

Parameters affecting phage display library design for improved generation of human antibodies

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by

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I hereby declare all works and writing contained within this thesis was conducted by myself, all references used are cited accordingly and any personal assistance acknowledged by name.

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Berlin, 2009

All the work for this PhD thesis was conducted from October 2006 to April 2009 in the working group of Dr. Zoltán Konthur at the Department of Vertebrate Genomics under the tutelage of Prof. Dr. Hans Lehrach in the Max Planck Institute for Molecular Genetics.

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To my parents, wife and beloved daughter.

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1.0 Introduction

The immune system represents the first line of defence in our bodies against the invasion of foreign entities. Antibodies can be regarded as the soldiers in the war against infections and foreign bodies. Over the years, antibodies have proven to be a desirable tool both in diagnostics and therapeutics. Their high specificity in recognition is the main factor why antibodies are most sought after. Periodically, the first antibodies prepared were of animal origin by Emil Behring and Shibasabura Kitasato against diphtheria toxin [1]. Since then, with major advances in molecular biology techniques and better understanding of the immune system, the major focus is now on the development of therapeutic antibodies.

1.1 Antibodies

Antibodies, also termed as Immunoglobulins (Ig) are gamma globulins that are secreted in the blood or other bodily fluids of vertebrates. They are hugely attributed to the humoral immune system and is an essential product of the adaptive immune system. The production of antibodies is accredited to a type of white blood cell called B-cell. When an antigen is presented on the surface, the B-cell becomes activated. The recognition of antigenic determinants by the B-cell antigen receptor will trigger the differentiation and proliferation of B-cells to yield antibody secretion in the blood. Activated B-cells will differentiate to either antibody producing cells (plasma cells) or memory cells. Antibodies exist in two forms, free/soluble antibodies or surface/membrane immunoglobulin. Free or soluble antibodies are secreted from activated B-cell (plasma cells) into the blood or other body fluids. Surface or membrane Ig is the membrane bound form which is part of the B-cell receptor (BCR)[2,3].

Structurally, antibodies are glycoproteins with a molecular weight in the region of 150 KDa. Antibodies have a Y shape like configuration with two identical halves made up of four polypeptide chains held together by inter-chain disulphide bridges. The two halves contain a heavy and a light chain. Each chain is made up of different structural domains called Ig domains. The domains are called the variable (IgV) domain and the constant (IgC) domain. Antibodies also have a unique feature which is the immunoglobulin fold in which two beta sheets are folded in a “sandwich” like shape. The structure is held together by cysteine-cysteine interactions between the heavy and the light chain (Figure 1.1) [2,3].

Although the general structure for all antibodies is similar, the upper region of each chain is responsible for antigen binding. This region is known as the Fab (fragment antigen binding) region. The Fab region consists of one constant and one variable domain each from both heavy and light chain. The base of the antibody Y shape is called the Fc (fragment crystallisable) region. The region between the Fab to the Fc region of heavy chains is separated by a Hinge region which gives the antibody flexibility (Figure 1.1). Antibodies contribute to the immune system in three main mechanisms. Antibodies prevent the penetration of pathogens by binding to them, stimulate the removal of pathogens by recruiting macrophages and prompt direct termination of pathogens via complement activation [2,3].

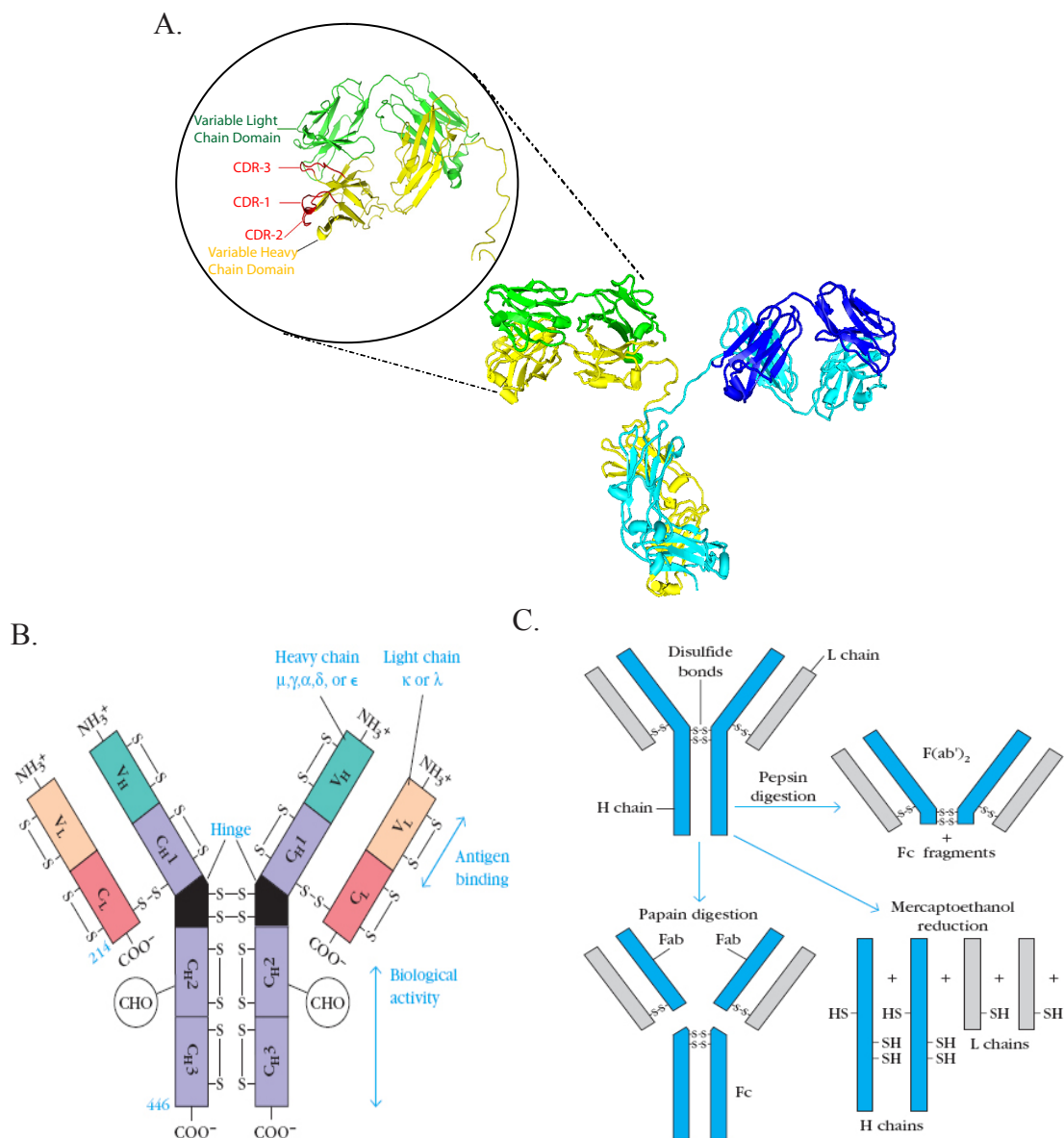


Figure 1.1: Schematic representation of typical antibody Y shape structure. (A) 3D model highlighting CDR regions of the variable heavy chain, (B) Disulphide bridges surrounding the Ig molecule and (C) Various forms of antibody fragments (Fab, F(ab')₂, Fc) [3].

The Fc region of the antibodies is responsible for complement activation via attachment of the first component of the complement cascade thus activating the classical complement system. This enables the assassination of bacteria in two processes. First is a process called opsonisation, where the antibody and complement molecules bind to mark the pathogen for ingestion by phagocytes. Another way is via membrane attack complexes that assist antibodies to terminate the pathogens directly. To battle pathogens outside the cells, antibodies work by binding the pathogens together causing agglutination. Antibodies by virtue of their paratopes will form a blanket to cover the pathogen and stimulate an effector response by recognition of the Fc region. The cells recruited for the effector response has Fc receptors on the cell surface. Phagocytes will trigger phagocytosis, mast cells and neutrophils will degranulate, natural killer cells will release cytokines and cytotoxic molecules [4].

For antibodies to be efficient, they must be able to trigger a response against all types of pathogens. A vast diversity in antibody binding sites is required for such a feat to be achieved. A complex recombinatorial process is responsible for the generation of adversely large antibody repertoires with a relatively small number of antibody genes. There are 51 functional V segments, 23 D segments and 6 J segments which can recombine to give 7038 different VH sequences [5]. For variable Kappa, there are 34 functional V segments with 5 J segments [6]. The Lambda light chain has 30 functional V segments and 7 J segments [7]. The total amount of different VL sequences possible is 360 [5]. The nature of random combination between the heavy chain repertoire with the light chain repertoire will generate a primary repertoire in excess of 10^{10} different antibody sequences [8]

The region (locus) of a chromosome that is responsible for encoding antibodies is made up of several distinct genes for each Ig domain. The locus with the heavy chain genes is found on chromosome 14q32.33 and the loci containing the lambda and kappa light chains are found on chromosomes 22 and 2 respectively [9,10,11].

The hypervariable regions or complementary determining regions (CDR) are responsible for the antigen binding abilities of the antibodies. The antibody recognition site on the antigen is termed an epitope. The competent binding ability of antibodies against a huge array of targets of diverged lineage is credited to the colossal diversity of the antibody paratopes (the recognition site on the antibody against epitopes). These epitopes bind to the paratopes by a highly specific interaction, called induced fit. The diversity on the variable domains is a direct result from the three CDR regions (CDR1, CDR2 and CDR3) hypervariability. A multitude of variable domain

genes (266 IGHC, 148 IGKV and 84 IGLV) via genetic recombination processes will rearrange to introduce regions of high diversity (CDR regions) and regions where the sequences are conserved (framework regions). This somatic recombination process is called the V(D)J recombination (Figure 1.2) [12,13].

V(D)J recombination accounts for the generation of the primary antibody repertoire. V(D)J recombination involves the rearrangement of several gene segments (variable (V), diversity (D) and joining (J)) to encode the entire variable region. In heavy chains, all three gene segments are present but only the V and D segments are found in light chains [8]. In the genome, multiple copies of each gene segment is present. The rearrangement of the gene segments takes place in the B-cell where random selection of each gene segment will result in a combination of one gene per segment to form a complete variable domain. Upon production of a functional antibody gene, the B-cell would undergo a process called allelic exclusion where no other variable region can be produced. This makes each B-cell a monoclonal antibody producing cell [1,9].

The secondary antibody repertoire is generated by a process called somatic hypermutation (SHM). This occurs upon the introduction of antigens to B cells which triggers the proliferation of B cells thus allowing mutation to occur in the variable domains. The rate of base exchange in SHM is one base change per gene, per cell division. The resulting effect would be daughter B cells presenting slight differences at amino acid level in the variable domains [10]. The impact of SHM is a two way influence. It can serve to increase the diversity of the antibody pool being generated which could result in higher binding affinities and also lower binding affinities [11,12].

Affinity maturation is a process where B-cells with higher affinity antibodies will out compete the weaker ones for survival. This process only takes place in mature B-cells that have undergone V(D)J recombination normally in germinal centres. T-cells has been shown to play a role in determining antibody affinity although the precise role played by T-cells is still unknown. The mechanism controlling affinity maturation is still not fully understood although it has been suggested that the selective proliferation of B-cells to secrete antibodies of a certain affinity is dependent on the amount of antigen present for competition. Thus, B-cells with high affinity receptors will preferentially be stimulated when antigen becomes scarce. Therefore the affinity of the antibody is inversely proportional to the amount of antigen present [13].

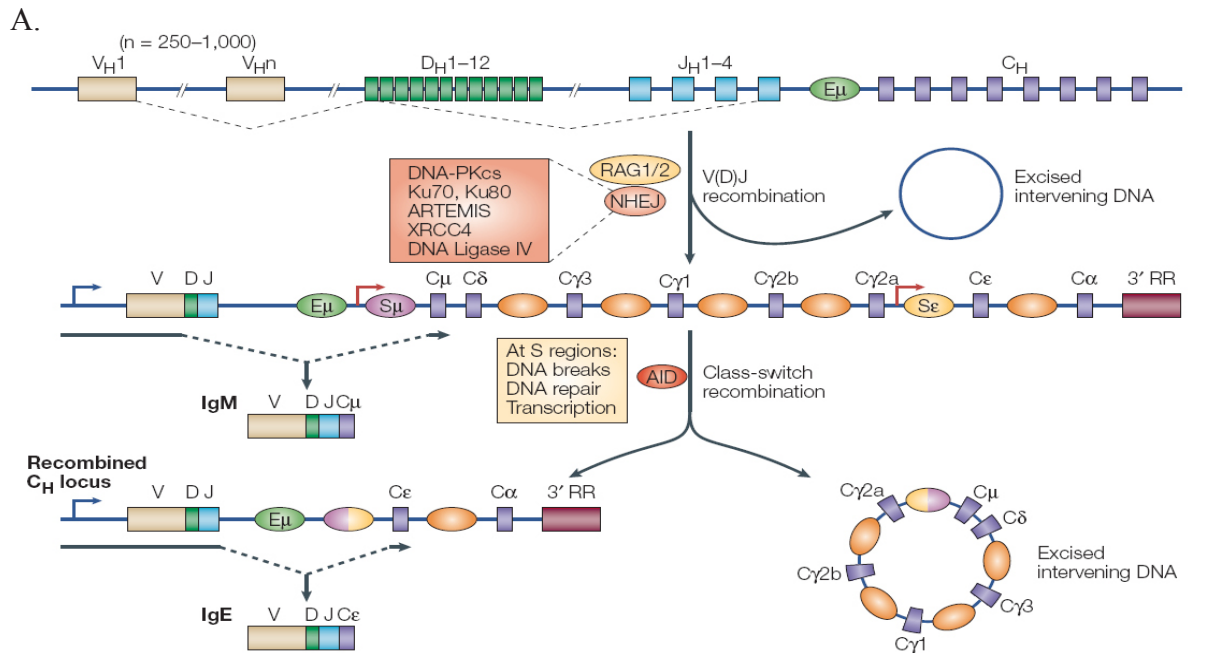


Figure 1.2: Mechanism of VDJ recombination and Class Switch Recombination. (A) Schematics of SHM and CSR process, and (B) Comparison of SHM and CSR. The variable region of the immunoglobulin heavy chain is assembled from component variable (V_H), diversity (D_H), and joining (J_H) gene segments by V(D)J recombination. The process of rearrangement involves cleavage of the recombination signal sequences in the DNA, which flank the rearranging gene segments, which is carried out by the recombination-activating gene 1 (RAG1)–RAG2 complex. Joining of the DNA ends requires nonhomologous end-joining (NHEJ) proteins, including Ku70, Ku80, ARTEMIS, X-ray repair cross-complementing protein 4 (XRCC4), DNA ligase IV and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). Transcription across the locus is driven by a promoter upstream of the rearranged VDJ segment (blue arrow), which facilitates the synthesis of a μ heavy chain. This then associates with a light chain, thereby forming an IgM molecule, which is displayed on the cell-surface of a B cell. Subsequently, secondary isotypes are produced by class-switch recombination (CSR), a process that exchanges the constant region of the heavy chain (CH) with a set of downstream constant-region genes (CSR to IgE is shown). This deletional-recombination reaction, which requires the enzyme activation-induced cytidine deaminase (AID), involves the generation of DNA breaks at switch (S) regions, which precede the constant-region genes, followed by the repair of DNA. This leads to a rearranged CH locus and deletion of the intervening sequence as an episomal circle. Cytokines stimulate transcription (red arrows) through the CH gene and determine the immunoglobulin isotype that the B cell will switch to. The rearranged variable regions of both the heavy and light chains also undergo a high rate of point mutation through the process of somatic hypermutation (SHM) (not shown). The E_μ and 3'-regulatory region (3'RR) enhancers influence V(D)J recombination and CSR, respectively [Figure taken from 14].

Antibodies are made up of several different types of classes or isotypes. There are five antibody heavy chain isotypes in humans, known as IgA (Ig- α), IgD (Ig- δ), IgE (Ig- ϵ), IgG (Ig- γ) and IgM (Ig- μ) (Figure 1.3). Each differs in its biological property, functional location and preferred type of antigen. The isotypes transformation of a B cell occurs during cell development and activation. A naïve B-cell (immature) expresses only IgM. Upon maturity, B-cells will co-express both IgM and IgD on the surface. Some daughter cells of an activated B-cell will undergo a process called class switch recombination which results in conversion of IgM / IgD to IgE, IgG or IgA [14]. The constant domain is identical in all antibodies of the same isotype and differs with different isotypes. The heavy chains of IgG, IgA and IgD have a constant region consisting of three tandem constant domains (CH1, CH2 and CH3) whereas IgM and IgE have four constant domains. There are two isotypes of light chains in humans, kappa (κ) and lambda (λ). The light chain has one variable and one constant domain. Each antibody has two identical light chains of one isotype present per antibody [1].

Class switching occurs in the heavy chain locus via a mechanism called Class Switch Recombination (CSR) (Figure 1.2). This mechanism relies heavily on the switch regions located upstream of each constant region, except for the δ -chain in the heavy chain locus. Switch regions are made up of tandem repeats of pentamers (GAGCT and GGGGT) or a 49 base pair sequence with varying degree of homology [15,16]. These switch regions will act as sites for cutting and joining during the process. A series of RNA editing enzymes (APOBEC, AID) will act to break and fuse an upstream switch region to a downstream switch region with the deletion of the intervening DNA. The joining of the variable domain exon is the final piece of the puzzle. Through a process called non-homologous end joining, the variable domains are rejoined to the desired constant region (α , ϵ or γ) resulting in a complete isotype specific antibody chain sequence [17,18].

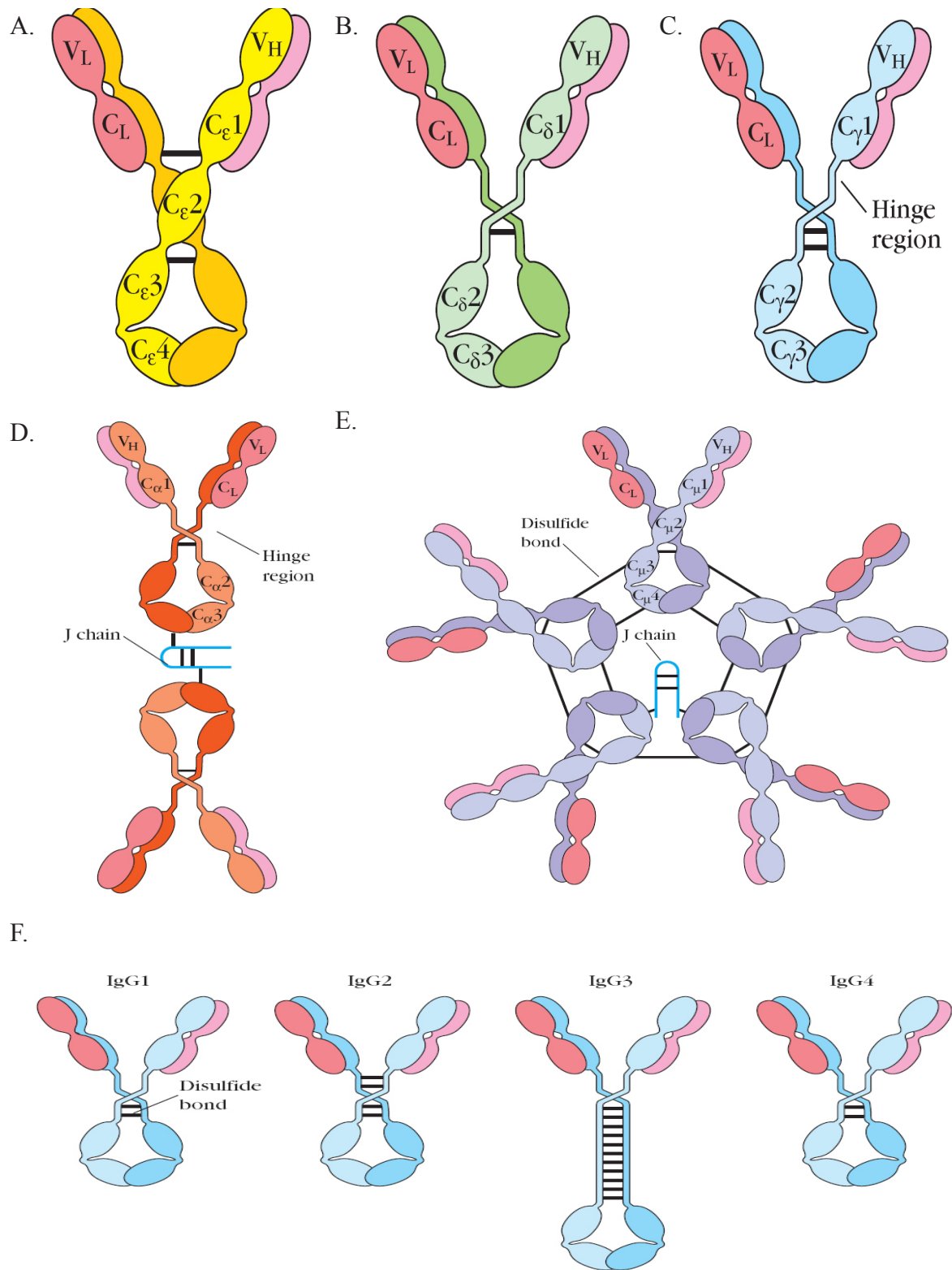


Figure 1.3: General structures of the five major classes of secreted antibodies. (A) IgE, (B) IgD, (C) IgG, (D) IgA - dimer, (E) IgM - pentamer and (F) Subclasses of human IgG [3].

1.2 Recombinant antibody technology

Paul Ehrlich first theorized the side chain theory to explain the reaction of the immune system against infections. It was from this he envisioned the “magic bullet” concept whereby a compound would be able to specifically target and kill disease cells [21,22]. The first true magic bullet was discovered in 1975 when Kohler and Milstein realised Ehrlich’s vision with the introduction of monoclonal antibodies [23]. A continuous culture of antibodies with predefined specificity was able to be produced. This paved the way to many daughter methods for monoclonal antibody (MAb) production (Figure 1.4). In 1984 they were rewarded with the Nobel Prize in Medicine for the breakthrough [24]. The advent of recombinant antibody technology would not have been possible without the discovery of Polymerase Chain Reaction (PCR) technology by Kary Mullis in 1984 [25]. The technology allowed scientist to amplify a single DNA via in vitro enzymatic replication. The Nobel Prize in Chemistry was awarded to Mullis in 1993 for his invention [26]. The contributions of antibodies has widen since, with applications spanning from detection assays to clinical therapies for humans.

MAb production involves the fusion of myeloma cells with spleen cells of a mouse that has been immunized with an antigen. The cells are fused together to form a hybridoma using polyethylene glycol. Taking advantage of the inability of myeloma cells to synthesize hypoxanthine-guanine-phosphoribosyl transferase (HGPRT), a selective medium containing hypoxanthine, aminopterin and thymidine (HAT) is used to distinguish between hybridomas and myeloma cells. This in turn makes the hybridomas immortal and can be grown indefinitely [23,27,28]. The bottleneck to this technology was the availability of myeloma cells for fusion. This meant that only mouse monoclonal antibodies could be generated. The antibodies derived from this technology was entirely of murine origin. Although the initial work was carried out on mice, the technology was adapted to rabbits later in 1995 when a new plasmacytoma fusion partner was developed for rabbits [29].

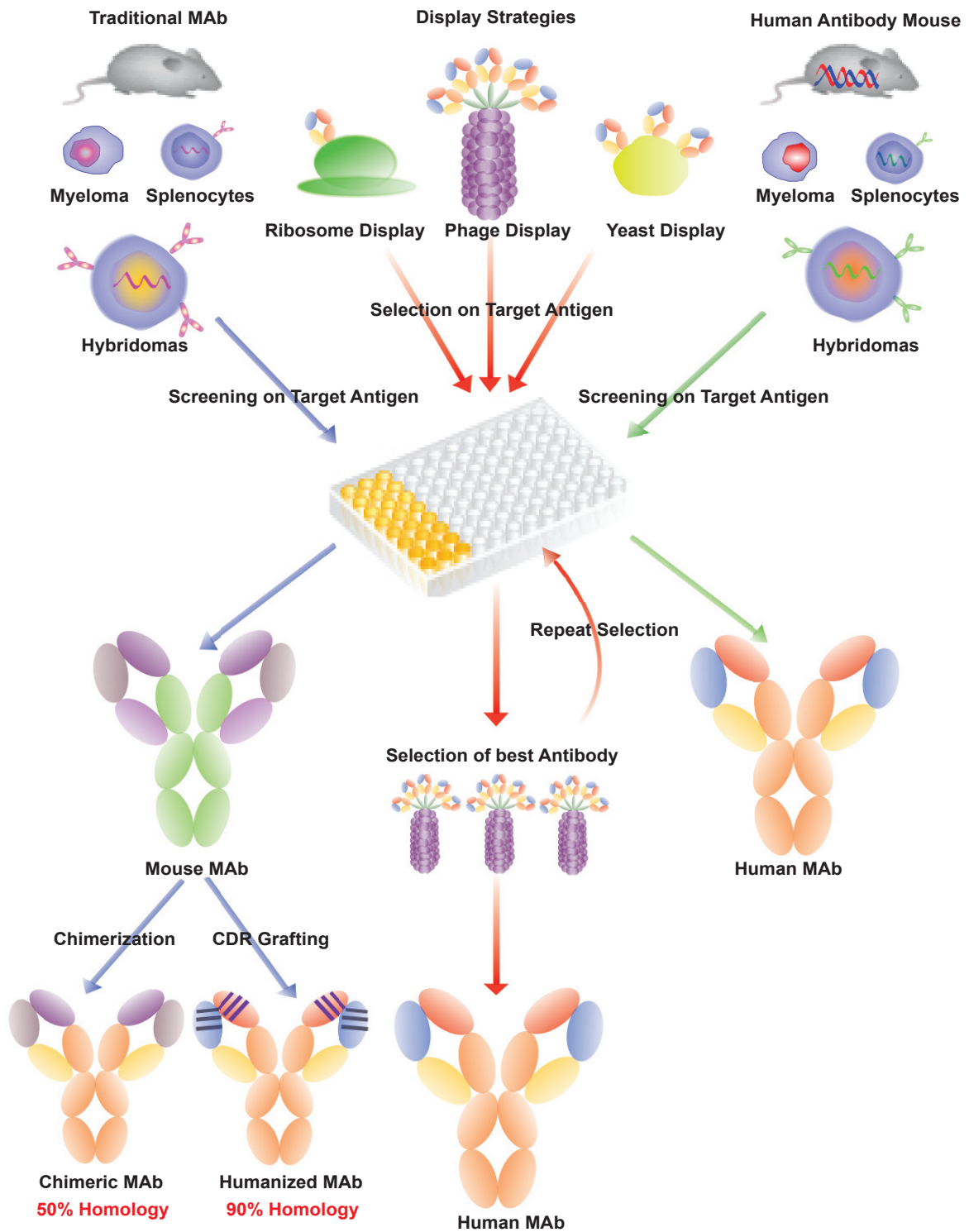


Figure 1.4: Conventional murine monoclonal antibody technology and daughter methods for the recombinant production of monoclonal antibodies.

Although mouse derived MABs was able to live up to its expectations as a diagnostic tool, it fell short in its promise in the field of therapeutics. This was due to the development of anaphylactic reactions by recipients of mouse MAb immunotherapy treatments [30,31,32]. It was later discovered that the human immune system reacted against the mouse MAb as a foreign protein hence targeting an immune response against it. This brought about the discovery of the HAMA (Human Anti Mouse Antibody) effect [33,34]. The need to eliminate such counter responses by the human immune system is pivotal for the success of MAb therapies. To overcome this dilemma, scientist resorted to engineering new antibody structures with hopes of minimizing the immunogenicity of antibodies for clinical applications [35].

The first post-MAb technology approach to lower immunogenicity was the introduction of chimeric antibodies. Chimeric antibodies are generated by linking the genes encoding mouse variable domains of the desired antibody with the constant domain genes from humans [36]. This resulted in an antibody with 60 % to 70 % homology to the human antibody [37,38]. One of the first chimeric antibodies approved by the FDA as a therapeutic agent is Rituximab, an anti-CD20 antibody for the treatment of relapsed B cell non-Hodgkin's lymphoma. The major concern was the fully mouse derived variable domains of the chimeric antibodies. Unfortunately the concern was warranted with the onset of the HACA (Human Anti Chimeric Antibodies) effect in patients [39,40].

To circumvent this problem, Jones *et al.* in 1986 experimented on the CDR grafting concept which resulted in the generation of Humanized Antibodies from mice [41]. The graft constitutes removing the hypervariable regions (CDRs) from a mice antibody which is responsible for antigen binding and transplanting them to another framework which is of human origin (42). This gives an antibody with 90 % to 95 % homology to human antibodies. Three antibody based therapies including Herceptin (anti-HER2/neu receptor) were generated using this approach (43).

The generation of fully human antibodies was possible with the developments in recombinant DNA technology [44,45,46]. Although the generation of large recombinant human antibody libraries are sought after, the main obstacle faced by users with such a huge library is the selection procedure. Phage display technology was seen as the ideal solution to screen a huge diverse library for single clones producing antibodies with the introduction of controlled selection pressures [47].

The major advantage of phage display is the simplicity, robustness and stability of phage particles which allows it to be applied on various surfaces and also for in vivo selection. A distinct feature of phage display allows the physical coupling of genotype and phenotype in bacteria thus requires the introduction of encoding DNA artificially [48]. This means the basic requirement for phage display based antibodies is a large diverse library encoding antibodies [49,50]. The first engineered antibody fragment was the single chain Fv format (scFv), initially used for expression in *E.coli* [51]. McCafferty *et al.* then demonstrated the presentation of scFv successfully on phage surfaces in 1990 [52]. A year later, Hoogenboom *et al.* then generated Fab based antibody format presentation on phage surface [53]. Various antibody formats will be discussed in the next chapter.

Other display technologies such as ribosome display and yeast display were then introduced for antibody generation [54,55]. Ribosome display, just as phage display requires a large diverse antibody library for selection. In ribosome display, the antibody is transcribed and translated in vitro using prokaryotic [56,57] or eukaryotic cell-free expression systems [58,59]. The combined conditions of an absent stop codon, elevated magnesium ion concentration and low temperatures will slow down the ribosome at the end of the mRNA while the emerging antibody will fold and present itself outside the ribosome tunnel for selection. A major difference between phage and ribosome display is the in vitro phenotype-genotype coupling and amplification which allows easier handling of a larger library as oppose to phage display [60]. Large libraries can be generated rapidly because there is no need to transform huge numbers of plasmids into a host [57]. The system allows easy mimicry of the affinity maturation process by introducing mutations at every round of selection because a PCR step is required rather than an in vivo amplification [61,62,63]. Although ribosome display could circumvent the drawbacks of library generation associated to in vitro display methods it also has some drawbacks [57]. Some problems with ribosome display is due to the meticulous handling that is required as mRNA in the ribosomal complexes are easily degradable by RNase. Another shortcoming is the premature termination of proteins translation consequence possibly of ribosome stalling prior to the end of the mRNA [56,57]. As the method is dependent on the success of retrieving

genotype information with phenotype interaction, the interaction between the synthesized polypeptide to the ribosomal complex must be maintained. Dissociation of this complex occurs in ribosome display thus resulting in the loss of genotype and phenotype coupling.

Yeast display technology has been used to display antibody formats on yeast *Saccharomyces cerevisiae*. The concept of yeast display for antibody production involves the presentation of the antibody as a fusion protein to the agglutinin adhesion receptor complex (Aga1 and Aga2) [64]. The Aga2p protein functions to mediate cell-cell interaction by yeast during cell mating. By presenting antibody proteins fused to Aga2p, it will direct antibodies away from the cell surface thus minimizing potential exchanges with other molecules on the yeast cell wall [65]. The main advantage of this platform is the high eukaryotic expression and processing mechanism, the assurance of affinity and stability maturation from secretion, minimal avidity effects and the gap repair ability of yeast. Gap repair is an endogenous recombination process in *S. cerevisiae* that allows the insertion of genes in plasmids at exact sites without the need of restriction enzymes. This allows easy library generation and affinity enrichment of CDR regions [66]. The major advantage is the coupling of Fluorescent-activated Cell Sorting (FACS) to yeast display. FACS can be used to monitor antibody expression or antibody-antigen binding efficiency with fluorescent labelled antigens. The main pitfall of this technology are the smaller antibody library sizes [65,67]. Another problem worth noting is the loss of activity of the selected antibody format after expression in soluble form. This was reported with the selection of mammalian calmodulin (CaM) protein. The investigators reported the possibility that the Aga-2 fusion contributes to the binding observed thus losing the binding characteristics when expressed without the Aga-2 fusion [68].

The essence for the generation of human antibodies is the amplification of genetic information encoding for antibody variable regions from human B cells. This genetic information is then cloned into appropriate vectors according to the desired platforms for selection. Although with various technological platforms being introduced, phage display still remains the preferred choice for antibody engineering due to the robustness and stability of the method coupled with the flexibility to incorporate conditions favoured by the user [69].

1.3 Antibody format engineering

With the introduction of phage display, it was then possible to engineer natural and synthetic antibody fragments for screening. The first antibody format to be generated from it was the single chain Fvs (scFv) by Huston *et al.* in 1988 [51]. The first monoclonal scFv presented on phage was generated by McCafferty *et al.* in 1990 [52]. This led to the successful presentation of monoclonal Fab format in 1991 by Hoogenboom *et al.* [53]. The following years led to the first successful use of phage display to present scFv libraries [70] and Fab libraries [71]. To date, there have been numerous antibody formats reported for phage presentation, such as scFv [52], Fab [72], scFab [73], domain antibodies [74,75], diabodies, triabodies, tetrabodies [76,77] and even alternative scaffolds [78]. Figure 1.5 shows some of the many different types of antibody formats available. The enigma commonly faced by antibody phage display users is the question of the best format.

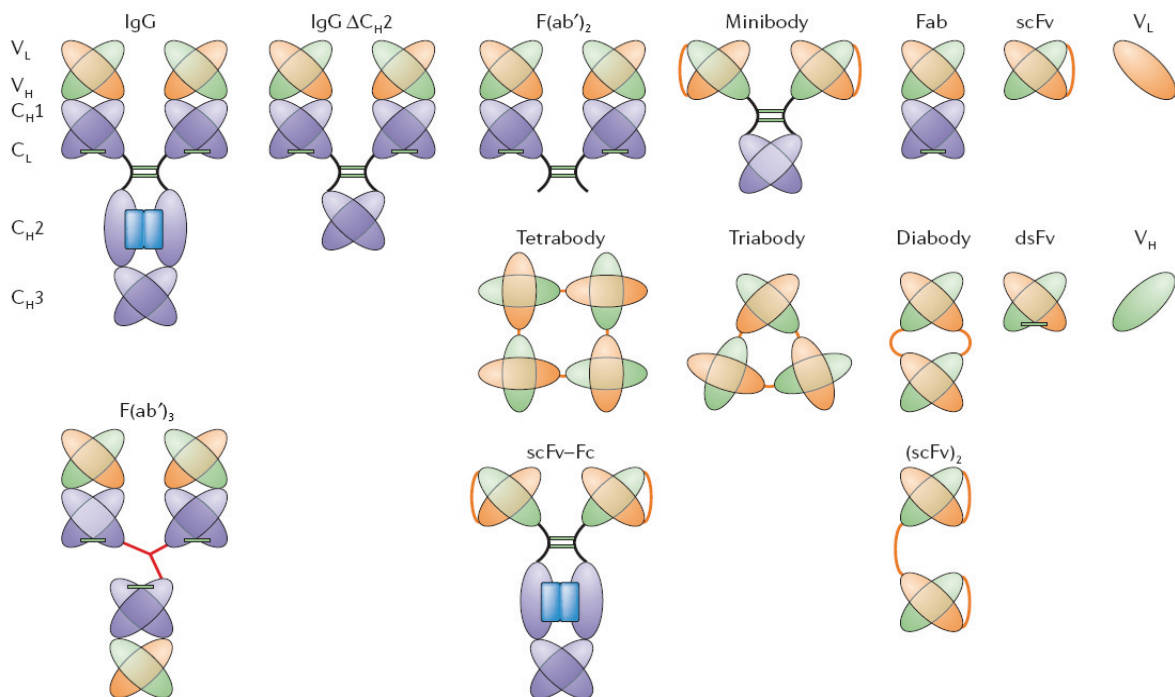


Figure 1.5 : The various types of antibody formats available for use or modification. The modular domain architecture of immunoglobulins has been exploited to create a growing range of alternative antibody formats that spans a molecular-weight range of at least 12–150 kDa and a valency (n) range from monomeric (n = 1), dimeric (n = 2) and trimeric (n = 3) to tetrameric (n = 4) and possibly higher [Figure taken from 43].

The scFv is basically both variable domains of the heavy and light chain held together by a flexible glycine / serine linker to prevent dissociation. The relative size of a scFv construct is about 28 kDa. This format proved to be a success with many libraries generated and tested with binders for multiple types of targets. There have been several adaptations to the way scFv's are being used. Bi-scFv, triabody, diabody, minibody and tetrabody are some of the models generated based on the scFv platform. The various formats introduce a bispecific, bivalent, trivalent and tetravalent environment [79]. The downstream applications of scFv are limited due to the absence of the constant domains and lower intrinsic stability.

scFv format stability is dependent exclusively on the intrinsic stability of the variable domains and the strength of the interface between the light and heavy variable domains. When both variable domains are intrinsically weak, the interaction between the two domains in a scFv is able to yield a more stable structure than either weak domains individually. If either one domain is predominantly more stable, the interaction will stabilize the weaker domain to a certain degree. The intermediate unfolding equilibrium may be observed depending on the degree of the interaction and differences in the intrinsic stability where one domain will be folded and the other remaining in the native form. If the intrinsic stability of both domains are high, the interface will not be able to stabilize the scFv beyond the stability of the more stable domain [80].

Fab based constructs, although a more favourable choice for downstream applications, has a major setback in display efficiency which has dimmed its popularity for use in phage display. Fab constructs are basically the variable and constant domain 1 of the heavy chain and the variable and constant domain of the light chain held together by a disulphide bond. The size of a Fab construct is approximately double that of a scFv at approximately 50 kDa [71]. The larger size of the Fab construct makes display efficiency on phage somewhat inefficient in comparison to scFv display. Unlike the scFv where both domains are expressed as a single fusion molecule due to the linker, a Fab construct depends on the successful disulphide formation of the light chain and heavy chain in the bacterial periplasmic space. This will contribute to the decrease in display efficiency on phage [53]. Although the use of Fab constructs on phage is not as efficient as scFv, the importance of Fab constructs has made the need to generate better presentation conditions for Fab constructs a major challenge.

Several factors should be considered when designing Fab phagemid vectors. The important element to be retained in phagemid vectors is the bridge between the recognition element with the instruction facet for its production [81]. The phagemid concept was used in various types of phage libraries ranging from peptide [82,83,84,85], proteins [86], scFv [52] and Fab [81]. The use of the phagemid vector for Fab requires some experimental considerations. First is the balanced expression of both antibody chains as either light or heavy chain can be fused to pIII. The use of a phagemid vector allows the down regulation of expression by glucose using the lacZ promoter. It can also allow control of the amount of antibody-pIII fusion protein being incorporated into the phage particle. The use of a phagemid vector also gives the user the choice of monovalent and multivalent display by using different helper phages. A comparison of available types of helper phages with their properties is shown in Table 1.1. Phage display vectors also allows the expression of soluble antibody fragments and the introduction of differences in antibody sequences for better yield in periplasm expression [87,88,89]. Although both, scFv and Fab formats, are widely used for the generation of full IgG molecules, Fab formats can be advantageous for this purpose. The major advantage of a Fab construct is the readiness to convert the Fab to a fully functional IgG construct without the loss of affinity due to the similarities in the natural format in comparison to the scFv which is more restricted. The conversion of scFv to IgG by grafting the variable domains can potentially lead to the loss of binding ability of the antibody due to the differences in structural fold of a scFv in a full IgG. The differences in structural fold will cause the surface of the binding pockets (paratope) to differ [90]. Various formats of Fab's has also been examined with the introduction of Fab2 (Bispecific Fab) and Fab3 (Trispecific Fab) [80].

The stability of Fab formats are not entirely influenced by the direct interaction between VL and CL domains, but a mutual stabilization occurs across the VH/VL and CH1/CL interface. The whole CH/CL and VH/VL unit had significant mutual stabilizing effect thus indicating a high degree of cooperation between VH/VL and CH1/CL interface. The interchain disulphide bond in the Fab plays an essential role in this form of stabilization. The Fab fragments also showed a lower unfolding rate in comparison to scFv. This form of kinetic stabilization offered by Fab formats may increase the resistance against short time exposure to adverse conditions that could be detrimental for scFv formats [53].

Table 1.1: Various types of helper phage available [Table taken from 91].

Helper phage name	Mutation/mechanism	^a Helper phage titers/ml	Display levels	^{a,b} Rescued phagemid titers/ml	Use in display	Helper phage propagation
M13K07		10 ^{11 c}	Low	2 × 10 ¹⁰⁻¹²	Standard infection	Growth
KM13	Trypsin site in g3p, elution with trypsin	10 ¹¹	Low	2 × 10 ¹⁰⁻¹²	Standard infection	Growth
	g3 deletion	10 ⁵⁻⁶	High	~10 ¹⁰	Standard infection	g3 plasmid expression under lac promoter
M13MDD3.2	g3 deletion	2 × 10 ⁹	NT	10 ⁹	Standard infection	g3p plasmid expression
R408d3	g3 deletion ^d	10 ¹⁰	NT	NT	Standard infection	g3p plasmid expression under pspA promoter
Hyperphage	g3 deletion (8-406)	10 ⁹	High	10 ⁹⁻¹⁰	Standard infection	g3p integrated into <i>E. coli</i> genome
CT helper phage	g3 N1 & N2 domains deleted ^e	3 × 10 ¹¹	Low	10 ¹¹ total 5 × 10 ⁸ infective	Standard infection	g3p plasmid expression
Ex-phage	Amber stop codon 5' g3	10 ^{12-13 f}	High	10 ¹⁰⁻¹¹	Non-suppressor strain	Suppressor strain
Phaberge	Amber stop codon 3' g3	10 ¹¹	High	10 ⁹⁻¹⁰	Non-suppressor strain	Suppressor strain

The mechanisms of action, as well as the reported titers of both packaged phage and helper phage are given. NT-not tested.

^aTiters are unconcentrated supernatant titers—i.e. not PEG precipitated.

^bRefers to the number of infectious phagemid particles, independent of whether they carry antibody or no.

^cData from our laboratory.

^dSome reversion due to packaging of plasmid expressing p3 observed—probably also occurs in other plasmid expression systems, but not examined.

^eRescued phagemid are two populations, displaying and infective, non-displaying and non-infective.

^fStandard M13K07 titers obtained in this laboratory tend to be 10–100-fold higher than obtained elsewhere.

1.4 Antibody library generation

The main component in antibody discovery via any means of technology is the antibody library. In phage display, libraries of remarkably large sizes (10¹⁰) have been reported [92]. The assumption is that the affinities of the antibodies are proportionate to the size of the libraries [93]. A clear distinction between the terms antibody library source and format is vital to understanding the concept of recombinant human antibody production. Antibody sources can be categorized to two main classes based on the origin of antibody sequences being either natural or synthetic repertoires (Figure 1.6). Natural source repertoire libraries are generally either naïve or immunized libraries [92,94,95]. Synthetic source repertoires are constructed entirely in vitro and is devoid of natural biases and redundancies of in vivo antibody repertoires which allows the control of genetic information and introduce diversity [95,96,97].

Antibody libraries are a collection of genes encoding antibody formats. Antibody libraries are constructed by obtaining genes encoding antibodies from B-cells, then subjected to amplification via PCR to obtain substantial amount of DNA for cloning [98,99]. How the diversity in these genes are derived is the essence to the differences in antibody libraries. Three common formats of antibody libraries noteworthy are the immune, naïve and synthetic libraries [100,101].

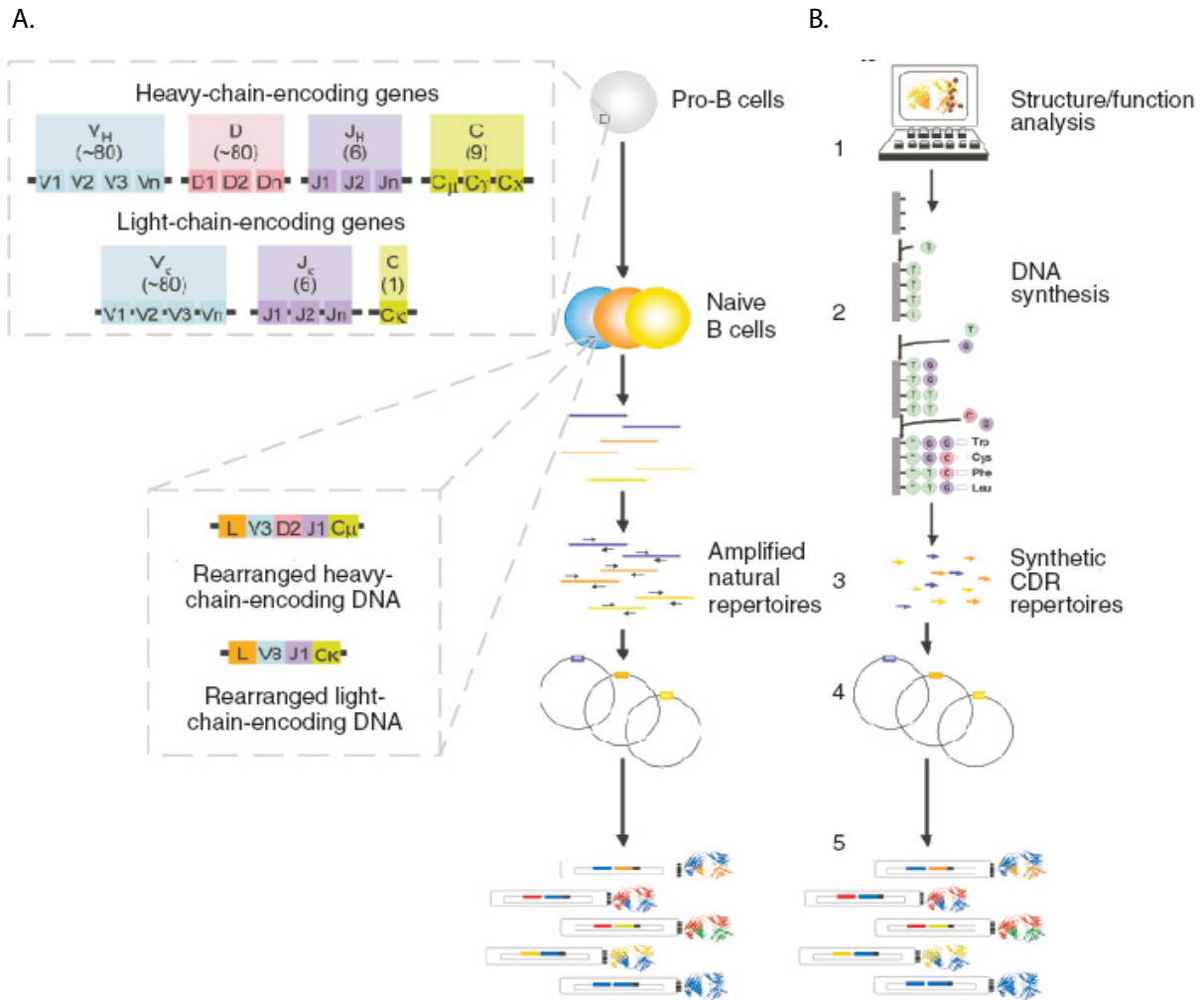


Figure 1.6: Comparison of the construction of antibody libraries from natural or synthetic sources. (A.) Antibody libraries from natural repertoires are derived by harvesting V_H and V_L genes from naive B cells. B-cell maturation (1) involves the rearrangement of germline antibody genes in pro-B cells to produce naive B cells that contain diverse, functional antibody genes. The gene encoding the heavy chain is formed first by the joining of three diversity elements (V_H , D and J_H), which together encode the variable domain, and a constant element (C_H), which encodes the constant region of immunoglobulin M (IgM). Subsequently, the gene encoding a κ (shown) or λ light chain is formed by the joining of two diversity elements (V_c and J_c , or V_L and J_L for λ light chains) that encode the variable domain and a constant segment (C_K or C_L) that encodes the constant domain. Gene segments that encode leader sequences (L) direct secretion of both chains. For library construction, mRNA from naive B cells is reverse transcribed to produce cDNA (2). V_H and V_L repertoires are amplified from the cDNA using PCR (3), and these are combined in a phage-display vector (4) to produce phage-displayed antibody repertoires (5). (B.) For the construction of synthetic antibody repertoires, insights from structural and functional analyses of functional antibodies (1) are used to design synthetic oligonucleotides (2) that introduce chemically and spatially defined diversity into the CDR loops (3). The synthetic CDR repertoires are incorporated into defined V_H and V_L framework genes in phage-display vectors (4) to produce phage-displayed antibody repertoires (5) [Figure taken from 98].

Immune libraries best uses repertoires from activated B-cells of the IgG family where the diversity has undergone in vivo maturation [102]. Naïve libraries derive repertoires from resting B-cells of the IgM family where the repertoire is immature [103]. Synthetic libraries on the other hand make use of cloned V genes and the diversity is created synthetically [104].

An immunized library, as the name would suggest, derives the antibody cDNA from patients that had prior exposure to a certain disease or infection and recovered. Here the natural antibodies which have reacted towards the infection are harvested from the B-cells. The Ig isotypes normally used for library generation is IgG [105] but other isotype libraries like IgA and IgE have been generated with reference to specific diseases such as coeliac disease [106,107] and latex allergic [108] patients respectively. Therefore the possibility to obtain antibodies against these antigens is higher as it is biased towards the given antigen. The size of an immunized library need not be as high as a naïve library due to the specialization of the immune reaction hence antibody production in vivo generated by the infection or disease [102]. Therefore, two main characteristics of an immunized library is the enriched population of antigen-specific antibodies and high affinity antibodies as some of these antibodies will have undergone affinity maturation [70,102]. The obvious complication to generate such libraries is the availability of B-cells as it is impossible to immunize humans. Some other set backs are such as the time required for recovery after exposure, tolerance to toxicity of certain antigens, lack of immune response to self antigens and most of all the need to generate a new library for a new antigen [105].

For the construction of naïve antibody libraries, IgM mRNA of B-cells from a pool of healthy donors without any exposure to any given antigen are normally used as sources for V-genes [103]. Even so, there have been cases where IgD, a subset of Ig family found to be expressed on B-cells together with IgM has been used for naïve antibody library generation [109,110]. B-cells are normally isolated from peripheral blood lymphocytes [111], bone marrow or tonsils [93]. Here a large library size is required for a better success rate due to the non-specificity of the antibodies possibly generated. A large library size is important for an increase chance of obtaining higher affinity antibodies and success rate of selection against a multitude of antigens [112,113]. Naïve libraries have a huge advantage as it is not biased to any antigen therefore it can be used to screen for any antigen with modest success [103]. Therefore, several large naïve libraries with average diversity of 10^{10} have been reported to successfully yield antibodies to various targets ranging from self, non-immunogenic, toxic antigens and chemical haptens [111,114,115].

Unlike the immunized libraries, the antibodies normally generated from naïve libraries have a tendency to exhibit lower affinities. To circumvent this problem, further diversification of the V-genes is required. When randomization of CDRs are carried out to increase the affinities of antibodies from naïve libraries, the resulting library would have a single framework with variations only in the CDRs. This library is called a semi-synthetic library as the main framework was derived from a naïve library subsequently modified *in vitro* to yield better antibodies [116,117,118].

The final class of antibody library is the synthetic library. With the tremendous developments and advancements in bioinformatics, it is now possible to study structural and amino acid preferences in antibody constructs. With the information generated bioinformatically regarding antibody epitopes [119], antibody-protein interactions [120], affinity maturation design [121], V-gene recombination patterns [122,123] and structural predictions of variable regions [124], a synthetic repertoire can then be generated. Studies on the variable gene repertoires have helped designers to better grasp the concept of antibody engineering. Work on structural repertoires helped to determine amino acid preferences and variability patterns in hypervariable loops [125,126,127,128]. Commonly, a stable antibody framework is used and randomisation is carried out at specific CDR regions to generate a synthetic repertoire [129]. CDR3 of the heavy chain is normally used as it is the region with the highest surface interaction with antigens [130]. As such, a lot of efforts have been invested to study the CDR3 of the heavy chain [131,132,133]. The extent of understanding led to the introduction of the H3-Rules where it governs the amino acid substitution at given positions within the CDR3 for better tertiary structure identification [134,135,136]. Other than differences in amino acid sequences in CDR3 of the heavy chain, the varying length of CDR3 also contributes to the diversity generated [132]. Such studies were also carried out for other CDRs to identify specificity-determining residues [137]. The gold standard for synthetic libraries would be the HuCAL antibody library where all six CDRs are randomized synthetically [138,139,140]. The different binding structures of antibodies correlates to the diversity due to the folds generated by varying amino acid sequences of both chains [141,142]. Work on light and heavy chain interactions have also assisted designers in designing better antibody structures [143,144,145]. Coupled with the innovations in mutagenesis technology, specific and accurate mutations can be introduced to a given antibody scaffold after proper designing. Multiple modifications and randomization can be carried out with high precision with new techniques in mutagenesis [92,146,147]. Besides generating a library, modifications could also be carried out post selection to increase the affinity of selected antibodies [147].

The obvious advantage of such libraries is the independence from natural immune system diversities. This would allow accessibility of higher diversity libraries for in vitro technologies. Synthetic in this instance would also function as naïve libraries thus allowing the user to screen for multiple targets without the limitations of the immune libraries. The use of a synthetic library is synonymous to a buffet dinner as the user would be spoilt for choices of antibody frameworks. Frameworks can be selected based on stability, high expression or lack of immunogenicity which is vital for therapeutics [96]. With all the promise in hand, the use of synthetic antibody libraries have not really taken off in comparison to naïve libraries. This is largely due to the complexity in designing and generating a synthetic library. For all the obvious advantages of synthetic libraries, it some what is the Achilles' heel of such libraries. This is because to generate a synthetic library would require extensive knowledge in molecular biology and great understanding of the antibody molecule. On top of that, the cost of generating such libraries is enormous, therefore not an ideal choice of library for most researchers. This is evident with the existing synthetic libraries belonging to four major financially sound institutions. This group made up of the Scripps Research Institute, the Medical Research Council, Morphosys AG and Genentech are responsible for majority of the work done in this field [96].

The main ingredient to ensure successful selection of useful antibodies is a primary phage library of high quality. Criterion for high quality would constitute a large functional size with a high antibody sequence diversity. This is to facilitate the selection of high affinity antibodies against a multitude of different antigens. Another important consideration is to ensure good expression levels of antibody genes to permit either miniaturization or automation of the panning process as well as up-scaling of the expression levels for production purposes. The library should also be designed to allow rapid engineering processes for optimization and lowering immunogenicity of lead candidates. Finally, the handling of the library and antibodies should be convenient [148].

1.5 Phage display panning procedures

Selecting binders to a specific target with a phage library is like looking for a needle in a molecular haystack. Therefore with a diverse amount of possible binders being selected, the ability to identify, isolate and recover the binder of interest is crucial. The main feature of phage display that makes it suitable for panning is the physical linkage between genotype and phenotype [47].

In phage display the panning is an iterative process where specific binder molecules are continuously enriched and multiplied from a pool of predominantly non-binders until the specific binders finally become the majority population [149]. This “panning“ method has been referred to the tool used by gold washers to isolate gold [83]. For panning, antigens can be anchored to various types of solid supports, such as magnetic beads [150], column matrix [151], nitrocellulose [152] or to a larger extent plastic surfaces in the form of polystyrene tubes [153] or 96 well polystyrene microtiter plates [154]. The selection process is an affinity-based enrichment process and involves multiple rounds of selection. The antibody presenting phage particles are incubated with the immobilized antigens to allow interactions to occur. Next, non-binders are removed from the selection matrix by washing off unbound phage particles. The bound phages are then used to infect *E. coli* and are subsequently re-amplified to be used in the following round. This selection cycle is normally repeated until a satisfactory enrichment is achieved. Normal panning protocols usually constitute between 2 to 4 rounds. A schematic representation of a conventional phage display panning process together with the life cycle of filamentous phage in *E. coli* is shown in Figure 1.7.

An attractive feature of phage display that makes it an ideal tool for selection of antibodies is the extent of flexibility it allows the user to determine conditions to conduct the selection in [155]. Each round of the panning procedure is divided to three main stages, binding, washing and elution / amplification. This is repeated normally from three to six rounds to obtain enrichment of phage presenting binders. Each stage of the panning procedure should be planned out before starting the selection process depending on the type of molecules presented and binding characteristics of the binder and target. Careful considerations should be taken into account for each of the panning stages and round. The amount of time for binding to occur should be higher in the earlier rounds and reduced in accordance with further rounds. This is due to the vast number of varied phage particles in the initial stages. Thus a longer incubation time could allow the minority binders to find the target and bind. As the propagation of phages

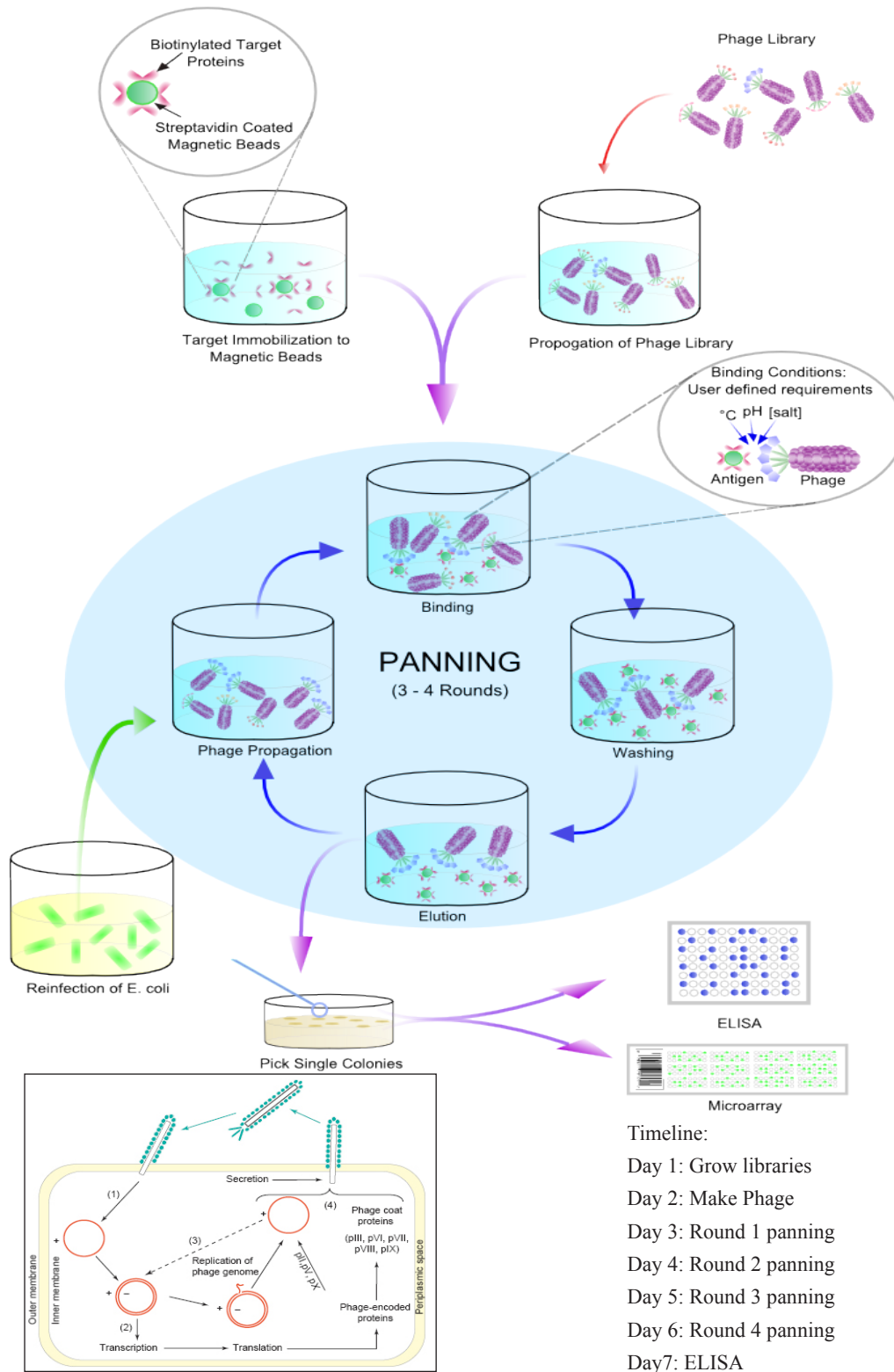


Figure 1.7: Phage display panning process with timeline with 4 panning rounds. Phage life cycle in *E. Coli* highlighted in box. The drawing is schematic and not to scale. (1) Phage bind to the *E. coli* cell through the pIII coat protein. The single-stranded viral genome (C strand, single circle) is injected into the cell and a complementary strand (K strand) is synthesized to form a double-stranded phage genome (double circle). (2) Subsequently, all ten phage-encoded proteins are produced by host-mediated protein synthesis, including coat proteins (pIII, pVI, pVII, pVIII and pIX), Proteins for replication (pII, pV and pX) and proteins involved in assembly and export (pI and pIV). (3) The phage genome is replicated using the (C)-strand as a primer and the (K)-strand as a template. (4) Virions are assembled and exported across the bacterial membranes [Phgae life cylce taken from155].

in each round is conducted, the number of phage particles with binders will proportionately increase with the number of rounds thus requiring shorter time periods to bind to the target. The stringency of the wash steps in the panning procedure is normally increased conversely to the panning round to efficiently remove false binders thus lowering background binding phage. As the selection rounds are carried out, the number of positive clones increases thus leaving a lower diversity in comparison to the initial rounds. Stringency of the wash step can be increased by multiple wash steps, increasing the wash times or by adding increasing concentrations of competing ligand to the wash buffer to remove binders with slower dissociation rate constants to be selected.

Elution/ amplification of selected phage particles is the final stage in each selection round. Elution of bound phage particles should be as complete as possible to ensure the maintenance of clonality. This directly effects the success of the re-amplification for the coming rounds. Several different strategies have been used to conduct phage recovery, such as change in pH [156], addition of mild chaotropic agents [157] or even with proteases although is dependent on the phage system used [129]. This is critical as sufficient phage particles should be eluted to ensure clones are re-amplified for good enrichment. The amplification process should be carried out with adequate excess of rapidly growing *E. coli* to successfully capture all the phage present in the pool. Coinfection with helper phage for the production of new phage particles for subsequent selection rounds is only required for phagemid based libraries [158,159].

The high throughput generation of antibodies via phage display could prove at times to work against it rather than for it. As the generation of high numbers of antibody clones is produced in bacterial hosts, this may also lead to loss of antibodies or to deletions/recombinations of the plasmid DNA caused by the metabolism of the bacteria. Another problem is to adapt the eukaryotic reading frames included in the plasmids to preferred codons in human genome [160]. Bacteria and mammals have varying populations of tRNAs which leads to varying preferences in codon usage for certain amino acids [161]. This can cause some clones to be lost due to weak or absent expression of the sequence in *E. coli*. An approach to overcome this bottleneck is to have codon based mutagenesis of clones in vitro. Expression levels can be predicted using codon bias thus allowing improvements of expression levels [162,163]. Codon optimization has been shown to increase expression of recombinant proteins [164,165]. Proper handling of library stocks is important as contamination with wild type filamentous phages or helper phages, whole libraries can be lost [160].

2.0 Objective

The generation of human antibodies by the immune system is a multi-stage process with each stage contributing to the overall success of antibody production in-vivo. In vitro mimicry of the antibody fabrication process is also bound by the same multi-level process complexities. Here, we considered the in-vivo processes for antibody production and superimposed them to conventional in-vitro antibody production to identify key parameters essential in the production of human antibodies - exclusively with phage display. Each parameter identified would be studied as a reflection of the actual in vivo provision.

As there are various antibody formats used for library generation, the need to ascertain the best format to generate a library should be determined first. As there are several different isotypes circulating in the blood, a method to replicate this flexibility would require the design of a series of equally flexible phagemid vectors. The phagemid vectors must be able to facilitate in-vitro class switching and format switching in the context of phage display. Therefore, phagemid vectors that allow easy conversion of scFv to IgD and IgG Fab will be generated. Two different isotypes of Fab formats of IgD and IgG origin will be generated using the repertoire from a semi-synthetic scFv library.

To simulate the antibody secretion by plasma cells, phage display is used to conduct selections against antigens. As the success of the panning is dependent on the successful presentation of antibody fragments on the phage surface, several formats would be used for analysis. To ensure reliable comparability, semi-synthetic IgD Fab and IgG Fab libraries is to be subcloned from a semi-synthetic scFv library for selection rather than a naïve library. As scFv formats are documented to thrive at presentation on phage surfaces, the challenge would be to compare the performance of two different Fab formats (IgD and IgG) on phage display.

Format switching of binding clones would be carried out to ascertain if functionality is maintained, improved, or lost after format switching. The best format for library generation would be evaluated based on downstream application, presentation efficiency and flexibility for format switching.

The immune repertoire generation would require designing a collection of V-gene primers with a high coverage of the natural immune repertoire present in the immune system. V-gene primers based on the best format would be designed and evaluated. An optimized protocol is also required to ensure the utmost amplification of the V-gene repertoire due to the high complexity of the natural repertoire. The generation of an antibody portfolio using the established protocol is to replicate the antibody diversity of circulating B-cells in-vivo. From a pool of donors, a collection of V-genes would be amplified using the protocol to attain the preface for an in-vitro antibody library generation.

3.0 Materials

3.1 Consumables

<i>Materials</i>	<i>Manufacturer</i>
RNA 6000 NanoChip®	Agilent Technologies, USA
Parafilm M	American National Can™
Vacutainer® EDTA tubes	BD Biosciences, Heidelberg, Germany
Falcon ® 96-well PVC (flexible) flat-bottom plate	
Whatman-paper 3 mm Chr	Biometra biomedizinische Analytik, Göttingen, Germany
Gene pulser elektroporation cuvettes (1mm)	BioRad Laboratories GmbH, Munich, Germany
Safe-seal Tips (10 µL, 200 µL, 300 µL and 1 mL)	BioZym GmbH, Oldendorf, Germany
1 mL UV cuvettes	Brand GmbH & Co KG, Wertheim, Germany
Sterile disposable pipettes 5 mL, 10 mL and 25 mL	Corning GmbH, Kaiserslautern, Germany
Centrifuge tubes CentriStar™ Cap (15 mL and 50 mL)	
50 mL reagent reservoir	
Reaction tubes (0.5 mL, 1.5 mL and 2.0 mL) UVette	Eppendorf AG, Hamburg, Germany
Hybond ECL Nitrocellulose membrane	GE Healthcare, Munich, Germany
100 mm Petri dishes	Greiner Bio-One GmbH, Frickenhausen, Germany
2 mL Cryo tubes	
PCR reaction tubes	
PP-tube (14 mL)	
Sterile pipette tips (20 µL, 200 µL and 1 mL)	
NuPAGE Novex 4-12 % Bis-Tris Midi Gel	Invitrogen GmbH, Karlsruhe, Germany
NuPAGE 4-12 % Bis-Tris Gel	
Immobilon-P PVDF Transfer Membrane	Millipore GmbH, Eschborn, Germany
MultiScreenHTS Plates with hydrophilic Durapore PVDF membrane with 0.65 µm pore size	
Lazy spreader	Sigma-Aldrich Chemie GmbH, Munich, Germany
AeraSeal breathable sealing film	
Pyrex Solid Glass beads (3 mm)	
Disposable inoculating loops	Thermo Fisher Scientific (Nunc GmbH & Co. KG), Langenselbold, Germany
96-well polypropylene MTP	
MaxiSorb, 96-well, flat-bottom MTP	

3.2 Laboratory equipment

Apparatus

Centrifuges

Micro Centrifuge SD (for PCR tubes)
Centrifuge 5415 C
Centrifuge 5810 R (with cooling system)

Manufacturer

Carl Roth GmbH, Karlsruhe, Germany
Eppendorf AG, Hamburg, Germany

Incubators

Peltier-Cooled Incubator IPP500
Microtitre plate incubator shaker iEMS

Memmert GmbH, Schwabach, Germany
Thermo Scientific, Rockford, USA

Shakers and Mixers

Thermomixer Comfort
Rocky, shaker for filters and gels
Vortex Genie 2-Mixer

Eppendorf AG, Hamburg, Germany
Fröbel Labortechnik, Wasserburg, Germany
Bender & Hobein AG, Zürich, Switzerland

General Laboratory Equipments

Agilent 2100 Bioanalyzer
Bio-Rad Gel Doc 2000
Bio-Rad Micropulser Electroporator
Peltier Thermal Cycler PTC-200
BioPhotometer
Water bath Grant Sub 14
Digital pH-/mV-/Thermometer GMH 3510
Electrophoresis Power Supply: EPS 200,
EPS 300, EPS 301, EPS 600
Branson Sonifier 250
Blotting device, semi-dry, for protein gels
Hofer TE 70
Gel electrophoresis equipment Hofer SE
200
Magnetic stirrer Hotplate MR 3001
X Cell4 Surelock™ Midi cell, X Cell Sure
Lock™ Mini-Cell
Magnetic Particle Concentrator for MPC-E
Magnetic Particle Concentrator for MPC-96

Agilent Technologies, USA
BioRad Laboratories GmbH, Munich,
Germany
Eppendorf AG, Hamburg, Germany
Grant Instruments, Cambridge, UK
Greisinger electronic GmbH, Regenstauf,
Germany
GE Healthcare, Munich, Germany
Heinemann GmbH, Schwäbisch Gmünd,
Germany
Hofer Inc, Holliston, USA
Heidolph, Frankfurt, Germany
Invitrogen GmbH, Karlsruhe, Germany

Apparatus

Manufacturer

General Laboratory Equipments

Autoklav Typ 23	Melag GmbH, Berlin, Germany
Weighing machine Adventure Pro AV812	Ohaus, USA
MTP spectrophotometer, SpectraMAX 250	Molecular Devices, Sunnyvale, CA, USA
Freezer, -80°C Forma	Thermo Scientific, Oberhausen, Germany
Microflow Laminar Flow Workstation	
NanoDrop ND-1000	

3.3 Softwares

Software

Manufacturer

Acrobat 7.0	Adobe Systems Incorporated, USA
Adobe Illustrator CS2	
Adobe InDesign CS2	
Adobe Photoshop CS2	
GraphPad Prism 5	GraphPad Software Inc, USA
Vector NTI Suite 10.0	Invitrogen GmbH, Karlsruhe, Germany
Microsoft Excel 2003	Microsoft Co, USA
Microsoft Word 2003	
EndNote 10.0	Niles Software Inc., USA
Ascent	Thermo Scientific, Oberhausen, Germany

3.4 Chemicals, buffers and solutions

Unless stated otherwise, all used chemicals have been bought at the companies Bio-Rad, New England Biolabs, Fermentas, peqlab, QIAGEN, Sigma-Aldrich, Merck, Roche, Thermo Scientific and Roth.

Common buffers

PBS	0.0027 M KCl, 0.137 M NaCl, 0.01 M phosphate buffer, pH 7.4. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na ₂ HPO ₄ and 0.24 g KH ₂ PO ₄ in 800 mL of ddH ₂ O. Adjust the pH to 7.4 with HCl. Add ddH ₂ O to 1 L.
4 % BSA	4 g BSA in 100 mL PBS.

4 % Milk Powder	4 g dry milk powder in 100 mL PBS.
10 x HMFM (freezing mix)	Dissolve 72 g KH_2PO_4 and 188 g K_2HPO_4 in 800 mL ddH ₂ O and autoclave. Dissolve 3.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 18 g $\text{Na}_3\text{-citrate} \cdot 2\text{H}_2\text{O}$, 36 g $(\text{NH}_4)_2\text{SO}_4$ and 1,760 g glycerol (1,645 ml of 87% glycerol) in 3.2 L ddH ₂ O, autoclave and add 800 ml of the phosphate solution.
40 % (w/v) glucose	400 g D-+-glucose monohydrate dissolved in ddH ₂ O to 1 L and sterilised by filtration through a 0.2 µm pore size filter.
70 % (v/v) Ethanol	700 mL of technical grade Ethanol dissolved in ddH ₂ O to 1 L.
1 M IPTG	1.19 g of Isopropyl-β-D-thiogalactoside in 5 mL ddH ₂ O. Store at -20°C.
0.5 M EDTA, pH 8.0	Dissolve 186.1g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ in 800 mL ddH ₂ O. Adjust pH to 8.0 with NaOH (~20g of NaOH pellets). Adjust volume to 1 L with ddH ₂ O.
8 M Urea	Dissolve 48 g of Urea in 100 mL of ddH ₂ O.
20% PEG 6000/ 2.5 M NaCl	Dissolve 200g of PEG 6000 and 146 g of NaCl to 1 L with ddH ₂ O.
7.5 M Ammonium acetate, pH 7.5	Dissolve 57.8 g ammonium acetate in approximately 50 mL ddH ₂ O. Adjust pH to 7.5 with ammonium hydroxide. Adjust to final volume of 100 mL.
1 M $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$	Dissolve 264 g of $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ in 1 L of ddH ₂ O.
100 % Ethanol	
1 M Tris-HCl (pH 6.8)	Dissolve 12.1 g Tris base in 50 mL of ddH ₂ O. Adjust pH with concentrated HCl and add ddH ₂ O to a final volume of 100 mL.
1 M DTT	Dissolve 770 mg Dithiothreitol in 10 mL ddH ₂ O. Store at -20°C.
GlycoBlue	Applied Biosystems, USA.
UltraPure Agarose	Invitrogen GmbH, Karlsruhe, Germany
Biocoll	Biochrom AG, Berlin, Germany.

SDS-PAGE Buffers

4 x SDS loading buffer	0.4 % bromophenol blue, 50 % (w/v) glycerol, 8 % SDS, 0.4 M DTT and 0.2 M Tris-HCl pH 6.8. To prepare 7.5 mL of buffer, dissolve 1.5 mL of 1 M Tris-HCl (pH 6.8), 3 mL of 1 M DTT, 0.6 g of SDS powder, 0.03 g of bromophenol blue and 3 ml of 100% glycerol.
Coomassie blue staining solution	Dissolve 1.25 g Coomassie Brilliant Blue G 250 in 225 mL technical grade ethanol, 225 mL ddH ₂ O and 50 mL acetic acid were added. The mixture was stirred for 2 hr and filtered through a folded filter paper.
Destain solution	20 mL of ethanol and 10 mL of acetic acid in a final volume of 100 mL with ddH ₂ O.
20x MES buffer	Dissolve 195.2 g of MES, 121.2 g of Tris Base, 20 g of SDS and 6 g of EDTA in 800 mL of ddH ₂ O. Adjust the pH to 7.3 and add ddH ₂ O to a final volume of 1 L. The final concentration would be 1 M MES, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA, pH 7.3.

Western Blot and ELISA Buffers

Transfer buffer	Dissolve 4.2 g of Tris Base with 19.6 g of Glycine in ddH ₂ O to a final volume of 1.4 L.
Blocking and dilution buffer (PTM)	Dissolve 2 g of Milkpowder and 1 mL (v/v) Tween 20 to 100 mL of PBS.
Wash buffer (PBS-T)	Dissolve 1 mL (v/v) Tween 20 in 1 L of PBS
Citrate buffer	10 mL of 50 mM Tri-sodium citrate with 10 mL of 50 mM citric acid
Development substrate hydrogen	1 ABTS tablet with citrate buffer and 10 μL of peroxide.

Gel Electrophoresis Buffers

Running buffer (10x TBE)	Dissolve 1 g NaOH, 108 g Tris Base, 55 g Boric Acid and 7.4 g EDTA 700 mL ddH ₂ O in 2 L flask with stirring. Add ddH ₂ O to bring up total volume to 1 L.
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3.5 Media

2YT-Broth

31 g premixed 2YT-Broth (16 g tryptone, 10 g yeast extract, 5 g NaCl) was dissolved in dH₂O to 1 L, sterilised by autoclaving at 120°C for 20 min and cooled to RT. Prior to use, the appropriate amounts of antibiotics, glucose and IPTG was added.

2YT-Broth Agar

46 g premixed 2YT-Broth Agar (16 g tryptone, 10 g yeast extract, 5 g NaCl, 15 g agar) was dissolved in dH₂O to 1 L, sterilised by autoclaving at 120°C for 20 min and cooled to 55°C in a waterbath. Agar medium was supplemented with appropriate amounts of antibiotics and glucose and poured into 90 mm diameter Petri dishes under a laminar flow. After solidification agar plates were packaged in plastic bags and stored at 4°C in a dark place until use, but for a maximal period of 20 days.

Top Agar

Dilute 100 mL of heated 2YT-Broth Agar with 100 mL of 2YT-Broth.

SOB Media

20 g of Tryptone, 5 g of yeast extract and 0.5 g of NaCl was dissolved in ddH₂O to 1 L, sterilised by autoclaving at 120°C for 20 min and cooled to rt.

SOC Media

For 100 mL of SOC media, 1 mL of 40% glucose, 1 mL of 1 M MgCl₂ and 1 mL of 1 M MgSO₄ was made up to 100 mL using SOB media.

3.6 Additives

Ampicilin :- Stock: 50mg/mL in 50% EtOH; Working concentration: 100µg/mL

Chloramphenicol :- Stock: 34mg/mL in ddH₂O; Working concentration: 17µg/mL

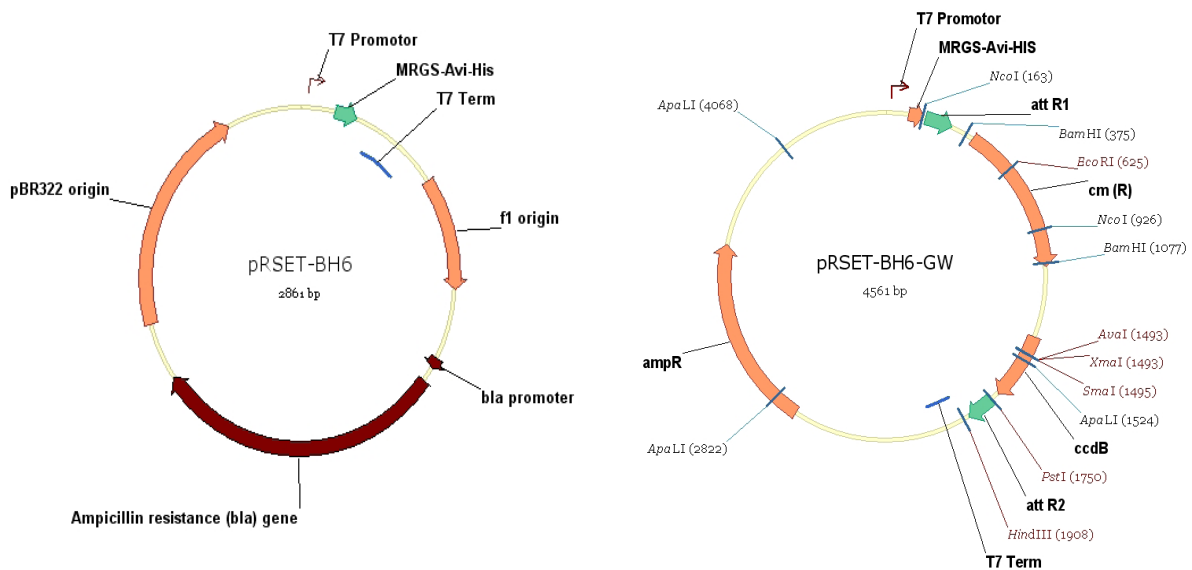
Kanamycin :- Stock: 30 mg/mL in ddH₂O ; Working concentration: 60 µg/mL

3.7 Microorganisms and Eukaryotic cell lines

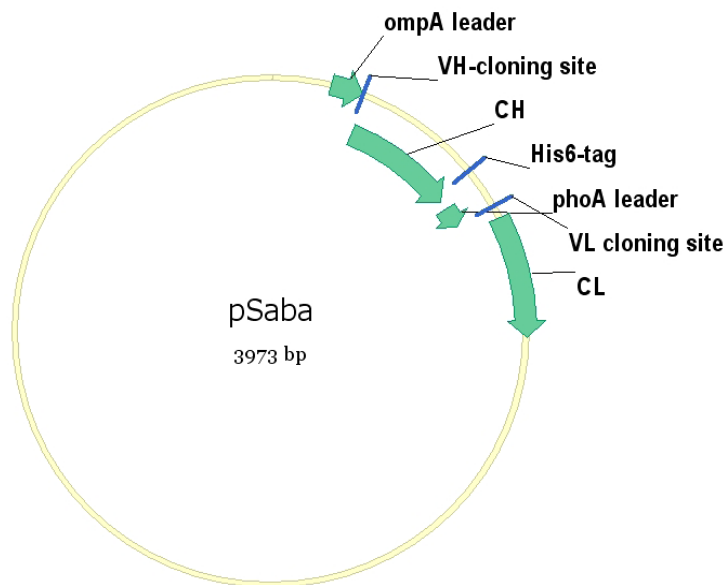
- DB3.1 F⁻ *gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(rB⁻, mB⁻) ara14galK2 lacY1 proA2 rpsL20(Smr) xyl5 Δleu mtl1-* from Invitrogen GmbH, Karlsruhe, Germany.
- DH10B F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ⁻ rpsL nupG* - from Invitrogen GmbH, Karlsruhe, Germany.
- TG1 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rK⁻ mK⁻) [F' traD36 proAB lacIqZΔM15]-* from Stratagene, Agilent Technologies, USA.
- HB2151 *nalr thi-1 ara (lac-proAB [F' proAB+ laciq lacZ(M15)]* - from Stratagene, Agilent Technologies, USA.
- BL21 Star™(DE3) F⁻ *ompT hsdSB(rB⁻, mB⁻) gal dcm rne131 (DE3)* - from Invitrogen GmbH, Karlsruhe, Germany.
- Helper phage M13-K07 - from New England Biolabs, Frankfurt, Germany.
- Hyperphage is courtesy of Prof Stephan Dübel, TU-Braunschweig.

3.8 Plasmids

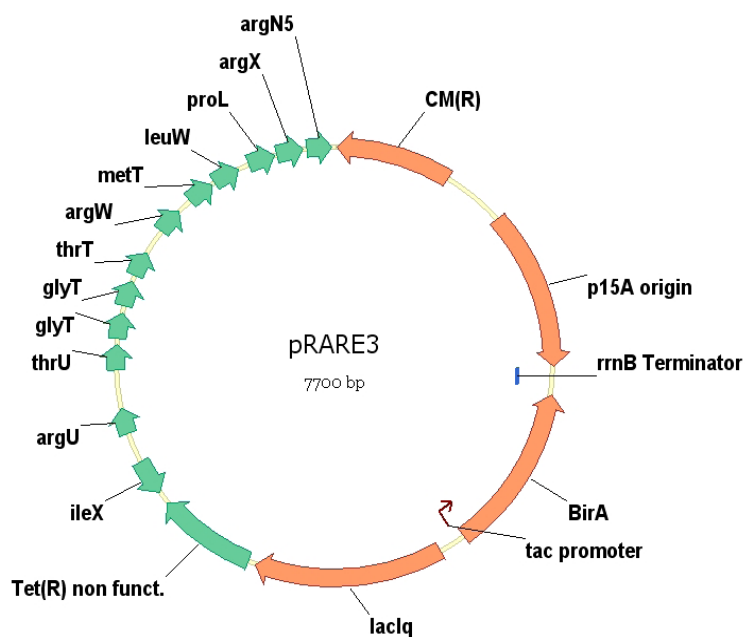
pRSET-BH6 was used as for the recombinant expression of in vivo biotinylated target antigens.
 pRSET-BH6-GW was used for cloning full length proteins by gateway recombination.



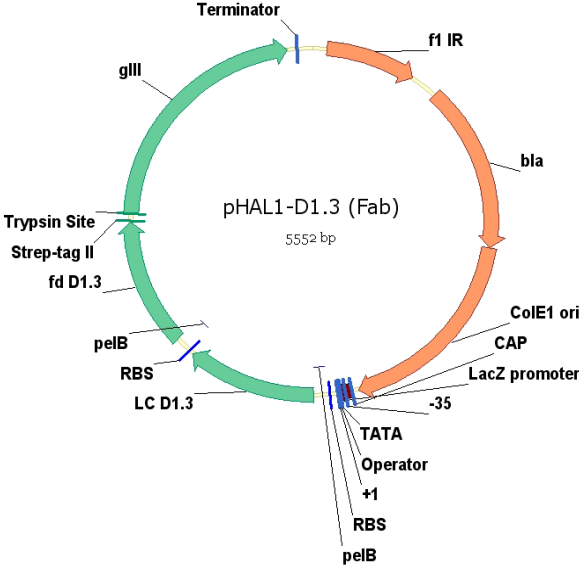
pSABA courtesy of Dr Bars was used as for the generation of the pTSL phagemid vector series.



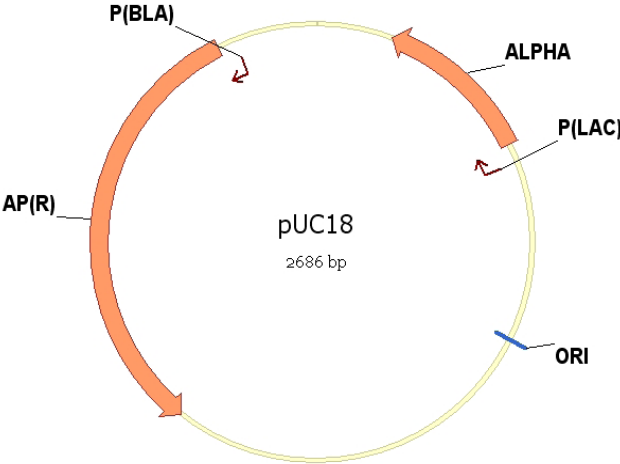
pRARE3 was used as a helper plasmid for the expression of recombinant proteins. It harbours the biotin ligase for co-expression with the target protein to have efficient in vivo biotinylation as well as 12 rare codon tRNA genes.



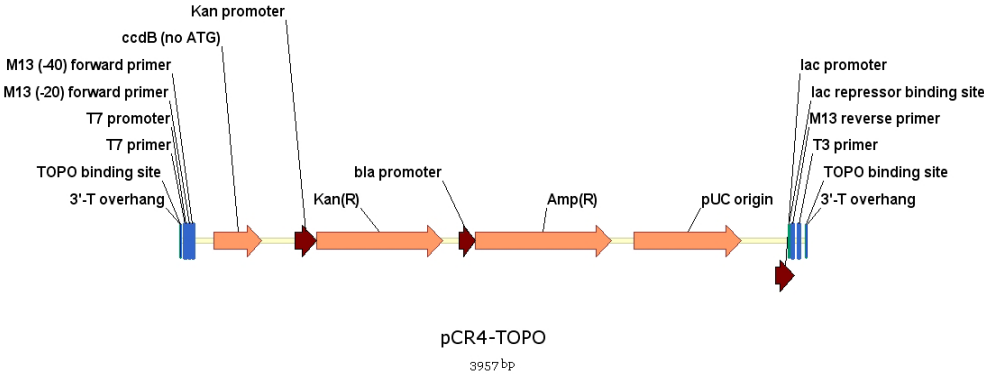
pHAL-D1.3 was used as for the generation of the pTSL phagemid vector series (Courtesy of Dr Hust, TU-Braunschweig).



pUC18 was used as for the generation of the pTSL phagemid vector series.



pCR4-TOPO was used as for the generation of the pTSL phagemid vector series.



3.9 Recombinant Proteins, Affinity Columns and Magnetic Beads

Biotinylated Protein L, Invitrogen GmbH, Karlsruhe, Germany

Protein L-HRP conjugated, Invitrogen GmbH, Karlsruhe, Germany

Streptavidin-HRP, Thermo Scientific, Oberhausen, Germany

Ni-NTA agarose beads, Qiagen GmbH, Hilden, Germany

Protino Ni-TED silica beads, Macherey-Nagel GmbH & Co. KG, Düren, Germany

MonoAvidin beads, Thermo Scientific, Oberhausen, Germany

Dynabeads® M-280 Streptavidin, Invitrogen GmbH, Karlsruhe, Germany

3.10 Antibodies

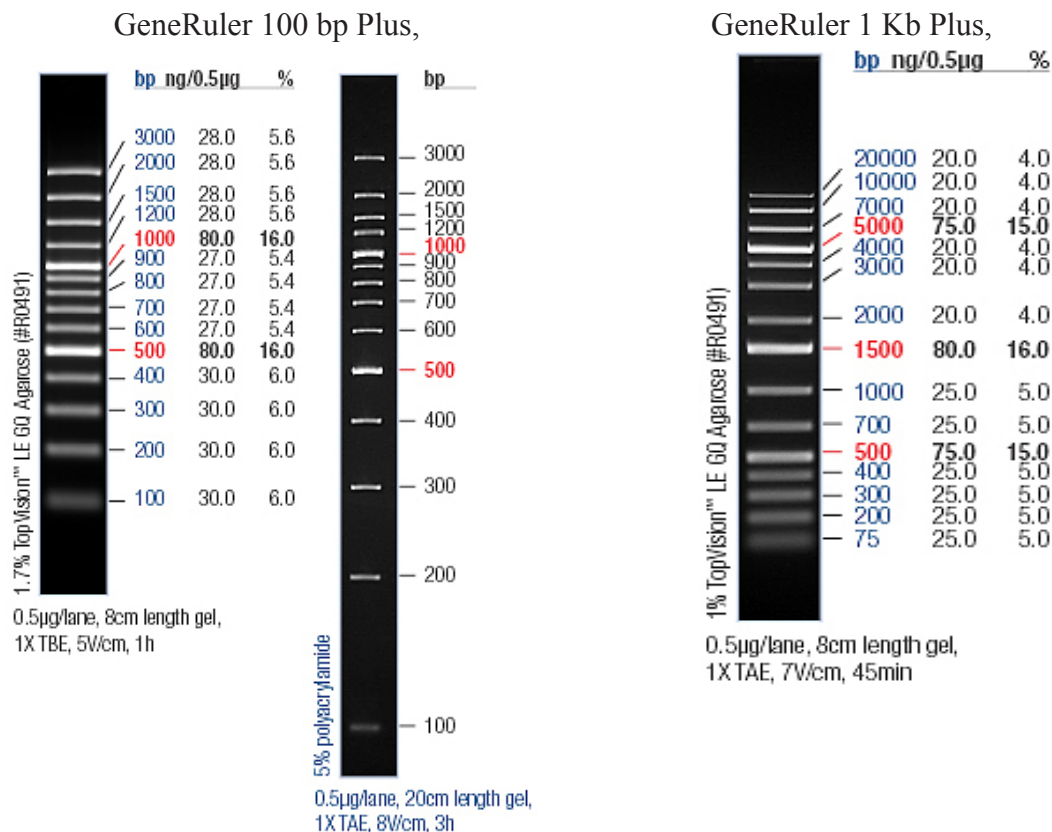
Anti-M13-HRP, mouse mAb, GE Healthcare, Munich, Germany

Anti-Mouse IgG-HRP, rabbit Sigma-Aldrich Chemie GmbH, Munich, Germany

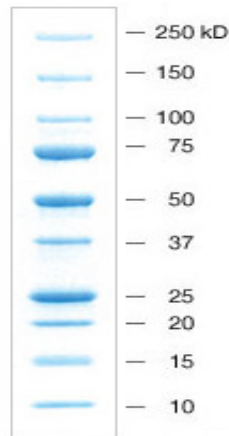
Anti-Penta•His antibody, mouse mAb Qiagen GmbH, Hilden, Germany

3.11 DNA and Protein Markers

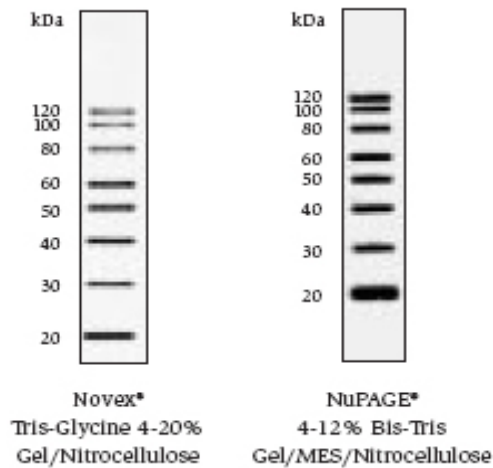
DNA markers from Fermentas GmbH, St. Leon-Rot, Germany



Pre-stained precision protein marker Bio-Rad Laboratories GmbH, München, Germany



MagicMark XP Western Protein Standard, Invitrogen GmbH, Karlsruhe, Germany



3.12 Kit Systems

Kit Name

PAGE Blue stain

Protino® Ni-TED Resin

SERVA Silver Stain Kit

Pierce 660 nm Protein Assay

Protein L Fast Flow Column

Monomeric Avidin Kit

RNeasy Kit

QIAprep Spin Miniprep Kit

QIAGEN Plasmid Maxi Kit

QIAquick PCR Purification Kit

QIAquick Gel Extraction Kit

Manufacturer

Fermentas GmbH, St. Leon-Rot, Germany

MACHEREY-NAGEL GmbH & Co. KG,
Düren, Germany

SERVA Electrophoresis GmbH, Heidelberg,
Germany

Thermo Scientific, Oberhausen, Germany

Qiagen GmbH, Hilden, Germany

3.13 Enzymes and Molecular Biology Materials

<i>Enzyme Name</i>	<i>Manufacturer</i>
<i>Restriction Endonuclease</i>	
BamHI	New England Biolabs, Frankfurt, Germany
BsrGI	
EcoRI	
HindIII	
MluI	
NcoI	
NotI	
SalI	
XhoI	
<i>Polymerase</i>	
Bio X Act Short	BioLine, Luckenwalde, Germany
High Fidelity PCR Enzyme Mix	Fermentas GmbH, St. Leon-Rot, Germany
Recombinant Taq Polymerase (House Taq)	Max Planck Institute For Molecular Genetics
Phusion Hot Start Polymerase	New England Biolabs, Frankfurt, Germany
REDTaq	Sigma-Aldrich Chemie GmbH, Munich, Germany
<i>Ligase</i>	
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
T4 DNA Ligase	Promega GmbH, Mannheim, Germany
<i>Phosphatase</i>	
Antartic Phosphatase	New England Biolabs, Frankfurt, Germany
<i>Reverse Transcriptase</i>	
SuperScript® II	Invitrogen GmbH, Karlsruhe, Germany
totalscript-OLS®	Omni Life Science, Hamburg, Germany
<i>Other Materials</i>	
ET SSB	New England Biolabs, Frankfurt, Germany
Oligo(dT)12-18 Primer	
RNaseOUT	
Ribonuclease H	

Enzyme Name***Manufacturer******Other Materials***

Random Primers	New England Biolabs, Frankfurt, Germany
10 mM dNTP mix	
S1 Nuclease	Roboklon GmbH, Berlin, Germany
DNAse	Sigma-Aldrich Chemie GmbH, Munich, Germany
Lysozyme	

3.14 Oligonucleotides

All oligonucleotides were ordered from Invitrogen GmbH, Karlsruhe, Germany with a synthesis scale of 50 nmol. All primary stocks were diluted to 100 µM and working stocks to 10 µM.

Primer Name***Sequence******Phagemid Cloning***

3'-ccdB-NotI	TGACTGCGGCCGCTAGTGTCATAGTCCTGAAAAT
3'-ccdB-XhoI	TGACTCTCGAGTAGTGTCATAGTCCTGAAAAT
5'-ccdB-Sall	ACTGTGCGACTATGCTATGAAGCAGCGT
5'-ccdB-BssHI-NcoI	ACTGCGCGCCATGGTATGCTATGAAGCAGCGT
BamHI/MluI His Tag Rv	GCGGGATCCGTGGTGTATGGTGGTGTATGACGCGT- GCAAGATTTGGATCGCTCAACTTTC
HindIII-VL-pSABA-Fw	CATCCCAAGCTTCCCGGGAGAAAATAAAGT- GAAACAAAG
IgD CH1 MluI Rv	GCCTCCACGCGTAGATTCTGGCCAGCGGAA- GATCTCCTTCTTACTCTTGCTG
IgD CH1 XhoI Fw	CTCGAGCCCAACCAAAGCAGATGTGTTTCCAATCAT- ATCAGGGTGCA
IgG CH1 MluI Rv	GCCTCCACGCGTGCAAGATTTGGGCTCAACTTTCTT- GTCCACCTTGGTGTGTC
IgG CH1 XhoI Fw	GCCTCCCTCGAGCACCAAGGGCCCATCGGTCTTC
LMB3 Fw	CAGGAAACAGCTATGAC
NcoI/XhoI His Tag Fw	GTAAACCCATGGCCGAGTAATAAGTCTCGAGCG
NheI/HindIII-CL-pSABA-Rv	CATGAAGCTTGCTAGCTTAACACTCTCCGCGGTT- GAAGCTCTTTGTGACGGGCG
pIII Rv	GTTAGCGTAACGATCTAA
pTSL HC Fw	GCTAGCAAGCTTCATGCAA
VH-Tom-NcoI-Fw	ACATGCCATGGCCGAGGTGCAGC
VH-Tom-XhoI-Rv	CCGCTCGAGACGGTGACCAGG
VL-Tom-Sall-Fw	AGCGGGTCGACGGACATCCAGATG

<i>Primer Name</i>	<i>Sequence</i>
<i>V-Gene Repertoire</i>	
<u>Variable Heavy Chain</u>	
VH1	CAGGTCCAGCTKGTRCAGTCTGG
VH157	CAGGTGCAGCTGGTGSARTCTGG
VH2	CAGRTCACCTTGAAGGAGTCTG
VH3	GAGGTGCAGCTGKTGGAGWCY
VH4	CAGGTGCAGCTGCAGGAGTCSG
VH4-DP63	CAGGTGCAGCTACAGCAGTGGG
VH6	CAGGTACAGCTGCAGCAGTCA
<u>Variable Light Kappa Chain</u>	
VK1	GACATCCRGDTGACCCAGTCTCC
VK246	GATATTGTGMTGACBCAGWCTCC
VK3	GAAATTGTRWTGACRCAGTCTCC
VK5	GAAACGACACTCACGCAGTCTC
<u>Variable Light Lambda Chain</u>	
VL1	CAGTCTGTSBTGACGCAGCCGCC
VL1459	CAGCCTGTGCTGACTCARYC
VL15910	CAGCCWKGKCTGACTCAGCCMCC
VL2	CAGTCTGYCTGAYTCAGCCT
VL3	TCCTATGWGCTGACWCAGCCAA
VL3(DPL16)	TCCTCTGAGCTGASTCAGGASCC
VL3(38)	TCCTATGAGCTGAYRCAGCYACC
VL6	AATTTTATGCTGACTCAGCCCC
VL78	CAGDCTGTGGTGACYCAGGAGCC

3.15 Antibody library repertoire

The semi-synthetic antibody repertoires used were of human origin and were cloned into phagemid vectors. The antibody fragments are displayed as single-chain variable Fragments (scFv). The library is courtesy of Dr. Ian M. Tomlinson and Dr. Greg Winter, MRC – Centre of Protein Engineering, Cambridge, UK. The randomised (DVT) positions in the side chains are based mainly on those positions which are diverse in the primary – germline – repertoire (total of 18 residues – H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96). 96% of the clones tested contain an insert and the library size is estimated to contain~ 1.5 x 10⁸ primary clones.

3.16 Magnetic Particle Processor

The Magnetic Particle Processor (Figure 2.1) was used to conduct the washing and incubation of magnetic particles. It is a prototype instrument of the KingFisher magnetic particle processor series of Thermo Scientific. It enables the handling of 96 magnetic pins, corresponding to the positions of a 96-well microtitre plate (MTP). The MPP has three predefined positions for 96-well plates and can accommodate three MTP's filled with the individual buffers for each wash or incubation step respectively. The magnetic particles are transferred between wells by a capture and release motion using the rod-shaped magnets covered with plastic caps. The modular system of the MPP allows the use of any number of 8-magnet columns between one and twelve. Each individual column position (1-12) on a MTP can be independently programmed. Instrument control is dependent on the Ascent software provided, and parameters such as time, position, frequency and strength of shaking movements is user-defined. Therefore, the MPP allows reproducible control of each step of the phage display selection protocol for as many as 96 parallel selections.

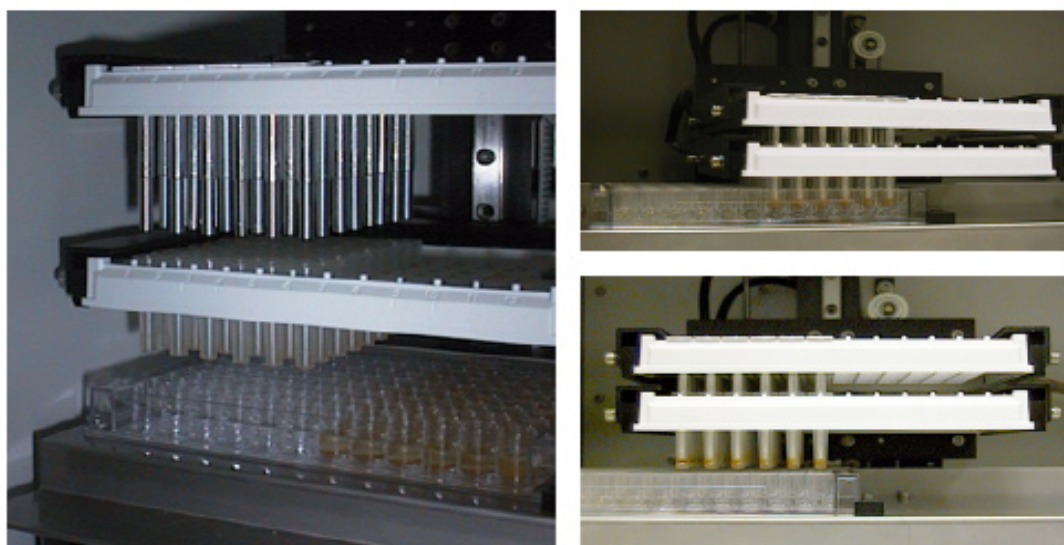


Figure 3.1: Magnetic particle processor. Left: rod-shaped magnets and plastic caps separated, magnetic particles in solution in microtitre plate wells. top right: magnets in plastic caps, collection of magnetic particles to plastic caps; bottom right: transfer of magnetic particles to new pre-filled microtitre plate wells.

4.0 Methods

4.1 Molecular Biology based methods

4.1.1 Medium for *E. coli* cultivation

The 2YT liquid- and solid media were sterilized for 20 min at 121°C and 1 bar. Antibiotics and glucose, if necessary, were added before use after sterilization. The medium was stored at rt.

4.1.2 Storage conditions and regrowth of bacterial cultures

Bacteria cultures can be stored at 4°C for a short time. For long-term storage at -80°C, an overnight culture was mixed with 80% glycerol (0.9 mL culture + 0.625 mL glycerol) and stored o/n at -20°C. For strain reactivation, a small amount of the frozen culture in the cryotubes was scraped off and put into fresh preheated 2YT medium.

4.1.3 OD measurements of cultures

For the evaluation of the correct cell density to make a suitable dilution, all optical densities have been measured at a wavelength of OD₆₀₀ against a blank value (media).

4.1.4 Preparation of electrocompetent cells

For the production of electro competent *E. coli* cells (strain DH10B, TG1, HB2151 and DB3.1) 50 mL 2YT medium in a 250 mL Erlenmeyer flask were inoculated with bacteria culture from glycerol stock and grown o/n at 37°C. On the following day 500 mL of preheated 2YT medium in a 2 L Erlenmeyer flask were inoculated with bacterial o/n culture and grown at 37°C until OD₆₀₀ 0.25-0.50 was reached. The culture was chilled on ice for 30 min, separated to 4 pre chilled 250 mL bottles and then centrifuged for 15 min at 4°C and 4000 rpm. The cell pellet was resuspended in 125 mL ice-cold Millipore water and kept on ice for 15 min. Another centrifugation step was done as before. The pellet was resuspended in 50 mL ice-cold Millipore water, transferred to 50 mL Falcon tubes and kept on ice again for 15 min. After another centrifugation step the pellet was resuspended in 25 mL ice-cold 10% glycerol and kept

on ice for 15 min. Once again the centrifugation was repeated. In a final step the pellet was resuspended in 2 mL ice-cold 10 % glycerol. 50 μ L aliquots were made and quick-frozen with liquid nitrogen in 0.5 mL tubes. Storage is done at -80°C.

4.1.5 Purification of Plasmid

When 5 mL of *E. coli* culture were purified to isolate the plasmid – DNA the “QIAprep Spin Miniprep” Kit of QIAGEN was used. In the purification of 100-200 mL bacteria culture, the “Plasmid Maxi Kit” of QIAGEN was used. The purifications were done to the manufacturer’s specifications.

4.1.6 Gel electrophoresis separation of DNA

The agarose gel electrophoresis is a method for size dependent separation of DNA. At first an agarose gel (0.7-1.2 % in 0.5x TBE) with ethidium bromide (1 % v/v) was prepared. The DNA was mixed with loading buffer and loaded into small pockets of the gel. The separation was done in an electrical field with 80-120 V and 150-400 mA. For the documentation and visualization of DNA – fragments the ‘Gel Doc 2000’ station (BioRad) was used.

4.1.7 DNA extraction from gel electrophoresis

The extraction of DNA-fragments out of an agarose gel was carried out with the ‘QIAquick Gel Extraction’ Kit of QIAGEN and to the manufacturer’s specifications.

4.1.8 DNA concentration with Ethanol precipitation

7.5 M of ammonium acetate was added at a 1/10 volume of the DNA sample together with 2 μ L of GlycoBlue. Then 100 % ethanol was added at 2.5 times of the total volume. The sample was snapped frozen in liquid nitrogen for 5 min. The frozen pellet was centrifuged at 14000 rpm for 15 min at 15°C. The supernatant was removed slowly by inverting the tube on a clean linen free towel. The pellet was stored at -20°C until use.

4.1.9 DNA concentration determination

1.5 μL of DNA was used for concentration determination using the NanoDrop ND1000. Duplicate readings are taken and the concentration is calculated as the average of the readings. The determinations were done to the manufacturer's specifications.

4.1.10 Restriction enzyme digestion

Restriction endonucleases cut plasmid DNA at specific sequences. All restriction enzymes were used from NEB with the supplied buffers. Digestions were done using 100 to 1000 ng of DNA with sufficient units of enzyme as recommended by the supplier. Double digestions are carried out using the best buffer conditions according to the manufacturer's specification. All digestions are incubated at 37°C o/n unless stated otherwise.

4.1.11 Dephosphorylation of digested plasmids

To avoid a religation of the digested plasmid, the vector was dephosphorylated with Antarctic Phosphatase (NEB). For this purpose 1/10 volume of 10x Antarctic Phosphatase buffer and 1 μL of Antarctic Phosphatase (5 Units) were added to 1 μg of digested DNA. The mixture was incubated for 15 min at 37°C in the case of 5' extensions or blunt-ends or 60 min in the case of 3' extensions. After dephosphorylation, the phosphatase and remaining restriction enzymes were heat inactivated at their necessary temperature. The plasmid was then stored at -20°C until use.

4.1.12 Ligation of DNA fragments

Ligation of two DNA fragments are carried out to combine the sequences together using T4 DNA Ligase. The amount of digested vector varies from 100-1000 ng. The applied vector-insert ratio was 1:3. This was calculated by the relation between amount of DNA to the length of the fragment. All ligation experiments are accompanied with two controls. First is the vector religation control where only the vector is used with ligase without the insert. The second control is the background control where the vector and insert are used without any ligase. Ligation experiments are carried out using the T4-ligase buffer (1x end concentration) with T4-DNA-ligase in ddH_2O . The end volume was conformed to the DNA-concentrations. The ligation was carried out o/n at 15°C .

4.1.13 Transformation of *E. coli* by electroporation

For the transformation, 1 μ L of the DNA was transferred to 50 μ L of ice-thawed electro competent cells. The mixture was pipetted to an ice-cold electroporation cuvette with a 1 mm electrode gap. The electroporation was performed at 1.5kV for 5 ms. Directly after the electroporation, 1 mL of SOC media at RT was added to the mixture. The mixture was then incubated for 1 hr at 37°C and 120 rpm for bacteria regeneration. Bacteria was pelleted by centrifugation for 4 min at full speed. The cell pellet was resuspended in 100 μ L of SOC media and plated onto 2YT agar-plates with appropriate antibiotics and 2% glucose. Plates were incubated o/n at 37°C. On the next day, grown colonies were picked to check for positive integration. The clonal background was analyzed through an empty vector as negative control.

4.1.14 DNA sequencing

For sequencing, at least 250 ng of plasmid DNA and 20 μ L of each primer (10 μ M) was given to the in-house service department of the institute, where sequencing was carried out. Sequencing traces were obtained electronically and analysed using Contig Express software of the VectorNTI software package.

4.1.15 PCR protocols

The polymerase chain reaction is a technique employing DNA-polymerase to amplify DNA to several orders of magnitude from the initial copy in a doubling process. A conventional PCR cycle can be divided into three steps:

1. Denaturation: double stranded DNA is dissociated to single stranded DNA at high temperatures (94-98°C).
2. Annealing: sequence-specific oligonucleotides (primer) complementing single-strands are introduced. The annealing temperature is about 3-5°C lower than the melting point of the primers (normally 50-65°C). A primer-template hybrid is then formed.
3. Elongation: the mechanism of building a new strand of DNA from 5' to 3' with the help of the polymerase and excess dNTPs at 72°C.

The steps from 1 to 3 are usually repeated for 25 to 30 cycles. At the end of the PCR a primer-flanked DNA region is multiplied countless times. For preparative amplification of DNA-fragments the polymerases Bio X Act Short, REDTaq and Phusion Hot Start were used for fragments up to 1 Kb in length. The High Fidelity PCR Enzyme Mix was used for fragments 3 Kb in length. For analytical purposes the House-Taq was used. All polymerases are used together with the supplied reaction buffer. Additives such as MgCl₂ and DMSO were applied, if necessary. The DNA amount differed from 20-50 ng. The concentration of the dNTP-mix was 2.5 mM and the primers was 10 nM. The total volume of a PCR reaction was in between 20-50 µL. All reactions were prepared on ice.

a. Conventional PCR amplification

This was carried out to amplify DNA material for downstream experiments. 1 µL of sufficient DNA material was used. Depending on the size of the fragments, different polymerases were used. All polymerases used are proof reading polymerase.

b. Gradient PCR

This process was conducted to identify optimal conditions for PCR amplification of certain DNA fragments. A set of 8 different temperature settings are used for temperature optimization. For initial optimization purposes, the HouseTaq was used. After identification of parameters, depending on the size of the fragments, different polymerases were used. All polymerases used are proof reading polymerase.

c. Ligation-Elongation PCR

Two individual PCR fragments were digested with appropriate restriction enzymes and purified using the Qiagen PCR Purification Kit to remove excess enzyme and short DNA fragments, and for buffer exchange to ddH₂O or elution buffer (TE buffer). The purified ligated fragments were subjected to PCR amplification using the outer most primers. Depending on the size of the fragments, different polymerases were used. All polymerases used are proof reading polymerase.

d. Colony PCR

To analyze numerous clones of a transformation to determine the success of a ligation. A small portion of the colony was tapped off using a pipette tip into 20 μL of ddH₂O and mixed well. 1 μL of this mix was used for PCR. All analytical PCR experiments were carried out using the HouseTaq.

e. Analytical PCR

Analytical PCR was used to analyze clones or plasmid preparations for the integration of specific DNA sequences into the vector backbone. Specific primers that anneal to the vector backbone are used. All analytical PCR experiments were carried out using the HouseTaq in a volume of 20 μL .

PCR reactions used are based on the following conditions highlighted in Table 4.1. Depending on the varying constructs and application purposes, modifications to the temperature settings and time are based on the optimization process. Table 4.2 shows the conditions for a typical PCR program used. The end volume of each reaction is dependent on the downstream requirements therefore the concentrations of buffer and materials are scaled accordingly.

Table 4.1: Typical PCR reaction mix with a final volume of 20 μL .

Materials	End Concentration / Units	Volume (μL)
Template	20 - 50 ng/ μL	1
dNTP mix	2.5 mM	1.6
Polymerase	Manufacturer's recommendations	x
Buffer	Dilute to recommendation	y
Forward Primer	0.1 μM	0.4
Reverse Primer	0.1 μM	0.4
ddH ₂ O	Top up to desired end volume	z
End Volume		20 μL

Table 4.2: Typical PCR program.

Step	Temperature (°C)	Time (min:sec)
a	95	1:30
b	95	0:30
c	55	0:30
d	72	0:45
e	Repeat steps b - d	30 times
f	72	5:00

f. Addition of additives to PCR experiments

- *MgCl₂ concentration optimization to PCR amplifications*

MgCl₂ concentration for polymerase optimization was performed by increasing each manufacturer's recommended MgCl₂ concentrations by 0.25 mM, 0.5 mM and 1.0 mM (final conc.), respectively. The manufacturer's recommendations are as follows: REDTaq 1.1 mM, BIO-X-ACT 1.0 mM, and Phusion 1.5 mM.

- *Addition of ETSSB to PCR amplifications*

Where applied, 0.2 µL of ET SSB (500 µg/mL) from New England Biolabs (Frankfurt, Germany) was added to each 20 µL PCR reaction. Supplied reaction buffers were added according to manufacturer's information.

g. Semi-quantitative analysis of PCR performance

Gels were photographed using a gel documentation station (Gel Doc 2000, Bio-Rad, Munich, Germany) and specific amplification bands were quantified using AIDA® image analyzer software 4.10 (Raytest, Straubenhardt, Germany). All gel images were analyzed using the 1D evaluation tool with a fixed lane width of 18 pixels. The band intensities were normalised using the 500 bp lane of the DNA ladder loaded at constant concentration for all gels.

4.1.16 Subcloning of Antibody Library Repertoire

The ligation products of the light and heavy chains together with the dephosphorylated pTSL vector was ligated using a 1:3 vector to insert ratio. 1 µg of vector was ligated to 900 ng of insert using T4 DNA ligase (4 U/µL) from Promega in a 50 µL reaction volume. Ligation was carried out o/n at 8°C. The ligation product was subjected to ethanol precipitation and reconstituted in a final volume of 5 µL with distilled water. The ligation product was electroporated in 50 µL of TG1 electrocompetent cells (Stratagene). The cells were reconstituted in 1 mL of pre-warmed SOC media and incubated at 37°C with constant shaking for 1 hr. Two transformations were spread on one 25 x 25 cm 2YT agar plates. The agar plates were incubated at 37°C o/n for colony formation.

4.1.17 First strand cDNA synthesis

Before proceeding to reverse transcription (RT) 1 µg of RNA per sample was treated with DNase (Sigma-Aldrich, Munich, Germany) for 15 min at rt followed by heat inactivation of the DNase at 70°C for 10 min. Using always the same batch of total RNA, two different RTases were evaluated in parallel; SuperScript® II (Invitrogen, Karlsruhe, Germany) and totalscript-OLS® (Omni Life Science, Hamburg, Germany). Total cDNA preparation was performed using oligo(dT)₁₂₋₁₈ primer and random primers together (Invitrogen, Karlsruhe, Germany). For gene specific amplification, gene specific primers were used. All RT reactions were carried out according to the manufacturer's recommendation containing equal amounts of total RNA (400 ng) as starting material.

4.1.18 PBMC preparation and RNA isolation

Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood of adult donors in Vacutainer® EDTA tubes (BD Biosciences, Heidelberg, Germany) within 10 min after blood drawing by density gradient separation. In detail, 15 mL of Biocoll (Biochrom AG, Berlin, Germany) separating solution was put in a 50 mL tube and 10 mL EDTA-blood was added above the Biocoll separating solution. After centrifugation for 15 min at 1800 rpm (652 rcf) the cell cloud was carefully removed with a pipette and placed in a fresh 50 mL Falcon tube. Sterile PBS was added up to a final volume of 50 mL and cells were centrifuged for 10 min at 1200 rpm. The supernatant was discarded and the cell pellet was resuspended in RTL buffer

for RNA isolation using the RNeasy kit from Qiagen (Hilden, Germany). RNA isolation was according to the supplied protocol. The RNA quality and concentration was determined using a RNA 6000 NanoChip® with an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). From all individuals included in this study informed consent was obtained.

4.1.19 Evaluation of RNA samples

a. Pre-analysis preparations

1. All reagent and reagent mix are stored in 4°C except for the RNA ladder (-20°C). All the reagents were re-equilibrate to room temperature for at least 30 minutes.
2. The dye and gel-dye mix was covered with aluminium foil and kept in the dark.
3. Turn on heating block and set to 70°C
4. Gel matrix preparation:- 550 uL of gel matrix was added to a spin filter and centrifuged for 10 min at 4000 rpm (1500g). 65 uL filtered gel was transferred into the provided 0.5 mL RNase free microfuge tubes. The aliquot can be stored at 4°C and used within one month.
5. Gel-dye matrix (for 2 Chips) preparation:- The dye was mixed by vortexing for 10 seconds and spun down. 1 uL of dye was added to 65 uL of gel matrix in a new 0.5 mL RNase free microfuge tubes. The matrix was vortexed thoroughly for 10 sec. The gel-dye matrix was centrifuge at maximum speed (14000 rpm) for 10 min.

b. RNA sample analysis

A new RNA6000 Nanochip was placed on the priming station. 9 uL of gel-dye matrix was loaded into the well marked as a “Highlighted G”. Set a timer for 30 sec and the plunger is position at the 1 mL mark. The chip priming station is closed; the plunger pressed until it is held by the syringe clip; and held for 30 sec. The plunger was released with the clip release mechanism and wait 5 sec. The plunger was slowly pulled back up to 1 mL and the chip priming station opened. 9 uL of the gel-dye matrix was loaded in the two additional wells marked G. 5 uL of RNA marker was added into the well marked ladder and each of the 12 RNA wells. 6 uL of the marker must be loaded into each unused well. RNA sample and RNA ladder was incubated at 70° C for 2 min and placed on ice for 5 min. Sample and ladder was briefly spun to concentrate solutions to the bottom. 1 uL of ladder was loaded into the well marked “ladder” and 1 uL of RNA into each of the 12 wells. The Chip was placed on the adapted vortexer and mixed for 1 min at 2400 rpm. Place the loaded Chip in the Bioanalyzer and run the RNA program as recommended by the manufacturer.

4.1.20 Estimation of library size

The size of a library is correlated to the number of individual colonies harvested to generate the library DNA pool. Four methods were used to estimate the size of the generated library.

a. Estimation by titration

10 µL from a 1 mL transformation culture was diluted with 90 µL of SOC media for a dilution factor of 10(-1). A 1:10 serial dilution was carried out on a 96 well plate until a final dilution of 10(-13). 10 µL of each dilution was spotted on the 2YT (100 µg/mL Amp + 2 % Glu) agar plate. Spot was left to dry and incubated o/n at 37°C. The number of colonies on each spot was calculated and extrapolated to estimate the number of colonies available. Formula used was:-

$$\text{Number of colonies} \times \text{dilution factor} \times 100 \text{ (1 mL volume)} \times \\ 2 \text{ (2 mL culture per plate)} \times 41 \text{ (number of plates)}$$

b. *Estimation by dilution on 10 cm agar plates*

10 μ L from the 1 mL transformation culture was diluted with 990 μ L of SOC media (100 x dilution) . A 1:10 serial dilution was carried out to three dilutions of 100, 1000 and 10000 times. 100 μ L of each dilution was plated on 2YT (100 μ g/mL Amp + 2 % Glu) agar plates. Agar plates were left to dry and incubated o/n at 37°C. The number of colonies on each spot was calculated and extrapolated to estimate the number of colonies available. Formula used was:-

$$\begin{aligned} & \text{Number of colonies} \times \text{dilution factor} \times 10 \text{ (1 mL final volume)} \\ & \times 2 \text{ (2 mL per plate)} \times 41 \text{ (number of plates)} \end{aligned}$$

c. *Estimation by dilution on 25 x 25 cm agar plates*

10 μ L from the 1 mL transformation culture was diluted with 990 μ L of SOC media (100 x dilution) . A 1:10 serial dilution was carried out to three dilutions of 100, 1000 and 10000 times. 100 μ L of each dilution was plated on 2YT (100 μ g/mL Amp + 2 % Glu) agar plates. 0.8 mL of each dilution was plated on the agar plate. Agar plates were left to dry and incubated o/n at 37°C. The number of colonies on each spot was calculated and extrapolated to estimate the number of colonies available. Formula used was:-

$$\begin{aligned} & \text{Number of colonies} \times \text{dilution factor} \times 2.5 \text{ (2 mL per plate)} \\ & \times 41 \text{ (number of plates)} \end{aligned}$$

d. *Estimation by plating lower volume on 25 x 25 cm agar plates*

50 μ L (20 x dilution) and 100 μ L (10 x dilution) from the 1 mL transformation culture was plated on the 25 x 25 cm 2YT (100 μ g/mL Amp + 2 % Glu) agar plates. Plates were left to dry and incubated o/n at 37°C. The number of colonies on each spot was calculated and extrapolated to estimate the number of colonies available. Formula used was:-

$$\begin{aligned} & \text{Number of colonies} \times \text{dilution factor} \times 2 \text{ (2 mL per plate)} \\ & \times 41 \text{ (number of plates)} \end{aligned}$$

4.2 Protein based methods

4.2.1 Expression of in-vivo biotinylated antigens

Plasmid DNA (pRSET-BH6) from *E. coli* was purified using the Qiagen MiniPrep DNA kit according to the manufacturer's instructions. The desired purified plasmid DNA was electroporated as described earlier using electrocompetent *E. coli* cells BL21 Star™(DE3) containing pRARE3 plasmid. Transformation protocol is as described earlier. 2YT agar plates supplemented with 100 µg/mL Amp, 17 µg/mL Cam and 2% Glu were used. Colonies were harvested the next day using 3 mL of 2YT (100 µg/mL Amp + 17 µg/mL Cam). 1 mL of the harvested colonies were cultivated in 50 mL 2YT (100 µg/mL Amp + 17 µg/mL Cam) until an OD₆₀₀ of 0.7. Protein expression in cells were induced with 1 mM (final conc.) of IPTG. Culture was left to incubate for 3 to 4 hr with constant shaking (180 rpm). Cells were pelleted in 50 mL fractions by centrifugation (4000 rpm, 30 min). Pellets were frozen at -20°C until use.

4.2.2 Preparation of proteins under native conditions

Frozen pellets of proteins were resuspend in 3 mL of native lysis buffer I with 3 µL of lysozyme (stock 20 mg/mL). Pellets were resuspend gently with slow pipetting movements and incubated for 30 min on ice. The lysate was sonicated 3 times for 20 sec with 20 sec intervals on ice. The lysate was transferred to 2 x 2 mL Eppendorf tubes. The tubes was centrifuged for 10 min at 12000 rpm. 50 µL of the supernatant was removed for SDS gel analysis and the remaining supernatant for purification. The remaining pellet was used for preparation under denatured conditions.

4.2.3 Preparation of proteins under denatured conditions

Pelleted cells was resuspend in 500 µL of 8 M Urea. The pellet was resuspended well and subjected to sonication for 15 min in a water bath. The lysate was centrifuged for 10 min at 12000 rpm to pellet cell debris. 50 µL of the supernatant was used for SDS gel analysis and the remaining supernatant for purification. The pellet was discarded.

4.2.4 Preparation of periplasmic proteins

Cell pellet was resuspended in 1 mL of ice cold 1x TES. Careful resuspension was carried out to avoid cell rupture. 1.5 mL of 1 to 5 dilution of ice cold 1x TES was added. Preparation was incubated on ice for 30 min. The supernatant was collected by centrifuging at 4000 rpm for 30 min. The supernatant was kept at 4°C until use.

4.2.5 His Tag purification using Ni-NTA columns under native conditions

1 mL bed volume of 50% Ni-NTA agarose (Qiagen) was packed in a 12 mL empty SPE column (Phenomenax). The column was equilibrated with 20 mL of native lysis buffer I. The supernatant from the native preparation or periplasmic preparation was added to the column. The supernatant was allowed to drain by gravitational flow until the last drop. Care was taken to ensure the column would not dry up. The column was subjected to 2 wash steps with 10 mL wash buffer per wash. Proteins were eluted in 4 x 2 ml fractions using the elution buffer.

4.2.6 His Tag purification using Ni-TED columns under native conditions

The Protino Ni-TED columns (1 mL bed volume) were equilibrated with 4 mL of supplied 1x LEW buffer. The supernatant from the native preparation or periplasmic preparation was added to the column. The supernatant was allowed to drain by gravitational flow until the last drop. Care was taken to ensure the column would not dry up. The column was subjected to 2 wash steps using 4 mL of 1x LEW buffer per wash. The proteins were eluted in 3 mL fractions (total of 3 fractions) using the supplied elution buffer.

4.2.7 Purification of biotinylated proteins using Monoavidin columns

The kit was equilibrated to room temperature. The Monoavidin column (Pierce) was equilibrated with 8 mL of PBS. 6 mL of Biotin Blocking and Elution Buffer was passed through the column to block any non-reversible biotin binding sites. 12 mL of Regeneration Buffer was added to remove biotin from the reversible binding sites. The column was washed with 8 ml of PBS. Add the supernatant to the column and allow it to pass by gravitational flow. After the entire sample had passed through the column, 0.25 mL of PBS was added to force sample

completely into the resin bed. Six 2.0 mL fractions of PBS was used as a wash step. For the elution of the bound biotinylated molecule, Biotin Blocking and Elution Buffer was added to the column and at least six 2.0 mL fractions were collected.

4.2.8 Protein L purification of antibody fragments

Periplasmic supernatant was either filtered using a syringe filter with low protein binding or centrifuge at 3500 rpm for 15 min to remove debris. The 1 mL Fast Flow Protein L column (Pierce) was equilibrated using 5 mL of PBS. Supernatant was passed through the protein L column drop wise using a syringe. The protein L column was washed using 5 mL of PBS. The bound antibodies were eluted using 4 to 5 column volumes of 100 mM Glycine-HCl, pH 3.0. The elution fraction was eluted into a tube containing 200 μ L of 1 M Tris-HCl, pH 7.8 to neutralize the pH.

4.2.9 Protein concentration determination

Determination of protein concentration was carried out using the Pierce 660 nm Protein Assay kit with a working range between 50-2000 μ g/mL for microplate procedure. 10 μ L of each standard (7 samples, ranging from 125 to 2000 μ g/mL, stored at 4°C) was added in replicates. 10 μ L of sample and a blank sample (PBS) was added to a microplate well (Falcon [®], 96-well PVC (flexible) flat-bottom plate). 150 μ L of the Protein Assay Reagent was added to each well and mixed by shaking. Plate was incubated at room temperature for 5 min. The absorbance was read at 660 nm wavelength using the SpectraMAX 250 MTP spectrophotometer.

4.2.10 SDS PAGE analysis

For the electrophoretic protein separation, ready-to-load gels from Invitrogen were used. A protein sample of 10 μ L was mixed with 2.5 μ L of 4x SDS loading buffer. The mixture was heated at 95°C for 5 min using a thermalcycler. The protein solution was left to cool to rt. 20 μ L of sample was pipetted into each pocket. The electrophoresis was carried out at a current of 200 mA and 400 V in a X Cell4 Surelock™ Midi cell or X Cell Sure Lock™ Mini-Cell depending on the gel size for about 35 min in 1x MES buffer. 5 μ L of the pre-stained Precision Plus Protein Standard (Fermentas) was added to the first lane of the gel.

4.2.11 SDS PAGE Staining and Destaining

After the electrophoretic separation of the proteins, the gel was transferred into the dye solution for staining. For analytical purposes, Coomassie brilliant Blue GLR 250 was used. The gel was incubated for 40 min at rt with slow shaking. The stained gel was destained with a destaining solution for 40 min at rt with slow shaking. The gel was transferred to ddH₂O and slowly shaken overnight at RT. The gel was submerged in 5 % aqueous glycerol for long term storage.

Alternatively, the PAGE Blue staining kit (Fermentas) was used to stain the gels. The gel was washed twice with ddH₂O by slowly shaking for 10 min per wash. The gel was then covered with sufficient PAGE Blue stain for 1 hr with slow shaking. The gel was destained using ddH₂O by shaking it for 10 min. The gel was submerged in 5 % aqueous glycerol for long term storage.

4.2.12 Silver Staining of SDS PAGE gels

For Silver staining, the SERVA Silver Staining Kit (SERVA Electrophoresis) was used. The gel was Fixed in fixing solution for 20 min with gentle agitation. Two wash steps with 30% ethanol was done for 10 min each. The gels were pre-treated in Sodiumthiosulfate pentahydrate (Supplied: 30 mg in 100 mL ddH₂O) for 1 min with gentle agitation. Three quick wash steps of 10 sec each was done with 30% ethanol. The gel was stained with silver nitrate solution (10 mL silver nitrate in 100 mL ddH₂O) for 15 min with gentle agitation. Two quick wash steps of 10 sec each was done with 30% ethanol. Development of the gel was done with the developing solution (20 mL Sodium carbonate solution with 100 mL ddH₂O and 50 μ L of 37% Formaldehyde) for approximately 1 to 5 min (best by visualization of bands). The gel was washed with 100 mL of ddH₂O for 10 sec. The development process was stopped using citric acid solution (20 mL in 100 mL ddH₂O) for 5 min with gentle agitation. The gel was submerged in 5 % aqueous glycerol for long term storage.

4.2.13 Western Blotting

Prior to performing Western immunoblots, proteins of interest were run on a NuPAGE 4-12 % Bis-Tris gel as described. Meanwhile, four sheets of Whatman® paper and one sheet of PVDF-membrane (Millipore) or Hybond ECL Nitrocellulose membrane (GE Healthcare) were cut according to the dimensions of the separation gel (5.5 cm x 9 cm). All sheets of Whatman® paper were soaked in transfer buffer at a time and placed onto the anode of the Western apparatus. The PVDF membrane was submerged for ~10 sec in 100 % ethanol and soaked for ~30 sec in transfer buffer before it was placed on top of the Whatman® paper on the apparatus. For the Hybond ECL Nitrocellulose membrane, it was soaked for ~30 sec in transfer buffer before being placed on top of the Whatman® paper. The separation gel was laid air-bubble free onto the membrane and, on top, the remaining Whatman® papers were placed. The cathode of the apparatus was connected and running the apparatus for 80 min at 40 mA to performed the electrophoretic transfer of the proteins from the gel to the PVDF membrane.

4.2.14 Immunostaining

The immunostaining technique offers a possibility for specific staining of membrane transferred proteins with conjugated antibodies or binding proteins. Firstly, the western blot was incubated in blocking buffer for 1 hr at rt. All wash steps in between incubation were done twice for 5 min in PBS-T. The blocked membrane was incubated for 1 hr at rt with the conjugated binding partner. Development was carried out using CN/DAB staining (Thermo Scientific). Conjugated secondary antibodies were used when conjugated primary antibodies were not available.

4.2.14 Mass spectrometry identification of proteins

The protein bands analyzed by SDS-PAGE were subjected to in-situ trypsinolysis and microcrystalline layers of α -cyano-4-hydroxycinnamic acid (CHCA) were prepared on AnchorChips 384/600 as described [166]. Mass spectrometric analysis was performed on a Scout MTP Ultraflex II MALDI-TOF-MS/MS equipped with a SmartBeam laser (Bruker Daltonics, Bremen, Germany), equipped with a solid-state SmartBeam laser. Positively charged ions in the m/z range 700 – 4,000 Da were detected and 4000 single-shot spectra were accumulated for each analysis. Fixed laser attenuation was used, the optimal value of which was determined prior to analysis by evaluation of a few fractions. Spectrum processing was performed automatically

with the software FlexAnalysis 3.0. Automatic detection of the peptide monoisotopic signals was performed using the algorithm SNAP with a signal-to-noise threshold of 6. Internal mass correction was performed using the signals of two reference peptides (Angiotensin I, MH+ 1,296.6853 (monoisotopic mass), and ACTH (18-39), MH+ 2,465.1989) included in the MALDI matrix solution.

Protein identification was performed using the Mascot software 2.2 (Matrixscience, London, UK) searching the UniProt/Swiss-Prot and UniProt/Trembl sequence databases. The following settings were used for the searches: mass error tolerance for peptide masses: 15 ppm; mass error tolerance for the fragment ions: 0.35 Da; fixed modification: carbamidomethylation; variable modification: methionine oxidation; number of missed cleavage sites: 1; type of instrument: MALDI-TOF-PSD. Identifications based on PMF were considered very likely to be correct if the score was greater than 80. For identification by MS/MS a different score is calculated and in this case above threshold for identification was set to 50.

All mass spectrometry analysis was conducted by the Mass Spectrometry team at the Max Planck Institute for Molecular Genetics (courtesy of Dr. Nordhoff).

4.3 Phage display based methods

4.3.1 Helper phage preparation

A 1:10 dilution series of M13K07 Helper phage stock was prepared in PBS by adding 10 μ L phage to 90 μ L PBS. Top Agar was heated up and left to cool down to 43°C in a water bath. Polypropylene tubes were filled with 4 mL of Top agar. 200 μ L of *E. coli* culture and 100 μ L of the phage dilution (10E-7 – 10E-11) were mixed and pour onto a 2YT Agar plate. Plates were left o/n at 37 °C. A single plaque was picked and inoculated in 4 mL 2YT (60 μ g/mL Kan). The culture was incubated at 37°C with constant shaking until turbid (3-4 hr). 1 mL of the culture was added to 50 mL 2YT (60 μ g/mL Kan) in an Erlenmeyer flask. Culture was grown o/n at 37°C. The next day, the *E. coli* was pelleted in a 50 mL Falcon tube (4000 rpm, 30 min). The supernatant was used for phage precipitation and pellet discarded.

4.3.2 Phage library preparation

A glycerol stock of the phage library was cultivated in 200 mL of pre-warmed 2YT (100 µg/mL Amp + 1 % Glu) until an OD_{600} of 0.4 to 0.5. Culture was separated to 2 x 50 mL Falcon tubes and 2×10^{11} cfu helper phage was added and left to incubate without shaking at 37°C for 30 min. The cells were pelleted by centrifugation (3800 rpm, rt) for 10 min. The pellet was resuspended in 200 mL of pre-warmed 2YT (100 µg/mL Amp + 60 µg/mL Kan + 0.1 % Glu). The culture was incubated overnight at 30°C with constant shaking (270 rpm). The next day, the *E. coli* was pelleted in a 50 mL Falcon tube (4000 rpm, 30 min, 8°C). The supernatant was used for phage precipitation and pellet discarded.

4.3.3 Phage precipitation

7.5 mL of ice cold 20% PEG / 2.5 M NaCl was added to 42.5 mL supernatant and left on ice for 1 hr. Tubes were centrifuged at 4000 rpm for 30 min. The supernatant discarded. Now, a white phage pellet should be visible. Pellet was left to dry for ~5-15 min up side down in a stand. The pellet was resuspended in 500 µL PBS and transferred to a new Eppendorf tube. The phage preparation was centrifuged in a bench top centrifuge at full speed for 15 min and the remaining *E. coli* pellet discarded. This washing process was repeated again (1-2 times). Helper phage was stored at 4°C until use.

4.3.4 Titration of phage particles

5 mL of 2YT was inoculated in a 15 mL PP tube with a single clone of TG1 from an agar plate and grown shaking overnight at 37°C and 250 rpm. 0.5 mL of overnight TG1 culture was inoculated in 50 mL 2YT using a 250 mL Erlenmeyer flask and incubate shaking at 37°C and 250 rpm until $OD_{600} = 0.4 - 0.5$. A 1:10 serial dilution (until 10^{-9}) was prepared from the enriched phage libraries from selection rounds by adding 10 µL phage to 90 µL PBS-T in a 96-well U-bottom PP microtiter plate. 100 µL of *E. coli* TG1 ($OD_{600} = 0.4 - 0.5$) was added to the phage dilutions $10^{-5} - 10^{-9}$, covered with plastic lid and incubated stationary for 30 min at 37°C. The infected *E. coli* cultures were mixed and 10 µL droplets of each dilution series were plated on a single 2YT-Amp + 2 % Glu and 2YT-Kan + 2 % Glu agar plates per enriched library. Once the droplets dried, the plates were incubated top-down o/n at 37 °C. In the next day, the number of colonies in the droplets on all plates were counted and the colony forming units (infectious

phage particles/mL) were extrapolated using the formula:

$$\text{c.f.u.} = \text{number of colonies} \times \text{dilution factor} \times 100$$

On average, phage preparations in microtiter plates (200 μL culture volume) produce 10^{10} – 10^{11} c.f.u. The c.f.u. values obtained on 2YT-Amp + 2 % Glu and 2YT-Kan + 2 % Glu agar plates for each phage library were compared. The helperphage genome containing population should be a minimum of 4 – 5 orders of magnitude smaller than the antibody fragment containing phagemids population.

4.3.5 Panning of phage antibody libraries

4.3.5.1 Loading of Magnetic Beads

1 mg (100 μL) Dynabeads M-280 Streptavidin magnetic beads were washed 3x 5 min with 1.5 mL PBS-T and 1x 5 min with 1.5 mL PBS at rt. At the mean time, (a) 100 – 200 μg biotinylated protein antigen, or alternatively (b) 1 – 2 μg biotinylated peptide antigen were dissolved in 1 mL PBS. The wash solution was discarded, the magnetic beads resuspended gently in the 1 mL antigen solution and incubated for 1 hr at rt on a rotator. Alternatively, the incubation can be performed o/n at 4°C on a rotator. The antigen solution was removed and the magnetic beads washed 3x 5 min with 1.5 mL PBS-T. Finally, the last wash solution was discarded, the magnetic beads were resuspended in 200 μL PBS and the antigen-loaded bead stock was stored until further use at 4°C.

4.3.5.2 Semi-automated Panning on Magnetic Particle Processor

For simplicity, this section would be described in point form.

1. 5 mL of 2YT were inoculated with a single clone of TG1 from an agar plate in a 15 mL polypropylene tube and grown shaking overnight at 37°C and 250 rpm.
2. 50 mL of 2YT were inoculated with 0.5 mL of the fresh o/n TG1 culture in a 250 mL Erlenmeyer flask and incubate shaking at 37°C and 250 rpm until the culture reaches an $\text{OD}_{600} = 0.4 - 0.5$.
3. Bead-plate was arranged. Positions A1 – A12 of a 96-well V-bottom PP (PP) microtiter plate were filled with 180 μL PTM and for each antigen 20 μL from corresponding antigen-loaded bead stocks were added to the specified position. Magnetic beads of antigen 1 were added to positions A1, beads of antigen 2 to positions A2, and so on.

For the first round selection continue with step 4. For later selection rounds, continue with step 7.

4. Unselected antibody phage library were pre-incubated with empty magnetic beads in PTM to deplete binders to the selection matrix. In a 15 mL PP tube, 2 mg (200 μ L) Dynabeads M-280 Streptavidin was added to $1 \times 10^{13} - 3 \times 10^{13}$ phage particles in 10 mL PTM. The mixture was incubated for 1 – 2 hr at rt on a rotator.
5. Beads were concentrated at the bottom of the tube by centrifugation for 2 min, 2000 rpm and the antibody phage library solution was carefully transferred to a new 15 mL PP tube. The magnetic beads were discarded.
6. The phage-plate for the first round was arranged. Positions A1 – A12 of a 96-well V-bottom PP microtiter plate were filled with 200 μ L of the antibody phage library solution.
Continue with step 8.
7. For the remaining rounds, the phage-plate were prepared. Positions A1 – A12 of a 96-well V-bottom PP microtiter plate were filled with 100 μ L PTM. 100 μ L of the amplified phage solutions of the previous round were added according to the same antigen order in positions A1 – A12.
8. Wash plate(s) were prepared. Positions A1 – A12 of a 96-well V-bottom PP microtiter plates were filled with 200 μ L PBS-T. The number of plates were adjusted according to Table 4.3.
9. The release plate was prepared by filling positions A1 – A12 of a 96-well V-bottom PP microtiter plates with 200 μ L PBS.
10. Plates were placed in the magnetic particle processor according to the plate positions in Table 4 and start the panning program. The program was set to move magnetic beads from plate to plate and incubate the beads in each plate as indicated in Table 4.4. During all incubations, the beads should be kept in suspension by moving plastic tips up-and-down in the wells at medium speed (30 – 50 mm/sec). The program ends by releasing the beads in the release plate.
11. Once the Panning program finishes, *E. coli* culture plates were prepared. Positions A1 – A12 of a 96-well U-bottom PP microtiter plates were filled with 200 μ L of *E. coli* TG1 ($OD_{600} = 0.4 - 0.5$), *E. coli* culture plate was placed in the instrument and started the Transfer Program. This program simply transfers the beads from the release plate to the *E. coli* culture plate.
12. The selection stock plate was removed from the instrument, covered with a plastic lid and incubated for 30 min at 37°C.

Table 4.3: Overview of Automated Magnetic Bead-Based Panning Procedure.

Plate	Panning Round 1	Panning Round 2	Panning Round 3	Panning Round 4
1	Bead plate	Bead plate	Bead plate	Bead plate
2	Phage plate	Phage plate	Phage plate	Phage plate
3	Wash plate	Wash plate 1	Wash plate 1	Wash plate 1
1	Release plate	Wash plate 2	Wash plate 2	Wash plate 2
2	<i>E. coli</i> culture plate	Release plate	Wash plate 3	Wash plate 3
3	-	<i>E. coli</i> culture plate	Release plate	Wash plate 4
1	-	-	<i>E. coli</i> culture plate	Release plate
2	-	-	-	<i>E. coli</i> culture plate
Time	~ 135 min	~ 145 min	~ 155 min	~ 165 min

Table 4.4: Automated Magnetic Bead-Based Panning Protocol (Round 4).

Plate	Plate name	Work step	Volume (μ L)	Time (min)
1	Bead plate	Blocking of antigen-loaded and control magnetic beads with PTM	200	60
2	Phage plate	Incubation of magnetic beads in antibody phage stocks of the selection rounds	200	60
3	Wash plate 1	Wash 1 of magnetic beads in PBS-T	200	10
4	Wash plate 2	Wash 2 of magnetic beads in PBS-T	200	10
5	Wash plate 3	Wash 3 of magnetic beads in PBS-T	200	10
6	Wash plate 4	Wash 4 of magnetic beads in PBS-T	200	10
7	Release plate	Waiting position for magnetic beads until <i>E. coli</i> culture plate is ready for infection ¹	200	5 - 10
8	<i>E. coli</i> culture plate	Infection of <i>E. coli</i> TG1 culture with bead-bound phage particles	200	- ²

Notes: ¹ At this stage, 10 μ L bead-bound phage solution can be collected for titration.

² Incubation takes place outside the instrument at 37°C.

4.3.5.3 Packaging of Phagemids

1. The selection stock plate was added with 200 μL of pre-warmed 2YT-Amp + 2 % Glu medium (37°C), mix thoroughly and 200 μL was transferred into a 96-well filtration plate (MultiScreenHTS Plates with a hydrophilic Durapore PVDF membrane). The selection stock plate was sealed again with breathable sealing film and the incubation continued in a microplate shaker overnight at 37°C and 1200 rpm.
2. To the filtration plate, 20 μL M13K07 helperphage ($\sim 10^9$ phage particles) was added, covered with a plastic lid and incubated stationary for 30 min at 37°C.
3. The filtration plate was placed on top of a 96-well U-bottom PP microtiter plate and fixed with sticky tape. A counter balance plate was prepared in similar fashion.
4. The bacterial culture was filtered by centrifugation in microtiter plate holders (swing out rotor) for 2 – 5 min at 2000 rpm.
5. The filtrate containing the remaining M13K07 helperphage was discarded.
6. Retained bacteria was resuspended in 220 μL pre-warmed 2YT-Amp + Kan + 2 % Glu and transferred to a fresh 96-well U-bottom PP microtiter plate. Phage production plate was sealed with breathable sealing film and incubated in a microplate shaker o/n at 30°C shaking at 1400 rpm.
7. The next day, 160 μL of glycerol solution was added to the selection stock plate, mix and stored as a glycerol stock at -80°C.
8. Bacteria in the phage production plate was pelleted by centrifugation for 10 min at 2000 rpm. Supernatant was carefully transferred without disturbing the pellet to a 96-well filtration plate.
9. The filtration plate was placed on top of a new 96-well U-bottom PP microtiter plate and fixed with sticky tape.
10. Antibody presenting phage particles were filtered to remove possible *E. coli* cells remaining by centrifugation for 2 – 5 min at 2000 rpm.
11. Filtrate (phage stock plate) was stored at 4°C until used.
12. 50 μL PBS was added to each well of the phage stock plate and mix thoroughly. For the next round of selection, 100 μL was used and 10 μL for phage titration. Phage stock plate was sealed carefully with sticky tape and stored until further use at 4°C.

4.3.5.4 Magnetic Particle ELISA of Polyclonal Antibody Phage

The polyclonal antibody phage ELISA for evaluation of enrichment success was performed using a magnetic particle processor to maintain similar conditions as the initial selection process. At a maximum, polyclonal ELISA for twelve independent selections over four rounds can be performed simultaneously with the appropriate negative control. Proposed plate layout for 12 ELISAs in parallel and an example of a polyclonal ELISA result are shown in Figure 4.1. The ELISA protocol takes 4 hours and is summarised in Table 4.5.

1. Bead-plate arranged by filling each position of a 96-well V-bottom PP microtiter plate with 180 μL PTM and 20 μL of antigen-loaded bead stock according to plate layout in Figure 4.1. Magnetic beads of antigen 1 was added to positions A1 – D1, beads of a antigen 2 to positions A2 – D2, and so on.
2. As a negative control, empty beads were used. 5 mg (500 μL) Dynabeads M-280 Streptavidin magnetic beads were washed 3x 5 min with 1.5 mL PBS-T and 1x 5 min with 1.5 mL PBS at rt. The last wash solution was discarded and beads resuspend in 1 mL PBS. 20 μL was added to positions E1 – H12.
3. Phage-plate was arranged by filling each position of a 96-well V-bottom PP microtiter plate with 150 μL PTM. 50 μL of phage solution from the phage stock plates of the individual rounds were added to the plate according layout in Figure 4.1. Phage stocks of selection rounds 1 – 4 was added on antigen 1 to position A1 – D1 and E1 – H1, respectively. Phage stocks of selection rounds 1 – 4 were added on antigen 2 to position A2-D2 and E2-H2, respectively, and so on.
4. Wash plates 1 – 3 were prepared. 96-well V-bottom PP microtiter plates were filled with 200 μL PBS-T.
5. Wash plate 4 was prepared by filling the 96-well V-bottom PP microtiter plates with 200 μL PBS.
6. For the antibody plate, 4 μL of mouse monoclonal anti-M13-HRP was added to 20 mL PTM (1:5000). The 96-well V-bottom PP microtiter plates were filled with 200 μL antibody solution.
7. Plates were placed on the magnetic particle processor and the magnetic bead-based ELISA program started. The program is set to move magnetic beads from plate to plate and incubate the beads in each plate as indicated in Table 4.5. During all incubations, the beads should be kept in suspension by moving plastic tips up-and-down in the wells at medium speed (30 – 50 mm/sec).

8. While the ELISA program was running, the substrate plate was prepared. One ABTS tablet (10 mg) was diluted in 20 mL substrate buffer. Shortly after the antibody plate incubation step in the ELISA process is finished, 10 μ L hydrogen peroxide was added to the substrate solution and 200 μ L pipetted to each well of the 96-well polystyrene microtiter plates and place plate in the instrument.
9. Once beads are incubated in the substrate and colour developed for 20 min, beads are removed from the substrate by transferring them back to wash plate 4.
10. Substrate plate was removed from the instrument and the absorbance was measured at 405 nm using the SpectraMAX 250 MTP spectrophotometer.
11. For each individual selection target, enrichment was evaluated by plotting the obtained values for antigen-loaded and control beads of each phage selection rounds next to each other as depicted in Figure 4.1.

Table 4.5: Automated Magnetic Bead-Based Panning Protocol (Round 4).

Plate	Plate name	Work step	Volume (μ L)	Time (min)
1	Bead plate	Blocking of antigen-loaded and control magnetic beads with PTM	200	60
2	Phage plate	Incubation of magnetic beads in antibody phage stocks of the selection rounds	200	60
3	Wash plate 1	Wash 1 of magnetic beads in PBS-T	200	10
4	Wash plate 2	Wash 2 of magnetic beads in PBS-T	200	10
5	Antibody plate	Incubation of antigen-loaded and control beads with mouse anti-M13-HRP-conjugated, 1:5000 in PTM	200	60
6	Wash plate 3	Wash 3 of magnetic beads in PBS-T	200	10
7	Wash plate 4	Wash 4 of magnetic beads in PBS-T	200	10
8	Substrate plate	Incubation of magnetic beads in ABTS-containing substrate buffer for horseradish peroxidase ¹	200	20

Notes: ¹ Prior measurement of extinction, beads are transferred back to Wash plate 4.

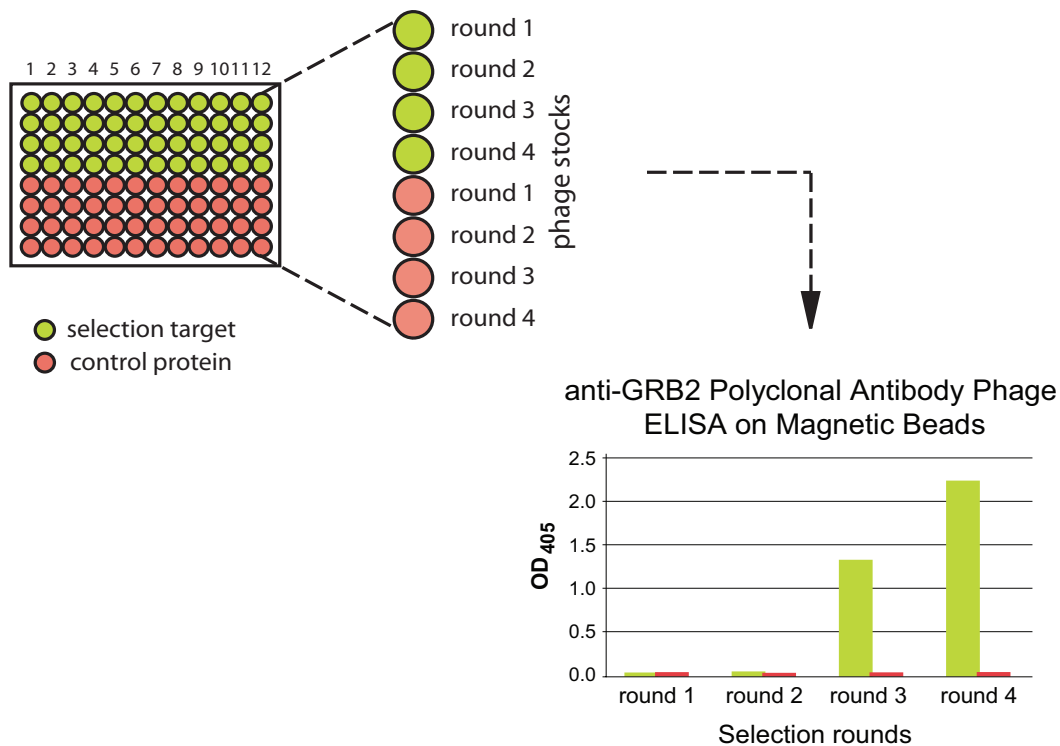


Figure 4.1: Magnetic particle ELISA for monitoring Polyclonal Antibody Phage enrichment. On the left, the plate layout for 12 individual selections is shown. For each selection the phage stock solutions are split and pipetted into the positions A – D and E – H of the same column, respectively. Next, selection target-coated or control protein-coated magnetic beads are added to the phage solutions as indicated. All steps of the ELISA are performed in a 96-well magnetic particle processor. On the right, the enrichment of specifically binding antibody phage during 4 rounds of selection on GRB2 is shown. Specific enrichment is seen in rounds 3 and 4.

4.3.6 Evaluation of phage selections

4.3.6.1 Picking monoclonal antibody fragment presenting cells

A 1:10 dilution series was prepared by diluting 10 μL of phage in 90 μL PBS, 10 μL of this in 90 μL on the subsequent well and so on (up to 10^{-12} dilution). 100 μL *E. coli* (TG1 for phage and HB2151 for expression) culture at $\text{OD}_{600}=0.4-0.5$ was added and incubated at rt for 30 min. 100 μL of dilutions $10^{-6}-10^{-8}$ was plated on a 2YT agar (100 $\mu\text{g}/\text{mL}$ Amp + 2 % Glu). Plates were incubated o/n at 37°C . 92 single phagemid colonies were picked into a MTP containing 200 μL of 2YT (Amp + 2 % Glu) in HMF media and grown o/n at 37°C , 1400 rpm. Positions H3, H6, H9 and H12 on the plate were left empty as negative controls. The plate was stored at -80°C as the monoclonal stock plate until used.

4.3.6.2 Monoclonal scFv expression and periplasmic preparation in microtitre plate format

A fresh plate of culture was prepared by inoculating 10 μ L of culture to 200 μ L of 2YT (Amp + 2 % Glu) in a 96-well U-bottom PP microtiter plate. Culture was grown o/n at 37°C, 1400 rpm. 10 μ L of the o/n culture was inoculated to 200 μ L of pre-warmed (37°C) 2YT (Amp + 1 mM IPTG) and incubated at 30°C, 1400 rpm o/n. The next day, 50 μ L of 3xPE buffer was added to the o/n culture. The culture was mixed by pipetting. The plate was incubate on ice for 20 min. The cell was pelleted by centrifugation at 2500 rpm for 15 min. The supernatant was used for further evaluations.

4.3.6.3 Monoclonal antibody fragment expression in culture

Phagemid DNA (pTSL phagemid series) from *E. coli* was purified using the Qiagen MiniPrep DNA kit according to the manufacturer's instructions. The desired purified plasmid DNA was electroporated as described earlier using electrocompetent *E. coli* HB2151 cells. Transformation protocol is as described earlier. 2YT agar plates supplemented with 100 μ g/mL Amp and 2% Glu were used. Colonies were harvested the next day using 3 mL of 2YT (100 μ g/mL Amp + 0.1 % Glu). 1 mL of the harvested colonies were cultivated in 50 mL 2YT (100 μ g/mL Amp + 0.1 % Glu) until an OD₆₀₀ of 0.4-0.5. Protein expression in cells were induced with 1 mM (final conc.) of IPTG. Culture was left to incubate for 3 to 4 hr with constant shaking (180 rpm). Cells were pelleted in 50 mL fractions by centrifugation (4000 rpm, 30 min). Pellets were kept at 4°C until use.

4.3.6.4 Preparation of monoclonal antibody fragment presenting phages

1. A replicate plate of the monoclonal stock plate was prepared prior.
2. 10 μ L of o/n culture from the replicate monoclonal stock plate was inoculated with 200 μ L of pre-warmed 2YT-Amp + 2 % Glu medium (37°C), sealed with breathable sealing film and incubated for 2.5 hr at 37°C and 1200 rpm. 200 μ L of pre-warmed 2YT-Amp + 2 % Glu medium (37°C) was added to the plate, mixed thoroughly and 200 μ L was transferred into a 96-well filtration plate (MultiScreenHTS Plates with a hydrophilic Durapore PVDF membrane). The selection stock plate was sealed again with breathable sealing film and the incubation continued in a microplate shaker overnight at 37°C and 1200 rpm.

3. 20 μ L M13K07 helper phage ($\sim 10^9$ phage particles) was added to the filtration plate, covered with a plastic lid and incubated stationary for 30 min at 37°C.
4. The filtration plate was placed on top of a 96-well U-bottom PP microtiter plate and fixed with sticky tape. A counter balance plate was prepared in similar fashion.
5. The bacterial culture was filtered by centrifugation in microtiter plate holders (swing out rotor) for 2 – 5 min at 2000 rpm.
6. The filtrate containing the remaining M13K07 helperphage was discarded.
7. Retained bacteria was resuspended in 220 μ L pre-warmed 2YT-Amp + 2 % Glu and transferred to a fresh 96-well U-bottom PP microtiter plate. Phage production plate was sealed with breathable sealing film and incubated in a microplate shaker o/n at 30°C shaking at 1400 rpm.
8. The next day, bacteria in the phage production plate was pelleted by centrifugation for 5 min at 2000 rpm. Supernatant was carefully transferred without disturbing the pellet to a 96-well filtration plate.
9. The filtration plate was placed on top of a new 96-well U-bottom PP microtiter plate and fixed with sticky tape.
10. Antibody presenting phage particles were filtered to remove possible *E. coli* cells remaining by centrifugation for 2 – 5 min at 2000 rpm.
11. Filtrate (antibody presenting phage) was stored at 4°C until used.

4.3.6.5 Monoclonal ELISA with soluble antibody fragments

Half of the NUNC 96 well MaxiSorb ELISA plate was coated with 100 μ L of antigen (10 μ g/mL in PBS) and the remaining half with PTM for 1 hr at rt. The solutions were discarded by tapping the plates on a linen towel. The plates were washed 3 x with PBS-T by gentle agitation. All the surfaces of the wells were blocked using 500 μ L PTM and incubated for 1 hr at rt. The blocking solution was discarded and plates washed 3 x with PBS-T. 100 μ L of PTM was added to the first half of the plate coated with antigen. 100 μ L of soluble antibody supernatant was added to the PTM, mixed and 100 μ L transferred to the PTM coated half (eg. A1 to A7, A3 to A9 and so forth). The plate was incubated for 1 hr at rt. The solutions were discarded again and the wash step repeated. 200 μ L of Protein L-HRP (1:5000 in PTM) was added to each well and incubated for 1 hr at rt. The solution was discarded and the wash step repeated again. 100 μ L of the developing substrate was added to each well and left to develop for 30 min at rt in the dark. The absorbance was measured using a wavelength of 405 nm with the SpectraMAX 250 MTP spectrophotometer.

4.3.6.6 Monoclonal ELISA with antibody fragment presenting phages

Half of the NUNC 96 well MaxiSorb ELISA plate was coated with 100 μ L of antigen (10 μ g/mL in PBS) and the remaining half with PTM for 1 hr at rt. The solutions were discarded by tapping the plates on a linen towel. The plates were washed 3 x with PBS-T by gentle agitation. All the surfaces of the wells were blocked using 500 μ L PTM and incubated for 1hr at rt. The blocking solution was discarded and plates washed 3 x with PBS-T. 100 μ L of PTM was added to the first half of the plate coated with antigen. 100 μ L of antibody presenting phage solution was added to the PTM, mixed and 100 μ L transferred to the PTM coated half (eg. A1 to A7, A3 to A9 and so forth). The plate was incubated for 1 hr at rt. The solutions were discarded again and the wash step repeated. 200 μ L of mouse anti-M13-HRP (1:5000 in PTM) was added to each well and incubated for 1 hr at rt. The solution was discarded and the wash step repeated again. 100 μ L of the developing substrate was added to each well and left to develop for 30 min at rt in the dark. The absorbance was measured using a wavelength of 405 nm with the SpectraMAX 250 MTP spectrophotometer.

4.3.7 Antibody fragment presentation assay with Protein L beads

The assay is based on the depletion of antibody presenting phages after repeated rounds of surface coating from the same loading solutions. It was adopted from the Friguet-Test for affinity determination of antibodies in solution [167]. As protein L will capture kappa light chains, the phages recovered by the protein L beads would have both the antibody heavy and light chain assembled properly and presented on the phage surface.

Protein L-Biotin was coupled to the magnetic beads using the antigen loading protocol described earlier. Using a phage library of known cfu, a 1:10 dilution series of the phage library in the titre range of 10^8 to 10^1 cfu was prepared in 100 μ L PBS. Add 100 μ L of the dilution series to the second row of the 96-well U-bottom PP microtiter plate filled with 100 μ L of PTM. 10 μ L of Protein L coupled beads were added to 200 μ L of PTM in the first row of the MTP. Using the MPP, beads were transferred from Row 1 to Row 2 containing phage. The beads were incubated in the phage solution for 1 hr using the MPP with constant magnet movements as described in the panning procedure. Beads were washed using the MPP with 200 μ L PBS-T. The beads were released in 100 μ L of PBS after the wash. 100 μ L of the phage dilutions in PBS were infected with 100 μ L of TG1 culture with an $OD_{600} = 0.4-0.5$. The culture was incubated at RT for 30 min without shaking. 100 μ L of infected TG1 cells were plated on 10 cm 2YT Agar (2% glu + 100 μ g/mL Amp) plates. Plates were incubated o/n at 37°C. The cfu was calculated.

4.4 The Diversity (DiVE) Assay

The assay was developed to have a method visually compare the diversity of various rounds of phage display panning.

a. Phage Display Panning

Phage display panning was carried out using the semi-synthetic scFv library based on a single human framework for VH (V3-23/DP-47 and JH4b) and V κ (O12/O2/DPK9 and J κ 1) with diversity in the side chain using a DVT coding at antigen binding sites. Panning was carried out using magnetic beads in a magnetic particle processor applying a semi-automated selection protocol as described earlier. *E. coli* glycerol stocks of selection rounds 2 to 4 were stored at -80°C for analysis.

b. PCR amplification of variable regions

DNA was prepared from overnight *E. coli* cultures of a single scFv clone (4IE3) and from selection rounds 2, 3 and 4 using a MiniPrep Kit (Qiagen, Hilden, Germany). The final volume for all PCR reactions was 20 μ L containing 0.4 μ L of cDNA, 200 μ M dNTPs, 0.2 μ M of forward primer, 0.2 μ M reverse primer (Invitrogen, Karlsruhe, Germany) and 1 x Phusion HF reaction buffer (New England Biolabs, Frankfurt, Germany). The amount of polymerase used was 0.2 μ L of 2 U/ μ L of Phusion Taq from New England Biolabs, per reaction. All reactions were carried out using a MJ Research PCT-200 PCR machine. After heating at 95°C for 45 sec, we performed 30 cycles (30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C) and ended after 5 min at 72°C. PCR amplification of the variable heavy chain region was carried out using VH-NcoI-Fw (ACATGCCATGGCCGAGGTGCAGC) and VH-XhoI-Rv (CCGCTCGAGACGGTGACCAGG) primers. PCR amplification of the variable light chain was carried out using VL-SalI-Fw (AGCGGGTCGACGGACATCCAGATG) and VL-NotI-Rv (AAGGAAAAAAGCGGCCCGCCCGTTTGATTTC) primers. All PCR products were purified using the PCR Purification Kit (Qiagen) and eluted in 30 μ L of distilled water. The band size was approximately between 350 to 370 bp. DNA concentration was determined using NanoDrop ND-1000 (Thermo Fischer Scientific, Dreieich, Germany)

c. Optimisation of the DiVE Assay to the study of PCR amplicons

For optimisation, 350 ng of VH amplicon from 4IE3, round two and round four of positive enrichment selection rounds was used. Amplicons were subjected to heat denaturation at 98°C for 10 min and re-annealing at 60°C for 5 min before nuclease treatment. For temperature optimization, 1 U S1 nuclease (Roboklon GmbH, Berlin, Germany) was used with 30 min incubation at 65°C. S1 nuclease titration was carried out using 0.02 U, 0.1 U and 0.2 U of S1 nuclease with 30 min incubation time. The reaction was stopped by adding 2 µL of 0.5 M EDTA (pH 8). Optimisation of incubation times was carried out at 65°C with three different time points (10, 20 and 30 min) using 0.2 U of S1 nuclease.

d. Application of DiVE Assay to monitor phage display panning rounds

Both positive enrichment selection rounds were PCR amplified using the V-gene primers for heavy and light chain as described above. A total amount of 350 ng of DNA was used in a 50 µL reaction volume. Each reaction mix prepared contains the 0.5 X S1 reaction buffer (20 mM NaCH₃COO⁻, 0.15 M NaCl, 1 mM ZnSO₄) and distilled water to a final volume of 46 µL. The reaction mix was subjected to the denaturing and re-annealing process using the thermocycler as described above. 0.04 U of S1 nuclease was added to each reaction and incubated further at 65°C for 10 and 20 min. 2 µL of 0.5 M EDTA was added to each sample to stop the enzyme activity.

e. Analysis of DiVE assayed amplicons using electrophoresis

A 1% agarose gel with ethidium bromide was prepared. A 1:10 dilution of the 100 bp plus DNA ladder (Fermentas, St. Leon-Rot, Germany) was prepared using 1 X S1 reaction buffer (Roboklon GmbH, Berlin, Germany). Equal amounts of DNA were loaded on the gel for separation.

f. Batch sequencing of amplicons from all rounds

PCR amplicons of the heavy and light variable regions was subjected to batch sequencing by conventional Sanger Sequencing method using VH-NcoI-Fw and VL-SallI-Fw for both variable heavy and variable light chain fragments respectively.

5.0 Results

5.1 Construction of pTSL phagemid vector series

5.1.1 Amplification of constant regions of IgD

cDNA was amplified according to the method previously described using IgD Hinge Rv primer. To amplify the CH1 region from the cDNA template, the primers IgD-CH1-XhoI-Fw and IgD CH1-MluI-Rv were used. A fragment of approximately 280 bp in size was amplified (Figure 5.1).

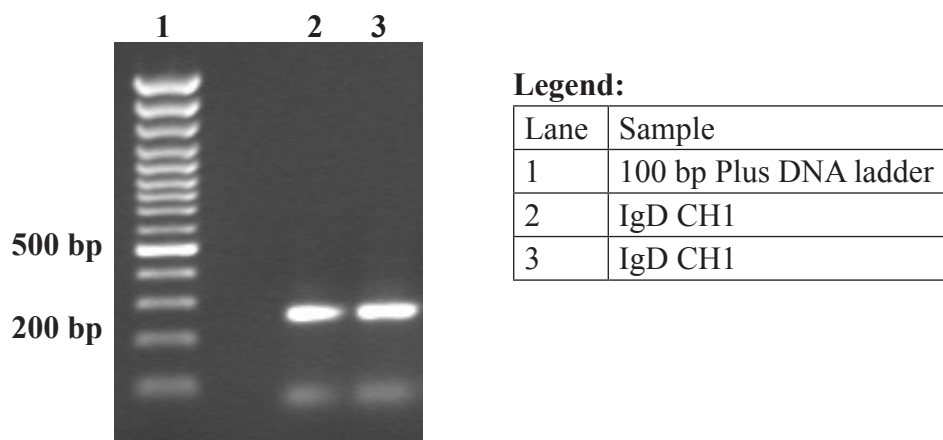


Figure 5.1: PCR amplicon of IgD CH1 fragment.

5.1.2 Amplification of ccdB cassette

The ccdB cassette required for cloning into the HC cloning site was amplified with primers 5'-ccdB-BssHI-NcoI and 3'-ccdB-XhoI. For LC, the ccdB cassette was amplified using 5'-ccdB-SalI and 3'-ccdB-NotI. A fragment of approximately 700 bp in size was amplified (Figure 5.2).

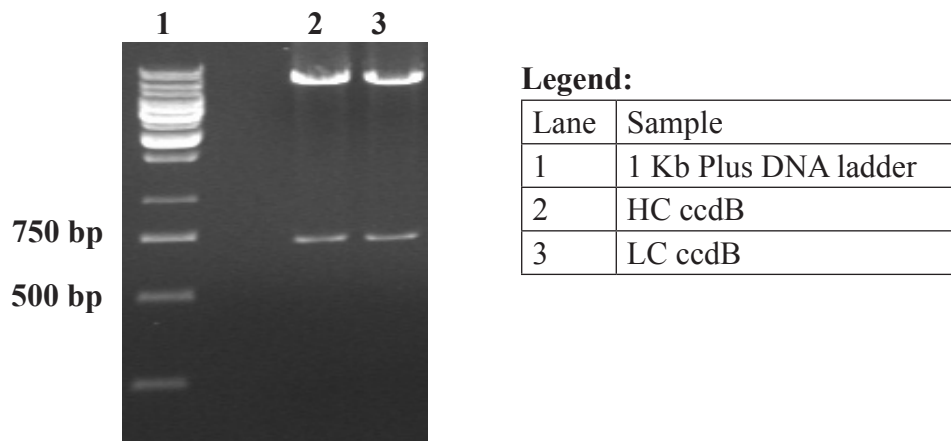


Figure 5.2: PCR amplicon of the ccdB cassette for heavy and light chain region.

5.1.3 Subcloning of inserts to phagemid vector

The phagemid vector backbone was derived from the pIT2 phagemid vector. Modifications using pSABA and pHAL vectors were used to incorporate required leader sequences and cloning sites. A maxi prep was prepared for all three vectors (pIT2, pHAL1, pSABA). pHAL1 was digested with NcoI and BamHI to remove the strep-tag and Fd region from the vector. pSABA was digested with NcoI and XmaI to obtain the HC fragment (VH cloning site, IgG CH1 and his-tag). The digested HC fragment from pSABA was PCR amplified using NcoI/XhoI His tag Fw and BamHI/MluI His tag Rv primers. The PCR amplified HC fragment was then digested with NcoI and BamHI and ligated to the digested pHAL vector resulting in pTSL Parking Vector.

pSABA vector was digested with XmaI and HindIII to obtain the LC fragment (PhoA leader, VL cloning site and Kappa constant region). The LC fragment was PCR amplified using HindIII-VL-pSABA-Fw and NheI/HindIII-CL-pSABA-Rv primers. The amplified LC fragment was digested with HindIII and ligated to a HindIII digested TOPO vector. The correct orientation of the fragment was confirmed using a NcoI and NotI digestion. pTSL parking and LC-Topo were digested with HindIII and ligated together. To confirm the orientation of the LC inserted, a test digestion with Sall and BamHI was carried out. The vector constructed was named pTSL Parking 2.

pTSL Parking 2 was digested with HindIII and EcoRI to obtain the HC-pIII fragment (RBS, PelB, VH Cloning site, IgG CH1, His-Tag and pIII). The same digestion was carried out on the pIT2 vector. Cloning with the HindIII and EcoRI cut site was carried out to insert the HC-pIII fragment to the pIT2 vector. This was named pTSL Parking 3. A second digestion was carried out using only HindIII with pTSL Parking 2 and 3. This removed the PhoA-LC fragment from pTSL Parking 2 and opened the pTSL Parking 3 at the HindIII cut site. The PhoA-LC fragment was ligated into the pTSL Parking 3 vector at the HindIII site. Orientation of clones was checked using a NotI and MluI digestion (correct orientation 754 bp, wrong orientation 540 bp). This was renamed as pTSL2.

The 5'-ccdB-Sall and 3'-ccdB-NotI amplified ccdB cassette together with pTSL2 was digested with Sall and NotI. The ccdB cassette was ligated to the pTSL vector using the Sall and NotI site. The vector was then digested with NcoI and XhoI and ligated to the 5'-ccdB-BssHI-NcoI and 3'-ccdB-XhoI amplified ccdB with NcoI and XhoI digestion resulting in pTSL2.

pTSL2 was digested with XhoI and MluI to exchange the IgG CH1 with IgD CH1. The IgD CH1 amplified template was also digested with XhoI and MluI for ligation. This ligation resulted in pTSL1. pTSL3 was constructed using pTSL2. pTSL2 was digested with Sall and NotI to remove the ccdB-CL region. A PCR amplified fragment of the ccdB cassette using 5'-ccdB-Sall and 3'-ccdB-NotI was ligated to the vector using Sall and NotI and called ccdB LC modified pTSL. For the HC cloning site, the ccdB cassette was amplified using 5'-ccdB-NcoI and 3'-ccdB-MluI. The amplicon and ccdB LC modified vector were both digested with NcoI and MluI for ligation. pTSL4 was created by conducting a BsrGI digest on pTSL3. The vector was gel purified and religated.

5.1.4 pTSL Vector series

Four phagemid vectors were created for the universal shuttling of scFv to Fab formats and vice versa. Figure 5.3 shows the chronology of the pTSL vector series.

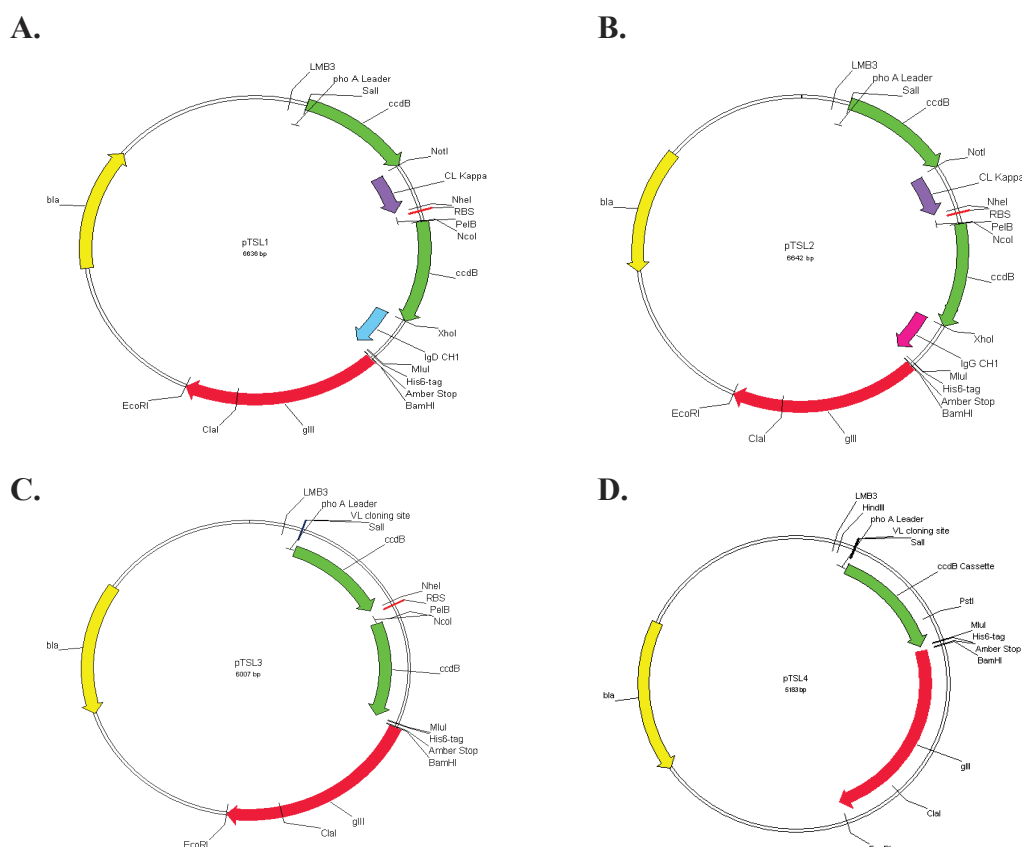


Figure 5.3: Chronology of pTSL phagemid vector series. (A.) pTSL 1 with a dual ccdB cassette for scFv conversion to IgD Fab, (B.) pTSL 2 with a dual ccdB cassette for scFv conversion to IgG Fab, (C.) pTSL3 with a dual ccdB cassette for the generation of full Fab constructs, (D.) pTSL4 with a single ccdB cassette for cloning pIII fusion constructs.

All phagemid vectors are based on the pIT2, pSABA and pHAL phagemid vectors. The pTSL vector series has two origins of replication being the pBR322 and fl origin for double- and single-stranded DNA synthesis (plasmid and filamentous phage origins). It also has an ampicillin resistant gene for selection of transformed bacteria. The pTSL vector series also contains a pIII fusion gene for expression of pIII fused proteins. This is under the control of a lac promoter. A signal sequence is fused to the pIII fused protein to direct secretion of the fused protein and subsequently cleaved by a signal peptidase. There are two signal peptides for pTSL1, 2 and 3. The signal peptides are PhoA and PelB, for light and heavy chain respectively. Each protein will be expressed with a His-Tag at the C-terminal end to allow easy purification using immobilized metal ion affinity chromatography (IMAC). The pTSL phagemid vectors allow conversion to infective phage by superinfection using phagemid-bearing cells containing helper phages. The ccdB suicide gene cassette included into the cloning sites helps to ensure selectivity for positive clones from blank clones to lower recombination background using non-ccdB sensitive strains. The cloning sites designed allow easy inter-conversion between formats and complements the pIT2 vector. Table 5.1 shows a summary of the restriction enzymes sites.

Table 5.1: Summary of cloning sites in pTSL vector series.

Vector	Ab format	Format Switch	Light Chain		Heavy Chain	
pTSL1	IgD Fab	scFv to IgD Fab	SalI	NotI	NcoI	XhoI
pTSL2	IgG Fab	scFv to IgG Fab	SalI	NotI	NcoI	XhoI
pTSL3	Full Fab	Any isotype Fab	SalI	NheI	NcoI	MluI
pTSL4 ¹	pIII-fusion	Any protein	SalI		MluI	

Note: ¹Vector was designed to insert one protein sequence.

5.1.5 Evaluation of CcdB cassette activity

Each vector clone was evaluated for ccdB suicide activity before use. The comparison of growth in DB3.1 and DH10B *E.coli* strains were carried out. For *E. coli* strains to be resistant to the ccdB activity, the presence of *gyrA462* in the bacteria genome is required [168]. DB3.1 strain has the *gyrA462* gene [168] whereas the DH10B strain does not [169] thus making the DB3.1 strain resistant to ccdB activity and DH10B non-resistant. The phagemids containing the dual ccdB cassettes was able to inhibit the growth on non-resistant strain (DH10B). The ability to inhibit growth in non-resistant strains makes this feature highly effective in lowering transformation backgrounds. As a control, pUC18 was used, where no growth inhibition was seen (Figure 5.4).

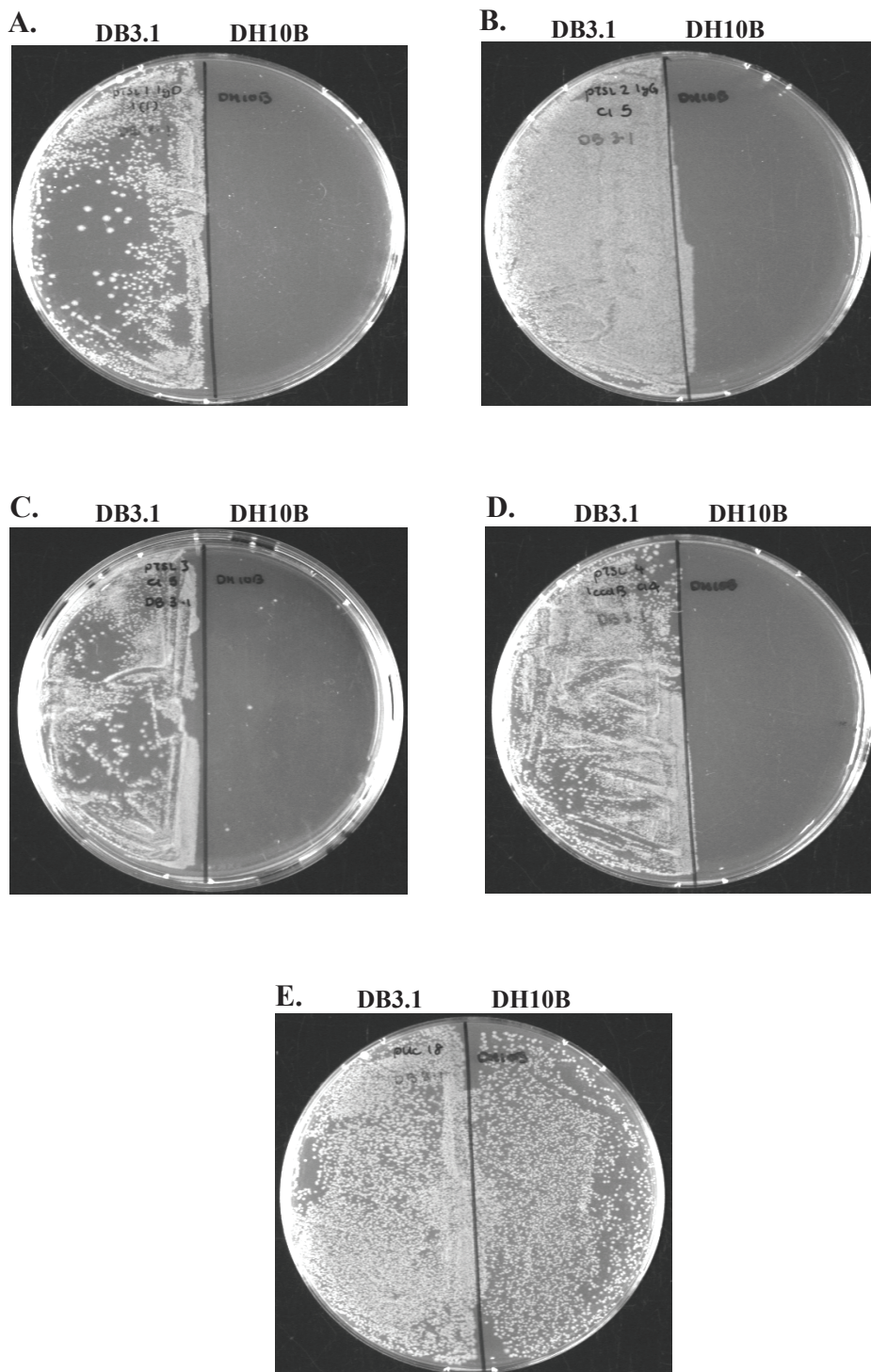


Figure 5.4: ccdB activity evaluation of pTSL phagemid vector series on DB3.1 and DH10B. (A.) pTSL1 in DB3.1 and DH10B, (B.) pTSL2 in DB3.1 and DH10B, (C.) pTSL3 in DB3.1 and DH10B, (D.) pTSL4 in DB3.1 and DH10B, (E.) Control plasmid pUC18 in DB3.1 and DH10B.

5.2 Generation of semi-synthetic antibody repertoire for library generation

The assembly of the semi-synthetic antibody repertoire was carried out by PCR assembly and one-step cloning. As a basis, the original Tomlinson I library was used.

5.2.1 Generation of Variable Region Repertoire

Variable regions for heavy and light chains were amplified via PCR from a Maxi preparation of the Tomlinson semi synthetic antibody library. The variable heavy chain region was amplified using the primer pair of VH-Tom-NcoI-Fw and VH-Tom-XhoI-Rv. The amplicon was approximately 370 bp in size. The variable light chain region was amplified using the primer pair of VL-Tom-SalI-Fw and VL-Tom-NotI-Rv. The VL amplicon was approximately 350 bp in size (Figure 5.5).

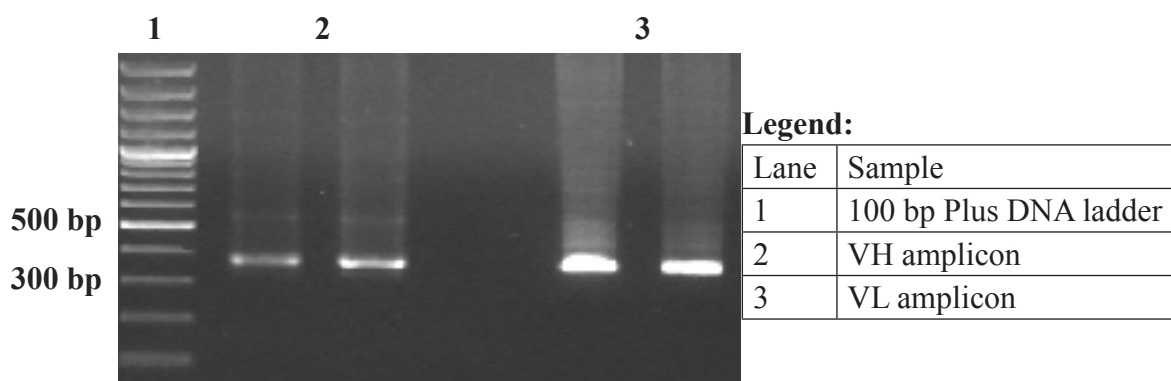


Figure 5.5: PCR amplification of heavy and light variable regions.

5.2.2 PCR assembled 1-step Cloning Strategy

To generate the required fragments for cloning, ligation-pcr amplification procedure was carried out. Fragments needed to be digested, then ligated and the ligation product amplified to increase the concentration of DNA material. This was divided out to 3 stages of work:-

a. Assembly of Variable light to kappa constant region with DNA linker

The LMB Fw primer with the pIII Rv primer was used to amplify the cloning cassette from pTSL2. A 2.4 Kb fragment consisting of the LC region ccdB, kappa constant region, HC leader peptide, HC region ccdB, IgG CH1 region and partial sequence of the pIII gene was amplified (Figure 5.6). The fragment was gel purified using the Qiagen Gel Extraction Kit and digested using NotI to remove the LC ccdB cassette. The remaining 1.6 Kb fragment was gel purified (Figure 5.7). The variable light chain fragments were also digested with NotI and gel purified. The 1.6 Kb fragment was ligated to the variable light fragment using T4 DNA ligase (400 U/ μ L) from NEB to form the LC fragment. The ligated fragment was purified using Qiagen PCR Purification kit. The purified LC fragment was then PCR amplified using VL-Tom-Sall-Fw and pIII Rv yielding a fragment approximately 1.9 Kb in size (Figure 5.8). The LC fragment was then gel purified.

b. Assembly of Variable heavy to light chain fragment

The gel purified LC fragment and PCR amplified VH fragment was digested using NcoI. The resulting 770 bp fragment from the LC fragment was excised from gel and purified (Figure 5.9). The VH fragment was purified using Qiagen PCR purification kit. Both fragments were ligated resulting in a 1.1 Kb size of variable regions fragment (VL-CL-DNA linker-VH). The ligation product was PCR amplified using outer primers, VL-Tom-Sall-Fw and VH-Tom-XhoI-Rv using the conditions described earlier (Figure 5.10). The PCR product was gel purified using the Qiagen gel extraction kit.

c. Final assembly product for library generation

IgD CH1 and IgG CH1 was amplified using pTSL1 (IgD) and pTSL2(IgG) as template. For the IgD CH1 fragments, primers IgD CH1 XhoI Fw and IgD CH1 MluI Rv were used. Primers IgG CH1 XhoI Fw and IgG CH1 MluI Rv were used to amplify IgG CH1 fragment. The IgD and IgG CH1 fragments were digested with XhoI. The PCR amplified VH-VL-cloning cassette was also digested using XhoI. The digested products were ligated and PCR amplified using VL-Tom-Sall-Fw with IgD CH1 MluI Rv (for IgD Fab) and IgG CH1 MluI Rv (for IgG Fab). This resulted in a fragment of approximately 1.4 Kb (Figure 5.11).

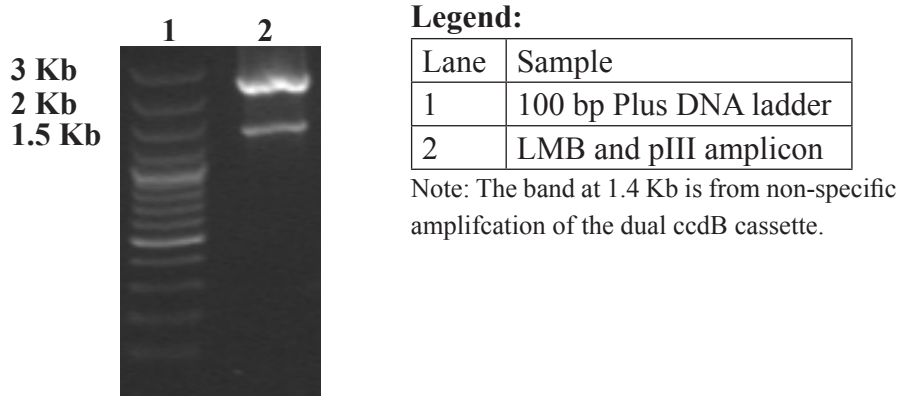


Figure 5.6: PCR amplification of amplify the cloning cassette from pTSL2 using LMB and pIII primers.

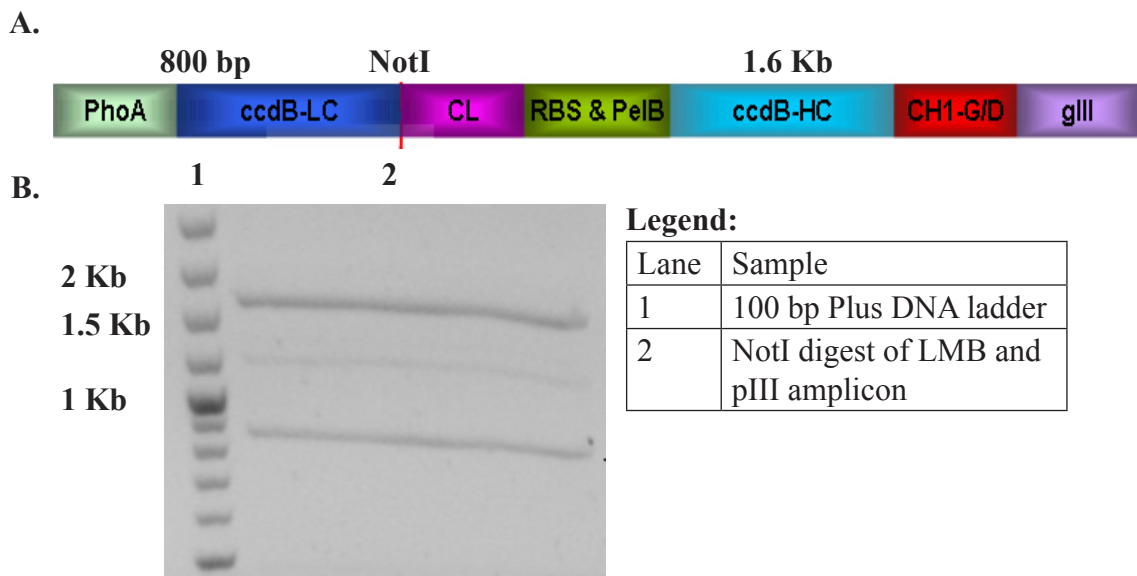


Figure 5.7: NotI digestion of cloning cassette from pTSL2. (A) Cartoon showing the fragments of the amplified cloning cassette. (B) Gel electrophoresis separation of NotI digestion of the cloning cassette.

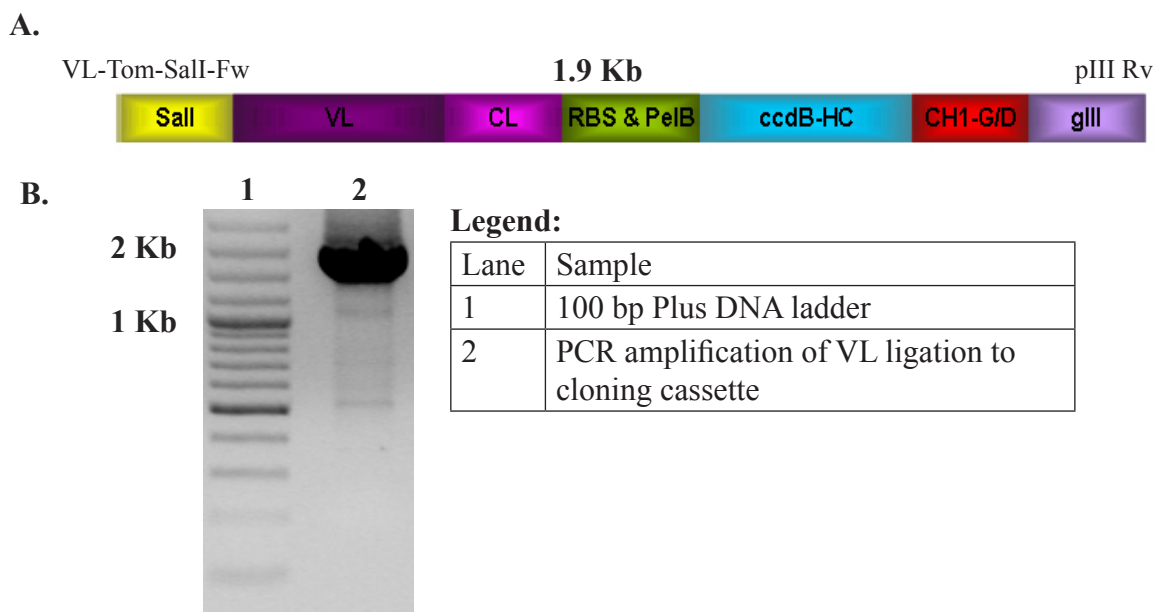


Figure 5.8: PCR amplification of VL ligated to cloning cassette from pTSL2. (A) Cartoon showing the fragments of the amplified VL-cloning cassette. (B) Gel electrophoresis separation of the VL-cloning cassette.

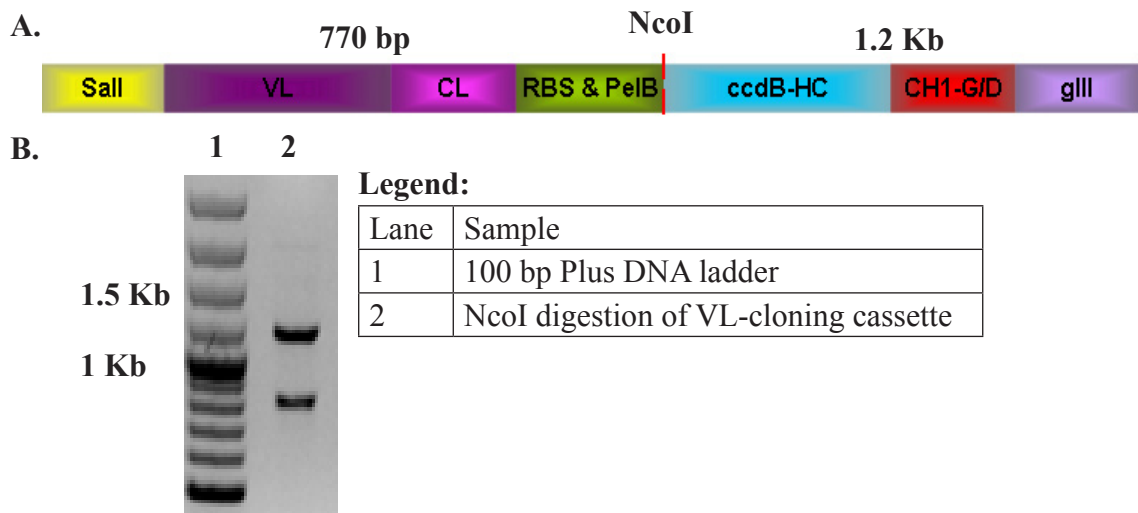


Figure 5.9: PCR amplification of VL ligated to cloning cassette from pTSL2. (A) Cartoon showing the fragments of the amplified VL-cloning cassette. (B) Gel electrophoresis separation of the VL-cloning cassette.

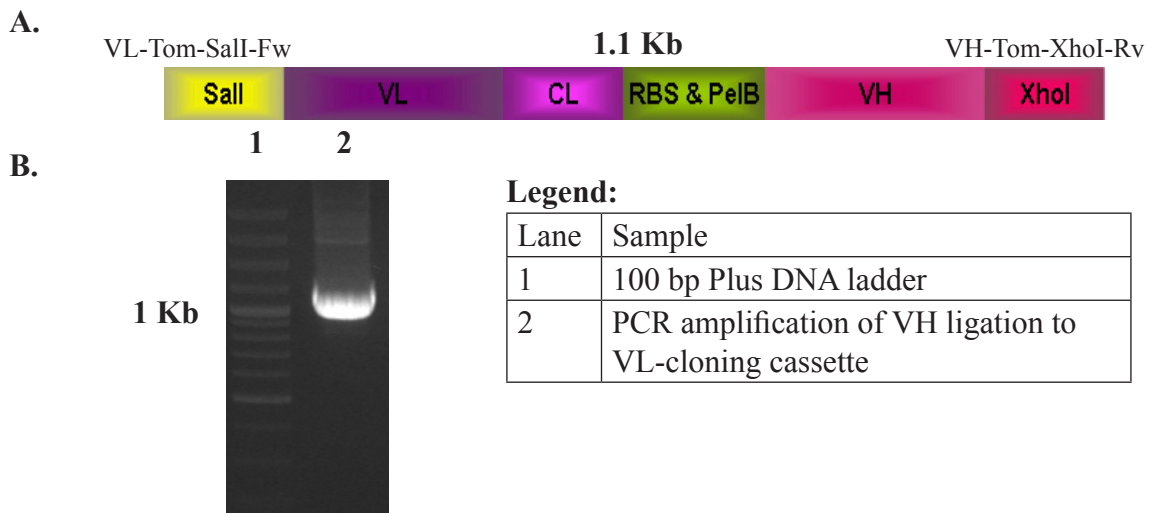


Figure 5.10: PCR amplification of VH ligated to VL-cloning cassette. (A) Cartoon showing the fragments of the amplified VH-VL-cloning cassette. (B) Gel electrophoresis separation of the VH-VL-cloning cassette.

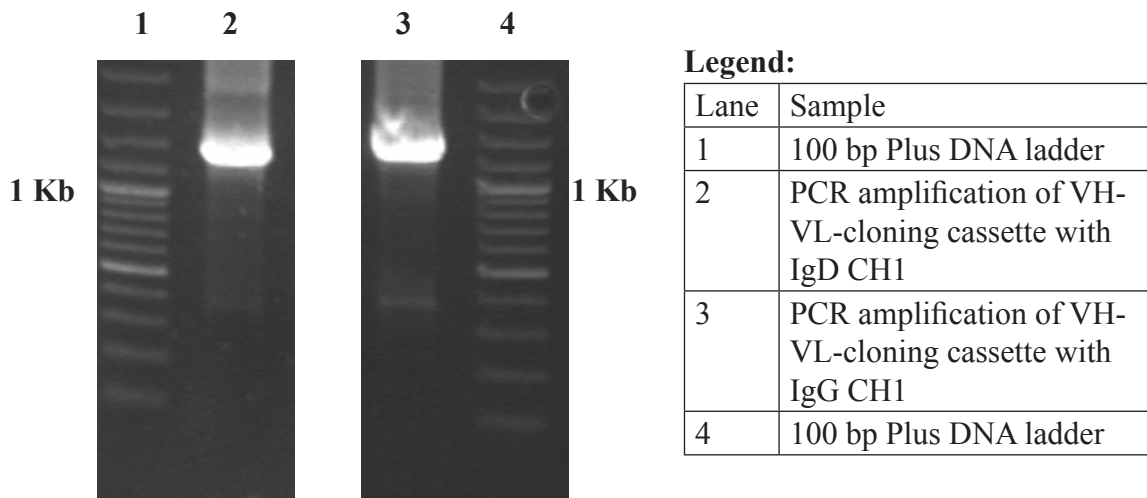


Figure 5.11: PCR amplification of VH -VL-cloning cassette ligated to IgD and IgG CH1 respectively.

5.2.3 Semi synthetic Library Generation

a. Vector preparation for sub-cloning

The vector pTSL2 and the VH-VL-cloning cassette for IgD and IgG Fab were digested with Sall and MluI. The VH-VL-cloning cassettes were purified using the PCR purification kit from Qiagen. As gel extraction of the digested vector reduced the transformation efficiency tremendously (data not shown), Gel extraction of the digested vector reduced the transformation efficiency tremendously (data not shown), therefore the entire digestion reaction of the vector pTSL2 was dephosphorylated using the Antarctic Phosphatase without any gel separation. In order to ensure low religation from the dephosphorylated vector, a test transformation with DH10B *E. coli* cells was carried out to determine the background of the dephosphorylated vector before proceeding (Figure 5.12).

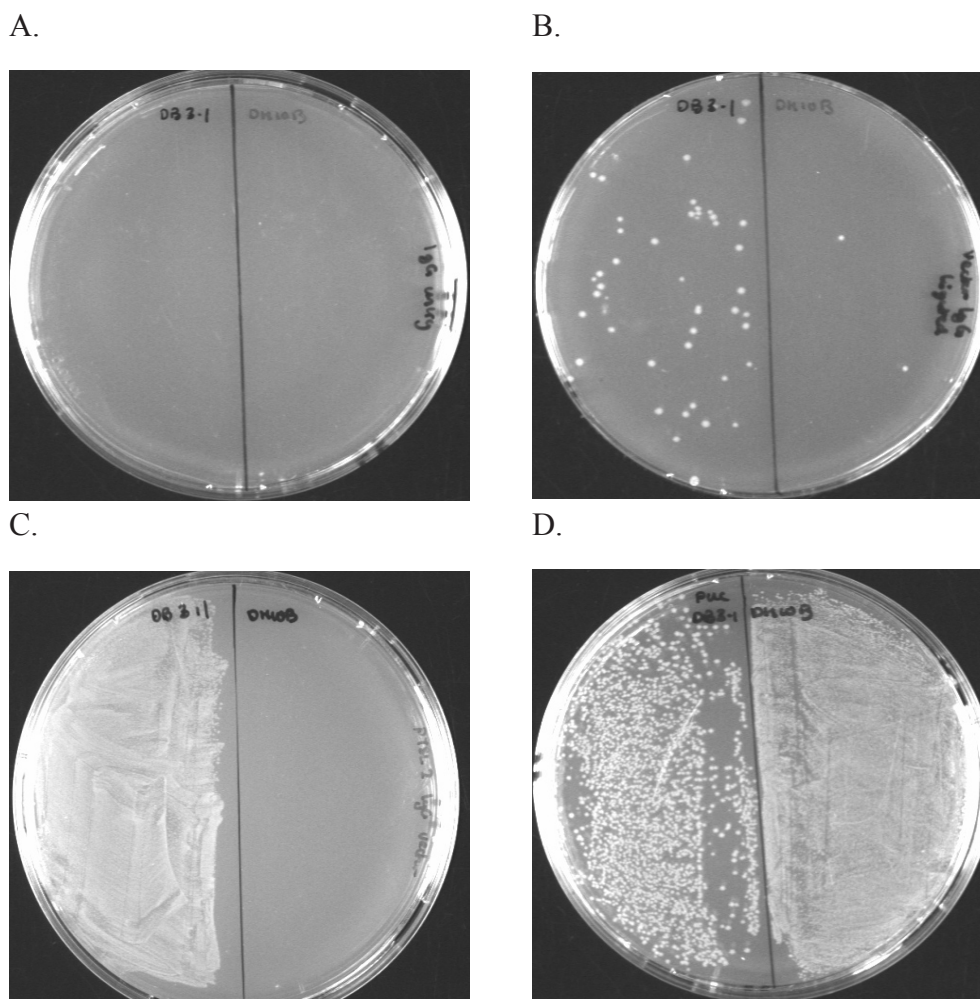


Figure 5.12: Test transformation of dephosphorylated vector pTSL2 for antibody library sub-cloning. (A.) Sall/MluI digested pTSL2 not ligated, (B.) Religation of Sall/MluI digested pTSL2, (C.) Uncut pTSL2, (D.) pUC18 Control. DB3.1 cells were used on the left panel and DH10B cells were used on the right panel.

b. *Cloning of semi synthetic antibody library*

The dephosphorylated vector pTSL2 and the VH-VL-cloning cassette for IgD and IgG Fab were ligated and transformed. A total of 85 ligation reactions per library were generated and each ligation was precipitated using the ethanol method as described. Individual ligation reaction tube was reconstituted with 2 μL of ddH₂O and transformed in 50 μL of TG1 *E. Coli* cells. The transformed cells were resuspended in 1 mL SOC media and grown for 1 hour at 37°C with constant shaking (650 rpm). 2 reactions (2 mL) were plated on one 25 x 25 cm 2YT (100 $\mu\text{g}/\text{mL}$ Amp + 2 % Glu) agar plates. A total of 41 agar plates were used for each IgD and IgG library.

c. *Determination of library diversity*

The diversity of the libraries were estimated using four different protocols as described earlier. The methods with results in close proximities were used. The average reading was used as the library diversity. The first method, by titrating the diversity was not able to show any results. No cell growth was possible and the colonies concentrated in a 10 μL droplet was uncountable. The method was not suitable. The second, third and fourth method using a dilution series and plating a certain volume on different surface sizes gave better estimates of the diversity. A summary of the estimated diversities of both libraries with the different methods are shown in Table 5.2.

Table 5.2: Summary of library diversity estimation using different protocols.

Method Dilution	IgD Fab Library				IgG Fab Library			
	1	2	3	4 ^a	1	2	3	4 ^a
100	- ^b	9.0x10 ⁶	8.3x10 ⁷	3.6x10 ⁷	-	2.4x10 ⁷	3.5x10 ⁷	2.2x10 ⁷
1000	-	6.6x10 ⁶	8.6x10 ⁷	3.2x10 ⁷	-	1.5x10 ⁷	2.9x10 ⁷	2.0x10 ⁷
10000	-	1.6x10 ⁷	4.5x10 ⁷	-	-	- ^c	2.2x10 ⁷	-
Average	-	1.1x10 ⁷	7.1x10 ⁷	3.4x10 ⁷	-	2.0x10 ⁷	2.9x10 ⁷	2.1x10 ⁷
Overall	3.9 x10 ⁷				2.3 x 10 ⁷			

Note: ^aThis method uses a different dilution series: 20 and 10.

^bNo clones were available with this method.

^cNo clones were available at this dilution.

d. Colony PCR evaluation of library clones

Colony PCR was carried out using LMB and pIII primer pair to amplify the entire VH-VL-cloning cassette for both IgD and IgG Fab libraries. 24 samples from each library were analyzed. Figure 5.13 shows 8 of the 24 samples from the colony PCR analysis of both IgD and IgG library. An expected size band of 1.6 Kb is of the VH-VL-cloning cassette with the CH1 regions. The colony PCR analysis showed that 92 % and 96 % of the clones from the IgD and IgG library, respectively had the correct insert.

e. Sequencing of clones from library preparation

48 clones were selected and sequenced using the sequencing primers for heavy and light chains. Sequences were analyzed using the ContigExpress of the VectorNTI package (Invitrogen). Of the 48 clones, 94 % of the clones contained inserts for the IgD library and 96 % of the IgG library contained inserts. Table 5.3 shows the summary of the number of clones containing inserts according to their respective libraries and chains.

The diversification of amino acids is restricted to pre-defined positions in the framework. Only CDR 2 and CDR3 are randomized in this library. For the variable region of the heavy chain, 7 positions out of 10 in CDR2 and 4 positions at CDR3 was randomized. For the light chain variable region, 2 out of 7 and 5 out of 8 positions were randomized for CDR2 and CDR3, respectively. The CDR1 on both chains were not randomized and remained constant. Randomization of the amino acid positions was initially carried out by using degenerate oligos for gene assembly. Table 5.4 shows the sequence diversity of each CDR from 8 selected clones from both libraries.

The DVT codon used in the generation of the antibody repertoire in the Tomlinson semi synthetic library allows 8 possible amino acids to be represented at the positions. Table 5.5 shows the allowed codons and the amino acids encoded by the codons. The amino acids allowed are alanine, asparagine, aspartic acid, cysteine, glycine, serine, threonine and tyrosine. Seven of the eight amino acids allowed are encoded by a single codon each except for serine where two codons are allowed. The distribution of each amino acid should be proportional for the seven amino acids with a slight higher percentage of occurrence for serine. The distribution of amino acids based on their positions for the heavy and light variable region are as shown in Figure 5.14. All positions were randomized by a minimum of 5 amino acid combinations.

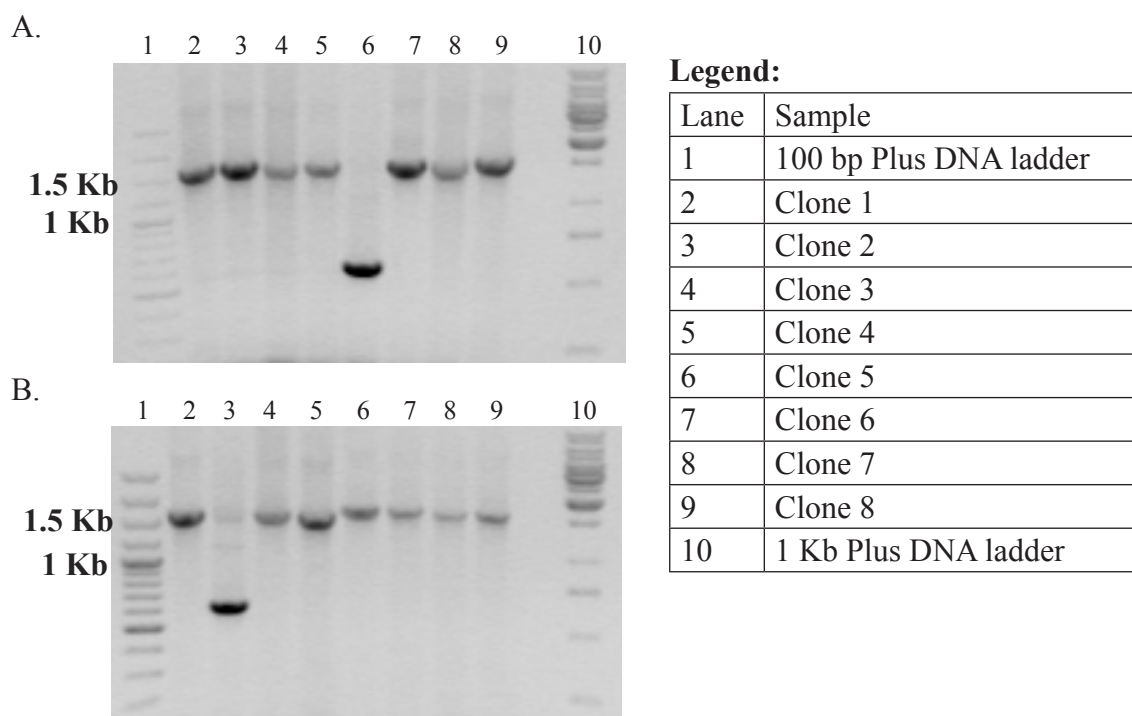


Figure 5.13: Colony PCR analysis showing 8 of 24 selected clones. (A.) IgD Fab library clones, (B.) IgG Fab library clones. The remaining 16 clones from the IgD library had 1 negative and 15 positives. The remaining 16 clones for the IgG library were positive.

Table 5.3: Summary of 48 sequenced clones from both sub-cloned libraries.

Library	IgD Fab		IgG Fab	
	Light Chain	Heavy Chain	Light Chain	Heavy Chain
No. of clones with insert	46	45	47	46
% of clones with insert per chain	96 %	94 %	98 %	96 %
Overall % ¹	94 %		96 %	

Note: ¹ Overall % is taken as the lowest % as both chains contribute to the entire construct.

Theoretically, there should not be any bias of codon usage at the randomized positions. Even so, there seems to be a preference for certain amino acids at given regions. From the list of 8 possible amino acids allowed by DVT, cysteine seems to be the un-preferred choice for the CDR regions. Alanine, serine and threonine are the preferred amino acids from the codon to be used. Asparagine, aspartic acid, glycine and tyrosine are moderately used. There does not seem to be a preference in amino acids for the different chains. The distribution over the light and heavy chain is random.

Table 5.4: Example of sequence diversity located in each CDR highlighted from 8 randomly selected clones. (A.) Clones from IgD Fab library, (B.) Clones from IgG Fab library.

A.

IgD	LC-CDR1	LC-CDR2	LC-CDR3	HC-CDR1	HC-CDR2	HC-CDR3
	RASQSISSLYN	*AS*LQS	QQ****P*	SYAMS	*I****G**T*	****
1	RASQSISSLYN	TASDLQS	QQDYATPA	SYAMS	AIGCSGTSTS	ADDD
2	RASQSISSLYN	TASYLQS	QQTSTYPA	SYAMS	SIAYAGDSTS	GTAD
3	RASQSISSLYN	TASNLQS	QQSDSNPN	SYAMS	TIAYGGNSTA	TTSY
4	RASQSISSLYN	SASNLQS	QQATSSPA	SYAMS	YINADGATTS	ACGA
5	RASQSISSLYN	AASNLQS	QQSDAAPC	SYAMS	TITSTGANTY	YATA
6	RASQSISSLYN	AASALQS	QQDITYAPD	SYAMS	SIGNSGASTS	SAST
7	RASQSISSLYN	SASSLQS	QQADYYPS	SYAMS	SICDTGGCTN	SDDS
8	RASQSISSLYN	TASALQS	QQTAYDPA	SYAMS	SIGTGGATTS	ASCG

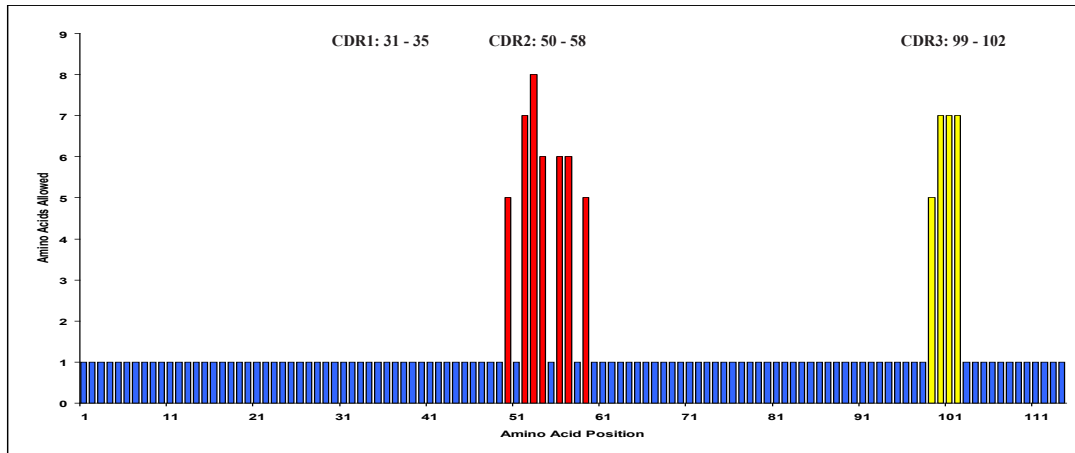
B.

IgG	LC-CDR1	LC-CDR2	LC-CDR3	HC-CDR1	HC-CDR2	HC-CDR3
	RASQSISSLYN	*AS*LQS	QQ****P*	SYAMS	*I****G**T*	****
1	RASQSISSLYN	GASDLQS	QQTADNPD	SYAMS	AIATSGNATS	SGTG
2	RASQSISSLYN	SASALQS	QQTYTAPT	SYAMS	TIGAAGNTTT	ATGS
3	RASQSISSLYN	AASSLQS	QQAANTPY	SYAMS	SIASAGSYTA	GDA A
4	RASQSISSLYN	AASYLQS	QQSYYNPT	SYAMS	SISGAGAATS	YDDT
5	RASQSISSLYN	SASNLQS	QQSYSTPN	SYAMS	TIDASGNNTT	SADN
6	RASQSISSLYN	SASSLQS	QQSANS PA	SYAMS	SIAACGDCTN	TDYS
7	RASQSISSLYN	GASGLQS	QQTSASPS	SYAMS	YIDYDGSATT	TYAS
8	RASQSISSLYN	YASSLQS	QQNADTPS	SYAMS	GIATSGTTTT	SSSS

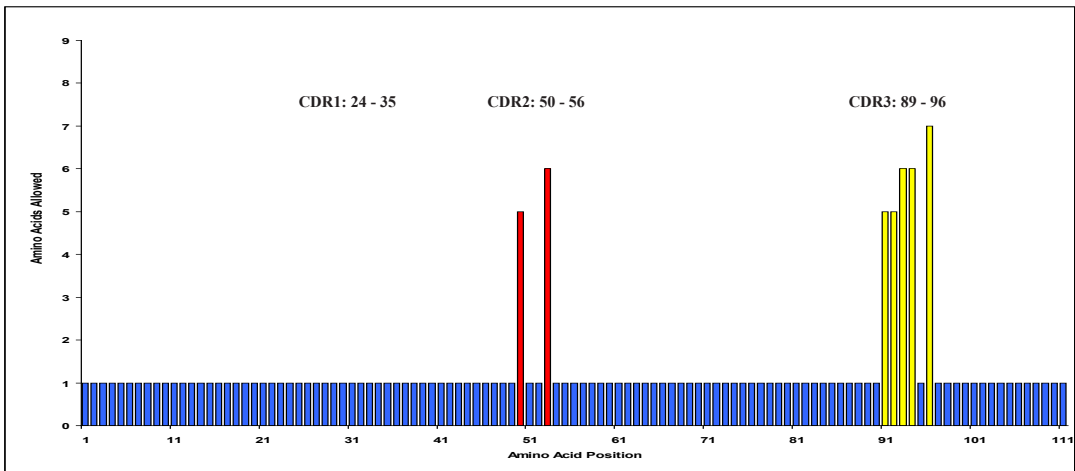
Table 5.5: Summary of allowed amino acids by DVT codon. Table summarizes the amino acids and codon usage allowed by DVT. The theoretical % of use is the expected % of use if no preference is allowed in the sequence.

Amino Acid (3 Letter Code)	Codon Usage	Theoretical % of use
Alanine (Ala)	GCT	12.5
Asparagine (Asn)	AAT	12.5
Aspartic Acid (Asp)	GAT	12.5
Cysteine (Cys)	TGT	12.5
Glycine (Gly)	GGT	12.5
Serine (Ser)	TCT or AGT	25.0
Threonine (Thr)	ACT	12.5
Tyrosine (Tyr)	TAT	12.5

A.



B.



C.

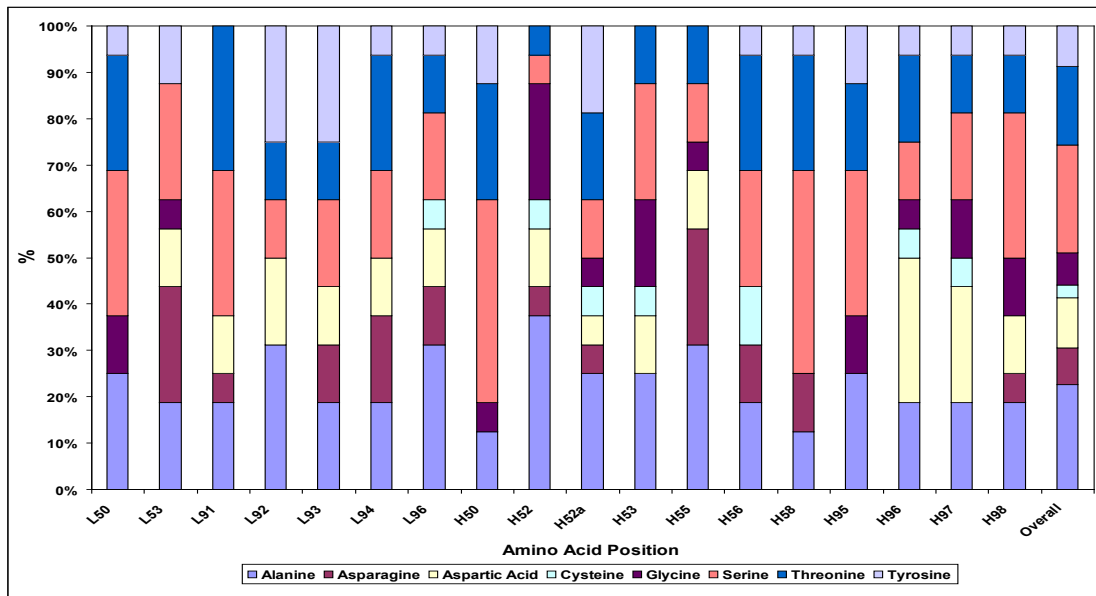


Figure 5.14: Distribution pattern of amino acids allowed by DVT codon at allowed positions in the CDR regions. (A.) Number of varying amino acids in the Variable Heavy Chain, (B.) Number of varying amino acids in the Variable Light Chain, (C.) Distribution pattern amino acid based. Red represents CDR2 and yellow for CDR3 positions where randomizations are allowed. Numbers represent positions. L is for the Light Chain and H is for the Heavy Chain. Overall represents the overall distribution of amino acids regardless of position.

5.3 Antigen preparation

The recombinant antigens expressed using the plasmid pRSET-BH6 and pRSET-BH6-GW are expressed with a AVI-Tag at the N-terminus, thus allowing in vivo biotinylation to occur. The *E. Coli* cells used were prepared with a helper plasmid pRARE3 which serves to increase the expression levels of the recombinant proteins in bacteria with the introduction of specific tRNAs. Other than the AVI-Tag, the proteins expressed also has a His-Tag at the N-terminal end of the protein. This will allow the use of immobilized metal affinity chromatography (IMAC) methods to purify the proteins. Various purification strategies were evaluated using different purification matrixes. The proteins were also subjected to identification by mass spectrometry and sequencing to ensure the correct proteins were used. As the employed antibody selection processes requires the antigens to be biotinylated, the success of the biotinylation was also evaluated before application to downstream processes. Antigens evaluated for use in the antibody selection process are shown in Table 5.6.

5.3.1 Evaluation of antigen purification strategies

The purification strategies evaluated makes use of either of the affinity tags available on the proteins (AVI-Tag and His-Tag). Model antigens GRB2 and PARK7 together with MAPK9 and Ubi8 were cloned in the *E. Coli* expression vector pRSET-BH6 and pRSET-BH6-GW, respectively. The use of Ni-TED, Ni-NTA and Monomeric Avidin for antigen preparation was evaluated. Each has specificity to different attributes on the proteins (Figure 5.15)

Table 5.6: List of antigens with their respective molecular weight evaluated for antibody selection process. A panel of 16 antigens were evaluated for use. Only purified, confirmed and successfully biotinylated proteins are used for antibody selection process.

Antigen	Molecular Weight (kDa)	Antigen	Molecular Weight (kDa)
ATF4-A3	47	GST	30
ATF4-E2	47	MAPK9	55
ATF4-MPMG	47	ROA1	25
ATX2	56 / 28 ^a	SH3GL1	44
Cyclophilin	25	SH3GL2	43
eGFP	32	SH3GL3	40
GRB2	30	TPI-SV	30
GPS2	40	Ubi8	20

Note: ^a Protein is subjected to in vivo degradation.

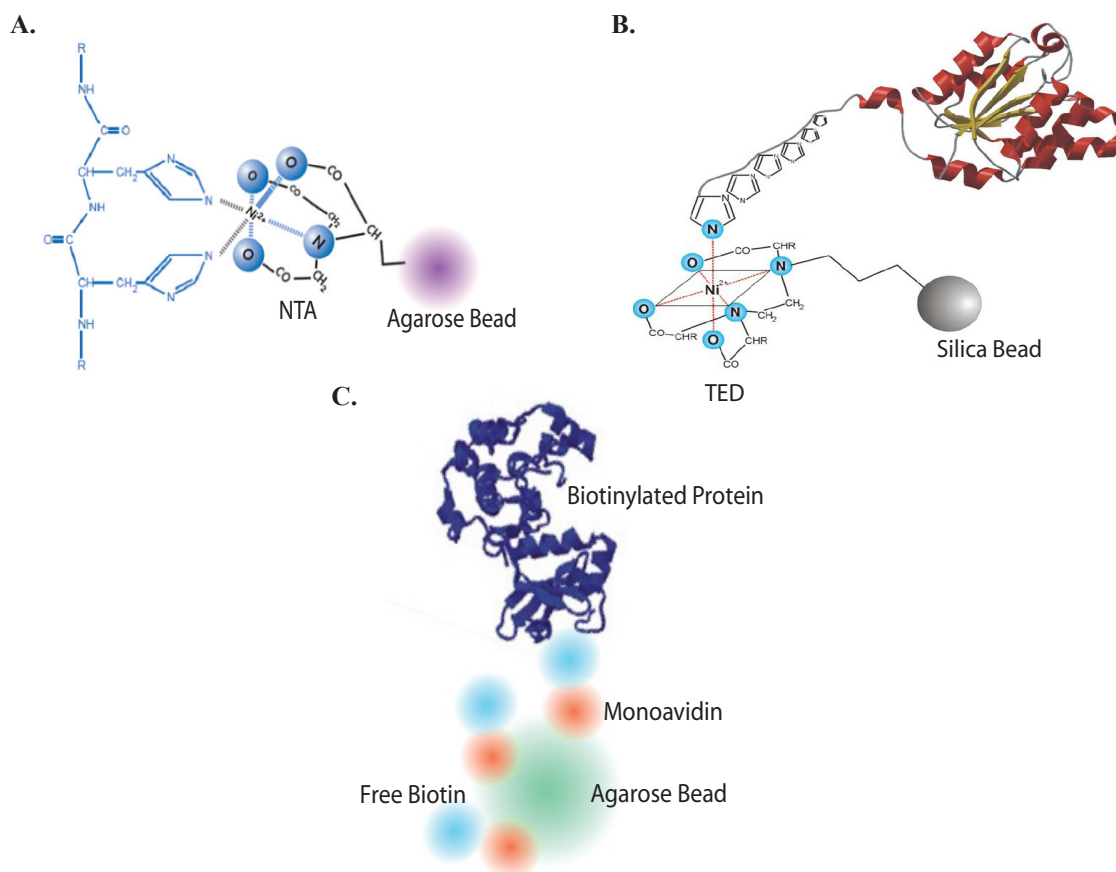
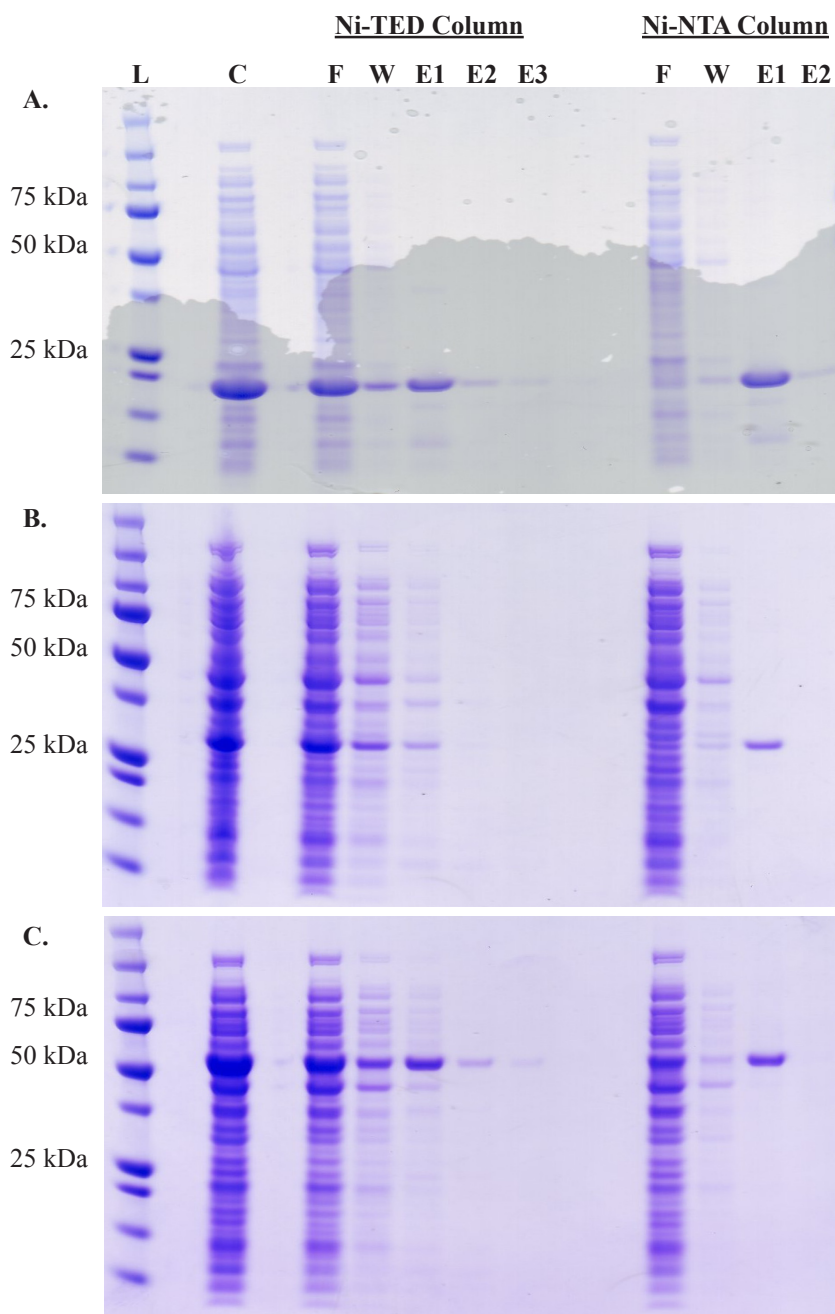


Figure 5.15: Protein purification strategies used. (A.) Ni-NTA (Nitrilotriacetic acid), (B.) Ni-TED (Tris(carboxymethyl) ethylendiamin), (C.) Monoavidin.

a. Purification via His-Tag

For His-Tag purification, two different matrixes were evaluated. Two IMAC columns; Ni-NTA from Qiagen and Ni-TED from Marchery Nagel were used for affinity purification coupling Ni^{2+} ions to the histidine from the His-Tag. Figure 5.16 shows the comparison of both purification methods using proteins of varying sizes on SDS gel.

Both Ni-NTA and Ni-TED columns were able to purify the antigens with a certain degree of specificity. Both columns were packed with equal bed volumes of 1 mL and the amount of protein loaded on each column was also similar. A direct comparison shows that the Ni-NTA column has a higher binding capacity per bed volume than Ni-TED. This was evident with the higher protein band in the Flow-Through fraction from Ni-TED instead of Ni-NTA. Ni-NTA also showed a higher retention of antigens in the wash fractions as compared to Ni-TED and, hence, the recovery of recombinant protein was higher using Ni-NTA rather than Ni-TED. The purity of the protein from both columns were comparable (Figure 5.16).



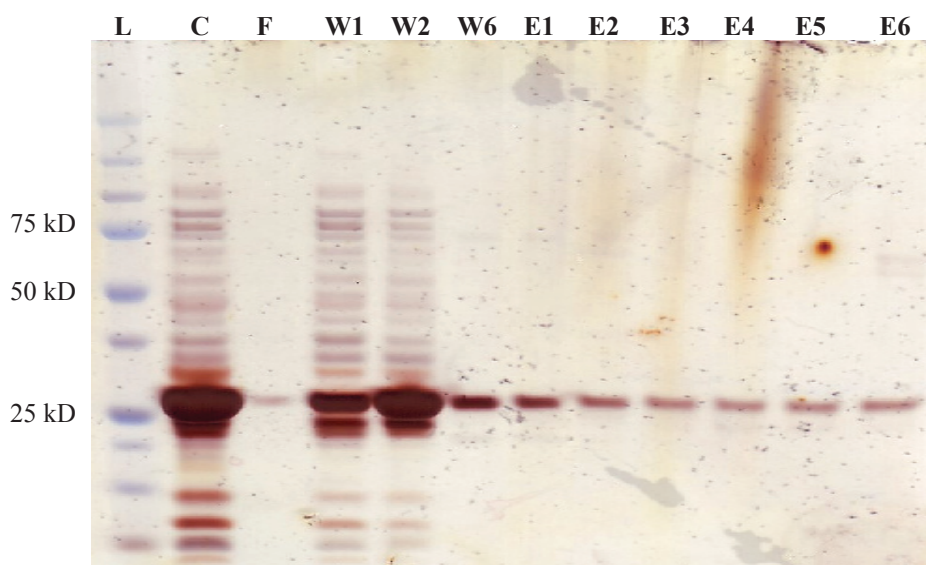
Legend:

Lane	Fraction	Lane	Fraction
L	Protein Ladder	E1	Elution 1
C	Crude Extract	E2	Elution 2
F	Flow-Through	E3	Elution 3
W	Wash		

Figure 5.16: Coomassie stained SDS gel comparison of Ni-TED and Ni-NTA purification of proteins. (A.) Ubi8 protein with an expected size of 20 kDa, (B.) GRB2 protein with an expected size of 30 kDa, (C.) MAPK9 protein with an expected size of 55 kDa.

b. *Purification via biotin (AVI-Tag)*

As all the antigens were in-vivo biotinylated, the use of monomeric avidin column for purification was also investigated. The loading capacity of the monomeric avidin column was lower than the Ni-NTA and Ni-TED columns, although able to purify the antigens. Majority of the purified protein was eluted during the wash step thus lowering the recovery of purified protein (Figure 5.17). The high number of elution fractions required to remove the bound proteins would result in lower concentrations of the purified protein.



Legend:

Lane	Fraction	Lane	Fraction
L	Protein Ladder	E1	Elution 1
C	Crude Extract	E2	Elution 2
F	Flow Through	E3	Elution 3
W1	Wash 1	E4	Elution 4
W2	Wash 2	E5	Elution 5
W6	Wash 6	E6	Elution 6

Figure 5.17: Silver stained SDS gel of PARK-7 using monomeric avidin. The expected size of PARK7 is 26 kDa.

5.3.2 Evaluation of recombinantly expressed antigens

a. *Evaluation of expression levels of recombinant antigens*

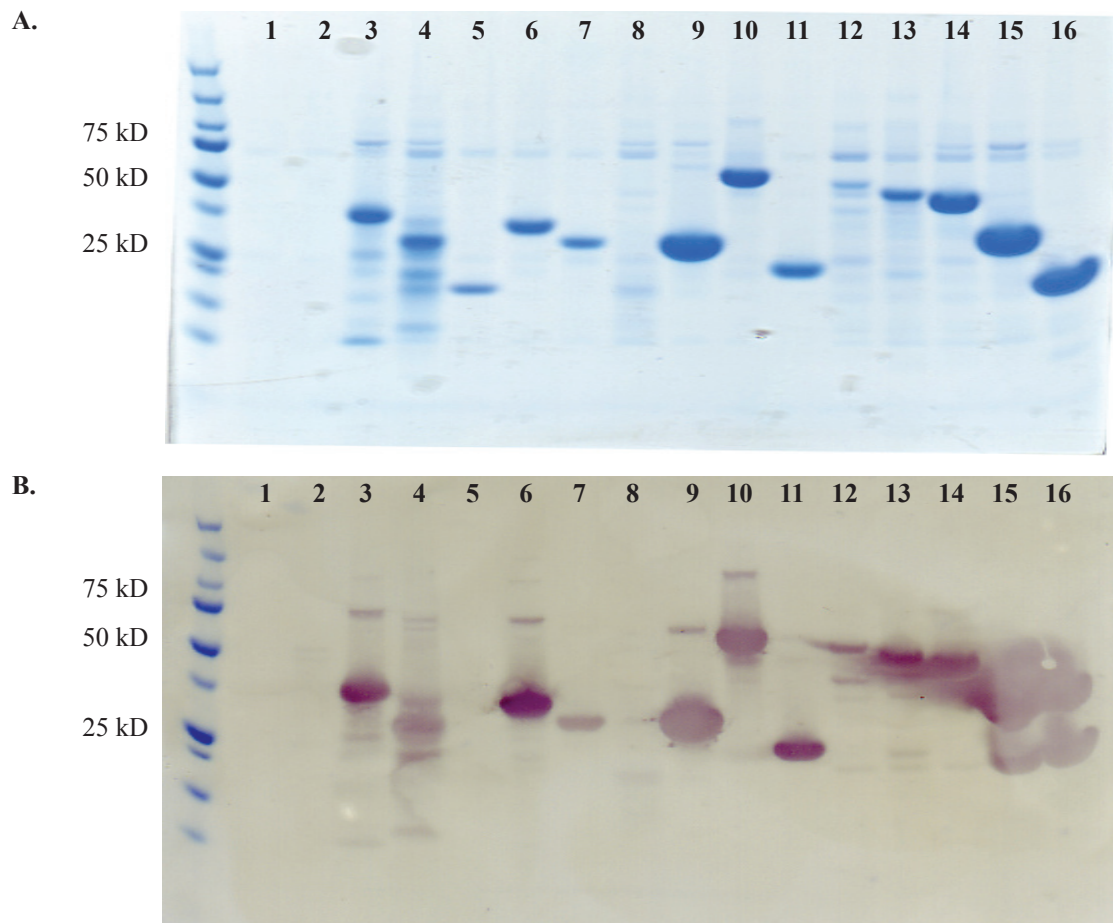
All 16 antigens considered for antibody selection process was evaluated for expression levels. All antigens were individually cloned, expressed and purified using the His-tag via Ni-NTA columns. Figure 5.18 (A.) shows the coomassie stained SDS-PAGE analysis of the expression levels after purification. From the list of 16 initial antigens, only ATF4-A3 and ATF4-E2 were not expressed. ATX2 and GPS2 showed low expression levels. The remaining antigens exhibited good expression levels.

b. *Evaluation of in vivo biotinylation analysis of recombinant antigens*

The purified in vivo biotinylated antigens were coupled to streptavidin beads for phage display panning. The ability to couple the antigens to streptavidin beads was evaluated using SDS PAGE and western-blot with streptavidin-HRP. From the coomassie stained SDS gel, we could see the overexpression of most of the antigens. Figure 5.18 (B.) shows the development of streptavidin-HRP western blots. The western-blot analysis of ATF4-A3 and ATF4-E2 was to ensure that expression was non existant as the sensitivity of western-blot would be higher than coomassie staining on SDS-PAGE. As no signal was visible the possibility of low level expression was discounted. Of the remaining antigens, Cyclophillin was the only protein not in-vivo biotinylated. The double bands in the MAPK9 and Ubi8 samples were due to the sample overflowing during loading of the gel.

b. *Evaluation of mass spectrometry and sequencing analysis of recombinant antigens*

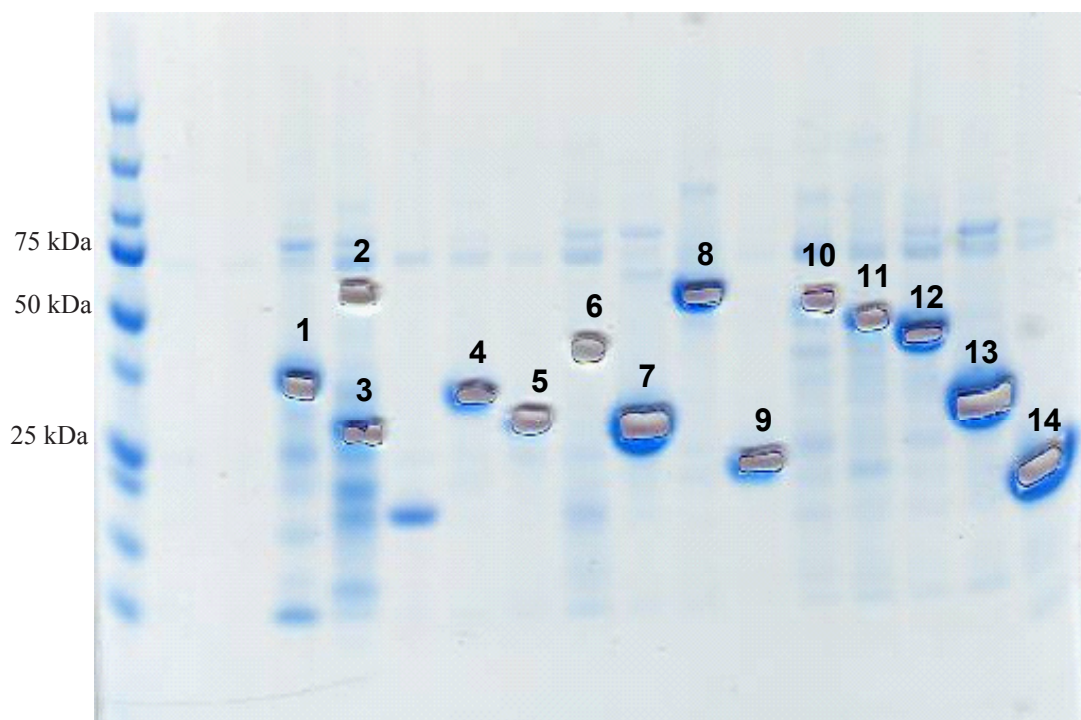
For MS and DNA sequencing analysis, only the proteins that were succesfully expressed and biotinylated were evaluated. The bands of the expected sizes were sent for mass spectrometry analysis. For ATX2, two bands at 56 kD and 28 kD were analyzed by mass spectrometry (Figure 5.19). The sequencing resutls showed all plasmids containing the inserts without any modifications. The mass spectrometry analysis of the proteins confirmed the proteins expressed are of the proteins expected. ATX2 was analysed on mass spectrometry using both suspected 56 kD and 28 kD bands and confirmed to be ATX2. Table 5.7 shows the summary of the evaluation and identification results of the antigens. After evaluation, from the initial 16 antigens, ATF4-A3, ATF4-E2 and Cyclophillin were removed from the list for antibody selection.



Lane	Antigen	MW (kDa)	Lane	Antigen	MW (kDa)
1	ATF4-A3	47	9	GST	30
2	ATF4-E2	47	10	MAPK9	55
3	ATF4-MPMG	47	11	UFM1	25
4	ATX2	56 / 28 ^a	12	SH3GL1	44
5	Cyclophilin	25	13	SH3GL2	43
6	eGFP	32	14	SH3GL3	40
7	GRB2	30	15	TPI-SV	30
8	GPS2	40	16	Ubi8	20

Note: ^a Protein is subjected to in vivo degradation.

Figure 5.18: Analysis of the 16 preliminary antigens for antibody selection process. (A.) Coomassie stained SDS-PAGE analysis for expression levels, (B.) Western-Blot analysis for successful in vivo biotinylation of antigens using Streptavidin-HRP.



Band	Antigen	MW (kDa)	Mascot Score	Band	Antigen	MW (kDa)	Mascot Score
1	ATF4-MPMG	47	54	8	MAPK9	55	275
2	ATX2	56	na	9	UFM1	25	79
3	ATX2	28 ^a	36	10	SH3GL1	44	64
4	eGFP	32	252	11	SH3GL2	43	101
5	GRB2	30	97	12	SH3GL3	40	201
6	GPS2	40	55	13	TPI-SV	30	113
7	GST	30	94	14	Ubi8	20	95

Note: ^a Protein is subjected to in vivo degradation.

na- No available match.

Figure 5.19: Protein bands used for mass spectrometry analysis and summary of mascot score for each protein.

Table 5.7: Summary of evaluation and selection process of the initial 16 antigens evaluated for antibody selection process.

Lane	Antigen	MW (kDa)	Expression	Biotinylation	DNA Sequencing ^b	Mass Spec. Identification ^b	Antibody Selection ^e
1	ATF4-A3	47	-	-	-	-	-
2	ATF4-E2	47	-	-	-	-	-
3	ATF4-MPMG	47	+	+	+	+	+
4	ATX2	56 / 28 ^a	+	+	+ ^c	+ ^d	+
5	Cyclophilin	25	-	-	-	-	-
6	eGFP	32	+	+	+	+	+
7	GRB2	30	+	+	+	+	+
8	GPS2	40	w	w	+	+	+
9	GST	30	+	+	+	+	+
10	MAPK9	55	+	+	+	+	+
11	UFM1	25	+	+	+	+	+
12	SH3GL1	44	+	+	+	+	+
13	SH3GL2	43	+	+	+	+	+
14	SH3GL3	40	+	+	+	+	+
15	TPI-SV	30	+	+	+	+	+
16	Ubi8	20	+	+	+	+	+

Note: ^a Protein was subjected to in vivo degradation.

^b Only proteins successfully expressed and biotinylated were evaluated.

^c Sequencing showed the entire gene was present without any modifications.

^d Protein was identified using both 56 kD and 28 kD bands.

^e Proteins successful for inclusion into antibody selection process.

+ Positive result for evaluation.

- Negative result for evaluation.

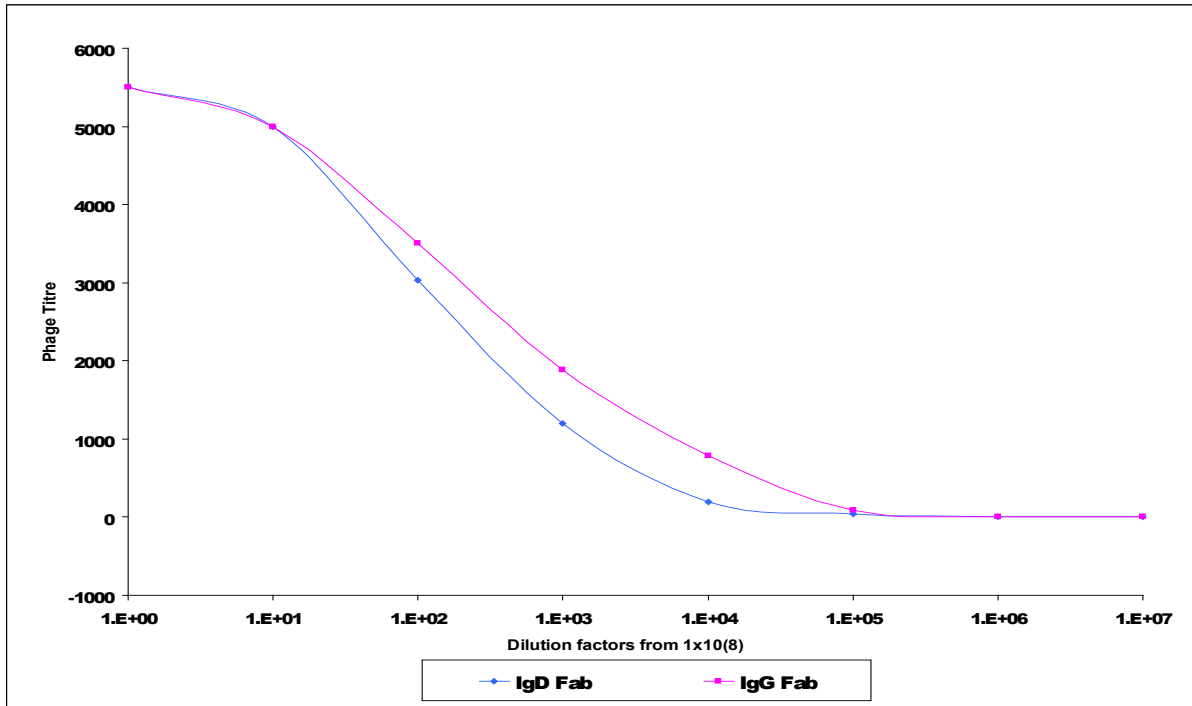
w Weak levels of expression or biotinylation.

5.4 Antibody fragment presentation assay

The antibody fragment presentation assay determines the amount of phage particles presenting functional Fab fragments from both IgD and IgG Fab libraries. The starting material used was 10^8 phage particles from each library. A series of 10 x dilution was prepared to a dilution factor of $1.E+07$ (10 phage particles). Figure 5.20 (A.) shows that at the first two points the protein L beads were saturated with Fab presenting phage particles. At the dilution factor $1.E+02$ (10^6 phage particles), the distribution of phage particles for both IgD and IgG started to separate. IgD Fab generally shows a lower presentation efficiency than IgG Fab. At the dilution $1.E+06$ (100 phage particles), the lower saturation point for both libraries was reached.

The maximum amount of phages presenting Fab for both libraries was estimated from Figure 5.20 (B.). For IgD, an estimated 4.9×10^6 phage particles presented functional Fab fragments on their surface. The amount of phages presenting IgG Fab was estimated at 4.1×10^7 phage particles. This is approximately 8 times higher presentation efficiency for IgG Fab than IgD Fab on phages. Overall, from a known amount of phage particles added as the starting material (10^8 phage particles), only 5 % and 41% from the IgD and IgG Fab library, respectively, have functional Fab molecules presented on the phage surfaces.

A.



B.

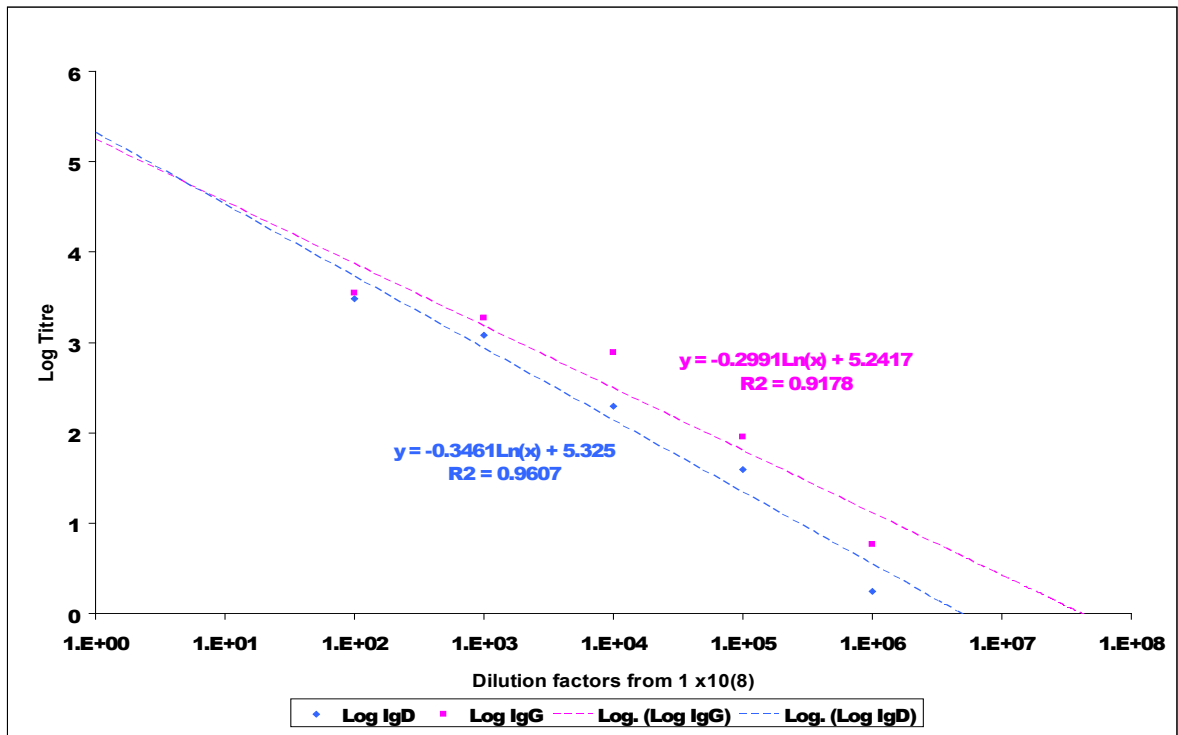


Figure 5.20: Antibody format presentation efficiency assay for IgD Fab and IgG Fab on phage surface. (A.) Dilution curve of the antibody format presentation efficiency, (B.) Log based linear regression of the dilution curves for the antibody format presentation assay.

5.5 Diversity Visualization by Endonuclease – A rapid assay to monitor diverse nucleotide libraries

Many experiments require a rapid and cost effective method to monitor nucleic acid sequence diversity. The DiVE (Diversity Visualisation by Endonuclease) assay, allows rapid visualisation of sequence diversity of PCR products based on DNA hybridisation kinetics coupled with the activity of a single-strand specific nuclease. Simple agarose gel electrophoresis was used to visualise the degree of diversity variation amongst samples, thereby eliminating the time and cost of other available methods. A schematic representation of the DiVE assay is shown in Figure 5.21. Double stranded PCR product was quantified before performing DiVE. The homoduplex dsDNA was denatured and reannealed under stringent conditions. The incubation with S1 nuclease was used to remove single stranded loops of heteroduplexes formed during reannealing. The sequence diversity is inversely proportional to the band intensities visualized on the gel. Therefore the differences in band intensities allows the user to visually monitor differences in diversity .

5.5.1 Optimisation of S1 nuclease incubation temperature

For the optimisation of the assay, we used a monoclonal scFv (4IE3) and round two of selection to simulate the extremes of high and low diversity. The temperature optimal for most single strand specific nucleases is in the range between 37°C to 70°C. At 45°C and 60°C incubation temperature, S1 nuclease activity on ssDNA was reported to increase by two- and three-fold, respectively [170]. Therefore we investigated four temperature points (60°C, 64°C, 67°C and 70°C) for incubation. The intensities of the survivors for 4IE3 at 60°C, 67°C and 70°C were weakly visible and at 64°C total depletion of the band was observed. There were no surviving bands visible for the round two amplicons at all temperatures. To determine the optimal temperature conditions for the assay, the results from the extreme minimum diversity using clone 4IE3 was referred to. As a high amount of enzyme (1 U) was used, 60°C was the most suitable condition as seen in Figure 5.22 (A.).

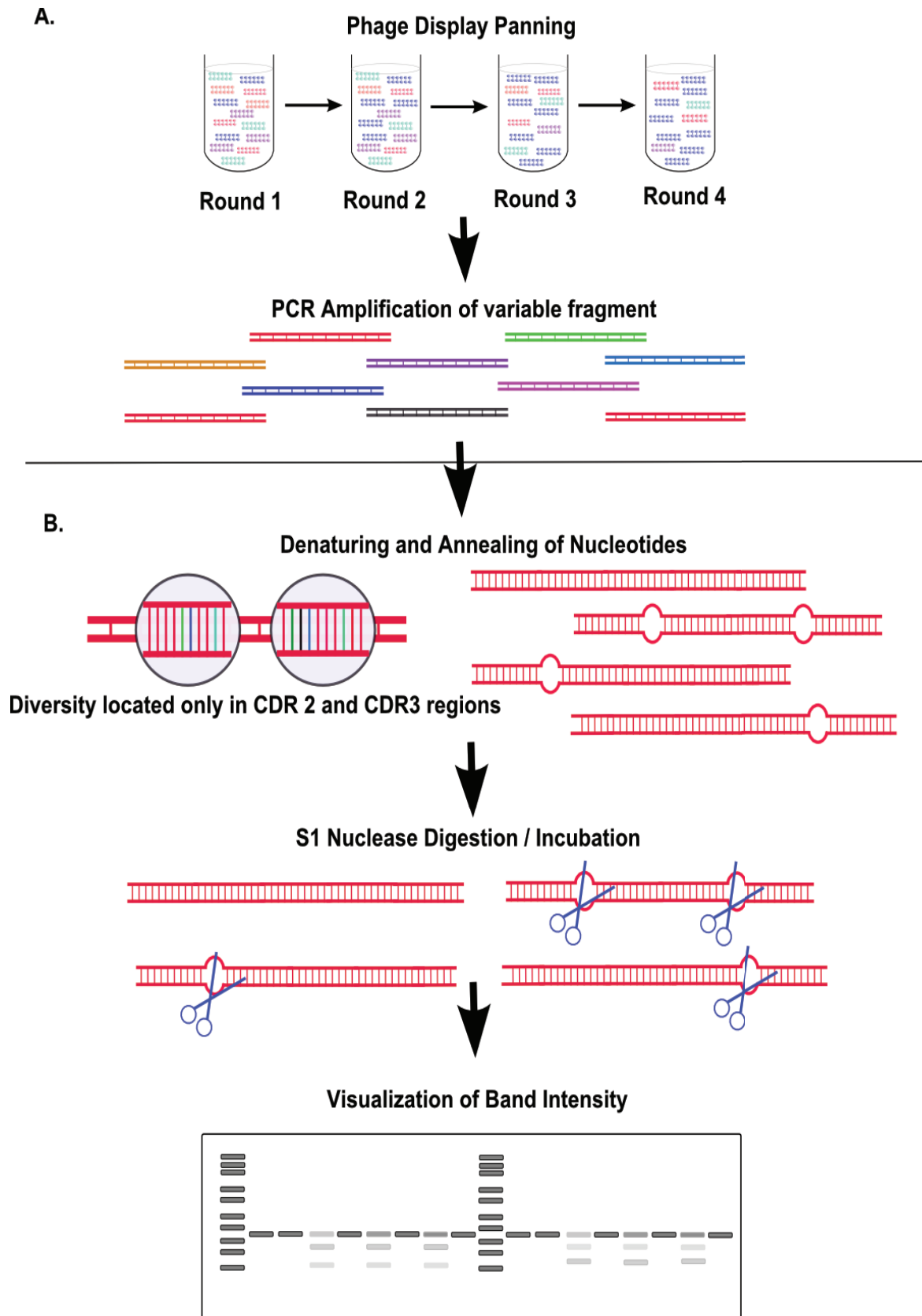


Figure 5.21: Principle of the DiVE Assay for visual evaluation of diversity from phage display library panning rounds. (A.) Phage panning to derive enriched pool of sequences for PCR amplification. (B.) Denaturation and annealing of PCR amplified fragments. Diversified regions are confined to CDR 2 and CDR 3. S1 nuclease will digest bubbles formed by mismatches. Electrophoresis to visualise diversity via variation in band intensities.

5.5.2 Titration of S1 nuclease enzyme units for digestion

The amount of enzyme used was optimized by titrating various units to prevent complete digestion by high enzyme units. All titration experiments were carried out at 60°C for 30 min. The amount of enzyme used was 0.02 U, 0.1 U and 0.2 U. For 4IE3, the best condition to yield a band intensity closest to that of the untreated sample was with 0.02 U of S1 nuclease only. The 350 bp band for the round 2 selection was visible at 0.02 U and 0.1 U. No surviving band was visible at 0.2 U as seen in Figure 5.22 (B.).

5.5.3 Optimisation of S1 nuclease incubation time

As the enzyme efficiency is influenced by incubation temperature, the amount of time to expose the samples to the enzyme was also important. From the enzyme titration, the optimal amount of enzyme required was found to be between 0.02 U to 0.1U with an incubation of 30 min at 60°C. To speed up the assay, 0.04 U of enzyme with 10 minute variations in incubation times (10 min, 20 min and 30 min) was examined. For this experiment clone 4IE3, round 2 and round 4 was used. Round 4 is used to simulate moderate diversity as the diversity at round 4 is not expected to reach monoclonal level but should contain only low diversity after several rounds of panning. Comparison of band intensities derived from the three incubation times is shown in Figure 5.22 (C.), a 10 min incubation was sufficient to show variations between intensities. At 20 min incubation a more distinct variation in intensities was seen between round 2 and round 4. After 30 min, no bands were visible for round 2 and round 4. Hence, we concluded that a 10 to 20 min incubation is suitable.

5.5.4 Application of DiVE Assay to monitor phage display panning rounds

To show the wide applicability of the DiVE assay, the assay was applied to different selections of scFv antibodies on heavy and light chain, with enriched and non-enriched rounds. Figure 5.23 shows the application of the DiVE assay for two selections from round 2, 3 and 4. Both variable regions were used independently with positive and negative enrichment on phage ELISA evaluations. The method is able to monitor the enrichment process of each variable chain. The method was also employed using phage PCR and colony PCR samples without the need of DNA purification to exemplify the robustness of the assay (data not shown). The method was successful at showing the decrease of diversity with the additional rounds of

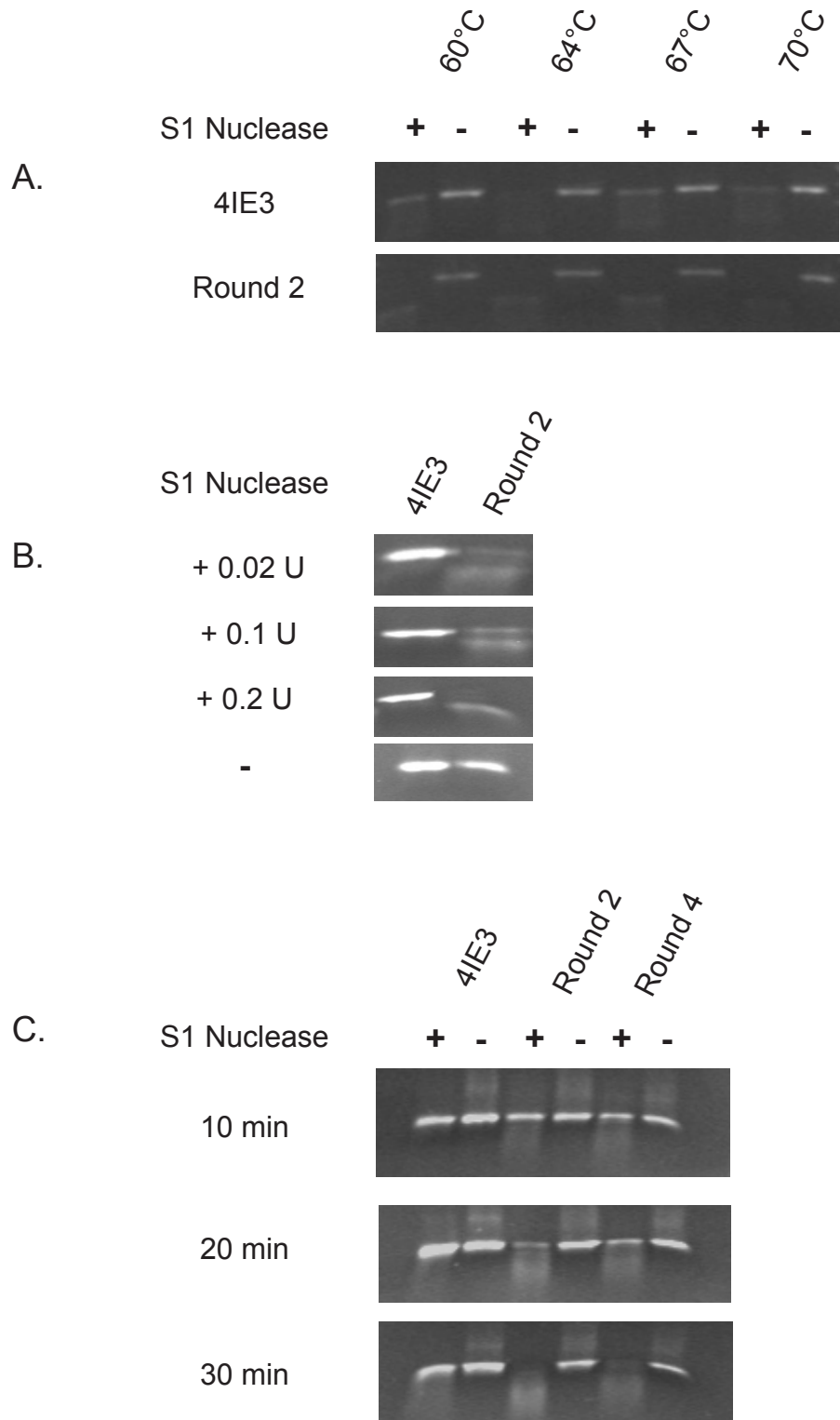


Figure 5.22: Optimisation of S1 nuclease digestion conditions. (A.) Incubation temperature variation with 1 U of S1 nuclease. (B.) Titration of S1 nuclease with 60°C incubation for 30 minutes. (C.) Incubation time variation with 0.4 U of S1 nuclease. The samples used are PCR amplified fragments of the variable heavy chain region of clone 4IE3, round 2 and round 4 of selection. Expected band size is approximately 360 bp. + and - represents the addition of S1 nuclease to the sample.

selection towards an enriched pool of binders. Side product bands of estimated 173 bp and 282 bp were observed for both heavy and light chain amplicons (Figure 5.23), respectively. The 173 bp band coincides with the length of the 5'-end to 3'-end of the heavy chain antisense framework whereas the 282 bp band corresponds to the sense strand length of the 5' end to the 3' end of the framework before CDR3 of the light chain (Figure 5.23).

5.5.5 Batch sequencing of amplicons from all rounds

As the variable region sequences of the library was designed to allow variations at certain positions, sequencing was carried out to ensure that mutations do not occur at framework regions. Batch sequencing using the Sanger sequencing method was conducted for the amplicons of the individual positive and negative selection rounds as well as the monoclonal scFv 4IE3. The framework regions are constant and variations are expected to occur only at designated locations. The sequencing results showed variations in the CDR 2 and CDR3 regions with no variations at the framework regions for both variable heavy and light fragments (Figure 5.24). The sequence traces for the selection rounds and single clone showed variations at the CDR regions for the former and a conserved sequence for the latter.

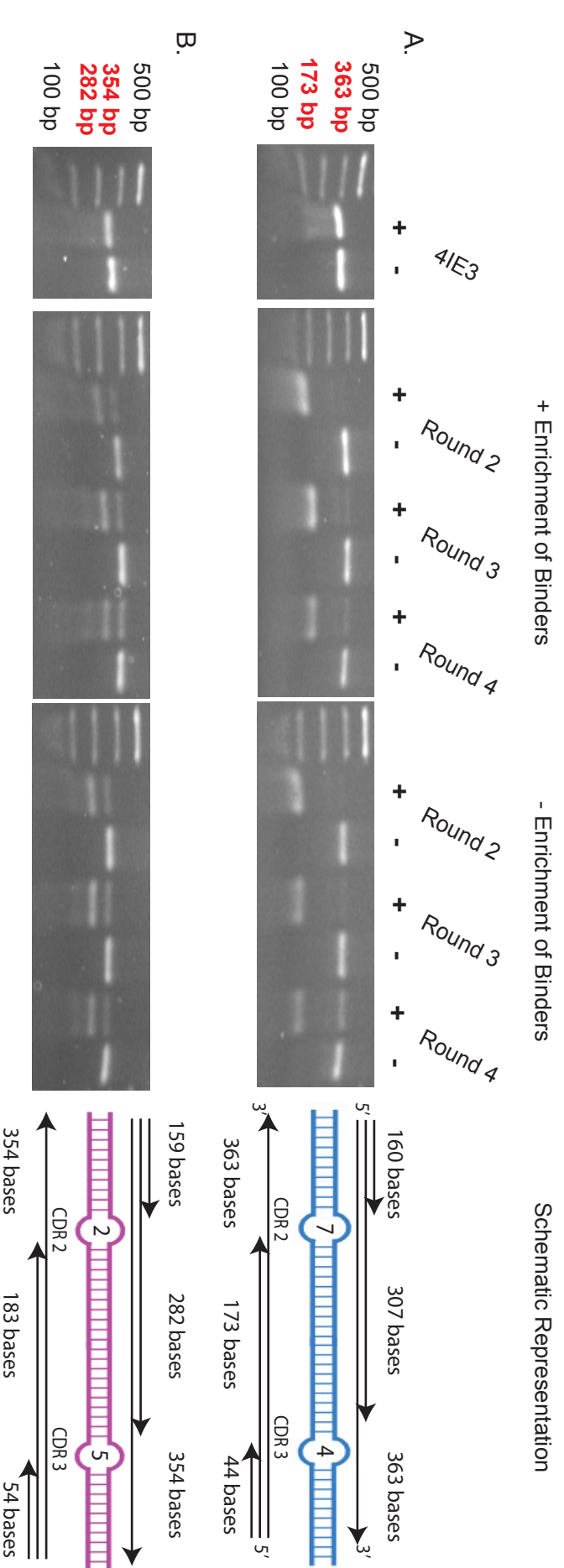


Figure 5.23: DiVE Assay of selection rounds from phage display panning procedure and probable fragments formed by the DiVE Assay. (A.) Variable heavy chain fragment. (B.) Variable light chain fragment. The samples used are PCR amplified fragments of the variable region of a single clone 4IE3 and various rounds of the library selection (positive and negative enrichment of binders). Expected band sizes are approximately 363 bp for heavy chain and 354 bp for light chain. On the right, schematic representation of the variable sequences. CDR's are represented as bubbles with the numbers indicating the frequency of varying amino acid positions. The 173 bp band coincides with the length of the 5'-end to 3'-end of the heavy chain antisense framework whereas the 282 bp band corresponds to the sense strand length of the 5' end to the 3' end of the framework before CDR3 of the light chain after S1 nuclease digestion.

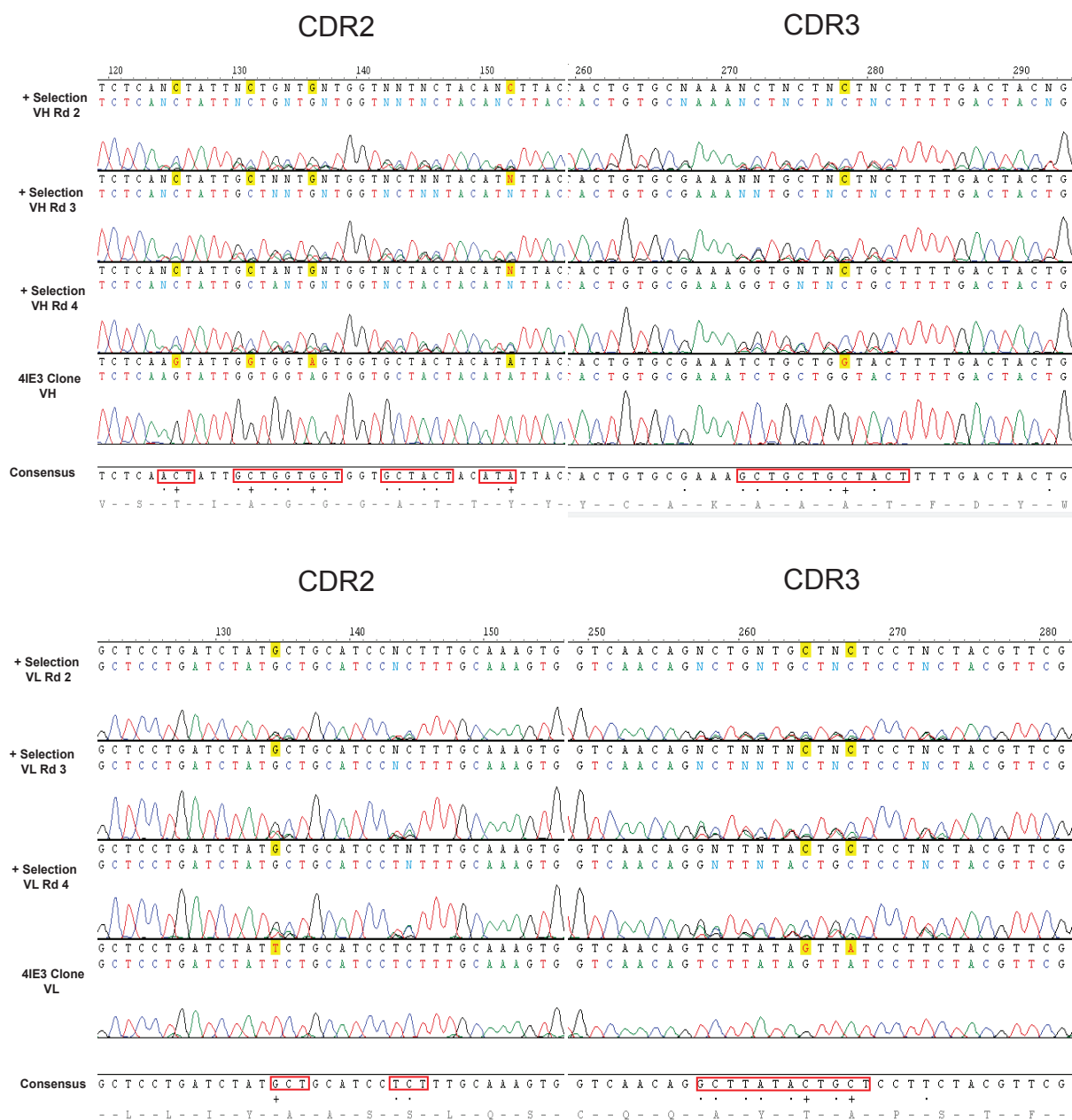


Figure 5.24: Batch sequencing of selection rounds and clone 4IE3. (A.) Variable heavy chain sequences. (B.) Variable light chain sequences. Sequences highlighted in red boxes are regions of diversification.

5.6 Antibody selection process

5.6.1. Coupling of biotinylated antigens to streptavidin beads

The semi-automated selection protocol requires the antigens to be biotinylated for easy coupling to streptavidin magnetic beads. The 13 antigens selected were coupled to streptavidin magnetic beads and the coupling process was evaluated by SDS-PAGE of the beads. Figure 5.25 shows the SDS-PAGE of the beads coupled with biotinylated antigens. From the gel, expected bands for ATX2-F4 and GPS2 were not visible with coomassie staining.

5.6.2. Semi-automated selection of antibodies

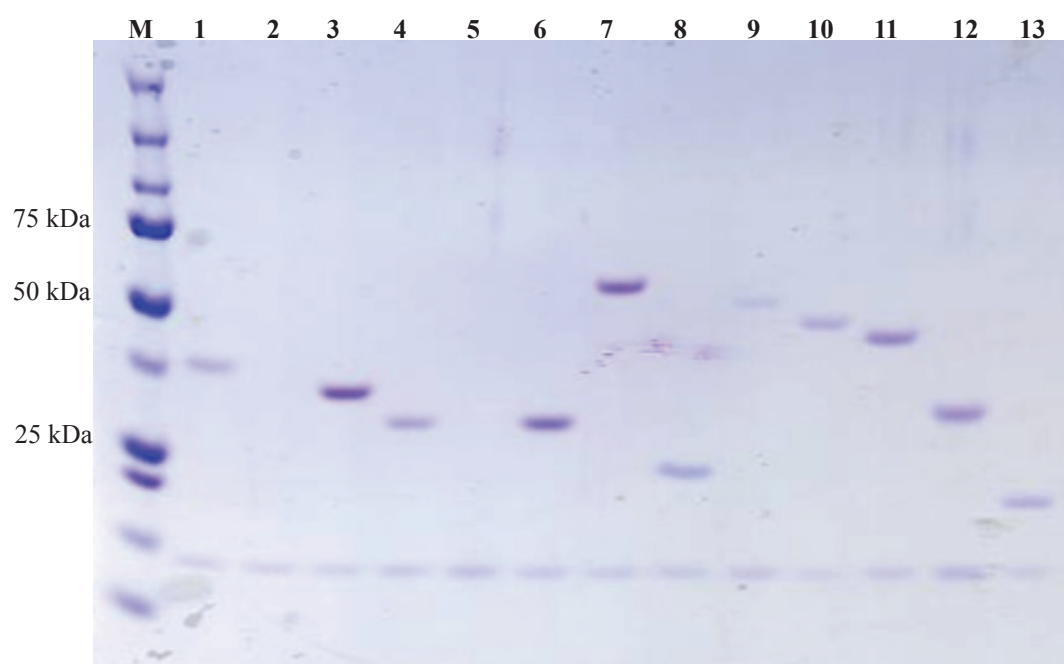
Selection was carried out using two different helper phage preparations (M13KO7 and Hyperphage). Four to five rounds of selection were carried out. Two different display strategies were used for antibody selection. First was a Exclusive Mono strategy where only monovalent display was used with M13KO7. The other was a Combo strategy utilizing a combination of multi- and mono-valent display by using Hyperphage for the first round of panning and subsequently with M13KO7. Table 5.8 shows the summary of display strategies used throughout the selection process for all libraries on all antigens.

5.6.3. Polyclonal ELISA evaluation of panning rounds

Polyclonal ELISA was carried out for all panning rounds for all 13 antigens. Figure 5.26 shows the enrichment of binding phage particles to each antigen. The scFv library was able to yield binders for all 13 antigens. No distinct variation between mono- and multivalent display was evident in the selection rounds. Selection using the Combo display method was able to exhibit enrichment of polyclonal ELISA for all 13 antigens with the scFv library. While the Exclusive Mono method only had 11 antigens enriched with no enrichments for ATX2 and GPS2. For low expressing antigens, ATX2 and GPS2 enrichment was visible only for Hyperphage and not for M13KO7. Majority of the enrichments appear after round 3 with most showing higher OD readings at round 4.

The IgG Fab library only showed 2 positive enrichments out of the 13 for TPI-SV and UBI8. The enrichment was successful when screened using the Combo method rather than the Exclusive Mono method. The IgD Fab library was not able to show any enrichment for both

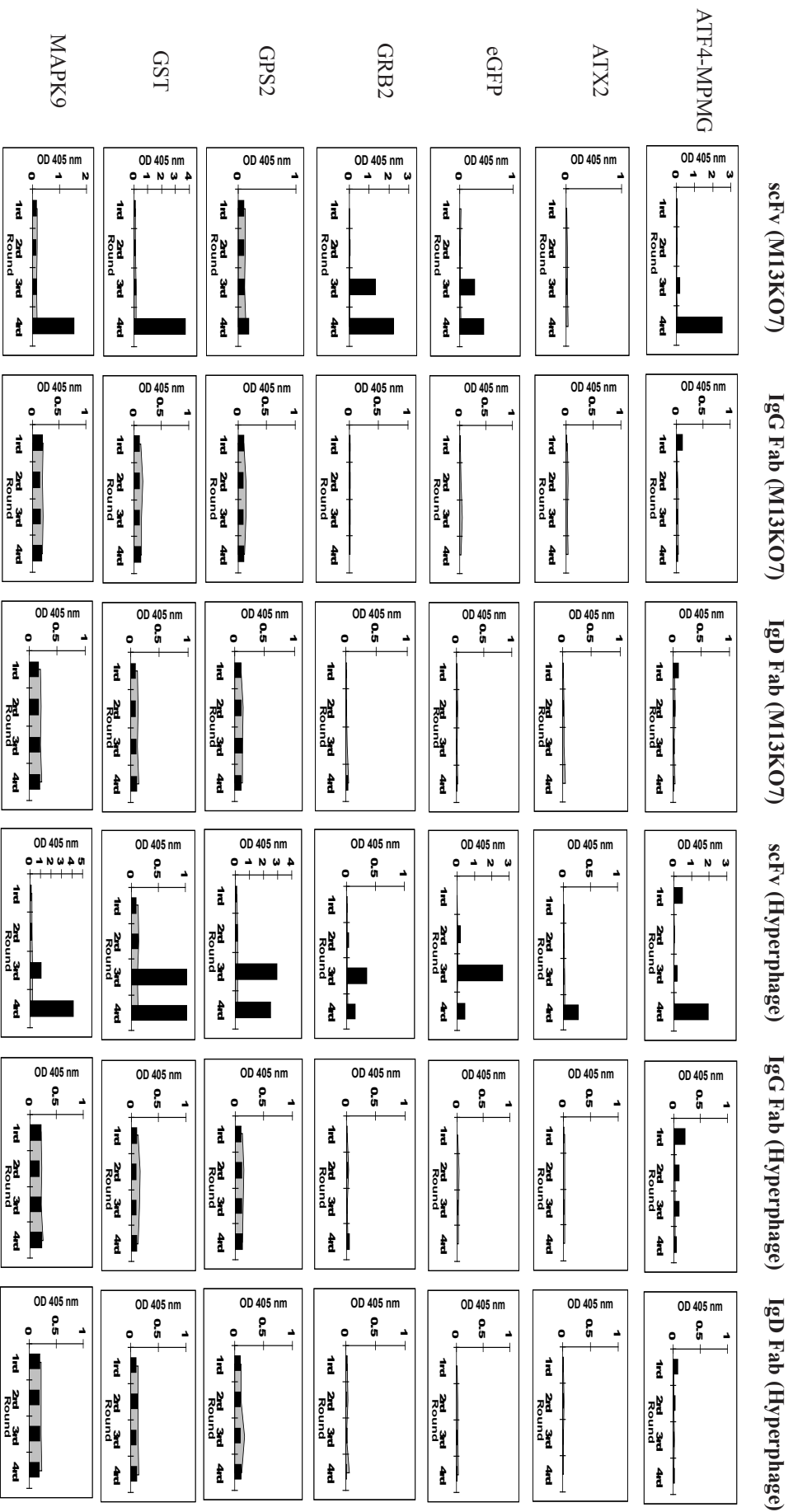
M13KO7 and Hyperphage for all the antigens. Therefore further rounds of panning were carried out for TPI-SV and UBI8 as both were the only antigens that showed enrichment in the IgG Fab with Hyperphage. The round 4 phage stocks of both M13KO7 and Hyperphage for both antigens were reinfected and selected for a further 2 rounds. The two additional rounds of panning managed to yield enrichment for UBI8 from the IgD library using the Exclusive Mono method. Figure 5.27 shows the polyclonal ELISA results for both antigens using both IgG Fab and IgD Fab libraries.



Lane	Antigen	MW (kDa)	Lane	Antigen	MW (kDa)
M	Protein Ladder		7	MAPK9	55
1	ATF4-MPMG	47	8	UFM1	25
2	ATX2	56 / 28 ^a	9	SH3GL1	44
3	eGFP	32	10	SH3GL2	43
4	GRB2	30	11	SH3GL3	40
5	GPS2	40	12	TPI-SV	30
6	GST	30	13	Ubi8	20

Note: ^a Protein is subjected to in vivo degradation.

Figure 5.25: SDS-PAGE analysis of the 13 selected antigens coupled to streptavidin beads for antibody selection process.



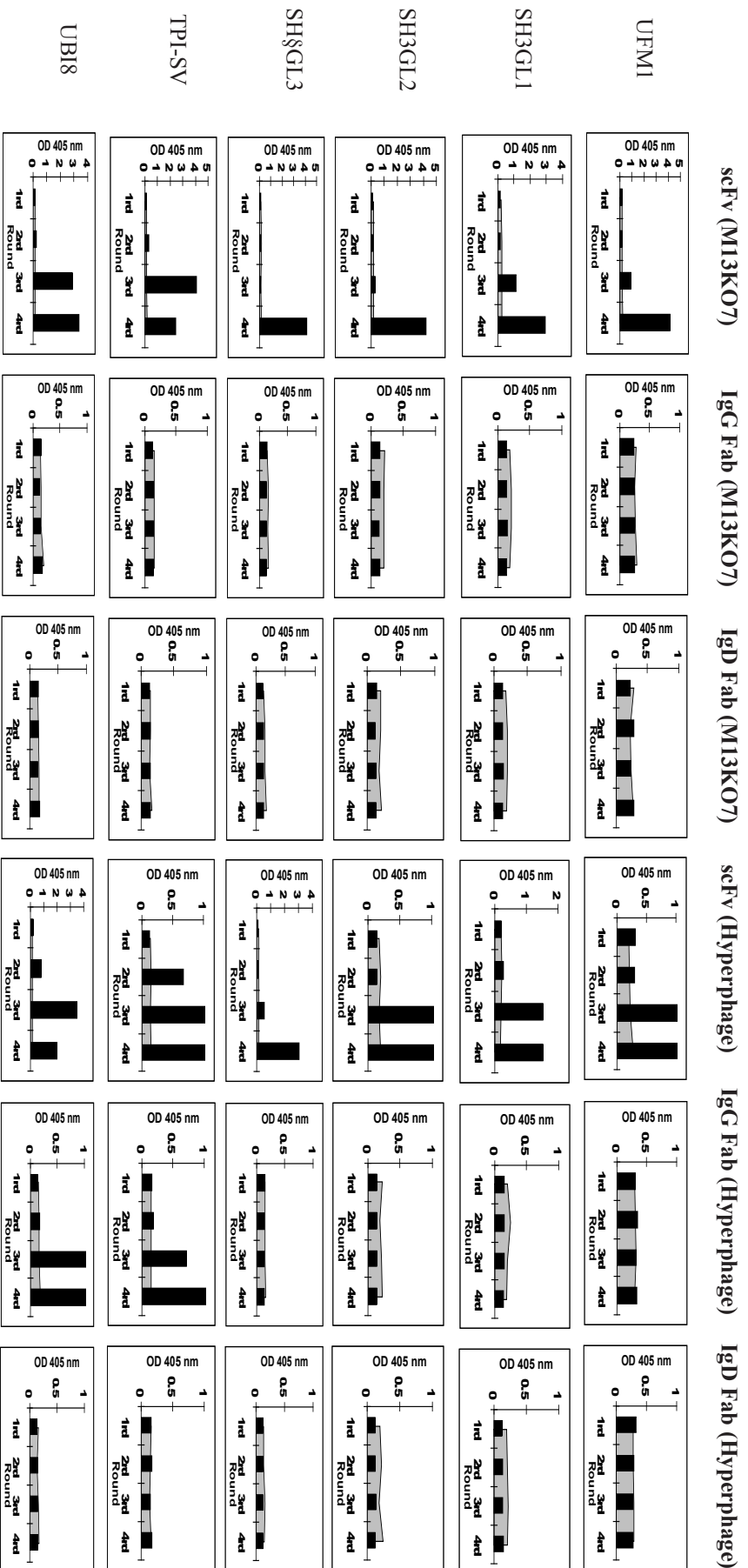


Figure 5.26: Polyclonal ELISA results of panning rounds with the scFv, IgG Fab and IgD Fab libraries with two different display methods. M13K07 represents library using the Exclusive Mono method and Hyperphage is for the Combo display method. Grey areas represent the background OD readings and black coloured bars represent the OD readings of samples.

Table 5.8: Description of the two different selection strategies used during the antibody selection process.

Strategy	Exclusive Mono		Strategy	Combo	
Round	Helper Phage	Display	Round	Helper Phage	Display
1	M13KO7	Monovalent	1	Hyperphage	Polyvalent
2	M13KO7	Monovalent	2	M13KO7	Monovalent
3	M13KO7	Monovalent	3	M13KO7	Monovalent
4	M13KO7	Monovalent	4	M13KO7	Monovalent
5	M13KO7	Monovalent	5	M13KO7	Monovalent

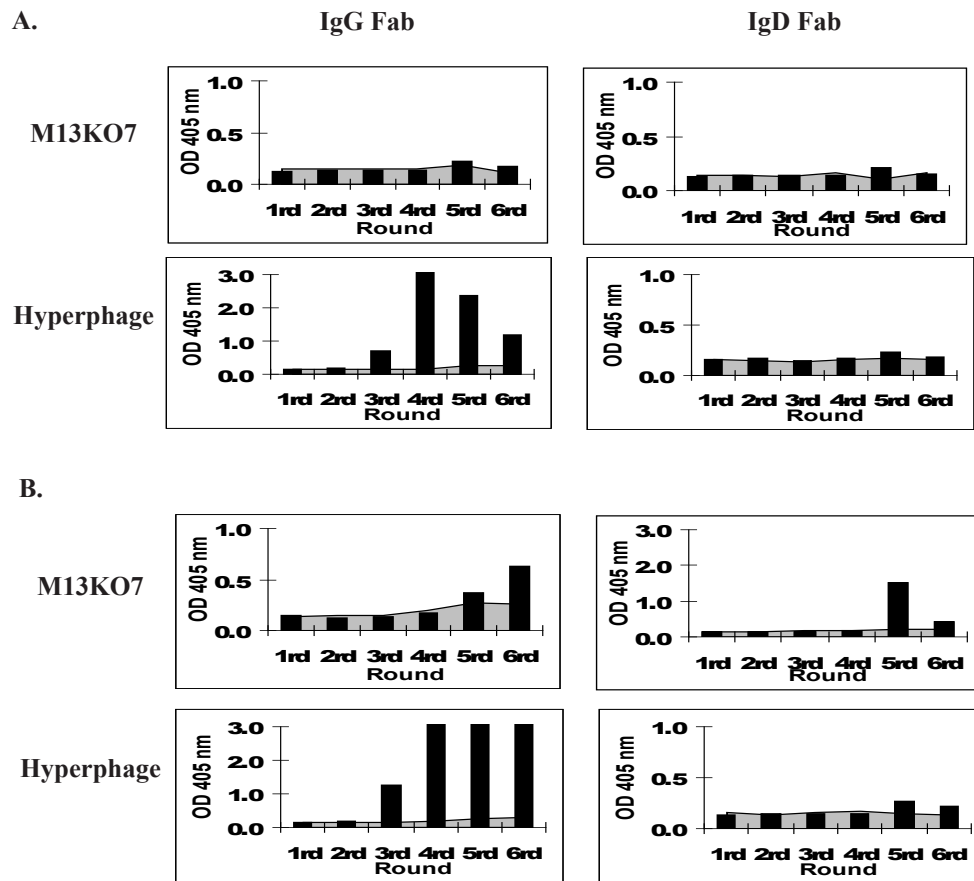


Figure 5.27: Polyclonal ELISA of the addition Round 5 and Round 6 selection for TPI-SV and UBI8. (A.) Results of the additional rounds including the previous rounds for TPI-SV for IgG Fab and IgD Fab library with both display methods, (B.) Results of the additional rounds including the previous rounds for UBI8 for IgG Fab and IgD Fab library with both display methods. Grey areas represent the background OD readings and black coloured bars represent the OD readings of samples.

5.5.4. Monoclonal ELISA evaluation of selected clones

From the enriched polyclonal rounds, single clones of the rounds were prepared and selected. Monoclonal ELISA was carried out for these monoclonal antibodies to identify monoclonal antibodies. As TPI-SV showed enrichment for scFv and IgG Fab, and UBI8 had enrichment for all the libraries, the selections from this two antigens were further evaluated for monoclonal antibodies. Table 5.9 shows the summary of the rounds from each library used for monoclonal antibody selection. From the polyclonal ELISA results, the scFv M13KO7 round 3 and round 4 of IgG Hyperphage was used for TPI-SV monoclonal selection. For the IgD library, as there was no enrichment on polyclonal level therefore both M13KO7 and Hyperphage libraries were used. For UBI8, round 4 of scFv M13KO7, round 5 of IgG Hyperphage and round 5 of IgD M13KO7 was used for monoclonal selection based on positive enrichments on polyclonal level.

The scFv plate for both antigens shows strong signals for majority of the monoclonal antibodies. The IgD Fab libraries showed more positive clones with stronger OD readings for Ubi8 than TPI-Sv. For IgG Fab, the pattern is the opposite with more positive clones for TPI-Sv than Ubi8 (Figure 5.28).

Single clones were selected from each antigen for further downstream investigation. Table 5.10 shows the sequences of the CDR regions belonging to the variable regions of the heavy and light chain from the selected clones. The monoclonal ELISA results are also shown in Table 5.10. All clones exhibit the highest OD readings for their selections. Although the allowed diversity with the DVT codon was 8 amino acids, 3 unexpected codons encoding tryptophan, valine and histidine was retrieved from 2 scFv clones and 1 IgG Fab clone. 7 clones in total were selected for format switching experiments.

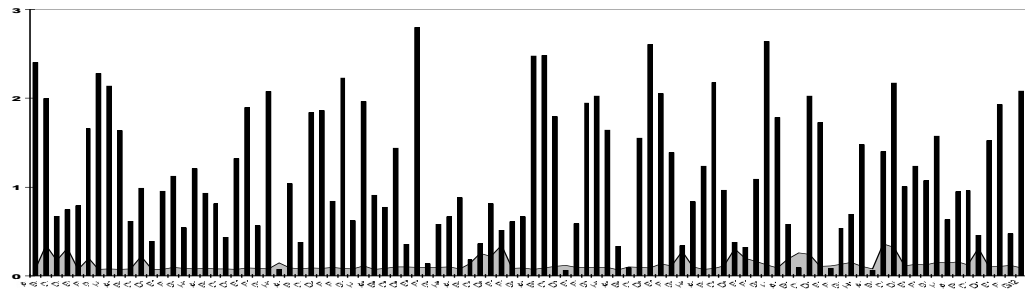
Table 5.9: Polyclonal enrichment rounds selected for monoclonal selection for TPI-SV and UBI8.

Antigen	TPI-SV		Antigen	UBI8	
Library	Helper Phage	Round	Library	Helper Phage	Round
scFv	M13KO7	3	scFv	M13KO7	4
IgG	Hyperphage	4	IgG	Hyperphage	5
IgD ^a	M13KO7	5	IgD	M13KO7	5
IgD ^a	Hyperphage	5			

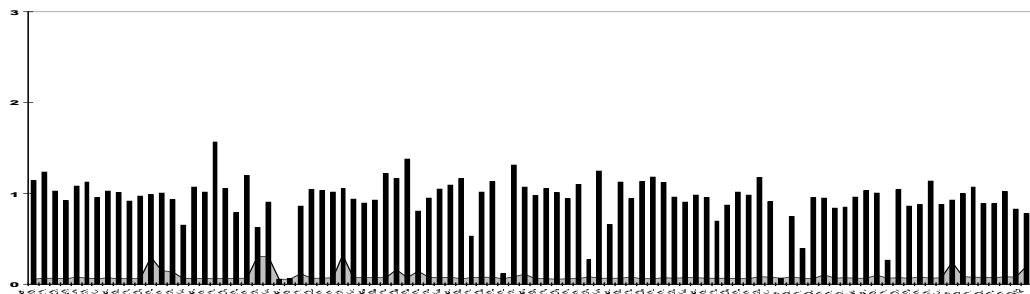
Note: ^a No enrichment on polyclonal level.

A.

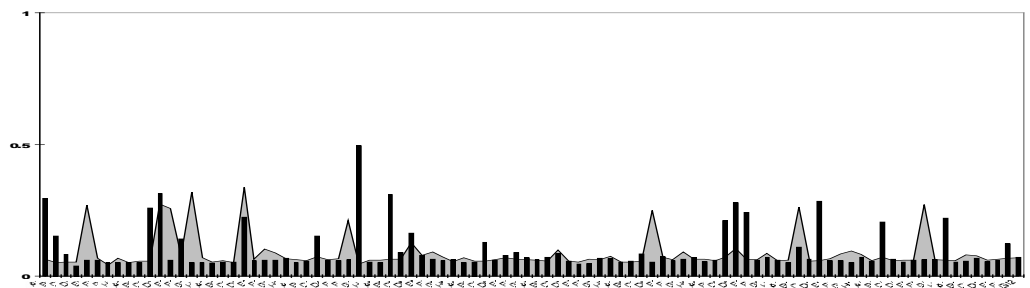
scFv M13KO7



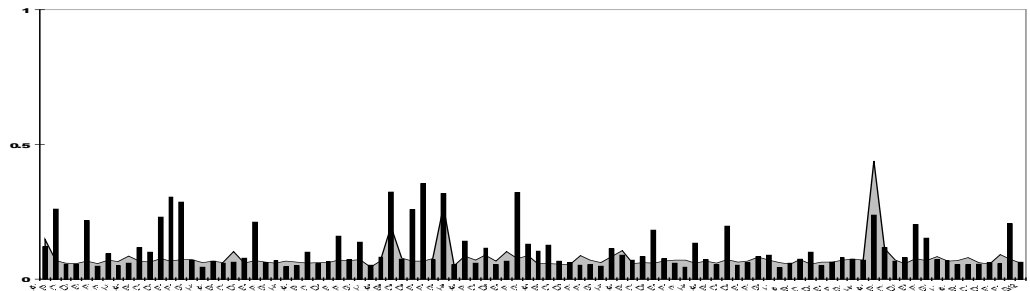
IgG Hyperphage



IgD M13KO7

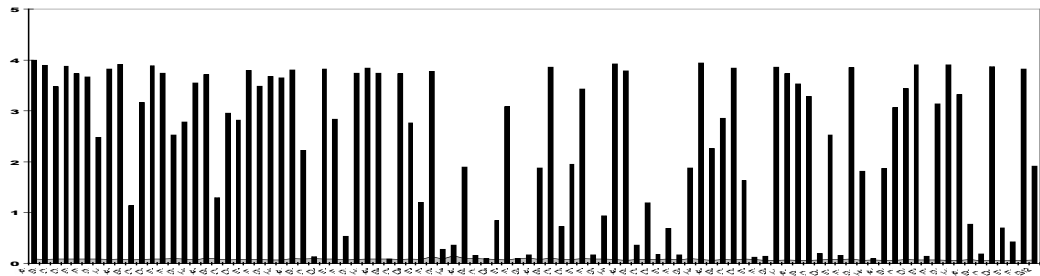


IgD Hyperphage

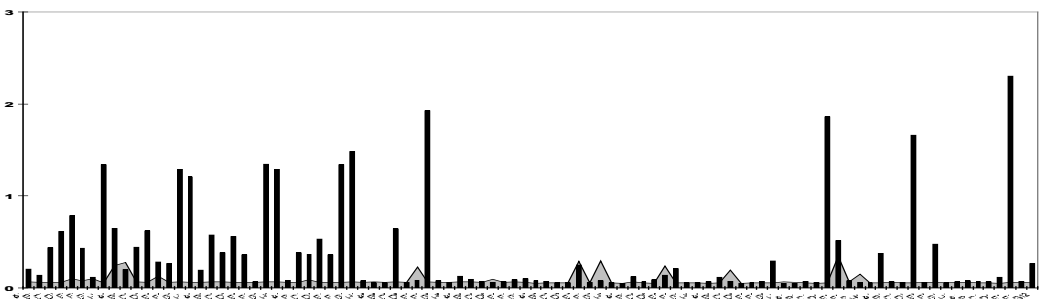


B.

scFv M13KO7



IgG Hyperphage



IgD M13KO7

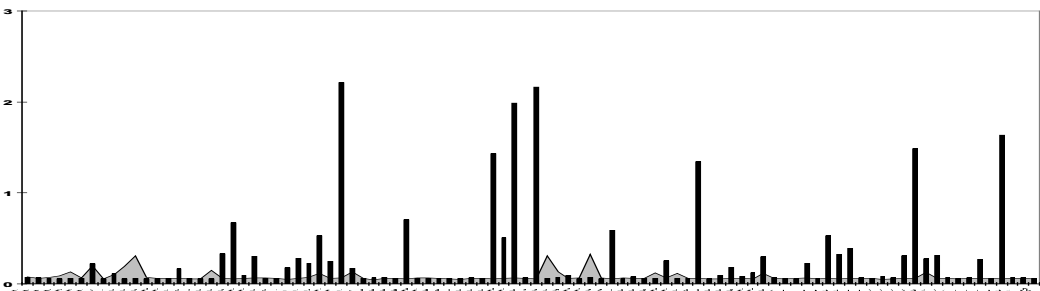


Figure 5.28: Monoclonal ELISA of selected clones for TPI-SV and UBI8. (A.) Results of the monoclonal ELISA for TPI-SV from all libraries, (B.) Results of the monoclonal ELISA for UBI8 from all libraries,. Grey areas represent the background OD readings and black coloured bars represent the OD readings of samples. TPI-SV IgD library was screened from both M13KO7 and Hyperphage.

Table 5.10: Summary of CDR sequences of clones selected for format switching. (A.) Variability of clones selected for format switching with their respective monoclonal ELISA readings, (B.) List of amino acids allowed by DVT codon and unexpected amino acids found in selected clones.

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A.

Clone	Antigen	ELISA	HC CDR 1	HC CDR 2	HC CDR 3	LC CDR 1	LC CDR 2	LC CDR 3
Tomlinson	--	--	SYAMS	*I**G**T*	***	RASQSISSLYN	*AS*LQS	QQ****P*
2 C scFv	Ubi8	3.165	SYAMS	SISSTGSM T S	SDNA	RASQSISSLYN	YASYLQ S	QQGY NTPT
2 F scFv	Ubi8	2.528	SYAMS	TIGGYGS NTH	GGNA	RASQSISSLYN	AASYLQ S	QQSY SYPS
6 E IgD	Ubi8	2.212	SYAMS	SIT AA GG TT TY	DDTD	RASQSISSLYN	SASTLQ S	QQY G SS YPT
5 D IgG	Ubi8	1.928	SYAMS	T IAYD G AYTD	DDVD	RASQSISSLYN	AASSLQ S	QQ NAN Y PY
1 A IgG	TPI-SV	1.144	SYAMS	T IGSGG Y TTY	TTTS	RASQSISSLYN	N ASAL Q S	QRSS AGPT
9 A scFv	TPI-SV	2.476	SYAMS	T IYSTG D STN	GAAG	RASQSISSLYN	A ASAL Q S	QQY D T APA
10 D scFv	TPI-SV	2.608	SYAMS	S ISSSSG S GTY	GAAA	RASQSISSLYN	YASYLQ S	QQ GD T APA

B.

AMINO ACIDS ALLOWED BY DVT CODON USAGE	CODON USAGE	POLARITY	COLOR CODE
ALANINE (ALA)	GCT	NONPOLAR	A
ASPARAGINE (ASN)	AAT	POLAR	N
ASPARTIC ACID (ASP)	GAT	POLAR	D
CYSTEINE (CYS)	TGT	NONPOLAR	C
GLYCINE (GLY)	GGT	NONPOLAR	G
SERINE (SER)	TCT AGT	POLAR	S
THREONINE (THR)	ACT	POLAR	T
TYROSINE (TYR)	TAT	POLAR	Y
UNEXPECTED CODON TRYPTOPHAN (TRP)	TGG	NONPOLAR	W
UNEXPECTED CODON VALINE (VAL)	GTT	NONPOLAR	V
UNEXPECTED CODON HISTIDINE (HIS)	CAT	POLAR	H

5.6 Format switching

To investigate the influence of different antibody formats on binding efficiency, individual positive clones were selected to sub clone the corresponding V-regions into different format backbones. Positive monoclonal clones from TPI-SV and UBI8 selections were sequenced and selected for format switching. Table 5.10 shows the clones selected for format switching. All formats were switched from the parental format to the other two daughter formats to have a representation of all three formats from one clone for better comparisons. The influence of parental format exchange to daughter format can also be observed in this way. Figure 5.29 shows the format switching schemes for all possible formats.

Phage ELISA of equal amounts of phage particles (approximately 10^{10}) was carried out for all daughter clones and parental clones to evaluate the binding ability of antibody fragments after format switching. The intensity of the OD is used as a reference of binding ability of each clone. From the phage ELISA, all parental clones maintain the highest OD reading in comparison to all subsequent daughter clones regardless of format. Figure 5.30 shows the OD readings of format switching phage ELISA. The error bars show the deviations of the readings in a 4 sample replication.

Conversion of scFv to both IgD and IgG Fab formats, is still able to maintain the binding ability but at a lower OD reading. There is no preference of Fab formats from scFv parental clones as both IgG and IgD outperform each other in terms of OD on separate occasions. This shows the maintenance of binding ability of scFv derived daughter clones but with a lower efficiency in Fab format. Only clone 2F when converted from scFv to IgG Fab showed binding characteristics quite similar to the parental clone. Clones 10D, 9A and 2C when converted showed lower binding characteristics than the parentals for both Fab formats. The average loss of binding was about 64% from the parental OD.

The IgG Fab parental clones showed almost equal OD readings after conversion to scFv formats. There seems to be compatibility for IgG Fab constructs for conversion to scFv without a significant loss of binding ability. Clone 1A and 5D when converted from their original IgG Fab format to scFv showed a drop of OD less than 10% of the parental. Conversions of IgG Fab to IgD Fab lead to about 56% loss of binding. A two fold loss of signal from IgG to IgD Fab could also account for the lower presentation efficiency of IgD Fab on phage. Conversions from IgG Fab to scFv seem to be a more favourable conversion than IgD Fab.

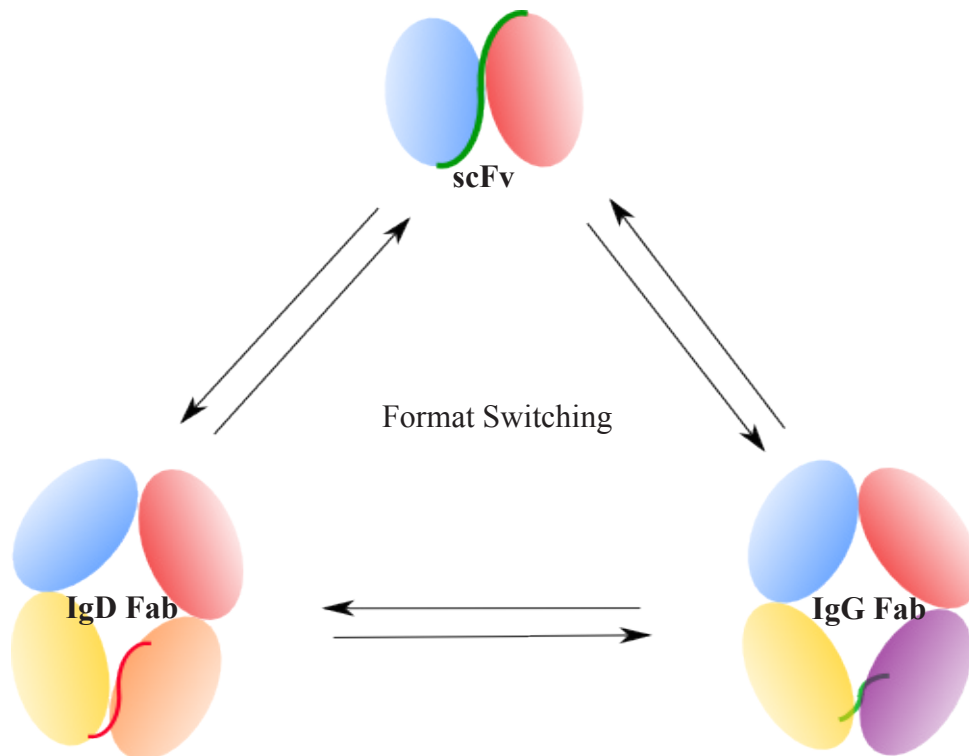


Figure 5.29: Format switching in-between the three formats. The original format of the clone is regarded as the parental format and the subsequent conversions are termed daughter formats. scFv is connected by a glycine serine linker whereas IgD and IgG Fab formats are formed by disulphide bonds at different positions.

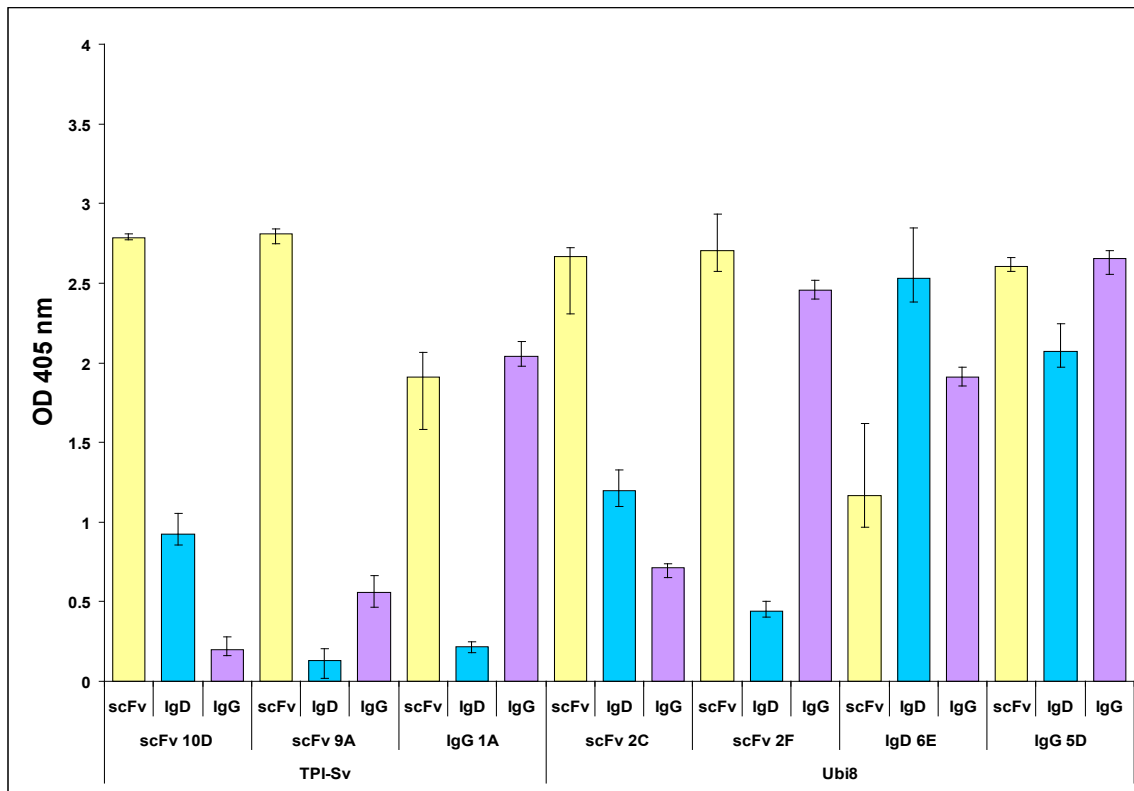


Figure 5.30: Format switch phage ELISA of selected clones. The parental format clones are named at the bottom for each subset. Each parental format was converted to the other possible two daughter formats. Wiskers represents error bars of quadruplicates.

IgD Fab parental clone, showed a slight decrease in OD for IgG Fab daughter clone whereas a higher drop in OD for scFv daughter clone. This implies the conversion from IgD Fab to IgG Fab would only result in slight decrease in binding ability of the daughter clone. The switch from IgD Fab to scFv is not a favourable strategy with significant drop in OD readings. The conversion to IgG Fab leads to a drop of 24% and 54% for scFv format.

5.7 Amplification of V-gene repertoire from B-cells

5.7.1 Bioinformatic analysis of V-gene specific primers

All 576 heavy chain and light chain (Kappa and Lambda) V-genes sequences were retrieved from VBASE2 and grouped according to their family assignment. Sequences of the individual families were aligned using VectorNTI AlignX software and the first N-terminal protein-coding nucleotides were compared with previously published primer sequences [171,172]. For ease of use, primers were named according their targeted V-gene families.

Table 5.11 shows the individual analysis result for each primer with family assigned genes. The total number of genes in any given V-gene family, the oligonucleotide sequence and fold degeneracy of the primer and the number of genes with 0 – 4 mismatches relative to the primer sequence respectively is given. If the primer shows less than four mismatches when compared to the reference V-gene sequence with a covered gene region of more than 18 bp in length, the gene is regarded as covered. Some primers show a certain degree of degeneracy at individual positions. Due to this, some primers match more than one V-gene family and some V-genes are matched by more than one primer. In such a case, each V-gene is only scored once per family and only with the best primer, i.e. with the lowest degree of mismatches. The percentages of mismatches for the sequences are also displayed.

Table 5.11: Bioinformatic analysis of oligonucleotides for amplification of V-genes based on family assigned genes from VBASE2.

Family	Number of covered (not covered) genes	Primer	Primer Sequence	Fold Degeneracy	0		1		1		2		3		4	
					0	1	1	1	2	2	3	3	4	4		
<i>Variable Heavy Chain</i>																
VH1	43 (1*)	VH1 VH157 VH1 & VH157	CAGGTCCAGCTGKTRCAGTCTGG CAGGTGCAGCTGGTGSARTCTGG	4	4	16	37	1	3	2	7	4	9	12	5	5
VH2	10	VH2	CAGRTCCACCTTGAAGGAGTCTG	2	7	70	3	30	15	12	6	5	1	0.8		
VH3	130 (5*)	VH3	GAGGTGCAGCTGKTGGAGWCY	8	81	62	27	21	19	15	12	6	5	1	3.2	
VH4	31	VH4	CAGGTGCAGCTGCAGGAGTCSG	2	21	68	6	6	3							
VH5	4	VH4-DP63	CAGGTGCAGCTGCAGGAGTGG	1	2	7	1	1	3							
VH6	2	VH157	CAGGTGCAGCTGGTGSARTCTGG	4	4	100	2	50								
VH7	6	VH6 VH157	CAGGTGCAGCTGCAGGAGTCA CAGGTGCAGCTGGTGSARTCTGG	1	2	83										
<i>Variable Light Kappa Chain</i>																
VK1	61 (6*)	VK1	GACATCCRGDTGACCCAGTCTCC	6	45	74	12	20	2	3	2	3	2	3		
VK2	29 (10*)	VK246	GATATTGTGMTGACBCAGWCTCC	12	15	52	7	24	2	2	7	4	14	1	3	
VK3	21 (4*)	VK3	GAAATGTGRTGACRCAGTCTCC	8	17	81	2	10	1	1	5					
VK4	1	VK246	GATATTGTGMTGACBCAGWCTCC	12		100								100		
VK5	1	VK5	GAAACGACACTCAGCAGTCTC	1	1	100										
VK6	4	VK246	GATATTGTGMTGACBCAGWCTCC	12										25		
<i>Variable Light Lambda Chain</i>																
VL1	13 (1*)	VL1	CAGTCTGTSBTGACGACGCCGCC	6	7	54										
		VL1459	CAGCCTGTGCTGACTCARYC	4												
		VL15910 &	CAGCCWGGKCTGACTCAGCCMCC	8												
		VL15910														
VL2	18	VL2	CAGTCTGYYCTGAYTCAAGCCT	8	16	89	2	46								
VL3	22 (1*)	VL3	TCCTATGWMGCTGACWCAGCCAA	4	2	9	6	11	27							
		VL3(DPL16)	TCCTCTGAGCTGASSTCAGGASCC	4	8	37	1	5	1	1	5	1	5	1	5	
		VL3(38)	TCCTATGAGCTGAYRCAAGCYACC	8	8	37	1	9	36							
		VL3 & VL3(38)														
VL4	4	VL1459	CAGCCTGTGCTGACTCARYC	4	1	25	2	50								
VL5	11	VL1459	CAGCCTGTGCTGACTCARYC	4	5	46	4	36								
		VL15910 &	CAGCCWGGKCTGACTCAGCCMCC	8	1	9										
		VL15910														
VL6	6	VL6	AATTTATGCTGACTCAGCCCC	1	5	83	1	17								
VL7	5	VL7	CAGDCTGTGGTGCACYCAGGAGCC	6	4	80	1	20								
VL8	5	VL78	CAGDCTGTGGTGCACYCAGGAGCC	6	5	100										
VL9	2	VL1459	CAGCCTGTGCTGACTCARYC	4												
		VL15910 &	CAGCCWGGKCTGACTCAGCCMCC	8	2	100										
		VL15910														
VL10	2	VL15910	CAGCCWGGKCTGACTCAGCCMCC	8			2	100								

*** Genes which are not covered by the primers:**

VH1: humIGHV140; VH3: humIGHV120, humIGHV144, humIGHV147, humIGHV218, humIGHV318; VK1: humIGKV057, humIGKV058, humIGKV064, humIGKV111, humIGKV118, humIGKV200;
 VK2: humIGKV038, humIGKV039, humIGKV042, humIGKV076, humIGKV100, humIGKV132, humIGKV138, humIGKV178, humIGKV195, humIGKV197; VK3: humIGKV119, humIGKV122,
 humIGKV176, humIGKV194; VL1: humIGLV163; VL3: humIGLV171

In total, 431 (93.9 %) out of 459 family-assigned V-genes are covered with the given primer set. 117 sequences in VBASE2 are not assigned to any V-gene family. The analysis of these V-genes with the given set of primers is presented in Table 5.12. In this set, the coverage is considerably lower – only 82 out of 117 (70.1 %) .

A detailed analysis of V-gene coverage by the oligonucleotide primer set applied in our study according to variable heavy and light chains including family- and non-assigned genes is presented in Table 5.13 (A.).

Altogether 89.1 % of all 576 human V-genes in VBASE2 are covered with the given primer set. However, VBASE2 offers the possibility to classify the V-gene sequences according to their functionality [173]: Class 1 V-genes have references for both genomic and rearranged sequences; Class 2 V-genes are based solely on genomic source informations, so this class holds pseudogenes (genes including stop codons), orphans (genes allocated to a different loci) as well as V-genes without proof of usage; Class 3 V-genes are supported only by rearranged sequences.

Based on the specific VBASE2 classification, a thorough analysis of the functionality of the not covered genes was conducted (Table 5.14). Thus, the coverage of the possible functional V-genes (after excluding the non covered pseudogenes and orphans) is 96.4 %. The coverage of the family- and non-assigned genes is 96.6 % and 95.3 % respectively. The detailed analysis regarding isotype of the chain and family assignment can be found in Table 5.13 (B). We designed 3 new primers to allow coverage of non-covered V-genes that exhibit binding interactions (Table 5.15).

Since the variable light chain coverage is lower, an additional analysis was made in order to construct new primers for genes of interest (the non-covered genes with exception of pseudogenes and orphans), whereas the criteria was to cover more than one gene. Moreover, a primer is suggested to cover the single Class 1 V-Gene – a functional one from the variable heavy chain VH3 family (Table 5.15). Thus, the total coverage has been further increased to 98.7 %, whereas the coverage by the family- and non-assigned genes rose to 98.9 % and 97.7 %, respectively. Details of the primers is shown in Table 5.13 (C).

Table 5.12: Bioinformatic analysis of oligonucleotides for amplification of V-genes based on non-assigned genes from VBASE2.

Genes with no family assigned	No. of covered (not covered) genes	Primer	Primer Sequence	Fold Degeneracy	Mismatches#															
					0	1	2	3	4	5	6	7	8	9						
Variable Heavy Chain																				
	21 (19*)																			
		VH1	CAGGTCCAGGCTKGTTCAGTCTGG	4																
		VH157	CAGGTGCAGGCTGTSARCTGG	4	1	5														
		VH3	GAGGTGCAGGCTGKTGGAGWCY	8	6	29														
		VH4	CAGGTGCAGGCTGCAAGGAGTCSG	2																
		VH1 & VH157																		
		VH1, VH3 & VH157																		
Variable Light Kappa Chain																				
	25 (6*)																			
		VK1	GACATCCRGDTGACCCAGTCTCC	6	5	20	4	16	2	8										
		VK246	GATATTGTGMTGACBCAGMCTCC	12			1	4	2	8										
		VK3	GAATTTGTRWTAACRCAGTCTCC	8					4	16										
Variable Light Lambda Chain																				
	36 (10*)																			
		VL1	CAGTGTGTSBTGACCGAGCCGCC	6	1	3														
		VL1459	CAGCCTGTGCTGACTCARYC	4	5	14	3	8	3	8										
		VL15910	CAGCCWGGKGTGACTCAGCCMCC	8																
		VL1, VL1459 & VL15910																		
		VL2	CAGTCTGYYCTGAYTCAGCCCT	8	2	6														
		VL3	TCCTATGMGCTGACWCAGCCAA	4			1	3												
		VL3(DPL16)	TCCTCTGAGCTGATCAGGASCC	4					1	3										
		VL3(38)	TCCTATGAGCTGAYRCAGCYACC	8			3	8		3										
		VL3 & VL3(38)																		
		VL78	CAGDCTGTGGTGACYCAGGAGCC	6	1	3	6	17												

* Genes which are not covered by the primers:

Variable Heavy Chain: humIGHV075, humIGHV091, humIGHV113, humIGHV156, humIGHV160, humIGHV166, humIGHV177, humIGHV180, humIGHV191, humIGHV205, humIGHV224, humIGHV235, humIGHV243, humIGHV254, humIGHV265, humIGHV276, humIGHV283, humIGHV302, humIGHV310. Variable Light Kappa Chain: humIGKV020, humIGKV112, humIGKV114, humIGKV117, humIGKV142, humIGKV153. Variable Light Lambda Chain: humIGLV030, humIGLV084, humIGLV092, humIGLV114, humIGLV123, humIGLV126, humIGLV132, humIGLV133, humIGLV138, humIGLV158

Table 5.13: Coverage of human V-genes from VBASE2 with analysed oligonucleotide primer sets.

(A.) Coverage of all V-genes from VBASE2 with initial primer set, (B.) Coverage of V-genes from VBASE2 without the non covered pseudogenes and orphans with the initial primer set, (C.) Coverage of V-genes from VBASE2 using the new primer set.

A.

Variable chain	Total no. genes*	No. of genes covered*	Coverage* (%)
Heavy (H)	232 (272)	226 (247)	97.4 (90.8)
Light Kappa (K)	137 (168)	117 (142)	85.4 (84.5)
Light Lambda (L)	90 (136)	88 (124)	97.8 (91.2)

B.

Variable chain	Total no. relevant# genes*	No. of genes covered*	Coverage* (%)
Heavy (H)	228 (249)	226 (247)	99.1 (99.2)
Light Kappa (K)	128 (155)	117 (142)	91.4 (91.6)
Light Lambda (L)	90 (128)	88 (124)	97.8 (96.9)

C.

Variable Chain	No. of genes covered	Coverage with All V-genes*	Coverage Excluding Pseudogenes & Orphans#
Heavy (H)	227 (248)	97.8 (91.2)	99.6 (99.6)
Light Kappa (K)	126 (151)	92.0 (89.9)	98.4 (97.4)
Light Lambda (L)	88 (127)	97.8 (93.4)	97.8 (98.4)

Note: * Family-assigned genes (all V-genes)

Possible functional genes (pseudogenes and orphans excluded)

Table 5.14: Bioinformatic analysis of the classification and the functionality of the non covered V-genes with the given primer sets (according to VBASE2).

Family	No. Non-covered V-genes	No. Class 1* V-genes	Pseudogenes	No. Class 2** V-genes	Others	No. Class 3*** V-genes
<u>Variable Heavy Chain</u>						
VH1	1		1			
VH3	5	1	3		1	
No family assigned	19		15	4		
<u>Variable Light Kappa Chain</u>						
VK1	6		5		1	
VK2	10		1		9	
VK3	4		3			1
No family assigned	6		4		2	
<u>Variable Light Lambda Chain</u>						
VL1	1					1
VL3	1				1	
No family assigned	10		7	1	2	

Note: * Functional genes

** Genes with only genomic reference(s)

*** Genes with only rearranged reference(s)

Table 5.15: Bioinformatic analysis of the new primers against V-genes un-covered by the initial primer set.

Family	Primer	Primer Sequence	Fold Degeneracy	No. of target genes
<u>Variable Heavy Chain</u>				
VH3	VH3N	TCAACACAACGGTTCCCAGTTA	1	1
<u>Variable Light Kappa Chain</u>				
VK2	VK2N1	AGATGCTGTGTGAMCCAGCCTC	2	4
	VK2N2	TCCCTCCAAGTTCACATCCTGAG	1	5
<u>Variable Light Lambda Chain</u>				
Not Assigned	VLNA	GTCCAGTTCCTCTATTATGRTAG	2	3*

Note: * One of the genes is a pseudogene

5.7.2 Evaluation of two different reverse transcriptases for cDNA synthesis

Reverse transcription (RT) efficiency of human V-genes from total RNA was compared using two reverse transcriptases (RTase). The commercially available SuperScript® II and totalscript-OLS® were applied in parallel to synthesize total cDNA from the same RNA sample using a combination of oligo(dT) and random hexamer primers. Additionally, RTases were used to synthesise IgD-specific cDNA with a gene-specific primer binding in the unique IgD hinge region from the same human RNA sample. Obtained cDNAs were employed as template in a PCR reaction to evaluate V-gene specific amplification and possible primer cross talk. Amplification was carried out with two primer-pair combinations of the same VH157 forward primer. For the amplification of IgD-specific and IgG-specific transcripts the IgD CH1-Rv and the IgG CH1-Rv reverse primers were taken, respectively. The comparison of the PCR results in Figure 5.31 demonstrates that IgD-specific cDNA synthesis was highly specific. As expected, IgD isotype specific amplicons were seen in both cDNA preparations. The IgG-specific primer pair gave no amplification product and showed no cross talk on IgD-specific cDNA.

At the same time, the efficiency of cDNA preparation with both RTase was assessed. The PCR results obtained from total cDNA as template showed significant amplification with both IgD- and IgG-specific primer combinations for SuperScript II but not for totalscript-OLS

prepared cDNA. When the PCR amplification yield on IgD-specific templates was compared, in both cases amplicons were seen. SuperScript II prepared cDNA yielded slightly more amplification product.

5.7.3 Evaluation of different polymerases for PCR amplification

Three commercially available polymerases – REDTaq, BIO-X-ACT short and Phusion were examined for their efficiency to amplify V-genes in a standard protocol. Figure 5.32 (A) shows the results of the amplification applying all heavy chain V-gene specific forward primers in combination with the IgD constant domain specific reverse primer. Poor amplification was recorded for REDTaq polymerase while BIO-X-ACT short gave amplicons for most primer combination, but with very low yield. Phusion gave the best amplification results of the three tested. The VH family pool (equimolar concentrations of all idiotype forward primers) resulted in very poor amplification, indicating that primer mixtures decrease amplification yield significantly. Attempts to improve the conditions by varying annealing temperatures and by addition of DMSO did not enhance amplification (result not shown). However, optimisation of the PCR condition by modifying the $MgCl_2$ concentration in the reaction buffer showed improvement in the amplifications for some polymerases. The manufacturer's recommended concentration were increased by 0.25 mM, 0.5 mM and 1 mM $MgCl_2$ (final conc.), respectively. Figure 5.32 (B) and Figure 5.32 (C) depict the amplification using two different V-gene primers (VH157 and VH3) in combination with the IgD CH1-rv reverse primer. Elevating the recommended $MgCl_2$ concentration of the manufacturer's by 0.25 mM of $MgCl_2$ gave slightly more yield in amplicon for REDTaq polymerase, while further increase of $MgCl_2$ concentration decreased product yield. For BIO-X-ACT, increasing the $MgCl_2$ concentration by 0.25 mM and 0.5 mM $MgCl_2$ showed minor improvement in amplicon yield. Addition of $MgCl_2$ had no significant effect on the performance of Phusion polymerase, which had the highest overall amplification yield in this study.

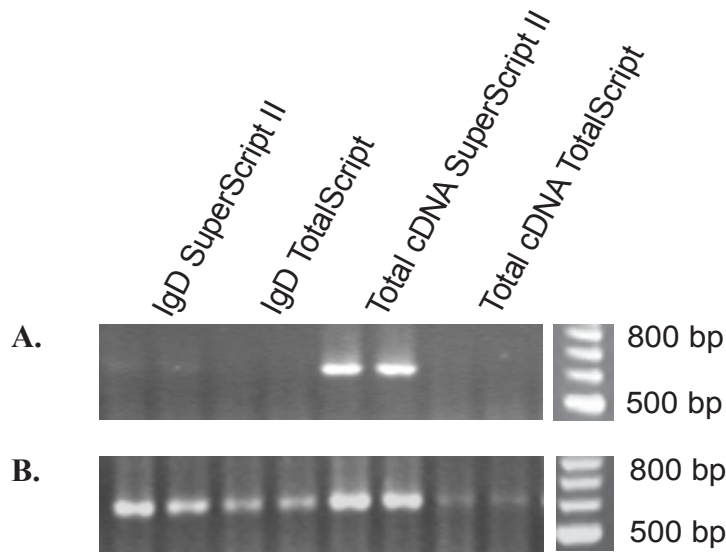


Figure 5.31: Comparison of reverse transcription efficiency and specificity of IgD gene specific primer cDNA synthesis. Amplification using VH 1257 forward primer. Reverse primers used are: (a) IgG CH1-Rv (b) IgD CH1-Rv. Expected band sizes range from 650 to 700 bp.

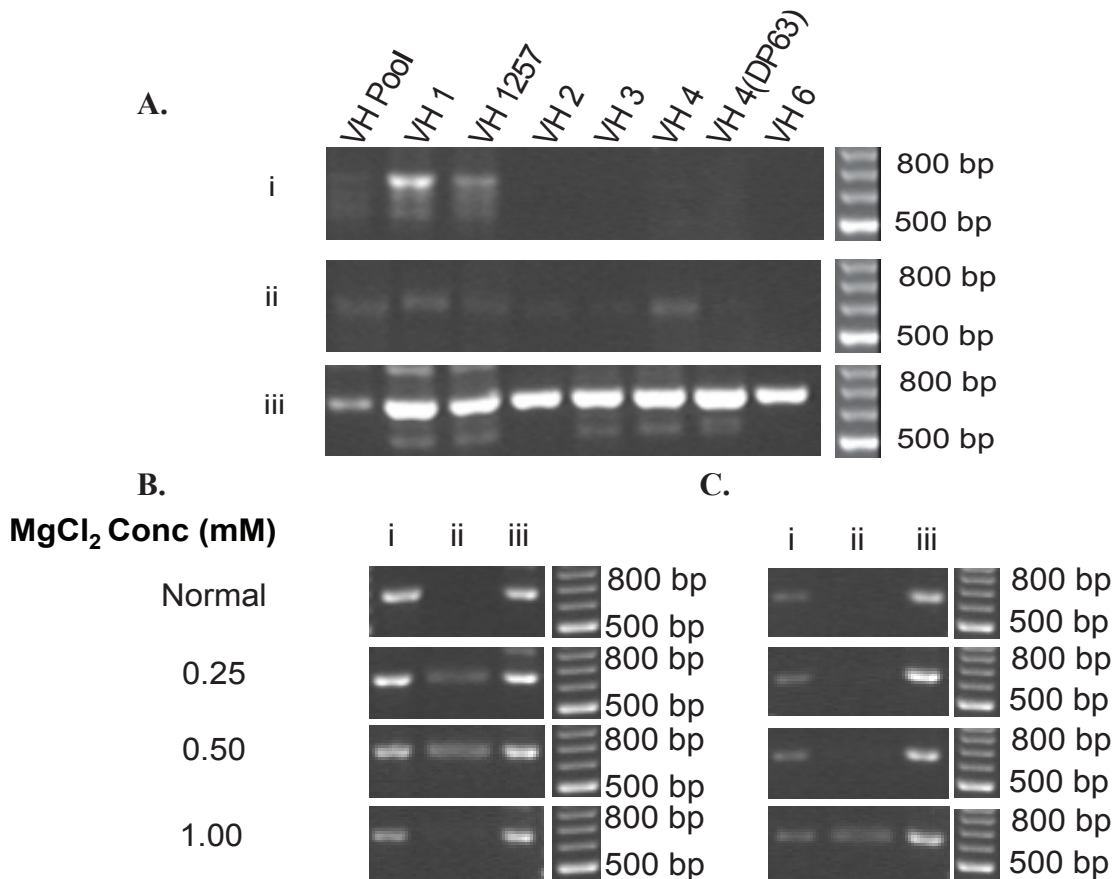


Figure 5.32: Efficiency of three different polymerases for antibody V-region idiotype amplification with a standard protocol and MgCl₂ optimization. The polymerases used are: (i) REDTaq Polymerase, (ii) Bio-X-Act Polymerase and (iii) Phusion Taq Polymerase. V-region cDNAs were amplified using the conventional PCR procedure described in the text. (A) all V-gene families with a standard protocol, (B) VH 1257 with MgCl₂ and (C) VH 3 with MgCl₂ forward primers. All amplifications was carried out using the IgD CH1 reverse primer. Expected band sizes range from 650 to 700 bp. Donor template for A differs from B and C, resulting in apparent differences in V-gene usage.

5.7.4 Effects of addition of Extreme Thermostable Single-Strand DNA binding protein (ET SSB) on PCR efficiency

The effect on PCR amplification yield after addition of ET SSB to the PCR reaction mix was examined. In Figure 5.33, PCR amplification results with or without the addition of ET SSB are shown for an equimolar pool of all V-gene forward primers (VH Pool) and the single primer VH157, both were used in combination with the IgD-specific reverse primer IgD CH1-Rv. Additionally, cDNA samples generated using either SuperScript II or totalscript-OLS RTases were assessed. For all cDNA templates a significant increase in DNA amplification with both primer sets was recorded after addition of ET SSB.

In the lower panel the relative intensities are shown as determined with the AIDA image analyzer software. Relative concentrations are based on the intensity of the 500 bp fragment from the DNA ladder. For both sets of primers a significant (up to 10-fold) increase in amplicon yield was seen in the ET SSB containing PCR samples. The amplification yield of IgD idiotypes increased ~6-fold for IgD-specific cDNA generated by both RTase. When total cDNA was used as template, a ~5-fold increase was seen for SuperScript II and a ~10-fold increase was seen for totalscript-OLS synthesised cDNA. The greatest positive effects of ET SSB was seen when applied in combination with totalscript-OLS synthesized cDNA preparations. A similar distribution pattern of yield was observed for multiplexed as well as single primer combinations.

5.7.5 Antibody isotype and idiomorph amplification

Amplification with the entire V-gene primer combination for IgD, IgG, Kappa and Lambda isotypes of a single donors was assessed. The optimised protocol uses cDNAs obtained by SuperScript II RT as template and Phusion polymerase with addition of ET SSB for efficient amplification in PCR. IgD-idiotype analysis was performed on IgD-isotype cDNA, while all other idiotypic analyses were done on total cDNA derived from oligo(dT) and random hexamer priming. The ability of the protocol to amplify all V-gene families using a single PCR protocol was evident and Figure 5.34 shows a typical antibody idiotypic amplification for the different isotypes. The optimised protocol was able to amplify the idiotypes of all four isotypes tested (IgD, IgG, Kappa and Lambda) using a combination of V-gene specific forward primers (Figure 5.34) and isotype-specific reverse primers. Differences in band intensity are likely due to the differences in V-gene usage of the donor.

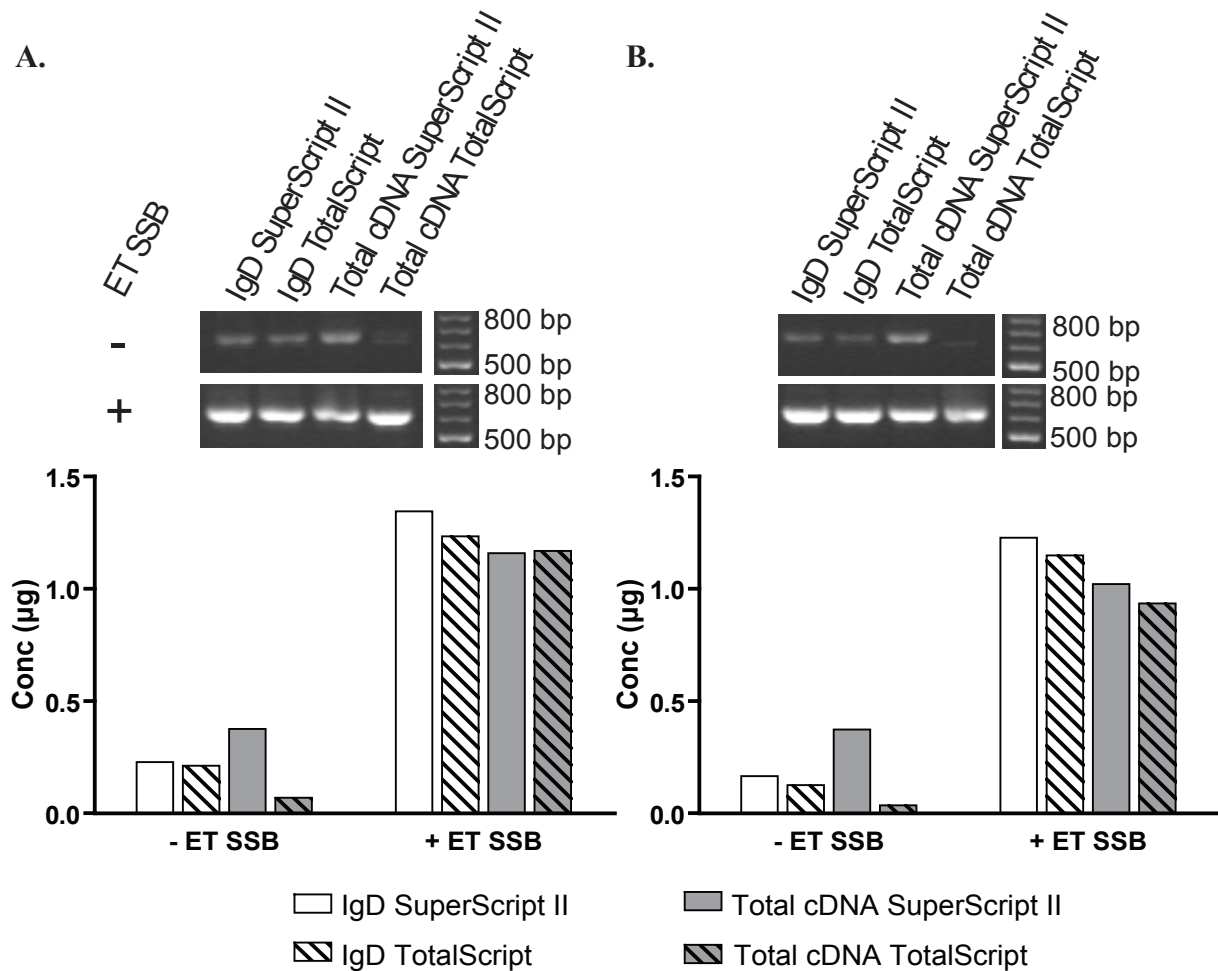


Figure 5.33: Influence of ET SSB in antibody idiotype V-gene amplification. cDNA of samples and ET SSB are as depicted. Primers are as follows: (a) VH Pool (b) VH 1257. All samples were amplified using IgD-CH1-Rv primer. Expected band sizes range from 650 to 700 bp. Bar chart shows the DNA concentration (μg) of the bands determined using AIDA software analysis. Sample orientation of bar chart is according to the cDNA sample orientation as depicted.

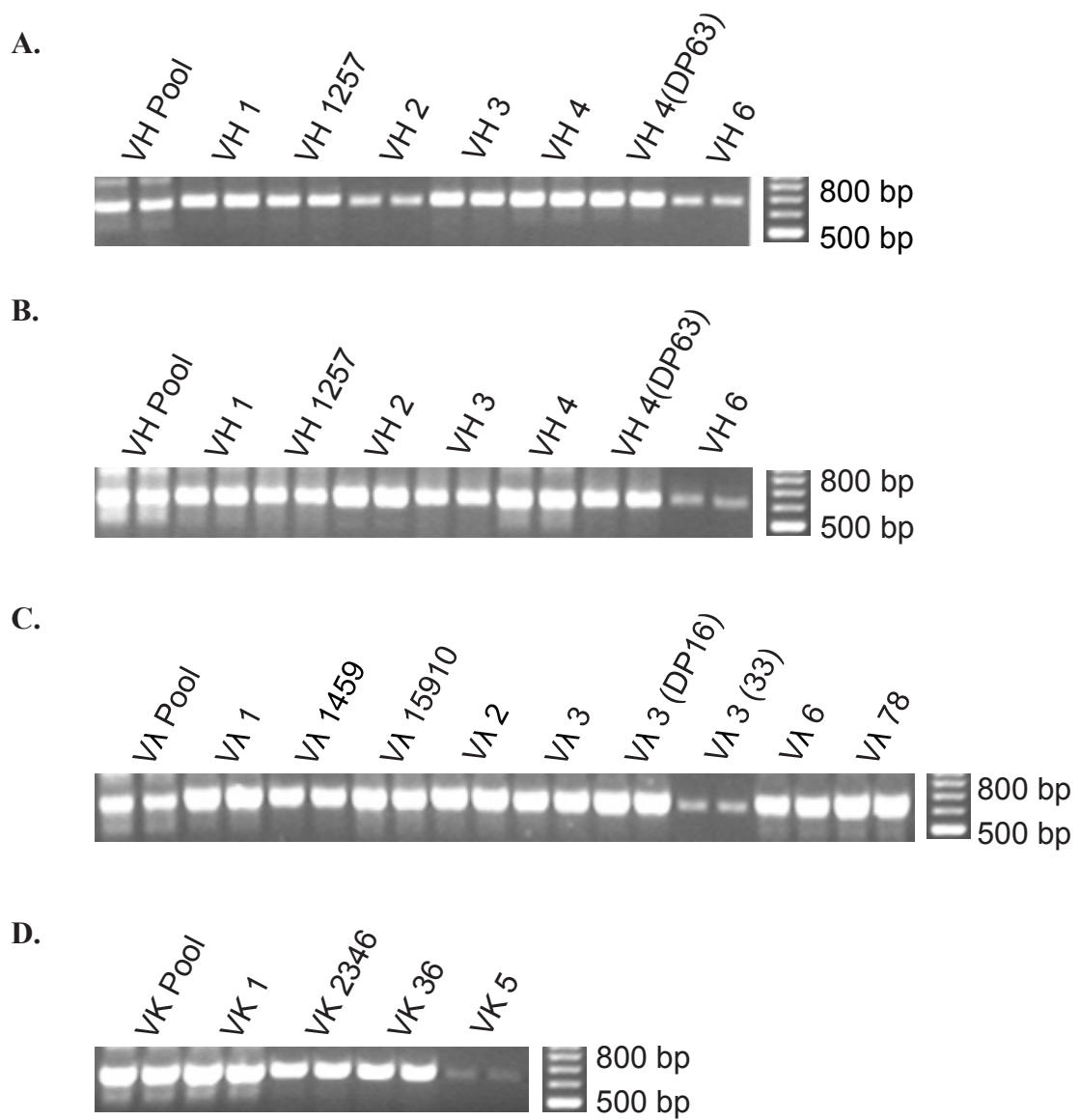


Figure 5.34: Full antibody isotype and idiotype amplification with a single donor sample. Amplification was carried out using isotype specific reverse primers: (A.) IgD (IgD-CH1-Rv), (B.) IgG (IgG-CH1-Rv), (C.) Lambda (Pool Lambda CL1-Rv & Lambda CL2-Rv), (D.) Kappa (Kappa CL-Rv). Idiotype specific forward primers are as depicted. Expected band sizes range from 650 to 700 bp.

5.7.6 V-gene repertoire optimization for library generation

Amplification using the entire V-gene primer combination for IgD, IgG, Kappa and Lambda isotypes of five individual donors was assessed. Individual antibody V-gene profiles for all five donors were successfully generated by the protocol. The distribution of V-genes amongst the donors show significant variations for certain V-gene families as expected. The unique V-gene usage of the individuals are shown in Figure 5.35.

As cDNA template function as the basis for V-gene repertoire amplification for antibody library generation, to generate a naive library with a significant diversity, a large set of donors would be required. This will result in an enormous number of PCR amplifications thus increasing the time and cost to generate a naive library.

To circumvent this bottleneck, several parameters to decrease the number of PCR amplification required were investigated. Firstly the use of a combination of pooled primers of all V-genes were used. Although able to amplify the resulting amplification was not able to distinguish the profiles based on their V-gene usage. As the V-gene profiles have certain preferences for V-gene families, this could result in a preference in amplification as the other lower expressed V-genes are not well represented in the pool.

Secondly, the effects of pooling cDNA template for V-gene repertoire generation was investigated. V-gene profile amplification from a pooled population of cDNA and a pooled amplification of the five donors was conducted. The pooled V-gene profile of the five donors showed a profile that is close to that of the individual profiles when amplified individually. This approach would then be able to overcome the complication of under representation of low expressing V-gene families. As the template is amplified with just a V-gene primer, less competition and preference for amplification would be expected. As there is no significant skewness in the V-gene usage after pooling, this would allow the amplification of a pool from five donors for a naive antibody library generation.

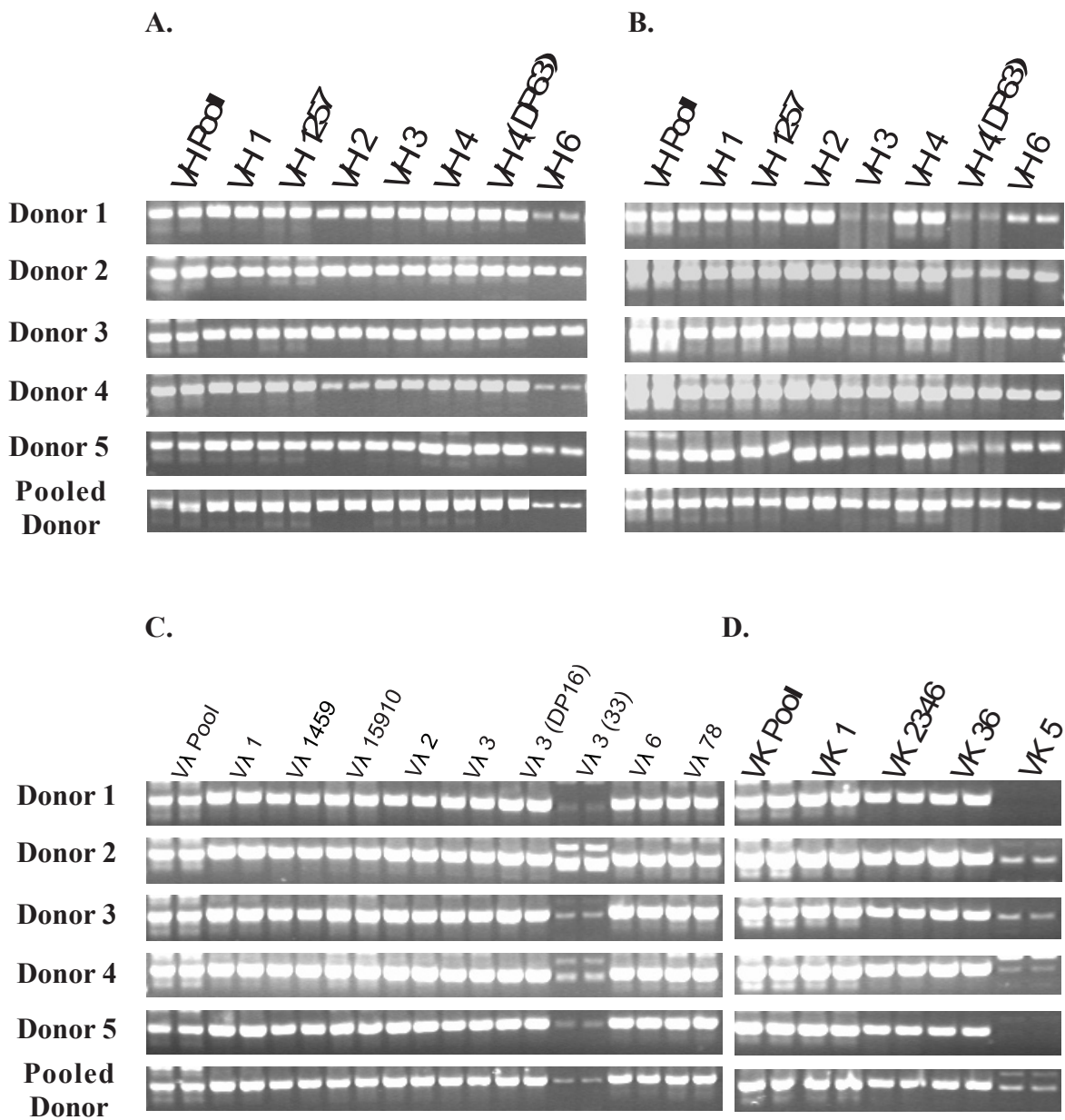


Figure 5.35: Full antibody isotype and idiotype amplification of five individual donors and a pool of five donor cDNAs. Amplification was carried out using isotype specific reverse primers: (A.) IgD (IgD-CH1-Rv), (B.) IgG (IgG-CH1-Rv), (C.) Lambda (Pool Lambda CL1-Rv & Lambda CL2-Rv), (D.) Kappa (Kappa CL-Rv). Idiotype specific forward primers are as depicted. Expected band sizes range from 650 to 700 bp.

6.0 Discussion

The emergence of antibodies as immunotherapeutic and diagnostic tools has caused a rush to develop better recombinant human antibodies. Antibodies offer unrivalled target specificity for conventional therapies and diagnostic tools. The benefit of antibodies especially in terms of therapy has made it a multi billion dollar business with the approval of 18 monoclonal antibody therapies for various conditions [174]. The production of antibodies is still dependent largely on the traditional hybridoma technology, which requires the immunization of animals. However, over the last 20 years, various combinatorial technologies have been introduced which allows the development and selection of antibodies from an *in vitro* immune repertoire without the need to immunize a living host [94,175,176,177]. Amongst the array of different display technologies, phage display is the most successful method to date for generating human therapeutic antibodies. Therefore, streamlining the phage display process is still of general importance to help improve the existing protocols for human antibody generation. As a result, investigations into library design and generation parameters affecting human antibody selection by phage display were carried out.

6.1 Phagemid vector construction

In phage display, the most commonly used phage coat protein for antibody fusion is the minor coat protein III [178]. In phagemid vectors, only the phage packaging signal and pIII gene is provided. For phagemid systems, a helper phage such as M13KO7 is required for successful phage packaging. This is due to the lack of other genes encoding the packaging proteins in phagemid vectors for phage generation [179]. The pTSL phagemid vectors were designed with an ampicillin resistant gene and a lac promoter for regulation of gene expression. The phagemid vector has two origins of replication to allow the formation of pIII fused antibody fragment for phage packaging and bacterial expression. Each cloning site for both heavy and light chain regions are incorporated with a *ccdB* cassette. The toxicity of the *ccdB* gene has been used successfully for positive selection for cloning experiments with different antibiotic resistance [180,181]. Low transformation background was achieved with the use of the *ccdB* gene that permits toxicity to inhibit growth of re-ligated clones. This proved to be an advantageous feature to include in the phagemid vector for use in antibody library generation, as high transformation background would render the library generation process ineffective. The cloning sites in the pTSL vectors allow easy conversion in between different antibody formats.

6.2 Generation of semi-synthetic antibody library

Common formats used for antibody libraries are scFv and Fab with the former being more preferred due to its smaller size, lack of an interchain disulphide bond and presentation as a single peptide via a glycine-serine linker. The smaller scFv at 30 kDa would allow it to be expressed with much greater ease in bacteria than the larger Fab (50 kDa). The scFv being only a single polypeptide chain also allows easier fusion to the minor coat proteins of phages thus allowing better presentation efficiency than Fab constructs. This is because Fab constructs have to be expressed as two individual chains with either one of the heavy or light chain being fused to the phage coat protein. By transportation to the periplasmic region, the two chains combine via disulphide bond formation to form the Fab construct [182]. The IgG Fab format is usually used due to the ease of conversion from Fab to full IgG molecule with the preservation of affinity and binding [183,184]. The study of presentation efficiency and performance of different Fab formats is important due to some downstream limitations posed by the scFv construct. Limitations such as the lack of stability, tendency to multimerize and a short serum half life will limit the efficacy of scFv formats as a tool for therapy. Therefore, the comparison of two different Fab constructs from IgD and IgG isotype was carried out.

The pTSL vector series were used to generate new semi-synthetic antibody libraries. The Tomlinson semi-synthetic scFv library I was used as a template to generate other libraries for this work to allow better comparison as the variable constructs originate from a single pool. A dual vector system where both heavy and light chain are cloned separately into two vectors and restriction enzyme ligation to sub-clone existing antibody libraries have been reported [185, 186]. The dual vector system would require two sets of transformation to generate individual sub-libraries, for both heavy and light chain. Here, the variable heavy and light repertoires were generated based on PCR assembly by amplifying the V regions of both chains, digesting with restriction enzymes, ligating and re-amplifying it to obtain the ligated fragments. This allowed the generation of the Fab constructs without the need of an intermediate transformation procedure or having to generate two sub-libraries which proves cumbersome. The transformation efficiency for both libraries using the pTSL phagemid vectors was comparable. The largest sized library is generally anticipated as theoretically it would yield more binders for a larger repertoire of antigens in comparison to smaller libraries [101]. Even so, large sized libraries does not necessary guarantee higher affinity antibodies [187]. The use of TG1 strains for library generation was able to generate a library size of approximately

5×10^7 for both IgD Fab and IgG Fab libraries. DNA sequencing of a random test set of antibody clones showed that the diversity of the clones was conserved.

To date, there has been only 3 known IgD derived antibody libraries made [109,188,189]. Raum *et al.* managed to show the durability of the IgD Fab construct by converting the enriched IgD Fab fragments to full IgG1 constructs successfully. The ability of both IgD and IgG Fab constructs to be converted to full IgG molecules makes it an ideal format for comparison. The IgD Fab differs from the IgG Fab by virtue of the disulphide bond formation. Although the antibody fold is kept much in place, the different location of the interchain disulphide bond cysteine in the heavy chain of IgD and IgG allows a possible variation in forming functional Fab constructs to be presented on phage surfaces (Figure 6.1). Rather than performing a single clone comparison, the comparison of libraries was carried out to provide a better understanding on the overall performance of both constructs.

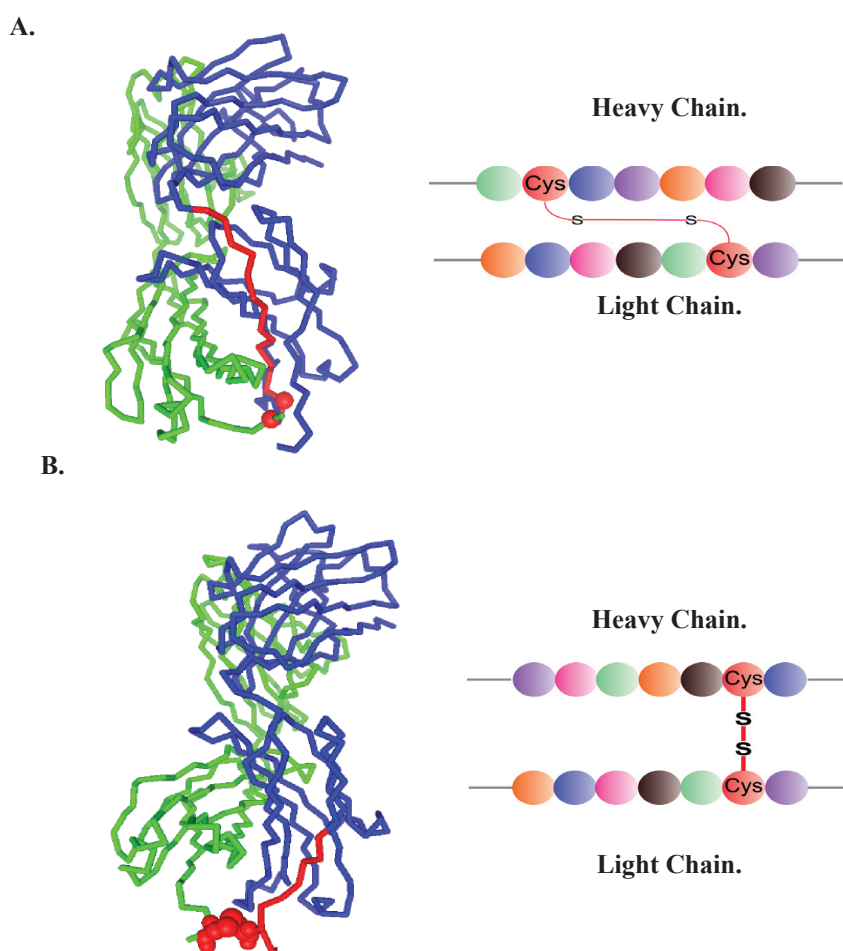


Figure 6.1: Tertiary and secondary structure of IgD Fab (PDB: 1ZVO) and IgG Fab (PDB: 1HZH). (A.) IgD Fab, (B.) IgG Fab. The red lines represent the beginning of the CH1 region for the IgD and the end region of the IgG CH1 chain. The red coloured spheres in the cartoon represents the cysteines on both heavy and light chains that forms the interchain disulphide bonds.

6.3 Antigen preparation

Before conducting antibody selection processes, antigens for selection must be prepared. The importance of high quality purified targets must not be overlooked as it can affect the selection process by enriching antibodies against contaminants. For the applied protocol, purified biotinylated antigens are required for coupling to magnetic beads. Antigens are recombinantly expressed using the pRSET-BH6 and pRSET-BH6-GW vectors, where all proteins are expressed with a AVI-Tag and His-Tag. The AVI-Tag allows *in vivo* biotinylation to occur and the His-Tag functions as an affinity purification tag using IMAC.

Several different purification strategies were evaluated to obtain the highest possible quality antigens for phage display selection. Ni-TED and Ni-NTA were compared for affinity purification using the His-Tag. The yield and purity of antigens from Ni-TED was lower than Ni-NTA. Ni-TED is a pentadentate metal chelator thus occupying five out of the six possible binding sites in the coordination sphere of the Ni²⁺ ion. Ni-NTA occupies only four sites on the Ni²⁺ ion thus having 2 active binding sites to capture proteins in comparison to only one free site with the Ni-TED. This extra binding site could contribute to the higher yield of the purification. Monomeric avidin was also investigated to study the possibility of using the biotinylation as a purification strategy. Although the proteins purified were clean of other contaminating proteins, a major setback was the final elution volume of the column. The strong binding interaction between biotin and avidin would require a strong dissociation to release the captured proteins. In order to elute the captured proteins a longer elution step was necessary. This resulted in lower concentrations of protein with the need to concentrate the protein after purification. As the purity and yield of the antigens purified using Ni-NTA was sufficient for selection, the Ni-NTA protocol was used for preparing antigens used for phage display.

6.4 Antibody format presentation efficiency assay

Two Fab libraries of different isotypes were prepared using the pTSL vectors. A novel protocol was established to study the presentation efficiency of the IgD and IgG Fab antibody formats by selecting against Protein L coupled beads. The presentation efficiency of scFv is known to be superior to Fab formats [190]. Both IgD and IgG Fab formats differ by the formation of disulphide bonds between the heavy and light constant regions (Figure 6.1). The rationale of using both Fab formats was to investigate if the variation would elicit a positive or negative impact to the presentation efficiency of Fab formats on phage surfaces.

A method using Protein L coupled beads with known Fab presenting phage titres in a serial dilution was conducted. The method was modified from the in solution affinity determination by Friguet [167]. The Friguet assay involves the measurement of binding affinity of antibodies to proteins by ELISA equilibrium titration. The main assumption is that equilibrium is achieved in solution without the separation of antibody-antigen complex [191]. Instead of a solid phase assay in microtitre plates, Protein L was bound to magnetic beads, which would enhance binding, reduction of non-specific binding (low signal to background ratios) and allow better binding equilibrium to be achieved at a shorter time with the increased surface area [192]. The specificity of Protein L to capture kappa light chains was exploited to capture only functional Fab presenting phages. This is important as formation of functional Fab constructs is dependent on the successful fusion of the light and heavy chain to form a disulphide bond. This method will ensure that non-fully formed Fab or wild type pIII phages are not captured to lower background. The determination of efficiency is based on the phage titre recovered by the beads after infection.

Based on the protocol established, IgG Fab showed better presentation efficiency than IgD Fab. Therefore, the differences of the disulphide bond formation between the IgD Fab and IgG Fab does influence the presentation efficiency. This could be due to the structural fold of both Fab formats as the positions of the cysteine in IgD could be harder to access than the IgG.

6.5 Diversity Visualization by Endonuclease (DiVE) assay

A rapid and cost effective method was described to monitor the distribution of diversity throughout the panning rounds during a phage display selection process. The basis of the assay is the reannealing kinetics of double strand DNA after denaturation. The reannealing of single strand DNA from a pool of diverse sequences would yield multiple combinations with multiple mismatches occurring. The single strand sensitivity of the S1 nuclease was used to remove any formation of nicks, heteroduplex loops and single stranded ends from the reannealing process. The stabilization of DNA duplex is a multi-factorial process which includes base sequences, concentration, length, thermodynamics, salt concentration and pH [193,194,195], therefore, several steps to optimize the S1 nuclease digestion was investigated.

Optimization of S1 nuclease digestion conditions

The basic parameters (incubation temperature, enzyme units and incubation time) were investigated first to optimize the S1 nuclease digestion. From the optimizations, the ideal temperature used was 60°C. This is within the reported optimal temperature for single-strand-specific nuclease at 37 – 70°C [196] with a two- and three-fold increase in activity on single strand DNA at 45°C and 60°C respectively [170]. An optimal temperature suitable to allow maximum enzyme activity and dsDNA stability is preferred. Higher temperatures were not suitable as they could destabilize the double strand as it nears the melting temperature.

The second factor investigated was the amount of enzyme units required to obtain a visible band on the agarose gel. The reaction buffer at a final concentration of 0.5x (100 mM NaCH₃COO, 0.75 M NaCl, 5mM ZnSO₄) is sufficient. The presence of Zn²⁺ ions in the buffer is critical as Zn²⁺ ions are important co-factors for the *Aspergillus Orzae* S1 nuclease [196]. The enzyme works best at high salt concentrations but as the goal is to ensure sufficient removal of ssDNA tails and ssDNA breaks in dsDNA, moderate conditions are sufficient for use.

A concentration in between the optimized range of 0.02 U and 0.1 U was used. 3 time points of 10 min variations was used with 0.04 U of enzyme. By reducing the incubation times and moderately using the enzyme, the band intensity patterns were varied to illustrate the best conditions for our use. An incubation time of 10 to 20 min was sufficient to distinguish the variation in diversity of each panning round.

Application of the DiVE Assay

The optimized protocol was applied to two different selections for both heavy and light chain amplicons where a positive and negative enrichment was observed on polyclonal level. This was to examine the durability of the protocol on amplicons with different sequences but of similar length. The assay proved its flexibility to adapt to both heavy and light chain amplicons successfully to monitor the diversity.

The S1 nuclease functions as an endo-exonuclease which produces 5'-mono-nucleotides as the end products of DNA and RNA hydrolysis [170]. Partial length fragments were observed resulting from the removal of ssDNA extensions from partial annealing of non-matching strands. CDR regions with lower frequency of diversification would pose a higher chance of surviving because of better re-annealing opportunities. This is evident with the side product bands of 173 bp and 282 bp from the heavy chain and light chain amplicons, respectively.

The ability of this protocol to generate the diversity variation pattern for both heavy and light chain reinforces the robustness of the DiVE assay. Selection technologies such as SELEX, yeast display, ribosome display and phage display which incorporate a set of diverse oligonucleotides could potentially benefit from the DiVE assay. All that would be required is a PCR amplicon generated from a fraction of the selection rounds to observe the pattern of enrichment. As selections of varying targets would perform differently, over-panning could result in the loss of a desired clone to those that exhibit a growth or codon usage preference. Hence this protocol could assist in deciding how many rounds of selection are truly required to generate a concentrated pool with sufficient monospecificity for the researchers' use. In conclusion, this protocol can be used as a yardstick to monitor the individual diversity of each selection round. The DiVE assay offers a rapid, cost effective and robust method to monitor diversity of selection rounds from oligonucleotide libraries.

6.6 Phage display selection with semi-synthetic libraries

Phage display panning procedure was carried out using a magnetic particle processor to ease the workload of screening 3 libraries with 14 antigens simultaneously. This semi-automated high-throughput selection process was according to the method by Konthur *et al.* [149]. The protocol was initially designed for use with scFv libraries but considerations to the stringency of the selection were taken into account to allow a simultaneous selection of both scFv and Fab libraries. The stringency of each round was increased with the addition of a single round of wash step with every selection round. The wash times for each wash step remained constant and the wash buffer used was PBS-T throughout, without the addition of any denaturants, organic solvents, increase of temperature or counter selection target. This is to allow the mildest selection condition to facilitate the Fab library selections without compromising specificity or selectivity. An array of high, medium and low expressing antigens was used in the selection to simulate varying amounts of antigen concentrations used.

The selection process was carried out using both monovalent display and multivalent display. Multivalent phage display allows the presentation of multiple copies of the antibody fragment per phage virion, whereas monovalent display allows the presentation of maximally one antibody copy per phage. For a long time, complication with multivalent display was the inability to enrich for high affinity binders due to the avidity effect of lower affinity binders competing for antigen [71,84]. The use of the Hyperphage system however overcomes this problem [89,197]. A multivalency of the presented antibodies occurs only in the 1st selection round. In the remaining rounds, M13KO7 is used for phage propagation. Both, scFv and Fab formats have been successfully used for selection in both mono- and multivalent display systems [198,199,200,201].

The panning protocol was carried out using both mono- and multivalent display for all three libraries in the first round followed by monovalent display in subsequent rounds. The combination of multi- and monovalent display was able to enrich for all the antigens, whereas the monovalent display approach was unable to enrich for ATX2 and GPS2. This could be due to the low concentrations of these two antigens in comparison to the other 11 antigens after purification. The success of multivalent display to enrich binders for these two antigens could be attributed to the avidity effect. This is in agreement with the rationale of the approach of different display strategies at round 1 panning. The choice of either mono- or multivalent display is subjected to various considerations. Generally, scFv formats perform well under both

conditions. In cases where a difficult or low concentration antigen is available, multivalent display for round 1 seems a more advantageous strategy.

The selection process yielded significant enrichment for the scFv libraries and lower enrichment for the Fab libraries. The size of the library is believed to coincide with the affinity and success rate of selections against a large set of antigens [93,115]. This concept was further strengthened by the isolation of more than 1000 antibodies against the BLYS protein from a large antibody library [202]. With a larger size library, the higher number of unique clones will allow a higher probability to isolate binders. This taken into account together with the advantage scFv formats have over the Fab constructs in terms of presentation efficiency, it is expected that the scFv would outperform the Fab libraries in concurrent selections[182]. This was evident with enrichments for all 13 antigens using the scFv library and only 2 antigens enriched from the IgG Fab library during the polyclonal ELISA. Majority of the enrichments occurred during round 3 and 4 of panning.

TPI-SV and UBI8 were further investigated for monoclonal selections due to positive enrichments in polyclonal ELISA for Fab libraries. For the first instance, soluble monoclonal antibody fragments were prepared by microtitre plate expression and tested on ELISA to obtain functional soluble antibody fragments. This was only possible for scFv formats as no signals were visible for both Fab libraries. There have been several strategies used to increase expression levels of scFv and Fab fragments by introduction of molecular chaperones, employing various leader peptides and varying expression protocols [203,204,205,206]. Although no signals were seen for Fab library monoclonal, it does not necessary mean that there are no binding clones. This is because there is a balance that has to be achieved between functional soluble Fab from phage bound Fabs. Not all phage clones displaying Fab can produce Fab efficiently in bacteria due to the differences in folding because of the varying amino acid sequences [207]. Although the framework used in the library is a synthetic framework, the CDR regions still allow much variation which could influence the ability to express a certain Fab. Therefore to overcome this, phage-Fabs are used for monoclonal selection [159]. Upon the change from soluble Fab expression to phage-Fab expression format, positive clones were visible for both antigens. To ascertain the possibility that due to the difficulty in enriching polyclonal phage-Fabs, monoclonal selections of negative enrichment from SH3GL1, SH3GL2 and SH3GL3 antigens were also carried out. The notion for this experiment was to investigate the possibility of positive clones being present but clouded by the non-binding clones in a polyclonal pool even after panning. This was taken into consideration as the semi-automated protocol used was not optimized for

a given antigen or antibody format but more of a general protocol to reduce the diversity and provide a more focused pool of binders. Monoclonal selections of round 4 monoclonals from the negative enrichments were carried out for both Fab libraries. Several monoclonals were found for SH3GL2 and SH3GL3.

The library panning using the semi-automated fashion could still be used with more success for Fab libraries with some optimizations of the selection condition. A series of optimization protocols could be carried out to improve the success rate of high-throughput panning of Fab libraries. Different selection strategies have been shown to yield different recovery results with the same library [208]. Another consideration is that in nature, the light chain preference of soluble IgD is about 87% lambda [209]. The kappa to lambda ratio normally reflects the ratio of the most abundant Ig family in serum, usually IgG reported at 1.5 to 2.0 [210,211]. The preference of IgD for lambda light chain might be an indication to stability for the IgD protein in soluble form, therefore the use of a kappa chain in the library may not be the best choice.

6.7 Format switching

The common formats used for antibody phage display is either scFv or Fab fragments [92]. The choice of format for antibody libraries depends on the downstream applications. As it is costly to generate multiple antibody libraries of different formats, an ideal solution would be to generate a library using a format with the most flexibility for format switching. All three constructs (scFv, IgG Fab and IgD Fab) studied, generally has the same Fv regions grafted into the main framework. The major variation of the scFv to the Fabs is the presence of a peptide linker normally between 15 to 20 peptides in length [51,212]. Variations in peptide linkers is of huge interest as it can be used to create variations of scFv modules with different sizes, flexibility and valency [213,214,215]. The IgD and IgG Fab fragments differ by the position of the cysteine in the CH1 region resulting in a different disulphide bond formation. Here inter-changing of formats was carried out to observe the influence of format variations of similar grafted Fv region on binding characteristics. The idea is to identify the best format to be employed for phage display antibody library generation in the context of maintaining functionality or minimal loss of binding characteristics following post-selection conversions to other formats.

There are several different factors that can contribute to the binding characteristics of antibody formats. The binding regions of antibodies are isolated to the CDR regions, where *in vivo* somatic hypermutation occurs to increase affinity of the antibodies. Representation of somatic hypermutation regions of antibodies suggest a binding pocket where diversity to regions around the periphery of the binding site contributes to the diversity and affinity of the antibody to the antigen [130]. The contribution of each heavy and light chain to antigen binding is a highly debatable enigma. There are reports suggesting that the heavy or light chain contributes to antigen binding regardless of the other [216,217]. However, it has also been shown that the relationship between both heavy and light chain is critical for antigen binding [218]. It is most likely that all the varying influences reported of the heavy and light chain is dependent on the antibody binding regions as not all residues in the CDR but also some conserved residues actually have contact with the antigen [130]. The CH1-CL and VH-VL pairing functions as an ideal connector for one another, resulting in an increase in apparent affinity and stability [219]. Failures to detect a signal with Fab fragments after scFv-to-Fab conversion have been suggested to be due to multimerisation of medium affinity scFv that led to a strong but multivalent binding. But the corresponding Fab cloned remained purely monomeric hence having a lower avidity effect. Another reason is the alteration to the conformation of the binding pocket by the change of format due to the removal of the VH-VL linker sequence of the scFv [139]. The fold generated by the peptide linker of scFv could vary from the fold generated by the constant regions of the daughter Fab hence disrupting the initial epicentre for binding. This will then lead to a loss in the binding aptitude.

Conversion of scFv to Fab

From the 4 scFv clones tested, only 1 clone (Clone 2F) retained high binding characteristics when converted to IgG Fab. This could be attributed to the amino acid sequence which could allow a favourable fold that still maintains the binding characteristics. IgG sub-clones of 10D, 9A and 2C exhibited significantly lower OD readings from their respective parental scFv clones. On the other hand, the conversion of scFv to IgD Fab formats showed a decrease of OD readings similar to that of IgG Fab. IgG Fab conversions from scFv have been shown to have a 76% success rate in maintaining the binding characteristics to the parental scFv [139]. Nothing is known about IgD Fab conversions as not much has been done using IgD constructs except of the generation of scFv libraries using IgD repertoires [109,188,189,]. The conversion to daughter scFv and IgD Fab clones from a parental IgG Fab clone has mixed results. In general, scFv formats still demonstrate good binding characteristics when converted from

IgG Fab but a drop in binding characteristics in IgD Fab. One cannot discount the presentation efficiency for the drop in binding as IgD Fab was shown to have a lower presentation efficiency of about 8 fold in comparison to IgG Fab. The presentation efficiency of scFv is better than Fab due to the smaller size and mono-chain peptide construction thus allowing easier expression [115].

Conversion of Fab to scFv

IgG Fab conversions to scFv have been reported to show a 10 fold decrease in affinity but in some cases also a higher affinity is seen [220, 221]. IgG Fab conversions to scFv for both antigens showed slight reduction in binding (less than 10% in OD intensities). The two daughter IgD Fab clones from IgG 1A and IgG 5D showed different binding characteristics after conversion. IgG 1A had a huge drop in OD readings and IgG 5D only showed a slight drop. The huge drop in signal does not necessary mean a huge loss of binding but could be due to the presentation efficiency of IgD on phage. As determined earlier, the presentation efficiency of IgD Fab on phages is much lower than IgG Fab. Lower presentation efficiency would directly result in a lower OD reading for IgD Fab. Conversion of IgD Fab parental clones showed a decrease in binding nature. As the selection was not very good for IgD Fab libraries, only one IgD Fab clone was used for format switching. The IgD clone when converted to IgG Fab only exhibited a small loss in OD readings but a bigger drop in OD when converted to scFv. This could be possible as IgD Fab and IgG Fab are rather similar in structure [222]. The higher loss in binding for scFv formats can be attributed to the distortion of the binding pocket generated by the linker as scFv fragments generally have a higher presentation efficiencies than Fab constructs.

Antibody format for library generation

The main considerations for the ideal format to be used for antibody library generation would require flexibility in format conversions and good presentation efficiency. In terms of presentation efficiency, IgD Fab format was poorer than IgG Fab. The presentation efficiency of scFv formats is much better than Fab formats in general hence giving scFv an advantage during in vitro selections. IgD Fab conversions to scFv and IgG Fab exhibited drops in binding characteristics. This is to be expected during format switching. But the loss of binding characteristics when converted to Fab format from scFv is much higher than that for IgG Fab to scFv. The drop of binding from IgD Fab to IgG Fab is much higher than conversions in reverse.

Overall, IgG Fab format appears to be the ideal choice of format for antibody library generation. The versatility of the IgG Fab in format conversions in comparison to scFv and IgD is the best with minimal loss of binding characteristics when converted to scFv and IgD in comparison to the reverse conversion orders.

It was observed from the performance of the parental and daughter antibodies that the selected monoclonals perform best in the format they were selected in originally. Any form of conversion will undoubtedly lower the binding characteristics either in terms of affinity or avidity which can be detrimental for downstream applications. A compromise between presentation efficiency and format conversion compatibility would mean IgG Fab is the most ideal format to be used for library generation.

6.8 V-gene primer design, analysis and repertoire generation from B-cells

To obtain V-gene repertoires from a pool of B-cells, an optimized protocol to generate a IgG Fab antibody library would be required. A series of protocols ranging from V-gene primer design, cDNA synthesis and V-gene amplification was investigated.

Bioinformatic analysis of V-gene primers

Most human V-gene specific primers applied to date are based on the germ-line sequences present in VBASE, a manually compiled database of all known human immunoglobulin V-genes at the time, which was developed more than a decade ago at the MRC Centre for Protein Engineering in Cambridge UK [223]. In 1998, Sblattero and Bradbury compiled a minimal set of primers for V-gene amplification [171] which was used as a basis for the study. Here, all primers were analysed using the collection of available V-gene sequences from the VBASE2 [173]. This database is regularly updated, it incorporates sequence information from genome sequencing projects included in EMBL-Bank/GenBank/DDBJ [224] and Ensembl [225] as well as additionally integrates the information of existing immunoglobulin sequence databases such as VBASE, the last version of publicly available Kabat database [226] or the IMGT/LIGM database [227, 228]. Detailed primer analysis using all 576 available entries from VBASE2 suggests, that by designing primers using reference sequences (family assigned genes) the coverage for all heavy and lambda V-genes excluding pseudogenes and orphans is in the region of 99 % and 97 %, respectively. The lower coverage of the kappa V-genes at 92 % is due to gene sequences with insertions or deletions compared to the primer sequence, which

resulted in too many mismatches and consequently did not meet the stringent criteria set for the analysis parameters. For this reason, new primers were suggested: two primers for nine light kappa chain genes, a primer for three light lambda chain genes and a primer for the single non-covered heavy chain functional gene. They were designed in order to increase the total coverage up to 97 %, but this is also an opportunity to check if the additionally covered genes are used in the V(D)J recombination, since only genomic references are available up to date. At present, no such V-genes could be analyzed as the primers used do not amplify those V-genes. The introduction of the new primers to the existing primer set would greatly improve the coverage. In essence, the primer set used in this protocol does give a diverse coverage for the respective families based on the current gene information available. Thus the primer set is able to cover the entire antibody repertoire with a confident coverage of V-genes.

Influence of different enzymes on cDNA synthesis and V-gene amplification

cDNA synthesis from total RNA is a decisive measure in obtaining cDNA material for gene expression analysis. The decision on which priming method used is critical as it will determine the outcome. The reverse transcription (RT) should reflect the true messenger RNA (mRNA) population for the transcript of interest. Conventional protocols require the synthesis of the first strand cDNA using MMLV reverse transcriptase that lacks RNase H activity [229]. In this study, SuperScript® II and totalscript-OLS® reverse transcriptases were evaluated for both, V-gene -specific and total cDNA synthesis. A previous comparison of the RTases showed that although conversion of RNA to cDNA of abundant transcript is equally efficient, the same did not apply for low abundance transcripts. SuperScript II has been shown to be more efficient with low transcript genes [230]. This is fully in concordance with our findings. IgD-specific transcripts are low abundant in RNA samples of PBMCs and only weak signals are obtained from the totalscript-OLS total cDNA as template compared to SuperScript II converted RNA. However, the addition of ET SSB to the PCR reaction mixture greatly enhances the PCR efficiency of totalscript-OLS cDNA. A fine balance of the amount of RNA used for RT reaction is important. When high amounts of RNA are used for RT, RTase could cause an overamplification of certain transcripts at certain PCR conditions, leading to misrepresentation of individual transcript populations [231]. A sensible ratio of RTase to RNA template should be preserved because a high ratio of RTase to RNA template could lead to polymerase inhibition [232]. We used 400 ng total RNA for every RT reaction. This is well within the recommended amount of RNA for use, and indeed, we saw neither signs of PCR inhibition nor false amplification. While both RTases tested, SuperScript II and totalscript-OLS, gave comparable results when used

with gene-specific primers, SuperScript II had however an advantage when used with generic oligo(dT) priming in having a consistently higher yield. For the synthesis of cDNA for V-gene amplification, hence, SuperScript II seems the ideal choice with ample yield and specificity. IgD-specific cDNA was synthesized with an anti-sense primer in the IgD hinge region which is unique over the hinge regions of other isotypes. The specificity of the cDNA synthesized from IgD-specific primer did not show cross reaction with IgG isotype primers. The amplification using IgG CH1 specific primers was not able to show any amplification leaving no doubt to the sensitivity of the primer used for cDNA synthesis. The control samples being the cDNA derived from oligo(dT) and random hexamer priming was able to demonstrate the success of the RT-PCR process. The choice to use either gene-specific primers, oligo(dT), random hexamers or a combination of oligo(dT) and random hexamers for priming is a delicate issue. RT efficiency depends on the priming strategy while the yield is dependent on the RNA concentration [233]. Specific primer priming, rather than random priming, has been reported to show an increase in sensitivity [234]. This is important in low transcript gene cDNA synthesis.

There are several commercially available polymerases in the market today. The choice of a polymerase, which is optimal for the application at hand, is an enigma in most cases. Therefore, a comparison of the performance was carried out for the three different polymerases (REDTaq, BIO-X-ACT and Phusion) in respect to V-gene amplification efficiency, which is the most important parameter as it determines the PCR product yield. Several other factors such as target length and sequence, primer design, buffer conditions, sample impurities, cycling conditions and polymerases can influence the amplification efficiency [235,236]. The recommended buffers were applied to minimize variations for an optimal condition. Additionally, the supplier recommended units of polymerases was also used. In the comparison, all three polymerases could be used for the amplification with partially only poor to moderate yield.

Effects of additives on cDNA synthesis and V-gene amplification

Additives are occasionally used in PCR reactions to increase the amplification efficiency. Hence, a MgCl₂ concentration optimization was carried out by adding three different MgCl₂ concentrations (0.25 mM, 0.5 mM and 1.0 mM) to the manufacturer supplied standard buffers, which already contained between 1.0 mM to 1.5 mM MgCl₂. Some improvement in amplification was visible for REDTaq and BIO-X-ACT in comparison to the respective standard MgCl₂

concentrations, while performance of Phusion remained consistent throughout the range. It is concluded that Phusion gives the best yield using standard conditions without any MgCl₂ optimization and hence may be the enzyme of choice to amplify all antibody isotype and idiotypes using the same PCR program for all primer pairs in a single run.

Further optimisation investigated the use of additives in the PCR reaction mixture. Addition of DMSO to reduce DNA secondary structures led to no improvement and therefore the influence of a thermostable single strand binding protein on the PCR reaction was assessed. SSB proteins destabilize DNA-complexes *in vivo* to allow higher accessibility to single stranded DNA. It enhances the fidelity of DNA synthesis, processivity of polymerases and promotes polymerase binding [237]. This allows better primer binding to single stranded DNA and augment PCR amplification. With both, gene-specific and total cDNA amplification in the presence of ET SSB, a marked increase in yield for both single and pooled primer amplifications was seen. Also, comparable efficiency between both single primer and primer pool amplification was visible with ET SSB. The availability of ET SSB would allow better functionality of multiplex PCR platforms and increased specificity as well as efficiency of conventional PCR. Without ET SSB, total cDNA derived from SuperScript II showed a major advantage over Totalscript-OLS. Total cDNA synthesis for both SuperScript II and Totalscript-OLS was comparable with the addition of ET SSB. The presence of ET SSB assisted the amplification of total cDNA derived from Totalscript-OLS. The influence of ET SSB was evident with the overall higher amplification pattern of both, gene-specific and total cDNA samples, treated with ET SSB to that without.

It is seen that the use of ET SSB is beneficial for V-gene amplifications and should be adopted when amplifying diverse sets of antibody sequences from cDNA in multiplexed and single PCR reactions equally.

Evaluation of optimised protocol

The optimised protocol for amplifying V-genes of various idiotypes to the IgD, IgG, kappa and lambda isotype was assessed. The procedure including all primer combinations worked to satisfaction and produced significant yield of DNA amplification. The variation seen in band intensities for the antibody profiles is due to the V-gene usage preferences in the analyzed individual. This preference is attributed to the high diversity in antibody repertoires. The diversity is credited to the antibody V-gene recombination processes [238] including receptor editing and somatic hypermutation [239]. This is important as antibodies of similar V-gene sequence but of different isotypes have demonstrated differences in specificity and idioype [240]. Therefore, being able to amplify an array of multiple isotypes and idiotypes would be convenient for such studies. Studies on V-gene usage patterns have also been of interest to understand if a certain idioype or isotype is preferred, which could be related to the cause of a certain condition. Hence the protocol could contribute to current V-gene usage studies conducted for various diseases such as systemic lupus erythematosus [241,242], Sjögren disease [243], rheumatoid arthritis [244,245] and even leukaemia [246]. The ability to amplify a significant amount of cDNA of V-genes from various antibody isotype and idioype could also have an advantage in recombinant antibody library generations [247,248,249].

In conclusion, an optimised procedure for human antibody isotype and idioype amplification was set up by revisiting all relevant steps for V-gene amplification in detail. The work included optimisation of amplification primers, assessment of reverse transcriptases for cDNA synthesis as well as evaluation of polymerases and additives for PCR amplification. Furthermore, the method is able to produce Ig-isotype specific cDNAs for idioype amplification. Therefore, it is proposed that by designing specific reverse primers the protocol can be adapted to amplify other isotypes such as IgA, IgE and IgM with their respective idiotypes.

Optimization of V-gene repertoire for library generation

With the optimized protocol, it would be possible to generate V-gene repertoires of a high diversity as templates for antibody library generation. However, the amplification of each V-gene family from a pool of individuals would require numerous rounds of amplification. In this work, the influence of pooling donor cDNAs for amplification was investigated with hopes to minimize the number of amplification required without sacrificing the diversity of the V-genes. As each individual exhibits different V-gene usage, a set of five individual V-gene profiles was generated for comparisons to a pool of the five donor cDNAs. Equal amounts of cDNA from each individual was pooled and amplified with the established method. The V-gene repertoire from the pooled cDNA corresponds to the individual profiles thus, ensuring no loss of diversity or preferences during amplification. Therefore, a pool of 5 individual cDNA at equal amounts can be used to amplify V-gene repertoires for antibody library generation without any loss in diversity. By applying the established protocol for V-gene amplification and pooling 5 donors together, this could facilitate the generation of a human antibody library with a high coverage of V-genes with less amplification rounds.

7.0 Summary

The main rationale for the titanic flexibility of the immune system to engage various types of entities deemed foreign by the body is down to the huge diversity of the antibody repertoire. In vivo antibody production is a complicated multi-stage process which involves complex DNA editing and mutational processes. The diversity of the human antibody repertoire is fashioned by combinatorial assortment of various DNA segments that encode for antibodies [92]. With the prospective potential of antibodies in therapy and diagnostics, more effective and efficient strategies to increase in vitro generation of human antibodies have been investigated. However, the process to generate human antibodies in vitro is also a complicated and multi-stage process. The quality of antibody libraries is influenced by several factors including antibody formats, display levels, sequence diversity, expression level, tendency to multimerize, compatibility with in vitro screening, affinity maturation and ease of format conversion to other antibody formats or display and selection systems [92].

The aim of the thesis was to investigate different parameters that contribute to the generation of human antibodies by phage display. A new series of pTSL phagemid vectors was constructed to handle library generation of various formats. The phagemid vectors allow bacterial expression of soluble antibody fragments and pIII fused antibody fragments packaged as phage. The phagemid vectors also ease the shuttling of antibody genes from one format to another. The use of ccdB gene cassettes in the cloning sites allows low background during initial library cloning.

To investigate the influence of different antibody scaffolds on display efficiency and antibody panning, a human semi synthetic scFv antibody library was subcloned into IgD and IgG Fab format rather than generating a completely naïve library of Fab formats. This is to allow a controlled comparative environment for better assessment of format performances. Both libraries generated for assessment consist of an estimated 5×10^7 individual clones. Taking advantage of the specificity of protein L to kappa light chain, a presentation efficiency assay was carried out to ascertain the presentation efficiency of both formats on phage surfaces. In terms of both Fab formats evaluated, IgG Fab performed better than IgD Fab with a 8-fold presentation efficiency.

A novel method was designed to monitor the distribution of diversity throughout the panning rounds of a phage display selection. The DiVE (Diversity Visualisation by Endonuclease) assay combines the reannealing kinetics of ds-DNA with the single strand specificity of S1 nuclease. The best conditions for the assay were determined by a series of optimization experiments. The DiVE assay was applied to various rounds of the phage selection rounds and was able to generate a diversity variation pattern for both heavy and light chain. The DiVE assay offers a rapid and cost effective method to monitor diversity of selection rounds from oligonucleotide libraries.

The panning process used was semi-automated to allow high-throughput selection of multiple antigens and libraries simultaneously. From both libraries, specific binding antibody fragments were obtained. The selection process was carried out using two different strategies employing a monovalent and multivalent display. It was observed that in cases where a difficult or low concentration antigen is available, multivalent display for round 1 appears a more advantageous strategy.

Next, the interchange of V-regions between individual formats and the influence on binding characteristics was evaluated. From the format conversion experiments, in between three formats - scFv, IgD Fab and IgG Fab - IgG Fab was the best format as it showed the lowest drop in binding characteristics when converted to either scFv or IgD Fab.

Once the best interchangeable format for antibody library design was determined, the amplification procedure to generate an IgG Fab library from human donor materials was assessed. First, a set of V-gene primers was designed and evaluated. The primer set was analyzed against V-gene sequences from the VBASE2 database. The primer set analyzed showed the primers providing a significant coverage of known V-gene sequences. Based on the specific VBASE2 classification, the coverage of the possible functional V-genes (after excluding the non covered pseudogenes and orphans) is 96.4 %. The coverage of the family-assigned genes is 96.6% and the non-assigned genes is 95.3 %. The light chain family showed the lowest coverage hence the need to increase the coverage by introducing additional primers. Four new primers were designed to increase the total coverage to 98.7 %, whereas the coverage by the family- and non-assigned genes increased to 98.9 % and 97.7 %, respectively. This would assist in allowing maximum coverage of all possible V-genes to permit amplification of the highest possible diversity.

Although primer design is vital in antibody library generations, an optimized protocol for amplification is also imperative as both go hand in hand. Other than V-gene specific primer design, key parameters affecting the amplification of full antibody repertoires include cDNA synthesis from total RNA extracts of peripheral blood mononuclear cells and ultimately the polymerase chain reaction (PCR).

Two different sources of RTase were analyzed for cDNA preparation. SuperScript II RTase performed better than the competitor in terms of yield for both gene specific and generic oligo(dT) priming. Several commercially available polymerases (REDTaq, BIO-X-ACT and Phusion) were analyzed in respect to V-gene amplification. Phusion performed most consistent with the entire range of V-gene primers used. The addition of additives such as DMSO and MgCl₂ did not affect the outcome. To further improve the amplification, the addition of a single-strand binding protein (ET SSB) was evaluated. It was found that ET SSB is beneficial for V-gene amplifications and should be adopted when amplifying diverse sets of antibody sequences from cDNA in multiplexed and single PCR reactions equally. The influence of pooling cDNA templates from donors were investigated to reduce the number of amplifications required for a naïve human antibody library generation. A pool of five donor cDNA was able to show a similar profile when amplified individually.

From this work, it can be concluded that by using the newly evaluated set of V-gene primers ample coverage of antibody genes can be accomplished. Together with the optimized protocol, it provides the user a rapid, specific and efficient method to generate preparatory materials for library generation. The pTSL phagemid vector series constructed supplements the production of antibody libraries for phage display with the possibility of switching in between formats with ease. A standard protocol for library cloning was also established during the generation of the semi-synthetic library which can accommodate the actual naïve or immunize library generation. The ideal format to be used for antibody library generation would be IgG Fab.

This work offers the possibility to support future studies in V-gene usage studies en route to understanding the mechanisms of immunological based diseases such as autoimmunity. The higher coverage and optimized protocol would greatly assist in the amplification of V-genes for such studies. While in this study, all integral steps for the optimal generation of human antibody libraries such as V-gene amplification, vector design and antibody format presentation were assessed, multiple downstream processes can be considered for optimization. With new antibody formats being introduced, evaluations of other formats such as the scFab [73] could also be considered. As recombinant antibody production depends on the ability to express soluble antibody fragments, efficient expression of antibody formats can be further evaluated. This could include the introduction of molecular chaperons and varying expression conditions to increase recombinant production of antibodies in *E. coli*. Alternatively, other than bacterial systems, primarily mammalian expression systems can also be considered for expression. The design of an optimised full Ig vector for cell culture can also be assessed. All antibodies based on the above described protocol would have the potential for further downstream applications with the possibility of format switching without loss in binding characteristics.

8.0 Zusammenfassung

Die gigantische Flexibilität des Immunsystems, die es in die Lage versetzt, verschiedenste Typen von Entitäten angreifen zu können, die als fremd erkannt werden, liegt in der enormen Diversität des Antikörper-Repertoires begründet. Die Antikörperbildung in vivo ist ein komplizierter, mehrstufiger Vorgang, der komplexes DNA-Editing und Mutationsprozesse umfasst. Die Diversität des menschlichen Antikörper-Inventars wird durch kombinatorische Auswahl verschiedener DNA-Segmente, die für Antikörper codieren, bewerkstelligt [92]. In Hinblick auf das prospektive Potenzial von Antikörpern in Therapie und Diagnostik wurden effektivere und effizientere Strategien untersucht, die zur Verbesserung der in vitro-Erzeugung menschlicher Antikörper führen sollen. Allerdings erweist sich auch der in vitro-Herstellungsprozess humaner Antikörper als komplizierter und mehrstufiger solcher. Die Qualität von Antikörper-Bibliotheken wird von verschiedenen Faktoren beeinflusst, darunter Antikörperformate, "display level", Sequenzdiversität, Expressionsstärke, Tendenz zur Multimerbildung, der Kompatibilität mit in vitro Screening-Verfahren, der Affinitätsreifung und der Leichtigkeit, mit der Formatkonversionen in andere Antikörperformate oder Display- und Selektionssysteme [92] möglich sind.

Das Ziel der vorliegenden Arbeit war, verschiedene Parameter zu untersuchen, die Einfluss auf die Erzeugung menschlicher Antikörper durch Phage-Display haben. Es wurde eine Serie neuer pTSL-Phagemidvektoren konstruiert, die der Erzeugung von Bibliotheken verschiedener Formate dienen. Diese Phagemid-Vektoren erlauben sowohl die Expression löslicher Antikörper-Fragmente als auch die von pIII-fusionierten Antikörperfragmenten, die als Phagen verpackt sind. Außerdem erlauben diese Vektoren das "shuttleing" von Antikörpergenen von einem Format in ein anderes. Die Verwendung von CcdB Genkassetten an den Klonierungsstellen ermöglicht einen geringen Hintergrund während der initialen Bibliotheksklonierung.

Um den Einfluss verschiedener Antikörpergerüste auf die Effizienz des Displays und die Antikörpersuche zu untersuchen, wurde eine menschliche halbsynthetische scFv-Antikörperbibliothek in IgD und IgG Fab-Formate subkloniert, anstatt solche Bibliotheken im Fab-Format komplett naiv zu erzeugen. So steht eine kontrollierte, vergleichbare Umgebung für eine bessere Überprüfung der Leistung der einzelnen Formate zur Verfügung. Beide zur Bewertung erzeugten Bibliotheken bestehen aus schätzungsweise etwa 5×10^7 unabhängigen Klonen. Um die Effizienz beider Formate zu testen, wenn sie auf Phagen-Oberflächen präsentiert werden, wurde die spezifische Bindung von Protein L an die kappa Leichte Kette ausgenutzt.

Im Kontext der beiden untersuchten Fab-Formate, zeigte IgG Fab eine bessere Leistung als IgD Fab mit einer 8-fach erhöhten Effizienz der Präsentation.

Es wurde eine neue Methode entwickelt, die es erlaubt, die Verteilung der Diversität über die einzelnen Panning-Runden einer Phage-Display-Selektion zu verfolgen. Der DiVE (Diversity Visualisation by Endonuclease) Assay verbindet Reannealing-Kinetik doppelsträngiger DNA mit der Einzelstrang-Spezifität der S1-Nuklease. Durch eine Reihe von Optimierungsexperimenten wurden die besten Bedingungen für diesen Test ermittelt. Der DiVE-Assay wurde auf verschiedene Phagen-Selektions-Runden angewandt und war in der Lage, sowohl für die Schwere als auch für die Leichte Kette ein Verteilungsmuster der Diversität zu erzeugen. Der DiVE-Assay bietet eine schnelle und kostengünstige Methode, um die Diversität von Selektionsrunden gegen Oligonukleotid-Bibliotheken zu verfolgen.

Der verwendete Panning-Prozess war teilautomatisiert, um die gleichzeitige Selektion verschiedener Antigene und Bibliotheken zu gestatten. Aus beiden Bibliotheken wurden spezifisch bindende Antikörperfragmente isoliert. Der Selektionsprozess wurde mittels zweier verschiedener Wege durchgeführt, einer monovalenten und einer polyvalenten Display-Strategie. Die Beobachtung zeigte, dass in Fällen schwieriger oder nur in geringer Konzentration verfügbarer Antigene, die multivalente Display-Strategie für die erste Runde der vielversprechendere Ansatz zu sein scheint.

Im nächsten Schritt wurde der Austausch von V-Regionen zwischen individuellen Formaten und der Einfluss auf die Bindungscharakteristik untersucht. In Format-Konversionsexperimente mit drei Formaten – scFv, IgD Fab und IgG Fab erwies sich IgG Fab als das geeignetste Format, da es den geringsten Abfall der Bindungscharakteristik aufwies, wenn es anschließend entweder nach scFv oder IgD Fab konvertiert wurde.

Nachdem das beste Austauschformat für das Antikörper Bibliotheksdesign ermittelt war, wurde die Amplifikationsprozedur, die zur Erzeugung einer IgG Fab-Bibliothek aus menschlichem Spendermaterial verwendet wurde, untersucht. Zunächst wurde ein Satz an V-Gen-Primern entworfen und bewertet. Der Primersatz wurde gegen V-Gen-Sequenzen aus der VBASE2-Datenbank abgeglichen. Es zeigte sich, dass die Primer aus dem Satz eine signifikante Abdeckung der bekannten V-Gen-Sequenzen gewährleisten. Basierend auf der spezifischen VBASE2-Klassifikation, betrug die Abdeckung der möglicherweise funktionalen V-Gene (nachdem verwaiste Einzelsequenzen und nicht erfasste Pseudogene ausgeschlossen wurden)

94,6%. Die Abdeckung familienweise zugeordneter Gene beträgt 96,6% und nicht zugeordneter Gene 95,3%. Die geringste Abdeckung zeigte sich in der Familie der Leichten Kette, daher musste die Abdeckung hier durch Hinzufügen weiterer Primer verbessert werden. Vier weitere Primer wurden entworfen, welche die Gesamtabdeckung auf 98,7% erhöhten, wobei die Abdeckung bei familienweise zugeordneten Genen auf 98,9% und bei nicht zugeordneten Genen auf 97,7% stieg. Eine möglichst maximale Abdeckung aller möglichen V-Gene ist Voraussetzung für die Amplifikation mit höchstmöglicher Diversität.

Obwohl das Primerdesign ein grundlegender Punkt bei der Erzeugung von Bibliotheken ist, stellt auch ein optimiertes Amplifikationsprotokoll eine unabdingbare Voraussetzung dar, denn beides muss Hand in Hand zusammen wirken, um den Erfolg sicherzustellen. Schlüsselparameter, welche die Amplifikation kompletter Antikörperrepertoires beeinflussen sind neben dem Entwurf der verwendeten Primer, die cDNA-Synthese aus Gesamt-RNA-Extrakten peripherer mononukleären Blutzellen und letztendlich die Polymerase-Kettenreaktion (PCR) selber.

Zwei RTasen zur cDNA-Präparation unterschiedlicher Herkunft wurden untersucht. SuperScript II RTase zeigte bessere Leistung als der Mitbewerber in Hinblick auf die Ausbeute sowohl bei genspezifischem als auch generischem oligo(dT)-Priming. Einige kommerziell verfügbare Polymerasen (REDTaq, BIO-X-ACT und Phusion) wurden in Hinblick auf die V-Gen-Amplifikation analysiert. Dabei zeigt die Phusion die konsistenteste Leistung mit der gesamten Auswahl an verwendeten V-Gen-Primern. Zusätze wie DMSO und MgCl₂ hatten keinen Einfluss auf das Resultat. Um die Amplifikation weiter zu verbessern, wurde der Zusatz von Einzelstrang-Bindungsprotein (ET SSB) untersucht. Es hat sich gezeigt, dass Zugabe von ET SSB hilfreich ist und angepasst werden sollte, wenn diverse Sätze von Antikörper-Sequenzen in Multiplex-PCR-Reaktionen gleichmäßig amplifiziert werden sollen. Der Einfluss der Vereinigung von cDNA-Templates verschiedener Spender wurde untersucht, um die Zahl der zur Erzeugung naiver menschlicher Antikörperbibliotheken notwendigen Amplifikationen zu minimieren. Eine Mischung von fünf Spender cDNAs war in der Lage ein ähnliches Profil zu erzeugen, wie die cDNAs einzeln amplifiziert.

Aus der vorliegenden Arbeit kann der Schluss gezogen werden, dass durch Verwendung eines neu bewerteten Satzes an V-Gen-Primern eine umfassende Abdeckung der Antikörpergene erreicht werden kann. Im Zusammenspiel mit dem optimierten Protokoll stellt sie dem Benutzer eine schnelle, spezifische und effiziente Methode zur Verfügung, um präparative Materialien zur Gewinnung von Bibliotheken zu erzeugen. Die pTSL-Serie von Phagemid-Vektoren unterstützt die Produktion von Antikörperbibliotheken durch die Möglichkeit, relativ einfach zwischen den Formaten zu wechseln. Außerdem wurde während der Erzeugung der teilsynthetischen Bibliothek ein Standard-Protokoll zur Klonierung von Bibliotheken etabliert, welches die jeweilige Generation naiver oder immunisierte Bibliotheken aufnehmen kann. Als ideales Format erwies sich IgG Fab.

Die vorliegende Arbeit ermöglicht die Unterstützung zukünftiger Untersuchungen zur V-Gen-Usage auf dem Wege zu einem tieferen Verständnis der Mechanismen immunologisch basierter Krankheiten wie etwa Autoimmunerkrankungen. Solche Studien dürften vom vorliegenden optimierten Protokoll sowie der erhöhten Abdeckung profitieren. Während in dieser Untersuchung alle integralen Schritte der optimalen Erzeugung menschlicher Antikörperbibliotheken auf dem Prüfstand standen, wie V-Gen-Amplifikation, Vektorentwurf oder Antikörperformate zur Präsentation, könnte auch die Untersuchung verschiedener weiter folgende Schritten in Hinblick auf ihre Optimierung lohnend sein. Mit der Einführung neuer Antikörperformate, könnte auch die Untersuchung anderer Formate, etwa scFab, in Betracht gezogen werden. Da die Produktion rekombinanter Antikörper von der Möglichkeit abhängt, lösliche Fragmente zu exprimieren, kann auch die Effizienz der Expression von Antikörperformaten weiter untersucht werden. In Frage kämen dabei die Einführung molekularer Chaperone und variierender Expressionsbedingungen um die rekombinante Produktion in *E. coli* zu erhöhen. Alternativ könnte auch auf andere, nicht-bakterielle Expressionssysteme, in erster Linie Säugerzelllinien, zurück gegriffen werden. Der Entwurf eines optimierten vollständigen IgG-Vektors für die Zellkultur könnte ebenso untersucht werden. Alle Antikörper, die auf dem oben beschriebenen Protokoll basieren, haben das Potenzial für weitere, nachfolgende Anwendungen, durch die Möglichkeit des Formatwechsels ohne den Verlust ihrer Bindungseigenschaften.

9.0 References

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10.0 List of publications

Poster Presentation

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11.0 Curriculum Vitae

12.0 Appendix

12.1 List of abbreviations

A.

Ab	Antibody
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium
Amp	Ampicillin
ampR	Ampicillin resistance gene (bla)
AVI-Tag	Avidin-Tag

B.

BCR	B-cell receptor
bp	Base pair
BSA	Bovine serum albumin

C.

Cam	Chloramphenicol
cDNA	Complementary DNA
CDR	Complementarity determining region
cfu	Colony-forming unit
CH1	Constant Heavy Region 1
CH2	Constant Heavy Region 2
C κ	Constant Kappa Light
C λ	Constant Lambda Light
CL	Constant Light
CSR	Class switch recombination
C-terminus	Carboxy-terminus

D.

Da	Dalton
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleosid-5'-triphosphate
dsDNA	Double stranded DNA
D segment	Diversity segment
DTT	Dithiothreitol

E.

E. coli	Escherichia coli
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

F.

Fab	Fragment antigen binding
FACS	Fluorescence assisted cell sorting
Fc	Fragment crystalline
Fv	Variable fragment

G.	
Glu	Glucose
H.	
HACA	Human-anti-chimeric response
HAMA	Human-anti-mouse response
HAT	Hypoxanthine, aminopterin and thymidine
HGPRT	Hypoxanthine-guanine-phosphoribosyl transferase
His-Tag	Histidine Affinity Tag
hr	Hour
HRP	Horseradish peroxidase
I.	
Ig	Immunoglobulin
Ig α / A	Immunoglobulin A
Ig δ / D	Immunoglobulin D
Ig ϵ / E	Immunoglobulin E
Ig γ / G	Immunoglobulin G
Ig μ / M	Immunoglobulin M
IG region	Intergenic region
IgV	Immunoglobulin variable domain
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl- β -D-thiogalactoside
J.	
J segment	Joining segment
K.	
Kan	Kanamycin
KanR	Kanamycin resistance gene
Kb	Kilo base pairs
kDa	Kilo Dalton
KD	Dissociation constant
M.	
M	Mole / litre
mAb	monoclonal Antibody
MCS	Multi-cloning site
Min	Minutes
mL	Millilitre
mm	Millimeter
MP	Milk powder
mRNA	Messenger RNA
MPP	Magnetic particle processor
MTP	Microtitre plate
MW	Molecular weight (in Dalton)

N.
nmol Nanomoles per litre
Ni-NTA Nickel-nitrilotriacetic acid
Ni-TED Nickel-tris-carboxymethyl ethylene diamine

O.
OD Optical Density
OD₆₀₀ OD at 600 nm wavelength
o/n Over night

P.
pI-pXI Phage proteins I to XI.
PAGE Polyacrylamide gel-electrophoresis
PBS Phosphate buffered saline
PBS-T Phosphate buffered saline with Tween 20
PBMC Peripheral blood mononuclear cells
PCR Polymerase chain reaction
PEG Polyethylene glycol
pfu Plaque-forming units
PP Polypropylene
PS Packaging signal
PTM 2 % milk powder, 1 % Tween 20 in PBS
PVDF Polyvinylidene difluoride

R.
RNA Ribonucleic acid
rt Room temperature
RT Reverse transcriptase
rpm Revolutions per minute

S.
scFv Single chain variable fragment
scFab Single chain fragment antigen binding
SDS Sodium dodecylsulfate
sec Seconds
SSB Single-strand DNA binding protein
ssDNA Single stranded DNA
SHM Somatic hypermutation

T.
Taq DNA polymerase from *Thermus aquaticus*
TEMED N,N,N',N'-tetramethylethylenediamine
Tris Tris(hydroxymethyl)-aminomethane
tRNA transfer RNA
Tween 20 Polyoxyethylenesorbitan monolaurate

U.	
U	Enzyme units
V.	
V	Volt
V-genes	Variable genes
VH	Variable domain of the immunoglobulin heavy chain
VL	Variable domain of the immunoglobulin light chain
V segment	Variable gene segment

Others

(v/v)	volume/volume
(w/v)	weight/volume
μg	Microgram
μL	Microlitre
μm	Micrometer
°C	Degree Celsius

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