCGGCCC- TGATGGCGGC
PAK69F              ---------- ---------- ---------- ---------- --------AG
PAK69R              CCGGTGTTT- CTCTTGAAAA --ATC..... .........C ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            460        470        480        490        500
R7V                 CGTAACGTTG GACGAGTCCG GGGGCGGCCT CCAGACGCCC GGAGGAGCGC
PAK69F              GC.CGTT... .G........ .C........ .......... ..........
PAK69R              .......... .......... .......... .......... ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            510        520        530        540        550
R7V                 TCAGCCTCGT CTGCAAGGCC TCCGGGTTCA CCTTCAGCAG TTATGGCATG
PAK69F              .......... .........G -......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            560        570        580        590        600
R7V                 GGCTGGGTGC GACAGGCACC CGGCAAGGGG CTGGAATACG TCGCGGGTAT
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            610        620        630        640        650
R7V                 TAGAAGTGAT GGTAGTAACC CAAACTACGG GGCGGCGGTG AAGGGCCGTG
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........
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                    ....|....|....|....|....|....|....|....|....|....|
                            660        670        680        690        700
R7V                 CCACCATCTC GAGGGACAAC GGGCAGAGCA CAGTGAGGCT GCAGCTGAAC
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

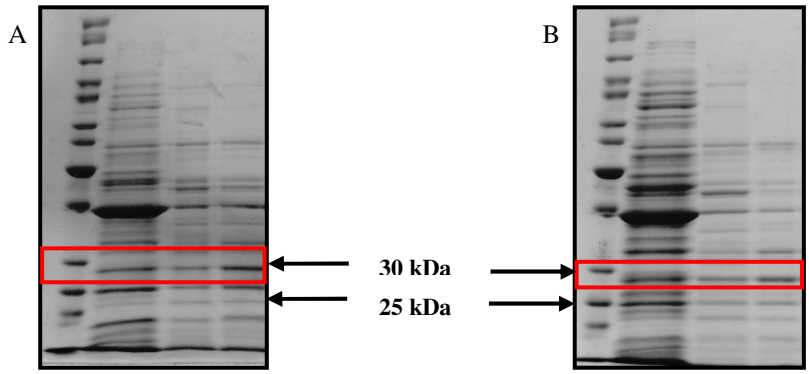
                    ....|....|....|....|....|....|....|....|....|....|
                            710        720        730        740        750
R7V                 AACCTCAGGG CTGAGGACAC CGGCACCTAC TACTGCGCCA AAAAGATGAA
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            760        770        780        790        800
R7V                 TAGGGCTTAT ATTATCGACG CATGGGGCCA CGGGACCGAA GTCATCGTCT
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

                    ....|....|....|....|....|....|....|....|....|....|
                            810        820        830        840        850
R7V                 CCTCCGGTGG AGGCGGTTCA GGTGGAGGTG GCTCTGGCGG AGGCGGATCG
PAK69F              .......... .......... .......... .......... ..........
PAK69R              .......... .......... .......... .......... ..........

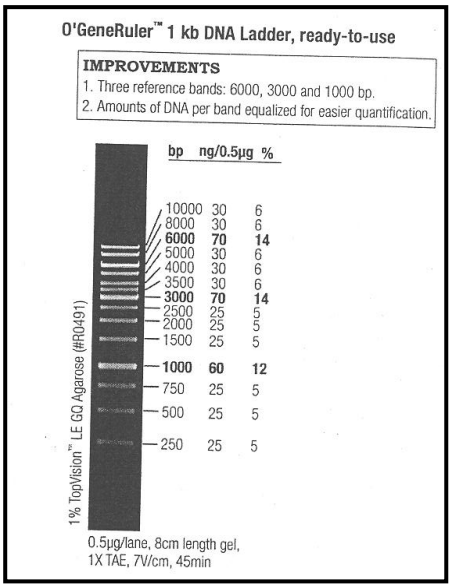
                    ....|....|....|....|....|..
                           1160       1170       1180
R7V                 CTAGGCCAGC CCAATGCGGC CGCAGAACA- --
PAK69F              .......... .......GGC CTCGGGGGGG CC
PAK69R              TG-------- ---------- ----------
```

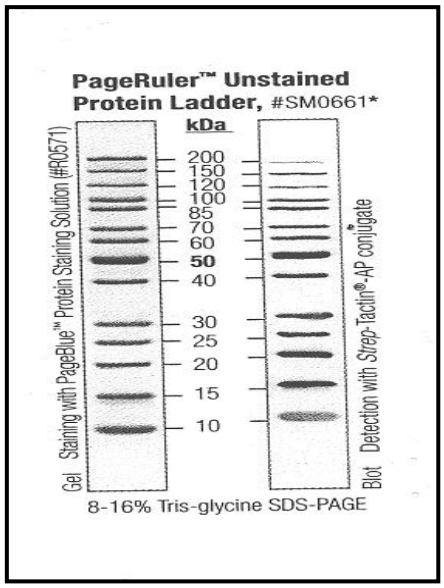
D: Anti-R7V Protein expression using pAK400 vector system at 24°C



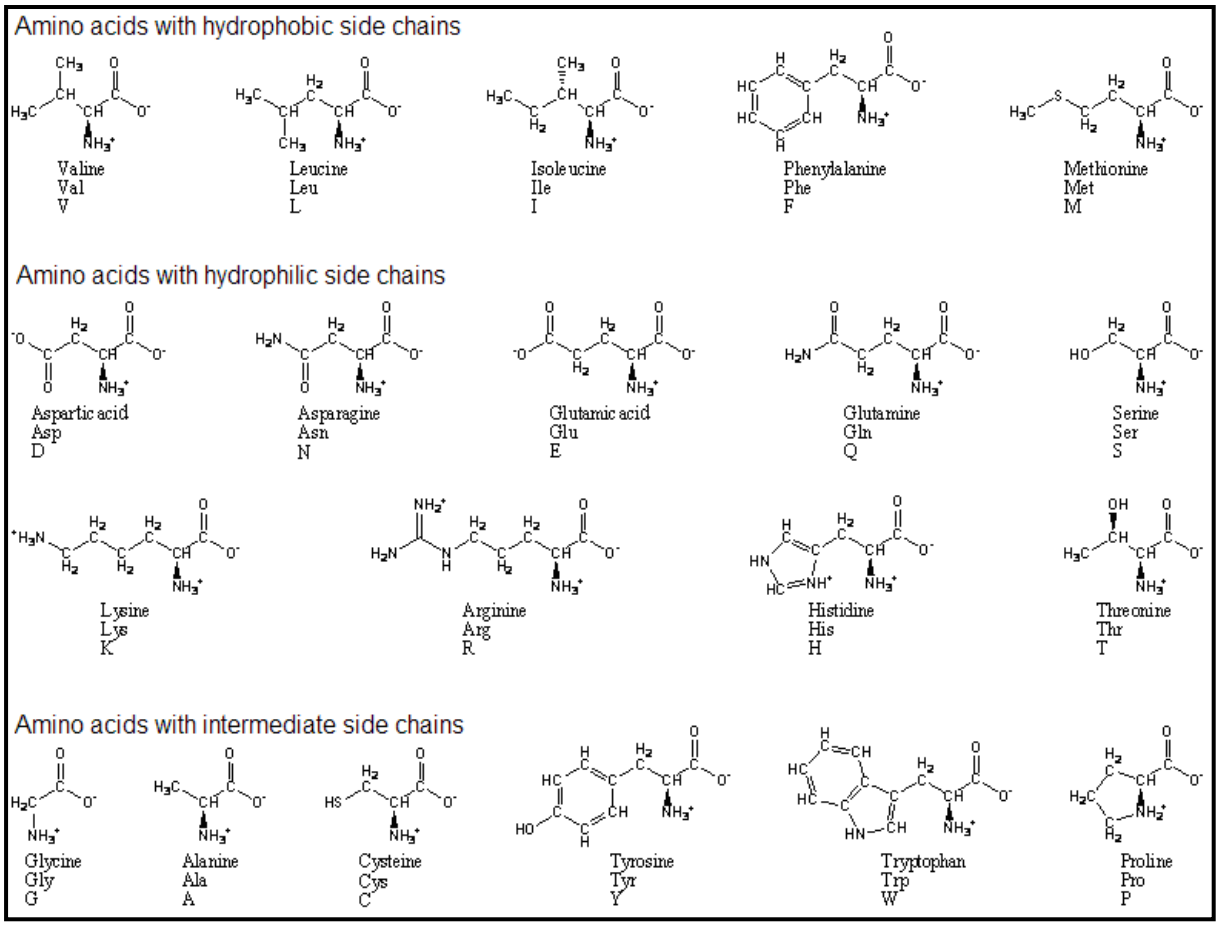
12% SDS-PAGE gel of anti-R7V antibody fragment
A: Anti-R7V Protein Expresion in *E.coli* HB2151 (Lane 1: Molecular Mass Marker (Page Ruler Unstained Protein Ladder), Lane 2: *E.coli* HB2151 (total cell lysate), Lane 3: scFv expressed in pAK400 (Induced), Lane 4: scFv expressed in pAK400 (Uninduced)
B: Anti-R7V Protein Expresion in *E.coli* TG1 (Lane 1: Molecular Mass Marker (Page Ruler Unstained Protein Ladder), Lane 2: *E.coli* TG1 (total cell lysate), Lane 3: scFv expressed in pAK400 (Induced), Lane 4: scFv expressed in pAK400 (Uninduced)

E: DNA and protein marker used in the study





F: Structure of amino acids



G: BSA standard curve used for the protein concentration determination