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***In vitro* selection and characterisation of human anti-HIV-1
antibody fragments**

Jie Tang

School of Life Sciences

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***In vitro* Selection and Characterisation of
Human Anti-HIV-1 Antibody Fragments**

Jie Tang

**A thesis submitted in partial fulfilment of the requirements of the University of
Westminster for the degree of Doctor of Philosophy**

May 2011

Abstract

Generation of neutralising antibodies with broad specificity would be one of the effective approaches to control HIV-1 spread. It is clear that a method that allows rapid generation of neutralising antibodies is needed. This project aims at developing a novel approach to rapidly access human anti-HIV-1 antibodies *in vitro* by using ribosome display and selection from DNA libraries of HIV-1 patients.

Two single-chain antibody libraries (M325 and K530) were constructed from two HIV-1 long-term non-progressors, whose sera showed cross-neutralising activities against various HIV-1 strains across a range of clades. In each library, total RNA was extracted from blood of each donor and used to synthesise cDNA. Families of 4 κ light chains, 9 λ light chains and 8 heavy chains were generated by using RT-PCR amplification. These fragments were then assembled with all possible combinatorial pairs to form diversified repertoires in the form of VL-link-VH-partial CH.

Both libraries were subjected to ribosome display for *in vitro* selection of functional antibodies. Ribosome display is a cell-free technique used to generate proteins that can bind to an immobilised antigen. During this process, the translated proteins are associated with their mRNAs, enabling a simultaneous selection of functional proteins and their gene. The employment of ribosome display facilitated rapid screening of two large libraries against recombinant gp120 (generated from patient K530).

Ten selected antibodies were expressed as single-chain variable fragments in *Escherichia coli*. High activity antibodies were purified from both total cell extract and periplasmic fraction using optimised expression and purification conditions. These antibodies showed various binding activities against gp120 and modest neutralising activities against a laboratory HIV-1 clone. Remarkably, an identical CDR3 sequence was observed in a number of selected antibodies from the two separated libraries, indicating a strong selection of functional antibodies by ribosome display.

This study has provided a novel, *in vitro* method to select potentially neutralising monoclonal antibodies against HIV-1.

Declaration

I hereby declare that the work described in this thesis is the result of my own original studies carried out in the School of Life Sciences, University of Westminster, except where published work has been cited and where collaborators have been acknowledged.

This work has not been submitted or accepted in substance, in part or in any form, for any other degree.

Jie Tang

Acknowledgements

I would like to express my most sincere gratitude to my supervisors Dr. Angray Kang, Dr. Mingyue He (The Babraham Institute) and Prof. Áine McKnight (Queen Mary, University of London) for their profound supervision and continuous support throughout my PhD. They have not only taught me many laboratory techniques, but also provided valuable discussions on this thesis. Without their guidance, help and encouragement, this PhD would not have been possible.

I would also like to thank Dr. Simon Jeffs (Imperial College) for providing recombinant gp120 and Dr. Hanna Dreja (Queen Mary, University of London) for testing patient sera and running neutralisation assays for the study.

I have enjoyed my lab work with many helps from Dr. Anatoliy Markiv, Bernard Anani, Armaghan Azizi and many other teachers, students and staffs from University of Westminster.

I am always grateful to my dear parents and husband for their endless love, care and encouragement. They are always there and more importantly, they have never stopped supporting me.

Finally, but not least, I would like to thank Cavendish Research Scholarship for the financial support of this project.

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Abbreviations

Abbreviation	Full name
ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immune deficiency syndrome
AP	Alkaline phosphatase
ARM	Antibody-Ribosome-mRNA
ART	Antiretroviral therapy
ARV	AIDS-associated retroviruses
bp	Base pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CA	Capsid
CD4	Cluster of differentiation 4
CDC	Centres for Disease Control and Prevention
cDNA	Complementary DNA
CDR	Complementarity-determining region
CH	Constant region of heavy chain
<i>cpx</i>	Complex
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
DIS	Dimerisation initiation site
DMEM	Dulbecco's modified Eagle medium
dNTP	Deoxyribonucleotide triphosphate
EBV	Epstein-Barr virus
<i>E. coli</i>	<i>Escherichia. Coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
ES	Elite suppressor
Fab	Antigen-binding fragment
Fc	Fragment crystallisable

FCS	Foetal calf serum
FR	Framework
Gag	Group-specific antigen
Gp120	Glycoprotein 120
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HTLV	Human T-cell leukemia virus
HVTN	HIV Vaccine Trials Network
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilised metal affinity chromatography
IN	Integrase
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KDa	Kilodaltons
LAV	Lymphadenopathy-associated virus
LB	Luria Bertani
LTNP	Long-term non-progressor
LTR	Long terminal repeat
MA	Matrix
MAb	Monoclonal antibody
MPER	Membrane-proximal external region
mRNA	Messenger RNA
NBT	Nitro blue tetrazolium
NC	Nucleocapsid
Nef	Negative factor
NIH	National Institutes of Health
NK	Natural killer
OD	Optical density
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
PNPP	p-Nitrophenyl phosphate

Pol	Polymerase
PR	Protease
RFP	Red fluorescent protein
Rev	Regulator of virion expression
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
ScFv	Single-chain variable fragment
SD	Shine-Dalgarno
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
Tat	Transactivator of transcription
TBST	Tris buffered saline Tween 20
TCLA	T-cell line adapted
TEMED	Tetramethylethylenediamine
TH	T helper
TNF	Tumour-necrosis factor
tRNA	Transfer RNA
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
VH	Variable region of heavy chain
Vif	Virus infectivity factor
VL	Variable region of light chain
V κ	Variable region of kappa chain
V λ	Variable region of lambda chain
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1

Introduction

1.1 AIDS/HIV Background

1.1.1 The epidemic of AIDS

The human immunodeficiency virus (HIV) is a member of the genus *Lentivirus* in the *Retroviridae* family that causes acquired immune deficiency syndrome (AIDS). The Joint United Nations Programme on HIV/AIDS (UNAIDS) reported that more than 25 million people have died from AIDS in the world, and an estimate of 33.3 million people have been infected by HIV, including 2.6 million new cases in 2009 (UNAIDS, 2010). Although antiretroviral therapy (ART) has greatly increased the survival rate and quality of life among infected individuals, only 5.2 million people can access, leaving 65% of the population who still need treatment today (UNAIDS, 2010). Moreover, an effective vaccine is still an elusive goal, despite considerable research efforts that have been attempted over the past three decades.

1.1.2 The discovery of HIV

The initial articles related to AIDS were published by the Centres for Disease Control and Prevention (CDC) in 1981 (CDC, 1981a, CDC, 1981b). AIDS symptoms were originally noticed in homosexual males. Subsequently, similar symptoms were observed in haemophiliacs, intravenous drug users and heterosexual Haitians in the following years (Selik *et al.*, 1984). The term AIDS designated by the CDC was first used in 1982, when 593 people were diagnosed with the syndrome, with 243 deaths (CDC, 1982).

HIV was first isolated in 1983, when Barré-Sinoussi and her colleagues at the Pasteur Institute recovered a virus from the lymph node of a French patient who had persistent generalised lymphadenopathy, a disease that was suspected to be associated with AIDS (Barre-Sinoussi *et al.*, 1983). This virus was later called lymphadenopathy-associated virus (LAV) by Montagnier (Montagnier *et al.*, 1984b), who shared the Nobel Prize in Physiology or Medicine 2008 with Barré-Sinoussi for their discovery of HIV. LAV contained reverse transcriptase (RT) activity and shared some characteristics of human

T-cell leukaemia virus (HTLV) (Barre-Sinoussi *et al.*, 1983), which made some early investigators to believe that HTLV was isolated from AIDS patients (Gallo *et al.*, 1983). However, LAV grew to substantial titre in CD4⁺ cells and killed them, instead of immortalising the lymphocytes in culture (Montagnier *et al.*, 1984a). In early 1984, Gallo and associates isolated another human retrovirus from the peripheral blood mononuclear cells (PBMCs) of AIDS patients (Gallo *et al.*, 1984). This virus was named HTLV-III but later was confirmed as the same as LAV (Chang *et al.*, 1993). At the same time, Levy and co-workers reported their identification of retroviruses from different AIDS groups, as well as from asymptomatic individuals (Levy *et al.*, 1984). These viruses were named the AIDS-associated retroviruses (ARVs), and it was the first time a healthy carrier state for the AIDS virus was observed. The three prototype viruses, LAV, HTLV-III and ARV, were soon recognised as belonging to the same group of retroviruses, which was given a separate name HIV by the International Committee on Taxonomy of Viruses in 1986 (Coffin *et al.*, 1986).

A second AIDS virus was later discovered from patients in West Africa, particularly the Cape Verde Islands and Senegal (Clavel *et al.*, 1986). This virus differs by more than 55% from the previously isolated HIV-1 strains, and was designated as HIV-2. The genome of HIV-2 is similar to that of HIV-1 (figure 1.1), except that the viral protein U (*vpu*) gene is restricted to HIV-1 and the viral protein X (*vpx*) gene is restricted to HIV-2 (Cohen *et al.*, 1988, Tristem *et al.*, 1990). HIV-2 is less pathogenic than HIV-1. Individuals infected by HIV-2 usually survive longer without showing the disease, maintain a higher level of CD4⁺ cells and show a reduced rate in transmission than those infected with HIV-1 (Kanki *et al.*, 1992, Reeves and Doms, 2002, Donnelly *et al.*, 1993). Therefore, most of the studies have been focused on HIV-1.

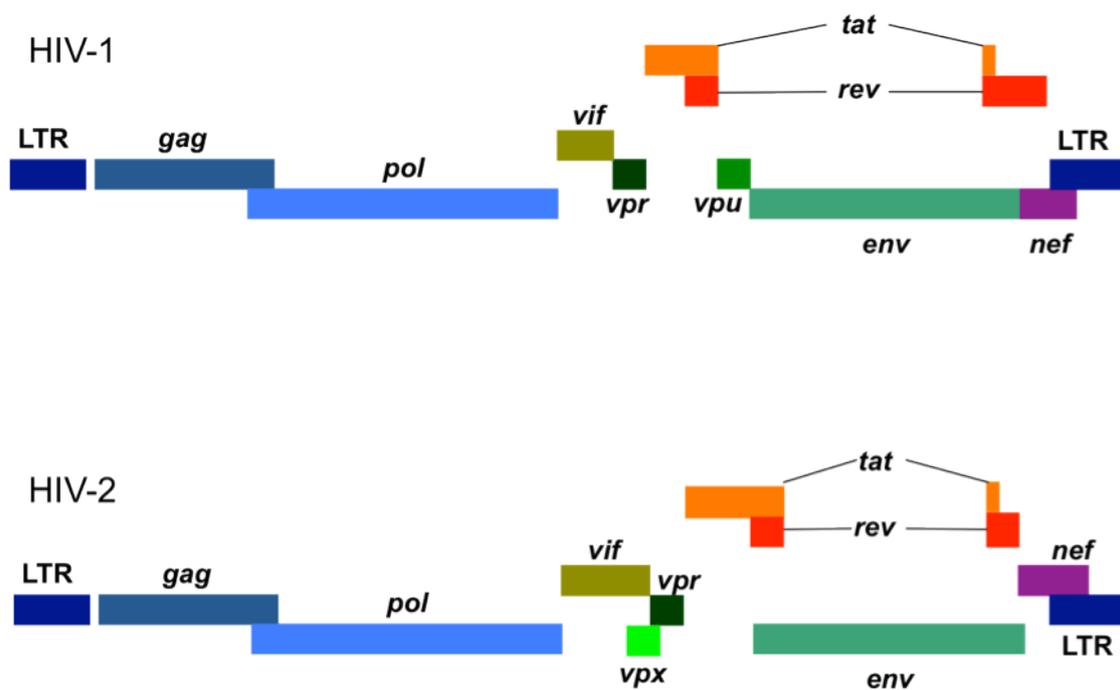


Figure 1.1 Genomic maps of HIV-1 and HIV-2

The genome of HIV-1 and HIV-2 are very similar, except that the *vpu* gene is restricted to HIV-1 and the *vpx* gene is restricted to HIV-2.

1.1.3 The heterogeneity of HIV

HIV is featured by its extensive heterogeneities in biology and serology, which is reflected by the variable genetic sequences of the virus. The viral RT is very error prone during replication (Preston *et al.*, 1988, Roberts *et al.*, 1988). Research by Coffin indicated that HIV replicates on almost a daily basis and up to 10 base changes in the HIV genome can occur in a single replicative cycle (Coffin, 1995). In addition, recombination of two or more different HIV strains may occur in a single cell, generating a mosaic DNA genome (McCutchan, 2006). The total viral genomic sequences can differ by 6% to 10% among different individuals (Levy, 2007). The high mutation rate and diverse genome give rise to enormous changes on viral phenotype, especially the regulatory and envelope proteins (Martins *et al.*, 1991). The envelope protein presented on the surface of HIV can vary up to 35% between subtypes and 20% within subtypes (Gaschen *et al.*, 2002).

Based on the full-length viral genome sequencing and amino acid analysis, HIV-1 can be classified into four groups: the “major” group M, the “Outlier” group O, a “non-M/non-O” group N and a recently discovered group P (Robertson *et al.*, 2000, Plantier *et al.*, 2009). More than 90% of HIV-1 infections belong to HIV-1 group M, which can be further divided into at least nine subtypes (clades): A, B, C, D, F, G, H, J and K (figure 1.2). Each subtype differs from the others by at least 15 to 20% in amino acid sequences in the group-specific antigen (Gag) and envelope glycoprotein (Env) region (Robertson *et al.*, 2000). In some cases, recombinant viruses become epidemiologically important branches and they are called circulating recombinant forms (CRFs) (McCutchan, 2006). Viruses recombining from four or more subtypes are called complex (*cpx*). Currently 16 CRFs have been recognised from the group M (Peeters *et al.*, 2003, Robertson *et al.*, 2000). Two previously designated HIV-1 subtypes, E and I, were renamed CRF_01AE and CRF_04cpx respectively when they were identified recombinants (Anderson *et al.*, 2000). The subtypes responsible for the majority of global infections are A, B, C, D, CRF01_AE and CRF02_AG (Robertson *et al.*, 2000). Group O individuals were initially found in Cameroon (Peeters *et al.*, 1997) and accounts for about 25% of viral isolates from Cameroon (Ayoubu *et al.*, 2001). Group N infections were only found in a few cases in Cameroon and this group appears to be more similar to chimpanzee simian immunodeficiency virus (SIV) than other HIV-1

groups (Peeters *et al.*, 2003). A new group of HIV-1 isolates (group P) was identified from a Cameroonian woman in 2009 (Plantier *et al.*, 2009). This virus is closely related to gorilla SIV and genetically distinct from HIV-1 groups M, N and O.

Although HIV-2 is very rare compared to HIV-1 and is located in a few countries in West Africa, eight distinct groups of HIV-2 (A to H) have been identified. Group A and B account for the most prevalence of HIV-2, while the other six groups have only one representative infection (Chen *et al.*, 1997, Yamaguchi *et al.*, 2000, Damond *et al.*, 2004). Differences between HIV-2 groups are nearly as much as that between the M, N, O and P groups of HIV-1, with up to 25% variation in the amino acid sequences of the Gag, Polymerase (Pol) and Env (Zagury *et al.*, 1988).

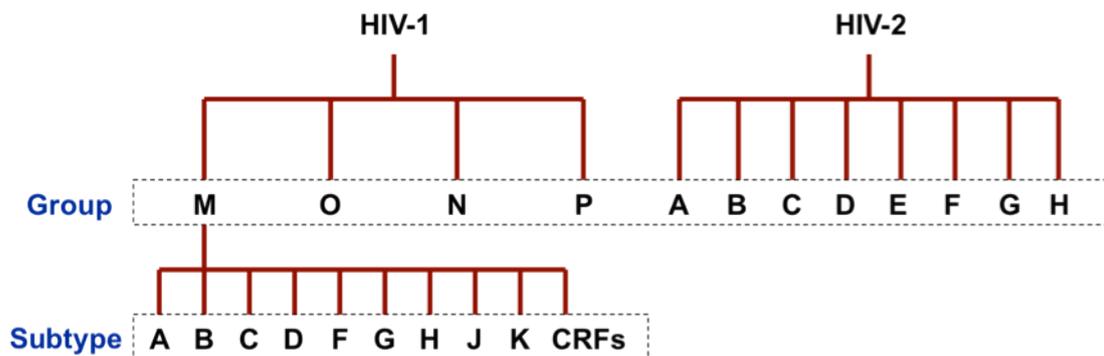


Figure 1.2 The different levels of HIV classification

1.1.4 The origin of HIV

Based on molecular evolutionary studies of primate lentiviruses, it has been proposed that HIV-1 came into the human population from primates about 30 to 100 years ago (Sharp *et al.*, 2001). Other investigators argued that this virus could have appeared in human population earlier (Leigh Brown and Holmes, 1994). Although there are several explanations and hypotheses for the origin of HIV-1 presented, the most common and generally accepted idea is that HIV-1 was derived from SIV found in chimpanzees (Gao *et al.*, 1999). It is generally proposed that the HIV-1 groups M, N and O entered the human population by three separate cross-species transmissions of chimpanzee SIV (Huet *et al.*, 1990, Corbet *et al.*, 2000). Studies on recently isolated SIV samples provided evidence that group M most likely originated from chimpanzees in South Eastern Cameroon, while group N originated from chimpanzees in South Central Cameroon (Keele *et al.*, 2006). By contrast, no direct evidence has been found to link a SIV isolate to HIV-1 group O yet (Keele *et al.*, 2006). Recently, viruses isolated from the Western Lowland Gorilla resembled group O (Van Heuverswyn *et al.*, 2006), suggesting that gorillas may be involved in the transmission of SIV to humans.

HIV-2 is genetically close to SIV isolates in monkeys in West Africa, especially sooty mangabeys (Marlink, 1996). It is believed that the two most prevalent groups A and B were originated from sooty mangabeys from Ivory Coast (Santiago *et al.*, 2005).

1.1.5 The structure of HIV

HIV exists as roughly spherical shape (figure 1.3) with a diameter of approximately 100 to 120 nm (Kuznetsov *et al.*, 2003). The viral proteins are designated with numbers reflecting the protein sizes in kilodaltons (kDa). A mature virus is surrounded by a lipid bilayer membrane, on which about 70 trimeric Envs are embedded (Chan *et al.*, 1997, Kuznetsov *et al.*, 2003). The Env consists of an external surface glycoprotein, gp120 and a transmembrane glycoprotein, gp41, both derived from a 160 kDa precursor glycoprotein, gp160 (McCune *et al.*, 1988).

Three structural Gag proteins are located inside the virus: matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7) (Freed, 1998). The MA forms an inner shell just inside the viral membrane; recent evidences suggested that MA might be a regulatory protein involved in enhancing HIV pathogenesis (Li *et al.*, 2010). The CA protein constitutes a conical core inside MA, coating two identical copies of single-stranded RNA. The NC interacts with viral RNA and is required for RNA splicing and RNA encapsidation (Zhang and Barklis, 1995). All these three Gag proteins are cleaved from a polyprotein precursor, p55, by the viral protease (PR) (Mervis *et al.*, 1988, Kohl *et al.*, 1988).

The two copies of RNA are located inside the capsid (p24) and are linked together at the 5' end (Jossinet *et al.*, 1999). The dimerisation initiation site (DIS) on the linkage is a hairpin structure and plays a role in virus maturation and recombination (Balakrishnan *et al.*, 2003). The 5' and 3' end of HIV RNA encode a long terminal repeat (LTR) sequence, which regulates integration and virus replication (Temin, 1981, Vicenzi *et al.*, 1994). There are three enzymes closely associated with the viral RNA: the reverse transcriptase (RT, p66, p51), the protease (PR, p10) and the integrase (IN, p32). RT is also called RNA-dependent DNA polymerase, and plays an important role in viral replication by transcribing the RNA into double-stranded DNA (Baltimore, 1970). The PR cleaves viral proteins into their functional forms. The IN incorporates the viral DNA into host cell chromosomal DNA (Brown *et al.*, 1989). All three enzymes are cleaved from Pol precursor polyprotein (Jacks *et al.*, 1988).

HIV also has two regulatory proteins, transactivator of transcription (Tat, p14) and regulator of virion expression (Rev, p19), which are essential for viral replication (Fisher *et al.*, 1986, Sodroski *et al.*, 1986). Tat is a major protein that up-regulates HIV replication. It also induces T cell apoptosis (Westendorp *et al.*, 1995) and co-receptors expression on cell surfaces (Huang *et al.*, 1998), and blocks natural killer (NK) cell activities (Zocchi *et al.*, 1998). Rev affects viral protein expression by regulating messenger RNA (mRNA) splicing and transporting unspliced mRNA to the cytoplasm of cell for protein translation (Malim *et al.*, 1988, Malim *et al.*, 1989).

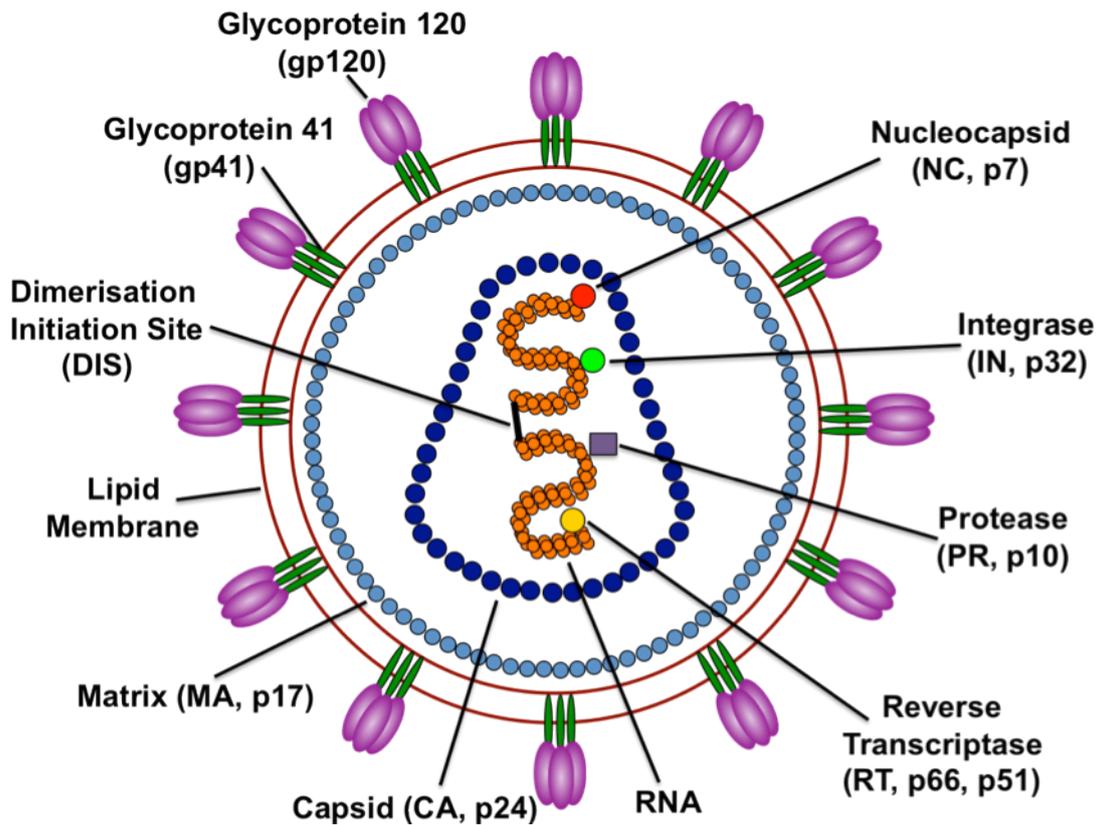


Figure 1.3 Structure of HIV

Trimeric glycoprotein gp120 and gp41 are embedded on HIV surface membrane. From outside to viral centre are structural proteins matrix (MA, p17) and capsid (CA, p24). Inside CA are two copies of single-stranded RNA, linked at their 5' end by dimerisation initiation site (DIS). Structure protein nucleocapsid (NC, p7) and three enzymes reverse transcriptase (RT, p66, p51), protease (PR, p10) and integrase (IN, p32) are presented inside the viral core.

In addition, two accessory proteins closely associated with the core, namely negative factor (Nef, p27) and virus infectivity factor (Vif, p23). Nef regulates virus replication (Garcia and Miller, 1991) and activates cellular proteins (Sawai *et al.*, 1994) while Vif increases virus infectivity (Strebel *et al.*, 1987) and cell-to-cell transmission (Fisher *et al.*, 1987), and helps in proviral DNA synthesis and assembly (Borman *et al.*, 1995). Other accessory proteins include viral protein R (Vpr, p15), viral protein U (Vpu, p16, only presents on HIV-1) and viral protein X (Vpx, p15, only presents on HIV-2), which mainly help in virus replication, virus release and viral infectivity, respectively (Greene and Peterlin, 2002).

1.1.6 The life cycle of HIV

The life cycle of HIV starts by gp120 attaching to its cellular receptor cluster of differentiation 4 (CD4) (Dalglish *et al.*, 1984). The CD4 receptor is expressed on the surface of T helper cells, macrophages and dendritic cells. It has four immunoglobulin like domains (D1 to D4), in which D1 region is involved in HIV binding (Arthos *et al.*, 1989). Gp120 consists of five variable domains (V1-V5) with five constant domains (C1-C5), and the outer surface of gp120 is heavily glycosylated (Wei *et al.*, 2003). A major binding site has been identified to locate on C4 domain near the 3' end of gp120 (Lasky *et al.*, 1987, Sweet *et al.*, 1991). Studies showed that other discontinuous, conserved regions also interact with the binding sites on CD4 (Thali *et al.*, 1993).

After attachment to the CD4 protein, a conformational change occurs on the gp120, leading to interactions between the gp120 and its co-receptors (figure 1.4). HIV generally needs chemokine receptors for additional attachment, the most common ones are CCR5 for macrophage-tropic isolates (Cheng-Mayer *et al.*, 1997) and CXCR4 for T cell line-tropic isolates. The viruses that use these co-receptors are referred to as R5 and X4 viruses, respectively (Berger *et al.*, 1998). The co-receptor binding and tropism are mainly determined by the V3 loop of gp120, as well as the V1 and V2 regions (Hartley *et al.*, 2005). Mutation studies showed that as few as three amino acid changes in the V3 loop were sufficient to change R5 virus to X4 virus (Shioda *et al.*, 1992). The attachment to co-receptors brings the virus closer to the cell, and exposes gp41 to a fusion domain on the cell (Sattentau and Moore, 1991).

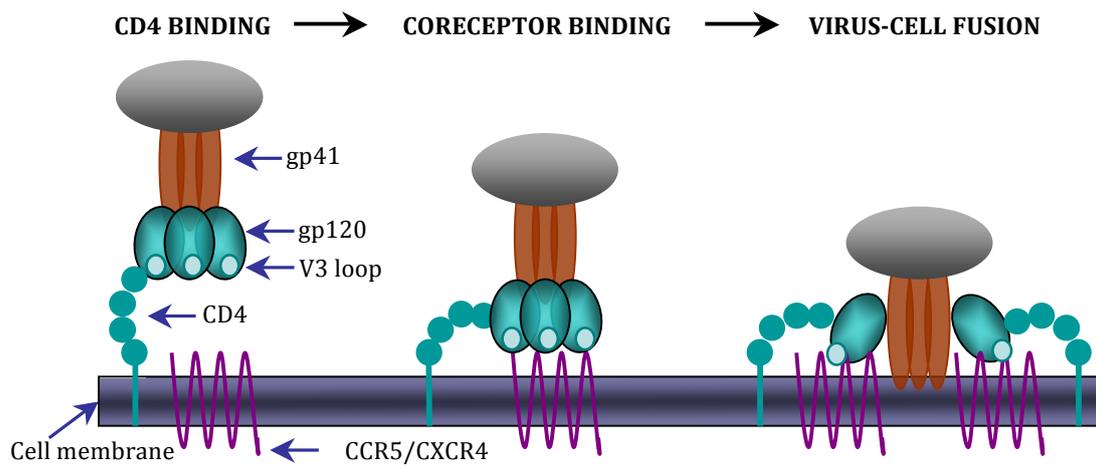


Figure 1.4 A schematic illustration of HIV entry

HIV entry can be divided into three steps: (1) gp120 attaches to CD4 receptor; (2) conformational change in gp120, which induces gp120 to bind to co-receptor, CCR5 or CXCR4; (3) structural rearrangement in gp41 allows the virus and cell membrane fusion and ultimately entry of the virus into the target cell.

Virus and CD4⁺ cell fusion is a critical step in HIV life cycle. The fusion between viral and the host cellular membranes allows the entry of viral capsid into the cell (Pascual *et al.*, 2005). Mutation studies indicated that several domains of gp120 (the V1/V2 domain, the V3 loop and the C4 domain) and gp41 could contribute to membrane fusion (Sullivan *et al.*, 1993, Page *et al.*, 1992, Suphaphiphat *et al.*, 2007). However, the exact mechanism for the nucleocapsid entry remains unclear. Hypotheses include intermixing of the outer lipid membranes of the virus and the cell (i.e. semifusion) (Haywood, 1994), and involvement of Gag protein in viral core entry (Spearman *et al.*, 1994).

After HIV has entered the cell, the reverse transcription of viral RNA starts by formation of a RNA-DNA hybrid helix (Baltimore, 1970), followed by the synthesis of a second strand of cDNA by RT. The double-stranded viral DNA is then transported to the cell nucleus, where it is integrated into host cell DNA by viral integrase (IN) (Brown *et al.*, 1989, Bushman *et al.*, 1990). The integrated viral DNA is known as proviral DNA, and it can be dormant for a long time. Upon activation, the proviral DNA is transcribed to generate mRNA by the host cell RNA polymerase II. The primary mRNA transcripts contain multiple introns and can be processed to yield more than 30 alternative mRNAs by various levels of splicing (Schwartz *et al.*, 1990). Doubly spliced mRNA can translate to the major regulatory proteins, particularly Nef, Tat and Rev (Greene and Peterlin, 2002); partially spliced mRNA has the potential to express the Env precursor (gp160), Vif, Vpu and Vpr; the unspliced mRNA can be expressed as Gag and Gag-Pol precursor proteins or serve as the genomic RNA. The gp120 and gp41 is in fact generated by an endoprotease cleavage of gp160 precursor before transportation to the plasma membrane of the cell (Hallenberger *et al.*, 1992). The two nascent viral RNA are also transported to the plasma membrane with Gag and Gag-Pol precursor proteins. Assembly and package of the virion takes place by budding through plasma membrane of the cell (Freed and Martin, 1996, Booth *et al.*, 2006). During this process, the Gag and Gag-Pol precursor proteins are cleaved by protease to form the proper HIV proteins, which result in a mature virion (figure 1.5).

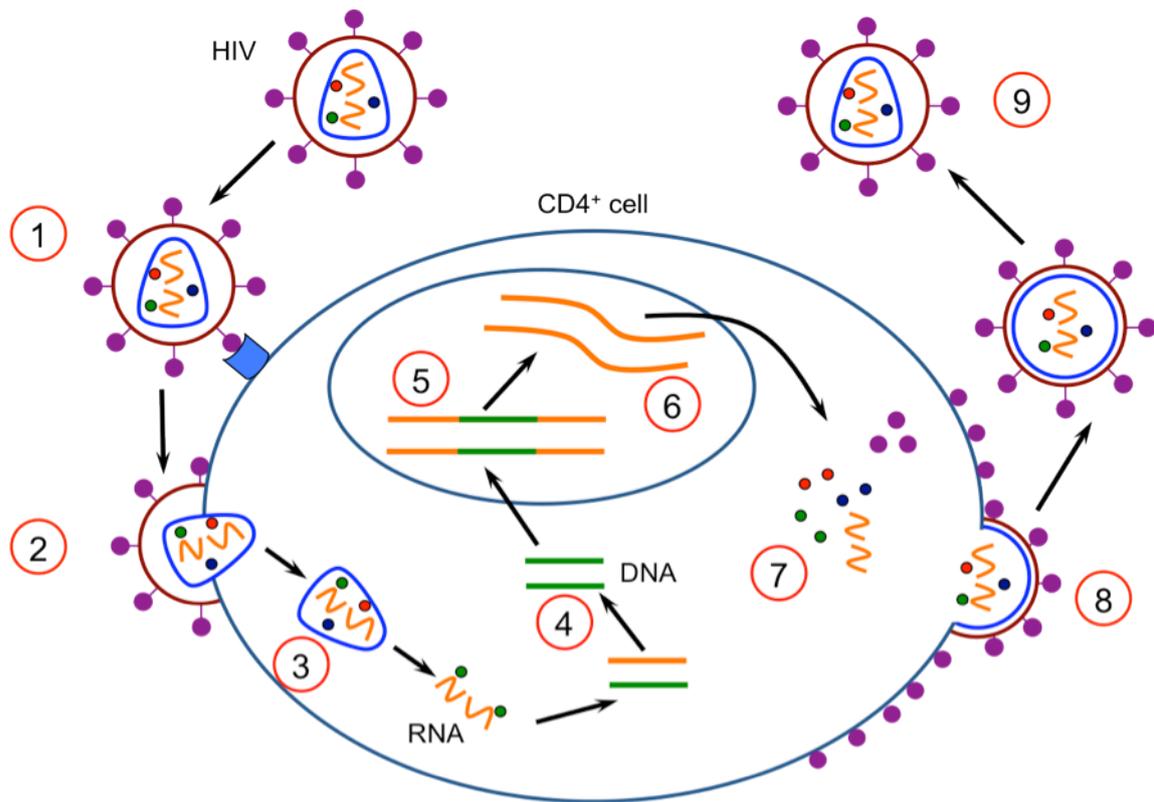


Figure 1.5 The life cycle of HIV

The life cycle of HIV includes multiple steps: (1) the viral surface envelope protein (purple cycle) attaches to cellular receptors (blue block); (2) the viral membrane fuses to the cell membrane; (3) the viral nucleoid enters the cell; (4) the viral RNA is reverse transcribed into double-strand DNA (green line) by RT (green cycle); (5) the viral DNA is integrated into host chromosome (orange straight line) by IN; (6) the proviral DNA is transcribed into mRNA; (7) the mRNA translates to various viral proteins (precursors); (8) viral RNA and envelope protein are assembled at the cell membrane; (9) a fully functional mature virion is produced.

1.1.7 The cell-mediated responses of HIV infection

HIV infection is controlled by anti-HIV-specific activities from both CD8⁺ and CD4⁺ cells. The HIV-specific CD8⁺ T cell response appears within the first few weeks of HIV infection, and expands rapidly to 10% of the total circulating CD8⁺ T cells when the viral load peaks (Borrow *et al.*, 1994, Wilson *et al.*, 2000). CD8⁺ T cells play an important role in controlling viral replication and disease progression. Clinical studies showed that patients with stronger CD8⁺ cytotoxic T lymphocyte (CTL) responses had a slower disease progression compared to those with lower CTL responses (Musey *et al.*, 1997). Furthermore, studies on asymptomatic long-term non-progressors (LTNPs) (Rinaldo *et al.*, 1995) and HIV-exposed but uninfected Gambian women (Rowland-Jones *et al.*, 1995) revealed the strong association between their lack of disease and a high level of CD8⁺ CTL activity. In an animal model experiment, the depletion of CD8⁺ lymphocytes during primary SIV infection resulted in a rapid and marked increase in viraemia, which was again suppressed with the presence of SIV-specific CD8⁺ T cells (Schmitz *et al.*, 1999). These observations suggest the importance of CD8⁺ T cells in effective control of viremia and could form the basis of an HIV-1 vaccine strategy.

HIV-specific CD8⁺ T cells can function by direct killing of HIV infected cells or by secreting a number of antiviral factors. Unlike the neutralising antibodies that only target viral envelope proteins, the CD8⁺ CTLs may recognise a variety of HIV peptides, such as Gag, RT, Env and some accessory proteins (McMichael and Rowland-Jones, 2001). As the infection progresses, CD8⁺ T cell responses decline and fail to suppress viral load persistently. CTLs are believed to exert a selective force on HIV *in vivo*, giving rise to viruses that have mutated critical peptides and eventually escape CTL recognition (McMichael and Rowland-Jones, 2001). This has been confirmed by several clinical studies where mutated epitopes have been observed to avoid CD8⁺ CTL responses (Phillips *et al.*, 1991, Borrow *et al.*, 1997, Price *et al.*, 1997).

CD4⁺ T cell response almost appears at the same time of CD8⁺ T cell response (Pitcher *et al.*, 1999). CD4⁺ T helper (TH) cells can be divided into TH1 and TH2 subsets, which both respond to HIV infection through production of cytokine (Mosmann *et al.*,

1986). TH1 produces interleukin (IL)-2, interferon (IFN)- γ and tumour-necrosis factor (TNF)- α that can facilitate cell-mediated immunity; TH2 produces IL-4, 5, 6, 10 and 13 that increase antibody production (humoral immunity). In addition, CD4⁺ T cells are important in the priming and maintenance of CD8⁺ T cells (Sun *et al.*, 2004). Mice studies indicated that CD8⁺ T cell memory development was impaired with the depletion of CD4⁺ T cells (Shedlock and Shen, 2003, Sun and Bevan, 2003). However, since CD4⁺ T cells are the principal targets of HIV, the CD4⁺ T cell response is diminished at the early stage of infection and its contribution to the immune system is severely compromised. Vaccines that can elicit both CD4⁺ and CD8⁺ T cell responses to HIV may be beneficial to control viral infection at the early stage.

1.1.8 The humoral responses of HIV infection

Antibodies usually appear within one to two weeks after the acute infection. Generally IgG1 antibody dominates in all the clinical stages, while levels of other antibody classes can vary on different clinical stages (Barker *et al.*, 1995). The early antibodies recognise Gag protein and are not neutralising (Busch *et al.*, 1995). They induce antibody dependent cellular cytotoxicity (ADCC) against gp120 and gp41 (Evans *et al.*, 1989), by which infected cells are recognised by effector NK cells or by monocytes/macrophages bearing fragment crystallisable (Fc) receptors and eventually destroyed (Yagita *et al.*, 1992).

Neutralising antibodies targeting Env normally appear after two to three months and their antiviral activities vary greatly between individuals (McKnight *et al.*, 1992, Li *et al.*, 2006). Most studies on neutralising antibodies indicated that a number of regions on gp120 and gp41 are sensitive to antibody neutralisation: the V3 loop, the V1, V2 region and the CD4 binding domain of gp120, the membrane-proximal external region (MPER) of gp41 and the carbohydrate moieties covering viral envelope (figure 1.6) (Chanh *et al.*, 1986, Muster *et al.*, 1993, Trkola *et al.*, 1996). While most of the identified neutralising antibodies recognise and neutralise a particular virus isolate, there are a few broadly neutralising monoclonal antibodies (mAb) that show cross-clade neutralising activity against different viral strains (see below).

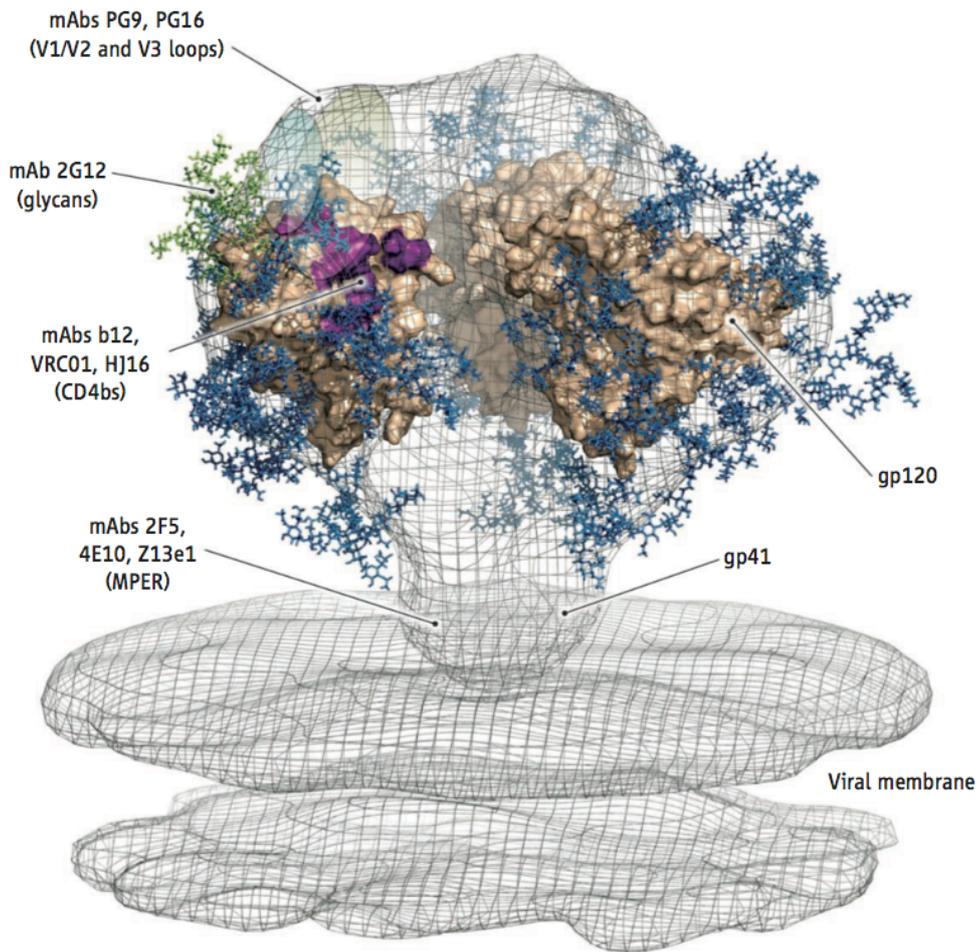


Figure 1.6 Broadly neutralising mAbs against HIV

A variety of broadly neutralising mAbs have been identified, allowing targeting different epitopes on the surface of HIV. The figure illustrates the bilayer viral membrane, the viral envelope spike gp41 and gp120, and the glycans (dark blue and green). Broadly neutralising mAbs recognise at least four distinct epitopes on the envelope spike: b12, VRC01 and HJ16 target the CD4 binding sites on gp120; PG9 and PG16 target the V1/V2 and V3 loop on gp120; 2G12 targets the outside glycans; 2F5 and 4E10 target the MPER of gp41. The figure is adapted from (Burton and Weiss, 2010).

The characterised neutralising mAbs are described in detail below:

b12

The b12 antibody, isolated in early 1990s by Burton and his colleagues from a phage display library assembled from 5 ml of bone marrow cells of a LTNP donor, was the first identified and most extensively characterised broadly neutralising mAb against HIV-1 (Burton *et al.*, 1991, Burton *et al.*, 1994). Epitope mapping identified an epitope (RPVVSTQLLLNGSLAEEEVV) that overlaps the CD4 binding site of gp120 (Barbas *et al.*, 1992). Therefore, the antibody could block HIV from attaching to CD4 receptor and thus prevent infection. Analysis of the b12 crystal structure in complex with gp120 showed its binding to the outer domain surface of gp120 with high affinity without additional gp120 conformational constraints (Zhou *et al.*, 2007). In comprehensive cross-clade neutralisation analysis b12 effectively neutralised 50% of 90 viruses crossing almost every subtypes, including primary isolates (Binley *et al.*, 2004). *In vivo* studies also showed that b12 protected macaques from simian human immunodeficiency virus (SHIV) challenge (Parren *et al.*, 2001).

2G12

The mAb 2G12 was produced from immortalised peripheral blood lymphocyte (PBL) by electrofusion (Buchacher *et al.*, 1994). 2G12 recognises a complex mannose-dependent epitope on the carbohydrate-covered silent face of the gp120 outer domain (Trkola *et al.*, 1996, Sanders *et al.*, 2002). 2G12 can neutralise HIV in subtype A, B and D, but not clade C nor CRF01_AE. This insensitivity of neutralising ability may be due to the absence of one or more glycans required for efficient 2G12 binding to gp120 (Binley *et al.*, 2004). In passive transfer studies 2G12 can protect macaques from viral infection, especially in combination with other broadly neutralising mAbs (Baba *et al.*, 2000, Mascola *et al.*, 1999). The crystal structure of 2G12 indicates that the antibody can achieve nanomolar-binding affinity to a glycan array because of its unusual configuration of the antigen-binding fragments (Fabs) (Calarese *et al.*, 2003). The variable heavy chains of the antibody have exchanged positions to interact with the light chain of the neighbouring Fab, resulting in a single, large antigen-binding site.

2F5 and 4E10

The 2F5 and 4E10 mAbs were also isolated by Buchacher and his colleagues (Buchacher *et al.*, 1994). These two broadly neutralising mAbs recognise two conserved linear epitopes (ELDKWAS and NWFDIT, respectively) on MPER of gp41. 2F5 and 4E10 can bind to the envelope trimer at various stages of infection, and neutralisation may occur by interrupting gp41 refolding (Crooks *et al.*, 2005). 4E10 showed very broad neutralising activity across all HIV clades tested, with modest potency relative to b12 (Binley *et al.*, 2004). However, 4E10 exhibits wide variation between different assay systems. 2F5 has a broad neutralising activity against clade A, B, D and CRF01_AE, but does not neutralise clade C viruses. Both 2F5 and 4E10 are unusual in having long complementarity-determining region 3 (CDR3) (22 and 18 amino acids respectively) (Zwick *et al.*, 2004, Cardoso *et al.*, 2005), which are very rarely present in infected patients. Recent experiments have shown that both 2F5 and 4E10 can protect macaques from mucosal challenge of SHIV (Hessell *et al.*, 2010).

PG9 and PG16

Walker and his colleagues identified two antibodies PG9 and PG16 with exceptional neutralising breadth and potency through a large-scale direct functional screen of approximately 30,000 B cells (Walker *et al.*, 2009). PG9 and PG16 were isolated from a clade A donor, and recognise an epitope encompassing V1/V2 and V3 variable loops on gp120. Interestingly, even though PG9 and PG16 efficiently neutralised more than 70% of the 162 viruses including primary isolates, they didn't bind to monomeric gp120 or gp41. This observation suggested that the epitopes targeted by these two antibodies might be preferentially expressed only on trimeric HIV envelope. A very long H-CDR3 loop of 28 residues was also defined on both antibodies and structural study revealed that the long CDR H3 loop forms a novel, sulphated "hammerhead" sub-domain that mediate potent neutralisation (Pejchal *et al.*, 2010).

HJ16

A broadly neutralising mAb HJ16 was discovered by Corti and his colleagues (Corti *et al.*, 2010). This antibody, isolated by improved Epstein-Barr Virus (EBV) immortalisation method (Traggiai *et al.*, 2004), recognises a novel epitope proximal to the CD4 binding site on gp120. HJ16 showed reactivity that was comparable in breadth, but distinct from another CD4 binding site-specific neutralising mAb b12. In addition, unlike most antibodies that preferentially neutralise tier-1 isolates

(homologous vaccine strains that are very sensitive to neutralisation), HJ16 preferentially neutralises tier-2 isolates (primary isolate Env clones) (Corti *et al.*, 2010).

VRC01 and VRC02

Wu and his colleagues successfully identified two broadly, potent neutralising mAbs VRC01 and VRC02 using a genetically engineered gp120. The newly designed gp120, showed no binding to most of non-neutralising antibodies, but preferentially bound to broadly neutralising antibodies (Wu *et al.*, 2010). Targeting specifically to the conserved CD4 binding site, VRC01 and VRC02 neutralised about 90% of 190 viral strains represented all major circulating HIV-1 subtypes. Both antibodies exhibited high levels of somatic mutations. Detailed analysis of the antibody sequences revealed 32% of variable region of heavy chain (VH) and 17% to 19% of variable region of kappa chain (V κ) nucleotides had mutated from the putative germline sequences. More importantly, the isolation of VRC01 and VRC02 from an HIV-1 infected donor using a rationally designed Env has demonstrated that such neutralising antibodies were indeed elicited in human (table 1.1).

Table 1.1

MAb	Target epitopes	Neutralising ability	Special features
b12	CD4-binding site	Potently neutralise 50% of 90 viruses across all subtypes	Long CDR3 loop
2G12	Complex mannose	Neutralise subtype A, B and D	VH domain swap
2F5	Membrane proximal region of gp41	Neutralise subtype A, B, D and CRF01_AE	Long CDR3 region
4E10	Membrane proximal region of gp41	Modestly neutralise 90 viruses across all subtypes	Long CDR3 region
PG9 PG16	V1/V2 and V3 domain	Potently neutralise more than 70% of 162 viruses across almost all subtypes	Long, sulphated, “hammerhead” H-CDR3 loop
HJ16	Proximal to CD4 binding site	Neutralising ability comparable to, and generally complementary to b12; preferentially neutralise tier-2 isolates	Preferentially neutralise tier-2 isolates
VRC01 VRC02	Conserved CD4 binding site	Neutralise 91% of 190 viral strains represented all major circulating HIV-1 subtypes	Highly mutated VH and Vκ

Table 1.1 Broadly neutralising mAbs

1.1.9 Long-term non-progressor (LTNP)

Individuals who have been HIV positive for 7 to 15 years (different authors use different time spans) but not on ART and have not developed AIDS are defined as long-term non-progressors (LTNPs). LTNPs normally maintain stable CD4⁺ counts of 500-600 cells/ μ l. A subset of LTNPs can even control their viral load to undetectable level and are named elite suppressors (ES). LTNPs have a number of characteristics that distinguish them from other HIV-1 infected individuals. A study of 68 LTNPs indicated that LTNPs maintain lower median plasma viral RNA than controls (6,000 vs. 40,000 RNA copies/ml), but the levels can vary greatly between individuals (Candotti *et al.*, 1999). Research showed that the CD4⁺ T cell-associated viruses were much lower in LTNPs. Moreover, a high level of anti-HIV-1 CD8⁺ memory CTL specific for viral protein Gag, Pol and Env was also associated with lack of AIDS progression in LTNPs, indicating that CD8⁺ memory CTL response may play an important role in controlling HIV replication and preventing disease development in LTNPs (Rinaldo *et al.*, 1995, Greenough *et al.*, 1999).

Many studies defining the viral genetic characteristics of LTNP have focused on deletion of *nef* gene of HIV. A cohort of LTNPs (one blood donor and six blood transfusion recipients) was well studied (Deacon *et al.*, 1995). HIV-1 sequences from their PBMCs had similar deletions of the *nef* gene. However, various results have been shown in subsequent studies, and *nef* deleted gene was only partially shown or absent in other LTNP studies (Greenough *et al.*, 1999). What role the *nef* gene plays in LTNP is not known.

Meanwhile, studies on human genetic variability that may affect HIV susceptibility have been focusing on CCR5 receptor. Several studies showed that a deletion of 32 base pairs in the CCR5 gene might be responsible to the resistance of HIV infection. The truncated protein cannot be detected by virus at the cell surface (Liu *et al.*, 1996). However, others showed that CCR5 might not be the only reason for the lack of disease in LTNP (Cohen *et al.*, 1997, Morawetz *et al.*, 1997). No differences of immunological and virologic parameters were found between LTNPs with deleted CCR5 and those with wild-type CCR5, thus indicating other factors are involved.

Although the factor which determines the HIV infection and disease progression in LTNPs remains unknown, LTNPs have become an important subject in HIV and AIDS research. Many studies on HIV pathogenesis, immunogenesis and vaccine design are based on this cohort of individuals. In this study, samples from more than 300 HIV-1 positive patients have been screened at Barts and The London Hospital, Queen Mary, University of London. Two serum samples from LTNPs M325 and K530 were found to have the wild type CCR5 and they displayed cross-clade neutralising activity (Weiss *et al.*, 1986) at high titres (see appendix 1), suggesting neutralising antibodies might have been generated by the two patients.

1.1.10 Ribosome display

It has been shown that broadly neutralising mAbs can be isolated by phage display (Burton *et al.*, 1991), electrofusion or EBV transformation (Buchacher *et al.*, 1994). Recently, a high-throughput functional screening approach has been used to isolate potent neutralising mAbs from B cell culture (Walker *et al.*, 2009).

In this study, ribosome display technology was investigated for *in vitro* antibody discovery from libraries made from HIV-1 LTNPs. Ribosome display is a cell-free system, in which DNA library can be rapidly screened without the need for cloning (He and Taussig, 1997). Like all other display technologies, ribosome display uses the same principle of linking proteins (phenotype) and DNA (genotype) for selection. Ribosome display produces stable Antibody-Ribosome-mRNA (ARM) complexes to link individual antibody fragments to their corresponding mRNA (He and Taussig, 2002). The formation of ARM complexes is achieved through deletion of the stop codon from the mRNA, which causes stalling of the translating ribosome at the end of mRNA with the nascent polypeptide not released. The linkage of protein-mRNA allows simultaneous selection of desirable antibodies with their encoding mRNA which can be recovered and amplified as DNA by RT-PCR (He *et al.*, 2004). Through repeated cycles, specific antibodies originally presented in rare species can be enriched and isolated from a very large population. Ribosome display enables screening libraries with up to 10^{12-13} members in a single reaction.

1.1.11 *E. coli* expression of proteins

Expression systems for producing recombinant protein (e.g. antibody) include those derived from bacteria (Baneyx, 1999), yeast (Cregg *et al.*, 2000), Baculovirus (Kost *et al.*, 2005) and mammalian cells (Rosser *et al.*, 2005). Among these, *Escherichia coli* (*E. coli*) is widely used for expression of recombinant proteins due to its relative simplicity, low cost, easy cultivation, the well-studied genetics and the availability of compatible tools (Sorensen and Mortensen, 2005). Moreover, varieties of plasmids and bacterial hosts including mutant strains are available, making it possible to express protein under a condition of choice. Although expression of soluble proteins in *E. coli* is still a bottleneck, a couple of empirical rules have been developed to guide the design and selection of an expression system (Makrides, 1996).

Recombinant expression plasmids contain a promoter to control protein synthesis; it should also exhibit a minimal level of basal transcriptional activity. The minimal basal transcription is controlled by a suitable suppressor, which in most cases is lac repressor, encoded by *lacI* gene or its mutants. Most widely used promoters can be induced by using either a thermal method or chemical inducers, e.g. Isopropyl β -D-1-thiogalactopyranoside (IPTG) (Hannig and Makrides, 1998). In this study, pSANG vector, adapted from pET26(+) vector (Novagen, Nottingham, UK), utilises a T7/lac hybrid promoter which combines the strong T7 promoter with the lac operator (figure 1.7). Basal expression of the protein is controlled by the lac repressor, encoded by the *lacI* gene. The T7 promoter requires the presence of the T7 RNA polymerase in the expression system, such as BL21(DE3) strain (Martin *et al.*, 2006). Another vector (pABEXT) used in this study was modified from pMAL-c2X vector (New England Biolabs, Hitchin, Herts, UK). pABEXT vector contains the tac promoter, which is also controlled by the lac repressor, encoded by the *lacI^q* gene (figure 1.8). The tac promoter is not as strong as T7 promoter, but it allows expression in *E. coli* without the need for T7 RNA polymerase.

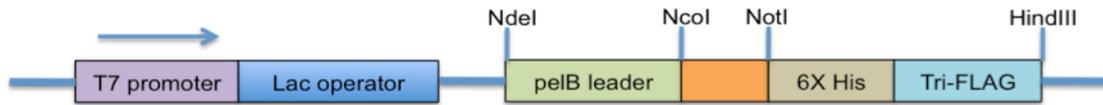


Figure 1.7 Schematic view of the expression cassettes of the pSANG10-3F vector
 The single-chain antibody encoding genes are sub-cloned at the *NcoI/NotI* sites.
 Picture is adapted from (Martin *et al.*, 2006).

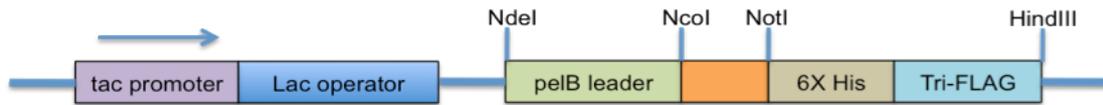


Figure 1.8 Schematic view of the expression cassettes of the pABEXT vector
 pABEXT vector is constructed using pMAL-c2X vector as backbone. The single-chain antibody encoding genes are sub-cloned at the *NcoI/NotI* sites.

Apart from the expression vector, the choice of a strain and its genetic background also affects protein expression. Some key features for an expression strain have been identified. These include deficiency in the most harmful proteases, maintenance of expression plasmid stability and the genetic elements relevant to the expression system (e.g., DE3) (Sorensen and Mortensen, 2005). *E. coli* BL21 is a robust host strain and has been used widely in recombinant expression applications. BL21 is deficient in two proteases, OmpT and Lon, reducing the possibility of protein degradation and thus allowing isolation of intact recombinant proteins. BL21(DE3) is the preferred host for vectors containing T7 promoter, as it encodes the T7 RNA polymerase gene under the control of lacUV5 promoter. To eliminate the rare codon bias in *E. coli* expression, BL21(DE3) strain was further modified to introduce a plasmid pRARE encoding some rare transfer RNAs (tRNAs) to overcome the shortage of the rare tRNA pools from bacteria (Novy *et al.*, 2001). In this study, the DNA ligation with pSANG vector was firstly used to transform XL1-blue strain due to its high transformation efficiency and blue/white screening; positive clones were subsequently transformed into BL21(DE3)pRARE strain for protein expression. Antibodies that are used in functional assays were produced by this method. An alternative strategy was also attempted in this study, in which the DNA ligation with pABEXT vector was transformed into XL1-blue strain for both cloning and expression without the second transformation step. This strategy was performed for a quick screening purpose. Figure 1.9 illustrates the two different approaches for expression of proteins in *E. coli*.

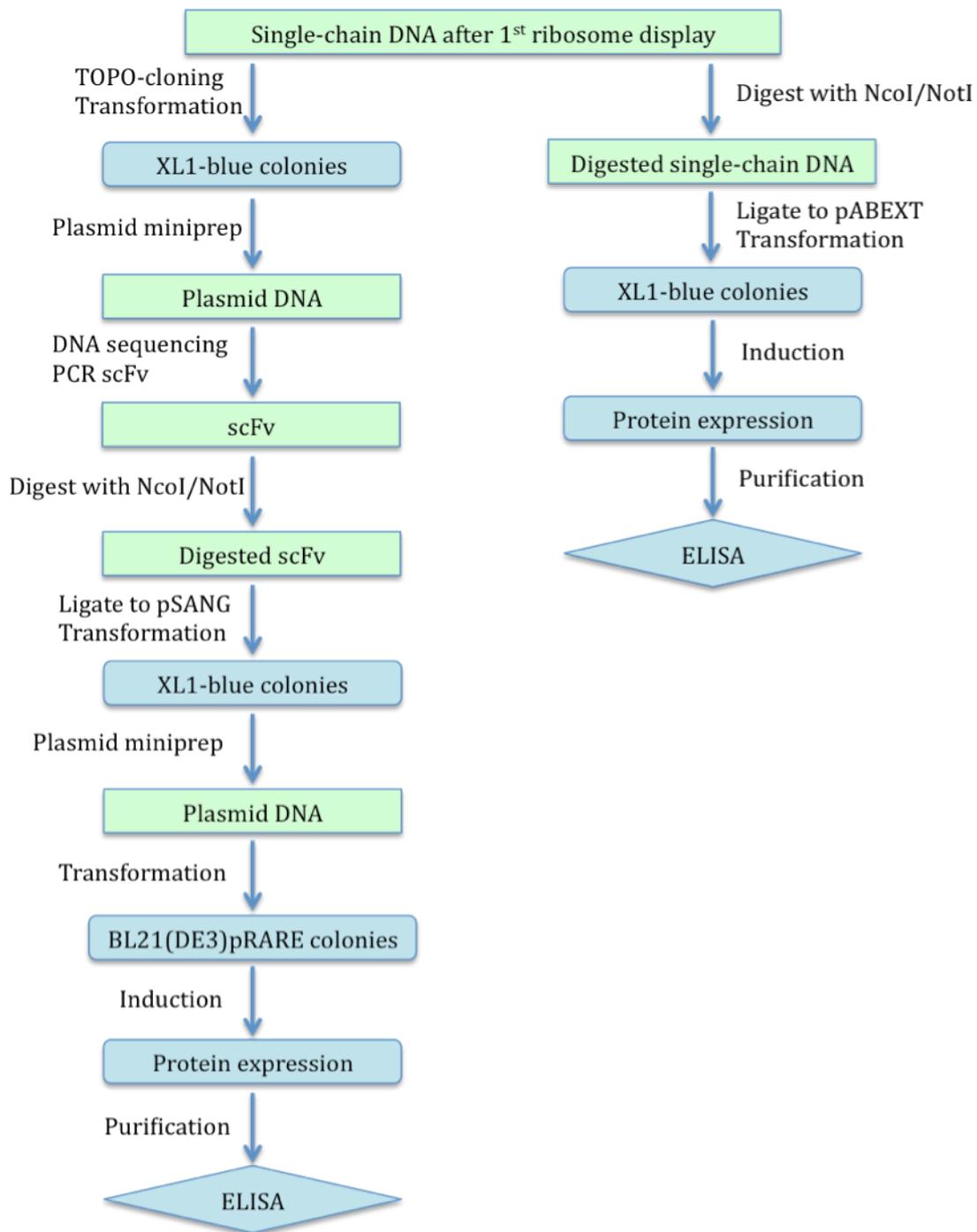


Figure 1.9 Two strategies of antibody expression

Two strategies were used in this study to express antibodies selected by ribosome display: (1) antibodies were cloned into pSANG vector and expressed in BL21(DE3)pRARE cells as illustrated to the left of the figure; (2) antibodies were cloned into pABEXT vector and expressed in XL1-blue cells as illustrated to the right of the figure.

The way to express proteins also affects protein yields. The expression strategies in general include synthesis of the proteins in (1) the reducing environment of the cytoplasm; (2) the oxidising environment of the periplasmic space, which is located between the cytoplasm and outer membranes, or (3) direct secretion of the protein into the culture medium (Gualerzi and Pon, 1990). The cytoplasmic expression approach benefits from a high expression level through a strong promoter, but most proteins produced form insoluble aggregates as inclusion bodies (Makrides, 1996). The advantages of recovering recombinant proteins from inclusion bodies are high yield (Zhuo *et al.*, 2005), protection from proteases (Grune *et al.*, 2004) and permitting production of proteins that are lethal to the host cells (Miroux and Walker, 1996). However, a tedious refolding process is needed to regain soluble active protein. Since refolded proteins often do not reproduce their original biological activities, efforts have been directed to avoid or minimise the formation of inclusion bodies (Schein, 1991). Secretion of proteins into periplasmic space (periplasmic expression) or extracellular medium (extracellular secretion) provides an alternative to facilitate correctly folded proteins. They are achieved by using a leader sequence, such as OmpT, OmpA, PelB and MalE (Blight *et al.*, 1994). Compared with cytoplasmic expression, this strategy allows simpler purification, protection of proteins from proteolysis, generation of higher N-terminus authenticity and enhancement of disulfide bond formation (Jonasson *et al.*, 2002).

Another factor affecting efficient protein expression in *E. coli* is the codon usage. Certain codons are rare in *E. coli* while abundant in heterologous genes from sources such as eukaryotes (Kane, 1995, Rosano and Ceccarelli, 2009). Expression of genes with rare codon in *E. coli* may lead to translational errors and severely impair the translation efficiency. The most problematic codons include Arginine (AGA, AGG, CGA and CGG), Glycine (GGA and GGG), Isoleucine (AUA), Leucine (CUA) and Proline (CCC) (Sorensen and Mortensen, 2005). Approaches to decrease codon bias include site-directed mutagenesis of the target sequence (Calderone *et al.*, 1996) and complement of gene encoding the tRNA cognate to the problematic codons (Dieci *et al.*, 2000). Assembly of rare tRNA genes into plasmid such as pRIG and pRARE in the host strain has shown to be able to significantly enhance the expression of some codon biased genes (Baca and Hol, 2000).

1.1.12 HIV-1 Antibody neutralisation assay

The anti-HIV-1 antibody activity resulting in the prevention of virus entry into cells is an important functional attribute. This may be achieved by binding to the viral surface protein envelope, preventing virus attachment and/or entry into cells (Wyatt and Sodroski, 1998). Neutralisation epitopes for HIV-1 include the CD4 binding domain (Burton *et al.*, 1991), the hypervariable regions (V1, V2 and V3) of gp120 (Haigwood *et al.*, 1990, Fung *et al.*, 1992, Gorny *et al.*, 1991, Pinter *et al.*, 1993), the MPER of gp41 (Buchacher *et al.*, 1994, Stiegler *et al.*, 2001) and the mannose residues on the outer face of gp120 (Trkola *et al.*, 1996, Scanlan *et al.*, 2002).

The HIV-1 neutralisation assay is designed to analyse the activity of neutralising antibodies. Many parameters may affect this neutralisation assay, and thus a standardised *in vitro* assay is important for meaningful comparisons of the quality and potency of neutralising antibodies. Early studies on HIV-1 neutralising antibody responses relied on the ability of T-cell line adapted (TCLA) viruses to infect cell lines. Sera or recombinant antibodies were used to co-culture with TCLA virus, and parameters such as syncytium-formation, viral proteins or cell survival were measured to evaluate reduction of infection (Nara *et al.*, 1987, Hanson *et al.*, 1990, Montefiori *et al.*, 1988). However, neutralisation of TCLA viruses poorly predicted primary isolate neutralisation because the adapted primary viruses in T-cell lines are highly sensitive to neutralisation (Wrin *et al.*, 1995). To mimic the *in vivo* environment, assays using patient viruses to infect seronegative PBMCs were subsequently developed. Primary viruses or clinical isolates were co-cultured with the test sera or mAbs, and viral protein or reverse transcription was measured to indicate viral replication (Mascola, 1999, Mascola *et al.*, 2002). However, these assays require the use of PBMCs from different individuals, which display differential susceptibility to HIV-1 infection and impair the experiment reproducibility (Daar *et al.*, 1990, Zhou and Montefiori, 1997).

Recently, a technology that uses pseudovirus in a neutralisation assay (Richman *et al.*, 2003) was adapted and refined by Montefiori. The pseudoviruses are generated by incorporating HIV-1 Env cloned from primary isolates into Env-deficient laboratory-adapted strains, which were only capable of a single round of infection (Montefiori,

2005). The neutralising activity of test sera or antibodies is measured by reduction of infectivity on reporter cell lines, such as TZM-bl that contains the β -galactosidase and luciferase as sensitive markers (Wei *et al.*, 2002). Compared to the PBMC assay, the pseudoviral system has advantages on the ability to produce generically identical virus in each stock, and rapid test for neutralisation against primary patient Envs from various clades (Polonis *et al.*, 2008). In general, this system greatly enhanced the consistency, accuracy and reproducibility of the neutralisation assay. Despite the discrepancies reported between reporter cell line-based pseudovirus assays and PBMC-based assays (Binley *et al.*, 2004), the pseudovirus assay has been recommended by the HIV Vaccine Trials Network (HVTN) and National Institutes of Health (NIH) as the standard assay for evaluating neutralising antibody responses (Mascola *et al.*, 2005).

1.2 Aims of the project

Despite of three decades of intensive research, an effective vaccine for the prevention of HIV infection has proved to be elusive. Significant efforts have been made in understanding the molecular biology of the HIV vaccines, in particular the role of viral encoded protease and reverse transcriptase. Moreover, an understanding of the molecular functions that allow viral whole cell recognition and cell entry has also being elucidated. With the detailed knowledge of the protease and the reverse transcriptase, considerable success has been achieved in developing drugs to target these molecules for the disruption of virus propagation.

The current approach for the treatment of the HIV positive individuals relies on a cocktail of these drugs. However, ideally what is required is a vaccine that may prevent the initial infection, or hold an infection in check. With the detailed understanding of the viral surface molecular architecture, molecules responsible for the attachment to the cell surfaces and viral entry have been identified. It may be possible to target some of these surface molecules to prevent either initial attachment or subsequent entry into the whole cell, such as gp120. However, since it is heavily glycosylated, the peptide sequences are possibly hidden from the immune surveillance molecules. Notwithstanding, it was shown that immune sera from HIV-1 LTNPs are capable of neutralising a broad range of HIV isolates. To date, a number of broadly neutralising human mAbs have been isolated. Analysis of these antibodies by epitope mapping facilitates to identify the amino acid sequences capable of inducing broadly protective HIV antibodies. Studies on such many different neutralising antibodies would help us to understand both the molecular mechanism and the nature of the broadly neutralising responses. It is clear that methods are needed to rapidly select many different neutralising antibodies for analysis and characterisation.

This study aimed to establish an approach to identify neutralising mAbs *in vitro* using cell-free ribosome display from cDNA libraries made from patient PBMCs, combined with homologous and heterologous gp120 selection. Secondly, the relationship between binding, neutralising activity and the antibody subclasses was also determined. The donors (M325 and K530) had previously been identified as LTNPs

with broadly neutralising activity against clade B, C, CRF02_AG. The hypothesis was to investigate the antibody repertoire to determine whether similar antibody solutions were used in this common broad neutralisation activity. It was reasoned that donor M325 should have antibodies directed against gp120 derived from K530.

The followings are the main steps involved in this project:

- (1) Construction of ribosome display libraries from two HIV-1 LTNPs;
- (2) Carrying out ribosome display to select antibodies against antigen gp120;
- (3) *E. coli* cloning and expression of selected population as single-chain variable fragment (scFv);
- (4) ELISA screening for potential gp120 binders;
- (5) Analysis and characterisation of binders.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Molecular biology reagents/kits

MP biomedical, Illkirch, France

Taq & Go Ready to Use PCR Mix

New England Biolabs, Hitchin, Herts, UK

10X NEBuffer 3, 10X T4 DNA ligase reaction buffer, 100 base pair (bp) DNA ladder, Protoscript ® First Strand cDNA Synthesis Kit, restriction Endonucleases *HindIII*, *NcoI*, *NdeI*, *NotI*, T4 DNA ligase

Promega, Southampton, Hampshire, UK

Bright-GLO Luciferase reagent, TNT® T7 Quick Coupled Transcription/Translation System

Qiagen, Crawley, West Sussex, UK

Ni-NTA Spin Columns, QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit, QIAexpress Kit

Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK

DNase I recombinant

Sigma-Aldrich, Poole, Dorset, UK

Amicon Centrifugal Filter Units

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, TOPO TA Cloning® Kit for Sequencing, TRIZOL® Reagent

2.1.2 Chemicals

Becton, Dickinson and Company, Oxford, UK

Bactotryptone, Yeast extract

BIO-RAD Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Precision plus protein dual Xtra standards

GBIOSCIENCES, Maryland Heights, MO 63043-3202, U.S.A.

Nickel chelating resin

Merck, Darmstadt, Germany

5-bromo-4-chloro-3-indolyl phosphate (BCIP), Nitro blue tetrazolium (NBT)

Sigma-Aldrich, Poole, Dorset, UK

Agarose, β -mercaptoethanol, dithiothreitol, Diethylaminoethyl (DEAE)-dextran, Mg acetate, Monoclonal anti-polyHistidine-alkaline phosphatase antibody produced in mouse

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

0.2 ml PCR tube, 50 ml centrifuge tube, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), 96 well flat bottom opaque Nunc plate, acetic acid glacial, acrylamide: bis-acrylamide 29:1 solution 40%, ammonium persulfate, Bovine serum albumin (BSA), Bromophenol blue, CaCl_2 , carbenicillin, chloramphenicol, Coomassie brilliant blue R-250, Diethylpyrocarbonate (DEPC)-treated H_2O , Dulbecco's modified Eagle medium (DMEM), F96 MaxiSorp Nunc-Immuno plate, glucose, glycerol, Glycine, guanidine hydrochloride, guanidine thiocyanate, imidazole, Immobilon-P transfer membrane, Isopropyl β -D-1-thiogalactopyranoside (IPTG), isopropyl alcohol, kanamycin, KCl, Luria Bertani (LB) agar, LB broth, methanol, MgCl_2 , MgSO_4 , NaCl, p-Nitrophenyl phosphate (pNPP), Sodium dodecyl sulphate (SDS), tetracycline, Tetramethylethylenediamine (TEMED), Tris base, Tween 20

2.1.3 Equipments

BIO-RAD Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Mini-PROTEAN Tetra Electrophoresis System, Mini Trans-Blot Cell

BMG Labtech, Aylesbury, UK

FLUOstar OPTIMA

Clare Chemical Research, Dolores, USA

Dark Reader Transilluminator DR-88X

Thermo Fisher Scientific, Loughborough, Leicestershire, UK

Nanodrop 1000 Spectrophotometer

Wallac, PerkinElmer, Cambridge, UK

VICTOR Plate Reader

2.1.4 Oligonucleotide primers

All the oligonucleotide primers used for antibody library construction, ribosome display and scFv expression are listed in the table 2.1. Primers/oligonucleotides were synthesised from Invitrogen (Paisley, UK).

Table 2.1

First strand cDNA and variable fragments (reverse)	
HuKF	TCC AGA TTT CAA CTG CTC ATC AGA TGG CGG
HuLF	GGC TTG GAG CTC CTC AGA GGA GGG YGG GAA
CH2F	GGG TRT CCT TGG GTT TTG GGG GGA A
Variable kappa (Vκ) (forward)	
VK1	GAC ATC CRG DTG ACC CAG TCT CC
VK2346	GAT ATT GTG MTG ACB CAG WCT CC
VK36	GAA ATT GTR WTG ACR CAG TCT CC
VK5	GAA ACG ACA CTC ACG CAG TCT C
Variable lambda (Vλ) (forward)	
VL1	CAG TCT GTS BTG ACG CAG CCG CC
VL1459	CAG CCT GTG CTG ACT CAR YC
VL15910	CAG CCW GKG CTG ACT CAG CCM CC
VL2	CAG TCT GYY CTG AYT CAG CCT
VL3A	TCC TAT GWG CTG ACW CAG CCA C
VL3B	TCC TAT GAG CTG AYR CAG CYA CC
VL3DLP16	TCC TCT GAG CTG AST CAG GAS CC
VL6	AAT TTT ATG CTG ACT CAG CCC C
VL78	CAG DCT GTG GTG ACY CAG GAG CC
Variable heavy (VH) (forward)	
VH1	CAG GTC CAG CTK GTR CAG TCT GG
VH1257	CAG GTG CAG CTG GTG SAR TCT GG
VH2	CAG RTC ACC TTG AAG GAG TCT G
VH3A	GAG GTG CAG CTG KTG GAG WCC
VH3B	GAG GTG CAG CTG KTG GAG WCT
VH4	CAG GTG CAG CTG CAG GAG TCS G
VH4DP63	CAG GTG CAG CTA CAG CAG TGG
VH6	CAG GTA CAG CTG CAG CAG TCA
T7-variable kappa (T7 Vκ) (forward)	
VK1T7	CTA TAG GAA CAG ACC ACC ATG GCC GAC ATC CRG DTG ACC CAG TCT CC
VK2346T7	CTA TAG GAA CAG ACC ACC ATG GCC GAT ATT GTG MTG ACB CAG WCT CC
VK36T7	CTA TAG GAA CAG ACC ACC ATG GCC GAA ATT GTR WTG ACR CAG TCT CC
VK5T7	CTA TAG GAA CAG ACC ACC ATG GCC GAA ACG ACA CTC ACG CAG TCT C
T7-variable lambda (T7 Vλ) (forward)	
VL1T7	CTA TAG GAA CAG ACC ACC ATG GCC CAG TCT GTS BTG ACG CAG CCG CC
VL1459T7	CTA TAG GAA CAG ACC ACC ATG GCC CAG CCT GTG CTG ACT CAR YC
VL15910T7	CTA TAG GAA CAG ACC ACC ATG GCC CAG CCW GKG CTG ACT CAG CCM CC
VL2T7	CTA TAG GAA CAG ACC ACC ATG GCC CAG TCT GYY CTG AYT CAG CCT
VL3AT7	CTA TAG GAA CAG ACC ACC ATG GCC TCC TAT GWG CTG ACW CAG CCA C
VL3BT7	CTA TAG GAA CAG ACC ACC ATG GCC TCC TAT GAG CTG AYR CAG CYA CC
VL3DLP16T7	CTA TAG GAA CAG ACC ACC ATG GCC TCC TCT GAG CTG AST CAG GAS CC
VL6T7	CTA TAG GAA CAG ACC ACC ATG GCC AAT TTT ATG CTG ACT CAG CCC C
VL78T7	CTA TAG GAA CAG ACC ACC ATG GCC CAG DCT GTG GTG ACY CAG GAG CC
Kappa link-heavy (κ link VH) (forward)	
VH1K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG GTC CAG CTK GTR

	CAG TCT GG
VH1257K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG GTG CAG CTG GTG SAR TCT GG
VH2K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG RTC ACC TTG AAG GAG TCT G
VH3AK	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA GAG GTG CAG CTG KTG GAG WCC
VH3BK	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA GAG GTG CAG CTG KTG GAG WCT
VH4K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG GTG CAG CTG CAG GAG TCS G
VH4DP63K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG GTG CAG CTA CAG CAG TGG G
VH6K	CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA CAG GTA CAG CTG CAG CAG TCA
Lambda link-heavy (λ link VH) (forward)	
VH1L	CCC TCC TCT GAG GAG CTC CAA GCC CAG GTC CAG CTK GTR CAG TCT GG
VH1257L	CCC TCC TCT GAG GAG CTC CAA GCC CAG GTG CAG CTG GTG SAR TCT GG
VH2L	CCC TCC TCT GAG GAG CTC CAA GCC CAG RTC ACC TTG AAG GAG TCT G
VH3AL	CCC TCC TCT GAG GAG CTC CAA GCC GAG GTG CAG CTG KTG GAG WCC
VH3BL	CCC TCC TCT GAG GAG CTC CAA GCC GAG GTG CAG CTG KTG GAG WCT
VH4L	CCC TCC TCT GAG GAG CTC CAA GCC CAG GTG CAG CTG CAG GAG TCS G
VH4DP63L	CCC TCC TCT GAG GAG CTC CAA GCC CAG GTG CAG CTA CAG CAG TGG G
VH6L	CCC TCC TCT GAG GAG CTC CAA GCC CAG GTA CAG CTG CAG CAG TCA
Combination (T7AB is forward, CH2Not is reverse)	
T7AB	GCA GCT AAT ACG ACT CAC TAT AGG AAC AGA CCA CCA TGG CC
CH2Not	CCG GGA TGC GGC CGC GGT RTC CTT GGG TTT TGG GGG GAA
Ribosome display (EP1, IP1 and LP1 are reverse, Kz1 is used on both directions)	
EP1	GCT ACC GCC TCC ACT CCC ACC GCC AGA TCC CCC ACC CGA GCC TCC CCC TGA ACC GCC TCC CCG GGA TGC GGC CGC RGT RTC CTT GG
IP1	GAA CAG ACC ACC ATG AG GAA GAC TGA YGG TCC
Kz1	GAA CAG ACC ACC ATG
LP1	GCTGCT ACC GCC TCC ACT CCC ACC GCC AGA TCC CCC ACC CGA GCC TCC CCC TGA ACC GCC TCC CCG GGA TGC GGC CGC GAA CAG ACC ACC ATG AG GAA GAC
scFv expression (reverse)	
TJ011	GCC CGC GGC CGC TGT GCC CCC AGA GGT G
TJlgG24	GCC CGC GGC CGC TGT GCT CTC GGA GGT G

Table 2.1 Primers used in PCR

Primers Degenerate codons used for synthesising variable regions are: M=A/C; R=A/G; W=A/T; S=G/C; Y=C/T; K=G/T; V=A/G/C; H=A/C/T; D=A/G/T; B=G/C/T; N=A/G/C/T. Primer directions are shown in brackets.

2.1.5 Plasmids

Protein expression vector pSANG10-3F (Martin *et al.*, 2006) was provided by Dr. John McCafferty (Department of Biochemistry, University of Cambridge). Plasmid pMal-c2X (used to construct vector pABEXT) was purchased from New England Biolabs (Hitchin, Herts, UK).

2.1.6 Cell culture

E. coli strain XL1-blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (*F'* *proAB lacI^qZΔM15 Tn10 Tet^r*)] competent cells used for plasmid transformation were purchased from Stratagene (California, USA). *E. coli* strain BL21(DE3)pRARE competent cells used for protein expression were made from *E. coli* BL21(DE3) [*F'* *dcm ompT hsdS*($r_B^- m_B^-$) *gal λ*(DE3)] (Merck, Nottingham, UK) with plasmid pRARE in Dr. Angray Kang's lab (School of Life Sciences, University of Westminster, UK).

PBMCs from two HIV-1 patients (M325 and K530) were kindly provided by Dr. Hanna Dreja (Centre for Infectious Disease, Institute of Cell and Molecular Science, Barts and The London, Queen Mary, University of London, UK). Serum neutralising activities of these two patients were tested by Dr. Hanna Dreja and are listed in the appendix 1.

Recombinant antigen gp120 generated from HIV-1 virus isolated from patient K530's serum was provided by Dr. Simon A. Jeffs (Wright-Fleming Institute, Division of Medicine, Imperial College London, UK). This was selected because it was homologous for use in screening the K530 library and heterologous for the M325 library.

2.2 Construction of human single-chain antibody libraries

From each of the two individual patient PBMCs (M325 and K530), a human single-chain antibody library was constructed respectively, and designated M325 and K530. First, total RNA was extracted from the corresponding patient PBMCs. The RNA was then used to synthesis first strand complementary DNA (cDNA) using specific primers followed by PCR using designed primers to amplify the variable regions. Finally single-chain antibody libraries were constructed by assembling all individual variable regions of light chains (VLs) with individual heavy chains (VH-CH1-hinge-partial CH2) by PCR. Detailed procedures are described below.

2.2.1 Total RNA extraction

PBMCs from patient M325 were isolated from 100 ml of the whole blood using Ficoll gradient centrifugation prior to the treatment with 5 ml of TRIZOL® Reagent for homogenisation, while PBMCs from patient K530 were recovered from 20 ml of the whole blood by the same way and the isolated PBMCs had been stored at -80°C for 3 years before use. To extract total RNA from the PBMCs K530, 2 ml of TRIZOL® Reagent was added to disrupt cells and dissolve cell components, as well as eliminate the potential hazards of HIV-1 infection.

Total RNA was isolated using TRIZOL® Reagent according to the manufacture's instruction. In brief, 0.2 ml of chloroform was added per 1 ml of TRIZOL® Reagent used for the initial homogenisation, and the sample was shaken vigorously for 15 seconds and incubated at room temperature for 15 minutes. The sample was then centrifuged at 12000 g for 15 minutes at 4°C, and the upper aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol for every 1 ml of TRIZOL® Reagent. After incubation at room temperature for 10 minutes, the mixture was centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was carefully removed, and the RNA pellet was washed with 1 ml of 75% ethanol per 1 ml of TRIZOL® Reagent, and centrifuged at 7500 g for 5 minutes at 4°C. The RNA was dried in air for 10 minutes, before re-dissolved by addition of 30 µl of DEPC-treated H₂O and stored at -80°C.

2.2.2 First strand cDNA synthesis

Three first strand cDNAs (V_{κ} , V_{λ} and heavy chain) were synthesised from total RNA using Protoscript® First Strand cDNA Synthesis Kit with specific primers (HuKF, HuLF and CH2F, respectively), according to the manufacturer's instructions. Briefly, 1 μg of total RNA was incubated at 70°C for 5 minutes with 30 pmol of primer, 40 nmol of dNTP mix and sufficient DEPC-treated H_2O to a final volume of 16 μl . The mixture was cooled down on ice for at least 30 seconds before adding 2 μl of M-MuLV Reverse Transcriptase Reaction Buffer (10X RT Buffer), 1 μl of M-MuLV Reverse Transcriptase (25 units) and 1 μl of RNase inhibitor (10 units). The mixture was incubated at 42°C for 1 hour, followed by 95°C for 5 minutes to inactivate the enzymes. 1 μl of RNase H (2 units) was then added to the mixture for 20 minutes at 37°C to degrade the remaining RNA. Finally the mixture was incubated at 95°C for 5 minutes to inactivate the enzyme. Synthesised first strand cDNAs were used to construct individual V_{κ} , V_{λ} and heavy chain fragments, respectively.

2.2.3 Single-chain antibody library construction

Individual fragments including 4 V_{κ} , 9 V_{λ} , and 8 heavy chains (VH-CH1-hinge-partial CH2) were amplified by PCR (figure 2.1). 5'-primers specific for individual V_{κ} , V_{λ} and VH families were designed to amplify all the functional variable regions of Immunoglobulin (Ig) G family (Sblattero and Bradbury, 1998). In order to identify the subclasses of neutralising mAbs, 3'-primers for heavy chains were designed to anneal at the beginning region of CH2 domain, thus amplifying VH-hinge-CH1 and partial CH2 which provides the information of immunoglobulin subclasses (IgG1, 2, 3 or 4). A secondary PCR was performed to introduce restriction endonuclease sites *NcoI* at the 5' end of the VL (V_{λ} and V_{κ}) and *NotI* at the 3' end of the CH2 for the cloning purpose, as well as a synthetic linker to join VL to heavy chain. Individual VL was linked with individual heavy chain through PCR to form V_{κ} -link-VH-CH1-hinge-partial CH2 and V_{λ} -link-VH-CH1-hinge-partial CH2. In this way, a total of 104 combinations of light chain and heavy chain families were generated (Figure 2.2).

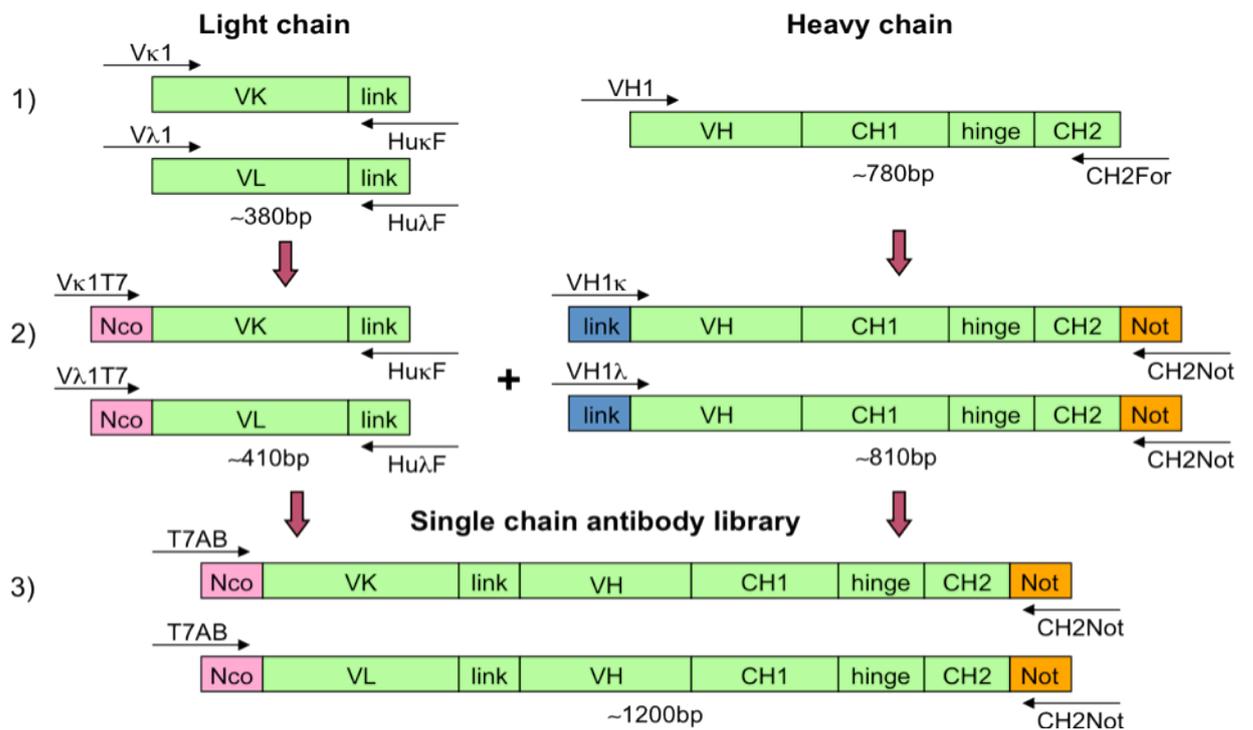


Figure 2.1 Illustration of single-chain antibody library construction

(1) Individual fragment (4 V_{κ} , 9 V_{λ} and 8 heavy chains) were generated by PCR; (2) each fragment was introduced restriction endonuclease sites and a linker sequence by PCR; (3) each light chain and heavy chain was assembled to each other, forming the single-chain antibody library.

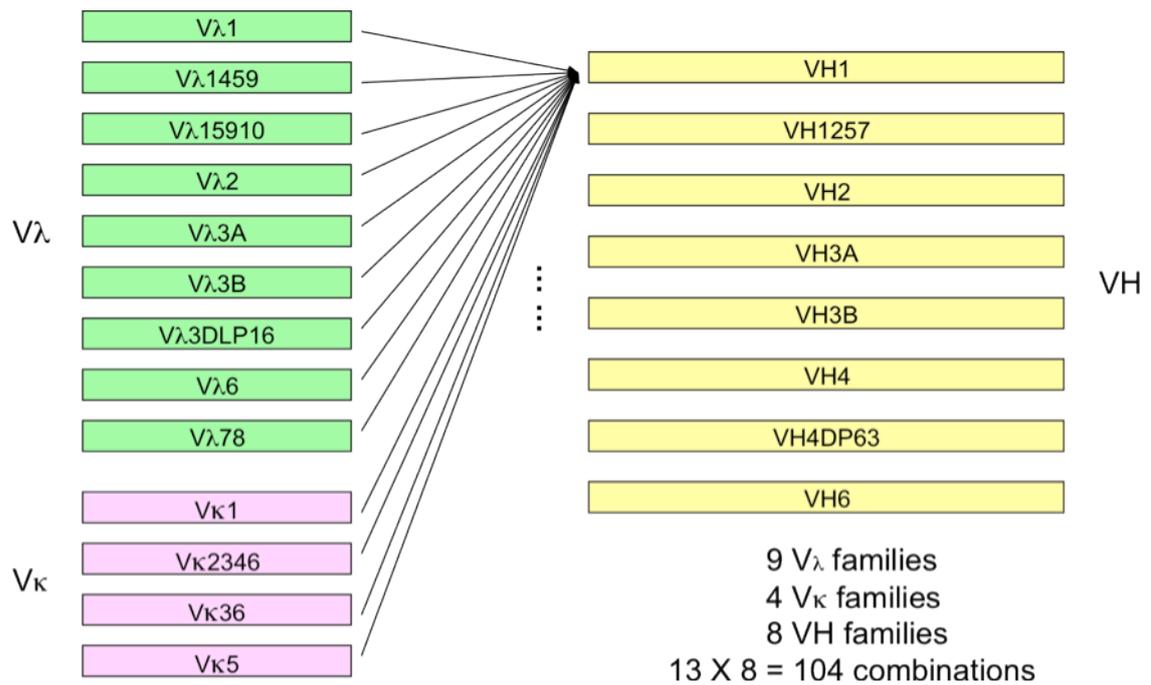


Figure 2.2 Illustration of combinations of light chains and heavy chains

In both libraries, individual light chain families and heavy chain families were randomly combined one by one by PCR assembly. 104 combinations were mixed for the use of ribosome display.

Taq & Go Ready to Use PCR Mix was employed in all PCR amplification. The following is the standard reaction mixture: 10 μ l of 5X Taq & Go Ready to Use PCR Mix, 25 pmol of each primer, 2 μ l of template and sufficient DEPC-treated H₂O to a final volume of 50 μ l. PCR was carried out using the condition of one cycle at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50-60°C (depending on the primer pairs) for 30 seconds and extension at 72°C for 1 minute, and one cycle of final extension at 72°C for 10 minutes.

20 μ l of PCR products were analysed by gel electrophoresis with 1.5% (w/v) agarose. DNA fragment size was determined by comparison with 100 bp DNA ladder. For purifying DNA from agarose gels, the piece of gel containing the desired DNA fragment was excised under Dark Reader Transilluminator DR-88X to eliminate ultraviolet (UV) damage and the DNA was extracted from the gel using QIAquick Gel Extraction Kit according to the manufacturer's instructions. Briefly, excised agarose gel was dissolved in 300 μ l of QG buffer (5.5 M guanidine thiocyanate, 20 mM Tris-HCl, pH 6.6) and incubated at 50°C for 10 minutes. The solution was mixed with 100 μ l of isopropyl ethanol before loaded to QIAquick spin column and centrifuged at 13000 g for 1 minute. The column was washed with 750 μ l of PE buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol, pH 7.5) and centrifuged at 13000 g for 1 minute. DNA was eluted in 30 μ l of DEPC-treated H₂O and stored at -20°C.

2.3 Ribosome display

Both single-chain antibody libraries were screened by *in vitro* ribosome display according to protocol described (He and Taussig, 2007) with slight modifications. The steps of ribosome display cycle are illustrated in figure 2.3.

2.3.1 Full-length generation of ribosome display construct

To display antibodies on the surface of ribosome, the 5' end of the library should contain T7 promoter and a eukaryotic translation initiation (Kozak) sequence (Kozak, 1987). This was achieved by designing a T7AB primer (5'- GC AGC TAA TAC GAC TCA CTA TAG GAA CAG ACC ACC ATG GCC -3'). On the other hand, to efficiently recover the cDNA from ribosome complexes after selection without prior mRNA isolation, a primer annealing at the position about 60-80 bp upstream of the 3' end is required as ribosome occupies about 60 nucleotides at the 3' end. This would lead to the generation of cDNA short of 60-80 nucleotides (He and Taussig, 2005). An extension primer (EP1) was thus designed (5'- GCT ACC GCC TCC ACT CCC ACC GCC AGA TCC CCC ACC CGA GCC TCC CCC TGA ACC GCC TCC CCG GGA TGC GGC CGC RGT RTC CTT GG -3'), which covers the missing 60-80 nucleotides. Using the primers T7AB and EP1, a full-length DNA construct was obtained by PCR. The generated full-length DNA is directly used for the subsequent cycle of ribosome display.

2.3.2 Antigen coating

10 µg of antigen (recombinant gp120) in a volume of 20 µl was used to coat a 0.2 ml PCR tube at 4°C overnight. After washing twice with 100 µl of phosphate buffered saline (PBS, 137 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4), the PCR tube was blocked with 100 µl of 10 mg/ml BSA in PBS at room temperature for 1 hour. The PCR tube was then washed by 100 µl of PBS three times followed by ribosome display washing buffer (PBS containing 0.01% Tween 20, 5 mM Mg acetate and 0.1% BSA, pH 7.4) and kept at 4°C before use.

Ribosome Display Flow Chart

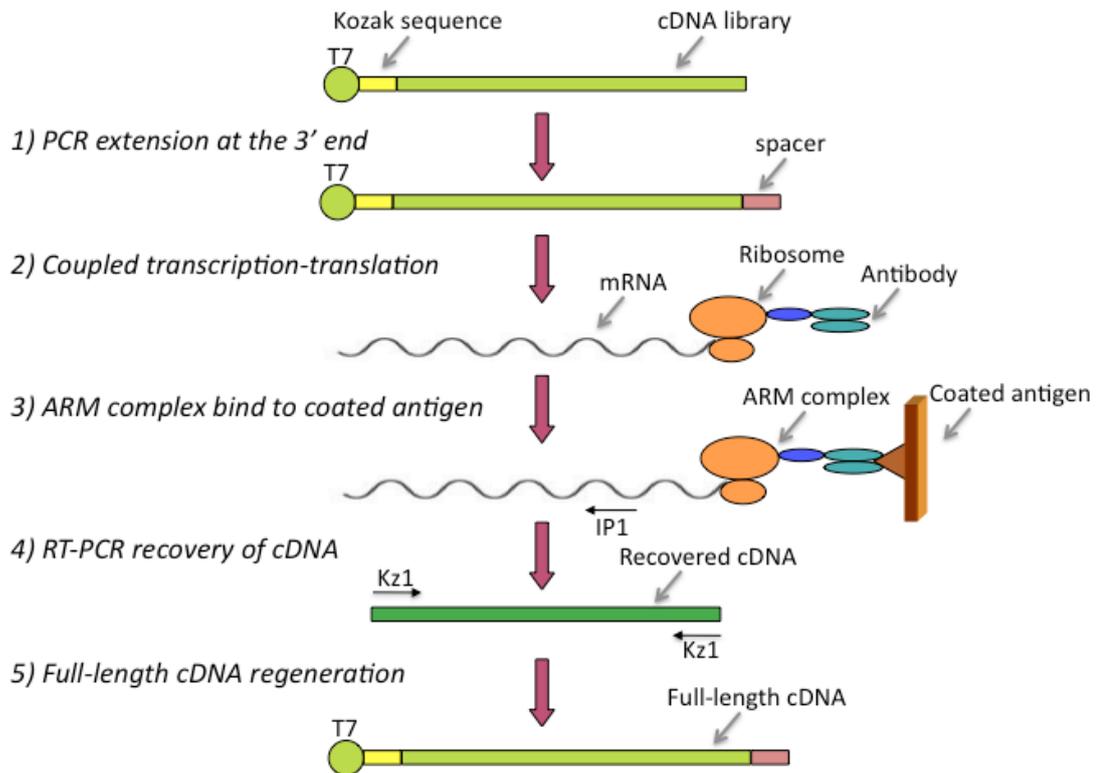


Figure 2.3 Ribosome display flow chart

(1) cDNA library mixture is extended at 3' end to generate the full-length construct by PCR; (2) *in vitro* coupled transcription/translation is set up, individual antibody fragments are generated through ribosome-directed protein synthesis and physically linked with its translating mRNA due to the absence of the stop codon; (3) Antibody-Ribosome-mRNA (ARM) complex is added to antigen gp120-coated tube for *in vitro* selection, unbound ARM complexes are washed away; (4) RT-PCR recovery of cDNA from selected mRNA sequences, single primer PCR is set up to amplify the sequences with primer Kz1; (5) full-length cDNA is regenerated by PCR, which can be used as template for the subsequent round of ribosome display or cloning for *E. coli* expression.

2.3.3 Coupled transcription/translation

In vitro coupled transcription/translation was performed with TNT® T7 Quick Coupled Transcription/Translation System. The reaction was set up by mixing 20 µl of TNT T7 Quick Master Mix, 0.5 nmol of methionine (both provided in the kit), 50 nmol of Mg acetate, 0.1-1 µg of DNA (product from section 2.3.1) and DEPC-treated H₂O making up to a final volume of 25 µl, and incubating at 30°C for 60 minutes. In order to remove input DNA, 60 units of DNase I were added to the TNT mixture and incubated at 30°C for 20 minutes. Then the mixture was diluted with 35 µl of cold PBS containing 5 mM of Mg acetate before transferring to antigen-coated PCR tube, and incubating at 4°C for 2 hours.

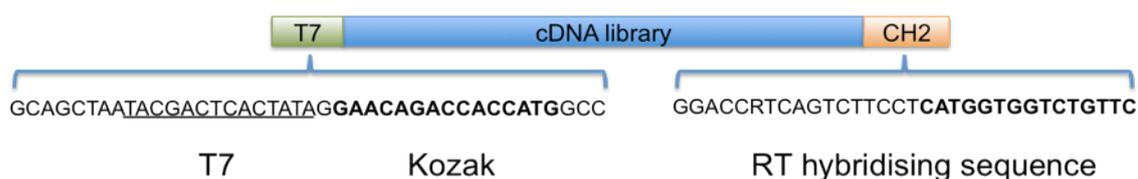
2.3.4 cDNA recovery by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After washing 5 times with ice cold ribosome display washing buffer and 2 times with cold distilled H₂O, the PCR tube was treated with 8 µl of DEPC-treated H₂O heated at 75°C for 10 minutes; then 20 nmol of deoxyribonucleotide triphosphate (dNTP, provided in ProtoScript® First Strand cDNA Synthesis Kit) and 20 pmol of primer IP1 were added to the tube and heated at 70°C for 5 minutes, followed by rapid cooling on ice for at least 30 seconds. RT-PCR recovery was performed with ProtoScript® First Strand cDNA Synthesis Kit, by adding 200 units of M-MuLV reverse transcriptase, 10 units of RNase inhibitor (both provided in the kit), 10 nmol of dithiothreitol and DEPC-treated H₂O to make up to a final volume of 20 µl. The mixture was incubated at 42°C for 75 minutes followed by 80°C for 5 minutes. The cDNA generated was ready for amplification by single primer PCR (figure 2.4).

2.3.5 Single primer PCR and generation of full-length cDNA construct

Single primer PCR was carried out with 10 µl of 5X Taq & Go Ready to Use PCR Mix, 50 pmol of primer Kz1, 2 µl of recovered cDNA as template and DEPC-treated H₂O to a final volume of 50 µl. The cycling was carried out by: one cycle of initiation

at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 1 minute, and finally, one cycle at 72°C for 10 minutes. 1 µl of the PCR product was used as the template to carry out another 30 cycles PCR under the same condition to further amplify the selected cDNA. The second PCR product was analysed by gel electrophoresis and purified with QIAquick Gel Extraction Kit. Purified cDNA product was used as template to extend the cDNA fragment to full-length and introduce restriction site *NotI* at the 3' end, with 25 pmol of each primer T7AB and LP1 and annealing temperature at 55°C. The regenerated full-length cDNA can be used as template in additional rounds of ribosome display or *E. coli* cloning.



IP1: GAACAGACCACCATGAGGAAGACTGAYGGTCC

Kz1: GAACAGACCACCATG

Figure 2.4 Primer design for *in situ* reverse transcription and single primer amplification

Primer IP1 was designed to introduce a 15 bp Kozak sequence to the 3' end of mRNA, leading to the generation of single-stranded cDNAs with a complementary flanking sequence at both 5' and 3' ends, which can be amplified with a single primer Kz1. Kozak sequence (bold) and T7 sequence (underlined) are shown.

2.4 Cloning and DNA sequencing

2.4.1 Chemically competent cell preparation

Chemically competent XL1-blue and BL21(DE3)pRARE cells were prepared using the Cohen method (Cohen *et al.*, 1972). In brief, 5 single colonies of *E. coli* cultured on Luria Bertani (LB) agar (containing 12.5 µg/ml of tetracycline for XL1-blue strain and 25 µg/ml of chloramphenicol for BL21(DE3)pRARE strain) were transferred into 10 ml of LB media and grown at 37°C, 250 rpm overnight. The culture was then added into 200ml of fresh LB media supplemented with the required antibiotics. The culture continued to grow at 37°C, 250 rpm until optical density (OD)₆₀₀ was 0.4 to 0.5. After cooling down on ice for 30 minutes, the culture was split in 50 ml volumes into four centrifuge tubes and subsequently centrifuged at 1000 g for 7 minutes at 4°C. Each pellet was resuspended in 12.5 ml of cooled 100 mM MgCl₂ and centrifuged at 1000 g for 7 minutes at 4°C. Then 25 ml of cooled 100 mM CaCl₂ was added to each pellet and the mixture was left on ice for 30 minutes, followed by centrifugation at 1000 g for 7 minutes at 4°C. Finally, each pellet was resuspended in 1 ml of 100 mM CaCl₂ containing 15% glycerol, and aliquots (50 µl) were flash frozen in dry ice and stored at -80°C.

2.4.2 Cloning, digestion and ligation

TOPO cloning was used to clone the PCR product selected by ribosome display. The reaction was set up with TOPO TA Cloning® Kit according to manufacturer's instructions. 4 µl of DNA recovered from ribosome display selection was ligated into 1 µl of TOPO vector and 1 µl of salt solution (provided in TOPO TA Cloning® Kit for Sequencing) and incubated at room temperature for 1 hour, before transforming into XL1-blue competent cells for blue/white colony screening. Plasmid DNA from positive colonies was extracted for sequencing (see below).

To express selected scFv in *E. coli*, restriction enzyme digestion followed by ligation into expression vector was performed as following: 30 µl of purified DNA was incubated with 5 units of each restriction enzyme *NcoI* and *NotI* in the presence of 1X NEBuffer 3, 100 µg/ml BSA and H₂O to a final volume of 50 µl. The reaction mixture was incubated at 37°C for 3 hours. Digested product was examined by gel electrophoresis on 2% (w/v) agarose gel, and subsequently purified with QIAquick Gel Extraction Kit, as described in section 2.2.3.

After digestion with restriction enzymes *NcoI* and *NotI*, the scFv was inserted into expression vector pSANG10-3F, which was also digested with the same restriction enzymes *NcoI* and *NotI*. Ligation reaction was set up by mixing 7 µl of purified scFv, 1 µl of purified pSANG10-3F, 1 µl containing 20 units of T4 DNA ligase and 1 µl of 10X T4 DNA ligase reaction buffer making up to a final volume of 10 µl. The reaction mixture was incubated at room temperature for 1 hour before transforming into XL1-blue competent cells. Positive plasmid DNA was identified and used to transform BL21(DE3)pRARE cells for protein expression.

Since the use of pSANG 10-3F vector requires two transformation steps prior to protein expression, an alternative vector was constructed to allow single transformation and immediate production. pABEXT vector was created by double digesting pMAL-c2X vector with restriction enzyme *NdeI* and *HindIII*, and ligating the insert between the *NdeI* and *HindIII* sites of pSANG10-3F vector (Figure 2.5). The constructed pABEXT vector encodes tac promoter (de Boer *et al.*, 1983) and PelB leader sequence (Milstein *et al.*, 1972). The tac promoter facilitates protein expression in *E. coli* XL1-blue strain, while the PelB leader sequence enables protein secretion into periplasm of *E. coli* therefore crude extract of secreted proteins can be tested quickly in ELISA. A full sequence of pABEXT vector is shown in the appendix 2.

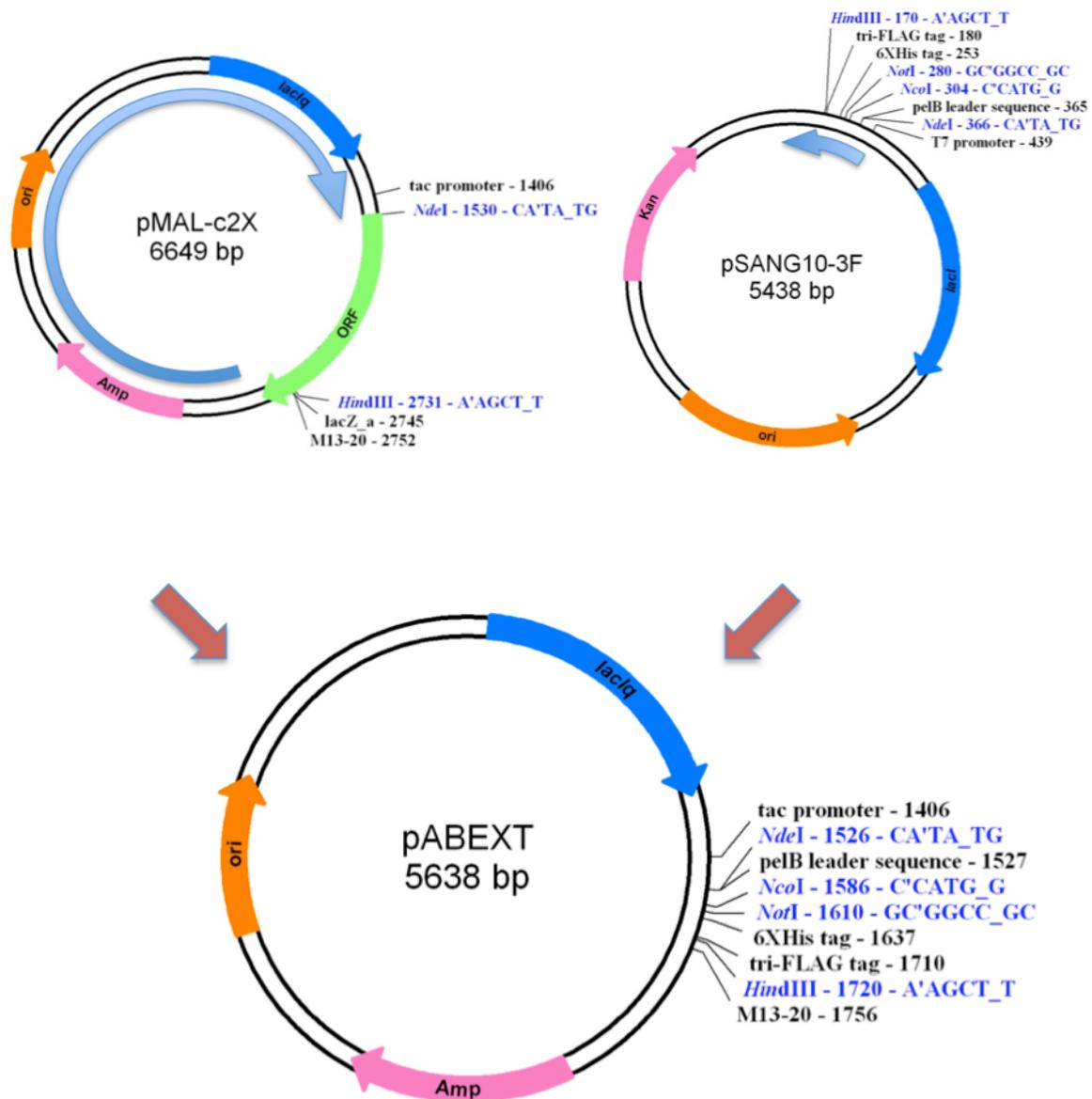


Figure 2.5 Construction of pABEXT vector

pABEXT vector was constructed by using pMAL-c2X vector as backbone, and inserting the fragment between *NdeI* and *HindIII* sites of pSANG10-3F vector. The constructed pABEXT vector consists tac promoter and PelB leader sequence, and both 6XHis-tag and tri-FLAG-tag for protein purification and detection.

2.4.3 *E. coli* transformation

For DNA cloning, TOPO ligation mixture was transformed into XL1-blue competent cells on X-gal plate to perform blue/white screening. 50 µl of XL1-blue competent cells were thawed on ice for 5 minutes and added 6 µl of TOPO ligation mixture. After incubation on ice for 5 minutes, the cells were heat shock in a 42°C water bath for 1 minute, followed by placing the tube on ice for 5 minutes. 250 µl of pre-warmed SOC (20 mg/ml Bactotryptone, 5 mg/ml Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂, 0.4% glucose, pH 7.0) was added to the tube and incubated at 37°C, 250 rpm for 1 hour. All the mixture was plated on X-gal plate (LB agar containing 40 µg/ml of X-gal, 200 µM of IPTG and 100 µg/ml of carbenicillin) and incubated at 37°C overnight.

For protein expression, the pSANG and scFv ligation mixture was transformed into XL1-blue competent cells on LB agar plate supplemented with 50 µg/ml of kanamycin using the same protocol as described above.

2.4.4 Plasmid DNA miniprep and DNA sequencing

Clones identified from X-gal plate was grown in 10 ml of LB media supplemented with 100 µg/ml carbenicillin at 37°C, 250 rpm overnight. The overnight culture was centrifuged at 1000 g for 10 minutes, and plasmid DNA was isolated from cells using QIAprep® Spin Miniprep Kit according to manufacturer's instructions. In brief, the cell pellet was resuspended in 250 µl of P1 buffer (50 mM Tris-HCl, 10 mM EDTA, 50 µg/ml RNase, pH 8.0) and transferred into a 1.5 ml micro-centrifuge tube. The cells were lysed by adding 400 µl of P2 buffer (0.2 M NaOH, 1% SDS) and inverting the tube 5 times. The solution was neutralised by adding 580 µl of N3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) and inverting the tube 5 times. The mixture was centrifuged at 13000 g for 10 minutes, and the supernatant was carefully transferred to QIAprep spin column and centrifuged at 13000 g for 1 minute. Then the column was washed once with 500 µl of PB buffer (5 M guanidine hydrochloride, 20 mM Tris-HCl, 38% ethanol, pH 6.6), followed by 750 µl of PE

buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol, pH 7.5). After discard the flow-through, the column was centrifuged at 13000 g for 1 minute again to remove residual wash buffer. Purified DNA was eluted by adding 30 μ l of DEPC-treated H₂O and centrifuged at 13000 g for 1 minute.

Purified plasmid DNA was sequenced at the Wolfson Institute for Biomedical Research, University College London using primers M13-20 (5'-GTA AAA CGA CGG CCA GT-3') and M13 rev (5'-GGA AAC AGC TAT GAC CAT G-3').

2.5 Protein expression and purification

2.5.1 Protein expression with pSANG vector

Clones of interest were expressed as scFv after DNA sequencing. DNA was amplified by PCR using primer T7AB (5'- GC AGC TAA TAC GAC TCA CTA TAG GAA CAG ACC ACC ATG GCC -3') and reverse primer TJ011 (5'- GCC CGC GGC CGC TGT GCC CCC AGA GGT G -3', for IgG1 sequences), or TJIgG24 (5'- GCC CGC GGC CGC TGT GCT CTC GGA GGT G -3', for IgG2, IgG4 sequences). After digestion with restriction enzymes *NcoI* and *NotI*, the scFv was inserted into expression vector pSANG10-3F and transformed into XL1-blue competent cells. Positive plasmid DNA was identified and used to transform BL21(DE3)pRARE cells for protein expression.

BL21(DE3)pRARE was chosen for expressing antibody scFv as mentioned in 1.1.11. 5 single colonies from LB agar supplemented with 50 µg/ml of kanamycin and 25 µg/ml of chloramphenicol were grown in 10 ml of LB media at 37°C, 250 rpm overnight with required antibiotics. Before induction, 700 µl of overnight culture was made a bacterial stock by mixing with 300 µl of 50% glycerol and stored at -80°C. The remaining culture was used for protein expression by adding into 200 ml of fresh LB media supplemented with appropriate antibiotics. The culture was grown at 37°C, 250 rpm until OD₆₀₀ reached 0.5, and then incubated on ice for 30 minutes. To make an uninduced sample as the control, 1 ml of culture was taken out and centrifuged at 13000 g for 1 minute. Protein expression was induced by adding IPTG into the culture at the final concentration of 0.3 mM and the culture was incubated at 20°C, 250 rpm for 20 hours. 1 ml of the induced culture was collected for protein analysis (section 2.5.5).

2.5.2 Protein expression with pABEXT vector

To facilitate a high throughput, an alternative expression strategy was performed using pABEXT vector. Selected cDNA from ribosome display was digested with *NcoI* and *NotI* directly (figure 2.6). Single-chain product (VL-link-VH-CH1-hinge-partial CH2) at the size of approximately 1200 bp was ligated into pABEXT vector. Proteins were expressed in 50 ml scale with the method described in section 2.5.1, and extracted by osmotic shock (see 2.5.3). The crude extract was used to perform ELISA against recombinant gp120 as described in 2.6.1. Positive clones were subsequently sequenced with primer M13-20, and standard protocol performed with these clones as described above.

2.5.3 Purification of proteins from *E. coli* periplasm (osmotic shock)

The proteins expressed in the periplasm of *E. coli* were extracted by osmotic shock, and subsequently purified with a column supplemented with Nickel chelating resin, as described in *The QIAexpressionistTM -- A handbook for high-level expression and purification of 6xHis-tagged proteins*, fifth edition. Basically, the cells from a 200 ml culture were collected by centrifugation at 3000 g for 20 minutes at 4°C. The cell pellet was resuspended in 5 ml of TES buffer [30 mM Tris base, 1 mM Ethylenediaminetetraacetic acid (EDTA), 20% sucrose, pH 8.0]. The cells were incubated on ice for 10 minutes and centrifuged at 9000 g for 20 minutes at 4°C. The supernatant (periplasmic fraction 1) was collected and stored on ice, while the cell pellet was resuspended in 5 ml of 5 mM MgCl₂. After incubating for 10 minutes on ice, the suspension was centrifuged at 9000 g for 20 minutes at 4°C. The supernatant (periplasmic fraction 2) was collected and mixed with periplasmic fraction 1. Remaining cell pellets were analysed for the non-secreted recombinant protein expression.

The periplasmic fractions were subsequently purified using a Ni-NTA column, which was equilibrated with lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 10 mM imidazole, pH 8.0). At the same time, 3.3 ml of 4X lysis buffer (200 mM NaH₂PO₄, 2

M NaCl, 40 mM imidazole, pH 8.0) was added to the supernatant, which was then loaded to the column. The column was washed with 5 ml of wash buffer (50 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, pH 8.0), and the protein was eluted with 0.5 ml of elution buffer (50 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 8.0) by 5 times. Purified proteins were flash frozen on dry ice and stored at -20°C.

2.5.4 Purification of proteins from *E. coli* total extracts

Recombinant proteins were also extracted from total cell lysate of *E. coli* and affinity purified with Ni-NTA spin columns using the protocol described in *Ni-NTA Spin Kit Handbook*, second edition and *The QIAexpressionistTM -- A handbook for high-level expression and purification of 6xHis-tagged proteins*, fifth edition. Briefly, the cells from 200 ml culture were collected by centrifugation at 3000 g for 20 minutes at 4°C. The cell pellet was resuspended in 5 ml of lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 10 mM imidazole, pH 8.0). 500 µg of lysozyme and 5 µmol of phenylmethanesulfonyl fluoride (PMSF) were added to the suspension, in order to break *E. coli* cell walls and inhibit protease. The cell membrane was disrupted by sonicating the suspension for 30 seconds by 6 times using an ultrasonic probe, followed by incubating on ice for 1 hour. Soluble protein was collected by performing centrifugation at 19000 g for 45 minutes at 4°C, and transferring supernatant to a fresh tube. Recombinant protein was subsequently purified by Ni-NTA spin column, which was equilibrated with 600 µl of lysis buffer before use. The supernatant was loaded to spin column and centrifuged at 2000 g for 2 minutes at 4°C. The column was washed with 600 µl of wash buffer (50 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, pH 8.0). Finally, protein was eluted in 100 µl of elution buffer (50 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 8.0) by 5 times.

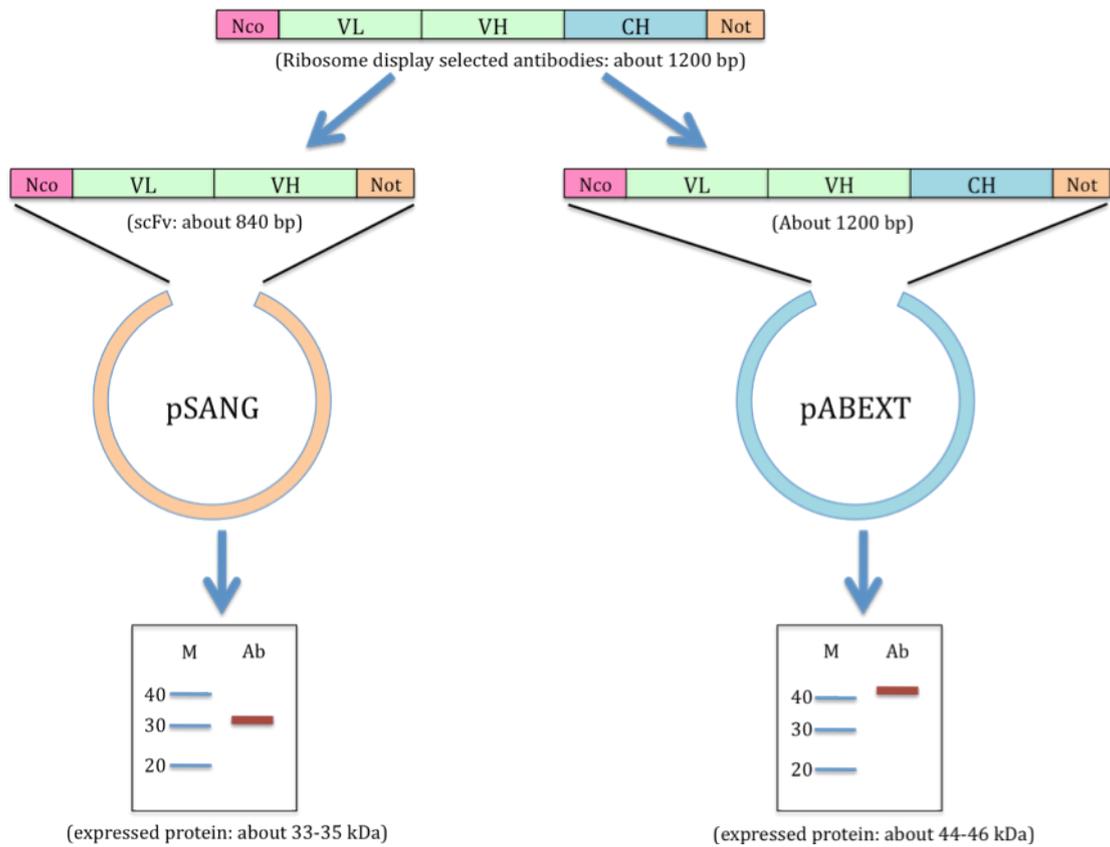


Figure 2.6 Antibody expression in two vectors

Ribosome display selected antibodies were expressed either in pSANG vector in the form of scFv, or in pABEXT vector in the form of single-chain for a quick screening purpose. Note antibodies were expressed at different sizes because of the two different methods.

To remove the excess salt from eluted proteins, centrifugal filter units was used to desalt and also concentrate the purified proteins according to the manufacturer's instructions. Eluted protein fractions were mixed and loaded to Amicon filter column, and centrifuged at 13000 g for 15 minutes at 4°C to remove most of the solvent. The remaining solution was diluted with 450 µl of PBS, followed by centrifugation at 13000 g for 15 minutes at 4°C. The column was inverted in a clean collection tube, and centrifuged at 1000 g for 2 minutes at 4°C to collect concentrated and desalted protein. Protein concentration was determined by Nanodrop 1000 spectrophotometer in accordance with manufacturer's instructions. The proteins were flash frozen and stored at -80°C.

2.5.5 Electrophoresis analysis of scFv proteins

Recombinant proteins purified by the methods described in section 2.5.3 and 2.5.4 were analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. SDS-PAGE was prepared using Laemmli system (Laemmli, 1970) with some modifications. 12% Resolving gel [12% acrylamide/bis (29:1), 360 mM Tris base, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED, pH 8.8] and 4% Stacking gel [4% acrylamide/bis (29:1), 60 mM Tris base, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED, pH 6.8] were used to detect protein fragments. Cell pellets (uninduced and induced samples from section 2.5.1) were dissolved in 50 µl of 4X protein gel loading buffer (62.5 mM Tris base, 2% SDS, 5% β-mercaptoethanol, 0.05% Bromophenol blue, 10% glycerol, pH 6.8) and heated at 95°C for 5 minutes. 36 µl of other samples from protein purification (soluble fraction, flow-through, wash fraction and elution1-4) were diluted with 12 µl of 4X protein gel loading buffer and heated at 95°C for 5 minutes. Typically 20 µl (10 µl for uninduced and induced samples) was loaded on each well of the gel, while 5 µl of protein marker was used as standards. Electrophoresis was performed using a Mini-PROTEAN Tetra Electrophoresis System with running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), and 200 volts were applied for 45 minutes. Gels after SDS-PAGE can be used for Coomassie blue staining or for western blotting.

For Coomassie blue staining (Meyer and Lamberts, 1965), the proteins were visualised on gels by dye staining using the solution containing 0.025% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid, in a microwave until boiling followed by 1 minute on rocking table. Finally, the gel was destained using 10% (v/v) acetic acid by boiling in a microwave.

For western blotting, the proteins were transferred from the gel to Immobilon-P transfer membrane using a Mini Trans-Blot Cell with transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) (Towbin *et al.*, 1979), and 100 volts were applied for 1 hour. The membrane was blocked with 2% milk powder (Tesco)/PBS at room temperature for 1 hour. After washing with Tris buffered saline Tween 20 (TBST, 50 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.4) twice, the membrane was incubated with monoclonal anti-polyHistidine-alkaline phosphatase antibody produced in mouse (1: 20000 diluted in 2% milk/PBS) at room temperature for 2 hours. The membrane was then washed 5 times with TBST, and protein bands were visualised by adding substrate [0.02% BCIP and 0.03% NBT in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, 0.05% Tween 20, pH 9.5)] and incubating at room temperature for 2 to 5 minutes.

Protein markers were used for SDS-gel and western blotting. They were made by mixing 4 recombinant proteins together: pET32a plasmid (20 kDa), Red Fluorescent Protein (RFP) in pSANG (30 kDa), MopB protein in pSANG (42 kDa) and Heat Shock Protein (HSP) 70 in pET16b (75 kDa). These protein standards were confirmed by comparing with commercial Precision plus protein dual Xtra standards in SDS-PAGE and western blotting.

2.6 Functional assays

2.6.1 ELISA

Antibody binding to recombinant gp120 was examined by Enzyme-linked immunosorbent assay (ELISA). F96 MaxiSorp Nunc-Immuno plates were coated with 100 μ l of 1 μ g/ml of gp120 in PBS overnight. As a negative control, 100 μ l of 1 μ g/ml of BSA/PBS was coated. Plates were blocked with 2% milk/PBS at room temperature for 1 hour, followed by washing with TBST 5 times. Purified antibodies diluted at 5-25 μ g/ml in PBS were incubated in the wells at room temperature for 2 hours. Plates were washed with TBST for 5 times before adding monoclonal anti-polyHistidine-alkaline phosphatase antibody (1: 10000 diluted in 2% milk/PBS) at room temperature for 2 hours. The assay was developed with 1 mg/ml pNPP in 0.2 M Tris (pH 8.0). After incubating at room temperature for 2 hours, the absorbance at 405 nm was measured using a VICTOR plate reader. Both sample and control were performed in triplicate.

2.6.2 Neutralisation assay

The neutralisation assay was performed at the Centre for Infectious Disease, Blizard Institute of Cell and Molecular Science, Barts and The London, Queen Mary, University of London. The neutralising activity was measured by luciferase-based assay in TZM-bl cell (Li *et al.*, 2005). Antibodies were tested at the starting concentration of 50 μ g/ml followed by two 4-fold dilutions in 50 μ l of Dulbecco's modified Eagle medium (DMEM) with 10% Foetal calf serum (FCS) in a 96 well flat bottom opaque Nunc plate. 50 μ l of virus prepared from replication competent HIV-1 molecular clones was added and left at 37°C for 1 hour. 10^4 TZM-bl cells in 100 μ l of DMEM with 10% FCS, containing 50 μ g/ml of Diethylaminoethyl (DEAE)-dextran, were added to the virus-antibody mixtures. Wells with protein elution buffer (i.e. PBS) were included as virus control (cell + virus), and wells with cells alone were included as the cell control. Neutralising plasma (1:20 dilution) from patient K530 was used as the positive control. After incubation at 37°C for 48 hours, 150 μ l of the medium was

removed from each well. 50 μ l of Bright-GLo Luciferase reagent was added to remaining cells and incubated for 2 minutes. Luminescence was measured using a FLUOstar OPTIMA. Neutralisation percentage was calculated as $100 \times [1 - (\text{sample luminescence} / \text{virus control luminescence})]$ after the cell alone control luminescence was subtracted.

Chapter 3

Results

3.1 Construction of single-chain antibody libraries

3.1.1 Construction of the single-chain antibody library from patient M325

A total of 98 µg RNA was isolated from 100 ml of blood of donor M325 and 20 µg RNA was from 20 ml of blood of donor K530. Both RNA samples were used as the template to generate required cDNA synthesis followed by PCR amplification of individual V λ , V κ and heavy chain using specific primers designed (see the materials and methods).

Figure 3.1 shows the PCR production of individual V κ and V λ light chain fragments from patient M325 using antibody family-specific primers. DNA bands with expected size were excised and recovered.

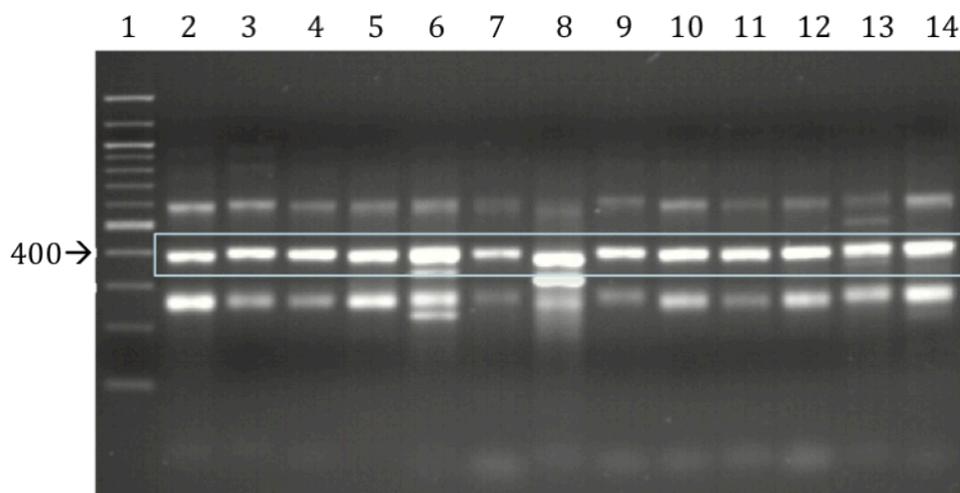


Figure 3.1 PCR products of 4 V κ chains and 9 V λ chains in library M325

Lane 1, 100bp ladder; lane 2, V κ 1; lane 3, V κ 2346; lane 4, V κ 36; lane 5, V κ 5; lane 6, V λ 1; lane 7, V λ 1459; lane 8, V λ 15910; lane 9, V λ 2; lane 10, V λ 3A; lane 11, V λ 3B; lane 12, V λ 3DLP16; lane 13, V λ 6; lane 14, V λ 78. The expected size of products (~380 bp) is indicated.

However, PCR generation of VH from the synthesised cDNA only led to production of about 50% of the VH families with the expected size, while the rest failed to produce detectable bands. To solve this problem, one-step RT-PCR, in which the reverse transcription is coupled with PCR, was performed, leading to the successful generation of all the VH families using the identical set of primers directly from mRNA template in the total RNA extract (figure 3.2). The heavy chains (780bp) were identified, based on their sizes. Despite some non-specific PCR bands were also detected on the gel, the correct-sized bands were eluted, pooled and used for DNA assembly (see below).

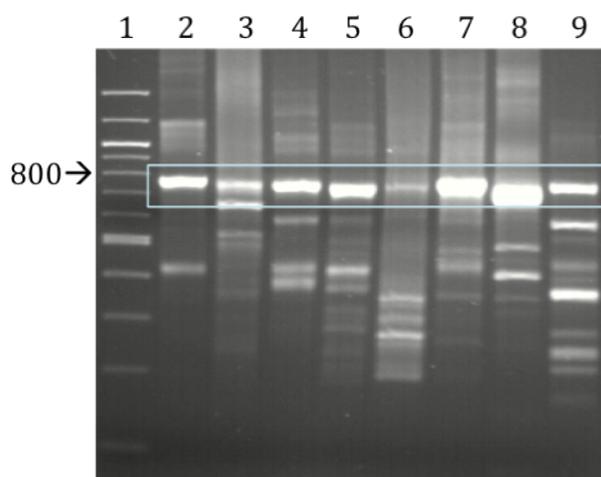


Figure 3.2 PCR products of 8 heavy chains in library M325

Lane 1, 100bp ladder; lane 2, VH1; lane 3, VH1257; lane 4, VH2; lane 5, VH3A; lane 6, VH3B; lane 7, VH4; lane 8, VH4DP63; lane 9, VH6. The expected size of the products (~780 bp) is indicated.

Both the eluted light chains and heavy chains were then combined and assembled individually. Analysis by gel electrophoresis shows the successful linkage of light chains with heavy chains. Interestingly, VHs linked with kappa chains gave stronger bands than the VHs linked with lambda chains. A representative gel picture (V κ and V λ linked with VH1) is shown as figure 3.3. The assembled fragments were pooled and stored for use. This library was called Library M325.

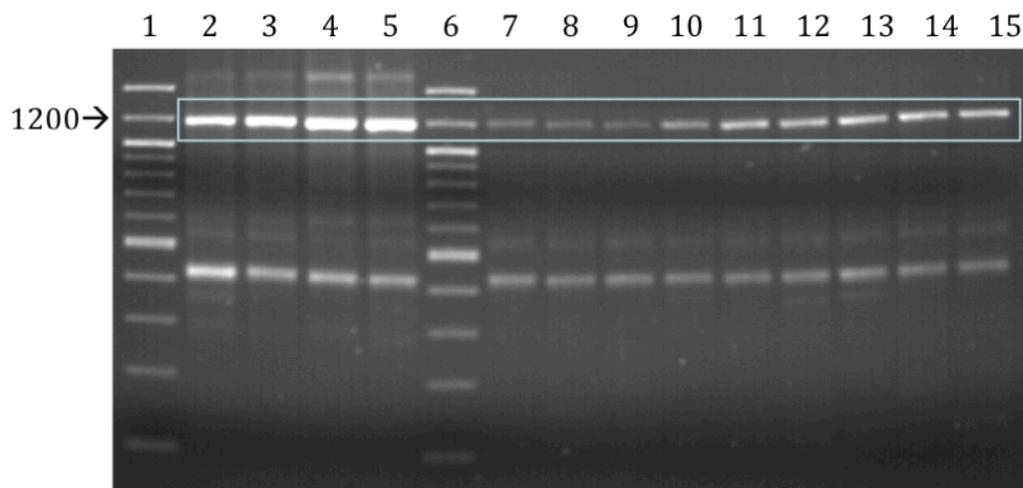


Figure 3.3 PCR products of V κ -VH1 and V λ -VH1 combinations in library M325

Combinations with VH1 families are chosen to illustrate the single-antibody library M325 construction. Lane 1, 100bp ladder; lane 2, V κ 1-VH1; lane 3, V κ 2346-VH1; lane 4, V κ 36-VH1; lane 5, V κ 5-VH1; lane 6, 100bp ladder; lane 7, V λ 1-VH1; lane 8, V λ 1459-VH1; lane 9, V λ 15910-VH1; lane 10, V λ 2-VH1; lane 11, V λ 3A-VH1; lane 12, V λ 3B-VH1; lane 13, V λ 3DLP16-VH1; lane 14, V λ 6-VH1; lane 15, V λ 78-VH1. The expected size of the products (~1200 bp) is indicated.

3.1.2 Construction of the single-chain antibody library from patient K530

An alternative library from patient K530 was also constructed using the same procedure described for the library M325 in 3.1.1, with following modifications: VL, VH fragments from first 30 cycles of PCR were separated on an agarose gel, purified and then used as the template for amplification by PCR for further 30 cycles using the same set of primers. Subsequent combinations were carried out using the same protocol as the library M325, using purified DNA from second round of PCR as templates. Figure 3.4, 3.5 and 3.6 show the gel analysis of the assembled DNA fragments with the expected sizes, indicating correct construction of the library in the form of VL-link-VH-CH1-hinge-partial CH2 as designed.

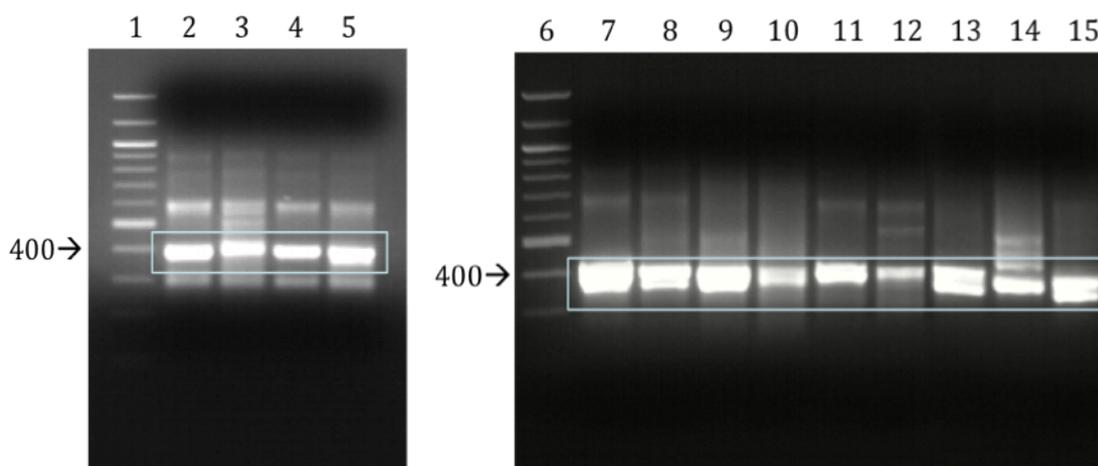


Figure 3.4 PCR products of 4 V κ chains and 9 V λ chains in library K530

Lane 1, 100bp ladder; lane 2, V κ 1; lane 3, V κ 2346; lane 4, V κ 36; lane 5, V κ 5; Lane 6, 100bp ladder; lane 7, V λ 1; lane 8, V λ 1459; lane 9, V λ 15910; lane 10, V λ 2; lane 11, V λ 3A; lane 12, V λ 3B; lane 13, V λ 3DLP16; lane 14, V λ 6; lane 15 V λ 78. Products are from two rounds of 30 cycles PCR. The expected size of products (~380 bp) is indicated.

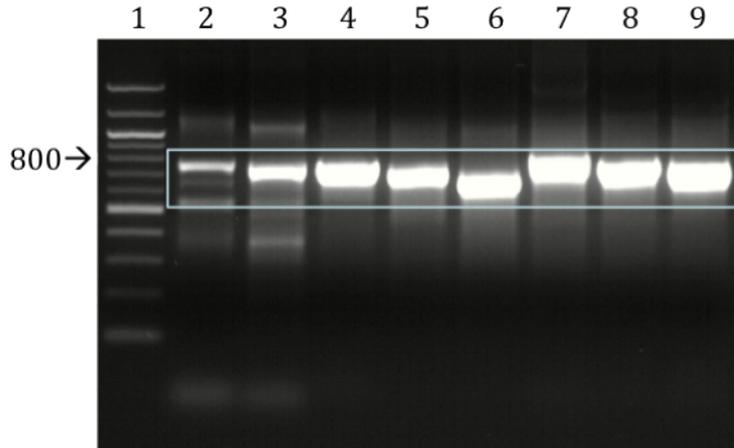


Figure 3.5 PCR products of 8 heavy chains in library K530

Lane 1, 100bp ladder; lane 2, VH1; lane 3, VH1257; lane 4, VH2; lane 5, VH3A; lane 6, VH3B; lane 7, VH4; lane 8, VH4DP63; lane 9, VH6. Products are from two rounds of 30 cycles PCR. The expected size of the products (~780 bp) is indicated.

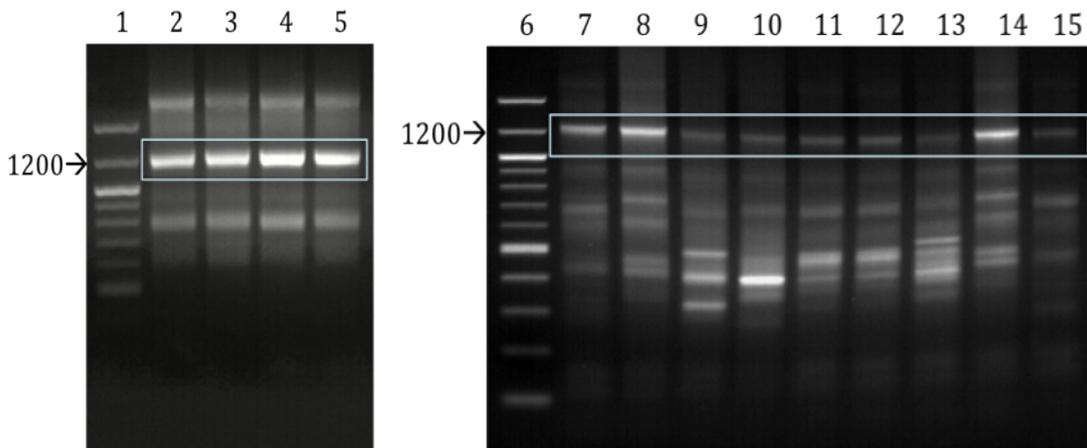


Figure 3.6 PCR products of V κ -VH1 and V λ -VH1 combinations in library K530

Combinations with VH1 families are chosen to illustrate the single-antibody library K530 construction. Lane 1, 100bp ladder; lane 2, V κ 1-VH1; lane 3, V κ 2346-VH1; lane 4, V κ 36-VH1; lane 5, V κ 5-VH1; lane 6, 100bp ladder; lane 7, V λ 1-VH1; lane 8, V λ 1459-VH1; lane 9, V λ 15910-VH1; lane 10, V λ 2-VH1; lane 11, V λ 3A-VH1; lane 12, V λ 3B-VH1; lane 13, V λ 3DLP16-VH1; lane 14, V λ 6-VH1; lane 15, V λ 78-VH1. The expected size of the products (~1200 bp) is indicated.

3.2 Ribosome display and antibody selection

Individual PCR libraries were combined and extended at the 3' end with a spacer for ribosome display as described in section 2.3.1. The full-length PCR products (about 1225 bp) were added into TNT coupled reticulocyte lysate and the mixture was incubated at 30°C for 1 hour to generate ARM complexes. The translation mixture was then added into wells coated with 0.5 mg/ml of antigen gp120 for additional one hour. After washing, the bound ARM complexes on wells were directly subjected to *in situ* RT-PCR to recover the selected genetic information. Recovered PCR product was analysed by agarose gel electrophoresis (Figure 3.7).

In a preliminary selection, no RT-PCR product was detected after 30 cycles of PCR, while product can be seen with 40 cycles of PCR. This indicates that the DNA fragment being selected is rare and thus, further cycles are required. However, stop codons were observed in the PCR products after 40 cycles, indicating that too many PCR cycles may have introduced mutations into the DNA fragments (i.e., when dNTPs in the PCR reaction become limiting, the error rate of the Taq polymerase increases). This problem has been avoided by performing the first PCR by 30 cycles, followed a second PCR of 30 cycles with replenished dNTPs. DNA sequencing shows no stop codon generated in this method.

In order to increase the recovery sensitivity, an *in situ* RT-PCR procedure developed by He and Taussig was performed using a single-primer (He and Taussig, 2007). In this method, an internal primer IP1 was designed to contain both a sequence for hybridising to the upstream region of 3' mRNA (to avoid the stalling ribosome) and a sequence identical to the 5' region of mRNA (as illustrated in figure 2.4). cDNA synthesis using IP1 leads to the generation of single-stranded cDNAs with a complementary flanking sequence at both 5' and 3' ends, which can be effectively amplified by PCR using a single primer (He and Taussig, 2007). As the internal primer IP1 anneals at 28 amino acids upstream of the C terminal, the PCR product recovered was 75bp shorter than original full-length size. In order to perform the subsequent ribosome display cycle, the recovered PCR fragment was then extended by second PCR with long primers T7AB and LP1 to add the T7 promoter and protein initiation

elements, as well as extend the 3' end into the full-length product (1225bp) (Figure 3.7, right).

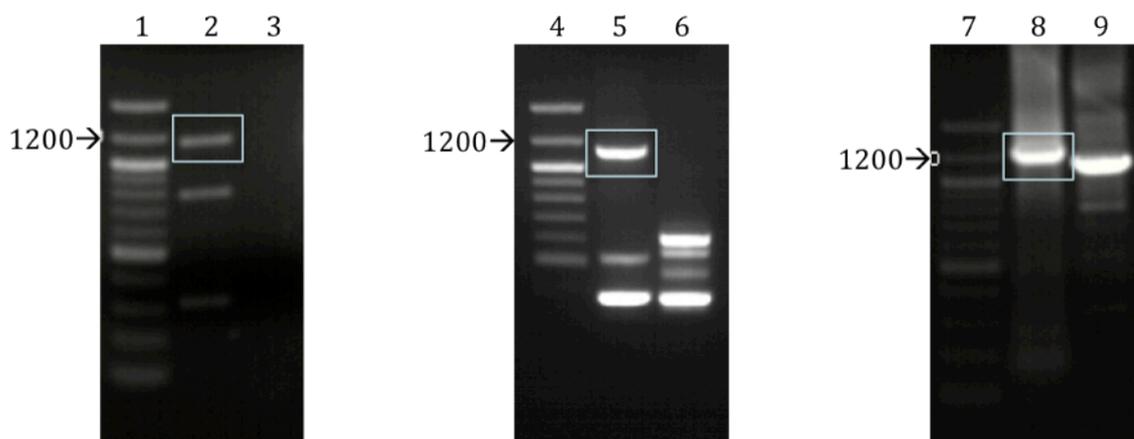


Figure 3.7 PCR products after one round of ribosome display

PCR products after 1 round of ribosome display were run on 1.5% (w/v) agarose gels. The expected size of the amplified product with single-primer Kz1 is ~1150 bp, and the expected size of the full-length product amplified with T7AB and LP1 is ~1225 bp. DNA bands shown in rectangles were excised from the agarose gel and purified as described in section 2.2.3. Lane 1, 100 bp ladder; lane 2, single-primer PCR product after 1 round of ribosome display against antigen gp120 from library M325; lane 3, single-primer PCR product after 1 round of ribosome display against BSA from library M325 (negative control); lane 4, 100 bp ladder; lane 5, single-primer PCR product after 1 round of ribosome display against antigen gp120 from library K530; lane 6, single-primer PCR product after 1 round of ribosome display against BSA from library K530 (negative control); lane 7, 100 bp ladder; lane 8, full-length PCR product using DNA purified from lane 2 as template (~1225 bp); lane 9, amplification of DNA purified from lane 2 with primer Kz1 (~1150 bp, to compare with lane 8).

To select antibodies against gp120, three rounds of ribosome display were initially performed using the library M325. However, sequence analysis of 3 random selected clones revealed the same sequence among them, indicating a strong enrichment of this sequence in the three-cycle selection. In order to select more diverse antibodies, a single round of ribosome display was carried out from both libraries M325 and K530. Ribosome selected cDNA products were cloned into *E. coli* XL1-blue and screened for blue/white clones on a X-gal plate. Table 3.1 lists the CDR3 sequence analysis of 10 representative clones (7 from library M325 and 3 from library K530), which reveals that most of selected clones fall into several groups with very similar CDRs in both light chains and heavy chains, suggesting the selection worked. Interestingly, a sequence with the identical CDR3 of both light and heavy chains were identified with two clones from each of the two libraries (clones 011, 1-5 from library M325 and clones 2-2, 2-4 from library K530) (Table 3.1).

IMGT website was used to analyse the sequences (Giudicelli *et al.*, 2004). When aligning with online database, all the V κ and V H domains have shown 99-100% similarities to published anti-HIV antibodies (Altschul *et al.*, 1990). In contrast, no similar sequence of anti-HIV antibody was found for V λ domains. Based on antibody subclasses, 7 out of 10 show the sequences of IgG1, and the rest belong to IgG2 or IgG4 families. The selected antibody families and subclasses are listed in table 3.2. Full DNA sequences of the selected 10 antibodies are shown in the appendix 3.

Table 3.1

Library	Clone	Light chain		Heavy chain	
		CDR3	FR4	CDR3	FR4
M325	011	QQYSASSST	FGQG	ARLAVDTVMVQGYFDL	WGQG
	1-1	QSYDNRNQI	FGGG	VRQSLDNYAYHLDY	WGQG
	1-5	QQYSASSST	FGQG	ARLAVDTVMVQGYFDL	WGQG
	1-7	QSYEASSHEWV	LGGG	ARDEVTGTGVLDY	WGQG
	I3	QRYGSSPRA	FGPG	ARDHVDTPMGLDY	WSQG
	I4	QRYGSSPRA	FGPG	ARDHVDTPMGLDY	WSQG
	M5	QAWDSSTVV	SGGG	ARQGYTHRDVLTRQKFYFYMDV	WGKG
K530	2-1	QSYDNRNQI	FGGG	VRQSLDNYAYHLDY	WGQG
	2-2	QQYSASSST	FGQG	ARLAVDTVMVQGYFDL	WGQG
	2-4	QQYSASSST	FGQG	ARLAVDTVMVQGYFDL	WGQG

Table 3.1 CDR3 sequences of the selected antibodies

DNA sequencing of the selected clones from one round of ribosome display reveals their CDR3 and framework (FR) 4 sequences. Clones 011, 1-5 from library M325 and clones 2-2, 2-4 from library K530 show identical CDR3 sequences (shaded in blue), and clone 1-1 from library M325 appears to have same CDR3 sequence as clone 2-1 from library K530 (shaded in yellow).

Table 3.2

Library	Clone	VL family	VH family	Subclass
M325	011	V κ 1	VH2	IgG1
	1-1	V λ 6	VH3A	IgG4
	1-5	V κ 1	VH2	IgG1
	1-7	V λ 6	VH6	IgG1
	I3	V κ 1	VH1	IgG1
	I4	V κ 1	VH1	IgG1
	M5	V λ 6	VH6	IgG4
K530	2-1	V λ 6	VH3A	IgG2
	2-2	V κ 1	VH2	IgG1
	2-4	V κ 1	VH2	IgG1

Table 3.2 VL, VH families and subclasses of the selected antibodies

DNA sequencing of the selected clones from one round of ribosome display reveals that the antibodies fall into several groups of VL, VH families and subclasses. E.g., clones 011, 1-5 from library M325 and clones 2-2, 2-4 from library K530 belong to the same VL, VH families and subclass (shaded in blue); clones I3 and I4 from library M325 belong to the same group (shaded in green); while clone 1-1 from library M325 and clone 2-1 from library K530 have the same VL, VH families, but different subclasses (shaded in yellow).

3.3 Protein expression and purification

3.3.1 Calibration of protein markers

In order to facilitate detection and monitoring protein size, especially in western blotting probed by an anti-His-tag antibody, His-tagged protein standard was created in our lab. Four known recombinant proteins [pET32a plasmid (20kDa), RFP (30kDa), MopB protein (42kDa) and HSP 70 (75kDa)] were fused to a 6X His-tag and produced in *E. coli*, followed by affinity purification. These His-tagged proteins were mixed with the same ratio and used as the markers for the SDS-PAGE and western blotting, as described in section 2.5.5. A comparison of this lab-made protein marker and commercial protein marker is shown in figure 3.8.

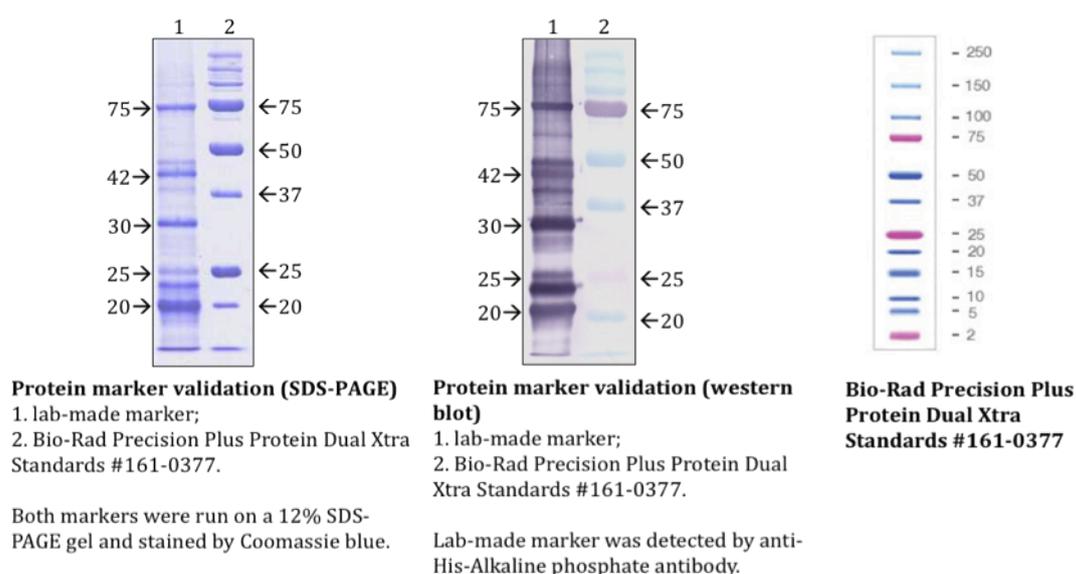


Figure 3.8 Comparison of lab-made protein marker with Bio-Rad standards

5 μ l of lab-made protein marker and 10 μ l of Bio-Rad Precision plus protein dual Xtra standards were run on a 12% SDS-PAGE gel. The left picture shows the comparison of the size on SDS-PAGE stained by Coomassie blue; the picture in the middle shows the comparison on western blot, detected by monoclonal anti-polyHistidine-alkaline phosphatase antibody; the picture on the right is the Bio-Rad Precision plus protein dual Xtra standards.

3.3.2 Analysis of antibody expression and solubility in *E. coli*

Protein expressed in pSANG vector was designed to secrete into the periplasmic space due to the presence of the PelB leader sequence at the 5' end of the antibody scFv. To determine whether or not the antibodies were expressed in *E. coli* BL21(DE3)pRARE strain, the expressed antibody fragments were first examined by purifying the scFvs by Ni-NTA column from the total bacteria extracts under a denaturing condition with 8 M urea. After the His-tagged scFv protein was eluted from Ni-NTA column by decreasing the pH from 8.0 to 4.5, SDS-PAGE was used to analyse the purity and quantity (figure 3.9, lane 7, 8 and 9). This shows that a dominated protein with expected scFv size was isolated and concentrated. Secondly, to determine whether the antibodies were soluble and correctly folded, the expressed antibody fragments were purified from the total cell extract under a native condition. 8 M urea was replaced by 50 mM NaH₂PO₄ in all the buffers but maintained the pH 8.0, and the scFv was eluted from the Ni-NTA column by increasing the imidazole concentration (figure 3.10, lane 7, 8 and 9). Finally, to examine whether the antibodies were secreted into the periplasmic space of *E. coli*, the periplasmic proteins were extracted by osmotic shock (see section 2.5.3), followed by Ni-NTA purification column (figure 3.11, lane 8 and 9). Figure 3.9-3.11 provides an example of the purification of the antibody pSANG-1-7, which was chosen to validate various purification strategies. The optimised purification conditions were then applied to other antibody fragments as shown in the appendix 4.

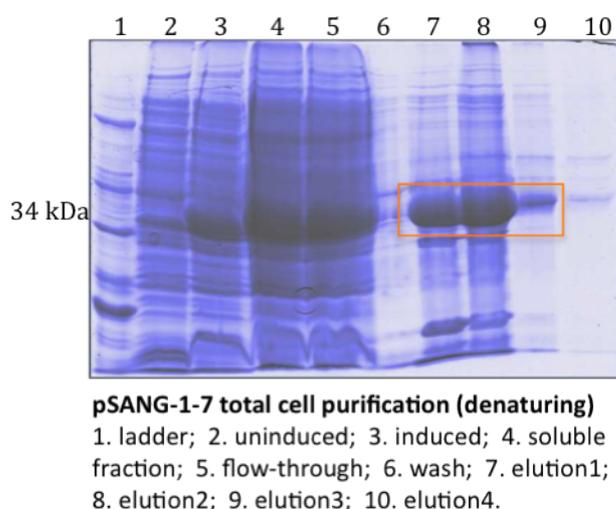
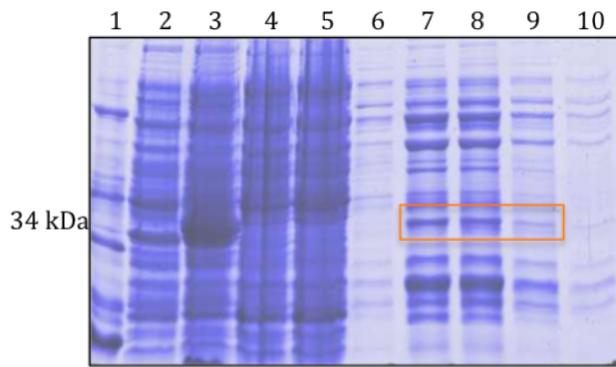
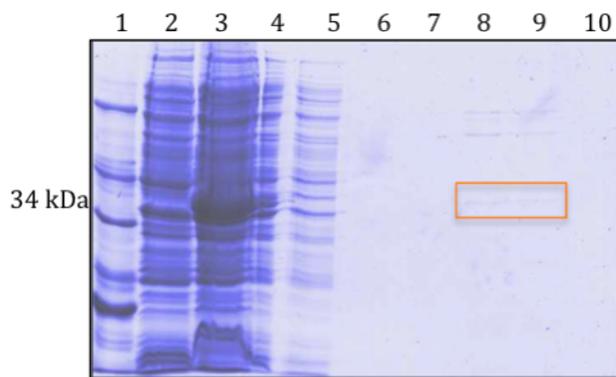


Figure 3.9 Antibody 1-7 purified from total cell extract under the denaturing condition



pSANG-1-7 total cell purification (native)
 1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.

Figure 3.10 Antibody 1-7 purified from total cell extract under the native condition



pSANG-1-7 periplasmic purification
 1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.

Figure 3.11 Antibody 1-7 antibody purified from periplasmic fraction

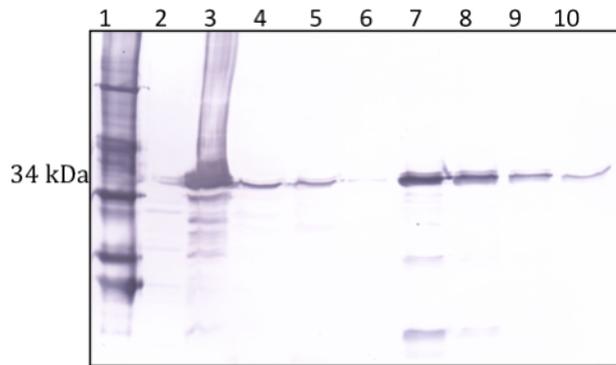
The above figures (3.9-3.11) reveal the successful expression and purification of recombinant antibody fragment from *E. coli*. By comparing the uninduced (lane 2) and induced (lane 3) protein samples, it is shown that the recombinant antibody fragment was expressed at the expected size of 34 kDa upon IPTG induction. While large quantity of scFv was recovered under the denaturing condition (Figure 3.9, lane 7, 8 and 9), only a small amount of the scFv was eluted under the native condition or from periplasm (figure 3.10 and 3.11), indicating that although recombinant scFv expressed in a large quantity, only a small fraction was soluble and secreted into periplasmic space. It was noticed that a number of contaminated proteins were also detected in the eluted samples (figure 3.10), which may reflect the inefficiency of IMAC purification under the native condition.

To confirm the successful expression and secretion of soluble proteins, western blotting was applied and probed by monoclonal anti-polyHistidine-alkaline phosphatase antibody. Figure 3.12 shows the detection of the expressed scFv protein at 34 kDa (lane 3) and eluted fractions (lane 7-10) from total cell extract purification under the native condition; while figure 3.13 shows the same protein expressed and purified from the periplasmic fraction.

Four antibodies from library M325 (011, 1-1, 1-5 and 1-7) and three antibodies from library K530 (2-1, 2-2, 2-4) were expressed in pSANG vector and purified from both total cell extracts and periplasmic spaces, as described in chapter 2. It was shown that all seven antibody fragments were expressed, with an expected size of 33-34 kDa, which was again confirmed by western blotting with monoclonal anti-polyHistidine-alkaline phosphatase antibody. These pictures also show that while more scFv protein was purified from total cell soluble fraction, secreted scFv protein recovered from the periplasmic fraction was much less. The antibodies 011, 1-1 and 2-1 did not show detectable scFv protein in the elution fractions when purified from periplasm by osmotic shock. See all SDS-PAGE and western blot pictures shown in the appendix 4.

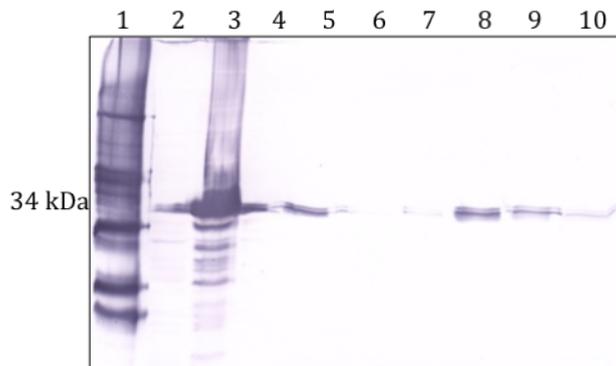
It has been estimated that an average yield of the scFv from the total soluble *E. coli* extract was about 100 – 500 µg from 200 ml of bacterial culture (i.e., 0.5-2.5 mg/L) after purification with Ni-NTA column followed by desalting with Centrifugal Filter

Units. However, the yield from the periplasm of *E. coli* is only 20 – 80 µg from 200 ml (i.e., 0.1-0.4 mg/L). Therefore, the total soluble cell fraction was used in the subsequent scFv purification.



pSANG-1-7 total cell purification (native)
1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.

Figure 3.12 Antibody 1-7 purified from total cell extract under the native condition (western blot)



pSANG-1-7 periplasmic purification
1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.

Figure 3.13 Antibody 1-7 purified from periplasmic fraction (western blot)

3.3.3 Quick screening of antibodies expressed in pABEXT vector

In order to rapidly screen antigen-binding antibodies, *E. coli* crude extract containing expressed antibodies without affinity purification was used directly in ELISA to test the binding to recombinant gp120 (as shown in figure 1.9). For this purpose, pABEXT vector was employed to express antibodies directly after one round of ribosome display in XL1-blue cells. In a preliminary study, total protein yield (purified under the denaturing condition) and soluble protein secretion (purified from the periplasm) were compared between pABEXT and pSANG vectors (figure 3.14, 3.15). Antibody 1-7 exhibits lower protein yield when expressed in pABEXT vector (figure 3.14, lane 7, 8 and 9), but comparable, if not more, soluble protein when it is expressed in pABEXT vector (figure 3.15, land 8 and 9).

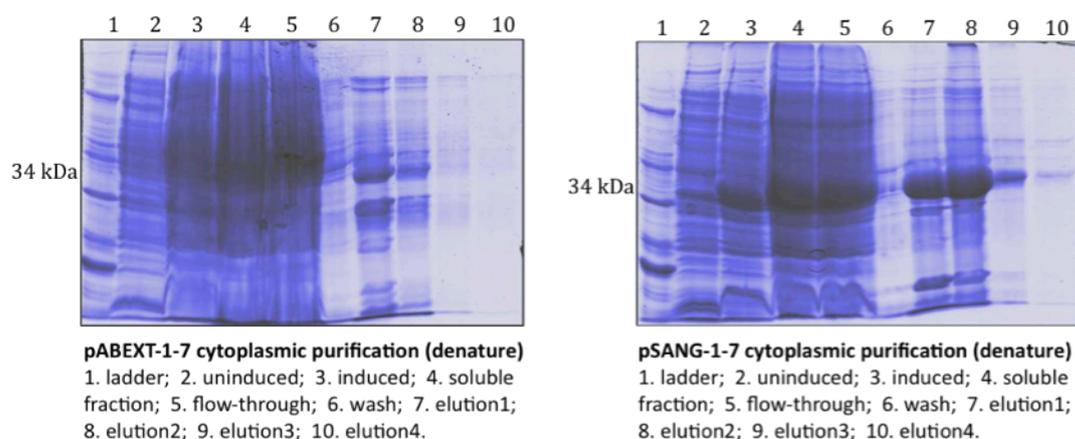


Figure 3.14 Comparison of protein yields between pABEXT and pSANG vectors

Antibody 1-7 was expressed in pABEXT and pSANG vectors and purified from total cell extract under the denaturing condition. pABEXT vector showed less protein production than pSANG vector.

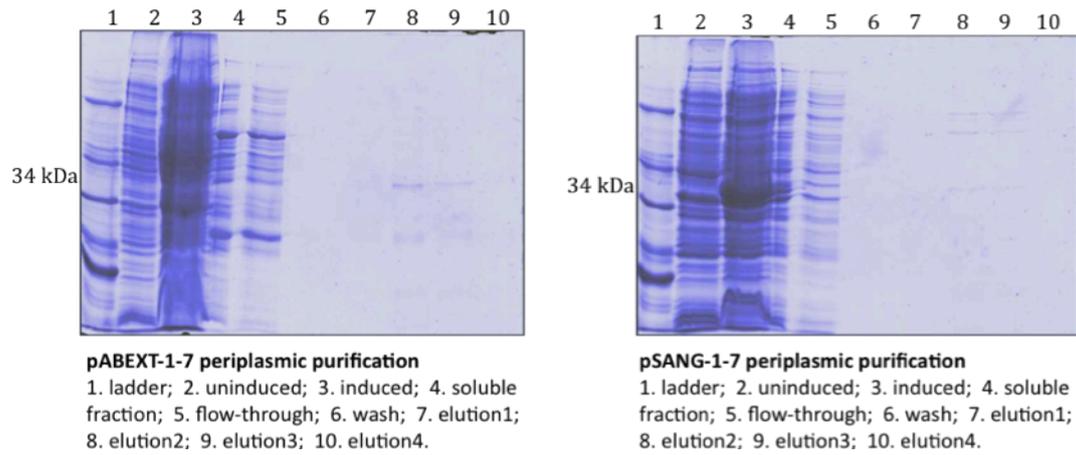


Figure 3.15 Comparison of soluble protein from periplasm between pABEXT and pSANG vectors

Antibody 1-7 was expressed in pABEXT and pSANG vectors and purified from periplasm using Ni-NTA column. pABEXT vector showed no less secreted protein than pSANG vector.

In general, DNA fragments recovered from one round of ribosome display were digested with *NcoI/NotI* and ligated into pABEXT vector. *E. coli* XL1-blue was used to express the single-chain antibodies which contained the partial constant region (expected size of 44 – 46 kDa) (figure 2.6). 300 clones selected from library M325 and 192 clones from library K530 were expressed in this method at 50 ml scale and the bacterially synthesised antibody fragments were extracted by osmotic shock and used directly for ELISA against recombinant gp120 and BSA (negative control). It has been shown that 20 clones from library M325 and 6 clones from library K530 demonstrated binding with varying degrees in the ELISA against gp120 derived from K530. Further test of these clones by ELISA in duplicate showed only 3 clones from library M325 (I3, I4 and M5) were reliably showing the antigen binding. Figure 3.16 shows single-chain antibody I3 was expressed at the expected size of 44 kDa (lane 3, 4 and 5). Compared with antibodies expressed in pSANG vector, antibodies I3, I4 and M5 expressed in pABEXT vector were more viscous and difficult to analyse on gel electrophoresis.

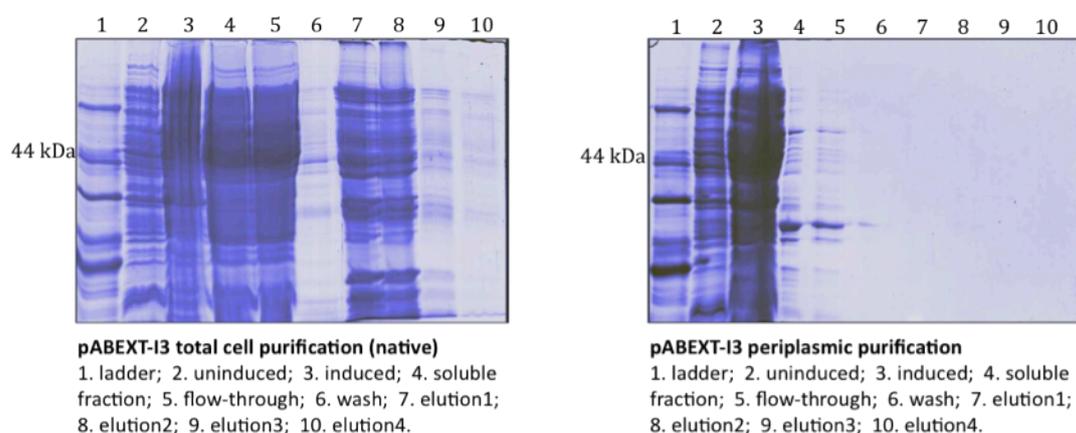


Figure 3.16 Single-chain antibody I3 expressed in pABEXT vector

After one round of ribosome display, the selected antibodies were cloned into pABEXT vector directly and expressed in XL1-blue cells. An example of antibody I3 expression and purification from both total cell extract and periplasm is shown here.

Subsequently, antibodies I3, I4 and M5 were sub-cloned and expressed in pSANG vector. These antibodies were again purified from both total cell extract and periplasm and analysed by SDS-PAGE and western blotting (figure 3.17, 3.18 and appendix 4).

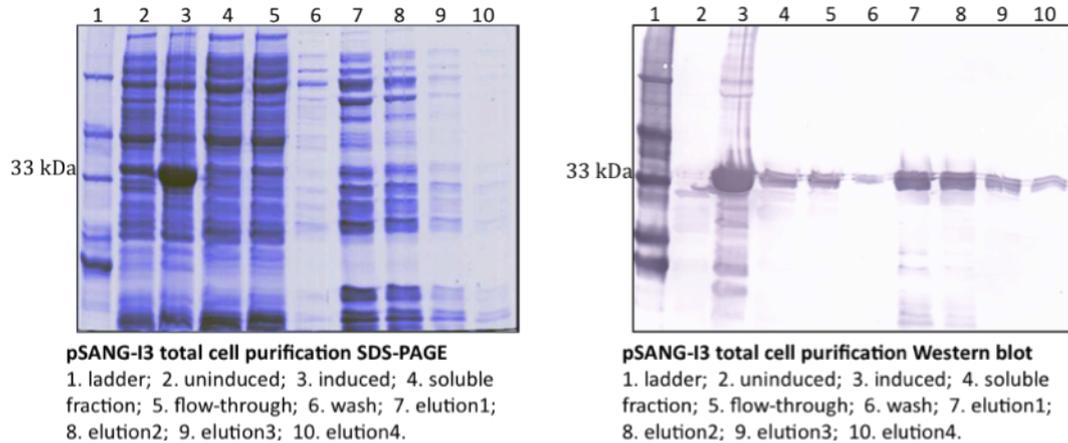


Figure 3.17 SDS-PAGE and western blot of antibody pSANG-I3 purified from total cell extract under the native condition

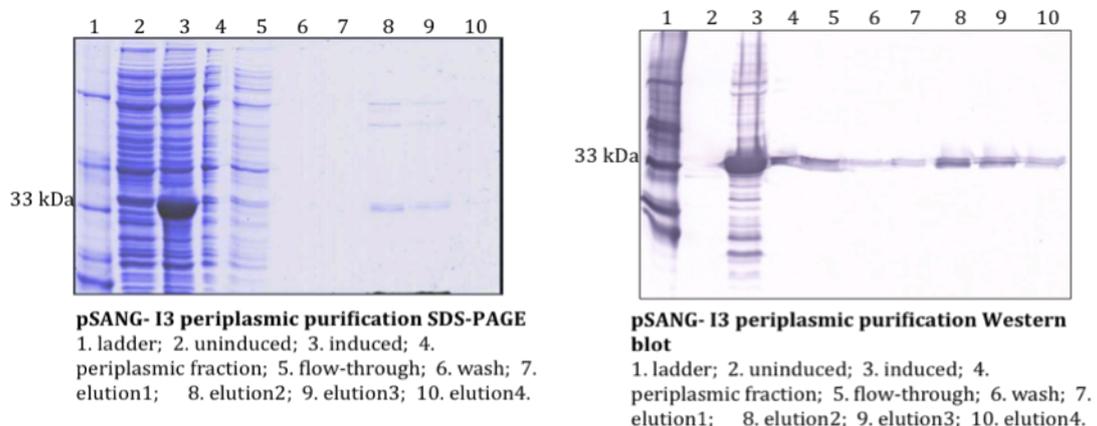


Figure 3.18 SDS-PAGE and western blot of antibody pSANG-I3 purified from periplasmic fraction

3.3.4 Effect of the codon following the initiation codon AUG

Some of the antibody fragments (such as pSANG-011 and pSANG 1-7) with distinct CDR sequences showed the expression at very low level (figure 3.19 and 3.20, left). Sequence studies also revealed that the codon following the initiation codon AUG was changed from GCC (Alanine) to GGC (Glycine). This could be the reason causing the poor protein expression (Bivona *et al.*, 2010). In order to test this, the GGC (Glycine) was converted back to GCC (Alanine) by PCR. This has resulted in an improved protein level by 5-10 folds (Figure 3.19 and 3.20, right).

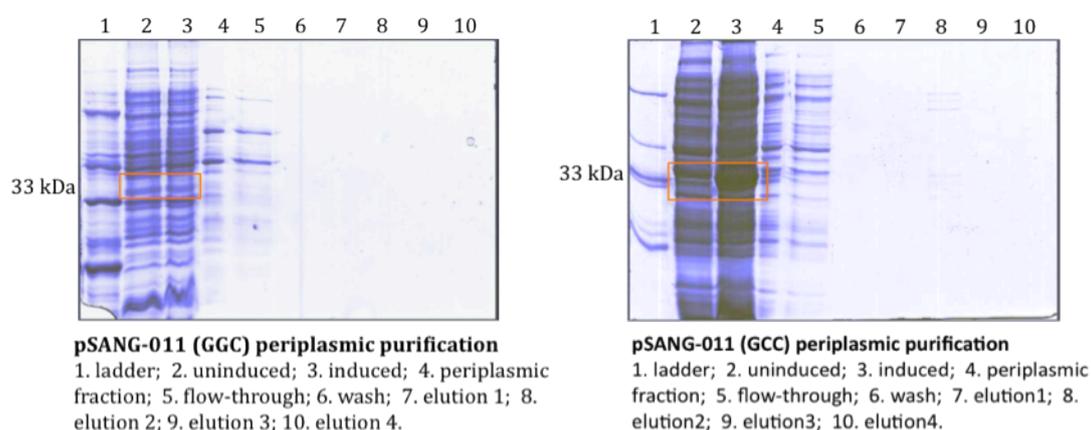


Figure 3.19 Comparison of antibody pSANG-011 with one codon difference

In left picture, an error codon of GGC (Glycine) was shown following the initiation codon AUG and the protein expression was very low after induction; in right picture, after correcting the error codon to GCC (Alanine), considerable protein expression was shown after induction.

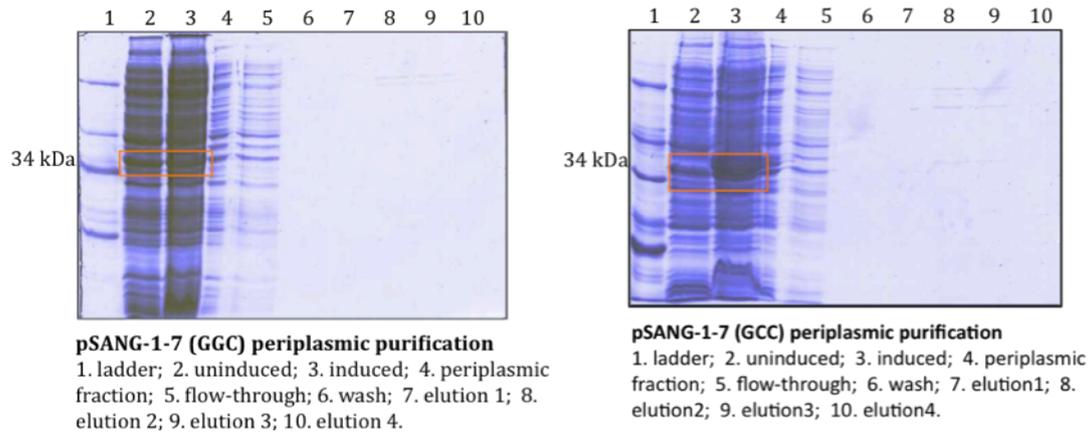


Figure 3.20 Comparison of antibody pSANG-1-7 with one codon difference

In left picture, an error codon of GGC (Glycine) was shown following the initiation codon AUG and the protein expression was very low after induction; in right picture, after correcting the error codon to GCC (Alanine), considerable protein expression was shown after induction.

3.3.5 Optimisation of protein expression

To optimise protein expression, a number of conditions have been examined, including induction temperature and codon usage. Antibody induction was performed at 20°C for 20 hours to minimise the formation of inclusion bodies and improve protein folding (Cabilly, 1989, Schein, 1993). However, it shows that protein solubility was not significantly improved when comparing to the protein expressed at 37°C for 3 hours (figure 3.21).

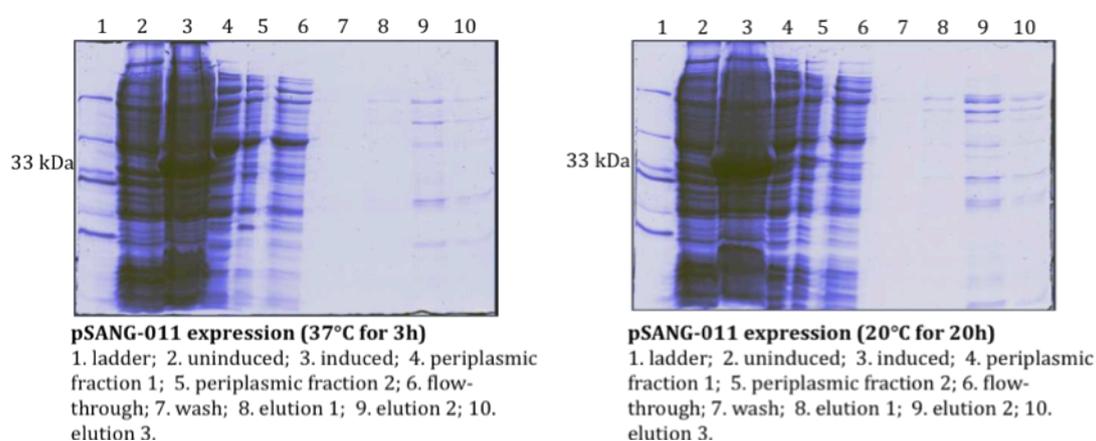


Figure 3.21 Comparison of antibodies expression at different temperatures

Antibody pSANG-011 was expressed at 37°C for 3 hours (left) and 20°C for 20 hours (right) separately. The improvement of protein yield and solubility was not significant.

The codon usage in the selected sequence (011, 1-1, 1-5, 1-7, I3, I4, M5, 2-1, 2-2 and 2-4) were analysed to see if there was rare codon that was not covered by pRARE plasmid. pRARE encodes tRNA genes for rare codons Arginine (AGG, AGA), Glycine (GGA), Isoleucine (AUA), Leucine (CUA) and Proline (CCC) (Novy *et al.*, 2001). However, a few codons that are also rare in *E. coli* were not included, such as Arginine (CGG, CGA) and Glycine (GGG). DNA sequencing shows that Arginine (CGG, CGA) and Glycine (GGG) presented in all the expressed proteins and consecutive rare codons presented in most sequences (1-1, 1-7, I3, I4, M5, 2-1, 2-2 and 2-4) as summarised in table 3.3. The presence of these rare codons may impede the translation of recombinant antibodies in *E. coli* (Kane, 1995).

Table 3.3

Antibodies	Rare codon CGA	Rare codon CGG	Rare codon GGG	Consecutive rare codons
011	2	3	6	0
1-1	2	1	8	5
1-5	2	3	6	0
1-7	1	1	4	4
I3	2	4	9	4
I4	2	3	9	4
M5	3	3	7	4
2-1	2	1	8	1
2-2	2	3	6	1
2-4	2	3	6	1

Table 3.3 Summary of rare codons excluded in pRARE plasmid and consecutive rare codons in expressed antibodies

Rare codons Arginine (CGA and CGG) and Glycine (GGG) that are not included in pRARE plasmid are shown in all the expressed antibodies; consecutive rare codons such as CGG CGA, CGG ACG are shown in most of the antibody sequences.

3.4 Functional assays

3.4.1 ELISA

To examine the antibody specificity and binding, the 10 selected antibodies expressed in pSANG vector were tested in ELISA against recombinant gp120 generated from patient K530. The ELISA was performed on a 96-well Nunc plate. BSA was used as the negative control. Other negative controls include mAb-free wells. Bacterially expressed antibody fragments after purification from the total cell extract under the native condition at the final concentration of 25 $\mu\text{g/ml}$ were incubated in each well (figure 3.22). As a comparison, antibodies purified from periplasm at the final concentration of 5 $\mu\text{g/ml}$ were also included (figure 3.23).

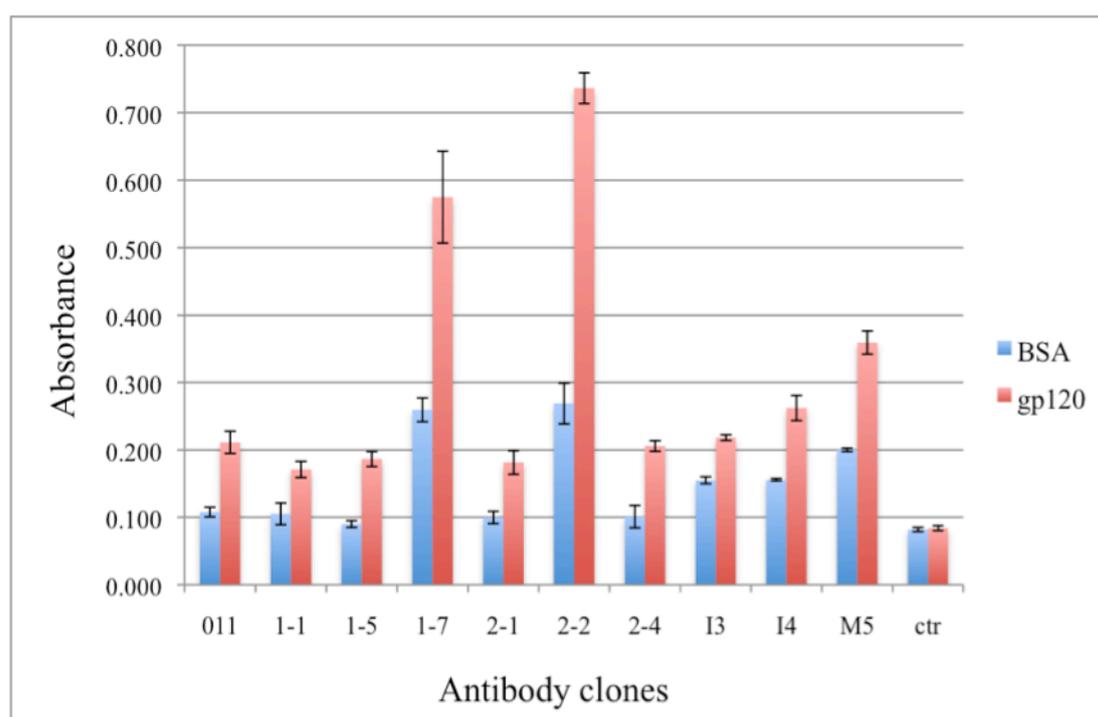


Figure 3.22 Antibody binding to gp120 after purification from total cell extract under the native condition

Antibodies purified from total cell extract showed binding activities to recombinant gp120 in ELISA (red columns), BSA was used as negative control (blue columns). MAb-free wells were also included as negative control (ctr).

While antibodies 1-7 and 2-2 showed highest binding affinity to gp120, other antibodies showed modest activity. However, they all bound specifically to gp120 (figure 3.22). Binding to BSA was also observed from some samples (1-7, 2-2, I3, I4 and M5), which may be caused by the use of BSA as blocking reagent in ribosome display. It was noticed that the signals of binding to gp120 were weaker when using the scFvs from the periplasm (figure 3.23) than that from total cell extract. The reduced binding observed with periplasmic scFvs was possibly due to a lower amount of antibody being recovered from the periplasm. Despite of less binding, the ELISA result using the antibodies from periplasm agrees with that from total cell extracts, confirming the binding activity of these antibodies. Based on figures 3.22 and 3.23, antibody 011, 1-7, 2-2, I3, I4 and M5 showed specific binding, while the other 4 antibodies had less or no binding.

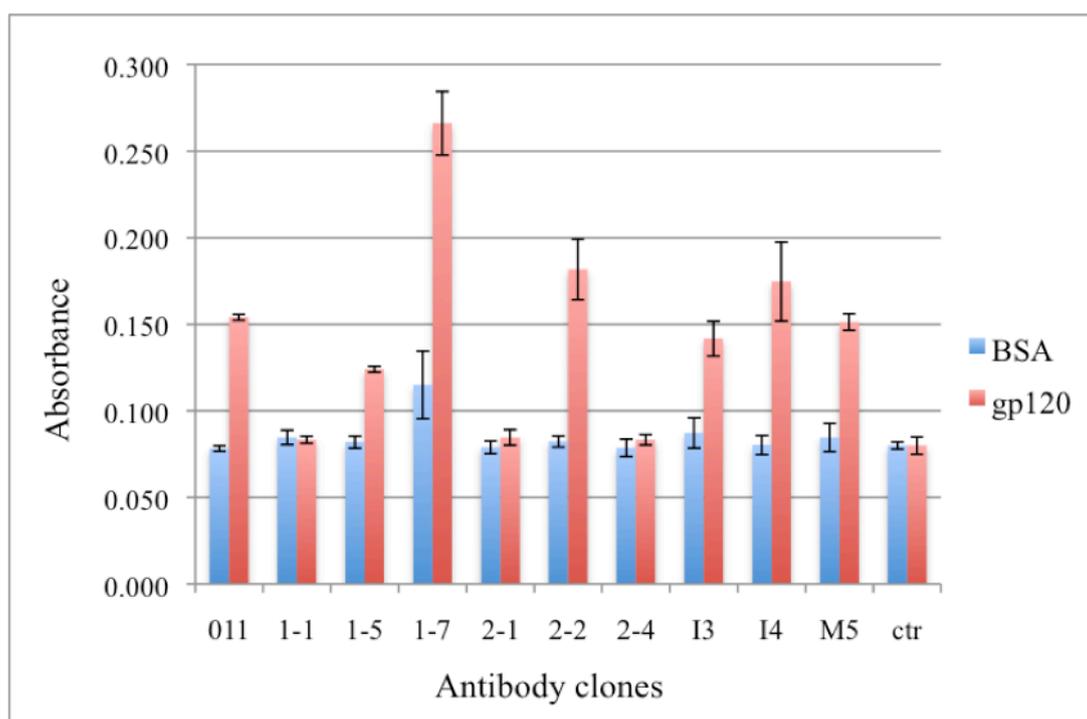


Figure 3.23 Antibody binding to gp120 after purification from periplasmic fraction

Antibodies purified from periplasmic fraction showed variable binding activities to recombinant gp120 in ELISA (red columns), BSA was used as negative control (blue columns). MAb-free wells were also included as negative control (ctr).

3.4.2 Neutralisation assay

To examine the neutralising activity of the selected antibodies, TZM-bl cells were chosen for the assay. The antibodies purified from total cell extract under the native condition were used as they provided a higher amount of protein. In total, 10 selected antibodies from both libraries were incubated with K530 gp160-derived infectious chimeric molecular clones before assaying on TZM-bl cells. The neutralisation activity was measured by the reduction of Luciferase luminescence. Percentage of neutralisation is illustrated in figure 3.24. While three antibodies (1-5, 2-1 and 2-2) resulted in about 50% luminescence reduction in the assay, another three mAbs (1-1, 1-7 and 2-4) displayed lower discrepancies compared to the control sample. This shows a modest neutralising activity, although they were not potent compared to plasma control.

Thus, taking the ELISA data and neutralisation results together, clones 1-7 and 2-2 showed best activities among 10 antibodies. Clones 1-1, 1-5, 2-1 and 2-4 showed modest function in both assays. The other four clones only showed binding in ELISA.

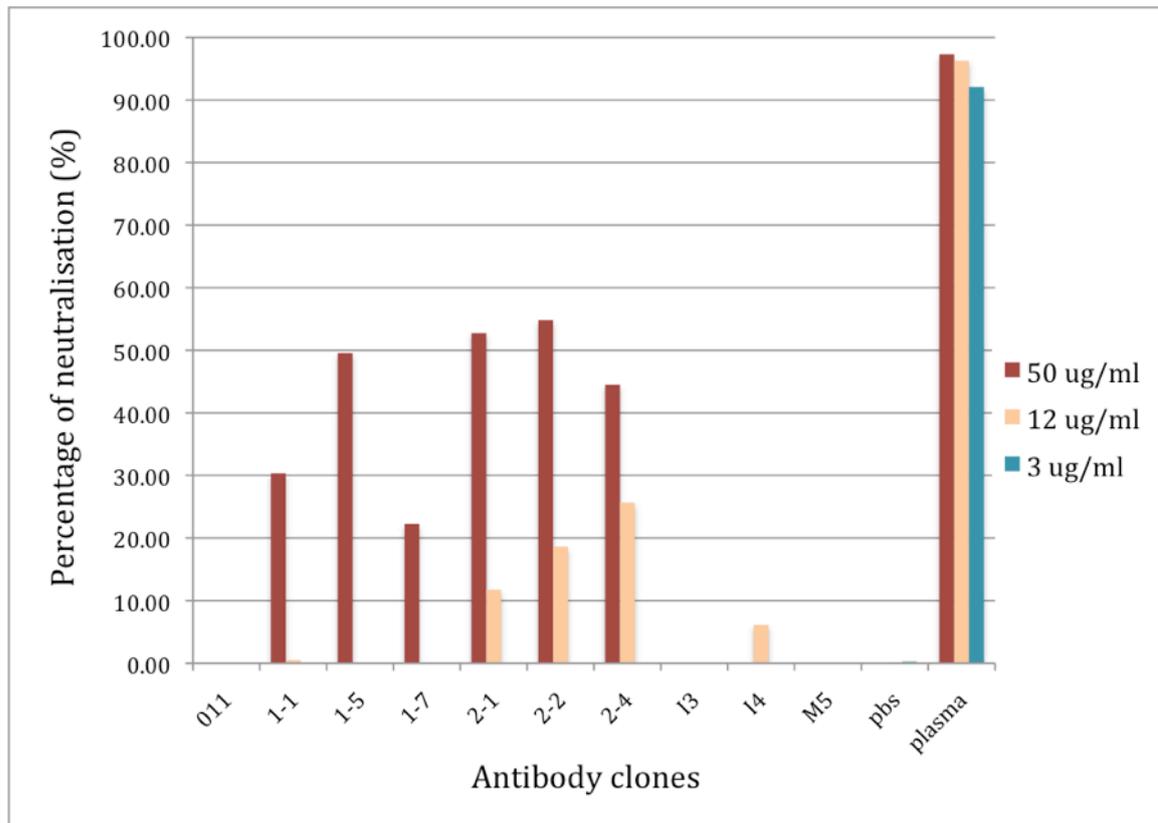


Figure 3.24 Percentage of neutralisation

Chimeric molecular clones of HIV-1 gp160 derived from patient K530 were neutralised with 10 mAbs, at 50, 12 and 3 $\mu\text{g/ml}$ respectively. Results were reported as percentage of neutralisation activity, relative to negative control with antibody-free wells. Each colour represents different antibody concentration, except plasma control was tested at 1:20, 1:80 and 1:320 dilutions, respectively.

Chapter 4

Discussion

Within 30 years of discovery, HIV has become one of the most pathogenic viruses circulating in human beings and caused more than 25 million deaths. With the increased understanding of the virus replication and infection mechanism, significant progress has been made to block the virus entry, suppress the virus replication and thus delay the disease progression. Despite seven classes of ART that are currently used in the treatment of HIV infection (nucleoside RT inhibitors, nonnucleoside RT inhibitors, protease inhibitors, fusion inhibitors, entry inhibitors, HIV integrase strand transfer inhibitors and multi-class combination products) (FDA, 2010), about 10 million infected individuals are still unable to access or afford to these therapies. Therefore, a protective vaccine with a relative low cost is urgently needed. To overcome the extraordinary mutability and genetic diversity of HIV, an effective vaccine needs to induce both T cell cytotoxic activity to recognise and eliminate infected cells and neutralising antibodies to prevent further viral replication (Johnston and Fauci, 2008). Current vaccine candidates generally focus on eliciting cytotoxic T cell responses (Letvin, 2005), yet only a limited number of broadly neutralising mAbs have been produced (Burton and Weiss, 2010). It will facilitate a deep understanding of the nature of HIV-induced immune responses if more broadly neutralising mAbs could be produced by a simple and practical method. This study aimed at establishing such a method to generate human antibodies *in vitro* by combining ribosome display selection with DNA libraries made from patient's blood.

4.1 Single-chain antibody library construction

Construction of the antibody libraries in this project was designed to achieve the followings: (1) to cover all possible rearranged variable regions, (2) to identify antibody subclasses, and (3) to use as little blood sample as possible. Two libraries M325 and K530 have been constructed by PCR to contain 13 VL families (9 V λ families and 4 V κ families) and 8 VH families, covering all the functional variable genes listed in human gene database, V BASE (Sblattero and Bradbury, 1998). This is in contrast with previously reported method in which only γ 1 (Fd region) and κ chains were amplified by PCR (Burton *et al.*, 1991). DNA sequencing of the functional antibody candidates after one round of ribosome display revealed both lambda chains and kappa chains, despite that most reported anti-HIV-1 neutralising mAb sequences are limited to V κ and VH chains. Unlike mouse immunoglobulin in which kappa chains predominant, both kappa and lambda chains are similarly presented in human (Das *et al.*, 2008). Isolation of functional lambda chains from two independent libraries in this study indicates that all families of variable regions should be included in the library for a selection.

The relationship between antibody function and its subclass was also investigated in this study. Previous research on neutralising mAbs was focusing on variable fragment or Fab, and the limited reports on antibody subclasses were restricted to IgG1 or IgG3 (Burton *et al.*, 1994, Buchacher *et al.*, 1994). Although IgG1 and IgG3 subclasses are generally believed to be much more effective activators of the classical complement pathway, some studies suggested that IgG2 is also effective in activating complement when the epitope density is high (Michaelsen *et al.*, 1991). Interestingly, subclasses IgG2 and IgG4 were found in several antibody candidates by this study, as well as IgG1 subclass that accounts for majority of the selected sequences. This agrees with the relative concentration of human immunoglobulin subclasses (IgG1>IgG2>IgG3=IgG4) (Meulenbroek and Zeijlemaker, 1996), whereas the fact that no IgG3 subclass was found could be probably due to the limited number of selected candidates. The role of immunoglobulin subclasses in HIV-1 neutralisation needs to be investigated further. In this study, we designed the library to genetically encode sequences that would permit the identification of the immunoglobulin subclass linked to its variable

heavy and light chains. We could readily differentiate human kappa and lambda light chains, IgG1, 2, 3 and 4 subclasses for the heavy chains without performing ELISA as previously described (Buchacher *et al.*, 1994). Although not carried out in this study, the approach could be further applied to elucidate IgA and IgE, providing complete coverage of the immune repertoire.

Importantly this study shows that large human antibody libraries could be constructed from 20 ml of blood, and possibly as little as 5 ml (this is achievable based on the amount of total RNA that was used in this study: to assemble a library requires 3 µg of total RNA; the amount of total RNA recovered approximate to 1 µg per ml blood). The libraries could be immediately used for selection of specific antibodies by *in vitro* ribosome display without the need for DNA cloning, thus maintaining the original library size and diversity.

4.2 Ribosome display selection

Ribosome display was used in this study to isolate functional single-chain antibodies. This technology could be developed as an alternative method to previously common used methods such as EBV immortalisation (Buchacher *et al.*, 1994, Corti *et al.*, 2010) or phage display (Burton *et al.*, 1991). Ribosome display is featured to allow efficient screening of a very large library without compromising the library size by transformation efficiency. The PCR amplified DNA library is readily selected against immobilised antigens before cloning and transforming into bacteria. Theoretically the library size is only restricted by the available ribosomes in the system. Ribosome display is also a time-saving method. One round of selection and recovery process can be accomplished in just one day, as no cell culture is involved. The initial ribosome display experiments using M325 library against gp120 derived from K530 employed three rounds of selection. This resulted in the recovery of an enriched population comprised of a single sequence for the scFv. This high degree of enrichment resulted in reduced diversity. Subsequently, it was decided to use a single round of ribosome display to enrich and recover more diverse candidates.

The antibodies from both libraries were affinity selected against the same antigen: recombinant gp120 derived from patient K530. Interestingly, clones with identical CDR3 region were found from the two separate libraries, e.g. mAbs 011, 1-5 were from library M325 while mAbs 2-2, 2-4 from library K530 (see table 3.1). The recovery of the similar sequences with identical H-CDR3 suggests the success of the selection from the libraries by ribosome display technology. Antibody CDRs, in particular H-CDR3, play an important role in determining antibody specificity and affinity. The selection of the identical H-CDR3 from the two different libraries suggests that this H-CDR3 region is a dominating functional sequence from the two HIV patients. By comparing with existing neutralising mAbs, the H-CDR3 selected in this study is shorter (only 15 amino acids). It was generally shown that the existing neutralising mAbs have a protruding long H-CDR3, e.g., while 2F5 has 22 residues, both b12 and 4E10 with 18 amino acids (Zwick *et al.*, 2004, Cardoso *et al.*, 2005, Sapphire *et al.*, 2001). Recent study identified a 28-residue H-CDR3 in two other neutralising mAbs PG9 and PG16 (Pejchal *et al.*, 2010). These H-CDR3s are usually longer than the average length of H-CDR3 in humans (13 residues), rabbits (11 to 12

residues) and mice (9-10 residues) (Wu and Cygler, 1993). In this study, only one long H-CDR3 (23 residues) was discovered from the patient libraries.

These antibodies selected from both libraries have specific binding activity in ELISA (especially mAb 1-7 from library M325 and mAb 2-2 from library K530) and neutralising activity, confirming *in vitro* selection from patient libraries can rapidly produce functional antibodies. This also agrees with the result of preliminary patient serum screening: Serum M325 (CRF02_AG) neutralises clade B, C and CRF02_AG, and serum K530 (clade C) neutralises clade B, C, CRF01_AE and CRF02_AG. The current study shows that antibodies capable of neutralising a recombinant clade C antigen could be isolated from the libraries (see results 3.4.2).

In the future, the selected antibodies could be tested with different HIV-1 subtypes to further characterise their neutralising activity. The breadth of antibody neutralisation is an important and interesting criterion of anti-HIV-1 neutralising antibodies. The current results encourage the continuation or extension of this work to generate a panel of different mAbs that would facilitate the discovery of potential candidates for therapeutic application.

4.3 Protein expression

The expression and purification of recombinant antibodies has proved to be a bottleneck in this study and many factors that may affect protein expression were investigated. A total of 10 antibodies (7 from library M325 and 3 from library K530) were tested and the soluble fractions either from whole cell lysate or periplasmic space were analysed. The recombinant scFv proteins with the correct sizes were confirmed by protein gel electrophoresis and western blotting analysis. Both the T7 promoter based pSANG and the lac promoter based pABEXT vectors have shown utility in producing a large amount of recombinant scFv proteins within hours following induction. However, it was noticed that inclusion bodies formed, possibly due to the foreign nature of human immunoglobulins, and/or the high expression rate (Arbabi-Ghahroudi *et al.*, 2005). One possible route to recover functional proteins from inclusion bodies is by protein refolding. This was not attempted in this study due to time constraints and the requirement to reengineer the scFv without a signal peptide for cytoplasmic expression (Arbabi-Ghahroudi *et al.*, 2005). Instead, efforts were made to identify the optimal conditions to produce soluble proteins in sufficient quantity for the subsequent studies. These included the use of different expression systems and optimising the expression conditions and codon usages, and protein recovery under different conditions.

A side-by-side comparison has shown that protein purification from the total cell lysate generated more recombinant proteins than that from periplasmic space. However, purification from the periplasmic space generated a better purity. Usually, oxidised periplasmic space facilitates the folding and assembly of recombinant scFv, and the secreted proteins can be extracted by a simple osmotic shock procedure with less contaminants from bacterial proteins (Makrides, 1996). However, in practical, a substantial amount of recombinant scFv was not extracted, possibly due to the inefficiency of osmotic shock that causes the poor recovery of the scFv. Isolation of scFv proteins from the total cell lysate may overcome this problem, thus resulting in a better recovery of scFv proteins (figure 4.1). In this study, antibodies purified from both methods have been compared, showing scFv proteins purified from both methods preserved the correct folding and function.

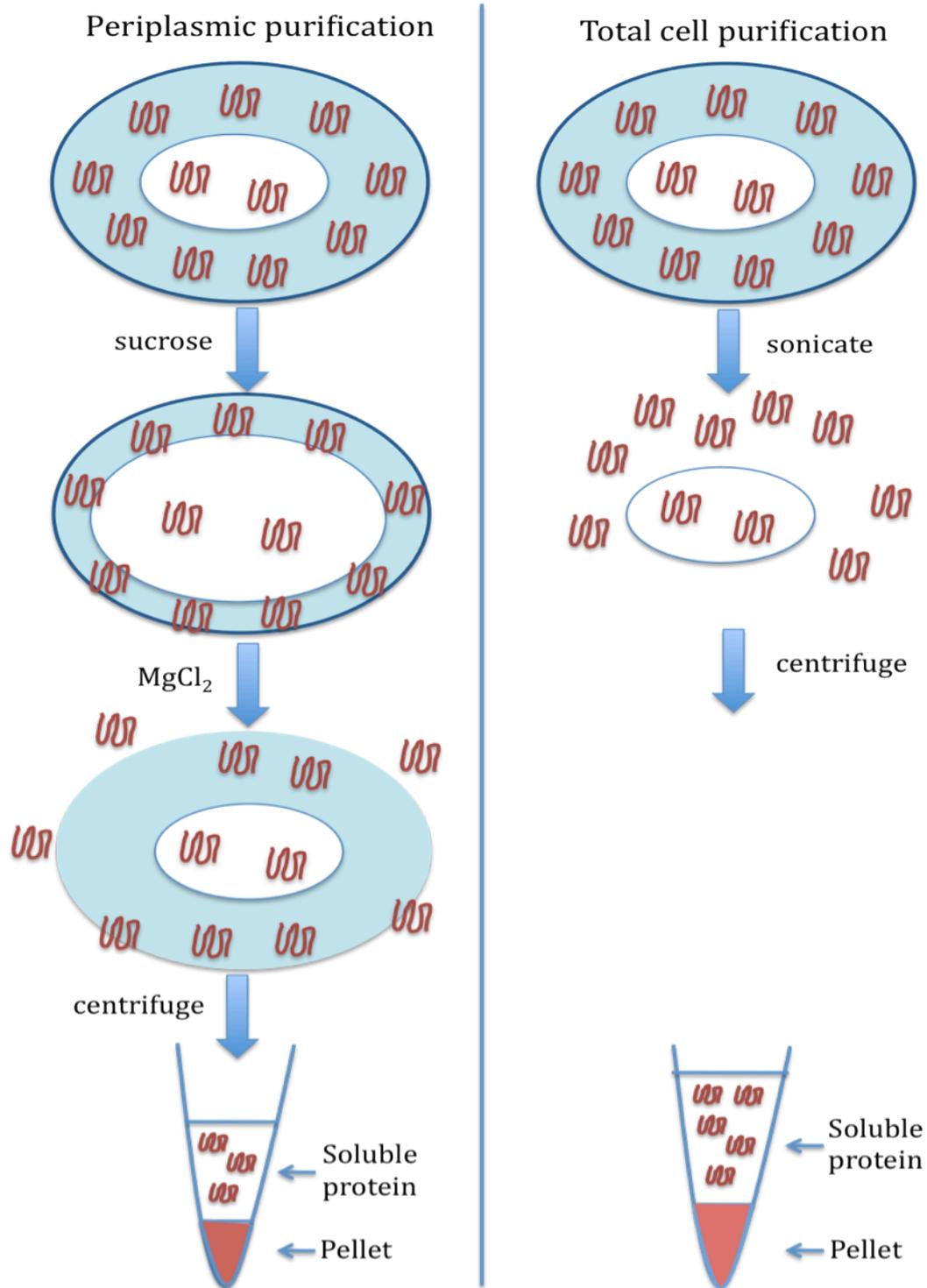


Figure 4.1 Illustration of two purification methods

Periplasmic protein was purified by osmotic shock, in which some scFv protein might stick to membranes and remain in the cell; Total cell purification generates more scFv protein by sonication, but it also showed more contaminating proteins. The cytoplasm is shown by the white circle, and the periplasmic space is shown by the blue area.

To further improve the scFv in the future, a screening procedure may be adapted, which has allowed identifying optimised conditions for protein expression in *E. coli* by a single experiment (Islam *et al.*, 2007). In addition, more suitable host strains such as those to enhance the disulfide bond formation in the bacterial cytoplasm could be tested (Prinz *et al.*, 1997), e.g., Rosetta-gami B strain.

The plasmid pABEXT vector contains the lac promoter. Unlike the pSANG vector, it does not require T7 RNA polymerase to express proteins. Therefore selected antibodies could be expressed in normal hosts such as XL1-blue cells, without the need for the extra steps of transforming the plasmid into host bacteria BL21(DE3)pRARE. Compared with pSANG vector, antibodies expressed in pABEXT vector showed a lower protein yield, but similar or more soluble protein could be obtained after purification from the periplasm of *E. coli*. However, proteins expressed in pABEXT vector are more viscous and difficult to analyse by SDS-PAGE. As both of the plasmid systems have advantages and disadvantages, combined use of these two systems was adapted. In general, the pABEXT vector was used when a quick screening of antibodies was required while the pSANG system was used when a large-scale production of the candidates was needed (see results 3.3.3).

It was shown that scFvs from the periplasmic extract without further purification could be directly used for functional test of the antibodies in ELISA. Thus this method was used to screen bacterial clones after ribosome display selection. This crude extract of scFv proteins may contain contaminated proteins/peptides that may affect the ELISA results. Therefore, potential candidates identified by this screening method were re-tested by His-tag affinity purification. In addition, some antibodies may be truncated resulting in shorter polypeptides from the pABEXT system, thus causing false positive in ELISA. These antibodies were ruled out by western blotting when they failed to show correct size or by directly DNA sequencing. A number of mutations were also detected; these may have been produced during multiple amplifying and cloning steps. In future, this could be reduced by using higher fidelity polymerases.

The expression temperature was also examined in this study. Cultivation at a reduced temperatures is a well-known technique to reduce the aggregation of recombinant proteins and improve soluble protein yields (Weickert *et al.*, 1996). It has shown effective in improving the solubility of many difficult proteins, such as bacterial luciferase (Vasina and Baneyx, 1997). Various strategies have been developed to express protein, especially toxic and unstable proteins at temperatures as low as 4°C (Ferrer *et al.*, 2003, Mujacic *et al.*, 1999). In a routine antibody production, the standard condition for protein expression was set to 20°C for 20 hours to reduce the cellular protein concentration and improve protein folding. However, in this study expression at a lower temperature for a longer period of time showed a similar yield of soluble proteins as that expressed at 37°C for 3 hours, indicated by SDS-PAGE results (figure 3.21).

Codon usage has also been tested to increase the expression level of soluble proteins. As the second codon following the initiation codon AUG has been known to be crucial to protein expression, which is explained by the influence of the composing nucleotides on the structure of the ribosomal binding site (Looman *et al.*, 1987). As Alanine located at the second position has shown to increase protein expression (Bivona *et al.*, 2010), the Glycine at second position was replaced by Alanine in the clones 011 and 1-7. This has led to the improvement of protein expression by 5-10 folds, agreeing with the previously published observation (Bivona *et al.*, 2010).

Rare codons were also detected across the sequences from the selected candidates due to the foreign nature of human immunoglobulin origin. To eliminate the rare codon bias in *E. coli*, the pRARE plasmid containing 6 rare codons was inserted into host bacteria BL21(DE3). This would facilitate expression of human scFvs in *E. coli*. However, there were 3 other rare codons Arginine (CGG, CGA) and Glycine (GGG) as well as consecutive rare codons (e.g. CGG CGA, CGG ACG) detected in the selected antibodies (table 3.3), which may cause the difficulty of producing soluble proteins. This problem could be reduced by using commercially available plasmids, such as pRARE2, which contain more rare codons. An alternative method could be to use Rosetta host strains such as Rosetta-origami B strains that are designed to enhance the expression of eukaryotic protein.

4.4 Functional assays

The selected antibody candidates have been demonstrated to be functional by both ELISA (figure 3.22, 3.23) and neutralisation assay (figure 3.24). Specific binding was observed in each antibody at 25 µg/ml against recombinant gp120 in ELISA (figure 3.22). Furthermore, modest neutralising activity was observed from 6 antibodies (3 from the library M325 and 3 from the library K530) at 50 µg/ml against recombinant gp160 (figure 3.24). The successful selection of functional antibody fragments by ribosome display technology from patient libraries suggests this could be an efficient route to rapidly generate specific antibodies *in vitro*. In addition, ribosome display is a powerful method for *in vitro* evolution of antibody fragments (He and Taussig, 2002), these selected antibodies can be further improved by ribosome display in a combination with mutagenesis.

Antibody fragments 1-7 and 2-2 showed the highest activities in ELISA. The binding activity specific to the antigen suggests the correct folding of these proteins in bacteria. The results also suggest that the presence of 6XHis-tag and tri-FLAG-tag has no substantial effects on the function of the antibodies. A number of antibodies with the lower activity were also selected. These antibodies may be a background selection or may not fold correctly in *E. coli*, as they were selected in the format of antibody-ribosome-mRNA complex produced in the rabbit reticulocyte lysate, a eukaryotic system. The functional antibodies purified from total cell or periplasm were functionally similar, showing either method could be used to produce the antibodies. However, in general, periplasmic space generates less scFvs but better folded fragments possibly due to the oxidizing environment and the presence of proteins that are important for folding and assembly of recombinant protein (Arbabi-Ghahroudi *et al.*, 2005).

Antibodies selected from the two libraries showed similar functions, suggesting the consistency of the method for the selection. It may also indicate these selected antibodies were functionally dominating molecules in the libraries. It would help to generate panels of different antibodies with more diversified sequences for a choice, especially for the therapy if more patient libraries could be generated in the future, and

selected with other defined antigens, such as trimeric gp120. Sequence studies show that the selected antibodies, despite very similar sequences and an identical CDR3 region (e.g., 2-2 and 2-4), their antibody binding activities are not the same, suggesting their binding sites are not entirely determined by their CDR3 sequence.

The neutralisation results suggest that antibodies with binding affinity to an antigen do not guarantee their function in neutralising the target *in vivo*. This has been observed by others working with HIV-1 neutralising mAbs (Burton *et al.*, 1994), where they found that only a small part of the affinity selected antibodies (i.e., b12) showed potent neutralising activity even though other antibodies were also directed to the same binding site and had similar affinity to b12. Generally, antibodies can target many epitopes on HIV-1 Env protein, but potent neutralising activity could only be achieved by recognising the conserved epitopes that are essential for virus attaching and entry the cells (Wu *et al.*, 2010). In this recent report, Wu and co-workers used molecular modelling to design the envelope protein to preserve only the CD4 binding site and eliminate extra antigenic regions of HIV-1. This “rational design of envelope”, which showed success in selecting antibody expressing B cells, could be used in our *in vitro* selection system in the future.

It is of interest to note the PBMCs from donor K530 used to construct the antibody library were taken 3 years prior to PBMCs being taken for K530 gp120 isolation. During this 3-year period the HIV-1 virus surface proteins probably underwent some degree of genetic variations. The ability to recover antibodies that neutralise K530 gp120 suggests these molecules were important in keeping the virus in check. It is possible that K530 gp120 emerged sooner or that the antibodies recognise a precursor to K530 gp120 that also recognise the current K530 gp120. The epitope may be a conserved functional epitope required by HIV-1. With the development of a method of capturing and accessing the antibody repertoire that in theory requires as little as 5 ml of blood, it should be possible to undertake longitudinal studies to follow the repertoire of the antibodies and the antigens. Such a study would facilitate a better understanding of HIV-1 escape and allow identification of broadly neutralising antibodies if recombinant antigen isolated from different timeline could be used, (e.g., recombinant gp120 from 0, 1, 3, 5 year of HIV-1 infection could be isolated from LTNP and used in

ribosome display, which could permit the production of broadly neutralising mAbs in the future).

The caveats with the scFv approach adopted in comparison with naturally occurring serum antibodies are in lack of avidity, effector functions, and polyclonal specificities. The antibodies (IgG) in serum have two binding sites, secondly they also have Fc domains that can confer complement activation and/or binding to Fc receptors and are a cocktail of specificities. The recombinant scFvs are monovalent, lack effector functions and are mono-specific. However, if the neutralisation is purely a function of inhibiting virus attachment or entry, which is independent of effector function and does not require receptor cross-linking, then potent neutralising antibodies discovered in the scFv format would probably be very effective when engineered back to full-length IgG. Conversely it may be that more than one antibody specificity is required for neutralising a broad range of HIV-1 virus clades, thus the activity of polyclonal serum may not be readily reproduced by monoclonal antibodies as reported by (Scheid et al., 2009, Mouquet et al., 2010).

4.5 Summary

In summary, the project described in this thesis has established a novel approach for rapid selection of anti-HIV-1 antibody fragments by *in vitro* ribosome display of patient libraries. It could provide a powerful discovery tool for identification of therapeutic antibodies. The followings list the major results obtained:

Two anti-HIV-1 antibody libraries (M325 and K530) have been constructed by PCR and can be used directly for ribosome display screening without the need for DNA cloning. It also shows that a library could be made using the blood sample of as little as 20 ml;

The eukaryotic ribosome display procedure has successfully led to the selection of functional human antibodies *in vitro*. Ten antibody candidates have been generated and their IgG subclasses have been identified by DNA sequencing.

The selected recombinant antibodies have been expressed as scFv in *E. coli*, and a number of expression and purification conditions have been examined to generate soluble proteins.

A rapid screening method has also been established using pABEXT vector, which facilitated *E. coli* expression and screening of individual clones for functional antibodies.

The functions of the selected antibodies were confirmed in both ELISA and neutralisation assays.

4.6 Future studies

In general, a procedure for the capturing and analysis of an immune repertoire that requires relatively small volumes of blood has been described. In the future, the approach will be refined and optimised to permit the construction of antibody libraries from as little as 5 ml of blood.

In this study, we utilised monomeric gp120 as a capture antigen. The availability of trimeric gp120 and gp140 provides additional capture antigens.

The scFvs isolated in this proof-of-concept study in future would be used to generate full-length IgG molecules produced in CHO cells using standard procedures. The recovered purified full-length IgG molecules would then be used in neutralising assays.

With these refinements, it will be possible to undertake longitudinal studies capturing the repertoire of antibodies and antigens from sero-conversion through disease progression or lack of.

These additional studies would permit a complete answer to the hypothesis that in a proportion of LTNP's the pre-existence of antibodies that are able to cross neutralise viruses capable of evolving from those currently replicating ensures that virus load is held fully in check. The effective control of virus replication by these antibodies prevents immune destruction and ensures the failure of disease to develop. Understanding the factors controlling this balance between host and pathogen will inform the design of better therapeutic treatments and effective prophylactic vaccines against HIV.

Appendix

1. Neutralising activity of patient sera

Sera from two LTNPs (M325 and K530) were tested by Dr. Hanna Dreja (Centre for Infectious Disease, Institute of Cell and Molecular Science, Barts and The London, Queen Mary, University of London, UK). It was tested against different HIV-1 clades in TZM-bl assay (reciprocal dilution at which at least 60% of input virus was neutralised). Their neutralising activities are listed as following:

Patient M325 (type CRF02_AG)

Clade	Titre
B	80
C	40
CRF02_AG	160

Patient K530 (type C)

Clade	Titre
B	160
C	160
CRF01_AE	160
CRF02_AG	1280

2. pABEXT vector sequence

(Tac promoter is shaded in grey; Restriction digestion sites *Nco*I, *Not*I are underlined)

CCGACACCATCGAATGGTGCAAACCTTTCGCGGTATGGCATGATAGCGCCCGGA
AGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCGCA
GAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCA
CGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGGCGATGGCGGAGCTGAATTAC
ATTCCCAACCGCGTGGCACAACAACCTGGCGGGCAAACAGTCGTTGCTGATTGGCG
TTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTGCGGGCGATTAAA
TCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTGATGGTAGAACGAAGCG
GCGTCGAAGCCTGTAAAGCGGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGG
GCTGATCATTAACCTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCT
GACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGT
ATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATT
GGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTC
TGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCG
GAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGC
TGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCT
GGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGGGATATCTCG
GTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCA
CCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCA
ACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTG
AAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGG
CCGATTCATTAATGCAGCTGGCAGCAGAGTTTCCCGACTGGAAAGCGGGCAGTG
AGCGCAACGCAATTAATGTAAGTTAGCTCACTCATTAGGCACAATTCTCATGTTTG
ACAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCG
GAAGCTGTGGTATGGCTGTGCAGGTCGTAATCACTGCATAATTCGTGTGCTCAA
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GAGCACTTACCAACAAGGACCATAGCATATGAAATACCTGCTGCCGACCGCTGC
TGCTGGTCTGCTGCTCCTCGCTGCCAGCCGGCCATGGCCAGGTGCAGCTGCAGG
CGGCCGATCCGCACATCATCATCATCACAAGCTGGACTACAAAGACCATGA
CGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTAATAA
AAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGCGTTA
CCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA
GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGC
AGCTTGGCTGTTTTGGCGGATGAGATAAGATTTTCAGCCTGATACAGATTAATCA
GAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGG
TCCCACCTGACCCCATGCCGAACCTCAGAAGTGAAACGCCGTAGCGCCGATGGTAG
TGTGGGTCTCCCCATGCGAGAGTAGGGAACCTGCCAGGCATCAAATAAAACGAAA
GGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCT
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CACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAACCTGGCGAACTA
CTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTG
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GGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTA
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ACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTG
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GTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCT
TGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCT
ACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAA
CTGGCTTCAGCAGAGCGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTA
GGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCT
GTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAA
GACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTTCGTGCAC
ACAGCCCAGCTTGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAG
CTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTA
AGCGGCAGGGTTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCC
TGATATCTTTATAGTCTGTGCGGTTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTT
TACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCC
TGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCA
GCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGA
TGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTACACCCGCATATATGGTGCAC
TCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATC
GCTACGTGACTGGGTCTATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCG
CCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTC
CGGGAGCTGCATGTGTCAGAGGTTTTACCCGTCATCACCGAAACGCGCGAGGCAG
CTGCGGTAAAGCTCATCAGCGTGGTCGTGCAGCGATTACAGATGTCTGCCTGTTC
ATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAA
AGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAA
GGGGGATTTCTGTTTATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCA
CGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAA
ACAACCTGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGC
CAGCGCTTCGTTAATAACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGC
GATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTT
ACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCCGACAGCTTTT
GCAGCAGCAGTCGCTTACGTTTCGCTCGCGTATCGGTGATTCAATTCTGCTAACCG
TAAGGCAACCCCGCCAGCCTAGCCGGTCTCAACGACAGGAGCAGCATCATGCG
CACCCGTGGCCAGGACCCAACGCTGCCCCGAAATT

3. Nucleic acid and amino acid sequences of the selected scFvs

(Link region and 6XHis-tag are shaded in grey)

>011 (33.46 kDa)

```
atgggcgacatccagttgaccagtcctccctcctcactgtctgcgtctgcaggagacaga
M G D I Q L T Q S P S S L S A S A G D R
gtcacccctcacttgctcgggagtcagggcattagcaatgatttagcccggcttcagcag
V T L T C R A S Q G I S N D L A R L Q Q
aaaccagggaaagcccctaagtcctgatctatgctgcatccagtttgcaaagtggggtc
K P G K A P K S L I Y A A S S L Q S G V
ccatcaaagttcagcggcagtgatctgagacagaattcactctcaccatcagcagcctg
P S K F S G S G S E T E F T L T I S S L
cagcctgatgatcctgcaacttactactgccaacagtacagtgcattcttcttcgacgctc
Q P D D P A T Y Y C Q Q Y S A S S S T F
ggccaagggaccaaggtggaaattaaacgaactgtggctgcaccatctgtcttcatcttc
G Q G T K V E I K R T V A A P S V F I F
ccgccatctgatgagcggctgaaatctggacagatcaccttgaaggagtctgggtccggcg
P P S D E R L K S G Q I T L K E S G P A
ctggtgaagcccacacagacgctcacactggcctgcgccgtctctgggctctcgcctcagc
L V K P T Q T L T L A C A V S G L S L S
acgagtgagtgcgagtggttggtccgcctcagccccaggggaaggccccggagtggtcta
T S G V R V G W L R Q P P G K A P E W L
gcacgcattgattgggacgatgacaagttctacaacacttctctgaagaccaggctcacc
A R I D W D D D K F Y N T S L K T R L T
ctctccaaggacacctccaaaaatcaagtgggtcttacaatgaccatcatggacccccgtg
L S K D T S K N Q V V L T M T I M D P V
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D T G T Y Y C A R L A V D T V M V Q G Y
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F D L W G Q G I M V T V S S A S T K G P
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S V F P L A P S S K S T S G G T A A A S
gcacatcatcatcaccatcacaagctggactacaagacatgacgggtgattataaagat
A H H H H H K L D Y K D H D G D Y K D
catgacatcgattacaaggatgacgatgacaag
H D I D Y K D D D D K
```

>1-1 (34.16 kDa)

atggccaattttatgctgactcagccccactctgtgtcggagtctccggggaagacggta
M A N F M L T Q P H S V S E S P G K T V
accatctcctgcacccgcaccagtggcagatttggcagcaagtatgtgcagtggtaccaa
T I S C T R T S G S I A S K Y V Q W Y Q
cagcggccggcagtgccccacaactgtgatgtttgaggatagtcaaagaccctctggg
Q R P G S A P T T V M F E D S Q R P S G
gtccctgatcggttctctggctccatcgacagctcctccaattctgccttcctcaccatc
V P D R F S G S I D S S S N S A F L T I
gctggactgcagcctgaggacgaggctgactactattgtcagctttatgataacagaaat
A G L Q P E D E A D Y Y C Q S Y D N R N
cagatcttcggcgggaccaaagttgaccgtccttaggtcagcccaaggctgccccctcg
Q I F G G G T K L T V L G Q P K A A P S
gtcactctgttcccaccctcctctgaggagctccaagccgaggtgcagctgttggagacc
V T L F P P S S E E L Q A E V Q L L E T
gggggaggcttagttcagcctggggggtccctgagactctcctgtgcagcctctagattc
G G G L V Q P G G S L R L S C A A S R F
agcttcaagaactactggatgcagtggtccgccaacctccaggggaaggggctggtgtgg
S F K N Y W M Q W V R Q P P G K G L V W
gtctcacgtatcaacaacgacggaaatcagaaaagatacgcggacggcgtgaagggccga
V S R I N N D G N Q K R Y A D G V K G R
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A E D T A V Y Y C V R Q S L D N Y A Y H
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L D Y W G Q G T L V T V S S A S T K G P
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S V F P L A P C S R S T S E S T A A A S
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>1-5 (33.45 kDa)

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V T L T C R A S Q G I S N D L A R F Q Q
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K P G K A P K S L I Y A A S S L Q S G V
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P S K F S G S G S E T E F T L T I S S L
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Q P D D P A T Y Y C Q Q Y S A S S T F
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G Q G T K V E I K R T V A A P S V F I F
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P P S D E R L K S G Q I A L K E S G P A
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L V K P T Q T L T L A C A V S G L S L S
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T S G V R V G W L R Q P P G K A P E W L
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L S K D T S K N Q V V L T M T N M D P V
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>1-7 (34.64 kDa)

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I I S C T R S T G T I A A N Y V Q W Y Q
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Q R P G N S P T T V I F E D N Q R P S G
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V P D R F S G S I D S S S N S A S L T I
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P R L K T E D E A D Y Y C Q S Y E A S S
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G L E W L G R T Y Y R S K W S K W Y N D
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Y A V S L K S R I T I N P D T S K N Q S
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S L H L N S V T P E D A A V Y Y C A R D
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>2-1 (34.19 kDa)

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T I S C T R T S G S I A S K Y V Q W Y Q
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Q R P G S A P T T V M F E D S Q R P S G
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V P D R F S G S I D S S S N S A F L T I
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S G L Q P E D E A G Y Y C Q S Y D N R N
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Q I F G G G T K L T V L G Q P K A A P S
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V T L F P P S S E E L Q A E V Q L L E T
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V S H I N N D G N Q K R Y A D G V K G R
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H D I D Y K D D D D K

>2-2 (33.44 kDa)

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P S K S S G S G S E T E F T L T I S S L
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L S K D A S K N Q V V L T M T D M D P V
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F D L W G Q G I M V T V S S A S T K G P
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S V F P L A P S S K S T S G G T A A A S
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>2-4 (33.48 kDa)

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V T L T C R A S Q G I S N D L A R F Q Q
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K P G K A P K S L I Y A A S S L Q S G V
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L V R P T Q T L T L A C A V S G L S L S
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L S E D T S K N Q V V L T M T N M D P V
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>I3 (33.5 kDa)

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Q K P G Q P P R L L I Y G A S K R A T G
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L E P E D F A V Y Y C Q R Y G S S P R A
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>I4 (33.32 kDa)

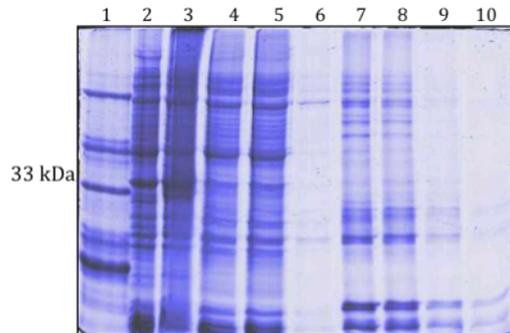
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>M5 (34.94 kDa)

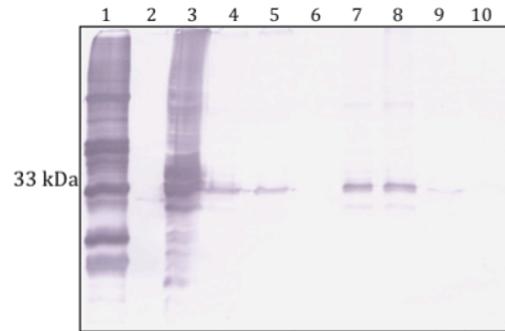
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4. SDS-PAGE and western blot of the expressed antibodies

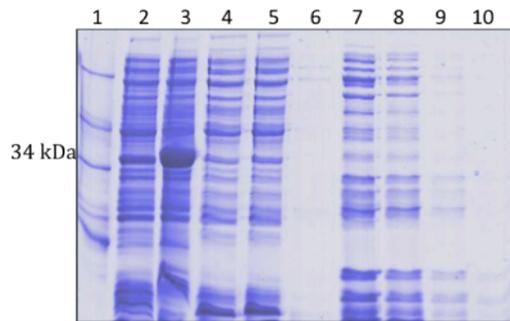
(pSANG-011, 1-1, 1-5, 1-7, 2-1, 2-2, 2-4, I3, I4 and M5)



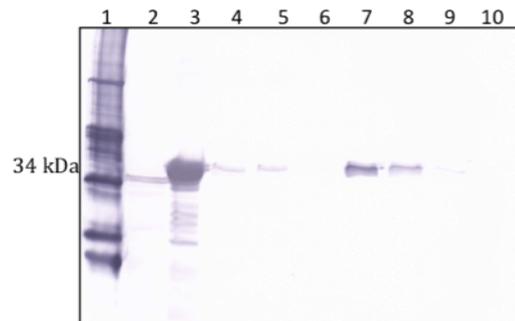
pSANG-011 total cell purification SDS-PAGE
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 5. flow-through; 6. wash; 7. elution 1; 8. elution
 2; 9. elution 3; 10. Elution 4.



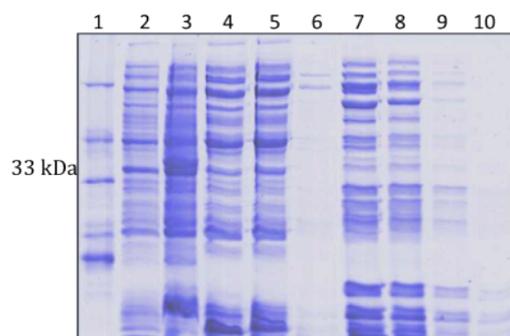
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 5. flow-through; 6. wash; 7. elution 1; 8. elution
 2; 9. elution 3; 10. Elution 4.



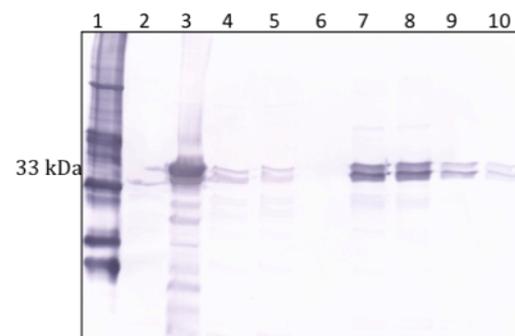
pSANG-1-1 total cell purification SDS-PAGE
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 8. elution2; 9. elution3; 10. elution4.



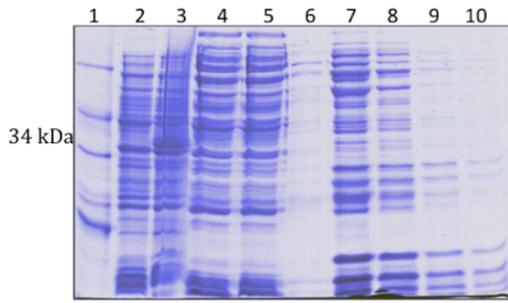
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 1. ladder; 2. uninduced; 3. induced; 4. soluble
 fraction; 5. flow-through; 6. wash; 7. elution1;
 8. elution2; 9. elution3; 10. elution4.



pSANG-1-5 total cell purification SDS-PAGE
 1. ladder; 2. uninduced; 3. induced; 4. soluble
 fraction; 5. flow-through; 6. wash; 7. elution1;
 8. elution2; 9. elution3; 10. elution4.

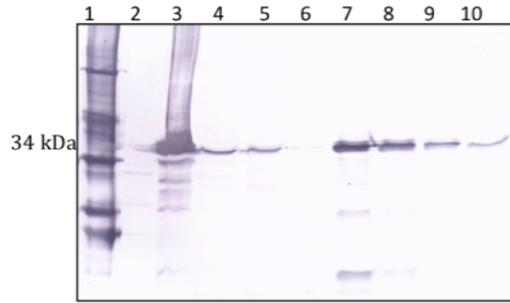


pSANG-1-5 total cell purification Western blot
 1. ladder; 2. uninduced; 3. induced; 4. soluble
 fraction; 5. flow-through; 6. wash; 7. elution1;
 8. elution2; 9. elution3; 10. elution4.



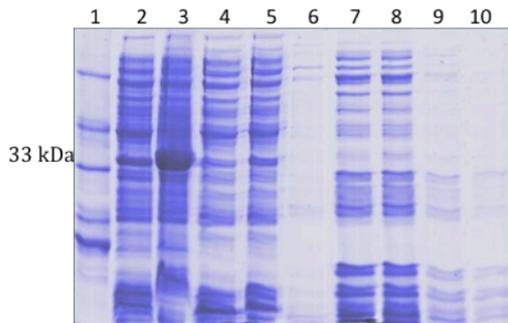
pSANG-1-7 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



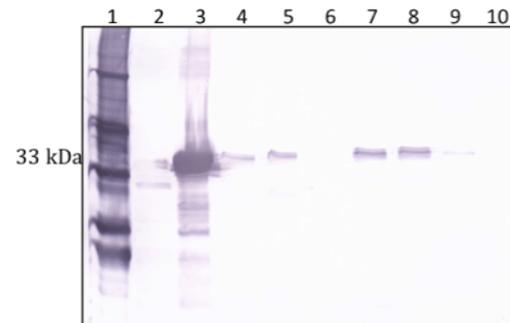
pSANG-1-7 total cell purification (native)

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



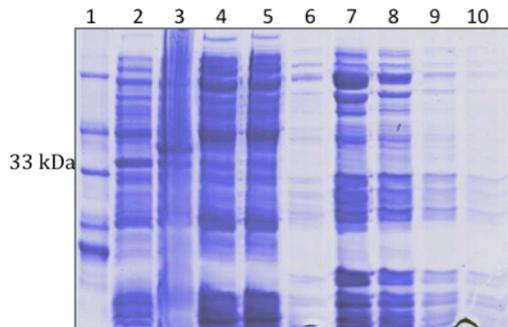
pSANG-2-1 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



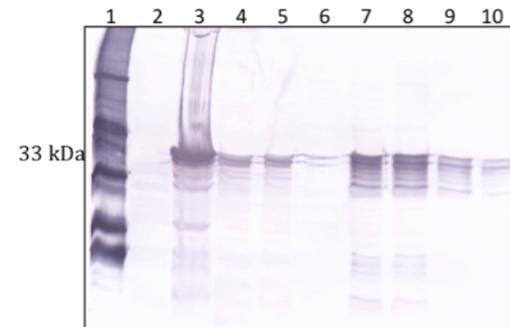
pSANG-2-1 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



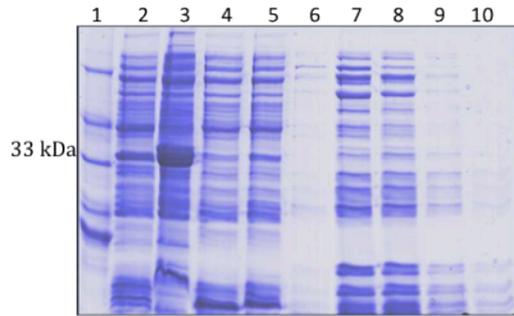
pSANG-2-2 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



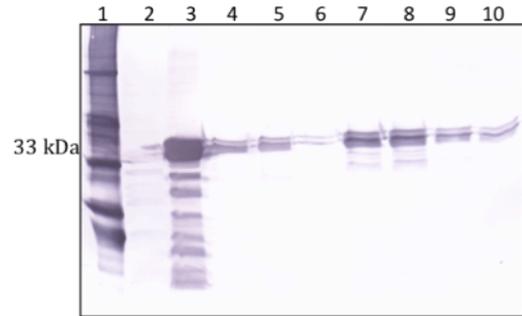
pSANG-2-2 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



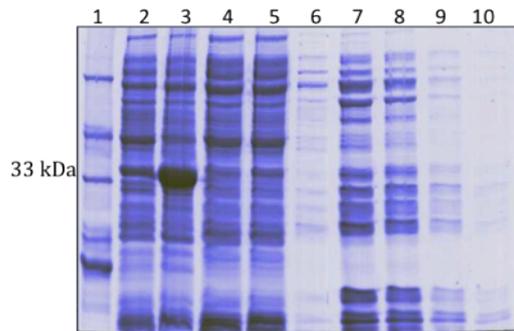
pSANG-2-4 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



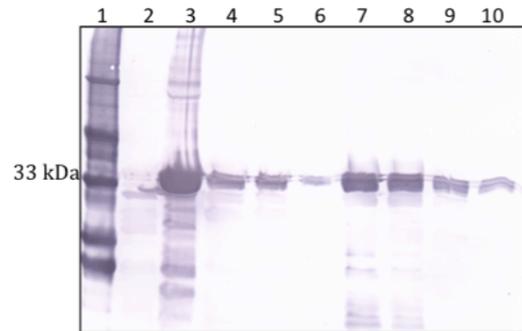
pSANG-2-4 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



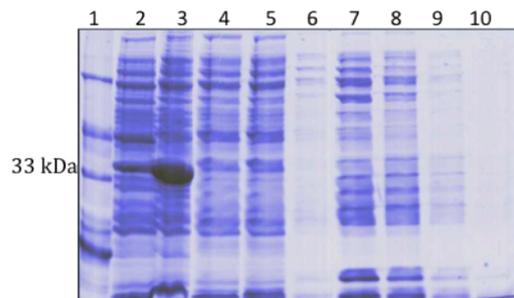
pSANG-I3 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



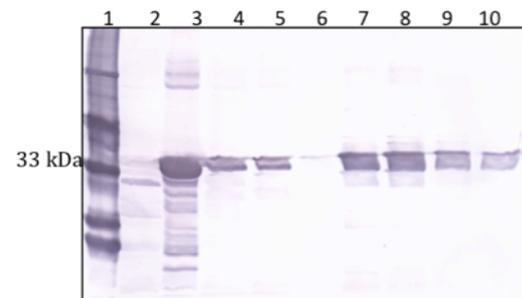
pSANG-I3 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



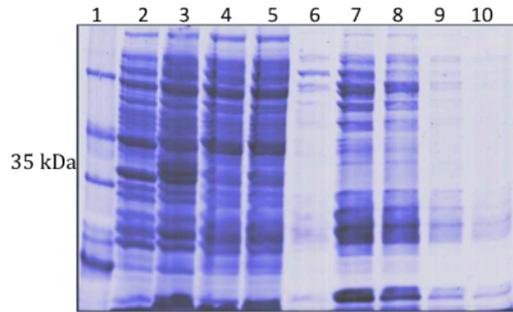
pSANG-I4 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



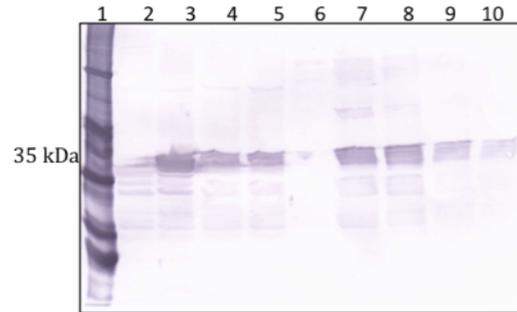
pSANG-I4 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



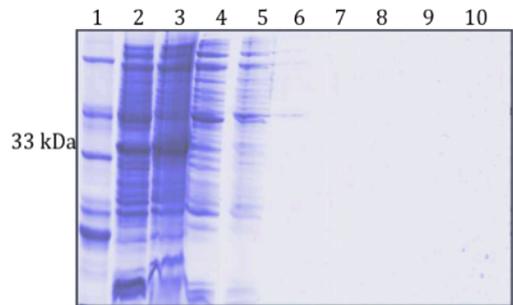
pSANG-M5 total cell purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



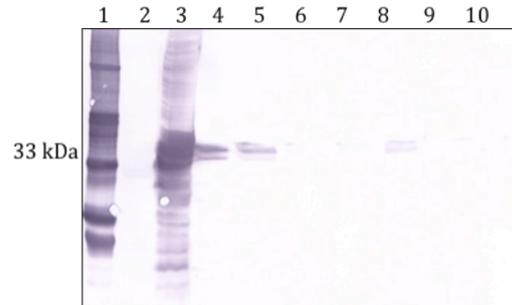
pSANG-M5 total cell purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. soluble fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



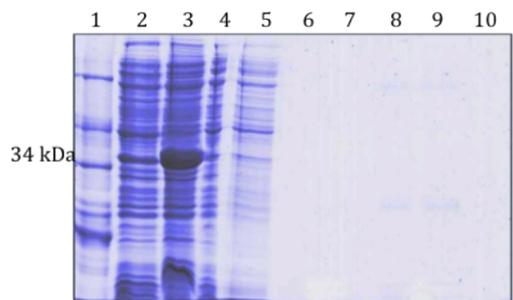
pSANG-011 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



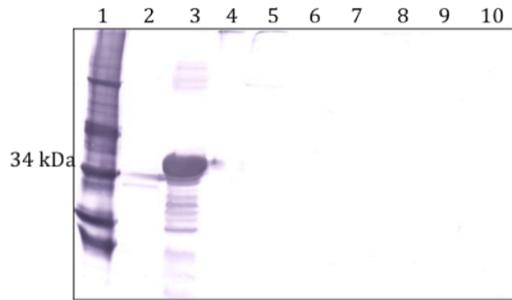
pSANG-011 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



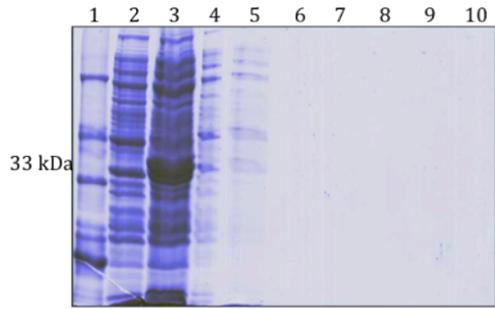
pSANG-1-1 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



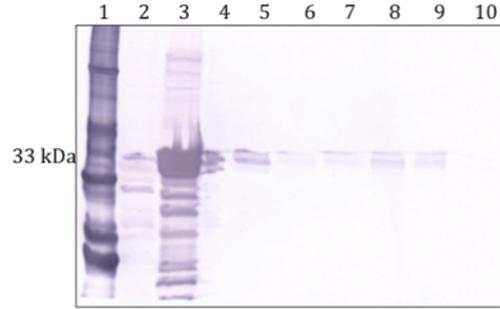
pSANG-1-1 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



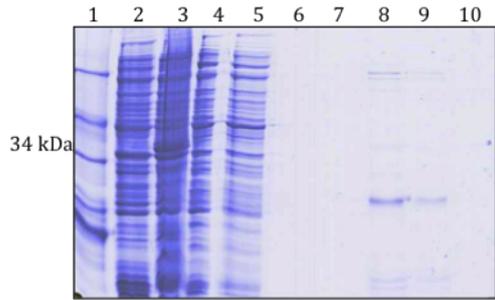
pSANG-1-5 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



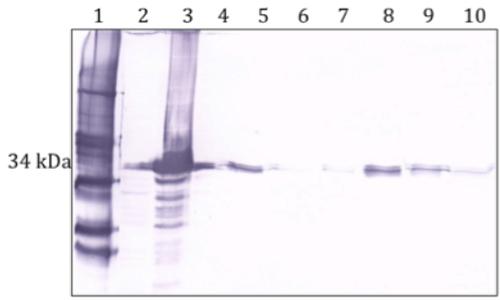
pSANG-1-5 periplasmic purification western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



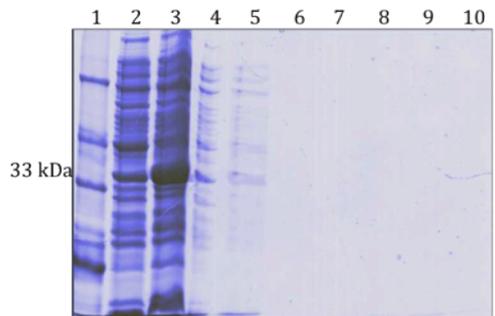
pSANG-1-7 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



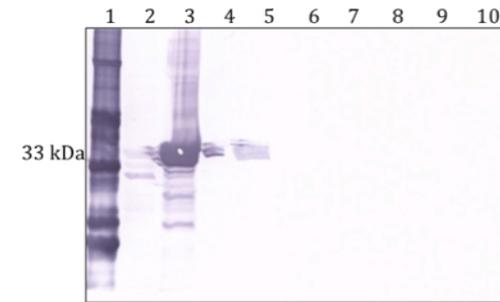
pSANG-1-7 periplasmic purification

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



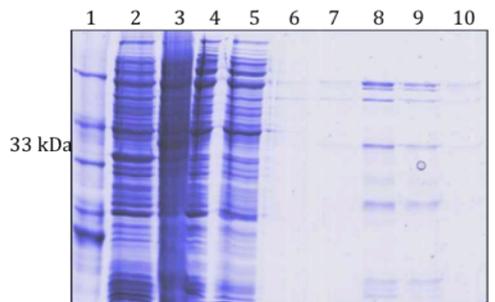
pSANG-2-1 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



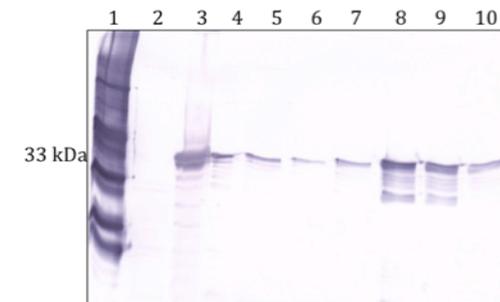
pSANG-2-1 periplasmic purification western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



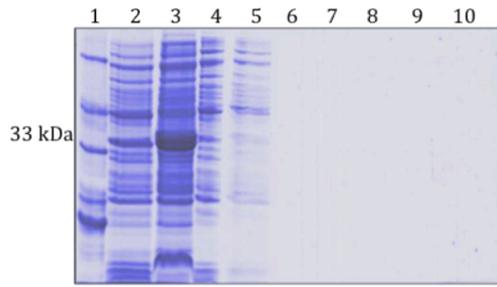
pSANG-2-2 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



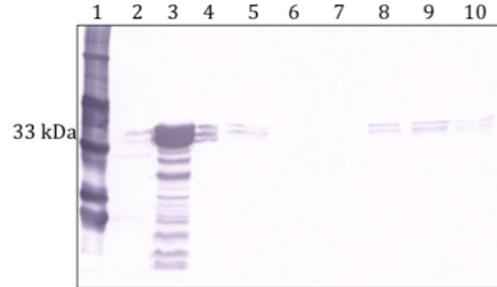
pSANG-2-2 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



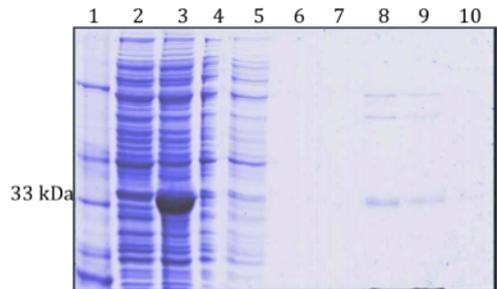
pSANG-2-4 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



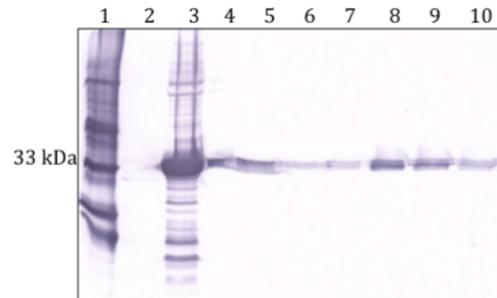
pSANG-2-4 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



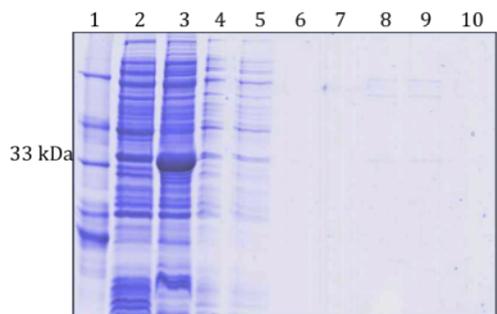
pSANG- I3 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



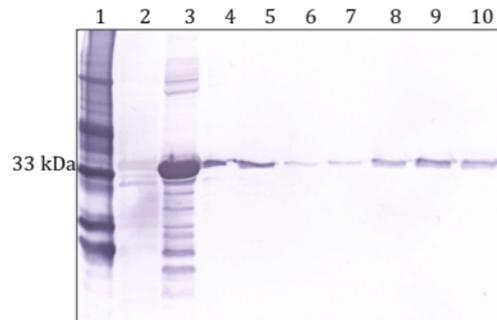
pSANG- I3 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



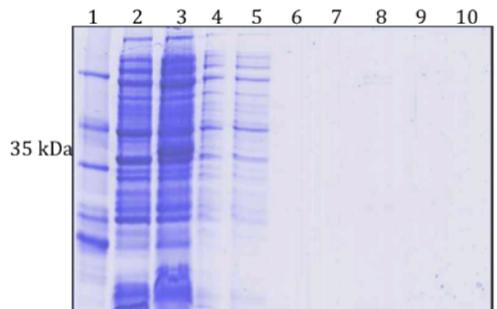
pSANG- 14 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



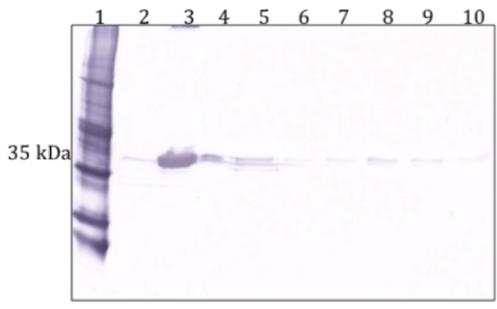
pSANG- 14 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



pSANG- M5 periplasmic purification SDS-PAGE

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.



pSANG- M5 periplasmic purification Western blot

1. ladder; 2. uninduced; 3. induced; 4. periplasmic fraction; 5. flow-through; 6. wash; 7. elution1; 8. elution2; 9. elution3; 10. elution4.

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