PRODUCTION OF ANTIBODY BY PHAGE DISPLAY TECHNOLOGY

Potjamas Pansri

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Degree of Doctor of Philosophy of Science in Biotechnology

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การผลิตแอนติบอดีด้วยเทคโนโลยีฟาจ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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PRODUCTION OF ANTIBODY BY PHAGE DISPLAY TECHNOLOGY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พจมาศ แพนศรี : การผลิตแอนติบอดีด้วยเทคโนโลยีฟาจ (PRODUCTION OF ANTIBODY BY PHAGE DISPLAY TECHNOLOGY) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 115 หน้า.

คลังของแอนติบอดีชนิด scFV ได้รับการสังเคราะห์เป็นผลสำเร็จ คลังชนิด Naïve นี้สังเคราะห์โดยอาศัยแหล่งพันธุกรรมของแอนติบอดีของมนุษย์ จากผู้บริจาคซึ่งไม่เคยได้รับการฉีดกระตุ้น จำนวน 140 คน ซึ่งนับเป็นแ หล่งพันธุกรรมของแอนติบอดีที่มากที่สุด ชิ้นส่วน scFv นี้สร้างมาจากส่วน variable ของ heavy chain และส่วน variable ของ light chain โดยสายเต็มของชิ้นส่วน scFv นั้นอาศัยการผสมรวมและจับคู่ของ heavy chain และ light chain โดยให้ครอบคลุมทุกรูปแบบของแอนติบอดีที่สามารถเกิดขึ้นได้จากทุก isotype ของ immunoglobulin วิธีที่ใช้ในการทดลองได้แก่ overlap extension PCR ร่วมกันกับชุดของ primer ที่มีความซับซ้อน วิธีการโคลนนิ่งชิ้นส่วน scFv แอนติบอดีนี้ส่งผลให้ได้คลังของแอนติบอดีที่มีขนาด $1.5x10^8$ ซึ่งจัดเป็นคลังขนาดกลาง คลังของแอนติบอดีชนิด scFv นี้ถูกนำมาใช้ในการทดลองเพื่อคัดเลือกหาแอนติบอดีที่คุณสมบัติสามารถจับจำเพาะ โดยทดสอบกับตัวอย่างโปรตีนหลายชนิด ทั้ง attenuated ไวรัส เซรุ่ม รวมสกัดจากงู โปรตีนบนผิวเซลล์ และสาร hapten จากนั้นแอนติบอดีซึ่งจับจำเพาะที่คัดเลือกได้ ถูกนำมาทดสอบหาความหลากหลายของรูปแบบของแอนติบอดี ด้วยวิธีเปรียบเทียบลำดับเบสของยีนและวิธีตรวจสอบลายพิมพ์ดีเอ็นเอ จากนั้นตัวอย่างซึ่งจับจำเพาะที่คัดเลือกได้ถูกนำมาผลิตเป็นแอนติบอดีในรูป soluble โดยผลิตใน *E.coli* ตัวอย่างของแอนติบอดีจำนวนสี่ตัวอย่างได้ผ่านการแยกให้บริสุทธิ์โดยใช้สาร Ni-NTA และปริมาณของแอนติบอดีบริสุทธิ์ที่แยกได้มีปริมาณที่เหมาะสม และยังคงมีคุณสมบัติในการจับจำเพาะดังเดิม การศึกษาครั้งนี้นอกจากจะมุ่งพัฒนาให้คลังของแอนติบอดีมีความหลากหลายของรูปแบบของแอนติบอดีจำนวนมากแล้ว วิธีการที่คัดเลือกแอนติบอดีต่อแอนติเจนที่แตกต่างกันก็มีส่วนสำคัญ ทำ ให้แอนติบอดีที่คัดเลือกโดยใช้คลังของแอนติบอดีชนิด naïve นี้มีคุณภาพดี

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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POTJAMAS PANSRI : PRODUCTION OF ANTIBODY BY PHAGE DISPLAY TECHNOLOGY. THESIS ADVISOR : ASST. PROF. MONTAROP YAMABHAI, Ph.D., 115 PP.

PHAGE DISPLAY/SCFV/PHAGE DISPLAY ANTIBODY LIBRARY/ NAÏVE LIBRARY/PANNING/MONOCLONAL ANTIBODY/RECOMBINANT ANTIBODY

A scFv antibody phage display library was successfully constructed. This naïve library was based on the widest possible human antibody gene repertoire, derived from 140 non-immunized donors. The scFv's were generated by recombining heavy chain and light chain variable regions. The full length scFv fragments were assembled in a process making use of all possible combinations of heavy and light chains, among all immunoglobulin isotypes. This was achieved by using a complex set of primers and overlap extension PCR. The resulting scFv gene repertoire was cloned to form a moderately sized library composed of $1.5x10^8$ individual clones. This naïve library was then used for selecting of specific binders by testing with different model proteins, attenuated viruses, crude venom extracts, cell surface antigens, and a hapten. Sequence variability and variable gene diversity among binders were proven by gene sequencing and DNA fingerprinting. A subset of the selected binders was then chosen for producing of soluble antibodies in *E. coli*. Four antibodies specimens were purified using Ni-NTA. The purifications yielded appropriate amounts of adequately pure scFv's, and the antibody specificity was retained. While the focus of this study has been the creation of the naïve human library, the selection of binders for

various profoundly different antigens has also played a key role. The naïve human antibody library described herein has been proven to constitute a reliable source of high quality antibodies.

School of Biotechnology

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Potjamas Pansri

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LIST OF ABBREVIATIONS

×*g*	=	times gravity
°C	=	degree Celsius
µg	=	microgram
µl	=	microlitre
bp	=	base pair
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide 5’ triphosphate
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
l	=	litre
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
N	=	normality
ng	=	nanogram
PCR	=	polymerase chain reaction
pmol	=	picomol
RNA	=	ribonucleic acid
rRNA	=	ribosomal ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

UV	=	ultraviolet
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER I

INTRODUCTION

Monoclonal antibodies have become an important tool in several fields, including molecular biology, pharmaceutical and medical research, as well as in the treatment of cancer and infectious diseases (Cavalli-Björkman et al., 2002; Baert et al., 2003; Plosker and Figgitt, 2003; Demelza et al., 2006). Monoclonal antibodies are produced from mice or rats using hybridoma technology (Gerhard et al., 1978; Köhler et al., 1978). By immunizing a target animal with a specific antigen, isolating the B-cells and fusing them with myeloma tumor cells, a hybridoma cell line is established. In order to acquire an antibody for a given antigen, mice will be repeatedly immunized over a period of 2-6 months. This procedure is followed by 1-2 months of laboratory work, before the hybridoma cells can be finally produced. Since the advent of antibody technology, antibody production has moved from hybridoma to recombinant methods. The advantages of these recombinant methods are several, (i) antibodies can be produced in bacteria, yeast, or plants (Miller et al., 2005; Almquist et al., 2006; Cabezas et al., 2008), (ii) immunization is rendered unnecessary and (iii) intrinsic properties of the antibodies, such as immunogenicity, affinity, specificity and stability, can be improved by various mutagenesis technologies (Gram et al., 1992; Valjakka et al., 2002; Wu et al., 2004). Numerous methods have been developed to obtain recombinant antibodies; a number of these has been reviewed (Bradbury et al., 2003b; Bradbury et al., 2003a). One of the key advances in recombinant antibody

technology has been the invention of phage display for isolation of antibodies directly from diverse repertoires of antibody genes. These antibody genes are expressed on the surface of filamentous bacteriophage as fusion proteins (Smith, 1985; McCafferty et al., 1990; Barbas et al., 1991; Clackson et al., 1991; Hoogenboom et al., 1991; Griffiths et al., 1993). Such a collection of phages is called a phage display antibody library, within which each phage particle displays a single antibody.

In order to construct an antibody library, antibody genes are fused to phage genes, thus creating a link between the antibody phenotype and its encoded genotype. Antibody genes can be isolated from B-lymphocytes of non-immunized donors, giving rise to a naïve library, a type of library which has been proven to be a reliable source of human antibodies (Marks and Marks, 1996). Two antigen-binding fragments, Fab and scFv have been used extensively for display on phage (de Haard et al., 1999; Little et al., 1999). One advantage of these smaller antibody fragments is that they have high tissue penetrability, while maintaining their affinity and specificity. They are also easier and faster to produce in recombinant form. Despite the usefulness of recombinant antibodies, successful construction of a human antibody phage library has been achieved by a few number of research groups (Vaughan et al., 1996; Goletz et al., 2002). The most critical aspect is the size of the antibody repertoire that is to be displayed. The larger the antibody repertoire of the library, the better the chance of obtaining binders for a particular antigen. According to previous studies, any single phage library cannot possibly be made to encompass the whole potential human antibody repertoire. Moreover, antibody genes are amplified by PCR, witch occasionally generates point mutations, which in turn might contribute to the library non-functional molecules. This effect reduces the chance of obtaining the

desired antibody sequence, rather than a non-productive clone, such as a clone with a frame shift mutation and/or a stop codon in the antibody sequence. Furthermore, non-productive chains are very well expressed, whereas the expression of scFv fusion produces a clone with a growth disadvantage (Strohal et al., 1987; Courtney et al., 1995). When growing the phages, the non-productive clones thus tend to achieve higher titers. Moreover, construction of a large library is limited by the efficiency of cloning and bacterial transformation. To obtain large numbers of recombinant clones, it is necessary to use large amounts of DNA for the ligation (Vaughan et al., 1996) and to perform multiple transformations.

In this work, our aim has been to produce a useful naïve human scFv antibody phage library with high diversity. The library was generated from a wide combination of variable region genes. The pool of genes derived from B lymphocytes of 140 non-immunized donors. The primer set that employed was designed to maximize retrieval of possible naïve variable genes within the donor antibody repertoire, during the antibody gene amplification step of the library construction. To verify the usefulness of the library, it was used for screening and selection of specific scFv binders to a variety of antigens, including protein antigens, live attenuated viruses, crude venom extracts, cell surface antigens and a hapten.

CHAPTER II

REVIEW AND LITERATURE

2.1 The antibody molecule

Antibodies or immunoglobulins (Ig) (Litman et al., 1993) are glycoproteins, produced by B- lymphocytes called plasma cells in immune system. Their job is to identify and neutralize foreign substances or antigens (Borghesi and Milcarek, 2006). There are five classes or isotypes of antibodies (IgM, IgG, IgE, IgA, IgD), which differ in the C-terminal portion of their heavy chain (Eleonora and Nina, 2003). The basic functional unit of each antibody is a monomer containing only one Ig unit. The secreted antibodies can also be dimer with two Ig units like IgA, or pentamer with five Ig units like IgM (Roux, 1999). The details of immunoglobulin isotypes is described in Table 1.

Table 1. Immunoglobulin classes.

Ig class	Ig subclass	H chain	Secreted form	Function
IgG		γ	Monomer	Produced in 2º response
	IgG1	γ1		
	IgG2	γ2		
	IgG3	γ3		
	IgG4	γ4		
IgM		μ	Pentamer	Produced in 1º response
IgA		α	Dimer	Mucoidal immunity
	IgA1	α1		
	IgA2	α2		
IgE		ϵ	Monomer	Allergic hypersensitivity: attach to FC receptor on mast cells and Basophils
IgD		δ	Monomer	B cell Ag receptor along with monomeric IgM

Note: Adapted from (Clancy, 2000)

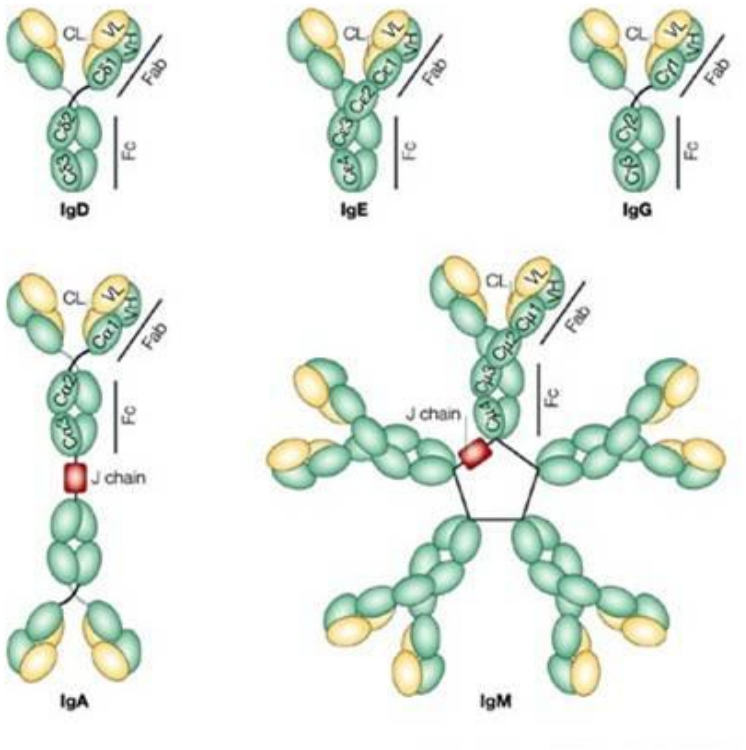


Figure 1. Isotypes of Immunoglobulin or antibody (Rojas and Apodaca, 2002).

2.1.1 Structure of antibody

The success of antibody cloning is based on an elementary understanding of antibody structure. All antibodies have a basic structure consisting of an identical pair of heavy chains and a pair of identical light chains held together by disulfide bridges and non-covalent interactions (Harlow and Lane, 1988; Woof and Burton, 2004). There are five isotypes of heavy chain (μ, γ, ϵ, α, δ) and two isotypes of light chain (κ, λ) (Charles et al., 2001). Each of the heavy chains is encoded by the variable (V_H), diversity (D), joining (J_H), and constant (C_H) genes (Nemazee, 2006), while each of the light chains encoded by V_L, J_L, and C_L genes. Pairing of the heavy chain V-D-J regions and light chain V-J regions creates an antigen-binding site, also called paratope. The DNA and the amino acid sequences of the C region are relatively

conserved within a given species, while those of the V region are antigen-dependent. Each V region consists of an alternating framework (FW), which is relatively, more conserved, and three hyper-variable or complementarity-determining regions (CDRs), with have the greatest sequence diversity. The CDRs and to a lesser extent the FW regions, interact with the antigen to form the antigen-binding site. The first two CDRs are encoded by the V segment, while the third CDR is the product of the junction of V-D-J for heavy chain or V-J for light chain (Janeway, 2001). This knowledge of antibody structure has facilitated the creation of antibody molecules outside their natural host (Bruggemann et al., 1989; Clackson et al., 1991).

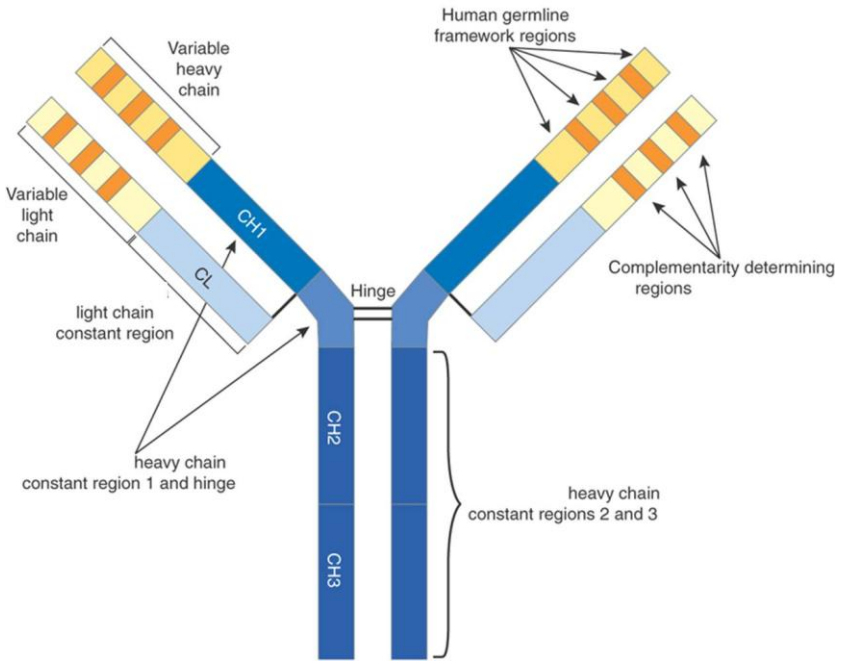


Figure 2. Structure of antibody or Immunoglobulin (Rother et al., 2007).

2.1.2 The diversity of antibodies

A large diversity of antibodies can be created by the immune system. Each antibody has a unique antigen-binding site at the N-terminal domains of heavy and

light chains. Several mechanisms are responsible for the diversity of the antigen-binding site (Padlan, 1994). A recombinatorial diversity created by random selection of one V_H gene, one D gene and one J_H gene, or one V_L gene and one J_L gene, to constitute the V_H and V_L domains, respectively (Diaz and Casali, 2002; Eleonora and Nina, 2003). Thus, a combinatorial diversity is generated by the assembly of the V_H and V_L domains. A functional diversity is generated by the imprecise joining mechanisms and by deletion or addition of random nucleotides at the location of the recombining V_H-D-J_H genes (Peter, 2005). Moreover, a maturation of the antigen-binding site caused by a somatic hypermutation, improves the shape of the CDRs of the antibody, further tuning them to mach the antigen (Honjo and Habu, 1985). The cumulative effect of these mechanisms, renders the antigenic affinity and specificity of the antibody (Or-Guil et al., 2007).

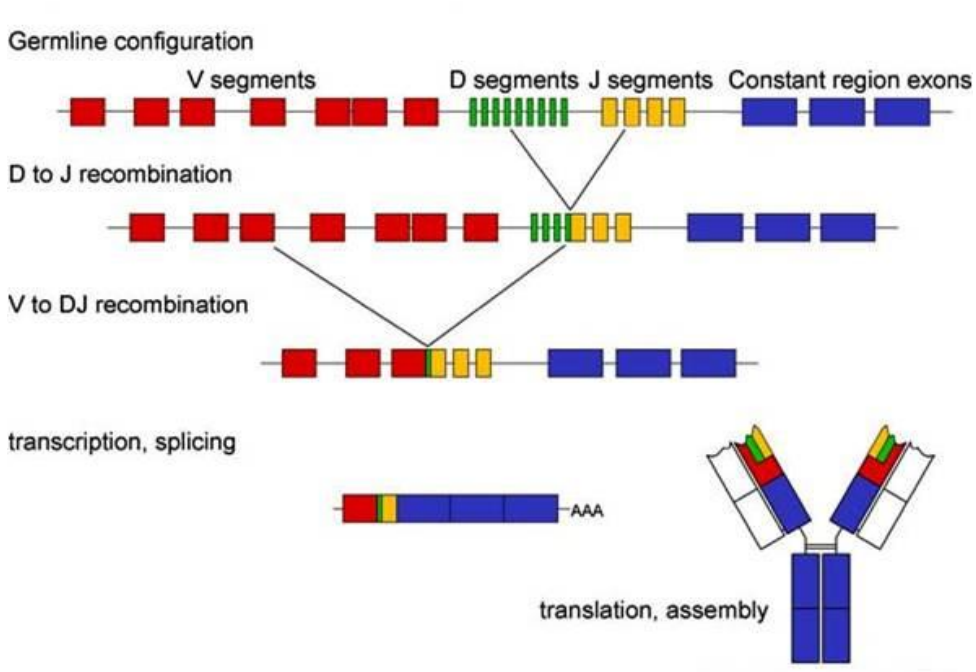


Figure 3. VDJ recombination (Inlay, 2002). The recombination is first occurred between one D and one J gene of heavy chain segment. Any gene between

these D and J genes is deleted, then following by the joining of one V gene to DJ recombination segment. A rearranged VDJ gene is then formed. All other genes between V and DJ segment are deleted from the genome. The VDJ region of the heavy chain and the constant chain are transcribed. The primary RNA is processed to add a polyadenylation (poly-A) tail after constant region. Translation of this mRNA leads to the production of the heavy chain of immunoglobulin. The light chain of immunoglobulin rearranges in a very similar way but the light chain lack a D segment.

2.1.3 Antibody fragments

Antibody molecules contain discrete protein domains that can be separated by protease digestion or produced by recombinant technology. Different antibody fragments incorporating the CDR have been devised, to facilitate the expression of antibodies in recombinant form. One such fragment is Fab (fragment antibody binding), which is composed of the V_H-C_H and V_L-C_L domains linked by disulfide bonds (Putnam et al., 1979). A smaller engineered antibody fragment is called Fv (fragment variable) consists of V_H and V_L domains. The recombinant form of Fv is called single-chain fragment variable (scFv), and is composed of V_H and V_L domains. The two separate variable domains in the scFv are joined by a flexible linker peptide, a 15 amino acid linker with the sequence (Gly4Ser)3 is commonly used (Huston et al., 1988). The linker allows expression as a single polypeptide string, and aids the association of the V_H and V_L, leading to the formation of the antigen-binding site. The linker peptide can significantly affect the properties of the scFv (Argos, 1990; Holliger et al., 1993), such as the stability. This property of the scFv can be improved

by the use of linkers with different lengths and sequences (Huston et al., 1988; Takkinen et al., 1991; Tang et al., 1996). A short linker (0-10 amino acids) can hinder the dimerisation of scFv (Holliger et al., 1993). A picture illustrating the antibody fragments is shown in Figure 4, and a summary of the characteristics of different antibody fragments is presented in the Table 2.

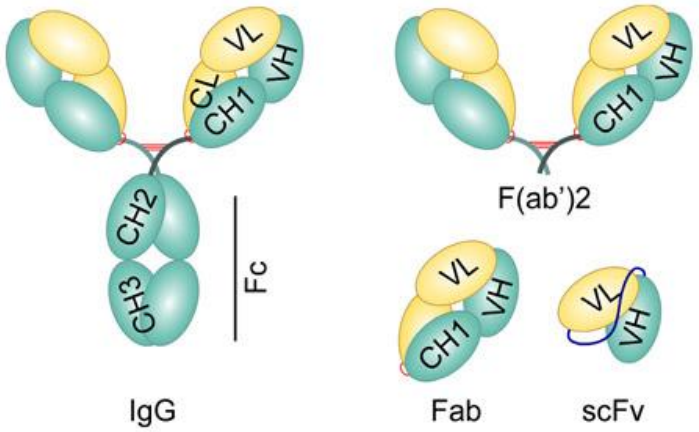


Figure 4. Immunoglobulin (IgG) or antibody fragments (Peterson et al., 2006).

Table 2. Characteristics of antibody fragments.

Antibody fragment	Size (kDa)	Paratopes (valency)	Structure
scFv	25-30	1	V_H and V_L domains are coupled by a linker peptide. Changing the linker length can lead to the formation of diabody (60 kDa), triabody (90kDa), or tetrabody (120 kDa)
Fv	25	1	V_H and V_L with no linker between the V domain
Fab	50	1	Two chains of V_H-C_H1 and V_L-C_L
$F(ab)_2$	100	2	Two Fab molecules
IgG	150	2	Parent antibody molecule consists of two heavy chain (V_H-C_H1-C_H2-C_H3) and two light chain (V_L-C_L)

Note: Adapted from (Azzazy and Highsmith, 2002)

2.2 Phage display technology

Phage display was first reported by George P. Smith (Smith, 1985). It is an effective tool for producing a large diversity of peptides and proteins, and from these selecting molecules that have specific binding properties. This technique can be particularly useful for studying (i) protein-ligand interactions (Cesareni, 1992), (ii) antigen-antibody interactions (Griffiths, 1993; Winter et al., 1994), and (iii) to improve the affinity of proteins for their binding properties (Burton, 1995; Neri et al., 1995). Phage display involves the expression of peptides, proteins, or antibody fragment on the surface of filamentous bacteriophage (Smith, 1985; Winter et al., 1994; Kay et al., 2000). The DNA sequence of interest is inserted into a location in the phage genome, in which it fuses to a gene encoding a phage coat protein. The protein encoded by the insert is expressed or displayed on the surface of the phage particle, fused to one of the phage coat proteins (Figure 5). The phenotype of the expressed protein is thus linked to its genotype, which is present in the genome of the phage.

Instead of engineering proteins or peptide variants one by one and then expressing, purifying, and analyzing each variant, one advantage of phage display is that a large diversity of variant proteins can be constructed. For example, phage display antibody libraries with diversities as high as 10^{10} have been established (Vaughan et al., 1996; de Haard et al., 1999). These libraries can be utilized for the selection and purification of phage particles bearing sequences with desired binding specificity, from a background of non-binding variants. Three key scientific breakthroughs paved the way for the development of the phage display technology. The first one is the construction of libraries of DNA sequences encoding fusion

proteins (Smith, 1985), The second breakthrough is the understanding of the structure and function relationship of the genes and the proteins of the phage particle (Roberto et al., 1998). Furthermore, the successful expression of functional proteins in the periplasmic space of *E.coli* played a major role (Better et al., 1988).

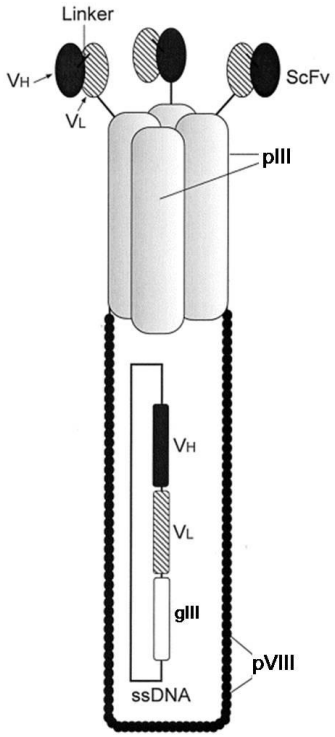


Figure 5. Diagram of filamentous phage displaying scFv molecules (Azzazy and Highsmith, 2002). The phage particle consists of circular ssDNA surrounded by coat proteins. The pVIII is the major coat protein, whereas pIII at the top of the phage is one of the minor coat proteins. The genes encoding the scFv and a linker are fused to gene III in the genome of the filamentous phage. The scFv is displayed as a fusion to pIII protein at the top of the phage. The scFv is not fused to all pIII protein molecules, and therefore the phage retains its ability to infect bacteria.

2.2.1 Biology of the filamentous bacteriophage

2.2.1.1 Structure

The filamentous bacteriophage has been identified as a virus, which infects a variety of Gram-negative bacteria using the bacterial pili as a receptor. The best characterized of these phage (M13, fl and fd) are collectively referred to as the Ff phage. These infect *E.coli* containing the F conjugative plasmid. The genomes of these three bacteriophage have been completely sequenced (van Wezenbeek et al., 1980; Beck and Zink, 1981; Hill and Petersen, 1982). The genome of Ff phage is a single-stranded covalently closed DNA molecule, which is approximately 7 nm wide by 900-2000 nm in length. It consists of 11 genes (6408 nucleotides), the products of which are listed in Table 3. The genes are grouped according to their functions in the life cycle of the phage. There are three groups of genes. The first group (gene II, V, and X) encodes proteins required for DNA replication (Rapoza and Webster, 1995). The second group (gene III, VI, VII, VIII and IX) encodes the proteins that make up the capsid proteins for phage particle (Simons et al., 1981; Rapoza and Webster, 1993; Endemann and Model, 1995). Protein pVIII is the major capsid protein, with approximately 2700 molecules present in each phage particle. The remaining four minor capsid proteins make up the ends of the phage particle. Protein pIII, which is commonly fused with recombinant proteins (Smith, 1985), is presented as approximately 3-5 molecules. The third group (gene I, IV, and XI) encodes three proteins involved in assembly of the phage. Gene products pI, pIV, and pXI are required for phage assembly (Horabin and Webster, 1988; Rapoza and Webster, 1995), but they are not part of the phage particle.

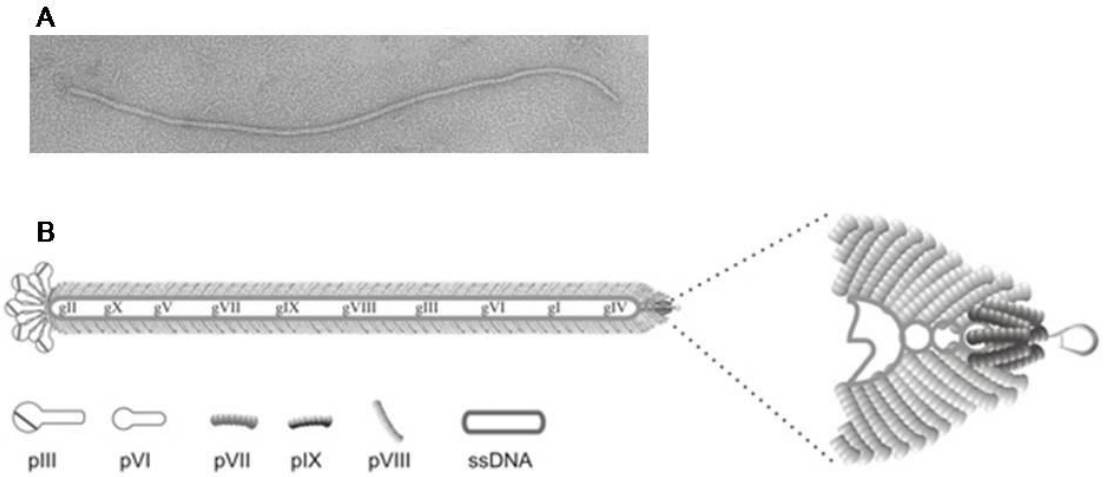


Figure 6. (A) Electron microscopic image of filamentous bacteriophage (Specthrie et al., 1992). (B) The schematic structure of filamentous bacteriophage (Dickerson and Janda, 2005) . The phage coats contain approximately 2800 copies of the major coat protein (pVIII) and 5 copies of each of the minor coat proteins (pIII, pVI, pVII, and pIX). The number of pVIII molecule adjusts to accommodate the size of the single stranded genome it packages. At one end of the phage particle contains the surface exposed minor coat protein (pIX) and a more buried minor coat protein (pVII). At the other end of the phage particle contains the surface exposed minor coat protein (pIII) and the less exposed protein (pVI). These form the rounded tip of the phage and pIII is the first protein to interact with the host cell during infection.

Table 3. Genes and gene products of fl bacteriophage (Webster, 1996).

Gene	Amino acid	Function
I	348	Assembly
II	410	DNA replication
III	406	Minor capsid protein
IV	405	Assembly
V	87	Binding of ssDNA
VI	112	Minor capsid protein
VII	33	Minor capsid protein
VIII	50	Major capsid protein
IX	32	Minor capsid protein
X	111	DNA replication
XI	108	Assembly

2.2.1.2 Life cycle

The general stages of a phage life cycle are infection, replication of the viral genome, assembly of new viral particles, and then release of the progeny particles from the host. During phage infection, the pIII end of the phage attaches to the F pilus of male *E.coli*. Then the pVIII major capsid proteins and other capsid proteins integrate into the inner bacterial membrane. The phage ssDNA is translocated into the cytoplasm of bacteria. This process requires the presence of the bacterial TolQRA protein (Russel et al., 1988). The TolQRA complex probably forms a membrane pore through which the phage DNA eventually enters the host cytoplasm. In the cytoplasm, the bacterial DNA replication machinery synthesizes the complementary strand, and converts the DNA into a supercoiled, double stranded replicative form (RF). This RF undergoes rolling circle replication to make ssDNA, and serves as a template for expression of phage capsid proteins, and other phage proteins involved in the assembly process. The phage particle is assembled by packaging of ssDNA into a casing of protein pVIII. One end of the particle is capped by protein pVII and pIX, while protein pIII and pVI fill the other end (Marvin, 1998). The assembly process is gradual, starting at one end of the ssDNA genome, and progressing until the end of

the ssDNA is reached and the pIII and pVI find their place in the cap. The complete phage particle is extruded through the bacterial envelop into the medium. Filamentous bacteriophage does not produce lytic infection in their host.

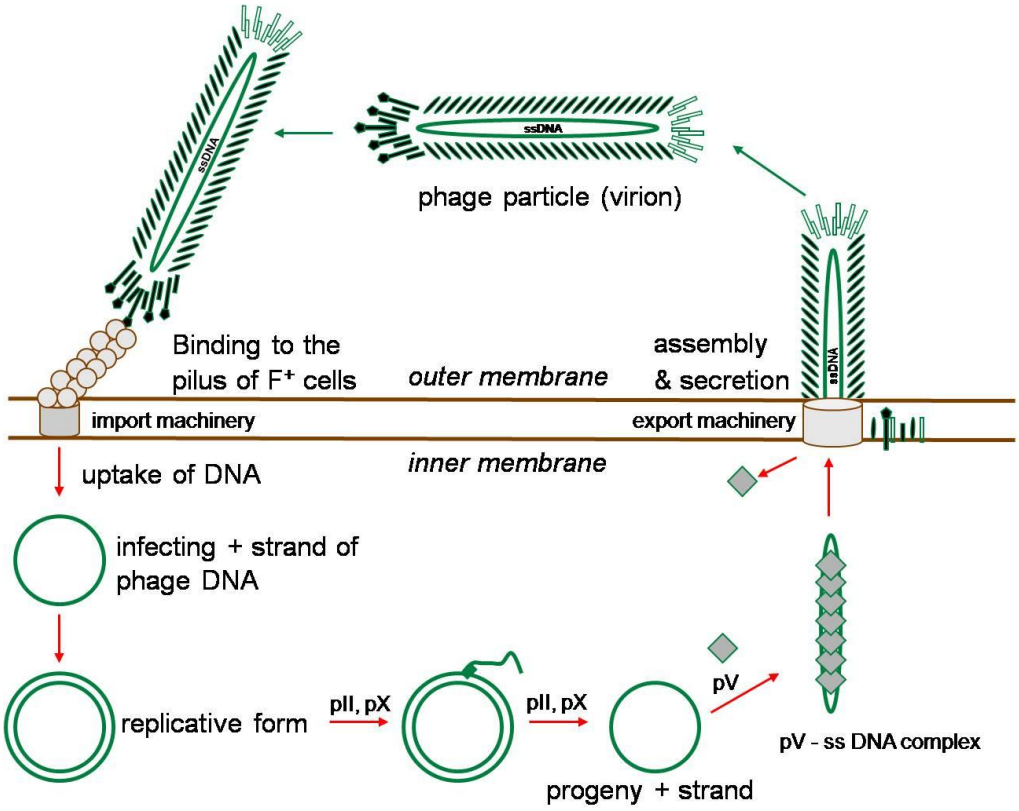


Figure 7. Life cycle of filamentous bacteriophage. Phage particle infects bacteria by contacting the TolQRA protein on the bacterial pilus with pIII tip. The phage genome is then transferred to the cytoplasm of the bacterial cell where the single stranded DNA is converted to a double stranded replicative form (RF). The pII nicks the double stranded form of the genome to initiate replication of the + strand. Host enzymes copy the replicated + strand, resulting in more copies of double stranded phage DNA. The pV competes with double stranded DNA formation by

sequestering copies of the + stranded DNA into a protein/DNA complex destined for packaging into new phage particles. The pX regulates the number of double stranded genomes in the bacterial host. The pI, pXI, pIV complex forms channels through which mature phage are secreted from the bacterial host. The pIX and pVII interact with the pV-single stranded DNA complex at a region of the DNA called the packaging sequence (PS). The pV proteins covering the single stranded DNA are then replaced by pVIII proteins and the growing phage particle is threaded through the pI, pXI, pIV channel. Once the phage DNA is fully coated with pVIII, the secretion terminates by adding the pIII/pVI cap. Finally, the new phage releases from the bacterial surface.

2.2.2 Phage display vectors

A special kind of cloning and expression vector called phagemid has been constructed by combining plasmid and the phage genome. Phagemids contain the origin of replication and packaging signal (PS) of M13 phage, together with the origin of replication and the expression system of the plasmid (Figure 7). Generally, expression plasmids consist of multiple cloning sites, an antibiotic resistance marker, epitope tags such as a hexahistidine tag or a c-myc tag (Sidhu, 2001), and a lacZ promoter (Mead and Kemper, 1988). Moreover, phagemids often contain an amber stop codon, to allow host specific expression of pIII fusion protein or suluble fusion partner, respectively (Hoogenboom et al., 1991), and a gene encoding one coat protein that will be fused to the foreign DNA that is to be expressed (Barbas et al., 1991; Kang et al., 1991). Phagemids can maintain themselves as plasmids, resulting in the

expression of the desired protein in the bacteria. However, they lack other genes that encode proteins necessary for phage assembly. To get production of viable phage, an infection with a helper phage is necessary. The helper phage provides the genes for the phage proteins that are missing on the phagemid. The packing signal in the helper phage genome has been altered to be less effective, and thus the recombinant phagemid ssDNA is packaged into phage particles using helper phage proteins (Bass et al., 1990).

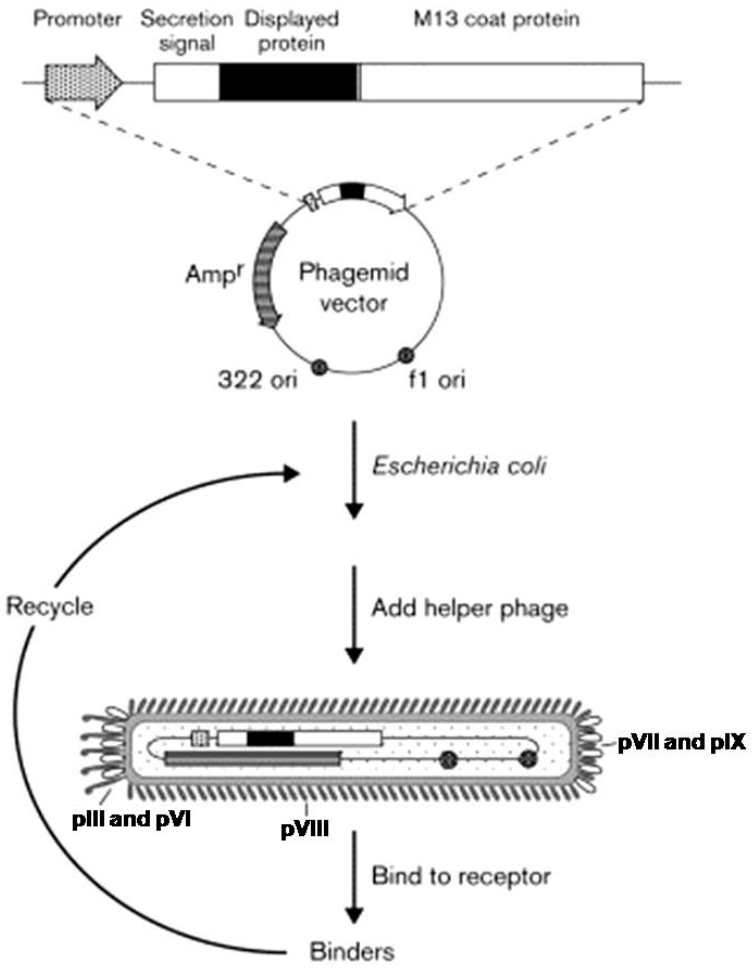


Figure 8. Schematic show an example of a phagemid vector designed for phage display (Sidhu, 2000). A phagemid vector contains origins of single-

stranded (f1 ori) and double-stranded (322 ori) DNA replication, and a selective marker, in this case the ampicillin resistance gene. The phagemid also contains a promoter that drives translation of the fusion protein. The vector replicates in *E. coli*, but co-infection with a helper phage is required for the production of phage particles. The fusion proteins can be displayed as either N-terminal fusions (with pIII, pVII, pVIII, or pIX) or C-terminal fusions (with pIII, pVI, or pVIII). The phage particles can be used in binding selections, and the binding clones can be multiplied through infection of *E. coli* host.

Using the phagemid vector system, large proteins can be displayed as N-terminal fusion proteins, by fusing them to pIII or pVIII (Bass et al., 1990), or pIX (Gao et al., 1999). Furthermore, C-terminal display has been achieved on pVI, pIII, and pVIII (Jespers et al., 1995; Fuh and Sidhu, 2000). A disadvantage of the phagemid systems, is that the number of recombinant fusion proteins displayed on each phage particle is reduced, because of competition for incorporation into the phage particle, between the wild-type coat protein and a fusion coat protein (Winter et al., 1994). The use of modified helper phage, that lacks the gene for the chosen coat protein (Rondot et al., 2001) has been attempted. Moreover, the development of mutated coat proteins and artificial coat proteins (Sidhu, 2001) has been reported. The number of recombinant proteins displayed on the individual phage is determined by several factors; the type of coat protein chosen as a fusion partner (pIII or pVIII), the display system chosen for expression (phage or phagemid), and the choice of helper phage in case a phagemid system is used. A protease sensitive helper phage KM13

(Kristensen and Winter, 1998) employs a trypsin cleavage sequence in pIII protein between its N-terminal domain and the C-terminal domain. The N-terminal domain of pIII is required for infection, therefore phages with cleaved off N-terminus are non-infectious. In this way the functionality of helper phage pIII can be destroyed, so that only phages displaying a fusion protein remain infectious. A similar approach was tried with the creation of a CT helper phage, which lacks the N1–N2 domains of pIII (Kramer et al., 2003). A refinement of some phage display systems is the use of an amber stop codon, located between the gene encoding the coat protein and the recombinant gene. This allows for a soluble version of the recombinant protein to be produced, if the phage are maintained in a non-suppressor strain of host bacteria (Winter et al., 1994).

2.2.3 Types of phage display systems

Phage display systems can be classified according to the rearrangement of foreign genes and coat protein genes, corresponding to where the fusion protein is displayed (McConnell et al., 1994). In a type 3 vector, the wild type gene III has been substituted for a recombinant gene, encoding of foreign protein fused to the N-terminal of pIII. The foreign protein is displayed on all five pIII molecules. If the foreign peptide is large, it is often removed from some or most of the pIII molecules by proteolytic enzymes in host bacterium. Similarly, a type 8 vector displays foreign proteins on every pVIII protein. However, only short foreign proteins can be displayed on pVIII molecules, at least when it comes to multivalent display (Il'ichev et al., 1989; Kishchenko et al., 1994).

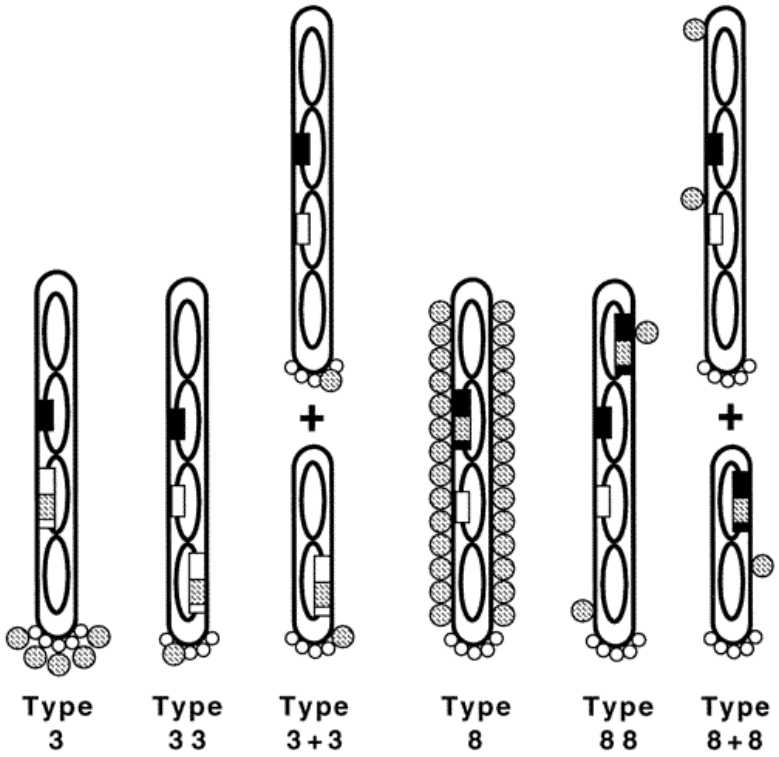


Figure 9. Types of phage display systems (Smith and Petrenko, 1997). The phages are represented by long cylinders, and the short cylinder represents phagemids. The twisted lines inside each virion represent the ssDNA. The genes encoding coat protein pVIII and pIII are represented by black and white boxes, respectively. The hatched boxes represent foreign genes fused to a coat-protein gene, and the hatched circles on the surfaces of the virions, represent the foreign proteins. The five white circles at the tip of the virions correspond to the N-terminal domains of the five pIII molecules. The foreign proteins displayed on pIII are either fused to the N-terminal domain (type 3 vector), or replaced pVIII (type 3+3 and most type 33 vectors). In a type 8 vector, the foreign peptide is displayed on all copies of pVIII, whereas in the type 88 and 8+8 vector, only minorities of the pVIII display the foreign peptide.

In a type 88 vector (Smith, 1993), the phage genome contains two gVIII, encoding two different types of pVIII molecule; one is recombinant and the other is wild type. This gives rise to a virion, containing both wild-type and recombinant pVIII molecules. This type of vector allows the expression of large foreign proteins on the virion surface. Similarly, a type 33 vector (McConnell et al., 1994) bears two gIII, one of which is recombinant.

A type 8+8 vector is different from a type 88 vector, in that the two gVIII are on separate genome. The wild-type gene is carried by the helper phage, while the recombinant gene is on the phagemid (Mead and Kemper, 1988). Similarly to plasmids used in recombinant DNA technology, a phagemid contains a plasmid origin of replication, which allows it to be replicated normally in an *E. coli*, and an antibiotic resistance gene that allows phagemid-bearing host cells to be selected. The phagemid also contains a phage origin of replication, which due to the lack of the DNA replication and particle assembly genes, is inactive until the cell is infected with the helper phage. Two types of virions are produced, particles carrying helper phage DNA and particles carrying phagemid DNA. Type 3+3 and 6+6 vector are similar to type 8+8 vector, in which the phagemid carries a recombinant gIII or gVI, respectively. The recombinant pIII encoded by the type 3+3 phagemid is usually missing the N-terminal domain, since cells expressing this domain are resistant to superinfection by helper phage.

2.2.4 Phage-displayed peptide libraries

The peptide phage display approach is based on the generation of random combinatorial libraries, that provide millions of variants from which desired peptides

can be isolated by affinity selection (Scott and Smith, 1990). The peptides displayed in these libraries are encoded by synthetic oligonucleotides, which range in length from five to twenty amino acids (Scott and Smith, 1990; Kay et al., 1993). These synthetic oligonucleotides are synthesized with each random residue encoded by a degenerate codon, either NNK or NNS (N=A, C, G, T; K=G, T; S=G, C) (Sondek and Shortle, 1992; Virnekas et al., 1994). To hamper the introduction of stop codons, the degenerate codon NNN is excluded. These synthetic oligonucleotides are ligated to the end of one of the M13 phage coat proteins, where they are expressed as peptide-capsid fusion proteins. Such a library often has a complexity of approximately 10^9 to 10^{10}, due to the limitation due to the limitation imposed by the transformation efficiency of *E.coli* (Dower et al., 1988). These random peptide libraries can be screened for binding to target molecules of interest. The display of random peptides on filamentous bacteriophage, as fusions to either pIII or pVIII coat proteins (Sternberg and Hoess, 1995), allows identification of peptides that specifically bind to a variety of targets (Turk and Cantley, 2003).

2.2.4.1 Applications for phage-displayed peptide libraries

Random peptide libraries are a source of binding partners for various targets, and they allow the selection of peptides that specifically bind to targets of interest, for example ribonuclease S (Smith et al., 1993), streptavidin (Devlin et al., 1990), and antibodies (Scott and Smith, 1990). Phage display libraries of random peptides have been used successfully for identifying antibody epitopes, in cases where the antigen has to be identified (Cortese et al., 1999). Moreover, phage particles displaying antigenic peptides can be used for mapping epitopes recognized by monoclonal and

polyclonal antibodies (Hill and Stockley, 1996). A highly useful application in immunology and in the medical field has been the use of peptides and protein fragments displayed on the M13 virus particle, to elicit a secondary immune response against the coat proteins of parasites and viruses (de la Cruz et al., 1988; Di Marzo Veronese et al., 1994; Azzazy and Highsmith, 2002). Furthermore, phage-displayed peptides that neutralize immunoglobulins may be employed as therapeutic agents for controlling autoimmune diseases (Blank et al., 1999). Phage display has also been used in the selection of organ-specific peptides *in vivo* (Pasqualini and Ruoslahti, 1996). Following the injection of a peptide library into mice, the organs of interest are harvested and washed, and the eluted phages used in subsequent rounds of injection and selection. Peptides selected in this manner, have been successfully used to specifically deliver drugs to tumor cells (Arap et al., 1998). Phage displayed peptide libraries have become practical tools for drug discovery (Riemer et al., 2005; Nishimura et al., 2008; Sulochana and Ge, 2007).

The conventional strategies for the phage display have so far failed to identify antagonists for protein–protein interactions, in the cases where these interactions involve large contact surfaces (Cochran, 2000). In contrast, phage displayed peptide libraries have successfully been used for generating both antagonists and agonists of receptors (Cortese et al., 1995) and intracellular targets (Doorbar and Winter, 1994; Sidhu et al., 2003). With regard to protein-protein interactions involving large contact surfaces, peptides can be isolated with sequence homology to the natural protein binding partner of the target (Kay et al., 2000). The potential of random peptide libraries to study various interactions has been presented, such as to develop substrate phage to identify substrate sites for different enzymes (Matthews and Wells, 1993), to

identify peptides binding to carbohydrate moieties (Szardenings, 2003), for identification of protein kinase substrate (Dente et al., 1997), for isolation of peptides binding to cell surface receptor (Wrighton et al., 1996), or to identify ligand receptor (Balass et al., 1997).

2.2.5 Phage-displayed antibody libraries

Phage display has been used for isolation of recombinant antibodies with a range of unique specificities (Barbas et al., 1991; Hoogenboom et al., 1998) and produced many high affinity antibodies without prior immunization. The binding properties of these antibodies has often been further manipulated *in vitro* (Hoogenboom et al., 1998). Antibody fragments can be displayed on the surface of filamentous bacteriophage in different formats, including Fab fragment (Cabilly, 1989), Fv fragment (Skerra and Plückthun, 1988), scFv fragment (McCafferty et al., 1990), and diabody fragments (Holliger et al., 1993). When constructing a phage antibody library, the method is similar to the one employed for construction of a peptide display library, the only difference being that the sequences encoding the displayed proteins are derived from antibody genes. For the display of antibodies, the filamentous bacteriophage M13 and phagemid vector are common choices. Three different types of antibody libraries are typically displayed on phages; (i) immunized, (ii) naïve, and (iii) synthetic antibody libraries (Hoogenboom et al., 1998). An immunized library is constructed from a host immunized with an appropriate target antigen (Clackson et al., 1991). A naïve or non-immunized antibody library is based on a repertoire of antibody genes derived from a non-immunized donor (Marks et al., 1991), whereas a synthetic antibody library is constructed by using synthesized

germline V-gene fragments with randomized CDRs (Knappik et al., 2000). Phage display antibody libraries can be a powerful source of antibodies. In the year 2000 it was estimated, that approximately 30% of all human antibodies at that point in clinical development, were obtained from phage display technology (Reichert, 2000).

2.2.5.1 Applications for phage displayed antibodies libraries

One of the most powerful applications of phage display, has been the isolation by affinity selection, of recombinant antibodies with a specific binding to a variety of antigens (Barbas et al., 1991; Hoogenboom et al., 1998). In this regard, phage display technology has many uses in the medical and pharmaceutical field. It is possible (i) to generate human monoclonal antibodies for use in cancer immunotherapy, (ii) to investigate and elucidate the specificity of autoimmune antibodies (Calcutt et al., 1993) (iii) to understand the immune response during infection from viruses such as HIV (Barbas et al., 1994) or Hepatitis B (Zebedee et al., 1992). A study has indicated that naïve phage display libraries are an excellent source of antibodies against cell surface receptors, as compared to immunized libraries (Rob C. Roovers et al., 2001). Thus, phage displayed antibody libraries have become practical tools for drug discovery (Hoet et al., 2005), and several phage-derived therapeutic monoclonal antibodies have been developed and are being tested in advanced clinical trials (Roovers et al., 2007; Tornetta et al., 2007; Fan et al., 2008).

Phage displayed recombinant antibodies have several advantages over monoclonal antibodies generated by hybridoma technology (Corisdeo and Wang, 2004). In comparison to the time-consuming hybridoma production, antibody genes can be cloned rapidly from B lymphocytes using recombinant DNA methods. A phage

displayed antibody library derived from variable gene repertoires can eliminate animal immunization and large-scale cell-culture of hybridoma. Finally, soluble recombinant antibodies expressed in bacteria, can be produced quickly and economically. Phage display is particularly useful in cases where monoclonal antibodies cannot be obtained by classical hybridoma techniques, as with antibodies against non-immunogenic or toxic antigens such as Aflatoxin, which is a hapten and also toxic (Moghaddam et al., 2001a). Phage display can also be used to clone and rescue monoclonal antibodies from genetically unstable hybridoma. Phage displayed antibody genes can be easily sequenced, mutated, and then screened, to discover those antibodies with improved antigen binding.

2.2.6 Selection by phage display library (Bio-panning)

Antibody libraries are screened for specifically binding clones and then amplified by a technique called bio-panning, in which phage displayed peptides or antibodies are incubated with an immobilized target or antigen of interest (Clackson et al., 1991; Nissim et al., 1994). Unbound phages are removed by washing, whereas phages that specifically bind the target are eluted, by changing the binding conditions or by proteolysis. Specifically bound phage can be eluted from the immobilized antigen with acidic solutions such as HCl or glycine buffer (Kang et al., 1991), with basic solutions such as triethylamine (Marks et al., 1991), by enzymatic cleavage of a protease site incorporated in the recombinant coat protein (Kristensen and Winter, 1998), or by competition with excess antigen (Clackson et al., 1991). In the next step, the eluted phages are amplified in *E. coli*. Ideally, only one round of selection should be required, however the inevitable presence of unspecific background phage, limits

the enrichment that can be achieved per round. In practice, several rounds of selection can often be necessary (approximately 2-4 rounds).

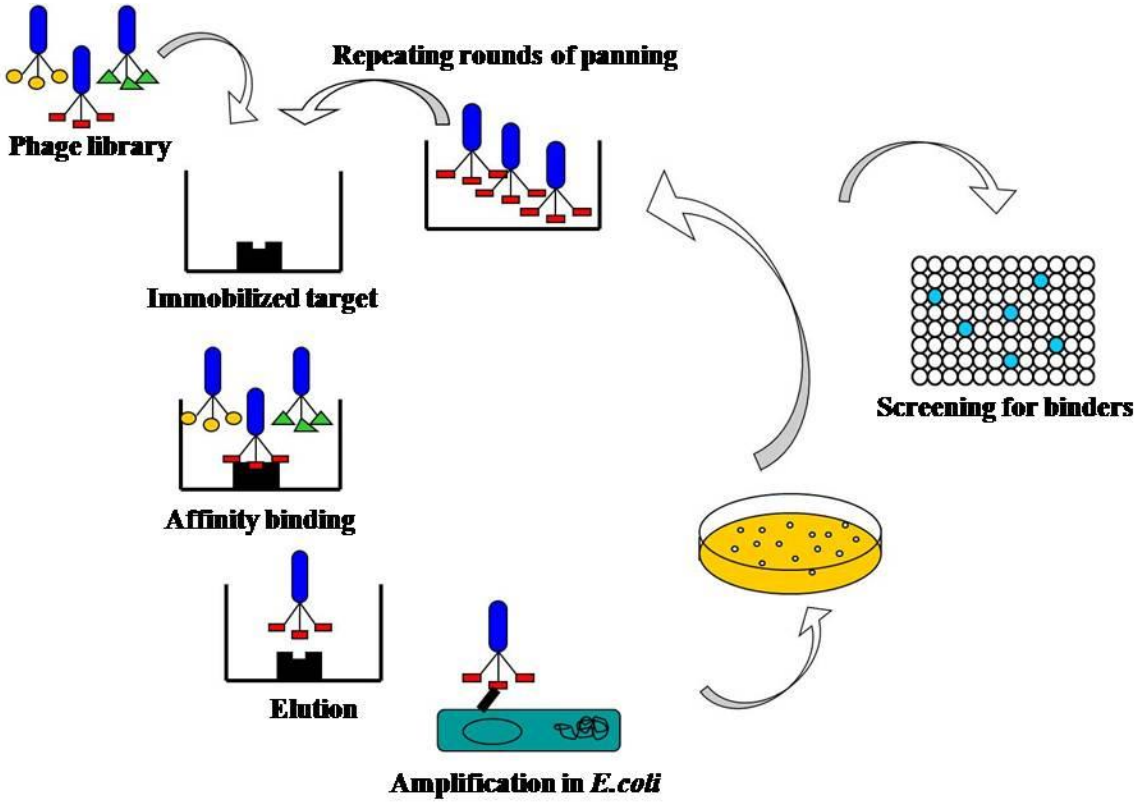


Figure 10. Diagram of bio-panning. The phage displayed peptides or antibodies are exposed to immobilized target molecules, and phages with specificity are bound. Non-binding phages are washed away, and this is followed by elution of bound phage by disruption of the interaction between the phage displayed polypeptides and the target antigen. The eluted phage are used to infect *E. coli* and thereby amplified. If the bio-panning rounds are repeated, the phages recognizing the target are gradually enriched. After one to several rounds of panning, the binders, that have at this point come to dominate the repertoire, are selected.

2.2.6.1 Selection using immobilized targets

Specific binders within a phage library can be isolated by performing a selection against a target molecule adsorbed onto plastic, such as Maxisorb Immuno Tubes, (Nunc, Denmark) or Immuno 96 MicroWell Plates (Nunc, Denmark) (Marks et al., 1991). Alternatively, antigen may be immobilized on chips of BIAcore sensors (Malmborg et al., 1996). It should be considered that some phage displayed antibodies selected against an immobilized antigen, may not be able to recognize the native form of the antigen. One way to overcome this problem, is to employ indirect antigen coating, through the use of antigen-specific antibodies (Sanna et al., 1995).

2.2.6.2 Selection using antigens in solution

This selection technique is performed when the antigen cannot be immobilized on a solid surface. The use of labeled soluble antigens is more accurate if a relatively high concentration of the antigen is used during selection (Hawkins et al., 1992). Following incubation of biotinylated antigen with phage displayed antibody, phage bound to the labeled antigen are recovered, using avidin or streptavidin-coated magnetic beads (Moghaddam et al., 2003). The binding phages are then dissociated from the antigen. One disadvantage of this technique is that anti-streptavidin antibodies are often isolated along with specifically bound phages.

2.2.6.3 Selection on cells

The selection of phage displayed antibodies against cell surface receptors is often carried out on either monolayer of adherent cells (Liu et al., 2005) or on cells in suspension (Mazuet et al., 2006). Unbound phage can be washed away by rinsing the

tissue culture flasks (monolayer) or by centrifugation (cell suspension). To maximize the isolation of specifically binding phage and minimize the background of non-binders, a positive and negative selection should be employed (de Kruif et al., 1995b; Mazuet et al., 2006). A competition is set up, between a small amount of positive cells and an excess of negative cells (absorber). The absorber cells capture the non-specific binders. A fluorescently labeled antibody against an antigen present only the target cells is added, and FACS is employed to isolate the target cells, and thus also the phage binding to the surface of these cells (Siegel et al., 1997; Mazuet et al., 2006). Selections may also be carried out on tissue sections (Liu et al., 2005).

2.2.6.4 *In vivo* selection

In this method, phage particles are injected directly into animals and then target tissues are collected. In this way, the phages displaying peptide or antibody that bind specifically to cellular receptors have been determined (Pasqualini and Ruoslahti, 1996; Johns et al., 2000).

2.3 Research objectives

1. To construct a naïve phage library displaying scFv antibody, which is the smallest structure of antibody that can interact specifically with antigens.
2. To produce human antibodies with high specificity and affinity to a wide variety of antigens by phage display technology.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria

Bacteria	**Strain**	**Genotype**
Escherichia coli	TG1	*(lac-proAB) Sup E thi hrd* D5*/F'*
	(suppressor)	*tra D36 pro* A^+B $lacI^q$ *lacZΔM15*
Escherichia coli	HB2151	K12 *ara Δ* (*lac-proAB*)
	(non suppressor)	*thi*/F'*pro*A^+B $lacI^q$ *lacZΔM15*
Escherichia coli	DH5α F'	F'/*end*A1 *hsdR17* (r_K^- mK^+)
		supE44 thi-1 recA1 gyrA (Nal^r)
		relA1 Δ(*lacZYA-argF*)*U169 deoR*
		(ϕ80 *dlac*Δ*(lacZ)M15)*

3.1.2 Antigens

Albumin from bovine serum (BSA) was natural purified protein obtained from Fluka, USA. Crude venom of cobra and green pit viper snakes were prepared from *Naja Kaouthia* and *Trimeresurus albolabris*, respectively, by the Thai Red Cross Society, Thailand. Aflatoxin B1-BSA conjugated and soluble Aflatoxin B1 were

prepared from *Aspergillus flavus* (Sigma, Germany). Amylase enzyme Type XII-A was prepared from *Bacillus licheniformis* (Sigma, Germany). Purified Chick Embryo Cell (PCEC) rabies vaccine strain flury LEP (Chiron Behring, India) are inactivated rabies viruses that are obtained from primary chicken fibroblast cells. Cholangiocarcinoma KKU-100 cell was porta hepatic-derived and the Opisthorchis-associated cholangiocarcinoma cell line, which was a kind gift from Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University.

3.1.3 Instruments

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Sorvall RC5C plus, Kendro laboratory Products, USA
	Eppendrof centrifuge 5810 R, Eppendrof, USA
Deep freezer -70 °C:	Heto, Ultra Freeze, Denmark.
ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA
Freezer -20 °C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel dryer	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA

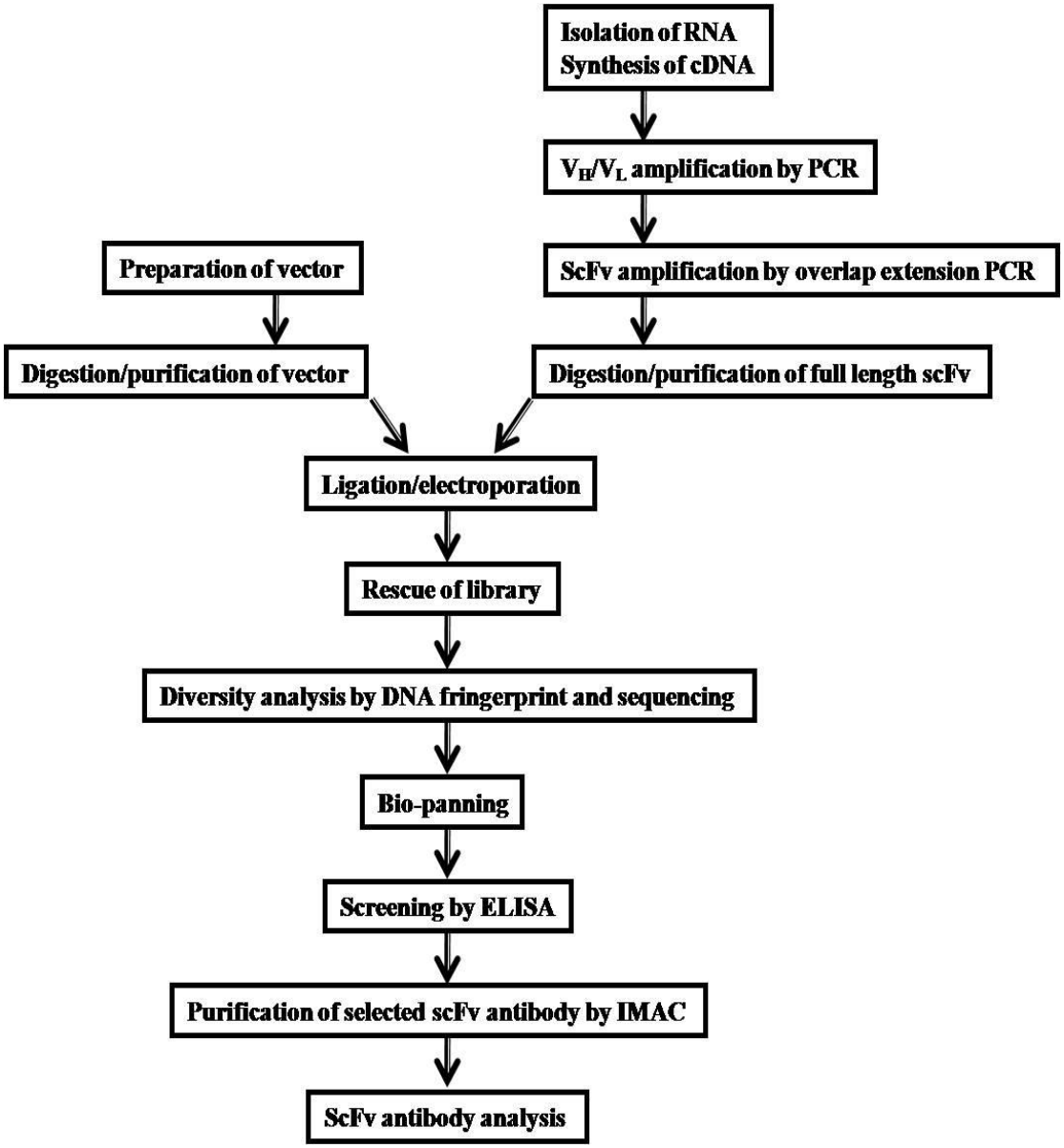
Gel electrophoresis apparatus:	Mini Protean® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood	Holten LaminAir HBB 2448, Denmark. BH2000 Series ClassII Biological Safety Cabinets, BHA120 & BHA180, Clyde-Apac,
Membrane transfer machine:	Semi Phor, Hoefer Scientific instruments, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA Eppendorf 54154, Eppendorf, Germany
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
Rotator:	Certomat TCC, B. Braun Biotech International, Germany
Sequencing machine:	ABI prism model 310 Genetic Analyzer, Applied Biosystems, USA
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Sonicator:	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK
Stirrer:	Variomag Electronicrührer Poly 15, Germany

Magnetic stirrer MSH300, USA

Thermomixer: Thermomixer compact, Eppendrof, USA

Vortex: Vortex-Genie 2 G506, Scientific Industries, USA

3.2 Methods



3.2.1 Phage displayed antibody library construction

3.2.1.1 Isolation of total RNA from leukocytes

Peripheral blood of non-immunized donors was obtained from the Thai Red Cross of Nakhon Ratchasima. B-lymphocytes were isolated from peripheral blood by using Ficoll paque reagent (Amersham, USA), then carefully laying the diluted blood sample on top of the Ficoll paque reagent. Then the two-phase solution was centrifuged at 400×*g* for 30 minutes. B lymphocytes were collected from the interface between the two phases. The interface contamination such as platelets and plasma proteins were removed by washing with PBS. Total RNA was extracted from B lymphocytes by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. B lymphocytes were resuspended in 1 ml TRIzol and incubated at 65°C for 15 minutes with occasional inversion of the tube. After adding 0.2 ml of chloroform, the tube was vortexed for 15 seconds and then centrifuged at 12,000×*g* for 15 minutes at 4°C. The aqueous phase was transferred to a new tube containing 1 µl of RnaseOut (40U/µl, Invitrogen, USA), and 0.5 ml of isopropanol was added to precipitate RNA. The tube was incubated at room temperature for 10 minutes. The precipitated RNA was pelleted by centrifugation at 12,000×*g* for 15 minutes at 4°C. The pellet was washed with 0.5 ml of 75% ethanol and then centrifuged at 12,000×*g* for 15 minutes at 4°C. After the supernatant was removed, the pellet was air dried for 5 minutes at room temperature and dissolved in sterile deionized water. Then 1 µl of RnaseOut (40U/µl, Invitrogen, USA) was added into total RNA and stored at -70°C.

3.2.1.2 Isolation of mRNA

Poly A^+ mRNA was isolated from total RNA with the oligotex kit (QIAGEN, Germany). The system is designed for rapid isolation of purified mRNA from total RNA, with the use of polystyrene-latex particles. The poly A^+ tail at the 3'-end of mRNA bound to the deoxy-thydimylate (dT) oligomers which are coupled to the surface of the Oligotex particle. The poly A^+ mRNA was released from the latex particle by lowering the ionic strength. The reaction was performed according to the manufacturer's protocol. The total volume of RNA was adjusted to be 250 µl with RNase free water and mixed with oligotex particles. The solution was incubated at 70°C for 3 minutes and then incubated at room temperature for further 10 minutes. The poly A^+ mRNA was pelleted by centrifugation at 12,000×*g* for 2 minutes. The pelleted mRNA was transferred to the column. After centrifugation at 12,000×*g* for 1 minute, the column was washed and then eluted. The mRNA was kept at -70°C.

3.2.1.3 First strand cDNA synthesis

The mRNA was converted to first strand cDNA using reverse transcriptase enzyme with oligo-dT_{18} and random hexamer primers. The first strand synthesis was started at the 3' end of poly $(A)^+$ mRNA by using the oligo-dT primer. The total volume of the reaction was 100 µl which consisted of 10 µg RNA, 20 µM oligo-dT primer, 8 ng of random hexamer primers, and 0.125 mM of dNTPs (Promega, USA), 200 units of MMuLV reverse transcriptase (200U/µl, NEB, USA) and 160 units of RNaseOut (40U/µl, Invitrogen, USA), all dissolved in 1 x RT buffer. The RNA was heated to at 90°C for 5 minutes and quickly chilled on ice before it was added to the reaction. The reaction was incubated at 42°C for 2 hours and then the reaction was

heated to 95°C for 3 minutes and quickly chilled on ice. After that, the PCR reaction was performed as described below. The second strand synthesis took place using the mRNA/DNA as a template. The cDNA was kept at -20°C.

3.2.1.4 Amplifying and reassembling heavy and light chain sequences

The heavy and light chain antibody sequences were amplified by PCR using *Taq* and *pfu* DNA polymerase with 2 sets of primers. The total volume of the of each reaction was 50 µl which consisted of 2.5-5 µl of reaction from previous step, 1 µM of each forward and reverses primers, 200 µM of dNTPs, 1x ThermoPol buffer, 2.5 units of *Taq* DNA polymerase enzyme (5U/µl, NEB, USA), 1.25 units of *pfu* DNA polymerase enzyme (3U/µl, Promega, USA) and 0.1 mg/ml of BSA. The PCR cycles were initiated by pre-denaturing at 94°C for 5 minutes, following by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57-65°C for 1 minute, extension at 72°C for 2 minutes. The final extension was performed, by heating the reaction to 72°C for 10 minutes. The heavy chain primers were modified to include a *Sfi*I site and linker sequence whereas the light chain primers were generated a *Not*I site and a linker sequence. All combinations of primers was listed in Table 4 were used in the PCR reactions. Thus, a total of 75 reactions were performed. The PCR products of each heavy chain and a light chain were pooled in this step for the next assembly step. Before assembly, the pooled PCR products were purified by gel electrophoresis. Low melting temperature agarose gel was used for fractionating the sample. The excised band was melted in LMT elution buffer (20 mM Tris pH 8.0, 1 mM EDTA pH 8.0). After that, DNA was purified by using phenol/chloroform extraction and then precipitated with 0.2 volume of 10 M ammonium acetate plus 2 volume of absolute

ethanol. The pooled heavy and light chain products were assembled at the linker sequence by PCR in the absence of primers. The total volume of the PCR reaction was 50 µl, which consisted of equal molar amounts of DNA, approximately 500-1,000 ng, 200 µM of dNTPs, and 1.25 units of *pfu* DNA polymerase enzyme (3U/µl, Promega, USA), all in 1x *pfu* DNA polymerase buffer. The separated heavy and light chain fragments were converted to scFv by amplification under the following conditions: 5 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 50 seconds and extension at 72°C for 1 minute. The correctly linked products from the assembly step were extended by PCR under the following conditions: 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes. This was followed by the final extension at 72°C for 10 minutes. Finally, the full length scFv fragment was obtained in PCR with two primers which anneal at the 5'and 3' ends of the assembled product. The primers that were used in this overlap extension PCR are listed below:

Table 4. Listing of primers for PCR amplification of human antibody heavy and light chain variable regions.

Primer	Sequence
V_H-	5’CCTTTCTATGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG3’
5’primers	5’CCTTTCTATGCGGCCCAGCCGGCCATGGCCGAGGTACAGCTGCAGCAGTCAGG3’
	5’CCTTTCTATGCGGCCCAGCCGGCCATGGCCCAGGTCAACTTAAGGGAGTCTGG3’
	5’GCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG3’
	5’GCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG3’
	5’GCCCAGCCGGCCATGGCCGAGGTGCAGCTGTTGCAGTCTGC3’
V_H-	’ACCAGAGCCGCCGCCGCCGCTACCACCACCACCTGAGGAGACGGTGACCAGGGTGCC3’
3’primers	5’ACCAGAGCCGCCGCCGCCGCTACCACCACCACCTGAGGAGACGGTGACCGTGGTCCC3’
	5’ACCAGAGCCGCCGCCGCCGCTACCACCACCACCTGAAGAGACGGTGACCATTGTCCC3’
	5’ACCAGAGCCGCCGCCGCCGCTACCACCACCACCTGAGGAGACGGTGACCAGGGTTCC3’
Vκ-	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGACATCCAGATGACCCAGTCTCC3’
5’primers	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGAAATTGTGCTGACTCAGTCTCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGATGTTGTGATGACTCAGTCTCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGAAATTGTGTTGACGCAGTCTCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGACATCGTGATGACCCAGTCTCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGAAACGACACTCACGCAGTCTCC3’
Vλ-	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCAATTTTATGCTGACTCAGCCCCA3’
5’primers	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAGTCTGTGTTGACGCAGCCGCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAGTCTGCCCTGACTCAGCCTGC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCTCCTATGTGCTGACTCAGCCACC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCTCTTCTGAGCTGACTCAGGACCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCACGTTATACTGACTCAACCGCC3’
	5’AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAGGCTGTGCTCACTCAGCCGTC3’
Vκ-	5’CAGTCATTCTCGACTTGCGGCCGCACGTTTGATTTCCAGCTTGGTCCC3’
3’primers	5’CAGTCATTCTCGACTTGCGGCCGCACGTTTAATCTCCAGTCGTGTCCC3’
	5’CAGTCATTCTCGACTTGCGGCCGCACGTTTGATCTCCAGCTTGGTCCC3’
	5’CTCGACTTGCGGCCGCACGTTTGATATCCACTTTGGTCCC3’
	5’CTCGACTTGCGGCCGCACGTTTGATCTCCACCTTGGTCCC3’
Vλ-	5’CAGTCATTCTCGACTTGCGGCCGCACCTAAAACGGTGAGCTGGGTCCC3’
3’primers	5’CTCGACTTGCGGCCGCACCTAGGACGGTGACCTTGGTCCC3’
	5’CTCGACTTGCGGCCGCACCTAGGACGGTCAGCTTGGTCCC3’
PTfw	5’CCTTTCTATGCGGCCCAGCCGGCCATGGCC3’
PTrv	5’CAGTCATTCTCGACTTGCGGCCGCACG3’

3.2.1.5 Construction of a novel phagemid vector (pMOD1) for the expression of scFv genes

The pHage 3.2 vector (Maxim Biotech Inc, USA) was used as a basic frame for the construction of a novel phagmid vector that allows the fusion of scFv with hexahistidine tag and myc epitope. The new multiple cloning sites DNA sequence consisting of *Nco*I, *Xho*I and *Not*I restriction sites and the hexahistidine tag, were introduced into pHage 3.2 vector for the generation of pMOD1 (Figure 11). Two oligonucleotides which were Mod1up 5' TCGACCCATGGCTCGAGGCGGCCGCA CATCATCATCACCATCACGGGGCCGCAGGGCC 3' and Mod2dn 5' CTGCGGC CCCGTGATGGTGATGATGATGTGCGGCCGCCTCGAGCCATGGG 3' were annealed and inserted into pHage 3.2 vector at *Apa*I/*Sal*I sites. The reaction was done by using T4 DNA ligase (400U/µl, NEB, USA) at 25°C for 1 hour. The pMOD1 vector was multiplied by transformation of *E. coli* DH5α F' and the phagemid was prepared using QIAprep miniprep extraction kit (QIAGEN, Germany) according to the manufacturer's protocol. The integrity of the pMOD1 was confirmed by DNA sequencing. The pMOD1 vector was used for the expression of scFv genes under the control of lacZ promoter. The vector had an ampicillin resistance selectable marker.

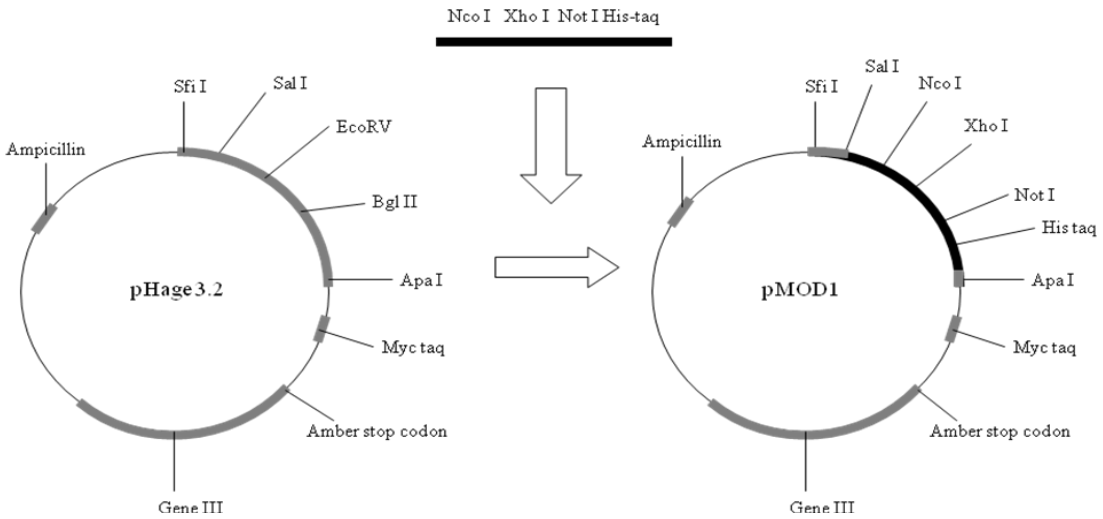


Figure 11. Construction of phagemid vector (pMOD1).

3.2.1.6 Cloning of scFv fragments into pMOD1 vector

The scFv fragments DNA were inserted into pMOD1 vector between *Sfi*I and *Not*I sites. The DNA of scFv fragments and pMOD1 vector was sequential digested with *Sfi*I (20U/µl, NEB, USA) and *Not*I (10U/µl, NEB, USA) enzymes, respectively, to generate compatible sticky ends. The digestion reactions of scFv fragments and pMOD1 vector were performed separately, each in a total volume of 500 µl. For the *Sfi*I digestions, the reaction mixtures consisted of 10 µg of insert DNA, 12 µg of vector DNA, 1x NEB buffer 2, 1 µg/ml BSA and 200U of *Sfi*I (20U/µl, NEB, USA). The reactions were incubated at 50°C for 16 hours. The *Sfi*I digested DNA was cleaned by Wizard clean up kit (Promega, USA) before the next digestion step. The *Not*I digestion mixtures consisted of 400 µl of purified *Sfi*I digested DNA, 1x NEB buffer 3, 1 µg/ml BSA and 100 U of *Not*I (10U/µl, NEB, USA). The reaction mixtures were incubated at 37°C for 16 hours. After the digestion, the *Sfi*I/*Not*I digested vector was dephosphorylated by adding 3 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37 ºC for 1 hour. After the dephosphorylation, the vector was heat

inactivated at 85ºC for 15 minutes. The inserts and vectors were separated from stuffer fragments by gel electrophoresis followed by Wizard clean up kit (Promega, USA). The scFv DNA was ligated into pMOD1 vectors at a 3:1 ratio. The total volume of ligation reaction was 200 µl, which consisted of 2.8 µg of inserted DNA, 5.5 µg of pMOD1 vector, 1x T4 DNA ligase buffer and 15 µl of T4 DNA ligase (400U/µl, NEB, USA). After 16 hours of incubation at 16ºC, the ligation reaction was concentrated to 40 µl by precipitating overnight with 3M sodium acetate pH5.2 plus absolute ethanol. The ligation reaction was then transformed into 600 µl of *E.coli* TG11 cells by electroporation. The reaction was done in two separate cuvettes by pipetting 20 µl of ligated sample into a 2 mm cuvette containing 300 µl of *E.coli* TG11 competent cells. The electroporation was performed at 2.5 kV, 25 µF, 200 Ω and τ approximately 4 msec using an electroporation machine (Eppendrof 2510, Eppendrof, USA). The cuvette was flushed immediately with 3 ml of SOC medium at room temperature, and the two separate transformation reactions were combined in 50 ml polypropylene tube. The 6 ml of combined transformed cells were incubated at 37ºC for 1 hour. After that the transformed cells were spread on eight 24x24 cm plates, containing TYE medium, 100 µg/ml ampicillin plus 1% glucose, and incubated overnight at 37ºC. The library size was quantified by spreading dilutions of the transformation reaction on separate plates. A volume of 100 µl from transformation reactions was taken and a four step 10-fold serial dilution was made. Then 100 µl of non-diluted and the four dilutions were plated on separate TYE agar plates containing 100 µg/ ml ampicillin and 1% glucose. The vector ligation control was performed in parallel to evaluate the background of library. The concentration of vector DNA in the ligation control was scaled down to 100 ng in a total volume of 10 µl. The large

plates of library were scraped into 20 ml of 2xYT with 20% glycerol and aliquoted into freezing vials. The glycerol stock of library was stored at -70ºC. To determine the diversity of the library, 20 of individual clones were picked and analyzed by DNA sequencing and *Bst*N1 digestion pattern.

3.2.2 Introducing diversity

3.2.2.1 *Phi*29 amplification

The percentage of the library diversity lost in the transformation step can be reduced by amplification of the circular DNA directly after the ligation reaction. The amplification was performed by using bacteriophage *Phi*29 polymerase. The principle is based on rolling circle replication and the formation of extended linear concatemers (Figure 12). Amplification was performed in a 200 µl volume using 175 ng of purified ligation reaction as a template and 10U of *Phi*29 polymerase (10U/ µl, NEB, USA). The reaction contained 1 mM dNTPs, 50 µM ramdom hexamer primer, 0.1 mg/ml BSA and 1x *Phi*29 polymerase buffer. The reaction was incubated overnight at 30ºC, and purified by Wizard SV Gel and PCR Clean-Up System (promega, USA). The purified concatemer was digested with *Not*I (NEB, USA) for 14 hours and purified using Wizard SV Gel and PCR Clean-Up System (promega, USA). Plasmids then were re-circularized by using 800U of T4 DNA ligase (400U/ µl, NEB, USA) in a total volume of 20 µl. After incubation at 25ºC for 2 hours, the ligation reaction was inactivated at 65ºC for 10 minutes and used to transformed 300 µl of *E.coli* TG11 competent cells by electroporation. The transformed cells were spread on a TYE agar

plate containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plate was incubated at 37ºC for overnight.

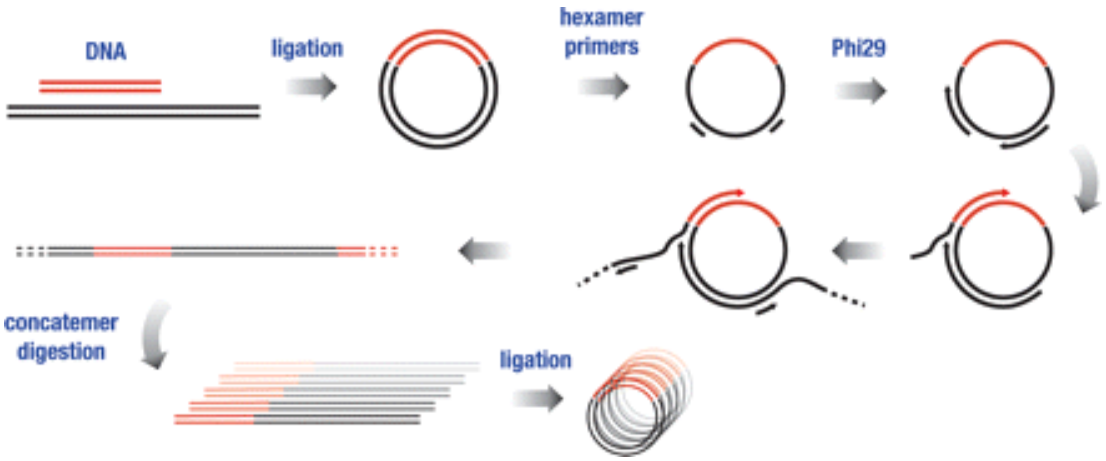


Figure 12. Amplification of circular DNA from the ligation reaction by using bacteriophage *Phi*29 polymerase. The DNA fragments are joined into circular DNA by ligation. Hexamer primers are annealed and *Phi*29 polymerase replicates the circular DNA through rolling-circle replication. This amplification leads to extended linear concatemers. The concatemers are cleaved by restriction digestion and re-circularized using DNA ligase.

3.2.3 Manipulation of library

3.2.3.1 Rescuing phagemid libraries

The library glycerol stock was diluted to an OD_{600} of 1.00, which represents approximately $8x10^8$ cells. Then 40 ml 2xYT containing 100 µg/ ml ampicillin and

1% (w/v) glucose was inoculated with 10 ml of diluted library stock. The OD_{600} of the initial culture was 1.227 which represents 6.14 x 10^8 cells. The culture was grown at 30ºC for 3 hours, until the cells got into mid-log phase, at which point the OD_{600} was about 0.56. The culture was infected with $2x10^{10}$ M13K07 helper phage (Maxim Biotech Inc, USA) to give a 1:1 ratio of bacteria : helper phage, and incubated at 37ºC for 1 hour. After that the culture was spun down at 3000×*g* for 10 minutes and resuspended in 500 ml 2xYT containing 100 µg/ ml ampicillin and 50 µg/ ml kanamycin in a 2 liter flask. The culture was incubated at 30ºC overnight with vigorous shaking. The phage particles expressing scFv antibody were harvest by centrifugation 3000×*g* for 30 minutes. The supernatant was mixed with 0.2 volumes of 20% (w/v) PEG/2.5 M NaCl solution. The mixture was chilled on ice for 1 hour, and then centrifuged at 3000×*g* for 10 minutes. The pellet was resuspended in 5 ml of 15% (v/v) glycerol in PBS. The phage library was dispensed into 500 µl aliquots in microcentrifuge tubes. To determine the phage titer, the PEG precipitated phage was diluted by making six 100-fold serial dilutions and adding 100 µl of diluted phage in to 900 µl of mid-log *E.coli* TG11. The infected *E.coli* TG11 cells were incubated at 37ºC for 30 minutes, and then plated on separate TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plates were incubated at 37ºC overnight.

3.2.3.2 Affinity selection of phage displayed scFv antibody (Bio-panning)

The phage antibody library was used for selecting the phage particles that specifically bind to immobilized antigen. The selection was done on Immuno 96 MicroWell™ Plates (Nunc, Denmark) with immobilized antigen. The immobilization conditions of each target protein were different with respect to buffer (PBS, 100 mM

$NaHCO_3$ pH 8.5), temperature (4ºC, 37ºC) and concentration. The microtiter well was treated with 50-1000 µg of target protein in 50 µl of PBS or 100 mM $NaHCO_3$, pH 8.5 buffer. The immobilizing was performed overnight at 4ºC or/and for 1-3 hour at 37ºC. The well was then blocked to avoid non-specific binding of phage particles with 2% (w/v) skimmed milk (2%MPBS) and incubated at room temperature for 1 hour. The blocking solution was poured off and the well was washed 3 times with PBS. Then 50 µl of 2%MPBS containing 10^{11}-10^{12} phages from the phage antibody library was added to the well and incubated at room temperature for 2 hours. The unbound phages were removed by washing the well with PBS containing 0.1% (v/v) tween 20 (PBST). The wash buffer was added to the well and the plate was vigorously rotated for 3 minutes. After shaking out the wash buffer and repeating this washing step for 10-15 times, the well was rinsed with PBS 10-15 times. The bound phages could be eluted by trypsinization or/and low pH condition using acidic elution buffer (50 mM glycine-HCl pH, 2.0). The trypsinization was performed by adding 50 µl of freshly prepared trypsin buffer (5 µl of 10mg/ml trypsin stock in 45 µl of PBS) to the well and leaving it for 10 minutes at room temperature. The low pH elution was performed by using 50 µl of 50 mM glycine-HCl pH, 2.0, to be elution buffer. After incubation at room temperature for 10 minutes, the acidic solution had to be neutralized by adding 50 µl of neutralization solution (200 mM $NaHPO_4$ pH 7.5). The recovered phages were amplified in *E.col*i TG1 cells by infecting 175 µl of mid-log phase *E.coli* TG1 at OD_{600} of 0.4 with 25 µl of eluted phages and incubating at 37ºC for 30 minutes. For the output titering, the eluted phages were diluted. Three 10-fold serial dilutions were performed. Then 100 µl of non-diluted and the three dilutions were separately spread

on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plates were incubated at 37ºC overnight.

3.2.3.3 Further rounds of selection

The infected *E.coli* TG1 colonies on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose were scraped and kept as 15% (v/v) glycerol stock at –70°C. Five µl of scraped bacteria was inoculated into 5 ml of 2xYT containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The culture was grown at 37ºC until the OD_{600} was 0.4, and then $5x10^{10}$ helper phages were added. The culture was incubated again at 37°C without shaking for 30 minutes. After spinning at 3000×*g* for 15 minutes, the supernatant was discarded and the pellet was resuspended in 5 ml of 2xYT containing 100 µg/ ml ampicillin, 50 µg/ ml kanamycin and 0.1% (w/v) glucose. The culture was incubated for at least 20 hours at 30°C with shaking. The next day, phage particles expressing scFv antibody were harvest by centrifugation (3000×*g* for 30 minutes). The supernatant was mixed with 0.2 volumes of 20% (w/v) PEG/2.5 M NaCl solution. The mixture was chilled on ice for 1 hour, and then centrifuged at 3000×*g* for 10 minutes. The pellet was resuspended in 200 µl of PBS. This precipitated phage was used for the next round of selection.

3.2.3.4 Monoclonal phage rescue

After selection, the single colonies of infected *E.coli* TG1 on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose were randomly picked and separately inoculated into each well of 96- well culture plate containing 100 µl of 2xYT with 100 µg/ ml ampicillin and 1% (w/v) glucose. The inocula were incubated

at 37°C with shaking overnight. The following day, 5 µl of overnight culture from each well was transferred to a second 96-well culture plate containing 100 µl of 2xYT with 100 µg/ ml ampicillin and 1% (w/v) glucose. The second culture plate was incubated at 37°C with shaking for 2-3 hours, until the culture started to get cloudy. Then 25 µl of 2xYT containing 100 µg/ ml ampicillin, 1% (w/v) glucose and 10^{10} KM13 helper phage (MRC HGMP Resource Centre, Cambridge, UK) was added to each well. The inocula were incubated at 37°C with shaking for 1 hour. Then the supernatant was aspirated off after centrifugation at 3000×*g* for 15 minutes. The bacterial pellet was resuspended in 150 µl of 2xYT, containing 100 µg/ ml ampicillin and 50 µg/ ml kanamycin. The cultures were incubated for at least 20 hours at 30°C with shaking. The culture supernatant containing the amplified phages was used in ELISA, to determine which individual clones bound to target protein. The original 96-well culture plate or master plate was treated by adding 95 µl of 40 % (v/v) glycerol to the remaining culture, to give a final concentration of 15%. The glycerol stock was stored at –70°C for later use.

3.2.3.5 Phage ELISA

Phage production from each round of selection was screened for binding by ELISA to identify monoclonal phage antibodies. The ELISA wells were coated with 5-600 µg of antigen in 50 µl PBS or 100 mM $NaHCO_3$ pH, 8.5. The coating was performed overnight at 4ºC or/and for 1-3 hour at 37ºC. The wells were rinsed 3 times with PBS. Non-specific binding was blocked by 2%MPBS at room temperature for 1 hour. Then the wells were rinsed 3 times with PBS. The 25 µl of supernatant from phage rescue of selected single colonies were added to the wells containing 25 µl of

4%MPBS. The binding was done at room temperature for 1-2 hours. After that, the wells were washed 3 times with PBST followed by 3 times of PBS. Secondary antibody was used for detecting the bound phages. A 1:5000 dilution of a mouse anti-M13 phage-horseradish peroxidase (HRP) conjugate (Amersham-Pharmacia Biotech, Sweden) in 50 µl of 2%MPBS was added into each well, and the plates were incubated at room temperature for 1 hour. The wells were washed 3 times with PBST followed by 3 times of PBS. The 50 µl of ABTS (2, 2-azino-di-3-ethyl-benzthiazoine-6-sulfonate) peroxidase substrate (Fluka, USA) was added, and the plates were incubated at room temperature for 20 minutes. The reaction was stopped with 50 µl of 1% (w/v) SDS. Detection was done by measuring the absorbance at 405 nm in an ELISA plate reader. The assay was performed in duplicate.

3.2.4 Expression and purification of scFv antibody

3.2.4.1 Soluble scFv antibody induction and expression

The soluble scFv antibody was expressed and secreted without pIII domain, by infecting to *E.coli* HB2151, which is non-suppressor bacterial strain. The 20 µl of phage particles of selected clones from the master glycerol stock, were used to infect 100 µl of mid-log phase *E.coli* HB2151 at an OD_{600} of 0.4, and incubated at 37ºC for 30 minutes. After that, the infected bacteria were streaked on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plates were then incubated at 37ºC overnight. The following day, single colonies were picked with toothpicks and used to inoculate 10 ml of 2xTY, containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The cultures were incubated with shaking at 30ºC overnight. Then

100 µl of each overnight culture was used to inoculate 10 ml 2xTY containing 100 µg/ml ampicillin plus 0.1% (w/v) glucose and incubated with shaking at 37ºC until OD_{600} reached 0.9. The induction was performed by adding 10 µl of 1 M isopropyl-β-d-thiogalactopyranoside (IPTG), achieving a final concentration of 1mM. The incubation was continued at 30ºC with shaking for further 6 hours or overnight. For overnight induction, the secreted antibody could be found in the supernatant, whereas the antibody fragments could be found in periplasm when a shorter incubation time was used.

3.2.4.2 Localization of expressed scFv antibody

The localization of expressed antibody in either the culture medium or a cellular compartment of *E.coli* HB2151 was determined by analyzing the different cellular fractions. *E.coli* HB2151 expressing antibody of the selected clone was grown and induced as described earlier. To facilitate localization of expressed scFv antibody, medium supernatant, periplasmic extract, lysed cells and inclusion bodies were prepared. The cells were harvested by centrifugation at 3000×*g* for 30 minutes. The supernatant was then collected. The periplasmic fraction was prepared by dissolving the cell pellet in 30 mM Tris-HCl, pH 8.0, with 20% (w/v) sucrose (80 ml per 1 g wet weight). After that 0.5 M EDTA, pH, 8.0 and phenylmethylsulfonyl fluoride (PMSF) were added and stirred slowly at room temperature for 10 minutes. The cells were collected by centrifugation at 10000×*g*, 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold 5 mM $MgSO_4$ or sterile water and slowly stirred at 4°C for 10 minutes. The mixture was centrifuged at 10000×*g*, 4°C for 10 minutes, after which a 1 ml of periplasmic fraction

was transferred from the supernatant. The remaining cell pellet was resuspended in ice-cold 20 mM Tris-HCl, pH 7.5 (2-5 ml per 1 g wet weight). The cells were lysed by adding 1 mg/ml lysozyme and then chilled on ice for 30 minutes. The lysed cells were then sonicated (Waken GE100 Ultrasonic processor, Japan) for a total of 1.5 minute (15x 3s, with 3s rest in between) at 30 amplitude. The lysate was then centrifuged at 12000×*g* for 20 minutes. The supernatant was transferred to a new microcentrifuge tube, for analysis of a cytosolic fraction. The pellet was kept to prepare the inclusion bodies fraction. The insoluble pellet containing the inclusion bodies was resuspended in 750 µl of 20 mM Tris-HCl, pH 7.5, and centrifuged at 10000×*g* for 5 minutes. The supernatant was discarded and the wash step repeated. The pellet from the final wash was resuspended in 1 ml 1% (w/v) SDS with heating and vigorous vortexing, after which a sample was kept as the inclusion bodies fraction. After preparation, all samples were analyzed under reducing conditions on a SDS polyacrylamide gel, consisting of 12 % running gel and 5 % stacking gel in SDS-PAGE electrophoresis buffer. The samples were boiled for 3 min before they were loaded onto the gel. The proteins were detected with Coomassie Brilliant Blue (CBB) staining solution for 15 minutes, followed by destaining with destaining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid). The gel was then dried using the Gel Drying System (Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific instruments, USA).

3.2.4.3 Optimization of expression

To optimize the expression, different IPTG concentrations were monitored and evaluated for the best quality and quantity of scFv antibody expression. The inductions were performed by varying IPTG ranging from 0-0.5 mM. The *E.coli* HB2151 cells carrying the phagemid encoding the scFv antibody were grown in 5 ml of 2xTY medium, containing 100 µg/ml ampicillin and 2% (w/v) glucose. After reaching an OD_{600} of 0.9, cells were harvested and grown for 6 hours at 30°C in glucose-free 2xTY medium containing 100 µg/ml ampicillin and an IPTG concentration ranging from 0-0.5 mM. The expression level of scFv antibody from each IPTG induction was evaluated and compared in medium supernatant, periplasmic extract, soluble protein fraction and inclusion body fraction by SDS-PAGE.

3.2.4.4 Large scale Production of soluble scFv antibody

After optimization of the expression, large scale production of scFv antibody from cell lysate was established. A colony of *E.coli* HB2151 carrying the phagemid encoding the scFv antibody, was inoculated into 5 ml of 2xTY medium containing 100 µg/ml of ampicillin and 2% (w/v) glucose. Then the culture was incubated overnight with shaking at 37°C. The following day, 1 ml of the overnight culture was transferred into 100 ml of 2xTY medium supplemented with 100 µg/ml of ampicillin and 2% (w/v) glucose. After shaking, the culture was incubated at 37°C until it reached an OD_{600} of 0.9. The *E.coli* cells were changed into 100 ml of fresh 2xTY medium containing 100 µg/ml of ampicillin and 0.1 mM IPTG. The culture was then incubated for 6 h at 30°C with shaking. Then the culture was harvested by

centrifugation at 3000×*g* for 30 minutes at 4°C, and all supernatant was discarded. The pellet was resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1mM PMSF, 10 mM imidazole; pH 8.0). To efficiently lyse the cells, lysozyme was added to a final concentration of 1 mg/ml, followed by 30 minutes of incubation on ice. The lysed cells were then sonicated on ice for a total of 10 minutes at amplitude of 30, and centrifuged at 12000×*g* for 30 minutes. The supernatant was kept for purification by Ni-NTA.

3.2.4.5 Purification of scFv antibody by immobilized metal affinity chromatography (IMAC) using Ni-NTA

ScFv antibody containing a hexahistidine tag was purified using Ni^{++} ions immobilized on resin by covalent linkage to nitrilotriacetic acid (NTA) (QIAGEN, Germany). The high affinity interaction between the histidine residues and the Ni^{++} ions allow the specific purification of histidine tagged recombinant proteins. The antibody fragments were eluted with imidazole, which competes for binding to the Ni^{++} ions. Prior to use of the Ni^{++} ions, the resin was equilibrated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Thus, the same pH and buffer composition with that of the Ni^{++} ions resin ensuring that the sample would bind properly. To allow the binding of hexahistidine tag to Ni-NTA resin, the antibody sample was incubated with the resin for 1 hour on an inverting platform at 4ºC to optimize binding. The mixture was centrifuged at low speed in a microcentrifuge for 15 seconds. The flow-through from the wash was collected for later analysis. The pellet was washed by resuspending it in wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0) and spun at low speed on a

microcentrifuge for 15 seconds. This wash step was repeated another two times. The wash fraction from each step was collected. The antibody was eluted by 1 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH8.0). After 10 minutes, the purified antibody was collected by centrifugation at low speed on a microcentrifuge for 15 seconds. The volume of initial culture was 100 ml but this protocol can be scaled up or down as appropriate. The elution fraction was washed with three sample volume of PBS containing 1 mM PMSF. Then the fraction was concentrated and imidazole was removed by a centriprep 10 filtration system, which has a MW cutoff of 10,000 daltons. To monitor antibody purity, SDS-PAGE electrophoresis was used. To determine the efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared by SDS polyacrylamide gel and western blot analysis. ELISA was used to detect the antibody binding.

3.2.4.6 Western blot analysis

The proteins on the SDS polyacrylamide gel were transferred to a PVDF membrane using a membrane transfer machine (Semi Phor, Hoefer Scientific Instruments, USA). After blocking the membrane with a 4% (w/v) skimmed milk or 1% (w/v) BSA in TBS containing 0.1% (v/v) tween 20 (TBST), the membrane was washed 3 times with TBST by rotating for 10 minutes. Mouse anti-myc (Invitrogen, USA) was used for detecting the myc tag on the scFv antibody. The membrane was incubated with mouse anti-myc in a dilution of 1:5000 in TBST for 1 hour, followed by 4 wash steps with TBST by rotating for 10 minutes. The goat anti-mouse conjugated HRP (Invitrogen, USA) diluted 1:5000 in TBST was added and membrane

incubated for 1 hour, before being developed. The 3,3',5-5'-Tetramethylbenzidine (TMB) substrate (Sigma, Germany) was used according to the manufacturers protocol for developing the color on the membrane.

3.2.4.7 ScFv antibody ELISA

The ability of scFv antibody to bind specifically to its antigen was determined by ELISA. The ELISA wells were coated with 5-600 µg of target protein in 50 µl PBS or 100 mM $NaHCO_3$, pH 8.5. The coating was performed overnight at 4ºC and/or for 1-3 hours at 37ºC. The wells were rinsed 3 times with PBS. Non-specific binding was blocked by 2% (w/v) MPBS or 3% (w/v) BSA at room temperature for 1 hour. Then the wells were rinsed 3 times with PBS. The 25 µl of soluble scFv antibody were added to the wells containing 25 µl of 4% (w/v) MPBS or 6% (w/v) BSA. The binding was done at room temperature for 1-2 hours. After that, the wells were washed 3 times with PBST followed by 3 times of washing with PBS. Secondary antibody against myc tag was used for detecting the bound scFv fragments. A 1:5000 dilution of mouse anti-myc antibody in 50 µl of 2% (w/v) MPBS or 3% (w/v) BSA was added to each well, and the plates were incubated at room temperature for 1 hour. The wells were washed 3 times with PBST followed by 3 times washing with PBS. Then, a 1:8000 dilution of goat anti-mouse conjugated HRP in 50 µl of 2% (w/v) MPBS or 3% (w/v) BSA was added to each well, and the plates were incubated at room temperature for 1 hour. The wells were washed 3 times with PBST followed by 3 times of PBS. The color of reaction was developed by adding 50 µl of ABTS substrate, and the plates were incubated at room temperature for 20 minutes. The reaction could be stopped with 50 µl of 1% (w/v) SDS. Detection was done by

measuring the absorbance at 405 nm in an ELISA plate reader. The assay was performed in duplicate.

3.2.4.8 Competition ELISA for anti Aflatoxin B1

Selection of antibodies from large repertoire phage display libraries has been shown to yields antibodies specific to certain haptens, but only when this hapten is conjugated to an immobilising molecule, such as BSA. To investigate the potential of the phage that expressed Aflatoxin antibody, for binding to the soluble Aflatoxin B1, a competition ELISA was performed. First, 10^{10} phages in PBS from the selected clones were pre-incubated with 0 to 5 µg of soluble Aflatoxin-B1 in a total volume of 100 µl. After incubation at 37ºC for 30 minutes, the competitor mixture was transferred to microtiter wells coated with 4 µg of BSA-conjugated Aflatoxin-B1 and incubated for further 1 hour. After that, unbound phages were washed away and the bound phages were detected as previously described. The assay was performed in duplicate.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Library construction

4.1.1 Construction of pMOD1 phagemid vector

Construction of phagemid expression vector pMOD1 was performed as represented in Figure 11. The new vector was based on the pHage 3.2 vector (Maxim Biotech Inc, USA) and modified to introduce hexahistidine residues and a new multiple cloning site. The *Sfi*I (5'-GGCCNNNN/NGGCC-3') and *Not*I (5'-GC/CGGCCGC-3') sites in the new multiple cloning site were used for cloning of scFv fragments into phagemid vector. The use of these specific restriction sites presents a unique advantage. The *Sfi*I and *Not*I are virtually never found in antibody sequences, and are very uncommon in most genes, thus eliminating internal digestion. Two different sticky ends were designed to allow cloning of scFv fragment in a directional manner (Hoogenboom et al., 1991; Orum et al., 1993). The palindromic sticky ends, 5' and 3' overhangs derived from *Sfi*I and *Not*I sites respectively, render impossible self dimerization by insert or vector. The pComb3 (Barbas et al., 1991), the commonly used phagemid vector (Kaplan et al., 1998; Bjorling et al., 1999; Kim et al., 2000), contains two couples of general enzyme cutting sites, where the heavy chain and light chain DNAs had to be sequentially cloned into the vector (*Xho*I and *Spe*I for heavy chain, *Sac*I and *Xba*I for light chain). This vector has limitations in the

cloning step and the stability of phagemid contained antibody genes (Burioni et al., 1997). Yang and his colleagues (Yang et al., 1995) constructed pComb3H to overcome the limitations of the original pComb3 vector, by using a single *sfi*I cloning step. To produce the soluble antibody derived from pComb3 and pComb3H vector requires the excision of gene III by restriction digestion, followed by self ligation of the remaining vector. Contrary to this, the pMOD1 vector incorporates an amber stop codon, which is placed between the myc-tag sequence and gene III leader sequence. This allows for the production of soluble antibody in non-suppressor host strains without excising the gene III fragment. The gene III leader sequence was fused to the N-terminus of gene III, so that the soluble antibody would be secreted into the periplasmic space (Kang et al., 1991). The fusion gene is under the control of a lacZ promoter. The pMOD1 vector also contained an ampicillin resistance gene, which permits growth and selection. Moreover, pMOD1 vector has a hexahistidine tag, directly fused with an scFv antibody, rendering purification by IMAC possible (Lindner et al., 1992). This hexahistidine tag also allows monitoring of full length scFv product formation by enzymes such as peroxidase or alkaline phosphatase. The nucleotide sequence of pMOD1 was determined using dideoxynucleotide chain-termination method (Sanger and Coulson, 1975). The sequence of new multiple cloning sites and the map of the novel phagemid pMOD1 are shown in Figure 13. The finished pMOD1 vector was then used successfully to clone the human scFv antibody genes.

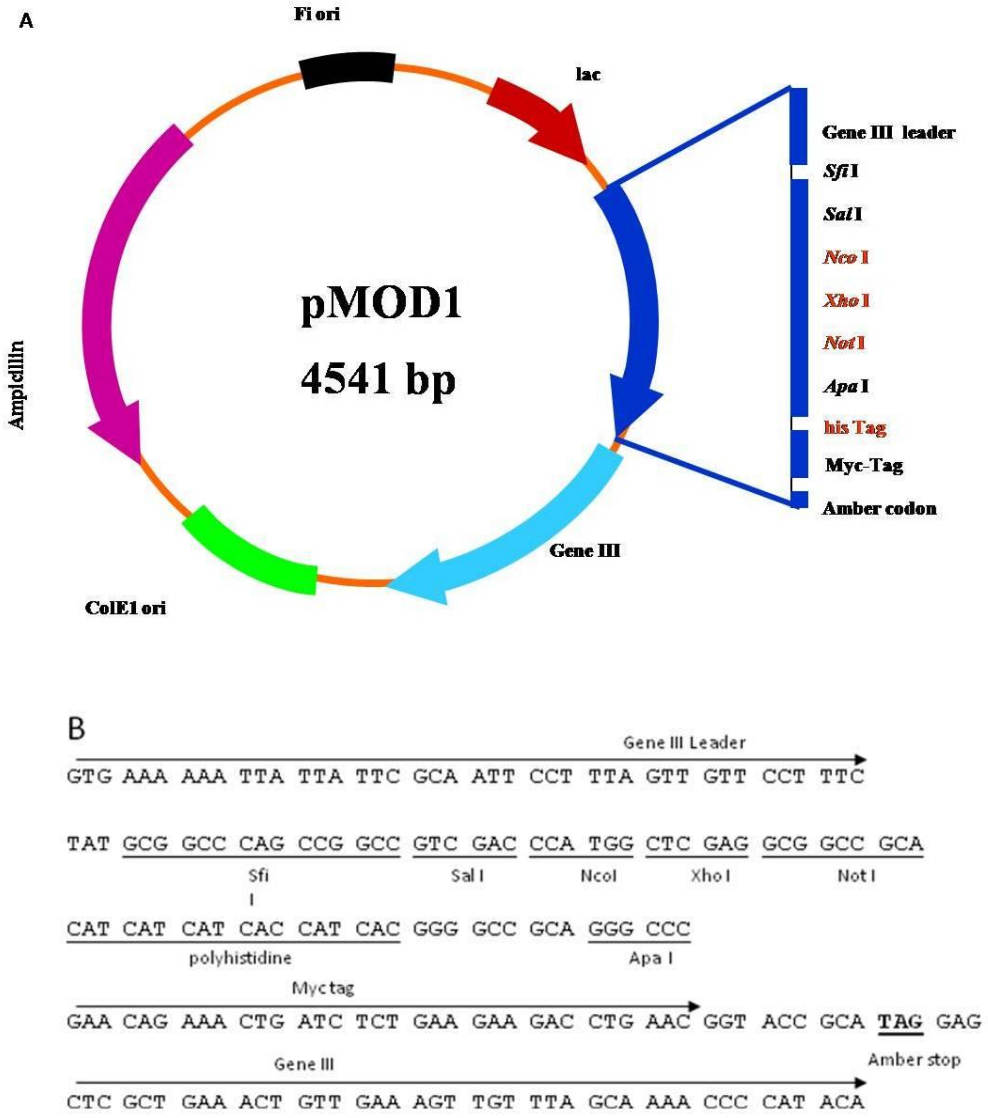


Figure 13. Map of pMOD1 phagemid vector. (A) The arrow shows the position of LacZ promoter, multiple cloning sites, gene III sequence and ampicillin resistance gene. (B) Sequence of the multiple cloning site of pMOD1.

4.1.2 RNA extraction and cDNA synthesis

To make the library as diverse as possible, the variable region genes were isolated from 140 non-immunized donors. The total RNA was prepared from whole peripheral blood samples. The RNA extracts were of high purity, free of DNA contamination (Figure 14) and allowed production of first strand cDNA by the use of MMuLV reverse transcriptase (NEB, USA) and a mix of oligo-dT_{18} and random hexamers primers. The primers used for amplification allowed for representation of all five antibody classes, whereas in previous studies (Marks et al., 1991; de Kruif et al., 1995a), antibody repertoires have been constructed using germline V_H IgG or IgM specific primers for generating cDNA.

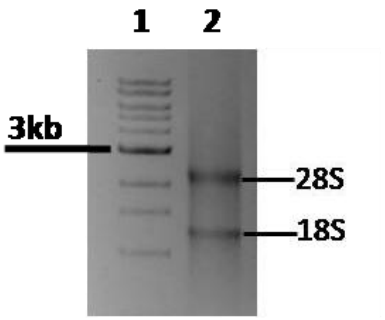


Figure 14. The total RNA sample showed discreet 18S and 28S ribosomal RNAs, which were approximately 1.3 kb and 2.6 kb, respectively, on 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide.

4.1.3 Amplifying and reassembling heavy and light chain sequences

In order to reduce amplification biases, we performed 75 independent PCRs, using all possible combinations within a designed primer set (Table 4) that encompasses the entire theoretical set of whole repertoires of human antibody genes. Six V_H forward primers paired with four V_H reverse primers, generating a total of twenty-four variants, six V_κ forward primers paired with five V_κ reverse primers generating a total thirty variants, and seven V_λ forward primers paired with three V_λ reverse primers generating a total twenty-one variants. The PCRs led to the representation in the repertoire of variable regions deriving from all conceivable framework assemblies. The V_κ amplification resulted in a sufficient amount of product, giving a sharp band on an argarose gel at the predicted size of approximately 350-400 bp. The V_H and V_λ genes amplification, however, did not result in comparable high yields (Figure 15).

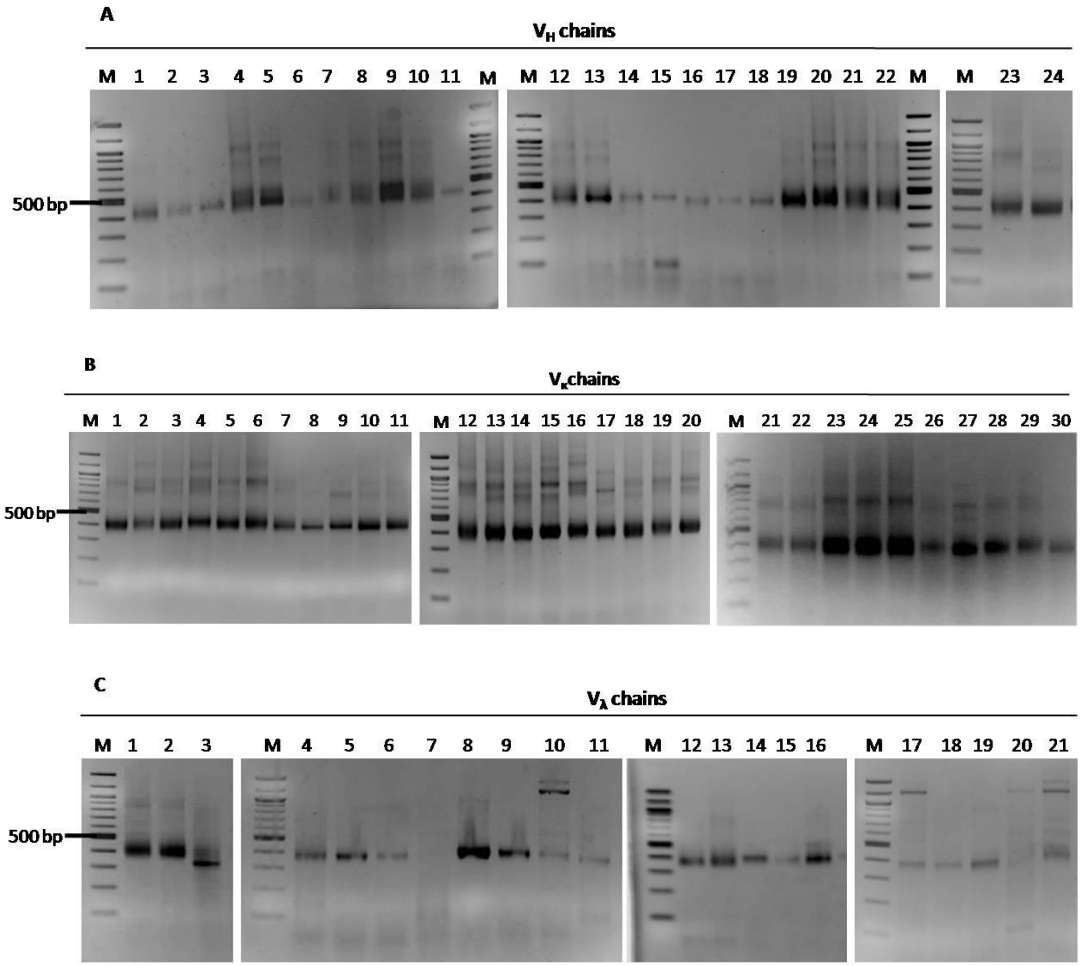


Figure 15. Agarose gel analysis of heavy and light chain DNA from first step PCR, using all combination of the primers within the primer sets. (A), Lane M, DNA markers (100 bp ladder); lane 1-24, PCR products of V_H DNA from 24 combination of six V_H forward primers paired with four V_H reverse primers. (B), Lane M, DNA markers (100 bp ladder); lane 1-30, PCR products of V_κ DNA from 30 combination of six V_κ forward primers paired with five V_κ reverse primers. (C), Lane M, DNA markers (100 bp ladder); lane 1-21, PCR products of V_λ DNA from 21 combination of seven V_λ forward primers paired with three V_λ reverse primers.

The bands of expected products that were not clearly visible in the agarose gel indicated that there were insufficient products for the next step. To overcome the problem, freshly prepared cDNA template and hot start PCR was tried, to improve yield, sensitivity, and specificity of PCR reaction. In hot start PCR, *Taq* polymerase which is an essential reaction component, was not added until the first denaturing step. The hot start PCR amplification yielded shaper bands of approximately 400-450 bp for V_H genes and 350-400 bp for V_λ genes (Figure 16). It should be noted that some variable genes were difficult to amplify (Figure 16A, lane 11; Figure 16B, lane 18 and 21), yielding significantly less product. They might be present in a too low number or they might be single copy genes. The result suggests that PCR amplification using the specified primers yields sufficient amounts of V_H, V_κ and V_λ gene DNA, which means these primers produce sufficient diversity for constructing a human scFv library.

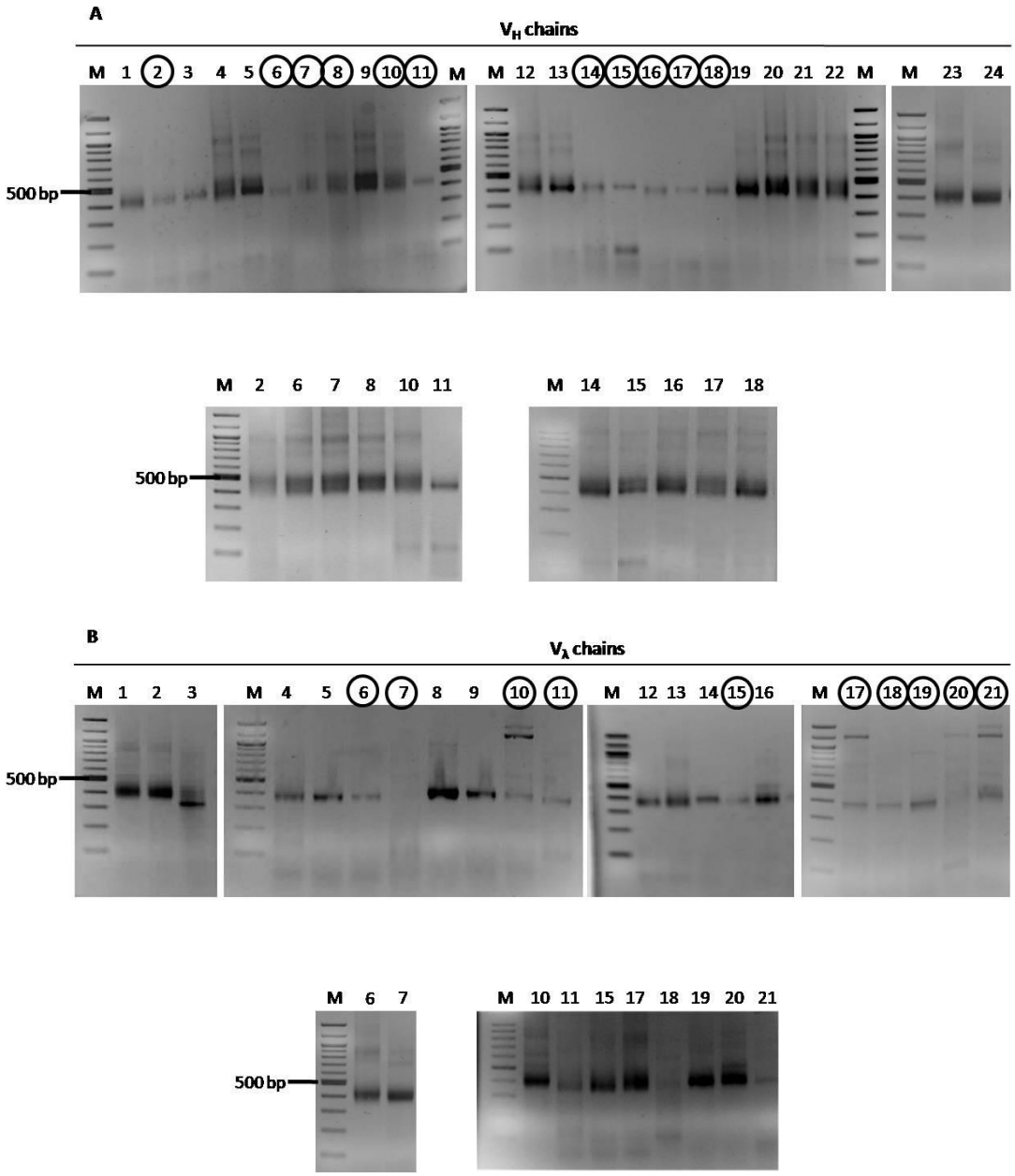


Figure 16. Agarose gel analysis of hot start PCR products of V_H genes (A) and V_λ genes (B).

In order to create scFv fragment genes of a V_H-linker-V_L type, the separate V_H and V_L genes were converted to a scFv gene by inserting a linker DNA sequence. The assembly of V_H, V_L, and linker fragment was carried out by PCR. The 3′ ends of V_H gene complementary to 5′ ends of linker sequence and the 3′ ends of this linker DNA

was complementary to 5′ ends of V_L gene (Clackson et al., 1991; Marks et al., 1991). In contrast to previous studies, the 3′ ends of V_H fragment genes were annealed to complementary 5′ ends of V_L fragment genes through a $(Gly_4Ser)_3$ linker peptide sequence, then V_H and V_L genes were assembled and amplified by overlap extension PCR. Linker was generated by the listed primers, (Table 4) modified from previous work (Okamoto et al., 2004) which constructed a non-immune mouse scFv library. This modification simplified the assembly step, by reducing the number of fragments involved from three to two. Moreover, to avoid the uncorrected overlaps during assembly PCR, the three $(Gly_4Ser)_3$ repeats in the single chain linker region were encoded by different codons (5' GGTGGTGGTGGCAGCGGCGGCGGCGGCTCT GGTGGTGGTGGATCC 3'). The linker used in this study was also different from commonly used linker (5' GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCG GTGGC GGATCG 3') described by Huston (Huston et al., 1988).

The generation of scFv fragments required the high concentration of V_H and V_L genes to drive the hybridization. The equimolar amounts of V_H and V_L genes were employed to create the full length scFv genes of approximately 800 bp (Figure 17).

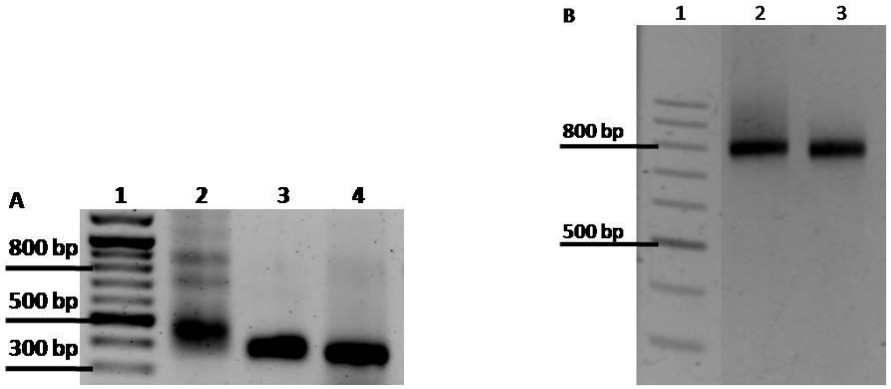


Figure 17. Agarose gel analysis of pooled PCR products of variable regions of heavy and light chains (A). Lane 1, DNA marker (100 bp ladder); lane 2 pooled V_H; lane 3, pooled V_κ; lane 4, pooled V_λ. The scFv products from Third step PCR (B). Lane 1, DNA marker (100 bp ladder); lane 2, pooled V_H-linker-V_κ products; lane 3, pooled V_H-linker-V_λ products.

4.1.4 Cloning of scFv fragments into pMOD1 vectors

Once full length scFvs had been prepared, they were ligated to pMOD1 vectors at *Sfi*I and *Not*I sites. To ensure the efficiency of cloning, importance was placed on allowing for extensive digestion. A small amount of undigested vector would lead to a large background of non-recombinant clones. The digested products of scFv fragments and pMOD1 vector were assessed on agarose gel (Figure 18).

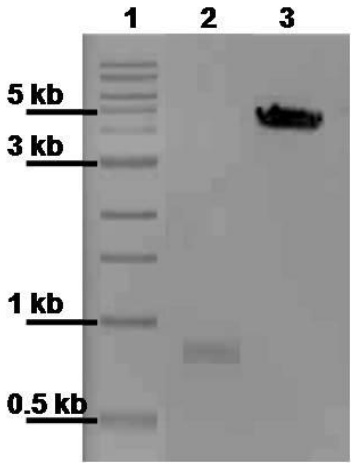


Figure 18. Agarose gel analysis of *Sfi*I and *Not*I digestion products. Lane 1, 1 kb DNA ladder; lane 2, scFv fragments; lane 3, pMOD1 vector.

A pilot experiment was set up before the large scale construction. The two ligation reactions containing a total of 2.1 µg of DNA were performed. One of the ligation samples was directly electroporated in to *E.coli* TG1, while the other one was amplified by *Phi*29 polymerase before electroporation. The library sizes of 1×10^7 and 3×10^4 were obtained from non *Phi*29 amplified and *Phi*29 amplified samples, respectively. Compared to previously reported results (Christ et al., 2006), the number of transformants achieved after the Phi29 amplification was lower by 10^3 fold. The reason may be due to the different set up of the *Phi*29 amplification step. The previous work was performed using a 500 µl starting volume. This reaction volume was doubled every 3 hours, with the reaction mixture reaching a final volume of 8 ml, whereas no additional volume was added in this study. During the incubation time, the available components in the reaction mixture were consumed and converted into copies of concatemer DNA. The apparently insufficient components hampered the efficiency of the *Phi*29 amplification.

A total of 8.3 µg of DNA was electroporated in to *E.coli* TG1. The background was determined in parallel using 100 ng of digested pMOD1 vector. The final scFv library, with a diversity determined to be 1.5×10^8 and with 0.04% of background, was successfully constructed. A larger library size of $1.2x10^9$ (Grabulovski et al., 2007), was obtained using 85 µg of DNA. In general it can be said that the higher the amount of DNA used, the larger a library size can be obtained. However, for a naïve antibody library, having a diversity of 1.5×10^8 is sufficient to encompass the whole antibody repertoire (~10^6-10^8) present in the human immune system at any one time.

4.2 Diversity analysis of antibody fragments

Since the aim of this study has been to amplify as many different variable regions as possible, the sequence diversity was confirmed in both the primary library and the binders retrieved after antigen selection. In order to analyze the diversity of scFv repertoire and the quality of the primary library, DNA segments encoding the scFv genes from fifteen randomly picked clones were examined. The phagemid DNA of these clones was digested with *Bst*NI, and their fingerprint patterns were compared. Thirteen different patterns were identified, showing all examined clones to be unique (Figure 19). The variation in patterns indicated the framework diversity in scFv phage library.

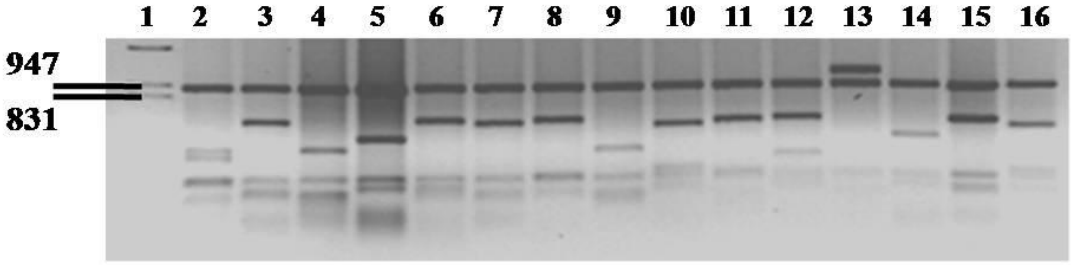


Figure 19. Fingerprint analysis of primary library. DNA of the fifteen scFv clones was digested with *Bst*N1 at 60°C for 3 hours. The restriction patterns were analyzed on 1% agarose gel. . Lane 1, DNA marker (100 bp ladder); lane 2-16, randomly picked clone 1-15 from primary library, respectively.

The variable regions of ten random clones were also sequenced. After sequencing, the origin of the V gene and complementarity determining region 3 (CDR3) lengths were determined using the V BASE immunoglobulin V gene database (Tomlinson et al., 1996) and IgBLAST program. DNA sequencing data indicate that variable regions derived from thirteen different V gene families, belonging to six V_H gene families (VH1, VH2, VH3, VH4, VH5, and VH6) and seven V_L gene families (Vκ2, Vκ3, Vκ4 and Vλ2, Vλ3, Vλ5, Vλ6). The variable regions derived from VH1, VH3, VH4, Vκ1, Vκ3, Vλ1, Vλ6 families have been commonly observed among antibody fragments from phage display libraries (Sheets et al., 1998; Loset et al., 2005), and human hybridomas (Ohlin and Borrebaeck, 1996). Besides variable regions derived from these gene families, sequencing results identified others from the less frequently used, VH2, VH5, VH6, Vκ2, Vκ4, Vλ2, Vλ3 and Vλ5 families. The library was thus diverse, with almost all families represented.

Table 5. Amino acids sequences of primary library clones.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VH									
1	QVQLQQSGPGLLKPSQTL SLTCAISGDSVS	SKGPAWN	WIRQSPSRGLEWLG	RTYYWSGWRHDY APSLQS	RITINPDTSKNQFSLQLN SVTPDDTAVYYCAK	GRDSGFDI	IGHV6-1*01 DP74	12	VH3
2	VQLVESGGGLVKPGESLR LSCEASGFA FS	SYNMN	WLRQAPGKGLEWVS	SMSPSGRDIFYPES LKG	RFTASRDNARNSLYLQ MNSLRVEDTAMYYCVR	STLLEIAGKSPGLD M	IGHV3-21*01 DP77	20	VH3
3	QVNLRESGPTQVKPTQAL TLTCTVSGVSLS	TSGVGVG	WLRQAPGKGLEWL A	LLYWDDDTRYNP SLKT	RLAITRGSSRDQVVLTV TNVDPSDTGTYFCAH	RRSGLLVFGIRDAF DI	IGHV2-5*09 S12-2	24	VH2
4	VQLQQSGAEVKKPGSSV KVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQK FQG	RVTITADESTSTAYMEL SSLRSEDTAVYYCAR	VRDTAMEFFDY	IGHV1-69*12 DP10	2	VH1
5	QVQLQQSGPGLVKPSETL SLTCIVSGGSIT	SYSWS	WTRQPAGQGLEWIG	RIYNGGSTNYNPS LKS	RVTMSLDTSKNQFSLRL SSVTAADTAVYYCAR	GPYGTGSQCHV FDP	IGHV4-4*07 VIV4	2	VH4
6	QVQMQESGAGLLNPLET LSLTCTVYGGSVS	NHYYYGS	RIRQPPGKGQEWFA	YIHYTETTNYNPS LKS	RVTISVDTSKNQFSLRLS SVTAADTAVYYC AR	VDILSGMRHF	IGHV4-61*08 DP66	19	VH4
7	EVQLVESGAEVRRPGESL TISCRGSGDTFP	KHWIA	WVRQMPGKGLEWLG	RIDPSDSYTNYNPS FQG	HVSISADQSISTVYLQW SSPKASDTALYYCAR	LTCRTTSCYTDNWS DA	IGHV5-a*01 VH32	17	VH5
8	QVQLQQSGPGLVKPSQTL SLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG	RTYYRSKWYNEY ALSVKS	RLTINPDTSKNQFSLILN SVTPEDSAVYYCAT	WRFDY	IGHV6-1*01 DP74	5	VH6
9	EVQLVESGGGLVQPGGSL RLSCAASGFTFS	SYEMN	WVRQAPGKGLEWVS	YISSSGSTIYYADS VKG	RFTISRDNAKNSLYLQM NSLRAEDTAVYYCAR	VSLDTAGDAFDI	IGHV3-48*03 DP58	0	VH3
10	VQLVQSGGGLVQPGGSL RLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYAD SVKG	RFTISRDNSKNTLYLQM NSLRAEDTAVYYCAK	DNTEMVRGVMLY YY YYMDV	IGHV3-23*04 DP47	1	VH3

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VL									
1	DIQMTQSPDSLAVSLGER ATINC	KSSQSVLFSSNN KHHLA	WYQQKRGLPPKLLIF	WASTRES	GVPDRFSGSGSGTDFTL TISSLQAEDVAVYYC	QQYQSIPHT	IGKV4-1*01 DPK24	9	VK4
2	SSELTQDPAVSVALGQTV RITC	QGDSLRTYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLT ITGAQAEDEADYYC	NSRDSSDNHVV	IGLV3-19*01 DPL16	2	VL3
3	EIVLTQSPGTVSLSPGQRV TLSC	RASQSVRGSFF A	WYQQKPGQAPRLL IH	GASSRAT	GIPDRFDGSGSGTDFTLS ISRLETEDFA VYYC	QQYGTSPYT	IGKV3-20*01 DPK22	12	VK3
4	QSALTQPASASGSPGQSV TISC	TGTISDIGAHDL VS	WYQQPPGKAPKLIIF	EVSKRAS	GVPDRFSGSKSGNAASL TISGLQADDEADYFC	CSSTNKNNFAVE	IGLV2-8*01 V1-2	19	VL2
5	QAVLTQPSSLSASPGSSAS LTC	TLRSDFDVRSY RIY	WYQQKPGSPPQYLLR	FKSDSEKHRGS	GVPSRFSGSKDASANAG ILLISGLQFDDEADYYC	MIYYNMASE	IGLV5-45*03 V4-2	16	VL5
6	EIVLTQSPLYLPVTPGEPA SISC	RSSQSLLHSNG YNYLD	WYVQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTL EISRVEAEDVGVYYC	MQALQTPK	IGKV2D-28*01 DPK15	5	VK2
7	QAVLTQPSSLSASPGASA SLTC	TLRSGIDVAAY RIY	WYQQKPGSPPQYLLR	YKSDSDKQQGS	GVPSRFSGSKDASANAG ILLISGLQSQDEADYYC	AIWHNSAWV	IGLV5-45*03 V4-2	6	VL5
8	LTQPPSVSVSAGQTVSITC	SGENLQKIYVS	WYQQKPGQSPILVIY	KDNKRPS	GIPERFSGSNSGNTATLT VSGA LAADEAEYYC	QTWDIDTAL	IGLV3-1*01 DPL23	23	VL3
9	SSELTQDPAVSVALGQTV TITC	QGESLRNYFAS	WYQQKPGQAPILVMY	DEDIRPS	GIPDRFSGSSSEKTASLTI TGVQA EDEATYYC	KCRGSGGDHLEIL	IGLV3-19*01 DPL16	19	VL3

4.3 Selection and analysis of high-affinity clones

The scFv antibody phage library of a 1.5×10^8 repertoire yielded binders to seven different model antigens [BSA, GST, Amylase, Aflatoxin B1, Cobra snake venom, attenuated rabies viruses, cholangiocarcinoma (KKU-100) whole cells]. One or two rounds of selection were performed on immobilized antigens (Table 6).

Table 6. Number of unique scFv derived from the selection rounds.

Antigens	Rounds of panning	Number of clones after 1st round	Number of binding phage after 1st round	Number of binding phage after 2nd round	Number of scFv producing clones	Number of different scFv producing clones
Aflatoxin B1	1	$2.5x10^2$	4/56	-	3/4	3/3
Attenuated Rabies viruses	2	$1.2x10^3$	1/96	7/96	8/8	2/8
Amylase	1	8.8x10	3/88	-	3/3	NA
BSA	1	$2.0x10^3$	6/96	-	NA	NA
Cobra snake venom	2	$1.4x10^4$	1/96	9/96	3/3	1/10
Cholangiocarcinoma KKU-100 cell	1	$2.2x10^3$	2/96	-	NA	2/2
Green pit viper snake venom	2	$5.1x10^4$	0/96	0/96	NA	NA
GST	1	$4.3x10^2$	6/28	-	NA	NA

Note: the number of identified clones / the number of screened clones. NA is an abbreviation for not applicable.

After each selection, individual clones from each antigen selection round were randomly picked and their binding to their antigen was tested by phage ELISA. The phages were scored as positive by ELISA, if the absorbance on antigen coated wells was at least two times higher than binding of phage to 2% (w/v) MPBS coated-wells. According to these criteria, nine hundred and forty clones from all selections were

examined and forty-nine clones among these were positive (Figure 20). The results indicate that these forty-nine positive clones specifically bound to their antigen. The number of different antibody fragments selected on each antigen, was in the same range as the corresponding numbers from selections made with other naïve scFv phage display libraries (Vaughan et al., 1996; Sheets et al., 1998), even though those respective libraries are 93 and 44 times larger than this scFv library.

The results in Table 6 show that one round of selection gave a significant number of specific binders from each antigen selection, while the phage output was below 10^4 cfu/ml. However, a second round of selection increased the number of specific binders from the selection of attenuated rabies virus and crude cobra snake venom.

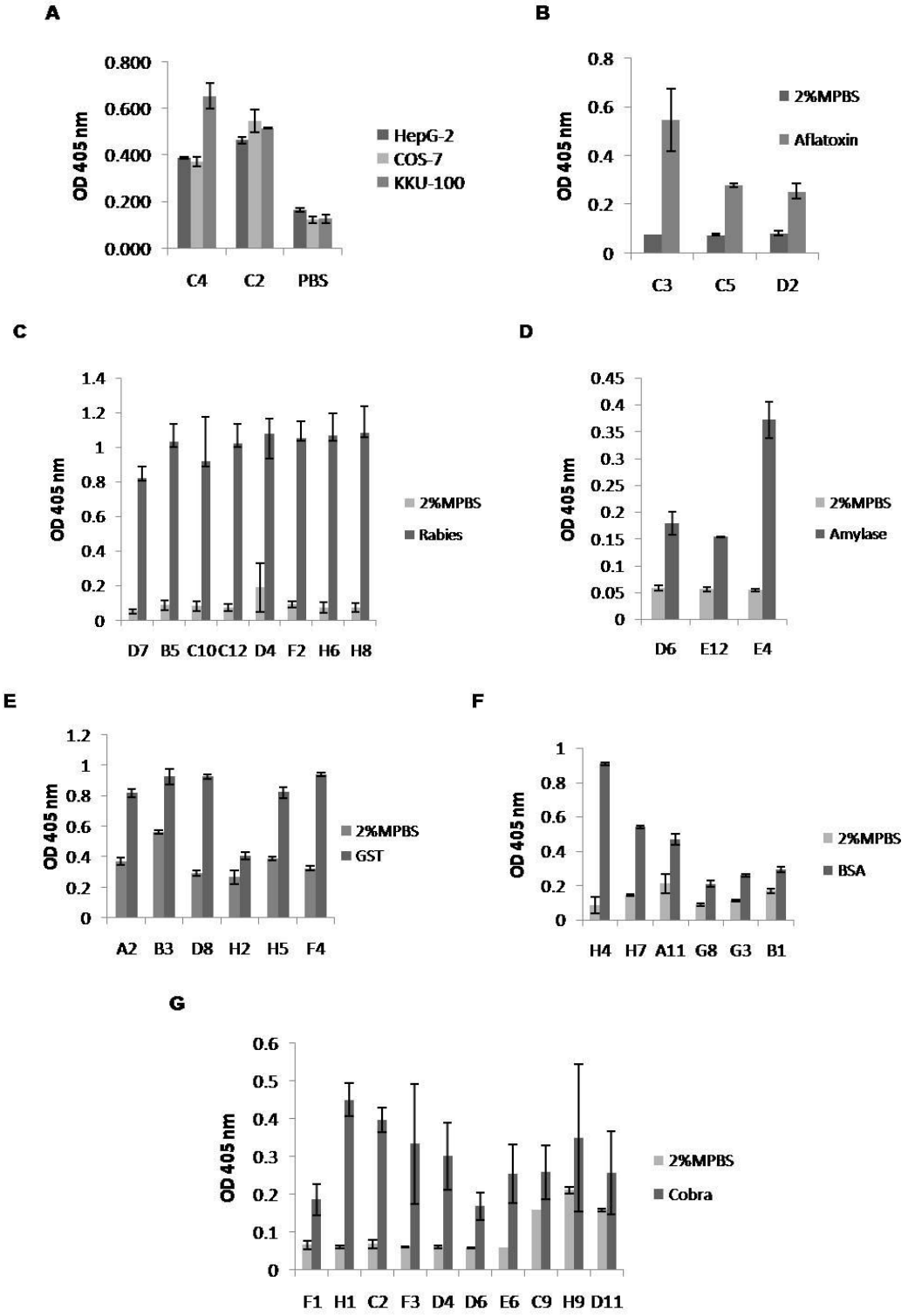


Figure 20. Binding of phage to seven antigen models; (A) Cholangiocarcinoma KKU-100 cells; (B) Aflatoxin B1; (C) Attenuated Rabies virures; (D) Amylase; (E) GST; (F) BSA; (G) Cobra snake (*Naja kaouthia*) venom.

The expressed scFv antibody phage particles against Cholangiocarcinoma KKU-100 cells and cobra snake (*Naja kaouthia*) venom were chosen for further confirmation of specificity (Figure 21). Phage displayed anti-Cholangiocarcinoma KKU-100 clone C4 specifically bound to KKU-100, with an absorbance value in ELISA two times higher than those of COS-7 and HepG-2 cells. A similar observation was made in phages displaying anti-cobra snake (*Naja kaouthia*) venom. All ten expressed phages specifically bound to cobra snake venom, giving values two to six times of the ELISA signal relative to the green pit viper snake (*Trimeresurus albolabris*) venom.

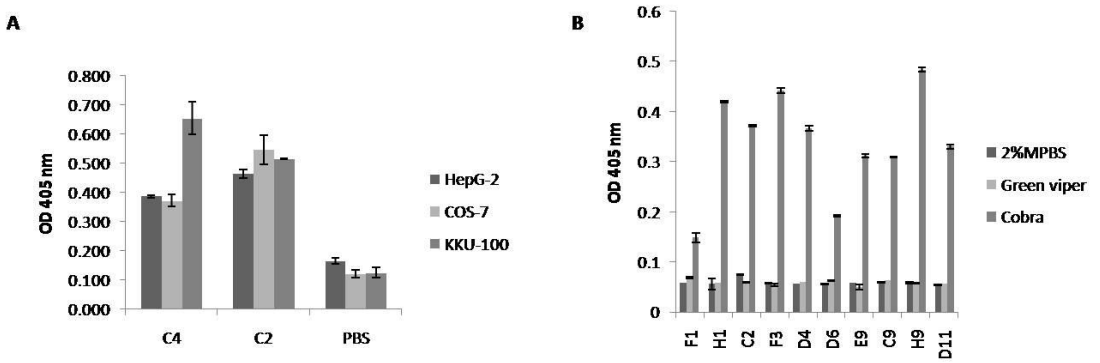


Figure 21. Cross reaction analysis of phages displaying anti- Cholangiocarcinoma KKU-100 cells (A) and phages displaying anti-cobra snake (*Naja kaouthia*) venom (B).

To characterize the ELISA positive clones, twenty-three representative clones from a total of forty-nine ELISA positive clones (Figure 20) were analyzed by sequencing. The DNA sequencing data revealed that all ten clones of phages displaying anti-cobra (*Naja kaouthia*) snake venom were identical. The seven clones of phages displaying anti-Rabies virus, D7, C10, C12, D4, F2, H6, and H8, were

identical, whereas the anti-Rabies virus clone B5 was unique. The three clones of phages displaying anti-Aflatoxin B1 and two clones displaying anti-Cholangiocarcinoma KKU-100 were all unique in nucleotide and amino acid sequences. However, the binding signal results for all ten clones varied. The reason may be due to differences in the expression level of antibody in each clone, even though the phagemids were identical (Figure 21B). The details of seven unique clones from a total of twenty-three clones are described in Table 7. The sequencing results of phage displaying anti-Rabies virus and anti-cobra snake (*Naja kaouthia*) venom suggest, that for the use of this naïve scFv antibody library, one round of bio-panning is enough, in case binders of diverse characteristics are desired. Increasing the number of panning rounds affected the yield of weaker binders, the phages displaying the strongest binders usually dominate in final output of the second panning round. The weaker binders, which may express unique biological activities, are being lost during the repeated bio-pannings and may not be recoverable (Bradbury and Marks, 2004).

The primary library had a prevalence of V_H genes from VH3 family, and V_L genes from Vλ3 family. However, the V_H and V_L genes found in antibodies recognizing selected antigens appeared to be more widely distributed, including VH1, VH3, VH4, and Vκ1, Vλ1, Vλ2, Vλ3, Vλ6, respectively (Table 7). The CDR3 of the binders had a distribution of lengths ranging between 6 to 19 amino acids for the V_H genes, and 9 to 11 for the V_L genes. None of these clones shared the same CDR3 sequence even when the genes came from the same germ line segments (Table 7). The number of different amino acids from germ line of the six selected clones varied from 1-16 amino acids, as seen in primary library. This result suggested that both germ line B genes and antibodies from secondary immune responses were selected from the bio-

panning. Moreover, of the eight binding clones, six (Aflatoxin C3, C5, D2; Rabies D7, B5; Cobra D11) were picked for DNA fingerprint analysis, and all these showed a unique pattern on the gel (Figure 22).

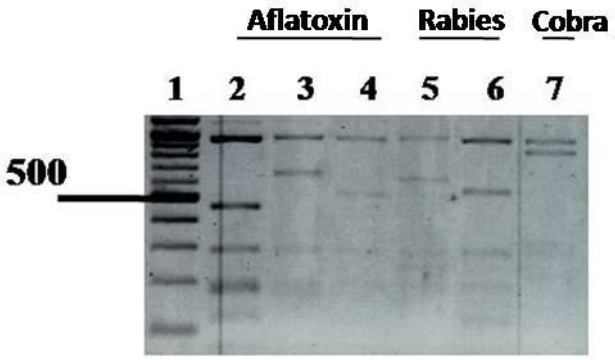


Figure 22. DNA fingerpring patterns of scFv genes from the selections against Aflatoxin B1, Cobra snake (*Naja kaouthia*) venom and attenuated Rabies viruses. The *Bst*NI digest of scFv genes identified six unique clones, out of the six clones tested. Lane 1, DNA marker (100 bp ladder); lane 2 Aflatoxin B1 clone C3; lane 3, Aflatoxin B1 clone C5; lane 4, Aflatoxin B1 clone D2; lane 5, Rabies clone D7; lane 6, Rabies clone B5; lane 7, Cobra snake (*Naja kaouthia*) clone D11.

Table 7. Amino acids sequences of antibody fragments selected against four model antigens.

Clone	Family	CDR3	Germline	Amino acids differences from germline
Aflatoxin C3	VH1	ADDYGSGSYGFDY	IGHV1-3*01(DP25)	1
Aflatoxin C5	VH3	SRVGLWGPRYYYYYGMDVW	IGHV3-23*04(DP47)	4
Aflatoxin D2	VH1	GGPLDY	IGHV1-45*02(DP4)	5
Rabies D7	VH1	GGNFDY	IGHV1-18*01(DP14)	2
Rabies B5	VH3	GYATFDY	IGHV3-23*01(DP47)	7
Cobra D11	VH4	HGRDTSGYTMDYFDS	IGHV4-59*07(H4)	14
Cholangiocarcinoma C2	VH3	DRGKYPGDGMGV	IGHV3-23*01(DP47)	6
Aflatoxin C3	VK1	QQSYSTPYA	IGKV1D-39*01(DPK9)	4
Aflatoxin C5	VL3	QVWDRDSRTIV	IGLV3-9*01(D2-6)	16
Aflatoxin D2	VL2	SSYAGSNNLV	IGLV2-8*01(D1-2)	3
Rabies D7	VL1	AAWDDSLSGPV	IGLV1-47*01(DPL3)	2
Rabies B5	VK1	QQYSYNPYT	IGKV1D-39*01(DPK9)	1
Cobra D11	VL6	QSYDSSNRV	IGLV6-57*01(V1-22)	5
Cholangiocarcinoma C2	VL1	AAWDDSLNGYV	IGLV1-44*01(DPL2)	2

Note. CDR3 (complementarity determining region 3) for both heavy and light chains were assigned by IgBLAST program.

Analysis of germ line family was done by using Ig BLAST, and V BASE immunoglobulin V gene database (shown in red font).

Three of the anti-Aflatoxin B1 selected phages, which specifically bound to BSA- conjugated Aflatoxin B1, (Figure 19B) were analyzed with regard to their specificity and affinity of binding against soluble Aflatoxin B1 by competitive ELISA (Figure 23). In a dose-response curve, 625 ng of soluble Aflatoxin B1 was found to displace 50% of the 10^{10} phages displaying anti-Aflatoxin B1 clone C3. The linear range of the inhibition curve was between 78 ng and 5000 ng of soluble Aflatoxin B1. On the other hand, neither of the phages clone C5 nor D2, displaying anti-Aflatoxin B1, were inhibited by soluble Aflatoxin B1. Moghaddam and his colleagues (Moghaddam et al., 2001b) demonstrated similar results from their phage selection against Aflatoxin B1. In their study, the eight scFv antibodies that specifically bound to BSA-conjugated Aflatoxin B1 and soluble Aflatoxin B1 were isolated from a human lymphocyte antibody library and a semi-synthetic antibody library after four rounds of panning.

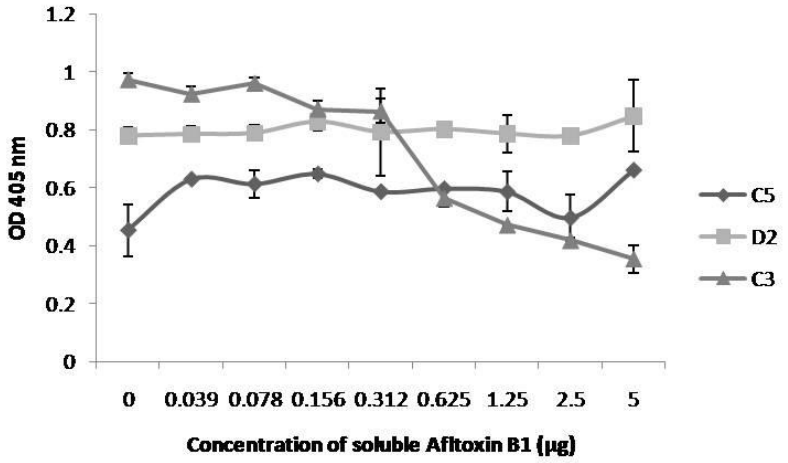


Figure 23. Specificity of binding of anti-Aflatoxin B1 expressed phages were determined by competitive ELISA. The binding of anti-Aflatoxin B1 expressing phages was reduced after treatment with varying concentration of soluble Aflatoxin B1.

4.4 Production of soluble antibody fragments

The pMOD1 phagemid vectors with their respective scFv genes were expressed in *E. coli* HB2151, which is non-suppressor strain. This expression host recognizes the amber stop codon (TAG) upstream of the gene III sequence in the pMOD1 vector. The amber codon is read as stop codon and only soluble ScFv antibody is produced. The scFv-gene III fushion is under the control of the LacZ promoter. The scFv antibody production is initiated by addition of IPTG, in the absence of helper phage. Six selected scFv antibody clones, the binders of Aflatoxin B1, Cobra snake (*Naja kaouthia*) venom, and attenuated Rabies virus, were chosen for expression in soluble form. Four clones out of six produced functional antibody fragments, which were specific to their antigen and readily, detected in cell culture supernatants by ELISA (Figure 24). The ability of each selected antibody to bind their antigen was varied. The strongest binding clone was Aflatoxin B1 C3, which derived from the IGHV1-3*01 germline (Table 7).

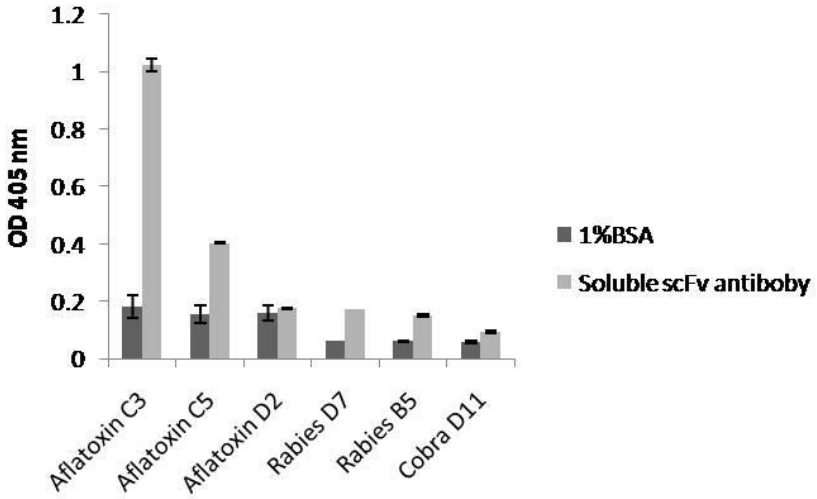


Figure 24. Specificity of soluble scFV antibody. ELISA for testing of the binding of antibody fragments to Aflatoxin B1, attenuated Rabies viruses and Cobra

snake (*Naja Kaouthia*) venom. Four scFvs out of six was specific to their antigen compare to BSA.

4.5 Purification of soluble scFv antibodies

The pMOD1 vector contains a gene III leader sequence, fused to the N-terminus of gene III-scFv construct. In this way, the scFv fusion protein is secreted into the periplasmic space (Kang et al., 1991). Moreover, the gene III-scFv fusion protein was fused to a hexahistidine tag, thereby permitting rapid affinity purification by IMAC. The best binder (anti-Aflatoxin B1 clone C3) and the weakest binder (anti-cobra snake venom clone D11) were chosen for purification. The inductuon was performed at 30ºC for 6 hours, using 1 mM IPTG in a total culture volume of 15 ml. The antibody fragments were purified from periplasmic fractions by using Ni-NTA resin, and analyzed by SDS-PAGE (Figure 25). Looking at the SDS-PAGE, the scFv-hexahistidine fusion protein of about 32 kDa was expressed, but the product band from both samples were not clearly visible. Either low expression yield or inefficient purification could be the case.

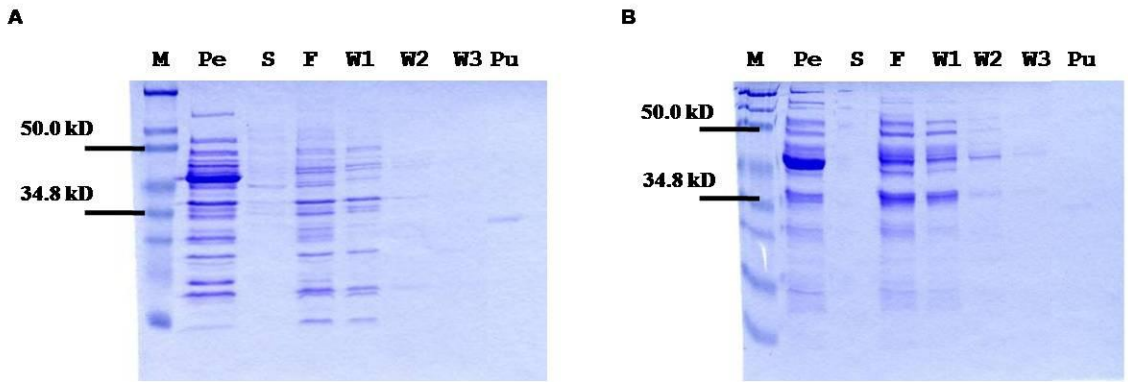


Figure 25. SDS-PAGE analysis of purified anti-Aflatoxin B1 clone C3 (A) and anti-cobra snake venom clone D11 (B) after induction with 1 mM IPTG. Lane

M, protein molecular weight marker; lane Pe, periplasmic fraction; lane S, culture supernatant fraction; lane F, flow-through fraction; lane W1-W3, wash fraction; lane Pu, IMAC purified scFv antibody.

In the phagemid vector, the expression of scFv-pIII fusion protein is driven by LacZ promotor (Hoogenboom et al., 1991). ScFv-pIII fusion protein is generated either from leaky expression after the glucose has been used up or by adding IPTG. Since both scFv and pIII are toxic to *E.coli*, overexpression of scFv-pIII fusion protein may lead to less, rather than more fusion protein (Freund et al., 1996; Krebber et al., 1996; Bothmann and Pluckthun, 1998). To identify the problem, scFv fusion protein localization was investigated. Four clones out of the six clones in Figure 23 were chosen for determination of fusion-protein localization. After induction at 30ºC for 6 hours, using 1 mM IPTG in a total culture volume of 15 ml, culture supernatant, periplasmic fraction, cell lysate, and inclusion body (insoluble) fraction, were prepared and collected. All fraction samples were compared by SDS-PAGE (Figure 26). The results obtained, show that scFv antibody products of the selected clones were sequestered into inclusion bodies (insoluble form).

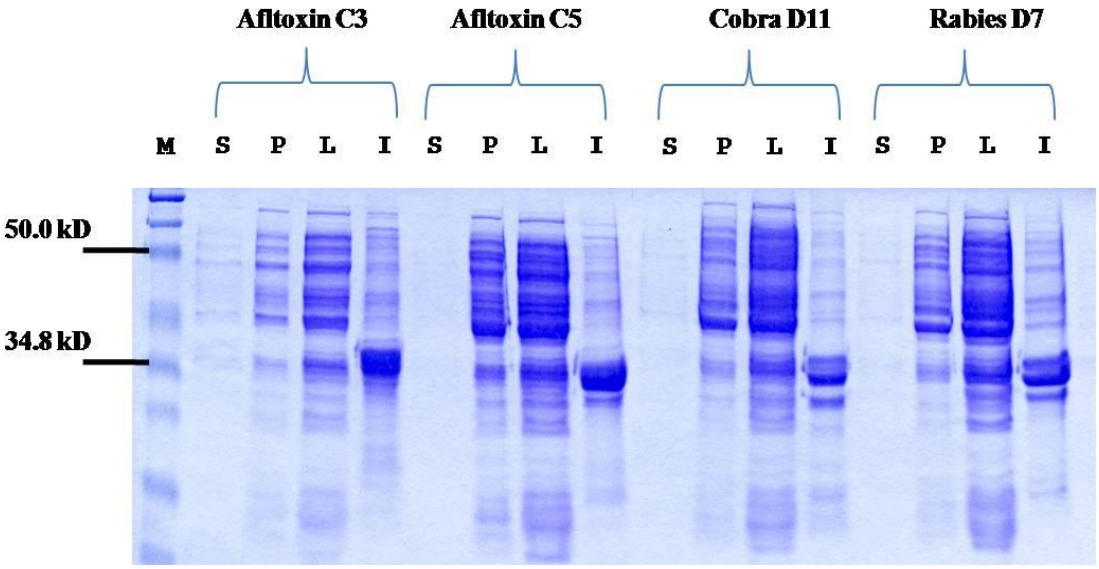


Figure 26. Localization of scFv antibody in different fractions. Lane M, protein molecular weight marker; lane S, culture supernatant; lane P, periplasmic fraction; lane L, cell lysate fraction; lane I, inclusion body (insoluble) fraction.

A case of recombinant protein expression has been reported, in which the product was lethal in *E.coli* (Barlow et al., 1987), unless the expression level was very low (White et al., 1990). One solution to this problem appears feasible, thus allowing the expression of scFv fusion protein. To reduce the expression level, and thereby obtaining sufficient expression of soluble scFv fusion protein, the expression was induced using a small amount of IPTG or in the absence of IPTG. The anti-Rabies virus was chosen for testing the low-IPTG induction. The inductions were performed at 30ºC for 6 hours using varying amounts of IPTG, ranging from 0-0.5 mM. The total volume of each expression culture was 15 ml. Then the expression level of scFv antibody from each IPTG induction was determined. Samples of medium supernatant, periplasmic fraction, cell lysate and inclusion bodies, were compared by SDS-PAGE.

The results obtained show that the scFv fusion protein in the cell lysate fraction, and present in inclusion bodies, even in the absence of IPTG. The expression yield of soluble anti-Rabies virus antibody was highest, when 0.05 mM of IPTG was used in the induction. The expression yields in cell lysate were reduced, whereas the amounts of inclusion bodies were increased when the concentrations of IPTG were raised (Figure 27).

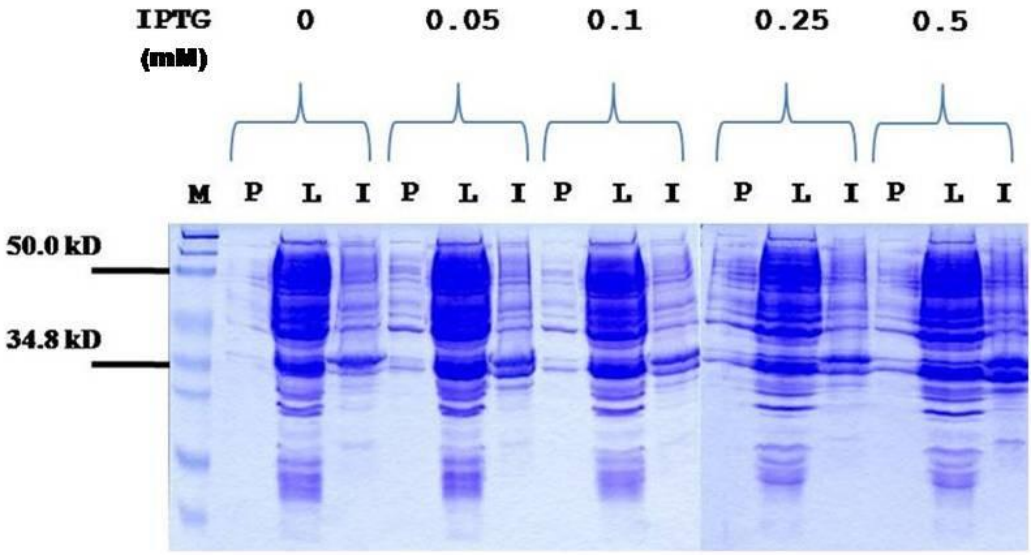


Figure 27. Induction of anti-Rabies virus scFv antibody with various concentrations of IPTG. Lane M, protein molecular weight marker; lane P, periplasmic fraction; lane L, cell lysate fraction; lane I, inclusion body (insoluble) fraction.

To qualify the functional anti-Rabies virus antibody, the specific binding to its antigen was tested by use of ELISA. The binding activity was found to be the highest, when the soluble scFv antibody was obtained from lysed cells from the 0.1 mM IPTG induction culture (Table 8).

Table 8. The ELISA results of scFv antibody activity from a variety of IPTG induction.

Rabies 300 microgram	Rabies	Rabies	2%MPBS	2%MPBS		Rabies	Rabies	2%MPBS	2%MPBS
P 0	0.097	0.088	0.075	0.068	**I 0**	0.07	0.065	0.061	0.066
P 0.05	0.096	0.093	0.068	0.06	**I 0.05**	0.066	0.063	0.063	0.063
P 0.1	0.113	0.112	0.074	0.063	**I 0.1**	0.081	0.074	0.069	0.077
P 0.25	0.098	0.098	0.062	0.071	**I 0.25**	0.087	0.088	0.075	0.08
P 0.5	0.099	0.085	0.06	0.062	**I 0.5**	0.079	0.084	0.084	0.078
L 0	0.101	0.082	0.069	0.074	**S 0**	0.089	0.092	0.62	0.62
L 0.05	0.144	0.102	0.069	0.067	**S 0.05**	0.09	0.084	0.067	0.066
L 0.1	0.149	0.128	0.081	0.071	**S 0.1**	0.088	0.088	0.067	0.069
L 0.25	0.122	0.098	0.071	0.07	**S 0.25**	0.086	0.088	0.071	0.07
L 0.5	0.09	0.09	0.063	0.061	**S 0.5**	0.082	0.084	0.069	0.073

Note: P, periplasmic fraction; L, cell lysate fraction; I, inclusion bodies (insoluble) fraction; S, supernatant culture

For the large scale production of soluble scFv antibody, three clones were chosen. These were anti-Aflatoxin B1 clone C3, anti-Rabies virus clone D7, and anti-cobra snake venom clone D11. The volumes of the expression cultures were between 100 and 200 ml each. The results of SDS-PAGE analysis (Figure 28) showed that the scFv-hexahistidine fusion antibodies with a size of about 32 kDa each, were successfully expressed and purified from lysed cells, using Ni-NTA spin columns, (Qaigen, Germany) according to the manufacturer protocol. However, additional proteins appeared to co-purifying along with the desired scFv antibodies. Similar observations have been reported about impurities in antibody purification (Das et al., 2005; Duellman and Burgess, 2006). A further purification step to remove contaminants will be necessary, in order to obtain antibody of sufficient purity. Anion exchange chromatography (Du Bois et al., 1998) and gel-filtration (O'Connell et al., 2002) could be attempted to overcome the problem.

ScFv antibodies have been reported by many research groups to form dimers and multimers, when short linker peptides are used (Desplancq et al., 1994; Whitlow et al., 1994; Alfthan et al., 1995). Dimerization of ScFv antibodies has been reported, even when long linker peptides were used (Iliades et al., 1998; Tan et al., 1998 ; Lee et al., 2002; Smulski et al., 2006). The explanation for this phenomenon may be that the linker presumably constraining the folding, hindering appropriate association of V_H and V_L fragments located on the same polypeptide. Then V_H-V_L interface exposes hydrophobic residues, thus increasing the likelihood of intermolecular associations, and resulting in multimerization. In this study, the western blot result (Figure 28) indicated that only monomer scFv was obtained and that it was functionally active.

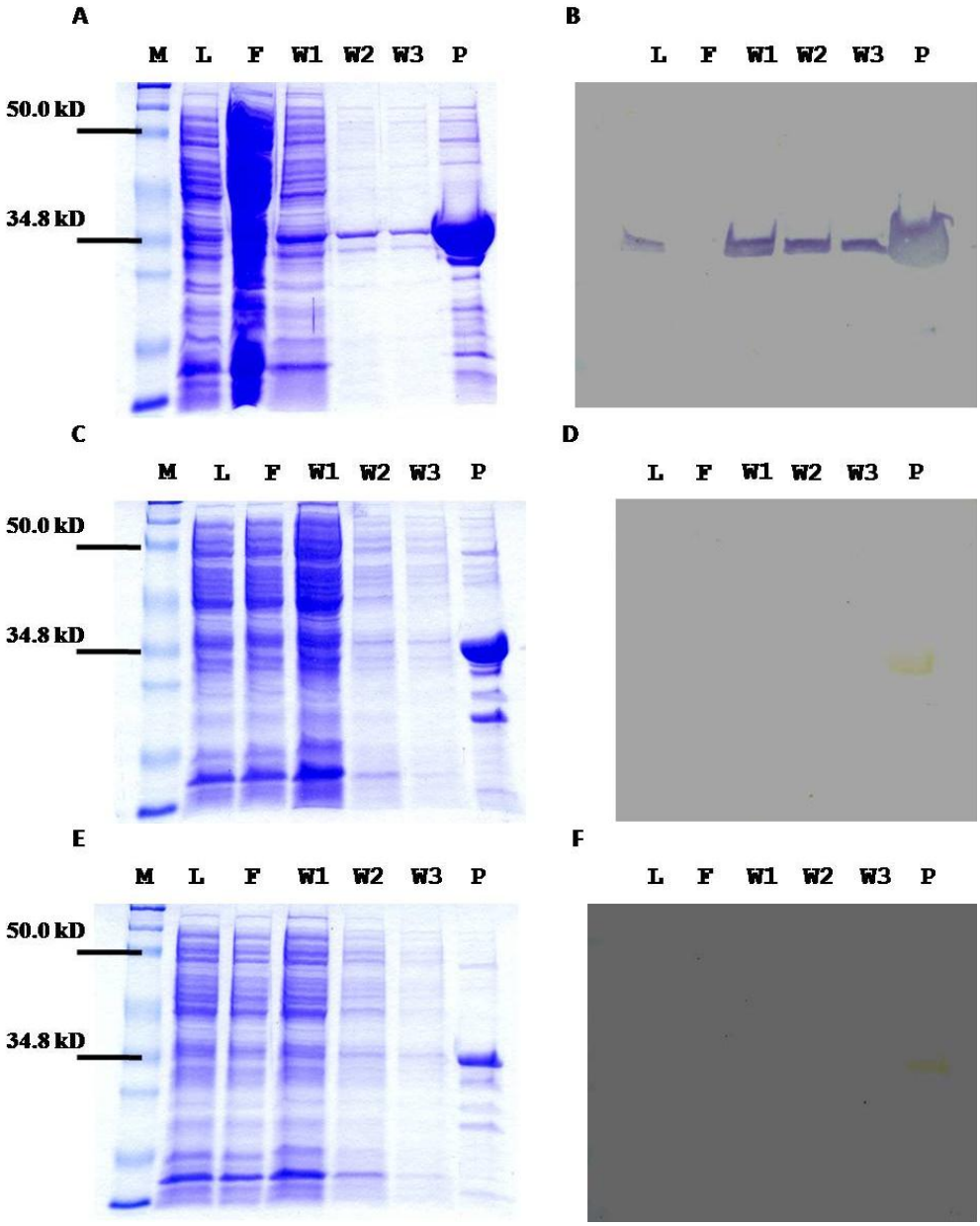


Figure 28. SDS-PAGE and western blot analysis of purified scFv antibody. SDS-PAGE stained with CBB; (A) anti-Aflatoxin B1 clone C3, (C) anti-Rabies virus clone D7, (E) anti-cobra snake clone D11; Lane M, protein molecular weight marker; lane S, culture supernatant fraction; lane F, flow-through fraction; lane W1-W3, wash fraction; lane P, purified scFv antibody by IMAC. Western blot stained with mouse anti-myc and goat anti-mouse conjugated HRP. Color was developed by using TMB

substrate. (B) anti-Aflatoxin B1 clone C3, (D) anti-Rabies virus clone D7, (F) anti-cobra snake clone D11.

The yields of purified scFv antibodies ranged from 3.51 mg/1L culture volume for the anti-cobra snake venom clone D11, to 3.91 mg/1L culture volume for the anti-Rabies virus clone D7, as shown in Table 9. These were in the same range as other recombinant antibodies that have been purified by using standard IPTG induction as 1 mM (Table 10). The comparison of antibody yields after purification by IMAC is presented in Table 10.

Table 9. Data obtained during induction and purification of scFv antibodies.

Antibody	Volume of bacteria culture (ml)	Fraction	Weight (mg)	Concentration (mg/ml)	Final volume (µl)
Anti-Aflatoxin C3	200	Wet weight of cells	1000		
		Total protein content	169.6	21.2	8000
		Yield of purified protein after IMAC	0.707	0.707	1000
Anti-Aflatoxin C5	200	Wet weight of cells	1300		
		Total protein content	144	18	8000
		Yield of purified protein after IMAC	0.764	0.764	1000
Anti-Rabies D7	100	Wet weight of cells	1230		
		Total protein content	96	24	4000
		Yield of purified protein after IMAC	0.391	1.565	250
Anti-cobra D11	100	Wet weight of cells	1040		
		Total protein content	80	22.5	4000
		Yield of purified protein after IMAC	0.351	1.405	250

Table 10. Comparison of antibody yields puified by IMAC.

Antibody	**Yields of purified antibody (mg/L culture volume)**	**Group**
Anti-Aflatoxin B1 C3	3.53	This study
Anti-Aflatoxin B1 C5	3.82	This study
Anti-Rabies virus D7	3.91	This study
Anti-cobra snake venom D11	3.51	This study
Anti-estradiol	10.1	Dorsam *et al.*,(1997)
Anti-testosterone	4.3	Dorsam *et al.*,(1997)
Anti-digoxigenin	0.84	Dorsam *et al.*,(1997)
Anti-progesterone	0.74	Dorsam *et al.*,(1997)
Anti-scorpion toxin	1.1	Quintero-Hernandez *et al.*, (2007)
Anti-CD3 surface antigen	4	Kipriyanov *et al.*, (1997)
Anti-breast tumor cell (SK-BR-3)	1	Goenaga *et al.*,(2007)

CHAPTER V

CONCLUSION

In summary, the strategy in this study has been improved variable region sampling, in order to construct a high diversity antibody repertoire. The successful construction of a high quality human antibody library can contribute to the expansion of the use of this technology. The library has proven useful for the selection of antibody fragments to various targets, including protein antigens, live attenuated viruses, crude venom extracts, cell surface antigens and a hapten. Regarding the binders obtained, useful antibodies such as anti-Rabies virus and anti-Aflatoxin B1 are currently being considered for further development, and could prove useful as either therapeutics or as diagnostic agents. Antibody therapy is likely to become more widely applied in future, and this development will be greatly facilitated by the increased knowledge of effective techniques for the isolation and purification of monoclonal antibodies. Even though the research undertaken in this work concerns specific antigens (Rabies virus, Aflatoxin B1), the methods used could be applied to any fields in which recombinant antibodies are required. In view of the potentially large harvest of monoclonal antibodies for diagnostic and therapeutic purposes, high throughput antibody selections can be envisaged, especially considering the spread of automation technology (Hallborn and Carlsson, 2002; Konthur and Walter, 2002). The time and effort invested in antibody production by phage display technology is an important accomplishment.

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