

**Expression and engineering of recombinant antibodies
against a heat-shock protein of
*Mycobacterium bovis***

by

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For my parents

The great tragedy of science is the slaying of a beautiful hypothesis by an ugly fact.

Thomas Huxley

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Summary

**Expression and engineering of recombinant antibodies against a heat-shock
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In the medical and veterinary diagnostic fields there is an ongoing need for stable and specific antibodies. There is also a requirement for simple, robust and cost-effective diagnostic assays to be used in the developing world. Recombinant antibodies from phage displayed libraries are economical to produce and can often be engineered to improve affinity, avidity and stability. While recombinant antibody fragments are useful in immunoassays, they are not strictly comparable to normal immunoglobulins and may under-perform in certain assays. Converting monovalent single-chain antibody fragments (scFvs) to bivalent immunoglobulin-like formats could conceivably provide a more suitable molecular scaffold for use in immunoassays. Two

scFvs that recognised the 65 kDa heat-shock protein (HSP65) of *Mycobacterium bovis* were used in this study. They were originally derived from the *Nkuku*® repertoire, a phage displayed antibody library based on the immune repertoire of the chicken, *Gallus gallus*. The genes coding for these scFvs were subcloned in expression vectors containing chicken IgY constant-heavy domains, to create bivalent constructs which were designated ‘gallibodies’. Expression of these constructs was attempted in three heterologous systems. While they were successfully produced in adherent mammalian cell cultures, the growth requirements of these cultures complicated subsequent purification. Bacteria and yeasts were investigated as alternative expression systems, but antibodies were not produced in either system. The gallibodies were compared to their monovalent scFv counterparts for stability as well as their applicability in ELISAs and gold-conjugated immunochromatographic lateral-flow assays. As gallibodies, both retained their functionality after exposure to different conditions and they were capable of immunocapture in ELISA. This was in contrast to their performance as scFvs. Furthermore, these antibody-like molecules could be stably conjugated to colloidal gold and used in lateral flow tests where positive and specific signals were obtained. This confirmed that recombinant single-chain monomeric antibody fragments could be reconstituted as bivalent immunoglobulin-like molecules and that they are a potentially useful platform for developing practical, robust immunodiagnostic reagents. It appeared from these experiments that the antibodies could act as a pair in which one captures, and the other detects HSP65. To find out whether they recognised discrete regions on the protein, their epitopes were mapped using a phage displayed peptide library in combination with computer-based algorithms. The presumptive epitope of one was mapped to residues 350 to 370 on HSP65 of *M. bovis*. The sequences selected from the peptide library by the other corresponded to three separate regions on the target protein. These recombinant antibody recognition sites are analogous to some of those that have been mapped by others using traditional monoclonal antibodies.

Abbreviations

Ab(s)	-	antibody(ies)
ADCC	-	antibody-dependant cellular cytotoxicity
AID	-	activation-induced cytidine deaminase
Amp	-	ampicillin
Amp ^R	-	ampicillin resistance
A _x	-	absorbance at <i>x</i> nanometres
bp	-	base pairs
BSA	-	bovine serum albumin
°C	-	degrees Celsius
C _{HL}	-	constant region heavy/light chain
CDR(s)	-	complementarity-determining region(s)
CMV	-	cytomegalovirus
C-region	-	antibody constant region(s)
3D	-	three-dimensional
ddH ₂ O	-	double-distilled water (sterilised)
DIVA	-	distinguishing infected from vaccinated animals
DMEM	-	Dulbecco's modified Eagle medium
DMSO	-	dimethylsulfoxide
DNA	-	deoxyribonucleic acid
dNTPs	-	deoxyribonucleotide triphosphates
ELISA	-	enzyme-linked immunosorbent assay
Fab	-	fragment antigen -binding
FBS	-	foetal bovine serum
Fig.	-	figure
<i>g</i>	-	relative centrifugal force
<i>gb</i>	-	gallibody
GHCl	-	guanidinium chloride
H-chain	-	heavy chain
HEK 293-H	-	human embryonic kidney cells
His-tag	-	histidine tag
HRP	-	horseradish peroxidase
HSP65	-	65 kDa heat-shock protein
ICT	-	immunochromatographic test
Ig(s)	-	immunoglobulin(s)
IgG	-	immunoglobulin class G

IgY	-	immunoglobulin class Y
IPTG	-	isopropylthiogalactoside
Kan	-	kanamycin
kDa	-	kilo Dalton
<i>l</i>	-	litre
LB	-	Luria-Bertani
L-chain	-	light chain
LHS	-	left-hand side
M	-	molar
mAb	-	monoclonal antibody
MCS	-	multiple cloning site
mer	-	denotes structure units of a polymer
mg	-	milligram
min	-	minute(s)
ml	-	milliliter(s)
mm	-	millimeter(s)
mM	-	millimolar
MP	-	milk powder
MPPBS	-	milk-powder in PBS
MWCO(s)	-	molecular weight cut-off(s)
N	-	normal (acids)
NaOAc	-	sodium acetate
Ni-NTA	-	nickel-nitrilotriacetic acid
nm	-	nanometres
OD _x	-	optical density at <i>x</i> nm
OmpA	-	outer-membrane protein A
OPD	-	<i>o</i> -phenylenediamine
pAb	-	polyclonal antibody
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate-buffered saline
PCR(s)	-	polymerase chain reaction(s)
PEG	-	polyethylene glycol
PelB	-	pectate lyase B
PSB	-	protein sample buffer
PVDF	-	polyvinylidene fluoride
rAb	-	recombinant antibody
RF	-	replicative form
RHS	-	right-hand side

RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RSS	-	recombination signal sequence
RT	-	room temperature
RVF	-	Rift Valley fever
scFv	-	single chain variable fragment
SDS	-	sodium dodecyl sulphate
sec	-	second(s)
SNF(s)	-	supernatant fluid(s)
SOE	-	splicing with overlap extension
ss	-	single-stranded
Strep-tag	-	streptavidin tag
SV40	-	simian virus 40
TAE	-	Tris-acetate-EDTA
TE	-	Tris-EDTA
Tris	-	Tris(hydroxymethyl)-aminomethane
tRNA	-	transfer RNA
V _{H/L}	-	variable region heavy/light chain
V-region	-	antibody variable region(s)
v/v	-	volume per volume
w/v	-	weight per volume
YCB agar	-	yeast carbon base agar
YPGal	-	yeast peptone containing galactose
YPGlu	-	yeast peptone containing glucose
μl	-	microlitre(s)

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Chapter 1

LITERATURE REVIEW

1.1 Background

Antibody technology was revolutionised about twenty years ago with the introduction of the technique of phage display which allowed researchers to bypass both immunisation and the complexities of hybridoma technology (Smith, 1985; Clackson *et al.*, 1991). This technology makes it possible to display antibodies on the surface of phage particles and to isolate potentially high affinity binders to a wide variety of desired targets. Antibodies can potentially be obtained rapidly and economically, and then produced in large amounts using *in vitro* expression systems. The entire process drastically minimises the use of animals and results in well-defined antibodies with less batch-to-batch variation than those obtained from *in vivo* systems. Phage display technology can be used to produce antibodies directed against toxic targets that would be impossible using immunisation. Furthermore, this system lends itself to the engineering of immunoglobulins to improve their key characteristics such as affinity, avidity and stability (Schimmele & Plückthun, 2005).

Mycobacterium bovis causes bovine tuberculosis in cattle and wildlife. In addition to being economically important in the animal trade, it is a zoonotic disease that is clinically identical to *M. tuberculosis* infection in humans (Pollock & Neill, 2002; Baker *et al.*, 2006). Overlaps in bovine and human tuberculosis infections have resulted in a recommendation that research on these two species should not exclude one or the other (Vordermeier *et al.*, 2006). In the absence of a vaccine that confers complete protection against tuberculosis in animals or human adults, there is a need for diagnostic methods that can discriminate between infected, uninfected and vaccinated individuals. Antibodies, in particular monoclonal antibodies, are likely to play an important role in developing such tests. Immunoassays using antibodies conjugated to enzymes or colloidal gold nanoparticles offer the advantages of economy, rapidity and robustness. Accordingly, this project aimed to investigate antibody formats in which recombinant antibody fragments directed against *M. bovis* are engineered to improve their usability in ELISA and immunochromatographic tests.

1.2 Bovine tuberculosis

Bovine tuberculosis (BTB) is caused by the acid-fast, Gram-positive bacillus *M. bovis* (Pollock & Neill, 2002; Cousins *et al.*, 2004) which was originally discovered by Robert Koch in 1882 (Sakula, 1983). It is an economically important disease that predominates in cattle but also affects wildlife (Michel *et al.*, 2006). Furthermore, *M. bovis* is zoonotic and may be indistinguishable from *M. tuberculosis* infection when it manifests disease in humans, although the burden in such infections is not accurately known (Baker *et al.*, 2006). Not surprisingly, genome sequencing has revealed that *M. tuberculosis* and environmental *M. bovis* are >99.95 % identical at the nucleotide level (Garnier *et al.*, 2003). BTB is believed to have existed on the Eurasian continent since antiquity (Cousins *et al.* 2004) and has recently been identified in ancient human remains for the first time (Taylor *et al.*, 2007). It was shown that the organism already contained the RD4 deletion two thousand years ago, a marker which is used today to differentiate *M. bovis* from *M. tuberculosis* (Taylor *et al.*, 2007). The disease was first documented in South Africa by Hutcheon in 1880, and was most probably introduced through the importation of certain breeds of cattle (Cousins *et al.*, 2004). Today, BTB occurs worldwide and its control remains a concern for both the developed and the so-called developing worlds (Cockle *et al.*, 2002; Cousins *et al.*, 2004). In countries with wildlife reservoirs, such as New Zealand, South Africa, Ireland and the USA, BTB has been difficult to curtail and remains widespread (Buddle *et al.*, 2002; Baker *et al.*, 2006; Michel *et al.*, 2006).

1.2.1 Prevention and diagnosis

Currently there is no vaccine that provides complete and consistent protection in animals. *M. bovis* bacillus Calmette-Guérin (BCG), a strain of *M. bovis* that was attenuated in France almost 100 years ago remains the only one available (Vordermeier *et al.*, 2006). In herds (e.g. cattle) BGC vaccination curbs the disease by reducing the burden of infection rather than preventing the disease altogether. However, vaccination in this manner is not always popular because it can interfere with diagnosis. (Buddle *et al.*, 1999; Suazo *et al.*, 2003). BCG is still used to vaccinate humans against tuberculosis (TB) but it has only been consistently successful in protecting children against forms of childhood TB such as meningitis (Vordermeier *et al.*, 2006). Two commonly employed serological techniques for BTB diagnosis are the purified protein derivative

(PPD, also called tuberculin) skin test and the interferon-gamma (IFN- γ) assay (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004).

Intradermal PPD delayed hypersensitivity remains the backbone of bovine TB diagnosis and disease control (Whipple *et al.*, 2001; Cockle *et al.*, 2002). Tuberculin, which is a *Mycobacterium* culture-filtrate containing a variety of antigenic peptides, is injected intradermally and hypersensitivity at the site of injection is assessed after seventy-two hours according to prescribed criteria (Whipple *et al.*, 2001; Cousins *et al.*, 2004). In the comparative tuberculin test, both bovine and avian PPDs are injected at different sites, as opposed to bovine tuberculin alone which is used in the single intradermal test (Cousins *et al.*, 2004). While infection can be diagnosed with a fair degree of accuracy in most instances (Wood & Jones 2001; Cousins *et al.*, 2004), the method has a number of shortfalls (Cockle *et al.*, 2002). The accuracy of the tuberculin skin test is often compromised by BCG vaccination rendering false positives. This results in unnecessary livestock losses where slaughter control programmes are in place, e.g. the UK (Vordermeier *et al.*, 2006). Occasionally infected animals do not react to PPD challenge and show no clinical symptoms (Pollock & Neill, 2002), but this is more common in advanced BTB (Cousins *et al.*, 2004). Furthermore, the skin test may only be repeated after a sixty-day respite which delays identification of suspected cases (Wood & Jones, 2001).

The INF- γ enzyme immunoassay (EIA) is a less invasive, blood-based comparative test that detects production of the cytokine in response to the overnight incubation of whole-blood samples with tuberculin (Whipple *et al.*, 2001). Samples are tested against bovine PPD, avian PPD and a blank (no antigen), the latter two serving as controls. Animals are considered positive for *M. bovis* infection if cytokine production in response to bovine PPD is higher than that of the controls (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004). Studies in several countries have confirmed that the assay detects *M. bovis* infections earlier and with greater sensitivity than PPD testing and within a shorter period of time (Wood & Jones, 2001). In most countries, a commercially available kit (BOVIGAM™) is used in conjunction with intradermal testing (Whipple *et al.*, 2001; Wood & Jones, 2001; Palmer *et al.*, 2006). It has been reported that animals injected with tuberculin for skin testing prior to whole-blood sampling show increased INF- γ levels even when blood samples were assayed twenty-four hours after collection (Whipple *et al.*, 2001). Although this may only be valid for infected individuals

(Palmer *et al.*, 2006), using both tests reduces the frequency of inconclusive results (Cousins *et al.*, 2004) and also extends the time that samples can be stored or transported before testing is carried out (Wood & Jones, 2001).

Additional methods used for *M. bovis* and *M. tuberculosis* diagnosis include identifying acid-fast bacteria by microscopy, culturing the organism, PCR amplification and animal-side or laboratory rapid-tests e.g. STAT-PAK and MAPIA (Lyashchenko *et al.*, 2000; OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004; Lyashchenko *et al.*, 2006; Lyashchenko *et al.*, 2007; Wernery *et al.*, 2007). Assays which detect nitric oxide (Pons, 2002), lipoarabinomannan (Chan *et al.*, 2000; Sharma *et al.*, 2006) or isocitrate dehydrogenase (Florio *et al.*, 2002) have also been investigated. The majority of studies have focused on antigen-based assays, specifically those that can distinguish infected from vaccinated individuals (DIVA). Most of these employ cocktails of immunodominant-antigens (either recombinant or natural) to detect antibodies in serum (Dillon *et al.*, 2000; Lyashchenko *et al.*, 2000; Amadori *et al.*, 2002; Weldingh *et al.*, 2005; Lyashchenko *et al.*, 2006; Wernery *et al.*, 2007). The lack of a DIVA test is a constraint on applying suitable disease control strategies (Cockle *et al.*, 2002). Moreover, effective diagnostic tests should be able to differentiate between *Mycobacterium* strains. If required to detect a variety of mycobacterial antigens (Buddle *et al.*, 1999; Cockle *et al.*, 2002; Hasegawa *et al.*, 2002; reviewed by Vordermeier *et al.*, 2006), such tests will require antibodies of exquisite specificity and sensitivity (Dillon *et al.*, 2000). Such a requirement would in all likelihood exclude polyclonal antibody preparations.

1.3 The 65 kDa heat-shock protein of *Mycobacterium*

The 60 kDa chaperonins (Cpn60) are omnipresent, highly conserved proteins that mediate the folding, assembly and transport of various proteins (Young & Garbe, 1991; Fink, 1999). In bacteria specifically, the expression of a number of chaperone proteins is up-regulated in response to external stress stimuli such as increased temperature, lack of nutrients or oxygen and attack by the host immune system (Young & Garbe, 1991; Qamra & Mande, 2004). Most of the information about the structure and function of Cpn60s has been gleaned from the GroESL molecule from *Escherichia coli* (Shinnick *et al.*, 1988; Qamra *et al.*, 2004). Tetradecameric *E. coli* GroEL (Cpn60; Braig *et al.*, 1994) and its heptameric co-chaperone GroES (Cpn10) are

expressed from a bicistronic operon (Qamra *et al.*, 2004). Three domains (equatorial, intermediate and apical) can be differentiated in each subunit of GroEL (Braig *et al.*, 1994) (Figs. 1.1 and 1.2). The equatorial domain binds ATP and is the ‘scaffold’ of the structure as monomers are linked together through these domains. The apical domain forms the opening of the cylindrical cavity and can accommodate large conformational changes related to polarity when binding the ligand. The intermediate domain acts as a hinge between the apical and equatorial domains (Braig *et al.*, 1994; Fink, 1999). GroEL functions in a complex with GroES (Cpn10), which is able to bind the 14-mer complex and isolate the binding cavity from the surrounding medium (Qamra & Mande, 2004). Interactions between these two chaperonins are ATP-dependant (de Bruyn *et al.*, 2000).

The mycobacterial homologue of the *E. coli* GroEL is the 65 kDa heat-shock protein (HSP65; Kong *et al.*, 1993), although Cpn60s from certain *Mycobacteria* may diverge slightly from the canonical model. In *M. tuberculosis* two forms of Cpn60 (Cpn60.1 and Cpn60.2) are expressed from separate gene loci. *Cpn60.1* and *cpn10* are arranged together on the operon as in *E. coli*, while *cpn60.2* is independently positioned further downstream (Kong *et al.*, 1993; Qamra *et al.*, 2004). The two gene products are approximately sixty percent identical (Kong *et al.*, 1993). *M. bovis* BCG *cpn60.2* was cloned and sequenced in the 1980’s (Thole *et al.*, 1985; Thole *et al.*, 1987) and various antigenic regions on the protein have been mapped using monoclonal antibodies (Shinnick *et al.*, 1987; Thole *et al.*, 1988; Hajeer *et al.*, 1992; Rambukkana *et al.*, 1992). HSP65 makes up almost ten percent of the total soluble proteins that can be extracted from culture filtrates of *M. bovis* BCG (de Bruyn *et al.*, 1987). Culturing this organism at temperatures in the 37 – 48 °C range increases the amount of 65 kDa protein that can be detected (Young & Garbe, 1991). Cpn60s are associated with the cytosol (de Bruyn *et al.*, 1987; Qamra & Mande, 2004) and may be secreted extracellularly, although the latter has only been shown with Cpn10 (Fossati *et al.*, 2003).

Qamra and Mande (2004) solved the crystal structure of *M. tuberculosis* HSP65 (Cpn60.2), using data from unliganded *E. coli* HSP60 (GroEL) as a model (Braig *et al.*, 1994; Fig. 1.3, LHS). Qamra *et al.* (2004) showed that Cpn60.1 and Cpn60.2 from *M. tuberculosis* do not exist as tetradecameric assemblies, but rather as dimers which they attributed to amino acid mutations at two critical conserved positions (Ala2→Ser2 and Glu76→Ser76) in the Cpn60.1 unit.

Consequently, the Cpn60 complex uncharacteristically lacks ATPase activity, although it retains the ability to bind protein intermediates through large surface-exposed hydrophobic regions (Qamra & Mande, 2004). The authors attribute this anomaly to an evolutionary energy-saving strategy; as mycobacteria grow vastly more slowly than most other bacteria, an ATP-independent pathway may preserve essential protein-folding mechanisms at no energy cost to the organism (Qamra *et al.*, 2004). In contrast to the canonical GroEL whose subunits dimerise through their equatorial domains, HSP65 dimerises unusually through its apical domains (Fig. 1.3, RHS). In this way the aforementioned hydrophobic areas are exposed on the protein surface, most likely so as to bind unfolded or immunogenic molecules (Qamra & Mande, 2004).

HSP65 is highly immunoreactive and is considered an important target antigen in vaccine development (Shinnick *et al.*, 1988). As the antigen is conserved in tubercle bacilli, a mAb directed against HSP65 is likely to be useful for the general diagnosis of mycobacterial infection or exposure (Shinnick *et al.*, 1987). Furthermore, it may provide an alternative to the PPD skin test for diagnosis.

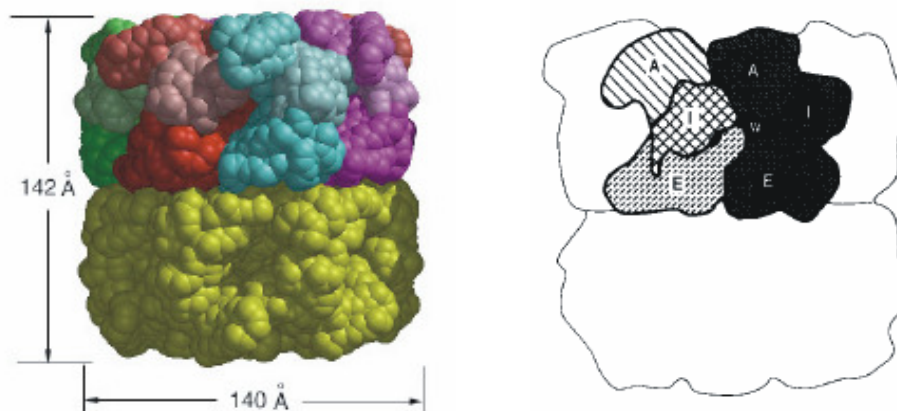


Figure 1.1 The structure of the canonical molecular chaperone GroEL. The molecule is made up of fourteen subunits, arranged in two rings of seven subunits each. These heptamers are illustrated by the three-dimensional structure on the LHS (one heptamer in multiple colours, the other in yellow). Each of the fourteen subunits contains three domains (apical or A, intermediate or I and equatorial or E), shaded in varying degrees of the same tone on the LHS. The drawing on the RHS indicates the position of these domains when subunits are oligomerised in the double-ring structure (Braig *et al.*, 1994 and Sigler *et al.*, 1998).

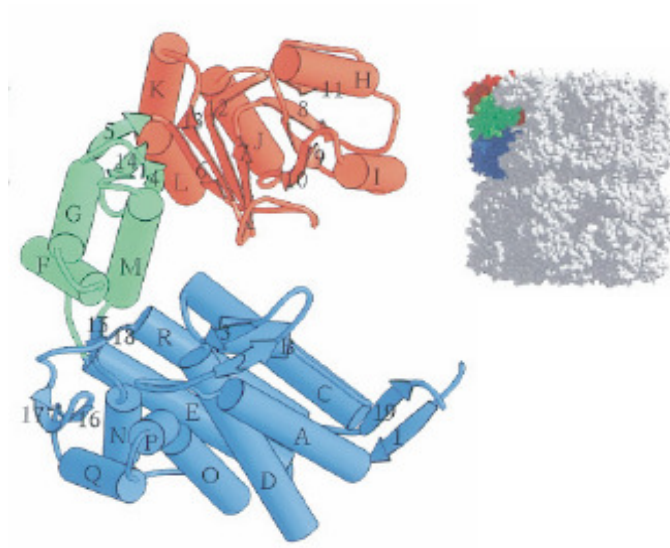


Figure 1.2 The secondary structure of an individual GroEL subunit is illustrated on the LHS. Each domain is shaded for differentiation: apical (red), intermediate (green) and equatorial (blue). The orientation of the subunit within one of the heptameric rings is indicated by the same colours in the space-filled insert on the RHS (Xu & Sigler, 1998).

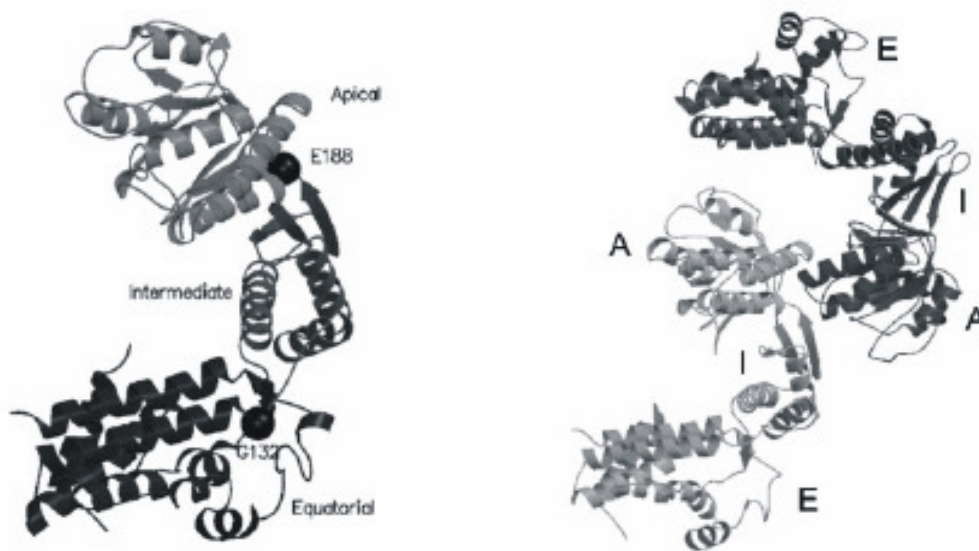


Figure 1.3 The structure of HSP65 (Cpn60.2) as solved by X-ray crystallography. The general structure of the molecule is illustrated by the ribbon-diagram on the left, showing the equatorial, intermediate and apical domains of the protein. The hinge points of the domains are specified by spheres. The unusual dimerisation of the molecule through its apical domains is illustrated on the right. The monomers are differentiated by the shade of grey and the domains are abbreviated A (apical), I (intermediate) and E (equatorial) (Qamra & Mande, 2004).

1.4 Antibodies

Antibodies (Abs) or immunoglobulins (Igs) are glycoproteins of conserved and characteristic structure which are produced by terminally-differentiated B-cells (plasma cells) in response to antigenic challenge. Each molecule of IgG, the best characterized and most abundant Ig in mammals, is roughly 150 kDa in size and is made up of four polypeptide chains: two identical heavy (H) (~55 kDa) and two identical light (L) chains (~25 kDa). Each of these chains contains both variable (V) constant (C) regions, linked by disulphide bonds (Edelman, 1969; recently reviewed by Lipman *et al.*, 2005). In mammals, five different antibody classes or isotypes exist (IgA, IgD, IgE, IgG, and IgM), each of which is associated with a particular type of immune response, as well as its duration. Papain digestion of IgG yields two identical fragments, each with an antigen binding site, known as Fab fragments and a third with no antigen binding ability, the Fc portion (Porter, 1959). The Fc can be cleaved from whole antibody at an alternative site by pepsin, which renders a large F(ab')₂ fragment (Roitt, 1988). This is illustrated in Fig. 1.4.

The Fc portion is an effector domain with a generally conserved sequence and includes the carboxy termini of the H chains. This region is important in complement activation and phagocytosis (Lipman *et al.*, 2005). On the other hand, the amino-termini of a heavy and a light chain associate to form the antigen-binding region, or paratope. Epitope-binding specificity is conferred within this domain by the complementarity-determining regions (CDRs) of the H and L chain V regions, which are unique regions of hypervariable sequences confined to three clusters on the L chain, and three on the H chain (Roitt, 1988). Framework regions bordering these CDRs need to be relatively conserved to maintain the structural integrity of the final Ab fold (Collins *et al.*, 2003). Recombinant antibody technology makes it possible to produce single-chain variable fragments (scFvs) through the heterodimeric linking of V_H and V_L domains with a flexible polypeptide linker. The linker, usually around fifteen amino acids long, joins the V_L carboxy-terminus to the V_H amino-terminus, or *vice versa* (Bird *et al.*, 1988; Huston *et al.*, 1988). Its properties can affect the avidity, aggregation (reviewed by Hudson & Kortt, 1999) and expression levels (Tang *et al.*, 1996; Trinh *et al.*, 2004) of the scFv. These molecules, although small (26 – 27 kDa), contain a complete binding site and are fully functional as target-binders (Fig. 1.5).

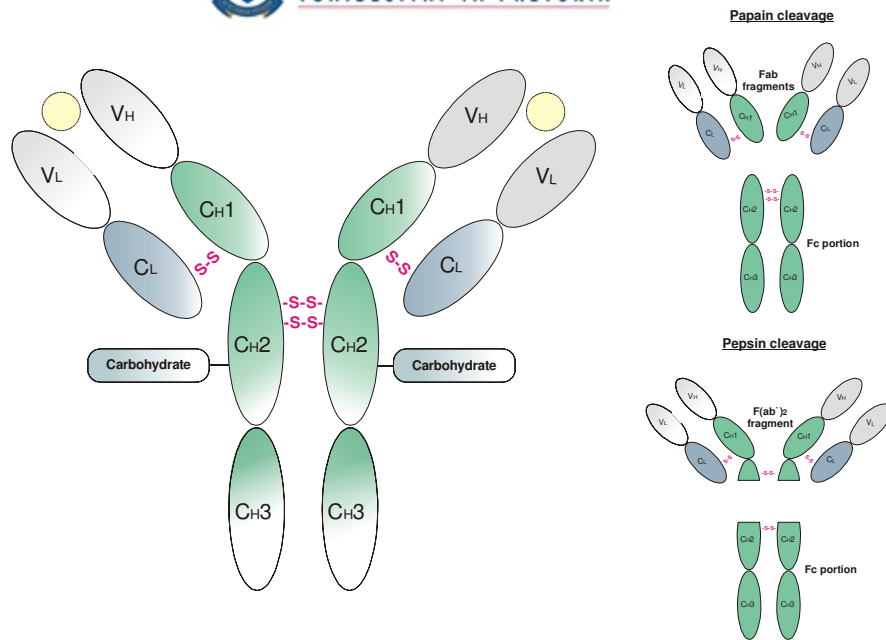


Figure 1.4 Diagram showing the structure of IgG and its proteolytic digestion products. The basic antibody structure is illustrated (left). V_H denotes variable heavy chains, V_L variable light chains, C_H constant heavy chains, C_L constant light chains. Yellow spheres indicate the sites of antigen binding. Fragments generated by enzymatic cleavage are depicted in illustrations on the right (adapted from Edelman *et al.*, 1969; Tonegawa, 1983; Roitt, 1988).

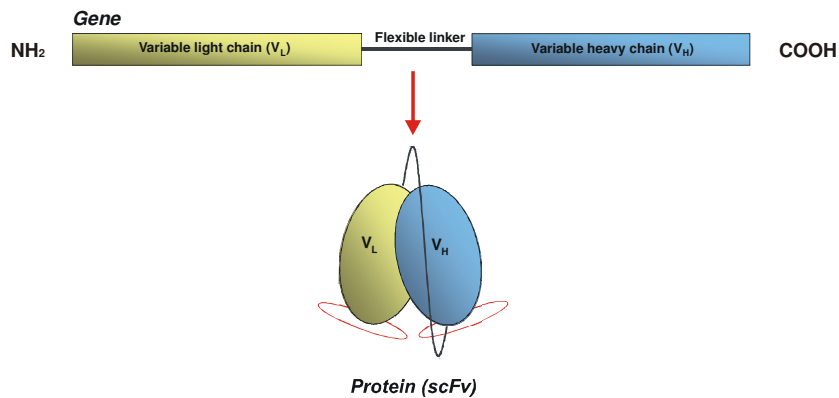


Figure 1.5 Diagram showing a gene construct (top) coding for an scFv. The juxtaposition of V_L and V_H domains joined by the flexible polypeptide linker is illustrated below. The scFv paratope encompasses the areas indicated by the red ellipses.

1.4.1 The generation of antibody diversity

The ability of the immune system to respond to an array of foreign antigens results from a series of tightly controlled antibody gene-segment recombinations which generate diversity at several stages during B-cell development. Were it not for these events, the genome would need to be impossibly large in order to accommodate enough information to allow the animal to respond to every conceivable antigen that could be encountered (Tonegawa *et al.*, 1974). In most vertebrates, V_H are assembled from three gene segments: these are the variable (V), diversity (D) and joining (J) segments (Fig. 1.6). V_L are made up of V and J segments only (Early *et al.*, 1980). All of these fragments occur clustered within their respective groups, close to one another on the same chromosome and just upstream of the nucleotides coding for the C region (Early *et al.*, 1980). Combinatorial joining of these segments in the developing B-cell is an ordered process (Yaoita *et al.*, 1983; Alt *et al.*, 1984) and occurs by site-specific recombination, initiated by double-stranded DNA breaks. This process requires gene segments to be flanked by a conserved recombination signal sequence (RSS) (Max *et al.*, 1979; Sakano *et al.*, 1979; Early *et al.*, 1980). To generate further diversity, the coding ends of exons are modified by the loss or gain of nucleotides when combinatorial joining occurs, generating different codons, a process called junctional diversity (Max *et al.*, 1979, Sakano *et al.*, 1979). Once the VJ_L or VDJ_H rearrangement has taken place, the gene is transcribed, continuing through the C region segment of the gene. The V, J or V, D, J and C regions are then joined by RNA splicing to create mRNA which will be translated into either light or heavy chains. Initially, all H chains are μ chains, resulting in the production of IgM, but class switching may occur through a second splicing event in the B-cell (Early *et al.*, 1980; Yaoita *et al.*, 1983).

After gene rearrangement has taken place, further variation is introduced by somatic hypermutation (Tonegawa 1983; Roitt, 1988). Point mutations are responsible for producing antibodies with high affinity (Collins *et al.*, 2003). Several such mutations are introduced at mutational ‘hotspots’ surrounding the rearranged variable regions, especially within the CDRs (Tonegawa, 1983; Levy *et al.*, 1989). These mutations occur at a high frequency, *i.e.* approximately 10^{-3} mutations per base pair per generation. A possible link between hypermutation and the enzyme activation-induced cytidine deaminase (AID) has been made; the V genes of humans with a recessive form of hyper-IgM syndrome in which the AID enzyme is lacking are not able to undergo hypermutation (Collins *et al.*, 2003).

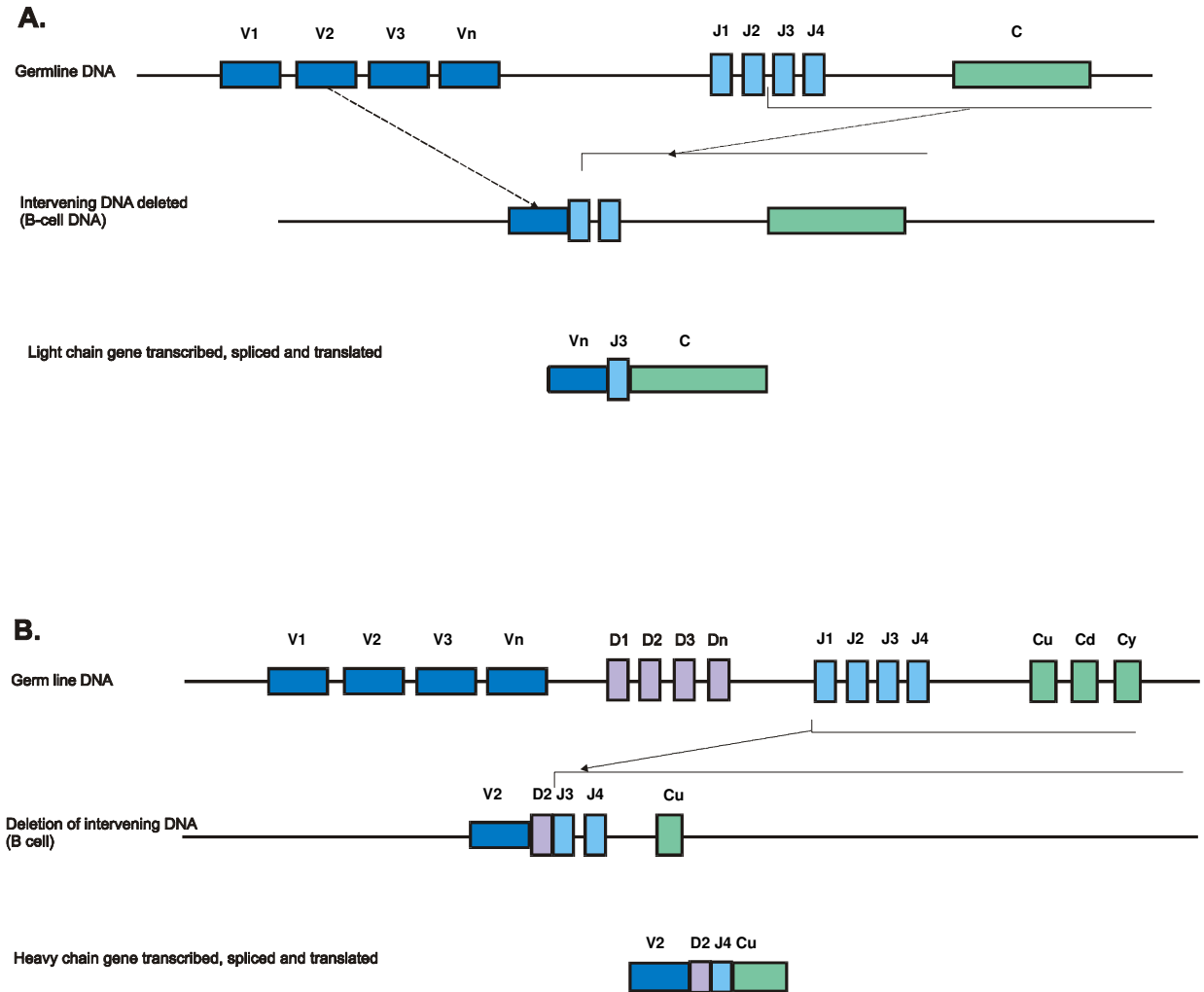


Figure 1.6 Immunoglobulin germline organization and gene rearrangement in mammalian light (A) and heavy (B) chains (adapted from Prescott *et al.* 1999; McCormack *et al.*, 1991).

In contrast to mammals which rely largely on gene-segment rearrangements, the avian immune system generates its Ab diversity by a process known as somatic gene conversion (Fig. 1.7). Chicken antibody L chain loci consist of only one functional V gene which undergoes rearrangement with a unique J segment (Reynaud *et al.*, 1985; Carlander *et al.*, 1999). In H chains J segments rearrange with one of approximately fifteen D segments, which are all very similar in sequence, before this complex recombines with the unique V segment (Reynaud *et al.*, 1989; Ratcliffe 2006). As with mammalian gene recombination, a degree of diversity is generated through the imprecise joining of segments and point mutations (Parvari *et al.*, 1990; Ratcliffe, 2006). Despite this, only minimal chain diversity is created compared to mammalian systems such as mice or humans that have a greater number of V and J segments available to be rearranged (McCormack *et al.*, 1991). Almost as if to compensate, further upstream on each V_H and V_L chain locus is found a family of pseudogenes designated by the Greek letter psi (ψ) (Fig. 1.7). These pseudogenes do not have the RSS required for combinatorial joining but they can donate sequences to rearranged germline VJ_L or VDJ_H genes by replacement of sequences within these rearrangements (Reynaud *et al.*, 1989). Generating diversity in this manner makes it relatively easy to use the polymerase chain-reaction (PCR) to access and amplify chicken antibodies. This is because the 5' and 3' termini of H- and L-chain coding genes always code for the same amino acid sequences. Consequently only two sets of primers (one for the V_H chain and one for the V_L chain) are required (Davies *et al.*, 1995).

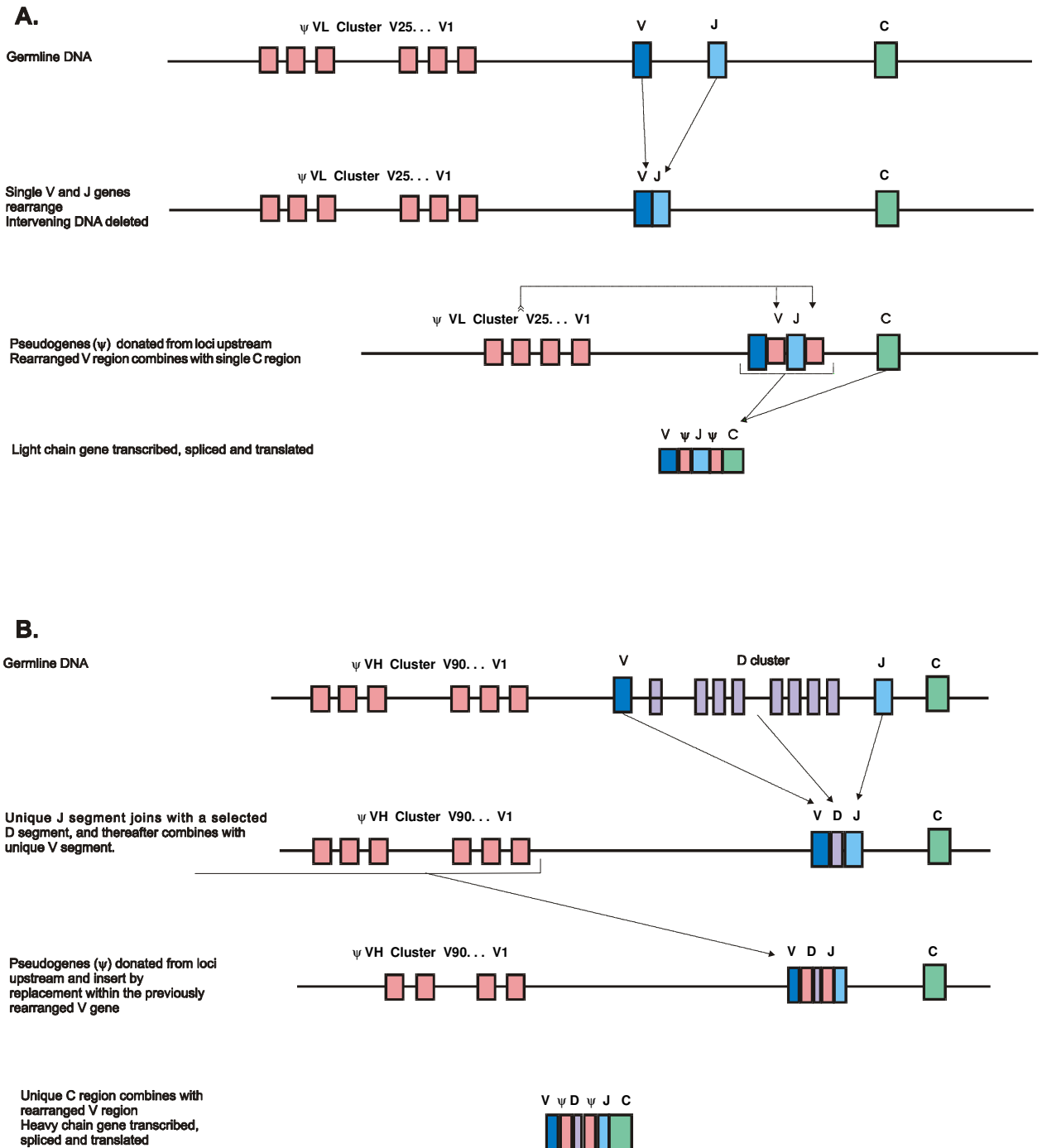


Figure 1.7 Chicken antibody light (A) and heavy (B) chains rearrange by somatic gene conversion in which pseudogenes (ψ) donate sequences to rearranged V genes (adapted from Prescott *et al.* 1999; McCormack *et al.*, 1991).

1.4.2 Polyclonal and monoclonal antibodies

Antibodies prepared from the serum of an animal immunised with a specific antigen are referred to as polyclonal antibodies (pAbs). They are in effect a mixture of all the antibodies produced in response to antigenic challenge. In 1975, Köhler and Milstein described hybridoma technology, a method in which cells from an immunised donor are fused with immortal mouse myeloma cells to provide a reliable source of continuously-secreted homogenous antibodies *in vitro*. The antibodies produced by a single ‘hybridoma’ are called monoclonal antibodies (mAbs) and are widely applied in a variety of immunoassays and immunodiagnostic tests. Although mAb are extremely useful in such applications because of their homogeneity and specificity, both pAbs and mAbs have their own advantages and disadvantages (reviewed by Lipman *et al.*, 2005). Genetic drift within cell lines is one of the most common problems experienced with mAb. While pAbs may provide greater sensitivity in immunoassays as they are produced by a large and diverse population of B cells recognising several epitopes, they are generally less specific than mAb, which as a rule preferentially recognise one particular epitope. This being said, mAbs often lack the avidity of pAbs, a problem which has been circumvented by combining several mAbs each directed against a different epitope (Andreotti *et al.*, 2003; Lipman *et al.*, 2005). Although classical mAb and pAb have certainly not been rendered obsolete, recombinant DNA technology has now provided an alternative way of producing antibodies with desirable characteristics in relatively large amounts and with little or no variability between batches. This approach has the potential to reduce the cost and effort of the traditional methods associated with mAb production (Andreotti *et al.*, 2003).

1.5 Recombinant antibodies by phage display

Phage display has now made it possible to exploit the immune system *in vitro* (Smith 1985; Clackson *et al.*, 1991). Derivatives of filamentous phages (Ff) such as M13, f1, Fd and ft (Fig. 1.8) are predominantly used for phage display (Arap, 2005). Gene III (*gIII*) codes for a minor coat protein (pIII) which consists of two functional domains (Smith, 1985). Its amino-terminal domain binds to the sex-pilus of *E. coli* during infection with the structurally important C-terminal domain being buried within the virus particle (Parmley & Smith, 1988; Arap, 2005). Smith (1985) discovered that foreign sequences could be inserted between these two domains allowing foreign peptides to be displayed as fusion-peptides on the phage surface. This made it

practical to select binding peptides from an enormous repertoire of irrelevant peptides using a screening process known as ‘panning’ (Parmley & Smith, 1988). Subsequently entire single chain antibody fragments were displayed in this way (McCafferty *et al.*, 1990; Clackson, 1991). During consecutive rounds of panning, phages displaying antibodies that recognise a desired target are retained, while non-specific binders are washed away (Fig. 1.9). With each round, the number of specific binders is enriched (Parmley & Smith, 1988; Laffly & Sodoyer, 2005). This seemingly simple idea, i.e. linking genotype and phenotype, has proven to be a potent tool for producing and selecting recombinant antibodies (rAbs) against a wide variety of targets (Carmen & Jermutus, 2002).

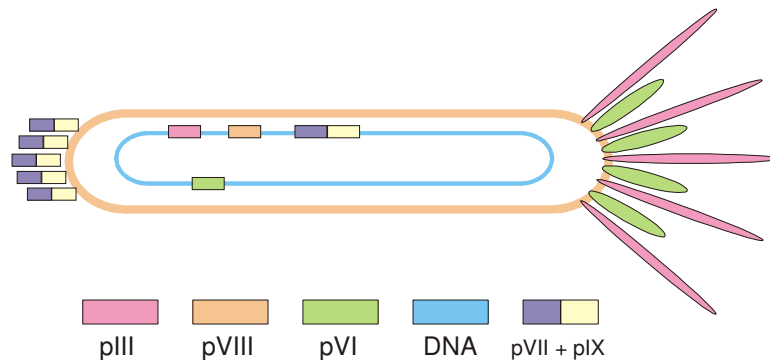


Figure 1.8 Schematic representation of the Ff bacteriophage particle. Capsid proteins are represented in different colours (see key). Corresponding small coloured blocks identify the encoding genes for each protein as they are arranged on the chromosome (adapted from Webster, 1996).

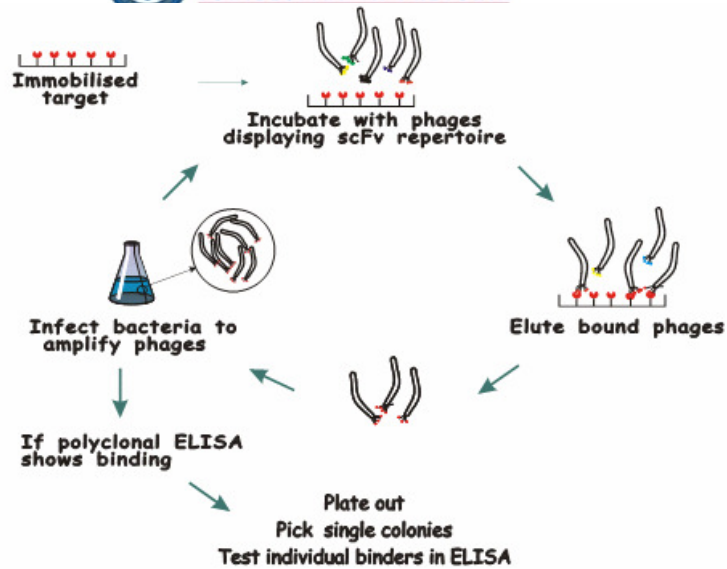


Figure 1.9 Diagram illustrating selection of binders from a phage display library. Phages displaying antibody fragments are subjected to multiple rounds of panning. The target of interest is usually immobilized on a plastic surface and incubated with phages displaying antibodies. Phages that remain bound after washing are eluted and used to re-infect bacteria. After successive rounds of panning, enrichment of specific binders occurs. These can be tested in polyclonal ELISA for reaction with the antigen of interest. If enrichment is successful, individual clones are picked and tested individually in ELISA (diagram courtesy of Dr. J. Fehrsen, Immunology Section, OVI).

1.5.1 Phage display antibody libraries

Antibody libraries can be constructed from either immunised or naïve (non-immunised) sources. Furthermore, non-immune libraries can be classified as semi-synthetic or synthetic, based on the degree of CDR randomisation introduced synthetically during library construction (Brichta *et al.*, 2005).

Immune libraries are constructed from donor animals previously immunised with a particular antigen (Williamson *et al.*, 1993; reviewed by Winter *et al.*, 1994). Such libraries are predisposed to yield antibodies against a specific antigen, most of which will already have undergone affinity maturation in the donor (Clackson *et al.*, 1991). Thus, such libraries need not be very large to be useful. Their diversity relies upon the immune response of the donor animal to the antigen and as a consequence, they require some time to produce (Brichta *et al.*, 2005). The resulting repertoire will tend to favour Abs with a certain specificity although it can also contain Ab against antigens that were not used for immunisation (Bradbury & Marks, 2004).

Naïve libraries are constructed from the germline V gene repertoires of non-immunised donors and are thus not restricted to any specific target (Gram *et al.*, 1992; Sheets *et al.*, 1998; de Haard *et al.*, 1999; Sblattero & Bradbury, 2000; van Wyngaardt *et al.*, 2004). Such libraries theoretically represent the primary repertoire of the donor animal and thus contain a broad range of specificities (Brichta *et al.*, 2005). Consequently, they are often referred to as ‘single pot’ repertoires (Nissim *et al.*, 1994). Once constructed, non-immune libraries can rapidly produce results, have no need of animals after their initial construction and can yield Ab to toxic, self-antigens or non-immunogenic substances (Brichta *et al.*, 2005). Such repertoires, however, require much effort, expense and technical expertise to construct and need to be exceedingly large to increase the likelihood of yielding high affinity Abs.

Whereas naïve libraries contain selections of genes rearranged *in vivo*, repertoires classified as semi-synthetic or synthetic can have their V genes rearranged *in vitro* and/or contain either partially or completely ‘artificial’ CDRs of varying loop length (Griffiths *et al.*, 1994; Nissim *et al.*, 1994; de Kruif *et al.*, 1995; Carmen & Jeremutis, 2002; Hanes *et al.*, 2000; van Wyngaardt *et al.*, 2004). The introduction of such synthetic sequences into selected CDRs can generate variants that would not be available naturally thereby increasing the diversity of the repertoire. CDR3 is usually targeted for randomisation as it is reportedly the most hypervariable region in the Ab of mice and humans (Barbas *et al.*, 1992; Xu & Davis, 2000).

1.5.2 Chicken antibodies and the *Nkuku*® library

As discussed previously, chickens rearrange their Ig genes by somatic gene conversion which generates H and L chain genes that have effectively identical amino acid sequences at their termini. Therefore, as mentioned earlier, only two sets of primers per V gene are required to access the chicken repertoire by PCR (Davies *et al.*, 1995). The *Nkuku*® library (van Wyngaardt *et al.*, 2004) is a semi-synthetic scFv phage display library derived from naïve chicken Ab V genes. This library, which was constructed at the Onderstepoort Veterinary Institute (OVI), comprises a combination of the naïve chicken Ab repertoire and a sub-library of synthetically-randomised H chain CDR3s of predetermined size. The primary library (approximately 3.75×10^8 phage clones) used the RNA from five chicken bursae. V region genes were amplified with primers that allowed the addition of a (Gly₄Ser)₃ linker *en bloc* to link the H and L chains. A semi-synthetic sub-library which includes a degenerate region representing CDR3, supplements

the natural repertoire. Once these two repertoires had been combined, the final library consisted of approximately 2×10^9 clones. The *Nkuku*® library is diverse enough to have generated Ab fragments that bind proteins, viruses and haptens.

1.6 Recombinant antibody engineering

As mentioned earlier, recombinant Abs may be engineered to improve their performance (*i.e.* affinity and avidity) by using a variety of different strategies (for reviews see Plückthun & Pack, 1997; Wörn & Plückthun, 2001; Schimmele & Plückthun, 2005). For example, the affinity of an Ab for its antigen can be enhanced by altering factors that influence its binding e.g. mutating specific individual residues or groups of residues vital to antigen recognition. Furthermore, where monovalent binding is a limiting factor (e.g. as with scFvs), avidity can be increased by altering inter-domain linkers or by appending additional domains. A handful of these individual approaches are outlined below. Antibodies, however, are often engineered by combining a number of these approaches to obtain the desired improvement (Jung *et al.*, 1999).

1.6.1 Mutagenesis

Combinatorial mutagenesis may be used to randomise CDRs by using degenerate oligonucleotides to increase the diversity of Ab libraries (Maynard & Georgiou, 2000). This kind of mutagenesis is referred to as ‘focussed’ since it is frequently directed at specific regions e.g. V-region CDR3 loops which contribute a great deal to binding (van Wyngaardt *et al.*, 2004). In contrast, random mutation introduces variations into entire genes (Maynard & Georgiou, 2000). This has been achieved *in vitro* by error-prone PCR which uses reaction conditions that reduce polymerase fidelity (Cadwell & Joyce, 1992) or *in vivo* by expressing foreign genes in mutator *E. coli* strains such as mutD5-FIT (Irving *et al.*, 1996).

1.6.2 Chain shuffling

Chain shuffling (Clackson *et al.*, 1991; Marks *et al.*, 1992) generates diversity through multiple L- and H-chain pairings, the aim being to find a combination that results in a suitably functional Ab. The technique is based on the concept of affinity maturation by light chain promiscuity (Maynard & Georgiou, 2000). Using phage display, libraries of L- and H- chains can be constructed and screened for optimal V- and H-domain combinations (Kang *et al.*, 1991). For

instance, Marks *et al.* (1992) engineered human Abs for improved affinity by reshuffling L-chain genes from an existing scFv with repertoires of H-chain genes from naïve donors, and *vice versa*.

1.6.3 CDR grafting

In 1986, Jones *et al.* used CDR grafting to successfully transplant the CDRs from mouse Ab into a human myeloma-derived Ab framework. The Ab retained the binding specificity of the donor Ab and the group suggested CDR grafting as a way of humanising Abs for therapeutics. Using a reciprocal approach, Jung & Plückthun (1997) grafted the loops of an Ab that had a tendency to aggregate into the framework of a scFv which is known to fold favourably. This reduced the aggregation and improved its expression. Furthermore, the study showed that grafting unstable loops onto stable frameworks was as good as grafting the actual binding regions.

1.6.4 Linker modification

Although the small size of scFvs (Bird *et al.*, 1988; Huston *et al.*, 1988) can be beneficial in certain applications, these fragments are limited by their monovalent binding (Greunke *et al.*, 2006). This can be overcome by modifying the polypeptide linker that connects the two V-domains. When linkers of 12 residues or less are used, scFvs are not able to fold into their typical configuration. As a result, the domains of individual scFv molecules associate with one another to form scFv dimers or trimers (reviewed by Hudson & Kortt, 1999). As each scFv retains its binding ability in this arrangement, the Ab's avidity is increased. Linkers can also be further engineered using phage display to improve or restore Ab function (Tang *et al.*, 1996).

1.6.5 Appended domains

In addition to linkers, avidity can be influenced by different Ab formats. Greunke *et al.* (2006) 'reformatted' monovalent scFvs as bivalent murine-avian chimeric IgYs by fusing them to chicken Ab-derived C_H domains. Each scFv-Fc fusion was secreted in mammalian cell culture as a single polypeptide chain. These individual chains then associated with one another by disulphide bonding in the culture supernatant to form functional, bivalent Abs. Similarly, Scallon *et al.* (2004) added domains to two IgG-format mAbs which bound monovalently due to steric hindrance. In an attempt to increase molecule flexibility, an extra C_{H1} domain was added between the native C_{H1} domain and the hinge region of each Ab. In this manner, the group restored bivalency and improved the binding ability and neutralising potential of both mAbs. After

converting scFvs into Fabs Quintero-Hernández *et al.* (2007) found that the stability of these Abs was increased in the new format. This was particularly true for structural stability, where scFv-Fabs showed greater resistance to GHI denaturation than scFvs. The authors proposed that adding C_H domains for stability could replace the additional maturation steps that would be needed to engineer the same scFv to a similar level of stability.

1.7 Epitope mapping using phage displayed peptide libraries

Jerne (1960, cited by Laver *et al.*, 1990) first defined an epitope as a particular ‘surface configuration’, ‘immunogenic element’ or ‘antigenic pattern’ on an antigen, a specific site on a molecule through which it may interact with another or other molecules. Generally, this interaction refers to the recognition of an immunoreactive determinant on an antigen by an antibody (Atassi, 1984). The success of this interaction may require one or many molecular contacts to occur and these associations may be rigidly specific or flexible (Wang & Yu, 2004).

Two groups of epitopes are defined: continuous (linear or sequential) and discontinuous (assembled or conformational) determinants (Sela, 1969; Barlow *et al.*, 1984; Wang & Yu, 2004) and both can be described at the molecular level using various techniques of epitope mapping (Atassi, 1984; Laver *et al.*, 1990; Wang *et al.*, 1995). Continuous epitopes are made up of successive adjacent amino acids arranged locally on the polypeptide chain. Discontinuous epitopes consist of residues that are not successively connected by peptide bonds, but are brought into juxtaposition in the folded three-dimensional (3D) structure (Atassi, 1984; Barlow *et al.*, 1986). Some authors (Sela, 1969; Benjamin *et al.*, 1984; Barlow *et al.*, 1986; Geysen *et al.*, 1986) have suggested that epitopes on native proteins are essentially discontinuous or ‘topographic’. In other words, with specific reference to antibody-antigen binding, linear epitopes which cross-react with a range of antibodies may mimic only the crucial contacts of the native recognition site (Barlow *et al.*, 1986). Laver *et al.* (1990) derived from structural data that epitopes on native proteins generally consist of 15 to 22 discontinuous residues, of which only five to six are essential for binding. While the remaining residues may not make direct contact, they are still needed for complementarity and altering them may abolish binding. Benjamin *et al.* (1984) document a similar trend in competitive binding assays using cytochrome *c* from various species.

The ‘induced fit’ theory described by Tainer *et al.* (1984), where initial contact with appropriate site(s) is sufficient for recognition, also parallels this model.

In 1986, Geysen *et al.* synthesized oligopeptides on polyethylene rods to map the binding of a mAb raised against foot-and-mouth disease virus. Here, synthesized peptides that reacted positively with the mAb in ELISA are described as ‘mimotopes’. These are peptides that are able to bind to ‘recipient’ molecules (e.g. an antibody) although their amino acid structures are not identical to that of the natural epitope (Geysen *et al.*, 1986). Based on this initiative, Parmley & Smith (1988) proposed the idea of ‘epitope libraries’. Using phage-display (Smith, 1985), millions of random peptides could be displayed on the surface of infectious phage and binders affinity purified by ‘biopanning’ (Fig. 1.9) (Parmley & Smith, 1988). In contrast to Geysen’s (1986) synthesis on pins, this system offered the added advantage of replicability due to the infective nature of phage viruses (Parmley & Smith, 1988). Within two years of this initiative, three phage displayed libraries for epitope mapping were constructed and published almost simultaneously (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott & Smith, 1990). Peptides displayed on the minor coat protein (pIII; Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott & Smith, 1990) and the major coat protein (pVIII; Felici *et al.*, 1991; Bonnycastle *et al.*, 1996) of filamentous phage remain archetypal, although peptides can be displayed on other phage proteins (e.g. pVI or pIX; Smith & Petrenko, 1997) or using alternative phage systems (e.g. bacteriophages λ , T7 and T4; Wang & Yu, 2004). There are fundamentally two types of peptide libraries: (i) random libraries, which can be further divided into linear or constrained libraries and (ii) gene-targeted libraries.

Randomised libraries use degenerate codons e.g. NNS or NNK, where N is an equal mixture of G, A, T and C; S is an equal mixture of G and C; and K is an equal mixture of G and T. This avoids bias at the amino acid level and lessens the occurrence of stop codons (Devlin *et al.*, 1990; Cwirla *et al.*, 1990; Wang & Yu, 2004). Linear or unconstrained random libraries display oligonucleotide chains on the surface of phage that generally do not have a three-dimensional structure (Smith & Petrenko, 1997). However, they have proved limiting as binders to all targets cannot always be isolated (Ladner, 1995). Geysen *et al.* (1986) had already noted a similar trend during his mapping experiments and suspected that a 3D structure may be required in for recognition in certain instances. To overcome this, up to three β -amino alanine residues were introduced between existing amino acids to increase the steric flexibility between them. This

altered the geometry of the peptide so that the residues required for binding were more optimally arranged (Geysen *et al.*, 1986). In constrained phage display libraries peptides are displayed in the form of secondary and/or tertiary conformations on the surface of phage (reviewed by Ladner, 1995). Peptides can be constrained into three-dimensional structures through disulphide bonds to form a loop (Lugazzo *et al.*, 1993; O'Neil *et al.*, 1992; Zhong *et al.*, 1994; Bonnycastle *et al.*, 1996), by α -helices (Petrenko *et al.*, 2002) or using a variety of scaffolds derived from the structural designs of existing molecules e.g. cytochrome b₅₆₂, ankyrin repeat domains, zinc fingers, charybdotoxin and knottins (van Zonneveld *et al.*, 1995; reviewed by Hosse *et al.*, 2006; Watt, 2006). Generally it is accepted that constrained libraries, in comparison to unconstrained, reduce the frequency of non-specific binding and provide better information about the epitope structure. Furthermore, their binders tend to be of higher affinity (Ladner, 1995). Several random libraries (constrained and unconstrained) have been used to map critical residues in molecule interactions and identify antigenic and immunogenic mimics for a variety of disease states (O'Neil *et al.*, 1992; Yu *et al.*, 2005; Casey *et al.*, 2006; Eshaghi *et al.*, 2005; Kolonin *et al.*, 2006). Additional examples and applications are reviewed in detail by Cortese *et al.* (1996) as well as Smith & Petrenko (1997).

Gene-targeted libraries contain DNase-digested genes of interest displayed on phage as native peptide sequences in contrast to mimotopes (Wang *et al.*, 1995). Although these types of libraries are not as diverse as the random peptide libraries discussed above, the display of relatively large and native sequence is advantageous for isolating epitopes with greater accuracy. This was shown in the first application which mapped an epitope on a bluetongue virus structural protein (Wang *et al.*, 1995). Fack *et al.* (1997) compared gene-targeted and random libraries to define the epitopes of four mAbs raised against different antigens. Epitopes were successfully found for all four mAbs using a gene-targeted library. In contrast, epitopes for only two of the mAbs could be delineated when two random libraries (6-mer and 15-mer) were panned. Panning on the gene-targeted library subsequently revealed that these two mAbs bound to epitopes larger than 6- or 15 amino acids respectively, and thus were consequently not represented in the random libraries. The group concluded that epitopes which spanned more than six amino acids were identified with greater consistency using gene-targeted libraries as opposed to random peptide display. Furthermore, structural epitopes could be detected. Matthews *et al.* (2002) performed a similar study to investigate whether vaccine targets isolated from a gene-derived library of the

bacteriophage T4 genome could initiate an effective immune response against native antigen ('immunogenic fitness'). The group concluded that gene-targeted libraries provided possible targets displaying greater immunogenicity than those isolated from random peptide libraries. Gene-derived peptide libraries have been used to locate various antigenic determinants (du Plessis *et al.*, 1995; Blüthner *et al.*, 1996; Fehrsen & du Plessis, 1999; Gupta *et al.*, 2001; Mullaney *et al.*, 2001; Matthews *et al.*, 2002) and to map receptor-ligand binding sites (van Zonneveld *et al.*, 1995; Jolly *et al.*, 2001).

1.7.1 The XCX₁₅ library

The XCX₁₅ library was one of 11 different pVIII-displayed libraries constructed by Bonnycastle *et al.* (1996) to investigate the influence of peptide conformation on antibody-peptide binding. Eight of these libraries were disulphide-constrained, while the remaining three were linear. The XCX₁₅ library is NNK randomised and is a 'half-Cys' library containing one cysteine residue at position two on the random peptide displayed at the N-terminus of pVIII (Bonnycastle *et al.*, 1996). Peptides from the XCX₁₅ library were displayed using the f88.4 vector (Zhong *et al.*, 1994) which is derived from fd-tet (Zacher *et al.*, 1980). The fd-tet vector was originally created as a DNA cloning vector and is derived from the genome of wild-type, non-lytic fd phage. A gene segment from the Tn10 transposon inserted into the minus-strand origin of replication confers tetracycline resistance. This selective pressure is continuously required for fd-tet phage to survive in infected/transfected cells. As a result of this insertion, fd-tet has flawed minus-strand replication, which in turn has resulted in the relatively low infectivity of fd-tet phage (Zacher *et al.*, 1980; Scott & Barbas, 2001). In f88.4 a synthetic pVIII gene cassette was introduced into the disrupted minus-strand origin, adjacent to the tetracycline-resistance genes (Scott & Barbas, 2001). This vector is thus a type '88' vector as described by Smith (1993), where two pVIII genes (one synthetic and one wild-type) are contained in the same phage genome. Although both of these genes encode similar protein sequences, their nucleotide sequences are different (Bonnycastle *et al.*, 1996; Enshell-Seijffers *et al.*, 2001). In '88' display systems, mosaic phage displaying recombinant and wild-type pVIII coat-protein are generated without the need for helper phage (Enshell-Seijffers *et al.*, 2001).

All the libraries constructed by Bonnycastle *et al.* (1996) were individually screened with 17 different Abs, each of which was directed against proteins of known structure (15 mAbs and 2

pAb). Antibodies directed against linear epitopes selected binders from several of the libraries, whereas panning with Abs raised against discontinuous epitopes was not always successful. These results confirmed that in some instances a specific epitope conformation was required, as such a constraint more effectively mimicked a discontinuous epitope. The panel of constrained and unconstrained peptide libraries provided an insightful tool for delineating structurally biased as well as polyspecific epitopes.

1.8 Expression systems

The discovery of restriction endonucleases (Linn & Arber, 1968) and recombinant DNA technology (Cohen *et al.*, 1973) enabled genes from one organism to be replicated and/or expressed in another organism (Chang & Cohen, 1974). Since then, several organisms and biological systems have been exploited for this purpose. Today, several engineered cell strains and vectors from different organisms have been ‘optimised’ and are available commercially. This section very briefly touches on heterologous protein expression in *E. coli*, mammalian and yeast cells, with particular focus on their use for Ab expression. Other systems have also been used to express Abs. These include insect cells, fungi, algae, transgenic plants and animals (Grosse-Hovest *et al.*, 2004; Ko & Koprowski, 2005; Mayfield & Franklin, 2005; Nevalainen *et al.*, 2005; Sethuraman & Stadheim, 2006; Johansson *et al.*, 2007; O’Connell *et al.*, 2007).

1.8.1 *Escherichia coli*

Of all the expression systems available, none has been better characterised than *E. coli*. Most of this organism’s popularity stems from its rapid doubling time, ease of manipulation, relatively low cost and ease of upscale (Yin *et al.*, 2007). Since the first functional Fv fragments were produced in *E. coli* (Skerra & Plückthun, 1988), various other Ab formats have been expressed e.g. full-length Abs, scFvs and single-chain Fabs (Kipriyanov *et al.*, 1997; Simmons *et al.*, 2002; Hust *et al.*, 2007; Mazor *et al.*, 2007). The only major disadvantage of producing Ab in *E. coli* is the absence of post-translational modifications, specifically glycosylation, which is needed for effector functions (Tao & Morrison, 1989; Yin *et al.*, 2007). Sometimes, however, aglycosylated Abs may be preferred (Simmons *et al.*, 2002; Mazor *et al.*, 2007). Broadly, heterologous proteins can be expressed in three different locations namely the cytoplasm, the periplasmic space or into the extracellular medium (reviewed by Makrides, 1996 and also Yin *et al.*, 2007). Proteins

expressed in the cytoplasm are commonly insoluble, whereas those in the periplasm and extracellular medium are generally soluble (Yin *et al.*, 2007). The periplasmic compartment is convenient for Ab expression primarily because the oxidative environment facilitates disulphide bonding which is important in Ab stability. Functional scFv ‘intrabodies’ have been expressed cytoplasmically in *E. coli* using mutant strains and specific chaperones or signal sequences (Sánchez *et al.*, 1999; Jurado *et al.*, 2002).

Proteins can be fused to a secretion signal (leader) sequence at their N-terminus to facilitate translocation from the cytoplasm, out through the inner membrane and into the periplasmic space (reviewed by Choi & Lee, 2004). Leader peptides are conserved in both prokaryotes and eukaryotes (Perlman & Halvorson, 1983). The positively-charged N-terminus is a stretch of two to 10 amino acids and precedes a hydrophobic core of 10 to 20 residues. The C-terminus is usually six residues long and often contains a Pro or Gly residue. Most importantly, it always contains a small neutral side-chain residue (e.g. Gly, Ala or Ser) at position -1 and also at -3, where the leader sequence is cleaved from the premature protein (Izard & Kendall, 1994; Choi & Lee, 2004). In this way, a mature protein has its native N-terminal sequence i.e. without an attached initiation residue (Met) that would otherwise be required for expression (Choi & Lee, 2004). Various different translocation systems exist (e.g. *sec*-translocase, twin-arginine pathway; Terpe, 2006) as well as several well-known leader peptides e.g. outer-membrane protein (Omp) A, C, F or T, pectate lyase (Pel) B, maltose-binding protein (MalE) or heat-stable enterotoxin 2 (StII) (reviewed by Choi & Lee, 2004). While no common guidelines exist, Sletta *et al.* (2007) have demonstrated that a compatible leader sequence can significantly increase the total yield of heterologous protein from *E. coli*.

Although protein expression directed to the periplasm or extracellular medium is considered ‘secretory’, most *E. coli* strains do not actually possess their own secretory machinery (Blight & Holland, 1994; Sørensen & Mortensen, 2005). ‘Secretion’ into the extracellular medium from the periplasm is mostly passive ‘leakage’, which is especially common under the control of a weak promoter e.g. *lac* promoter (Sørensen & Mortensen, 2005; Terpe, 2006). Active extracellular protein secretion is poorly understood, although some bacterial strains have been engineered to perform this process and contain transplanted secretion apparatus (Blight & Holland, 1994; Majander *et al.*, 2005). Other methods of encouraging transport out of the cell include fusing the

target protein to naturally secreted proteins or to signal sequences (Angkawidjaja *et al.*, 2006). Another way is to co-express the protein of interest with chaperones or simply to supplement the medium with glycine (Makrides, 1996).

1.8.2 Mammalian cells

Mammalian cell culture is viewed as costly and technically demanding. It nevertheless remains the expression system of choice for almost three-quarters of the therapeutic Abs currently produced. Eukaryotic systems, specifically mammalian cell lines, possess advanced protein folding, secretion and post-translational modification pathways in comparison to their prokaryotic counterparts. Most significantly, these systems can perform post-translational modifications (Sethuraman & Stadheim, 2006; Schirrmann *et al.*, 2008). Glycosylation of Abs is paramount not only for downstream biological effector functions *i.e.* complement activation and Ab-dependant cellular cytotoxicity (ADCC), but also for molecular stability *i.e.* resistance to protease degradation and heat-induced denaturation (Werner *et al.*, 2007). Suspension cultures at high cell-densities are generally used for large-scale production with the proteins being secreted into the medium. Secretion requires a secretion signal sequence, similar to that described above for *E. coli* (Sakaguchi, 1997). In recent years there has been a trend toward serum-free culture to reduce cost and avoid contamination by pathogens or prions (Chu & Robinson, 2001; Chiba & Jigami, 2007). Although extensive developments have occurred in eukaryotic cell systems over the last decade, novel mammalian production cell lines and expression vectors are continually being engineered and optimised to improve consistency and stability. Yields of up to 5 g/l can be achieved (Schirrmann *et al.*, 2008).

Mammalian cell lines or their engineered derivatives are frequently used to express therapeutic proteins. Examples are Chinese hamster ovary cells (CHO), CV-1 origin, SV40 (COS) cells, murine lymphoid cells and human embryonic kidney (HEK) cells. A variety of Ab formats directed against a variety of targets have been successfully expressed in these lines (Trill *et al.*, 1995; Birch & Racher, 2006; Schirrmann *et al.*, 2008). A CHO cell culture that stably produces chicken antibodies was reported by Shimamoto *et al.* (2005). IgYs derived from a chicken hybridoma have been produced at ten-fold higher concentrations in CHO-K1 cells than in the parent hybridoma. Anti-parasitic Abs against *Plasmodium falciparum* (Lundquist *et al.*, 2006) and anti-Shiga toxin 2 Abs (Akiyoshi *et al.*, 2005) have also been produced in these cells.

Furthermore, a CHO *FUT8*-knockout strain engineered by Yamane-Ohnuki *et al.* (2004) stably produced defucosylated recombinant IgG1 Abs which demonstrated increased ADCC compared to their fucosylated equivalents. COS cells have been used to produce bi-specific Abs recognizing cardiovascular markers (Shen *et al.*, 2006) as well as humanised C_{H3}-fusion anti-tumour Abs which were more stable than those produced in a prokaryotic system (Shi *et al.*, 2007). Murine lymphoid (NS0 and Sp2/0) suspension cultures have been used to manufacture therapeutic human-mouse chimeric Abs e.g. Synagis™ and Zenapax™ (Chu & Robinson, 2001). Single-chain Fv fragments have been reconstituted to functionally-glycosylated IgG, IgE and IgY Abs in HEK 293 cells (Greunke *et al.*, 2006; Braren *et al.*, 2007a). Recently, this cell line was used to generate a ‘bifunctional fusion inhibitor’ of HIV-1 consisting of an anti-CCR5 co-receptor mAb and two covalently attached fusion inhibitors (Kopetzki *et al.*, 2008). In addition to the above examples, baby hamster kidney (BHK) cells and human retinal (Per.C6) cells have also been used to produce rAb (Schirrmann *et al.*, 2008). These examples illustrate that despite perceptions to the contrary, mammalian cells are among the more successful Ab production systems available at present.

1.8.3 Yeasts

Yeasts are versatile organisms for heterologous protein expression. Not only are they economical, considered by the majority as straightforward to use and are generally regarded as safe (*i.e.* they do not sustain pathogens, nor produce harmful by-products such as pyrogens), but they also grow to high densities in basic yeast medium and are capable of post-translational processing similar to eukaryotes (Böer *et al.*, 2007; Gach *et al.*, 2007). Although glycosylation is limited to the addition of mannose (Gellissen, 2000), glycoengineering to produce therapeutic proteins with humanised carbohydrate moieties appears to be a promising development (Li *et al.*, 2006; also reviewed by Chiba & Jigami, 2007). Foreign proteins produced in yeasts are by and large fused to leader sequences, such as the α -mating factor gene (α -MF1) and secreted into the extracellular medium (Gellissen, 2000; van Ooyen *et al.*, 2006). Since the lysis of yeast cells requires enzyme digestion due to the composition of the cell wall, cytoplasmic expression is not popular as it increases the time and cost involved in isolating the protein of interest. In yeasts, integrative expression vectors are preferred over episomally replicating types. Even though episomal plasmids are available at higher copy numbers for expression, genome integration results in more stable cultures which are more consistent protein producers (Gellissen, 2000; van Ooyen *et al.*,

2006). Indeed, a single vector, engineered to integrate into the chromosomes of a variety of different yeasts has now been developed (Terentiev *et al.*, 2004).

The production of functional, recombinant IgG1 and Fab fragments in lower eukaryotes was first described by Horwitz *et al.* (1988), who expressed full-length and Fab fragment anti-tumour Abs into the growth medium of the ‘bakers yeast’ *Saccharomyces cerevisiae*. This was the first yeast to be evaluated as a functional heterologous protein expression system. However, limitations of its use (e.g. hyperglycosylation and allergenic α 1,3-mannose terminal bonds) led to the investigation of alternative yeast species (Terentiev *et al.*, 2004; Böer *et al.*, 2007). Antibody production has been attempted in ‘less conventional’ yeasts such as *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe* and methylotrophic yeasts such as *Pichia pastoris* or *Hansenula polymorpha* (Joosten *et al.*, 2003). Presently, *P. pastoris* seems to be the most popular for Ab production. Several Abs have been produced with this system, including scFvs (Freyre *et al.*, 2000), full length IgGs with human-specific glycans (Li *et al.*, 2006), recombinant (Lange *et al.*, 2001), chimeric (Gach *et al.*, 2007) and humanised Fab fragments (Takahashi *et al.*, 2000; Ning *et al.*, 2005) and scFv-Fc fusions (Powers *et al.*, 2001; Braren *et al.*, 2007b). There are few examples of Abs expressed from *K. lactis*, although scFvs can be secreted in milligram amounts per litre (Swennen *et al.*, 2002; van Ooyen *et al.*, 2006). Swennen *et al.* (2002) produced the same scFv in both *K. lactis* and *Y. lipolytica*. Although not a common expression system for Abs, *S. cerevisiae* has been used by Frenken *et al.* (2000) to secrete heavy-chain Ab fragments (V_{HH}). This group achieved yields over 100 mg/l and further large-scale expression of V_{HH} Abs by fed-batch fermentation was later reported by Thomassen *et al.* (2002). In addition, intracellular, catalytic Fabs have been expressed in *S. cerevisiae* to restore chorismate mutase activity (Bowdish *et al.*, 1991). However, low yields of active Ab were achieved. Recently, the lesser known methylotrophic yeast *Ogataea minuta* was used by Kuroda *et al.* (2008) to express full-length Abs.

1.9 Recombinant antibodies in immunoassays

Traditionally, immunoassays have relied upon Abs from immunised animals or hybridomas (Finlay *et al.*, 2006). Recently, however, rAbs have become increasingly popular in diagnostics and therapeutics research, with those from chickens being particularly attractive (reviewed by Narat, 2003).

Chicken Ig genes are rearranged from a single V-region gene segment (discussed in 1.4.1) making the immune repertoire relatively easily accessible (Davies *et al.*, 1995). Being phylogenetically distinct from mammals, chickens react strongly when immunised with mammalian proteins. This is a particular advantage when generating Abs against molecules which are known to be poor immunogens in mammals (Nakamura *et al.*, 2004). The chicken IgG homologue (designated IgY) is inherently robust and can be stored under varied conditions without a significant decrease in activity (Narat 2003; Nilsson & Larsson, 2007). It does not cross-react with mammalian antibodies e.g. rheumatoid factors (RF), bind to protein A or G, nor does it activate the complement system. It is therefore suited to immunoassays where unwanted cross-reactions may cause 'background' signals or false-positives (Larsson *et al.*, 1991; Shimamoto *et al.*, 2005). The eggs of immunised chickens are a cheap and abundant source of native Ab that places comparatively little burden on the experimental animals (Narat, 2003). Native IgY from the serum or egg-yolks of immunised donors has been used widely in ELISAs to detect e.g. allergens (Blais *et al.*, 2002), viruses (Juliarena *et al.*, 2007) and biological (Phillips *et al.*, 1995) as well as disease-specific markers (Lee *et al.*, 2004). Several recombinant chicken Abs, mostly scFvs, have been used in ELISAs to detect prion proteins (Nakamura *et al.*, 2004; Miyamoto *et al.*, 2007), viral antigens (Fehrsen *et al.*, 2005; Foord *et al.*, 2007; Lee *et al.*, 2007), disease-markers (Sato *et al.*, 2008) and toxins (Finlay *et al.*, 2006). Despite the success of rAbs in assays such as ELISA, this resource has not yet been exploited in gold-conjugated immunochromatographic tests (ICTs) and there are no reports at all in the scientific literature regarding recombinant chicken Abs in gold-conjugated ICTs.

ICTs are similar to ELISAs in that they can be performed in either a sandwich- or an indirect format (Fig. 1.10). Basic sandwich ICT tests use two Abs which recognise different epitopes on the same antigen (Yuhi *et al.*, 2006) with the capture Ab being immobilised on a nitrocellulose or other suitable membrane. The Ab used to detect the captured antigen is conjugated to nanometer-

scale colloidal gold particles and is initially dried onto a glass fibre ‘conjugate pad’ which is attached to one end of the membrane. When a liquid test sample is applied to a ‘sample pad’, it diffuses through the conjugate pad, rehydrating the detection Ab which binds the sample analyte. This complex is eventually immobilised by the capture Ab as it migrates up the strip. The accumulation of gold particles on the membrane forms a clearly visible red band (Chandler *et al.*, 2000; Yuhi *et al.*, 2006). A control line is usually also included. These tests are useful as field tests since they are rapid and generally stable over long periods under various conditions. They are also portable, user-friendly and inexpensive to manufacture (Shyu *et al.*, 2002).

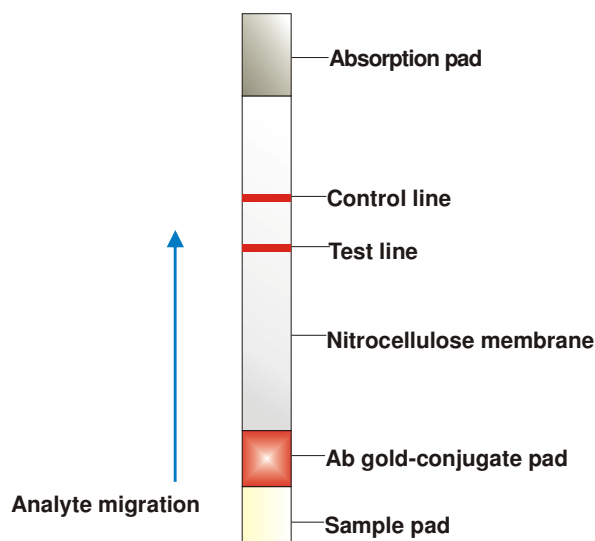


Figure 1.10 Basic ICT format. Antibodies for capture are immobilised on a nitrocellulose membrane (capture and control lines). Antibodies conjugated to gold particles are available in a dried state on the conjugate pad. Liquid test sample is applied to the sample pad, and migrates through the conjugate pad towards the absorbent pad by diffusion. Conjugated Ab will bind analyte present in the sample and be trapped on the capture line, creating a red signal line (adapted from Chandler *et al.*, 2000).

In contrast to their ease of use, ICTs are complicated to develop and require extensive optimisation of several parameters including analyte buffers, blocking agents, test membranes, preservatives, surfactant content, conjugate concentration, gold colloid size and total protein concentration (Robinson, 2002). The colloidal gold used in ICTs is a particle suspension or ‘sol’, based on electron-dense negatively charged hydrophobic metal particles stabilised by electrostatic repulsions which allow them to repel one another continuously and thus remain in

suspension (Geoghegan & Ackerman, 1977; Daumas *et al.*, 2002). The suspended particles can be electrostatically coupled to proteins, including Ab and antigens, to form an ‘immunogold’ complex. Electrostatic coupling is advantageous for immunodiagnostics as it reportedly does not distort macromolecules (Geoghegan & Ackerman, 1977), which is often a shortfall of solid-phase immunoassays (Butler *et al.*, 1992).

Good-quality gold sols are paramount in developing sensitive and reproducible ICTs. They are available from commercial sources and are evaluated ultrastructurally using transmission electron microscopy to check for uniformity of shape and size. Irregular particles will not repel other gold particles uniformly, nor will they coat evenly with protein. This may lead to an unstable conjugate (Chandler *et al.*, 2000). Generally, particles between 40 and 100nm can be successfully conjugated to IgG to produce a visible signal. Particles of 40nm are the most widely used for conjugating to antibodies or antigens, but sizes of 20nm can be used for smaller molecules (Robinson, 2002). Conjugation takes place at a pH near the isoelectric point of a protein (Geoghegan & Ackerman, 1977; Gasparyan, 2005). The amino acids Lys, Trp and Cys are important for adsorption. Lys is positively charged and is inherently attracted to the negatively charged gold particles, drawing the gold and protein molecules closer together. Trp is involved in hydrophobic interactions while Cys creates strong, permanent dative bonds by creating sulphur bridges such that electrons are shared (Robinson, 2002; Chandler *et al.*, 2000). When Abs are conjugated to gold they tend to adsorb via their Fc regions. This leaves the Fab available to bind analyte. Certain Ab formats are easier to couple to gold than others and the most success has been had with IgG, specifically IgG1 (Robinson, 2002). Smaller proteins, or proteins containing very few of these amino acids, can be pre-conjugated to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) to improve coupling and sensitivity. Alternatively, the signal can be enhanced using silver deposition (Robinson, 2002).

Gold-particle conjugation has been used routinely in electron microscopy (EM) for a number of years (Kleymann *et al.*, 1995; Hainfield & Powell, 2000). Only recently has it found its way into immunodiagnostics in the form of ICTs such as lateral flow assays or flow-through assays. In this format, immunogold complexes can detect viruses (Nielsen *et al.*, 2007; Peng *et al.*, 2007), toxins (Shyu *et al.*, 2002; Klewitz *et al.*, 2006), parasites (Zhang *et al.*, 2006) and other molecules (Wang *et al.*, 2005). Most of these examples utilise full-length mAbs or pAbs for conjugation and

it appears that there are very few tests that use rAbs (Nielsen *et al.*, 2007). Chicken IgY has been reported to produce colloids of even higher stability than IgGs (Gasparyan, 2005) and thus may be a useful alternative in such assays. However, although IgY has proved its worth in immunoassays such as the ELISA, only one example of an ICT using gold-conjugated IgY can be found in the literature (Kalvatchev *et al.*, 1993). This example uses egg-yolk derived IgY and not a rAb. Greater demands for sensitivity, reproducibility and rapidity in diagnostic assays have made gold the label of choice for ICTs. The focus is now on developing stable, high-affinity rAb to be used in these formats in the future.

1.10 Conclusions and objectives

In both the medical and veterinary diagnostic fields there is an ongoing need for stable and specific antibodies which can be economically produced. From the foregoing it is clear that platforms such as phage display, heterologous protein expression systems and rAb engineering have a major role to play in the development of robust, new-generation immunochemical reagents. Preliminary tests using a number of the scFvs isolated from the *Nkuku*® library have revealed that the scFv format can be problematic in some ICT immunoassays (Mr. N. Borain and Mr. A. Bohms, personal communication). It is hypothesised therefore that single-chain Ab reconstituted into complete IgY or an IgY-like format (Greunke *et al.*, 2006) will be more generally useful than monovalent scFvs in both enzyme-linked immunosorbent assays (ELISAs) and ICTs. Accordingly, this project focuses on determining whether two scFvs derived from the *Nkuku*® library can be converted into molecules that can be used as practical immunodiagnostic reagents. This study seeks to establish: (i) whether anti-HSP65 scFvs previously derived from the *Nkuku*® repertoire are sufficiently stable and robust to be used as practical immunoreagents; (ii) whether scFvs can be reconstituted into IgY-like (bivalent) formats and whether these formats can be successfully produced in mammalian cells; (iii) the specificity and sensitivity of these reconstituted bivalent Abs in ELISA; (iv) whether these bivalent Abs can be expressed and purified from alternate heterologous systems and (v) how scFvs and ‘improved scFv’ constructs perform in ICT format.

Chapter 2

MATERIALS AND METHODS

Materials

2.1 General Reagents

See Appendix: Buffers, Stock solutions and media.

2.2 Specific Reagents

2.2.1 Cell strains, cell lines and plasmid vectors

Electrocompetent TG1 *E. coli* (F⁻ and Amp resistant) were purchased from Stratagene (La Jolla, USA). The mammalian expression vector scFvIgY(CH₂₋₄)His (Greunke *et al.*, 2006) which contained chicken H-chain Ig genes was kindly provided by Dr. Edzard Spillner of the University of Hamburg, Germany. The bacterial expression vectors QIAexpress pQE-TriSystem Vectors (Amp and Kan resistant) and M15 *E. coli* (F⁻ and Kan resistant) used for bacterial expression were supplied by QIAGEN® (Hilden, Germany). The pUC19 plasmid vector was from Invitrogen™ (Carlsbad, USA). The yeast expression vector pKLAC1 and *K. lactis* GG799 competent cells were components of the *K. lactis* Protein Expression Kit from New England Biolabs (Ipswich, USA).

2.2.2 Antibodies

Anti-HSP65 and anti-16 kDa scFvs were derived from the *Nkuku*® library (van Wyngaardt *et al.*, 2004) by Mr. W. van Wyngaardt and Ms C. Mashau of the Immunology Section, OVI. Single-chain Fvs were affinity-purified and supplied by Ms J. Frischmuth of the National Bioproducts Institute (Pinetown, South Africa). Anti-c-myc Ab (9E10) was produced in-house from a hybridoma culture by Mr. van Wyngaardt. HRP-conjugated polyclonal rabbit anti-mouse, unconjugated rabbit anti-*Mycobacterium bovis* and HRP-conjugated swine anti-rabbit Abs were from DakoCytomation (Glostrup, Denmark). HRP-conjugated polyclonal goat anti-chicken IgG

was acquired from Serotec (Kidlington, UK). HRP-conjugated polyclonal rabbit anti-chicken IgG Fc-fragment and unconjugated affinity-purified rabbit anti-chicken IgG Fc-fragment Abs were from Rockland Immunochemicals (Gilbertsville, USA). Egg-yolk derived chicken IgY was provided by Dr. J. Fehrsen (Immunology Section, Onderstepoort Veterinary Institute). A mouse mAb directed against the pVIII of filamentous phage (B62-FE2) was acquired from Progen Biotechnik (Heidelberg, Germany).

2.2.3 Antigens

Recombinant HSP65 from *Mycobacterium bovis* BCG was acquired from Aalto Bio Reagents (Dublin, Ireland) or from Stressgen (Michigan, USA). The *M. tuberculosis* 16 kDa antigen was from a proprietary source. Both antigens were a generous gift from Vision Biotech (Pty) Ltd. (Cape Town, South Africa).

2.2.4 Peptide library and phage stocks

Glycerol stocks of the XCX₁₅ peptide library (Bonnycastle *et al.*, 1996) both harboured in bacteria (K91 *E. coli*) and as precipitated phage particles (f88.4) were supplied by Dr. J. Fehrsen (Immunology Section, OVI).

2.2.5 Oligonucleotides

Custom oligonucleotides were obtained from Inqaba Biotech (Pretoria, South Africa) and also from Invitrogen™ (Carlsbad, USA). These are listed in Table 2.1.

Table 2.1 Oligonucleotides used for sub-cloning, sequencing and site-directed mutagenesis.

Oligo Name	Sequence
CH2-4 Yeast For	5' GGGGGTACCCGCCTGTAGCCCCAGAGG 3'
CH2-4rev	5' AGGAGGAGGG GTGGAGGACC 3'
CH3rev 1	5' TGGTGATGGTGATGGTGATGCGTGTTCCTGTAGACGCTCT 3'
CH3rev 2	5' CCCCCCGCGGCCCGCCTAGTGATGGTGATGGTGATGGTGATGGTGATGC 3'
CHpUC Cterm	5' CCCCCATGGTCAGTGATGGTGATGTTTACCAGCC 3'
EcoRV-ompA 1	5' GCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCCGCCGTGACGTTGGACGAG 3'
EcoRV-ompA 2	5' CCGGATATCATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCT 3'
EcoRV-pelB 1	5' GGATTGTTACTACTCGCGGCCAGCCGGCCATGGCCGCCGTGACGTTGGACGAG 3'
EcoRV-pelB 2	5' GGCGATATCATGAAATACCTATTGCCTACGGCAGCCCGTGGATTGTTACTACTCGCGGC 3'
Integration Primer 1	5' TACCGACGTATATCAAGCCCA 3'
Integration Primer 2	5' ATCATCCTTGTGACGCGAAAGC 3'
Integration Primer 3	5' CAGTGATTACATGCATATTGT 3'
M13 for	5' GTTTTCCAGTCAGGAC 3'
M13 rev	5' CAGGAAACAGCTATGAC 3'
Mut Yeast For	5' GTCACCCTGAGCTGCCTGGTACGAGGCTTCCGCCCA 3'
Mut Yeast Rev	5' ACCAGGCAGCTCAGGGTGACGCGGGAGAGGG 3'
NotI-Cterm	5' AACTCGGCGGCCGCTTTACCAGCCTGTTTCTGCA 3'
Nterm CH2-4	5' GGGGGTACCCGCCTGTAGCCCCAGAGGTG 3'
pKLAC 1 seqFor	5' GAAGAAGCCTTGATTGGATTC 3'
pKLAC1 seqRev	5' TTATCGCACAAGACAATCGGC 3'
pQEsFor	5' GTTATTGTGCTGTCTCATC 3'
pQEsRev	5' CACAAATACCACTGAGATCGA 3'
scFv-Cterm Kpn	5' CCCGGTACCCTAGGACGGTCAGGGTTG 3'
T7for	5' TAATACGACTCACTATAGGG 3'
VHBsiW	5' GATCCGTACGGCCGTGACGTTGGACG 3'
VLAsc	5' GATCGGCGCGCCACCTAGGACGGTCAGGG 3'
VLF1	5' GACTCAGCCGTCCTCGGTGTCAG 3'
XCXseq2	5' AGTAGCAGAAGCCTGAAGA 3'
Yeast For	5' GGGGTGACAAAAGAGCCGTGACGTTGGACGAG 3'
Yeast Rev	5' CCCCCGCGGCCGCTCAGTGATGGTGATGGTGATGGTGATGGTGATGTTTACCAGC 3'

Methods

Unless specified, molecular biological methods were as described by Sambrook & Russel (2001).

2.3 Plasmid extraction and purification

Bacteria were grown overnight at 37 °C with shaking (220 rpm) in LB medium containing the appropriate antibiotic. Cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C and plasmids were extracted using the QIAGEN® Plasmid Midi Kit or QIAFilter™ Plasmid Midi Kit according to the manufacturer's (QIAGEN®, Hilden, Germany) instructions. Essentially, harvested cells were resuspended in buffer and subjected to alkaline lysis. This lysate was neutralised and cleared by centrifugation, or in the case of the QIAFilter™ kit, by filtration. Cleared lysate was subjected to anion-exchange chromatography and the eluted DNA was recovered by ethanol precipitation.

2.4 DNA precipitation

DNA was precipitated by adding 2.5 volumes of absolute ethanol and a one-tenth volume of NaOAc to the DNA samples. The suspensions were left at -20 °C for 1 hour to overnight and then centrifuged at 14 000 rpm using a Hettich (Tuttlingen, Germany) EBA 12R microcentrifuge for 20 min at 4 °C. Pellets were air-dried and resuspended in the desired volume of buffer or ddH₂O.

2.5 Determining DNA concentration

DNA was diluted appropriately and volumes of 1 to 2 µl were analysed with a NanoDrop® ND-1000 Spectrophotometer using the associated 'Nucleic Acids' software application module (NanoDrop Technologies Inc., Wilmington, USA). Concentration was expressed in ng/µl, based on absorbance at 260 nm, with an extinction coefficient of 50 ng-cm/ml. The NanoDrop® has a pathlength of 0.2 mm.

2.6 Agarose gel electrophoresis

DNA samples were separated in 1 % agarose slab gels containing 0.5 to 1 µg/ml ethidium bromide. Electrophoresis was carried out in 1 x Tris-acetate-EDTA (TAE) buffer according to standard methods (Sambrook & Russel, 2001). Gels were viewed using transmitted UV light and a digital record was made using the UVP AutoChemi™ System (Upland, USA). The Hyperladder I DNA molecular mass standards (Bioline, London, UK) were used as a reference for DNA fragments larger than 1 kb. The Fermentas O'GeneRuler™ 50 bp DNA ladder (Burlington, Canada) or the Promega 100 bp Ladder (Madison, USA) were the standards for DNA fragments smaller than 1 kb.

2.7 Sub-cloning genes into plasmid vectors

Relevant genes were amplified from plasmid vector templates using the Fast Start High Fidelity PCR System (Roche, Mannheim, Germany) or *Ex Taq*™ polymerase (Takara, Shiga, Japan) Suitable oligonucleotide primers (Inqaba Biotech, Pretoria, South Africa and Invitrogen™, Carlsbad, USA) added restriction enzyme cleavage sites to the relevant ends of the amplicon (Table 2.1). Vectors and inserts were digested with the appropriate restriction endonucleases while vectors were further digested with Antarctic Phosphatase (New England BioLabs, Ipswich, USA) to remove 5' phosphoryl groups and prevent vector re-ligation.

2.8 Restriction endonuclease digestion

The restriction endonucleases *Acc65 I*, *Asc I*, *Bsi W*, *EcoRV*, *Kpn I*, *Not I*, *Sac I*, *Sac II*, *Sal I* and *Xho I* were obtained from New England Biolabs (Ipswich, USA). Restriction digestion was as directed by the manufacturer in the supplied buffers. The amount of DNA digested was dictated by need and the reaction was scaled up appropriately each time. Where two enzymes were optimally active in the same buffer, target DNA was digested with both enzymes simultaneously. An aliquot of each digest was electrophoresed on an agarose gel to verify that digestion was successful. The digested DNA was purified and recovered as described below.

2.9 DNA purification after PCR amplification and/or endonuclease digestion

2.9.1 Crystal violet agarose gel electrophoresis

To reduce exposure to UV light, DNA fragments were separated in crystal-violet agarose gels as described by Rand (1996). Alternatively, samples were separated on slab-gels containing a one-tenth concentration of ethidium bromide.

2.9.2 Recovery of DNA

PCR amplicons and restriction endonuclease digests were purified and concentrated using commercial kits according to the manufacturer's instructions (QIAGEN®, Hilden, Germany and Invitex, Berlin, Germany). DNA fragments separated by ethidium bromide agarose gel electrophoresis were recovered using Takara Recochips (Shiga, Japan) or by gel extraction (QIAGEN®, Hilden, Germany).

2.10 Ligation

DNA ligation was done using the Rapid DNA Ligation Kit from Roche (Mannheim, Germany). Plasmid and insert DNA were mixed in suitable molar ratios and diluted to a final volume of 10 µl. An equal amount of ligation buffer was added, the reaction was mixed and the DNA ligase was added. The reaction was allowed to stand at RT for at least 5 min.

2.11 Colony PCR

Colony PCR was essentially as described by Güssow & Clackson (1989). Bacterial colonies were picked and resuspended in 20 µl H₂O. Cells were lysed at 100 °C for 10 min then centrifuged at 14 000 rpm for 2 min. Ten microlitres of the supernatant fluid (SNF) was used in a standard 25 µl PCR reaction with GoTaq® Green Master Mix (Promega, Madison, USA) and 10 pmol of the applicable primers to amplify inserts.

2.12 DNA sequencing

Nucleic acids were sequenced on the Applied Biosystems ABI PRISM® 3100 Genetic Analyser (Foster City, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) which is based on the dideoxy chain-termination DNA sequencing method of Sanger *et al.* (1977). Sequence alignment was carried out using BioEdit (Hall, 1999).

2.13 Site-directed Mutagenesis

2.13.1 GeneTailor™ System

Mutations were introduced into DNA sequences using the GeneTailor™ Site Directed Mutagenesis System (Invitrogen™, Carlsbad, USA). Essentially this entailed subcloning the DNA of interest into a vector of choice, methylation and amplification using AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen™, Carlsbad, Germany) or *Ex Taq*™ polymerase (Takara, Shiga, Japan). The primers used for mutagenesis were designed to have a 15 – 20 nucleotide overlap at their 5' ends (Mut Yeast For/Rev, Table 2.1). Only one of these primers contained the mutated sequence, which flanked the overlap downstream. Extra nucleotides were included downstream of the mutated site to ensure correct annealing. The linear, double-stranded amplicon was used to transform One-Shot® MAX Efficiency® DH5 α ™-T1^R *E. coli* cells. The kit is designed such that methylated template DNA within DH5 α ™-T1^R cells is degraded by a host endonuclease, leaving only unmethylated mutated product. Transformants were selected and screened for inserts using colony PCR. Positive transformants were sequenced to verify that mutagenesis had taken place.

2.13.2 Mutagenesis by overlap extension in PCR

Where the commercial system (above) was not successful, mutagenesis was carried out according to the methods described by Ho *et al.* (1989) and Horton *et al.* (1989). The overlapping mutagenesis primers described above were used, in addition to two flanking region primers which appended restriction digestion sites to the respective 5' and 3' ends of the amplicon. Essentially, two amplicons with overlapping ends are generated in two separate PCRs, using a corresponding mutagenesis primer and flanking primer per reaction. In a third PCR, the two overlapping amplicons are annealed, and each one serves as a primer for the 3' extension of the other (illustrated in Fig. 3.43).

2.14 ELISA

2.14.1 Indirect ELISA

Maxisorp Immuno™ Plate (Nunc™, Roskilde, Denmark) wells were coated with 50 μ l of 10 μ g/ml antigen either overnight at 4 °C or for 1 hour at 37 °C. Unoccupied binding sites were blocked with 2 % (w/v) milk-powder in 1 \times PBS solution (MPPBS) at 37 °C for 1 hour. Blocking solution was removed and incubation with primary Ab diluted in 2 % (w/v) MPPBS followed.

Wells were washed three times with 1 x PBS containing 0.05 (v/v) % Tween 20. Secondary Ab, diluted appropriately in 2 % (w/v) MPPBS was incubated followed by three washes. HRP-conjugated detecting Ab diluted in 2 % (w/v) MPPBS was added and incubated under the same conditions as previously. Wells were washed as described. Substrate solution containing 5 mg OPD and 2.5 μ l H₂O₂ in 5 ml citrate buffer was made up fresh. Fifty microlitres was added to each well and the colour reaction was allowed to develop. The reaction was stopped using 50 μ l of 1 M H₂SO₄ per well. Absorbance was read at 492 nm using an ELISA plate reader (BDSL Immunoskan MS, Labsystems). In control experiments, the immobilised antigen was replaced with 2 % (w/v) MPPBS. All determinations were performed in duplicate.

2.14.2 Sandwich ELISA

Sandwich ELISAs were as above, except that Polysorp Immuno™ Plate (Nunc™, Roskilde, Denmark) wells were coated with 10 μ g/ml capture Ab diluted in 1 x PBS. Antigen (10 μ g/ml) was incubated for 1 hour at 37 °C after blocking with 2 % (w/v) MPPBS. Wells were washed as described above and conjugated detecting Ab was added, diluted as required in 2 % (w/v) MPPBS. After 1 hour at 37 °C, wells were washed once again. Colour development was read as described in Section 2.14.1. Control experiments were the same, except that immobilised Ab was replaced with 2 % (w/v) MPPBS. All determinations were performed in duplicate.

2.15 PAGE analysis of proteins

PAGE was carried out according to standard protocols (Sambrook & Russel, 2001). Briefly, protein samples diluted 1:1 with 2 x protein-sample buffer (PSB) were denatured at 100 °C for 5 min and separated on the basis of molecular weight in a pre-cast 4 – 12 % gradient Bis-Tris gel in 1 x MOPS running buffer (both Bio-Rad, Hercules, USA). Electrophoresis was carried out in a Criterion™ Cell vertical gel system (Bio-Rad, Hercules, USA) at 200V for approximately 1 hour. Proteins were stained with Coomassie Blue at RT for 15 to 30 min. The gel was destained overnight at RT in 4 % (v/v) glacial acetic acid. Precision Plus Kaleidoscope™ Protein Standard (Bio-Rad, Hercules, USA) was used as a molecular weight marker for all PAGE analyses.

2.16 Western Blotting

Electrophoretically separated proteins were transferred to Invitrolon™ polyvinylidene difluoride (PVDF) membranes (Invitrogen™, Carlsbad, USA) in an EC140 Mini Blot Module apparatus (E-C Apparatus Corporation, Holbrook, USA) in 1 x transfer buffer for 1 ½ hours at 15 V. The blot was blocked in 2 % (w/v) MPPBS for 1 hour at RT with gentle rocking. Protein was detected using the appropriate primary and/or secondary Abs diluted in 2 % (w/v) MPPBS. The membrane was washed three times for 1 min in 1 x PBS containing 0.05 % (v/v) Tween 20 after incubation with each Ab. HRP-conjugated Ab was added next, diluted and incubated as described, and the membrane was once again washed. Pierce SuperSignal® West Pico Chemiluminescent Substrate (Rockford, USA) was added and incubated for 5 min. The blot was viewed with the SYNGENE GeneGnome HR BioImaging system and its Analysis Software (Cambridge, UK) or otherwise exposed and developed on photo-reactive film (Roche, Mannheim, Germany). Alternatively, a HisDetector™ Western Blot Kit was used according to the manufacturer's (KPL, Gaithersburg, USA) instructions to colorimetrically detect histidine-tagged proteins.

2.17 Spot Blotting

Protein samples were spotted directly onto PVDF or nitrocellulose membranes. One microlitre of sample was used per spot. Proteins were detected as for western blotting (Section 2.16).

2.18 Protein purification

2.18.1 Ni-NTA affinity purification

Histidine-tagged proteins were diluted in 1 x lysis buffer (supplied with kit) and allowed to bind to Ni-NTA agarose at RT for at least one hour (QIAGEN®, Hilden, Germany). The slurry was rotated slowly throughout the binding period after which it was allowed to settle in a polypropylene column. The flow-through was collected and passed through the matrix a second time. The column was washed twice with washing buffer (supplied with kit) and proteins were eluted in 1ml fractions using elution buffer (supplied with kit). Small-scale Ni-affinity purifications were done using the QIAGEN® Ni-NTA Spin Kit (Hilden, Germany) according to the manufacturer's instructions.

2.18.2 Immunoaffinity purification

Anti-IgY affinity columns were constructed using the AminoLink® Plus Immobilisation Kit (Pierce, Rockford, USA) and polyclonal anti-IgY Abs (Rockland Immunochemicals, Gilbertsville, USA). Both coupling and purification were carried out at pH 7.2 according to guidelines provided by the manufacturer. IgY was diluted appropriately in coupling buffer, added to the coupling gel slurry along with sodium cyanoborohydride and mixed gently for at least four hours. Any remaining reactive sites were blocked using quenching buffer and sodium cyanoborohydride with gentle rotation for 30 min. After extensive washing to remove non-coupled protein, the column was equilibrated with binding buffer. The sample was twice passed through the column by gravity flow. Non-bound sample was removed by washing. Proteins were eluted in 1ml fractions at pH 2.5 - 3 and neutralised with 1 M Tris-HCl at pH 8.5 - 9 after each collection.

2.18.3 Polyethylene glycol (PEG) precipitation of IgY

Attempts to purify IgY-like molecules by PEG precipitation were adapted from Polson (1985) for the isolation of IgY from egg-yolk. Briefly, a sample with a volume x was added to a solution of $[(5 \times 5x)/100]$ g PEG 6000 dissolved in $4x$ ml PBS. The solution was mixed and centrifuged at $5000 \times g$ for 20 min. The SNF (volume y) was removed and $[(y \times 8.5)/100]$ g PEG was dissolved in this fluid. The sample was centrifuged at $5000 \times g$ for 25 min. The SNF was discarded and the pellet was resuspended in $2.5x$ ml PBS and $[(12 \times 2.5x)/100]$ g of PEG was added and dissolved. The solution was incubated at RT for 10 min and then centrifuged as described previously. The SNF was again discarded and the pellet was re-centrifuged to remove all traces of SNF. The final pellet was resuspended in $0.25x$ ml PBS.

2.18.4 Ion exchange chromatography

Whatman® (Kent, England) DE52 pre-swollen DEAE-cellulose was equilibrated in decreasing concentrations of PBS ($5 \times$, $1 \times$, and $0.5 \times$) by suspending it in buffer, allowing the particles to settle and reading the pH of the SNF. This was repeated until the pH was equal to that of the starting buffer, before moving to the next (lower) concentration. Once the slurry had been equilibrated to $0.5 \times$ PBS, the SNF was removed and discarded. Serum-supplemented cell culture medium containing the secreted proteins was diluted 1 : 1 with ddH₂O and added to the remaining sediment. The suspension was agitated intermittently over the course of 15 min, after

which the DE52 was allowed to settle. The SNF was removed to a new tube and analysed by SDS-PAGE (Section 2.15).

2.19 Determination of protein concentration

Purified protein was quantitated spectrophotometrically using the Bio-Rad Protein Assay, based on the method of Bradford (1976). Three dilutions of protein sample were prepared (1/10, 1/100, 1/1000) in protein buffer (usually 1 x PBS). One part of Assay Dye Reagent was added to four parts of sample and mixed well. A blank was set up in the same way using protein buffer only, in place of protein sample. The samples were left for 5 min and the A_{595} was read on a Unicam He λ ios α UV-Visible spectrometer (Cambridge, UK) using a pre-programmed standard curve for relative quantitation.

2.20 Protein dialysis and buffer changing

Buffer in which purified proteins were resuspended was changed by ultracentrifugation using Vivaspin 20 (50 000 MWCO) or Vivaspin 6 (10 000 MWCO) columns (Sartorius, Goettingen, Germany). Alternatively, purified proteins were dialysed using Slide-A-Lyzer[®] Dialysis Cassettes with appropriate MWCOs (Pierce, Gilbertsville, USA).

2.21 Protein expression

2.21.1 *Escherichia coli*

E. coli cells were made chemically competent according to the method of Inoue *et al.* (1990). Briefly, the bacterial cells were inoculated into 250 ml LB medium containing the relevant antibiotics and grown at 18 °C until $OD_{600} = 0.6$. The culture was left on ice for 10 min and centrifuged at $2\ 500 \times g$ for 10 min at 4 °C. After the SNF had been discarded, the resulting cell pellets were gently resuspended in 80 ml ice-cold transformation buffer, incubated on ice for 10 min and centrifuged as previously. Pellets were once again resuspended in 20 ml transformation buffer and DMSO was added to a final concentration of 7 % (v/v). Finally, the cells were incubated on ice for 10 min before being aliquotted and stored in liquid N₂.

Chemically competent *E. coli* were transformed using the methods of Inoue *et al.* (1990). After thawing at RT, a 200 μ l aliquot of competent cells was added to each ligation reaction in a 2 ml tube (not glass), mixed and incubated on ice. After 30 min, the cells were heat-shocked at 42 °C

for 30 sec and snap-cooled on ice. Eight hundred microlitres of LB medium was added and the cells were grown at 28 °C for 1 hour, then plated on LB agar containing the relevant antibiotics followed by overnight incubation at 37 °C.

Bacterial clones for scFv production were grown in 2 x TY medium supplemented with 2 % (v/v) glucose and 100 µg/ml ampicillin. Bacterial clones used for producing bivalent constructs were grown in LB medium supplemented with the appropriate antibiotic. In both instances, cultures were incubated at 37 °C with shaking (220 rpm) until $OD_{600} = 0.6$ then centrifuged at 4000rpm for 10 min and the SNFs discarded. Pellets were resuspended in one-fifth of the original volume of LB medium supplemented with the appropriate antibiotic and 1mM IPTG and shaken overnight at 30°C. After centrifugation at $2\ 500 \times g$ for 10 min SNFs containing secreted Abs were removed and stored in aliquots at -20°C, either with or without 4 % (w/v) sucrose. Alternatively, proteins were expressed using Overnight Express™ Instant TB Medium according to the manufacturer's (Merck Biosciences, Darmstadt, Germany) instructions.

Cytoplasmic-, periplasmic- and inclusion body protein fractions were extracted from bacteria using BugBuster® Protein Extraction Reagent and Lysonase™ Bioprocessing Reagent according to instructions provided by the manufacturer (Merck Biosciences, Darmstadt, Germany). Cell pellets from cultures induced overnight were resuspended in 5ml BugBuster per gram of cell pellet supplemented with 10 µl of Lysonase™ per gram of pellet. Samples were incubated at RT with gentle rotation for 10 min and then centrifuged at $16\ 000 \times g$ for 20 min at 4 °C. SNFs (both the cytoplasmic and periplasmic fractions) were transferred to fresh tubes and evaluated by spot-blot and/or ELISA. For extracting inclusion bodies, pellets were resuspended in the same volume of BugBuster® used previously. Lysonase™ was then added to a final concentration of 1000 units/µl and the suspensions were gently mixed then incubated at RT for 5 min. Six volumes of one-tenth strength BugBuster® was added, mixed by vortexing for 1 min and centrifuged at $5\ 000 \times g$ for 15 min at 4 °C. The inclusion bodies (pellets) collected were twice resuspended in half of the original culture volume of one-tenth strength BugBuster® and centrifuged as previously. The pellet was resuspended a third time and recentrifuged at $16\ 000 \times g$ for 15 min at 4 °C. Inclusion bodies were resuspended in $1 \times$ PBS.

2.21.2 Human embryonic kidney (HEK 293-H) cells

HEK293-H cells were cultivated in DMEM with L-glutamine and glucose, supplemented with 10% (v/v) heat-inactivated FBS (all Gibco™, Invitrogen™, Carlsbad, USA). Opti-MEM I was substituted when adapting cells to low-serum conditions (Gibco™, Invitrogen™, Carlsbad, USA). Cell stocks were grown in 175 cm² flasks or 145 cm² cell culture-treated plates (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in 5 % CO₂ and cultures were given fresh medium on average every two to three days as required. Confluent monolayers were removed in 1 x PBS with 0.01 % (w/v) EDTA after 5 to 10 min incubation at 37 °C. Stocks were frozen at an estimated 60 to 80 % confluency, in freezing medium consisting of culture medium, FBS and DMSO and stored in liquid N₂.

Zeocin™ (Invitrogen™, Carlsbad, USA) concentrations for selection were optimised on untransfected 24-hour HEK 293-H cultures in 6-well plates (Greiner Bio-One, Frickenhausen, Germany). Varying concentrations of Zeocin™ were added to culture plate wells and cells were monitored for changes over a period of 1 to 2 weeks. Control wells received medium containing no Zeocin™. The concentration of Zeocin™ that killed the majority of cells within 1 to 2 weeks was chosen as being optimal for selecting transfected cells.

HEK 293-H cultures at 50 – 80 % confluence in DMEM supplemented with 10 % (v/v) FBS were transfected with plasmid DNA using GeneJuice® Transfection Reagent (Merck Biosciences, Darmstadt, Germany) or TransIT®-293 Transfection Reagent (Mirus Bio Products, Madison, USA) according to each manufacturer's instructions. Cultures were split 24 to 72 hours after transfection and fed with fresh medium containing Zeocin™ at the predetermined concentration. Once visible, individual foci were removed, transferred to fresh tissue culture plates containing DMEM with 10 % (v/v) FBS and Zeocin™ and expanded.

2.21.3 *Kluyveromyces lactis*

Genes were subcloned in the plasmid vector pKLAC1 as described (Section 2.7). The vector was then linearised by digestion with *SacII*, after which the vector was purified and concentrated (Section 2.9). One microgram of linearised pKLAC1 was added to thawed *K. lactis* GG799 cells containing 620 µl of Yeast Transformation Reagent. The tube was inverted to mix thoroughly and incubated at 30° C for 30 min. Cells were heat-shocked at 37 °C for 1 hour and then pelleted by

centrifugation at 7000 rpm for 2 min. The SNF was discarded and the pellet was resuspended in 1 ml sterile ddH₂O. Centrifugation was repeated and this time the pellet was resuspended in 1 ml YPGlu medium and incubated at 30° C with shaking at 250 rpm. After 30 min, the transformation mix was pelleted and resuspended in 1 ml sterile ddH₂O as before. Dilutions of 1, 10 and 50 µl of transformed cells in 75 µl of sterile ddH₂O were plated on YCB Agar Medium plates containing 5mM acetamide. The plates were incubated inverted at 30 °C. Individual colonies apparent after three to four days were streaked onto fresh YCB Agar Medium plates and incubated for a further one to two days before screening for correctly integrated and/or multiple tandem-copy expression fragments.

Yeast cells were lysed with Lyticase (Sigma®, St. Louis, USA) and DNA was purified using the QIAGEN® DNAeasy Tissue and Blood extraction Kit (Hilden, Germany) according to the manufacturer's instructions. Positive clones were identified by PCR using the Integration Primers 1 and 2 supplied in the *K. lactis* Protein Expression Kit (Table 2.1). A 1.9 kb PCR-product was expected from transformants that contained correctly integrated expression cassettes. Additionally, multi-copy transformants (*i.e.* with more than one copy of the expression cassette per cell in tandem) could be identified by PCR amplification of a 2.8 kb product using Integration Primers 2 and 3 (Table 2.1).

YPGal medium was inoculated with expression fragment-positive colonies and incubated at 30 °C with shaking (250 rpm) for a minimum of two days or until a saturated culture was obtained ($OD_{600} \geq 30$). The saturated culture was centrifuged at 4000 rpm for 15 min. SNFs were removed to a fresh tube and analysed in ELISA (Section 2.14) for protein expression.

2.22 Gold-conjugated immunoassays

2.22.1 Gold-colloid salt-protection assay

The assay was carried out in a 96-well (Corning, New York, USA) format using methods derived from Geoghegan and Ackerman (1977). Purified Ab or antigen was diluted to a final concentration of 50 µg/ml in PBS. A series of aliquots of 40nm colloidal gold ($OD_{525} = 1$) (Vision Biotech (Pty) Ltd., Cape Town, South Africa) were adjusted from pH 6.5 to pH 9 in increments of 0.5 using 30 mM boric acid or 30 mM sodium tetraborate. This series was repeated with aliquots of 5 mM borate buffer. Abs or antigen were diluted in borate buffer in individual

microwells to yield final protein concentrations of 0.25, 0.5, 10, 1, 1.25 and 2.5 $\mu\text{g/ml}$ in a total of 25 μl per well. This was done for each pH of buffer made. Two-hundred and fifty microlitres of colloidal gold of the corresponding pH was added to each corresponding well and allowed to stand for 10 to 20 min, after which 25 μl 2 M NaCl was added and incubated at RT for a further 10 to 15 min. The protection point was determined visually and by spectrophotometry. Wells with an OD_{525} closest to 1 were regarded as optimal.

2.22.2 Conjugating antibodies or antigen to gold nano-particles

The methods of Geoghegan and Ackerman (1977) were also used for conjugation. Ab or antigen was diluted in 5 mM borate buffer to a final volume equal to 10 % of the total conjugation volume (*i.e.* if 30 ml of colloidal gold was used for conjugation, then 10 % of this volume is 3 ml). The optimal pH and concentration of this dilution was determined previously in the salt-protection assay. Forty-nanometre colloidal gold ($\text{OD}_{525} = 1$) (Vision Biotech Pty. (Ltd), Cape Town, South Africa) was added and the mixture was gently swirled every five min for a total of 20 min at RT. A 10 % (w/v) BSA solution was added to a final concentration of 0.5 %, and the solution was again mixed. The suspension was spilt into two pre-weighed centrifuge tubes and centrifuged at $6000 \times g$ for 30 to 40 min at 4 °C. The SNF was carefully removed by aspiration. The pellets were weighed and combined. A small sample of pellet was diluted and measured spectrophotometrically at 525nm. The pellet was then resuspended in conjugation resuspension buffer to give a final OD proportional to the total OD of the volume of colloid used for conjugation *i.e.* 1 ml of 40nm colloidal gold has $\text{OD}_{525} =$ approximately 1. If 30 ml colloid was used for conjugation, a theoretical final OD_{525} of approximately 30 was required.

2.23 Epitope mapping using the XCX₁₅ random peptide library

K91 bacterial stocks harbouring the XCX₁₅ library were thawed and resuspended in 100 ml LB medium containing 20 $\mu\text{g/ml}$ tetracycline. Dilutions of this suspension were analysed spectrophotometrically at A_{600} , where an $\text{OD} = 1$ corresponds to approximately 8×10^8 cells/ml. The XCX₁₅ library contains approximately 1×10^9 clones. To ensure that all clones contained in the library were represented during cultivation, the final culture volume(s) needed to contain at least 1×10^{11} cells. Cells were diluted correctly in 1 l volumes of LB medium with tetracycline and grown overnight at 37 °C with shaking (230 – 250 rpm).

Phage particles were recovered by PEG/NaCl precipitation according to the methods of Bonnycastle *et al.* (2001). Briefly, phages were precipitated twice in a final concentration of 2.5 % (w/v) PEG/0.5 M NaCl and centrifuged at $6\ 200 \times g$ for 40 min at 4 °C. After the final precipitation phages were resuspended in 1 x PBS. NaN₃ was added as preservative to a final concentration of 0.02 % (w/v). Phages were stored with 15 - 20 % (v/v) glycerol at minus 70 °C.

The concentration of phage particles per millilitre was determined using the method described by Bonnycastle *et al.* (2001). Dilutions of phage were made in 1 x PBS and scanned from 220 to 320 nm using the Unicam Helios α UV-Visible spectrophotometer (Cambridge, UK). The ‘adjusted’ A₂₆₉ was calculated by subtracting the OD at A₃₂₀ from the OD at A₂₆₉. Using this adjusted A₂₆₉, phages per millilitre were calculated using the formula:

$$(\text{Adjusted } A_{269}) \times (6 \times 10^{16}) / (\text{Number of nucleotides in the phage genome})$$

Bonnycastle *et al.* (2001)

High-density K91 *E. coli* were grown according to Bonnycastle *et al.* (2001). A volume of two millilitres of LB medium was inoculated with a single colony of K91 cells and incubated overnight at 37 °C with shaking (250 rpm). Fifty millilitres of fresh LB medium was inoculated with 500 µl of this overnight culture and grown until the OD₅₉₅ was between 1.0 and 1.5. The cells were incubated for a further 10 min with slow (100 rpm) shaking to allow the bacterial pili to regenerate. The OD₅₉₅ was checked again to ensure that it had not exceeded 2.0.

Panning was essentially as described by Bonnycastle *et al.* (1996). For the first round, 10 µg/ml of scFv-IgY was immobilised on the surface of an Immuno™ Tube (Nunc™, Roskilde, Denmark) overnight at 4 °C or for 1 hour at 37 °C. After coating, the tube was washed three times with 1 x PBS and then blocked with 2 % (w/v) MPPBS for 1 hour at 37 °C. XCX₁₅ f88.4 phage were added at a final concentration of 10¹¹ particles and incubated as previously. Unbound phages were removed by washing five times with 1 x PBS containing 0.5 % (v/v) Tween 20 and then five times with 1 x PBS containing 0.05 % (v/v) Tween 20. The tube was then rinsed with 1 x PBS to remove residual Tween 20. Phages were eluted in 1 ml phage elution buffer at RT for 10 min and neutralised in 150 µl phage neutralisation buffer. The eluate was used to infect a fresh

5 ml high-density culture of K91 cells for 10 min at RT. Four millilitres of LB medium was added to the culture together with tetracycline to give a final concentration of 0.02 µg/ml. The suspension was incubated for 1 hour at 37 °C with shaking and dilutions of this culture were plated on LB agar with 40 µg/ml tetracycline to determine the phage titre in terms of transforming units. The remainder of the culture was centrifuged at $3\ 300 \times g$ for 10 min. The cell pellet was resuspended in a maximum of 5 ml LB medium, plated as described and incubated overnight at 37 °C. Bacterial colonies were scraped off the agar plates into LB medium, pooled and pelleted by centrifugation. Phages were PEG precipitated from the SNF as described. This entire 'output' was used for the next round of panning. The Ab concentration was reduced to 5 µg/ml for the fourth round of panning. Colonies from the third and fourth rounds were picked and analysed in phage-ELISA (described below). In addition phage DNA was isolated and sequenced (described below).

For phage-capture ELISAs, cultures of each clone were grown overnight in LB medium that contained 15 µg/ml tetracycline and centrifuged the next day. Cell pellets were retained for sequencing and SNFs were removed to fresh tubes. Polysorp Immuno™ Plate (Nunc™, Roskilde, Denmark) wells were coated with 10 µg/ml of Ab diluted appropriately in 1 x PBS. SNFs containing phage were incubated for 1 hour at 37 °C after blocking with 2 % (w/v) MPPBS. Wells were washed as described previously (Section 2.14) and detected with B62-FE2 Ab and HRP-conjugated rabbit anti-mouse Ab (DakoCytomation, Glostrup, Denmark). Both Abs were diluted appropriately in 2 % (w/v) MPPBS. Substrate, control wells and colour reaction evaluation were also as described previously in this section (Section 2.14).

Double-stranded replicative-form (RF) DNA for each clone was extracted from the pellets that had been retained after culturing overnight (see above) using the QIAGEN® Plasmid Midi Kit (Section 2.3). DNA from the clones was sequenced (Section 2.12) using XCXseq2 primer (Table 2.1). Alternatively, ssDNA was isolated from phage as described by Sambrook & Russel (2001). Phages were PEG-precipitated once (0.2 % (v/v) PEG) from 1.5 ml of SNFs at RT for 15 min, and centrifuged at 14 000 rpm for 10 min at 4 °C using a Hettich (Tuttlingen, Germany) EBA 12R microcentrifuge. SNFs were removed and the tubes were recentrifuged briefly to facilitate the removal of any fluid that remained. Phage pellets were re-suspended in 100 µl of Tris-EDTA (TE) buffer and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to

each tube. The samples were vortexed thoroughly, allowed to stand at RT for 1 min and then vortexed again before being centrifuged at maximum speed for 5 min in a microfuge. The upper aqueous layer was removed to a fresh tube and the ssDNA was precipitated. After centrifugation, the dried nucleic acid pellets were resuspended in ddH₂O. Nucleic acid concentration was determined spectrophotometrically (2.5). The XCXseq2 primer was once again used for sequencing.

Presumptive Ab binding sites on the HSP65 protein were predicted using computer-based algorithms with default parameters. LALIGN (Myers & Miller, 1989) is a pairwise, linear alignment tool available at the Swiss European Molecular Biology Network (EMBnet) node server (<http://www.ch.embnet.org>). The Pepitope server (Mayrose *et al.*, 2007a; <http://pepitope.tau.ac.il/>) is a web-based tool for 3D-mapping. Two algorithms are available on this server: PepSurf (Mayrose *et al.*, 2007b) and Mapitope (Bublil *et al.*, 2007). Peptides that produced signals lower than controls in phage-capture ELISA and peptides which did not follow the XCX₁₅ motif were excluded from 3D analyses. The crystallographic data of the HSP65 antigen from *M. bovis* (Qamra & Mande, 2004) is accessible from the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>; Berman *et al.*, 2000) under the file name 1sjp.

Chapter 3

RESULTS

3.1 Single-chain variable fragments

Four scFvs that had previously been isolated from the *Nkuku*® library were used in this study. The scFvs designated E1 and G8 bind the HSP65 antigen of *M. bovis*, whereas B5 and C10 are directed against the 16 kDa protein of *M. tuberculosis*. All four were tested for stability while subsequent reconstitution studies focussed on scFvs E1 and G8 only. These fragments are comprised of chicken Ab variable heavy- and light-chain genes, linked via a poly-glycine linker (van Wyngaardt *et al.*, 2004). Clones obtained by panning were expressed in TG1 cells in the phagemid vector pHEN1 under IPTG-induction (Hoogenboom, 1991; 2.21.1). The scFv gene is cloned downstream of a PelB leader sequence which directs protein expression towards the periplasm. Fragments expressed in their entirety have a carboxy-terminal *c-myc* epitope tag and can therefore be either affinity-purified from the culture medium or used in the crude SNF form. A database of scFvs isolated from the *Nkuku*® library is being created. It is hoped that comparisons of characterised Abs in this collection may be used to study particular residues or positions within the primary structure that may confer particular characteristics on scFvs (e.g. stable binding, high-temperature stability, etc.)

Immunochromatographic tests (ICTs) have the potential to provide simple, rapid and cost-effective diagnostic assays for the developing world. Such tests are created specifically with field and rural applications in mind, where for example cold-storage facilities are not available. Consequently, ICTs rely heavily on the use of robust immunoreagents. In order to determine which of four different *Nkuku*® scFvs were likely to be appropriate for such applications, three physical properties were examined. These were freeze-thaw survivability, high-temperature stability and the ability to refold after chemical denaturation.

3.1.1 Stability of scFvs directed against antigens of *M. bovis* and *M. tuberculosis*

3.1.1.1 Freeze-thaw survivability

Each of the four scFvs were subjected to consecutive freeze-thaw cycles while suspended in bacterial cell culture SNFs. After each cycle, an aliquot was removed and tested for its capacity to recognise the target antigen in indirect ELISA (Section 2.14.1). Single-chain fragments which bound the antigen coated to the surface of the well were detected with the 9E10 anti-c-myc tag monoclonal Ab followed by HRP-conjugated rabbit anti-mouse Ab and OPD/H₂O₂ substrate. All the Ab fragments survived up to 10 cycles without a significant decrease in binding capacity (Fig. 3.1). Disaccharides are known to act as cryoprotectants (Nilsson & Larsson, 2007). In an identical experiment performed with 2 % (w/v) sucrose added to the SNFs, the results did not differ significantly (data not shown). The scFvs could therefore be used repeatedly after freezing, without the addition of a cryoprotectant.

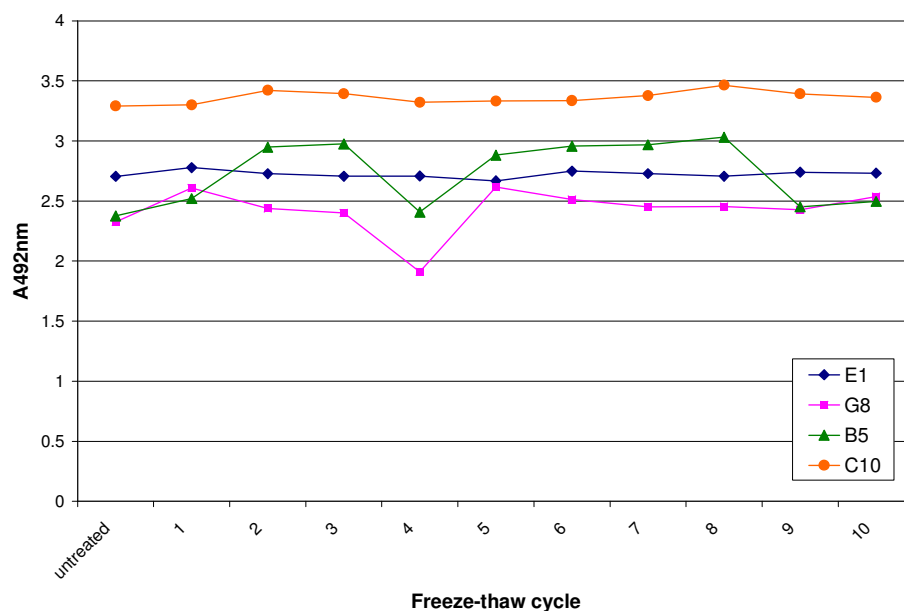


Figure 3.1 Indirect ELISA illustrating the freeze-thaw durability of scFvs E1 (blue), G8 (pink), B5 (green) and C10 (orange). All four scFvs retained the ability to recognise their cognate antigens immobilised to the surface of an ELISA plate. The A_{492nm} values of the MP control wells (ranging from 0.04 to 0.07) were subtracted from the corresponding values plotted.

3.1.1.2 Temperature stability of scFvs

Stability at elevated temperatures may indicate an scFv's potential usefulness under field conditions. Accordingly, 12 aliquots of each of the four scFvs were incubated at different temperatures for a period of 16 hours. This was accomplished using the temperature gradient programme on an Eppendorf™ MasterCycler which makes it possible to vary the temperature at different positions across the heating block. After overnight incubation, duplicates were tested using indirect ELISA (as detailed in Section 3.1.1.1) to determine whether Ag-binding had been affected. Arbitrarily, an ELISA absorbance value of 1 at 492nm was used as a cut-off value. The anti-HSP65 scFvs E1 and G8 remained functional after exposure to temperatures up to approximately 47 °C. At temperatures above this, scFv function started to decline. In contrast, the anti-16 kDa scFvs B5 and C10 were less robust. ELISA results showed that B5 had a maximum temperature tolerance of approximately 37 °C (Fig. 3.2). C10 did not survive incubation overnight at any temperature above 30 °C, thereby showing that it had little promise as a useful immunoreagent (Fig. 3.2). Freshly grown SNFs of E1 and G8 were tested for medium term storage-stability at different temperatures (Fig 3.3). Baseline signals were determined for each scFv before treatment. E1 gave an absorbance signal of approximately 2.8 when measured at 492nm and G8 produced a signal of 2.0 (not shown). Both retained most of their functionality after four weeks under the different conditions, with RT and 4 °C having the greatest effect on binding.

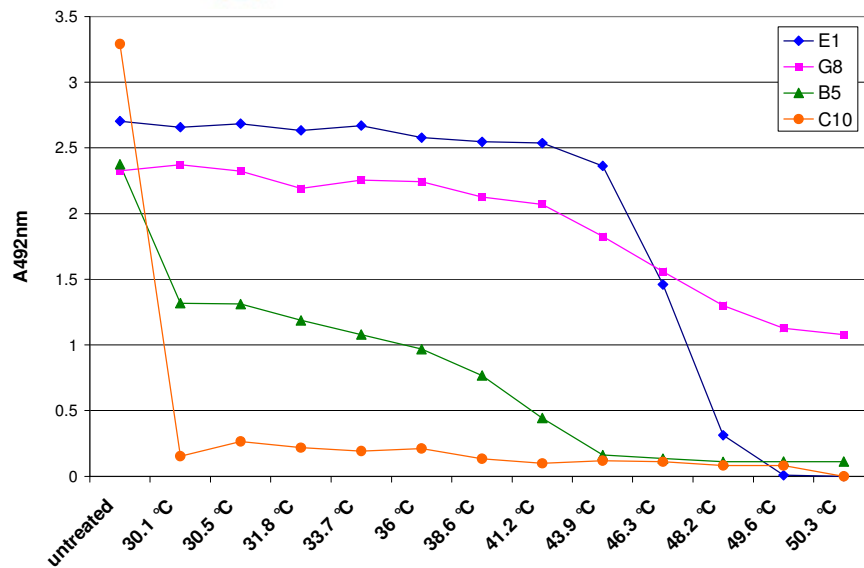


Figure 3.2 The affect of elevated temperatures on scFvs E1 (blue), G8 (pink), B5 (green), and C10 (orange). SNFs containing secreted scFvs were incubated for 16 hours at various temperatures and thereafter evaluated in indirect ELISA. The scFvs were detected with 9E10 anti-*c-myc* tag mAb (1 in 1000 in 2% (w/v) MPPBS) followed by HRP-conjugated rabbit anti-mouse Ab (1 in 1000 dilution in 2 % (w/v) MPPBS). The A_{492nm} of control wells (ranging from 0.04 to 0.07) was subtracted from the plotted values.

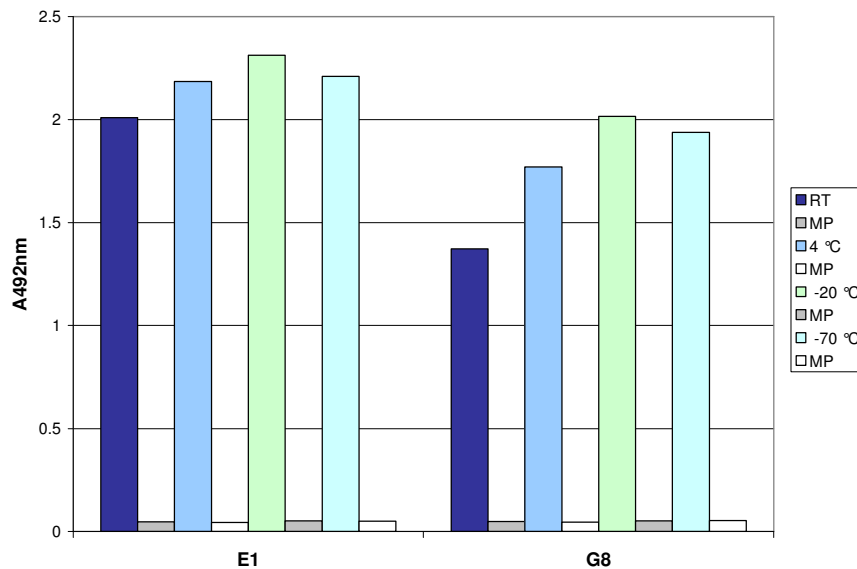


Figure 3.3 Indirect ELISA illustrating medium term storage of scFvs in SNF form. SNF aliquots of 1.5 ml were stored at different temperatures for four weeks. Tetracycline was added as a preservative at 15 mg/ml, and tubes were protected from light. ELISA plates were coated with 10 µg/ml HSP65. MP controls were coated with MP instead of HSP65. The scFvs were detected with 9E10 anti-*c-myc* tag mAb (1 in 1000 in 2% (w/v) MPPBS) followed by HRP-conjugated rabbit anti-mouse Ab (1 in 1000 dilution in 2 % (w/v) MPPBS).

3.1.1.3 Ability of scFvs to refold after chemical denaturation

The chaotropic agent guanidinium chloride (GHC1) was used to evaluate the propensity of the four scFvs to refold after denaturation. They were each exposed overnight to the denaturant at concentrations of 3 M and 6 M in separate tubes. The GHC1 was removed by ultrafiltration and substituted with at least three volumes of fresh PBS (Section 2.20). Samples were concentrated to their original starting volumes (approximately 3 ml) and the Abs in both the buffer-changed and the denaturant-containing solutions were evaluated by indirect ELISA. As expected, the scFvs tested in the presence of the denaturant produced no signal in ELISA. Of the four, only the HSP65-specific G8 could still recognise its cognate antigen once the GHC1 had been removed (Fig. 3.4). In addition, HSP65 coated on the surface of an immunoplate (indirect ELISA) was incubated for one hour at 37 °C with 1 to 6 M concentrations of GHC1 in order to determine the effects of denaturant on this Ag. The denaturant was removed by washing and the HSP65 was detected with rabbit anti-*M. bovis* Ab followed by HRP-conjugated anti-rabbit Ab. Immobilised HSP65 was not affected by GHC1 and the ELISA signals were similar to those produced by untreated control wells (Fig 3.5). In the presence of denaturant therefore, abrogation of binding may be attributed to the effect of the GHC1 on the paratope, and not the epitope.

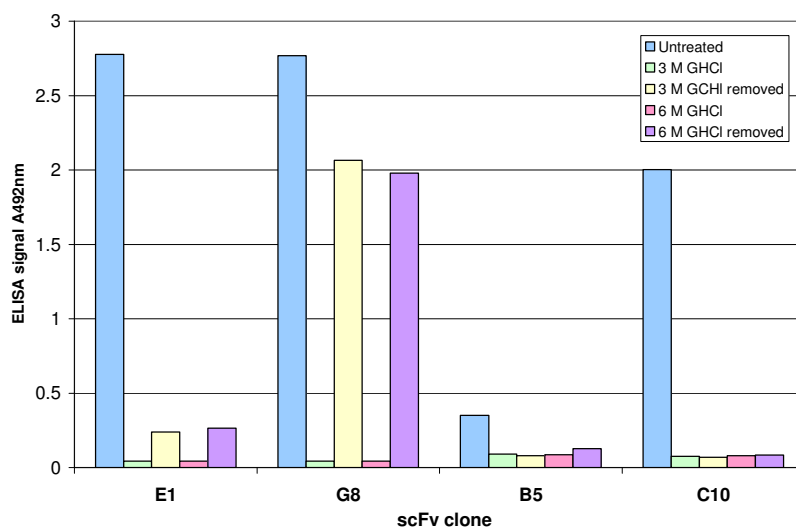


Figure 3.4 The effect of GHC1 on four anti-*Mycobacterium* scFvs. Abs were incubated overnight in 3 M or 6 M concentrations of GHC1 and then tested in indirect ELISA in the presence and absence of denaturant. None of the scFvs could bind their cognate antigen in the presence of GHC1. Only G8 bound cognate antigen after the denaturant had been removed, indicating that it was able to refold.

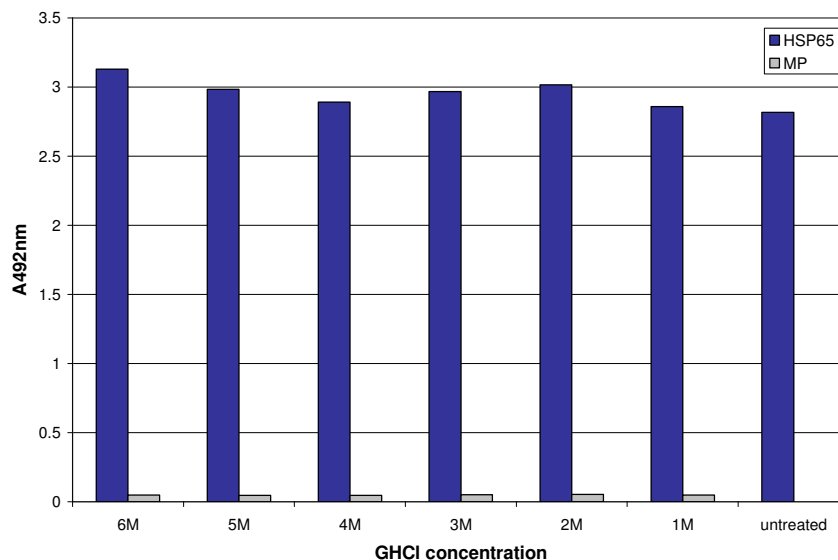


Figure 3.5 Immobilised HSP65 antigen treated with increasing concentrations of GHCl in ELISA. MP is a negative control in which 2 % (w/v) MPPBS was used instead of antigen. An untreated well was included as a control. Treatment with denaturant did not significantly decrease the signals obtained in ELISA.

3.1.1.4 Stability of the scFv paratope

A stable paratope that does not distort when the Ab molecule is adsorbed to a solid surface is a valuable attribute for an immunocapture reagent. Accordingly, affinity-purified E1 and G8 scFvs were evaluated in a sandwich ELISA format (Section 2.14.2) in which the immunoplates were coated with 12 µg/ml of purified scFv. HSP65 antigen was added at 10 µg/ml and then detected with rabbit anti-*M. bovis* polyclonal Ab (1 in 1000 dilution in 2 % (w/v) MPPBS) followed by HRP-conjugated anti-rabbit polyclonal Ab (1 in 1000 dilution in 2 % (w/v) MPPBS). Negative (no antigen) and positive (antigen adsorbed directly to the well surface, no scFv) controls were included. The results are depicted in Figure 3.6. The immobilised scFvs produced an ELISA signal that was barely above background (negative control), suggesting very little paratope stability in either Ab. In a similar experiment, 2 µl of each purified scFv were immobilised onto nitrocellulose or PVDF membranes at a concentration of 10 µg/ml, and detected as described. No chemiluminiscent signal was obtained (not shown). Therefore, it seemed unlikely that these scFvs could be used in antigen capture immunoassays without some sort of modification.

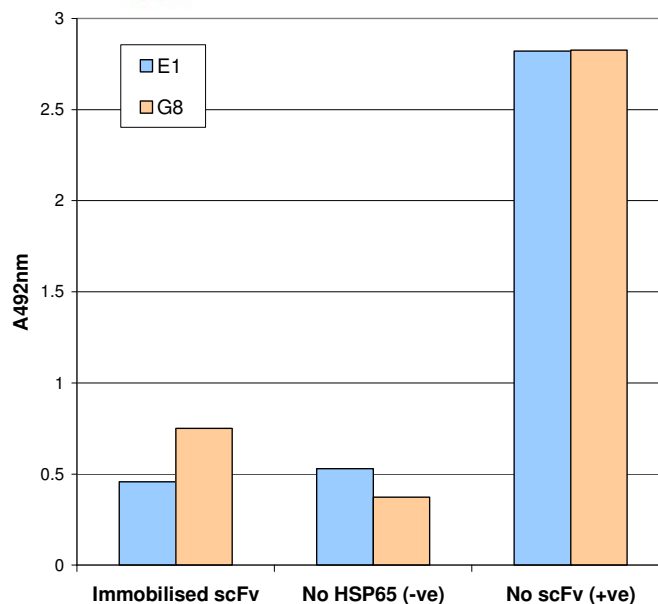


Figure 3.6 Purified scFvs E1 (blue) and G8 (orange) used as immunocapture reagents in ELISA.

3.1.2 Sequence comparison of E1 and G8 scFv

The sequences of anti-*Mycobacterium* scFvs isolated from the *Nkuku*[®] library (van Wyngaardt *et al.*, 2004) are shown in Fig 3.7. E1, G8 and D8 are anti-HSP65 scFvs. E1 and G8 are used in this study, while D8 is known to be a poor binder (results not shown) and is included for comparative purposes. C10 and B5, anti-16 kDa antigen Abs that did not perform well in temperature stability studies, are also included for comparative purposes. There were several amino acid exchanges in the framework residues (Fig. 3.7, see legend). Some of the substitutions that are unique to E1 (e.g. Gly {G} at V_H76 where Ser {S} is common) or G8 (e.g. Leu {L} at V_L45 where Pro {P} is common) amount to changes in side chain properties e.g. non-polar to polar or charged side chains. At other positions similar exchanges are noted, but are common to two or more scFvs which have contrasting characteristics. For example, G8 and D8 have Asp (N) at V_L71 where Ala (A) is common in the other scFvs. However, G8 is a stable, strong binder, whereas D8 is a poor binder. Thus, by comparison, it is not likely that this particular position is important for stability in these scFvs. In other cases, the replacement is a similar amino acid e.g. E1 and D8 have Ser (S) at V_H80 where Asn (N) is more common (both are uncharged polar residues).

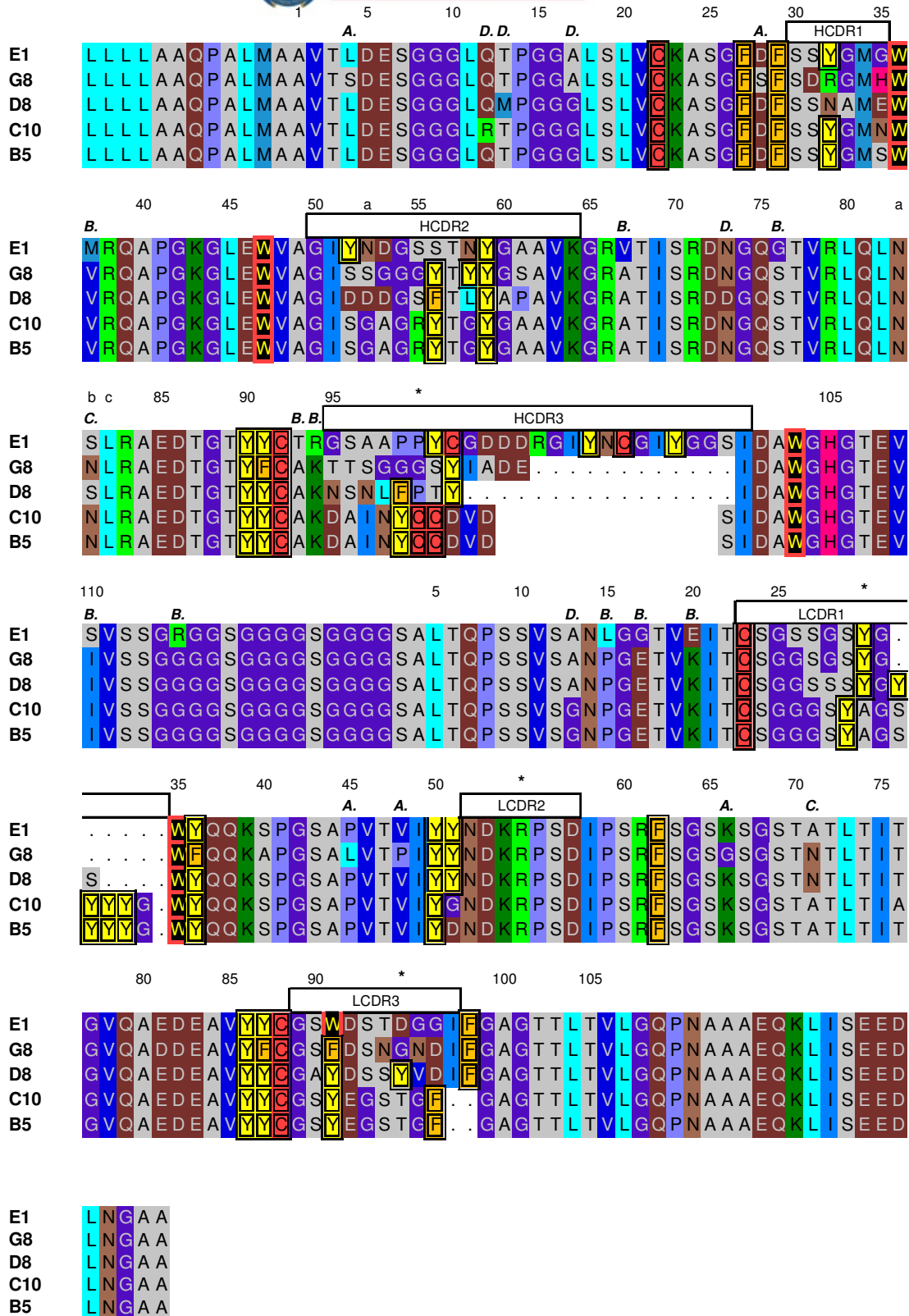


Fig 3.7 Amino acid sequences of anti-*Mycobacterium* scFvs (used with permission of Dr. J. Fehrsen, unpublished). E1, G8 and D8 are anti-HSP65 scFvs. D8 is included for comparative purposes and is a poor binder. C10 and B5 are anti-16 kDa Ag scFvs which did not perform well in temperature stability studies. Residue exchanges are indicated:

(**A**) exchanges unique to the G8 framework; (**B**) exchanges unique to the E1 framework; (**C**) exchanges common to more than one of the scFvs, which are not likely to influence Ab attributes; (**D**) exchanges in scFvs other than E1 or G8 that may comparatively contribute to undesirable properties. LCDR indicates L-chain CDRs and HCDR indicates H-chain CDRs. The scFvs are numbered according to the Kabat system, using the work of Aburatani *et al.* (2002) as a guideline. Where Kabat numbering does not follow on exactly in these chicken scFvs, it is indicated by an asterisk (*).

3.2 Expressing scFvs reconstituted as recombinant IgY-like fusions in mammalian cell culture

Recombinant scFvs can be engineered to improve their fundamental characteristics (affinity, avidity and stability). This, together with their small size (~22 kDa), can make the use of scFvs potentially advantageous in most applications, particularly therapeutics (recent examples are Bhatti *et al.*, 2008 and Wuertzer *et al.*, 2008) and immunocytochemistry (Kleymann *et al.*, 1995). As shown previously in ELISA (Section 3.1.1.4), scFvs may not function well as immunocapture reagents. Furthermore, not all scFvs couple consistently to gold nanoparticles (personal communication, Mr. N. Borain and Mr A. Bohms, Vision Biotechnology (Pty) Ltd.). These performance inconsistencies may perhaps result from the small size and rigidity of the scFv, the location of certain amino-acids/charges on/in the molecule (Robinson, 2002; Spitznagel & Clark, 1993) and its overall stability (investigated in Section 3.1). It was hypothesised that adding bulk in the form of Ab C_H-domains may (i) decrease molecular distortion during adsorption to solid surfaces and (ii) 'standardise' the gold-labelling process since gold adsorption occurs through specific interactions with amino-acids such as Lys, Trp or Cys (Chandler *et al.*, 2000). Reconnecting the chicken scFv with domains derived from its parent IgY may therefore improve its stability and functionality. Egg-yolk derived IgY is known to be inherently more tolerant to high temperatures and chaotropic agents than serum immunoglobulins and has a noteworthy stabilising effect on gold colloids (Narat, 2003; Gasparyan, 2005).

The scFvIgY(CH₂₋₄)His plasmid vector used for IgY-format expression in HEK 293-H cell monolayer culture (Fig. 3.8) was obtained from the University of Hamburg. This approximately 6.7 kb plasmid contains avian IgY C_H-domains two, three and four (CH₂₋₄), derived from a chicken cDNA library with tetra-histidine tags for purification attached to the carboxy termini. It is based on the mammalian expression vector pcDNA3.1-zeo (Invitrogen™, Carlsbad, USA) and contains both ampicillin and Zeocin™ resistance genes for selection (Greunke *et al.*, 2006). Essentially, this 'CH₂₋₄' format lacks two constant domains (C_{H1} and C_L) in comparison to the complete IgY molecule (Fig. 3.9). An N-terminal rat immunoglobulin κ signal upstream of the cloning site directs the secretion of individual polypeptide chains into the cell medium, where

they assemble into divalent IgY-like molecules that are stabilised by disulphide bonding. In this dissertation, these scFv-IgY fusions are designated ‘gallibodies’ (abbreviated *gb*), a word derived from the Latin binomial nomenclature for the domestic chicken (*Gallus gallus*).

The human embryonic kidney (HEK) 293 cell line is stably transformed with DNA from a strain of adenovirus type 5 (Graham *et al.*, 1977). HEK 293-H is a derivative of this original line which is specifically adapted to grow in a specially-developed commercial medium (293 SFM II, Invitrogen™), although it also grows well in ‘traditional’ media. The plasmid contains a cytomegalovirus (CMV) promoter which drives foreign gene transcription (Thomas & Smart, 2005) as well as the Simian-virus 40 (SV40) early-promoter and origin of replication to ensure high expression of the selectable marker. Therefore under Zeocin™ selection, episomal replication is maintained and constitutively expressing cell lines can be established (Thomas & Smart, 2005; Piechazek *et al.*, 1999).

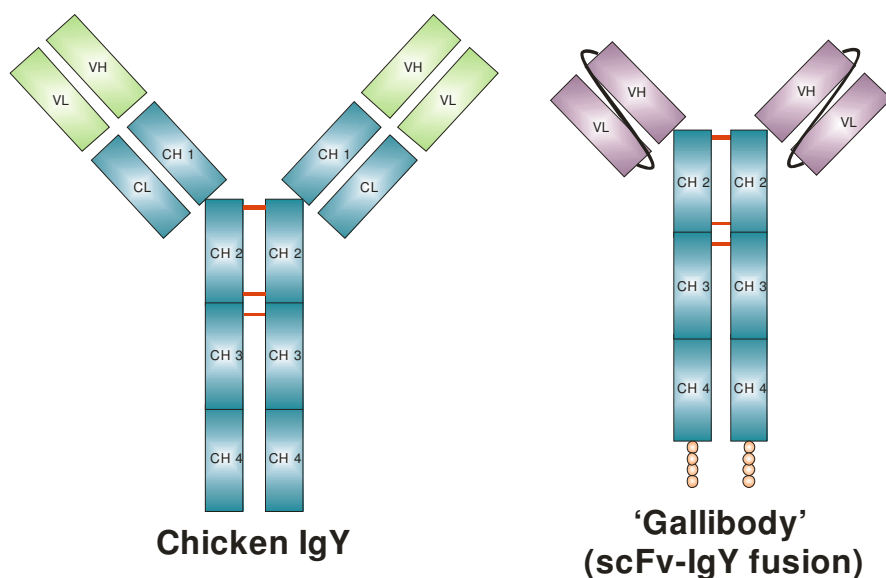


Figure 3.8 The structure of the scFvIgY(C_{H2-4})His molecule (right) compared to complete IgY (left). Constant (C) domains are indicated in blue, variable (V) domains in green and reconstituted scFvs in purple. Interchain disulphide bonds are indicated in red. The scFvIgY(C_{H2-4})His molecule (gallibody) is a bivalent IgY-like format consisting of reconstituted scFv sans the C_{H1} and C_L domains on each Ab chain when compared to the complete IgY molecule. The C_{H2-4} format has a carboxyl-terminal tetra-His tag (orange spheres) for purification (adapted from Warr *et al.*, 1995 and Greunke *et al.*, 2006).

3.2.1 Cloning scFv genes into the scFvIgY(CH₂-4)His vector

When received from Dr Spillner in Hamburg, the expression plasmid (Fig. 3.9) contained a ‘dummy’ Ab fragment gene (*scFv27*). It was therefore digested (Section 2.8) with *AscI* and *Acc65I* (*KpnI* isoschizomer) to release this ‘stuffer fragment’. The scFv genes for E1 and G8 scFvs were recovered from pHEN using primers VHBsiW and VLAsc (Table 2.1) that were designed to introduce *BsiW* and *AscI* cleavage sites. Both inserts and vector were digested with the corresponding enzymes and ligated to each other (Section 2.10). *Acc65I*- and *BsiW*-digested fragments have compatible ends and can thus be coupled. Ultracompetent M15 *E. coli* cells (Section 2.21.1) were transformed using the ligation mixtures. A control transformation, containing only linearised vector and no insert was performed concurrently. Ten transformants were selected for each construct (dubbed *gbE1* and *gbG8*) and subjected to colony PCR (Section 2.11) with the T7for and CH2-4rev primers (Table 2.1) to check for inserts. An amplicon of 700 to 800 bp was expected for experimental ligations (representative examples are shown in Fig 3.10 and 3.11), whereas no insert (or a very small insert in the case of vector re-linearisation) was expected for the control (not shown). DNA from individual clones containing inserts of the correct size was sequenced (Section 2.12). Clones *gbE1.3* and *gbG8.8* were selected for the subsequent transfection of HEK 293-H cells.

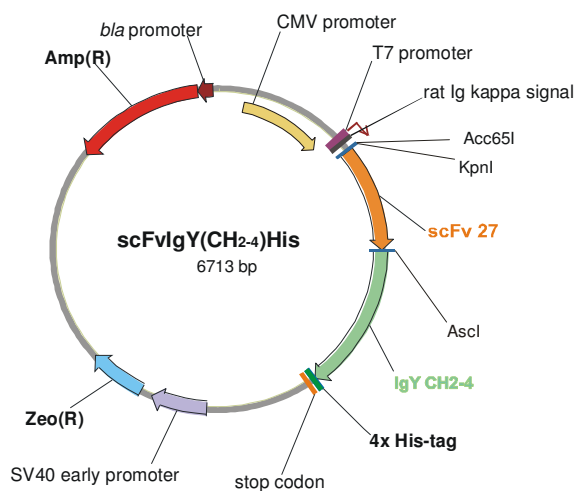


Figure 3.9 Vector map of the mammalian expression vector for scFv-IgY fusion Abs. The *scFv27* ‘stuffer fragment’ is indicated, as is the IgY C_H gene-fragment. Restriction enzyme cleavage sites used in cloning are labelled (Adapted from information provided by Dr. E. Spillner).

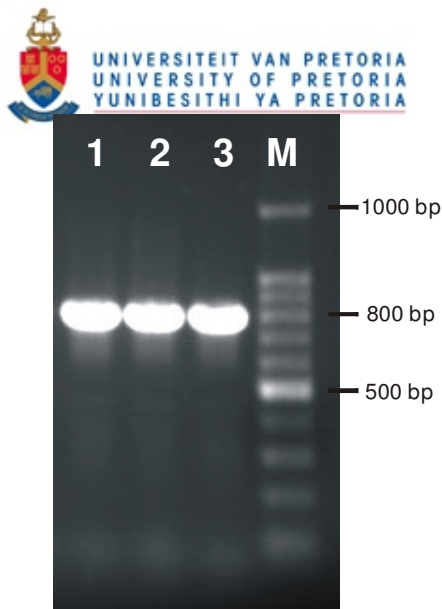


Figure 3.10 Colony PCR of individual colonies picked for the *gbE1*-variant. Lanes 1, 2 and 3 illustrate examples of amplified inserts of the correct size (approximately 800 bp). Fragment sizes are indicated on the right by the 100 bp ladder. Five microlitres of each sample, including marker, was loaded. The clone in lane 3 was grown up for transfection of mammalian cell cultures (*gbE1.3*). Colony PCR of negative control colonies are not shown, but these yielded no amplicons.

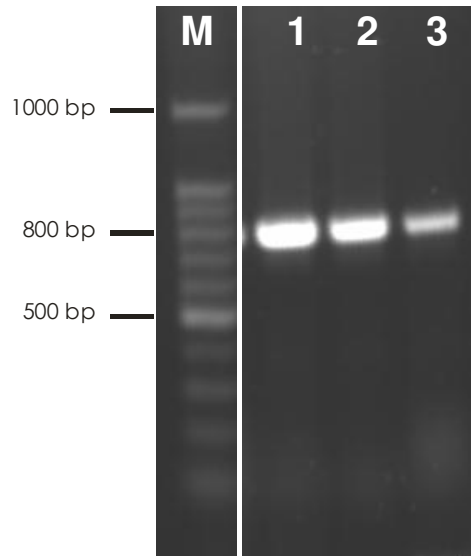


Figure 3.11 Colony PCR of individual colonies picked for the *gbG8*-variant. Three examples of inserts of the correct size (approximately 800 bp) are shown. Fragment sizes are indicated on the left by the 100 bp ladder. The clone in lane 1 (clone 8; *gbG8.8*) was grown up for transfection of mammalian cell cultures. Colony PCR of negative control colonies are not shown but yielded no amplicons.

3.2.2 Death curve for HEK 293-H culture

A 'kill curve' (Section 2.21.2) was carried out using Zeocin™ to determine the lowest concentration of the selection agent that would kill untransfected HEK 293-H cells within a 'reasonable period of time'. Ideally, the majority of cells should be killed within one week. Commonly, when treated with lethal doses of antibiotics, mammalian cells round up and detach from the plate. Cells treated with Zeocin™, however, first develop uncharacteristic shapes including pseudopodium-like appendages as the plasma membrane disintegrates. Large empty vesicles may be observed in the cytoplasm as organelles and scaffolding proteins disassemble, and sub cellular particles may be visible in the medium when they are released from completely disintegrated cells (Zeocin™ Instruction Manual). For this experiment, HEK 293-H cultures (passage 4) were fed with medium containing between 50 and 1000 µg/ml of Zeocin™. A control well was fed with antibiotic-free medium. Morphological changes were recorded at three and seven days after treatment. By the third day, cells that had been fed with medium containing ≥ 400 µg/ml Zeocin™ were no longer viable. At concentrations below 400 µg/ml cells were still viable although their morphology had changed (Fig 3.12). Zeocin™ supplementation at 100 µg/ml killed the majority of cells within the desired time frame, and was therefore chosen as the optimal concentration for selecting HEK 293-H transformants.

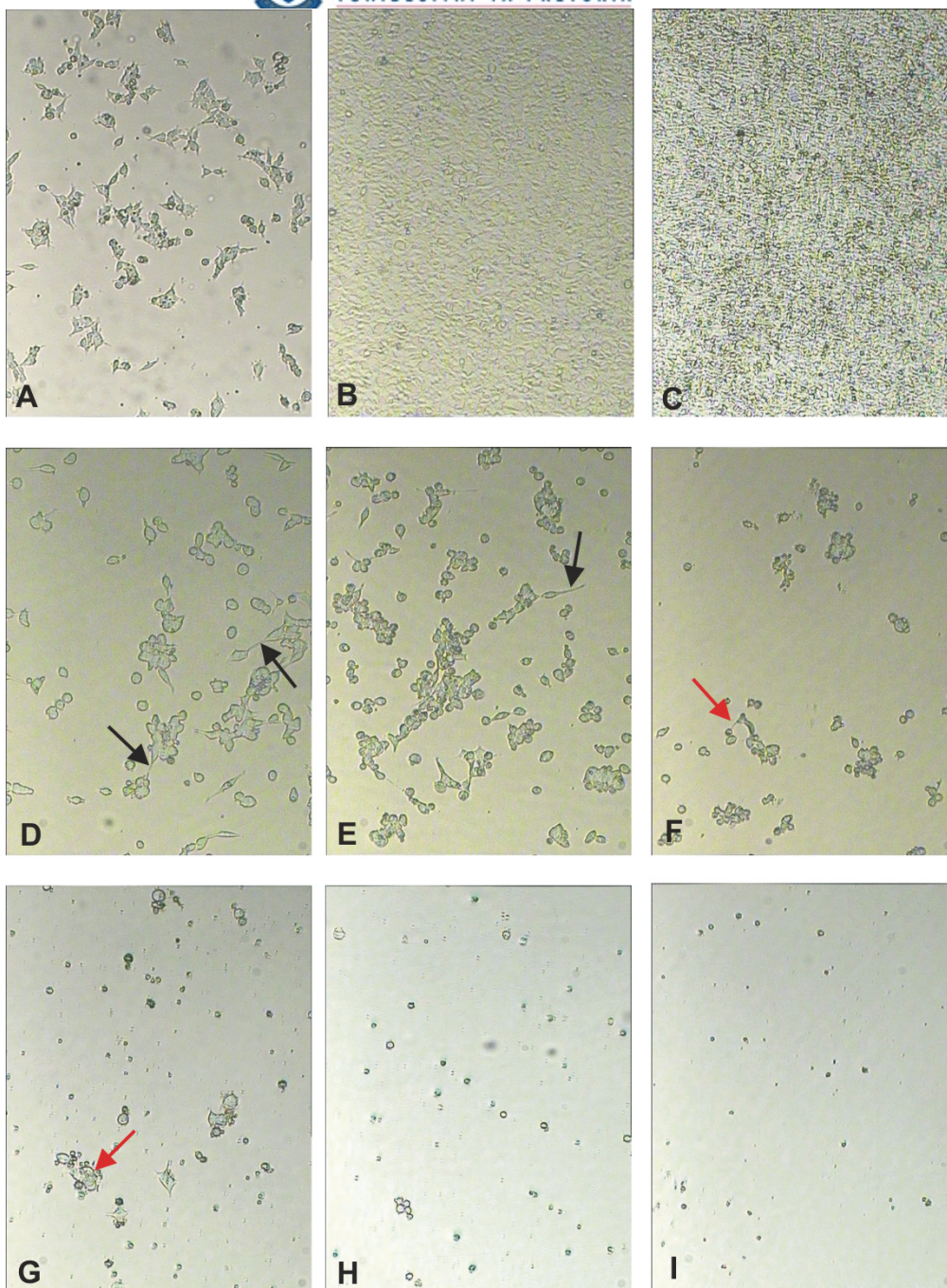


Figure 3.12 The effect of Zeocin™ on HEK 293-H cells over a period of one week (100X magnification). A confluent HEK 293-H culture was split into 35mm wells at 25 % confluency and grown for 24 hours (A) before treatment with Zeocin™. The growth of a control culture, not supplemented with Zeocin™, was recorded after three days (B) and one week (C). Individual wells, treated with 50, 100 or 200 µg/ml Zeocin™, were recorded at three days (D, E and F) and one week (G, H and I). Higher concentrations of Zeocin™ killed all cells within three days (not shown). Pseudopodium-like appendages were visible within three days (black arrows), as were vacuole-like structures (red arrows). A concentration of 100 µg/ml killed cells within the one week time frame required (H), and was chosen as the optimal concentration for selection of transfected clones.

3.2.3 Expressing scFvIgY(CH₂₋₄)His gallibodies in HEK 293-H cell culture

HEK 293-H cultures in six-well plates were transfected with varying ratios of plasmid DNA and one of two different transfection reagents (Section 2.21.2). Stable cultures of the *gbG8.8* clone (passage 9) were established first. The *gbE1.3* clone (passage 9) foci that had been propagated from transfections done using GeneJuice® Transfection Reagent did not survive. Accordingly, TransIT®-293 Transfection Reagent was later used to transfect cells with DNA from this clone (passage 5) and stable *gbE1.3* cultures were eventually established. Using either reagent, 2 to 3 µg of DNA and 8 to 10 µl of transfection reagent were sufficient. Cell foci were observed approximately one week after transfection under selection with 100 µg/ml Zeocin™ (Fig 3.13). Once individual foci became apparent, the concentration of Zeocin™ was halved. Foci were propagated to confluence and re-expanded to establish stable cultures. The next step was to determine whether gallibodies expressed by the surviving transfected cells were secreted into the cell culture medium as soluble and functional proteins.

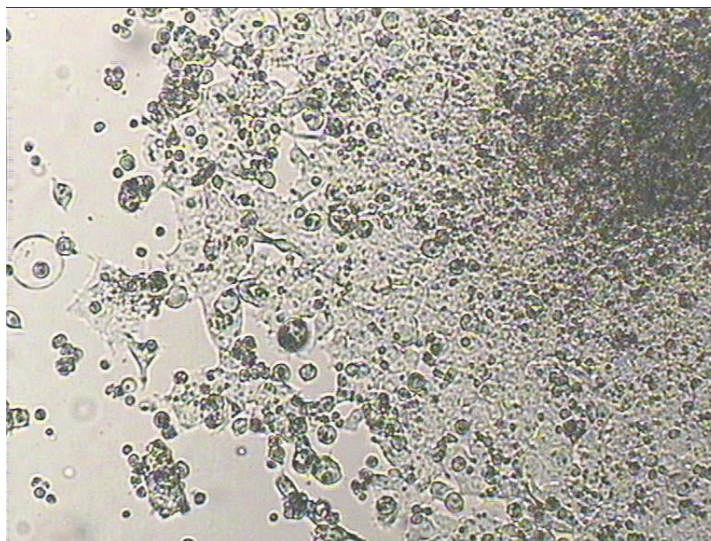


Figure 3.13 Edge of HEK 293-H *gbG8.8*-clone cell-focus (100X magnification). Cells surviving at this stage had successfully been transfected with plasmid DNA and were transiently expressing Ab under Zeocin™ selection (100 µg/ml).

3.2.4 Detecting bivalent Abs in cell culture medium

Once foci had become apparent, three to four day old cell culture medium was tested for transient expression of the Abs in a sandwich ELISA (Section 2.14.2). Any bivalent gallibodies that had been secreted into the cell culture medium were trapped with unlabelled anti-IgY Ab and detected with HRP-conjugated anti-IgY Ab (Fig. 3.14). Similarly, indirect ELISA (Section 2.14.1) was used to test for Ab functionality. This test showed that there were bivalent Abs present that bound to their cognate HSP65 antigen (Fig. 3.15). Furthermore, the Abs were able to recognise denatured HSP65 antigen in western blotting (Fig. 3.16). Once transient expression had been confirmed, foci were expanded. Confluent cultures derived from these foci were pooled and tested for their ability to secrete functional scFv-IgY fusions in the same manner (data not shown).

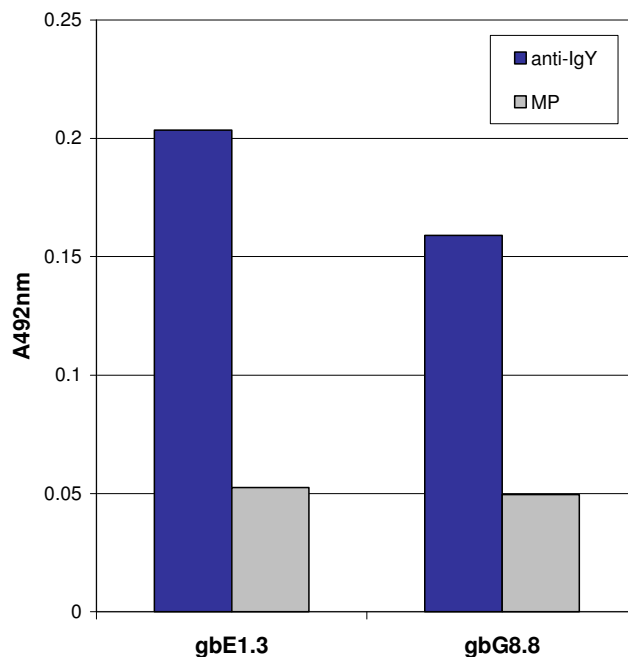


Figure 3.14 Sandwich ELISA to detect transient expression of gallibody Ab secreted into cell culture medium (100 µg/ml Zeocin™) 3 days post-transfection in 6-well plates (GeneJuice® Transfection Reagent). Microwells were coated with 10 µg/ml of polyclonal anti-IgY Ab. Cell culture medium containing transiently expressed gallibodies was added undiluted and detected with HRP-conjugated anti-IgY (1 in 10 000 dilution in 2 % (w/v) MPPBS). MP is a negative control, where microwells were coated overnight with 2 % (w/v) MPPBS in place of anti-IgY.

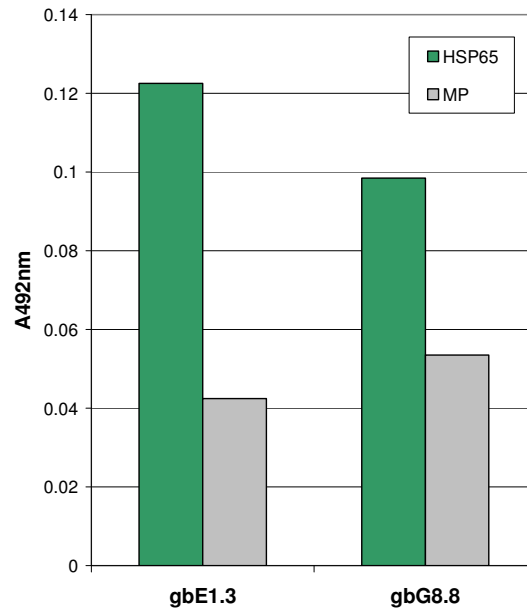


Figure 3.15 Indirect ELISA to detect functionality of transiently expressed IgY-like molecules in 3 day-old selection medium (100 µg/ml Zeocin™). Microwells were coated with 10 µg/ml of recombinant HSP65, undiluted cell culture SNFs were added and functional gallibodies were detected with anti-IgY-HRP diluted 1 in 10 000 in 2 % (w/v) MPPBS. Cultures were growing in 6-well plates at the time of sampling. MP is a negative control (2 % (w/v) MPPBS used instead of antigen).

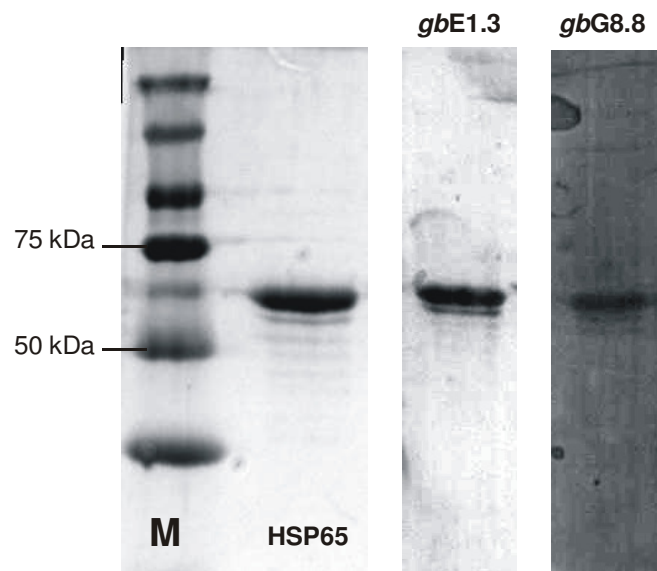


Figure 3.16 Gallibodies in western blot. Recombinant HSP65 was separated by SDS-PAGE as pictured on the LHS. M denotes the protein standard and molecular weights are indicated. Similar gels were blotted onto PVDF membranes. The antigen was detected with cell culture medium from constitutively expressing cultures of either *gbE1.3* (middle) or *gbG8.8* (RHS) followed by detection of the histidine tag (HisDetector™).

3.3 Purifying gallibodies produced in monolayer cell cultures

Stable cell cultures were first established with the G8.8 gallibody construct. Most of the initial purification experiments were therefore carried out using harvested *gbG8.8* culture medium. Medium from an up-scaled culture was collected, pooled, aliquotted and stored at -20 °C until needed. Consequently, all purification experiments were done using the same batch of medium. While the gallibodies were labelled with a penta-histidine tag at their carboxy terminal ends, not all purification methods were targeted toward this affinity-tag as other histidine-rich, contaminating proteins purified along with the target protein. Additionally, the presence of BSA in the medium further complicated the purification process. The reduced gallibody H-chain (approximately 63 - 65 kDa) was roughly the same size as this abundant serum protein (66.2 kDa), making it very difficult to distinguish between the two. Ultimately the purification experiments uncovered the need to develop culture conditions and/or alternative expression systems that would facilitate downstream processing.

3.3.1 PEG precipitation approach

Because of their similarity to chicken IgY, PEG precipitation of gallibodies from serum-supplemented cell culture supernatant was first attempted using the classic method described by Polson (1985) for egg-yolk Abs (Section 2.18.3). Ten millilitres of *gbG8.8* SNF from a confluent culture was used with the final pellet being resuspended in 1 ml PBS. This sample had an $OD_{280} = 16$, which corresponds to roughly 16 mg/ml of protein. After electrophoresis in a reducing SDS-PAGE gel no purified product in the range of 63 – 65 kDa could be distinguished (Fig. 3.17). In addition, western blots (Section 2.16) of the same samples using either anti-IgY HRP-conjugate (Fig. 3.18) or anti-His Abs (not shown) showed no target protein after precipitation. As a negative control, precipitation was performed using the same volume of conditioned culture medium from untransfected cells (Lane 4, Fig. 3.17 and Fig. 3.18). A similar yield of protein was obtained, suggesting that the molecule of interest had in fact not been previously precipitated. Consistent with this observation, both the transfected and untransfected PEG-precipitated samples separated identically in reducing SDS-PAGE analysis (Lane 2 and Lane 4, Fig. 3.17 and

Fig. 3.18). Thus, a simple PEG-precipitation proved to be unsuitable for purifying the recombinant Abs.

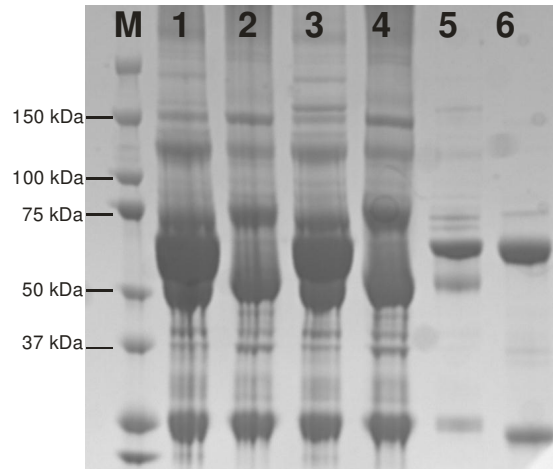


Figure 3.17 SDS-PAGE analysis of PEG-precipitated gallibodies (pre-cast 4 – 12 % Bis-Tris, Bio-Rad). Lane M: molecular weight standard. Lane 1: *gbG8.8* cell medium (10 μ l). Lane 2: PEG-precipitated *gbG8.8* medium (10 μ l). Lane 3: cell medium from untransfected HEK 293-H cells. Lane 4: PEG-precipitated untransfected medium. Untransfected medium served as a negative control for the experiment. FBS diluted 1:100 in PBS in Lane 5 and egg-yolk derived IgY 1.5 mg/ml in Lane 6 served as negative and positive controls.

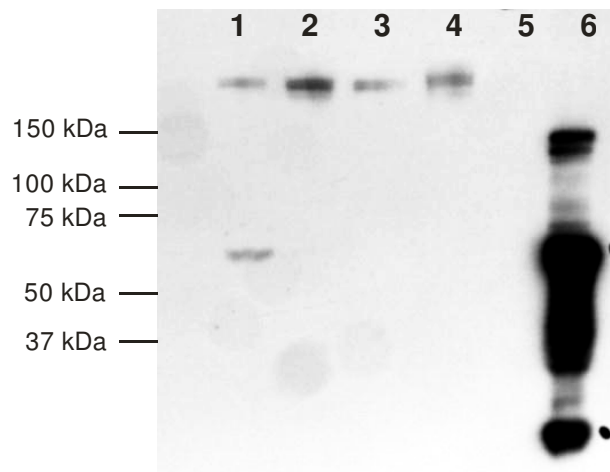


Figure 3.18 Western blot of PEG-precipitated recombinant Abs detected with rabbit anti-chicken polyclonal serum (1:1000 dilution in 2 % (w/v) MPPBS) followed by HRP-conjugated swine anti-rabbit polyclonal Ab (1:1000 dilution in 2 % (w/v) MPPBS) after five minutes exposure using chemiluminescence. Samples were loaded as described in Figure 3.17 and approximate molecular weights are indicated. A protein present in the cell medium of transfected and untransfected HEK 293-H cultures (~250 kDa) cross reacted with the polyclonal serum.

3.3.2 Nickel-affinity purification

3.3.2.1 Nickel spin-column purification

Two 20 ml aliquots of serum-supplemented cell culture medium were removed from a HEK 293-H culture expressing *gbG8.8* Ab, diluted 1: 1 with binding buffer and concentrated (Section 2.20) to 2 ml. Each concentrate was adsorbed to a Ni-NTA Spin column (designated A and B) (Section 2.18.1) and combined eluates were then adsorbed to a third spin-column (designated C). Pooled eluate was analysed by SDS-PAGE (Section 2.15) and in sandwich ELISA (Section 2.14.2). ELISA indicated that a large portion of purified product was lost in the flow-through after initial binding (Fig. 3.19, FT A+B and FT C). This was possibly due to overloading. While G8.8 gallibody could indeed be detected after spin column purification, a number of contaminants remained (Fig. 3.20, Lanes 8 and 9). The actual amounts recovered were marginal and purification on a larger scale was required.

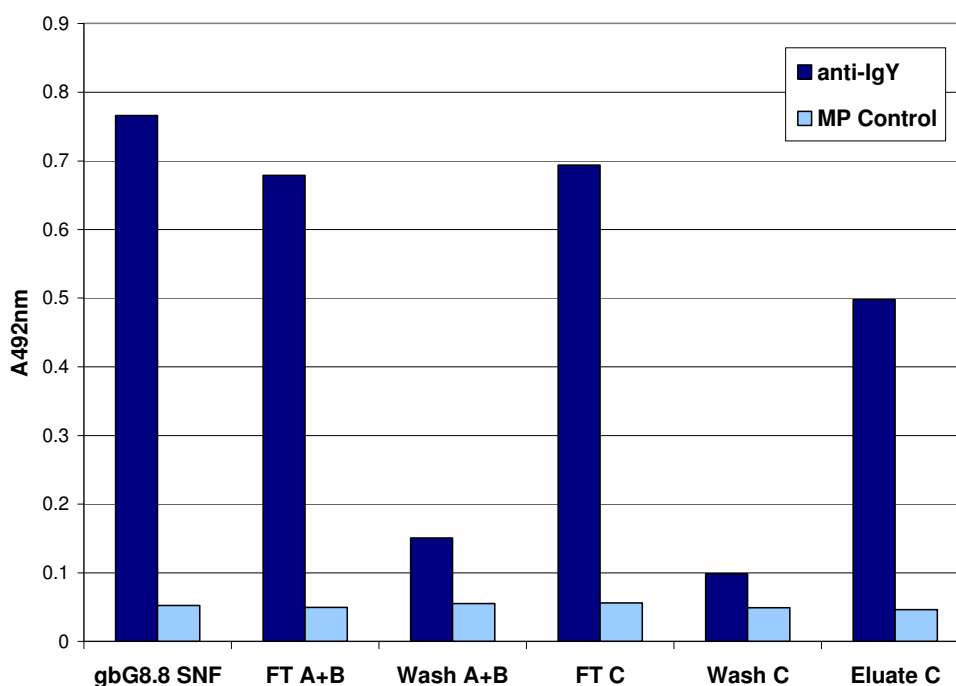


Figure 3.19 Sandwich ELISA evaluation of nickel-affinity spin-column purification. All samples from the purification were pooled. Gallibodies were captured with anti-IgY polyclonal Ab (10 µg/ml) and detected with HRP-conjugated anti-IgY diluted 1 in 10 000 in 2 % (w/v) MPPBS. MP control is a negative control (2 % (w/v) MPPBS replaced the capture Ab).

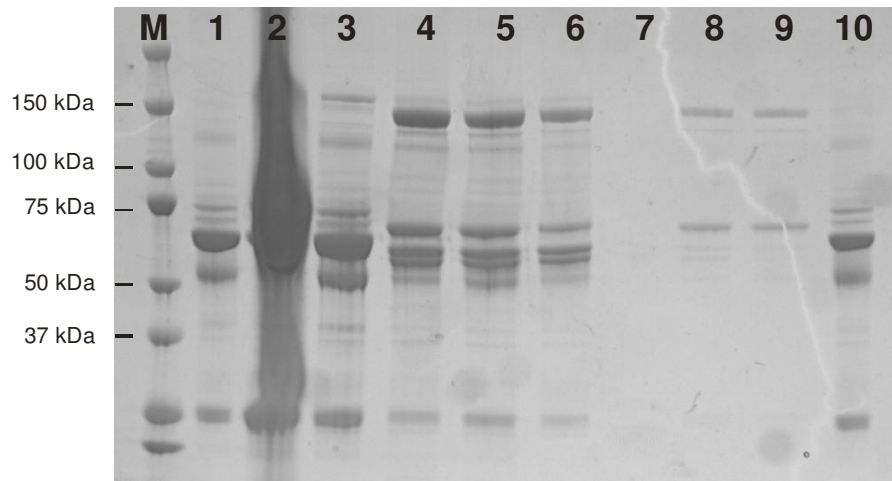


Figure 3.20 Ni-NTA Spin purification of *gbG8.8* analysed in reducing SDS-PAGE (pre-cast 4 – 12 % Bis-Tris, Bio-Rad). Protein molecular weight standard was loaded in Lane M. Lane 1: untreated *gbG8.8* cell culture SNF 1 : 1 with binding buffer (5 μ l). Lane 2: pooled flow-through after binding columns A and B (5 μ l). Lane 3: pooled washes A and B (5 μ l). Lane 4: pooled eluate from column A (5 μ l). Lane 5: pooled eluate from column B (5 μ l). Lane 6: flow-through from column C (5 μ l). Lane 7: pooled washes column C (5 μ l). Lane 8: elution 1 and 2 (pooled) from column C (10 μ l). Lane 9: elution 3 from column C (10 μ l). Lane 10: FBS diluted 1:100 in PBS as a control (5 μ l).

3.3.2.2 Batch-purification with Nickel-affinity resin

Since the spin-column method was patently not suitable for large sample volumes, batch purification with the charged resin was attempted. Forty millilitres of serum-supplemented cell culture SNF from a *gbG8.8* culture was equilibrated to a final concentration of 1 x binding buffer with 10mM imidazole, mixed with 5 ml of Ni-NTA slurry and allowed to bind. In order to capture as much protein as possible out of the mixture, the flow-through was run through the column for a second time using gravity-flow. After five washes of 4 ml each, bound proteins were eluted in 1 ml fractions using increasing concentrations of imidazole. A pH value of between 8 and 8.5 was maintained throughout the process. Samples were analysed by SDS-PAGE (Section 2.15) and ELISA (Section 2.14.2). Very little gallibody was detected in the flow-through (F/T), indicating that most of the protein of interest had been sequestered out of solution (Fig. 3.21, F/T). The same applied for the wash (W) steps, where only small amounts of the Ab were lost under low imidazole (20 mM) conditions (Fig. 3.21, W1 to W5). Most non-specific contaminants were removed within the first three washes (Fig. 3.21A and 3.22, W1 to W3). The recombinant Ab was detected on the SDS-PAGE gel only after the third elution (150 mM

imidazole) (Fig. 3.21B, E3) although it was shown to be present in the first elution by ELISA (Fig. 3.22, E1). Generally, 40 ml of cell culture SNF from confluent, serum-supplemented HEK 293-H cultures yielded between 1 to 3 mg of protein as determined by the Bradford (1976) method (Section 2.19) when batch-purified with Ni-resin as described (data not shown). However, not all of this protein represented gallibodies as SDS-PAGE showed that some recalcitrant contaminants still remained (Fig. 3.21B).

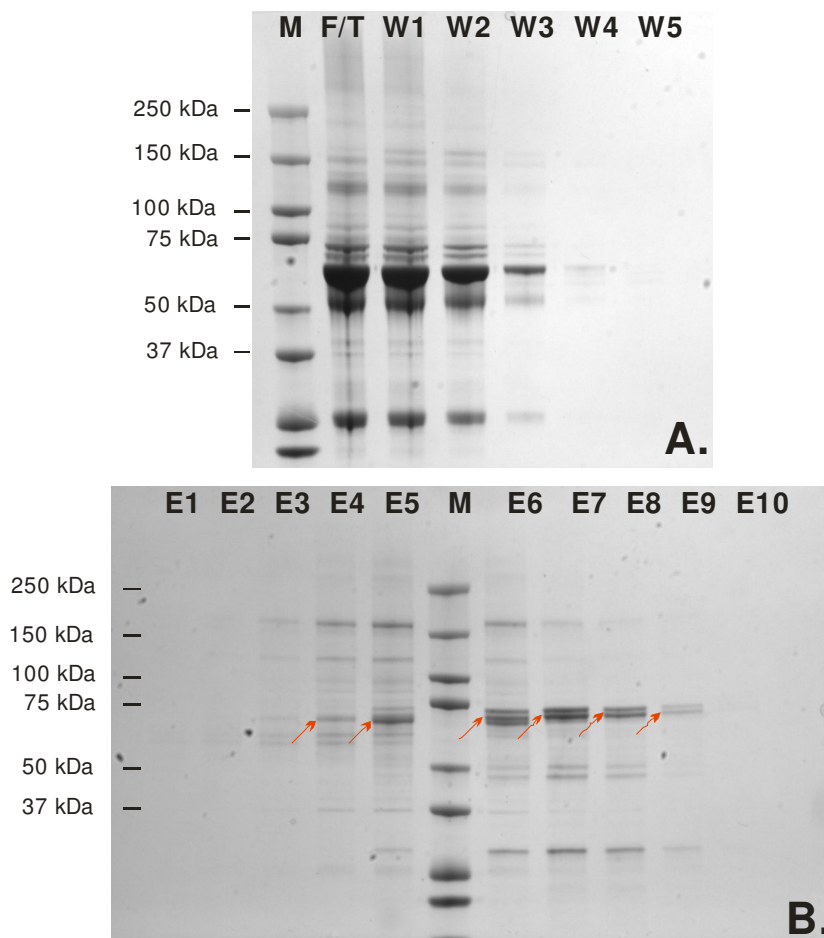


Figure 3.21 SDS-PAGE analysis of Ni-NTA batch-purified *gbG8.8*. An aliquot of the initial 40 ml sample was not loaded on the gel. **A:** Protein molecular weight standard was loaded in Lane M, F/T is the column flow-through collected after initial sample binding (2.5 μ l loaded), W designates a 4 ml wash (2.5 μ l loaded). **B:** E designates a 1 ml elution fraction (5 μ l sample loaded). M is molecular weight standard as described above. The band of interest is indicated in each fraction (red arrows).

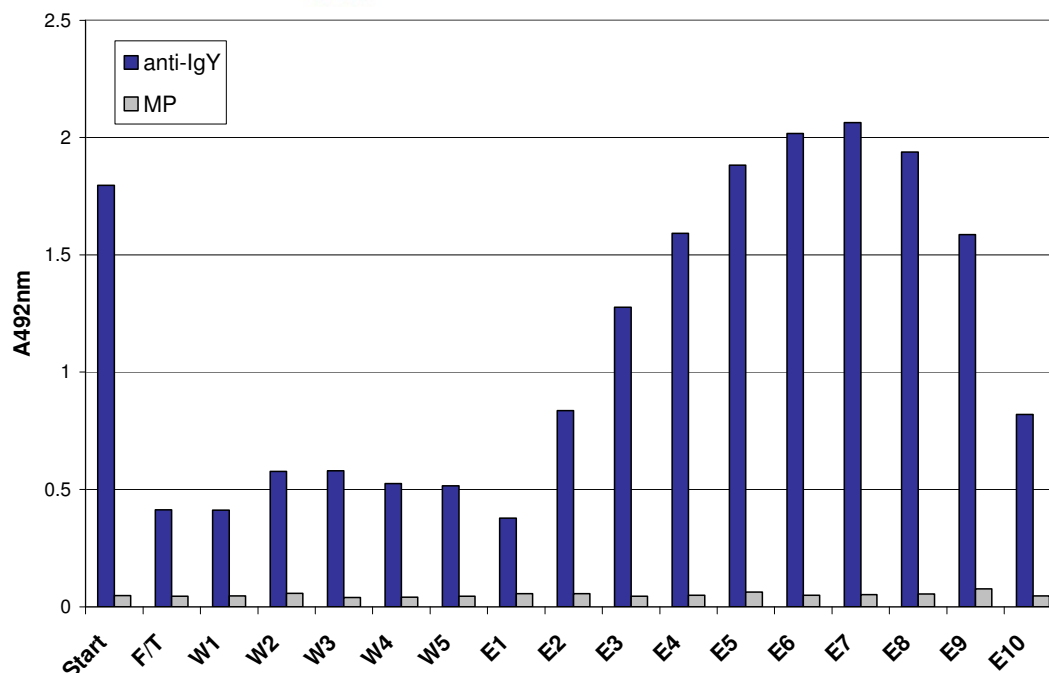


Figure 3.22 Sandwich ELISA of Ni-NTA batch-purified *gbG8.8* detected with polyclonal HRP-conjugated anti-IgY. Fifty microlitres of each sample was tested undiluted. MP is a negative control (2 % (w/v) MPPBS used instead of capture Ab). ‘Start’ is an aliquot of the sample before binding, F/T designates the column flow-through after binding, W designates a 4ml wash and E indicates a 1 ml elution fraction.

3.3.3 Immunoaffinity chromatography

Since metal-ion (His-tag) chromatography was not able to purify gallibodies to homogeneity, immunoaffinity chromatography was tried as an alternative. For this approach, 5 mg of unconjugated polyclonal anti-IgY Ab was coupled to an AminoLink® Plus column (Section 2.18.2). Thirty millilitres of *gbG8.8* serum-supplemented cell culture SNF was equilibrated to give a final concentration of 1 x PBS (pH 8) and passed through the affinity matrix twice. The column was then washed and Ab was eluted at pH 2.8 in eight 1 ml fractions which were neutralised on collection. Samples were taken after each step during purification and analysed in SDS-PAGE (Section 2.15) and indirect ELISA (Section 2.14.1). A large amount of protein did not bind to the column at all (F/T) or was lost during washing (W1 to W4) (Fig. 3.23A and 3.24). Purified Abs were eluted during the first three fractions (E1 to E2, Fig. 3.23B and Fig 3.24) and the final purified product contained fewer contaminants (specifically BSA from medium

supplementation) when compared to nickel-affinity purification. The yield was, however, relatively low (Fig. 3.23B).

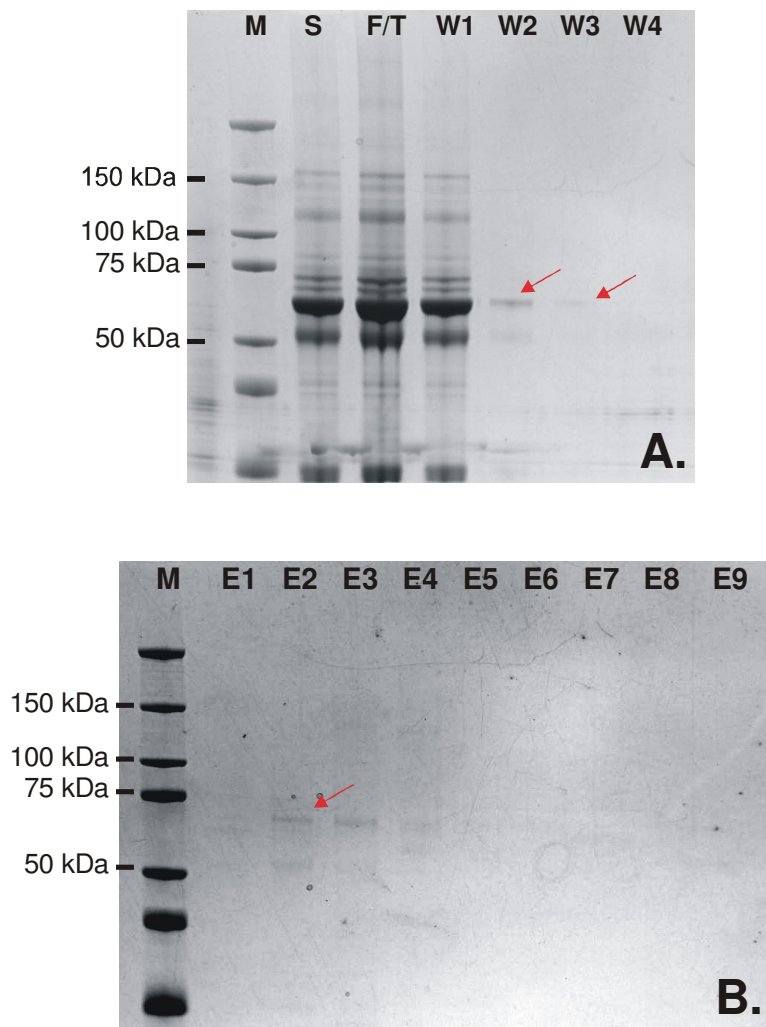


Figure 3.23 SDS-PAGE analysis of gallibody G8.8 purified by immunoaffinity chromatography. **A:** Protein molecular weight standard was loaded in Lane M, S is untreated sample ('Start'), F/T is pooled flow-through collected after sample binding (2.5 μ l loaded), W designates a 3 ml wash (2.5 μ l loaded). **B:** E designates a 1 ml elution fraction (5 μ l sample loaded). M is molecular weight standard as described above. The band of interest is indicated in each fraction (red arrows).

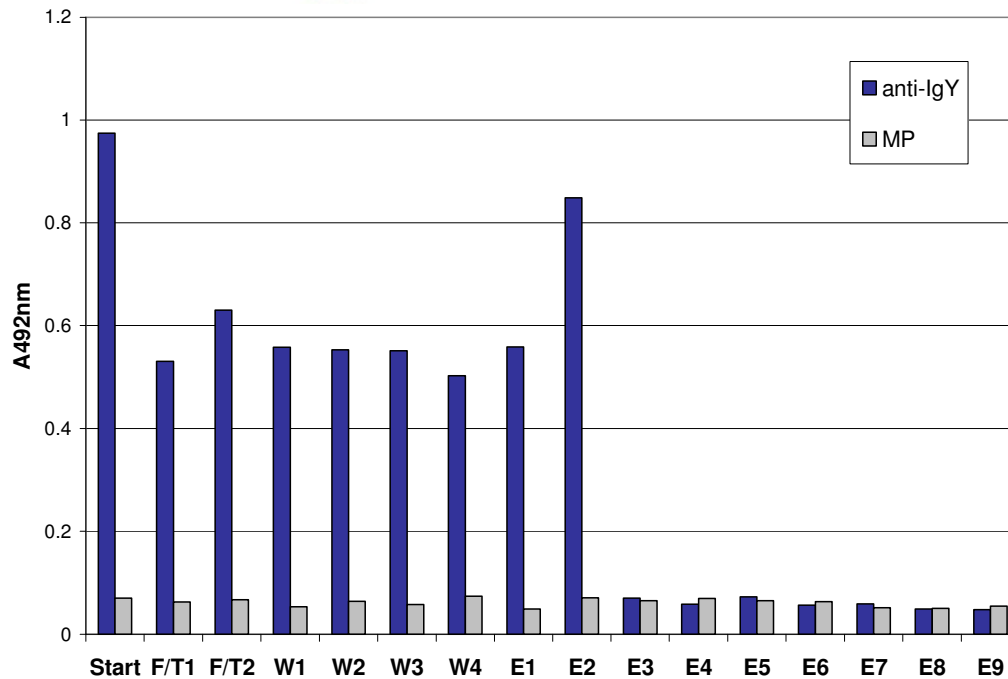


Figure 3.24 Sandwich ELISA of immunoaffinity chromatography purification detected with polyclonal HRP-conjugated anti-IgY (1 in 10 000 dilution in 2 % (w/v) MPPBS). Fifty microlitres of each sample was tested, undiluted. MP is a negative control (2 % (w/v) MPPBS used instead of capture Ab). ‘Start’ is an aliquot of the sample before binding, F/T designates the column flow-through after binding, W designates a 4ml wash and E indicates a 1 ml elution fraction.

Polyclonal anti-IgY should contain species of Abs directed against all four constant domains of the IgY molecule. As a consequence, binding to this affinity-column may not have been optimal as the recombinant gallibodies lacked the C_{H1} and C_L domains present in native IgY. Abs directed against these domains would thus not have been able to bind the construct. This supposition was validated by subsequent work which showed that gallibodies could be more exclusively detected in ELISA with an Ab directed against the Fc-region (Fig. 3.25). Attempts to purify these molecules with an anti-(IgY-Fc) column were not, however, successful (not shown).

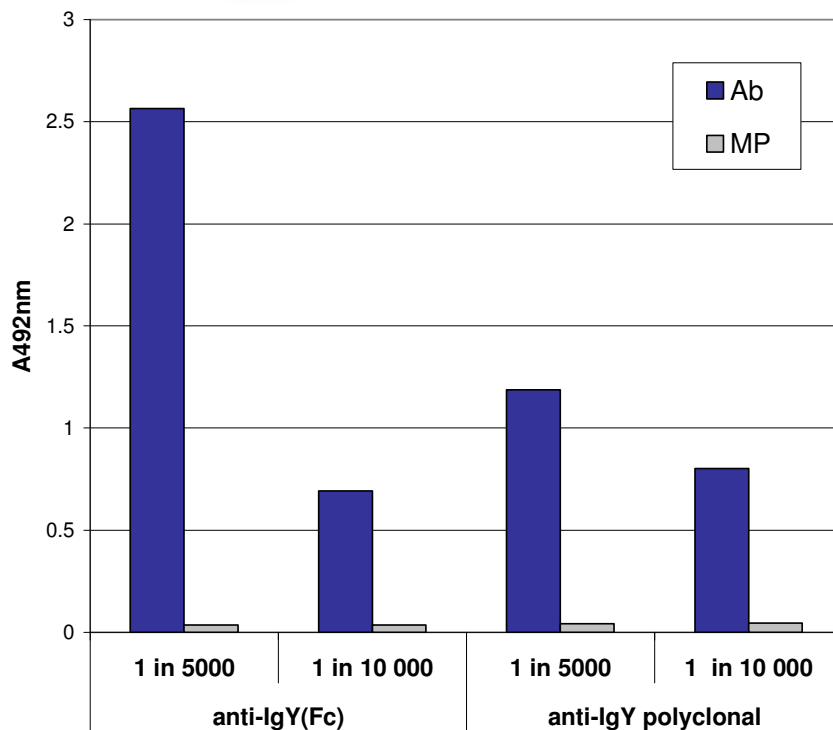


Figure 3.25 Detection of gallibodies in sandwich ELISA using HRP-conjugated polyclonal anti-IgY Ab or affinity-purified anti-IgY(Fc) Ab diluted in 2 % (w/v) MPPBS. The same sample of *gbG8.8* cell culture SNF was used. MP is a negative control (2 % (w/v) MPPBS used instead of capture Ab).

3.3.4 Ion-exchange chromatography to reduce contaminating BSA

FBS-supplementation of cell cultures proved problematic in purification, specifically because BSA remained the major contaminant after the various purification attempts. Ion-exchange chromatography (Section 2.18.4) was therefore attempted in an effort to remove residual BSA from harvested scFv-IgY culture medium. SDS-PAGE showed that this additional step made no worthwhile difference to the amount of albumin present in the sample (Fig. 3.26).

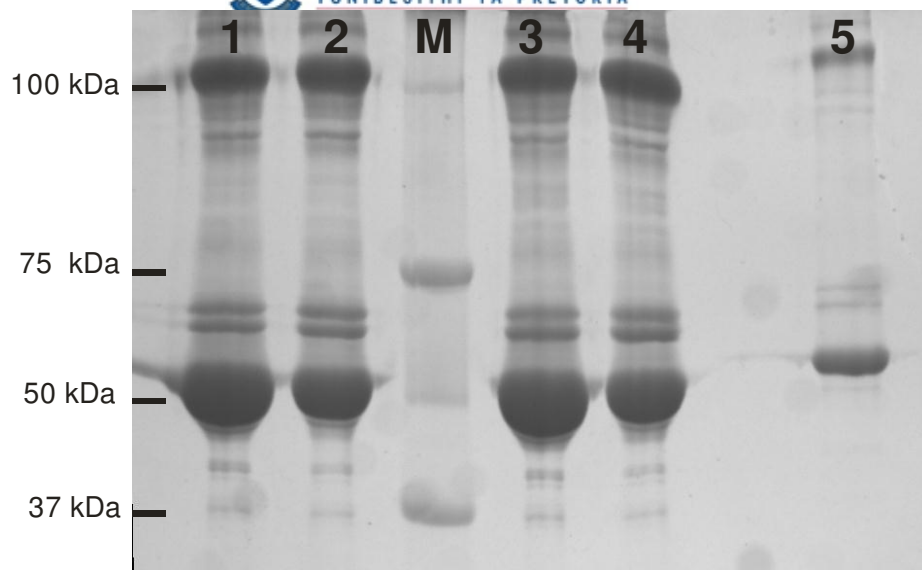


Figure 3.26 Non-reducing SDS-PAGE (4 % stacking, 10 % separating) analysis of cell culture medium subjected to ion-exchange chromatography using DE52-cellulose. Lane M contains a protein standard molecular weight marker. Lane 1: 10 μ l *gbG8.8* cell medium untreated. Lane 2: 10 μ l *gbG8.8* medium DE52-treated. Lane 3: 10 μ l untransfected cell medium, untreated (control). Lane 4: 10 μ l untransfected cell medium DE52-treated (control). Lane 5: 5 μ l FBS diluted 1:100 in PBS (control).

3.3.5 Serum-free cell culture

In order to directly address the problem of contaminating BSA, attempts were made to adapt HEK 293-H cells to growth in serum-free or serum-reduced conditions. This was done by either sequential reduction in FBS supplementation or by feeding with a 50:50 mixture of reconditioned complete medium and either Opti-MEM I (low-serum) or DMEM as outlined in Table 3.1. The same confluent culture, split equally, was used for each approach. ZeocinTM concentration was reduced to 25 μ g/ml from Combination 2 onwards (Table 3.1A).

Cultures adapted by reconditioning did not survive. Those adapted sequentially fared better for the first few passages but then started to show signs of stress once feeding with Combination 2 began. To preserve these cultures, FBS was reduced by 10 % (v/v) in place of 25 % (v/v) each time (Table 3.1B). Cultures were either grown in a combination of complete medium and DMEM, or complete medium and Opti-MEM I. Serum was reduced sequentially based on observation (culture confluence, cell vitality, and cell morphology). In this manner, cultures were

readily adapted to a final concentration of 3 % (v/v) FBS. However, adaptation slowed growth significantly and consequently decreased the level of recombinant gallibodies expressed. Anomalies in cell morphology were observed and the cells became ‘stickier’ making them difficult to work with. The consequent decrease in growth rate and protein expression from low-serum adapted cells precluded this approach as a practical method for medium- to large-scale production of Abs. Alternative expression systems were investigated instead.

Table 3.1 Adapting HEK 293-H cell cultures to growth in serum-free conditions.

A.	Sequential^a		Conditioned^a		
	Combination (C)	Complete medium	Opti-MEM I / DMEM	Complete medium ^b	-MEM I / DMEM
C1	75%	25%	100%		
C2	50%	50%	50 % from C1	50%	
C3	25%	75%	50 % from C2	50%	
C4	100%	0%	50 % from C3	50%	
C5		100%			

B.	
Complete ^c medium	DMEM or Opti-MEM I
50%	50%
40%	60%
30%	70%
20%	80%
10%	90%

^a Cells were passaged 2 to 3 times in each combination
^b Reconditioned from previous feeding by filtration
^c DMEM + 10 % FBS

3.4 Expressing gallibodies in bacteria

Expressing recombinant antibodies in bacteria potentially has, in theory at least, several advantages over the use of mammalian systems. Bacterial cultures grow more rapidly and are less laborious to work with; they do not require serum-supplementation or expensive medium and the host organism is well characterised and easily manipulated with a myriad of strains and vectors being commercially available. Accordingly, bacterial expression of gallibodies was attempted using the QIAexpress pQE-TriSystem His•*Strep* 1 Vector together with the M15 *E. coli* strain (Section 2.21.1). The pQE TriSystem vectors are designed for single-vector protein expression in three different systems (bacterial, mammalian and insect). His•*Strep* Vector 1 contains adjacent *Strep*-tag and eight-His tag coding sequences just downstream of the multiple cloning site for the expression of C-terminus, dual-tagged proteins (Fig. 3.27). A β -lactamase gene confers ampicillin resistance (Amp^{R}) for selection. Expression is under the control of an optimised T5 promoter/*lac* operator element which is efficiently regulated by high levels of the *lac* repressor. Multiple copies of the pREP4 low-copy plasmid present in M15 *E. coli* constitutively express this repressor in the presence of kanamycin. This means that the plasmid and cell strain work in tandem to drive high-level expression (The QIAexpressionist™ Handbook, 2003). It is well known that secretion of proteins into the periplasm or growth medium simplifies downstream processing (Choi & Lee, 2004). The periplasm in particular provides an oxidative environment which promotes disulphide bond formation and therefore correct folding of the polypeptide chain. Considering that antibody molecules are inherently disulphide-rich, it seemed logical to direct expression toward the periplasm by fusing a secretion-signal to the N-terminus of the heterologous protein. The initial strategy endeavoured to clone the scFv- $\text{C}_{\text{H}2-4}$ fusion, preceded by a secretion-signal sequence, as a single gene-fragment (Fig. 3.28). This approach was, however, not successful perhaps because of the relatively large size (approx. 2 kb) of the fragment (results not shown). As an alternative to the approach described above, an expression cassette containing the $\text{C}_{\text{H}2-4}$ region only was created. The scFv genes and a secretion-signal sequence could then be introduced in a second cloning procedure.

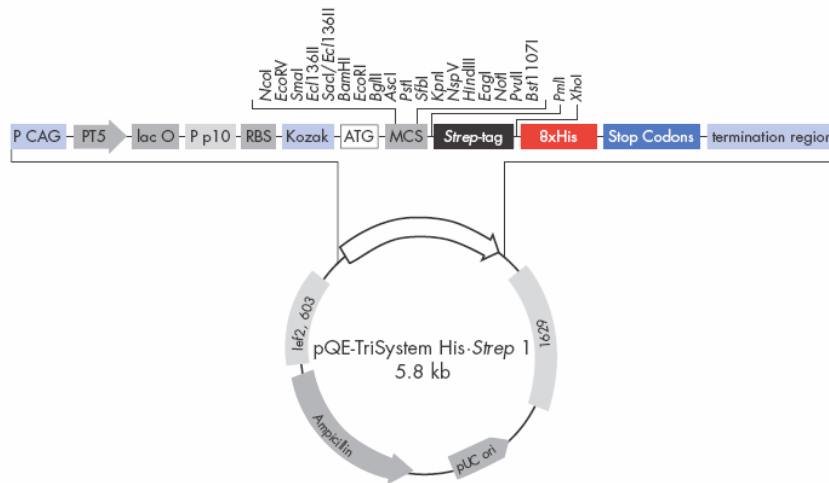


Figure 3.27 Schematic representation of the His•Strep I plasmid vector (Illustration taken from the QIAexpressionist™ Handbook, 2003).

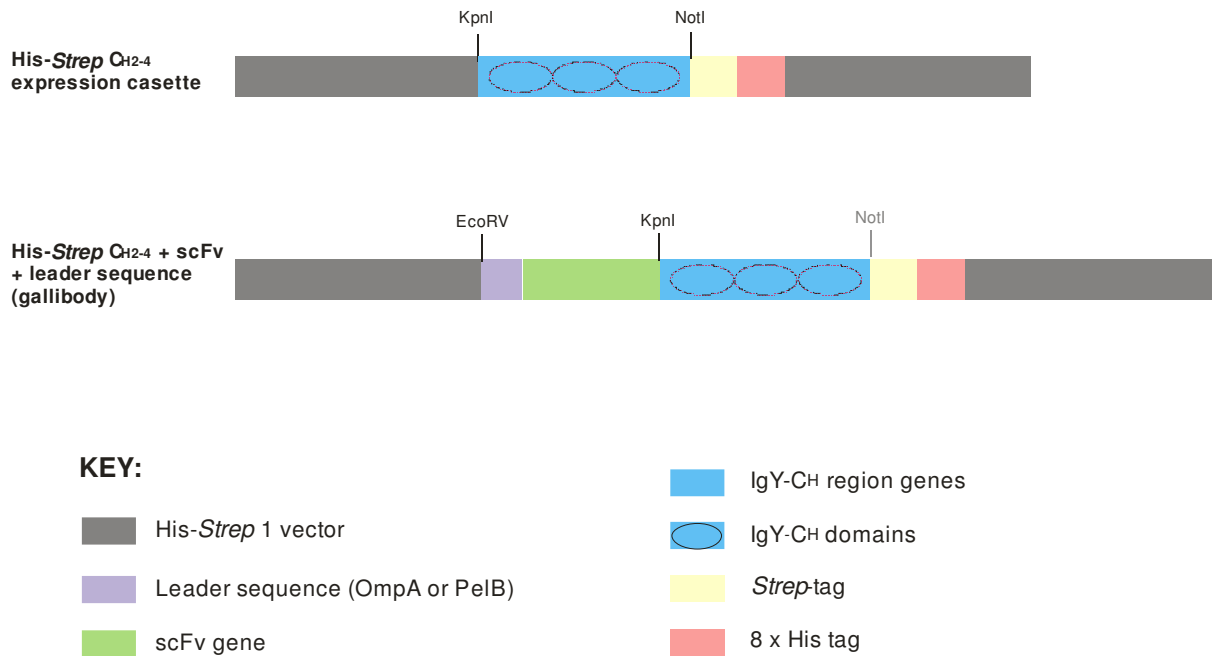


Figure 3.28 Sub-cloning IgY-C_{H2-4} and scFv genes into the His•Strep I vector. First, an expression cassette containing only the IgY-C_{H2-4} region was created (top). Thereafter, scFv genes, together with secretion signal leader sequences (either OmpA or PelB) were introduced to create gallibody constructs (bottom).

3.4.1 Generating an IgY C_{H2-4} expression cassette

The IgY C_{H2-4} fragment (approx. 1 kb) was amplified out of the scFvIgY(CH₂₋₄)His plasmid by PCR, using the primers NotI-Cterm and Nterm CH2-4 (Table 2.1) which added *NotI* and *KpnI* cleavage sites to the ends of the gene fragment. The C_{H2-4} amplicon was purified (Section 2.9) and then digested with these two enzymes (Section 2.8) as was the His•Strep 1 Vector (Fig. 3.29A). Both insert and vector were purified after restriction digestion, ligated (Section 2.10) and introduced into chemically competent M15 *E. coli* (Section 2.21.1). A control ligation containing only digested vector with no insert was included. Ten transformants were randomly selected and as colonies were present in the control experiment, one colony from this plate was also picked. Colony PCR was performed (Section 2.11) using the primers pQEsFor and pQEsRev (Table 2.1). This resulted in an amplicon of roughly 1.5 kb from successfully transformed colonies (Fig. 3.29B, Lanes 1 to 5, 8, 9 and 10). DNA from five of these transformants was sequenced (Section 2.12) and based on alignments with the parent sequence, the His•StrepC_{H2-4} clone 2 was selected for use in subsequent experiments.

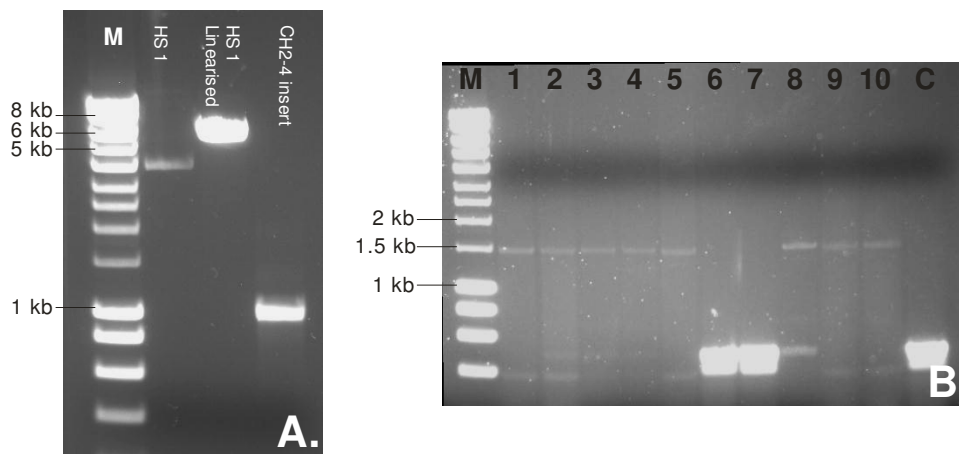


Figure 3.29 Evaluation of insert and vector prepared in the construction of a bacterial expression cassette containing IgY-C_H regions (A). PCR amplicons of approximately 1.5 kb were expected from positive transformants after colony PCR (B). A transformant picked from the control ligation is indicated by lane C. Both gels are 1.5 (w/v) agarose slab gels. Lanes labelled M contain the 1 kb DNA ladder.

3.4.2 Cloning scFv genes into the CH₂₋₄ expression cassette for the expression of secreted scFv-IgY fusions

In an attempt to direct protein expression towards the periplasmic space, secretion-signal coding sequences were appended to the 5' termini of the scFv gene (either *E1* or *G8*). Two commonly-used secretion signals were chosen: the outer-membrane protein A sequence (OmpA; MKKTAIAIAVALAGFATVAQA) and the pectate lyase B sequence from *Erwinia carotovora* (PelB; MKYLLPTAAAGLLLLAAQPAMA) (Choi & Lee, 2004). Single-chain-Fv genes were first amplified out of the mammalian expression constructs (Section 2.7). Primers EcoRVpelB 1 and 2 then introduced an *EcoRV* restriction site flanking a PelB sequence 5' to the scFv gene; while primers EcoRV-ompA 1 and 2 introduced the same site, but with a flanking OmpA sequence. The scFvCterm KpnI common primer appended a *KpnI* restriction site onto the 3' end of the scFv gene (Table 2.1). Figure 3.30 shows DNA inserts (A) and plasmid vectors (B) after the first restriction digest. The E1scFv (Lanes 1 and 2, Fig. 3.30) is slightly bigger than the G8scFv (Lanes 3 and 4, Fig. 3.30) as the CDR3 of E1scFv contains a larger number of residues. A second digestion followed, after which the fragments were purified, ligated and introduced into M15 *E. coli*. Transformants were selected and subjected to colony PCR using primers pQETsFor and scFvCterm Kpn (Table 2.1). Those clones which produced an amplicon of 1 kb had been transformed successfully and the DNA was sequenced. In six E1 (OmpA), two G8 (OmpA), four E1 (PelB) and one G8 (PelB), the constructs had been successfully inserted in the correct reading-frame. The colony PCR of selected OmpA clones is shown in Figure 3.31.

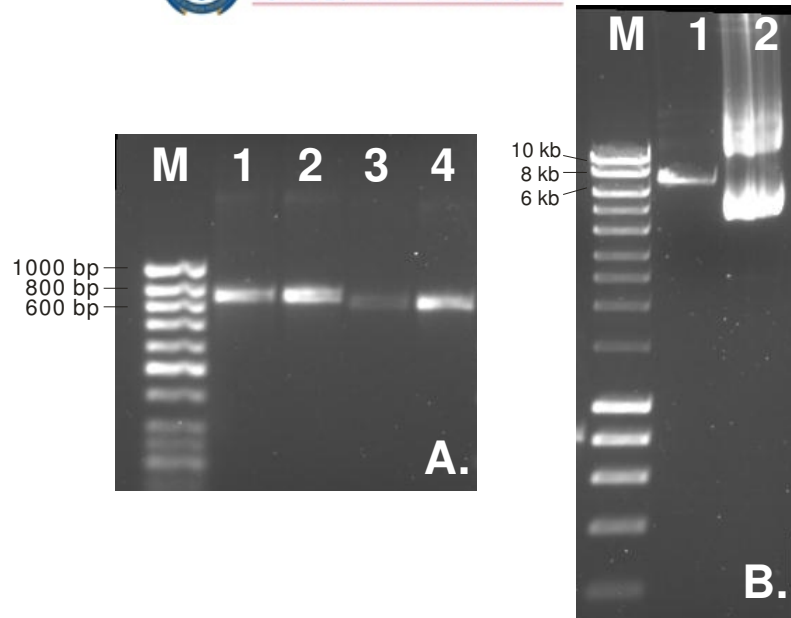


Figure 3.30 Preparation of scFv-genes preceded by secretion-signal coding sequences (A) and His•StrepC_{H2-4} expression cassette (B) for ligation. Gel A: E1scFv + OmpA (Lane 1); E1scFv + PelB (Lane 2); G8scFv + OmpA (Lane 3); G8scFv + PelB (Lane 4). Gel B: the His•StrepC_{H2-4} expression cassette was digested with the appropriate endonucleases (Lane 1) and separated alongside a sample of undigested vector (Lane 2) to ensure that it had linearised. In each case samples were separated on 1 % (w/v) agarose slab gels and a 100 bp (A) or 1 kb (B) DNA ladder was loaded in Lane M.

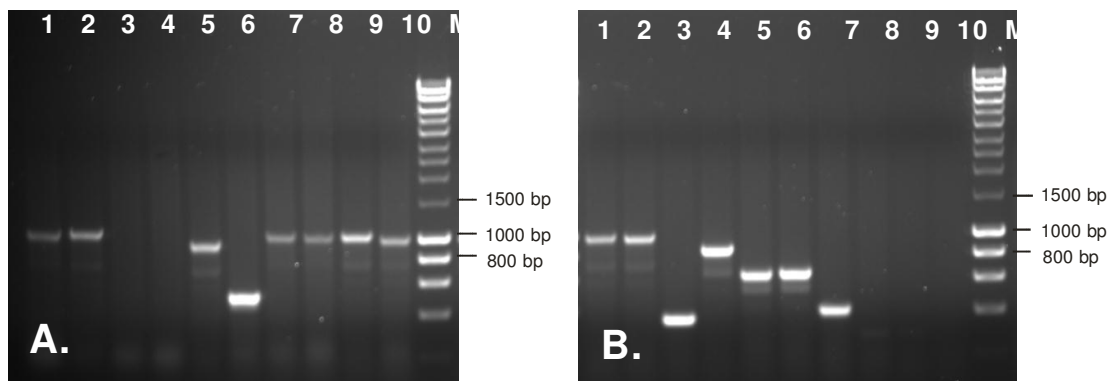


Figure 3.31 Colony PCR on selected E1 + OmpA (A) and G8 + OmpA (B) transformants. Colonies that yielded an amplicon of 1 kb contained the correct insert and were sequenced.

3.4.3 Pilot protein expression of gallibodies in M15 *E. coli*

Clones with the correct sequence reading frame were grown in 10 ml Overnight Express™ Instant TB Medium in order to induce protein expression. Figure 3.32 shows SDS-PAGE (Section 2.15) analysis of the SNFs and cytoplasmic/periplasmic fractions of untransformed M15 cells and M15 cells transformed with His•*Strep* 1 Vector (original), which were induced in the same way to serve as negative controls. All of the clones secreted a protein of approximately 55 kDa into the culture medium (SNF) when induced. This is an inherent property of the M15 cell line (The QIAexpressionist™, 2003). No significantly over-expressed proteins within the 63 kDa range were seen in SDS-PAGE analyses of cytoplasmic, periplasmic or inclusion body extraction samples of these clones (not shown). Very faint signals were obtained when some of the clones were tested in indirect ELISA but background signals were high (not shown) and no conclusive evidence for expression was obtained. Spot-blot (Section 2.17) of the same samples, detected with anti-IgY(Fc) Ab did not produce any signal (not shown). Similarly, no blot signal was observed when SNFs were purified using Ni-NTA spin columns (Section 2.18.1) (not shown). Expression of gallibody constructs in M15 *E. coli* was thus not further pursued.

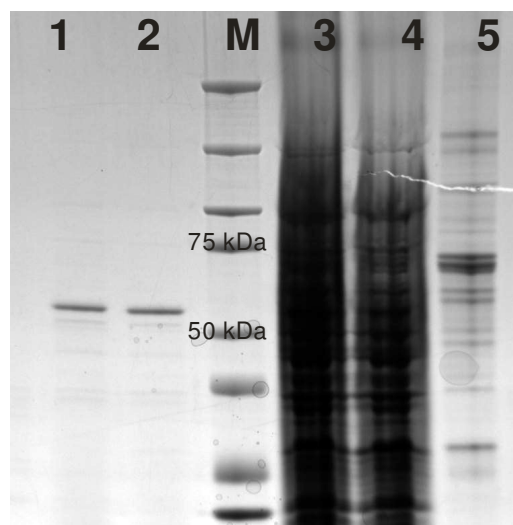


Figure 3.32 SDS-PAGE analysis of M15/ His•*Strep* 1 expression control. Untransformed M15 cells were induced overnight in 10 ml Overnight Express™ Instant TB Medium and SNFs (Lane 1) as well as cytoplasmic/periplasmic fractions (Lane 3) were analysed by SDS-PAGE. M15 cells transformed with unmodified His•*Strep* 1 vector were analysed in the same way (Lane 2 and 4). Lane 5 contains *gbG8.8*, the reconstituted scFv molecule expressed in HEK 293-H cells, as a positive control. M15 cells inherently secrete an approximately 55 kDa protein into the growth medium when induced. M contains protein molecular weight marker and significant sizes are indicated.

3.5 Investigating gallibody expression in yeast

After the attempts to produce gallibodies in bacteria were not successful, yeasts were evaluated as a possible means of expressing these scFv-IgY fusions. Yeasts are attractive organisms for heterologous protein expression as they grow easily in liquid batch culture like bacteria yet, like the higher eukaryotes, they can express complex proteins. GG799 *K. lactis* is a wild-type isolate which is used in the food industry and is capable of industrial-scale secretion of heterologous proteins and rapid, high-density growth (Colussi & Taron, 2005; van Ooyen *et al.*, 2006). It can be purchased in kit-form together with the integrative shuttle vector pKLAC1, which is linearised by restriction enzyme cleavage with *Sac II* or *BstX*. This produces an expression cassette that integrates into the native *LAC4* promoter region of the yeast genome by homologous recombination (van Ooyen *et al.*, 2006). In yeasts, integrative transformants are genetically more stable and more productive than episomal transformants (Smith *et al.*, 1985; van Ooyen *et al.*, 2006; Read *et al.*, 2007). High-level production of heterologous protein is often as a result of multicopy integrants, in which more than one cassette has integrated per genome. Acetamide selection of transformants on nitrogen-free, acetamide-supplemented medium is known to increase the incidence of multi-copy transformants (Read *et al.*, 2007). The pKLAC1 vector (Fig. 3.33) contains the *amdS* gene from *Aspergillus* which encodes for the enzyme acetamidase. Correctly integrated pKLAC1 transformants are able to produce this enzyme to catabolise acetamide into ammonia for use by the cell as a source of nitrogen (Colussi & Taron, 2005) which then can survive on nitrogen-free agar. pKLAC1 also contains a β -lactamase gene (Amp^{R}) and a bacterial origin of replication, as well as a mutant form of the *LAC4* promoter ($\text{PLAC}_{4\text{-PBI}}$) which reduces gene expression in bacteria, but continues to strongly drive high-level expression and secretion in *K. lactis* (Colussi & Taron, 2005). The α -mating factor, a secretion leader-sequence located just upstream of the multiple cloning site (MCS), facilitates secretion of the mature protein into the growth medium.

As a prerequisite for expression, the gene of interest to be cloned in pKLAC1 needs to be free of *SacII* and *BstX* sites since these enzymes are used to linearise the vector for subsequent integration into the yeast genome. As IgY-C_{H4} contains a *SacII* cleavage site, site-directed

mutagenesis was used to alter the coding sequence at this particular point. A construct lacking the fourth C_H domain was also constructed (C_{H2-3}) as an alternative (Fig 3.34). As with previous (Section 3.4) attempts, it was not possible to sub-clone scFv-C_H fusions as single gene fragments. Once again, therefore, an expression cassette containing only the IgY-C_H fragment was first created and the scFv genes were introduced during a second round of sub-cloning. To determine whether this was in fact a practical system for expressing IgY-derived genes, only the C_H regions of chicken IgY were first cloned in the vector (Fig 3.34).

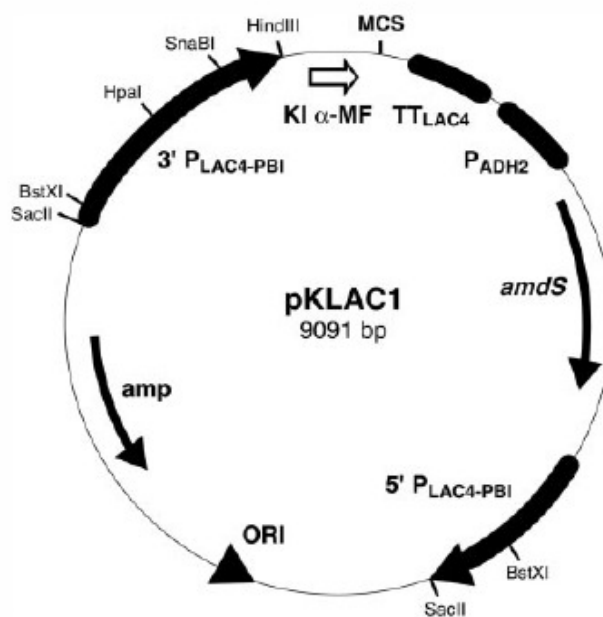


Figure 3.33 The pKLAC1 integrative vector (diagram from Colussi & Taron, 2005). The vector sequence is available on GenBank as AY968582 (Benson *et al.*, 2000; accessible at www.ncbi.nlm.nih.gov).

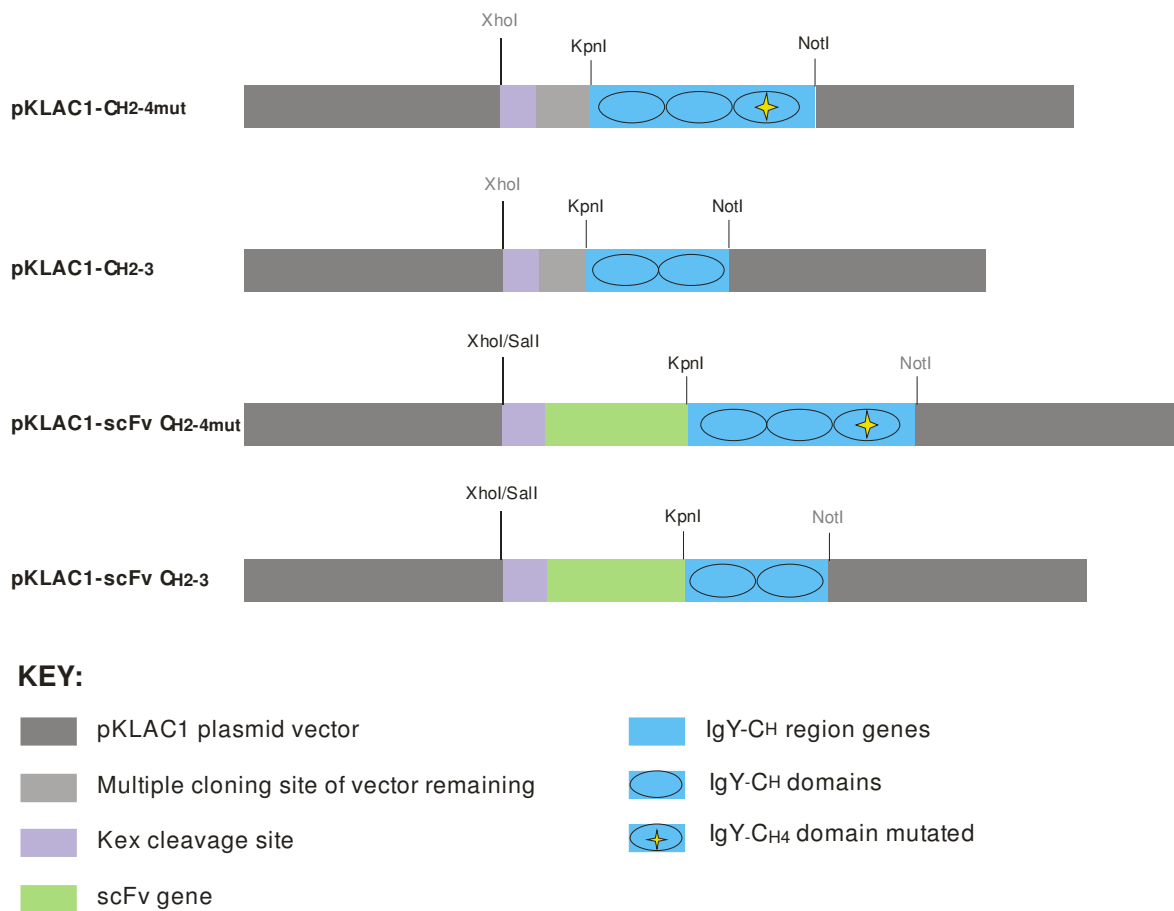


Figure 3.34 Recombinant Ab expression cassettes constructed for use in the *K. lactis* system. Restriction enzyme sites used in the construction of each cassette are in black typeface, those available but not used at the time are indicated in grey. In cassettes containing only the C_H domains (pKLAC1-C_{H2-4mut} and pKLAC1-C_{H2-3}), a small area of MCS remained for cloning of the scFv gene (light grey block). Proteins encoded by these cassettes would include a small translated portion of this MCS at their N terminus.

3.5.1 Site-directed mutagenesis to remove *SacII* site from IgY-C_{H4} domain

3.5.1.1 Site-directed mutagenesis in scFvIgY(CH₂₋₄)His plasmid using GeneTailor™

The GeneTailor™ Site Directed Mutagenesis System kit was used to mutate the *SacII* cleavage site in the C_{H4} domain of IgY by changing two of the base-pairs located within the cleavage site (CCGCGG to ACGAGG), without altering the translated amino acid sequence (Fig 3.35, also Section 2.13.1). The scFvIgY(CH₂₋₄)His plasmid (6.7 kb) originally obtained from Germany was methylated and amplified by PCR using a high-fidelity *Taq* polymerase and specifically designed primers (Mut Yeast For and Mut Yeast Rev, Table 2.1). Initial attempts using AccuPrime™ *Taq* DNA Polymerase High Fidelity did not yield amplicons of the correct size (Fig 3.36). Consequently, the experiment was repeated using *Ex Taq*™ polymerase. An approximately 7 kb amplicon was then obtained, although the DNA appeared to be heterogeneous in agarose gel electrophoresis, as shown by a smear (Fig 3.36, Lane 2). This DNA was nevertheless used to transform One-Shot® MAX Efficiency DH5α™-T1^R *E. coli* cells. Ten transformants were picked for analysis in colony PCR (Section 2.11) using the NotI-Cterm and NtermCH2-4 primers (Table 2.1). All ten of these clones contained inserts, yielding amplicons of roughly 1 kb (Fig 3.37). DNA from three of these (Fig. 3.37, Lanes 3, 4 and 6) were sequenced (Section 2.12) but only one (clone 3) translated correctly. Due to errors within this sequence at the site of mutation, however, it was not certain whether the base-pair exchanges had in fact been incorporated. Enzymatic cleavage (Section 2.8) with *SacII* was used as an alternative analysis. This indicated that the cleavage site had not been removed (Fig 3.38). Since the polymerase needs to copy the entire plasmid during the PCR used for mutation (Fig 3.35), a combination of polymerase fidelity and an unusually large plasmid (almost 7 kb) may conceivably have contributed to the failure of the mutation attempt. For this reason, the C_{H2-4} region was cloned (Section 2.7) in a smaller vector as described in the following sections.

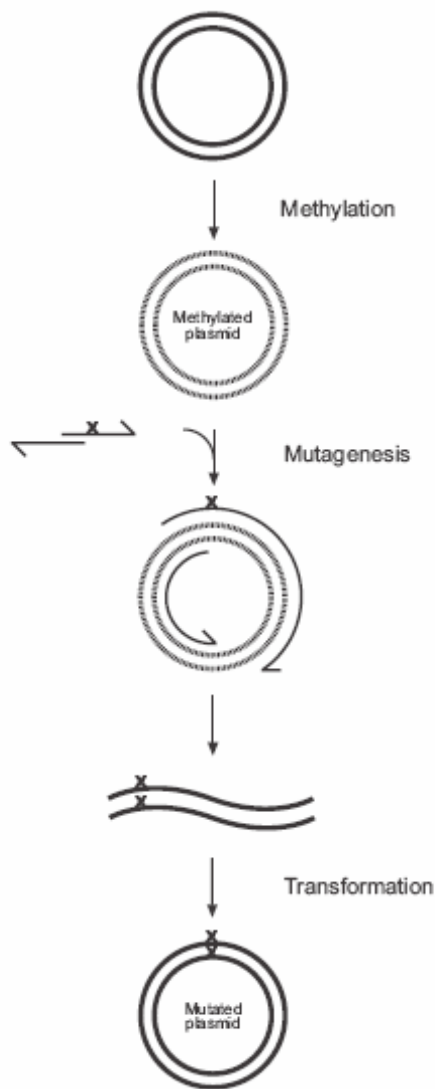


Figure 3.35 Flow-diagram illustration of the GeneTailor™ Site Directed Mutagenesis System process. Target plasmid DNA is methylated and then amplified in a mutation PCR using two specially designed mutagenesis primers. Only one of these primers contains the desired mutation. Once the primers bind, they copy around the entire plasmid. The unmethylated, linear dsDNA that results is transformed into *E. coli* where it is circularized. Remaining methylated template DNA is degraded by host endonucleases (Diagram from GeneTailor™ Instruction Manual, Version G).

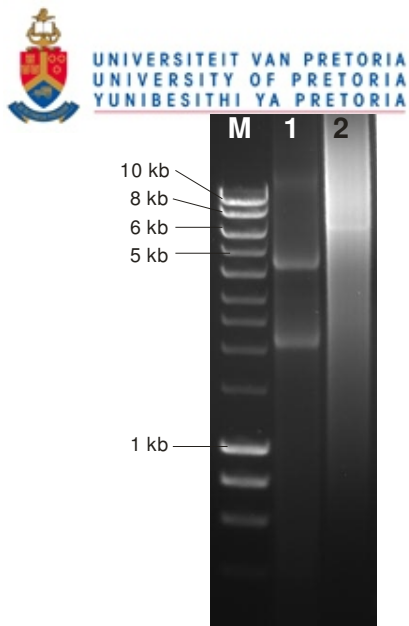


Figure 3.36 Mutagenesis PCR using Accuprime™ polymerase (Lane 1) and Ex Taq™ polymerase (Lane 2). DNA fragments were separated in 1 % (w/v) agarose and Lane M contains a 1 kb DNA ladder.

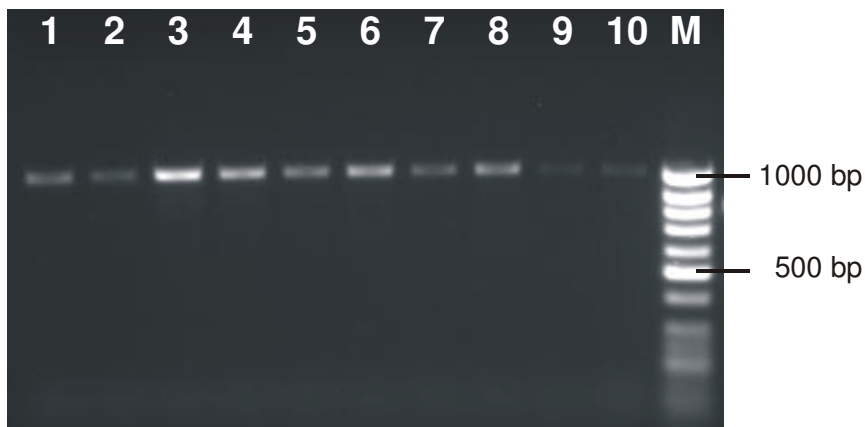


Figure 3.37 Colony PCR on transformants selected after site-directed mutagenesis using the GeneTailor™ System generated amplicons of 1 kb. Samples were separated in 1 % (w/v) agarose and Lane M contains a 100 bp DNA ladder.

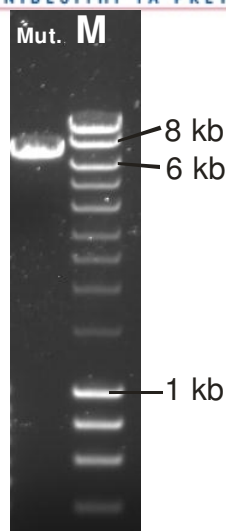


Figure 3.38 Restriction digest of clone 3 plasmid DNA with *SacII* separated in 1 % (w/v) agarose. Plasmid DNA (Mut.) was linearised, indicating that cleavage had occurred. Therefore, the *SacII* site had not been removed in the mutagenesis reaction.

3.5.1.2 Site-directed mutagenesis in the pUC19 vector

The C_{H2-4} fragment was amplified out of the scFvIgY(CH₂₋₄)His plasmid by PCR for sub-cloning into pUC19, a smaller plasmid than scFvIgY(CH₂₋₄)His (approximately 2.7 kb). The primers CH2-4Yeast For and CHpUC Cterm (Table 2.1) could be used to add *KpnI* sites to both ends of the amplicon as directional cloning was not important in this instance. Both the insert and vector were digested with *KpnI* after which the purified fragments (Fig 3.39) were ligated and used to transform chemically competent M15 *E. coli* (Section 2.21.1). Control ligations which did not include the potential insert were performed simultaneously. Of ten transformants selected, only one contained an insert of the correct size (approximately 1.25 kb, Fig 3.40) as shown by colony PCR (using M13 for and M13 rev, Table 2.1). Plasmid DNA from this clone was amplified by PCR using the GeneTailor™ kit, and the amplicon was used to transform One-Shot® MAX Efficiency® DH5 α ™-T1^R *E. coli* cells as previously described (Section 2.13). All ten transformants selected for colony PCR (using the primers CH2-4 Yeast For and Yeast Rev) yielded fragments of the anticipated size (1 kb, Fig 3.41). Plasmid DNA was extracted from six of these clones (Fig. 3.41, Lanes 1 to 6) and digested with *SacII* as previously. All six plasmids linearised after digestion (Fig 3.42) indicating that the *SacII* site had once again not been mutated. Therefore, use of the kit was discontinued and mutation using overlap-extension PCR (Fig 3.43) was attempted instead.

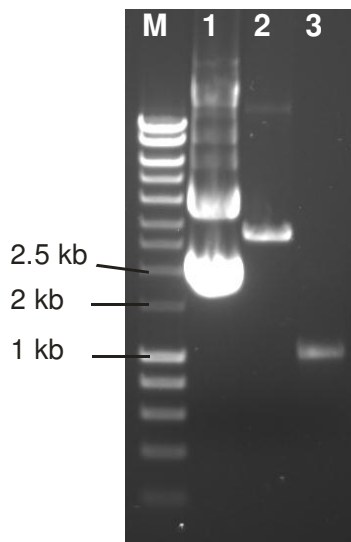


Figure 3.39 Restriction-digested C_{H2-4} insert and pUC vector separated in 1 % (w/v) agarose. Uncut vector (Lane 1) served as a control for correct cleavage of pUC19 (Lane 2). Digested insert was loaded in Lane 3. Fragment sizes are indicated in Lane M.

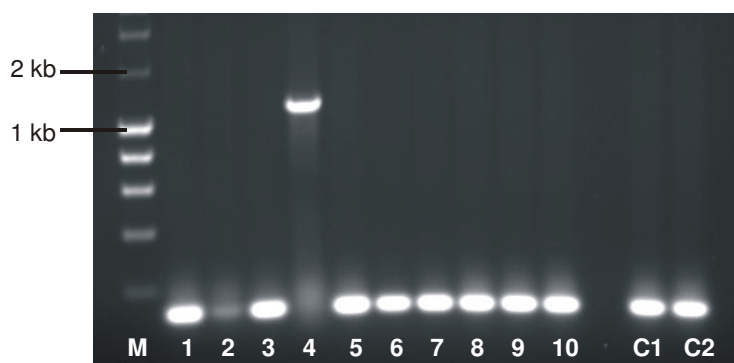


Figure 3.40 Colony PCR of pUC19- C_{H2-4} transformants. Only clone contained an insert of the correct size (Lane 4) and was propagated for further mutation work. C1 and C2 are transformants picked from control plates. Fragments were separated in 1 % (w/v) agarose.

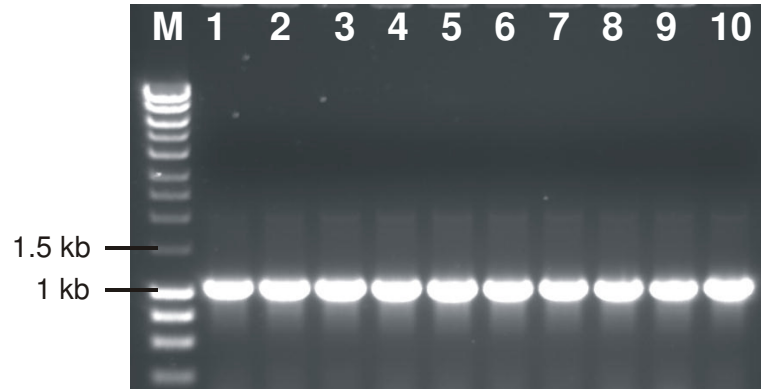


Figure 3.41 Colony PCR of mutated pUC-C_{H2.4} transformants separated in 1 % (w/v) agarose. Lane M contains a 1 kb DNA marker.

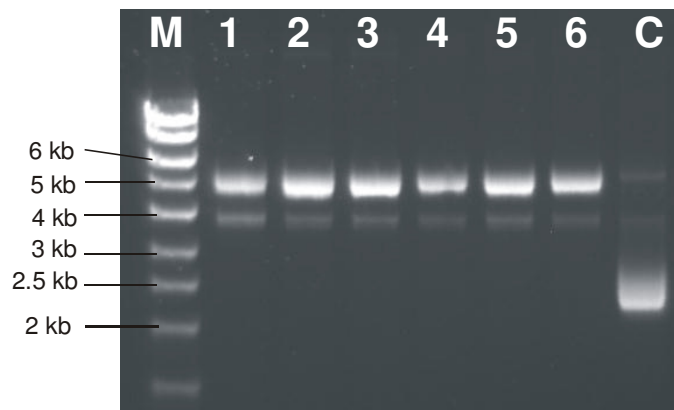


Figure 3.42 *SacII* digestion of plasmid DNA from six mutated pUC19-C_{H2.4} clones in 1 % (w/v) agarose. Lane C contained undigested plasmid from clone 5 as a control. Lane M contains a 1 kb DNA ladder.

3.5.1.3 Site-directed mutagenesis using overlap-extension PCR

Site-directed mutagenesis by overlap extension PCR (Section 2.13.2, Fig 3.43) was attempted using the scFvIgY(CH₂₋₄)His plasmid as template. The first reaction using primers Mut Yeast Rev and CH2-4 Yeast For (Table 2.1) generated a 250 - 300 bp fragment with a *KpnI* cleavage site at the 5'-end, whereas the second PCR using Mut Yeast For and Yeast Rev (Table 2.1) generated a fragment of roughly 700 bp with a 3'-end *NotI* cleavage site (not shown). After gel extraction and purification the amplified fragments were used in a 'pull-through' PCR with the flanking primers (CH2-4 Yeast For and Yeast Rev). In this PCR, consisting of a concentrated and a dilute reaction, the fragments served as primers for the 3' extension of one another and generated 1 kb amplicons as anticipated (Fig 3.44). These products were pooled and digested with *SacII*. A small aliquot was analysed in agarose gel electrophoresis. This time the fragment remained intact (Fig 3.45), indicating that the base-pair exchanges had indeed been successfully accomplished.

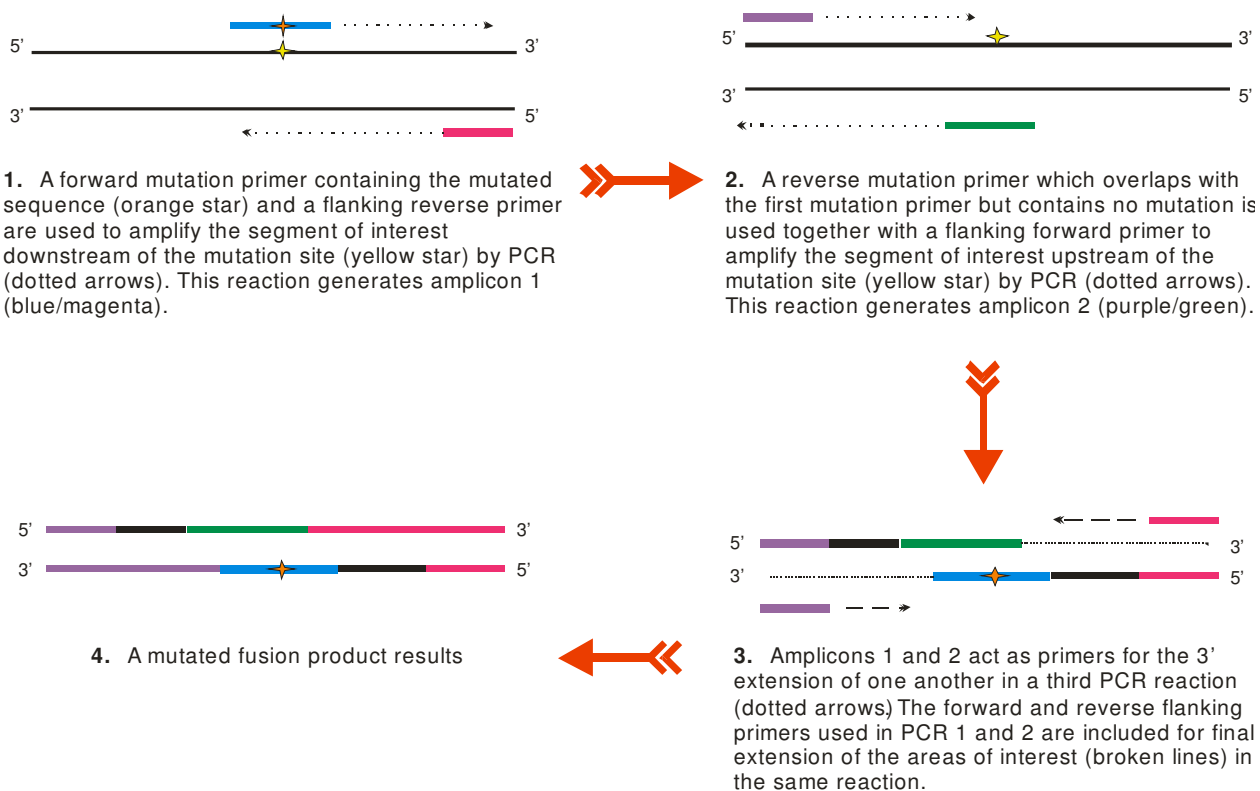


Figure 3.43 Mutagenesis by overlap extension (adapted from the method described Ho *et al.*, 1989).

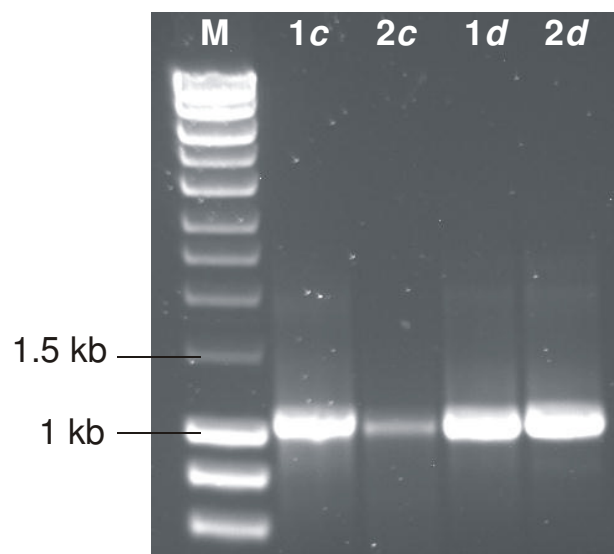


Figure 3.44 Overlap extension PCR to generate mutated C_{H2-4} inserts. In this reaction, overlapping fragments generated in previous PCRs (not shown) served as primers for one another and generated amplicons of 1 kb, as indicated in Lane M by the DNA ladder. The dilute reactions (1d and 2d) were carried out using aliquots of the concentrated reaction (1c and 2c) which had been amplified for 15 cycles.

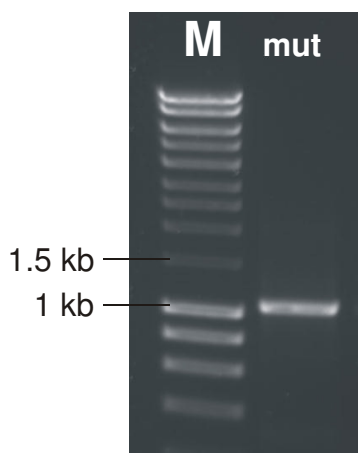


Figure 3.45 The overlap-extension mutated C_{H2-4} fragment (Fig 3.34) remained intact (1 kb) after *SacII* cleavage, indicating that the enzyme site had been successfully removed. Lane M contained DNA markers and the relevant fragment sizes are indicated.

3.5.2 Sub-cloning mutated IgY-C_{H2-4} into pKLAC1 to create an expression cassette

The yeast expression vector pKLAC1 and the mutated C_{H2-4} fragment were each digested with *NotI* followed by *KpnI*. Ligation into these two sites left a small region of the MCS between the Kex cleavage site (protease processing site) and the C_{H2-4} gene fragment (Fig 3.34), to allow subsequent cloning. Fragments were purified, then ligated and used to transform M15 *E. coli*. Control reactions contained no insert. Three out of the 10 transformants that were selected contained inserts of the expected size (approximately 1.3 kb, Fig 3.46) as was revealed by colony PCR using the primers pKLAC1 seqFor and pKLAC1 seqRev (Table 2.1). Clone number 8 was selected for subsequent experiments. It was designated as pKLAC1-C_{H2-4mut}.

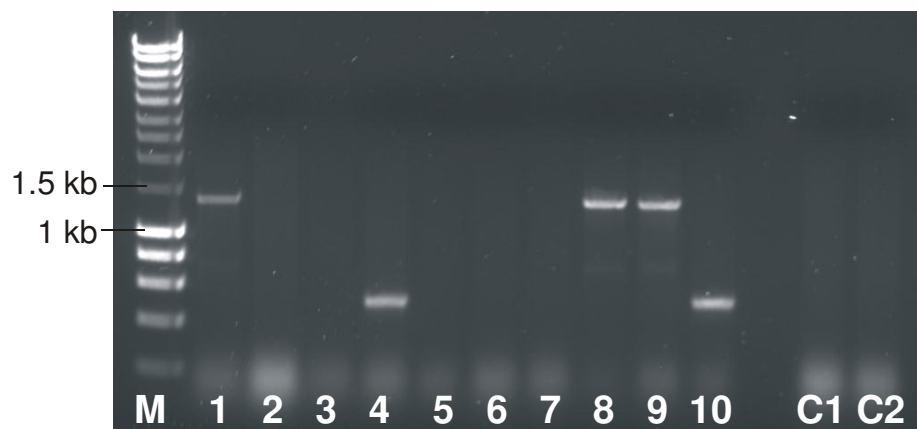


Figure 3.46 Colony PCR of clones picked from pKLAC1-C_{H2-4} transformation. Clones 1, 8 and 9 generated correctly-sized amplicons of 1.3 kb, as indicated by the DNA ladder in Lane M. C1 and C2 are transformants picked from the negative control.

3.5.3 Sub-cloning IgY-C_{H2-3} into pKLAC1 to create an expression cassette

The region C_{H2-3} (i.e. *sans* domain 4) was amplified out of scFvIgY(CH₂₋₄)His using primers designed according to the domain sequences published by Parvari *et al.* (1988). The primers CH2-4 Yeast For, CH3rev 1 and CH3rev 2 (Table 2.1) added *NotI* and *KpnI* sites to the terminal ends of the fragment in two PCRs, generating a final fragment of approximately 700 bp (Fig 3.47). Similar to the C_{H2-4mut} cassette (Section 3.5.2), a small region of the MCS remained between the Kex cleavage site and the gene fragment (Fig 3.34). This fragment and pKLAC1

were digested with *NotI* and *KpnI* (Fig 3.47), purified and ligated before being introduced into M15 *E. coli* for propagation. Control ligations containing no insert were carried out simultaneously. Colony PCR with pKLAC1 seqFor and pKLAC1 seqRev yielded eight out of ten clones with the correctly sized inserts (approximately 950 bp, Fig 3.48). The DNA of four clones was sequenced. Clone number 6 was chosen for use in the experiments that followed. This clone was designated pKLAC1-C_{H2-3}.

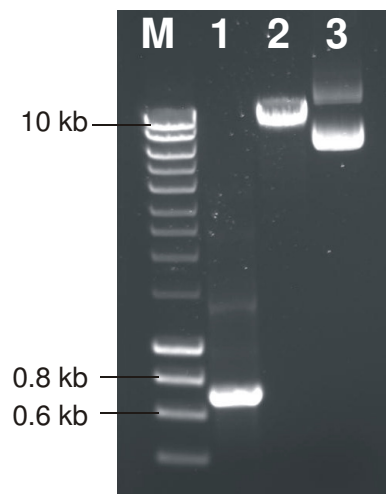


Figure 3.47 C_{H2-3} insert (Lane 1) and pKLAC1 vector (Lane 2) recovered after double digestion. Lane 3 contained undigested pKLAC1 to serve as a control for cleavage. Relevant molecular weights are indicated by the DNA ladder in Lane M.

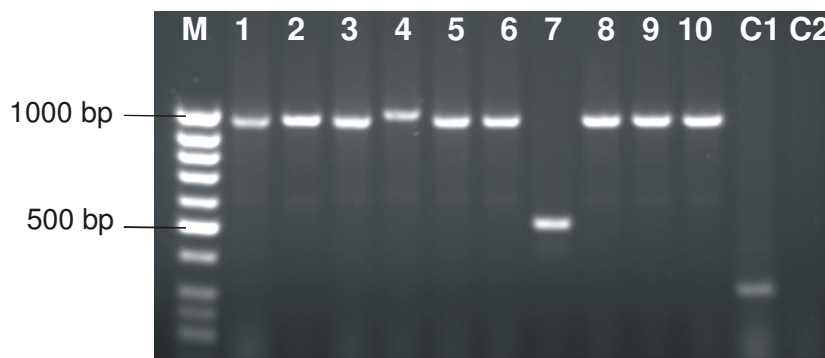


Figure 3.48 Colony PCR of pKLAC1-C_{H2-3} transformants. Eight out of the ten clones generated amplicons of 950 bp. Clones 2, 3, 6 and 9 were propagated and the plasmid DNA sequenced. Relevant fragment sizes are indicated by the DNA ladder in Lane M.

3.5.4 Gene expression in GG799 *K. lactis* with pKLAC1-C_{H2-4mut} and pKLAC1-C_{H2-3}

To investigate whether this system could express IgY-derived genes, purified plasmid DNA from overnight cultures of pKLAC1-C_{H2-3} and pKLAC1-C_{H2-4mut} were linearised with *SacII* (Fig 3.49) and used to transfect GG799 *K. lactis* cells (2.21.3). Protein expressed from these constructs should contain a small translated portion of the MCS at the N terminus. A positive control plasmid (pMalE) containing the *E. coli* maltose binding protein gene was supplied with the commercial kit and was treated identically. Yeast colonies were visible after three days at 30 °C on all plates. Ten clones were picked representing each different construct, patched onto fresh YP agar plates and grown for a further two days. Each clone was then propagated in 2 ml of YPGal medium at 30 °C for two days to induce protein expression. The cultures were centrifuged and the SNFs were analysed in a sandwich ELISA (Section 2.14.2) aimed at detecting C_{H2-3} regions. Readings at A₄₉₂ were relatively low, but at least 2 × higher than background (Fig 3.50 and 3.51).

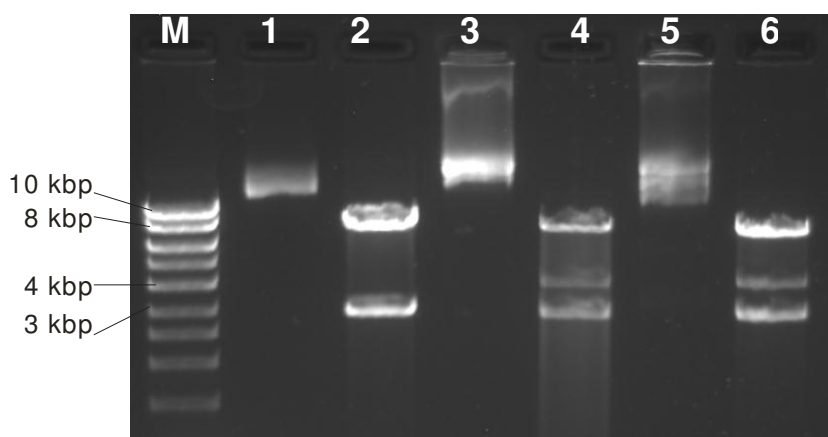


Figure 3.49 *SacII* digestion of pKLAC1-C_H region constructs for transfection into *K. lactis* cells. M denotes the DNA ladder and fragment sizes are indicated. Lane 1: undigested pMalE control plasmid; Lane 2: linearised pMalE plasmid; Lane 3: undigested pKLAC1-C_{H2-4mut}; Lane 4: linearised pKLAC1-C_{H2-4mut}; Lane 5: undigested pKLAC1-C_{H2-3}; Lane 6: linearised pKLAC1-C_{H2-3}. After *SacII* digestion each plasmid yielded two fragments: one larger than 6 kbp and one of approximately 2.8 kbp, as expected. A third fragment of approximately 4 kbp is the pREP4 plasmid inherent to the M15 *E. coli* cells in which the pKLAC1-C_H constructs were propagated.

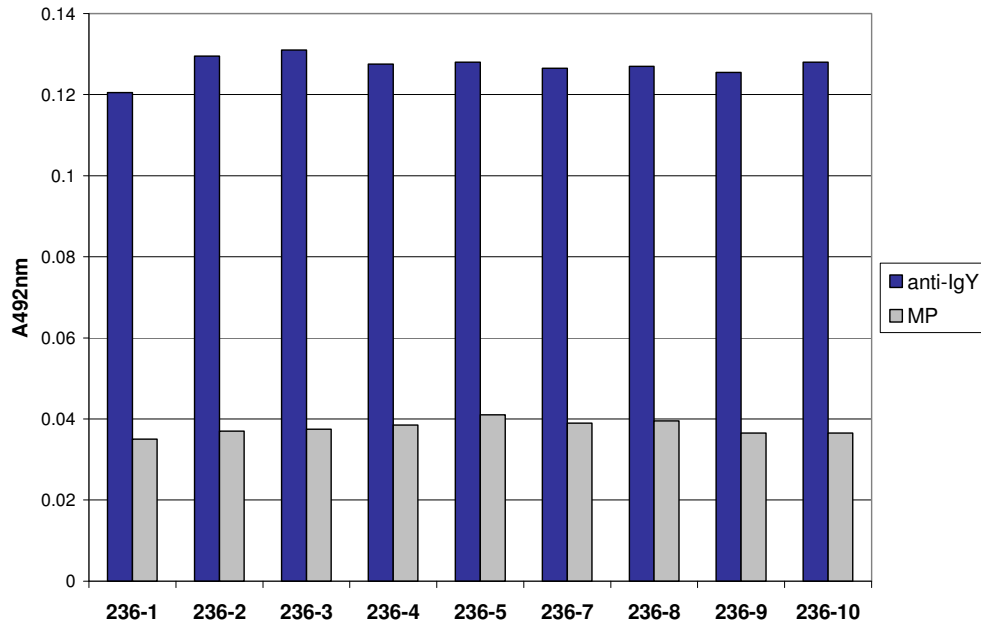


Figure 3.50 Sandwich ELISA to detect expression of C_{H2-3} region genes in *K. lactis*. IgY-derived domains expressed into the SNF were trapped with polyclonal anti-IgY Ab (10 μ g/ml), and detected with HRP-conjugated anti-IgY(Fc) Ab (1 in 5000 in 2 % (w/v) MPPBS). MP controls were not coated with anti-IgY trapping Ab.

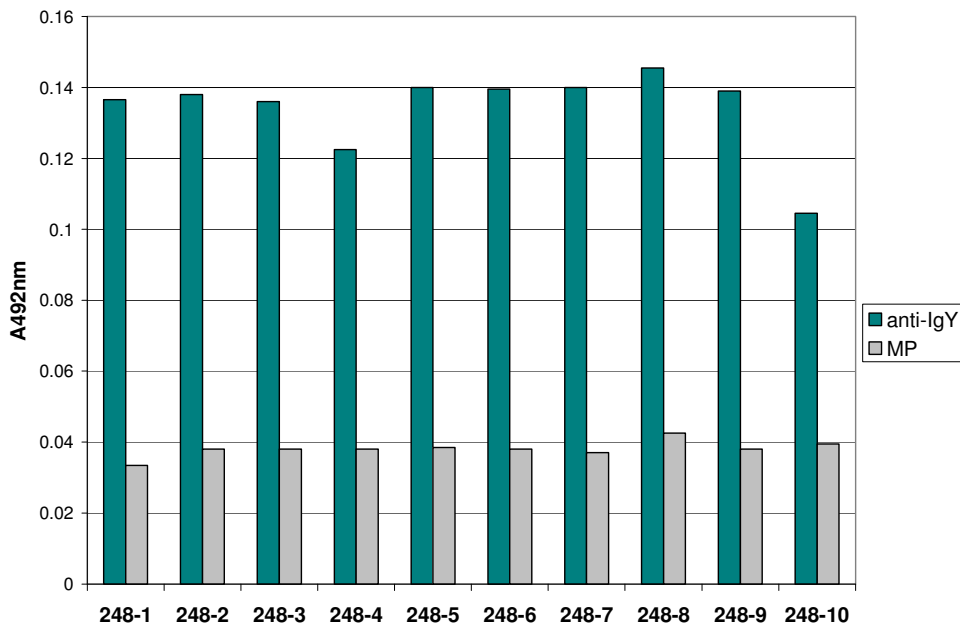


Figure 3.51 Sandwich ELISA to detect expression of $C_{H2-4mut}$ region genes in *K. lactis*. IgY-derived domains expressed into the SNF were trapped with polyclonal anti-IgY Ab (10 μ g/ml), and detected with HRP-conjugated anti-IgY(Fc) Ab (1 in 5000 in 2 % (w/v) MPPBS). MP controls were not coated with anti-IgY trapping Ab.

3.5.5 Sub-cloning scFv genes into pKLAC1-C_{H2-4mut} and pKLAC1-C_{H2-3}

The results described in the previous section suggested that the *K. lactis* system was able to produce at least low levels of IgY-derived domains. To determine whether this was true of functional constructs containing binding sites (Fig 3.34), the scFv genes for E1 and G8 were amplified out of the plasmid scFvIgY(C_{H2-4}) using the primers scFv Yeast Rev and Yeast For to add *Sall* and *KpnI* sites. Single-chain Fv inserts were then cleaved by restriction digestion (Fig 3.52). In the case of the pKLAC1-C_{H2-4mut} and -C_{H2-3} expression cassettes, digestion was with *XhoI* and *KpnI*, as *Sall* and *KpnI* digestion yields compatible ends. Purified, ligated digests for each combination (E1 + C_{H2-4mut}, E1 + C_{H2-3}, G8 + C_{H2-4mut} and G8 + C_{H2-3}) were then used to transform M15 *E.coli*. Control ligations containing the expression cassettes, but without the potential insert were performed concurrently. Eight colonies were picked for each construct and subjected to colony PCR using the primers Yeast For and scFv Yeast Rev (Table 2.1). Two colonies from each control plate were treated identically. Amplicons of approximately 800 bp were expected from transformants containing scFv gene-inserts (Fig 3.53). Three clones of each variety were propagated. The plasmid DNA was purified and sequenced which revealed that the clones pKLAC1-E1 C_{H2-3}(3), pKLAC1-G8 C_{H2-3}(1), pKLAC1-E1 C_{H2-4mut}(2) and pKLAC1-G8 C_{H2-4mut}(1) all contained the correct insert sequence (not shown).

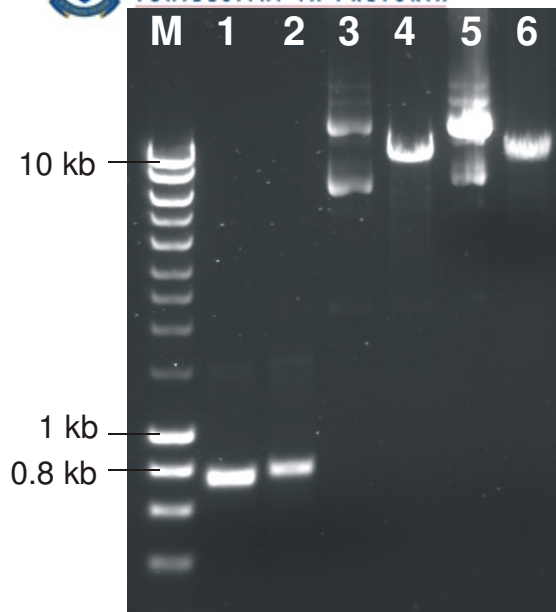


Figure 3.52 Single-chain Fv inserts G8 (Lane 1) and E1 (Lane 2) and pKLAC1-C_{H2-3} (Lane 4) and -C_{H2-4} (Lane 6) expression cassettes recovered after double digestion. Undigested pKLAC1-C_{H2-3} (Lane 3) and -C_{H2-4} (Lane 5) were included as controls.

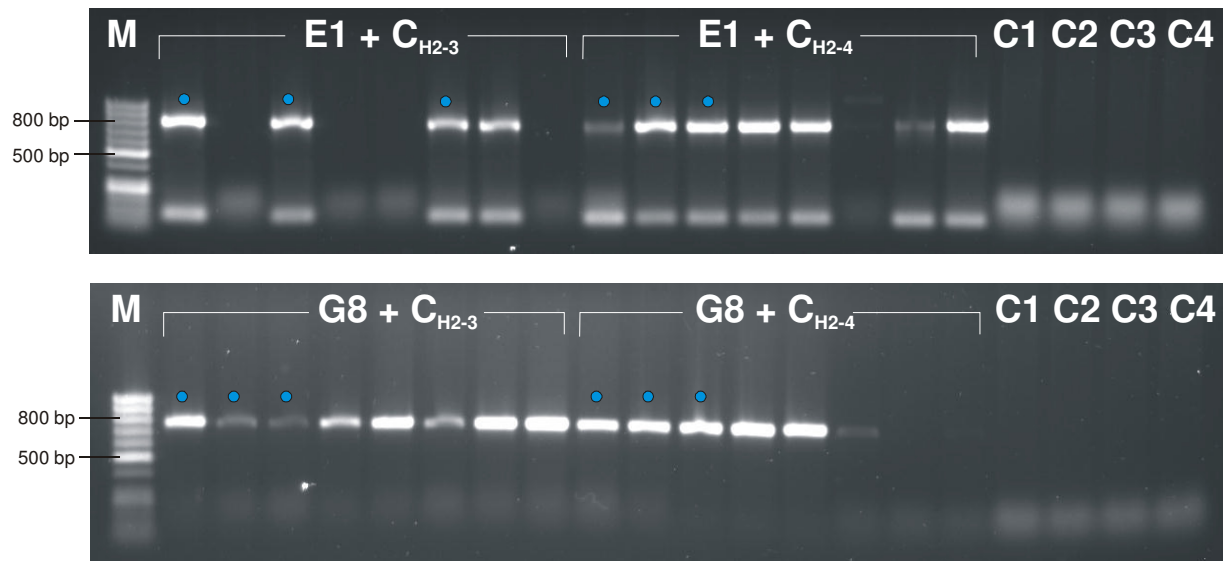


Figure 3.53 Colony PCR of scFv + pKLAC1-cassette transformants. In all cases, an 800 bp amplicon was expected from positive transformants. Clones marked above with a blue dot were propagated and the plasmid DNA sequenced. C1 and C2 are control colonies from the pKLAC1-C_{H2-3} control ligation. C3 and C4 are controls picked from the pKLAC1-C_{H2-4} control ligation.

3.5.6 Gallibody expression in GG799 *K. lactis*

Bacterial cultures of pKLAC1-E1 C_{H2-3}(3), pKLAC1-G8 C_{H2-3}(1), pKLAC1-E1 C_{H2-4mut}(2) and pKLAC1-G8 C_{H2-4mut}(1) were grown overnight and plasmid DNA was purified for transfecting chemically competent *K. lactis* cells. The plasmids pMalE and pUC19 were included as positive and negative controls respectively. *SacII*-linearised plasmids are depicted in Fig 3.54. Colonies were visible on all YP agar-plates after three days at 30 °C and resembled the positive control. Growth on the pUC19 plate was not expected and was suspicious. Nevertheless, ten colonies from each construct were picked, patched onto fresh plates and incubated for a further two days. Small-scale protein expression was carried out as described in Section 3.5.4, but this time 3 ml cultures were propagated. The cultures were centrifuged and SNFs collected. Two millilitres of each SNF was concentrated four times to improve detection, as previous experimentation with SNFs from 2 ml cultures (Section 3.5.4) had yielded very low signals. Four SNFs from each construct were then tested in sandwich ELISA to detect IgY-C_H regions. The SNF's signals were not much higher than background (Fig. 3.55).

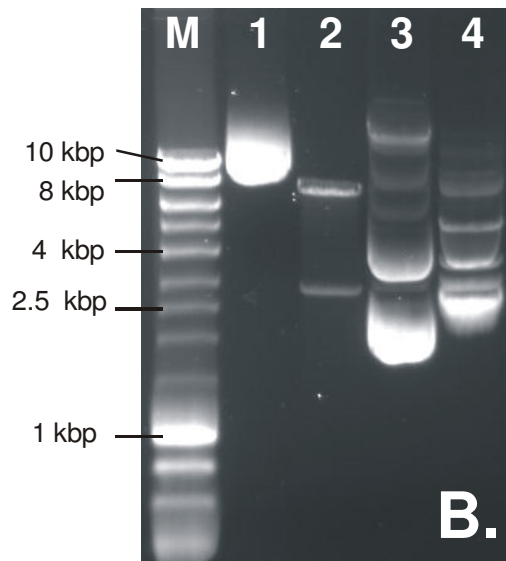
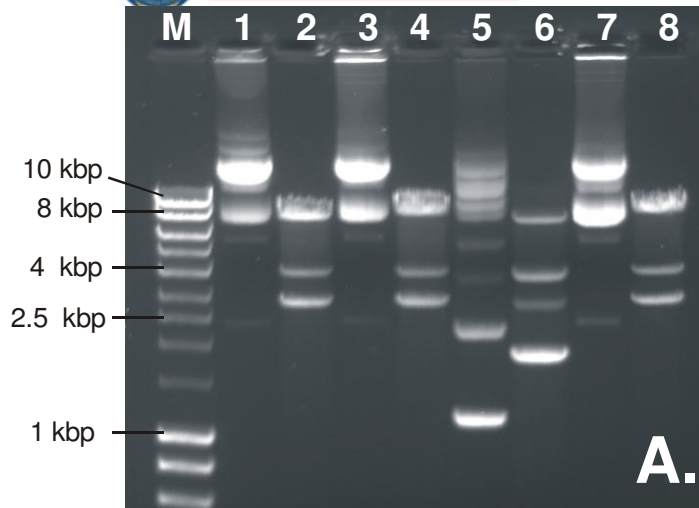


Figure 3.54 *SacII* digestion of pKLAC1 scFv- C_H region constructs for transfection into *K. lactis* cells. M denotes the DNA ladder and the relative fragment sizes are indicated for both agarose gels. Constructs for scFv-IgY expression are depicted in A. Lane 1: undigested pKLAC1-E1 $C_{H2-3}(3)$; Lane 2: linearised pKLAC1-E1 $C_{H2-3}(3)$; Lane 3: undigested pKLAC1-E1 $C_{H2-4mut}(2)$; Lane 4: linearised pKLAC1-E1 $C_{H2-4mut}(2)$; Lane 5: undigested pKLAC1-G8 $C_{H2-3}(1)$; Lane 6: linearised pKLAC1-G8 $C_{H2-3}(1)$; Lane 7: undigested pKLAC1-G8 $C_{H2-4mut}(1)$; Lane 8: linearised pKLAC1-G8 $C_{H2-4mut}(1)$. After *SacII* digestion each plasmid yielded two fragments: one larger than 6 kbp and one of approximately 2.8 kbp, as expected. A third fragment of approximately 4 kbp is the pREP4 plasmid inherent to the M15 *E. coli* cells in which the pKLAC1- C_H constructs were propagated. The construct in lane 5 migrated differently, but the digestion pattern is similar to the other constructs. Control plasmids were loaded on gel B. Lane 1: undigested pMalE; Lane 2: linearised pMalE; Lane 3: undigested pUC19; Lane 4: pUC19 remains circular after *SacII* digestion, as it contains no such enzyme site. Equal amounts of pUC19 were not loaded in lanes 3 and 4, which may explain the difference in migration between these two samples.

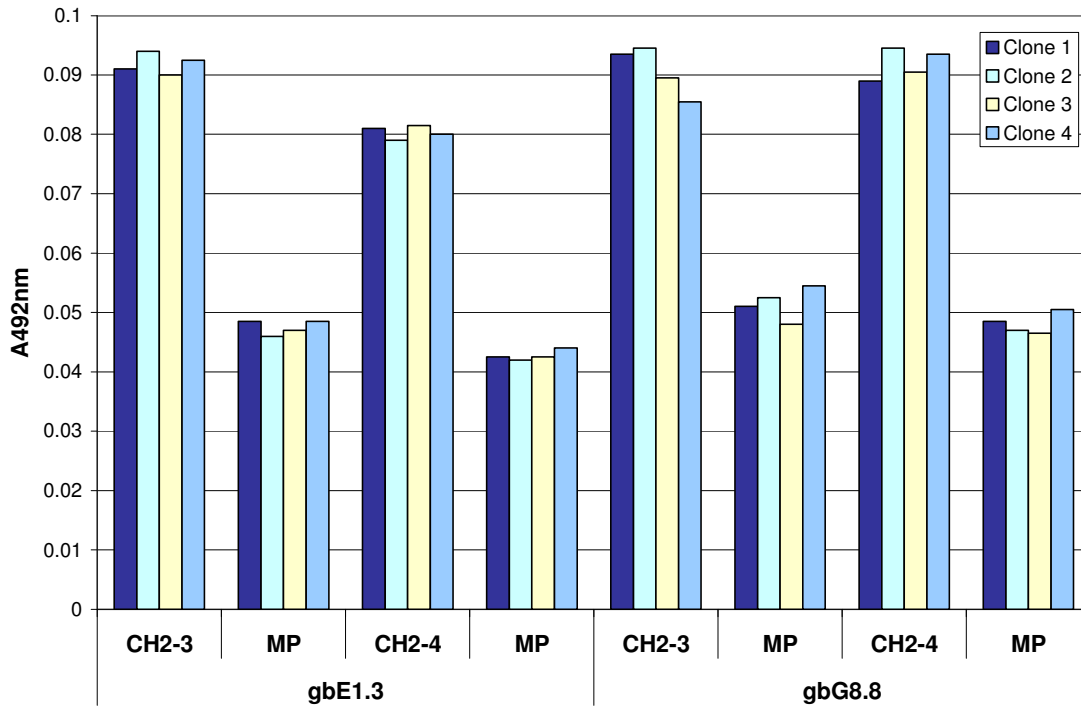


Figure 3.55 Sandwich ELISA to detect expression of various scFv-IgY genes in *K. lactis*. IgY-derived domains expressed into the SNF were trapped with polyclonal anti-IgY Ab, and detected with HRP-conjugated anti-IgY (Fc-region) Ab. MP controls were not coated with anti-IgY trapping Ab. As these background signals were high, no conclusive information could be gathered from this experiment.

As a final evaluation, the same clones were subjected to colony PCR to detect integration of the scFv-C_H genes (Section 2.21.3). Cultures of the pMalE and pUC19 control plasmids were also evaluated. Yeast genomic DNA was extracted from 500 µl of culture after lyticase digestion. PCR using Integration Primers 1 and 2 did not yield the expected amplicons for any of the samples (not shown), indicating that no *SacII*-linearised inserts had integrated. Attempts to express gallibody Abs in this system were therefore terminated.

3.6 Gallibodies as immunodiagnostic reagents

The merits and shortfalls of recombinant scFvs as immunoreagents were discussed previously (Section 3.2). To reiterate, it has been observed that (i) not all scFvs can be immobilised or remain functional after immobilisation on solid surfaces and (ii) not all scFvs couple to gold in a ‘universal’ fashion. This potential for underperformance was a key motivation for reconstituting scFvs to produce recombinant gallibodies.

An Ab that is to be used for immunocapture needs to bind, and consequently immobilise or capture, molecules out of solution when it is adsorbed to a solid phase such as plastic or nitrocellulose. This attribute forms the basis of many immunodiagnostic tests. In ICTs for instance, immunocapture Abs are adsorbed onto nitrocellulose and used to trap the diagnostic target out of suspension. It has long been known that Abs and Ab fragments may lose functionality when randomly adsorbed to solid supports (Butler *et al.*, 1992; Lu *et al.*, 1996; Butler *et al.*, 1997; Torrance *et al.*, 2006). Many have sought solutions using orientated or covalent coupling strategies that modify either the Ab or the support surface (Jönsson *et al.*, 1985; Matson & Little, 1988; Orthner *et al.*, 1991; Lu *et al.*, 1996; Torrance *et al.*, 2006). In this study it was hypothesised (Section 3.2) that an IgY-C_H ‘scaffold’ for the scFvs may decrease molecular distortion resulting from adsorption to solid surfaces.

ICTs based on colloidal gold require very stable Abs. As gold-adsorption occurs through specific interactions with amino acid residues such as Lys, Trp or Cys (Chandler *et al.*, 2000), these residues should preferably be located in the Fc region of full-length Abs to ensure that binding is retained after conjugation (Robinson, 2002). Cys and Trp are known to be located in conserved positions within IgY (Parvari *et al.*, 1988) and other immunoglobulin sequences (Fig. 3.56). Vision Biotechnology (Pty) Ltd. has reported that while some scFvs are easily coupled to gold, not all perform equally well (personal communication, Mr. N. Borain and Mr A. Bohms). Others have noted that small proteins do not couple well to colloidal gold and have turned to covalent conjugation and orientation techniques, similar to those described for immunocapture (Hainfield & Powell, 2000).

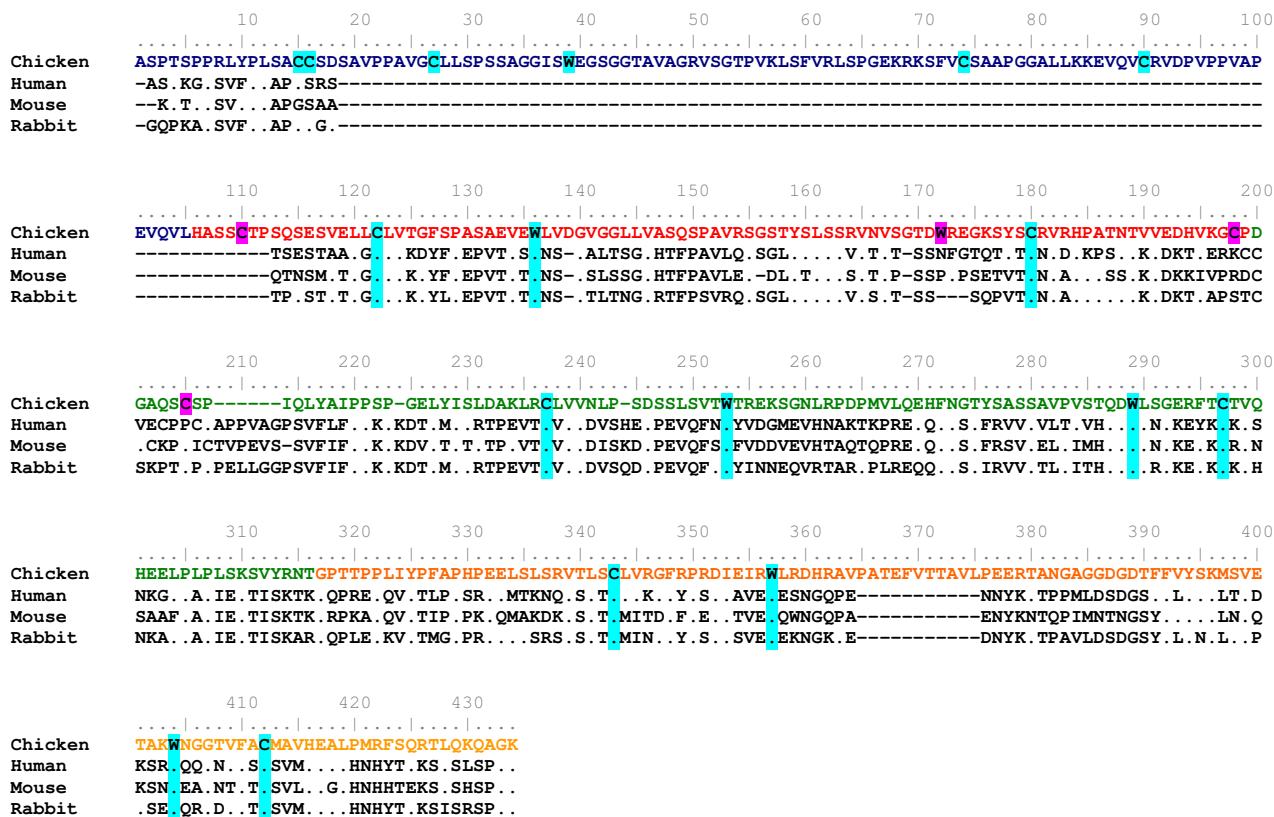


Figure 3.56 Position of Cys (C) and Trp (W) throughout IgY C_H-regions in comparison to the C_H-regions of other IgGs. All sequences were aligned with CLUSTAL W using default parameters (Thompson *et al.*, 1994). Chicken-IgY domains are coloured: domain 1 (blue), domain 2 (red), domain 3 (green) and domain 4 (orange). Conserved C and W residues identified in IgY are highlighted in blue (Parvari *et al.*, 1988), as are those which are similarly conserved in aligned mammalian Igs. Non-conserved C and W residues in the chicken Ig sequence are highlighted in magenta. Dots (.) indicate residues that are identical to the chicken IgY sequence. Dashes (-) indicate gaps in the alignments. Ig sequences were from GenBank (Benson *et al.*, 2000). Accession numbers are: chicken, S00390 (*Gallus gallus* Ig γ -chain, clone 36 fragment); human, CAC12842 (*Homo sapiens* IgG H-chain); mouse, AAB59656 (*Mus musculus* Ig γ -chain); rabbit, (*Oryctolagus cuniculus* Ig γ -chain C-region).

3.6.1 Immunocapture with gallibodies

It was previously established that the scFvs E1 and G8 could not be used for antigen capture when adsorbed to polystyrene microwells (Section 3.1.1.4), however, ELISA signals were greatly improved when the same experiment was repeated using purified gallibody constructs immobilised to the microwell surface (Fig. 3.57). In this experiment recombinant HSP65 antigen (10 µg/ml) trapped with purified *gbE1.3* or *gbG8.8* (10 µg/ml) was detected using polyclonal anti-*M. bovis* Ab followed by HRP-conjugated anti-rabbit Ab. Added C_H domains did not, however, improve the scFvs ability to capture HSP65 when adsorbed to nitrocellulose or PVDF (Section 3.1.1.4). This was revealed in spot-blot experiments (2 µl) using the two gallibodies (10 µg/ml). No captured HSP65 (10 µg/ml) could be detected with anti-*M. bovis* polyclonal and anti-rabbit-HRP Abs (blot not shown).

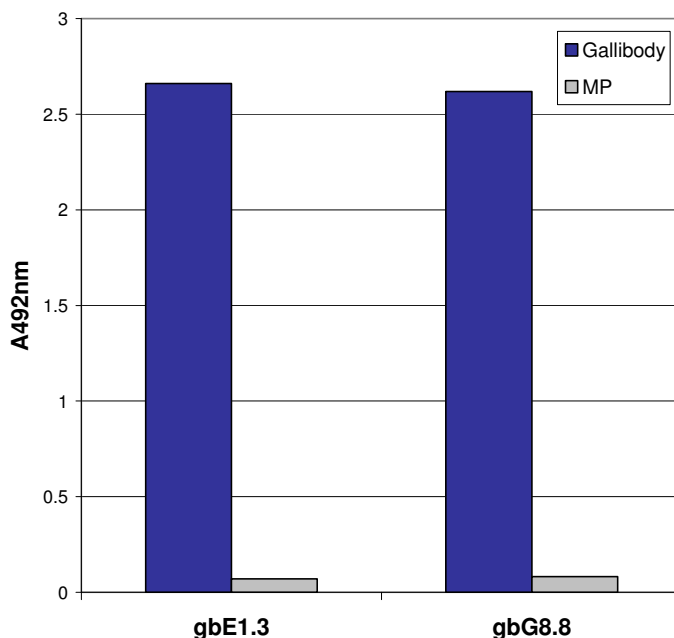


Figure 3.57 Sandwich ELISA to evaluate purified *gbE1.3* and *gbG8.8* (10 µg/ml) as immunocapture reagents when immobilised to a plastic surface. MP is a negative control, where HSP65 antigen was replaced with 2 % (w/v) MPPBS. Antigen (10 µg/ml) captured by each gallibody was detected with polyclonal anti-*M. bovis* Ab (1 in 1000 dilution in 2 % (w/v) MPPBS).

3.6.2 Temperature and denaturation stability

Previously, scFv molecules were evaluated for stability at varying temperatures and in the presence of denaturants (Section 3.1.1). Their IgY-like counterparts were subjected to a similar assessment. Gallibodies in both crude and purified forms had already proved their resilience to freezing and thawing during their day-to-day use in the laboratory (results not shown).

3.6.2.1 Temperature stability

Thermostability experiments also showed very little loss of functionality. Even after overnight incubation at 50 °C the gallibodies still produced signals above 1.5 in ELISA (Fig. 3.58). Both Abs retained their ability to bind to recombinant HSP65. Similar results were obtained after storage for four weeks at RT, 4 °C, -20 °C or -70 °C (Fig. 3.59). These results are an improvement on the scFv formats, which showed decreases in signal after 47 °C incubation (Section 3.1.1.2) and larger relative decreases in signal after storage at RT and 4 °C (3.1.1.2). In fact, harvested SNFs of both gallibody constructs remained functional after being at -20 °C for one year. There was no significant loss in signal when tested in indirect ELISA (results not shown).

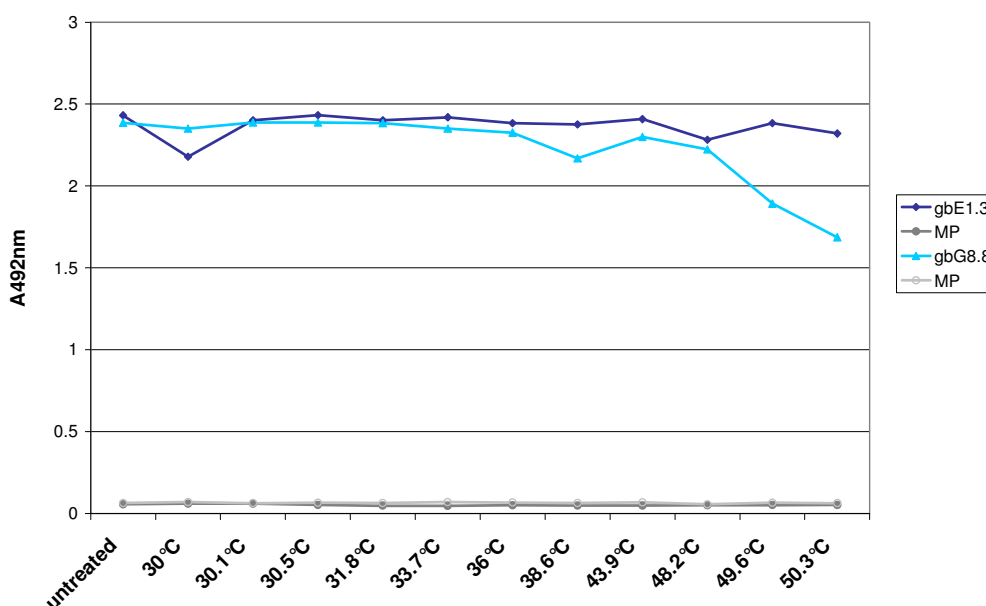


Figure 3.58 The effect of elevated temperatures on gallibodies *gbE1.3* (dark blue) and *gbG8.8* (light blue). Cell culture SNFs containing secreted gallibodies were incubated for 16 hours at various temperatures and thereafter evaluated in indirect ELISA. MP control wells were coated with 2 % (w/v) MPPBS instead of HSP65. The gallibodies were detected with HRP-conjugated rabbit anti-IgY(Fc) Ab (1 in 5000 in 2 % (w/v) MPPBS).

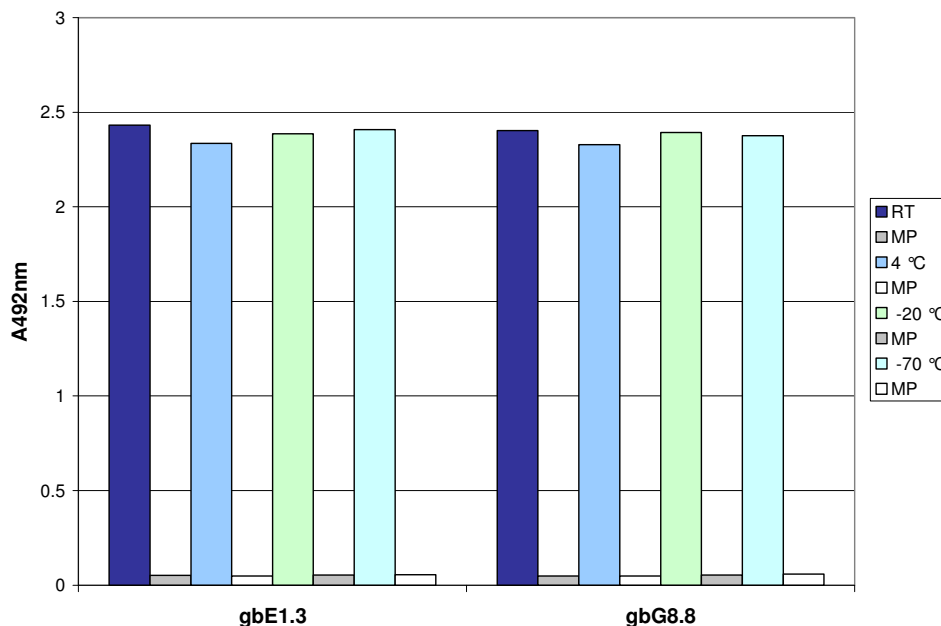


Figure 3.59 Indirect ELISA illustrating medium term storage of gallibodies in cell-culture SNFs. SNF aliquots of 1.5 ml were stored at different temperatures for four weeks. Tetracycline was added as a preservative at 15 mg/ml, and the tubes were protected from light. ELISA plates were coated with 10 µg/ml HSP65. MP controls were coated with MP instead of HSP65. The Abs were detected with anti-IgY (Fc region) Abs.

3.6.2.2 Refolding experiments

The denaturation experiments that had been performed previously with scFvs (Section 3.1.1.3) were repeated with harvested gallibody SNFs. The experiment was identical except that this time only the effect of 6 M GHI was investigated. Binding was evaluated by indirect ELISA. Results are shown in Fig. 3.60. As before (Section 3.1.1.3), no binding occurred in the presence of the chaotropic agent. Both gallibodies re-folded after GHI was removed by ultrafiltration. However, *gbE1.3* refolded more efficiently than *gbG8.8*, with a decrease of approximately 30 % in ELISA signal compared to that of roughly 60 % for *gbG8.8*. This is contrary to the scFv experiments in which E1 did not refold at all, and G8 regained at least 75 % of its original signal in ELISA (Section 3.1.1.3). Thus, adding the C_H domains to the scFvs did in fact appear to improve conformational stability after denaturation.

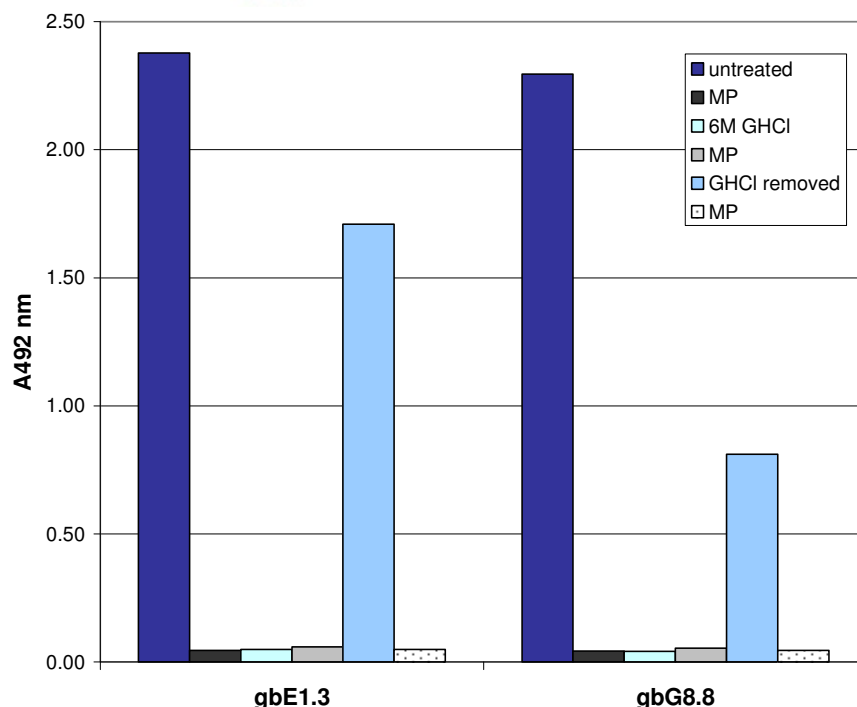


Figure 3.60 Indirect ELISA to investigate refolding after treatment with 6 M GHCl. MP is a negative control, where HSP65 antigen was replaced with 2 % (w/v) MPPBS. Gallibody Abs were detected with anti-IgY (Fc region) Abs.

3.6.3 Gold-conjugated ICTs using scFvs and scFv-IgYs

3.6.3.1 Salt-protection assays for Ab-gold conjugation

Salt protection assays determine the pH and the concentration of bound protein at which a gold colloid is protected from collapse due to ionic fluctuation (Geoghegan & Ackerman, 1977). The pH values of six aliquots of 5 mM Borate buffer and 40 nm gold colloid were each adjusted in 0.5 unit increments starting at pH 6. Immunoaffinity purified E1 and G8 scFvs (Ms Janine Frischmuth, National Bioproducts Institute) and Ni-NTA purified (Section 2.23.1) *gbG8.8* and *gbE1.3* gallibodies were diluted to 50 and 100 μ g/ml in PBS and added to microwells with borate buffer to yield the concentrations outlined in Table 3.2. This was repeated for each pH value in the range. HSP65 antigen was treated the same except that it was diluted to 15 μ g/ml stock as only limited quantities were available (Table 3.2).

Table 3.2 Dilution series of scFvs, gallibodies and HSP65 antigen for microwell-format salt-protection assay.

[HSP65 working stock]	15 µg/ml				
HSP65 working stock (µl)	5	10	15	20	25
5 mM Borate buffer (µl)	20	15	10	5	0
[Final HSP65] µg/ml	0.08	0.15	0.23	0.30	0.38

[Ab working stock]	50 µg/ml					100 µg/ml
Ab working stock (µl)	5	10	15	20	25	25
5 mM Borate buffer (µl)	20	15	10	5	0	0
[Final Ab] µg/ml	0.25	0.50	0.75	1.00	1.25	2.50

Gold colloid at the corresponding pH was added to each well, followed by 250 µl 2 M NaCl. The protection point of each well was measured at A_{525} , using the absorbance of unconjugated gold-colloid as the standard. Wells with A_{525} readings closest to the standard were considered optimal. The scFv designated E1 did not protect well across the range and the colloid destabilised at lower concentrations throughout the pH series. The optimal conditions for this Ab were determined to be 1.25 µg/ml at pH 9 (Fig. 3.61). G8 was very poorly protected with the colloid collapsing at low pH and Ab concentration (Fig. 3.61). A concentration of 1.25 µg/ml at pH 8.5 provided the closest possible A_{525} reading to that of unconjugated gold, but was not optimal (Fig. 3.61). The E1.3 gallibody conjugate was well-protected throughout the range and was optimal at 1.25 g/ml at pH6.5 (Fig. 3.61). Gallibody G8.8 was not as well protected with the colloid collapsing at low concentration (purple wells, Fig. 3.61). However, the conjugation was stable at 2.5 g/ml at pH 6.5 (Fig. 3.61). A repeat of the protection-assay with *gb*G8.8 at higher concentrations did not yield significantly different results (not shown). HSP65 itself was poorly protected, but it is assumed that this is due to the low protein concentration used. Optimum conjugation in this instance was determined at 0.38 g/ml at pH 7.5 (Fig. 3.61).

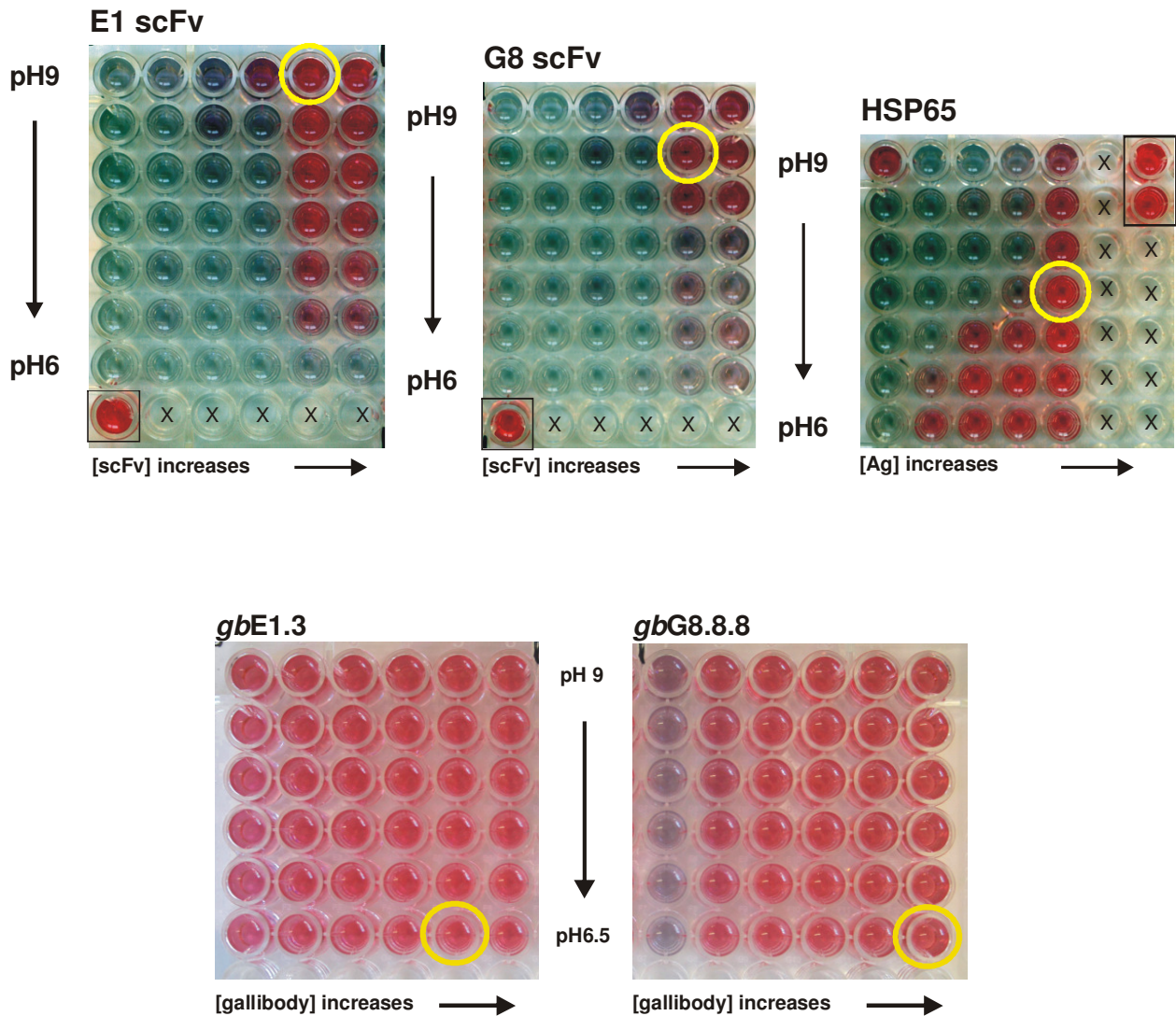


Figure 3.61 Salt-protection assay using scFvs, recombinant HSP65 antigen (top) and gallibodies (bottom). All wells were read at A_{525} , blanked on 5 mM borate buffer. Unconjugated 40 nm colloidal gold served as the standard (blocked, shown for scFvs and antigen). Wells circled in yellow indicate the best concentration and pH combination for conjugating the particular molecule to gold particles. These wells had A_{525} values closest to the standard.

3.6.3.2 Conjugating recombinant anti-HSP65 Abs to 40 nm colloidal gold

Antigen and Abs were conjugated to 40nm gold colloid (Section 2.22.2) under the conditions determined above. The conjugated pellets of each were resuspended to produce an $OD_{525} = 30$. Different volumes of each were spotted onto the glass-fibre sample-pad of a nitrocellulose strip and allowed to dry at RT. Recombinant HSP65 antigen, purified scFvs and purified gallibodies at 10 $\mu\text{g/ml}$ had previously been adsorbed to nitrocellulose strips (prepared by Mr. A. Bohms, Vision Biotechnology) for use in a variety of assay configurations (Fig. 3.62). These were tested on three different types of proprietary membranes, designated A, B and C (supplied by Vision Biotechnology (Pty) Ltd.). Fifty-microlitres of running buffer was added to the sample pad and allowed to migrate up the nitrocellulose, taking the gold-conjugate with it. Up to 100 μl was used if necessary. Where sandwich assays were attempted, HSP65 antigen was included in the running buffer at 10 $\mu\text{g/ml}$. The ability of gallibody Abs to directly detect immobilised HSP65 was also evaluated on a separate occasion (Fig. 3.64). The membrane supplied by Vision Biotechnology (Pty) Ltd. for this experiment was from an unspecified proprietary source.

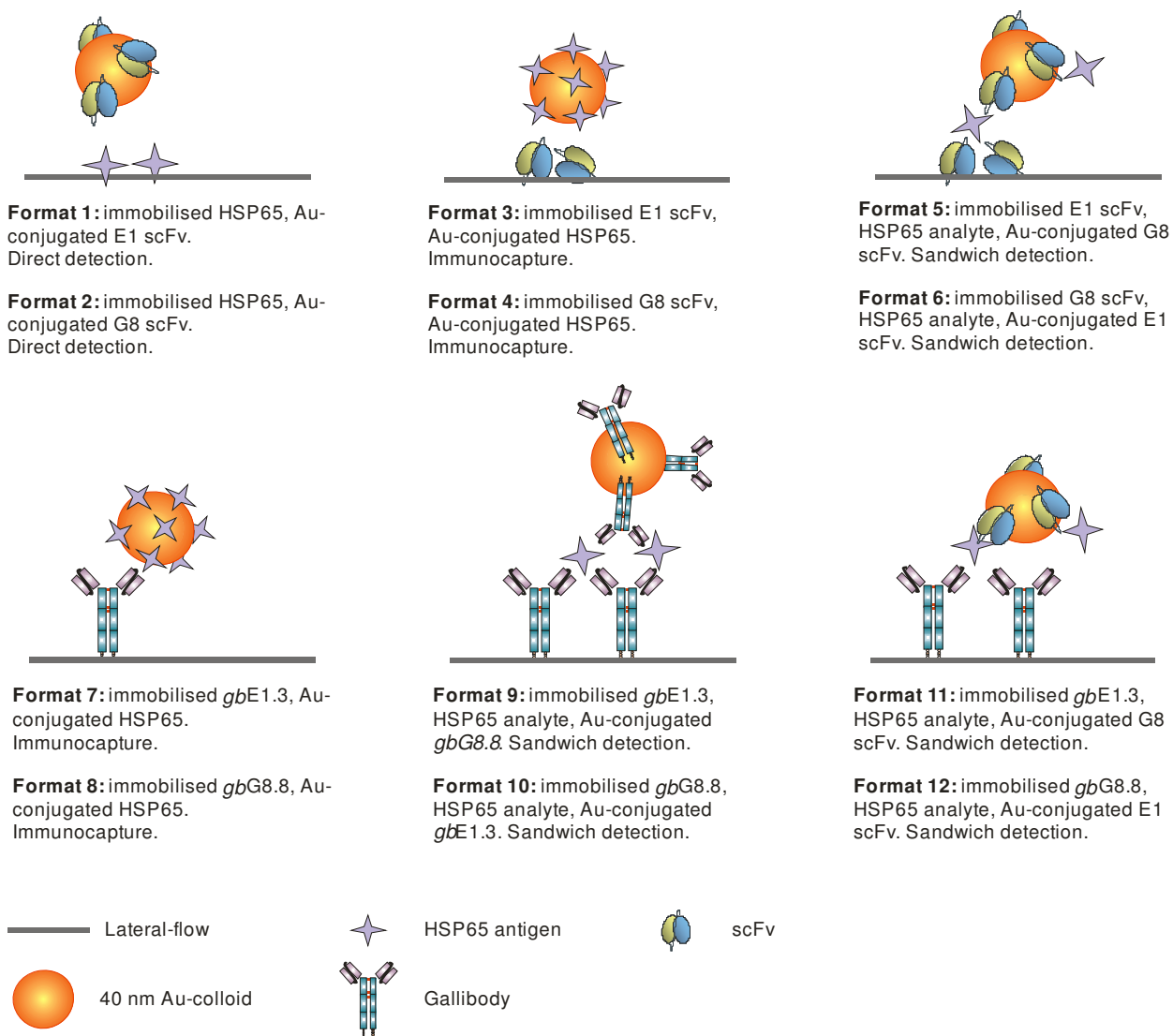


Figure 3.62 Gold-conjugated (Au-conjugated) lateral-flow assay configurations tested using recombinant anti-HSP65 Abs and various nitrocellulose membranes.

The results of ICTs in the various formats are given in Table 3.3. Three assays are pictured to illustrate the signal obtained (Fig 3.63). Membrane C was not optimal for detecting HSP65 in any of the tested configurations. Generally, but not without exception (assay 10), membranes A and B gave similar results. Interestingly, the E1 scFv and E1.3 gallibody appeared to be capable of immunocapture in gold-conjugated ICT format (Formats 3, 5, 7, and 11), contrary to previous experiments (Sections 3.1.1.4 and 3.6.1). The Abs could recognise HSP65 as a pair, where one Ab captures and the other detects the antigen, but only if E1 scFv was used for capture and G8 scFv for detection (Format 5). These results were confirmed using *gbE1.3* and G8 scFv (Format 11). Positive results could not be obtained using the G8 scFv or G8.8 gallibody for capture.

Table 3.3 The detection of HSP65 antigen in lateral-flow format using a variety of configurations. Positive (+), strongly positive (++) , negative (-) and inconclusive (?) results are indicated for each arrangement. Membranes that were not tested for a particular assay are indicated. All assays were performed with 10 μ l of gold-conjugate, except for assay 10(*) where 20 μ l gave a positive result.

Format	Membranes		
	A	B	C
1	+	+	-
2	+	+	-
3	+	+	-
4	-	-	-
5	++	++	-
6	-	-	-
7	+	+	-
8	+	+	-
9	-	-	-
10*	+	-	Not done
11	+	+	-
12	?	?	-

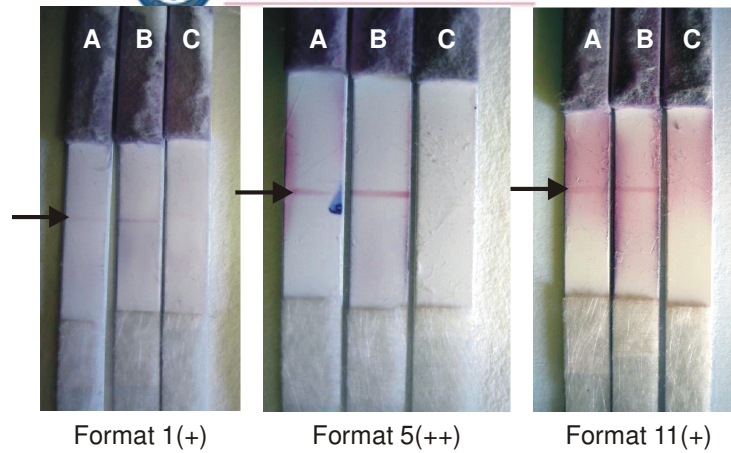


Figure 3.63 Signal strength in various ICT configurations detecting HSP65 antigen. Positive (+) and strongly positive (++) reactions are illustrated. The arrows indicate the position of the bands. The three membranes tested are labelled A, B and C. Membrane C results were negative in all three examples.

Gold-conjugated gallibodies tested separately (Fig. 3.64) produced a positive reaction in the form of a red line on the corresponding strip (Fig. 3.65). A 5 to 10 μl volume of *gbE1.3* conjugate was sufficient to produce a clear, positive signal (numbers 3 and 4 on LHS, Fig. 3.65). However, an aliquot of 25 μl was required for a clear signal using the *gbG8.8* conjugate (number 4 RHS, Fig. 3.65).

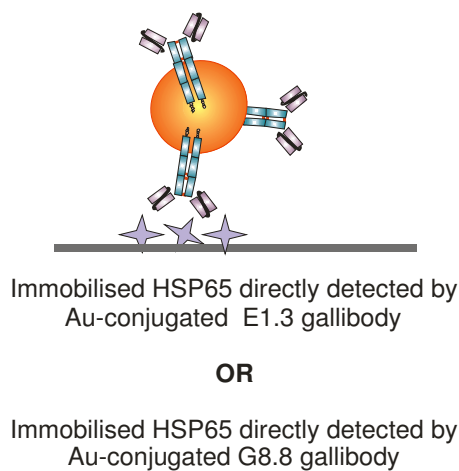


Figure 3.64 Evaluating gallibody Abs in gold-conjugated ICTs.

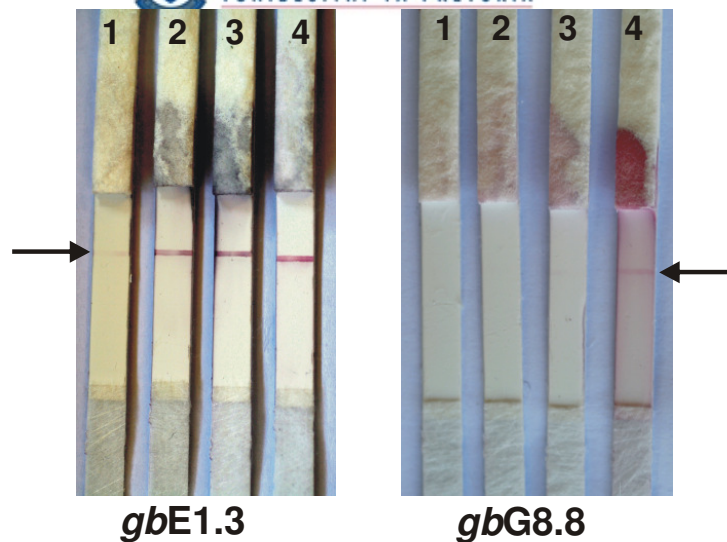


Figure 3.65 Recombinant HSP65 antigen is recognised by gallibody Abs E1.3 (left) and G8.8 (right). The arrows indicate the position of the bands. The E1.3 gallibody was tested using 1 μ l (strip 1), 5 μ l (strip 2) and 10 μ l (strips 3 and 4) of conjugate. Strip 3 was run in buffer made in the lab and strip 4 used running buffer supplied with a test kit that was used as a control. Gallibody G8.8 produced a clear signal when 25 μ l (strip 4) of conjugate was used, but not with 1 μ l (device 1), 5 μ l (device 2) or 10 μ l (device 3).

An ICT for Rift Valley Fever (RVF), also developed by Vision Biotechnology and the OVI, was used as a control to exclude non-specific binding of the Ab variable regions. In this test, gold particles on the sample pad are tagged with both RVF-antigen and IgY. Sample serum containing RVF-specific Abs will bind this conjugate, as well as a second RVF-antigen immobilised on the strip ('stripe 1'). As a control, anti-IgY is adsorbed further up on the nitrocellulose ('stripe 2'). An unused test has one blue line (number 4, Fig. 3.66). A negative test has one red line where anti-IgY traps gold-conjugated IgY out of solution. A RVF-positive sample would show two parallel lines (not shown). Control tests were allowed to run with buffer only (number 3, Fig. 3.66), as well as with gallibody Ab in running buffer (numbers 1 and 2, Fig. 3.66). There was no non-specific binding and the tests were negative as would be expected in the absence of anti-RVF Abs.



Figure 3.66 An ICT for RVF was used as a negative control and is described in the text. Device 4 is an unused test. Only running buffer was added to device 3 (negative reaction). Gallibodies were diluted to their conjugation concentrations in running buffer and added as sample to device 1 (*gbE1.3*) and device 2 (*gbG8.8*).

3.7 Mapping the epitopes of E1 and G8

Sandwich-format ICTs using E1 and G8 indicated that the V-regions of these two Abs might conceivably bind to different epitopes on HSP65 (Section 3.6.3.2). In lateral-flow tests, the two Abs were able to bind to their cognate antigen provided that E1 (whether in the scFv or gallibody format) was used as the capture antibody and G8 was used as an scFv for detection. Two different approaches were applied in order to investigate this further. The first was an attempt to reproduce the sandwich ICT findings (Section 3.6.3.2) in an ELISA *i.e.* to confirm that both Abs could bind HSP65 at the same time. Secondly, phage-display and computer-aided analysis were combined in an attempt to locate the possible regions on the protein that were recognised by each of the Abs.

3.7.1 Cross-capture sandwich ELISA

Single-chain Fvs converted to the gallibody format could be used for immunocapture in ELISA (Section 3.6.1). Accordingly, wells were coated with either *gbE1.3* or *gbG8.8* at 10 µg/ml, after which E1 and G8 scFvs which had been pre-incubated with antigen and 9E10 mAb were added (*gbE1.3* detected by G8 scFv and *vice versa*). The rationale was that by allowing the scFv to complex with the antigen first, only certain regions would be left accessible for the gallibody to bind to. If the paratopes of E1 and G8 recognised the same epitope or epitopes in close proximity to one another, no binding site would be available to capture the Ab-antigen complex and it would not be detected in ELISA. HSP65 antigen trapped by gallibody was included as a positive control while the negative controls contained scFv and Ab ‘premix’ without antigen. Very low signals were produced by both of the Ab pairs, indicating in each case that the scFv-antigen complexes could not be bound by the corresponding gallibodies (Fig 3.67).

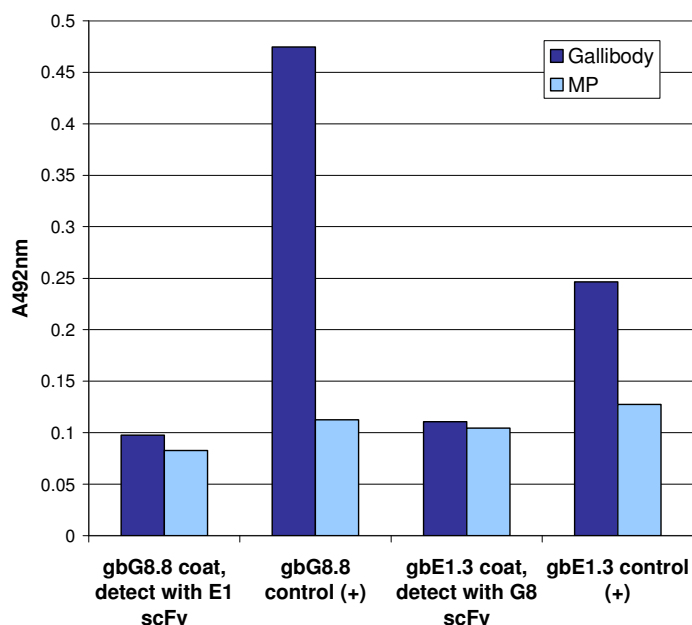


Figure 3.67 Testing antibody pairs in sandwich ELISA. Wells were coated with gallibody formats. Trapped antigen was detected using the alternate scFv. This in turn was detected using anti-c-myc mAb (9E10) and HRP-conjugated anti-mouse Ab (1 in 1000 dilution with 2 % (w/v) MPPBS). HSP65 (10 µg/ml) trapped with gallibody and detected with anti-*M. bovis* Ab (1 in 1000 dilution in 2 % (w/v) MPPBS) was included as a positive control (+). MP controls contained scFv and 9E10 mAb but no antigen.

3.7.2 Isolating peptide mimotopes by panning the XCX₁₅ library

In an attempt to physically locate the sites recognised by E1 and G8, the XCX₁₅ phage-displayed library (Bonnycastle *et al.*, 1996) was panned against both gallibodies. Four rounds of panning were carried out in immunotubes coated with gallibodies at a concentration of 10 µg/ml for rounds one to three. In round four the concentration was reduced to 5 µg/ml. Twenty clones were picked from the final two rounds of each panning and their binding to gallibodies was tested in phage-capture ELISA (Section 2.23, Fig. 3.68 and Fig. 3.69). SNF from an overnight culture of the filamentous phage-display vector fd-tet 8.53 in DH5α *E. coli* was used as a negative control. All but five of the 20 phage clones from the panning using *gbE1.3* were conclusively recognised in the immunoassay (Fig. 3.68). In contrast, none of the clones selected from the *gbG8.8* panning produced convincing signals (Fig. 3.69).

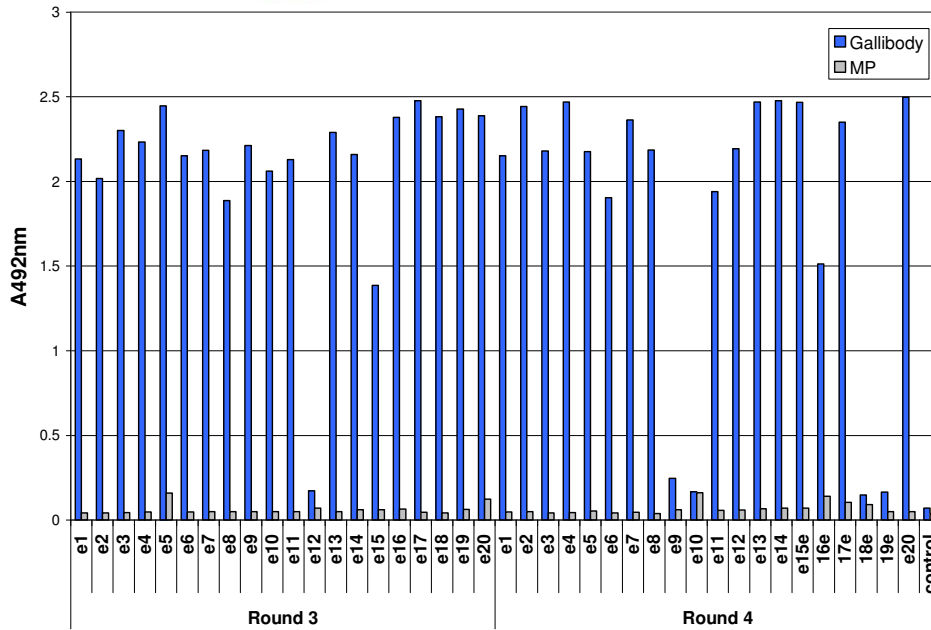


Figure 3.68 Phage-capture ELISA of clones selected from XCX₁₅ panning on *gbE1.3*. MP is a control where wells were not coated with gallibody. Clones were analysed in duplicate.

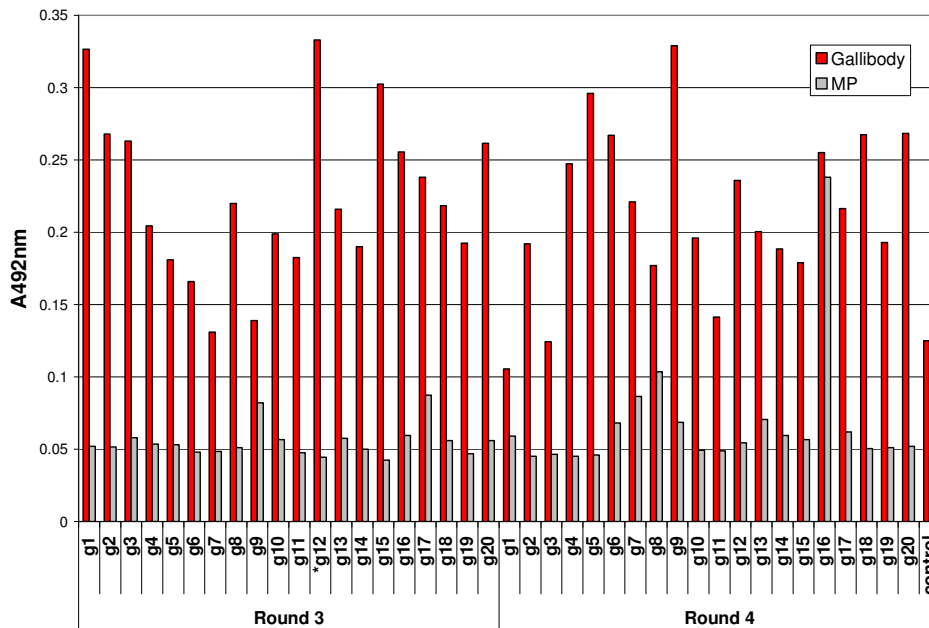


Figure 3.69 Phage-capture ELISA of clones selected from XCX₁₅ panning on *gbG8.8*. MP is a control where wells were not coated with gallibody. Clones were analysed in duplicate. The asterisk (*) denotes a reading based on one well only, due to an artefact.

To identify the actual amino acid sequences that were recognised, the RF DNA was extracted and the inserts were sequenced. Some of the non-binding clones were also sequenced for comparison. When *gbE1.3* was used for panning in-frame sequences were obtained for 15 clones from round 3 and 15 clones from round 4 (Table 3.4, overleaf). Certain peptides were found more frequently than others in both rounds. Panning on *gbG8.8* yielded 14 sequences from round 3 and 12 from round 4 (Table 3.4). In contrast to the situation with E1, there was little consensus between the peptides selected by this Ab. All of the non-binding sequences from both pannings conformed to the XCX₁₅ motif with its artificially introduced cysteine (Cys) at position 2 (indicated with asterisks, Table 3.4). Surprisingly, peptides with an amino acid other than Cys at position 2 were isolated from the XCX₁₅ library. These particular sequences were obtained for more than one clone (E1-b and G8-a, Table 3.4) and bound in phage-capture ELISA (not shown).

Table 3.4 Peptide sequences of clones selected from the third and fourth rounds of XCX₁₅ panning on *gbE1.3* and *gbG8.8*. The peptides are listed in decreasing frequency for each Ab. Peptides which did not bind in phage-capture ELISA are indicated by an asterisk (*). Cys (C) residues that have the potential to pair with fixed Cys residues are outlined in black.

Peptide	Sequence	Frequency		
		Round 3	Round 4	Total
E1 Panning				
E1-a	ECSEHCWSRQSKLATN	10	2	12
E1-b	DYNSKDIKWQPQLSQVG	2	4	6
E1-c	YCANNTLWDRPHDQTN	0	4	4
E1-d	ICRHRLQNAQYPRRKWV	0	2	2
E1-e*	KCMLPHYHRR TQGTSCGG	1	1	2
E1-f	HCEHSN CNLLQAHS LPY	1	0	1
E1-g*	DCPMSHE YCSAAFFS QY	1	0	1
E1-h	ECSEHCWWRQSKLATN	0	1	1
E1-i	HCEHRGCN LHEHRDM TT	0	1	1
E1-j	PCKRRECRVQ NQNA STG	0	1	1
G8 Panning				
G8-a	NSPRRSQKSRTLAKQH G	3	1	4
G8-b	MCSLGTSREKPAQSKRT	1	1	2
G8-c	ICRHRLQNAQYPRRKWV	2	0	2
G8-d	LCTTFQSHYRFKPSQHA	1	0	1
G8-e	RCQM QNSSYRTTQLPGT	1	0	1
G8-f	PCKSMQFRVQIPNTGPV	1	0	1
G8-g	MCQSEPC P QPSTWMAVS	1	0	1
G8-h	TCTCTQYYRPLSFGHSK	1	0	1
G8-i	LCGHFPLQFQQSTWNKP	1	0	1
G8-j	ACNHLYRSKIFQPAPLD	1	0	1
G8-k	DCNQTSFRRPPDRNEN	1	0	1
G8-l*	LCQSAVLEVSAA YLLLP	0	1	1
G8-m	QCTTNEQWGRSSQQPSY	0	1	1
G8-n	QCTPLEDNVMRIKSMES	0	1	1
G8-o	NCNSNCGLD TSTHVSQR	0	1	1
G8-p	QCQAQLMRSQDMSQMRP	0	1	1
G8-q	LCFWAQINMERAPQVHI	0	1	1
G8-r	KCMAQEKRFNTARRESP	0	1	1
G8-s*	HCKTKRSGLRRK LQTHD	0	1	1
G8-t	QC PHPSLQSPYTKLDEY	0	1	1
G8-u	NCTHSRSAKTPHRRPGD	0	1	1
<div style="display: flex; justify-content: space-around; margin-top: 10px;"> <div style="background-color: #ADD8E6; padding: 5px; border: 1px solid black;"> positively charged, hydrophilic negatively charged, hydrophilic </div> <div style="background-color: #FFFF00; padding: 5px; border: 1px solid black;"> polar side chain, hydrophilic non-polar side chain, hydrophobic </div> </div>				

3.7.3 Mapping epitopes using peptide mimics derived from a phage-displayed library

3.7.3.1 Mapping the epitope of E1

All the peptides that had been selected by E1 from the XCX₁₅ library (Table 3.4) were aligned to the HSP65 amino acid sequence using the programme LALIGN (Myers & Miller, 1989). Local alignments using default parameters did not, however, yield any results. Using a global alignment without gaps and with default parameters revealed that most of the E1 peptides corresponded with the region covered by residues 356 to 370 (RHS, Fig. 3.70). Residues with similar side-chains were found in conserved positions. This similarity was based upon similar side-chain or charge properties and positions were considered conserved only if peptides participating in the overlap contained a similar residue at that particular position.

When the first 14 mer of each peptide was analysed using the PepSurf algorithm (Mayrose *et al.*, 2007b), a programme which makes it possible to view where on the 3D structure individual sequences match best, some of the residues participating in Cluster 1 (calculated ‘best-fit’) mapped within and just N-terminal of the region that had been matched linearly *i.e.* residues 350 to 370 (Fig. 3.71). Peptides E1-a and E1-h were common to both the linear and 3D alignments. The sequences were also subjected to analysis with Mapitope (Bublil *et al.*, 2007), and a ‘combined’ algorithm. The former matches sequences to the 3D structure based on common attributes identified in a data set, whereas the latter calculates where the results of PepSurf and Mapitope intersect. As the Mapitope algorithm produced identical results for E1 and G8 peptides, this analysis was considered meaningless (not shown). Similarly, the ‘combined’ Pepitope analysis did not generate any useful results (not shown).

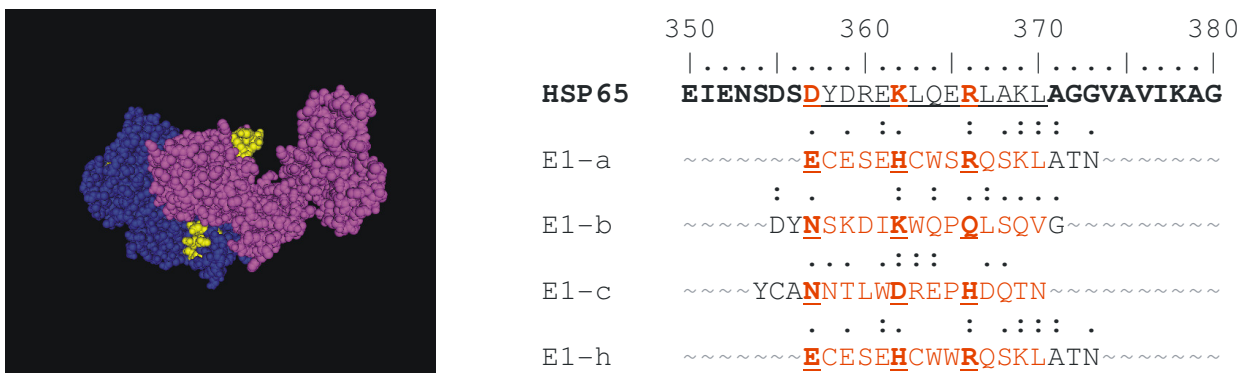


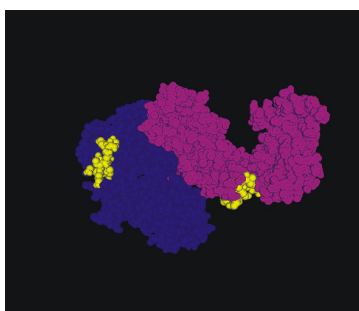
Figure 3.70 Linear alignments of E1 mimotopes to the HSP65 sequence. LALIGN was used to align each peptide to the protein sequence individually (Myers & Miller, 1989). The algorithm aligns amino acids generally *i.e.* charged/uncharged, polar/non-polar or acidic/basic. Identity (:) and similarity (.) of individual amino acids to HSP65 is specified. Most of the E1 peptides overlapped with the protein sequence in the region shown (RHS). Similar residues at conserved positions are highlighted, based on side-chain or charge properties (RHS; bold and underlined red text). The presumptive linear epitope (underlined on RHS) is mapped on the HSP65 molecule in yellow (LHS), to show that this area is exposed on the protein surface. The two HSP65 monomers are coloured pink and blue for differentiation and the epitope is mapped on each one. The illustration was generated using Cn3D (<http://www.ncbi.nlm.nih.gov>).

3.7.3.2 Mapping the epitope of G8

Linear alignments of peptides selected by G8 revealed three areas of correspondence with the target polypeptide. These represented residues 134 to 149, residues 433 to 474 and residues 512 to 522 (underlined HSP65 sequence, Fig. 3.72). A fourth presumptive overlap was observed which included residues 209 to 225. This one, however, was excluded from the analysis since the peptides used to identify it did not overlap convincingly with one another. Moreover, one of them did not bind in ELISA. In the case of the others though, the corresponding regions on the protein were mostly hydrophilic and exposed on the protein surface (LHS, Fig. 3.72). As before, similarity was determined by the side-chain or charge. The PepSurf results were difficult to interpret clearly (not shown) and as a result the presumptive G8 epitopes must be treated with caution.

3.7.4 Antigenic regions on HSP65

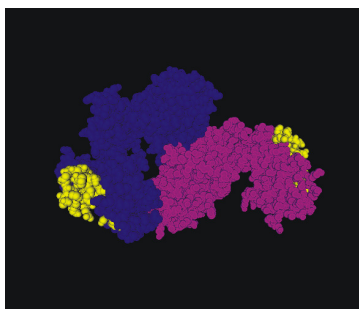
Notwithstanding difficulties in interpreting the computer-generated models shown above, the phage-display results with Ab E1 in particular indicate that residues 350 to 370 may include sites recognised by its paratope (Figs. 3.70 and 3.71). Peptides acquired from G8 panning showed evidence of possible linear matches in three regions, none of which corresponded to the putative epitope of E1. For comparison, Figure 3.73 shows the positions of the E1 presumptive epitope and the G8 linear matches relative to a number of other traditional mAbs' epitopes mapped by others using various techniques (Shinnick *et al.*, 1987; Hajeer *et al.*, 1992; Rambukkana *et al.*, 1992). The putative epitope recognised by E1 is just N-terminal of a previously predicted mAb recognition site spanning residues 375 to 383. The G8 linear segment spanning residues 134 to 149 overlaps with the published mAb epitope between residues 129 to 139. Similarly, the G8 linear segment spanning residues 512 to 522 overlaps with a known epitope composed of residues 497 to 520. Where they do not overlap with published epitopes, the three supposed G8 'epitopes' still align in close proximity to these regions (Fig. 3.73).



```

130           140           150
|...|...|...|...|...|
HSP65 LLKGAKEVETKEQIAATAAISAGDQS
      : . . : : .
G8-f  ~~~PCKSMQFRVQIPNTGPV~~~~~
      : . : . : .
G8-g  ~~~MCQSEPCOPSTWMAVS~~~~~

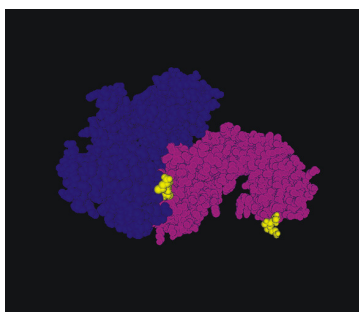
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430           440           450           460           470           480
|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
HSP65 EGDEATGANIVKVALEAPLKQIAFNSGLEPGVVAEKVRNLPAGHGLNAQTG
      : . . : : . : : : : :
G8-a  ~~~~~~NSPRRSQKSRTLAKQHG~~~~~
      : . . : : .
G8-e  ~~~~~~RCQMQNSSYRTTQLPGT~~~~~
      : . . : : .
G8-h  ~~~~~~TCTCTQYYRPLSFGHSK~~~~~
      : . : : :
G8-j  ~~~ACNHLYRSKIFQPAPLD~~~~~
      : . : : : :
G8-o  ~~~~NCNSNCGLDTSTHVSQR~~~~~

```



```

           510           520           530
...|...|...|...|...|...|...|
HSP65 LQNAASIAGLFLTTEAVVADKPEKEKASVP
      : . . : : . : .
G8-b  ~~~~~~MCSLGTSREKPAQSKRT~~~~~
      : : . : : .
G8-d  ~~~~~~LCTTFQSHYRFKPSQHA~~~~~
      : : : : . : :
G8-i  ~~~~~~LCGHFPLQFQQSTWNKP~~~~~

```

Figure 3.72 Linear alignments of G8 mimotopes to the HSP65 sequence. LALIGN was used to align each peptide to the protein sequence individually (Myers & Miller, 1989). The algorithm aligns amino acids generally *i.e.* charged/uncharged, polar/non-polar or acidic/basic. Identity (:) and similarity (.) of individual amino acids to HSP65 is specified. The G8 peptides overlapped in three regions (RHS). Similar residues at conserved positions were identified based on side-chain or charge properties (RHS, bold text). The presumptive linear epitopes (underlined, RHS) are mapped on the HSP65 molecule in yellow (LHS) to show that these regions are exposed on the protein surface. The two HSP65 monomers are coloured pink and blue for differentiation and the epitope is mapped on each one. The illustration was generated using Cn3D (<http://www.ncbi.nlm.nih.gov>).

N-terminus



- HSP65 protein
- Previously mapped mAb epitopes
- G8 alignments (linear)
- E1 presumptive epitope

Figure 3.73 Schematic representation of the HSP65 polypeptide sequence and the relative positions of epitopes predicted in this and previous studies. The epitopes numbered 2 and 6 are from data published by both Shimmick et al. (1987) and Hajeer et al. (1992). Epitopes 1 and 8 are from Shinnick et al. (1988) and 3 and 4 are from Hajeer et al. (1992). Epitopes 5 and 7 are from Rambukkana et al. (1992).

Chapter 4

DISCUSSION

Phage display can be used to generate robust immunoreagents with high affinity and specificity for use in diagnostics development. This technology enables Ab fragments such as scFvs to be produced rapidly and economically. Where specialised functions or characteristics are desired, scFvs can be improved by standard recombinant DNA methods. This is particularly useful when, for example, the size or other inherent properties of scFvs may limit their performance in certain assay formats. In this study, two chicken scFvs were ‘reconstituted’ back to divalent ‘almost-full-size’ Abs by adding three of the four IgY-derived H-chain constant domains (C_{H2-4}). The resulting ‘gallibodies’ could be immobilised by adsorption on solid substrates to capture their target antigen and in addition, were able to be more stably conjugated to colloidal-gold than their scFv counterparts.

4.1 Single-chain Fv antibodies isolated from the Nkuku® library

A number of high-affinity scFv antibodies directed against a variety of different classes of target molecules have been isolated from the *Nkuku*® repertoire constructed at the OVI (van Wyngaardt *et al.*, 2004). A database of the scFv sequences derived from this library is being compiled as more binders are found. Four scFvs designated E1, G8 (anti-HSP65 of *M. bovis*), B5 and C10 (anti-16 kDa of *M. tuberculosis*) isolated previously from the *Nkuku*® repertoire showed that this library contained potentially useful Abs against antigens of *Mycobacterium*. Two of these, E1 and G8, were tested for their temperature durability, freeze-thaw survivability and denaturant susceptibility (Section 3.1.1). Experiments such as these give a fair idea of an scFv’s stability (Wörn & Plückthun, 2001) and are thus a valuable indication of its suitability in immunoassays such as ICTs and ELISAs. For instance, its thermostability can directly indicate whether the performance of an Ab in an immunoassay will be compromised by the conditions that an assay may be exposed to prior to use. Moreover, medium to long-term storage experiments under various conditions can predict the ‘shelf-life’ that an immunoassay can be expected to have. Both the anti-HSP65 scFvs E1 and G8 maintained their affinity for antigen after exposure to

temperatures up to 47 °C (Section 3.1.1.2). They also withstood ten cycles of freezing and thawing (Section 3.1.1.1). B5 and C10 were included for comparison even though they were directed against a different antigen (16 kDa protein). With the exception of their performance in the freezing and thawing assessments, these two scFvs were not as stable as E1 and G8. Accordingly, B5 and C10 are probably not suitable for use in immunoassays without some sort of improvement. E1 and G8 are, by contrast, potentially good candidate Abs for assays that require robust reagents.

Antibodies often need to be purified for use in immunodiagnostics. Generally, this process involves conditions that could potentially destroy their functionality e.g. precipitation and elution at acidic pH. Additionally, there are upstream processing factors (e.g. addition of buffers to the sample) specific to every assay which could alter the sample environment (e.g. pH, ionic strength etc.). GHI is a strong denaturant and is therefore useful for determining the specific limitations of an Ab's structural stability. When all four scFvs were subjected to denaturation by GHI and refolding, only G8 regained its functionality, albeit with a small decrease in signal (Section 3.1.1.3). Although denaturant stability and thermostability are sometimes considered to be proportional (Wörn & Plückthun, 2001), E1 deviated from this postulate. This was shown by the experiments in which E1 demonstrated its thermostability by remaining functional after overnight incubation at 47 °C (Section 3.1.1.2), but was unable to regain its ability to bind the target antigen after chemical denaturation (Section 3.1.1.3). The loss of functionality was an effect of the denaturant on the paratope itself and not the epitope. This antigenic stability may be as a result of the ELISA format used: the antigen was first coated to the surface of an immunoplate well and then treated with GHI, so perhaps no major unfolding took place because the protein was stabilised by being adsorbed to plastic. In retrospect, experiments that included determining at what molarity of GHI the Ab could still remain functional would have been useful. Nevertheless, these experiments revealed that the two anti-HSP65 scFvs, E1 and G8, were quantitatively more stable than several others that had been isolated from the same library (Dr. J. Fehrsen & Mr. W. van Wyngaardt, personal communication).

As with any protein, the stability of each individual scFv is a function of its amino acid sequence. Comparing the primary structures of the four chicken scFvs revealed a number of variations in the framework regions (Fig. 3.7). In the case of mammalian scFvs, certain point mutations that

can supposedly contribute to their stability, affinity and avidity have been reported. For example, mutations in the V_H-domain of the Lys at position 66 to an Arg and at position 52 of an Asn to a Ser are apparently a stabilising influence (Wörn & Plückthun, 2001; Brockmann *et al.*, 2005). Having a Pro residue at position 8 in κ V_L-chains has also been associated with an Ab's stability (Spada *et al.*, 1998), while having Glu in place of wild-type Gln at position V_H6 in type IIA frameworks impacts negatively on the 'immunoglobulin fold' as well as expression levels in bacteria (de Haard *et al.*, 1998). The scFvs shown in Fig. 3.7 were numbered according to the Kabat system, using the scFvs of Aburatani *et al.*, (2002) as a guideline. As the chicken framework differs somewhat from human and murine frameworks, their numbering is relative to conserved motifs. All of the *Nkuku*® scFvs examined so far contain the Arg for Lys exchange at V_H66 (see Fig. 3.7). Since their stability varies greatly, this implies that this position has no influence in the context of chicken scFv frameworks. The situation is identical for the Pro at position V_L8 and the Glu in place of Gln at V_H6. Accordingly, this present study has shown that only the exchange of an Asn for a Ser at V_H52a correlates with the stability of chicken scFvs. An interesting observation is that E1 contains an Asn residue at V_H66. In comparison, G8 which was able to refold after chemical denaturation has a Ser residue at this position. In contrast, all other, less stable scFvs listed have a Gly or Asp at this position. That these findings have any direct relevance to their robustness remains to be proven. Firstly, the stability data gleaned from the 'stress-incubation' experiments are not comprehensive enough to provide evidence for more than broad assumptions. Furthermore, all other studies which report the effects of point mutations cite murine or human frameworks. Until a more comprehensive chicken Ab database becomes available, parallels between these and mammalian frameworks remain somewhat speculative. The *Nkuku*®-scFv database which is currently being compiled at the OVI may enable better comparisons between sequences and physical properties to be made in the future. The effect of point mutations on the performance of *Nkuku*®-derived scFvs has recently been investigated using random mutagenesis (Ms. J. Sixholo, publication in preparation) and data derived from such studies may help to shed further light on the factors which influence the stability of chicken scFvs.

4.2 The benefits of 'redesigning' scFvs as gallibodies

Two problems were faced when the two anti-HSP65 scFvs were used as immunoreagents. Firstly, they could not be used for immunocapture *i.e.* they stopped recognising the antigen when they

were adsorbed to the plastic surface of a microplate well. Neither E1 nor G8 could trap antigen out of solution when used as the capture Ab in ELISA (Section 3.1.1.4). Secondly, based on communication with staff at Vision Biotech (Pty) Ltd, a Cape Town-based company with extensive experience in gold-labelling of Abs, native scFvs couple inconsistently to gold with efficiencies that vary greatly between different scFvs. Reconstituting chicken-derived scFvs into Ab-like molecules ('gallibodies') did, however, improve the behaviour of scFvs in these immunoassays. A likely reason is that the IgY molecule provides an ideal 'scaffold' with its extra C_H domain and rigid structure. Another factor is that this scaffold converts monovalent scFvs into bivalent molecules, thereby increasing their functional affinity (Greunke *et al.*, 2006). Lastly, IgYs are inherently stable (Gasparyan, 2005; Nilsson & Larsson, 2007) and such a scaffold could conceivably improve the structural and thermal stability of the chicken scFvs E1 and G8, as has been shown with humanised scFvs (Quintero-Hernández *et al.*, 2007).

When molecules are adsorbed to a solid support, structural distortion usually occurs (Butler *et al.*, 1992). In Abs, where antigen binding depends on the 'immunoglobulin-fold', this can result in altered specificity or even a complete loss of function. Because of their small size, the effects of distortion are likely to be more pronounced in scFvs when compared to full-length Abs or other larger proteins. Immobilisation onto polystyrene (e.g. immunoplates) occurs principally through hydrophobic interactions (Butler *et al.*, 1992) and it is alleged that this causes greater distortion than adsorption to nitrocellulose (Shields *et al.*, 1991). This adsorption is random and the orientation of the immobilised Abs is not known. The experiments of Dávalos-Panjota *et al.* (2001) suggest, however, that IgY will adsorb to polystyrene microspheres via its more hydrophobic Fc region at pH 8 in a low ionic strength environment with the antigen-binding regions being orientated away from the solid support and hence accessible to the solvent. It seems likely therefore that the Fc domains of E1 and G8 could have adsorbed in the same way after being converted to gallibodies since in this format they indeed proved to be successful as immunocapture reagents (Section 3.6.1). Although the scFvs were originally selected using adsorbed, and therefore probably also distorted HSP65, both gallibodies were able to sequester the antigen out of solution. This finding implies that either very little distortion of HSP65 occurs during adsorption, or that only one subunit of the protein dimer adsorbs and gets distorted, leaving the other exposed to the solvent in its native configuration.

In addition to improving functionality after adsorption, the gallibody format also had an effect on structural and thermal stability. After overnight incubation at 50 °C, *gbE1.3* and *gbG8.8* still produced signals above 1.5 in ELISA (Section 3.6.2.1). This is an improvement on the scFv formats, where the signal declined rapidly when the temperature was increased beyond 47 °C. Very little decrease was noted after storage at different temperatures for four weeks (Fig 3.59). This is in contrast to the same experiment performed with scFvs, where the reduction in signal was greatest after medium-term storage at RT and 4 °C. After denaturation with GHCl, scFv E1 was not able to refold (Section 3.1.1.3). In the gallibody format by contrast, this Ab could refold to an extent, retaining at least three-quarters of its original binding ability as measured in ELISA (Fig 3.4 and Fig 3.60). Perhaps unexpectedly, G8 seemed to refold better as an scFv than as a gallibody (Fig. 3.4 and Fig. 3.60). While the structural stability of E1 was certainly improved as a gallibody, the same was not true for G8. This observation suggests that the G8 paratope is relatively plastic but is constrained within the IgY-derived scaffold where it adopts a less pliable fold. In contrast, the structure of E1's paratope is possibly better preserved by the gallibody structure.

The potential benefit of the IgY-scaffold for coupling to colloidal gold was examined using the gallibodies in gold-conjugated ICT assays (Section 3.6.3). IgY is known to have a stabilising effect on gold sols (Gasparyan, 2005) *i.e.* it has a wide zone within which it will protect colloidal gold nanoparticles from the electrolyte fluctuations that can cause a sol to collapse. In addition, Lys, Trp and Cys interact with gold particles in very specific ways (Section 1.9) and the conserved distribution of these residues in the C-domains of IgY (Figure 3.56) suggested that it might combine well with 40 nm-sized gold particles. Gold-conjugation takes place near to a protein's isoelectric point and for this reason the coupling optima of proteins do vary. This was shown by E1 and G8 which required different concentrations and pHs for coupling (Section 3.6.3.1). Neither scFv coupled to gold in a very stable manner as indicated by the large range across which the colloid collapsed (Fig. 3.61). More stable optima may have been achieved by testing higher concentrations of scFv in the salt-protection assay, but in a commercial setting this is not cost effective. Smaller gold-particles (e.g. 20 nm) may also sometimes yield more stable conjugates, but for most immunoassays optimal visibility with minimal steric hindrance is achieved with 40nm particles (Chandler *et al.*, 2000).

When tested as gallibodies, E1 and G8 both adsorbed to gold at the same pH. Overall, the gallibodies protected the gold colloid from ionic flocculation across the pH/[Ab] range far better than the scFvs (Fig. 3.61). In other words, the scFvs are brought into a more favourable ‘coupling range’ when converted into gallibodies. However, despite efforts to ‘standardise’ the behaviour of scFvs during gold adsorption, it still seems that their performance is strongly influenced by the charge-composition of their individual amino acid sequences, which of course vary greatly in their CDRs.

4.3 Purifying gallibodies from mammalian cell culture

Reconstituted scFvs were efficiently expressed as single polypeptide scFv-IgY fusions in HEK 293-H cell culture (Section 3.2.4) using the University of Hamburg’s scFvIgY(CH₂-4)His vector (Greunke *et al.*, 2006) and cultures constitutively expressing the gallibodies were established with relative ease. On the whole, the cells were easy to work with, grew rapidly and produced usable quantities of protein. Even though cell culture is a relatively expensive and time-consuming process, the principal disadvantage of the system remained its requirement for FBS supplementation which complicated the various downstream efforts to purify the gallibodies (Section 3.3). New protein-depletion products that have recently reached the market e.g. Seppro™ (Genway Biotech Inc.) may successfully remove some of the contaminating BSA and could be tried in the future.

PEG-precipitation is a simple, rapid method that is normally used to extract complete IgY from egg-yolks (Polson *et al.*, 1985). Accordingly, this method was first applied to *gbG8.8* cell culture. Several proteins were precipitated out of the SNF using PEG 6000 and were visible in SDS-PAGE (Fig. 3.17). However, *gbG8.8* itself was not able to be detected amongst these by western blotting (Fig. 3.18). Fundamentally, PEG causes proteins in solution to undergo a change in solubility which in turn causes them to aggregate and thus precipitate. What is more, the precipitation efficiency of PEG increases with an increase in molecular weight (Bhat & Timasheff, 1992; Shulgin & Ruckenstein, 2006). Shulgin & Ruckenstein (2006) have reported that temperature, pH and the cosolvent (e.g. PEG) concentration can also influence protein solubility in a water-cosolvent mixture. Obviously, therefore, the combination of pH and PEG concentration that is used to precipitate complete IgY out of solution is not optimal for gallibodies built on an IgY-scaffold which is missing its C_{H1}-domain. Further experimentation

may yet reveal conditions which will make it possible to successfully purify gallibodies from cell culture SNFs using simple precipitation protocols.

Since precipitation was not successful, the tetra-His tagged gallibodies (Fig. 3.8) had to be purified from HEK 293 cell culture SNFs using nickel-ion (Ni^{2+}) immunoaffinity chromatography (Greunke *et al.*, 2006). Small spin-columns (QIAGEN®) could not handle large sample volumes and significant amounts of the target protein were lost upon binding as a result of overloading (Section 3.3.2.1). A small amount of purified product was nevertheless obtained which indicated that the C-terminal tag was in all likelihood not buried within the protein. Batch purification was better suited to larger volumes with minimal losses in the flow-through (Section 3.3.2.2). In all cases, a number of contaminating proteins co-purified along with the gallibodies (Fig. 3.20 and Fig. 3.21B). Imidazole was included in the binding, washing and elution buffers to reduce this non-specific binding of His-rich proteins to the Ni^{2+} -affinity matrix. This molecule has a similar structure to the side chain of histidine and at low concentrations it is able to compete with non-specific or low-affinity proteins for binding to Ni^{2+} (Janknecht *et al.*, 1991). When imidazole was used, most of the contaminants were removed by washing although a few still eluted with the gallibodies (Fig 3.21). Nevertheless, the purity and yield of gallibodies from HEK 293-H cell culture SNFs was satisfactory for use in gold-coupling studies. In future studies, the efficiency of Ni-NTA purification step may perhaps be able to be improved by further optimising each stage of the process *viz.* binding, washing and elution. All five washes were performed at the same concentration of imidazole (20 mM), but if the concentration of this reagent is increased stepwise in each wash up to a certain threshold (*i.e.* below the concentration of imidazole used to elute tagged proteins), then additional non-specific proteins should in theory be removed before elution. In addition, it may be advantageous to increase the size of the His-tag to six residues. A 6-mer His-tag may bind more avidly to Ni^{2+} than one comprising only four residues. The NTA component of the Ni-NTA matrix already occupies four out of the six ligand binding sites available on the Ni^{2+} ion. Therefore, each immobilised Ni-NTA molecule can bind the side-chains of two His molecules. Given that some of the His-rich contaminants may already contain two or three tandem His residues, they are likely to bind to and be eluted from the matrix with comparable efficiency to the tetra-His tagged gallibodies. The aim should accordingly be to increase the avidity of the His-tag, so that the matrix retains gallibodies at higher concentrations of imidazole and non-specific proteins are released from the column first.

Using an anti-IgY Ab (Section 3.3.3) for immunoaffinity purification was a logical alternative to Ni-NTA chromatography. Although the gallibodies were certainly purer, the yields obtained were comparatively low (Fig. 3.23). A substantial amount of gallibody was lost during the initial binding process and during each of the four washes as could be detected in the corresponding flow-throughs using ELISA (Fig. 3.24). In contrast to the Ni-NTA spin-column purification results (Section 3.3.2.1), this was probably not as a result of overloading. Five milligrams of anti-IgY antibody was coupled to the Amino Link® matrix. If a binding ratio of 1:1 (anti-IgY: gallibody) is assumed, this should have been more than sufficient to sequester all of the gallibodies in 30 ml of cell culture SNF, bearing in mind that between 1 to 3 mg of protein was purified by Ni-NTA chromatography using 40 ml of the same SNF stock (Section 3.3.2.2). ELISAs using anti-IgY (Fc-region) Abs for detection confirmed the suspicion that polyclonal anti-IgY Abs did not detect the gallibodies with maximal efficiency (Fig. 3.25). Since several ‘species’ of Abs exist within a polyclonal serum, each may be directed at a different component of the target molecule. It is possible, therefore, that a number of the Abs in the polyclonal anti-IgY ‘mix’ were directed at the C_{H1} domain of IgY and consequently these were incapable of capturing gallibodies which recognise this region. Although, theoretically, gallibodies should be captured out of solution more effectively using anti-Fc Ab for immunoaffinity chromatography, in practice this was not a fruitful approach (results not shown). This was possibly a result of ‘over-processing’ of the anti-Fc Ab, which itself had been affinity purified during production by the supplier.

From the foregoing, it became clear that the problem of background protein contamination could not be addressed using downstream processes. Therefore, a solution was sought further upstream, at the level of protein production. Attempts were made to adapt the stable HEK 293-H cultures that expressed gallibodies to a low-serum or serum-free medium using two slightly different strategies, namely ‘conditioning’ and ‘sequential adaption’ (Table 3.1A). ‘Conditioning’ used reconditioned medium mixed equally (v/v) with low-serum or serum-free media. Reconditioned medium is two to three day old medium which is removed from a growing culture, filtered, and re-used. Since it contains growth factors that were previously secreted by the growing culture, its inclusion usually helps to minimise cell shock when cultures are split or fed. Despite this, the conditioned cultures could not adapt to such a quick decrease in FBS and they did not survive (Section 3.3.5). It is important to remember that not all of the chemical signals that are secreted

by cells encourage growth and if the cells were under stress at any time, biomolecules signalling this stress would have been secreted into the growth medium. These would then have been passed on during reconditioning. Hence, this way of reconditioning can actually be detrimental.

During ‘sequential reduction’, the amount of FBS in the growth medium was lessened by 25 % (v/v) every two to three passages (Table 3.1A). Passaging the cells allowed them to become accustomed to whichever combination was current, before the FBS concentration was further reduced. This could not be done for conditioned adaptation because the growth medium needed to be recycled each time that the cells were fed. The sequentially adapted cultures fared better in the long-term only because FBS was decreased in smaller increments after cultures that had been fed with equal amounts of complete : low/no-serum media (Combination 2, Table 3.1) began to look distressed. The HEK 293 cultures were eventually adapted to grow in medium containing 3 % (v/v) FBS as opposed to the 10 % (v/v) used originally. Even though purification was indeed facilitated (not shown), the cultures grew very slowly and accordingly, gallibody production was decreased. Taken as a whole, the exercise was actually counterproductive and further studies in this direction were not pursued. Attempts to adapt untransfected HEK 293-H cells yielded similar results (not shown). Since this mammalian cell line reliably expresses gallibodies when supplemented with FBS, further research aimed at improving Ab yields and purity may well be fruitful. For instance, chemically-defined, serum- and protein-free media are available for HEK 293-H cell culture (e.g. 293 SFM II or CD 293 from Invitrogen™). These reagents can, however, negatively affect transfection. They are also expensive and therefore may not be suitable for upscaled production. Alternatively, HEK 293 cells that have been pre-adapted to serum-free growth in traditional medium could be tried, although it remains to be seen how the use of these cells will affect the growth rate, the ease with which stable lines are established and the quantity of gallibody secreted.

4.4 Alternative heterologous protein expression systems for gallibodies

Applying an ‘upstream’ approach to purification meant that alternative heterologous protein expression systems had also to be explored. The bacterium *E. coli* and the yeast *K. lactis* were chosen for these investigations. *E. coli* was an obvious choice as it is the most well-known heterologous expression system with a number of well-documented advantages (discussed in Section 1.8 and Section 3.4). *K. lactis* was a more novel choice; antibodies have been produced

most successfully in the yeast *P. pastoris* but few studies have used *K. lactis* even though it is widely used in the food and pharmaceutical industries (van Ooyen *et al.*, 2006). Furthermore, in preference to the familiar *S. cerevisiae*, *K. lactis* has an unusual metabolism in that it does not form ethanol under aerobic conditions. Where ethanol is produced under aerobic conditions, ATP and consequently biomass is reportedly reduced (Schaffrath & Breunig, 2000).

Two QIAexpress pQE-TriSystem His•Strep Vectors are available, one of which was used in the attempts to express gallibodies in *E coli*. Although these vectors can also drive expression in mammalian or insect cells, this aspect was not investigated in the current study. Proteins that are expressed from the His•Strep vectors are tagged with a streptavidin- and an 8 × His-tag in tandem at either their N- or C-terminus. The His•Strep Vector 1 (Fig. 3.27) tagged the gallibodies at their C-termini. This meant that they would only be detected or purified if they had been expressed in their entirety. Furthermore, since the scFv-domains were located at the N-terminus, the tags would in all likelihood be more accessible at the C-terminus after protein folding. To direct proteins towards the periplasm and promote proper folding, the leader peptides OmpA and PelB were cloned immediately upstream of the scFv gene fragment (Fig. 3.28). Attempts to clone the entire leader-scFv-C_{H2-4} DNA fragment (nearly 2 kb) *en bloc*, were not successful (not shown), possibly due to its large size. For this reason, an expression cassette (His•StrepC_{H2-4}) was constructed by first inserting the IgY-derived C_{H2-4} regions into the plasmid (Fig. 3.28). This made the subsequent DNA ligations straightforward and created a vector that could be used to clone any scFv and its chosen leader sequence. Although the transformed bacterial clones contained plasmids with the correct DNA sequence, none produced detectable amounts of gallibodies (Section 3.4.3).

These results, while disappointing, were perhaps not unexpected. It is well known that not every protein can be successfully or optimally expressed in every system. Additionally, a system that succeeds in producing one protein may fail for another. Gene expression and protein folding are complex processes that involve several specialised constituents of the host machinery. A bottleneck, influenced by intrinsic (e.g. gene sequence features, promoter or operator elements and mRNA stability) and extrinsic factors (e.g. growth temperature, pH and growth medium) could exist at any stage. If any gallibodies had been produced, but could perhaps not fold correctly, they should have accumulated in the cytoplasm. Similarly, a non-functional leader

peptide would stop them from being translocated from the cytoplasm to the periplasmic space. However, no gallibodies were detected in either the cytoplasm or the periplasm (Section 3.4.3). This indicates that there was a constraint prior to periplasmic translocation. Considering that the scFv-C_{H2-4} fusion polypeptide could not be identified in either of the cellular compartments, it is also possible that it was in fact produced, but was degraded *in vivo*. Generally, recombinant protein expression overwhelms the host machinery, setting off a chain of events that can trigger protein misfolding and induce a stress response in *E. coli*. This ultimately triggers degradation of the foreign protein (Makrides, 1996; Sørensen & Mortensen, 2005). Two of the approaches used in this study, namely using leader peptides to direct expression toward a specific compartment and protein expression at low temperature, can sometimes overcome both misfolding and degradation, but not in this case. Alternatives, such as the use of protease-deficient strains or strains engineered to facilitate disulphide bonding (e.g. Origami™, Merck) may yield better results.

Another possibility is that rare codon usage might explain the absence of the foreign gene products. Even though the use of degenerate codons makes it possible for a single protein to be translated from slightly different DNA sequences, the genes of certain organisms may be biased toward using codons that are rarely used by others. This can result in either low or no gene expression in a heterologous host because it is unable to interpret the template (Gustafsson *et al.*, 2004). Humans and *E. coli*, for example, ‘prefer’ significantly different codons and *E. coli* is often not the best host in which to express human proteins. (Gustafsson *et al.*, 2004). It is not known whether avian genomes, specifically that of the chicken, prefer any particular nucleotide triplets. Highly expressed genes within an organism reportedly exhibit greater codon bias than those expressed at low levels (Makrides, 1996). In any vertebrate, Abs can be considered to be highly expressed, particularly when the immune system is responding to antigenic challenge. It therefore seems likely that the C-region sequence of IgY will be predisposed to using certain triplets which are not supported by the tRNAs of *E. coli*. Several engineered vectors and strains (e.g. pRARE and Rosetta™ cells, Merck) that over-express rare tRNAs are now obtainable commercially and may help to address the problem of poor expression resulting from inadequate host-tRNA species.

Efforts to express gallibodies in the yeast *K. lactis* fared similarly to the attempts made in *E. coli*. For these attempts the IgY-derived C_H regions were cloned in the expression vector pKLAC1 (Fig. 3.33) in order to first create an expression cassette (Fig. 3.34) which would facilitate subsequent cloning of the scFv gene. The scFv gene could then be cloned (Section 3.5.5) immediately downstream of the α -mating factor signal peptide (Kex cleavage site) which is inherent to the pKLAC1 vector. A *SacII* site in the gene coding for the C_{H4} domain proved problematic and when the vector was linearised to permit homologous recombination with the yeast genome, this domain was cut into two. Various attempts at modification using a commercially available site-directed mutagenesis kit were ineffective (Section 3.5.1.1 and 3.5.1.2). The site was eventually altered after a ‘pull-through’ PCR (Fig. 3.43) was devised. This resulted in the construct called pKLAC1-C_{H2-4mut} (Section 3.5.1.3). Another expression cassette, pKLAC1-C_{H2-3}, was also constructed. This was different from pKLAC1-C_{H2-4} in that it omitted the fourth C_H domain (Fig. 3.34). It obviated the problems experienced with the *SacII* site in C_{H4} and could also theoretically produce Fab fragments.

Gene expression was first evaluated using both cassettes (Section 3.5.4). Sandwich ELISAs using induced culture SNFs from a number of cassette-transformed clones suggested that the C_H-region sequences were in fact being translated and expressed (Fig.3.50 and Fig. 3.51), albeit at low levels. The scFv genes were therefore subcloned into these cassettes to create ‘complete’ constructs for gallibody expression. After transfection with these constructs, however, growth that resembled the positive control was observed on all plates, including the negative controls (Section 3.5.6). This made the success of the transformation difficult to gauge. The pUC19 negative control for *K. lactis* transfection is different to the vector-only negative control of *E. coli* where vector re-ligation can occur and colonies are observed. If transfection does not work in *K. lactis*, no growth is expected at all. Since pUC19 has no *SacII* site it cannot be linearised and integrated into the yeast genome. Furthermore, this plasmid does not contain the *amdS* gene that is required for growth on nitrogen-free YP agar. Therefore, even if pUC19 had managed to replicate episomally within the yeast cells, they should not have been able to survive. Further experiments were therefore interpreted with a degree of circumspection. A sandwich ELISA to detect gallibodies in the growth medium was inconclusive (Fig. 3.55). Consequently, the ‘transformed’ yeast clones were screened for integrated scFv-C_H fusions by using PCR. When this failed to show integrated inserts, the *K. lactis* experiments were terminated. These

experiments illustrate that no single system can express every protein despite the fact that it, like many others, is marketed as an instant ‘clone-and-express’ kit. In retrospect, it may have been better to use an ‘Ab-familiar’ yeast system, such as *P. pastoris*. However, chicken Abs may have a particular codon bias and since these preferences can vary between genera, a different system would not have guaranteed a different outcome.

4.5 Epitope mapping

Lateral-flow ICTs revealed that *gbE1.3* and *gbG8.8* could apparently both bind to HSP65 at the same time (Section 3.6.3.2). Although this was not true in ELISA (Section 3.7.1) or dot-blot (Section 3.6.1, results not shown), it suggested that the two gallibodies possibly recognised different epitopes and could act in immunoassays as a pair in which one Ab would capture the antigen and the other would detect it. Accordingly, the XCX₁₅ phage displayed peptide library (Bonnycastle *et al.*, 1996) was panned against the *gbE1.3* and *gbG8.8* in an attempt to locate their recognition sites (Section 3.7.2). This experiment would not have been possible using the original scFvs, as neither was capable of immunocapture until their conversion to IgY-like molecules.

Generally, the first step towards characterising an epitope is to determine whether the Ab recognises a continuous or discontinuous region of the polypeptide chain (Zhou *et al.*, 2007). It has generally been accepted that recognition of a denatured macromolecule in western blot indicates a continuous epitope *i.e.* one which is comprised of successive residues on the primary structure of the protein. Both gallibodies bound to HSP65 in western blotting (Fig. 3.16) but this may not conclusively prove that they recognised linear epitopes (Fack *et al.*, 1997; Wang & Yu, 2004; Zhou *et al.*, 2007). This is because: (i) discontinuous epitopes may require only a few of the residues within the peptide to be recognised and these may be available in the denatured state or (ii) a conformation resembling the native epitope may still exist due to incomplete denaturation of the antigen; or (iii) a certain degree of renaturation may occur after a protein is blotted onto a membrane. For this reason, the peptides that were isolated by panning were mapped using both linear and 3D techniques. It is important to bear in mind that epitope mapping from a random peptide library generates ‘mimotopes’ which may efficiently bind to the paratope even though their amino acid sequence is not identical to the native epitope. Hence, like all epitopes, mimotopes are ‘conformational’ (van Regenmortel, 1989).

It is not clear why the gold-conjugated ‘Ab-pair’ results (Section 3.6.3.2) could not be repeated in ELISA. A possible explanation lies in the basic differences between the two assay formats: Firstly, ICTs provide less opportunity for steric hindrance compared to ELISAs. When the Ab pair was examined in ELISA, the gallibody was adsorbed to the surface of the plate to capture unlabelled antigen which was then detected using an scFv. This complex was in turn detected with 9E10, a mAb specific for the c-myc tag on the scFv, and finally by HRP-conjugated anti-mouse Ab. In this format, there may simply not have been enough space to accommodate the secondary detection reagents (Butler *et al.*, 1997). Another factor may be that ICTs detect antigen with greater avidity than ELISAs. In these assays, gallibodies were adsorbed to nitrocellulose to capture unlabelled antigen, which in turn was detected by gold-particles covered with scFvs or gallibodies. A single Ab-coated gold ‘sphere’ has a greater concentration of binding sites than, for instance, a single HRP-conjugated Ab. This means that an individual Ab-labelled gold particle should be able to combine with more than one antigen particle *i.e.* with greater avidity, thereby producing a stronger signal.

Even though proteins adsorbed to membranes may be less distorted than those attached to plastic (Shields *et al.*, 1991), E1 and G8 did not function as a capture/detection pair in any combination *i.e.* gallibody capture/scFv detection or gallibody capture/gallibody detection (Section 3.6.1, results not shown). They were, however, able to do so when adsorbed to nitrocellulose in lateral-flow tests (Section 3.6.3.2). This could be due to the increased avidity of Ab-labelled gold-particles as discussed above, or it could be a result of renaturation of the capture Ab during or after blotting (Zhou *et al.*, 2007). Gallibodies that were spot-blotted onto nitrocellulose or PVDF were tested for immunocapture as soon as the spots had dried. In contrast, the ICT membranes were assembled at a remote location and then dispatched to OVI. This meant at least a week’s delay before use. It is perhaps not inconceivable that refolding may have occurred during this time, rendering the Abs on those membranes functional again.

Panning each Ab against the XCX₁₅ library (Section 3.7.2) yielded a number of different mimotopes (Table 3.4). While some were more frequently isolated than others, there was little consensus between the peptides that were selected by G8. Additionally, these peptides bound very weakly in ELISA compared to those selected by E1 (Fig. 3.68 and Fig. 3.69). This once again seems to imply that the paratope of G8 has a somewhat difficult ‘fit’ (discussed in Section

4.2) *i.e.* the energetic environment of the binding-pocket is perhaps less reliant on strong, ionic forces and more reliant on weaker hydrogen bonding or van der Waals' forces. In any event the XCX₁₅ display library peptides were not especially suited to this paratope (Bonnycastle *et al.*, 1996). In fact, it may be useful in future to pan at least one other library of a different format against both of these Abs. Unexpectedly, each Ab selected one peptide that diverged from the XCX₁₅ format, namely E1-b and G8-a (Table 3.4). Both were encountered frequently and still bound in ELISA (not shown). In peptide E1-b, the fixed Cys is replaced by Tyr and in G8-b a Ser occupies this position. All three of these residues have uncharged, polar side chains. However, a Cys at position two is required for 'loop' formation, so these two peptides were excluded from the 3D analyses.

As mentioned previously, presumptive epitopes were located using both linear and 3D approaches. While linear alignments can be used to locate continuous epitopes, these kinds of recognition sites may indeed form only a small component of a larger, conformational epitope, the recognition of which will be influenced not only by the direct association of contact residues, but also by the interactions of residues within the perimeter (Atassi, 1984). Each peptide selected by the Abs during panning was aligned individually to the HSP65 amino acid sequence, using a global LALIGN alignment (Section 3.7.3). The peptides were also mapped in 3D using two algorithms available at the Pepitope server. PepSurf maps all the individual peptide mimotopes in a set onto presumptive epitope locations on the folded 3D structure (Mayrose *et al.*, 2007a; Mayrose *et al.*, 2007b). In contrast, Mapitope first searches the data set for shared determinants and then predicts epitopes based on common features (Bublil *et al.*, 2007; Mayrose *et al.*, 2007a). When the PepSurf and Mapitope algorithms are used simultaneously ('combined' analysis), the predicted 'fit' contains only residues that overlap between the two calculations. The presumptive epitope of E1 was mapped to a region that included residues 350 to 370, based on a general consensus between results obtained when both approaches were applied individually (Section 3.7.3.1). The peptides E1-a and E1-h were common to both the linear and the 3D alignments. Both of these contain an additional Cys residue that could possibly form a disulphide loop with the Cys in position two of the XCX₁₅ repertoire. Interestingly, all but three of the peptides selected by E1 contained an additional Cys at position seven in the 17 mer peptide (Table 3.4). Those clones which did not bind in ELISA had an additional Cys at a different position. Therefore, it appears that the mimotope recognised by E1 has a particular constrained

conformation. Non-binders in phage-capture ELISA (E1-e and E1-g, Table 3.4) and peptides which did not follow the XCX₁₅ motif (E1-b, Table 3.4) were excluded from all Pepitope analyses as this skewed the outputs (data not shown). The peptides selected by G8 corresponded to three different locations on the HSP65 polypeptide, namely residues 134 to 149, residues 433 to 474 and residues 512 to 522 (Fig. 3.72). These areas, however, were determined linearly and are therefore remain merely presumptive. Very few of the peptides selected by G8 contained an additional Cys. In the absence of usable 3D data, no assumptions can be made about this epitope's conformational requirements. Nonetheless, the information obtained by these analyses is in accordance with the suggestion that E1 and G8 recognise different locations on HSP65. A limitation of Pepitope algorithms is that only 14 residues can be mapped at a time. As a result, the peptides selected by each Ab had to be aligned to the HSP65 crystal structure once using the first 14-mer of the peptide sequence, and then again using the last 14-mer. These two calculations did not correspond with one another. The Mapitope algorithm is not really suitable for data obtained from a source such as the XCX₁₅ library which has a conserved arrangement. When the peptides selected by E1 and G8 were analysed using this algorithm, they produced very similar results because the algorithm regarded XCX₁₅ as a common feature. Consequently, the 'combined' calculations were also meaningless.

A few studies (Shinnick *et al.*, 1987; Hajeer *et al.*, 1992; Rambukkana *et al.*, 1992) have mapped linear epitopes on the HSP65 protein using mAbs. Thole (1988) for instance, identified four epitopes on the polypeptide. This made it feasible to identify five main epitope 'tracts' using collective data (Fig. 3.73). The published epitopes were identified using biologically-derived Abs. Most occur in hydrophilic regions (data not shown), hinting strongly at 'accessibility'. In a sense, therefore, these are 'expected' epitopes and it would not be unforeseen if other Abs were also able to recognise these same regions. Indeed, the epitopes predicted for E1 or G8 lie close to the previously mapped mAb epitopes (Fig. 3.73). A distinction should nevertheless be made between mAbs and *Nkuku*®-derived scFvs. The mAbs used in the previous studies were raised in animals that had been immunised with HSP65. The immune system of an animal will respond to an antigen by manufacturing Abs to the regions which it perceives to be antigenic only if they are also immunogenic. Abs raised against the same antigen but in different animals will in all likelihood recognise slightly different epitopes (reviewed by Benjamin *et al.*, 1984). The scFvs E1 and G8, on the other hand, were isolated from a naïve, semi-synthetic chicken Ab library.

Despite the ‘minimal diversity’ supposedly displayed by the chicken immune repertoire, when compared to mammals, these two scFvs recognised regions not entirely removed from the ones identified by the murine mAbs. Moreover, although the repertoire contains a sub-library which encodes for a synthetic, randomised H-chain CDR3, the H- chain CDR3’s of E1 and G8 appear to be of naïve (germline) origin (Dr. J. Fehrsen, personal communication). These observations confirm accessibility as an overriding factor in antigenicity. It is interesting that Uray *et al.* (2000) identified serum Abs in humans that recognised epitopes shared by human Hsp60 and the Hsp65 from *M. bovis*. These individuals, who were ‘naïve’ to infection, raised Abs against common determinants without being immunised. Taken together, the observations of this and the current study suggest that the two scFvs used in this study may cross-react with other proteins. This remains to be investigated. Before this can be done, further experiments need to be carried out to authenticate that epitopes predicted in this study are in fact those recognised by E1 and G8. This can be evaluated in inhibition assays using synthetic peptides that represent the predicted regions. Should these peptides inhibit the binding of their corresponding Ab to the antigen, then there is a strong likelihood that they represent an actual antigenic region.

4.6 Concluding remarks

Over the last few years, recombinant Abs have shown themselves to be practical immunoreagents which can perform as well as those derived from ‘traditional’ sources e.g. murine hybridomas. This applies particularly to chicken scFvs, not only because of their performance, but also because their Ig repertoire can be accessed far easier than that of any mammal with the exception of camelids (Muyldermans, 2001). In this study, IgY-derived C_H-domains were added to chicken scFvs in an attempt to alter their performance in ELISAs and ICTs. This platform complements the aforementioned benefits of chicken scFvs. This means that recombinant chicken Abs are now available to the researcher or assay developer in a choice of formats ranging from single-chain fragments to molecules which are analogous to the complete IgY from egg yolk. Converting chicken scFvs to Ig-like molecules was found to be a practical way of improving stable, specific scFvs which may under-perform in certain areas e.g. in immunocapture.

Although the ‘domain adding’ approach to improve Abs is not new (Scallon *et al.*, 2004; Di Niro *et al.*, 2007; Quintero-Hernández *et al.*, 2007), most published examples have used mammalian Ab domains. In contrast, Greunke *et al.* (2006) used chicken IgY-derived C_H-regions to create

bivalent molecules from monovalent scFvs. In the current study, which is based on their findings, the effect of adding IgY-C_H domains on stability and preservation of affinity after adsorption was established. The performance of two stable scFvs in two immunoassay formats was significantly improved. The C_H-scaffold improved Ab-gold conjugation by ‘focussing’ the range within which favourable coupling conditions could be achieved and it also converted the scFvs into Abs that could be used for immunocapture in ELISAs. While the exercise can be considered successful, the overall practicality of using gallibodies would be enhanced if simpler systems for expression and purification were available. As this study has illustrated, the success of either often depends on the individual protein. Besides those examined here, a large choice of alternative expression systems remains. In fact, transient expression of *Nkuku*® scFvs has successfully been carried out in plants (Rybicki & Maclean, University of Cape Town, unpublished).

The two Abs examined in this study are stable and specific, particularly when used as gallibodies. They appear to recognise different regions on the HSP65 polypeptide, making them useful as an Ab pair in ICTs. They can potentially be used as components in tests aimed at general *Mycobacterium* diagnosis e.g. assays based on detecting multiple antigens, or even as an alternative to the PPD test. Their potential to cross-react with other antigens from the *Mycobacterium* complex will need to be studied first. There is little doubt that gallibody-style Abs are appropriate for ELISA and ICT assays and there seems little reason to assume that they would not function equally well in novel formats such as antibody arrays.

APPENDIX

All chemicals used were of analytical grade.

Ampicillin	Make up as 100 mg/ml stock solution in ddH ₂ O. Filter-sterilise and freeze away at -20 °C in aliquots.
5 mM Borate Buffer	A. 500ml 0.1 M Boric Acid (H ₃ BO ₄) in ddH ₂ O B. 500ml 0.1 M Sodium Tetraborate (Na ₂ B ₄ O ₇ · 10 H ₂ O) in ddH ₂ O Add B to A (acidic pH range) or A to B (basic pH range) until desired pH is reached. Dilute to 5 mM with ddH ₂ O.
10 % (w/v) BSA	10 g of BSA Fraction V dissolved in 80 ml ddH ₂ O. Adjust to pH 9 with NaOH and make volume up to 100ml.
0.1 M Citrate Buffer pH 4.5	A. 500 ml 0.1 M Na ₃ C ₆ H ₅ O ₇ · 2H ₂ O in ddH ₂ O B. 500 ml 0.1 M H ₃ C ₆ H ₅ O ₇ · H ₂ O in ddH ₂ O Add B to A until pH reaches 4.5. Bottle and store at 4 °C.
Conditioned cell medium	Two to three day old growth medium (DMEM with 10 % (w/v) FBS), filter sterilised (0.02 µm pore-size).
Coomassie Blue	2.5 g Coomassie Brilliant Blue 450 ml methanol 100 ml acetic acid 400 ml ddH ₂ O Adjust volume to 1 l with ddH ₂ O and store at RT°.
Crystal violet agarose gels	Dissolve agarose in 1 x TAE buffer and add crystal violet to a final concentration of 10 µg/ml just before pouring. Add to the running buffer at the same concentration. Load samples in 30 % (v/v) glycerol and 0.002 % (v/v) xylene cyanol (6 x concentrated) (Rand 1996).
1% EDTA (w/v) stock solution	Add 1 g of EDTA to 100 ml ddH ₂ O. Filter sterilise and store at 4 °C.

Ethidium Bromide 10 mg/ml	Dissolve 1 g ethidium bromide completely in 100 ml ddH ₂ O. Protect from light and store at RT.
Freezing Medium	7.2 ml culture medium (50 % (v/v) fresh and 50 % (v/v) conditioned) 2 ml undiluted FBS 750 µl DMSO Filter sterilise and prepare fresh. Store at 4 °C until use.
Glucose stock solution	20 % (w/v) solution: 20 g D-Glucose dissolved in ddH ₂ O to a final volume of 100 ml. Autoclave or filter sterilise. Store at RT. 40 % (w/v) solution: 40 g D-Glucose dissolved in ddH ₂ O to a final volume of 100 ml. Autoclave or filter sterilise. Store at RT.
40 % (w/v) Galactose stock solution	20 g Galactose dissolved in ddH ₂ O to a final volume of 100 ml. Autoclave or filter sterilise. Store at RT.
Gold-conjugation resuspension buffer	10 mM Tris-base 0.2 % BSA 0.2 % Tween 20 20 % sucrose Dissolve in 80 ml ddH ₂ O. Adjust pH to 8 with HCl and make up to final volume of 100 ml.
Gold-conjugation running buffer	25 mM Tris-base 1 % Triton X100 1 % BSA 150 mM NaCl Dissolve in 80 ml ddH ₂ O. Adjust pH to 8 with HCl and make up to final volume of 100 ml.
2 N H₂SO₄	Add 29 ml concentrated H ₂ SO ₄ to 493 ml of ddH ₂ O, with mixing. Work on ice at all times. Store at RT.
Heat-inactivated FBS	Incubate in a water bath at 56 °C for half an hour.

1 M IPTG stock solution	2.383 g IPTG dissolved in ddH ₂ O to a final volume of 10 ml. Sterilise by filtration, aliquot and store at -20 °C.
Kanamycin	Make up as 50 mg/ml stock solution in ddH ₂ O. Filter-sterilise and store at -20 °C.
LB Medium	10 g Tryptone 5 g Yeast extract 10 g NaCl ddH ₂ O Adjust pH to 7.0 using 10M NaOH. Make volume up to 1 l with ddH ₂ O. Autoclave and store at RT°. For LB-agar, add 15 g Agar-agar for every 1 l. Add antibiotics and/or glucose when cool. Store plates at 4 °C.
80 mM NaCl	0.47 g NaCl dissolved in a final volume of 100 ml ddH ₂ O.
1 % (w/v) NaN₃	Dissolve 1 g of NaN ₃ in a final volume of 100 ml ddH ₂ O. Filter sterilise.
10 x PBS stock solution	80 g NaCl 2 g KCl 26.8 g Na ₂ HPO ₄ ·7H ₂ O 2.4 g KH ₂ PO ₄ pH to 7.4 with HCl. Adjust volume to 1 l with ddH ₂ O and sterilize by autoclaving. Store at RT.
1 x PBS – 0.01 % (v/v) EDTA solution	Add 1 ml 1% (w/v) EDTA solution per 100 ml 1 x PBS. Store at 4 °C.
16.7% (w/v) PEG / 3.3M NaCl	100 g PEG 8000 116.9 g NaCl Dissolve in 475 ml ddH ₂ O, with brief heating if necessary. The solution may be autoclaved. Store at 4 °C.
Phage Elution Buffer	Dissolve 1.5 ml glycine in 90 ml ddH ₂ O and pH to 2.2 with HCl. Add 0.1 g BSA and make up to 100 ml. Filter sterilise and store at 4 °C.



Phage Neutralisation Buffer

1 M Tris adjusted to pH 9 with HCl.

2 x PSB

10 ml 1.5M Tris pH 6.8

6 ml 20 % SDS

30 ml glycerol

1 ml β -mercaptoethanol

1.8 mg bromophenol blue

Make up to final volume of 100ml in ddH₂O. Aliquot and store at -20

°C. Store working solution at 4 °C.

50 x TAE stock solution

242 g Tris-base

37.2 g Na₂EDTA

57.1 ml glacial acetic acid

ddH₂O

Make up to final volume of 1 l with ddH₂O and store at RT.

TE buffer

2.5 ml 1 M Tris in ddH₂O pH 7.4

0.5 ml 0.5 M Na₂EDTA in ddH₂O pH 8

Mix and make up to a final volume of 250 ml with ddH₂O.

Autoclave and store at RT.

Tetracycline

Make up as 10 mg/ml stock solution in absolute ethanol. Filter-sterilise and store at -20 °C. Protect from light.

1 x Transfer Buffer

20 mM Tris-base

150 mM Glycine

ddH₂O

Adjust pH to 8.0 with HCl and make up to final volume of 1 l. Make up fresh and store at 4 °C.

Transformation Buffer

10 mM HEPES

15 mM CaCl₂

250mM KCl

55 mM MnCl₂

Adjust pH to 6.7 with KOH before adding MnCl₂. Make up to 1 l with ddH₂O, filter -sterilize and store at 4 °C.



1 M Tris-HCl stock solution

Dissolve 121.14 g Tris-base in 800 ml ddH₂O.

Adjust pH to 7.0 using concentrated HCl. Make up to 1 l with ddH₂O.

Autoclave and store at RT.

2 x TY medium

8 g NaCl

10 g Tryptone

5 g Yeast extract

Dissolve in 1 l ddH₂O and autoclave. Store at RT°.

To make 2 x TY agar, add 15 g Agar-agar for every 1 l. Add antibiotics and/or glucose when cool. Store plates at 4 °C.

Yeast carbon base (YCB) Agar

15 ml 1 M Tris-HCl buffer stock solution

5.85 g YCB medium powder

10 g Agar-agar

Dissolve in total volume of 495 ml ddH₂O. Autoclave and add 5 ml 100 x acetamide solution (supplied with *K. lactis* kit) when cool.

YP Gal Medium

10 g Yeast extract

20 g Agar-agar

Dissolve in 950 ml ddH₂O. Autoclave and cool to RT. Add 50 ml 40 % (w/v) galactose when cool.

YP Glu medium

10 g Yeast extract

20 g Agar-agar

Dissolve in 950 ml ddH₂O. Autoclave and cool to RT. Add 50 ml 40 % (w/v) glucose when cool.

Aburatani, T., Ueda, H. & Nagamune, T. (2002). Importance of CDR H3 basal residue in V_H/V_L interaction of human antibodies. *Journal of Biochemistry* **132**, 775 - 782.

Akiyoshi, D.E., Rich, C.M., O'Sullivan-Murphy, S., Richard, L., Dilo, J., Donohue-Rolfe, A., Sheoran, A.S., Chapman-Bonofiglio, S. & Tzipori, S. (2005). Characterization of a human monoclonal antibody against Shiga toxin 2 expressed in Chinese hamster ovary cells. *Infection & Immunity* **73**, 4054 - 4061.

Alt, F., Yancopoulos, G.D., Blackwell, T.K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S. & Baltimore, D. (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments. *The EMBO Journal* **3**, 1209 - 1219.

Amadori, M., Lyashchenko, K.P., Gennaro, M.L., Pollock, J.M. & Zerbini, I. (2002). Use of recombinant proteins in antibody tests for bovine tuberculosis. *Veterinary Microbiology* **85**, 379 - 389.

Andreotti, P.E., Ludwig, G.V., Puruski, A.H., Tuite, J.J., Morse, S.S. & Peruski, L.F. (2003). Immunoassay of infectious agents. *BioTechniques* **35**, 850 - 859.

Angkawidjaja, C., Kuwahara, K., Omori, K., Koga, Y., Takano, K. & Kanaya, S. (2006). Extracellular secretion of *Escherichia coli* alkaline phosphatase with a C-terminal tag by type I secretion system: purification and biochemical characterization. *Protein Engineering, Design & Selection* **19**, 337 - 343.

Arap, M.A. (2005). Phage display technology - Applications and innovations. *Genetics and Molecular Biology* **28**, 1 - 9.

Atassi, M.Z. (1984). Antigenic structures of proteins. Their determination has revealed important aspects of immune recognition and generated strategies for synthetic mimicking of binding sites. *European Journal of Biochemistry* **145**, 1 - 20.

Baker, M.G., Lopez, L.D., Cannon, M.C., De Lisle, G.W & Collins, D.M. (2006). Continuing *Mycobacterium bovis* transmission from animals to humans in New Zealand. *Epidemiology and Infection* **134**, 1068 - 1073.

Barbas III, C.F., Bain, J.D., Hoekstra, D.M. & Lerner, R.A. (1992). Semisynthetic combinatorial antibody libraries: A chemical solution to the diversity problem. *Proceedings of the National Academy of Sciences USA* **89**, 4457 - 4461.

Barlow, D.J., Edwards, M.S. & Thornton, J.M. (1986). Continuous and discontinuous protein antigenic determinants. *Nature* **322**, 747 - 748.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E. & Wilson, A.C. (1984). The antigenic structure of proteins: A reappraisal. *Annual Reviews in Immunology* **2**, 67 - 101.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A. & Wheeler, D.L. (2000). GenBank. *Nucleic Acids Research* **28**, 15 - 18.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N. & Bourne, P.E. (2000). The Protein Data Bank. *Nucleic Acids Research* **28**, 235 - 242. Abstract only.

Bhat, R. & Timasheff, S.N. (1992). Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Science* **1**, 1133 - 1143.

Bhatti, M., Yahioğlu, G., Milgrom, L.R., Garcia-Maya, M., Chester, K.A. & Deonarain, M. P. (2008). Targeted phytotherapy with multiply-loaded recombinant antibody fragments. *International Journal of Cancer* **122**, 1155 - 1163. Abstract only.

Birch, J.R. & Racher, A.J. (2006). Antibody production. *Advanced Drug Delivery Reviews* **58**, 671 - 685.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S., Lee, T., Pope, S.H., Riordan, G.S., Whitlow, M. (1988). Single-chain antigen-binding proteins. *Science* **242**, 423 - 426.

Blais, B.W., Omar, M. & Phillippe, L. (2002). Detection of Brazil nut proteins in foods by enzyme immunoassay. *Food and Agricultural Immunology* **14**, 163 - 168.

Blight, M.A. & Holland, I.B. (1994). Heterologous protein secretion and the versatile *Escherichia coli* hemolysin translocator. *Tibtech* **12**, 450 - 455.

Blüthner, M., Bautz, E.K. & Bautz, F.A. (1996). Mapping of epitopes recognized by PM/Scl autoantibodies with gene-fragment phage display libraries. *Journal of Immunological Methods* **198**, 187 - 198. Abstract only.

Böer, E., Steinborn, G., Kunze, G. & Gellissen, G. (2007). Yeast expression platforms. *Applied Microbiology and Biotechnology* **77**, 513 - 523.

Bonnycastle, L.L.C., Mehroke, J.S., Rashed, M., Gong, X. & Scott, J.K. (1996). Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. *Journal of Molecular Biology* **258**, 747 - 762.

Bonnycastle, L.L.C., Menendez, A. & Scott, J. (2001). General phage methods. In *Phage display. A laboratory manual*. Edited by C.F. Barbas III, D.R. Burton, J.K. Scott and G.J. Silverman. Cold Spring Harbour Laboratory Press: Cold Spring Harbor, New York.

Bowdish, K., Tang, Y., Hicks, J.B. & Hilvert, D. (1991). Yeast expression of a catalytic antibody with chorismate mutase activity. *Journal of Biological Chemistry* **266**, 11901 - 11908.

Bradbury, A.R.M. & Marks, J.D. (2004). Antibodies from phage antibody libraries. *Journal of Immunological Methods* **290**, 29 - 49.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248 - 254.

Braig, K., Otwinowski, Z., Hegde, R., Bolsvert, D.C., Joachimiak, A., Horwich, A.L., & Sigler, P.B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371**, 578 - 586.

Braren, I., Blank, S., Seismann, H., Deckers, S., Ollert, M., Grunwald, T. & Spillner, E. (2007a). Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries. *Clinical Chemistry* **53**, 837 - 844.

Braren, I., Greunke, K., Umland, O., Deckers, S., Bredehorst, R. & Spillner, E. (2007b). Comparative expression of different antibody formats in mammalian cells and *Pichia pastoris*. *Biotechnology and Applied Biochemistry* **47**, 205 - 214.

Brichta, J., Hnilova, M. & Viscovic, T. (2005). Generation of hapten-specific recombinant antibodies: antibody phage display technology: a review. *Veterinary Medicine - Czech* **50**, 231 - 252.

Brockmann, E., Cooper, M., Strömsten, N., Vehniäinen, M. & Saviranta, P. (2005). Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions. *Journal of Immunological Methods* **296**, 159 - 170.

Bublil, E.M., Freund, N.T., Mayrose, I., Penn, O., Roitburd-Berman, A., Rubinstein, N.D., Pupko, T. & Gershoni, J.M. (2007). Stepwise prediction of conformational discontinuous B-cell epitopes using the Mapitope algorithm. *Proteins* **68**, 294 - 304.

Buddle, B.M., Parlane, N.A., Keen, D.L., Aldwell, F.E., Pollock, J.M., Lightbody, K. & Andersen, P. (1999). Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. *Clinical and Diagnostic Laboratory Immunology* **6**, 1 - 5.

Buddle, B.M., Skinner, M.A., Wedlock, D.N., Collins, D.M. & Lisle, G.W. (2002). New generation vaccines and delivery systems for control of bovine tuberculosis in cattle and wildlife. *Veterinary Immunology and Immunopathology* **87**, 177 - 185.

Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenburg, B., Chang, J., Brown, W.R. & Cantarero, L.A. (1992). The physical and functional behaviour of capture antibodies adsorbed on polystyrene. *Journal of Immunological Methods* **150**, 77 - 92.

Butler, J.E., Navarro, P. & Sun, J. (1997). Adsorption-induced antigenic changes and their significance in ELISA and immunological disorders. *Immunological Investigations* **26**, 39 - 54.

Cadwell, R.C. & Joyce, G.F. (1992). Randomization of genes by PCR mutagenesis. *PCR Methods and Applications* **2**, 28 - 33.

Carlander, D., Stålberg, J. & Larsson, A. (1999). Chicken antibodies: A clinical chemistry perspective. *Upsala Journal of Medical Science* **104**, 179 - 190.

Carmen, S. & Jermutus, L. (2002). Concepts in antibody phage display. *Briefings in Functional Genomics and Proteomics* **1**, 189 - 203.

Casey, J.L., Coley, A.M., Street, G., Parisi, K., Devine, P.L. & Foley, M. (2006). Peptide mimotopes selected from a random peptide library for diagnosis of Epstein-Barr virus infection. *Journal of Clinical Microbiology* **44**, 764 - 771. Abstract only.

Chan, E.D., Reves, R., Belisle, J.T., Brennan, P.J. & Hahn, W.E. (2000). Diagnosis of tuberculosis by a visually detectable immunoassay for lipoarabinomannan. *American Journal of Respiratory and Critical Care Medicine* **161**, 1713 - 1719.

Chandler, J., Gurmin, T. & Robinson, N. (2000). The place of gold in rapid tests. *IVD Technology* **6**, 37 - 49.

Chang, A.C.Y. & Cohen, S.N. (1974). Genome construction between bacterial species *in vitro*: replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **71**, 1030 - 1034.

Chiba, Y. & Jigami, Y. (2007). Production of humanized glycoproteins in bacteria and yeasts. *Current Opinion in Chemical Biology* 11, 670 - 676.

Choi, J.H. & Lee, S.Y. (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. *Applied Microbiology and Biotechnology* 64, 625 - 635.

Chu, L. & Robinson, D.K. (2001). Industrial choices for protein production by large-scale cell culture. *Current Opinion in Biotechnology* 12, 180 - 187.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D. & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature* 352, 624 - 628.

Cockle, P.J., Gordon, S.V., Lalvani, A., Buddle, B.M., Hewinson, R.G. & Vordermeier, H.M. (2002). Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infection and Immunity* 70, 6996 - 7003.

Cohen, S.N., Chang, A.C.Y., Boyer, H.W. & Helling, R.B. (1973). Construction of biologically functional bacterial plasmids *in vitro*. *Proceedings of the National Academy of Sciences USA* 70, 3240 - 3244.

Collins, A.M., Sewell, W.A. & Edwards, M.R. (2003). Immunoglobulin gene rearrangement, repertoire diversity and the allergic response. *Pharmacology and Therapeutics* 100, 157 - 170.

Colussi, P.A. & Taron, C.H. (2005). *Kluyveromyces lactis* LAC4 promoter variants that lack function in bacteria but retain full function in *K. lactis*. *Applied and Environmental Microbiology* 71, 7092 - 7098.

Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., Cortese, I., Fortugno, P., Galfré, G., Nicosia, A. & Felici, F. (1996). Selection of biologically active peptides by phage display of random peptide libraries. *Current Opinion in Biotechnology* 7, 616 - 621.

Cousins, D.V., Huchzermeyer, H.F.K.A., Griffin, J.F.T., Brückner, G.K., van Rensburg, I.B.J. & Kriek, N.P.J. (2004). Tuberculosis. In *Infectious Diseases of Livestock*, 2nd Ed, pp 1973 - 1993. Edited by J.A.W. Coetzer & R.C Tustin. Cape Town: Oxford.

Cwirala, S.E., Peters, E.A., Barrett, R.W. & Dower, W.J. (1990). Peptides on phage: A vast library of peptides for identifying ligands. *Proceedings of the National Academy of Sciences USA* 87, 6378 - 6382.

Daumas, F., Mazarguil, H., Millot, C., Lopez, A. & Salomé, L. (2002). Probing functionalized gold colloids for single particle tracking experiments. *Biochemical and Biophysical Research Communications* 295, 610 - 615.

Dávalos-Panjota, L., Ortega-Vinuesa, J.L., Bastos-González, D. & Hidalgo-Álvarez, R. (2001). Colloidal stability of IgG- and IgY-coated latex microspheres. *Colloids and Surfaces B: Biointerfaces* **20**, 165 - 175.

Davies, E.L., Smith, J.S., Birkett, C.R., Manser, J.M., Anderson-Dear, D.V. & Young, J.R. (1995). Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of Immunological Methods* **186**, 125 - 135.

de Bruyn, J., Bosmans, R., Turneer, M., Weckx, M., Nyabenda, J., van Voortem, J., Falmagne, P., Wiker, H.G. & Harboe, M. (1987). Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. *Infection and Immunity* **55**, 245 - 252.

de Bruyn, J., Soetaert, K., Buyssens, P., Calonne, I., de Coene, J.L., Gallet, X., Brasseur, R., Wattiez, R., Falmagne, P., Montrozier, H., Lanéle, M.A. & Daffé, M. (2000). Evidence for specific and non-covalent binding of lipids to natural and recombinant *Mycobacterium bovis* BGC Hsp60 proteins, and to the *Escherichia coli* homologue GroEL. *Microbiology* **146**, 1513 - 1524.

de Haard, H.J.W., Kazemier, B., van der Bent, A., Oudshoorn, P., Boender, P., van Gemen, B., Arends, J. & Hoogenboom, H.R. (1998). Absolute conservation of residue 6 of immunoglobulin heavy chain variable regions of class IIA is required for correct folding. *Protein Engineering* **12**, 1267 - 1276.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J. & Hoogenboom, H.R. (1999). A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *Journal of Biological Chemistry* **274**, 18218 - 18230.

de Kuif, J., Boel, E. & Logtenberg, T. (1995). Selection and amplification of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *Journal of Molecular Biology* **248**, 97 - 105.

Devlin, J.J., Panganiban, L.C. & Devlin, P.E. (1990). Random peptide libraries: A source of specific protein binding molecules. *Science* **249**, 404 - 406.

Dillon, D.C., Alderson, M.R., Day, C.H., Bement, T., Campos-Neto, A., Skeiky, Y.A.W., Vedvick, T., Badaro, R., Reed, S.G. & Houghton, R. (2000). Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. *Journal of Clinical Microbiology* **38**, 3285 - 3290.

Di Niro, R., Ziller, F., Florian, F., Crovella, S., Stebel, M., Bestagno, M., Burrone, O., Bradbury, A.R.M., Secco, P., Marzari, R. & Sblattero, D. (2007). Construction of miniantibodies for the *in vivo* study of human autoimmune diseases in animal models. *BMC Biotechnology* **7**.

du Plessis, D.H., Romito, M. & Jordaan, F. (1995). Identification of an antigenic peptide specific for bluetongue virus using phage display expression of NS1 sequences. *Immunotechnology* **1**, 221 - 230.

Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell* **19**, 981 - 992.

Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishauser, U. & Waxdal, M.J. (1969). The covalent structure of an entire γ G immunoglobulin molecule. *Proceedings of the National Academy of Sciences USA* **63**, 78 - 85.

Enshell-Seiffers, D., Smelyanski, L. & Gershoni, J.M. (2001). The rational design of a 'type 88' genetically stable peptide display vector in the filamentous bacteriophage fd. *Nucleic Acids Research* **29**, e50.

Eshaghi, M., Ta, W.S. & Yusoff, K. (2005). Identification of epitopes in the nucleocapsid protein of Nipah virus using a linear phage-displayed random peptide library. *Journal of Medical Virology* **75**, 147 - 152. Abstract only.

Fack, F., Hügle-Dörr, B., Song, D., Queitsch, I., Petersen, G., Bautz, E.K.F. (1997). Epitope mapping by phage display: random versus gene-fragment libraries. *Journal of Immunological Methods* **206**, 43 - 52.

Fehrson, J. & du Plessis, D.H. (1999). Cross-reactive epitope mimics in a fragmented-genome phage display library derived from the rickettsia, *Cowdria ruminantium*. *Immunotechnology* **4**, 175 - 184.

Fehrson, J., van Wyngaardt, W., Mashau, C., Potgieter, A.C., Chaudhary, V.K., Gupta, A., Jordaan, F.A. & du Plessis D.H. (2005). Serogroup-reactive and type-specific detection of bluetongue virus antibodies using chicken scFvs in inhibition ELISAs. *Journal of Virological Methods* **129**, 31 - 39.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. & Cesareni, G. (1991). Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *Journal of Molecular Biology* **222**, 301 - 310.

Fink, A.L. (1999). Chaperone-mediated protein folding. *Physiological Reviews* **79**, 425 - 449.

Finlay, W.J.J., Shaw, I., Reilly, J.P. & Kane, M. (2006). Generation of high-affinity chicken single-chain Fv antibody fragments for measurement of *Pseudonitzschia pungens* toxin domoic acid. *Applied and Environmental Microbiology* **72**, 3343 - 3349.

Florio, W., Bottai, D., Batoni, G., Esin, S., Pardini, M., Maisette, G. & Campa, M. (2002). Identification, molecular cloning and evaluation of potential use of isocitrate dehydrogenase II of *Mycobacterium bovis* BCG in serodiagnosis of tuberculosis. *Clinical and Diagnostic Laboratory Immunology* **9**, 846 - 851.

Foord, A.J., Muller, J.D., Yu, M.Y., Wang, L., & Heine, H.G. (2007). Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. *Journal of Immunological Methods* **321**, 142 - 151.

Fossati, G., Izzo, G., Rizzi, E., Gancia, E., Modena, D., Moras, M.L., Niccolai, N., Giannozzi, E., Spiga, O., Bono, L., Marone, P., Leone, E., Mangili, F., Harding, S., Errington, N., Walters, C., Henderson, B., Roberts, M.M., Coates, A.R.M., Casette, B & Mascagni, P. (2003). *Mycobacterium tuberculosis* chaperonin 10 is secreted in the macrophage phagosome: is secretion due to dissociation and adoption of a partially helical structure at the membrane? *Journal of Bacteriology* **185**, 4256 - 4267.

Frenken, L.G.J., van der Linden, R.H.J., Hermans, P.W.J.J., Bos, J.W., Ruuls, R.C., de Geus, B., & Verrips, C.T. (2000). Isolation of antigen specific Llama V_{HH} antibody fragments and their high level secretion by *Saccharomyces cerevisiae*. *Journal of Biotechnology* **78**, 11 - 21.

Freyre, F.M., Vázquez, J.E., Ayala, M., Canaán-Haden, L., Bell, H., Rodríguez, I., González, A., Cintado, A., Gavilondo, J.V. (2000). Very high expression of anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. *Journal of Biotechnology* **76**, 157 - 163.

Gach, J.S., Maurer, M., Hahn, R., Gasser, B., Mattanovich, D., Katinger, H. & Kunert, R. (2007). High level expression of a promising anti-idiotypic antibody fragment vaccine against HIV-1 in *Pichia pastoris*. *Journal of Biotechnology* **128**, 735 - 746.

Garnier, T., Eiglmeier, K., Camus, J., Medina, N., Mansoor H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Dogget, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V. & Hewinson, R.G. (2003). The complete genome sequence of *Mycobacterium bovis*. *Proceedings of the National Academy of Sciences USA* **100**, 7877 - 7882.

Gasparyan, V.K. (2005). Hen egg immunoglobulin Y colloidal gold agglutination assay: Comparison with rabbit immunoglobulin G. *Journal of Clinical Laboratory Analysis* **19**, 124 - 127.

Gellissen, G. (2000). Heterologous protein production in methylotrophic yeasts. *Applied Microbiology and Biotechnology* **54**, 741 - 750.

GeneTailor™ Site-Directed Mutagenesis System Instruction Manual. For standard and high-throughput in vitro site-directed mutagenesis. Version G, 8 April 2004.

Geoghegan, W.D. & Ackerman G.A. (1977). Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new methods, theory and application. *The Journal of Histochemistry and Cytochemistry* **25**, 1187 - 1200.

Geysen, H.M., Rodda, S.J. & Mason, T.J. (1986). *A priori* delineation of a peptide which mimics a discontinuous antigenic determinant. *Molecular Immunology* **23**, 709 - 715.

Goodchild, S.A., Hopkins, N.A.E., Love, T.E., Patel, V., Houghton, B.C. & Mayers, C. (2004). Engineering recombinant antibodies to detect biological warfare agents. Wiltshire: Porton Down Defence Science and Technology Laboratory.

Graham, F.L., Smiley, J., Russel, W.C. & Nairn, R. (1977). Characteristics of human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology* **36**, 59 - 72.

Gram, H., Marconi, L., Barbas III, C.F., Collet, T.A., Lerner, R.A. & Kang, A.S. (1992). *In vitro* selection and affinity maturation of antibodies from a naïve combinatorial immunoglobulin library. *Proceedings of the National Academy of Sciences USA* **89**, 3576 - 3580.

Greunke, K., Spillner, E., Braren, I., Seismann, H., Kainz, S., Hahn, U., Grunwald, T. & Bredehorst, R. (2006). Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody formats. *Journal of Biotechnology* **124**, 446 - 456.

Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, .E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E. & Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO Journal* **13**, 3245 - 3260.

Grosse-Hovest, L., Müller, S., Minoia, R., Wolf, E., Zakhartchenko, V., Wenigerkind, H., Lassnig, C., Besenfelder, U., Müller, M., Lytton, S.D., Jung, G. & Brem, G. (2004). Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. *Proceedings of the National Academy of Sciences USA* **101**, 6858 - 6863.

Gupta, S., Arora, K., Sampath, A., Singh, S.S., Gupta, A. & Chaudhary, V.K. (2001). Mapping of HIV-1 Gag epitopes recognized by polyclonal antibodies using gene-fragment phage display system. *Preparative Biochemistry and Biotechnology* **31**, 185 - 200. Abstract only.

Güssow, D. & Clackson, T. (1989). Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Research* **17**, 4000.

Gustafsson, C., Govindarajan, S. & Minshull, J. (2004). Codon bias and heterologous protein expression. *Trends in Biotechnology* **22**, 346 - 353.

Hainfield, J.F. & Powell, R.D. (2000). New frontiers in gold labelling. *The Journal of Histochemistry and Cytochemistry* **48**, 471 - 480.

Hajeer, A.H., Worthington, J., Morgan, K. & Bernstein, R.M. (1992). Monoclonal antibody epitopes of mycobacterial 65-kD heat-shock protein defined by epitope scanning. *Clinical and Experimental Immunology* **89**, 115 - 119.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95 - 98.

Hanes, J., Schaffitzel, C., Knappik, A. & Plückthun, A. (2000). Picomolar affinity antibodies from a fully synthetic naïve library selected and evolved by ribosome display. *Nature Biotechnology* **18**, 1287 - 1292.

Hasegawa, N., Miura, T., Ighii, K., Yamaguchi, K., Lindner, T., Merrit, S., Matthews, J.D. & Siddiqi, S.H. (2002). New simple and rapid test for culture confirmation of *Mycobacterium tuberculosis* complex: a multicenter study. *Journal of Clinical Microbiology* **40**, 908 - 912.

Ho, S.N., Hunt, H.D., Horton, R. M., Pullen, J.K. & Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51 - 59.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P. & Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Research* **19**, 4133 - 4137.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. & Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61 - 68.

- Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. & Robinson, R.R. (1988).** Secretion of functional antibody and Fab fragment from yeast cells. *Proceedings of the National Academy of Sciences USA* **85**, 8678 - 8682.
- Hosse, R.J., Rothe, A. & Power, B.E. (2006).** A new generation of protein display scaffolds for molecular recognition. *Protein Science* **15**, 14 - 27.
- Hudson, P.J. & Kortt, A.A (1999).** High avidity scFv multimers: diabodies and triabodies. *Journal of Immunological Methods* **231**, 177 - 189.
- Hust, M., Jostock, T., Manzel, C., Voedisch, B., Mohr, A., Brenneis, M., Kirsch, M.I., Meier, D. & Dübel, S. (2007).** Single chain Fab (scFab) fragment. *BMC Biotechnology* **7**.
- Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M., Novotný, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. & Opperman, H. (1988).** Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **85**, 5879 - 5883.
- Inoue, H., Nojima, H & Okayama, H. (1990).** High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23 - 28.
- Irving, R.A., Kortt, A.A. & Hudson, P.J. (1996).** Affinity maturation of recombinant antibodies using *E. coli* mutator cells. *Immunotechnology* **2**, 127 - 143.
- Izard, J.W. & Kendall, D.A. (1994).** Signal peptides: exquisitely designed transport promoters. *Molecular Microbiology* **13**, 765 - 773.
- Janknecht, R., de Martynhoff, G., Lou, J., Hipskind, R.A., Nordheim, A. & Stunnenberg, H.G. (1991).** Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proceedings of the National Academy of Sciences USA* **88**, 8972 - 8976.
- Johansson, D.X., Drakenberg, K., Hopmann, K.H., Schmidt, A., Yari, F., Hinkula, J. & Persson, M.A.A. (2007).** Efficient expression of recombinant human monoclonal antibodies in *Drosophila* S2 cells. *Journal of Immunological Methods* **318**, 37 - 46.
- Jolly, C.L., Huang, J. & Holmes, I.H. (2001).** Selection of rotavirus VP4 cell receptor binding domains for MA104 cells using a phage display library. *Journal of Virological Methods* **98**, 41 - 51. Abstract only.
- Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. & Winter, G. (1986).** Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522 - 525.

- Jönsson, U., Malmqvist, M. & Rönnberg, I. (1985).** Immobilization of immunoglobulins on silica surfaces. *Biochemistry Journal* **227**, 363 - 371.
- Joosten, V., Lokman, C., Van Den Hondel, C.A.M.J.J. & Punt, P.J. (2003).** The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. *Microbial Cell Factories* **2**.
- Juliarena, M., Gutierrez, S. & Ceriani, C. (2007).** Chicken antibodies: a useful tool for antigen capture ELISA to detect bovine leukaemia virus without cross-reaction with other mammalian antibodies. *Veterinary Research Communications* **31**, 43 - 51.
- Jung, S. & Plückthun, A. (1997).** Improving in vivo folding and stability of a single-chain Fv antibody fragment by loop grafting. *Protein Engineering* **10**, 959 - 966.
- Jung, S., Honegger, A. & Plückthun, A. (1999).** Selection for improved protein stability by phage display. *Journal of Molecular Biology* **294**, 163 - 180.
- Jurado, P., Ritz, D., Beckwith, J., de Lorenzo, V. & Fernández, L.A. (2002).** Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*. *Journal of Molecular Biology* **320**, 1 - 10.
- Kalvatchev, Z., Alexandrov, E., Dontcheva, R. & Madjurova, A. (1993).** Use of immunogold assay for rapid evaluation of antigens of spotted fever group of *Rickettsiae*. *Acta Virologica* **37**, 184 - 186.
- Kang, A.S., Jones, T.M. & Burton, D.R. (1991).** Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proceedings of the National Academy of Sciences USA* **88**, 11120 - 11123.
- Kipriyanov, S.M., Moldenhauer, G. & Little, M. (1997).** High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *Journal of Immunological Methods* **200**, 69 - 77.
- Klewitz, T., Gessler, F., Beer, H., Pflanz, K. & Scheper, T. (2006).** Immunochromatographic assay for determination of botulinum neurotoxin type D. *Sensors and Actuators B* **113**, 582 - 589.
- Kleymann, G., Ostermeier, C., Heitmann, K., Haase, W. & Michel, H. (1995).** Use of antibody fragments (Fv) in immunocytochemistry. *The Journal of Histochemistry and Cytochemistry* **43**, 607 - 614.
- Ko, K. & Koprowski, H. (2005).** Plant biopharming of monoclonal antibodies. *Virus Research* **111**, 93 - 100.
- Köhler, G. & Milstein, C. (1975).** Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495 - 497.

Kolonin, M.G., Bover, L., Sun, J., Zúrita, A.J., Do, K., Lahdenranta, J., Cardó-Vila, M., Giordano, R.J., Jaalouk, D.E., Ozawa, M.G., Moya, C.A., Souza, G.R., Staquicini, F.I., Kunyiasu, A., Scudiero, D.A., Holbeck, S.L., Sausville, E.A., Arap, W. & Pasqualini, R. (2006). Ligand-directed surface profiling of human cancer cells with combinatorial peptide libraries. *Cancer Research* **66**, 33 - 40. Abstract only.

Kong, T.H., Coates, A.R.M., Butcher, P.D., Hickman, C.J. & Shinnick, T.M. (1993). *Mycobacterium tuberculosis* expresses two chaperonin-60 homologues. *Proceedings of the National Academy of Sciences USA* **90**, 2608 - 2612.

Kopetzki, E., Jekle, A., Ji, C., Rao, E., Zhang, J., Fischer, S., Cammack, N., Sankurarti, S. & Heilek, G. (2008). Closing two doors of viral entry: Intramolecular combination of a coreceptor- and fusion inhibitor of HIV-1. *Virology Journal* **5**.

Kuroda, K., Kobayashi, K., Kitagawa, Y., Nakagawa, T., Tsumura, H., Komeda, T., Shinmi, D., Mori, E., Motoki, K., Fujii, K., Sakai, T., Nonaka, K., Suzuki, T., Ichikawa, K., Chiba, Y. & Jigami, Y. (2008). Efficient antibody production upon suppression of *O*-mannosylation in the yeast *Ogataea minuta*. *Applied and Environmental Microbiology* **74**, 446 - 453.

Ladner, R.C. (1995). Constrained peptides as binding entities. *Tibtech* **13**, 426 - 430.

Laffly, E. & Sodoyer, R. (2005). Monoclonal and recombinant antibodies, 30 years after....*Human Antibodies* **14**, 33 - 55.

Lange, S., Schmitt, J. & Schmid, R.D. (2001). High-yield expression of recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*. *Journal of Immunological Methods* **255**, 130 - 114.

Larsson, A., Karisson-Parra, A. & Sjöquist, J. (1991). Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. *Clinical Chemistry* **37**, 411 - 414.

Laver, W.G., Air, G.M., Webster, R.G. & Smith-Gill, S.J. (1990). Epitopes on protein antigens: misconceptions and realities. *Cell* **61**, 553 - 556.

Lee, D.Y., Cho, Y.W., Kang, S.G., Shin, S.J. & Yoo, H.S. (2004). Development of a novel antigen capture-ELISA using IgY against porcine interleukin-6 and its application. *Journal of Veterinary Science* **5**, 337 - 343.

Lee, Y., Leu, S.C., Hung, H., Wu, H., Huang, I., Hsieh, W., Chiu, W., Hsieh, M., Cheng, T. & Yang, Y. (2007). A dominant antigenic epitope on SARS-CoV spike protein identified by an avian single-chain variable fragment (scFv)-expressing phage. *Veterinary Immunology and Immunopathology* **117**, 75 - 85.

Levy, N.S., Malipiero, U.V., Lebecque, S.G. & Gearhart, P.J. (1989). Early onset of somatic mutation in immunoglobulin V_H genes during the primary immune response. *Journal of Experimental Medicine* **169**, 2007 - 2019.

Li, H., Sethuraman, N., Stadheim, T.A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y., Kim, N., Mansfield, R., Nett, J.H., Rios, S., Strawbridge, R., Wildt, S. & Gerngross, T.U. (2006). Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nature Biotechnology* **24**, 210 - 215.

Linn, S. & Arber, W. (1968). Host specificity of DNA produced by *Escherichia coli*, X. *In vitro* restriction of phage fd replicative form. *Proceedings of the National Academy of Sciences USA* **59**, 1300 - 1306.

Lipman, N.S., Jackson, L.R., Trudel, L.J. & Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information resources. *ILAR Journal* **46**, 258 - 268.

Lu, B., Smyth, M.R. & O'Kennedy, R. (1996). Orientated immobilization of antibodies and its application in immunoassays and immunosensors. *The Analyst* **121**, 29R - 32R.

Lugazzo, A., Felici, F., Tramontano, A., Pessi, A. & Cortese, R. (1993). Mimicking of discontinuous epitopes by phage-displayed peptides, I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene* **128**, 51 - 57.

Lundquist, R., Nielsen, L.K., Jafarshad, A., SoeSoe, D., Christensen, L.H., Druilhe, P. & Dziegiel, M.H. (2006). Human recombinant antibodies against *Plasmodium falciparum* merozoite surface protein 3 cloned from peripheral blood leukocytes of individuals with immunity to malaria demonstrate antiparasitic properties. *Infection and Immunity* **74**, 3222 - 3231.

Lyashchenko, K.P., Singh, M., Colangeli, R. & Gennaro, M.L. (2000). A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods* **242**, 91 - 100.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Olsen, J.H., Ball, R., Dunoncaux, G., Dunker, F., Buckley, C., Richard, M., Murray, S., Payeur, J.B., Andersen, P., Pollock, J.M., Mikota, S., Miller, M., Sofranko, D., & Waters, W.R. (2006). Tuberculosis in elephants: antibody response to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology* **13**, 722 - 732.

Lyashchenko., K.P., Greenwald, R., Esfandiari, J., Greenwald, D., Nacy, C.A., Gibson, S., Didier, P.J., Washington, M., Szczerba, P., Motzel, S., Handt, L., Pollock, J.M., McNair, J., Andersen, P., Langermans, J.A.M., Verreck, F., Ervin, S., Ervin, F. & McCombs, C. (2007). PrimaTB STAT-PAK assay, a novel, rapid lateral-flow test for tuberculosis in non-human primates. *Clinical and Vaccine Immunology* **14**, 1158 - 1165.

Majander, K., Anton, L., Antikainen, J., Lång, H., Brummer, M., Korhonen, T.K. & Westerlund-Wikström, B. (2005). Extracellular secretion of polypeptides using a modified *Escherichia coli* flagellar secretion apparatus. *Nature Biotechnology* **23**, 475 - 481.

Makrides, S.C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiological Reviews* **60**, 512 - 538.

Marks, J.D., Griffiths, A.D., Malmqvist, M., Clackson, T.P., Bye, J.M. & Winter, G. (1992). By-passing immunization: building high affinity human antibodies by chain shuffling. *Nature* **10**, 779 - 783.

Matson, R.S. & Little, M.C. (1988). Strategy for the immobilization of monoclonal antibodies on solid-phase supports. *Journal of Chromatography B* **458**, 67 - 77. Abstract only.

Matthews, L.J., Davis, R. & Smith, G.P. (2002). Immunogenically fit subunit vaccine component via epitope discovery from natural peptide libraries. *Journal of Immunology* **169**, 837 - 846.

Max, E.E., Seidman, J.G. & Leder P. (1979). Sequences of five potential recombination sites encoded close to an immunoglobulin κ constant region gene. *Genetics* **76**, 3450 - 3454.

Mayfield, S.P. & Franklin, S.E. (2005). Expression of human antibodies in eukaryotic micro-algae. *Vaccine* **23**, 1828 - 1832.

Maynard, J. & Georgiou, G. (2000). Antibody engineering. *Annual Review of Biomedical Engineering* **2**, 339 - 376.

Mayrose, I., Penn, O., Rubinstein, N.D., Shlomi, T., Tarnovitski Freund, N., Bublil, E.M., Ruppim, E., Sharan, R., Gershoni, J.M., Martz, E. & Pupko, T. (2007a). Pepitope: epitope mapping from affinity-selected peptides. *Bioinformatics* **23**, 3244 - 3246.

Mayrose, I., Shlomi, T., Rubinstein, N.D., Gershoni, J.M., Ruppim, E., Sharan, R. & Pupko, T. (2007b). A graph-based algorithm for epitope mapping using combinatorial phage-display libraries. *Nucleic Acids Research* **35**, 69 - 78.

- Mazor, Y., van Blarcom, T., Mabry, R., Iverson, B.L. & Georgiou, G. (2007). Isolation of engineered full-length antibodies from libraries expressed in *Escherichia coli*. *Nature Biotechnology* **25**, 563 - 565.
- McCafferty, J., Griffiths, A.D., Winter, G. & Chiswell, D.J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552 - 554
- McCormack, W.T., Tjoelker, L.W. & Thompson, C.B. (1991). Avian B-Cell development: Generation of an immunoglobulin repertoire by gene conversion. *Annual Review in Immunology* **9**, 219 - 241.
- Michel, A.L., Bengis, R.G., Keet, D.F., Hofmeyr, M., De Klerk, L.M., Cross, P.C., Jolles, A.E., Cooper, D., Whyte, I.J., Buss, P. & Godfroid, J. (2006). Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Veterinary Microbiology* **112**, 91 - 100.
- Miyamoto, K., Shimamoto, T., Aosasa, M., Kimura, S., Nakamura, N., Okubo, Y., Yokoyama, T., Horiuchi, H., Furusawa, S. & Matsuda, H. (2007). Development of recombinant chicken IgY from single chain fragment variable region for diagnosis of BSE. *Biologicals* **35**, 31 - 34.
- Mullaney, B.P., Pallavicini, M.G. & Marks, J.D. (2001). Epitope mapping of neutralising botulinum neurotoxin A antibodies by phage display. *Infection and Immunity* **69**, 6511 - 6514.
- Muyldermans, S. (2001). Single domain camel antibodies: current status. *Reviews in Molecular Biology* **74**, 277 - 302.
- Myers, E.W. & Miller, W. (1989). Optimal alignments in linear space. *Computer Applications in the Biosciences: CABIOS* **4**, 11 - 17. Abstract only.
- Nakamura, N., Shuyama, A., Hojyo, S., Shimokawa, M., Miyamoto, K., Kawashima, T., Aosasa, M., Furusawa, S. & Matsuda, H. (2004). Establishment of a chicken monoclonal antibody panel against mammalian prion protein. *Journal of Veterinary Medical Science* **66**, 807 - 814.
- Narat, M. (2003). Production of antibodies in chickens. *Food Technology and Biotechnology* **41**, 259 - 267.
- Nevalainen, K.M.H., Te'o, V.S.J. & Bergquist, P.L. (2005). Heterologous protein expression in filamentous fungi. *Trends in Biotechnology* **23**, 468 - 474.
- Nielsen, K., Yu, W.L., Lin, M., Davis, N., Elmgren, C., MacKenzie, R., Tanha, J., Li, S., Dubuc, G., Brown, E.G., Keleta, L. & Pasick, J. (2007). Prototype single step lateral flow technology for detection of avian influenza

virus and detection of chicken antibody to avian influenza virus. *Journal of Immunoassay and Immunochemistry* **28**, 307 - 318.

Nilsson, E. & Larsson, A. (2007). Stability of chicken IgY antibodies freeze-dried in the presence of lactose, sucrose and threolose. *The Journal of Poultry Science* **44**, 58 - 62.

Ning, D., Junjian, X., Qing, Z., Sheng, X., Wenyin, C., Guirong, R. & Xunzhang, W. (2005). Production of recombinant humanized anti-HBsAg Fab fragment from *Pichia pastoris* by fermentation. *Journal of Biochemistry and Molecular Biology* **38**, 294 - 299.

Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D. & Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *The EMBO Journal* **13**, 692 - 698.

O'Connell, K.P., Kovaleva, E., Campbell, J.H., Anderson, P.E., Brown, S.G., Davis, D.C., Valdes, J.J., Welch, R.W., Bently, W.E. & van Beek, N.A. (2007). Production of a recombinant antibody fragment in whole insect larvae. *Molecular Biotechnology* **36**, 44 - 51.

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.3: Bovine tuberculosis. Last updated 23.07.2004. Downloaded from http://www.oie.int/eng/normes/MANUAL/A_00054.htm on 02.05.2007 at 14:53 pm.

O'Neil, K., Hoess, R.H., Jackson, S.A., Ramachandran, N.S., Mousa, S.A., DeGrado, W.F. (1992). Identification of novel peptide antagonists for GpIIa/IIIa from a conformationally constrained phage peptide library. *Proteins: Structure, Function, and Bioinformatics* **14**, 509 - 515. Abstract only.

Orthner, C.L., Highsmith, F.A., Tharakan, J., Madurawe, R.D., Morcol, T. & Velander, W.H. (1991). Comparison of the performance of immunosorbents prepared by site-directed or random coupling of monoclonal antibodies. *Journal of Chromatography B* **558**, 55 - 70. Abstract only.

Palmer, M.V., Waters, W.R., Thacker, T.C., Greenwald, R., Esfandiari, J. & Lyashchenko, K.P. (2006). Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with *Mycobacterium bovis*. *Clinical and Vaccine Immunology* **13**, 387 - 394.

Parmley, S.F. & Smith, G.P. (1988). Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* **73**, 305 - 318.

Parvari, R., Avivi, A., Lentner, F., Ziv, E., Tel-Or, S., Burstein, Y. & Schechter, I. (1988). Chicken immunoglobulin γ -heavy chains: limited V_H gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. *The EMBO Journal* **7**, 739 - 744.

Parvari, R., Ziv, E., Lantner, F., Heller, D. & Schechter, I. (1990). Somatic diversification of immunoglobulin light chains by point mutations. *Proceedings of the National Academy of Sciences USA* **87**, 3072 - 3076.

Peng D., Hu, S., Hua, Y., Xiao, Y., Li, Z., Wang, X. & Bi., D. (2007). Comparison of a new gold-immunochromatographic assay for the detection of antibodies against avian influenza virus with hemagglutination inhibition and agar gel immunodiffusion assays. *Veterinary Immunology and Immunopathology* **117**, 17 - 25.

Perlman, D. & Halvorson, H.O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptidases. *Journal of Molecular Biology* **167**, 391 - 409.

Petrenko, V.A., Smith, G.P., Mazooji, m.M. & Quinn, T. (2002). α -Helically constrained phage display library. *Protein Engineering* **15**, 943 - 950.

Phillips, A.O., Steadman, R., Donovan, K.D. & Williams, J.D. (1995). A new antibody capture enzyme linked immunoassay specific for transforming growth factor beta. *International Journal of Biochemistry and Cell Biology* **27**, 207 - 213.

Piechaczek, C., Fetzer, C., Baiker, A., Bode, J. & Lipps, H.J. (1999). A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. *Nucleic Acids Research* **27**, 426 - 428.

Plückthun, A. & Pack, P. (1997). New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* **3**, 83 - 105.

Pollock, J.M. & Neill, S.D. (2002). *Mycobacterium bovis* infection and tuberculosis in cattle. *The Veterinary Journal* **163**, 115 - 127.

Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E. & van der Merwe, K.J. (1985). Improvements in the isolation of IgY from the yolks of eggs laid by immunised hens. *Immunological Investigations* **14**, 323 - 327.

Pons, L. (2002). An easy, inexpensive test detects tuberculosis in livestock and wildlife. *Agricultural Research* **Nov**, 20 - 21.

Porter, R.R. (1959). The hydrolysis of rabbit γ -globulin and antibodies with crystalline papain. *Biochemistry Journal* **73**, 119 - 126.

Powers, D.B., Amersdorfer, P., Poul, M., Nielsen, U.B., Shalaby, M.R., Adams, G.P., Weiner, L.M. & Marks, J.D. (2001). Expression of single-chain Fv-Fc fusions in *Pichia pastoris*. *Journal of Immunological Methods* **251**, 123 - 135.

Prescott, L.M., Harley, J.P. & Klein, D.A. (1999). *Microbiology*, 4th Ed. USA: McGraw Hill.

Qamra, R. & Mande, S. (2004). Crystal structure of the 65-kilodalton heat shock protein, chaperonin 60.2, of *Mycobacterium tuberculosis*. *Journal of Bacteriology* **186**, 8105 - 8113.

Qamra, R., Srinivas, V. & Mande, S.C. (2004). *Mycobacterium tuberculosis* GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. *Journal of Molecular Biology* **342**, 605 - 617.

Quintero-Hernández, V., Juárez-González, V.R., Ortíz-León, M., Sánchez, R., Possani, L.D. & Becerril, B. (2007). The change of the scFv into the Fab format improves the stability and *in vivo* toxin neutralization capacity of recombinant antibodies. *Molecular Immunology* **44**, 1307 - 1315.

Rambukkana, A., Das, P.K., Burggraaf, J.D., Faber, W.R., Teeling, P., Krieg, S., Thole, J.E.R., & Harboe, M. (1992). Identification and characterization of epitopes shared between the Mycobacterial 65-kilodalton heat shock protein and the actively secreted antigen 85-complex: Their *in situ* expression on the cell wall surface of *Mycobacterium leprae*. *Infection and Immunity* **60**, 4517 - 4527.

Rand, K.N. (1996). Crystal violet can be used to visualize DNA bands during gel electrophoresis and to improve cloning efficiency. *Elsevier Trends Journals Technical Tips Online* **1**. <http://biomednet.com>.

Ratcliffe, M.J.H. (2006). Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Developmental and Comparative Immunology* **30**, 101 - 118.

Read, J.D., Colussi, P.A., Ganatra, M.B. & Taron, C.H. (2007). Acetamide selection of *Kluyveromyces lactis* cells transformed with an integrative vector leads to high-frequency formation of multicopy strains. *Applied and Environmental Microbiology* **73**, 5088 - 5096.

Reynaud, C., Anquez, V., Dahan, A. & Weill, J. (1985). A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. *Cell* **40**, 283 - 291.

Reynaud, C., Dahan, A., Anquez, V. & Weill, J. (1989). Somatic hyperconversion diversifies the single VH gene of the chicken with a high incidence in the D region. *Cell* **59**, 171 - 183.

Roitt, I.M. (1988). *Essential Immunology*, 6th Ed. London: Blackwell Scientific Publications.

Robinson, N. (2002). Immunogold conjugation for IVD applications. *IVD Technology* **8**, 33 - 37.

Sakaguchi, M. (1997). Eukaryotic protein secretion. *Current Opinion in Biotechnology* **8**, 595 - 601.

Sakano, H., Hüppi, K., Heinrich, G. & Tonegawa, S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature* **280**, 288 - 294.

Sakula, A. (1983). Robert Koch: Centenary of the discovery of the tubercle bacillus, 1882. *Canadian Veterinary Journal* **24**, 127 - 131.

Sambrook, J. & Russel, D.W. (2001). *Molecular Cloning. A laboratory manual*, 3rd Ed. New York: Cold Spring Harbor Laboratory Press.

Sánchez, L., Ayala, M., Freyre, F., Pedroso, I., Bell, H., Falcón, V. & Gavilondo, J.V. (1999). High cytoplasmic expression in *E. coli*, purification, and *in vitro* refolding of a single chain Fv antibody fragment against hepatitis B surface antigen. *Journal of Biotechnology* **72**, 13 - 20.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**, 5463 - 5467.

Sato, Y., Nishimichi, N., Nakano, A., Takikawa, K., Inoue, N., Matsuda, H. & Sawamura, T. (2008). Determination of LOX-1 ligand activity in mouse plasma with a chicken monoclonal antibody for ApoB. *Atherosclerosis*, article in press doi:10.1016/j.atherosclerosis.2008.02.001.

Sblattero, D. & Bradbury, A. (2000). Exploiting recombination in single bacteria to make large phage antibody libraries. *Nature Biotechnology* **18**, 75 - 80.

Scallon, B., Cai, A., Radewonuk, J. & Naso, M. (2004). Addition of an extra immunoglobulin domain to two anti-rodent TNF monoclonal antibodies substantially increased their potency. *Molecular Immunology* **41**, 73 - 80.

Schaffrath, R. & Breuning, K.D. (2000). Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. *Fungal Genetics and Biology* **30**, 173 - 190.

Schimmele, B. & Pluckthun, A. (2005). Engineering proteins for stability and efficient folding. In *Protein Folding Handbook. Part II*, pp. 1281 - 1333. Edited by J. Buchner & T. Kiefhaber. Weinheim: Wiley-VCH Verlag GmbH & Co.

Schirrmann, T., Al-Halabi, L., Dübel, S. & Hust, M. (2008). Production systems for recombinant antibodies. *Frontiers in Bioscience* **13**, 4576 - 4594.

Scott, J.K. & Barbas, C.F. III (2001). Phage-display vectors. In *Phage display - A laboratory manual*, pp 2.1 - 2.19. Edited by C.F. Barbas III, D.R. Burton, J.K. Scott & G.J. Silverman. Cold Spring Harbor Laboratory Press, New York.

Scott, J.K. & Smith, G.P. (1990). Searching for peptide ligands with an epitope library. *Science* **249**, 386 - 390.

Sela, M. (1969). Antigenicity: some molecular aspects. *Science* **166**, 1365 - 1374.

Sethuraman, N. & Stadheim, T.A. (2006). Challenges in therapeutic glycoprotein production. *Current Opinion in Biotechnology* **17**, 341 - 346.

Sharma, A., Saha, A., Bhattacharjee, S., Majumdar, S. & Das Gupta, S.K. (2006). Specific and randomly derived immunoactive peptide mimotopes of mycobacterial antigens. *Clinical and Vaccine Immunology* **13**, 1143 - 1154.

Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindquist, E., Schier, R., Hemingsen, G., Wong, C., Gearhart, J.C. & Marks, J.D. (1998). Efficient construction of a large nonimmune phage antibody library: The production of high-affinity single-chain antibodies to protein antigens. *Proceedings of the National Academy of Sciences USA* **95**, 6157 - 6162.

Shen, J., Vil, M.D., Jimenez, X., Iacolina, M., Zhang, H. & Zhu, Z. (2006). Single variable domain-IgG fusion. A novel recombinant approach to Fc domain-containing bispecific antibodies. *Journal of Biological Chemistry* **281**, 10706 - 10714.

Shi, J., Chang, X., Feng, J., Cheng, Y., Cheng, H., Guo, H., Ye, X. & Cui, H. (2007). Expression of an ovarian cancer anti-idiotypic antibody (6B11VLVHCH3) in Chinese hamster ovary (CHO) cells with improved immunoactivity and stability over proteins expressed in prokaryotic cells. *Hybridoma* **26**, 289 - 295.

Shields, M.J., Siegel, J.N., Clark, C.R., Hines, K.K., Potempa, L.A., Gewurz, H. & Anderson, B. (1991). An appraisal of polystyrene- (ELISA) and nitrocellulose-based (ELIFA) enzyme immunoassay systems using monoclonal antibodies reactive toward antigenically distinct forms of human C-reactive protein. *Journal of Immunological Methods* **141**, 253 - 261.

Shimamoto, T., Nishibori, N., Aosasa, M., Horiuchi, H., Furusawa, S. & Matsuda, H. (2005). Stable production of recombinant chicken antibody in CHO-K1 cell line. *Biologicals* **33**, 169 - 174.

Shinnick, T.M., Sweetser, D., Thole, J., van Embden, J. & Young, R.A. (1987). The etiologic agents of leprosy and tuberculosis share an immunoreactive protein antigen with the vaccine strain *Mycobacterium bovis* BCG. *Infection and Immunity* **55**, 1932 - 1935.

Shinnick, T.M., Vodkin, M.H. & Williams, J.C. (1988). The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. *Infection and Immunity* **56**, 446 - 451.

Shulgin, I.L. & Ruckenstein, E. (2006). Preferential hydration and solubility of proteins in aqueous solutions of polyethylene glycol. *Biophysical Chemistry* **120**, 188 - 189.

Shyu, R., Shyu, H., Liu, H. & Tang, S. (2002). Colloidal gold-based immunochromatographic assay for detection of ricin. *Toxicon* **40**, 255 - 258.

Sigler, P.B., Xu, Z., Rye, H.S., Burston, S.G., Fenton, W.A. & Horwich, A.L. (1998). Structure and function in GroEL-mediated protein folding. *Annual Reviews in Biochemistry* **67**, 581 - 608.

Simmons, L.C., Reilly, D., Klimowski, L., Raju, T.S., Meng, G., Sims, P., Hong, K., Shields, R.L., Damico, L.A., Rancatore, P. & Yansura, D.G. (2002). Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *Journal of Immunological Methods* **263**, 133 - 147.

Skerra, A. & Plückthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038 - 1041.

Sletta, H., Tøndervik, A., Hakvåg, S., Aune, T.E.V., Nedal, A., Aune, R., Evensen, G., Valla, S., Ellingsen, T.E. & Brautaset, T. (2007). The presence of N-terminal secretion signal sequences leads to strong stimulation of the total expression levels of three tested medically important proteins during high-cell-density cultivations of *Escherichia coli*. *Applied and Environmental Microbiology* **73**, 906 - 912.

Smith, G.P. (1985). Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315 - 1316.

Smith, G.P. (1993). Surface display and peptide libraries. *Gene* **128**, 1 - 2.

Smith, G.P. & Petrenko, V.A. (1997). Phage display. *Chemical Reviews* **97**, 391 - 410.

Smith, R.A., Duncan, M.J. & Moir, D.T. (1985). Heterologous protein secretion from yeast. *Science* **229**, 1219 - 1224.

Sørensen, H.P. & Mortensen, K.K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology* **115**, 113 - 128.

Spada, S., Honegger, A. & Plückthun, A. (1998). Reproducing the natural evolution of protein structural features with selectively infective phage (SIP) technology. The kink in the first strand of antibody kappa domains. *Journal of Molecular Biology* **283**, 395 - 407.

Spitznagel, T.M. & Clark, D.S. (1993). Surface-density and orientation effects in immobilized antibodies and antibody fragments. *Bio Technology* **11**, 825 - 829.

Suazo, F.M., Escalera, A.M.A, Torres, R.M.G. (2003). A review of *M. bovis* BCG protection against TB in cattle and other animal species. *Preventive Veterinary Medicine* **58**, 1 - 13.

Swennen, D., Paul, M., Vernis, L., Beckerich, J., Fournier, A. & Gaillardin. (2002). Secretion of an active anti-Ras single-chain Fv antibody by the yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis*. *Microbiology* **148**, 41 - 50.

Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J., Lerner, R.A. & Hendrickson, W.A. (1984). The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. *Nature* **312**, 127 - 134.

Takahashi, K., Yuuki, T., Takai, T., Ra, C., Okumura, K., Yokota, T., Okumura, Y. (2000). Production of humanized Fab fragment against human high affinity IgE receptor in *Pichia pastoris*. *Bioscience, Biotechnology and Biochemistry* **64**, 2138 - 2144.

Tang, Y., Jiang, N., Parakh, C. & Hilvert, D. (1996). Selection of linkers for a catalytic single-chain antibody using phage display technology. *Journal of Biological Chemistry* **271**, 15682 - 15686.

Tao, M. & Morrison, S.L. (1989). Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector function mediated by the human IgG constant region. *Journal of Immunology* **143**, 2595 - 2601.

Taylor, G.M., Murphy, E., Hopkins, R., Rutland, P. & Christov, Y. (2007). First report of *Mycobacterium bovis* DNA in human remains from the iron age. *Microbiology* **153**, 1243 - 1249.

Terentiev, Y., Pico, A.H., Böer, E., Wartmann, T., Klabunde, J., Breuer, U., Babel, W., Suckow, M., Gellissen, G. & Kunze, G. (2004). A wide-range integrative yeast expression vector system based on *Arxula adenivorans*-derived elements. *Journal of Industrial Microbiology and Biotechnology* **31**, 223 - 228.

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology* **72**, 211 - 222.

The QIAexpressionist™. A handbook for high-level expression and purification of 6 x His-tagged proteins. Fifth Edition, June 2003.

Thole, J.E.R., Dauwerse, H.G., Das, P.K., Groothuis, D.G., Schouls, L.M. & van Embden, J.D.A. (1985). Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. *Infection and Immunity* **50**, 800 - 806.

Thole, J.E.R., Keulen, W.J., Kolk, A.H.J., Groothuis, D.G., Berwald, L.G., Tiesjema, R.H. & van Embden, J.D.A. (1987). Characterization, sequence determination and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *E. coli* K-12. *Infection and Immunity* **55**, 1466 - 1475.

Thole, J.E.R., van Schooten, W.C.A., Keulen, W.J., Hermans, P.W.M., Janson, A.A.m., de Vries, R.R.P., Kolk, A.H.J. & van Embden, J.D.A. (1988). Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. *Infection and Immunity* **56**, 1633 - 1640.

Thomas, P. & Smart, T.G. (2005). HEK293 cell clone: A vehicle for the expression of recombinant proteins. *Journal of Pharmaceutical and Toxicological Methods* **51**, 187 - 200.

Thomassen, Y.E., Meijer, W., Sierkstra, L. & Verrips, C.T. (2002). Large-scale production of V_{HH} antibody fragments by *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology* **330**, 273 - 278.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673 - 4680.

Tonegawa, S., Steinberg, C., Dube, S. & Bernardini, A. (1974). Evidence for somatic generation of antibody diversity. *Proceedings of the National Academy of Sciences USA* **71**, 4027 - 4031.

Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature* **302**, 575 - 581.

Torrance, L., Ziegler, A., Pittman, H., Paterson, M., Toth, R. & Eggleston, I. (2006). Orientated immobilisation of engineered single-chain antibodies to develop biosensors for virus detection. *Journal of Virological Methods* **134**, 164 - 170.

Trill, J.J., Shatzman, A.R. & Ganguly, S. (1995). Production of monoclonal antibodies in COS and CHO cells. *Current Opinion in Biotechnology* **6**, 553 - 560.

Trinh, R., Gurbaxani, B., Morrison, S.L. & Seyfzadeh, M. (2004). Optimization of codon pair use within the (GGGS)₃ linker sequence results in enhanced protein expression. *Molecular Immunology* **40**, 717 - 722.

Uray, K., Hudecz, F., Füst, G. & Prohászka, Z. (2003). Comparative analysis of linear epitopes on human and mycobacterial 60-kDa heat shock proteins using samples of healthy blood donors. *International Immunology* **15**, 1229 - 1236.

van Ooyen, A.J.J., Dekker, P., Huang, M., Olstroom, M.M.A., Jacobs, D.I., Colussi, P.A. & Taron, C.H. (2006). Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Research* **6**, 981 - 392.

van Regenmortel, M.H.V. (1989). Structural and functional approaches to the study of protein antigenicity. *Immunology Today* **10**, 266 - 272.

van Wyngaardt, W., Malatji, T., Mashau, C., Fehrsen, J., Jordaan, F., Miltiadou, D & du Plessis, D.H. (2004). A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnology* **4**.

van Zonneveld, A., van den Berg, B.M.M., van Meijer, M. & Pannekoek, H. (1995). Identification of functional interaction sites on proteins using bacteriophage-displayed random epitope libraries. *Gene* **167**, 49 - 52.

Vordermeier, H.M., Chambers, M.A., Buddle, B.M., Pollock, J.M. & Hewinson, R.G. (2006). Progress in the development and control of vaccines and diagnostic reagents to control tuberculosis in cattle. *The Veterinary Journal* **171**, 229 - 244.

Wang, L. & Yu, M. (2004). Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. *Current Drug Targets* **5**, 1 - 5.

Wang, L., du Plessis, D.H., White, J.R., Hyatt, A.D. & Eaton, B.T. (1995). Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. *Journal of Immunological Methods* **178**, 1 - 12.

Wang, S., Zhang, C., Wang, J. & Zhang, Y. (2005). Development of colloidal gold-based flow-through and lateral-flow immunoassays for the rapid detection of the insecticide carbaryl. *Analytica Chimica Acta* **546**, 161 - 166.

Warr, G.W., Magor, K.E. & Higgins, D.A. (1995). IgY: clues to the origins of modern antibodies. *Immunology Today* **16**, 392 - 398.

Watt, P.M. (2006). Screening for peptide drugs from the natural repertoire of biodiverse protein folds. *Nature Biotechnology* **24**, 177 - 183.

Webster, R.E. (1996). Biology of the filamentous bacteriophage. In *Phage Display of Peptides and Proteins. A laboratory Manual*, pp 1 - 34. Edited by B.K. Kay, J. Winter & J. McCaffery. Academic Press: San Diego.

Weldingh, K., Rosenkrands, I., Okkels, L., Doherty, T.M. & Andersen, P. (2005). Assessing the serodiagnostic potential of 35 *Mycobacterium tuberculosis* proteins and identification of four novel serological antigens. *Journal of Clinical Microbiology* **43**, 57 - 65.

Werner, R.G., Kopp, K. & Schlueter, M. (2007). Glycosylation of therapeutic proteins in different production systems. *Acta Paediatrica* **96**, 17 - 22.

Wernery, U., Kinne, J., Jahans, K.L., Vordermeier, H.M., Esfandiari, J., Greenwald, R., Johnson, B., Ul-Haq, A. & Lyashchenko, K.P. (2007). Tuberculosis outbreak in a dromedary racing herd and rapid serological detection of infected camels. *Veterinary Microbiology* **122**, 180 - 115.

Whipple, D.L., Palmer, M.V., Slaughter, R.E. & Jones, S.L. (2001). Comparison of purified protein derivatives and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. *Journal of Veterinary Diagnostic Investigation* **13**, 117 - 122.

Williamson, R.A., Burioni, R., Sanna, P.P., Partridge, L.J., Barbas III, C.F. & Burton, D.R. (1993). Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. *Proceedings of the National Academy of Sciences USA* **90**, 4141 - 4145.

Winter, G., Griffiths, A.D., Hawkins, R.E. & Hoogenboom, H.R (1994). Making antibodies by phage display. *Annual Reviews in Immunology* **12**, 433 - 455.

Wood, P.R. & Jones S.L. (2001). BOVIGAM™: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis* **81**, 147 - 155.

Wörn, A. & Plückthun, A. (2001). Stability engineering of antibody single-chain Fv fragments. *Journal of Molecular Biology* **305**, 898 - 1010.

Wuertzer, C.A., Sullivan, M.A., Qiu, X. & Federoff H.J. (2008). CNS delivery of vectored prion-specific single-chain antibodies delays disease onset. *Molecular Therapy* **16**, 481 - 486. Abstract only.

Xu, J.L. & Davis, M.M. (2000). Diversity in the CDR3 region of V_H is sufficient for most antibody specificities. *Immunity* **13**, 37 - 45.

Xu, Z. & Sigler, P.B. (1998). GroEL/GroES: Structure and function of a two-stroke folding machine. *Journal of Structural Biology* **124**, 129 - 141.

Yamane-Ohnuki, N., Kinoshita, S., Inoue-Urakubo, M., Kusunoki, M., Iida, S., Nakano, R., Wakitani, M., Niwa, R., Sakurada, M., Uchida, K., Shitara, K. & Satoh, M. (2004). Establishment of *FUT8* knockout Chinese hamster ovary cells: an ideal host line for producing completely defucosylated antibodies with enhanced antibody-dependant cellular cytotoxicity. *Biotechnology and Bioengineering* **87**, 614 - 622.

Yaoita, Y., Matsunami, N., Choi, C.Y., Sugiyama, H., Kishimoto, T. & Honjo, T. (1983). The D-JH complex is an intermediate to the complete immunoglobulin heavy-chain V-region gene. *Nucleic Acids Research* **11**, 7303 - 7316.

Yin, J., Li, G, Ren, X. & Herrler, G. (2007). Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *Journal of Biotechnology* **127**, 335 - 347.

Young, D.B. & Garbe, T.R. (1991). Heat shock proteins and antigens of *Mycobacterium tuberculosis*. *Infection and Immunity* **59**, 3086 - 3093.

Yu, X., Gilden, D.H., Ritchie, A.M., Burgoon, M.P., Keays, K.M. & Owens, G.P. (2005). Specificity of recombinant antibodies generated from multiple sclerosis cerebrospinal fluid probed with a random peptide library. *Journal of Neuroimmunology* **172**, 121 - 131. Abstract only.

Yuhi, T., Nagatani, N., Endo, T., Kerman, K., Takata, M., Konaka, H., Namiki, M., Takamura, Y. & Tamiya, E. (2006). Gold nanoparticle based immunochromatography using a resin modified micropipette tip for rapid and simple detection of human chorionic gonadotrophin hormone and prostate-specific antigen. *Science and Technology of Advanced Materials* **7**, 276 - 281.

Zacher, A.N. III, Stock, C.A., Golden II, J.W. & Smith, G.P. (1980). A new filamentous phage cloning vector: fd-tet. *Gene* **9**, 127 - 140.

Zeocin™ Instruction Manual. Version J, August 22, 2002.

Zhang, G-P., Guo, J-Q., Wang, X-N., Yang, J-X., Yang, Y-Y., Li, Q-M., Li, X-W., Deng, R-G., Xiao, Z-J., Yang, J-F., Xing, G-X. & Zhao, D. (2006). Development and evaluation of an immunochromatographic strip for trichinellosis detection. *Veterinary Parasitology* **137**, 286 - 293.

Zhong, G., Smith, G.P., Berryl, J. & Brunham, R.C. (1994). Conformational mimicry of Chlamydial neutralization epitope on filamentous phage. *Journal of Biological Chemistry* **269**, 24183 - 24188.

Zhou, Y., Chen, Z., Purcell, R.H. & Emerson, S.U. (2007). Positive reactions on western blots do not necessarily indicate the epitopes on antigens are continuous. *Immunology and Cell Biology* **85**, 73 - 78.