Antibody phage-displayed libraries derived from chicken immunoglobulin genes: a source of highly specific diagnostic antibodies

By

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A dissertation submitted to the Faculty of Veterinary Science of University of Pretoria in partial fulfillment of the requirements for the degree of

Master of Science (Veterinary Science)

DECLARATION

I, Thamsanqa Emmanuel Chiliza, do hereby declare that this dissertation is my own work. It has been submitted to the University of Pretoria for the degree of Master of Science. It has not been submitted before for any degree at any other University.

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Thamsanqa Emmanuel Chiliza

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ABBREVIATIONS

AID	-	activation induced cytidine deaminase
ALDO	-	aldolase
A_x	-	absorbance at x nanometre
ATP	-	adenosine-5'-triphosphate
bp	-	base-pair
˚C	-	degrees Celsius
CI-ELISA	-	competitive inhibition ELISA
cDNA	-	complementary DNA
CDR	-	complementarity determining region
dC	-	2'-deoxy-cytidine
dG	-	2'-deoxy-guanosine
DNA	-	deoxyribonucleic acid
dNTP	-	2'-deoxynucleoside-5'-triphosphate
dU	-	2'-deoxy-uracil
e.g	-	for example
et al.	-	et alii (and others)
etc	-	continuing in the same way
ELISA	-	enzyme linked immunosorbent assay
Ff	-	filamentous phage
Fig.	-	figure
FW	-	frame-work
G	-	glycine

HAT	-	hypoxanthine-, aminopterin- and thymidine
h	-	hour
HRPII	-	histidine-rich protein 2
i.e.	-	in other words
Ig	-	immunoglobulin
IgY	-	immunoglobulin class Y
kDa	-	kilodalton
kg	-	kilogram
LDH	-	lactate dehydrogenase
mAb	-	monoclonal antibody
MCFV	-	malignant catarrhal fever virus
mg	-	miligram
min	-	minute
ml	-	milliliter
MP	-	milk powder
mRNA	-	messenger ribonucleic acid
ng	-	nanogram
OD_x	-	optical density at x nm
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
RBC	-	red blood cells
RDT	-	rapid diagnostic tests
RSS	-	recombination signal sequence

RT	-	room temperature
scFv	-	single-chain variable fragment
SDS-PAGE	-	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
s	-	seconds
S	-	serine
ssDNA	-	single-stranded DNA
µl	-	microlitre
µg	-	microgram
VAT	-	variable antigen
V_H	-	variable heavy
V_L	-	variable light
vol	-	volume
VSG	-	variant surface glycoprotein
WHO	-	world health organisation
wt	-	weight
xg	-	gravity

ABSTRACT

Antibody phage-displayed libraries derived from chicken immunoglobulin genes: a source of highly specific diagnostic antibodies

by

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Degree : Master of Science

In meeting the high demand for monoclonal antibodies, the chicken immunoglobulin system was exploited to generate recombinant antibodies against multiple target antigens. Following simultaneous immunisation of two chickens with a mixture of *Plasmodium falciparum* recombinant lactate dehydrogenase (LDH), histidine rich protein II (HRPII) and aldolase (ALDO), recombinant trypanosome variable surface glycoprotein (VSG) and malignant catarrhal fever virus (MCFV) each chicken produced egg yolk antibodies (IgY) against four of the five antigens.

Using phage display technology, two single-chain variable fragment (scFv) antibody libraries, one with the immunoglobulin V_H and V_L chain regions joined by a single amino acid (G) and the other with a 15 amino acid flexible linker [$(G_4S)_3$] were constructed using pooled splenic RNA. The single amino acid-linked scFv repertoire was evaluated as a source of highly specific diagnostic antibodies by panning against each of the five different antigens. After two rounds of panning, polyclonal phage ELISA showed the presence of antigen-specific phage antibodies against three (LDH, HRPII and VSG) of the five antigens. Five different anti-LDH and six different anti-HRPII scFvs were identified by sequence analysis. Evidence of high levels of antigen-driven gene conversion events was found in the framework and complementary determining regions and the V_L chain pseudogene donors were identified. Stability of the selected scFvs was determined by incubation at different times and at different temperatures. The specificity and potential use of an LDH-specific scFv as a diagnostic reagent was shown in sandwich and competitive inhibition ELISAs.

CHAPTER 1: LITERATURE REVIEW

1.1 Antibody technology

An antibody directed against a specific epitope (i.e. antigenic determinant) and produced by a single clone of B cells is called a monoclonal antibody (mAb). Monoclonal antibodies are widely used in the diagnosis of disease, biomedical research, identifying pathogens and locating potentially protective antigens for vaccine development. Since monoclonal antibodies usually recognise a single epitope with high specificity, they are particularly powerful diagnostic tools. There are two methods which are generally used to generate mAbs: (1) traditional hybridoma technology (Köhler and Milstein, 1975) and (2) antibody phage display technology (McCafferty *et al.*, 1990).

1.1.1 Hybridoma technology

A process known as hybridoma technology has been widely used to generate mAbs since its introduction by Köhler and Milstein in 1975. This method is laborious, often inefficient and is usually used to generate murine antibodies. This means that when used therapeutically they are likely to be immunogenic in humans. Another limitation is the number of clones that can be accessed from a single host animal. The essentials of the method are that splenic B cells from an immunised mouse are fused with suitable myeloma (cancer) cells to form hybridomas which secrete antibodies of a single specificity. The hybridomas are cultured and selected for in hypoxanthine-, aminopterin- and thymidine-containing (HAT) medium. Unfused spleen and myeloma cells die and only the fused myeloma-spleen cell hybrids (hybridomas) survive (Köhler and Milstein, 1975). Hybridomas expressing mAbs are then distributed to 96 well plates for expansion and the supernatant fluid produced is screened by ELISA to identify antigen-specific

clones. The positive hybridomas are then re-screened in a second test. The clones from the second test may in turn be subjected to tertiary screening. Antigen-specific mAbs are then purified from a clone of hybridoma cells which produces a single specific antibody population. Appropriately characterised mAbs can be used in diagnostic assays or other applications. Fig. 1.1 shows the essential steps in hybridoma production.

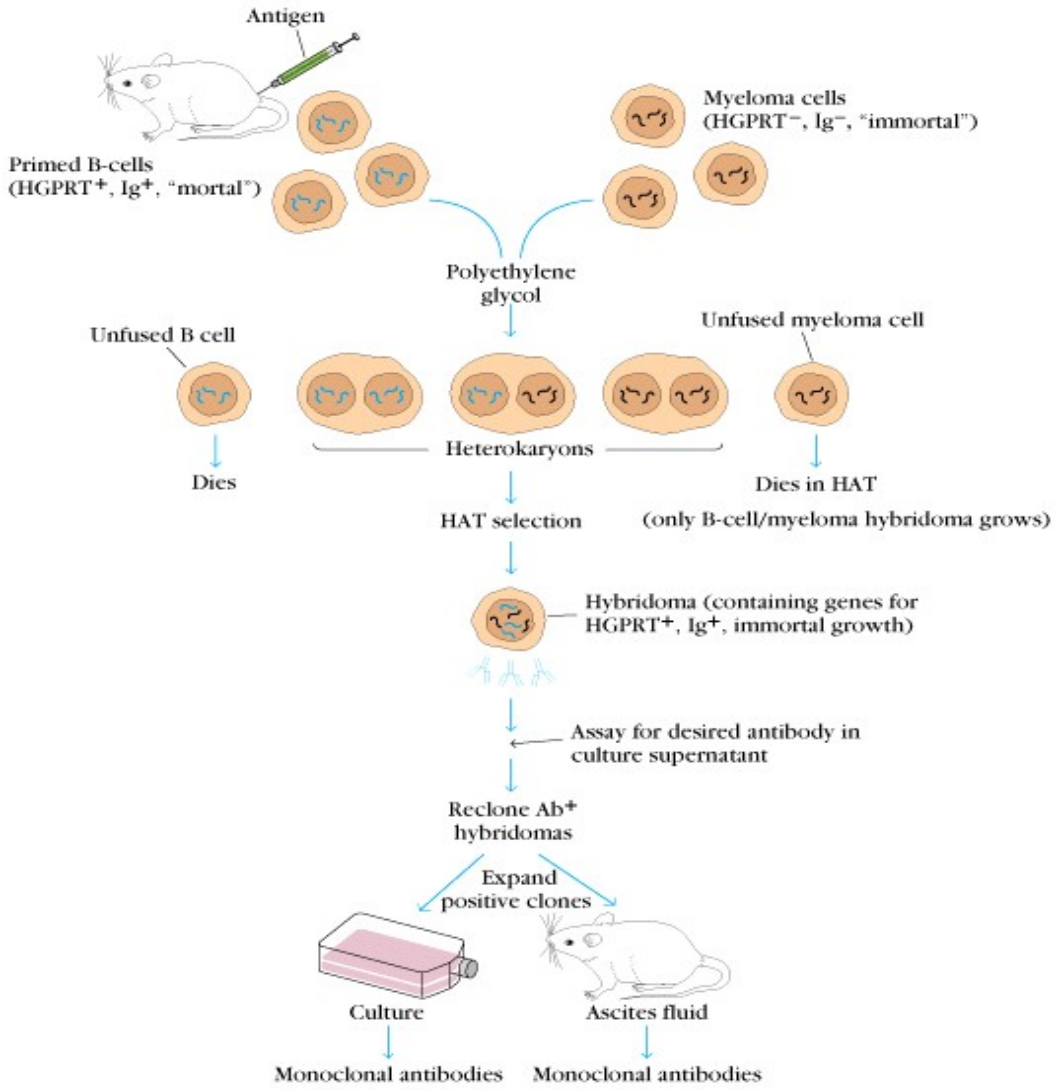

Figure 1.1: Principle steps in the production of a hybridoma (from http://home.inje.ac.kr /~lecture /immunobiotech/ch9/ antibodyeng.htm).

1.1.2 Phage display technology

The use of filamentous bacteriophages as peptide-displaying vectors was first introduced by George Smith in 1985 (Smith, 1985). This method physically links phenotype and genotype thereby allowing both selection and amplification of binding peptides. It was initially described as a method for mapping epitopes, but after the successful display of antibody fragments by McCafferty *et al.* (1990) interest in phage display increased tremendously since this technology has the potential to replace hybridomas in some cases.

The Ff filamentous phages are non-lytic, single stranded DNA (ssDNA) phages infecting susceptible F+ (male) *Escherichia coli* strains via their minor coat protein (pIII) which is located at the tip of the phage particle. This protein binds to the bacterial F+ sex pilus**.** The *pIII* gene of a typical filamentous phage encodes a polypeptide of 406 amino acid residues which is responsible for both infection of the host and normal morphogenesis of the phage particle (Smith, 1985). In phage display, exogenous peptide sequences can be cloned in one of several capsid proteins, namely the abovementioned pIII (predominantly), pIV, pVIII or pIX (Hoogenboom *et al.*, 1998; Gao *et al.*, 1999; 2002). The foreign peptide is expressed on the phage surface as a fusion protein and hence can specifically interact with cognate molecules (Smith, 1985; McCafferty *et al.*, 1990; Hoogenboom *et al.*, 1991; 1998). The phage particles expressing the protein or peptide of interest are selected by “panning” on an immobilised target as will be described later (Fig. 1.4).

To construct an antibody phage library, an mRNA is isolated from a secondary lymphoid organ such as the spleen. Genes coding for the variable regions of immunoglobulins are accessed by PCR, linked and introduced into a display vector (McCafferty *et al.*, 1990). The variable heavy (V_H) and variable light (V_L) chain genes containing antigen-binding regions are usually linked via a synthetic flexible linker to construct genes that code for a single-chain variable fragment (scFv) (Fig. 1.2) repertoire which is cloned so as to form a fusion with pIII, the attachment protein (Fig. 1.3).

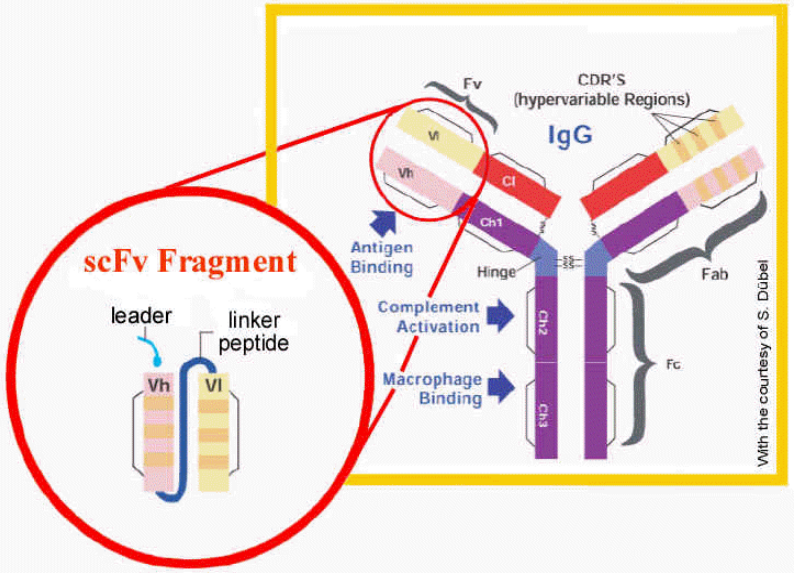

Figure 1.2: Diagram showing the structure of an antibody and single-chain variable fragment (scFv) (from http://www.gene-therapeutics.com/.../ Abb-scFv.gif).

The amplified antibody variable regions are fused to the amino terminus of pIII while a signal sequence downstream of gene *III* directs the antibody fragments to the bacterial periplasm during morphogenesis of phage particles (Hoogenboom *et al.*, 1998). Two types of vectors are generally used to construct phage display libraries, namely phage and phagemid vectors. Phage vectors are based entirely on the Ff filamentous phage genome

while the phagemid genome consists of plasmid DNA and some phage intergenic regions (O'Connell *et al*., 2002). In a phage vector the expression of the antibody-coat protein fusion is under the control of a natural promoter and a strong transcriptional terminator (Hoogenboom *et al*., 1991). In the phagemid vector on the other hand, the *lac* Z promoter drives the antibody-pIII expression (Barbas III *et al*., 1991; Hoogenboom *et al*., 1991). For the successful display of antibody fragments by phagemids, a leader sequence for secretion, a weaker promoter, a selection marker and both bacterial and viral replication origins are all required (Hoogenboom *et al*., 1998).

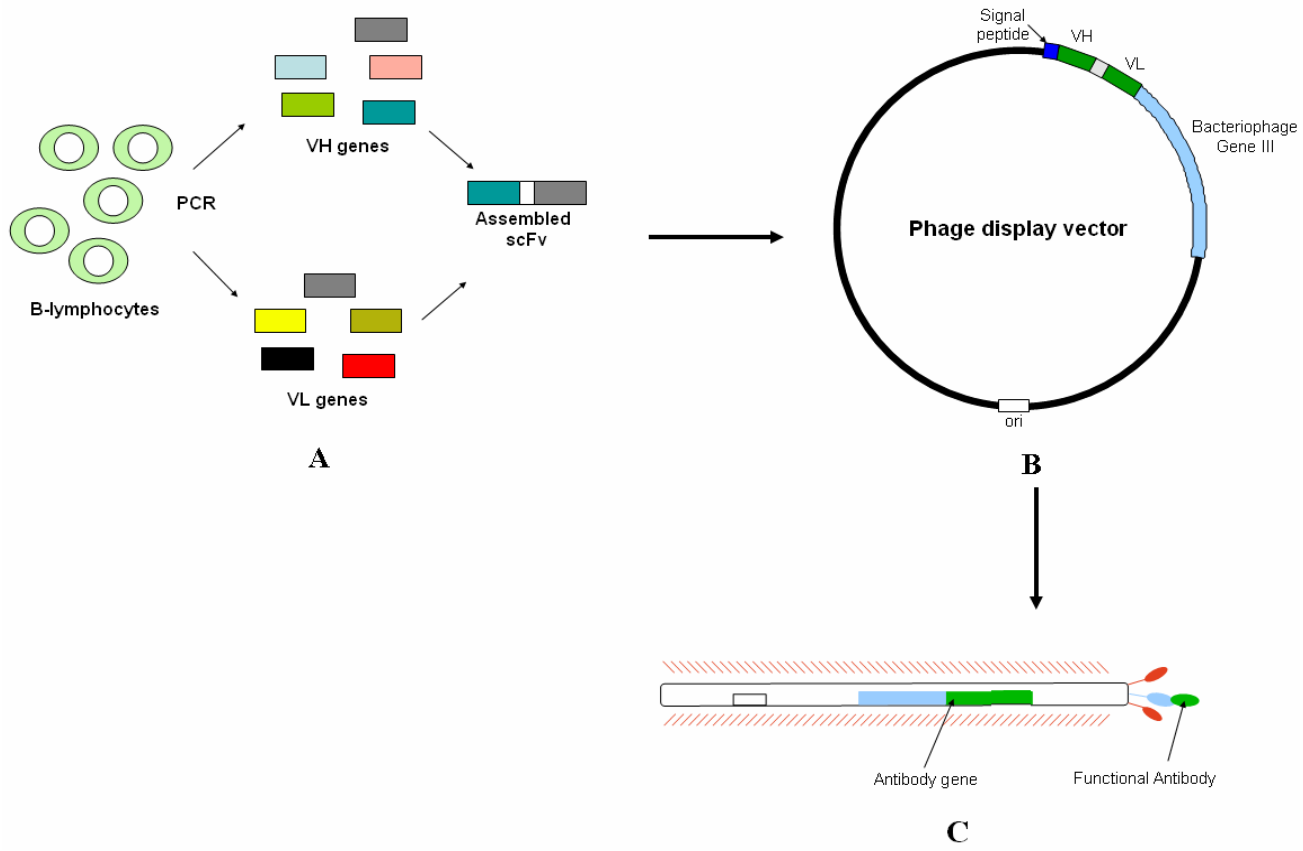

Figure 1.3: Diagram showing the steps involved in constructing an antibody phage display library: (A) amplification and assembly of scFv fragments by PCR, (B) cloning of scFv genes into vector DNA and (C) display of a functional scFv on the phage surface.

Packaging the DNA of a phagemid into a phage particle requires a helper phage to supply all the structural proteins required. The wild-type pIII from the helper competes with the antibody-pIII fusion for display on the phage surface. This means that most of the particles will probably not have antibody-pIII fusions (O'Connell *et al*., 2002). In fact, only about 10% of rescued phage particles are likely to display a single fusion particle on their surface (Hoogenboom *et al*., 1998). The rate of phagemid rescue differs for each member in the phage library due to the different effects exerted on the host *E. coli* by each scFv gene and/or its gene product. High levels of pIII fusion protein expression can inhibit infection with helper phage resulting in a loss of some of the library members due to failure in rescuing them (O'Connell *et al*., 2002). These potential drawbacks have, however, not prevented large antibody libraries from being made. Phagemid vectors are usually preferred over phages for antibody display due to higher transformation efficiencies and because of their monovalent display of the antibody fragment (Hoogenboom *et al*., 1998).

Phage libraries, on the other hand, display more copies of each scFv per particle and can as a consequence yield high levels of selection on antigen as compared to phagemid libraries. Many of the selected antibodies may, however, have a low intrinsic affinity for their antigens. This can be overcome by converting a multivalent phage display repertoire to a monovalent phagemid display library after a single round of selection if required. Monovalent display enables high-affinity antibodies to be more efficiently selected, especially against a less complex antigen while multivalent display enhances selection of rare antibodies (O'Connell *et al*., 2002). Although monovalent display virtually guarantees the selection of high affinity antibodies, multivalent display may be preferred

when a limited number of phage particles per clone are to be selected from a huge antibody library (Hoogenboom *et al*., 1998). Phage libraries have so far not been used as widely as phagemid repertoires for displaying antibody fragments.

Phage-display libraries are screened for scFv fragments that bind to a chosen antigen by a process called “panning” (Barbas III *et al*., 1991). The surface-immobilised target antigen is incubated with the phage antibody library and the unbound phage particles are washed away. Bound particles are then eluted, usually at high or low pH. The eluted phage particles are amplified by infecting *E. coli* TG1 cells. The panning cycle may be repeated, thus enriching the library with antigen-specific clones (Fig. 1.4).

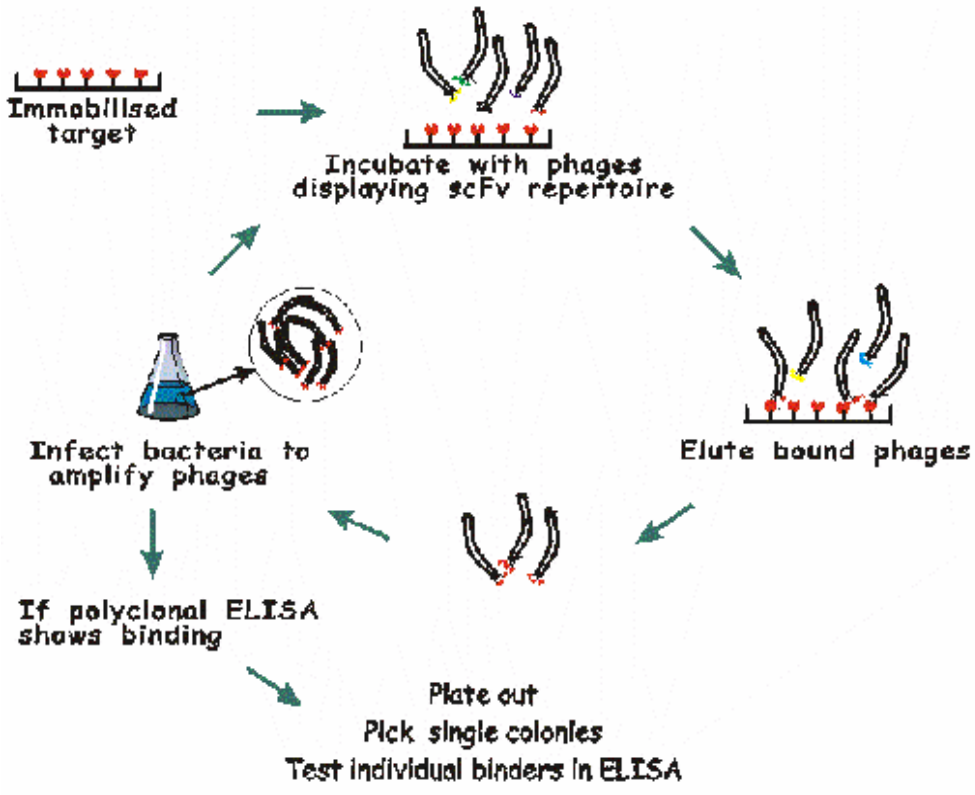

Figure 1.4: Diagram illustrating selection of phage particles expressing an antibody (scFv fragment) of interest by panning on an immobilised target.

1.1.2.1 Polypeptide linker effect

In the design of scFv antibodies, the carboxy terminus of one V-domain is joined to the amino terminus of the other V-domain by a polypeptide linker. Usually the linker consists of a serine (Ser) residue which improves the hydrophobicity of the peptide linker to enable hydrogen bonding and a number of glycine (Gly) residues which gives the linker some flexibility (Argos, 1990). The use of flexible linkers allows the V_H and V_L domains to assemble in the natural Fv orientation (Plückthun and Pack, 1997; Hudson and Kortt, 1999; Todorovska *et al.*, 2001). The linker length between V_H and V_L domains determines the flexibility, valency and size of the scFv. ScFvs are likely to be monomeric when the V_H and V_L domains are joined by a flexible polypeptide linker of 12 or more amino acid residues (15 residues are widely used), providing the minimal antigen binding site. By shortening the linker to less than 12 residues, the V_H and V_L domains cannot fold to return the natural Fv orientation, but instead two or more separate complementary scFv molecules associate to form bi-, tri- or tetrameric complexes. The complexes are referred to as multibodies i.e. multivalent antibodies. By shortening the linker to between zero and two residues, triabodies or tetrabodies can be formed by the association of three or four scFv molecules. A linker of three to ten residues can result in the association of two scFvs to form diabodies (Fig. 1.5). These multivalent antibodies are mono-specific and have higher avidity than the parent scFv (Hudson and Kortt, 1999; Atwell *et al*., 1999).

Multivalent antibodies can also be formed on the phage surface by association of soluble fragments with intact antibody-pIII fusion. These soluble fragments probably result from proteolysis of antibody-pIII fusion proteins in the periplasmic space of *E. coli* during phage particle morphogenesis (Andris-Widhopf *et al*., 2000).

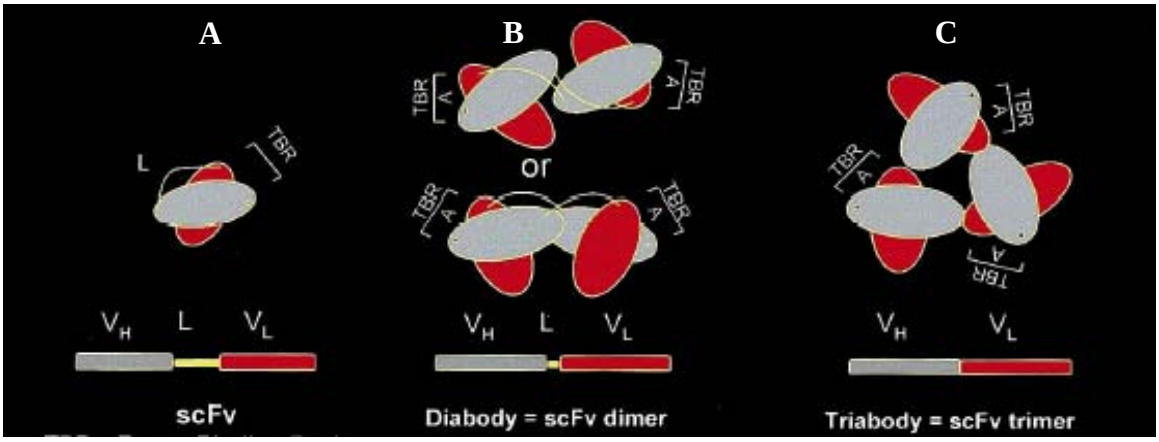

Figure 1.5: ScFv gene constructs and encoded protein products, depending on a linker length (A) scFv monomer, (B) diabody and (C) triabody (from Hudson and Kortt, 1999).

1.1.2.2 Types of antibody phage libraries

There are two types of antibody phage libraries that can be constructed from animals namely: (i) *"Naïve" antibody libraries*: "Naïve" antibody libraries are constructed using mRNA isolated from B cells of a non-immunised animal (Nissim *et al.*, 1994; Davies *et al*., 1995; van Wyngaardt *et al*., 2004). They contain the primary V-gene repertoire of the immune system and as a result can recognise a wide variety of antigens (Hoogenboom *et al.*, 1998). The disadvantages associated with these libraries are the large size required to make for the successful selection of high affinity antibodies and the cost of constructing such repertoires. (ii) *Immune antibody library*: An immune antibody library is constructed from the mRNA of B cells of an immunised animal and therefore does not need to be so large to successfully yield high affinity antibodies. This is because the V-genes have undergone affinity-maturation and as a result, the library is highly bias toward the expression of antigen-specific V-genes. Compared to a "naïve" library, an immune phage antibody repertoire is thus enriched in affinity-matured antibodies specific for those antigens against which an immune response was elicited (Yamanaka *et al.*, 1996;

Andris-Widhopf *et al.*, 2000; Sapats *et al*., 2003; Finlay *et al.*, 2005). The disadvantages of immune libraries include the time required for immunisation, the possibility of non-immunogenicity or toxicity of some antigens, and the fact that for each antigen a new library must be constructed. Antibodies against more than one immunogen can, however, be successfully isolated from the same library (Finlay *et al*., 2005). This finding is the basis of the present study.

For use in sensitive diagnosis, *in vitro* affinity-maturation of recombinant antibodies may sometimes be necessary. This is a two step process: firstly, the introduction of diversity in V-genes using a variety of methods including error-prone PCR and chain shuffling. The second step is the selection and screening of affinity matured antibodies from a new sub-library of variants (Hoogenboom *et al*., 1998). Since affinity maturation has already taken place in the animal, this process is less likely to be necessary in the case of recombinant antibodies from immune sources.

1.2 Immunoglobulin genes

When constructing antibody libraries, and in particular to understand the advantages of the avian immune system, it is important to know how different species generate antibody diversity. In this sub-chapter; the widely used laboratory animal, the mouse is compared to an alternative, namely the chicken (*Gallus domesticus*).

1.2.1 The mouse immunoglobulin gene locus

In the mouse the source of the primary Ig repertoire is a number of variable (V), diversity (D) and joining (J) gene segments (Tonegawa *et al.*, 1983). The Ig heavy (H) chain locus consists of approximately 140 V_H gene segments that can be grouped into 15 families. One-hundred-and-four of these segments are believed to be functional (de Bono *et al.*, 2004). There are also 13 active D_H (diversity) gene segments that can be grouped into four families and four functional J_H (joining) gene segments (reviewed by Schroeder, 2006). The Ig light (L) chain locus is divided into two sub-IgLs; the Igκ and Igλ. The Vκ locus consist of 93 expressed Vκ gene segments that belong to 18 families. There are 5 members in the Jκ locus. One of these is non-functional and there is one constant κ (Cκ) element. The λ locus is made up of three Vλ gene segments and two Cλ segments each associated with a single Jλ gene segment (Lefranc, 2003).

Three types of framework (FW) 1 structure is found in the V_H gene segments. These can be used to group V_H families into clans, each with its own unique FW3 structure (Kirkham *et al.*, 1992). The FW1 of the V-domain links the V to the first C-domain and the FW2s encoded in V-region and FW4s in J-regions of both V_H and V_L chains constitute the hydrophobic core of the united V-domain (Schroeder, 2006). The antigen-

binding domains, complementarity-determining regions (CDRs) 1 and 2 are encoded by V-gene segments and CDR3s are generated during the V(D)J joining. The diversity of CDR3s thus depends on the multiplicity of VDJ combinations (Padlan, 1994; Xu and Davis, 2000) (see Fig. 1.6). The light chain CDR3 consists of V_L and J_L sequences only, thus L chain variability is encoded in the germline (Schroeder, 2006).

1.2.1.1 Mouse B cell development

The IgH chain locus undergoes rearrangement before the IgL locus (Burrows and Cooper 1997; Burrows *et al*., 2002). There are four stages involved in Ig gene assembly: (1) pro-B cell, (2) pre-B cell, (3) immature B cell, and (4) mature B cell stage (Schroeder, 2006). The D_H-J_H joining and in-frame V_H-DJ_H rearrangement define the pro-B cell and pre-B cell stages, respectively. During late pre-B cell stage the L chain rearrangement is triggered by reactivation of the recombination activating genes (RAG) 1 and RAG2. The Vκ-Jκ recombination occurs before the Vλ-Jλ joining. The immature B cell stage is defined by surface expression of IgM which depends on the successful association of an in-frame L chain and an endogenous μ chain. The cells expressing IgM molecules on their surface are selected to undergo splicing of the Cμ-Cδ locus leading to surface co-expression of IgD marking the mature B cell stage (Burrows *et al.*, 2002).

The matured B cells in the bone marrow migrate through the blood into the secondary lymphoid organs (spleen, lymph node and Peyer's patches). In the presence of a cognate antigen in the lymphoid organ, the B cells undergo rapid proliferation in the lymph follicles. In the mouse, there are two types of genetic modification that follow productive V gene rearrangement; namely class-switch recombination and somatic hypermutation.

These two modifications are region-specific recombination and multiple single nucleotide substitutions on rearranged V-gene segments (Harris *et al*., 2002). The activated B cells are further diversified by activation induced cytidine deaminase (AID) -mediated somatic hypermutation of the V-domains and class switching which enables replacement of the constant (C) domain by means of switch sequence non-homologous recombination with a downstream C exon. B cell development takes only three weeks. By day 12 and days 16-17, pre-B cells and B cells expressing surface IgM are present in the foetal liver. This organ thus acts as a primary lymphoid organ for more than 7 days (Harris *et al*., 2002). As will be seen, the way in which the mouse or human immune system generates diversity (Fig. 1.6) has practical implications when attempts are made to access this diversity.

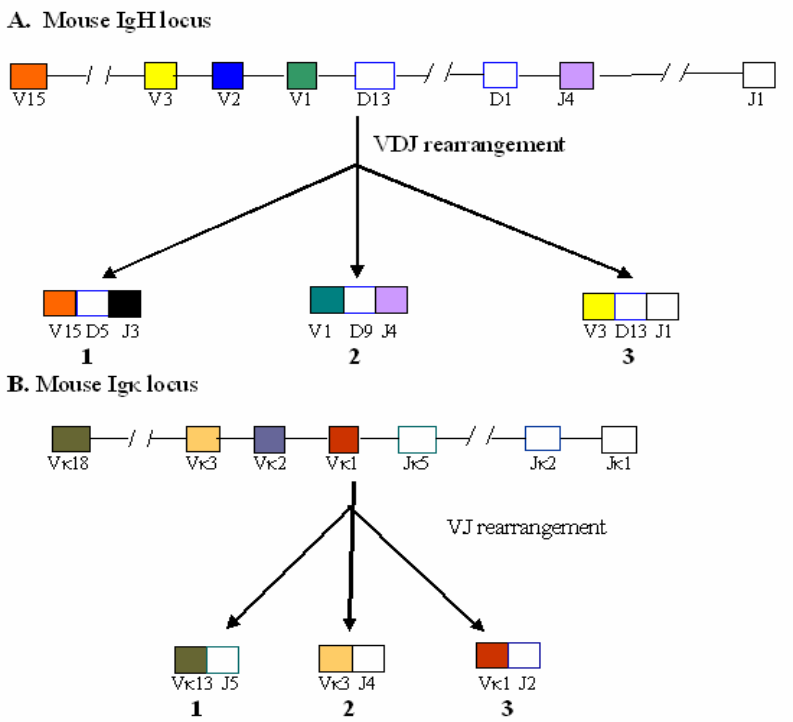

Figure 1.6: Diagram illustrating IgH VDJ (A) and Igκ VJ (B) rearrangement; 1, 2 and 3 are examples of possible V (D) J products for each case in mouse.

1.2.2 The chicken (*Gallus domesticus*) immunoglobulin gene locus

In the chicken, Igs are divided into four classes, IgM, IgG, IgA and IgY (from egg yolk). As with the mouse, IgM is generated during the primary antibody response and expressed on the surface of B lymphocytes. IgG is mainly generated during the secondary antibody response while IgA is normally present in serum and secretions such as bile (reviewed by Ratcliffe, 2006). The egg yolk immunoglobulins (IgY) are transferred in the female bird from the serum to the egg yolk to confer passive immunity to the embryo and neonate (Jukes *et al*., 1934). Three Igs are homologous to mammalian IgM, IgG and IgA and have some similarities in their structure and function. Chickens, however, do not have homologues of mammalian IgD and IgE. IgY may be the ancestor of both IgG and IgE due to its ability to serve the same functions as IgG in the defence against systematic infections. Like IgE, it can mediate anaphylactic reactions. IgY has two antigen-binding sites, binds strongly to multivalent antigens and can neutralise pathogenic viruses (Warr *et al*., 1995).

Unlike the mouse, the chicken IgH chain locus has only one unique functional V_H and one J_H gene segment. A total of 15 D_H regions have been identified. All fifteen D_H segments are functional and have very similar sequences. The D segments are flanked by V_H and J_H segments. The joining of D-J_H and V-D_H is homology-mediated based on a single base pair and 3 base pair overlaps in J_H and V_H, respectively (Reynaud *et al.*, 1989; 1991). The chicken IgL locus (consist of only the Vλ) also has only one unique functional V_L and J_L gene segment (Reynaud *et al*., 1985). Upstream of both functional V_H and V_L gene segments are a number of pseudogenes (ψ) (Reynaud *et al*., 1987; 1989). These pseudogenes (100ψV_H and 25ψV_L) contain the same gene sequences as the functional V_H

and V_L gene respectively. Both ψV_H and ψV_L have either 5' or 3' truncations. This means that both lack the promoter and recombination signal sequences (RSSs) at their 5' and 3' ends. Functional RSSs are a prerequisite for gene rearrangement. They therefore cannot be expressed unless they donate their sequences to rearranged VDJ_H or VJ_L genes (Reynaud *et al*., 1987; 1989). This process is called gene conversion. The chicken V_H and V_L chain diversity is mainly generated by multiple rounds of gene conversion involving the upstream pseudogenes. This mechanism, which is clearly distinct from that employed by mice and humans, generates the diversified avian antibody repertoire (Reynaud *et al*., 1987; McCormack *et al*., 1993).

Nine of 25 ψV_L segments are orientated in an inverted fashion (opposite polarity) to the functional V_L gene, the majority in an alternating orientation (Reynaud *et al*., 1987). Due to homologous recombination among adjacent ψV_L segments, the alternating orientation has probably evolved to prevent pseudogenes from being lost due to homologous recombination. This also applies to the IgH ψV_H genes (McCormack *et al*., 1993). Since the V_H gene locus is "rich" in pseudogenes, this contributes to generating a large heavy chain repertoire (Cohen and Givol, 1983; Reynaud *et al*., 1989). The V_H pseudogenes have D segment sequences at the 3' end. During recombination, the germline D element sequences act as an acceptor exploiting sequence homology to the D region-like sequences found in V_H pseudogenes. Base pair modifications may also occur in the D segment during gene conversion (Reynaud *et al*., 1989; 1991). The addition of base pairs at the V-D_H and D-J_H adds to diversity at the junction yielding an increased variability of CDR3, which is encoded in the V-D-J_H junction (McCormack *et al*., 1991). A single pseudogene can contribute more than once during an antibody response to a certain

antigen. As a result, a single V gene can be converted into different end products (Cohen and Givol, 1983). The germ-line element contribution to the chicken IgH and IgL chain diversification is limited to one V_L, J_L and one V_H, J_H functional gene (Parvari *et al.*, 1988; 1990). As with the V_H chain, gene conversion of rearranged functional V_L gene with non-functional pseudo-V_L genes also creates diversity in the pre-immune repertoire of the chicken L chain with 25 pseudogenes acting as sequence donors (Reynaud *et al.*, 1987). The gene conversion mechanism is shown schematically in Fig. 1.7 and described in more details in the next section (1.2.2.1).

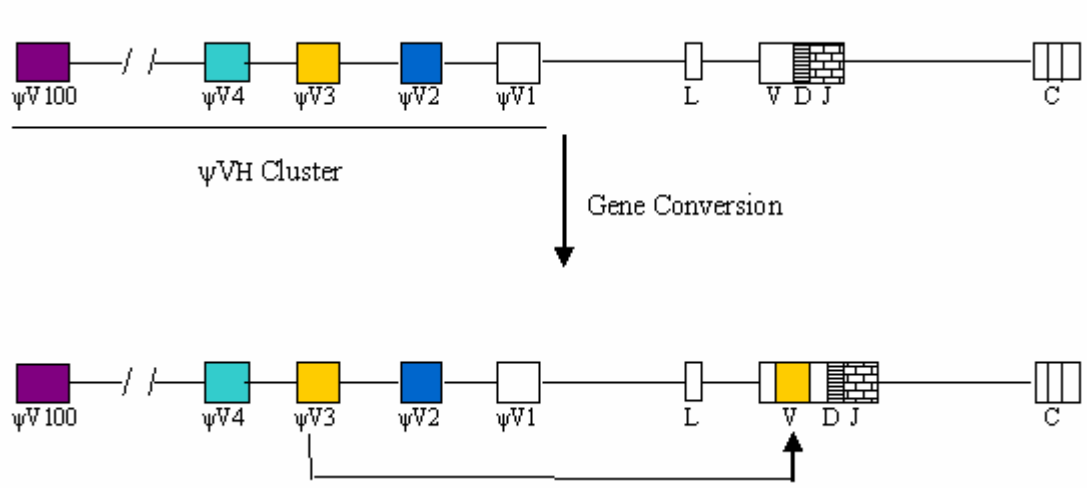

Figure 1.7: The chicken IgH chain locus organisation, the diagram is illustrating the process of Ig diversification by gene conversion. Essentially the same process introduces diversity into the IgL chain locus (adapted from Michael *et al.*, 1998).

1.2.2.1 Chicken B cell development

In birds, the bursa of Fabricius develops during embryonic life. This organ is the site of B cell development. The embryonic bursa is colonised by dividing stem cells at day 8-12. Lymphoid stem cells colonise the bursa by the eighth day of embryogenesis and by day

12 the cells start to express surface immunoglobulin (sIg). Prior to hatching more than 95 percent of the stem cells express sIg (Thompson and Neiman, 1987). The colonisation of the embryonic bursa is followed by rapid B cell expansion in the bursal follicles (Reynaud *et al*., 1985). Gene rearrangement occurs once during early embryonic development and it in turn triggers gene conversion. The productive rearranged cell-surface expressing sIg genes are selected before the onset of gene conversion to undergo clonal expansion. Non-productive rearranged Ig genes are eliminated (Reynaud *et al*., 1987; 1991; Paramithiotis and Ratcliffe, 1993). The Ig gene rearrangements generate sIg receptors with specificity which is mostly determined by the germline (V_H and V_L) encoded genes. The sIg receptor is thought to transduce signals to the bursal cells which then produce receptor specific chemokines that attract the sIg receptor-expressing cells into the bursal follicle (reviewed by Ratcliffe, 2006).

It has been suggested that affinity maturation by gene conversion is antigen driven. The B cells migrate into the follicular cortex after encountering their cognate antigen. In the follicular cortex, rapid cell division induces gene conversion in the presence of locally expressed activation induced cytidine deaminase (AID) which is responsible for the diversification of Ig genes. Therefore gene conversion is AID-dependent (Arakawa *et al*., 2002; Harris *et al*., 2002; Withers *et al*., 2005). The germline V gene and pseudogenes sequence homology promotes genetic exchange during gene conversion (Reynaud *et al*., 1987). During the Ig locus transcription process, a transcription “bubble” is generated which renders the DNA single stranded (ssDNA). AID recognises and acts on ssDNA in the “bubble” by directly deaminating dC to dU yielding the so called initiating lesion. It then nicks the DNA strand. The nicked DNA is homologously inserted into the target

upstream pseudogene sequence. DNA polymerase then accurately replicates the pseudogene sequence and when elongation is completed, the elongated strand re-associates with the functional V gene introducing sequences encoded by the pseudogene (Fig. 1.8). The base pair mismatch is repaired by gene conversion using the replicated strand as the template to repair the dU:dG mismatches in the non-replicated DNA strand (Ratcliffe, 2006).

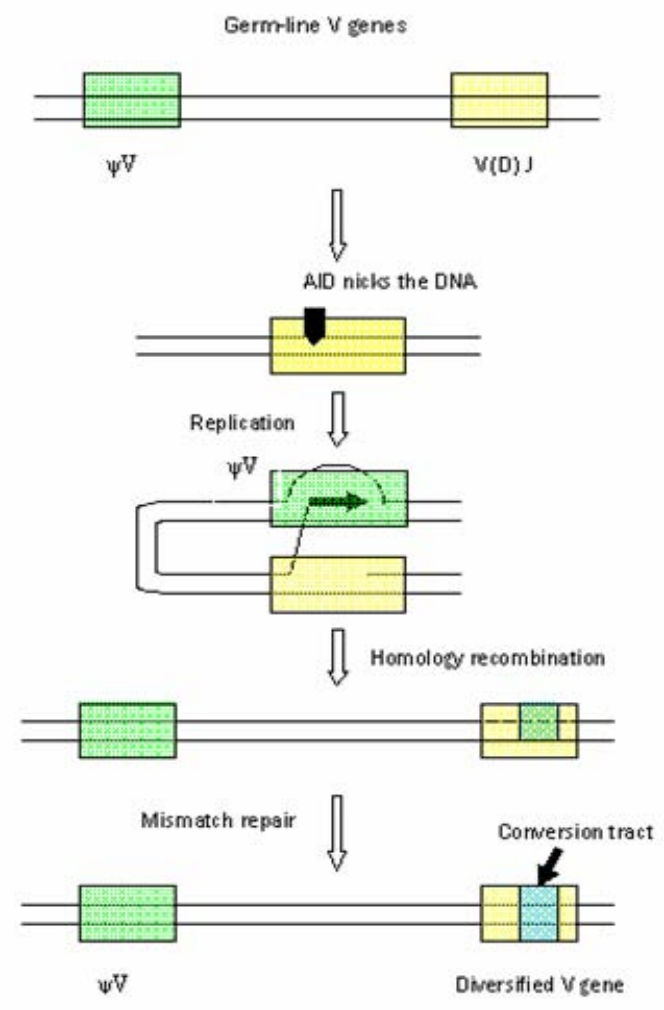

Figure 1.8: Diagram illustrating AID involvement in the diversification of Ig genes by gene conversion (adapted from Arakawa *et al*., 2004).

The diverse B cell repertoire confers specificity for the target antigen, but this repertoire is not restricted only to encountered antigens. The presence of gut-derived materials (antigens, bacterial mitogens or superantigens) and expression of sIg receptor is required for the bursal B cell development after hatching. It has been proposed that these later stages are triggered by individual gut-derived antigen-antibody interactions (Ratcliffe, 2006).

As with mammals, somatic hypermutations also contribute to affinity-maturation of the immune response. Somatic hypermutations or gene conversion events increase with time from pre- to post-hatch. Up to seven of these events may occur (Reynaud *et al*., 1987). Pre-immune repertoire generation in the bursa ends 21 days after hatching and somatic point mutations take-over to further diversify the V genes, resulting in antibodies with matured affinity. High affinity antibodies are predominantly produced during the late immune response (Parvari *et al.*, 1990). The bursa continuously matures after hatching while B cells rapidly proliferate in each bursal follicle and the cells migrate to the secondary lymphoid organs (eg. spleen) for further specific development. When the chicken is between the ages of 8 and 12 weeks the bursa reaches complete maturity which results in involution. Twenty-four months after hatching, only a sclerotic remnant of the original organ is present (Thompson and Neiman, 1987).

1.3 Disease targets

For the development of mAb-based diagnostic immunoassays, it is crucial to identify suitable target antigens. For field use, tests based on immunochromatographic principles have the potential to replace existing techniques. The present study focuses on recombinant antibodies that can potentially be used in such immunoassays. Several target antigens which had been previously identified were of interest to this study.

1.3.1 Malaria

As early as 1880, the causative agent of malaria was identified as a single-celled parasite called *Plasmodium*. There are four *Plasmodium* species which cause human malaria. These are *P. vivax*, *P. ovale*, *P. malaria*, and *P. falciparum*. The parasite is fatal in less than 1% of cases. The parasites are transmitted to human hosts from the infected female *Anopheles* mosquito during blood meals. The disease occurs worldwide, mostly in tropical and sub-tropical regions. More than 100 countries around the world are affected with sub-Saharan Africa being the most affected region (see Fig. 1.8). At least one million people die from malaria every year and most of these deaths occur in sub-Saharan Africa (World Health Organisation [WHO]).

The human malaria parasite life cycle consists of two separate cycles, the asexual and sexual. The asexual life cycle takes place in the human host's erythrocytes (red blood cells) while the sexual life cycle occurs in the mosquito gut. Within 48 hours of red blood cells (RBC) being infected, the parasite develops into trophozoites and divides asexually producing schizonts. The accumulation of schizonts forces the RBC to burst and release daughter merozoites which reinitiate the cycle by invading more RBC. Death results from

anaemia and obstruction of blood flow by clogging of capillaries in vital organs such as the brain (cerebral malaria) (WHO).

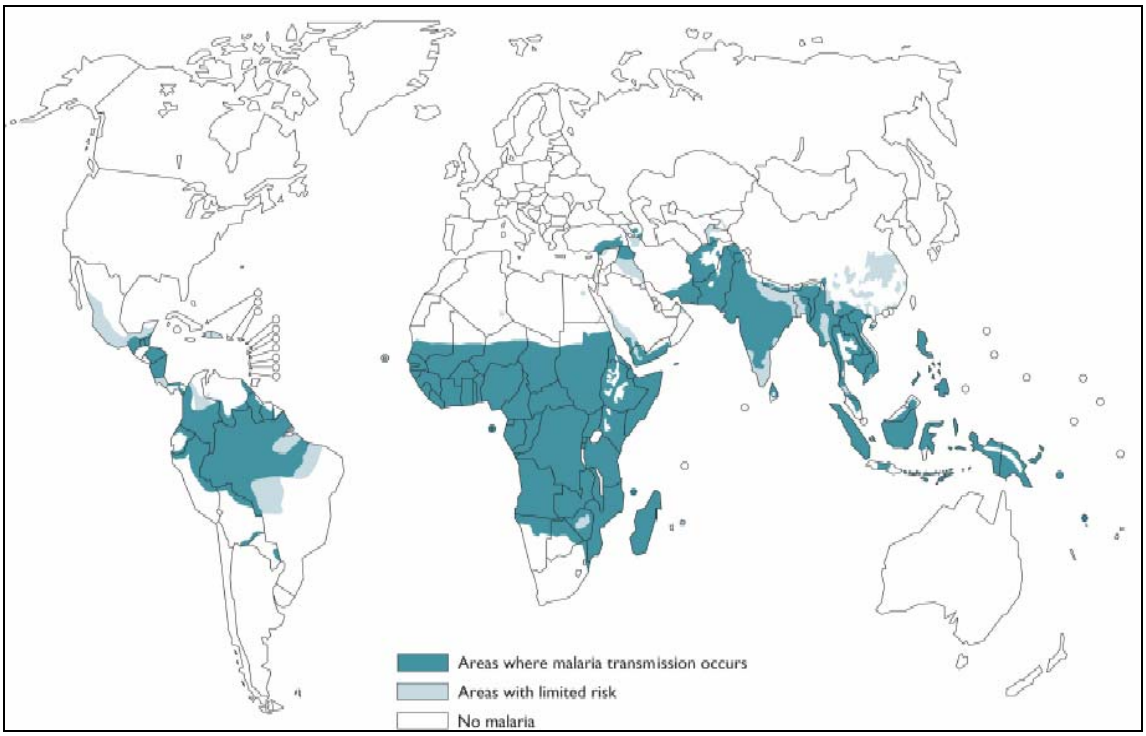

Figure 1.9: The world map showing the distribution of malaria (from WHO).

The most widely used method for malaria diagnosis is microscopic blood-smear examination. There are limitations associated with this method including the time (up to 60 minutes) required to prepare the blood film. A skilled microscopist is required to interpret results, particularly at low levels of parasitaemia. This method may be impractical when large numbers of blood samples need to be accurately examined, more especially in developing countries where expertise may be scarce (Snounou *et al*., 1993; Srinivasan *et al*., 2000; Moody, 2002). In an attempt to overcome challenges in malaria diagnosis Snounou and collegues (1993) developed a PCR based on species-specific

sequences of the small subunit ribosomal RNA genes (ssrRNA) to detect all four human malaria parasites. Although PCR can be more sensitive than routine microscopic analysis (Snounou *et al.*, 1993), it does not meet the recommended criteria of a rapid diagnostic method since it usually takes more than an hour to obtain results (Moody, 2002). The World Health Organisation has identified a need for easy to perform, rapid, sensitive and cost-effective malaria diagnostic tests. Antibody-based malaria rapid diagnostic tests (RDT) appear to meet the WHO recommendations (Moody, 2002). These test kits are based on the detection of parasite-specific antigens using mAbs immunochromatographic capture procedures.

Immunochromatographic technology is based on two phases; the mobile and stationary phase. In the mobile phase the antigen-specific mAb captures the target antigen in the patient's blood and migrates across the nitrocellulose membrane surface. There are usually three different mAbs in the stationary phase. The first mAb to be encountered by the migrating antigen-antibody complex is usually specific to *P. falciparum*. The second mAb is pan-specific and recognises a highly conserved antigen among the four malaria parasites (*P. vivax, P. ovale, P. malarae* and *P. falciparum*). The third mAb serves as a control recognising the migrating mAb whether antigen bound or non-bound (see Fig. 1.9) (Moody, 2002).

Several malaria parasite antigens have been identified as potential candidates for the development of either malaria RDT and/or anti-malarial drugs. These are the histidine-rich protein II (HRPII), lactate dehydrogenase (pLDH), and *Plasmodium* aldolase (ALDO). The suitability of these antigens for malaria diagnosis is based on the fact that

they are produced by the malaria parasites during their asexual life cycle in the human and are unique to the parasite (Leech *et al*., 1984; Howard *et al*., 1986; Rock *et al*., 1987; Knapp *et al*., 1990; Döbeli *et al*., 1990; Meier *et al*., 1992).

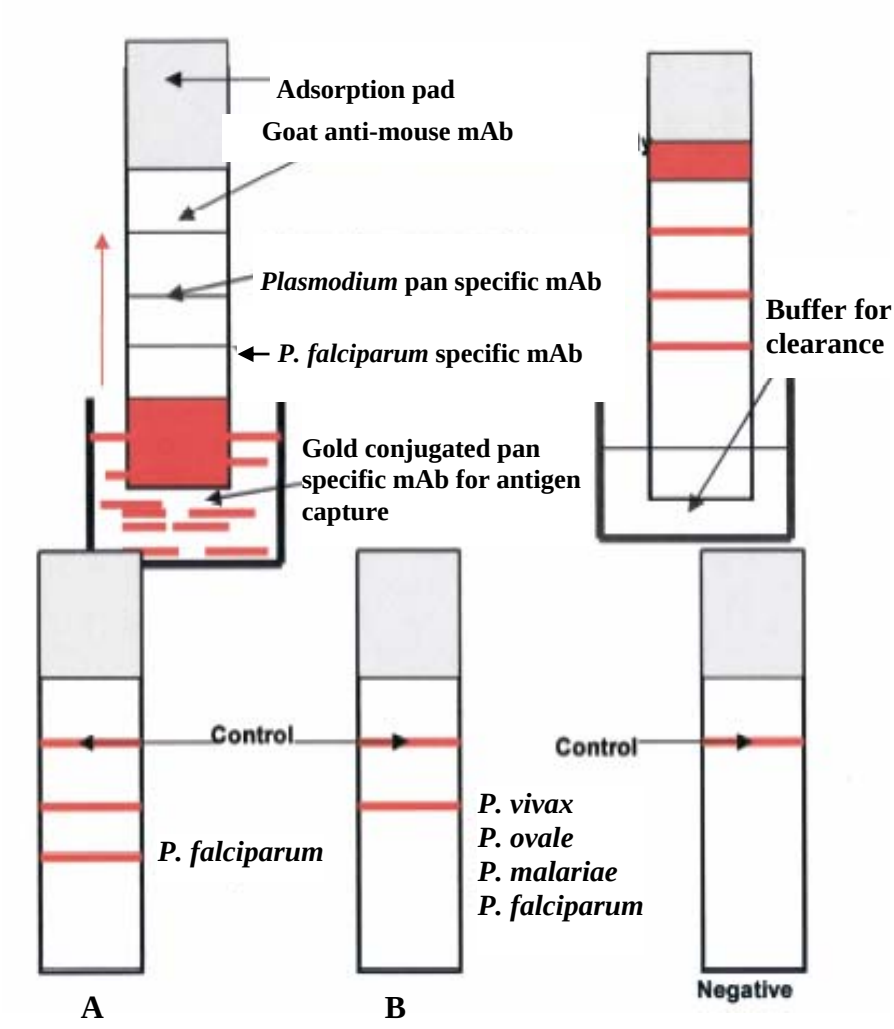

Figure 1.10: The principle of immunochromatographic RDT (from Moody, 2002). The antigen-specific mAb is mixed with patient's blood sample and allowed to migrate across the stationary phase. The positive tests (A) *P. falciparum* and (B) one of the four malaria parasites infection. In the negative test only the control gives a signal confirming the migration of antigen-specific antibody across the immobile phase.

Three proteins rich in histidine residues (HRPI, HRPII, and HRPIII) are produced by *P. falciparum*, but HRPII was shown to be unique and dominant in this parasite. HRPII is synthesised by the intracellular parasite and expressed on the surface membrane of the infected RBC (IRBC) during its asexual life cycle (Leech *et al*., 1984; Howard *et al*., 1986; Rock *et al*., 1987). During the erythrocytic life cycle the parasite requires energy in the form of ATP for the synthesis of RNA, DNA and proteins to ensure its survival. The only source of the required energy is the glycolytic pathway. Two well-characterised enzymes LDH and ALDO play a key role in the glycolytic pathway and have also been identified as target antigens for the development of enzyme-based immunoassays (Knapp *et al*., 1990; Döbeli *et al*., 1990; Meier *et al*., 1992; Gomez *et al*., 1997).

1.3.2 Trypanosomiasis

Trypanosomiasis is a disease of human and domestic livestock. It is caused by blood parasites called African trypanosomes. Three species (*Trypanosoma brucei, T. congolense and T. vivax*) are of economic importance to the agriculture sector. In humans, *Trypanosoma brucei, T. b. gambiense* and *T. b. rhodesiense* cause sleeping sickness. *T. congolense* and *T. vivax* affect livestock causing a disease known as Nagana. Trypanosomes are transmitted to mammalian host by tsetse flies (*Glossina* sp.) and reside in the bloodstream (Donelson, 2003).

Microscopic blood-smear examination is commonly used to diagnose trypanosomiasis, but cyclical parasitaemiac fluctuation can limit the usefulness of this method (Kashiwazaki *et al*., 1994). Other diagnostic approaches include PCR and the detection of parasite antibodies and antigens (Masake and Nantulya, 1991; Masiga *et al*., 1992; Singh

et al., 1995; Eisler *et al.*, 1998; Masake *et al.*, 1997; 2002). Trypanosome-specific antibodies in the blood may indicate past infections. On the other hand, current infections can be identified by detecting circulating parasite antigens and diagnostic immunoassays based on immunochromatographic principles that have been developed to detect species-specific antigens (Kashiwazaki *et al.*, 1994).

In the bloodstream, the trypanosome surface is coated with approximately 10 million identical copies of the variant surface glycoprotein (VSG). Several hundred different VSG genes are encoded in the parasite genome and expressed one at a time (antigenic variation), thus preventing immune recognition of the parasite (Mehlert *et al.*, 1998). VSG is made up of an N-terminal domain and a C-terminal domain and is anchored to the parasite surface via covalent attachment of the C-terminal residues (Carrington and Boothroyd, 1996). VSG determines the variable antigen type (VAT) of the individual trypanosome which elicits a specific immune response. VATs are predominantly expressed at early stages of infection and specific antibodies are found in the blood within 6 to 32 days after infection. The majority, if not all different strains of trypanosomes express VAT, thus making it a good candidate for development of diagnostic tests for detection of anti-VAT antibodies (Verloo *et al.*, 2000; 2001; Urakawa *et al.*, 2001; Lejon *et al.*, 2005). The potential of VSG (VATs) as an antigen for detection of specific antibodies has led to cloning and expression of recombinant VSG for use in diagnosis (Urakawa *et al.*, 2001). Other potential candidates such as the single conserved *N*-linked carbohydrate ($GlcNAc_2$-Man_{5-9}) moiety has been found in C-terminus of all VSGs studied so far (Mehlert *et al.*, 1998). This carbohydrate appears to play a key role in the process of binding and uptake of host macromolecules required for survival and

growth. It therefore, offers a great advantage over the expression of multiple specific proteins. The $GlcNAc_2$-Man_{5-9} moiety is found exposed at the flagellar pocket (Magez *et al*., 2001), and also makes a good target for diagnostic immunoassays (Magez *et al*., 2001; Stijlemans *et al*., 2004).

1.3.3 Malignant catarrhal fever

Malignant catarrhal fever (MCF) is generally a fatal disease syndrome of domestic and wild ruminants as well as pigs (Løken *et al*., 1998; Li *et al.*, 2005). MCF in cattle is caused by two related viruses; wildebeest-associated alcelaphine herpesvirus-1 (AlHV-1) (Bridgen *et al*., 1989) and sheep-associated infection caused by ovine herpesvirus-2 (OvHV-2) (Dunowska *et al*., 2001). MCF viruses belong to the *Rhadinovirus* genus of the *Gammaherpesvirinae* subfamily of the *Herpesviridae*. Similarities between AlHV-1 and OvHV-2 have been shown at the genomic level (Ensser *et al*., 1997; Dunowska *et al*., 2001). Recently, a new member of MCF virus associated with goats has been described and designated caprine herpesvirus-2 (CpHV-2) (Li *et al*., 2001).

The clinical signs of MCF include sudden fever, discharge from eyes and nose with matting of facial hair, inflammation toward mucosal surfaces and blood vessels, body temperature of 40.6 to 42°C and death, resulting in significant economic losses in cattle and other susceptible domestic ruminants (Pierson *et al*., 1973; Bridgen *et al*., 1989; Løken *et al*., 1998). In the past, viral antigen was detected in both living and dead infected cells (Patel and Edington 1980) and other serological tests such as virus neutralisation, complement fixation, indirect immunofluorescence, direct-binding ELISA and competitive-inhibition ELISA (CI-ELISA) are also used for MCF diagnosis. The

identification of MCF affected animals has also been based on the detecting of antibodies to AlHV-1. AlHV-1 infected cells were used as antigens in ELISA or indirect immunoflorescence (Rossiter, 1981, Løken *et al*., 1998). The sensitivity and reliability of results obtained using this approached remained questionable due to the fact that MCF viruses share common antigens with other herpesviruses. Some animals may not develop detectable levels of antibody prior to death which may result in false results; therefore, the presence of antibody alone may not be reliable for diagnosis of MCF infection (Washington State University website notes). Currently PCR is used for diagnosis of MCF from tissue samples collected from the infected animal using a virus specific primer set (Wiyono *et al.*, 1994; Li *et al.*, 2001). Histopathology remains the most recognised diagnostic procedure (Müller-Doblies *et al.*, 1998).

At least 11 MCF virus proteins have been reported to elicit immune response in animals suffering from MCF (Adams and Hutt-Fletcher, 1990; Li *et al.*, 1995a). Among the conserved glycosylated proteins found in AlHV-1 and OvHV-2 (Dunowska *et al.*, 2001), a potential candidate (130 kDa protein) for the development of diagnostic immunoassays specific to MCF viruses only, had been shown to exist. A monoclonal antibody against this conserved glycosylated protein epitope among MCF viruses has been used to develop specific, rapid and economical CI-ELISA for detection of MCF virus antibodies in sheep and other ruminants (Li *et al*., 1994; 1995b). The present study aims at selecting recombinant antibodies that may be used in future for the development of such immunoassays. Such monoclonal antibodies can potentially replace the monoclonal antibody used in the CI-ELISA of Li *et al.* (1994).

1.4 Motivation and strategy

Chickens pass their immunity to the developing embryo by relocating maternal antibodies from the serum to the egg-yolk and neonates (Jukes *et al*., 1934; Patterson *et al*., 1962). Immunising laying chickens therefore enables antibody titres to be determined by simply collecting the eggs and isolating antibodies (IgY) from the yolk. Other advantages of using the chicken as an immunisation host include the fact that a relatively small quantity of antigen is required to trigger an immune response and the relative tolerance of the bird to complete Freund's adjuvant (Gassmann *et al*., 1990; Tini *et al*., 2002). The chicken is nowadays therefore often used to produce antibodies for diagnostic and immunotherapeutic applications. Of relevance to this project is the fact that combinatorial antibody library can be constructed from immunised chickens (Davies *et al*., 1995; Yamanaka *et al*., 1996; Andris-Widhopf *et al*., 2000; Sapats *et al*., 2003; Finlay *et al*., 2005) and used to select antigen-specific clones.

As mentioned earlier, the mouse IgH locus consists of 15 V_H gene families which are grouped into clans on basis of their FW1 sequence composition. While the IgL locus is divided into two Igκ and Igλ which contain 18 Vκ and 3 Vλ family members, respectively. To construct an antibody library using mouse genes, the IgH and IgL gene repertoires are amplified using a mixture of 23 IgH specific primers (19 sense primers {V_H 5'} and 4 reverse primers {V_H 3'}) and 22 IgL specific primers (18 sense primers {17 Vκ 5' and one Vκ 5'} and four reverse primers {three Vκ3' and one Vκ3' specific}) (Andris-Windpof *et al.,* 2001; Burmester and Plückthun, 2001). In contrast, the technical advantage of using chicken genes in the construction of antibody libraries is that only one set of primers for each V_H and V_L gene segment is required to successfully access the

whole immunoglobulin repertoire (Davies *et al*., 1995; Yamanaka *et al*., 1996; Andris-Widhopf *et al*., 2000; Sapats *et al*., 2003; van Wyngaardt *et al*., 2004; Finlay *et al.*, 2005). This is because the 3' region of FW 4 in J_L or J_H is highly conserved and only one functional V_H and V_L gene segment exists (Reynaud *et al*., 1987; 1989; Thompson and Neiman, 1987) (see Fig. 1.6 and 1.7). Once an antibody-phage library is constructed, it can be used as a source of antigen-specific antibody clones without further use of animals.

This project was aimed at determining whether high-affinity chicken scFvs directed against five different diagnostic targets can be derived from a single immune bird. Accordingly, two chickens were immunised with a mixture of LDH, HRPII, ALDO, VSG and MCF virus. Its splenocytes were used as a source of mRNA from which filamentous phage libraries displaying single-chain Fv antibody fragments was constructed. The library was screened for binders which can potentially be incorporated into a variety of immunoassay formats. The approach described here yielded useful affinity-matured binders and provided a focussed alternative to large "universal" repertoires (Nissim *et al.*, 1994; Davies *et al*., 1995; van Wyngaardt *et al*., 2004) as a source of useful antibody fragments.

1.5 Aim, objectives and potential benefits

1.5.1 Aim

To establish whether phage display libraries can be constructed using splenic mRNA from a chicken immunised with five different proteinaceous antigens, namely *Plasmodium falciparum* lactate dehydrogenase, histidine-rich protein II and aldolase; *Trypanosoma sp.* variant surface glycoprotein and lastly, malignant catarrhal fever virus.

1.5.2 Objectives

To stimulate splenic B cell proliferation by the simultaneous immunisation of a chicken with five different antigens; isolate spleens, prepare mRNA and convert mRNA into cDNA; construct a phage library displaying affinity-matured chicken scFvs; and to select antibody clones with sufficiently high specificity for future use in diagnostic methods.

1.5.3 Potential benefits

Once an antibody-phage library is constructed, it can be used as a potential source of antigen-specific antibody clones by panning against any one of the chosen immunogens. The resulting antibodies can also be used in research and for the diagnosis of human or animal diseases. This study will contribute to the development and application of recombinant antibody technology in the field of veterinary science.

CHAPTER 2: MATERIALS AND METHODS

2.1 Antigens for immunisation

Recombinant lactate dehydrogenase (LDH), aldolase (ALDO) and histidine-rich protein II (HRPII) of *Plasmodium falciparum*; and recombinant variant surface glycoprotein (VSG) of *Trypanosoma* spp. were a gift from Vision Biotech Pty (Western Cape). Malignant catarrhal fever virus (MCFV) was cultured and purified using a modified method of Schloer and Breese (1982). WC11 virus strain (cell culture passage strain of AlHV-1) was propagated on foetal bovine cells at the 8th passage level in BioWhittaker's minimum essential medium eagle (EMEM) supplemented with 7% serum. When an estimated 70 to 90% of the cells exhibited cytopathic effects, the supernatant fluid was harvested and centrifuged at 4 500 xg (Sorvall SLA 3000 rotor, Beckman) for 30 min. Virus particles were pelleted from the supernatant by ultracentrifugation through a 7 ml cushion of 35% sucrose (wt/wt) in Beckman SW 28 rotor (120 000 xg, 90 min at 4˚C), and resuspended in phosphate buffer saline pH 7.4 (PBS). Purified virus was obtained following equilibrium density-gradient ultracentrifugation in a 20-40% sucrose gradient at 70 000 xg at 4˚C in a Beckman SW 28 rotor for 40 min. The pellet was resuspended in PBS and centrifuged at 120 000 xg in a Beckman SW 28 rotor at 4˚C for 90 min, resuspended in PBS and examined by electron microscopy.

2.2 Immunisation of chickens

Ethical approval to use two chickens in this study was granted by the Onderstepoort Veterinary Institute Animal Ethics committee. Two specific pathogen-free adult laying hens were immunised by intra-muscular inoculation of a mixture of LDH, HRPII, ALDO, VSG and MCFV antigens (50-100 µg of each in 500 µl of PBS), emulsified with an equal

volume of Montanide ISA 70 adjuvant (Seppic, France). At three week intervals the chickens received the first, second and third booster immunisations. Two weeks after the third booster, a fourth inoculation with the same mixture of antigens but this time emulsified in Freund's incomplete adjuvant (Sigma) using the method described by Vulliet (1996) was performed. After the third booster, the eggs were collected and the egg yolk antibodies (IgY) were prepared using the polyethylene glycol (PEG) precipitation method of Polson (1990) and analysed by SDS-PAGE using 10% polyacrylamide gels. The gel was stained with Coomassie Brillant Blue and the samples were incubated at 100˚C for 5 min prior to loading on the electrophoretic gel. The bird's immune response to the target antigens was confirmed in ELISA using IgY prior to the collection of spleens. Twenty-one days after the fourth booster, chickens were euthanased by administering 1 ml/kg of pento-barbitone intraperitoneally. The spleens were removed and used for RNA extraction (see 2.4).

2.3 IgY ELISA

Wells of a Nunc Polysorp ELISA plate were coated overnight at 4ºC either with target antigen (10 µg/ml) or with 2% milk powder in PBS as a negative control. The following day non-specific binding was blocked with 300 µl/well of 2% MP at 37C for 1 h. Plates were washed three times (3x) with PBS containing 0.05% Tween, then 3x with PBS. Egg-yolk IgY was diluted in PBS containing 0.5% Tween to 100 µg/ml and 50 µl was added to each well. After 1 h incubation at 37ºC, plates were washed as described above and IgY was detected with 1:10 000 dilution of horseradish peroxidase-conjugated goat anti-chicken IgG (seroTec, USA) in PBS containing 2% MP and incubated at 37ºC for 1 h. Wells were washed as described above and colour was developed by adding 50 µl

(1mg/ml) of the enzyme substrate O-phenylenediamine dihydrochloride (Sigma, Germany) and 10 µl of hydrogen peroxide. The absorbance was measured at 492 nm using BDSL Immunoskan MS (Labsystems) after stopping the reaction with 50 µl of 1M H_2SO_4.

2.4 Western immunoblotting

Western immunoblotting was performed by Dr. Christine Vroon (OVI-Biotechnology Division). Partially purified MCFV and uninfected foetal bovine testes (FBT) cells were separated in SDS-PAGE using a 10% polyacrylamide gel and the proteins were transferred to an Immobilon-P Transfer membrane (Millipore). The membrane was treated with blocking buffer (PBS containing 5% skim milk and 0.05% Tween) for 1 h and exposed to 1/100 dilution of IgY in blocking buffer for 1 h and washed four times for eight minutes with PBS-0.05% Tween. The membrane was exposed to a 1000 times diluted horseradish peroxidase-conjugated anti-chicken IgG in blocking buffer for 1 hr and bound anti-chicken antibodies were detected with substrate solution (10 ml PBS, 6 mg of 3,3'-diaminobenzidine and 10 µl hydrogen peroxide).

2.5 Phagemid preparation

The phagemid vector, pHEN1 was used for phage display and expression as soluble fragments of scFv antibodies. The vector is designed to allow cloning of inserts using *Sfi1/Not1* restriction sites on gene *III* (Hoogenboom *et al*.; 1991) (see Fig 2.1). Vector DNA was isolated from an overnight culture of *E. coli* TG1 bacterial stock cells using a Plasmid Midi Prep kit (Qiagen) and digested with *Sfi1* and *Not1* restriction endonucleases as described by van Wyngaardt *et al*. (2004). The digested vector was first purified with a

PCR purification kit (Qiagen). It was then purified by electrophoresis in 1% agarose gel and extracted using a QIAquick gel extraction kit (Qiagen). The concentration of DNA was determined by measuring absorbance at 260 nm (A_{260} = 50 ng/µl).

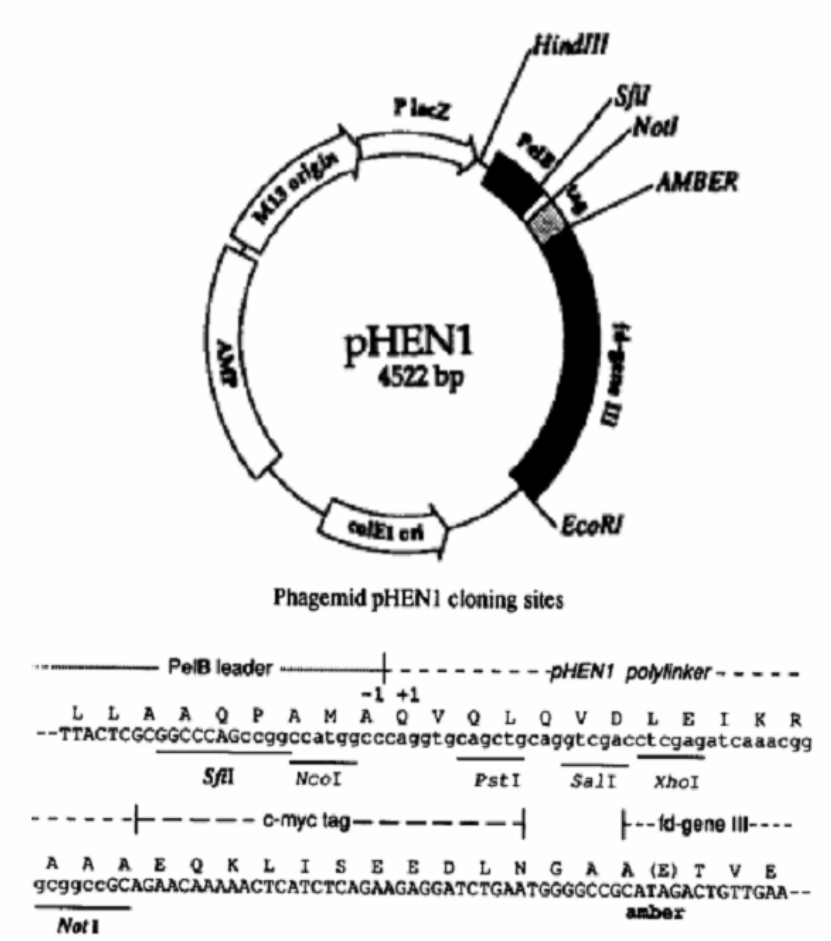

Figure 2.1: The structure and cloning sequence of the pHEN vector used for phage display and expression of scFv antibodies (from Hoogenboom *et al.*, 1991).

2.6 Antibody library construction

2.6.1 Preparation of RNA and synthesis of cDNA

Total spleen RNA was prepared as described by Andris-Widhopf *et al.* (2001). Chicken spleens were minced and homogenised through a fine stainless steel mesh in 30 ml TRI

reagent (Sigma). The homogenate was centrifuged for 10 min at 2500 xg and the supernatant fluid was transferred to a clean COREX (15 ml) glass tube, mixed with 3 ml of 1-bromo-3-chloropropane (BCP) (Sigma) and incubated at room temperature (RT) for 15 min, then centrifuged at 10 000 xg for 15 min at 4ºC. The resulting clear aqueous phase was transferred to a clean tube and 15 ml of isopropanol was added, mixed and incubated at RT for 10 min. The mixture was centrifuged at 10 000 xg for 30 min at 4ºC to pellet the RNA. The RNA pellet was washed with 75% ethanol and centrifuged at 10000 xg for 30 min at 4ºC. The total RNA pellet was air-dried and resuspended in 500 µl of nuclease-free water (stored at -80ºC). The nucleic acid concentration was determined by measuring absorbance at 260 nm (A_{260} = 40 ng/µl). A total amount of 6 mg was obtained from both chickens. Complementary DNA (cDNA) was synthesized from approximately 25 µg of total RNA using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 following the manufacture's instructions. Fifty individual and identical cDNA synthesis reaction mixtures were prepared each consisting of 5 mM $MgCl_2$; 1x RT buffer; 1mM dNTPs; RNase inhibitor (1 unit/µl); reverse transcriptase (0.25 unit/µl); 0.125 µM oligo dT-adapter primer and 500 ng of total RNA. Nuclease free-water was added to make a final volume of 10 µl for each reaction. The cDNA was synthesised by incubating for 10 min at 30ºC, 60 min at 42ºC, 5 min at 95ºC and 5 min at 5ºC.

2.6.2 PCR amplification of V_H and V_L chain genes

The VHF1/VHR1 and VLF1/VLR1 sets of primers (see Table 1) were used for PCR amplification of V_H and V_L gene segments in two separate reactions. PCR was performed using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 in a total volume of 50 µl in which 10 µl of cDNA synthesis reaction was mixed with 40 µl reaction mixture consists of 1x PCR

buffer, 20 pmol of forward and reverse primers, 1.25 units of TaKaRa *Ex Taq* HS and distilled water. The PCR conditions consisted of 30 cycles of 1 min at 94ºC, 1 min at 60ºC, 1 min at 72ºC, followed by a final extension of 5 min at 72ºC. This was performed on a PERKIN ELMER GeneAmp PCR system 2 400 thermocycler. A total of 25 separate PCR's were performed for each V_H and V_L gene amplification. The products were pooled; purified using a QIAquick PCR purification kit (Qiagen) and then further by gel electrophoresis in a 2% agarose gel containing 10 µg/ml of crystal violet to make the DNA visible (van Wyngaardt *et al.*, 2004). DNA fragments of 390 and 350 base pairs (bp) respectively (Davies *et al*., 1995) were extracted using a QIAquick gel extraction kit (Qiagen), and stored at -20ºC in elution buffer (EB).

2.6.3 Assembly of scFv gene

The Hend1/Lstart1 and NewlVarH/NewlVarL set of primers (see Table 1) were used to introduce overlapping sequences which enabled the scFv gene fragments to be assembled by overlap extension PCR (Horton *et al*., 1989; Huston *et al*., 1988). The Hend1/Lstart1 primers encode a linker consisting of a single amino acid (G) residue. While the NewlVarH/NewlVarL primers encode a 15 amino acid linker sequence $(G_4S)_3$. The overlap extension PCR consisted of preheating for 2 min at 94˚C followed by the first 15 cycles of 1 min at 94ºC; 1 min at 60ºC; 2 min at 72ºC and a final extension of 5 min at 72ºC. The resulting product was diluted 25x in a 100 µl pull-through PCR reaction mixture made-up of 20 pmol of VHF2 and VLR1 primers. The VHF2 and VLR1 primers introduced the *Sfi1* and *Not1* restriction sites at the both ends of scFv gene fragment respectively (Sapats *et al.*, 2003; van Wyngaardt *et al*., 2004). The assembled scFv fragments (~750 bp) were purified in 1.2% agarose gel as previously described and

digested with *Sfi1* and *Not1* restriction endonucleases under appropriate conditions to yield an insert with sticky-ends. The digested insert DNA was purified using a Qiagen PCR purification kit and stored at -20ºC until used.

Table 1: Nucleotide sequences of primers used.

Primer	Nucleotide sequence (5'-3')
[a] VHF1	CTGATGGCGGCCGTGACGTTGGAC
[b] VHF2	GTCCTCGCAACTGCGGCCCAGCCGGCCCTGATGGCGGCCGTGACG
[a] VHR1	CCGCCTCCGGAGGAGACGATGACTTCG
[a] VLF1	GACTCAGCCGTCCTCGGTGTCAG
[b] VLR1	TGATGGTGGCGGCCGCATTGGGCTG
[c] Hend1	CCGGAGGAGACGATGACTTCGG
[c] Lstart1	GCGCTGACTCAGCCGTCCTCGG
[b] NewlVarH	CCGCCAGAGCCACCTCCACCTGAACCGCCTCCACCGGAGGAGACGATGACTTCGG
[b] NewlVarL	TCAGGTGGAGGTGGCTCTGGCGGAGGCGGATCGGCGCTGACTCAGCCGTCCTCGG
[b] OP52F	CCCTCATAGTTAGCGTAACG
[b] M13R	CAGGAAACAGCTATGAC

All primers were synthesised by Invitrogen, [a] derived from Yamanaka *et al*., 1996 and Andries-Widhopf *et al*., 2000; [b] as published by van Wyngaardt *et al*., 2004; [c] designed by Jeanni Fehrsen unpublished.

2.6.4 Electroporation and library size estimation

Ligation of *Sfi1/Not1*-digested scFv inserts (1.6 µg) and vector DNA (3.2 µg) was carried out using a rapid T4 DNA ligation kit (Roche), using a 2:1 molar ratio of vector: insert following the manufacturer's instructions. The ligated scFv phagemid library was desalted using the method of Atrazhev and Elliot (1996). The DNA was then electroporated (Gene-pulser; Biorad) into electrocompetent *E. coli* TG1 cells (Stratagene). Five electroporations for each library were performed at 2.5 kV, 25 µF, and 200 Ω. Serial dilutions of transformed cells were made to determine the library size. The cells were cultured on TYE agar plates containing 100 µg/ml ampicillin and 2% glucose (A/G). The remaining bacterial culture was plated out onto 15 TYE (A/G) agar plates (150 mm) and incubated overnight at 30ºC. The resulting lawns of bacterial cells were scraped into 2x TY medium. A 15% glycerol stock of the bacteria was prepared and stored at -70ºC. The library was re-amplified by diluting 1ml stock in 2x TY (A/G) to give OD_{600} reading of <0.1 and grown to OD_{600} 1. Cells were pelleted and a sub-stock of cells in 15% glycerol was prepared and stored at -70ºC. The phage antibody stock was prepared by diluting 1 ml of the sub-stock to OD_{600} 0.05 in 2 litre of 2x TY (A/G), grown at 37ºC with shaking to an OD_{600} of 0.5 and infected with M13-K07 helper phage at a ratio of phages: bacteria of 20:1. It was then incubated for 30 min at 37ºC in a water-bath, a further 30 min at 37ºC with shaking at 100 rpm. The cells were pelleted and resuspended in 2xTY containing 50 µg/ml ampicillin, 25 µg/ml kanamycin and shaken at 270 rpm overnight at 30ºC. Phage particles from supernatant of an overnight culture were precipitated with 1:5 volumes of 20% PEG/2.5 M NaCl and resuspended in PBS. A 15% glycerol stock of antibody-phage particles was prepared and stored at -70ºC. Twenty clones were randomly chosen from titration plates. Each clone was tested for scFv insert

by colony PCR using the OP52/M13 primer set (Table 1) that flanks the cloning site of the vector (94ºC for 2 min, then 30 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s and final extension at 72ºC for 4 min).

2.7 Biopanning of antibody libraries against target antigens

Maxisorp Immunotubes (Nunc, Denmark) were coated overnight at 4ºC with 10 µg/ml of the target antigen in 2 ml PBS and the following day blocked for 1 h at 37ºC by adding PBS containing 2% milk powder (MP) (wt/vol). Tubes were washed three times with PBS-0.1% Tween and 3 times with PBS. Panning was carried out by diluting 1 ml of the PEG-precipitated phage stock with an equal amount of PBS-4% MP/0.2% Tween and then added to the immunotube. The tube was rotated for 30 min at room temperature, and then allowed to stand for 1 h. The tube was then washed 20 times with PBS-0.1% Tween (PBS-T) and 20 times with PBS. Bound phage particles were eluted by adding 1 ml of 100 mM triethylamine (pH 12) and incubated at RT for 10 min with slow horizontal rotation. The triethylamine was neutralised by adding 500 µl of Tris-HCl pH 7.4. One ml of the eluted phage was used to infect 5 ml of freshly grown *E. coli* TG1 cells (OD_{600} 0.5) at 37ºC. The culture volume was increased to 10 ml by adding 4 ml of 2x TY. It was then incubated at 37ºC in a water bath for 30 min to allow infection. The phage infected cells were pelleted, resuspended in 1 ml of 2x TY, plated out on three TYE (A/G) agar plates (150 mm) and incubated overnight at 30ºC. The output phage titre was determined by plating 100 µl of infected *E. coli* TG1 cells with serial dilutions of phage on TYE (A/G) agar and incubating overnight at 30ºC. The resulting lawn of cells was scraped off and a glycerol stock was prepared. For phage rescue, bacterial stock harbouring the phagemid was diluted to an OD_{600} of 0.05, grown to an OD_{600} of 0.5 in 25 ml 2x TY (A/G) at 37ºC

(shaking). Five ml of the culture was infected with M13KO7 helper phage at a phage: bacterium ratio of 20:1 and incubated at 37ºC for 30 min in a water-bath to allow infection. The cells were then pelleted by centrifugation and resuspended in 25 ml of 2x TY containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (A/K), and subsequently incubated at 30ºC overnight with shaking. Bacteria in overnight culture were pelleted and phage particles were collected from the supernatant by 20% PEG/2.5 M NaCl precipitation on ice for 1 h. The phages were pelleted by centrifugation and resuspended in 1 ml PBS. Half of precipitated phage particles were used for the next round of panning. A total of four rounds of panning were performed.

2.8 Screening of phage pools

2.8.1 Polyclonal phage ELISA

Pooled phage inputs for each round of panning were tested for antigen-specific binding by indirect ELISA. Wells of a Nunc Polysorp ELISA plate were coated overnight at 4ºC either with target antigen dissolved in PBS at 10 µg/ml concentration or 2% milk powder as a negative control. The following day coating solution was removed and non-specific binding was blocked with 300 µl/well of 2% MP at 37ºC for 1 h. Plates were washed three times with PBS-T, then 3x with PBS. The input phage preparations were diluted 200x in PBS-2% MP/ 0.1% Tween (PBS-MP/T) and 50 µl of the dilution was added to each well. After 1 h incubation at 37ºC, plates were washed as described above and bound phage particles were detected with a 1:1 000 dilution of mouse anti-M13 monoclonal antibody (Progen, Germany) in PBS-MP/T and incubated at 37ºC for 1 h. The plate was washed as previously described and probed with horseradish peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin (DakoCytomation, Denmark)

in PBS-MP/T, incubated at 37ºC for 1 h and after washing with PBS-T, the wells were developed with 50 µl (1 mg/ml) of enzyme substrate O-phenylenediamine dihydrochloride (Sigma, Germany). The absorbance was measured at 492 nm after stopping the reaction with 50 µl of 1M H_2SO_4.

2.8.2 Monoclonal phage ELISA

Individual colonies were randomly chosen from the output of the third or fourth round of panning and inoculated into a 96-well flat-bottom culture plate (Costar, USA) containing 100 µl of 2x TY (A/G). Plates were incubated overnight at 30ºC with shaking at 220 rpm. A 96-well replica plater (Sigma, Germany) was used to transfer inoculum from the overnight culture (master) plate to a fresh 96-well culture plate containing 150 µl of 2x TY (A/G) per well. This new plate was then incubated for 2.5 h at 37ºC with shaking, then 50 µl of 2x TY (A/G) containing $2x10^9$ pfu M13KO7 helper phage was added to each well to rescue the phage particles and further incubated at 37ºC for 30 min without shaking; then centrifuged at 600 xg for 10 min. The supernatant was replaced with 2x TY (A/K) and the plate was incubated overnight at 30ºC with shaking at 220 rpm. The supernatant liquid containing the phage particles was collected and used for ELISA as previously described (van Wyngaardt *et al.*, 2004). The bacterial stock was prepared by adding glycerol to the master plate and stored at -70ºC.

2.8.3 Expression of soluble scFv antibody fragments

Monoclonal soluble scFv fragments were expressed in *E. coli* TG1 cells. Positive phagemid clones identified by ELISA were tested for their ability to bind as soluble antibody fragments. Inocula were transferred from the master plate using a replica plater

to a 96-well culture plate containing 100 µl of 2x TY supplemented with 100 µg/ml ampicillin and 0.1% glucose per well and grown at 37ºC for 2-3 h with shaking. To induce expression of soluble scFv protein antibodies, 50 µl of 2x TY containing 100 µg/ml ampicillin and 3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each well. ScFv induction was overnight at 30ºC with shaking. Cells were pelleted by centrifugation and supernatant fluid containing soluble antibody fragments was used in ELISA as described above except that PBS containing 0.05% Tween was used to wash the ELISA plate (van Wyngaardt *et al*., 2004).

2.8.4 DNA sequencing of single scFv clones

Single stranded phagemid DNA was isolated from an overnight culture of bacterial cells harbouring antigen binding scFv antibodies. The M13 and OP52 primers (Table 1) were used for sequencing and the DNA sequence was analysed using BioEdit Sequence Alignment Editor and protein sequences were deduced using Gene Runner program and CLC Free Workbench 3.2.2 program was used to align the sequences.

2.8.5 Introduction of a flexible linker

The V_H and V_L gene fragments were amplified from phage DNA using the NewlVarH/VHF2 and NewlVarL/VLR1 primer sets that encode for a flexible linker; the PCR products were pooled and joined by SOE-joining as described by Huston and colleagues (1988). Restriction enzyme sites (*Sfi1/Not1*) were introduced as described above and the modified scFv gene was cloned back into the pHEN display vector and transformed into chemically competent TG1 cells by heat-shock at 42˚C for 45 s.

Chemically competent *E. coli* TG1 cells were prepared using a one-step method as described by Chung and colleagues (1989).

2.9 Immunoassays

2.9.1 Sandwich ELISA

A Nunc Polysorp plate was coated with 50 µl of LDH-specific IgY (100µg/ml) in PBS (pH 7.4) and 2% MP as a negative control at 4˚C overnight. Three times washing was performed with PBS-0.05% Tween between each step. Non-specific binding was blocked with 2% MP for 1 h at 37˚C. The plate was washed and 10 µg/ml of LDH in PBS containing 0.05% Tween 20 was added to each well and incubated at 37˚C for 1h. Then a 1:1 dilution of scFv LC9 containing supernatant with 4% MP was added and incubated at 37˚C for 1 h. The scFv binding was detected as described in section 2.8.3.

2.9.2 Competitive inhibition ELISA

The ELISA plate was coated with LDH as described section 2.7. LDH-specific IgY (provided by Wouter van Wyngaardt) was added at 100 µg/ml and 1 mg/ml and “cocktail” IgY (used in section 2.3) was added at 100 µg/ml and 2 mg/ml concentration in PBS containing 0.5% Tween. The scFv LC9 was added and detected as described above.

CHAPTER 3: RESULTS

3.1 Antigens for immunisation

Of the antigens used in this study, all except MCF virus were recombinant proteins obtained in purified form by sucrose-gradient ultracentrifugation (Fig. 3.1 A). The virus was examined by electron microscopy an integument zone between the viral envelope (VE) and the nucleocapsid (NC) was observed with the capsids being arranged to form ring-like or hexagonal shapes as described by Todd and Storz (1983) (Fig. 3.1 B).

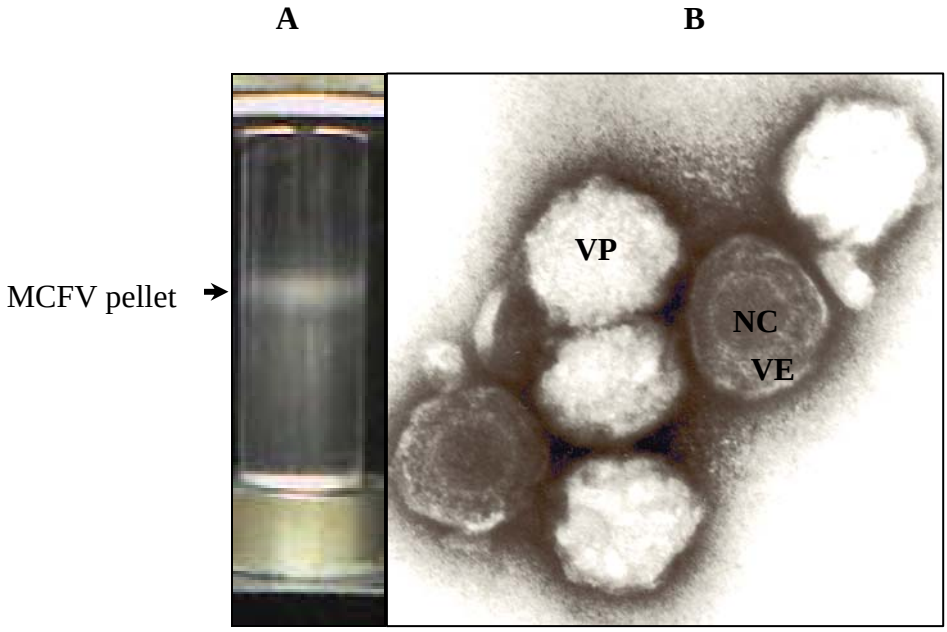

Figure 3.1: Picture A shows the sucrose density-gradient MCF virus pellet and an electron microscope image in B shows MCFV particles (VP), viral envelope (VE) and nucleocapsid (NC).

3.2 Immunisation of chickens and IgY ELISA

The egg yolk antibody (IgY) titre of laying hens should in theory, directly correlate with B-cell proliferation in the spleen. This organ therefore is expected to have a high concentration of immunoglobulin mRNA. In order to monitor the two chickens' immune

response to the different target antigens, IgY was purified from eggs collected after the third booster immunisation and analysed by SDS-PAGE (Fig. 3.2). Chicken “A” had antibodies against ALDO, HRPII, VSG and MCFV (Fig. 3.3) while chicken “B” had antibodies against LDH, ALDO, VSG and MCFV antigen (Fig. 3.4) as shown by indirect ELISA (triplicate reading averages). The foetal bovine cells were also included in the ELISA test to measure possible background as the MCFV was cultivated in these cells. After the fourth booster immunisation, spleens were harvested from both chickens and the total RNA was isolated.

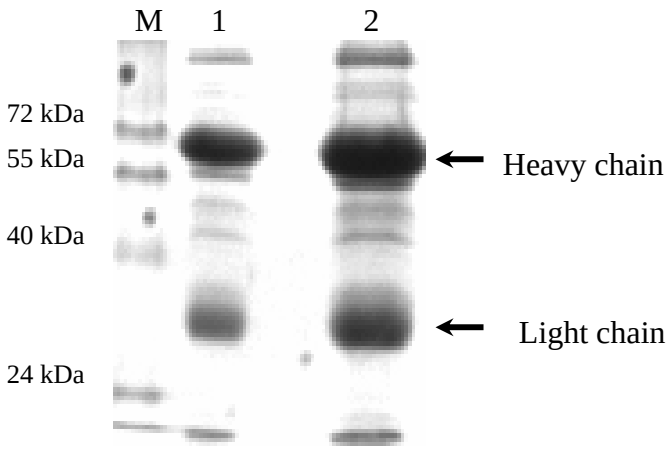

Figure 3.2: Analysis of IgY (lane 1 and 2) by SDS-PAGE. Lane M is the protein molecular mass marker IV (peQLab). Bands at approximately 68 kDa and 32 kDa represent the immunoglobulin’s heavy and light chain respectively.

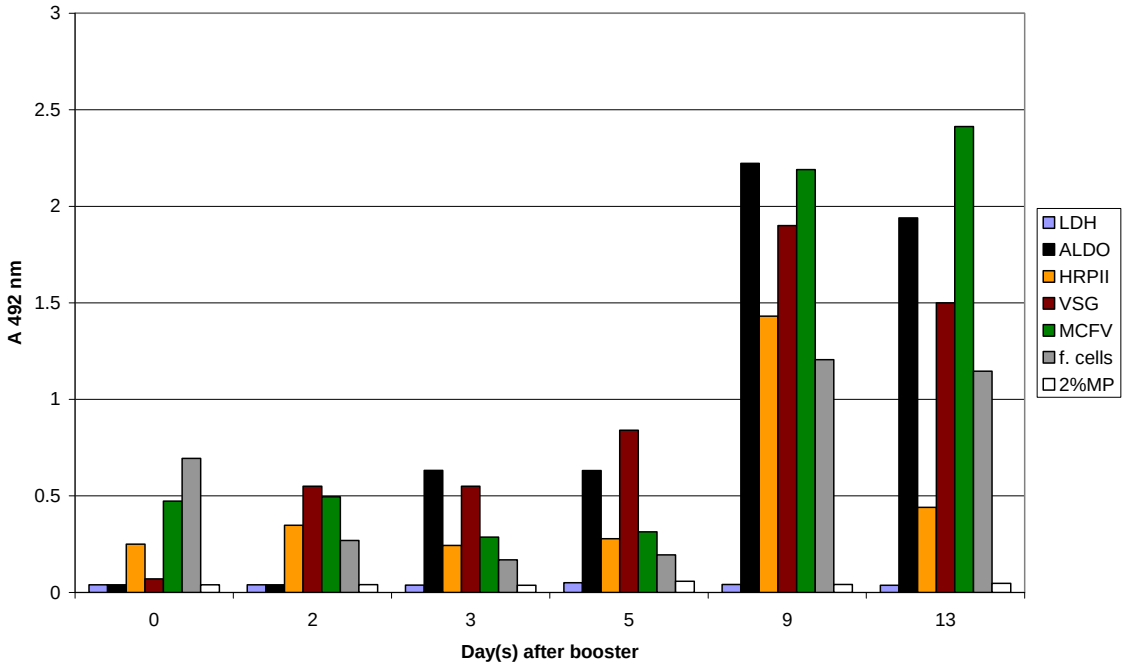

Figure 3.3: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “A” after a third booster immunisation.

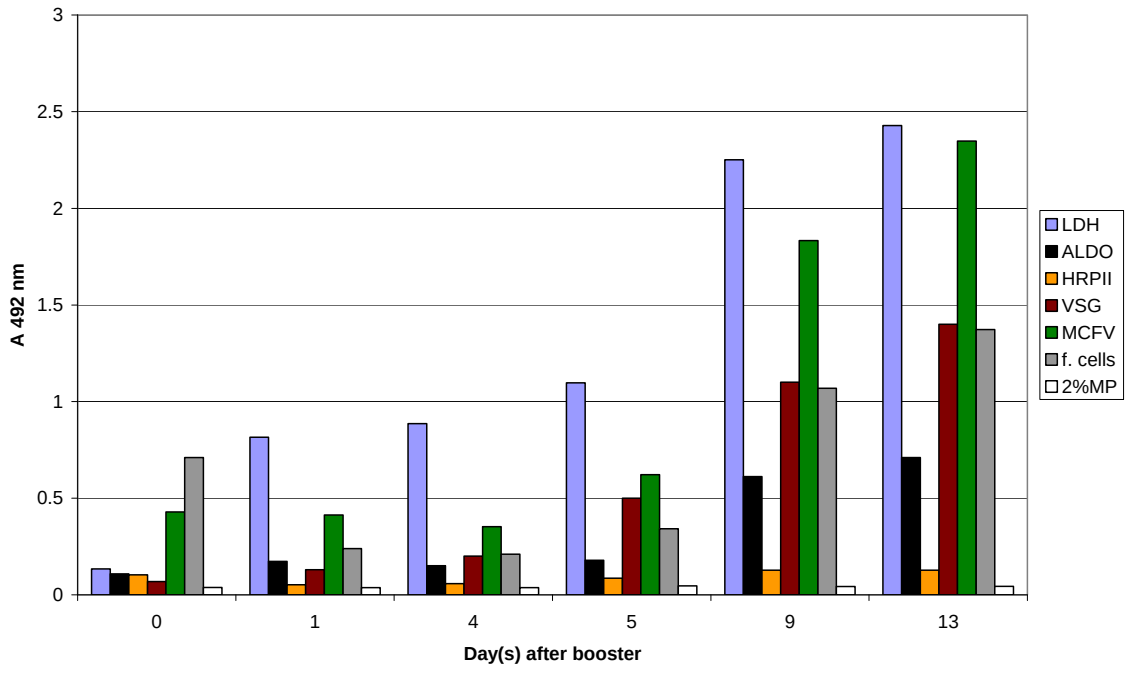

Figure 3.4: Indirect ELISA analysis of antigen specific IgY titre in eggs collected from chicken “B” after a third booster immunisation.

3.3 Antibody library construction

Two phage-displayed scFv antibody libraries, one with the immunoglobulin V_H and V_L regions joined by a single amino acid (G) (the "directly" linked library) and the other with a normal 15 amino acid flexible linker [$(G_4S)_3$] were constructed using RNA isolated from the spleens of two immunised chickens (Fig 3.5). In the case of both libraries, cDNA was synthesised using oligo-dT primers from both chickens and then pooled and directly used as a template for PCR amplification of the V_H and V_L chain gene fragments. The gene fragments from both chickens were pooled in one reaction tube together with primers that contain overlapping linker sequences which allow the V_H and V_L gene segments to be assembled by overlap extension to form single-chain variable region fragments (scFvs) (Fig. 3.6). The resulting scFv genes were inserted into the phagemid pHEN. After electroporation of the gene repertoire into electro-competent *E. coli* TG1 cells, the resulting libraries had calculated sizes of 6.7×10^7 for the single amino acid ("directly" linked) and 1.7×10^7 transformants for the flexibly linked repertoire (Fig. 3.5). Colony PCR of 20 randomly chosen clones showed that between 90 and 100% could be expected to contain the scFv DNA insert of ~ 750 bp (Fig. 3.7 and 3.8). Vector self-ligation background was estimated to be ≤ 10% of the library.

Library diversity. DNA from ten randomly chosen clones from each of the libraries was sequenced. Their amino acid sequences were deduced and three complementary-determining regions (CDRs) and four framework (FW) regions were identified in each of the heavy (V_H) and light (V_L) chain fragments. A single amino acid (G) linker ("directly" linked library) or a 15 amino acid [$(G_4S)_3$] "normal" linker was also present. Following

alignment with each other, significant diversity in the V_H and V_L chain was observed especially in the CDRs. Variability was also noted in the framework regions. No two clones had identical V_H or V_L fragments. The aligned amino acid sequences of five clones from each library are shown in Fig. 3.9 and 3.10.

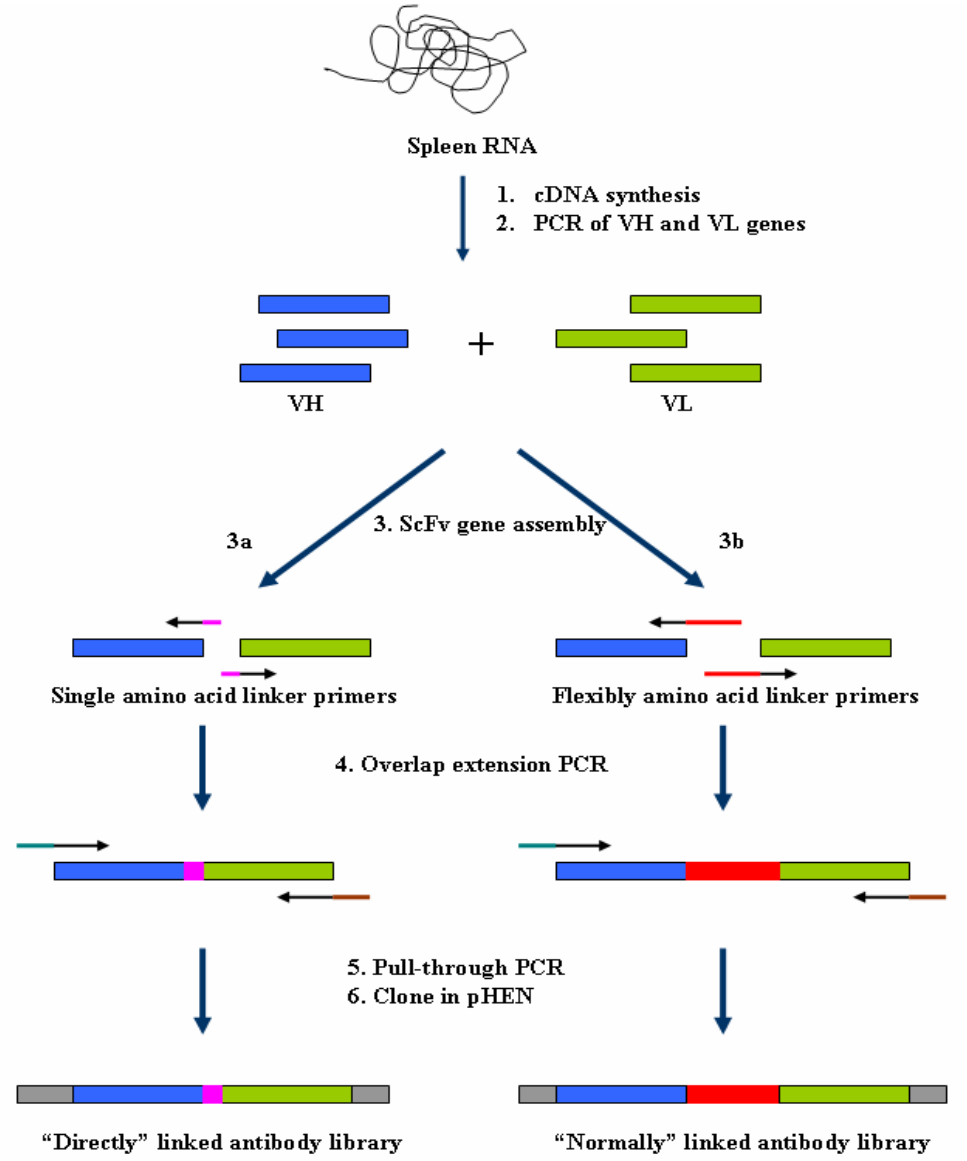

Figure 3.5: Flow diagram showing the construction of two antibody phage displayed libraries: (1) reverse transcription-PCR of RNA to cDNA, (2) amplification of V_H and V_L gene segments, (3-4) assembly of scFv gene by extension PCR, (5) pull-through PCR to introduce restriction sites at the ends and (6) cloning scFv gene in pHEN vector DNA.

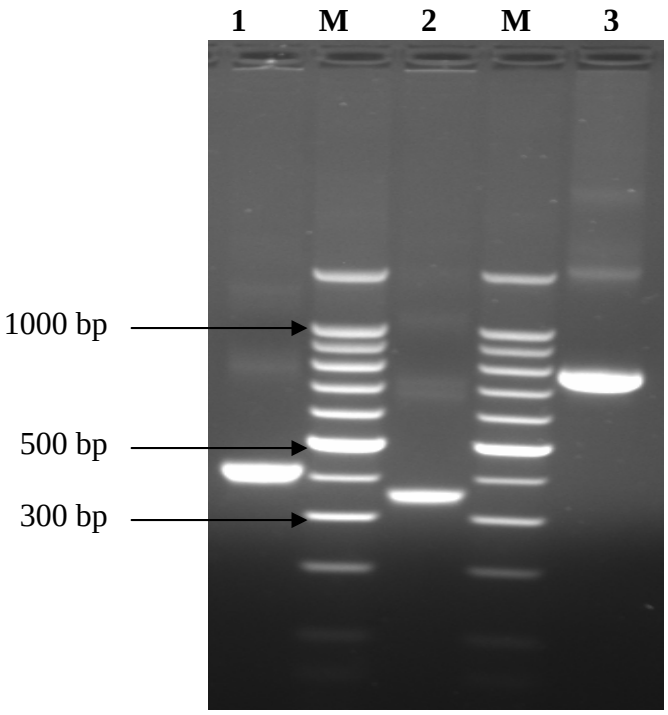

Figure 3.6: PCR amplicons lane 1, V_H; lane 2 V_L; and lane 3 the scFv gene assembled by pull-through PCR. M is a 100 bp ladder DNA molecular mass marker.

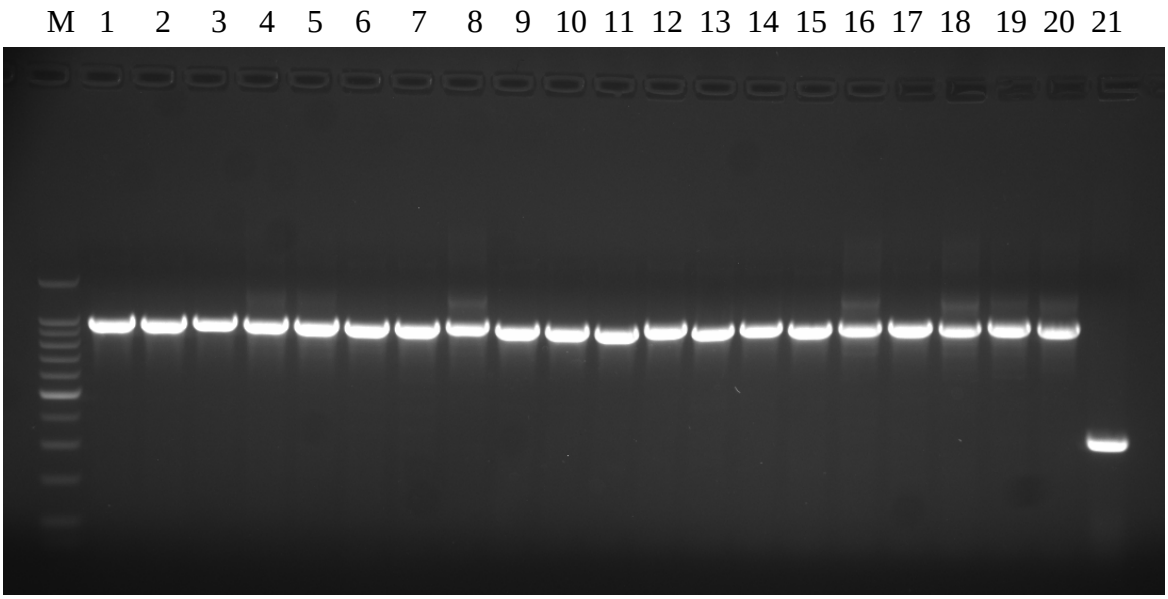

Figure 3.7: PCR amplicons obtained after colony PCR (“directly” linked library) using vector-specific primers that recognise insert flanking regions. M is a 100 bp ladder DNA molecular mass marker. Lanes 1-20 show amplicons from randomly picked colonies and lane 21 is the negative control (pHEN DNA).

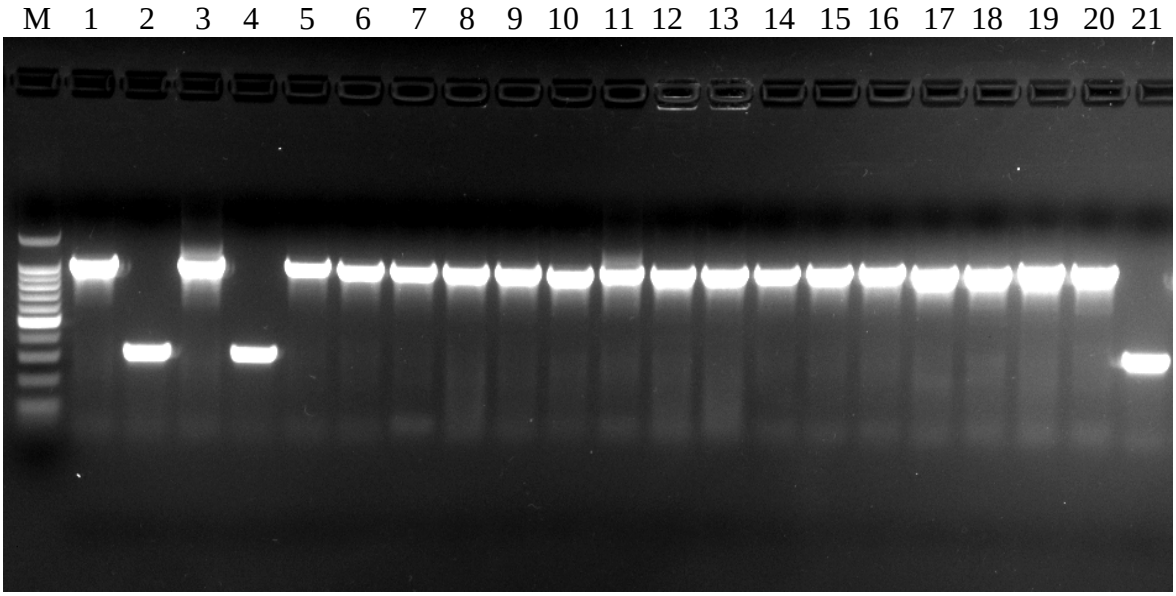

Figure 3.8: PCR amplicons obtained after colony PCR (“normally” linked library) using vector specific primers that recognise insert flanking regions. M is a 100 bp ladder DNA molecular mass marker. Lanes 1-20 show amplicons from randomly picked colonies and lane 21 is the negative control (pHEN DNA).

```
          ←---------------------------|HCDR1|-------------|    HCDR2     |------------------------
D1  LAAQPALMAAVTLDESGGGLQTPGGALSLVCEASGFPFSSYAMLWVRQAPGKGLEFVAGISTSGSNTAYGPAVKGRATISRDDGQNIVRLQLNNLRAEDR
D2  ........................G.....KG...T..T.N.G.........Q...S..ND..Y.Y..T...........N..ST...........T
D3  ........................T.....K....FIG....Q.............DDDSTF.L.A.............ST...........T
D4  ........................G.....K....T..N.Q.G.............I.DDD.R..D..S...........N..S...........T
D5  ............................K....D...NG.A......N....W..N.GDD....N..S...........N..STL..........T

    -------|      HCDR3       |---------→ ←------------------| LCDR1 |--------------| LCDR2 |--------
D1  GIYYCTRSSCGACWSH-----TGVIDAWGHGTEVIVSSGALTQPSSVSANLGGTVKITCSGGGNYDYGWYQQKSPGSAPVTVIYDDDKRPSNIPSRFSGS
D2  AT...AKE.GSGG---RGSYIA.-..S....-.................P.E..R.....-.DSH...F.............NNNN...D-.......
D3  AT...AK-.VYGDITYRERLSAT-..T.....................P.E........E...F.............L..NN.Q............
D4  .T...AK..SNSYCDYAVPCSADE.......................P.E..E.I...DN..-.........D.P......NNS....D........
D5  ...F...G.----------TADDV.......................P.E........TPAS-..........GPL.....W..E...D........

    ----------------------|  LCDR3    |---------→
D1  SSGSTATLVITGVQAEDEAVYYCGTVDDTAN-GMFGAGTALSVLGQPNAAAEQKLISEEDLNG
D2  L....N..T.....V......F..SF.SDI.S.I.....T.T.....................
D3  R....N..T......D.....F...R.SDY-V.I.....T.T.....................
D4  T.......T.....V......F..SYE..--SSI.....T.T.....................
D5  K.......T............F..SA.---YKHI.....T.T.....................
```

Figure 3.9: Amino acid sequence alignment of randomly chosen clones from “directly” linked antibody library. A single amino acid linker (G) separates the V_H and V_L chains.

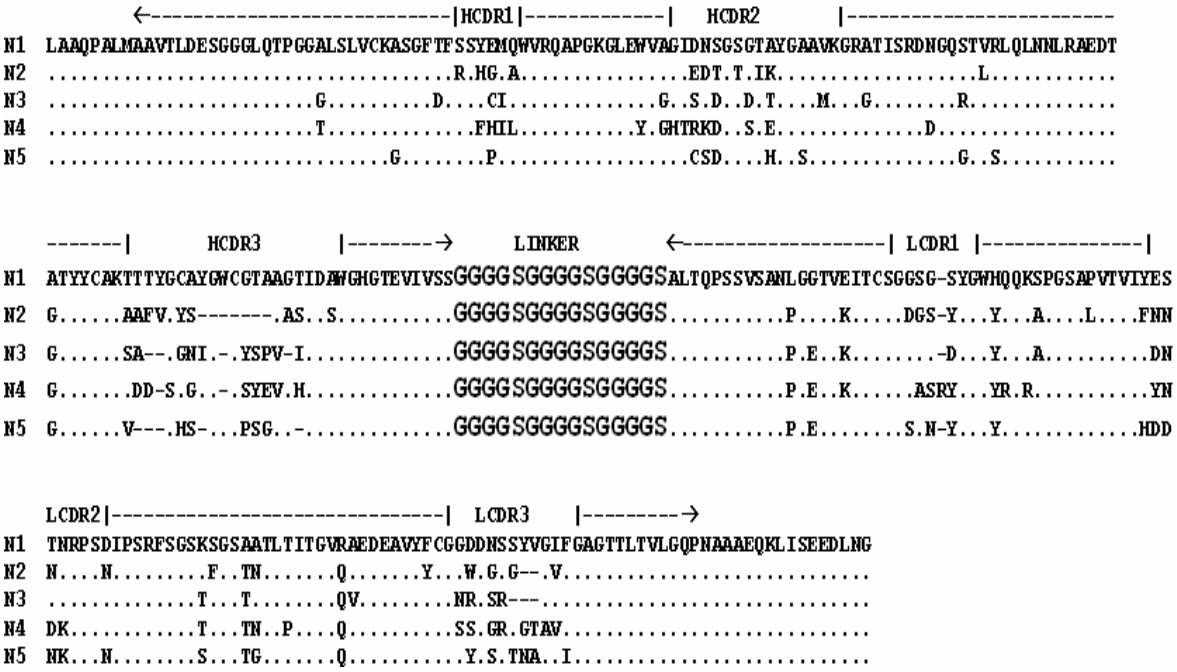

Figure 3.10: Amino acid sequence alignment of randomly chosen clones from “normal” linked antibody library. A normal 15 amino acid linker [$(G_4S)_3$] joins the V_H and V_L chains.

3.4 Biopanning

Since this was the first study in which a chicken antibody library was constructed by linking of heavy and light chain with a single amino acid (G) residue, the normally linked library was kept as an alternative in case panning failed with the “directly” linked library. Therefore antigen-specific antibodies were selected from the “directly” linked antibody library (6.7×10^7 clones). Four rounds of panning against each individual antigen were performed. Polyclonal phage ELISA indicated an enrichment of anti-LDH, -HRPII and -VSG phage antibodies with each round of panning. All the antigens except ALDO and MCFV showed ELISA signals after only the second round (Fig. 3.11).

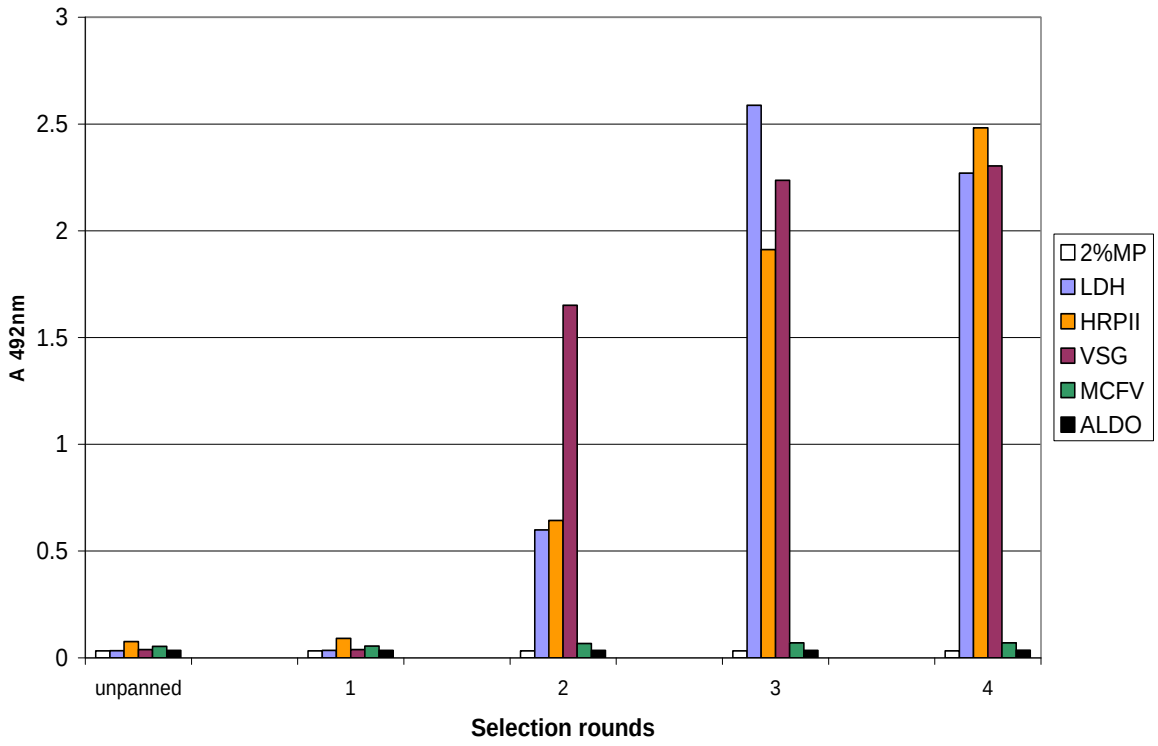

Figure 3.11: Polyclonal phage ELISA showing an increase in ELISA absorbance values from round 2 to 4 indicating an enrichment of LDH-, HRPII- and VSG-specific phage antibodies. No apparent enrichment occurred with ALDO and MCFV.

3.4.1 Selection of anti-LDH scFvs

Ninety individual clones resulting from the round 3 panning on LDH were cultured in a 96-well culture plate and screened for anti-LDH activity in a monoclonal phage ELISA. Of the 90 phage clones, 21 reacted with LDH (Fig. 3.12). After IPTG induction they were then all tested in soluble antibody fragment form using the supernatant directly in ELISA. Of 21 positive phage clones, only 12 reacted as scFvs (Fig. 3.13).

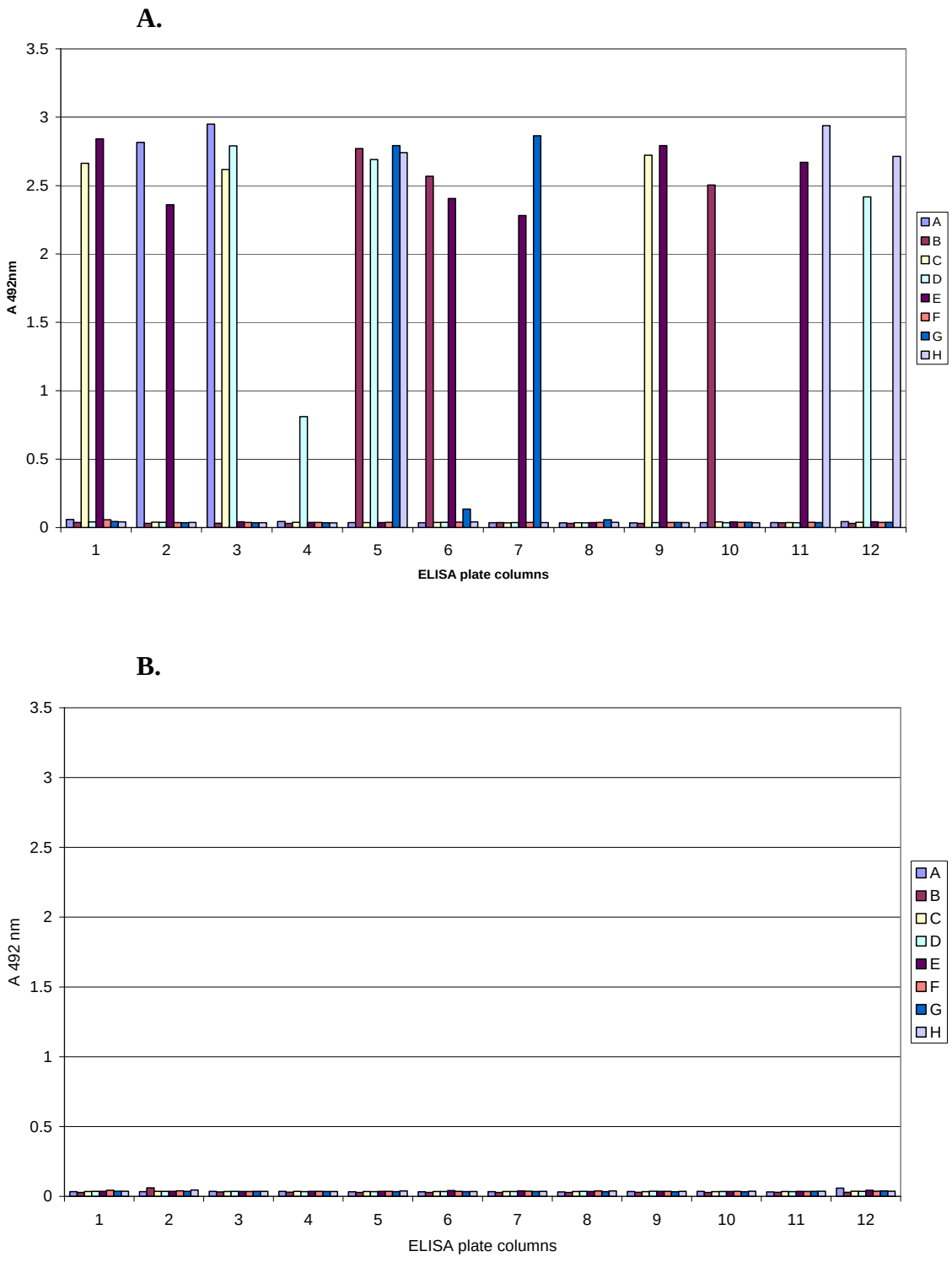

Figure 3.12: Monoclonal phage ELISA (A) on LDH with clones randomly chosen from LDH selection 3. The negative control 2% MP is shown in B.

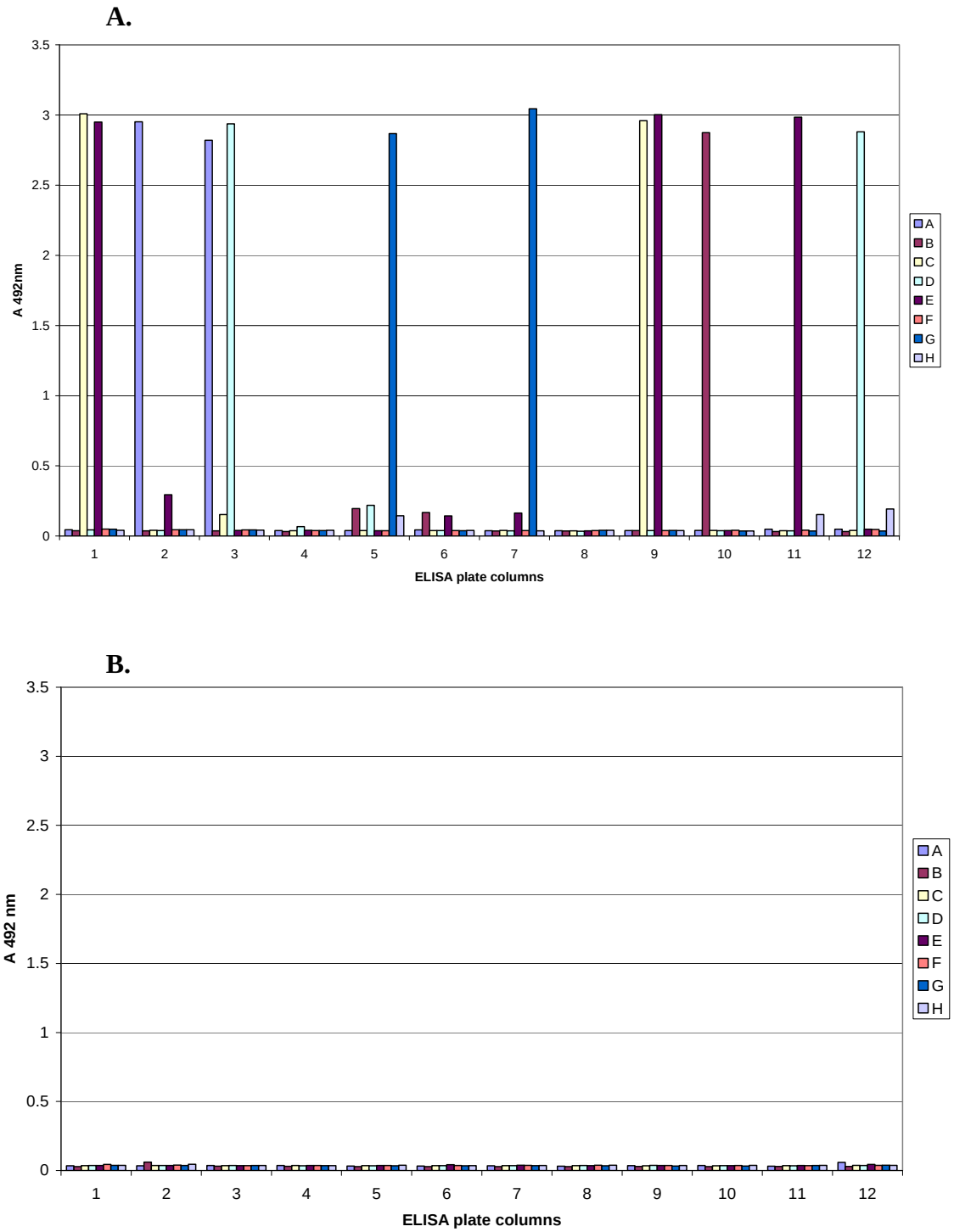

Figure 3.13: Soluble antibody fragment ELISA (A) corresponding to monoclonal phage clones shown in Fig. 3.12. The negative control 2% MP is shown in B.

Sequence analysis. The DNA coding for ten of the scFv binders was sequenced. Their amino acid sequences were deduced and the CDRs and FW regions were identified in each of the V_H and V_L chain fragments. Following alignment of the sequences with each other, five unique clones were identified. They were designated LA2, LB10, LC9, LD3 and LE 11. Although there were differences in the V_H and V_L chain CDRs, similarities were also noted between particular clones with some having identical V_H fragments. Clone LA2 and LD3 had identical V_H and V_L chain fragments with a major difference found in the FW region 1 of the V_L chain. Two clones, LB10 and LE11, expressed the same V_H chain but with two different V_L chains.

```
                ←---------FW1--------------|  CDR1 |-----FW2----|       CDR2       |----------FW3--------
Germline  --------AAVTLDESGGGLQTPGRALSLVCKASGFTFS  SYNMG  WVRQAPGKGLEFVA  GIDN-TGRYTGYGSAVKG  RATISRDNGQSTVRLQLNNLRAED
LA2       LAAQPALM.............G.............  DRG.H  ..........W..  Y.TSGD.S..Y..A....  ..................S.....
LD3       LAAQPALM.............G............G  DRG.H  ..........W..  Y.TSGD.S..Y..A....  ..................S.....
LB10      LAAQPALM.............G.......G...D..  ..E.Q  ..........W.G  L.NS-D.SD.Y..A....  ........R...............
LE11      LAAQPALM.............G.......G...D..  ..E.Q  ..........W.G  L.NS-D.SD.Y..A....  ........................
LC9       LAAQPALM............A..G.......G...SI.  ..A.N  ..........DY..  ..N-GD.SR.Y.AP..R.  .......D...............
                ←-------------------------------------------------------------VH--------------------------------

          --------|      CDR3         |--FW4--→                ←--------FW1------|    CDR1   |------FW2------|
Germline  TGTYYCAK  AAGT-------AGSIDA  WGHGTEVIVSS----------------ALTQPSSVSANPGGTVKITC  SGDSSYYG  WYQQKAPGSAPVTVIY  DNT
LA2       ..I.....  DGNGGYG----VDDV..  ...........G----------------.............E..EL..  ..GGR...  .....S..........  E.N
LD3       ..I.....  DGNGGYG----VDDV..  ...........GALTQPSSVSANLGGTV.............E..EL..  ..GGR...  .....S..G.......  E.N
LB10      ........  S.DSSYWYIDY.ED..T  ...........G----------------.P...........E..E...  ..SD....  .F...S.......L..  ..D
LE11      ........  S.DSSYWYIDY.ED..T  ...........G----------------.............E......  ..S.NN..  .............L..  A..
LC9       .AI.....  .S.SGGGW--G.SN...  ...........G----------------.............E......  ..GYID..  .....S..........  ..D
          ------------------------------------→                ←-----------------------------------------------

          CDR2 |------------FW3--------------|     CDR3     |---FW4--→
Germline  NRPS  NIPSRFSGSKSGSTATLTITGVRADDNAVYYC  ASTDSST-AGI  FGAGTTLTVL------------------
LA2       ....  D.....................Q.E.E...F.  GGY....NS-.  ...........GQPNAAAEQKLISEEDLNG
LD3       ....  D.....................Q.E.E...F.  GGY....NS-.  ...........GQPNAAAEQKLISEEDLNG
LB10      K...  .........F...H.......Q.E.E.....  GTE.NNY-S-.  ...........GQPNAAAEQKLISEEDLNG
LE11      ....  .........G............HV..E...F.  G.Y..NI---.  ........S.PGQPNAAAEQKLISEEDLNG
LC9       K...  G.............H.......QV..EG..F.  G.F.DNAGYV.  ...........GQPNAAAEQKLISEEDLNG
          ----------VL-----------------------------------------------→
```

Figure 3.14: Amino acid sequence alignment of anti- LDH scFvs LA2, LB10, LC9, LD3 and LE11 with the chicken germline Ig V_H and V_L. Diversity was found predominantly in the CDR regions.

In comparison with the germline sequence (Reynaud *et al*., 1983; 1989), up to seven additional amino acids were found in VHCDR3 of all identified fragments indicating that the gene conversion process had taken place (McCormack *et al*., 1993). Evidence of extensive somatic hypermutation was also found in the FW regions. This is shown by amino acid substitutions in the FW regions (Fig. 3.14).

ELISA. The identified clones were further tested in ELISA against both LDH and ALDO to further establish their specificity. All bound to LDH but not ALDO (Fig. 3.15a). The stability and hence potential usefulness of these fragments was then tested by incubating aliquots (supplemented with 2% sucrose) at room temperature (RT), 4ºC and -20ºC; and testing for binding in ELISA after one month. ScFv A2 and C9 were stable under all the tested conditions while others had partially or totally lost their binding ability depending on incubation temperature (Fig. 3.15b).

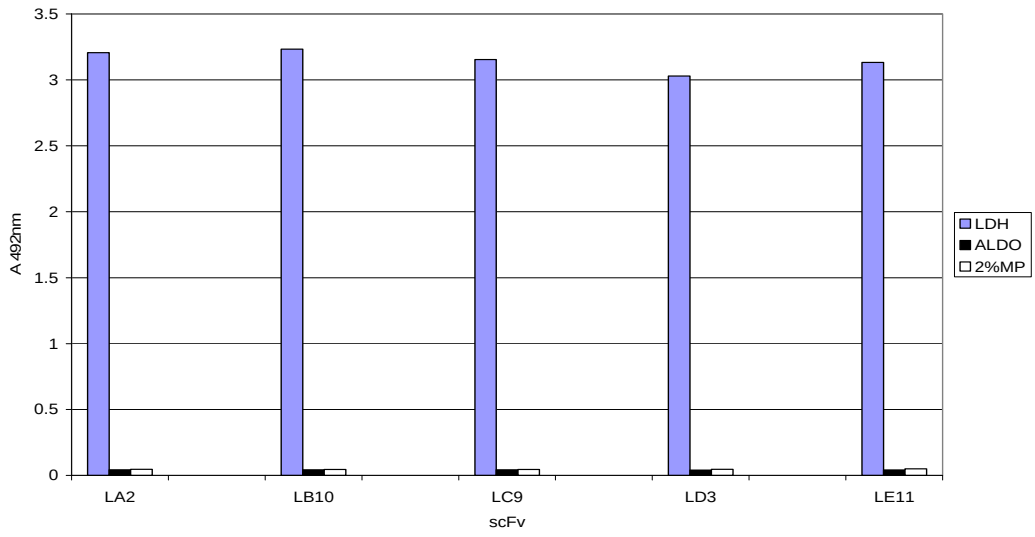

Figure 3.15(a): ELISA of selected anti-LDH scFvs showed specificity to LDH with no signal against ALDO and the negative control (2% MP).

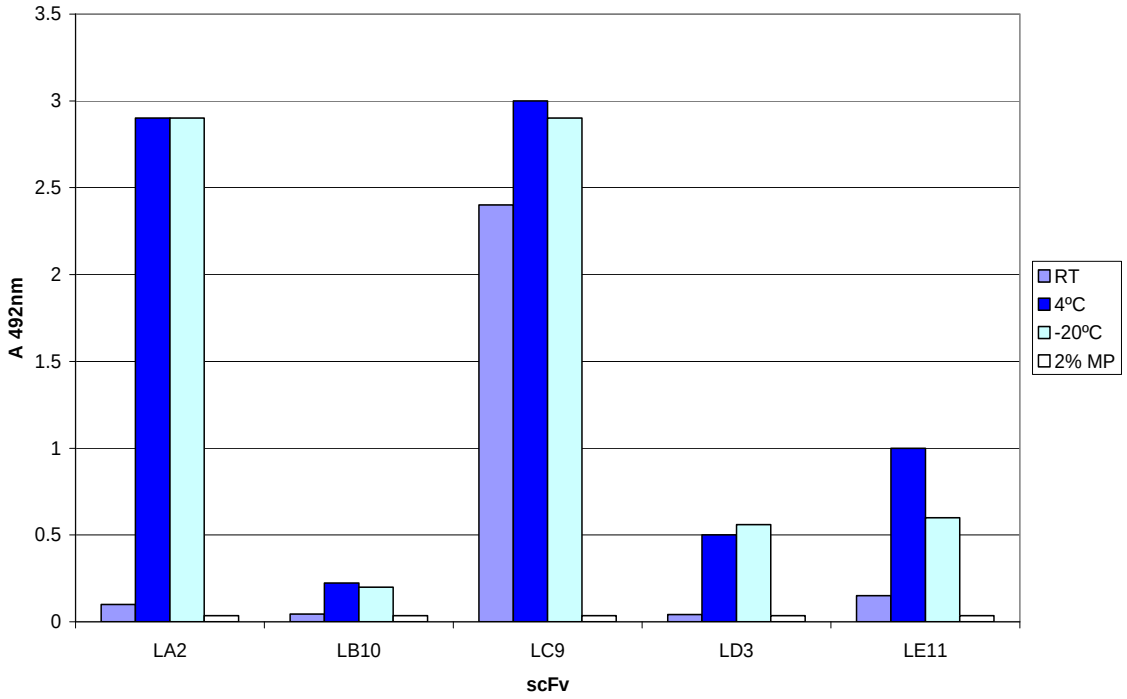

Figure 3.15(b): Stability test ELISA of anti-LDH scFvs that were stored for one month at RT, 4ºC and -20ºC. LA2 and LC9 were shown to be stable compared to other clones.

3.4.2 Selection of anti-HRPII scFvs

Ninety fusion phage clones from selection rounds 3 and 4 of the HRPII panning experiment were cultured in separate 96-well culture plates and tested for their binding to the target antigen in monoclonal phage ELISA. Of the 90 clones, 63 from selection round 3 and 59 from selection round 4 reacted with the antigen (Fig. 3.16 and 3.17). They were then all tested in the soluble antibody fragment form. All the positive phage clones from round 3 and round 4 also reacted as scFvs (Fig.3.18 and 3.19).

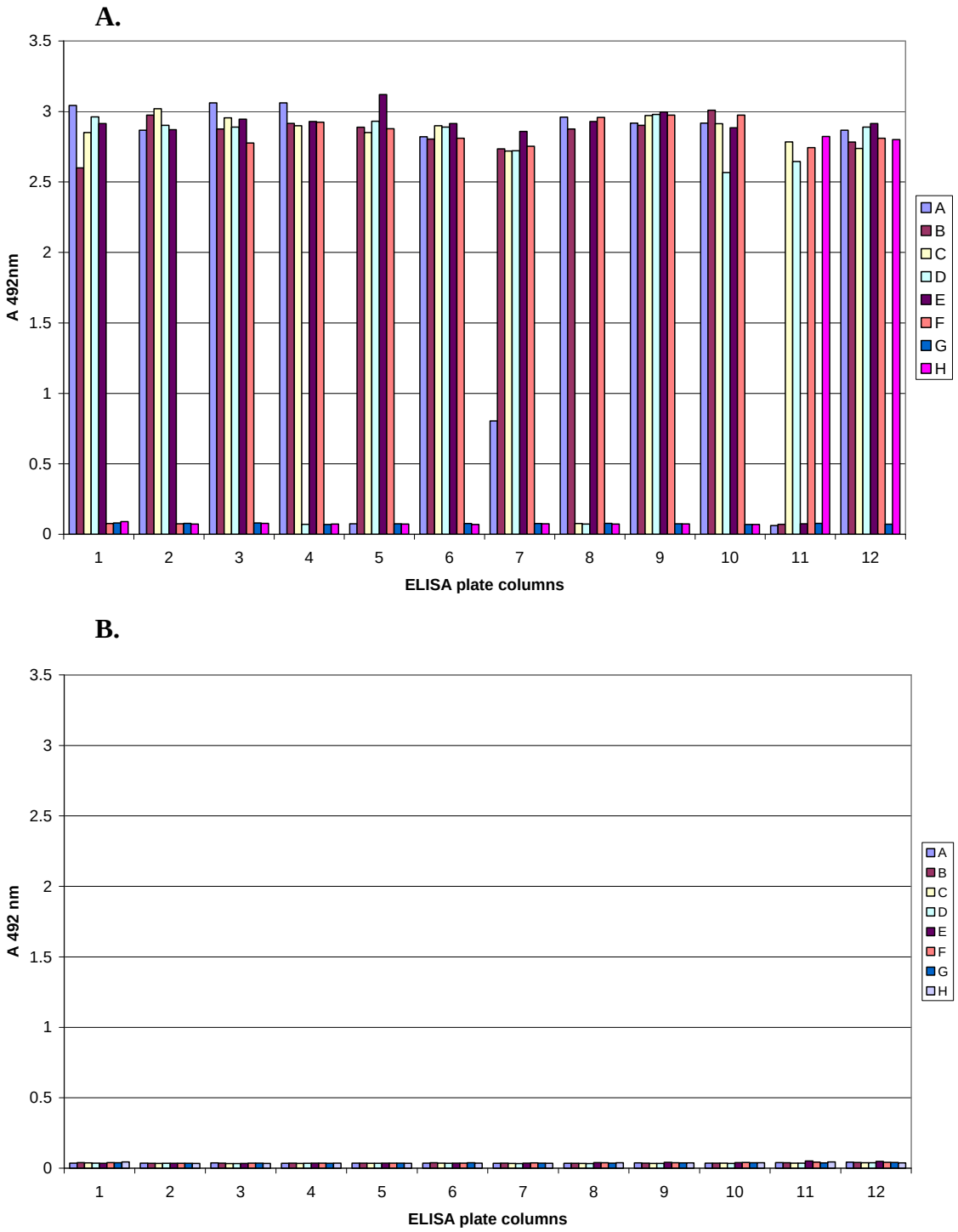

Figure 3.16: Monoclonal phage ELISA (A) on HRPII with clones randomly chosen from HRPII selection 3. The negative control 2% MP is shown in B.

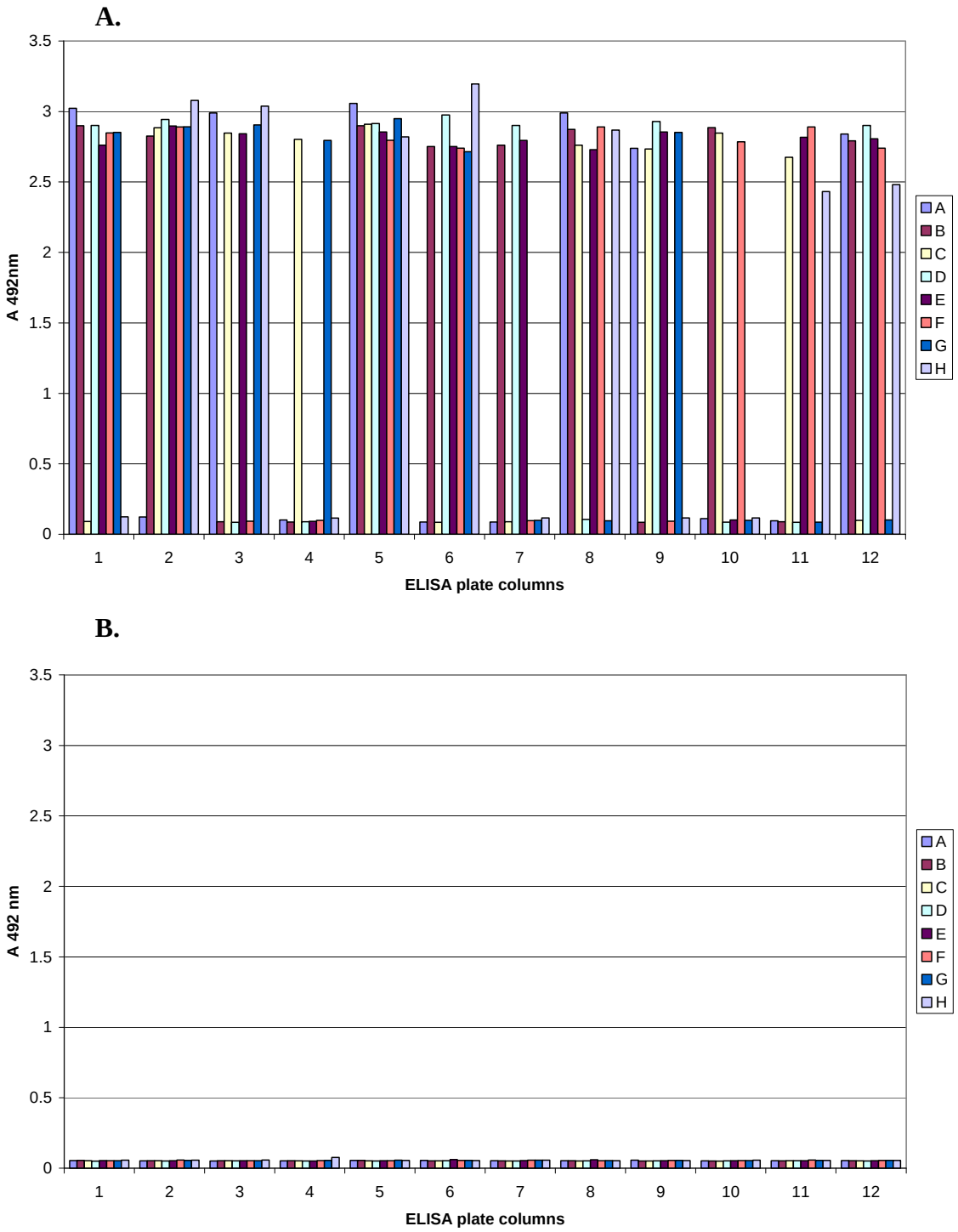

Figure 3.17: Monoclonal phage ELISA (A) on HRPII with clones from HRPII selection 4. The negative control 2% MP is shown in B.

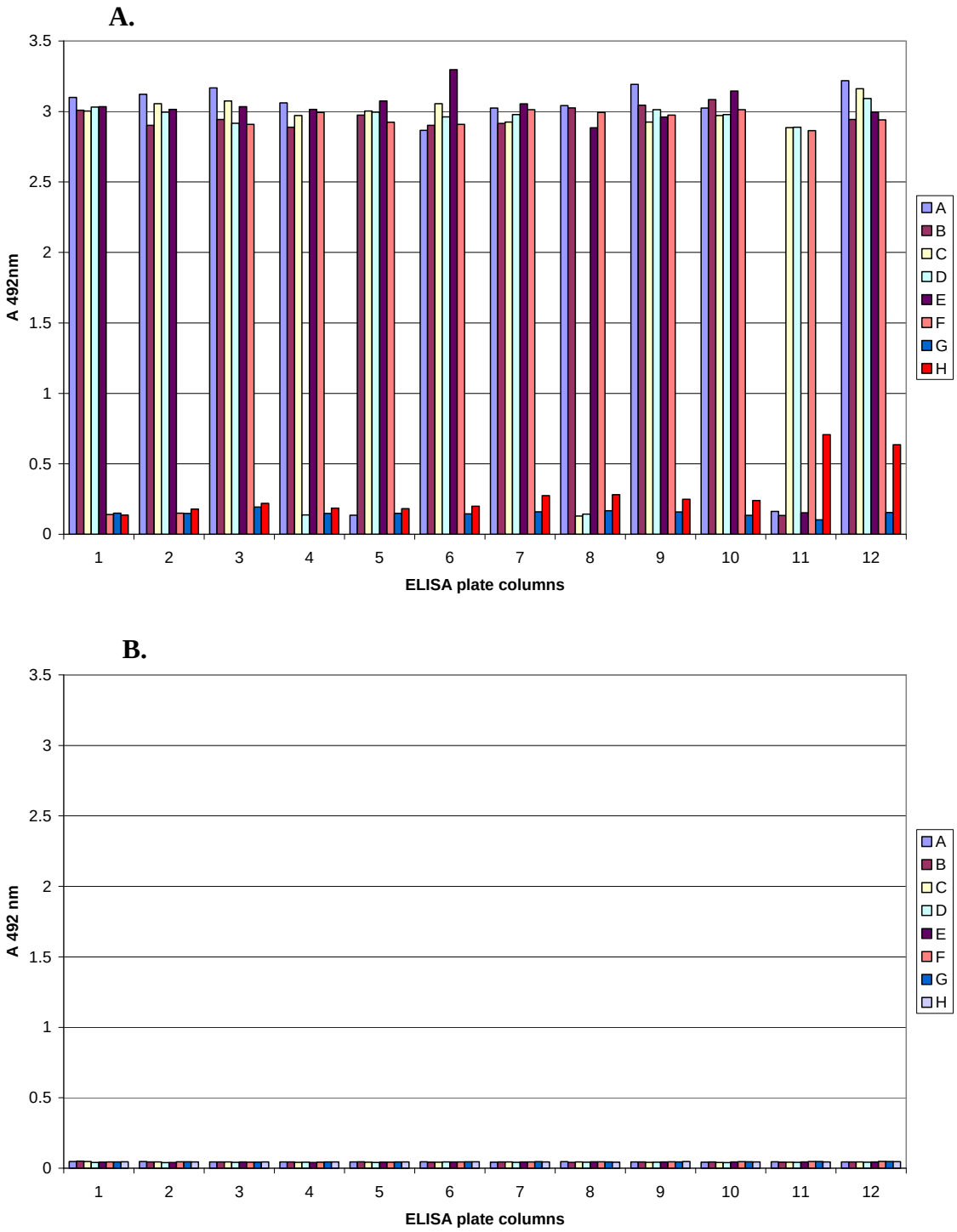

Figure 3.18: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 3. The negative control 2% MP is shown in B.

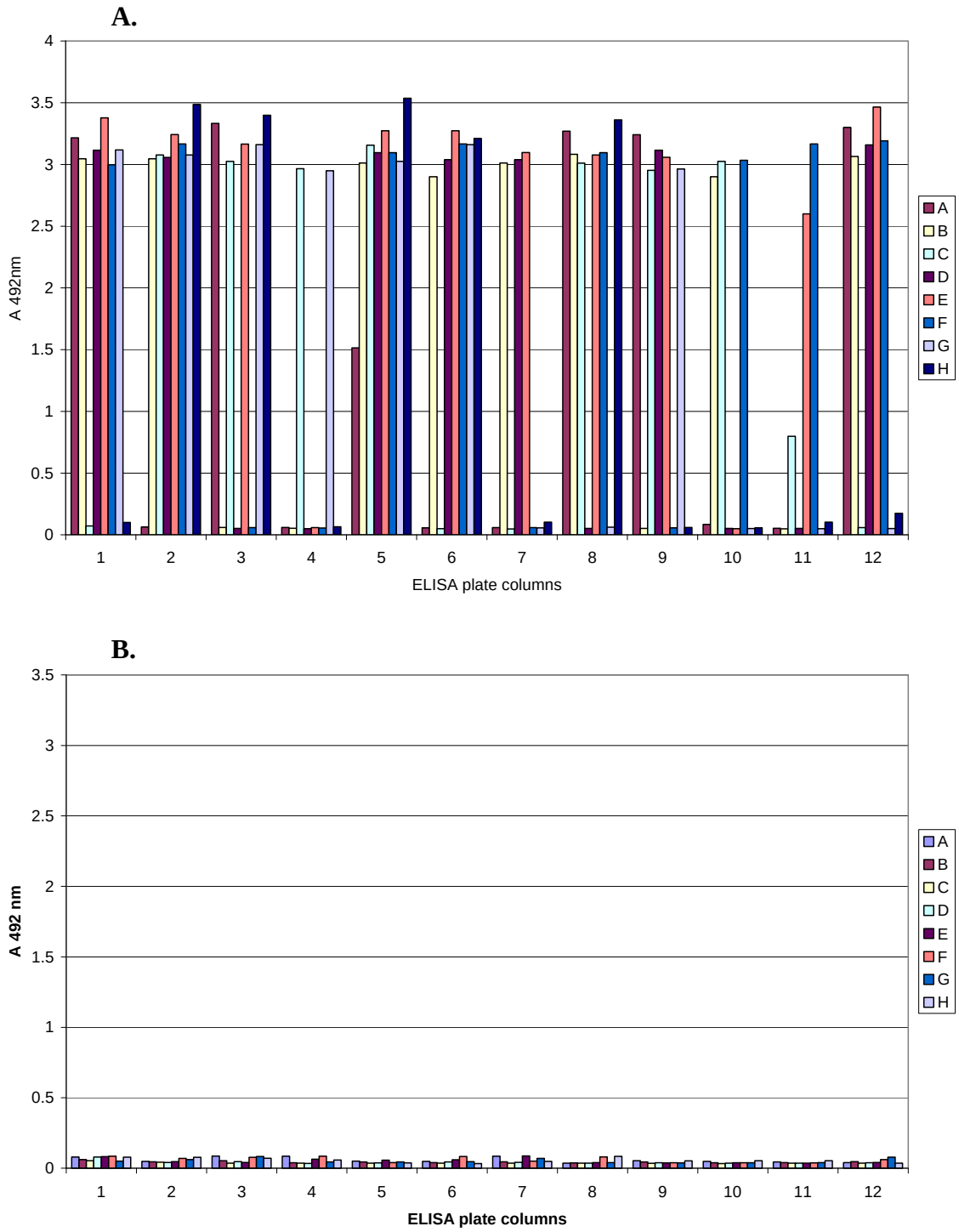

Figure 3.19: Soluble antibody fragment ELISA on HRPII (A) corresponding to monoclonal phage clones selected in round 4. The negative control 2% MP is shown in B.

Sequence analysis. DNA coding for 10 of the round 3 and the same number of round 4 scFv binders which had been randomly selected were sequenced. The amino acid sequences were deduced and the three CDRs and four FW regions were identified in each of the V_H and V_L chain fragments as expected. Following alignment, six unique clones were identified. They were designated HA2, HB5, HD2, HD6, HF11 and H4A5. Significant differences in the V_H and V_L chain CDRs were observed. In comparison with the germline sequence (Reynaud *et al*., 1983; 1989), there were up to eleven additional amino acids in VHCDR3 indicating that gene conversion had taken place (McCormack *et al*., 1993). There was evidence of somatic hypermutation in the FW regions; 1 to 3 amino acid variability was found among the clones (Fig. 3.20).

```
                ←---------FW1-------------|  CDR1 |---FW2-----|      CDR2        |-------------FW3-------
Germline  --------AAVTLDESGGGLQTPGRALSLVCKASGFTFS  SYMMG  WVRQAPGKGLEFVA  GIDNTGRYTGYGSAVKG  RATISRDNGQSTVRLQLNNLRAEDT
HA2       LAAQPAIM.................GG..........S.H  -...F  ..............  D.....GAW-.AP....  .......D.................
HD6       LAAQPAIM.................GG............R  .FG.F  ...........Y..  ...DD.AE.A..A....  ..................D......
HB5       LAAQPAIM.................GG..............  ..P.N  .......R..W..  ..ST-AS.PL.AP....  ............M............
H4A5      LAAQPAIM.................GV..........S..  DHG..  ...........I.  S.SDG.AR.N.......  ........................
HF11      LAAQPAIM.................GG.....XG...D..  R.E.H  ...........W..  ..SGS..T.Y.AP....  ........................
HD2       LAAQPAIM.................G...........D..  ..D..  ..............  ..HDD.SSAW.AT..Q.  .....K.........R.........
                ←-------------------------------------------------------------VH-----------------------------------

          -------|         CDR3          |---FW4--→ ←-----FW1---------|   CDR1   |------FW2------|   CDR2   |--------
Germline  GTYYCAK  AA----GTAG-------SIDA  WGHGTEVIVSS-ALTQPSSVSANPGGTVKITC  SGDSSYYG  WYQQKAPGSAPVTVIY  DNTNRPS  NIPSRFSGS
HA2       .......  TICDSY.WCSAG-----N...  ...........G.............E......  ..GRYK..  .F...S.........  E.NK...  D........
HD6       .......  S.YVCY.-C.-D-----....  ...........G.............E......  F.ATYS..  .....S.........  Y.DK...  D........
HB5       A......  S.YTGS.SSDGSVYSTPY..V  ...........G.........Y..E..R...  ..G.GS..  .....S.........  ..S....  .........
H4A5      .......  N.Y--A..LDGSL-----...  ...........G.............E......  ..GGN...  .....S.........  .......  .........
HF11      AI.F...  TTCI-N.WCSDAAL----...  ...........G.............E......  ..GG....  .F...S.........  GS.S...  .........
HD2       A....TR  GSSAGYSG.S--------...  ...........G.............E..E...  ..GDD-..  .....S.........  S.DK...  D........
          ----------------------------------------→ ←-------------------------------------------------VL-------------

          ----FW3---------------|      CDR3      |---FW4-→
Germline  KSGSTATLTITGVRADDNAVYYC  ASTDSSTA---GI  FGAGTTLTVL-------------------
HA2       .....H.......Q.E.E.....  GNA.DNDT----.  ..........GQPNAAAEQKLISEEDLNG
HD6       T............Q.E.E...F.  GTY.N.DT----.  ..........GQPNAAAEQKLISEEDLNG
HB5       A............Q.E.E.....  GGY..TD.GYV.T  ..........GQPNAAAEQKLISEEDLNG
H4A5      T....S.......Q.E.E...F.  G.Y...-.GYV..  ..........GQPNAAAEQKLISEEDLNG
HF11      .............Q.E.E...F.  GGY...D.----V  ..........GQPNAAAEQKLISEEDLNG
HD2       T............QVE.E...F.  GTAG.A---AV..  ..........GQPNAAAEQKLISEEDLNG
          ------------------------------------------------→
```

Figure 3.20: Amino acid sequence alignment of anti-HRPII scFvs HA2, HB5, HF11, HD2, HD6 and H4A5 with the chicken germline Ig V_H and V_L chain. All clones were different from one another and diversity was mainly concentrated in the CDR regions.

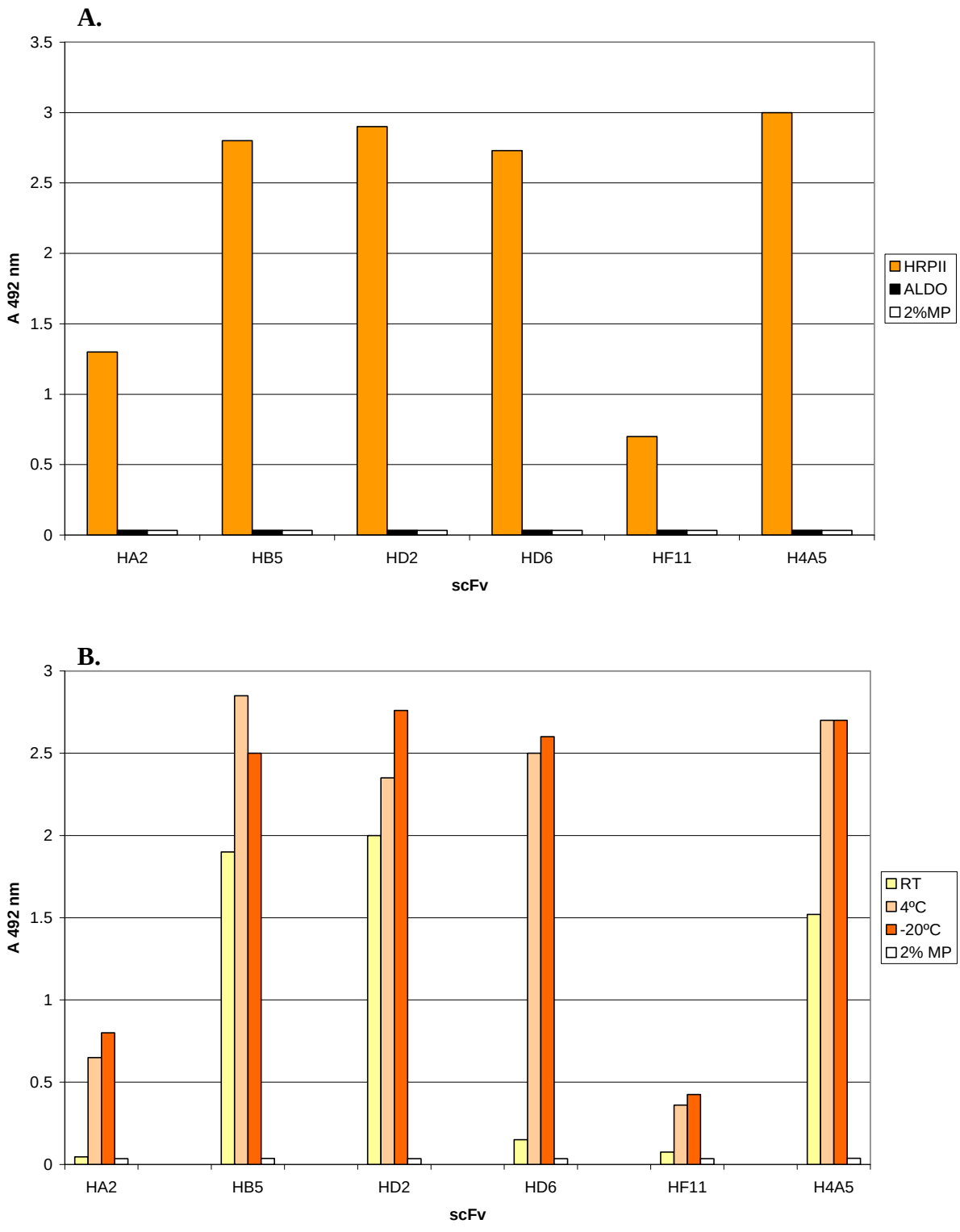

Figure 3.21: ELISA of selected scFvs on HRPII showed specificity to HRPII with no signal against immunising partner ALDO (A) and four scFvs showed stability following incubation at RT, 4ºC and -20ºC for one month (B).

ELISA. The identified clones were further tested in ELISA against HRPII and ALDO to determine their specificity. Each scFv showed specificity for HRPII and did not recognise ALDO or the negative control (2% MP) (Fig. 3.21 A). The stability at different temperatures (RT, 4ºC and -20ºC) was examined by ELISA after one month incubation. ScFv HB5, HD2 and H4A5 were stable under at all conditions. HD6 lost its binding ability after incubation at RT. The other scFvs showed a partial loss of binding ability at 4ºC and -20ºC while a complete failure to bind was observed after incubation at RT (Fig. 3.21 B).

3.4.3 Introduction of a flexible linker

A fifteen amino acid residue linker sequence was introduced into the DNA of selected anti-LDH clones in a two step PCR reaction (Fig. 3.22).

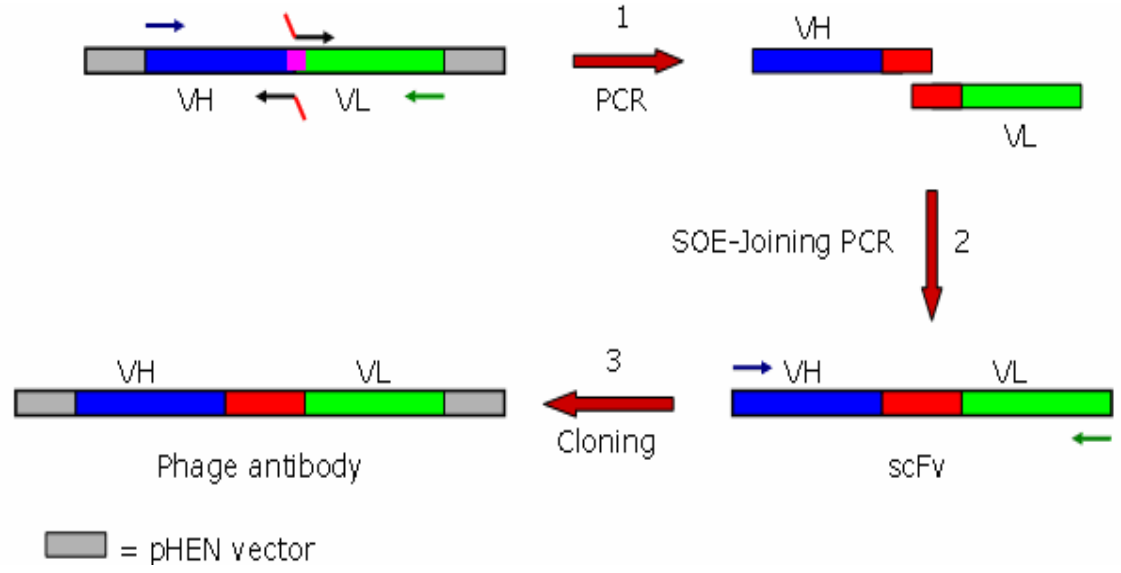

Figure 3.22: Diagram illustrating conversion of "directly" linked V_H-G-V_L scFvs to flexible linked V_H-$(G_4S)_3$-V_L scFvs using PCR and cloning into the pHEN displaying vector using *Sfi1/Not1* restriction sites.

Sequence analysis. DNA coding for the newly constructed anti-LDH scFvs was sequenced. Their amino acid sequences were deduced but N-B10 and N-E11 was found to be out-of-frame. N-A2, N-C9 and N-D3 were aligned to their parent clone amino acid sequences (results not shown). N-A2 and N-C9 had similar amino acid sequence to their parent clones with a flexible linker of fifteen residues joining V_H and V_L chains. One amino acid was missing in the VH chain of N-D3.

ELISA. The newly constructed scFvs designated N-A2, N-B10, N-C9, N-D3 and N-E11, were tested in ELISA (Fig. 3.23). N-A2, N-C9 and N-D3 retained their ability to recognise the target LDH, while N-B10 and N-E11 failed to recognise the target and N-D3 partially lost its binding ability.

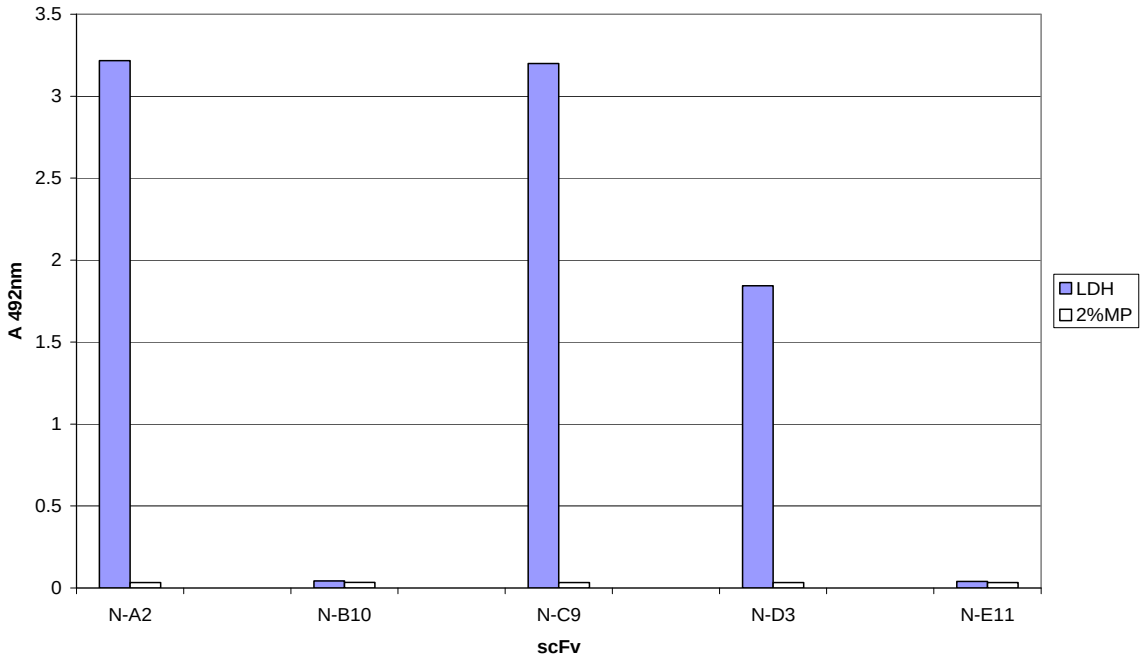

Figure 3.23: ELISA of newly constructed anti-LDH scFvs N-A2, N-B10, N-C9, N-D3 and N-E11. All scFvs retained their binding ability except the two scFvs N-B10 and N-E11 both of which failed to bind to LDH.

3.5 Identification of pseudo ψVL gene donors

The pseudo ψVL genes that donated their sequences during gene conversion were identified by comparing the amino acid sequences of selected scFvs to 25 known pseudo ψVL genes (Reynaud *et al*., 1987). Up to 15 pseudo ψVL genes appeared to have donated their sequences during gene conversion events. Between two to five pseudo ψVL genes were involved in the maturation of anti-LDH and anti-HRPII light chain clones. The list of pseudo ψV genes that appear to have donated their sequences during the diversification of VL chain sequences is shown in Table 2. Where more than one candidate pseudogene appeared to have been involved, all are listed (Fig. 3.24 and 3.25).

Table 2: The list of pseudo ψVL genes that were involved during gene conversion events.

Clone	FW1	CDR1	FW2	CDR2	FW3	CDR3
LA2	5*	5*	5*/10*	5*/10*	10*	10*
LD3	10* & 5*	5*	5*/10*	5*/10*	10*	10*
LB10	24*	4	10*	4	1/19	1/19
LC9	14*	13	7*	7*/8	8/14*	14*
LE11	5*	23	24*	17/GL	17/24*/5*	5*
HA2	13	13	6	12	12/19	12
HB5	13	10*	13	GL	GL/10*	10*
HD2	24*	18*	4	4	18*/8	8
HD6	14*	14*	7*	7*	5*/10*	5* or 10*
HF11	5*	5*	6	6	6/10*	10*
H4A5	5*	5*/23	23	GL	5*	5*

(* indicates donors sequences orientated in an inverted fashion; GL = germline)

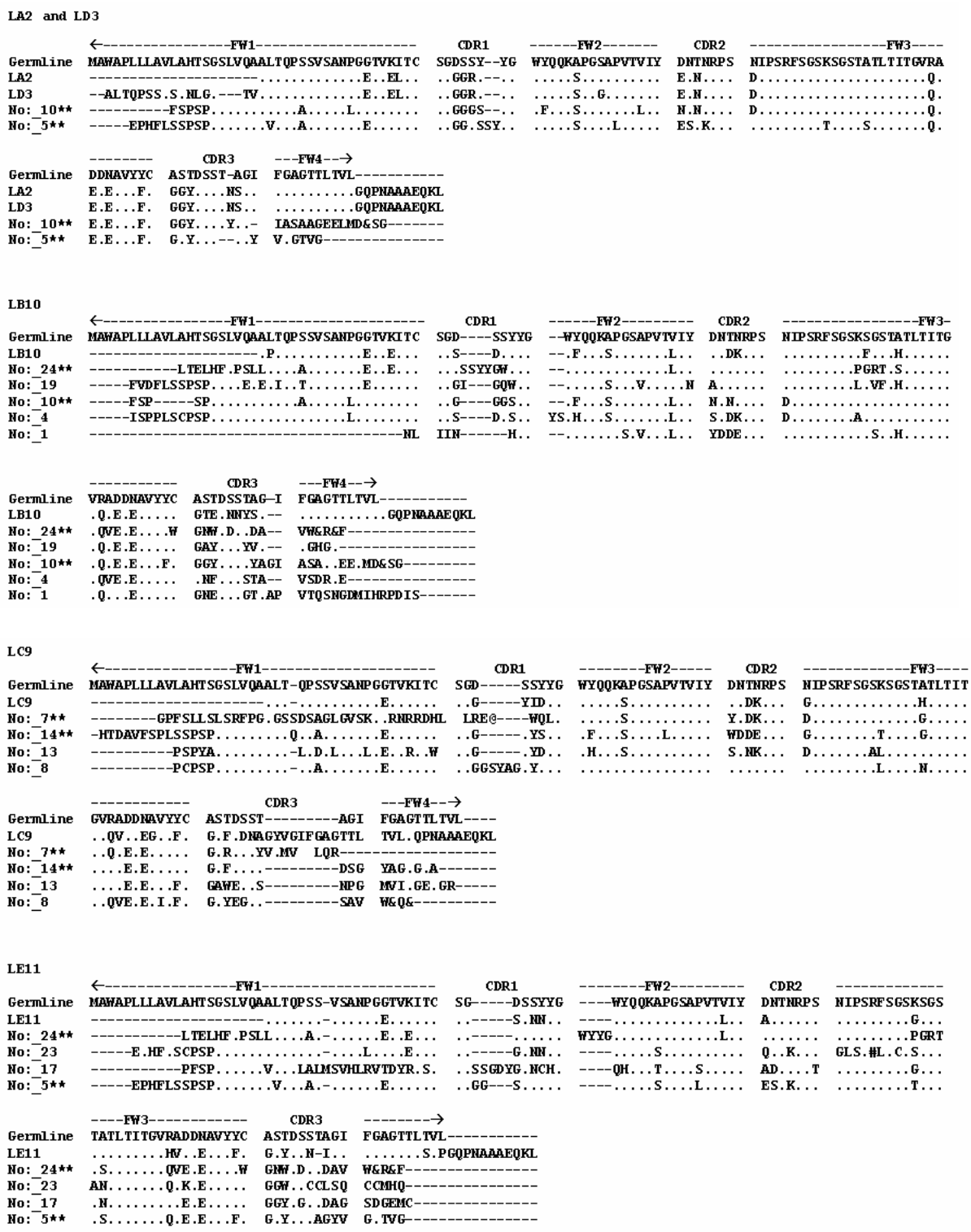

Figure 3.24: Alignment of anti-LDH V_L chain sequences to pseudo ψV_L gene donors. The V_L chain amino acids were assigned to their corresponding donor sequences.

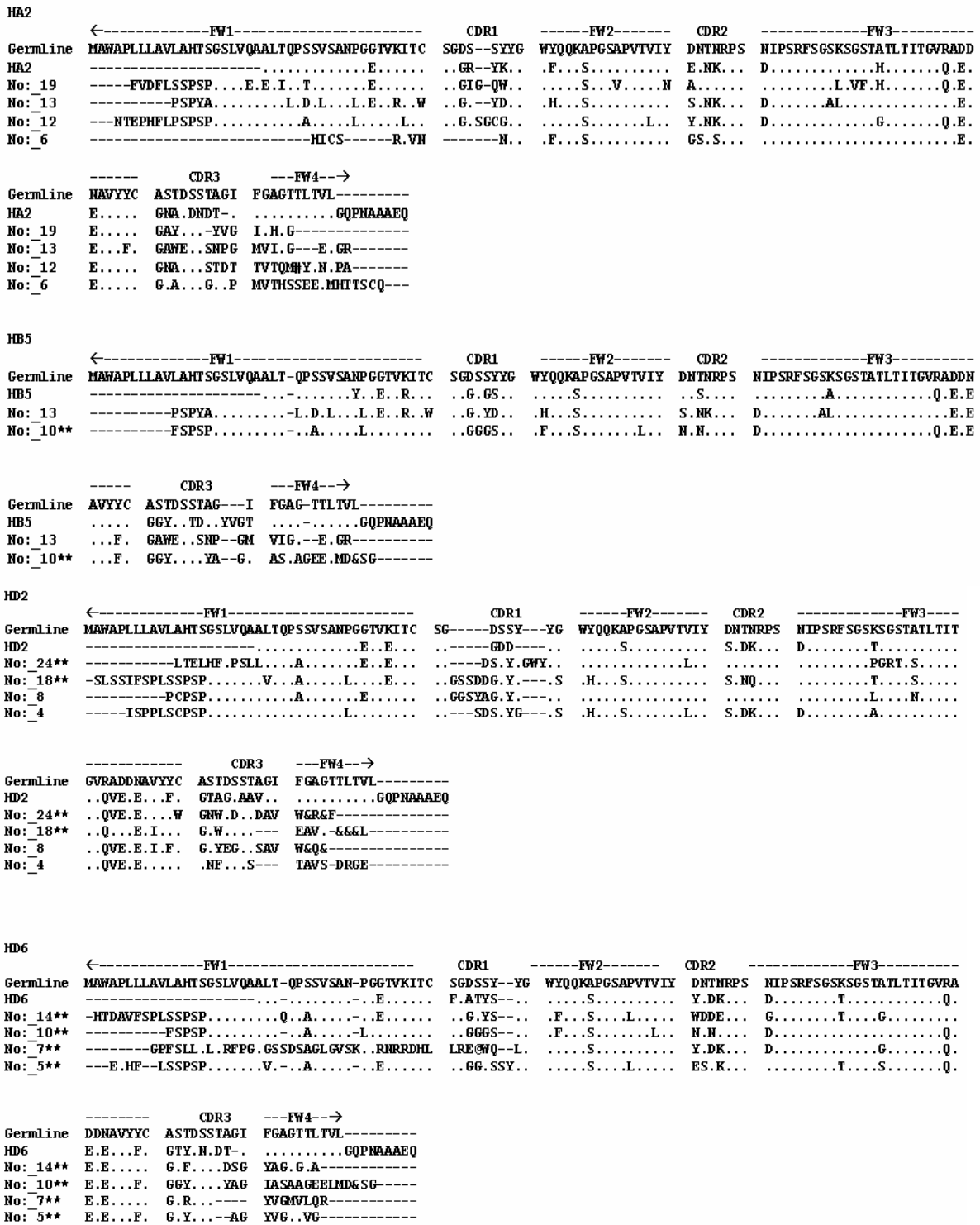

Figure 3.25 continues on the next page

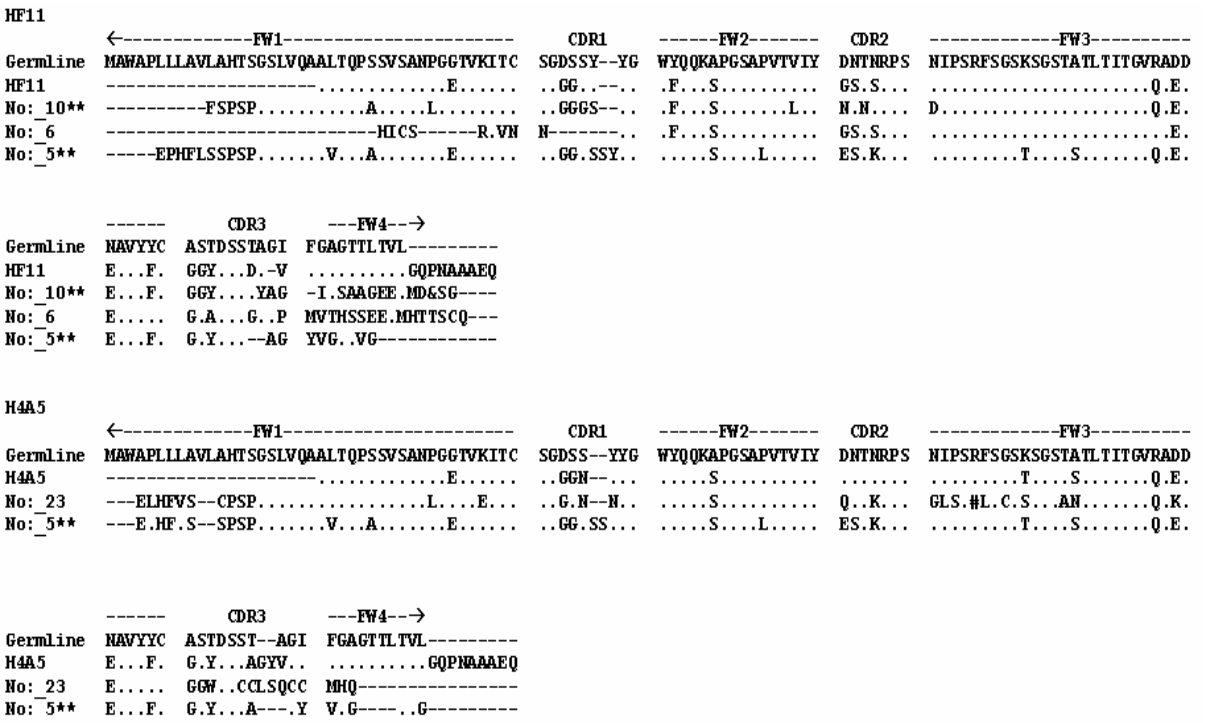

Figure 3.25: Alignment of anti-HRPII V_L chain sequences to pseudo ψV_L gene donors. The V_L chain amino acids were assigned to corresponding donor sequences.

3.6 Potential use of selected scFvs

Egg yolk antibodies (IgY) specific for LDH and the anti-LDH scFv LC9 were used to determine whether an scFv from the library could be used in modern system as a useful reagent in immunoassay development. Using chicken IgY as a capture antibody and LC9 as the detection antibody, a sandwich ELISA was set up for the detection of LDH antigen (Fig. 3.26). The same IgY was used to inhibit the binding of LC9 to LDH antigen in competitive inhibition ELISA (CI-ELISA). Up to 90% inhibition of LC9 was obtained when the IgY concentration was increased to 1 mg/ml (Fig. 3.27). The IgY directed against all antigens (“cocktail” IgY) was unable to either capture LDH (Fig. 3.26) or block LC9 binding to LDH (Fig. 3.28).

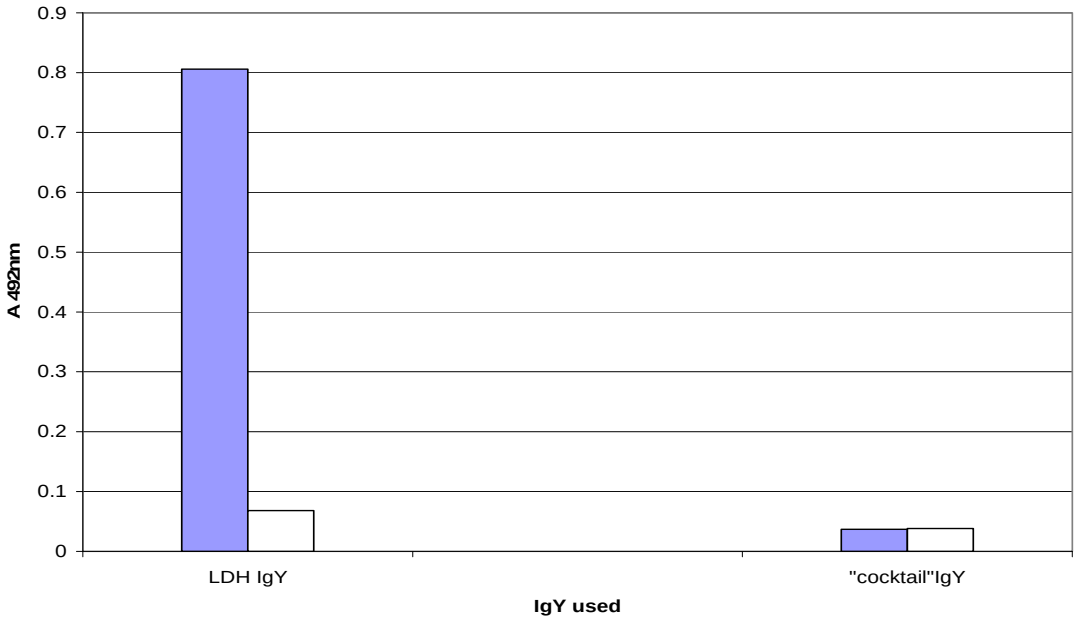

Figure 3.26: Sandwich ELISA using IgY as capture LDH and scFv LC9 specific for LDH as a detection antibody. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.

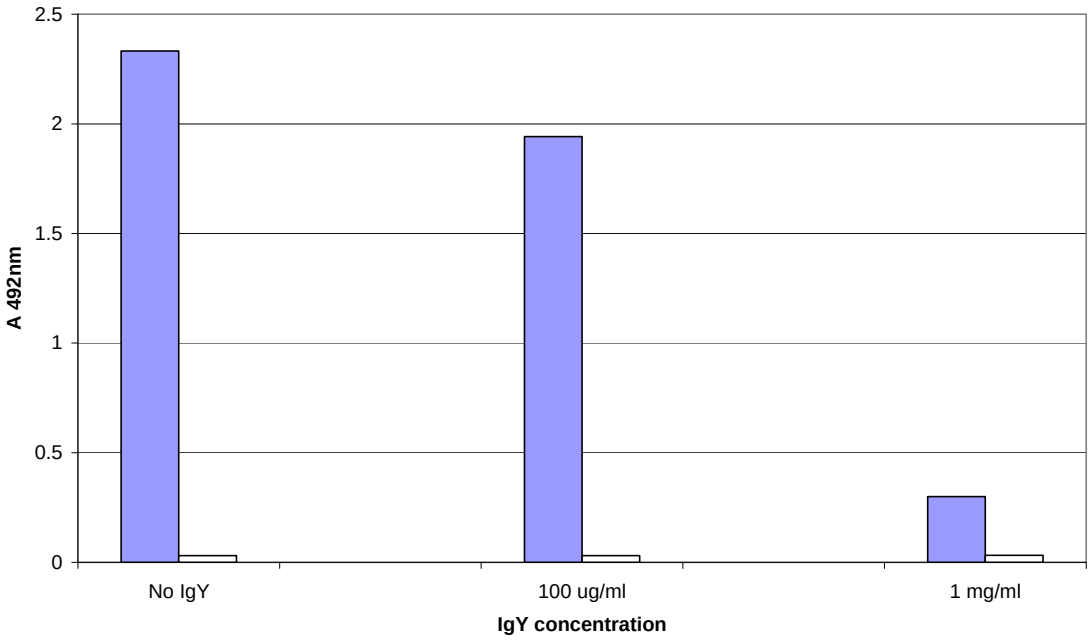

Figure 3.27: Competitive inhibition ELISA using LDH-specific IgY at different concentrations to block LC9 binding to immobilised LDH. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.

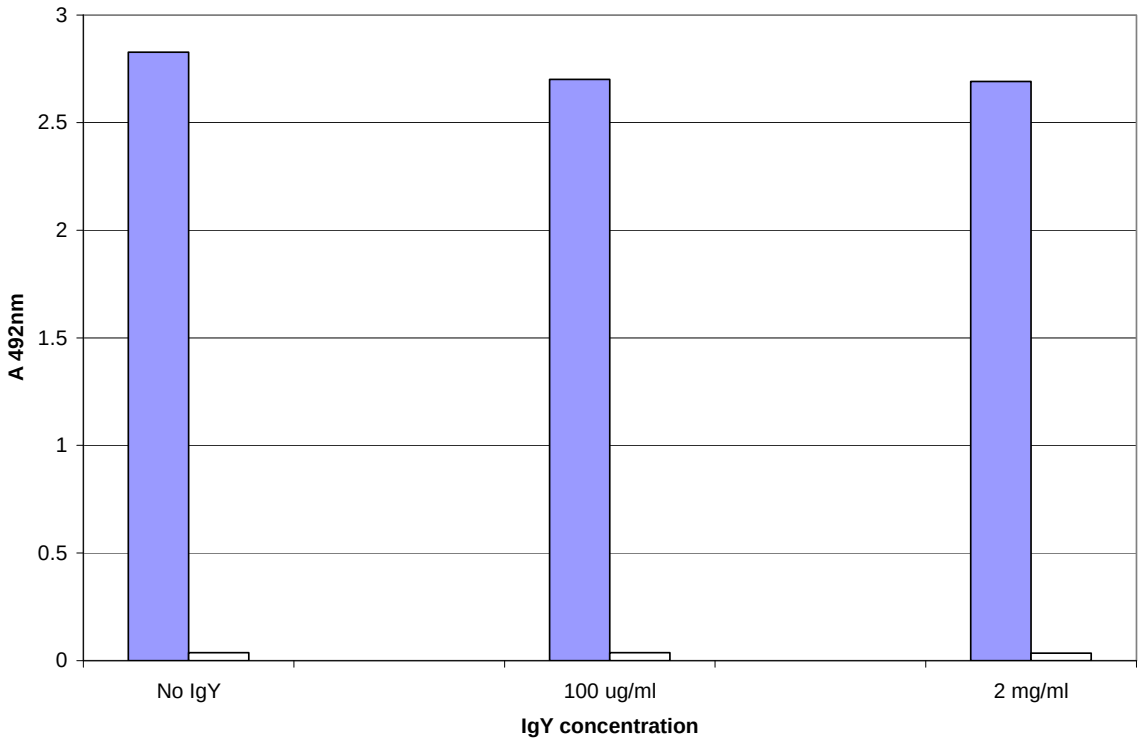

Figure 3.28: Competitive inhibition ELISA using "cocktail" IgY at different concentrations to block the LC9 binding to immobilised LDH. The blue bar represents ELISA reaction and white bar shows the negative control milk powder.

3.7 IgY immunoblotting of MCFV proteins

Selection of anti-MCFV scFv antibodies was unsuccessful, therefore IgY polyclonal antibodies were used to detect immunoblotted MCFV proteins to establish if the chicken produced antibodies to any of the known protein epitopes. Of eleven MCFV proteins known to elicit immune responses in animals, only four proteins with molecular weights of 130 kDa, 78 kDa, 24 kDa and ~55 kDa proteins respectively were predominantly recognised by the IgY in the immunoblot (Fig. 3.29).

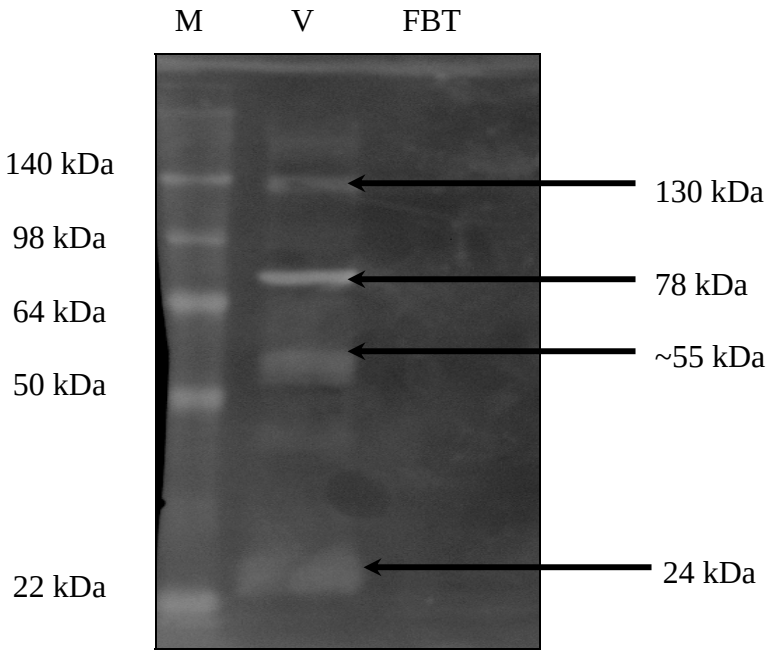

Figure 3.29: Western blot analysis of virus proteins of partially purified MCFV proteins immunoblotted with chicken IgY. The Pre-Stained Standard protein marker (Seeblue Plus2, Invitrogen) is loaded into lane M, V is the lane loaded with viral proteins and FBT is the lane loaded with the uninfected foetal bovine testes cells.

3.8 Other use of IgY

As with ALDO, initial attempts to enrich phage antibodies against HRPII failed. A new batch of HRPII was purchased (Immunology Consultant Lab, US) and tested in ELISA using “cocktail” polyclonal IgY. The new batch gave a six-fold higher ELISA signal than the old batch (Fig. 3.30) and anti-HRPII antibodies were successfully enriched using the new batch (see Fig. 3.11).

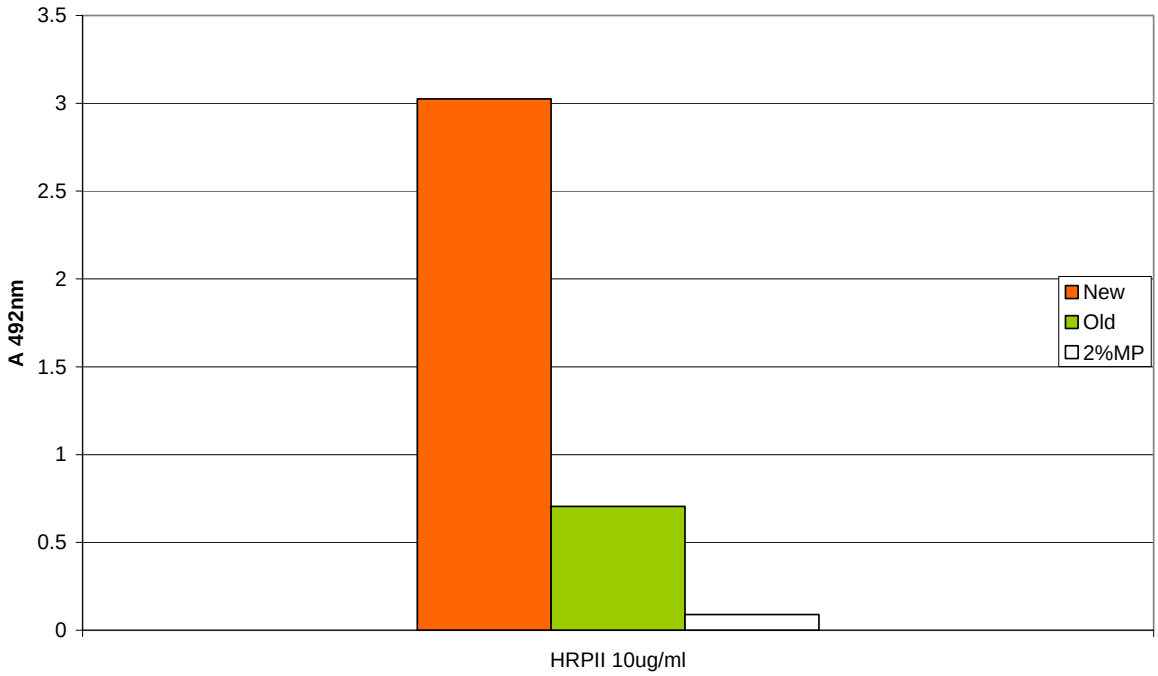

Figure 3.30: ELISA of the new batch of HRPII and comparison to the old batch.

CHAPTER 4: DISCUSSION

4.1 Chicken immune response to antigens

Two chickens, designated “A” and “B” were each immunised with a mixture of five different antigens (LDH, HRPII, ALDO, VSG and MCFV), all of which were emulsified in ISA70 adjuvant. ELISA was used to determine the amount of egg-yolk IgY (Fig. 3.2) produced in response to each of the antigens. Chicken “A” failed to produce antibodies to LDH (Fig. 3.3) while chicken “B” did not produce any antibodies to HRPII (Fig. 3.4) that were detectable in ELISA. This finding suggests that there may be limitations to the extent to which the avian immune system can be exploited and correlates with the prediction by Kaufman (2000) that some chickens infected with pathogens will not present all protective peptides to T cells even though appropriate peptides may in fact exist. In the chicken, only one of each class I and class II major histocompatibility complex (MHC) molecules is expressed. By immunising a chicken with many different target antigens its immune system may therefore become “overloaded” and be unable to simultaneously present peptides representing all the different antigens.

Class I molecules bind antigens that are present in the cell cytoplasm, while class II is associated with extracellular peptides. The human MHC locus has at least 280 genes and up to six different class I molecules and several different class II molecules are expressed. As a result, a large number of different peptides can be presented (Engelhard, 1994). In contrast, the chicken MHC locus, known as the B-locus is simple and compact. It consists of only 19 genes and is therefore approximately 20 times smaller than the human MHC. The chicken MHC determines the resistance and susceptibility to small and large pathogens (Kaufman *et al*., 1999a**;** Kaufman, 2000) and as mentioned, there are only two

copies of each class I and class II molecule genes in the B-locus. Only one of each is dominantly expressed (Kaufman *et al*., 1999a). This means there are fewer chances of an MHC molecule binding a foreign peptide to present to T cells which affects a number of important cell-mediated immune functions and also determines life or death in response to a pathogen (Kaufman *et al*., 1999b; Kaufman, 2000; Davison, 2003). The preferential dominant expression of a single class I molecule is said to be a 'suicidal strategy' which may sometimes be fatal to the chicken (Kaufman, 2000). For example, in the study by Sapats and her colleagues (2006), they reported the isolation of chicken scFvs specific for a very virulent infectious bursal disease virus. They infected chickens with live virus, but could only use spleens from chickens that survived. The bird's inability to present protective peptide antigen may be a reason for the death of the other chickens. Another example may be evident in the present study where it was found that only four MCF viral proteins including the proposed 130 kDa target protein earmarked for developing a diagnostic test out of eleven (Li *et al.*, 1995a; Adams and Hutt-Fletcher, 1990) were recognised by polyclonal antibodies (IgY) in immunoblotting (Fig. 3.29). The chickens produced antibodies against at least four viral antigens and three unrelated recombinant proteins. This antigenic load may represent a limit to the number of different antigens that the chicken immune system can handle simultaneously.

4.2 Antibody library construction

Only two sets of primers were necessary to access the diversified avian immunoglobulin genes by PCR amplification of the V_H and V_L gene fragments from the splenic RNA of both chickens as described in previous studies (Davies *et al*., 1995; Yamanaka *et al*., 1996; Andris-Widhopf *et al*., 2000; Sapats *et al*., 2003; van Wyngaardt *et al*., 2004;

Finlay *et al.*, 2005). In order to compensate for the failed production of antibodies to one antigen by each of the two chickens, the amplified V_H and V_L chain gene fragments were pooled. Two immune phage-displayed scFv libraries were constructed by artificial and random pairing of the V_H and V_L gene segments (Fig. 3.6). The essential difference between the two libraries was the length of the polypeptide linker sequence. A "standard" antibody library with a 15 amino acid [$(G_4S)_3$] linker joining the V_H and V_L chains was constructed as a benchmark (size of ~ 1.7 x 10^7 transformants), but was not further evaluated. The other library had the V_H and V_L chains linked by a single amino acid (G) residue. It had ~ 6.7 x 10^7 primary transformants. Both libraries consisted of highly diverse populations of scFv clones with the expected linker length separating the V_H and V_L chains (Fig. 3.9 and Fig. 3.10). The scFvs with normal-length flexible linkers are likely to be monomeric, providing the minimal antigen binding site (Plückthun and Pack, 1997). On the other hand, shortening a linker sequence allows the scFv to form diabodies, triabodies or tetrabodies which are multivalent but mono-specific, thereby increasing the avidity (Hudson and Kortt, 1999; Atwell *et al.*, 1999). On the phage surface multivalent antibodies are likely to be formed by association of soluble fragments with intact antibody-pIII fusion protein. These soluble fragments probably result from proteolysis of antibody-pIII fusion protein in the periplasmic space of *E. coli* during phage particle morphogenesis (Andries-Widhopf *et al.*, 2000). ScFvs with a shorter amino acid linker are known to perform better than normally linked scFvs when tested in ELISA and are generally advantageous in selection against epitopes associated with a low intrinsic affinity per binding site. The higher avidity is due to the potential for multivalent interaction with separate antigenic determents (Plückthun and Pack, 1997; Hudson and Kortt, 1999; Ravn *et al.*, 2004). While described previously for the construction of a

mouse sub-library by Ravn and colleagues (2004) for panning, this is the first chicken scFv antibody library known to have been constructed by joining V_H and V_L chains with a single amino acid residue. This study mainly focuses on evaluating the short-linker repertoire as a source of antigen-specific scFvs specific for five different antigens.

4.3 Antibody selection

There was enrichment of antigen-specific phage antibodies directed against LDH, HRPII and VSG after only two rounds of panning (Fig. 3.11). The rapid selection of specific antibodies suggests that antigen-specific clones are well-represented within the scFv population (Finlay *et al*., 2005). With "naïve" antibody libraries, it usually takes three to four or more rounds of panning to produce highly specific signals (van Wyngaardt *et al*., 2004). Although the IgY ELISA indicated the presence of antigen-specific antibodies, no ALDO and MCFV-specific phage antibodies could be obtained even though colony count calculations indicated enrichment after each successive round of panning. The failure to enrich specific phage antibodies against ALDO (Fig. 3.11) may simply have been an artefact due to poor handling of the target enzyme during shipment from the supplier. Initial panning against HRPII also failed, but phage antibodies were obtained after a new batch of antigen was used. Since MCFV is lipid-enveloped, stringent washing with the detergent during panning may be the cause of the failure to enrich viral-specific phage antibodies. In the immunoblot, polyclonal antibodies (IgY) did indeed recognise several viral proteins. Other approaches such as panning on expressed recombinant viral protein may therefore enable anti-MCFV antibodies to be successfully selected and the library may yet be useful in the future as a source of highly specific anti-MCFV scFvs.

Selection of specific scFvs against VSG was suspended due to the limited availability of antigen. Nevertheless, the library yielded several antigen-specific (LDH and HRPII) antibodies that recognised their cognate antigens. DNA sequencing of clones found to be positive in ELISA allowed unique clones to be identified. Five scFvs against LDH and six scFvs against HRPII were identified. Following alignment of their amino acid sequences, considerable hypermutation and extension of CDR regions was noted in comparison with the germline sequence of the V_H and V_L chains (Fig. 3.14 and 3.21). Compared to CDR3 of both the V_H and V_L chains, the clones exhibited less variability in their CDR1 and CDR2 regions. Amino acid residues that appear to be antigen-specific were found in CDR2 region of all anti-LDH V_H chains (Fig. 3.14), suggesting that antigen-driven gene conversion events had occurred during the *in vivo* immune response. The length of the anti-LDH HCDR3 varied with up to seven additional amino acid residues compared to the germline HCDR3 (Fig. 3.14). The HRPII binders had up to eleven amino acid residues more in their HCDR3s. The amino acid residue extensions found in the HCDR3 regions differed from antigen to antigen suggesting that these regions could be the major determining factor for the antigen binding specificity (Reynaud *et al.*, 1989; McCormack *et al.*, 1993; Nissim *et al.*, 1994; Xu and Davis, 2000; Finlay *et al.*, 2005).

The V_H chain of the LDH-binding clones, LA2 and LD3 CDR regions are identical with three more residues in CDR3 and there is one amino acid change in the FW1. The major difference in the V_L chain was in the FW1 region; a repeat of the first 16 amino acid sequences of the FW1 region was present in the V_L chain of LD3 (Fig. 3.14). When aligned, they differed in positions 12 and 14. The two amino acid differences suggest that

the final V_L chain (LD3) product could have resulted from multiple rounds of gene conversion events with different pseudogene donors (Cohen and Givol, 1983; Reynaud *et al*., 1987; McCormack *et al*., 1993, Yamanaka *et al*., 1996). If true, then the LA2 V_L chain resulted from the inner primer binding site found in the parent hybrid V_L chain clone (LD3) as a result of sequence repeats. The CDR regions of the LA2 and LD3 V_L chains are identical with only one amino acid change in FW2 region. This could possibly have resulted from a PCR error during amplification. Although the role of V_H chain in antigen binding is well documented, clone LA2 and LD3 V_L chains may be important in defining the fine antigen binding (Sapats *et al.*, 2003) and the stability of the selected scFv (Fig. 3.15 b). The V_H chain of LB10 and LE11 were similar in their CDR regions with seven more residues in CDR3 and one amino acid change in the FW3 region. Their V_L chains differed significantly in both CDR and FW regions. The LC9 scFv had its own unique V_H (five more amino acids in CDR3) and a V_L chain that differed from the other scFvs. The anti-HRPII scFvs' entire V_H and V_L chains differed significantly from one another in both CDR and FW regions (Fig. 3.20). HA2 and HF11 have six, HB5 have eleven and HD6 and H4A5 have four more amino acid residues than the germline in HCDR3 (Fig. 3.20).

4.4 Stability testing

The supernatant fluids containing the LDH and HRPII binders was stored at different temperatures, namely room temperature, 4°C and -20°C for one month and then tested in ELISA to determine their ability to recognise their antigens. Four (LA2, LB10, LD3 and LE11) anti-LDH scFvs completely lost binding ability after a month at RT. Significant loss (66% reduction of ELISA signal) of binding at 4°C and -20°C occurred in LB10,

LD3 and LE11 while LA2 remained stable. LC9 continued to recognise antigen under all the conditions suggesting the antibody remained stable (Fig. 3.15(b)). Three anti-HRPII scFvs HA2, HD6 and HF11 lost binding ability at RT with a partial loss at 4°C and -20°C for HF11. HB5, HD2 and H4A5 were stable at all tested conditions. The stability of selected scFvs is a key determinant of good candidates for development of a diagnostic immunoassay for field application in areas where storage facilities are not available.

4.5 Donor sequences

The V_L chain pseudogene (ψV_L) donor sequences have been well characterised for the chicken (Reynaud *et al*., 1987). This made it feasible to trace those pseudogenes which had donated their sequences during V_L chain gene conversion. By comparing the CDR and FW region amino acid sequences to the germline, non-germline amino acids were assigned to ψV_L donors where possible. More than one ψV_L donor was involved in diversification of each of the selected V_L chain clones indicating that the selected clones had undergone several rounds of gene conversion during *in vivo* affinity maturation. Based on the number of potential donors, two for LA2, LD3, HB5 and H4A5; three for HF11; four for LE11, HA2, HD2 and HD6; and five for LB10 and LC9 gene conversion events had occurred (Fig. 3.24 and 3.25). The boundary between donor sequences and germline sequences is not well-defined. In some cases germline sequences were found in CDR2 regions (LE11, HB5 and H4A5). An inability to trace the origins of other amino acid residues is likely to be due to extensive somatic hypermutation that resulted in mutations occurring evenly around the rearranged V genes (Gearhart and Bogenhagen, 1983; Parvari *et al*., 1990). An overlap of donor sequences from different ψV_L donors in a single region (either CDR or FW) was found. McCormack and Thompson (1990) also

reported an overlap between two gene conversion events which prevented an accurate description of either ends of the V_L chain sequences. A sixteen amino acid residue repeat was found at the beginning of FW1 region of clone LD3. The origin of these sequences was traced using amino acids at positions 12 and 14 to enable allocation of possible donors. Corresponding sequences belonged to ψV_L 10 and ψV_L 5. The fact that the LD3 clone consists of amino acids from only two ψV_L donors supports the suggestion that FW1 is a hybrid of two donor sequences formed during two separate gene conversion events.

An examination of the V_L chains amino acid sequences showed that the pseudo ψV_L gene donors that were closer, and those in an inverted orientation regardless of their location (closet or distant) with respect to the functional V_L locus, were predominantly used during gene conversion. Ten of the eleven selected V_L clones had at least one donor sequence in an inverted orientation. The V_L chain gene conversion events that took place in this study are therefore similar to those reported previously (Reynaud *et al*., 1987; McCormack and Thompson, 1990; McCormack *et al*., 1993). Although the origin of V_H chain sequences could not be traced because the ψV_H sequence had not been characterised, evidence of gene conversion events is found in the HCDR3 (Fig. 3.14 and 3.20) and HCRD2 of LDH clones (Fig. 3.14).

4.6 Introduction of a flexible linker

In order to evaluate the reported superiority of scFvs joined with a single amino acid residue, a flexible fifteen amino acid linker was introduced into the scFvs. N-A2 and N-C9 recognised LDH in ELISA (Fig. 3.23) since these scFvs were in-frame and have

correct parent sequences. N-D3 had partially lost its binding ability with N-B10 and N-E11 unable to bind LDH. N-D3 had a missing codon in V_H and N-B10 and N-E11 were completely out-of-frame. Therefore no conclusion could be drawn due to the fact that sequence analysis revealed changes in nucleotide sequences which may have resulted from PCR errors.

4.7 Potential use of selected scFvs

The usefulness of selected scFvs in diagnostic reagents was tested by setting-up sandwich and CI-ELISAs using an LDH-specific IgY prepared by Wouter Wyngaardt (Fig. 3.26 and 3.27) and anti-LDH LC9 scFv. The “cocktail” IgY failed to either trap LDH or block scFv binding (Fig. 3.26 and 3.28). This may be due to the broader spectrum of this serum as it consists of antibodies against three different proteins and at least four other viral proteins. The stable scFv LC9 was chosen from the selected anti-LDH antibodies stored at 4˚C or -20˚C. The fact that binding to LDH by LC9 was inhibited, also demonstrates the specificity of the selected scFvs from the constructed library. Therefore, these scFvs have the potential to be of use in developing diagnostic immunoassays aimed at capturing antigens or detecting serum antibodies.

4.8 Conclusion

The successful isolation of recombinant antibodies from a single multiple targeted immune library illustrates the power of phage-display technology for producing potentially useful diagnostic and therapeutic reagents. The rapid enrichment of antigen-specific phage antibodies suggests that the spleen may have been enriched in affinity matured antibody clones or could have been due to the multivalency of tri- or tetrabodies

that resulted from joining of V_H and V_L chain with a single amino acid linker. The avian immune system has been shown to have some potential use in raising recombinant antibodies against multiple target antigens. This can be achieved by immunising a single chicken with a cocktail of different target antigens. Depending on the combination and complexity of antigens used, useful and stable antigen-specific scFvs to each of the immunising partners can be selected in a relatively short time frame. Several unique and stable antibodies were identified that might be useful for diagnostic applications. The ability of selected scFvs for use in development of diagnostic immunoassays was demonstrated in sandwich ELISA and CI-ELISA. The resulting libraries are likely to remain a rich source of highly-specific single chain antibody fragments to a number of MCFV proteins as shown in the immunoblot using IgY (Fig. 3.28). In addition, the polyclonal antibodies (IgY) raised in the course of the study can be used in research. This is the first study in which it was demonstrated that a single chicken can be used to raise recombinant antibodies against more than two different target antigens. This study therefore extends work by Finlay and colleagues (2005) where a single chicken was used to raise antibodies against two target antigens. The approach described here can be seen as an alternative to the traditional construction of a single immune antibody library for a single target antigen. It makes minimal use of animals and may therefore be cost-effective. Lastly, high levels of antigen-driven gene conversion events were shown in this study. In support of the previous reports, it was found that there is indeed a preferential usage of pseudogene donor sequences during gene conversion which appears to be determined by its proximity and its orientation in relation to the functional variable gene locus.

Future studies should establish more precisely the number of recombinant antigens that the chicken immune system can handle simultaneously. The immunised birds are a potentially important source of antibodies as a result of *in vivo* affinity maturation. For therapeutic applications chicken antibodies can be converted to complete human antibodies by the process called humanisation (Nishibori *et al*., 2006).

CHAPTER 5: APPENDICES AND REFERENCES

5.1 Appendices

5.1.1 Appendix A: Buffers and stock solutions

Ampicillin stock (100 mg/ml)

Dissolve 1 g of ampicillin in 10 ml dH_2O. Filter sterilise using 0.2 µm filter. Aliquot and store at -20˚C.

Coomassie brilliant blue

1.25 g Coomassie blue

225 ml methanol

50 ml acetic acid

225 ml dH_2O

Destaining stock solution

400 ml acetic acid

600 ml dH_2O

20% Glucose

200 g glucose

600 ml dH_2O

Adjust volume to 1l.

1M H_2SO_4

Add 29 ml of concentrated H_2SO_4 in 493 ml dH_2O to make 2M H_2SO_4 and use to stop ELISA reaction.

0.1M IPTG

Dissolve 1.19 g isopropyl β-D-thiogalactopyranoside in 40 ml dH_2O and adjust volume to 50 ml. Filter sterilise and store at -20˚C.

Kanamycin stock (25 mg/ml)

Dissolve 250 mg kanamycin in 10 ml dH_2O. Filter sterilise, aliquot and store at -20˚C.

2% MP

Dissolve 2 mg of Elite milk powder in 10 ml of 1x PBS.

5M NaCl

Dissolve 29.22 g of NaCl in 80 ml dH_2O and adjust volume to 100 ml with dH_2O, autoclave and store at RT.

10x PBS stock solution

80 g NaCl

2 g KCl

26.8 g Na_2HPO_4-. 7 H_2O

2.4 g KH_2PO_4

800 ml dH_2O

Adjust pH to 7.4 with HCl and adjust volume to 1l. Autoclave and store at RT.

PBS-0.5% Tween 20

Add 500 µl Tween to 100 ml of 1x PBS and store at RT.

20% PEG/2.5M NaCl

200 g polyethylene glycol

500 ml 5 M NaCl

Adjust volume to 1l with dH_2O and autoclave. Store at RT.

Sucrose gradients

Dissolve sucrose in PBS and store at -20˚C.

[ ] %	sucrose (g)	1xPBS (g)
20	8	32
25	10	30
30	12	28
35	70	130
40	16	24

10x SDS running buffer

10 g sodium dodecyl sulphate

30.3 g Tris

144.1 g glycine

Dissolve in 800 ml dH_2O and adjust volume to 1l. Store at RT.

1 M Tris pH 7.4

Dissolve 12.11 g of Tris (hydroxymethyl) aminomethane in 80 ml of dH_2O. Adjust pH to 7.4 with HCl and then adjust to 100 ml with water. Autoclave and store at RT.

5.1.2 Appendix B: Media

2xTY broth

Prepare 1 litre:

16 g Tryptone

10 g Yeast extract

5 g NaCl

Adjust to 1 litre with distilled water. Autoclave and store at RT.

TYE plates:

10 g Tryptone

5 g Yeast extract

8 g NaCl

15 g Agar

Dissolve in 600 ml dH_2O and adjust to 900 ml with dH_2O. Autoclave, cool to 50˚C, then add 100 ml of 20% glucose to make 1 litre and 1 ml of ampicillin (100 µg/µl), then pour onto plates and store at 4˚C.

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5.3 Publication

Paper:

Chiliza, T.E. and D.H. du Plessis. Generation of a single immune scFv antibody library against multiple target antigens: a source of highly-specific antibodies (in preparation).